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CORRELATION OF INSULIN PRETREATMENT AND INSULIN BINDING OF *AMOEBA PROTEUS*—A NEW TECHNIQUE FOR EVALUATION

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Abstract—1. Insulin binding sites of *Amoeba proteus* were analysed. The developed technique helped to compare equivalent pseudopodia membranes, according to their fluorescein-isothiocyanate (FITC)–insulin binding capacity.
2. The number of insulin binding sites decrease in the control conditions as a fraction of time.
3. The down regulation of binding sites (or similar phenomenon) lasts ~48 hr.
4. Only low concentrations of insulin can induce a binding site elevation in the long term.
5. Hormonal imprinting could be presented in *Amoeba*.
6. The developed technique is suitable for detection of membrane dynamics in *Amoeba proteus*.

INTRODUCTION

In protozoa a series of special effects is produced by various hormone molecules. These biologically active molecules can induce and modify the activity of physiological processes such as phagocytosis (Csaba and Lantos, 1975; Csaba *et al.*, 1984), growth (Csaba and Németh, 1982; Kovács and Csaba, 1988) and metabolical pathways (Csaba and Lantos, 1976). Their induction mechanisms (second messengers, protein kinase or Ca-dependent processes) are very similar to those intracellular cascade reactions which are described in higher organisms (Csaba and Nagy, 1976; Kőhidai *et al.*, in press).

Though the peptide type hormones which can lead to these physiological effects are universal in multicellular systems, many of them are present (LeRoith *et al.*, 1982) in unicellular organisms too. The strange phenomenon is that these single cells are not only able to synthesize the hormones, but the same cells also have the capacity to bind them (LeRoith *et al.*, 1980). The role of both the endogenous hormones and the hormone binding is unclear. There are speculations about the extracellular signal role of the produced peptides (Csaba and Kovács, 1987). The bound hormone molecules are considered as real hormones with their special intracellular agonist pathways and effects (Roth *et al.*, 1982) or only simple food molecules which have been selected according to their inductive character during evolution (Csaba, 1986).

The phenomenon of hormonal imprinting has related points to the above mentioned problem. The phenomenon itself means that cells treated by different hormones will produce different, generally higher, reactions when the cells meet the same hormone again. The progeny cells also possess this capacity (Csaba, 1980). The phenomena requires a special type of "memory" which is responsible for the selectively changed activity/sensitivity. The background of the outlined process is known only in parts. Some experiments showed that the integrity of the membrane (Kovács *et al.*, 1984; Kőhidai *et al.*, 1986), or the condition of the intracellular second messenger mechanisms (Kovács and Csaba, 1987; Csaba *et al.*, 1987) can influence the efficiency of hormonal imprinting. The induction of the nucleus also has an important role (Csaba *et al.*, 1982; Kőhidai *et al.*, 1985).

There are many hormones checked with respect to hormone binding, evoking hormonal imprinting. Among these, insulin is one of the most frequently examined (Kovács *et al.*, 1984a and b, 1988; Kovács and Csaba, 1988; Kőhidai *et al.*, 1990). There are many publications concerning insulin effects on ciliates e.g. Tetrahymena, but the other class of protozoa, *Amoebae* is a relatively unexplored but important field of hormone-receptor research.

The purpose of the present study was to test the insulin binding of *Amoeba proteus* cells and to compare the hormone binding capacity of control cells to insulin pretreated (imprinted) cells. During this work we had to develop a new, proportional method for evaluating the fluorescent hormone binding of the pseudopodia membrane. In this way, we intended to characterize the dynamics of insulin effect in time and concentration course. The results collected could also characterize the hormonal imprinting in a new model system.

MATERIALS AND METHODS

Amoeba proteus strain G model cells of the present experiment were grown in mass culture in KCM solution (Oshima *et al.*, 1986) by the Prescott method (Prescott and James, 1955).

Cells of exponential cultures were treated in graded doses (10^{-10}; 10^{-8}; 10^{-6} M) of insulin (Semilente MC, Novo; Copenhagen, Denmark), the untreated group of cells served as the control group of the experiment. Following treatment, the cells were washed with KCM solution.

