RESEARCH COMMUNICATION

Molecular cloning of a rat κ opioid receptor reveals sequence similarities to the μ and δ opioid receptors

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By screening a rat brain cDNA library using a cloned μ opioid receptor cDNA as probe, a clone was identified that is very similar to both the μ and δ opioid receptor sequences. Transient expression of this clone in COS-7 cells showed that it encodes a κ opioid receptor, designated KOR-1, which is capable of high-affinity binding to κ -selective ligands. Treatment of transfected cell membranes with bremazocine, a κ -selective agonist, resulted in a 53 % decrease in adenylate cyclase activity, indicating that

this κ opioid receptor displays inhibitory coupling to adenylate cyclase. Thus, one member from each of the three opioid receptor types, μ , κ and δ , has been molecularly cloned. Analysis of sequence similarities among these opioid receptors, as well as between opioid receptors and other G-protein-coupled receptors, revealed regions of sequence conservation that may underlie the ligand-binding and functional specificities of opioid receptors.

INTRODUCTION

Opioids include endogenous peptides such as endorphins, enkephalins and dynorphins, as well as exogenous compounds which include natural alkaloids from plants and synthetic analogues of these alkaloids and peptides. When administered, these compounds exert powerful effects throughout the body, regulating such complex physiological functions as nociception, neuroendocrine secretion, neurotransmitter release, respiratory depression and decreases in gastrointestinal motility [1]. Opioids trigger these effects through their interactions with endogenous cell-surface receptors. Extensive pharmacological studies over the past two decades have demonstrated the presence of three major types of opioid receptors, μ , κ and δ [2]. It has also been shown that each type of opioid receptor possesses distinct, yet overlapping, ligand-binding properties and functional characteristics [3].

All three types of opioid receptors mediate the inhibition of adenylate cyclase through a pertussis-toxin-sensitive mechanism, suggesting that these receptors may belong to the family of membrane receptors that couple to G proteins. Receptors within this family share the structural feature of seven transmembrane domains [4,5]. Recently, both the δ and μ opioid receptor cDNAs have been cloned [6–8]. They display the structural motif of seven hydrophobic regions as well as the functional feature of inhibiting adenylate cyclase activity. In this paper we report the molecular cloning of a κ opioid receptor. The availability of one member from each of the three types of opioid receptors also allows an analysis of sequence similarity among these receptors.

MATERIALS AND METHODS

Isolation of cDNA clones with structural similarity to both δ and μ opioid receptors

A 1.4 kb *Hind*III cDNA fragment containing the complete protein-coding region of a rat μ opioid receptor [8] was used to screen a rat brain cDNA library [9] under low stringency.

Hybridization and the final wash were performed at 55 °C using previously described conditions [8]. Twenty clones were isolated. Sequencing revealed that one clone was highly similar to both the δ and μ opioid receptor sequences [6,8], although it was truncated in the 5' end of its open reading frame. To isolate a full-length protein coding sequence, this clone was used to rescreen the same cDNA library. Two independent clones were isolated and sequence determination from both strands of one clone indicated that it contained the entire protein-coding region. Hydropathy analyses were performed and putative post-translational modification sites of the receptor protein were identified using the PCGENE program. Similarity comparisons between KOR-1 and other G-protein-coupled receptors were performed by the BLAST program.

Expression of KOR-1 cDNA in COS-7 cells, binding analysis and adenylate cyclase assays

A 2.0 kb *Xba*I fragment encompassing the entire protein-coding region was cloned downstream of the human cytomegalovirus promoter in a mammalian-cell expression vector, pRc/CMV (Invitrogen). COS-7 cells were electroporated with supercoiled plasmid DNA and the membranes prepared as described [8]. Binding of membrane protein (10–20 μ g/reaction) was performed at room temperature for 1 h in 50 mM Tris/HCl, pH 7.4 and 0.2 % BSA. A range of 0.04–1.1 nM [³H]diprenorphine was used for saturation binding and 0.5 nM was used for displacement experiments. Reactions were terminated by vacuum filtration through Whatman GF/B filters that had been pretreated with 1 % (w/v) polyethylenimine. Non-specific binding was determined using 10 μ M naloxone. The total binding without any opioid ligand was more than 2-fold higher than the non-specific binding in the displacement experiments. Adenylate cyclase activities using membranes from cells expressing the receptor were determined as described by Salomon et al. [10] and values

Abbreviations used: DAMGO, [D-Ala²,N-Me-Phe⁴,Gly-ol⁵]enkephalin; DPDPE, [D-Pen^{2,5}]enkephalin; DSLET, [D-Ser²,Leu⁵,Thr⁶]enkephalin; DADLE, [D-Ala²,D-Leu⁵]enkephalin.

