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Primary structures and expression from cDNAs of rat opioid receptor δ-and μ-subtypes

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The complete amino acid sequences of rat opioid receptors (designated as ROR-A and ROR-B) have been deduced by cloning and sequencing the cDNAs. The ligand-binding properties of ROR-A and ROR-B expressed from the cloned cDNAs in Chinese hamster ovary cells correspond most closely to those of the pharmacologically defined δ- and μ-opioid receptor subtypes, respectively. RNA blot hybridization analysis revealed that cerebrum and brainstem contain both ROR-A and ROR-B mRNAs, whereas neither ROR-A nor ROR-B mRNAs can be detected in cerebellum.

Opioid receptor; cDNA cloning; cDNA expression; RNA blot hybridization analysis; Rat brain

1. INTRODUCTION

The opioid receptors exhibit a widespread distribution throughout central and peripheral nervous systems and mediate pharmacological actions of opioid analgesics, such as morphine, and physiological effects of endogenous opioid peptides derived from proopiomelanocortin, preproenkephalin A and preprodynorphin [1]. At the molecular level, activation of the opioid receptors leads to inhibition of adenylate cyclase, activation of potassium channels and inhibition of calcium channel activity through the action of guanine nucleotide-binding regulatory proteins (G-proteins) [2,3]. Pharmacologically, the opioid receptors have been classified into at least three subtypes (μ, κ and δ) on the basis of their difference in apparent affinity for opioid ligands [1,2]. However, there has been no direct evidence for the presence of molecularly distinct opioid receptor subtypes. Recently, the cDNA cloning of the mouse δ-opioid receptor has been reported [4,5]. In the present investigation, we have cloned two cDNAs encoding opioid receptors (designated as ROR-A and ROR-B) from rat cerebrum cDNA libraries and analyzed their ligand-binding properties by expression of the cloned cDNAs in Chinese hamster ovary (CHO) cells. The results obtained indicate that ROR-A and ROR-B represent the δ- and μ-opioid receptor subtypes, respectively, and provide evidence indicating that distinct gene products underlie pharmacologically different opioid receptor subtypes.

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Abbreviations: G-proteins, guanine nucleotide-binding regulatory proteins; CHO cells, Chinese hamster ovary cells; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DPDPE, [D-penicillamine^2, D-penicillamine^5]enkephalin; DSLET, [D-Ser^2,Leu^5]enkephalin-Thr; DAGO, [D-Ala^2,MePhe^4,Glyol^5]enkephalin.

2. MATERIALS AND METHODS

2.1. *Cloning of cDNAs*

A randomly primed and an oligo(dT)-primed cDNA library were constructed in phage λgt10 using poly(A)$^+$ RNA prepared [6,7] from adult Wistar rat cerebrum. They were screened with the mouse δ-opioid receptor cDNA probe prepared as follows. Polymerase chain reaction (PCR) amplification was carried out using cDNA synthesized from poly(A)$^+$ RNA prepared from adult ICR mouse brain and synthetic primers corresponding to nucleotide residues 77–101 (sense) and 400–424 (antisense) of the mouse δ-opioid receptor cDNA [4]. The amplified PCR product was blunt-ended and cloned into the *Sma*I site of pBluescript SK(−) (Stratagene) to yield the plasmid, pMOR1. The 0.35-kilobase pairs (kb) *Bam*HI–*Pst*I fragment from pMOR1 was used as a probe. cDNA inserts from clones positive to the probe were subcloned into pBluescript SK(−) and further analyzed. Five clones, including pROR1 and pROR10, carried common *Sac*I- and *Sma*I fragments, indicating that they are derived from identical mRNAs (ROR-A). cDNA inserts of 3 clones, including pROR15, share a common restriction endonuclease map (*Bam*HI, *Xho*I, *Hin*dIII, *Pst*I and *Eco*RV) and are regarded as transcripts of identical mRNAs (ROR- B). cDNA clones pROR10 and pROR15 carry the entire protein- coding sequences and were used for sequence analysis [8,9].

2.2. *Expression of cDNAs and binding assay*

The ~ 2.1-kb *Eco*RV–*Xba*I fragment from pROR10 and the 1.24-kb *Aat*I–*Hin*dIII fragment from pROR15, containing the entire protein-coding sequences, were inserted into the *Hin*dIII site of an expression vector pKNH [10] to yield the plasmids, pRORS10-1 and pRORS15-1, respectively. CHO cells in culture were transfected with pRORS10-1 or pRORS15-1 cleaved with *Pvu*I [11]. Clones expressing ROR-A (CROR10–17) or ROR-B (CROR15–27) were isolated by

screening G418-resistant clones by RNA blot hybridization analysis using each cDNA probe.

