Effects of atrial natriuretic peptide on the unicellular *Tetrahymena pyriformis* model

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Abstract

The vertebral hormone, atrial natriuretic peptide (ANP) has characteristic effects in *Tetrahymena* cells. ANP is able to induce the release of sodium ions, probably *via* the membrane rather than the contractile vacuole. The hormone is a potent inhibitor of Chinese ink-phagocytosis. A 10^{-12} M concentration of ANP has the maximum positive effect on the division of cells but long-term treatments indicate the presence of a down-regulation-like mechanism. ANP has a strong chemoattractant characteristic between 10^{-13} and 10^{-11} M concentrations. Significant concentration dependent FITC-ANP binding at pretreatments of 10^{-12} M suggest the presence of special receptor-dependent mechanisms which are responsible for the development of hormonal imprinting. The similarities between the most effective concentrations ($10^{-13}-10^{-12}$ M) and the human serum level of ANP shows that the effects of ANP have a more evolutionary background than previously considered.

Introduction

Molecules of the atrial natriuretic peptide (ANP) composed of 21—73 amino acids were detected in various vertebrate tissues (Seidah *et al.*, 1984; Kanagawa and Matsuo, 1984). These molecules have important roles in the recently described hormonal complex system controlling volume and pressure homeostasis. The main physiological effects of ANP are: (1) increasing natriuresis and diuresis (de Bold, 1985); (2) a parallel inhibition of aldosterone (Kudo and Baird, 1984), vasopressin (Samson, 1985) and renin secretion (Burnett, 1984); (3) stimulation of testosterone synthesis (Bex and Corbin, 1985); and (4) relaxation of smooth muscles (Fiscus *et al.*, 1985).

These effects are dependent upon receptor-mediated intracellular processes, where the membrane receptor of ANP itself has a well-characterized role. Low and high affinity binding sites were demonstrated in several vertebrate cell types and these experiments (Leitman *et al.*, 1988) showed the presence of a 66 kD protein monomer which formed at least three different structures in the membrane. The receptors of natriuretic peptide are grouped into three classes: NPR_A, NPR_B and NPR_C (Nakao *et al.*, 1992a). While members of classes A and B act by a cGMP second messenger system the C class of binding site has a truncated intramembrane component and the inositol lipid system is the second messenger system generated (Nakao *et al.*, 1992b). The heterogeneity of receptors has a good correlation with the newly described

natriuretic peptides, BNP and CNP, besides the 'stem' molecule of ANP (Lang *et al.*, 1992). Some cross-reactions (*e.g.* ANP acts on NPR_A and NPR_C receptors) make the above described system complicated (Lang *et al.*, 1992).

Although previous experiments have demonstrated the presence of ANP binding proteins in a wide range of cell types, there were very essential types, *e.g.* white blood cells (Strom *et al.*, 1987), kidney epithelial cells (Leitman *et al.*, 1988) or red blood cells (Strom *et al.*, 1987), which were found to lack these proteins. This fact led to our interest in the phylogenetic aspects of the problem. Indeed the question arose as to whether the eukaryotes appearing very early in the phylogeny possess recognition systems for ANP molecules such as signal peptides.

Our model cell was the unicellular ciliate *Tetrahymena pyriformis* GL. This organism is frequently used in cell and molecular biology since it has all the essential parameters (well-defined membrane structure, intracellular second messenger systems with vulnerable enzyme cascades; metabolic pathways; nuclear organization and DNA activation). The ANP-induced physiological events were the main factors investigated, since peptide type hormones (*e.g.* insulin, TSH, FSH) and amino acid type substances (histamine, serotonin, oxytocin and vasopressin) could induce very characteristic physiological effects like phagocytosis (Csaba and Lantos, 1975), chemotaxis (O'Neill *et al.*, 1988), contractile vacuolar activity (Csaba and Kovács, 1992) or division (Csaba and Németh, 1980) in *Tetrahymena*. Such activities have possible connections with the fundamental mechanisms of ANP functions in higher animals.

