Volume 330, number 2, 146–150 FEBS 12953 September 1993

Purification and reconstitution of the δ opioid receptor

K.G. Gomathi, Shail K. Sharma*

Department of Biochemistry, All India Institute of Medical Sciences, New Delhi 110029, India

Received 12 June 1993; revised version received 20 July 1993

The δ opioid receptor has been purified, in an active form, by succinylmorphine affinity chromatography. The receptor was purified partially from bovine frontal cortex and to apparent homogeneity from neuroblastoma × glioma hybrid NG108-15 cells as observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining. Antiserum to the purified bovine receptor inhibited ligand binding to membranes and immunoprecipitated a 58 kDa protein from NG108-15 cells. Reconstitution of the receptor with lipids enhanced binding by 9-fold. The 58 kDa band protein after electroelution and reconstitution with lipids also showed specific binding, indicating that the receptor could be renatured even after SDS-PAGE in an appropriate lipid environment.

δ Opioid receptor; NG108-15; Reconstitution

1. INTRODUCTION

Opioid receptors are classified into μ, δ and κ types based on ligand specificity and tissue distribution. However, the molecular basis of this receptor heterogeneity still remains unclear. The frontal cortex of bovine brain is relatively enriched in delta receptors [1]. The neuroblastoma × glioma hybrid NG108-15 cells express exclusively the δ receptors on their cell surface [2,3]. Attempts to purify the μ [4,5] and κ receptor [6,7] have been reported. There is also a report on the purification of the δ opioid receptor [8] but in an inactive form. Recently, the delta opioid receptor has been cloned [9,10]. In this report, we describe purification and reconstitution of the functionally active δ opioid receptor from the bovine frontal cortex and NG108-15 cells.

2. MATERIALS AND METHODS

2.1. *Cell growth*

Neuroblastoma × glioma hybrid NG108-15 cells were grown as previously described [11] in 162 cm^2 tissue culture flasks. C6 cells were grown in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal calf serum (Biological Industries, Israel), streptomycin (100 μg/ml) and penicillin (100 units/ml) in 75 cm^2 flasks.

2.2. *Affinity purification*

Membranes were prepared from frontal cortex and NG108-15 cells as described by Simonds et al. [12]. Membranes were washed by incubating in 50 mM Tris-HCl, pH 7.5, containing 1 mM PMSF (Sigma, USA) for 30 min at 37°C and solubilized using 10 mM CHAPS (Sigma, USA) as described by Simonds et al. [12] with minor modifications as described by Zukin and Maneckjee [13]. 6-Succinylmorphine was synthesized from morphine (Govt. Opium and Alkaloid Works, India) and coupled to Sepharose 4B (Pharmacia, Sweden) using an ethylenediamine spacer arm [14]. CHAPS solubilized extract was diluted to 1 mM CHAPS and applied to the succinylmorphine-Sepharose column (1.2 × 13 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 1 mM CHAPS and 0.1 mM PMSF. The column was washed with 250 ml of the buffer and eluted with 100 ml of KCl (1 M) in the same buffer. Protein was monitored at 280 nm, the peak fractions pooled, dialysed and concentrated.

2.3. *Opioid binding*

Binding to membranes (750 μg) was carried out by incubating in 50 mM Tris-HCl, pH 7.5, with [^{3}H]DADLE (New England Nuclear, USA; 30 Ci/mmol) for 60 min at 37°C in a final volume of 300 μl. Non-specific binding determined in the presence of DADLE (National Institute on Drug Abuse, USA), 10 μM was 25–30% of total binding. Incubation was terminated by rapid filtration through Whatman GF/B filters. Filters were washed with 3 × 5 ml of ice-cold buffer, dried and the radioactivity determined in a toluene based scintillation fluid.

