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Interspecific effect of Er-1 and Er-2 *Euplotes* pheromones in *Tetrahymena* *

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Abstract Pheromones are chemicals released externally by an organism that have intraspecific messenger effects on physiological functions of the cell/organism. Molecules belonging into this group are widely distributed in phylogeny; their specific activity has been described in unicellular organisms, insects and vertebrates. Pheromonal communication in vertebrates is proved to be an olfactory-dependent process and 7TM-receptors are considered as transducers in signalling. At the lower unicellular level (e.g. *Euplotes raikovi*) membrane bound forms of the pheromone isoforms are working as effective binding sites of the molecule. The present study investigated whether basic cell-physiological effects of unicellular pheromones (Er-1 and Er-2) are limited to the species producing the pheromone or they can modulate activity of other protozoa (e.g. *Tetrahymena*) or phylogenetically higher ranked models (MRC5 fibroblast or J774 macrophage). Results of investigation in *Tetrahymena pyriformis* GL show that the growth modulator effect of the two pheromones altered significantly: at 10^{-11} mol/L Er-1 had a positive effect, Er-2 an inhibitory one. Chemotactic effects of the two ligands were also distinct: Er-1 had a wide range chemorepellent character, while Er-2 had a two-peak chemoattractant character. Chemotactic selection with Er-2 showed that the receptor for this pheromone has a "short-term" character; Er-1 failed to select any subpopulation which supports our previous data, that signalling of the two pheromones is endogenously diverse in *T. pyriformis* GL. Pheromone-specificity of the elicited response indicates that *Tetrahymena* can distinguish small differences between closely related ligands (e.g. charges in Er-1 and Er-2) [*Acta Zoologica Sinica* 52 (6): 1125－1132, 2006].

Key words *Euplotes*, *Tetrahymena*, Pheromone, Swimming, Chemotaxis, Proliferation

游仆虫信息素 Er-1 和 Er-2 对四膜虫的种间作用*

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摘 要 信息素是生物体向外释放的化学物质，在细胞及生物体中具有种内信息传递的生理学功能。信息素这一类分子广泛分布于系统发生史中，它们的特异活性在单细胞生物、昆虫以及脊椎动物中均有报道。脊椎动物中信息素的信号传输已被证实是一嗅觉依赖过程，7TM-受体被认为是信号传输过程中的信号转换器。在低等单细胞生物（例如：来可夫游仆虫）的细胞膜上存在有信息素异构体，作为信息素分子的有效结合位点而行使其功能。本研究主要探讨单细胞的信息素（Er-1 和 Er-2）的基础细胞生理学作用是仅限于产生该信息素的物种，还是对其它的原生动物（例如：四膜虫）或对系统发育中分类地位较高的细胞（例如：MRC5 成纤维细胞或 J774 巨噬细胞）均具有调节活性。研究结果表明，游仆虫的两种信息素对梨形四膜虫 GL 的生长调节有显著不同的作用：当信息素浓度为 10^{-11} M 时，Er-1 具有正调控作用，而 Er-2 具有抑制剂的作用。这两种配体的趋化作用也有很不同：Er-1 具有一种广范的化学排斥特性，而 Er-2 具有一个双峰的化学吸引剂的性质。计算机检测发现，与 Er-2 的作用不同，Er-1 可略微降低被测细胞的游动速率。趋化现象的选择特性表明 Er-2 信息素的受体

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有一种“短期”的特性；而 Er-1 是不能选择任何亚种群的，这也支持了我们先前的研究数据，即这两种信息素在四膜虫 GL 内产生两种不同的信号。四膜虫对信息素特异性的反应表明四膜虫能辨别非常近似但带有微小差异的配体（如 Er-1 和 Er-2 的电荷差异）[动物学报 52 (6): 1125－1132, 2006]。

关键词 游仆虫，四膜虫，信息素，游动，趋化性，增殖

Intercellular signalling has a significant role in unicellular systems. Several substances produced by autocrine or paracrine activity serve as chemical messengers. The biological potency of these substances is rather complex: these endogenously synthesised products potentially influence metabolic activities, mating, migration or other behavioural changes of the cells. Chemical character of these molecules is also very diverse, some ligands are typically unicellular type messengers (e.g.bacterial formyl tri-and tetrapeptides), while others are good examples for the phylogenetical continuity as similar substances are present in invertebrates or vertebrates, including human beings (Vallesi et al., 1998). Appearance of the latter molecules in unicellular level is still not well described, however, there are more theories about their development. Some researchers suggest that these ligands represent a direct continuity of molecular phylogeny in signalling. Others consider these organic compounds as first trials of evolution to form a relatively wide assortment of signal molecules for selection or simply surplus materials of the biochemical pathways (Stoka, 1999).

