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SHORT COMMUNICATION

EFFECT OF VASOACTIVE PEPTIDES ON *TETRAHYMENA*. CHEMOTACTIC PROPERTIES OF ENDOTHELINS (ET-1, ET-2, ET-3, FRAGMENT 11–21 OF ET-1 AND BIG ENDOTHELIN-1): A SHORT-TERM INDUCIBLE SIGNALLING MECHANISM OF CHEMOTAXIS

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The unicellular *Tetrahymena* is a sensitive model for the study of chemotaxis induced by endothelins. In short-term chemotactic responses, ET-2 and ET-3 were chemorepellent, compared to the referent control (culture medium) and chemoattractant, ET-1. These differences suggest that the change of some aromatic residues in the loop region (residues 5–9) of the ET-1 abrogates its chemoattractant character. The response of *Tetrahymena* is highly selective, since substitution of two amino acids are enough to cause this alteration in (behavioural) response. Such a change seems to be more important than the loss of the entire first 10 amino acids (in ET-1 fragment 11–21), since after this it acquires some chemoattractive effect of ET-1. Selection with ET-3 rigorously stimulated the cell's responsiveness to the medium, this ability was abolished by the repeated ET-3 treatment. Big endothelin-1 was repellent in all concentrations. These experiments demonstrate the very sensitive discriminating capacity of chemotactic responsiveness at a low level of phylogeny. Chemotactic selection with endothelins underlines the possibility that there may be separate mechanisms responsible for the short-term chemotactic responses and the long-lasting effects of chemotactic selection.

KEYWORDS: *Tetrahymena*; chemoattraction; selection; endothelins; phylogeny; hormones.

INTRODUCTION

The ability to recognise and select different molecules had a significant role in the early evolution of molecular development in living organisms. Because the detection of chemical signals was essential to both unicellular and multicellular organisms, the development of ligands and their proper receptors represented an interaction-dependent event of molecules. According to Lenhoff's theory (Lenhoff, 1968), molecular selection played a key role in this process and only the efficiently 'selected' signal molecules were able to induce metabolic or other pathways. This mechanism is rather complex, involving structural matching between ligand and receptor. The present day ligand–receptor complex interactions are thought to result from the above mentioned process (Csaba, 2000).

In the study of the evolution of signalling, one of the most frequently applied model cells is the eukaryotic, ciliated protozoan *Tetrahymena*. It is homologous to higher ranked organisms with membrane receptors (e.g. insulin receptor, Christopher and Sundermann, 1995; Csaba, 2000), second messenger systems, such as cAMP (Csaba and Lantos, 1976), cGMP (Kőhidai *et al*., 1992), inositol lipids (Kovács and Csaba, 1994) or Ca^{2+}-calmodulin system (Schultz *et al*., 1983). A wide range of signalling molecules, such as peptide

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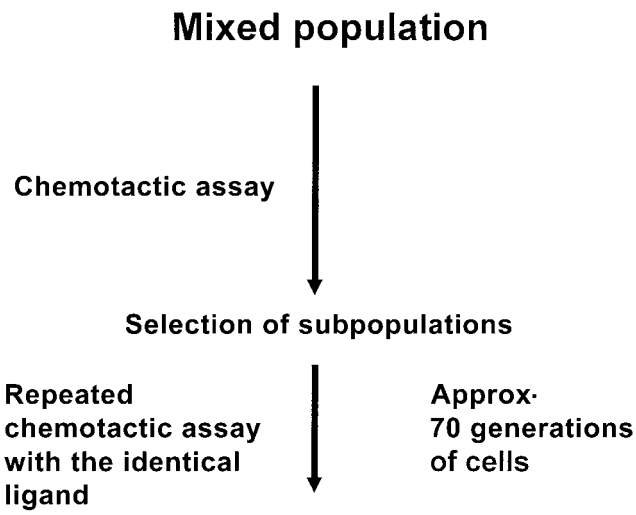


Fig. 1. General scheme of chemotactic selection.

hormones, cytokines, lectins or volatile oils can elicit chemotactic effects in *Tetrahymena* (Kőhidai, 1999). Other results showed that chemotaxis has an essential and discriminatory role in nourishment of free swimming ciliates (Kőhidai, 1999). On the basis of these observations, a new technique, 'chemotactic selection' was developed (Kőhidai *et al.*, 2000) (Fig. 1). This technique applies chemotactic assay as a probe for selection of cells possessing enhanced chemotactic responsiveness. The selected subpopulations are cultivated for 10 s of generations and with repeated chemotaxis assays, provide the possibility to evaluate whether the ligand-induced chemotactic response is a short or long-lasting character of the organism tested.

