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Agonists and antagonists bind to different domains of the cloned κ opioid receptor

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ABSTRACT **Opium and its derivatives are potent analgesics that can also induce severe side effects, including respiratory depression and addiction. Opioids exert their diverse physiological effects through specific membrane-bound receptors. Three major types of opioid receptors have been described, termed δ, κ, and μ. The recent molecular cloning of these receptor types opens up the possibility to identify the ligand-binding domains of these receptors. To identify the ligand-binding domains of the κ and δ receptors, we have expressed in COS-7 cells the cloned mouse δ and κ receptors and chimeric δ/κ and κ/δ receptors in which the NH_2 termini have been exchanged. The opioid antagonist naloxone binds potently to wild-type κ receptor but not to wild-type δ receptor. The κ/δ chimera bound [^{3}H]naloxone with high affinity. In contrast, the κ-specific agonist [^{3}H]U-69,593 did not bind to the κ/δ chimera. These findings indicate that selective agonists and antagonists interact with different recognition sites in the κ receptor and localize the antagonist-binding domain to the NH_2 terminus. Consistent with the results of radioligand-binding studies, the κ/δ chimera did not mediate κ-agonist inhibition of cAMP formation. In contrast, the δ/κ chimera did mediate κ-agonist inhibition of cAMP formation, but this effect was not blocked by naloxone. Furthermore, a truncated κ receptor lacking its NH_2 terminus was able to mediate agonist inhibition of cAMP accumulation in a naloxone-insensitive manner. This result further indicates that the NH_2 terminus of the κ receptor contains the selective antagonist-binding domain. The ability to dissociate agonist- and antagonist-binding sites will facilitate the development of more specific κ agonists, which could have analgesic properties devoid of side effects.**

Opioids such as morphine are used clinically for the management of pain (1). However, the use of opioids has undesirable side effects—including respiratory depression, decreased gastrointestinal motility, sedation, nausea, and mood changes. Other major limitations include abuse potential, tolerance, and physical dependence. Morphine and the endogenous opioid peptides, the enkephalins, endorphins, and dynorphins, exert their physiological effects through membrane-bound receptors expressed in the central and peripheral nervous systems and in target tissues.

The three major types of opioid receptors, δ, κ, and μ, have recently been cloned and functionally characterized (2–5). They belong to the Asp-Arg-Tyr (DRY)-containing subfamily of seven transmembrane-spanning receptors. There is ≈60% amino acid identity among the sequences of the δ, κ, and μ opioid receptors. The sequences of the putative membrane-spanning segments (TM I–VII) and the three intracellular loops connecting these segments are highly conserved, whereas the sequences of the extracellular NH_2-termini segments, second and third extracellular loops, and the intracellular COOH termini are divergent. It seems reasonable to assume that these divergent extracellular regions may be responsible for the distinct ligand-binding profiles of the δ, κ, and μ receptors. To test this hypothesis, we have exchanged the extracellular NH_2 termini of the mouse δ and κ receptors (3, 4) and examined the abilities of these chimeric receptors to bind δ- and κ-selective agonists and antagonists, as well as to mediate inhibition of adenylyl cyclase activity.

METHODS

Generation of Chimeras. To exchange NH_2 termini between the mouse δ and κ opioid receptors, a common restriction site, *Spe* I, was generated at an equivalent position in the cDNAs in the region encoding the first transmembrane domain without altering the amino acid sequence of either receptor. Site-directed mutagenesis was done by using the Altered Sites *in vitro* mutagenesis system (Promega) and 27-mer oligonucleotides containing the *Spe* I site (δ-receptor oligonucleotide, CTGGGCAACGTACTAGTCATGTTTG-GC, and κ-receptor oligonucleotide, GTGGGCAATTCAC-TAGTCATGTTTGTC). After digestion with *Spe* I and the appropriate 5′ and/or 3′ enzymes, the cDNA fragments encoding the NH_2 and COOH termini of δ and κ were isolated from a 1.2% low-melting-point agarose gel. Fragments encoding the NH_2 terminus of δ receptor and the COOH terminus of κ receptor and vice versa were ligated together and cloned into the mammalian expression vector pCMV-6c. Truncated δ and κ receptors were generated by ligating the fragments encoding the COOH termini directly into the expression vector. Translation of the receptor sequences in these constructs was predicted to begin at a conserved ATG just distal to the *Spe* I site.

