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Quantitative evaluation of human delta opioid receptor desensitization using the Operational Model of Drug Action

by

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Running title: Quantitative evaluation of receptor desensitization

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Abbreviations: DOR, delta opioid receptor; hDOR, human delta opioid receptor; GPCR, G protein-coupled receptor; GTPγS, guanosine 5'[γ-thio] triphosphate; PKA, protein kinase A; GRK, G protein-coupled receptor kinase; PBS, phosphate buffered saline; PD, Ca^{2+}, Mg^{2+} deficient phosphate-buffered saline; TE, Tris-EDTA; IMDM, Iscove's modified Dulbecco's medium; CHO, chinese hamster ovary; HEK 293, human embryonic kidney 293; Fmoc, fluorenylmethyloxycarbonyl; GFP, green fluorescent protein.

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ABSTRACT

Agonist-mediated desensitization of the opioid receptors is thought to function as a protective mechanism against sustained opioid signaling and therefore may prevent the development of opioid tolerance. However, the exact molecular mechanism of opioid receptor desensitization remains unresolved, due to difficulties in measuring and interpreting receptor desensitization. In the present study, we investigated deltorphin II-mediated rapid desensitization of the human delta opioid receptors (hDOR) by measuring GTPγ[^{35}S] binding and inhibition of cAMP accumulation. We developed a mathematical analysis based on the Operational Model of Agonist Action (Black et al., 1985) to calculate the proportion of desensitized receptors. This approach permits a correct analysis of the complex process of functional desensitization by taking into account receptor-effector coupling and the time dependence of agonist pre-treatment. Finally, we compared hDOR desensitization with receptor phosphorylation at S363, the translocation of β-arrestin2, and hDOR internalization. We found that in Chinese hamster ovary (CHO) cells expressing the hDOR, deltorphin II treatment leads to phosphorylation of S363, translocation of β-arrestin2 to the plasma membrane, receptor internalization and uncoupling from G proteins. Interestingly, mutation of the primary phosphorylation site S363 to alanine had virtually no effect on agonist-induced β-arrestin2 translocation and receptor internalization, yet significantly attenuated receptor desensitization. These results strongly indicate that phosphorylation of S363 is the primary mechanism of hDOR desensitization.

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INTRODUCTION

A major obstacle in clinical use of the opioids is the development of analgesic tolerance. It is hypothesized that agonist-mediated desensitization of the opioid receptors may protect the organism from sustained opioid signaling and thus prevent the development of tolerance. Opioid agonist-mediated receptor desensitization is classified on the basis of the duration as rapid or prolonged, and on the basis of the mechanism triggering desensitization as homologous (caused by activity of the same receptor molecule) or heterologous (caused by activation of other receptors). In this study we examined the molecular mechanisms of the rapid homologous desensitization of the human delta opioid receptor (hDOR).

Stimulation of the delta opioid receptor (DOR) by an agonist, leads to activation of inhibitory Gi/o proteins, and dissociation of Giα subunits from βγ dimers (Quock et al., 1999). Released βγ dimers facilitate the binding of G protein-coupled receptor kinases (GRK) to the activated receptor (Li et al., 2003). GRKs then phosphorylate the activated receptor at several Ser/Thr residues. Among these residues S363 within the C-terminus of the DOR has been identified as the primary phosphorylation site (Kouhen et al., 2000). Agonist-bound phosphorylated receptors display high affinity for the cytosolic adaptor proteins, β-arrestins. Binding of β-arrestins facilitates the targeting of the receptor to clathrin-coated pits and receptor internalization via the endocytosis of the clathrin-coated vesicles. The internalization process may lead either to the degradation of the receptor in lysosomes, thereby terminating receptor function; or to re-sensitization by recycling of

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the dephosphorylated receptor to the plasma membrane. The functional consequences of these regulatory mechanisms is the modulation of receptor signaling (Daaka et al., 1997; Hall and Lefkowitz, 2002).

Therefore, rapid homologous desensitization of the DOR involves multiple regulatory mechanisms such as GRK-mediated receptor phosphorylation, β-arrestin binding and receptor internalization. However, since these regulatory events are all causally dependent on each other, it is not clear which is the key process that is directly responsible for DOR desensitization. In earlier studies, it was found that phosphorylation of the endogenous hDOR in human neuroblastoma SK-N-BE cells correlates with receptor desensitization (Hasbi et al., 1998) and that DOR desensitization occurs under conditions impairing receptor internalization (Hasbi et al., 2000; Willets and Kelly, 2001). In contrast, Law and coworkers demonstrated that both phosphorylation and internalization contribute to DOR desensitization in HEK 293 cells (Law et al., 2000). High affinity binding of β-arrestin to the receptor is expected to sterically interfere with G protein coupling, thereby attenuating G protein signaling (Gurevich and Gurevich, 2004). In some studies β-arrestin recruitment has been considered a hallmark of the homologous receptor desensitization (Barak et al., 2006). However, the direct role of β-arrestin binding in DOR desensitization has not yet been studied in detail.

As reviewed above, significant inconsistencies exist in the interpretation of the relationship between different DOR regulatory events and DOR desensitization. In addition, there is no clear agreement on the definition of the term “receptor

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desensitization". Usually, receptor desensitization is interpreted as molecular changes occurring at the level of the receptor, but it is measured at the level of the receptor's function. The functional effect, however, depends not only on the functional status of the receptor but also on the signal transduction amplification between the receptor and the effector (Trzeciakowski, 1999). We hypothesize that some of the difficulties in interpreting desensitization experiments are due to the non-linearity in receptor/effector coupling and could be prevented if the number of desensitized receptors is correctly determined.

The goal of the present study was: 1) to quantitatively evaluate the proportion of hDOR molecules desensitized after treatment with a delta opioid agonist, deltorphin II; and, 2) to investigate the role of delta opioid receptor phosphorylation at S363, β-arrestin binding and receptor endocytosis in rapid hDOR desensitization. To perform detailed analyses of hDOR functions, we used a well characterized cellular model system, Chinese hamster ovary (CHO) cells stably expressing either the wild-type hDOR or a phosphorylation deficient mutant, in which S363 was mutated to alanine [hDOR(S363A)]. Receptor desensitization by deltorphin II, was measured in two second messenger assays: stimulation of GTPγ[^{35}S] binding and inhibition of forskolin-stimulated cAMP production. The results were analyzed using a modified version of the Operational Model of Agonist Action (Black et al., 1985). Our data demonstrate that: 1) the developed mathematical model can be used to analyze a complex phenomenon, such as the time-course of receptor desensitization, and correctly calculate the proportion of desensitized receptors; and, 2) although a mutation of the primary phosphorylation site, S363, does not

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prevent β-arrestin2 recruitment and receptor internalization, it significantly attenuates hDOR desensitization.

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MATERIALS AND METHODS

Mutagenesis of the cloned hDOR and generation of the recombinant CHO cell lines. A Ser363Ala mutation was introduced into the human delta opioid receptor cDNA using the QuikChange site directed mutagenesis kit (Stratagene). Chinese hamster ovary (CHO) cell lines stably expressing the wild type hDOR or mutant hDOR(S363A) were generated and characterized as previously described [clone 1-209-19hDOR/CHO, (Malatynska et al., 1996), clone hDOR(S363A)/CHO#13, (Navratilova et al., 2004), respectively]. Receptor expression, determined by [^{3}H] naltrindole radioligand binding, reached 1.8 pmol/mg protein, for the wild type hDOR and 2.0 pmol/mg protein, for the mutant hDOR(S363A).

Cell culture and deltorphin II treatment. Recombinant Chinese hamster ovary cells expressing the wild-type or (S363A) mutant hDOR were grown and maintained in Ham's-F12 medium (Sigma) containing 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and either 800 µg/ml hygromycin or 500 µg/ml G418, respectively, at 37°C in a 5% CO_2 humidified atmosphere. For desensitization studies, the cells were pretreated with 100 nM of (D-Ala^2) deltorphin II (Tocris) for 0-60 min in serum free IMDM medium (Invitrogen).

GTPγ[^{35}S] binding. Deltorphin II-stimulated binding of GTPγ[^{35}S] to crude membranes prepared from control and agonist pretreated hDOR/CHO or hDOR(S363A)/CHO cells was determined as previously described (Quock et al., 1997). Briefly, cell monolayers

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were washed with Ca^{2+}, Mg^{2+} deficient phosphate-buffered saline (PD) and harvested in PD buffer containing 0.02% EDTA. After centrifugation at 1500 × g for 10 min, the cells were homogenized in ice-cold 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 buffer. A crude membrane fraction was collected by centrifugation at 40,000 × g for 15 min, and resuspended in GTPγS assay buffer (25 mM Tris-HCl, 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 μM GDP, 30 μM bestatin, 10 μM captopril and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). Approximately 50 μg of membrane preparation were incubated at 30°C for 90 min in 1 ml GTPγS assay buffer, with appropriate concentrations of deltorphin II, in the presence of 0.1 nM guanosine 5'[γ-[^{35}S] thio] triphosphate (GTPγ[^{35}S]) (1250 Ci/mmol, PerkinElmer Life Sciences). The reaction was filtered using a Brandel Cell Harvester through Whatman GF/B glass fiber filters (Brandel, Inc.). The filters were washed three times with 4 ml ice-cold 25 mM Tris-HCl, 120 mM NaCl, pH 7.4. Filter-bound radioactivity was measured in EcoLite scintillation cocktail (ICN Biochemicals) using a Beckman LS 6000SC liquid scintillation counter.

Inhibition of forskolin-stimulated cAMP formation. Cyclic AMP assays were performed according to a method modified from Gilman (Gilman, 1970). CHO cells expressing the wild-type or S363A mutant hDOR were plated in 24-well plates and grown in Ham's F12 media containing 10% fetal calf serum and antibiotics to ~ 200,000 cells/well confluency. On the day of the experiment, cells were rinsed with IMDM and incubated in the presence or absence of 100 nM deltorphin II. After the appropriate time, the cells were washed three times (10 min each) with IMDM, to remove the agonist. Deltorphin II inhibition curves were measured by stimulating the cells with 100 μM

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water-soluble forskolin (7-deacetyl-7-[O-(N-methylpiperazino)-γ-butyryl]-dihydrochloride, Calbiochem) in the presence of various concentrations of the agonist and 5 mM 3-isobutyl-methylxanthine (Sigma) for 20 min, at 37°C, in 5% CO_2 humidified atmosphere. The reaction was terminated by rinsing once with ice-cold IMDM, placing the plates on ice and replacing the media with ice-cold TE buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.5). Cells were dislodged from the wells using a Costar cell scraper, transferred to microcentrifuge tubes and lysed by boiling for 10 min. Tubes were centrifuged in a bench top centrifuge for 3 min. 50 µl of each supernatant and 50 µl of a standard (0.125-128 pmol of cAMP) were separately incubated with 50 µl (0.9 pmol/50 µl) of [^{3}H]cAMP (PerkinElmer Life Sciences) and 100 µl of protein kinase A solution (6 µg PKA/100 µl in TE buffer with 0.1% BSA) on ice for 2 h. Bound and free [^{3}H]cAMP was separated by adding 100 µl of activated charcoal in ice-cold TE buffer containing 2% bovine serum albumin to each tube, and centrifuging at 5600 × g for 1 min. The radioactivity in a 200 µl aliquot from each supernatant was counted in a Beckman LS 6000SC liquid scintillation counter. The amount of cAMP in the samples was determined by interpolating the cAMP standard curve using GraphPad Prizm 4 software.

