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Monoclonal antibody to the δ opioid receptor acts as an agonist in dual regulation of adenylate cyclase in NG108-15 cells

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Abstract Monoclonal antibodies generated against multiple antigenic peptides of the N-terminal sequence (^{3}LVPSARAELQSSPLV^{17}) of the cloned δ opioid receptor immunoprecipitated a 58 kDa protein from CHAPS-solubilized NG108-15 membranes. The immunoprecipitates bound [^{3}H]DPDPE – but not [^{3}H]DAMGO – with a K_d of 6.4 nM and a B_{max} of 75 pM. Western blot analysis revealed a distinct band of 58 kDa. The antibodies inhibited basal and PGE_1-stimulated cAMP levels, and mimicked the effect of agonists manifest in a compensatory increase in cAMP formation. The antibody will be potentially useful in the analysis of functional epitopes on the δ opioid receptor.

Key words: Adenylate cyclase; Antibody; cAMP; G protein; Opioid receptor; Signal transduction

1. Introduction

The opioid receptors mediate a host of behavioral effects including antinociception, reward and reinforcement as well as a number of neuroendocrine responses. Three major classes of opioid receptors designated μ, δ and κ have been cloned and identified as members of the G protein family of seven transmembrane domain receptors which are negatively coupled to adenylate cyclase [1–6]. Extensive efforts in the past were made by several investigators to purify the receptor proteins for elucidating their molecular properties [7–10]. However, all such attempts in the last two decades to obtain adequate amounts of homogeneous and functionally competent receptor proteins have been, by and large, unsuccessful. Studies on site-directed mutagenesis [11,12] and receptor chimeras [13] had suggested the involvement of different receptor regions in the multiple specificity for a variety of ligands responsible for complex behavioral manifestations. The availability of monoclonal antibodies will help in the delineation of functional epitopes on the receptor and provide a valuable tool for the quantitation of the receptor protein present in the cells under different physiological conditions. It will also fulfil a long-felt need for a convenient method for the purification of the receptor protein for structural studies. In this paper we report the generation of a functional monoclonal antibody to the multiple antigenic peptide (MAP) of the N-terminal of the cloned δ opioid receptor. Significantly, the antibody acts as an agonist in the dual regulation of adenylate cyclase in NG108-15 cells.

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Abbreviations: cAMP, cyclic adenosine-3′,5′-monophosphate; CHAPS, 3-[(3-cholamidopropyl) dimethyl ammonio] 1-propane-sulfonate; DAMGO, [D-Ala^2, *N*-methyl-Phe^4, Gly-ol] enkephalin; DPDPE, D-Pen^2,D-Pen^5-enkephalin; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-[2-hydroxyethy1]piperazine-*N*′-[2-ethanesulfonic acid]; HRPO, horseradish peroxidase; K_d, equilibrium dissociation constant; MAP, multiple antigenic peptide; Nt-Ab, N-terminal monoclonal antibody; PBS, phosphate-buffered saline; PGE_1, prostaglandin E_1; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TTBS, Tris-buffered saline with Tween-20 (0.1%)

2. Materials and methods

2.1. Cell growth

Neuroblastoma×glioma hybrid NG108-15 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with hypoxanthine aminopterin thymidine, 10% fetal calf serum (Biological Industries, Israel), streptomycin (100 μg/ml) and penicillin (100 U/ml) as previously described [1,10].

2.2. Production of monoclonal antibodies

MAP to the N-terminal sequence (^{3}LVPSARAELQSSPLV^{17}) of the cloned δ opioid receptor was obtained from Research Genetics, USA. BALB/c mice were injected intraperitoneally (i.p.) with MAP (10 μg) emulsified in Freund's complete adjuvant. Booster i.p. injections were given every 15 days with MAP emulsified in Freund's incomplete adjuvant. Three days prior to fusion 10 μg MAP was injected intravenously (i.v.). Mice were bled and serum collected for polyclonal antisera. The spleens from the immunized mice were fused with SP2/0-Ag14 myeloma cells as described by Dastidar and Sharma [14]. The positive secreting clones were selected by an ELISA using pure MAP and NG108-15 cells.

