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Identification of the Critical Domains of the δ-Opioid Receptor Involved in G Protein Coupling Using Site-Specific Synthetic Peptides

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SUMMARY

A large body of evidence implicates the second and third intracellular loops and the carboxyl-terminal portion of many G protein-coupled receptors as sites responsible for the interaction to G proteins. We synthesized a number of peptides from selected sites of the murine δ-opioid receptor and measured their ability to modify ligand-stimulated G protein activation and ^{3}H agonist binding to the receptor. In membranes from Rat-1 fibroblasts transfected to express the murine δ-opioid receptor stably (clone D2 cells), the δ-opioid agonist [D-Ser^2-Leu^5-Thr^6]enkephalin (DSLET) stimulated high affinity GTPase activity, which was inhibited by peptides that are derived from the proximal (i3.1) and the distal portions (i3.3) of the third intracellular loop with IC_{50} values of 15 ± 5 and 50 ± 4 μM, respectively. Peptides i3.1 and i3.3 inhibited DSLET-stimulated [^{35}S]guanosine 5′-*O*-thiotriphosphate binding in the same membranes. However, a peptide designated i4, which was derived from a juxtamembranous region of the carboxyl-terminal tail of the δ-opioid receptor, failed to alter agonist-mediated high affinity GTPase activity or agonist-driven [^{35}S]guanosine 5′-*O*-thiotriphosphate binding. Specific binding of [^{3}H]DSLET to membrane preparations from clone D2 was reduced by peptides i3.1 and i4. Combinations of these peptides abolished detectable [^{3}H]DSLET binding in the same membranes. Peptides i3.1 and i3.3 also destabilized the high affinity state of the receptor as assessed in ^{3}H agonist binding on membranes from neuroblastoma X glioma (NG108-15) hybrid cells, which express the δ-opioid receptor endogenously; furthermore, δ-opioid receptor-stimulated GTPase activity in the same membranes was inhibited by peptides i3.1 and i3.3 but i4 was inactive. In contrast, peptides derived from the second intracellular loop (i2.1 and i2.2), an intermediate portion of the third intracellular loop (i3.2), and the extreme amino-terminal region of the receptor were without effect in these assays. These observations indicate that although peptides i3.1, i3.3, and i4 act via different mechanisms, they provide evidence that at least two sites of the third intracellular loop and part of the carboxyl-terminal tail of the δ-opioid receptor are important in the interaction between this receptor and cellular G proteins. Collectively, these results provide novel information about regions of the δ-opioid receptor that are involved in G protein coupling and high affinity agonist binding.

Opioid receptors belong to the large superfamily of GPCRs and modulate distinct signal transduction pathways via coupling to multiple G proteins (1). Over the past few years, all of the three pharmacological classes of opioid receptors (δ, μ, and κ) have been cloned from a range of species (2, 3). A comparison of amino acid sequences of these receptors reveals high sequence similarity in the seven putative transmembrane domains as well as the second and third intracellular loops, suggesting that they might interact with similar G proteins (4). Analyses of opioid receptor interactions with specific G proteins in both native brain membranes and various cell lines have been reported (5–7). However, the structural basis for the interaction of opioid receptors and G proteins has not been defined.

Although most of the GPCRs seem to share a common multiple-membrane-spanning conformation, the functional significance of this configuration has not been elucidated (8, 9). Previous studies investigating receptor regions that specify coupling to particular G proteins and therefore to specific

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ABBREVIATIONS: GPCR, G protein-coupled receptor; DSLET, [D-Ser^2,Leu^5,Thr^6]enkephalin; NTI, naltrindole; DIP, diprenorphine; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate: Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

effector systems suggest that there is a variation among receptor families. Site-directed mutagenesis studies with the α- and β-adrenergic receptors indicate that the second and third intracellular loops are critical regions for specific G protein interactions (8–11). In contrast, studies with the human neutrophil *N*-formyl peptide receptor indicate that the second intracellular loop and the carboxyl-terminal tail serve as major contact sites with G proteins (12). Furthermore, as the splice variation in the carboxyl-terminal region of the EP3 prostanoid receptor results in the regulation of distinct combinations of effector systems, this implies a crucial role for the carboxyl-terminal tail in this receptor (13). Competition studies with peptides predicted to represent segments of receptor sequence have been used extensively to examine effects on ligand binding and G protein activation and have provided evidence for a critical role of these regions in G protein coupling (14–17).

To delineate regions of the δ-opioid receptor involved in G protein coupling, we synthesized a series of peptides corresponding to amino acid sequences of the second and third intracellular loops and the cytoplasmic tail of the murine δ-opioid receptor and examined their ability to modify the affinity of the receptor for ^{3}H opioid agonist ligands and to interfere with the opioid receptor/G protein coupling in both Rat-1 fibroblasts transfected to express the murine δ-opioid receptor and NG108-15 neuroblastoma X glioma hybrid cells, which endogenously express this receptor. We provided evidence that indicates that two regions in the third intracellular loop and part of the carboxyl-terminal tail of the δ-opioid receptor serve as major contact sites with G proteins.

Experimental Procedures

Materials. [γ-^{32}P]GTP (6000 Ci/mmol) was obtained from Amersham International (Buckinghamshire, UK); [^{3}H]DSLET (57 Ci/mmol), [^{3}H]DIP (39 Ci/mmol), [^{3}H]NTI (32 Ci/mmol), and [^{35}S]GTPγS (1150 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). Opioid ligands were from Sigma Chemical (Poole, Dorset, UK). GTP, ATP, Gpp(NH)p, phosphocreatine, creatine phosphokinase, and all other reagents used were of analytical grade and were from Sigma.

Peptide synthesis. Peptides were synthesized by an Applied Biosystems peptide synthesizer (model 430 A, Norwalk, CT) using *tert*-butyloxycarbonyl chemistry and purified by high performance liquid chromatography on an MCH-10 reverse-phase C-18 preparative column with a Varian 5000 LC instrument. The purity of the peptides was verified by analytical high performance liquid chromatography and amino acid analysis. Peptides from the second and third intracellular loops and from the extreme NH_2-terminal regions of the murine δ-opioid receptor were generated from the indicated regions (Fig. 1) of the published sequence (2, 3). These were designated i2.1, residues 144–153, VDRYIAVCHP; i2.2, residues 154–165, VKALDFRTPAKA; i3.1, residues 236–248, MLLRLRSVRLLSG; i3.2, residues 248–256, GSKEKDRSL; i3.3, residues 256–264, LRRITRMVL; i4, residues 322–333, DENFKRCFRQLC; and NH_2 terminus, residues 1–8, MELVPSAR.