Radioligand-Binding Assays. The chimeras $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ and $\delta_{1\text{-}69}/\kappa_{79\text{-}380}$ (Fig. 1) were generated and transfected into COS-7 cells in parallel with either wild-type κ or δ receptor by the calcium phosphate precipitation method as described (4, 6). For receptor-binding studies, COS-7 cells expressing the receptors were harvested 72 hr after transfection in 50 mM Tris·HCl, pH 7.8/1 mM EGTA/5 mM $MgCl_2$/leupeptin at 10 μg/ml/pepstatin at 10 μg/ml/bacitracin at 200 μg/ml/aprotinin at 0.5 μg/ml (buffer 1) and centrifuged at 24,000 × *g* for 7 min at 4°C. The pellet was homogenized in buffer 1 using a Polytron. The homogenate was centrifuged at 48,000 × *g* for 20 min at 4°C, and the pellet was resuspended in buffer 1 and used in the radioligand-binding assay. Cell membranes (10–20 μg of protein) were incubated with [^{3}H]U-69,593 (2 nM, specific activity 47.4 Ci/mmol; 1 Ci = 37 GBq), [^{3}H]naloxone (6 nM, specific activity 72.1 Ci/mmol), [^{3}H][D-Pen^2, D-Pen^5]enkephalin, where Pen is penicillamine ([^{3}H]-DPDPE; 2 nM, specific activity 34.3 Ci/mmol), or [^{3}H]nal-

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Abbreviations: DPDPE, [D-Pen^2, D-Pen^5]enkephalin, where Pen is penicillamine; DSLET, [D-Ser^2, D-Leu^5]enkephalin-Thr.

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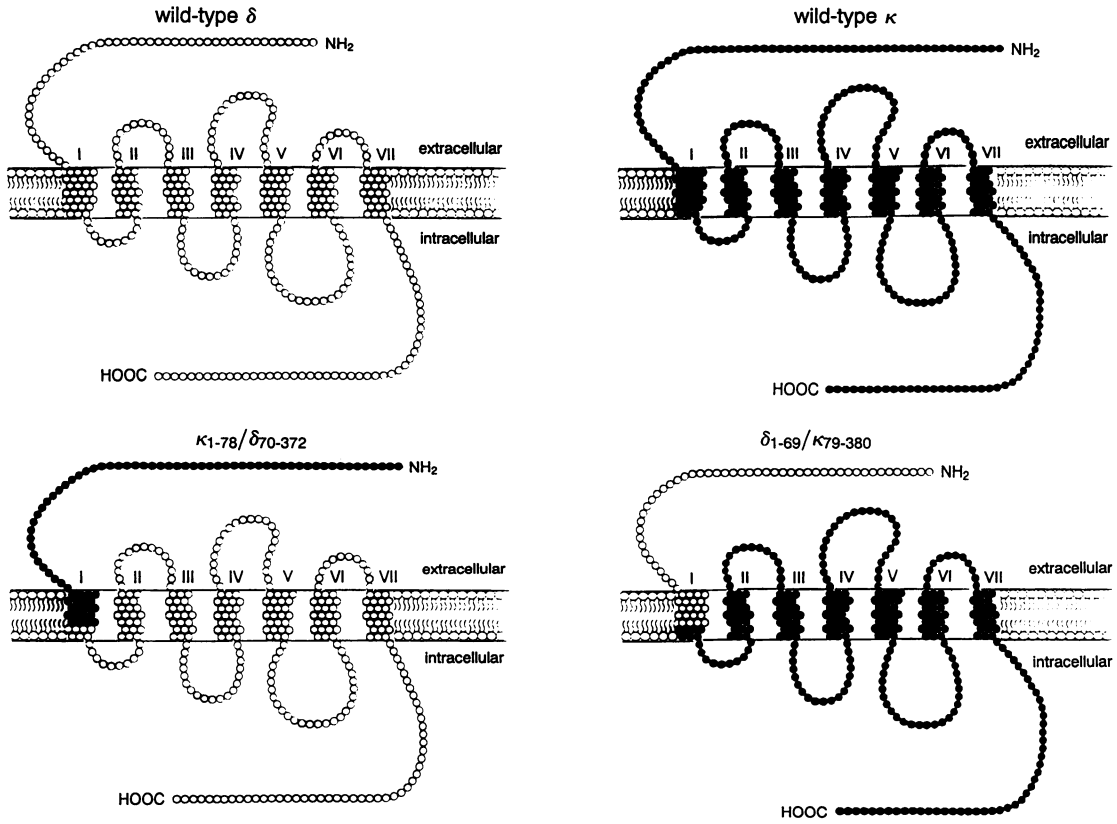

