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Agonist-specific down-regulation of the human δ-opioid receptor

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Abstract

Down-regulation of the δ-opioid receptor contributes to the development of tolerance to δ-opioid receptor agonists. The involvement of the carboxy terminus of the mouse δ-opioid receptor in peptide agonist-mediated down-regulation has been established. In the present study, we examined the down-regulation of the truncated human δ-opioid receptor by structurally distinct δ-opioid receptor agonists. Chinese hamster ovary (CHO) cells, expressing the full-length or truncated epitope-tagged human δ-opioid receptors were incubated with various δ-opioid receptor agonists (100 nM, 24 h), and membrane receptor levels were determined by [^{3}H]naltrindole saturation binding. Each δ-opioid receptor agonist tested down-regulated the full-length receptor. Truncation of the carboxy terminus abolished down-regulation by all δ-opioid receptor agonists, except SNC80 ((+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]*N*,*N*-diethylbenzamide). In addition, truncation of the C-terminus completely attenuated [D-Pen^2-D-Pen^5]enkephalin (DPDPE), but not SNC80-mediated [^{32}P] incorporation into the protein immunoreactive with an anti-epitope-tagged antibody. These findings suggest that SNC80-mediated phosphorylation and down-regulation of the human δ-opioid receptor involves other receptor domains in addition to the carboxy terminus. Pertussis toxin treatment did not block SNC80-mediated down-regulation of the truncated Et-hDOR, indicating that the down-regulation is independent of $G_{i/o}$ protein activation and subsequent downstream signaling.

Keywords: δ-Opioid receptor, human; Down-regulation; Agonist-directed trafficking; Phosphorylation; Pertussis toxin

1. Introduction

Opioid agonists acting at the δ-opioid receptor have strong antinociceptive activity with relatively weak side effects (Rapaka and Porreca, 1991). For this reason, δ-opioid receptor agonists are attractive pharmacological agents for the clinical control of pain. A major complication of using agonists as therapeutic agents is that chronically stimulated receptors become less responsive to the agonist (desensitization). This loss of receptor responsiveness is manifested as drug tolerance, requiring increased doses of the drug over time in order to maintain the same effect.

Desensitization of agonist-mediated receptor signaling is a complex process (Bohm et al., 1997). Within seconds to minutes of agonist binding, the functional response is reduced, with minimal reduction of the cell surface expression of receptors (uncoupling). Agonist binding also promotes rapid (minutes) translocation of the receptors to membrane compartments inaccessible to ligand (sequestration) and into intracellular vesicles (internalization). In the continued presence of agonist, generally over a period of hours, the total cellular receptor concentration declines (down-regulation). It is likely that δ-opioid receptor down-regulation contributes to tolerance since chronic δ-opioid receptor agonist treatment was shown to reduce receptor levels in rodent brain (Tao et al., 1988; Zhao and Bhargava,

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1997). Better understanding of the mechanism of the down-regulation of the human delta opioid receptor should lead to the development of opioid analgesics that produce limited analgesic tolerance.

The carboxy terminal tail of the opioid receptors was shown to have a critical role in receptor desensitization and internalization. Truncation of the last 15 or 37 residues of the carboxy terminus of the mouse δ-opioid receptor caused complete blockade of [D-Pen^2-D-Pen^5]enkephalin (DPDPE)-mediated internalization in CHO cells (Trapaidze et al., 1996). The importance of the phosphorylation sites in the carboxyl terminal tail in [D-Ala^2,D-Leu^5]enkephalin-mediated down-regulation of the mouse δ-opioid receptor has been demonstrated (Cvejic et al., 1996). The role of hierarchical phosphorylation of Ser/Thr residues in the C-terminal tail of the mouse δ-opioid receptor in DPDPE-mediated desensitization and internalization has recently been shown, with Ser^{363} being the critical primary phosphorylation site (Maestri-El Kouhen et al., 2000). Recent data, however, indicate that in addition to the cytoplasmic tail, other intracellular domains may also be involved in the homologous regulation of the δ-opioid receptors (Murray et al., 1998; Kramer et al., 2000; Lowe et al., 2002). Furthermore, it is not clear whether the molecular mechanism of receptor down-regulation is the same for all, structurally different, δ-opioid receptor agonists.

