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Isosteric Replacement of Acidic with Neutral Residues in Extracellular Loop-2 of the κ-Opioid Receptor Does Not Affect Dynorphin A(1−13) Affinity and Function

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Chimeric receptor studies have suggested that the binding and selectivity of the endogenous opioid peptide, dynorphin A, arise from the interaction of its "address" with the second extracellular loop (EL-2) of the κ-opioid receptor.[1,2] For example, μ/κ-chimera, with only EL-2 of the κ-receptor, have been shown to bind dynorphin A with the same affinity as the wild-type κ-receptor. Attempts to explain this phenomenon have centered mainly on the presence of negatively charged residues within EL-2 of the κ-receptor. It has become generally accepted that key basic residues (Arg^6, Arg^7, Arg^9) in the dynorphin A "address" recognize the EL-2 acidic residues through ionic interactions.[3] Recent modeling studies, however, have suggested the charges in the "address" may not form direct salt links.[4] Based on a sequence analysis of the κ-receptor and an experimental NMR structure of dynorphin A,[5] a structural model was developed in which helical segments of EL-2 and the dynorphin A "address" interact through hydrophobic contacts at the helix−helix interface. The primary recognition points for dynorphin A binding were tentatively explained on the basis of the hydrophobic effect, and not through direct interaction of counterions. A schematic of EL-2 showing the negatively charged residues and the putative site of interaction is shown in Figure 1.

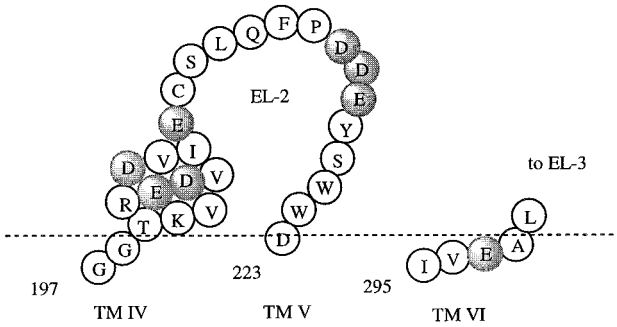

Figure 1. Schematic diagram of EL-2 of the κ-opioid receptor showing the putative helical segment. Charged residues are shown in gray; the dashed line represents the TM boundary.

To experimentally evaluate the role played by the anionic residues of EL-2 in the binding and selectivity of dynorphin A, we have created a series of mutants in which the clustered negative charges in this domain have been neutralized. All substitutions were isosteric so as to minimize the steric effects of mutation. The point mutations were introduced in the κ-opioid receptor by polymerase chain reaction (QuikChange Site Directed Mutagenesis Kit, Stratagene) and confirmed by DNA sequencing. Charge neutralizations were performed sequentially by first creating the triple-point mutant, E203QD204ND206N, with subsequent modifications to contain four (E203QD204ND206NE209Q) and finally seven (E203QD204ND206NE209QD216ND217NE218Q) point mutations. To further evaluate the effect of charged residues at alternate positions on EL-2, two more mutants were also created, D216ND217NE218Q and E203QD204ND206ND216ND217NE218Q. The mutants were transiently expressed in HEK cells and prepared for competitive binding experiments using [^{3}H]diprenorphine (Supporting Information). Attempts to express the six- and seven-point mutants, however, failed suggesting that a residual charge on EL-2 is required to maintain structural integrity (perhaps through charge repulsion).

