ABSTRACT

Activation of kappa receptors inhibits adenylate cyclase, enhances K⁺ conductance and reduces Ca²⁺ conductance via pertussis toxin-sensitive G proteins. We recently cloned a human kappa opioid receptor and stably expressed it in Chinese hamster ovary (CHO) cells. In this study, the effects of activation of the human kappa receptor by agonists on [³⁵S]GTPγS binding to CHO cell membranes were examined. The presence of GDP and Mg²⁺ was essential for the kappa agonist (-)-U50,488H-induced increase in [³⁵S]GTPγS binding to be observed and the optimal concentration was 3 μM and 5 mM, respectively. The presence of 100 mM Na⁺ was necessary to produce the maximal signal-to-background ratio. (-)U50,488H-induced increase in [³⁵S]GTPγS binding was time- and tissue-concentration-dependent. (-)U50,488H increased [³⁵S]GTPγS binding in a dose-dependent manner with an EC₅₀ of 3.1 nM. (+)-U50,488H had no effect, which indicates that this effect is stereospecific. Naloxone (1 μM) or norbinaltorphimine (10 nM) shifted the dose-response curve of (-)-U50,488H to the right by 100-fold. These results indicate that enhancement of [³⁵S]GTPγS binding by (-)-U50,488H is a kappa receptor-mediated event. Pretreatment of the cells with pertussis toxin, but not cholera toxin, abolished the (-)-U50,488H-induced increase in [³⁵S]GTPγS binding, which indicates the involvement of Gₛ and/or Gᵢ₃ proteins. [³⁵S]GTPγS binding induced by (-)-U50,488H had a Kᵢ₃ value of 0.34 ± 0.08 nM and a Bₘ₃₃₃ value of 431 ± 29 fmol/mg protein. The rank order of potencies of opioid ligands tested in stimulating [³⁵S]GTPγS binding was dynorphin A 1-17 > (±)-ethylketocyclazocine > β-funaltrexamine, (−)-U50,488H, tifluadom > nalorphine > pentazocine, nalbuphine > buprenorphine. Dynorphin A 1–17, (±)-ethylketocyclazocine, (−)-U50,488H, tifluadom and β-funaltrexamine were full agonists, but nalorphine and pentazocine were partial agonists producing maximal responses of 68% and 23% of those of full agonists, respectively. Nalbuphine and buprenorphine had low levels of agonist activities. Norbinaltorphimine and naloxone were antagonists devoid of activities. Enhancement of [³⁵S]GTPγS binding by kappa agonists provides a simple functional measure for receptor activation and can be used for determination of potencies and efficacies of opioid ligands at the kappa receptor.

Opioid receptors play important roles in many physiological functions. The presence of multiple types of opioid receptors, at least mu, delta, kappa and epsilon, in the nervous system has been established by pharmacological and binding studies as well as differential anatomical localization in the brain (for a review, Chang, 1984). Activation of kappa opioid receptors produces many effects including analgesia (von Voigtlander et al., 1983), dysphoria (Pfieffer et al., 1986) and water diuresis (Leander, 1983a; Dykstra et al., 1987). Dynorphins are thought to be endogenous ligands for kappa receptors (Chavkin and Goldstein, 1981). (-)-U50,488H is the prototype of selective kappa agonists (von Voigtlander et al., 1983), whereas norbinaltorphimine is a selective kappa antagonist (Portoghese et al., 1987).

There is ample evidence supporting the notion that the kappa opioid receptor belongs to the superfamily of GPCRs. Activation of kappa opioid receptors leads to inhibition of adenylate cyclase (Attali et al., 1989; Konkoy and Childers, 1989, 1993; Lawrence and Bidlack, 1993; Lawrence et al., 1995; Prather et al., 1995), activation of low-Kᵢ₃ GTPase (Clark et al., 1986; Clark and Medzihradszky, 1987; Lawrence et al., 1995), enhancement of incorporation of [³²P]azidoanilido-GTP into Gₛ subunits (Prather et al., 1995), increase in inward-rectifying K⁺ conductance (Ma et al., 1995; Henry et

ABBREVIATIONS: CHO cells, Chinese hamster ovary cells; CHO-hkors, Chinese hamster ovary cells stably expressing human k opioid receptor; DAMGO, Try-o-Ala-Gly-(Me)Phe-Gly-ol; DPDPE, Tyr-o-Pen-Gly-Phe-o-Pen-OH; EDTA, ethylenediaminetetraacetic acid; G protein, guanosine triphosphate-binding regulatory protein; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid; GDP, guanosine diphosphate; GPCRs, G protein-regulated receptors; GTP-γS, guanosine 5’-O-(3-thio)triphosphate; (-)-U50,488H, (trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide; hkor, human κ opioid receptor; HEPES, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid; TEL buffer, 50 mM Tris-HCl buffer, 1 mM EGTA and 10 μM leupeptin, pH 7.5.
Kappa Agonists Enhance \[^{35}S\]GTP\(\gamma\)S Binding

Methods and Materials

Materials. \[^{3}H\]Diprenorphine (35 Ci/mmole) and \[^{35}S\]GTP\(\gamma\)S (1000–1300 Ci/mmole) were obtained from Amersham Corp. (Arlington Heights, IL) and NEN Life Sciences Co. (Boston, MA), respectively. Naloxone was a gift from DuPont/Merck (Wilmington, DE), and (-)U50,488H was provided by Upjohn (Kalamazoo, MI). DNP-phenol, DPDPE and DAMGO were purchased from Peninsula Laboratories (Belmont, CA). GDP and GTP\(\gamma\)S were purchased from Sigma Chemical Co. (St. Louis, MO). (+)-Ethylketocyclazocine, tifluadom, nalorphine, naltbuphine, pentazocine, \(\beta\)-famitrexamine, nornalbuphine and buprenorphine were provided by the National Institute on Drug Abuse.

