

CD3/T-cell receptor coupling to a pertussis and cholera toxin-insensitive G-protein

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Received 11 May 1989

We have analyzed the effect of CD3/T-cell receptor stimulation on GTP hydrolysis and GTP binding. We show that stimulation of Jurkat, T-cell, membranes with OKT3 results in a 50% increase in GTP hydrolysis which is specifically inhibited by GDP. Pretreatment of the membranes with neither pertussis toxin nor cholera toxin inhibited the GTP hydrolysis. We also show that stimulation with OKT3 increases the binding of GTP γ S to Jurkat membranes. These data strongly implicate the involvement of a G-protein in CD3/T-cell receptor signalling.

T-lymphocyte; Signal transduction; GTP hydrolysis; GTP binding

1. INTRODUCTION

G-proteins are a group of GTP hydrolysing, membrane associated, proteins that link receptor stimulation to several effectors including adenylate cyclase [1]. Receptors can be coupled to the adenylate cyclase via a stimulatory (and cholera toxin-sensitive) G_s-protein or via an inhibitory (and pertussis toxin-sensitive) G_i-protein. Recently, the involvement of a G-protein, termed G_p, in the breakdown of phosphatidylinositol (PI) was suggested [2]. The G-protein that stimulates phospholipase C (PLC) can be either sensitive or insensitive to pertussis toxin depending on the cell type studied. Thus, a pertussis toxin-sensitive G-protein has been shown to couple the receptor for f-Met-Leu-Phe (fMLP) to activation of PLC in neutrophils [3,4] whereas the G-protein that links the TRH receptor on GH₃ pituitary cells [5] and

the Ig-receptor on B-cells [6] to PLC is pertussis toxin-insensitive.

The human T-cell receptor consists of the clonotypic antigen-specific Ti α - β heterodimer and the monomorphic CD3 complex. The CD3 complex comprises at least four, and possibly five, integral membrane proteins and is suggested to be involved in transmembrane signalling following antigen binding to the T-cell receptor. Stimulation of the CD3/T-cell receptor by antigen or specific antibodies leads to an increased inositol phosphate formation and to increased intracellular Ca²⁺ [7]. The evidence for the involvement of G-proteins in T-cell activation is so far only indirect. The fact that G-protein modulation by bacterial toxins were able to block T-cell activation [8,9] might be due to indirect action on i.e. cAMP. Similarly, the fact that nonhydrolysable GTP analogues in permeabilized cells or cell membranes mimic cellular events, such as inositol phosphate formation and serine esterase release, involved in T-cell activation [10,11] is suggestive but not conclusive evidence since the effect could be mediated by G-proteins that are involved in a parallel signal transduction pathway. Moreover, GTP may have effects that are independent of membrane based G-proteins in-

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Abbreviations: PLC, phospholipase C; cAMP, cyclic adenosine 3',5'-monophosphate

cluding the recently reported ability to release Ca^{2+} from intracellular stores [12]. The study of GTP hydrolysis or GTP binding upon stimulation of specific receptors on cellular membranes appears to be a more direct way to investigate G-protein coupling to receptors. In this report we demonstrate that stimulation of the CD3/T-cell receptor complex results in increased hydrolysis and binding of GTP to T-cell membranes.

2. EXPERIMENTAL

2.1. Cells

The human leukemic T-cell line, Jurkat, was maintained in RPMI-1640 supplemented with 7.5% fetal calf serum, l-glutamine and penicillin/streptomycin.

2.2. Chemicals

The monoclonal anti-CD3-antibody, OKT3, was from a hybridoma obtained from the American Type Culture Collection (no. CRL 800) and purified on protein A-Sepharose. Pertussis toxin was a kind gift from Dr Per Askelöf at Statens Bakteriologiska Laboratorium, Stockholm, Sweden. Cholera toxin was purchased from Sigma. $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and $[\text{S}^{35}]\text{GTP}\gamma\text{S}$ were purchased from NEN, DuPont.

