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INHIBIN

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INTRODUCTION

Mottram & Cramer (1) laid the foundations of the inhibin (IB) concept six decades ago when they observed severe morphological changes in the anterior pituitary gland of a male rat whose germinal elements in the testes had been selectively damaged by radiation. This observation led to the suggestion that the pituitary gland is in some way regulated by a testicular factor other than steroid hormones. This hypothesis was further supported by experiments involving parabiotic rats. These parabiotic pairs consisted of a normal and a castrated rat. The hyperactivity of the pituitary gland in the castrated partner was prevented by treatment with water-soluble testicular extracts (2). Subsequently, McCullagh (3) coined the name "inhibin" for this hypothetical testicular factor. The inhibin concept remained an unresolved riddle in endocrine physiology for a long time (4–7). During this time the two gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] were successfully isolated and characterized (8). Insensitive bioassays of FSH and LH were replaced by more specific and sensitive radioimmunoassays. Using these assays, more definite quantitative information about the hypothalamo-pituitary-gonadal axis was derived. Several lines of evidence from clinical and experimental data suggested the divergent secretions of FSH and LH. All of these studies supported unequivocally the concept of inhibin (9–11). In addition, inhibin (which was originally described in the male) has now been found in the female. Inhibinlike activity has been detected in a variety of gonadal-related fluids and extracts (12–21). Inhibin may be defined as a proteinaceous material of gonadal origin that is involved in the regulation of pituitary FSH secretion in both sexes.

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BIOASSAY

Detection and quantitation methods are essential in isolation and purification of biologically important substances. These assay procedures should meet the criteria of simplicity, specificity, sensitivity, precision, and reproducibility. Unfortunately, no such assay methods are available for measuring inhibin activity; many only detect the activity. A standard reference preparation of inhibin is not available to compare the activity and potency ratios from different laboratories. Although a detailed discussion of these assays is beyond the scope of this article, we attempt to point out some common problems in these assays.

In Vivo Methods

Several in vivo methods for assaying inhibin activity have been developed and critically evaluated (21–30). Most of these methods rely on the suppression of circulating levels of FSH after the injection of inhibin-enriched preparations. In many instances the endogenous circulating levels of FSH must be elevated before a significant FSH-suppressing effect is observed.

We have used an in vivo rat system for assay of inhibin activity. Briefly, immature male rats (34-days old) are injected subcutaneously with the appropriate amounts of test sample in 0.2 ml of saline at two different time intervals [8 hours (hr) and 11 hr]. The synthetic LH-releasing hormone (LHRH) at a dose of 5 μg/rat is administered subcutaneously at 14 hr, and at 15 hr the animals are sacrificed under light ether anesthesia and blood samples are withdrawn. The serum content of FSH and LH are analyzed by specific radioimmunoassays (RIA).

Alternatively, male rats (34-days old) are bilaterally castrated under light ether anesthesia, and the test samples are immediately injected subcutaneously in appropriate amounts in 0.2 ml of saline. The second dose of the same sample is administered on day 35, and the blood samples are collected after 6 hr. The serum levels of FSH and LH are analyzed by RIA. A twofold increase of FSH and LH levels is usually observed in these immature castrated rats, as compared to those in the control animals of the same age group. The IB-containing fraction, when tested at different concentrations, suppresses FSH in a dose-dependent manner. The maximum FSH suppression in this model system ranged from 45–75%. Although the fraction specifically suppresses FSH in this model system, occasionally a small degree (20–30%) of LH suppression occurs.

The in vivo methods are usually not very sensitive, and FSH-suppressing activity varies from assay to assay. Therefore, this type of assay is not practical for routine use in screening the large number of fractions obtained during isolation procedure. Hence, a simple in vitro assay to measure inhibin activity must be developed.

In Vitro Methods

The rat anterior pituitary culture assay has been used to assay inhibin activity (31, 32). This assay procedure is reasonably sensitive and can be used routinely to screen the large number of fractions obtained during the isolation procedure. Also, it has a good index of precision and gives acceptable dose-response curves. However, this method is time consuming and requires at least 72 hr to detect significant FSH suppression after the addition of test material. Under the conditions of LHRH stimulation, it requires additional 5–8 hr to block the release of FSH. In addition, both FSH and LH levels are suppressed, and the system then becomes less specific (33–35). Hence, it is more appropriate to depend on the basal suppression of FSH secretion in the in vitro culture system.

Pituitary halves obtained from adult male rats, when incubated in vitro in an appropriate medium, respond well to synthetic LHRH and release significant amounts of FSH and LH. The pituitary tissue retains its morphological integrity under these conditions. Addition of inhibin-containing preparations markedly reduces FSH, but not LH, in this system (5). Based on this method, an in vitro mouse pituitary incubation assay has been developed (36). The immature mouse pituitary responds well to synthetic LHRH, its agonists, and its antagonists (37, 38). The amounts of FSH and LH released by the mouse pituitary into the medium are analyzed by specific radioreceptor assays, which measure the biologically active hormones. Inhibin-containing preparations from human seminal plasma, bull seminal plasma, ovine testicular extract, bovine follicular fluid, and human follicular fluid show a selective suppression of FSH secretion without a significant effect on LH secretion in this test system. A slightly modified mouse pituitary incubation system has been applied to suppress basal levels of FSH secretion using inhibin from porcine follicular fluid. The mouse pituitary gives maximal response to LHRH when tested in a cumulative experiment; similar response may be difficult to produce in a cell culture system (39–41).

