

Src promotes delta opioid receptor (DOR) desensitization by interfering with receptor recycling

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Abstract

An important limitation in the clinical use of opiates is progressive loss of analgesic efficacy over time. Development of analgesic tolerance is tightly linked to receptor desensitization. In the case of delta opioid receptors (DOR), desensitization is especially swift because receptors are rapidly internalized and are poorly recycled to the membrane. In the present study, we investigated whether Src activity contributed to this sorting pattern and to functional desensitization of DORs. A first series of experiments demonstrated that agonist binding activates Src and destabilizes a constitutive complex formed by the spontaneous association of DORs with the kinase. Src contribution to DOR desensitization was then established by showing that pre-treatment with Src inhibitor PP2 (20 μ M; 1 hr) or transfection of a dominant negative Src mutant preserved DOR signalling following sustained exposure to an agonist. This protection was afforded without interfering with endocytosis, but suboptimal internalization interfered with PP2 ability to preserve DOR signalling, suggesting a post-endocytic site of action for the kinase. This assumption was confirmed by demonstrating that Src inhibition by PP2 or its silencing by siRNA increased membrane recovery of internalized DORs and was further corroborated by showing that inhibition of recycling by monensin or dominant negative Rab11 (Rab11S25N) abolished the ability of Src blockers to prevent desensitization. Finally, Src inhibitors accelerated recovery of DOR-G α i3 coupling after desensitization. Taken together, these results indicate that Src dynamically regulates DOR recycling and by doing so contributes to desensitization of these receptors.

Keywords: opioid receptors • analgesia • tolerance • Src • recycling • desensitization/resensitization • trafficking

Introduction

Opiates are among the most effective analgesics known but their clinical use is limited by their potential for abuse and frequent side effects such as nausea, vomiting and constipation. Most if not all of these unwanted actions arise from the activation of the mu opioid receptors (MOR), and cannot be dissociated from MOR-mediated analgesia [1]. In contrast, delta opioid receptor (DOR) activation produces antinociception [2, 3] with reduced respiratory depression [4], low constipation [5], minimal physical dependence [6] and attenuated addictive potential [7, 8]. However, a

major problem with DORs is their rapid desensitization [9, 10] leading to the development of analgesic tolerance [11, 12].

Receptor desensitization is an important adaptive mechanism that protects the cell from excessive activation by environmental stimuli. For most G protein coupled receptors (GPCRs), including opioid receptors, the process is initiated by phosphorylation of serine/threonine residues located at the receptor's C-terminus and/or third intracellular loop. This post-translational modification results in β -arrestin recruitment, which inhibits any further stimulation by uncoupling the receptor from its G protein and triggering subsequent internalization [11, 12]. Because internalization basically removes receptors from the cell surface, sequestration was initially considered as a step in signal termination. However, functional consequences of GPCR internalization cannot be fully understood unless considered in the light of post-endocytic trafficking.

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If sequestration is associated with recycling of the receptor back to the cell surface, internalization allows to restore a pool of functional membrane receptors that enable to prolong or recover signalling [13–15]. In contrast, if the receptor is preferentially directed towards the lysosomal compartment, proteolytic degradation and rapid desensitization are the major consequences of internalization [16–18]. DORs display this second type of profile, since only a small percentage of internalized receptors recycles back to the membrane while the great majority is committed for proteolysis soon after endocytosis [18, 19]. Despite early commitment to degradation, DORs do not immediately traffic to lysosomes remaining withheld in the endosomal compartment [19]. The creation of this pool of intracellular receptors raises the possibility of influencing DOR sorting so as to redirect retained receptors back to the cell surface, providing a means of prolonging DOR signalling and delaying or avoiding tolerance.

A major determinant for lysosomal sorting of GPCRs is covalent modification by ubiquitination, a post-translational modification which tags the protein for recognition by the endosomal sorting complex required for transport (ESCRT) machinery [20, 21]. DORs, however, are an interesting exception to this pattern since ubiquitination is not required for their lysosomal trafficking [15]. Instead, they rely on 'sorting' proteins of the G protein coupled receptor associated sorting protein (GASP) family [18, 22]. The mechanisms by which GASP proteins may ensure DOR sorting within the endosomal-lysosomal system is not known, but GASP1 interaction with alpha helix VIII is sufficient to promote lysosomal targeting of these receptors [18, 22]. Unlike post-translational modifications that allow to modulate receptor trafficking in an activation-dependent manner, the sequence that recognizes GASP is permanently incorporated into the amino-acid sequence of the receptor. The presence of this constitutive tag raises the question as to whether dynamic regulation of sorting events is possible or, on the contrary, whether post-endocytic trafficking of DORs is rigidly predetermined by primary structure.

We have previously shown that duration of DOR signalling could be considerably prolonged by Src blockers [23]. In the present study, we extended these findings by assessing whether Src could influence duration of DOR signalling *via* modulation of post-endocytic trafficking. Using pharmacological and molecular blockers for this kinase, we were able to show that Src promotes desensitization by preventing DOR recycling. These results constitute the first evidence that post-endocytic sorting of DORs may be dynamically regulated and that this regulation has functional relevance.

Materials and methods

Reagents

Buffer chemicals, protease inhibitor, DPDPE, forskolin, isobutylmethylxanthine, cycloheximide, pertussis toxin (PTX), sucrose, monensin sodium,

anti-FLAG M2 affinity resin and FLAG peptide were purchased from Sigma-Aldrich (Oakville, ON, Canada). 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) was from VWR (Mont-Royal, QC, Canada), BSA from EMD chemicals (Gibbstown, NJ, USA) and [³H]adenosine from PerkinElmer Life Sciences (Waltham, MA, USA). G418, DMEM, foetal bovine serum, glutamine, penicillin and streptomycin were purchased from Wisent (St-Bruno, QC, Canada).

Cell culture and transfection

Experiments were carried out in HEK293 cells stably expressing murine DORs or its truncated mutant [24, 25], with the exception of experimental series requiring co-transfection of wild-type DORs either with dominant negative c-Src (DNM-Src: K295R/Y527F) [26] or dominant negative Rab11 (DNM-Rab11: Rab11S25N) [27]. Cells stably expressing wild-type DORs or truncated DOR mutants (DOR344T) were transfected with 6 µg/100 mm petri dish of corresponding DNA using lipofectamine (Invitrogen, Burlington, ON, Canada) as transfection agent, followed by selection with G418 (500 µg/ml). Receptor expression levels in DOR clones was assessed by means of [³H]Naltrindole binding yielding a Bmax of 605 ± 42 fmol/mg protein. Membrane expression of DORs and DOR344T was similar, as verified by ELISA. For transient expression, DORs were co-transfected either with DNM-Src (10 µg), DNM-Rab11 (15 µg) or the empty vector (pcDNA3) using poly-ethylenimine as transfection agent. Receptor expression was verified by ELISA so as to obtain similar membrane expression across transfection conditions. Cells were grown and maintained in complete DMEM containing 10% (v/v) foetal bovine serum, 1000 units/ml penicillin, 1 mg/ml streptomycin and 5 mM glutamine, in a humidified atmosphere of 5% CO₂ at 37°C.

Src knock-down by siRNA

A pool of four desalted, deprotected siRNA oligonucleotide duplexes specifically targeted to human Src (GenBank NM_005417) and bearing UU overhangs were purchased from Dharmacon Research (Lafayette, CO, USA) and introduced into DOR-expressing cells performed with DharmaFECT1 according to manufacturer's specifications. In order to achieve optimal Src, knock-down cells were transfected with increasing concentrations of siRNA (100, 50 and 25 nM) and Src expression subsequently verified by Western blot analysis 48, 64 and 72 hrs after transfection. Non-targeting DNA in equivalent concentrations was used to control for off-target changes in Src expression. 25 nM siRNA and 48 hrs recovery post-transfection produced maximal Src knock-down, and were therefore chosen for the study.

