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Radioligand-Dependent Discrepancy in Agonist Affinities Enhanced by Mutations in the κ-Opioid Receptor

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SUMMARY

A series of κ/μ receptor chimeras and a number of κ receptors substituted in the second transmembrane segment (TM-II) were investigated using as radioligands, respectively, the κ-selective agonist [^{3}H]CI977 and the nonselective opioid antagonist [^{3}H]diprenorphine (DIP). All of the receptor constructs bound [^{3}H]DIP with similar and high affinity, whereas the apparent affinity of the nonpeptide agonist CI977, when estimated in competition binding with the antagonist [^{3}H]DIP, was impaired between 42- and >500-fold in the κ/μ chimeras and between 64- and 153-fold in three of the κ receptor mutants that had been substituted in the TM-II segment. However, homologous competition binding experiments, using [^{3}H]CI977 as radioligand, showed that the high affinity binding of this nonpeptide agonist was in fact not impaired in four of the κ/μ chimeras and in three TM-II substituted κ receptors compared with the wild-type κ receptor. In all cases in which mutations decreased the apparent affinity of CI977 without affecting its actual affinity, as determined in homologous assays using [^{3}H]CI977, the calculated number of receptor sites (B_{max}) was decreased. In three of the κ/μ constructs, binding of [^{3}H]CI977 was undetectable, indicating that in these chimeras the affinity of the nonpeptide agonist had actually been affected. Also, for the κ-selective peptide agonist dynorphin A(1–8), the measured affinity for the receptor mutants was strongly dependent on whether it was determined using the antagonist [^{3}H]DIP or the agonist [^{3}H]CI977 in that ≤800-fold higher K_i values were determined in competition with the antagonist. It is concluded that mutations in the κ-opioid receptor can cause large discrepancies between the affinity determined for agonists in homologous versus heterologous competition binding assays and that this pattern, which is compatible with a partial uncoupling of receptors, is observed in surprisingly many types of receptor mutations.

The opioid receptors (μ, κ, and δ are the primary members) form a distinct subfamily within the G protein-coupled family of receptors (1–4). In addition to the natural peptide agonists and the nonpeptide antagonists that have been developed in recent years for a number of 7-TM peptide receptors, the opioid system features several nonpeptide agonists, of which morphine represents the prototype (5). Highly selective nonpeptide ligands have been developed, particularly for the κ-opioid receptor. Examples are the nonpeptide antagonist nor-BNI (6, 7) and the structurally dissimilar acrylacetamide agonist compounds U69,593 [(5α,7α,8β)-(−)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide] and CI977 {(5*R*)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro (4,5)dec-8-yl]4-benzofuranacetamidemonohydrochloride} (8–10).

Mutational studies, particularly through chimeric analysis, of the μ-, κ-, and δ-opioid receptors suggest that there are distinct molecular interactions for each of the ligand categories of nonpeptide agonists, nonpeptide antagonists, and peptide agonists (11–19). For example, in the κ receptor, the exchange of a segment including the EC loop 3 and TM-VII were shown to affect the high affinity binding of the κ-selective antagonist nor-BNI, whereas the ability to bind the nonselective compounds naloxone and DIP or nonpeptide agonists was unaffected (15, 18). For the peptide agonist dynA(1–8), EC loop 2 has been hypothesized to contribute to the κ-selective binding profile of this peptide (12, 18). Thus, in analogy to findings in other 7-TM peptide receptor systems (e.g., tachykinin and angiotensin receptors), the essential receptor epitopes for peptide agonists and nonpeptide antagonists seem to be (at least partially) nonoverlapping (20–24).

