Binding *in vitro* to rat liver receptors does not correlate with activities *in vivo* of bovine somatotropin

Use of chemically modified derivatives as probes

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Bovine somatotropin with an increasing number of its carboxylate groups modified by reaction with glycine methyl ester in the presence of a water-soluble carbodi-imide was tested for its activity in different bioassays. Only those derivatives which were known to be active in the body-weight-increase bioassay were able to compete with ^{125}I -labelled bovine somatotropin for their specific binding sites *in vivo*. No difference was found in the rate of clearance of a poorly active derivative as compared with that of native somatotropin. In contrast, both active and inactive derivatives were found to be equally effective in displacing the tracer from its binding sites present in isolated cells and membrane preparations from rat liver. These results suggest that the liver somatogenic receptors studied *in vitro* are less discriminating than those detected *in vivo*.

The saturability of the uptake *in vivo* of somatotropins by rat liver (Turyn & Dellacha, 1978) is mainly due to the formation of specific hormone-binding protein complexes (Bonifacino *et al.*, 1983). The results derived from these studies are in accordance with binding studies *in vitro* which give evidence for the existence of two types of receptors in rat liver with either lactogenic or somatogenic specificity (Posner *et al.*, 1974; Ranke *et al.*, 1976a,b; Postel-Vinay, 1976).

Somatogenic binding sites, probably related to the anabolic actions of the hormone, were identified *in vivo* in the liver of normal and hypophysectomized rats (Turyn & Dellacha, 1978; Bonifacino *et al.*, 1983) and were also detected in rat liver membrane preparations (Postel-Vinay, 1976; Etzrodt *et al.*, 1976; Baxter *et al.*, 1980; Kyle *et al.*, 1982) and in hepatocytes from normal (Ranke *et al.*, 1976a,b; Postel-Vinay & Desbuquois, 1977) as well as hypophysectomized (Turyn *et al.*, 1976) rats.

Although the interaction of somatotropins with their cellular receptors has been extensively studied, the physiological significance of the occupancy of these binding sites and the molecular mechanisms involved in the expression of the resulting biological effects are still unknown.

In an attempt to investigate this point, several derivatives of bovine somatotropin, obtained by the specific and gradual blocking of some of its free carboxylate groups with glycine methyl ester (Delfino *et al.*, 1983), were tested for their ability to displace ^{125}I -labelled bovine somatotropin from its liver binding sites by using assays *in vivo* and *in vitro*. The biological potency of these derivatives, as measured by the weight-gain test, diminishes with an increasing degree of modification (Delfino *et al.*, 1983). Hence, they were considered useful probes to study the relationship between binding and biological activity.

Materials and methods

Materials

Bovine somatotropin was prepared as described by Dellacha & Sonenberg (1964). Other chemicals were obtained as indicated previously (Delfino *et al.*, 1983).

Iodination of bovine somatotropin

^{125}I -labelled bovine somatotropin was prepared by the method of Greenwood *et al.* (1963) using a limiting amount of chloramine-T (Roth, 1975) in order to introduce on average 1 mol of I/mol of hor-

none. The reaction mixture contained 10 μ l of 0.3M-potassium phosphate buffer, pH 7.4, 1 mCi of Na¹²⁵I, 4 μ l of a solution of bovine somatotropin (1 mg/ml) in 5mM-NaOH, and 3 μ l of a solution of chloramine-T (72 μ g/ml) in 50mM-potassium phosphate buffer, pH 7.4. The order of addition of the reagents to the tube was as indicated. After 8 min of incubation at 20°C, the reaction mixture was diluted with 200 μ l of 50mM-Tris/HCl buffer, pH 7.4, with 1% (w/v) of bovine serum albumin and the solution was filtered through a Sephadex G-100 column (5mm $\times$ 600mm) equilibrated and eluted with the same buffer at 4°C. The labelled monomeric hormone was recovered as a symmetrical peak well resolved from aggregated hormone and free iodine. Its specific radioactivity ranged usually between 80 and 120 μ Ci/ μ g.

