δ -Opioid-receptor-mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding protein G_{i2}

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Mouse neuroblastoma $\times$ rat glioma hybrid cells (NG108-15) express an opioid receptor of the δ subclass which both stimulates high-affinity GTPase activity and inhibits adenylate cyclase by interacting with a pertussis-toxin-sensitive guanine-nucleotide-binding protein(s) (G-protein). Four such G-proteins have now been identified outwith photoreceptor-containing tissues. We have generated anti-peptide antisera against synthetic peptides which correspond to the C-terminal decapeptides of the α -subunit of each of these G-proteins and also to the stimulatory G-protein of the adenylate cyclase cascade (G_s). Using these antisera, we demonstrate the expression of three pertussis-toxin-sensitive G-proteins in these cells, which correspond to the products of the G_{i2}, G_{i3} and G_o genes, as well as G_s. G_{i1}, however, is not expressed in detectable amounts. IgG fractions from each of these antisera and from normal rabbit serum were used to attempt to interfere with the interaction of the opioid receptor with the G-protein system by assessing ligand stimulation of high-affinity GTPase activity, inhibition of adenylate cyclase activity and conversion of the receptor to a state which displays reduced affinity for agonists. The IgG fraction from the antiserum (AS7) which specifically identifies G_{i2} in these cells attenuated the effects of the opioid receptor. This effect was complete and was not mimicked by any of the other antisera. We conclude that the δ -opioid receptor of these cells interacts directly and specifically with G_{i2} to cause inhibition of adenylate cyclase, and that G_{i2} represents the true G_i of the adenylate cyclase cascade. The ability to measure alterations in agonist affinity for receptors following the use of specific antisera against a range of G-proteins implies that such techniques should be applicable to investigations of the molecular identity of the G-protein(s) which interacts with any receptor.

INTRODUCTION

Pretreatment of cells or tissues with pertussis toxin produces an attenuation of receptor-mediated inhibition of adenylate cyclase activity [1]. Thus the guanine-nucleotide-binding protein (G-protein) involved, G_i, was defined to be a substrate for ADP-ribosylation catalysed by this toxin. This polypeptide was identified, by the use of pertussis toxin and [³²P]NAD⁺ followed by SDS/PAGE and subsequent autoradiography, as a 40 kDa protein [2]. From cDNA cloning studies it is now clear that there are multiple G-proteins which are substrates for this toxin, the α -subunits of which are all close to 40 kDa. These are the products of the G_o, G_{i1}, G_{i2} and G_{i3} genes [3,4]. All of these polypeptides are highly similar in amino acid sequence and much recent research has attempted to address the question of which of these G-proteins represents the true G_i. Pertussis toxin cannot discriminate between receptor contacts with each of these proteins and therefore novel approaches have had to be devised to address this question.

Considerable evidence implicates the extreme C-terminus of G-protein α -subunits as representing a key site of contact between receptors and G-proteins. This conclusion is based upon the fact that the site of pertussis-toxin-catalysed ADP-ribosylation of its substrates is a cysteine residue located only four amino acids from the C-terminus of the α -subunit [1]. Furthermore, the molecular defect of the so-called *unc* mutation of murine S49 lymphoma cells, in which a β -adrenergic receptor is unable to interact with G_s, is the substitution of a proline residue for an arginine six amino acids from the C-terminus of G_s α [5,6].

The neuroblastoma $\times$ glioma hybrid cell line, NG108-15, has been widely used as a model system to study signal transduction processes [7]. Both δ -opioid and α_2 -adrenergic receptors inhibit adenylate cyclase in these cells by interacting with and activating a pertussis-toxin-sensitive G-protein(s), which must therefore be G_i [8–10]. Herein we identify each of the pertussis-toxin-sensitive G-proteins expressed in these cells by immunoblotting membranes of NG108-15 cells with selective antisera which identify the C-terminal decapeptides of each G-protein [11–14]. We demonstrate that the true G_i of the adenylate cyclase cascade must be the product of the G_{i2} gene, as antibodies which selectively identify this G-protein both prevent opioid-receptor stimulation of high-affinity GTPase activity and inhibition of adenylate cyclase in membranes from these cells, and convert the opioid receptor to a state with reduced affinity for opioid agonists.

MATERIALS AND METHODS

Materials

[³²P]NAD⁺ (20–30 Ci/mmol) and [³H]DADLE ([³H][D-Ala², D-Leu⁵]enkephalin) (39.3 Ci/mmol) was purchased from New England Nuclear, Darmstadt, Germany. [γ -³²P]GTP (> 10 Ci/mmol) was from Amersham International, Cardiff, Wales, U.K. Pertussis toxin was from Porton Products, Porton Down, Wiltshire, U.K. Horseradish-peroxidase-coupled donkey anti-rabbit IgG was from the Scottish Antibody Production Unit, Carlisle, Scotland, U.K. All other reagents were from Sigma or BDH and were of the highest grade available.

Abbreviations used: G-protein, guanine-nucleotide-binding protein; DADLE, [D-Ala², D-Leu⁵]enkephalin; Gpp[NH]p, guanosine 5'-[β -imido]triphosphate.