Previous data showed that endothelin-1 (ET-1) has also characteristic effects on *Tetrahymena* (Kőhidai and Csaba, 1995): it has a dose-dependent inhibitory effect on growth and phagocytotic activity of cells. Endothelin-1 acts as a chemoattractant in a wide range (10^{-15}–10^{-9} M) in *Tetrahymena*. Our study also demonstrated that these single celled ciliates also have the ability to synthesise ET-1-like substances (Kőhidai and Csaba, 1995).

Small molecules (amino acids and short peptides), oligopeptides and proteins all represent significant steps in the phylogeny of signal molecules. In the present study, we used the 21 amino acid residue-containing endothelin (ET) family to collect data about the compositional requirements of signal molecule structures. The objectives of our study were to elucidate whether (1) the members of endothelin family (ET-1, -2, -3, fragment 11–21 of ET-1 and big endothelin-1) have characteristic chemotactic effects at a lower eukaryotic level on the model cell *Tetrahymena*. (2) progenies of subpopulations selected with chemotaxis to ET have an altered response to the identical ET isoform.

MATERIALS AND METHODS

Tetrahymena pyriformis GL cells were cultured in axenic cultures containing 1% tryptone and 0.1% yeast extract (Difco, Michigan, U.S.A.). Cells of logarithmic growth phase (48 h) were assayed. Cell density was 10^4 cell/ml. The hormones used were endothelin-1 (ET-1), endothelin-2 (ET-2), endothelin-3 (ET-3), fragment 11–21 of endothelin-1 and big endothelin-1 (big ET-1) (Sigma, USA); NaCl-phosphate buffer (PBS) 0.05 M phosphate buffer containing 0.9% NaCl at pH 7.2 was also used. The chemotactic activity of *Tetrahymena* cells was evaluated with a two-chamber, capillary chemotaxis assay modified by ourselves (Kőhidai and Csaba, 1995). The incubation time was 20 min, a relatively short time necessary to measure the pure chemotactic responses and prevent the contamination of samples by chemokinetic responder cells. In the concentration course study, the chemotactic responses were tested in the range 10^{-15}–10^{-9} M. Fresh culture medium was applied as the control substance since the inorganic environment has not demonstrated controlled effects on the growing of cilia (rapid swimmer cells; Nelsen and Debault, 1978) and can significantly alter the composition of the surface membrane (Csaba *et al.*, 1992). After incubation, the samples of the inner chamber were fixed in 4% formaldehyde solved in PBS. The number of cells was determined using a Neubauer haemocytometer. Chemotactic selection is a technique which deals with the chemotactic capacity of different signal molecules to form subpopulations from mixed cultures of cells (Kőhidai *et al.*, 1998). First we applied the chemotaxis assay described above. At the end of incubation, the positive responder cells were transferred to fresh culture medium for cultivation. Both cultures selected with a known endothelin isoform (E) and fresh culture medium as control (C) were consecutively transferred in every 48 h. The chemotactic response of cultures was determined again in the following combinations: responses of cultures selected with