Obtention of methoxyglycylated derivatives of bovine somatotropin

Methoxyglycylated derivatives were prepared by reaction of bovine somatotropin with a water-soluble carbodi-imide in the presence of an excess of glycine methyl ester, according to the method employed by Delfino *et al.* (1983). Derivatives 0.5, 1, 3 and 5 were obtained after 0.5, 1, 3 and 5 min of reaction and contained 1.9, 2.8, 5.8 and 7.6 methoxyglycyl residues incorporated/molecule of the hormone, respectively.

Assays in vivo

The specific uptake *in vivo* of native bovine somatotropin or two methoxyglycylated derivatives (1 and 5) by rat liver was determined by the method of Turyn & Dellacha (1978) modified as follows. Long-Evans female rats (80–140 g body wt.), anaesthetized with sodium thiopental (6 mg/100 g body wt.), were injected via one jugular vein with ¹²⁵I-labelled bovine somatotropin (1 $\times$ 10⁶ c.p.m.) alone or together with different doses of native hormone or its derivatives. After 20 min a sample of blood was removed from the other vein and two slices from different lobes of the liver were quickly obtained. The blood was allowed to clot and two aliquots of serum were placed in plastic tubes and weighed. The liver slices were rinsed with 0.15M-NaCl, blotted with filter paper and weighed in plastic tubes. Radioactivity was measured in a well scintillation gamma counter. The results were expressed as the ratio c.p.m./g of liver: c.p.m./g of serum.

The growth-promoting activity of the native hormone and its derivatives (1 and 5) was measured by the body-weight gain assay (Marx *et al.*, 1942) according to the following procedure. Hypophysectomized Long-Evans rats (110–130 g

body wt.) were injected intraperitoneally twice a day for 5 days. The response of each rat was measured by the difference between its weight at day 9 and the average weight at days 1, 2 and 3, days 4 and 8 being the first and the last days of the injections, respectively.

Assays *in vivo* were carried out employing the native hormone as the standard. Estimates of the relative biological potency of the unknowns were calculated by statistical analysis for parallel line assays for a factorial-balanced 2 $\times$ 2 doses design (Finney, 1964).

Binding assays in vitro

Binding studies *in vitro* were performed with isolated cells (Turyn *et al.*, 1976) and membrane preparations from rat liver.

Hepatocytes from hypophysectomized female Long-Evans rats (80–100 g body wt.) were obtained by perfusion of the liver with collagenase (Sigma, type I) according to the method described by Berry & Friend (1969). The cell suspension obtained was washed and resuspended in Hanks' balanced salt solution (Hanks & Wallace, 1949), pH 7.4, containing 1% (w/v) bovine serum albumin. The interaction of ¹²⁵I-labelled bovine somatotropin with receptors from isolated cells was determined by incubating, for 2 h at 25°C, about 12 ng of labelled hormone (120000 c.p.m.) in the presence of increasing amounts of unlabelled hormone or its methoxyglycylated derivatives with 4 $\times$ 10⁶ hepatocytes in a total volume of 0.5 ml of Hanks' balanced salt solution, pH 7.4, with 1% (w/v) bovine serum albumin. After incubation, samples of the reaction mixture (150 μ l, in duplicate) were layered on the top of 700 μ l of 5% (w/v) cold sucrose solution contained in conical plastic tubes. The tubes were centrifuged at 1500 g for 5 min at 4°C and the supernatants were discarded. The tips of the tubes containing the cell pellets were cut and counted for radioactivity. Under these conditions, the specific binding ranged from 5.0 to 8.5% of the total counts initially placed in each tube. Non-specific binding, determined in the presence of an excess (1 μ g) of unlabelled hormone, ranged from 2.5 to 4.0% of the total amount of radioactivity added.