Generation and isolation of clones of Rat-1 fibroblasts expressing the murine δ-opioid receptor. Clone D2 was obtained by transfection of a cDNA encoding the mouse δ-opioid receptor (4, 18) as described previously (19). Briefly, clone D2 was obtained through cotransfection with the use of Lipofectin reagent (Life Technologies, Grand Island, NY) of Rat-1 fibroblasts with a cDNA encoding the murine δ-opioid receptor in the expression vector pCMV-ms12 and the plasmid pBABEhygro. Clones were selected based on their resistance to hygromycin B (200 μg/ml) and were expanded,

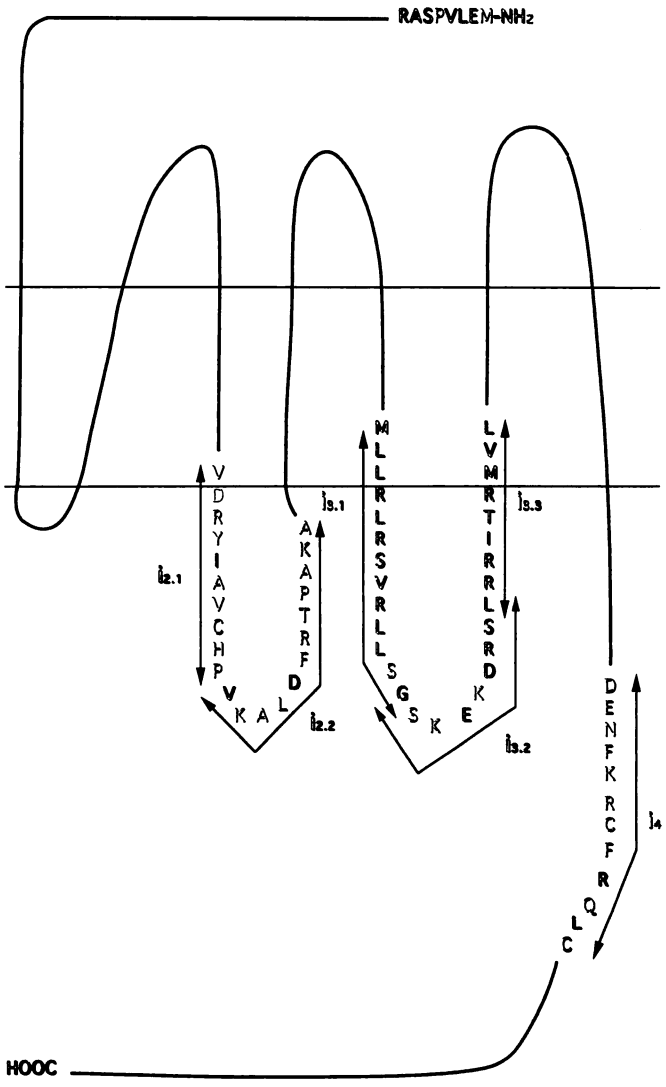

Fig. 1. The topography and sequences of the synthetic peptides from the δ-opioid receptor. Peptides i2.1 (144–153) and i2.2 (154–165) correspond to the NH_2- and COOH-terminal regions of the second intracellular loop, respectively. Peptides i3.1 (236–248) and i3.3 (256–264) correspond to the NH_2- and COOH-terminal regions of the third intracellular loop, respectively, and peptide i3.2 (248–256) corresponds to an intermediate part of the third intracellular loop. Peptide i4 (322–333) was derived from the cytoplasmic carboxyl-terminal region, whereas the octapeptide NH_2 (1–8) was derived from the extreme NH_2 terminus of the δ-opioid receptor (2–4).

and the specific binding of the ^{3}H-opioid ligands was subsequently determined (19).

Cell culture. Cells of the clone D_2 were grown in Dulbecco's modified Eagle's medium supplemented with 5% donor calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in 5% CO_2 at 37°. Membranes from these cells were prepared by homogenization as described for a variety of other cells (20). NG108-15 hybrid membranes (passage 36–41) were prepared as described previously (21).

Binding experiments. In experiments designed to define ligand specificity D2 cell membranes (10 μg) were incubated at 30° for 45 min in buffer A (20 mM Tris·HCl, pH 7.5, 50 mM sucrose, 20 mM $MgCl_2$) with varying concentrations of [^{3}H]DSLET (0.2–20 nM). Nonspecific binding was defined with the use of parallel tubes containing 10 μM DSLET or 10 μM naloxone. Nonspecific binding from each opioid ligand was 7–10% of the total binding. Binding was terminated by rapid filtration through Whatman GF/C filters, followed by extensive (three times with 4 ml) washes with ice-cold buffer A.

Filters were maintained overnight in Aquasol before liquid scintillation counting. In experiments in which uncoupling of opioid receptors from G proteins was performed, the membranes (10 μg of protein) were preincubated with different peptides (100 μM), as described in legend to Fig. 1; 100 μM Gpp(NH)p; or buffer for 60 min at 30° before the addition of ligands. Analysis of the binding data was performed with the computer program EBDA (Biosoft, Milltown, NJ) (22).

High affinity GTPase. GTPase activity in D2 and NG108-15 cell membranes was determined as described previously (21) at 37° using [γ-^{32}P]GTP (0.5 μM at 60,000 cpm). Low affinity GTPase (5–10% of the total counts) was assessed by parallel assays containing 100 μM GTP and was subtracted from the total activity to give the amount of high affinity GTPase activity. Membranes in 10 mM Tris, pH 7.5, 0.1 mM EDTA, and 5 mM $MgCl_2$ were preincubated in the presence of the peptides (100 μM) or buffer for 60 min at 30° before assessment of DSLET-stimulated high affinity GTPase activity.

