Foetal-calf serum stimulates a pertussis-toxin-sensitive high-affinity GTPase activity in rat glioma C6 BU1 cells

Graeme MILLIGAN

Molecular Pharmacology Group, Departments of Biochemistry and Pharmacology, University of Glasgow, Glasgow G12 8QQ, U.K.

Cellular proliferation of rat glioma C6 BU1 cells in tissue culture is dependent on the presence of either calf or foetal-calf serum in the medium. Foetal-calf serum stimulated a high-affinity GTPase in membranes derived from C6 BU1 cells. Pretreatment of the cells with pertussis toxin decreased the high-affinity GTPase activity substantially, and attenuated the foetal-calf-serum-stimulated increase in this GTPase activity. Cholera toxin, in contrast, did not modulate the response to foetal-calf serum. Foetal-calf serum did not inhibit adenylate cyclase activity in membranes of these cells, indicating that the G-protein that was stimulated by foetal-calf serum was not G_i (the inhibitory one). Although the nature of the specific component of foetal-calf serum responsible for this pertussis-toxin-sensitive receptor-mediated stimulation of high-affinity GTPase activity has not been identified, it was mimicked neither by bombesin, which can stimulate inositol phospholipid turnover via a guanine nucleotide binding protein, nor by platelet-derived growth factor, which is present in substantial concentrations in foetal-calf serum. This report represents the first demonstration of a pertussis-toxin-substrate-mediated response in this cell line and provides further evidence that G-proteins other than G_i can be functionally inactivated by pertussis toxin.

INTRODUCTION

Mammalian cell lines in tissue culture require exogenously added growth factors to maintain growth and proliferation. This is most frequently achieved by the addition of serum to the medium. The nature of the growth-factor requirement from serum is poorly defined, as is the biochemistry of response to serum stimulation. It has been shown, nevertheless, that the receptors for a number of growth factors, e.g. epidermal growth factor, platelet-derived growth factor and insulin, can stimulate tyrosine-specific protein phosphorylation [1,2] and that growth factors such as bombesin can modulate inositol phospholipid metabolism [3,4].

It is now appreciated that a family of guanine nucleotide binding proteins (G-proteins) are involved in the transduction of information from agonist-receptor complexes to effector systems for a wide range of receptors, including those which modulate the activity of adenylate cyclase [5,6], inositol phospholipid turnover [7,8] and even ion channels [9]. In a number of cases, interaction of the pertinent G-protein with an agonist-occupied receptor has been shown to stimulate the rate of synthesis of GTP from a binding site on the G-protein [10]. This enhanced GTPase activity is thus a convenient indicator of G-protein activation [11].

Clones of rat glioma C6 cells have previously been shown to express large amounts of a 40 kDa pertussis-toxin substrate [6], which has been assumed to represent the inhibitory G_i . However, no receptor linked to this transducing protein has been identified. In the present report I demonstrate that foetal-calf serum stimulates high-affinity GTPase activity, but does not inhibit adenylate cyclase in cells of the rat glioma C6 BU1. This stimulation of GTPase activity is attenuated by prior treatment of the cells with pertussis toxin, but is unaffected by similar treatment with cholera toxin. These results indicate that a component of foetal-calf serum can

activate a pertussis-toxin-sensitive G-protein which is not G_i .

EXPERIMENTAL

C6 BU1 cells, which were kindly given by Dr. G. Wilkin, Imperial College, London, were grown routinely in 80 cm² tissue-culture flasks in Dulbecco's Modified Eagle's Medium containing 5% (v/v) foetal-calf serum (Gibco) and 100 units each of penicillin and streptomycin/ml. The cells were sub-cultured after brief trypsin treatment by splitting 1:10. Pertussis-toxin (kindly given by Dr. J. Freer, Department of Microbiology, University of Glasgow) and cholera-toxin (Sigma) pretreatment of the cells was performed by incubating the appropriate toxin (100 ng/ml) in growth medium with the cells for 16 h before cell harvest. Cells were harvested at confluency, and cell pastes and membranes prepared as in [12] and stored at -70°C until use.

