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Investigations of receptor-mediated phagocytosis by hormone-induced (imprinted) *Tetrahymena pyriformis*

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Abstract. Receptor-mediated endocytosis by *Tetrahymena pyriformis* was studied using tetramethylrhodamine isothiocyanate-labeled concanavalin A (TRITC-Con A) with fluorescence and confocal microscopy. In the presence of insulin, or 24 h after insulin pretreatment (hormonal imprinting), the binding and uptake of TRITC-Con A increased when compared to controls, owing to the binding of TRITC-Con A to sugar oligomers of insulin receptors. Mannose inhibited the binding of Con A, thus demonstrating the specificity of binding. Histamine, a phagocytosis-promoting factor in mammals and *Tetrahymena*, and galactose, did not influence the uptake of TRITC-Con A.
Key words. Insulin; imprinting; *Tetrahymena*; phagocytosis; insulin receptor.

Hormonal imprinting develops during the first encounter between a target cell and a hormone molecule, and elicits changes in physiological and biochemical conditions, which last in mammals for the individual's lifetime, and in unicellular organisms for several generations[1,2]. These changes are expressed mainly as a response to repeated encounters with the hormone. Significant changes following hormonal imprinting have been demonstrated in hormone binding capacity[3-5], in functional activity and levels of second messengers[6,7], in the rate of proliferation, and in storage of PAS-positive material[3].

Fundamental physiological events in protists, such as proliferation, changes in cell size, action of the contractile vacuole, and phagocytosis, give useful information about the condition of a cell, whichcan be changed considerably by hormonal imprinting. In this report the relationship between phagocytosis and hormonal imprinting was investigated. *Tetrahymena* sp., a ciliated protist, can take up nutrients in three ways: a) by micropinocytosis[8]; b) by transport through the plasma membrane[9] and c) by formation of food vacuoles (FVs) through the oral field[10]. Some results indicate that FV formation is a receptor-mediated process; the formation of FVs is induced[11] or inhibited[12] by ligands possessing receptors. According to Ricketts[11] endocytosis is affected by several substances, for example amino acids, peptides, and proteins are active inducers of the formation of FV. With latex beads, the size influences the quantity of particles taken up[13].

When Tryptone medium is sterilized, particles form. *Tetrahymena* cultured in this boiled medium form FVs, but they do not do so when the medium is filtered particle-free[14]. In particle-free, synthetic medium, in which the rate of cell proliferation is very high, pinocytosis is the mechanism of nutrient uptake[8]. These observations suggest that the inducer of FV formation is a mechanical stimulus. The number of FVs can change in the presence of some bioactive materials e.g., histamine[15], lectins[16], endorphins[12], cAMP[17], and in the presence of inactive materials that are localized in FVs during phagocytosis and easily observed with light microscopy e.g., Chinese ink[17], metal colloids[12] and trypan blue[18].

The degree of binding of a ligand can be altered by hormonal imprinting[1,2,19]. Insulin imprinting increases both the insulin and the concanavalin A binding capacity of the *Tetrahymena* membrane, since the insulin and Con A binding sites are similar[20]. Insulin imprinting enhances the rate of proliferation, and the increased rate of proliferation necessarily means increased nutrient uptake[16]. In the present study we followed the binding of TRITC-Con A to investigate whether the hormonal imprinting with insulin alters the binding characteristics of the cytopharyngeal membrane of *Tetrahymena*, and to observe the response of phagocytosis to the treatment.

Materials and methods

Logarithmic phase populations of *Tetrahymena pyriformis* Zeuthen (ATCC 30327) were grown in 1% Tryptone (Difco, MI, USA) containing 0.1% yeast extract at 28 °C. For assays of phagocytosis the cells were treated with tetramethylrhodamine isothiocyanate-labelled Con A (TRITC-Con A, 10 μg/ml; Sigma Chemical Co., St. Louis, MO, USA). At the end of the incubation time phagocytosis was halted and the cells