FIG. 1. Schematic of wild-type and chimeric δ and κ opioid receptors.

trindole (1 nM, specific activity 31.2 Ci/mmol) in a final volume of 200 μl for 40 min at 25°C in the presence or absence of competing agents. All radioligands were obtained from DuPont/NEN. Nonspecific binding was defined as radioactivity remaining bound in the presence of 1 μM naltrindole or naloxone for δ- and κ-selective ligands, respectively. The binding reaction was terminated by the addition of ice-cold 50 mM Tris·HCl, pH 7.8 and rapid filtration over Whatman GF/B glass fiber filters pretreated with 0.5% polyethyleneimine and 0.1% bovine serum albumin. The filters were washed with 12 ml of ice-cold buffer and soaked overnight in scintillation fluid. The bound radioactivity was determined by using a scintillation counter. IC_{50} values were analyzed as described (6) and obtained using the curve-fitting program FITCOMP on the National Institutes of Health-based Prophet system. Saturation analysis of [^{3}H]naloxone binding was used to determine K_d values and densities of receptors expressed in the COS-7 cells, and these K_d values were used to convert the IC_{50} values to K_i values as described (6) using the Cheng–Prusoff equation. Binding to the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera (Fig. 2*A*) was expressed as a percentage of binding to wild-type δ for [^{3}H]DPDPE and [^{3}H]naltrindole or wild-type κ for [^{3}H]U-69,593 and [^{3}H]naloxone.

cAMP Accumulation Assays. cAMP accumulation in COS-7 cells expressing the wild-type or mutant receptors was measured as described (7). Briefly, COS-7 cells were subcultured in 12-well culture plates. The cells were transfected 72 hr before the cAMP experiments. Culture medium was removed from the wells and replaced with 500 μl of fresh medium containing 0.5 mM isobutylmethylxanthine. Cells were incubated for 20 min at 37°C. Medium was removed and replaced with fresh medium/0.5 mM isobutylmethylxanthine with or without 10 μM forskolin and various opioid agonists and antagonists. The cells were incubated for 30 min at 37°C. Medium was removed, and cells were sonicated in the wells in 500 μl of 1 M HCl. The HCl was removed under vacuum, and the cAMP was quantified by using an RIA kit from DuPont/NEN, as described (4).

RESULTS

The radioligand-binding properties of the chimeras $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ and $\delta_{1\text{-}69}/\kappa_{79\text{-}380}$ and the mouse δ and κ receptors were examined. As shown (4, 6), the wild-type κ receptor could be labeled with the κ-selective agonist [^{3}H]U-69,593 and the antagonist [^{3}H]naloxone, whereas the wild-type δ receptor was labeled with the δ-selective agonist [^{3}H]DPDPE and antagonist [^{3}H]naltrindole. These κ-selective and δ-selective ligands have minimal cross-reactivity (4, 6). Although naloxone and dynorphin A also have high affinity for the μ receptor (6), in the context of these studies they are referred to as κ-selective because they have very low affinities for wild-type δ receptor (6). Only subtype-selective ligands were used in our studies because we anticipated that at least one of the chimeric receptors may contain a mixture of κ and δ ligand-binding sites. Only through the use of subtype-selective ligands were we able to discern these different sites on the same chimeric receptor.

The two chimeric opioid receptors had specific agonist- and antagonist-binding properties. The $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ receptor bound the antagonist [^{3}H]naloxone (which poorly labels the wild-type δ receptor) and the δ-selective agonist and antagonist, [^{3}H]DPDPE and [^{3}H]naltrindole, respectively, but did not bind the κ-receptor-selective agonist [^{3}H]U-69,593 (Fig. 2*A*). Saturation analysis of [^{3}H]naloxone binding to the wild-type κ and $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ receptors revealed similar K_d values (2 and 3 nM, respectively) and B_{max} values (1320 fmol/mg of protein for wild-type κ and 1008 fmol/mg of protein for $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$). The similar densities of κ and κ/δ receptors indicates that the lack of [^{3}H]U-69,593 binding to the κ/δ chimera was not due to low expression of the mutant receptor. Similar K_d values were obtained for [^{3}H]naltrindole binding to wild-type δ and $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ receptors (0.6 nM). In

