ACTA PROTOZOOLOGICA
Vol. 24, No. 3-4, pp. 259-264 (1985)

Effect of Inhibitors of Protein Synthesis and Endocytosis on Hormonal Imprinting in the *Tetrahymena*

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Received on 28 December 1984

Synopsis. Methylamine, which inhibits clustering in the process of receptor-mediated endocytosis, did not interfere with insulin-induced hormonal imprinting in the *Tetrahymena*. Dimethylsulphoxide (DMSO) had no appreciable influence on imprinting by insulin either. However, hormonal (insulin) imprinting was inhibited by cytochalasine B, which acts on the microfilament system, by colchicine acting on the microtubular system, by the lysosomal enzyme inhibitor chloroquine, and by the Ca-inhibitor Ni^{2} as well. Among agents acting at transcription-translation level, actinomycin D and cycloheximide inhibited imprinting, whereas puromycin did not. In this light is appears that the inhibition of imprinting may differ between the levels of binding and functional response. Another conclusion emerging from the present study is that injury of the membrane of the *Tetrahymena* by inhibitors persists long in the progeny generations.

Hormonal imprinting, whose mechanism is the subject of this study, is essentially a far-reaching change which occurs at the first interaction of the hormone and its target cells, and accounts for an increased responsiveness of the cell to the given hormone throughout the further stages of cell life (C s a b a 1981). Imprinting also alters the hormone binding capacity and certain functional states of the cell, and is demonstrable not only in those cells directly involved in the first interaction, but also in their progeny generations (C s a b a et al. 1981, 1984). Several details of the phenomenon termed as hormonal imprinting are

Supported by the Scientific Research Council, Ministry of Health, Hungary, 16/1-04, 432/Cs

still obscure, but evidence has been accumulating on the important role of membrane receptors in the establishment of imprinting. Since the membrane receptors actively participate in certain transmembraneous transport processes, they occur not only in the cellular membrane, but also inside the cell, which they enter via endocytosis in coated vesicles, and become thus involved in recirculation processes which result in their reincorporation into the membrane. Thus the membrane receptors practically form a chain of information between the cell-coating membrane, and the cytoplasmic intracellular spaces (Brown et al. 1983, Fehlman et al. 1982, Steinman et al. 1983). The information transfer furnished by the membrane receptors is a highly complex process, which is controlled by several cellular-level factors. It follows that, presumably, imprinting is not limited either to membrane-level processes, being rather the issue of a chain of events.

The better understanding of the interrelated, viz. interacting events involved in imprinting can be facilitated if inhibitors are used to "blot out" one or another important cellular function, to learn about its involvement or non-involvement in the mechanism of hormonal imprinting.

In the living world, the key hormone of cellular metabolism is insulin, which can induce imprinting not only in multicellular organisms, but also in unicellular ones (Csaba 1980, 1981). We therefore used *Tetrahymena pyriformis* as model cell in the experiments whose description is forthcoming.

Material and Methods

Tetrahymena pyriformis GL cells, maintained in 0.1% yeast extract containing 1% Bacto trypton (Difco, Michigan) medium at 28°C, were used in the logarithmic phase of growth. The 24-h cultures were treated with 10^{-6} M insulin and/or with the following inhibitors, at the final concentrations specified: chloroquine (Chinoin, Budapest), 25 μmol : methylamine-HCl (Merck, Darmstadt, FRG), 10 μmol; cytochalasine-B (EGA-Chemie, Steinheim, FRG), 5 μg/ml; dimethyl sulphoxide (DMSO, Reanal, Budapest, 0.5%; DMSO also serves as solvent of cytochalasine-B); colchicine (BDH; Poole, England), 10 μmol; $NiCl_2$ (Reanal, Budapest), 1 μg/ml; cycloheximide (Koch-Light, Colnbrook, Bucks, England), 20 μg/ml; actinomycin D (Merck-Sharp-Dohme, N. J., USA), 3 μg/ml.

Paired samples were set up for parallel treatment with insulin + inhibitor and inhibitor alone, and further to the absolute control series an insulin-treated control group was also set up.

After treatment the *Tetrahymena* cells were returned to plain medium for further 24 h, were fixed in 4% neutral formaline, and washed in two changes of phosphate buffer (PBS 0.05 M phosphate buffer, pH 7.2; 0.9 M NaCl). Insulin

