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Glucose and Cyclic Adenosine Monophosphate Stimulate Activities of Adenylate Cyclase and Guanylate Cyclase of *Tetrahymena Pyriformis* Infusoria

A. O. Shpakov, K. V. Derkach, and Z. I. Uspenskaya*

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The sensitivities of cyclase enzymes adenylate cyclase and guanylate cyclase to glucose and extracellular cAMP were studied in *Tetrahymena pyriformis* infusoria. Glucose effectively stimulated activities of both cyclase enzymes, while cAMP more effectively stimulated adenylate cyclase. It was shown that [6-^{14}C]glucose specifically bound to *Tetrahymena pyriformis* infusoria at dissociation constant (K_D) and number of binding sites (B_{max}) 43 nM and 7.53 fmol glucose per 100,000 cells and [8-^{3}H]cAMP bound at 19 nM and 4.46 fmol cAMP per 100,000 cells, respectively. Hence, glucose and cAMP specifically bound to *Tetrahymena pyriformis* cells and stimulated activities of cyclases in these infusoria.

Key Words: *adenylate cyclase; glucose; guanylate cyclase; infusoria; cyclic adenosine monophosphate*

Enzymes with cyclase activities, adenylate cyclase (AC) and guanylate cyclase (GC), play the key role in the regulation of vital processes in unicellular organisms [2,14]. They catalyze the synthesis of second messengers cAMP and cGMP, through which they regulate activities of cyclic nucleotide-dependent effector systems of the cell responsible for gene expression, growth and metabolic processes, food-getting behavior, mobility, and chemotaxis. Cyclase activities in unicellular organisms is regulated by a wide spectrum of extracellular signals including hormones and growth factors, related to those of the higher eukaryotes, and the natural compounds – sugars, nucleotides, amino acids, fatty acids, terpenoids, alkaloids. Glucose (an essential energy substrate) specifically binds to receptors located in the plasma membrane and coupled with heterotrimeric G-proteins and stimulates activities of membrane-bound AC forms in the yeast fungi [8,10]. Other fungi also have serpentine receptors homologous to glucose receptors in yeast-like fungi. Through these receptors glucose regulates activities of intracellular effector proteins [13]. Cyclic AMP, a second messenger in the majority of organisms, functions as the first messenger (hormone) in *Dictyostelium discoideum* ameba, *Neurospora crassa* fungus, and *Dileptus anser* infusoria [3,7,9,11]. It specifically binds to serpentine receptors and regulates (through heterotrimeric G-proteins) cyclase enzymes.

We previously found that AC of *Tetrahymena pyriformis* infusoria were regulated by some vertebrate hormones [1,4,5]. However, there are no data on the regulation of AC and GC by natural substances and

Laboratory of Molecular Endocrinology, I. M. Sechenov Institute of Evolutional Physiology and Biochemistry, Russian Academy of Sciences; *Laboratory of Cytology of the Unicellular, Institute of Cytology, the Russian Academy of Sciences, St. Petersburg, Russia. ***Address for correspondence:*** alex_shpakov@list.ru. A. O. Shpakov

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metabolites with which infusoria permanently contact throughout their vital activity and which modulate their food-getting behavior and mobility [12]. We studied the sensitivity of cyclases to glucose and extracellular cAMP in *Tetrahymena pyriformis* infusoria and characterized the binding sites for these substances on infusorian cell surface. Importantly, we know only two signal molecules regulating GC activity in the unicellular organisms: cAMP and folic acid stimulating soluble and receptor GC in *D. discoideum* ameba [11,15].

MATERIALS AND METHODS

The study was carried out on cultures of *T. pyriformis* infusoria, 150-300 thousand cells/ml. The following reagents were used: creatine phosphate, creatine phosphokinase from rabbit muscles (EC 2.7.3.2), ATP, GTP, cGMP, β,γ-imidoguanosine-5'-triphosphate (GppNHp; Sigma), other reagents from Sigma and Fluka. Radioisotope experiments were carried out using D-[6-^{14}C]glucose (45 Ci/mmol) and [8-^{3}H]cAMP (30 Ci/mmol; Amersham Biosci. Int.), and [α-^{32}P]ATP (1000 Ci/mmol) and [α-^{32}P]GTP (6000 Ci/mmol; Institute of Reactor Materials).

Enzyme activities were measured in homogenized infusoria. Infusorian cells were precipitated by centrifugation (600*g*, 3 min), washed 3 times in Tris-HCl buffer (pH 7.5), and homogenized by ultrasonic treatment on an USDN-2T device at 20 kHz for 1 min with cooling. The percent of destroyed cells was at least 95% of their total content.

