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Effects of steroid hormones on five functional parameters of *Tetrahymena*: evolutionary conclusions

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The unicellular *Tetrahymena pyriformis* was studied for chemotaxis, chemotactic selection, phagocytosis, growth and body shape changes in the presence of water soluble (β-cyclodextrin-coupled) steroid hormones (testosterone, estradiol, progesterone, hydrocortisone and dexamethasone). Testosterone was chemoattractant over a wide range of concentrations, while progesterone and dexamethasone were active only at one concentration (10^{-5} and 10^{-6} mg ml^{-1} respectively) and were either neutral or repellent at other concentrations. Hydrocortisone and estradiol were unambiguously chemorepellent. Chemotactic selection enhanced the effect of testosterone and estradiol, while in the case of hydrocortisone the action was reversed. The other parameters were mildly influenced by the steroid hormones. The results call attention to the fine molecular recognition capacity of *Tetrahymena* and to the possible rapid effects of steroid hormones at membrane receptors at a very low evolutionary eukaryotic level.

KEY WORDS — *Tetrahymena*; chemotaxis; steroid hormones; phagocytosis; growth

INTRODUCTION

Steroid molecules belong to the ancient 'bioregulators', i.e. molecules which appeared prior to eukaryotic organisms. Some of these steroids show wide appearence in phylogeny: such molecules as testosterone and progesterone have been reported not only in vertebrates but also in the haemolymph of fleshfly, *Sarcophaga*.[1] Progesterone is also found in plants.

Steroid-receptor research demonstrates the role of these structures in early morphogenesis and in the development of anterior–posterior polarity both in avian and Drosophila models.[2] Further data suggest a very ancestral origin, as cDNA analysis detects highly conserved regions in amino acid sequence of DNA binding domains of steroid receptors in invertebrates, e.g. *Cenorabditis elegans, Drosophila melanogaster* and also in humans.[3]

Although the most accepted theory of steroid signalling is still concerned with cytosolic/nuclear receptors,[4,5] there is also evidence for the presence of steroid receptors in the plasma membrane,[5] the stimulation of which results in rapid, non-genomic effects of steroid hormones.

In the research of phylogenetic backgrounds of signalling by steroid hormones, the protozoan *Tetrahymena pyriformis* has a significant role. At this low level of eukaryotic phylogeny, cells still possess endogenous steroids (dehydroepiandrosterone, testosterone and estradiol),[6] but the equivalent receptors are not detectable using binding assays. However, pretreatments with steroids (prednisolone, estradiol, dexamethasone) provoke the appropriate binding structures in the cytoplasm[6] and these steroids also act on the protozoa, e.g. prednisolone treatment results in euchromatinization of the nucleus[7] and increase in RNA synthesis.[8] Pretreatments also have significant physiological effects as prednisolone induces the clonal growth rate of these cells[9] and dexamethasone interferes with carbohydrate metabolism.[10]

In spite of the facts mentioned above, the details of steroid action are rather obscure in *Tetrahymena*. However, this unicellular model offers several advantages: the cells have several well-characterized (vertebrate type) membrane receptors,[11] second messenger

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systems (cAMP,[12] cGMP,[13] Ca-calmodulin,[14] IP3[15]), and the induction of physiological responses (i.e. growth,[16] metabolism,[17] chemotaxis,[18] phagocytosis[19]) have been studied for several signal molecules. Considering these facts, testing steroid hormone effects in *Tetrahymena* could be useful for two purposes. First, the short cell cycle of 150 min, allows follow-up studies on hundreds of generations, which seems to be very advantageous for steroid research, while the unicellular organization minimizes the intercellular interactions, each cell can be considered as an individual biological experimental subject. Second, *Tetrahymena* is considered to be an appropriate model of ligand–receptor interactions.[20] Its chemotactic responses are unequivocally physiological and it can distinguish biologically-active ligands on the basis of their slight structural diversities or physicochemical characteristics.[21] Steroids provide a group of ligands whose supposed chemoattractant or repellent features have still not been investigated in eukaryotic ciliates. The only practical problem is that steroids are not soluble in water.