2.3. ELISA

ELISA was carried out by a modification of the method described by Sharma et al. [15]. Wells of the ELISA plate were coated with 1 μg MAP/1×10^5 NG108-15 cells in 50 mM Tris-HCl, pH 7.5 by incubating overnight at 4°C. Non-specific sites were blocked with 3% BSA in phosphate-bufferd saline (PBS) for 1 h at 37°C. Wells were incubated overnight at 4°C with hybridoma culture supernatant or growth medium from Sp2/0-Ag14 as control. Plates were washed thrice with PBS and incubated with goat anti-mouse IgG coupled to peroxidase for 1 h at room temperature. Color was developed using *ortho*-phenylenediamine (0.5 mg/ml in 0.15 M citrate phosphate buffer, pH 5.0) and H_2O_2 (0.03%) and the reaction was terminated after 10 min by addition of 5 N H_2SO_4.

2.4. Purification of monoclonal antibodies

2.4.1. Ammonium sulfate precipitation. Saturated ammonium sulfate solution was added to the hybridoma culture supernatant to bring the final concentration to 50%. The solution was stirred gently at 4°C overnight and centrifuged at $3000\times g$ for 30 min. The pellet was dissolved in 0.1 volume of the starting volume in PBS, dialyzed extensively against PBS, centrifuged and the protein concentration was determined by Bradford's method [16].

2.4.2. Purification on protein A-Sepharose CL-4B column (high salt). Monoclonal antibodies were purified by a modification of the method of Ey et al. [17]. Concentration of NaCl in the hybridoma culture supernatant was adjusted to 3.3 M and 1/10 volume of 1 M

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sodium borate (pH 8.9) was added. The solution was passed through a protein A-Sepharose CL-4B (Pharmacia, Sweden) bead column. The beads were washed with 10 column volumes each of 3 M NaCl, 50 mM sodium borate (pH 8.9) and 3 M NaCl, 10 mM sodium borate (pH 8.9). Antibody was eluted with 100 mM glycine (pH 3.0). The eluates were collected in tubes containing 50 μl of 1 M Tris-HCl (pH 8.0). Fractions containing immunoglobulin were identified by absorbance at 280 nm, and protein was determined by Bradford's method [16].

2.5. Solubilization of the δ opioid receptor

Membranes from NG108-15 cells were prepared and the receptor solubilized as described by Gomathi and Sharma [10]. In brief, membranes were washed by incubating with 50 mM Tris-HCl, pH 7.5 containing 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, USA) for 30 min at 37°C and solubilized using 10 mM 3-[(3-cholamidopropyl) dimethyl ammonio] 1-propane-sulfonate (CHAPS) (Sigma, USA). For immunoprecipitation the CHAPS-solubilized extract was diluted to 1 mM CHAPS with 50 mM Tris-HCl, pH 7.5.

2.6. Immunoprecipitation

Protein (A+G)-agarose beads (Calbiochem, USA), 10 μl, were suspended in 1 ml TBS (20 mM Tris-HCl, pH 7.4 with 150 mM NaCl) and washed three times in TBS by centrifugation at 10 000 rpm for 1 min. To 100 μl of beads suspended in TBS, 100 μg monoclonal antibody (purified by ammonium sulfate precipitation) or 100 μg mouse IgG was added. The tubes were incubated for 1 h at room temperature with gentle shaking, followed by centrifugation at 10 000 rpm for 1 min and washing with TBS three times. To the antibody- or mouse IgG-coated beads, 70 μl of solubilized receptor (1 mM CHAPS, 200 μg protein) was added. Receptor-antibody complexes were incubated overnight at 4°C with mild shaking. Samples were centrifuged at 10 000 rpm for 1 min and supernatant was saved for assay of residual binding. The beads were washed three times with TBS and three times with buffer A (50 mM Tris-HCl, pH 7.5 with 1 mM CHAPS and 1 mM PMSF). The pellet containing the immunoprecipitates was used for binding of opiates.

2.7. Opioid receptor binding

Immunoprecipitates obtained as described above or NG108-15 membranes (500–750 μg protein) were suspended in 50 mM Tris-HCl, pH 7.5 and binding was measured with [^{3}H]DPDPE (32.4 Ci/mmol)/[^{3}H]DAMGO (48.9 Ci/mmol) (New England Nuclear, USA) for 60 min at 37°C in a final volume of 300 μl. Non-specific binding in the presence of cold DPDPE/DAMGO (National Institute of 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 Tris-HCl (50 mM, pH 7.5), dried and radioactivity determined in a toluene-based scintillation fluid.

