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European Journal of Pharmacology 403 (2000) 37–44

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Selectivity of μ-opioid receptor determined by interfacial residues near third extracellular loop

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Received 27 April 2000; received in revised form 20 July 2000; accepted 28 July 2000

Abstract

We hypothesized that the selectivity profile of the rat μ-opioid receptor for opioid receptor-selective ligands is determined by the nature of the amino acid residues at highly divergent sites in the ligand-binding pocket. To determine which characteristics of these residues contribute to opioid receptor ligand selectivity, we made various mutant receptors that replaced the Lys^{303} and Trp^{318} residues near the extracellular interface of transmembrane domains VI and VII, respectively. Ligand binding determinations using transiently transfected monkey kidney epithelial (COS-1) cells show that Lys^{303} mutations cause little change in the receptor binding profile, whereas the Trp^{318} mutant receptors have considerably lower affinity for μ-opioid receptor-selective ligands and greatly increased affinity for δ-opioid receptor-selective ligands. The nature of these mutations show that this effect is not due to sterics or charge alone. [^{35}S]guanosine-5′-*O*-(3-thio)-triphosphate ([^{35}S]GTPγS) activity assays show that these residues may influence functional, as well as binding selection. We conclude that a primary role for Trp^{318} is to form a basis for ligand selectivity.

Keywords: Opioid receptor; Ligand selectivity; Mutagenesis; [^{35}S]GTPγS

1. Introduction

Due to their sequence homology and to the availability of a wide range of ligands, the G-protein-coupled opioid receptors offer an excellent opportunity for studying the molecular basis of ligand selectivity and activity. Little work has been done in determining the contribution of divergent amino acid residues within the transmembrane domains of opioid receptors in determining selectivity or function. Few sites in the transmembrane domains of these receptors demonstrate convincing divergence in amino acid chemical functionality (Meng et al., 1993; Thompson et al., 1993; Kieffer et al., 1992; Bunzow et al., 1994). One such site is the Lys^{303} in the rat μ-opioid receptor. This residue corresponds to Trp^{284} in the rat δ-opioid receptor, Glu^{297} in the rat κ-opioid receptor, and Gln^{286} in the rat orphan-opioid receptor. Among this set of amino acids, there is considerable diversity in chemical functionality (i.e., positively charged, aromatic hydrophobic, negatively charged, and polar neutral residues).

This current analysis was undertaken in an effort to determine the functional implications of amino acid divergences of this type. One possibility is that the nature of the amino acid does not matter at these sites, and that any amino acid will suffice. Alternatively, these amino acids may form the basis of opioid receptor ligand selectivity. If the latter argument is true, then one should be able to substitute the amino acid of one receptor type for the other and show that it leads to altered, or even reversed opioid receptor ligand selectivity. For example, if one were to replace Trp^{318} in the μ-opioid receptor with leucine (the homologous δ-opioid receptor residue) to yield the W318L-μ receptor mutant, and subsequently show that this mutant binds δ-opioid receptor-selective ligands with higher affinity, and μ-opioid receptor-selective ligands with lower affinity, then that would be clear evidence in support of the argument that the residue serves an important role in discriminating between ligands of different opioid receptor selectivities.

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00578-1

Site-directed mutagenesis studies which yield a "gain-of-function" of this type have been reported previously by this laboratory — the orphan-opioid receptor was converted into a receptor which recognized opioid receptor-selective antagonists (Meng et al., 1996). In the present work, two divergent sites (Lys^{303} and Trp^{318} in the μ-opioid receptor) were chosen in an effort to determine their importance in producing opioid receptor selectivity. Mutations at these sites were made and assayed for their ability to bind opioid receptor-selective ligands as determined by radioligand competition, and for their ability to transduce message, as determined by agonist-induced [^{35}S]guanosine-5′-*O*-(3-thio)-triphosphate ([^{35}S]GTPγS) binding.