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Cell culture

Neuroblastoma × glioma hybrid NG108-15 cells were grown in tissue culture as described previously [15]. Cells were harvested when some 80% confluent, and membrane fractions were prepared as in [15]. In some cases the cells were treated with pertussis toxin (25 ng/ml, 24 h) prior to harvest. Membranes were stored at -70°C before use.

Rat glioma C6 BU1 cells were grown as in [16]. Membranes from these cells were prepared as above. Plasma membranes from white adipocytes derived from rat epididymal fat pads were prepared as in [17].

Immunological studies

The generation and specificities of the various antisera used in this study are defined in Table 1. Each antiserum was produced in a New Zealand White rabbit using a conjugate of a synthetic peptide and keyhole-limpet haemocyanin (Calbiochem) as antigen. The details of this process have previously been recorded [11]. Membrane samples were resolved by SDS/PAGE [12.5% acrylamide/0.0625% bis-acrylamide (w/v)] in 20 cm × 20 cm slab gels overnight at 100 V. Proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 2 h at 37°C with 3% gelatin in Tris-buffered saline [TBS; 20 mM-Tris/HCl (pH 7.5)/500 mM-NaCl]. Primary antiserum (1:200 dilution unless otherwise noted) in 1% gelatin/TBS was then added and left overnight. The primary antiserum was then removed and the blot was washed extensively with distilled water followed by washes with TBS containing 0.1% (v/v) Tween 20 (TTBS) and then TBS. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase) in 1% gelatin/TBS was added and left for 2 h. Removal of the secondary antiserum was followed by the same series of extensive washes of the nitrocellulose as detailed following removal of the primary antiserum. *o*-Dianisidine hydrochloride (Sigma) was employed as the substrate for detection of the antibody complex as described previously [12,13].

Pertussis-toxin-catalysed ADP-ribosylation

Membranes from NG108-15 cells were ADP-ribosylated as described in [15] using thiol-preactivated pertussis toxin and [^{32}P]NAD $^{+}$. Samples were resolved, following alkylation [18], in identical SDS/PAGE slabs to those used for the immunoblotting experiments. The gels were then dried and autoradiography was performed for 48 h using Kodak X-O-Matt X-ray film.

Generation of oligodeoxynucleotide probes

Synthetic 33-mer oligodeoxynucleotides (generated on an Applied Biosystems DNA synthesizer) were complementary to bases of the mRNAs which encode amino acids 125–135 of the

α -subunits of each of G_{i1} , G_{i2} and G_{i3} . This region corresponds to one of the highly divergent areas of the coding sequence of these G-proteins as defined by analysis of rat cDNA clones [3]. Labelling of probes at the 5'-end was achieved with ^{32}P (6000 Ci/mmol, Amersham International) and T4 polynucleotide kinase (Boehringer-Mannheim). Incorporation of radioactivity averaged 7×10^8 d.p.m./ μg of probe.

Detection of mRNA species corresponding to G_{i1} , G_{i2} and G_{i3}

NG108-15 cells were harvested and then ground to a fine powder in liquid N_2 , and subsequently homogenized in 6 ml of GIT buffer [4 M-guanidinium thiocyanate/25 mM-sodium acetate (pH 6.0)/120 mM-2-mercaptoethanol] as in [13], followed by extrusion through 18 and 23 G needles. The homogenate was then centrifuged (10000 g, 10 min) to remove particulate material, and the supernatant was layered over 6 ml of CsCl buffer [5.7 M-CsCl/25 mM-sodium acetate (pH 6)] in polyallomer ultracentrifuge tubes (Beckman). Samples were centrifuged overnight in a Beckman SW40 rotor (170000 g, 20 h, 20°C). Total RNA was recovered from the bottom of the tubes, resuspended in GIT buffer, extracted once against each of chloroform/phenol (1:1, v/v) and chloroform and finally precipitated with ethanol. The concentration of precipitated RNA was determined by absorbance at 260 nm.

Northern blotting

Total RNA was denatured by incubation at 65°C with 2 M-formaldehyde and 50% deionized formamide. It was resolved in 1% agarose gels containing 2.2 M-formaldehyde before transfer to Hybond N nylon filters (Amersham) [13]. Filters were prehybridized at 42°C for 4 h in hybridization solution [50% deionized formamide, 5 × SSPE (1 × SSPE = 150 mM-NaCl/10 mM- NaH_2PO_4 /1 mM-EDTA, pH 7.4), 5 × Denhardt's solution (5 × = Ficoll 400, bovine serum albumin and polyvinylpyrrolidone, each at 1 mg/ml), 0.1% SDS and 100 μg of denatured salmon-testes DNA/ml] and were then hybridized (42°C , 16 h) in hybridization solution containing the relevant oligodeoxynucleotide probes (5 × 10^6 c.p.m./ml). The probe for $G_{i1\alpha}$ was 5'-GCTGTCCTCCACAGTCTCTTTATGAC-GCCGGC-3', that for $G_{i2\alpha}$ was 5'-ATGGTCAGCCCAGAG-CCTCCGGATGACGCCCGA-3' and that for $G_{i3\alpha}$ was 5'-GCCATCTCGCCATAAACGTTTAATCACGCCTGC-3'. Each probe is derived from information encoding amino acids 125–135 for each G-protein as expressed in the rat. We have previously used these probes to demonstrate the expression of each of $G_{i1\alpha}$, $G_{i2\alpha}$ and $G_{i3\alpha}$ in rat adipocytes [13].

