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Identification and purification of inhibin and inhibin-related proteins

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Introduction

Within the past 5 years the existence of 'inhibin' has been proven conclusively. After many decades of uncertainty surrounding this putative non-steroidal mediator of gonadal negative feedback, inhibin has been purified to homogeneity, its physico-chemical and biological properties defined, the genes encoding its subunits cloned and sequenced and its physiological role(s) examined in detail. A useful working definition recently proposed by Burger (1988) states that "inhibin is a glycoprotein hormone consisting of two dissimilar disulphide-linked subunits termed α- and β- which inhibits pituitary gonadotrophin production and/or secretion, preferentially that of FSH". The literature relating to the discovery, isolation and characterization of inhibin has been reviewed extensively in the past few years and the reader will find much additional information in comprehensive reviews by Findlay (1986), de Jong (1987, 1988), Ying (1988) and Lincoln *et al.* (1989).

Inhibin

Identification of inhibin

Whilst the concept that the gonads secrete a non-steroidal factor capable of suppressing pituitary gonadotrophin secretion arose over sixty years ago (Mottram & Cramer, 1923; McCullagh, 1932), interest in inhibin remained at a relatively low level for many years. The advent of radioimmunoassays for the measurement of LH and FSH in the early 1970s prompted, amongst other things, renewed interest in the 'inhibin-concept' and it was soon established that various gonadally-derived fluids of both male (e.g. rete testis fluid, testicular lymph, seminal plasma) and female (e.g. ovarian follicular fluid) origin contain demonstrable inhibin-like biological activity (ILA). These early observations prompted several research groups to attempt to purify the active principle from one or other of the sources identified. However, it was not until 1985 that this task was accomplished.

Purification of inhibin

It is worth considering, with the benefit of hindsight, why the isolation of inhibin presented such a formidable challenge. In the mid-1970s there was no generally accepted 'standard' bioassay procedure for the reliable detection of inhibin-like biological activity (ILA). Rather, the various groups attempting to purify inhibin utilized a range of in-vivo and in-vitro bioassay procedures, each purporting to detect ILA, yet each relying on a different set of assumptions as evidence of their validity (see Baker *et al.*, 1981; de Jong, 1987). A gradual convergence of opinion as to which of these bioassays afforded the most effective method for the detection and quantitation of ILA (as then defined) led to the widespread adoption of the rat pituitary cell monolayer culture system in which ILA induces a selective fall in the basal release and/or cellular content of FSH. It is

noteworthy that each of the groups which eventually succeeded in isolating gonadal inhibin used such an assay system for screening chromatographic fractions generated during their purification schemes. In view of the more recent identification in gonadal fluids of molecules other than inhibin which suppress (i.e. follistatin, see below) or stimulate (i.e. activin, see below) pituitary FSH secretion, these rat pituitary cell bioassays should no longer be regarded as specific for inhibin as was once assumed. A second level of complexity with the purification of inhibin arose due to selection by different research groups of a range of ILA-containing gonadal fluids (e.g. porcine, bovine or human FF, ovine rete testis fluid, ovine testicular lymph, seminal plasma) as their respective starting materials. Thirdly, investigators had to contend with the apparent presence in these gonadal fluids of multiple forms of ILA displaying different physico-chemical properties and therefore eluting as confusing multiple peaks and/or poorly resolved components during chromatography. Much of this complexity is now readily explained by proteolytic processing of the high M_r precursor molecules for inhibin to generate biologically-active inhibin molecules differing in size and charge (Mason *et al.*, 1985, 1986; Forage *et al.*, 1986). Finally, inhibin is now recognized as a rather hydrophobic molecule which, unless measures are taken to prevent it, tends to associate not only with other proteins present in crude fluids/extracts but also with certain chromatographic stationary phases, thus giving rise to anomalous and often unpredictable behaviour during chromatography. Whether gonadal and other body fluids contain 'carrier proteins' which selectively bind inhibin has not yet been established.

A crucial development came in the early 1980s with the recognition that ILA was associated with a remarkably stable molecule capable of regaining its full biological activity following denaturation using harsh dissociating solvents (e.g. containing 8 M-urea, 4 M-acetic acid or 1% sodium dodecyl sulphate; SDS). The use of such protein-denaturing solvents to inhibit protein–protein interactions during gel-permeation, ion-exchange and hydrophobic interaction chromatography and the introduction of reversed phase high performance liquid chromatography (RP-HPLC) and preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) greatly facilitated the development of effective purification schemes and led directly to the successful isolation of inhibin in 1985 by four independent groups (Robertson *et al.*, 1985; Miyamoto *et al.*, 1985; Ling *et al.*, 1985; Rivier *et al.*, 1985).

