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Effects of Trimethyltin on Pinocytosis of *Dictyostelium discoideum*

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Summary. The effect of trimethyltin (TMT) on viability, morphology and pinocytosis in the cellular slime mould *Dictyostelium discoideum* were examined. Pinocytotic activity was determined using a fluorescent dye, Lucifer yellow (LY), as a marker of fluid phase endocytosis and quantitative image cytometry with epi-fluorescence computer-aided methods. Treatment of cells with trimethyltin chloride at concentrations from 5 to 20 μM causes inhibition in the pinocytotic uptake of LY in growing *D. discoideum* cells in a dose-dependent and time-dependent manner without changing the cell viability, as assessed with the FDA and ethidium bromide viability test. These results are discussed in the light of known actions of organotin compound that affect various cellular functions.

Key words: *Dictyostelium discoideum,* organotin compounds, pinocytosis, trimethyltin chloride.

Abbreviations: FDA - fluorescein diacetate, LY - Lucifer yellow, PBS - phosphate-buffered saline, TMT - trimethyltin

INTRODUCTION

Organotin compounds are extensively used in industry and as biocides in agriculture (Attar 1996, Fent 1996, Huang *et al.*1996). Usage of organotins in industry includes heat stabilisers for polyvinyl chloride, catalysts for polyurethane foam, and silicone rubber and antifouling paints for ships, boats, or fishing nets (Wilkinson 1984, Forsyth *et al.* 1993). About 10 to 30 % of organotin compounds are introduced directly to the environment as biocides (Crowe 1987). The use of organotin compounds in agriculture began in the 1950s. and early 1960s. (Blunden *et al.* 1985). Since that time these compounds have been extensively used as bactericidal, fungicidal, and herbicidal agents (Crowe 1987, Fent 1996). Organotin compounds and products of their degradation accumulate in the environment and might be expected to affect both protista and cells of higher organisms.

Studies carried out on the cellular level indicate that organotin compounds interact with cell membranes causing cell fusion, aggregation, blebbing, and membrane rupture (Heywood *et al.* 1989, Musmeci *et al.* 1992, Kleszczyńska *et al.* 1997). Organotin compounds are

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localized in the phospholipid bilayer and it is generally accepted that they interact with the membrane lipids (Gabrielska *et al.*1997). They alter the mechanical and electrical properties of biological membranes (Butterfield *et al.*1991, Harkins and Armstrong 1992) and membrane models (Cullen *et al.* 1997, Kuczera *et al.*1997, Różycka-Roszak *et al*.1997). Recent studies have shown that in isolated mammalian brain neurones organotins act through modification of voltage-dependent Na^+ current (Oyama 1992) and have a suppressive effect on the K^+-induced release and synthesis of acetylcholine in the mouse cerebral cortex (Kobayashi *et al*.1996). It was reported that they reduced the cell viability of thymocytes and yeast *Candida maltosa* (Pieters *et al.* 1994, Tobin and Cooney 1999), changed the cell shape and morphology of guinea pig cochlear outer hair cells (Clerici *et al*.1993), and cell proliferation in lymphocytes and V79 Chinese hamster cells (Jensen *et al.* 1991, Ganguly 1995), suppressed chemotactic response to chemoatractants in neutrophils (Arakawa and Wada 1984), altered phagocytosis and exocytosis in polymorphonuclear leukocytes (Elferink *et al.*1986), and phagocytosis in aquatic organisms *Tapes philippinarum* and *Ciona intestinalis* (Cooper *et al*.1995, Cima *et al.*1998). They changed mitochondrial function (Zazueta *et al.*1994) and calcium homeostasis by inhibiting the Ca^{2+} release channel in the endoplasmic reticulum in thymocytes and sarcoplasmic reticulum in skeletal muscle (Chow *et al*.1992, Kang *et al.* 1997).

Although organotin compounds are found in the natural environment and protista are exposed to these compounds in their natural habitats, to our knowledge no reports have appeared on the effects of organotins on these cells. In our experiments we used *Dictyostelium discoideum* myxamoebae to study the effect of trimethyltin on cell morphology, viability and pinocytotic activity.

