Biochem. J. (1988) 249, 653–659 (Printed in Great Britain)

Antibodies which recognize the *C*-terminus of the inhibitory guanine-nucleotide-binding protein (G_i) demonstrate that opioid peptides and foetal-calf serum stimulate the high-affinity GTPase activity of two separate pertussis-toxin substrates

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We investigated the mechanisms of receptor-mediated stimulation of high-affinity GTPase activity in response to opioid peptides and to foetal-calf serum in membranes of the neuroblastoma × glioma hybrid cell line NG108-15. Increases in GTPase activity in response to both of these ligands was abolished by prior exposure of the cells to pertussis toxin. Pertussis toxin in the presence of [^{32}P]NAD^+ catalysed incorporation of radioactivity into a broad band of approx. 40 kDa in membranes prepared from untreated, but not from pertussis-toxin-pretreated, cells. Additivity studies indicated that the responses to opioid peptides and to foetal-calf serum were mediated by separate guanine-nucleotide-binding proteins (G-proteins). Whereas opioid peptides produced an inhibition of adenylate cyclase in membranes of untreated cells, foetal-calf serum did not. Affinity-purified antibodies which recognize the *C*-terminus of the inhibitory G-protein identified a 40 kDa polypeptide in membranes of NG108-15 cells. These antibodies attenuated opioid-stimulated high-affinity GTPase activity, but did not markedly affect the response to foetal-calf serum. We conclude that receptors for the opioid peptides function via the inhibitory G-protein (G_i), whereas foetal-calf serum activates a second pertussis-toxin-sensitive G-protein, which has a *C*-terminal sequence significantly different from that of G_i.

INTRODUCTION

The majority of cell-surface receptors communicate with second-messenger effector systems via specific members of a family of heterotrimeric guanine-nucleotide-binding proteins (G-proteins) [1,2]. These include G_s and G_i, respectively the G-proteins of stimulation and inhibition of adenylate cyclase, and G_o, a novel G-protein of unknown function [1,2].

Upon activation by an agonist-occupied receptor, GDP bound to the α-subunit of a G-protein is replaced by GTP. Subsequent deactivation of the system involves the hydrolysis of the terminal phosphate of this nucleotide by a GTPase activity which is integral to the α-subunit of the transducer protein. Thus receptor stimulation of GTPase activity is indicative that a particular receptor functions via activation of a G-protein [3–5].

Appreciation of the role of G-proteins in linking hormone receptors to their second-messenger effector systems has been significantly enhanced by the recognition that certain bacterial toxins can catalyse the mono-ADP-ribosylation of particular G-proteins and hence modify the function of these transducers [2,5]. Pertussis toxin, for example, is able to ADP-ribosylate G_i, and this modification attenuates receptor-mediated inhibition of adenylate cyclase [5–7]. However, it is now appreciated that G_i is not the sole substrate for pertussis toxin, and thus abolition of a receptor-mediated response by treatment with pertussis toxin cannot be considered to define a role for G_i in the response.

The neuroblastoma × glioma hybrid cell line NG108-15 has been widely used as a model system to examine signal transduction from receptors to their effector systems [7–11]. This cell line is convenient for studies of this nature because, as well as possessing receptors which modulate adenylate cyclase [4,5] and which stimulate inositol phospholipid turnover [10,11], it also expresses a number of different G-proteins [5,12].

Opioid receptors of the δ subclass have previously been shown to stimulate high-affinity GTPase activity and to inhibit adenylate cyclase in membranes prepared from NG108-15 cells [3,8]. Both of these responses were prevented by prior exposure of the cells to pertussis toxin [5]. These results were interpreted as evidence that the opioid response was mediated via G_i. Here we directly examine this hypothesis, by using affinity-purified antibodies which recognize the *C*-terminal region of G_i [13]. We show that preincubation of membranes of NG108-15 cells with these antibodies leads to an abolition of opioid-peptide stimulation of a high-affinity GTPase activity. A similar pertussis-toxin-sensitive GTPase activity was stimulated in these membranes by foetal-calf serum, but this stimulation was not modulated by preincubation with the antibodies. We conclude that the

Abbreviations used: G-protein, guanine-nucleotide-binding protein; DADLE, [D-Ala^2][leucine]enkephalin.

