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EFFECTS OF DIPEPTIDES CONTAINING THE AMINO ACID, PROLINE ON THE CHEMOTAXIS OF *TETRAHYMENA PYRIFORMIS*. EVOLUTIONARY CONCLUSIONS ON THE FORMATION OF HORMONE RECEPTORS AND HORMONES

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Our investigations demonstrate that proline-containing dipeptides can provoke a chemosensory response from the unicellular *Tetrahymena pyriformis*. The chemotactic effects of the dipeptides have a close relationship with the side chain and the lipophilicity of the amino-terminal amino acid. Comparison of 'mirror' variants of proline-containing dipeptides points to the fact that dipeptides with small side chain and non-polar character amino acids (Gly-Pro, Ala-Pro) are preferred on the amino-terminal end. In the case of amino acids with very variable side chains, small (Pro-Gly) and the large side chain and non-polar character amino acids (Pro-Leu, Pro-Phe) on the carboxyl-terminal end can induce significant chemotactic responses. With valine on any terminus the proline-containing dipeptide induced a weak repellent effect.

KEYWORDS: dipeptide; hormone evolution; proline; receptor evolution; *Tetrahymena*

INTRODUCTION

According to the current literature, the molecular evolution of signalling required a bilateral fitting of signal molecules and binding sites (receptors). Lenhoff's theory on this process (Lenhoff, 1974) suggests that at the very basic levels of recognition molecules of nourishment were the only molecules recognized by the cells. Consecutive selection of these food molecules—and their binding sites—resulted in the selection of different groups of signal molecules possessing a special effect by binding sites in the surface membrane. At the early stages of this process it was postulated that the small molecular 'units', like short peptide chains, might have dual capacity: they were consumed as nourishment but their identical structure to the acting parts of longer peptides possibly provided special signal character to these molecules (Leick, 1992; Wheatley *et al.*, 1993).

Previous results revealed that the unicellular ciliate, *Tetrahymena* is a good model for investigation of backgrounds of signalling and the evolutionary aspects of it. The suitability of the model was demonstrated on different levels of signal transmission: hormone binding at a receptor level (Christopher and Sundermann, 1995*a*,*b*; Kovács and Csaba, 1990; Sekiya and Nozawa, 1983), composition of its membrane (Renaud *et al.*, 1991), essential membrane-linked processes such as receptor down regulation (Csaba and Köhidai, 1986); changes of membrane potential induced by signal molecules (Köhidai *et al.*, 1986); presence and inducibility of second messenger systems cAMP (Csaba and Lantos, 1976), cGMP (Köhidai *et al.*, 1992), Ca-calmodulin (Schultz *et al.*, 1983), IP_3 (Kovács and Csaba, 1994) and other key enzymes like PKC (Hegyesi and Csaba, 1994). Moreover, the basic physiological responses such as growth (Wheatley *et al.*, 1993), phagocytosis (Orias and Rasmussen, 1979) and metabolism (Köhidai and Csaba, 1985) also point to this unicellular organism may be being a useful reference in receptor and hormone evolution research.

In addition to the activities listed above, the chemosensitive character of *Tetrahymena* provides a useful physiological tool to investigate the study of the evolution of signal molecules. According to previous studies our model cell is highly sensitive (by attractant or repellent response) to the real

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signal molecules, e.g. ACTH, TSH, FSH (Köhidai *et al.*, 1994; Köhidai and Csaba, 1996); it can distinguish slight differences in the molecular forms acting, e.g. insulin (Csaba *et al.*, 1994) vasopressin and oxytocin (Köhidai and Csaba, 1996); and it fails to present adequate chemotactic response to non-hormone molecules, e.g. protamine sulphate (Köhidai *et al.*, 1994).

In our previous experiments, we discovered the significant role of proline in hormonal imprinting (Csaba, 1980; Christopher and Sundermann, 1995*a*,*b*; Csaba, 1994). A synthetic opioid pentapeptide could provoke imprinting of Chang liver cells, while the prolin-free tetrapeptide could not do it (Csaba *et al.*, 1987). In other experiments—in *Tetrahymena*—certain proline dipeptides developed imprinting except Pro-Pro (Csaba and Kovács, 1994). If we suppose that during evolution imprinting had a role in the selection of signal molecules the presence of proline must be considered. This is supported by the experiments of Ishii (Ishii, 1988), when studying the evolution of gonadotropic hormones, he found that the progressive increase of proline residues in the luteotropic hormone is responsible for the species specificity of the hormone. These were the results which directed us to study the chemosensory effects of proline dipeptides.

