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PERSISTENCE OF RECEPTOR "MEMORY" INDUCED IN *TETRAHYMENA* BY INSULIN IMPRINTING

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Tetrahymena cells treated (imprinted) with insulin on a single occasion bound significantly more insulin than the control cells for as many as 70 days, i.e. over 664 generation changes. Although late reexposure to insulin reduced the binding of labeled hormone for 24 h, the binding value of the imprinted cells still increased significantly over the control. Maintenance under anaerobic conditions for 80 days accounted for a temporary suspension of the effect of imprinting which was, however, recovered within a week of return to aerobic conditions, in an even stronger form than observed in cultures maintained without an anaerobic episode. The experiments demonstrated that the imprinting-induced receptor "memory" lasted long, but was vulnerable to treatment with another polypeptide hormone.

The cells receive chemical or other environmental signals by means of membrane-associated or cytosol-associated highly sensitive, complex configuration, the receptors, whose structure varies with the nature of the specific signal molecule. The mechanism provides for a selective processing of the different impulses which act on the cells in biological systems, and it accounts not only for an immediate cellular response, but also for the induction of a cellular-level "memory" of the event, to judge from the consistent recurrence of the signal-evoked response pattern over a relatively long period. This ability of cells contributes to the evolvement of highly complex reaction patterns in multicellular organisms.

Biochemical memory, which operates at all levels of phylogenesis from unicellular organisms to high vertebrates, presupposes the coordinated function of all cells and all protective mechanisms of the organism. Although many details of cellular "memory" are still obscure, evidence has been accumulated that its physiological operation and changes presuppose intactness of the cell membrane and nucleus, and its modulations depend on the protein synthesis.

The foregoing considerations can also offer an explanation for the phenomenon of hormonal imprinting, which has been shown to occur in both unicellular [1] and multicellular organisms [2]. Primary interaction (imprint-

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ing) of a given target cell with a hormone accounts for a changed — usually increased — response to the latter on reexposure, as if the cell "remembered" the primary event. The changed response is shown not only by the cells directly involved in primary interaction, but also by many subsequent generations. The underlying mechanism is a chain of reactions, which take place at several levels and are, for the most part, still obscure. The external membrane of the cell, the intracellular membrane systems, the interactions between intracellular compartments [3, 4], certain intranuclear changes and functional states of the cellular nucleus [5] may all be involved in the mechanism of imprinting.

The imprinting-induced modifications of cellular response can be assessed from changes in several cellular functions, such as mitotic activity [6], phagocytic activity [7], hormon binding capacity [8, 9] and membrane potential [10].

The purpose of the present study was to investigate how long the imprinting-induced increase in response to insulin was transmitted to subsequent generations of the model cell *Tetrahymena* [11]. It was also examined in this context, whether imprinting-induced receptor "memory" was influenced by later treatment with insulin itself or another polypeptide hormone (TSH), or by lasting inhibition of mitosis under anaerobic conditions of maintenance.

Materials and methods

Culture. Tetrahymena pyriformis GL cells, propagated in 0.1% yeast extract (Difco, USA) and 1% Tryptone (Oxoid, England) containing medium at 28 °C were used in the exponential phase of growth.

Treatment. Part of the mass culture was treated with 10^{-6} M insulin for 1 h, the untreated part served as control. After insulin treatment, the experimental culture was returned to plain medium for 24 h. The culture medium was subsequently changed every 48 h.

Determination of hormone binding. Samples taken from the cultures at predetermined time intervals (every seventh day between generations 9–323, and every fourteenth day between generations 323–664) were fixed in 4% formalin solution (in PBS), washed in PBS, exposed to fluorescein-isothiocyanate (FITC, BHD, England) labeled insulin for 1 h, and examined for binding of the label with a Zeiss Fluoval cytofluorimeter.

Statistical evaluation. The analogous signals of the cytofluorimeter were transformed to digital signals for assessment of mean values, standard deviation and significance of intergroup differences by means of a HP-41CX calculator connected with the cytofluorimeter. Six replica assays were done, and 20 cells were assayed in each group for intensity of fluorescence.

Second hormone treatment. Cells cultured as above were treated with 10^{-6} M insulin or thyrotropin for 1 h, were returned to plain medium for 24 h, and were finally reexposed to FITC-insulin for 1 h. Hormone binding was then assayed by cytofluorimetry, as above.

