# Inhibin binding sites in bovine pituitary membranes

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*Membranes derived from bovine pituitary glands free of the neural lobe were used to investigate the presence of binding sites for inhibin, a glycoprotein produced by the ovarian granulosa cells capable of selectively suppressing FSH secretion from the pituitary gland. Optimal concentration of membranes (400 μg prot) and $^{125}$I-bovine inhibin (2 nM) were incubated in a medium containing 50 mM Tris-HCl pH 7.4, 0.01 M $MgCl_2$ and BSA 0.01% in a final assay volume of 200 μl at 37° C for different time intervals. Non-specific binding was estimated using unlabelled inhibin in excess.*

*The time course of specific $^{125}$I-bovine inhibin (2 nM) binding to bovine pituitary membranes is slow with 50% binding at approximately 20 min of incubation and reaching equilibrium at 90 min of incubation. The kinetic analysis shows an apparent pseudo first order association rate constant ($K_{ob}$) equivalent to 4 x $10^{-2}$ $min^{-1}$. Following equilibrium with the tracer, a large excess of unlabelled inhibin (1.27 μM) was able to displace 84% of the specific binding within 120 min of incubation and 50% of the binding at approximately 40 min The analysis under displacing conditions showed an apparent dissociation rate constant ($K_2$) equals to 1.5 x $10^{-2}$ $min^{-1}$ and an apparent association rate constant ($K_1$) equals to 1.3 x $10^9$ M $min^{-1}$. Thus, the estimation of the apparent kinetic equilibrium dissociation constant ($K_d = K_2/K_1$) of the binding of inhibin to bovine pituitary membranes was 1.2 nM.*

*These results show for the first time the existence of bovine inhibin specific binding sites in bovine pituitary, and also that such a binding can take place in the absence of either gonadal and/or hypothalamic influences. They also contribute to the better understanding of the role of non-steroidal hormones such as inhibin, in the regulation of gonadotrophin secretion.*

***Key terms:*** *binding sites, bovine inhibin, bovine pituitary membranes, dissociation constant.*

## INTRODUCTION

It has been demonstrated that the hormone inhibin, a glycoprotein formed by two subunits and produced by the ovarian granulosa cells, is capable of selectively suppressing follicle-stimulating hormone (FSH) secretion in several species such as human (McLachlan *et al*, 1987), bovine (Robertson *et al*, 1985), porcine (Miyamoto *et al*, 1985) and rat (Fujii *et al*, 1983). Following the isolation of inhibin from different fluids and species, the understanding of its physiological role in the regulation of the gonadotrophin secretion specially FSH, reached interesting dimensions and questions such as the site of production of the hormone, target organs, *etc*, started to be elucidated.

At present, the mechanism by which inhibin is capable of exerting its suppressive effect on FSH secretion is not clearly understood and some early evidence showed that ram inhibin administered to the third ventricle decreased gonadotrophin-releasing

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hormone (GnRH) secretion from the hypothalamus and consequently affected FSH production from the pituitary gland (Lumpkins *et al*, 1981). Similarly, Condon *et al* (1983) showed that porcine follicular fluid administered to the dorsal anterior hypothalamic area decreased FSH plasma levels in rats. Based on the aforementioned evidence, it was quite tempting to suggest a possible site of action of inhibin at the hypothalamic level. In contrast, de Greef (1983, 1987) reported that bovine follicular fluid did not affect GnRH release *in vivo* in the rat despite of a pituitary FSH suppression.

Recent evidence shows that inhibin is capable of suppressing GnRH stimulation of its own binding sites at the gonadotroph (Wang *et al*, 1989). In contrast, Laws *et al* (1990) show that highly purified bovine or porcine inhibin increase GnRH binding to ovine dispersed gonadotrophs as a consequence of an increase in the number of GnRH receptor. Despite of the apparent controversy about the sort of relationship between inhibin and GnRH, it has been suggested that the mechanism of action of inhibin on gonadotrophin secretion is exerted at least in part, by modulating GnRH mechanism of action at the pituitary level.

