# In vitro inactivation of gonadotropin receptors, a membrane-associated action?[1]

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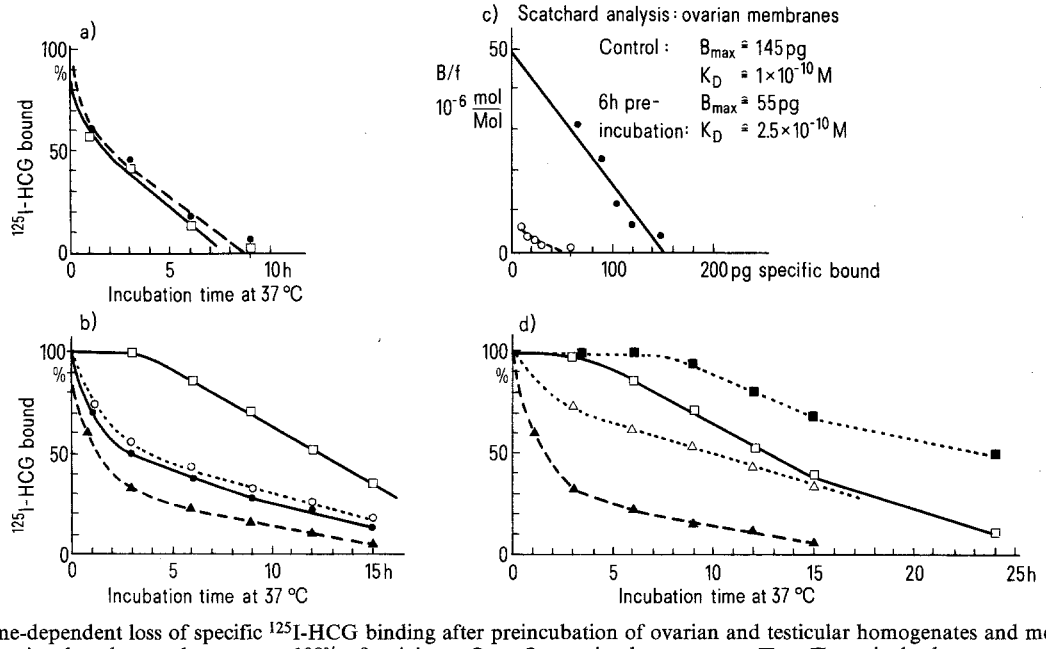
*Summary.* Under in vitro conditions the time-dependent inactivation process of LH/HCG receptors is nearly identical in ovarian and testicular homogenates but different in gonadal membrane preparations. In the ovarian membranes the loss of binding sites during the first preincubation time is faster than in testicular membranes, especially in membranes of luteinized rat ovaries. Compared with the homogenates, however, the receptor-inactivation in the membranes is generally delayed. The inhibitory effect of metabolic agents on receptor inactivation indicates that membrane-associated actions are involved in this process.

The action of luteinizing hormone (LH) or human chorionic gonadotropin (HCG) is mediated by specific receptors located in the plasma membrane of target cells[3]. In contrast to the testis and the interstitial ovarian cells the synthesis of LH/HCG-receptors in preantral follicles and in corpora lutea is regulated by several hormones like gonadotropic hormones, estrogen and prolactin[4]. Besides receptor synthesis the degradation of the LH/HCG receptor is an important step in the regulation of binding sites[5]. Generally it can be assumed that after ligand binding there exists a processing of ligand-receptor complexes during hormone action, which depends on the mobility of these complexes within the plasma membrane. The most clearly demonstrated mechanism for the LH/HCG receptor down-regulation after hormone binding is the clustering of receptors into surface aggregates which are internalized and lysosomally metabolized[5,6]. It has been suggested that not only the hormone-receptor complex but also the free receptors might be processed in the same way. Degradation kinetics of pulse chase labelled acetylcholin-receptors have shown that junctional and extra-junctional receptors are degraded similarly[7]. The mechanism of LH/HCG receptor inactivation, however, has not yet been described, as the isolation and specific labelling of these receptors is still a difficult problem. On the other hand it is well known that under in vitro conditions the loss of binding capacity of gonadal membranes for LH or HCG is dependent on time and temperature[8]. The present investigation was undertaken to clarify whether this inactivation process is an unspecific temperature effect or the result of actions related to cytoplasmatic or membrane factors. For this reason we tested the inactivation of free LH/HCG receptors in ovarian and testicular membranes under in vitro standard conditions and in the presence of metabolic and protease inhibitors and of a lysosomotropic agent.