Mutational mapping of ligand binding sites is, however, a difficult task, especially when using binding assays in which the radioligand and the unlabeled competitor are dissimilar. Thus, in the tachykinin neurokinin NK1 receptor system, it was recently found that a series of mutations located on a presumed inward-facing patch on TM-II seriously impaired the binding affinity of the agonist, Substance P, as determined in a heterologous competition binding assay with radiolabeled nonpeptide antagonist. However, the mutations had only a minor or even no effect on the actual high affinity

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ABBREVIATIONS: TM, transmembrane; EC, extracellular; nor-BNI, nor-binaltorphimine; DIP, diprenorphine; dynA(1–8), dynorphin A(1–8).

binding of the peptide as determined in a homologous binding assay using instead the peptide agonist, Substance P, as radioligand (25). In fact, the results from the homologous binding experiments were in good agreement with signal transduction experiments, which demonstrated that several receptor mutants could be activated by Substance P stimulation and that the EC_{50} observed was only slightly affected compared with the wild-type receptor (26). It was proposed that these mutations in the TM-II segment had impaired the ability of the receptor to interchange between receptor conformations that each bind agonist and antagonist radioligands with high affinity. In some systems (e.g., the tachykinin NK2 receptor), it is possible even with the wild-type receptor to observe a >100-fold difference between the high affinity binding of the radiolabeled peptide agonist itself and the binding of the peptide as observed in heterologous competition with a radiolabeled nonpeptide antagonist (25, 26). Thus, the choice of radioligand can affect the measured affinity dramatically in the wild-type receptors but, importantly, to an even greater degree in certain mutants.

In the current study, we reinvestigated a series of chimeric κ/μ-opioid receptor constructs that bind the antagonist [^{3}H]DIP with similar and high affinity (15); in this study, we also used the radiolabeled nonpeptide agonist [^{3}H]CI977. In addition, we tested a series of κ receptor mutants with point substitutions in TM-II corresponding to positions that had been studied in the NK1 receptor (25). The apparent binding affinity [not only for the nonpeptide agonist CI977 but also for the peptide agonist dynA(1–8)] was found to be critically dependent on the choice of radioligand.

Materials and Methods

Construction of mutant opiate receptor genes. The receptor chimeras were synthesized by the polymerase chain reaction overlap extension technique (27) using as templates the wild-type rat κ- and μ-opiate receptor cDNAs (15, 24). The points of chimeric exchange in the mutant receptor constructs were placed immediately after the following amino acid positions in the κ receptor: κμ1, Ile61; μκ3, Tyr119; μκ5 and κμ5, Thr199; and μκ6 and κμ6, Leu251. The chimera κμκ was constructed through the introduction of a *Bgl*II/*Mlu*I fragment of the μ wild-type receptor into the κ receptor (15). The structure of the mutant receptor cDNAs was verified by DNA sequencing of the polymerase chain reaction-generated fragments (Sequenase; United States Biochemical, Cleveland, OH).

Expression of receptor cDNAs in COS cells. COS-7 cells were grown in Dulbecco's modified Eagle's medium 1885 supplemented with 10% fetal calf serum, 1% glutamine, 1% sodium-pyruvate, 1% nonessential amino acids, and 0.1% gentamycin. The wild-type κ and μ receptors and the mutant constructs were transfected transiently into COS-7 cells by the calcium-phosphate precipitation method (28).

Ligands. The nonselective antagonist [^{3}H]DIP (specific activity, 30 Ci/mmol; TRK.730) and [^{3}H]CI977 (specific activity, 43 Ci/mmol; TRK.945) were obtained from Amersham International (Buckinghamshire, UK). Nor-BNI (no. B-109) and dynA(1–8) (no. D-164) were purchased from Research Biochemicals (Natick, MA). CI977 was kindly provided by Dr. M. Pritchard (Parke-Davis, Cambridge, UK).

Binding experiments. Radioligand binding assays were performed using intact cells. At 1 day after transfection, the cells were seeded in 12–24-well plates at a density of 1–10 × 10^4 cells/well. The competition binding studies were carried out 24 hr thereafter using as radioligand either the antagonist [^{3}H]DIP (250 pM) or the agonist [^{3}H]CI977 (200 pM). Unspecific binding was determined in the presence of 1 μM (−)naloxone. The binding was performed at 4° for 16 hr in 0.5 ml of 25 mM Tris·HCl, pH 7.4, with 5 mM $MgCl_2$, 0.1% bovine serum albumin, and 100 μg/ml bacitracin (15). All determinations were done in triplicate. Binding data were analyzed using the computer program Inplot 4.0 (GraphPAD, San Diego, CA).

