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THE MOLECULAR MECHANISMS OF CELLULAR TOLERANCE TO δ-OPIOID AGONISTS*

A MINIREVIEW

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Chronic treatment with δ-opioid agonists, similar to other agonist drugs, causes tolerance. Tolerance is a complex adaptation process that consists of multiple, cellular and neural-system adaptations. Cellular tolerance to δ-opioid agonists involves feedback-regulation of the function, concentration, and localization of the δ-opioid receptors (receptor desensitization) as well as of intracellular effectors (functional desensitization). We are using a recombinant Chinese hamster ovary cell line expressing the human δ-opioid receptors (hDOR/CHO) to investigate the molecular mechanisms of cellular tolerance We found that the structurally distinct δ-opioid agonists mediate receptor down-regulation by different mechanisms. Thus, truncation of the last 35 C-terminal amino acids of the hDOR completely abolished DPDPE, but not SNC 80-mediated receptor down-regulation. In addition, down-regulation of the wild type-, and the truncated hDORs exhibited different inhibitor sensitivity-profile. Chronic δ-opioid agonist treatment also causes functional desensitization of forskolin-stimulated cAMP formation and cAMP overshoot in the hDOR/CHO cells. We have demonstrated that chronic SNC 80 treatment also causes concurrent phosphorylation of the adenylyl cyclase (AC) VI isoenzyme hDOR/CHO cells. Both AC superactivation and AC VI phosphorylation were SNC 80 dose-dependent, naltrindole-sensitive, and exhibited similar time course-, and protein kinase inhibitor-sensitivity profile. We hypothesize that phosphorylation of AC VI plays an important role in δ-opioid agonist-mediated AC superactivation in hDOR/CHO cells.

Keywords: Human δ-opioid receptor – cellular tolerance – receptor down-regulation – adenylyl cyclase superactivation – adenylyl cyclase phosphorylation

INTRODUCTION

δ-selective opioid agonists are attractive analgesic agents since these drugs exhibit strong antinociceptive activity but cause fewer side effects than the morphine-derivatives that predominantly activate the μ-opioid receptor type [31]. However, similarly to other agonists, chronic δ-selective analgesics also cause tolerance: the chronically stimulated δ-receptors become less responsive, and the drug doses have to be

* Dedicated to Professor Maria Wollemann on the occasion of her 80th birthday.

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increased to maintain the therapeutic effect. Tolerance is a complex compensatory adaptation process. Cellular tolerance involves regulation of the concentration and activity of the opioid receptors (receptor desensitization), second messenger-generating enzymes, ion channels, as well as other intracellular proteins (functional desensitization) in the receptor-expressing cells [6]. In addition, as a consequence of these intracellular adaptations, long-term δ-agonist treatment also leads to neuroplasticity and neural network remodeling (neural-system tolerance) [38].

Better understanding of the molecular mechanisms underlying cellular and neural-system tolerance to δ-opioid agonists should aid the development of long-acting analgesics with minimal side effects. These studies were facilitated by the molecular cloning of the human δ-opioid receptor (hDOR) [16]. Sequence analysis of the cloned cDNA indicated that the open reading frame encodes a 372-amino acid protein, with 93% homology to the rat-, and mouse δ-opioid receptors. The cloned cDNA was subsequently expressed in mammalian cells and the pharmacological properties of the recombinant receptor have been characterized [30]. In Chinese hamster ovary cells expressing the human δ-opioid receptor (hDOR/CHO) δ-opioid agonists stimulated [^{35}S]GTPγS binding, inhibited forskolin-stimulated cAMP formation, stimulated inositol-lipid hydrolysis [33] and phosphorylation of ERK1/2 mitogen activated protein kinases (MAPK, unpublished observation). Agonist treatment also caused δ-opioid receptor phosphorylation [28], internalization, and down-regulation [22, 27] in the hDOR/CHO cells. Finally, chronic agonist treatment led to adaptive subsensitivity to δ-agonist-mediated inhibition of forskolin-stimulated cAMP formation in the hDOR/CHO cells [22]. Therefore, the recombinant hDOR/CHO cell line can serve as useful model system to study the molecular mechanisms of cellular opioid tolerance.

I. Molecular mechanisms of δ-opioid receptor desensitization

The majority of prior studies on cellular δ-opioid tolerance investigated the biochemical changes that occur in the opioid receptor/G protein signal transduction complex. Thus, uncoupling of the opioid receptors from G-proteins; sequestration and internalization of cell membrane receptors; and down-regulation of the total cellular receptor level have been implicated in the development of cellular tolerance [45].

