

# Quantitative Evaluation of Human $\delta$ Opioid Receptor Desensitization Using the Operational Model of Drug Action

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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 because of 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 guanosine 5'-O-(3-[ $^{35}$ S]thio)triphosphate 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 desensi-

tization by taking into account receptor-effector coupling and the time dependence of agonist pretreatment. Finally, we compared hDOR desensitization with receptor phosphorylation at Ser363, the translocation of  $\beta$ -arrestin2, and hDOR internalization. We found that in Chinese hamster ovary cells expressing the hDOR, deltorphin II treatment leads to phosphorylation of Ser363, translocation of  $\beta$ -arrestin2 to the plasma membrane, receptor internalization, and uncoupling from G proteins. It is noteworthy that mutation of the primary phosphorylation site Ser363 to alanine had virtually no effect on agonist-induced  $\beta$ -arrestin2 translocation and receptor internalization yet significantly attenuated receptor desensitization. These results strongly indicate that phosphorylation of Ser363 is the primary mechanism of hDOR desensitization.

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 by duration as rapid or prolonged, and by 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  $G_{i/o}$  proteins, and dissociation of  $G\alpha_i$  subunits from  $\beta\gamma$  dimers (Quock et al., 1999). Released

$\beta\gamma$  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, Ser363 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,  $\beta$ -arrestins. Binding of  $\beta$ -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 resensitization by recycling of the dephosphorylated receptor to the plasma membrane. The functional consequence 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-me-

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**ABBREVIATIONS:** hDOR, human  $\delta$  opioid receptor; DOR,  $\delta$  opioid receptor; GRK, G protein-coupled receptor kinase; CHO, Chinese hamster ovary; HEK, human embryonic kidney; IMDM, Iscove's modified Dulbecco's medium; GTP $\gamma$ [ $^{35}$ S], guanosine 5'-O-(3-[ $^{35}$ S]thio)triphosphate; TE, Tris/EDTA buffer; GFP, green fluorescent protein.

diated receptor phosphorylation,  $\beta$ -arrestin binding, and receptor internalization. However, because these regulatory events are all causally dependent on each other, the key process directly responsible for DOR desensitization remains unclear. 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 et al. (2000) demonstrated that both phosphorylation and internalization contribute to DOR desensitization in HEK 293 cells. High-affinity binding of  $\beta$ -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,  $\beta$ -arrestin recruitment has been considered a hallmark of the homologous receptor desensitization (Barak et al., 2006). However, the direct role of  $\beta$ -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 desensitization." Receptor desensitization is usually interpreted as a molecular change 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 nonlinearity in receptor/effector coupling and could be prevented if the number of desensitized receptors were correctly determined.

The goals of the present study were: 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 Ser363,  $\beta$ -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 Ser363 was mutated to alanine [hDOR(S363A)]. Receptor desensitization by deltorphin II, was measured in two second messenger assays: stimulation of GTP- $\gamma$ [ $^{35}$ 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, Ser363, does not prevent  $\beta$ -arrestin2 recruitment and receptor internalization, it significantly attenuates hDOR desensitization.

## Materials and Methods

**Mutagenesis of the Cloned hDOR and Generation of the Recombinant CHO Cell Lines.** A Ser363Ala mutation was intro-

duced into the human  $\delta$  opioid receptor cDNA using the QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA). CHO cell lines stably expressing the wild-type hDOR or mutant hDOR(S363A) were generated and characterized as described previously [clone 1-209-19hDOR/CHO, Malatynska et al. (1996); clone hDOR(S363A)/CHO#13, Navratilova et al. (2004)]. Receptor expression, determined by [ $^3$ H]naltrindole radioligand binding, reached 1.8 pmol/mg of protein for the wild type hDOR and 2.0 pmol/mg of 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, St. Louis, MO) containing 10% fetal calf serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and either 800  $\mu$ g/ml hygromycin or 500  $\mu$ g/ml G418, respectively, at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. For desensitization studies, the cells were pretreated with 100 nM (D-Ala<sup>2</sup>)deltorphin II (Tocris Cookson Inc., Ellisville, MO) for 0–60 min in serum-free IMDM (Invitrogen, Carlsbad, CA).

**GTP- $\gamma$ [ $^{35}$ S] Binding.** Deltorphin II-stimulated binding of GTP- $\gamma$ [ $^{35}$ S] to crude membranes prepared from control and agonist pretreated hDOR/CHO or hDOR(S363A)/CHO cells was determined as described previously (Quock et al., 1997). In brief, cell monolayers were washed with Ca<sup>2+</sup>, Mg<sup>2+</sup>-deficient phosphate-buffered saline and harvested in the same buffer containing 0.02% EDTA. After centrifugation at 1500g for 10 min, the cells were homogenized in ice-cold 10 mM Tris-HCl and 1 mM EDTA, pH 7.4 buffer. A crude membrane fraction was collected by centrifugation at 40,000g for 15 min, and resuspended in GTP- $\gamma$ S assay buffer (25 mM Tris-HCl, 150 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10  $\mu$ M GDP, 30  $\mu$ M bestatin, 10  $\mu$ M captopril, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). Approximately 50  $\mu$ g of membrane preparation were incubated at 30°C for 90 min in 1 ml of GTP- $\gamma$ S assay buffer, with appropriate concentrations of deltorphin II, in the presence of 0.1 nM guanosine 5'-O-(3-[ $^{35}$ S]thio)triphosphate (GTP- $\gamma$ [ $^{35}$ S]; 1250 Ci/mmol; Perkin-Elmer Life and Analytical Sciences, Boston, MA). The reaction was filtered using a Brandel Cell Harvester through Whatman GF/B glass fiber filters (Brandel Inc., Gaithersburg, MD). The filters were washed three times with 4 ml of ice-cold 25 mM Tris-HCl and 120 mM NaCl, pH 7.4. Filter-bound radioactivity was measured in EcoLite scintillation cocktail (MP Biomedicals, Irvine, CA) using a Beckman LS 6000SC liquid scintillation counter (Beckman Coulter, Fullerton, CA).