Beta-cyclodextrin carriers are dedicated molecules that bind hydrophobic molecules, such as steroids, into the torus-shaped cavities of the molecule and then, in the aqueous environment, they facilitate the dissolution of the ligand. Thus these carriers are able to induce a steroid-rich micro-environment around the cells. In the present work our goal was to test the effect of these water-soluble steroid complexes, as capable ligands for both membrane and cytosolic action.

The problems to be solved were as follows:

(1) Have these substances structure-specific and concentration-dependent effects on the chemotactic behaviour of *Tetrahymena*?
(2) Is there any characteristic capacity of rapid induction with water-soluble steroids to select subpopulations with altered chemotactic responses?
(3) What is the correlation of chemotactic responses with other physiological responses (e.g. phagocytic activity, body shape changes, growth) induced by the same steroid?

MATERIALS AND METHODS

The effects were evaluated on the basis of two very essential physiological responses of ciliates: signal molecule-dependent chemotaxis and its target action of phagocytosis. In addition, 'chemotactic selection'[22] was applied to analyse the specificity of any chemotactic effect elicited. As the size and shape of the model cell is highly dependent on the stage of the cell cycle and is associated with the presence of subpopulations, the experiments were also conducted with computer assisted morphometry to study the homogeneity of subpopulations. In the case of chemotactically effective, chemoattractant ligands, their effect on the growth of cultures was also evaluated.

Cells and culture

Tetrahymena pyriformis GL strain was used in the logarithmic phase of growth. The cells were cultured at 28°C in tryptone medium (Difco, Michigan, USA) containing 0.1% yeast extract. The density of *Tetrahymena* cultures studied was 10^4 cell ml^{-1}.

Chemicals

The water-solubile steroid derivatives were conjugates of 2-hydroxypropyl-β-cyclodextrin. The tested steroids were: β-estradiol, hydrocortisone, progesterone, testosterone and dexamethasone, all obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Parameters studied

The effects of water-soluble-steroids were investigated in five ways: (a) concentration dependence of chemotaxis; (b) chemotactic selection and re-exposure of subpopulations; (c) phagocytic activity, (d) computer-assisted morphometry and (e) analysis of growth in selected cultures.

Concentration dependence of chemotaxis. Chemotactic activity was determined with our modified version[23] of Leick's capillary chemotaxis assay.[24] In this assay an outer and an inner chamber are connected by a capillary tube. The cells are placed into the outer chamber, while the inner chamber contains the test substance. In our set-up, tips of a multi-8-channel pipette were used as the inner chamber, and the cells were placed into the wells of microtitration plates (Nalge Nunc Int., Rochester, NY, USA). The steroids were tested in 10^{-9}–10^{-3} mg ml^{-1} range. In control groups, fresh culture medium was substituted for the reference substance. The incubation time was 15 min. After this the samples were fixed in 4% formaldehyde containing phosphate buffer (PBS, pH 7.2). The number of cells was determined with a light microscope and Neubauer haemocytometer.

Chemotactic selection and re-exposure of subpopulations. In these experiments we applied the chemotaxis assay mentioned above to select positive responder

subpopulations. This test had two steps: the first was for the chemotactic selection with the maximal chemotactic concentrations of each steroid in the dose–response study. In parallel there was a control group, where fresh culture medium was used as chemoattractant. At the end of selection, samples were transferred into fresh medium and they were cultured with consecutive transfers every 48 h, for 168 h. In the second step the chemotactic activity of samples selected with steroids or control substances was measured in four groups: C/C, subpopulations selected with the culture medium-responsiveness tested with culture medium; C/S, subpopulations selected with the culture medium-responsiveness tested with the actual steroid; S/C, subpopulations selected by steroid responsiveness tested with culture medium; S/S, subpopulations selected by steroid-responsiveness tested with the identical steroid (hereafter in place of S, the abbreviation of the actual steroid will be used).

