Agonist-stimulated high-affinity GTPase in *Dictyostelium* membranes

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GTP hydrolysis in *Dictyostelium discoideum* membranes is caused by a low ($K_m > 1$ mM) and a high affinity (K_m 6.5 μM) GTPase. cAMP enhances GTP hydrolysis apparently by increasing the affinity of the high affinity GTPase (stimulated K_m 4.5 μM); the low affinity GTPase was not affected by cAMP. Stimulation of GTP hydrolysis by cAMP was maximal at early time points and declined thereafter. A half-maximal stimulation of GTPase occurred at 3 μM cAMP and the specificity of cAMP derivatives for stimulation of GTPase activity showed a close correlation with the specificity for binding to the cell surface cAMP receptor. Treatment of *D. discoideum* cells with pertussis toxin decreased the cAMP-induced stimulation of GTPase from $42 \pm 6\%$ in control cells to $17 \pm 9\%$ in pertussis toxin-treated cells. These results suggest that the interaction of cAMP with its surface receptor leads to stimulation of high affinity GTPase in *D. discoideum* membranes. At least one of those enzymes may represent a guanine nucleotide-binding protein sensitive to pertussis toxin.

Enzyme activity; GTPase; GTP hydrolysis; cyclic AMP; Pertussis toxin; G-protein

1. INTRODUCTION

In the cellular slime mold *Dictyostelium discoideum* cAMP acts as a first and second messenger and is involved in chemotaxis [1], morphogenesis [2], and cell differentiation [3]. cAMP binds to highly specific surface receptors, which activate several enzymes, including adenylate cyclase and guanylate cyclase [4–6]. The produced cAMP is secreted [7] and relays the chemotactic signal to the more distal cells. The cGMP produced remains largely intracellular and is probably in-

volved in the chemotactic reaction [8–10]. Prolonged stimulation of *D. discoideum* cells with constant cAMP concentrations induces desensitization of guanylate and adenylate cyclase within a few seconds and a few minutes, respectively [11–14].

In vertebrates the effector molecules are coupled to the surface receptors via signal transducing G-proteins [15–17]. These proteins not only bind guanine nucleotides but also hydrolyze GTP. When affected by hormone-activated receptors, GTP hydrolysis is increased, due to an increase in the turnover of these proteins from the inactive GDP-bound to the active GTP-bound states. The hormone-stimulated GTP hydrolysis in different systems is inhibited following ADP-ribosylation by cholera and/or pertussis toxin [15,18–21]. The existence of a G protein in *D. discoideum* membranes has been suggested previously by Leichtling et al. [22], who showed that a 42-kDa protein binds GTP and can be ADP-ribosylated by the cholera toxin.

Recent results [23,24] suggest the presence of fast and slowly dissociating forms of the cell surface cAMP receptor. cAMP induces the inter-

conversion of binding forms in vivo [25,26], which is promoted by guanine nucleotides in vitro [26–29]. This suggests the involvement of guanine nucleotide-regulatory proteins in chemosensory transduction. This view is further supported by the recent observation that guanosine triphosphates stimulate adenylate cyclase in vitro [30,31], and by the finding that treatment of cells with pertussis toxin affects activation of adenylate cyclase in vitro [31] and in vivo (Snaar-Jagalska, B.E., unpublished). Finally, cAMP increases the binding of [³H]GTP to isolated membranes and at the same time accelerates the dissociation rate of bound [³H]GTP [32].

An essential function of G-proteins is the agonist-stimulated hydrolysis of GTP. Therefore, we investigated whether the GTP hydrolysis is stimulated by cAMP in *D. discoideum*. The results show the presence of agonist stimulated high-affinity GTPase, which is partly sensitive to pertussis toxin.

2. EXPERIMENTAL

2.1. *Materials*

[γ-³²P]GTP (37.94 Ci/mmol) was purchased from New England Nuclear. cAMP, ATP, ATPγS, AppNHp, (Sp)-cAMPS, GTP, creatine phosphate, creatine kinase and cAMP derivatives were obtained from Boehringer Mannheim. DTT was from Sigma. Pertussis toxin was purchased from List.