[^{35}S]GTPγS binding assay. GTPγS binding of the D2 cell membranes (15 μg) was performed in a reaction mixture (100 μl) containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 10 μM GDP, 0.2 mM ascorbate, and 0.3–0.5 nM [^{35}S]GTPγS (50 nCi). Before assay, membranes in 10 mM Tris/0.1 mM EDTA were preincubated in the presence of 3 mM $MgCl_2$ and the presence of peptides or buffer for 60 min at 30°. The reaction was initiated after the preincubation period by the addition of the reaction mixture in the presence or absence of DSLET (10 μM), and then samples were incubated at 4° for 1 hr. The reaction was terminated by rapid filtration through GF/C Whatman filters, followed by extensive (three times with 4 ml) washes with ice-cold washing buffer consisting of 20 mM HEPES, pH 7.4, and 3 mM $MgCl_2$. Bound radioactivity was quantified by counting the filters in an Aquasol scintillation cocktail. Specific binding was calculated by subtracting the amount of [^{35}S]GTPγS bound in the presence of 10 μM unlabeled GTPγS from total bound [^{35}S]GTPγS. [^{35}S]GTPγS binding to synthetic peptides was negligible.

Statistical analysis. All data are expressed as the mean ± standard error of three or more individual experiments, each with a different membrane preparation. Each experiment was carried out in triplicate. Statistical analysis was performed with one-way analysis of variance and Student's *t*-test, with $p < 0.05$ indicating significance.

Results

To determine whether peptide fragments of the δ-opioid receptor would be able to interfere with effective δ-opioid receptor interaction with the cellular G protein population, a series of peptides that were 8–13 amino acids long were synthesized. The composition and sequence disposition of the synthetic peptides from the various regions of the cloned murine δ-opioid receptor are indicated in Fig. 1. These sequences were selected to correspond to the NH_2 and COOH termini of the second and third intracellular loops, as well as to a conserved region of the COOH-terminal tail of the murine δ-opioid receptor (2–4). Some of the peptides contained residues of the predicted transmembrane domains thought to be crucial for membrane interaction and the formation of a biologically active conformation (15, 23). A peptide corresponding to the extreme NH_2-terminal portion of the δ-opioid receptor sequence was also generated.

The δ-opioid-selective peptide DSLET stimulated high affinity GTPase activity in membranes of a clone (D2) of Rat-1 fibroblasts stably transfected to express the murine δ-opioid receptor (19). Maximal increase in high affinity GTPase activity was achieved using 10 μM DSLET. Subsequently, membranes from D2 cells were preincubated with the individual peptides (each at 100 μM) listed in Fig. 1 for 1 hr before measurement of GTPase activity and its regulation by DSLET. Such preincubations reduced high affinity GTPase activity, but stimulation of this activity by DSLET was still observed. Fig. 2 summarizes the effect of the various peptides used on δ-opioid receptor-stimulated GTPase activity. Treatment of clone D2 membranes with peptides i3.1 and i3.3, which correspond to the NH_2- and COOH-terminal domains of the third intracellular loop, significantly reduced the DSLET-stimulated high affinity GTPase. In contrast, peptide i3.2, which was generated from an intermediate portion of the third intracellular loop; the peptide corresponding to the carboxyl-terminal tail i4; and peptides i2.1 and i2.2, which were from the second intracellular loop and the octapeptide corresponding to the extreme NH_2-terminal region, failed to alter DSLET-stimulated high affinity GTPase activity. On the other hand, no synergism in the reduction of agonist-mediated GTPase activity was observed when peptides i3.3 and i4 were added together. Peptide i3.1 was the most effective of those used and inhibited the ability of DSLET to stimulate high affinity GTPase activity with an IC_{50} value of 15 ± 5 μM (Fig. 3, *top*). In contrast, the potency of peptide i3.3 on DSLET-stimulated high affinity GTPase activity was lower than that of peptide i3.1. Concentration-response curves for this peptide indicated an IC_{50} value of 50 ± 4 μM (Fig. 3, *bottom*).

To examine whether peptides i3.1 and i3.3 would also be able to modify opioid-binding affinity at the δ-opioid receptor of clone D2 cells, similar preincubations of the membranes with each of the peptides were performed, and subsequently the specific binding of various opioid agonists and antagonists was examined. As demonstrated in Fig. 4, reductions of 40 ± 2% (five experiments) and 20 ± 3% (three experiments)

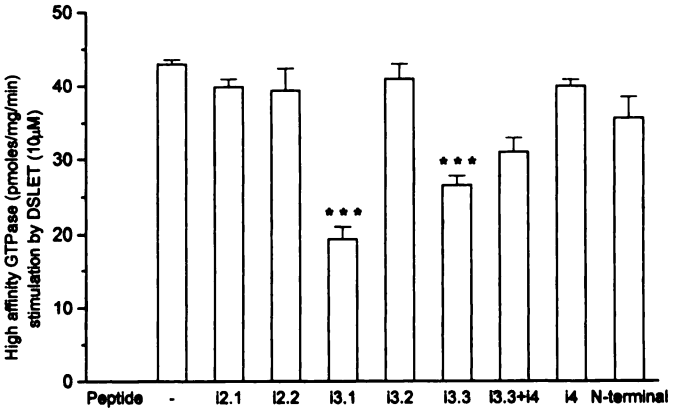

Fig. 2. The effect of peptides on DSLET-stimulated high affinity GTPase activity in membranes from Rat-1 fibroblasts expressing the δ-opioid receptor. Membranes (5 μg) of clone D2 cells were preincubated with buffer, the various peptides described in the legend to Fig. 1 (100 μM), or combinations of the peptides for 60 min at 30° as described in Experimental Procedures. Basal and DSLET (10 μM)-stimulated high affinity GTPase activities were then measured. Basal high affinity GTPase was 15 ± 1.3 pmol/min/mg of membrane protein, and the presence of 10 μM DSLET stimulated the activity to 43 ± 0.6 pmol/min/mg of membrane protein. Low affinity GTPase activity was determined by the addition of 100 μM GTP. Data are presented as DSLET-stimulated high affinity GTPase activity (pmol/min/mg of membrane protein). Basal GTPase activity was unaffected by the presence of the peptides. Results are mean ± standard error from three independent experiments performed in triplicate. ***, $p < 0.005$ compared with the corresponding DSLET-stimulated high affinity GTPase activity in the absence of peptide (Student's *t* test).