**Inhibition of Forskolin-Stimulated cAMP Formation.** Cyclic AMP assays were performed according to a method modified from 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 confluence. 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  $\mu$ M water-soluble forskolin (7-deacetyl-7-[O-(N-methylpiperazino)- $\gamma$ -butyryl]-dihydrochloride; Calbiochem, San Diego, CA) in the presence of various concentrations of the agonist and 5 mM 3-isobutyl-1-methylxanthine (Sigma) for 20 min, at 37°C, in 5% CO<sub>2</sub> 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 and 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. Fifty microliters of each supernatant and 50  $\mu$ l of a standard (0.125–128 pmol of cAMP) were separately incubated with 50  $\mu$ l (0.9 pmol/50  $\mu$ l) of [ $^3$ H]cAMP (PerkinElmer Life and Analytical Sciences) and 100  $\mu$ l of protein kinase A solution (6  $\mu$ g of protein kinase A/100  $\mu$ l in TE buffer with 0.1% bovine serum albumin) on ice for 2 h. Bound and free [ $^3$ H]cAMP was separated by adding 100  $\mu$ l of activated charcoal in ice-cold TE

buffer containing 2% bovine serum albumin to each tube and centrifuging at 5600g for 1 min. The radioactivity in a 200- $\mu$ l 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 Prism 4 software (GraphPad Software, San Diego, CA).

**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. 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 gels and transferred to nitrocellulose membranes. The membranes were incubated with an antibody raised against a synthetic peptide corresponding to residues around phospho-Ser363 of the human DOR [phospho-hDOR(Ser363) antibody; Cell Signaling Technology Inc. (Danvers, MA)]. Immuno-reactive bands were detected using the SuperSignal West Dura chemiluminescent kit (Pierce, Rockford, IL).

**Synthesis of a Fluorescent Deltorphin II Analog.** [Gln<sup>4</sup>]-deltorphin-rhodamine was synthesized by solid phase synthesis using the *N* $\alpha$ -9-fluorenylmethoxycarbonyl strategy. [Gln<sup>4</sup>]-deltorphin (Tyr-D-Ala-Phe-Gln-Val-Val-Gly) was conjugated to a fluorescent label, tetramethylrhodamine isothiocyanate (Molecular Probes) via the *N*<sup>ε</sup>-Lys residue present in the  $\beta$ -Ala-Gly- $\beta$ -Ala-Gly-Lys spacer that was included at the C terminus of [Gln<sup>4</sup>]-deltorphin. In whole-cell radioligand binding assays, the rhodamine labeled [Gln<sup>4</sup>]-deltorphin exhibited a high affinity for the hDOR with an IC<sub>50</sub> = 11 ± 2 nM.

**Confocal Microscopy.** For visualization of  $\beta$ -arrestin2 recruitment, CHO cells expressing the wild-type or S363A mutant hDOR were grown on glass-bottomed chamber slides and transiently transfected with a green fluorescent protein-tagged  $\beta$ -arrestin2 construct ( $\beta$ -arrestin2-GFP; a gift from Dr. R. Lefkowitz, Duke University Medical Center, Durham, NC) using the FuGENE 6 transfection protocol (Roche Diagnostics, Indianapolis, IN). Forty-eight hours 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 with 1  $\mu$ M [Gln<sup>4</sup>]-deltorphin-rhodamine for 30 min at 37°C. Selective DOR antagonist naltrindole (1  $\mu$ M, IC<sub>50</sub> = 40 pM; Sigma/RBI, Natick, MA) was added to some wells to determine DOR specificity. After treatment, the cells were rinsed with ice-cold phosphate-buffered saline, fixed with 4% paraformaldehyde, and mounted in a Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Slides were examined under a Zeiss LSM520 laser scanning confocal microscope equipped with a 100 $\times$ /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 (Adobe Systems, Mountain View, CA).

**Data Analysis.** To quantitatively analyze the magnitude of hDOR desensitization, the measured levels of GTP $\gamma$ [<sup>35</sup>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 GTP $\gamma$ [<sup>35</sup>S] binding experiments, deltorphin II-stimulated levels of bound GTP $\gamma$ [<sup>35</sup>S] (*B*) were first adjusted by subtracting the basal levels (*B*<sub>0</sub>) and then normalized to the percentage of adjusted maximum stimulation (*B*<sub>max</sub>) in control cells according to the equation: Effect = (*B* - *B*<sub>0</sub>)/[*B*<sub>max</sub>(con) - *B*<sub>0</sub>(con)] × 100%.

In cAMP experiments, the residual level of cAMP production in control cells, which was not inhibited by saturating concentrations of deltorphin II (*P*<sub>max</sub>(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*<sub>0</sub>) and expressed as an increase in cAMP inhibition, rather than a decrease

in cAMP production, in accordance with the equation: Effect = (*P*<sub>0</sub> - *P*)/[*P*<sub>0</sub> - *P*<sub>max</sub>(con)] × 100%. The average maximum level of forskolin-stimulated cAMP was 37 ± 12 and 30 ± 9 pmol/10<sup>5</sup> cells in hDOR/CHO and hDOR(S363A)/CHO cells, respectively. The residual level of cAMP was 12 ± 5 and 14 ± 5 pmol/10<sup>5</sup> 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 et al. (1985). This model describes the correlation between a biological effect *E* and agonist concentration [A] as a function of three parameters: *E*<sub>m</sub>, *K*<sub>A</sub>, and  $\tau$ :

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

where *E*<sub>m</sub>, or the operational maximum, represents the maximum possible effect in the tissue, *K*<sub>A</sub> is the dissociation constant of the agonist and  $\tau$  is the operational efficacy or the transducer ratio. Parameter  $\tau$  is defined as:

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

where [R]<sub>T</sub> is the total concentration of receptors and [K<sub>E</sub>] is the concentration of occupied receptors required to produce half of the maximum *E*<sub>m</sub>. The operational model describes how both *E*<sub>max</sub> and EC<sub>50</sub> change when either the total concentration of receptors [R]<sub>T</sub>, or the concentration of receptors [K<sub>E</sub>] necessary to produce half of the operational maximum (*E*<sub>m</sub>) changes. The dependence of the effect on [R]<sub>T</sub> was used earlier to estimate the proportion of  $\beta_2$  adrenergic (Lohse et al., 1990) or  $\mu$  opioid receptors (Osborne and Williams, 1995) desensitized by agonist pretreatment.

Using the approach of Lohse et al. (1990), a paired set of dose-response curves obtained before and after desensitization was fitted simultaneously using eq. 1. The parameters *E*<sub>m</sub> and *K*<sub>A</sub> 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 postdesensitization curves is the operational efficacy ( $\tau$ ). Because  $\tau$  is proportional to the number of functional (nondesensitized) receptors, the fraction of the desensitized receptors *D* can be calculated as:

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

In this equation,  $\tau_{\text{control}}$  is the initial operational efficacy in the nondesensitized state and  $\tau_{\text{desensitized}}$  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 Prism 4 software.

