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Mapping the receptor domains critical for the binding selectivity of δ-opioid receptor ligands

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Abstract

While a good deal has been learned about determinants of high affinity ligand/receptor interactions in G-protein-coupled receptors, less is known about mechanisms of ligand selectivity. The opioid receptors offer an excellent opportunity to study the mechanisms whereby structurally very similar receptors discriminate between different but structurally highly related ligands. In the current study, we use a series of chimeric constructs between the δ-opioid receptor and either the μ- or the κ-opioid receptors to investigate the structural basis of binding selectivity of multiple classes of δ-opioid receptor selective ligands. Our results demonstrate that a region containing the sixth transmembrane domain (TM6) and the third extracellular loop (EL3) in the δ-opioid receptor is absolutely critical for δ-opioid receptor selectivity. The introduction of this region into the κ-opioid receptor is sufficient to impart a δ profile for δ-opioid receptor selective alkaloids such as naltrindole and naltriben. In order to locate the amino acid residues that may be involved in ligand selectivity in TM6 and EL3 of the δ-opioid receptor, several mutations were introduced into that region. These mutations showed differential effects on peptide and alkaloid ligands. In addition, none of the individual mutations alone could account for the changes exhibited by the chimeric receptors. We conclude that the selectivity of most δ-opioid ligands is achieved through their interaction with many different residues in the TM6/EL3 region. Our results also support a view that the extracellular domains of peptide receptors may provide the basis of a sorting mechanism for ligand selectivity.

Keywords: Opioid receptor; Chimeric receptor; Mutagenesis; Structure-function relationship; δ-Opioid receptor ligand

1. Introduction

The recent cloning of opioid receptors offers a unique opportunity to understand the structural basis of ligand selectivity (Reisine and Bell, 1993; Uhl et al., 1994). Not only do opioid receptors exhibit a high degree of sequence homology, but their ligands are also highly structurally related (Simon, 1991), and the question of how the receptors choose among these highly related ligands is of great physiological and pharmacological importance. All endogenous opioid ligands possess a common N-terminal sequence Tyr-Gly-Gly-Phe- but they have different C-terminal extensions, which may allow them to discriminate between the multiple opioid receptors. Furthermore, there exists a wealth of synthetic opioid ligands with different structure and selectivity, greatly facilitating the analysis of receptor-ligand interactions.

Since the pioneering work on the cloned adrenoceptors, extensive progress has been made in elucidating the structure-function relationships of G-protein-coupled receptors (Ostrowski et al., 1992; Strader et al., 1994). For example, it is now known that charged amino acid residues in the transmembrane domains are often critical in high affinity ligand binding and/or receptor activation. Accordingly, charged residues in the transmembrane domains conserved across the three opioid receptors have found to be important for opioid binding (Kong et al., 1993; Surratt et al., 1994), thereby revealing some of the mechanisms for high affinity binding common to the opioid receptor family.

However, locating the regions of the receptors responsible for *ligand selectivity* remains more elusive. This issue is of particular relevance when there are multiple endogenous ligands interacting with multiple receptors – a situation seen for many peptide families. Emerging results in

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the opioid receptor field have begun to reveal the complexity of the mechanisms of high affinity selective binding (Kong et al., 1994; Wang et al., 1994; Xue et al., 1994; Chen et al., 1995; Fukuda et al., 1995; Hjorth et al., 1995; Meng et al., 1995; Minami et al., 1995; Onogi et al., 1995; Wang et al., 1995 Xue et al., 1995; Shahrestanifar et al., 1996; Watson et al., 1996; Zhu et al., 1996). While the studies to date suggest that the selective binding of certain opioid ligands, such as the μ-opioid receptor selective [D-Ala^2,N-Me-Phe^4,Gly-ol^5]-enkephalin (DAMGO) or the κ-opioid receptor selective dynorphin A, may be achieved through interactions with unique receptor domains, the critical domains have not been systematically specified and the generalizability of these findings across multiple ligands of the same selectivity remains unclear. Even less is known about the structural elements that are responsible for the selectivity of different categories of ligands, especially as we contrast opioid peptides and opiate alkaloids. The δ-opioid receptor offers an excellent opportunity to address these issues, as it interacts with several categories of highly selective ligands, including the mammalian enkephalins, the amphibian deltorphins, and the δ-opioid receptor selective alkaloids (Knapp and Yamamura, 1992). The purpose of the present study was to attempt to localize critical sites for δ-opioid receptor selectivity by proceeding in two phases: first by using chimeric constructs to localize to relatively small domains the structural elements responsible for the selectivity of different categories of δ-opioid ligands; then, based on the results from the chimeric study, by creating several δ-opioid receptor mutants in an attempt to achieve a more punctuate identification of the molecular basis of binding selectivity.