Stable expression of hkor in CHO cells. CHO cells were transfected with the hkor cDNA in the vector pBK-CMV (Zhu et al., 1995) and clonal cell lines stably expressing hkor (CHO-hkor) were established with Geneticin selection as described (Sambrook et al., 1989).

\[^{35}S\]GTP\(\gamma\)S binding. Determination of \[^{35}S\]GTP\(\gamma\)S binding to G proteins was carried out with a modified procedure of Traynor and Nahorski (1995).

After removal of the growth medium, CHO-hkor cells were washed twice with 100 mM phosphate-buffered saline, harvested in Versene solution (0.54 mM EDTA, 0.14 mM NaCl, 2.7 mM KCl, 8.1 mM Na\(_2\)HPO\(_4\), 1.46 mM KH\(_2\)PO\(_4\), 1 mM glucose), centrifuged at 500 \(\times\) g for 3 min and washed once with phosphate-buffered saline. The cell pellet was suspended in a buffer of 50 mM TEL/0.1 mM phenylmethylsulfonyl fluoride, sonicated and centrifuged at 1000 \(\times\) g for 10 min. The pellet was sonicated and centrifuged again. Combined supernatant was centrifuged at 46,000 \(\times\) g for 30 min. The pellet was suspended 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 -600 \(\mu\)g protein/ml, frozen in dry ice/ethanol and stored at -70°C until use. All procedures were performed at 4°C.

Immediately before \[^{35}S\]GTP\(\gamma\)S binding assay, membranes were thawed at 37°C, chilled on ice, passed through a 22-gauge needle and diluted with 50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl\(_2\), and 1 mM EDTA with 1 mM dithiothreitol and 0.1% bovine serum albumin freshly added (buffer A). Membranes (-7.5 \(\mu\)g protein) were incubated in buffer A containing \[^{35}S\]GTP\(\gamma\)S (100,000–150,000 dpm, -80 \(\mu\)M) and GDP with or without an opioid ligand (10 \(^{-10}\) to \(^{-5}\) M) in a total volume of 0.5 ml for 60 min at 30°C. Non-specific binding was defined by incubation in the presence of 10 \(\mu\)M GTP\(\gamma\)S. Non-specific binding was found to be similar in the presence and absence of (-)U50,488H and was subtracted from total stimulated and total basal binding. Bound and free \[^{35}S\]GTP\(\gamma\)S were separated by filtration with GF/B filters under reduced pressure. Radioactivity on filters was determined by liquid scintillation counting.

The routine assay conditions, which included 100 mM NaCl, 5 mM MgCl\(_2\), and 3 \(\mu\)M GDP in the binding buffer, and performed with 80 to 120 pM \[^{35}S\]GTP\(\gamma\)S and 7.5 to 10 \(\mu\)g membrane protein for 60 min at 30°C, yielded 3,500 to 5,500 dpm and 1,000 to 1,500 dpm for maximal stimulated and basal \[^{35}S\]GTP\(\gamma\)S binding, respectively. EC\(_{50}\) values of drugs were determined by curve fitting to the equation for a sigmoidal curve \(E = E_{max} \cdot [D]/(D^\alpha + EC_{50}^\alpha)\), where \(E\) is effect produced by a certain concentration of the drug, \(D\), \(E_{max}\) is the maximal response elicited by the drug and \(n\) is a fitting parameter.

Opioid receptor binding. Membranes were prepared from CHO-hkor cells as described previously (Zhu et al., 1996). Opioid receptor binding was conducted with \[^{3}H\]Diprenorphine according to our published procedure (Zhu et al., 1995). Binding was carried out in 50 mM Tris-HCl buffer containing 1 mM EDTA and 10 \(\mu\)M leupeptin (pH 7.4 at room temperature) (TEL buffer) or in \[^{35}S\]GTP\(\gamma\)S binding buffer at 25°C for 1 h in duplicate in a volume of 1 ml with 30 to 60 \(\mu\)g protein. Binding data were analyzed with the EBDA program (McPherson, 1983). \(K_i\) values were determined by use of the equation of Cheng and Prusoff (1973). Comparison of \(K_i\) values of each drug in the two buffers were performed with Student’s \(t\) test, with \(P < .05\) being considered significantly different.

Determination of protein content. Protein contents of membranes were determined by the BCA method of Smith et al. (1985) with bovine serum albumin as the standard.

