MINIREVIEW

Paracrine Regulation of Reproductive Function by Inhibin and Activin (43473)

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The term inhibin was first suggested by McCullagh in 1932 (1) to describe the active principle of an extract of bovine testis that could inhibit the appearance of "castration cells" in the pituitary after irradiation of the testis. The discovery of the existence of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) as distinct pituitary hormones led to the definition of inhibin as a substance of gonadal origin that specifically decreases FSH release from the pituitary without altering LH release. Data continued to accumulate for the existence of a nonsteroidal feedback regulator from the gonads to the pituitary (2–5). However, the isolation of this activity continued to prove extremely difficult (for review, see Ref. 5).

The purification and cloning of inhibin in 1985 has allowed confirmation of inhibin as a factor produced in the testis and ovary that regulates FSH release and synthesis by the pituitary *in vitro* and *in vivo*. The last 6 years have seen rapid advances in our understanding of the biochemistry and physiology of inhibin and the structurally related activin peptides (for reviews, see Refs. 6–13).

Inhibin is a 32-kDa dimeric protein composed of an α-subunit and one of two related β-subunits (βA and βB). Both inhibin A (α-βA) and inhibin B (α-βB) suppress FSH release from the anterior pituitary. The α-subunit is produced as a pre-pro-α having a 20-amino-acid signal sequence (pre) followed by an upstream region. The mature α-subunit (called αC) follows dibasic proteolytic cleavage sites. Alternative cleavage products can be secreted as monomers (called αN and pro αC) (6, 14, 15) (Fig. 1). Pro-α monomers have been shown to inhibit FSH binding, but they have no direct effect on FSH synthesis or release (16).

A higher molecular weight form (58 kDa) of dimeric inhibin, which is also bioactive in the FSH-release assay, has been purified from bovine follicular fluid (17). Bovine 58-kDa inhibin is a dimer of αN-αC and βA. Larger molecular weight forms of inhibin-related molecules have been identified in bovine (18), porcine, and human (19) follicular fluid. Inhibin-related species of 120-kDa, 108-kDa, 88-kDa, and 65-kDa are detected in bovine follicular fluid. The inhibin-related molecules inhibit FSH release from anterior pituitary cells. Furthermore, they react with antisera directed to determinants belonging to both the α- and βA-subunit of 32-kDa inhibin (18).

Inhibin β-subunit mRNAs are translated into pre-pro-β forms, which are proteolytically processed to the mature β-subunits. In addition to forming inhibins, the β-subunits can assemble into three hormones: activin A (βAβA), activin AB (βAβB), and activin B (βBβB) (Fig. 1). Activin was purified from follicular fluid as a stimulator of FSH release from pituitary cell cultures (20, 21) and has more recently been shown to stimulate FSH release *in vivo* (22, 23). Subsequently, activin A was isolated from conditioned medium of the human leukemia cell line THP-1 as an erythroid differentiation factor (24). Activin is now recognized as a regulator of a variety of actions, including nerve survival (25), neuronal differentiation (26), and mesoderm induction in Xenopus (27, 28). Moreover, exogenous activin A can induce axial structures in Xenopus and chick embryos (29, 30). Activin A mRNA is expressed early in rat embryonic development (31) in a number of tissues, including the brain, and is likely to play an important part in mammalian embryonic development of non-gonadal tissues.

All three inhibin subunits are highly conserved in evolution (14). Between the two β-subunits, there is a

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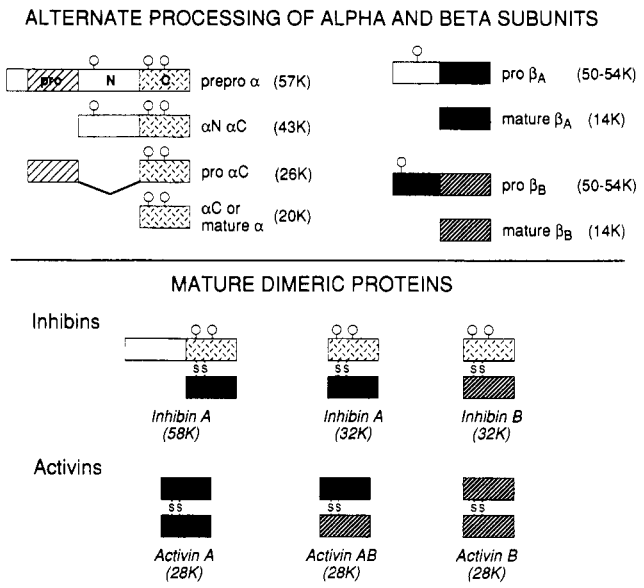

Figure 1. Schematic representation of inhibin α- and β-subunit processing and combination to form mature inhibin and activin polypeptides. Potential glycosylation sites for human inhibin are indicated by the open circle.

high degree of conservation of both the mature polypeptides and the prohormone forms across human, bovine, porcine, and murine species. For example, the mature βA is 100% conserved between the human and rat. There is also a strong degree of conservation between the sequences of the human and Xenopus βA (27, 28). In addition, within a given species, the mature βA and βB subunits are also closely related, having 70% homology in both pigs and humans (32, 33).

The homology in the mature α-subunits is in the range of 86–95% among the human, ovine, bovine, porcine, and rat sequences (13, 14). Two glycosylation sites exist on the human α-subunit and one on the α-subunit from other species. The βA- and βB-subunits are not glycosylated. There are seven cysteine residues in the mature α-subunit and nine each in the β-subunits. The α- and β-subunits show homology in the region of the cystine residues (14). Disulfide cross-linking of the subunits is required for FSH regulating activity.

Inhibin and activin are recognized to be part of a larger superfamily that includes transforming growth factor (TGF)-β, müllerian inhibiting substance, the decapentaplegic gene complex of Drosophila, the bone morphogenic proteins, and the vegetal growth factor gene of Xenopus (34–38).

