Tetrahymena cells distinguish insulin preparations according to either their amorphous and crystalline form or their bovine and porcine origin: aspects of hormone binding and chemotaxis in relation to imprinting

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Abstract

Bovine insulin had a strong negative effect on the chemotaxis of *Tetrahymena* at the first encounter with the hormone. The crystalline porcine insulin proved to be neutral, while the amorphous porcine insulin had a significant positive chemotactic effect. At the second encounter with the hormone the presence of imprinting was detectable with bovine insulin and induced a significant positive chemotaxis. The chemotactic effect of amorphous porcine insulin was depressed while crystalline porcine insulin expressed the most significant positive chemotactic effect. In the binding of hormones there was unambiguous positive imprinting independently from the species specificity and from the physicochemical condition of the insulins. *Tetrahymena* discriminated at both the first and second encounters between the bovine and porcine insulins which differed in two amino acids. The imprinter capacity evident in binding was independent of the chemotactic preference and the imprinting embodied in chemotaxis.

Introduction

The unicellular *Tetrahymena* has the capacity to detect and to bind the signal molecules of its environment at receptor level (Csaba, 1985). The signal molecules can be (among others) amino acids, oligo- and polypeptides and hormones composed of these molecules. At the first encounter with the signal molecule the hormonal imprinting is developed resulting in altered binding capacity and responsiveness of the cell at subsequent encounters with the same molecule (Csaba, 1980, 1986, 1994). The receptor memory developed by imprinting is transferred to the daughter cells and it persists after several hundred generations of the cell (Csaba *et al.*, 1982).

Tetrahymena has a good discriminating capacity at the first encounter with the signal molecule, but it becomes more specific following imprinting. In this way the unicellular organism is able to distinguish hormones and hormone analogues in the same way as it perceives the position of the amino acid (COOH-terminal or NH_2-terminal) in short sequences such as dipeptides (Csaba and Kovács, 1993; Csaba *et al.*, 1986). Previous experiments (Kovács *et al.*, 1989) have shown that minimal modification of the insulin molecule does not influence the effect of imprinting in binding. In consequence the

present experiments were carried out in order to answer the following questions: (1) can *Tetrahymena* distinguish insulins which are different in their physicochemical characters (crystalline and amorphous); (2) can *Tetrahymena* distinguish between bovine and porcine insulins which differ in only two amino acids; and (3) is there any dependence on the chemotactic effect (preference) and binding capacity when applied to different forms of the same hormone?

Materials and methods

In the experiments the cells of *Tetrahymena pyriformis* GL strain were investigated. The cells were cultured in 1% Bacto tryptone (Difco, Michigan, U.S.A.) medium containing 0.1% yeast extract at 28°C for 24 h.

Assay of chemotaxis

In order to measure chemotaxis we used the 8-channel-automatic-pipette modified method of Leick and Helle (1983). The cell density used was 10^4 cells/ml and the incubation time was 15 min. In every group there were ten parallel experiments. Following the assay the cells were fixed in PBS containing 4% formaldehyde and the samples were counted in a Neubauer haemocytometer. The statistical analysis was done using a Sigmaplot program.

Another group of cells was treated with 10^{-6} M insulin. The insulins used were: Actrapid (100% crystalline porcine insulin); Semilente (100% amorphous porcine insulin, 0.08 mg Zn per 10 ml); Monotard (70% crystalline and 30% amorphous porcine insulins, 0.08 mg Zn per 10 ml); Lente (mixture of 70% crystalline bovine and 30% amorphous porcine insulins); and Ultralente (100% crystalline bovine insulin). All the hormones used were monocomponent insulins (Novo, Copenhagen, Denmark). Insulins (10^{-12} to 10^{-6} M) were used to test the concentration course of chemotaxis. Another group of cells was treated with 10^{-6} M insulin for 1 h, and then transferred to normal medium. The cells were tested, 24 h later, for chemotaxis with the insulins in concentrations of 10^{-12} to 10^{-6} M.