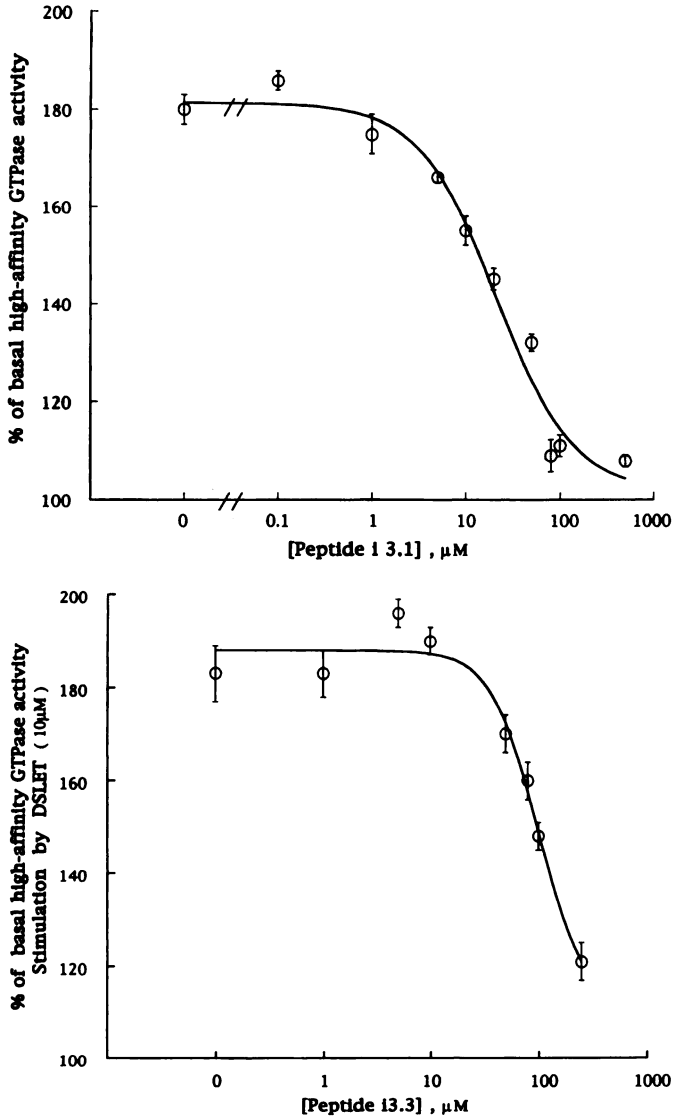

Fig. 3. Concentration-effect of peptides i3.1 and i3.3 on agonist-stimulated GTPase activity in membranes of clone D2. Membranes from clone D2 cells were preincubated with the indicated concentrations of peptides i3.1 (*top*) or i3.3 (*bottom*) and subjected to GTPase assays in the presence of 10 μM DSLET as described in the legend to Fig. 2. Basal GTPase activity was 22.4 ± 2 (mean ± standard error). The IC_{50} values for inhibition of DSLET-stimulated high affinity GTPase activity by peptides i3.1 and i3.3 were 15 ± 5 μM (three experiments) and 50 ± 4 μM (three experiments), respectively. The IC_{50} values were determined by fitting the data to competition curves with the computer program Kaleidagraph (Synergy Software, Reading, PA).

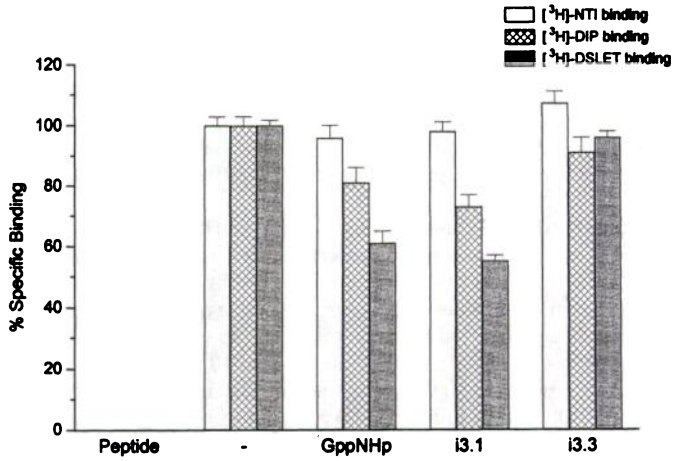

Fig. 4. Effect of peptides i3.1 and i3.3 on [^{3}H]DSLET, [^{3}H]DIP, and [^{3}H]NTI binding to D2 membranes. Membranes from D2 cells were preincubated for 60 min at 30° in the presence of Gpp(NH)p (100 μM) or the peptides i3.1 and i3.3 (100 μM). The binding of a single concentration of [^{3}H]DSLET (2 nM), [^{3}H]DIP (5 nM), and [^{3}H]NTI (3 nM) close to the K_d value for each ligand was measured, and nonspecific binding was defined by parallel incubations in the presence of 10 μM naloxone. Data are mean ± standard error of three individual experiments.

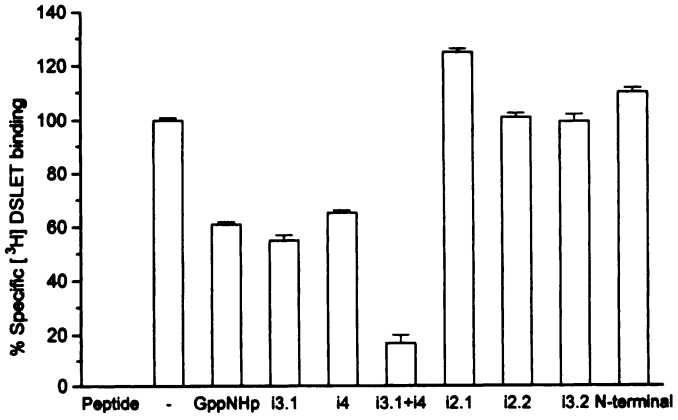

Fig. 5. Effect of peptides on the binding of [^{3}H]DSLET to clone D2 cell membranes. Membranes were preincubated with buffer, Gpp(NH)p (100 μM), and some of the peptides described in legend to Fig. 1 (i2.1, i2.2, i3.2, i4, and the NH_2-terminal octapeptide) or combinations of the peptides for 60 min at 30° before specific [^{3}H]DSLET (2 nM) binding, which was performed as described in Experimental Procedures. Nonspecific binding was assessed by the presence of 10 μM naloxone. Results are pooled from three or more individual experiments and are mean ± standard error.