We have shown recently (Okura et al., 2000) (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]*N*,*N*-diethylbenzamide (SNC80), a nonpeptidic δ-opioid receptor agonist, is still able to down-regulate the human δ-opioid receptor truncated at Gly^{338} (trunchDOR) in recombinant CHO cells (Okura et al., 2000). In this study, we have examined down-regulation of the truncated human δ-opioid receptor by other structurally distinct δ-opioid receptor agonists that activate the human δ-opioid receptor with various efficacies. We also investigated whether agonist-specific differences also exist in agonist-mediated phosphorylation of the truncated human δ-opioid receptor. Finally, we have tested whether second messenger regulated protein kinases are involved in SNC80-mediated down-regulation of the truncated human δ-opioid receptor by measuring receptor down-regulation after uncoupling the receptor from $G_{i/o}$ proteins by pertussis toxin treatment.

2. Materials and methods

2.1. Drugs

The structures of peptide and nonpeptide δ-opioid receptor-agonists used in this study are shown in Fig. 1. SNC80 ((+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]*N*,*N*-diethylbenzamide) and (−)TAN67 ((−)2-methyl-4*a*-(3-hydroxyphenyl)-1, 2, 3, 4, 4*a*, 5, 12, 12*a*-octahydro-quinolino[2, 3, 3-*g*]isoquinoline) were synthesized at the National Institute of Health (Bethesda, MD) and Toray Industry (Kamakura, Japan), respectively. DPDPE and pCl-DPDPE were synthesized in the laboratory of Victor J. Hruby, as previously described (Toth et al., 1990). Naltrexone was purchased from Sigma (St. Louis, MO). [^{3}H]naltrindole (30.5 Ci/mmol) and [^{32}P]orthophosphate (3000 Ci/mmol) were purchased from DuPont NEN (Boston, MA). All other compounds were obtained from commercial sources.

2.2. Cell culture and cell treatment

The full-length human δ-opioid receptor with a mutated stop codon, and truncated human δ-opioid receptor missing 34 carboxyl-terminal amino acids after Gly^{338}, were ligated

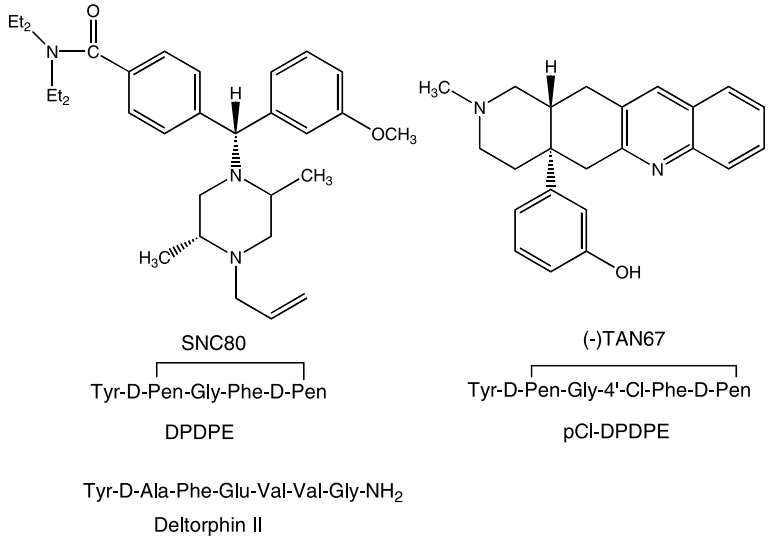

Fig. 1. The chemical structures of the δ-opioid receptor agonists used in the present study.

in-frame into the pcDNA3.1/Myc-His mammalian expression vector to create the eptitope-tagged wild-type (Et-hDOR) and tail-truncated (TruncEt-hDOR) human δ-opioid receptor constructs. The structures of the constructs are shown in Fig. 2. The mutants were stably transfected into Chinese hamster ovary (CHO) cells to create the previously described Et-hDOR/CHO and TruncEt-hDOR/CHO cell lines (Okura et al., 2000). Transfected CHO cells were grown in HAM's-F12 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and 500 μg/ml G418. Cells were grown at 37 °C under 5% CO_2 atmosphere. The transfected cells were grown to approximately 90% confluence, washed with phosphate buffer and pretreated with Iscove's Modified Dulbecco's Medium (IMDM) in the absence or presence of δ-opioid receptor agonists, SNC80 (100 or 500 nM), DPDPE (100 or 500 nM), pCl-DPDPE (100 nM), TAN67 (100 nM) or deltorphin II (100 nM) for 24 h. Pertussis toxin treatment was carried out for 24 h, by the addition of pertussis toxin (50 ng/ml), with or without 500 nM SNC80 in the last 12 h of the treatment.