2.8. Western blot

Western blots were performed as described by Dastidar and Sharma [14]. NG108-15 cells were washed with sterile PBS three times and preheated sample buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 10% glycerol, 0.1% bromophenol blue) at 100°C was added. The samples were kept at 100°C for 3 min, followed by centrifugation at 10 000×*g* for 5 min. The supernatant was electrophoresed on sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels at 125 V for 90 min. The protein bands were electrotransferred onto polyvinylidene difluoride Immobilon-P membranes (Millipore, USA) for 90 min at 150 mA. The membranes were blocked for 1 h in TTBS (20 mM Tris-HCl, pH 7.4, and 150 mM NaCl containing 0.2% (v/v) Tween-20) at room temperature. Membranes were incubated with Nt-Ab (polyclonal antiserum) to MAP or normal mouse IgG (1:100 in TTBS) for 16 h at 4°C and washed five times (5 min each) in TTBS followed by exposure to secondary antibody (sheep anti-mouse conjugated to HRPO, 1:1000 in TTBS) (Gibco, USA) for 1 h at room temperature. Membranes were washed five times (5 min each) with TTBS followed by incubation for 1 h at room temperature with streptavidin-HRPO conjugate (1:1500 in TTBS) (Gibco, USA). Membranes were washed 5 times with TTBS (5 min each) and developed using ECL Kit (Amersham, UK). Western blots were also performed using 5 μg affinity-purified N-terminal antibody in TTBS as the primary antibody and goat anti-mouse conjugated with alkaline phosphatase (1:5000 dilution of 0.4 mg/ml stock in TTBS) as the secondary antibody. In this case membranes were incubated in 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (chromogenic) reagent (Kirkegaard and Perry Laboratories, USA) to visualize the immunoreactive bands.

2.9. Immunofluorescence

NG108-15 cells (1×10^3) were plated on poly-L-lysine-coated coverslips (Sigma, USA) and immunofluorescence was performed as described by Goswami et al. [18] using Nt-Ab (polyclonal antiserum) as the primary antibody (1:20 in PBS) and fluorescein isothiocyanate-labelled anti-mouse as the secondary antibody (1:100 in PBS).

2.10. Assay of cAMP in intact cells

Confluent cells (in 60 mm petri dishes) were washed three times with 5 ml of medium A (DMEM with 25 mM HEPES, pH 7.4) and incubated in medium A containing 0.5 mM Ro20-1724 for 10 min at 37°C. The reaction was initiated by the addition of 30 μl of opiate in water/30 μl of PGE_1 in ethanol/solvent. Ethanol (0.5% when Ro20-1724 was used and 1% when Ro20-1724 and PGE_1 were used) had no effect upon cAMP formation. After incubation the medium was discarded and 2 ml 5% TCA with [^{3}H]cAMP (3000 cpm) was added at 4°C. cAMP was purified and assayed by the method of Gilman [19] as modified by Sharma et al. [1,2]. Values reported for triplicate dishes were corrected to 100% recovery of cAMP.

3. Results

In these studies three preparations of antibody (Nt-Ab) were used: (I) the polyclonal antisera (Section 2.2), (ii) monoclonal antibody concentrated by ammonium sulfate precipitation of the hybridoma culture supernatant (Section 2.4.1) and (iii) monoclonal antibody purified on protein A-Sepharose CL-4B columns (Section 2.4.2).

The δ opioid receptor from NG108-15 membranes was solubilized in 10 mM CHAPS; the extract diluted to 1 mM CHAPS was incubated with Nt-Ab-coated protein (A+G)-agarose beads. The immunoprecipitates thus obtained were used for binding of [^{3}H]DPDPE and [^{3}H]DAMGO. Equivalent amounts of the solubilized receptor protein were also used as a suitable reference. The immunoprecipitates bound more than 70 fmol of [^{3}H]DPDPE, with only 20 fmol of residual binding left in the supernatant. [^{3}H]DAMGO, the μ opioid receptor-specific ligand, did not bind to the immunoprecipitated receptor. Immunoprecipitates obtained with nor-

Table 1
Binding of [^{3}H]DPDPE/[^{3}H]DAMGO to the receptor in Nt-Ab immunoprecipitates

Receptor	Specific binding (fmol)	
	[^{3}H]DPDPE	[^{3}H]DAMGO
Receptor-Nt-Ab		
Pellet	78.6	1.26
Supernatant	20.7	2.21
Receptor-mouse IgG		
Pellet	5.52	0.72
Supernatant	90.5	3.36
CHAPS solubilized receptor	154.7	5.13

