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Effects of chronic nociceptin/orphanin FQ exposure on cAMP accumulation and receptor density in Chinese hamster ovary cells expressing human nociceptin/orphanin FQ receptors

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Abstract

Nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand for the G_i-coupled N/OFQ receptor (NOP). We have examined the effects of chronic exposure of Chinese hamster ovary cells expressing the recombinant human NOP receptor (CHO_{hNOP}) to 1 nM N/OFQ for up to 48 h in the absence and presence of the NOP selective antagonist [Nphe^1]N/OFQ (1–13)NH_2 ([Nphe^1]). Then, either a concentration–response curve for N/OFQ inhibition of cAMP formation was constructed or the cells were homogenized and membrane receptor density was determined using [^{125}I]Y^{14}N/OFQ. There was a time-dependent reduction in pEC_{50} (without a change in maximum) for N/OFQ with significant differences observed following >24 h of exposure (control pEC_{50} ~9.5; 48 h pretreatment ~8.7). In cells co-exposed to N/OFQ+[Nphe^1] for 48 h, there was no reduction in pEC_{50}. There was a compensatory (~2.5-fold), [Nphe^1]-sensitive increase in cAMP mass in cells exposed to N/OFQ for 24–48 h. N/OFQ pretreatment also resulted in a time-dependent [Nphe^1]-sensitive loss of cell surface receptors. At 48 h, B_{max} was reduced from ~2.0 to ~1.3 pmol mg^{-1} protein without a change in pK_d for N/OFQ. There was a positive correlation between pEC_{50} for cAMP inhibition and B_{max}. The lack of effect on maximum cAMP response probably results from receptor overexpression and the creation of a receptor reserve.

Keywords: Nociceptin/orphanin FQ; [Nphe^1]N/OFQ (1–13)NH_2; Desensitization; cAMP formation; [^{125}I]Y^{14}N/OFQ binding

1. Introduction

Nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand for the opioid receptor like receptor-1 (Meunier et al., 1995; Reinscheid et al., 1995), hereafter referred to as the N/OFQ receptor (NOP) (Cox et al., 2000). Based on structural and coupling similarities, the N/OFQ–NOP receptor system is considered to be a novel member of the opioid receptor family (Calo' et al., 2000b; Mogil and Pasternak, 2001). Activation of this G_i-coupled "inhibitory" receptor produces an inhibition in the formation of cAMP, closure of voltage-sensitive Ca^{2+} channels, and stimulation of an outward K^+ conductance in a variety of preparations (Hawes et al., 2000; Calo' et al., 2000b; Meunier, 2000; Mogil and Pasternak, 2001). At a systems level, activation of this receptor produces a diverse array of physiological responses including modulation of nociception, feeding, cardiovascular system, and the production of anxiolysis (Calo' et al., 2000b; Meunier, 2000; Mogil and Pasternak, 2001). If this system is to be utilized with effect in man, then it is necessary to characterize and understand the response to continued activation, i.e., possible desensitization.

Continuous exposure to agonist results in loss of agonist responsiveness (Lohse, 1993). Indeed, exposure of classical opioid receptors to opioid agonists results in reduced agonist responsiveness with receptor–G protein uncoupling or loss of membrane-bound receptors resulting in a diminished ability to inhibit cAMP accumulation. This has been demonstrated for μ-opioid (MOP) (e.g., Yabaluri and Medzihradsky, 1997), δ-opioid (DOP) (e.g., Allouche et al., 1999), and κ-opioid (KOP) receptors (e.g., Raynor et al., 1994).

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Consistent with these observations for classical opioid receptors, several studies have been performed with NOP (Hawes et al., 2000). Acute exposure of NOP to N/OFQ results in attenuation of N/OFQ-induced: (a) increase in extracellular acidification in Chinese hamster ovary (CHO) cells stably expressing human NOP (Pei et al., 1997), (b) inhibition of cAMP formation in human neuroblastoma SK-N-SH cells (Cheng et al., 1997), and in NG108-15 hybrid cells (Ma et al., 1997), (c) inhibition of Ca^{2+} channel currents in NG108-15 cells (Morikawa et al., 1998), (d) increase in inwardly-rectifying K^+ currents in locus caeruleus neurons (Connor et al., 1996), and (e) activation of mitogen-activated protein kinase (Hawes et al., 1998). The effects of chronic N/OFQ exposure are largely unknown.

