Agonist-Induced Desensitization and Down-regulation of the Human Kappa Opioid Receptor Expressed in Chinese Hamster Ovary Cells

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ABSTRACT

In this study, we examined whether the human kappa opioid receptor stably expressed in Chinese hamster ovary cells underwent desensitization and down-regulation after prolonged exposure to the agonist (−)-U50,488H. Pretreatment with (−)-U50,488H led to a reduction in the magnitude of increase in [35S]GTPγS binding by the subsequent application of (−)-U50,488H. The extent of desensitization was related to duration of exposure and (−)-U50,488H concentration. Pretreatment with (−)-U50,488H also reduced the potency of (−)-U50,488H in inhibiting forskolin-stimulated adenylate cyclase. In membranes of (−)-U50,488H-pretreated cells, the affinity of (−)-U50,488H was lower than that in the untreated control, and GTPγS had no effect on (−)-U50,488H affinity, consistent with the notion of uncoupling of the receptor-G protein complex by (−)-U50,488H treatment. Down-regulation of the kappa opioid receptor also occurred on exposure to (−)-U50,488H. Higher (−)-U50,488H concentrations and/or longer incubation periods were required for down-regulation than for desensitization. The degree of down-regulation depended on the agonist concentration and incubation time. (−)-U50,488H-induced desensitization and down-regulation were blocked by naloxone. (+)U50,488H, an inactive stereoisomer, did not cause desensitization or down-regulation. These results indicate that both processes were receptor-mediated. After incubation with (−)-U50,488H and removal of (−)-U50,488H, both (−)-U50,488H-induced [35S]GTPγS binding and receptor number returned to the control level, which indicates that both processes were reversible. Thus, desensitization and down-regulation of the kappa opioid receptor occur after agonist exposure and represent two different adaptation mechanisms.

Opioid receptors play important roles in many physiological functions and mediate pharmacological effects of opiate and opioid compounds. The presence of multiple types of opioid receptors (mu, delta, kappa and epsilon) in the peripheral and central nervous system has been established by pharmacological and binding studies as well as differential anatomical localization (for a review, Chang, 1984). Activation of opioid receptors couples to various effectors via pertussis toxin-sensitive G protein, including adenylate cyclase and K+ and Ca++ channels (for reviews, Loh and Smith, 1990; Childers, 1991).

Activation of kappa opioid receptors produces many effects including analgesia (Von Voigtländer et al., 1983; Dykstra et al., 1987), dysphoria (Pfeiffer et al., 1986; Dykstra et al., 1987), water diuresis (Von Voigtländer et al., 1983; Dykstra et al., 1987) and hypothermia (Adler and Geller, 1993). After the cloning of the mouse delta opioid receptor (Kieffer et al., 1992; Evans et al., 1992), we (Zhu et al., 1995), Mansson et al. (1994) and Simonin et al. (1995) reported cloning of the human kappa opioid receptor. Deduced amino acid sequences of these clones display the motif of seven transmembrane helices, characteristics of G protein-coupled receptors.

Many G protein-coupled receptors show attenuated responsiveness to agonists after prolonged or repeated activation. Three processes are involved in response to agonists occurring across a time scale ranging from seconds to days: desensitization (seconds to hours), sequestration (minutes to hours) and down-regulation (hours to days). These processes were best studied in the beta-2 adrenergic receptor (for a