The blots were washed to a final stringency of 0.5 × SSC (1 × SSC = 150 mM-NaCl/15 mM-trisodium citrate, pH 7.0)/0.1% SDS (55°C , 30 min). Autoradiographic localization of the

Table 1. Generation and specificities of anti-G-protein antisera

The antisera were generated in New Zealand White rabbits using a conjugate of the synthetic peptide and keyhole limpet haemocyanin as detailed in the Materials and methods section. The specificities of the antisera were demonstrated in the references noted in the Table. $G_{i1\alpha}$ and $G_{i2\alpha}$ have identical C-terminal decapeptides and thus antiserum AS7 identifies each of these polypeptides equally. Transducin α is also identified by antiserum AS7, but as this G-protein is restricted in distribution to photoreceptor-containing tissues, the antiserum can be used as a probe for G_{i1} or G_{i2} in all other locations.

Antiserum	Peptide employed	Corresponding G-protein sequence	Antiserum identifies:
AS7	KENLKDCGLF	Transducin α 341–350	Transducin, G_{i1} , G_{i2} [11]
OC1	ANNLRGCGLY	$G_{i\alpha}$ 345–354	$G_{i\alpha}$ [14]
I3B	KNNLKECGLY	$G_{i3\alpha}$ 345–354	G_{i3} [13]
CS1	RMHLRQYELL	$G_{i\alpha}$ 385–394	$G_{i\alpha}$ [12]
LE2	LERIAQSDYI	$G_{i2\alpha}$ 160–169	G_{i2} [11]

bound probes was performed by exposure to presensitized X-ray film at -80°C .

Adenylate cyclase assays

Forskolin ($10\text{ }\mu\text{M}$)-amplified adenylate cyclase activities and opioid-peptide-modulation of this activity were measured as described [19] by the use of a binding protein to determine amounts of cyclic AMP generated. In a number of cases, neuroblastoma $\times$ glioma NG108-15 cell membranes were preincubated for 60 min at 30°C with reconstituted IgG fractions generated (see below) from either normal rabbit serum or antiserum AS7.

High-affinity GTPase assays

These assays were performed essentially as in [7,20]. In a number of cases, membranes from NG108-15 cells were incubated with reconstituted IgG fractions of the various antisera for 60 min at 37°C in the presence of all the reagents used in the GTPase assay except for $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and receptor agonist. Following the preincubation period, both $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ ($0.5\text{ }\mu\text{M}$, 60000 c.p.m.) and DADLE ($1\text{ }\mu\text{M}$) were added and the samples were incubated for a further 20 min before analysis of the generation of free ^{32}P .

Binding experiments

These were performed at 25°C for 30 min in 10 mM-Tris/HCl (pH 7.5)/50 mM-sucrose/20 mM-MgCl₂ (buffer B). In saturation experiments using $[\text{H}]\text{DADLE}$, the concentration of ligand was varied between 0.5 and 20 nM. Non-specific binding was defined in all cases by parallel assays containing $10\text{ }\mu\text{M}$ -DADLE. Non-specific binding increased with $[\text{H}]\text{ligand}$ concentration in a linear manner but was never greater than 20% of the total binding. Binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes of the filter with ice-cold buffer B (5 ml). Specific binding data was manipulated according to Scatchard [22].

Generation of IgG fractions from anti-G-protein antisera

A column of Protein A-Sepharose 4B (Pharmacia) (0.5 ml) was equilibrated with 1.5 M-glycine (pH 8.9)/3 M-NaCl (buffer C). A 5 ml portion of antiserum in buffer C was added to the column and allowed to equilibrate with gentle mixing. The column was washed with buffer C until the eluate had an A_{280} of 0.0 and was then eluted with 100 mM-citric acid, pH 4.0, into 2 M-Tris/HCl, pH 7.5. The eluted IgG fractions were dialysed overnight against 1000 vol. of 10 mM-Tris/HCl/0.1 mM-EDTA (pH 7.5) (buffer A), then lyophilized and reconstituted with buffer A prior to use.

RESULTS

We treated membranes of neuroblastoma $\times$ glioma hybrid NG108-15 cells with thiol-activated pertussis toxin and $[\text{P}^{32}]\text{NAD}^{+}$ and then resolved the membranes on SDS/PAGE slab gels. Three distinct polypeptides were identified which had apparent molecular masses of 40.5, 40 and 39 kDa (Fig. 1). As cDNA cloning experiments have demonstrated the potential expression, outwith photoreceptor-containing tissues, of four distinct G-proteins (G_{i1} , G_{i2} , G_{i3} and G_o) which are substrates for pertussis toxin [3], we performed immunoblots of NG108-15 cell membranes with a series of anti-peptide antisera generated against the C-terminal decapeptides of each of these polypeptides (see Table 1 for details of antiserum generation and specificity). Antiserum OC1 specifically identifies the α -subunit of G_o , and identified a single polypeptide of 39 kDa in NG108-15 membranes which comigrated with an equivalent polypeptide in

Fig. 1. Pertussis-toxin-catalysed ADP-ribosylation of membranes from neuroblastoma $\times$ glioma hybrid NG108-15 cells

