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DIRECT CHEMOTACTIC EFFECT OF BRADYKININ AND RELATED PEPTIDES—SIGNIFICANCE OF AMINO- AND CARBOXYTERMINAL CHARACTER OF OLIGOPEPTIDES IN CHEMOTAXIS OF *TETRAHYMENA PYRIFORMIS*

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The chemotactic character of the nonapeptide bradykinin (BK1–9) and its derivatives was studied in the eukaryotic ciliated model *Tetrahymena pyriformis*. The results demonstrate that BK1–9 has a direct and ligand-specific chemoattractant effect (maximal at 10^{-11} M) without any intermediate substance as is essential in some mammalian test systems. Evaluation of the chemotactic effect elicited by derivatives showed that the presence of N- and C-terminal arginines can influence chemotactic potency of the molecule via expression of pyrrolidine and aromatic ring structures of terminal amino acid residues. Removal of the N-terminal Arg (expression of Pro) results in a significant decrease in chemotaxis (BK2–9), while further truncation of the C-terminal, causing expression of the aromatic ring of Phe (BK2–8), results in a highly chemoattractant variant. A single pyrrolidine ring on the C-terminus BK1–7 also has a positive effect on the chemotactic character, however further truncation (BK1–6, BK1–5) causes the chemoattractant character to become chemorepellent. Study of chemotactic selection with BK derivatives supports our previous findings that only phylogenetically selected ligands or their close derivatives are able to induce long-term selection with chemotaxis. © 2002 Academic Press

KEYWORDS: chemotaxis; chemotactic selection; bradykinin; *Tetrahymena*; phylogeny.

INTRODUCTION

The nonapeptide bradykinin (BK) is one of the most frequently tested kinin-derivatives. Its formation from two precursors, the high and low molecular weight kininogens converted by kallikrein, occurs in several tissues. Characteristic physiological effects of BK (vasodilatation (Yamawaki *et al.*, 1993), contraction of smooth muscle (Rocha e Silva *et al.*, 1949), direct and indirect effects on cardiac endothelium (Parratt *et al.*, 1995), histamine release (Bueb *et al.*, 1993) and hyperalgesic effects (Haley *et al.*, 1989)) are mediated by two main subtypes of receptor B1 and B2. While B2 receptors are constitutively expressed by several cell types (Burch, 1991), B1 receptors are upregulated with their most effective ligand des-Arg^9-BK in tissues following infections and trauma (Marceau, 1995). Activities of BK are complex, since as well as receptor-mediated pathways, a variety of extracellular messenger systems (e.g. products of cyclooxygenase and lipooxigenase pathways, interleukins, NO) are generated (Geppetti *et al.*, 1995). As well as the multifocal extracellular effects of BK, its characteristic intracellular messenger pathway is G protein-coupled (Perney and Miller, 1989) and activates both IP_3 linked Ca^{2+} release (Bascands *et al.*, 1991) and PKC activation by diacylglycerol (Dixon *et al.*, 1989).

According to the above mentioned roles, BK is considered to be a significant effector molecule of inflammation *via* its hyperaemic effect and induction of local pain; however, some references also point to its indirect chemotactic effect (Pasquale *et al.*, 1991). In this effect prostaglandins and leukotrienes have been demonstrated as effector molecules of BK (Coutant *et al.*, 1997). The novel chemotactic potency of BK is underlined by its direct inducer character on the actin network of

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migrating cells (Coutant *et al*., 1997). The effects of BK on migratory cells in tissues is made more plausible by considering the special position of the amino acids (Arg, Pro and Phe) composing the native molecule and the effects of proteolytic enzymes of inflammatory tissues, which provide the possibility of producing shorter BK derivatives with non-characterized effects.