Results

Stable expression of the human kappa opioid receptor cDNA in CHO cells. The human kappa opioid receptor cDNA was expressed stably in CHO cells. Saturation binding of \[^{3}H\]Diprenorphine to membranes of CHO-hkor cells was carried out in 50 mM TEL buffer. \(K_d\) and \(B_{max}\) were determined to be 0.12 ± 0.02 nM and 1292 ± 52 fmol/mg protein (mean ± S.E.M., \(n = 3\), respectively. \[^{3}H\]Diprenorphine
binding to the membranes was potently inhibited by kappa selective ligands, but not by mu or delta ligands (see table 1).

Effects of GDP on $[^{35}S]$GTPγS binding. Experiments were performed with 5 mM Mg$^{++}$, 100 mM Na$^+$ and various concentrations of GDP in the presence and absence of 3 μM (−)-U50,488H (fig. 1). Without GDP, no increase in $[^{35}S]$GTPγS binding by the kappa receptor agonist (−)-U50,488H was observed. GDP decreased $[^{35}S]$GTPγS binding in a dose-dependent manner both in the presence and absence of (−)-U50,488H (fig. 1). With ≥0.3 μM GDP, the magnitude of reduction in $[^{35}S]$GTPγS binding was larger in the absence than the presence of (−)-U50,488H and, thus, the increase in $[^{35}S]$GTPγS binding caused by the agonist could be observed. The presence of 3 μM GDP produced the maximal stimulatory response. At concentrations of GDP ≥ 100 μM, effects of (−)-U50,488H were greatly diminished or obliterated. In this study, 3 μM GDP was used in all other experiments.

Effects of Mg$^{++}$ concentration on $[^{35}S]$GTPγS binding. The importance of Mg$^{++}$ in $[^{35}S]$GTPγS binding was determined in the absence and presence of 3 μM (−)-U50,488H with 3 μM GDP and 100 mM Na$^+$ (fig. 2). At concentrations below 5 μM and 0.5 mM, there was no significant difference in $[^{35}S]$GTPγS binding between with and without (−)-U50,488H. At 5 or 15 mM, the basal level of $[^{35}S]$GTPγS binding was doubled. From 1.5 to 15 mM, binding in the presence of (−)-U50,488H was greatly increased and a plateau was reached at 5 and 15 mM. At the plateau, agonist-induced binding was 2.5- to 3.5-fold of basal binding. At 50 mM, both basal level and agonist-induced binding decreased. Based on these results, 5 mM Mg$^{++}$ in the form of MgCl$_2$ was used in all other experiments.

Effects of Na$^+$ on $[^{35}S]$GTPγS binding. Effects of Na$^+$ on $[^{35}S]$GTPγS binding were investigated in the presence of 3 μM GDP and 5 mM Mg$^{++}$ with or without 3 μM (−)-U50,488H (fig. 3). Without Na$^+$, (−)-U50,488H-stimulated increase in $[^{35}S]$GTPγS binding was evident, although the magnitude of the increase was relatively small (not shown, but similar to 0.1 mM NaCl of fig. 3). At ≤1 mM, Na$^+$ did not affect basal or agonist-induced $[^{35}S]$GTPγS binding. Basal $[^{35}S]$GTPγS binding was decreased by [Na+] ≥ 10 mM, whereas binding in the presence of (−)-U50,488H was reduced at [Na$^+$] ≥ 100 mM, both in a concentration-dependent manner. Binding in the presence of 100 mM Na$^+$ provides the best signal-to-background difference. At this concentration, the basal binding amounted to approximately 28% of the maximal binding achieved by (−)-U50,488H. In all other experiments, binding was performed in the presence of 100 mM NaCl.
Time course of basal and (−)-U50,488H-stimulated [35S]GTPγS binding. Time courses of [35S]GTPγS binding were conducted in the presence and absence of 3 μM (−)-U50,488H at 30°C. Basal [35S]GTPγS binding increased with time, whereas (−)-U50,488H-stimulated [35S]GTPγS binding (difference between with and without (−)-U50,488H) reached a plateau at ~90 min.

Relationship between [35S]GTPγS binding and the amount of CHO-hkor membrane. [35S]GTPγS binding increased linearly with or without 3 μM (−)-U50,488H up to 40 μg of membrane proteins in each assay tube. Routinely, 7.5 to 10 μg of membrane proteins were used, which gave 3,500 to 5,500 dpm and 1,000 to 1,500 dpm for stimulated and basal [35S]GTPγS binding, respectively.

Effect of (−)-U50,488H on [35S]GTPγS binding to CHO-hkor membranes. [35S]GTPγS binding to CHO-hkor membranes with various concentrations of (−)-U50,488H was examined in the presence of 3 μM GDP, 5 mM Mg2+ and 100 mM Na+ (fig. 4). Binding of [35S]GTPγS was increased by (−)-U50,488H in a concentration-dependent manner with an EC50 of 3.1 nM. The maximal response, which represented ~3.5-fold of the basal level, was reached at 0.3 μM (−)-U50,488H (fig. 4). Ten micromolar (−)-U50,488H failed to stimulate [35S]GTPγS binding to membranes of untransfected CHO cells (not shown). (−)-U50,488H up to 1 μM did not increase [35S]GTPγS binding, compared with the basal level (not shown), which indicated stereospecificity of receptor activation. Naloxone (1 μM) or norbinaltorphimine (10 nM) shifted the concentration-effect curve of (−)-U50,488H to the right by about 100-fold (fig. 4). Kd values of naloxone and norbinaltorphimine calculated from these data were 10 nM and 0.1 nM, respectively, indicating that their potencies in the CHO-hkor system are similar to those in other kappa opioid receptor assays (Portoghese et al., 1987). The mu agonist DAMGO and the delta agonist DPDPE had no effect (fig. 4). Taken together, these results indicate that (−)-U50,488H-induced increase in [35S]GTPγS binding is mediated by specific activation of the kappa opioid receptor.

