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Research Article

Agonist-induced internalization and desensitization of the human nociceptin receptor expressed in CHO cells

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Abstract. In this study, we examined agonist-induced internalization, recycling and signalling (measure of cAMP levels) of the cloned human nociceptin receptor (hNOP) expressed in CHO-K1 cells. Internalization was proven by a receptor-binding assay on viable cells. The agonist nociceptin/orphanin FQ (NC) promoted rapid internalization of the hNOP receptor (≈78% of cell surface receptors were lost after 2 min exposure to 1 μM NC) in a clathrin- and ATP-dependent manner. Internalization was more rapid and marked in CHO-K1 cells than, as we previously reported, in SK-N-BE cells. This difference may be related to higher levels of β-arrestin isoforms detected in CHO-K1 than in SK-N-BE cells. hNOP receptor internalization was partially reversible and recycling occurred in the presence of the agonist; receptor recycling was dependent on okadaic acid-sensitive phosphatases and was blocked by monensin. Confocal microscopy analysis confirmed the internalization and the recycling back to the plasma membrane of an epitope-tagged hNOP receptor expressed in CHO-K1 cells. These receptors underwent rapid desensitization upon agonist challenge: NC efficacy in inhibiting forskolin-stimulated cAMP production was significantly reduced 10 min after exposure and correlated with the rate of receptor internalization. Moreover, we observed that blockade of hNOP receptor recycling by monensin would cause a more prolonged and relevant desensitization of this receptor. Thus, the dynamic cycle between hNOP receptor activation, internalization and recycling determines the activity of this receptor on the cell surface.

Key words. Internalization; recycling; CHO-K1; nociceptin; nociceptin receptor; desensitization; cAMP.

Prolonged activation of G protein-coupled receptors (GPCRs) leads to a greatly decreased sensitivity of the receptor to a subsequent agonist challenge [for a review see ref. 1]. This phenomenon, termed desensitization, is a general physiological mechanism by which receptors adapt to a changing environment. The molecular mechanisms that lead to agonist-dependent desensitization of GPCRs are not fully defined, but several distinct events are involved, including uncoupling of receptors from their heterotrimeric G proteins and reduction in the number of receptors at the cell surface by either internalization or down-regulation which may be associated with degradation [for a review see ref. 2]. This event also promotes the activation of G protein-coupled receptor kinases (GRKs) that phosphorylate the activated receptor, enabling the GPCR to bind arrestins, proteins that sterically preclude further coupling to heterotrimeric G proteins [3–6]. Several studies have proposed that the binding of arrestins to GRK-phosphorylated receptors triggers their sequestration into endosomal recycling compartments (receptor internalization). This is one mechanism by which activated receptors are dephosphorylated and then partially resensitized [1, 2]. Receptor internalization (i.e. the loss of receptors from the cell surface) is generally envisioned as a rapid, agonist-in-

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duced movement of the receptor into a cell compartment distinct from the plasma membrane, where it is unavailable for binding hydrophilic ligands but remains detectable by hydrophobic ligands [7]. These processes have been extensively investigated for the β_2-adrenergic receptor [6] and for receptors binding opioids [8] or other neuropeptides [9]. However, the dynamic relationships between internalization, desensitization and down-regulation are far from being well understood.

Desensitization and internalization processes of the nociceptin receptor (NOP; also known as ORL1 or OP_4), a recently identified member of the GPCR family [10–12], have been poorly investigated. The endogenous peptide nociceptin/orphanin FQ (NC) binds this receptor with high affinity [13]. Numerous studies have shown that this peptidergic system is involved in the control of a variety of biological functions including nociceptive transmission [13, 14], regulation of rewarding and reinforcing properties of drugs of abuse [15] and adaptive behavioral fear responses to stress [16]. Recently, we reported NC-promoted internalization of the human NOP (hNOP) receptor occurring in the neuroblastoma cell line SK-N-BE, by measuring the loss of binding sites for the hydrophilic ligand [^{3}H]-NC in viable cells, and β-arrestin 2 was involved in this process [17]. In this study, we investigated desensitization of the hNOP receptor expressed in CHO-K1 cells following prolonged exposure to NC. This event has been related to internalization and recycling of this receptor in intact cells. We found that the mechanism for NC-induced rapid desensitization of the hNOP receptor requires the loss of receptors from the cell surface.

Materials and methods

Materials

Cell culture reagents were from Gibco Life Technologies (Milan, Italy). NC and [Nphe^1]nociceptin(1–13)-NH_2 (Nph) were a kind gift of Prof. S. Salvadori (University of Ferrara, Italy). [^{3}H]-NC was from Amersham-Pharmacia (Milan, Italy). Antimycin, aprotinin, bacitracin, cycloheximide, forskolin, geneticin, leupeptin, monensin, okadaic acid, phenylmethanesulfonylfluoride and 2-deoxyglucose were purchased from Sigma-Aldrich (Milan, Italy); all other reagents were of analytical grade or of the highest purity available.

Cell culture

CHO K-1 cells (No. CCL61; American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (50:50, v/v) supplemented with 10% fetal calf serum (FCS), 100 IU penicillin, 0.1 mg streptomycin and 0.25 µg amphotericin B/ml and maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. The medium was replaced at 2-day intervals. Cells were grown to ≈80% confluence in 75-cm^2 flasks and were detached from the flask surface by incubating monolayers for 5 min with phosphate-buffered saline (PBS; pH 7.4), then using a cell scraper. Cell viability was assessed using the trypan blue dye exclusion method: ≈90% of the cells were viable.

The cDNA encoding the hNOP receptor (a kind gift of Prof. O. Civelli) was subcloned into CHO-K1 cells using the mammalian expression vector pcDNA 3 (Invitrogen) harboring a neomycin-resistant gene as a selection marker. Transfection was achieved using 10 µg of hNOP-pcDNA 3/0.5 × 10^6 cells and using a cationic liposome formulation-mediated transfer (LipofectAMINE; Life Technologies, Milan, Italy). Clonal cell lines stably expressing the hNOP receptor (CHO-hNOP) were established with geneticin selection (0.5 mg/ml) added for 1 month. The NOP receptor was detected by receptor binding on intact cells.