Cells were washed with phosphate-buffered saline twice and homogenized with a Dounce homogenizer in 50 mM Tris-HCl, pH 7.5. The homogenate was centrifuged at 1,000 × *g* for 10 min and the precipitate suspended in the same buffer, homogenized and centrifuged at 1,000 × *g* for 10 min. The two supernatants were combined and centrifuged at 20,000 × *g* for 30 min. The pellet was suspended in the same buffer and used for binding assays. [^{3}H]DADLE binding reaction was performed with membrane preparations (40–70 μg of protein per ml) in 0.2 or 1.0 ml of 50 mM Tris-HCl, pH 7.5, at 37°C for 45 min. After incubation, the samples were collected on GF/B filters (Whatman) and washed with 10 ml of 50 mM Tris-HCl, pH 7.5. The filters were then counted for radioactivity.

2.3. *RNA blot hybridization analysis*

Poly(A)$^+$ RNAs prepared from adult Wistar rats were analyzed as described previously [12], except that a 0.8% agarose gel was used and the filters were washed with 0.3 × SSC containing 0.1% SDS. The hybridization probes used were the ~ 3.0-kb cDNA insert of pROR1 containing 1.08 kb of the protein-coding sequence of ROR-A and the ~ 2.8-kb cDNA insert of pROR15. The probes were labelled with [α-^{32}P]dCTP by the random primer method [13]. Autoradiography was performed at −80°C for 10 days with an intensifying screen. An RNA ladder (Bethesda Research Laboratories) and the *Hin*dIII cleavage products of phage λ DNA were used as size markers.

3. RESULTS AND DISCUSSION

cDNA libraries derived from rat cerebrum poly(A)$^+$ RNA were screened by hybridization with a DNA fragment comprising a part of the mouse δ-opioid receptor cDNA [4]. Two classes of cDNA clones (ROR-A and ROR-B) were isolated (see section 2.1) and sequence analysis revealed open reading frames that encode sequences of 372 and 398 amino acids, respectively. Fig. 1 shows the alignment of the deduced amino acid sequences of the ROR-A and ROR-B proteins. Amino acid sequence comparison with each other revealed 61% identity. The ROR-A protein showed 97% sequence identity compared with the mouse δ-opioid receptor [4,5]. Seven transmembrane segments can be predicted in both of the ROR-A and ROR-B proteins, which is the characteristic structural feature of the G-protein-coupled receptors [14]. Furthermore, as with many of the G-protein-coupled receptors, the amino-terminal region preceding the predicted transmembrane segment, TM-I, of these proteins contains potential sites of *N*-glycosylation [15]; 2 sites are found in ROR-A and 5 sites in ROR-B. The putative cytoplasmic region between TM-V and TM-VI, which has been shown to be involved in selective effector coupling of the muscarinic acetylcholine receptor [16] and the adrenergic receptor [17], is conserved well between ROR-A and ROR-B. This observation may suggest that ROR-A and ROR-B couple with a similar type of G-protein.

To examine whether the cloned cDNAs encode the opioid receptors, we performed expression studies. CHO cells were stably transformed with the cDNAs, and membrane preparations from the transformed clones were tested for the opioid ligand-binding properties (Table I). Saturation analysis of [^{3}H]DADLE binding (Fig. 2) revealed that ROR-A and ROR-B are capable of binding DADLE, an opioid agonist with a high affinity for both δ- and μ-opioid receptor subtypes, with similar high affinities. The apparent K_d values for the other opioid ligands were obtained by measuring displacement of [^{3}H]DADLE binding by increasing concentrations of the ligands. As expected from the 97% amino acid sequence identity observed between ROR-A and the mouse δ-opioid receptor, ROR-A showed ligand-binding specificity of the δ-opioid receptor subtype. DPDPE and DSLET, agonists with the highest affinity for the δ-opioid receptor subtype, showed high affinities for ROR-A (K_d = 28 nM and 12 nM, respectively), whereas the affinity of ROR-A for DAGO, an

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ROR-A  MEPVPSARAELQFSLLANVSDTFPSA-------FPSASANASGSPG--------------------ARSASSLALAIAITALYSAVCAV  62
ROR-B  MDSS--------TGP-GNTSDCSDPLAQASCSPAPGSWLNLSHVDGNQSDPCGLNRTGLGGNDSLCPQTGSPSMVTAITIMALYSIVCVV  81
                                                                                          TM-I