The cellular slime mould *D. discoideum* was chosen as a model organism commonly used not only in research into mechanisms of cell locomotion and chemotaxis, but also in investigations concerning endocytosis (Gonzalez *et al.* 1990, Aubry *et al.* 1993, Hacker *et al.* 1997, Reddy and Chatterjee 1997, Titus 2000). This soil amoeba seems to be a suitable model organism in research on biological effects of environmental pollutants. In many of its activities, such as locomotion, chemotaxis, endocytosis, divisions, cell-to-cell interactions and differentiation, it resembles activities of vertebrate cells, particularly the highly motile mammalian polymorphonuclear leukocytes.

MATERIALS AND METHODS

Materials

The following chemicals were purchased as indicated: trimethyltin chloride TMT [$(CH_3)_3SnCl$] (Alfa, Karlsruhe), LY and maltose (Sigma St. Louis, USA), bacteriological peptone and yeast extract (Bio Merieux SA, France), PBS (Wytwórnia Surowic i Szczepionek, Lublin, Poland), NaCl, KCl, $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot H_2O$, KH_2PO_4, $CaCl_2$, $K_2HPO_4 \cdot 12H_2O$, glucose (POCh, Gliwice, Poland). Culture flasks were purchased from Corning NY, UK and microscopic slides from Chance LTD, Warley, UK.

Cells

Dictyostelium discoideum strain AX-2, obtained from professor Jan Michejda (University of Poznań), was grown axenically at 21°C with shaking at 150 rpm in peptone-yeast extract medium (14,30 g/l bacteriological peptone; 7,15 g/l yeast extract; 1,28g/l $Na_2HPO_4 \cdot 12\ H_2O$; 0,48 g/l KH_2PO_4; 18,00 g/l maltose; pH 7.4). Cells were used in their logarithmic phase of growth. Growing cells ($5x10^6$ cells/ml) were washed twice in Chalkley medium pH 6.8 before being used for the experiments.

The cell counts were made in a Bürker haemocytometer. Viability was determined by the fluorescein diacetate test (FDA test) and ethydium bromide test, based on the preservation of esterase activity in living cells and nucleic acid staining in dead cells (Szydłowska *et al.* 1978), recommended for determination of cell survival by Kemp *et al.* (1983).

Assay for pinocytosis

Pinocytosis was determined by fluorescent microscopy using fluorescent day Lucifer yellow (LY) as a fluid-phase marker. About 0.5 ml cell suspension in Chalkley's medium, pH 6.8, permitted their attachment to a microscope slide. To induce pinocytosis the medium was removed by suction and replaced by 0.125M NaCl dissolved in phosphate buffer at pH 6.8, containing 100 μg/ml LY, unless otherwise stated. Cells were observed under an inverted Olympus IMT-2 microscope using phase contrast or fluorescent optics for 45 min. After 15 min, 30 min, or 45 min incubation pinocytosis was stopped by six rinses with ice-cold phosphate buffer at pH 6.8, after which the cells were washed twice in the Chalkley medium without LY at 21°C. The intensity of fluorescence was measured with a Leitz Orthoplan microscope working in an epifluorescence mode equipped with a photomultiplaier attachment (excitation wavelength 428 nm, emission wavelength 535 nm).

Organotin treatment

TMT was diluted in the Chalkley medium or in the pinocytotic medium from stock solution in water to the working concentration of 5, 10, 15 and 20 μM respectively.

Statistical analysis

For each value measured at least 100 cells were analysed. Mean and standard deviation for each parameter were calculated. The statistical significance was determined by the nonparametric Mann-Whitney U-test and T-student test with $p<0.05$ considered significant.