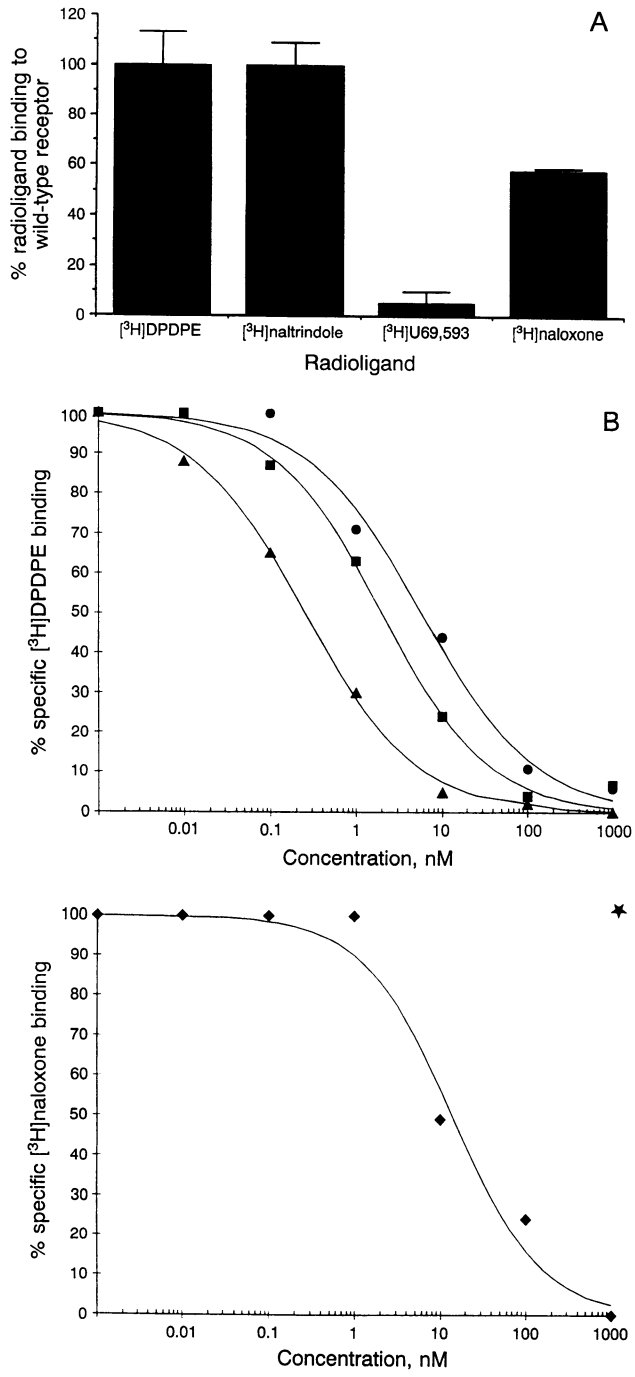

FIG. 2. Binding properties of the chimeric $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ receptor. (*A*) Binding of δ- and κ-selective agonists and antagonists to the chimeric $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ receptor. COS-7 cells were transfected by the calcium phosphate precipitation method with $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ or wild-type δ- or κ-receptor cDNAs. δ- and κ-selective agonists ([^{3}H]-DPDPE and [^{3}H]U-69,593, respectively) and antagonists ([^{3}H]naltrindole and [^{3}H]naloxone, respectively) were tested for their abilities to bind to the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ receptor. Values are expressed as percentage of [^{3}H]DPDPE and [^{3}H]naltrindole binding to wild-type δ receptors, and [^{3}H]U-69,593 and [^{3}H]naloxone binding to wild-type κ receptors. These are the average results of three to four different experiments. (*B*) Inhibition of [^{3}H]DPDPE (*Upper*) and [^{3}H]naloxone (*Lower*) binding to the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera by κ- and δ-selective agents. The δ-selective agonists [D-Ser^2,D-Leu^5]enkephalin-Thr (DSLET) (■) and DPDPE (●) and the δ-selective antagonist naltrindole (▲) were tested for their abilities to inhibit [^{3}H]DPDPE binding to this chimera (*Upper*). IC_{50} values for inhibition of [^{3}H]DPDPE binding were 5.8, 2.0, and 0.25 nM for DPDPE, DSLET, and naltrindole, respectively. The K_i value for inhibition of [^{3}H]naloxone binding by naloxone (◆) was 6 nM, but the κ-selective agonist U-50,488 (★) did not inhibit [^{3}H]naloxone binding to the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera (*Lower*). These are the average results of three to four different experiments.