Receptor binding on intact cells

Cell surface NOP receptors were measured adopting a radioligand-binding assay [17]. The cells were treated with NC for varying times at 37 °C, washed thoroughly, and then incubated at 4 °C with the radioligand. Physiological temperatures are required for receptor endocytosis whereas a low temperature is required to prevent further endocytosis and recycling of receptors during the quantitative procedure. CHO-hNOP cells were seeded out into 60-mm dishes at a density of 2.5 × 10^5 cells per dish and grown for 48 h; the medium was then removed and each dish was washed with 2 ml of binding buffer (130 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, 1 mg/ml bovine serum albumin, 25 mM HEPES; with addition of peptidase inhibitors: 1 mM phenylmethanesulfonylfluoride, 10 µg/ml leupeptin, 80 mg/ml bacitracin, 10 µg/ml aprotinin, pH 7.4). To each dish, 2 ml of binding buffer was added containing [^{3}H]-NC (0.0625–4 nM in saturation binding assays or 4 nM to measure receptor internalization). Non-specific binding was determined in the presence of 10 µM unlabelled NC. Binding assays were carried out in triplicate adopting the procedure described by Toll [18] and cells were maintained at 4 °C. At the end of the incubation time, the buffer was aspirated and the cells were washed three times with ice-cold binding buffer. After the third wash, 1 ml of 0.5 M NaOH was added to each dish to digest the cells. NaOH was left on the cells for 2 h, after which 0.8 ml was placed in vials with 5 ml of scintillation cocktail (Packard, Milan, Italy) and counted by scintillation proximity assay. Protein content was evaluated on 0.05-ml aliquots of cell extract by the BCA method (Perbio Science, UK). The Ligand program was used to estimate receptor density (B_{max}), Hill slopes and ligand affinity (K_d) [19].

Internalization of the hNOP receptor

CHO-hNOP cells were cultured in 60-mm dishes as described and exposed, in binding buffer containing the peptidase inhibitors, to different peptides or to the medium alone (control) at 37 °C. At the time indicated, the medium was removed and the cells were treated with 2 ml of acidic ice-cold PBS (pH 6.5) for 2 min. Dishes were then washed with a further 2 × 2 ml of acid wash buffer and finally incubated in the binding buffer. The number of hNOP receptors on the cell surface was evaluated by binding assay, as described above. Receptor internalization was measured as a reduction in the binding sites for 4 nM [^{3}H]-NC and data were expressed as percentage of loss of cell surface receptors in comparison to untreated cells (control). In one set of experiments, the cells were exposed to monensin (50 µM) at 37 °C before the binding assay; this ionophore prevents acidification of intracellular compartments and blocks the membrane recycling process by raising the pH within endosomes and, thereby, preventing the dissociation of receptor and ligand [2]. Cells were depleted of ATP by incubation with 50 nM antimycin and 50 mM 2-deoxyglucose at 37 °C for 30 min in binding buffer before adding NC [4].

Construction of the hemagglutinin-tagged hNOP receptor cDNA and expression in CHO-K1 cells

The human influenza virus hemagglutinin (HA) epitope-tagged hNOP receptor was constructed using oligonucleotides and the polymerase chain reaction (PCR) method to add nine amino acids (YPYDVPDYA) to the amino terminus of the hNOP receptor between the N-terminal methionine and the second amino acid. The pcDNA 3 plasmid containing the hNOP receptor cDNA was used as PCR template. The 5′ end of the PCR product carried a *Hin*d III restriction site. The forward primer was 5′-ATG **AAG CCT** ATC ATG TAC CCA TAC GAC GTC CCA GAC TAC GCT GAG CCC CTC TTC CCC GCG -3′, where the *Hin*d III site is indicated in bold and the HA-tag is underlined. The SP-6 primer was the reverse primer. The 300-bp fragment located at the 5′ region of the PCR product was sequenced to confirm addition of the epitope-coding region to the amino terminus of the hNOP receptor. This fragment, after PCR amplification, was digested with *Hin*d III and *Ssp*I (this restriction endonuclease cuts a unique restriction site of the hNOP receptor cDNA which is located ≈260 bp downstream of the start codon) and ligated to the remainder of the wild-type hNOP receptor cDNA (≈1.2 kb of the *Ssp*I/*Xho*I 3′ portion of the hNOP receptor cDNA) in the pcDNA 3 vector. HA-tagged hNOP receptor cDNA (10 µg) was transiently transfected in CHO-K1 cells (≈60% confluent – these cells were maintained as previously described) and cultured in 100-mm dishes (Falcon) – using PerFect Lipid Pfx-7 (Invitrogen). In preliminary experiments, by monitoring fluorescence of green fluorescence protein (GFP) expressed by a GFP control vector co-transfected with the above-described plasmid, we ascertained that transfection efficiency was approximately 60%. After transfection (≈4 h), the cells were incubated with fresh medium and allowed to recover for 8 h before being seeded on sterile glass coverslips and then treated with NC 48 h later.

Immunofluorescence microscopy

At the end of peptide exposure, transiently transfected HA-tagged CHO-hNOP cells were washed at 4 °C with 50 mM phosphate buffer (PB; pH 7.4) three times, fixed with 4% paraformaldehyde for 20 min, washed twice with PB, and three times with PBS. Cells were then permeabilized for 20 min in 10% non-fat dry milk and 0.1% saponin in PBS at room temperature. After non-specific-site blockade (1 h in the presence of PBS/0.2% BSA), cells were washed with PBS and incubated overnight at 4 °C with mouse monoclonal anti-HA antibody (1 : 500 dilution; Roche Bioscience). After washing with PBS/0.2% BSA for 5 min once and PBS alone six times, bound primary antibody was detected by incubation with cyanine 3 (cy3)-conjugated goat anti-mouse antiserum (1 : 1500 dilution; Amersham Biosciences) for 60 min. After further washing (PBS six times) for 10 min, the coverslips were dried and treated with anti-fade (ProLong Antifade kit; Molecular Probes) before being fixed onto glass slides. Slides were examined by laser scanning confocal microscopy (Bio-Rad MRC 1000 confocal microscopy). The estimated depth of optical sections under the confocal imaging conditions used was 0.5–1.0 µm. The images were processed with Adobe Photoshop (Adobe). Control cells in which the primary antibody was omitted never showed cy3 fluorescence signals.

