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Phosphatidylinositol 3-kinase-like Activity in *Tetrahymena*. Effects of Wortmannin and LY 294002

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Summary. Insulin has many different effects on *Tetrahymena*, as e.g. on the glucose uptake, cell division, survival, phosholipase D activity and insulin production. PI 3-kinase is one of the key enzymes in the action of insulin. Thus it was supposed that similarly to the higher eukaryotes, PI 3-kinase activity plays fundamental role in the insulin action also in *Tetrahymena*. Here we report that PI 3-kinase-like activity is immunoprecipitated from *Tetrahymena* cell lysate with anti-IRS 1 and anti-p85 antibodies. Both immunoprecipitates contain higher PI 3-kinase activity from lysate of insulin treated cells than the lysate of untreated ones. *In vivo* treatments with PI 3-kinase inhibitors wortmannin (100-500 nM) and LY 294002 (10-20 µM) elevated the PI 3-kinase activity in the IRS 1-antibody precipitable material, while in anti-p85 antibody precipitate this activity was lower than in the controls. *In vitro*, wortmannin proved to be an effective PI 3-kinase inhibitor. Immunostaining revealed that p85 immunoreactivity localized to the cortex of cells, while IRS 1 localized cytoplasmically. *In vivo* treatments with both PI 3-kinase inhibitors elevated the amount of IRS 1, while p85 immunoreactivity was increased only after wortmannin treatments. Both PI 3-kinase inhibitors reduced the F-actin content of cells. Wortmannin caused a forward cytoplasmic stream, which translocate the nucleus towards cytopharynx. These treatments inhibited the phagocytotic activity significantly. On the basis of the results, we propose that in *Tetrahymena* a PI 3-kinase like activity is functioning; the ability of both PI 3-kinase inhibitors and insulin to influence the synthesis or association of subunits of PI 3-kinase, and to influence F-actin remodelling and F-actin dependent processes (e.g. phagocytosis) indicate the supposed activity of PI 3-kinase in *Tetrahymena*.

Key words: IRS 1, LY 294002, PI 3-kinase, phagocytosis, signalling, *Tetrahymena*, wortmannin.

INTRODUCTION

Phosphatidylinositol (PtdIns), the basic building block for the intracellular inositol lipids in eukaryotic cells, consists of D-*myo*-inositol-1-phosphate (Ins1P) linked *via* its phosphate group to diacylglycerol. The inositol head group of PtdIns has five free hydroxyl groups, as many as three of which (except 2 and 6 position) have been found to be phosphorylated in cells, in different combination. PtdIns and its phosphorylated derivatives (phosphoinositides, PIs) all reside in membranes and are substrates for kinases (Vanhaesbroeck *et al.* 2001).

Phosphatidylinositol 3-kinase (PI 3-kinase) transfers the terminal phosphate of ATP to the D-3 position of PtdIns, PtdIns-4-monophoshate, or PtdIns-4,

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5-bisphosphate to yield the products PtdIns-3-P, PtdIns-3,4-bisphosphate, or PtdIns-3,4,5-trisphosphate, respectively (Fruman *et al.* 1998). These PIs are not in the pathway for hormone-stimulated inositol 1,4, 5 trisphosphate production and are not substrates for PtdIns-specific phospholipase C enzymes (Serunian *et al.* 1989). PI 3-kinase has a heterodimeric structure, consisting of a regulatory 85 kD (p85) subunit and a catalytic 110 kD (p110) subunit. In addition two distinct forms of p85 subunit have been described: p85α and p85β (Otsu *et al.* 1991). The isoforms of PI 3-kinases can be divided into three classes. All PI 3-kinase catalytic subunits share a homologous region that consists of a catalytic core domain (HR1) linked to PI kinase homology domain (HR2) and a C2 domain. *In vitro*, class I PI 3-kinases can utilize PtdIns, PtdIns-4-P and PtdIns-4,5-P_2 as substrates. In cells, however, their preformed substrate appears to be PtdIns-4,5-P_2 (Vanhaesbroeck *et al.* 2001). The 3-phosphorylated inositol lipids (3-PIs) fulfil roles as second messengers by interacting with the lipid binding domains of a variety of cellular proteins. 3-PIs have been shown to impinge on many different aspects of cell biology, including vesicle trafficking, growth, DNA synthesis, regulation of apoptosis and cytoskeletal changes (Vanhaesbroeck *et al.* 2001). PI 3-kinase acts as a direct biochemical link between its phosphorylated products and a number of proteins containing intrinsic or associated tyrosine kinase activities, including the receptor for PDGF (Whitman *et al.* 1987), or insulin (Endemann *et al.* 1990). N-terminal SH2 domain of PI 3-kinase associates e.g. with insulin receptor substrate 1 (IRS 1), after insulin treatment of liver and muscle cells 10 to 20 fold increase in PI 3-kinase activity was immunoprecipitated with anti-IRS1-antibody (Folli *et al.* 1992).

The unicellular *Tetrahymena pyriformis* has receptors for signal molecules (e.g. hormones) and can respond to them (Csaba 1994). It utilizes many signalling pathways analogous to those of mammalian cells e.g. the inositol phospholipids (Kovács and Csaba 1990a), and provides evidence for the activity of enzymes such as PLA_2, PLC and PLD, which play important roles in the generation of second messengers (Kovács and Csaba 1997, 1999; Kovács *et al.* 1997).