contrast, the $\delta_{1\text{-}69}/\kappa_{79\text{-}380}$ receptor bound only the κ-selective agonist [^{3}H]U-69,593, although at low levels (46 fmol/mg of protein) compared with wild-type κ receptor. Furthermore, little specific radioligand binding to NH_2-terminally truncated forms of κ and δ, $\kappa_{79\text{-}380}$ and $\delta_{70\text{-}372}$, was detected. Therefore, minimal binding data could be reported for the $\delta_{1\text{-}69}/\kappa_{79\text{-}380}$ chimera and the truncated receptors due to poor labeling by all compounds tested. The low labeling may have been due to low expression or diminished affinites of the receptors for the radioligands. However, as indicated in Fig. 3, these receptors were functionally active and could be stimulated by high concentrations of agonists, indicating that they were expressed.

The initial binding results indicate that agonist- and antagonist-binding domains of the κ receptor are separable and located in different regions of the protein. The antagonist-binding domain of the κ receptor is localized to the region of amino acids 1–78, which includes the NH_2-terminal extracellular domain. In contrast, the antagonist-binding domain of the δ receptor is not located in the NH_2 terminus.

To further examine the binding properties of the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera, inhibition studies were conducted (Fig. 2*B*). [^{3}H]Naloxone binding to the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera was not inhibited by the κ-selective agonist U-50,488, but U-50,488 could inhibit [^{3}H]naloxone to wild-type κ receptor with a K_i of 0.1 nM. [^{3}H]Naloxone binding was dose-dependently and potently inhibited by the antagonist naloxone with a K_i value of 6 nM (Fig. 2*B*) and by the κ-selective antagonist norbinaltorphimine with a K_i value of 0.10 nM (data not shown), which are the same potencies for binding to the wild-type κ receptor (6). Levorphanol and ethylketocyclazocine, which are nonselective agonists, bound to the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera and wild-type κ receptor in identical manners. The K_i values calculated for inhibition of [^{3}H]naloxone by levorphanol were 0.6 and 0.4 nM for $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ and wild-type κ, respectively. Ethylketocyclazocine inhibited [^{3}H]naloxone binding to $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ and wild-type κ with K_i values of 3.0 and 1.4 nM, respectively. The calculated K_i values for inhibition of [^{3}H]-naltrindole by DSLET and naltrindole were identical between wild-type δ and the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera (4.8 and 1.7 nM, respectively, for DSLET and 0.85 and 0.86 nM, respectively, for naltrindole). This result implies that both agonist- and antagonist-binding sites in the δ receptor are in residues 70–372. In addition, we further characterized the interaction of the κ-selective antagonist-binding site with the δ-selective agonist- and antagonist-binding sites within the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera and found that these sites were in close proximity to each other because naltrindole and deltorphin II were both able to potently inhibit [^{3}H]naloxone binding to the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera with K_i values <0.1 nM. Deltorphin does not bind to the wild-type κ receptor, and naltrindole is very impotent. Furthermore, norbinaltorphimine could inhibit binding of [^{3}H]naltrindole to the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera.

Fig. 3 shows that both chimeras were functionally active and could mediate selective agonist inhibition of forskolin-stimulated cAMP accumulation (5, 6). The inhibition of cAMP accumulation by the κ-selective agonist U-50,488 via the $\delta_{1\text{-}69}/\kappa_{79\text{-}380}$ chimera was not blocked by naloxone (Fig. 3), consistent with the naloxone-binding site residing in the NH_2 terminus of the κ receptor. The potency of U-50,488 to inhibit cAMP formation was ≈1 nM, which is similar to its potency at interacting with wild-type κ receptor. Furthermore, dynorphin A was able to inhibit cAMP formation via the $\delta_{1\text{-}69}/\kappa_{79\text{-}380}$ chimera, and its effect was not blocked by naloxone. We also expressed a truncated version of the κ receptor, $\kappa_{79\text{-}380}$, in