Western blot analysis of β-arrestin in CHO-K1 and SK-N-BE cells

Confluent CHO-K1 and SK-N-BE cells (cultured as previously reported [17]) were pelleted by centrifugation, resuspended in 0.2 ml cold lysis buffer (NE-PER protein extraction kit; Perbio Science), then lysed; 60 µg of cytosolic proteins was electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, then immunoblotted using a 1 : 1000 dilution of a β-arrestin 1 antibody (K-16; Santa Cruz) specific for β-arrestin 1 or using a 1 : 1500 dilution of a β-arrestin 2 antibody (H-9; Santa Cruz) which does not cross-react with β-arrestin 1. The blots were washed, incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz), and visualized by chemiluminescence (ECL; Amersham-Pharmacia). The protein concentration was determined by the BCA method. To quantify the intensities of the bands, membranes were scanned and

analyzed by an imaging densitometer (model GS-700; Bio-Rad, Hercules, Calif.). The bands were quantitated with the Molecular Analyst Image Analysis software (Bio-Rad).

cAMP assay

hNOP receptor coupling to adenylyl cyclase was investigated by measuring, in whole CHO-hNOP cells, the concentration-dependent inhibitory effect of NC on forskolin-stimulated cAMP accumulation [20].

Three days before the experiments, ≈50,000 CHO-hNOP cells were seeded into 24-well plates. On the day of experiments, the culture medium was removed and the cells were washed with 0.5 ml PBS (in mM: 137 NaCl, 2.7 KCl, 0.9 $CaCl_2$, 0.5 $MgCl_2$, 6.5 Na_2HPO_4, 1.5 KH_2PO_4, pH 7.2), and then incubated at 37 °C for 10 min with 0.5 ml assay buffer (in mM: 150 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 10 HEPES, 10 mg/ml BSA, pH 7.4). After removal of buffer, 0.45 ml of fresh buffer containing 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma-Aldrich) was added for 10 min and cells were exposed to 10 µM forskolin without and with NC (10^{-12}–10^{-4} M) for 10 min at 37 °C. The reaction was stopped by addition of 1 vol of ice-cold 0.2 M HCl. Cells were further disrupted by sonication and suspensions were centrifuged at 14,000 g for 15 min. The resulting supernatants were stored at –20 °C until determination of cAMP by radioimmunoassay (Amersham-Pharmacia). To study agonist-dependent desensitization of the inhibition of adenylyl cyclase activity, cells were preincubated at 37 °C either with or without NC. The cells were then washed with incubation medium to remove unbound NC and, thereafter, assayed as above using different concentrations of NC for inhibition of forskolin-stimulated cAMP accumulation. In experiments in which the cells were pretreated with monensin, the stock solution in 95% ethanol was added to individual wells in 10-µl aliquots 1 h before the addition of NC, in the presence of the assay buffer used to evaluate cAMP production. The values are presented as percent of NC-induced inhibition of forskolin-stimulated cAMP production or as absolute levels of cAMP (pmol/mg protein) and refer to the mean ± SE of the determinations from a minimum of three separate experiments done in triplicate.

Statistical analysis

Statistical comparisons were performed by analysis of variance (ANOVA) and post hoc Dunnett's multiple-comparison test with differences of $p < 0.05$ being considered significant; EC_{50} values were determined by non-linear regression (GraphPad).

Results

Characterization of the recombinant hNOP receptor in CHO-K1 cells

The presence of hNOP receptors was evaluated by receptor-binding assay on viable cells still attached to the dishes. [^{3}H]-NC bound with high affinity to CHO-hNOP cells and specific binding was routinely ≈80–85% of total binding. Under these conditions, ligand (4 nM) association reached the equilibrium within 90 min at 4 °C (data not shown). Dissociation of the labelled peptide on washing the dishes with fresh binding buffer and then adding cold NC (10 µM) was completed within 45 min (data not shown); on the whole, ≈90% of [^{3}H]-NC specifically bound to hNOP receptors was removed by cold NC while the remaining (≈10%) was associated with the cells. Therefore, only a minimal amount of hNOP receptors are internalized at 4 °C; binding assays were routinely performed at this temperature for 120 min.

[^{3}H]-NC binding to CHO-hNOP cells was saturable and the peptide apparently bound to a single site (Hill slopes were not significantly different from 1; data not shown); the K_d was 0.95 ± 0.18 nM and B_{max} 110 ± 5 fmol/mg of protein (n = 3) (fig. 1). In this study, we selected a clone of CHO-hNOP cells expressing a relatively low level of NOP receptors on their cell surface in order to better relate their function with the native receptors that are expressed in neuronal cells at a low concentration [17]. In wild-type CHO-K1 cells, the specific binding of [^{3}H]-NC was less than 5% (data not shown).

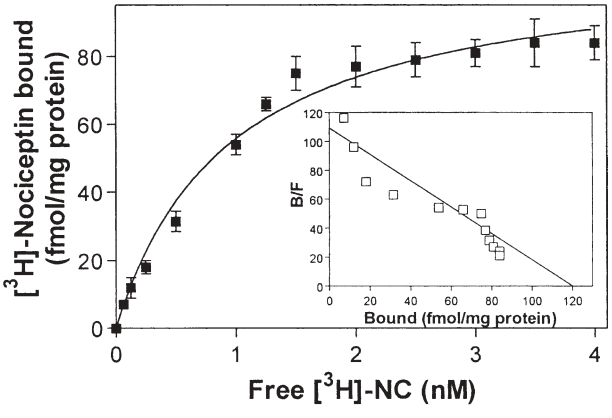

Figure 1. Saturation binding of [^{3}H]-NC to intact CHO-hNOP cells incubated with increasing concentrations of the radioligand (0.0625–4 nM) for 120 min at 4 °C as described in the Materials and methods. Inset, Scatchard transformation of specific [^{3}H]-NC binding. Incubation was stopped as reported in Materials and methods. Non-specific binding was determined in the presence of 10 µM unlabelled NC. Only specific binding is shown, for the sake of clarity. Values are the mean ± SE of three experiments done in triplicate.

Effect of NC on hNOP receptor internalization and recycling

Exposure of CHO-hNOP cells to NC (1 μM at 37 °C) promoted rapid internalization of the hNOP receptor, as measured by loss of cell surface receptors for the hydrophilic ligand [^{3}H] NC (fig. 2). NC-induced receptor internalization was maximal at 2 min of exposure, extending to 78 ± 5% of surface receptors. Thereafter, partial recovery of cell surface NOP receptors (≈30 % of the internalized receptors) was observed within 60 min in the continued presence of the agonist; however, this event was not observed in cells exposed to monensin (fig. 2). Internalization of the hNOP receptor was substantially decreased by depletion of ATP with antimycin and 2-deoxyglycose (fig. 2), indicating that NC-induced endocytosis occurs via energy-dependent mechanisms [4].