Few mutational analyses have been performed on these two sites or their homologues in the other opioid receptors. Published reports include a κ-opioid receptor mutant (E297K), that was shown to have decreased affinity for the κ-opioid receptor-selective ligand norbinaltorphimine, yet unchanged affinity for the less opioid receptor-selective ligands diprenorphine and naloxone (Hjorth et al., 1995). The wild-type κ-opioid receptor was also shown to be convertible to one which bound with high affinity and was activated by the μ-opioid receptor-selective agonist peptide [D-Ala^2, *N*-$MePhe^4$,Gly-ol]enkephalin (DAMGO) by changing only four residues, two of which being homologous to the ones reported in this work (Seki et al., 1998). More attention has been paid to Trp^{284} in the δ-opioid receptor (Varga et al., 1996; Valiquette et al., 1996), although these latter two studies produced somewhat disparate results, possibly due to the difference in the amino acid to which the target residue was mutated. Recently, it was reported that a W318A mutation of the μ-opioid receptor yields a receptor in which specific binding was nearly undetectable (Xu et al., 1999). However, only a very limited number of ligands was tested, and the gain-of-function towards non-μ-opioid receptor-selective ligands was not explored.

Thus, although there is scattered evidence that these sites are important for determining the pharmacological profiles of opioid receptor-selective ligands, these sets of data have yet to reveal a full picture of how the opioid receptor amino acid residues influence ligand characteristics such as affinity, selectivity, and potency. The μ-opioid receptor has a complex set of determinants for these ligand characteristics (Onogi et al., 1995; Seki et al., 1998). When one is making mutant opioid receptors, one should evaluate carefully the nature of the amino acid residue of the wild-type and consider the nature of the amino acid to which the residue will be mutated. In the present study, Lys^{303} was mutated to glutamine, glutamic acid, and tryptophan. This chemically diverse set of amino acids at this position were chosen to help reveal the chemical requirements which determine μ-opioid receptor-selective ligand affinity, activity, and selectivity. These mutated residues represent the amino acids present at the homologous positions in the other (non-μ) opioid receptors. Similarly, Trp^{318} was mutated to leucine and lysine, both to elucidate the chemical requirements at this site for high affinity ligand binding, and to determine whether this residue acts as a selectivity switch. For both sets of μ-opioid receptor mutants (K303 and W318), the homologous δ-opioid receptor residue is represented. If these mutant receptors bind a class of molecules which are traditionally considered as selective for the δ-opioid receptor, then there is compelling reason to conclude that the mutated residues are important in determining selectivity.

2. Materials and methods

2.1. Drugs and chemicals

Dulbecco's modified essential medium, fetal calf serum, and phosphate-buffered saline (PBS) were from Gibco/BRL Life Technologies (Gaithersburg, MD, USA). FuGene6 was from Roche (Indianapolis, IN, USA) Naltrexone, naloxone, naloxonazine, bremazocine, and naltrindole were from Research Pharmaceuticals International (Natick, MA, USA). DAMGO, [D-Pen^2, D-Pen^5]enkaphalin) (DPDPE, and D-Phe-c[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2 (CTAP, where Pen = Penicillamine (β,β′-dimethylcysteine)) were from Peninsula (Belmont, CA, USA). (+)-4-[(α *R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC-80) was purchased from Tocris (Ballwin, MO, USA). Titrated bremazocine and [^{35}S]GTPγS were from New England Nuclear (Boston, MA, USA).

2.2. Construct creation

The cDNA encoding the rat μ-opioid receptor was cloned in this laboratory (Thompson et al., 1993). This receptor gene was subcloned into an expression vector under the control of the cytomegalovirus promoter, courtesy of Dr M.D. Uhler (Huggenvik et al., 1991). Mutations of this construct, which also contained the gene for ampicillin resistance, were accomplished using a double-stranded mutagenesis protocol (Deng and Nickoloff, 1991). Mutations were confirmed by restriction enzyme analysis (where possible) and DNA sequencing.

2.3. Radioligand competition assay procedure

Plasmid DNA containing the mutant or wild-type receptor (25 μg) was used to transfect monkey kidney epithelial (COS-1) cells using the calcium phosphate method (Chen and Okayama, 1987). Following receptor expression, membrane preparations which contained the receptor were used to determine receptor binding (Goldstein and Naidu, 1989). Approximately 1.5 nM [^{3}H]bremazocine was used to label the receptors. The labeled receptors were incubated for 1 h at room temperature in 50 mM Tris buffer

(pH 7.4) in the presence of varying concentrations of competing, unlabeled ligand. Competing ligands were present at nine different concentrations, and in duplicate. At the end of the incubation, free ligand was separated from membrane-bound ligand using a Brandel cell harvester (Brandel, Gaithersburg, MD).