Pheromones represent a special group of chemical messenger molecules, possessing well described physiological effects. Their intraspecific physiological effects involve primer, releaser, modulator and signalling type activities. However, in some capacities-especially as repellent ligands-they can act in interspecific relations, too. Several structurally closely related pheromones have also been described in protozoa, one of the best characterised group (Er-1, -2 etc.) is produced by *Euplotes raikovi*. Former studies on these molecules characterised their molecular structure, structure/function relationships and cell-physiological activities (Mronga et al., 1994; Ottiger et al., 1994).

Chemotaxis is one of the most basic cell-physiological responses, which has a close relation to simple feeding and mating processes alike. For this reason investigation of chemotaxis has high potential significance also for evaluating possible interspecific pheromonal effects. The unicellular eukaryotic ciliate, *Tetrahymena* is frequently used as a model in cell and molecular biology. The applicability of *Tetrahymena* is supported by its vertebrate type membrane receptors e.g. for insulin (Christopher and Sundermann, 1995) or formyl-peptides (Köhidai et al., 2003b), the inducibility of second messenger systems, such as cAMP (Csaba and Nagy, 1976), Ca^{2+}-calmodulin (Schultz and Klumpp, 1984) or IP3 (Kovács and Csaba, 1990) and the possession of metabolic pathways which also occur in more complex organisms (Connett and Blum, 1972; Köhidai and Csaba, 1985). While sensitive screening of the environment and chemotaxis is one of the most essential reactions of free swimming cells, the chemotactic responses of these ciliate model cells are considered rather physiological. A wide range of ligands can act as selective inducers of chemotaxis in *Tetrahymena*. Inorganic ions (Tanabe et al., 1980), amino acids (Köhidai et al., 2003a), as well as oligopeptides (Csaba and Kovács, 1997) or volatile oils (Köhidai et al., 1995) may function as chemotactic substances. High ligand selectivity and sensitivity of chemotactic responses in *Tetrahymena* has been proven by slight modifications (i.e. amidation, formylation) of ligands. Relevant responsiveness of *Tetrahymena* to pheromones (tricosene and bornyl acetate) has also been detected by Csaba and Kovács (1993).

In the present work our purpose was to analyse whether pheromones released by *E. raikovi* (Er-1 and Er-2) have interspecific effects on another protozoon, *Tetrahymena pyriformis* GL, with special respect to chemotaxis and other basic physiological activities. The main objectives of our work were: to characterise the potential chemotactic ability of *Euplotes* pheromones in another protozoon, *T. pyriformis* GL; to analyse other cell-physiological effects of the investigated two pheromones, whether they possess only chemotactic responsiveness or also other activities, and to compare interspecific effects of pheromones in model-cells representing different levels of phylogeny.

1 Materials and methods

1.1 Cells and culturing

Cells of *T. pyriformis* GL strain were maintained in axenic cultures containing 1% tryptone (Difco, Michigan, USA) and 0.1% yeast extract, at 28℃. Cultures were in logarithmic phase of growth, density of samples was 10^4 cell/ml.

Two type of model cells were tested as representatives of higher ranks of phylogeny: MRC-5 human fibroblast and J774 murine monocyte cell lines. MRC-5 cultures were grown in Dulbecco MEM (low glucose) medium (DMEM) (Sigma Ltd., St.

Louis, MO, USA) completed with nonessential amino acids (NEA) and 10% FCS. J774 cultures were grown in RPMI-1640 medium (Sigma Ltd., St. Louis, MO, USA) containing 10% FCS. Both types of cultures were kept at 37℃ and 5% CO_2, 16 mg/100 ml gentamycine was added to the cultures.