2. Materials and methods

2.1. Construction of the chimeric receptors

The cDNAs encoding the rat μ-, δ- and κ-opioid receptor were cloned in our laboratory (Thompson et al., 1993; Meng et al., 1993). The rat δ-opioid receptor sequence from our laboratory is not published but the coding region is identical to the sequence reported by (Fukuda et al., 1993). A total of six unique restriction enzymes sites, BsrG I, Afl III, BstE II, Bgl II, BstB I and Bsu36 I were used to construct various receptor chimeras. The Afl III sites are conserved at the corresponding positions in native μ-, δ-, κ-opioid receptor cDNAs. The Bgl II sites are present in both wild type δ- and κ-opioid receptor cDNAs but not in the μ-opioid receptor cDNA. All the necessary restriction sites were introduced into the μ-, δ-, κ-opioid receptor cDNAs at the corresponding positions in their sequences. Only the mutations that created the BstE II sites in the μ- and κ-opioid receptor were not silent. The introduction of a BstE II site in the μ-opioid receptor changed Thr^{207} to the corresponding Val^{188} in the δ-opioid receptor. Similarly, the creation of a BstE II site changed Gly^{198} in the κ-opioid receptor to the corresponding Val^{188} residue in the δ-opioid receptor. The location of these restriction sites is shown in Fig. 1. All the opioid receptors were divided into seven segments at the corresponding positions, labeled as 'A' to 'G' from N-terminal to C-terminal (Fig. 1). Series of chimeric κ/δ- and μ/δ-opioid receptors were constructed by subcloning. Extensive restriction enzyme mappings were conducted to ensure that the arrangement of different segments is correct and to ascertain that there are no incorrect bases at the ligation sites.

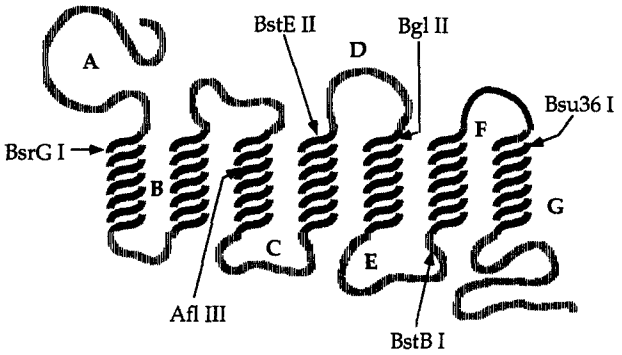

Fig. 1. The location of the restriction enzymes used in the construction of chimeric opioid receptors. BsrG I: at the A/B boundary (δ 55–56, κ 65–66, μ 74–75); Afl III: at the B/C boundary (δ 131–132, κ 141–142, μ 150–151); BstE II: at the C/D boundary (δ 187–188, κ 197–198, μ 206–207); Bgl II: at the D/E boundary (δ 214–215, κ 227–228, μ 233–234); BstB I: at the E/F boundary (δ 256–257, κ269–270, μ 275–276); Bsu36I: at the F/G boundary (δ 305–306, κ 317–318, μ 323–324).

2.2. Nomenclature of the chimeric receptors

In naming a construct, the first letter denotes the original receptor which represents the majority of the chimera; the second letter represents the second receptor donating a single (or multiple) domains, and the last letter designates the name of the domain (e.g. μ/δ-A means the μ-opioid receptor was altered by replacing its A domain with the corresponding A domain from the δ-opioid receptor).

2.3. Construction of δ-opioid receptor mutants

After the characterization of the chimeric receptors, several δ-opioid receptor mutants were made using a double stranded site-directed mutagenesis protocol (Deng and Nickoloff, 1991).

