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Peripheral and intragonadal actions of inhibin-related peptides

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Introduction

Inhibin is a glycoprotein hormone consisting of two dissimilar, disulphide-linked subunits, termed α and β, which inhibits the synthesis and/or secretion by the pituitary of gonadotrophins, preferentially FSH (Burger, 1988). When we reviewed the physiology of inhibin in ruminants at the previous meeting on reproductive physiology in domestic ruminants (Findlay & Clarke, 1987), inhibin had only recently been purified from bovine follicular fluid and its cDNA primary sequence deduced by cloning methods. This paper reviews recent progress during which time considerable advances have been made in our understanding of the physiology of inhibin as a peripheral hormone controlling FSH secretion and the use of inhibin as a fecundity vaccine. We also discuss a number of inhibin-related peptides which have been isolated only recently, some of which have intraovarian actions of potential importance. The fact that this review is limited to the physiology of inhibin in domestic ruminants should not detract from the important contribution made from investigating other species such as the rat, pig and human (see Ying, 1988; de Kretser & Robertson, 1989).

Isolation and characterization of inhibin-related peptides

Inhibin has been purified to homogeneity from follicular fluid of the cow (Robertson *et al.*, 1985, 1986; Fukuda *et al.*, 1986; Knight *et al.*, 1987) and sheep (Leversha *et al.*, 1987), and from ovine rete testis fluid (Bardin *et al.*, 1987), and shown to be composed of two disulphide-linked subunits, termed α and β. Two molecular weight forms of bovine inhibin have been isolated (M_r 31 000 and M_r 58 000), consisting of either an M_r 20 000 α-subunit (αC) or an extended M_r 43 000 α-subunit (α-43) and an M_r 15 000 β-subunit (Robertson *et al.*, 1985, 1986) (Fig. 1). Two molecular weight forms of inhibin in ovine follicular fluid have also been described (M_r 32 000 and M_r 67 000; Leversha *et al.*, 1987), and larger molecular weight forms have been identified in bovine follicular fluid (M_r 65 000, 88 000, 108 000, 120 000; Miyamoto *et al.*, 1986). Based on the cDNA structures of

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the inhibin subunits (see Ying, 1988), these higher molecular weight inhibins are likely to be dimers of various sized fragments of the subunit precursors. Activin, consisting of two β-subunits, has been isolated from bovine follicular fluid and purified to homogeneity (McLachlan *et al.*, 1987). In contrast to inhibin, activin stimulates FSH secretion by the pituitary, and it has local actions in the gonad (Findlay *et al.*, 1990a).

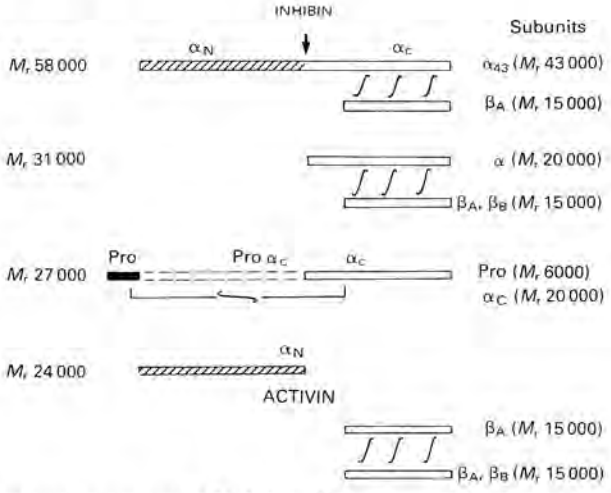

Fig. 1. The subunit structures of inhibin, derivatives of the α-subunit and activin.

Three inhibin genes have been identified so far; namely, the α gene and two β genes, termed β_A and β_B, coding from two forms of inhibin ($\alpha\beta_A$, $\alpha\beta_B$) and activin ($\beta_A\beta_A$, $\beta_A\beta_B$) (see Ying, 1988). Unlike the human, rat and pig (see Ying, 1988), only the α and β_A genes are expressed in detectable amounts in ruminants, since no β_B subunit sequence (Robertson *et al.*, 1985, 1986; Leversha *et al.*, 1987; Knight *et al.*, 1987; Bardin *et al.*, 1987) or cDNA (Forage *et al.*, 1986; Crawford *et al.*, 1987) has been identified in sheep or cattle. The β_B subunit gene has been identified in the ovine genome (Crawford *et al.*, 1987; Rodgers, 1989) and shows high homology (>90%) with the rat, human and pig β_B genes (R. J. Rodgers, unpublished observations). Southern analysis of the sheep genome revealed one α and β_A gene (Crawford *et al.*, 1987) and evidence for 6 other β_A-related genes, one of which is the ovine β_B gene (Rodgers, 1989).