1.2 Pheromones and buffers

Euplotes pheromones (Er-1 and Er-2) were generous gifts of Prof. P. Luporini. In the experiments NaCl-phosphate buffer (PBS) 0.05 mol/L, phosphate buffer containing 0.9% NaCl at pH 7.2 was also used.

1.3 Chemotaxis assays

The two-chamber capillary chemotaxis assay of Leick and Helle (1983) was modified by us (Köhidai et al., 1995). In this assay we used a multichannel micropipette, where the tips of pipette filled with test substance served as inner chambers, while 96-well microtiter plates (Microtest plate 96, Sarstedt Inc. Newton, USA) -filled with *Tetrahymena* cultures (cell density 10^4 cell/ml) -served as outer chambers. Concentration dependence of chemotaxis induced hy Er-1 and Er-2 was tested in $10^{-12}-10^{-6}$ mol/L range. Effects of plain, fresh culture media as controls were tested on the chemotaxis in parallel runs. The incubation time was 20 minutes. According to our pilot experiments this is the optimal incubation time when the concentration gradient required for chemotaxis is still present in the chamber. The shorter times provided not enough cells in the sample, while at times longer than 20 min. we cannot distinguish chemotactic-responder cells from chemokinetic-responder cells. Then the samples were fixed in 4% formaldehyde containing PBS. The number of cells was determined oculometrically in a Neubauer cytometer by light microscopy.

Chemotactic behaviour of monocytes and fibroblasts was evaluated by a modified Boyden chamber technique in a Neuro Probe chamber (Neuro Probe, Cabin John, USA). This technique provides the analysis of chemotactic responsiveness of 96 identical samples. The upper chamber of the this system was filled with the suspension of cells (density of cells was 10^5/ml (J774) and 5×10^4/ml (MRC5); lower chamber — wells of a microtitration plate—were filled with the dilutions of Er-1 and Er-2 ($10^{-12}-10^{-6}$ mol/L). Adhesive, PC filters (5 μm) were used to separate the two chambers. For dilution of the test substances, fresh culture media (RPMI-1640 or DMEM) were used. Cells were incubated in an optimal environment (37℃ and 5% CO_2), for 3 h for J774 and for 4 h for MRC5 cells. After incubation, the plates were centrifuged at 2 000 r/min for 5 min, then 150 ml supernatant was removed. The number of cells was determined by the 3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide (MTT) assay (Park et al., 1987). MTT was obtained from Sigma (St. Louis, MO, USA). This assay is based on the measurements of mitochondrial succinyl dehydrogenase activity. Samples were evaluated with Multiscan ELISA reader (Labsystems), at 540 and 620 nm.

1.4 Chemotactic selection of ciliates

This technique deals with the chemotactic capacity of different signal molecules to form subpopulations of mixed cultures of cells. Investigation of chemotactic selection is a unique technique developed by us (Köhidai and Csaba, 1998). This method of study helps to distinguish the two main characteristic groups of chemotaxis receptors in the surface membrane: long-term receptors, which are genetically determined and constitutively expressed in the membrane, and short-term receptors which are induced by the presence of ligand.

In this case, the chemotaxis assay described above was applied by the pheromone proved to be chemoattractant (Er-2) in the concentration course study. Culture medium was used as negative control. After chemotaxis assay with 10^{-11} mol/L Er-2 the positive responder cells were transferred into fresh culture medium and these cultures were consecutively transferred every 48 h for 7 days. After this, the selected cultures were assayed again in the following combinations: C/C cells selected with the control substance in the first run and assayed to the control substance in the second run C/C; C/Er-2-cells selected with the control substance in the first run and assayed to Er-2 in the second run; Er-2/C-cells selected with Er-2 in the first run and assayed to the control substance in the second run; Er-2/Er-2-cells selected with the Er-2 in the first run and assayed to the Er-2 in the second run.