2.4. Expression of the receptor and binding assay

A mammalian expression vector containing the cytomegalovirus immediate-early promoter, courtesy of Dr Mike Uhler (Huggenvik et al., 1991), was used to express the chimeric and wild type receptors in COS-1 (a simian

cell line expressing the SV40 large T-antigen) cells. Twenty-five micrograms of plasmid DNA were transfected into each 100 mm dish of COS-1 cells using the method of Chen and Okayama (1987). Receptor binding of the membrane preparation of the transfected cells was performed according to Goldstein and Naidu (1989). About 1.5 nM of [^{3}H]ethylketocyclazocine (24.8 Ci/mmol, NEN) was used to label the receptors in chimeric study. For the mutagenesis study, about 1 nM of [^{3}H]bremazocine (31.3 Ci/mmol, NEN) was used to label the receptors. This was done because one of the mutants exhibited decreased affinity to [^{3}H]ethylketocyclazocine, whereas all the mutants maintained wild-type like affinity to bremazocine. All assays were conducted in 50 mM Tris buffer (pH 7.4) at room temperature. The affinity of the labeling ligand [^{3}H]ethylketocyclazocine toward these receptors was determined by two independent self-competition assays. The average value of two assays was used to calculate the K_i of other ligands competing with ethylketocyclazocine. For each competing ligand, nine different concentrations were used and all the chimeric receptors in the same data table (see below) were assayed in the same batch in duplicates. Due to the number of the receptor constructs and ligands involved in the study, we did not repeat the binding assay for all the chimeric receptors. Based on our binding study with the wild type opioid receptors, the variation in apparent K_i values between different assays is within two-fold in most cases and rarely exceed three-fold. Instead, we chose to study more constructs and ligands in order to extract more information from these studies. In the assays using peptide ligands, a mixture of protease inhibitors was added to the binding buffer. The final concentration of each component in the cocktail is as follows: 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 1 mM iodoacetamide. This mixture did not influence the affinity of ethylketocyclazocine toward the κ- and δ-opioid receptors (unpublished observations). Receptor binding results were analyzed with the LIGAND program (Munson and Rodbard, 1980).

3. Results

3.1. Construction of the chimeric receptors

We chose to use the single-segment-replacement chimeras in our study because their overall conformations may be closer to those of the wild type receptors than the conformations of most of the stepwise-segment-replacement chimeras (Meng et al., 1995). Eleven out of fourteen of the single-segment-replacement chimeric δ/κ-opioid receptors were found to bind [^{3}H]ethylketocyclazocine with very good affinity. The remaining three chimeras, δ/κ-C, δ/κ-D and κ/δ-B did not bind the non-selective ligand [^{3}H]ethylketocyclazocine or [^{3}H]bremazocine. Since it is important to assess the role of the same segment in at least two different pairs of receptors, we constructed several additional chimeras, δ/κ-BC, δ/κ-ABCD, δ/κ-ABC, κ/δ-BCD and κ/δ-CD. As a result, the difference between any pair of corresponding δ and κ segments can be assessed under two different combinations with other receptor segments. To further facilitate the analysis of the origin of δ-selectivity, seven μ/δ or δ/μ chimeras that could bind [^{3}H]ethylketocyclazocine with good affinities were also chosen to study the role of the δ segments in a different structural environment.

3.2. Pharmacological profiles of the chimeric receptors

The binding results are summarized in Tables 1–3. All these chimeric receptors bound non-selective ligands eth-

Table 1
Pharmacological profile of chimeric δ/κ-opioid receptors (apparent K_i, nM) [a]