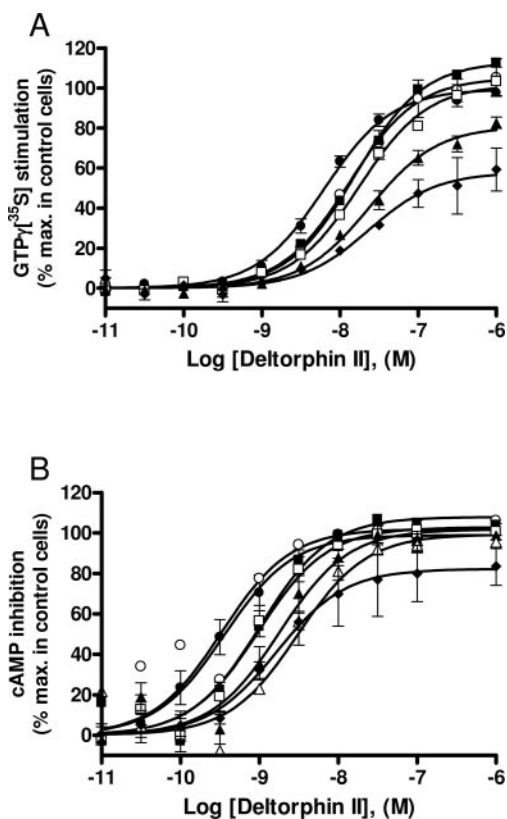
## Results

**Agonist-Mediated hDOR Desensitization.** To study rapid desensitization of the human  $\delta$  opioid receptor, we used recombinant 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 GTP $\gamma$ [<sup>35</sup>S] binding in cell membranes isolated from untreated (nondesensitized) cells or cells that were pretreated (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  $\text{GTP}\gamma^{[35\text{S}]}$  binding shifted to the right upon deltorphin II pretreatment of hDOR/CHO cells. In addition, at longer agonist treatment times, maximal  $\text{GTP}\gamma^{[35\text{S}]}$  bind-



**Fig. 1.** Pretreatment of hDOR/CHO cells with deltorphin II leads to time-dependent desensitization of deltorphin II-stimulated  $\text{GTP}\gamma^{[35\text{S}]}$  binding and inhibition of cAMP production. hDOR/CHO cells were pretreated for the indicated times with 100 nM deltorphin II in serum-free IMDM at 37°C. A, cell membranes were prepared and deltorphin II-stimulated  $\text{GTP}\gamma^{[35\text{S}]}$  binding was measured as described under *Materials and Methods*. Deltorphin II concentration curves were normalized to percentage of maximum effect in control (untreated) cells. B, after pretreatment, the cells were washed and deltorphin II-mediated inhibition of forskolin (100  $\mu\text{M}$ , 20 min)-stimulated cAMP accumulation was measured as described under *Materials and Methods*. Dose-response curves were plotted as percentage 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 duplicate. 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.

**TABLE 1**

$E_{\text{max}}$  and  $\text{EC}_{50}$  values of deltorphin II-mediated stimulation of  $\text{GTP}\gamma^{[35\text{S}]}$  binding and inhibition of cAMP production in hDOR/CHO cells pretreated for indicated times with deltorphin II

Deltorphin II concentration responses for  $\text{GTP}\gamma^{[35\text{S}]}$  stimulation and cAMP inhibition in hDOR/CHO cells pretreated for different time intervals with deltorphin II were fitted with sigmoidal curves (Hill coefficient  $n_H = 1$ ).

	Time						
	0 min	2 min	5 min	10 min	30 min	45 min	60 min
$\text{GTP}\gamma^{[35\text{S}]}$							
$E_{\text{max}}$ (%)	100 ± 1	105 ± 2	113 ± 1	102 ± 2	81 ± 2		58 ± 4
$\text{EC}_{50}$ (nM)	6.3 ± 0.4	13 ± 1	16 ± 1	18 ± 2	24 ± 3		23 ± 7
cAMP							
$E_{\text{max}}$ (%)	100 ± 3	102 ± 7	108 ± 5	103 ± 3	102 ± 4	99 ± 6	82 ± 6
$\text{EC}_{50}$ (nM)	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

ing was also reduced compared with the control cells. The deltorphin II-mediated shift in  $\text{EC}_{50}$ , and the reduction of  $E_{\text{max}}$  were dependent on the time of pretreatment (Table 1). Likewise, Fig. 1B demonstrates that in agonist pretreated cells, deltorphin II was less efficacious and less potent in inhibiting forskolin-stimulated cAMP production compared with the control cells. This effect was also dependent on the time of deltorphin II pretreatment (Table 1). The reduced cellular response to deltorphin II, as measured by either  $\text{GTP}\gamma^{[35\text{S}]}$  binding or inhibition of cAMP accumulation, reflects a decreased ability of the pretreated 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.** Because, in our system, receptor desensitization leads to a reduction in  $E_{\text{max}}$  as well as an increase in  $\text{EC}_{50}$ , both of these changes need to be considered 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 used the operational model of drug action, which calculates the operational efficacy  $\tau$  (and thus the receptor number) based on changes in both  $E_{\text{max}}$  and  $\text{EC}_{50}$ , as explained under *Materials and Methods* (eq. 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  $\tau$ . In the simplest model, we can assume that the number of functional receptors  $[R]_T$ , and consequently  $\tau$ , decreases exponentially as a function of the time of the pretreatment, according to:

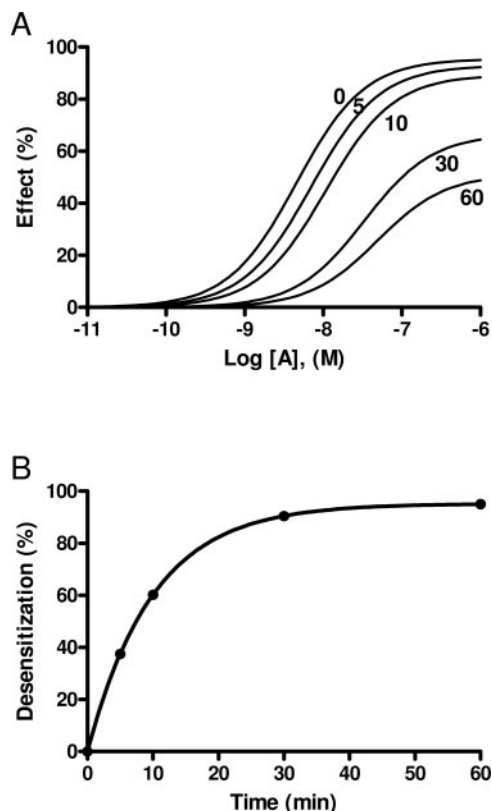
$$\tau(t) = \tau_0 \exp(-kt) + \tau_\infty \quad (4)$$

where  $k$  is the rate constant of desensitization,  $\tau_\infty$  is the asymptotic value of  $\tau$  approached when the maximum number of receptors has been desensitized, and  $(\tau_0 + \tau_\infty) = \tau_{\text{control}}$  is the initial  $\tau$  value in nondesensitized cells. We can now introduce this restriction on parameters  $\tau$  into eq. 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 eq. 4 into eq. 1 yields.