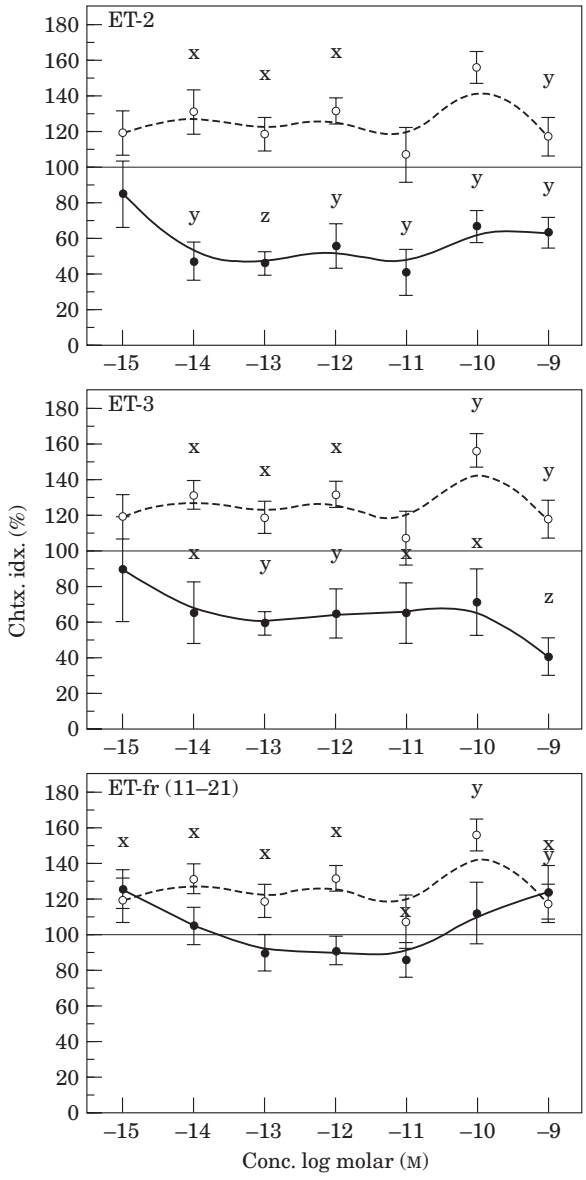


Fig. 2. Concentration range study of chemotactic responsiveness of *Tetrahymena* to ET-2, ET-3 and fragment 11–21 of ET-1. Dotted line represents chemotactic potency of referent ET-1 derivative. (x, $P<0.05$; y, $P<0.01$; z, $P<0.001$).

endothelins (E) or the control substance (C) were tested in relation to the identical endothelins (E/E; C/E) or to the control (E/C; C/C). Statistica and Origin 4.0 were used to analyse data. Values of t-probe are shown in the figures: x, $P<0.05$; y, $P<0.01$; z, $P<0.001$.

RESULTS AND DISCUSSION

There were significant differences in the chemotactic activity of the three endothelin derivatives (Fig. 2) In contrast to the wide-range chemoattractant character of ET-1 which was demonstrated previously (Kőhidai and Csaba, 1995), ET-2 and ET-3 are chemorepellent in the 10^{-14}–10^{-9} M range. Fragment 11–21 of ET-1 was chemoattractant at 10^{-15} M and 10^{-9} M. However, in the range of 10^{-14}–10^{-10} M, this peptide was neutral. Big endothelin-1, the precursor molecule of the hormone, had a chemorepellent character in wide ranges (10^{-12}–10^{-10}; 10^{-8}–10^{-6} M) of the tested concentrations (Fig. 3).