2.4. Activity assay procedure

The [^{35}S]GTPγS assay was performed as described previously (Befort et al., 1996). COS-1 cells were transfected with DNA (8 μg) containing the receptor gene using FuGene6 (Roche). About 64 h after transfection, the cells were harvested in ice-cold PBS and centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was removed and the cells were suspended in 5 ml of lysis buffer (5 mM Tris base pH 7, 5 mM EDTA, 2.5 mM EGTA, 0.1 mM paramethylphenylsulfonyl fluoride). This suspension was homogenized for 10 s. This material was then centrifuged at 2500 rpm for 10 min at 4°C. The supernatant was then poured into a new tube and re-centrifuged at 15,000 rpm for 20–30 min at 4°C. The supernatant was removed and the membrane pellet was suspended in 560 μl of suspension buffer (50 mM Tris base, pH 7.0, 0.32 M sucrose, 1 mM EDTA). This suspension was then passed several times through a 26-gauge needle. This homogenate (500 μl) was added to 940 μl of reaction buffer (50 mM Tris base, pH 7.0, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, and 1 mM dithiothreitol), and 160 μl of 3-[(3-cholamidopropyl)dimethylammonoi]-1-propane sulfonate (CHAPS) buffer. This preparation was then added to tubes which contained eight different concentrations of test drug (or no drug to determine basal activity), [^{35}S]GTPγS, GDP (or GTPγS, to determine non-specific binding), and reaction buffer on ice. The final mixture was then removed from the ice–water bath and incubated for 1 h at room temperature (Emmerson et al., 1996). Final concentrations were 20 μM GTPγS, 30 μM GDP, 0.1 nM [^{35}S]GTPγS, and drug concentrations ranging from 10 μM to 1 pM. Membrane bound [^{35}S]GTPγS was then separated from free [^{35}S]GTPγS using a Brandel cell harvester (Brandel, Gaithersburg, MD).

2.5. Data analysis

The raw data were fitted to curves using the least-squares program of Prism (GraphPad Software, San Diego, CA) These data were then converted to K_d (for Scatchard analysis), K_i (radioligand competition), or EC_{50} (activity assays). The values in the tables are the means ± the S.E.M. of three experiments.

3. Results

3.1. Ligand binding characteristics of mutant and wild-type receptors

Table 1 shows the results of the radioligand competition assays for the mutant and wild-type rat μ-opioid receptors. Inspection of the data reveal that there is little difference in ligand binding properties between the wild-type μ-opioid receptor and the K303 mutant receptors. The affinity constants for bremazocine determined by Scatchard analysis did not differ dramatically from that of the wild-type μ-opioid receptor. Substitution for the tryptophan in position 318, however, caused a marked reduction in their affinity for tritiated bremazocine (about four-fold for the W318L receptor and six-fold for the W318K receptor), although binding is still in the high affinity range. These results are very consistent with the apparent inhibition constants determined for bremazocine by homologous competition assays.

Table 1
Binding affinities of opiate ligands (apparent K_i ± S.E.M.), in nanomolar