The nucleotide sequence of the rat κ opioid receptor reported here has been submitted to the EMBL, Genbank and DDBJ databases with the accession number of L22001.

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are expressed relative to the basal level control group arbitrarily set at 100%.

RESULTS AND DISCUSSION

Cloning of a rat κ opioid receptor

All three types of opioid receptors, μ , κ and δ , display overlapping ligand-binding profiles and similar cellular functions, including inhibitory coupling to adenylate cyclase and modulatory effects on Ca^{2+} and K^{+} channels [11,12]. These functional similarities suggest structural similarities and the successful cloning of a μ opioid receptor cDNA using sequence information from a δ opioid receptor confirmed this assumption [8]. Using the strategy of reduced hybridization stringency and the rat μ opioid receptor cDNA as a hybridization probe to screen a rat brain cDNA

Figure 1 Saturation binding of [^3H]diprenorphine to COS-7 cell membranes transfected with KOR-1

Saturation binding of [^3H]diprenorphine to the rat κ opioid receptor was determined using membranes from COS-7 cells transfected with the KOR-1 cDNA. Data from a representative experiment are expressed as means $\pm$ S.E.M. The inset shows the Scatchard analysis of the binding data.

Table 1 Ligand binding to the cloned rat κ opioid receptor expressed in COS-7 cells

Abbreviation: Nor-BNI, nor-binaltorphimine dihydrochloride.

Ligands	K_i (nM)
κ selective ligands	
Bremazocine	0.06
Nor-BNI	0.02
ICI 204,448	1.7
U62,066	0.9
U50,488	5.8
U69,593	9.0
Other opioid ligands	
Naloxone	20
Morphine	195
DAMGO	> 10000
DEDPE	> 10000
DADLE	> 10000
DSLET	> 10000

library, a clone was isolated with sequence similarity to both the μ and δ opioid receptors. This cDNA clone, designated KOR-1, was transiently expressed in COS-7 cells and membranes were prepared. Saturation binding was performed using [^3H]diprenorphine, a non-selective opioid antagonist with high affinity for all three types of opioid receptors, which displayed specific binding with a K_d value of 0.1 nM and a B_{max} value of approx. 354 fmol/mg of protein (Figure 1). The pharmacological profile of KOR-1 was characterized using various opioid ligands to displace [^3H]diprenorphine binding. IC_{50} values obtained from displacement curves were converted into K_i values using the Cheng and Prusoff equation [13] and are shown in Table 1. The six κ -selective ligands tested all displaced [^3H]diprenorphine binding with subnanomolar or nanomolar affinities. The μ -selective agonist [$\text{D-Ala}^2, \text{N-Me-Phe}^4, \text{Gly-ol}^5$]enkephalin (DAMGO) and the δ -selective agonists [$\text{D-Pen}^{2,5}$]enkephalin (DPDPE), [$\text{D-Ala}^2, \text{D-Leu}^6$]enkephalin (DADLE) or [$\text{D-Ser}^2, \text{Leu}^5, \text{Thr}^6$]enkephalin (DSLET) did not effectively displace [^3H]diprenorphine binding (K_i values > 10 μM). The rank order of potency for these ligands and the high affinity for κ -selective compounds indicate that KOR-1 encodes a κ opioid receptor.

All three types of opioid receptors are believed to couple to adenylate cyclase [4,12]. To examine whether the cloned κ receptor is coupled to this signalling pathway, adenylate cyclase activity was determined using membranes from transfected cells. Treatment with the κ -selective agonist, bremazocine (10 nM), resulted in the reduction of basal adenylate cyclase activity by 53% ($n = 2$, S.D. = 9.5% for the untreated control group; $n = 9$, S.D. = 2.1% for the bremazocine-treated group; $P < 0.0001$ by Student t test), indicating that this κ opioid receptor is coupled to the inhibition of adenylate cyclase. Such an inhibitory effect on adenylate cyclase has also been shown for both of the cloned opioid receptors, μ and δ [6,8].