Phagocytic activity. Phagocytic activity was measured with Chinese ink phagocytosis. Prior to experiment, *Tetrahymena* cultures were transferred to and starved in Losina-Losinsky medium[25] containing inorganic salts (0.01% NaCl, 0.001% $MgCl_2$, 0.001% $CaCl_2$, 0.001% KCl, 0.002% $NaCO_3$) for 3 h. The cells were treated with the steroids using optimal chemotactic concentrations of each. Chinese ink test particles were added in the ratio of *Tetrahymena* suspension: steroid solution: Chinese ink = 2 : 1 : 1. The incubation time was 5 min and then the samples were fixed in 4% formaldehyde dissolved in Losina-Losinsky solution. The samples were dropped onto slides and the number of test particles was counted in a 100-cell sample. Since each experiment had four parallels, histograms represent the phagocytic activity of 400 cells.

Computer-assisted morphometry. In this experiment we analysed the subpopulations formed by chemotactic selection. Fixed samples were taken and stained with toluidine blue. The digitalized images of samples were evaluated with Biomorph 1.1. This program allowed us to analyse the individual area of cells, their perimeter and the characteristic *w/l* factor (ratio of shortest and longest axis) which indicates the degree of elongation of cells.

Analysis of growth in chemotactically selected cultures. Steroids possessing positive selector ability (testosterone, progesterone and dexamethasone) were used in this analysis. The cells were selected with the most chemotactic concentration of each ligand, as described above. After selection, equal numbers of cells were transferred to fresh culture medium. The cultures were maintained in 10-ml tubes with consecutive transfers every 48 h, for 168 h. In each group, 10 parallel samples were tested. At the end of the experiment the density of cultures was determined by light microscopy with the same technique as described above in the section 'concentration dependence of chemotaxis'.

Statistical analysis

The data obtained were statistically evaluated with ANOVA of Microcal Origin 2.8 which provided values for Student's *t*-test and standard deviation. In histograms the statistical identity of interconnected values was analysed with chi-square tests.

RESULTS AND DISCUSSION

In the present experiments, steroids had characteristic, dose-dependent chemotactic and repellent effects in the model cells (Figure 1). In the case of testosterone, the range of chemotactic response was wide in that low concentrations (10^{-9}–10^{-7} mg ml^{-1}) could induce chemotaxis (Figure 1a) as well as higher concentrations (10^{-4}–10^{-3}) except for the 10^{-5} mg ml^{-1} concentration, which was neutral. Progesterone had significant chemotactic—120.4% (SD ± 3.12)—character at 10^{-5} mg ml^{-1} (Figure 1b). In the lower (10^{-9}–10^{-6} mg ml^{-1}) and higher (10^{-3} mg ml^{-1}) concentrations, this hormone had a characteristic, repellent effect. Dexamethasone also had a single significant positive—120.9% (SD ± 3.86)—peak, at 10^{-6} mg ml^{-1} (Figure 1c). Lower (10^{-8}–10^{-9} mg ml^{-1}) and higher (10^{-5}–10^{-3} mg ml^{-1}) concentrations had weak repellent character. Two steroids, β-estradiol and hydrocortisone had no chemoattractant effect, however their dose-dependencies were different. β-Estradiol had the most characteristic negative—40.4% (SD ± 8.72)—peak at 10^{-6} mg ml^{-1}. Lower and higher concentrations were also repellent, except for the neutral 10^{-8} mg ml^{-1} dose (Figure 1d). Hydrocortisone was also repellent, but this was the only molecule with an almost flat dose–response curve (Figure 1e).