*Materials and methods.* Iodination of highly purified HCG (biological activity of 11,000 IU/mg) (a gift from Schering AG, Berlin-West) was performed according to the modified method C of Leidenberger and Reichert[9]. The specific activity of the $^{125}$I-HCG was 30–50 Ci/mg. Phenylmethylsulfonylfluoride and Tos-Lys-$CH_2Cl$ were purchased from Sigma (München, FRG), Trasylol® from Bayer AG (Leverkusen, FRG). All other reagents were from the most convenient commercial source.

Gonadal plasma membranes were prepared from immature, adult and pregnant female and adult male SIV 50 rats. A 1:10 (w/v) tissue homogenate was prepared in cold Tris-HCl buffer (40 mM/l, pH 7.4, containing 1 mM $CaCl_2$). The homogenates were centrifuged at 200×g for 10 min and the supernatants (crude membranes plus cytosol) were




*a* and *b:* Time-dependent loss of specific $^{125}$I-HCG binding after preincubation of ovarian and testicular homogenates and membranes at 37 °C. Non-preincubated controls represent 100% of activity. *a* ●——●, ovarian homogenate; □——□, testicular homogenate. *b* ●——●, ovarian membranes of adult rats; ○——○, ovarian membranes of immature rats; ▲——▲, ovarian membranes of pregnant rats; □——□, testicular membranes. *c* Scatchard analysis of control (●——●) and 6-h preincubated (○——○) ovarian membranes of adult rats. *d* Time course study of receptor inactivation, control membranes and membranes treated with 10 mM $NaN_3$. ▲——▲, control ovarian membranes of pregnant rats; △——△, ovarian membranes of pregnant rats in the presence of $NaN_3$; □——□, control testicular membranes; ■——■, testicular membranes in the presence of $NaN_3$.

tested for $^{125}$I-HCG binding under standard binding conditions. For the preparation of crude membranes (without cytosol) supernatants were centrifuged at 10,000 × g for 15 min and the pellets were washed 3 times with Tris-HCl buffer. Partially purified membranes were obtained from the crude membranes by a discontinuous sucrose gradient centrifugation as described by Bramley and Ryan[10].

The specific HCG-binding capacity of homogenates and membrane preparations was analyzed by a filter method, according to Siebers et al.[11]. The membranes were incubated at 37 °C, 30 min with 10 ng $^{125}$I-HCG. For the estimation of unspecific binding an excess of 1000-fold unlabelled HCG was added. All determinations were done in triplicate.

Ovarian and testicular homogenates from adult rats and gonadal membrane preparations from immature, adult and pregnant female and adult male rats, in amounts corresponding to 10 mg wet wt, were incubated in 0.9 ml Tris HCl buffer containing 0.1% bovine serum albumine (BSA) at 37 °C. At different time intervals (3, 6, 9, 12 and 24 h) the incubations were stopped and the $^{125}$I-HCG binding capacity was tested. Test tubes without preincubation represented 100% activity. The number of $^{125}$I-HCG binding sites in the control and the 6-h preincubated crude membranes of adult rat ovaries were calculated by Scatchard analysis[12].

In another experiment crude membranes of adult rat ovaries corresponding to 10 mg wet wt were incubated in 0.9 ml Tris-HCl buffer for 5 h at 37 °C alone or with the following additions: 10 µM, 100 µM, 10 mM and 100 mM sodium fluoride; 10 µM, 100 µM, 10 mM and 100 mM sodium azide; 10 mM and 100 mM $NH_4Cl$; 10 µM, 100 µM and 1 mM phenylsulfonylfluoride; 10 µM, 100 µM and 1 mM Tos-Lys-$CH_2Cl$; 10 IU and 100 IU Trasylol. Phenylmethylsulfonylfluoride and Tos-Lys $CH_2Cl$ were dissolved in 95% ethanol (in the assays the ethanol concentration was below 1%) and the other agents in the incubation buffer. After preincubation the HCG binding capacity of the membranes was tested. The results are expressed in cpm ± SD specifically bound $^{125}$I-HCG. Furthermore a time course study with 10 mM sodium azide was performed using ovarian and testicular membranes of pregnant female and adult male rats. The $^{125}$I-HCG binding capacity was studied after incubation for 3, 6, 9, 12 and 24 h at 37 °C.