Hormonal imprinting (Csaba, 1980, 1984, 1985), another aspect of hormonal action, was also investigated. This phenomenon is present at both higher and lower phylogenetic levels. Several substances (hormones, drugs and other chemicals) are able to develop a special type of 'memory'. Following the first encounter with a given molecule the cell, or the progeny cells, will respond in an altered, and generally more sensitive way at the second encounter. This changed responsiveness followed by significant changes at receptor level were tested as to whether or not the ANP molecule was able to develop hormonal imprinting.

Our aim in the present work was to increase our knowledge of ANP at the evolutionary level and to determine some occasional activities of the molecule at the unicellular level.

Materials and methods

Cell culture conditions

Tetrahymena pyriformis strain GL was used in the logarithmic phase of growth at 28°C in 0.1% yeast extract containing 1% tryptone (Difco, Michigan, U.S.A.) medium. The cell density of *Tetrahymena* cultures was determined by a Neubauer haemocytometer. The density was 10^4 cells/ml in each experiment. All trials were repeated in five consecutive experiments.

Materials

Our purpose was to characterize the concentration relationships (10^{-13}—10^{-7} M) of the supposed ANF actions developed at receptor level. The ANP used was hANP 1-28 (Bissendorf Peptide GmbH, Wedemark, Germany). The following substances were used: FITC labelled hANP (prepared in our laboratory), FITC labelled protein G (Sigma Chemical Co., St Louis, U.S.A.), and antibody to hANP.

Effects on the Na-K pump mechanisms

The cells were treated with 10^{-12} M ANP for 15, 30, 60, 90, 120, 180, 300 s. Each step was arrested by fixation in 4% formaldehyde diluted in PBS. The supernatant of the cells was immediately removed. The samples were measured by an IL-943 flame photometer.

Pulsation of contractile vacuole

Aliquots of cell suspensions were treated for 15 min with 10^{-12} M ANP, and the control group received cell free culture medium in the same volume. Following incubation the cells were placed onto 1.5% agar-film coated slides, and 5 min were given for adaptation and 10 min for measurement. The period of two contractile vacuole systoles were determined in 200 cells in each group.

Phagocytic activity

Tetrahymena cells were washed in inorganic salt containing Losina Losinsky (L-L) solution and sustained (starved) in it for 3 h. Afterwards suspended Chinese ink with or without ANP was added to the cell suspension (v/v/v) for 10 min. The cells were fixed with formaldehyde, and the number of food vacuoles was microscopically determined in 200 cells/group.

Growth of cells

Cells cultured in tube conditions were used. The duration of treatments with ANP were short-term (1 h) and long-term (24 h), respectively. The number of cell samples was determined and controlled by a Neubauer haemocytometer after 24 h of treatments.

Chemotactic activity

To determine chemotactic activity of cells we used a quantitative assay according to Leick and Helle (1983) in which the cell density was 10^4 cell/ml. *Tetrahymena* cells (in culture medium) were placed into the outer container of the capillary chamber, and the inner container was filled with ANP containing culture medium. The time of incubation was 15 min. Samples were taken from the inner container and fixed with formaldehyde. The number of cells demonstrating positive chemotaxis was determined by haemocytometer. In addition in the short-term test we analysed the chemotactic activity of cells treated for 24 h using the same ANP concentrations.