2.3. Membrane preparations

After appropriate pretreatment, the cells were gently rinsed three times (15 min each, 37 °C) with fresh IMDM solutions. The cells were detached in 5.0 mM EDTA–phosphate-buffered saline solution. The harvested cells were homogenized in 10 ml Tris–Mg buffer (50 mM Tris–HCl, 5.0 mM $MgCl_2$, pH 7,4) using a Teflon-glass tissue grinder. The homogenates were centrifuged at 40,000*g* for 10 min. The pellet was resuspended in ice-cold Tris–Mg buffer and centrifuged again at 40,000*g* for 10 min. The final pellet was resuspended in Tris–Mg buffer for the binding assay.

2.4. Radioligand binding studies

Radioligand binding studies with [^{3}H]naltrindole were performed as previously described (Knapp et al., 1994). Briefly, for saturation binding experiments, cell membranes were incubated with [^{3}H]naltrindole (0.03–0.5 nM) for 90 min at 30 °C in Tris–Mg buffer (pH 7.4) containing 1.0 mg/ml bovine serum albumin, 50 μg/ml bacitracin, 30 μM bestatin, 10 μM captopril and 0.1 mM phenylmethylsulfonyl fluoride. The reaction was terminated by rapid filtration through Whatman GF/B glass fiber filters. The filters were rinsed three times with 4 ml ice-cold buffer. Membrane-bound radioactivity was measured in a liquid scintillation counter. Nonspecific binding was determined in the presence of 10 μM naltrexone. All assays were conducted in duplicate. Protein concentrations were measured by the method of Lowry et al. (1951). Inhibition of [^{3}H]NTI binding by SNC80 and DPDPE was also measured to determine the K_i values of the agonists in Et-hDOR/CHO and TruncEt-hDOR/CHO cell membranes. Radioligand binding inhibition experiments were performed as previously described (Knapp et al., 1994).

2.5. Metabolic [^{32}P]labeling and immunoprecipitation

CHO cells, expressing the full-length or the truncated Et-hDORs, were phosphate-starved by incubating in phosphate-free Dulbecco's modified Eagle's medium (DMEM) including 5% dialyzed fetal calf serum for 2 h. Following metabolic labeling with 200 μCi/ml [^{32}P]orthophosphate for 1 h, the cells were incubated with SNC80 or DPDPE (500 nM) for a further 1 h. The cells were scraped into ice-cold homogenization buffer (50 mM Tris, 250 mM sucrose, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaF, 10 mM Na-pyrophosphate and 10 μl/ml protease inhibitor cocktail, 0.2 mM Na-orthovanadate and 100 nM okadaic acid, pH 7.4). After centrifugation, cells were resuspended in a solubilization buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% Igepal CA-630, 0.5% Triton X-100. 0.2% digitonin, 5 mM EDTA, 10 mM NaF, 10 mM β-glycerol-phosphate and 10 μl/ml protease inhibitor cocktail, 0.2 mM Na-orthovanadate and 100 nM okadaic acid, pH 7.4) and incubated on ice for 4 h. The lysates were precleared by incubation with 1 μg goat IgG and 10 μl protein G-agarose slurry (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h on ice. Protein content of the lysate was measured by the Bradford method and aliquots of the lysates were incubated on ice with 10 μl Anti-myc antibody (Invitrogen, La Jolla, CA) and protein G-agarose overnight. The immunoprecipitates were thoroughly washed (three times, 15-min incubation each) in wash buffer (solubilization buffer with the concentration of the detergents reduced to 0.075% Triton X-100, 0.05% Igepal CA-630 and 0.1% digitonin) at 4 °C. The final pellet was boiled in 15 μl Laemmli sample buffer for 5 min and resolved by sodium

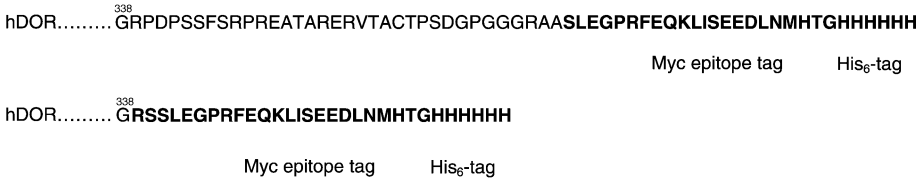

Fig. 2. Amino acid sequences of the C-termini of the full-length and the truncated epitope-tagged hDOR constructs. Bold fonts indicate amino acids derived from the pcDNA3.1Myc/His vector.

dodecylsulphate polyacrylamide (10%) gel electrophoresis (SDS-PAGE). Relative protein concentrations were determined by scanning densitometry of the silver-stained gel. [^{32}P] incorporation into the immunoreactive band was determined by autoradiography followed by scanning densitometry of the film. Receptor phosphorylation was quantified as a ratio between the densities of the silver-stained band and the autoradiography band.