NC-induced hNOP receptor internalization (10 nM–5 μM) was concentration dependent; the EC_{50} (concentration of NC which causes half-maximal internalization of hNOP receptors) was 113 ± 12 nM (n = 3) (fig. 3 A). These experiments were carried out on CHO-hNOP cells exposed for 10 min to NC. This exposure time was chosen because, as shown in figure 2, hNOP receptor internalization was still high; moreover, as described below, confocal microscopy analysis of HA-tagged CHO-hNOP cells confirmed that internalized hNOP receptors are clearly visible at this time.

The purported antagonist Nph (10 μM) induced only modest internalization of hNOP receptors and significantly reduced NC-induced internalization (fig. 3 B). Hypertonic sucrose (0.4 M) is known to inhibit the sequestration of some GPCRs that employ a clathrin-coated-vesicle mechanism [4]; this compound blocked NC activity in CHO-hNOP cells (fig. 3 B). Moreover, NC-induced internalization of hNOP receptors was prevented in cells kept at 4 °C for 120 min (fig. 3 B).

To assess whether the internalized receptors could return to the cell surface, CHO-hNOP cells were initially exposed to NC (1 μM) for 10 min at 37 °C as previously described. The cells were then extensively washed with acid ice-cold PBS (see Materials and methods) to remove NC and then allowed to recover at 37 °C without the agonist for 30, 60, 90 and 120 min (considered as wash-out time). Thereafter, the cell culture medium was removed, the cells were incubated at 4 °C in binding buffer in the presence of 4 nM [^{3}H]-NC and cell surface hNOP receptors were measured by receptor binding as described in Materials and methods. In these conditions, about 30% of hNOP receptors gradually returned to the cell surface in 90 min (fig. 4). Prolonging the recovery up to 120 min did

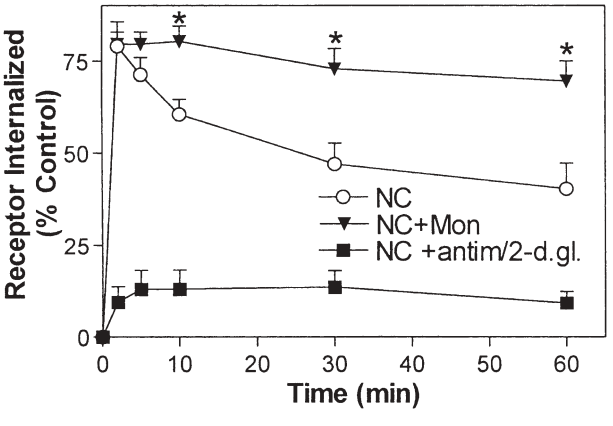

Figure 2. Time course of the effect of NC (1 μM) on internalization of the hNOP receptor in the presence of antimycin (50 nM) + 2-deoxyglucose (50 mM) or of monensin (50 μM) as described in Materials and methods. CHO-hNOP cells were exposed to NC at 37 °C for different times and internalized receptors were determined as described in Materials and methods. Each value is the mean ± SE of five experiments done in triplicate. * $p < 0.05$ vs NC-treated cells (Dunnett's test after ANOVA).

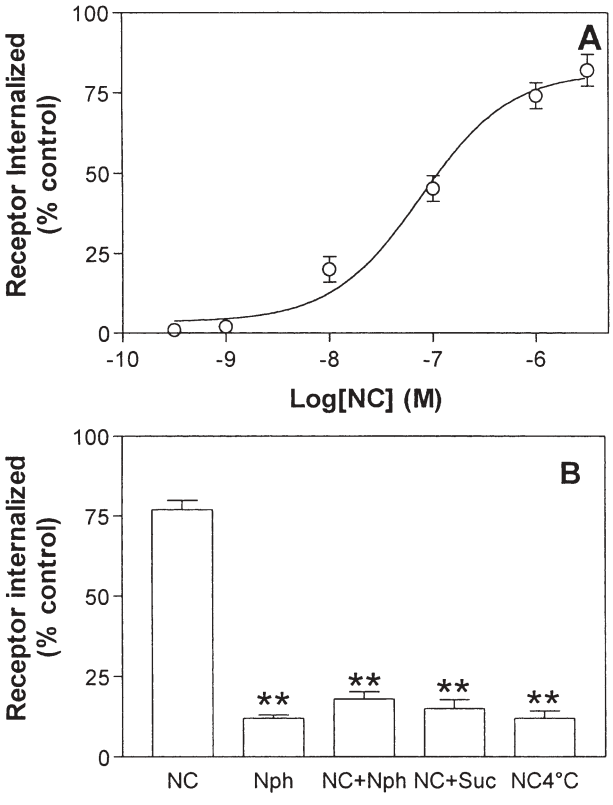

Figure 3. (*A*) Concentration-response curve of NC-induced internalization of hNOP receptors expressed in CHO-K1 cells. Cells were exposed to NC for 10 min at 37 °C and internalized receptors were determined as described in Materials and methods. Each value is the mean ± SE for five experiments done in triplicate. (*B*) Effect of NC, Nph, hypertonic sucrose (Suc; 0.4 M) and low temperature (4 °C) on internalization of hNOP receptors. CHO-hNOP cells were exposed to 1 μM NC for 10 min at 37 °C in the presence or absence of 10 μM Nph or sucrose. Alternatively, the cells were exposed to 1 μM NC at 4 °C for 120 min. Internalized receptors were determined as described in Materials and methods. Each value is the mean ± SE of five experiments done in triplicate. ** $p < 0.01$ vs NC-treated cells (Dunnett's test after ANOVA).

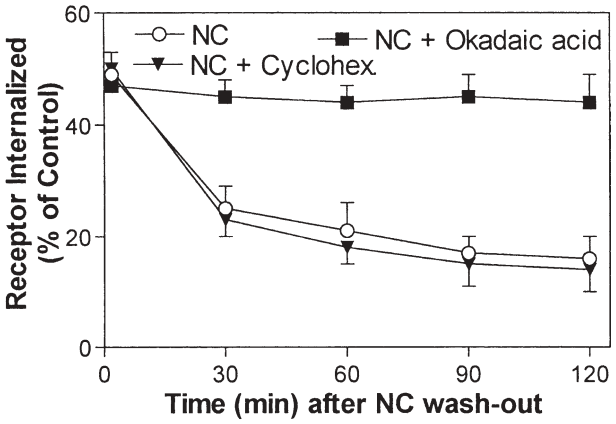

Figure 4. Recycling of the hNOP receptor on the cell surface. CHO-hNOP cells were exposed to NC (1 μM) for 10 min; then the cells were thoroughly washed and incubated for 30, 60, 90 and 120 min (considered as wash-out time) in fresh medium without (control) or with 10 nM okadaic acid at 37 °C or in the presence of cyclohexymide (70 μM). Thereafter, the cell culture medium was removed and the cells were incubated at 4 °C in binding buffer in the presence of 4 nM [^{3}H]-NC. Cell surface hNOP receptors were measured by receptor binding as described in Materials and methods. Each value is the mean ± SE of four experiments done in triplicate.