In the consecutive 24, 48 and 96 hr, we tested the FITC (fluorescein-isothiocyanate)–insulin binding of cells.

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Because of the variable cell shape and thickness, we developed radiate shaped (Grebecki and Grebecka, 1978) *Amoeba*. The formed pseudopodia represent an identical membrane/cytoplasm ratio in contrast with the variable ratio of the normal cell body. We tried to find a physiologically and chemically relatively mild and harmless technique, so as not to injure the cell membrane during this process. These pseudopodia contained less crystals and other optically active components which could potentially disturb the evaluation of hormone binding to the membrane.

The main steps of experimental induction of pseudopodia formation were (a) weak and durable period (2 min) centrifugation in small tubes. (b) Washing of cells by distilled water twice (second time only in the half volume of the total). (c) Let the cells rest for 5 min then shake them. (d) Fixation by 1:1 volume Carnoy's solution for 15 min. (e) Shake the cells again to remove them from the wall of the tube.

The cells were then washed with phosphate buffered saline, pH 7.4 three times. The washed cells were incubated by FITC–insulin (FITC/protein ratio 0.41; protein concentration 0.3 mg/ml) for 1 hr. Following the incubation, the cells were washed by PBS two times and replaced by distilled water.

Intensity of fluorescence was measured cytofluorimetrically (Zeiss Fluoval cytofluorimeter connected to a HP 41CX calculator equipped with a special biometric program). Only the pseudopodia having the same size (length and width) were measured in the centre.

In each group, 15 cells, about 75 pseudopodia were measured; the experiments were repeated three times.

Data were treated by biometrical programs of Excel 3.0 and Plot 40. Histograms were drawn with the help of Harvards Graphic program 2.12 where the abscissa represents the intensity of fluorescence and the ordinate the number of cells.

RESULTS

The FITC–insulin binding capacity of the pseudopodial surface membrane of the control and insulin pretreated *Amoeba* was differed according to the time lapsed. We followed the changes of histograms from group to group and time to time in line with the average values related to the actual control (Table 1).

Table 1. Mean binding values of FITC-labelled insulin 24, 48 and 96 hr after treatment with different concentrations of insulin, related to the control as 100

	10^{-6} M	10^{-8} M	10^{-10} M
24 hr	108.48	93.98	94.61
48 hr	101.3	88.33	84.63
96 hr	88.17	142.01	114.75

In the case of untreated, control cells, after 24 and 48 hr of culturing we found very similar histogram profiles (Figs 1a, 2a), which showed a mild shift to the left. However a striking difference was observed in the case of the 96-hr cultures (Fig. 3a). In this group, low FITC–insulin binding capacity was characteristic, making them significantly different to the previous cultures.

The dynamics of this shift to the lower binding capacity is not seen in the insulin pretreated groups according to the concentration course.

In the case of 10^{-10} M insulin pretreated cells, the 24-hr sample was identical to the control group (94.61%). After a time, this similarity disappeared and in the 48-hr group there was a significant ($P < 0.05$) decrease of the average (84.63%; Fig. 2b). In the 96-hr sample, the above-mentioned shift goes and most of the cells represent a low intensity of fluorescence (Fig. 3b). In the case of control cells the average intensity was 114.75%. This is a significant ($P = 0.05$) increase to the identical control.

After pretreatment with 10–8 M insulin the 24-hr results showed no difference between the control and the pretreated group (93.98%; Fig. 1c). The