Molecular characterization of inhibin

Initially, Robertson *et al.* (1985) isolated from bovine FF an M_r 56 000 form of inhibin which, upon reduction, dissociated into two subunits of M_r 44 000 (α44) and 14 000 (β). Other groups working with porcine FF (Miyamoto *et al.*, 1985; Ling *et al.*, 1985; Rivier *et al.*, 1985) isolated an M_r 31–32 000 form(s) of inhibin which after reduction yielded α- and β-subunits of M_r 18–20 000 and 14–15 000, respectively. Robertson *et al.* (1986) subsequently reported that inclusion of an acidic precipitation step in their purification scheme for bovine FF inhibin resulted in the generation of an M_r 31 000 form of inhibin which was successfully isolated along with the M_r 56 000 form. Fukuda *et al.* (1986), using a complex purification scheme identical to that used by their group to isolate pig FF inhibin (Miyamoto *et al.*, 1985), and Knight *et al.* (1987), using a simplified purification scheme, also isolated an M_r 32 000 form of inhibin from bovine FF and confirmed that the molecule consisted of two disulphide-linked subunits similar in size to those reported for M_r 31–32 000 pig inhibin. An M_r 30 000 form of inhibin (M_r 21 000 α-subunit and M_r 16 000 β-subunit) was also isolated from ovine follicular fluid (Leversha *et al.*, 1987). Ling *et al.* (1985) actually isolated two isoforms of M_r 32 000 inhibin from pig FF (termed inhibin A and inhibin B) which shared an identical α-subunit but had slightly different β-subunits termed β_A and β_B respectively. It was subsequently established that both forms of inhibin β-subunit are also expressed in human and rat ovary although the β_B form has not yet been detected in bovine ovary.

Amino-terminal sequence analysis of the isolated inhibin subunits (α, β_A and β_B) permitted the synthesis of oligonucleotide probes which were then used to isolate from ovarian mRNA libraries,

cDNA species coding for the α- and β-subunits of porcine (Mason *et al.*, 1985) and bovine (Forage *et al.*, 1986) inhibin. The cloning and sequencing of these cDNA molecules, accomplished less than 1 year after inhibin was first isolated, enabled the complete amino acid sequence to be predicted for the α- and β-subunits of porcine (Mason *et al.*, 1985), bovine (Forage *et al.*, 1986), human (Mason *et al.*, 1986) and rat (Woodruff *et al.*, 1987) inhibin. Whilst native gonadal inhibin has not yet been isolated from rat or man, Mason *et al.* (1987) have reported the expression in Chinese hamster ovary cells of recombinant DNA-derived human inhibin which was biologically active when tested in a rat pituitary cell bioassay.

Although the inhibin concept first arose from studies in the male, progress towards isolating and characterizing testicular inhibin lagged behind that for ovarian inhibin. However, Bardin *et al.* (1987) and Vaughan *et al.* (1989) have reported the isolation from ovine rete testis fluid of an M_r 32 000 form of inhibin which, upon reduction, dissociated into α- and β-subunits of M_r 21 000 and 14 000 respectively. Amino-terminal sequencing revealed only minor differences compared to the corresponding sequences for pig or bovine ovarian inhibin. Moreover, Bardin *et al.* (1987) cloned and characterized cDNA encoding the α-subunit of inhibin in human testis to reveal a nucleotide sequence identical to that for human ovarian inhibin. Antisera raised against synthetic fragments of ovarian inhibin have been used for immunolocalization studies in testis of several species (Bardin *et al.*, 1987), providing further evidence that ovarian and testicular inhibins are highly homologous.

These molecular characterization studies revealed a striking degree of interspecies homology with respect to inhibin with approximately 85% homology in the primary structure of the α-subunit, ~100% homology for the β_A subunit and >95% for the β_B subunit. Moreover, α-, β_A- and β_B-subunits display similar distribution patterns for cysteine residues, suggesting that they are each derived from a common ancestral gene (Mason *et al.*, 1986). It has also emerged that the β-subunits of inhibin share considerable homology with a range of proteins implicated in cell differentiation and developmental processes including transforming growth factor β (TGFβ), Müllerian-inhibiting substance (MIS), an erythroid differentiation factor and activin (see below), giving rise to the concept of the 'inhibin-related peptide family' (see Ying, 1988).