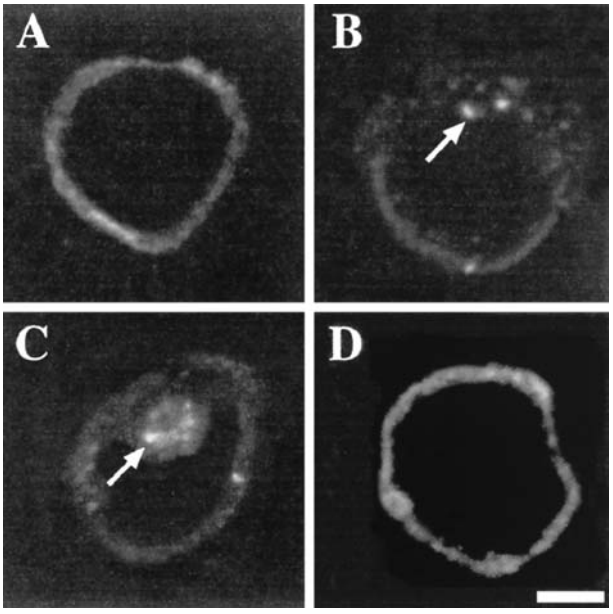

Figure 5. Changes in hNOP receptor cell distribution evaluated by confocal immunofluorescence microscopy. Transiently transfected HA-tagged CHO-hNOP cells were incubated without (control; *A*) or with the agonist NC (1 μM) at 37 °C for 5 min (*B*) or 10 min (*C*). Alternatively, the cells were exposed to 1 μM NC for 10 min; then the cells were thoroughly washed and incubated in fresh medium for 60 min (*D*). In the absence of the agonist, receptors reside at the plasma membrane. Agonist-induced internalization of receptors occurred rapidly at 37 °C. The internalization event is reversible, as incubation of cells in fresh medium resulted in recycling of hNOP receptors to the plasma membrane. After fixation with formaldehyde and permeabilization with saponin, hNOP receptors were visualized using a mouse monoclonal anti-HA antibody and then with a fluorescein-conjugated goat anti-mouse secondary antibody. The white arrows indicate examples of intracellular localization of the hNOP receptor. Only cells with detectable fluorescence were included. Data are representative of three different experiments in which 20–25 cells/experimental group were evaluated (bar, 5 μm).

not raise this percentage. Okadaic acid (10 nM) abolished the recovery (fig. 4). Finally, to evaluate if de novo synthesis of hNOP receptors may contribute to recovery of cell surface receptors following internalization, CHO-hNOP cells were exposed to NC (1 μM) for 30 min, followed by 120 min of recovery in the presence of 70 μM cycloheximide, an inhibitor of protein synthesis [2]. In these conditions, hNOP receptors recycled to the cell surface (fig. 4). In preliminary experiments, we ascertained that exposure to 70 μM cycloheximide for 2 h did not modify the number of hNOP receptors expressed on the cell surface or NC-induced internalization (data not shown).

hNOP receptor internalization – confocal microscopy of HA-tagged CHO-hNOP cells

To obtain direct information about internalization and recycling of the hNOP receptor, changes in intracellular receptor distribution were evaluated by confocal microscopy of CHO-K1 cells transiently transfected with an epitope-tagged receptor. The nine-amino-acid HA epitope was inserted into the N terminus of the cDNA coding for the hNOP receptor subcloned in the pCDNA 3 vector which was then used to transfect CHO-K1 cells (see Materials and methods). In preliminary experiments, HA-tagged CHO-hNOP cells were shown to have a similar pharmacological profile as the CHO-hNOP cells. [^{3}H]-NC binding to these cells (these experiments were carried out as described in Materials and methods) was saturable and the K_d was 0.90 ± 0.11 nM (n = 3). Moreover, exposure of HA-tagged CHO-hNOP cells for 5, 10 and 30 min to NC (1 μM at 37 °C) promoted internalization of the hNOP receptor (evaluated by loss of cell surface receptors for the hydrophilic ligand [^{3}H]-NC) similar to that previously described for the CHO-hNOP cells (data not shown).

In untreated cells (control), confocal microscopy revealed fluorescence almost exclusively restricted to the cell surface, suggesting that most of the receptors were localized at the plasma membrane (fig. 5A). Exposure to NC (1 μM) induced a rapid redistribution of hNOP receptors. In cells exposed to NC for 5 min, several receptors were localized inside the cell with a punctuate distribution in submembrane regions (fig. 5B). After 10 min exposure to this agonist, hNOP receptors were localized in structures distributed throughout the cytoplasm (fig. 5C). After exposure to 1 μM NC for 10 min, the cells were extensively washed with acidic ice-cold PBS to remove the peptide and then incubated in fresh cell culture medium for 60 min at 37 °C to allow recycling of the receptors to the cell surface. In these conditions, receptor

internalization appeared to be reversible and, as shown in figure 5D, the fluorescence was mainly localized at the cell surface, a feature indicating that hNOP receptors were again present only on the plasma membrane.

β-Arrestin expression in CHO-K1 and SK-N-BE cells

β-Arrestin 1 and 2 are ubiquitously expressed in all cell types, although in various proportions [21, 22] and play a highly conserved role in promoting endocytosis of various GPCRs [23]. In a previous study [17], we found that over-expression of β-arrestin 2 increased NC-promoted internalization of the hNOP receptor expressed in SK-N-BE cells. This finding suggests that NOP receptor internalization could be related, at least partially, to the cellular expression of β-arrestins. To confirm this hypothesis, we determined the relative expression of β-arrestin 1 and 2 in CHO-K1 and SK-N-BE cells by Western blot analysis. As shown in figure 6, both isoforms were detected in these cells; however, CHO-K1 cells contained higher levels of the two arrestins. Densitometric analysis of the immunoblotted bands showed that β-arrestin 1 was ≈1.7-fold higher in CHO-K1 than in SK-N-BE cells (7.5 ± 0.3 vs. 4.3 ± 0.4 arbitrary units of optical density, n = 4, $p < 0.01$) whereas β-arrestin 2 was ≈2.0-fold higher in CHO-K1 than in SK-N-BE cells (16.3 ± 0.5 vs. 7.8 ± 0.6 arbitrary units of optical density, n = 4, $p < 0.01$).