Our purpose was to characterize the direct chemotactic behaviour of BK and its related peptides in the unicellular ciliated eukaryote model *Tetrahymena pyriformis* GL. The model is widely applied in the research of signal transduction (Csaba, 1985), as its membrane composition and receptors, e.g. insulin receptor (Christopher and Sundermann, 1995), and intracellular second messenger pathways, e.g. cAMP (Csaba and Lantos, 1976), cGMP (Kőhidai *et al*., 1992), Ca-calmodulin (Kovács *et al*., 1989), and IP_3 (Kovács and Csaba, 1990) are highly homologous to mammalian vertebrates. Several physiological responses, such as cell growth and death (Christensen *et al*., 1995; Wheatley *et al*., 1993), phagocytosis (Kőhidai *et al*., 1995*b*), and metabolic activity (Kőhidai and Csaba, 1985) of *Tetrahymena* are characteristically induced with signal molecules. Chemotaxis is considered to be one of the most physiological responses of these cells (Kőhidai, 1999). It is a good index to investigate signal molecules in this model cell; inorganic salts (Tanabe *et al*., 1979), amino acids (Levandowsky *et al*., 1984), short and longer chain peptides (Kőhidai *et al*., 1997; Leick, 1992), lectins (Kőhidai and Csaba, 1996) or volatile oils (Kőhidai *et al*., 1995*a*) can modulate the characteristic responses of these cells.

The objectives of our study were to assess: (1) whether and how BK influences the chemotaxis of *Tetrahymena pyriformis*, without any intermediate substance; (2) how the chemotactic character of the reference molecule (BK1–9) is modified by the alterations of the amino- or carboxyl-terminated part or inner structure of the molecule; and (3) whether the BK or its fragments have the chemotactic potential to select subpopulations possessing higher responsiveness from the mixed cultures.

MATERIALS AND METHODS

Cells and culturing

Populations of *Tetrahymena pyriformis* GL in the logarithmic phase of growth were cultured in 1% Tryptone medium (Difco, Michigan, U.S.A.) containing 0.1% yeast extract.

Chemicals and peptides

The applied bradykinin and related peptides were:
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (BK1–9)
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe (BK1–8)
Arg-Pro-Pro-Gly-Phe-Ser-Pro (BK1–7)
Arg-Pro-Gly-Phe-Ser (BK1–6)
Arg-Pro-Pro-Gly-Phe (BK1–5)
Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (BK2–9)
Pro-Pro-Gly-Phe-Ser-Pro-Phe (BK2–8)
Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Des Pro^2 BK)
Arg-Pro-Pro-Gly-Phe-Ser-Phe-Pro-Phe-Arg (D-Phe^7BK)

Chemotaxis assay

The two-chamber capillary chemotaxis assay of Leick and Helle (1983) was modified by us (Kőhidai *et al*., 1995*a*). In this assay we used a multichannel micropipette, where the tips of the pipette filled with test substance served as inner chambers, while 96-well microtiter plates filled with *Tetrahymena* cultures (cell density 10^4 cell/ml) served as outer chambers. The incubation time was 20 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 did not provide enough cells in the sample, while at times longer than 20 min we could not distinguish chemotactic-responder cells from chemokinetic-responder cells. Then the samples were fixed in 4% formaldehyde containing phosphate-buffered saline (PBS). The number of cells was counted in a Neubauer cytometer by light microscopy. Following the relevant findings in the literature, derivatives were determined (in narrow and wide ranges alike) as chemoattractants (or chemorepellents) when their chemotactic ability was significantly higher (or lower) than the corresponding controls.

Chemotactic selection

The chemotaxis assay described above was used to select chemotactically-high previously-identified responder subpopulations from cultures of logarithmic phase of growth. Only chemotactic BK analogues were used in this part of the study. These analogues were: BK1–9 (10^{-11} M), BK1–8 (10^{-11} M), BK1–7 (10^{-8} M), BK2–8 (10^{-9} M), Des Pro^2 BK (10^{-8} M); culture medium was used as a negative control. After the chemotaxis assay with optimal concentrations of the substances, the positive responder cells were transferred into fresh