Effects of pretreatment with pertussis toxin or cholera toxin on [35S]GTPγS binding. To assess the G proteins that were involved in the action of (−)-U50,488H, CHO-hkor cells were treated with pertussis toxin (100 ng/ml) or cholera toxin (20 μg/ml) for 24 h before membrane preparation. Pertussis toxin pretreatment completely abolished (−)-U50,488H-induced increase in [35S]GTPγS binding and reduced basal binding of [35S]GTPγS by 60% (fig. 5A). In contrast, cholera toxin pretreatment did not affect basal or (−)-U50,488H-induced [35S]GTPγS binding (fig. 5B). Thus, (−)-U50,488H-induced [35S]GTPγS binding was most likely caused by binding to pertussis toxin-sensitive G proteins, i.e., Gi and/or Go.

Determination of Kd and Bmax of (−)-U50,488H-induced [35S]GTPγS binding. Displacement of [35S]GTPγS binding with unlabeled GTPγS was performed in the absence of Gi and/or Go. Basal [35S]GTPγS binding was most likely caused by binding to pertussis toxin-sensitive G proteins, i.e., Gi and/or Go.

Kappa Agonists Enhance [35S]GTPγS Binding
and presence of 10 \( \mu M \) (-)-U50,488H for 180 min (fig. 6) to determine \( K_d \) and \( B_{max} \) of \( [35S] \)GTP\( \gamma \)S binding that could be maximally stimulated by (-)-U50,488H. Scatchard analysis of the difference between the two curves revealed a \( K_d \) of 0.34 ± 0.08 nM and a \( B_{max} \) of 431 ± 29 fmol/mg protein for \( [35S] \)GTP\( \gamma \)S binding (mean ± S.E.M., \( n = 3 \)).

Determination of potencies and efficacies of opioid ligands on \( [35S] \)GTP\( \gamma \)S binding. Several opioid receptor ligands were examined for their potencies and efficacies in stimulating \( [35S] \)GTP\( \gamma \)S binding to membranes. \( EC_{50} \) values and maximal responses were determined and compared with those of (-)-U50,488H (fig. 7, table 1). Dynorphin A 1–17, (±)-ethylketocyclazocine, tifluadom and \( \beta \)-funaltrexamine produced maximal responses similar to (-)-U50,488H and were full agonists. Dynorphin A 1–17 and (±)-ethylketocyclazocine had higher potencies than (-)-U50,488H with \( EC_{50} \) values of 0.18 and 0.57 nM, respectively, whereas \( \beta \)-funaltrexamine and tifluadom were potent similar to (-)-U50,488H with \( EC_{50} \) values of 1.7 nM and 3.9 nM, respectively. Nalorphine and pentazocine acted as partial agonists with the maximal stimulation of \( [35S] \)GTP\( \gamma \)S binding at 68% and 23% of that of (-)-U50,488H, respectively. \( EC_{50} \) values of nalorphine and pentazocine, determined as the dose that elicited 50% of the maximal response of each drug, were 17.9 nM and 103 nM, respectively. Nalbuphine and buprenorphine had some stimulatory effects at ≥100 nM, but no plateau in maximal response was detected at up to 30 \( \mu M \). The rank order of potencies of ligands tested for stimulation of \( [35S] \)GTP\( \gamma \)S binding was dynorphin A 1–17 > (±)-ethylketocyclazocine > \( \beta \)-funaltrexamine, tifluadom, (-)-U50,488H > nalorphine > pentazocine, nalbuphine > buprenorphine. Although norbinaltorphimine and naloxone had high affinity for the kappa receptor, they did not increase \( [35S] \)GTP\( \gamma \)S binding. In addition, naloxone and norbinaltorphimine shifted the dose-response curve of (-)-U50,488H to the right (fig. 4), indicating that they are antagonists.

Binding affinities of opioid ligands to the human kappa opioid receptor. Competitive inhibition of \( [3H] \)diprenorphine binding by opioid ligands to CHO-hkor membranes was conducted to determine binding affinities of opioid ligands tested in \( [35S] \)GTP\( \gamma \)S binding. Binding was performed in \( [35S] \)GTP\( \gamma \)S binding buffer as well as in TEL buffer, because Tris-HCl buffer is the most commonly used buffer for binding studies. \( K_d \) values of these ligands were listed in table 1. There was no significant difference in \( K_d \) values of each drug in these two buffers. These values determined in membranes of CHO-hkor cells were similar to those determined in membranes of COS-1 cells transiently expressing the kappa receptor (Zhu et al., 1995). Ligands selective for kappa receptor ((−)-U50,488H, norbinaltorphimine, dynorphin A 1–17) had much higher affinity than the mu selective ligand DAMGO or the delta selective ligand DP-DPE. For the five full agonists (dynorphin A 1–17, (±)-ethylketocyclazocine, tifluadom, \( \beta \)-funaltrexamine and (-)-U50,488H), the \( K_d \) values were very similar to the \( EC_{50} \) values for stimulation of \( [35S] \)GTP\( \gamma \)S binding (table 1).