Data for chemotactic selection suggest a distinct molecule-dependent effect of water-soluble steroids in *Tetrahymena* (Figure 2). In the case of testosterone, the selection of chemotactically responsive cells formed a subpopulation with enhanced chemotactic behaviour compared with cells selected from the medium containing no steroid (T/C). The cells had a significantly higher chemotactic response to the selector molecule (T/T). Progesterone was the only steroid

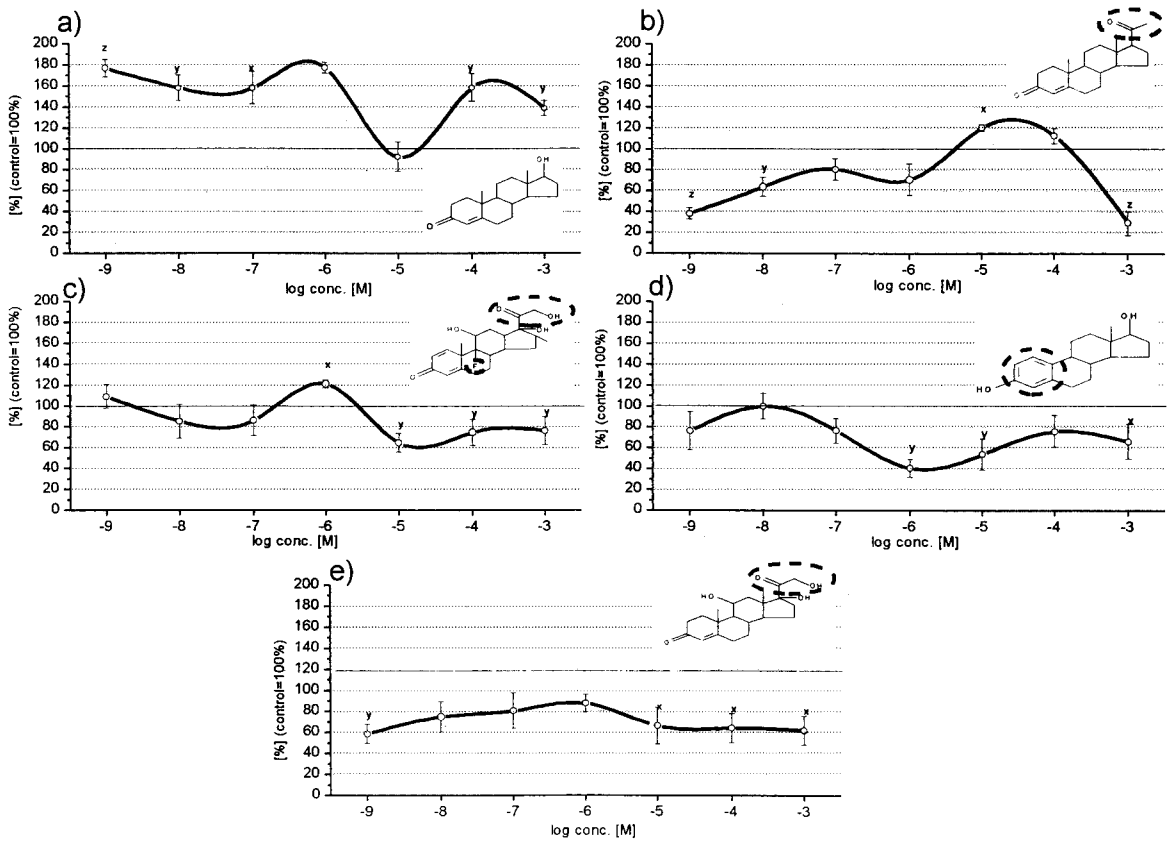

Figure 1. Concentration dependence of the chemotactic effect of steroid hormones in *Tetrahymena*: (a) testosterone; (b) progesterone; (c) dexamethasone; (d) β-estradiol; (e) hydrochortisone. x, $p<0.05$; y, $p<0.01$; z, $p<0.001$. SD values of data points are less than 8.72. Formulas of tested steroids are shown as inserts; a ring is drawn around the characteristic features of each molecule

which had an inverse tendency with respect to medium–steroid preference. The cells selected with progesterone had enhanced chemotactic activity towards the control medium, but exposure to the progesterone-containing substance could not provoke enhanced chemotaxis of these 'selected' cells.