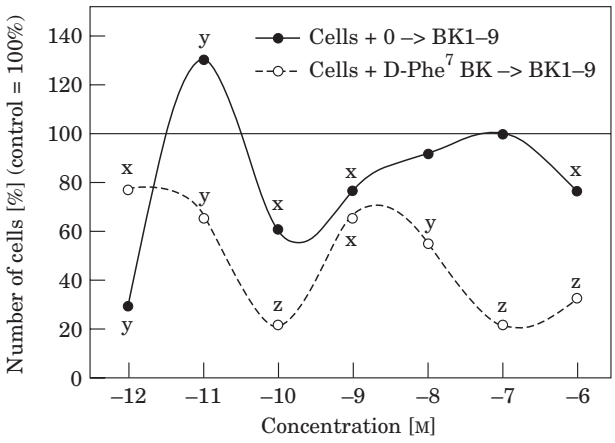

Fig. 1. Concentration course study of chemotactic effects elicited by bradykinin (BK1–9) in *Tetrahymena* (solid line). Dotted line represents the suppressor effect of pretreatment with the competitive B2 receptor antagonist D-Phe^7 BK- on chemotaxis induced with BK1–9 (x=P<0.05; y=P<0.01; z=P<0.001, SD is lower than ± 7.52).

culture medium and these cultures were consecutively transferred every 48 h for 7 days. After this, the cultures were tested in an identical chemotaxis assay. The theoretical groups of samples were: B/B—cells selected with the bradykinin analogue in the first run and assayed for the bradykinin analogue in the second run; B/C—cells selected with the bradykinin analogue in the first run and assayed for the control substance in the second run; C/B—cells selected with the control substance in the first run and assayed for the bradykinin analogue in the second run; and C/C—cells selected with the control substance in the first run and assayed for the control substance in the second run.

Statistical analysis

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

RESULTS

Concentration course study

The chemotactic character of the reference molecule (BK1–9) was detected at only one peak, 10^{-11} M, whilst at higher concentrations the effect was neutral, and around the chemotactic concentration (10^{-12} and 10^{-10} M) we detected the repellent character of the molecule (Fig. 1). Following treatment of cells with D-Phe^{7}BK, a competitive antagonist of BK1–9, the reference molecule did not elicit any chemotactic effect (Fig. 1).

The shorter peptide fragments had different chemotactic effects up to their carboxy- or amino-terminal truncation. The chemotactic concentration range of BK1–8 (with carboxyl-terminal Phe) was wider, as it was chemotactic in the low (10^{-12}–10^{-11} M) and high (10^{-7} M) concentrations (Fig. 2a). The effect of BK2–9 (with amino-terminal Pro) was chemorepellent along the tested concentration range (10^{-12}–10^{-6} M) (Fig. 2b). BK2–8 (with amino-terminal Pro and carboxy-terminal Phe) was chemoattractant, with maximal effect at 10^{-9} M (Fig. 2c). Intramolecular modification of the basic structure, particularly with prolines on the amino-terminal part of the molecule, also resulted in changes to the chemotactic potency of the molecules compared to BK1–9. Removal of Pro^2 in Des Pro^2 BK resulted in a molecule with a wide range of chemotactic effects (maximal at 10^{-8} M) (Fig. 2d).

Further truncation of the carboxy-terminal part of the molecule, in BK1–7 (with carboxy-terminal Pro), resulted in a chemotactic molecule possessing chemoattractant effect in the 10^{-12}–10^{-6} M range (Fig. 3a). In the case of the fragment BK1–6 (with carboxy-terminal Ser) there was a dramatic change in chemotactic character as its most effective concentration was at 10^{-10} M with only a neutral effect; below and above this concentration, the substance had chemorepellent potential for *Tetrahymena* (Fig. 3b). BK1–5 fragment (with carboxy-terminal Phe) had a chemorepellent character at the concentrations tested.

Study of chemotactic selection

In this part of our work we tested those analogues that proved to be chemotactic in the concentration course study. Previously it has been shown that the control medium itself has selector capacity (Kőhidai and Csaba, 1998), therefore co-efficients of chemotactic selection (Ch_{sel}) were calculated as ratios of CC/CS and SC/SS for each substance. In the case of good selector capacity the absolute values of SC/SS are over 1.0 and they are significantly higher than values of CC/CS (Kőhidai *et al.*, 2000).

