Insulin Binding Sites Induced in the *Tetrahymena* by Rat Liver Receptor Antibody

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Tetrahymena cells treated with purified rabbit antibodies to rat hepatocellular membrane exhibited a considerable increase in binding capacity on reexposure to the antibody 24 h later. Insulin binding was similarly enhanced by preexposure to the antibody, and *vice versa*, preexposure to insulin enhanced the later binding of rat liver receptor antibodies. This suggests that (1) the *Tetrahymena* and the rat possess similar insulin receptors, and (2) the receptor antibody is also able to induce imprinting for itself as well as for insulin. Concanavalin-A, noted for binding overlap with insulin, failed to induce imprinting either for insulin or for antibodies to receptors, whereas the latter did induce imprinting for Concanavalin-A.

At present state of knowledge it seems most likely that the evolution of hormones and hormone receptors had taken place independently [1], and the chance interaction of a potential hormone molecule with a membrane pattern capable of acting as its receptor accounted – if it had selection advantage – for the establishment of a stable, phylogenetically founded receptor-hormone relationship [2]. Of the structures involved in that relationship the hormone-like molecules seem to be more variable than their potential binding sites.

Earlier studies along this line have shown that the unicellular Tetrahymena is able to bind the hormones of higher animals by a receptor-like interaction, and is usually also capable of a specific response to these [3]. The first interaction with a hormone gives rise to hormonal imprinting also at the unicellular level, exactly as does perinatal hormonal influence in vertebrates [4]. Thus imprinting accounts at all levels of phylogenesis for a greater sensitivity – responsiveness – of the receptor at later interaction(s) with the hormone. It is, however, still unclear whether the main events of imprinting take place at membrane or genic level. The present experiments were performed to throw a light on that problem.

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Tetrahymena cells were treated with gamma-globulin obtained from rabbits hyperimmunized with rat liver membrane receptors, and were examined for binding fluoresceinisothiocyanate-(FITC)-labeled insulin, as well as binding receptor antibody (RAB) after insulin treatment. The gamma-globulin preparation was purified and checked for specific activity before use. Concanavalin A (Con A), noted for overlap with insulin, was also examined for influence on the unicellular.

1. Hepatocellular plasma membrane preparation

Membrane preparations were obtained by Neville's (5) method from the liver of CFY rats of both sexes, 250 g in weight. The protein content of the membrane preparations was determined by the method of Lowry [6].

2. Preparation of antibodies to heptacellular membrane (insulin receptor)

New Zealand rabbits, on average 3 kg in weight, were hyperimmunized with 5 mg membrane preparation per animal. The first dose was suspended in Freund adjuvant; subsequently 4 booster doses were given at weekly intervals. Two days after the last treatment (5th injection) the rabbits were killed by bleeding, the serum IgG fraction was isolated by precipitation with $(NH_4)_2SO_4$, and was purified on DEAE cellulose column.

3. Testing of the immune serum for specificity

The freshly prepared hepatocellular plasma membrane preparation was examined for insulin binding capacity in presence of the immune serum, using [^{125}I]monoiodine labeled porcine insulin (Isotope Institute, Hungarian Academy of Sciences, Budapest; spec. act. 5550 GBq/g).

0.25 mg/ml membrane preparation (membrane protein) was incubated in presence of 5 mg/ml immune serum protein, in Krebs-Ringer phosphate buffer (pH 7.5), with or without 4×10^3 ng/ml non-labeled insulin, for 1 h at 37 °C. After 1 h, ^{125}I-labeled insulin was added to the system at 0.1 ng/ml final concentration, and incubation was continued for another hour at 30 °C. The buffer used for dissolution of labeled and non-labeled insulin contained 20 mg/ml BSA. Each sample was tested in 3 to 5 replicates.

Table I. [^{125}I]insulin binding capacity of hepatocellular membrane in presence of hepatocellular membrane antibody.

Immune serum no.	Total binding	Non-specific binding	Specific binding[a] (total binding minus non-specific binding)	
	[cpm]	[cpm]	[cpm]	[%]
Control (5 mg/ml BSA)	1750	130	1620	100
1	2020	1750	270	15
2	2400	1200	1200	75
3	3240	1090	2150	133
4	2790	1190	1600	100
5	1910	1540	370	22
6	2750	1600	1150	72

[a] The lower the specific binding of insulin, the higher the specificity of the serum, which displaces insulin on the membrane binding sites.

After incubation the samples were centrifuged at $10\,000 \times g$ for 1 min at 4 °C, and the pellet was assayed for radioactivity. The scintillation counts measured in the samples also containing non-labeled insulin were regarded as non-specific binding, and were subtracted from the scintillation counts of samples containing only labeled insulin. The results are shown in Table I. The preparations designated as no. 1 and no. 3 were used for further study.

4. Binding of RAB to the Tetrahymena

Tetrahymena pyriformis GL cells, maintained in 1‰ yeast extract containing 1% Bacto-trypton medium (Difco, Michigan, USA) at 27 °C were used in the logarithmic phase of growth. The cells were treated for 4 h with RAB (RAB-1 or RAB-3), insulin (Insulin, Semilente, Novo, Denmark) or Concanavalin-A (Con-A; Serva, Heidelberg, GFR), each added to the medium at 10^{-6} M concentration. After treatment the cells were returned to normal medium for 24 h, fixed in 4% neutral formaline (pH 7.2, in PBS) for 5 min, washed in three changes of PBS, and examined for binding FITC-labeled RAB-1, RAB-3, insulin and Con-A, each added at 0.4 mg/ml concentration. The cells preexposed to insulin and Con-A were reexposed to RAB-1 and RAB-3, respectively, whereas those preexposed to RAB-1 and RAB-3 to the corresponding labeled antibody, insulin or Con-A.