An excess of α over β_A mRNAs has been detected in cow (Forage *et al.*, 1986; Torney *et al.*, 1989; Rodgers *et al.*, 1989) and sheep (Crawford *et al.*, 1987; Rodgers *et al.*, 1989) ovaries and sheep testes (Crawford *et al.*, 1987; Bardin *et al.*, 1987). RNA from sheep adrenal and placenta, but not kidney, pituitary or liver, also hybridized with α-subunit cDNA, and there was also detectable β_A mRNA in ovine placenta but not in the other tissues tested (Crawford *et al.*, 1987). There have been no reports of expression in brain or bone marrow of ruminants, tissues which express the inhibin genes in rats (Meunier *et al.*, 1988).

Additional proteins related to the α-subunit of inhibin have been identified in bovine follicular fluid (Sugino *et al.*, 1989; Robertson *et al.*, 1989; Knight *et al.*, 1989) (Fig. 1). A Pro-αC subunit (M_r 27 000) was shown to consist of two subunits (M_r 20 000 and M_r 6000), suggesting a disulphide linkage (Sugino *et al.*, 1989; Robertson *et al.*, 1989). The two subunits, based on their molecular masses and *N*-terminal amino acid sequences, are consistent with the pro-region of the α-subunit precursor sequence and the αC sequence, respectively. Pro-αC is immunologically but not biologically active in inhibin assays and is likely to be present in follicular fluid (Robertson *et al.*, 1989; Sugino *et al.*, 1989). A second protein (αN) (Fig. 1) was identified as an M_r 24 000 non-reducible protein with an *N*-terminal amino acid sequence identical to the α43 subunit (Robertson *et al.*, 1989). The α43 subunit (αN + αC) is cleaved to form the αC subunit (M_r 20 000) and αN. The αN

protein has no inhibin immuno- or bioactivity (Robertson *et al.*, 1989) but has other actions in the ovary (Findlay *et al.*, 1989a; see also below). A third protein, described by Knight *et al.* (1989), is an M_r 25–26 000 form of the inhibin α-subunit isolated from bovine follicular fluid and is present in ovarian venous plasma of the cow but not the sheep. The relationship between this protein and Pro-αC (Robertson *et al.*, 1989; Sugino *et al.*, 1989) is not clear at present.

Another class of proteins, termed follistatin (Ueno *et al.*, 1987) or FSH-suppressing protein (FSP; Robertson *et al.*, 1987), has been isolated from pig and cow follicular fluids. Bovine FSP consists of 3 proteins (M_r 31 000, 35 000 and 39 000) with identical *N*-terminal amino acid sequences all of which have inhibin-like activity but are structurally distinct from inhibin. The pig FSPs are products of a single gene and arise by gene splicing (Esch *et al.*, 1987). The inhibin-like biological activities, based on in-vitro pituitary cell assays, range from 5–10% (bovine) to 30% (pig) that of inhibin. Bovine FSP can modulate FSH-induced differentiation of rat granulosa cells (Xiao *et al.*, 1990), consistent with a local action in the ovarian follicle (Findlay *et al.*, 1990a). FSP has recently been shown to act as a binding protein for activin (Nakamura *et al.*, 1990). FSP is also a product of stellate cells derived from the bovine pituitary gland (Gospodarowicz & Lau, 1989), suggesting a possible paracrine role to control FSH release by gonadotrophs (Allaerts & Denef, 1989).

Development and application of radioimmunoassays for inhibin

Radioimmunoassays have been described for ovine (Schanbacher, 1988; McNeilly *et al.*, 1989; Findlay *et al.*, 1990b) and bovine (McLachlan *et al.*, 1986; Hasegawa *et al.*, 1987; Schanbacher, 1988) inhibin, using either antisera directed against *N*-terminal fragments of the α-subunit (Schanbacher, 1988; McNeilly *et al.*, 1989) or against purified bovine M_r 32 000 inhibin (McLachlan *et al.*, 1987; Hasegawa *et al.*, 1987; Findlay *et al.*, 1990b).

Hasegawa *et al.* (1987) measured parallel increases in peripheral concentrations of inhibin and oestradiol after FSH treatment of cows; inhibin decreased after the peak of the preovulatory LH surge. Data across the oestrous cycle were presented for only 1 cow. Inhibin concentrations rose during the luteal phase, fell after luteolysis, reaching a nadir before the onset of the preovulatory surge of LH, and then rose again during ovulation and for the 2–3 days thereafter. Inhibin concentrations appeared to be inversely related to oestradiol values and were unrelated to the pattern of FSH in peripheral plasma.