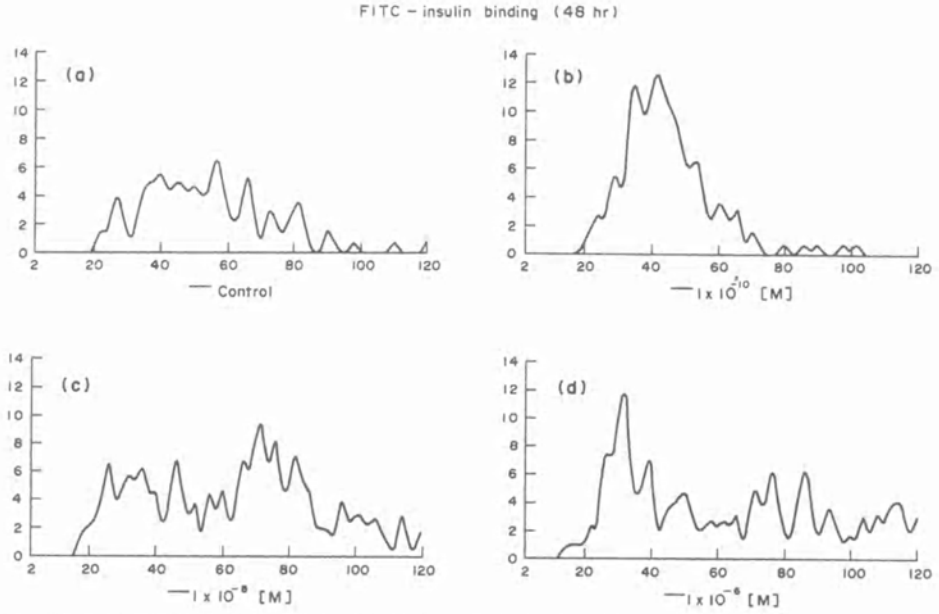

Fig. 1. FITC insulin binding of *Amoeba* 24 hr after insulin treatment (imprinting). Abscissa represents the intensity of fluorescence, ordinate demonstrates the number of cells.

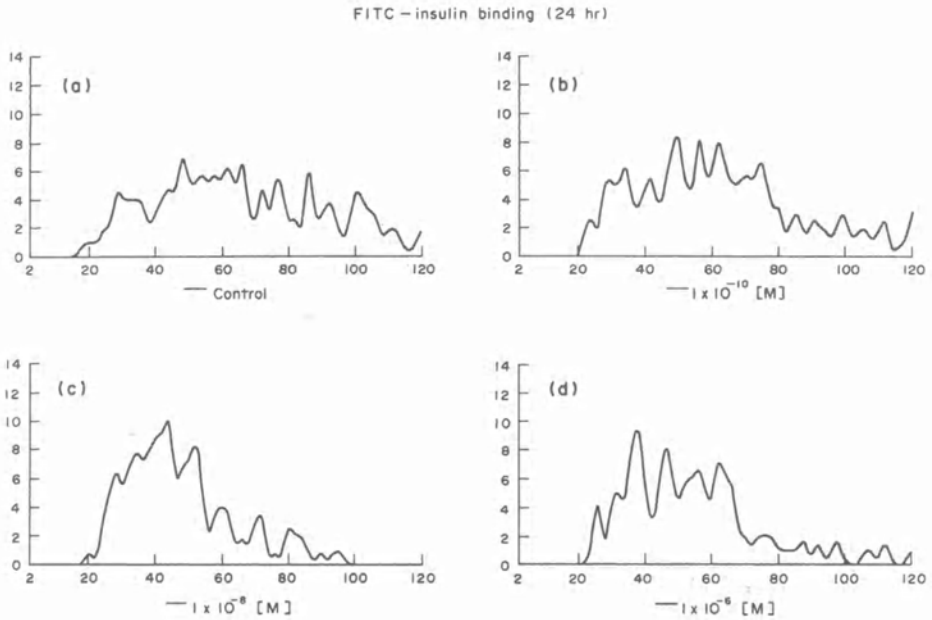

Fig. 2. FITC insulin binding of *Amoeba* 48 hr after insulin treatment (imprinting). Abscissa represents the intensity of fluorescence, ordinate demonstrates the number of cells.

48-hr group represents a strong accumulation of cells around the low values (Fig. 2c). The values related to the control mirror this relation (88.33%; $P < 0.05$). After 96-hr cultivation there is a shift to the higher values, which is represented by the different profile of histogram (Fig. 3c) and the average value (142.01%; $P < 0.05$), alike.

The 10^{-6} M concentration was the highest we used. This provoked a restless histogram after 24 hr of treatment, with a spike at the low values (Fig. 1d). The control related average was 108.48% but there was no significant difference. After 48 hr a more compact histogram appears with a mass of values around the control (101.3%; Fig. 2d). The 96-hr cultures contained cells with a low intensity of fluorescence (88.17%; $P = 0.05$; Fig. 3c) significantly differing from the control.