Since the in vitro mouse pituitary system has been used in our laboratory to isolate α-inhibins from human seminal fluid, we describe this assay procedure here. The pituitary tissue obtained from a 20–22-day-old mouse is incubated in the Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin (BSA) at 37°C in an atmosphere of 95% O_2 and 5% CO_2. Appropriate amounts of test samples are added in 0.5 ml of the above medium, and the tissue is incubated for 1 hr, after which synthetic LHRH (3 μg/pituitary) is added and the incubation is continued for 3 hr. Thus, the total incubation period is 4 hr. In a second set of incubation assays, the mouse pituitary is challenged at different time intervals with appropriate amounts of the test material and LHRH. The medium is separated from the pituitary tissue, and levels of the mouse pituitary gonadotropins are estimated by specific

radioreceptor assays. FSH radioreceptor assays are performed with bull testes preparation and ovine FSH as the ligand, whereas the LH receptor assays use rat testes homogenate and ovine LH as the ligand.

INHIBINS FROM SEMINAL FLUID

The concept of inhibin regulation of pituitary gonadotropin secretion was originally described in the male (1–3). Several attempts have been made to isolate inhibin from testes-related fluids and extracts from various species (15), as well as from human seminal plasma (42–44).

Human Inhibinlike Peptide

Ramasharma and coworkers (45, 45a) isolated and determined the structure of a peptide with inhibinlike activity from human seminal plasma. It was named inhibinlike peptide (ILP). Briefly, ILP was obtained from ethanol precipitates of sperm-free human ejaculates by a combination of procedures (44) including ion-exchange chromatography on sulphopropyl-Sephadex C-50 and DEAE-Sephadex A-25, gel filtration on Sephadex G-50, and HPLC on Waters C_{18} micro-Bondapak column. The peptide is shown to behave as a single substance in HPLC as well as in polyacrylamide gel electrophoresis at pH 4.5.

The amino acid analyses of ILP give the following composition in molar ratio: Lys_5, His_7, Arg_3, Asp_4, Ser_{1-2}, Glu_{2-3}, Gly_6, Ala_1, Val_2, Ile_1, Phe_1 (45). The primary structure of the peptide has been determined (45, 45a), as shown in Figure 1. The peptide is very basic and consists of 7 histidine, 5 lysine and 3 arginine residues, with one residue each of aspartic and glutamic acids.

Incubation of whole mouse pituitaries with ILP inhibited LHRH-induced FSH release. The inhibitory action of ILP on FSH release was dose-dependent, and it had no effect on LH release (45), as shown in Figure 2, part *A*. When injected into castrated male rats (34-days old), ILP inhibited the rise in circulating FSH levels, whereas no effect on LH levels was observed (Figure 2, part *B*). ILP has been synthesized by the solid-phase method, and the synthetic product was shown to be homogeneous in HPLC and paper electrophoresis (46). In both the in vitro mouse pituitary assay and the in vivo LHRH-induced FSH release in immature male rats, the FSH-suppressing activities of the synthetic and natural ILP are comparable (46).

Liu et al (47) employed an impure synthetic ILP to demonstrate that "synthetic 31-amino acid inhibin-like peptide lacks inhibin activity." As stated by these authors, "only 15–30% of the material (synthetic peptide) had the complete correct sequence. The remaining peptides had one or more amino acids missing, mainly the histidine residues at positions 1, 9 or 17." Any conclusions drawn from experiments using such crude peptide mixtures are meaningless.

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                    5                   10
H-His-Asn-Lys-Gln-Glu-Gly-Arg-Asp-His-Asp-

                   15                   20
  Lys-Ser-Lys-Gly-His-Phe-His-Arg-Val-Val-

                   25                   31
  Ile-His-His-Lys-Gly-Gly-Lys-Ala-His-Arg-Gly-OH
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Figure 1 Amino acid sequence of ILP.

Antisera raised in rabbits to synthetic ILP afford a highly specific and sensitive RIA for the peptide (48) using a synthetic [Tyr^4]-ILP analog as primary radioiodinated ligand. Synthetic ILP completely displaces antiserum binding of [^{125}I-Tyr^4]-ILP with half maximal displacement at 36-fmoles ILP/tube. ILP, [Tyr^4]-ILP and ILP-(9–31) had essentially equal potency, while ILP-(1–25), ILP-(1–23) had reduced activity. Apparently, the antiserum recognizes the COOH-terminal segment of ILP.

Human α-Inhibin-92

Using the RIA for ILP, two new peptides structurally related to ILP have been isolated and characterized from human seminal plasma (49). One consists of 52 amino acids and the other of 92 amino acids. They are designated α-IB-52 and α-IB-92, respectively. Sequence analyses show that the NH_2-terminal 31 amino acids of α-IB-52 are identical to those of ILP, and the COOH-terminal

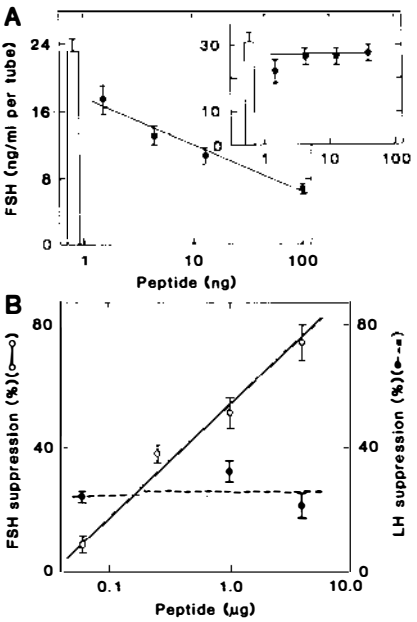

Figure 2 (*A*) Action of ILP on gonadotropin release by mouse pituitary (open bars = control values). (*B*) Effect of ILP on FSH and LH release in castrated male rats.