Five BK analogues had different chemotactic selector capacities (Fig. 4a and b). The basic molecule BK1–9 selected a high responder subpopulation (Ch_{sel}=1.79). This chemotactic activity was only relatively strong, as the control substance selected subpopulation had a decreased response compared to the absolute control. The two BK

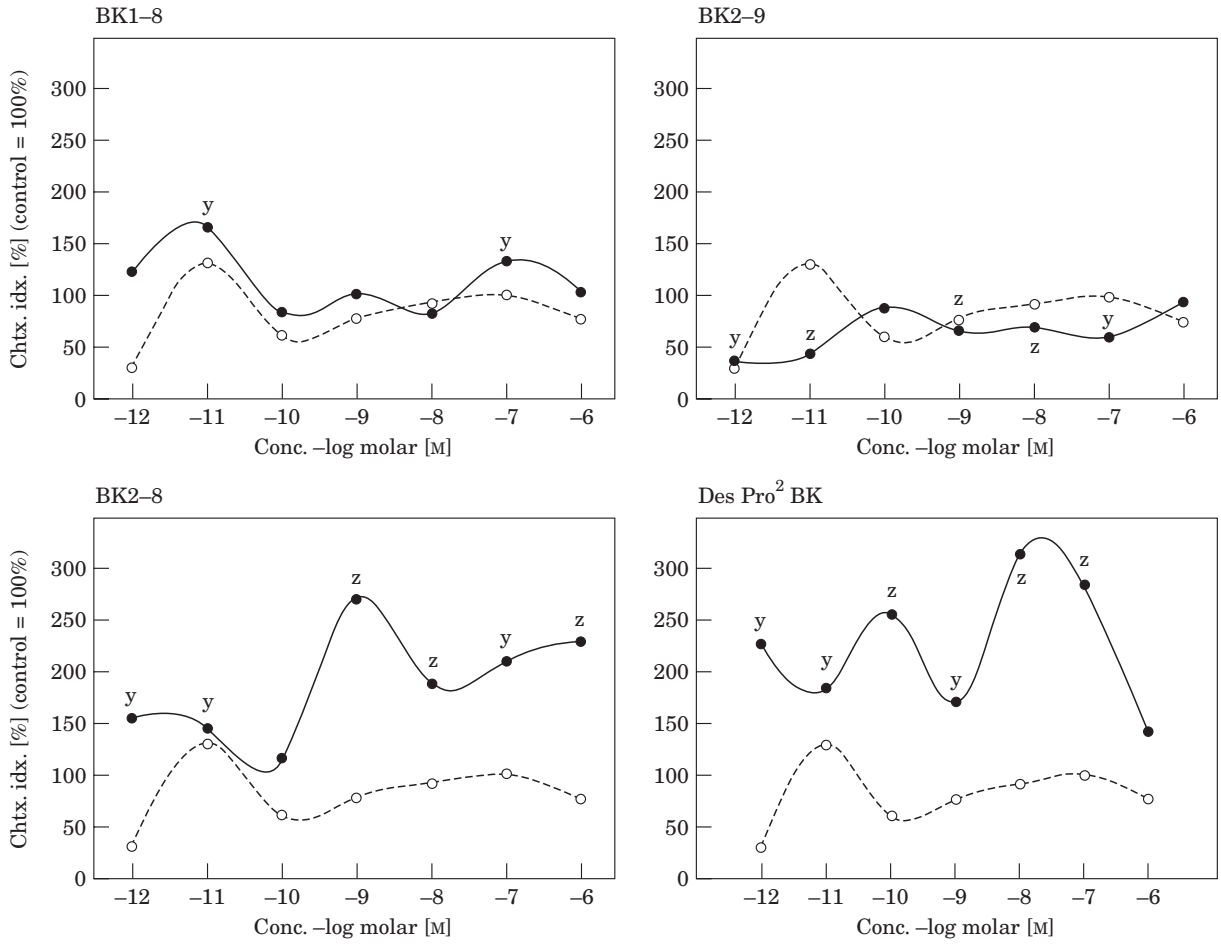

Fig. 2. Effect of removal of the terminal Arg residues from bradykinin molecule in a concentration course study of chemotaxis elicited in *Tetrahymena*. Dotted line represents the concentration-dependence of reference molecule BK1–9 (x=P<0.05; y=P<0.01; z=P<0.001, SD is lower than ± 15.32).