CHAPS solubilized protein was precipitated using polyethylene glycol 6000, centrifuged at 40,000 × *g* for 30 min and [^{3}H]DADLE binding to the pellet was studied in the presence of CHAPS, 1 mM as described by Chow and Zukin [15] in a final volume of 300 μl. Binding to the affinity purified receptor (15 μg) was determined in the presence of CHAPS, 1 mM by incubating with [^{3}H]DADLE or [^{3}H]DPDPE (Amersham, UK) in a final volume of 500 μl for 60 min at 37°C. Non-specific binding was determined in the presence of 10 μM of the respective unlabelled ligand and was 20–25% of total binding. After cooling on ice, 50 μl of ice-cold 1% γ-globulin was added and the mixture applied to Sephadex G-25M column (9.1 ml bed volume, Pharmacia PD10) equilibrated with Tris-HCl buffer containing 1 mM CHAPS. Initial 4.5 ml eluate was collected, mixed with 12 ml of Triton X-100–toluene scintillation fluid and the radioactivity determined.

2.4. *Production of antiserum*

Four BALB/c mice were bled, serum prepared, pooled and stored as pre-immune mouse serum (PIMS). The mice were then immunized

*Corresponding author. Fax: (91) (11) 686-2663.

Abbreviations: CHAPS, 3-[(3-cholamidopropyl) dimethyl ammonio]1-propane-sulfonate; DADLE, D-Ala^2, D-Leu^5-enkephalin; DPDPE, D-Pen^2,D-Pen^5-enkephalin; ELISA, enzyme-linked immunosorbent assay; IMS, immune mouse serum; K_d, equilibrium dissociation constant; kDa, kilodaltons; PIMS, pre-immune mouse serum; PMSF, phenyl methyl sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin.

 Published by Elsevier Science Publishers B.V.

with affinity-purified receptor preparation (20 μg) from bovine frontal cortex emulsified in Freunds adjuvant, subcutaneously at fortnightly intervals. The mice were bled after four injections, the sera pooled and stored as immunized mouse serum (IMS).

2.5. *Immunoprecipitation*

Surface proteins of NG108-15 cells were labelled with ^{125}I (Bhabha Atomic Research Centre, India) [16] and solubilized as described above. 1 ml of the diluted extract (CHAPS concentration 1 mM) was mixed with 200 μl of serum (diluted 1:50) at 4°C overnight. 100 μl of goat anti-mouse IgG + IgM conjugated to microspheres (Kirkegaard and Perry Lab., USA) was added and mixed at room temperature for 2 h. The antigen–antibody complexes were collected by centrifugation. The microspheres were washed with 3 × 1 ml of phosphate-buffered saline containing 1% bovine serum albumin and treated with SDS-PAGE sample buffer.

2.6. *SDS-PAGE and electroelution*

Electrophoresis was carried out by the method of Laemmli [17] as modified by Ogita and Markert [18]. Gels were stained with silver by the method of Morrisey [19]. Protein was electroeluted [20] from gel slices by sealing in dialysis bags with 500 μl of Laemmli electrode buffer and placing the bags in a horizontal electrophoretic chamber. The tank was then filled with buffer and elution carried out at 20 mA for 5 h at room temperature. SDS was dialysed out and the protein concentrated by lyophilization.

2.7. *WGA-Sepharose chromatography*

Affinity purified receptor (400 μg) was applied on a WGA-Sepharose column (bed volume 10 ml) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 1 mM CHAPS and 0.1 mM PMSF. The column was washed with 100 ml of buffer and eluted with 50 ml of 0.5 M *N*-acetyl D-glucosamine. The protein peaks were pooled, dialysed and concentrated.

2.8. *ELISA methods*

ELISA was carried out as decribed by Sharma et al. [21]. Wells of the ELISA plate were coated with cells/protein in 50 mM Tris-HCl, pH 7.5, by incubating at 37°C for 4 h. Non-specific sites were blocked with 3% bovine serum albumin. PIMS or IMS diluted 1:100 was added and incubation carried out for 2 h at room temperature. The plates were washed thrice with phosphate-buffered saline, pH 7.2, and incubated with goat anti-mouse IgG + IgM coupled to peroxidase for 1 h at room temperature. Colour was developed using orthophenylene diamine (0.5 mg/ml in 0.15 M citrate-phosphate buffer, pH 5.0) and H_2O_2 (0.03%) and terminated after 10 min by 5 N H_2SO_4.