Functional activity of the hNOP receptor expressed in CHO-K1 cells

To evaluate whether the transfected hNOP receptor was functional, coupling to adenylyl cyclase was assessed by measuring the inhibition of forskolin-stimulated cAMP accumulation following receptor activation (fig. 7). In control CHO-K1 cells, NC (up to 10 µM) had no effect, confirming the absence of endogenously expressed functional hNOP receptors (data not shown). NC inhibited, in a concentration-related manner, cAMP accumulation induced by forskolin. The antagonist Nph (1 µM) induced a parallel rightwards shift in the NC concentration-response curve with no change in the maximal effect (fig. 7), consistent with competitive antagonism (EC_{50} shifted from 5.46 to 92.55 nM).

Time-dependent loss of the agonist-induced inhibition of forskolin-induced cAMP accumulation upon NC pretreatment

The significance of receptor internalization and recycling on the rate of hNOP receptor desensitization was investigated in a time course study reported in figure 8. A decrease in NC inhibition of cAMP production by forskolin was observed in cells pretreated with 1 µM NC (fig. 8). After 10 and 20 min of NC exposure, an initial and significant desensitization of the hNOP receptor was shown (this effect could be related to the rapid internalization of the receptor; see fig. 3). However, in cells pretreated with NC for 30 and 45 min, NC regained the ability to inhibit forskolin-induced cAMP accumulation; this action, in fact, was not significantly different from that of cells not previously exposed to this peptide (fig. 8), thus indicating that significant receptor desensitization was absent (fig. 8). A more prolonged exposure to NC (60 and 120 min) induced, again, a significant decrement in the inhibitory effect of NC upon forskolin-stimulated cAMP production

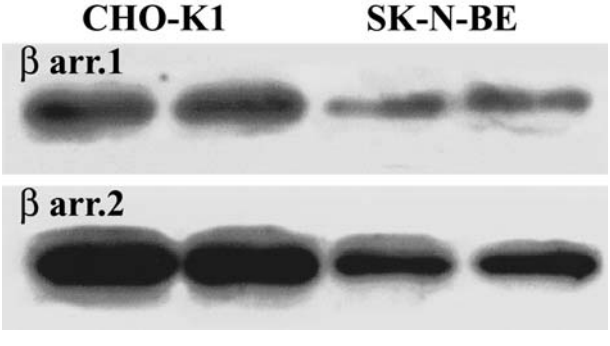

Fig. 6. Expression of β-arrestin 1 and β-arrestin 2 in CHO-K1 and SK-N-BE cells. Sixty micrograms of total cellular protein was resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose. Representative autoradiograms of two different samples, demonstrating the migration position of β-arrestin 1 (≈50 kDa [see ref. 22]) and of β-arrestin 2 (≈45 kDa [see ref. 22]) in both cell lines are shown. The apparent molecular weight (kDa) of the bands was determined by comparison with a molecular-weight standard (Santa Cruz No. sc-2035) run in the same gel. These blots are representative of four independent experiments, all of which gave identical results. In agreement with a previous study [59], more β-arrestin 2 than β-arrestin 1 was found in CHO-K1 cells.

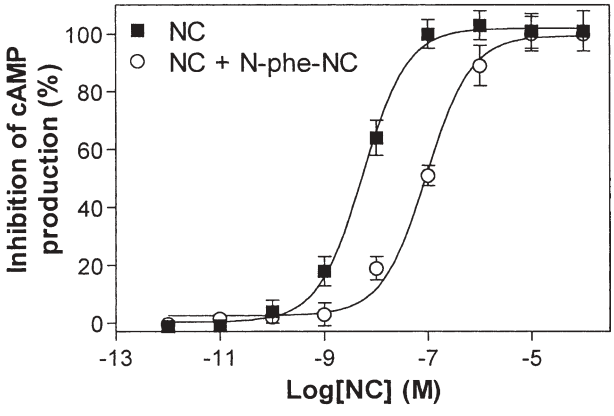

Figure 7. NC inhibits forskolin-stimulated cAMP formation in whole CHO-hNOP cells in the presence or absence of the competitive antagonist Nph (1 µM). cAMP levels were evaluated as described in Materials and methods. Each value (percentage inhibition) is the mean ± SE of four experiments done in triplicate. In these experimental conditions, forskolin-stimulated cAMP production in control cells was 181 ± 6 pmol/mg protein (n = 12); maximal inhibition of forskolin-stimulated cAMP production by 10^{-5} M NC was 29 ± 5 pmol/mg protein (n = 12) and basal production of cAMP in untreated cells was 17 ± 3 pmol/mg protein (n = 12).

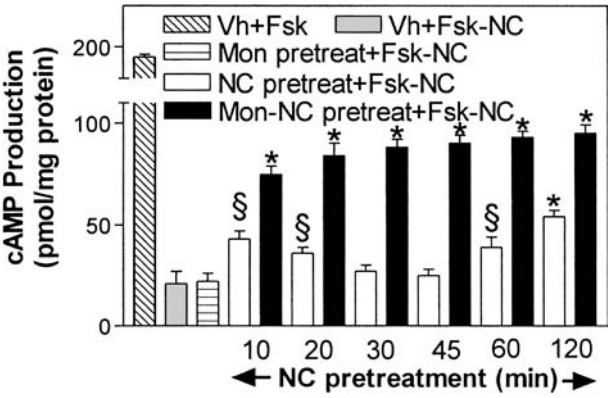

Figure 8. Receptor recycling and hNOP receptor desensitization in CHO-hNOP cells in the presence or absence of monensin. CHO-hNOP cells were pretreated with 1 μM NC for various periods of time as indicated; then, the culture medium was removed, the cells were washed with PBS, and the ability of 1 μM NC to inhibit forskolin-stimulated cAMP production was determined as described in Materials and methods. When the effect of 50 μM monensin was measured, the cells were pretreated with this compound 1 h before addition of 1 μM NC (Mon-NC pretreat + Fsk-NC). Control cells were not pretreated with NC and were exposed to forskolin in the presence of 1 μM NC (Vh + Fsk-NC). A group of control cells was exposed only to monensin; then, the cells were treated with forskolin in the presence of 1 μM NC (Mon pretreat + Fsk-NC). Forskolin-stimulated cAMP production in control cells (Vh + Fsk) is also shown. Each value is the mean ± SE of four experiments done in triplicate; $^{*}p < 0.01$, $^{\S}p < 0.05$ in comparison with control cells not pretreated with NC (Vh + Fsk-NC) or in comparison with cells pretreated with monensin alone (Mon pretreat + Fsk-NC) (Dunnett's test after ANOVA).