ABBREVIATIONS: CHO cells, Chinese hamster ovary cells; CHO-hkor cells, Chinese hamster ovary cells stably transfected with the cloned human x opioid receptor; G protein, guanine nucleotide-binding regulatory protein; GDP, guanosine diphosphate; GTPγS, guanosine-5′-O-(3-thiotriphosphate); hkor, human x opioid receptor; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; NaF, sodium fluoride; PBS, phosphate-buffered saline; (−)-U50,488H, (−)-trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetylamide.
Review, Hausdorf et al., 1990). Desensitization is thought to be mediated partly through the uncoupling of the high-affinity receptor-G protein complex, which explains the loss of receptor responsiveness to agonists. Down-regulation involves a reduction in the number of receptors. Compared with mu and delta opioid receptors, relatively few studies have been conducted on desensitization and down-regulation of the kappa opioid receptor and reports are conflicting (Attali et al., 1989; Attali and Vogel, 1990; Joseph and Bidlack, 1995; Raynor et al., 1994; Blake et al., 1997; Jin et al., 1997). Attali et al. (1989) showed that pretreatment of rat spinal cord-dorsal root ganglion co-cultures with etorphine or U50,488H led to heterologous desensitization of kappa opioid, muscarinic and alpha-1 adrenergic receptors. After 72 h exposure to 1 μM etorphine, a slight down-regulation of the kappa opioid receptor was observed (Attali and Vogel, 1990). Incubation of R1.1 cells with 0.1 μM U50,488H for 24 h or 48 h resulted in 50% reduction of Bmax of 3H]U69,593 and 3H]naloxone (Joseph and Bidlack, 1995). However, there was no change in the inhibition of forskolin-stimulated adenylate cyclase activity by U50,488H in terms of potency and maximal response (Joseph and Bidlack, 1995). Incubation of COS-7 cells transiently expressing the cloned mouse kappa opioid receptor with 1 μM U50,488H for 4 h diminished kappa opioid agonist-induced inhibition of forskolin-stimulated adenylate cyclase, with no change in the total receptor number (Raynor et al., 1994). Treatment of CHO cells stably expressing the rat kappa opioid receptor with U69,593 up to 10 μM for 4 h did not lead to reduction of the capacity of the agonist to inhibit forskolin-stimulated adenylate cyclase (Avidor-Reiss et al., 1995). Pretreatment of HEK-293 cells stably transfected with the human kappa opioid receptor with 1 μM U50,488H for 3 h led to a 6-fold increase in the EC50 value, with no change in the maximal response, of U50,488H in inhibition of forskolin-stimulated adenylate cyclase activity (Blake et al., 1997). Concomitantly, the Bmax value of 3H]diprenorphine binding was reduced by 31% with no change in its IC50 value (Blake et al., 1997). U69,593 elicited a large K+ current in Xenopus oocytes injected with mRNA of the kappa opioid receptor and a G protein-linked inwardly rectifying potassium channel, and this effect was quickly desensitized with a T1/2 of 10.5 min (Henry et al., 1997). Chronic in vivo administration of U50,488H shifted the dose-response curve of U69,593-induced electrophysiological responses to the right by 3-fold and reduced the maximal effect in guinea pig hippocampal slices in vitro (Jin et al., 1997).

We recently established a cell line of CHO-hkor cells (Zhu et al., 1995). Hkor expressed in CHO cells exhibited binding affinity and specificity for opioid ligands expected of the kappa opioid receptor (Zhu et al., 1997). In addition, we demonstrated that activation of hkor by kappa opioid agonists enhanced 35S]GTPyS binding. Pretreatment with pertussis toxin abolished agonist-induced increase in 35S]GTPyS binding, which indicates the coupling of the kappa opioid receptor to G1 and/or G3 proteins. This assay provides a sensitive functional measure for interaction between kappa opioid receptors and pertussis toxin-sensitive G proteins (Zhu et al., 1997). In this study, we examined whether desensitization and down-regulation of the human kappa opioid receptors expressed in the CHO cell line occurred after exposure to the kappa opioid agonist (–)U50,488H, by determining 35S]GTPyS binding, adenylate cyclase and receptor binding activities.

Materials and Methods

Materials. 3H]Diprenorphine (35 Ci/mmol) was obtained from Amersham Corp (Arlington Heights, IL). 3H]GTPyS (1,000–1,200 Ci/mmol), 3H]adrenine (30 Ci/mmol) and 11C]cAMP (40 Ci/mmol) were purchased from NEN Life Sciences (Boston, MA). Naloxone was a gift from DuPont/Merck Co. (Wilmington, DE). (–)U50,488H and (+)U50,488H were provided by Upjohn Co. (Kalamazoo, MI). GDP, GTPyS and Dubelcco’s modified Eagle’s medium were purchased from Sigma (St. Louis, MO). Genetin was purchased from Mediatech Co. (Herndon, VA); fetal calf serum from Hyclone Co. (Logan, UT) and penicillin and streptomycin from GIBCO-BRL Co. (Gaithersburg, MD).

Stable expression of the human kappa opioid receptor in CHO cells. CHO cell lines stably expressing the human kappa opioid receptor (CHO-hkor) were established as described previously (Zhu et al., 1997).

Pretreatment of CHO-hkor cells with the kappa opioid agonist (–)U50,488H. CHO-hkor cells were cultured in 100-mm culture dishes in Dulbecco’s modified Eagle’s medium F12 HAM supplemented with 10% fetal calf serum, 0.2 mg/ml G418, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO2 and 95% air at 37°C. At ~90% confluence, the cells were washed once with 100 mM PBS and treated with the kappa opioid agonist (–)U50,488H in the medium mentioned above for a time at a concentration as indicated. Cells were harvested and membranes prepared by a procedure similar to that described previously (Zhu et al., 1997). Cells were washed twice with 100 mM PBS, harvested in Versene solution, centrifuged at 500 × g for 3 min and washed once with PBS. The cell pellet was resuspended in 50 mM Tris-HCl buffer containing 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N’,N’-tetraacetic acid, 5 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM NaF and 10 mM Na pyrophosphate, sonicated and centrifuged at 46,000 × g for 30 min. The pellet was resuspended in 50 mM Tris, pH 7.0, and centrifuged again. The membrane pellet was resuspended in 50 mM Tris, pH 0.32 M sucrose, pH 7.0, aliquoted at ~100 μg protein/ml, frozen in dry ice/ethanol and stored in ~70°C until use. All procedures were performed at 4°C.