2.6. Data analysis and statistics

The apparent dissociation constant (K_d) of [^{3}H]naltrindole and the maximal number of binding sites (B_{max}) were estimated by nonlinear regression analysis using the Prism Ver. 2 software (GraphPad, San Diego, CA). Statistical significance was determined by one-way analysis of variance (ANOVA) and Newman–Keuls tests. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Characteristics of [^{3}H]naltrindole-specific binding to CHO cell membranes expressing the epitope-tagged full-length or truncated human δ-opioid receptors

[^{3}H]naltrindole-specific binding was saturable in both Et-hDOR/CHO and TruncEt-hDOR/CHO cell membranes. Rosenthal transformation of the data revealed a single population of binding sites (Fig. 3, filled circles) with B_{max} values of 1410 ± 190 and 153 ± 15 fmol/mg protein and K_d values of 117 ± 6 and 86 ± 17 pM (means ± S.E., $n = 4$) in Et-hDOR/CHO and TruncEt-hDOR/CHO cell membranes, respectively.

3.2. Down-regulation of the full-length and truncated Et-hDORs by different δ-opioid receptor agonists

Inhibition of [^{3}H]NTI binding by SNC80 and DPDPE was used to ascertain that epitope tagging and/or truncation did not interfere with agonist binding to the hDOR. The K_i values of SNC80 and DPDPE were 0.5 and 1.7 nM in Et-hDOR/CHO and 0.7 and 3.0 nM in TruncEt-hDOR/CHO cell membranes (data not shown). The K_i values of SNC80 and DPDPE at the mutant receptors were similar to their K_i

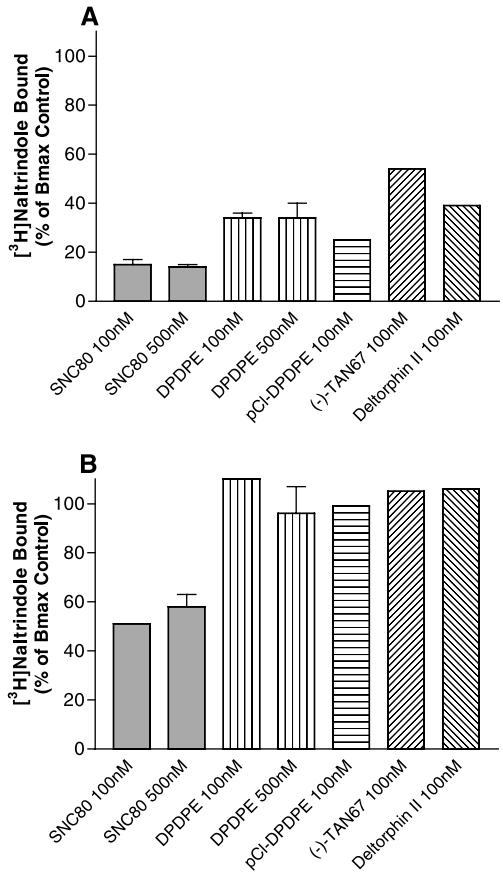

Fig. 4. Effect of chronic δ-opioid agonist pretreatment on the B_{max} values of [^{3}H]naltrindole in (A) Et-hDOR/CHO and (B) TruncEt-hDOR/CHO cell membranes. Cells were preincubated with SNC80 (100 or 500 nM), DPDPE (100 or 500 nM) or *p*Cl-DPDPE, (−)TAN67 or deltorphin II (100 nM) for 24 h. After extensive washing, cell membranes were prepared and saturation studies were performed using different concentrations of [^{3}H]naltrindole (0.03–0.5 nM). The bars represent the means of the calculated B_{max} values as percentage of B_{max} in control cells, treated with IMDM only.