Results

The chimeric κ/μ receptor constructs used in the current report were previously described in an analysis aimed specifically at the identification of epitopes important for the binding of the κ-selective antagonist nor-BNI (15). In addition, four point mutated κ receptors were analyzed. These constructs were substituted in and around TM-II at positions that have previously been shown in the tachykinin NK1 receptor to affect the ability of agonist to compete for antagonist binding without affecting the actual high affinity binding of agonist (25).

Binding analysis of chimeric κ/μ constructs. The nonselective antagonist DIP was chosen to determine expression and general structural integrity of the receptor constructs when expressed transiently in COS-7 cells. DIP binds with high affinity and in a nonselective manner to the κ and the μ wild-type opioid receptors (κ, K_d = 0.21 nM; μ, K_d = 0.18 nM) and to the entire set of chimeras (Table 1). The K_d values ranged from 0.18 to 1.45 nM, indicating that these constructs were suitable for the investigation of binding properties of subtype-selective ligands (Table 1).

For the wild-type κ receptor, similar B_{max} values were obtained using either of the two radioligands: 157 fmol/10^5 cells with [^{3}H]DIP and 130 fmol/10^5 cells with [^{3}H]CI977. However, for chimeras that could be analyzed with both radioligands, the B_{max} values were consistently higher for the antagonist radiolabel than for the agonist (Tables 1 and 2).

Heterologous competition binding analysis was performed with the nonpeptide agonist CI977, using the antagonist [^{3}H]DIP as radioligand (Table 1). In this analysis, high affinity binding of CI977 was seemingly impaired in all of the receptor chimeras as reflected by the 42- to >500-fold reduction in K_i values compared with the wild-type κ receptor (Table 1, *left*).

Despite the low apparent affinity for CI977, as determined in the heterologous binding experiment with the antagonist [^{3}H]DIP, the constructs were also studied with [^{3}H]CI977 used as radioligand. For the wild-type κ receptor, high affinity CI977 binding was seen regardless of the choice of radiolabel, although the affinity estimated by homologous binding analysis ([^{3}H]CI977/CI977) yielded a 4-fold lower value compared with the affinity estimated in the heterologous binding analysis (K_d = 0.70 nM, K_i = 2.78 nM; Table 1). Surprisingly, in this analysis, five of the chimeric constructs (Table 1, *top right*) bound the [^{3}H]CI977 compound well. Thus, the homologous binding analysis demonstrated that the affinity for CI977 was in fact similar to that of the wild-type κ receptor (F_{mut} = 1.7–3.5). Therefore, although the ability of the agonist CI977 to compete for [^{3}H]DIP binding was clearly impaired, the high affinity binding of CI977 itself was essentially unaffected in these chimeric constructs. The number of receptors binding the nonpeptide agonist was, however, reduced (B_{max} in Table 2).

Three of the chimeric κ/μ receptor constructs (κμ1, μκ5, μκ6) did not show detectable specific binding of the [^{3}H]CI977 radioligand (Table 1). These constructs and the

TABLE 1

Binding affinities for the nonpeptide agonist C1977 using either antagonist [^{3}H]DIP or agonist [^{3}H]C1977 as radioligand

K_d, K_i, B_{max}, and Hill coefficient (shown in parentheses) values were calculated as described in Experimental Procedures. Mean ± standard error values from n independent experiments are shown for the wild-type κ receptor and a series of chimeric κ/μ constructs expressed transiently in COS-7 cells. The effect of mutation on the binding affinity for the C1977 agonist ligand is expressed for each of the chimeric constructs according to: F_{mut} = K_i (mutant)/K_i (wild-type) or, for the homologous competition experiments, F_{mut} = K_d (mutant)/K_d (wild-type). The difference between the apparent affinity and the actual affinity obtained using either antagonist or agonist radioligand is expressed by the K_i/K_d ratio for CI977, which is calculated according to: K_i ([^{3}H]DIP)/K_d([^{3}H]CI977). NB, no binding; ND, not determined.

