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The Isolation and Physiology of Inhibin and Related Proteins

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ABSTRACT

Inhibin, a glycoprotein that preferentially suppresses follicle-stimulating hormone (FSH) secretion, has been isolated from follicular fluid as a heterodimer of two dissimilar subunits linked by disulphide bonds. The larger subunit is termed α and the smaller is designated β. Two forms of inhibin termed A and B have been isolated, the differences being due to variations in the amino acid sequence of the β-subunit; Inhibin A consists of α-β_A and Inhibin B of α-β_B. Dimers of the β-subunit, termed activins, have also been found in follicular fluid; these stimulate pituitary FSH secretion.

Inhibin is produced in the female by the granulosa cell and corpus luteum under the control of FSH and luteinizing hormone (LH), respectively. The levels in serum rise to peak at mid-cycle and in the mid-luteal phase of the human menstrual cycle, and decline prior to menstruation. In pregnancy, the late-luteal phase decline in inhibin does not occur and the levels increase slowly. Studies suggest that the levels in pregnancy arise from an embryonic source, particularly the placenta.

In the male, inhibin is produced by the Sertoli cells under the control of FSH by mechanisms involving cyclic adenosine 3′, 5′-monophosphate. Testosterone exerts a minor inhibitory control at supraphysiological levels (10^{-5} M), but human chorionic gonadotropin stimulation results paradoxically in a rise in serum inhibin levels. Disruption of spermatogenesis in the rat by cryptorchidism, heat treatment, or efferent duct ligation results in a decline in inhibin levels and a rise in FSH levels, findings consistent with the negative feedback action of inhibin on FSH secretion.

As well as their roles in the reproductive system, inhibin and activin have more widespread actions in the haemopoietic, immune and nervous systems as evidenced by the finding of mRNA for its subunits in a range of tissues. Other studies have shown actions on erythroid differentiation and on mitotic activity in thymocytes. These actions suggest that inhibin and activin may function as growth factors as well as regulators of FSH.

INTRODUCTION

The term "inhibin" was proposed originally by McCullagh in 1932 to denote the activity of an aqueous extract of the testis that has the capacity to suppress castration cell formation in the anterior pituitary gland. The concept of inhibin emerged from studies (Mottram and Cramer, 1923) showing that castration cells appear in the pituitary following damage to the seminiferous tubules. It is important to realize that the term "inhibin" was proposed before the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), were defined as separate entities and indeed before testosterone was isolated as a pure substance.

In the intervening years, the term inhibin has been used to designate a nonsteroidal gonadal product with the capacity to specifically suppress FSH secretion. Though considerable circumstantial evidence for inhibin emerged from studies using specific bioassays and immunoassays to measure FSH and LH (de Kretser et al., 1977; Main et al., 1978, Franchimont et al., 1980), the isolation of this molecule was a slow process and did not occur until 53 years after the original postulate.

Isolation and Characterization of Inhibin

Direct evidence for the existence of inhibin emerged from several studies between 1970 and 1980 when extracts of seminal plasma (Franchimont, 1972), rete testis fluid (Setchell and Sirinathsinghi, 1972; Setchell and Jacks, 1974; Baker et al., 1976), testis extracts (Keogh et al., 1976), and ovarian follicular fluid (de Jong and Sharpe, 1976) were shown to have the capacity to suppress FSH secretion. Subsequently, the isolation of inhibin proved difficult due to

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the nature of the molecule and its hydrophobicity, the use of a variety of assays with differing specificities—each with the potential for nonspecific inhibition, and the failure of investigators to consider the recoveries and specific activities of their extracts.

The initial isolation of inhibin was achieved from bovine follicular fluid (bFF) as a 58 kDa glycoprotein consisting of two disulphide-linked subunits of apparent molecular masses 43 kDa and 15 kDa (Robertson et al., 1985). The introduction of a pH precipitation step during purification led to the isolation of a 31 kDa form consisting of two subunits of 20 kDa and 15 kDa, leading to the postulate that the 31 kDa form results from cleavage of the 43 kDa subunit (Robertson et al., 1986a). A similar form from bFF was also isolated by Fukuda et al. (1986). Miyamoto and colleagues (1985) also reported the isolation of a 32 kDa glycoprotein from porcine follicular fluid (pFF) consisting of two subunits of 20 kDa and 13 kDa, a finding confirmed by Ling et al. (1985) and Rivier et al. (1985). The larger subunit has been termed α, and the smaller, the β-subunit. Ling and colleagues (1985) isolated two forms of inhibin, termed Inhibin A and Inhibin B, identified by the differing NH_2-terminal amino acid sequences of their β-subunits, now termed β_A and β_B.

In sheep, a 30 kDa form with 20 and 16 kDa subunits has been isolated from follicular fluid (Leversha et al., 1987) and from rete testis fluid, the latter representing the first purification of inhibin from a male source (Bardin et al., 1987). Bardin et al. (1987) found two forms of inhibin characterized by different α-subunits, one of which was 16 amino acids shorter at the NH_2-terminal end, due presumably to differential processing of the α-subunit.

Structural Analysis and Homologies

Details of the entire structure of inhibin emerged from the cloning of the genes controlling the production of the inhibin subunits from porcine and bovine sources (Mason et al., 1985; Forage et al., 1986). Separate genes code for the precursors to the α, β_A, and β_B-subunits that are subsequently processed at sites of paired arginine residues to yield the 43 kDa α-subunit and the 15 kDa β-subunit (Fig. 1). The 43 kDa α-chain is further processed to render the smaller 20 kDa α-chain sequence found in 31 kDa inhibin. Whether the latter processing occurs prior to or after dimerization is unknown, but contact with serum results in conversion of 58 kDa inhibin to the 31 kDa form (McLachlan et al., 1986b).