The chemotactic selection also had a partially adverse effect in the case of hydrocortisone. Cells selected with this steroid expressed reduced chemotactic behaviour towards the control medium (H/C), but had significant chemotactic potency towards the hormone (H/H). This result is surprising since without selection, hydrocortisone was the most repellent steroid of all the molecules studied. This was not the only case of reversal by selection: cells selected with dexamethasone had no altered responsiveness to the control medium (D/C), but we could detect a positive selection to the identical signal molecule (D/D). We did not find the same in the case of estradiol, the other repellent steroid: cells selected with this molecule recognized fresh culture medium as a strong repellent substance (E/C), and this property did not change significantly in the case of exposure to the identical molecule (E/E).

Phagocytic activity of starved cells was characterized in two ways. First we used the phagocytic index, which is the average number of test particles found in the cells. The results showed that there was no significant difference in this factor compared to the control group. The values are: control, 3.08 (SD $\pm$ 0.85); testosterone, 2.99 (SD $\pm$ 0.93); progesterone, 2.93 (SD $\pm$ 0.88); hydrocortisone, 3.24 (SD $\pm$ 1.01); dexamethasone 2.97 (SD $\pm$ 0.66); β-estradiol, 3.09 (SD $\pm$ 0.71). On the distribution curves one can observe the weak effect of the treatments (Figure 3).

According to the morphometric data the chemotactic selection with different steroids was able to select different subpopulations of cells. Over the subpopulations characterized by the altered sensitivity to the identical signal molecule (see above) there were

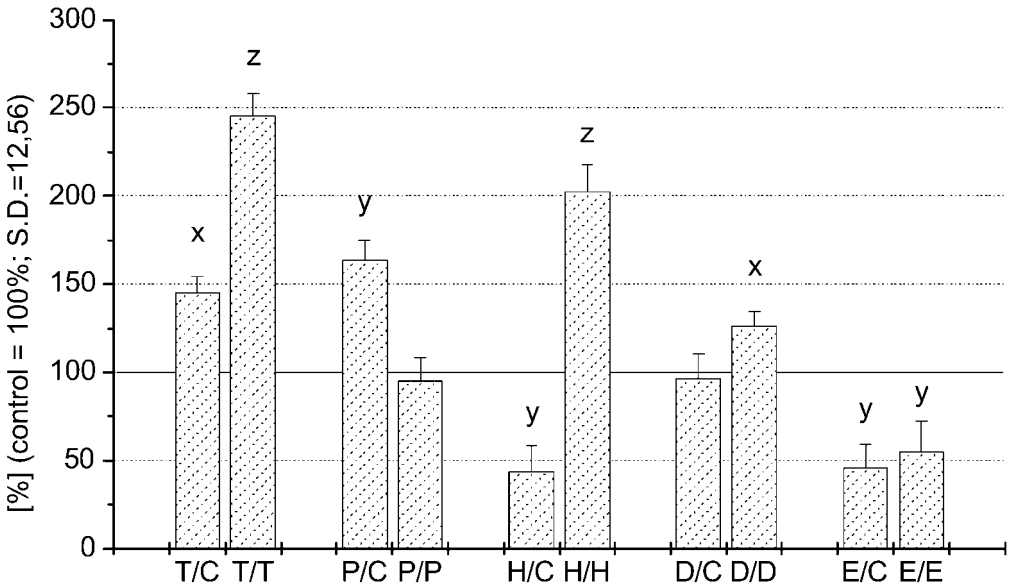

Figure 2. Effect of chemotactic selection with steroids using the maximal chemotactic concentration (C/S) of the identical steroid (see Figure 1) in *Tetrahymena* cultures. In the abbreviations the first letter represents the type of selected subpopulation, the second letter represents the steroid applied as a probe. T, testosterone; P, progesterone; H, hydrocortisone; D, dexamethasone; E, β-estradiol; C, control. Values are correlated with chemotactic activity C/C groups. x, $p<0.05$; y, $p<0.01$; z, $p<0.001$; SD values of data points are less than 13.02 in each group