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                    5                   10                  15
H-Thr-Tyr-His-Val-Asp-Ala-Asn-Asp-His-Asp-Gln-Ser-Arg-Lys-Ser-

                   20                  25                  30
Gln-Gln-Tyr-Asp-Leu-Asn-Ala-Leu-His-Lys-Thr-Thr-Lys-Ser-Gln-

                   35                  40                  45
Arg-His-Leu-Gly-Gly-Ser-Gln-Gln-Leu-Leu-His-Asn-Lys-Gln-Glu-

                   50                  55                  60
Gly-Arg-Asp-His-Asp-Lys-Ser-Lys-Gly-His-Phe-His-Arg-Val-Val-

                   65                  70                  75
Ile-His-His-Lys-Gly-Gly-Lys-Ala-His-Arg-Gly-Thr-Gln-Asn-Pro-

                   80                  85                  90
Ser-Gln-Asp-Gln-Gly-Asn-Ser-Pro-Ser-Gly-Lys-Gly-Ile-Ser-Ser-

    92
Gln-Tyr-OH.
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Figure 3 Amino acid sequence of α-IB-92. Residues 41–92 constitute α-IB-52, and residues 41–71 constitute ILP.

52 amino acids of α-IB-92 are identical to the structure of α-IB-52 (see Figure 3). Bioassay data (Figure 4) in mouse pituitaries in vitro shows that α-IB-52 is 3.4 times, and α-IB-92 over 40 times, more active than ILP peptide in suppressing follitropin release (49).

Lilja & Jeppsson (50) reported a basic protein in hSP with the same amino acid sequence as α-IB-52. These authors believed that the basic protein is the major degradation product of the gel-forming protein secreted by the seminal vesicles.

α-IB-92 has been synthesized by the thiocarboxyl segment strategy (51). Three segments were synthesized by the solid-phase method, purified, and characterized: [GlyS^{34}]-α-IB-92-(1–34) (I), CF_3CO-[GlyS^{65}]-α-IB-92-(35–

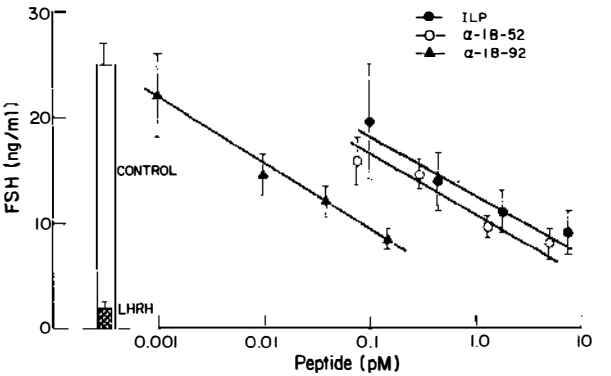

Figure 4 Effect of ILP, α-IB-52, and α-IB-92 on LHRH-induced FSH secretion by mouse pituitary; means ± SEM (n = 5).

65) (II), and Msc-α-IB-92-(66–92) (III), where Msc is 2-(methylsulfonyl)-ethyloxycarbonyl. All were reacted with citraconic anhydride followed by removal of the Msc group in III to give Ia, IIa, and IIIa, respectively. Peptide IIIa was coupled to IIa by the silver nitrate–*N*-hydroxysuccinimide procedure, and after removal of uncoupled segments and the trifluoroacetyl group, Ia was coupled, followed again by removal of uncoupled segments. Final deblocking to remove citraconyl groups was accomplished under exceptionally mild conditions in aqueous acetic acid. The synthetic product was identical to natural α-IB-92 in amino acid analysis, reversed-phase high-performance liquid chromatography (HPLC), gel electrophoresis, and tryptic mapping. The synthetic peptide was indistinquishable from natural α-IB-92 in a radioimmunoassay and in an in vitro mouse pituitary assay (see Table 1). In the mouse pituitary assay system, a dose of 0.001 nM of either synthetic or natural α-IB-92 causes significant inhibition of FSH release in the presence of 10 ng of LHRH.