Insulin has many different effects on *Tetrahymena*. Insulin is one of the hormones produced by *Tetrahymena* (LeRoith *et al.* 1980) as well as being recognized by its receptors (Kovács and Csaba 1990b). Exogenously administered insulin promotes glucose uptake by *Tetrahymena* (Csaba and Lantos 1975) and increases its cell division capacity (Hegyesi and Csaba 1997). It was found that insulin altered the protein composition in ciliary membrane extracts (Christopher and Sundermann 1995); the new proteins may be candidates for cellular activities in insulin imprinting, in which phenomenon *Tetrahymena* cells memorize their first encounter with insulin and then alter their sensitivity to these molecules (Csaba 1980). Insulin at micro- and nanomolar levels activates the survival and proliferation of *Tetrahymena* (Christensen *et al.* 1996, 1998). On the basis of the above-mentioned facts it is presumable that also in *Tetrahymena* PI 3-kinase is functioning properly, similarly to that of the higher eukaryotic cells.

In this study, the presence and activity of PI 3-kinase in *Tetrahymena* was examined. We investigated the possibility that PI 3-kinase is activated by insulin treatment in this unicellular organism. Effective inhibitors of PI 3-kinase may help to define the role of PI 3-kinase and its products in these cells. To study whether PI 3-kinase inhibitors wortmannin and LY294002 act on the different - presumably PI 3-kinase-dependent - physiological phenomena of *Tetrahymena*, we investigated the immunoprecipitable PI 3-kinase activity, the localization and amount of p85 and IRS 1, the phagocytotic activity and the cytoskeletal system in this ciliate organism in the presence of these inhibitors.

MATERIALS AND METHODS

Materials. Antibodies against IRS 1, p85α, acetylated tubulin, FITC-labelled anti rabbit and anti-mouse IgG, wortmannin, LY294002, FITC-labelled Con A, TRITC-labelled phalloidin, daunorubicin, protein A - agarose, chromatographic phospholipid and inositol phospholipid standards, and tryptone were obtained from Sigma (St. Louis, MO. USA). Silica gel G TLC aluminium sheets were obtained from Merck (Darmstadt, Germany). [γ-^{32}P]-ATP (specific activity 30 TBq/mM) was purchased from Institute of Isotope Kft. (Budapest, Hungary). Yeast extract was obtained from Oxoid (Unipath, Basingstoke, UK). Insulin Semilente MC is a product of Novo (Copenhagen, Denmark). All other chemicals used were of analytical grade available from commercial sources.

***Tetrahymena* cultures**. In the experiments, *T. pyriformis* GL strain was tested in the logarithmic phase of growth. The cells were cultivated at 28°C in 0.1 % yeast extract containing 1 % tryptone medium. Before the experiments the cells were washed with fresh culture medium and were resuspended at a concentration of 5×10^4 cells ml^{-1}.

Enzymatic assay for PI 3-kinase. **Preparation of cell lysates**. *Tetrahymena* cells were treated with 500 nM wortmannin, 20 μM LY 294002, or 10^{-6} M insulin for 1 h at 28°C. Untreated cells served as controls. After three washings with fresh culture medium, the cells were lysed in lysis buffer (1 % Triton X 100, 150 mM NaCl, 10 mM

Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 0.5 % Nonidet NP-40, 10 µg/ml aprotinin) for 60 min at 4°C with constant agitation. The lysates were centrifuged (10 000 x g, 4°C for 15 min). The supernatant (total cell lysate) were frozen at -80°C. (In the case of measurement of *in vitro* PI 3-kinase activity to the lysate of insulin treated cells 500 nM wortmannin were added.)

Immunoprecipitation with anti IRS 1- and anti p85- antibodies. The lysates [50 µl, 100 µg protein (measured the absorbance at 280 nm)] were incubated with either anti-IRS 1 antibody (15 µl, 5 µg) or anti-p85α antibody (15 µl, 5 µg) for 1 h and then for 30 min with 10 µl 50 % protein A - agarose at 4°C. The immunoprecipitates were washed three times with PBS/ 1% NP-40, twice with 0.5 mM LiCl/ 100 mM Tris HCl, pH 7.6, and twice with 10 mM Tris-HCl, pH 7.4/ 100 mM NaCl/ 1 mM EDTA.

Assay of PI 3-kinase activity in immunoprecipitates. PI 3-kinase activity was measured by *in vitro* phosphorylation of phosphatidylinositol (PtdIns). The pellets of immunoprecipitates were resuspended in 50 µl of 10 mM Tris (pH 7.4) containing 100 mM NaCl and 1 mM EDTA. To each pellet 10 µl of 100 mM $MgCl_2$ and 10 µl of PtdIns (2 µg/ml; sonicated in 10 mM Tris with 1 mM EDTA) were added. The reaction was started by the addition of 10 µl of 440 µM ATP containing 30 µCi of [γ-^{32}P]-ATP. After 15 min at room temperature with constant shaking, the reaction was stopped by the addition of 20 µl 8 N HCl and 160 µl of $CHCl_3$: methanol [1 : 1]. The samples were centrifuged, and the organic phase was applied to a silica gel TLC plate coated with 1 % potassium oxalate. In order to separate the highly phosphorylated phosphatidylinositols from the non-specific radioactivity remaining at the origin of the TLC plate, the plates were developed in 1-propanol/ 2 M acetic acid - 63 : 35 (v/v). After development, 0.5 cm strips were cut from the chromatogram and transferred into scintillation vials, and the radioactivity was measured by liquid scintillation counting. The individual inositol phospholipids were identified by a parallel run of authentic standards. The experiments were carried out in triplicate.