Discussion

Opioid receptors mediate functional effects of agonists via activation of G proteins. In the present study, we demonstrated the ability of kappa agonists to enhance binding of \( [35S] \)GTP\( \gamma \)S to membranes of CHO-hkor cells. The presence of GDP and Mg\(^{2+} \) was essential for agonist-stimulated \( [35S] \)GTP\( \gamma \)S binding. Na\(^+ \) was necessary for maximal signal-to-background ratios. The extent of (-)-U50,488H-induced increase in \( [35S] \)GTP\( \gamma \)S binding was dependent on agonist concentration, incubation time and tissue concentration. The effect of (-)-U50,488H was stereospecific and reversed by naloxone or norbinaltorphimine, which indicates that this effect is mediated by the kappa receptor. Pertussis toxin-, but not choleratoxin-sensitive G proteins were involved. In this system, \( [35S] \)GTP\( \gamma \)S binding assay allowed classification of high-affinity kappa opioid ligands into full agonists, partial agonists and antagonists. Although full agonists elicit maximal effects, partial agonists and antagonists produce sub-maximal and no effects, respectively. This biochemical assay thus permits determination of efficacies of kappa opioid ligands. In addition, the CHO-hkor cell, being devoid of other receptors, is an excellent system for this purpose. \( EC_{50} \) values of five full agonists tested in stimulating \( [35S] \)GTP\( \gamma \)S binding were in the nanomolar range, very similar to their \( K_d \) values in inhibiting \( [3H] \)diprenorphine binding, which strongly suggests that there is no or little spare receptors in this system.

In addition to its relatively large stimulated signals and its allowing determination of ligand efficacy, \( [35S] \)GTP\( \gamma \)S binding assay has some practical advantages. Membranes can be prepared and frozen for convenience. The assay itself is easy, quick and reproducible. Recently, Befort et al. (1996) reported use of \( [35S] \)GTP\( \gamma \)S binding to evaluate activity of delta opioid receptors transiently expressed COS cells. The utility of this assay for transiently expressed receptors will facilitate examination of effects of mutations on receptor functional activity.

Stimulation of \( [35S] \)GTP\( \gamma \)S binding by agonists allows one to examine G protein activation by ligand-occupied receptors regardless of the types of G proteins and effector systems.
These EC\textsubscript{50} values are more than 100-fold greater than their respective receptors, respectively (Clark and Medzihradsky, 1987). 

phin peptides stimulated low-$K_m$-U50,488H was observed at $(\pm)$-U50,488H and dynorphin A 1–17 were in the nanomolar range, similar to their receptor (Prather et al., 1995). Activation of a cloned rat kappa opioid receptor stably expressed in CHO cells increased the incorporation of $[^{32}P]azidoanilido-GTP into four G\textsubscript{\alpha} subunits with maximal increases of 20 to 44%, and EC\textsubscript{50} values of $^{(32)}P$azidoanilido-GTP at 30°C for 60 min as described under "Methods and Materials." Each value represents mean ± S.E.M. of three independent experiments in duplicate.

TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>$EC_{50}$ (nM)</th>
<th>Maximal effect</th>
<th>$K_i$ (nM)</th>
<th>$EC_{50}$ S binding buffer (nM)</th>
<th>$EC_{50}$ S binding buffer (nM)</th>
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<tbody>
<tr>
<td>(-)-U50,488H</td>
<td>3.1 ± 0.1</td>
<td>1.00</td>
<td>4.1 ± 1.2</td>
<td>3.4 ± 1.0</td>
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<tr>
<td>Dynorphin 1-17</td>
<td>0.18 ± 0.04</td>
<td>1.00 ± 0.10</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
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<td>(-)-Ethylketocyclazocine</td>
<td>0.57 ± 0.01</td>
<td>0.97 ± 0.07</td>
<td>0.34 ± 0.10</td>
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<td>$\beta$-FNA</td>
<td>1.7 ± 0.1</td>
<td>1.03 ± 0.02</td>
<td>3.3 ± 0.3</td>
<td>3.7 ± 0.7</td>
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<tr>
<td>Tifluadom</td>
<td>3.9 ± 0.5</td>
<td>0.93 ± 0.01</td>
<td>1.6 ± 0.3</td>
<td>1.5 ± 0.1</td>
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<tr>
<td>Nalorphine</td>
<td>17.9 ± 2.0$^a$</td>
<td>0.68 ± 0.04</td>
<td>12.2 ± 2.3</td>
<td>9.2 ± 1.3</td>
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<tr>
<td>Nalbuphine</td>
<td>0.38 ± 0.04$^b$</td>
<td>(at 30 $\mu$M)</td>
<td>39.7 ± 8.1</td>
<td>30.9 ± 6.3</td>
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</tr>
<tr>
<td>Pentazocine</td>
<td>103 ± 30$^a$</td>
<td>0.23 ± 0.03</td>
<td>16.8 ± 3.4</td>
<td>13.4 ± 0.9</td>
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</tr>
<tr>
<td>Buprenorphine</td>
<td>0.22 ± 0.02$^b$</td>
<td>(at 30 $\mu$M)</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.03</td>
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<tr>
<td>Naloxone</td>
<td>No effect</td>
<td>No effect</td>
<td>4.5 ± 1.1</td>
<td>4.0 ± 0.4</td>
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<tr>
<td>Norbinaltorphimine</td>
<td>No effect</td>
<td>No effect</td>
<td>0.01 ± 0.01</td>
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<tr>
<td>DAMGO</td>
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<td>No effect</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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<tr>
<td>DPDPE</td>
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<td>No effect</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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</table>

$^a$ Determined as the dose that elicited 50% of the maximal response of the drug.