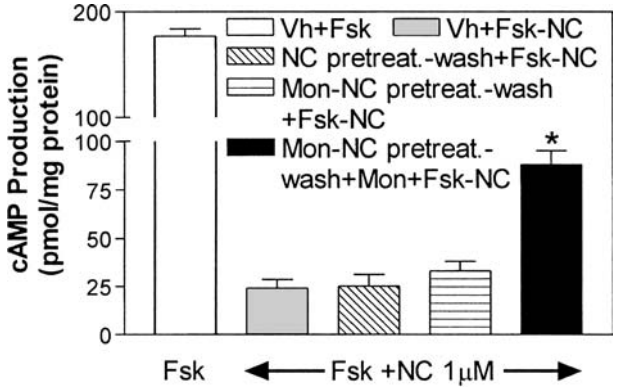

Figure 9. Effect of monensin on the desensitization and resensitization of the hNOP receptor expressed in CHO-K1 cells. Cells were exposed or not to monensin (50 μM) for 1 h followed by 1 μM NC for 30 min; then the cells were washed three times with acid ice-cold PBS (pH 6.5) to remove NC and monensin. After washing, the cells were incubated in fresh medium at 37 °C for an additional 30 min and the ability of 1 μM NC to inhibit forskolin-stimulated cAMP production was then measured. Five different treatments were done: (i) cells were incubated in culture medium alone and then treated with forskolin (Vh + Fsk); (ii) cells were exposed to culture medium alone and treated with forskolin and NC (Fh + Fsk-NC); (iii) cells were exposed to NC and, after washing and incubation in fresh medium for 30 min, treated with forskolin and NC (NC pretreat.-wash + Fsk-NC); (iv) cells were exposed to monensin and NC and, after washing and incubation in fresh medium for 30 min, treated with forskolin and NC (Mon-NC pretreat.-wash + Fsk-NC); (v) cells were exposed to monensin and NC and, after washing and incubation in the presence of monensin for 30 min, treated with forskolin and NC (Mon-NC pretreat.-wash + Mon + Fsk-NC). Each value is the mean ± SE of four experiments done in triplicate. $^{*}p < 0.01$ in comparison with cells exposed to monensin and NC and, after washing and incubation in fresh medium for 30 min, treated with forskolin and NC (Mon-NC pretreat.-wash + Fsk-NC) (Dunnett's test after ANOVA).

(fig. 8). When NC pretreatment was done in the presence of monensin, the rate of hNOP receptor desensitization was greatly increased in comparison to that observed in cells exposed to NC alone. This pronounced desensitization was rapid and slightly increased over exposure time to NC (fig. 8).

To investigate any receptor resensitization in CHO-hNOP cells exposed to monensin and NC for 30 min, the cells were extensively washed to remove these compounds and incubated in fresh medium for 30 min; then, the ability of 1 μM NC to inhibit forskolin-stimulated cAMP production was measured. Cells, treated as described above, regained the ability to inhibit forskolin-induced cAMP accumulation as well as control cells which were not exposed to monensin and NC (fig. 9). In contrast, in CHO-hNOP cells exposed to monensin and NC and then incubated for 30 min in fresh medium containing monensin, the ability of NC to inhibit forskolin-stimulated cAMP production showed a minimal recovery (fig. 9).

These results indicate that the removal of monensin allows the recycling of the internalized receptor to the cell surface of CHO-hNOP cells and NC is again capable of regulating adenylyl cyclase activity.

Discussion

We examined the internalization and recycling events of the cloned hNOP receptor expressed in CHO-K1 cells. We employed the hydrophilic peptide NC to measure the receptors on the cell surface of intact cells by a binding assay and found that NC induced a time- and concentration-dependent internalization of the hNOP receptor. The number of receptors on the cell surface has been proposed as critical for the overall activities of GPCRs [2, 8, 21]; when cloned receptors are expressed in host cells, an artificial over-expression of receptors is often achieved and this may affect agonist-induced receptor endocytosis [2–4]. Therefore, in this study, we selected a clone of CHO-hNOP cells expressing as low a density of hNOP receptors as found in neuronal cells [17]. However, hNOP internalization was more rapid and marked in CHO-K1 cells (≈ 78% of cell surface receptors are lost after 2-min exposure to 1 μM NC) than in neuroblastoma cells (≈ 57% of cell surface receptors are lost after 30 min exposure to 1 μM NC) [17]. This difference could be attrib-

utable to cellular factors like β arrestins or G protein kinases which may be differentially expressed in different cell lines [1, 2, 5, 22–24]. In agreement with this idea, we found higher levels of β-arrestin isoforms in CHO-K1 than in SK-N-BE cells.

NC-induced internalization is significantly reduced by the peptide Nph, a selective NOP receptor antagonist [13], which by itself does not affect receptor internalization. Thus, this compound may offer a useful tool for investigating cellular events triggered by this receptor. NOP receptor internalization is prevented by low temperature and hyperosmolar sucrose which inhibit agonist-mediated receptor endocytosis [4]. Receptor internalization through clathrin-coated pits is blocked by hypertonic external solutions due to an abnormal formation of microcages of clathrin into normal coated pits resulting in inhibition of endocytosis [25].

We found that okadaic acid, a selective inhibitor of protein phosphatases type 1 (PP1) and 2A (PP2A) [26] suppresses hNOP receptor recycling to the plasma membrane, probably by blockade of dephosphorylation of the internalized receptor. In fact, the current idea is that acidification within the endosome leads to dissociation of the ligand from its receptor and this process facilitates dephosphorylation of the receptor by neutral phosphatases (most likely PP2A) [27, 28]. Moreover, hNOP receptor recycling is not affected by cycloheximide which prevents the delivery of receptors to the cell surface due to new synthesis [4].

The present study reports that the hNOP receptor does internalize and recycle to the cell surface of CHO-hNOP cells. The decrease in the rate of recycle of cell surface receptors in the presence of monensin supports the hypothesis of the contribution of Golgi transport in maintaining the steady state of the membrane receptor pool [4]. Recycling of hNOP receptors to the cell surface occurs in the continued presence of the agonist as shown in our measurements of the amounts of cell surface receptor following NC exposure. Recycling is generally thought to occur after the agonist has been washed out, but there is evidence for GPCR recycling back to the plasma membrane both in the presence and absence of agonist [29, 30]. In our experiments, we could not determine whether the agonist NC is recycled with the receptor back to the cell surface or whether it is degraded in lysosomes [9, 31].