Receptor	Antagonist radioligand: [^{3}H]DIP					Agonist radioligand: [^{3}H]CI977					K_i ratio for C1977
	B_{max}	DIP		CI977		B_{max}	DIP		CI977		
		K_d (n)	F_{mut}	K_i (n)	F_{mut}		K_i (n)	F_{mut}	K_d (n)	F_{mut}	
	fmol/10^5 cells	*nM*		*nM*		*fmol/10^5 cells*	*nM*		*nM*		
κ-wt	157 ± 26	0.21 ± 0.03 (15) (−1.04 ± 0.03)	=1.0	2.78 ± 0.42 (10) (−0.82 ± 0.06)	=1.0	130 ± 22	0.23 ± 0.02 (12) (−1.26 ± 0.07)	=1.0	0.70 ± 0.10 (16) (−0.98 ± 0.06)	=1.0	4
κμ6	626 ± 44	0.39 ± 0.12 (4) (−1.23 ± 0.13)	1.8	121 ± 41 (3) (−0.65 ± 0.17)	42	110 ± 49	0.79 ± 0.29 (3) (−1.33 ± 0.03)	3.4	1.79 ± 0.33 (3) (−0.90 ± 0.08)	2.3	70
κμκ	487 ± 125	0.39 ± 0.10 (5) (−1.18 ± 0.14)	1.6	139 ± 18 (4) (−0.91 ± 0.06)	50	37 ± 10	1.38 ± 0.39 (3) (−0.95 ± 0.04)	6.0	1.29 ± 0.35 (5) (−0.76 ± 0.08)	1.7	180
κμ5	419 ± 256	0.90 ± 0.55 (3) (−1.45 ± 0.29)	4.3	455 ± 100 (8) (−0.78 ± 0.08)	169	13 ± 2	0.34 ± 0.15 (3) (−1.35 ± 0.10)	1.5	2.68 ± 0.54 (7) (−0.86 ± 0.06)	3.5	170
μκ3	109 ± 47	1.44 ± 0.23 (3) (−1.07 ± 0.03)	6.8	183 ± 48 (4) (−0.77 ± 0.13)	63	7 ± 1	0.29 ± 0.11 (3) (−0.88 ± 0.07)	1.3	1.16 ± 0.20 (5) (−0.73 ± 0.08)	1.5	160
κμ1	190 ± 27	0.26 ± 0.13 (3) (−1.21 ± 0.05)	1.2	>1000 (6)	>500		ND		NB		
μκ5	58 ± 15	0.54 ± 0.11 (6) (−1.07 ± 0.09)	2.6	796 ± 263 (5) (−0.90 ± 0.09)	275		ND		NB		
μκ6	53 ± 8	1.45 ± 0.24 (7) (−1.02 ± 0.08)	6.5	627 ± 240 (4) (−0.96 ± 0.25)	215		ND		NB		
μ-wt	110 ± 21	0.18 ± 0.02 (7) (−1.16 ± 0.06)	0.9	>1000 (5)	>500		ND		NB		

TABLE 2

Radioligand binding analysis for the nonpeptide agonist CI977 in the wild-type κ receptor and receptor mutants with point substitutions in TM-II

The effect on binding affinity is expressed for each of the chimeric constructs relative to the affinity of the compound for the wild-type receptor in the F_{mut} factor, and the difference in affinity estimated using either agonist or antagonist radioligand is expressed by the K_i/K_d ratio for CI977 (as defined in Table 1). Values in parentheses are Hill coefficients.