Confocal scanning laser microscopic (CSLM) analysis of *Tetrahymena* cells labelled with monoclonal anti-acetylated tubulin, anti-IRS 1, anti-p85a and TRITC-phalloidin. To localize tubulin containing structures, wortmannin (100, 500 and 1000 nM; 1 h) - treated, and untreated (control) cells were fixed in 4 % paraformaldehyde dissolved in PBS, pH 7.2. After washing with wash buffer (WB; - 0.1 % BSA in 20 mM Tris-HCl; 0.9 % NaCl; 0.05 % Tween 20, pH 8.2) the cells were incubated with monoclonal anti-acetylated tubulin antibody diluted 1 : 500 with antibody (AB) buffer (1 % BSA in 20 mM Tris-HCl, 0.9 % NaCl, 0.05 % Tween 20; pH 8.2) for 45 min at room temperature. After three washings with WB the anti-tubulin antibody treated cells were incubated with FITC-labelled anti-mouse goat IgG (diluted to 1 : 500 with AB buffer) for 45 min at room temperature. After the immunocytochemical labelling - to localize the nucleus - the cells were treated with daunorubicin (0.01 mg/ml, dissolved in PBS) for 10 min. After this incubation, the cells were washed four times with WB, and were mounted onto microscopic slides. The mounted cells were analyzed in a Bio-Rad MRC 1024 confocal scanning laser microscope (CSLM) equipped a krypton/ argon mixed gas laser as a light source. Excitation carried out with the 480 nm line (for FITC) and 530 nm (for daunorubicin) from the laser.

In the case of localization of the binding of anti-p85 and anti-IRS 1-antobodies the *Tetrahymena* cultures were treated with 500 nM wortmannin, 20 µM LY 294002 or 1 µM insulin for 60 min. After three washings with WB, the cells were treated as above. Both antibodies were diluted with AB to 1 : 500. The secondary antibody (FITC-labelled anti- rabbit goat IgG) was diluted with AB to 1 : 750.

To localize F-actin, 500 nM wortmannin- and 20 µM LY 294002-treated cells were fixed (in 4% paraformaldehyde dissolved in PBS, pH 7.2) and incubated with 0.1 µM TRITC-phalloidin (diluted in AB) for 30 min. After three washings with AB, the cells were mounted onto microscope slides and analysed in CSLM.

Flow-cytometric (FACS) analysis of the binding of anti-p85- and anti-IRS 1 antibodies to *Tetrahymena*. *Tetrahymena* populations were treated with 500 nM wortmannin, 20 µM LY 294002 or 1 µM insulin for 60 min. Untreated cells served as controls. After treatments, the cells were fixed in 4 % paraformaldehyde dissolved in PBS, pH 7.2. After washing with WB, the cells were incubated with anti-p85 or anti-IRS 1 - antibody diluted 1 : 300 with AB for 45 min at room temperature. After three washings with WB, the cells were incubated with FITC-labelled anti-rabbit IgG for 45 min at room temperature. After these incubations, the cells were washed four times with WB. To determine the non-specific binding of secondary antibodies the primary antibodies were omitted as absolute controls. The measurement of fluorescence intensity was done in a FACS Calibur flow cytometer (Beckton Dickinson, San Jose, USA), using 10^4 cells for each measurement. For the measurement and analysis a CellQuest 3.1 program was used. During the evaluation, cell populations had been separated on the basis of size defined by „gating". In the identical cell populations, the FITC-labelled second antibody content inside the cells was measured. The evaluation of the results was done by the comparison of percentage changes of geometric mean channel values to the control groups. Each experiment was repeated three times.

Analysis of the phagocytotic activity. *Tetrahymena* populations were treated with wortmannin (100 or 500 nM) or LY 294002 (10 or 20 µM) in the presence of FITC-labelled Con A (0.01 mg/ml, dissolved in fresh culture medium). Untreated cells served as controls. Samples were taken after 5, 15 and 30 min. The cells were fixed in 4 % paraformaldehyde solution (in pH 7.2 PBS), washed in two changes of PBS, and the number of fluorescent food vacuoles was quantified by a Zeiss Fluoval fluorescent microscope. In each sample, 100 cells were counted. The experiments were carried out in triplicate.

Statistical treatment of the data. Student's *t*-test was used for the evaluation of all data, with $p<0.05$ accepted as the level of statistical significance.

RESULTS

Assay of PI 3-kinase activity. Upon *in vivo* addition of both PI 3-kinase inhibitors (wortmannin and LY 294002) and insulin to *Tetrahymena* cells, a marked increase was observed in anti-IRS 1- immunoprecipitable PI 3-kinase activity, measured by PtdIns as substrate (Fig. 1). The results indicate that 1 µM insulin activates the appearance of PI 3-kinase in *Tetrahymena* lysate significantly. In this experiment, 500 nM wortmannin was shown to be nearly as effective as 1 µM insulin. LY 294002 was only ~ 30 % as effect as wortmannin

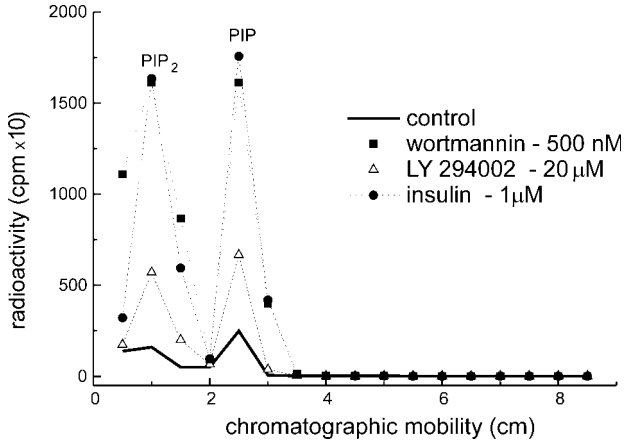


Fig. 1. Anti-IRS 1-immunoprecipitated PI 3-kinase activity of control (untreated); *in vivo* wortmannin (500 nm); LY 294002 (20 μM) and insulin (1 μM) treated *Tetrahymena*. Incorporation of [γ-^{32}P] ATP into the inositol phospholipids. The labelled lipids were extracted, separated by TLC and measured by liquid scintillation counting. The experiments were done in triplicate with a representative experiment shown.