In the present study, we examined the effects of chronic (up to 48 h) N/OFQ exposure in CHO cells expressing the recombinant human NOP receptor (CHO_{hNOP}). As a measure of receptor activity/desensitization, we monitored the well-described inhibition of forskolin-stimulated cAMP formation and as a crude measure of down-regulation loss of [^{125}I]Y^{14}N/OFQ binding sites. In addition, we have utilized the highly selective and competitive NOP antagonist [Nphe^1]N/OFQ (1–13)NH_2 ([Nphe^1]) in these studies.

2. Methods

2.1. Sources of chemicals and reagents

N/OFQ and [Nphe^1] were synthesized at one of our institutes. All cell culture media and supplements were from Life Technologies (Paisley, UK). [2,8-^{3}H]-cAMP (28.4 Ci·mmol^{-1}) and [^{125}I]Y^{14}N/OFQ (2000 Ci·mmol^{-1}) were from NEN DuPont (Boston, MA) and Amersham (Little Chalfont, UK), respectively. Phosphoramidon was from peptide institute (Osaka, Japan). Amastatin, bestatin, and captopril were from Sigma (Poole, UK). All other reagents were of the highest purity available. CHO cells stably expressing the human ORL1 receptor were obtained from Dr. F. Marshall and Mrs. N. Bevan of Glaxo-Wellcome, Stevenage, Herts, UK.

2.2. Cell culture and pretreatment

CHO_{hNOP} cells were maintained in Dulbecco's medium/Hams F12 medium (v/v 50:50) supplemented with foetal bovine serum 5% (v/v), G418 200 μg·ml^{-1} (to maintain expression of NOP-containing plasmid), and hygromycin B 200 μg·ml^{-1} (to maintain expression of a plasmid used for a reporter gene assay *not* used in the present study) at 37 °C in 5% carbon dioxide-humidified air. These cultures were subcultured twice weekly and used for pretreatment when confluent (2–3 days).

Monolayers of CHO_{hNOP} were pretreated with 1 nM nociceptin in medium as described above for 2–48 h. In some experiments, cells were treated for 48 h with a combination of 1 nM N/OFQ and 1 μM [Nphe^1]. The medium for pretreatment also contained peptidase inhibitors: amastatin, bestatin, captopril, and phosphoramidon (30 μM each). For longer incubations, the supplemented culture medium was changed every 24 h. Pretreated cells were rapidly harvested with 10 mM HEPES-buffered saline/0.05% EDTA, pH 7.4. The harvested cells were immediately washed three times and resuspended in either ice-cold Krebs/HEPES buffer of the following composition (in mM): Na^+ (143.3), K^+ (4.7), Ca^{2+} (2.15), Mg^{2+} (1.2), Cl^- (125.6), $H_2PO_4^{2-}$ (1.2), SO_4^{2-} (1.2), glucose (11.7), and HEPES (10), bovine serum albumin 0.5%, pH 7.4 with 10 M NaOH (whole cells) or 50 mM Tris buffer with 5 mM $MgSO_4$ and bovine serum albumin 0.5%, pH 7.4, with 10 M KOH (membranes).

2.3. Measurement of cAMP formation

cAMP formation was measured in 0.3 ml volumes of whole cell suspensions in the presence of isobutylmetylxanthine (IBMX: 1 mM), forskolin (1 μM), and N/OFQ (1 pM–1 μM). After 15 min, at 37 °C, reactions were terminated and cAMP was extracted by the addition of HCl (10 M). The reaction mixture was neutralized with NaOH (10 M) and Tris–HCl (1 M, pH 7.4), and mass of cAMP was measured in the supernatants using a protein-binding assay (Okawa et al., 1999).

2.4. [^{125}I]Y^{14}N/OFQ binding

Binding assays were as previously described (Okawa et al., 1999). Briefly, harvested cells were homogenized with an Ultra Turrax for 30 s at 13 500 rpm and the homogenate was centrifuged at 13 500 rpm, 4 °C for 10 min. The resulting pellet was resuspended in Tris–HCl buffer. This procedure was repeated twice more, as above. All binding assays were performed in 1 ml of Tris buffer for 30 min at room temperature with 3–4 pM [^{125}I]Y^{14}N/OFQ and ~2 μg of membrane protein (to ensure % [^{125}I] bound was <10%) in the presence of peptidase inhibitors cocktail (10 μM). Nonspecific binding was defined in the presence of 10^{-6} M N/OFQ. Following incubation, bound and free radioactivities were separated by rapid vacuum filtration using a Brandel cell harvester onto Whatman GF/B filters and washed with 3×4 ml aliquots of cold Tris buffer. The filters were presoaked in polyethylenimine (0.5%) to reduce nonspecific binding and were loaded onto the harvester wet. For determination of the maximal binding capacity (B_{max}) and the equilibrium dissociation constant (K_d), a pseudo-isotope dilution was performed (i.e., competition between native N/OFQ and [^{125}I]Y^{14}N/OFQ).