Immunopurification of FLAG-tagged receptors

This procedure was adapted from a previously described method [23] and was used to assess receptor interaction with endogenous Src or Gα subunits. Briefly, cells were incubated overnight in serum-free medium and the day of the experiment were exposed to PP2 (20 µM) or vehicle (DMSO) for 1 hr prior to treatment with DPDPE (1 µM) for the indicated periods of time. In experiments in which PTX was used the toxin (100 ng/mL) was introduced 16 hrs prior to the experiment. After agonist stimulation, the reaction was stopped on ice by washing cells with cold PBS. Cells were then suspended in lysis buffer (5 mM Tris, 3 mM MgCl₂, 2 mM EDTA, 1 mM NaF, 1 mM Na₃VO₄, 5 µg/ml leupeptine, 5 µg/ml soybean trypsin inhibitor and 10 µg/ml benzamide) and homogenized using an ultraturax (IKA, Wilmington, NC,

USA). Following a short centrifugation at 1500 rpm, the supernatant was centrifuged at 18,500 rpm for 20 min. and the resultant pellet was resuspended in lysis buffer for a second round of centrifugation (18,500 rpm; 20 min.). The pellet obtained was then solubilized in 0.5% *n*-dodecyl-maltoside, 25 mM Tris pH 7.4, 140 mM NaCl, 2 mM EDTA, 1 mM NaF, 1 mM Na₃VO₄, 5 μg/mL leupeptine, 5 μg/ml soybean trypsin inhibitor and 10 μg/mL benzamide. Following agitation at 4°C for 2 hrs, the solubilized fraction was centrifuged at 10,000 rpm for 60 min., and the receptor was immunopurified from the supernatant fraction using an anti-FLAG M2 antibody resin. 20 μl of antibody-coupled resin equilibrated in solubilization buffer and supplemented with 0.1% bovine serum albumin (w/v) were used to purify the receptor overnight at 4°C under gentle agitation. The next morning the resin was pelleted, washed twice with 500 μl of solubilization buffer and four times with 500 μl of modified solubilization buffer (containing 0.1% instead of 0.5% *n*-dodecyl-maltoside (w/v)). The receptor was then eluted by incubating the resin for 10 min. at 4°C with 100 μl of modified solubilization buffer containing a FLAG peptide (150 μg/ml). This elution was repeated three times, and the eluates were combined and concentrated by membrane filtration over Microcon-30 concentrators (Millipore). SDS sample buffer was then added and samples were used for SDS-PAGE.

SDS-PAGE and Western blotting

SDS-PAGE was performed as previously described [23] using a 4% stacking gel and 9% separating gel. Proteins resolved in SDS-PAGE were then transferred (50 mA, 16 hrs, Bio-Rad Mini-Trans Blot apparatus) from the gels onto nitrocellulose (GE Healthcare, Piscataway, NJ, USA). c-Src polyclonal antibody (SRC2 : sc-18; Santa Cruz Biotech, Santa Cruz, CA, USA) and Gαi3 antibody (Sc-262; Santa Cruz Biotech.) were used at a dilution of 1:1000 to determine, respectively, the amount of total Src and of Gαi3 proteins that had precipitated along with DORs, followed by secondary anti-rabbit horseradish-conjugated antibodies (1:10,000 or 1:40,000; Amersham Biosciences, Piscataway, NJ, USA). The amount of FLAG-DORs present in each sample was evaluated with antisera directed against the FLAG epitope (anti-FLAG M2 antibody; 1:2000; Sigma-Aldrich). Horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:4000; Amersham Biosciences) and chemiluminescence detection reagents (GE Healthcare) were used to reveal the blotted proteins and relative intensities of the labelled bands were analysed by densitometric scanning performed with MCIID (Imaging Research Inc).

Src activation was assessed as previously described [23], after a 5-min. exposure to DPDPE. Samples were separated in SDS-PAGE and anti-phospho-Src polyclonal antibody (Tyr416; 1:1000; Cell Signaling, Danvers, MA, USA) was used to determine the presence of activated Src. Total amount of protein loaded was detected by probing with anti-Src polyclonal antibody (SRC2 : sc-18; 1:5000; Santa Cruz Biotech.). In siRNA knock-down samples, total Src levels were normalized to the amount of β-actin present in each sample, detected by using a monoclonal antibody (1:20000; SantaCruz Biotech).

cAMP accumulation assays

cAMP accumulation assays were carried out according to a previously described protocol [23]. Desensitization of DORs (DES) was achieved by 30-min. exposure to DPDPE (1 μM). Cells were then washed three times in cold PBS and the efficacy of washing was confirmed by showing no difference in naltrindole Ki in [³H]naltrindole displacement experiments carried

out in control and DPDPE-treated cells. To study the effect of a pharmacological inhibition of Src, cells were pretreated for 1 hr with PP2 (20 μM) prior to DPDPE exposure. For experiments using monensin as recycling inhibitor, the drug (50 μM) was added together with PP2 (or vehicle for non-treated cells). For sucrose treatment, cells were pre-incubated in the presence of 0.4 M sucrose (2 hrs), which was maintained throughout successive treatment with PP2 (1 hr) and DPDPE (30 min.). [³H]ATP and [³H]cAMP were separated by sequential chromatography on Dowex exchange resin and aluminium oxide columns and the results were expressed as the ratio of [³H]cAMP/[³H]ATP plus [³H]cAMP. Effects of the DOR agonist DPDPE were expressed as percentage of change with respect to cAMP produced in the absence of ligand (values given in table). When agonist actions were compared across different experimental conditions (*e.g.* following desensitization, Src blockade, inhibition of internalization or recycling), results were normalized to the maximal effect of DPDPE in corresponding non-treated controls (values in figures).

Internalization assays

Measurement of surface-expressed FLAG-tagged DORs and quantification of receptor internalization was assessed using an ELISA method adapted from [28, 29]. Cells were seeded at a density of 10⁵ cells/well and grown on 24-well polylysine-coated plates for 48 hrs. The day of the experiment, DPDPE (1 μM) or vehicle were introduced in new incubation medium containing DMEM/HEPES 20 mM for the indicated times. When PP2 (20 μM) or sucrose (0.4 M) were used, these pre-treatments were, respectively, introduced 1 hr and 3 hrs prior to the agonist. The internalization reaction was stopped by addition of cold PBS. After three PBS washes, cells were fixed for 15 min. at 4°C in paraformaldehyde (3%) and non-specific binding was blocked by incubation with PBS/BSA 1%/CaCl₂ 1 mM at room temperature for 30 min. Cells were subsequently incubated with anti-FLAG M1 antibody (1:1000; Sigma-Aldrich) for 1 hr at room temperature, washed three times and incubated with peroxidase-conjugated (HRP) anti-mouse antibody (1:8000; Amersham Biosciences) for 30 min. After extensive washing, 200 μl of the HRP substrate o-phenylenediamine dihydrochloride (*SIGMA FAST™ OPD*, Sigma-Aldrich) was added to each well. The reaction was allowed to proceed for 8 min. and stopped using 3N HCl. 200 μl of the mix were then transferred to a 96-well plate for optical density (OD) evaluation at 492 nm in a microplate reader (Victor3; Perkin Elmer). OD reading corresponded to the signal generated by receptors at the cell surface. The amount of surface receptors internalized following exposure to DPDPE (1 μM) was calculated by subtracting OD obtained in absence of agonist from the one obtained after agonist exposure. Results were expressed as percentage of receptors initially present at the membrane according to the following calculation: $100 \times (OD^{Basal} - OD^{Stimulated}) / (OD^{Basal})$ where OD^{Basal} and OD^{Stimulated} correspond to the signal obtained in presence or absence of DPDPE, respectively.

Surface recovery assays

Recycling of internalized receptors back to the plasma membrane was estimated by an assay which is a variant of the one described above. Protein synthesis was blocked using 10-μM cycloheximide introduced 1 hr before a single dose of DPDPE (1 μM; 30 min.) and allowed to remain in the medium throughout the duration of the experiment. The agonist was removed by extensive washing with DMEM and cells were subsequently incubated at 37°C for increasing periods of time in an agonist-free medium (DMEM/HEPES/cycloheximide). In experiments using PP2 (20 μM) and/or

monensin (50 μ M), pre-treatments were introduced 1 hr prior to DPDPE exposure. The experiments were stopped by addition of cold PBS and the steps to label membrane DORs proceeded as described in the previous section. The amount of internalized receptors that recycled back to the surface was expressed as percentage of receptors internalized following exposure to DPDPE. When recovery from agonist-induced internalization was compared across different experimental conditions (*e.g.* Src silencing with siRNA, Src inhibition by PP2, inhibition of recycling by DNM-Rab11 or monensin), results were normalized to the recovery observed in corresponding untreated controls.

Data analysis

Statistical analysis and curve fitting were done using Prism 4 (GraphPad, San Diego, CA, USA).

Results

DORs and Src form a constitutive complex that dissociates upon receptor activation

We have previously shown that Src activity regulates the extent and duration of DOR responsiveness to different ligands [23]. To start to examine the nature of this regulation, we first investigated functional and physical interactions between the two proteins. As previously observed, the agonist DPDPE (1 μ M; 5 min.) stimulated Src activity [23], an effect that could be blocked by pre-exposure to PTX (Fig. 1A). Physical interaction between Src and the receptor was next monitored by immunopurifying FLAG-tagged DORs and performing Western blot analysis to measure the total amount of kinase recovered. As shown in Fig. 1B, Src copurified with the receptor in the absence of ligand, suggesting a spontaneous association of both proteins. Addition of DPDPE (1 μ M) to the incubation medium induced a rapid destabilization of the complex, as indicated by more than 30% reduction in the amount of Src recovered within the first 5 min. of agonist exposure (Fig. 1B). This rapid destabilization was followed by a much slower dissociation that progressed during the remaining 25 min. of agonist treatment. Maximal dissociation of the DOR-Src complex was not modified by PP2 (Fig. 1C) but was blocked by PTX (Fig. 1D) indicating that disruption of the complex was dependent upon activation of the G-protein but not of Src.