1.5 Computer assisted analysis of swimming behaviour

The micro-chamber was inserted onto the base of a stereomicroscope, coupled to a TV camera, lying on a thick marble shelf, deeply embedded in a thick wall to exclude both vibrations and other unwanted stimuli. Observations and recordings were made under dark-field conditions at 20 − 30 x. The cells were added by glass pipette: 1 − 2 min elapsed before recording the cellular locomotion, to let them settle down and get acquainted with their new environment. To prevent errors, a stage micrometer slide was recorded for each different enlargement on every tape used to record the steps of the experiment. The TV recorded track (Fig.1A) was recorded by hand on a triacetate sheet. The acquired track was then transferred onto a digitising pad (Fig.1B), where the successive positions of *Tetrahymena* cells were

acquired by the magnetic pen and evidenced on the PC monitor (Fig.1C). The experimental populations were recorded from 0 to 30 min and, then, left to settle; the next recording was made from 1 h to 1 h 20 min from onset and so on.

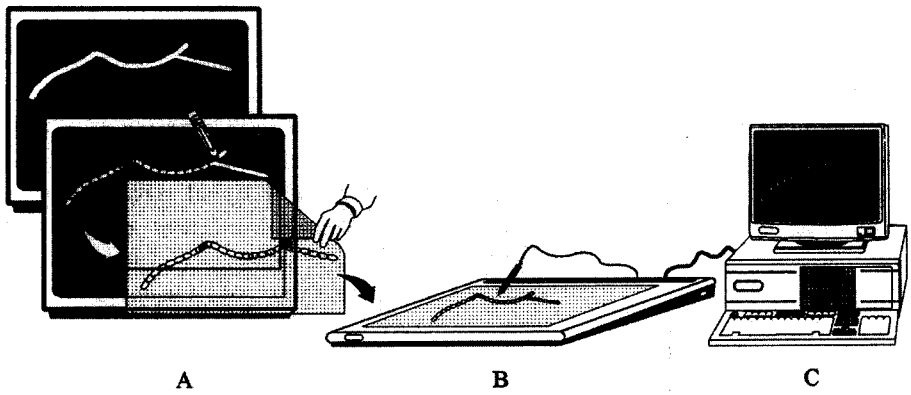

Fig.1 Micro-video-recording technology used as detection system

This procedure is very convenient because 1) it covers long periods of time, 2) it saves conspicuous quantities of videotape, 3) information loss is not significant and it must be noted that a 20 min videotape recording session contains an almost endless amount of data, certainly sufficient to analyse and draw an ethogram.

1.6 Proliferation assay

Low-density cultures of *Tetrahymena* (10 cell/ml) were treated with 10^{-12} – 10^{-6} mol/L pheromones (Er-1 and Er-2). Groups of the treated cells were kept in sterile conditions, at 28℃. The density of control and pheromone-treated samples was determined in 48-hour cultures by MTT assay (Park et al., 1987). Each data set of the experiment represented the average of counts of 8 individual parallels.

1.7 Statistical analysis of data

Experiments evaluating effects of *Euplotes* pheromones were done in 8 parallels. ANOVA method of Origin 4.0 was used to evaluate statistical significance. In the figures the following marks refer to the significant differences, x: $P<0.05$; y: $P<0.01$; z: $P<0.001$.

2 Results

2.1 Chemotaxis and chemotactic selection

Chemotactic responsiveness of cells was tested in a two-chamber capillary system, which provides a good model for the development of chemotactic environment in the case of free swimming ciliates. As Figure 2 shows the two Er pheromones elicited significantly diverse responses, Er-1 had a wide range (10^{-12} – 10^{-6} mol/L) chemorepellent effect, while Er-2 was strong chemoattractant in low and high concentrations, however, at 10^{-8} mol/L the pheromone possess significant chemorepellent character.

Chemotactic ability of the two pheromones was tested also on two model systems (MRC5 fibroblast and J774 rodent macrophage cultures) representing higher phylogenetical ranks. In these model cells the