Antiserum of high titer (1:40,000 final dilution) was obtained from one rabbit that had been immunized with α-IB-52 (52). It cross-reacted with ILP and α-IB-92 with equal affinity. The antiserum showed no cross-reaction to any of the known pituitary or hypothalamic peptides. Using radioiodinated α-IB-92 as the primary ligand, a specific and sensitive RIA for α-IBs has been developed with the ED_{50}, and the slope of 10 different assays (mean ± SE) ranged 2.23 ± 0.1 ng/tube and 0.57 ± 0.03, respectively (52). The minimal detectable dose was found to be 0.1 to 0.2 ng/tube. The recovery of added α-IB-92 to the blank serum at two concentrations (10 and 50 ng) was 85.5% (n = 20) in three different experiments. The intra- and interassay coefficient of variations for a pool of unidentified human serum was 9.49% and 14.0%,

Table 1 Comparison of natural (N) and synthetic (S) α-IB-92 in a mouse pituitary assay (MPA) and radioimmunoassay (RIA)

Assay	Peptide	ED_{50} (nM[a])	Slope	Relative potency
	α-IB-92 (N)	0.0959 (0.075–0.12)	0.620	1
MPA				
	α-IB-92(S)	0.0766 (0.052–0.112)	0.870	1.25 (0.67–2.3)
	α-IB-92 (N)	0.140 (0.13–0.15)	0.640	1.0
RIA				
	α-IB-92 (S)	0.128 (0.119–0.13)	0.552	1.1 (0.88–1.4)

[a]95% confidence limits in parentheses.

respectively. The pool of human serum showed a dose-dependent response. Human pituitary and hypothalamic extracts also showed a concentration-dependent response; the dose-response curve for the pituitary extract shifted to the left after fractionation on Sephadex G-100 column. The amounts of immunoreactive material (52) in human pituitary, hypothalamus, and serum, as expressed in ng per gram wet weight of tissue or ml, are estimated to be pituitary, 70.3; hypothalamus, 12.9; and serum (normal adult male), 7.2. The presence of immunoreactive α-IB-92 (12.9 ng/ml) in human follicular fluid was also shown (K. Ramasharma, unpublished data).

When the human serum sample was fractionated on a Sephadex G-100 column, the immunoreactive material eluted earlier than did the authentic α-IB-92 or α-IB-52. When α-IB-92 was mixed with blank human serum and chromatographed on the same column, the immunoreactivity again appeared in the void volume. Under identical chromatographic conditions, the immunoreactive material in the human pituitary and hypothalamic extracts was also found in the high-molecular-weight region (52).

The immunoreactive fraction obtained from gel filtration and further fractionated on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was resolved into several bands. These bands were electrophoretically transferred to nitrocellulose paper. Immunoreactive material from human pituitary extract appeared to behave as α-IB-92 and α-IB-52. The hypothalamic extract and human serum showed α-IB-92-related material (52).

A surprising finding was that pituitary and hypothalamic extracts contain immunoreactive α-IB-related peptides (52). Preliminary studies demonstrated the existence of α-IB in sheep and rat pituitary glands as examined by RIA. When these extracts were subjected to gel filtration, the immunoreactivity appeared mostly in the high-molecular-weight region. However, blotting analysis clearly indicates the presence of immunoreactive α-IB-92 and α-IB-52. The significant amounts of immunoreactivity were found in the anterior pituitary, but not in the posterior lobe. The amount of α-IB immunoreactivity found in the hypothalamus is lower than that observed in the pituitary (52).

Although the pituitary gland is the primary site of action of inhibin, several other organs such as the hypothalamus, pineal gland, and gonads have also been implicated (15). Results obtained using partially purified inhibin preparations suggested that inhibin must bind to the cell membrane before it evokes the cellular response in FSH suppression (53–55). The binding ability of α-IB-92 has been verified using human or ovine pituitary membrane preparations (56). Human pituitary membrane homogenate was prepared in 50-mM Tris-HCl buffer (pH 7.4), containing 0.3-M sucrose and 0.01% bacitracin. The binding studies were carried out using 50-mM Tris-HCl (pH 7.4) containing 0.1% BSA and 0.01% bacitracin at 4°C for 16 hr. The

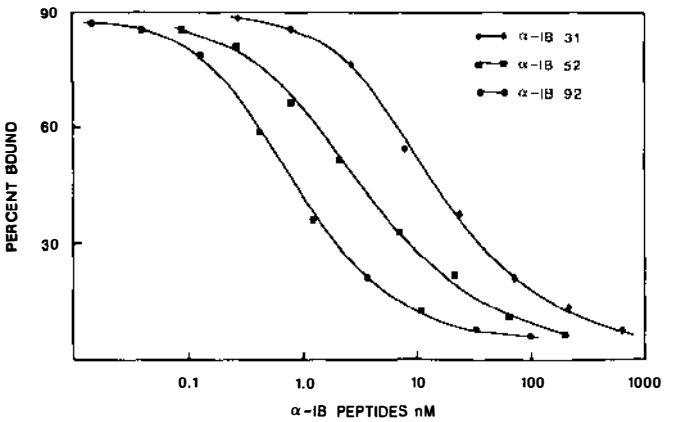

Figure 5 Competition of the binding of [^{125}I]α-IB-92 to human pituitary membrane preparation by α-IB-92, α-IB-52 and ILP (α-IB-31).

membrane-bound radioactivity was separated by Whatman filter paper (GF/B) and counted in the Beckman gamma counter. The results were computed for curve fitting in a computer.

The binding of labeled α-IB-92 to the human pituitary membrane was concentration dependent. The [^{125}I]-α-IB-92 binding to the human pituitary membrane can be displaced by the unlabeled peptide in a dose-dependent manner (Figure 5). The binding is specific, as most of the pituitary hormones and hypothalamic peptides failed to displace the bound label. α-IB-52 and ILP exhibit lesser binding affinities in displacing the label (Figure 5), compared to that of α-IB-92. These data demonstrate the existence of α-IB-92 binding sites on the human pituitary membrane.