in the specific binding of [^{3}H]DSLET (2 nM) and the partial agonist [^{3}H]DIP (5 nM), respectively, were observed when the membranes were preincubated in the presence of the poorly hydrolyzed guanine nucleotide analog Gpp(NH)p (100 μM), whereas the binding of the opioid antagonist [^{3}H]NTI (3 nM) was unaffected. Similarly, preincubations of the D2 membranes with the peptide i3.1 reduced [^{3}H]DSLET and [^{3}H]DIP binding with a decrease to the level seen in the presence of Gpp(NH)p without altering ^{3}H antagonist binding. In contrast, peptide i3.3 was unable to produce a significant reduction in the specific binding of [^{3}H]DSLET and [^{3}H]DIP. The only other peptide that inhibited [^{3}H]DSLET binding was peptide i4 (35 ± 1%; three experiments) (Fig. 5).

Peptide i4 had an IC_{50} value of 100 μM for reducing [^{3}H]DSLET binding, indicating that the effect of inhibition on agonist binding was specific (data not shown). However, peptides i3.2 and i2.2, as well as the amino-terminal peptide, had no effect on [^{3}H]DSLET binding. In contrast to the effects of the other peptides, peptide i2.1 produced a 20% stimulation of [^{3}H]DSLET binding. A combination of the effective peptides i3.1 and i4 at high concentrations (100 μM each) reduced [^{3}H]DSLET binding to as low as 20% of the initial value (Fig. 5).

To examine in greater detail the significance of the inhibition in binding produced by the δ-opioid receptor-derived peptide i3.1, we analyzed its ability to induce an alteration in the K_d value for [^{3}H]DSLET binding. As illustrated in Fig. 6, saturation analysis of the specific [^{3}H]DSLET binding to

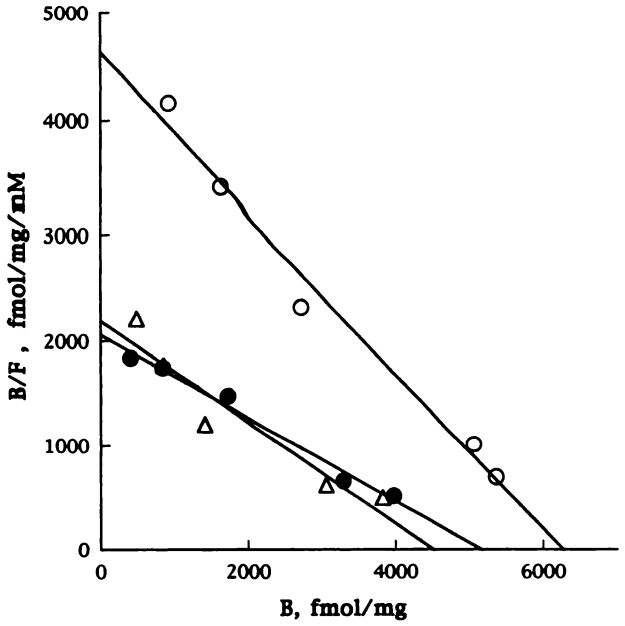

Fig. 6. Binding of [^{3}H]DSLET to membranes of clone D2 cells. Clone D2 cell membranes (15 μg) were preincubated with peptide i3.1 (100 μM) (●), Gpp(NH)p (100 μM) (△), and buffer (○) for 1 hr at 30° before the addition of varying concentrations of [^{3}H]DSLET (0.3–20 nM). Nonspecific binding was defined by the presence of 10 μM DSLET as described in Experimental Procedures. Binding parameters for membranes from untreated cells were B_{max} = 5800 ± 500 fmol/mg of protein and K_d = 1.4 ± 0.2 nM; for membranes incubated in the presence of Gpp(NH)p, B_{max} = 4800 ± 100 fmol/mg of protein and K_d = 2.6 ± 0.3 nM; and for membranes preincubated with peptide i3.1, B_{max} = 5300 ± 700 fmol/mg of protein and K_d = 2.8 ± 0.2 nM (mean ± standard error). Data are from one representative experiment. *B/F*, bound/free.

