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Pertussis toxin inhibits cAMP surface receptor-stimulated binding of [^{35}S]GTPγS to *Dictyostelium discoideum* membranes

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GTP-binding activity to *Dictyostelium discoideum* membranes was investigated using various guanine nucleotides. Rank order of binding activities was: GTPγS > GTP > 8-N_3-GTP; the binding of GTPγS and GTP, but not of 8-N_3-GTP, was stimulated by receptor agonists. [^{3}H]GTP binding to *D. discoideum* membranes has been described previously by a single binding type (K_d = 2.6 μM, B_{max} = 85 nM). More detailed studies with [^{35}S]GTPγS showed heterogeneous binding composed of two forms of binding sites with respectively high (K_d = 0.2 μM) and low (K_d = 6.3 μM) affinity. cAMP derivatives enhanced GTPγS binding by increasing the affinity and the number of the high-affinity sites, while the low-affinity sites were not affected by cAMP. The specificity of cAMP derivatives for stimulation of GTPγS binding showed a close correlation with the specificity for binding to the cell surface cAMP receptor. Pretreatment of *D. discoideum* cells with pertussis toxin did not affect basal GTP and GTPγS binding, but eliminated the cAMP stimulation of GTP and GTPγS binding. These results indicate that *D. discoideum* cells have a pertussis toxin-sensitive GTP-binding protein that interacts with the surface cAMP receptor, suggesting the functional interaction of surface receptor with a G-protein in *D. discoideum*.

GTP binding; cyclic AMP receptor; Pertussis toxin; G-protein

1. INTRODUCTION

In *Dictyostelium discoideum* cAMP functions as a hormone-like signal during chemotaxis [1], morphogenesis [2] and cell differentiation [3]. Extracellular cAMP binds to specific surface receptors [4], and induces many biochemical and biophysical responses, including alteration of the extracellular concentration of H^+, Ca^{2+} and K^+, the activation of adenylate cyclase, guanylate cyclase (reviews [5,6]), phospholipase C [7], and phosphorylation of cAMP receptors [8], and myosin heavy and light chains [9].

In vertebrates effector molecules are coupled to the surface receptors via signal transducing G-proteins [10]. A family of GTP-binding proteins in vertebrates mediate the regulation of adenylate cyclase, phospholipase C, cGMP phosphodiesterase, and ion channels [10–12]. G-proteins are heterotrimeric proteins with an αβ-subunit structure. The α-subunits (45–39 kDa) contain a single high-affinity guanine nucleotide-binding site and often a site for NAD-dependent ADP-ribosylation catalyzed by cholera or pertussis toxin [13].

Several investigations in *D. discoideum* suggest that the surface cAMP receptor is coupled to an intracellular effector via G-proteins. It has been shown that cAMP binding to membranes is altered by guanine nucleotides [14], and alternatively cAMP increases [^{3}H]GTP binding to isolated membranes, at the same time accelerating the dissociation rate of GTP [15]. In addition, cAMP

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Abbreviations: 8-N_3-GTP, 8-azidoguanosine 5′-triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); (Sp)-cAMPS, adenosine 3′,5′-monophosphorothioate, Sp-isomer; 8-Br-cAMP, 8-bromoadenosine 3′,5′-monophosphate; dcAMP, 2′-deoxyadenosine 3′,5′-monophosphate; cIMP, inosine 3′,5′-monophosphate; 5′-AMP, adenosine 5′-monophosphate; Ado, adenosine; cGMP, guanosine 3′,5′-monophosphate; DTT, dithiothreitol

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stimulates high-affinity GTPase activity in *D. discoideum* membranes [16]. Furthermore, in *D. discoideum* adenylate cyclase is stimulated [17] or inhibited [18] by GTP, depending on the conditions used. Treatment of cells with pertussis toxin affects the signal-transduction pathway from cAMP receptors to adenylate cyclase in vitro [18] and in vivo [19]. Inhibition of adenylate cyclase by GTP is blocked by pretreatment of cells with pertussis toxin. Moreover, desensitization to persistent cAMP stimulus is blocked after pertussis toxin treatment in vivo [19]. Recently it has been shown that GTP stimulates IP_3 formation in permeabilized cells [7].

Here, the interaction between surface cAMP receptor and putative G-protein was investigated in more detail by analysis of the binding of GTP and GTP analogs to *D. discoideum* membranes. The results show that receptor agonists stimulate GTP and GTPγS binding to native membranes, and that pertussis toxin alters the interaction between receptor and a putative G-protein.

2. MATERIALS AND METHODS

2.1. *Materials*

[8-^{3}H]GTP, sodium salt (10.6 Ci/mmol), was obtained from Amersham, [^{35}S]GTPγS (1355 Ci/mmol) was purchased from New England Nuclear, and 8-N_3-[α-^{32}P]GTP (6.8 Ci/mmol) was from ICN Radiochemicals. The cAMP derivatives, ATP, ATPγS and GTPγS were from Boehringer. DTT was obtained from Sigma. Pertussis toxin was purchased from List Biological Laboratories.