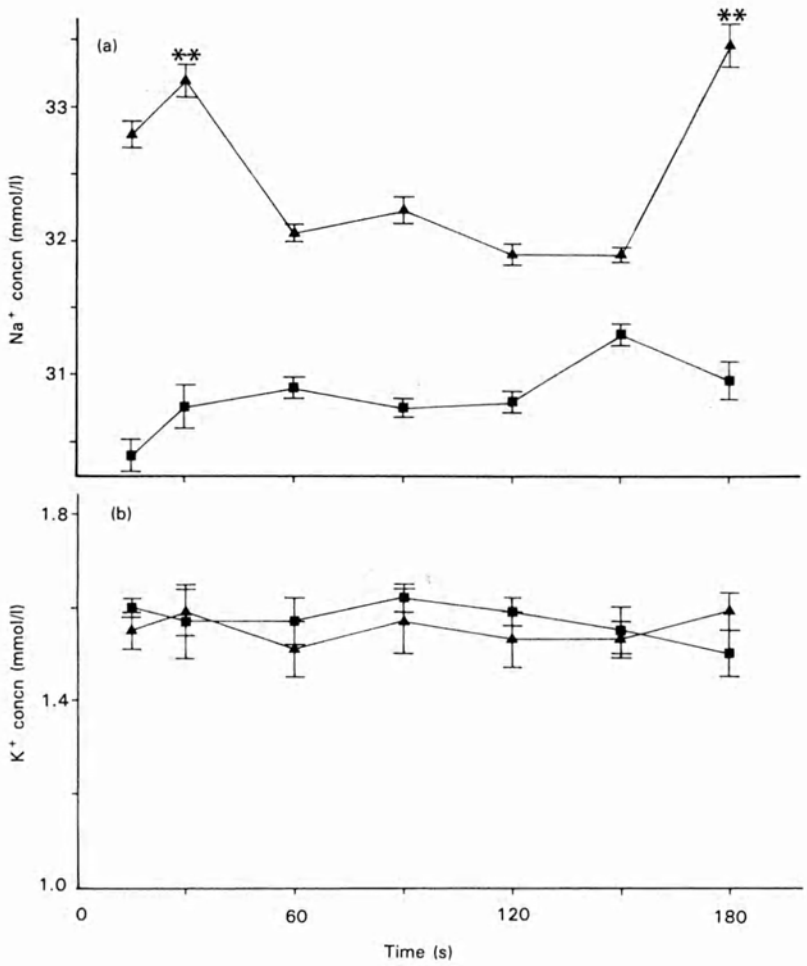

Figure 1 Effect of ANP treatment on the (a) sodium and (b) potassium release. Significance in Figures 1–9: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ■, Control; ▲, ANP.

Effects of hormonal imprinting on chemotaxis

The experiments were carried out in four groups. (1) Control/control (C/C) cells had no pretreatment and the culture medium was applied as the chemoattractant in the chemotaxis assay. (2) Control/ANP (C/A) cells also had no pretreatment but 10^{-13} M ANP was applied as the chemoattractant in the assay. (3) ANP/control (A/C) cells were pretreated with 10^{-13} M ANP for 1 h. The pretreated cells were washed and transferred into fresh culture medium for 24 h. In the chemotaxis assay fresh culture medium served as the chemoattractant. (4) ANP/ANP (A/A) cells were pretreated and cultured as described for group 3. In the chemotaxis assay 10^{-13} M ANP served as the chemoattractant. The techniques for the measurement of chemotaxis were as previously described.

ANP binding sites

The binding pattern and the binding capacity of *Tetrahymena* cells were investigated by FITC labelled ANP. In the hormonal imprinting study we used 1 h pretreatments with different concentrations of ANP. Then the cells were cultured for 24 h in fresh culture medium. The formaldehyde fixed cells were washed with PBS, then incubated in FITC-ANP for 1 h. The intensity of fluorescence was measured with a Zeiss Fluoval cytofluorimeter. A Hewlett Packard HP 41CX microcomputer registered and analysed the data with the help of an analogue-digital converter. The intensity of fluorescence was determined in 30 cells in each group.

Immunostaining of endogenous ANP

A special microwave fixation technique (Köhidai *et al.*, 1992) was used to obtain the best immunostaining in *Tetrahymena*. The cells were stained with antibodies to hANP. These antibodies were applied in different concentrations in the dilution range of 1:20,000—1:200,000 at room temperature. The sensitivity of the antibody was tested by RIA and its usable range was between 5 and 500 pg/tube. The detector system was FITC labelled protein G. For evaluation of covered samples we used the previously mentioned Zeiss Fluoval cytofluorimeter HP 41CX microcomputer system. In each group 20—30 cells were measured and the experiment was repeated three times.

The following were used as negative controls: (a) substitution of primary antibodies with non-immune serum; and (b) substitution of primary antibody with the same primary antibody preincubated overnight with hANP at 4°C.

Analysis of data

All measurements were carried out and evaluated in double blind experiments. Data gained by cytofluorimetry were statistically analysed by an HP 41CX microcomputer built-in program which provided the significance analysis (Student t test) and other standard statistical procedures. Data from other experiments were treated and evaluated by the statistical computer programs Sigma plot 4.01 and NCSS.

Results

Two experiments which were focused on the comparative physiological aspects of ANP function confirmed our hypothesis, that ANP is able to develop its characteristic actions not only in vertebrates but also in unicellular eukaryotes.

