Influence of divalent cations on the detection of somatogenic and lactogenic binding sites in mouse liver cells

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Specific binding of ^{125}I -labelled human somatotropin was demonstrated in isolated hepatocytes from male mice. In the presence of divalent cations (Ca^{2+} and Mg^{2+}) the binding of ^{125}I -labelled human somatotropin was competitive with ovine prolactin. Scatchard analysis of competition data indicated a K_D of $1.4 \pm 0.2 \text{ nM}$ and a binding capacity of 13000 ± 2000 sites/cell. In the absence of divalent cations and in the presence of EDTA, human and bovine somatotropins were found to be equally effective to displace bound ^{125}I -labelled human somatotropin, while ovine prolactin showed a weak competition. In this case, the binding capacity was 8400 ± 1500 sites/cell and the K_D was $1.1 \pm 0.1 \text{ nM}$.

We have demonstrated the specific uptake in mouse liver *in vivo* not only of ^{125}I -labelled human somatotropin (with growth-promoting and lactogenic effects in rodents) but also of ^{125}I -labelled bovine somatotropin, which exhibits exclusively somatogenic activity (Ciccía-Torres *et al.*, 1983). The results derived from this study suggest the presence in mouse liver of distinct somatogenic and lactogenic binding sites.

On the other hand, when binding studies with ^{125}I -labelled human somatotropin were carried out in isolated mouse liver cells, only sites with lactogenic specificity were detected (Fouchereau-Péron *et al.*, 1980).

The present investigation was carried out in an attempt to establish the conditions *in vitro* in which the lactogenic and somatogenic receptors observed *in vivo* could be detected. For this purpose, binding of ^{125}I -labelled human somatotropin to isolated mouse cells was assayed in incubation media of different composition. We report here that the lactogenic sites were revealed in an assay medium containing Ca^{2+} and Mg^{2+} , whereas the somatogenic sites were detected in the presence of a divalent cation-chelator.

Abbreviation used: HBSS, Hanks' balanced salt solution.

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Materials and methods

Materials

The majority of the hormones and chemicals used in this work were prepared, purified or obtained as indicated previously (Ciccía-Torres *et al.*, 1983). Crude collagenase (type I, no. C-0130) was from Sigma.

Iodination of human somatotropin

^{125}I -labelled human somatotropin was prepared as already described (Turyn *et al.*, 1976).

Preparation of cells

Hepatocytes, from normal adult male mice (24–26 g body wt.) of the Swiss strain, were prepared by a modification of the method described by Berry & Friend (1969). The liver was perfused *in situ* through the portal vein at a rate of 8 ml/min. About 50 ml of HBSS (Hanks & Wallace, 1949) without Ca^{2+} and Mg^{2+} was first perfused, followed by 60 ml of the same solution containing 0.05% collagenase and finally another 50 ml of the first solution. All media were kept at 37°C and gassed with O_2/CO_2 (19:1). At the end of the perfusion, the liver was carefully removed and gently shaken for about 10 min in 50 ml of HBSS without Ca^{2+} and Mg^{2+} . The suspension was then filtered through a nylon cloth and centrifuged at 50 g for 2 min. The supernatant was removed and cells

were washed twice and then counted in a Neubauer haemocytometer. This procedure yielded about $(4-5) \times 10^7$ cells/liver. Viability, as judged by exclusion of 0.4% Trypan Blue, was 80–90%.

Incubation media

The following media were used: (a) HBSS with Ca^{2+} and Mg^{2+} (pH 7.4), consisting of 137 mM-NaCl, 5 mM-KCl, 0.3 mM- $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.4 mM- KH_2PO_4 , 4 mM- NaHCO_3 , 0.4 mM- MgSO_4 , 1.26 mM- CaCl_2 , 0.5 mM- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5 mM-glucose, 0.01 g of Phenol Red/l and 0.1% bovine serum albumin; (b) HBSS without Ca^{2+} and Mg^{2+} (pH 7.4) with the same composition as (a) except for the omission of divalent cations; (c) HBSS without Ca^{2+} and Mg^{2+} (pH 7.4) containing 2 mM-EDTA and 0.1% bovine serum albumin.