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δ-opioid receptor functions via G_i, whereas the foetal-calf serum response is mediated by a second pertussis-toxin-sensitive G-protein.

MATERIALS AND METHODS

Materials

[γ^{32}P]GTP was synthesized as previously described [14]. [D-Ala^2][leucine]enkephalin (DADLE) and [D-Ala^2]-[methionine]enkephalin amide (DALAMID) were purchased from Sigma. Foetal-calf serum was from Gibco. Pertussis toxin was kindly given by Professor J. Freer, Department of Microbiology, University of Glasgow.

Cell growth

NG108-15 neuroblastoma × glioma hybrid cells, which were kindly given by Dr. W. A. Klee, National Institutes of Health, Bethesda, MD, U.S.A., were grown in 80 cm^2 tissue-culture flasks as previously described [8,12], except that penicillin and streptomycin (100 i.u./ml) were routinely included and that foetal-calf serum was heat-inactivated by treatment at 56°C for 1 h before use. The cells were harvested at confluency, and cell pastes and membranes prepared [8], which were maintained at −70°C until they were assayed. In some cases, pertussis toxin (100 ng/ml) was added to the cultures 16 h before harvest.

Antibody preparation and immunoblotting

Antisera AS6 and AS7 were produced in rabbits against the *C*-terminal decapeptide of rod transducin as described by Falloon *et al.* [13]. Antibodies which recognize the α-subunit of G_i were affinity-purified from these antisera on a column of bovine transducin [15]. Assessment of the specificity of these antibodies for the α-subunit of G_i was conducted [15] against purified G_i and G_o, which were isolated from bovine brain [7]. Immunoblotting of NG108-15 membranes with these antisera was carried out as previously described [16,17].

GTPase assays

GTPase assays were routinely performed essentially as described by Koski & Klee [3], except that the concentration of 5′-adenylyl imidodiphosphate was lowered to 0.1 mM. In the preincubation experiments, affinity-purified antibodies were diluted to the appropriate concentration with normal rabbit serum and then incubated at 37 °C with NG108-15 membranes in the presence of the GTPase assay components for up to 1 h. After this time, receptor ligands and [γ-^{32}P]GTP were added, and the assays were incubated for a further 20 min at 37 °C before being processed as in [3].

Adenylate cyclase assays

Adenylate cyclase was assayed as in [18]. Foetal-calf serum was observed to modify the cyclic AMP standard curve, and thus had to be included at appropriate concentrations in the standard curve to correct for this anomaly.

Pertussis-toxin-catalysed ADP-ribosylation

Pertussis-toxin-catalysed ADP-ribosylation of NG108-15 membranes was performed as described previously [12,19].