Anaerobic culturing conditions. Part of the cultures were, after propagation for 3 months, reduced to anaerobic conditions by spreading a 1 mm thick layer of paraffin oil onto the surface the nutrient medium. Eighty days later the cells were returned to aerobic conditions for one week, during which they were assayed for insulin binding on days 4 and 7. Anaerobic conditions were then provided again for 6 months, after which the cells were returned to aerobic conditions and assayed for insulin binding on day 7.

The cells were treated with 10^{-6} M insulin or TSH on day 7 after the first anaerobic episode of 80 days.

Results

In the experimental series we followed up the changes induced by primary interaction with a hormone (hormonal imprinting) in the hormone binding capacity of the interacting cells and their offspring generations (Fig. 1). The generation time of *Tetrahymena* being about 150 min, the weekly examination detected FITC-insulin binding in about every 60th generation. The hormone binding capacity of the insulin-pretreated cells differed significantly from the control throughout. Binding increase over the control was on average 110% in the initial period (generations 9–323) and 115% in the 664th generation.

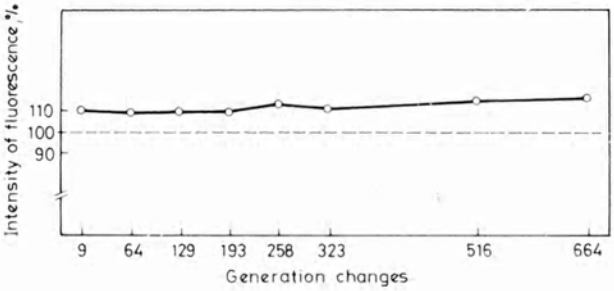

Fig. 1. FITC-insulin binding in the offspring generations of *Tetrahymena* cells treated with hormone on a single occasion (control = 100%)

In the second experimental series we studied the late effects of hormonal imprinting and the response of the cells to a second treatment with the same or another polypeptide hormone (Fig. 2). This series covered the following groups: control/control (C/C); control/insulin (C/I); control/TSH (C/T); insulin/control (I/C); insulin/insulin (I/I); insulin/TSH (I/T).

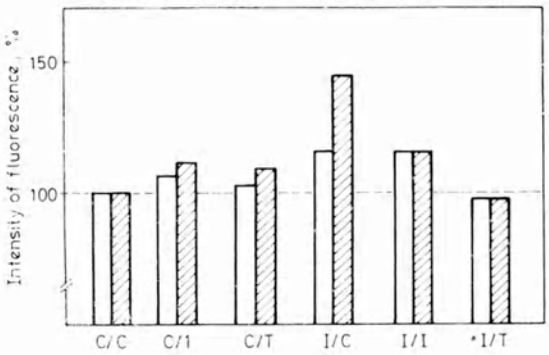

Fig. 2. Late effects of insulin-imprinting in *Tetrahymena* cells reexposed to insulin or TSH at different time intervals in the course of 664 generation changes (open columns), and 7 days after maintenance under anaerobic conditions for 80 days (shaded columns); (C = control; I = insulin; T = TSH)

The binding of FITC-insulin was low (105%) in the cells not preexposed to the hormone (C/I), and that of TSH was still lower (102%) under the same conditions of treatment (C/T). The cells preexposed, but not reexposed to insulin (I/C) and those both preexposed and reexposed to it (I/I) equally showed a significant (115%) binding increase over the control, whereas those preexposed to insulin and reexposed to thyrotropin (I/T) did not appreciably differ from the control in respect of insulin binding (98%).

The imprinted cells reduced to anaerobic conditions for 80 days recovered their insulin binding capacity gradually, to judge from a relative decrease (91%) on day 4 of return to aerobic conditions, and a significant relative increase (140%) on day 7 (Fig. 3).

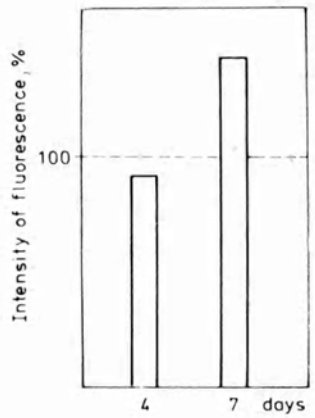

Fig. 3. FITC-insulin binding in *Tetrahymena* cells at different times after return from anaerobic to aerobic conditions (control = 100%)

Membrane-level changes shown in response to exposure to insulin or TSH after anaerobic conditions of culturing were also assessed in cultures treated on the schemes described above. Although the absolute values of FITC-insulin binding were slightly increased on day 7 after return to aerobic conditions, the bindings profile itself did not appreciably differ from the pre-anaerobic one. Although the binding values of the groups C/I and C/T increased appreciably (112, respectively 108%) over the control, they were still considerably lower than those shown by the I/C (142%) or I/I cells (116%). Mixed hormone treatment (I/T) had no influence on insulin binding (98%) in this experimental series. The cultures returned to anaerobic conditions for further 6 months showed the same patterns of response as after 80 days.