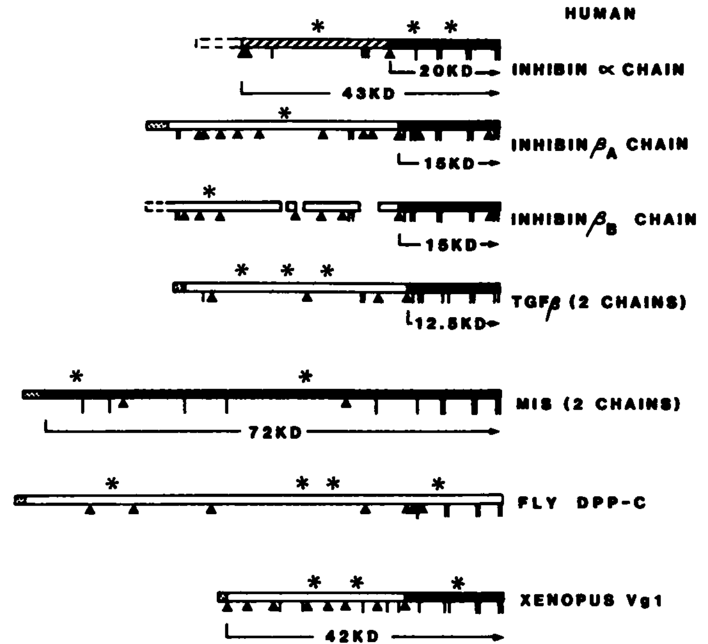

FIG. 1. This diagrammatic representation shows the structure of inhibin, transforming growth factor β (TGFβ), Müllerian Inhibiting Substance (MIS), the decapentaplegic gene complex of drosophila (DPP-C), and the Xenopus VG_1 gene. The *solid bar* indicates the isolated portion of the molecule. Note the cysteine residues *(two vertical black bars)* the dibasic processing sites (β), and potential glycosylation sites (*). The extended α-subunit of inhibin forming part of the 58 kDa form of inhibin is shown *(diagonal stripes)*.

The structure of the genes encoding for the inhibin subunits of human, ovine, and rat inhibin has been obtained recently (Mason et al., 1986; Mayo et al., 1986; Stewart et al., 1986; Bardin et al., 1987; Woodruff et al., 1987). In humans, sheep, and rats, the presence of the α, β_A, and β_B-subunits have been identified, but in cattle, only mRNA for the β_A subunit has been found, although the β_B-subunit sequence is present in genomic libraries.

Significant homology exists between the structure of the α- and β-chain within and between species (Forage et al., 1987). The sequences of the α-chain show 85% homology between cattle, humans, and pigs. Even greater conservation is seen with the β_A-subunit, in that a single amino acid difference was found in the ovine from that seen in the bovine, porcine, rat, and human molecules. Where it is present, the β_B-subunit shows over 85% conservation of structure.

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Inhibin β-Subunit Dimers (Activin A and AB)

Recently, two proteins that stimulate FSH secretion from the pituitary gland were isolated independently from pFF. These substances were shown to be dimers of the β-subunits of inhibin and have been termed Activin A ($\beta_A \beta_A$) and Activin AB ($\beta_A \beta_B$) (Ling et al., 1986; Vale et al., 1986). The concentrations required to half-maximally stimulate FSH are 0.4–1 ng/ml, and the EC_{50} is comparable to inhibin (0.3–1 ng/ml) and tenfold less than that found for gonadotropin-releasing hormone (GnRH) (Vale et al., 1986). Activin A also has been isolated from bFF and shows similar in vitro characteristics (McLachlan et al., 1987b). No data are available to indicate whether activin is secreted into the bloodstream or whether it acts only locally as a regulatory molecule (Hutchinson et al., 1987). However, it should be noted that castration in both males and females removes negative feedback and not positive feedback on pituitary gonadotrophin secretion, suggesting that if activin circulates, its action on FSH secretion is obscured by inhibin.

The structure of the inhibin subunits also has significant homology to a series of other proteins (Fig. 1), namely transforming growth β (TGF β), Müllerian Inhibiting Substance (MIS), the decapentaplegic gene complex in Drosophila, and the VG_1 gene in Xenopus (Derynck et al., 1985; Mason et al., 1985; Cate et al., 1986; Padgett et al., 1987; Weeks and Milton, 1987). The homology is particularly evident in domains surrounding the highly conserved cysteine residues. The homology of inhibin to proteins with widespread actions on a number of tissues raises the possibility—for which evidence is emerging—that inhibin has actions that extend beyond its proposed role in the feedback regulation of gonadotropin secretion.

Other Peptides with FSH-Inhibiting Properties

Seminal plasma inhibins. The existence of inhibin-like activities in seminal plasma has been found in several studies from a number of species (Franchimont, 1972; Franchimont et al., 1975; Thakur et al., 1978). Two distinct proteins showing inhibin-like activity in some bioassays have been isolated. From human seminal plasma, Thakur et al. (1978) isolated a glycosylated 19 kDa protein. This protein, termed β-inhibin has been shown to be structurally identical to that of a sperm-coating antigen called β-microseminoprotein of prostatic origin (Johansson et al., 1984; Akiyama et al., 1985). Recent studies have raised doubts about its capacity to suppress FSH (Gordon et al., 1987).

The second protein, also isolated from human seminal plasma, has been termed α-inhibin. Three molecular mass species (35, 52 and 92 kDa) have been identified and all three suppress the GnRH-stimulated FSH rise by whole mouse pituitaries in culture and also suppress the FSH levels in castrated rats (Seidah et al., 1984; Li et al., 1985). Conflicting claims regarding the bioactivity of a synthetic 31 amino acid peptide have been made (Yamashiro et al., 1984; Liu et al., 1985). The sequence of this peptide has been shown to be the NH_2-terminal part of a larger molecule, namely the major degradation product of the gel-forming protein of semen secreted by the seminal vesicle (Lilja and Jeppsson, 1984). Recent studies have shown that the larger molecule has FSH-suppressing activity in vivo (Yu et al., 1988).