The 10^{-12} M ANP concentration had its characteristic effect on the release of Na and K ions (Figures 1a and 1b). In the culture medium ANP treatment selectively increased the concentration of Na released while the concentration of K ions released was identical to the control group.

The periodicity of pulsation of contractile vacuoles demonstrated that this essential physiological process which is responsible for ion and volume regulation is sensitive to 10^{-12} M ANP treatments in *Tetrahymena* cells (Figures

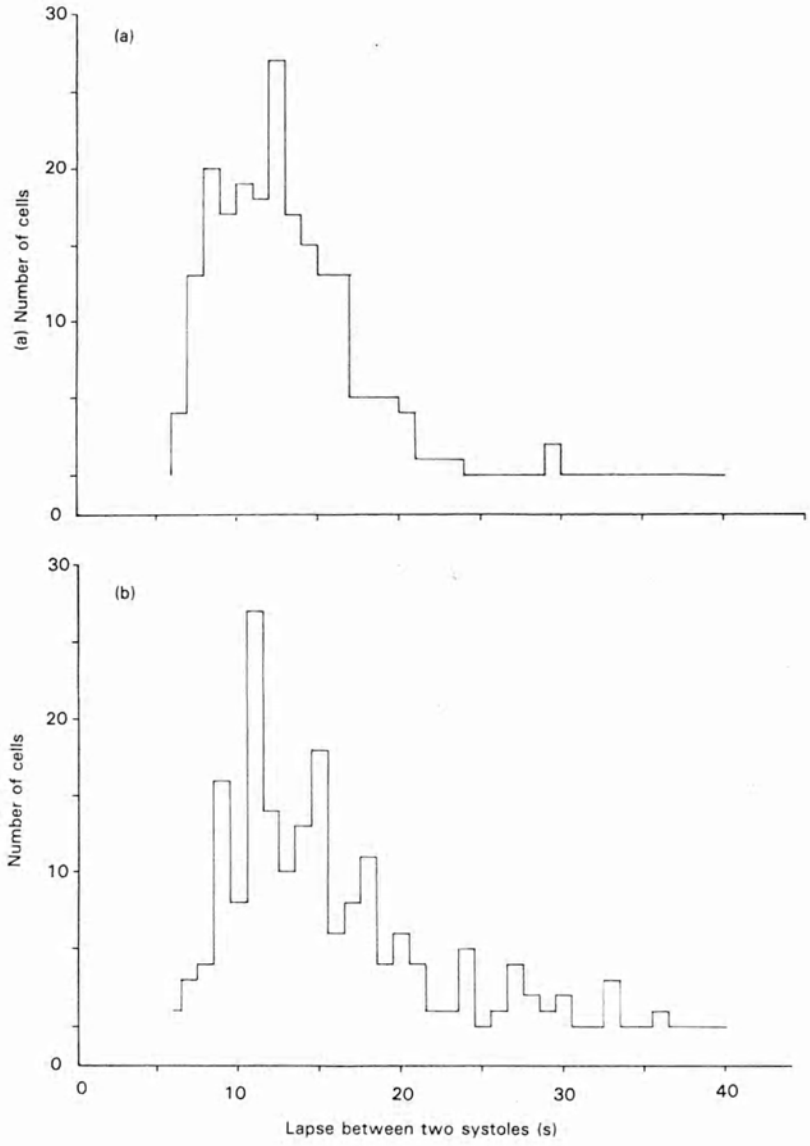

Figure 2 Histogram of contractile vacuolar activity of (a) control and (b) 10^{-12} M ANP-treated cells.

2a and 2b). The observed increase (110%) of lapse between two systoles of the contractile vacuoles indicates the agonist function of ANP.

The phagocytic activity of *Tetrahymena* cells was significantly depressed in a wide concentration range (10^{-13} to 10^{-10} M) and the phagocytic activity remained at the same level as the control only at the highest concentration (10^{-7} M) (Figure 3).

Growth of *Tetrahymena*

In this part of the experiments there was a marked difference in short- and long-term ANP treatments. While the short-term treatment increased the intensity of divisions significantly (Figure 4) in a wide range of concentrations (10^{-13} to 10^{-7} M), the long-term effect of ANP was almost ineffective (Figure 5), and had some positive but statistically insignificant effects only in the high (10^{-8} to 10^{-7} M) concentrations.