Kappa opioid receptor binding assay. Receptor binding was conducted with 3H]diprenorphine in 50 mM Tris-HCl buffer containing 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N’,N’-tetraacetic acid and 5 μM leupeptin (pH 7.4) as described previously (Zhu et al., 1995). (~)Naloxone (10 μM) was used to define nonspecific binding. Saturation experiments were performed with various concentrations of 3H]diprenorphine (ranging from 0.02 nM to 2 nM). Competitive inhibition of 3H]diprenorphine binding by (~)U50,488H was performed with 3H]diprenorphine at a concentration close to its IC50 (~0.2 nM) and various concentrations of (~)U50,488H. Binding was conducted at 25°C for 60 min in duplicate in a volume of 1 ml with 30 to 40 μg protein. Bound and free ligands were separated by rapid filtration under reduced pressure over GF/B filters presoaked with 0.2% polyethyleneimine and 0.01% bovine serum albumin in 50 mM Tris-HCl (pH 7.4) for 1 h. Binding data were analyzed with EBDA and LIGAND programs (McPherson, 1983).

35S]GTPyS binding assay. 35S]GTPyS binding assay was performed as described previously (Zhu et al., 1997). Before assay, membranes were thawed at 37°C, chilled on ice, passed through a 22-gauge needle and diluted with 50 mM HEPES, 100 mM NaCl, 5 mM MgCl2 and 1 mM ethylenediaminetetraacetic acid with 1 mM diithiothreitol and 0.1% bovine serum albumin freshly added (pH 7.4). Membranes (~10 μg protein) were incubated in the buffer described above containing 35S]GTPyS (~0.000–150,000 cpm, ~80 pM), GDP (3 μM) and varying concentrations of the kappa opioid agonist (~)U50,488H (10–11 to 10–5 M) in a total volume of 0.5 ml for 60 min at 30°C. Nonspecific binding was defined by incubation in the
CHO-hkor cells grown in 6-well plates were incubated with [3H]adenosine for 24 h at 37°C for the last 2 h. After removal of the medium, cells were washed three times with PBS, and serum-free medium containing 1 mM 3-isobutyl-1-methylxanthine was added. Cells were then treated with vehicle, forskolin (10 μM), and forskolin (10 μM) with (-)U50,488H at 37°C for 10 min. The reaction was stopped with the addition of 0.1 N HCl. [14C]cAMP (3–3000 dpm) was added as the recovery standard. Radiolabeled cAMP was separated from other labeled nucleotides by the dual-column method of Salomon (1979). The radioactivities of eluted [14C]cAMP and [14C]cAMP were determined by two-channel scintillation counting. [14C]cAMP formed was calculated as percent of total adenine nucleotides taking into account the cross-over and recovery.

**Protein assay.** Protein contents of membranes were determined by the biocinchonic acid method of Smith et al. (1985) with bovine serum albumin as the standard.

**Statistical analysis.** For comparison of multiple groups, data were analyzed with analysis of variance to determine whether there were significant differences among groups. If so, Sheffe F-test was performed to determine whether there was significant difference between the control and each treatment group. For comparison of two groups, Student’s t test was performed. P < .05 was used as the level of significance.

### Results

**Effect of (-)U50,488H pretreatment time on (-)U50,488H-induced increase in [35S]GTPγS binding and the number of kappa opioid receptors.** CHO-hkor cells were pretreated with 1 μM (-)U50,488H for various periods of time and examined for their responses to the subsequent application of (-)U50,488H. When membranes were prepared without 10 mM NaF and 10 mM Na pyrophosphate, desensitization was not observed consistently (data not shown). The levels of (-)U50,488H-induced [35S]GTPγS binding were similar in untreated membranes prepared in the presence and absence of 10 mM NaF and 10 mM Na pyrophosphate. Hence, membranes for desensitization experiments were prepared in the presence of 10 mM NaF and 10 mM Na pyrophosphate.

Pretreatment with 1 μM (-)U50,488H for ≥15 min reduced the maximal response of (-)U50,488H-induced [35S]GTPγS binding with or without increasing the EC50 value (fig. 1A and table 1). Maximal [35S]GTPγS binding induced by (-)U50,488H was significantly decreased to 76%.