Western blot analysis. hDOR/CHO or hDOR(S363A)/CHO cells were treated for indicated time intervals with 500 nM deltorphin II at 37°C in a serum-free IMDM medium. After agonist treatment the cells were harvested in 20 mM Tris buffer, containing 2 mM EDTA, 4 mM EGTA, protease- and phosphatase inhibitors (Sigma), sonicated and boiled in reducing NuPAGE sample buffer (Invitrogen). Equal amounts of sample proteins (as determined by Bradford assay) were resolved on 10% polyacrylamide

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gels and transferred to nitrocellulose membranes. The membranes were incubated with an antibody raised against a synthetic peptide corresponding to residues around phospho-S363 of the human DOR (phospho-hDOR(S363) antibody, Cell Signaling). Immuno-reactive bands were detected using the SuperSignal West Dura chemiluminescent kit (Pierce).

Synthesis of a fluorescent deltorphin II analog. [Gln^4]deltorphin-rhodamine was synthesized by solid phase synthesis using the Nα-Fmoc strategy. [Gln^4]deltorphin (Tyr-D-Ala-Phe-Gln-Val-Val-Gly) was conjugated to a fluorescent label, tetramethylrhodamine isothiocyanate (Molecular Probes) via the N$^{\varepsilon}$-Lys residue present in the β-Ala-Gly-β-Ala-Gly-Lys spacer that was included at the C-terminus of [Gln^4]deltorphin. In whole cell radioligand binding assay the rhodamine labeled [Gln^4]deltorphin exhibited a high affinity for the hDOR with an IC_{50} = 11 ± 2 nM.

Confocal microscopy. For visualization of β-arrestin2 recruitment, CHO cells expressing the wild-type or S363A mutant hDOR were grown on glass bottom chamber slides and transiently transfected with a green fluorescent protein-tagged β-arrestin2 construct (β-arrestin2-GFP, gift from Dr. Lefkowitz) using the FuGENE 6 transfection protocol (Roche Diagnostics). 48 h after transfection, the cells were left untreated or treated with 500 nM deltorphin II for 5 min at 37°C. For visualization of receptor internalization, the hDOR/CHO or hDOR(S363A)/CHO cells were incubated in serum-free IMDM medium with 1 μM [Gln^4]deltorphin-rhodamine for 30 min at 37°C. Selective DOR antagonist naltrindole (1 μM, IC_{50} = 40 pM, Research Biochemicals

International) was added to some wells to determine DOR specificity. After treatment, the cells were rinsed with ice-cold PBS, fixed with 4% paraformaldehyde and mounted in a Vectashield Mounting Medium (Vector Laboratories). Slides were examined under a Zeiss LSM520 laser scanning confocal microscope equipped with a 100×/1.40 oil objective using excitation/emission filter sets 488/505 nm or 543/560 nm. Single optical sections were acquired through the trans-nuclear plane. The acquisition parameters were constant in all parallel experiments. The images were processed using the Adobe Photoshop 7.0 software.

Data analysis. In order to quantitatively analyze the magnitude of hDOR desensitization, the measured levels of GTPγ[^{35}S] binding or cAMP formation were normalized to 0% in the absence of the agonist and to 100% at saturating amounts of the agonist in untreated (control) cells. In GTPγ[^{35}S] binding experiments, deltorphin II-stimulated levels of bound GTPγ[^{35}S] (B) were first adjusted by subtracting the basal levels (B_0) and then normalized to the percent of adjusted maximum stimulation (B_{max}) in control cells according to the equation: Effect = $(B - B_0)/ [B_{max}(con) - B_0(con)] \times 100\%$.

In cAMP experiments, the residual level of cAMP production in control cells, which was not inhibited by saturating concentrations of deltorphin II ($P_{max}(con)$), was first subtracted from all dose-response curves for deltorphin II-mediated inhibition of cAMP production (P). Subsequently, each dose response curve was normalized to 100% forskolin-stimulated levels in the absence of the agonist (P_0) and expressed as an increase in cAMP inhibition, rather than a decrease in cAMP production, in accordance with the equation:

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Effect = $(P_0 - P)/[P_0 - P_{max}(con)] \times 100\%$. The average maximum level of forskolin-stimulated cAMP was 37 ± 12 and 30 ± 9 pmol/100,000 cells in hDOR/CHO and hDOR(S363A)/CHO cells, respectively. The residual level of cAMP was 12 ± 5 and 14 ± 5 pmol/100,000 cells in hDOR/CHO and hDOR(S363A)/CHO cells, respectively. During the selected agonist treatment time interval (0-60 min), opioid agonist treatment did not lead to measurable augmentation of maximal forskolin-stimulated cAMP formation (i.e. did not cause adenylyl cyclase superactivation) (Varga et al., 2003).

To evaluate the proportion of desensitized receptors we used the Operational Model of Drug Action developed by Black and collaborators (Black et al., 1985). This model describes the correlation between a biological effect E and agonist concentration [A] as a function of three parameters: E_m, K_A and τ:

$$E = \frac{E_m \tau [A]}{K_A + [A] + \tau [A]} \quad (1)$$

where E_m, or the operational maximum, represents the maximum possible effect in the tissue, K_A is the dissociation constant of the agonist and τ is the operational efficacy or the transducer ratio. Parameter τ is defined as:

$$\tau = [R]_T / [K_E] \quad (2)$$

where $[R]_T$ is the total concentration of receptors and $[K_E]$ is the concentration of occupied receptors required to produce half of the maximum E_m. The operational model describes how both E_{max} and EC_{50} change when either the total concentration of receptors $[R]_T$, or the concentration of receptors $[K_E]$ necessary to produce half of the operational maximum (E_m) changes. The dependence of the effect on $[R]_T$ was used earlier to

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estimate the proportion of β_2 adrenergic (Lohse et al., 1990) or μ opioid receptors (Osborne and Williams, 1995) desensitized by agonist pretreatment.

Using the approach of Lohse (Lohse et al., 1990), a paired set of dose-response curves obtained pre- and post-desensitization was fitted simultaneously using equation (1). The parameters E_m and K_A characterize the cellular system and the agonist and therefore do not change with the number of desensitized receptors. The only parameter that distinguishes the pre- and post-desensitization curves is the operational efficacy (τ). Since τ is proportional to the number of functional (non-desensitized) receptors, the fraction of the desensitized receptors D can be calculated as:

$$D = 1 - \frac{\tau_{desensitized}}{\tau_{control}} \quad (3)$$

In this equation, $\tau_{control}$ is the initial operational efficacy in the non-desensitized state and $\tau_{desensitized}$ is the operational efficacy after a fraction of receptors have been desensitized (Lohse et al., 1990). All data were fitted and statistically analyzed using GraphPad Prizm 4 software.

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RESULTS

Agonist-mediated hDOR desensitization. To study rapid desensitization of the human delta opioid receptor, we used recombinant Chinese hamster ovary (CHO) cells expressing the human delta opioid receptor (hDOR/CHO, (Malatynska et al., 1996)) and measured the effects of deltorphin II pretreatment on two different cellular functions. (1) We measured concentration-response curves for deltorphin II-stimulated GTPγ[^{35}S] binding in cell membranes isolated from untreated (non-desensitized) cells or cells that were pre-treated (desensitized) for increasing time intervals with deltorphin II (100 nM). (2) In separate sets of experiments, we measured deltorphin II dose-response curves for the inhibition of forskolin-stimulated cAMP production in untreated and deltorphin II pretreated cells.

As seen in Fig. 1A, the concentration curves of deltorphin II-stimulated GTPγ[^{35}S] binding shifted to the right upon deltorphin II pretreatment of hDOR/CHO cells. In addition, at longer agonist treatment times maximal GTPγ[^{35}S] binding was also reduced compared to the control cells. The deltorphin II-mediated shift in EC_{50}, and the reduction of E_{max} were dependent on the time of pretreatment (Table 1). Similarly, Fig. 1B demonstrates that in agonist pretreated cells, deltorphin II was less efficacious and less potent in inhibiting forskolin-stimulated cAMP production as compared to the control cells. This effect was also dependent on the time of deltorphin II pre-treatment (Table 1). The reduced cellular response to deltorphin II, as measured by either GTPγ[^{35}S] binding or inhibition of cAMP accumulation, reflects a decreased ability of the pretreated

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receptors to couple to G proteins. Our results are in agreement with previous studies, which found that DOR signaling is desensitized upon agonist treatment. However, although qualitatively important, this observation does not provide any quantitative measure of the number of desensitized receptors.

Modification of the Operational Model of Drug Action to calculate the kinetics of receptor desensitization. Since, in our system, receptor desensitization leads to a reduction in E_{max} as well as an increase in EC_{50}, both of these changes need to be considered in order to correctly evaluate the proportion of desensitized hDOR. In this circumstance, the reduction of the maximal effect after desensitization is not proportional to the number of desensitized receptors. Therefore, changes occurring at the receptor level, like receptor phosphorylation or internalization, cannot be directly correlated with the measured changes in the maximal effect. To obtain a quantitative estimate of the number of desensitized receptors we employed the Operational Model of Drug Action, which calculates the operational efficacy τ (and thus the receptor number) based on changes in both E_{max} and EC_{50}, as explained in "Materials and Methods" (equation (1)). To analyze data from experiments involving the time-course of receptor desensitization, we have modified the operational model by adding restrictions that should be fulfilled for the parameter τ. In the simplest model, we can assume that the number of functional receptors $[R]_T$, and consequently τ, decreases exponentially as a function of the time of the pretreatment, according to:

$$\tau(t) = \tau_0 \exp(-kt) + \tau_\infty \qquad (4)$$

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where k is the rate constant of desensitization, τ_∞ is the asymptotic value of τ approached when the maximum number of receptors has been desensitized. ($\tau_0 + \tau_\infty$) = $\tau_{control}$ is the initial τ value in non-desensitized cells. We can now introduce this restriction on parameters τ into equation (1) and obtain more meaningful estimates of the time course of receptor desensitization, which will allow us to calculate the rate of receptor desensitization and the maximum level of desensitization at equilibrium. Substituting $\tau(t)$ from equation (4) into equation (1) yields:

$$E = \frac{E_m\{\tau_0 \exp(-kt) + \tau_\infty\}[A]}{K_A + [A]\{1 + \tau_0 \exp(-kt) + \tau_\infty\}} \qquad (5)$$