2.9. *Reconstitution into lipid vesicles*

Total lipids were extracted from bovine brain [22]. The extract was dissolved in chloroform, dried and resuspended in 50 mM Tris-HCl, pH 7.5. Vesicles were prepared and receptor reconstituted by the method of Scheideler and Zukin [23]. Lipid vesicles were prepared by direct probe sonication (Heat Systems–Ultrasonics Inc., USA). One second pulses of 80–100 W were given for 10 min and titanium shed by the probe was removed by centrifugation at 1000 × *g* for 10 min. Affinity-purified receptor in 50 mM Tris-HCl, pH 7.5, containing 10 mM CHAPS was added to the suspension of lipid vesicles (protein:lipid, 1:5), mixed and left for 1 h at 40°C. CHAPS was then diluted to a concentration of 1 mM and the vesicles collected by centrifugation at 100,000 × *g* for 5 h. Binding to the lipid vesicles was studied as described for the affinity-purified receptor.

2.10. *Protein estimation*

Protein was estimated in the membrane preparation by the method of Lowry [24] and in the CHAPS solubilized extract and affinity-purified receptor by the modified Bradford microassay [25].

3. RESULTS

Affinity chromatography of the CHAPS solubilized protein from bovine frontal cortex on the succinylmorphine-Sepharose column yielded a single peak which corresponded to about 0.5% of the protein in the membrane preparation. The peak showed significant DADLE binding activity (689 fmol/mg protein). In the case of NG108-15 cells too a single protein peak was eluted from the affinity column which corresponded to 0.28% of the membrane protein. The peak showed significant binding to DADLE and had a specific activity of 1170 fmol/mg protein. In terms of binding, the yield was 11% from frontal cortex and 3% from NG108-15 cells. Affinity chromatography performed with more than 10 preparations of bovine frontal cortex membranes and six different preparations of NG108-15 cells gave similar values. Parameters obtained from the saturation curves for the binding of [^{3}H]DADLE at 1–20 nM are reported in Table I. [^{3}H]DADLE was found to bind to a single class of binding sites (as shown by Scatchard plots). Though the affinities of DADLE for the membranes and the affinity-purified receptor were of the same order of magnitude, a significant increase in K_d was observed both in the case of frontal cortex and NG108-15 cells.

In order to determine the purity of the affinity eluate, the purified protein from frontal cortex and NG108-15 cells was analyzed by SDS-PAGE. Fig. 1 shows the pattern obtained in the case of the purified receptor from frontal cortex. Two bands, one of 58 kDa and the other of 30 kDa were seen. The affinity-purified protein from NG108-15 cells, however, showed a single band of 58 kDa on SDS-PAGE followed by silver staining (Fig. 2, lane 1). Since the opioid receptor is known to be a WGA binding glycoprotein, the affinity purified receptor from NG108-15 cells was further subjected to WGA-Sepharose chromatography. The receptor bound

Table I

Parameter estimates of [^{3}H]DADLE binding to receptors from bovine frontal cortex and NG108-15 cells

	K_d (nM)	B_{max} (fmol/mg protein)	Hill coefficient
Frontal cortex			
Membranes	2.9	37	0.9
CHAPS extract	3.8	40	1.0
Affinity-purified receptor	5.3	1,600	1.0
NG108-15 cells			
Membranes	2.8	162	0.9
CHAPS extract	4.0	180	1.0
Affinity-purified receptor	9.5	3,640	1.0

Values shown represent means of three experiments performed in duplicate for frontal cortex and two experiments performed in duplicate for the NG108-15 cells. Standard error was less than 10% in case of the membranes and CHAPS extract and less than 15% for the affinity purified protein.

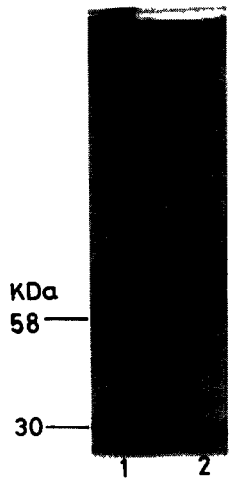

Fig. 1. Analysis of affinity-purified receptor from bovine frontal cortex by SDS-PAGE on 7.5% gels. The gels were stained with silver. (Lanes 1 and 2) 25 and 5 μg of the affinity-purified receptor.

to WGA and could be eluted with the competing sugar, *N*-acetyl-D-glucosamine. The WGA eluate showed a single band of 58 kDa (Fig. 2, lane 2) and retained the capacity to bind [^{3}H]DADLE (data not shown).