Inhibin and Activin as Paracrine Factors

In the last decade, there have been rapid advances in our understanding of the importance of paracrine factors in regulating testicular (39–42) and ovarian (43–47) function. These factors include substances thought to be unique to the gonads, such as müllerian inhibiting substance (48), seminiferous growth factor (49), and P-Mod-S (50), and those more widely implicated in cell-cell communication, including fibroblast growth factor (51–54), nerve growth factor (55–57), insulin-like growth factors (47, 58–60), TGF-α (61), and interleukins (62).

Inhibins and activins were initially isolated from the gonads based on their abilities to modulate pituitary function *in vitro*. However, the hypothesis that these factors act solely as negative or positive feedback regulators of FSH secretion *in vivo* is inadequate to explain all of the available data. For example, serum inhibin levels and ovarian inhibin subunit mRNA expression are clearly inversely proportional to serum FSH levels during the secondary FSH surge of the rat estrous cycle, but not during the primary FSH surge (63, 64). Similarly, immunoneutralization of serum inhibin results in increased serum FSH levels only in immature male rats; it has no effect in adults (65, 66) with normal serum testosterone levels (67). Finally, there is little correlation between inhibin and FSH levels in disease states in the male (68).

Inhibin and activin are produced in, and act at, multiple sites in the testis (69–71). These observations, along with the widespread distribution of α- and β-subunit mRNA in a number of different organ systems (72) and the homology of the activins and inhibins with known paracrine regulators of cell growth and function (such as TGF-β), have led to the suggestion that inhibins and activins may act as paracrine regulators within the gonads, as well as other tissues (69–72).

Most recently, we and others have demonstrated specific binding of inhibin and activin in the ovary and testis, as well as in several other tissues in which inhibin subunit mRNA and inhibin-related proteins are produced (43, 73–77). This has provided additional support for the hypothesis that a significant, perhaps the major, role of activins and inhibins in the reproductive system is that of paracrine and/or autocrine regulatory factor controlling gonadal, pituitary, and placental function.

Technical Considerations

Several technical considerations need to be taken into account in interpreting the literature, particularly in cases in which conflicting reports exist. Inhibin and activin have been measured using a number of different methodologies, each of which is complicated by the unique relationship of these two factors, which are closely related biochemically but often have diametrically opposite biological activities.

A necessary first step in determining whether a factor can exert a paracrine action in an organ is to determine its site of production. Where the gene of interest has been cloned, this can be done using RNA blot (Northern) analysis or *in situ* hybridization, which allows more precise localization of mRNA to a specific cell type. Although this reflects the potential sites of

protein production, the presence of mRNA does not necessarily correspond to a particular activity. The situation is further complication by the fact that the existence of mRNA coding for the α- and β-subunits of inhibin cannot tell us whether that tissue is producing free α-subunit, inhibin, activin, or a combination of these.

Immunodetection is also widely used to localize or quantitate inhibin and activin production. This can be in the form of immunocytochemistry, Western blot assays, or antibody-based quantitative assays, such as radioimmunoassays. Here, too, the close biochemical relationship between inhibins and activins and the conservation of the molecules have made the task of identification a formidable one. Antibodies of high affinity that exhibit specificity for inhibin or activin alone have been difficult to obtain. Sufficient quantities of purified inhibin A, inhibin B, pro-α-derived peptides, activin A, activin B, and activin AB have not been available to determine the complete cross-reactivities of the available antisera with all members of the family. Different molecular weight forms of inhibin may also have different affinities for a given anti-inhibin antiserum (78). The most widely available antiserum (As. No. 1989) was raised to purified inhibin (79) and has been recently shown to have significant cross-reactivity with the free α-subunit of inhibin (80, 81). Since free α-subunit may circulate (81), data collected using this antiserum need to be reinterpreted in this light.

The final technical consideration is the detection of bioactive inhibin or activin in complex biological fluids or cell culture medium. The methods for detection and estimation of biologically active inhibin have been extensively reviewed by de Jong (9). The most commonly used of these is the suppression of FSH release by pituitary cells *in vitro*. This is a sensitive measure of inhibin capable of detecting picomolar levels. It can also be used as a bioassay for activin, which increases the release of FSH in these cultures.

Recent work in our laboratory using purified recombinant activin and inhibin has shown the net effect on FSH release in pituitary cell cultures to be a nonlinear sum of the concentration of the two factors (L. A. Krummen, unpublished data). The assay is strongly biased toward the detection of inhibin, which would explain why follicular fluid has an inhibin-like activity even though it is the source for the purification of activin. This creates obvious difficulties in using this assay to determine the absolute levels of bioactive inhibin in biological fluids containing mixtures of the two proteins.

Another bioassay for activin employs the human erythroleukemic cell line K562. Activin causes this cell line to differentiate along the hematopoetic pathway (82). A number of markers of differentiation (e.g., hemoglobin synthesis) may be used as the end point for the assay of activin (83). Inhibin alone has no effect in this assay, but will neutralize the effects of activin in a quantitatively linear fashion. However, even though this assay appears to be less biased toward measurement of inhibin than the pituitary cell assay, it is still difficult to obtain accurate quantitative measurements of activin in samples containing unknown concentrations of inhibin. Serum, or any heme-containing tissue extract, will interfere in this assay. The interaction of both hormones in these bioassay systems also presents difficulties in measuring changes in the ratio of biologically to immunologically active inhibin or activin in biological samples in which concentrations of both hormones might be altered as a result of treatment.

Finally, it has recently been recognized that at least two binding proteins exist for inhibin and activin: α-2 macroglobulin (84; L. A. Krummen, T. K. Woodruff, P. Cossum, and J. P. Mather, unpublished data) and follistatin (85). α-2 Macroglobulin is a protein present in high levels in human serum known to bind a number of growth factors, including TGF-β and nerve growth factor (86). It is presently unknown whether binding of activin or inhibin to α-2 macroglobulin alters the bioactivity or immunoreactivity of activin or inhibin.