After reexposure the cells were washed in three changes of PBS, were spread on slides, dried, and examined for intensity of fluorescence in a Zeiss Fluoval cytofluorimeter. The results were recorded by means of a Hewlett-Packard-42A calculator, using a digital processor. Twenty cells were examined in each group. All experiments were performed in triplicate, and the mean value of the three assays was considered for evaluation. The inter-group differences were analyzed for significance with Student's t-test.

In principle the hormone binds to the receptor with a detail complementary to the receptor configuration, and the receptor antibody represents the counterpart of the complementary receptor pattern. Supposing that the receptors studied were similar, it was expected that imprinting with RAB will enhance the binding of labeled RAB or insulin added to the system 24 h later. As shown in Fig. 1, RAB and insulin binding did in fact increase over the control to a similar – considerable – degree. The two immune globulins (RAB-1 and RAB-3) differing in specific activity on rat hepatocellular receptor showed qualitatively similar, but quantitatively different binding relations, conform to the difference in specificity (we deliberately used the least specific and the most specific antibody in this study, to obtain evidence on the specificity of binding also from the difference between the two extremes). Con-A, which overlaps insulin on the latter's receptor, failed to induce imprinting, as

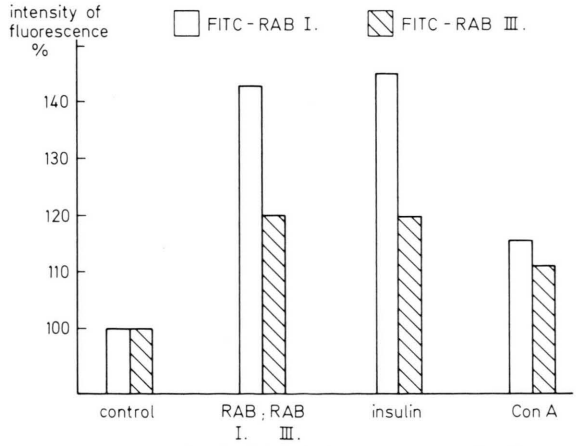

Fig. 1. Binding of FITC-labeled receptor antibodies to control and pretreated *Tetrahymena* cells (abscissa). Significance to controls: $p < 0.01$, except Con-A.

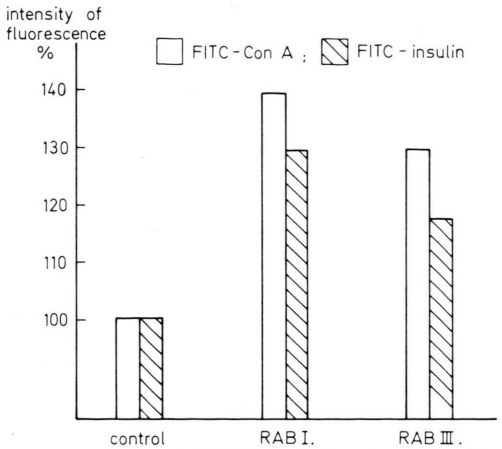

Fig. 2. Binding of FITC-labeled Con-A and insulin to RAB pretreated *Tetrahymena* cells. Significance to controls: $p < 0.01$.

demonstrated also earlier in pertinent studies. This can be explained by the circumstance that Con-A binds to simple sugars, while amplification of the receptor – imprinting – presupposes binding to oligomeres at the least [9].

Treatment with hepatocellular receptor antibody enhanced considerably the binding of FITC-labeled insulin, and of Con-A as well. It follows that RAB establishes – amplifies – binding sites for insulin, and *vice versa* (Fig. 2). It was shown earlier that imprinting with insulin enhanced the binding of Con-A; the present experiments served evidence of a similar influence of RAB.

It appears that the structure of the insulin receptor is universal [7, 8], being of similar configuration in the *Tetrahymena* and rat. Hormonal imprinting thus seems to depend not so much on the nature of the hormone, as on the first interaction with a given configuration, since the antibody (RAB) had induced it as efficiently as the hormone itself. Evidence that the "receptor-like" membrane pattern of the *Tetrahymena* is a genuine receptor, has also emerged from the present experiments, in that interaction took place not only with the hormone, but also with the antibody carrying a configuration complementary to a "genuine" receptor operative at a higher phylogenetic level.

The *Tetrahymena* divides about 5 times within 24 h [10]. Consequently the FITC-labeled active molecules used for reexposure had interacted with an offspring generation of the cells originally pre-exposed to the corresponding non-labeled molecules. It follows that imprinting by an antibody is transmitted to the progeny exactly as hormonal imprinting.

Since it does not seem likely that the antibodies acted at genic level, the hypothetical explanation remains that the information conveyed by the antibodies was generated – and transmitted – either at membrane level, or by retrograde feedback to the genic level. Both hypotheses remain to be substantiated by further study.

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