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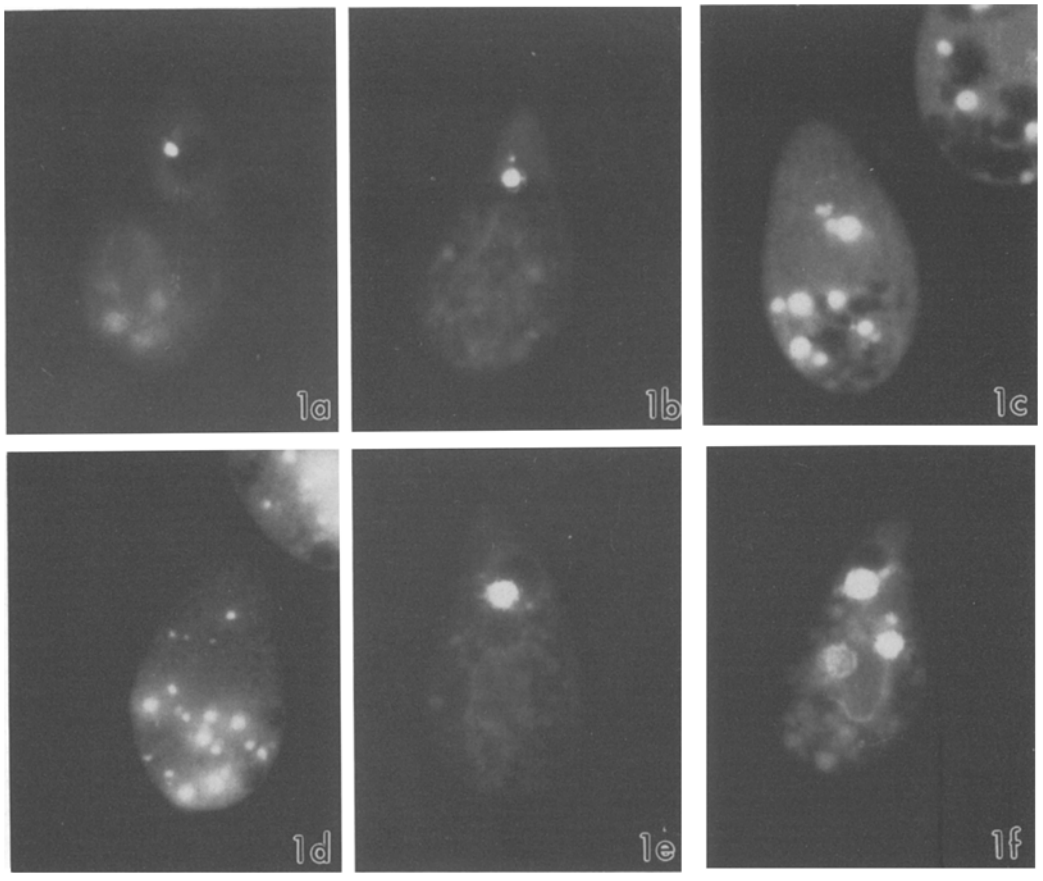

Figure 1. Binding of TRITC-Con A to control (*a*–*d*) and insulin-imprinted cells after (*a*) 30 s; (*b*, *e*) 1 min; (*f*) 10 min; (*c*) 30 min and (*d*) 60 min incubation, (×900).

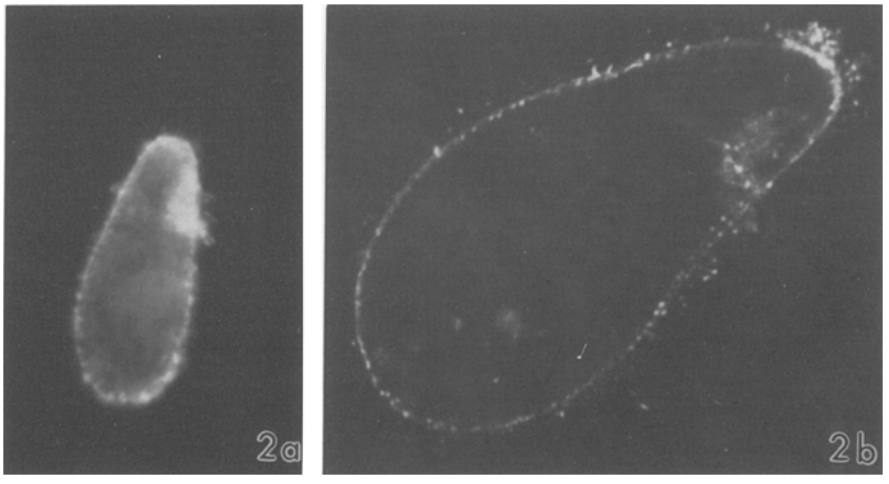

Figure 2. Binding of TRITC-Con A to formalin-fixed, insulin-imprinted cells. Fluorescent light microscopy (*a*: ×750) and confocal microscopy (*b*: ×1200).

fixed with 8% (v:v) formalin (diluted in phosphate buffered saline, PBS, pH 7.2). After fixation the cells were washed thrice with PBS and dropped onto slides, and the cells were examined for the number of vacuoles and for the localization of TRITC-Con A.