We measured inhibin in peripheral and ovarian venous blood of ewes during the oestrous cycle (Findlay *et al.*, 1990b). There was very little hour-to-hour variation in inhibin concentrations in peripheral blood. During the follicular phase, inhibin in the ovarian vein appears to be secreted in pulses coincident with the pulses of androstenedione that follow LH pulses (Murray *et al.*, 1989a). Inhibin in follicular fluid was 10^4-fold higher than that in ovarian venous plasma, which was 3-fold higher than that in peripheral plasma. Cautery of visible follicles resulted in a 35% reduction in inhibin and an 81% reduction in oestrogen concentrations in the ovarian vein within 10 min, confirming that the ovarian follicle is the major, if not the only, source of inhibin in the sheep ovary. The continued secretion of inhibin after cautery is most probably coming from small follicles below the surface of the ovary which escaped cautery. During the luteal and follicular phases of the cycle, inhibin and FSH were inversely related ($r = -0{\cdot}69$ and $-0{\cdot}45$, respectively, $P < 0{\cdot}001$). Inhibin and LH were not related in the luteal phase but were positively correlated in the follicular phase ($r = 0{\cdot}31$, $P < 0{\cdot}01$). Inhibin and oestrogen concentrations in the follicular phase were also correlated ($r = 0{\cdot}30$, $P < 0{\cdot}01$). Furthermore, inhibin concentration approximately doubled across the follicular phase and decreased within 3–6 h of the preovulatory surges of gonadotrophin, reaching a nadir around the time of the second rise in FSH 24–28 h later (Figs 2 & 3).

McNeilly *et al.* (1989) measured inhibin concentrations and secretion rates during the oestrous cycle of ewes with ovarian transplants, and Mann *et al.* (1989a) applied the same inhibin assay to

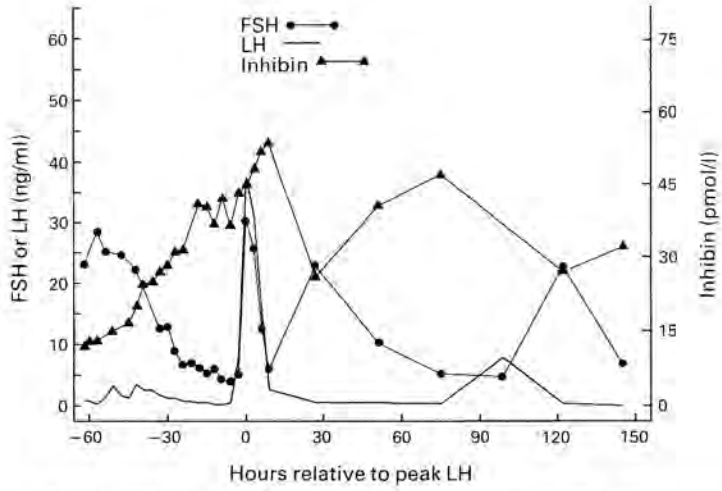

Fig. 2. Peripheral concentrations of FSH, LH and inhibin in a ewe after induction of luteolysis with cloprostenol at −62 h. (After Findlay *et al.*, 1990b.)