	δ/κ-A	δ/κ-B	δ/κ-BC	δ/κ-ABC [b]	δ/κ ABCD [c]	δ/κ-E	δ/κ-F	δ/κ-G	δ Wild	κ Wild
Non-selective opioid receptor ligands										
Ethylketocyclazocine	11	3.6	3.4	5.2	3.1	7.3	9.1	16	9.6 ± 3.3	5.1 ± 2.0
Bremazocine	1.0	0.46	0.57	0.55	0.26	0.90	1.4	1.3	1.3 ± 0.6	0.35 ± 0.22
Naltrexone	20	13	11	19	14	24	18	35	24 ± 8.1	10 ± 3.9
δ-Opioid receptor ligands										
[Leu^5]enkephalin	5.2	37	18	8.6	104	5.8	> 10000	5.6	4.2 ± 2.4	> 10000
[Met^5]enkephalin	3.9	44	14	5.7	57	2.8	2600	3.5	3.0 ± 1.8	> 10000
DPDPE	14	430	260	160	440	17	> 10000	29	21 ± 4.0	> 10000
JOM13 [d]	7	1.9	3.5	2.4	9.4	4.2	> 10000	4.0	2.1 ± 0.43	> 10000
Deltorphin II	2.3	6.9	20	9.9	250	1.9	2500	1.3	2.0 ± 0.24	> 10000
TIPP [e]	2.4	1.8	1.9	2.9	9.2	2.1	> 10000	1.2	1.0 ± 0.36	> 10000
Naltrindole	0.11	0.38	0.44	0.18	0.10	0.22	50	0.11	0.30 ± 0.13	74 ± 29
Naltriben	0.19	0.33	0.16	0.18	0.11	0.21	82	0.14	0.39 ± 0.21	92 ± 31

[a] The K_i values of the chimeric constructs toward the same ligand were determined in the same batch of binding assay. Data for the wild type opioid receptors were from three independent assays. They are expressed as mean ± S.D. [b]δ/κ ABC is the same as κ/δ DEFF. [c]δ/κ ABCD is the same as κ/δ EFG. [d]JOM13: Tyr-c [Δ-Cys-Phe-Δ-Pen] OH. [e]TIPP: Tyr-Tic-Phe-Phe.

Table 2
Pharmacological profile of chimeric κ/δ-opioid receptors (apparent K_i, nM) [a]

	κ/δ-A	κ/δ-BCD	κ/δ-CD	κ/δ-C	κ/δ-D	κ/δ-E	κ/δ-F	κ/δ-G	δ Wild	κ Wild
Non-selective opioid receptor ligands										
Ethylketocyclazocine	3.1	16	8.1	4.0	4.7	3.7	4.7	3.7	9.6 ± 3.3	5.1 ± 2.0
Bremazocine	0.38	1.8	0.52	0.24	0.68	0.46	0.75	0.42	1.3 ± 0.6	0.35 ± 0.22
Naltrexone	13	14	5.8	7.8	11	20	22	4.0	24 ± 8.1	10 ± 3.9
δ-Opioid receptor ligands										
[Leu^5]enkephalin	> 10000	9700	> 10000	> 10000	9500	> 10000	100	4900	4.2 ± 2.4	> 10000
[Met^5]enkephalin	> 10000	4900	> 10000	> 10000	4700	> 10000	130	9300	3.0 ± 1.8	> 10000
DPDPE	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	620	> 10000	21 ± 4.0	> 10000
JOM13	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	31	> 10000	2.1 ± 0.43	> 10000
Deltorphin II	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	650	> 10000	2.0 ± 0.24	> 10000
TIPP	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	12	> 10000	1.0 ± 0.36	> 10000
Naltrindole	88	48	37	34	68	210	0.30	58	0.30 ± 0.13	74 ± 29
Naltriben	110	84	28	71	84	210	0.36	110	0.39 ± 0.21	92 ± 31

[a] The K_i values of the chimeric constructs toward the same ligand were determined in the same batch of binding assay. Data for the wild type opioid receptors were from three independent assays. They are expressed as means ± S.D.

ylketocyclazocine, bremazocine and naltrexone with very good affinity (naltrexone is non-selective in terms of discriminating κ- and δ-opioid receptors). Therefore the basic binding pocket for opioid alkaloids is most likely well-preserved in these constructs. This gives us a good starting point for further structure-function analysis.

Table 1 shows the effect of replacing a δ segment with its corresponding κ segment in a predominantly δ-opioid receptor environment. It can be seen from the data that the replacement of δ-A, δ-C, δ-E and δ-G segments by their corresponding κ regions did not significantly alter the binding affinities of any of the δ ligands tested. However, the substitution of δ-B region with κ-B caused a 10–20-fold drop in the affinities of the peptides [Leu^5]enkephalin, [Met^5]enkephalin and cyclic [Δ-penicillamine^2,Δ-penicillamine^5]enkephalin (DPDPE). The binding of other δ-opioid ligands was not greatly influenced by this substitution. The replacement of the δ-D region with κ-D (compare δ/κ ABCD with δ/κ ABC) lowered the affinities of the enkephalins toward the receptor by about 10-fold, although this time, the binding of DPDPE was not significantly altered. However, the affinity of the amphibian δ-selective peptide deltorphin II was lowered by over 20-fold by the replacement of the δ-D segment. All tested δ-opioid receptor ligands significantly lost their affinities toward chimera δ/κ-F. In fact, the affinity values of most of the δ-opioid receptor ligands toward δ/κ-F were indistinguishable from those toward a wild type κ-opioid receptor.