Sequence analysis of the κ opioid receptor and comparison with the μ and δ opioid receptor types

The rat κ opioid receptor is a protein of 380 amino acids (Figure 2) as deduced from a 1140 bp open reading frame in the cDNA sequence. A mouse κ receptor has been cloned by Bell, Reisine and colleagues [14]. Comparison of the rat κ receptor with the mouse κ receptor showed a 97% similarity at the amino acid sequence level, indicating the conserved relationship between this κ receptor from these two species. Using the PCGENE software package, hydropathy analysis of the rat κ opioid receptor indicated the presence of seven hydrophobic domains, characteristic of G-protein-coupled membrane receptors [4,5,12]. Two potential glycosylation sites are present within the N-terminus, which is believed to be on the extracellular side of the cell membrane. There are three potential protein kinase C phosphorylation sites. One of these putative sites is present in the third intracellular loop between the fifth and sixth transmembrane domains, often called 'G-protein loop' because of its importance in G-protein coupling [15], suggesting that phosphorylation may play a role in modulating signal transduction of the κ receptor through G-protein coupling. The other two potential phosphorylation sites are in the C-terminus of the κ receptor. Similar phosphorylation sites in the C-terminal region of the β -adrenergic receptor have been shown to play a pivotal role in regulating receptor desensitization [16,17].

Thus one member from each of the three receptor types, μ , κ and δ has been molecularly cloned. This provides, for the first time, the opportunity to examine critically sequence similarities among opioid receptors. Such an analysis may yield important clues as to the structural basis of opioid receptor pharmacology and physiology. Figure 2 shows the amino acid sequence align-

Rat κ Opioid Receptor (KOR)	MESPIQIFRGEPTCAPSA	20
Rat μ Opioid Receptor (MOR)	MDSSTGPGNTSDCSDPLAQASCSPAPG-W	29
Mouse δ Opioid Receptor (DOR)	MELVPS-RAE	10
Rat Somatostatin Receptor (SOM)	MELT	4
KOR	CLLPNSSSWFPNWAESDSNGSVGSEDQQLPAHISPAIPVITAVYSVVF	70
MOR	LN-SHVDGNQSDPCGLNRT-LG-NDSLCPQTGSP-MVTAIT-M-L--I-C	79
DOR	LQSSPLVNLSDAFPSAFPAGANASGSPGARSAS-L-LAIA---L--A-C	60
SOM	SEQF-G-QVWIPSPFDLNGSLGP-NGSNQTEPYDMSNAVL-FI-F--C	54
KOR	VVGLVGN ⁺ SLVMFVIRYTKMKATNIYIFNLALADALVTTMPFQSAVYL	120
MOR	---F--F--Y--V-----A-S-L---VN--	129
DOR	A---L--V---G--V---L-----A-S-L---K--	110
SOM	---C--T--IY--L-A---I---L---I---E-FMLGL--LAMQVA	104
KOR	MNSWPF ⁺ GDVLC ⁺ KIVISIDYYNMTSIFTLT ⁺ MTMSVD ⁺ RYIAVCH ⁺ PVKALDFR	170
MOR	-GT---TI-----CT-----	179
DOR	-ET---EL---A-L-----	160
SOM	LVH---KAI-RV-MTV-GI-Q---C--V--I---L--V--I-SAKW-	154
KOR	TPLKAKIINICINLLASSVGISAIVLG ⁺ GKTVREDVDVIECSLQFPDDEYS	220
MOR	--RN---V-V-N-I-S-AI-LPVMFMAT--Y- QGS-D-T-T-SHPTWY	227
DOR	--A--L-----V---G--VPIM-MAV-QP- DGAVV-M---SPSWY	208
SOM	R-RT--M--VAV-GVSL--LPIPIYA-LRSNQGRSS -TINW-GESGA	203
KOR	W [#] WDLFMKICVFVFAFV [#] IPVLIIVCYTLMILRLKSVRLLSGSREKDRNLR	270
MOR	-EN-L-----I---IM-I---T--G-----M---K-----	276
DOR	-DTVT-----L---V-I---T--G--L---R-----K---S---	257
SOM	-YTG- I-YA-ILG-LV-LT--CL--LFI- [#] IKV--SGIRV--SKRKSEK	252
KOR	RITKLVLVVAVF ⁺ ICWT ⁺ PIHIFILVEALGSTSHSTAVLS SYYFCIAL	318
MOR	---RM-----V-----YVIK--I -IPE-TFQTV -WH-----	324
DOR	---RM-----GA-VV--A-----VI-WT-V DINRRDP-VVAALHL---	306
SOM	KV-RM-SI-----F--L-FY--N-SSVSV AISP-PALKGMDFEVV--	300
KOR	GYTNSSLN ⁺ FPVLYAF ⁺ LDENFKRCFRDF CFP ⁺ IKMRMERQSTNRVRNTVQDP	367
MOR	---C-----E- -I-TSSTI-Q-NST---QNTREH	373
DOR	--A-----QL -RTPCG-Q-PG-LR-P-QATTRE	355
SOM	T-A--CA--I--A--SD---KS-QNVL-LVKVSGA-DGERSDSKQDKSRL	350
KOR	ASMRDVGGMNKPV	380
MOR	P-TANTVDRTNHQLENLEAETAPLP	398
DOR	RVTACTPSDGPGGGAA	372
SOM	NETTETQRTLLNGDLQTSI	369