Neither Inhibin-α nor Inhibin-β bears any structural similarity to inhibin as isolated from follicular fluid and their low biological activity raises serious questions as to their role in the control of FSH. Furthermore, since inhibin is regarded as a product of the gonads, the use of the term inhibin in the nomenclature of these seminal plasma-derived proteins is unwise.

FSH-suppressing protein (FSP or follistatin). During the purification of inhibin from bFF and pFF, a fraction was found that suppressed FSH in the inhibin bioassay but had no immunological activity in specific inhibin radioimmunoassays. We have isolated three molecular mass species of 31, 35, and 39 kDa from bFF, all with a common NH_2-terminal sequence that show no homology to the α, β_A, or β_B-subunits of inhibin (Robertson et al., 1987). In studies using pFF, Ueno et al. (1987) identified two molecular mass species of 32 and 35 kDa that also show no homology to inhibin. The bioactivity of these substances is 10–30% that of inhibin.

The porcine molecule, termed follistatin, has been cloned and shown to be the product of one gene, with the deduced amino acid sequence demonstrating homology to a human pancreatic trypsin inhibitor (Esch et al., 1987). The role of this protein in the physiology of the ovary and the feedback regulation of FSH must await further studies.

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Assays for Inhibin

Attempts to isolate inhibin depended on the development of specific assays based on the suppression of FSH. Many of the bioassays developed were not adequately defined in terms of specificity, sensitivity, and precision and failed to take into account that the use of the inhibition of FSH as the endpoint of the assay could lead to nonspecific effects. Following the purification of inhibin, radioimmunoassays have now been developed that offer relatively rapid, practical, and sensitive methods to measure inhibin.

In vitro bioassay. The rat pituitary cell bioassay for inhibin has been used in a number of studies measuring either the release of FSH into the medium (Eddie et al., 1979) or the changes in the FSH cell content (Scott et al., 1980) as the endpoint in the system. Recently, a sensitive bioassay has also been developed that used ovine pituitary cells in culture, which enables the measurement of bioactive inhibin levels in serum in some physiological situations (Tsonis et al., 1986). The demonstration that follicular fluid, and potentially serum, may contain activin or FSP raises significant problems with biaossay potencies, since the presence of activin could lead to the underestimation of inhibin potencies and the presence of FSP would result in an overestimation.

Radioimmunoassay. The demonstration that the synthesis of the inhibin subunits are controlled by separate genes raises strong possibilities that free α- and β-subunits may eixst at the site of production of inhibin or may pass into the circulation. Furthermore, the identification of β-subunit dimers of inhibin also raises questions of specificity in the radioimmunoassays for the inhibin molecule. It is important that any assay system developed should clearly specify the cross-reactivities of free α- or β-subunit and inhibin-related proteins. Two approaches have been used in the development of radioimmunoassays for inhibin—immunization with the native molecule and immunization with short synthetic peptides derived from the sequence of inhibin. The first radioimmunoassay for bovine and human inhibin was generated with antisera developed to 58 kDa and 31 kDa bovine inhibin (McLachlan et al., 1986b,c). Two further assay systems with increased sensitivity have been developed in our laboratory with the 31 kDa form of bovine inhibin used as the immunogen (McLachlan et al., 1987a,b; Robertson et al., 1988b). These antisera have been used in heterologous system, 31 kDa bovine inhibin acting as the tracer in the measurement of human, rat, ovine, and porcine inhibin. These assays do not cross-react to a significant degree with activin A, TGFβ, MIS, or the free α- and β-subunits of inhibin obtained after reduction and alkylation of the native molecule. More recently, Hasegawa and colleagues (1987) used 32 kDa porcine inhibin and partially purified bovine inhibin to develop specific radioimmunoassay systems that are applicable to the measurement of rat, porcine, and bovine inhibin in serum.

Several investigators have used synthetic peptides to develop assays for inhibin; the most common is immunization with a peptide consisting of the first 26 amino acids of the α-subunit of porcine inhibin (Rivier et al., 1986). These assays have been capable of measuring the concentrations of rat and porcine inhibin, the results being reported in terms of the mass of synthetic peptides rather than the use of a native inhibin standard. While excluding cross-reactivity to free β-subunit and activin, these assays have the potential to significantly cross-react with free α-subunit.

Mechanism of Action

Although a number of investigators have demonstrated suppression of FSH in vivo and in vitro by impure preparations of inhibin, these results should be viewed with caution since activin and FSH-supporting protein (FSP) are present in follicular fluid, which commonly was used as the source of the impure inhibin preparations. However, in a recent study, the infusion of pure 31 kDa bovine inhibin to castrated sheep demonstrated a dose-related FSH suppression (Findlay et al., 1987).

Similarly, a number of investigators have explored the action of inhibin in vitro on pituitary cells or on pituitary halves in culture but have utilized impure preparations from both male and female sources (see Hudson et al., 1979, for review). As with the in vivo studies, these results should be interpreted with caution due to the presence of activin, inhibin, and FSP in the active fractions. The studies showed that these preparations have an inhibitory effect on basal FSH release, cell content, and synthesis with minor effects on LH, thyroid-stimulating hormone (TSH), prolactin, and growth hormone. Furthermore, they demonstrated proportionally greater effects on FSH release following GnRH stimulation, but many studies also showed an effect on LH release under the

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same conditions. Recently, two studies using purified inhibin from follicular fluid have confirmed these effects on pituitary cells in culture (Robertson et al., 1986b; Fukuda et al., 1987).