	Antagonist radioligand: [^{3}H]DIP				Agonist radioligand: [^{3}H]CI977				K_i/K_d ratio for CI977
	B_{max}	DIP	CI977	F_{mut}	B_{max}	DIP	CI977	F_{mut}	
	fmol/10^5 cells	*K_d nM (n)*	*K_i nM (n)*		*fmol/10^5 cells*	*K_d nM (n)*	*K_i nM (n)*		
Wild-type κ	157 ± 26	0.21 ± 0.03 (14)	2.78 ± 0.42 (10)	=1.0	130 ± 22	0.24 ± 0.03 (12)	0.70 ± 0.12 (16)	=1.0	4
		(−1.05 ± 0.03)	(−0.82 ± 0.06)			(−1.26 ± 0.07)	(−0.97 ± 0.06)		
[S123A]κ	14 ± 4	0.24 ± 0.04 (6)	3.75 ± 0.96 (5)	1.3	8 ± 2	0.12 ± 0.01 (6)	0.49 ± 0.11 (6)	0.6	8
		(−0.90 ± 0.10)	(−0.99 ± 0.12)			(−0.96 ± 0.03)	(−0.83 ± 0.06)		
[Y119A]κ	356 ± 110	0.49 ± 0.14 (5)	178 ± 74 (3)	64	63 ± 19	0.30 ± 0.05 (5)	1.49 ± 0.61 (4)	1.9	119
		(−1.15 ± 0.06)	(−0.83 ± 0.16)			(−1.16 ± 0.13)	(−0.87 ± 0.08)		
[T109A]κ	292 ± 78	0.24 ± 0.07 (7)	191 ± 42 (4)	80	54 ± 13	0.52 ± 0.08 (6)	0.91 ± 0.12 (4)	1.2	245
		(−0.99 ± 0.07)	(−0.68 ± 0.12)			(−1.13 ± 0.05)	(−0.86 ± 0.05)		
[D105N]κ	552 ± 103	0.16 ± 0.04 (7)	510 ± 298 (3)	153	24 ± 3.8	0.46 ± 0.10 (6)	0.33 ± 0.06 (6)	0.4	1300
		(−1.01 ± 0.03)	(−0.88 ± 0.07)			(−1.17 ± 0.09)	(−0.82 ± 0.08)		

wild-type μ receptor therefore did not exhibit high affinity binding of the agonist.

Binding analysis of TM-II κ receptor mutants. A series of residues that were presumed to be positioned on an inward face of the κ receptor TM-II segment (Asp105, Thr109, Tyr119) and one residue positioned immediately above TM-II (Ser123) were exchanged with asparagine or alanine (Table 2). Substitution of the Asp105 position with alanine yielded a construct that exhibited a prohibitively low level of expression; thus, the alternative exchange (D105N) was chosen for further analysis.

For the three receptor mutants (Asp105, Thr109, and Tyr119), the apparent affinity for CI977 obtained with the [^{3}H]DIP radioligand was reduced by 64–153-fold relative to the wild-type κ receptor affinity, whereas the actual affinity as determined in the homologous binding assay using [^{3}H]CI977 was essentially unaffected (Table 2 and mutant D105N in Fig. 1). Also, in these constructs, the B_{max} value was consistently lower (24–63 fmol/10^5 cells) for the agonist radioligand than for the antagonist radioligand (292–552 fmol/10^5 cells) (Table 2).

The construct S123A showed a binding pattern for CI977 similar to that observed in the wild-type κ receptor using either radioligand.

Binding analysis of κ-selective peptide agonist dynA(1–8). In the wild-type κ receptor, dynA(1–8) bound with similar high affinity independent of whether the radiolabeled DIP or CI977 was used (K_i = 0.37 and 0.41 nM, respectively; Table 3). In contrast, in the four chimeric κ/μ constructs in which binding could be assessed using either radioligand, the affinity of dynA(1–8) seemed to be markedly lower when determined with the radiolabeled antagonist [^{3}H]DIP than when determined with the nonpeptide agonist [^{3}H]CI977 (Table 3). In one of the constructs, κμ5, an 810-fold difference in affinities was observed. Interestingly, in this construct, the affinity for the peptide dynA(1–8) was even considerably lower than that seen for the wild-type μ receptor (K_i = 2021 versus 24.9 nM, respectively), indicating the presence of some form of structural incompatibility between the κ and μ receptor segments in this chimera. Notably, this chimeric effect for dynA(1–8) was only obvious in competition with radiolabeled [^{3}H]DIP (Table 3).

In the mutant κ receptors substituted in the TM-II segment, the affinity of dynA(1–8) was 4–36-fold lower when determined with [^{3}H]DIP than with the radiolabeled CI977 [K_i(DIP)/K_i(CI977) ratio; Table 3].