GTPase activities of the membranes were measured as described by Koski & Klee [13], except that adenylyl imidodiphosphate was omitted from the reaction mixture. Usually less than 10% of the labelled nucleotide was hydrolysed during the assay. Low-affinity GTPase was subtracted from the total by the inclusion of parallel assays containing 100 μM -GTP. Absolute values of the basal high-affinity GTPase activity varied between different membrane preparations over the period of these experiments by some 2-fold, but a similar relative stimulation by foetal-calf serum was observed in all cases. Adenylate cyclase assays were performed as described in [14], except that the diterpene forskolin (10 μM) was used to elevate the basal adenylate cyclase activity. Foetal-calf serum was observed to modulate the cyclic AMP standard curve. This was corrected for by inclusion of foetal-calf serum, at appropriate concentrations, into the standard curve.

Fig. 1. High-affinity GTPase activity in membranes of rat glioma C6 BU1 cells: modulation by foetal-calf serum

Foetal-calf serum, between 0.1 and 30% (v/v), was added to GTPase assays of C6 BU1-cell membranes. A low-affinity GTPase activity, which was not affected by foetal-calf serum, was subtracted from all points by analysing parallel assays containing 100 μ M-GTP. Points are means $\pm$ S.E.M. for quadruplicate assays. The experiment was repeated eight times with essentially identical results, except that the absolute basal activities of high-affinity GTPase varied over an approx. 2-fold range.

ADP-ribosylation of C6 BU1-cell membranes by pertussis toxin was as previously described [15], except that the toxin concentration was 25 μ g/ml. Cholera-toxin-mediated ADP-ribosylation was performed identically. Protein was measured as described by Lowry *et al.* [16].

Calf serum and foetal-calf serum were from Gibco, and were heat-inactivated by treatment at 56 °C for 1 h before use. [γ - 32 P]GTP was prepared as described elsewhere [17]. [32 P]NAD⁺ was from New England Nuclear. All other materials were obtained from commercial sources and were of the highest grade available.

RESULTS

The release of [32 P]P_i from [γ - 32 P]GTP by membranes prepared from the rat glioma cell line C6 BU1 was enhanced by the addition of foetal-calf serum (Fig. 1) or calf serum (results not shown). The basal GTPase activity could be divided into two components, a high-affinity component which was sensitive to the presence of foetal-calf serum, and a low-affinity component which was not. In all experiments, the low-affinity component was subtracted from the total. Over the range of foetal-calf-serum concentrations which it was possible to use, the stimulation of high-affinity GTPase activity approached, but did not reach, a maximum (Fig. 1). In the experiments reported, time courses of the high-affinity GTPase activity were linear over a 20 min period, in both the absence and the presence of foetal-calf serum (results not shown).

Basal high-affinity GTPase activity was characterized

Fig. 2. High-affinity GTPase activity in C6 BU1-cell membranes: rate of hydrolysis versus GTP concentration

Values for high-affinity GTPase activity were calculated by subtracting hydrolysis by low-affinity GTPases and correcting for the specific radioactivity of [γ - 32 P]GTP at each concentration. Assays were for 10 min at 37 °C in the absence (○) or presence (●) of 10% (v/v) foetal-calf serum. Points represent means $\pm$ S.E.M. for quadruplicate assays. Inset; Eadie-Hofstee transformation of the data.

by an apparent K_m of 0.42 μ M and a $V_{max.}$ of 134 pmol/min per mg of protein (Fig. 2). The addition of foetal-calf serum to 10% (v/v) increased $V_{max.}$ to 294 pmol/min per mg of protein (2.2-fold), and also slightly increased the apparent K_m , to 0.61 μ M (1.5-fold). For both basal and foetal-calf-serum-stimulated high-affinity GTPase activity, the data were adequately described by a single function (Fig. 2, insert).

The growth factors bombesin (up to 3.2 μ M) and platelet-derived growth factor (up to 1.3 μ g/ml) were unable to mimic foetal-calf serum in stimulating high-affinity GTPase activity (results not shown). Although the nature of the factor in the serum which was responsible for this activity has not been characterized, it was stable to the process of heat inactivation of the serum (56 °C, 1 h) and also to further boiling for 5 min (results not shown). The factor was not removed by overnight dialysis against 1000 vol. of phosphate-buffered saline [12] (results not shown).