ROR-A  GLLGNVLVMFGIVRYTKLKTATNIYIFNLALADALATSTLPFQSAKYLMETWPFGELLCKAVLSIDYYNMFTSIFTLTMMSVDRYIAVCH 152
ROR-B  GLFGNFLVMYVIVRYTKMKTATNIYIFNLALADALATSTLPFQSVNYLMGTWPFGTILCKIVISIDYYNMFTSIFTLCTMSVDRYIAVCH 171
                                    TM-II                                  TM-III

ROR-A  PVKALDFRTPAKAKLINICIWVLASGVGVPIMVMAVTQPRDGAVVCTLQFPSPSWYWDTVTKICVFLFAFVVPILIITVCYGLMLLRLRS 242
ROR-B  PVKALDFRTPRNAKIVNVCNWILSSAIGLPVMFMATTKYRQGSIDCTLTFSHPTWYWENLLKICVFIFAFIMPVLIITVCYGLMILRLKS 261
                              TM-IV                                    TM-V

ROR-A  VRLLSGSKEKDRSLRRITRMVLVVVGAFVVCWAPIHIFVIVWTLVDINRRDPLVVAALHLCIALGYANSSLNPVLYAFLDENFKRCFRQL 332
ROR-B  VRMLSGSKEKDRNLRRITRMVLVVVAVFIVCWTPIHIYVIIKALITIPET-TFQTVSWHFCIALGYTNSCLNPVLYAFLDENFKRCFREF 350
                                    TM-VI                                  TM-VII

ROR-A  CRAPCGGQEPGSLRRPRQATARERV-TACTPSDGPGGGAAA          372
ROR-B  CIPTSSTIEQQNSTRVRQNT-REHPSTANTVDRTNHQLENLEAETAPLP 398
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Fig. 1. Alignment of the amino acid sequences of ROR-A (top) and ROR-B (bottom). The one-letter amino acid notation is used. Gaps (–) have been inserted to achieve maximum homology. Numbers of the amino acid residues at the right-hand end of the individual lines are given. The positions of the predicted transmembrane segments (TM-I–VII) are indicated. The nucleotide sequences of ROR-A and ROR-B will appear in the DDBJ/GenBank/EMBL Nucleotide Sequence Databases under the accession numbers D16348 and D16349, respectively.

agonist selective for the μ-opioid receptor subtype, was quite low (K_d = 2,240 nM). On the other hand, ROR-B exhibited much higher affinity for DAGO (K_d = 4.0 nM) and lower affinities for DPDPE (K_d = 1,900 nM) and DSLET (K_d = 142 nM) than ROR-A. Furthermore, morphine and naloxone, which preferentially interact with the μ-opioid receptor subtype, exhibited affinities for ROR-B higher than those for ROR-A. U50488, selective for the κ-opioid receptor subtype, had low affinities for both ROR-A and ROR-B. The K_d values obtained for ROR-A and ROR-B compare well with the reported K_d values for the δ- and μ-opioid receptor subtypes [18,19], respectively. These results indicate that ROR-A and ROR-B correspond most closely to the pharmacologically defined δ- and μ-opioid receptor subtypes, respectively.