NG108-15 membranes ($20\text{ }\mu\text{g}$) were ADP-ribosylated with thiol-preactivated pertussis toxin and $[\text{P}^{32}]\text{NAD}^{+}$. The sample was resolved on SDS/PAGE (12.5% acrylamide/0.0625% bisacrylamide, $20\text{ cm} \times 20\text{ cm}$ resolving gel) and the dried gel was autoradiographed for 24 h with intensifying screens. None of the three polypeptides identified incorporated radioactivity in the absence of activated pertussis toxin. The identification of the three polypeptides as $\text{G}_{i3\alpha}$, $\text{G}_{i2\alpha}$ and $\text{G}_o\alpha$ is based on their relative mobility and the immunological studies described in Fig. 2.

rat brain (Fig. 2a), a tissue known to express high levels of this polypeptide. Antiserum AS7 interacts equally with the α -subunits of both G_{i1} and G_{i2} as these two G-proteins have identical C-terminal decapeptides. This antiserum identified two polypeptides of 41 and 40 kDa in both rat brain and rat adipocytes, but identified only a single polypeptide in NG108-15 membranes (Fig. 2d). This polypeptide comigrated with the polypeptide of greater mobility in adipocytes and brain, which we have shown previously to be G_{i2} [13]. A $\text{G}_{i2\alpha}$ -specific antiserum (LE2) also identified the 40 kDa polypeptide in each of the three tissues but did not identify the 41 kDa polypeptide of brain and adipocytes (results not shown). We have previously used a G_{i1} -specific antiserum to demonstrate that the 41 kDa polypeptide of brain and adipocytes corresponds immunologically to G_{i1} [13]. Antiserum I3B identifies $\text{G}_{i3\alpha}$; this antiserum also identified a single polypeptide of 40.5 kDa in NG108-15 membranes which comigrated with a polypeptide in rat glioma C6 BU1 cells and in rat adipocytes (Fig. 2c), which we have previously demonstrated to be $\text{G}_{i3\alpha}$ [13]. Thus NG108-15 cells express detectable levels of G_{i2} , G_{i3} and G_o , but not of G_{i1} .

Synthetic oligodeoxynucleotide probes derived from the coding sequence for amino acids 125–135 of the α -subunits of each of G_{i1} , G_{i2} and G_{i3} were generated and used in Northern blots of total RNA isolated from NG108-15 cells. These probes confirmed the presence of mRNA coding for $\text{G}_{i2\alpha}$ and $\text{G}_{i3\alpha}$ in these cells (Fig. 3). However, mRNA corresponding to $\text{G}_{i1\alpha}$ could not be detected (Fig. 3). We further demonstrated the presence of $\text{G}_o\alpha$ in NG108-15 membranes by immunoblotting with antiserum CS1 which is directed against the C-terminal decapeptide of forms of $\text{G}_o\alpha$ (Fig. 2b). In these membranes, $\text{G}_o\alpha$ was represented predominantly by a 44 kDa polypeptide.

Receptor activation of a G-protein produces an increase in the rate of release of GDP from the nucleotide-binding site and thus an increase in binding and subsequent hydrolysis of GTP. Activation of any receptor which is coupled to a G-protein should thus theoretically produce a stimulation of GTPase activity. Assays of this nature report directly functional interactions of receptors and G-proteins. As demonstrated previously, the synthetic opioid DADLE ($1\text{ }\mu\text{M}$) stimulated high-affinity GTPase activity in membranes of NG108-15 cells [8,9]

Fig. 2. Expression of multiple pertussis-toxin-sensitive G-proteins in neuroblastoma $\times$ glioma hybrid cells: demonstration of their molecular identities

Neuroblastoma $\times$ glioma hybrid NG108-15 cells were grown in culture, harvested when 80% confluent and stored as a cell paste. Membranes were prepared as described in the Materials and methods section. Membranes from NG108-15 cells and from rat glioma C6 BU1, rat cerebral cortex and rat white adipocytes were resolved on SDS/PAGE as in Fig. 1 and transferred to nitrocellulose. The choice of tissue to compare with NG108-15 membranes was based on our previous identification of particular G-proteins in those tissues [11,13]. Immunoblotting was performed with 1:200 dilutions of (a) antiserum OC1 (lane 1, 100 μ g of NG108-15 membranes; lane 2, 10 μ g of rat cerebral cortex), (b) antiserum CS1 (lane 1, 100 μ g of NG108-15 membranes; lane 2, 50 μ g of rat white adipocyte membranes), (c) antiserum I3B (lane 1, 100 μ g of NG108-15 membranes; lane 2, 50 μ g of rat white adipocytes; lane 3, 100 μ g of rat glioma C6 BU1 cell membranes) and (d) antiserum AS7 (lane 1, 100 μ g of NG108-15 membranes; lane 2, 50 μ g of rat cerebral cortex; lane 3, 50 μ g of rat glioma C6 BU1 membranes). Detection of the primary antiserum was achieved using an IgG fraction from a donkey anti-rabbit antiserum which was coupled to horseradish peroxidase. The substrate for the enzyme was *o*-dianisidine.