Since the physiological role of inhibin can not be fully understood through its suggested relationships with GnRH at the pituitary level, we have attempted to investigate the presence of binding sites for inhibin in membranes from bovine pituitary glands in the absence of any possible relationship with some of the most important hormones involved in the regulation of gonadotrophin secretion.

## MATERIALS AND METHODS

Cows pituitaries free from their neural part were collected from the local abattoir and homogenized in $NaHCO_3$ 1 mM containing EGTA 2 mM, $MgCl_2$ 5 mM and PMSF 0.2 mM. Following filtration of the homogenate with a gauze it was centrifuged at 2,500 g for 5 min at 4° C. The supernatant was discarded and the pellet submitted to a saccharose density gradient and the membranes collected in a band following centrifugation at 37,000 g for 3 h at 4° C. Membranes were resuspended in Tris HCl 50 mM and centrifuged at 37,000 g x 45 min at 4° C. The pellet obtained was resuspended in buffer Tris HCl 50 mM, pH 7.4, containing saccharose 0.25 M, PMSF 0.2 mM, Pepstatin-A 7.3 mM and Leupeptin 52.5 mM, and sonicated for 30 min. The protein content was estimated by the method of Bradford and the membranes aliquoted for further use.

### *Inhibin preparation*

Inhibin from bovine follicular fluid was purified after successive chromatographic steps which included gel filtration in Sephacryl S-200 HR in the presence of ammonium acetate 0.05 M pH 7.0 at 4° C and in the presence of glacial acetic acid 25% V/V at 4° C. Fractions containing inhibin-like activity (ILA) from the previous steps of purification were submitted to ion-exchange chromatography in DEAE-Sepharose CL 6B (Sigma) at 4° C in the presence of 8 M urea and then to RP-HPLC (RP-HPLC chrospher 100 RP-18/15um, Merck) eluted with a multilineal gradient of acetonitrile 10-60% (Table I).

The estimation of ILA (*i.e.*, capacity to suppress FSH production *in vitro*) throughout the purification scheme (Fig 1) was carried out using a rat pituitary cell dispersion bioassay. The levels of FSH were estimated by radioimmunoassay (NIAMMD, Bethesda, MD, USA). Rat FSH-I-7 and NIH - rat FSH-RP2 were used as tracer and standard, respectively.

### *Iodination procedure*

Five micrograms of bovine inhibin in 50 µl of PBS 0.04 M, pH 7.4, were incubated with 0.5 mCi $Na^{125}I$ (10 µl) (Chilean Nuclear Energy Commission) in the presence of 10 µl Chloramine T 0.25% for 60 s. The reaction was stopped by the addition of 250 µl metabisulfite 0.1% and 200 µl of elution buffer (PBS 0.04 M, pH 7.4), containing BSA 0.5% and azide 0.1%. The free iodine was separated from bound by absorption on a Sephadex G-25 (Sigma Chemicals Co)

TABLE I

Summarising results of the different steps of the purification of inhibin, including biological potencies ($ED_{50}$), purification factors and percentage of recovery

| Purification steps | Proteins (µg) | ED ng/ml | ED ng/well | Purification factor | % of Recovery |
|---|---|---|---|---|---|
| Bovine Follicular Fluid (bFF) | $4.16 \times 10^6$ | $2.37 \times 10^3$ | $7.00 \times 10^2$ | 1.0 | 100.00 |
| Sephacryl S-200 HR: | | | | | |
| Native Conditions | $3.50 \times 10^4$ | $4.20 \times 10^2$ | $1.18 \times 10^2$ | 5.9 | 4.99 |
| Dissociating Conditions | $8.25 \times 10^3$ | $1.56 \times 10^2$ | $4.60 \times 10^1$ | 15.2 | 3.01 |
| DEAE-Sepharose CL-6B | $1.20 \times 10^3$ | $3.05 \times 10^1$ | $9.00 \times 10^0$ | 77.8 | 2.24 |
| RP-HPLC | $1.19 \times 10^2$ | $5.09 \times 10^0$ | $1.50 \times 10^0$ | 466.7 | 1.33 |