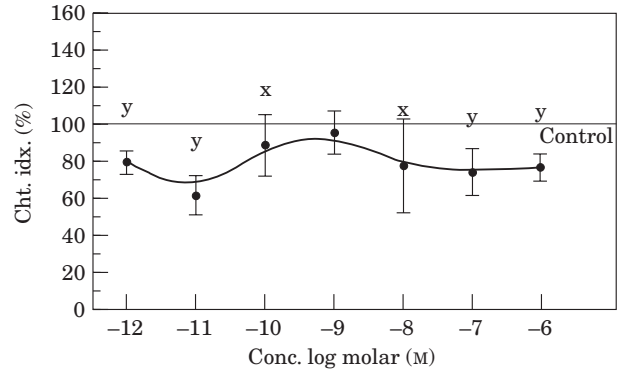
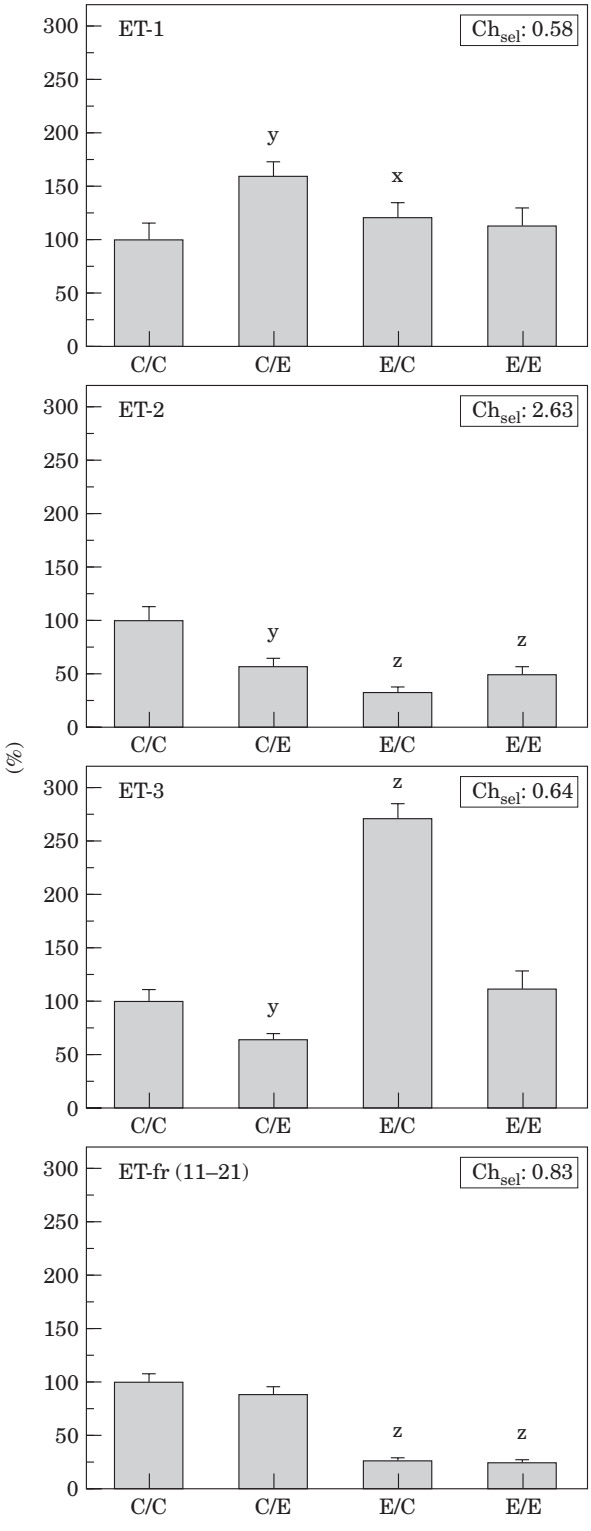


Fig. 3. Concentration range study of chemotactic responsiveness of *Tetrahymena* to big ET-1. (x, $P<0.05$; y, $P<0.01$; z, $P<0.001$).

Investigations of chemotactic selection with endothelins showed that although their selector ability was based upon induction of hypothetical chemotactic receptors, the long-lasting effects of these selections in chemotaxis might be different from the short-term (acute) effects of the molecules (Fig. 4). For evaluation of these differences, the calculation of chemotactic selection coefficient (Ch_{sel}) was used, which was calculated from ratios of chemotactic responsiveness of cells from selected and non-selected groups, considering whether they were compared with control substance or medium containing the selector endothelin (Kőhidai *et al.*, 2000). This means that Ch_{sel} considers each relation between the four groups studied with one of the ET molecules. In the case of ET-1 and fragment 11–21 of ET-1, the chemotactic responsiveness in the offspring generation (about the 70th generation after selection) was reduced, which is portrayed with low Ch_{sel} values (0.58 and 0.83 respectively). In the case of ET-3, the selection itself did not reduce (in fact, slightly enhanced) the responsiveness in the ET-3 selected cells to the identical endothelin isoform, but responsiveness of these subpopulations to other ETs was also enhanced in a significantly higher value. These shifts of responsiveness explain the low Ch_{sel} value (0.64) of this

endothelin. Although the concentration course study showed that ET-2 is chemorepellent, selections with the hormone showed that in mixed populations there are always cells, although in low number, which prefer substances considered as repellent ones. Selection of these cells seems to be not only possible but, according to their chemotactic responses and high Ch_{sel} value (2.63), this is a long-lasting characteristic of the subpopulation.