2.2. *Culture conditions*

D. discoideum NC4 (H) cells were grown [20], harvested in the late logarithmic phase with 10 mM KH_2PO_4/Na_2HPO_4, pH 6.5 (buffer A), at 5°C, washed, and starved on non-nutrient agar at a density of 1.5×10^6 cells/cm^2. After 4–5 h cells were collected by centrifugation, washed twice with buffer A, and the pellet was resuspended in buffer B (40 mM Hepes-NaOH, 0.5 mM EDTA, 250 mM sucrose, pH 7.7) to a density of 10^8 cells/ml. Homogenization was performed by pressing of the cell suspension through a Nucleopore filter (pore size 3 μm) at 0°C. The lysate was centrifuged at $10000 \times g$ for 5 min, the pellet was washed once with buffer B, and the final pellet was resuspended in buffer B to a density equivalent to 2×10^8 cells/ml.

2.3. *Pertussis toxin treatment*

Cells were starved in buffer A at a density of 10^7 cells/ml in the absence or presence of 0.1 μg/ml pertussis toxin. After 5 h, cells were washed three times in buffer A, and the final pellet was resuspended in buffer A, and used for GTPγS-binding assays.

2.4. *GTPγS-binding assay*

Binding of [^{35}S]GTPγS to membranes was measured in a total volume of 100 μl containing 0.1 nM [^{35}S]GTPγS, different concentrations of GTPγS, 10 mM $MgCl_2$, buffer A and 80 μl membranes. The stimulation of GTPγS binding by cAMP and cAMP derivatives was measured at binding equilibrium. DTT, an inhibitor of phosphodiesterase [21], was used to prevent degradation of cAMP derivatives during incubation with membranes. The endogenous production of cAMP after addition of 10 mM DTT was 1.63 nM; this concentration is too low to affect GTPγS binding.

Binding was measured after 30 min incubation at 0°C. Samples were centrifuged for 3 min at $10000 \times g$ at 2–4°C, and the supernatant was aspirated. The pellet was dissolved in 80 μl of 1 M acetic acid, 1.2 ml emulsifier (Packard) was added and radioactivity was determined. Blank values were determined in the presence of 0.1 mM unlabeled GTP and were subtracted from all data shown. Binding of [^{3}H]GTP and 8-N_3-[α-^{32}P]-GTP was performed at a final concentration of 0.1 μM.

3. RESULTS

D. discoideum membranes were incubated with the same amount of radioactive [^{3}H]GTP, [^{35}S]GTPγS and 8-N_3-[α-^{32}P]GTP in the presence or absence of 10 μM cAMP, and equilibrium binding was established (table 1). Nonspecific binding was essentially identical for the three ligands. Specific binding of [^{3}H]GTP was about 2% of the input radioactivity and stimulation about 22% by 10 μM cAMP. These data are similar to those presented previously. The binding of [^{35}S]GTPγS is about 5-fold higher than that of [^{3}H]GTP, and the stimulation of binding by cAMP (37%) is also higher than for [^{3}H]GTP binding. 8-N_3-[α-^{32}P]GTP has very low binding activity and cAMP induces only a slight stimulation of binding. This low binding affinity eliminates possible identification of the GTP-binding protein coupled to cAMP receptors using photoaffinity labelling with 8-N_3-GTP.

The binding of [^{35}S]GTPγS was characterized in more detail because this binding was most effectively stimulated by cAMP. According to the kinetics of association (fig.1) the [^{35}S]GTPγS binding reached an equilibrium after 30 min incubation with membranes at 0°C. Analysis of the association rate of GTPγS binding (inset) indicated fast (33%) and slow binding types (67%) with a half-life of about 0.35 and 5.3 min, respectively. The binding at equilibrium was enhanced by cAMP without an obvious change in the association kinetics.

Table 1

The GTP binding activity in *D. discoideum* membranes

	[^{3}H]GTP	Binding (% of input)	
		[^{35}S]GTPγS	8-N_3-[α-^{32}P]-GTP
Nonspecific binding	1.01 ± 0.09	1.01 ± 0.07	1.03 ± 0.10
Specific binding	2.08 ± 0.09	10.31 ± 0.28	0.29 ± 0.02
Specific binding + cAMP	2.54 ± 0.11	14.12 ± 0.38	0.30 ± 0.02
% stimulation	22.11 ± 7.48	37.95 ± 5.24	3.45 ± 9.92