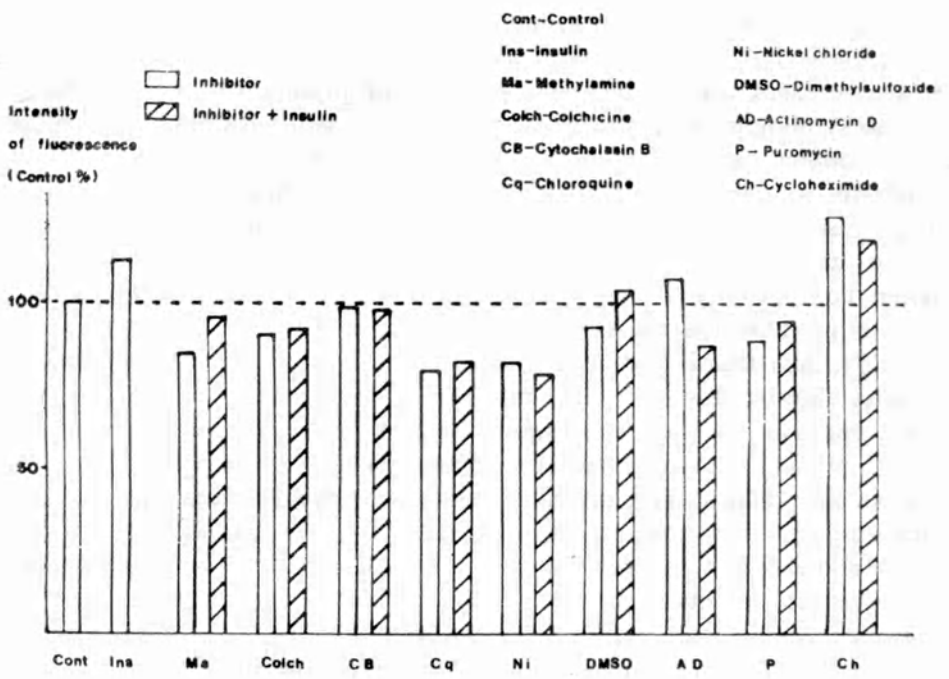

Fig. 1. Binding of FITC-insulin to *Tetrahymena* cells 24 h after pretreatment with insulin alone, inhibitor alone, or inhibitor + insulin, relative to the control as 100

binding was assessed by cytofluorimetry, after 1-h incubation of the samples in presence of fluoresceine-isothiocyanate. The results were evaluated with a HP 41C calculator, connected with the cytofluorimeter.

Twenty cells were assayed for fluorescence in each group, and 5 replica assays were performed with each sample, to calculate mean values for 100 cells in each series.

Results

We demonstrated earlier that methylamine and actinomycin D depressed, whereas colchicine did not influence, the imprinting effect of diiodotyrosine (T_2), as assessed from changes in the mitotic rate. All three inhibitors depressed, however, cell division rate induced by T_2 without imprinting (C s a b a et al. 1982).

Part of the inhibitors used in the present experiments interfere somehow with endocytosis, whereas the other part with the transcription-translation processes. The inhibitors acting on endocytosis influenced imprinting to different degrees and by different modes of action. Methylamine depressed the binding of insulin, but did not fully abolish im-

printing. It follows that although inhibition of clustering in the coated pits (Maxfield et al. 1979, Michael et al. 1980, Poole et al. 1976) decreases the binding capacity (in all probability the number of receptors), it apparently does not prevent the establishment of imprinting. However, disturbance of the microtubular-microfilamentary system, which transports the coated pit into the cell, hampered imprinting, to judge from the experimental fact that the post-imprinting binding of insulin was practically the same in cultures treated with colchicine (Wunderlich and Speth 1970) or cytochalasine B (Nilsson 1977) as in those treated with inhibitor alone. The minor fluctuations of plus or minus trend were not significant statistically, and therefore negligible. Similar results were obtained after treatment with the lysosomal enzyme inhibitor chloroquine (Steinman et al. 1983). At the same time DMSO, which inhibits endocytosis by an obscure mechanism (Nilsson 1976, 1977), behaved similar to methylamine, inasmuch as imprinting did take place in its presence.

It follows from the foregoing observations that while receptor clustering is no essential prerequisite of hormonal imprinting in the *Tetrahymena*, normal operation of the intracellular transport mechanism(s) and of the lysosomal system is in fact indispensable for imprinting. Caution should be, however, exercised in interpreting this statement, since both colchicine and cytochalasine B act on the *Tetrahymena* in a manner slightly different from their action on other organisms. For example, colchicine does not damage the ciliary and cortical tubules (Wunderlich and Speth 1970), and cytochalasine B inhibits only the formation of nutrient vacuoles, but not that of pinocytotic vacuoles (Nilsson 1977). Moreover, the conclusions drawn above apply exclusively to the binding of the hormone, which may not necessarily show a parallelism with the functional response to it. For example, colchicine appeared to be indifferent for the *Tetrahymena* — and for cultured mammalian cells as well — when imprinting was assessed by impact on mitosis (Csaba et al. 1982).

Of the inhibitors acting at transcription-translation level, actinomycin D (Peters 1976) did not usually inhibit the binding of insulin, but inhibited the induction of imprinting by that hormone, as a rule to such degree that labelled insulin binding was even lower than after inhibitor treatment alone so that the inhibitor effect appeared to be potentiated by the hormone.

The two protein synthesis inhibitors differed remarkably in action. While puromycin inhibited the binding of insulin to the cell, but did not inhibit imprinting, cycloheximide enhanced rather than inhibited binding, but hampered the imprinting action of the hormone. At present

state of knowledge we could hardly explain this phenomenon. It appears that cycloheximide interferes with the very event of protein synthesis, which is essential for imprinting but non-essential for receptor synthesis, to judge from the circumstance that cycloheximide developed no inhibitory action in itself, since in its presence binding was highest relative to the other experimental series, and increased even over the control; this high binding value showed a minor decrease, if insulin was simultaneously present (imprinting).

Since 1-h exposure to the inhibitor or inhibitor + insulin had been followed by return to plain medium for 24 h, and insulin binding was assessed thereafter, it is obvious that the *Tetrahymena* cells had in the meantime divided several (at least 4-5) times, whence the effect of the inhibitors came into display in the progeny generations. In these, nearly all inhibitors tested accounted in themselves for a decrease in labelled insulin binding. This substantiates the conclusion emerging from earlier studies that injury of the *Tetrahymena* membrane (receptor) persists long in the progeny generations (C s a b a et al. 1982).

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