Discussion

In the wild-type κ-opioid receptor, the affinity of the nonpeptide agonist CI977 is 3–4 fold lower when determined in heterologous competition binding assays against the antagonist [^{3}H]DIP than when determined in homologous binding assays against [^{3}H]CI977. This discrepancy between apparent and actual affinity for the nonpeptide agonist, as defined in heterologous versus homologous competition binding assays, is increased to several hundredfold by a number of receptor mutants. The underestimation of agonist affinity is not restricted to the nonpeptide agonist in that the affinity of the peptide agonist dynA(1–8) in these receptor mutants was found to be ≤800-fold lower when measured in competition with the radiolabeled antagonist than against the radiola-

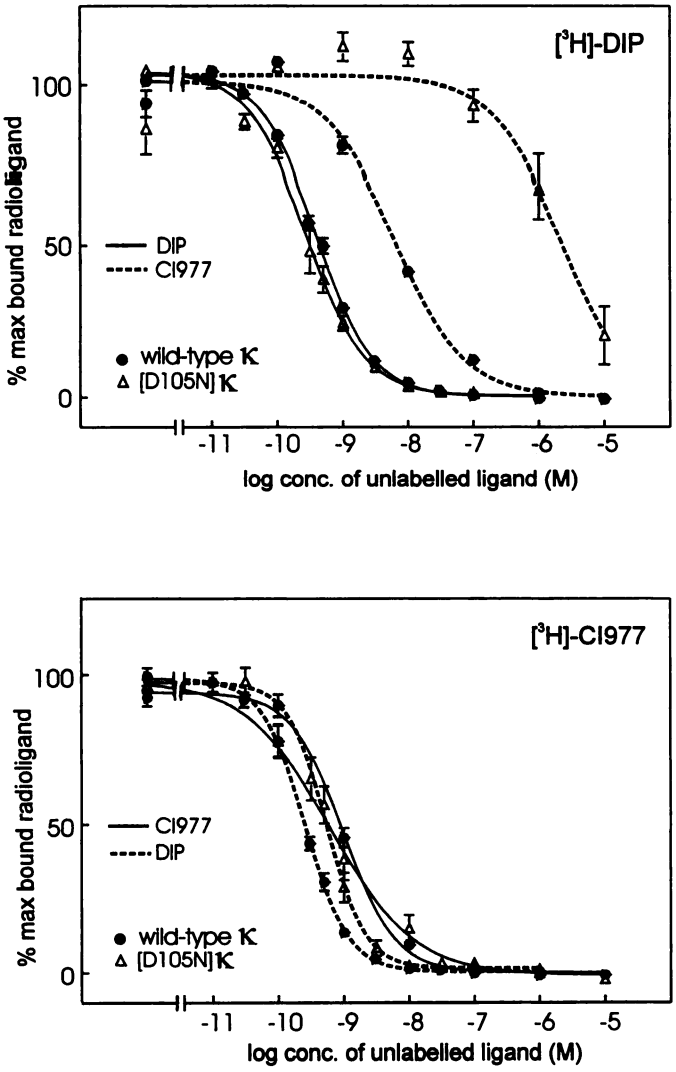

Fig. 1. Competition binding analysis of the nonpeptide agonist CI977 for the wild-type κ receptor and the [D105N] κ receptor mutant. The antagonist [^{3}H]DIP (A) and the agonist [^{3}H]CI977 (B) were used as radioligands in competition binding analysis of the agonist CI977 to transiently transfected COS-7 cells. *Unbroken lines*, homologous binding analysis ([^{3}H]DIP/DIP or [^{3}H]CI977/CI977). *Dotted lines*, results from heterologous binding analysis. Data represent at least three independent experiments and are presented as percentage of maximally specifically bound radioligand. Binding affinities are listed in Table 2.

beled agonist. These observations extend previous findings in the tachykinin NK1 receptor, in which mutations on a presumed inward face of TM-II impaired the apparent affinity of the agonist Substance P by >1000-fold as determined in heterologous competition with nonpeptide antagonists (25, 26). However, in fact, the mutant NK1 receptors bound Substance P with almost wild-type affinity when measured in homologous competition binding assays (26). The current study shows that this phenomenon (that mutations may dramatically increase the discrepancy between the apparent and the actual affinity for agonists) could be more general than expected. Thus, the phenomenon is observed here in TM-II mutants in a different receptor system, and importantly, it is also observed in relation to other types of mutants, in this case a number of chimeric constructs. Recently, we made similar observations in connection with point mutations located in the TM-VII segment of the κ receptor.[1]

