Guanine nucleotides modulate the ligand binding properties of cell surface folate receptors in *Dictyostelium discoideum*

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Dictyostelium discoideum cells show 2 distinct classes of cell surface binding sites for folates. One type is non-specific, i.e., binds folic acid (FA), 2-deaminofolic acid (DAFA), and methotrexate (MTX) with similar affinity ($K_{0.5} \simeq 140$ nM). Scatchard analysis of this non-specific binding type suggests either heterogeneity or negative cooperativity. Isolated *D. discoideum* membranes show similar binding characteristics. Guanine nucleotides changed the binding levels of [^{3}H]MTX. In the presence of 0.1 mM GTP, the number of binding sites remains unchanged, while the affinity decreases. GDP and guanylyl imidodiphosphate (GPPNP) are required at about 20-fold higher concentration than GTP, which elicits a half-maximal effect at 15 μM. Other guanine and adenine nucleotides are ineffective up to 1 mM. These results suggests that the non-specific cell surface receptor for folic acid interacts with a guanine nucleotide regulatory (G-) protein.

Folate receptor *Guanine nucleotide* *G-protein* Dictyostelium discoideum

1. INTRODUCTION

Intercellular communication in vertebrates as well as in lower organisms is receiving increasing attention. Well studied components of such communication systems are signal molecules (hormones), receptors, trans-membrane processing of signals, production of second messengers and adaptation (desensitization).

D. discoideum provides an accessible model system for studying cell communication. cAMP and FA have been identified as extracellular signalling agents [1,2], and for both compounds specific cell surface receptors have been detected [3–8]. The cAMP receptor is apparently subjected to rapid ligand-induced conversions [9]. Both signalling molecules elicit cAMP and cGMP synthesis [10–12] and, in the presence of a constant stimulus, cells become rapidly insensitive [13,14]. In addition, cAMP and FA mediate chemotaxis [15] and differentiation [16–18].

Many characteristics of the signal transduction in *D. discoideum* are closely related to those observed for the action of hormones or neurotransmitters in vertebrates. However, in contrast to vertebrate cells, trans-membrane processing of a signal has not been studied in the slime mold. In higher organisms a G-protein seems to be a universal component of the signal transductionpathway across the plasma membrane [19]. Also in *D. discoideum* membranes, the presence of a protein similar to the vertebrate G-protein has been shown [20]. However, a functional interaction between this G-protein and a cell surface receptor has not been demonstrated.

Here, we show that guanine nucleotides affect the binding of folates to cell surface receptors. Similar results have been reported for e.g. β-adrenergic receptors [21] and chemotactic peptide receptors [22]. Our data suggest that a G-protein is involved in the transduction of FA signals in *D. discoideum*.

2. EXPERIMENTAL

2.1. *Culture conditions*

D. discoideum NC 4 (H) was cultivated together with *E. coli* 281 on a solid medium containing 3.3

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g peptone, 3.3 g glucose, 4.5 g KH_2PO_4, 1.5 Na_2HPO_4. H_2O and 15 g agar per l. Cells were harvested after 40 h of growth at 21°C, just before clearing of the bacterial lawn occurred. The cells were washed 3 times in cold harvesting buffer (10 mM Na^+/K^+ phosphate buffer pH 6.5) by centrifugation for 4 min at $150 \times g$. The cell density was adjusted to 2×10^8 cells/ml and the suspension was aerated at 0°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 particulate fraction was washed once by centrifugation at $10000 \times g$ for 2 min at 0°C, and resuspended at a density equivalent to 10^8 cells/ml. This membrane preparation was kept on ice during the experiments (usually less than 1 h).

2.2. *Materials*

[7,3′,5′-^{3}H]MTX (10.7 Ci/mmol) was purchased from the radiochemical Centre (Buckinghamshire). FA was from BDH Biochemicals (Poole) and 8-azaguanine was from Fluka A.G. (Buchs). Silicon oil AR 20 and AR 200 were from Wacker Chemie (München). All nucleotides and MTX were purchased from Sigma Co. (St. Louis, MO). DAFA was synthesized by enzymatic deamination of FA and purified as in [23].