The follistatins are three monomeric glycoproteins (molecular mass, 32, 35, and 39 kDa) originally isolated as inhibitors of FSH release (85, 87, 88). These proteins were then found to bind both activin (89, 90) and inhibin (91) through the β-subunit. Binding of activin to follistatin inactivates the bioactivity of this molecule *in vitro* (90); the biological activity of follistatin-bound inhibin has not been studied. Follistatin mRNA has been detected by Northern analysis in the ovary, uterus, testis, pituitary, cerebral cortex, kidney, adrenal, thymus, pancreas, heart, skeletal muscle, and lung (92), and follistatin protein has been isolated from both porcine and bovine follicular fluids (87, 88). Many of these tissues also express the inhibin subunit messages during embryogenesis (31) or in the adult (72). The secretion of follistatin *in vivo* may, thus, act locally as a terminator of activin or inhibin activity by binding free dimer. It is of note that FSH has been shown to stimulate bovine granulosa cell follistatin secretion *in vitro* (93) and that both follistatin mRNA and immunoreactive protein exhibit cyclic changes during the rat ovarian cycle (94). Thus, it seems that an understanding of the regulation of secretion of follistatin may be important in obtaining a complete picture of the physiological control of paracrine effects of activin and inhibin in reproductive, as well as other, tissues. Moreover, the ability of such binding proteins to interfere in immunodetection methods for both inhibins and activins needs to be carefully assessed in order to obtain a true picture of the physiologically important levels of these proteins in reproductive tissues and fluids.

The assay of inhibin and activin mRNA, protein, and biological activity all have their unique difficulties, which have contributed to some degree of confusion in

the literature. It is hoped that technical advances and the availability of more completely characterized reagents in the future will help resolve some of the questions that will arise below in the discussion of the available literature.

The remainder of this review will concentrate on the paracrine roles of inhibin and activin in the pituitary, testis, ovary, and placenta. Evidence will be reviewed regarding the site and mode of regulation of inhibin and/or activin production, the functional consequences of inhibin and/or activin activity within each tissue, and the distribution and specificity of local inhibin and/or activin binding sites. It should be kept in mind that both inhibin and activin may be produced at different sites and have different roles in regulating testicular and ovarian function during sexual differentiation in the embryo, in pubertal development, and in the adult.

Inhibins in the Testis

The testis was the first organ suggested as the source of inhibin (1). Indeed, inhibin has been partially purified from ram rete testis fluid and mRNA for all three subunits ($\alpha > \beta B \gg \beta A$) has been demonstrated in the testis of embryonic, prepubertal, and adult rats using either measurements of mRNA levels in testicular homogenates or by *in situ* hybridization (31, 95–99). These observations predict that the testis may produce both bioactive inhibin and activin throughout life. However, the cellular distribution of messages for the various inhibin/activin subunits, as well as the relative levels of inhibin and activin proteins present at critical times during prenatal and pubertal development, suggest that both the production and role of inhibin and activin in the testis may change during sexual development.

Inhibin and Activin Production and Paracrine Activity in the Testis: Embryonic Development

Expression of α- and βB-mRNA is seen using *in situ* hybridization in the gonad of the embryonic rat from Day 14 of gestation to birth (31). The α-subunit is localized in both the tubular and interstitial tissue, whereas the βB-subunit is seen only in the tubular tissue. At 20 days of gestation, a strong signal for βA-mRNA can be seen over the interstitial tissue. Activin can cause a rapid and specific re-aggregation of Sertoli-germ cell monolayers into tubule-like structures *in vitro* (74). It has been suggested that this may reflect a role of activin in tubular organization in development (74). An elucidation of the exact role of activin in testicular development awaits further study.

Prepubertal and Adult Testis, Sertoli Cell Production of Inhibin and Activin

Sertoli cells were thought to be the sole source of inhibin production in the testis for several decades, based on evidence of bioactivity derived from cultured Sertoli cells (3). Soon after the purification and cloning of the inhibin subunits, Sertoli cells derived from immature rats were shown to express mRNA for the α- and β-subunits of inhibin *in vitro* (19, 69, 96, 98, 100). Immunohistochemical localization of α- and βB-subunit protein (65, 99, 101) and *in situ* identification of α- and β-mRNA (99) in the seminiferous epithelium of prepubertal and mature rats also predict inhibin production by Sertoli cells in the adult testis.

Inhibin protein has been identified by immunoactivity and bioactivity in conditioned medium from Sertoli cell cultures derived from immature rats (78, 102–105) and from seminiferous tubule cultures from mature rats (106, 107). Most conclusively, inhibin has been purified from conditioned medium of cultured Sertoli cells from 21-day-old rats and shown to be 32-kDa inhibin B (104).

Initial studies suggested that inhibin secretion by Sertoli cells had a strong vectorial component, with the great majority of the inhibin being secreted apically (108–110). However, more recent studies suggest that this "vectorial secretion" is an artifact of the Millipore HA membrane used in the dual chamber culture system in these experiments and that secretion is actually bidirectional, with a more even distribution (111). Therefore, inhibin produced and secreted by the Sertoli cell *in vivo* may be available for interaction with surface receptors on cell types isolated inside the blood-testis barrier, as well as in the testicular interstitium.

The question of whether activin is produced by Sertoli cells *in vivo* has proven to be a more difficult one to answer. Sertoli cells in the adult contain the mRNA for both the α- and β-subunits and should thus be capable of secreting both activin and inhibin. However, it is possible that the expression of these subunits *in vivo* could be regulated in such a way as to favor the secretion of only one of these factors. Most studies to date on Sertoli cell inhibin secretion have used antisera specific for the α-chain of inhibin and would, therefore, not be expected to detect activin secretion. The pituitary bioassay, as discussed above, is strongly biased toward the detection of inhibin. Activin would have to be present in great excess to be detected using this assay. Recently, a 25-kDa protein that increases FSH release from pituitary cell cultures was partially purified from medium conditioned by Sertoli cells derived from 22-day-old animals (78). This strongly suggests that, at least in immature rats, Sertoli cells secrete both activin and inhibin.