Ligand		Receptor					
		Wild-type	K303E	K303Q	K303W	W318L	W318K
Non-selective	Bremazocine[a]	0.51 ± 0.07	0.28 ± 0.02	0.74 ± 0.04	0.43 ± 0.06	2.19 ± 0.33	3.20 ± 1.04
	Bremazocine	0.48 ± 0.07	0.23 ± 0.02	0.62 ± 0.11	0.29 ± 0.06	1.84 ± 0.13	2.34 ± 0.51
	Naloxone	3.44 ± 0.6	2.63 ± 0.4	5.10 ± 1.0	5.4 ± 2.3	16.8 ± 3.5	23.3 ± 4.7
Mu-selective	Naltrexone	0.92 ± 0.19	1.15 ± 0.47	2.32 ± 0.41	1.13 ± 0.12	3.30 ± 0.19	6.40 ± 1.30
	Naloxonazine	3.80 ± 0.64	1.62 ± 0.07	4.60 ± 0.29	3.55 ± 0.82	8.87 ± 1.48	12.10 ± 0.23
	Morphine	19.6 ± 3.0	17.3 ± 2.8	42.7 ± 1.4	7.8 ± 0.7	89.0 ± 19.0	187.0 ± 85.0
	CTAP	2.36 ± 0.46	5.06 ± 1.14	5.2 ± 1.33	5.06 ± 1.14	15.9 ± 2.40	42.5 ± 6.70
	Fentanyl	14.5 ± 8.6	16.3 ± 11.6	27.7 ± 15.5	42.3 ± 22.3	26.5 ± 10.8	7.0 ± 0.7
	DAMGO	14.3 ± 4.7	14.6 ± 2.2	20.8 ± 1.1	8.9 ± 0.8	14.3 ± 4.2	27.6 ± 5.6
Delta-selective	Naltrindole	18.9 ± 1.2	3.8 ± 0.1	11.2 ± 0.9	9.2 ± 1.8	0.3 ± 0.1	1.10 ± 0.5
	DPDPE	580 ± 108	570 ± 42	1111 ± 170	577 ± 105	69 ± 15	20 ± 5
	SNC-80	> 1000	> 1000	> 1000	> 1000	196 ± 20	995 ± 71

Results from Scatchard analysis, heterologous competition, and homologous competition for opioid receptor-selective ligands with wild-type and mutant rat μ-opioid receptors transiently expressed in monkey kidney epithelial (COS-1) cells. Experimental values are from three independent experiments performed in duplicate. Binding data were analyzed using GraphPad Prism software.

[a] K_d.

This general tendency for K303 substitutions to cause little effect, and W318 substitutions to cause reduced affinity for μ-opioid receptor-selective and nonselective opioid receptor ligands is also seen in the apparent K_i values of other ligands. Naloxone, a non-selective alkaloid opioid receptor antagonist, is bound slightly more tightly by the K303E mutant receptor, and with slightly lower affinity by the K303Q and K303W mutant receptors compared to the wild-type receptor. Substitutions for Trp^{318} led to receptors which have lower affinity for naloxone by five- to seven-fold. Similar results are obtained for the more μ-opioid receptor-selective alkaloid antagonists naltrexone and naloxonazine, although the shift in affinity for the W318 mutant receptors were less dramatic in these cases. These trends were also conserved in the binding of the μ-opioid receptor-selective alkaloid agonist morphine. Morphine affinity was changed about two-fold or less in the K303 mutant receptors compared to wild-type receptors, whereas the W318 mutant receptors had morphine affinities that were reduced four-fold (W318L) and nine-fold (W318K).

The effect of substitution on the μ-opioid receptor-selective antagonist CTAP was similar. Binding affinity is decreased only about two-fold in each of the K303 mutant receptors, but by about seven-fold for the W318L mutation, and more than an order of magnitude for the W318K mutant receptor (about 18-fold).

Mutations at these two sites had differential effects on the binding of the partial μ-opioid receptor-selective agonist fentanyl. Fentanyl had similar binding affinity for the K303E receptor, about a two-fold reduction in affinity at the K303Q and W318L receptors, and about a three-fold reduction at the K303W receptor relative to wild-type. Only the W318K receptor bound fentanyl with higher affinity than did wild-type (about two-fold).

The μ-opioid receptor-selective peptide agonist DAMGO was evidently influenced little by any of these mutations. DAMGO affinity was shifted less than two-fold in all the mutant receptors compared to wild-type, although the W318K receptor had nearly a two-fold reduction in affinity for DAMGO.