Fig. 3. Detection of mRNA corresponding to $G_i2\alpha$ and $G_i3\alpha$ but not $G_i1\alpha$ in NG108-15 cells

Northern blots of total RNA isolated from NG108-15 cells were performed as described in the Materials and methods section using the probes defined in Table 2. The $G_i2\alpha$ probe (lane 2) identified a single band of some 2.4 kb and the $G_i3\alpha$ probe (lane 3) identified a single band of some 3.4 kb. Nothing was identified with the $G_i1\alpha$ probe (lane 1), although we have demonstrated previously that this probe will specifically identify $G_i1\alpha$ mRNA [13]. Positions of the 18 and 28 S rRNA species are indicated. Autoradiograms were exposed for 72 h.

following a 60 min preincubation at 37°C; in the present experiments by some 4.6 ± 0.4 pmol/min per mg of protein [basal, 13.0 ± 0.2 pmol/min per mg of membrane protein; DADLE-stimulated, 17.6 ± 0.4 pmol/min per mg of membrane protein (means $\pm$ S.E.M., $n = 4$)] but not in membranes of cells which had been pretreated with pertussis toxin (25 ng/ml, 16 h) (basal, 12.4 ± 0.3 pmol/min per mg of membrane protein; DADLE-stimulated, 12.4 ± 0.3 pmol/min per mg of membrane protein) confirming that the δ -opiate receptor interacts with a pertussis-toxin-sensitive G-protein(s). Based upon the expression of the various G-proteins in these cells, this G-protein must be G_i2 , G_i3 , G_o or some combination thereof.

Fig. 4. IgG purified from antiserum AS7 displays the same specificity for G-proteins as the crude antiserum

IgG purified from antiserum AS7 by passage over Protein A-Sepharose was used as the primary reagent in an immunoblot equivalent to that displayed in Fig. 2(d). Each of the IgG fractions generated from the various antisera displayed the same selectivity of G-protein identification as displayed in Fig. 2 for the crude antisera (results not shown).

To address the specificity or otherwise of this interaction, we purified IgG fractions from normal rabbit serum and from antisera AS7, I3B, OC1 and CS1 by passage over Protein A-Sepharose 4B (see the Materials and methods section), and then incubated membranes of untreated NG108-15 cells with these IgG fractions for 60 min at 37°C (concentrations corresponding to 1:100 dilutions of the original antiserum). These IgG fractions displayed the same specificity of G-protein recognition as the crude antisera (Fig. 4 and results not shown). Subsequently we ascertained the ability of a receptor-saturating concentration of DADLE (1 μ M) (EC_{50} 5 nM) to stimulate high-affinity GTPase activity. Following treatment of the membranes with the IgG fraction from non-immune rabbits, DADLE stimulated high-affinity GTPase activity over the basal level

(4.7 ± 0.3 pmol/min per mg of membrane protein, $n = 4$, mean $\pm$ S.E.M.) to the same degree as in the absence of the non-immune IgG fraction. IgG fractions from each of OC1 (anti-G_o α) (4.1 ± 0.3 pmol/min per mg of membrane protein, $n = 4$), I3B (anti-G_i3 α) (4.6 ± 0.4 pmol/min per mg of membrane protein, $n = 4$) and CS1 (anti-G_o α) (4.3 ± 0.3 pmol/min per mg of membrane protein, $n = 4$) did not affect the function of the opioid peptide. However, the IgG fraction purified from antiserum AS7 [anti-(G_i1 α and G_i2 α)] completely attenuated the ability of the agonist to stimulate the high-affinity GTPase activity (0.1 ± 0.1 pmol/min per mg of membrane protein, $n = 4$) (Fig. 5). In contrast to receptor-stimulated GTPase activity, the IgG fraction of antiserum AS7 did not modulate the basal high-affinity GTPase activity [AS7, 10.5 ± 0.2 pmol/min per mg of membrane protein ($n = 4$); normal rabbit serum, 9.9 ± 0.2 pmol/min per mg of membrane protein ($n = 4$)] or the non-G-protein related low-affinity GTPase activity of these membranes (results

Fig. 5. δ -Opioid receptor-stimulation of high-affinity GTPase activity in membranes from neuroblastoma $\times$ glioma hybrid cells: the receptor interacts specifically with G_i2

Neuroblastoma $\times$ glioma NG108-15 hybrid cells, were grown in tissue culture as described in the Materials and methods section. Following harvest of the cells and overnight storage at -80°C , a membrane preparation was produced. IgG fractions from both normal rabbit serum and from the various antisera described in Table 1 were produced by chromatography on Protein A-Sepharose (Pharmacia). These fractions were incubated with membranes from NG108-15 cells for 60 min at 37°C in the presence of all reagents used in the GTPase assay except [γ - ^{32}P]GTP and receptor agonist. Following the preincubation period, both [γ - ^{32}P]GTP and DADLE ($1\ \mu\text{M}$) were added, and samples were incubated at 37°C for a further 20 min. Results are presented to display levels of both the basal high-affinity GTPase activity ($\square$) and the high-affinity GTPase activity in the presence of agonist ($\boxtimes$). Results are means $\pm$ S.E.M. from four independent experiments. Quadruplicate determinations were performed for each point in each experiment. The S.E.M. values in each independent experiment were less than 6% of the mean.