The mature, fully-processed forms of α- (M_r 18–20 000) and β- (M_r 14 000) subunits of inhibin constitute the carboxy-termini of much larger precursor molecules composed of about 360 and 420 amino acid residues, respectively (Fig. 1). Each precursor molecule contains a number of potential proteolytic cleavage sites and the locations of these are consistent with the generation of the different M_r forms of inhibin and its subunits identified in gonadal fluids (Robertson *et al.*, 1986; Miyamoto *et al.*, 1986; Knight *et al.*, 1989) and granulosa cell-conditioned culture media (Bicsak *et al.*, 1988). For instance, Miyamoto *et al.* (1986) used the technique of immunoblotting to demonstrate the presence in bovine FF of M_r 65 000, 55 000 and 32 000 forms of inhibin dimer. These size differences were attributed to differential processing of the α-subunit precursor since, upon reduction, these disulphide-linked dimers yielded immunoreactive α-subunits of M_r 52 000, 42 000 and 20 000 together with an immunoreactive β-subunit of constant size (M_r 14 000). These authors also noted the presence of higher M_r forms of inhibin (120 000, 108 000 and 88 000) which they proposed represented disulphide-linked trimers composed of two processed β-subunit precursor sequences linked to an α-subunit of variable size. These different M_r forms of inhibin appear to possess ILA when eluted from SDS-PAGE gels and tested in an in-vitro inhibin bioassay (Miyamoto *et al.*, 1986) although the possibility exists that, for ILA to be expressed, further processing occurs in the serum-containing culture medium used for the inhibin bioassay, to yield the mature, fully-processed M_r 32 000 dimer. In this regard, McLachlan *et al.* (1986) showed that, in the presence of serum, M_r 55 000 bovine inhibin is converted to the M_r 31 000 form which is generally regarded to be the smallest and most predominant form of bioactive inhibin likely to reach the anterior pituitary *in vivo*.

The mature M_r 18–20 000 α-subunit of inhibin contains one (bovine, porcine) or two (human) potential N-glycosylation sites in contrast to the mature β-subunit which lacks such sites (Mason *et*

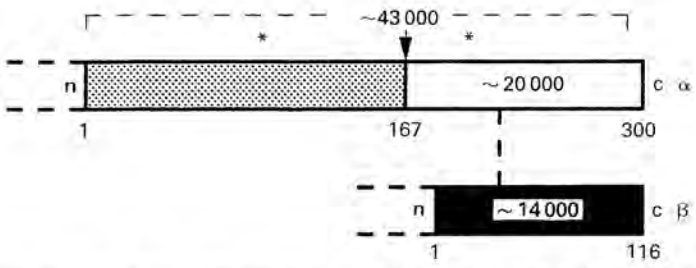


Fig. 1. Diagram showing basic subunit structure of bovine inhibin. Numbers refer to amino acid residues and asterisks indicate approximate positions of potential N-glycosylation sites. α- and β-subunits are linked by at least one interchain disulphide bond represented by the dashed vertical line. (After Forage *et al.*, 1986.)

al., 1985, 1986; Forage *et al.*, 1986). This factor not only explains the 10–20% discrepancy between the predicted and observed M_r values for the α-subunit but also confirms earlier proposals, based on the behaviour of ILA during lectin affinity chromatography, that inhibin is a glycoprotein (see de Jong, 1988).

Monomeric forms of inhibin α-subunit

Knight *et al.* (1989) exploited the technique of immunoaffinity chromatography to isolate from bovine FF an abundant and hitherto unrecognized form of inhibin which was highly reactive with antisera raised against either M_r 32 000 inhibin or a synthetic α-subunit fragment but which lacked inhibin-like biological activity. Upon SDS-PAGE, this material had an apparent M_r of 25 000 under non-reducing conditions, and 21 000 under reducing conditions. During amino-terminal sequencing of the isolated material only one amino acid residue was identified at each cycle of Edman degradation and the sequence (1–14) revealed was identical to that reported by Forage *et al.* (1986) for the amino terminus of the M_r 20 000 α-subunit of bovine inhibin. The authors attributed the apparently greater M_r value shown by the non-reduced material to anomalous behaviour during SDS-PAGE and concluded that it was a native form of monomeric M_r 21 000 α-subunit. Independent reports from Sugino *et al.* (1989) and Robertson *et al.* (1989) confirmed the presence in bovine FF of 'free' α-subunit (M_r 25 000 under non-reducing conditions and 21 000 under reducing conditions) devoid of biological activity in inhibin bioassays. Contrary to the conclusion of Knight *et al.* (1989) however, SDS-PAGE and microsequencing revealed that the isolated material was actually dimeric in nature comprising an M_r 21 000 α-subunit linked by a disulphide bond to the much smaller 'pro'-region (40 residue amino terminal fragment) of the 'parent' α-subunit precursor molecule. Sugino *et al.* (1989) and Robertson *et al.* (1989) reasoned that the generation of this molecular species (referred to by the latter group as the 'pro-α_c' fragment) must entail the release by proteolytic excision of the intervening 167 residue fragment. This fragment (referred to as 'αN') was successfully isolated from bovine FF by Robertson *et al.* (1989), confirming the accuracy of this deduction (see Fig. 2).

Such findings emphasize the complexity of potential post-translational processing of inhibin subunit precursor molecules which may generate multiple mono- and dimeric forms, perhaps subserving different biological roles, which have yet to be identified. Indeed, the recent report by Findlay *et al.* (1989b) that active immunization of ewes against the 'αN' fragment impaired fertility suggests it may have a biological role quite distinct from that of inhibin αβ dimer.