FSH has been shown to be a major regulator of both immunoreactive and bioactive inhibin secretion and steady state levels of inhibin α (but not βB)-subunit mRNA in the seminiferous epithelium of immature rat *in vitro* (100, 102, 103, 111, 112). A regulatory role for FSH in controlling testicular production of inhibin α-subunit mRNA in adult animals has also been dem-

onstrated *in vivo* (96, 97). These data correlate well with reports that seminiferous tubules from adult animals release bioactive and immunoreactive inhibin into the media in response to FSH (106). Other factors that have been implicated in the control of Sertoli cell inhibin secretion include epidermal growth factor (102, 113), insulin (114), and opioids (115).

Spermatogenesis in the testis has been divided into a number of discrete developmental stages. In the rat, there is synchronous development of the germinal epithelium in any given cross-section of a seminiferous tubule. Thus, various stages of development can be recognized and dissected out for study using transillumination-assisted microdissection (116, 117). Such studies have led to the hypothesis that Sertoli cells do not act merely as "nurse" or support cells for developing spermatocytes, but that significant cross-talk between germ and Sertoli cells is requisite to establishing the microenvironments within the seminiferous epithelium which are necessary for the ordered development of germ cells.

This hypothesis is based on the observation that many of the biochemical and secretory functions of Sertoli cells are regulated in a stage-dependent manner during the cycle of the seminiferous epithelium (117). It is thought that this phenomenon is largely due to the regulatory influence of the various subpopulations of germ cells on neighboring Sertoli cells. As an extension of this hypothesis, stage-specific regulation of a Sertoli-cell-secreted protein is usually taken to suggest that the protein has a paracrine role in regulating the progression of germ cell development.

Identification of stage-specific expression of inhibin α- and βB-subunit mRNA (118) and secretion of immunoreactive inhibin protein (107) represent the first observations that expression of inhibin by the seminiferous epithelium of adult rats was regulated in a paracrine manner by cell to cell interactions with, or secretory factors from, the germinal compartment. Since then, Allenby *et al.* (119) have shown that depletion of late spermatids using a single dose of methoxy acetic acid is associated with a marked decrease of circulating levels of inhibin *in vivo*. Finally, the secretion of inhibin by Sertoli cell cultures is increased by the addition of spermatids or spermatid-conditioned medium (120). Taken together, these data suggest that inhibin expression and secretion may be regulated by germ cells or products secreted by these cells and may play a role in the paracrine regulation of spermatogenesis.

Leydig Cell Production of Inhibin and Activin

Initially, the Leydig cell was not considered as a possible site of production of activin or inhibin. However, a Leydig cell line, TM3, derived from normal immature mouse testis was shown to express only the β-subunits of inhibin, which suggests that this cell line produced activin (19). It was confirmed that conditioned medium from this line, and from primary cultures of interstitial cells from immature rats and pigs, contain an activity capable of stimulating FSH secretion by pituitary cell cultures, and that reacts with an antiserum that recognizes both inhibin and activin (19, 69). This activity is increased by the addition of forskolin (a stimulator of adenylate cyclase) or pregnant mare's serum gonadotropin to the cultures, which suggests that activin secretion is under gonadotropin regulation. These data suggest that activin, produced by immature Leydig cells, might act as a paracrine factor in the immature testis. The identification of β-subunit mRNA in fetal testis also suggests a role for activin in early gonadal development.

Ogawa *et al.* (121), using histochemical localization, have detected βA and βB, but not α, subunit protein in rat Leydig cells of adult as well as immature animals. However, other groups have detected only bioactive inhibin, immunoreactive inhibin-α, or inhibin α-subunit mRNA in purified Leydig cells prepared from adult rats (122, 123). Two different rat Leydig tumor-derived cell lines (I10A and RC2) contain mRNA for α-, but not the β-, subunits (W. Lee and J. P. Mather, unpublished data). Immunoreactive α-subunit protein has also been detected by immunohistochemical staining in the testis of normal men, in men with testicular disorders, and in a human Leydig cell tumor (124).

Stimulation of Leydig cells, prepared by Percoll gradient enrichment from adult rat interstitium, with LH results in a marked increase in the expression of inhibin α-subunit mRNA (L. A. Krummen, unpublished observations), as well as immunoreactive inhibin secretion (122, 123). These results may explain the ability of LH to partially restore testicular inhibin α-subunit mRNA concentrations after hypophysectomy in the adult rat (125), as well as the increase in serum immunoreactive inhibin noted after injections of human chorionic gonadotropin (hCG) to men receiving testosterone to inhibit pituitary function (126). Similar increases in serum immunoreactive inhibin in rats receiving hCG treatment have also been reported (127). However, controversy exists over whether the LH-stimulated increase in α-subunit mRNA or immunoreactive inhibin levels seen either *in vivo* or *in vitro* reflect production of bioactive inhibin molecules or inactive free α-inhibin subunits. In any case, the preponderance of available data are consistent with the hypothesis that interstitial cells from the immature testis produce activin, whereas Leydig cells from the adult interstitium produce primarily inhibin or free α-subunits.

Recent work from our laboratory (L. A. Krummen, unpublished observations) has confirmed that immunoreactive and bioactive activin, but not inhibin, is made by an interstitial cell population from immature rat testes. This activin production disappears between 19 and 21 days of age and is not detectable either by

bioassay or immunoassay in the adult rat interstitium. This would suggest that Leydig-cell-derived activin may play a paracrine role in the embryonic and immature rat, but not in the adult.

In vivo studies have been reported in which circulating inhibin levels were measured after destruction of Leydig cells by ethane dimethane sulphonate to determine what proportion of the total circulating inhibin might be contributed by Leydig cells (128). The authors found no significant correlation between changes in inhibin levels in interstitial fluid or testicular or spermatic venous blood and the presence or absence of Leydig cells. Sharpe *et al.* (128) conclude that Leydig cells make little contribution to the intratesticular and blood levels of inhibin in the adult rat. The question of whether inhibin or α-derived proteins from Leydig cells play a significant paracrine role in the adult testis remains to be answered.