Src blockade prevents desensitization

The next series of experiments was directed at evaluating the possibility that agonist-dependent activation of Src may contribute to functional desensitization of DORs. To do so we determined whether Src inhibition by a pharmacological blocker or by transfection of a dominant negative Src mutant modified reduction in DOR

signalling efficacy that characteristically results from sustained exposure to an agonist. cAMP accumulation assays carried out in control cells stably expressing wild-type DORs showed that DPDPE produced a maximal inhibition of $61\% \pm 2$ in basal cAMP production ($n = 22$, pooled control curves). As expected, 30-min. exposure to this same agonist (1 μ M) diminished its subsequent ability to inhibit cAMP production. In particular, DPDPE potency was reduced by eight fold (EC_{50} CTL (4 ± 1) versus EC_{50} DES (32 ± 2 nM)) while maximal inhibition only reached $72\% \pm 10$ ($n = 4$) of that observed in parallel controls (Fig. 2A, left panel). However, when exposure to DPDPE was carried out in presence of PP2 at a concentration (20 μ M) that abolished Src activation (Inset Fig. 2A), desensitization was no longer observed since DORs exposed to DPDPE in the presence of PP2 maintained similar signalling efficacy as non-desensitized receptors (Fig. 2A, right panel).

Src participation in DOR desensitization was confirmed by repeating similar experiments in cells co-transfected with DORs and a dominant negative Src mutant (K295R/Y527F; DNM-Src) [26] (Fig. 2B). Transfections were optimized to achieve similar membrane expression of DORs as in the previous experimental series, and DNM-Src levels were titrated to obtain complete inhibition of endogenous Src activity (Inset Fig. 2B). Results confirmed that in cells overexpressing DNM-Src, signalling efficacy of desensitized DORs (DPDPE; 1 μ M; 30 min.) did not differ from that of the corresponding controls (Fig. 2B, right panel). In contrast, in cells where DORs were co-transfected with the empty vector (pcDNA3) agonist pre-treatment significantly reduced subsequent ability of DPDPE to inhibit cAMP production (Fig. 2B, left panel).

Src inhibitors prevent DOR desensitization without modifying internalization, but maximal protection requires normal sequestration

Internalization contributes to rapid desensitization of DORs [30] and Src activity has been implicated in the sequestration of various GPCRs [31, 32]. Thus, it was of interest to determine whether each of the experimental approaches that reduced DOR desensitization by blocking Src activity had any effect on DOR internalization. To do so, Src inhibition was achieved as in the previous experiments using PP2 or DNM-Src and DOR internalization by DPDPE was assessed by monitoring loss of membrane receptors at different treatment intervals. In cells stably expressing wild-type DORs, DPDPE-dependent internalization was in reasonable agreement with previous reports [33, 34], displaying a half-life of 14 min. and a maximal sequestration of $87\% \pm 3$ of surface receptors within the first 2 hrs of agonist exposure. In transiently transfected cells expressing DOR/pcDNA3, a half-life of 19 min. was observed with a maximal sequestration of $64\% \pm 3$ of surface receptors. As shown in Fig. 3, neither of the experimental conditions that afforded protection from desensitization interfered with DOR endocytosis.

Having established that Src inhibitors protect against desensitization without influencing internalization, the next series of experiments was ran to explore whether the converse was also true, *i.e.* if

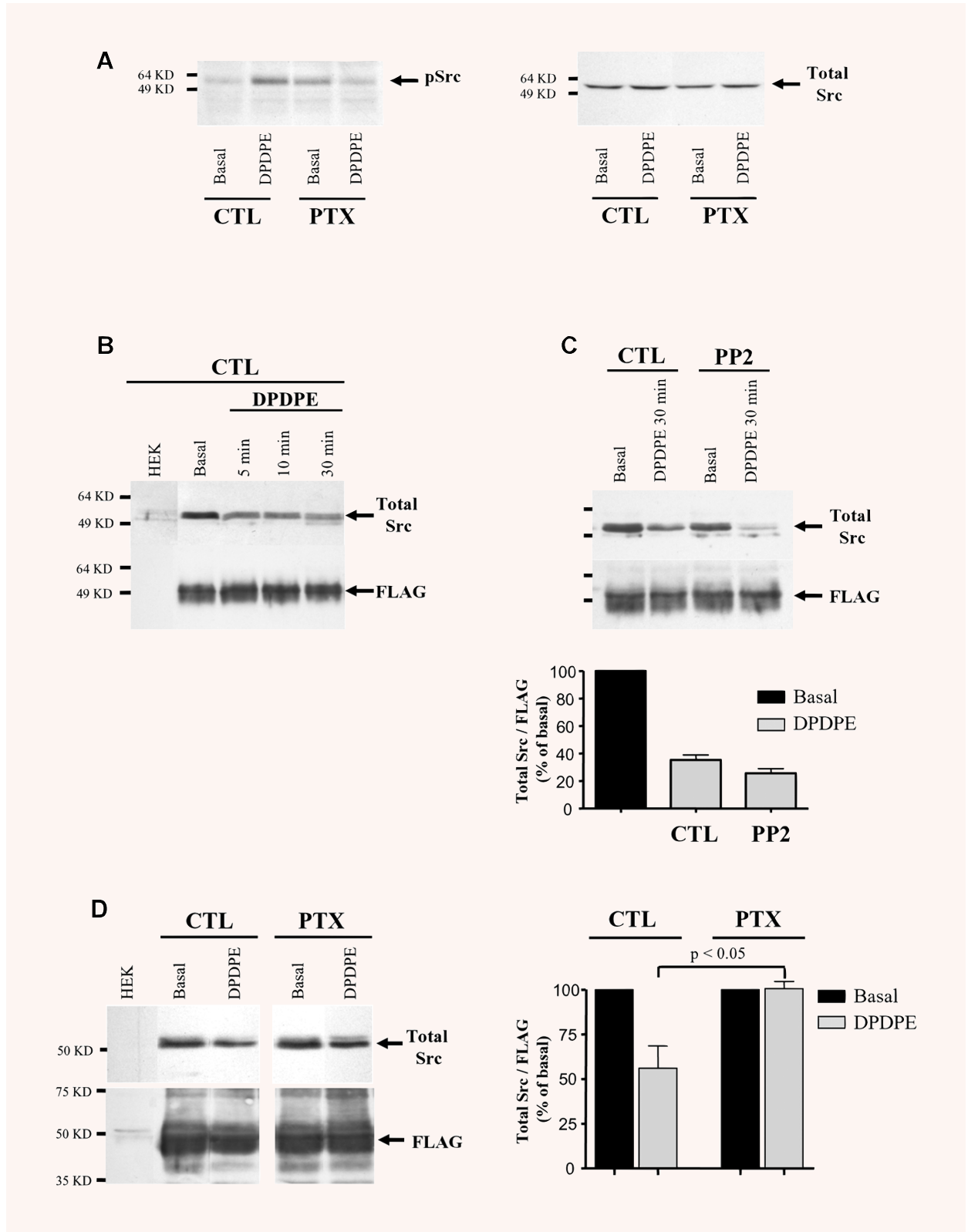




Fig. 1 DOR stimulation activates Src and reduces its spontaneous interaction with the receptor. **(A)** Cells stably expressing FLAG-tagged DORs were incubated overnight in serum-free medium containing either PTX (100 ng/ml) or vehicle (CTL) and exposed to DPDPE (1 μ M; 5 min.). Src activation was assessed by measuring phospho-Tyr416 immunoreactivity (pSrc). Total amount of protein loaded was detected by probing with anti-Src antibody (Total Src). Results correspond to a representative example of at least four times. Samples come from the same blot. **(B)** Cells were treated or not with DPDPE (1 μ M) for the indicated time periods. DORs were immunopurified as described in the experimental section and the amount of Src recovered with immunopurified receptors was revealed by immunoblot with anti-Src. Total amount of receptor recovered was assessed with anti-Flag M2 antibodies. Western blot shows a representative example. **(C)** Cells were exposed to PP2 (20 μ M; 1 hr) or vehicle (CTL) prior to treatment with DPDPE (1 μ M; 30 min.). Effect of PP2 on agonist-induced DOR-Src complex dissociation was monitored as in B. Histograms below show complex dissociation following 30 min. exposure to DPDPE and correspond to six independent experiments. **(D)** Cells were exposed or not to PTX (100 ng/mL; 16 hrs) and treated with DPDPE (1 μ M) for 5 min. DOR-Src interaction was assessed as in B. Western blots corresponds to a representative example. All samples shown were obtained from the same blot. Histograms to the right correspond to four experiments. Statistical analysis by Student's *t*-test, *P*-value appears in the figure.