Table 2 displays the change induced by the replacement of a κ segment with its corresponding δ segment in a largely κ environment. As can be readily seen, only the presence of δ-F region could significantly increase the affinities of the κ-opioid receptor for δ-opioid receptor ligands. The presence of all other δ segments in a κ-opioid receptor did not increase the affinities of most δ-opioid receptor ligands to an experimentally determinable range. Most strikingly, the presence of δ-F in a κ-opioid receptor

Table 3
Pharmacological profile of chimeric μ/δ-opioid receptors (apparent K_i, nM) [a]

	μ/δ-A	δ/μ-B	μ/δ-C	μ/δ-D	μ/δ-E	δ/μ-F	μ/δ-G	μ Wild	δ Wild
Non-selective opioid receptor ligands									
Ethylketocyclazocine	10	6.9	9.8	4.6	9.6	23	7.1	8.6	12
Bremazocine	0.43	0.38	0.99	0.45	0.90	0.45	0.13	0.97	0.83
Naltrexone	0.60	11	0.94	1.4	1.4	1.7	1.2	1.4	31
δ-Opioid receptor ligands									
[Leu^5]enkephalin	100	3.9	120	68	55	390	39	49	2.7
[Met^5]enkephalin	46	2.4	31	18	22	76	8.6	15	1.0
DPDPE	> 10000	22	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	20
JOM13	2000	5.5	1400	1300	780	> 10000	780	900	1.9
Deltorphin II	> 10000	5.9	5500	4600	5000	> 10000	4700	4800	1.8
TIPP	> 10000	2.2	> 10000	> 10000	> 10000	> 10000	> 10000	> 10000	1.1
Naltrindole	14	0.07	26	16	40	30	28	29	0.10
Naltriben	26	0.10	40	18	41	34	38	48	0.12

[a] The K_i values of the chimeric constructs and the wild type receptors toward the same ligand were determined in the same batch of binding assay.

Table 4
Pharmacological profile of δ-opioid receptor mutants (apparent K_i, nM) [a]

	$VI^{(281-2)}$-IL	$R^{(291)}$-P	$R^{(292)}$-E	$RR^{(291-2)}$-PE	$D^{(293)}$-T	$AAL^{(298-300)}$-VSW	δ Wild
Bremazocine	1.8 ± 0.7	1.3 ± 0.1	1.4 ± 0.3	0.9 ± 0.5	1.2 ± 0.6	1.6 ± 0.8	1.5 ± 0.3
[Leu^5]enkephalin	43 ± 5 [d]	87 ± 50	55 ± 25	160 ± 56 [d]	12 ± 6	51 ± 12 [d]	20 ± 9
TIPP	11 ± 7	6.4 ± 2.4	2.4 ± 1.2	6.5 ± 1.9 [d]	1.5 ± 1.0	6.1 ± 2.6	1.8 ± 0.7
Deltorphin II	4.6 ± 1.2	11 ± 3 [d]	3.3 ± 1.0	17 ± 4 [d]	2.2 ± 1.0	13 ± 8	3.2 ± 1.3
Naltrindole	0.57 ± 0.09 [d]	0.19 ± 0.04	0.09 ± 0.04	0.12 ± 0.02	0.15 ± 0.04	0.83 ± 0.13 [d]	0.16 ± 0.03
BWB373	0.44 ± 0.05 [d]	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.00	0.20 ± 0.02	2.8 ± 0.4 [d]	0.16 ± 0.02

[a] Data are expressed as means ± S.D. ($n = 3$). [b] Single letter amino acid residue abbreviations are used to label the δ-opioid receptor mutants. [c] About 1 nM [^{3}H]bremazocine was used as a labeling ligand in the mutagenesis study, because [^{3}H]ethylketocyclazocine does not label the D^{293}-T mutant very well. [d] Indicates significant difference from wild type, $P < 0.05$.

was sufficient to completely restore the affinities of naltrindole and naltriben to the wild type δ-opioid receptor level, although δ-F could not do the same for other δ-opioid receptor ligands tested.