The binding affinities of the δ-opioid receptor-selective ligands were surprising. The δ-opioid receptor-selective antagonist alkaloid naltrindole had greatly increased affinity for the W318 mutant receptors. The affinity for this ligand increased to the nanomolar (W318K) or even sub-nanomolar range (W318L), exhibiting approximately 17- and 60-fold increases in affinity, respectively. Binding affinity was increased for the K303 mutant receptors as well, although not as dramatically. Similarly, the affinity of these mutant receptors for the δ-opioid receptor-selective agonist peptide DPDPE was increased. The W318L receptor had about an eight-fold increase in affinity and the W318K receptor had a 29-fold higher affinity for DPDPE. The K303 mutant receptors' affinities were either unchanged (K303E and K303W) or slightly decreased (less than two-fold reduction for the K303Q receptor). The δ-opioid receptor-selective ligand SNC-80 showed poor affinity for the wild-type and all K303 mutant receptors, but the ligand's affinity was increased upon mutation of Trp^{318}. Affinity was increased perhaps only slightly for W318K, but significantly for W318L (greater than five-fold). Thus, the W318 mutant receptors exhibited substantial loss of affinity towards most μ-opioid receptor-selective and non-selective opioid receptor ligands and increased affinities towards δ-opioid receptor-selective ligands. For example, the wild-type μ-receptor preferred CTAP over DPDPE by a factor of 240, whereas the W318K receptor preferred CTAP over DPDPE by only a factor of two. By contrast, μ-opioid receptor-selective and non-selective opioid receptor ligand binding was relatively unchanged by mutation of Lys^{303}. Even among δ-opioid receptor-selective ligands, only the binding of naltrindole by the K303E receptor showed greater than four-fold affinity shift compared to wild-type.

3.2. Signal transduction properties of mutant and wild-type receptors

Wild-type and mutant opioid receptors were tested for activity using the opioid receptor selective agonists DAMGO, morphine, fentanyl, and DPDPE. The results of these [^{35}S]GTPγS assays are presented in Table 2. Although mutations at the K303 site did not generally affect ligand binding in a dramatic way, some large changes were seen in receptor activation. When assaying for DAMGO-induced receptor activation, all mutant receptors had greatly increased EC_{50} values (Table 2A). The W318K mutant receptors was least-changed compared to wild-type, although its EC_{50} value was increased by nearly an order of magnitude. The most dramatic shift in EC_{50} was seen in the K303E mutant receptor, whose EC_{50} value increased nearly 400-fold. These EC_{50} values are increased more than would have been expected, based only on change in affinity. Consequently, the EC_{50}/K_i ratios for all mutant receptors increased from about an order of magnitude (W318L) to about 400-fold (K303E) relative to wild-type (Table 2B).

The results for morphine are not nearly as dramatic. In this case, the location of the mutation dictated its effect on receptor activation. The K303 mutant receptors had only slightly changed EC_{50} values, whereas the W318 mutant receptors had EC_{50} shifts of seven-fold (W318L) and 15-fold (W318K) (Table 2A). These changes in EC_{50} were determined primarily by the altered affinity of the ligand for the receptor. Consequently, the EC_{50}/K_i ratios were all essentially equivalent (Table 2B). Values for K303W are not included because morphine-induced increase in [^{35}S]GTPγS binding was small, making a meaningful EC_{50} undeterminable.

The EC_{50} values for fentanyl at the mutant receptors were not changed dramatically in any case. Only W318K

Table 2
Activities ($EC_{50} \pm$ S.E.M., nM) of opiate agonists and their activity/affinity ratios

	Ligand	Receptor					
		Wild-type	K303E	K303Q	K303W	W318L	W318K
(A) EC_{50}	DAMGO	1.53 ± 0.69	584 ± 228	56.7 ± 22.0	118 ± 37.6	25.4 ± 16.0	15.0 ± 4.5
	Morphine	51.1 ± 10.5	86.7 ± 31.2	92.5 ± 16.1	ND[a]	357 ± 59	753 ± 54.8
	Fentanyl	23.3 ± 6.5	14.0 ± 4.2	28.0 ± 11.3	17.4 ± 1.6	36.5 ± 7.7	52.1 ± 4.5
	DPDPE	> 1000	> 1000	> 1000	ND	> 1000	187 ± 9.1
(B) Ratio EC_{50}/K_i	DAMGO	0.11	40	2.7	13.3	1.7	0.54
	Morphine	2.6	5.0	2.2	ND	4.0	3.9
	Fentanyl	1.6	0.86	1.0	0.41	1.4	7.5
	DPDPE	ND	ND	ND	ND	ND	9.3

