

Effect of Hormones on the Concentration of a Digoxin-like Material in *Tetrahymena*

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Summary. The unicellular *Tetrahymena pyriformis* contains digoxin-like materials (digoxin, digitoxin, digoxigenin). The effect of two amino-acid-type hormones (histamine and serotonin) and a peptide hormone (insulin) was studied on the level of the hormone-like steroid, digoxin, in the concentrations of 10^{-6} and 10^{-9} by using immunocytochemistry and flow cytometric and confocal microscopic analysis. Each hormone elevated significantly the digoxin concentration at the end of the 30 min treatment. As *Tetrahymena* was maintained in a tryptone-yeast extract medium during the treatment, it seems to be likely that steroid synthesis was enhanced. The most effective was histamine, the effect of which was significantly more expressed than that of the others and the less effective (however significant), was serotonin. The results call attention to hormonal interactions inside the unicellular organism and on the possibility of mutual intercellular influences, considering the low effective hormone concentration which was needed for the striking effect.

Key words: digoxin, evolution, histamine, hormonal interactions, insulin, serotonin, *Tetrahymena*.

INTRODUCTION

Many members and characteristics of the hormonal system of higher ranked animals can be demonstrated in the unicellular *Tetrahymena*. It contains receptor-like structures in the plasma membrane and cytosol (Csaba 1980, 1985; Christopher and Sundermann 1995; Christensen *et al.* 2003), hormones similar to mammalian ones (LeRoith *et al.* 1980, 1983; Lenard 1992;

Kőhidai and Csaba 1995; Kőhidai *et al.* 2000, 2002a, b) and signal transduction pathways (Kuno *et al.* 1979; Muto *et al.* 1983; Kovács and Csaba 1990, 1997) using the same components which are working in mammals. Hormones, received by the receptors can influence a lot of physiological processes of the unicellular animal, e.g. cell division, phagocytosis, chemotaxis etc. (Wheatley *et al.* 1993, Csaba 2000), nevertheless our knowledge on the interactions of these hormones inside the *Tetrahymena* is very scarce. Recently we studied the effect of three amino acid- or peptide-type hormones (which are present in *Tetrahymena*) on the concentration of epidermal growth factor (EGF), a peptide hormone, also present in *Tetrahymena* (Csaba *et al.* 2004). As this

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was demonstrated, in the present experiment the effect of the same hormones on digoxin, a hormone-like steroid molecule, is studied.

MATERIALS AND METHODS

Populations of *Tetrahymena pyriformis* GL were grown axenically in a 0.1% yeast extract containing 1% tryptone medium at 28°C. In the experiments cells in the mid-exponential growth phase (48h cultures; $\sim 5 \times 10^5$ cells/ml) were used. The cells were washed with fresh culture medium and harvested by centrifugation at ~ 400 g for 3 min at room temperature, and were adjusted to $\sim 5 \times 10^5$ cells/ml with fresh culture medium. Five samples were prepared for each measurement. The samples contained *Tetrahymena* in fresh medium and the hormones (except control) in 10^{-6} and 10^{-9} M concentrations for 30 min. The hormones were the following: histamine dihydrochloride (Sigma, St. Louis, USA); serotonin HCl (Sigma) or insulin (Semilente MC, Novo, Copenhagen, Denmark). After treatment the cells were studied by flow cytometry and confocal microscopy.

Flow cytometric analysis

Samples of cells were fixed with 4% paraformaldehyde solution (dissolved in pH 7.2 PBS) for 5 min, and then washed twice in wash buffer (0.1% BSA; 20 mM Tris-HCl; 0.9% NaCl; 0.05% Nonidet NP-40; pH 8.2). To block nonspecific binding of antibodies the cells were treated with blocking buffer (1% BSA in PBS) for 30 min at room temperature. Aliquots from cell suspensions (50 ml) were transferred into tubes, and 50 ml primary antibody (monoclonal anti-digoxin developed in mouse ascites; [Sigma, D-8156, Clone Di-22; dilution 1:200 in antibody buffer (1% BSA in wash buffer)]) was added for 30 min at room temperature. Negative controls were carried out with 50 ml PBS containing 10 mg/ml BSA instead of primary antibody.

After washing four times with wash buffer to remove excess primary antibody the cells were incubated with secondary antibody (FITC-labeled monoclonal anti-mouse IgG developed in goat; Sigma, St. Louis USA; dilution 1: 50 with antibody buffer) for 30 min at room temperature.

For controlling the specificity, autofluorescence of the cells and aspecificity of the secondary antibodies were detected. The measurement was done in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA), using 25,000 cells for each measurement. In the cell populations the digoxin content (concentration) had been compared. For the measurement and analysis CellQuest Pro program was used. The numerical comparison of detected values was done by the comparison of percentual changes of geometric mean channel values to the appropriate control groups by using Origin program and Student t-test.