NG108-15 membranes were solubilized using CHAPS. To 200 μg of solubilized protein 200 μg of Nt-Ab (ammonium sulfate precipitate)/normal mouse IgG coupled to protein (A+G)-agarose beads was added. The receptor antibody complex bound to the beads was collected by centrifugation and diluted with the binding buffer. Binding assays were performed with aliquots of the immunoprecipitate, supernatant and equivalent amount (200 μg) of CHAPS solubilized receptor protein as described in Section 2. Concentrations of [^{3}H]DPDPE and [^{3}H]DAMGO were 5 nM and of cold DPDPE/DAMGO 10 μM. Values shown are the average of two experiments performed in triplicate.

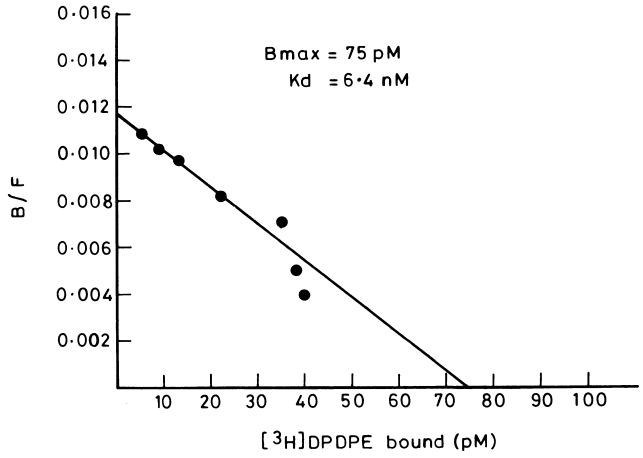

Fig. 1. Scatchard analysis of [^{3}H] DPDPE specific binding to Nt-Ab immunoprecipitates. Binding assays with the δ-specific ligand [^{3}H]DPDPE (final concentration 1–20 nM) were carried out with the immunoprecipitates obtained from CHAPS solubilized NG108-15 membranes and Nt-Ab (ammonium sulfate precipitate) as described in Section 2. Non-specific binding was determined in the presence of cold DPDPE (10 μM) and was 20–30% of the total binding. Each value represents the average of two experiments performed in triplicate.

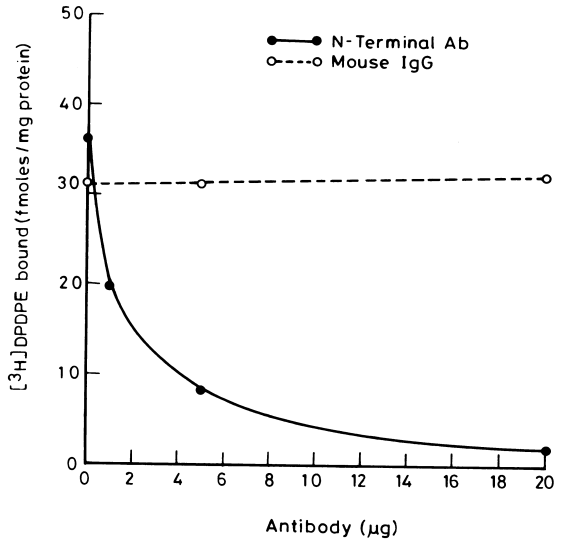

Fig. 2. Competitive inhibition of [^{3}H]DPDPE binding to δ opioid receptors by Nt-Ab. Specific binding of [^{3}H] DPDPE (final concentration 10 nM) to NG108-15 membranes (750 μg) was carried out in the presence of varying amounts of Nt-Ab (affinity-purified) (●); normal mouse IgG (○). Non-specific binding was determined in the presence of cold DPDPE (10 μM) and was 20–30% of the total binding. Each value is the average of two experiments performed in triplicate.

mal mouse IgG did not show any binding with [^{3}H]DPDPE (Table 1). The K_d and B_{max} for [^{3}H]DPDPE binding to the Nt-Ab immunoprecipitates were estimated to be 6.4 nM and 75 pM, respectively, from the Scatchard plot shown in Fig. 1.

Fig. 2 shows the inhibitory effect of Nt-Ab on the binding of [^{3}H]DPDPE to NG108-15 membranes as a function of its concentration. Maximal inhibition by the antibody under the conditions of our experiment was discernible at 15 μg of the antibody, with 50% inhibition at 4 μg. Normal mouse IgG, serving as a control, elicited no inhibition as shown by the dotted line.