(A) Results of [^{35}S]GTPγS assay. Values represent agonist-induced binding of radiolabelled non-hydrolyzable GTP in membrane preparations of monkey kidney epithelial (COS-1) cells transiently transfected with wild-type and mutant rat μ-opioid receptor DNA. Values represent three independent experiments performed in duplicate. Data analysis was performed using GraphPad Prism software. (B) Values represent the simple ratio of the mean EC_{50} seen in 2A and the corresponding mean K_i value (from Table 1).

[a] ND = Not determined.

had an EC_{50} shift of greater than two-fold. (Table 2A). However, both the W318K and K303W receptors appeared to have a different EC_{50}/K_i ratio. The W318K mutant receptor has slightly increased binding for fentanyl (about two-fold), and also has a decreased ability to transduce fentanyl message (about two-fold), and hence has an EC_{50}/K_i ratio which is greater than four-fold higher than that of the wild-type receptor. Practically, the opposite holds true for the K303W receptor, and hence its EC_{50}/K_i ratio is four-fold lower than wild-type.

The results of DPDPE-induced activation show that this δ-opioid receptor-selective agonist is a poor activator of the wild-type and mutant receptors. A meaningful EC_{50} was not determinable for DPDPE at the wild-type or most mutant receptors. DPDPE did activate the receptors, but the EC_{50} values are generally greater than 1 μM. Only the W318K mutant receptor had an EC_{50} value that was determined with confidence.

The mutant receptors were screened also for their ability to be activated by many other opiate ligands. Each ligand present in Table 1, and some others, was tested for its ability to stimulate [^{35}S]GTPγS binding at high concentrations. The results showed that in no case was any ligand that is traditionally considered an agonist for the μ-opioid receptor converted to an antagonist, or vice versa (data not shown).

4. Discussion

The hypothesis that Lys^{303} and Trp^{318} of the rat μ-opioid receptor — sites which display varying amino acid residue functionality at homologous sites across the opioid receptor class — serve as key sites for binding μ-opioid receptor-selective ligands, and excluding δ-opioid receptor-selective ligands was confirmed in part and refuted in part. Substitution of Lys^{303} with amino acids present in the homologous position of the other opioid receptors led to little change in ligand affinities. Only few exceptions to this generality were seen (morphine bound slightly better to the K303W receptor and naltrindole bound the K303E receptor better, for example). We therefore conclude that the high variability at this site across the opioid receptors, at least as it applies to the rat μ-opioid receptor, is neither maintained as a mechanism of selection for μ-opioid receptor-selective ligands, nor as a means to exclude δ-opioid receptor-selective ligands. Hence, the Lys^{303} position acts as a "promiscuous" site — the receptor is evidently able to bind non-selective opioid ligands and μ-opioid receptor-selective ligands irrespective of which amino acid is present at that site. This does not mean that the homologous amino acids at this position in the other opioid receptors are not serving to maintain selectivity. It is clear from published reports that the homologous Trp^{284} in the δ-opioid receptor is serving at least as a basis to discriminate between classes of molecules (Valiquette et al., 1996; Varga et al., 1996), although no information has been provided concerning its role in excluding μ-opioid receptor-selective ligands, for example. The promiscuous nature of this site can be understood by observation of computer-generated molecular models of the μ-opioid receptor (Pogozheva et al., 1998). In this model, the Lys^{303} residue does not point inward in the direction of the putative ligand-binding pocket, but rather is pointed along the line of the α-helical backbone. In this position, the residue is not positioned to serve as a force in repelling ligands of certain types. It is possible that the function of the lysine in this position is not related to ligand binding per se, but rather is used to anchor the receptor into the membrane. This function could be mediated by ion pairing between the positively charged lysine side-chain group near the extracellular surface of transmembrane domain VI and the negatively charged head groups of the lipid bilayer. The importance of this placement of positively charged amino acid residues near the membrane interface is well-known — transmembrane domains characteristically have a posi-

tively charged amino acid residue at its interface with the aqueous milieu (Ballesteros and Weinstein, 1995).