Confocal microscopic analysis

After the flow cytometric analysis the cells were subjected to confocal microscopic analysis in a BioRad MRC 1024 confocal laser scanning microscope, equipped with krypton-argon mixed gas-laser as a light source, at an excitation wavelength of 480 nm line. Three

independent series of experiments were carried out with identical results.

RESULTS AND DISCUSSION

Tetrahymena is able to synthesize steroid molecules. One of them is the tetrahymanol for the synthesis of which squalene tetrahymanol synthase enzyme is responsible (Saar *et al.* 1991). The unicellular eukaryotes also contain 20 α -hydroxysteroid dehydrogenase (Inazu *et al.* 1994)). In addition to the synthesized steroid hormones (as testosterone and estradiol: Csaba *et al.* 1998) digoxin, a cardioactive glycoside can be found, which also has a steroid structure. In earlier experiments (Kovács *et al.* 1998) the presence of digoxin, digitoxin and digoxigenin was demonstrated in *Tetrahymena*. The monoclonal antibody used in the present experiments was produced against digoxin however, it had a high affinity also to digoxigenin.

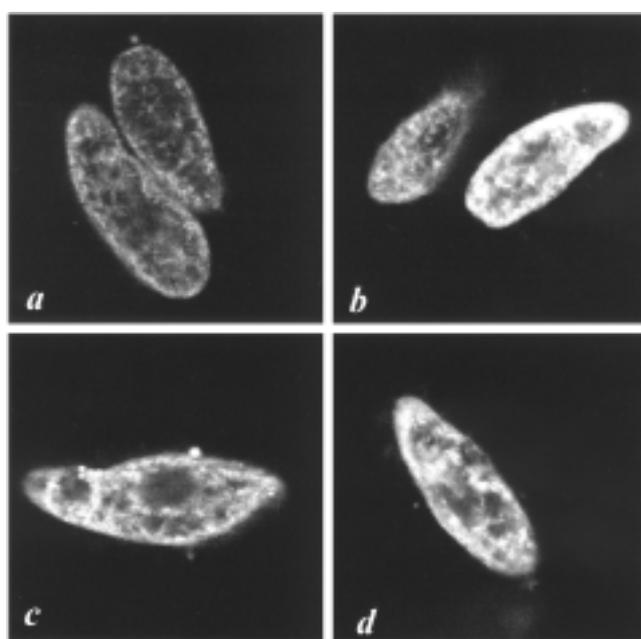
The flow-cytometric results unanimously show that the hormones used for treatment significantly elevated the concentration of the digoxin-like materials in *Tetrahymena* (Table 1). The confocal microscopic pictures support the flow cytometric quantitative results. However, there is no difference between the control and hormone treated cells in the localization of the digoxin-like material (Fig. 1).

As simple tryptone-yeast extract medium was administered for maintaining *Tetrahymena* it is not likely that complete digoxin was taken up from it. This means that the (amino-acid and peptide) hormones which are also physiologically present in *Tetrahymena*, enhanced the steroid synthesis. The most effective hormone was histamine which was similarly effective in the EGF-experiments done earlier (Csaba *et al.* 2004). However, in these earlier experiments serotonin was as effective as histamine and now serotonin was the less active, and in the present case the difference between the effects of histamine and the other two hormones is significant. This emphasizes the important role of histamine at this low level of phylogeny, which is supported by the presence of histamine and histamine synthesizing enzyme (HDC) genes already in bacteria (Ruby *et al.* 2002, Darvas and Falus 2004), and in *Tetrahymena* (Hegyesi *et al.* 1999). The fact, that there was no significant difference between the two concentrations used shows the strong influence of histamine and the other two hormones in a very low (nanomolar) concentration. Considering this,

Table 1. Effect of hormones on the content of a digoxin-like material in *Tetrahymena*.

Treatment	Concentration	Geo-mean	Signif. to control	Signif. to insulin	Signif. to serotonin
Untreated control		108.65 ± 14.62			
Histamine	10 ⁻⁶	163.0 ± 13.18	p<0.001	p<0.05	p<0.01
	10 ⁻⁹	150.96 ± 7.07	p<0.001	p<0.05	p<0.01
Serotonin	10 ⁻⁶	128.28 ± 10.7	p<0.05	n.s.	
	10 ⁻⁹	131.2 ± 6.6	p<0.05	n.s.	
Insulin	10 ⁻⁶	141.63 ± 13.82	p<0.01		n.s.
	10 ⁻⁹	136.118 ± 7.46	p<0.01		n.s.

There are no significant differences between the results of treatments (with the same hormone) in 10⁻⁶ and 10⁻⁹ M concentrations.



Figs 1a-d. Confocal microscopic pictures (median sections) of *Tetrahymena*. **a** - control; **b** -histamine treated; **c** - serotonin treated; **d** - insulin treated. Hormone concentrations 10⁻⁹ M. There is no striking differences in the localization of the digoxin-like molecule however, the quantitative differences can be observed. Magnification ×1400.

not only the influence of these hormones on the inside hormone metabolism can be imagined, but the effect to other members of the population by the secretion of hormone-like materials.

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