The binding of ¹²⁵I-labelled bovine somatotropin to receptors from hypophysectomized female rat liver microsomes, prepared as indicated by Bonifacino *et al.* (1981), was measured by incubating, for 48 h at 4°C, about 1 ng of labelled hormone (100000 c.p.m.) in the presence of increasing amounts of unlabelled hormone or its methoxyglycylated derivatives with 1.0–1.4 mg of microsomal protein in a total volume of 0.5 ml. Incubation medium consisted of 25mM-Tris/HCl, pH 7.4,

10mM-CaCl₂ and 0.08% (w/v) bovine serum albumin. The reaction was stopped by dilution with 4ml of cold incubation medium followed by centrifugation at 1400g for 25min at 4°C. Supernatants were discarded and the pellets were counted for radioactivity. The specific binding varied from 5.5 to 9.5% and non-specific binding, determined in the presence of an excess (1 µg) of unlabelled hormone, varied from 7.1 to 10.5%.

Serum clearance of native bovine somatotropin and derivative 5 injected into rats

Long-Evans female rats (140–160g) were injected via one jugular vein, under sodium thiopental anaesthesia, with 20 µg of bovine somatotropin or derivative 5 dissolved in 0.2ml of 50mM-potassium phosphate buffer, pH 7.4. Samples of blood (0.25ml) were extracted from the other jugular vein after variable periods of time following injection. Blood was allowed to clot and the sera were stored at –20°C until analysis.

Concentration of immunoreactive native hormone or derivative 5 in serum samples (50 µl) were determined by radioimmunoassay employing a rabbit antiserum to bovine somatotropin (Poskus *et al.*, 1976) and ¹²⁵I-labelled bovine somatotropin as the tracer according to the procedure described by Peña *et al.* (1980) with the following modifica-

tions. (a) The reaction mixture was incubated for 2 days at 4°C. (b) The tubes of the standard curves of bovine somatotropin and derivative 5 were incubated in the presence of 50 µl of normal rat serum. Therefore, the addition of serum previous to the separation step was avoided.

The concentration values were interpolated in a logit–log plot (Fig. 1) (Feldman & Rodbard, 1971). The results were expressed as the amount of hormone relative to that present at the time of the injection (C/C_0).

Results

Specific binding in vivo to rat liver and growth-promoting activity of bovine somatotropin and two selected methoxyglycylated derivatives

The ability of native bovine somatotropin and two methoxyglycylated derivatives to compete with ¹²⁵I-labelled bovine somatotropin for specific binding to liver somatogenic receptors *in vivo* is shown in Table 1. The average liver-to-serum ratio obtained for derivative 1, which has about 3 methoxyglycylated residues incorporated/molecule, was similar to that measured for the native hormone. In contrast, derivative 5, with approx. 8 residues modified, caused only minimal inhibition of the uptake. On the other hand, when these derivatives were tested in the body-weight-increase bioassay, it was demonstrated that derivative 1 kept full growth-promoting activity whereas derivative 5 was almost inactive (Table 2). Thus, the relative biological potencies observed for derivatives 1 and 5 in this bioassay agree with those determined by binding assays *in vivo*.

Specific binding of bovine somatotropin and its methoxyglycylated derivatives to isolated cells or microsomal preparations from hypophysectomized rat liver

Fig. 2 shows the competition curves obtained when the specific binding of ¹²⁵I-labelled bovine somatotropin to hypophysectomized rat hepatocytes is inhibited by the unlabelled hormone and different methoxyglycylated derivatives. All the derivatives had displacing potencies equal or slightly higher than that of the native hormone. Similar results were obtained when the binding assay was performed with microsomal preparations from hypophysectomized rat liver (Fig. 3).

Serum clearance of native bovine somatotropin and derivative 5

The rate of serum disappearance of derivative 5 as compared with that of native bovine somatotropin was studied. The kinetics shown in Fig. 4 revealed no significant differences between both species.