Binding studies

The assay procedure for binding determination was as follows: ^{125}I -labelled human somatotropin (10–20 ng) was incubated with liver cells $[(1-2) \times 10^6]$ in 400 μl of the indicated buffers containing 0.1% bovine serum albumin. Incubations, in triplicate, were carried out in plastic tubes (12 mm $\times$ 75 mm) at 25°C with constant shaking for 120 min. Bound and free hormone were separated by centrifugation as follows. Two portions (150 μl) of each incubation mixture were layered over 700 μl of a cold 5% (w/v) sucrose solution in conical plastic tubes and immediately centrifuged at 1500g for 5 min at 4°C. After the supernatant was aspirated, the tip of the tubes containing the cell pellet was cut with a razor blade and radioactivity bound to the pellet was measured in a γ -radiation counter. Parallel incubations were performed in the presence of an excess of unlabelled human somatotropin (5 μg /tube). Specific binding was taken as the difference between the total uptake of radioactivity by the cells and that uptake which was not displaced by an excess of native hormone. Under these conditions the specific binding represented 3–7% and the non-specific binding 1.5–3.5% of the total amount of radioactivity added in each tube.

The reversibility of the binding was determined as follows. ^{125}I -labelled human somatotropin was incubated with liver cells at 25°C for 120 min, as described above. The reaction was stopped by dilution with 4 ml of cold incubation medium followed by centrifugation at 1500g for 5 min at 4°C. Supernatants were aspirated and the cell pellets were suspended in 4 ml of incubation medium or 4 ml of incubation medium plus 50 μg of unlabelled human somatotropin and incubated at 25°C for a variety of time intervals before centrifugation (1500g for 5 min at 4°C). The new cell pellets were counted for radioactivity.

Results

Optimization of the assay conditions

The specific binding of ^{125}I -labelled human somatotropin to isolated hepatocytes carried out in the different incubation media already indicated was time- and temperature-dependent. At 25°C an apparent equilibrium of bound human somatotropin was attained within 120 min of incubation and was maintained for at least another 120 min. The process was reversible; approx. 50% of specifically bound hormone was dissociated after 75 min at 25°C and the rate was unaffected by the presence of unlabelled hormone (2500-fold molar excess) (results not shown).

Little variation of binding was noted between pH 7.1 and 7.6. Specific binding of ^{125}I -labelled human somatotropin was linearly related to the cell concentration over the range $(1-8) \times 10^6$ cells/ml (results not shown).

Fig. 1. Effect of ^{125}I -labelled human somatotropin concentration on binding to hepatocytes isolated from normal male mice

(a) Cells (1.25×10^6 /tube) were incubated with increasing amounts of ^{125}I -labelled human somatotropin as indicated on the abscissa for 2 h at 25°C in HBSS (with Ca^{2+} and Mg^{2+}). (b) Scatchard plot of the binding data obtained from the experiment shown in (a).

Effect of hormone concentration on the specific binding

Fig. 1(a) shows that the specific binding of ^{125}I -labelled human somatotropin to hepatocytes was a saturable process. Scatchard (1949) analysis of the binding data from Fig. 1(a) yielded a linear plot (Fig. 1b). Linear regression analysis of saturation experiments defined a binding capacity of 15000 ± 2600 sites/cell with a dissociation equilibrium constant (K_D) of 1.3 ± 0.2 nM (means $\pm$ S.E.M., $n = 3$).

Displacement of ^{125}I -labelled human somatotropin from hepatocytes by unlabelled hormones: influence of divalent cations

Native human somatotropin readily inhibited ^{125}I -labelled human somatotropin binding to liver cells (Fig. 2). The values of K_D and binding capacity calculated from competition experiments were 1.4 ± 0.2 nM and 13000 ± 2000 sites/cell respectively (means $\pm$ S.E.M., $n = 4$). These parameters were similar to those obtained from saturation curves.

The specificity of the human somatotropin binding to mouse liver cells was studied by comparing the ability of unlabelled ovine prolac-

tin, human or bovine somatotropin to inhibit the binding of ^{125}I -labelled human somatotropin. When the incubation medium was HBSS (with Ca^{2+} and Mg^{2+}) a lactogenic hormone such as ovine prolactin caused substantial displacement of bound ^{125}I -labelled human somatotropin, whereas bovine somatotropin had minimum effect (Fig. 2). Non-related hormones (insulin, follitropin) did not compete for the specific binding (Fig. 2).

In an attempt to detect the somatogenic binding sites whose existence was indicated by our findings *in vivo* (Ciccia-Torres *et al.*, 1983), the composition of the incubation media was modified considering that the environmental requirements for maximum binding might not be the same for both sites.

The competition experiments performed with HBSS (without Ca^{2+} and Mg^{2+}) + 2 mM-EDTA are shown in Fig. 3. In this case the potencies of human and bovine somatotropins to inhibit ^{125}I -labelled human somatotropin binding to male mouse hepatocytes were almost identical, while ovine prolactin competed weakly.

The values of K_D and the binding sites/cell calculated from the competition experiments shown in Fig. 3 were 1.1 ± 0.1 nM and 8400 ± 1500 respectively (means $\pm$ S.E.M., $n = 4$).