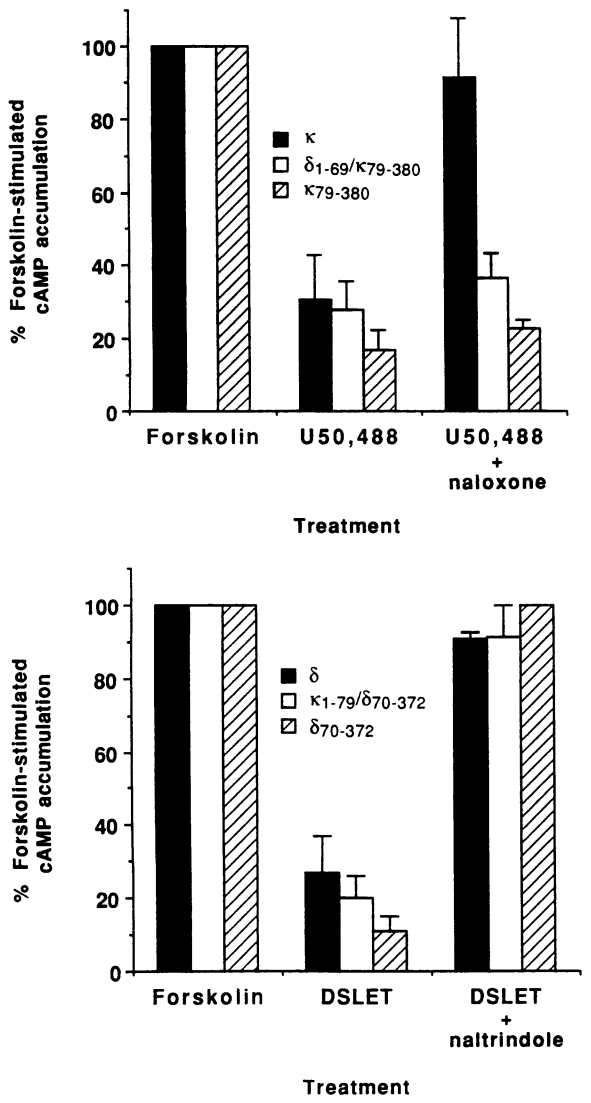

FIG. 3. Inhibition of forskolin-stimulated cAMP accumulation. COS-7 cells were transfected by the calcium phosphate precipitation method with wild-type (solid bars), chimeric (open bars), or truncated (hatched bars) receptor cDNA. κ- and δ-selective agonists (1 μM U-50,488 and DSLET, respectively) were tested for their abilities to inhibit 10 μM forskolin-stimulated cAMP accumulation. The abilities of κ- and δ-selective antagonists (1 μM naloxone and naltrindole, respectively) to block the effects of agonists were also examined. Results were calculated as a percentage of forskolin-stimulated cAMP accumulation (173 pmol per well for wild-type δ receptor, 244 pmol per well for wild-type κ receptor, 172 pmol per well for $\delta_{1\text{-}69}/\kappa_{79\text{-}380}$ receptor, 205 pmol per well for $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ receptor, 100 pmol per well for $\delta_{70\text{-}372}$ receptor, and 51 pmol per well for $\kappa_{79\text{-}380}$ receptor). Basal cAMP levels, which were <5% of forskolin-stimulated cAMP levels, were subtracted from all values. Results are the mean ± SEM of three different experiments.

which the extracellular NH_2-terminal domain is missing. Cells transfected with a construct encoding this truncated κ receptor could mediate U-50,488 inhibition of forskolin-stimulated cAMP formation (Fig. 3 *Upper*). This effect was not blocked by the κ-selective antagonist naloxone. This result confirms the necessity of residues 1–78 of the κ receptor for antagonism by naloxone but not in agonist activation. Furthermore, as seen with cells expressing the $\delta_{1\text{-}69}/\kappa_{79\text{-}380}$ chimera, the δ-selective agonist DSLET had no effect on cAMP formation in cells expressing the truncated κ receptor (data not shown), further demonstrating the specific effect of U-50,488.