Characteristic changes in FITC–insulin binding of tip regions of the pseudopodia in the 24 hr experiment (Fig. 4) were observed.

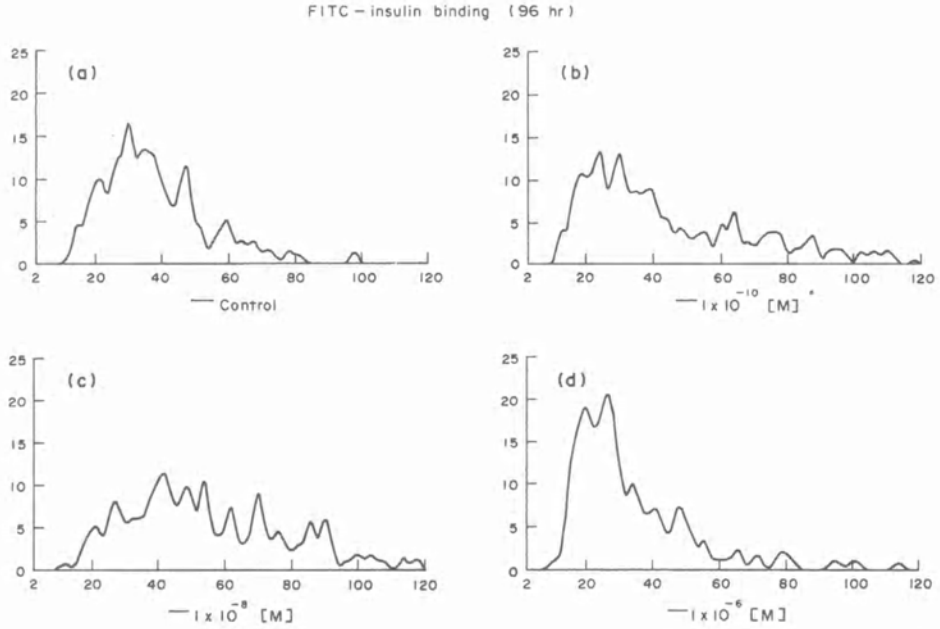

Fig. 3. FITC insulin binding of *Amoeba* 96 hr after insulin treatment (imprinting). Abscissa represents the intensity of fluoresence, ordinate demonstrates the number of cells.

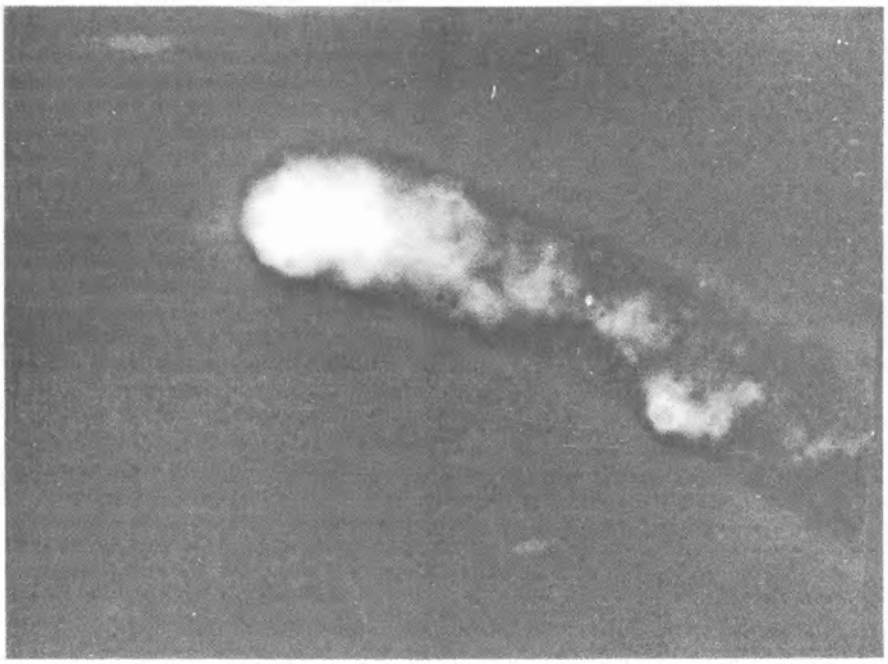

Fig. 4. FITC insulin binding of the pseudopodial tip region in *Amoeba* treated with 10^{-6} M insulin 24 hr before.