Human β-Inhibin

Sheth et al (57) reported the isolation of a polypeptide with inhibinlike activity from human seminal plasma. This polypeptide has an amino acid content of nearly 130 residues, with serine as the NH_2-terminal amino acid. The amino acid sequence of the first 30 residues has also been determined (57). The polypeptide was later designated β-inhibin, and was completely sequenced (58). The primary structure of β-inhibin (shown in Figure 6) confirms the earlier data (57) on the NH_2-terminal sequence. It consists of 94 residues with one each of methionine and histidine, two each of arginine, leucine, phenylalanine, and tryptophan, and 5 disulfide bridges. β-Inhibin contains no alanine and is chemically distinct from α-IB-92.

One of the disulfide bridges is linked between Cys-73 and Cys-87, and the others have not been identified. Sheth and coworkers (59) further indicate that the inhibinlike activities of both β-inhibin-(1–94) and β-inhibin-(67–94) are

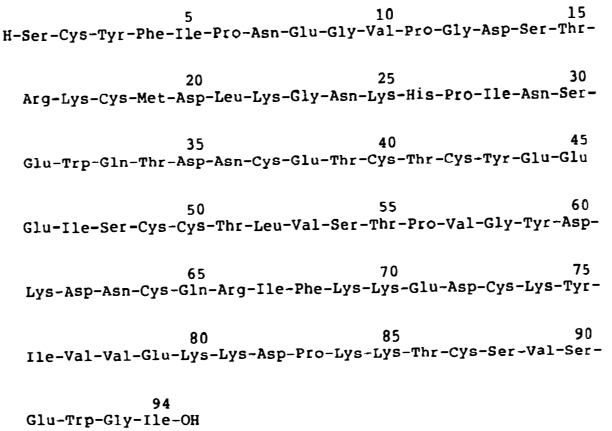

Figure 6 Amino acid sequence of β-inhibin.

equipotent on a mole-to-mole basis. A 10-ng dose of β-inhibin inhibits FSH release in rat pituitary in vitro assay, whereas 100 ng are required for the in vivo adult male rat assay (57). Neither α-IB-92 nor β-inhibin exhibits inhibin activity in the rat pituitary culture assay.

Bovine Inhibin

A highly acidic protein with an isoelectric point of pH = 2.2 has been obtained from bovine seminal plasma (60). This protein has a molecular weight of 18,200 and a Stokes radius of 1.90 nm, and is capable of suppressing human chorionic gonadotropin (hCG)-induced uterine weight increase in immature mice. The relationship of this protein to human α-IB-92 or β-inhibin is undetermined, as no structural data are presented.

INHIBINS FROM FOLLICULAR FLUID

Porcine Follicular Inhibin

Miyamoto et al (61) were the first investigators to isolate and characterize inhibin from porcine follicle fluid using cultured cells of rat anterior pituitary for bioassay. Purification steps were performed in the presence of urea to eliminate noncovalent protein–protein interactions. The purified inhibin had a molecular weight of 32,000. It behaved as a homogeneous protein in HPLC and SDS-PAGE. The hormone suppressed specifically the secretion of FSH from the pituitary cells, with an ED_{50} value of 0.9 ng/ml medium. After reduction with β-mercaptoethanol it gave rise to two subunits with molecular weights of 20,000 and 13,000, respectively. The NH_2-terminal sequence of

Figure 7 Deduced amino acid sequence of pfIB-α.

the large subunit was Ser-Thr-Ala-Pro and of the other, Gly-Leu-Glu-Cys.

Subsequently, two groups of investigators (62, 63) confirmed the data of Miyamoto et al (61) in obtaining the same inhibin molecule from porcine follicular fluid. In addition, the amino acid composition (278 residues) of the hormone (62) was given as residues for the 32-kd inhibin: Asx_{21}, Thr_{14}, Ser_{18}, Gly_{23}, Gly_{24}, Ala_{22}, Val_{14}, Met_6, Ile_{10}, Leu_{28}, Tyr_{11}, Phe_{10}, His_8, Trp_4, Lys_5, Arg_{16}, Cys_{14}, Pro_{30}. The first 10 NH_2-terminal residues of the 20-kd and 13-kd subunits were, respectively, Ser-Thr-Ala-Pro-Trp-Pro-Trp-Ser and Gly-Leu-Glu-Cys-Asp-Gly-Arg-Thr-Asn-Leu. In addition, Ling et al (62) isolated two forms (A and B) of inhibin with an identical α-subunit but a slightly different β-subunit. However, both forms have similar inhibin activity.

Using the NH_2-terminal-sequence data, Mason et al (64) identified cloned complementary DNAs encoding the biosynthetic precursors of the two subunits (α and β) of porcine follicular inhibin (pfIB). From the cDNA sequences, the primary structures of pfIB-α, and pfIB-β_A or pfIB-β_B, were deduced (shown in Figures 7, 8, and 9). One glycosylation site was predicted to occur in the α-subunit. Thus the α subunit has 134 amino acids with 7 cysteine residues, and the β_B subunit has 115 amino acids with 9 cysteine residues. The authors (64) suggest that the two subunits are derived from one ancestral gene, as the alignment of half of the cysteine residues in the two subunits indicates a similar distribution and some sequence homology. When compared with the amino acid sequence of human transforming growth factor-β (TGF-β) (65), pfIB-β shows some homology (see Figure 10). Sur-