2.2. *Culture conditions and membrane isolation*

D. discoideum cells (strain NC-4) were grown as described in [13], harvested in 10 mM KH$_2$PO$_4$/Na$_2$HPO$_4$, pH 6.5, washed and starved in phosphate buffer by shaking at a density of 10^7 cells/ml. After 5–6 h, cells were collected by centrifugation, washed twice with phosphate buffer, and the pellet was resuspended in 40 mM Hepes/NaOH, 0.5 mM EDTA, 250 mM sucrose, pH 7.7, to a density of 2 × 10^8 cells/ml. Homogenization was performed by pressing the cell suspension through a Nuclepore filter (pore size 3 µm) at 0°C. The lysate was centrifuged at 10000 × g for 5 min, the pellet washed once with 10 mM triethanolamine-HCl, pH 7.4, containing 0.5 mM EDTA, and the final pellet resuspended in 10 mM triethanolamine-HCl, pH 7.4, to the equivalent of 1 × 10^8 cells/ml.

2.3. *GTPase assay*

GTPase activity of the *D. discoideum* membranes was determined with a reaction mixture containing [γ-³²P]GTP (0.1 µCi/assay), 2 mM MgCl$_2$, 0.1 mM EGTA, 0.2 mM AppNHp, 0.1 mM ATPγS, 10 mM DTT, 5 mM creatine phosphate (Tris salt), 0.4 mg/ml creatine kinase and 2 mg/ml bovine serum albumin (purified) in 50 mM triethanolamine-HCl, pH 7.4, in a total volume of 100 µl. After 5 min prein-

cubation of the reaction mixture at 25°C, the reaction was initiated by the addition of 30 µl membranes (10–40 µg protein/tube) to 70 µl of a reaction mixture and conducted for 3 min, if not otherwise indicated. The reaction was terminated by the addition of 0.5 ml sodium phosphate buffer (50 mM), pH 2.0, containing 5% (w/v) activated charcoal. The reaction tubes were centrifuged at 4°C for 5 min at 10000 × g and the radioactivity of 0.4 ml of the supernatant was determined using Cerenkov radiation.

Release of ³²P$_i$ from [γ-³²P]GTP in the absence of membranes was 0.5–2.5% of added [γ-³²P]GTP. High-affinity GTPase was defined as the difference between total GTPase and low-affinity GTPase activity. Low-affinity GTPase activity was determined in the presence of 50 µM GTP [13,33], and was about 40–50% of total GTPase. Maximal hydrolysis of GTP did not exceed 10–15% of added GTP. GTPase assays were performed in triplicates, with intra-assay variation of less than 3% of the means. Experiments were repeated at least twice, with results comparable with those shown.

3. RESULTS

The aim of the present study is to investigate GTPase activity in *D. discoideum* membranes as a function of the interaction between cell-surface cAMP receptors and putative G-protein(s). To determine GTP-specific nucleoside triphosphatase a low concentration of GTP (0.25 µM) and maximal suppression of the non-specific nucleoside triphosphatases activity was used [33]. Addition of AppNHp, an inhibitor of a number of ATPases [34] decreased the rate of GTP hydrolysis from 101.0 ± 4.2 to 51.4 ± 1.6 pmol P$_i$·min^{-1}·mg protein^{-1} (table 1). Redistribution of radioactivity

Table 1

Hydrolysis of GTP in *D. discoideum* membranes by high-affinity GTPase

Addition	[γ-³²P]GTP hydrolysis (pmol P$_i$·min^{-1}·mg protein^{-1})	
	Control	+ cAMP
None	101.0 ± 4.2	104.2 ± 7.6
0.2 mM AppNHp	51.4 ± 1.6	55.1 ± 2.2
0.2 mM AppNHp + RS	28.2 ± 3.1	32.5 ± 1.0
0.2 mM AppNHp + RS + 0.1 mM ATP	18.2 ± 0.7	21.8 ± 1.2
0.2 mM AppNHp + RS + 0.1 mM ATPγS	11.8 ± 1.2	16.1 ± 1.1

Membranes were incubated with 0.25 µM GTP in the presence or absence of 3 µM cAMP for 10 min. The ATP regeneration system (RS) was 5 mM creatine phosphate and 0.4 mg/ml creatine kinase. The data are means of three experiments

among guanine and adenine dinucleotides was prevented by a nucleoside triphosphate regeneration system and by ATPγS. Under this condition the liberation of $^{32}P_i$ was suppressed to 6–8% and effectively stimulated by cAMP. The not easily hydrolysed derivatives of ATP, ATPγS and AppNHp, were used to suppress production of cAMP by adenylate cyclase.

