RESEARCH COMMUNICATION

Molecular cloning and expression of a rat κ opioid receptor

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At least three types of opioid receptors have been identified in the nervous system. In this paper we report molecular cloning and expression of a rat κ opioid receptor. PCR was performed on double-stranded cDNA derived from poly(A)⁺ RNA of the rat striatum with primers similar to those of Libert and co-workers [Libert, Parmentier, Lefort, Dinsart, Van Sande, Maenhaut, Simons, Dumont and Vassart (1989) *Science* **244**, 569–572]. One of the PCR products, which had 65% sequence similarity to the mouse δ opioid receptor, was used to screen a rat striatum cDNA library. Two positive clones were isolated and found to be identical. The clone had a 2.1-kb insert, which was termed RKOR-1. RKOR-1 has an open reading frame of 1140 bp and

encodes a 380-amino-acid protein. Hydropathy analysis indicates that RKOR-1 has seven putative transmembrane domains with short intra- and extra-cellular loops. Membranes of Cos-7 cells transfected with RKOR-1 exhibited high specific binding for [³H]diprenorphine ([³H]DIP), a non-selective opioid ligand. Naloxone inhibited [³H]DIP binding with stereospecificity. [³H]DIP binding was potently inhibited by selective κ opioid ligands, with K_i values in the nanomolar or subnanomolar range, but much less potently inhibited by drugs selective for μ or δ receptors. Thus, RKOR-1 represents an opioid receptor with κ characteristics.

INTRODUCTION

Opiates and opioid compounds act on receptors on the surface of cell membranes to produce their effects. Opioid receptors play important roles in many physiological functions, including modulation of pain perception, regulation of respiratory, gastrointestinal, cardiovascular, urinary and immune functions, and regulation of body temperature [1]. Development of tolerance and dependence to opioid compounds has been thought to be mediated through opioid receptors [1]. The presence of three classes of opioid receptors, μ , κ , δ , in the peripheral and central nervous system has been established by pharmacological and binding studies (for a review see [2]). Receptor autoradiography studies performed *in vitro* indicate that different types of opioid receptors have differential regional distributions in the central nervous system (for a review see [3]). Three groups of endogenous opioid peptides have been identified, enkephalins for δ receptors, dynorphins for κ receptors, and presumably β -endorphin for μ receptors. κ opioid receptors have been shown to mediate many physiological and pharmacological effects, including analgesia, diuresis, hypothermia and dysphoria.

There is ample evidence supporting the notion that opioid receptors belong to the superfamily of G-protein-coupled receptors. In neuronal cells, activation of opioid receptors is coupled, via pertussis-toxin-sensitive G proteins, to various effectors including adenylate cyclase and K⁺ and Ca²⁺ channels (for a review see [4]). In recent years, many G-protein-coupled receptors have been cloned. Analysis of the deduced amino acid sequences has indicated that they have a motif of seven transmembrane regions. A putative κ -receptor cDNA was published previously [5] that has the features of a G-protein-coupled receptor. However, upon expression, this clone exhibits low affinities for opioid compounds and does not display the ligand selectivity expected

of a κ receptor. More recently, Evans et al. [6] and Kieffer et al. [7] cloned and expressed a mouse δ opioid receptor. The deduced amino acid sequence of this clone displays the motif of seven transmembrane domains. When transiently expressed, the clone binds opioid compounds selective for δ receptors with much higher affinities (K_i values in the nanomolar range) than it does selective μ or κ drugs. Opioid binding to the expressed receptor is stereospecific. Activation of the expressed receptor leads to inhibition of adenylate cyclase. Subsequently, using screening under low stringency conditions, Chen et al. [8] cloned a rat μ opioid receptor. When looking for novel somatostatin receptors, Yasuda et al. [9] cloned two G-protein-coupled receptors from mouse brain, which were identified as δ and κ opioid receptors.