In our recent studies, we demonstrated that to understand the effects of inhibin on FSH and LH secretion, a knowledge of the behavior of pituitary cells in culture is required (Farnworth et al., 1988a,b). These studies showed that during prolonged culture there is a continued, steady release of basal FSH that is tenfold greater than that of LH. Although the FSH cell content decreases, there is a net increase in the accumulation of FSH in the culture well. In contrast, both LH release and LH cell content decrease markedly during prolonged culture, leading to a net decrease of LH in the culture well. The addition of 31 kDa bovine inhibin to the culture results in a progressive and dose-dependent suppression of basal FSH release that is totally inhibited within 6 h and is the most sensitive parameter to the action of inhibin. These results support previous studies using crude bovine follicular fluid on rat pituitary halves (Jenner et al., 1983). This suppressive effect on FSH release could be maintained in culture for at least 25 days. At fivefold higher inhibin concentrations, the FSH cell content is also suppressed, frequently to levels below that of control cultures, indicating that inhibin promotes intracellular degradation of gonadotrophins. At similar concentrations of inhibin, LH release and cell content–though low are also suppressed by inhibin in a dose-dependent manner.

These results indicate two separate mechanisms of the action of inhibin on FSH secretion: at low concentrations, inhibin rapidly suppresses FSH release and synthesis; at a higher concentration, the cell content of both gonadotropins is affected by the degradation of intracellular stores of FSH and LH. In a recent study, Fukuda et al. (1987) demonstrated that inhibition of protein synthesis by cycloheximide can mimic the action of inhibin in causing a decrease in FSH secretion, supporting the view that inhibin causes an inhibition of FSH biosynthesis. Earlier studies had demonstrated that these effects are reversible after the removal of inhibin from the culture medium (Scott et al., 1980; Jenner et al., 1983).

Several studies using impure preparations of inhibin have demonstrated that inhibin decreases the GnRH-stimulated release of FSH and LH (de Jong et al., 1979; Eddie et al., 1979; Scott et al., 1980; Jenner et al., 1983). Recent studies using pure inhibin preparations have also confirmed that inhibin exerts suppressive effects on the release of FSH/LH following GnRH stimulation (Robertson et al., 1986b; Farnworth et al., 1988b). The mechanism of this inhibition is interesting because it also involves a decrease in the sensitivity of cells to GnRH and a decrease in the maximal GnRH-stimulated FSH and LH secretion (Farnworth et al., 1988b). It is of interest that this effect of inhibin is antagonised by the GnRH agonist, Buserelin, raising questions as to the manner in which inhibin affected GnRH-stimulated gonadotropin release. Recent studies by Wang et al. (1988a,b) have shown that inhibin decreases the number of GnRH receptors on pituitary cells in culture and also diminishes the up-regulation of GnRH receptors by GnRH.

STUDIES IN THE FEMALE

Source of Inhibin

The demonstration that follicular fluid is a potent source of inhibin activity (de Jong and Sharpe, 1976) occurred simultaneously with the demonstration that rat granulosa cells in culture produce bioactive inhibin (Erickson and Hsueh, 1978). Subsequently, a number of studies using both in vitro bioassay and radioimmunoassay methods have confirmed these results in rats (Hermans et al., 1982; Croze and Franchimont, 1984; Sander et al., 1984; Bicsak et al., 1986; Zhang et al., 1987a), cattle (Henderson and Franchimont, 1981), pigs (Channing et al., 1982), humans (Channing et al., 1984), and other primates (Noguchi et al., 1987). Several studies have shown that the amount of inhibin produced by granulosa cells from large follicles is greater than that from small follicles (Channing et al., 1982), and this fact is reflected in the concentrations of inhibin in the follicular fluid (Tsonis et al., 1983). In their study, Tsonis et al. (1983) showed that inhibin activity correlates significantly with the estradiol concentrations and aromatase activity of the granulosa cells. Recent radioimmunoassay studies have demonstrated that a similar good correlation can be obtained between the circulating levels of estradiol and inhibin in women undergoing ovarian hyperstimulation for in vitro fertilization (IVF) (McLachlan et al., 1986c). That study also demonstrated that the circulating inhibin levels reflect the mass of active granulosa cells

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in the ovary, since they correlate significantly with the number of follicles detected by ultrasound.

Recent studies have also shown that the corpus luteum is a significant source of inhibin in the luteal phase of the menstrual cycle (McLachlan et al., 1987a,b). This view has been supported by in vitro studies demonstrating that human granulosa cells allowed to luteinize in culture have the capacity to produce inhibin: these cells can no longer be stimulated by FSH but respond to LH and testosterone (Tsonis et al., 1987). Additional evidence that the luteal cells can produce inhibin comes from the demonstration that the rat and human luteal cells contain mRNA for the α-subunit of inhibin (Davis et al., 1986, 1987) and that inhibin can be detected in these cells by immunocytochemistry using an antiserum to a synthetic fragment of the porcine inhibin α-subunit (Cuveas et al., 1987; Merchenthaler et al., 1987).

Confirmation that the ovary is the major source of circulating inhibin comes from the rapid disappearance of serum inhibin levels following oophorectomy (Fig. 2) (Robertson et al., 1988a) and is of importance since mRNA for the α- and β-subunits of inhibin has now been demonstrated in a number of extra-gonadal tissues (Meunier et al., 1988). The initial half-time of disappearance of inhibin in these studies is 15 min; this value correlates well with that obtained by the bioassay measurements of inhibin after ovarectomy in pregnant mare's serum gonadotropin (PMSG)-stimulated rats (Lee et al., 1982).