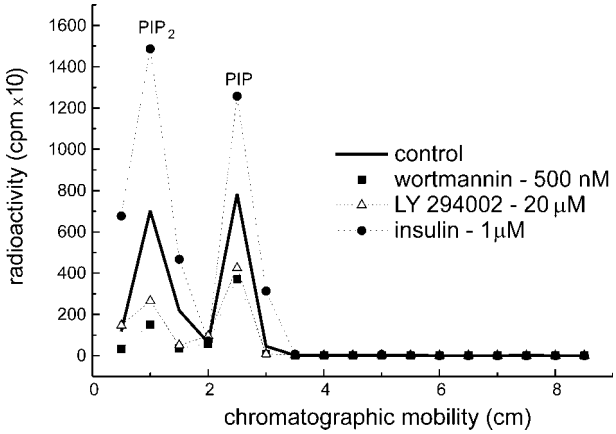


Fig. 2. Anti-p85 immunoprecipitated PI 3-kinase activity of control (untreated); *in vivo* wortmannin (500 nm); LY 294002 (20 μM) and insulin (1 μM) treated *Tetrahymena*. Incorporation of [γ-^{32}P] ATP into the inositol phospholipids. The labelled lipids were extracted, separated by TLC and measured by liquid scintillation counting. The experiments were done in triplicate with a representative experiment shown.

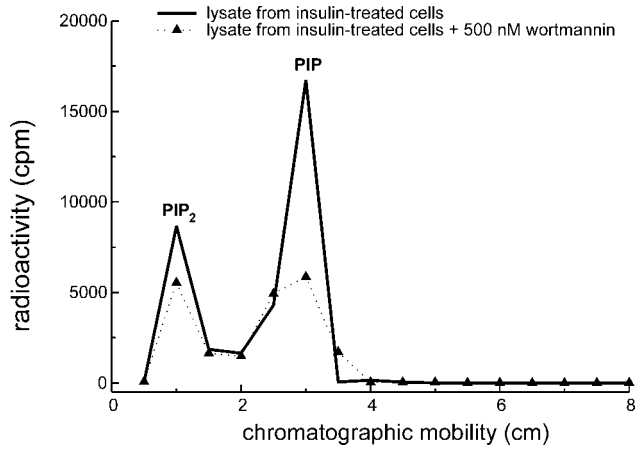


Fig. 3. *In vitro* inhibition of anti-IRS 1-immunoprecipitated PI 3-kinase activity of lysate of insulin (1 μM) treated *Tetrahymena* with 500 nM wortmannin. The experiments were done in triplicate with a representative experiment shown.

stimulating the IRS 1-antibody immunoprecipitable PI 3-kinase activity. The ability of the anti-p85 antibody to immunoprecipitate the PI 3-kinase was different compared to the anti-IRS 1. Upon addition of insulin, a marked increase was observed, but in contrast with IRS 1-immunoprecipitation, both PI 3-kinase inhibitors decreased the activity of this enzyme in the *Tetrahymena* lysate (Fig. 2).

The activity of PI 3-kinase in the lysate of insulin treated cells *in vitro* was inhibited with 500 nM wortmannin. The amount of ^{32}P-labeled PtInsP decreased significantly, while the amount of labelled $PtInsP_2$ decreased to a lesser degree (Fig. 3).

Localization of IRS 1 and p85. p85 immunoreactivity was localized in the cortex of *Tetrahymena* revealed by confocal images (Figs 4 a-d). The ciliary basal bodies (in the basal body cages) and the oral apparatus were strongly labelled with p85 antibody. Treatments with insulin and both PI 3-kinase inhibitors resulted in a significant increase of the labelling of cortical elements. In each experiment the wortmannin treatment influenced the localization of the macronucleus: the nucleus was localized forwards, near or around the cytopharynx (Figs 4, 6-8).

Immunostaining of the IRS 1 was diffusely punctuated, it was localized in the cytosol and in the oral apparatus; the cortical structures remained unlabeled (Figs 4 e-h). The localization and pattern of both immunoreactivity remained similar after the PI 3-kinase inhibitor treatments, but in the amount of labelling arose differences. These differences are clearly visible in the FACS measurements.

Measurement of binding of p85- and IRS 1-antibodies to the *Tetrahymena* by FACS. In accordance with PI 3-kinase activity measurements, on the basis of the FACS measurements, the IRS 1-immunore-