changes in internalization influenced the ability of Src blockers to counter desensitization. DOR internalization was modified by two alternative approaches: (i) using an internalization-deficient DOR mutant (DOR344T) that lacks C-terminal Ser/Thr residues [35, 36] and (ii) by incubating cells expressing wild-type DORs in hypertonic medium (0.4 M sucrose), a procedure that interferes with normal clathrin polymerization [37]. Changes in DOR internalization appear in Fig. 4A, which shows that agonist-dependent internalization was reduced by 30% in truncated DORs, while sucrose abolished sequestration for the first 10 min. of agonist exposure and reduced it by more than 60% for the remainder of treatment. Table 1 shows how pre-exposure to DPDPE (30 min.; 1 μ M) modified receptor responsiveness under the different internalization conditions. The influence of changes in internalization on the ability of Src blockers to interfere with desensitization was then established by repeating PP2 pre-treatment (20 μ M; 1 hr) and exposure to DPDPE (1 μ M; 30 min.) in cells expressing DOR344T (Fig. 4B) or in cells expressing wild-type DORs that had been pre-incubated with sucrose (Fig. 4C). As is apparent from the figures, the protective effect of PP2 was progressively reduced by treatments that increasingly interfered with internalization. In particular, the ability of PP2 to maintain DPDPE signalling in desensitized mutants became apparent at higher agonist concentrations than in wild-types ($P = 0.02$; $n = 4$; Fig. 4B) while in presence of sucrose PP2 protective actions were not only right shifted but also reduced in magnitude.

Src contributes to receptor desensitization by interfering with recycling

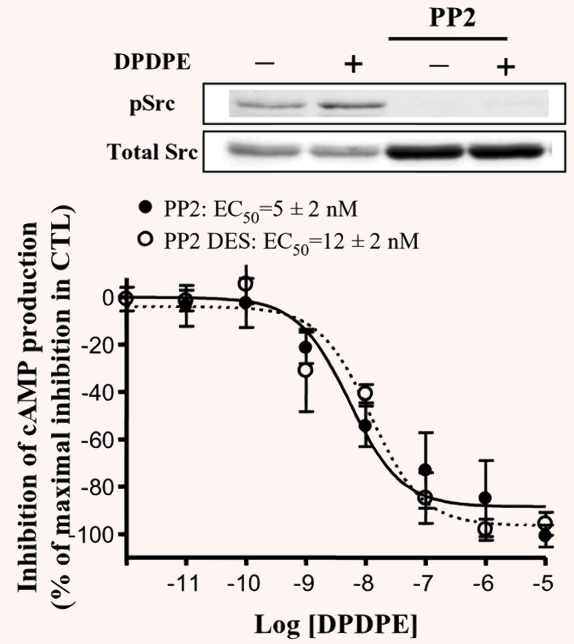
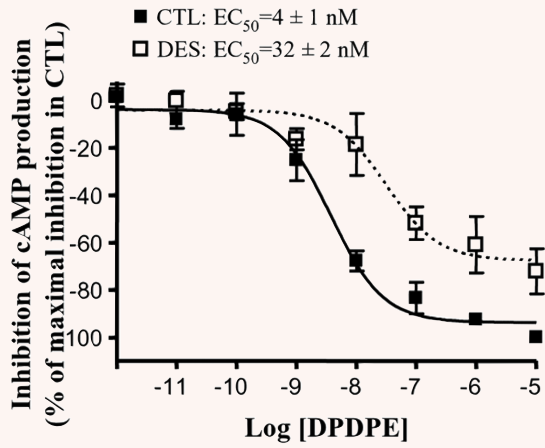
The fact that optimal sequestration was necessary for Src inhibitors to counter agonist-dependent desensitization suggested that the kinase negatively influenced DOR responsiveness *via* regulation of a post-endocytic event. Previous studies have demonstrated that a small proportion of internalized DORs are recycled to the membrane, while the bulk of them are rapidly trapped in an endocytic compartment from where they are slowly targeted for lysosomal degradation [19, 38]. Since the functional consequence of this sorting pattern is a reduction in receptor signalling [39, 40], we reasoned that Src inhibitors could counter desensitization by

redirecting internalized receptors to the recycling pathway. To assess this possibility, we determined how Src silencing by siRNA or its pharmacological blockade by PP2 influenced membrane recovery of internalized DORs (Fig. 5). In experiments assessing the effect of the pharmacological inhibitor, control and PP2-treated cells both displayed internalization of approximately 65% surface receptors following 30 min. exposure to DPDPE. Consistent with previous reports [15, 41], agonist removal allowed $36 \pm 3\%$ of internalized receptors to recycle back to the membrane of control cells. Most interestingly, a similar PP2 pre-treatment as the one that protected DORs from desensitization (20 μ M; 1 hr) increased basal membrane recovery by almost 25%, implying that after Src inhibition 45% of internalized receptors were recycling back to the membrane (Fig. 5A).

We next examined whether knock-down of the Src gene would similarly enhance DOR recycling. For this purpose, cells stably expressing DORs were transfected with on-target or off-target siRNA for human Src. Forty-eight hours after transfection with a pool of four siRNA duplexes specifically directed against human Src (concentration range 25–100 nM), expression of the protein was reduced by 90–95% (Fig. 5B). Since knock-down by 25 nM of target siRNA was not different from the one observed at higher concentrations, 25 nM was chosen for additional study. Knock-down specificity was corroborated by lack of effect of similar concentrations of non-targeting siRNA. In addition, the fact that β -actin levels remained stable across different transfections indicates absence of interferon response, which would otherwise be evidenced by reduced protein synthesis. Receptor recycling was then assessed in cells transfected with targeting or non-targeting siRNA. Exposure to DPDPE (1 μ M; 30 min.) induced similar internalization ($\cong 75\%$) of surface receptors in absence or presence of Src knock-down. In cells expressing Src normally, $36 \pm 5\%$ of sequestered receptors was recycled back to the membrane. This amount was increased by 20% following Src knock-down (Fig. 5C), reproducing results obtained with pharmacological inhibitors and directly implicating Src in the regulation of DOR recycling.

Results obtained with pharmacological inhibition or gene silencing of Src confirm this tyrosine kinase as a regulator of post-endocytic sorting, but they do not indicate whether the small

A



B

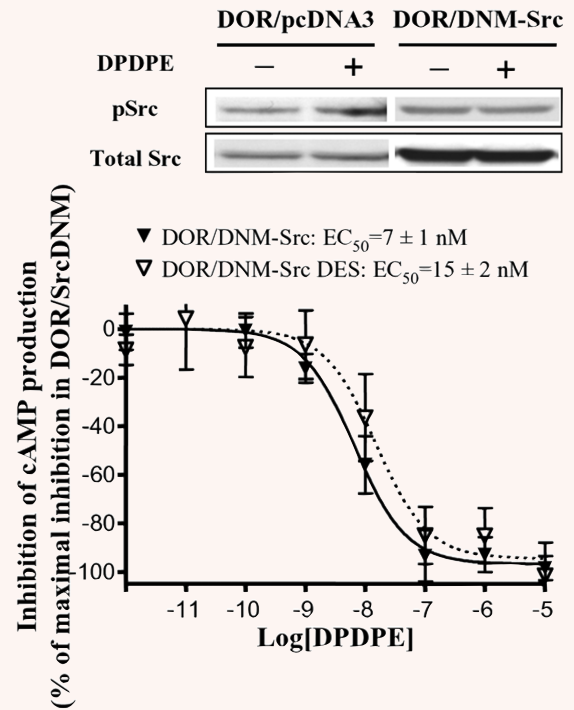
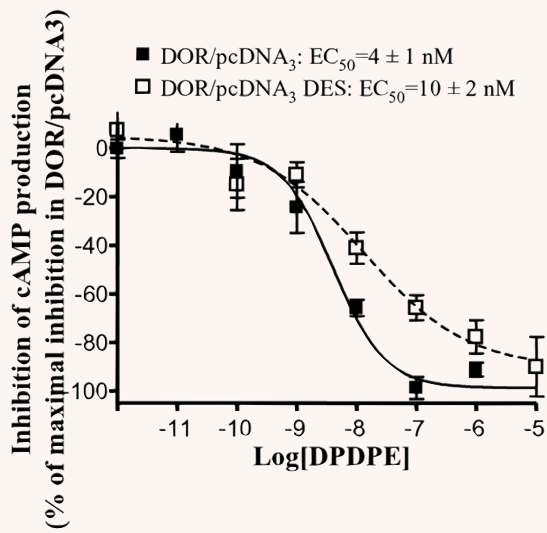