Phagocytosis of TRITC-Con-A was assayed for the following groups:

A) An 'absolute control' group of untreated cells, incubated in TRITC-Con A for 30 s, 1 min, 5 min, 10 min, 20 min, 30 min, and 60 min.

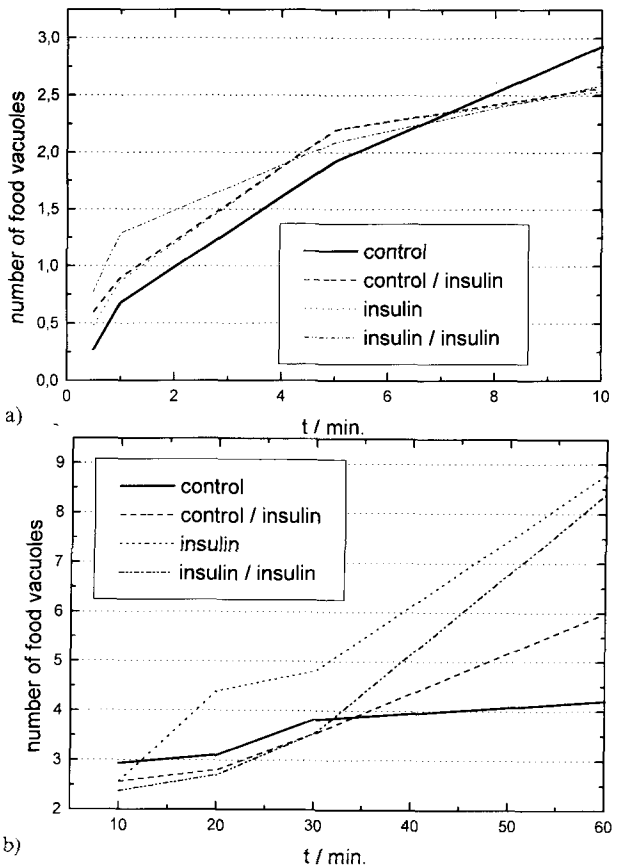

Figure 3. Formation of FVs in control cells in the absence and in the presence of insulin (control/insulin) and of imprinted cells in the absence (insulin) and presence of insulin (insulin/insulin) over two different time-periods.

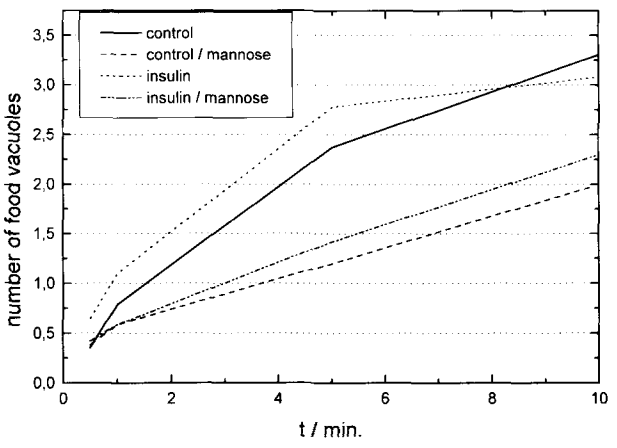

Figure 4. Effect of mannose on FV formation in control and insulin-imprinted (insulin) *Tetrahymena*.

B) Insulin-imprinted cells. Imprinting was with 10^{-6} M insulin (porcine, crystalline; Sigma, St. Louis, MO, USA) for 1 h followed by 3 washes in culture medium and then a final transfer to fresh culture medium. After imprinting, incubation in TRITC-Con A began 6 generations later (about 24 h). It was carried out for 30 s, 1 min, 5 min, 10 min, 20 min, and 30 min.

C) Control and insulin-imprinted cells, with 10^{-6} M insulin present during incubation with TRITC-Con A.

D) Control and insulin-imprinted cells in the presence of 250 µg/ml D(+) mannose or D(+) galactose. The incubation time with TRITC-Con A was 30 s, 1 min, 5 min, and 10 min.

E) Control cells in the presence of 10^{-6} M histamine: (i) histamine and TRITC-Con A were applied to cells simultaneously; (ii) TRITC-Con A was applied to cells following a 1 min treatment with histamine; (iii) TRITC-Con A was applied to cells following a 30 min treatment with histamine.

F) Fixed control and insulin-imprinted cells. Following a 5 min fixation with 4% formalin (in PBS, pH 7.2) the cells were washed thrice with PBS and then incubated for 5 min in 25 µg/ml TRITC-Con A at 27 °C. The cells were observed with a Bio-Rad MRC 600 confocal microscope.