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

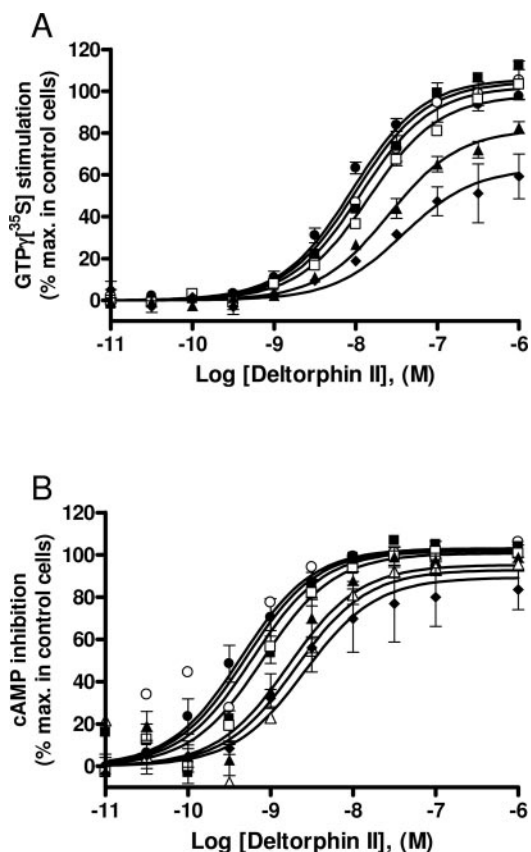
This is a five-parameter equation that describes a whole series of dose-response curves as a function of one variable, the time of deltorphin II pretreatment. Figure 2 illustrates the theoretical dependence of the dose-response curves on the time of pretreatment as predicted from eq. 5. The theoretical curves were generated using arbitrary values  $E_m = 100$ ,  $\tau_0 = 20$ ,  $\tau_\infty = 1$ ,  $K_A = 1$  nM, and  $k = 0.1/\text{min}$ , which were chosen to describe a realistic cellular model. The selected value of  $\tau_0 = 20$  describes a cellular system with significant receptor reserve. In this situation,  $E_{\text{max}}$  of the dose-response curve for nondesensitized receptors approaches the theoretical operational maximum  $E_m$ , and the  $EC_{50}$  is smaller than  $K_A$ . As  $\tau$  decreases with increasing time of pretreatment,  $EC_{50}$  increases, initially with little change in the  $E_{\text{max}}$ . After an extended pretreatment period, a decrease in  $E_{\text{max}}$  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 ( $E_{\text{max}}$ ) does not reach the theoretical maximum  $E_m$  in the nondesensitized state. Therefore, an attenuation of the  $E_{\text{max}}$  is already apparent when only a small proportion of the receptors has been desensitized. How-



**Fig. 2.** Mathematical simulation of the effect of agonist pretreatment on concentration-response curves. A, the concentration-response curves for an agonist [A] were generated using eq. 5, with parameters  $E_m = 100$ ,  $\tau_0 = 20$ ,  $\tau_\infty = 1$ ,  $K_A = 1$  nM and  $k = 0.1/\text{min}$ . The time of treatment for the individual curves was set to: 0, 5, 10, 30, and 60 min. B, the time course of receptor desensitization was simulated with eq. 4 using the same values of parameters  $\tau_0$ ,  $\tau_\infty$ , and  $k$  as in graph (A).

ever, because the reduction in  $E_{\text{max}}$  is always accompanied by an increase in  $EC_{50}$ , the reduction in  $E_{\text{max}}$  alone will underestimate the proportion of desensitized receptors. This emphasizes the need for using changes in both  $E_{\text{max}}$  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 G protein-coupled receptor.

**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 eq. 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),  $\tau$  is defined by eq. 4, in which  $\tau_0$  and  $\tau_\infty$  were fitted shared parameters; for the 60 min curve,  $\tau = \tau_{60}$  was fitted independently because at this time long-term desensitization mechanisms may already be present. In Fig. 3, A and B, the data obtained for the time course of hDOR desensitization (Fig. 1, A and B, respectively)



**Fig. 3.** Time course of deltorphin II-stimulated desensitization of hDOR fitted by the modified operational model (eq. 5). A, the concentration-response curves for deltorphin II-stimulated GTP $\gamma$ [ $^{35}\text{S}$ ] binding from the graph in Fig. 1A were fitted simultaneously using eq. 5. B, the concentration-response curves of deltorphin II-mediated inhibition of forskolin (100  $\mu\text{M}$ , 20 min)-stimulated cAMP accumulation plotted in graph 1B were fitted simultaneously using eq. 5. Deltorphin II pretreatment:  $\bullet$ , 0 min;  $\circ$ , 2 min;  $\blacksquare$ , 5 min;  $\square$ , 10 min;  $\blacktriangle$ , 30 min;  $\triangle$ , 45 min;  $\blacklozenge$ , 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  $\tau$  is defined by eq. 4, in which  $\tau_0$  and  $\tau_\infty$  are fitted shared parameters; for the 60 min curve, the  $\tau$  was designated  $\tau_{60}$  and was fitted independently. The fitted parameters  $E_m$ ,  $K_A$ ,  $k$ ,  $\tau_0$ , and  $\tau_\infty$  for A and B are summarized in Table 1.