Figure 2 Sequence alignment of the rat κ opioid receptor with the μ and δ opioid receptors and somatostatin receptor

The amino acid sequence of the rat κ opioid receptor, as deduced from the cDNA sequence, is aligned with that of a rat μ opioid receptor [8], a mouse δ receptor [6] and a rat somatostatin receptor [19]. The seven putative hydrophobic transmembrane domains are underlined and labelled from TM1 to TM7. Amino acids identical with the κ opioid receptor are denoted as ---. Gaps are introduced for alignment. Other symbols: +, conserved aspartic acid residues possibly involved with disulphide-bond formation; *, conserved aspartic acid residues that may interact with the primary amine group found in ligands; Δ , potential N-linked glycosylation sites; #, putative protein kinase C sites; $\blacklozenge$, potential palmitoylation site; overlining of amino acids indicates conserved residues among most G-protein-coupled receptors.

ment of the rat κ opioid receptor with the μ and δ opioid receptors as well as a rat somatostatin receptor [18], which is the most similar to opioid receptors among all G-protein-coupled receptors. The seven membrane-spanning regions, identified through hydropathy analysis and by comparison with alignments of other G-protein-coupled receptors [19], are well conserved among the opioid receptors with the most similar being the second, third and seventh transmembrane domains. Both the second and third transmembrane regions contain an aspartic acid residue that is present in all three opioid receptors as well as the somatostatin receptor. These aspartic acid residues may interact with the protonated amine group on the ligands [20]. A cysteine residue conserved among all three opioid receptors occurs in the C-terminus and might function as a target site for palmitoylation. This site of post-translational modification is thought to be involved with the ligand-induced conformational

Table 2 Amino-acid similarity profile of the κ , μ and δ opioid receptors and the somatostatin receptor

Division of structural regions is based on the assignment of transmembrane domains (TM) shown in Figure 2. The similarity value is calculated for each region, with the numerator indicating the number of amino acids which are identical between the two sequences in the region and the denominator indicating the length of overlapping sequence in that region. The percentage similarity is shown in parentheses. The conserved palmitoylated (abbreviated as 'palm.') cysteine residue divides the C-terminus into two regions, the N-terminal portion (before palm.) and the C-terminal portion (after palm.). Abbreviation: SOM, somatostatin receptor.

Structural region	Amino acid similarity		
	κ/μ	κ/δ	κ/SOM
Extracellular regions			
N-terminus	6/63 (10%)	5/53 (9%)	4/47 (9%)
First loop	12/18 (67%)	13/18 (72%)	5/18 (28%)
Second loop	12/28 (43%)	11/28 (39%)	5/29 (17%)
Third loop	5/14 (36%)	2/14 (14%)	1/14 (7%)
Transmembrane domains			
TM1	14/22 (64%)	13/22 (59%)	13/22 (59%)
TM2	19/22 (86%)	19/22 (86%)	12/22 (55%)
TM3	20/22 (91%)	20/22 (91%)	12/22 (55%)
TM4	7/22 (32%)	12/22 (55%)	5/22 (23%)
TM5	16/22 (73%)	16/22 (73%)	8/22 (36%)
TM6	16/22 (73%)	14/22 (64%)	13/22 (59%)
TM7	19/22 (86%)	18/22 (82%)	11/22 (50%)
Cytoplasmic regions			
First loop	7/7 (100%)	6/7 (86%)	6/7 (86%)
Second loop	20/22 (91%)	21/22 (95%)	12/22 (55%)
Third loop	21/24 (88%)	20/24 (83%)	5/24 (21%)
C-terminus (before palm.)	11/12 (92%)	11/12 (92%)	5/12 (42%)
C-terminus (after palm.)	7/35 (20%)	5/35 (14%)	1/35 (3%)

changes observed in other G-protein-coupled receptors [21,22]. Conserved cysteine residues also occur in the first and second extracellular loops, possibly linking them with a disulphide bond. This also suggests that this secondary structure may be important for ligand-receptor interaction. Since transmembrane domains are thought to be involved in forming the hydrophobic 'pockets' into which ligands fit, the structural similarity in these regions might explain the overlap in the pharmacological profiles of the opioid and somatostatin receptors. Variations in the primary amino acid sequence within these domains may also contribute to the unique binding capabilities of each opioid receptor.