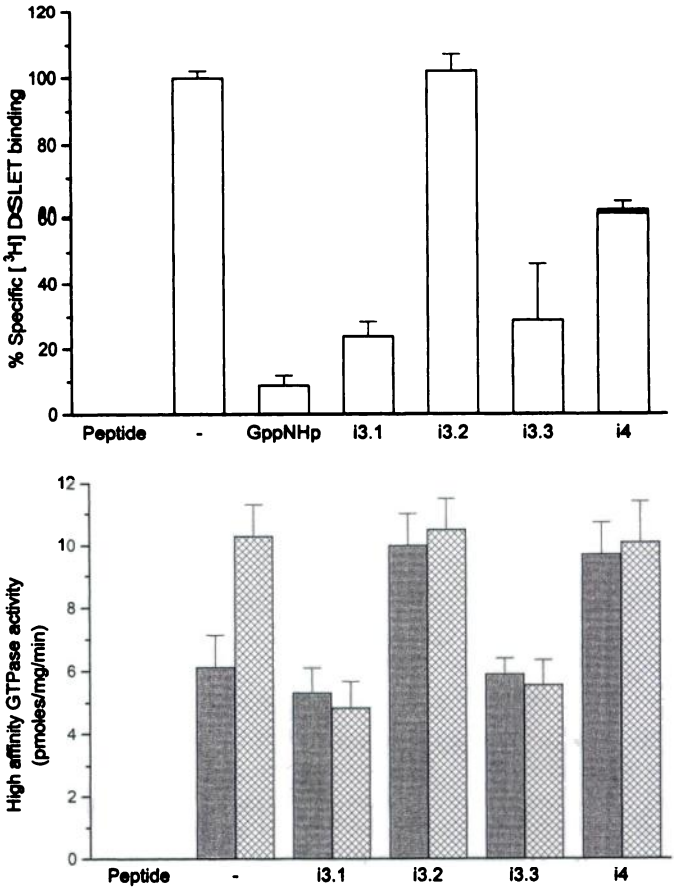

Fig. 7. Effect of peptides on [^{3}H]DSLET binding (*top*) and of high affinity GTPase activity (*bottom*) in membranes from NG108-15 cells. *Top*, Neuroblastoma X glioma NG108-15 hybrid cells were grown in tissue culture as described previously (21). After harvest of the cells and overnight storage at −80°, a membrane preparation was made. Membranes from NG108-15 cells (80 μg) were preincubated with buffer or peptides i3.1, i3.2, i3.3, and i4 for 60 min at 30°; subsequently, the binding of a single concentration of [^{3}H]DSLET (3 nM) close to the calculated K_d for this ligand was measured, and the nonspecific binding was defined in parallel incubations in the presence of 10 μM DSLET. Values are mean ± standard error from three experiments. *Bottom*, Membranes (8 μg) from NG108-15 cells were preincubated with buffer or with peptides i3.1, i3.2, i3.3, and i4 (100 μM) for 1 hr at 30°. After the preincubation period, both [γ-^{32}P]GTP and DSLET (10 μM) were added, and samples were incubated at 30° for an additional 20 min. Results are presented to display levels of both the basal high affinity GTPase activity (*shaded bars*) and the high affinity GTPase activity in the presence of agonist (*hatched bars*). Results are mean ± standard error of three experiments.

membranes from the D2 cell yielded linear plots, reflecting a single class of high affinity δ-opioid receptor binding sites with a K_d value of 1.4 ± 0.2 nM (five experiments) and a B_{max} value of 5.8 ± 0.5 pmol/mg of membrane protein. The ability of guanine nucleotides to cause a significant decrease in agonist binding affinity is a characteristic of most GPCRs. [^{3}H]DSLET binding to D2 cell membranes was analyzed in the presence of the nonhydrolyzable analog of GTP, Gpp(NH)p. Scatchard analysis showed that the reduction in binding was a consequence of decreased receptor affinity and a change in receptor number (B_{max} = 4.8 ± 0.1 pmol/mg of membrane protein, K_d = 2.6 ± 0.3 nM) (Fig. 6). Similar results were observed when the same preincubations were performed in the presence of peptide i3.1, which displayed a K_d value of 2.8 ± 0.2 nM and a B_{max} value of 5.3 ± 0.7 pmol/mg of membrane protein for [^{3}H]DSLET binding.

To further evaluate the influence of peptides i3.1, i3.2, i3.3, and i4 on δ-opioid receptor/G protein coupling, we tested the effect of these peptides on the binding of [^{3}H]DSLET to membranes of neuroblastoma X glioma hybrid NG108-15 cells, which are known to endogenously express a δ subtype of opioid receptor. This cell line expresses only a fraction of the levels of this receptor present in clone D2 cells. As shown in Fig. 7, *top*, peptides i3.1, i3.3, and i4 were able to significantly reduce specific [^{3}H]DSLET binding, whereas peptide i3.2 had no effect. Also, although in membranes of NG108-15 cells both peptides i3.1 and i3.3 were able to completely abolish DSLET-stimulated high affinity GTPase activity, peptides i3.2 and i4 were unable to produce any effect (Fig. 7, *bottom*).

To provide additional evidence for the functional significance of these regions in the interactions of the δ-opioid receptor with cellular G proteins, we examined the ability of all of the peptides to modulate DSLET stimulation of specific [^{35}S]GTPγS binding to membranes of clone D2 cells. The addition of 10 μM GDP reduced the total levels of basal specific [^{35}S]GTPγS binding; however, the presence of this nucleotide was required to observe a clear stimulation of basal [^{35}S]GTPγS binding on the addition of DSLET, as has been previously observed for other receptor systems (19, 24). As indicated in Table 1, 10 μM DSLET produced a consistent stimulation (250–300%) of [^{35}S]GTPγS binding that was sig-

TABLE 1

DSLET-stimulated [^{35}S]GTPγS binding to membranes of D2 cells: the effect of δ-opioid receptor-derived peptides

The ability of DSLET (10 μM) to stimulate the rate of guanine nucleotide exchange to membranes prepared from clone D2 cells was measured. Membranes (15 μg) were preincubated in the presence or absence of peptides (i2.1, i2.2, i3.1, i3.2, i3.3, i4, and the NH_2 octapeptide) with 3 mM $MgCl_2$ (as described in Experimental Procedures) before binding at 30° for 60 min. The membranes were then incubated at 4° for 60 min with 50 nCi [^{35}S]GTPγS in the presence or absence of 10 μM DSLET. Basal [^{35}S]GTPγS binding was 103 ± 6 fmol/mg. Data are presented as DSLET-stimulated [^{35}S]GTPγS binding in mean ± standard error values from four experiments.

Peptide	DSLET-stimulated [^{35}S]GTPγS binding
	fmol/mg protein
	302 ± 5
i2.1	280 ± 15
i2.2	290 ± 8
i3.1	230 ± 10[a]
i3.2	200 ± 9[a]
i3.3	215 ± 5[a]
i3.1 + i3.3	195 ± 5[a]
i4	288 ± 3
i4 + i3.1	200 ± 6
NH_2 terminal	290 ± 5

[a] $p < 0.01$ compared with the DSLET-stimulated value in the absence of peptide taken as control (Student's *t* test).

nificantly reduced by peptides i3.1, i3.2, and i3.3. Similar preincubations of the D2 cell membranes with the peptide i4 failed to alter agonist-stimulated [^{35}S]GTPγS binding. However, when preincubation of D2 cell membranes was performed with combinations of peptides i3.1, i3.3, and i4, a more profound attenuation of agonist-stimulated [^{35}S]GTPγS binding was observed, indicating that the participation of more than one effective peptides was required to provide maximal effect. In contrast, the peptides from the second intracellular loop, as well as the NH_2 terminal, failed to produce a reduction in the DSLET-stimulated binding of [^{35}S]GTPγS to G proteins.

