



Effects of the mammalian vasoconstrictor peptide, endothelin-1, on *Tetrahymena pyriformis* GL, and the immunocytological detection of endogenous endothelin-like activity

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The vasoconstrictor endothelin-1 (ET-1) is shown to have significant physiological effects on a unicellular organism, *Tetrahymena pyriformis*. These responses include: (1) A significant increase in intracellular $[Ca^{2+}]$ induced by 10^{-10} M ET-1; (2) Increased chemotaxis, maximal at 10^{-10} M; and (3) A small inhibition of proliferation at the 10^{-13} – 10^{-12} M concentration range. Immunocytochemical detection of endogenous ET-1 using rabbit antibodies directed against human or porcine ET-1 indicates that this is a further example of the widening group of vertebrate hormones now known to be synthesized by *Tetrahymena*. These observations suggest that hormones are of considerable antiquity in their phylogenetic appearance and have been highly conserved throughout evolution.

Key words: Endothelin-1; *Tetrahymena*; Evolution; Chemotaxis; Growth; Immunocytology; Microwave fixation.

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Introduction

The 21 amino acid polypeptide, endothelin-1 (ET-1), is among the most potent mediators of vasoconstriction in mammals (Yanagisawa *et al.*, 1988). Further investigations have revealed a new family of endothelins (endothelin-1, -2, -3, and a vasointestinal contractor). These peptide hormones have been isolated from vascular endothelial cells of heart, liver, kidney, uterus and the lungs. On the basis of receptor level studies, at least two types (ET_A and ET_B) of well-defined seven-looped membrane receptors have been found (Miller *et al.*, 1993). Their tissue specificity is not precisely worked out, but clear dominance has been found for ET_A in the heart (Davenport and Maguire, 1994). Signal transduction is by a G protein-phospholipase C system (Hay *et al.*, 1993), and one product of

this familiar pathway, IP₃, induces intracellular $[Ca^{2+}]$ release, while the other product, diacylglycerol, possibly activates intracellular protein kinase C linked mechanisms (Marsden *et al.*, 1989). Some other kinases (β -adrenoceptor-kinase or cAMP-dependent kinase) are also thought to play a role in this signal transduction, with cGNP being involved in some tissues (Reiser, 1990). It has also been reported that endothelin can open $[Ca^{2+}]$ -channels in the plasma membrane (Miasiro *et al.*, 1988).

These physiological events show that endothelin-dependency may be involved in such responses as spasmogenesis (Hay *et al.*, 1993) or mitogenesis (Noveral *et al.*, 1991), but so far, all reports have been exclusively concerned with mammals. The significance of the endothelin family has become of direct interest in clinical conditions in man, since elevated endothelin levels have already been associated within such diseases as pulmonary hypertension (Vanhoutte, 1988), diabetes mellitus (Miller *et al.*,

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1993), and malignant growth (Yamashita *et al.*, 1991).

The effectiveness of hormones or hormone-like substances of "higher" order animals on "lower" eukaryotic cells is not uncommon. Molecules such as ACTH, FSH, TSH, insulin, ANF, histamine, serotonin, T3, and T4 also act as signal molecules in unicellular organisms. In our well-acknowledged model cell system, *Tetrahymena pyriformis* (Csaba, 1985), responses can be detected in membrane fluidity (Kovács *et al.*, 1984), membrane receptor distribution (Kóhidai and Csaba, 1985), and alterations in membrane potential (Kóhidai *et al.*, 1986), as well as hormone-specific changes in metabolic activity (Kóhidai *et al.*, 1985), growth rate (Christensen and Rasmussen, 1992) and chemotaxis (Kóhidai *et al.*, 1994). On the other hand, many of the unicellular organisms not only receive and respond to these signals from "mammalian" hormones, but are capable of synthesizing these molecules or closely allied ones themselves, e.g. *Tetrahymena* not only responds to insulin, but produces a remarkably similar insulin-like protein (Le Roith *et al.*, 1980). This also is true for somatostatin (Berelowitz *et al.*, 1982), ACTH (LeRoith *et al.*, 1982) and relaxin (Schwabe *et al.*, 1983).