Expression in COS-7 cells of the chimeric $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ conferred functional properties indistinguishable from the wild-type δ receptor (Fig. 3 *Lower*). The δ-selective agonist DSLET inhibited forskolin-stimulated cAMP formation, and this effect was blocked by naltrindole (Fig. 3), a result consistent with localization of the agonist- and antagonist-binding domains of the δ receptor to residues 70–372. Similar results were obtained with a truncated δ receptor, $\delta_{70\text{-}372}$. In COS-7 cells expressing the $\delta_{70\text{-}372}$ receptor, DSLET inhibited cAMP formation, and this effect could be blocked by naltrindole. The κ-selective agonist U-50,488 did not have any functional effect on the truncated δ receptor, $\delta_{70\text{-}372}$, or the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ receptor (data not shown), further demonstrating the specificity of the agonism of DSLET via these receptors. Thus, in contrast to the κ receptor, the NH_2 terminus of the δ receptor is not necessary for antagonist or agonist binding.

DISCUSSION

The results of these studies indicate that agonists and antagonists bind to physically separable sites on the κ receptor. This was shown by interchanging the NH_2 termini of the κ and δ receptors to generate chimeras. κ antagonists bound to the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera but did not interact with the $\delta_{1\text{-}69}/\kappa_{79\text{-}380}$ chimera or a truncated κ receptor. In contrast, κ agonists interacted with the $\delta_{1\text{-}69}/\kappa_{79\text{-}380}$ chimera and the truncated κ receptor but not the κ/δ chimera. The lack of κ agonist binding to the $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera is not due to insufficient expression of the receptor because the densities of the κ and $\kappa_{1\text{-}78}/\delta_{70\text{-}372}$ chimera were similar and δ agonist and antagonist binding to this chimera was indistinguishable from that of wild-type δ receptor. Furthermore, the receptor was associated with G proteins and was functionally active because a δ-selective agonist specifically inhibited cAMP formation in cells expressing the chimera.

Our findings suggest that antagonists interact with the NH_2 terminus of the κ receptor, whereas agonists bind to more distal regions, possibly the second and third extracellular loops because these are the only other extracellular domains that differ in amino acid sequence between the κ and δ receptors. Richardson *et al.* (8) have reported that modification of κ receptors of the guinea pig cerebellum with the carbodiimide, 1-ethyl,3-(3-dimethylaminoethyl)carbodiimide (EDAC), could be prevented by selective agonists but not by antagonists. They have suggested that κ-selective agonists and antagonists bind to distinct sites in the receptor because agonists, but not antagonists, were able to protect the receptor from modification by EDAC. These findings are consistent with our results, which suggest that agonists and antagonists bind to separate recognition sites in the κ receptor.

Ligands have been shown to bind to the NH_2 terminus of other receptors, such as the follicle-stimulating hormone and choriogonadotropin receptors (9, 10). However, for those receptors, agonists have been shown to bind to the NH_2 terminus. In contrast, antagonists selectively bind to the NH_2 terminus of the κ receptor, indicating a specific characteristic of this opioid receptor.

Previous studies on the β-adrenergic receptor and tachykinin receptor have suggested that agonists and antagonists bind to distinct domains (11–13). However, for these receptors, the agonist- and antagonist-binding domains physically overlap. This is not the case for the κ receptor, in which agonists and antagonists bind to physically separable regions. In the cases of the β-adrenergic and tachykinin receptors, overlapping domains allow for agonists and antagonists to compete for common sites within the receptor. The unusual aspect of the κ receptor is that the NH_2 terminus must be able to fold upon the receptor to bring the antagonist- and agonist-binding domains in close-enough proximity to allow antagonists and agonists to compete for binding.

Whereas these chimeras distinguish the agonist- and antagonist-binding domains of the κ receptor, this is not so for the δ receptor. However, our recent mutagenesis studies (14) have shown that selective agonists and antagonists interact differently with the δ receptor, suggesting that their binding domains may, therefore, be distinct but overlapping. Future mutagenesis studies will provide a more precise delineation of antagonist-binding domains of both the δ and κ receptors.

The results presented here indicate an unexpected difference between the κ and δ receptors with respect to the locations of agonist- and antagonist-binding domains and the important role played by the NH_2-terminal 78 residues of the κ receptor in antagonist binding. Furthermore, recent studies (H.K., K.R., and T.R., unpublished work) indicate that the second extracellular loop of the κ receptor is a recognition site for the binding of selective agonists. The demonstration that agonists and antagonists bind to different regions of the κ receptor should facilitate development of more selective κ ligands. This is an area of considerable interest because κ receptor-selective agents have limited abuse potential and respiratory-depressant effects (1). The structural analysis of the ligand-binding domains of the opioid receptors will provide the basis for the rational design of another generation of therapeutically useful analgesics with limited side effects.

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