By contrast, Trp^{318} has a significant effect on ligand binding. Mutation of this site causes almost universal reduction of ligand binding affinity of non-selective opioid receptor ligands and μ-opioid receptor-selective ligands. Perhaps more importantly, mutation at this site leads to dramatically increased affinities for δ-opioid receptor-selective ligands. It was with this possibility in mind that the W318L receptor mutant was made. In the analogous position in the δ-opioid receptor there is a leucine residue (Leu^{300}). It was hypothesized that by changing this Trp^{318} to Leu^{318} — the "δ-like" residue — it might be possible to produce a mutant receptor that better recognizes δ-opioid receptor-selective ligands. This hypothesis is confirmed by the present data, as naltrindole and DPDPE binding affinities are dramatically increased in W318 mutant receptors compared to the wild-type receptors. Clearly, this residue forms a basis for molecular opioid receptor selectivity. The structural nature of this effect too, can be understood by inspection of the molecular model. In this model, the Trp^{318} residue is in the binding pocket, and in very close proximity to the bound ligand. It would appear that Trp^{318} serves both to repel δ-opioid receptor-selective ligands and to attract μ-opioid receptor-selective ligands. This conclusion is based on the observation that the nature of W318 substitution has little bearing on its concomitant ligand-binding abilities. Whether the aromatic tryptophan is substituted with a positively charged lysine or an aliphatic leucine, the results are the same, namely reduced binding for non-selective opioid receptor ligands and μ-opioid receptor-selective ligands and an increased affinity for δ-opioid receptor-selective ligands. The molecular mechanism for this effect is unclear, but is possibly mediated by simple steric occlusion. Unfortunately, it is not possible to test this hypothesis by mutagenesis due to the lack of a larger encoded amino acid.

Generally, the [^{35}S]GTPγS assays demonstrate that the mutant receptors can be activated by opioid receptor-selective ligands. This lends further evidence that the mutations do not cause profound conformational changes which would otherwise suggest that the influence of the single residue changes could not be ascribed as the source of the receptors' changed pharmacological profiles. The results of the activity assays generally show that for the K303 mutant receptors, their ability to be activated did not change significantly, whereas the W318 mutant receptors showed a greater change in EC_{50} for the opioid receptor-selective ligands.

As should be expected when surveying the ligand-binding and signal transduction properties of several ligands across several mutant receptors, the results do not lend themselves to a simple explanation. Agonist signal transduction is a complex, concerted reaction. The receptor must be able to propagate a signal (ligand binding) that occurs on or near the extracellular surface (Onogi et al., 1995; Seki et al., 1998) through the membrane to the proteins which are in association with the receptor amino acid residues present on or near the intracellular surface. This process most likely consists of a series of rearrangements of the amino acid side-chain groups and/or the helix bundles as a group, since no amino acid residue near the binding pocket is at the same time near the coupled G-proteins. The signal can only reach the opposite side of the membrane by sequential residue interactions that are probably ionic, hydrophobic and steric in nature (Gether, 2000). A detailed knowledge of the reaction pathway will only be determined when the effect of ligand binding is known for each of these parameters. It is difficult to assess these influences based on an analysis of a small number of mutations, because each mutation creates multifaceted changes in the receptor. No amino acid substitution will change only ionic interactions without concomitant changes in hydrophobic interactions, for example. Deconvolution of the data will be difficult even when all amino acid residues have been substituted at the target site. Therefore, one can expect variations in binding affinity and ligand activity when simultaneously assessing the effects of different mutations at the same site.

The importance of hydrophobic interaction is seen in the case of morphine. The addition of a hydrophobic residue at position 303 increases its affinity and a loss of this hydrophobic residue at position 318 causes a reduction in its affinity. The activity of morphine across these receptors can be predicted essentially by the receptors' affinities for the ligands alone. However, the K303W mutant receptor exhibits a loss of morphine-induced activity that is surprising considering that the affinity for morphine by that receptor is higher than that of the wild-type receptor. At this mutant receptor, morphine acts as a very weak or partial agonist within the concentration range tested.