There is considerable sequence similarity in transmembrane domains among previously cloned G-protein-coupled receptors. Taking advantage of the sequence similarity, Libert et al. [10] devised a strategy for cloning new G-protein-coupled receptors. They designed two sets of degenerate oligonucleotide primers, corresponding to part of putative transmembrane domains III and VI respectively, and performed PCR on cDNA derived from the dog thyroid. They were able to identify four PCR products representing partial sequences of potentially new G-protein-coupled receptors. The respective full-length clones of these four PCR products were identified as A₁ and A₂ adenosine receptors (RDC7 and RDC8) [11,12], vasoactive intestinal polypeptide receptor (RDC1) [13] and related to 5-hydroxytryptamine receptor (RDC4). We employed a similar approach to clone new G-protein-coupled receptors from the rat striatum. The rat striatum was used because it expressed relatively high levels of all three classes of opioid receptors. One of the PCR products, which had putative transmembrane domains IV and V, displayed 65% amino acid sequence identity with the mouse δ opioid receptor [6,7]. In this study we isolated the full-length clone of this

Abbreviations used: ds, double-stranded; [³H]DIP, [³H]diprenorphine; [³H]EKC, [³H]ethylketocyclazocine; EGTA, [ethylenedis(oxonitrilo)]tetraacetate; DAMGO, [D-Ala²,MePhe⁴,Gly-ol⁵]enkephalin; DPDPE, [D-Pen²,D-Pen⁵]enkephalin; D-Pen, D-penicillamine; 1 × SSC, 150 mM NaCl/15 mM sodium citrate.

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The cDNA sequence of RKOR-1 has been submitted to GenBank (accession number L22536).

fragment. This clone encodes a 380-amino-acid protein, which has seven putative transmembrane domains. When transiently expressed in Cos-7 cells, this clone exhibits characteristics of a κ opioid receptor.

EXPERIMENTAL

PCR

RNA was extracted from fresh striata of Sprague–Dawley rats according to the method described by Chomczynski and Sacchi [14]. Poly(A)⁺ RNA was isolated from total RNA with Poly A Tract mRNA Isolation System (Promega, Madison, WI, U.S.A.) by use of biotinylated oligo(dT) and streptavidin–paramagnetic particles. Double-stranded (ds) cDNA synthesis was performed with SuperScript Choice System for cDNA synthesis (GIBCO BRL, Gaithersburg, MD, U.S.A.). The first-strand cDNA synthesis was performed with RNAase H⁻ reverse transcriptase using oligo(dT)_{12–18} as the primer and poly(A)⁺ RNA as the template. The second-strand cDNA synthesis was conducted by nick translational replacement of the mRNA according to Gubler and Hoffman [15] with *Escherichia coli* DNA polymerase I, RNAase H and DNA ligase. PCR was performed with *Taq* DNA polymerase on ds cDNA in a DNA thermal cycler (Bellco Glass, Vineland, NJ, U.S.A.) (93 °C for 3 min, 55 °C for 2.5 min, and 72 °C for 3 min for 30 cycles). Sequences of the PCR primers are as follows: 5' primer: CTGTG(C/T)G(C/T)(C/G)AT(C/T)-(A/G)CIIT(G/T)GAC(C/A)G(C/G)TAC and 3' primer: AG(T/A)AGGGCAICCGAGAI(G/C)(G/A)(T/C)GAA.

These primers are modified versions of those of Libert et al. [10] without restriction enzyme linkers. PCR products ranged from 300 to 900 bp and were fractionated by PAGE into three fractions: 300–400, 400–615, and 615–900 bp. Each fraction was eluted from the gel and cloned into TA cloning vector (Invitrogen, San Diego, CA, U.S.A.), which had β -galactosidase selection. DNA sequences of PCR products were determined with the dideoxynucleotide-chain-termination method of Sanger et al. [16] using Sequenase version 2.0 sequencing kit (United States Biochemical, Cleveland, OH, U.S.A.) and [α -³⁵S]dATP (> 1000 Ci/mmol) (Dupont-NEN, Boston, MA, U.S.A.). Nucleotide sequence analysis was performed for similarity to previously cloned G-protein-coupled receptors by searching GenBank with the fasta program [17]. Among 187 clones that were sequenced and analysed, we found 12 previously cloned G-protein-coupled receptors (RDC4, D₁ and D₂ dopamine, α_{1a} and β_2 adrenergic, cannabinoid, rat GPCR, A₁, A₂ and A₃ adenosine, rat CGPCR, 5HT_{1D}, endothelin A) and 20 unknown clones. One of the unknown clones, which was 427 bp in length, exhibited 65% sequence similarity to the mouse δ receptor [6,7]. The 403-bp *EcoRI*–*HindIII* fragment of this clone (due to the presence of an internal *HindIII* site) was subcloned into pGEM3zf(+) (Promega) and used as the probe to screen a cDNA library and for Northern-blot analysis.