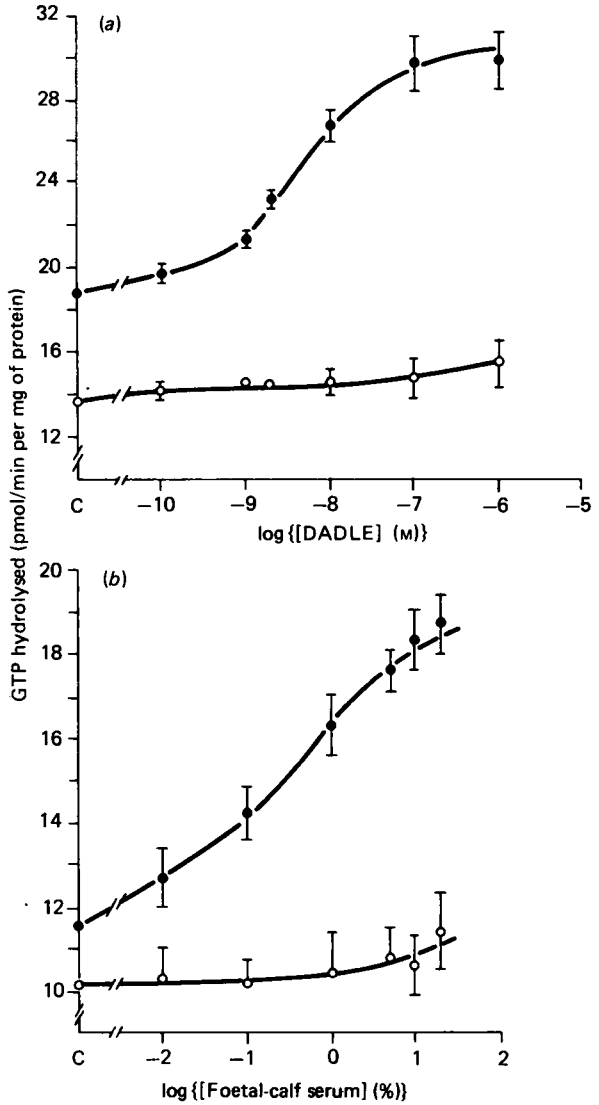

Fig. 1. Stimulation by DADLE (*a*) and foetal-calf serum (*b*) of high-affinity GTPase activity in NG108-15 membranes: the effect of pertussis toxin

(*a*) NG108-15 cells were treated either with pertussis toxin (○) or with vehicle (●) as described in the Materials and methods section. High-affinity GTPase activity was measured in membranes of these cells in response to different DADLE concentrations (0.1 nM–1 μM). Data represent means ± S.D. of quadruplicate assays. The experiment was performed three times with similar results except that the absolute values of basal GTPase activity varied over an approx. 2-fold range. (*b*) NG108-15 cells were treated with pertussis toxin (○) or with vehicle (●) for 16 h before harvest as described in the Materials and methods section. High-affinity GTPase activity was measured in membranes prepared from these cells in response to differing concentrations of foetal calf serum (0.01–20%). Points represent means ± S.D. for quadruplicate assays. The experiment was repeated three times with similar results. C, control (no added stimulant).

RESULTS

The opioid peptides DADLE (Fig. 1*a*) and DALAMID (results not shown) stimulated the rate of hydrolysis of GTP by high-affinity GTPases in membranes prepared

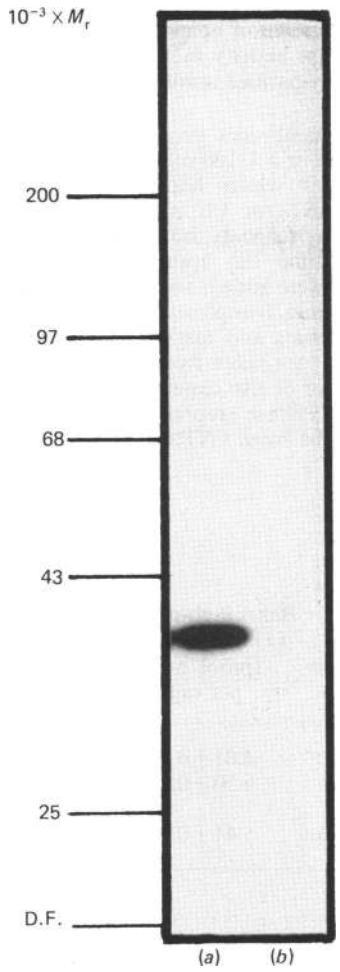

Fig. 2. ADP-ribosylation of membranes of control and pertussis-toxin-treated cells

NG108-15 cells were treated with either pertussis toxin or vehicle as described in the Materials and methods section, and membranes were prepared. These membranes (48 μg) were then challenged with fresh pertussis toxin (25 μg/ml) and [^{32}P]NAD$^+$ as previously described [11]. The samples were resolved on a SDS/10%-polyacrylamide gel, which was stained with Coomassie Blue, dried and autoradiographed. (*a*) Control membranes + pertussis toxin; (*b*) membranes of pertussis-toxin-pretreated cells + pertussis toxin; D.F., dye front.