Discussion

To successfully perform receptor signaling via G proteins, specialized binding regions must exist both on the surface of the activated receptor and on the G protein. There has been significant interest recently in the structural determinants of receptor/G protein interactions and the requirements for direct activation or inhibition of G proteins by synthetic peptides. For these reasons, we synthesized and used a series of peptides that correspond to parts of the intracellular loops of the δ-opioid receptor in an attempt to identify for the first time the sites of contact for this receptor and its associated G proteins. The opioid receptors are prototypic examples of receptors that interact with members of the family of pertussis toxin-sensitive G_i-like G proteins to cause inhibition of adenylate cyclase and to regulate a variety of ion channels (1, 7, 25). Little has been known about the way the selectivity of these interactions is achieved, particularly because no specific point-to-point contact sites between an opioid receptor and its corresponding G protein or proteins have been identified with mutagenesis or related studies.

In a number of studies, particularly those involving examination of receptors for catecholamines, a range of approaches have implicated the third intracellular loop of many GPCRs as being required for G protein recognition and activation and to confer selectivity among G proteins and, thus, second messenger activation (10, 11, 26, 27). However, the second intracellular loop also seems to be central to receptor folding, activation, and G protein coupling (12, 15, 28), and for a number of receptors, a key role has been implicated for elements within the carboxyl-terminal tail in G protein coupling and specificity (13, 15, 17, 29). It was thus anticipated that peptides derived from some of these regions might provide useful probes for opioid receptor/G protein interactions. We therefore synthesized a number of peptides corresponding to the proximal and distal elements of the second cytoplasmic loop, three peptides derived from the third cytoplasmic loop, one peptide derived from the amino terminus, and one peptide derived from a conserved region of the COOH-terminal tail of the δ-opioid receptor (Fig. 1).

Receptor peptide i2.1 contains the highly conserved GPCR motif [DRYXXV(I)XXPL] and could represent a major site involved in G protein activation, in particular because molecular modeling studies have suggested that the conserved R may adopt a distinctly different conformation when an agonist binds to a receptor. Peptide i3.3 contains the sequence BBXXB (where B is a basic residue and X is any nonbasic residue) at its carboxyl terminus, which seems to be involved in many receptors in G protein activation (27, 28). However, the peptide derived from the carboxyl-terminal tail of the δ-opioid receptor (i4) contains conserved stretches of amino acid residues proximal to Cys328 and Cys333, which bear fatty acid residues (4). Palmitoylated cysteines may anchor the tail in the membrane and thereby constitute additional intracellular loops between the seventh transmembrane helix and Cys328 or Cys333. In addition, peptides i2.1, i3.1, and i3.3 retain 1–3 amino acids of the predicted transmembrane α-helical regions of the receptor.

Alignment of the amino acid sequences of the δ-opioid receptor with the μ-opioid receptor indicated complete identity in these regions, with the exception of three amino acids of the amino-terminal portion of the third intracellular loop, two amino acids of the carboxyl-terminal region of the second intracellular loop, and replacement of a glutamine and leucine by a glutamate and phenylalanine, respectively, in the COOH-terminal peptide i4 of the μ-opioid receptor. Such similarity suggests that the δ- and μ-opioid receptors are likely to interact to very similar subsets of G proteins. It is noteworthy in this regard that all of the peptides exert effects similar to those observed in the current study for the δ-opioid receptor in membranes of cells transfected to express the μ-opioid receptor.[1]

To test whether these peptides could mimic or interfere with δ-opioid receptor/G protein interactions, we initially measured the ability of these peptides to modify both basal and agonist-stimulated high affinity GTPase activity in membranes of both neuroblastoma X glioma hybrid NG108-15 cells, which express this receptor endogenously but at rather low levels, and a clone of Rat-1 cells (D2), which we transfected to stably express relatively high levels of this receptor. In both of these cell lines, the vast majority of agonist stimulation of high affinity GTPase activity is eliminated by prior treatment of the cells with pertussis toxin,

[1] Z. Georgoussi, M. Merkouris, I. Mullaney, G. Megaritis, C. Carr, C. Zioudrou, and G. Milligan, manuscript in preparation.

indicating that the major interactions with G proteins are with members of the G_i subfamily (21).

The results presented in Fig. 2 indicate that only the peptides corresponding to sections of the third intracellular loop (i3.1 and i3.3) could interfere with functional δ-opioid receptor activation of the G protein population and that none of the peptides derived from the third intracellular loop had an ability to directly activate the G protein population. None of the peptides from the second intracellular loop, the carboxyl-terminal tail, or the NH_2-terminal octapeptide had an effect on the basal or agonist-stimulated high affinity GTPase activity. Concentration-response curves showed that the two third intracellular loop peptides (i3.1 and i3.3) produced this effect with different potencies (compare Fig. 3, *top* and *bottom*). The effects of peptides i3.1 and i3.3 are also supported from results on NG108-15 cells except that both peptides almost equipotently reduced the agonist-stimulated high affinity GTPase activity.

When we examined the effects of the synthetic peptides on ligand binding to the δ-opioid receptor, we observed that peptides i3.1 and i4 produced a significant inhibition of the specific [^{3}H]DSLET binding in clone D2 membranes. In addition to the effects of [^{3}H]DLSET binding, peptides i3.1 and i3.3 had a lesser effect on [^{3}H]DIP binding, whereas ^{3}H antagonist binding was unaffected.