This equation is a five-parameter equation, which describes a whole series of dose-response curves as a function of one variable, the time of deltorphin II pretreatment. Fig. 2 illustrates the theoretical dependence of the dose-response curves on the time of pretreatment as predicted from equation (5). The theoretical curves were generated using arbitrary values E_m = 100, τ_0 = 20, τ_∞ = 1, K_A = 1 nM and k = 0.1 min^{-1}, which were chosen to describe a realistic cellular model. The selected value of τ_0 = 20 describes a cellular system with significant receptor reserve. In this situation, E_{max} of the dose response curve for non-desensitized receptors approaches the theoretical operational maximum E_m and the EC_{50} is smaller than K_A. As τ decreases with increasing time of pretreatment, EC_{50} increases, initially with little change in the E_{max}. After an extended pretreatment period, a decrease in E_{max} becomes apparent and is accompanied by further increase in EC_{50} to the asymptotic value K_A. In systems with no receptor reserve the maximum effect (E_{max}) does not reach the theoretical maximum E_m in the non-desensitized state. Therefore, an attenuation of the E_{max} is already apparent when only a

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small proportion of the receptors have been desensitized. However, since the reduction in E_{max} is always accompanied by an increase in EC_{50}, the reduction in E_{max} alone will underestimate the proportion of desensitized receptors. This emphasizes the need for using changes in both E_{max} and EC_{50} when evaluating desensitization experiments. The presented analysis expands the Operational Model of Agonism to calculate the kinetics of receptor desensitization. A similar approach can also be applied to calculate desensitization under various experimental conditions, for any GPCR.

Quantitative evaluation of the kinetics of hDOR desensitization using the modified Operational Model of Drug Action. The experimental data for the time-course of receptor desensitization were analyzed using equation (5), with the parameters E_m and K_A shared for all time points (0-60 min). For curves corresponding to times (0-45 min), τ is defined by equation (4) in which τ_0 and τ_∞ were fitted shared parameters; for the 60 min curve $\tau = \tau_{60}$ was fitted independently since at this time long-term desensitization mechanisms may already be present. In Figs. 3A and 3B the data obtained for the time course of hDOR desensitization (Figs. 1A and 1B, respectively) were fitted using equation (5). All five parameters (E_m, τ_0, τ_∞, K_A and k) were shared among all dose-response curves for GTPγ[^{35}S] binding and again in a separate calculation using inhibition of cAMP production. The values obtained for these parameters for the GTPγ[^{35}S] and the cAMP assays are summarized in Table 2. The initial value of $\tau_{control} = (\tau_0 + \tau_\infty)$ is reported in Table 2 instead of the fitted τ_∞ value.

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From Table 2 we can observe the fitted operational maximum E_m is 120 ± 5% and 106 ± 4% for the GTPγ[^{35}S] and cAMP assays, respectively. In GTPγ[^{35}S] measurements the operational efficacy in control cells $\tau_{control} = (\tau_0 + \tau_\infty)$ was 7.8 ± 2.4. Since by definition $\tau = [R]_T/[K_E]$, we can conclude that the expression of the receptors in our cells is approximately 7.8 times higher than the number of receptors necessary to produce half of the operational maximum E_m. Consequently, some receptor reserve exists in our cellular system for the GTPγ[^{35}S] stimulation assay. After deltorphin II pretreatment, the operational efficacy τ is reduced and, at infinity, would reach the value 1.4 ± 0.5. The operational efficacy measured by the cAMP assay $\tau_{control} = (\tau_0 + \tau_\infty) = 36 \pm 21$ is higher than that measured by the GTPγ[^{35}S] assay. The expression of the receptors in our cells is approximately 36 times higher than the number of receptors necessary to produce half of the operational maximum E_m in the cAMP assay. This supports the notion that signal amplification measured by the cAMP assay occurs at signal transduction steps downstream of G protein activation. Consequently, even when a significant portion of the receptors is desensitized upon agonist treatment, only a small reduction in E_{max} is observed in cAMP assay and the predominant effect of desensitization is an increase of the EC_{50} values. Importantly, the desensitization (D) calculated using equation (3) correlates very well when comparing the two assays (82% for both GTPγ[^{35}S] and cAMP assays), confirming that this parameter is not dependent on the choice of the measured effect. The half-life of receptor desensitization ($t_{1/2}$) was estimated using equation $t_{1/2} = 0.693/k$, and was 9.8 min and 8.4 min, in the GTPγ[^{35}S] and cAMP assays, respectively. The theoretical dissociation constant (K_A) of deltorphin II estimated from the two assays was 81 ± 16 nM and 16 ± 8 nM, respectively. Values of K_A are about 10 -50 fold larger

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than the EC_{50} values measured in untreated cells, again supporting the presence of spare receptors.

S363 in the hDOR is phosphorylated upon deltorphin II treatment. The molecular mechanisms underlying receptor desensitization remain controversial. It is currently accepted that several mechanisms may contribute to rapid receptor desensitization including receptor phosphorylation, binding of β-arrestin and receptor internalization. In HEK 293 cells phosphorylation of the mouse DOR is hierarchical with S363 in the C-terminal region being the primary phosphorylation site (Kouhen et al., 2000). In addition, mutation of S363 to Ala was shown to reduce agonist-mediated uncoupling of the receptor from adenylyl cyclase signaling (Law et al., 2000). Since receptor phosphorylation leads to β-arrestin recruitment and receptor internalization, it is not clear which of these mechanisms is crucial for hDOR desensitization.

To investigate the role of S363 phosphorylation in the desensitization of the human DOR we mutated S363 to alanine and created a CHO cell line stably expressing the mutant hDOR(S363A) receptor (Navratilova et al., 2004). We used Western blot analysis with a specific phospho-hDOR(S363) primary antibody (Cell Signaling) to investigate agonist-mediated S363 phosphorylation. Fig. 4A shows that treatment of hDOR/CHO cells in increasing time intervals with deltorphin II (500 nM) leads to the phosphorylation of the hDOR at S363. Receptor phosphorylation was detected as an increased intensity of several immunoreactive bands in the molecular weight range of 50-70 kDa, which correspond to differently (N-linked and/or O-linked) glycosylated forms of the hDOR

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(unpublished data). We have demonstrated previously (Navratilova et al., 2005) that this phosphorylation is completely blocked by concomitant treatment of the cells with a selective delta opioid receptor antagonist, naltrindole (1μM). As expected, no immunoreactive bands were observed upon treatment of the mutant hDOR(S363A) expressing cells with deltorphin II (Fig. 4B). The intensities of the 50-70 kDa bands were analyzed using the Image J software (NIH) and plotted as a function of the time of deltorphin II treatment. The early phase (0-30 min) of receptor phosphorylation was fitted with an exponential association curve (Fig. 4C). The half-life of receptor phosphorylation estimated from the fitted curve is $t_{1/2}$ = 1.7 ± 0.2 min. These experiments provide evidence that deltorphin II promotes time dependent phosphorylation of hDOR at S363 and confirm that this phosphorylation is eliminated by mutating S363.

S363A mutation attenuates hDOR desensitization. Next we investigated the effect of S363A mutation on the hDOR desensitization. Using the GTPγ[^{35}S] binding assay, we showed that hDOR(S363A) mutant receptor was desensitized by deltorphin II pretreatment to a much lesser extent than the wild-type hDOR, as evident from a smaller shift of the dose-response curve (Fig. 5A). This finding was reproduced by measuring deltorphin II dose-response curves for inhibition of cAMP production in hDOR/CHO and hDOR(S363A)/CHO cells (Fig. 5B). In order to evaluate the statistical significance of these findings we fitted the observed data with sigmoidal concentration-response curves using the Hill coefficient equal to 1, and compared all curves using the extra sum-of-squares F test calculated with GraphPad Prizm 4. The resulting E_{max} and EC_{50} values for the individual curves are shown in Table 3. We found that the control dose-response

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curves for untreated wild-type and mutant receptors were not significantly different in either GTPγ[^{35}S] or cAMP assay, confirming that the S363A mutation had no effect on acute receptor signaling. Importantly, in hDOR/CHO cells the shift in dose-response curves upon deltorphin II pretreatment was significant (EC_{50} increased 4.5-fold from 6.9 to 31 nM in GTPγ[^{35}S] assay, $p<0.0001$ and 5.5-fold from 1.4 to 7.5 nM in cAMP assay, $p<0.05$). Conversely, in hDOR(S363A)/CHO cells the shift upon deltorphin II pre-treatment was much smaller (2.3-fold from 5.8 to 13 nM and 1.4-fold from 2.0 to 2.8 nM in GTPγ[^{35}S] and cAMP assay, respectively). This shift was statistically significant in GTPγ[^{35}S] assay ($p<0.001$) but did not reach statistical significance in cAMP assay ($p=0.48$). Finally, the difference between the deltorphin II-mediated shift in hDOR/CHO cells and hDOR(S363A)/CHO cells was statistically significant for both assays ($p<0.0001$). These analyses clearly show that pretreatment of the hDOR/CHO cells for 60 min with 100 nM deltorphin II causes desensitization of G protein signaling which is significantly attenuated in hDOR(S363A)/CHO cells. This finding identifies S363 as an important residue for hDOR desensitization.

To estimate the proportion of the wild-type and S363A mutant receptors desensitized by agonist pretreatment, we applied the Operational Model of Agonist Action (Black et al., 1985) as explained in "Materials and Methods". For this analysis, we assumed that the dose-response curves for the hDOR and hDOR(S363A) under control conditions (untreated) are described by the same equation and therefore, share the parameter $\tau = \tau_{control}$. This assumption is justified by the results of the regression statistical comparison. The fitted curves for GTPγ[^{35}S] binding and inhibition of cAMP production experiments

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in cells expressing the wild-type and S363A mutant receptors are presented in Figs. 6A and 6B, respectively, and the calculated values for the fitted parameters are summarized in Table 4. From the fitted parameters τ, desensitization of the wild type and S363 mutant hDOR was computed using equation (3). Desensitization of the hDOR achieved after 60 min of deltorphin II treatment was 85% and 81% in GTPγ[^{35}S] and cAMP assays, respectively; values comparable to the maximal desensitization calculated from the desensitization time course (82% in both assays). On the other hand, desensitization of the S363A mutant was markedly lower, reaching only 20% and 18% in GTPγ[^{35}S] and cAMP assays, respectively. Because the S363A mutation significantly reduces the number of receptors desensitized by deltorphin II, S363 must play a crucial role in receptor desensitization.

One possibility for the role of S363 in hDOR desensitization may be that S363 phosphorylation hinders the interaction of the receptor with G proteins. Alternatively, other events dependent on S363 phosphorylation may also be necessary for complete receptor uncoupling. These events may include: phosphorylation of other residues; binding of GRKs or β-arrestins to the receptor; receptor sequestration into clathrin-coated pits, and receptor internalization into endosomes. Since deltorphin II-mediated desensitization of the hDOR is significantly reduced by the mutation of S363 to alanine, we hypothesized that every molecular event involved in receptor desensitization would also be significantly attenuated by this mutation.