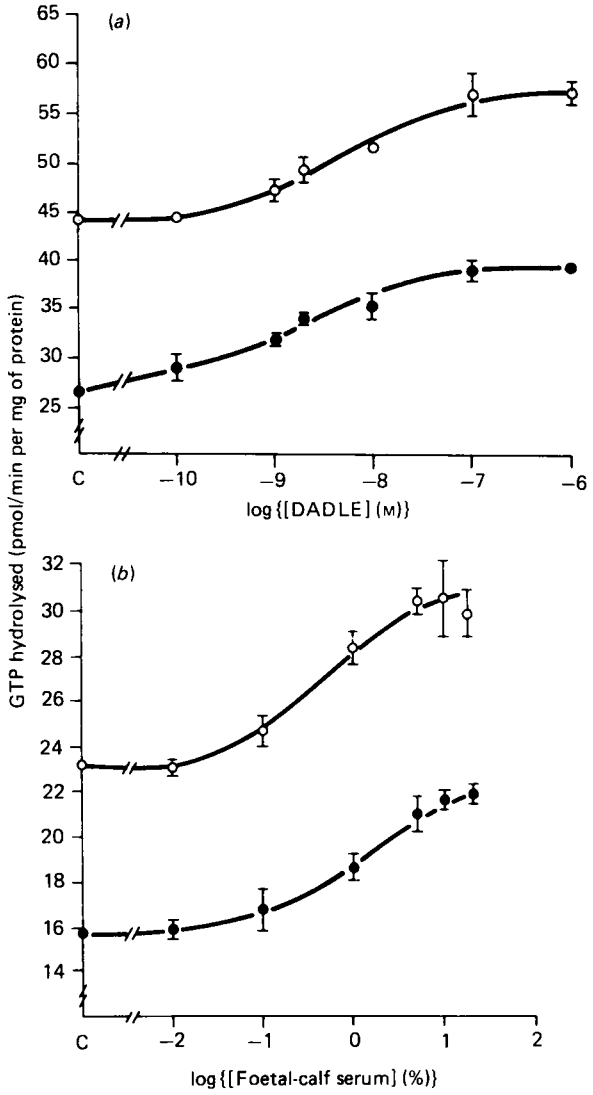

Fig. 3. High-affinity GTPase activity in NG108-15 membranes: additivity studies with DADLE and foetal-calf serum

(*a*) DADLE (0.1 nM–1 μM) stimulation of high-affinity GTPase activity was measured in the presence (○) and absence (●) of a saturating concentration of foetal-calf serum (20%). Points are means ± S.D. of quadruplicate assays from a single experiment, which was replicated three times. (*b*) Foetal-calf serum (0.01–20%) stimulation of high-affinity GTPase activity was measured in the presence (○) and absence (●) of a saturating concentration of DADLE (10 μM). Points are means ± S.D. of quadruplicate assays. The experiment was performed three times. C, control (no added stimulant).

from cells of the neuroblastoma × glioma hybrid cell line NG108-15. Stimulation occurred over concentrations between 0.1 nM and 1 μM and was half-maximal at 50 nM. Absolute values of the high-affinity GTPase activity were increased by some 12.5 ± 0.8 pmol/min per mg of membrane protein by maximal concentrations of the opioid peptides.

Foetal-calf serum also stimulated high-affinity GTPase activity in these same membranes (Fig. 1*b*). In this case stimulation occurred between 0.01 and 20% (v/v) foetal-calf serum and was half maximal at 0.8%. Maximal increases in high-affinity GTPase activity in response to foetal-calf serum were 6.3 ± 1.0 pmol/min per mg of membrane protein.

Membranes of pertussis-toxin-treated cells displayed decreased basal high-affinity GTPase activity, and the residual high-affinity GTPase activity was no longer stimulated by either the opioid peptide DADLE (Fig. 1*a*) or foetal-calf serum (Fig. 1*b*).

Treatment of membranes of NG108-15 cells with activated pertussis toxin and [^{32}P]NAD$^+$ led to the incorporation of radioactivity into a single broad band of 40 kDa (Fig. 2). When the cells were treated with pertussis toxin (100 ng/ml) for 16 h before harvest and membranes were prepared from these intoxicated cells, then further challenge with fresh pertussis toxin and [^{32}P]NAD$^+$ was unable to incorporate label into this band (Fig. 2).