![Fig. 1](image)

**Fig. 1.** Effect of pretreatment time with 1 μM (-)U50,488H on (A) [35S]GTPγS binding induced by (-)U50,488H and (B) [3H]diprenorphine binding. CHO-hkor cells were treated at 37°C with or without 1 μM (-)U50,488H for different periods of time, washed extensively and membranes prepared. [35S]GTPγS binding in response to (-)U50,488H and saturation [3H]diprenorphine binding were performed as described under “Materials and Methods.” (A) Each point represents the mean ± S.E.M. of three to six experiments. Basal [35S]GTPγS binding ranged from 83 to 88 fmol/mg protein and did not differ among the control and the treatment groups. (B) Scatchard analysis of [3H]diprenorphine binding after each treatment was performed. Each curve represents one of the three to six experiments performed. EC50 and maximal level values of [35S]GTPγS binding and Kd and Bmax of [3H]diprenorphine binding are presented in table 1.

<table>
<thead>
<tr>
<th>Times</th>
<th>[35S]GTPγS binding</th>
<th>[3H]Diprenorphine binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>EC50</td>
<td>Maximal</td>
</tr>
<tr>
<td>15 min</td>
<td>3.8 ± 0.5</td>
<td>191 ± 3</td>
</tr>
<tr>
<td>1 h</td>
<td>5.4 ± 0.7</td>
<td>144 ± 7</td>
</tr>
<tr>
<td>24 h</td>
<td>10.4 ± 1.4</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>4 h</td>
<td>23.5 ± 7.4</td>
<td>89 ± 6</td>
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</tbody>
</table>

*P < .05 compared with the control.*
62%, 53% and 47% of the control level after preincubation for 15 min, 1 h, 4 h and 24 h, respectively. EC_{50} values were significantly increased to 2.9- and 6.6-fold of that of the untreated control (3.8 ± 0.5 nM) for 4 h and 24 h pretreatment, respectively. 

Effect of pretreatment with 1 μM (−)U50,488H for different times on the opioid receptor number was determined (fig. 1B and table 1). Previous exposure to 1 μM (−)U50,488H for 4 h or 24 h significantly reduced B_{max} of [3H]diprenorphine binding (by 19–26%) without changing its K_{d} value. Pretreatment for 15 min or 1 h did not affect K_{d} or B_{max} of [3H]diprenorphine binding.

Thus, desensitization, but not down-regulation, occurred after exposure to 1 μM (−)U50,488H for 15 min or 1 h. After 4 h or 24 h incubation, the kappa opioid receptor was desensitized and down-regulated.

**Effect of (−)U50,488H pretreatment concentration on (−)U50,488H-induced increase in [35S]GTPyS binding and the number of kappa opioid receptors.** Pretreatment for 1 h with 10 nM, 100 nM or 1 μM (−)U50,488H, but not 1 nM, reduced [35S]GTPyS binding elicited by (−)U50,488H (fig. 2A and table 2). Pretreatment with 10 nM and 100 nM (−)U50,488H reduced maximal [35S]GTPyS binding to 82% and 64% of the control level, respectively, without affecting the EC_{50} value. A 1 μM pretreatment increased the EC_{50} value to 2.4-fold of the control and reduced the maximal response to 62% of the control level. One hour exposure to 100 nM or 1 μM (−)U50,488H did not affect K_{d} or B_{max} value of [3H]diprenorphine binding (fig. 2B and table 2). Thus, 1 h pretreatment with ≥1 μM (−)U50,488H led to desensitization, but not down-regulation, of the kappa opioid receptor.

Previous exposure for 4 h with ≥10 nM (−)U50,488H shifted downward the dose-response curve of (−)U50,488H-induced [35S]GTPyS binding (fig. 3A and table 3). Maximal [35S]GTPyS binding was reduced to 67%, 57% and 53% of the control level after 10 nM, 100 nM and 1 μM (−)U50,488H pretreatment, respectively. In addition, EC_{50} values were increased to 2.6- and 2.9-fold of the control value in the 100 nM and 1 μM pretreatment groups, respectively. Treatment with 10 nM, 100 nM and 1 μM (−)U50,488H reduced B_{max} values of [3H]diprenorphine binding by 25 to 30%, whereas K_{d} values were unchanged (fig. 3 and table 3). The extent of down-regulation was similar among 10 nM, 100 nM and 1 μM (−)U50,488H treatment groups. In contrast, 1 nM pretreatment did not affect receptor responsiveness or number (fig. 3 and table 3). Thus, 4 h preincubation with 10 nM, 100 nM or 1 μM (−)U50,488H led to both desensitization and down-regulation of the kappa opioid receptor.

**Role of the kappa opioid receptor in (−)U50,488H-induced desensitization and down-regulation.** Pretreatment with 1 μM (−)U50,488H, an inactive isomer of (−)U50,488H, for 4 h did not have any effect on...
Table 3

Effect of 4-h pretreatment with different (−)U50,488H concentrations on (−)U50,488H-stimulated [35S]GTPγS binding and [3H]diprenorphine binding to the κ receptor

CHO-hkor cells were treated for 4 h with various concentrations of (−)U50,488H and washed; the (−)U50,488H-induced increases in [35S]GTPγS binding and [3H]diprenorphine binding to the receptor were carried out as described under "Materials and Methods." Each datum represents mean ± S.E.M. of three to six experiments. Data were analyzed by analysis of variance followed by Sheffe F test.