Ovarian membranes of adult rats preincubated on their own for 6 h at 37 °C or in the presence of the substances indicated. Specific $^{125}$I-HCG-binding was tested at a concentration of 10 ng $^{125}$I-HCG. Mean values cpm ± SD

| Substances | | $^{125}$I-HCG bound cpm ± SD |
|---|---|---|
| Control membranes without preincubation | | 27,000 ± 400 |
| Preincubated membranes | | 10,300 ± 1,100 |
| NaF | 10 µM | 10,600 ± 1,600 |
| | 100 µM | 11,700 ± 1,200 |
| | 10 mM | 24,000 ± 1,500 |
| | 100 mM | 26,900 ± 1,400 |
| $NaN_3$ | 10 µM | 14,200 ± 400 |
| | 100 µM | 14,500 ± 900 |
| | 10 mM | 22,500 ± 1,700 |
| | 100 mM | 23,000 ± 400 |
| $NH_4Cl$ | 10 mM | 13,300 ± 1,200 |
| | 100 mM | 17,100 ± 1,100 |
| Phenylmethylsulfonylfluoride | 10 µM | 11,300 ± 1,000 |
| | 100 µM | 10,900 ± 1,400 |
| | 1 mM | 10,000 ± 1,000 |
| Tos-Lys-$CH_2Cl$ | 10 µM | 10,000 ± 400 |
| | 100 µM | 9,900 ± 1,500 |
| | 1 mM | 12,000 ± 1,800 |
| Trasylol® | 10 IU | 10,600 ± 1,100 |
| | 100 IU | 10,100 ± 800 |

*Results and discussion.* The time-dependent loss of HCG binding capacity in testicular and ovarian homogenates from adult rats during incubation at 37 °C is shown in the figure, a. Half of the binding capacity is lost within 3 h in the testis and ovary. After 6–8 h no binding of labelled hormone is detectable. These results correspond well to those found by Bellisario and Bahl[8] in testicular homogenates. It must be assumed that in part cytoplasmatic enzymes are responsible for this rapid receptor degradation. To eliminate this cytosol effect crude and partially purified gonadal membranes were tested. If membranes of immature, adult and pregnant rat ovaries were incubated at 37 °C the receptor activity decreased in a similar way to that in ovarian homogenates within the first 3 h; the further inactivation process, however, was considerably delayed. After 15–20 h, the binding activity is completely destroyed (figure, b). There exists no difference, whether a crude membrane preparation or purified membranes (receptor 7-fold concentrated per mg protein) are taken (data not shown). Compared with the membranes of immature and adult animals the receptor degradation in ovarian membranes of pregnant animals is accelerated. After an incubation interval of 6 h the ovarian membranes of adult and immature animals still have 40–50% binding capacity, whereas in the membranes of pregnant animals only about 20% will be found.

Scatchard plot analysis of the binding data revealed similar dissociation constants ($K_D$) in preincubated and control crude membranes of adult rat ovaries (figure, c) indicating that the low binding capacity after preincubation is not due to a decreased binding affinity but due to a loss of binding sites. In contrast to the ovary, the kinetics of receptor inactivation in testicular crude membranes during the experimental period are more linear. The rapid degradation at the beginning of the incubation period cannot be observed (figure, b). The data show a half-life of the receptors of about 12 h. As in the ovary, no difference is seen if purified membranes are used (data not shown).

From the fact that in the testis only the interstitial cells are endowed with LH/HCG receptors and from the linear inactivation process of these receptors it can be concluded that all LH/HCG-binding sites in the testis are inactivated in the same way. In the ovary, on the other hand, the non-linear inactivation process of the LH/HCG-receptors indicates either the presence of at least 2 receptor populations or receptors differing according to different cell types. 1 group of these receptors is degraded very fast with a half-life of about 2–4 h and the other one at a rate similar to that found in the testicular membranes. From the observation that in pregnant rat ovaries most of the high-numbered binding sites are inactivated in a very short time, it can be assumed that the rapidly-degraded receptor group is located in luteal cells and cells of medium follicles only.

The differences in receptor inactivation make it likely that membrane actions rather than an unspecific temperature effect are involved in the loss of LH/HCG binding sites of the membranes. This assumption is supported by the observation of Ascoli and Puett[13] that the processing and degradation of the hormone-receptor complexes can be inhibited by several steps: the aggregation of the hormone receptor complex in the cell membrane by metabolic agents and the lysosomal degradation by lysosomotropic agents and protease inhibitors.

To test the action of these agents on the inactivation of free receptors, crude ovarian membranes were treated during

the preincubation period with metabolic inhibitors (NaF and $NaN_3$), protease inhibitors (Tos-Lys-$CH_2Cl$, phenylmethylsulfonylfluoride, Trasylol®) and a lysosomotropic agent ($NH_4Cl$). None of these agents influenced HCG binding (data not shown). Compared with untreated control membranes NaF and $NaN_3$ are potent inhibitors of the LH/HCG-receptor inactivation at a concentration of 10 and 100 mM. A similar effect was accomplished with $NH_4Cl$. The other agents tested did not have any effect on the receptor inactivation process (table).