To examine whether both ligands caused stimulation

Table 1. Adenylate cyclase activity in membranes of NG108-15 cells: effect of DADLE and foetal-calf serum

Basal adenylate cyclase activity in membranes of NG108-15 cells was amplified by forskolin (0.1 mM) and then measured in the absence or presence of either DADLE (10 μM) or foetal-calf serum (20%, v/v). Data are means ± S.D. of triplicate determinations from two separate experiments, which were repeated.

Treatment	Adenylate cyclase (pmol/min per mg of protein)
Control	40.0 ± 4.3
DADLE	19.5 ± 0.8
Control	37.5 ± 1.0
Foetal-calf serum	43.6 ± 0.9

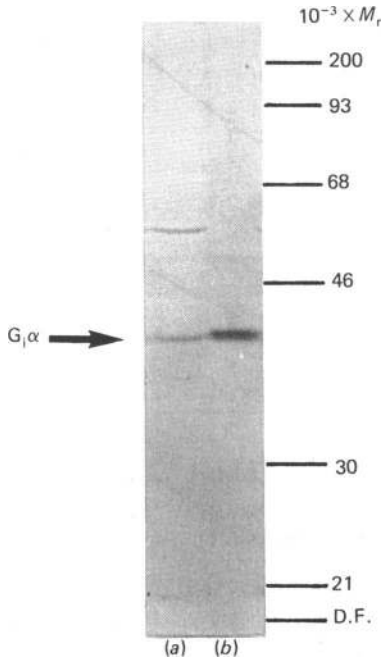

Fig. 4. Immunoblotting of NG108-15 membranes with affinity-purified antibodies from antiserum AS7

A 150 μg sample of NG108-15 membranes (*a*) or a 100 ng sample of G_i ($\alpha\beta\gamma$) purified from bovine brain (*b*) was separated on a SDS/10% polyacrylamide gel at 50 V overnight. The resolved proteins were transferred to nitrocellulose and blocked and immunoblotted [12,15] with a 1:100 dilution of antibodies which had been affinity-purified from antiserum AS7. After overnight incubation, the first antibody was replaced with a horseradish-peroxidase-coupled anti-(rabbit IgG) antibody. *o*-Dianisidine was used as substrate to detect the enzyme-linked antibody. D.F., dye front.

of the GTPase activity of a single G-protein, we performed additivity studies. When dose–response curves of GTPase stimulation by foetal-calf serum were carried out in the absence or presence of a single concentration of DADLE (10 μM) which had previously been shown to produce a maximal increase in GTPase

Table 2. Attenuation of opioid-receptor-stimulated high-affinity GTPase activity in membranes of NG108-15 cells by affinity-purified antibodies from antiserum AS7

NG108-15 membranes were incubated with water, normal rabbit serum or a 1:100 dilution, in normal rabbit serum, of antibodies which had been affinity-purified from antiserum AS7, for 1 h at 37 °C in the presence of the GTPase assay reagents, but in the absence of [γ-^{32}P]GTP. After this time the appropriate receptor ligand and [γ-^{32}P]GTP were added, and incubation was continued for a further 20 min. Samples were then processed as described in the Materials and methods section. Data, which are means ± S.D., are taken from a single experiment which is representative of five experiments performed. 'Receptor-stimulated GTPase' represents agonist stimulation over and above the basal GTPase activities.

Addition to pre-incubation	Basal high-affinity GTPase activity (pmol/min per mg)	Receptor-stimulated high-affinity GTPase activity (pmol/min per mg	
		DADLE (1 μM)	Foetal-calf serum (10%, v/v)
Water	4.01 ± 0.29	3.01 ± 0.15	4.22 ± 0.13
Normal rabbit serum	6.50 ± 0.10	2.64 ± 0.09	6.27 ± 0.11
AS7 antibodies	5.41 ± 0.69	0.19 ± 0.11	4.21 ± 0.16

activity, then the response to foetal-calf serum was unaltered (Fig. 3*a*). The response was merely shifted to higher high-affinity GTPase activity, owing to the stimulation produced by the opioid ligand. In the reciprocal experiment, high-affinity GTPase activity was measured in response to increasing concentrations of DADLE, in the absence or presence of 20% foetal-calf serum. This produced similar results (Fig. 3*b*), indicating that stimulation of high-affinity GTPase activity by foetal-calf serum and opioid peptides was fully additive.