Discussion

Hormonal imprinting takes place at primary interaction between target cell and hormone. In unicellular organisms, non-specific membrane patterns transform to persistent receptor structures [12] in presence of hormone, whereas in higher organisms the genetically encoded receptor is amplified by hormonal influence [1]. Hormonal imprinting alters — usually increases — the hormone binding capacity and the specific response of the cell at later interactions [13, 14].

In earlier studies along this line, diiodothyrosine imprinting still operated in *Tetrahymena*, i.e. a "memory" of it was still demonstrable by elevation of the mitotic index, after as many as 500 generation changes [6]. Such persistent and transmissible receptor "memory" could arise either by a structural alteration of the receptor, or by a regular or hormone-induced recycling to the external membrane of receptors associated with the cytoplasmic membrane pool. A third alternative explanation is that the memory persists at post-receptorial rather than receptor level, and the changes induced by it in the post-receptorial mechanisms (e.g. cAMP elevation) account for an increased cellular response on reexposures to the hormone and usually for an increased hormone binding capacity as well.

The purpose of the present experiments was exactly to identify, whether the persistence over many generations of the functional changes associated with hormonal imprinting [6] was due to receptor-level or post-receptorial changes.

A single interaction with insulin was in fact sufficient to induce a "memory" of imprinting, and, consequently, an increased binding capacity for insulin in as many as 664 subsequent generations of *Tetrahymena*. The binding capacity was fairly uniform at the different sampling times, and tended to increase rather than to decrease towards the end of the experimental period. Although this does not exclude a periodic recurrence of negative binding results, the binding values unequivocally portrayed the persistent impact of imprinting. The present observations cannot disclose, whether imprinting had changed the structure or the number of the membrane receptors in *Tetrahymena*, but the fact remains that the insulin binding capacity was durably increased in the progeny generations of the imprinted cells.

Part of the cells were reexposed to insulin or TSH after 70 days of culturing, i. e. after 664 generation changes, to clarify whether or not the imprinting elevated binding capacity was further increased by the second treatment, and whether the response was specific. It is known that hormones or hormone-like materials may increase the binding capacity for another hormone also by nonspecific influence [15–18]. TSH was used to exclude this alternative.

The second hormone treatment gave rise to down-regulations, probably because the assay was done 24 h after insulin reexposure, when increase over the

control was appreciable, but not significant in the C/I cells, and significant relative to C/C but markedly decreased relative to I/C, in the I/I cells, owing in all probability to the down-regulating effect of the second insulin treatment. TSH was less active in the C/T group than insulin in the C/I group, and it abolished altogether the effect of insulin imprinting in the I/T series. This suggests that late reexposure to a polypeptide hormone destroys the imprinting effect of primary interaction with another polypeptide hormone. Special emphasis is laid in this context on the length of the time interval between the primary and secondary treatments, because no such effect was observed if one polypeptide hormone was employed shortly after, or simultaneously with, the other [19]. It follows that, although the effect of imprinting is strong, it can be extinguished by certain adverse conditions.

Part of the cells were, after 70 days (664 generation changes), reduced to anaerobic conditions for 80 days, and again for 6 months, to inhibit division and thereby the transmission of imprinting-induced memory to daughter cells. Since no exchange of medium was made during anaerobic culturing, the cells had to use up a considerable part of their membranes for nutrition. They indeed recovered their potentials slowly after return to aerobic conditions in fresh medium. There was no indication of the effect of imprinting after 4 days, but a significant binding increase over the original level took place by day 7. Response to reexposure to insulin or TSH on day 7 was similar to, or even greater, compared to that of the cells maintained in aerobic conditions throughout. It follows that the imprinted cells were not only able to transmit the "memory" of imprinting to daughter cells, but, as demonstrated already earlier [20] also to store it, and to use it again after return to physiological conditions. Thus, further to earlier functional evidence of receptor memory, additional evidence of it emerged from the present binding studies.

The long persistence of imprinting in both the functional and binding activities of *Tetrahymena* supports the hypothesis of a nuclear-level transmission of imprinting-induced "memory" from one generation to the other.

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