Table 3 demonstrates the effect of the presence or absence of various δ segments in a series of chimeric μ/δ- or δ/μ-opioid receptors. One of the purposes of testing this series of chimeras was to examine the role of various δ segments in different receptor environments (i.e. in κ vs. δ backgrounds), in order to achieve a better understanding of the origin of δ-opioid receptor ligand selectivity. The data suggest that the presence of δ-A, δ-C, δ-D, δ-E and δ-G segments in the μ-opioid receptor did not increase the affinities of δ-opioid receptor ligands toward these receptors. Due to the relatively better affinities of these ligands toward the μ-opioid receptor than the κ-opioid receptor, more data were in the experimentally determinable range. These data agreed very well with the results of Table 2. Again, the replacement of the δ-F segment by μ-F in the δ receptor significantly decreased the affinities of all δ-opioid receptor selective ligands. Interestingly, in contrast to the substitution of δ-B with κ-B, the replacement of the δ-B segment with the μ-B segment did not significantly influence the binding of [Leu^5]enkephalin, [Met^5]enkephalin or DPDPE.

3.3. Pharmacological profile of the δ receptor mutants

Since three families of δ-opioid receptor ligands with differing structures all rely on TM6/EL3 region to achieve their selectivity, we were interested in determining whether a common set of amino acid residues is involved in mediating their binding selectivity. Furthermore, we wanted to learn whether δ-opioid receptor selectivity is achieved through a few residues or a relatively large number of residues. Since it is difficult to predict a priori which specific sites may be critical for physical/chemical interactions between the receptor and the various ligands, we began by mutating several amino acid residues at the interface of extracellular loop/transmembrane domain and in the middle of EL3 in the δ-opioid receptor, and studied the consequences on the binding of δ-opioid receptor selective ligands. Table 4 summarizes the results of the mutagenesis study. Two of the mutants, Val^{281}-Leu^{282} to Ile-Leu (as in κ-opioid receptor) and Ala^{298}-Ala^{299}-Leu^{300} to Val-Ser-Trp (as in μ-opioid receptor), led to a significant decrease in the affinity of many tested δ-opioid receptor ligand ($P < 0.05$). The Arg^{291}-Arg^{292} to Pro-Glu (as in μ-opioid receptor) mutation, appeared to only influence the binding of the three peptide ligands tested, without altering the affinity of the alkaloids. However, none of the mutations produced the magnitude of affinity changes seen in the chimeric receptors.

4. Discussion

The current study with δ-opioid receptor chimeras and mutants demonstrates the following. (1) A region comprising the sixth transmembrane domain (TM6) and the third extracellular loop (EL3) (the F region) is absolutely critical for δ-opioid receptor selectivity. The introduction of this region in the κ-opioid receptor is necessary and sufficient to impart a δ-opioid receptor profile where the small ligands like naltrindole and naltriben are concerned; this region is necessary but *not* sufficient to impart high affinity binding of the endogenous δ-opioid receptor selective peptides. (2) The presence of the κ-D region (the second extracellular loop) in a δ-opioid receptor background reduces the binding affinity of enkephalins and deltorphin II. (3) The κ-B region, consisting of the first and second transmembrane domains and the first extracellular loop, may interfere with the selective binding of leucine enkephalin, methionine enkephalin and DPDPE, whereas the corresponding μ-B region does not produce such an effect. (4) Within the highly discriminating region subsuming TM6 and EL3, there are many residues which appear to participate in producing δ-opioid receptor selectivity, with the peptides apparently relying on a larger number of interactions than do the alkaloids.

It is important to note that while all tested δ-selective ligands strongly rely on TM6/EL3 for selective binding, they also exhibit unique requirements as a function of the chemical class to which they belong. In particular, the selective binding requirements of peptides appear to be more extensive than those of the alkaloids. Whereas nal-

trindole and naltriben rely exclusively on TM6/EL3 for selectivity, the enkephalin peptides show an additional requirement for the second extracellular loop of the δ-opioid receptor to achieve their high affinity binding. This appears to be the case in the chimeric δ/κ-opioid receptors when K_is fell in experimentally derminable range. This is of great interest because the corresponding EL2 of κ-opioid receptor has been shown to be critical for dynorphin A selectivity. It therefore appears that this second loop, which shows a great deal of heterogeneity across the three opioid receptors, plays a key role in ensuring that these receptors discriminate between the naturally occurring enkephalins versus dynorphins. Thus, physiologically, selectivity of the endogenous ligands for the κ- versus δ-opioid receptors may be ensured via a double selection mechanism mediated in part by the EL2's of both receptors.