Receptors for Inhibin and Activin in the Testis

Inhibin and activin have proved to be difficult to iodinate in a bioactive form (73). However, we have found that these proteins can be labeled with a fluorescent probe, fluorescein isothiocyanate (FITC), while maintaining full biological activity (43, 73). FITC-activin and FITC-inhibin binding to receptors can then be detected using fluorescence-activated cell analysis to determine specific binding (43, 73, 74, 129) to mixed cell populations. This method allows for a more accurate estimate of which cells in a mixed cell population bind hormone, but it is more difficult to quantitate than radiolabeled hormone binding.

Such studies have shown that specific receptors exist for both activin and inhibin on both Sertoli cells and Leydig cells cultured from immature rats (T. K. Woodruff, G. Rice, and J. P. Mather, unpublished data). Studies using cocultures of Sertoli and germ cells have identified activin binding to spermatogonia, late pachytene spermatocytes, and spermatids (129). No binding was seen, however, to leptotene/zygotene spermatocytes. All germ cell types specifically bind inhibin (74, 129). The binding of FITC-activin A to testicular cells is competed by activin A, partially competed by activin B, but not competed by a 1000-fold excess of inhibin A. Similarly, inhibin binding is competed by inhibin, but not by activin A or B (129). These data suggest that, in the testis, two separate receptors exist for activin and inhibin, and multiple activin receptors may exist.

Recently, an activin receptor was cloned from the AtT20 mouse pituitary tumor-derived cell line (77). The receptor sequence predicts a transmembrane serine/threonine-specific protein kinase. Activin A binds to this receptor with an affinity of 180 p*M* and can be competed by activin A, activin B, and inhibin A, but not TGF-β. mRNA coding for the receptor has been detected in mouse brain, testis, intestine, liver, and kidney. More work is needed to determine the relationship of the cloned receptor and the receptors detected by the binding studies mentioned above. However, these studies lend further strength to the hypothesis that activin and inhibin have a significant paracrine role in the testis.

Response of Testicular Cells to Activin and Inhibin

A number of investigators have studied the local effects of activin and inhibin on various testicular cell types. Many of these studies have examined the effects of purified activin or inhibin added *in vitro* to cultures enriched for the cell type of interest. In mixed testis cell cultures from neonatal or adult rats, inhibin increased, and activin decreased, LH-stimulated testosterone production. Neither hormone affected basal testosterone in these cultures (71). In contrast, other studies using more purified rat Leydig cell cultures have shown that activin stimulates testosterone release (130).

A more recent study on purified immature porcine Leydig cell cultures showed that activin A reduced hCG-stimulated dehydroepiandrosterone accumulation, but increased the conversion of pregnenolone and dehydroepiandrosterone to testosterone. This suggests that activin may alter the activity of either cholesterol side chain cleavage enzyme or 3β-hydroxysteroid dehydrogenase/isomerase (131). While further investigation is needed to determine the exact mechanism of action, it seems clear that activin, and perhaps inhibin, can act directly on the Leydig cell. It is unclear whether this is an autocrine (e.g., activin produced by the Leydig cell) or a paracrine (e.g., Sertoli-cell-produced activin) effect. This may vary with the developmental stage of the animal.

The stage-specific secretion of inhibin by the seminiferous tubules suggests that Sertoli-cell-derived inhibin may, in turn, regulate the development of the germ cells. This hypothesis is supported by studies that demonstrate an effect of locally administered inhibin on hamster testis. Inhibin-injected testes showed a decrease in spermatogonial numbers (132). These data suggest that inhibin may inhibit some stages of spermatogenesis and support the hypothesis that inhibin acts as a local regulator of spermatogenesis.

In contrast, *in vitro* data suggest that activin can stimulate spermatogonial proliferation in cocultures of Sertoli and germ cells prepared from 21-day-old rats (74). Inhibin had no effect in this *in vitro* system. Thus, if inhibin inhibits and activin stimulates spermatogenesis, they seem to act on different cell types or at different stages of germinal cell development. The existence of receptors for both proteins on germ cells (129) would allow for a direct action of activin and inhibin on these cells, although neither the *in vivo* studies nor the current *in vitro* coculture models allow definitive proof of a direct action.

The data summarized above lend strong support

to the hypothesis that activin and inhibin play a major role in the paracrine and autocrine regulation of testicular function. A schematic representation of possible paracrine interactions is presented in Figure 2. The existence of multiple related proteins in the inhibin family, and technical difficulties in measuring these proteins, has led to extensive, and sometimes conflicting, literature. The exact mechanism of action of these factors during development and in the adult testis is not yet clearly understood but promises to be an exciting and rapidly expanding field in the coming years.

Inhibin and Activin in the Ovary

Reproductive cyclicity in the female is maintained by endocrine, paracrine, and autocrine interactions. The central role of pituitary FSH and LH is to direct ovarian follicular recruitment, growth, atresia, and ovulation (133). Intragonadal factors act to attenuate or accentuate the actions of the gonadotropins or to independently regulate cellular functions (134). This system of interactive hormones provides the reproductive axis with complicated controlling mechanisms (hypothalamus ↔ pituitary ↔ ovary), redundancy, and fail-safe systems (ovarian hypertrophy following unilateral ovariectomy) to ensure accurate and faithful replication of productive cycles.

We will review the data that address the production of the inhibin subunit mRNA and proteins during the estrous and menstrual cycle, the availability of receptors on the ovary, and the effects of exogenous manipulation of inhibin or activin levels. It is important to note that inhibin has been studied primarily as an endocrine regulator of FSH secretion and not for its paracrine regulation of ovarian events.