variants shorter in the carboxy-terminal part proved to be 'classic' positive selectors: in subpopulations of BK1–8 the chemotactic responsiveness was slightly enhanced (Ch_{sel}=1.39), while BK1–7 had the strongest capacity to select high responder subpopulations for long distance evaluation among the derivatives (Ch_{sel}=1.65). In the case of the amino- and carboxy-terminal truncated BK2–8, selection did not cause any detectable difference at the second encounter with the substance (Ch_{sel}=0.96), while intramolecular modification of the selector in the case of Des Pro^2 BK resulted in decreased chemotactic responsiveness in the progeny (Ch_{sel}=0.63) (Fig. 4b).

DISCUSSION

Chemotactic ability of different peptides is highly structure dependent both in prokaryotic and eukaryotic organisms. Investigation of chemoattractant or chemorepellent effects of BK and related molecules provided us with the ability to characterize the structure–function relationships of oligopeptide structures (from pentapeptides to nonapeptides) derived from BK. These molecules are good candidates for research, focused on intramolecular approaches to ligand–chemotaxis–receptor interactions, as BK1–9 contains three physicochemically characteristic amino acids—arginine, proline and phenylalanine. The long R group possessing arginines is present in both the amino- and carboxy-terminal positions of BK, and previous investigations have proved that this amino acid provides significant chemotactic character both to prokaryotic peptides and complement derivatives (C5des-Arg) (Fernandez and Hugli, 1978). In the intramolecular structure of BK, three prolines provide high variability in structural interactions. These amino acids are composed of a