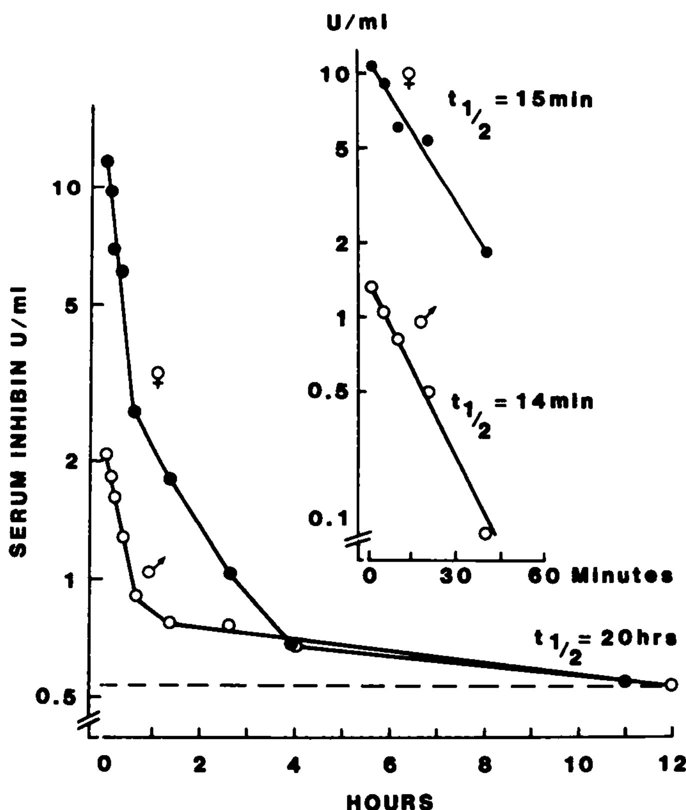

FIG. 2. The disappearance of inhibin from the circulation after castration is shown with radioimmunoassay measurements. (Reproduced with permission from Robertson et al., 1988, Mol. Cell. Endocrinol. In press.)

Control of Inhibin Secretion by Granulosa Cells

Several studies by Lee and colleagues (1981, 1982), using bioassay measurements of inhibin in the circulation and in ovarian cytosols, have demonstrated that FSH has the capacity to stimulate inhibin production. These data have now been confirmed by a number of studies using granulosa cells in culture (Bicsak et al., 1986; Zhang et al., 1987a). The latter studies demonstrated that in addition to FSH, IGF-1 can stimulate inhibin secretion by the granulosa cells in vitro, but the cells are not responsive to LH or prolactin. However, if the granulosa cells are kept in culture for 2 days in the presence of FSH, the induction of LH receptors results in the capacity of LH or human chorionic gonadotropin (hCG) to further stimulate inhibin production (Bicsak et al., 1986). Using bovine granulosa cells in culture, Henderson and Franchimont (1983) showed that in addition to FSH, testosterone also stimulates inhibin secretion, and that this effect can be reversed by anti-androgens. These effects of FSH on inhibin secretion appear to be mediated through a cyclic adenosine 3', 5'-monophosphate (cAMP) mechanism, since inhibin production by rat granulosa cells can be stimulated by cAMP analogues, phosphodiesterase inhibitors, and by forskolin (Bicsak et al., 1986). Inhibin secretion by both bovine and rat granulosa cells can be suppressed by epidermal growth factor (Franchimont et al., 1986; Zhang et al., 1987b).

Levels of Inhibin during Reproductive Cycles

Menstrual cycle. The availability of sensitive radioimmunoassays has enabled the measurement of circulating levels of inhibin during the menstrual cycle. Studies by McLachlan et al. (1987a,b) demonstrated that inhibin increased late in the follicular phase, reaching a peak coincident with the LH surge.

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The levels subsequently declined slightly, then rose to peak in mid-luteal phase to levels approximately twice those seen at mid-cycle. Late in the luteal phase, consistent with the decline in corpus luteum function, the levels decrease prior to the onset of the next menstrual cycle. This study demonstrated significant inverse relationships between FSH and inhibin during both the follicular and luteal phases of the cycle, observations consistent with the concept that inhibin is a feedback regulator of FSH secretion. The levels of inhibin correlate positively with both estradiol and progesterone levels in the luteal phase, providing circumstantial evidence that the corpus luteum produces inhibin. The declining levels of inhibin prior to the next menstrual cycle, rather than the declining levels of estradiol and progesterone, may provide the major signal for the rise in FSH that occurs prior to the onset of menstruation and presumably is involved in the stimulation of follicular development for the next cycle.

The inverse relationships between inhibin and FSH are in accord with the concept of a tropic hormone and its feedback substance. This relationship is supported by a number of studies (Stouffer and Hodgen, 1980; di Zerega et al., 1981) using follicular fluid as an impure source of inhibin showing that the administration of such extracts to rhesus monkeys in the follicular phase of the menstrual cycle decreases FSH and interferes with folliculogenesis, leading to reduced follicular phase levels of estradiol and lower luteal phase levels of progesterone. It is also in keeping with the inhibition of the post-castration rise of FSH and, to a lesser extent, of LH in female rhesus monkeys by follicular fluid extracts (Schenken et al., 1984). Studies in other species have also demonstrated that impure preparations of inhibin can suppress FSH during proestrus and estrus (de Paolo et al., 1979; Hermans et al., 1982a; Hoffman et al., 1979) and also have shown that this produces a decrease in FSH release in response to GnRH (de Paolo et al., 1979).

Estrus cycle. Using a heterologous radioimmunoassay system, Hasegawa et al. (1987) noted that inhibin levels in the circulation of rats during the estrus cycle are high during the follicular phase but drop sharply during proestrus, suggesting that the proestrus rise of FSH can be attributable to a fall in inhibin secretion. This view would be in accord with a recent study by Woodruff et al. (1988) demonstrating that mRNA for the α- and β-subunit of inhibin rises steadily during diestrus to peak late in the afternoon of proestrus. However this study did not show the presence of mRNA for the inhibin subunits in the corpus luteum from cycling animals in contrast to earlier studies (Davis et al., 1986; Cuevas et al., 1987; Woodruff et al., 1987) that detected inhibin subunit mRNA and inhibin by immunocytochemistry in the rat corpus luteum.