Endocytosis and recycling properties of the hNOP receptor were also investigated by immunofluorescence confocal microscopy [4], in order to visualize the internalization of an epitope (HA)-tagged receptor in transfected CHO-K1 cells. This approach allowed us to confirm that hNOP receptors undergo agonist-induced endocytosis, associated with receptor redistribution within the plasma membrane and in the cytoplasm. These receptors do not seem to be rapidly proteolyzed after agonist-induced endocytosis. On the contrary, they are partially recycled back to the plasma membrane. Several recent studies have identified GPCR-interacting proteins that specify the preferential sorting of GPCRs for either recycling or intracellular degradation as they interact with specific sequences located in the cytoplasmatic domain of GPCRs, especially in the carboxyl terminus [32]. For example, interaction of the β_2-adrenergic receptor with N-ethylmaleimide-sensitive factor-1 is important for intracellular membrane trafficking of this receptor [33]. Recently, Whistler et al. [34] identified a protein defined GPCR-associated sorting protein that plays a key role in the lysosomal sorting and functional down-regulation of δ opioid receptor and probably of other GPCRs, whereas this protein does not bind to the μ opioid receptor that is rapidly recycled back to the plasma membrane. Therefore, the hNOP receptor seems to behave like the μ opioid receptor and other GPCRs which, even in the continuous presence of the agonist, are recycled back to the cell surface [35, 36]. Further studies aimed to characterize any interaction of the hNOP receptor with the above-mentioned proteins will allow us to better understand the recycling and the intracellular signalling of this receptor.

In the present study, we showed that hNOP receptors undergo rapid desensitization upon agonist challenge. NC efficacy to inhibit forskolin-stimulated cAMP production is significantly reduced 10 min after exposure to 1 μM NC and it correlates with the rate of hNOP receptor internalization; however, desensitization is absent 30 min after exposure to NC when partial recycling of the hNOP receptor to the cell surface is detected (see fig. 2). Moreover, we observed that the blockade of hNOP receptor recycling in the presence of monensin would cause a more prolonged and relevant desensitization of this receptor. In CHO-hNOP cells, after short exposure to NC, internalization and desensitization of the hNOP receptor are reversible after removal of the agonist.

These data are in agreement with previous studies showing that a brief NC pretreatment may desensitize the inhibitory cAMP response of native NOP receptors expressed at a low density in neuroblastoma-derived cell lines [37–40]. Recently, Dautzenberg et al. [41] found that a novel, non-peptide NOP receptor agonist may desensitize and reduce the number of NOP receptors on the surface of HEK 293 cells. Moreover, application of NC has been reported to elicit homologous desensitization of NC-mediated Ca^{2+} channel inhibition in NG 108-15 cells [42] and in freshly dissociated hippocampal neurons [43]. In contrast, Hawes et al. [44] have found that NC pretreatment reduces, in CHO cells expressing the hNOP receptor, NC-induced mitogen-activated protein kinase activity but not inhibition of cAMP production by forskolin. These discrepant data cannot be clearly explained; however, they could be related to the different expression level of the hNOP receptor in this cell line. Hawes and colleagues, in fact, used CHO cells expressing threefold

more hNOP receptors than ours. Interestingly, as regards other GPCRs, Law et al. [8, 21] have elegantly demonstrated that a rapid desensitization of μ and δ opioid receptors heterologously expressed in cell lines cannot be observed unless the receptor level has been decreased to a critical level. Moreover, Hawes and colleagues exposed these cells to a low concentration of NC (10 nM) which, according to our study, causes only a modest internalization of hNOP receptors and could be insufficient to significantly desensitize this receptor.

We observed that a more prolonged exposure to NC (60 and 120 min) will result in a significant desensitization of the receptor and this event could also be due to receptor down-regulation. This latter process has been investigated in other GPCRs and may involve cellular mechanisms other than that regulating receptor internalization: enhanced degradation of the receptor [45–47], a decrease in the stability of receptor mRNA and a corresponding reduction in de novo receptor synthesis [for a review see ref. 48].

Desensitization analysis of the hNOP receptor is still in its infancy [37–44], whereas desensitization of opioid [8, 21, 49–51] and other neuropeptide [9, 31, 52] receptors has been very widely described in in vitro studies. Pretreatment with different opioid agonists may decrease the ability of a further agonist challenge to inhibit forskolin-induced cAMP accumulation, consistent with an agonist-dependent desensitization of the adenylyl cyclase responses [1]. The relationship between G protein activation and receptor internalization in GPCRs is unclear and appears to vary among receptors. Receptors are generally internalized in response to agonist binding [1], although antagonists or antibodies can trigger internalization of some receptors [53–55]. Studies on β-adrenergic and muscarinic receptors have demonstrated that partial agonists cause less internalization than full agonists and the amount of receptor internalization correlates with coupling efficacy [56–58].

From extensive studies on other GPCRs, desensitization has been shown to involve at least receptor phosphorylation and a decrease in the number of receptors at plasma membrane following internalization and, eventually, degradation in lysosomes [1]. It is tempting to correlate hNOP receptor desensitization with the kinetics of receptor internalization. Indeed, internalization of receptors has been suggested to constitute the major mechanism for desensitization of somatostatin receptors [52]. On the other hand, rapid desensitization of the δ opioid receptor involves multiple pathways, including agonist-induced receptor phosphorylation and rapid internalization [21]. However, receptor phosphorylation is not necessary for agonist-induced δ opioid receptor internalization and association of arrestin with the non-phosphorylated δ opioid receptor has been suggested to trigger receptor internalization and uncoupling of the G protein from the receptor [21]. Agonist-induced rapid desensitization of the δ opioid receptor is abolished only when both of these processes are blocked [21]. Although desensitization and internalization of the hNOP receptor could be two separate phenomena, the rapid loss of cell surface receptors might account for the rapid functional desensitization that we observed. This supports the idea that rapid receptor internalization represents a physiological adaptative mechanism preventing cellular over-stimulation. Therefore, the dynamic cycle between hNOP receptor activation, internalization and recycling determines the activity of this receptor on the cell surface. Further studies are in progress to ascertain the role played by receptor phosphorylation in desensitization and internalization events of the hNOP receptor.

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