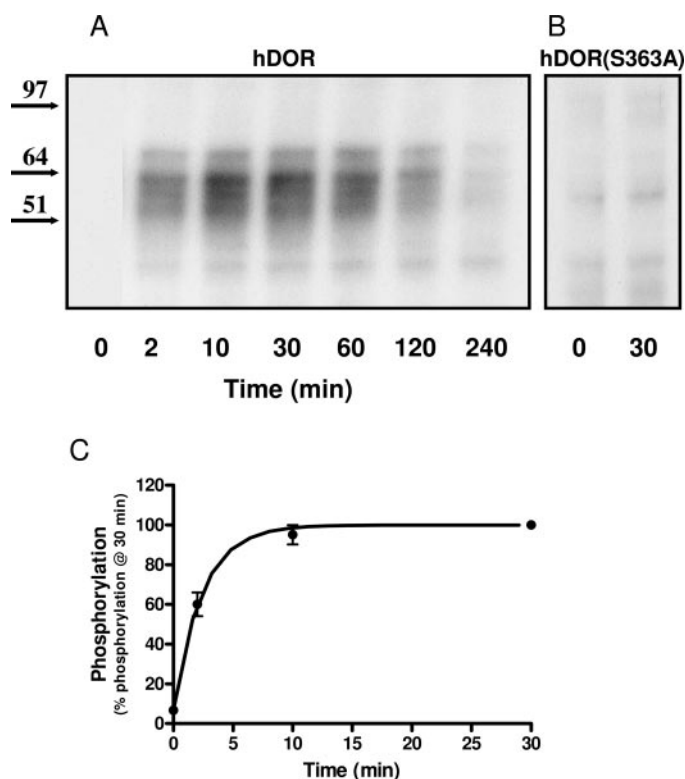
were fitted using eq. 5. All five parameters ( $E_m$ ,  $\tau_0$ ,  $\tau_\infty$ ,  $K_A$ , and  $k$ ) were shared among all dose-response curves for GTP $\gamma$ [ $^{35}$ S] binding and again in a separate calculation using inhibition of cAMP production. The values obtained for these parameters for the GTP $\gamma$ [ $^{35}$ S] and the cAMP assays are summarized in Table 2. The initial value of  $\tau_{\text{control}} = (\tau_0 + \tau_\infty)$  is reported in Table 2 instead of the fitted  $\tau_\infty$  value.

From Table 2 we can observe the fitted operational maximum  $E_m$  is  $120 \pm 5\%$  and  $106 \pm 4\%$  for the GTP $\gamma$ [ $^{35}$ S] and cAMP assays, respectively. In GTP $\gamma$ [ $^{35}$ S] measurements, the operational efficacy in control cells  $\tau_{\text{control}} = (\tau_0 + \tau_\infty)$  was  $7.8 \pm 2.4$ . Because 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 GTP $\gamma$ [ $^{35}$ S] stimulation assay. After deltorphin II pretreatment, the operational efficacy  $\tau$  is reduced and, at infinity, would reach the value  $1.4 \pm 0.5$ . The operational efficacy measured by the cAMP assay  $\tau_{\text{control}} = (\tau_0 + \tau_\infty) = 36 \pm 21$  is higher than that measured by the GTP $\gamma$ [ $^{35}$ 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 was desensitized upon agonist treatment, only a small reduction in  $E_{\text{max}}$  was observed in cAMP assay, and the predominant effect of desensitization was an increase in the  $EC_{50}$  values. It is noteworthy that the desensitization (D) calculated using eq. 3 correlates very well when comparing the two assays (82% for both GTP $\gamma$ [ $^{35}$ 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 GTP $\gamma$ [ $^{35}$ S] and cAMP assays, respectively. The theoretical dissociation constant ( $K_A$ ) of deltorphin II estimated from the two assays was  $81 \pm 16$  nM and  $16 \pm 8$  nM, respectively. Values of  $K_A$  are approximately 10- to 50-fold larger than the  $EC_{50}$  values measured in untreated cells, again supporting the presence of spare receptors.

**Ser363 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  $\beta$ -arrestin, and receptor internalization. In HEK 293 cells, phosphorylation of the mouse DOR is hierarchical; the Ser363 in the C-terminal region is the primary phosphor-

ylation site (Kouhen et al., 2000). In addition, mutation of Ser363 to Ala was shown to reduce agonist-mediated uncoupling of the receptor from adenylyl cyclase signaling (Law et al., 2000). Because receptor phosphorylation leads to  $\beta$ -arrestin recruitment and receptor internalization, it is not clear which of these mechanisms is crucial for hDOR desensitization.

To investigate the role of Ser363 phosphorylation in the desensitization of the human DOR, we mutated Ser363 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(Ser363) primary antibody (Cell Signaling Technology) to investigate agonist-mediated Ser363 phosphorylation. Figure 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 Ser363. Receptor phosphorylation was detected as an increased intensity of several immunoreactive bands in the molecular mass range of 50 to 70 kDa, which correspond to differently (N-linked and/or O-linked) glycosylated forms of the hDOR (D. Stropova, unpublished data). We have demonstrated previously (Navratilova et al., 2005) that this phosphorylation is completely blocked by concomitant treatment of the cells with a



**Fig. 4.** Treatment of hDOR/CHO cells with deltorphin II leads to phosphorylation of hDOR at Ser363. 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-Ser363 hDOR antibody. A, a representative immunoblot showing the time course of Ser363 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 ImageJ software, and averages from three independent experiments  $\pm$  S.E. were plotted. The early phase of receptor phosphorylation (0–30 min) was fitted with an exponential association curve.

TABLE 2

Parameters of deltorphin II-mediated desensitization of hDOR calculated by eq. 5

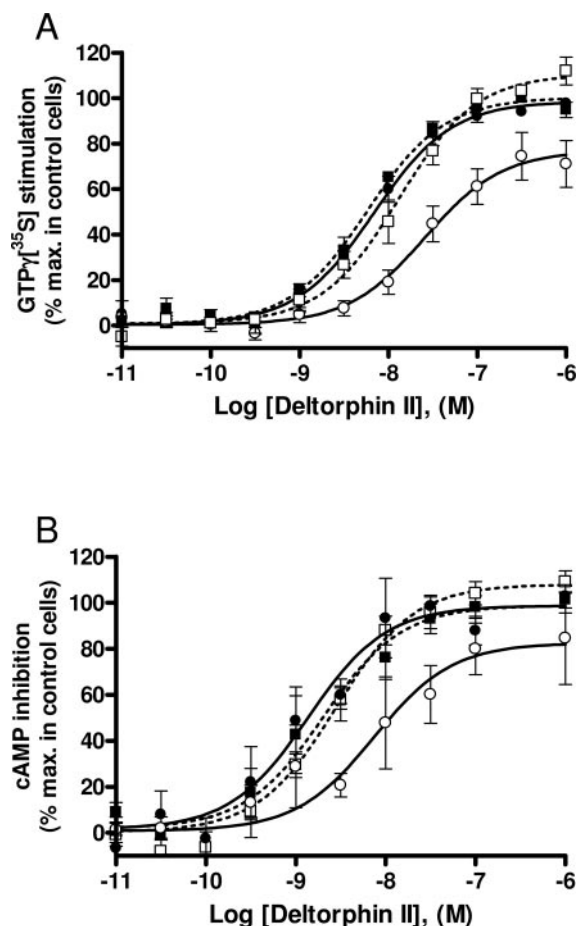
Five parameters of eq. 5 ( $E_m$  = operational maximum,  $\tau_{\text{control}} = (\tau_0 + \tau_\infty)$ , and  $\tau_\infty$  the operational efficacies before and after desensitization,  $k$  = desensitization rate constant,  $K_A$  = dissociation constant) were calculated by fitting the dose response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