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S363A mutation does not eliminate agonist-mediated recruitment of β-arrestin. We first investigated the role of S363 in β-arrestin binding to hDOR. To study agonist-mediated translocation of β-arrestin to the plasma membrane we transiently transfected the hDOR/CHO or hDOR(S363A)/CHO cells with a green fluorescent protein tagged β-arrestin2 construct (β-arrestin2-GFP, gift from Dr. Lefkowitz). 48 h after transfection, the cells were treated for 5 min with saturating concentrations of deltorphin II (100 nM). Images acquired on a Zeiss laser confocal fluorescent microscope (Figs. 7A, 7B) clearly show that deltorphin II treatment promoted the translocation of β-arrestin2-GFP to the plasma membrane in CHO cells expressing the wild-type hDOR. Interestingly, a similar pattern of β-arrestin2-GFP translocation was observed in cells expressing the S363A mutant receptor (Figs. 7C, 7D). Using a semi-quantitative analysis of β-arrestin2-GFP translocation, we did not observe any detectable difference in the magnitude and the time course of β-arrestin2-GFP translocation between the wild type and the hDOR(S363A) mutant receptor expressing cells. These results provide evidence that S363 in hDOR is not required for agonist-mediated β-arrestin2 translocation to the plasma membrane. In addition, these results indicate that even though β-arrestin2 binds to the hDOR(S363A) receptor, this binding is not sufficient to uncouple the receptor from its cognate G proteins.

S363A mutation does not eliminate agonist-mediated hDOR internalization. Next we studied the effect of S363A mutation on agonist-mediated internalization of the receptor. Internalization of the wild type and mutant hDOR was determined using confocal fluorescent microscopy by measuring the ability of the receptor to internalize a

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fluorescent analogue of deltorphin II ([Gln^4]deltorphin-rhodamine). In hDOR/CHO cells, after 30 min of agonist treatment, the fluorescent marker was concentrated in intracellular punctuated compartments resembling endosomes (Fig. 8A). Internalization of the fluorescent agonist was receptor-mediated since it was completely blocked by a delta-selective opioid receptor antagonist, naltrindole (1 μM, Fig. 8B). In cells expressing the S363A mutant receptor we observed virtually indistinguishable pattern of fluorescent deltorphin internalization. Therefore, S363A mutation in the hDOR did not eliminate the ability of the receptor to internalize [Gln^4]deltorphin-rhodamine. From these results we conclude that S363 is not necessary for agonist-mediated hDOR internalization and hDOR internalization is not sufficient to completely uncouple the receptor from G proteins.

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DISCUSSION

A major obstacle in understanding the exact mechanism of rapid homologous DOR desensitization is the inability to correctly determine the fraction of desensitized receptors. In our present study we used a mathematical analysis based on the Operational Model of Agonist Action (Black et al., 1985) to calculate the proportion of desensitized hDOR by deltorphin II pretreatment in two functional assays: stimulation of GTPγ[^{35}S] binding and inhibition of cAMP accumulation. To determine the molecular mechanism of rapid hDOR desensitization we investigated the role of deltorphin II-mediated phosphorylation of S363, translocation of β-arrestin2 to the plasma membrane and hDOR internalization. The results of these experiments demonstrate that β-arrestin binding and receptor internalization are not sufficient to bring about hDOR desensitization without phosphorylation of S363.

As emphasized in "Materials and Methods", receptor desensitization results not only in an attenuation of the maximum effect but also in a rightward shift of the concentration response curve (Fig. 2). Consequently, the measured reduction of a functional effect is not directly proportional to the number of desensitized receptors, particularly in the presence of spare receptors (Borgland et al., 2003; Connor et al., 2004). To minimize this disproportionality, many desensitization studies have been performed either in cell lines which express low levels of the DOR, such as SK-N-BE (Hasbi et al., 1998), or in cells where receptor level was experimentally manipulated (Law et al., 2000). In other studies, a mathematical analysis was used to calculate the proportion of desensitized receptors.

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Lohse adapted the Operational Model of Agonism (Black et al., 1985) and, assuming that desensitization represents a loss of signal-transduction efficacy of the receptor/effector system, derived an equation to calculate the number of desensitized receptors (Lohse et al., 1990). Similarly, Whaley and coworkers (Whaley et al., 1994) adapted models of receptor/G protein activation of adenylyl cyclase to derive expressions that predict changes in EC_{50} and E_{max} as the receptor number varies. It can be demonstrated that after transformation, the two methods yield identical equations. Since the Operational Model (Black et al., 1985) is not limited to activation of adenylyl cyclase but describes generally any relationship between receptor activation and effector function, we used this method in our study. In addition, we expanded the method of Lohse (Lohse et al., 1990), and assuming that the time dependence of receptor desensitization can be approximated by an exponential function, we derived an equation which allows us to estimate the half-life of hDOR desensitization.

Results of our analysis indicate that in recombinant CHO cells the hDOR is desensitized by deltorphin II treatment in a time-dependent manner with a half-life of approximately 10 min. Desensitization after 60 min of agonist treatment reached maximal levels that corresponded to about 80% of receptors desensitized. The same treatment desensitized only 20% of a mutant hDOR in which the primary phosphorylation site, S363, was mutated to alanine. The obtained rate of DOR desensitization corresponds to results reported by other investigators using cell lines with low expression levels of DOR (Allouche et al., 1999; Law et al., 2000). These results suggest that the use of the

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Operational Model enables us to correctly analyze receptor desensitization even in high receptor expression systems containing spare receptors.

Agonist-mediated phosphorylation of the DOR by high affinity agonists was demonstrated in our laboratory (Okura et al., 2000) as well as by other investigators (Allouche et al., 1999; Eisinger et al., 2002; Li et al., 2003; Willets and Kelly, 2001). Based on mutational analysis, S363 in the C-terminus of the mouse DOR was identified as the primary phosphorylation residue (Kouhen et al., 2000). In this study, we found using a phospho-hDOR(S363) primary antibody that S363 of the human DOR is phosphorylated upon deltorphin II treatment in a time-dependent manner with a half-life of about 2 min. This half-life is shorter than the half-life of receptor desensitization (~10 min), indicating that the relationship between receptor phosphorylation and desensitization is not direct. Indeed, elimination of S363 phosphorylation by mutation of this residue to alanine did not completely prevent hDOR desensitization produced by 60 min deltorphin II treatment, but only reduced the number of desensitized receptors from 80 to 20%. Therefore, the hDOR is desensitized by a S363 dependent and by a S363 independent mechanism. Phosphorylation-dependent and independent mechanisms of desensitization were also identified for rhodopsin (Xu et al., 1997) as well as for the DOR (Law et al., 2000). In addition, it was reported that binding of a kinase-negative mutant (K220R) of GRK2 could desensitize the parathyroid hormone receptor (Dicker et al., 1999), and the endothelin A and B receptors (Freedman et al., 1997) even in the absence of receptor phosphorylation. However, the phosphorylation-independent mechanism of DOR desensitization likely does not involve GRK, since binding of GRK2 alone to a

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phosphorylation deficient mutant of the DOR did not cause desensitization of GTPγ[^{35}S] binding in HEK 293 cells (Li et al., 2003).

It has been demonstrated that binding of β-arrestin to GPCRs requires phosphorylation of several S/T residues in the intracellular domains of the receptors (Gurevich and Gurevich, 2004). Accordingly, receptor phosphorylation was found to be the rate-limiting step for β-arrestin and β2-adrenergic receptor interaction (Krasel et al., 2004). GPCR- β-arrestin interaction in turn, is considered to be a key step in the uncoupling of the receptors from G proteins (Barak et al., 2006). In agreement with this concept, cotransfection of β-arrestin1 or 2 with GRK3 was required for desensitization of DOR-coupled inwardly rectifying potassium channel (Kir3) in Xenopus oocytes (Lowe et al., 2002). Surprisingly, our confocal microscopy images show that mutation of the primary phosphorylation site S363 in the hDOR that significantly impairs receptor desensitization did not prevent the recruitment of β-arrestin2-GFP to the plasma membrane. No visually apparent differences were observed in the time course and the extent of β-arrestin2-GFP translocation to the hDOR or hDOR(S363A). Fluorescent microscopy does not allow quantification of the β-arrestin-receptor interaction, however, β-arrestin2-GFP translocation clearly occurs after 5 min of deltorphin II treatment in the hDOR(S363A) mutant receptor. Therefore, changes in the interaction between β-arrestin2-GFP and the hDOR(S363A) compared to the wild type hDOR are not sufficient to explain an almost complete blockade of receptor desensitization observed after 60 min of agonist treatment. These results demonstrate that β-arrestin2 binding to hDOR alone is not sufficient for receptor desensitization without S363 phosphorylation. In support of this conclusion, it

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was reported recently using locus coeruleus neurons from β-arrestin2 knock-out mice that lack of β-arrestin2 expression has no effect on the rate or the magnitude of the mu opioid receptor desensitization (Dang, 2006). Nevertheless, in our system β-arrestin binding may still function as an essential mechanism in the residual S363 phosphorylation-independent desensitization, probably in conjunction with receptor internalization. Indeed, phosphorylation independent, β-arrestin2-dependent internalization of the DOR was reported in recombinant HEK 293 cells (Zhang et al., 2005). In addition, a study by Burns et al. found that visual arrestin is able to quench nonphosphorylated rhodopsin (Burns et al., 2006). Recently, Marion and coworkers (Marion et al., 2006) identified a common β-arrestin binding site formed by ten residues of the second intracellular loop of most GPCRs which is independent of GRK phosphorylation but dependent on agonist activation.

Desensitization of the DOR was reported in some studies to be independent of receptor internalization (Hasbi et al., 2000; Willets and Kelly, 2001). In contrast, Law et al. reported that both receptor phosphorylation and receptor endocytosis contribute to DOR desensitization in HEK 293 cells (Law et al., 2000). Our results support the idea that the primary mechanism of hDOR desensitization is S363 phosphorylation-dependent. In addition, a small fraction of receptor desensitization is S363 phosphorylation-independent. Further studies are needed to determine whether phosphorylation of other residues is responsible for the residual desensitization or whether it is indeed caused by phosphorylation-independent and β-arrestin-dependent internalization.