Prior intoxication of C6 BU1 cells with pertussis toxin (100 ng/ml for 16 h) prevented incorporation of radioactivity into a 40 kDa pertussis-toxin substrate when membranes of these pretreated cells were challenged *in vitro* with [32 P]NAD⁺ and fresh pertussis toxin (Fig. 3). Basal activities of the high-affinity GTPase were considerably decreased in these membranes, and it was now only weakly stimulated by foetal-calf serum (Fig. 4). In contrast, prior exposure of C6 BU1 cells to cholera toxin (100 ng/ml, 16 h), although eliminating the ability of further challenge with cholera toxin with [32 P]NAD⁺ to cause ADP-ribosylation of proteins of 45 and 42 kDa in membranes of these cells (Fig. 3), did not decrease basal high-affinity GTPase activities, nor did it diminish the stimulation of the GTPase activity by foetal-calf serum (Table 1).

Forskolin-activated adenylate cyclase activity was not inhibited by foetal-calf serum over the range of concentrations that produced stimulation of the high-

Fig. 3. ADP-ribosylation of membranes of control and toxin-treated cells

C6 BU1 cells were treated with cholera toxin or pertussis toxin or vehicle as described in the Experimental section, and membranes were prepared. These membranes (6 μ g) were then challenged with fresh toxin (25 μ g/ml) and [32 P]NAD $^{+}$ (10 μ M) as described previously [14]. Samples were resolved on a SDS/10% polyacrylamide gel, which was dried and autoradiographed. (a) Control membranes + pertussis toxin; (b) pertussis-toxin-pretreated membranes + pertussis toxin; (c) control membranes + cholera toxin; (d) cholera-toxin-pretreated membranes + cholera toxin. Abbreviation: D.F., dye front.

affinity GTPase activity (Fig. 5). Foetal-calf serum did, however, modify the cyclic AMP standard curve used in the quantification of the data, and as such produced an apparent inhibition of adenylate cyclase activity (results not shown). This was corrected for by inclusion of foetal-calf serum, at appropriate concentrations, in the cyclic AMP standard curve.

DISCUSSION

A family of heterotrimeric G-proteins are involved in signal transduction across the plasma membrane of target cells from receptors to their appropriate effector systems. Although the nature of the specific G-protein is dependent on the receptor and effector system, all known members of the G-protein family display the ability to bind and hydrolyse GTP [10]. This GTPase activity is inherent to the α -subunit of the G-protein and appears to regulate the association-dissociation cycle of the G-protein subunits [10]. The GTPase activity of G-proteins is stimulated by agonist occupation of a relevant receptor and hence activation of the G-protein. Thus ligand-stimulated GTPase activity is frequently a convenient indicator that a particular receptor stimulates a G-protein.

Mammalian cells in tissue culture require a poorly defined assortment of growth factors, and this mixture is generally provided by serum, with foetal-calf serum frequently being used as the source. In this paper, I show that both foetal-calf serum and calf serum stimulate a

Fig. 4. High-affinity GTPase activity in membranes of C6 BU1 cells: effect of pertussis toxin

C6 BU1 cells were treated with pertussis toxin (○) or vehicle (●) as described in the Experimental section. Membranes were prepared and high-affinity GTPase activity was measured as a function of foetal-calf-serum concentration. The points represent the means of quadruplicate assays from a typical experiment.

Table 1. Effects of cholera-toxin pretreatment on foetal-calf-serum stimulation of high-affinity GTPase activity of C6 BU1-cell membranes

C6 BU1 cells were treated with cholera toxin or vehicle as described in the Experimental section. After cell harvest and preparation of membranes, high-affinity GTPase activity was measured in the absence or presence of 10% foetal-calf serum.

Foetal-calf serum	GTPase activity (pmol/min per mg of protein)	
	Control	Cholera-toxin-pretreated
0	50.2 $\pm$ 0.8	57.0 $\pm$ 1.6
10%	119.5 $\pm$ 3.3	138.4 $\pm$ 4.0
Fold increase	2.38	2.42

high-affinity GTPase activity in membranes of rat glioma C6 BU1 cells. Calf serum has previously been shown to stimulate inositol phosphate production in NIH-3T3 cells transformed by overexpression of the P21 gene product of the N-ras proto-oncogene [4], and such a response was also observed for the growth factor bombesin. However, in those studies bombesin did not mimic the ability of foetal-calf serum to stimulate the high-affinity GTPase in membranes derived from C6 BU1 cells. Neither did platelet-derived growth factor, which is present in foetal-calf serum in significant amounts.