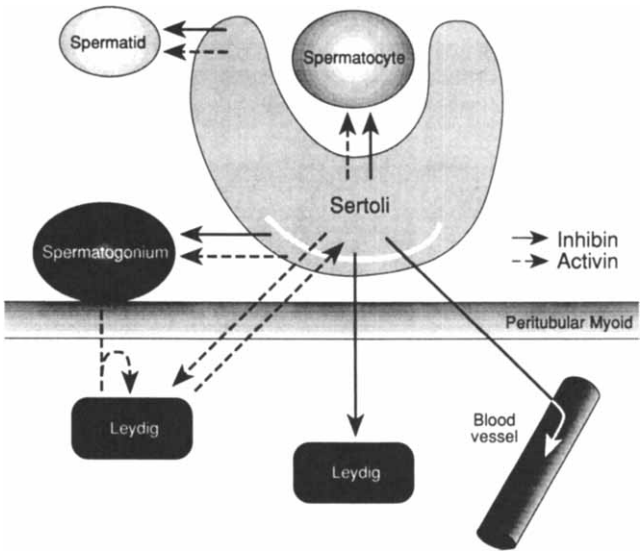

Figure 2. Schematic representation of the potential paracrine actions of inhibin and activin in the testis. Activin produced by Leydig cells may act to promote biochemical or morphological changes in Sertoli cell function, as well as stimulate spermatogonial proliferation. Receptors for both hormones on germ cells at later stages of development also suggest a role for these hormones in regulating more distal stages of spermatogenesis. The steroidogenic activity of Leydig cells may be affected by inhibins and activins derived from either Leydig (autocrine) or Sertoli (paracrine) sources. Available data suggest that circulating inhibin is derived from the Sertoli cell.

Ovarian Production of Inhibin

de Jong and Sharpe (2) and Schwartz and Channing (4) first recognized the ovary as the source of an FSH-regulating activity. Ten years later, inhibin and activin were isolated from the follicular fluid of cows, sheep, and pigs (12). Recently, inhibin has been isolated from human follicular fluid (135).

The level of endogenous inhibin in various biological fluids or tissues of rats and humans has been estimated using the dispersed pituitary cell bioassay or by radioimmunoassay (12, 79, 135). Inhibin has been identified as a product of rat granulosa cells that have been isolated throughout the estrous cycle. The granulosa-derived inhibin products are low early in the cycle and increase slightly in cells isolated on proestrus (136, 137). Measurement of inhibin in rodent ovarian homogenates and in ovarian vein plasma during the estrous cycle parallels the *in vitro* data; that is, inhibin ovarian content and venous levels increase from estrus to proestrus and decline during the periovulatory time (138–141). Follicular fluid isolated from the rat shows an increase in detectable inhibin from proestrous morning to afternoon (142). Inhibin in the peripheral plasma of the rat parallels the data described above, which suggests that the major source of inhibin is ovarian (143). Additionally, removal of the ovary results in no detectable serum inhibin.

In the intact rodent, the pattern of circulating hormone parallels inhibin subunit mRNA production (63, 64, 143, 144). Inhibin subunit mRNA expression is turned on in newly recruited follicles on the morning of estrus (63, 143, 144). As the follicle grows, inhibin subunit mRNA increases, reaching maximum levels by the afternoon of proestrus in fully developed follicles. Similarly, serum inhibin increases during metestrus to proestrus (64, 143). On the evening of proestrus, both mRNA levels and serum inhibin levels decline. This is caused by the stimulation of the follicles by the primary gonadotropin surge (145). Neither mRNA nor protein is detectable until the period of follicular recruitment that corresponds to the secondary FSH surge on the morning of estrus. In addition to initiating follicular growth, FSH turns on inhibin subunit mRNA expression (146). The result of stimulation of inhibin production at this time of the cycle is the suppression of pituitary FSH secretion. The inverse relationship between inhibin and FSH during the secondary FSH surge is consistent with our model of inhibin to FSH control. However, the elevated levels of inhibin and FSH during the primary gonadotropin surge are unexpected. Because of this apparent discrepancy, we hypothesized that, in the rat, inhibin has an endocrine function

during the secondary FSH surge and a paracrine function during follicular growth.

Similar to the rat, primate primary granulosa cell cultures derived from follicles at progressive stages of preovulatory development produce increasing amounts of immunoreactive inhibin (147). Inhibin has also been measured in human ovarian vein plasma and follicular fluid (148).

In contrast to the rat, the pattern of immunoreactive inhibin in the human and primate suggests an important luteal function. McLachlan and Robertson and others (149–151) have demonstrated that, in the human, immunodetectable inhibin parallels the progesterone profile. Localization of inhibin subunit mRNA again parallels serum findings: α- and βA-inhibin subunit mRNA are found in developing follicles as well as in the corpus luteum (152). The role of inhibin during the luteal phase is not understood. Whether paracrine or endocrine events are being modulated is under active investigation.

Ovarian Inhibin and Activin Receptors

The effects of inhibin and activin on the ovary are mediated by cell surface receptors. Inhibin or activin binding has been studied on a variety of cells (153–155) including granulosa cells from the rat (43) and rhesus monkey (T. K. Woodruff, unpublished data). Unlike the receptor cloned by Mathews and Vale (77), which binds both activin and inhibin (with lesser affinity), granulosa cells have separate receptors specific for activin or inhibin (43). Granulosa cells may have other binding sites for inhibin-related products. For example, there is an approximate 10-fold excess inhibin α-subunit mRNA versus inhibin β_A-subunit mRNA in the rat ovary. Free inhibin α-subunit has also been shown to be produced by the granulosa cell (156). This may be relevant in view of the data of Hillier *et al.* (157), who describe specific binding of the inhibin α-subunit to rat granulosa cell gonadotropin-releasing hormone (GnRH) receptors. Schneyer *et al.* (16) have also described specific binding of inhibin α-subunit with the granulosa cell FSH receptor. The inhibin α-subunit is expressed (43, 144) and produced (15, 18, 80, 81) as a free monomer. Thus, it is possible that the intragonadal activities of the free inhibin α-subunit may be as important as those of dimeric inhibin and activin. The relevance of these findings will require further investigation.