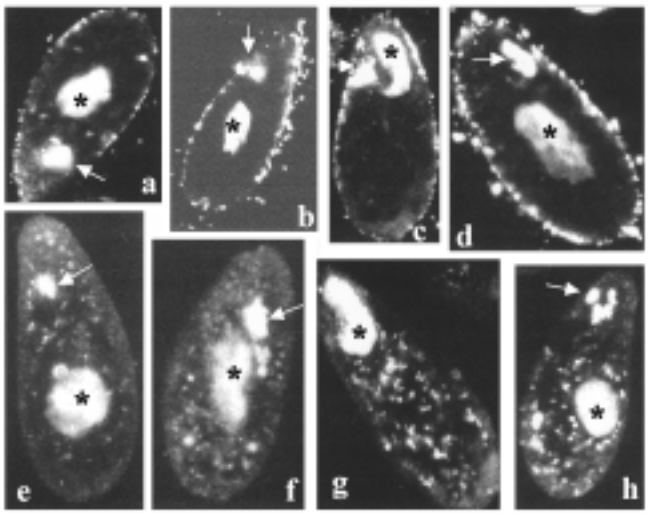
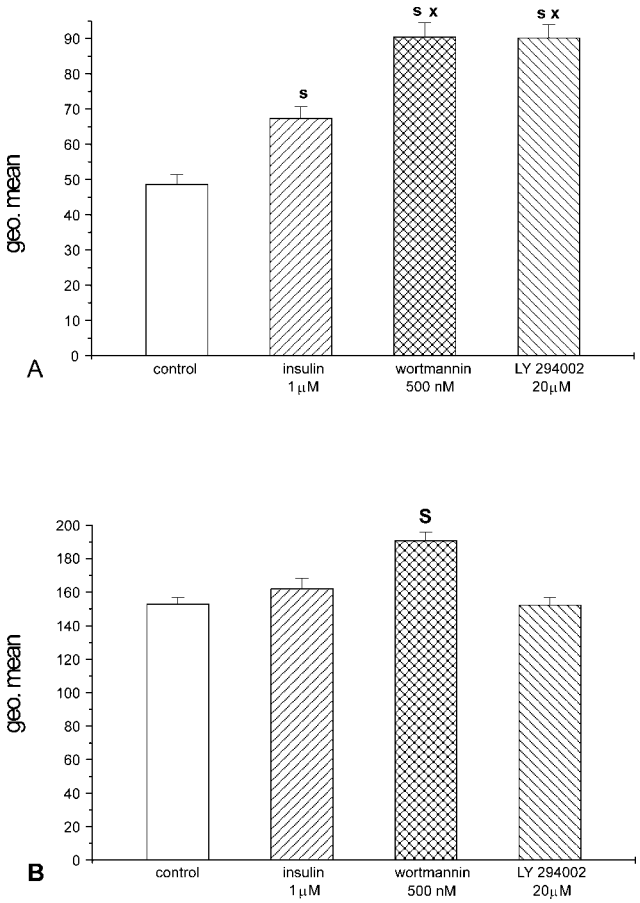


Fig. 4. Localization of p85 - (a-d) and IRS 1-antibody (e-h) in *Tetrahymena* demonstrated by FITC-labelled anti rabbit goat IgG. **a, e** - untreated (control) cells; **b, f** - 1 μM insulin treated cells; **c, g** - 500 nM wortmannin treated cells; **d, h** - 20 μM LY294002 treated cells. Confocal scanning laser microscopic pictures. Magnification x950. Asterisk - nucleus (labelled with daunorubicin); arrows - oral apparatus.



Figs 5. A, B. Flow cytometric analysis of the binding of anti-IRS 1- (**A**) and anti-p85 (**B**) antibody to the untreated; *in vivo* wortmannin (500 nm); LY 294002 (20 μM) and insulin (1 μM) treated *Tetrahymena*. The data represent the mean (±SD) derived from three separated experiments. S - $p<0.01$ to the controls; X - $p<0.01$ to the insulin treated cells.

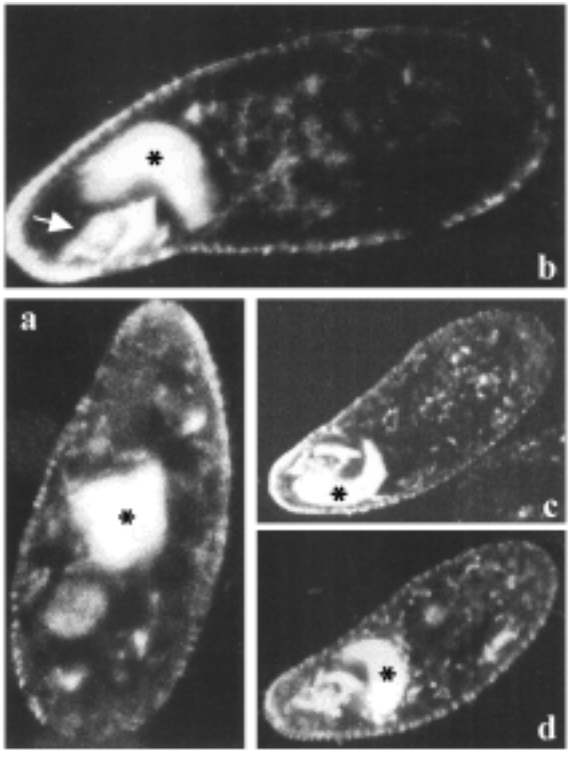


Fig. 6. Confocal scanning laser microscopic pictures of control (**a**) and 500 nM wortmannin treated cells (**b-d**). Binding of anti-acetylated tubulin antibody to the tubulin containing structures, and daunorubicin to the nuclei of *Tetrahymena*. Asterisk - nucleus (labelled with daunorubicin). arrow - oral apparatus. Magnification a - x1100; b - x1300; c, d - x850.

activity was significantly higher in the wortmannin, LY294002 and insulin treated cells than in the controls. In addition, in the wortmannin and LY 294002 treated groups further increase appeared compared with the insulin treated ones (Fig. 5 A). In the case of p85-antibody only the wortmannin treatment caused significantly higher labelling compared to the controls (Fig. 5 B).