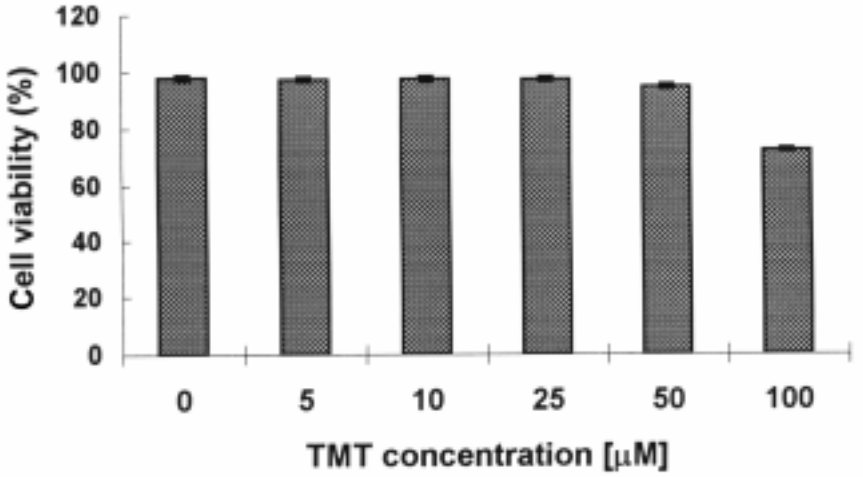

Fig. 1. Viability of trimethyltin-treated *D. discoideum* cells. Cells were incubated in cultured medium (control) and in the same medium supplemented with TMT at concentration 5, 10, 25, 50 or 100 μM for 2 hours. Viability of cells was checked using the FDA/ethidium bromide viability test. At least 200 cells were analysed for each experimental point. The presented results are the mean of at least three separate experiments

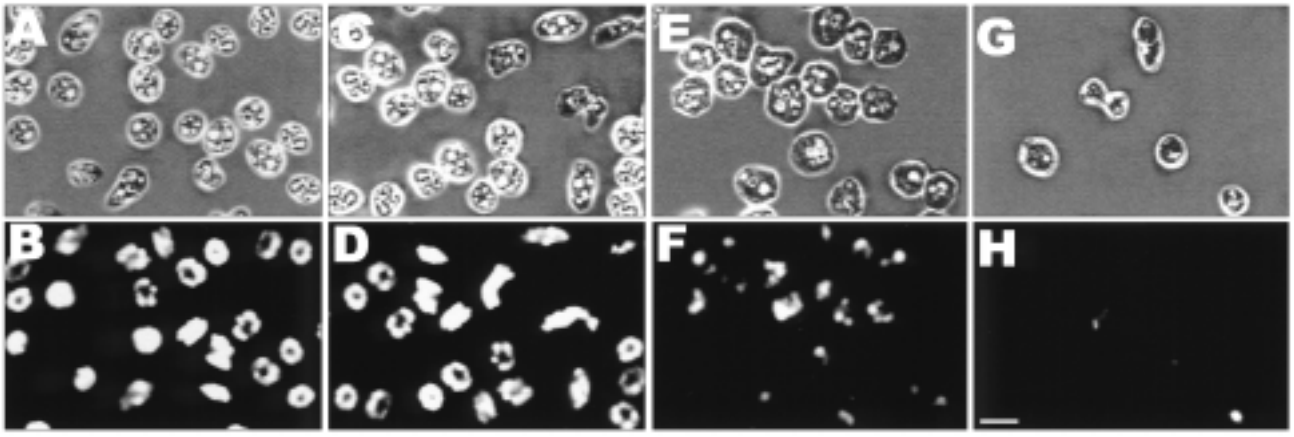

Fig. 2. Effects of trimethyltin on morphology and pinocytotic activity of *D. discoideum* cells (in a population). Cells were incubated in pinocytotic medium supplemented with 100 μg/ml LY for 30 min without (A, B) or with TMT at concentration 5 μM (C, D), 10 μM (E, F), or 20 μM (G, H). Cells were photographed in an Olympus IMT inverted microscope using contrast phase (A, C, E, G) or fluorescence optics (B, D, F, H). Scale bar - 10 μm