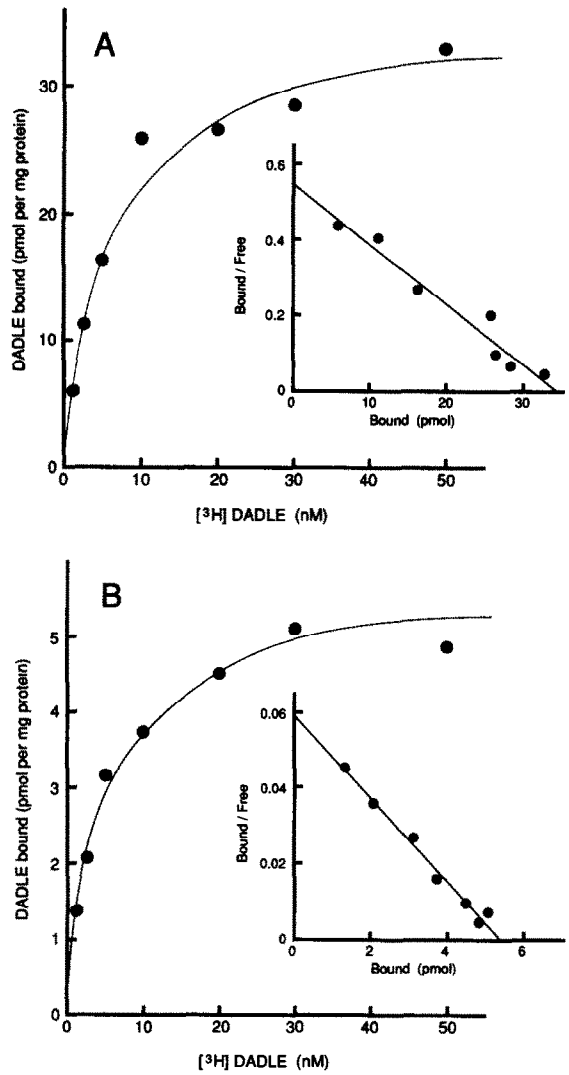

Fig. 2. Saturation analysis of [^{3}H]DADLE binding to membrane preparations from CROR10-17 cells expressing ROR-A (A) and CROR15-27 cells expressing ROR-B (B). Results of representative experiments are shown. The insets show Scatchard plots of the data. The non-specific binding (measured in the presence of excess amount of unlabelled DADLE) was less than 10% of the total radioactivity bound. No significant [^{3}H]DADLE binding could be detected in membrane preparations from non-transfected CHO cells.

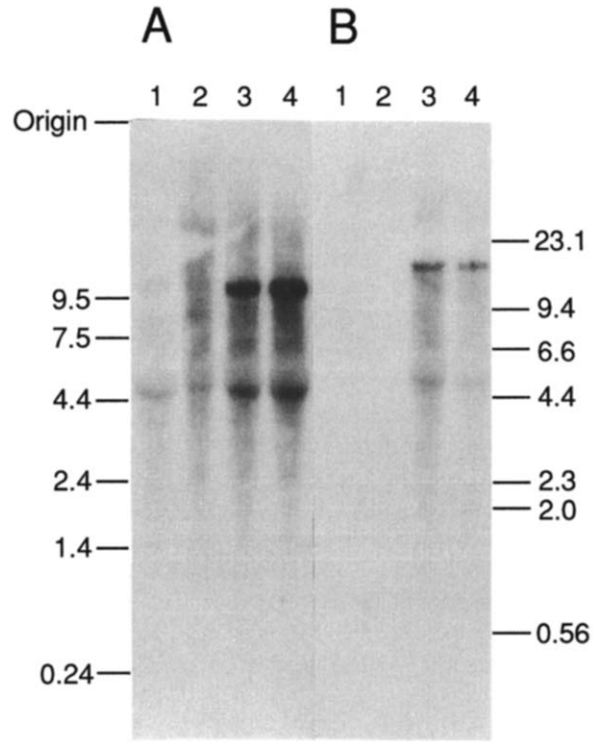

Fig. 3. Autoradiograms of blot hybridization analysis of poly(A)$^+$ RNA from rat tissues with the opioid receptor subtype ROR-A (A) and ROR-B (B) cDNA probes. Poly(A)$^+$ RNA (30 μg each) prepared from kidney (lane 1), cerebellum (lane 2), brainstem (lane 3) and cerebrum (lane 4) was analyzed as in section 2.3. The size markers are indicated.

Poly(A)$^+$ RNA preparations from rat tissues were subjected to blot hybridization analysis (Fig. 3). Cerebrum and brainstem contain two RNA species of ~ 4.5 and ~ 11 kb hybridizable with the ROR-A cDNA

Table I

Ligand-binding properties of opioid receptor species expressed from cDNAs

Ligand	K_d (nM)	
	ROR-A	ROR-B
DADLE	5.0	7.9
DPDPE	28	1,900
DSLET	12	142
DAGO	2,240	4.0
Morphine	1,260	5.3
Naloxone	67	1.8
U50488	>10,000	710

K_d values for DADLE were obtained by Scatchard analysis using [^{3}H]DADLE (see Fig. 2). K_d values for the other ligands were obtained by measuring displacement of [^{3}H]DADLE binding.

probe and an RNA species of ~ 16 kb hybridizable with the ROR-B cDNA probe. In contrast, neither ROR-A nor ROR-B mRNAs can be detected in cerebellum. In accord with this result are previous results obtained by autoradiographic studies with radioligands [20], which showed that the δ- and μ-opioid receptor subtypes distribute throughout the rat central nervous system except cerebellum. Multiple transcripts could be detected by the ROR-A cDNA probe, which is consistent with a recent report [5].

Our results obtained in this investigation indicate that the molecularly distinct opioid receptor subtypes, ROR-A and ROR-B, represent the pharmacologically defined δ- and μ-opioid receptor subtypes, respectively, and provide evidence indicating that pharmacologically different opioid receptor subtypes are distinct gene products. Pharmacologically, further refined classification of the opioid receptor subtypes has been suggested; δ is classified into δ_1 and δ_2 [21], and μ into μ_1 and μ_2 [22]. Whether these sub-classifications are derived from distinct genes or based on differences in post-translational modifications of ROR-A and ROR-B remains to be elucidated.

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