Two forms of 'free' inhibin α-subunit (M_r 25 000 and 44 000) have been identified in bovine utero-ovarian and jugular venous blood, indicating that these molecules are secreted into the peripheral circulation (Knight *et al.*, 1989). Moreover, these inhibin forms probably represent the dominant immunoreactive species detectable in bovine plasma by currently available inhibin radioimmunoassays. Thus it is now recognized that antisera against synthetic α-subunit fragments

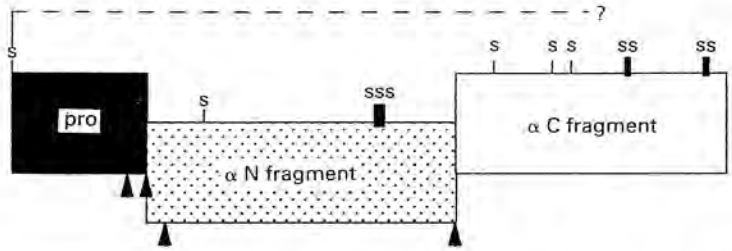


Fig. 2. Processing of inhibin α-subunit precursor to yield 'pro' 'αC' and 'αN' fragments isolated from bovine follicular fluid. Solid triangles indicate potential cleavage sites and S indicates positions of cystein residues. (Redrawn from Robertson *et al.*, 1989.)

(e.g. Knight *et al.*, 1989; Beard *et al.*, 1990) as well as purified native dimeric inhibin (e.g. Hasegawa *et al.*, 1988; Robertson *et al.*, 1988, 1989) cross-react extensively (>200%) with these more recently identified native forms of 'free' α-subunit. Clearly, the immunoreactivity of these different forms is strongly influenced by the molecular conformation since 'free' α-subunit obtained by reduction and alkylation of purified native inhibin dimer reportedly shows minimal cross-reaction with the same antisera to native inhibin (Robertson *et al.*, 1988).

Attempts in this laboratory to obtain physiologically relevant measurements of circulating concentrations of inhibin in cattle using radioimmunoassay have been hindered not only by the abundant presence of 'free' inhibin α-subunit in plasma (Knight *et al.*, 1989) but by the surprising finding that this material persists in the circulation of chronically ovariectomized heifers (see Figs 3 and 4) and steers. Paradoxically plasma levels of immunoreactivity were similar in gonadectomized and intact cattle, suggesting a major extra-gonadal source of this material (Fig. 3). Attempts to identify the source (J. H. M. Wrathall & P. G. Knight, unpublished observations) have so far been inconclusive although several extra-gonadal tissues of cattle were found to contain immunoassayable levels of inhibin (Fig. 5). Such findings accord with recent reports for other species that mRNA coding for α, and to a lesser extent, β-subunits of inhibin is expressed in various extra-gonadal tissues including adrenal, pituitary, central nervous system and placenta (Crawford *et al.*, 1987; Meunier *et al.*, 1988).