Isolation of cDNA clones

The probe was labelled with [α -³²P]dCTP (3000 Ci/mmol) (Dupont-NEN) to a specific radioactivity of 1×10^9 c.p.m./ μ g by use of random oligonucleotides as primers and the Klenow fragment of DNA polymerase I (GIBCO BRL). Screening of a rat striatum λ gt10 cDNA library was performed according to Benton and Davis [18]. Prehybridization was carried out in $1 \times$ Denhardt's solution/100 μ g/ml salmon sperm DNA/50 mM NaCl/5 mM Tris/HCl (pH 8.0)/0.5 mM EDTA at 65 °C for 2 h. Hybridization was performed with labelled probe in $6 \times$ SSC

($1 \times$ SSC = 150 mM NaCl/15 mM sodium citrate)/ $5 \times$ Denhardt's solution/0.1% sodium pyrophosphate at 65 °C for 18 h. A final wash was conducted with $6 \times$ SSC/0.1% sodium pyrophosphate three times at 50 °C. Two independent clones were isolated and subcloned into pGEM3zf(+) for dsDNA sequence determination [16]. Three fragments of 2.1, 1.5 and 0.6 kb were identified after *EcoRI* digestion. Subsequent sequence determination revealed that an internal *EcoRI* site was present and the sequences of the two clones were identical. The 2.1-kb insert was termed RKOR-1. Hydropathy analysis on the deduced amino acid sequence was performed according to the method of Kyte and Doolittle [19]. Similarity to previously cloned proteins were determined by searching GenBank with the fasta program [17].

Northern-blot analysis

Poly(A)⁺ RNA was isolated from the rat brain as described above. mRNA (5 μ g) was run on a 1% (w/v) agarose gel containing formaldehyde and then transferred to a nitrocellulose filter. The probe was labelled with [α -³²P]dCTP as described above. Hybridization of radiolabelled probe to RNA on nitrocellulose filter was performed at 42 °C overnight in 50% formamide/ $5 \times$ Denhardt's reagent/0.1% SDS/100 μ g/ml salmon sperm DNA/100 μ g/ml yeast tRNA. The blot was washed with $1 \times$ SSC/0.1% SDS at 55 °C for 30 min, air-dried and autoradiographed at –70 °C for 2 days.

Transfection of Cos-7 cells with pcDNA3-RKOR-1

The 2.1-kb insert (RKOR-1), resulting from partial digestion with *EcoRI*, was subcloned into the *EcoRI* site of the mammalian expression vector pcDNA3 (Invitrogen). Cos-7 cells were obtained from American Type Culture Collection (Rockville, MD, U.S.A.) and grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (Hyclone), penicillin G (100 units/ml) and streptomycin (100 μ g/ml) at 37 °C in 5% CO₂. When cells reached 50–70% confluency, transfection was performed with Lipofectin reagent (1 mg/ml) (GIBCO BRL) at 30 μ l of Lipofectin and 10 μ g of DNA per 85 mm-diam. dish according to the method of Felgner et al. [20]. After 2–2.5 days, cells were harvested. Membranes were prepared by hypotonic lysis with 20 mM Tris/HCl buffer (pH 7.5)/5 mM MgCl₂, followed by sonication, homogenization and centrifugation at 40000 *g* for 30 min at 4 °C.