Binding of guanosine triphosphates to *D. discoideum* membranes was investigated using [^{3}H]GTP, [^{35}S]GTPγS and 8-N_3-[α-^{32}P]GTP. The same specific activities and concentrations (20 nM) of ligands were used (input about 80000 cpm). Binding was measured after 30 min incubation in the presence or absence of 10 μM cAMP. Nonspecific binding was determined in the presence of 0.1 mM unlabeled GTP and was subtracted from total binding to obtain specific binding. Means ± SD of 3 experiments are presented

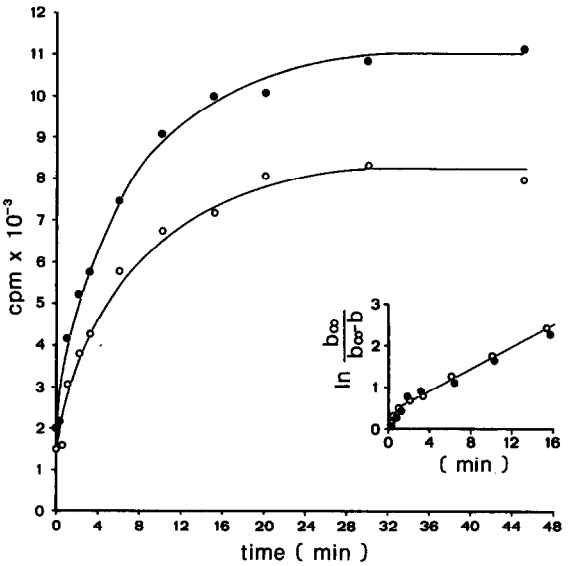

Fig.1. Association of 0.1 nM [^{35}S]GTPγS to membranes in the absence (○) or presence (●) of 10 μM (Sp)-cAMPS. (Inset) b_∞ equals the specific binding at equilibrium (30 min) (b at t min). Results are means of 3 experiments performed in triplicate.

The [^{35}S]GTPγS binding was also monitored at various ligand concentrations in the absence or presence of 10 μM (Sp)-cAMPS. The results were analysed by computer-assisted curve fitting (fig.2). Scatchard analysis suggests that the GTPγS binding activity in *D. discoideum* membranes is heterogeneous. Two components can be deduced with apparent K_d values of 0.22 ± 0.03 and 6.3 ± 1.15 μM, respectively. The high-affinity sites represented 7.4% of the total number of binding sites. The receptor agonist (Sp)-cAMPS did not affect the low-affinity sites, but altered significantly ($n = 4$, $P < 0.05$, t-test) the high-affinity sites as follows: the apparent K_d decreased from 0.22 ± 0.03 to 0.16 ± 0.02 μM and the concentration of binding sites increased from 31.45 ± 3.85 to 55.90 ± 1.96 nM.

The specificity of the cell surface cAMP receptors has been determined by measuring the inhibition of [^{3}H]cAMP binding by different concentrations of cAMP derivatives [22]. The effects of cAMP derivatives on [^{35}S]GTPγS binding are shown in fig.3. The specificity of cAMP derivatives for stimulation of GTPγS binding shows the following order cAMP > dcAMP (Sp)-cAMPS > 8-Br-cAMP > cIMP; cGMP and 5′-AMP are inactive. This order of cAMP derivatives is very similar to the order of binding to the cell surface cAMP receptor [22], but very dissimilar to binding to the regulatory subunit of cAMP-dependent protein kinase [23]. This suggests that the stimulation of GTPγS binding by cAMP is the consequence of interaction between cAMP receptor and GTPγS-binding protein.

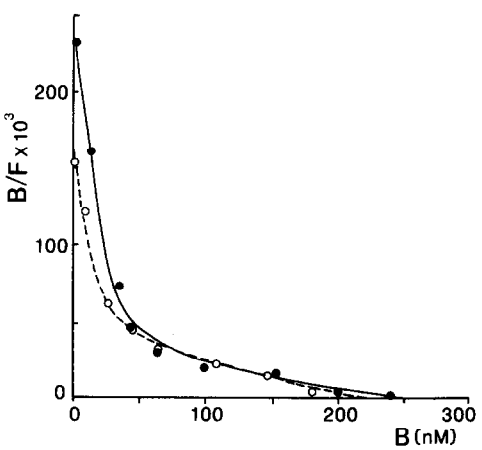

Fig.2. Scatchard plot of [^{35}S]GTPγS binding to membranes in the absence (○) or presence (●) of 10 μM (Sp)-cAMPS after incubation for 30 min; B, concentration of bound [^{35}S]GTPγS; F, free radioligand concentration. Binding of different [^{35}S]GTPγS concentrations (0.01, 0.1, 1, 2, 5, 10, 50, 100 μM) was measured.