Fig. 2 Src contributes to functional desensitization of DORs. **(A)** Cells stably expressing wild-type DORs were exposed to PP2 (20 μ M; 1 hr) or vehicle and then submitted (DES) or not (CTL) to desensitization by DPDPE (1 μ M; 30 min.). Cells were then washed and used in cAMP accumulation assays to generate dose response curves for DPDPE. Results are expressed as percentage of maximal cAMP inhibition obtained in non-desensitized controls and correspond to at least three experiments carried out in triplicate. Statistical comparison between desensitized and non-desensitized curves was determined by two-way ANOVA. *Left panel:* $P < 0.0001$; $n = 4$; *Right panel:* $P = 0.7$; $n = 3$. Non-desensitized curves carried out in presence of PP2 were not significantly different from corresponding controls. *Inset:* representative example of the effect of PP2 pre-treatment (20 μ M; 1 hr) on Src activation by DPDPE, as measured by phospho-Src immunoreactivity. **(B)** Cells were transiently transfected with DOR/pcDNA3 or DOR/DNM-Src and cAMP assays were performed 48 hrs after transfection. Desensitization, expression of results and statistical analysis as in A. *Left panel:* $P = 0.0002$; $n = 5$. *Right panel:* $P = 0.7$; $n = 5$. Non-desensitized curves carried out in DOR/pcDNA3 and DOR/DNM-Src were not significantly different from each other. *Inset:* representative example of the effect of DNM-Src overexpression on Src activation by DPDPE, measured by Phospho-Src immunoreactivity.

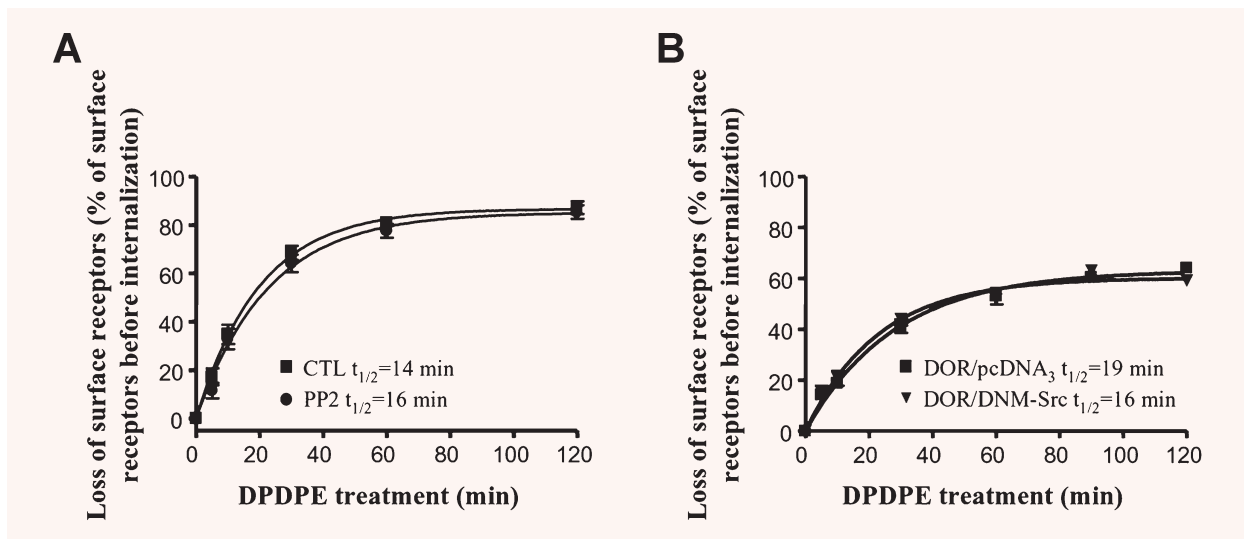


Fig. 3 Src blockers protect from desensitization without modifying DOR internalization. Internalization of surface receptors was measured following incubation with DPDPE (1 μ M) for the indicated periods of time. Presence of DORs at cell surface was measured by ELISA as explained in 'experimental procedures'. Results are expressed as a loss of surface receptors (percentage of surface receptors observed before internalization) and represent mean \pm S.E.M. of seven independent experiments carried out in triplicate. **(A)** DOR internalization was assessed in cells stably expressing wild-type DORs, following exposure to PP2 (20 μ M; 1 hr) or vehicle. **(B)** DOR internalization was assessed in transiently transfected cells expressing DOR/pcDNA3 or DOR/DNM-Src.

increase in recycling might have any consequences concerning desensitization. So we reasoned that if the increase in recycling were to play a significant role in the protective effect of Src blockers, then interfering with recycling would prevent the protective effect exerted by Src inhibitors. Thus, we determined whether protective action of PP2 could be affected by interfering with recycling. In a first series of experiments, we tested the effect of monensin, an ionophore that blocks receptor recycling by trapping internalized receptors within endosomes [38, 42]. Results showed that at a concentration that reduced DOR recycling (50 μ M; Inset Fig. 6A), monensin abolished PP2-mediated protection from desensitization (Fig. 6A). To confirm this observation, cells were transfected with a mutant form of Rab11 (DNM-Rab11) in which substitution of Ser 25 by Asn interferes

with GTP-binding capacity of this small G protein and inhibits recycling [27, 43]. Similar to monensin, a decrease in DOR recycling by DNM-Rab11 (Inset Fig. 6B) abolished the protective effect of Src-blockers upon desensitization (Fig. 6B), confirming that recycling is essential for Src modulation of DOR signalling efficacy.

Src activity interferes with the recovery of DOR-G α interaction after desensitization

Functional desensitization involves uncoupling of the receptor from the G protein [44] while resensitization requires recovery of a physical and functional interaction between the two proteins [40, 45]. Recycling is thought to contribute to this recovery by