It has been proposed that these same hormones of higher order animals are working as early messengers of intercellular communication throughout the animal kingdom (LeRoith *et al.*, 1987), but there are several unsolved questions. In this regard, we have explored the effects of endothelin-1 on *Tetrahymena*, and used it to answer three questions.

- (i) Is endothelin-1 a relatively recent signal molecule of only higher animals, or does it also have some effect on "lower" unicellular organisms?
- (ii) Is endothelin-1, or a close homologue, produced endogenously by *Tetrahymena*?
- (iii) What relationship exists between the presumed effects of endothelin-1 on *Tetrahymena* and those on the "higher" cell systems which might be seen as common hormonal responses?

Materials and Methods

Cells

Tetrahymena pyriformis GL strain was used in the logarithmic phase of growth. The cells were cultured at 28°C in tryptone medium (Difco, Michigan, U.S.A.) containing 0.1% yeast extract.

Chemicals

Endothelin-1 was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rabbit

anti-endothelin-1 (human, porcine) serum was purchased from Peninsula Labs (Belmont, CA, U.S.A.) (cross-reactivity: endothelin-1 100%; endothelin-2 7%; endothelin-3 7%).

Parameters studied

The effects of ET-1 were evaluated in four ways by dose dependent analysis of (a) intracellular $[Ca^{2+}]$ release, (b) growth, (c) chemotaxis and (d) phagocytosis. The density of *Tetrahymena* cultures was usually 10^4 cell/ml in each case, except (b) (see data below).

(a) *Effect on intracellular $[Ca^{2+}]$ release.* Cells were treated with 10^{-10} M ET-1 for 15 min before 50 mM Quin2-AM was applied for 25 min for detection of intracellular trapped $[Ca^{2+}]$. Samples were dried on slides, rinsed three times with PBS (0.9% phosphate buffered saline solution, pH 7.2) and once with distilled water. The control group underwent the same process, except the first step where the solvent (culture medium) of ET-1 was administered. The intensity of fluorescence of food vacuole-free cytoplasm was measured by means of a Zeiss Fluoval cytofluorimeter, in which the analogue signals were converted by an analogue digital processor before being registered by a Hewlett Packard HP41 CX microcomputer. The standard deviations and the significance values (Student's *t*-test) were given by a built-in algorithm.

(b) *Dose-dependent effect on growth.* 0.2 ml cells of logarithmic phase cultures were transferred to 2.8 ml fresh culture medium. The cells were treated with 10^{-15} – 10^{-10} M ET-1 for 24 hr, with controls being treated with equal volumes of culture medium. After fixation in 4% formaldehyde dissolved in PBS, cell density was determined by electronic counting (Christensen *et al.*, 1992), each measurement being the average of five parallel determinations of 70 ml cell suspension, with small standard deviations. Each experiment was repeated three times.

(c) *Dose-dependent effect on chemotaxis.* To determine the chemotactic activity of *Tetrahymena* cells, we applied a modified version of Leick's quantitative capillary assay (Leick and Helle, 1983). *Tetrahymena* cells were placed into an outer container, while concentrations of 10^{-15} – 10^{-9} M of ET-1 or control (culture medium) were placed in a multi-8-channel automatic pipette that ensured uniformity of sampling. The incubation time was 15 min. Samples were taken from the pipette and fixed as above. The number of cells demonstrating positive chemotaxis was determined, with each experiment being repeated five times.

(d) *Dose-dependent effect on phagocytosis.* Phagocytotic activity was measured by Chinese ink phagocytosis. *Tetrahymena* cultures were

transferred to and starved in Losina-Losinsky medium (Losina-Losinsky, 1931) containing inorganic salts for 3 hr. The cells were then treated with different concentrations of 10^{-15} – 10^{-10} M ET-1 and Losina-Losinsky solution containing Chinese ink was added in the ratio *Tetrahymena* suspension:ET-1 solution:Chinese ink = 1:1:1 for 15 min. Samples were fixed with formaldehyde and washed twice in Losina-Losinsky solution before the samples were placed for drying on slides. The number of intracellular ink particles was counted in 200 cells. Since each experiment was carried out in triplicate, each histogram represents the phagocytotic activity of 600 cells.