2.5. Data analysis

All data are expressed as mean ± S.E.M. The concentration of N/OFQ producing half-maximal inhibition of cAMP formation (EC_{50}) was obtained by computer-assisted

curve fitting (PRIZM-V2.0, GraphPad, San Diego, USA) of individual curves. In radioligand-binding studies, receptor density (B_{max}) and radioligand equilibrium dissociation constant (pK_d) were obtained using isotope dilution and semi-log transformation of the specific binding data (PRIZM-V2.0, GraphPad). Statistical comparisons were made by one-way analysis of variance followed by Bonfferoni's test, unpaired *t*-test, or simple regression as appropriate. A value of $P<0.05$ was considered significant.

3. Results

3.1. Preliminary studies

As it is possible that N/OFQ may "stick" in our desensitizing challenges, we initially determined how much of a 1 nM N/OFQ solution, spiked with 15 pM of [^{125}I]Y^{14}N/OFQ and incubated for 2 h, could be washed away using the protocol described in Methods. [^{125}I]Y^{14}N/OFQ present on or in the harvested cells was assessed using a Gamma counter. The residual activity of incubated N/OFQ was up to 3.7±0.3% of control (results from four different experiments) equivalent to ~30 pM. At this concentration, we do not observe any inhibition of cAMP formation in N/OFQ naive cells (this study and Okawa et al., 1999).

We also determined whether peptidase inhibitors were necessary in cAMP and receptor binding studies as N/OFQ is readily metabolized by aminopeptidases and endopeptidases (Terenius et al., 2000). As shown in Fig. 1, peptidase inhibitors did not affect the cAMP response in whole cells, but there was a reduction in B_{max} (1584±82 vs. 2208±145 fmol mg protein^{-1}, in the absence and presence of 10 μM inhibitors, respectively) in membranes.

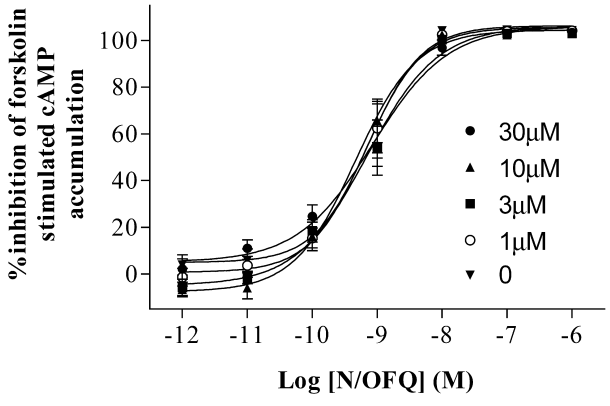

Fig. 1. Effects of peptidase inhibitors on nociceptin-induced inhibition of cAMP accumulation by N/OFQ in whole CHO_{hNOP} cells. Various concentrations of peptidase inhibitors (1–30 μM of amastatin, bestatin, captopril, and phosphoramidon as a cocktail) were incubated with N/OFQ for 15 min at 37 °C. Data are expressed as mean±S.E.M. (n=5).

Table 1
Potency (pEC_{50}) and efficacy (E_{max}) for N/OFQ inhibition of forskolin-stimulated cAMP formation in CHO_{hNOP} exposed to 1 nM N/OFQ or 1 nM N/OFQ plus 1 μM [Nphe^1]

Duration of exposure (h)	pEC_{50}	EC_{50} (pM)	E_{max} (% inhibition)
0	9.40±0.07	398	103.1±0.7
2	9.55±0.17	282	101.9±1.1
6	9.25±0.09	562	104.6±0.2
24	8.98±0.06	1047	103.1±0.7
48	8.73±0.04[a]	1863	103.1±0.2
48+[Nphe^1]	9.55±0.02	282	102.3±0.3

CHO_{hNOP} cells were pretreated with 1 nM N/OFQ for 0, 2, 6, 24, and 48 h and then harvested and inhibition of forskolin-stimulated cAMP formation was assessed as described in Methods. In some experiments, cells were pretreated with a combination of 1 nM N/OFQ and 1 μM [Nphe^1] for 48 h. Values are mean ± S.E.M. (n=4–6). Data were analysed using analysis of variance with Bonfferoni's correction.