How can mutations affect the apparent and not the actual affinity for an agonist? Receptors seem to interconvert between several conformations for which ligands do not have identical affinity. It is, for example, well known that agonists will bind with higher affinity to G protein-coupled conformations of the receptor than to G protein-uncoupled conformations (29–31). For peptide agonists (e.g., tachykinins), the difference in affinity can be considerable (60–100-fold) (32, 33). In contrast, it has been known for years that antagonists in the monoamine systems in general bind with similar affinity to the G protein-coupled and -uncoupled forms of these receptors (32–37). Thus, agonists and antagonists seem to differ in their preference for binding to distinct populations of receptor conformations.

The κ-opioid receptor mutations of the current study affected the apparent affinity of not only the nonpeptide agonist CI977 but also the peptide agonist dynA(1–8), as determined in competition with the antagonist DIP while barely affecting the affinity of the peptide as determined in competition with radiolabeled CI977. Because dynA(1–8) and CI977 are chemically very distinct, the pharmacological homology seen here seems to overrule the chemical heterology between the two ligands and suggests that G protein coupling has been affected by the mutations.

Importantly, we have not observed a similar phenomenon with antagonists (i.e., a large discrepancy between affinity determined in heterologous versus homologous binding assays). A slight tendency toward higher affinity has also been observed for antagonists in homologous binding assays (e.g., in the tachykinin wild-type receptors) (25), yet even in the mutant receptors, the difference is rather small, and similar affinities are generally estimated for antagonists regardless of the nature of the competition analysis.

Are the receptors uncoupled from G proteins by the mutations? The facts that the mutations affect specifically the apparent affinity of agonists as opposed to antagonists and that agonists of distinct chemical structures are affected suggest that an effect on G protein coupling is involved. This notion is also supported by the observation that mutations that enhance the discrepancy between the affinity for agonists determined in heterologous versus homologous competition binding analysis also decrease the B_{max} of the agonist (Tables 1 and 2). Similarly, treatment with, for example, nonhydrolyzable GTP analogs generally uncouples the receptors from G proteins and thereby leads to a decrease in affinity specifically for agonists (29, 30), as was observed in the current study for the mutant receptors. Because the low affinity of agonists for the uncoupled receptor often will prevent binding of radiolabeled agonists, the uncoupling will be observed as a decrease in B_{max} as determined by radiolabeled agonists. Consequently, the low affinity of the agonist can be determined only in heterologous competition binding assays using radiolabeled antagonist. Importantly, in several receptor systems, even very high concentrations of GTP analogs will result in only a partial uncoupling of the receptor (38, 39). Thus, as a proportion of the receptors become uncoupled, a decrease in the B_{max} for the the agonist will be observed. Importantly, the remainder of the receptors bind agonist

[1] K. Thirstrup, S. A. Hjorth, and T. W. Schwartz, unpublished observations.

TABLE 3

Radioligand binding analysis for the peptide agonist dynA(1–8)

The binding affinity for the peptide agonist dynA(1–8) was estimated in competition with either the antagonist radioligand [^{3}H]DIP or the agonist radioligand [^{3}H]C1977. The discrepancy in the affinity estimated using either analysis is expressed by the ratio between the K_i values obtained using the antagonist radioligand divided by the affinity determined using the agonist radiolabel: K_i([^{3}H]DIP)/K_i([^{3}H]CI977). Values in parentheses are Hill coefficents.