DISCUSSION

Amoeba proteus, like other protozoa, has different complexes of molecules in the plasma membrane which are able to bind special ligands (Kukulies *et al.*, 1986). These sites have connections not only to the processes of locomotion and phagocytosis but they are possibly also working as receptors (Csaba *et al.*, 1984; Kukulies *et al.*, 1986). Signal coupling of the already tested materials (histamine, prostaglandin) showed the specificity of this effect.

In another unicellular organism, *Tetrahymena*, we could demonstrate the phenomenon of hormonal imprinting (Kovács and Csaba, 1986), which requires a special memory of the receptor systems and the intracellular second messenger mechanisms, alike (Kőhidai *et al.*, 1990; Kovács and Csaba, 1987; Csaba *et al.*, 1987). In this experiment, we aimed to study insulin binding capacity of *Amoeba* met insulin first and different time intervals after this first encounter, making hormonal imprinting.

The study followed the FITC-labelled insulin binding to the plasma membrane of control and insulin pretreated cells in 24, 48 and 96-hr cultures. The newly developed technique helped to compare similar membrane areas of the pseudopodia in each cell.

The results demonstrated a decrease in hormone binding capacity after 96 hr of culturing in the controls. There may be different reasons for this: (1) an environmental effect caused by the increased density of cells in the dish; (2) the ageing of the cells and the related intracellular processes; (3) some extracellulary liberated materials which could modify the binding.

Our data showed that the plasma membrane of *Amoeba proteus* has different binding capacities according to the concentration of insulin at pretreatment. After 24 or 48 hr there was no significant positive difference between the control and the pretreated cells. This is understandable considering that in this period a very characteristic phenomenon is the down regulation for insulin binding sites in the case of other protozoa (Csaba and Kőhidai, 1986).

The characteristic changes are detected in the late time point, 96 hr. These cells are about the 8th to 9th generations of the pretreated cells. In this case the 10^{-8} M insulin pretreatment was optimal as this could provoke the maximal positive insulin binding considering all of the experiments. In the background we can find a high insulin binding site concentration in the plasma membrane caused by cytoplasmic or nuclear processes increasing the synthesis of insulin receptor components. Only the relatively low agonist concentrations were able to induce the possible mechanisms mentioned above, where the 10^{-10} M insulin treatment could induce a similar effect.

According to other papers (Jeon and Bell, 1964; Stockem, 1972), there is a membrane flow in *Amoeba* and there are differences between the tip region of the pseudopodia and the cell body; the tip region represents the "new" membrane field. There is a delay in the recovery of the tip by elements of glycocalyx. Perhaps this delay is the reason for the more intensive insulin binding in the tip region (Fig. 4) and shows that the presence of these components on the extracellular face of the plasma membrane has an important role in the signal recognition. The number or position of intracellular fluorescent particles were not determined in this experiment. The dynamics of formation and traffic of these particles is the next step to be investigated.

The characteristics of *Amoeba* plasma membrane show a close similarity to those which were described in *Tetrahymena* and higher organisms (Csaba *et al.*, 1986) after hormonal imprinting. In case of membrane receptors (like insulin receptor), these references report an increased hormone binding which is characterized by two periods. The first early phase is when the direct effects are present: the cell showed a changed physiological, metabolical etc. (Csaba, 1985) activity and down-regulation of binding sites (Csaba and Kőhidai, 1986). The late phase when the long term effects are observed: modified, generally increased, hormone binding (Kőhidai *et al.*, 1990) and the above mentioned early physiological or other activities are more intense than the early induction could provoke them. Behind these physiological activities there is also a triggered binding site/receptor function. The *Amoeba* cells observed could also present very similar characteristics.

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