Activity of AC (ATP-pyrophosphate liase cyclizing, EC 4.6.1.1) was measured at 30°C after 10-min incubation as described previously [4] in incubation medium of the following composition: 50 mM Tris-HCl (pH 7.5), 1 mM ATP, 20 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase, 5 mM $MgCl_2$, and [α-^{32}P]ATP (1 μCi per sample). The reaction was started by adding 50-100 μg protein. Activity of AC was expressed in pmol cAMP/min/mg protein.

Activity of GC (GTP-pyrophosphate liase cyclizing, EC 4.6.1.2) was measured by a previously described method [6] with some modifications. Homogenized infusorian cultures were incubated for 10 min at 30°C in a medium of the following composition: 50 mM Tris-HCl (pH 7.5), 1 mM GTP, 0.1 mM cGMP, 20 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase, 5 mM $MgCl_2$, [α-^{32}P]GTP (0.5-1 μCi per sample). The reaction was started by adding 50-100 μg protein and arrested by adding 100 μl 0.5 M HCl, after which the samples were plunged into boiling water for 6 min, and 100 μl 1.5 M imidasole was added to each sample. The samples were then layered onto a column with aluminum oxide. The resultant cGMP was eluted by 10 ml 10 mM imidasole-HCl-buffer (pH 7.4). Radioactivity in the eluate was measured on a Rackbeta counter (LKB). Activity of GC was expressed in pmol cGMP/min/mg protein.

Binding of D-[6-^{14}C]glucose and [8-^{3}H]cAMP was carried out in 20 mM Tris-HCl buffer (pH 7.5) with 10 mM dithiotreitol. The cells (100 μl) were incubated with [6-^{14}C]glucose or [8-^{3}H]cAMP (0.5-200 nM) for 30 min at 25°C and the incubation mixture was then transferred onto GF/B filters; the filters were washed 3 times with Tris-HCl buffer (5 ml each time), dried, put into vials with scintillation mixture, and radioactivity was measured on a Rackbeta counter. The specific binding of [6-^{14}C]glucose and [8-^{3}H]cAMP was calculated by subtraction of nonspecific binding measured in the presence of unlabeled ligands (10^{-3} M) from total [6-^{14}C]glucose and [8-^{3}H]cAMP binding. The dissociation constants (K_D) and number of binding sites (B_{max}) were calculated by Scatchard transformation of the saturation curves [13].

The data were statistically processed by ANOVA. Each experiment was repeated 3 times. The data were presented as *M*±*SEM* from several independent experiments. The differences between control samples and samples treated by glucose and cAMP were evaluated as significant at $p<0.05$.

RESULTS

Basal activity of AC in *T. pyriformis* cultures was 22.6±1.3 pmol cAMP/min/g protein. Enzyme activity increased in the presence of NaF (10 mM) and nonhydrolyzed GTP analog GppNHp (10^{-5} M) by 122 and 81%, respectively, which was in line with the previous data [1] and confirmed the *T. pyriformis* AC conjugation with the heterotrimeric G proteins, similarly as in higher eukaryotes. Basal activity of GC in *T. pyriformis* was 3.6±0.2 pmol cGMP/min/mg protein. It was stimulated by Mn^{2+} and Mg^{2+}, by manganese more effectively than magnesium: 10 mM Mn^{2+} stimulated *T. pyriformis* GC by 841%, while 10 mM Mg^{2+} by only 272% (enzyme activity without Mn^{2+} and Mg^{2+} was 1.2±0.2 pmol cGMP/min/mg protein). Hence, GC in infusoria, similarly as in vertebrates, is a Mg-dependent enzyme, effectively stimulated by Mn^{2+} in millimolar concentrations.