<table>
<thead>
<tr>
<th>4-h pretreatment concentration</th>
<th>[35S]GTPγS binding</th>
<th>[3H]Diprenorphine binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50 (nM)</td>
<td>Maximal binding (fmol/mg protein)</td>
</tr>
<tr>
<td>Control</td>
<td>3.8 ± 0.5</td>
<td>191 ± 3</td>
</tr>
<tr>
<td>1 nM</td>
<td>5.8 ± 2.2</td>
<td>184 ± 12</td>
</tr>
<tr>
<td>10 nM</td>
<td>8.0 ± 1.4</td>
<td>127 ± 5</td>
</tr>
<tr>
<td>100 nM</td>
<td>9.3 ± 1.6</td>
<td>110 ± 4</td>
</tr>
<tr>
<td>1 μM</td>
<td>10.4 ± 1.4</td>
<td>101 ± 5</td>
</tr>
</tbody>
</table>

*P < .05 compared with the control.

Fig. 3. Effect of pretreatment concentration with (−)U50,488H-induced for 4 h on (A) [35S]GTPγS binding induced by (−)U50,488H and (B) [3H]diprenorphine binding. CHO-hkor cells were treated at 37°C for 4 h with different concentrations of (−)U50,488H for 4 h, washed extensively and membranes prepared. [35S]GTPγS binding in response to (−)U50,488H and saturation [3H]diprenorphine binding were performed as described under "Materials and Methods." (A) Each point represents the mean ± S.E.M. of three to six experiments. Basal [35S]GTPγS binding ranged from 77 to 87 fmol/mg protein and did not differ among the control and the treatment groups. (B) Scatchard analysis of [3H]diprenorphine binding after each treatment was performed. Each curve represents one of the three to six experiments performed. EC50 and maximal level values of [35S]GTPγS binding and Kd and Bmax of [3H]diprenorphine binding are presented in table 3.

(−)U50,488H-induced [35S]GTPγS binding or the Kd and Bmax values of [3H]diprenorphine (fig. 4 and table 4). Although naloxone (10 μM) alone did not affect responsiveness or number of the kappa opioid receptor, naloxone (10 μM) blocked 0.1 μM (−)U50,488H (4 h)-induced desensitization of [35S]GTPγS response and reduction in Bmax of [3H]diprenorphine binding (fig. 4 and table 4). These results indicate that both processes are mediated by receptor activation.

Reversibility of (−)U50,488H-induced desensitization and down-regulation. CHO-hkor cells were treated with 0.1 μM (−)U50,488H for 4 h to induce desensitization and down-regulation. Twenty-four hours after removal of (−)U50,488H, both [35S]GTPγS binding induced by (−)U50,488H and [3H]diprenorphine binding returned to the control levels (fig. 5 and table 4).

Effect of (−)U50,488H pretreatment on (−)U50,488H-induced inhibition of forskolin-stimulated adenylate cyclase activity. Exposure of CHO-hkor cells to 0.1 μM (−)U50,488H for 1 h shifted the dose-response curve to the right of (−)U50,488H-elicited inhibition of forskolin-stimulated adenylate cyclase activity, compared with the control treatment (fig. 6A). The EC50 value of (−)U50,488H of the pretreated cells was about 2-fold of that of the control (control, 9.7 ± 1.1 nM; 1 h pretreatment, 21.0 ± 3.4 nM, n = 3, P < .05 by Student's t test). Maximal inhibition did not differ significantly (control, 16.6 ± 2.6%; 1 h pretreatment, 24.1 ± 2.6%, n = 3).

Pretreatment for 24 h with 0.1 μM (−)U50,488H produced a higher degree of desensitization (fig. 6B). EC50 values of (−)U50,488H were determined to be 6.8 ± 1.8 nM for the control cells and 32.8 ± 5.6 nM for the cells treated for 24 h (n = 3 each, P < .05 by Student's t test). However, the maximal inhibition did not differ significantly between the control (14.8 ± 4.4%) and the treated cells (21.0 ± 2.9%) (n = 3). (−)U50,488H pretreatment did not affect forskolin-stimulated adenylate cyclase activity. Thus, with adenylate cyclase activity as the functional endpoint, 0.1 μM (−)U50,488H pretreatment for 1 h or 24 h induced desensitization of the kappa opioid receptor.