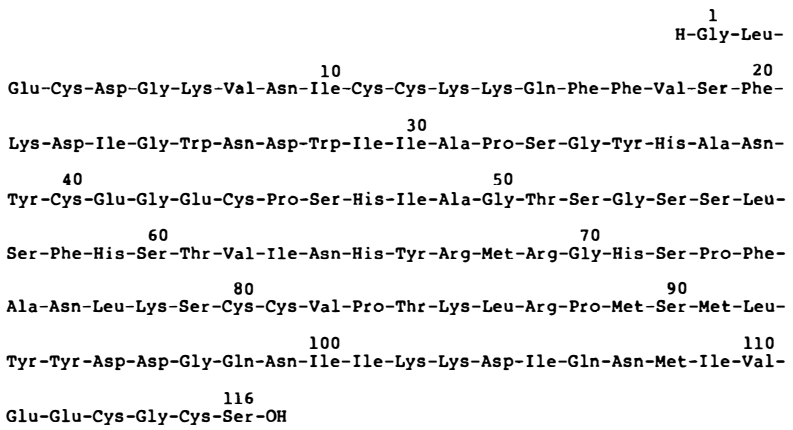

Figure 8 Deduced amino acid sequence of pfIB-β_A.

prisingly, TGF-β has recently been shown (66) to act as an inhibitor of FSH-induced aromatase activity in cultured rat granulosa cells.

Bovine Follicular Inhibin

Using a combination of procedures including exclusion chromatography, HPLC, and SDS-PAGE, Robertson et al (67) isolated a protein with inhibin activity from bovine follicular fluid collected in the presence of aprotimin and phenylmethyl-sulphonyl fluoride. Under nonreducing conditions, the protein gave a single band in SDS-PAGE with a molecular weight of 56,000. In the presence of mecaptoethanol, the protein gave two bands with molecular weight of 44,000 and 14,000 with NH_2-termini of Asn-Ala-Val and Tyr-Leu-

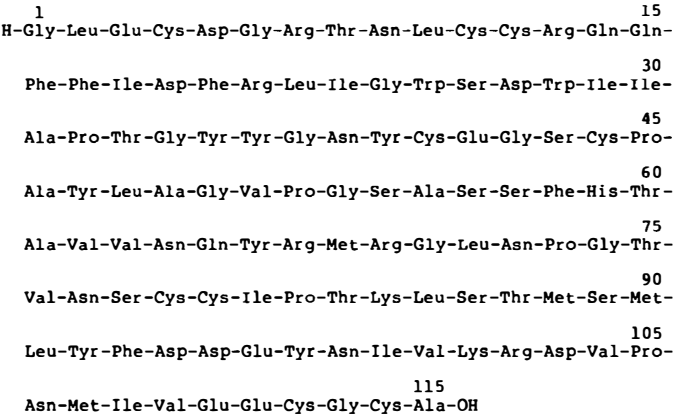

Figure 9 Deduced amino acid sequence of pfIB-β_B.

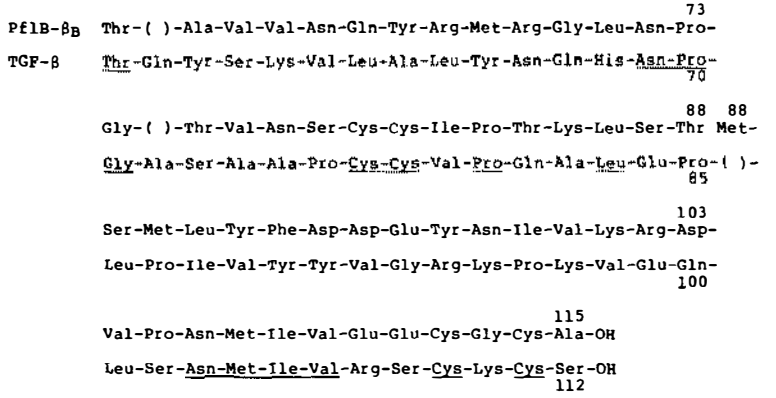

Figure 10 Comparison of pflB-β_B and TGF-β sequences.

Gln, respectively. Thus, bovine follicular inhibin (bfIB) is a 56-kd protein consisting of disulfide-linked 44-kd and 14-kd polypeptide chains.

Robertson et al also isolated a 31-kd form of inhibin from bovine follicular fluid (68). The 31-kd protein gave 2 bands after reduction on SDS-PAGE, with molecular weights of 20 kd and 15 kd. An antiserum to 56-kd inhibin can neutralize the bioactivity of both the 56-kd and 31-kd preparations. This ability may suggest that 31-kd inhibin is derived from the 56-kd molecule.

Independently, Fukuda et al (69) described the isolation of 32-kd inhibin from bovine follicular fluid using procedures similar to those for their work on pfIB (61). The 32-kd bfIB described by Fukuda et al (69) is apparently the same protein as the 31-kd inhibin described by Robertson et al. It consists of two polypeptide chains (20 kd and 13 kd) with NH_2-termini of Ser-Thr-Pro-Pro and Gly-Leu-Glu-Cys, respectively. Thus, the 31-kd bovine inhibin is very closely related to the porcine 32-kd hormone (61–63).