Receptor	DynA(1–8)				
	[^{3}H]DIP		[^{3}H]CI977		K_i([^{3}H]DIP)/K_i([^{3}H]C1977) for dynA(1–8)
	K_i (n)	F_{mut}	K_i (n)	F_{mut}	
	nM		nM		
κ-wt	0.37 ± 0.05 (7) (−1.05 ± 0.03)	=1.0	0.41 ± 0.11 (8) (−1.31 ± 0.47)	=1.0	1
κμ6	1.68 ± 0.4 (4) (−0.80 ± 0.16)	4.5	0.25 ± 0.02 (3) (−0.92 ± 0.04)	0.6	7
κμκ	3.28 ± 1.4 (4) (−1.06 ± 0.16)	8.9	0.13 ± 0.02 (3) (−0.96 ± 0.03)	0.3	25
κμ5	2,021 ± 499 (5) (−0.60 ± 0.10)	5,400	2.46 ± 1.0 (3) (−1.0 ± 0.06)	6.9	810
μκ3	0.96 ± 0.28 (4) (−0.72 ± 0.10)	2.6	0.11 ± 0.03 (4) (−1.14 ± 0.26)	0.3	9
[S123A]κ	35.5 ± 7.5 (3) (−0.84 ± 0.21)	96	3.24 ± 0.8 (3) (−0.88 ± 0.17)	7.9	11
[Y119A]κ	27.7 ± 2.7 (3) (−0.78 ± 0.01)	75	1.45 ± 0.45 (3) (−0.86 ± 0.03)	3.5	19
[T109A]κ	11.3 ± 5.2 (5) (−0.79 ± 0.08)	31	2.92 ± 1.8 (4) (−0.96 ± 0.08)	7.1	4
[D105N]κ	17.4 ± 7.2 (3) (−0.74 ± 0.07)	47	0.48 ± 0.14 (3) (−1.13 ± 0.22)	1.3	36
μ-wt	24.9 ± 6.9 (3) (−0.50 ± 0.05)	68	NB		

with high and normal affinity. This picture is very similar to that observed for the receptor mutants in the current study.

Unfortunately, we have not been able to directly address the question of receptor coupling in the κ-opioid system due to the lack of a reliable coupling assay for this receptor. However, in the corresponding mutant receptors in the NK1 receptor system, Huang *et al.* (26) found that except for the substitution of the acidic residue deep in TM-II (corresponding to D105 of the current study), all of the TM-II mutants coupled to phospholipase C. Although the E_{max} value was somewhat reduced, Substance P did stimulate phosphatidylinositol turnover with an EC_{50} value that was shifted only slightly to the right compared with the that for wild-type receptor (26); i.e., it corresponds to the IC_{50} value determined in homologous binding assays (25). Thus, it could be argued that the mutations probably lead to an uncoupling of a proportion of the receptors just as GTP analogs in some systems only partially uncouple receptors as determined in binding assays. If this is the case, it is noteworthy and surprising that so many mutations, including some located at the exterior

end of the TM helices, would uncouple these receptors. Furthermore, a large proportion of the receptors apparently were not uncoupled because they bound the agonist well and could be stimulated. Furthermore, in the TM-II mutants in the NK1 receptor system, the apparent affinity of the agonist Substance P is reduced 1,000–10,000-fold as determined in competition binding assays with radiolabeled antagonists. This is considerably more than the 60–100-fold decrease in affinity observed after treatment with GTP analogs (32, 33). Thus, although a partial uncoupling of the receptors from G proteins probably is part of the phenotype of these mutants, it is likely that this is not the entire story.

Implications for mutational mapping of agonist binding sites. Regardless of the actual molecular mechanism, receptor mutations clearly may affect the binding of agonists as measured in heterologous binding assays without actually disturbing the high affinity binding site of the agonists. In the current study, the substitutions in TM-II could have been misjudged to constitute part of the binding site for CI977 and dynA(1–8) if only an antagonist had been used as radiolabel. Thus, the use of homologous binding assays is important when attempting to map agonist binding sites. Similarly, it should be emphasized that mutations also may lead to a decrease in expression level, as suggested by the B_{max} values estimated in the current study for the agonist. Consequently, an even further decrease in expression level might have entirely prevented detection of any binding of the radiolabeled agonist. In conjunction with the low affinity measured in competition with radiolabeled antagonist, this would most certainly have been interpreted as having selectively affected agonist affinity by the mutation and thus be considered a presumed interaction site for the agonist. Thus, a low expression level of this type of mutation would lead to a false-positive result in a mutational mapping of binding site for an agonist.

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