On the basis of ligand-specific results of chemotactic selection described previously (Kőhidai and Csaba, 1998) and the data described above, we propose the possibility that in the signalling mechanisms of endothelins that resulted in chemotaxis, there are partially or basically different mechanisms facilitating the short-term effects of the hormone or its long-lasting selection in *Tetrahymena*. Although the mechanisms mentioned above are still proposed, biological characteristics of ciliates present some areas for the advanced study of the phenomenon. In the background effects of the short-term action, we should mention the changes of membrane fluidity induced by ligands (Kőhidai *et al.*, 1986) or short-term modulatory effects on the endogenous ET-1 synthesis of *Tetrahymena*.

Observations on the signalling mechanisms of *Tetrahymena* can be used as evolutionary models. From the above mentioned experiments, it seems clear that the discriminating capacity of *Tetrahymena* is excellent and similar to mammalian cells. Difference in two amino acids of the 21 amino acid containing molecule is enough for the reversion of the response (between ET-1 and ET-2). However, this reversion is not stronger after the change of more (six) amino acids (between ET-1 and ET-3). Considering the quality of the two amino acids (in position 6,7) changed in ET-2 related to ET-1 (Leu-Met to Trp-Leu), there was no difference in the effect of the first ET treatment, if in this position Tyr-Lys appeared (in ET-3). This makes it likely that Leu-Met was the key amino acid combination causing attraction, and the change of this pair to any of the other amino acids causes repellance. This suggests that sequences upstream from the characteristic helical structure of the molecule are important in interaction with chemoreceptors, and maybe even one aromatic residuum might decrease the chemotactic character of the molecule.

These two amino acids seem to be more important in causing chemokinesis than the loss of the first 10 amino acids (i.e. in fragment 11–21 of ET-1) since this ET-1 fragment yet can provoke chemoattraction (in quantity similar to ET-1) at the two ends of the concentration curve studied, and the

Fig. 4. Chemotactic selection of *Tetrahymena* with ET-derivatives. C, control, E, endothelin derivative; first letters represent substance applied at chemotactic selection, second letters represent substance of second chemotactic assay (x, $P<0.05$; y, $P<0.01$; z, $P<0.001$).

repellent effect by the concentrations between the ends is not significant. This also suggests that not only the loop bordered by disulphide bonds between Cys3–Cys11 is required for induction of chemotaxis and not exclusively the two amino acids mentioned have a role in the chemoattractive effect of the molecule, since the helical part of ET-1 molecule itself is able to induce chemotactic responses even in low (10^{-15} M) concentration. In mammals the carboxy-terminal residues 18–21 are also needed for ETB receptor binding (Forget *et al.*, 1996; Rovero *et al.*, 1998).

Since big ET-1 is a hormone precursor, we did not study selection. However, this relatively large molecule (38 amino acid containing) was consequently repellent, in contrast to the attractive effect of ET-1. This result is in agreement with observations in mammalian cell, where the cleavage of big endothelin molecule is essential for its vasoconstrictor effect (Brooks and Ergul, 1998; Cronin and Wallace, 1999). Nevertheless, we cannot explain why the big precursor was repellent and not a neutral one, as would have been expected.

It seems to be interesting that only ET-3 selected cells appeared to be significantly attracted to the medium itself. This increase of responsiveness was so remarkable that it allows us to surmise the formation of a new property (possibly a receptor) under the effect of the first ET-3 treatment. However, it is very difficult to explain the abolition of this property at the second encounter with endothelin.

The results demonstrate that at a 'low' level of phylogeny, *Tetrahymena* has a highly sensitive discriminatory capacity for recognising related molecules, and there is a possibility of selection of cells for chemotactic properties. The data gained should help the understanding of the evolution of the signalling system, however the authors expect more information concerning the mechanisms from their ongoing molecular genetical investigations, in the near future.

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