2.3. GTP hydrolysis assay

Jurkat cell membranes were prepared by sonication in 25 mM Tris, 5 mM MgCl_2 , 1 mM PMSF (pH 7.6). Cell debris was removed by centrifugation at $150 \times g$ for 5 min. The supernatant was collected and soluble proteins were removed by centrifugation at $10000 \times g$ for 10 min. The fractions were analysed by a 5'-nucleotidase assay as described before [13]. All of the nucleotidase activity remained in the membrane fraction demonstrating an almost complete recovery of plasma membranes (homogenate 94.2 nmol/mg per h; membrane fraction, 418.5 nmol/mg per h and soluble fraction, 0 nmol/mg per h). The pellet was resuspended and protein concentration assayed. This crude membrane fraction was kept on ice until used. Incubations were carried out at 30°C in a final volume of 100 μl . The reaction buffer consisted of 10 mM Tris (pH 7.8), 10 mM MgCl_2 , 1 mM EDTA, 0.2% BSA, 0.5 mM ascorbic acid, 0.5 mM adenylyl-5-yl imidodiphosphate, an ATP regenerating system (0.1 mM ATP, 3 mM creatine phosphate and 75 U/ml creatine phosphokinase), 100 nM $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ with GDP and OKT3 as indicated. The reaction was started by the addition of 20 μg of membrane protein and stopped by the addition of 700 μl of 5% Norit A in 0.15 M potassium phosphate buffer (pH 7.0). After centrifugation at $10000 \times g$ for 15 min 500 μl of supernatant was collected and the released $^{32}\text{P}_i$ counted.

2.4. GTP binding assay

Binding reactions were carried out at 30°C in 50 μl of 10 mM Tris (pH 7.8), 10 mM MgCl_2 , 1 mM EDTA, 0.2% BSA, 0.5 mM ascorbic acid, 0.5 mM adenylyl-5-yl-imidodiphosphate, 10 nM $[\text{S}^{35}]\text{GTP}\gamma\text{S}$ and 20 $\mu\text{g}/\text{ml}$ of OKT3 as indicated. The reaction was started by the addition of 50 μg membrane protein and stopped by the addition of 450 μl of icecold stopbuffer con-

taining 10 mM Tris (pH 7.4), 100 mM NaCl and 25 μM $\text{GTP}\gamma\text{S}$. Bound ligand was separated from free by filtration through Whatman GF/C filters with icecold stopbuffer without $\text{GTP}\gamma\text{S}$.

2.5. Toxin treatment of Jurkat membranes

Pertussis toxin and cholera toxin were preactivated with 15 mM dithiothreitol at 37°C for 30 min. The membranes (2 mg/ml) were then treated with either 25 $\mu\text{g}/\text{ml}$ of pertussis toxin or 50 $\mu\text{g}/\text{ml}$ of cholera toxin in 0.15 M potassium phosphate (pH 7.5), 1 mM NAD, 0.5 mM ATP, 10 mM thymidine and 50 μM GTP at 30°C for 30 min. Following treatment the membranes were resuspended in 10 mM Tris (pH 7.6) and kept on ice until used.

3. RESULTS AND DISCUSSION

Receptor-mediated activation of a G-protein regulated effector system is initiated by the replacement of bound GDP by GTP [14] and also associated with the activation of a high affinity GTPase as originally shown for adenylate cyclase [15]. Therefore, in order to obtain direct evidence for regulation of a G-protein by the CD3/T-cell receptor complex we have examined the effect of CD3 stimulation on GTPase activity in Jurkat membranes.

A significant GTP hydrolysis activity is present in control Jurkat membranes. Stimulation with OKT3 was found to increase GTP hydrolysis by 50% (fig.1) whereas stimulation with a nonactivating anti-CD4 antibody did not affect the hydrolysis (not shown). Hydrolysis was linear throughout the 15 min interval as described before [16]. The maximal hydrolysis rate was 3.7 pmol per mg membrane protein min^{-1} which is similar to the activity described in S49 lymphoma cells [17] but significantly lower than reported in CT6 T-cells [16]. The reason for this difference is unclear but may reflect a relatively low G-protein content in Jurkat membranes. It should also be noted that crude membrane preparations as used here and by others [16,17] contain a substantial amount of mitochondria making our estimation only relative.

A dose-response curve shows that the increase in GTP hydrolysis was half maximal at an antibody concentration of 2.8 $\mu\text{g}/\text{ml}$ (fig.2). This is comparable with antibody concentrations sufficient for Ca^{2+} influx and inositol phosphate formation in T-lymphocytes [11,18].