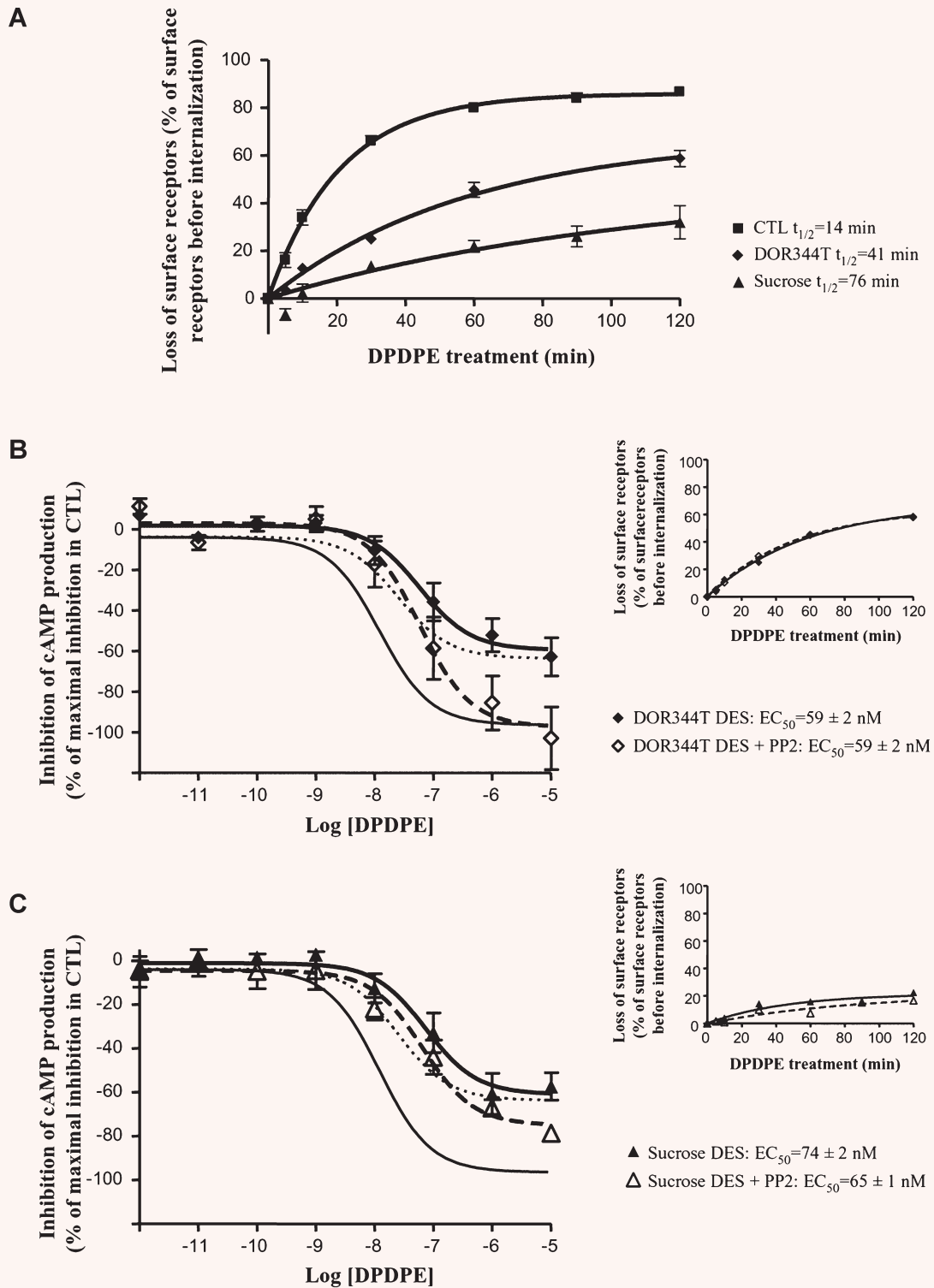




Fig. 4 Normal sequestration is required for Src blockers to optimally prevent desensitization. **(A)** Internalization of surface receptors by DPDPE (1 μ M) was measured in cells stably expressing wild-type DORs that were exposed (sucrose) or not (CTL) to sucrose (0.4 M) as well as in cells stably expressing similar amount of truncated DOR mutants (DOR344T). Internalization assays were performed as in Fig. 3 and results were expressed as a loss of surface receptors (percentage of surface receptors observed before internalization). Values correspond to mean \pm S.E.M. from at least four independent experiments carried out in triplicate. Statistical differences between curves were established by two-way ANOVA. CTL *versus* DOR344T: $P < 0.0001$; $n = 5$; CTL *versus* Sucrose: $P < 0.0001$; $n = 4$. **(B)** Effect of PP2 pre-treatment (20 μ M; 1 hr) on DPDPE-dependent desensitization (1 μ M; 30 min.) was assessed in cells stably expressing truncated DORs (DOR344T). cAMP accumulation assays were performed as in Fig. 2. Results are expressed as percentage of maximal cAMP inhibition obtained in corresponding naive, wild-type controls, and represent values of at least four experiments carried out in triplicate. Statistical comparison between DPDPE dose response curves in cells desensitized in presence or absence of PP2 was done by two-way ANOVA. DOR344T DES *versus* DOR344T DES + PP2: $P = 0.01$; $n = 4$. *Inset*: representative example of the effect of PP2 on internalization of DOR344T. Results were expressed as a loss of surface receptors (percentage of surface receptors observed before internalization). **(C)** Effect of PP2 pre-treatment (20 mM; 1 hr) on DPDPE-dependent desensitization (1 μ M; 30 min.) was assessed in cells stably expressing wild-type DORs that were exposed to sucrose (0.4 M) for the length of the experiment. Results, obtained from at least four experiments carried out in triplicate, are expressed as in B. Statistical analysis: sucrose DES *versus* sucrose DES + PP2: $P = 0.03$; $n = 6$. *Inset*: representative example of the effect of PP2 on internalization of wild-type DORs exposed to sucrose. Results were expressed as a loss of surface receptors (percentage of surface receptors observed before internalization). Thin lines superimposed on graph B and C correspond to dose response curves obtained in naive cells, expressing wild-type DORs, following desensitization in the absence (dashed line) or presence (full line) of PP2.

Table 1 Signalling and desensitization in wild-type DORs, truncated DORs and wild-type DORs exposed to sucrose

	CTL		DES		
	EC ₅₀ (nM)	E _{max} (% of inhibition)	EC ₅₀ (nM)	E _{max} (% of inhibition)	Two-way ANOVA CTL <i>versus</i> DES
CTL ($n = 7$)	5.5 \pm 1.4	64.0 \pm 2.8	31.8 \pm 1.5	42.5 \pm 2.4	$P < 0.0001$
DOR344T ($n = 9$)	5.9 \pm 1.4	60.5 \pm 2.7	19.7 \pm 2.7	43.0 \pm 6.1	$P < 0.0045$
Sucrose ($n = 3$)	74.5 \pm 3.1	42.2 \pm 7.1*	53.4 \pm 1.8	33.2 \pm 3.2	$P < 0.0280$

cAMP accumulation assays were performed in cells stably expressing wild-type DORs that were exposed to sucrose (0.4M) or not (CTL), as well as in cells stably expressing similar amount of truncated DOR mutants (DOR344T). Results are the mean \pm S.E.M. from at least three experiments carried out in triplicate. For each condition, E_{max} values represent the percentage of change with respect to cAMP production in the absence of ligand.

*Two-way ANOVA CTL *versus* sucrose: $P = 0.0007$, $n = 3$

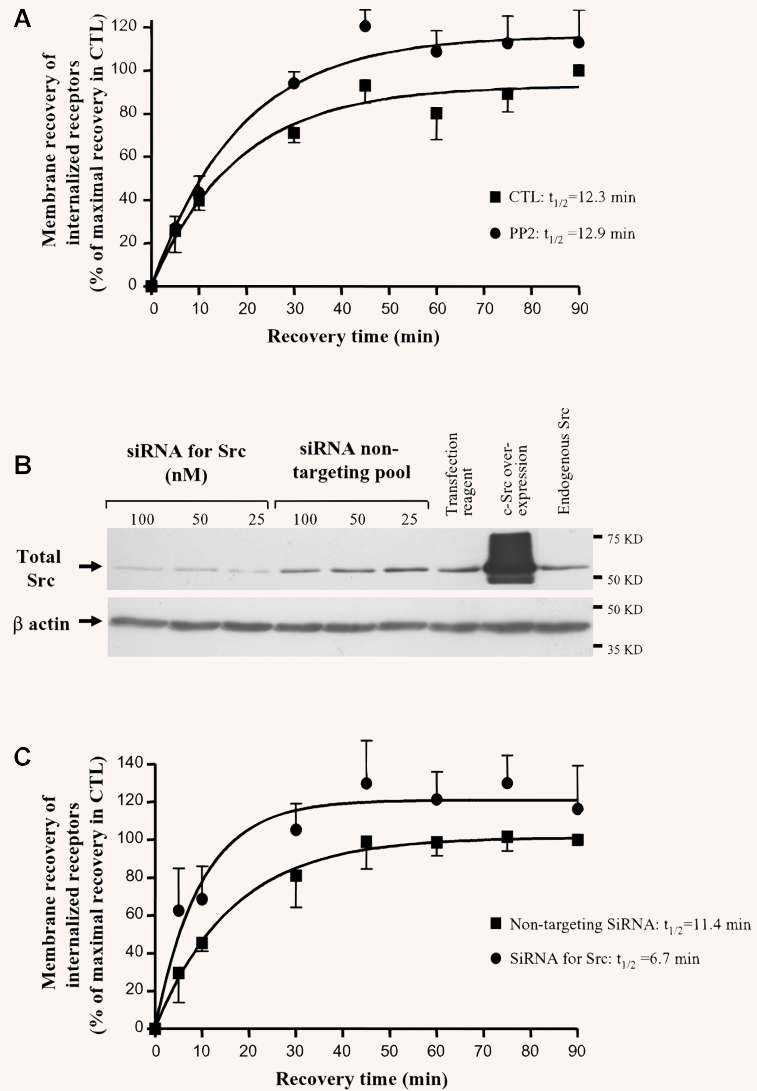
allowing internalized receptors to return to the membrane, and making them available for a new cycle of interaction with their signalling partners [46–48]. Thus, if membrane recycling of DORs is enhanced by interfering with Src function (Fig. 5), Src blockade would be expected to improve recovery of DOR interaction with the α subunit. In order to examine this issue, FLAG-tagged DORs were immunopurified and the amount of G α recovered with the receptor was determined by Western blot analysis. As shown in Fig. 7A, α i3 spontaneously associated with DORs and was susceptible to modulation by the agonist [49]. In particular, exposure to DPDPE for increasing time periods progressively reduced the amount of G α i3 recovered with the receptor (Fig. 7A), but the interaction recovered upon agonist removal (Fig. 7B). Surprisingly, PP2 did not modify DOR- α i3 uncoupling during exposure to DPDPE (1 μ M), but the Src blocker accelerated the recovery of DOR- α i3 interaction after agonist removal (Fig. 7B).

Discussion

Results obtained in this study indicate that the non-receptor tyrosine kinase Src inhibits DOR recycling and by doing so contributes to the desensitization of this receptor. It is well established that only a discrete portion of internalized DORs normally recycles back to the membrane while the great majority of them is targeted for lysosomal degradation [15, 19]. This particular sorting phenotype is encoded in the receptor's primary sequence, which predetermines non-covalent interactions between DORs and lysosomal sorting proteins [18, 22, 50]. By showing that its encoded sorting pattern may be modified by changes in Src activity, our results indicate that post-endocytic sorting of DORs is not irreversibly determined by the primary sequence.