Monoclonal antibodies to bfIB 32-kd subunits (20 kd and 13 kd) have been prepared by Miyamoto et al (70). Using these antibodies, six different forms of inhibin with molecular weights of 120,000, 108,000, 88,000, 65,000, 55,000, and 32,000 have been identified in bovine follicular fluid. These forms are further divided into two groups: one (120 kd, 108 kd, and 88 kd) consists of three polypeptide subunits with disulfide linkages, and the other (65 kd, 55 kd, and 32 kd) consists of two subunits also with disulfide bridges. These bioactive forms of bfIB contain segments that are 20-kd and 13-kd polypeptide chains of 32-kd inhibin. The authors (70) suggest the largest form of bfIB has the structure shown in Figure 11. Restricted proteolytic processing of the largest form would give rise to different forms of bioactive inhibin.

Cloning and sequence analysis of cDNA for the 56-kd bfIB of Robertson et

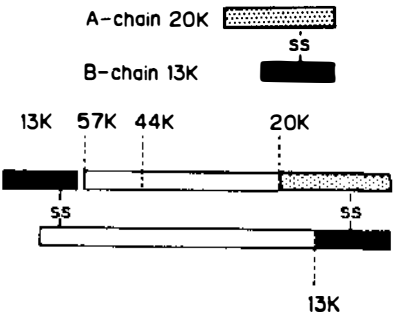

Figure 11 Proposed structure for the largest form of bfIB (From Ref. 70, Figure 4).

al (67) has been described (71). [Forage et al (71) stated that α-inhibin is "a protein of prostatic origin." In a paper by Li et al (49), the authors did not mention the origin of α-inhibin. In fact, recent data from a cytoimmunochemical technique indicate that α-inhibin-92 is located in the testis (D. T. Lau, unpublished data).] From the NH_2-terminal-sequence data of the two subunits, it is possible to obtain the oligonucleotide probe for the isolation of the cDNA of two 56-kd inhibin subunits (71). The amino acid sequence of the subunits is deduced from sequence analysis of cDNA. The α subunit (see Figure 12) consists of 300 amino acids with two potential *N*-glycosylation sites at Asn residues in positions 80 and 202. The β subunit consists of 116 amino acids with no sites for *N*-glycosylation and is identical to the porcine β_A subunit (see Figure 8). In agreement with Miyamoto et al (70), the 32-kd form of bfIB is derived by combination of 56-Kβ with a segment of the α subunit [56 Kβ-(167–300)] (71).

Human Follicular Inhibin

A comparison of chromatographic behavior of inhibin activity from human and bovine follicular fluid has been presented by van Dijk et al (72). Both inhibins are retained by immobilized lectins. The human hormone is apparently somewhat more basic (pI = 5.1–5.7) than bovine IB (pI = 4.75–5.25). Human and bovine inhibins behave similarly in various chromatographic assays.

Using porcine inhibin α, β_A and β_B cDNA as hybridization probes (64), Mason et al (73) identified cDNA clones for inhibins from human ovary mRNA. The primary structures of the subunits as deduced from the complete nucleotide sequence of cDNAs are nearly identical to their porcine equivalents. Human follicular fluid has two forms of inhibin (73) as does the porcine ovary (62, 63). These two forms have an identical α subunit but somewhat different β subunits.

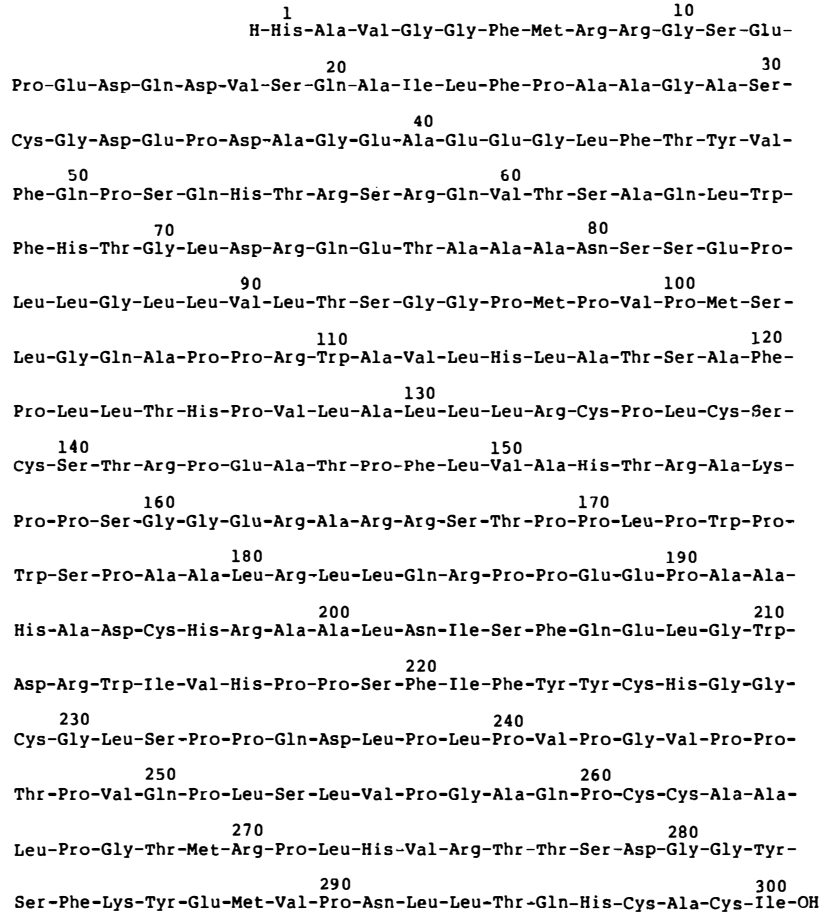

Figure 12 Deduced amino acid sequence of α chain of 56-kd bfIB.