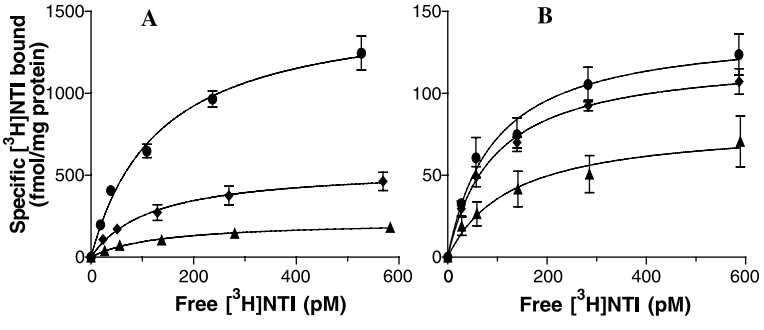

Fig. 3. Truncation of the C terminal tail of the human δ-opioid receptor attenuates DPDPE-, but not SNC 80-mediated receptor down-regulation in CHO cells. Saturation isotherms for [^{3}H]naltrindole binding to (A) Et-hDOR/CHO and (B) TruncEt-hDOR/CHO cell membranes after pretreatment with SNC 80 (▲) or DPDPE (♦) (500 nM, 24 h). The control cells (●) were incubated in IMDM alone for 24 h.

values at the untagged, wild-type human δ-opioid receptor (Malatynska et al., 1996). In addition, we have also verified that both DPDPE and SNC80 were able to stimulate [^{35}S]GTPγS binding with high potencies in both Et-hDOR/CHO and TruncEt-hDOR/CHO cell membranes (data not shown).

Treatment of the CHO cells expressing full-length Et-hDOR with SNC80 or DPDPE (100 nM for 24 h) significantly decreased the B_{max} of [^{3}H]naltrindole-specific binding (by 85% and 66%, respectively), with no change in the K_d values (Fig. 3A). Treatment of the cells with 500 nM of SNC80 or DPDPE caused a similar decrease in [^{3}H]naltrindole-specific binding (B_{max} values: 15% and 34% of untreated control, respectively) (Fig. 4A). Treatment of Et-hDOR cells with 100 nM of pCl-DPDPE, (−)TAN67 and deltorphin II reduced the B_{max} values by 75%, 46% and 61%, respectively (Fig. 4A).

On the other hand, treatment of CHO cells expressing the truncated Et-hDOR with DPDPE (100 or 500 nM), pCl-DPDPE (100 nM), (−)TAN67 (100 nM) and deltorphin II (100 nM) did not lead to significant reduction of [^{3}H]naltrindole-specific binding (Figs. 3B and 4B). Conversely, pretreatment of TruncEt-hDOR/CHO cells with SNC80 still caused significant receptor down-regulation (Fig. 3B). The B_{max} value of [^{3}H]naltrindole-specific binding in TruncEt-hDOR/CHO cell membranes was reduced by 49% and 42% after pretreatment with 100 and 500 nM SNC80, respectively (Fig 4B).

3.3. Phosphorylation of the full-length and truncated Et-hDOR

After metabolic [^{32}P] labeling and immunoprecipitation with an anti-myc antibody, three protein bands were obtained in silver-stained polyacrylamide gels with apparent molecular weights of approximately 28, 54 and 65–70 kDa. The 28- and the 54-kDa bands correspond to the light and heavy chains, respectively, of the antibody and accordingly are not visible on the autoradiography film. Only the 65–70-kDa protein band was apparent after [^{32}P] autoradiography. A single protein band with similar molecular weight was isolated from Et-hDOR/CHO cell lysates using metal affinity chromatography (data not shown). Since the Talon™ immobilized cobalt affinity resin (Clontech, Palo Alto, CA) specifically isolates polyhistidine-tagged recombinant proteins, this result supports the identity of the 65–70-kDa protein band as a polyhistidine-tagged human δ-opioid receptor. Phosphorylation of the immunoreactive band was quantified by calculating the ratio between [^{32}P] incorporation and protein content as determined by the densitometry of the autoradiography film and the silver-stained gel, respectively. As shown on Fig. 5A–C, treatment with SNC80 or DPDPE (500 nM, 1 h) led to 2.9- and 3.1-fold increase, respectively, in [^{32}P] incorporation into the immunoreactive protein from CHO cells expressing the full-length Et-hDOR. As expected, DPDPE treatment did not increase [^{32}P] incorporation into the immunoreactive band