Our mutagenesis results clearly indicate that although the δ-F region is critical for the binding of all tested δ ligands, different families of δ ligands rely on distinct interaction sites within this domain to achieve their selectivity. The small alkaloid antagonist naltrindole and the small alkaloid agonist (±)-4-[[α-*R*]-α-[[2*S*,5*R*]-4-allyl-2,5-dimethyl-1-piperazinyl]-3-hydroxybenzyl]-*N*,*N*-diethylbenzamide (BWB373) appear to interact with the residues at the interface of EL3/TM6 and EL3/TM7, but neither of them interacts with the residues in the middle of EL3. In contrast, while all three peptide ligands tested interacted to a certain degree with the EL/TM interface residues, the two Arg residues in the middle of EL3 proved to be the most important sites for these larger ligands. These findings are consistent with the idea that small alkaloid ligands may bind deeper into the binding pocket formed by the transmembrane domains than the peptide ligands. It also illustrates the view that peptide ligands may rely on more interaction sites, particularly the residues in the extracellular domains, to achieve higher selectivity than exhibited by the small alkaloid ligands.

It is notable that, for all five ligands tested, the effects seen with any one mutant are never large enough to explain the full magnitude of change seen in the chimeric receptors (Table 4). It seems that either there is a very critical interaction site yet to be uncovered or that they simply interact with a large number of sites on the δ-opioid receptor.

Our findings agree very well with the recent reports by other groups using different constructs. Fukuda et al. (1995) demonstrated that the major binding determinant of DPDPE resided in the δ-opioid receptor region spanning form TM5 to TM7. Wang et al. (1995) also located the region responsible for the selective binding of [D-Ser^2,D-Leu^3]enkephalin-Thr (DSLET) and naltrindole to a region containing δ-F in a stepwise domain replacement experiment. Furthermore, Wang et al. (1995) also discovered that the same two Arg residues in the EL3 may largely responsible for the selective binding of DSLET. These results emphasize the idea that although the δ-F segment is mainly responsible for the selectivity of many δ-opioid receptor ligands, different sets of amino acid residues in this small region are responsible for the selectivity of different δ ligands.

It is difficult at this stage to propose a three dimensional scheme for how the various residues on multiple extracellular, and possibly intracellular, domains of the δ-opioid receptor participate in ensuring binding selectivity. Based on the importance of EL2, and the high degree of sequence heterogeneity between the extracellular loops, as well as on the mutagenesis findings reported here, it is reasonable to implicate these loops in ligand selectivity. One possibility is that a pocket is formed near the outer surface of the receptor, involving EL3 and the top of TM6, EL2, and possibly EL and adjacent sequences. In the δ-opioid receptor, this pocket would be critical for interacting with enkephalins and their analogs; one aspect of it, the EL3/TM6 stretch would be primarily responsible for interacting with the small ligands. Clearly, additional experimental work is needed to determine if such a 'pocket' is the actual binding site or a sorting site defining selectivity. However, evidence does exist suggesting that peptide agonist binding reaches relatively deeply within the pocket formed by the transmembrane domains. Mutation of the Asp in TM2 of the δ-opioid receptor dramatically reduces agonist binding (Kong et al., 1993); in addition, work from our group has shown that residues deep in TM6 and TM7 of the μ-opioid receptor are critical for the binding of a large number of ligands including peptides (Mansour et al., unpublished observations). Similarly, a tyrosine residue in TM2 of the endothelin receptor subtype A is a critical component for the subtype-selective binding of peptide agonists (Krystek et al., 1994). Such evidence would lead us to actively consider the view that the primary site of agonist binding may lie within the barrel formed by the transmembrane domains, but that the regions described in this study may be critical for interactions which orient the appropriate ligands correctly for the deeper interactions, while 'rejecting' the inappropriate ligands.