Binding experiments

Opioid receptor binding was conducted with [³H]DIP (35 Ci/mmol) (Amersham, Arlington Heights, IL, U.S.A.) or [³H]ethylketocyclazocine ([³H]EKC) (24.5 Ci/mmol) (Dupont-NEN) at room temperature in 50 mM Tris/HCl buffer (pH 7.4) containing 1 mM EGTA, 10 μ M leupeptin and 0.2% BSA for 60 min. Naloxone (10 μ M) was used to define non-specific binding. Each binding assay was carried out in duplicate in a final volume of 1 ml was 25–60 μ g of protein/tube. Saturation binding experiments were performed with nine concentrations of [³H]DIP (ranging from 0.05 nM to 2.5 nM) in the presence or absence of 10 μ M naloxone. Competitive inhibition of [³H]DIP binding was performed with 0.4 nM [³H]DIP and nine concentrations of the unlabelled drug. After incubation, bound and free ligand were separated by rapid filtration over GF/B filters under vacuum. GF/B filters were pre-soaked with 0.1% polyethyleneimine for 20 min. Filters were washed three times with

	1	a	a	41		
RKOR-1	MESPIQIFRGEPTCAPSACILNENSSWFPNW.....AESDSNGS					
MDOR-1	MELV---RAELQ---PLVNLS....DAFPSPFP-A-A					
RMOR-1	MDSSTGPGNTSDCSPLAQ-SCS-APG--LNLSHVDGNQSDQGLNRT-L					
	42	TM1		91		
RKOR-1	VGSEDDQLEPAHISPAIPVITAVYSVVEVLGVNLSLMEFVIIRYTKMK					
MDOR-1	NA-GSPGARS-S.-L-LATA---L-A-CA---L-V---G-V---L-					
RMOR-1	G-NDSLCPQTGSP-MVTAIT-M-L---I-C---F-F---Y-V---					
	92	b	TM2	c	b	141
RKOR-1	TATNTIYIFNLALADALVTTIMFQSAVYLMNSWPFQDVLCIVISIDYYN					
MDOR-1	-----A-S-L---K-ET---EL---A-L---					
RMOR-1	-----A-S-L---VN-GT---TI---					
	142	TM3		191	TM4	
RKOR-1	MFTSIFTLTMSVDRIYAVCHPVKALDFRTPKAKIINICINLLASSVGI					
MDOR-1	-----A-L---V-G-V---					
RMOR-1	-----CT-----RN---V-V-N-I-S-AI-L					
	192	c		241	TM5	
RKOR-1	SAIVLGGTKVREDVDVIECSLQFPDDEYSWDLFMKICVEFAFVIPVLI					
MDOR-1	PIM-MAV-QP-D..GAVV-M---SPSW.Y-TVT---L---V-I---					
RMOR-1	PVMFMAT--Y-Q..GS-D-T-T-SHTW.Y-ENLL---I---IM-I---					
	242	d	d	d	TM6	291
RKOR-1	IIVCYTLMILRLKSVRLLSGSREKDRNLRRITKLVLVVAVFIQWTFIH					
MDOR-1	-T-G-L-R---K-S---RM---CA-VV-A---					
RMOR-1	-T-G-----M-K---RM---V---					
	292	TM7		e	340	
RKOR-1	IFILVEALGSTSHSTA.VLSSYYFCIALGYTNSSINFLVAFIDENFKRC					
MDOR-1	--VI-WT-VDINRDEL-VAAHL---A---					
RMOR-1	-YVIK--ITI..PE-TFQIV-NH---C---					
	341	dd	d	380		
RKOR-1	FRDFYFPKIMMERQSTNFRV.NTVQDPASMRQVGMKPV.....					
MDOR-1	--QLCRTPOG-Q-PG-IR-P-QA-IRERVACTPS.....DGGGGRAA					
RMOR-1	--E-CI-TSSTI-Q-NST---Q-REH-STANT-DRT-HOLENLEAETAP					
RKOR-1	..					
MDOR-1	..					
RMOR-1	IP					

Figure 1 Amino acid sequence of a rat κ opioid receptor (RKOR-1) and its alignment with mouse δ (MDOR-1) and rat μ (RMOR-1) opioid receptors [6-8]