I.A. Molecular mechanisms of agonist-mediated δ-opioid receptor phosphorylation

Similarly to other G protein-coupled receptors, homologous desensitization of the δ-opioid receptor appears to involve receptor phosphorylation. Increased phosphorylation of the δ-opioid receptor upon binding of peptide-, and nonpeptide agonists has been demonstrated [28]. G protein-coupled receptor kinases (GRK) are thought to

play a major role in agonist-mediated δ-opioid receptor phosphorylation [29]. The physiological importance of receptor phosphorylation by second messenger-regulated protein kinases on the other hand is not clear, since both DOR internalization-, and down-regulation are pertussis toxin (Ptx) insensitive [18]. Ptx-insensitivity indicates that receptor internalization and down-regulation can happen even when the receptor is uncoupled from Gi/o protein-mediated intracellular signal transduction cascades. It was also shown, that only the carboxy-terminus of the mouse δ-opioid receptor is required for DPDPE-, or DADLE-mediated mDOR phosphorylation in NG108,15 cells [46]. Conversely we have recently demostrated [28], that while DPDPE-mediated phosphorylation of the hDOR is attenuated by truncation of the last 35 carboxy-terminal residues, SNC 80 (a non-peptide δ-selective agonist) is still able to phosphorylate the truncated hDOR. Therefore we hypothesize that protein kinases are able to recognize additional phosphorylation sites in the intracellular loop regions in the SNC 80-bound conformation of the hDOR.

I.B. Molecular mechanisms of δ-opioid receptor internalization

Numerous investigators have shown that internalization of the δ-opioid receptor occurs predominantly through clathrin-coated vesicles [20]. In this pathway, agonist binding to G protein-coupled receptors initiate receptor phosphorylation, the recruitment of β-arrestins and sequestration of the arrestin-receptor complex into clathrin-coated pits. Subsequent internalization by dynamin-dependent budding and fission delivers the ligand-bound receptor complex into the early endosomes. From the early endosomes the receptors are sorted into either recycling endosomes or late endosomes [6]. Fusion of the late endosomes with lysosomes leads to proteolytic degradation of the receptor protein [23]. Recent data however indicate that the primary role of receptor internalization is not the desensitization, but rather the resensitization of opioid-mediated signaling [44]. Thus, it was suggested that the high propensity of morphine to induce tolerance is related to its failure to promote internalization (and thus, resensitization) of the μ as well as the δ-opioid receptors [15].

The carboxy-terminal tail of the opioid receptors is thought to have a critical role in receptor internalization. Truncation of the last 15 or 37 residues of the carboxy terminus of the mouse δ-opioid receptor was shown to block of DPDPE-mediated mouse DOR internalization in CHO cells [37]. Hierarchical phosphorylation of Ser/Thr residues in the C-terminal tail of the mouse DOR in DPDPE-mediated internalization was recently demonstrated, with Ser^{363} being the critical primary phosphorylation site [21]. On the other hand, other intracellular domains may also be involved in δ-opioid receptor internalization. Thus, it was shown that etorphine and DADLE are still able to internalize the C-terminal truncated mouse δ-opioid receptor in HEK293 cells [26].

I.C. Molecular mechanisms of δ-opioid receptor down-regulation

The molecular mechanism of δ-opioid receptor down-regulation is less clear. We have recently tested the involvement of the arrestin-dependent, and arrestin-independent pathways in the down-regulation of the hDOR using several experimental approaches.

I.C.1. Is β-arrestin involved in agonist-mediated hDOR down-regulation? It was shown that COS-7 cells contain relatively low concentration of endogenous β-arrestins [24]. Thus acceleration of receptor down-regulation by recombinant β-arrestin in hDOR/COS-7 cells would indicate that an arrestin-dependent pathway is involved in hDOR down-regulation. A red fluorescent protein tagged β-arrestin-1 (a generous gift from V. Gurevich, Vanderbilt University, Nashville, TN) fusion protein was used to monitor β-arrestin overexpression in transiently transfected COS-7 cells. The construct was made by site directed mutagenesis of the stop codon in the β-arrestin-1 cDNA to a Sal I restriction site. An EcoRV/Sal I fragment of the mutant arrestin cDNA was ligated into a digested pDs-Red mammalian expression vector (Invitrogen). COS-7 cells were co-transfected with hDOR- and the β-arrestin-1-red constructs. In the control cells empty pDs-Red vector was used instead of the β-arrestin construct. 48 h after transfection, the co-transfected cells were treated with either SNC 80, or DPDPE for different time intervals (0–24 h). After agonist treatment, cell membranes were prepared and [^{3}H]NTI specific binding was measured. Surprisingly, we found that overexpression of β-arrestin-1 had no effect on the rate of hDOR down-regulation in COS-7 cells (Fig. 1).