$^b$ Could not be determined.

$^c$ No plateau was observed.

involved. In this regard, it is similar to kappa agonist-induced enhancement of GTPase activity of G proteins (Clark et al., 1986; Clark and Medzihradsky, 1987; Lawrence et al., 1995) and increase in labeling of G\textsubscript{\alpha} subunits by $[^{32}P]azidoanilido-GTP (Prather et al., 1995). (-)-U50,488H and dynorphin peptides stimulated low-\textsubscript{K_m} GTPase activity by 10 to 20% with $EC_{50}$ values of 3 to 23 $\mu$M in guinea pig cerebellum membranes (Clark et al., 1986) and in rat and monkey brain membranes pretreated with $\beta$-funaltrexamine and cis-(-)-3-methylfentanyl isothiocyanate to alkylate the $\mu$ and $\delta$ receptors, respectively (Clark and Medzihradsky, 1987). These $EC_{50}$ values are more than 100-fold greater than their $K_i$ values of binding to the kappa receptor. In three mouse thymoma cell lines, stimulation of low-\textsubscript{K_m} GTPase activity by (-)-U50,488H was observed at $\geq 10$ nM and had a 21 to 53% increase over the basal level at 30 $\mu$M (-)-U50,488H (Lawrence et al., 1995).
very potent in inhibiting forskolin-stimulated adenylate cyclase with EC$_{50}$ values in the nanomolar range (Prather et al., 1995). In contrast, in many other systems, EC$_{50}$ values of (−)-U50,488H, U69,593 and dynorphin A 1–17 for the inhibition of forskolin-stimulated adenylate cyclase activity were in the micromolar range, 100- to 1000-fold of their $K_{i}$ values of binding to the kappa opioid receptor. These included the rat spinal cord and spinal cord-dorsal root ganglion culture (Attali et al., 1989), guinea pig cerebellar membranes (Konkoy and Childers, 1989), guinea pig brain membranes (Konkoy and Childers, 1993) and mouse thymoma cells (Lawrence et al., 1995; Lawrence and Bidlack, 1993). Maximal inhibition that could be achieved by (−)-U50,488H or dynorphin A 1–17 was 40% in guinea pig brain membranes (Konkoy and Childers, 1993), 37 to 66% in mouse thymoma cell lines (Lawrence et al., 1995; Lawrence and Bidlack, 1993) and 56 to 75% in CHO cells expressing a rat kappa receptor (Prather et al., 1995). Only (−)-U50,488H, U69,593 and dynorphin peptides were examined in the studies on inhibition of adenylate cyclase. Whether partial agonists were active in these systems was not determined.

The presence of GDP is essential for (−)-U50,488H-induced increase in [$^{35}$S]GTPγS binding. Optimal signals were obtained with 1 to 10 μM GDP. The requirement of GDP for the agonist-induced increase in binding of [$^{35}$S]GTPγS has also been demonstrated for many other receptors including muscarinic, A1 adenosine and mu opioid receptors (Hil et al., 1989; Lorenzen et al., 1993; Traynor and Nahorski, 1995). For the alpha-2D adrenergic receptor, GDP is not required for agonist-induced increase in [$^{35}$S]GTPγS binding, but it increased the magnitude of stimulation caused by an agonist (Tian et al., 1994). The mechanism of action of GDP, however, is not fully understood. From the data in figure 1, we propose the following hypothesis. In the absence of an agonist, GDP inhibits [$^{35}$S]GTPγS binding in a dose-dependent manner with an IC$_{50}$ value of ~0.8 μM. With no or a low concentration of GDP, [$^{35}$S]GTPγS competes favorably over GDP for binding to guanine nucleotide binding sites of G$_{a}$ subunits. As a result, the agonist-induced increase in [$^{35}$S]GTPγS binding is obscured. Addition of GDP in the micromolar range results in the binding of GDP to most guanine nucleotide binding sites of G$_{a}$ subunits. Binding of an agonist to the receptor increases dissociation of GDP from the interacting G proteins and their association of [$^{35}$S]GTPγS (Gilman, 1987) and, therefore, allows the increase in [$^{35}$S]GTPγS binding to be observed.