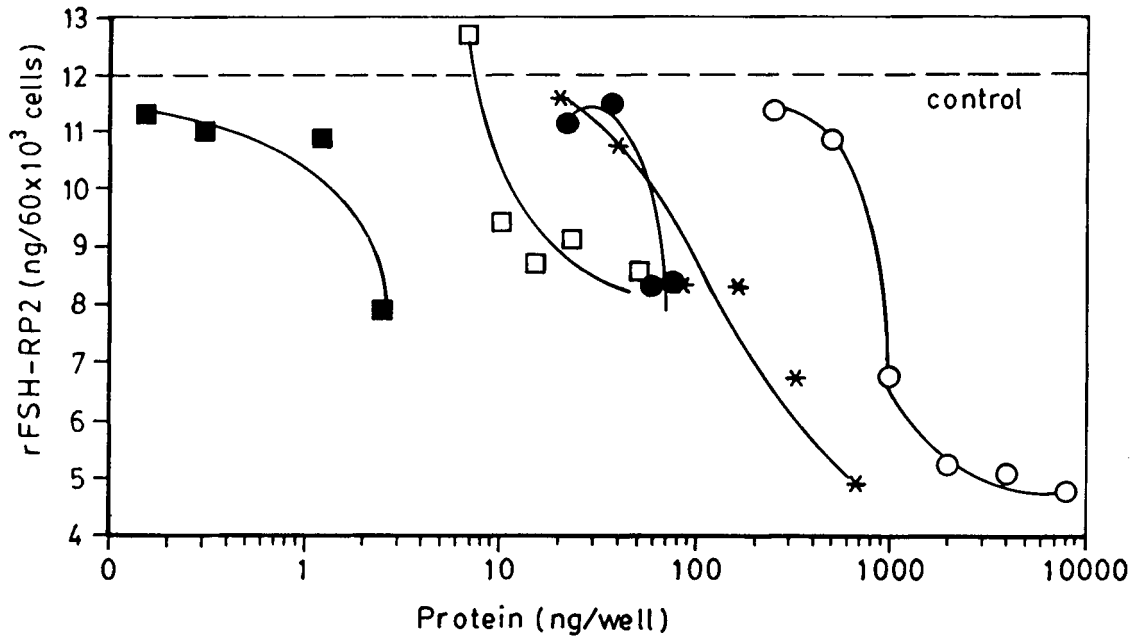


Fig 1. Bioassay dose-response curves for crude bovine follicular fluid (bFF) and for various inhibin-enriched fractions obtained during the purification. Inhibin-like activity is associated with the suppression of basal production of FSH by cultured rat pituitary cells. ○, crude bFF (starting material). *, inhibin-enriched fraction after first chromatographic step. •, inhibin-enriched fraction after second chromatographic step. □, inhibin-enriched fraction after third chromatographic step. ■ final preparation of inhibin after RP-HPLC.

column and the iodinated hormone eluted from the column and stored at 4° C until used.

### *Binding assay*

Optimal concentration of membranes (400 µg) and $^{125}$I-inhibin (2 nM) were incubated in a medium containing Tris-HCl 50 mM, pH 7.4, $MgCl_2$ 0.01 M and BSA 0.01% in a final assay volume of 200 µl at 37° C for different time intervals. Non specific binding was estimated using unlabelled inhibin in excess (633 times the concentration of the $^{125}$I-inhibin used).

The reaction was terminated by the addition of 1 ml of ice-cold Tris buffer, pH 7.4 and immediately filtered under reduced pressure over borosilicate GC-50 filters (Microfiltration system) previously soaked with BSA (0.1%). Filters were washed with 10 ml of ice-cold Tris buffer, pH 7.4 over a

period of 10 seconds and the radioactivity retained in the filters counted in a Gamma Counter (Auto Logic Abbot Laboratories).

### *Data analysis*

The specific binding data for the association and dissociation studies were analyzed with the computer programme LIGAND. Each point was assayed in triplicate and the standard deviation was omitted for simplicity.