I.C.2. Down-regulation studies using full length- or truncated human δ-opioid receptors. Most investigators agree that the cytoplasmic tail of the δ-opioid receptor has a fundamental role in DOR down-regulation. Cvejic et al. [9] identified the tail residue

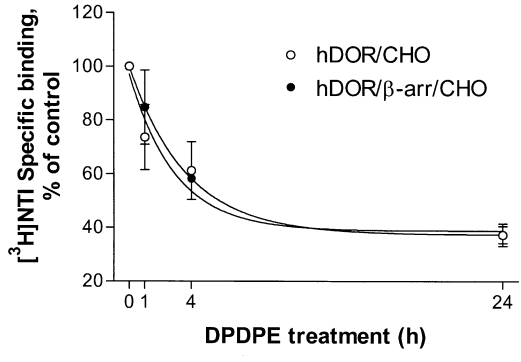

Fig. 1. Overexpression of β-arrestin-1 has no effect on the rate of hDOR down-regulation in COS-7 cells. Time-course of SNC 80-mediated reduction of [^{3}H]NTI binding to COS-7 cell membranes transiently transfected with hDOR alone (F) or cotransfected with hDOR and β-arrestin-1 (M). The figure is a representative from n = 3 experiments

Thr^{353} as crucial to DADLE-mediated down-regulation of the mouse δ-opioid receptor. The homologous residue however is an aliphatic amino acid (Ala) in the human δ-opioid receptor, yet we have previously shown that the human receptor is capable of agonist-mediated down-regulation [22]. This finding indicates that species differences may exist in the mechanism of DOR down-regulation and illustrates the necessity to study the regulation of the human receptor.

We have constructed an epitope (Myc-His)-tagged variant of the full length-, and the C-terminal tail-truncated human δ-opioid receptors [27]. The full-length epitope tagged hDOR (EthDOR) was prepared by mutating the stop codon to a Kpn I restriction site and ligating the Bam HI/Kpn I-digested mutant into a pcDNA3.1/Myc-His expression vector (Invitrogen). The truncated epitope-tagged human δ-opioid receptor was constructed by ligating a Bam HI/NotI restriction fragment (N-terminus to Gly^{338}) of the wild type human δ-opioid receptor into the pcDNA3.1/Myc-His expression vector. The constructs were transfected into CHO cells. To examine receptor down-regulation the cells expressing the epitope tagged receptor constructs were incubated in the presence or absence of either DPDPE or SNC 80 (500 nM) for 24 hours. After agonist pretreatment, cell membrane receptor levels were determined by [^{3}H]NTI saturation binding. We found [27] that while DPDPE-mediated down-regulation was completely abolished in the CHO cell line expressing the truncated epitope tagged human δ-opioid receptor, SNC 80 still mediated significant (42%) down-regulation of the truncated receptor.

I.C.3. The effect of lysosomal and proteasomal inhibitors on the down-regulation of the full length-, and truncated hDOR. In addition to clathrin-dependent lysosomal degradation pathways, membrane proteins can also be marked for degradation by ubiquitination. Ubiquitinated receptors are subsequently degraded in either proteasomes or lysosomes [13]. The involvement of both lysosomal-, and proteasomal degradation pathways has been demonstrated in mouse DOR down-regulation in HEK293 cells [8]. Therefore, we tested the involvement of lysosomal-, and proteasomal degradation pathways in SNC 80- and DPDPE-mediated hDOR down-regulation.

hDOR/CHO or trunchDOR/CHO cells were pretreated for 30–60 min with the ZLLL (25 mM) or chloroquine (25 mM). After inhibitor pretreatment, the cells were incubated with SNC 80 or DPDPE (500 nM, 24 h), washed and cell membranes were prepared as previously described. The total cellular hDOR concentration was determined by [^{3}H]NTI radioligand binding assay. As shown in Fig. 2A, preincubation of hDOR/CHO cells with a lysosomal inhibitor (chroroquine) had no effect on hDOR down-regulation. Conversely, proteasomal inhibitor (ZLLL) attenuated both SNC 80-, and DPDPE-mediated down-regulation.

Interestingly, however the residual SNC 80-mediated down-regulation of the truncated hDOR was sensitive to lysosomal (chloroquine), but not to proteasomal (ZLLL) inhibitors (Fig. 2B). Earlier data from our laboratory have already indicated that DPDPE and SNC 80 may interact with distinct activated conformations of the hDOR [14]. Therefore we hypothesize that structurally distinct δ-opioid agonists