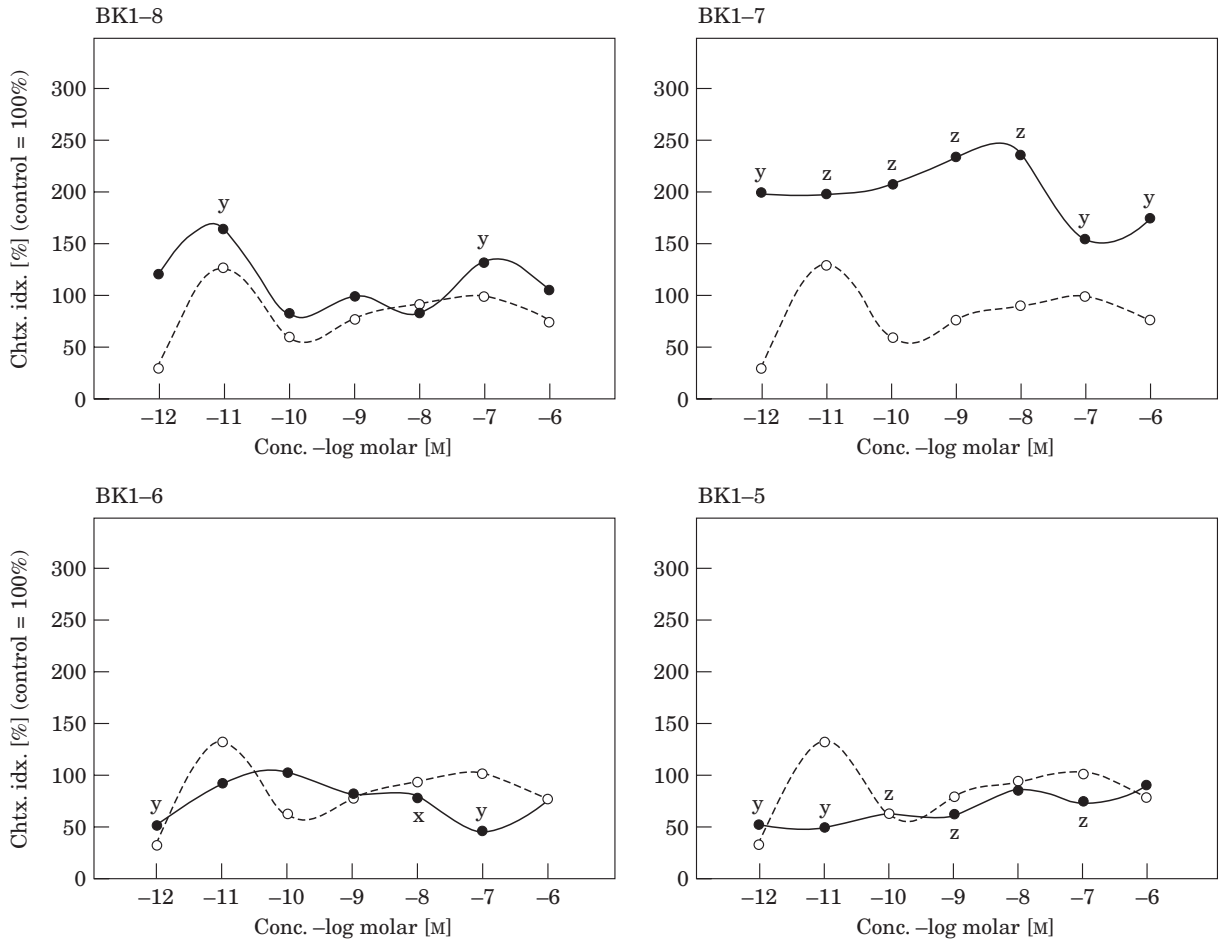

Fig. 3. Effect of truncation of carboxy-terminal region of bradykinin on chemotactic behaviour. Dotted line represents the concentration-dependence of reference molecule BK1–9 (x=P<0.05; y=P<0.01; z=P<0.001, SD is lower than ± 14.02).

characteristic-NH- (amino) component in the rigid pyrrolidine ring, which makes the whole molecule rigid and prevents the rotation of N-Cα, which is unusual in alpha-helical structures. However, proline is responsible for the reverse turns in several proteins (Lehninger *et al.*, 1993). The third significant component is phenylalanine with the characteristic aromatic ring. Its physicochemical character, size, charges, large solvent exposed area, and high pKa values make it significant as a building unit close to the C-terminus of BK1–9.

Our results show that the chemotactic responses of the unicellular ciliate model are highly sensitive to C- and N-terminal structures of the test molecules. When the molecule is characterized by two arginines possessing long R groups on the two terminals (BK1–9) or an aromatic phenylalanine is expressed on the C-terminus (BK1–8), this results in a chemotactic molecule acting over narrow concentration ranges (10^{-12} to 10^{-11} M). The presence of arginine on the N-terminus seems to be significant with respect to the chemoattractant character of the molecule, as removal of arginine from this terminus in BK2–9—and expression of the pyrrolidine ring of proline—results in a very strong repellent character in the molecule. The significance of the N-terminus is also underlined in the case of the analogue with the pyrrolidine ring of proline (BK2–8) on the N-terminus and aromatic ring of phenylalanine on the C-terminus. The wide range and strong chemotactic effect of this bradykinin analogue suggests that the heptapeptide characterized by the two rings on the two termini might be considered by the cells to be a very chemoattractant substance however the presence of phenylalanine does not result in characteristic changes to the chemoattractant character of the molecule.