In each treatment/group 100 cells were observed with a Nikon fluorescent microscope, and the mean vacuole number calculated. Statistical analysis was done by Student's t-test and analysis of variance (see 'Results').

Results

Binding of TRITC-Con A to the cytopharyngeal membrane was very intense after 30 s of incubation and fluorescent FVs were observed immediately after binding (fig. 1). Fixed cells (group F) also displayed a high degree of binding of TRITC-Con A to the cytopharyngeal membrane, which was more prominent in insulin-imprinted cells (fig. 2).

The number of red fluorescent FVs in untreated, control cells (group A) increased continuously; however, the increase slowed after 10 min incubation (figs 3a and 3b). Addition of insulin for 30 s or 1 min to non-imprinted cells increased the number of FVs significantly ($p < 0.01$), and this number still increased after 5 min of incubation ($p < 0.05$) (fig. 3a). The number of FVs was reduced when compared to the control cells after 10 min, 20 min, and 30 min incubation; it was significantly depressed in the 20 min sample ($p < 0.05$; fig. 3b). After a 60 min incubation in insulin, the number of FVs was again higher than in the control group. In the case of insulin-imprinted cells (group B), incubations of 30 s, 1 min, and 5 min increased the number of FVs (30 s = $p < 0.01$; 1 min and 5 min = $p < 0.05$) while 10 min incubation decreased it ($p < 0.05$; fig. 3a). Following the longer incubations (20 min, 30 min, and 60 min) there was again a higher value ($p < 0.01$) compared to the control group. The number of FVs was especially enhanced after 60 min incubation (fig. 3b). In imprinted cells in the presence of insulin (group C) the number of FVs was increased after 30 s and 1 min incubation, and this difference was considerable ($p < 0.01$) when compared to control cells (group A) or

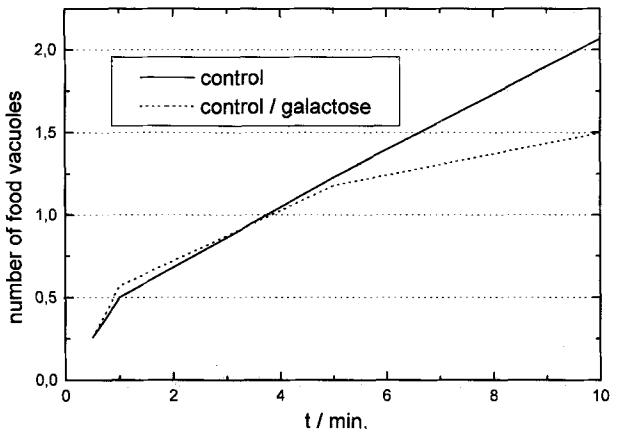

Figure 5. Effect of galactose on FV formation in *Tetrahymena*.

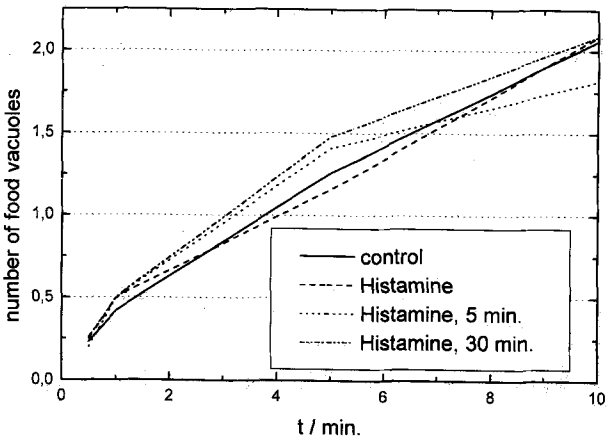

Figure 6. Effect of histamine and histamine pretreatment on FV formation in *Tetrahymena*.

imprinted cells in the absence of insulin (group B; $p < 0.01$). Following 10 min, 20 min, and 30 min incubations, the number of FVs was less than that of both control cells with or without insulin, and insulin-imprinted cells without insulin (group B), while after 60 min incubation there was a great increase (fig. 3b). In the presence of insulin, the size of FVs was greater than that of the control. This was especially noticeable in the insulin-imprinted cells (figs 1e and 1f). After 60 min incubation the size of the FVs was considerably reduced (fig. 1d).

In both control and imprinted cells the phagocytosis of TRITC-Con A was reduced in the presence of 250 µg/ml mannose (group D) after 1 min incubation ($p < 0.05$) and the reduction continued at the longer incubation periods ($p < 0.05$; fig. 4). The number of FVs was not influenced notably by the 250 µg/ml galactose treatment; there was a slight decrease after 10 min incubation only ($p < 0.05$; fig. 5). The presence of histamine (group E) did not influence the formation of FVs labelled with TRITC-Con A (fig. 6).

