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FOLATE AND cAMP MODULATE GTP BINDING TO ISOLATED MEMBRANES OF *DICTYOSTELIUM DISCOIDEUM*. FUNCTIONAL COUPLING BETWEEN CELL SURFACE RECEPTORS AND G-PROTEINS

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In membrane preparations from *D.discoideum* cells GTP-binding activity is observed. The lack of GTP binding to intact cells suggests that the binding sites are localized inside the cell. The GTP-binding activity also remains in the particulate fraction in the presence of 1 mM Ca^{++}. This excludes membrane-associated microtubuli to be responsible for the observed GTP binding. Scatchard analysis suggests the existence of one type of binding site (K_d = 2.6 μM and 3.6 x 10^5 sites per cell). The kinetics of association as well as dissociation, however, suggest that GTP binding is more complex than binding to a single type of site. GDP and guanylyl imidodiphosphate are potent competitors of GTP binding (respectively 5- and 10-fold worse than GTP) while GMP, cGMP and several adenine nucleotides are ineffective up to 1 mM. The chemoattractants cAMP and folic acid both increase the equilibrium binding level of GTP, while dissociation of GTP is accelerated. These data indicate the functional coupling between cell surface receptors and G-proteins. © 1985 Academic Press, Inc.

The cellular slime mold *D.discoideum* provides a good model system for studying transmembrane signal transduction. Folic acid and cAMP have been identified as extracellular hormone-like signalling agents (1-3). These compounds are detected by cell surface receptors (4-8) and induce several intracellular responses, such as rapid transient activation of guanylate cyclase and adenylate cyclase (9,10). In addition, chemotaxis is induced and repeated pulses of the signal compounds accelerate differentiation (2, 11). Cell surface binding of cAMP as well as folic acid is heterogeneous and ligand-induced receptor conversions occur (12-14). Similar properties have been reported for the action of hormones and neurotransmitters in vertebrates. In those systems binding heterogeneity and interconversion of binding sites has been shown to reflect distal steps in the signal trans-

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duction pathway, i.e. interactions between receptors and G-proteins (15-21). Also in *D.discoideum* the presence of a G-protein has been proposed based on the cholera toxin-induced ADP-ribosylation of a 42 kD protein (22). These data were substantiated further by the effects of guanine nucleotides on the cell surface receptors for cAMP and folic acid (23,24). Thus, a functional interaction between receptors and G-proteins has been demonstrated. However, still no evidence is available for such a coupling between G-protein and adenylate cyclase, since the *D.discoideum* adenylate cyclase is not activated in vitro by NaF, GTP or forskolin (25-27).

In this report the attention has been focussed on the putative G-protein and its interaction with cell surface receptors. It has been shown that ligands for the receptors modulate GTP binding to membranes from *D.discoideum*.

Materials and Methods

Culture conditions. *D.discoideum* cells NC4(H) were grown as described (12), harvested in the late logarithmic phase with 10 mM sodium/potassium phosphate buffer pH 6.5 (Pb buffer), and freed from bacteria by centrifugation at 100 x g for 4 min. Cells were starved on non-nutrient agar at a density of 1.5×10^6 cells/cm^2 for 4-5 h, harvested, washed, and suspended in Pb buffer at a density of 2×10^8 cells/ml and the suspension was aereted at 20°C for 10 min. Homogenization was performed by rapid elution of the cell suspension through a Nucleopore filter (pore size 3 µm) at 0°C. The filter was washed with 2 ml buffer, and the lysate was centrifuged at 10,000 x g for 2 min at 0°C, and resuspended at a density equivalent to 10^8 cells/ml. This membrane preparation was kept in ice during the experiments which were completed within 1 h after membrane preparation.

Materials. [8-^{3}H]GTP, sodium salt (10.6 Ci/mmol) was obtained from Amersham, folic acid from BDH Biochemicals. All nucleotides were purchased from Sigma, except ADP, which was from Boehringer. Silicon oil AR-20 and AR-200 were from Wacker Chemie (München, F.R.G.). Cellulose PEI-F flexible sheets for thin layer chromatography were purchased from Baker.

Binding assay. Binding of [^{3}H]GTP to membranes was measured at 0°C in an incubation volume of 150 µl containing 100 nM [^{3}H]GTP, 1 mM ATP, 10 mM $MgCl_2$ and 100 µl membranes. The final density of membranes in 150 µl incubation mixture was equivalent to 6.7×10^7 cells/ml. Bound [^{3}H]GTP was separated from free [^{3}H]GTP by centrifugation of the incubation mixture through silicon oil at 10,000 x g for 30 s (13). Blank values were determined in the presence of 0.1 mM unlabeled GTP.