## RESULTS AND DISCUSSION

The time course of specific $^{125}$I-inhibin (2 nM) binding to bovine pituitary membranes is shown in Figure 2. The binding is slow (50% binding reached at approximately 20 min of incubation) and specific, reaching equilibrium at 90 min of incubation and remaining as such for further 30 min.

The kinetic analysis of the binding shows an apparent pseudo first order association rate constant ($K_{ob}$) equivalent to 4 x $10^{-2}$ $min^{-1}$. Following equilibrium with 2 nM of $^{125}$I-inhibin a large excess of unlabelled inhibin (1.27 µM) was able to displace 84% of the specific binding within 120 min of incubation and 50% of the binding at approximately 40 min (Fig 3).

The analysis of the specific binding under displacing conditions showed an apparent dissociation rate constant ($K_2$) equals to 1.5 x $10^{-2}$ $min^{-1}$ and with this information it was possible to estimate the apparent association rate constant ($K_1$) as 1.3 x $10^9$ M $min^{-1}$. Thus, computer assisted analysis made possible to determine the apparent kinetic equilibrium dissociation constant ($K_d$) of the binding of inhibin to bovine pituitary membranes as 1.2 nM. Such a value is within the range described for other peptidic hormones involved in the regulation of the reproductive function in mammals.

The total and non-specific binding of $^{125}$I-inhibin to bovine pituitary membranes was determined using a range of ligand concentrations from 0.5-2 nM as shown in Figure 4. The highest specific binding was observed at a ligand concentration of 2 nM.

These studies describe, for the first time, inhibin binding sites in bovine pituitary membranes and provide support to the hypothesis that the mechanism of action of inhibin on FSH secretion is likely to be, at least in part, by a direct action on the pituitary gland and independent from

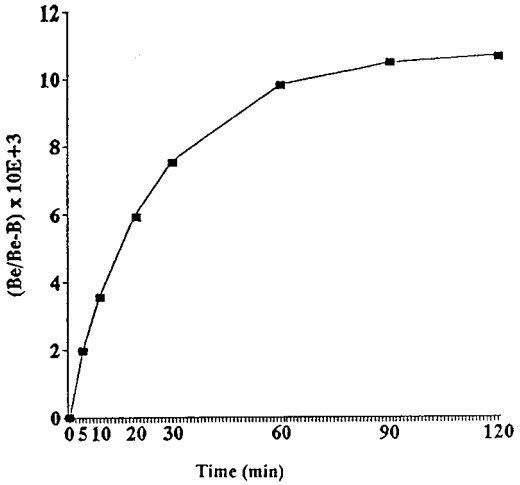


**Fig 2.** Time course of association of $^{125}$I-inhibin (2 nM) binding to bovine pituitary membranes at 37° C. 50% binding was reached at approximately 20 min and equilibrium occurred at 90 min. Apparent pseudo first order association rate constant ($K_{ob}$) = 4 x $10^{-2}$ $min^{-1}$. **Be**, amount of ligand bound at equilibrium; **B**, amount of inhibin bound at indicated time.