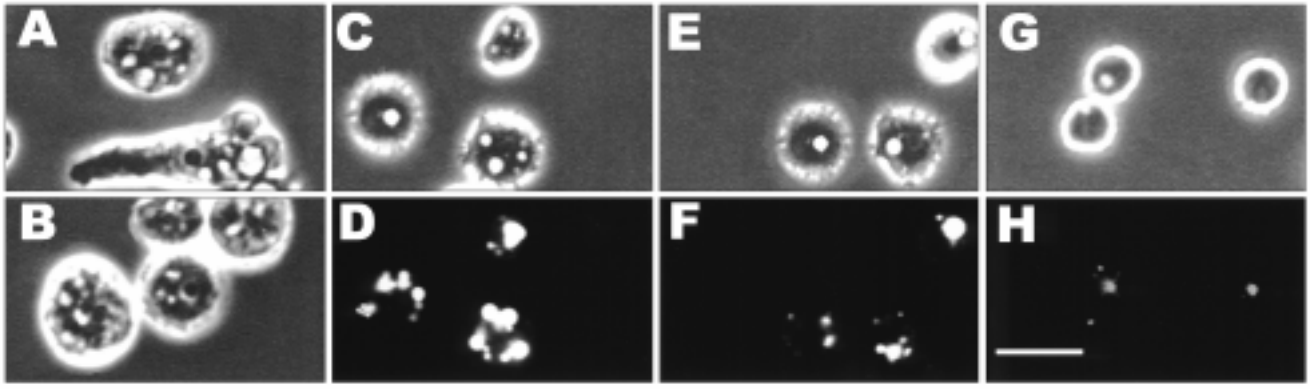

Fig. 3. Effects of trimethyltin on morphology and pinocytotic activity of *D. discoideum* cells (within individual cells). Cells were incubated in Chalkley's medium (A, B) or in pinocytotic medium supplemented with 100 μg/ml LY for 30 min (C, D, E, F, G, H), without (A, C, D) or with TMT at concentration 10 μM (B, E, F), or 20 μM (G, H).Cells were photographed in an Leitz Orthoplan microscope using contrast phase (A, B, C, E, G) or fluorescence optics (D, F, H). Scale bar - 10 μm

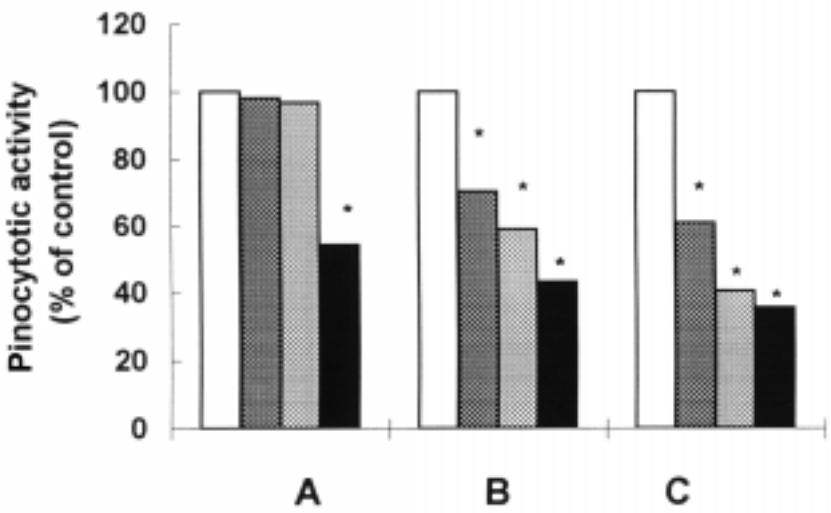

Fig. 4. Inhibition of pinocytotic activity of *D. discoideum* cells induced by trimethyltin. Cells were incubated with pinocytotic medium containing 100 μg/ml LY (white columns) or in the same medium supplemented with 5μM (dark grey columns), 10 μM (light grey columns), or 20 μM TMT (black columns) for 15 min (A), 30 min (B) or 45 min (C), respectively. The pinocytotic activity for each of the experimental conditions was determined and is presented as percentage of control. At least 100 cells were calculated for each experimental point. The presented results are the mean of at least five separate experiments. Values significantly different from control are indicated by (*)

Microphotography

Microphotographs were taken under a Olympus TMT inverted miscoscope using contrast phase or fluorescence optics or Leitz Orthoplan microscope with epifluorescence and phase contrast optics, equipped with a Nikon FX-35DX camera. High sensitivity Kodak TMAX 3200 films or Fomapan 800 films were used.