Discussion

In this study, TRITC-Con A was the marker ligand for the visualization of FVs. It binds to structures containing mannose and mannopyranoside, which are also components of the saccharide oligomer domain of receptors. The insulin receptor also binds Con A[20–22], which suggests that these saccharides are present in the insulin receptor.

Hormonal imprinting with insulin alters the insulin binding capacity of *Tetrahymena*. Presumably, altered receptor structures of the plasma membrane and alveolar sacs play a role in the increased binding capacity. There is a dynamic relation between the alveolar sacs and the plasma membrane, and the turnover between the two structures might affect down- and up-regulation of binding sites[23–25]. In *Paramecium* the membranes that enter the cell by internalization of FVs are replaced by turnover between the identical vesicles and the cytopharyngeal membrane[26]. The results of our experiments support the assumption that there are similar processes acting in *Tetrahymena*[24]. After insulin imprinting, the Con A binding capacity of the oral field and cytopharyngeal membranes was enhanced, and could be clearly observed by fluorescence and confocal microscopy (fig. 2). The first step in receptor-mediated phagocytosis is the specific connection of the ligand to the binding sites of the cytopharyngeal membrane, followed by internalization of FVs. The membrane fragments which enter the cell by this process are retrieved by the membranes of vesicles persisting around the oral field[27]. After insulin imprinting, more bindinq sites are present, which results in a more rapid phagocytosis than in non-imprinted cells. The retrieval of these internalized membrane structures requires time, which might account for the transient decrease in the number of FVs in the imprinted cells, or when insulin is present at 1 min incubation, a time at which the formation of FVs is very fast. On the other hand it is possible that FVs consume more membrane in the imprinted cells or when insulin is present, and this results in a longer time for retrieval of membrane. Nevertheless, following the longer periods of incubation the intensity of phagocytosis and number of vacuoles is enhanced again with membrane turnover comparable to that in the control group.

Similar changes were observed in the imprinted group in the presence of insulin. The overlap of insulin and Con A binding sites might explain this observation; there is good reason to suppose that the simultaneous presence of Con A and insulin results in an additive effect on phagocytosis. This possibility is confirmed by the very active formation of FVs after 30 s and 1 min incubation. On the day following imprinting, the phagocytosis of Con A is high compared to that of controls

after longer incubation times, which indicates an effect of insulin imprinting on the membrane system.

Mannose, a ligand of Con A, inhibits the formation of FVs considerably. Galactose, which is not a ligand of Con A, has no such influence. Mannose does not totally interrupt phagocytosis, of course, since the Con A molecule is also a glycoprotein and can induce phagocytosis by way of other receptors, too. Histamine is known to elicit increased phagocytosis of ink by *Tetrahymena*[15], but had no effect on the phagocytosis of Con A. According to the literature all internalization processes through the oral field are classified as phagocytosis[11–16]; however in the case of uptake of Con A, a soluble substance, pinocytosis also occurs. Histamine as an inducer of phagocytosis may be specific for mammalian cells[28,29], and this could be the reason why it has no effect on the uptake of Con A.

We can draw the following conclusions from our results. Firstly, TRITC-Con A is a good tool for the investigation of receptor-mediated phagocytosis, especially in a system where the effect of insulin or insulin imprinting is being studied. A short incubation time (30 s) is enough to demonstrate the binding capacity of the cytopharyngeal membrane.

Secondly, insulin can also activate phagocytosis at longer incubation times. This might explain the 'growth factor' role of insulin in *Tetrahymena*, and its capacity to induce clones in cases when cells would die without insulin[30]. Thirdly, insulin imprinting elicits significant changes in TRITC-Con A phagocytosis when the imprinted cells are compared with non-imprinted cells, incubated with or without insulin.

According to the literature, the membrane of FVs does not have a plasma membrane origin, but is similar in composition to the microsomal membrane system[27]. Membrane fragments that form the walls of the FVs are of microsomal origin, and fused to the cytopharyngeal membrane. Following longer incubations – when internalization and recirculation of the cytopharyngeal membrane has presumably occurred several times – there is a significant difference in phagocytosis by control and imprinted cells. During imprinting the receptor quality of the microsomal system itself also changes, and the changes are similar to those in the plasma membrane. Increased phagocytosis is characterized by a membrane pool of receptors, in increased quantity and/or with greater affinity, and by an increased velocity of recirculation. Further study is required to determine which of these three parameters are affected in imprinted *T. pyriformis*.

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