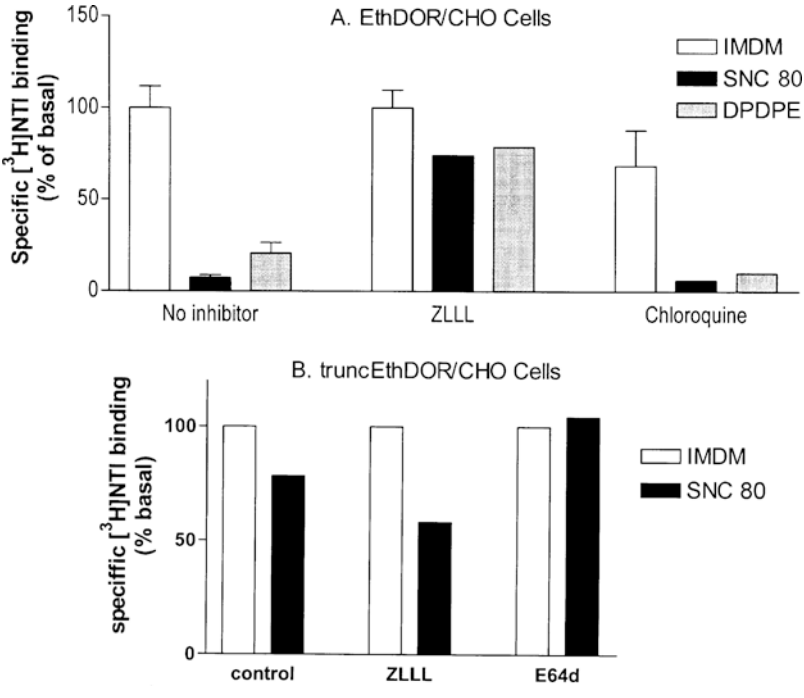

Fig. 2. The effect of proteasomal and lysosomal inhibitors on agonist-mediated hDOR down-regulation in CHO cells. A. [^{3}H]NTI (0.5 nM) specific binding to wild type hDOR/CHO cell membranes after 24 h treatment with 500 nM SNC 80 (solid bars), DPDPE (dotted bars) or IMDM alone (open bars) in the absence (n = 5) or presence of proteasomal (ZLLL, n = 2) and lysosomal (chloroquine, n = 3) inhibitors. B. [^{3}H]NTI (0.5 nM) specific binding to truncated hDOR/CHO cell membranes after 2 h treatment with 500 nM SNC 80 (solid bars) or IMDM alone (open bars) in the presence of proteasomal (ZLLL) and lysosomal (E64d) inhibitors

mediate receptor down-regulation through different mechanisms. Thus, DPDPE mediated down-regulation of the hDOR relies solely on proteasomal targeting motif(s) present in the C-terminal tail of the hDOR. In the SNC 80-bound receptor conformation however, additional lysosomal targeting motifs are also exposed, leading to more efficient down-regulation of the hDOR by SNC 80.

II. Functional desensitization

Cellular tolerance to agonists is not mediated only at the receptor/G protein level. Inhibitory feedback mechanisms regulate the assembly of the signal transduction complexes, the activity of second messenger generating enzymes (such as adenylyl cyclase) and ion channels, and the rate of protein synthesis and degradation (functional tolerance) [25].

Chronic activation of receptors coupled to the $G_{i/o}$ proteins frequently causes compensatory increase in cAMP formation after the inhibitory agonist has been removed (cAMP overshoot). We hypothesize that cAMP overshoot upon chronic opioid receptor stimulation is one of the molecular mechanisms that contribute to the development of functional tolerance to δ-opioid agonists. Although cAMP overshoot upon chronic δ-opioid receptor activation has been demostrated decades ago [34], its molecular mechanism is still not entirely understood. Down-regulation of the hDOR itself is probably not the primary reason for AC superactivation since the cAMP overshoot is apparent only after agonist removal. Chronic agonist treatment-mediated down-regulation of $G_{i/o}$ protein level [39], translocation of $G_{i/o}$ proteins into caveolar compartments [3], depalmitoylation of Gs proteins [2], inhibition of phosphodiesterase activity [19] and a GM_1 ganglioside-mediated switch from $G_{i/o}$-mediated signaling to Gs signaling [10] have been suggested as possible mechanisms leading to cAMP overshoot upon opioid withdrawal.

II.A. The molecular mechanism of adenylyl cyclase superactivaton upon chronic δ-opioid agonist stimulation in hDOR/CHO cells

II.A.1. The characteristics of cAMP overshoot in hDOR/CHO and hDOR/B82 cells. We have previously demonstrated that chronic treatment of hDOR/CHO cells δ-opioid agonists leads to AC superactivation in recombinant hDOR/CHO cell line. cAMP formation in the recombinant cells was determined by measuring the displacement [^{3}H]-cAMP from the regulatory units of PKA by recombinant cell lysates, as previously described [22, 33]. Maximal forskolin-stimulated cAMP formation after 4 h pretreatment with 100 nM SNC 80 was 472 ± 91% of IMDM treated control in

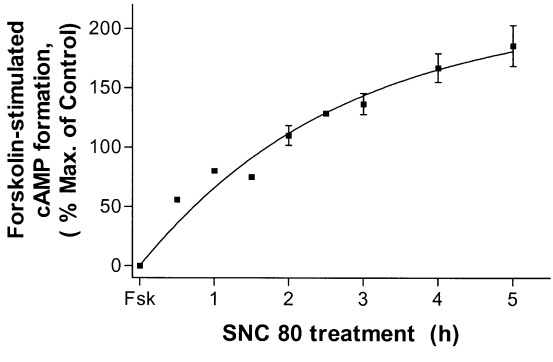

Fig. 3. The time course of SNC 80-mediated cAMP overshoot in hDOR/CHO cells. hDOR/CHO cells were treated with a maximally-stimulating concentration of SNC 80 (100 nM) for different time intervals. After agonist washout, the 100 μM forskolin-stimulated cAMP formation was measured. The half-life of SNC 80-mediated adenylyl cyclase superactivation was 1.90 ± 0.75 hours with a maximal value of 216 ± 22% of forskolin-stimulated IMDM-treated control (n = 2). The baseline of 100% has been subtracted from both E_{max} values