CONCLUDING REMARKS

Feedback effects of gonadal steroids, as well as LHRH, on secretion of gonadotropins from the pituitary gland have been firmly established (74). After inhibin was observed nearly 60 years ago (1–3), inhibins have now been isolated and characterized from human seminal plasma and porcine or bovine follicular fluid. Figure 13 summarizes the present knowledge on the control FSH release from the pituitary. Whether a separate molecule from the hypothalamus also controls FSH secretion remains to be investigated.

Inhibins from human seminal plasma (hSP) are more simple peptides; they

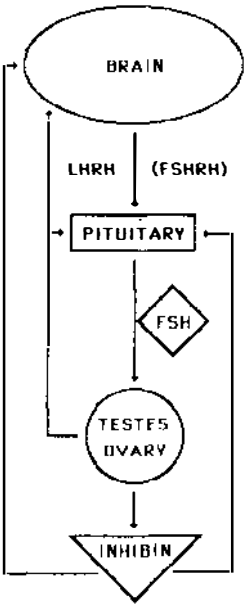

Figure 13 Diagram indicating the control of FSH secretion.

are active in vivo and in vitro assay systems in the presence of LHRH but not active in cultured pituitary cells (45, 49, 57–59). On the other hand, follicular inhibins, consisting of two subunits of glycoproteins with disulfide bridges, are active in pituitary cells in culture, but no data on them are available from in vivo bioassay systems (61–64, 67–71, 73). α-IB-92 from hSF is present in human serum, pituitary, hypothalamic (52), and follicular fluid (K. Ramasharma, unpublished data). In addition, specific binding sites in human and ovine pituitary membranes have been demonstrated (56).

The pituitary site of inhibin action is well documented in several in vivo and in vitro model systems (75). The female inhibin requires at least 72 hr to reduce FSH secretion in an in vitro pituitary cell culture system. On the other hand, male inhibin requires a relatively short time, but requires LHRH. These differential effects of inhibin in suppressing FSH raise the possibility that two types of modulating agents may be present in the gonad.

The hypothalamus was suggested as an additional site of action of inhibin (76). Endogenous LHRH levels in hypothalamic tissue were effectively reduced when incubated in vitro in the presence of seminal plasma inhibin preparations (15). On the contrary, de Greef et al (77) reported suppression of circulating FSH levels without changes in LHRH levels in the portal blood. If these results are correct, they probably indicate the existence of a separate FSH-releasing hormone (FSHRH) (78). The exact nature of these interactions

between inhibin and LHRH or FSHRH remains to be established. Furthermore, certain inhibin preparations affect the gonadal function by directly inhibiting the synthesis of DNA in the testis (79). Such local regulatory mechanisms are important for paracrine control of germ-cell development and follicular maturation.

The mechanism of action of inhibin remains unexplored. How inhibin modulates the intracellular events and effects differential secretions of FSH and LH is not clear. As indicated earlier, the anterior pituitary cultures must be exposed to inhibin-containing preparations for 72 hr for a significant FSH suppression to occur. Considering this time lag one could suggest that inhibin is somehow involved in the blockade of one of the molecular events (transcriptional or translational) leading to the biosynthesis of FSH molecules. The nature of the interplay of LHRH, steroid hormones, and inhibin (see Figure 13) in suppressing the release of FSH without affecting LH release remains unexplained. Whether cyclic nucleotides, particularly cyclic GMP, are involved in mediating the action of inhibin should be investigated.

The physiological role of inhibin is to convey the feedback signal(s) to the pituitary gland regarding the peripheral concentrations of FSH and subsequently to modulate FSH-dependent events in the testes, as well as in the ovary. The precise role of FSH in the adult male in regulating spermatogenesis is not completely resolved (80). However, if the rate of sperm production is a function of circulating levels of FSH, then inhibin could convey these quantitative signals to the pituitary gland. Preliminary data available indicate that reduction of inhibin activity occurs concomitantly to a reduction in the activity of germinal elements (81–84). On the other hand, if the immature male rats are treated with bfIB preparation, the development of spermatogenesis is delayed (85). Administration of hSP inhibin preparation to immature rats resulted in a reduction of ^{3}H-thymidine incorporation into testicular DNA (79, 86). These experimental results support a predominant role for inhibin in prepubertal males rather than in the adult male.

In the female, there is a good correlation of circulating levels of FSH and ovarian function. Inhibin might play a physiologically important role in those situations where there is a divergent secretion of LH and FSH (87–89). Studies in monkeys suggest that inhibin plays an important role in the follicular development (90). However, it is not possible to ascertain the quantitative contribution of inhibin in the follicular development.

As pointed out earlier, much of the supporting evidence for the inhibin concept comes from clinical and pathological situations associated with primary disorders of spermatogenesis. Inhibin might be a marker to determine the etiology of puberty and menopause. Circulating levels of inhibin might be a good index for assessing the functional status of granulosa cells in the ovary (15, 16). It has also been suggested that inhibin might be a potential con-

traception agent (5). Inhibin can certainly help in understanding the hypothalamo-pituitary-gonadal axis in the complex biological phenomena of mammalian reproduction.

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