RESULTS

Effect of trimethyltin (TMT) on *Dictyostelium discoideum* amoebae

The effect of TMT on cell morphology and cation-induced fluid phase endocytosis in *D. discoideum* amoebae was studied. TMT was used within a range of concentrations which did not impair cell viability, as estimated by a viability test. The FDA and ethidium bromide viability test (Szydłowska *et al.* 1978) showed that in cultures of *D. discoideum* cells treated with TMT in concentrations up to 25 μM, about 98% of cells (97.5 ± 1%) remained viable for 2 h (Fig.1).

Phase-contrast and fluorescent microscopic observations of cells incubated for 45 min in the pinocytosis-inducing medium without or with TMT revealed that the cells changed their pinocytotic activity in a TMT dose-dependent manner. Solutions of NaCl are commonly exploited to stimulate the induced pinocytosis in amoebae (Stockem 1966, Prusch 1981, Rivero *et al.* 1999), and LY is extensively utilised as a marker of fluid-phase pinocytosis (Maeda and Kawamoto 1986, Camacho *et al.* 1996, Catizone *et al.* 1996). As shown in Figs. 2 and 3, the incubation of amoebae, both in Chalkley's medium and pinocytotic medium, in the presence of 10 μM TMT for 30 min caused no changes in the morphology of *D. discoideum* cells in comparison with untreated cells (Figs. 2 A, C, E and 3 A, B). Treatment with 20 μM TMT caused contraction and rounding of some cells accompanied by their detachment from the substratum (Figs. 2 G, 3 G). Fluorescent microscope observations revealed that the incubation of *D. discoideum* cells (control cells) in the pinocytosis-inducing medium supplemented with Lucifer yellow dye resulted in a fluorescent pattern typical of fluid phase endocytosis (Figs. 2 B, 3 D). On the other hand, the addition of TMT to the medium resulted in a decrease in fluid phase endocytosis in a dose-dependent manner (Figs. 2 D, F, H; 3 F, H). 20 μM TMT dramatically reduced LY uptake into *D. discoideum* cells (Figs. 2 H, 3 H).

Cytophotometric data

Microscopic observations were confirmed by quantitative single-cell analysis. Cation-induced fluid-phase endocytosis of *D. discoideum* cells was determined by the uptake of the fluorescent dye Lucifer yellow. Maeda