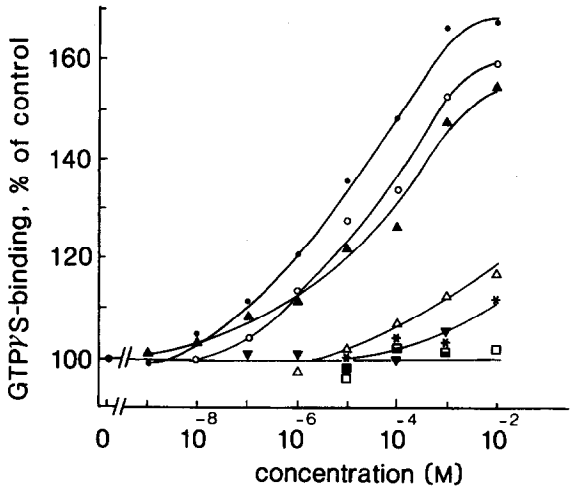

Fig.3. Specific modulation of GTPγS binding by cAMP derivatives was measured after 30 min incubation at 0°C. cAMP derivatives and 10 mM DTT were present during the incubation. Means of 3 experiments are presented. (●) cAMP, (○) dcAMP, (▲) (Sp)-cAMPS, (△) 8-Br-cAMP, (*) cIMP, (▼) cGMP, (■) Ado, (□) 5′-AMP.

cAMP-mediated stimulation of [^{3}H]GTP and [^{35}S]GTPγS binding in *D. discoideum* was also investigated in membranes derived from pertussis toxin treated cells (fig.4). Basal [^{3}H]GTP and [^{35}S]GTPγS binding was not affected. In contrast, enhancement of [^{3}H]GTP and [^{35}S]GTPγS binding by cAMP was no longer observed in membranes derived from toxin-treated cells, suggesting that

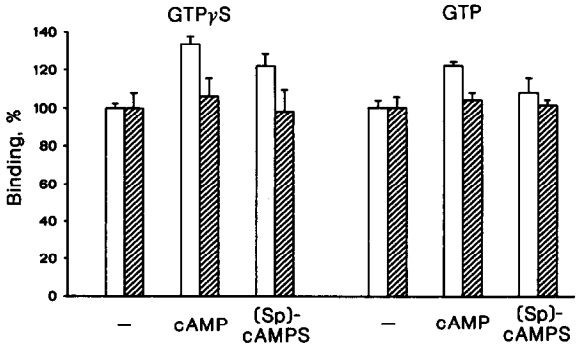

Fig.4. [^{35}S]GTPγS and [^{3}H]GTP binding to control membranes (open bars) and membranes derived from pertussis toxin-treated cells (shaded bars) in the absence or presence of 10 μM cAMP and 10 μM (Sp)-cAMPS. Means ± SD of 3 experiments are shown.

pertussis toxin inhibited the coupling of a GTP-binding protein to surface cAMP receptors.

4. DISCUSSION

In this report the functional interactions between cell surface cAMP receptors and GTP-binding protein(s) in *D. discoideum* native membranes and in membranes derived from pertussis toxin-treated cells are described. Previously, we have shown GTP binding activity on *D. discoideum* membranes with a single K_d = 2.6 μM and complex dissociation kinetics [15]. Furthermore, it was reported that chemoattractants increase the equilibrium binding of [^{3}H]GTP to membranes and accelerate its dissociation. The present results suggest that [^{35}S]GTPγS binding is heterogeneous with respectively high- (K_d = 0.2 μM) and low- (K_d = 6.3 μM) affinity binding sites. The addition of ligands enhances GTPγS binding due to an increase in affinity and the number of the high-affinity binding sites (fig.2). As judged from the activity of cAMP derivatives, the cell surface receptor is involved in modulation of GTPγS binding. However, the concentrations of cAMP derivatives required for stimulation of GTPγS binding are rather high. Furthermore, this stimulation shows non-Michaelian kinetics with respect to cAMP; the concentration-response curves for cAMP stimulation of GTPγS binding are very shallow. The latter may be expected, taking into account that GTP-binding protein may exist in several dynamic states of interaction with the cell surface receptors and that the ligand dissociation-association kinetics are strongly altered by cAMP and guanine nucleotides [14].

In vertebrates pertussis toxin catalyses the ADP-ribosylation of inhibitory (G_i) guanine nucleotide-binding regulatory protein and blocks the inhibition of adenylate cyclase by GTP [13] and stimulation of GTPase by the agonist [13]. In *D. discoideum* we have previously observed that inhibition of adenylate cyclase by GTPγS was absent in membranes derived from pertussis toxin-treated cells [18] and that pertussis toxin treatment partly reduced stimulation of a high-affinity GTPase by cAMP [16].

The present observation that stimulation of [^{3}H]GTP and [^{35}S]GTPγS binding by cAMP is blocked by pretreatment of cells with pertussis tox-

in suggests the functional interaction of surface cAMP receptors with pertussis toxin-sensitive G-protein(s) in *D. discoideum* membranes.

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