[a] $P<0.01$ vs. control (0 h).

In the remainder of studies reported here, peptidase inhibitors were used in all preincubations and during the experimental incubation protocol in binding studies only.

3.2. Time course for desensitization

As shown in Table 1, less than 6 h pretreatment with 1 nM of N/OFQ did not alter the concentration–response curve (pEC_{50} or E_{max}) of N/OFQ, while more than 24 h of pretreatment with N/OFQ shifted the concentration–response curve (control pEC_{50} ~9.5; 48 h pretreatment ~8.7) of N/OFQ to the right, reducing the pEC_{50} but not affecting the maximum response. The rightward shift after 48 h pretreatment was prevented by co-incubation with 1 μM [Nphe^1] (Fig. 2). This reduction in pEC_{50} was clearly time-dependent. In cells pretreated for 24 and 48 h, there was a compensatory increase (~2.5-fold) in both basal and forskolin-stimulated cAMP formation (Fig. 3). It is possible that the reduction of N/OFQ potency in the cAMP assay

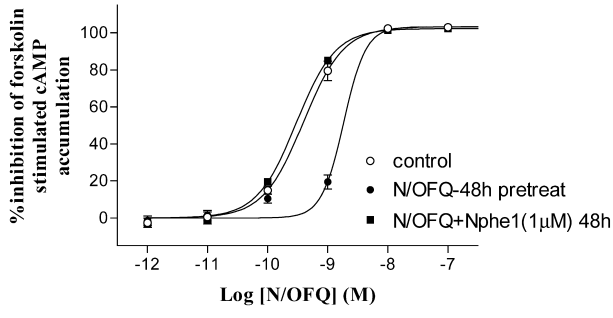

Fig. 2. Effects of 48 h of 1 nM N/OFQ pretreatment in the absence and presence of 1 μM [Nphe^1] on N/OFQ inhibition of forskolin-stimulated cAMP formation. Following pretreatment, cells were washed three times with Krebs/HEPES buffer and inhibition of forskolin-stimulated cAMP formation was measured in response to N/OFQ for 15 min at 37 °C. Data are expressed as mean ± S.E.M. (n=6). These and data for other time points are also summarized in Table 1.

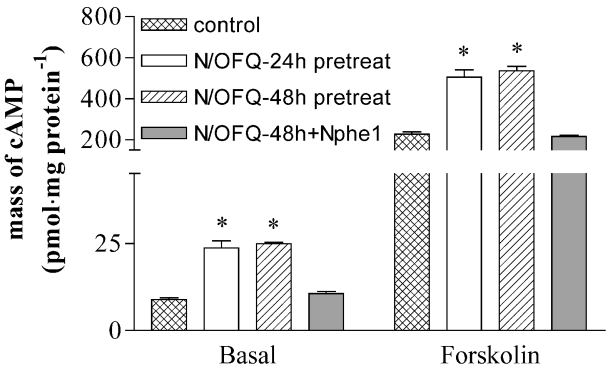

Fig. 3. Basal- and forskolin-stimulated (1 μM) cAMP mass accumulation in control, 24 and 48 h (with and without 1 μM [Nphe^1]) 1 nM N/OFQ-treated cells. Data are expressed as mean ± S.E.M. (n=5). *(P<0.0001) significant compared to control.

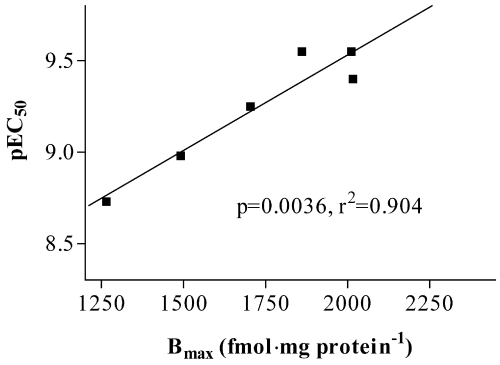

Fig. 4. Relationship between B_{max} for [^{125}I]Y^{14}N/OFQ binding and pEC_{50} for N/OFQ-induced cAMP inhibition. Data were analysed using simple regression test. Each point indicates the mean from at least four experiments.