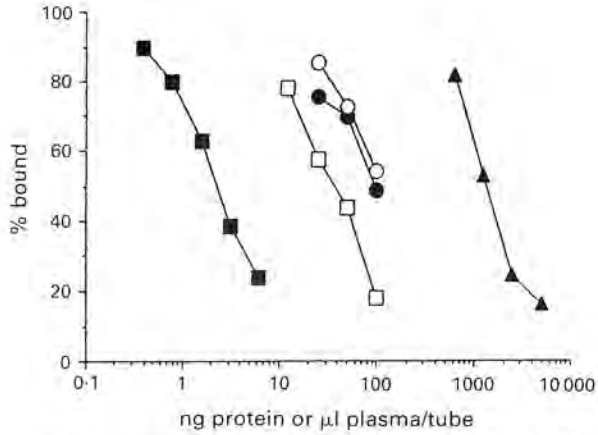
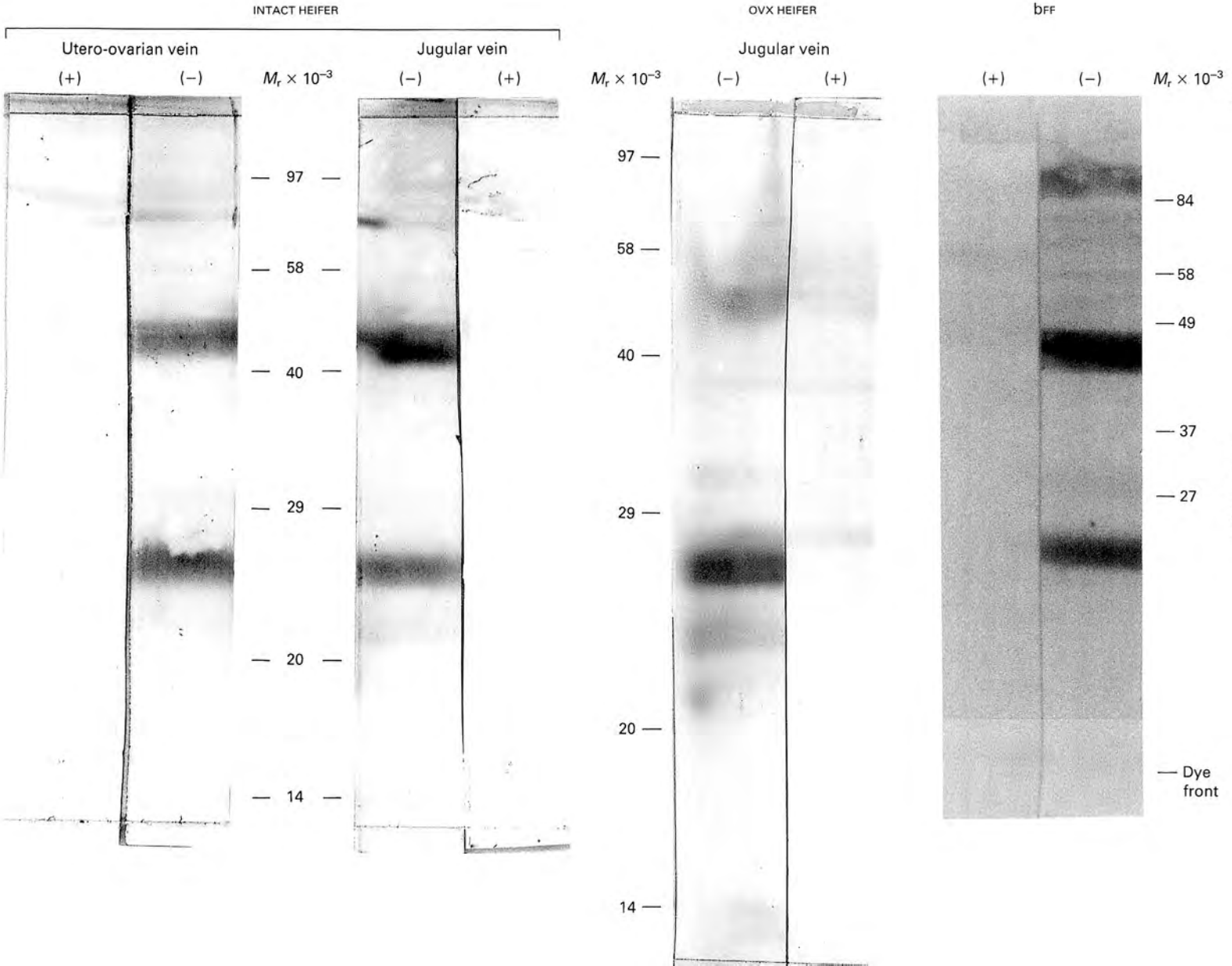


Fig. 3. Comparison of radioimmunoassay displacement curves for highly-purified M_r 32 000 bovine inhibin (■), bFF (▲), bovine utero-ovarian vein plasma (□) and jugular venous plasma from intact (●) and ovariectomized (○) heifers. The assay is based on an antiserum raised in sheep against the N-terminal sequence (1–32) of the α-subunit of human inhibin and radioiodinated M_r 32 000 bovine inhibin is used as the tracer (see Beard *et al.*, 1990).


INTACT HEIFER
Utero-ovarian vein
(+)
(−)
$M_r \times 10^{-3}$
97
58
40
29
20
14
Jugular vein
(−)
(+)
OVX HEIFER
Jugular vein
$M_r \times 10^{-3}$
(−)
(+)
97
58
40
29
20
14
bFF
(+)
(−)
$M_r \times 10^{-3}$
84
58
49
37
27
Dye front

It should be possible to resolve these difficulties associated with the immunochemical measurement of circulating inhibin in cattle, and probably other species, by developing a 2-site immunoradiometric assay for inhibin in which the specific detection of the analyte (i.e. biologically active inhibin dimer) depends on the recognition of two different antibodies directed against an appropriate pair of epitopes, one on the mature α-subunit and the other on the mature β-subunit (Fig. 6). Whilst excellent α-subunit specific antisera are already available, the generation of suitable β-subunit specific antibodies, which must cross-react with native inhibin dimer, has so far been unsuccessful.

Effects of highly-purified inhibin on gonadotrophin secretion *in vivo* and *in vitro*

Whilst the recent availability of highly-purified inhibin has encouraged detailed in-vitro investigations into its actions on cultured rat pituitary cells, the prospect of undertaking similarly detailed 'whole animal' studies is hindered by the strictly limited amounts of inhibin currently available. However, three in-vivo studies, involving intact rats (Ying *et al.*, 1987), ovariectomized ewes (Findlay *et al.*, 1987) and ovariectomized heifers (Beard *et al.*, 1990) have now confirmed that highly-purified inhibin preparations do indeed suppress plasma FSH concentrations with little or no effect on plasma LH.