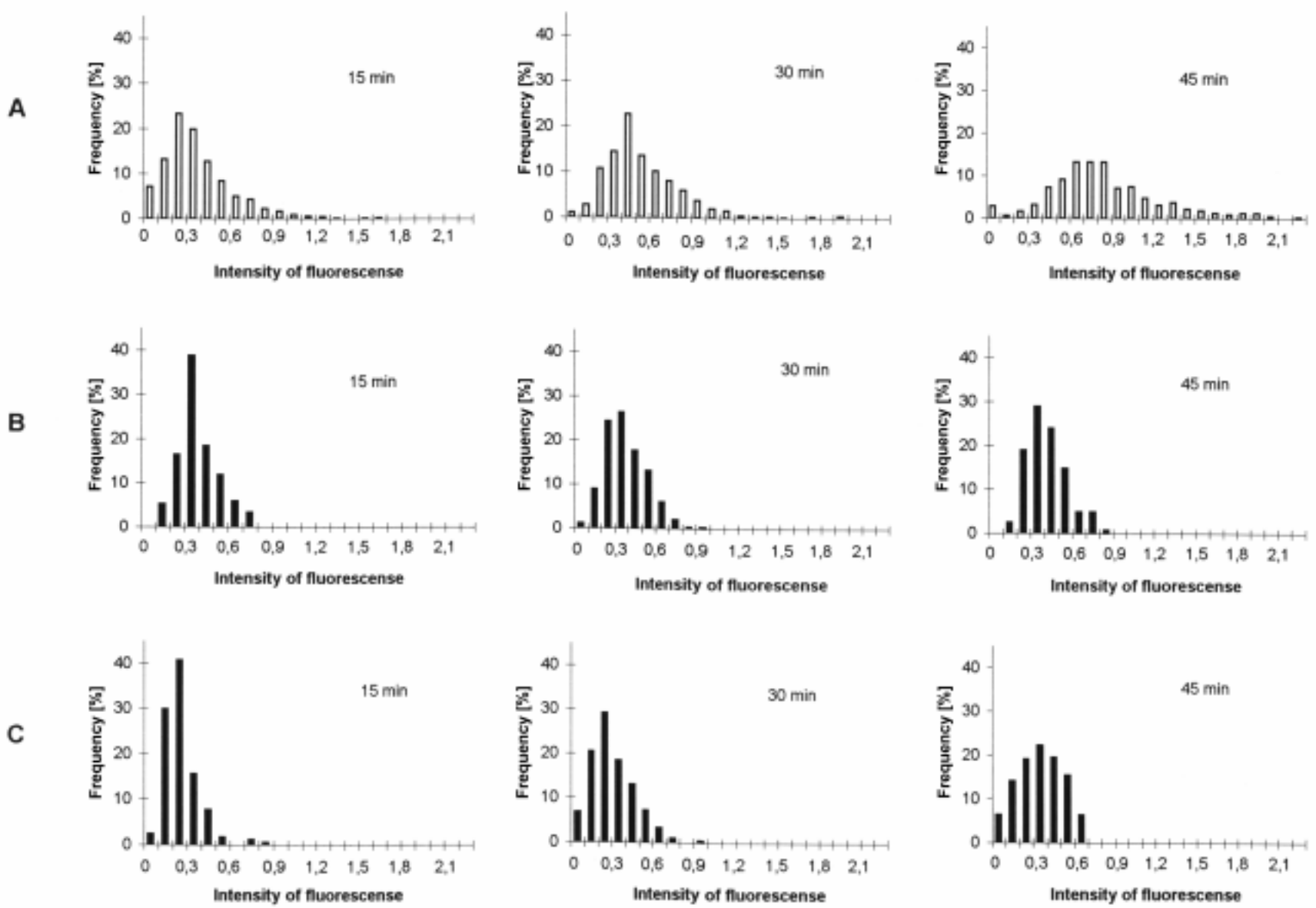

Fig. 5. Histograms of the LY uptake distribution of *D. discoideum* cells after cation-induced endocytosis. Cells were incubated with pinocytotic medium containing 100 μg/ml LY (A) or in the same medium supplemented with 10 μM (B) or 20 μM TMT(C) respectively, for 15 min, 30 min, or 45 min. The data presented are from the representative experiments where a total of 300 cells were counted for each experimental conditions

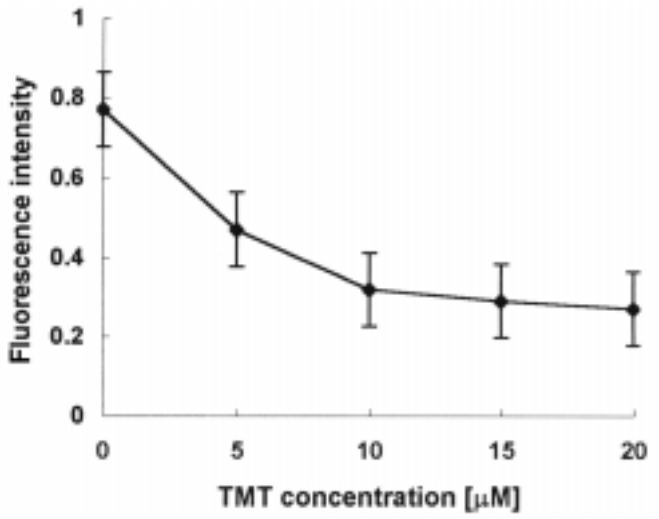

Fig. 6. LY uptake inhibition by trimethyltin in *D. discoideum* cells. Cells were incubated with pinocytotic medium containing 100 μg/ml LY or in the same medium supplemented with 5, 10, 15, or 20 μM TMT respectively, for 45min. The fluorescence intensity for each of the experimental conditions was determined. At least 100 cells were calculated for each experimental point. The presented results are the mean of at least five separate experiments

and Kawamoto (1986) reported that the pinocytotic activity of *D. discoideum* amoebae increases almost linearly in cells incubated at 12 °C to 28 °C and extracellular pH in the range 4.9-7.0. All the present experiments were performed at 22 °C at pH 6.8, unless other- wise noted.