hDOR/CHO cells ($n = 10$, $P < 0.01$) [33]. SNC 80 (100 nM)-mediated adenylyl cyclase superactivation was half maximal after 1.9 ± 0.8 h agonist treatment (Fig. 3). SNC 80 exhibited high potency (EC_{50} of 1.3 ± 1.7 nM) in adenylyl cyclase superactivation (Fig. 4). No cAMP overshoot was observed when the cells were treated with 100 nM SNC 80 in the presence of the δ-selective opioid antagonist, naltrindole (1 μM). Pertussis toxin pretreatment (18–24 h with 50–75 ng/ml) completely attenuated cAMP overshoot (Fig. 5), forskolin-stimulated cAMP formation after SNC 80-treatment in Ptx pretreated cells was $119 \pm 6\%$ of IMDM treated control, $n = 4$, $P > 0.5$).

It was suggested earlier that cAMP overshoot might be due to chronic opioid-mediated up-regulation of cellular adenylyl cyclase protein levels [32]. However, we found that SNC 80 (100 nM) treatment in the presence of the protein synthesis inhibitor cycloheximide (10 μM) still causes AC superactivation in hDOR/CHO cells. Cells not treated with cycloheximide exhibited a cAMP overshoot of $172 \pm 15\%$ of control. cAMP overshoot in cycloheximide treated cells on the other hand, was $184 \pm 0.13\%$ of control ($p > 0.50$). Cycloheximide-insensitivity of AC superactivation in hDOR/CHO cells indicates that new protein synthesis is not likely to be the most important mechanism involved in this process.

Conversely, pretreatment of a recombinant mouse fibroblast cell line (hDOR/B82) with SNC 80 did not cause cAMP overshoot even after 24 h pretreatment with maximal SNC 80 dose (Fig. 5).

II.A.2. Free G protein βγ-subunits are necessary for the development of chronic δ-opioid agonist-mediated AC superactivation in hDOR/CHO cells. Earlier we have shown that co-expression of a putative scavenger of free G-protein βγ-subunits, the α-subunit of transducin (αt1) attenuates AC superactivation cells in hDOR/CHO cells [33]. Therefore we hypothesized that similar to other inhibitory receptors,

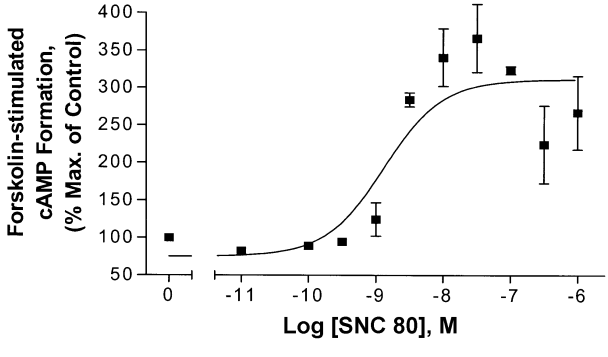

Fig. 4. Dose-response curves for SNC 80-mediated cAMP overshoot in hDOR/CHO cells. hDOR/CHO cells were pretreated with increasing concentrations of SNC 80 for 4 hours, extensively washed, and cAMP formation was determined, as previously. Maximal (100 μM) forskolin-stimulated cAMP formation was $311 \pm 20\%$ of forskolin-stimulated IMDM control. The EC50 of SNC 80 was 1.30 ± 1.7 nM ($n = 2$)

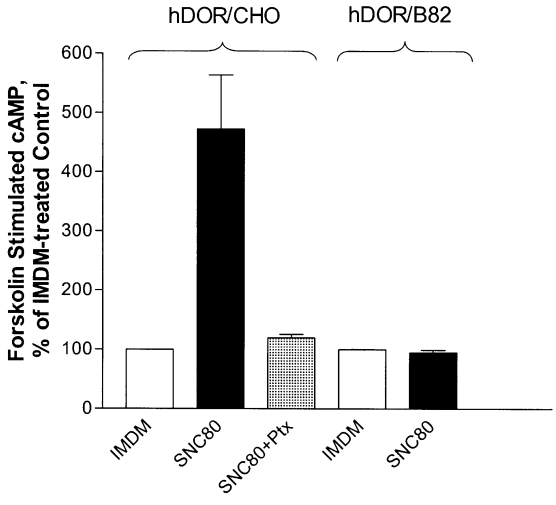

Fig. 5. Forskolin-stimulated cAMP formation after chronic SNC 80-treatment (100 nM, 24 h) in hDOR/CHO and hDOR/B82 cells. Maximal forskolin-stimulated (100 μM) cAMP formation after SNC 80 (100 nM) treatment was 472 ± 91% of IMDM treated control in hDOR/CHO cells. Pertussis toxin (18–24 h with 50–75 ng/ml) pretreated cells on the other hand after exhibited identical forskolin-stimulated cAMP formation after IMDM- or SNC 80 (119 ± 6% of IMDM treated control, n = 4, P > 0. 5) pretreatment. Forskolin (100 μM)-stimulated cAMP formation after chronic SNC 80 (100 nM, 24h)-treatment in hDOR/B 82 cells was not significantly different from IMDM treated controls (94.8 ± 4% of control, n = 4)