could be due to the observation that forskolin-stimulated cAMP levels are higher in pretreated than in control cells (see Fig. 3). To test this hypothesis, we examined the effect of N/OFQ against the stimulatory effect of 3 μM (in place of 1 μM) forskolin in N/OFQ naive cells. At this concentration (3 μM), forskolin produced an increase of cAMP comparable to that induced by 1 μM forskolin in N/OFQ-pretreated cells. Under these conditions, there were no differences in either pEC_{50} or E_{max} of N/OFQ (data not shown).

3.3. Loss of cell surface receptors

N/OFQ pretreatment also resulted in a time-dependent loss of [^{125}I]Y^{14}N/OFQ binding (Table 2). At 48 h, the B_{max} was reduced from ~2.0 to ~1.3 pmol mg^{-1} protein without change in pK_d for N/OFQ. The loss of cell surface receptors following 48 h pretreatment was prevented by co-incubation with 1 μM [Nphe^1] (Table 1). There was a positive correlation between pEC_{50} for cAMP inhibition and B_{max} (Fig. 4).

Table 2.
Maximal membrane binding capacity (B_{max}) and equilibrium dissociation constant (pK_d) for [^{125}I]Y^{14}N/OFQ in CHO_{hNOP} exposed to 1 nM N/OFQ or 1 nM N/OFQ plus 1 μM [Nphe^1]

Duration of exposure (h)	B_{max} (fmol mg·protein^{-1})	Percent of reduction in B_{max}	pK_d
0	2016±133	–	9.63±0.11
2	1860±178	7.7	9.75±0.10
6	1704±155	15.5	10.00±0.10
24	1491±90	26.1	9.69±0.10
48	1264±40[a]	47.4	9.83±0.13
48+[Nphe^1]	2011±207	0.2	9.58±0.08

CHO_{hNOP} cells were pretreated with 1 nM N/OFQ for 0, 2, 6, 24, and 48 h and then homogenized and washed three times with Tris–HCl buffer. In some experiments, cells were pretreated with a combination of 1 nM N/OFQ and 1 μM [Nphe^1] for 48 h. B_{max} and pK_d were determined by pseudo-isotope dilution using [^{125}I]Y^{14}N/OFQ for 30 min at room temperature. Values are mean ± S.E.M. (n=6). Data were analysed using analysis of variance with Bonfferoni's correction.

[a] P<0.01 vs. control (0 h).

4. Discussion

In this study utilizing CHO_{hNOP} cells, we have shown that acute (up to 6 h) treatment with 1 nM N/OFQ is not sufficient to induce significant desensitization of response at the level of adenylyl cyclase or loss of cell surface receptors. However, chronic (>24 h) treatment results in a rightward shift in the concentration–response curve to subsequent N/OFQ rechallenge, an up-regulation of cAMP formation, and loss of cell surface receptors. The rightward shift in the cAMP concentration–response curve, up-regulation of cAMP formation, and loss of cell surface receptors at 48 h were prevented by the NOP antagonist [Nphe^1]. During these longer N/OFQ challenges, there was no loss of maximum response.

In these studies, we have carefully controlled for (a) peptide carry-over from the desensitization to the rechallenge period and (b) the need for peptidase inhibitors. These are important considerations for peptides where "stickiness" and metabolism (Terenius et al., 2000) are possible in different assay systems. It is interesting that in whole cells, peptidase inhibitors are not necessary, yet in membranes, they appear to be required, as in their absence, binding is reduced by some ~30% indicating that peptidases are either released during homogenization or are present on the inner leaflet of the plasma membrane or vesicle membranes. Indeed, the use of peptidase inhibitors in some tissues is essential. For example, in the human vas deferens, N/OFQ does not inhibit electrically evoked twitches in the absence of the peptidase inhibitor cocktail (Bigoni et al., 2001).