Results of the Western blot analysis are shown in Fig. 3A,B. Two distinct bands – a major one at 58 kDa and a minor one at 30–38 kDa – are clearly identifiable (Fig. 3A) using the Nt-Ab (polyclonal antisera). Affinity-purified monoclonal antibody also revealed the presence of the same two bands (Fig. 3B).

We next examined the effects of the monoclonal antibody (Nt-Ab) on intact NG108-15 cells, with particular reference to its modulatory influence on the basal and PGE_1-stimulated cAMP formation. As seen in Table 2 Nt-Ab (ammonium sulfate precipitate) inhibited the basal level of cAMP by 38% and the PGE_1-stimulated levels by 49%. Naloxone partially reversed the Nt-Ab-induced inhibition by 25% in the case of basal and by 35% in the case of PGE_1-stimulated cAMP levels. The presence of both morphine and Nt-Ab elicited no additive inhibition of basal and PGE_1-stimulated cAMP levels. Normal mouse IgG did not show any effect. The inhibitory effect of Nt-Ab on PGE_1-stimulated cAMP formation was found to be concentration-dependent (Fig. 4). Opioids – morphine and etorphine – are known to lead to a compensatory increase in the levels of cAMP in NG108-15 cells. Data presented in Table 3 indicated that the effect of the Nt-Ab follows similar trend. Addition of 5μg of affinity-purified monoclonal antibody resulted in 38% compensatory increase in cAMP formation in comparison to 63% increase in the presence of morphine.

Further confirmation of the fact that the antibody (Nt-Ab) is indeed directed specifically towards δ receptors came from immunofluorescence studies on NG108-15 and C_6 glioma cells. As seen in Fig. 5, while NG108-15 cells gave strong membranous fluorescence (A), C_6 glioma cells lacking δ receptors gave no signal (B). Control cells treated with preimmune sera also showed no fluorescence (C).

Table 2
Inhibition of basal and PGE_1-stimulated cAMP formation in NG108-15 cells by Nt-Ab and its reversal by naloxone

Additions	pmol of cAMP formed/mg protein
No addition	167
Nt-Ab	104
Nt-Ab+naloxone	144
Morphine	94
Nt-Ab+morphine	102
PGE_1	3900
Nt-Ab+PGE_1	1980
Nt-Ab+PGE_1+naloxone	3200
Morphine+PGE_1	1990
Nt-Ab+morphine+PGE_1	1930
Mouse IgG+PGE_1	3680

NG108-15 cells (P15) were plated in 60 mm petri dishes. When 80% confluent, the medium was removed, cells were washed quickly with DMEM-HEPES and incubated with different effector agents as described in Section 2. Concentrations of effector agents were: Nt-Ab (ammonium sulfate precipitate); 200 μg; normal mouse IgG, 200 μg; morphine, 10 μM; PGE_1, 10 μM; naloxone, 10 μM. Each value is the average of two experiments performed in triplicate.

4. Discussion

We report the generation of a monoclonal antibody against the amino-terminal 3–17 sequence of the cloned δ opioid receptor, which immunoprecipitated the δ opioid receptor in

CHAPS-solubilized NG108-15 cellular membranes. The immunoprecipitates retain specific binding of [^{3}H]DPDPE with a K_d of 6.4 nM and a B_{max} of 75 pM, which was in the same range as reported by us earlier [10]. NG108-15 cells express only the δ opioid receptors and the immunoprecipitates did not bind to μ-specific ligands like [^{3}H]DAMGO. This is highly reassuring as regards the specificity of the binding of [^{3}H]DPDPE to the immunoprecipitates. A detailed investigation revealed a dose-dependent inhibition of basal and PGE_1-stimulated cAMP formation by the Nt-Ab. Naloxone reversed this inhibition by 25 and 35%, respectively. It is interesting to note that the monoclonal antibody (Nt-Ab) also mimicked the effect of opioid agonists in bringing about a compensatory increase in cAMP. The N-terminal of the δ-opioid receptor is known to be in the extracellular domain, along with several loops [23]. Therefore, the observed inhibitory effect of the monoclonal antibody (Nt-Ab) on the binding of [^{3}H]DPDPE to NG108-15 cells is not entirely unexpected. However, this does not necessarily imply that the epitope recognized by Nt-Ab coincides with the binding site of [^{3}H]DPDPE. The antibodies, being bulky molecules, may influence the ligand binding by steric hindrance. This question can only be settled by the delineation of the epitope recognized by Nt-Ab, and of the binding sites for the ligands. The point we wish to emphasize here is that monoclonal antibody (Nt-Ab) acts as an opioid agonist and facilitates signal transduction as inferred from its modulatory influence on the levels of cAMP, by bringing about subtle conformational changes in the receptor. Cucumel and Cupo [20] showed that anti-idiotypic antiserum (against antibodies directed against DSLET) inhibited the binding of [^{3}H]DADLE and mimicked inhibitory effects of the agonist on cAMP levels in NG108-15 cells, but did not discriminate between μ and δ opioids. On the other hand, Nt-Ab obtained in this study is not only specific for δ receptors but also acts as a potent opioid agonist. The reversal of its inhibitory effect by naloxone is understandable, in view of the high affinity of the antagonist for the receptor.