The short-term effect of ANP on chemotaxis had a biphasic profile, there was a maximal effect at 10^{-13} M but in the higher range (10^{-8}—10^{-7} M) cells also showed high chemotactic activity (Figure 6). The long-term treatments with ANP perturbed the effect on the profile (Figure 7). Although some data in the lowest concentrations are missing due to technical problems, it is evident that this 'restless' profile is completely different.

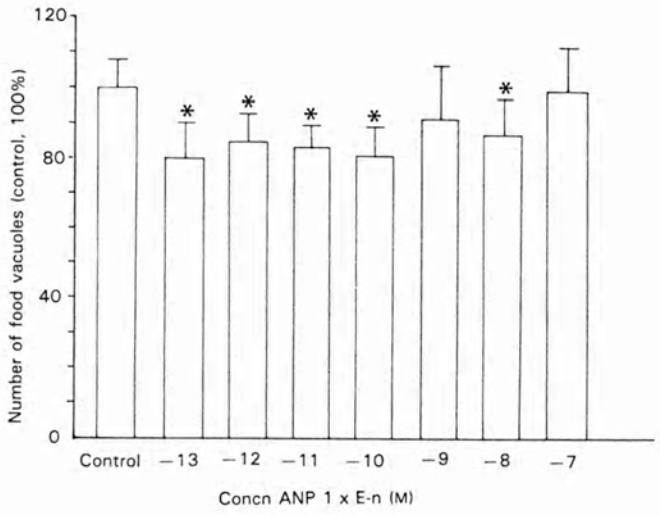

Figure 3 ANP treatment of Chinese-ink phagocytic activity.

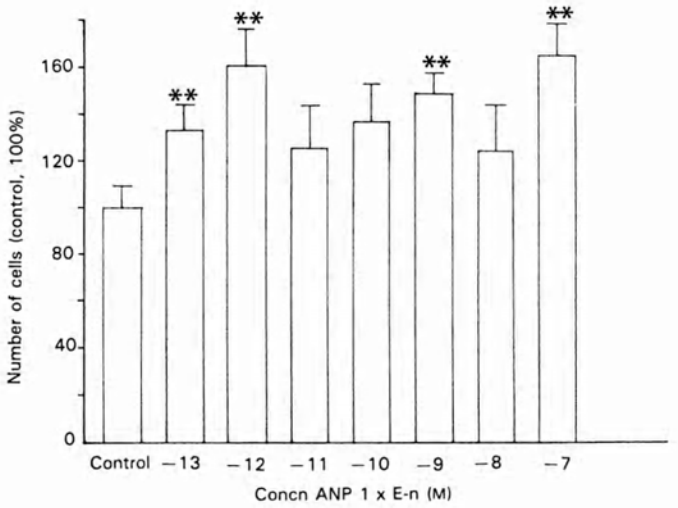

Figure 4 Short-term (1 h) treatment with ANP on the growth of cells.

The effects of ANP imprinting on chemotaxis were very characteristic in *Tetrahymena* (Figure 8). The C/A group verified our previous results that ANP had a significant chemoattractant character at 10^{-13} M concentration. Although chemoattraction increased, it was not so evident as in the short-term experiment using 10^{-13} M (Figure 6). This is not surprising as the two groups were not identical and they were not totally comparable while the age of the cultures differed: the cells of the C/A group were 24 h older than

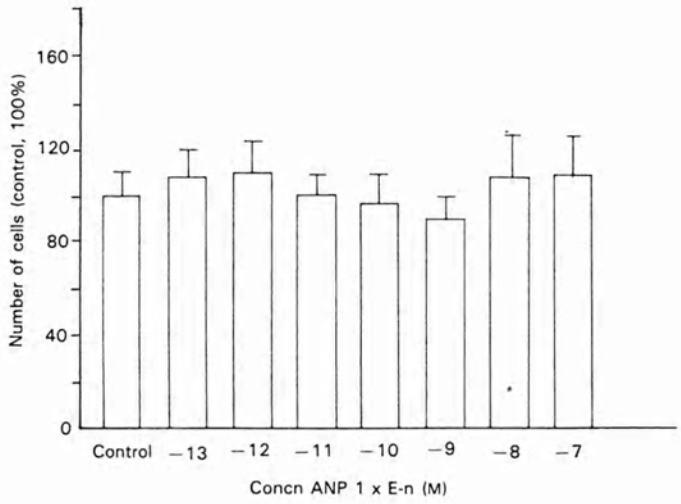

Figure 5 Long-term (24 h) treatment with ANP on the growth of cells.