Binding-assay of hormones

The cells were treated for 1 h with 10^{-6} M insulin preparations used in the chemotaxis experiments. Cells were thoroughly washed and transferred into insulin-free medium for 24 h. After this *Tetrahymena* cells were fixed with PBS containing 4% formaldehyde and incubated with FITC (BDH, Poole, England) labelled insulins (Semilente or Actrapid; FITC-protein ratio 0.43; protein content 0.015 mg/ml). The samples were dried on slides and the FITC-insulin binding was measured by a Zeiss fluoval cytofluorimeter. The signals of the cytofluorimeter were converted by a digital processor and these were registered by a Hewlett Packard 41CX computer which supplied mean values, standard deviations and values of inter-group significance (by Student

t test). All experiments were repeated in four replicates and twenty cells were measured in each group. In this way one column of results represented the mean values of 80 cells.

Results and discussion

The insulin forms chosen were crystalline, amorphous (precipitate) and mixtures of crystalline and amorphous as well as bovine and porcine insulins. In this way it was possible to determine (1) the differences in chemotactic activity and receptor level binding between the crystalline and amorphous insulins; (2) the differences in chemotactic activity and binding of the crystalline forms of porcine and bovine insulins; and (3) the differences in chemotactic activity and binding of insulin of pure porcine origin, or dominantly bovine crystalline and amorphous mixtures.

Whether porcine or bovine insulin was the origin of the crystalline insulin it had a neutral or negative (repellent) chemotactic effect at the first encounter (Figures 1 and 5). It never increased significantly above the control levels. The bovine insulin had a negative effect, but this was compensated for by adding 30% amorphous porcine insulin to the mixture (Figure 4). The amorphous porcine insulin itself (Figure 2) or in a mixture (30%) evoked a positive chemotactic effect particularly in high concentrations (Figure 3).

The bovine insulin produced a negative chemotactic effect at the first encounter, but chemotaxis was positive at the second encounter (following the imprinting). The difference between the effects at the first and second encounters exceeded 100% in some concentrations. The absolute values of porcine insulin following imprinting were similar to the absolute values of bovine insulin, but the difference was far less between the first and second

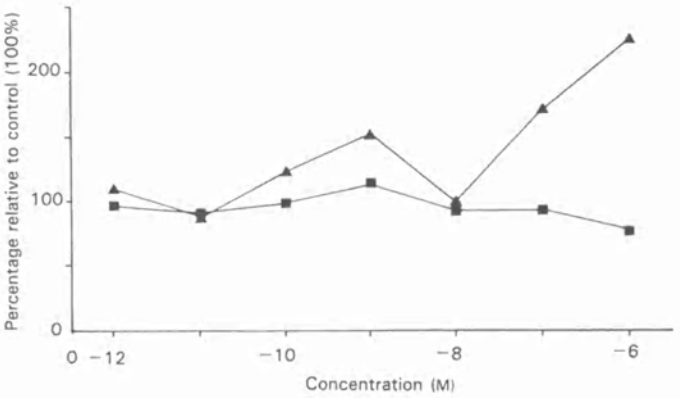

Figure 1 Chemotactic effect of Actrapid (pure porcine crystalline) insulin (■) to *Tetrahymena* on the first occasion, and (▲) after pretreatment (imprinting).

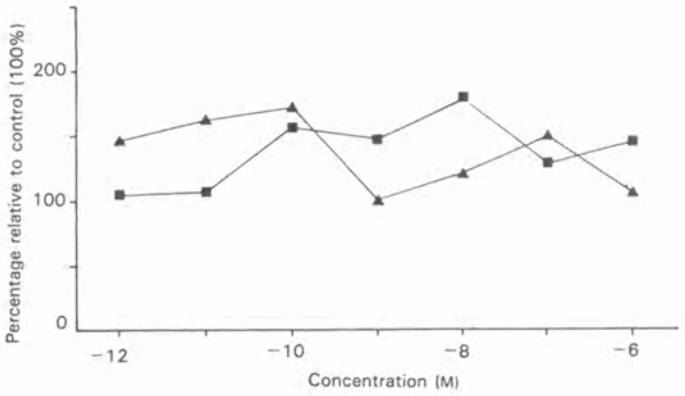

Figure 2 Chemotactic effect of Semilente (amorphous porcine) insulin (■) to *Tetrahymena* on the first occasion and (▲) after imprinting.

encounters. There was one exception in the case of crystalline insulin, where at 10^{-6} M concentration (which was used for imprinting the cells in both our previous and present experiments) there was ~150% difference in the chemotactic effect between the first and the second encounters (Figure 1).