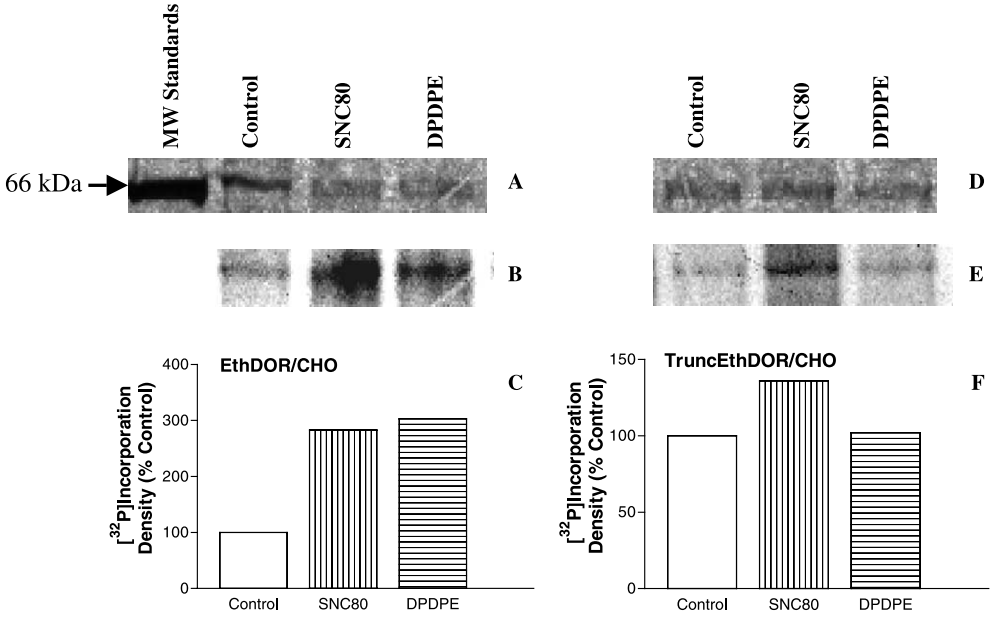

Fig. 5. SDS-PAGE of Et-hDOR/CHO (A, B, C) and TruncEt-hDOR/CHO (D, E, F) cell lysates after metabolic [^{32}P]labeling, chronic SNC80 or DPDPE treatment and immunoprecipitation. Following metabolic labeling with [^{32}P]orthophosphate in phosphate-free DMEM, recombinant CHO cells were incubated in the presence or absence of 500 nM SNC80 or DPDPE for 1 h. The cell lysates were immunoprecipitated with an anti-myc antibody and protein G-agarose. The precipitates were resolved on 10% SDS-PAGE gel. Relative protein concentrations were determined by silver staining of the gel (A, D). [^{32}P] incorporation was determined by autoradiography (B, E). Agonist-mediated phosphorylation was quantified (C, F) by calculating the ratio between the optical densities of the 65–70-kDa band in the autoradiography film and in the silver-stained gel, respectively. The figure shows a representative result from $n=3$ experiments.

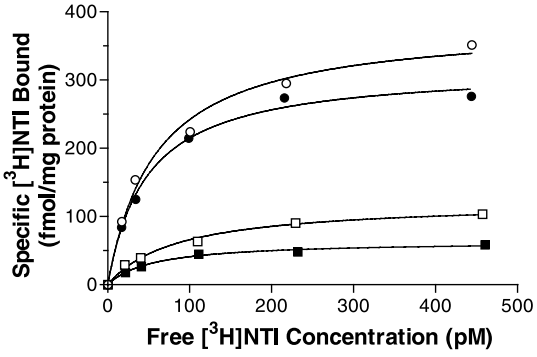

Fig. 6. Saturation isotherms of [^{3}H]naltrindole binding in TruncEt-hDOR/CHO cell membranes in the presence and absence of pertussis toxin. CHO cells expressing the epitope-tagged truncated hDOR were incubated for 24 h in the absence (○□) or presence (●■) of pertussis toxin (50 ng/ml). For the last 12 h of the treatment, aliquots of both control and pertussis toxin-treated cells were incubated with 100 nM SNC80 (□■).

isolated from TruncEt-hDOR/CHO cell lysates (Fig. 5B). Interestingly, however, SNC80 treatment still increased phosphorylation of the immunoreactive protein band by 39% in TruncEt-hDOR/CHO cells (Fig. 5D–F).

3.4. The effect of pertussis toxin on SNC80-mediated down-regulation of the truncated Et-hDOR

Pertussis toxin treatment alone had no significant effect on the B_{max} value of [^{3}H]naltrindole binding to either Et-hDOR/CHO or TruncEt-hDOR/CHO cell membranes (88% and 94% of untreated control, respectively). As expected, SNC80 (500 nM)-mediated down-regulation of the full-length Et-hDOR protein was not attenuated by pertussis toxin treatment (data not shown). The extent of SNC80 (500 nM, 24 h)-mediated down-regulation was also similar in pertussis toxin-treated and -untreated CHO cell membranes expressing the truncated human δ-opioid receptor. The B_{max} of [^{3}H]naltrindole-specific binding after pretreatment of the TruncEt-hDOR/CHO cells with SNC80 (100 nM, 12 h), were 29% and 17% of the untreated control in the absence or presence of pertussis toxin, respectively (Fig. 6).