2.3. *Binding assay*

The final density of membranes in 150 μl incubation mixture was equivalent to 6.7×10^7 cells/ml. During the incubation at 0°C, the samples were layered on top of 180 μl silicon oil (AR 20:AR200 = 11:4) and 10 μl 10% sucrose. Incubation was terminated by centrifugation at $10000 \times g$ for 40 s in a swing-out rotor. Consequently the samples were frozen in liquid nitrogen and cut through the oil layer. The tips containing pelletted cells or membranes were mixed with 1.5 ml Scintillator 299 (Packard) and measured for radioactivity. Blank values were obtained in the presence of 20 μM unlabeled MTX.

3. RESULTS

The kinetics of association of 5 nM [^{3}H]MTX to intact cells are shown in fig.1. A semilogarithmic plot of these association data reveals a biphasic process (fig.1B). The faster component equilibrated within 3 s, while a slower component required about 60 s for equilibration (K_{app} = 0.1 s^{-1}). The binding of [^{3}H]MTX to isolated membranes reached equilibrium within 5 s, and also in the presence of 0.1 mM GTP only this fast component was observed. However, the binding level of [^{3}H]MTX in the presence of GTP was only 65% of the level obtained without GTP. As shown in fig.1, the inhibiting effect of GTP on [^{3}H]MTX binding was complete within 15 s. Semilogarithmic plotting of the GTP-induced decrease of binding yields an apparently first-order rate constant of 0.2 ± 0.1 s^{-1} (fig.1C). As a consequence of the relatively small drop in [^{3}H]MTX binding due to 0.1 mM GTP, the standard deviations in these data points are too large to obtain an accurate kinetic constant. Also, the effect of 1 mM GTP was examined, yielding a comparable rate constant of 0.2 ± 0.1 s^{-1}. Evidently, a difference exists between the equilibrium binding levels of [^{3}H]MTX after two reciprocal procedures; (1) preincubation with GTP followed by incubation with the radioligand, or (2) preincubation with [^{3}H]MTX followed by addition of GTP. The latter method yielded significantly higher binding levels of [^{3}H]MTX than the former method.

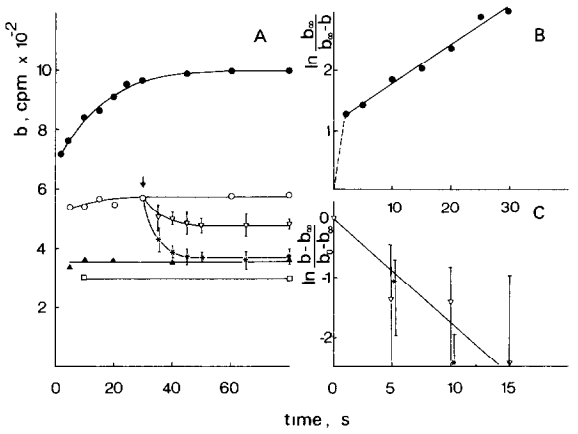

Fig.1. (A) Kinetics of association of 5 nM [^{3}H]MTX to intact cells (●), membranes (○), membranes after 60 s preincubation with 10^{-4} M GTP (▲) or with 10^{-3} M GTP (□). 10^{-4} M GTP (▽) or 10^{-3} M GTP (*) was added to the membrane preparation (arrow) after 30 s of incubation with 5 nM [^{3}H]MTX and the binding of [^{3}H]MTX was monitored for 50 s. (B,C) Semilogarithmic plots of the data from panel A. b_∞ represents the equilibrium binding level, b_0 is the binding level just before the addition of GTP.

