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SOLUBILIZATION OF THE LACTOGENIC RECEPTORS FROM RAT LIVER MICROSOMES

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SUMMARY

The receptors that bind lactogenic hormones have been solubilized from adult female rat liver microsomes with 1 % (wt/v) Triton X-100. The receptors remained in the supernatant after a centrifugation at 190,000 x g_{av} for 8 h. They could be detected by gel filtration on Sephadex G-100 and by precipitation with 12 % (wt/v) Polyethylene Glycol. The specific binding of [^{125}I] hGH to the solubilized receptors reached the equilibrium within 8 h at 25ºC. The solubilized binding sites retained their specificity for lactogenic hormones. The equilibrium constant for the dissociation of the complex was 8 x 10^{-10} M. These properties are not significantly different from those reported for the particulate preparation.

INTRODUCTION

The existence of specific binding sites with high affinity for hGH has been demonstrated in microsomal membranes from adult female rat liver(1). Further studies have characterized the properties of the interaction between hGH and these binding sites (2-4) and their physiological regulation (5-12). On the basis of some of the forementioned studies it has been established that these binding sites have rather strict lactogenic specificity.

In the present report we describe the solubilization of these receptors by using the nonionic detergent Triton X-100.

ABBREVIATIONS : hGH: human Growth Hormone; bGH: bovine Growth Hormone; eGH: equine Growth Hormone; oPRL: ovine Prolactin; hCS: human Chorionic Somatomammotrophin; oLH: ovine Luteinizing Hormone; oFSH: ovine Follicle -stimulating Hormone; bTSH: bovine Thyroid-stimulating Hormone; PEG: polyethylene Glycol.

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MATERIALS AND METHODS

Materials : hGH was prepared according to Roos et al. (13) and purified by gel filtration; bGH was prepared by the method of Dellacha and Sonenberg (14) and eGH according to Conde et al. (15). The chemical purity and homogeneity of these hormones were established by aminoacid analysis, C-terminal determinations and polyacrilamide gel electrophoresis. Their biological activity was 1.1 , 1.0 and 1.6 IU/mg respectively. oPRL, oLH, oFSH and bTSH were obtained from the NIAMDD, Bethesda, Md. hCS was purchased from Nutritional Biochemicals;bovine insulin, serum albumin and gamma-globulin from Sigma; carrier-free [^{125}I] NaI from New England Nuclear; PEG (Carbowax 6000) from Union Carbide and Sephadex G-100 and Blue Dextran from Pharmacia.

Animals : Adult (210-230 g), female rats of the Long-Evans strain were used in the estrous phase of the sexual cycle.

Iodination of hGH : Labeling of hGH with [^{125}I] to specific activities of 80-100 μCi/μg was carried out as described by Roth (16).

Microsomes preparation : Microsomes were obtained according to Posner et al. (1) except that the liver homogeneization was done in 0.3 M Sucrose, 5 mM Tris-HCl (pH 7.5) and 0.5 mM $CaCl_2$.

Solubilization procedure : Microsomes were resuspended in 1 % (wt/v) Triton X-100, 2 mM EDTA, 25 mM Tris-HCl (pH 7.5) to give a protein concentration of 5 to 7 mg of protein per ml. The suspension was stirred at room temperature (20ºC) for 30 min and then centrifuged at 190,000 x g_{av} for 8 h at 4ºC in a MSE Prepspin 50 ultracentrifuge fitted with a swing-out rotor in 14 ml tubes. After centrifugation 10 ml from the top of each tube were carefully aspirated, aliquoted and kept frozen at -20ºC. No membranes were detected in this extract by electron microscopy with negative staining. Proteins were determined by the procedure of Lowry et al. (17) adapted (18) to the presence of Triton X-100 in the sample. Approximately 47 % of the membrane protein was solubilized by this procedure.

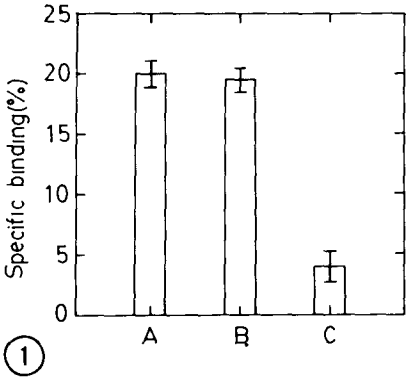
Binding Assays : Hormone binding to microsomes was measured according to Posner et al. (1); binding to the solubilized receptors was determined by incubating [^{125}I] hGH (100 to 150 x 10^3 cpm) with 150 μg of solubilized proteins in a final volume of 500 μl containing 25 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ and 0.08 % (wt/v) of bovine serum albumin at 25ºC overnight (14 h).

Nonspecific Binding was determined in the presence of 5 μg of unlabeled hGH. After incubation two methods were used to detect binding activity: gel filtration on Sephadex G-100 columns and PEG precipitation.