Inhibin in pregnancy. A striking difference has been seen in the inhibin levels in women on an IVF program when pregnancy occurred. The decline in inhibin levels late in the luteal phase of normal menstrual cycles is not seen in conception cycles, the levels of inhibin being maintained and in fact increasing significantly (McLachlan et al., 1987a,b). The difference between the patterns of conception and nonconception cycles first become discernible on the ninth day after oocyte retrieval and coincides with the first detectable rise in hCG levels in these women. Studies in three women with no ovaries who achieved pregnancy after oocyte donation showed that the inhibin levels during early pregnancy are similar to those in women with ovaries who achieve pregnancy (McLachlan et al., 1987b). These results strongly suggest that the rise of inhibin during the latter part of conception cycles do not involve continued corpus luteum secretion but result from the production of inhibin by the early embryo or the decidua. An embryonic source of inhibin is supported by our studies demonstrating that the placenta has the capacity to produce both immunoactive and bioactive inhibin (McLachlan et al., 1986c). Observations by Mayo et al. (1986) and Davis et al. (1987) indicating that the placenta contained mRNA for the α- and β_A-subunits and of inhibin strongly support this view. In subsequent studies, Petraglia et al. (1987) showed that placental cultures have the capacity to produce inhibin and that this substance can be localized immunocytochemically to the cytotrophoblast of the placenta, a view confirmed by Merchenthaler et al. (1987). Although the role of inhibin early in pregnancy remains to be elucidated, the significant homology that was noted between inhibin and the decapentaplegic gene complex in Drosophila and the VG_1 gene of Xenopus, both of which are involved in early embryogenesis, raises the possibility that inhibin may also be involved in embryonic differentiation. This view is supported by the recent demonstration that TGFβ, a protein with significant homology to inhibin, plays an important role in various embryonic events (Heine et al., 1987).

Inhibin levels during sexual maturation. Using

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radioimmunoassay, Rivier and Vale (1987) demonstrated a slow increase in inhibin levels in the circulation of female rats until Day 17, after which levels rise dramatically, reaching values at Day 30 that are not statistically different from those of adult female rats. The dramatic rise between Days 15 and 30 correlates inversely with the FSH levels, which fall during this period. In girls passing through puberty, the levels of inhibin rise progressively during pubertal maturation and correlate positively with stage of puberty and the levels of FSH (Burger et al., 1989). The latter observation suggests that the pubertal transition involves a reestablishment of new feedback relationships between FSH and inhibin.

Pathophysiological conditions in the female. Several investigators have speculated that in patients with polycystic ovarian syndrome elevated serum inhibin levels may be involved in producing the elevated LH and somewhat suppressed FSH levels (Tanabe et al., 1983; Franks et al., 1985). In a recent study of five subjects with polycystic ovarian disease, Buckler et al. (1988) demonstrated that inhibin levels are not significantly different in the early or mid-follicular phase and that the inhibin levels in the late-luteal phase, at mid-cycle, and during the mid-luteal phase are significantly lower than in normal cycles. Furthermore, the normal subjects and those with polycystic ovarian disease show no differences in inhibin levels following the endogenous gonadotropin rise and effective gonadotropin withdrawal that occurs after continued administration of luteinizing hormone-releasing hormone (LhRH) agonists. These studies strongly suggest that there is no primary defect of inhibin secretion in the polycystic ovarian disease syndrome.

STUDIES IN THE MALE

Source of Inhibin

The study by Steinberger and Steinberger (1976) showing that immature Sertoli cells in culture produce a substance capable of suppressing FSH has now been extended by extensive bioassay and immunoassay data confirming that these cells are the site of inhibin production in the testis (Le Gac and de Kretser, 1982; Verhoeven and Franchimont, 1983; Ultee van-Gessel et al., 1986; Bicsak et al., 1987). These observations confirm earlier studies that seminiferous tubules in culture (Eddie et al., 1978) and mouse organ cultures (Demoulin et al., 1979) can produce inhibin. The increase observed by Au et al. (1984a) in the inhibin content of the testis 24 h after efferent duct ligation also confirms that the Sertoli cells are the site of production of inhibin in vivo. That the testis is the major source of circulating inhibin in the male can be concluded from the demonstration that castration leads to a rapid decline of serum inhibin levels (Fig. 2) with an initial half time of 15 min (Robertson et al., 1988a). Further data supporting the view that the Sertoli cells are the major source of inhibin arise from the demonstration that mRNA preparations from the testis contain mRNA for the α-, β_A-, and β_B-subunits (Meunier et al., 1988; Toebosch et al., 1988).

Control of Sertoli Cell Production of Inhibin

Immature Sertoli cells in culture produce inhibin for prolonged periods (15–20 days) in the absence of hormonal stimulation (Le Gac and de Kretser 1982; Ultee van-Gessel et al., 1986). These cultures respond to FSH stimulation in a dose-dependent manner with an increase in inhibin levels detected by both bioassay and radioimmunoassay (Le Gac and de Kretser, 1982; Ultee van-Gessel et al., 1986; Bicsak et al., 1987). More recently, Gonzales et al. (1988), using segments of seminiferous tubules from adult rats in culture, showed that the adult Sertoli cell has the capacity to respond to FSH secretion in a dose-dependent manner. The demonstration that Sertoli cell production of inhibin is stimulated by analogues of cAMP and phosphodiesterase inhibitors strongly suggests that FSH stimulates inhibin by a cAMP-dependent mechanism (Bicsak et al., 1987). The decline in testicular bioactive inhibin levels following hypophysectomy in the rat is consistent with the requirement for FSH stimulation (Au et al., 1985). Au et al. (1985) have shown that the testicular content of inhibin after hypophysectomy can respond to FSH stimulation in vivo but does not respond to testosterone.