Functional significance of DOR regulation by Src is emphasized by the remarkable ability of PP2 and DNM-Src to eliminate

Fig. 5 Src negatively regulates DOR recycling to the membrane. **(A)** Cells stably expressing wild-type DORs were incubated with PP2 (20 μ M; 1 hr) or vehicle prior to DPDPE exposure (1 μ M; 30 min.) in order to induce internalization. The agonist was then removed and cells allowed to recover for the indicated periods of time before membrane receptors were assessed using an ELISA-based method. Results are expressed as percentage of maximal recovery of internalized receptor in control cells. The data represent mean \pm S.E.M. from seven independent experiments carried out in triplicate. Statistical comparison between curves (CTL *versus* PP2) was assessed using two-way ANOVA ($P = 0.0006$). **(B)** Cells stably expressing wild-type DORs were transfected with the indicated concentrations of siRNA targeted to Src. Non-targeting siRNA in equivalent concentrations was used as control. Src expression was verified by Western blot analysis 48 hrs after transfection, with anti-Src antibody. Last three lanes correspond to additional controls corresponding to Src expression in presence of transfection agent, in cells overexpressing c-Src and in non-transfected HEK cells. The blot was stripped and reprobed for β -actin as a loading control. All samples shown were obtained from the same blot. **(C)** Recycling assays in cells stably expressing wild-type DORs transfected either with 25 nM of targeting or non-targeting Src siRNA. Experiments were carried out as in A. The data represent mean \pm S.E.M. from four independent experiments carried out in triplicates. Statistical comparison between curves (Src targeting siRNA *versus* non-targeting siRNA) was assessed by two-way ANOVA ($P = 0.0054$).



short-term desensitization. The fact that this protection was abolished by monensin and DNM-Rab11 directly links the protective effects of Src blockers to the recycling process, and the observation that Src knock-down by siRNA increased membrane recovery of DORs specifically implicates this kinase in DOR recycling. Specific involvement of Src in DOR regulation is also supported by Western blot data showing that DORs spontaneously interact with a 60-kD protein that is released upon receptor activation and which is selectively recognized by anti-Src antibodies.

The possibility that Src inhibition could enhance membrane targeting of newly synthesized receptors is unlikely since membrane recovery of DORs was assessed in cells whose protein synthesis had been blocked by cycloheximide. On the other hand, the idea that Src contributes to DOR desensitization by regulating their post-endocytic sorting is supported by two observations. First, PP2 ability to prevent desensitization of an internalization-deficient DOR mutant was lower than the ability of this blocker to prevent desensitization of normally internalizing receptors. Second, in cells in which pre-incubation with sucrose produced a more

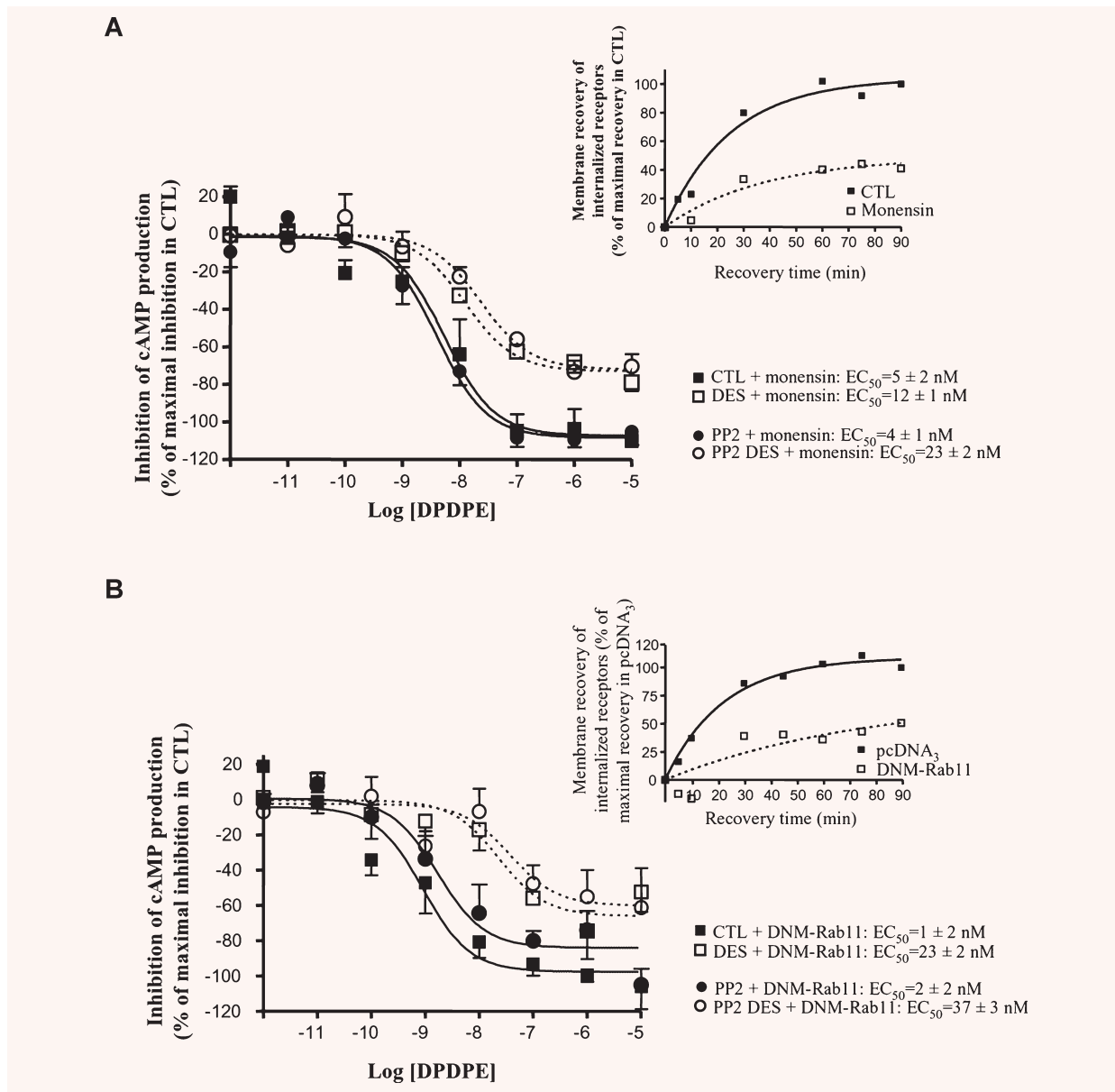


Fig. 6 Src inhibitors require recycling to counter DOR desensitization. **(A)** The effect of PP2 on desensitization of wild-type DORs was assessed as in previous figures with the experiment being conducted in the presence of monensin (50 μ M). Results are expressed as percentage of maximal cAMP inhibition obtained in non-desensitized, non-monensin-treated controls and correspond to three experiments carried out in triplicate. Statistical comparison of DPDPE dose response curves obtained from cells desensitized in presence or absence of PP2 was carried out using two-way ANOVA. DES + monensin *versus* PP2 + DES + monensin: $P = 0.2$; $n = 3$. *Inset*: representative example of DOR membrane recovery in presence or absence of monensin. Experiments were carried out as in Fig. 4 and results are expressed as percentage of maximal recovery of internalized receptor in cells that were not exposed to monensin. **(B)** cAMP assays were performed in transiently transfected cells co-expressing DORs and DNM-Rab11. Results are expressed as in A. DES + DNM-Rab11 *versus* PP2 + DES + DNM-Rab11: $P = 0.7$; $n = 4$. *Inset*: representative example of DOR membrane recovery in presence or absence of DNM-Rab11. Results are expressed as percentage of maximal recovery of internalized receptor in cells expressing the empty vector (pcDNA3).