Examination of the pinocytotic activity of *D. discoideum* cells by measurement of LY uptake revealed striking differences between the TMT-treated and the control cells. Results are presented in Fig. 4. After 15 min incubation in 5 μM or 10 μM TMT-containing pinocytotic medium no effect was observed in the uptake of LY (Fig. 4 A). Although the average value of pinocytotic activity for the control cells and 5 μM or 10 μM TMT-treated cells did not change significantly after 15 min incubation, the distribution of *D. discoideum* cells according to LY uptake differed between TMT-treated cells and the control amoebae (comp. Figs. 4 and 5). The histograms showed a lower frequency of cells with high LY uptake for TMT-treated cells than for the control cells (Figs. 5 A, B). In the presence of 20 μM TMT the average LY uptake after 15 min pinocytosis decreased by approximately 45% in comparison with that of the control cells (Fig. 4 A), and the frequency of cells with high LY uptake was significantly reduced (Fig. 5 C). The effect of TMT upon the cation-induced endocytotic activity was more pronounced after 30 min and 45 min. The average LY uptake after 30 min and 45 min incubation of *D. discoideum* amoeba in TMT-containing pinocytotic medium decreased in a time-dependent (Figs. 4 B, C) and dose-dependent manner (Fig. 6). In agreement with this the distribution of *D. discoideum* cells according to LY uptake was changed significantly. The histograms showed that the greater the TMT concentration the greater was the number of cells with no pinocytotic activity and the smaller the number of cells with high pinocytotic activity (comp. Figs. 5 A, B, C).

DISCUSSION

The presented results demonstrate that trimethyltin (TMT), a common component of herbicides, pesticides, and industrial pollutants, strongly affects the vital functions of soil myxamoebae *D. discoideum*. The acute toxicity of TMT, i.e. decrease in the viability of cells growing for 2 h in its presence, is significant only when TMT is present at 100 μM concentration. This corresponds to the results of experiments showing that at this concentration TMT modifies the properties of artificial black membranes.

Kuczera *et al.* (1997) observed that trimethyltin in milimolar concentrations caused calcium desorption from lecithin liposome membranes. Heyewood *et al.* (1989) found that thrimethyltin chloride at 25 μM concentration caused fusion, aggregation, blebbing, and total rupture of egg yolk vesicles (containing mostly phosphatidylcholine).

Krug (1992) reported that thrimethyltin chloride at a concentration of 500 μM does not significantly change the survival of HL60-cells differentiated with dimethyl sulphide to mature granulocytes, as tested with the trypan blue exclusion test.

Cooper *et al.* (1995) and Cima *et al.* (1998) reported that organotin compounds reduced the phagocytosis in aquatic organisms. A decrease in phagocytosis in polymorphonuclear leukocytes was observed by Elferink *et al.* (1986) but Hioe and Jones (1984) noted no changes in phagocytosis of peritoneal macrophages.

When we measured the fluid phase endocytosis of LY in myxamoebae, already 5 and 10 μM concentration of TMT caused a statistically significant decrease in the endocytotic cell activity (in 30 min) while 20 μM of TMT reduced endocytosis in as soon as 15 min. In addition, amoebae exposed to 20 μM TMT changed morphology,

becoming rounded and detached from the substratum. These results evidence that TMT strongly affects cell activity at concentrations, which do not affect cell viability.

It remains to be examined whether TMT at low concentration (and other herbicides and pesticides) affects other cell functions. The tests, which concern solely cell growth and viability, are inadequate for estimation of the effects of such compounds upon unicellular organisms living in the soil. In addition, it cannot be excluded that this also concerns the cells of the immune system of animals and humans since these cells show many activities similar to amoebae of *D. discoideum*, including endocytotic and locomotory activity. Since most of the organotin compounds are soluble in organic solvents and these substances react with phosphate groups of phospholipids (Gabrielska *et al.* 1997, Heywood 1989, Różycka-Roszak *et al.* 1997, Sarapuk and Przestalski 1998, Przestalski *et al.* 2000) it may be supposed that their interaction with lipoprotein membranes could be a primary site of their effects upon living cells.

The results presented permit us to postulate that *D. discoideum* amoebae may be employed as a suitable model organism in investigations concerning biological effects of environmental pollutants upon activities of cells, both unicellular organisms and cells of vertebrates.

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