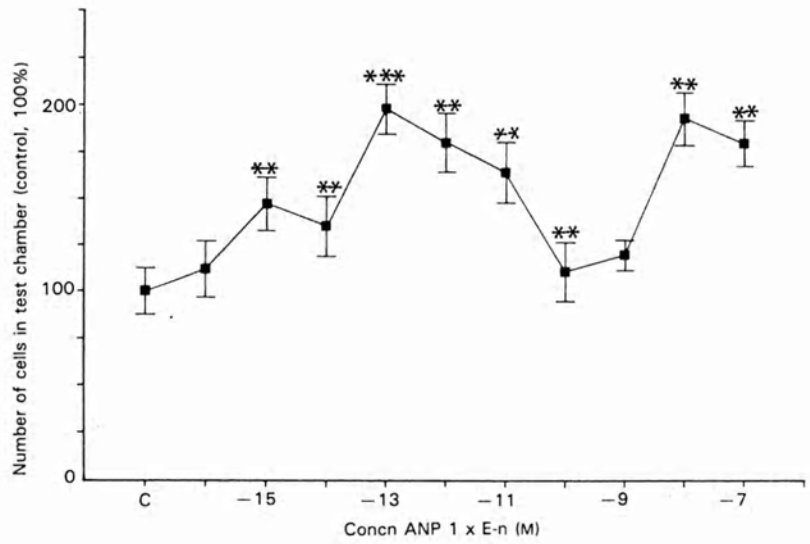

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Figure 6 ANP induced chemotactic activity.

the cells of groups in Figure 6. Cells which were pretreated with ANP 24 h before (A/C group) the chemotaxis assay expressed a very strong chemotactic activity in the pure culture medium. Those cells pretreated with ANP and which encountered this hormone again (A/A group) presented a less striking but significantly increased chemotaxis.

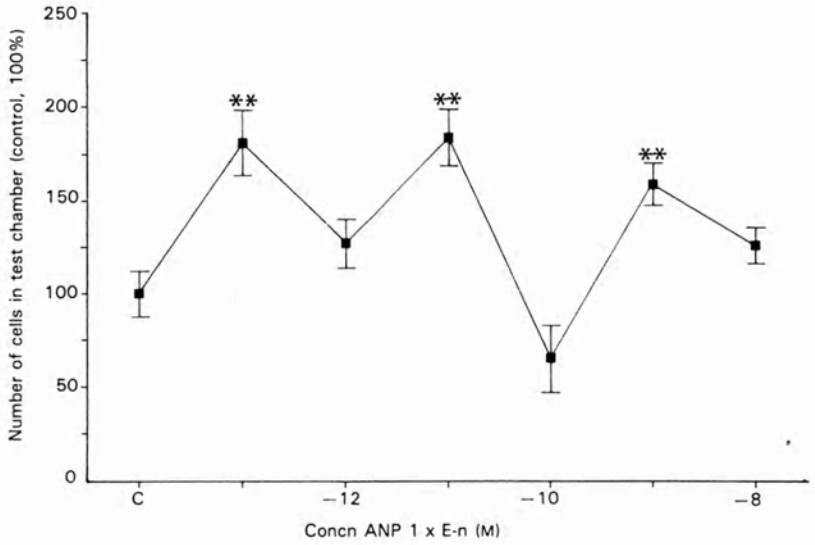

Figure 7 Long-term (24 h) treatment with ANP on chemotactic activity.