Results

The degradation of 100 nM [^{3}H]GTP during incubation with the membranes of *D.discoideum* is shown in Fig.1. Obviously, GTP was hydrolyzed

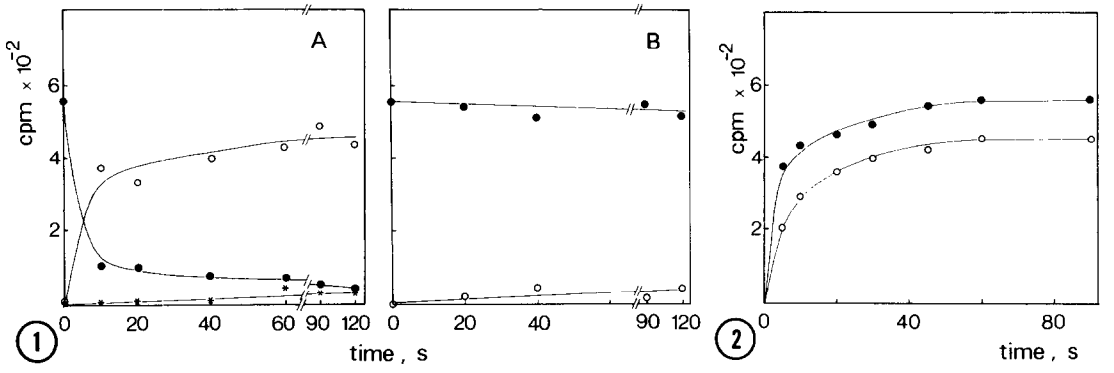

Fig. 1. Degradation of 100 µM [^{3}H]GTP during incubation with membranes in the absence (A) or presence (B) of 1 mM ATP.
(●) GTP; (o) GDP; (*) GMP.
Incubation was terminated by adding 600 µl methanol to the 150 µl samples and boiling them for 10 min. After centrifugation (8000 x g; 2 min) 10 µl of each sample was analyzed by TLC on cellulose PEI-F. Mobile phase: 1.5 M LiCl.

Fig. 2. Association of 100 nM [^{3}H]GTP to membranes in the absence (o) or presence (●) of 10 µM cAMP.

rapidly to GDP, which was only slowly converted to GMP. The addition of 1 mM ATP during incubation prevented GTP degradation to a large extent (Fig.1B). During 2 min less than 10% of GTP was hydrolyzed.

The kinetics of association of 100 nM [^{3}H]GTP are presented in Fig.2. Binding equilibrium was reached within 60 s. When the radioactive material which was bound to the membranes at 60 s was analyzed by TLC, 75% of the associated radioactivity comigrated with GTP and 25% with GDP. Equilibrium binding of [^{3}H]GTP was not affected by 1 mM $CaCl_2$. Intact cells, at a density equivalent to that of the membranes, did not bind detectable amounts of [^{3}H]GTP. The kinetics of dissociation of [^{3}H]GTP are shown in Fig.3. Obviously, the results suggest a complex process, since a semi-logarithmic plot (inset) is non-linear. The chemoattractant cAMP affects equilibrium [^{3}H]GTP binding as well as the rate of [^{3}H]GTP dissociation (Figs.2 and 3). The [^{3}H]GTP binding level is enhanced about 27% without an obvious change in the association kinetics, while dissociation is accelerated by 10 µM cAMP. Also the chemoattractant folic acid has these effects on GTP binding (Table 1). 5'AMP and cGMP, however, were ineffective at 10 µM.

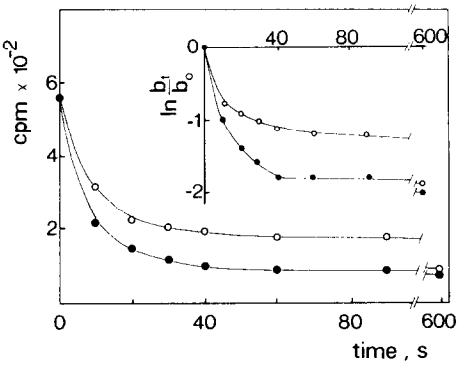

Fig. 3. Dissociation of 100 nM [^{3}H]GTP from membranes. Membranes were pre-incubated 90 s with 100 nM [^{3}H]GTP. Then at t = 0 rapidly mixed with 0.1 mM GTP (o), or 0.1 mM GTP and 10 µM cAMP (•). The cell-associated radioactivity was determined at the indicated times (b_t). Inset: semi-logarithmic plot of the data from Fig.3; b_o stands for the binding level before dissociation.

Figure 4 shows a Scatchard plot of the equilibrium GTP binding to isolated membranes. Apparently, [^{3}H]GTP binding may be described by a single binding type with K_d = 2.6 µM and a concentration of binding sites of 85 nM. This value corresponds to 3.6 x 10^5 GTP-binding sites per cell.

The nucleotide specifity of the observed binding sites is presented in Fig.5. GDP and guanylyl imidodiphosphate inhibited 100 nM [^{3}H]GTP binding for 50% at 5 µM and 10 µM, respectively. Thus, these compounds are slight-

Table 1. Effects of chemoattractants and related compounds on GTP binding

Compound	equilibrium binding, % of control	binding after 60 s chase, % of control
control	100	100
10 µM cAMP	127 ± 8	60 ± 8
10 µM 5'AMP	n.d.	98 ± 10
10 µM cGMP	n.d.	95 ± 11
10 µM folic acid	120 ± 6	70 ± 4

Data are presented as means from 15 experiments ± SEM.
Control equilibrium binding levels (60 s incubation) were 553 ± 30 cpm and after 60 s chase 166 ± 18 cpm.
[^{3}H]GTP concentration was 100 nM. The chase experiments were performed as described for Fig.3; cAMP, 5'AMP, cGMP or folic acid were only present during the chase.