There was a significant difference between the post-imprinting chemotactic character of porcine crystalline and amorphous insulins (Figures 1 and 2). In the case of crystalline insulin there was no distinction between the first and second encounters at 10^{-12} M and 10^{-6} M. However, in the presence of amorphous insulin there was a significant difference when the molecule was present alone or in a mixture with the crystalline form. In the case of crystalline insulin the 150% difference was in the imprinted group at 10^{-6} M. There was a narrow range of advantage for the first treatments in reactions induced by amorphous insulins or substances containing amorphous insulin.

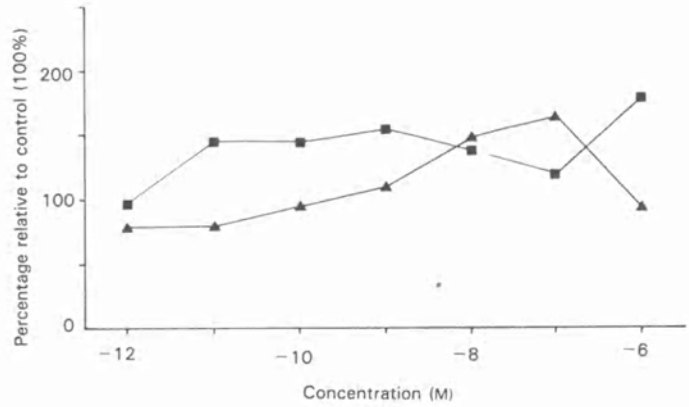

Figure 3 Chemotactic effect of Monotard (70% crystalline and 30% amorphous porcine) insulin (■) to *Tetrahymena* on the first occasion and (▲) after imprinting.

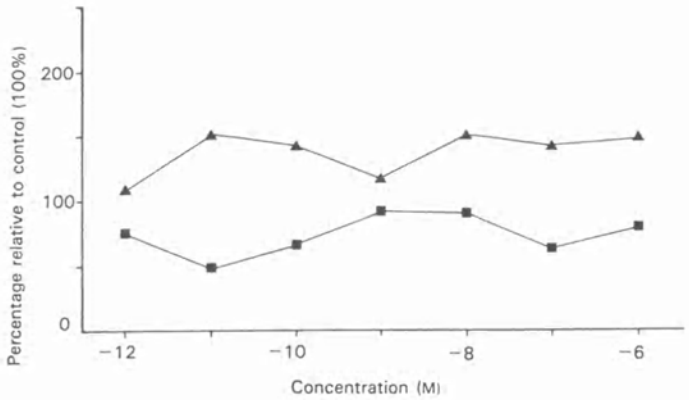

Figure 4 Chemotactic effect of Lente (70% crystalline and 30% amorphous bovine) insulin (■) to *Tetrahymena* on the first occasion and (▲) after imprinting.

The binding assays showed clearly that imprinting with each insulin significantly increased the binding of both the amorphous and crystalline insulins (Figure 6). Thus there was no significant difference between the insulin forms in regard to imprinting.

The bovine and porcine insulins differ in the amino acids of the A chain as there are alanine and valine in the bovine type, and threonine and isoleucine in the porcine insulin (Frieden, 1976). The experiments demonstrate that *Tetrahymena* is able to 'sense' the differences between the two amino acids which seems to be enough to turn the neutral chemotactic behaviour of porcine insulin to a negative (repellent) one in the case of bovine insulin.

