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Preparation of δ-Opioid Receptor-Specific Antibodies Using Molecular Cloned Genes

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We re-cloned mouse δ-opioid receptor from NG108-15 cells using RT-PCR, and confirmed it by restriction analysis and by sequencing the beginning and end part of the amplified DNA. When transiently expressed in COS-7 cells, cloned δ-opioid receptor showed saturable and specific binding to [^{3}H]naloxone with very similar binding parameters to originally reported ones. To make antibodies specific for the δ-opioid receptor, the carboxy tail of the δ-opioid receptor, which is unique to the δ-opioid receptor compared with other opioid receptors, was expressed in bacteria as a fusion proteinwith glutathione S-transferase. Purified fusion protein was injected into rabbits to produce antibodies. The antibodies produced were highly selective for δ-opioid receptor when tested by western blotting using membrane proteins prepared from transfected COS-7 cells. Cloned δ-opioid receptor and antibodies specific for δ-opioid receptor are going to be valuable tools for studying pharmacological actions of the δ-opioid receptor and morphine dependence.

Key words : δ-Opioid receptor, Antibodies, Cloning, Binding study

INTRODUCTION

Effects of morphine and its derivatives have been extensively studied because of their importance both in medical application and drug abuse (Lord *et al.*, 1977; Wood *et al.*, 1981; Holaday *et al.*, 1985; North, 1986; Childers, 1991). Furthermore recent cloning of cDNAs for opioid receptors is accelerating our understanding at the molecular level (Evans *et al.*, 1992; Kieffer *et al.*, 1992; Fukuda *et al.*, 1993; Chen *et al.*, 1993; Yasuda *et al.*, 1993).

Opioid receptors are widely distributed in the brain and peripheral tissues and they mediate various physiological actions such as analgesia, gastrointestinal movements, and respiration. Opioid receptors belong to GTP-binding protein coupled receptor family with seven transmembrane domains and they modulate cellular events like inhibition of adenylyl cyclase, activation of potassium channels, and inhibition of calcium channels (For review, see North, 1986; Childers, 1991).

Physical and mental dependence are representative adverse actions in chronic use of opioid drugs (Way *et al.*, 1969; Martin and Eades, 1966; Bickel *et al.*, 1988; Yano and Takemori, 1977). The mechanisms of opioid tolerance, dependence,and reinforcement have been intensively studied (Koob and Bloom, 1988; Meunier, 1991; Brase, 1990; Meunier, 1992; Neugus *et al.*, 1993; Collin and Cesselin, 1991; Nestler *et al.*, 1988). However, the molecular basis for these side effects has not been studied thoroughly.

To understand the precise mechan isms underlying these side effects, it is necessary to conduct well controlled cellular and molecular biological studies. The purpose of this study was to make tools to study the δ-opioid receptor. We re-cloned δ-opioid receptor and prepared the δ-opioid receptor-specific antibodies.

MATERIALS AND METHODS

Cell cultures

NG108-15 cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco) and HAT (0. 1mM hypoxanthine, 16 μM thymidine, 1 μM aminopterin). COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum. Cultures were kept at

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Table I. Primers used to amplify δ-opioid receptor and C-terminal tail

Primer	No.
ATATAAGCTTGGAGACGGACACGGCGGCGGC	(1)
AGTAGGATCCTTAAGGGGAAGGTCGGGTAGG	(2)
AATTGGATCCCAGCTCTGTCGCACGCCC	(3)
AATTGAATTCTCAGTAGAGCGCGGTGATGGC	(4)
ATATGGATCCATGGAGCTGGTGCCCTCTGCCCC	(5)

Underlined parts represent restriction enzyme sites.

10% CO_2 atmosphere, 37°C in saturated humidity.

δ-Opioid receptor cloning

Total RNA was prepared from a monolayer of NG 108-15 cells using the guanidium isothiocyanate method (Sambrook *et al.*, 1989). About 3μg of total RNA was reverse transcribed using primer-2 (Table I) and a reverse transcription kit (Invitrogen) to produce single stranded cDNA. δ-opioid receptor cDNA was amplified by a polymerase chain reaction (PCR) through 30 cycles of protocol (94°C, 1 min; 55°C, 1 min; 72°C, 2 min). Amplified DNA was subcloned into pBluescript SK^+ (Stratagene) and sequencing was done for the beginning and the end part of the δ-opioid receptor using a sequencing kit (USB).

Expression in COS-7 cells

An eukaryotic expression vector, pRC/CMV (Invitrogen), was cut with BstXI and blunted with T4 DNA polymerase. The cDNA part of the δ-opioid receptor which had correct sequences in the beginning and end parts was also blunt ended and subclo-ned into pRC/CMV. The clones containing the right orientation were selected by restriction analysis using Hind III/ Xba I and Bgl II/Not I.

Calcium phosphate method was used for DNA transfection into COS-7 cells. About 10 μg of DNA was mixed with HEPES buffer, pH 7.1 and $CaCl_2$. Mixture was applied spread onto the COS-7 cells drop by drop. Next morning, cells were subjected to glycerol shock and were split.