Several investigators have used antibodies against opioid receptors which elicited non-specific interactions with several proteins as revealed by Western blot analysis [20–22]. In contrast, both polyclonal antisera and the monoclonal Nt-Ab

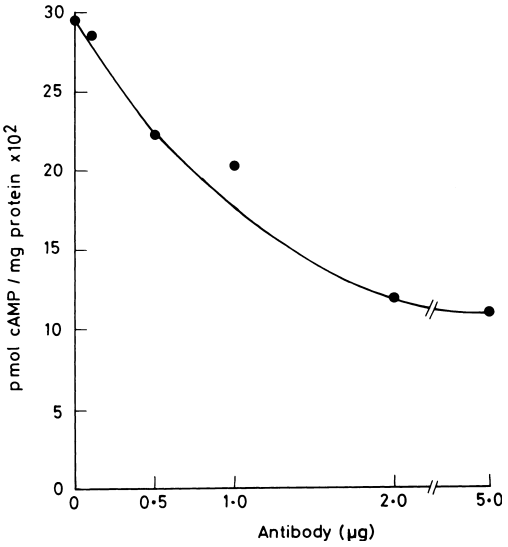

Fig. 4. Inhibition of PGE_1-stimulated cAMP formation as a function of Nt-Ab concentration. NG108-15 cells (P15) were plated in 60 mm petri dishes. When 80% confluent, the medium was removed, cells were washed quickly with DMEM-HEPES and incubated as described in Section 2. Concentrations of effector agents were: Nt-Ab (affinity-purified), 0–5 μg; PGE_1, 10 μM. Each value is the average of two experiments performed in triplicate.

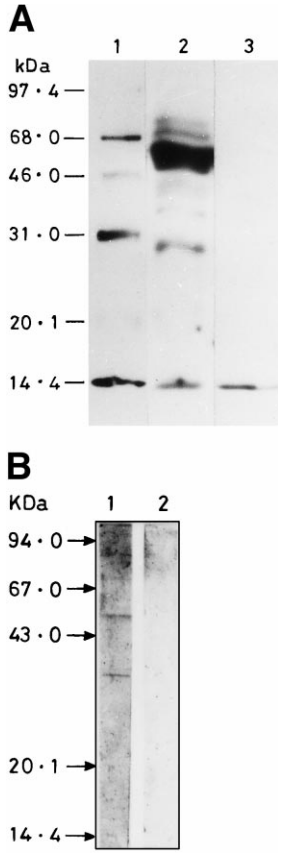

Fig. 3. Western blot analysis of CHAPS solubilized NG108-15 membranes. Whole NG108-15 cell lysates (A) or NG108-15 membranes solubilized with CHAPS (B) were subjected to SDS-PAGE followed by transfer to Immobilon-P membranes as described in Section 2. A: Lane 1, ECL markers; 2, Nt-polyclonal antiserum; 3, pre-immunized mouse serum. B: Lane 1, Nt-Ab (affinity-purified); 2, normal mouse IgG.

Table 3
Effect of Nt-Ab on opiate-induced compensatory increase in cAMP formation in NG108-15 cells

Additions	pmol cAMP formed/mg protein
No addition	1359
Morphine	2218
Nt-Ab	1871
Nt-Ab+morphine	2148
Etorphine	2529
Nt-Ab+etorphine	2353

NG108-15 cells (P15) were plated in 60 mm petri dishes. When 80% confluent, medium was removed and 2 ml fresh medium with morphine, 10 μM; Nt-Ab (affinity-purified) 5 μg; etorphine, 1.6 μM was added. Fresh Nt-Ab was added every 24 h. After 48 h the medium was removed, the monolayer was washed quickly in DMEM-HEPES three times and incubated in DMEM-HEPES with naloxone, 10 μM for cAMP formation as described in Table 2. Normal mouse IgG, 5 μg, when added during growth had no effect (data not shown). Each value is the average of three experiments performed in duplicate.