In general, the results of studies concerning the role of testosterone in the control of inhibin secretion are confusing. Le Gac and de Kretser (1982) showed no action of testosterone on inhibin secretion by immature Sertoli cells in vitro, but contradictory evidence was obtained by Verhoeven and Franchimont (1983) using a slightly different experimental design. More recent studies, extending over a large range of testosterone doses, have not demonstrated a significant effect of testosterone on basal

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inhibin secretion but, at doses of 10^{-5} and 10^{-6} M, a suppression of the FSH-induced rise of inhibin was found (Bicsak et al., 1987; Bardin et al., 1987; Gonzales et al., 1988). This failure of testosterone to significantly influence inhibin production in vitro is in accord with the failure of testosterone to cause a rise in the testicular inhibin levels 30 days after hypophysectomy (Au et al., 1985). Even if testosterone is given immediately after hypophysectomy, the testicular inhibin levels can not be stimulated, although the production of seminiferous tubule fluid is restored to normal (Au et al., 1986a). The rise in inhibin levels in serum following the destruction of Leydig cells by the cytotoxin ethane dimethane sulphonate (EDS) is in accord with an inhibitory influence of testosterone on inhibin secretion (de Kretser DM et al., unpublished results). However, restoration of testosterone levels in the circulation to the physiological range by the use of Silastic implants of testosterone that maintain normal FSH levels does not alter the rise in serum inhibin levels after the use of EDS. These data further strengthen the view that the rise in inhibin following EDS is related to the destruction of the Leydig cells or, alternatively, results from changes in the seminiferous epithelium induced by testosterone deprivation.

Although the above studies suggest that the role of testosterone is, if anything, inhibitory, we recently demonstrated that stimulation of the Leydig cells by hCG results in a significant rise in inhibin levels 24 h after the single injection (Drummond AE et al., unpublished results). This rise in inhibin does not occur if hCG is given to rats following the destruction of Leydig cells by EDS, indicating that the hCG-mediated rise of inhibin is due to stimulation of Leydig cells. This observation, together with the ineffective action of testosterone on inhibin production, strongly suggests that the Leydig cells may influence inhibin secretion by other mechanisms.

The results of a number of studies in men support the view that pituitary gonadotrophin support is vital for the maintenance of inhibin secretion; the levels of inhibin are low in patients with hypogonadotrophic hypogonadism and rise following stimulation by FSH and LH or, alternatively, when stimulation is achieved by pulsatile GnRH administration (Scheckter et al., 1988; Burger HG et al., unpublished results). Furthermore, when men are given clomiphene citrate, there is a significant rise of serum inhibin levels, presumably via the rise in FSH and LH (Tenover et al., 1988), and, in elderly men, the testes' ability to respond to clomiphene citrate in terms of inhibin secretion is impaired relative to the response in young men.

Removal of FSH and LH stimulation of the testis by the long-term administration of testosterone to normal men results in a decline in serum inhibin levels, emphasizing the importance of this stimulation in maintaining inhibin secretion by the testis (McLachlan et al., 1988). When FSH or LH is coadministered with testosterone, the suppressed levels of inhibin rise significantly, thereby specifically delineating a role for both FSH and LH in maintaining inhibin secretion; the stimulation by LH, presumably via the Leydig cells, is in accord with the rise in inhibin levels seen following hCG stimulation of rat Leydig cells (Drummond AE et al., unpublished results).

Local Actions of Inhibin in the Testis

The potential that inhibin may exert a significant local action has been explored using the incorporation of ^{3}H-thymidine by dividing a testicular cells in vitro. Using this model, Franchimont et al. (1981) and van Dissel-Emiliani et al. (1988) have demonstrated an inhibition of the incorporation of tritiated thymidine into spermatogonia using partially purified preparations of inhibin. This concept of a paracrine action requires further evaluation with purified inhibin preparations. The potential for inhibin to exert a local action on the meiotic process in the male also requires exploration in view of our recent demonstration that inhibin can inhibit germinal vesicle breakdown in mouse oocytes (O et al., 1989).

The concept that inhibin and activin may act on the Leydig cells to modify steroidogenesis has been explored by several groups. Hsueh et al. (1987) showed that inhibin stimulates and activin suppresses steroidogenesis in crude cultures of Leydig cells from immature rats and cultures of testicular cells from hypophysectomized rats. Using both crude and Percoll-purified Leydig cells, we have been unable to demonstrate any action of inhibin, activin, or FSP on basal or hCG-stimulated testosterone production (Risbridger GP et al., unpublished results). The reasons for these differing results is yet to be determined.

Inhibin Levels during Sexual Maturation

Our bioassay studies demonstrated that the testicular content of inhibin rises slowly over the first 10

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days, in parallel to testis weight (Au et al., 1986). This increase is probably due to the mitotic activity of Sertoli cells, resulting in an increase in the total number of Sertoli cells. Subsequently, inhibin levels increase markedly between 20 and 35 days of age; this increase correlates with the rising levels of FSH, in particular, but also with LH and testosterone. The levels subsequently decline slightly between 60 and 80 days of age. These results are in keeping with those demonstrated during the study of inhibin levels duirng pubertal maturation of boys, which show a strong positive correlation with FSH levels and stage of puberty. Correlations have also been noted with LH and testosterone levels (Burger et al., 1989). The data support a resetting of the relationship of FSH and inhibin after pubertal maturation.

Inhibin Levels after Spermatogenic Damage

The induction of spermatogenic damage by heat, cryptorchidism, or pressure atrophy following prolonged efferent duct ligation causes a decline in the bioassayable testicular inhibin levels (Au et al., 1983, 1984b, 1987). Recent studies using radioimmunoassay have confirmed that serum inhibin levels decline following the induction of cryptorchidism in rats, confirming the previous bioassay measurements. The decrease in inhibin levels closely parallel the decline in other parameters of Sertoli cell function, such as those of seminiferous tubule fluid production and androgen-binding protein secretion (Hagenas and Ritzen, 1976; Jegou et al., 1983).

In our study (Au et al., 1987), which utilized a single exposure of the rat testis to heat (43°C for 15 min), we demonstrated that inhibin levels in the testis, along with other markers of Sertoli cell function, do not change for 7 days after the heat treatment. They subsequently decline reaching a nadir 21 days after the treatment, and then rise in conjunction with the recovery of spermatogenesis. That we did not observe a change in these parameters of Sertoli cell function, including inhibin, for 7 days after the heat treatment strongly suggests that these parameters are not directly due to the acute detrimental effect of heat on Sertoli cell function. The changes suggest that the alteration in Sertoli cell function may well result from the loss of a germ cell type from the epithelium. The pattern of results correlates strongly with the loss of spermatids from the seminiferous epithelium. Further studies are clearly necessary to investigate the paracrine mechanisms that may be involved in the control of inhibin production by the Sertoli cell in vivo.