Since lipids are known to be required for optimal ligand binding to opioid receptors, reconstitution of the affinity-purified receptor was carried out in lipid vesicles prepared from bovine brain lipid extract. Reconstitution of the affinity-purified receptor from NG108-15 cells into lipid vesicles resulted in a 9-fold enhancement of [^{3}H]DADLE binding (Table II) suggesting that membrane lipid environment is important for ligand binding to the delta receptor. The 58 kDa band seen on SDS-PAGE was electroeluted (recovery was 48%) and reconstituted into lipid vesicles. Significant [^{3}H]DADLE binding was observed after reconstitution into lipid vesicles confirming the 58 kDa protein to be the δ opioid receptor.

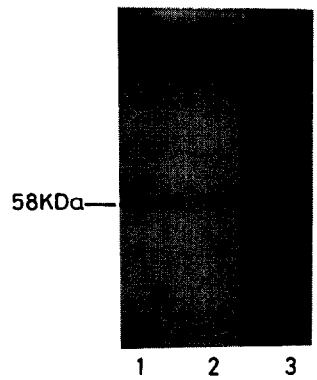

Fig. 2. Analysis of the receptor from NG108-15 cells by SDS-PAGE on 10% gels. Lanes 1 and 2 were stained with silver. (Lane 1) 5 μg of affinity purified receptor; (lane 2) 5 μg of WGA–Sepharose eluted receptor; (lane 3) autoradiographic pattern of ^{125}I-labelled antigen immunoprecipitated by IMS from NG108-15 cells.

Reactivity by ELISA of the antiserum raised to the affinity-purified receptor from frontal cortex is shown in Fig. 3. Strong cross-reactivity was seen with NG108-15 cells while no reactivity was seen with C6 glioma cells which lack opioid receptors. [^{3}H]DADLE binding to both frontal cortex and NG108-15 cell membrane preparation could be inhibited by the antiserum raised (Fig. 4). At a 1:100 dilution, IMS inhibited [^{3}H]DADLE binding to frontal cortex membrane preparation by 63% and to NG108-15 cell membrane preparation by 44% (Fig. 4). At 1:10 dilution of serum, a non-specific inhibition of 30% was observed even with PIMS.

In an attempt to immunoprecipitate the antigen, surface proteins of NG108-15 cells were labelled with ^{125}I, membranes prepared and solubilized using 10 mM CHAPS. The antigen was precipitated by incubating the extract (diluted 1:10) with the serum and collecting the antigen–antibody complexes on goat anti-mouse IgG + IgM coupled to microspheres. On SDS-PAGE and autoradiography, a single band was seen (Fig. 2, lane 3) which co-migrated with the affinity purified receptor and had a molecular weight of 58 kDa. Incubation with PIMS did not show any band (data not shown). Immunoprecipitation studies were also carried out with unlabelled NG108-15 cells. [^{3}H]DADLE binding to the supernatant, after immunoprecipitation of the antigen, was studied to look for residual binding sites. 44 fmol of [^{3}H]DADLE were bound per mg protein in case of IMS and 74 fmol in the case of PIMS. This corresponded to a 41% decrease in binding after precipitation with IMS compared to PIMS (data not shown).

Table II

[^{3}H]DADLE and [^{3}H]DPDPE binding to the affinity-purified receptor from NG108-15 cells

	Specific binding (fmol/mg protein)	
	[^{3}H]DADLE	[^{3}H]DPDPE
Affinity-purified receptor	1,187	2,226
Affinity-purified receptor reconstituted in lipid vesicles	10,304	ND
58 kDa band protein* reconstituted in lipid vesicles	4,360	ND

[^{3}H]DADLE and [^{3}H]DPDPE, 5 nM were used to study binding to affinity-purified receptor or affinity-purified receptor reconstituted into lipid vesicles (26 μg) as described in section 2.