DADLE produced inhibition of adenylate cyclase activity in membranes of NG108-15 cells with a similar concentration-dependence to that required for stimulation of GTPase activity. In contrast, however, foetal-calf serum did not modulate adenylate cyclase activity at concentrations up to 20% (v/v) which produced a maximal stimulation of high-affinity GTPase activity (Table 1).

Immunoblotting of membranes of NG108-15 cells with antisera AS6 and AS7 detected a polypeptide of some 40 kDa, which migrated identically with the α-subunit of purified bovine brain G_i. The antiserum also detected a polypeptide of 60 kDa in these membranes. Affinity-purified antibodies isolated from antiserum AS7 also recognized the 60 kDa protein as well as the α-subunit of G_i (Fig. 4). The nature of this 60 kDa polypeptide is unclear, although it is routinely observed in a range of tissues (results not shown).

Preincubation of NG108-15 membranes for 1 h at 37°C with a 1:100 dilution in normal rabbit serum of antibodies which had been affinity-purified from antiserum AS7 completely abolished the ability of DADLE to stimulate high-affinity GTPase activity, but had little

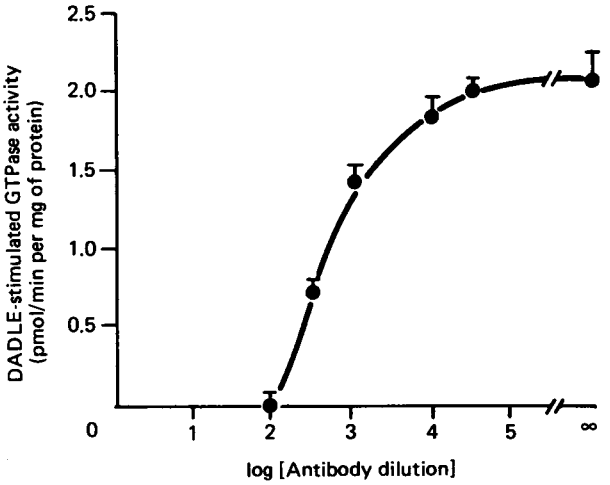

Fig. 5. Affinity-purified antibodies: inhibition of δ-opioid-receptor-stimulated GTPase activity

Antibodies which were affinity-purified from antiserum AS7 were diluted with normal rabbit serum. NG108-15 membranes (5 μg) were incubated with the GTPase assay mixture, except for [γ-^{32}P]GTP, and various concentrations of the antibodies for 1 h at 37°C. After this time, the samples were placed on ice, and DADLE (10 μM) and [γ-^{32}P]GTP were added. The samples were then incubated for a further 20 min at 37 °C before being processed as described in the Materials and methods section. Points represent means ± S.D. of quadruplicate assays from a single experiment, which was repeated four times.

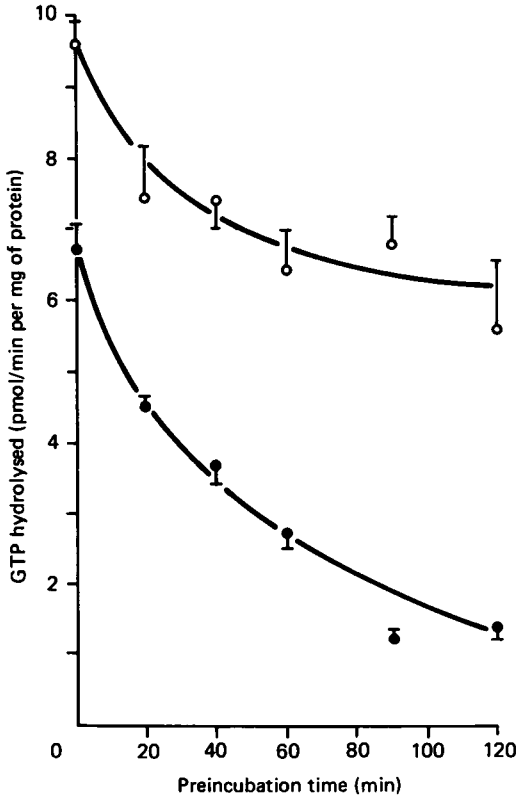

Fig. 6. Time course of the loss of basal and δ-opioid-receptor-stimulated high-affinity GTPase activity during the preincubation