Initial claims that the effect of highly purified inhibin on cultured rat gonadotrophs is confined to a specific reduction in FSH cell content and release (Ling *et al.*, 1985; Fukuda *et al.*, 1986; Robertson *et al.*, 1986) have now been revised following more recent demonstrations that both basal (Farnworth *et al.*, 1988a; Castillo, 1989) and GnRH-induced (Fukuda *et al.*, 1987; Farnworth *et al.*, 1988b; Campen & Vale, 1988; Castillo, 1989) LH release may also be suppressed, albeit to a lesser extent than FSH. The latter observation is in accordance with the observation by Wang *et al.* (1988) that exposure of rat pituitary cells to inhibin reduces the number of GnRH receptors.

Whilst it would be tempting to extrapolate these in-vitro findings for the rat to other species, including ruminants, we have shown (Muttukrishna & Knight, 1990) that exposure of cultured sheep pituitary cells to highly-purified bovine inhibin actually enhances GnRH-induced LH release without affecting basal LH release; basal and GnRH-induced FSH release were suppressed by inhibin, in a manner similar to that observed in rats (Fig. 7). There are already indications, therefore, that the actions of inhibin and related proteins on the ruminant pituitary may differ from those identified for the rat, a view reinforced by our subsequent observation (see Fig. 8) that activin stimulates basal LH release but suppresses GnRH-induced LH release from ovine pituitary cells *in vitro*.

Activin

Identification and purification of activin

Whilst developing procedures to isolate inhibin from pig FF two independent groups (Ling *et al.*, 1986; Vale *et al.*, 1986) observed that certain fractions obtained during gel permeation chromatography and HPLC appeared to stimulate, rather than suppress, FSH secretion *in vitro*.

Fig. 4. Analysis by 'Western blotting' of crude bovine follicular fluid (bFF) and immunoaffinity-purified serum extracts from intact and ovariectomized (OVX) heifers. Samples were subjected to SDS-PAGE under non-reducing conditions and immunoblotting was performed using an antibody raised in a rabbit against the N-terminal sequence (1–32) of the α-subunit of human inhibin. Specificity of immunostaining was confirmed by incubating each sample with antibody in the absence (−) and presence (+) of an excess of a synthetic peptide mimicking the N-terminal sequence (1–29) of the α-subunit of bovine inhibin. The positions of molecular weight markers are indicated.

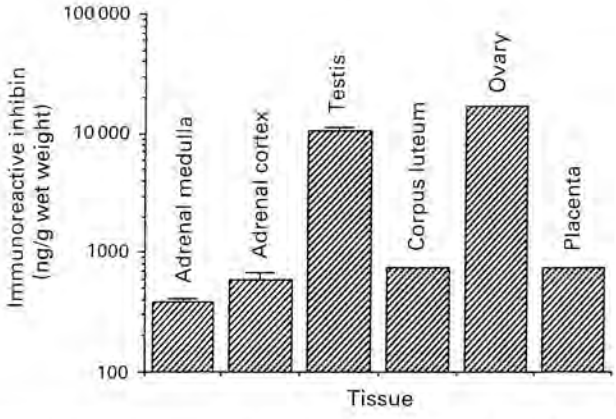


Fig. 5. Immunoreactive inhibin contents of various bovine tissues. With the exception of corpus luteum, ovary and placenta which represent single observations, values are means ± s.e.m. ($n = 6$). Other tissues analysed, including hypothalamus, pituitary, thymus, heart, kidney, liver and spleen ($n = 6$), had inhibin levels below the limit of detection of the assay (<80 ng/g). (Unpublished observations of J. H. M. Wrathall & P. G. Knight.)

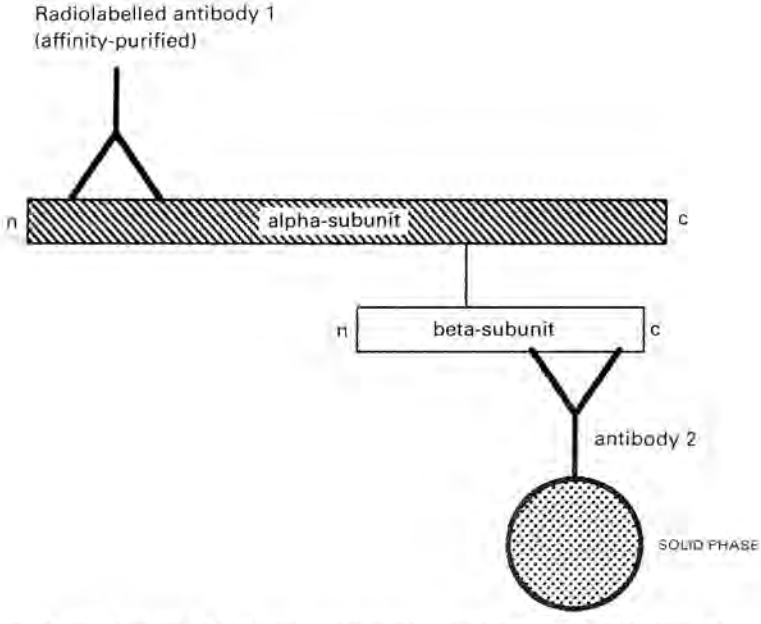


Fig. 6. Potential application of a 2-site immunoradiometric assay for the specific quantitation of dimeric (i.e. bioactive) inhibin.