D-Glucose in concentrations of 10^{-5}-10^{-3} M clearly stimulated activities of both cyclases of *T. pyriformis*, its maximum stimulatory effects on AC and GC activities being close (Fig. 1). EC_{50} for the stimulatory effects of glucose on AC and GC were 16 and 9.3 μM, respectively. In the presence of suramine (selective blocker of heterotrimeric G-proteins) in a concentration of 10^{-5} M, the stimulatory effects of glucose (10^{-4} M) on AC and GC decreased by 42 and 29% of the

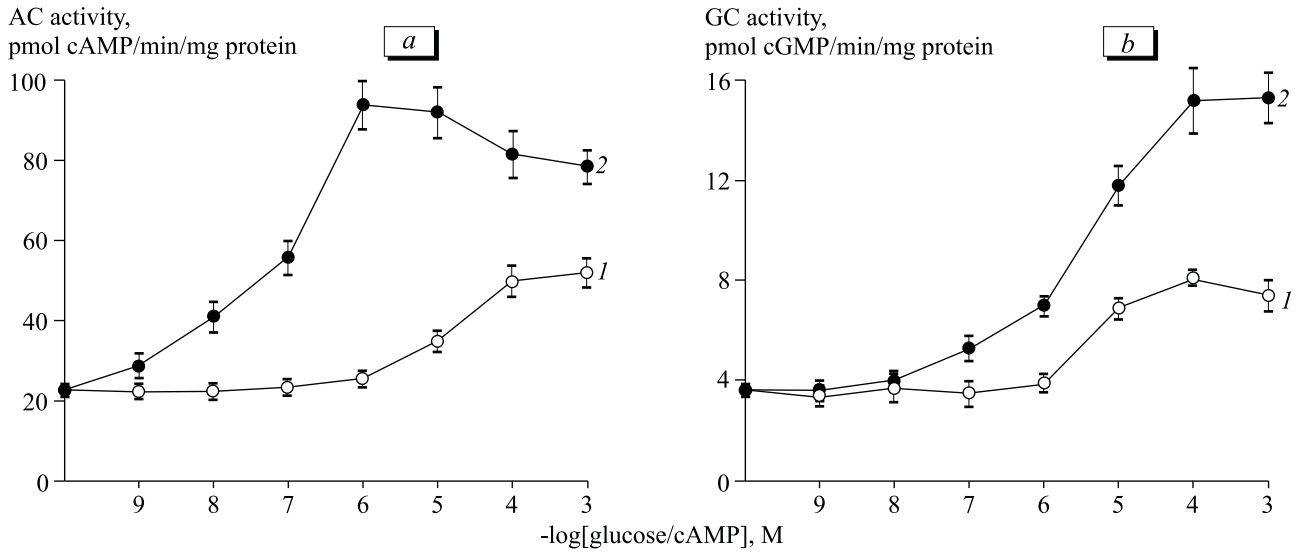

Fig. 1. Stimulatory effect of D-glucose (*1*) and cAMP (*2*) on activities of AC (*a*) and GC (*b*) in *T. pyriformis* infusoria cultures.

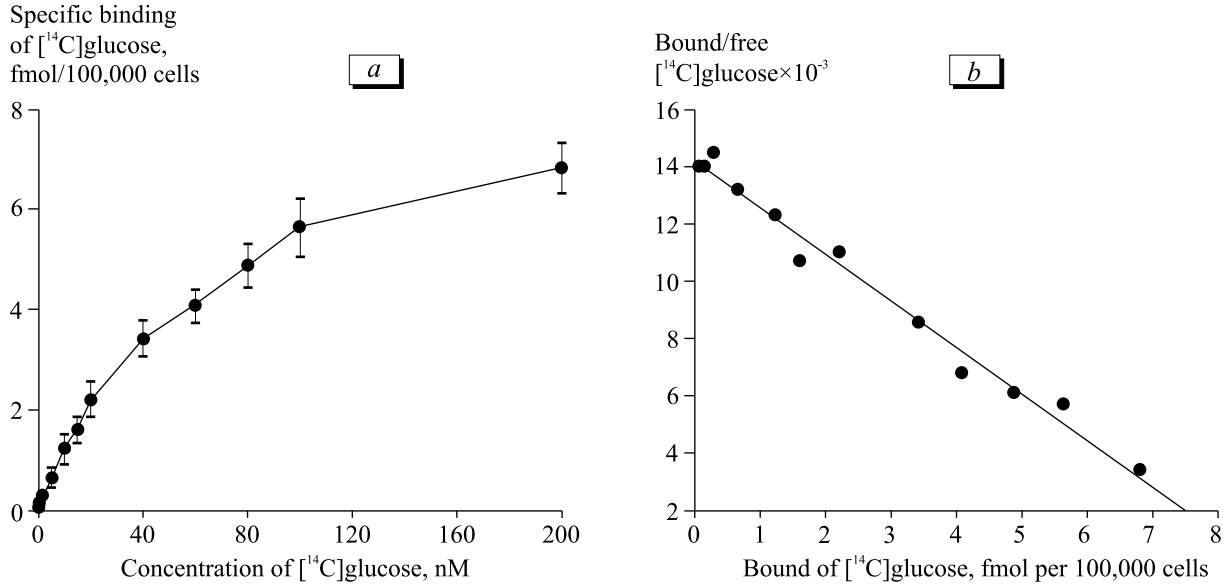

Fig. 2. Specific binding of D-[6-^{14}C]glucose to *T. pyriformis* cells (*a*) and its transformation in Scatchard's coordinates (*b*).