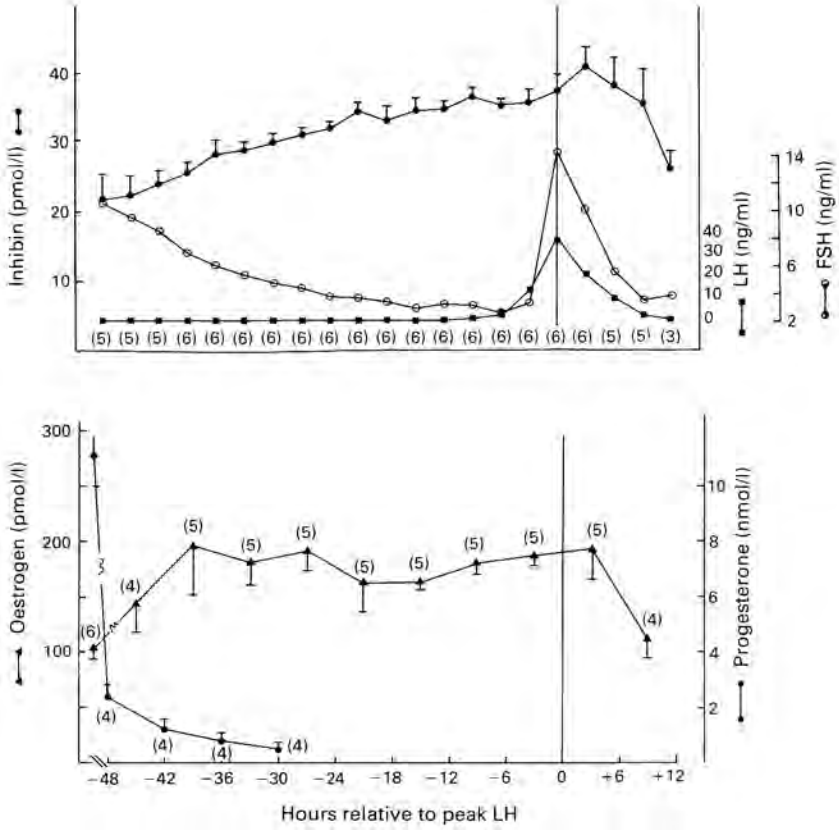

Fig. 3. Mean ± s.e.m. concentrations of hormones in 6 ewes after induction of luteolysis with cloprostenol. (After Findlay *et al.*, 1990b.)

samples of ovarian vein and peripheral plasma of ewes under anaesthesia. The patterns of inhibin described by this group are different from our data. They observed no significant changes in inhibin concentrations in ovarian and peripheral plasma and/or in secretion rates after luteolysis; their observations using a bioassay showed that inhibin secretion either decreased (Tsonis *et al.*, 1988a) or did not change (Mann *et al.*, 1989a). They did not find a correlation between inhibin and oestradiol, and after the LH surge inhibin concentrations either remained constant or appeared to increase. On the other hand, neither assay detected inhibin immunoreactivity in ovariectomized ewe plasma, and the 2–6-fold differences between ovarian and jugular venous concentrations are in good agreement. Both assays measured an increase in inhibin after treatment with FSH preparations (FSH: McNeilly *et al.*, 1989; PMSG: Murray *et al.*, 1989a, b).

The reasons for the differences in patterns of inhibin measured by the two assays are not clear. Using a value of 1 pg peptide standard equivalent to approximately 4 mU ovine rete testis fluid inhibin standard (McNeilly *et al.*, 1989; Campbell *et al.*, 1989), the concentrations measured by the peptide fragment assay are approximately 3–10-fold higher than our values. To what extent this represents differences in cross-reactivity with the various molecular weight forms of inhibin and its α-subunit is not known. The peptide fragment assay does generate values of immunoreactive inhibin which show a consistently good correlation with bioactivity ($r = 0{\cdot}71$–$0{\cdot}80$; McNeilly *et al.*, 1989; Campbell *et al.*, 1989; Mann *et al.*, 1989a) and Mann *et al.* (1989a) found no significant differences in the B/I ratio between the luteal and follicular phases. Another reason for the different results between the two radioimmunoassays could relate to the breed of sheep studied. For example, we have recently shown that inhibin concentrations in peripheral blood of ewes carrying the F (fecundity) gene (Booroola) are significantly lower than in control ewes, whereas ewes selected for multiple births (T flock) have higher inhibin concentrations (B. M. Bindon, M. A. Hillard, L. R. Piper, J. K. Findlay, unpublished observations).

The metabolic clearance rate (MCR) of immunoreactive inhibin has been calculated to be 21 (McNeilly *et al.*, 1989) and 43 (Mann *et al.*, 1989a) ml/min in intact ewes. We have calculated the half-life ($t_{\frac{1}{2}}$) values of the first component after injection of bovine follicular fluid (20 min), pure bovine inhibin (25 min) and human recombinant inhibin (16 min) into ovariectomized ewes. The $t_{\frac{1}{2}}$ values for these first components were similar, and in reasonable agreement with the MCR values. Immunoreactive inhibin remained detectable for up to 4 h after injecting the pure inhibins into ovariectomized ewes and up to 10 h after injecting follicular fluid. This indicates that there was a second component of inhibin activity in follicular fluid, some effect of follicular fluid on inhibin metabolism, or the presence of a binding protein in follicular fluid (Findlay *et al.*, 1986; Tsonis *et al.*, 1986).