The expected inverse correlations that occur between FSH and inhibin levels in the rat during testicular damage are consistent with the concept that inhibin acts as a feedback substance for FSH secretion. However, our results, from radioimmunoassay measurements of inhibin levels in serum in men with testicular disorders, have not confirmed this relationship (Burger et al., 1989; de Kretser et al., 1989). The mean levels of inhibin in serum in groups of men with testicular disorders of varying severity do not change. No correlation has been observed with serum FSH levels, testicular size, or sperm count. The failure to demonstrate a close relationship between FSH and inhibin levels may result from the fact that FSH is controlled by both testosterone and inhibin levels (see below). Several studies have suggested that Leydig cell function is impaired in men with testicular disorders of increasing severity, as evidenced by elevated LH levels and marginally low testosterone levels (de Kretser et al., 1972, 1975; Hunter et al., 1974). The recent study by Booth and colleagues (1987) has confirmed that the production rates of testosterone in men with testicular disorders decline by 30%. It is possible that this decline in testosterone production causes a rise in FSH that in turn acts on responsive Sertoli cells to stimulate inhibin levels: these changes would therefore obscure any direct relationship between inhibin and FSH. Alternatively, a decline in inhibin levels, transiently resulting from testicular damage, could result in an elevation of FSH levels, which in turn stimulate the impaired Sertoli cell to produce normal levels of inhibin but at a higher setting of FSH, namely a state of compensated Sertoli cell failure. More radically, the recent demonstration by Lee and colleagues (1988) that the Leydig cell may have the capacity to produce activin, which—if released into circulation—may stimulate FSH, requires a reevaluation of the pituitary testicular relationships during spermatogenic disruption.

The Role of Inhibin and Testosterone in the Control of FSH

Much of the early argument against the existence of inhibin was that testosterone had been demonstrated to inhibit FSH secretion, as shown by progressive suppression of both FSH and LH by increasing doses of testosterone (Lee et al., 1972; Baker et al.,

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1976; Decker et al., 1981; de Kretser et al., 1987). However, in studies in rats, the use of very high doses of testosterone in castrated animals—while suppressing LH levels to the undetectable range—does not lower FSH levels below the normal range (Decker et al., 1981; de Kretser et al., 1987). These results support a differential effect of testosterone on LH and FSH secretion. The rise in FSH that accompanies the lowering of testosterone levels when Leydig cells are destroyed with the drug EDS strongly supports the view that testosterone can suppress FSH secretion (Jackson and Morris, 1977; Jackson et al., 1986). However, it should be noted that FSH levels in these normal rats treated with EDS rises only to 50% of levels in castrated rats, indicating the existence of an inhibitory effect of the testis on FSH levels. This view would be consistent with the demonstrated rise in serum inhibin levels following treatment of normal rats with EDS (de Kretser DM et al., unpublished results). The concept of a dual role of inhibin and testosterone in the control of FSH secretion is further supported if EDS is given to rats that are already cryptorchid. In this experimental model, the already elevated FSH levels, induced by a decrease in inhibin levels following the induction of cryptorchidism (Au et al., 1983), rise to the range exhibited by castrated rats following the removal of testosterone by the action of EDS (O'Leary et al., 1986).

Further support for a dual control on FSH secretion comes from the studies of Plant (1982) who demonstrated that testosterone could completely abolish the rise in FSH and LH in rhesus monkeys following castration. However, during a recent reevaluation of this relationship, Dubey et al. (1987) noted that if GnRH secretion is maintained by pulsatile administration in monkeys with arcuate nucleus lesions, testosterone alone can not control the post-castration rise of FSH. These results support a role for inhibin in the control of FSH, a postulate that was recently supported by the experiments of Abeywardene et al. (1988), which demonstrated that infusions of porcine follicular fluid are able, in this model, to suppress the post-castration rise of FSH.

Extra-Gonadal Actions of Inhibin and Activin

Observations by Meunier et al. (1988) have supported the view that inhibin and activin may have more widespread actions than those in relationship to the reproductive system, showing that the α- and β-subunits of inhibin can be demonstrated in a large number of tissues such as the brain, spleen, adrenal, pituitary, kidney, and bone marrow.

Action on hemopoiesis. Eto and colleagues (1987), during investigations of a factor causing erthryroid differentiation of leukemic cell lines, noted that the isolated active protein has the same structure as Activin A. This is consistent with the demonstration that the β-subunits of inhibin can be identified in bone marrow cells (Meunier et al., 1988). More recently, the action of Activin A on erythroid differentiation in normal bone marrow cells has been shown by Yu and colleagues (1987), who noted that activin synergized with erythropoietin in stimulating erythroid differentiation. These investigators also noted that inhibin opposes this action of activin.

Lymphoid tissue. We have recently shown that inhibin stimulates the uptake of ^{3}H-thymidine into rat thymocytes both in the presence and absence of the lectins phytohaemagglutinin and concanavalin A (Hedger et al., 1989). This action occurs with an ED_{50} of 0.74 nM and is inhibited by bovine Activin A (ID_{50}, 0.35 nM) and by porcine TGFβ (ID_{50}, 3.7 pM). The observation that bovine Activin A stimulates ^{3}H-thymidine incorporation into 3T3 fibroblasts contrasts with its action on thymocytes and indicates that the effects on cell proliferation are cell-type-specific (Hedger et al., 1989). The actions of inhibin and activin described above support an immunomodulatory role for these proteins.

Neural tissue. The demonstration of mRNA for inhibin in the brain and spinal cord strongly suggests a regulatory role for these proteins in the central nervous system (Meunier et al., 1988). This view has been supported by the demonstration that the neurons of the nucleus tractus solitarius that project to the paraventricular nucleus contain the β-subunit of inhibin as shown by immunocytochemical localization (W. Vale, personal communication).

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