3.1. Time course and kinetics of GTP hydrolysis

The hydrolysis of GTP in *D. discoideum* membranes was multiphasic, and cAMP stimulated the initial hydrolysis of GTP (fig.1). Stimulation of P_i release by 3 μM cAMP was routinely measured at 3 min of incubation because at this time point P_i production and stimulation by cAMP were sufficiently large for accurate determination (inset of fig.1). The relationship between membrane protein and GTP hydrolysis was linear in the range of 10–40 μg membrane protein per assay for the incubation at 25°C for 3 min (not shown).

The hydrolysis of different concentrations of [γ-^{32}P]GTP in the absence and presence of 3 μM cAMP is shown in fig.2. Hydrolysis of [γ-^{32}P]GTP was potently reduced by increasing concentrations of unlabeled GTP (fig.2A). At all GTP concentra-
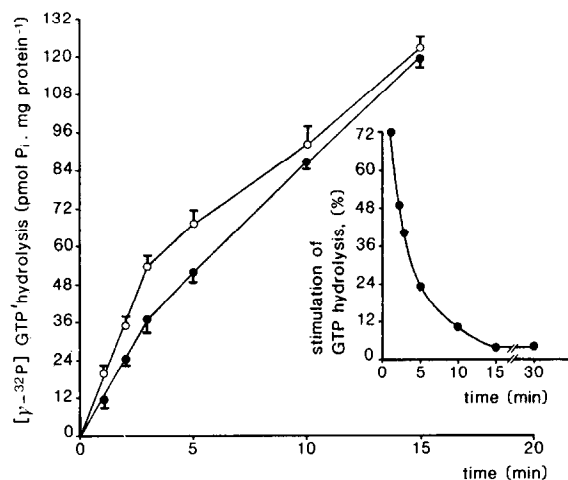
Fig.1. Time course of GTP hydrolysis in the absence and presence of cAMP. Membranes were incubated as described in section 2, but in a final volume of 1 ml. At the indicated times, aliquots of 100 μl were added to 500 μl of charcoal suspension. GTP hydrolysis was determined with 0.1 μM GTP in the absence (●) and presence (○) of 3 μM cAMP. Inset represents % stimulation of GTP hydrolysis by 3 μM cAMP.
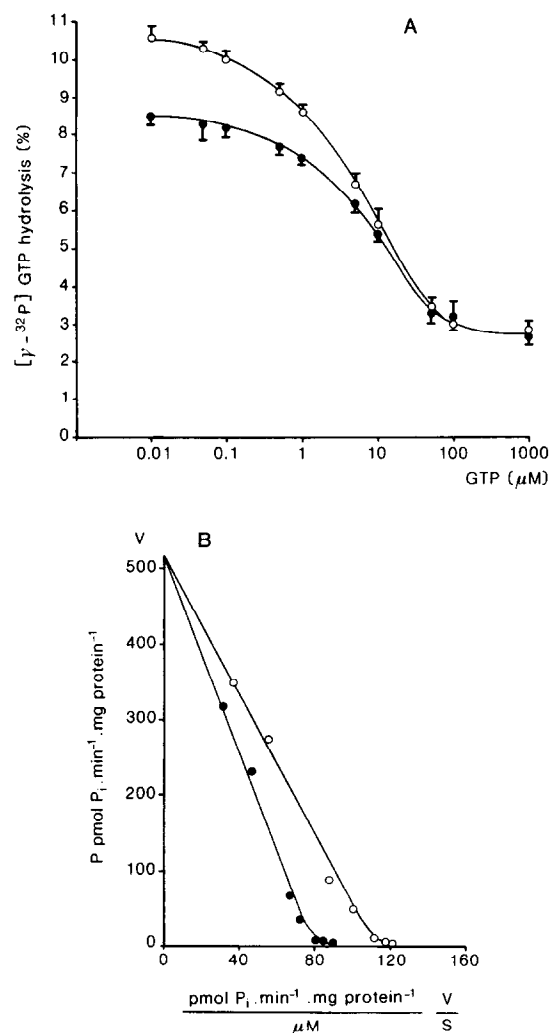
Fig.2. Kinetics of GTP hydrolysis in *D. discoideum* membranes. (A) Hydrolysis of [γ-^{32}P]GTP was determined at various concentrations of unlabeled GTP in the absence (●) and presence of 3 μM cAMP (○); (B) Eadie-Hofstee plot of a high-affinity GTPase. Low-K_m GTPase was subtracted from the total GTPase activity and the apparent K_m values (4.5 μM and 6.5 μM) of a high-affinity GTPase were extrapolated from the linear part of the curves.