Gel filtration was performed by applying the samples to a Sephadex G-100 column (600 x 5 mm) and eluting with 25 mM Tris-HCl (pH 7.5); 10 mM $CaCl_2$; 0.08 % (wt/v) bovine serum albumin and 0.1 % (wt/v) Triton X-100. Fractions of 0.5 ml were collected and counted in a gamma-counter.

The PEG precipitation method (19) was done as follows: at the end of the incubation time the tubes were placed in ice and 0.5 ml of cold 0.1 % (wt/v) bovine gamma-globulin were added, followed by 1 ml of cold 24 % (wt/v) PEG, both dissolved in 25 mM Tris-HCl (pH 7.5) buffer, mixing vigorously. The tubes were kept in ice for 10 min and then centrifuged at 1,400 x g_{av} for 25 min. The supernatants were drained off, and the tubes counted in a gamma-counter. No dissociation of the hormone-receptor complex was detected up to 1 h in ice after precipitation.

The concentration of membrane protein used in all experiments was in the zone of linear dependence of the specific binding.



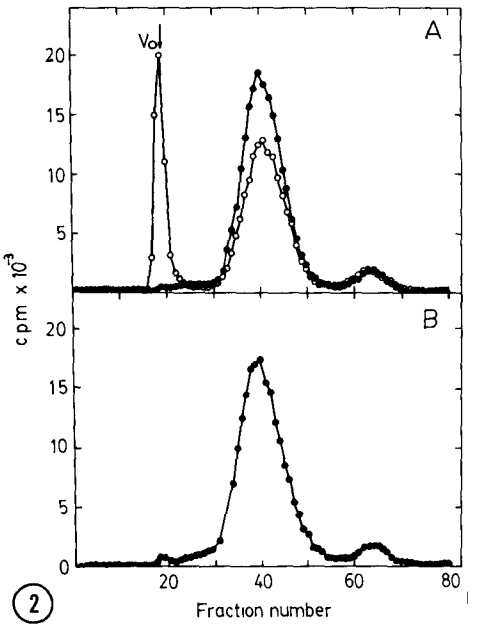


Fig. 1 : Effect of pretreatment of microsomes with Triton X-100 on the binding of [^{125}I] hGH. A) Non treated microsomes. B) Treated as C) but without Triton X-100. C) Microsomes treated with Triton X-100 as described under "Materials and Methods". After centrifugation the pellets were resuspended in fresh buffer and their binding activity determined. Specific Binding (%) is defined as (TB-N) x 100/T where TB is the total radioactivity bound, N is the nonspecifically bound radioactivity and T is the total radioactivity put into the tube. The height of each bar represents the mean of triplicate determinations. The SD is shown.

Fig. 2 : Gel filtration on Sephadex G-100 of [^{125}I] hGH incubated with no additions (A, ●—●); incubated with soluble receptors (A, o—o) or with soluble receptors and 5 µg of unlabeled hGH (B, ●—●).

RESULTS

The extraction of the microsomal membranes with Triton X-100 caused a loss of 80 per cent of the original specific binding of hGH (Fig.1). This phenomenon was paralleled by the appearance of specific hGH-binding activity detected by gel filtration in the extract supernatant (Fig.2). The elution profile of [^{125}I] hGH showed a major peak with a Ve/Vo ratio of 1.87 and a minor retarded peak probably due to degradation products. When the labeled hormone was incubated with the extract, part of the radioactivity appeared in a new peak, coincident with the void volume

of the column. This peak disappeared when the incubation was done in the presence of a 5,000-fold excess of unlabeled hormone. The amount of degradation products did not change in any of these experimental conditions.

It is clear that hGH was bound specifically to a soluble macromolecule present in the high-speed supernatant to form a complex with a molecular weight higher or equal to 150,000. The specific binding activity recovered in the extract was about 50 per cent of that initially present in the microsomes.

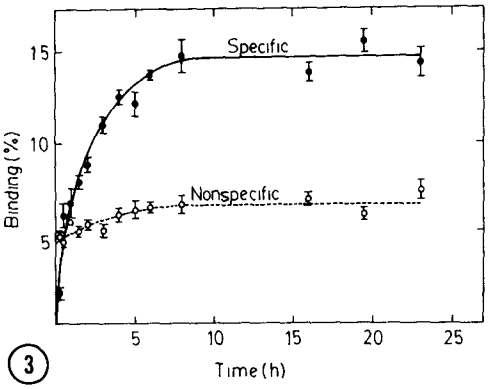
The hormone-receptor complex was also separated from the free hormone by the PEG precipitation method. The percentage of specific binding found by this procedure was similar to that obtained by gel filtration. Negligible nonspecific binding was observed when the assay was done by gel filtration, but about 6 per cent of the added radioactivity precipitated with PEG in the presence of an excess of unlabeled hGH. Control experiments without added receptor revealed that it was mainly due to precipitation of free $[^{125}I]$ hGH.

Various treatments of the microsomes with 2 M NaCl, 2 M KI, 3 M Urea, 2 mM EDTA, 30 % (v/v) Ethanol, or 1 % (wt/v) SDS under the same conditions as used with Triton X-100 did not solubilize any receptor activity.