In the present work our purpose was to characterize the chemoeffector character of 'mirror variants' of dipeptides. In these short molecules the unique amino acid, proline was the constant composing unit while the five other amino acids (alanine, glycine, leucine, phenylalanine and valine) were the partners. This was reasoned by earlier observations, when peptides having proline in different ends of the chain behaved disparately (Kovács and Csaba, 1994).

The main problems to be answered were:

(i) is there any characteristic chemoeffector effect of the amino acid proline composing dipeptides when it is in the carboxyl- or amino-terminal position?
(ii) how can the partner amino acid express an influence to the chemoeffector character of dipeptide with a constant component, proline?
(iii) is there any physico-chemical character of the dipeptide (or composing amino acid) which correlates with the chemoeffector effect elicited?

MATERIALS AND METHODS

Populations of *Tetrahymena pyriformis* GL in the logarithmic phase of growth were cultured in 0.1% yeast extract containing Tryptone medium (Difco, MI, U.S.A.).

The applied dipeptides were:

proline on the amino-terminal: Pro-Ala, Pro-Gly, Pro-Leu, Pro-Phe, Pro-Val; proline on the carboxyl-terminal: Ala-Pro, Gly-Pro, Leu-Pro, Phe-Pro, Val-Pro.

Previous experiments demonstrated that 10^{-6} M is one of the most suitable concentrations for provoking changes in binding, second messenger activity and chemotactic responses, by peptide hormones, e.g. insulin (Csaba *et al.*, 1994; Kovács and Csaba, 1994; Kovács *et al.*, 1989). The dipeptides were applied in a 10^{-6} M concentration. All dipeptides were obtained from Sigma Chemicals (St Louis, MO, U.S.A.).

We modified the two-chamber capillary chemotaxis assay of Leick and Helle, 1983) (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 microtitration plates—filled with *Tetrahymena* cultures—served as outer chambers. The incubation time was 15 min. 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 15 min we could not distinguish chemotactic-responders from chemokinetic-responder cells. Then the samples were fixed in 4% formaldehyde containing PBS. The number of cells was counted in a Neubauer cytometer by light microscopy.

All experiments were repeated five times. Data were evaluated by the statistical routines of SigmaPlot 4.0 and Origin 2.8, using Student's *t*-test.

RESULTS

Dipeptides with carboxyl-terminal proline

Chemotactic potency of molecules differed according to the 'variable' amino-terminal region of these molecules (Fig. 1). Dipeptides possessing chemotactic potency were Gly-Pro (290%), Ala-Pro (262%), Pro-Pro (210%) and Leu-Pro (162%). Effect of Phe-Pro was neutral (100%), while Val-Pro had a slight, however statistically not significant repellent effect (90%).

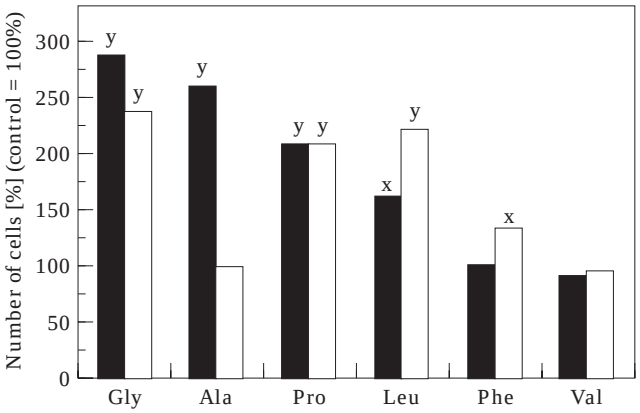

Fig. 1. Comparison of chemotactic potency of dipeptides with corresponding 'mirror' peptides. Filled columns—dipeptides with proline on the C-terminus; open columns—dipeptides with proline on the N-terminus. (x $P<0.05$; y $P<0.01$; SD is lower than 10%.)

Dipeptides with amino-terminal proline

When the standard component of the molecule—proline—was on the amino-terminal position, the chemotactic activities were also registered (Fig. 1). The most chemotactic dipeptides were Pro-Gly (240%) and Pro-Leu (222%), Pro-Pro (210%) but Pro-Phe also elicited chemotactic responses (133%). Two dipeptides Pro-Ala (100%) and Pro-Val (95%) had a neutral chemotactic effect.