tions below 50 μM, cAMP increased [γ-^{32}P]GTP hydrolysis (fig.2A). Both curves reached a plateau at about 50 μM GTP. These observations indicate that *D. discoideum* membranes contain a high-affinity, cAMP-sensitive GTPase and a low-affinity cAMP-insensitive GTPase ($K_m > 1$ mM). The high-affinity GTPase exhibited an apparent K_m value of about 6.5 μM (fig.2B). The stimula-

tory effect of cAMP on GTP hydrolysis by the high-affinity GTPase occurred without a change in the V_{max} value and was apparently caused by an increase of enzyme affinity for GTP from 6.5 μM to 4.5 μM (fig.2B).

3.2. Agonist stimulation of GTPase activity

The stimulatory effect of cAMP on GTP hydrolysis by the high-affinity GTPase in *D. discoideum* membranes was half-maximal at 3 μM cAMP and reached a maximum of 65% stimulation (fig.3). Specificity of cAMP derivatives for GTPase stimulation is shown in table 2. The order of GTPase stimulation was as follows: cAMP > 2'-dcAMP > (Sp)-cAMPS > 8-Br-cAMP, and cGMP and 5'-AMP were inactive. These derivatives bind to the chemotactic cAMP receptor with the same relative potencies [35], which is quite different from the binding specificity of cAMP-dependent protein kinase [36], indicating a functional coupling between the cell-surface cAMP receptor and a high-affinity GTPase.

3.3. Influence of pertussis toxin on cAMP-stimulated GTP hydrolysis

The effects of pertussis toxin treatment in vivo on the GTPase(s) activity in the membranes are shown in table 3. GTPase activity was measured at 0.01, 0.1 and 1 μM. After pertussis toxin treatment the basal GTPase activity was as in control membranes at all GTP concentrations (NS, $p > 0.5$). cAMP stimulated GTPase activity at 0.01 μM GTP by 42 $\pm$ 6%. This stimulation was significant-
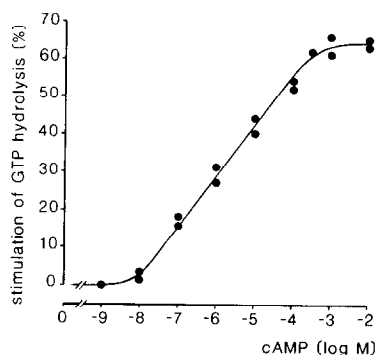

Fig.3. Concentration-response curve for cAMP stimulation of GTP hydrolysis. GTP hydrolysis by high-affinity GTPase was determined in *D. discoideum* membranes at the indicated concentration of cAMP with 0.1 μM GTP.

Table 2

Specificity of cAMP derivatives for stimulation of high-affinity GTPase in *D. discoideum* membranes

Derivative	Stimulation of P_i release (%)
No addition	0
cAMP	53.2 $\pm$ 0.9
2'-dcAMP	37.4 $\pm$ 2.4
(Sp)-cAMPS	29.9 $\pm$ 1.2
8-Br-cAMP	12.2 $\pm$ 1.8
cGMP	-1.6 ± 2.1
5'-AMP	1.4 $\pm$ 0.9

Membranes were incubated as described in section 2 with 0.1 μM GTP for 3 min in the presence or absence of 10 μM cAMP derivatives

ly (*, $p < 0.01$) reduced by pertussis toxin treatment to 17 $\pm$ 9%. Similar results were obtained at higher GTP concentrations: no reduction of basal GTPase activity by pertussis toxin and a significant reduction of the cAMP-mediated stimulation of GTPase. Enhancement of GTPase by cAMP in membranes derived from pertussis toxin-treated cells was not completely lost ($p < 0.01$), suggesting that pertussis toxin inhibited at least one component of the high-affinity GTPase activity that is