	$E_m$	$\tau_{\text{control}}$	$\tau_\infty$	$k$	$K_A$
	%			$\text{min}^{-1}$	nM
GTP $\gamma$ [ $^{35}$ S]	$120 \pm 5$	$7.8 \pm 2.4$	$1.4 \pm 0.5$	$0.070 \pm 0.018$	$81 \pm 16$
cAMP	$106 \pm 4$	$36 \pm 21$	$6.4 \pm 3.4$	$0.082 \pm 0.034$	$16 \pm 8$



selective  $\delta$  opioid receptor antagonist, naltrindole (1  $\mu$ M). 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- to 70-kDa bands were analyzed using the NIH ImageJ software (<http://rsb.info.nih.gov/ij/>) 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 was  $t_{1/2} = 1.7 \pm 0.2$  min. These experiments provided evidence that deltorphin II promoted time dependent phosphorylation of hDOR at Ser363 and confirmed that this phosphorylation was eliminated by mutating Ser363.

**S363A Mutation Attenuates hDOR Desensitization.** Next, we investigated the effect of S363A mutation on the



**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 for 60 min at 37°C. A, cells were harvested, cell membranes were prepared, and deltorphin II-stimulated GTP $\gamma$ [ $^{35}$ S] binding was measured, as described under *Materials and Methods*. Deltorphin II concentration curves were normalized to percentage 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  $\mu$ M, 20 min)-stimulated cAMP accumulation were measured as described under *Materials and Methods*. The inhibition curves were plotted as the percentage of cAMP inhibition in control cells. The normalized data from three (A) and five (B) independent experiments (performed in duplicate) 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.

hDOR desensitization. Using the GTP $\gamma$ [ $^{35}$ 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). 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 Prism 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 curves for untreated wild-type and mutant receptors were not significantly different in either GTP $\gamma$ [ $^{35}$ S] or cAMP assay, confirming that the S363A mutation had no effect on acute receptor signaling. It is noteworthy that 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 GTP $\gamma$ [ $^{35}$ 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 pretreatment was much smaller (2.3-fold from 5.8 to 13 nM and 1.4-fold from 2.0 to 2.8 nM in GTP $\gamma$ [ $^{35}$ S] and cAMP assay, respectively). This shift was statistically significant in GTP $\gamma$ [ $^{35}$ S] assay ( $p < 0.001$ ) but not 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 Ser363 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 under *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  $\tau = \tau_{\text{control}}$ . This assumption is justified by the results of the regression statistical comparison. The fitted curves for GTP $\gamma$ [ $^{35}$ S] binding and inhibition of cAMP production experiments in cells expressing the wild-type and

TABLE 3

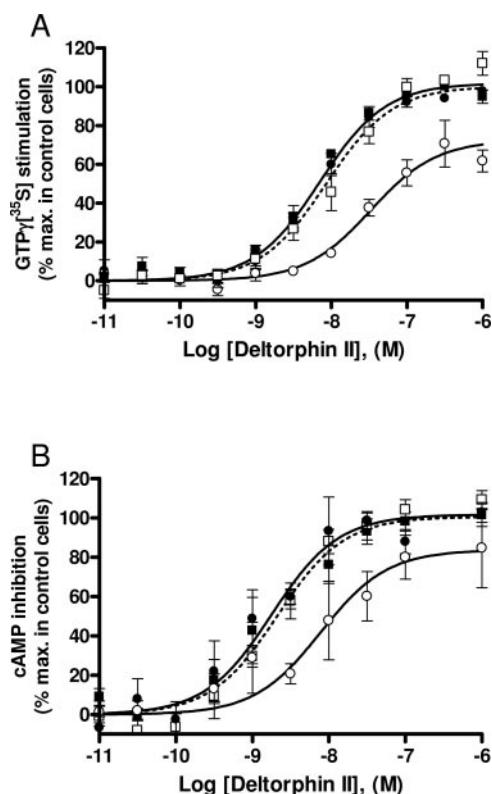
$EC_{50}$  values of deltorphin II-mediated stimulation of GTP $\gamma$ [ $^{35}$ S] binding and inhibition of cAMP production in naive and deltorphin II pretreated hDOR/CHO or hDOR(S363A)/CHO cells

Deltorphin II concentration responses for GTP $\gamma$ [ $^{35}$ S] stimulation and cAMP inhibition in naive and deltorphin II pretreated hDOR/CHO or hDOR(S363A)/CHO cells were fitted with sigmoidal curves (Hill coefficient  $n_H = 1$ ).

	hDOR		S363A	
	Control	Deltorphin	Control	Deltorphin
GTP $\gamma$ [ $^{35}$ S]				
$E_{\max}$ (%)	99 $\pm$ 2	77 $\pm$ 5	100 $\pm$ 2	110 $\pm$ 3
$EC_{50}$ (nM)	6.9 $\pm$ 0.9	31 $\pm$ 7	5.8 $\pm$ 0.7	13 $\pm$ 2
cAMP				
$E_{\max}$ (%)	99 $\pm$ 6	83 $\pm$ 9	99 $\pm$ 5	108 $\pm$ 5
$EC_{50}$ (nM)	1.4 $\pm$ 0.5	7.5 $\pm$ 4.0	2.0 $\pm$ 0.7	2.8 $\pm$ 0.8

S363A mutant receptors are presented in Fig. 6, A and B, respectively, and the calculated values for the fitted parameters are summarized in Table 4. From the fitted parameters  $\tau$ , desensitization of the wild-type and Ser363 mutant hDOR was computed using eq. 3. Desensitization of the hDOR achieved after 60 min of deltorphin II treatment was 85% and 81% in GTP $\gamma$ [<sup>35</sup>S] and cAMP assays, respectively; these values are comparable with 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 GTP $\gamma$ [<sup>35</sup>S] and cAMP assays, respectively. Because the S363A mutation significantly reduced the number of receptors desensitized by deltorphin II, Ser363 must play a crucial role in receptor desensitization.