**Kappa agonist-stimulated binding of [$^{35}$S]GTPγS** depends on the presence of Mg$^{2+}$ and the maximal signal-to-background ratio was observed between 5 and 15 mM. Mg$^{2+}$ has multiple effects on signal transduction of G protein-coupled receptors (for reviews, Gilman, 1987; Birnbaumer et al., 1990). The effects that require Mg$^{2+}$ are as follows: 1) formation of agonist-receptor-G protein ternary complex; 2) activation of G proteins by agonist-occupied receptors; 3) stimulation of GTP binding to G proteins and GDP dissociation by an agonist-receptor complex; 4) reduction of dissociation of GTPγS from G proteins to near zero; 5) $\beta\gamma$-stimulated GTP dissociation and GTPγS-induced subunit dissociation; 6) GTP-ase activity. The net results are that Mg$^{2+}$ promotes dissociation of oligomeric G proteins and the formation of an “activated” state of G$_{a}$. Effects of Mg$^{2+}$ on [$^{35}$S]GTPγS binding are likely caused by a combination of effects 1 through 5.

Na$^{+}$ is not required for kappa agonist stimulation, because (−)-U50,488H can increase [$^{35}$S]GTPγS binding even without Na$^{+}$. However, better stimulated-to-basal difference was obtained in the presence of >30 mM [Na$^{+}$]. Similar observations have also been reported in studies of G protein activation by muscarinic (Hilf et al., 1989), formyl peptide (Gierschik et al., 1989), A1 adenosine (Lorenzen et al., 1993) and alpha-2D adrenergic (Tian et al., 1994) receptors. It appears that Na$^{+}$ prevents activation of G proteins by unoccupied receptors (Gierschik et al., 1989). This effect of Na$^{+}$ is in accord with the findings that Na$^{+}$ was required for kappa agonist-induced increase in low-k$_{m}$ GTPase activity (Clark et al., 1986; Clark and Medzihradsky, 1987; Lawrence et al., 1995), inhibition of forskolin-stimulated adenylate cyclase (Attali et al., 1989; Konkoy and Childers, 1989, 1993; Lawrence and Bidlack, 1993; Lawrence et al., 1995) and increase in [$^{32}$P]azidoanilido-GTP into G$_{a}$ subunits (Prather et al., 1995).

Enhancement of [$^{35}$S]GTPγS binding by the kappa opioid agonist (−)-U50,488H was completely blocked by pretreatment of the cells with pertussis toxin, but not with cholera toxin, which confirms that the event is mediated entirely through pertussis toxin-sensitive G proteins. This result agrees with the findings that actions of kappa opioid receptors are mediated through pertussis toxin-sensitive G$_{a}$ and/or G$_{g}$ proteins (Lawrence and Bidlack, 1993; Prather et al., 1995; Ma et al., 1995). Inhibition of adenylate cyclase by kappa receptor activation was blocked by pertussis toxin pretreatment (Lawrence and Bidlack, 1993). Activation of a cloned rat kappa opioid receptor increased the incorporation of [$^{32}$P]azidoanilido-GTP into four G$_{a}$ subunits, three of which were identified as G$_{a_{13a}}$, G$_{a_{2a}}$, and G$_{a_{2a}}$ (Prather et al., 1995). Pertussis toxin treatment abolished kappa agonist-induced increase in K$^{+}$ conductance through an inward rectifying K$^{+}$ channel (Ma et al., 1995). Basal [$^{35}$S]GTPγS binding was also lowered by pertussis toxin treatment (see fig. 5A). A similar reduction of basal [$^{35}$S]GTPγS binding by pertussis toxin was observed in SHSY-5Y cells (Traynor and Nahorski, 1995). This may be caused by active coupling of unoccupied receptors to pertussis toxin-sensitive G proteins and, thus, stimulation of [$^{35}$S]GTPγS binding to G proteins at resting condition, similar to alpha-2D adrenergic receptor (Tian et al., 1994). In this CHO-hk2 system, kappa opioid receptors are not coupled to cholera toxin-sensitive G proteins. Coupling of the kappa opioid receptor to cholera toxin-sensitive G proteins was reported in the dorsal root ganglion-splanchnic dorsal horn culture (Crain and Shen, 1990) and in myenteric plexuses (Gintzler and Xu, 1991).

Potencies and efficacies of opioid ligands in stimulating mu opioid receptor-mediated [$^{35}$S]GTPγS binding to SHSY-5Y cell membranes correlated very well with those in other functional assays, such as an in vivo antinociceptive test and inhibition of contraction in guinea pig ileum in vitro (Traynor and Nahorski, 1995). In the present study, opioid agonists had different potencies in kappa receptor-mediated enhancement of [$^{35}$S]GTPγS binding and produced varying maximal responses, indicative of their efficacies. The rabbit vas deferens contained kappa opioid receptor, but not mu and delta receptors (Oka et al., 1981). Inhibition of field-stimulated contraction of the rabbit vas deferens was used to determine efficacies and potencies of ligands on kappa opioid receptors (Hayes and Kelly, 1985; Miller et al., 1986; Verlinde and De...
Ranter, 1988). Our finding that ethylketocyclazocine, tifluadom and (−)-U50,488H were full agonists in stimulating [35S]GTPγS binding is consistent with the observation that these three compounds were full agonists in the rabbit vas deferens (Romer et al., 1982; Hayes and Kelly, 1985; Verlinde and De Ranter, 1988). (−)-U50,488H and tifluadom were equally potent in stimulating [35S]GTPγS binding, whereas tifluadom was 7 to 400 times more potent than (−)-U50,488H in the rabbit vas deferens (Hayes and Kelly, 1985; Miller et al., 1986; Verlinde and De Ranter, 1988). Although ethylketocyclazocine was about 10 times more potent than (−)-U50,488H, in stimulating [35S]GTPγS binding it is 6 or 60 times more potent than (−)-U50,488H in the rabbit vas deferens (Hayes and Kelly, 1985; Miller et al., 1986). The observation that β-funaltrexamine enhances [35S]GTPγS binding in CHO-hkr cells is consistent with the original findings that it is a kappa agonist, in addition to having irreversible mu antagonist activities (Portoghese et al., 1980).