Fig. 6. Binding of [^3H]DADLE to membranes of NG108-15 cells: effect of Gpp[NH]p

Membranes from NG108-15 cells were incubated with various concentrations of [^3H]DADLE ($0.5\text{--}20\ \text{nM}$), as described in the Materials and methods section in the absence ($\square$) or presence ($\blacklozenge$) of Gpp[NH]p ($100\ \mu\text{M}$). A further series of incubations were performed in the presence of $10\ \mu\text{M}$ -DADLE to define non-specific binding. Specific binding data were treated as in [22]. In this experiment, binding parameters were: B_{max} 309 ± 7 fmol/mg of protein, K_d $3.2\ \text{nM}$ (in the absence of Gpp[NH]p) and B_{max} 283 ± 15 fmol/mg, K_d $6.4\ \text{nM}$ in the presence of Gpp[NH]p. Two further experiments produced similar results.

not shown). The same was true of the IgG fractions of all of the antisera employed in this study (Fig. 5). As such, the effect of the IgG fraction of antiserum AS7 was solely to prevent receptor activation of high affinity GTPase activity.

Opioid peptide ($10\ \mu\text{M}$ -DADLE)-mediated inhibition of forskolin-stimulated adenylate cyclase activity, which was some 35% in both the presence and absence of IgG derived from normal rabbit serum, was also attenuated completely by preincubation of the membranes of NG108-15 cells with a 1:100 dilution of the IgG fraction from antiserum AS7 (Table 2).

In many cases, identification of an interaction of a G-protein with a receptor has first been demonstrated by noting a reduction in agonist-binding affinity produced by poorly hydrolysed analogues of GTP. In saturation binding experiments, in the presence of Mg^{2+} ($20\ \text{mM}$), specific binding of [^3H]DADLE to

Table 2. Antibodies from antiserum AS7 attenuate opioid-receptor-mediated inhibition of adenylate cyclase

Membranes ($20\ \mu\text{g}$) from NG108-15 cells were preincubated for 60 min with IgG from either normal rabbit serum or antiserum AS7 (equivalent to a 1:100 dilution of the original antiserum). After this period, forskolin ($10\ \mu\text{M}$)-amplified adenylate cyclase activity was assessed by use of a binding protein for the determination of cyclic AMP as described [19], as was the effect of DADLE ($10\ \mu\text{M}$) on this activity. Results are means $\pm$ S.E.M. for assays performed in quadruplicate. The results are taken from a single experiment but two further experiments produced qualitatively similar data.

Incubation condition (source of IgG)	Forskolin-stimulated adenylate cyclase activity (pmol/min per mg of membrane protein)		Inhibition by $10\ \mu\text{M}$ -DADLE (%)
	– Opiate	+ Opiate	
Normal rabbit serum	83.6 ± 5.6	54.8 ± 4.9	34.5
Antiserum AS7	79.1 ± 2.6	80.2 ± 1.1	–1.4

Fig. 7. Binding of [3 H]DADLE to membranes of untreated and pertussis-toxin pretreated NG108-15 cells

Membranes from untreated ($\square$) or pertussis-toxin-pretreated (25 ng/ml, 24 h) ($\blacksquare$) NG108-15 cells were incubated with various concentrations of [3 H]DADLE, in the absence of Gpp[NH]p, as described in the legend to Fig. 6. Non-specific binding was defined as in Fig. 6. Binding parameters in the experiment displayed were: membranes from untreated cells, $B_{\max}$ 331 ± 6 fmol/mg of protein, K_d 1.8 nM; membranes from pertussis-toxin-pretreated cells, $B_{\max}$ 293 ± 16 fmol/mg of protein, K_d 4.2 nM. Two other experiments gave qualitatively similar results.

membranes of neuroblastoma $\times$ glioma hybrid NG108-15 cells could be adequately defined as being to a single population of high-affinity sites. Scatchard analysis of the data indicated the presence of some 400 fmol of receptor/mg of membrane protein, with an apparent dissociation constant (K_d) for the ligand of 2.2 ± 0.2 nM (mean $\pm$ S.D., $n = 9$) (Fig. 6). In a series of membrane preparations from individual cultures, the apparent number of receptors varied from 290 to 580 fmol/mg of protein. When a similar assay was performed in the presence of Gpp[NH]p (guanosine 5'-[β -imidotriphosphate) (100 μ M), Scatchard analysis of the specific binding indicated a reduction in the affinity of [3 H]DADLE binding but no alteration in the total number of available sites (Fig. 6).

Specific binding of [3 H]DADLE to membranes of NG108-15 cells which had been pretreated with pertussis toxin (25 ng/ml, 24 h) also produced a reduction in [3 H]ligand affinity in comparison with that in membranes from untreated control cells, but did not alter the total number of sites (Fig. 7). These two pieces of data confirmed previous studies [15,16] and indicated that the δ -opioid receptor in these cells interacts with a pertussis toxin-sensitive G-protein(s) [14–17].