GDP inhibits GTP hydrolysis by competing with GTP binding [14]. GDP was found to inhibit

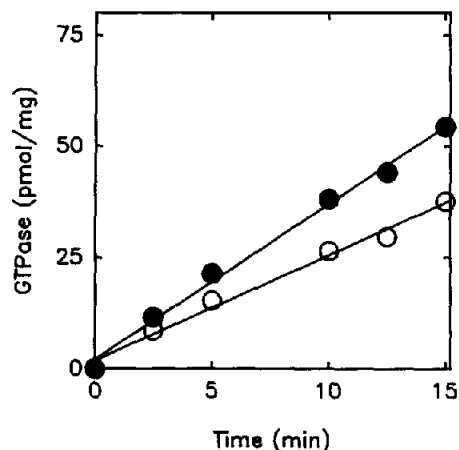


Fig. 1. Stimulation of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ hydrolysis by OKT3. Jurkat membranes (20 μg) were incubated with 100 nM $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ at 30°C for the indicated times in the presence (closed circle) and absence (open circle) of 20 $\mu\text{g}/\text{ml}$ of OKT3. GTP hydrolysis was determined as the release of ^{32}P . Each point is the mean of triplicate determinations. Standard deviations were less than 10%. The result is representative of five experiments.

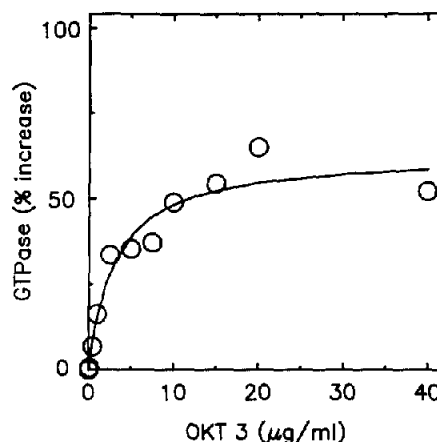


Fig. 2. Hydrolysis of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ at different concentrations of OKT3. Jurkat membranes (20 μg) were incubated with 100 nM $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ at 30°C for 10 min with the indicated concentrations of OKT3. Each point is the mean of triplicate determinations. Standard deviations were less than 10%. The result is representative of two experiments.

OKT3 stimulated GTP hydrolysis (fig. 3) whereas ADP did not (not shown). A half-maximal inhibition was seen at 19 μM of GDP.

Several G-proteins, including G_0 and G_i , are substrates for pertussis toxin catalyzed ADP-ribosylation that inhibits the G-protein [14]. If the CD3/T-cell receptor coupled G-protein is similarly pertussis toxin-sensitive, one would expect that effects induced by CD3 stimulation should be inhibited by toxin pretreatment. Pertussis toxin has been reported to inhibit CD3-stimulated Ca^{2+} influx [8,19]. However, others have reported that pertussis toxin affects PLC by a mechanism distinct from direct G-protein inhibition in T-lymphocytes [20]. Our data do not support the involvement of a pertussis toxin-sensitive G-protein in CD3/T-cell receptor signalling (table 1). Pretreatment of Jurkat membranes with 25 $\mu\text{g}/\text{ml}$ of pertussis toxin did not inhibit OKT3 stimulated GTP hydrolysis although we have found pertussis toxin substrates of molecular masses between 39 and 41 kDa using ADP-ribosylation in Jurkat membranes (not shown). In pertussis toxin-sensitive systems a significant inhibition of agonist-stimulated GTPase activity has been detected at toxin concentrations of 10 $\mu\text{g}/\text{ml}$ [17]. We have also not been able to inhibit either OKT3-stimu-

lated inositol phosphate turnover or GTPase activity when preincubating intact Jurkat cells with pertussis toxin for 20 h (not shown). We have found that also cholera toxin stimulates ADP-ribosylation in Jurkat membranes as well as increases the formation of cAMP in intact cells (not shown). However, pretreatment of Jurkat membranes with 50 $\mu\text{g}/\text{ml}$ of cholera toxin did not affect OKT3-stimulated GTP hydrolysis. This is in apparent contrast with a previous report showing that cholera toxin inhibits Ca^{2+} influx and IP_3 formation in Jurkat cells [9]. However, the cholera toxin-induced activation of G_s -proteins leads to an increased formation of cAMP which could affect IP_3 formation at a step subsequent to the G-protein as recently reported [21]. The toxin pretreatment caused a slight increase in basal and stimulated GTPase activity. This may be due to the membrane binding and mitogenic effect of these toxins [22,23]. However, pretreatment of intact cells or short term membrane stimulation did not cause an increased GTPase activity (not shown) making this explanation unlikely.