Assay of endogenous ET-1

Twenty 20 ml samples (density 10^4 cells/ml) were placed on to specifically surface-treated slides. Our microwave fixation method developed for this protozoa was applied to minimize the non-specific effects of fixation (Köhida *et al.*, 1992). Cells were washed thrice with PBS at pH 7.2, and the samples incubated with a dose range ET-1 antibody at room temperature in a moist chamber for 1 hr. Titres of the tested ET-1 antibody solutions were 1/20,000, 1/15,000, 1/10,000, 1/5,000 and 1/1,000 (the sensitivity of the applied antibody was 1/21,000, $IC_{50} = 18$ pg/tube). The antibody was dissolved in an assay buffer as described in (Köhida *et al.*, 1992).

The control groups were: (i) cells treated with the assay buffer, and (ii) cells treated with different concentrations (1/20,000–1/1,000) of rabbit serum. Samples were washed three times in PBS. FITC-labelled protein-G system was used to detect the binding of ET-1 antibodies. For cytofluorimetric evaluation of the samples, the same equipment was used as described in (a), and for statistical analysis, the above-mentioned built-in tests, SigmaPlot 4.0 and Origin 2.8 were used.

Results

Effect on $[Ca^{2+}]$ release

We detected a significantly higher $[Ca^{2+}]$ -content in ET-1 treated *Tetrahymena* cells compared with control cells ($P < 0.05$; Fig. 1). The distribution of fluorescence of the detector molecule Quin2-AM was homogenous in both control and ET-1 treated cells.

Effects on growth

Tetrahymena cultures in logarithmic phase showed a concentration-dependent response in growth rate (Fig. 2). The inhibitory effect was maximal (and significant) at concentrations of

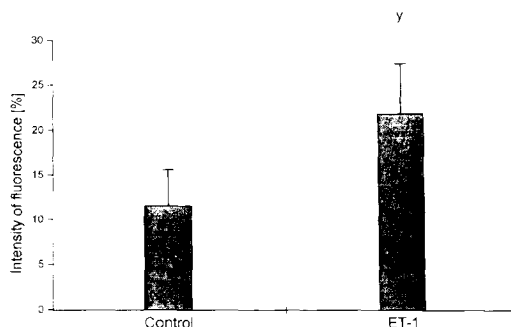


Fig. 1. ET-1 induced intracellular $[Ca^{2+}]$ release detected by Quin2-AM technique. n represents the total number of measurements per group; $y = P < 0.01$.

10^{-13} – 10^{-12} M ET-1. No other concentrations produced significant effects on growth.

Effects of chemotaxis

ET-1 possesses chemoattractant potency to *Tetrahymena* cells (Fig. 3). The maximal significantly effective concentration was at 10^{-10} M, but even the lowest tested concentration was effective.

Effects on phagocytosis

Phagocytotic activity was depressed to a slight degree by ET-1. This effect was observed across the whole ET-1 concentration range of 10^{-15} – 10^{-10} M (Fig. 4a and b), with a decrease in the number of multiparticle cells.

Immunoreactivity of the cells to ET-1 ABs

Both of our microscopic observations and measurements demonstrated the presence of an immunologically reactive endogenous form of ET-1 in *Tetrahymena pyriformis* GL cells. The distribution of ET-1 has showed no particular localization, with a diffuse reactivity throughout the cytoplasm, but especially close to the plasma membrane. Relative to both controls, immunodetection was positive and significant ($P < 0.05$), even at the lowest titre (1/20,000; Fig. 5).

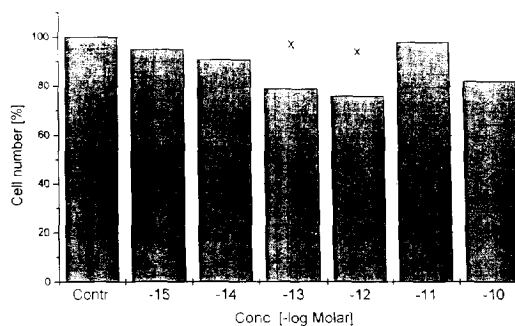


Fig. 2. Dose-dependent effect of ET-1 on the growth of *Tetrahymena pyriformis*. n represents the total number of measurements per group; $x = P < 0.05$.