Response of Ovarian Cells to Inhibin and Activin *In Vitro*

Inhibin and activin act on granulosa and theca cell steroidogenesis *in vitro*. Ying *et al.* (158) observed that porcine inhibin reduced FSH-stimulated aromatase activity and progesterone production in rat granulosa cell primary culture. Hutchinson *et al.* (159), however, failed to reproduce this result using bovine inhibin-treated rat granulosa cells. Xiao *et al.* (160) showed that activin enhanced progesterone and inhibin production as well as FSH-induced aromatase activity. Activin alone stimulated basal inhibin secretion and had no effect on progesterone or estradiol production. Follistatin attenuated both aromatase activity and inhibin production induced by FSH (160).

In vitro regulation of theca cell androgen production by activin and inhibin has been examined by Hillier *et al.* (161). Inhibin was shown to increase the production of androgen in a serum-free primary culture of human theca cells (161). The accumulation of androgen was greatest in cultures coincubated with inhibin and insulin-like growth factor-1. Activin inhibits LH + insulin-like growth factor-1-induced androgen synthesis in a comparable system (162).

A third type of *in vitro* experiment involves the culture of graafian follicles. Unlike the purified culture systems described above, the graafian follicle culture allows the interaction of theca and granulosa cells. Progesterone production was measured in this system and was unaffected by addition of inhibin. Estradiol and androstenedione production were not examined (163).

Regulation of Folliculogenesis by Inhibin

Inhibin is produced by follicles as they develop from small antral to preovulatory stages. Two models exist for the role of the inhibin produced by these follicles. First, inhibin may be actively maintaining tonic FSH levels. This hypothesis is supported by immunoneutralization experiments conducted by Rivier and Vale (164) in female rats. When anti-inhibin α-subunit antiserum was infused on diestrus, serum FSH rose significantly and remained elevated through the following day. Treated animals had increased ovulation rates and increased litter sizes. Similar reports of altered ovulation rates following passive immunization to inhibin in ewes have been published (165–167). Importantly, no effect on serum FSH is reported in sheep. An alternative interpretation of the data is that inhibin has a local role in the development of follicles in the ovulatory pool.

Factors dictating the ultimate fate of the follicle may include the capacity to synthesize and respond to steroids (168, 169), the levels of circulating gonadotropins, and the availability of gonadotropin receptors (170, 171). While these theories are adequate to explain the selection process in the intact animal, they do not explain the reliable increased selection of follicles that occurs in the unilaterally ovariectomized animal (ULO). In the ULO animal, the functions of two ovaries are carried out by the remaining ovary, and the total complement of follicles is maintained per cycle (172, 173). If a preset number of follicles per ovary are at the correct stage of maturation or have the appropriate receptors, the ULO animal should be able to maintain

only a normal complement for that ovary per cycle. The idea of selection based on the levels of circulating gonadotropin is also an insufficient explanation for the maintenance of healthy follicles in the ULO model, because the pattern of gonadotropin release is the same in cycles after the initial FSH elevation 9 hr after surgery.

This evidence strongly suggests that an interovarian signal exists which relays information on the total number of follicles between ovaries. This model predicts that the signal must be follicularly derived to allow for an assessment of the total number of follicles recruited. The signal must be produced at discreetly defined levels so that alternations in circulating levels allow either maintenance or selective degeneration of follicles. Ideally, the factor should be necessary for follicular development so that some type of feedback mechanism would lead to its own down-regulation and ultimately to atresia. Inhibin may fit this role of "follicular selection signal."

Inhibin subunit mRNA and protein production are temporally associated with the growth of follicles (43, 144), but are not associated with atretic follicles (174). Granulosa cells also have receptors for inhibin (43, 73). Taken together, these data suggest a role for inhibin (and activin) in the regulation of follicular determination and growth. In an effort to identify the role of inhibin in directing folliculogenesis, we injected immature female rat ovaries directly with human recombinant inhibin or activin (43). The contralateral ovary served as an internal control. Inhibin-treated ovaries showed a visible increase in follicular diameter, indicating follicular development. In contrast, activin-treated ovaries were smaller than the contralateral control ovary. Ovarian sections were examined to determine the classification of the follicles. Strikingly, inhibin promoted follicular development similar to the effects of systemic FSH on the follicles. Activin or activin with FSH caused follicular atresia. These data may reflect the ability of the hormones to locally direct aspects of folliculogenesis (Fig. 3). However, we do not yet know what the endogenous levels of inhibin and activin are in the ovary and how these levels change after the addition of recombinant hormones.

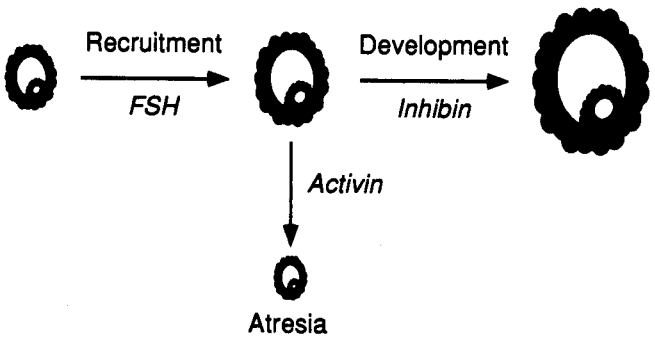

Figure 3. Schematic of follicular development. A follicle is recruited to begin growth by FSH. Once recruited, follicles either continue to develop (potentially mediated by inhibin) or undergo atresia (potentially mediated by activin).

In the human follicular phase, inhibin is detected in plasma of women undergoing ovarian hyperstimulation with clomiphene citrate and human menopausal gonadotropin in preparation for *in vitro* fertilization (175, 176). Plasma inhibin levels coincide with estrogen concentration as well as with the number of follicles detected by ultrasonography and the number of oocytes recovered at laparoscopy. This suggests that inhibin is produced coincident with follicular development.

In the normal primate reproductive cycle, it appears that inhibin is produced in the luteal phase. Temporally, the inhibin surge is associated with hormones involved in regulation of LH and progesterone (177–179). Moreover, activin can promote proliferation of luteinized granulosa cells (180).