4. Discussion

In this study, we examined the role of the carboxyl terminal tail of the human δ-opioid receptor in down-regulation, mediated by structurally distinct δ-opioid receptor agonists. Down-regulation was evaluated by saturation analysis of [^{3}H]naltrindole-specific binding to the P_1 fractions of cell membranes isolated from CHO cells expressing the full-length or the carboxyl terminal tail-truncated epitope-tagged human δ-opioid receptors. The dissociation constant of [^{3}H]naltrindole was similar in Et-hDOR/CHO and TruncEt-hDOR/CHO cell membranes and agreed with the K_d value obtained from CHO cell membranes expressing the wild-type human δ-opioid receptor without epitope tags (Malatynska et al., 1996). Inhibition of [^{3}H]NTI binding by SNC80 and DPDPE was used to ascertain that epitope tagging and/or truncation did not interfere with agonist binding to the hDOR. The K_i values SNC80 and DPDPE were similar at the epitope-tagged, truncated epitope-tagged and the untagged, wild-type human δ-opioid receptor indicating that the mutations did not change the agonist binding pocket of the hDOR. The mutations also had no effect on receptor function since both DPDPE and SNC80 were able to stimulate [^{35}S]GTPγS binding with high potencies in both Et-hDOR/CHO and TruncEt-hDOR/CHO cell membranes (data not shown). These results indicate that neither the attachment of the epitope tags nor the truncation of the carboxyl terminal tail caused major conformational changes in the ligand-binding domain or the activation mechanism of the receptor.

In Et-hDOR cell membranes, chronic SNC80 or DPDPE treatment (500 nM, 24 h) led to 86% and 66% reduction in the B_{max} values of [^{3}H]naltrindole, respectively. The loss of [^{3}H]naltrindole-specific binding was not due to the insufficient removal of the agonists, since the K_d value of [^{3}H]naltrindole was similar before agonist treatment and after agonist treatment and three washes. Receptor down-regulation was maximal at 100 nM SNC80 or DPDPE concentrations and higher agonist concentration did not lead to increased down-regulation.

As we have shown earlier (Okura et al., 2000), truncation of the C terminus of the human δ-opioid receptor at Gly^{338} completely abolished DPDPE-mediated receptor down-regulation in CHO cells. Attenuation of DPDPE-mediated down-regulation was not due to lower receptor density in the TruncEt-hDOR/CHO cell line, since DPDPE was able to down-regulate the wild-type human δ-opioid receptor in a CHO cell line expressing similar (200 fmol/mg protein) low receptor density (data not shown).

Agonist-specific differences were previously demonstrated in the homologous regulation of δ-, as well as μ- and κ-opioid receptors. (Arden et al., 1995; Sternini et al., 1996; Bot et al., 1998; Keith et al., 1998; Allouche et al., 1999; Li et al., 1999; Whistler and von Zastrow, 1999; Zhang et al., 1999). In this study, we examined the down-regulation of the truncated human δ-opioid receptor by structurally distinct δ-opioid receptor agonists, such as enkephalin (DPDPE and pCl-DPDPE) and deltorphin analogues (deltorphin II) and nonpeptide octahydroisoquinoline-((−)TAN67, Nagase et al., 1998) and diarylmethylpiperazine (SNC80, Calderon et al., 1997) derivatives. Similarly to DPDPE, truncation of the carboxy terminus also attenuated receptor down-regulation by pCl-DPDPE, deltorphin II and (−)TAN67. Conversely, SNC80 was still able to down-regulate the truncated Et-hDOR significantly ($p = 0.035$), by 42–49%. These findings indicate that while DPDPE-, pCl-DPDPE-, (−)TAN67- and deltorphin II-mediated down-regulation of the human δ-opioid receptor is dependent solely on residues in the carboxyl terminal tail, down-

regulation by chronic SNC80 also involves other receptor domains.