The further truncation of the C-terminus indicates that the C-terminal pyrrolidine ring is a significant determinant of chemoattractant character, while the presence or absence of a long N-terminal R chain is almost unnecessary (see

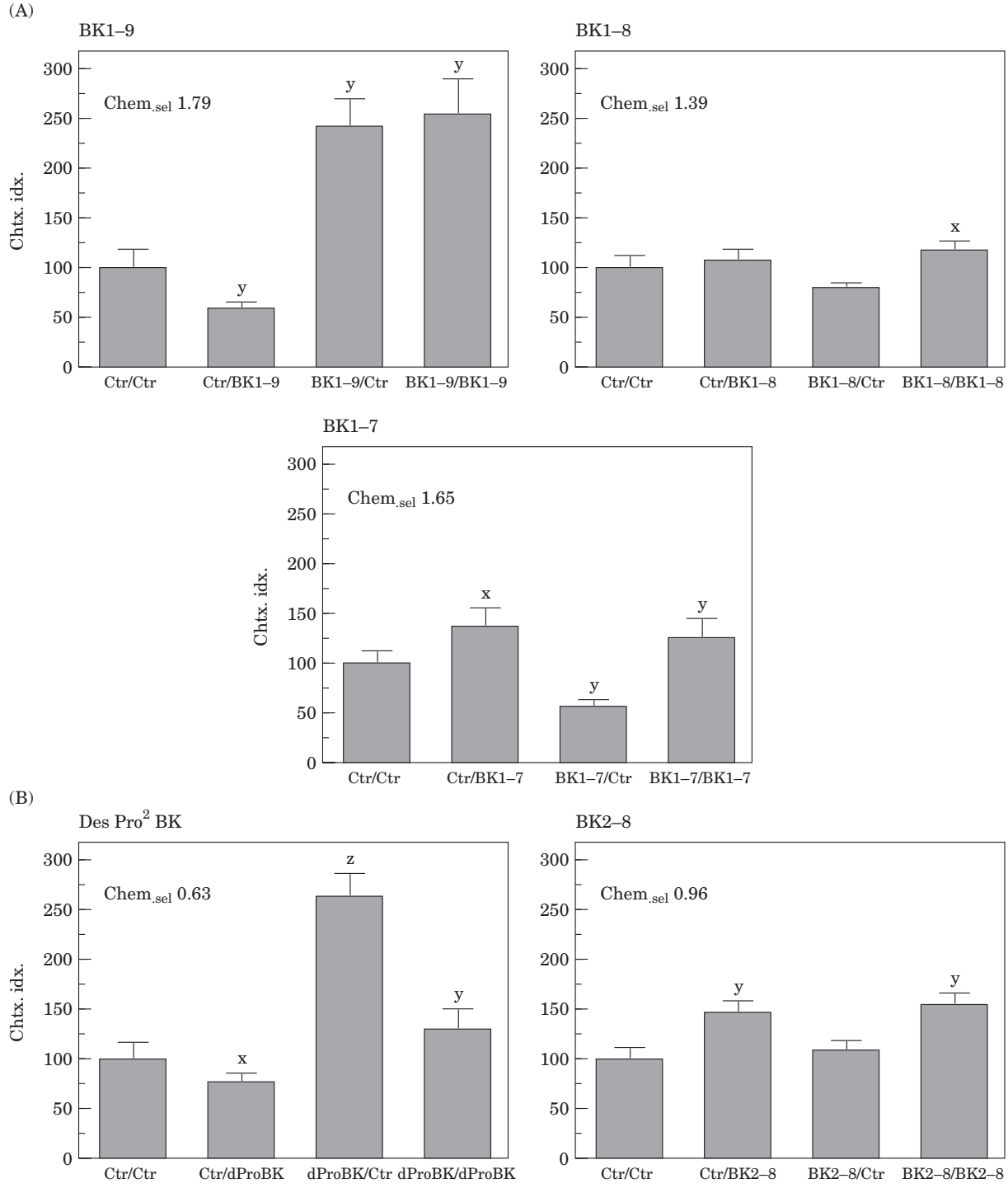

Fig. 4. Chemotactic selection with bradykinin and its derivatives resulting in (A) high-responder and (B) low-responder *Tetrahymena* subpopulations (x = $P<0.05$; y = $P<0.01$; z = $P<0.001$).

chemotactic effect of BK2–8). The significance of the N-terminal pyrrolidine ring to the chemoattractant effects of the molecule is more conceivable when we compare the observed effects to the repellent ability of BK1–6 with an N-terminal basic hydroxyl group or the more pronounced repellent character of BK1–5, where the aromatic ring is in the N-terminal position of the short pentapeptide.