It is important to note that the antibody used to identify immunoreactive inhibin recognizes free α-subunit rather than the heterodimer (80, 81). Free circulating α-subunit may not reflect dimeric inhibin and, therefore, it may result in the assignment of inhibin activity to inappropriate times in the cycle. Moreover, the biological activity of free α-subunit in controlling luteal phase events has not been explored.

Inhibin and Activin as Paracrine Regulators of Oocyte Maturation

A factor that regulates meiotic maturation of oocytes has been sought extensively. Members of the inhibin family have been implicated as regulators of this process. Specifically, TGF-β stimulates meiotic maturation, perhaps via an effect on cumulus cells (163). O *et al.* (181) showed that inhibin was able to inhibit meiotic maturation with or without cumulus cells, whereas activin did not affect oocyte meiosis. The activin-binding protein follistatin was able to accelerate meiotic maturation. This data may be related to one study in humans in which the inhibin levels were correlated positively with the maturation of the oocyte (182).

The hypothesis that inhibin is the oocyte maturation inhibitor is attractive in that the expression and production of inhibin decreases immediately preceding ovulation, thereby removing the oocyte from its meiotic restraint. This theory, however, would not apply to immature follicles housing arrested oocytes. Inhibin is not produced in these follicles. Further investigation must be done to provide a unified theory in this area.

Inhibin and Activin as Paracrine Factors in the Pituitary

As discussed above, inhibin, and later activin, was isolated on the basis of its effect on pituitary FSH release *in vitro*. These effects have been confirmed *in vivo* (for review see Ref. 10) where inhibin decreases and activin

increases (22, 23) circulating FSH levels. However, evidence is accumulating that activin may also act as a paracrine factor in the pituitary. Both α- and βB-mRNA have been detected in the pituitary (183), which suggests that inhibin B, or activin B, and possibly free pro-α, might be produced in the pituitary. Follistatin, the activin-binding protein, has been isolated from bovine pituitaries (91). Follistatin will inhibit activin-stimulated or basal FSH release from pituitary cultures. However, when bound to equimolar amounts of activin, the complex has neither stimulatory nor inhibitory activity in the pituitary bioassay (91).

A neutralizing monoclonal antibody directed against activin B has recently been shown to decrease the basal secretion of FSH, but not LH, in pituitary cell cultures (184). This antibody, which does not cross-react with activin A, did not change the EC_{50} for activin-A-stimulated FSH release. Immunoneutralization of inhibin did not alter basal or GnRH-stimulated FSH release (184). These findings suggest that activin B is involved in the paracrine or autocrine regulation of FSH secretion by the pituitary (Fig. 4).

Inhibin and Activin as Paracrine Factors in the Placenta

It has been shown that the ovaries are not the primary source of circulating inhibin during pregnancy. The presence of bioactive and immunoreactive inhibin in human placenta has recently been shown (185, 186).

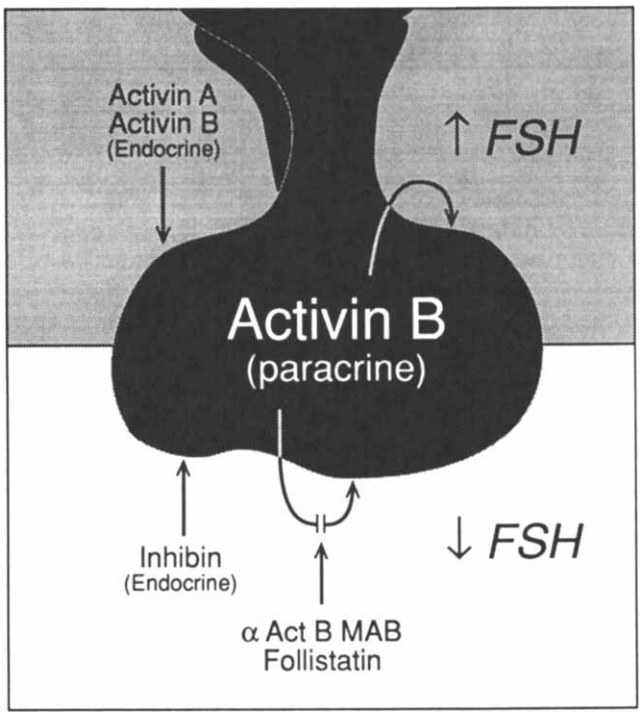

Figure 4. Schematic of paracrine and endocrine effects of activin and inhibin in the pituitary. Activins increase FSH release (upper panel) while inhibin decreases FSH release. Anti-activin antibodies or follistatin can decrease FSH release by interfering with paracrine stimulation by endogenous activin B.

Using a specific antiserum raised against the α-inhibin subunit, McLachlan *et al.* (185) recently described the presence of immunoreactive-inhibin in extracts of human placental tissue. Inhibin α- and βA-mRNA have also been localized in human placenta and co-localized with mRNA for GnRH (183, 187). Using primary placenta culture, the possible paracrine action of inhibin on placental hormone secretion has been evaluated. The addition of an antiserum to inhibin to the placental cell cultures significantly increases hCG release (186). The effect was dose related and time dependent, without changes in human placental lactogen concentrations. The addition of inhibin antisera caused an increase in GnRH release from cultured placental cells, thus suggesting an inhibitory action of inhibin on this hormone. The regulation of placental function may be differentially regulated during the development of the fetoplacental unit (188).

Conclusions

Inhibin has been confirmed as a factor produced in the gonads and capable of specifically regulating FSH release by the pituitary. However, inhibin and the related activins seem to play important paracrine roles in locally regulating testicular and ovarian function. They are also involved in paracrine regulation in the placenta, the pituitary, and other nonreproductive tissues. The local effects of these factors may be modified by the presence of binding proteins and the receptor or receptors expressed.

The specific type of activin or inhibin produced and the net effect locally may vary as a function of the sexual development of the organism or the stage of the reproductive cycle. A more complete understanding of these complex relationships will develop with advances in our ability to measure the proteins, their binding proteins, and cell surface receptors.

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