Fig.2 presents Scatchard plots of the equilibrium

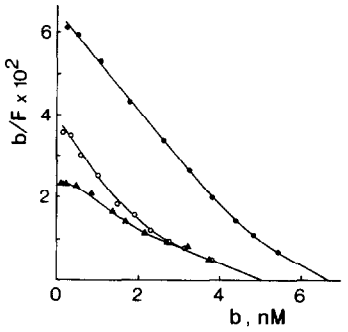

Fig.2. Scatchard plots of [³H]MTX binding to intact cells (●), to membranes (○), or to membranes after 60 s preincubation with 10^{-4} M GTP (▲). Incubation with [³H]MTX was for 60 s. A binding level of 5 nM corresponds to 88000 molecules bound per cell or cell equivalent. The final density of cells or membranes was equivalent to 6.7×10^7 cells per ml.

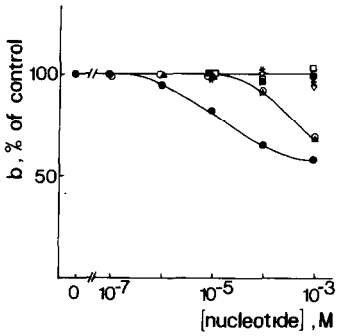

Fig.4. Dose-response curves for the effects of various nucleotides on the binding of [³H]MTX at 5 nM. The membranes were preincubated with the nucleotides for 60 s, and consequently incubated with the radioligand for 60 s. (●) GTP; (○) GPPNP; (▲) GDP; (▽) GMP; (□) ATP; (■) ADP; (*) cGMP.

binding of [³H]MTX to cells and isolated membranes. All curves are more or less concave upward, suggesting either binding site heterogeneity or negative cooperativity. The apparent affinity of cells ($K_{0.5}$ = 140 nM) was slightly higher than that of the membrane preparation ($K_{0.5}$ = 240 nM). In the presence of GTP, the binding at low radioligand concentrations was clearly diminished. In contrast, the number of the MTX-binding sites was not significantly affected by GTP. Comparison of the data for intact cells and isolated membranes in fig.2 shows that during the preparation of the membranes about 75% of the binding sites were retained.

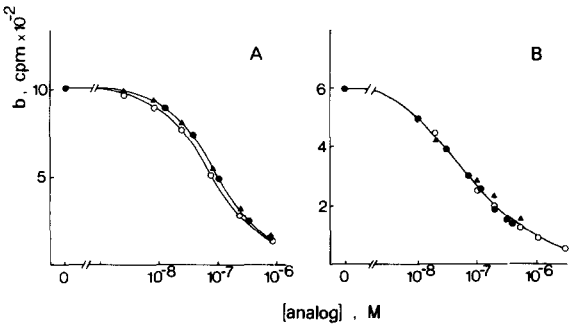

Fig.3. Incubation of [³H]MTX binding at 5 nM by unlabeled MTX (●), DAFA (○) or FA (▲) 0.33 mM 8-azaguanine was included in the samples containing FA in order to prevent enzymatic deamination of this compound. (A) Intact cells. (B) Isolated membranes.

The inhibition of [³H]MTX binding by FA, DAFA and MTX, as shown in fig.3, demonstrates that the ligand specificity of the bindding sites on isolated membranes resembles the specificity of the sites on intact cells. Half-maximal inhibition occurred at 80 nM for DAFA and at 100 nM for FA and MTX for intact cells, while for the isolated membranes 75 nM was obtained for DAFA, FA as well as MTX. Thus, the studied binding sites are non-specific with respect to FA, DAFA and MTX, which is clearly different from the 'specific' folate receptors as reported before [8].

Fig.4 shows the effects of several guanine and adenine nucleotides on the binding of [³H]MTX. Decreased binding was observed in the presence of GTP, GPPNP and GDP. None of the other compounds at concentrations up to 1 mM affected [³H]MTX binding. GTP showed a half-maximal effect at 15 μM. GPPNP and GDP did not reach a plateau up to 1 mM, but assuming that the maximal effects of GDP and GPPNP are similar to that of GTP, half-maximal inhibition of [³H]MTX binding is effected by 300 μM GPPNP or GDP.