In the present study, nalorphine and pentazocine were partial agonists at the kappa receptor in stimulating [35S]GTPγS binding and nalbuphine had low level of agonist activity. Our findings are consistent with those of Miller et al. (1986) that pentazocine and nalorphine inhibited electrically induced contraction of guinea pig ileum by acting on the kappa opioid receptor with a Kᵣ value of naloxone of ~20 nM. In contrast, pentazocine, nalbuphine and nalorphine were inactive in vas deferens preparations of the rat, mouse and rabbit, but they could antagonize the action of ethylketocyclazocine in these preparations (Hayes and Kelly, 1985; Miller et al., 1986). The variations in potencies of these compounds in these in vitro preparations was hypothesized to be caused by the difference in the number of spare receptors in these models (Hayes and Kelly, 1985; Miller et al., 1986).

Our observations that (−)-U50,488H, tifluadom and ethylketocyclazocine are full agonists and nalorphine and nalbuphine are partial agonists are in accord with results from in vivo pharmacological studies. (−)-U50,488H, tifluadom and ethylketocyclazocine were found to be highly efficacious kappa agonists in kappa receptor-mediated antinociception (Von Voightlander et al., 1983; Piercey et al., 1982; France et al., 1994; Dykstra et al., 1987) and diuresis (Von Voightlander et al., 1983; Leander, 1983a; Dykstra et al., 1987; Takemori et al., 1988). In drug discrimination procedures, these drugs substitute completely for kappa agonists ethylketocyclazocine, bremazocine and spiradoline (Holtzman et al., 1991; France et al., 1994; Dykstra et al., 1987; Picker, 1994b; Smith and Picker, 1995). On the contrary, maximal effects produced by nalorphine were less than those of full agonists in kappa receptor-mediated diuresis (Leander, 1983a, b). Nalorphine and nalbuphine substituted partially or failed to substitute for bremazocine in drug discrimination tests (Smith and Picker, 1995; Picker, 1994a). In addition, nalorphine and nalbuphine antagonized the stimulus effect of bremazocine (Picker, 1994a; Smith and Picker, 1995). Nalorphine and nalbuphine antagonized the diuretic effect of bremazocine (Leander, 1983b) as well as the antinociceptive effect of U50,488H (Dykstra, 1990).

Naloxone and norbinaltorphimine shifted the dose-response curve of (−)-U50,488H to the right, but neither had any effect on the basal [35S]GTPγS binding. These results indicate that both compounds are pure antagonists without positive or negative intrinsic activity in this system.

For five full agonists, the EC50 values in stimulating [35S]GTPγS binding were similar to their Kᵣ values in inhibiting [3H]diprenorphine binding. This finding indicates that there are few or no spare receptors in this CHO-hkr system.

The binding affinities of ligands examined, including full agonists, partial agonists and antagonists, for the kappa receptor were similar in [35S]GTPγS binding buffer and in commonly used Tris-based receptor binding buffer. [35S]GTPγS binding buffer contains 100 mM Na+, 5 mM Mg2+ and 3 μM GDP, whereas the Tris-based receptor binding buffer does not. We found that the addition of 100 mM Na+, 5 mM Mg2+, 3 μM GDP or all three to Tris-based receptor binding buffer did not change the apparent Kᵣ value of (−)-U50,488H (Li, J.-G. and Liu-Chen, L.-Y., unpublished observation). Based on results in table 1, it is likely that Na+, Mg2+, GDP or all three at the concentrations used did not affect binding affinities of other ligands examined. Contrary to our observations, Lawrence and Bidlack (1992) showed that in R1.1 thymoma cell membranes, 100 mM Na+ and 10 mM Mg2+ inhibited 50 pM (−)[3H]bremazocine binding by 45% and 37%, respectively. Although 100 μM GDP did not have an effect on (−)[3H]bremazocine binding, a combination of 100 μM GDP and 30 mM Na+ inhibited binding by 54%. Thus, effects of Na+, Mg2+ and GDP on kappa agonist binding may also depend on the ligand examined. This issue needs further investigation.

In conclusion, stimulation of [35S]GTPγS binding to membranes of CHO cells stably expressing the human kappa opioid receptor by kappa agonists provides a useful functional measure for interaction between kappa opioid receptors and pertussis toxin-sensitive G proteins. In addition, this assay distinguishes ligands of full agonists, partial agonists and antagonists, which, in general, agrees with results obtained from in vitro tissue preparations and in vivo pharmacology.

Acknowledgments

We thank Drs. Alan Cowan and Ronald J. Tallarida for helpful discussions.

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