Specific [^{3}H]DSLET binding to the δ-opioid receptors was not significantly inhibited by peptide i3.3. This apparent discrepant result raises the possibility that the receptor region represented by peptide i3.3 binds G protein to a limited extent and that this interaction is not able by itself to influence high affinity opioid receptor/G protein coupling in D2 membranes. Simultaneous application of two or more peptides corresponding to different sites of the receptor is expected to amplify the effects either additively or synergistically. Indeed, as illustrated in Fig. 5, the combination of the effective peptides i3.1 and i4 almost completely decreased agonist binding, indicating a synergistic rather than merely an additive effect. However, both third intracellular loop peptides (i3.1 and i3.3) had a marked effect in membranes of NG108-15 cells. These results might be explained by the markedly different levels of expression of the δ-opioid receptor in the two cell systems studied and the lower coupling efficiency of D2 cells compared with that of the NG108-15 cells. The elevated levels of receptor expression on a per-cell basis in clone D2 might result in a much higher level of spare receptors in these cells compared with NG108-15 cells. Thus, the effect of peptides i3.1, i3.3, and i4 mediated by the less "efficiently" coupled δ-opioid receptor is less obvious in D2 cells than that of more "efficiently" coupled δ-opioid receptor in NG108-15 cells. Another possible explanation is that although a single opioid receptor type can be examined in a cell line such as NG108-15, any cell line contains a particular set and proportion of G proteins that may be very different from those present in another system used to study this opioid receptor subtype. Indeed, Rat-1 fibroblasts have a different population of $G_{i\alpha}$ proteins and lack $G_{o\alpha}$.[2]

The effects of the various peptides that we observed were specific, and their differences cannot be accounted by the net charge of those peptides. For example, peptides i2.1, i3.2, and i4 have a net charge of 1+ but responded differently in all of the assays. Analysis of the results of agonist binding, high affinity GTPase activity, and [^{35}S]GTPγS binding modulation allows some further specification of the presumed functional interactions of the third intracellular loop and the carboxyl-terminal tail regions. Effects caused by the amino- and carboxyl-terminal peptides of the third intracellular loop indicate the essential role of these regions for the signal transfer in the δ-opioid receptor. Supplementary studies, with a peptide representing part of the insert in the third intracellular loop, were without effect other than [^{35}S]GTPγS binding; this discrepancy is due to a nonspecific effect because even at higher concentrations of the peptide used (data not shown), peptide i3.2 could not modify the GTPase activity in either D2 or NG108-15 hybrid cell membranes. These results imply that the insert does not engage in direct interactions with a binding site on a G protein. Analyses of the individual assays tested support the notion that peptides i3.1 and i3.3 seem to destabilize the high affinity state of the δ-opioid receptor by binding directly to G_i, thus preventing it from coupling to the receptor under both binding and GTPase assay conditions, albeit with different potencies. The most likely mechanism of the effects of peptides i3.1 and i3.3 is that they mimic the receptor and compete for the receptor binding region of the G protein. In contrast, the peptide derived from the carboxyl-terminal tail (i4) does not seem to be working through the same mechanism because it reduces high affinity agonist binding in a G protein-dependent manner but its interaction with the receptor and the G protein(s) is different in that it does not affect activation of the G protein as observed in agonist-mediated GTPase activity and [^{35}S]GTPγS binding. It should be borne in mind that proper receptor/G protein coupling involves several intracellular receptor domains but also different sites of the α and βγ subunits (14, 30). It is therefore likely, given the inability of peptide i4 to alter G protein activation, that there are different determinants of receptor/G protein coupling and direct G protein activation and that this peptide acts on a different site on the G protein. The second intracellular loop and the intermediate portion of the third intracellular loop apparently are not involved as discrete sites of G protein contact. This is evident from the failure of these peptides to either inhibit G protein-regulated high affinity binding to the δ-opioid receptor or interfere in G protein activation. In conclusion, although peptides i3.1, i3.3, and i4 produced different effects in the various assays tested, they strongly indicate the involvement of the third intracellular loop and COOH-terminal region in δ-opioid receptor/G protein coupling. This information contributes to the better understanding of the conformation of the interacting G protein sites and represents the first evidence that verifies structural determinants responsible for G protein coupling to the δ-opioid receptor. These data will serve as a useful anchoring point for the delineation of structural models of the opioid receptor/G protein interface.

Our data and related studies from other laboratories can be treated as special cases of a more general strategy to confer selectivity and specificity to receptor/G protein coupling. To meet these requirements, all receptors share distinct G protein binding domains that reflect structural similarities of G protein subtypes. There is a need for multiple points of interaction, and the organization of these domains may vary among different classes of receptors. In view of

[2] E. Hatzilaris and Z. Georgoussi, unpublished observations.

these considerations, the current study can make a valuable contribution to the evaluation of the topology of δ-opioid receptor/G protein interaction sites.

Our findings are in agreement with other results showing that sites of the third cytoplasmic intracellular loop and part of the carboxyl-terminal tail have a critical role in the functional coupling of G proteins. The results of mutagenesis studies of rhodopsin (34) and adrenergic (10, 35) receptors have suggested that mainly the amino-terminal portion of the cytoplasmic tail plays a role in maintaining productive receptor/G protein coupling. On the other hand, Prossnitz *et al.* (36) provided evidence that only the second intracellular loop modulates the selectivity of G protein coupling for neutrophil *N*-formylpeptide receptor. Elegant work by Konig *et al.* (17) and Münch *et al.* (15) with peptides from the second and third intracellular loops as well as the cytoplasmic tail of rhodopsin and β-adrenergic receptors, respectively, indicate that all three regions participate directly in receptor/G protein coupling. These observations suggest that multiple intracellular domains might form a three-dimensional binding site of the receptor for its specific G protein and effector systems. Our data extend these observations by determining that multiple regions within the δ-opioid receptor are critical in binding to probably different sites on the α or βγ subunits, which may be important in discriminating the cellular responses elicited by a receptor. It is therefore anticipated that specific amino acid sequences in multiple cytoplasmic regions play a role in determining the selectivity of receptor/G protein interaction. The lack of sequence homology in the third intracellular loop among different receptors does not allow prediction of a consensus sequence for coupling to specific G proteins. However, it is likely that there is not a single consensus structure within this receptor superfamily that dictates G protein binding but rather that individual receptors use different intracellular domains to achieve this goal.

The specificity of the peptide effects observed in the current study is reflected with various assays in two different cell systems expressing the δ-opioid receptor at differing levels. These results provide important insights concerning the actions of opioid receptor/G protein coupling mechanisms; however, to more precisely define the sites that are required for this association, we are performing site-directed mutagenesis on specific amino acids of the third intracellular loop and the carboxyl-terminal tail. Mapping of the sites of interaction of such receptor-derived peptides should aid in modeling the receptor/G protein interface and in the development of specific activators or inhibitors of G protein-mediated signal transduction.

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