This view, at first blush, fits very well with Schwyzer's message/address theory (Schwyzer, 1977); one would propose that the extracellular loops would act as the readers of the addresses, while the message is delivered deeper within the receptor. However, we would suggest that the theory needs some modification if it is to fully accommodate the data. A direct application of the theory would lead us to expect that the carboxyl-terminal leucine residue in [Leu^5]enkephalin or the equivalent methionine residue in [Met^5]enkephalin would be solely responsible for the selectivity of the enkephalins toward the δ-opioid receptor, while Tyr-Gly-Gly-Phe would contribute in a consistent manner to the binding of all endogenous opioids across the three opioid receptors. As a result, we would expect that receptor regions responsible for enkephalin selectivity should be fairly limited. In other words, if we assume that [Leu^5]enkephalin or [Met^5]enkephalin adopt a

particular conformation upon binding, it is not possible for the single C-terminal residue to interact with as many disparate regions as was uncovered by our series of chimeric δ/κ-opioid receptors. Thus, a modification of the original message/address hypothesis may be needed. One possibility is that the definition of address within the enkephalins is too limited, and that more than the C-terminal methionine or leucine defines the address. For instance, the enkephalin pentapeptides may have a unique conformation, distinctive from the conformations of C-terminally more extended peptides; this unique conformation would itself help in discrimination. Thus the 'message' and the 'address' would be interwoven. Interestingly, this idea bears a certain resemblance to the modified message/address concept proposed by Portoghese and colleagues from a different perspective (Portoghese, 1993). They suggested that the Phe residue in the opioid consensus sequence is also part of 'address' which contributes to ligand selectivity. It is conceivable that even more of the opioid core actually contributes to selectivity as well as to binding affinity of the enkephalins. Alternatively, one would propose that a ligand can adopt multiple conformations upon binding to a receptor, and may fit at multiple interaction sites in a receptor; thus a single residue of the peptide ligand may interact with several different and possibly distant regions of a receptor. This would lead to a dynamic view of receptor-ligand interactions, with multiple configurations of the flexible peptide ligands interacting with multiple sites on the receptor, thereby achieving selectivity through multiple mechanisms. Such a view would also give us new ways to explain differences between full agonists and partial agonists; in this view, full agonists could interact at multiple sites to bring about the allosteric changes needed for full efficacy in signal transduction (e.g. alterations in G-protein coupling). On the other hand, partial agonists may only access some of the sites and not be as efficacious.

The present findings also help us to address an apparent discrepancy between previous functional studies of the three opioid receptors, and the results of molecular cloning. It is well established from pharmacological studies that the binding profiles of the μ- and the δ-opioid receptors are relatively similar to each other, while the profile of the κ-opioid receptor is unique. However, the primary sequences of the opioid receptors exhibit an equal level of overall homology among themselves. It was therefore of interest to determine the structural elements that are responsible for the similarity between the μ- and the δ-opioid receptors, in contradistinction to the κ-opioid receptor. Our results indicate that although both κ-F and μ-F regions are detrimental to the binding of δ-opioid receptor ligands, the κ-B and κ-D regions exhibited additional selection against [Leu^5]enkephalin, [Met^5]enkephalin, DPDPE or deltorphin II while the μ-B and μ-D regions did not interfere with the binding of these δ-opioid receptor selective ligands. In addition, the first mutant in Table 4 (a δ to κ mutation) was effective in lowering the affinity of several δ-opioid receptor ligands (naltrindole, BWB373 and [Leu^5]enkephalin); since this site is identical across the δ- and μ-opioid receptors, but different in the κ-opioid receptor, this points to a unique site that the κ-, but not the μ-opioid receptor, can use to discriminate against δ-opioid receptor ligands. Therefore, the similarity in the structural properties of the B, D regions and even within the F region may partly account for the closeness of the δ- and the μ-opioid receptors in pharmacological profile.

Finally, the present findings may suggest novel strategies for the development of new families of specific opioid ligands. Since all the current δ-opioid receptor ligands achieve their selectivity mainly through their interactions with the TM6 and/or EL3 domains, it is conceivable that once we learn the orientation of the ligands in the δ-opioid receptor, one can devise new selective ligands designed to take advantage of unique interactions with other regions of the δ-opioid receptor which exhibit low levels of homology to the other two receptor types.

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