The effect of wortmannin on the microtubular system of *Tetrahymena* cytoskeleton. After treatments with both PI 3-kinase inhibitors, no visible changes were observed in the anti-acetylated tubulin-antibody binding. The basal body cages and the components of

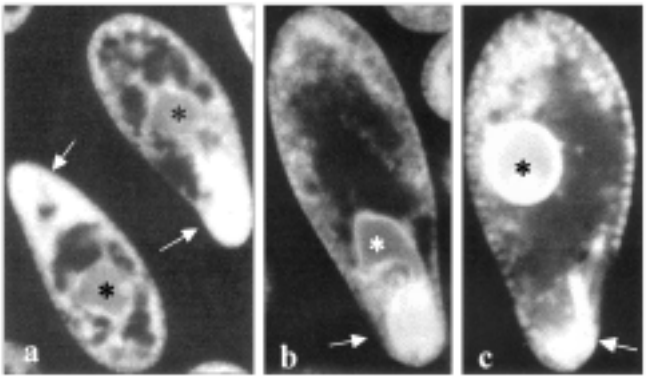


Fig. 7. Binding of TRITC-phalloidin to the F-actin containing structures of untreated (**a**), 500 nM wortmannin (**b**) and 20 µM LY 294002 (**c**) treated *Tetrahymena*. Asterisk - nucleus (labelled with daunorubicin); arrows - oral apparatus. Confocal scanning laser microscopic pictures. Magnification a - x850; b, c - x1100.

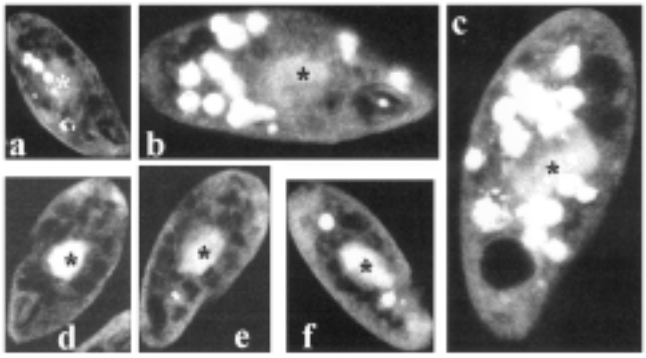


Fig. **9.** Phagocytotic activity of control (**a-c**), 10 µM (d-f) LY 294002 treated *Tetrahymena*. Incubation with FITC-Con A for 5 min (**a, d**); 15 min (b, e) and 30 min (c, f). Confocal scanning laser microscopic pictures. Asterisk - nucleus (labelled with daunorubicin). Magnification x850.

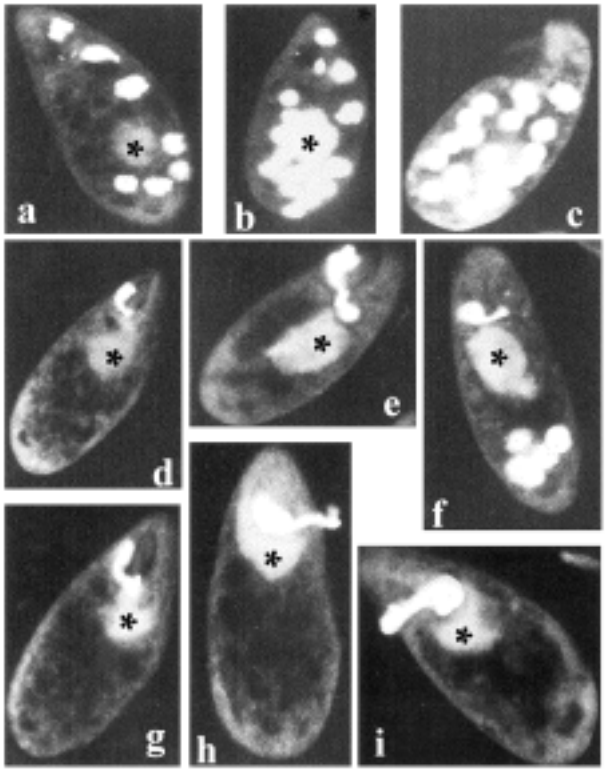


Fig. 8. Phagocytotic activity of control (**a-c**), 100 nM (**d-f**) and 500 nM (**g-i**) wortmannin treated *Tetrahymena*. Incubation with FITC-Con A for 5 min (a, d, g); 15 min (b, e, h) and 30 min (c, f, i). Confocal scanning laser microscopic pictures. Asterisk - nucleus (labelled with daunorubicin). Magnification x850.

oral apparatus (undulating membrane and adoral membranelles) were strongly labelled in each concentration of wortmannin used. These pictures clearly show the translocation of the nucleus to the cytopharynx (Fig. 6). In contrast, the other PI 3-kinase inhibitor LY 294002, failed to alter the position of nucleus.

The effect of wortmannin and LY 294002 on the localization of F-actin. In contrast to tubulin, the pattern of localization of F-actin was different in the treated cells, compared to the controls, revealed by TRITC-phalloidin labelling. In the control cells, the basal body cages and the elements of oral apparatus were strongly labelled with TRITC-phalloidin, and also in the cytoplasm, mostly around the food vacuoles and nucleus, considerable amount of labelling was visible (Fig. 7 a). After wortmannin treatments, the internal part of the cells was free from the F-actin, but these treatments did not alter the labelling of basal body cages and the oral apparatus with phalloidin (Fig. 7 b). After LY 294002 treatment, the cytoplasmic F-actin content was limited to the caudal part of the cells, but these treatments also did not alter the labelling of cortical structures and oral apparatus. The strong nuclear fluorescence was remarkable (Fig. 7 c).