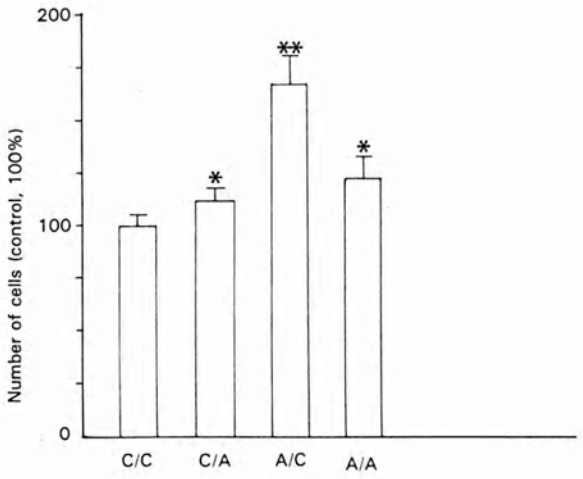

Figure 8 Effects of 10^{-13} M ANP pretreatments (imprinting) on chemotactic activity. C/C, control cells with chemoattractant in the culture medium; C/A, control cells and chemoattractant 10^{-3} M ANP; A/C, cells pretreated with 10^{-13} M ANP for 1 h and chemoattractant 10^{-13}M ANP; A/A, cells pretreated with 10^{-13} M ANP for 1 h and chemoattractant 10^{-13} M ANP.

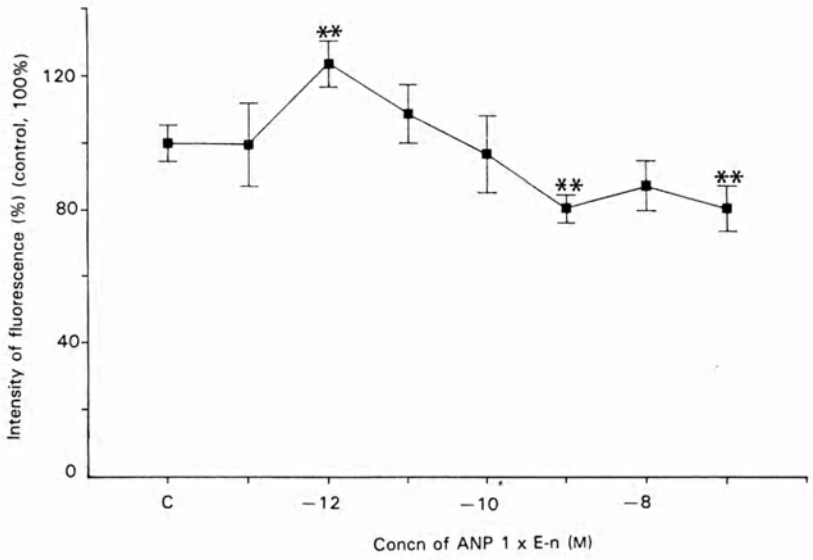

Figure 9 Effect of ANP pretreatments (imprinting) on FITC labelled ANP binding.

Our immunocytological experiments which were intended to detect the possible endogenous ANP of *Tetrahymena* did not reveal any significant data which could demonstrate the presence of ANP in any part of this type of eukaryotic cell. The data from our binding studies showed that the intensity of fluorescence in arbitrary units (AU) was (a) in the control group, 40.99; (b) in the 1:20,000 group, 40.04; (c) in the 1:100,000 group, 40.30; and (d) in the 1:200,000 group, 40.33.

The ANP binding capacity of *Tetrahymena* cells was evaluated using a FITC labelled form of the molecule. The cells imprinted with different concentrations of ANP showed a concentration dependent binding, where the maximal binding capacity was observed at 10^{-12} M ANP pretreatment (Figure 9).

Discussion

The aim of the present study was to evaluate the effects of the intensively investigated vertebrate hormone atrial natriuretic peptide (ANP) on invertebrate, unicellular cells. *T. pyriformis* GL model cells are very suitable for this kind of research as they possess both the essential second messenger mechanisms such as cGMP dependent processes (Köhidai *et al.*, 1992) and intracellular enzyme cascades (Kovács and Csaba, 1987) which are also present in higher animals. On the other hand the results may eventually lead to observations of new, still not investigated aspects of ANP.

Since the result of our trials to detect endogenous ANP in *Tetrahymena* cells was negative we suggest that ANP influences *Tetrahymena* as an extraneous substance.