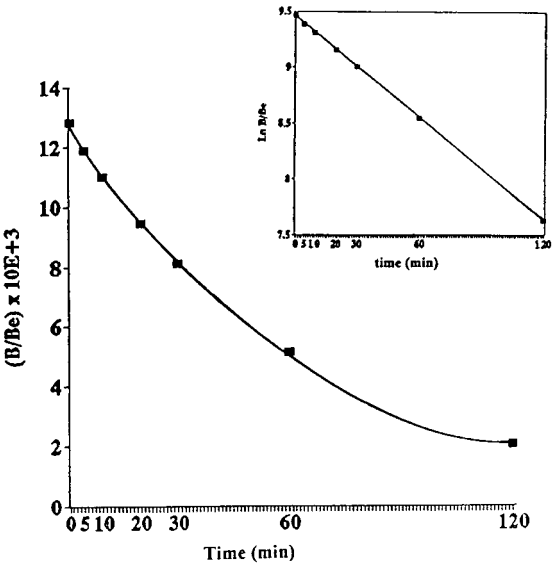


**Fig 3.** Time course of dissociation of $^{125}$I-inhibin (2 nM) binding to bovine pituitary membranes at 37° C in the presence of a large excess of unlabelled inhibin (1.27 µM). Only 84% of the specific binding was displaced by the cold tracer in 120 min of incubation. Apparent dissociation rate constant ($K_2$) = 1.5 x $10^{-2}$ $min^{-1}$; estimated apparent association rate constant ($K_1$) = 1.3 x $10^9$ M $min^{-1}$. Apparent kinetic equilibrium dissociation constant $K_d$ = 1.2 nM.

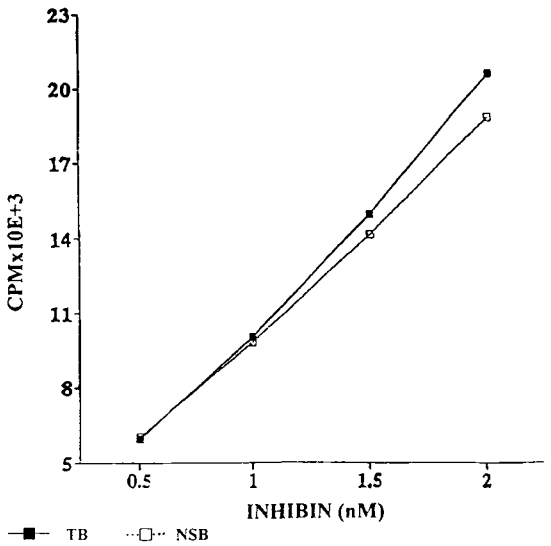


**Fig 4.** Total and non-specific binding of $^{125}$I-inhibin to bovine pituitary membranes over a range of ligand concentrations.

hypothalamic and gonadal influences, as early suggested by Castillo and Knight (1991). These authors reported that inhibin was capable of suppressing FSH basal secretion and cellular content at a greater extent than for LH in gonadotrophs derived from rats chronically exposed to a GnRH analogue *in vivo*. In addition, the reported evidence that the combinations of human inhibin with either a GnRH antagonist or oestradiol are more effective in suppressing FSH plasma levels in the rat (Rivier and Vale, 1991) strengthen even more the direct action of inhibin on the pituitary gland.

Early evidence reported by Chefeitz *et al* (1988) showed that both inhibin and activin could effectively compete for TGF-b1 binding to a novel type of cell surface TGF-b binding protein in $GH_3$ rat pituitary tumor cells that show undetectable levels of the well recognized TGF types of receptors. However, both inhibin and activin lacked of the ability to bind to TGF-b receptor types I, II and III in mink lung epithelial cells and human skin fibroblast, suggesting that the presence of the already known TGF-b receptors could eventually mask the existence of receptors capable of binding either peptides or inhibin alone.

Thus, it has been suggested that since inhibin and activin have a great degree of similarity with a complex group of biologically active polypeptides, and due to the functional similarities between these gonadal peptides and TGF-b, their mechanisms of actions might partially overlap, and the possibility that TGFs-b and both inhibin and activin might share a common cell surface receptor at the pituitary level is also suggested.

In conclusion, the evidence reported here contributes to the better understanding of the way by which non steroidal gonadal hormones –such as inhibin– regulate gonadotrophin secretion, and points out the fact that the physiological role of inhibin on FSH secretion can not be understood only as a result of an interaction with either GnRH, oestradiol or other biologically related peptides at the pituitary level, but also as a direct interaction between inhibin and its specific binding sites at the pituitary gland. Moreover, it is likely that cell surface components exist that can specifically recognize multiple members of the TGF-b family of bioactive polypeptides such as inhibin. Clearly, further studies are required to elucidate whether the other hormones involved in regulating the reproductive function do have a modulatory role on the binding of inhibin to the pituitary gland, either by sharing a common cell surface receptor, interacting with a separate but functionally homologous receptor, or by modifying the affinity or the specificity of such interaction.

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