*SDS-PAGE of the affinity-purified receptor was carried out on 10% gels. A lane was cut out and stained with silver. Gel from another lane corresponding to the 58 kDa band was excised, the protein electroeluted and reconstituted in lipid vesicles. Binding of [^{3}H]DADLE was carried out as described in section 2. Control lipid vesicles without protein did not show any specific binding. Non-specific binding was determined in the presence of DADLE, 10 μm and was 20–30% of total binding. Values shown are averages from two experiments performed in duplicate. ND = not done.

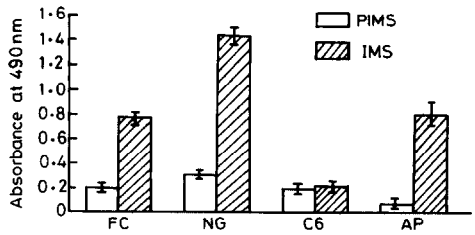

Fig. 3. Reactivity by ELISA of immunized mouse serum (IMS) and pre-immune mouse serum (PIMS). Frontal cortex membranes (FC), 100 μg; NG108-15 cells (NG), 1×10^5; C6 glioma cells (C6), 1×10^5; or affinity-purified receptor from frontal cortex (AP), 15 μg was coated per well of the ELISA plate and ELISA performed as described in section 2. Values shown are mean ± S.E.M. from 3 experiments performed in triplicate.

4. DISCUSSION

We describe purification in an active form of the δ opioid receptor from the bovine frontal cortex and NG108-15 cells. The receptor has been purified to apparent homogeneity as seen on SDS-PAGE followed by silver staining from the NG108-15 cells and partially from the bovine frontal cortex. The affinity purified receptor bound both [^{3}H]DADLE and [^{3}H]DPDPE specifically though with much lower specific activity than expected. This could be explained by the loss of the guanyl nucleotide binding protein (G-protein) during purification since no effect of guanyl nucleotides could be seen on [^{3}H]DADLE binding to the affinity-purified receptor (data not shown) nor bands in the molecular weight range of 40–43 kDa (molecular weight of G-proteins) observed on SDS-PAGE. G-proteins are known to be important for high affinity agonist binding to the receptor and complete uncoupling results in the

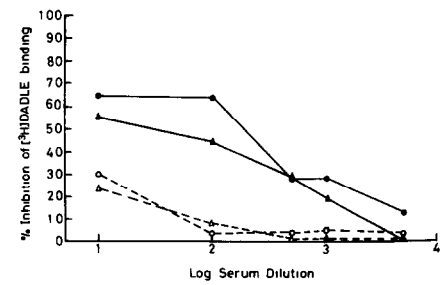

Fig. 4. Inhibition of [^{3}H]DADLE specific binding to frontal cortex membranes ○,●) and to NG108-15 cells (△,▲) by immunized mouse serum (●–●, ▲–▲) and pre-immune mouse serum (○–○, △–△). Frontal cortex/NG108-15 cell membranes (750 μg) were incubated with different dilutions of the sera (100 μl) for 1 h at 37°C. [^{3}H]DADLE (1.0 nM) was then added and incubation carried out further for 1 h at 37°C. Non-specific binding was determined in the presence of DADLE, 10 μM and was 25–30% of total binding. Rest of the assay details are as given in section 2 for membranes. Each point shown represents mean of values from 3 experiments performed in duplicate. Standard error was less than 10%.

conversion of the receptors into a low affinity state [26]. Reconstitution of the purified μ receptor with G_i protein has been reported by Ueda et al. [27] to increase agonist binding to the receptor by 215-fold.

In this study, reconstitution of the purified receptor into lipid vesicles resulted in enhanced [^{3}H]DADLE binding suggesting that membrane lipid environment plays a major role in maintaining the δ receptor in an active state. Similar observations have been made with both the μ [28] and the κ [7] receptors.

The 58 kDa band protein observed on SDS-PAGE when electroeluted and reconstituted into lipid vesicles retained the capacity to bind [^{3}H]DADLE. These observations confirmed that the 58 kDa protein was the receptor. Simonds et al. [8] have by convalent labelling with 3-methylfentanyl isothiocyanate obtained a molecular weight of 58 kDa for the δ receptor. The δ receptor purified by us also had a molecular weight of 58 kDa which agrees well with that reported by them. Our study is the first report on the reconstitution and renaturation of the δ opioid receptor after SDS-PAGE.