Fig. 5. Adenylate cyclase activity in membranes of C6 BU1 cells: effect of foetal-calf serum

Foetal-calf serum, between 0.1 and 20% (v/v), was added to membranes (30 μ g) prepared from C6 BU1 cells. The diterpene forskolin (10 μ M) was also added to all tubes. Adenylate cyclase activity was then assessed as described previously [14]. Points are the mean of triplicate assays.

Toxins derived from *Vibrio cholerae* and *Bordetella pertussis* have been invaluable tools in the characterization of G-proteins. Both toxins are able to catalyse mono-ADP-ribosylation of the α -subunits of specific G-proteins, and in the process modify their functions. Cholera toxin catalyses ADP-ribosylation of G_s , the G-protein interacting with receptors which stimulate adenylate cyclase, causing irreversible activation of G_s . Although prior treatment of C6 BU1 cells with cholera toxin caused ribosylation *in vivo* of the total available pool of G_s (Fig. 3), it did not inhibit the basal high-affinity GTPase activity of the membranes, nor its stimulation by foetal-calf serum (Table 1). This indicates that the G-protein responding to stimulation by foetal-calf serum in these membranes was neither G_s nor another cholera-toxin substrate. However, in contrast, pretreatment of the cells with pertussis toxin led to both a decrease in the basal high-affinity GTPase activity of the membranes and the attenuation of foetal-calf-serum stimulation of this activity. By these criteria, the relevant G-protein is a substrate for pertussis-toxin-catalysed ADP-ribosylation. Although pertussis toxin was originally demonstrated to be capable of specifically modifying G_i , the G-protein linking receptors to inhibition of adenylate cyclase [6], it is now realized that this toxin can modify other substrates besides G_i , including transducin, the G-protein of vertebrate rod outer segments which functions in rhodopsin-linked photon reception, and G_o , a G-protein of unknown function [18–20], which is widely distributed and is particularly prevalent in brain [21–22].

C6 BU1 cells have previously been shown to contain an abundant pertussis-toxin substrate of 40 kDa [15]. Initial studies indicated that this species was not G_i , G_o or transducin, as antibodies which recognized each of these species in rat tissues showed little or no immunochemical cross-reactivity with this protein [16]. Experiments, utilizing an affinity-purified antiserum raised against the C-terminal 10 amino acids of rod transducin, which differs in only one position within this region from the sequence of a clone of rat G_i [23], have

shown that this antiserum recognizes the C6 BU1-cell pertussis-toxin substrate (G. Milligan, C. Unson & A. Spiegel, unpublished work). As transducin is limited in distribution to photoreceptor-containing tissues, these observations therefore indicate that the C6 BU1-cell pertussis-toxin substrate is likely to represent a ' G_i -like' protein. Foetal-calf serum failed to inhibit forskolin-stimulated adenylate activity in membranes of these cells, providing further evidence that this protein is not G_i . It has previously been demonstrated that, although chemotactic responses to formyl-methionyl-leucyl-phenylalanine in leucocytic cells are abolished by pertussis toxin, this ligand does not mediate inhibition of adenylate cyclase [24]. Thus pertussis-toxin-sensitive G-proteins are expressed that have functions other than to couple receptors to this second-messenger system. For example, it is well known that receptors linked to enhanced turnover of polyphosphoinositides function via a pertussis-toxin substrate in neutrophils [25] and that this protein is not G_i [26], although it may be closely related [27].

Although it has been recognized for some years that clones of the C6 cell line express large amounts of a pertussis-toxin substrate [6], no receptors coupled to this transduction system have previously been described. Thus foetal-calf-serum stimulation of this novel pertussis-toxin-sensitive high-affinity GTPase activity affords the first indication of a specific function for this G-protein in C6 BU1 cells and provides a potential ligand with which to study receptor interactions in this cell line. Initial experiments indicate that the factor in foetal-calf serum which mediates the stimulation of GTPase activity is heat-stable but that it is too large to pass freely across a dialysis membrane. Further studies are now required to elucidate the chemical nature of this factor.

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