effects observed without suramine. This indicated that glucose stimulated the *T. pyriformis* cyclase enzymes through signal cascades dependent and independent on G-proteins. Monosaccharides structurally related to glucose, D-mannose and D-galactose, in concentrations of 10^{-5}-10^{-3} did not modify cyclase activities (data not presented). This attested to specific stimulatory effect of D-glucose on the *T. pyriformis* AC and GC.

In contrast to glucose, cAMP stimulated AC activity in a low concentration (10^{-9} M). The maximum stimulation was observed at a concentration of 10^{-6} M and the effect decreased with increasing its concentration (Fig. 1). These results were in line with the data on high sensitivity of AC of other unicellular organisms (*D. discoideum* ameba and *D. anser* infusoria) to cAMP in nanomolar concentrations [3,7,11]. On the other hand, the stimulatory effect of cAMP on GC was detected only at a concentration of 10^{-7} M and was maximum at concentrations of 10^{-4}-10^{-3} M (Fig. 1). Similarly as with glucose, the maximum stimulatory effects of cAMP on AC and GC were similar, though were attained with different concentrations of the cyclic nucleotide. EC_{50} for cAMP stimulation of AC and GC activities were 38 nM and 4.9 μM, respectively. Only one organism in which cAMP stimulated GC activity is known, *D. discoideum* ameba, and, similarly as for cAMP effect on AC, the heterotrimeric G-proteins acted as messengers in the signal transmission from cAMP receptors to GC [11,15]. Suramine (10^{-5} M) reduced cAMP stimulation of AC and GC (10^{-4} M) by 77 and 69%, respectively.

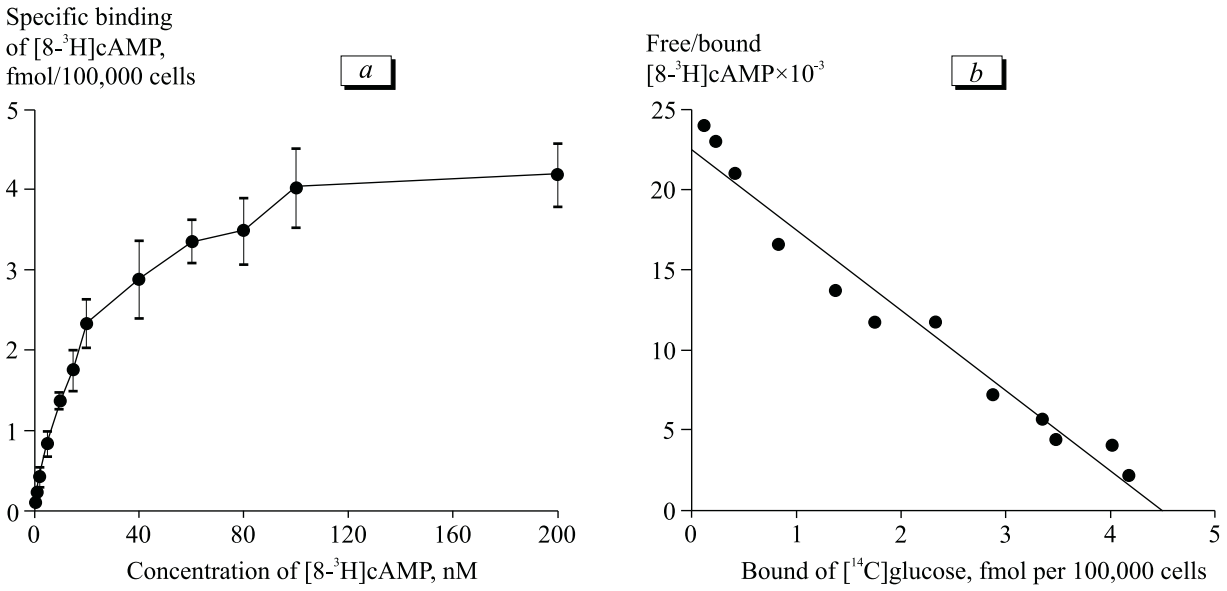

Fig. 3. Specific binding of [8-^{3}H]cAMP to *T. pyriformis* cells (*a*) and its transformation in Scatchard's coordinates (*b*).