The time course of these effects are interesting as acute exposure to N/OFQ has been shown to desensitize the NOP in several preparations/assay systems (Connor et al., 1996;

Pei et al., 1997; Cheng et al., 1997; Ma et al., 1997; Morikawa et al., 1998; Hawes et al., 1998). Longer time courses have not been extensively studied. The lack of effect at shorter times in this study and the inability to reduce the maximum effect most likely result from overexpression of NOP and transfection of an "artificial" receptor reserve. The functional consequences of that receptor reserve are such that even a loss of ~50% of receptors leaves sufficient to obtain a full maximum response. It would have been desirable to pretreat for >48 h, but due to the growth kinetics of these cells, that was not possible. As the concentration–response curve to N/OFQ was shifting rightwards with increasing time, we would predict that at longer times, a classical loss of maximum response might have been observed. Additional studies with cells expressing lower levels of NOP are needed, but these are not available to us, and in the absence of irreversible "alkylating-type" antagonists, we are unable to remove a larger proportion (>50%) of the cell surface NOP. In a recent study, Spampinato et al. (2001) reported that N/OFQ produced a time- and concentration-dependent and [Nphe^1]-sensitive internalization of NOP receptors in SK-N-BE human neuroblastoma cells (no measurements of cAMP were made). In these cells, the pretreatment with N/OFQ (1 μM) was for only 30 min. It should be noted that this cell line only expresses 38 fmol/mg protein NOP (with a very low pK_d of 8.9). In a desensitizing challenge, it is not merely the time of pretreatment that is important but the overall exposure to agonist, i.e., the concentration of "desensitizer" should be taken into account. In the study of Spampinato et al. (2001), the desensitizing challenge was three orders of magnitude higher than that used here, and hence, this could explain the kinetics of the response, but low levels of expression are clearly important.

In the present study, we have shown that receptor down-regulation (loss of cell surface receptors) was the predominant mechanism responsible for the desensitization of NOP function, resulting in attenuation of the N/OFQ-induced inhibition of cAMP accumulation. Similar results were obtained by Dautzenberg et al. (2001) in HEK293 cells expressing ~1.3 pmol mg^{-1} protein hNOP using the non-peptide agonist Ro64-6198 as the desensitizing agonist. In the present study, exposure of N/OFQ only reduced receptor density, with the pK_d remaining unaffected, indicating that it is unlikely that the receptor has uncoupled from the G-protein. If this was the case, then the pK_d might be expected to reduce. However, when receptors are lost, there was an up-regulation of both basal and forskolin-stimulated cAMP formation. These data may indicate constitutive activity of the NOP in this expression system, as we have suggested for the μ-opioid receptor also expressed in CHO cells (Harrison et al., 2000). At variance with these observations is the study of Dautzenberg et al. (2001), who reported a reduction in maximum cAMP response *and* a reduction in the pK_d of [^{3}H]N/OFQ. However, it should be noted, as we have discussed in the past (Harrison et al., 2000), that cell background is important in desensitization studies and there are differences between CHO and HEK cells.

In a previous study using CHO cells expressing the recombinant rat μ-opioid receptor, we have performed a similar study. In this study, we examined the effects of pretreatment with endomorphin-1. Pretreatment for 11–18 h reduced the maximum inhibition of cAMP formation and up-regulated cAMP formation. However, in this preparation, there was a maximum reduction in cell surface receptors of ~30% that occurred at 30 min. Clearly, in this preparation, there was a temporal distinction between the desensitization and down-regulation phenomena (Harrison et al., 2000). In HEK293 cells also transfected with the rat μ-opioid receptor, Pak et al. (1999) have shown both G-protein-dependent and -independent down-regulation.

Until recently, studies of N/OFQ–NOP pharmacology have been hampered by the lack of a selective and competitive antagonist. Recently, we described the activity of the first selective competitive peptide NOP antagonist (Calo' et al., 2000a,b). This molecule has been shown to antagonise the actions of a range of peptide and nonpeptide agonists in a range of tissues expressing both endogenous and recombinant NOP receptors (Calo' et al., 2000a; Hashimoto et al., 2000). In these studies, the pA_2/pK_B values were between 6 and 7. In this study, we have utilized [Nphe^1] in chronically N/OFQ-treated cells and have clearly shown a complete block of desensitization, up-regulation of cAMP formation, and loss of cell surface receptors, indicating that these phenomena are triggered by NOP receptor activation.

In conclusion, we suggest that a reduction in number of cell surface NOP (supported by a strong correlation between receptor density and pEC_{50}) seems to be the predominant mechanism underlying the functional desensitization of the NOP when overexpressed in CHO_{hNOP} cells. Further studies are warranted at lower levels of receptor expression, with higher concentrations of peptide and nonpeptide agonists and with NOP in the same cellular environment.

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