NG108-15 membranes were preincubated at 37 °C for various times in the presence of all the components of the GTPase assay mixture except [γ-^{32}P]GTP. After this preincubation, [γ-^{32}P]GTP, and where appropriate DADLE (1 μM), were added, and incubation was allowed to proceed for a further 20 min. Samples were processed as described in the Materials and methods section. Basal (○) and opioid-stimulated above basal (●) high-affinity GTPase activities are presented.

effect on the foetal-calf serum stimulation of high-affinity GTPase activity (Table 2). Basal GTPase activity of the membranes was not inhibited by the affinity-purified antibodies, however. In fact, in a number of experiments, the basal GTPase activity was higher after preincubation in the presence of normal rabbit serum than in its absence (see data in Table 2). Preincubation of the membranes with normal rabbit serum alone did not produce an attenuation of the response to the opioid peptide (Table 2). Greater dilutions of the affinity-purified antibodies into normal rabbit serum decreased the antibody-mediated attenuation of opioid stimulation of the GTPase activity (Fig. 5); half-maximal effects of the antibodies affinity-purified from antiserum AS7 were achieved at a 1:1000 dilution and were completely abolished at a 1:10000 dilution. Incubation in the absence of antiserum produced a time-dependent decrease in opioid-stimulated and in basal GTPase activity (Fig. 6), suggesting that a proportion of the G-protein pool was being inactivated with time.

DISCUSSION

A number of signal-transducing G-proteins have recently been identified; these include G_s and G_i, which are involved in stimulatory and inhibitory control of adenylate cyclase [1,2], transducin, which couples rhodopsin to a cyclic GMP phosphodiesterase in photoreceptor-containing tissues [20], and G_o, a novel G-protein of undefined function [21–24].

Neuroblastoma × glioma hybrid NG108-15 cells are known to express a range of G-proteins, including both G_i and G_o [12]. A recent report has suggested that δ-opioid receptors on these cells may be coupled to the modulation of calcium channels via G_o [25]. However, it has also been demonstrated that the α-subunit of G_o does not cause inhibition of adenylate cyclase, whereas the α-subunit of G_i does so [26].

It has previously been demonstrated that opioid peptides can both stimulate GTPase activity and inhibit adenylate cyclase activity, in a pertussis-toxin-sensitive fashion in NG108-15 cells [5]. In the present report we confirm these results and further show that foetal-calf serum is also able to stimulate a pertussis-toxin-sensitive high-affinity GTPase activity in membranes of these cells. Although the chemical nature of the factor present in foetal-calf serum which is responsible for the stimulation of GTPase activity is unknown, it is heat-stable and non-diffusible (results not shown), as recently reported for the rat glioma cell line C6 BU1 [27]. As such it may represent a peptide growth factor. We also demonstrate, for the first time, that the opioid-peptide and foetal-calf-serum responses are mediated via separate distinct G-proteins, which are both substrates for pertussis-toxin-catalysed ADP-ribosylation. This conclusion is based upon (1) the observation that the stimulated GTPase

activities associated with each ligand are fully additive, (2) the fact that antibodies which specifically recognize the *C*-terminal 10 amino acids of G_i inhibit interaction between the δ-opioid receptor and its associated G-protein, whereas it does not uncouple foetal-calf serum from its associated stimulated GTPase activity, and (3) the fact that, unlike the opioid peptides, foetal-calf serum does not produce inhibition of adenylate cyclase activity.

A toxin isolated from cultures of *Bordetella pertussis* has been of particular use in the study of receptors which mediate inhibition of adenylate cyclase [5,6]. Thus pertussis toxin catalyses mono-ADP-ribosylation of G_i, and so causes attenuation of receptor-mediated inhibition of adenylate cyclase. Attenuation of a response after pretreatment of a system with pertussis toxin has often been considered diagnostic in implicating a role for G_i in that response. It is now recognized that G_i is not the sole substrate for pertussis-toxin-catalysed ADP-ribosylation. Certain other G-proteins, including G_o and transducin, can also be modified and functionally inactivated by this toxin. Thus caution must be exercised that the designation of a pertussis-toxin substrate as 'G_i' is not made on the basis of ADP-ribosylation alone. The situation is further complicated because evidence has recently been presented that multiple 'G_i-like' proteins may be expressed [13,28,29].