Effect of wortmannin and LY 294002 on the phagocytotic activity of *Tetrahymena*. The food vacuoles were labelled strongly with FITC-labelled Con A; thus the vacuoles were easily counted in fluorescent microscope. The phagocytotic activity was inhibited considerably by both wortmannin concentrations (100 and 500 nM) (Table 1). During wortmannin treatments, the cavity of cytopharynx was markedly labelled with FITC-Con A, and the labelled material often was hanging out from the cytopharynx. In presence of wortmannin,

Table 1. Phagocytotic activity of control, wortmannin (100 and 500 nM) and LY 294002 (10 and 20 μM) treated *Tetrahymena*. The data represent the mean (±SD) derived from three separated experiments. **a** - labelling only in the cytopharynx; **b** - very small vacuoles under the cytopharynx. **c** - p<0,01 to the controls.

	5 min	15 min	30 min
control	6.3 ± 0.49	13.4 ± 1.34	18.3 ± 1.76
wortmannin 100 nM	**a**	**a**	4.7 ± 0.51**c**
wortmannin 500 nM	**a**	**a**	**a**
LY 294002 10 μM	1.6 ± 0.2**c**	2.3 ± 0.4**c**	4.9 ± 0.37**c**
LY 294002 20 μM	0	1.4 ± 0.22**bc**	2.1 ± 0.29**c**

food vacuoles were perceptibly separate with difficulty from the cytopharynx-membrane (Fig. 8). LY 294002 treatments also inhibited significantly the formation of food vacuoles (Table 1), the separated vacuoles were often very small, but did not affect the labelling of cytopharynx, as wortmannin did (Fig. 9).

DISCUSSION

Although phosphatidylinositol (PtdIns) represents only a small percentage of total cellular phospholipids, it plays a crucial role in signal transduction as the precursor of several second messenger molecules. The products of different PI-kinases, among others the 3-phosphorylated inositol lipids produced by PI 3-kinases, fulfil roles as second messengers by interacting with the lipid-binding domains of a variety of cellular proteins. Similarly to the higher eukaryotic cells, the ciliated unicellular organism *Tetrahymena* also contains PtdIns, which participates in its signalling mechanism (Kovács and Csaba 1990a).

Insulin has many different effects on *Tetrahymena*, influencing glucose uptake (Csaba and Lantos 1975), cell division (Hegyesi and Csaba 1997), survival (Christensen *et al.* 1996, 1998), phosholipase D activity (Kovács *et al.* 1996), insulin production (Csaba *et al.* 1999) and on the alteration of the protein composition in ciliary membrane extracts (Christopher and Sundermann 1995). Thus we supposed that also in *Tetrahymena* a PI 3-kinase like activity is functioning properly, one of the key enzymes in the action of insulin. In the present study, the presence, localization and activity of PI 3-kinase and the effects of PI 3-kinase inhibitors wortmannin and LY 294002 on different physiological phenomena of *Tetrahymena* were investigated.

Wortmannin and LY 294002 are structurally unrelated, cell-permeable compounds. Wortmannin binds covalently to the PI 3-kinase catalytic subunit, whereas LY 294002 is a competitive inhibitor of ATP binding-site (Vlahos *et al.* 1994). Probably these different properties lead to the divergences in the effects of PI 3-kinase inhibitors on *Tetrahymena*.

In most cell types, insulin stimulates tyrosine phosphorylation of insulin receptor substrate 1 (IRS 1). IRS 1 contains 14 potential tyrosine phosphorylation sites; the tyrosine phosphorylation generates docking sites for several SH2-containing proteins (White 1998). Among these, the predominant partner seems to be the p85 regulatory subunit of the PI 3-kinase. This enzyme is stimulated by a number of growth factors, including insulin, insulin-like growth factor and EGF (Rudermann *et al.* 1990).

In the present experiments using IRS 1- and p85 antibodies immunoprecipitable PI 3-kinase activity was found in *Tetrahymena* (Figs 1-3). These activities have been stimulated significantly with *in vivo* insulin treatment (Figs 1, 2). *In vivo* administration of wortmannin and LY 294002 caused also alterations in the immunoprecipitable PI 3-kinase activity. In case of IRS-1- antibody-precipitated material both PI 3-kinase inhibitors elevated the activity significantly (albeit the LY 294002 resulted in a lower value of this activity). It seems to be very difficult to explain the stimulatory effect of these molecules in the light of their inhibitory effects in mammalian cells. One of the possible explanations is that these treatments induce an enzyme-and/or IRS 1-overproduction in *Tetrahymena*; the higher amount of these molecules enables these organisms to carry out the appropriate functions. In contrast with IRS 1-antibody precipitation, lysate of both inhibitor-treated cells contained less PI 3-kinase activity in the anti-p85 immunoprecipitate than the controls. Similarities were found also in the FACS measurements: the binding of anti-IRS 1 antibody to the insulin, wortmannin and LY 294002-treated cells was significantly higher than to the controls, while the binding of p85 antibody was significantly higher only in the wortmannin-treated cells. These phenomena can derive also from the SH2-domain of p85 regulatory subunit of PI 3-kinase. If the complete enzyme has internal SH2-domain, it is capable to bind to the IRS 1; thus if the p85 regulatory and p110 catalytic subunits are associated, with IRS 1-antibody can immunoprecipitate higher PI 3-kinase activity; whereas p85 antibody can detect without respect to the association of this subunit (or integrity of SH2 domains of p85). In

accordance with these premises, in *Tetrahymena* the above mentioned treatments in all probability influence the association of subunits of PI 3-kinase, and leave the amount of synthesized molecules nearly unchanged. Another additional possibilities can be considered to approach this problem: (a) PI 3-kinase of *Tetrahymena* may not follow mechanisms of action described for mammalian enzymes; (b) phosphorylation and dephosphorylation of IRS-1 occurring likely in a response to insulin and PI 3-kinase inhibitors may change its reactivity with antibodies affecting its immunoprecipitation and intensity of its fluorescence.