It is easy to understand the significance of pyrrolidine ring structures of prolines in the determination of the chemotactic moiety of ligands. As discussed above, this component of peptides

is responsible for the termination of α-helical structures and therefore responsible for reverse turns in molecular structure. Bradykinin with its three prolines provides a good model for the investigation of these effects and the consequences to the ligand–receptor interaction. Our results showed that ring structures—both pyrrolidine and aromatic—can influence the chemotactic character of molecules, but in different ways. While the aromatic rings alone have almost no effect on chemotaxis (BK1–8 *vs.* BK1–9 or BK1–5 *vs.* BK1–6), synchronous expression of N-terminal pyrrolidine ring (BK2–8) makes the molecule more attractant, perhaps due to some novel intramolecular interaction between the two termini or altered distribution of net charges. The enhanced efficacy of BK2–8 might be explained by the changed biological activity: the 'cryptic' prolines possessing secondary amino groups become expressed only after removal of the N-terminal arginine. The high significance of proline is portrayed with other results gained with Des Pro^2 BK (Naruse *et al.*, 1981). Investigation of this analogue points to the lack of a proline close to the active part of the N-terminus. In this case the ligand can modify the biological activity of the molecule. Despite the fact that the terminal residues are intact, the intramolecular alteration of the molecule resulted in a chemoattractant ligand, which points to the fact that not only is the absence or presence of the long R chain of arginine decisive, but the orientation of arginine can also determine the chemotactic character of the molecule.

Evaluation of chemotactic responses on the basis of receptor specificity propounds the possibility that both B1 and B2 bradykinin receptors or homologous signaling mechanisms are present in the lower eukaryotic levels of phylogeny. Pretreatments with the B2 receptor antagonist D-Phe^7 BK (Vavrek and Stewart, 1985) proved that the chemotactic effect elicited with BK1–9 is B2 receptor-mediated, however the B1 receptor-specific BK1–8 (Vianna and Calixto, 1998) also has a wide and significant chemoattractant character in our model. Heterogeneity of B2 receptors (B3, B4, B5) (Regoli and Gobeli, 1995) provides more speculation concerning the specificity of BK actions in *Tetrahymena*.

In this project our purpose was not only to characterize chemotactic characters and the structural dependence of responses, but also the ability of the chemoattractant ligands to select high responder subpopulations via chemotaxis. The results showed that truncation of the C-terminus of BK has no strong effect on the efficiency of chemotactic selection, all three chemotactic substances could select high responder subpopulations compared to their controls. Nevertheless, it was observed that the pyrrolidine ring on the C-terminus (BK1–7) was highly preferred over the native BK1–9, and subpopulations selected with ligands possessing the pyrrolidine-containing ring on the C-terminus were high responders in the 70th progeny (the aromatic ring of BK1–8 also provides a positive selection). Chemotactic selection showed that some ligands have no good selector capacity in the long term, even if they were chemoattractant in the short term. Among these molecules there were differences, as both the pyrrolidine ring and aromatic ring containing BK2–8 had no selector potency for the subpopulations, whilst the effect of intramolecular modification in Des Pro^2 BK was rather strange, as it had a chemotactic effect at its first encounter with the 'virgin' cells, but the ability to recognize or respond to a ligand in the chemotactically-selected subpopulation instead gave the selector ligand a repellent character.

In conclusion, our results underline the direct chemotactic ability of bradykinin (BK1–9), even in the phylogenetically lower eukaryotic model, *Tetrahymena*. A structure–function study of the chemotactic character of bradykinin derivatives shows that both carboxyl- and amino-termini of the oligopeptide might be responsible for the determination of the chemotactic character of ligands. Terminal ring structures providing characteristic size and charge to the terminus are recognized preferentially in chemotaxis.

Chemotactic selection studies confirmed our previous finding, that receptors induced in chemotaxis might have different subsets, and only the phylogenetically-selected ligands or their close derivatives are able to induce long-term selection of the responsible signaling mechanism via chemotaxis.

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