Receptor binding studies

COS-7 cells transiently transfected between 48 to 72 hours were harvested in 50 mM Tris HCl, pH 8.0, containing 1 mM EDTA and 1 mM PMSF (Phenylmethylsulfonyl Fluoride). Cells were homogenized using Polytron and centrifuged at 48,000 x g for 10 min at 4°C. The same procedure was repeated. Pellet was resuspended in 50 mM Tris HCl, pH 8.0, containing 5 mM $MgCl_2$. For saturation studies, membrane proteins (80 μg protein/tube) were incubated for 1hrat 37°C with increasing amount of $[^3H]$naloxone. Nonspecific binding was defined as that determined in the presence of 10 μM of naloxone in the incubation mixture. Binding parameters were analyzed using LIGAND program.

Bacterial expression of fusion proteins

The carboxy tail of cloned δ-opioid receptor was expressed in the fusion protein with glutathione S-transferase (GST). The carboxy tail of δ-opioid receptor was PCR amplified and subcloned into pGEX-2T (Pharmacia). Saturated bacterial culture was diluted 10 times and was grown for 1 hour to reach the log phase. Proteins were induced by adding IPTG (Isopropyl β-D-Thiogalactopyranoside) in the log phase at the final concentration of 0.5 mM. Cells were harvested after, additional two hours incubation. Expressed protein was purified using glutathione beads (Pharmacia or Sigma).

Immunization of rabbits

Purified carboxy tail of δ-opioid receptor was injected into rabbits. For initial immunization, 500 μg of purified protein was mixed with an equal volume of complete adjuvant (Gibco or Sigma). For subsequent boostering, 200 μg of purified protein was injected with an equal amount of incomplete adjuvant (Gibco or Sigma). Boostering was done every three weeks and the rabbit was sacrificed after the 5th boostering.

RESULTS AND DISCUSSION

δ-Opioid receptor cloning

The role of the δ-opioid receptor in the development of morphine tolerance and dependence is quite clear from previous studies (Abdelhamind *et al.*, 1991; Miyamoto *et al.*, 1993). In addition, there are complicatd interactions between μ-opioid receptor and δ-opioid receptor both in binding and pharmaco-logical actions of opioids (Jiang *et al.*, 1990; Frank-Huijzen *et al.*, 1990).

NG108-15 cells, a hybrid of neuroblastoma and glioma, are known to express mouse δ-opioid receptors. The original cloning of δ-opioid receptor was also reported from these cells (Evans *et al.*, 1992; Kieffer *et al.*, 1992). To conduct systemic studies on δ-opioid receptor, we re-cloned δ-opioid receptor using the sequence information from the original works. By taking advantage of the power of PCR, we conducted RT-PCR using cDNA from these cells. We first amplified δ-opioid receptor cDNA using primers located at 3'-, and 5'-untranslated regions which contain start and stop codons (primer-1/primer-2, Table I). The PCR products were reamplified by combination of primers located at the beginning and end of the open read-ing frame. Amplified DNAs are shown in Fig.1; product shown in the left lane (primer-3/primer-

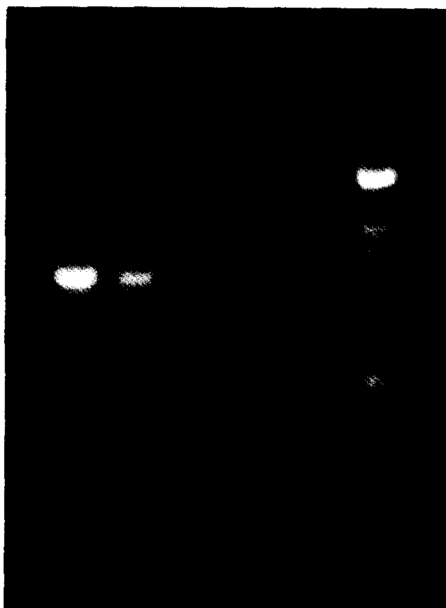

Fig. 1. Amplification of δ-opioid receptor from NG108-15 cells. One hundred base pair ladder (DNA molecular weight marker, Gibco) is shown in right-hand side of 1% agarose gel. Three bright positions represent 2 kb, 1.5 kb, and 600 bp, respectively. Amplified δ-opioid receptor cDNA was around 1.2 kb. Left lane represents δ-opioid receptor cDNA amplified using primer-3/primer-2 and right lane represent δ-opioid receptor cDNA amplified using primer-3/primer-4.

2) and product shown in the right lane (primer-3/primer-4). Amplified DNA was subcloned into pBluescript SK^+ for further characterization. The sequence of the beginning and the end part of the clone was identical with that of previously reported mouse δ-opioid receptor.

Pharmacological characterization of cloned δ-opioid receptor

To functionally characterize cloned δ-opioid receptor, we made an expression construct using an eukaryotic expression vector, pRC/CMV. Expressed δ-opioid receptor in COS-7 cells showed saturable specific binding to [^{3}H]naloxone with the K_d value of 20 nM, which is very similar to those previously reported (29 nM, Kieffer *et al.*, 1992; 17 nM, Raynor *et al.*, 1993). B_{max} value was 1,350 fmol/mg protein.