Seven putative transmembrane domains (TM) are underlined with = and numbered TM1 to TM7. ... indicate spaces introduced for alignment. - indicates identical amino acid to RKOR-1. Key to markers: a, putative Asn-linked glycosylation site; b, Asp residues within TM2 and TM3, which may be involved in the interaction with amine or basic moiety of the ligand; c, potential disulphide-bond formation between two Cys residues within the first and second extracellular loops; d, potential phosphorylation sites for cyclic AMP-dependent protein kinase and protein kinase C within the third intracellular loop and the C-terminal domain; e, potential palmitoylation site within the C-terminal domain.

ice-cold 50 mM Tris/HCl buffer. Radioactivity in the filters was determined by liquid-scintillation counting. Protein contents were determined by use of the bicinchoninic acid protein assay reagent [21] (Pierce, Rockford, IL, U.S.A.). Binding data were analysed with the EBDA program [22]. K_i values were calculated according to the equation of Cheng and Prusoff [23].

RESULTS AND DISCUSSION

Two positive clones were identified. DNA sequences of both strands of the two clones were determined and the sequences were identical. The clone has a 2.1-kb insert, which was termed RKOR-1. RKOR-1 contains the Kozak nucleotide sequence CCATGG surrounding ATG for translation initiation. RKOR-1 has an open reading frame of 1140 bp, which encodes a protein of 380 amino acids with a molecular mass of 42 748 Da. The

Figure 2 Northern-blot analysis of RKOR-1 in the rat brain

A Northern blot of Poly(A)⁺ RNA (5 μ g) from the rat brain hybridized with the probe (as described in the Experimental section) shows a single transcript of 5.5 kb.

amino acid sequence of RKOR-1 is shown in Figure 1. Hydrophathy analysis [19] reveals the presence of seven putative transmembrane domains separated by short intra- and extracellular loops. Thus this protein appears to be very hydrophobic. Two consensus asparagine-linked glycosylation sites (Asn²⁵ and Asn³⁹) are present within the N-terminal domain. A pair of cysteine residues (Cys¹³¹ and Cys²¹⁰) may form a disulphide bond between the first and second extracellular loops, similar to many G-protein-coupled receptors. There are several potential phosphorylation sites for cyclic AMP-dependent kinase and protein kinase C: three within the third intracellular loop (Ser²⁵⁶, Ser²⁶⁰, Thr²⁷³) and three within the C-terminal domain (Ser³⁵⁶, Thr³⁵⁷, Ser³⁶⁹). Phosphorylation of these potential sites may play an important role in receptor desensitization. Similar to catecholamine receptors, there is an aspartate residue (Asp¹³⁸) within the third transmembrane domain, which may interact with the amino group or other basic moieties of the ligand. An aspartate residue (Asp¹⁰⁵) in the second transmembrane domain may also be important in ligand binding. A potential cysteine-linked palmitoylation site is present within the C-terminal domain (Cys³⁴⁰). In the rat κ receptor cloned by Chen et al. [24] and the mouse κ receptor cloned by Yasuda et al. [9], a cysteine-linked palmitoylation site was assigned to Cys³⁴⁵, at which position is Tyr in our sequence. Whether this phenotypic variation has any functional consequence remains to be determined.

RKOR-1 displays amino acid sequence similarity to many G-protein-coupled receptors. The sequence identity of RKOR-1 to other receptors is as follows: μ opioid (60%), δ opioid (59%), somatostatin (33%), AT_{1B} angiotensin II (27%), AT_{1A} angiotensin II (26%), FMLP-related-II (24%), NPY₃ neuropeptide Y (23%), interleukin 8 (23%), NPY₁ neuropeptide Y (21%), H₂ histamine (21%) and thrombin (20%). Amino acid sequence alignment of RKOR-1 with a mouse δ opioid receptor [7], and a rat μ opioid receptor [8] is depicted in Figure 1. As evident from this figure, μ , δ and κ receptors are highly homologous, particularly in the sequence from transmembrane domains 1 to 7. In contrast, the N-terminal domain, the C-terminal domain and the second extracellular loop are very diverse among these receptors. These divergent sequences may play an important role in the ligand selectivity of these receptors.