Acknowledgements: This work was supported by a grant from the Indian Council of Medical Research (NO-5/3-1(8)BMSII) to S.K.S. The NG108-15 and the C6 cells were the kind gift of Dr. Marshall Nirenberg, NIH, USA.

REFERENCES

[1] Ninkovic, M., Hunt, S.P., Emson, P.C. and Iversen, L.L. (1981) Brain Res. 214, 163–167.
[2] Klee, W.A. and Nirenberg, M. (1974) Proc. Natl. Acad. Sci. USA 71, 3474–34677.
[3] Chang, K.J. and Cuatrecasas, P. (1979) J. Biol. Chem. 254, 2610–2618.
[4] Cho, T.M., Hasegawa, J., Ge, B.L. and Loh, H.H. (1986) Proc. Natl. Acad. Sci. USA 83, 4138–4142.
[5] Ueda, H., Harada, H., Misawa, H., Nozaki, M. and Takagi, H. (1987) Neurosci. Lett. 75, 339–344.
[6] Simon, J., Benyhe, S., Hepp, J., Khan, A., Borsodi, A., Szucs, M., Medzihradsky, K. and Wollemann, M. (1987) Neuropeptides 10, 19–28.
[7] Simon, J., Benyhe, S., Hepp, J., Varga, E., Medzihradsky, K., Borsodi, A. and Wollemann, M. (1990) J. Neurosci. Res. 25, 549–555.
[8] Simonds, W.F., Burke, T.R., Rice, K.C., Jacobson, A.E. and Klee, W.A. (1985) Proc. Natl. Acad. Sci. USA 82, 4974–4978.
[9] Evans, C.J., Keith, D.E., Morrison, H., Magendzo, K. and Edwards, R.H. (1992) Science 258, 1952–1955.
[10] Kiepper, B.L., Befort, K., Gaveriaux-Ruff, C. and Hirth, C.G. (1992) Proc. Natl. Acad. Sci., USA 89, 12048–12052.
[11] Sharma, S.K., Nirenberg, M. and Klee, W.A. (1975) Proc. Natl. Acad. Sci. USA 72, 590–594.
[12] Simonds, W.F., Koski, G., Streaty, R.A., Hjelmeland, L.M. and Klee, W.A. (1980) Proc. Natl. Acad. Sci. USA 77, 4623–4627.
[13] Zukin, R.S. and Maneckjee, R. (1986) Methods Enzymol. 124, 172–190.
[14] Simon, E.J., Dole, W.P. and Hiller, J.M. (1972) Proc. Natl. Acad. Sci. USA 69, 1835–1837.
[15] Chow, T. and Zukin, R.S. (1983) Mol. Pharmacol. 24, 203–212.
[16] Hynes, R.O. and Humphrey, K.C. (1974) J. Cell Biol. 62, 438–448.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.

[18] Ogita, Z.I. and Markert, C.L. (1979) Anal. Biochem. 99, 233–241.
[19] Morrisey, J.H. (1981) Anal. Biochem. 117, 307–310.
[20] Hames, B.D. and Rickwood, D. (1990) Gel Electrophoresis of Proteins, A Practical Approach, IRL Press, New York.
[21] Sharma, E., Kochhar, K.S. and Sharma, S.K. (1987) J. Biosci. 11, 423–433.
[22] Folch, J., Lees, M. and Sloane, S. (1957) J. Biol. Chem. 226, 497–509.
[23] Scheideler, M.A. and Zukin, R.S. (1990) J. Biol. Chem. 265, 15176–15182.
[24] Lowry, O.H., Rosebrough, N.J., Farri, S. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[25] Stoscheck, C.M. (1990) Methods Enzymol. 182, 50–68.
[26] Gilman, A. (1987) Annu. Rev. Biochem. 56, 615–649.
[27] Ueda, H., Harada, H., Nozaki, M., Katada, T., Ui, M., Satoh, M. and Takagi, H. (1988) Proc. Natl. Acad. Sci. USA 85, 7013–7017.
[28] Cho, T.M., Ge, B.L., Yamato, C., Smith, A.P. and Loh, H.H. (1983) Proc. Natl. Acad. Sci. USA 80, 5176–5180.