Table 3

Effect of pertussis toxin on GTPase activity in *D. discoideum* membranes

GTP (μM)	cAMP (3 μM)	GTPase activity (%)		
		Control		PT
0.01	$-$	a $\{$ 100	NS	101 $\pm$ 5 $\}$ a
0.01	$+$	142 $\pm$ 6	a	117 $\pm$ 9 $\}$
0.1	$-$	a $\{$ 100	NS	101 $\pm$ 3 $\}$ a
0.1	$+$	129 $\pm$ 4	a	108 $\pm$ 3 $\}$
1.0	$-$	a $\{$ 100	NS	100 $\pm$ 5 $\}$ a
1.0	$+$	117 $\pm$ 4	a	107 $\pm$ 1 $\}$

D. discoideum cells were starved for 5 h in the absence or presence of 0.1 μg/ml pertussis toxin, washed and used for membrane preparation. GTP hydrolysis by high-affinity GTPase was determined in membranes of control and pertussis toxin-treated cells in the absence ($-$) and presence of 3 μM cAMP ($+$). Three concentrations of GTP, 0.01, 0.1 and 1.0 μM, were used. The results are means of three independent experiments normalized in each experiment to basal GTPase values (100%) in the control membranes. NS, the difference is not significant at $p > 0.5$; [a], the differences are significant at $p < 0.01$

coupled to the cAMP receptor. Alternatively, pertussis toxin was not used at a saturated concentration (see section 4).

4. DISCUSSION

In vertebrates GTPase activity associated with guanine nucleotide-binding protein leads to inactivation of the hormone-stimulated effector enzyme by hydrolysis of G-protein-bound GTP to GDP and P_i [15,18–21].

In *D. discoideum* the existence of G-protein(s) has been suggested [23–31] but the stimulation of GTPase activity by receptor agonist was not observed. In this report we show the presence of high-affinity and low-affinity GTPase in *D. discoideum* membranes. Significant cAMP stimulation of a high-affinity GTPase could be detected only at low concentrations of GTP and by maximal suppression of the non-specific nucleoside triphosphatase activity. The specificity of GTPase stimulation by cAMP derivatives strongly supports the conclusion that cAMP interaction with a specific surface receptor leads to stimulation of a GTPase enzyme.

The present results, which characterise GTPase in *D. discoideum* membranes, are at least in part different from what has been observed in vertebrate cell membranes. The K_m of the high-affinity GTPase in other cell membranes is in the range $0.2–0.6 \mu M$ GTP [18,19], while in *D. discoideum* it is 10–20-fold higher (6.5 μM). GTP inhibits cAMP binding to *D. discoideum* membranes also at 2 μM, which is also 10-fold higher than in vertebrates [27,37–39]. In vertebrate membranes hormonal agents increase the V_{max} value of the enzyme without a major change in its substrate affinity [18,19], while in *D. discoideum* cAMP apparently stimulates the enzyme by increasing the affinity of the GTPase for its substrate GTP. This difference could be related to the long evolutionary distance between *D. discoideum* and vertebrate cells, and could be useful to elucidate the model of action of G-proteins.

In vertebrates pertussis toxin catalyses the ADP-ribosylation of a specific G_i and blocks the inhibition of adenylate cyclase by GTP [15] and stimulation of GTPase by agonist [15,19]. In *D. discoideum* we have previously observed that inhibition of adenylate cyclase by GTPγS was absent

in membranes derived from pertussis toxin-treated cells [31]. The present observation that pertussis toxin treatment in vivo reduced stimulation of a high-affinity GTPase by cAMP supports our hypothesis that *D. discoideum* membranes contain G_i-like activity. The cAMP-stimulated effect was not completely lost after pertussis toxin treatment, suggesting that other G-proteins could be involved or that pertussis toxin was not used at a saturated concentration. The latter possibility seems unlikely since we have shown that treatment of cells with 100 ng/ml pertussis toxin completely abolished GTP inhibition of adenylate cyclase [31]. This raises the question about the nature and function of the other GTP-hydrolyzing protein which is affected by the cAMP receptor but not by pertussis toxin. It is possible that this GTP-hydrolyzing protein is involved in cAMP stimulation of adenylate cyclase [31] or phosphoinositide metabolism [40].

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