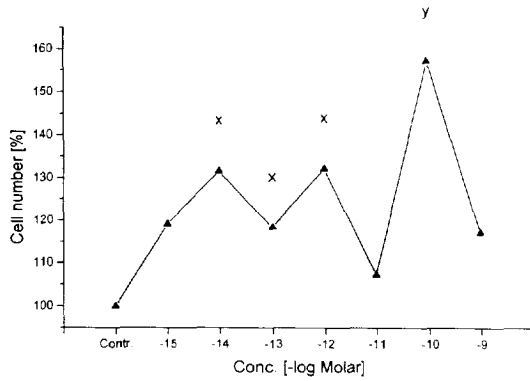


Fig. 3. Dose-dependent effect of ET-1 induced chemotaxis in *Tetrahymena pyriformis*. *n* represents the total number of measurements per group; *x* = *P* < 0.05, *y* = *P* < 0.01.

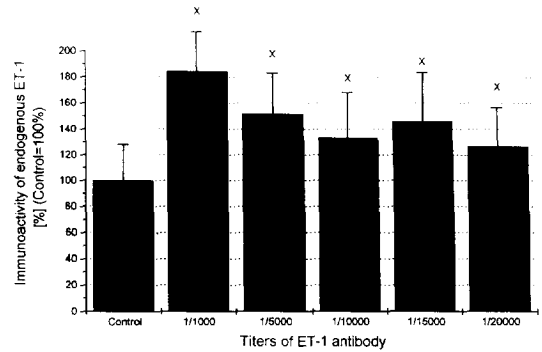


Fig. 5. Dose-dependent study (1/20,000–1/1000) of endogenous immunoreactive ET-1 content of *Tetrahymena pyriformis*. *n* represents the total number of measurements per group; *x* = *P* < 0.05.

Discussion

Our results demonstrate that the potent mammalian vasoconstrictor ET-1 has characteristic effects on a unicellular organism. An increase of intracellular [Ca²⁺] is known to be an IP₃-dependent (Hay *et al.*, 1993), or a membrane-channel dependent (Miasiro *et al.*, 1988), effect of ET-1. In *Tetrahymena*, both mechanisms are present (Kőhidai *et al.*, 1987; Kovács and Csaba, 1990) and have been characterized as mechanisms

responsive to hormonal action (Renaud *et al.*, 1991). Our positive results on intracellular [Ca²⁺] increase indirectly support and imply the presence of the appropriate pathway (receptor, signal transmitter system/second messenger, etc) in this unicellular organism.

Protozoa, especially *Tetrahymena*, are very sensitive to many different substances (Wheatley *et al.*, 1993, 1994), physiological factors (Christensen *et al.*, 1992) and physical agencies exerted through environmental disturbances (Zeuthen, 1971). Twenty-four-hour culturing was considered as a test-point to characterize the effects of ET-1 on growth as our previous experiments showed that the end of logarithmic phase is around this time in *Tetrahymena* GL cultures (Kőhidai *et al.*, 1987). ET-1 not only affects mammalian endothelial cells, but is also known to affect the mitotic activity of others, e.g. tumor cells (Nakaki, 1989). The significant depression of the growth of *Tetrahymena* at low (10⁻¹³–10⁻¹² M) concentrations suggests that this essential function of cells is also influenced by ET-1, but here (Fig. 2), however, it is a different response from that seen with cells of “higher” phylogenetic ranking.

Previous data showed that *Tetrahymena* shows characteristic responses to hormones such as ACTH, insulin, T2, histamine, serotonin (Kőhidai *et al.*, 1994) and other signal molecules (László *et al.*, 1988). The strong chemotactic response at all applied concentrations demonstrates that this relatively small ET-1 molecule has not only the potency to induce specific intracellular pathways, but is a true “attractant”, with cells being able to recognize the signal and respond to it by changing their direction of swimming. Since chemoattraction is often closely connected with nutritive activity (Leick and Hellung-Larsen, 1992), the accompanying depression of phagocytosis over a wide dose-range of ET-1 is noteworthy. Although the