To define further which of the pertussis-toxin-sensitive G-proteins expressed by these cells was linked to the δ -opioid receptor, we incubated membranes of NG108-15 cells either with Gpp[NH]p (100 μ M) or with IgG fractions isolated from normal rabbit serum and from antisera AS7 [anti-($G_{i1\alpha}$ and $G_{i2\alpha}$)], OC1 (anti- $G_{o\alpha}$) and I3B (anti- $G_{i3\alpha}$) for 60 min at 37 $^{\circ}$ C, and then assessed the ability of a single concentration of [3 H]DADLE (2 nM), which was close to the apparent K_d for the ligand, to bind

Fig. 8. Antiserum AS7 specifically reduces the binding of [3 H]DADLE to membranes from NG108-15 cells

Membranes from NG108-15 cells were incubated for 60 min at 37 $^{\circ}$ C in the presence of Gpp[NH]p (100 μ M) or the IgG fractions (equivalent to a 1:100 dilution of the original antiserum) isolated from normal rabbit serum (control), antiserum AS7 [anti-($G_{i1\alpha}$ and $G_{i2\alpha}$)], antiserum OC1 (anti- $G_{o\alpha}$) or antiserum I3B (anti- $G_{i3\alpha}$) as shown. The binding of a single concentration of [3 H]DADLE (2 nM) close to the calculated K_d for this ligand was measured and the non-specific component of this binding was defined by parallel incubations in the presence of 10 μ M-DADLE. Only the IgG fraction isolated from antiserum AS7 was able to mimic Gpp[NH]p in reducing the specific binding of the [3 H]DADLE. Data are presented as means $\pm$ S.E.M. for quadruplicate determinations in a single experiment. Similar results were obtained in two further experiments.

specifically to the δ -opioid receptor. The preincubation phase itself had only a small inhibitory effect on [3 H]DADLE binding ($< 20\%$), which Scatchard analysis of the specific binding demonstrated to be due to a reduction in the total number of receptors available without an effect on the affinity of the [3 H]ligand for the remaining receptors [incubation for 60 min on ice; $B_{\max}$ 578 ± 11 fmol/mg of protein, K_d 2.5 ± 0.2 nM; incubation for 60 min at 37 $^{\circ}$ C. $B_{\max}$ 429 ± 9 fmol/mg of protein, K_d 2.4 ± 0.2 nM (all data given as means $\pm$ range, $n = 2$)]. As anticipated, Gpp[NH]p reduced the specific binding of this concentration of the opioid ligand; however, of the various IgG fractions, only that derived from antiserum AS7 was able to produce a similar inhibition (Fig. 8). The IgG fractions from normal rabbit serum and from antisera OC1 and I3B did not affect the binding of [3 H]DADLE (Fig. 8).

The effects of Gpp[NH]p and the IgG fraction of antiserum AS7 in reducing [3 H]DADLE binding were not additive; indeed, no further reduction in binding of [3 H]DADLE was achieved by the combination of these agents in comparison with the effects of either in isolation (Fig. 9).

We then assessed whether the inhibitory effect of IgG from antiserum AS7 on the binding of [3 H]DADLE was due to an alteration in the affinity of the ligand for the receptor, as would be anticipated if the antibodies were producing a functional uncoupling of the receptor from the G-protein signalling system. Scatchard analysis of the specific binding of a range of concentrations (0.5–20 nM) of [3 H]DADLE to membranes from NG108-15 cells following preincubation in the presence or

Fig. 9. Effects of Gpp[NH]p and antiserum AS7 on [³H]DADLE binding to membranes from NG108-15 cells: lack of additivity

Membranes of NG108-15 cells were pre-incubated for 60 min at 37 °C with the IgG fraction of normal rabbit serum (control) (concentration equivalent to a 1:100 dilution of the original serum), the IgG fraction from normal rabbit serum + Gpp[NH]p (100 μM), the IgG fraction of antiserum AS7 (equivalent to a 1:100 dilution of the crude antiserum), or the IgG fraction of antiserum AS7 + Gpp[NH]p (100 μM) as indicated. Following the preincubation, specific binding of a single concentration of [³H]DADLE (2 nM) was assessed as in Fig. 8. Results are presented as means ± S.E.M. for quadruplicate assays derived from a single experiment. A second experiment produced similar results.

absence of the IgG fraction of antiserum AS7 indeed indicated that the affinity of the receptor for ligand was reduced, but that the total number of binding sites was not affected (Fig. 10). Equivalent results were produced when each of the experiments detailed above was repeated using [³H]diprenorphine as ligand (results not shown).

DISCUSSION

The α-subunits of the various pertussis-toxin-sensitive G-proteins show a high degree of similarity with each other [3], which varies from 94% identity between G_i1 and G_i3 to 68% between G_i2 and G_o. On this basis, it is pertinent to ask whether each of these G-proteins act to couple distinct receptors to different effectors or whether they might be more appropriately viewed as isoenzymic forms.

In many biochemical reconstitution experiments, a range of these pertussis-toxin-sensitive G-proteins, produced either via conventional protein purification schemes [21,22], or more recently by the expression of relevant cDNA clones [23], have demonstrated similar abilities either to interact with agonist-activated receptors or to control effector systems. Reconstitution studies, however, can only suggest possibilities and do not unambiguously identify the normal operating mechanism. To do this, it is necessary to either block or delete the normal pathway. In these studies we have attempted to block the interaction of the δ-opioid receptor of neuroblastoma × glioma hybrid NG108-15 cells with antisera which are directed against the extreme C-termini of the α-subunits of the various G-proteins. We decided to focus on this region because covalent modification of these G-

Fig. 10. Antiserum AS7 converts δ-opioid receptors in membranes from NG108-15 cells from high affinity for agonist binding to low affinity