Fig. 1. Immunoreactivity of bovine somatotropin and derivative 5

Standard curves for bovine somatotropin (○) and derivative 5 (▲) were performed as indicated in the Materials and methods section. Results are expressed as logit B/B_0 [$= \ln(B/B_0)/(1 - B/B_0)$] versus log dose for both competitors, where B is the specific binding at each concentration of unlabelled antigen added and B_0 is the specific binding in the absence of additions. Three independent experiments were performed and each point of the curves shown is the mean of three determinations. S.D. was always less than 3% of each value.

Table 1. *Competition between ^{125}I -labelled bovine somatotropin and either the native hormone or methoxyglycylated derivatives for binding in vivo to rat liver*

The specific uptake of the native hormone or its derivatives by the rat liver *in vivo* was determined by the method of Turyn & Dellacha (1978) modified as indicated in the Materials and methods section. Liver-to-serum ratios are means $\pm$ S.D. for the numbers of animals shown in parentheses. Binding displacement activity estimates *in vivo* are expressed as the relative potency referred to that of native hormone, according to the statistical procedure for parallel line assays (Finney, 1964). The 95% confidence limits are shown in parentheses. The ratio obtained with an excess of native hormone (48 μg) indicates the non-specific binding.

Unlabelled competitor injected together with ^{125}I -labelled bovine somatotropin	Number of methoxyglycyl residues incorporated per molecule of competitor	Dose (μg)	Liver/serum ratio	Binding displacement activity <i>in vivo</i>
—	—	0	3.77 ± 0.71 (6)	
		2	2.65 ± 0.48 (6)	
Native bovine somatotropin	0	20	1.69 ± 0.44 (5)	1.00
		48	1.22 ± 0.29 (3)	
Derivative 1	2.8	2	2.37 ± 0.21 (5)	1.42
		20	1.75 ± 0.37 (4)	(0.45–5.52)
Derivative 5	7.6	2	3.77 ± 0.43 (6)	0.12
		20	2.56 ± 0.25 (5)	(0.03–0.29)

Table 2. *Body-weight-increase bioassay of native bovine somatotropin and two methoxyglycylated derivatives*
The body-weight-increase bioassay was performed by the method of Marx *et al.* (1942), modified as indicated in the Materials and methods section. A balanced factorial assay design employing six animals per dose was used. Weight increase values are means $\pm$ S.D. Growth-promoting activity estimates are expressed as the relative potency referred to the native hormone according to the statistical procedure for parallel line assays (Finney, 1964). The 95% confidence limits are given between parentheses.

Sample assayed	Number of methoxyglycyl residues incorporated per molecule	Total injected dose (μg)	Body-weight increase (g)	Growth-promoting activity
Native bovine somatotropin	0	29	7.1 ± 1.0	
		228	15.9 ± 2.0	1.00
Derivative 1	2.8	34	7.5 ± 1.7	
		273	16.2 ± 1.6	0.91 (0.61–1.37)
Derivative 5	7.6	599	10.2 ± 2.5	
		4793	16.5 ± 0.9	0.08 (0.05–0.14)

Discussion

The present results strongly suggest the existence of a functional nexus between binding *in vivo* of bovine somatotropin and its growth-promoting activity (Tables 1 and 2), whereas they demonstrate a dissociation between assays *in vivo* and binding *in vitro* to isolated cells (Fig. 2) or microsomal preparations (Fig. 3) from rat liver. An apparent correlation between assays *in vivo* and *in vitro* for human somatotropin was already shown by Tsushima & Friesen (1973), who demonstrated that different human somatotropin preparations displaced the tracer bound *in vitro* to liver somatogenic receptors proportionately to their biological potencies, as estimated by the tibia test assay. Similarly, the behaviour of somatotropin preparations treated with alkali or performic acid also showed a parallelism between binding *in vitro* and growth-promoting activities (Tsushima *et al.*,

1973). However, both results may be criticized on the ground that the relative potency of the samples is probably due to the presence of variable amounts of remaining native hormone. In contrast, the methoxyglycylated derivatives of bovine somatotropin employed by us are devoid of native hormone, as judged by the absence of the corresponding band in isoelectrofocusing analysis (J. M. Delfino, H. N. Fernández & J. A. Santomé, unpublished work), and maintain their native conformation (Delfino *et al.*, 1983).