4. DISCUSSION

Folates bind to 2 different populations of receptors on the cell surface of *D. discoideum* [8]. These 2 classes of folate binding sites are distinguishable by their ligand specifity. The 'folate-specific' type shows a more than 100-fold higher affinity for FA

than for DAFA, while the 'non-specific' type binds these ligands with identical affinity.

As reported here, the 'non-specific' receptor also binds MTX with an affinity similar to that of FA and DAFA. Since [^{3}H]FA binds to the 'non-specific' as well as the 'specific' receptor, this is an unsuitable ligand for studying one of these binding types specifically. However, [^{3}H]MTX showed a high affinity for the 'non-specific' sites, while binding to the 'specific' receptors could not be detected. This was derived from the observation that low concentrations of DAFA could effectively inhibit [^{3}H]MTX binding.

Scatchard analysis of [^{3}H]MTX binding to intact cells as well as to isolated membranes resulted in concave upward curves, suggesting either heterogeneity or negative cooperativity. GTP decreased the apparent affinity of the binding sites, while the number of sites was unchanged. Hence, a high-affinity state of the receptor may be converted to a low-affinity state by GTP. Also GPPNP and GDP modulated the proportioning of the high- and low-affinity states, though less effectively than GTP. Assuming that [^{3}H]MTX binding may occur to 2 binding types with different affinity, the number of both binding sites and their affinity may be estimated from the experimental data. Table 1 shows the results for intact cells, membranes without GTP and membranes in the presence of GTP. Apparently the K_d value for the low-affinity receptor state is 340–400 nM, while for the high-affinity site this value is 47–60 nM. For intact cells the highest amount of high-affinity sites was derived (54%); for membranes this value was lower (30%). GTP at 0.1 mM reduced the fraction of high-affinity sites to 18%.

Though binding to both types of sites was observed with cells as well as with membranes, the kinetics of association of [^{3}H]MTX were clearly different for these preparations. With cells about 30% of the radioligand associated slowly (K_{app} = 0.1 s^{-1}), while with membranes no significant contribution by this slow process was observed. It is therefore unlikely that this slow process was caused by association to either the high- or the low-affinity binding type. Moreover, this process may reflect a ligand-induced conversion of low-affinity sites into high-affinity sites on intact cells. This process may not occur in isolated membranes.

The equilibrium level of [^{3}H]MTX binding to membranes in the presence of GTP was dependent of the sequence of addition of [^{3}H]MTX and GTP. Preincubation with GTP resulted in a lower level than observed after preincubation with [^{3}H]MTX. These data suggest that the effect of GTP is stronger with unoccupied receptors than with MTX-occupied receptors. Possibly, MTX-occupied receptors are less easily converted to the low-affinity state by GTP. In addition, once a receptor is converted to the low-affinity state, MTX cannot reverse this transformation within 60 s.

The cell surface cAMP receptor of *D. discoideum* shows properties remarkably similar to those of the folate receptor, as described here. At least 3 binding types are detected, of which 2 types interconvert upon addition of ligand [9]. Furthermore, guanine nucleotides affect the proportionality of these 2 binding types (P.J.M. Van Haastert and P.M.W. Janssens, unpublished). In other organisms, this specific action of certain guanine nucleotides (GTP, GPPNP and GDP) on ligand-receptor binding has led to the hypothesis that a G-protein

Table 1

Proportionality and dissociation constants of the high- and low-affinity binding sites

	High-affinity site		Low-affinity site	
	%	K_d (nM)	%	K_d (nM)
Cells	54	60	46	340
Membranes	30	47	70	400
Membranes preincubated with 10^{-4} M GTP	18	60	82	360

These data were derived from the Scatchard plots in fig.2 by hyperbola curve-fitting

mediates signal transduction across the plasma membrane [19,21]. Previously, in *D. discoideum* a membrane-bound protein was described showing properties, which are characteristic for vertebrate G-proteins [20]. The data presented here, show that a G-protein may be functionally linked to the 'non-specific' folate receptors.

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