Northern-blot analysis of poly(A)⁺ RNA from the rat brain showed a single transcript of approx. 5.5 kb (Figure 2). The

Figure 3 Competitive inhibition of [3 H]DIP binding to membranes of Cos-7 cells transfected with RKOR-1 by (a) CTAP ($\blacklozenge$), naltrindole ($\diamond$), Nor-BNI ($\blacktriangle$), ($-$)-naloxone ($\circ$) and ($+$)-naloxone ($\square$); (b) DAMGO ($\diamond$), DPDPE ($\blacktriangle$), U50,488H ($\circ$), dynorphin (1–17) ($\bullet$), dynorphin (2–17) ($\square$) and α -neoendorphin ($\blacksquare$)

K_i values of these compounds are listed in Table 1. Each point represented the mean of three independent experiments, each performed in duplicate, and variation from the mean was less than 10%. In each experiment, total binding ranged from 2500 to 3500 d.p.m., non-specific binding ranged from 150 to 300 d.p.m., and specific binding represented 90–95% of total binding.

difference between the mRNA transcript size (5.5 kb) and the insert size (2.1 kb) is most likely due to two factors. First, fortuitous priming in cDNA synthesis may occur in the AG-rich region of mRNA, instead of the poly(A) $^+$ tail, by oligo(dT). Secondly, there may be a long 5'-untranslated region.

Membranes of Cos-7 cells transfected with pcDNA3-RKOR-1 exhibited high specific [3 H]DIP and [3 H]EKC binding. In contrast, membranes of Cos-7 cells transfected with pcDNA3 did not show any specific binding. Binding of [3 H]DIP was saturable and K_d and B_{max} were calculated to be 0.48 nM and 1069 fmol/mg of protein ($n = 2$) respectively. While ($-$)-naloxone inhibited 0.4 nM [3 H]DIP binding with a K_i value of 10.5 nM, ($+$)-naloxone did not inhibit binding even at 1 μ M (Figure 3a). This result indicates stereospecificity of opioid binding to RKOR-1. [3 H]DIP binding was potently inhibited by naltorphimine, a selective κ antagonist, but much less by naltrindole, a selective δ antagonist, or cyclic D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH $_2$, a selective μ antagonist (Figure 3a). U50,488H, a selective κ agonist, was much more potent than [D-Ala 2 ,MePhe 4 ,Gly-ol 5]enkephalin (DAMGO), a selective μ

Table 1 K_i values of binding of opioid compounds to RKOR-1 transiently expressed in Cos-7 cells

Data were expressed as means $\pm$ S.E.M. for each compound. Each K_i value was derived from three independent experiments. Abbreviations: CTAP, cyclic D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH $_2$; Nor-BNI, naltorphimine.

Compound	K_i value (nM)
μ -Selective ligands	
DAMGO	> 1000
CTAP	> 1000
δ -Selective ligands	
DPDPE	> 1000
Naltrindole	12.9 $\pm$ 2.0
κ -Selective ligands	
U50,488H	2.5 $\pm$ 0.5
Nor-BNI	0.04 $\pm$ 0.01
Dynorphin A (1–17)	0.05 $\pm$ 0.01
α -Neoendorphin	0.10 $\pm$ 0.01
Others	
($-$)-Naloxone	10.5 $\pm$ 1.4
($+$)-Naloxone	> 1000
Dynorphin A (2–17)	> 1000

agonist, or [D-Pen 2 ,D-Pen 5]enkephalin (DPDPE), a selective δ agonist, in inhibiting [3 H]DIP binding (Figure 3b). In addition, dynorphin A (1–17) and α -neoendorphin had high affinities for RKOR-1, whereas dynorphin A (2–17) did not bind (Figure 3b). The K_i values of these drugs are listed in Table 1. These K_i values are in general agreement with those in the literature [25–28]. In addition, [3 H]EKC binding was potently inhibited by U50,488H, but not at all by DAMGO or DPDPE (results not shown). These results indicate that RKOR-1 represents an opioid receptor with κ characteristics, most likely κ_1 type due to its high affinity for U50,488H. The cloning of this κ opioid receptor will facilitate studies on its structure–function relationship, signal transduction, desensitization, down regulation and regulation of gene expression. Computer modelling of the structure of this protein may help in the design of selective drugs. Understanding of regulation of the receptor at the DNA, RNA and protein levels will better elucidate the mechanism of tolerance and dependence.

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