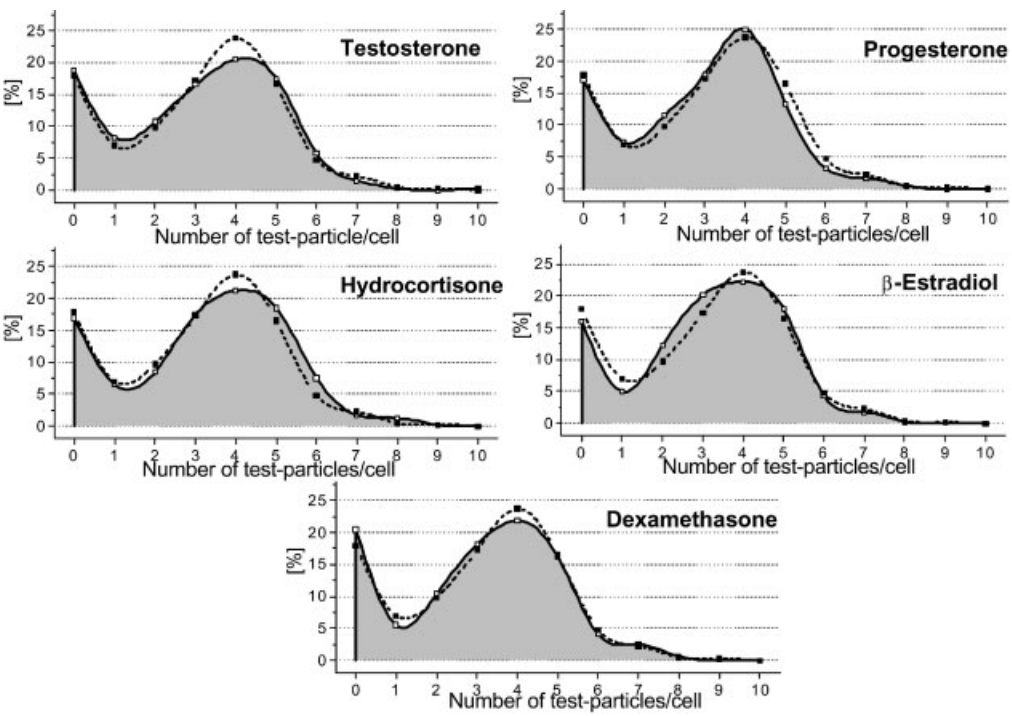

Figure 3. Phagocytotic activity of *Tetrahymena pyriformis* cultures treated with steroids. Dotted lines represent the distribution of the control group. SD values of data are less than 3.11 at each data point

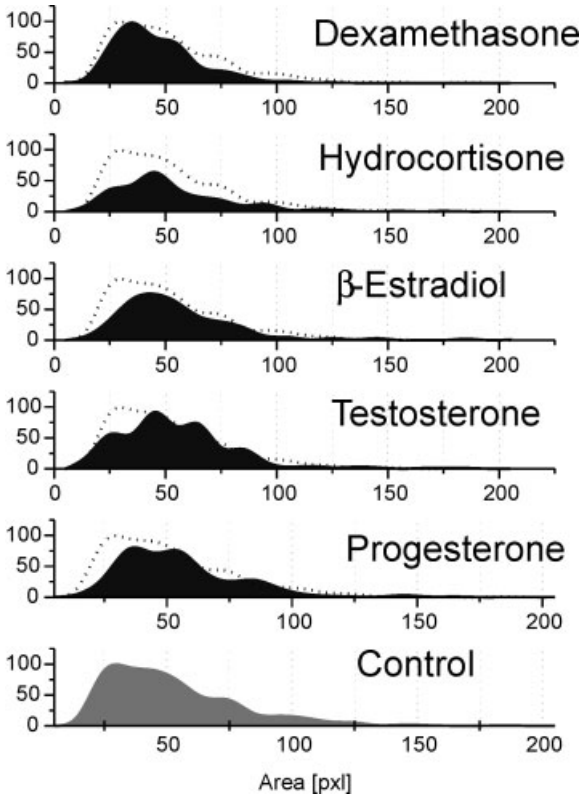

Figure 4. Computer-assisted morphometry of the area of selected subpopulations of *Tetrahymena*. Dotted lines represent the control value. SD values of data are less than 4.86 at each data point