chronic δ-opioid agonists mediate AC superactivation hDOR/CHO cells by release of G protein βγ-subunits. The cytosolic phosphoprotein phosducin is a physiological regulator of cellular free G protein βγ-subunit concentration. In the present work therefore we confirmed our hypothesis by overexpressing phosducin in hDOR/CHO cells. A Kpn I/Xba I fragment, containing the coding region of phosducin (ATCC, Rockville, MD), was ligated into the pcDNA 3.1 mammalian expression vector, and CHO cells were transiently co-transfected with hDOR/pREP10 and phosducin/pcDNA3.1 constructs. Doubly transfected cells were selected by their co-resistance to both geneticin and hygromycin. Control CHO cells were transfected with hDOR and an empty pcDNA3.1 vector. 48 h after transfection, the cells were treated with δ-opioid agonist and forskolin-stimulated cAMP formation-, or inositol lipid hydrolysis were measured.

As shown in Fig. 6, adenylyl cyclase superactivation in hDOR/CHO cells was significantly attenuated by transient co-transfection of phosducin. Transiently transfected control hDOR/CHO cells demonstrated an SNC 80-mediated cAMP overshoot of 947 ± 65% of control. Doubly transfected hDOR/phos/CHO cells on the other hand, exhibited cAMP overshoot of 540 ± 35% of control ($P < 0.01$, n = 3).

In order to verify that transient overexpression of phosducin indeed interferes with βγ-subunit-mediated δ-opioid signaling, we measured dose-response curves for SNC

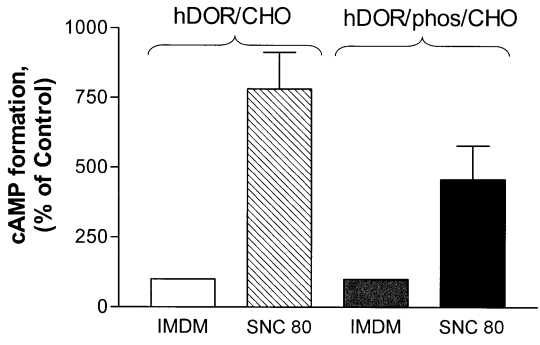

Fig. 6. Transient co-transfection of phosducin attenuates SNC 80-mediated cAMP overshoot in hDOR/CHO cells. CHO cells were transiently co-transfected with hDOR/pREP10 and phosducin/pcDNA3.1 constructs. Doubly transfected cells were selected by co-resistance to both geneticin and hygromycin. Control CHO cells were transfected with hDOR and an empty pcDNA3.1 vector. 48 h after transfection, the cells were treated with δ-opioid agonist and forskolin-stimulated cAMP formation was measured. Transiently transfected control hDOR/CHO cells demonstrated an SNC 80-mediated cAMP overshoot of 947 ± 65% of control. Doubly transfected hDOR/phos/CHO cells on the other hand, exhibited cAMP overshoot of 540 ± 35% of control ($P < 0.01$, $n = 3$)

80-stimulated [^{3}H]IP_1 formation in transiently co-transfected CHO cells, as previously described [33]. CHO cells transfected with the human δ-opioid receptor cDNA and an empty vector demonstrated 316 ± 20% maximal SNC 80-mediated IP_1 formation. The hDOR/phos/CHO cells on the other hand displayed a diminished maximal SNC 80-mediated IP_1 formation of 218 ± 12% of basal (**$p < 0.01$, $n = 3$). Basal IP_1 formation and the potency of SNC 80 (15 ± 20 vs.12 ± 20 nM) was not significantly different between the two cell lines.

II.B. Phosphorylation of AC VI upon chronic δ-opioid agonist treatment in hDOR/CHO cells

The βγ subunits of the G-proteins regulate the activity of a number of protein kinases. Therefore, we decided to test the effect of chronic SNC 80 treatment on the phosphorylation state of adenylyl cyclase in hDOR/CHO cells. To this end, first we determined which AC isoform is a likely to be resposible for the overshoot of forskolin-stimulated cAMP formation in hDOR/CHO cells. We compared the cellular AC isoenzyme [40] pool in cell lines where AC superactivation does (hDOR/CHO) or does not (hDOR/B82) occur, and found that a major difference between these two cell lines is the presence of the AC VI isoenzyme in CHO cells. Therefore, we used an ACV/VI specific antibody (Santa Cruz Biotechnologies) for immunoprecipitation after metabolic [^{32}P]-labeling and chronic δ-opioid agonist treatment of the hDOR/CHO cells [41]. The immunocomplex was resolved on 7.5% SDS-PAGE. The gels were silver-stained, dried and subjected to autoradiography. The amounts of

immunoprecipitated protein-, and the extent of [^{32}P]-incorporation, were quantified by scanning densitometry. We found that concurrent with AC superactivation, chronic SNC 80 treatment of the hDOR/CHO cells augments [^{32}P]-incorporation into the immunoreactive protein. Chronic SNC 80 treatment-mediated [^{32}P]-incorporation was SNC 80 dose-, and pretreatment-time dependent [43] and was attenuated by naltrindole, a selective δ-opioid receptor antagonist [41]. The time course of SNC 80-mediated AC superactivation-, and AC phosphorylation were remarkably similar. Interestingly, simultaneously Chakrabarti et al. [7] have shown that adenylyl cyclase phosphorylation is augmented by chronic morphine treatment in ileum longitudinal muscle myenteric plexus (LMMP) preparations.