It was previously suggested that down-regulation of the wild-type δ-opioid receptor is dependent on agonist efficacy (Remmers et al., 1998; Zaki et al., 2001). We have shown previously that DPDPE, pCl-DPDPE, deltorphin II, SNC80 and (−)TAN67 are all full agonists in stimulation of [^{35}S]GTPγS binding in CHO cells expressing the human δ-opioid receptor (Quock et al., 1999). Their relative efficacy values, however, were different, following the order: deltorphin II ≈ pCl-DPDPE ≥ DPDPE ≥ SNC80 > (−)TAN67 (Quock et al., 1999). Thus, no correlation is apparent between the abilities of δ-selective agonists to down-regulate the truncated human δ-opioid receptor and their relative efficacy values.

Earlier data from our laboratory indicated that DPDPE and SNC80 interact with distinct activated conformations of the hDOR (Li et al., 1995). These agonist-specific receptor conformations may lead to agonist-specific interactions between agonist-bound receptor and downstream effectors, such as G proteins, protein kinases or arrestins. Importantly, we found that in [^{35}S]GTPγS binding assay mutation Trp^{284}Leu in the third extracellular loop of the human δ-opioid receptor reveals functional differences in G protein activation by SNC80 and DPDPE (Hosohata et al., 2001). Therefore, in this study we investigated whether DPDPE- and SNC80-bound conformations of the hDOR also interact differently with protein kinases that target the receptor for protein degradation pathways.

Truncation at Gly^{338} eliminates all possible serine/threonine kinase phosphorylation sites from the cytoplasmic tail of the human δ-opioid receptor. It was found previously that the truncated rat δ-opioid receptor is not phosphorylated upon DADLE or DPDPE treatment (Zhao et al., 1997). Similarly, we found that truncation of the carboxyl terminal tail of the human δ-opioid receptor completely eliminated DPDPE-mediated receptor phosphorylation. Interestingly, however, we found that SNC80 was still able to phosphorylate the truncated human δ-opioid receptor in CHO cells. Based on these results, we suggest that while DPDPE-mediated receptor phosphorylation relies solely on residues in the C terminal tail of the receptor, other receptor domains may also serve as protein kinase substrates in the SNC80-occupied conformation of the hDOR. Since the data shown on Fig. 5 were obtained by a semiquantitative method (scanning densitometry), numerical calculation of ^{32}P incorporation per mole of receptor was not feasible. On the other hand, we have carefully calibrated protein and [^{32}P]orthophosphate concentrations, and worked in the linear range of the densitometry method. Therefore, the percentage values on Fig. 5A and B can be considered as semiquantitative indicators of ^{32}P incorporation into equal amounts of immunoprecipitated protein. As seen on Fig. 5, SNC80 treatment increased ^{32}P incorporation by 290% in the full-length, and by 140% in the truncated δ-opioid receptors. These values indicate that possibly only one, critical, non-C terminal residue is involved in SNC80-mediated phosphorylation of the truncated human δ-opioid receptor in CHO cells.

A number of different protein kinases are able to phosphorylate the δ-opioid receptor after heterologous (agonist-independent) kinase activation (Quock et al., 1999). The physiological importance of receptor phosphorylation by second messenger-regulated protein kinases, however, is not clear, since both internalization and down-regulation of the wild-type δ-opioid receptor are pertussis toxin-insensitive processes (Law et al., 1985; Chakrabarti et al., 1997; Zaki et al., 2001). The finding that receptor down-regulation still occurs when the receptor is uncoupled from Gi/o proteins argues against the involvement of second messenger regulated kinases in DOR down-regulation. In the present study, we found that similar to the down-regulation of the full-length hDOR, pertussis toxin treatment did not block SNC80-mediated down-regulation of the truncated human δ-opioid receptor, indicating that second messenger regulated protein kinases also have no role in the SNC80-mediated residual down-regulation of the truncated hDOR.

In summary, truncation of the C-terminus of the human δ-opioid receptor attenuated receptor down-regulation by several structurally distinct peptide (DPDPE, pCl-DPDPE, deltorphin II) and nonpeptide ((−)TAN67) δ-opioid receptor agonists. Interestingly, however, we found that SNC80 is still able to down-regulate the C-terminal tail truncated human delta opioid receptor. We have also demonstrated that treatment with SNC80, but not DPDPE, led to phosphorylation of the truncated Et-hDOR. Pertussis toxin treatment did not block SNC80-mediated down-regulation of the truncated Et-hDOR indicating that SNC80-mediated down-regulation of truncated hDOR is independent of $G_{i/o}$ protein activation and downstream second messenger regulated pathways. The molecular mechanism of SNC80-mediated down-regulation of the human δ-opioid receptor, therefore, is different from that of other δ-opioid receptor agonists, and involves other receptor domains in addition to the carboxyl terminal tail.

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