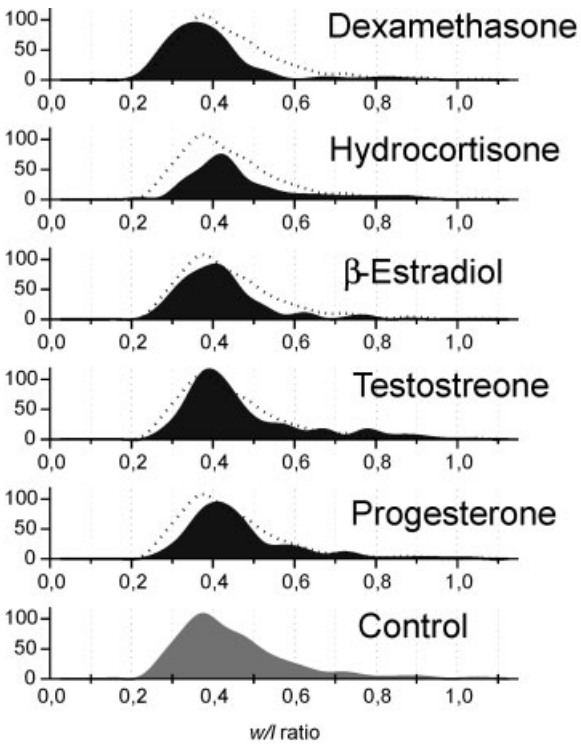

Figure 5. Computer-assisted morphometry of *w/l* ratios of selected subpopulations of *Tetrahymena*. Dotted lines represent the control value. SD values of data points are less than 0.02 at each data point

subpopulations with characteristic morphological features. In the morphometry of oval-shaped unicellular organisms, such as *Tetrahymena*, the measurement of surface area and calculation of the ratio of shortest and longest axes (*w/l*) are accepted indices. Distribution curves of computer-assisted morphometry data show (Figure 4) that the control group itself has at least two subpopulations. Dexamethasone selection has almost no morphological effect on the populations, however, the subpopulations found in the control are still present. Selection with progesterone and estradiol results in similar diversity of the population, with an increased cell size. Testosterone resulted in a very different and characteristic, 'restless' profile with three or four subpopulations. Hydrocortisone had a poor potency for selection and therefore it was difficult to identify any subgroups.

Distribution curves of the *w/l* ratios indicate the degree of elongation or roundness of the cell (Figure 5). Compared to the control we could detect that hydrocortisone and estradiol had no effect on this index, but selection with testosterone resulted in small subgroups of rounded cells. Progesterone-selected cells had a slight shift to the right which means that, on average, these cells are less elongated. There are also subgroups of rounded cells. Dexamethasone selection had an adverse effect i.e. the distribution of the *w/l* ratio is monophasic and shifted to the left, which means that this selection results in more elongated cells than in the control.

The results show that chemotactic selection with steroids has effects not only on chemotaxis, but that the growth characteristics of the subpopulations selected are also diverse (Figure 6). Although being the chemotactically most active, testosterone and progesterone had no significant effects on the growing dynamics of subpopulations (112% SD $\pm$ 3.91 and 108% SD $\pm$ 3.75 respectively), the effects of dexamethasone proved to be a slight, although it was significant inducer (135% SD $\pm$ 4.15).