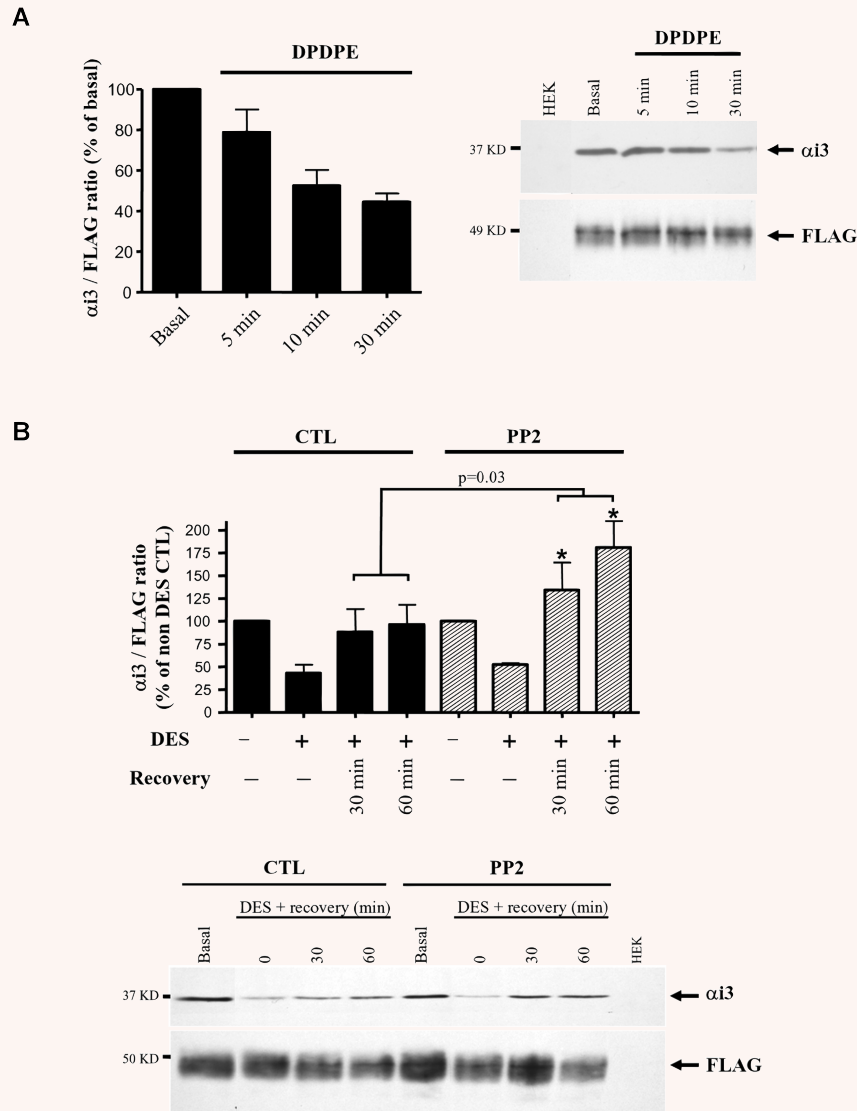


Fig. 7 Src activity interferes with recovery of DOR-G protein interaction upon agonist removal. **(A)** Cells stably expressing FLAG-tagged DORs were exposed to DPDPE (1 μ M) for the indicated time periods prior to DOR immunopurification. The purification product was then separated by electrophoresis SDS-PAGE and subject to successive Western blot analyses with anti- α i3 and anti-FLAG antibody. DOR- α i3 interaction was assessed by calculating the ratio between α i3 and FLAG-immunoreactivity present in each sample. Results were expressed as percentage of α i3/FLAG ratio obtained in basal conditions and represent mean \pm S.E.M. of at least four experiments. Right panel shows a representative example of the time course corresponding to samples ran on the same gel. **(B)** Recovery of DOR- α i3 interaction after desensitization (DES) by DPDPE (1 M; 30 min.) was assessed by monitoring the amount of G α i3 co-purified with DORs following removal of desensitizing agonist for 30 and 60 min. Results represent mean \pm S.E.M. of at least four experiments and are expressed as percentage of α i3/FLAG ratio obtained in non-desensitized cells. Statistical comparison of recovery values in presence or absence of PP2 was performed using a two-way ANOVA and *P*-values appear in the figure. Differences between α i3/FLAG ratio in desensitized *versus* recovery conditions within each group were established by one-way ANOVA followed by Neumann–Keuls *post hoc* test (**P* < 0.05). A representative immunoblot is shown below the histogram, samples were run in parallel and correspond to a single blot.

dramatic decrease in receptor internalization than truncation of the C-terminus, reduction in PP2 protective actions was even more remarkable. Indeed, sucrose produced a short period of complete inhibition of internalization (10 min.) followed by a progressive recover to attain internalization of almost 40% of surface receptors at the end of 2 hrs of agonist exposure. This observation is in agreement with previous reports showing that DORs are internalized through clathrin-dependent and -independent pathways [36], and sequestration *via* the latter most likely provided the source of receptors necessary for maintaining residual protection by PP2 in sucrose-treated cells. Sucrose also reduced DOR responsiveness to acute agonist exposure (Table 1), an observation that is not surprising given that hypertonic medium interferes with constitutive DOR internalization [34], which is necessary for maintaining a steady-state level of active membrane receptors [51, 52]. Interestingly, despite its ability to enhance recycling, PP2 did not modify DOR internalization in steady state assays. Since dynamin phosphorylation by Src has been shown to contribute to internalization of other G-protein coupled receptors [31], one possible explanation for the lack of effect of Src blockers on internalization is that the latter inhibited internalization to the same extent as they enhanced recycling. Another possibility is that steady state internalization assays are not sensitive enough to detect a small increase in recycling. Independent of these alternatives, the functional relevance of the observed increase in recycling was established in experiments in which monensin or Rab11 interfered with the ability of Src blockers to prevent DOR desensitization.

Apart from its negative impact on DOR ability to maintain agonist-dependent signalling, Src had a deleterious effect on physical coupling of the receptor to the α subunit. This was particularly evident when evaluating the effect of Src inhibitor PP2 on the recovery of DOR- α 3 interaction after desensitization. In contrast, given the ability of Src inhibitors to protect DORs from agonist-dependent desensitization it was surprising to find out that PP2 failed to prevent DOR uncoupling from α 3. This apparent contradiction could be explained by the mechanism underlying desensitization. Indeed, Tran and coworkers [48] have recently shown that GPCRs remain phosphorylated in the presence of agonist and have also established that resensitization does not require receptor dephosphorylation. These observations led them to propose that resensitization relies on β -arrestin dissociation and not on removal of phosphate groups. Within this model, resensitization of DOR signalling by recycling would entail membrane reinsertion of receptors that are β -arrestin-free but remain phosphorylated. Being free of β -arrestin allows recycled receptors to interact once again with the G protein. However, their phosphorylation status makes them highly prone to re-binding β -arrestin [53] and compromises the stability of DOR interaction with α 3 subunits. Hence, it is possible that by enhancing recycling Src blockers would promote the formation of DOR- α 3 complexes capable of sustaining signalling but that are too weak to endure sample preparation for immunopurification.

DOR interaction with lysosomal sorting proteins like GASP1 and SNX-1 takes place few amino acids downstream of the NPX_nY domain [18, 22, 50]. The tyrosine residue within this sequence has been identified as the major target for DOR phosphorylation

by Src [54]. Hence, modifications in the phosphorylation status of the NPX_nY are expected to influence receptor interaction with either of the lysosomal sorting proteins, providing a biochemical substrate for Src modulation of DOR recycling. Src activity may also be directed towards sorting proteins themselves, a possibility that is consistent with results from the present study showing that agonist binding destabilizes the constitutive association between DORs and the kinase. A plausible substrate for free Src is Hrs (Hepatocyte growth factor-regulated tyrosine kinase substrate) [55], which has been recently shown to participate in GPCR recycling and resensitization [56, 57]. A major goal for future study is to determine if and how Src regulates Hrs activity and DOR interaction with sorting proteins.

Immunopurification of unstimulated DORs allowed to recover both Src and α 3 subunits, indicating that the three proteins form part of a constitutive complex. The ability to form spontaneous multimeric arrays containing receptors and their signalling partners is being increasingly recognized as a general characteristic of GPCRs [58, 59], and this property has been recently confirmed for DORs [60]. However, spontaneous association between Src and DORs distinguishes them from other GPCRs whose interaction with the kinase only takes place upon receptor activation [32, 61–63]. As a matter of fact, DOR activation rapidly disrupts its association with Src, an observation that is highly reminiscent of the way agonist binding causes calmodulin to be released from this receptor [64]. Src release from DORs was concomitant with its activation and both events were sensitive to PTX, suggesting that organization within a constitutive complex provides physical proximity to ensure rapid Src activation by G α i/o proteins. Within this context, a possible scenario for Src activation would entail the following series of events: agonist binding to the receptor induces conformational changes that modify protein–protein interactions within the DOR-Src-G protein complex leading to G α i/o activation, Src stimulation and loosening of DOR interaction with the kinase. Released from the sterical constraints imposed by its association with the complex, Src is now able to phosphorylate its substrates, which may include sorting proteins and/or DORs themselves [23]. In addition to modifying agonist-mediated responses incubation with PTX also increased basal Src activity, but this effect was not accompanied by any visible changes in complex stability. Src is a target for PKA [65] and the observed increase in basal Src stimulation could be related to an increase in basal cAMP levels following G α i/o protein inactivation by PTX. However, it should be noted that Src activation by PTX was consistently lower than its activation by DPDPE (\cong 1/3), excluding the possibility that the absence of agonist-mediated responses could be linked to pre-existing maximal Src activity.

In conclusion, this study showed that Src modulates post-endocytic sorting of DORs, contributing to rapid desensitization of these receptors. The idea that DOR trafficking may be dynamically regulated raises the possibility of pharmacologically altering its sorting pattern to enhance recycling and counter desensitization. Analgesic tolerance to opiates is intrinsically linked to desensitization and any intervention that could prevent this adaptive response should improve clinical efficacy of these powerful analgesics.

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