DISCUSSION

Short peptide chains, such as dipeptide molecules, provide very special tools for the investigation of signalling concerning the requirements of size, polarity or acting surface of the ligand. In 'mirror variants' of dipeptides, only the carboxyl-terminal and amino-terminal position of the two amino acids was changed. This way we could study the effects of proline in two terminal positions and by this, there was a possibility of distinguishing which position (amino- or carboxyl-terminal) is more advantageous for the successful signalling. Building special amino acids into these dipeptides provides further advantages, this technique making the evaluation of effects detected less ambiguous.

Proline fulfils our requirement mentioned above. Physicochemical features of proline (it has the lowest hydropathy index—1.6—among the amino acids tested) are special. The structure of proline differs sharply from that of other amino acids in that its side chain is bonded to the nitrogen as well as to the α-carbon of the central compound. Its -NH- (imino) component in the rigid, pyrrolidine ring makes the rotation of N-Cα bound impossible, therefore proline is very rare in alpha-helical structures (Lehninger *et al.*, 1993). However, proline is responsible for reverse turns in proteins (Rawn, 1989). These features make this amino acid a good candidate to be a constant (reference) component of dipeptides in investigations of short chain peptides.

Table 1.
Residual volumes and lipophilicity of amino acids tested

Amino acid	*Residual volume Φ (ml/mole)*	*Lipophilicity*
Glycine	36.1	− 3.0
Alanine	53.2	− 2.67
Proline	73.6	− 2.62
Leucine	100.1	− 1.72
Phenylalanine	113.9	− 1.17
Valine	83.9	− 2.29

Changes in the volume of the amino-terminal part of dipeptides resulted in a gradual decrease of the chemoeffector character of the molecule. In respect of residual volumes (Φ) (Zamyatin, 1972) the smallest amino acids Gly and Ala (36.15 and 53.2 ml/moles, respectively) could elicit the strongest chemoattractant responses and the value of the next Pro-Pro dipeptide (residue volume 73.6) also fits to this activity–volume relation. Increasing volumes of the amino-terminal part of molecules Leu-Pro and Phe-Pro (100.1 and 113.9, respectively) follows the above-described reciprocity of chemotactic effect and residual volume, however, these molecules have a significantly depressed—but chemotactic—effect on *Tetrahymena*. The responses detected have good correlation not only with the size of the amino-terminal part of the dipeptide but there is also a close relation between the lipophilicity (van de Waterbeemb *et al.*, 1994) and the chemoeffector character of the molecule (Table 1).

Valine is the only amino acid which does not elicit responses in accordance with the trends described above (residual volume 83.9). Either it is seated on the carboxyl-terminal or amino-terminal position of the dipeptide valine itself or the intramolecular interactions of valine and proline result in a weak, but repellent moiety. We have no evidence about this effect since the size, lipophilicity, molecular charge or solubility indexes of the molecules do not point to any such special character.

In the C-terminal variants we could not detect the gradual relationships between chemoeffector character and the length of the side chain or lipophilicity as previously mentioned with the amino-terminal variants. Nevertheless there were also dipeptides with chemotactic potency. Both Pro-Gly and Pro-Leu had a strong chemotactic capacity. In respect of Gly we can conclude that amino-terminal position is the more favoured, while dipeptides with Leu on the carboxyl-terminal position possessed a higher chemotactic potency. The size of these two amino acids (Gly and Leu) differs, Gly being small but Leu large (residue volumes are 36.1 and 100.1, respectively), although their non-polar character is similar. Comparison of Pro-Ala and its amino-terminal variant Ala-Pro provides a more expressed trend which was observed between Pro-Gly and Gly-Pro. It seems to be significant that while the small, non-polar amino acids (Gly, Ala) are preferred on the amino-terminal part, on the carboxyl-terminal position the large, non-polar amino acids (Leu, Phe) are active concerning chemotaxis. In addition to the chemoattractant character of the dipeptides mentioned above, Pro-Pro, where a relatively small (residue volume 73.6) but polar amino acid is placed onto both terminal positions might point to the very variable characters of the carboxyl-terminal position of dipeptide and demonstrate a positive chemoeffector feature of this dipeptide.

The experiments demonstrate that proline-containing dipeptides can provoke a (positive or negative) chemosensory response from *Tetrahymena*. This demonstrates the outstanding role of proline in the signalling mechanism (hormone evolution?). The partner amino acid profoundly influences the effectivity and direction of the chemosensory response. This latter influence can be explained by the physicochemical characteristics of the amino acids. In the study of the evolution of signalling these facts have to be considered, as amino acids for peptide hormones could have been selected according to these aspects.

While receptor-mediated mechanisms are the focus of this project the future objective is to study the chemotactic effect after pretreatment with dipeptides or co-incubation of dipeptides with different side-chains.

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