Fig. 2. Specificity of binding of ^{125}I -labelled human somatotropin to hepatocytes from male mice

Cells were incubated in HBSS (with Ca^{2+} and Mg^{2+}) with ^{125}I -labelled human somatotropin for 2 h at 25°C in the presence of increasing amounts of native human somatotropin ($\circ$), ovine prolactin ($\triangle$) and bovine somatotropin ($\bullet$). Insulin ($\square$) and follitropin ($\blacktriangle$) were assayed at a single dose. Results are expressed as the ratio of the specific binding at each concentration of unlabelled hormone (B) to the specific binding in the absence of additions (B_0). The points represent mean values for four experiments.

Fig. 3. Hormonal specificity of binding of ^{125}I -labelled human somatotropin to hepatocytes from male mice in HBSS (without Ca^{2+} and Mg^{2+}) + 2 mM-EDTA

Cells were incubated with ^{125}I -labelled human somatotropin in the presence of increasing amounts of native human somatotropin ($\circ$), ovine prolactin ($\triangle$) and bovine somatotropin ($\bullet$). Insulin ($\square$) and follitropin ($\blacktriangle$) were assayed at a single dose. Results are expressed as the ratio of the specific binding at each concentration of unlabelled hormone (B) to the specific binding in the absence of additions (B_0). The points represent mean values for four experiments.

Discussion

We have previously studied the distribution *in vivo* of ^{125}I -labelled human and bovine somatotropins in different tissues of adult mice and demonstrated the specific uptake of these hormones by the liver. On the basis of these studies we suggested, in the mouse liver, the presence of somatogenic and lactogenic binding sites (Ciccia-Torres *et al.*, 1983).

On the other hand, Fouchereau-Peron *et al.* (1980) described in isolated mouse liver cells that human somatotropin and prolactin interact with the same type of binding site. Though the above findings support the presence of lactogenic sites, the authors consider they observed somatogenic ones.

Binding of protein hormones to specific receptors is a reversible and saturable process which depends on the amount and characteristics of hormone and receptor preparation, as well as the environment in which association and dissociation occurs. Considering this point, we decided to establish those conditions in which the binding sites observed *in vivo* could be detected *in vitro*.

In the present work we have identified, in isolated mouse liver cells, sites that bind human somatotropin. The cell-somatotropin interaction was shown to be a time-, temperature- and dose-dependent process.

When the specificity of somatotropin binding to male mouse hepatocytes was studied in a medium containing divalent cations, lactogenic binding sites were predominantly detected (Fig. 2). These results, as well as the dissociation equilibrium constant and the binding capacity obtained, are in agreement with the data reported by Fouchereau-Peron *et al.* (1980), who used Krebs-Ringer bicarbonate buffer (with divalent cations) as incubation medium.

On the contrary, a significant change in hormonal specificity was observed when the binding of somatotropin to mouse liver cells was studied in a medium containing EDTA in the absence of Ca^{2+} and Mg^{2+} (Fig. 3). In this case bovine somatotropin inhibited the binding of ^{125}I -labelled human somatotropin, suggested the existence of somatogenic binding sites. Analysis of the Scatchard plots showed that, although the addition of EDTA had no effect on affinity, it did result in a lower amount of available membrane binding sites. These results are in accordance with our findings *in vivo*, which give evidence for the presence in the mouse liver of a mixed population of both somatogenic and lactogenic binding sites (Ciccia-Torres *et al.*, 1983).

On the other hand Posner (1976), using Tris/Mg^{2+} buffer as incubation medium, detected binding of ^{125}I -labelled human somatotropin to

liver microsomes from non-pregnant female and male mice with only lactogenic specificity. More recently, Herington *et al.* (1983) described in liver membrane preparations from normal and dwarf mice the presence of binding sites for labelled prolactin, human and bovine somatotropins. When binding studies were carried out with ^{125}I -labelled human somatotropin in the presence of Ca^{2+} , lactogenic hormones displaced the tracer more strongly than did somatogenic ones.

Alternatively, the facts reported in the present paper could also be explained by the existence of a single type of binding site which binds human somatotropin in the presence or absence of Ca^{2+} and Mg^{2+} , bovine somatotropin only when these cations are absent and ovine prolactin only when they are present. Such an interpretation is supported by evidence from other species that suggests the existence of combined binding sites for both lactogenic and somatogenic hormones (Hughes, 1979; Cadman & Wallis, 1981; Fix *et al.*, 1981).

Therefore, an important fact to be kept in mind is the composition of the incubation media, since divalent cations such as Ca^{2+} and Mg^{2+} favour in mouse liver the binding of hormones with lactogenic activity, while the presence of EDTA (in the absence of divalent cations) allows the recognition of binding sites with somatogenic specificity.

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