Fentanyl shows the least variation in affinity across the receptors. None of the mutant receptors show a greater than three-fold shift in affinity for this drug, although K303W demonstrates nearly this much change versus wild-type. The EC_{50} values for fentanyl-induced receptor activation are all quite similar, as are the K_i values, and hence, fentanyl is relatively unaffected by the position or nature of the current mutations. The changes in EC_{50}/K_i ratios seen for the K303W and W318K mutant receptors indicate that the binding properties of fentanyl may be secondary to the intrinsic signal transduction pathway of the receptor. The differential interaction of the mutated amino acids with the membrane polar head groups may have a greater effect on signal transduction than their interaction with the ligand per se.

DAMGO has the most complex activity profile across the receptors. Although its binding affinity is relatively unchanged across the receptors, its EC_{50} varies widely. Because DAMGO is a larger, more complex, and flexible ligand, its differential effects would be predicted to be greater (Onogi et al., 1995; Seki et al., 1998). For the

K303 mutant receptors, it appears that ionic charge has a large effect. The reversal of charge from the positively charged lysine in the wild-type receptor to the negatively charged residue in the K303E mutant receptor leads to a drastically reduced DAMGO activity. Thus DAMGO-induced signal transduction in this mutant receptor seems to be impaired by a set of changes of ionic interactions that propagate its signal towards the G-proteins coupled to its intracellular residues. For the W318 mutant receptors, DAMGO signal transduction seems to be impaired by an alteration in the hydrophobic/aromatic interactions that also help propagate signal to the G-proteins similar to that seen for morphine and fentanyl.

The activity of DPDPE in these mutant receptors can be explained by a rationale similar to that used for fentanyl. The addition of a positively charged residue at position 318 reduces its ability to transduce signal, possibly due to the mutant receptor's newly formed ionic interaction with the membrane polar groups. Poor activity is seen in K303 mutant receptors simply due to very poor affinity for the ligand by this mutant receptor. The presence of a leucine at position 318 leads to a receptor which is poorly activated by DPDPE. Evidently, the relatively large side-chain group of the leucine residue interferes with the concerted signal transduction mechanism. This steric blocking of transduction is not apparent when using the smaller ligands, leading to a receptor that is free to change its conformation to propagate the agonist signal.

The results of this study indicate a possible basis for the divergence of amino acid residues across the opioid receptors at the positions homologous to the Lys^{303} in the μ-opioid receptor — there is simply no evolutionary pressure to maintain a single amino acid residue at this site. Evidently, amino acids of highly varying nature are tolerated well in the context of ligand binding. These homologous sites may, however, form a basis of a functional discrimination between opioid receptor types. DAMGO signal transduction is altered dramatically upon substitution for the wild-type Lys^{303}, although its binding is largely unchanged. Mutations at the homologous sites in the other opioid receptors may yield similar results. For example, the Trp^{284} in the δ-opioid receptor may have its effect not by preventing μ-opioid receptor-selective ligands from binding, but rather by preventing μ-opioid receptor-selective ligands from activating the receptor. This possibility remains untested as there have been no reports describing μ-opioid receptor-selective agonists' abilities to activate the W284K-δ-opioid receptor. The current results underscore the importance of performing activity assays as well as ligand binding experiments. If ligand binding only was determined in this work, then one would have been misled into concluding that the wild-type and mutant receptors are equivalent where DAMGO is concerned, whereas the [^{35}S]GTPγS assays demonstrate that this is clearly not the case. By contrast, the tryptophan in position 318 appears to serve an important function in selectivity by simultaneously enhancing the binding of μ-opioid receptor-selective ligands and in inhibiting the binding of δ-opioid receptor-selective ligands.

Acknowledgements

We wish to thank Professor James Woods of the Department of Pharmacology, University of Michigan, for providing various alkaloid ligands used in this study. We also thank Dr. Qiang Wei for helpful discussions about assay protocol and pharmacological characterization of the ligands presented in this study. We also thank Linda M. Gates for her assistance in tissue culture. This work was supported by grants from the National Institute on Drug Abuse, Grant numbers RO1-DA08920 (H. Akil) and RO1-DA02265 (H. Akil).

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