In previous experiments we demonstrated that one amino acid deviation in di- or oligopeptides resulted in significant changes in the potency of imprinting. Other experiments indicated that increasing the amount of one

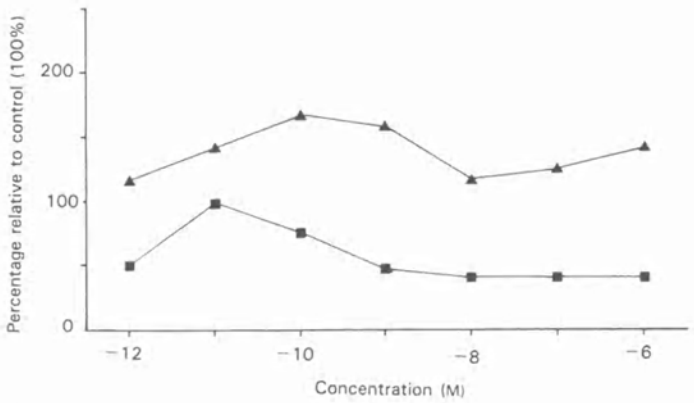

Figure 5 Chemotactic effect of Ultralente (pure bovine crystalline) insulin (■) to *Tetrahymena* on the first occasion and (▲) after imprinting.

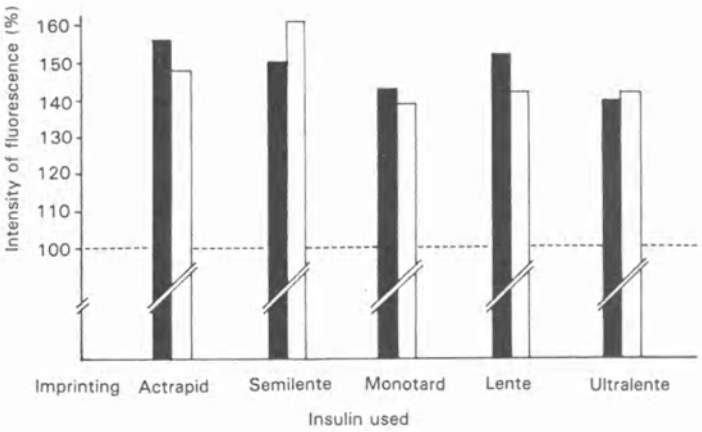

Figure 6 Binding of FITC-Actrapid (open columns) or FITC-Semilente (solid columns) insulin to *Tetrahymena* after imprinting. Control shown by broken line at 100% intensity of fluorescence.

amino acid in the hormone resulted in specification (Ishii, 1990). In view of this the present results are not surprising but they express the very selective quality of the recognition character(s) of *Tetrahymena*. However, we have to consider that the A8 amino acid has an important role in binding to receptors in mammals (Gammeltoft, 1984), and that the interspecies variability of the A8-A10 region is very high (Murray-Rust *et al.*, 1992). This raises the possibility that the amino acid preference, which is already present at this level, is embodied later as receptor affinity during evolution. Such a view must be regarded with scepticism until other amino acids have been tested in relation to chemotaxis.

Our experiments clearly show that the physicochemical condition of the hormone influences the chemotaxis induced by insulin of the same species specificity (*i.e.* the same amino acid sequence). It is surprising that the crystalline insulin-hexamer with the lower molecular weight (Murray-Rust *et al.*, 1992) is neutral at the first encounter, while the amorphous precipitate with its large mass has a positive chemotactic effect. It is possible that this enhanced effect is due to the increased molecular weight of the insulin conglomerate (Huckett *et al.*, 1991). In experiments with mammals, Cuatrecasas (1969) proved that the binding of Sepharose-bound insulin was higher, and Suzuki *et al.* (1972) came to the same conclusion using insulin-dextran complex *in vitro* and *in vivo*. Nevertheless, considering that in *Tetrahymena* the conditions are not exactly the same as in mammals, and that cilia of the oral apparatus and the body are not equal (Hufnagel, 1992), it is conceivable that there are two distinct working mechanisms.

In the present case the significant alterations are manifest in chemotaxis and not in the binding points so that there is a difference in binding and function, either at the first encounter with the hormone or as the result of imprinting. On the other hand the imprinting embodied in binding is not the result of a preference which is manifest in chemotaxis.

Our experiments have directed attention to the fact that different forms of insulin molecule are able to develop imprinting alike but from the aspect of function there are diversities in the degree of imprinting. The species specificity of the hormone (alterations in the amino acid sequence) is able to influence the primary effect of the imprinting and this is concerned with the physicochemical condition of the hormone.

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