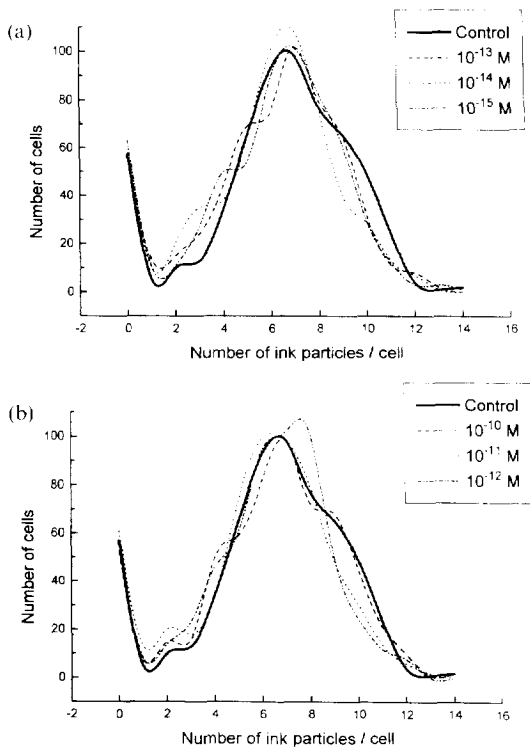


Fig. 4. Dose-dependent effect of ET-1 on phagocytosis in *Tetrahymena pyriformis*. Histograms (a) 10⁻¹⁵–10⁻¹³ M and (b) 10⁻¹²–10⁻¹⁰ M ET-1 treatment. *n* represents the total number of measurements per group.

response is low grade, it suggests nevertheless, that the observed positive chemoattraction must be induced through an independent pathway from phagocytosis. It also suggests that the mechanisms responsive to ET-1 may not be in the main stream of metabolic pathways, but only through connections with cell signalling in Protozoa.

The overall results with *Tetrahymena* serve to emphasize the ever-increasing evidence for the universality of hormones as intercellular signals throughout the eukaryotes, and by inference of the phylogenetical unity of their signal receptor/receiver systems. It has yet to be shown that pure hormones from protozoa will induce the same characteristic responses in mammalian cells that is observed in the opposite situation. The physiological responses elicited by ET-1 over the effective concentration range 10^{-10} – 10^{-15} M correlates well with the picomolar levels of ET-1 in vertebrate sera (King *et al.*, 1990). This fact points to a great antiquity and highly conserved mechanism in all receptor-signal interactions.

The presence of an endogenous ET-1-like molecule in *Tetrahymena* should no longer come as a surprise to workers in this field since several hormones, including insulin (LeRoith *et al.*, 1980), ACTH (LeRoith *et al.*, 1982), and relaxin (Schwabe *et al.*, 1983) have been described in this organism. The distribution of an ET-1-like molecule in this organism seems to be widespread throughout the cytoplasm, but we know little about that of other hormone-like molecules in this species, or how the pattern might change with physiological and environmental circumstances, and with cell growth. It would appear that ET-1 may have an autocrine, as well as a paracrine function, but this has not been established by the present experiments. If this proves to be the case, it would be in accord with Blalock who surmised a common genetic background to both signal molecule and their receptors (Swords *et al.*, 1990).

Our positive results gained from physiological experiments with ET-1 and immunocytochemical detection of endogenous ET-1-like activity point also to a virtual contradiction: how can the exogenous ET-1 induce responses in cells synthesizing ET-1-like molecules themselves? In this aspect, we should consider the following significant aspects. Several other hormones or hormone-like endogenous substances, e.g. insulin and ACTH, were also detected in *Tetrahymena* (LeRoith *et al.*, 1980, 1982); however, the vertebrate forms of these hormones could elicit significant responses on biochemical and physiological levels (Csaba, 1994). It is also worth noting that there is a possible difference in the well characterized exogenous ET-1, and

the ET-1-like substance found in *Tetrahymena*. The molecule found in *Tetrahymena* requires further biochemical characterization to clarify the chemical identity of the substance over the immunocytochemical similarities with vertebrate ET-1. The other aspect is that, in our experiments, the applied ET-1 concentrations covered a wide range however high the sensitivity of the immunocytochemical detection was. In this way, there is no evidence that the endogenous ET-1 has the potency to induce or modify the tested activities of the cell, however real are the supposed theories about its autocrine and paracrine activities.

The future objective of our work is to compare the endogenous ET-1-like content of cells cultured in axenic conditions and in chemically defined medium, e.g. Szablewski's medium (Szablewski *et al.*, 1991).

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