The two main groups of experiments carried out involved: (1) detection of well-known, receptor-dependent ANP effects on unicellular animals , and (2) detection of supposed ANP effects on new, still uncharacterized processes. Studies on the time-dependent action of ANP on sodium and potassium release demonstrated that the molecule was able to develop a fast sodium release at the unicellular level. The potassium concentration which was unchanged suggested that there was a specific ANP effect on sodium since we have no evidence for the cotransport of potassium as was described earlier by O'Donell and Owen (1986).

The volume and ion regulatory functions of *Tetrahymena* cells were investigated when we tried to find correlations between the activity of the contractile vacuole and ANP treatments. Contractile activity which was delayed by ANP suggested that this mechanism was not responsible for increased extracellular sodium concentration, and that modified membrane transport processes were involved in this response. Although the volume of the cells was not determined, it is probable that decreased activity of the contractile vacuoles was not strong enough to significantly modify the cell volume.

Among the investigated, still uncharacterized physiological responses of ANP the consequent and general inhibitory effect on Chinese ink phagocytosis was valuable as this process has a well-defined regulation at both membrane-receptor level (Csaba and Lantos, 1976) and intracellular levels (Darvas and Csaba, 1990). It is very hard to determine the responsible mechanism, but the wide range of inhibition suggests that ANP has a strong chemical effect on the membrane and on its receptors.

The modified growth characteristics indicated that the actions of ANP were very time-dependent. The agonist-like effect of short-term treatment (1 h) was also observed in the 24 h cultures, which showed that the alterations induced were relatively stable. The negative effect of long-term treatments may have a close connection with down-regulation mechanisms. This phenomenon was demonstrated in *Tetrahymena* by Csaba and Köhidai (1986) and it is strong evidence for selective binding of hormones and biologically active molecules. Negative results at the physiological level support the view that *Tetrahymena* has receptive structures for ANP.

The third physiological response which we observed involved chemotaxis. Like the rate of division, experiments on chemotaxis also presented differences between short- and long-term treatments. The chemoattractant character of ANP molecules was strongest at 10^{-13} M concentration which correlated well with the observed maximal effects on growth. Pretreatments for 24 h with ANP did not produce down-regulation, but there was a 'restless' curve which indicated a perturbed mechanism.

From studies of imprinting by ANP some characteristic information about the supposed ANP receptors in *Tetrahymena* cells has been obtained. The presence of hormonal imprinting presumes specific, amplifiable structures at membrane (Köhidai *et al.*, 1986a), cytoplasmic (Csaba *et al.*, 1987) or nuclear (Köhidai *et al.*, 1986b) levels. The first encounter with the hormone or other signal molecule induces and modifies one or more of the above-mentioned levels, resulting in a 'memory'-like phenomenon (Csaba, 1980, 1984, 1985). The altered responses are present not only in the originally induced cells but also in the cells of the offspring generations (Köhidai *et al.*, 1990).

The results of chemotaxis on ANP imprinted cells are difficult to interpret. As the ANP pretreatment (A/A) did not induce a significantly higher response when compared with the control conditioned cells (C/A) we cannot deduce that the mechanisms involved are due to imprinting. However, the other group of ANP pretreated cells (A/C) showed a characteristic effect of receptor mediated processes, when the increased chemotactic activity was compared with the C/A group. Nevertheless, it was not possible to determine whether the mechanisms of imprinting were affected by imprinting, or whether cell-cycle or other physiological phenomena masked such mechanisms.

The maximal binding (200%) of FITC labelled ANP (at 10^{-12} M) to ANP pretreated cells showed that there was probably a concentration-sensitive

population of binding sites in the membrane. These components were present after 24 h in the membrane of the cells. At this time only about 5% of the cells were identical with the pretreated cells. This suggests that ANP has not only binding sites on the membrane of the unicellular cells but this substance is able to induce membrane linked other processes which are responsible for intracellular regulation.

The ANP form we investigated is one of the three described in this hormone-receptor system and this fact obviously determines further studies, but our results may help to characterize the hormone. It should be noted that the optimal ANP concentrations which produce the maximal chemotaxis and maximal division rate were in the same range (10^{-13}—10^{-12} M) as the maximal ANP-FITC binding. This coincidence of results is more significant when we consider the similarities with the 10^{-12} M human serum concentration of ANP. The concordance at the level of fundamental physiological functions (division, chemotactic migration) and binding sites (receptors) suggests that ANP has greater implications in hormone evolution than previously considered.

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