As shown in Table 1, the remaining three mutants showed K_i values comparable to those of the wild-type κ-receptor. While a slight decrease in affinity (∼4-fold)

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Table 1. Binding and Functional Data for κ-EL-2 Mutants Expressed in HEK Cells[a]

	[3H]dipre-norphine	dynorphin A(1−13)		U-69,593
mutant	K_D (pM)	K_i[b] (nM)	EC_{50}[c] (nM)	K_i[b] (nM)
wild-type	29 ± 1.7	0.8 ± 0.12	18 ± 5.6	1.4 ± 0.4
E203QD204ND206N	50 ± 5.1	0.6 ± 0.3	6 ± 1	2.4 ± 1.1
E203QD204ND206N-E209Q	38 ± 4.1	1.1 ± 0.51	ND[d]	3.0 ± 2.1
D216ND217NE218Q[e]	50 ± 6.4	3.4 ± 2.0	8 ± 5	2.0 ± 1.1

[a] Values expressed as mean ± standard error from at least three independent determinations in triplicate. [b] Determined in competition experiments with [3H]diprenorphine. [c] Determined in [35S]GTPγS assay with dynorphin A(1−13) stimulation. [d] Not determined due to low expression. [e] This mutant was also prepared by T. Reisine who found it to bind dynorphin A with affinity similar to that of the wild-type receptor (personal communication).

is noted for the triple-point mutant, D216ND217NE218Q, the significance of this result is unclear. It is important to point out that all three mutants (including wild-type) bind dynorphin A(1−13) with nanomolar affinity. As a further test of charge neutralization, the experiments were repeated using the non-peptide κ-agonist U-69,593.[6] Once again, no significant difference in binding affinity was noted between the wild-type and mutant receptors. This is not surprising, however, since this relatively small agonist is thought to bind within the receptor cavity.[7]

Functional experiments were also performed using the [35S]GTPγS assay[8] to evaluate the agonist properties of dynorphin A(1−13) at the mutant receptors (Table 1). Although expression of the D216ND217NE218Q mutant was high enough to perform competitive binding experiments, the level was too low to adequately determine its EC_{50} value. However, the two remaining mutants possessed EC_{50} values that were similar to that of dynorphin A(1−13) stimulation at the wild-type receptor. The maximal degree of stimulation was also similar suggesting that charge neutralization in the putative binding domain of dynorphin A in EL-2 does not substantially alter function.

Taken overall, the data suggests that charge−charge interactions between EL-2 and the dynorphin "address" may not be as important as generally believed in determining the selectivity and binding affinity of dynorphin A to the κ-opioid receptor. This runs counter to the common implication that the greater negative charge on EL-2 of the κ-receptor (as compared to μ and δ) is the primary determinant in dynorphin A recognition, raising new questions regarding the role of EL-2 or other structural domains in selective binding and activation. It also does not appear these charges are involved in binding non-peptide κ-agonists (albeit our results are limited). A previous modeling study[4] has suggested dynorphin A recognition may be driven more by hydrophobic interactions at EL-2 than by ion pairing. In this case, the charges of EL-2 may function as a screening device, allowing the positively charged peptide to dock more efficiently to the κ-receptor. This may, in part, explain the limited effect noted upon charge neutralization. It has also been proposed in a previous report that the extracellular loops may mediate selectivity through an exclusionary mechanism.[9] We should point out, however, that the mutants reported in this study do not show an increased affinity toward μ- or δ-selective peptides (such as DAMGO, β-endorphin, and Leu-enkephalin),[10] suggesting the charges of EL-2 are not involved in screening μ- or δ-ligands.

An alternative, more provocative explanation for the retention of binding and function may involve the nonconserved Glu^{297} residue in the κ-receptor that has been associated with the binding and selectivity of the κ-antagonist, norbinaltorphimine (nor-BNI).[11] This residue occupies a position at the top of helix VI (near the EL-3−transmembrane domain interface) that is opposite to the putative EL-2 binding site. All mutants expressed here contain this key site and, as expected, bind nor-BNI with high affinity.[10] Recently, this residue has been shown to play a role in the binding of dynorphin A as well.[12] This potential contact may therefore represent a secondary recognition site whose presence becomes amplified in the absence of charged residues on EL-2. Such a mechanism may explain the failure of charge neutralization to greatly diminish dynorphin A(1−13) binding and further suggests potential sites to exploit in the design of dynorphin A analogues.

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Supporting Information Available: Experimental Section. This material is available free of charge via the Internet at http://pubs.acs.org.

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