Affinity and GTPγS sensitivity of (−)U50,488H binding to desensitized kappa opioid receptors. Affinity of (−)U50,488H binding and effect of 100 μM GTPγS on (−)U50,488H binding affinity to control and desensitized receptors were compared. Cells were treated with or without 1 μM (−)U50,488H for 1 h, which causes desensitization, but not down-regulation. Competitive inhibition by (−)U50,488H of [3H]diprenorphine binding to membranes of control and treated cells was conducted in the presence or absence of 100 μM GTPγS. For membranes of cells pretreated with 1 μM (−)U50,488H for 1 h, the Kd value of (−)U50,488H in inhibiting [3H]diprenorphine binding was 2.5 ± 0.5 nM (n = 3), which was significantly higher than that of the control cells (0.7 ± 0.1 nM, n = 3) (P < .05, analysis of variance followed
by Sheffe F-test). In the presence of GTPγS, $K_i$ values of (-)U50,488H binding were determined to be 2.9 ± 0.6 nM and 3.9 ± 0.7 nM for the pretreated and control receptors, respectively, and there was no significant difference. Thus, (-)U50,488H pretreatment lowered the affinity of (-)U50,488H for the receptor and abolished the effect of GTPγS on the agonist affinity, which indicates that desensitized receptors are uncoupled from G proteins.

![Graph A](image1.png)

**A**

![Graph B](image2.png)

**B**

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### Table 4

<table>
<thead>
<tr>
<th>Condition</th>
<th>$K_i$ (nM)</th>
<th>Maximal Binding (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.1 ± 0.6</td>
<td>183 ± 8</td>
</tr>
<tr>
<td>(-)U50,488H</td>
<td>2.9 ± 1.3</td>
<td>182 ± 9</td>
</tr>
<tr>
<td>Naloxone, 10 μM</td>
<td>3.3 ± 0.5</td>
<td>193 ± 10</td>
</tr>
<tr>
<td>(+)Naloxone, 0.1 μM</td>
<td>3.4 ± 1.0</td>
<td>189 ± 8</td>
</tr>
</tbody>
</table>

### Discussion

In this study, we demonstrated that exposure to (-)U50,488H led to a reduction in responsiveness to the agonist (desensitization) and a decrease in the receptor number (down-regulation) of the kappa opioid receptor. During desensitization, maximal response of (-)U50,488H-induced [35S]GTPγS binding was reduced with or without an increase in the EC50 value. In addition, agonist affinity was decreased and GTPγS effect on agonist affinity was abolished, yet affinity and receptor number of the antagonist [3H]diprenorphine binding were unchanged. With a longer incubation time and/or a higher agonist concentration, both desensitization and down-regulation occurred. During this phase, the EC50 value of (-)U50,488H in enhancing [35S]GTPγS binding was increased and the maximal response was reduced. In addition, the receptor number of [3H]diprenorphine binding was decreased, with no change in affinity. Both desensitization and down-regulation were mediated by receptor activation and were reversible after removal of the agonist.

In the present study, we have clearly shown that, temporally, desensitization precedes down-regulation. Initially the kappa opioid receptor undergoes desensitization without down-regulation, and later down-regulation concomitant with desensitization occurs. After ≤1 h incubation with ≤1 μM (-)U50,488H or 4 h incubation with 1 nM (-)U50,488H, desensitization, but not down-regulation, occurred. There were two different types of desensitization: one with a reduction in maximal response with no change in the EC50 value of (-)U50,488H and the other with a reduction in maximal response and an increase in the EC50 value of (-)U50,488H.

To the best of our knowledge, this study is the first one to use agonist-induced increase in [35S]GTPγS binding as the functional measure of the kappa opioid receptor responsiveness after agonist exposure. [35S]GTPγS binding is a direct measure of enhanced receptor-G protein coupling by receptor agonists. Basal levels of [35S]GTPγS binding in CHO-hkor membranes were about 85 fmol/mg protein and did not differ among the control and various treatment groups. The data shown in figures 1 to 5 and tables 1 to 4 are net stimulated...
increases with basal levels already subtracted. Thus, the maximal responses elicited by U50,488H represented about a 2-fold increase over the basal level, which were sufficient to allow examination of desensitization. Agonist-induced increase in [35S]GTP\(\gamma\)S binding has been used to examine desensitization of \(\mu\) and \(\delta\) opioid receptors (Breivogel et al., 1997). Our results that pretreatment with 0.1 \(\mu\)M \((-\)U50,488H for 1 h led to similar desensitization of both agonist-induced increase in [35S]GTP\(\gamma\)S binding and inhibition of forskolin-stimulated adenylate cyclase indicate that [35S]GTP\(\gamma\)S binding is a valid functional endpoint for examination of receptor desensitization.