In order to substantiate further the association of a G-protein to the CD3/T-cell receptor we analyzed the binding of the nonhydrolysable GTP analogue $\text{GTP}\gamma\text{S}$ to Jurkat membranes after stimulation with OKT3. We found that OKT3

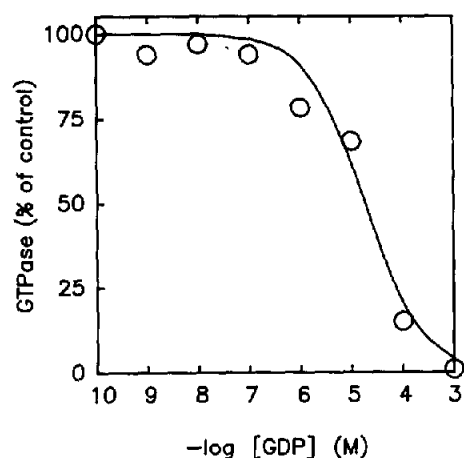


Fig. 3. Inhibition of OKT3-stimulated hydrolysis of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ by GDP. Jurkat membranes (20 μg) were incubated with 100 nM $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ at 30°C for 10 min with the indicated concentrations of GDP and 20 $\mu\text{g}/\text{ml}$ of OKT3. Each point is the mean of triplicate determinations. Standard deviations were less than 10%. The result is representative of two experiments.

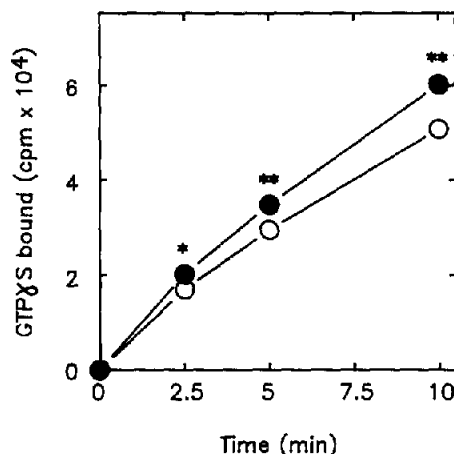


Fig. 4. Stimulation of $[\text{S}^{35}]\text{GTP}\gamma\text{S}$ binding by OKT3. Jurkat membranes (50 μg) were incubated with 10 nM $[\text{S}^{35}]\text{GTP}\gamma\text{S}$ (10^6 cpm) at 30°C with (closed circles) or without (open circles) 20 $\mu\text{g}/\text{ml}$ of OKT3 for the indicated times. Binding of $[\text{S}^{35}]\text{GTP}\gamma\text{S}$ was measured as described in section 2. Non-specific binding was less than 10% in all determinations. Each point is the mean of triplicate determinations. Standard deviations were less than 10%. * and ** indicate statistical significance ($p < 0.1$ and $p < 0.05$ by a two-tailed Student's t -test). The result is representative of three experiments.

causes a minor, but statistically significant, increase in the binding of $\text{GTP}\gamma\text{S}$ by approximately 20% (fig. 4). This is in line with the stimulation of $\text{GTP}\gamma\text{S}$ binding in HL-60 cells by fMLP [4]. In contrast to CT6 cells [17], $\text{GTP}\gamma\text{S}$ binding did not reach equilibrium within the 10 min period studied. Instead incubations for as long as 60 min

were necessary for equilibrium (not shown). This is compatible with a lower rate of GTP turnover in Jurkat cells and agrees with the already noted lower GTPase activity.

Our data suggest the involvement of a pertussis toxin- and cholera toxin-insensitive G-protein in signalling via the CD3/T-cell receptor complex. It seems likely that this G-protein is of the G_p type since it is toxin insensitive and associated with a phospholipase C linked receptor. The product of the growth-promoting ras-protein has been proposed to be a PLC coupled G-protein [24]. It is an intriguing possibility that the G-protein involved in CD3/T-cell receptor signalling may be similar to p21^{ras} .

Table 1
Effect of pretreatment of membranes with pertussis or cholera toxin

Pretreatment	Stimulation	GTPase activity (pmol/mg per 10 min)
—	—	17.8 $\pm$ 1.1
—	OKT3	26.6 $\pm$ 0.5
PTX	—	21.2 $\pm$ 0.4
PTX	OKT3	31.8 $\pm$ 0.7
CT	—	23.7 $\pm$ 2.0
CT	OKT3	32.0 $\pm$ 1.2

Effect of pertussis toxin and cholera toxin on OKT3-stimulated hydrolysis of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$. Jurkat membranes were pretreated with 25 $\mu\text{g}/\text{ml}$ of pertussis toxin or 50 $\mu\text{g}/\text{ml}$ of cholera toxin at 30°C for 30 min. The membranes were then resuspended and analyzed for GTPase activity in the presence or absence of 20 $\mu\text{g}/\text{ml}$ of OKT3. The results are expressed as mean $\pm$ SE of triplicate determinations. The result is representative of three experiments

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