Membranes of NG108-15 cells were pre-incubated at 37 °C for 60 min with an IgG fraction from either normal rabbit serum (□) or antiserum AS7 (◆) as described in the legend to Fig. 6. The specific binding of a range of concentrations of [³H]DADLE was then assessed. Binding parameters in this experiment were: B_{max} 481 ± 12 fmol/mg of protein, K_d 2.4 nM for membranes incubated with normal rabbit serum, and B_{max} 510 ± 23 fmol/mg of protein, K_d 5.6 nM for membranes incubated with antiserum AS7.

proteins by pertussis toxin produces an uncoupling of receptors from pertussis-toxin-sensitive G-proteins [2,15], and the site of this modification is a cysteine residue located four amino acids from the C-terminus [1]. The choice of a clonal cell line for these experiments was based upon the fact that, in such a homogeneous cell population, any G-proteins expressed must be in all of the cells and hence be available, at least theoretically, to interact with a receptor. The opioid receptor of the NG108-15 cell line has previously been well characterized and is known to cause both a stimulation of high-affinity GTPase activity and an inhibition of adenylate cyclase [8,9]. These two parameters are very highly correlated, in terms of both the potency and the efficacy of a series of opioid ligands [8], and hence are believed to represent subsequent steps of the same transduction pathway. Thus interference with one of these processes would be anticipated to be reflected in a similar modification in the other.

We have demonstrated that these cells express three distinct pertussis-toxin-sensitive G-proteins (Fig. 1) and that these are the products of the G_i2, G_i3 and G_o genes (Fig. 2). G_i1 could not be detected at the level of either protein (Fig. 2) or mRNA (Fig. 3). This situation is particularly fortuitous for these studies as G_i1α and G_i2α have identical C-terminal decapeptides, and therefore antiserum AS7 is able to identify these two G-proteins equally. Although immunoblotting conditions can be arranged to resolve these two G-proteins (Fig. 2), if both were expressed in the same cell it would not be possible to differentiate between them in functional assays using this antiserum. However, as we have shown, AS7 can be used as a functional probe for G_i2 in these cells due to the lack of G_i1.

Both opioid-inhibition of adenylate cyclase and opioid-

stimulation of high-affinity GTPase activity were completely abolished in membranes of these cells by pretreatment with an IgG fraction from antiserum AS7. IgG fractions from normal rabbit serum, and from the anti-G_i3, anti-G_o and anti-G_s C-terminal antisera had no effect. These data argue strongly that G_i2 must be the true G_i of the adenylate cyclase cascade, at least in these cells. We cannot rule out the possibility that G_i1 might function in a similar manner in other tissues in which it is expressed. It should be noted, however, that the expression of G_i2 (and G_i3) appears to be universal in mammalian tissues, whereas G_i1 is limited in distribution [24] and therefore does not mirror the distribution of adenylate cyclase which is also essentially universal.

Both opioid peptides and noradrenaline acting at an α_2 -adrenergic receptor can act to inhibit Ca²⁺ currents in NG108-15 cells. This can only be recorded, however, in cells which have been differentiated by prior exposure to agents which as a common mode of action increase levels of cyclic AMP [7]. Hescheler *et al.* [25] have suggested that opioid-peptide control of Ca²⁺ currents in these differentiated cells is transduced via G_o, and we have demonstrated recently that the anti-(G_o C-terminal) antiserum used in these studies (OC1) antagonizes the ability of noradrenaline to inhibit Ca²⁺ currents in prostaglandin-E₁-differentiated NG108-15 cells [14]. By contrast, antiserum AS7 was without effect in these assays [14]. It is thus possible that a single receptor may stimulate multiple G-proteins to modify distinct effector systems.

Although it would be anticipated that receptor-mediated stimulation of high-affinity GTPase activity could be recorded for all receptors coupled to G-proteins, in practise this is often difficult to demonstrate [1]. Further, in studies on G-protein-linked signalling cascades other than adenylate cyclase, such as receptor activation of phosphoinositidase C, it is often difficult to prepare cell-free systems which possess adequate coupling of receptor to effector. With such considerations in mind, we have also attempted to devise assays which should be applicable to any receptor-G-protein interaction. These have been based on the conversion of receptors between 'high' and 'low' affinity states for agonist, dependent upon the degree of interaction between the receptor and its G-protein. We postulated that these antisera would be able to interfere with the coupling of particular G-proteins with receptors which interact with them and hence produce a low-affinity state of the receptor for agonists which could be detected in ligand-binding assays.

These experiments were performed in the presence of a high concentration of Mg²⁺ (20 mM), as under such conditions all the receptors in untreated membranes are converted into a high-affinity state for agonists and hence are all presumably coupled to a G-protein. Under such conditions Scatchard analysis of [³H]agonist binding is monophasic. In the uncoupling experiments only antibodies derived from antiserum AS7 converted the opioid receptor to a low-affinity state. These results are in complete accord with those produced with the other

assays (GTPase and adenylate cyclase) for the function of the δ -opioid receptor.

This is the first report to use a panel of antisera directed against each of the pertussis-toxin-sensitive G-proteins which are expressed in a particular cell in an attempt to assess the selectivity of the contacts of a receptor with the various G-proteins. This approach is likely to be amenable to further studies on the selectivities/specificities of receptor-G-protein interactions.

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