Fig.3 shows the kinetics of association between $[^{125}I]$ hGH and the solubilized receptors at 25ºC. Nonspecific binding was maximal almost instantaneously, whereas specific binding reached equilibrium after 8 h of incubation.

As shown in Table 1 there was significant inhibition of $[^{125}I]$ hGH - binding by hormones with lactogenic activity like hGH, oPRL and hCS. Other hormones devoid of this property like bGH, eGH, oLH, oFSH, bTSH and bovine insulin did not compete with $[^{125}I]$ hGH.

Fig. 4 shows the competition curve between $[^{125}I]$ hGH and native hGH for



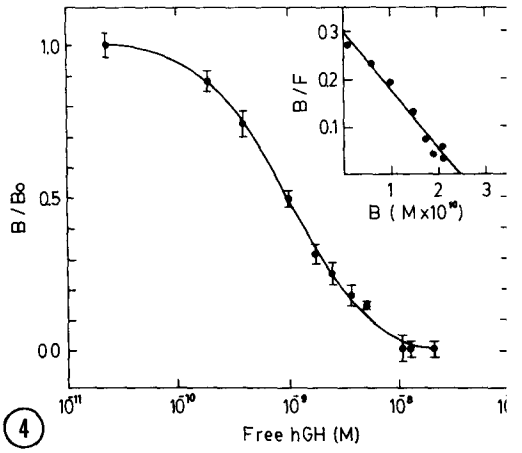


Fig. 3 : Kinetics of $[^{125}I]$ hGH association to the soluble receptors at 25°C. Specific and Nonspecific binding at different times were determined by PEG precipitation. Each point represents the mean ± SD of triplicate determinations.

Fig. 4 : Competition between $[^{125}I]$ hGH and native hGH for the soluble receptors. $[^{125}I]$ hGH was incubated with soluble receptors in the presence of increasing amounts of unlabeled hGH. B / Bo is the ratio between specific binding of $[^{125}I]$ hGH at each concentration of unlabeled hGH and specific binding in the absence of unlabeled hGH. Values are the mean ± SD of triplicate determinations. Inset : Scatchard plot of the competition data. B / F is the ratio between hGH specifically bound (B) and free hGH (F).

the solubilized receptors. Scatchard analysis (20) of these data yielded a linear plot (Fig. 4, inset). The calculated dissociation constant was 8×10^{-10} M and the binding capacity 1026 fmol per mg of protein.

DISCUSSION

Nonionic detergents and bile salts are useful tools for the solubilization of membrane proteins without affecting their biological activities (21). From the data obtained in the present experiments it is clear that Triton X-100 is able to solubilize the lactogenic receptors from rat liver microsomes with retention of their hGH-binding property. However, not all the binding activity disappearing from the microsomes is recovered in the supernatant. The reason for this is unclear : it may be due to partial inactivation of the receptors during the solubilization process or the

TABLE 1 Specificity of binding of $[^{125}I]$ hGH to the soluble receptors.

Additions	Binding (%)
None	30.6 ± 0.7
hGH	9.8 ± 0.2
oPRL	8.7 ± 0.0
hCS	13.2 ± 0.4
bGH	28.4 ± 0.7
eGH	31.8 ± 1.3
oLH	30.3 ± 1.1
oFSH	30.5 ± 0.7
bTSH	30.3 ± 1.1
bovine Insulin	30.2 ± 1.0

$[^{125}I]$ hGH was incubated with the soluble receptors in the presence of 5 μg of each indicated hormone. Binding was measured by the PEG precipitation method. Binding (%) is defined as (TB/T) x 100 where TB is the total radioactivity bound and T is the total radioactivity put into the tube. Values are expressed as the mean ± SD of triplicate determinations.

result of an inhibitory effect on the binding assay of the Triton X-100 present in the system.

Herington et al. (4) reported maximal specific binding of $[^{125}I]$ hGH to microsomal membranes in 16 h at 22ºC. This slow rate of association was attributed to vesiculization of their particular preparation. However, microsomes obtained by us in the presence of Ca^{2+} and in a buffered medium reached equilibrium within 6 h at 25ºC[#], this value being quite similar to that found in the present experiments. Apparently, then, the solubilization procedure does not lead to any significant change in the kinetic behavior of the receptors.

[#]S.H.Sánchez, J.S.Bonifacino and A.C.Paladini Manuscript in preparation.

Their specificity for lactogenic hormones is also retained.

The dissociation constant of the soluble receptors found here is in close agreement with those reported in the literature for the microsomal ones: 9 x 10^{-10} M (2); 12.4 x 10^{-10} M (3) and 7 to 9 x 10^{-10} M (5). No change in affinity seems to occur with the solubilization process.

The obtention in soluble form of the rat liver lactogenic receptors, with their binding properties intact, is the necessary step previous to further purification and their physical, chemical and immunological characterizati

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