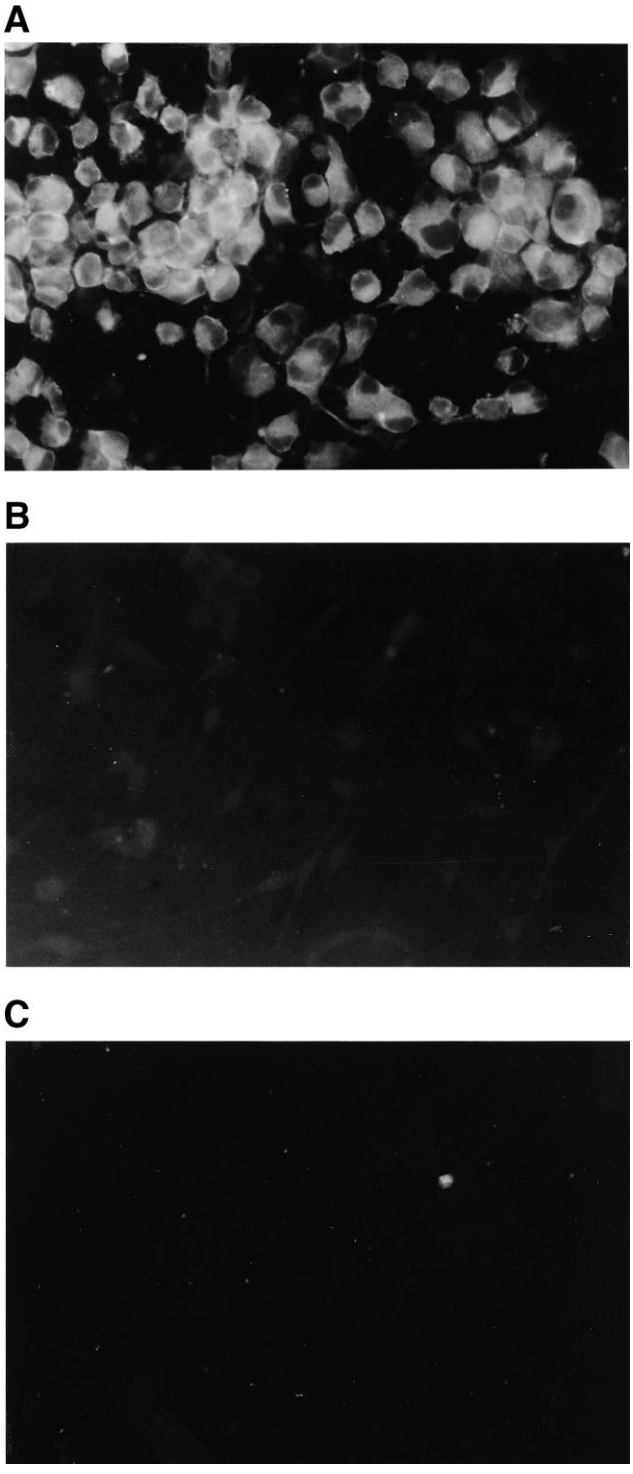

Fig. 5. Indirect immunofluorescence with Nt-Ab (polyclonal antiserum) 1:20, and NG108-15 cells/c6 glioma cells. Nt-Ab (polyclonal antiserum) (A) NG108-15 cells; (B) C_6 glioma cells; (C) NG108-15 cells with pre-immune mouse serum. Magnification ×400.

(affinity-purified) to the δ opioid receptor reported here recognized a major protein of 58 kDa (Fig. 3A,B) in NG108-15 membranes. These observations were further substantiated by strong membranous fluorescence exhibited by the Nt-Ab on NG108-15 cells (Fig. 5A). In view of its high specificity and strong binding to δ opioid receptor protein, the Nt-Ab is potentially useful in the preparation of large amounts of the receptor by affinity columns. In addition, it provides a valuable tool for studying the expression of the opioid receptor in brain under different physiological conditions.

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