Both groups went on to isolate and characterize the active substance(s), revealing them to be disulphide-linked dimeric proteins actually composed of two inhibin β-subunits (either a $\beta_A\beta_B$ heterodimer, termed 'activin' or a $\beta_A\beta_A$ homodimer now termed 'activin-A'). Activin-A was subsequently isolated from bovine FF (Hutchinson *et al.*, 1987). It also emerged that an erythroid differentiation factor isolated from a human leukaemia cell line is, in fact, identical to activin-A (Eto *et al.*, 1987).

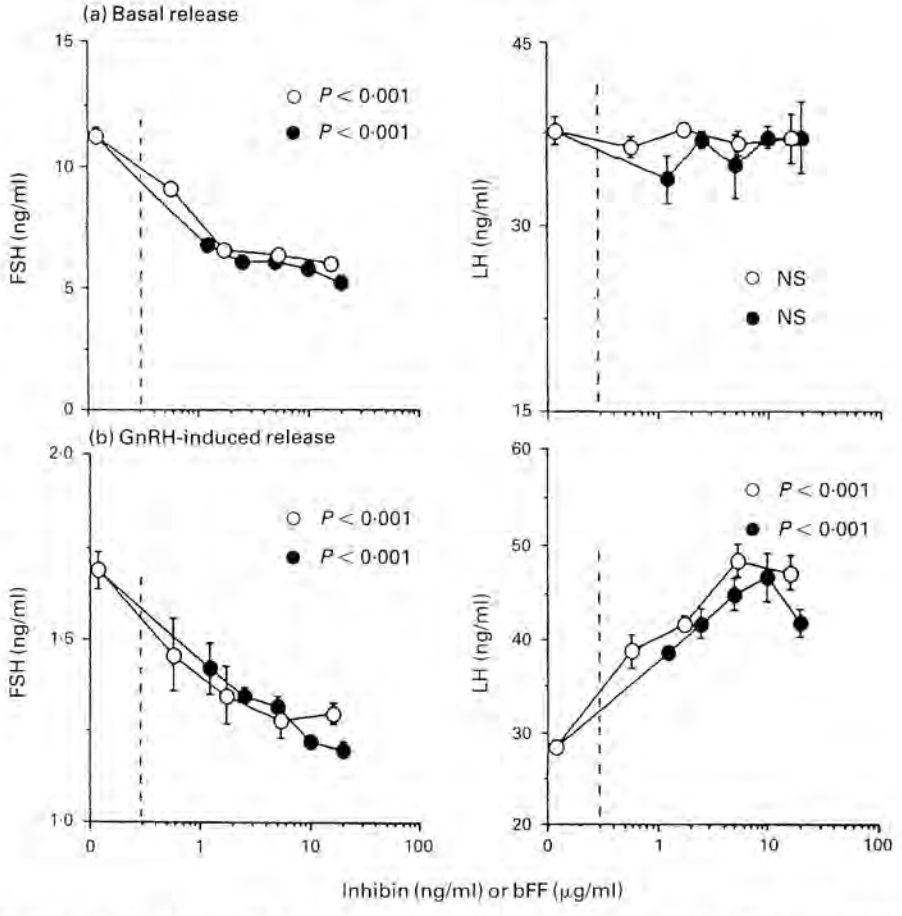


Fig. 7. Effects of highly purified M_r 32 000 bovine inhibin (●) and bovine follicular fluid (bFF, ○) on (a) basal and (b) GnRH-induced release of FSH and LH by ovine pituitary cells *in vitro*. Values are means ($n = 4$) and vertical bars indicate s.e.m. where this exceeds the symbol width. P values indicate a significant dose-dependent effect of each treatment (ANOVA). NS, not significant by ANOVA. (From Muttukrishna & Knight, 1990.)

Biological properties

In terms of their biological activity activin and activin-A are approximately equipotent, stimulating basal FSH secretion by cultured rat pituitary cells ($ED_{50} \sim 1$ ng/ml) without affecting the secretion of LH, TSH or prolactin. This effect of activin on pituitary FSH secretion is antagonized by inhibin. Schwall *et al.* (1989) recently reported the production of recombinant human activin-A which stimulates FSH secretion by rat pituitary tissue *in vitro* and *in vivo* without affecting LH release. Regarding the question of whether activin affects gonadotrophin secretion in ruminants, we have shown that, as in rats, recombinant human activin-A stimulates both basal and GnRH-induced secretion of FSH by sheep pituitary cells *in vitro* (S. Muttukrishna & P. G. Knight, unpublished observations). However, whilst activin-A also stimulated basal LH release, we were surprised to find that it actually suppressed GnRH-induced LH release, a novel action at variance with reports for the rat (Fig. 8). In view of the functional antagonism between inhibin and activin, this observation adds credence to our earlier finding reported above that inhibin enhances GnRH-induced LH release by sheep pituitary cells *in vitro* (see Fig. 7). These observations highlight the need for further comparative investigations in species other than the rat, which has, to date, been