The observation that in these CHO-hkor cells, agonist-induced increase in [35S]GTP\(\gamma\)S binding was desensitized readily and the maximal response was lowered on desensitization suggests that there are few, if any, spare \(\kappa\) opioid receptors for this response. A similar conclusion was reached in our previous study (Zhu et al., 1997). In this system, [35S]GTP\(\gamma\)S binding assay allowed classification of high-

**Fig. 5.** Reversibility of 0.1 \(\mu\)M \((-\)U50,488H-induced \(\kappa\) receptor desensitization and down-regulation. CHO-hkor cells were cultured at 37°C for 4 h in medium with or without 0.1 \(\mu\)M \((-\)U50,488H, washed and then grown in medium without \((-\)U50,488H for 24 h. Membranes were prepared and [35S]GTP\(\gamma\)S binding in response to \((-\)U50,488H and saturation \([3\text{H}]\text{diprenorphine binding were performed as described under "Materials and Methods."} (A) Each point represents the mean ± S.E.M. of three to six experiments. Basal [35S]GTP\(\gamma\)S binding ranged from 78 to 88 fmol/mg protein and did not differ among the control and the treatment groups. (B) Scatchard analysis of [3H]diprenorphine binding after each treatment was performed. Each curve represents one of the three to six experiments performed. EC\(_{50}\) and maximal level values of [35S]GTP\(\gamma\)S binding and \(K_d\) and \(B_{\text{max}}\) of [3H]diprenorphine binding are presented in table 4.

**Fig. 6.** Effect of \((-\)U50,488H preincubation on inhibition of forskolin-stimulated adenylate cyclase by \((-\)U50,488H. CHO-hkor cells were incubated without or with 100 nM \((-\)U50,488H for 1 h (A) or 24 h (B) and washed extensively with PBS buffer. \((-\)U50,488H-induced inhibition of forskolin-stimulated adenylate cyclase was determined as described under "Materials and Methods." In both control and \((-\)U50,488H-treated cells, after forskolin stimulation, [3H]cAMP represented approximately 2% of total [3H]adenine nucleotides. Data are expressed as percent of forskolin-stimulated adenylate cyclase activity. Each point represents the mean ± S.E.M. of three determinations in triplicate.
affinity kappa opioid ligands into full agonists, partial agonists and antagonists, depending on the maximal response produced.

Although 0.1 μM (−)U50,488H pretreatment for 1 h reduced maximal [35S]GTPγS binding by (−)U50,488H, it did not affect maximal inhibition of forskolin-stimulated adenylate cyclase activities by (−)U50,488H. These results suggest that, although there are few or no spare kappa opioid receptors for G proteins, there are spare inhibitory G proteins for adenylate cyclase.

The affinity and number of receptors determined by [3H]diprenorphine binding was not changed by several different (−)U50,488H pretreatment paradigms (1 μM for 15 min, 1 nM for 1 h, 10 nM for 1 h, 100 nM for 1 h, 1 μM for 1 h) followed by extensive washing. These results indicate complete removal of (−)U50,488H with our washing procedure.

Our finding that (−)U50,488H treatment led to desensitization of the kappa opioid receptor is contrary to the reports of Joseph and Bidlack (1995) and Avidor-Reiss et al. (1995). We demonstrated that desensitization of (−)U50,488H-induced enhancement of [35S]GTPγS binding occurred as early as 15 min after 1 μM (−)U50,488H incubation. In addition, pretreatment with 0.1 μM (−)U50,488H for 1 h or 24 h led to a desensitization in (−)U50,488H-induced inhibition of forskolin-stimulated adenylate cyclase. Joseph and Bidlack (1995) observed no desensitization of U50,488H-elicited inhibition of forskolin-stimulated adenylate cyclase in R1.1 cells after 24 h or 48 h treatment with 0.1 μM U50,488H. In addition, culturing of R1.1 cells with 10 nM bremazocine for 24 h did not affect inhibition of adenylate cyclase by U50,488H (Joseph and Bidlack, 1995). This apparent discrepancy may be the result of the different systems used. Murine thymoma R1.1 cells were used by Joseph and Bidlack (1995), whereas we used CHO cells stably transfected with the human kappa opioid receptor. Different in vitro systems may vary in their responses to exposure to the kappa opioid agonist, because of the different levels of key proteins involved in desensitization. Joseph and Bidlack (1995) speculated that the lack of kappa opioid receptor desensitization in R1.1 cells might be caused by low levels of components involved in desensitization, such as beta adrenergic receptor kinase. Our finding is also different from that of Avidor-Reiss et al. (1995), who found that incubation of CHO cells stably transfected with the rat kappa opioid receptor with U69,593 up to 10 μM for 4 h inhibited forskolin-stimulated adenylate cyclase to the same extent as a 10-min incubation. The reason for this difference is not apparent. Their treatment paradigms were different from ours. Avidor-Reiss et al. (1995) did a 4-h incubation with U69,593 and measured inhibition of forskolin-stimulated adenylate cyclase activity, whereas we preincubated with (−)U50,488H followed by removal of (−)U50,488H with washes and determined inhibition of forskolin-stimulated adenylate cyclase activity by the subsequent application of U50,488H. In addition, this discrepancy might reflect species difference in the kappa opioid receptor properties, i.e., rat versus human. Comparison of the C-terminal domain sequences of the rat and human kappa opioid receptors shows Ser358 in the human receptor instead of Asn358 in the rat. Serine residues can be phosphorylated, whereas Asn cannot. Whether this amino acid difference contributes to differential desensitization will be investigated.