Pertussis toxin in the presence of [^{32}P]NAD^+ catalysed ADP-ribosylation only of a band of 40 kDa in membranes of NG108-15 cells. Although the autoradiogram (Fig. 2) might be taken to imply that this represents a single polypeptide, such a conclusion is not justified, as the known substrates for pertussis toxin are all of 39–40 kDa. Thus the resolution of the gel is likely to be insufficient to allow for separation of two (or more) pertussis-toxin substrates of very similar molecular mass. Pretreatment of the cells with pertussis toxin prevented the incorporation of radioactivity into this band (Fig. 2) and also completely prevented both opioid-peptide and foetal-calf-serum stimulation of high-affinity GTPase activity (Fig. 1), indicating that this polypeptide(s) represented the relevant G-protein(s).

In response to activation by a relevant agonist-occupied receptor, all of these signal-transducing G-proteins have the capacity to bind GTP with high affinity and to hydrolyse this nucleotide. GTPase assays have thus been widely utilized to confirm that a receptor is coupled to its effector system via a G-protein (see [3] and [5] for examples).

Measurement of a receptor-stimulated GTPase activity, however, can provide no direct evidence about the molecular nature of the specific G-protein involved. In this report we provide conclusive evidence that the δ-opioid receptor in NG108-15 cells interacts with G_i. We have shown that antibodies which recognize the *C*-terminal region of the α-subunit of 'G_i-like' proteins, but not that of G_o [30], are able to uncouple the opioid receptor from its associated G-protein, by estimating opioid-peptide stimulation of high-affinity GTPase activity in the presence and absence of affinity-purified antibodies which were diluted into normal rabbit serum (Table 2). Normal rabbit serum alone did not mimic the effect of the affinity-purified antibodies. It has been postulated that the *C*-terminal domain of G-proteins is important for their interaction with specific receptors [31,32]. As such, antibodies directed against epitopes within this region might be expected to interfere with receptor–G-protein coupling. It might further be expected that antibodies which bind to this region of the G-protein would not modulate the binding of GTP to the G-protein, and also would not affect the basal rate of GTPase activity. This prediction is confirmed by the results, as the basal rate of GTP turnover was not decreased by the presence of the antibodies (Table 2). In fact, it was frequently noted that preincubation in the presence of normal rabbit serum gave higher values for the basal GTPase activity than did preincubation in its absence (Table 2). The added protein may thus act to protect the membrane G-protein from inactivation during the preincubation (see Fig. 6). These results also suggest that the opioid receptor interacts only with G_i, as no residual opioid-stimulated GTPase is observed after preincubation of membranes with the antibodies. It might be expected that a proportion of the opioid-stimulated GTPase activity would remain after preincubation with G_i-specific antibodies if the opioid receptors were able to interact with G_o as well as with G_i. That the GTPase response to foetal-calf serum was not inhibited by these antibodies indicates that this factor does not function via G_i or via a G-protein with a similar *C*-terminal sequence (G_i-like protein). As NG108-15 cells also express G_o, it is possible that the responses to foetal-calf serum are coupled to this signal-transducing protein. Further studies are now required to test this hypothesis. These might involve the production of antisera which recognize the *C*-terminal domain of G_o.

Many recent studies (for examples, see [33–35]) have attempted to reconstitute purified G-proteins with purified, or partially purified, receptors in phospholipid vesicles. The surprising conclusion of a number of these studies is that many receptors can interact to some extent with a range of G-proteins. The data presented in this study argue strongly against non-selective interactions between receptors and different species of G-proteins. Reconstitution studies in artificial systems may thus only poorly mimic the situation in the native membrane.

This work was supported by a grant from the Medical Research Council to G.M. F.R.M. thanks the M.R.C. for a research studentship.

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Received 4 June 1987/24 August 1987; accepted 25 September 1987