The cloned δ-opioid receptor was identified as δ_2-opioid receptor based on the pharmacological characteristics. This receptor showed much higher affinity for NTB (δ_2-selective) than for BNTX (δ_1-selective) (Sofuoglu *et al.*, 1991; Mattia *et al.*, 1991; Jiang *et al.*, 1991;Evans *et al.*, 1992; Kieffer *et al.*, 1992; Raynor *et al.*, 1993). The δ_2-opioid receptor was shown to play an important role in the development of morphine dependence (Miyamoto *et al.*, 1993).

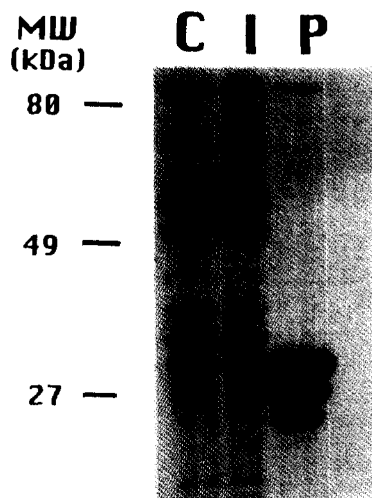

Fig. 2. Bacterial expression of carboxy tail of δ-opioid receptor. 'C' represents 'control', 'I' represents 'induced', and 'P' represents 'purified'. Protein expression was induced by IPTG at the final concentration of 0.5 mM. For control and induced lanes, the whole bacterial protein was resuspended in sample buffer and loaded on to the 10% polyacrylamide gel. Purified lane represents purified fusion protein through glutathione-agarose affinity chromatography.

Bacterial expression of carboxy tail of δ-opioid receptor

So far, kappa, mu, and one of delta (subtype seems to exist) opioid receptors have been cloned. Alignment of opioid receptor amino acids showed that they have very short 3rd cytoplasmic loops, but have relatively long carboxy tails (Evans *et al.*, 1992; Kieffer *et al.*,1992; Fukuda *et al.*, 1993; Chen *et al.*, 1993; Yasuda *et al.*, 1993). Potential sites in opioid receptors which can provide specificity in antibody production are carboxy tails because these regions are distinct from those in other G-protein coupled rece-ptors, and are long enough to raise antibodies.

To make antibodies specific for the δ-opioid receptor, we PCR amplified the carboxy tail of the δ-opioid receptor using primer-5/primer-4 (Table I), and subcloned into pGEX-2T vectors to produce fusion proteins (Smith and Johnson, 1987). This method was proven to be convenient and powerful in terms of time and effort. Milligrams of fusion proteins per liter of bacterial culture were obtained. Fig. 2 shows the fusion protein of the carboxy tail of δ-opioid receptor and GST in the SDS polyacrylamide gel. The Calculated carboxy tail of the δ-opioid receptor is about 4.5 kDa protein, and produced fusion protein was about 32 kDa including 27.5 kDa GST and linker portion. Purified protein usually gave three bands in SDS gel, indicating that it was cleaved in the process of purification.

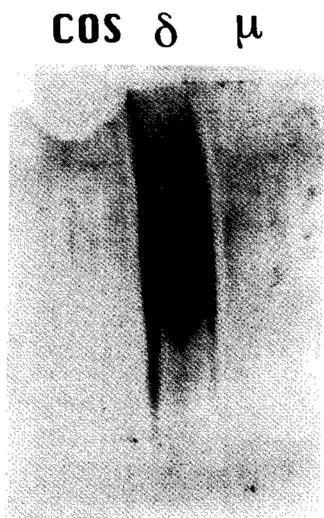

Fig. 3. Characterization of antibodies using immunoblotting. Membrane proteuns were prepared from COS-7 cells, and those which were transfected for 72 hours with [μ-and δ-opioid] receptor constructs. One hundred microgram of protein was loaded onto the 9% native polyacrylamide gel, and transferred to nitrocellulose filter (Bio Rad). The lane labelled 'COS' represent membrane proteins prepared from COS-7 cells which were not transfected with DNA. 'δ ' and 'μ ' represent membrane proteins prepared from COS-7 cells which were transfected with constructs containing δ-and μ-opioid receptor cDNAs, respectively. Primary antibodies were diluted 500 times and anti-rabbit alkaline phosphatase conjugated secondary antibodies (Sigma) were 20,000 times diluted.

Characterization of antibodies raised against δ-opioid receptor

When we conducted immune blotting with denatured SDS gel, the background was very high and the signal was not high enough. However, when immunoblotting was conducted using native polyacrylamide gel, antibodies strongly and specifically detected the δ-opioid receptor (Fig. 3). This result was expected because the injected antigen was not denatured (antigen was purified by affinity chromatography).

The tools obtained in this study are expected to be quintessential to conduct more sophisticated studies to elucidate the mechanisms of morphine tolerance and dependence.

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