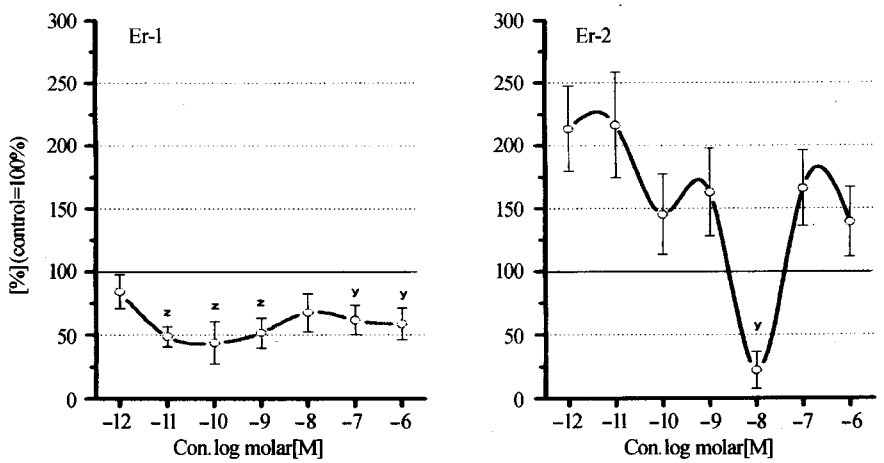

Fig.2 Chemotaxis induced by Er pheromones (Er-1 and Er-2) in *Tetrahymena pyriformis* GL cells shows high sensitivity to the close derivatives of pheromones of *Euplotes*, even in interspecific relations

tested pheromones could not modulate either positive or negative chemotactic responses (Fig.3-data gained on MRC-5 fibroblasts are shown on the figure due to the high level of the overlapping tendency of results in J774 monocytes). These results suggest, however, that interspecific chemotactic effects of Er pheromones are detectable between ciliates (see the above described chemotactic effects), the overlapping character of chemotactic effectiveness of these molecules is limited to lower-perhaps unicellular-phylogenetic levels.

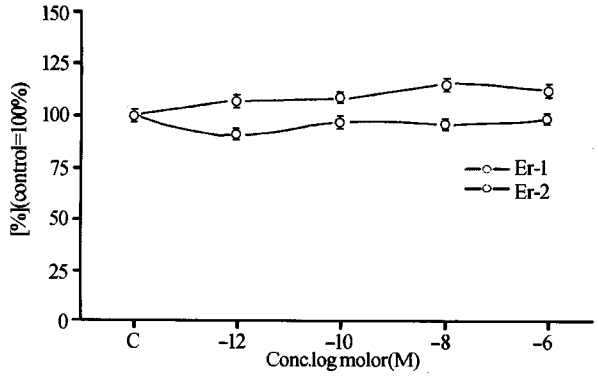

Fig.3 Chemotaxis induced by Er pheromones (Er-1 and Er-2) in vertebrate cell lines
Representative results show no responsiveness to the ciliate pheromones in MRC-5 fibroblasts.

Data obtained now support the view that receptor-level dynamics of the two pheromones are significantly different (Fig.4). Er-2 selected subpopulations show decreased chemotactic responsiveness in the 70 th generation, indicating that Er-2 receptors are working as short-term ones. In contrast, Er-1 proved to belong to the small group of ligands (e. g. melatonin) which are not only chemorepellent in general, but which form no small sub-population ready for positive selection in mixed cultures (data not shown).

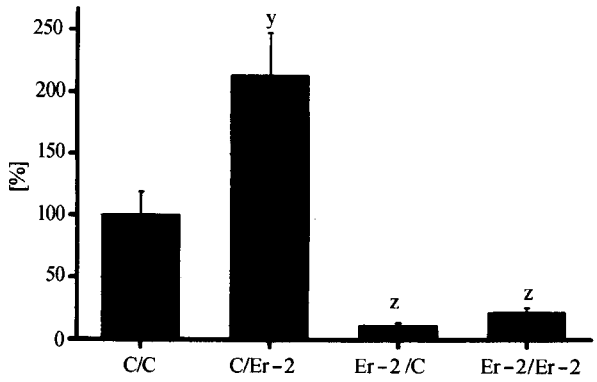

Fig.4 Chemotactic selection of *T. pyriformis* GL elicited by 10^{-12} mol/L Er-2 pheromone indicates that short-term chemotactic signalling pathways are induced by Er-2 pheromone

2.2 Computer assisted motion analysis

The data, gained by evaluation of swimming tracks of individual cells, had a good correlation with chemotaxis results. Diversities were detected by this technique: swimming velocity is weakly depressed by 10^{-12} mol/L Er-1 treatment, while 10^{-12} mol/L Er-2 could induce the velocity of the model cells (Fig.5).