One possibility for the role of Ser363 in hDOR desensitization may be that Ser363 phosphorylation hinders the interaction of the receptor with G proteins. On the other hand, other events dependent on Ser363 phosphorylation may also be necessary for complete receptor uncoupling. These events may include phosphorylation of other residues, binding of GRKs or  $\beta$ -arrestins to the receptor, receptor sequestration into clathrin-coated pits, and receptor internalization into



**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 at 37°C. A, the concentration-response curves of deltorphin II-stimulated GTP $\gamma$ [<sup>35</sup>S] binding from graph 5A were fitted simultaneously using eq. 1. B, the concentration-response curves of deltorphin II-mediated inhibition of forskolin (100  $\mu$ M, 20 min)-stimulated cAMP accumulation from graph 5B were fitted simultaneously using eq. 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  $\tau$  for A and B are summarized in Table 4.

endosomes. Because deltorphin II-mediated desensitization of the hDOR is significantly reduced by the mutation of Ser363 to alanine, we hypothesized that every molecular event involved in receptor desensitization would also be significantly attenuated by this mutation.

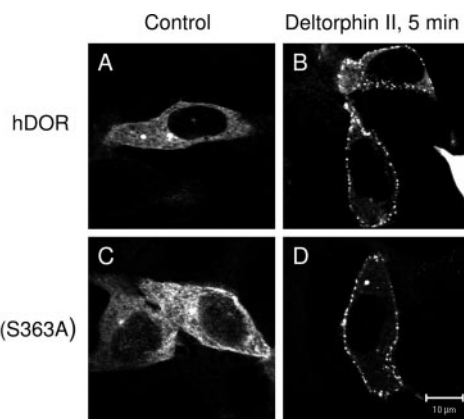
**S363A Mutation Does Not Eliminate Agonist-Mediated Recruitment of  $\beta$ -Arrestin.** We first investigated the role of Ser363 in  $\beta$ -arrestin binding to hDOR. To study agonist-mediated translocation of  $\beta$ -arrestin to the plasma membrane, we transiently transfected the hDOR/CHO or hDOR(S363A)/CHO cells with a green fluorescent protein tagged  $\beta$ -arrestin2 construct ( $\beta$ -arrestin2-GFP). Forty-eight hours 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 (Fig. 7, A and B) clearly show that deltorphin II treatment promoted the translocation of  $\beta$ -arrestin2-GFP to the plasma membrane in CHO cells expressing the wild-type hDOR. It is noteworthy that a similar pattern of  $\beta$ -arrestin2-GFP translocation was observed in cells expressing the S363A mutant receptor (Fig. 7, C and D). Using a semiquantitative analysis of  $\beta$ -arrestin2-GFP translocation, we observed no detectable difference in the magnitude and the time course of  $\beta$ -arrestin2-GFP translocation between the wild-type and the hDOR(S363A) mutant receptor-expressing cells. These re-

TABLE 4

Parameters of deltorphin II-mediated desensitization of hDOR and hDOR(S363A) calculated using the operational model

Parameters of the operational model (eq. 1) ( $E_m$  = operational maximum;  $\tau_{\text{control}}$ ,  $\tau_{\text{del-wt}}$ , and  $\tau_{\text{del-S363A}}$  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 GTP $\gamma$ [<sup>35</sup>S] binding or inhibition of cAMP production.

	$E_m$	$\tau_{\text{control}}$	$\tau_{\text{del-wt}}$	$\tau_{\text{del-S363A}}$	$K_A$
	%				nM
GTP $\gamma$ [ <sup>35</sup> S]	110 $\pm$ 4	13 $\pm$ 5	1.9 $\pm$ 0.5	10 $\pm$ 4	97 $\pm$ 33
cAMP	107 $\pm$ 7	19 $\pm$ 18	3.6 $\pm$ 2.3	16 $\pm$ 15	36 $\pm$ 30



**Fig. 7.** Deltorphin II stimulates translocation of  $\beta$ -arrestin2-GFP in CHO cells expressing wild-type or S363A mutant hDOR. CHO cells expressing the wild type (A and B) or S363A mutant (C and D) hDOR were transfected with a green fluorescent protein tagged  $\beta$ -arrestin2 construct ( $\beta$ -arrestin2-GFP). Forty-eight hours after transfection, the cells were treated in the absence (A and C) or presence (B and D) of 100 nM deltorphin II for 5 min at 37°C. After agonist treatment, the cells were fixed and examined under a laser scanning confocal microscope (LSM520; Carl Zeiss Inc., Thornwood, NY) equipped with a 100 $\times$ /1.40 numerical aperture oil objective. Single optical sections were acquired through the *trans*-nuclear plane. Scale bar, 10  $\mu$ m. Images were processed in Adobe Photoshop 7.0.



sults provide evidence that Ser363 in hDOR is not required for agonist-mediated  $\beta$ -arrestin2 translocation to the plasma membrane. In addition, these results indicate that even though  $\beta$ -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 fluorescent analog of deltorphin II ([Gln<sup>4</sup>]deltorphin-rhodamine). In hDOR/CHO cells, after 30 min of agonist treatment, the fluorescent marker was concentrated in intracellular punctate compartments resembling endosomes (Fig. 8A). Internalization of the fluorescent agonist was receptor-mediated because it was completely blocked by a  $\delta$ -selective opioid receptor antagonist, naltrindole (1  $\mu$ M; Fig. 8B). In cells expressing the S363A mutant receptor, we observed virtually indistinguishable patterns of fluorescent deltorphin internalization. Therefore, S363A mutation in the hDOR did not eliminate the ability of the receptor to internalize [Gln<sup>4</sup>]deltorphin-rhodamine. From these results, we conclude that Ser363 is not necessary for agonist-mediated hDOR internalization, and hDOR internalization is not sufficient to completely uncouple the receptor from G proteins.