Based on the seven-transmembrane-helices model, G-protein-coupled receptors can be divided into three groups of structural domains: extracellular regions including the N-terminus and the extracellular loops, the seven transmembrane domains, and the intracellular loops including the cytoplasmic loop and the C-terminus. A comparison of the sequences among the opioid and somatostatin receptors in each of these domains reveals an uneven distribution of sequence similarity (Table 2). Of the four extracellular regions, the first extracellular loop contains the highest level of similarity among the opioid receptors and differentiates them from the somatostatin receptor. If extracellular regions are involved in ligand binding, this loop would probably contribute to the overlap in the binding properties of opioid receptors. Transmembrane domains TM2 and TM3 are highly conserved among the opioid receptors, supporting the hypothesis that they play a role in ligand binding [20]. Transmembrane domain TM7 is also very similar among the three opioid receptors. Although its function has not been thoroughly assessed, TM7 may maintain such sequence conservation due to

Figure 3 Schematic diagram of protein structure for, and similarity distribution among, the three opioid receptors

The N-terminus is located extracellularly and the C-terminus is located intracellularly. The N- and C-termini of each opioid receptor are marked by arrows and the corresponding receptor name. The putative disulphide bond is depicted as (-S-S-) and the palmitoylation site is denoted as P. The membrane lipid bilayer is shown as an open box. Key to shadings: ●, identical among all three receptors; ◐, identical in two out of three receptors; ○, unique for each receptor.

its proximity to the N-terminal portion of the C-terminus. This stretch of amino acid sequence is believed to form a 'fourth' intracellular loop by an anchor of the fatty acid chain of the palmitoylated cysteine residue in the cytoplasmic side of the membrane. Such an anchored structure has been shown to be critical for the functional regulation of rhodopsin and the β -adrenergic receptor [21,22]. Another striking feature among the opioid receptors is the similarities in the second and third intracellular loops. Since the third loop has been shown to be necessary for interactions with G-proteins, this similarity would indicate a similarity in G-protein-mediated signal transduction by all three opioid receptors, a fact substantiated by the cloning of μ [8], δ [6] and now κ opioid receptors.

To provide visual representation for the similarity distributions among the three opioid receptors, sequence identities are depicted graphically (Figure 3). The most conserved regions are the transmembrane domains TM2, TM3 and TM7, the first extracellular loop, and the second, third and 'fourth' cytoplasmic loops. The high levels of sequence similarity suggest that these structures may contribute to shared properties among the three types of opioid receptors, including ligand binding, G-protein coupling and receptor regulation. Transmembrane domains TM1, TM4-6, and the second and third extracellular loops are less conserved at the sequence level. Specific roles, if any, for these domains remain to be determined. The greatest divergence among the opioid receptors lies at the N- and C-terminal ends. This is reflected not only in the lowest levels of sequence similarity, but also in the difference in length among the three opioid receptors. This might imply that the most distinguishing features among these receptors may depend on structural differences located in these areas. In the β -adrenergic receptor, for example, regulation of the receptor has been shown to require the C-

terminal region and a substrate-selective protein kinase [23,24]. If the opioid receptors are regulated in a similar fashion, differences in the C-terminal region may act to generate substrate diversity and provide a means of modulating receptor function in a type-specific manner.

We have described the cloning of a rat κ opioid receptor which belongs to the G-protein-coupled family of membrane receptors and displays inhibitory coupling to adenylate cyclase. The sequence information from the κ receptor has enabled comparison to be made among all three types of opioid receptors. The structural similarities and differences outlined above thus provide a basis for postulating specific hypotheses of structure-function relationships among opioid receptors. The availability of the receptor clones therefore allows vigorous testing of these models by systematic mutagenesis. These studies would facilitate molecular modelling of opioid-receptor structure, and aid in the rational design of novel opioid ligands that have greater selectivity and efficacy, with fewer undesirable side effects.

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