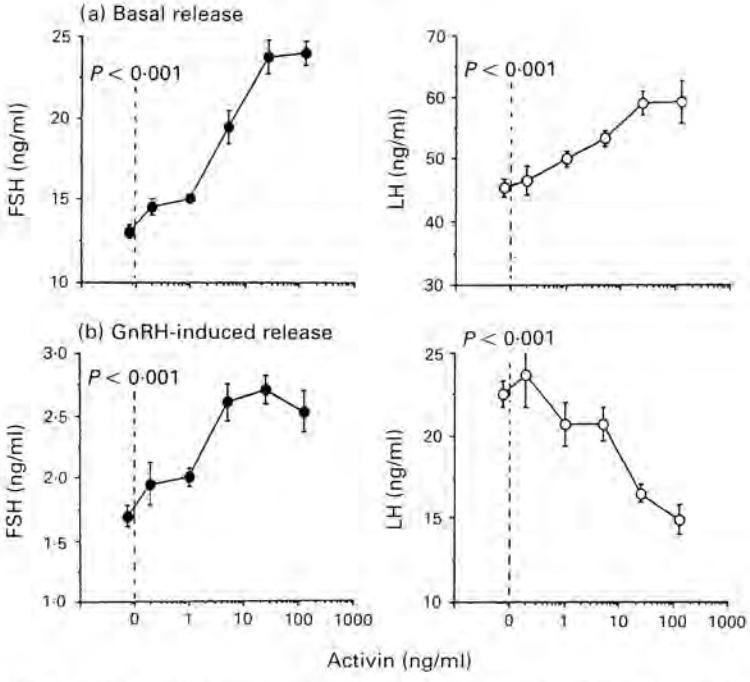


Fig. 8. Effect of recombinant human activin-A on (a) basal and (b) GnRH-induced release of FSH (●) and LH (○) by ovine pituitary cells *in vitro*. Values are means ($n = 4$) and vertical bars indicate s.e.m. *P* values indicate a significant dose-dependent effect of treatment. (Unpublished observations of S. Muttukrishna & P. G. Knight.)

used almost exclusively for in-vitro testing of inhibin and inhibin-related proteins. It is not yet known for any species whether activin of ovarian origin circulates as a hormone to modify pituitary gonadotrophin secretion. There is, however, ample evidence, at least for the rat, that it acts at the intraovarian level to modulate granulosa cell function (Ying, 1988; la Polt *et al.*, 1989).

Follistatin

Identification and purification

Ueno *et al.* (1987) noted that a further zone of ILA, distinct from the inhibins, was resolved by the first reversed-phase HPLC fractionation step of their inhibin-purification scheme. After three more HPLC steps they isolated a monomeric M_r 35 000 protein which was found to share no homology with either α- or β-subunits of inhibin and which they termed follistatin. A less abundant M_r 32 000 molecular variant of follistatin was also isolated from the same source and found to have an amino terminal sequence identical to the M_r 35 000 molecule. Molecular cloning techniques allowed the complete primary structure of the pig follistatin precursor to be deduced (Esch *et al.*, 1987) and the pig cDNA probes were subsequently used to characterize the human follistatin precursor, revealing only 6 amino acid substitutions between the two species (Shimasaki *et al.*, 1988). Regarding the production of follistatin by the ruminant ovary, Robertson *et al.* (1987) isolated from bovine FF three closely-related molecules with FSH-suppressing activity which were each shown to have the same amino terminal sequence as porcine follistatin. Moreover, Leversha *et al.* (1987) reported the presence of a non-reducible M_r 36 000 molecule with ILA (presumed to be follistatin) in ovine FF.

Biological properties

Porcine and bovine follistatins specifically suppress FSH production (release or cell content) by cultured rat pituitary cells *in vitro* but with a potency only 10–30% that of purified inhibin (Robertson *et al.*, 1987; Ueno *et al.*, 1987; Esch *et al.*, 1987). It remains to be established whether follistatin exerts similar effects on pituitary gonadotrophs from ruminant species. Furthermore, it is not known whether this putative hormone reaches the peripheral circulation in amounts capable of influencing pituitary gonadotroph function *in vivo* although there are recent indications that it acts intragonadally to modify ovarian steroid output (Findlay *et al.*, 1989a). Attempts to measure circulating concentrations of follistatin must await the development of specific radioimmunological assays since, as mentioned above, conventional bioassay procedures, as well as lacking sensitivity, are unable to discriminate between inhibin and follistatin.

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