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In summary, this study demonstrates that the Operational Model of Agonism provides an accurate mathematical approach to quantify the number of receptors desensitized by agonist treatment. By using this model on the wild-type and a phosphorylation-deficient mutant of the hDOR we were able to correlate desensitization with receptor phosphorylation, β-arrestin2 translocation and receptor internalization. We have demonstrated that in CHO cells expressing the hDOR, deltorphin II treatment leads to phosphorylation of S363, translocation of β-arrestin2 to the plasma membrane, receptor internalization and uncoupling from G proteins. Interestingly, the S363A mutation completely eliminates phosphorylation of this residue, yet it has virtually no effect on β-arrestin2 translocation and receptor internalization. On the other hand, S363A mutation significantly attenuates receptor desensitization. These results provide evidence that phosphorylation of S363 is required to uncouple the receptor from G proteins. Recruitment of β-arrestin2 and receptor internalization, on the other hand, are not sufficient to desensitize the hDOR without S363 phosphorylation. Therefore, we conclude that phosphorylation of S363 represents the primary mechanism of the human delta opioid receptor desensitization.

REFERENCES

Allouche S, Roussel M, Marie N and Jauzac P (1999) Differential desensitization of human [delta]-opioid receptors by peptide and alkaloid agonists. *Eur J Pharmacol* **371**(2-3):235-240.

Barak LS, Gilchrist J, Becker JM and Kim K-M (2006) Relationship between the G protein signaling and homologous desensitization of G protein-coupled receptors. *Biochem Biophys Res Commun* **339**(2):695-700.

Black JW, Leff P, Shankley NP and Wood J (1985) An operational model of pharmacological agonism: the effect of E/[A] curve shape on agonist dissociation constant estimation. *Br J Pharmacol* **84**(2):561-571.

Borgland SL, Connor M, Osborne PB, Furness JB and Christie MJ (2003) Opioid agonists have different efficacy profiles for G protein activation, rapid desensitization, and endocytosis of mu-opioid receptors. *J Biol Chem* **278**(21):18776-18784.

Burns ME, Mendez A, Chen CK, Almuete A, Quillinan N, Simon MI, Baylor DA and Chen J (2006) Deactivation of phosphorylated and nonphosphorylated rhodopsin by arrestin splice variants. *J Neurosci* **26**(3):1036-1044.

Connor M, Osborne PB and Christie MJ (2004) Mu-opioid receptor desensitization: is morphine different? *Br J Pharmacol* **143**(6):685-696.

Daaka Y, Luttrell LM and Lefkowitz RJ (1997) Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390**(6655):88-91.

Dang VC, Christie, M.J. (2006) Beta-arrestin-2 independent regulation of mu opioid receptor in locus coeruleus neurones, in *International Narcotics Research Conference* p 48, St. Paul, Minnesota.

Dicker F, Quitterer U, Winstel R, Honold K and Lohse MJ (1999) Phosphorylation-independent inhibition of parathyroid hormone receptor signaling by G protein-coupled receptor kinases. *Proc Natl Acad Sci U S A* **96**(10):5476-5481.

Eisinger DA, Ammer H and Schulz R (2002) Chronic Morphine Treatment Inhibits Opioid Receptor Desensitization and Internalization. *J Neurosci* **22**(23):10192-10200.

Freedman NJ, Ament AS, Oppermann M, Stoffel RH, Exum ST and Lefkowitz RJ (1997) Phosphorylation and Desensitization of Human Endothelin A and B Receptors. Evidence for G Protein-coupled Receptor Kinase Specificity. *J Biol Chem* **272**(28):17734-17743.

Gilman AG (1970) A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proc Natl Acad Sci U S A* **67**(1):305-312.

Gurevich VV and Gurevich EV (2004) The molecular acrobatics of arrestin activation. *Trends Pharmacol Sci* **25**(2):105-111.

Hall RA and Lefkowitz RJ (2002) Regulation of G protein-coupled receptor signaling by scaffold proteins. *Circ Res* **91**(8):672-680.

Hasbi A, Allouche S, Sichel F, Stanasila L, Massotte D, Landemore G, Polastron J and Jauzac P (2000) Internalization and Recycling of delta -Opioid Receptor Are Dependent on a Phosphorylation-Dephosphorylation Mechanism. *J Pharmacol Exp Ther* **293**(1):237-247.

Hasbi A, Polastron J, Allouche S, Stanasila L, Massotte D and Jauzac P (1998) Desensitization of the delta-opioid receptor correlates with its phosphorylation in SK-N-BE cells: involvement of a G protein-coupled receptor kinase. *J Neurochem* **70**(5):2129-2138.

Kouhen OM, Wang G, Solberg J, Erickson LJ, Law PY and Loh HH (2000) Hierarchical phosphorylation of delta-opioid receptor regulates agonist-induced receptor desensitization and internalization. *J Biol Chem* **275**(47):36659-36664.

Krasel C, Vilardaga JP, Bunemann M and Lohse MJ (2004) Kinetics of G-protein-coupled receptor signalling and desensitization. *Biochem Soc Trans* **32**(Pt 6):1029-1031.

Law PY, Kouhen OM, Solberg J, Wang W, Erickson LJ and Loh HH (2000) Deltorphin II-induced rapid desensitization of delta-opioid receptor requires both phosphorylation and internalization of the receptor. *J Biol Chem* **275**(41):32057-32065.

Li J, Xiang B, Su W, Zhang X, Huang Y and Ma L (2003) Agonist-induced formation of opioid receptor-G protein-coupled receptor kinase (GRK)-G beta gamma complex on membrane is required for GRK2 function in vivo. *J Biol Chem* **278**(32):30219-30226.

Lohse MJ, Benovic JL, Caron MG and Lefkowitz RJ (1990) Multiple pathways of rapid beta 2-adrenergic receptor desensitization. Delineation with specific inhibitors. *J Biol Chem* **265**(6):3202-3211.

Lowe JD, Celver JP, Gurevich VV and Chavkin C (2002) mu-Opioid receptors desensitize less rapidly than delta-opioid receptors due to less efficient activation of arrestin. *J Biol Chem* **277**(18):15729-15735.

Malatynska E, Wang Y, Knapp RJ, Waite S, Calderon S, Rice K, Hruby VJ, Yamamura HI and Roeske WR (1996) Human delta opioid receptor: functional studies on stably transfected Chinese hamster ovary cells after acute and chronic treatment with the selective nonpeptidic agonist SNC-80. *J Pharmacol Exp Ther* **278**(3):1083-1089.

Marion S, Oakley RH, Kim K-M, Caron MG and Barak LS (2006) A beta-Arrestin Binding Determinant Common to the Second Intracellular Loops of Rhodopsin Family G Protein-coupled Receptors. *J Biol Chem* **281**(5):2932-2938.

Navratilova E, Eaton MC, Stropova D, Varga EV, Vanderah TW, Roeske WR and Yamamura HI (2005) Morphine promotes phosphorylation of the human delta-opioid receptor at serine 363. *Eur J Pharmacol* **519**(3):212-214.

Navratilova E, Varga EV, Stropova D, Jambrosic JC, Roeske WR and Yamamura HI (2004) Mutation S363A in the human delta-opioid receptor selectively reduces down-regulation by a peptide agonist. *Eur J Pharmacol* **485**(1-3):341-343.

Okura T, Cowell SM, Varga E, Burkey TH, Roeske WR, Hruby VJ and Yamamura HI (2000) Differential down-regulation of the human [delta]-opioid receptor by SNC80 and [D-Pen2,D-Pen5]enkephalin. *Eur J Pharmacol* **387**(2):R11-R13.

Osborne PB and Williams JT (1995) Characterization of acute homologous desensitization of mu-opioid receptor-induced currents in locus coeruleus neurones. *Br J Pharmacol* **115**(6):925-932.

Quock RM, Burkey TH, Varga E, Hosohata Y, Hosohata K, Cowell SM, Slate CA, Ehlert FJ, Roeske WR and Yamamura HI (1999) The delta-opioid receptor: molecular pharmacology, signal transduction, and the determination of drug efficacy. *Pharmacol Rev* **51**(3):503-532.

Quock RM, Hosohata Y, Knapp RJ, Burkey TH, Hosohata K, Zhang X, Rice KC, Nagase H, Hruby VJ, Porreca F, Roeske WR and Yamamura HI (1997) Relative efficacies of delta-opioid receptor agonists at the cloned human delta-opioid receptor. *Eur J Pharmacol* **326**(1):101-104.

Trzeciakowski JP (1999) Stimulus amplification, efficacy, and the operational model. Part I--binary complex occupancy mechanisms. *J Theor Biol* **198**(3):329-346.

Varga EV, Rubenzik MK, Stropova D, Sugiyama M, Grife V, Hruby VJ, Rice KC, Roeske WR and Yamamura HI (2003) Converging protein kinase pathways mediate adenylyl cyclase superactivation upon chronic delta-opioid agonist treatment. *J Pharmacol Exp Ther* **306**(1):109-115.

Whaley BS, Yuan N, Birnbaumer L, Clark RB and Barber R (1994) Differential expression of the beta-adrenergic receptor modifies agonist stimulation of adenylyl cyclase: a quantitative evaluation. *Mol Pharmacol* **45**(3):481-489.

Willets J and Kelly E (2001) Desensitization of endogenously expressed [delta]-opioid receptors: no evidence for involvement of G protein-coupled receptor kinase 2. *Eur J Pharmacol* **431**(2):133-141.

Xu J, Dodd RL, Makino CL, Simon MI, Baylor DA and Chen J (1997) Prolonged photoresponses in transgenic mouse rods lacking arrestin. *Nature* **389**(6650):505-509.

Zhang X, Wang F, Chen X, Li J, Xiang B, Zhang YQ, Li BM and Ma L (2005) Beta-arrestin1 and beta-arrestin2 are differentially required for phosphorylation-dependent and -independent internalization of delta-opioid receptors. *J Neurochem* **95**(1):169-178.

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FIGURE LEGENDS

Fig. 1. Pretreatment of hDOR/CHO cells with deltorphin II leads to time-dependent desensitization of deltorphin II-stimulated GTPγ[^{35}S] binding and inhibition of cAMP production. hDOR/CHO cells were pretreated for the indicated times with 100 nM deltorphin II in serum free IMDM medium at 37ºC. (A) Cell membranes were prepared and deltorphin II-stimulated GTPγ[^{35}S] binding was measured as described in "Materials and Methods". Deltorphin II concentration curves were normalized to percent of maximum effect in control (untreated) cells. (B) After pretreatment, the cells were washed and deltorphin II-mediated inhibition of forskolin (100 μM, 20 min)-stimulated cAMP accumulation was measured as described in "Materials and Methods". Dose-response curves were plotted as percent increase of cAMP inhibition relative to control cells. The data points in (A) and (B) represent the averages and standard errors obtained from three independent experiments performed in duplicates. Deltorphin II pretreatment: (●) 0 min, (○) 2 min, (■) 5 min, (□) 10 min, (▲) 30 min, (△) 45 min, (◆) 60 min. The dose-response curves for each time point of pretreatment in (A) and (B) were fitted to sigmoidal curves with a Hill coefficient equal to 1.