II.C. Involvement of protein kinases in AC superactivation in hDOR/CHO cells

II.C.1. The effect of protein kinase inhibitors on SNC 80-mediated phosphorylation of AC VI. Specific protein kinase inhibitors were used to determine which kinase(s) is/are involved in the SNC 80-mediated phosphorylation of AC VI in hDOR/CHO cells. The inhibitors were added 1 h before the start of the δ-opioid agonist (1 μM

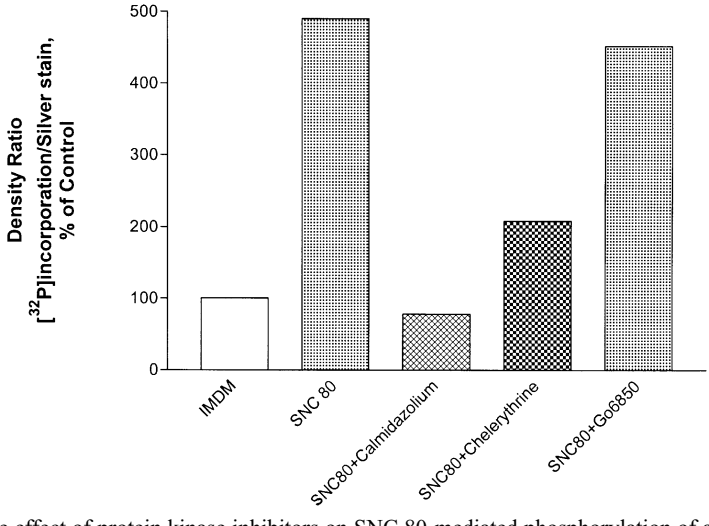

Fig. 7. The effect of protein kinase inhibitors on SNC 80-mediated phosphorylation of adenylyl cyclase VI in hDOR/CHO cells. hDOR/CHO cells were metabolically labeled with 200 μCi/ml [^{32}P]-orthophosphate in the presence of the protein kinase inhibitors, calmidazolium (0.5 μM), chelerythrine (5 μM), and Go6850 (10 μM) for 1 h, then treated with SNC 80 (1 μM) for additional 4 h. The cell lysates were immunoprecipitated, and immunoreactive proteins were resolved and quantitated as previously described [41]. Phosphorylation of AC VI increased to 490% of IMDM treated control after 4 h SNC 80 pretreatment. The density ratio ([^{32}P]-incorporation/protein) remained similar (452% of control) in the presence of the PKC-inhibitor Go 6850. SNC 80-mediated cAMP overshoot was completely attenuated (78% of control) in calmidazolium-, and partially reduced (208% of control) in chelerythrine-treated cells

SNC 80, 4 h) treatment. As seen in Fig. 7, the calmodulin antagonist, calmidazolium (0.5 μM) completely attenuated SNC 80 induced phosphorylation of AC VI. On the other hand, PKA and PKC inhibitors had only partial (chelerythrine, 5 μM) or no (KT 5720 and Go 6850) effect. Chakrabarti et al. [7] have found that chronic morphine treatment-mediated phosphorylation of adenylyl cyclase in guinea pig LMMP preparation is attenuated by chelerythrine pretreatment. Chelerythrine is a non-selective PKC-inhibitor that interacts with all known PKC isoenzymes. Conversely, a PKC inhibitor that interacts only with diacylglycerol (DAG)-sensitive PKC isoforms (Go 6850, calphostin C) had no effect on chronic SNC 80-mediated AC VI phosphorylation (Fig. 7) or AC superactivation (not shown). The mechanism of opioid receptor-mediated activation of DAG-insensitive (atypical) PKC isoforms is presently not clear. Stimulation of either PI-3 kinase or phospholipase A_2 by SNC 80 may lead to activation of the atypical PKC isoenzyme present in CHO cells (PKCζ). Stimulation of PI-3 kinase and arachidonate release [12] by opioid agonists in recombinant CHO cells has been previously shown. Importantly, it was also demonstrated earlier that contrary to other isoenzymes, PKCζ remains permanently activated during long-term opioid treatment [17].