In vitro, wortmannin seems to be a potent PI 3-kinase inhibitor: it strongly inhibits PI 3-kinase activity in the lysate of insulin-stimulated *Tetrahymena* (Fig. 3). This means that *in vivo* and *in vitro* methods can produce divergent results, as it was demonstrated earlier by us in other systems of *Tetrahymena* (Kovács and Csaba 1999).

To investigate the role of PI 3-kinase activity in the function of cytoskeleton and membrane-traffic dependent processes (e.g. phagocytosis), phagocytotic activity (formation of food vacuoles) was measured in wortmannin and LY 294002 treated cells (Table 1).

In *Tetrahymena* which has a more or less fixed cell surface area, because of the presence of a pellicle, endocytosis cannot occur by an invagination of the cell membrane. In this case, new membrane must be incorporated into the cytopharyngeal membrane to provide the limiting membrane for a new food vacuole. The source of the food vacuolar membrane may be newly synthesized, or recycling-membrane vesicles derived from older food vacuoles or from the Golgi complex (Nilsson 1979). Both possibilities require an intensive membrane-flow - mostly from caudal part of the cell towards cytopharynx. On the basis of experimental results obtained by using of different drugs it may be concluded that actin microfilaments play an important role in the formation of food vacuoles and in the membrane recycling processes (Nilsson 1979).

In connection with endocytosis, a variety of phosphoinositide functions have been discovered. In this process, phosphoinositides provide signals for targeting vesicles to specific fusion sites for membrane trafficking and have crucial roles in cytoskeletal reorganization (Takenawa and Itoh 2001).

In contrast to tubulin (where no visible changes appeared after treatments with both PI 3-kinase inhibitors), in the pattern and localization of F-actin revealed differences in the untreated and PI 3-kinase-inhibitor treated cells. The cortex contains F-actin also in the treated cells, whereas these treatments were accompanied by dramatic deficit in a subset of F-actin enriched structures in the inner part of cytoplasm (Fig. 7). This finding suggests that these treatments influence the remodelling of actin cytoskeleton by changing PI levels. In these cells the position of nucleus became irregular: the nucleus localized near or around the cytopharynx (Fig. 6). In wortmannin-treated cells the mechanism that allows cells maintain the normal localization of organelles, was significantly impaired. Perceptibly, a cytoplasmic stream pushed the nucleus forward. Also the inhibited phagocytosis refers to this stream. This phenomenon is maybe due to the localization of nucleus near or around the cytopharynx, which obstructs the traffic of food vacuoles. The FITC-labelled Con A binds to the content of food vacuoles of *Tetrahymena*. In wortmannin-treated cells the FITC-labelled Con A sometimes hanged out from the cavity of cytopharynx, indicating the forward stream of cytoplasmic elements (Fig. 8).

Treatments with LY 294002 also inhibit phagocytotic activity of *Tetrahymena* significantly, notwithstanding in these cells we did not find alterations in the localization of nucleus, and also the formation of food vacuoles seems to be normal, but the number and size of vacuoles became restricted (Fig. 9). These phenomena refer to the different effect of the wortmannin and LY 294002; this latter exerts its effect on the cytoskeleton probably through some other means than wortmannin.

The PI 3-kinase localization is limited to the cortical structures of *Tetrahymena*, such as basal body cages and the oral apparatus. PI 3-kinase associates with γ and α tubulin. After stimulation the cells with some growth factors and insulin, PI 3-kinase associates also with γ-tubulin (Kapeller *et al.* 1995). Presumably the cortical labelling of *Tetrahymena* with p85 antibody - on the basis of this phenomenon - is due to the γ-tubulin content of basal bodies; γ-tubulin is permanently associated with basal bodies in ciliates (Liang *et al.* 1996) (Figs 4 a-d). Another possibility for the cortical localization of p85 antibody is that this subunit has an inter-SH2 domain, which binds to PtdIns-4, 5. This property may provide a mechanism for concentrating PI 3-kinase at membranes rich in these lipids (Fruman *et al.* 1998).

The localization of IRS 1 is not limited to the cortex; the labelling with IRS 1-antibody was restricted to the oral apparatus and spot-like manner in the cytoplasm (Figs 4 e-h). Localization of both antigen (p85 and IRS 1) is unchanged after PI 3-kinase-inhibitor and

insulin treatments, while the amount of immunoreactive materials was changed.

From the results of the present experiments several conclusions can be drawn: (a) in *Tetrahymena* a PI 3-kinase-like activity functions; a material with PI 3-kinase activity can be immunoprecipitated with p85 and IRS 1-antibodies; (b) *in vivo* treatments with PI 3-kinase inhibitors and insulin alter the amount or activity of this enzyme; (c) *in vitro* this activity can be inhibited with wortmannin; (d) inhibitors of PI 3-kinase activity (wortmannin and LY 294002) and insulin alter the immunoreactivity of p85 and IRS 1-antibodies of *Tetrahymena*; (e) treatments with inhibitors decreased the amount of F-actin, inhibited the mechanism which allows the cells maintain the normal position of organelles, and inhibited the phagocytotic activity.

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