## Discussion

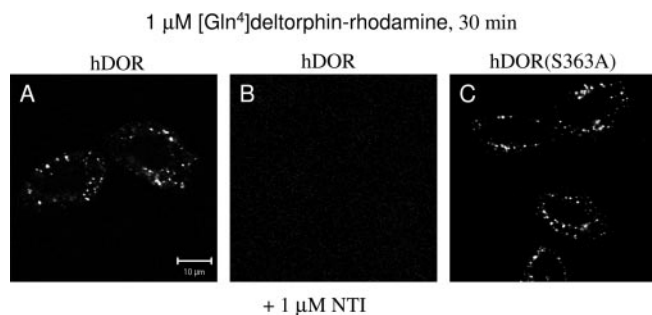
A major obstacle in understanding the exact mechanism of rapid homologous DOR desensitization is the inability to correctly determine the fraction of desensitized receptors. In our present study, we used a mathematical analysis based on the operational model of agonist action (Black et al., 1985) to calculate the proportion of desensitized hDOR by deltorphin II pretreatment in two functional assays: stimulation of GTP $\gamma$ [<sup>35</sup>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 Ser363, translocation of  $\beta$ -arrestin2 to the plasma membrane, and hDOR internalization. The results of

these experiments demonstrate that  $\beta$ -arrestin binding and receptor internalization are not sufficient to bring about hDOR desensitization without phosphorylation of Ser363.

As emphasized under *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 that express low levels of the DOR, such as SK-N-BE (Hasbi et al., 1998) or in cells in which receptor level was experimentally manipulated (Law et al., 2000). In other studies, a mathematical analysis was used to calculate the proportion of desensitized receptors. 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). Likewise, Whaley et al. (1994) adapted models of receptor/G protein activation of adenylyl cyclase to derive expressions that predict changes in EC<sub>50</sub> and E<sub>max</sub> as the receptor number varies. It can be demonstrated that after transformation, the two methods yield identical equations. Because 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 et al. (1990) and, assuming that the time dependence of receptor desensitization can be approximated by an exponential function, we derived an equation that allowed us to estimate the half-life of hDOR desensitization.

Results of our analysis indicated that in recombinant CHO cells the hDOR was 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 approximately 80% of receptors desensitized. The same treatment desensitized only 20% of a mutant hDOR in which the primary phosphorylation site, Ser363, 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 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; Willets and Kelly, 2001; Eisinger et al., 2002; Li et al., 2003). Based on mutational analysis, Ser363 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(Ser363) primary antibody that Ser363 of the human DOR is phosphorylated upon deltorphin II treatment in a time-dependent manner with a half-life of approximately 2 min. This half-life is shorter than the half-life of receptor desensitization (~10 min), indicating that the relationship between receptor phosphorylation and desensi-



**Fig. 8.** [Gln<sup>4</sup>]deltorphin-rhodamine is internalized in CHO cells expressing either wild-type or S363A mutant hDOR. For internalization studies, hDOR/CHO (A and B) or hDOR(S363A)/CHO (C) cells were incubated with [Gln<sup>4</sup>]deltorphin-rhodamine (1  $\mu$ M) for 30 min at 37°C. In B, 1  $\mu$ M 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 $\times$ /1.40 numerical aperture oil objective. Single optical sections were acquired through the *trans*-nuclear plane. The acquisition parameters were constant in all parallel experiments. Scale bar, 10  $\mu$ m. Images were processed in Adobe Photoshop 7.0.

tization is not direct. Indeed, elimination of Ser363 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 Ser363-dependent and -independent mechanisms. 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 probably does not involve GRK, because binding of GRK2 alone to a phosphorylation deficient mutant of the DOR did not cause desensitization of GTP $\gamma$ [<sup>35</sup>S] binding in HEK 293 cells (Li et al., 2003).

It has been demonstrated that binding of  $\beta$ -arrestin to GPCRs requires phosphorylation of several Ser/Thr residues in the intracellular domains of the receptors (Gurevich and Gurevich, 2004). Accordingly, receptor phosphorylation was found to be the rate-limiting step for  $\beta$ -arrestin and  $\beta_2$ -adrenergic receptor interaction (Krasel et al., 2004). G protein-coupled receptor- $\beta$ -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  $\beta$ -arrestin1 or -2 with GRK3 was required for desensitization of DOR-coupled inwardly rectifying potassium channel (Kir3) in *Xenopus laevis* oocytes (Lowe et al., 2002). Our confocal microscopy images show that mutation of the primary phosphorylation site Ser363 in the hDOR that significantly impairs receptor desensitization did not prevent the recruitment of  $\beta$ -arrestin2-GFP to the plasma membrane. No visually apparent differences were observed in the time course and the extent of  $\beta$ -arrestin2-GFP translocation to the hDOR or hDOR(S363A). Fluorescent microscopy does not allow quantification of the  $\beta$ -arrestin-receptor interaction; however,  $\beta$ -arrestin2-GFP translocation clearly occurs after 5 min of deltorphin II treatment in the hDOR(S363A) mutant receptor. Therefore, changes in the interaction between  $\beta$ -arrestin2-GFP and the hDOR(S363A) compared with 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  $\beta$ -arrestin2 binding to hDOR alone is not sufficient for receptor desensitization without Ser363 phosphorylation. In support of this conclusion, it was reported recently using locus ceruleus neurons from  $\beta$ -arrestin2 knockout mice that lack of  $\beta$ -arrestin2 expression has no effect on the rate or the magnitude of the  $\mu$  opioid receptor desensitization (Dang and Christie, 2006). Nevertheless, in our system,  $\beta$ -arrestin binding may still function as an essential mechanism in the residual Ser363 phosphorylation-independent desensitization, probably in conjunction with receptor internalization. Indeed, phosphorylation independent,  $\beta$ -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. (2006) found that visual arrestin is able to quench nonphosphorylated rhodopsin. Marion et al. (2006) identified a common  $\beta$ -arrestin binding

site formed by 10 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. (2000) 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 Ser363 phosphorylation-dependent. In addition, a small fraction of receptor desensitization is Ser363 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  $\beta$ -arrestin-dependent internalization.

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 hDOR and a phosphorylation-deficient mutant of the hDOR, we were able to correlate desensitization with receptor phosphorylation,  $\beta$ -arrestin2 translocation and receptor internalization. We have demonstrated that in CHO cells expressing the hDOR, deltorphin II treatment leads to phosphorylation of Ser363, translocation of  $\beta$ -arrestin2 to the plasma membrane, receptor internalization, and uncoupling from G proteins. It is noteworthy that the S363A mutation completely eliminated phosphorylation of this residue yet had virtually no effect on  $\beta$ -arrestin2 translocation and receptor internalization. On the other hand, S363A mutation significantly attenuated receptor desensitization. These results provide evidence that phosphorylation of Ser363 is required to uncouple the receptor from G proteins. Recruitment of  $\beta$ -arrestin2 and receptor internalization, on the other hand, are not sufficient to desensitize the hDOR without Ser363 phosphorylation. Therefore, we conclude that phosphorylation of Ser363 represents the primary mechanism of the human  $\delta$  opioid receptor desensitization.

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