II.C.2. The effect of protein kinase inhibitors on chronic SNC 80-mediated AC superactivation in hDOR/CHO cells. In the next set of experimente we tested the effect of selective protein kinase inhibitors on adenylyl cyclase superactivation. Similar to SNC 80-mediated AC VI phosphorylation, AC superactivation was also completely attenuated (108 ± 3% of control) by pretreatment with the calmodulin antagonist, calmidazolium (0.5 μM). The PKC inhibitor chelerythrine had only minimal effect on cAMP overshoot in δ-opioid agonist treated hDOR/CHO cells. Forskolin-stimu-

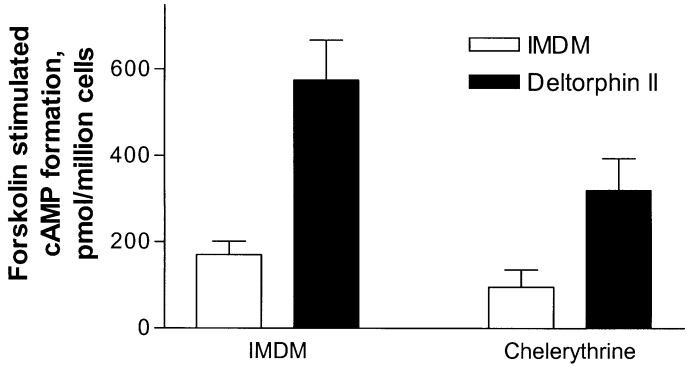

Fig. 8. The effect of chelerythrine pretreatment on δ-opioid agonist-mediated adenylyl cyclase superactivation in hDOR/CHO cells. hDOR/CHO cells were pretreated (0.5–1 h) in the absence or presence of 5 μM chelerythrine. The treatment continued for a further 4 h in the presence or absence of 100 nM deltorphin II. The cells were throroughly washed and forskolin (100 μM)-stimulated cAMP formation was determined. Data are represented pmol cAMP/million cells (mean ± SEM). Chronic deltorphin II-mediated cAMP overshoot was 355 ± 65% of control in IMDM treated-, and 375 ± 87% of control in chelerythrine pretreated cells ($P > 0.05$)

lated cAMP formation after deltorphin II treatment was 533 ± 186 pmol/million cells in IMDM treated-, and 324 ± 126 pmol/million cells in chelerythrine-pretreated cells (Fig. 8, $P > 0.05$).

II.C.3. The role of the protein kinase Raf-1 in chronic SNC 80-mediated AC superactivation. Interestingly, it was recently suggested that PKCζ may contribute to phosphorylation and activation of Raf-1 protein kinase in CHO cells [11]. On the other hand, Tan et al. [35] have recently demonstrated that phosphorylation of AC VI by Raf-1 leads to functional sensitization of the enzyme to different stimulators (forskolin, Gsα). Consequently, we tested the involvement of this mechanism in chronic δ-opioid agonist-mediated AC superactivation in hDOR/CHO cells. Chronic (4 h) deltorphin II treatment augmented forskolin (100 μM)-stimulated cAMP formation to 570 ± 286% (n = 7) of the control in hDOR/CHO cells. On the other hand, pretreatment (30 min, 37 °C) of the cells with a selective inhibitor of Raf-1 (GW5074, 10 μM) attenuated chronic deltorphin II-mediated AC superactivation by 40% (cAMP formation: 351 ± 197% of control, $p < 0.01$, n = 7) [42].

II.C.4. Multiple parallel signal transduction pathways activate Raf-1 to mediate upon AC superactivation in hDOR/CHO cells: Raf-1 acts downstream of activated tyrosine kinases in the MEK1/2 mitogen activated protein kinase signal transduction cascade. The catalytic activity of Raf-1 is modulated by multiple independent mechanisms [5]. Thus, it was shown earlier that only simultaneous inhibition of PKC and tyrosine kinases attenuates opioid agonist-mediated MEK1/2 phosphorylation in recombinant CHO cells [12]. Similarly, we found that simultaneous application of a tyrosine kinase inhibitor (genistein), and a PKC inhibitor (chelerythrine) blocks AC superactivation in hDOR/CHO cells [43], while the inhibitors individually have no significant effect.

Furthermore, it was suggested earlier [4] that opioid-mediated MAPK activation involves a calmidazolium-sensitive step. Similarly, we found that the calmodulin antagonist, calmidazolium, is able to attenuate both chronic SNC 80-mediated AC superactivation and AC VI phosphorylation, indicating the involvement of a calmodulin-sensitive step in the pathway leading to AC VI phosphorylation and AC superactivation in hDOR/CHO cells.

SUMMARY

We have demostrated that chronic δ-opioid agonist treatment of hDOR/CHO cells leads to down-regulation of the hDOR by agonist-specific molecular mechanisms. Chronic δ-opioid agonist treatment also causes AC superactivation and phosphorylation of the AC VI isoenzyme in the hDOR/CHO cells. AC superactivation and AC VI phosphorylation exhibits similar time course and similar protein kinase inhibitor-sensitivity profile. We therefore hypothesize that Raf-1-mediated AC VI phosphorylation plays an important role in δ-opioid agonist-mediated superactivation in hDOR/CHO cells.

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