A markedly reduced activity *in vivo* of a given derivative might be readily explained by its increased rate of disappearance from circulation with respect to the active species. An example of such situation has been described for the asialo human chorionic gonadotropin, which showed a great reduction of biological activity evaluated by both binding *in vivo* (Tsuruhara *et al.*, 1972b) as well as conventional bioassays (Van Hall *et al.*,

Fig. 2. Inhibition of ¹²⁵I-labelled bovine somatotropin binding to hypophysectomized female rat hepatocytes by native bovine somatotropin and its methoxyglycylated derivatives

The binding assays were performed under the conditions described in the Materials and methods section. Results are expressed as the ratio of the specific binding at each concentration of unlabelled hormone added (B) to the specific binding in the absence of additions (B_0); ○, native bovine somatotropin; ●, derivative 0.5; □, derivative 1; △, derivative 3; ▲, derivative 5. Three independent experiments were performed and each point of the curves shown is the mean of three determinations. S.D. was always less than 3% of each value.

1971a) while keeping full binding ability *in vitro* (Dufau *et al.*, 1971; Tsuruhara *et al.*, 1972b). However, this differential behaviour could be due to a faster plasma decay of the asialo hormone (Van Hall *et al.*, 1971b; Tsuruhara *et al.*, 1972a). This was not so in our case, since the serum clearance of an almost inactive derivative (namely derivative 5) was similar to that of the native bovine somatotropin (Fig. 4).

Different hypotheses may be proposed to explain the discrepancy between results *in vivo* and *in vitro*. It is possible that the receptors that become available *in vitro* trigger other effects not directly associated with the growth-promoting activity of the hormone, as suggested by Hughes (1979), Cadman & Wallis (1981) and Fix *et al.* (1981), using membrane preparations from rabbit liver. Alternatively, the facts reported in the present paper could be explained by alterations in the receptors during their isolation, leading to the maintenance of the binding unit but the lack of other units essential for the next step in the biological action of the hormone. The binding unit integrated in the supramolecular structure of the receptor *in vivo* would only recognize the native hormone, and active derivatives, which would be

Fig. 3. Inhibition of ¹²⁵I-labelled bovine somatotropin binding to hypophysectomized female rat liver microsomes by native bovine somatotropin and its methoxyglycylated derivatives

The binding assays were performed under the conditions described in the Materials and methods section. Results are expressed as indicated in Fig. 2; ○, native bovine somatotropin; ●, derivative 0.5; □, derivative 1; △, derivative 3; ▲, derivative 5. Three independent experiments were performed and each point of the curves shown is the mean of three determinations. S.D. was always less than 3% of each value.

Fig. 4. Serum disappearance of native bovine somatotropin or derivative 5 following their intravenous injection into rats. Bovine somatotropin (20 µg) (circles) or derivative 5 (triangles) dissolved in 0.2 ml of 50 mM-potassium phosphate buffer, pH 7.4, were injected via one jugular vein into female rats. Blood samples were taken at different times from the opposite vein and the concentration of immunoreactive hormone in serum was determined by radioimmunoassay as indicated in the Materials and methods section. Each curve was obtained in a different experimental animal. Results are expressed as the ratio of the concentration of hormone at each time (C) to that extrapolated at the time of the injection (C_0). Measurement of C was done in triplicate and the mean value obtained was used to calculate C/C_0 .

immediately processed further. In this connection it can be mentioned that the complexes formed *in vivo* between ^{125}I -labelled human somatotropin and liver somatogenic receptors have a greater Stokes' radius than the similar complexes obtained *in vitro* (Bonifacino *et al.*, 1983). This fact has been interpreted as a possible processing occurring *in vivo* after the specific binding step.

Our results indicate that the binding of bovine somatotropin to somatogenic receptors *in vitro*, unlike its counterpart *in vivo*, is not unequivocally linked to its growth-promoting activity.

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