Fig. 2. Mathematical simulation of the effect of agonist pre-treatment on concentration-response curves. (A) The concentration-response curves for an agonist [A] were generated using equation (5), with parameters $E_m = 100$, $\tau_0 = 20$, $\tau_\infty = 1$, $K_A = 1$ nM and $k = 0.1$ min^{-1}. The time of treatment for the individual curves was set to: 0, 5, 10,

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30, and 60 min. (B) The time course of receptor desensitization was simulated with equation (4) using the same values of parameters τ_0, τ_∞, and k as in graph (A).

Fig. 3. Time course of deltorphin II-stimulated desensitization of hDOR fitted by the modified operational model (equation (5)). (A) The concentration-response curves for deltorphin II-stimulated GTPγ[^{35}S] binding from graph 1A were fitted simultaneously using equation (5). (B) The concentration-response curves of deltorphin II-mediated inhibition of forskolin (100 μM, 20 min)-stimulated cAMP accumulation plotted in graph 1B were fitted simultaneously using equation (5). Deltorphin II pretreatment: (•) 0 min, (○) 2 min, (▪) 5 min, (▫) 10 min, (▲) 30 min, (△) 45 min, (◆) 60 min. The parameters E_m and K_A were shared for all time points (0-60 min); for times 0-45 min the parameter τ is defined by equation (4) in which τ_0 and τ_∞ are fitted shared parameters; for the 60 min curve the τ was designated τ_{60} and was fitted independently. The fitted parameters E_m, K_A, k, τ_0, and τ_∞ for (A) and (B) are summarized in Table 1.

Fig. 4. Treatment of hDOR/CHO cells with deltorphin II leads to phosphorylation of hDOR at S363. hDOR/CHO or hDOR(S363A)/CHO cells were treated for indicated time intervals with 500 nM deltorphin II at 37°C in a serum-free medium. Equal amounts of cell lysates were resolved on 10% NuPAGE Bis-Tris gels, transferred onto nitrocellulose membranes and immunoblotted with phospho-S363 hDOR antibody. (A) A

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representative immunoblot showing the time course of S363 phosphorylation in hDOR/CHO cells. (B) A representative immunoblot demonstrating that no specific immunoreactivity was detected after deltorphin II treatment in hDOR(S363A)/CHO cells. (C) Band intensities were quantified using the Image J software (NIH) and averages from three independent experiments ± standard errors were plotted. The early phase of receptor phosphorylation (0-30 min) was fitted with an exponential association curve.

Fig. 5. S363A mutation attenuates deltorphin II-mediated hDOR desensitization. hDOR/CHO (solid line) or hDOR(S363A)/CHO (dotted line) cells were pretreated or not with 100 nM deltorphin II in IMDM medium for 60 min at 37ºC. (A) Cells were harvested, cell membranes prepared and deltorphin II-stimulated GTPγ[^{35}S] binding measured, as described in "Materials and Methods". Deltorphin II concentration curves were normalized to percent of maximum effect in control (untreated) cells. (B) Cells were washed three times to remove the agonist, and dose-response curves for deltorphin II-mediated inhibition of forskolin (100 μM, 20 min)-stimulated cAMP accumulation were measured as described in "Materials and Methods". The inhibition curves were plotted as the percent of cAMP inhibition in control cells. The normalized data from three (A) and five (B) independent experiments (performed in duplicates) were combined and fitted by sigmoidal dose-response curves with a Hill coefficient set to 1. (•) hDOR/CHO cells, no pretreatment, (○) hDOR/CHO cells, deltorphin II pretreatment, (▪) hDOR(S363A)/CHO cells, no pretreatment, (▫) hDOR(S363A)/CHO cells, deltorphin II pretreatment.

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Fig. 6. Desensitization of the hDOR and hDOR(S363A) fitted by the operational model. The hDOR/CHO (solid line) or hDOR(S363A)/CHO (dotted line) cells were pretreated for 60 min with 100 nM deltorphin II in IMDM medium at 37ºC. (A) The concentration-response curves of deltorphin II-stimulated GTPγ[^{35}S] binding from graph 5A were fitted simultaneously using equation (1). (B) The concentration-response curves of deltorphin II-mediated inhibition of forskolin (100 μM, 20 min)-stimulated cAMP accumulation from graph 5B were fitted simultaneously using equation (1). The parameters E_m and K_A are shared for all curves. (•) hDOR/CHO cells, no pretreatment, (○) hDOR/CHO cells, deltorphin II pretreatment, (▪) hDOR(S363A)/CHO cells, no pretreatment, (▫) hDOR(S363A)/CHO cells, deltorphin II pretreatment. The fitted parameters E_m, K_A, and τ for (A) and (B) are summarized in Table 4.

Fig. 7. Deltorphin II stimulates translocation of β-arrestin2-GFP in CHO cells expressing wild-type or S363A mutant hDOR. CHO cells expressing the wild type (A, B) or S363A mutant (C, D) hDOR were transfected with a green fluorescent protein tagged β-arrestin2 construct (β-arrestin2-GFP, gift from Dr. Lefkowitz). 48 h after transfection the cells were treated in the absence (A, C) or presence (B, D) of 100 nM deltorphin II for 5 min at 37°C. After agonist treatment, the cells were fixed and examined under a Zeiss LSM520 laser scanning confocal microscope equipped with a 100×/1.40 oil objective. Single optical sections were acquired through the trans-nuclear

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plane. The magnification bar represents 10 μm. Images were processed in Adobe Photoshop 7.0.

Fig. 8. [Gln^4]deltorphin-rhodamine is internalized in CHO cells expressing either wild-type or S363A mutant hDOR. For internalization studies, hDOR/CHO (A, B) or hDOR(S363A)/CHO (C) cells were incubated with [Gln^4]deltorphin-rhodamine (1μM) for 30 min at 37°C. In (B) 1 μM naltrindole was added together with the agonist. After treatment, the cells were fixed and examined under a Zeiss LSM520 laser scanning confocal microscope equipped with a 100×/1.40 oil objective. Single optical sections were acquired through the trans-nuclear plane. The acquisition parameters were constant in all parallel experiments. The magnification bar represents 10 μm. Images were processed in Adobe Photoshop 7.0.

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TABLES

Table 1. E_{max} and EC_{50} values of deltorphin II-mediated stimulation of GTPγ[^{35}S] binding and inhibition of cAMP production in hDOR/CHO cells pretreated for indicated times with deltorphin II

Time (min)	0	2	5	10	30	45	60
E_{max} (GTPγ[^{35}S])	100 ±1	105 ±2	113 ±1	102 ±2	81 ±2	-	58 ±4
EC_{50} (GTPγ[^{35}S])	6.3 ±0.4	13 ±1	16 ±1	18 ±2	24 ±3	-	23 ±7
E_{max} (cAMP)	100 ±3	102 ±7	108 ±5	103 ±3	102 ±4	99 ±6	82 ±6
EC_{50} (cAMP)	0.36 ±0.06	0.33 ±0.15	1.0 ±0.2	0.98 ±0.18	1.8 ±0.4	3.1 ±1.0	1.7 ±0.6

Deltorphin II concentration responses for GTPγ[^{35}S] stimulation and cAMP inhibition in hDOR/CHO cells pretreated for different time intervals with deltorphin II were fitted with sigmoidal curves (Hill coefficient $n_H = 1$). Calculated EC_{50} ± SE are presented in nM concentrations, E_{max} ± SE are in %.

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Table 2. Parameters of deltorphin II-mediated desensitization of hDOR calculated by equation (5)

	E_m (%)	$\tau_{control}$	τ_∞	k (min^{-1})	K_A (nM)
GTPγ[^{35}S]	120 ±5	7.8 ±2.4	1.4 ±0.5	0.070 ±0.018	81 ±16
cAMP	106 ±4	36 ±21	6.4 ±3.4	0.082 0.034	16 ±8

Five parameters of equation (5) (E_m = operational maximum, $\tau_{control} = (\tau_0 + \tau_\infty)$, and τ_∞ the operational efficacies before and after desensitization, k = desensitization rate constant, K_A = dissociation constant) were calculated by fitting the dose response curves of GTPγ[^{35}S] or cAMP assays.

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Table 3. EC_{50} values of deltorphin II-mediated stimulation of GTPγ[^{35}S] binding and inhibition of cAMP production in naïve and deltorphin II pretreated hDOR/CHO or hDOR(S363A)/CHO cells

	hDOR(control)	hDOR(deltorphin)	S363A(control)	S363A(deltorphin)
E_{max} (GTPγ[^{35}S])	99 ±2	77 ±5	100 ±2	110 ±3
EC_{50} (GTPγ[^{35}S])	6.9 ±0.9	31 ±7	5.8 ±0.7	13 ±2
E_{max} (cAMP)	99 ±6	83 ±9	99 ±5	108 ±5
EC_{50} (cAMP)	1.4 ±0.5	7.5 ±4.0	2.0 ±0.7	2.8 ±0.8

Deltorphin II concentration responses for GTPγ[^{35}S] stimulation and cAMP inhibition in naïve and deltorphin II pretreated hDOR/CHO or hDOR(S363A)/CHO cells were fitted with sigmoidal curves (Hill coefficient $n_H = 1$). Calculated EC_{50} ± SE are presented in nM concentrations, E_{max} ± SE are in %.

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Table 4. Parameters of deltorphin II-mediated desensitization of hDOR and hDOR(S363A) calculated using the operational model (equation (1))

	E_m (%)	$\tau_{control}$	$\tau_{del\text{-}wt}$	$\tau_{del\text{-}S363A}$	K_A (nM)
GTPγ[^{35}S]	110 ±4	13 ±5	1.9 ±0.5	10 ±4	97 ±33
cAMP	107 ±7	19 ±18	3.6 ±2.3	16 ±15	36 ±30

Parameters of the operational model (equation (1)) (E_m = operational maximum; $\tau_{control}$, $\tau_{del\text{-}wt}$, $\tau_{del\text{-}S363A}$ are operational efficacies for untreated cells and deltorphin II pretreated hDOR/CHO or hDOR(S363A)/CHO cells, respectively; K_A = dissociation constant) were calculated by fitting the dose response curves of stimulation of GTPγ[^{35}S] binding or inhibition of cAMP production.