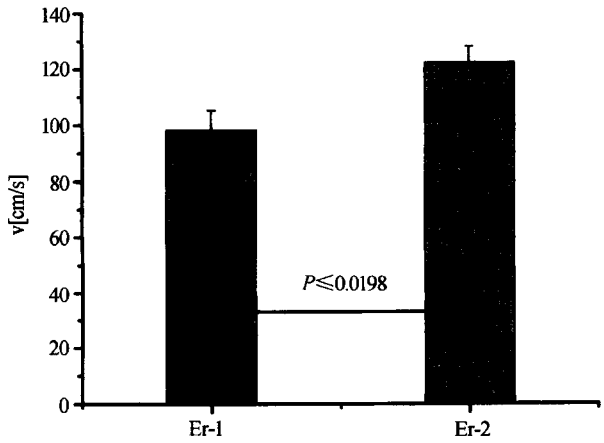

Fig.5 Computer assisted motion analysis of *T. pyriformis* GL cells treated with 10^{-11} mol/L Er-1 and Er-2 pheromones
Inserts show representative swimming tracks of cells treated with the identical Er pheromone.

2.3 Proliferation

Besides the effects on migration, another essential cell-physiological activity, proliferation was also tested. In this regard the two ligands proved to be also diverse. In this case Er-1 was a potent growth inducer (10^{-11} mol/L), while Er-2 had reverse, significant proliferation blocker effects at 10^{-11} mol/L (Fig.6).

3 Discussion

Effect of pheromones on the migratory character of cells has been reported previously in vertebrates such as horseshoe crabs (Ferrari and Targett, 2003) as well as in unicellular organisms (Kuhlmann et al., 1997). Until now, mating and related phenomena (aggregation, changed behaviour etc.) provided the main focus of such experiments. In the present study the objective of our work was to evaluate whether two structurally close Er pheromones possess also interspecific effects and whether the supposed effects are limited to the lower levels of phylogeny.

Chemotactic effects of Er pheromones in *Tetrahymena* proved to be ligand specific, as Er-1 could elicit significant chemorepellent responses, while the profile of its close derivative (Er-2) was significantly different. Studies of other chemoattractant ligands (e.g.endothelins, bradykinins) have previously

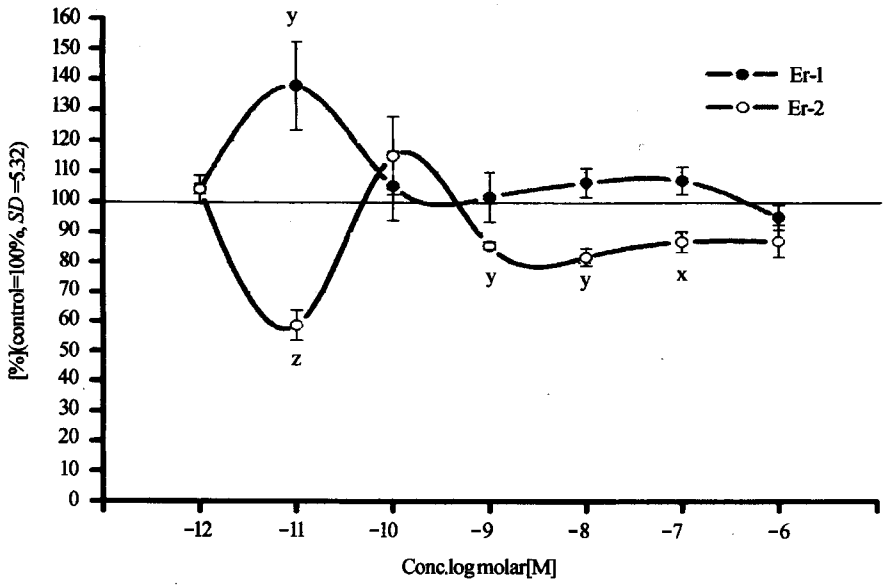

Fig.6 Diverse effects of Er-1 and Er-2 pheromones on the proliferation of *T. pyriformis* GL

demonstrated that chemotactic ligands can act via different subpopulations of receptors in *Tetrahymena* (Köhidai, 1999). The above described moiety of the receptor-ligand interaction is also conceivable for Er-2 as the ligand has characteristically different effectiveness at low (10^{-12} – 10^{-9} mol/L), medium (10^{-8} mol/L) and high (10^{-7} – 10^{-6} mol/L) concentrations.