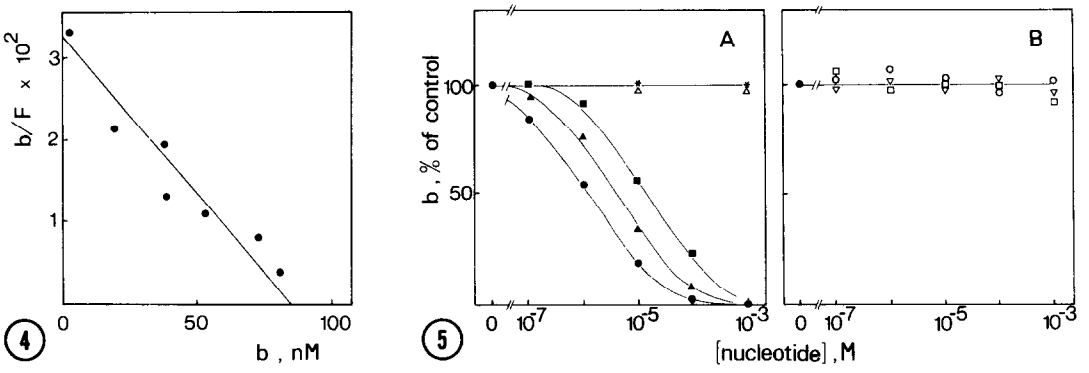

Fig. 4. Scatchard plot of [^{3}H]GTP binding to membranes after an incubation period of 60 s; b is the concentration of bound [^{3}H]GTP, F is the free radioligand concentration.

Fig. 5. Competition of nucleotides for 100 nM [^{3}H]GTP binding to membranes after an incubation period of 60 s.
A) (●) GTP; (▲) GDP; (■) guanylyl imidodiphosphate; (△) GMP; (*) cGMP.
B) (o) Adenosine; (▽) AMP; (□) ADP.

ly worse ligands than GTP (about 5-fold for GDP and 10-fold for guanylyl imidodiphosphate). GMP, cGMP and adenine nucleotides were ineffective up to 1 mM, i.e. more than 10,000-fold worse than GTP. Also ATP, which was usually present during incubation at a concentration of 1 mM, should possess a low affinity for the GTP-binding sites.

Discussion

In the present report the existence of membrane-bound GTP-binding sites in *D.discoideum* has been demonstrated. These sites specifically bind guanine nucleotides with an affinity in the micromolar range. Furthermore, it has been shown that chemoattractants (but not chemotactically inactive analogs) modulate the binding of GTP to these sites. Therefore, it is proposed that the observed binding activity corresponds to a membrane-bound G-protein, which interacts with cell surface receptors for cAMP and folic acid.

Another GTP-binding protein, which may be present in the particulate fraction of cell lysates, is tubulin. However, it is known that in the presence of Ca^{++} microtubuli dissociate into their subunits (28). As a

consequence of our assay method (sedimentation through oil) GTP binding measured in the presence of Ca^{++} cannot be caused by tubulin.

The GTP-ase activity of our membrane preparations was rather high. Fortunately, GTP degradation could be prevented by the addition of excess ATP. ATP apparently competes with GTP for the phosphatase activity, but not for the particulate GTP-binding sites. Thus, [^{3}H]GTP was stable long enough to achieve binding equilibrium.

The observed GTP-binding sites show a ligand specificity, which is remarkably similar to the specifity of the guanine nucleotide effects on the binding of cAMP and folic acid to their cell surface receptors (23,24). The number of G-proteins per cell (3.6×10^5) is higher than the sum of the cell surface receptors for cAMP and those for folic acid (both approx. 1×10^5). Thus, for each receptor-molecule at least one G-protein-molecule should be available.

Our kinetic data suggest that the GTP binding is of a complex nature. Neither association nor dissociation are first-order processes. This is also expected, taking into account that a G-protein may exist in several states of interaction with cell surface receptors and possibly adenylate cyclase, and that this G-protein may also hydrolyze a bound GTP molecule. Due to this complexity it should be premature to analyze the kinetic data in detail. However, it may be concluded that about 14% of the bound GTP is apparently non-dissociable (within 10 min). cAMP only affects the dissociation rate of the dissociable 86%. Without cAMP the GTP release seems biphasic with half-times of approx. 6 s and 2.5 min, while in the presence of 10 µM cAMP only one half-time component of 6 s is observed.

Having established that a functional coupling between cell surface receptors and G-proteins occurs, it is the in vitro activation of adenylate and guanylate cyclase that awaits further examination in *D.discoideum*.

Acknowledgements

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