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Fig. 1

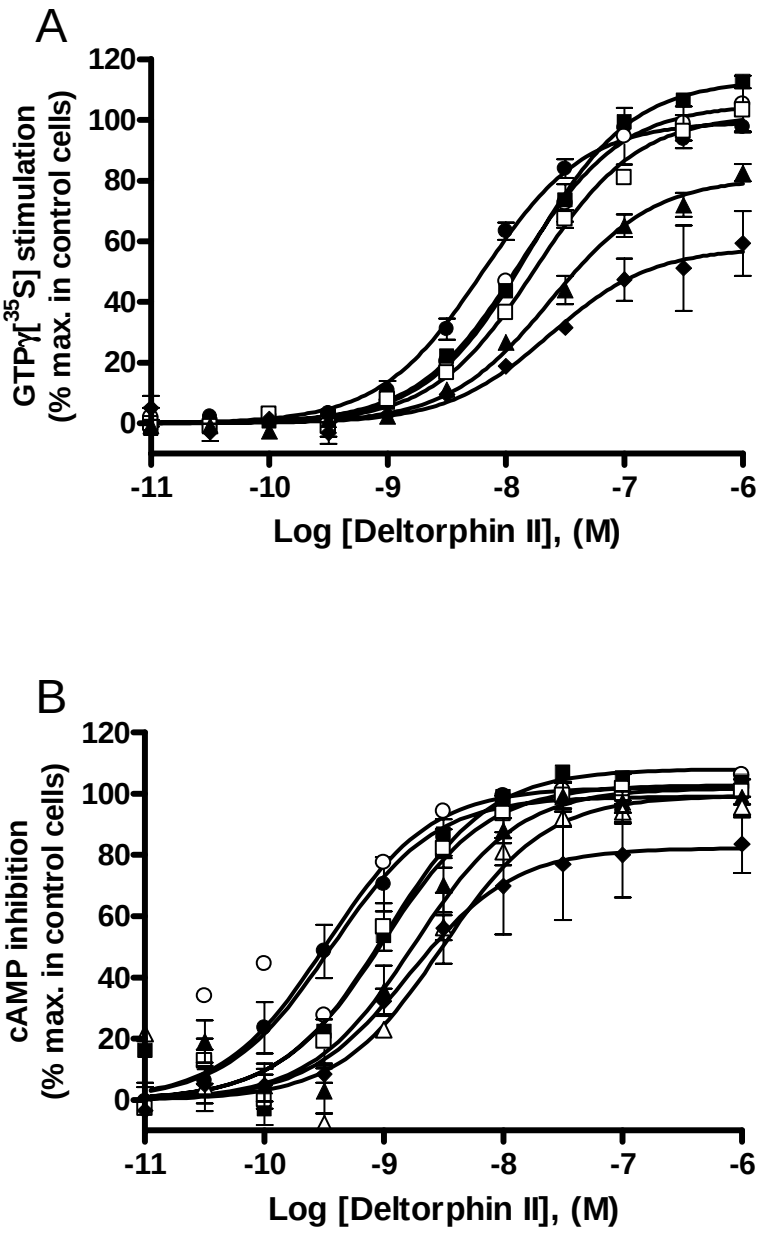

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Fig. 2

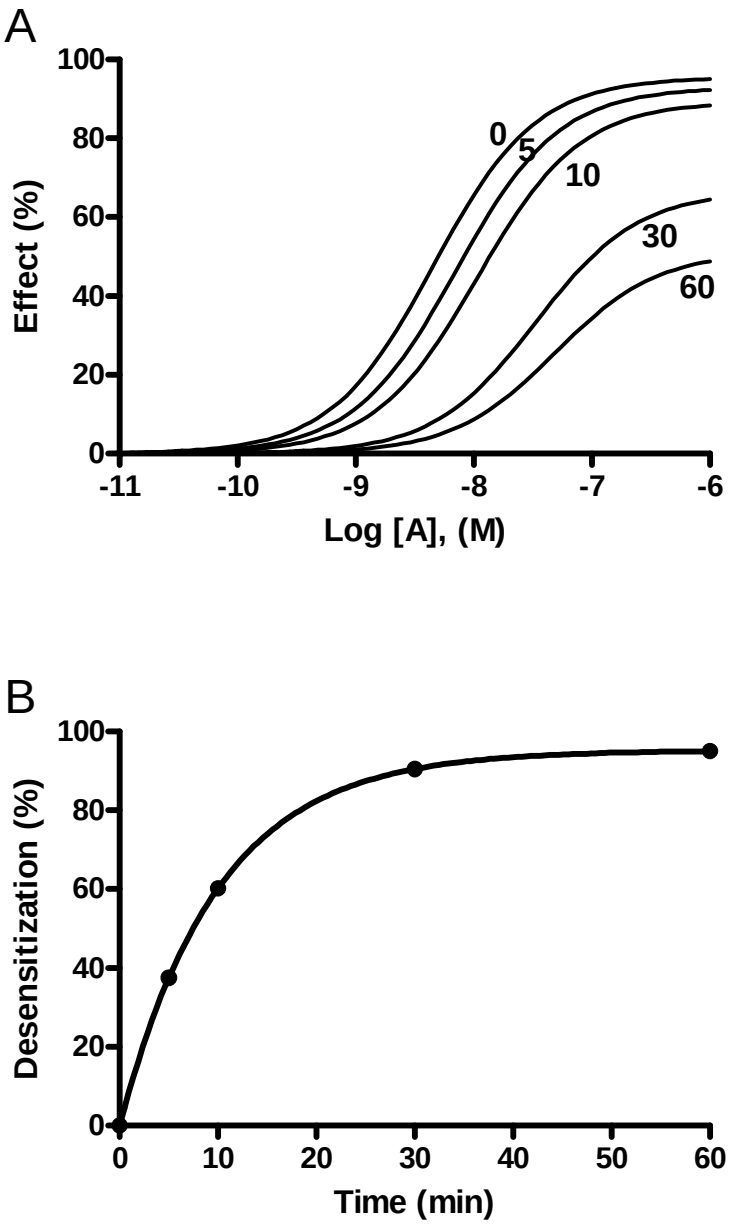

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Fig. 3

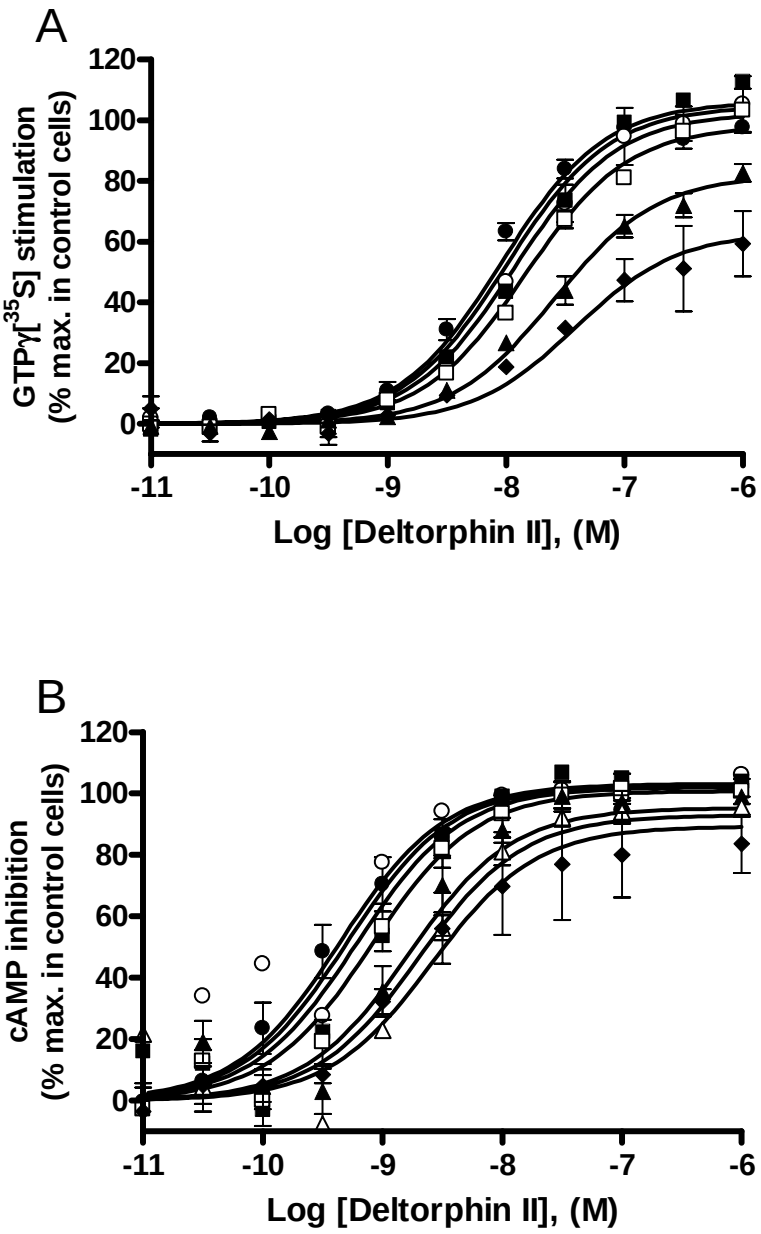

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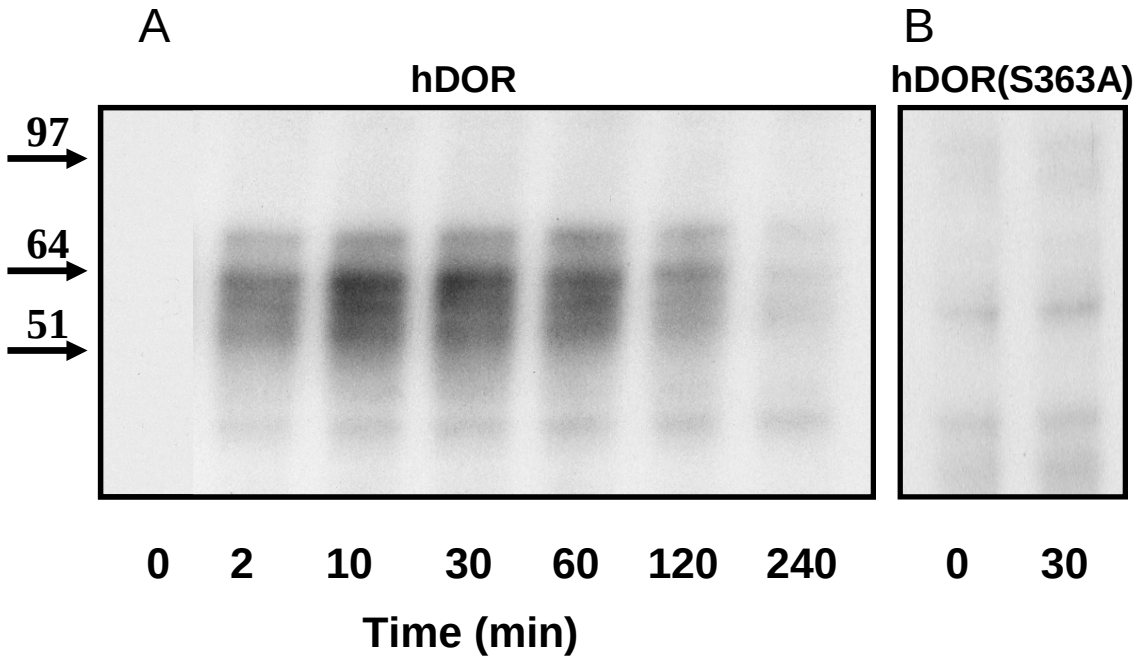
Fig. 4