As the two pheromones investigated possess rather close structural homology (Raffioni et al., 1992), the significant diversity detected may indicate that modifications in the structure of pheromones can reverse their biological effects. Furthermore this diversity also indicates that *Tetrahymena* has an underlying ability to sense and to distinguish between "non-self" signal molecules.

Our results underline the possibility of interspecific actions of the unicellular pheromones investigated. While the two *Euplotes* pheromones we tested can elicit interspecific cell-physiological activities in *Tetrahymena*, both ligands were neutral in the tested concentration (10^{-12} – 10^{-6} mol/L) in the model cells of vertebrates (fibroblasts and monocytes). In other words, it can be affirmed that there are limits to the interspecific activity of Er-pheromones: they could modulate only other protozoa, while phylogenetically higher ranked models did not react to these ligands.

Mating pheromones may also be considered as selector signals encouraging competition between gametes and selection of the fittest cells for fertilization (Pagel, 1993). The results presented here, on the selector ability of Er pheromones support the above-mentioned role of these ligands. Moreover they show that even positive or negative chemotactic character of the investigated pheromones seems to be a short term one, because its genetically determined structures are missing in the interspecific way of signalling.

Computer assisted analysis provides a significant method of measuring locomotion in unicellular organisms (Ricci et al., 1995). Following the pre-treatment of cells with the two pheromones, the identical pheromones could induce significantly higher responses-speed of cells in the Er-2 pre-treated group than in the Er-1 pre-treated one. This observation provides further support for the conclusion that the two structurally related pheromones possess diverse behavioural effects.

Our data on the proliferation promoter effects of Er-1 agree with previous results describing the autocrine mitogenic effects of Er-1 (Ortenzi et al., 2000). On the other hand, diverse effects of Er-1 and Er-2 on the proliferation and chemotaxis of *Tetrahymena* underlines the significance Er pheromones as potential, membrane bound transmitters of pheromone action. In this context however, it is suggested that both Er ligands are bound to the membrane, but their mediator ability is significantly diverse in respect of chemotaxis (Er-2 works as an attractant) and in proliferation (Er-1 elicits positive intracellular responses) (Ortenzi et al., 2000).

Where the effects of the two *Euplotes* pheromones are concerned, we should also discuss possible correlations between the fine molecular structure and the chemoattractant moiety of the two ligands. However, the two pheromones are closely related structurally; the up-down-up bundle of three short helices of Er-1 and Er-2 proved to be highly homologous, some parts of Er-2 have been described as unique structural features by NMR (Ottiger et al.,

1994). Among these characters a well-defined N-cap of the first helix and a deletion in the second helix have been described; both are potentially responsible for modification the constitutional alpha helix of Er-1 to 3_{10}-helix. Over these changes two conformational variants of the C-terminal tetrapeptide might also result in changes in biological activity of the Er-2 (Ottiger et al., 1994).

Significance of the above described interspecific signalling character of Er-pheromones seems to exceed the overlapping chemotactic effects of two peptide type ligands or understanding of their pheromonal activity based on proteomics. Pheromones represent principal members in the phylogeny of signal molecules. The chemically diverse group of these ligands (short and longer chain peptides, linear and ring structures of lipids etc.) elicits a variety of biological responses. Their production and release provides the possibility of the most simple, however, still persisting signalling mechanisms: autocrine activity elicits self-inducer (e.g. mitotic) effects, while paracrine actions of these molecules represent the first steps in the development of mating behaviour. Cellular differentiation (Starr and Jaenicke, 1974), aggregation (Van Haastert et al., 1982) and effects on the sexual behaviour (Dulka et al., 1987) are also induced or influenced by this family of signal molecules on different levels of phylogeny ranging from unicellular organisms to mammals.

On the basis of these phylogenetic backgrounds, the diverse and specific activities of the two closely related pheromones are more underlined. Present results show that even the most ancient cell-physiological activities-chemotaxis, proliferation of protozoa are based on highly specific recognition of the ligands. Diverse dynamics of the responsible Er-receptors (subclasses of receptors) in *Tetrahymena* call attention to the possibility of a parallel, overlapping development of pheromone signalling systems in different taxa of protozoa.

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