This indicated the involvement of G-proteins in cAMP-induced stimulation of *T. pyriformis* cyclases. The effect of cAMP was specific, as structurally related AMP and adenosine in fact did not change AC and GC activities (data not presented).

In order to characterize binding sites on *T. pyriformis* cell surface mediating the regulatory effects of D-glucose and cAMP on AC and GC, we studied their specific binding to infusoria *in vivo* (Figs. 2, 3). It was shown that [6-^{14}C]glucose specifically bound to infusoria cells at K_D and B_{max} of 43 nM and 7.53 fmol/100,000 cells, respectively, while [8-^{3}H]cAMP at 19 nM and 4.46 fmol/100,000 cells. Nonspecific binding in the presence of unlabeled glucose or cAMP (10^{-3} M) was 35-50% of total binding. One *T. pyriformis* cell had about 1250 binding sites for glucose and 750 binding sites for cAMP, on average, which was comparable with the number of cAMP receptors on *D. anser* infusoria [3], but was significantly lower than the number of glucose receptors on intensely growing yeast fungi [8,10] and number of cAMP receptors on *D. discoideum* ameba during the stage of aggregation into a multicellular formation [7,11]. Lower density of binding sites on the infusoria was presumably due to the fact that in contrast to the yeast fungi and *D. discoideum* ameba, glucose and cAMP did not play the key role in regulation of the infusoria vital activity, being just two of the numerous external signals determining the organism's food-getting behavior and intraspecies chemocommunication.

Hence, glucose and cAMP in micromolar concentrations stimulated activities of cyclase enzymes in *T. pyriformis* infusoria; cAMP in nanomolar concentrations stimulated activity of AC. Specific binding of glucose and cAMP to *T. pyriformis* cells *in vivo* seemed to be a mechanism triggering their stimulation of enzymes with cyclase activities.

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REFERENCES

1. K. V. Derkach, A. O. Shpakov, L. A. Kuznetsova, *et al.*, *Zh. Evolyuts. Biokhim. Fiziol.*, **39**, No. 4, 332-338 (2003).
2. A. O. Shpakov, *Tsitologiya*, **49**, No. 8, 617-630 (2007).
3. A. O. Shpakov, K. V. Derkach, Z. I. Uspenskaya, and M. N. Pertseva, *Dokl. Rossiisk. Akad. Nauk*, **424**, No. 2, 270-272 (2009).
4. A. O. Shpakov, K. V. Derkach, Z. I. Uspenskaya, *et al.*, *Zh. Evolyuts. Biokhim. Fiziol.*, **40**, No. 4, 290-297 (2004).
5. A. O. Shpakov, K. V. Derkach, Z. I. Uspenskaya, *et al.*, *Tsitologiya*, **46**, No. 4, 317-325 (2004).
6. D. L. Garbers and F. Murad, *Adv. Cyclic Nucl. Res.*, **10**, 57-67 (1979).
7. J. Y. Kim, J. A. Borleis, and P. N. Devreotes, *Dev. Biol.*, **197**, No. 1, 117-128 (1998).
8. L. Kraakman, K. Lemaire, P. Ma, *et al.*, *Mol. Microbiol.*, **32**, No. 5, 1002-1012 (1999).
9. S. Krystofova and K. A. Borkovich, *Eukaryot. Cell*. **5**, No. 9, 1503-1516 (2006).
10. K. Lemaire, S. van de Velde, P. van Dijck, and J. M. Thevelein, *Mol. Cell.*, **16**, No. 2, 293-299 (2004).
11. C. L. Manahan, P. A. Iglesias, Y. Long, and P. N. Devreotes, *Annu. Rev. Cell Dev. Biol.*, **20**, 223-253 (2004).
12. L. F. Rodgers, K. L. Markle, and T. M. Hennessey, *J. Eukaryot. Microbiol.*, **55**, No. 1, 27-33 (2008).
13. G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660-675 (1949).
14. A. O. Shpakov and M. N. Pertseva, *Int. Rev. Cell Mol. Biol.*, **269**, 151-282 (2008).
15. D. W. Veltman, J. Roelofs, R. Engel, *et al.*, *Mol. Biol. Cell*, **16**, No. 2, 976-983 (2005).