Previous results[18] showed that several groups of hydrophilic signal molecules, e.g. peptide hormones (insulin, ACTH, FSH, TSH, endothelin-1, atrial natriuretic hormone, oxytocin, vasopressin), amino acid

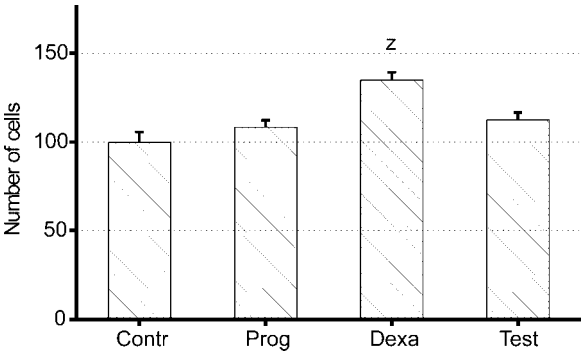

Figure 6. Growth characteristics of subpopulations of *Tetrahymena* selected with steroids and maintained for 168 h after chemotactic selection. x, $p<0.05$; y, $p<0.01$; z, $p<0.001$; SD values of data points are less than 5.69 in each group

type molecules (histamine, serotonin), lectins (ConA, Lens, Glycine max) and hydrophobic volatile oils are specific inducers or blockers of chemotaxis, which is one of the most significant membrane receptor-linked physiological responses of our model cell. In the present work our purpose was to clarify whether steroid molecules have specific signal molecule character with respect to the chemotactic responsiveness at a unicellular level of phylogeny.

Beta-cyclodextrin complexes are able to increase both the drug solubility and also its permeability through biological membranes.[26] The ligand release of these carriers provides highly specific ligand–receptor interactions.[27] It was essential therefore to apply a carrier which is able to present a ligand-rich microenvironment for the cells in the study of hydrophobic steroid molecules acting on *Tetrahymena* living in aqueous conditions.

Our results showed that a range of water-soluble steroids is able to modify the chemotactic responses of the unicellular, eukaryotic *Tetrahymena*. As chemotaxis is a direct and rapid response of the cells, our data raise the possibility that the new class of membrane-associated steroid receptors is involved in the induction of chemotaxis with steroids.[28] Our model cell was able to recognize hydroxylations of the common ring structure as well as the presence of double bounds inside and to produce different responses (see inserts of Figure 1). Thus our study of the potency of steroid molecules in which dose–response studies and molecular structures were compared, suggest that *Tetrahymena* is able to select between closely-related steroid molecules (hormones). Considering chemotactic selection we do not find the same property: except for testosterone and estradiol the behaviour (direction of chemotaxis) of selected cells differed from that of unselected cells. This is unusual compared with responses to other (amino acid or polypeptide) types of hormones.

Tetrahymena contains a steroid, tetrahymanol, in its membrane[29] in addition to dihydroepiandrosterone and minimal amounts of testosterone and estradiol.[6] Hydroxysteroid dehydrogenase is active in *Tetrahymena*.[30] After treatment with hydrocortisone, testosterone or estradiol, the prolonged presence of these hormones can be observed in the cells and even 1 week after treatment the two latter hormones can still be detected.[6] There is also a transformation of testosterone to estradiol, supporting the presence of cytochrome P_{450} aromatase enzyme at this low level of phylogeny.[31,32] Dexamethasone can interact with the regulation of enzymes in *Tetrahymena*.[33,34] These data (and those which have been mentioned in the Introduction[6–10]) make it likely that steroid hormones are not foreign to *Tetrahymena* and their characteristic effect, irrespective of the direction of this, is not surprising. However, the ability to make fine selections between the closely related steroid molecules is convincing and interesting and extends our knowledge of the selective recognition capacity of *Tetrahymena* which is already known in the case of amino acid and peptide hormones.

Summarizing the results it can be stated that steroid hormones strongly influence (positively or negatively, dependent on the structure of the hormone) chemotaxis as well as chemotactic selection of *Tetrahymena*. At the same time these hormones have a moderate, but not decisive, impact on phagocytosis, cell shape and cell growth. As testosterone and estradiol are physiological components of *Tetrahymena* and they also act in very low concentration, their chemotactic effect could be used by the cell for regulating chemotaxis in natural conditions. The experiments call attention to the selective response to steroids at a low level of evolution.

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