A time course study under inhibiting conditions (10 mM $NaN_3$) was performed using crude testicular membranes and ovarian membranes of pregnant animals. Under this condition the inactivation process is extremely delayed and reduced, but there is no complete inhibition of receptor loss (figure, d). The incomplete inhibition of receptor degradation could be explained by an additional unspecific temperature effect. Since sodium azide has been shown as an inhibitor of many enzymes, the effect of the metabolic agents tested on the receptor inactivation might be related to an inhibition of membrane associated enzyme-action and/or to an inhibition of receptor aggregation in the plasma membrane. If the free receptor is inactivated in the same way as the hormone-receptor complex, the aggregation is a necessary step for the processing and degradation of the receptor.

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## Continuous optical assay of sucrase and other glucosidases

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*Summary.* A continuous optical method for the assay of glucose-releasing hydrolases is reported. Particular emphasis is given to the assay of purified sucrase from rabbit small intestine. The procedure requires glucose dehydrogenase and mutarotase. In the presence of the latter enzyme, the initial lag is substantially shortened when glucose is released as $\alpha$-anomer. Under the test conditions used, the method shows a good proportionality up to an activity of 0.2 units/3 ml and may also be applied for measuring the activity in crude homogenates.

The possibility of a continuous optical measurement of an enzymic reaction rate by recording the formation or disappearance of a chromogen with a recording photometer generally offers advantages in accuracy, precision and rapidity and allows a better measurement of kinetic behaviour. The methods most used until now for the assay of disaccharidases and other glucosidases are discontinuous 2-step[4-7] or 1-step[8] methods, in which the reaction is stopped at suitable times and the glucose released is measured by glucose oxidase/peroxidase[4-8], hexokinase/glucose-6-phosphate dehydrogenase[6] or glucose dehydrogenase[7]. A method of isomaltase assay with a coupled system glucose-6-phosphate dehydrogenase/NADP has been described in the past[9], but it has not been used since. With the most commonly used glucose oxidase method, lags of up to 15 min have been observed before the reaction attains a constant rate[8]. Such lags are principally due to the non-enzymic conversion of glucose from the $\alpha$-form to $\beta$-form, the latter being the substrate of glucose oxidase.

Purified preparations of NAD-dependent glucose dehydrogenase and mutarotase for the assay of glucose have recently been made commercially available[10]. In the present paper we describe a method which uses such preparations for the measurement of the activity of the enzyme sucrase-isomaltase. The same procedure may be employed for the determination of other glucosidases, either in crude extracts or in purified preparations.

*Materials and methods.* $\alpha$-glucosidase and $\beta$-fructosidase from yeast, $\beta$-glucosidase from sweet almonds, hexokinase/glucose-6-phosphate dehydrogenase from yeast, ATP and $NADP^+$, were purchased from Boehringer, Mannheim, FRG. Glucose dehydrogenase, mutarotase, $NAD^+$, sucrose, palatinose, maleic acid and other chemicals were from Merck, Darmstadt, FRG. D-salicin was purchased from Fluka, Buchs, Switzerland.

For the preparation of the small intestine crude extracts, frozen rabbit small intestine was homogenized twice for 30 sec at 20% (w/v) in 50 mM sodium maleate buffer, pH 6.8+5 mM EDTA in a Waring Blendor at 4 °C. The homogenate was filtered through a cheese-cloth and the measurements of the enzyme activities were carried out on the filtrate. The protein content was determined according to Lowry et al.[11].

The sucrase-isomaltase complex was isolated from rabbit

Activities of some disaccharidases in a crude homogenate of rabbit small intestine

| Substrate | Continuous method (U/mg protein) | Discontinuous methods (HK+G6PD) | (MR+GD) (U/mg protein) |
|---|---|---|---|
| Sucrose | 0.228±0.012 | 0.228±0.019 | 0.239±0.012 |
| Maltose | 0.518±0.037 | 0.610±0.026 | 0.660±0.020 |
| Trehalose | 0.102±0.008 | 0.120±0.007 | 0.122±0.007 |

Test conditions for continuous assay are as indicated in figure 2; for discontinuous methods as indicated under 'materials and methods'. HK = hexokinase; G6PD = glucose-6-phosphate dehydrogenase; MR = mutarotase; GD = glucose dehydrogenase. Each value is the mean of 4 measurements ± SD.