Our results on kappa opioid receptor desensitization, on the other hand, are consistent with those of Raynor et al. (1994) and Blake et al. (1997). They demonstrated that chronic exposure to U50,488H induced kappa opioid receptor desensitization in Cos-7 cells transiently expressing the mouse kappa opioid receptor and HEK-293 cells stably expressing the human kappa opioid receptor, respectively. Inhibition of forskolin-stimulated adenylate cyclase activity was used as the functional endpoint. However, there are some differences. Raynor et al. (1994) observed that pretreatment with 1 μM U50,488H for 4 h did not reduce the number of sites of [3H]naloxone binding, whereas we did not find receptor down-regulation after the same treatment.

Prolonged exposure to (−)U50,488H reduced the B_max value of [3H]diprenorphine binding with no change in the affinity. The extent of decrease in receptor number after a 4-h exposure to 10 nM (−)U50,488H was ~30%. Incubation for 4 h with a higher concentration of (−)U50,488H up to 1 μM or for 24 h with 1 μM did not lead to further reduction in the receptor number. A similar degree of down-regulation was reported by Blake et al. (1997) after a 3-h pretreatment with 1 μM U50,488H. However, this down-regulation was less than the 50% reduction observed by Joseph and Bidlack (1995) after incubation with 0.1 μM U50,488H for 24 h in R1.1 thymoma cells. This difference may be a reflection of difference in the levels of molecules required for the down-regulation process between the two systems.

GTP or its analog uncouples G proteins from the receptor and thus lowers the affinity of the receptor for agonists. The observations that desensitized kappa opioid receptors exhibited lower affinity for U50,488H and that the ability of GTPγS to lower kappa opioid agonist affinity was abolished indicate that uncoupling of the kappa opioid receptor from G proteins occurs after incubation with U50,488H. These findings agree with those of Raynor et al. (1994). Similar observations have been reported for mu and delta opioid receptors (Puttfarcken et al., 1988; Werling et al., 1989; Law et al., 1983).

Both sodium fluoride and sodium pyrophosphate are phosphatase inhibitors. They have been used extensively in receptor phosphorylation studies to prevent de-phosphorylation (for example, Liggett et al., 1992; Pei et al., 1995; Zhang et al., 1996; Arden et al., 1995). The G protein-coupled receptor phosphatase was reported recently, and this activity was a latent form of protein phosphatase type 2A (Pitcher et al., 1995). The finding that the presence of sodium fluoride and sodium pyrophosphate during membrane preparation was necessary for (−)U50,488H-induced desensitization of [35S]GTPγS binding to be observed suggests that (−)U50,488H-induced kappa opioid receptor desensitization is associated with phosphorylation of certain proteins involved in signal transduction, possibly of the kappa opioid receptor. Whether the kappa opioid receptor is phosphorylated after prolonged exposure to an agonist and thus lowers the affinity of the receptor for agonists is currently under investigation. Mu and delta opioid receptors have undergone phosphorylation under conditions that cause desensitization (Pei et al., 1995; Zhang et al., 1996; Arden et al., 1995).

Pretreatment with 0.1 μM (−)U50,488H for 1 h led to desensitization of (−)U50,488H-induced inhibition of forskolin-stimulated adenylate cyclase without the presence of phosphatase inhibitors. The difference may be caused by a lengthy process of membrane preparation that had to be performed before [35S]GTPγS binding assay, during which
many enzymes may be liberated. In contrast, adenylate cyclase assay was carried out in whole-cell preparations immediately after pretreatment with \((-\text{U50,488H})\) and three washes of the cells.

In conclusion, we have demonstrated that the human kappa opioid receptor undergoes desensitization and down-regulation after prolonged exposure to the opioid agonist \((-\text{U50,488H})\). Temporally, desensitization occurs first, and if agonist exposure persists, down-regulation along with desensitization ensues. Both processes are mediated by receptor activation and are reversible after removal of the agonist. Because kappa opioid agonist-induced enhancement of [\(^{35}\text{S}\)]GTPγS binding involved only receptor and G proteins, biochemical changes during desensitization must occur at the level of receptor and/or G proteins. With the availability of cloned kappa opioid receptors, we can start to delineate biochemical mechanisms of desensitization and down-regulation.

References