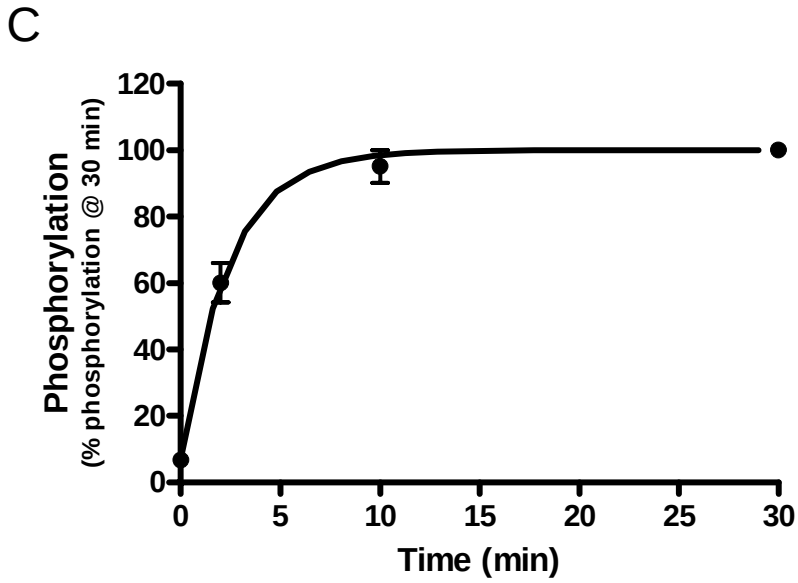


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Fig. 5

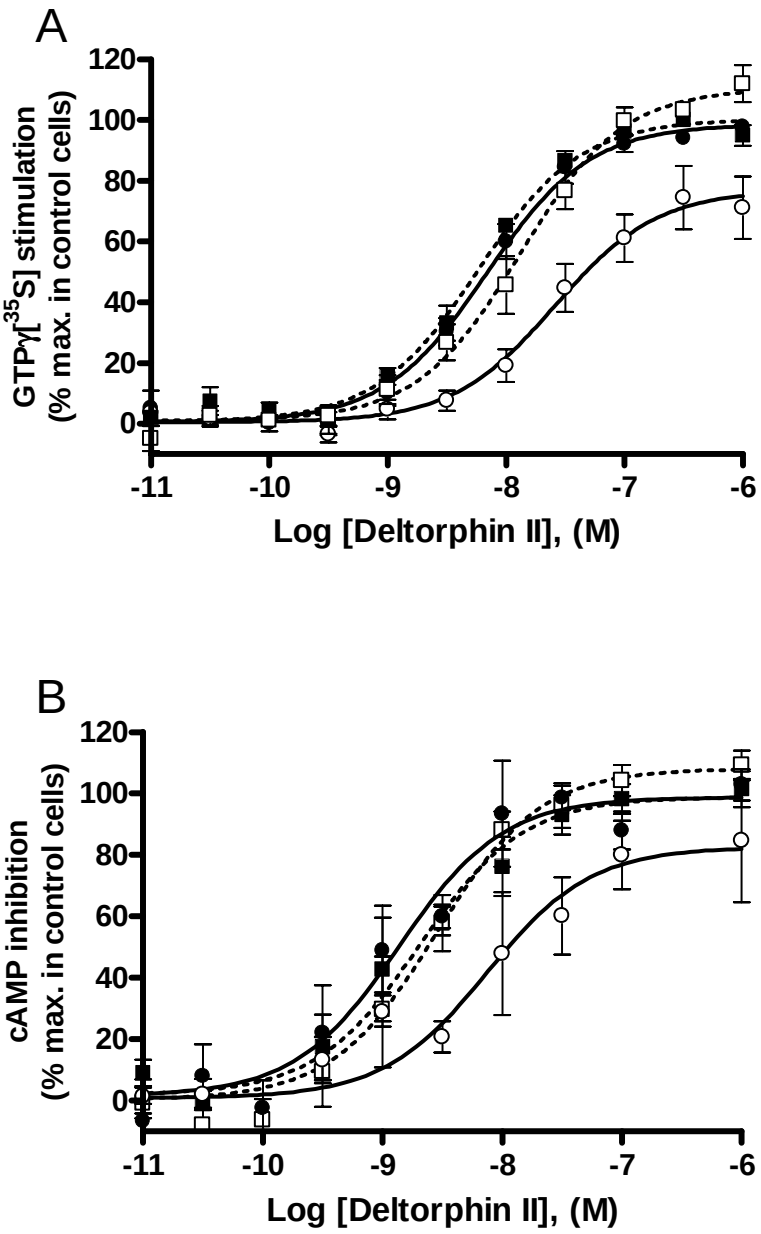

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Fig. 6

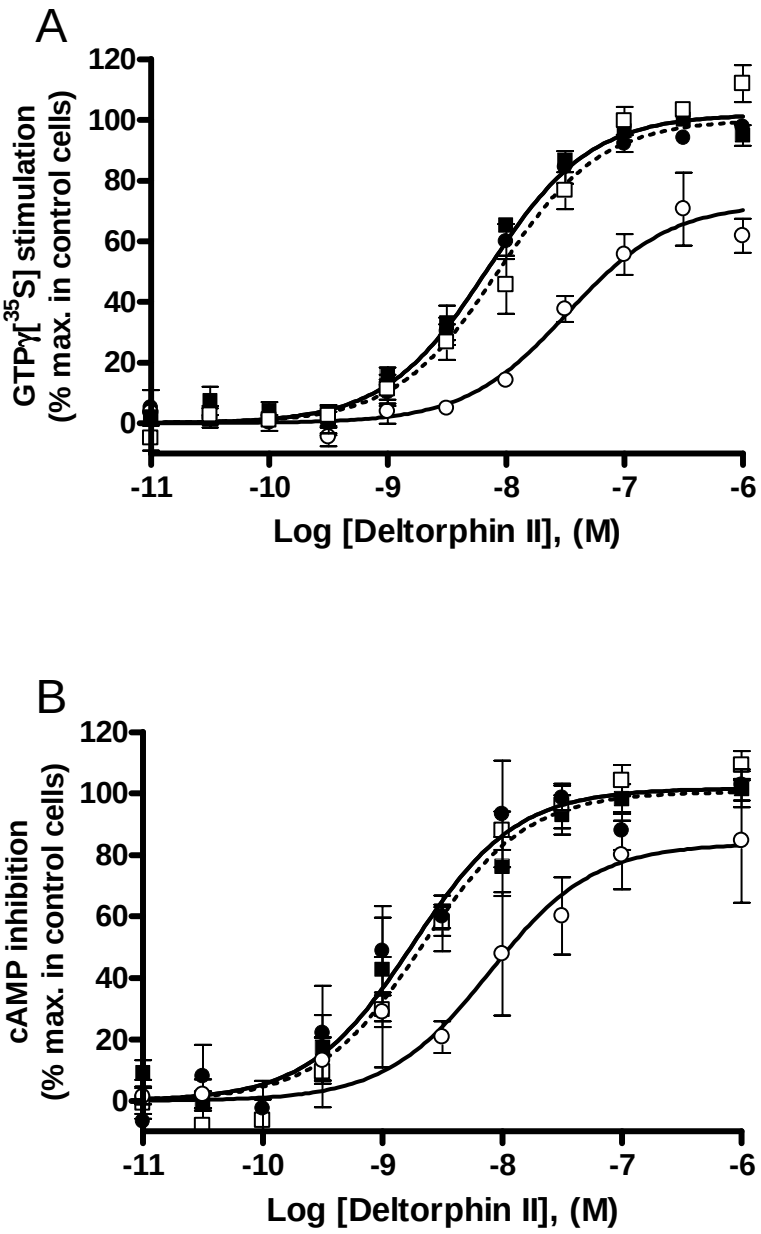

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Fig. 7

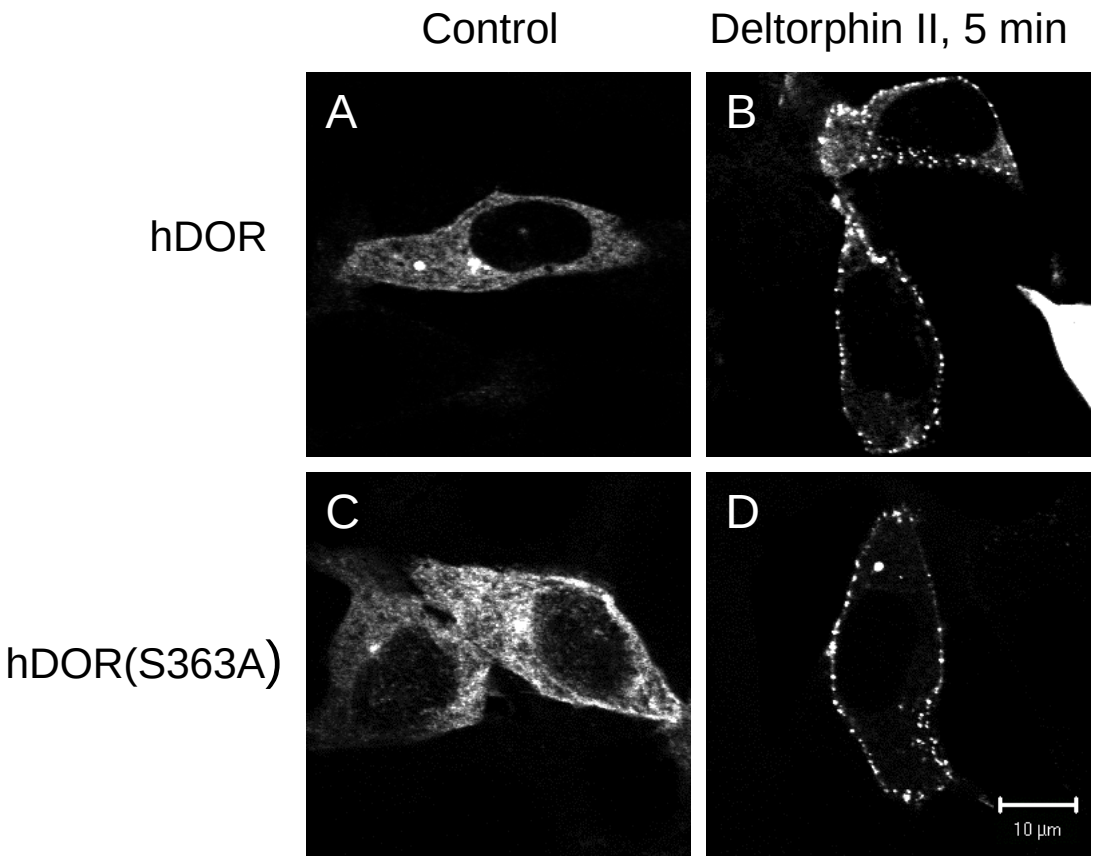

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Fig. 8