Sites of gene expression and production in the ovary

Northern blot analyses (Rodgers *et al.*, 1989) and in-situ hybridization studies (Torney *et al.*, 1989) showed that α and β_A inhibin gene expression occurs in follicles but not in fully developed cyclic or pregnancy corpora lutea, or in ovarian stromal tissues of sheep and cattle. The levels of α inhibin mRNA are much greater than β_A inhibin mRNA in ovine and bovine follicles (Rodgers *et al.*, 1989). Therefore, the β_A inhibin genes are either expressed at a much lower rate or the β_A-inhibin mRNA has a much shorter half-life relative to that of α-inhibin mRNA, suggesting that inhibin production may be regulated largely by the availability of β_A inhibin mRNA in follicles. The data also suggest that there is independent regulation of the subunits. This proposition is supported by an in-situ hybridization study (Torney *et al.*, 1989) in which the α-subunit mRNA was detected in granulosa cells of bovine follicles $\geq 0{\cdot}36$ mm diameter (excluding the theca), whereas β_A-subunit mRNA was only detectable in follicles $\geq 0{\cdot}80$ mm. The study also demonstrated that very small bovine antral and preantral follicles ($< 1{\cdot}4$ mm) were capable of expressing mRNA for inhibin. Torney *et al.* (1989) also noted expression of α-subunit mRNA in theca cells of bovine follicles $\geq 0{\cdot}80$ and $\leq 3{\cdot}00$ mm in diameter, but not in large preovulatory follicles (15 and 20 mm). The

significance of this expression of α-inhibin mRNA in the theca is not understood. There was also evidence that expression of inhibin mRNA was more intense in periantral granulosa cells (Torney *et al.*, 1989), confirming an immunohistochemical study of inhibin localization in pig and cattle ovaries (Rokukawa *et al.*, 1986).

Studies on the effects of electrocautery on bioactive inhibin secretion in ovarian lymph (Findlay *et al.*, 1986), and measurements of secretion rates of immuno- (Scaramuzzi *et al.*, 1989; Mann *et al.*, 1989a) and bio- (Mann *et al.*, 1989a) active inhibin in the ovarian vein draining ovaries with and without corpora lutea confirm that large antral follicles of the sheep are responsible for most, if not all, of the secretion of inhibin by the ovary at all stages of the oestrous cycle, and that the corpus luteum secretes little or no immunoreactive or bioactive inhibin. An earlier report (Tsonis *et al.*, 1988b) that the sheep corpus luteum secreted bioactive inhibin has therefore been refuted on the grounds mentioned above and also because of possible breed differences and confounding effects of previous treatments of the animals used in that study (Mann *et al.*, 1989a). Likewise in cattle, the luteal cells do not produce bioactive inhibin *in vitro* (Henderson & Franchimont, 1981, 1983), consistent with the lack of expression of the α and β_A-subunit genes in bovine corpora lutea (Rodgers *et al.*, 1989; Torney *et al.*, 1989). However, Rodgers *et al.* (1989) showed that, during the transition from follicle to early corpus luteum (Stage 1), there was differential regulation of expression of the inhibin genes in that α-subunit mRNA was detected in Stage 1 corpora lutea whereas β_A-subunit mRNA was not. This agrees with earlier observations on the secretion of bioactive inhibin (Henderson & Franchimont, 1981) by bovine granulosa cells *in vitro* which fell rapidly after the first day in culture, and was generally not detectable thereafter (but see Franchimont *et al.*, 1986). Using bovine granulosa cells in serum-free cultures, Luck *et al.* (1990) observed the fall in inhibin, measured by immunoassay, but found that immunoreactive inhibin remained measurable for up to 4 days in culture. This correlated with continued expression of the α-subunit mRNA in the luteinized granulosa cells, albeit at a much lower level than in freshly harvested cells. The β_A-subunit mRNA fell to non-detectable levels over the same period. The antiserum used cross-reacts with Pro-αC (Robertson *et al.*, 1989) and continued expression of α-subunit mRNA would be consistent with production of Pro-αC or some other product (e.g. αC?) of the α gene which cross-reacts in the radioimmunoassay (except αN). Whether or not the products of expression of the α-subunit gene play a role during the follicular–luteal transition period is not known, except that αN may be involved in the process of ovulation (Findlay *et al.*, 1989c).

The expression of the β_A subunit gene by ruminant granulosa cells (Rodgers *et al.*, 1989; Torney *et al.*, 1989; Luck *et al.*, 1990) implies that these cells are the only source of activin in the ovary. The definitive experiments showing production of biologically active activin by granulosa cells have not been reported, due largely to the difficulties in measuring activin.

Our knowledge about the site(s) of production of FSP in the ovary is fragmentary. FSP was isolated from follicular fluid (cattle: Robertson *et al.*, 1987; pig: Ueno *et al.*, 1987), and the gene has been characterized from cDNA libraries derived from pig (Esch *et al.*, 1987) and rat (Shimasaki *et al.*, 1989) ovarian mRNA indicative of mRNA production. PMSG treatment of immature rats increased ovarian FSP mRNA levels and in-situ hybridization studies showed that the FSP mRNA expression was low in primordial follicles, but dramatically increased in the granulosa cells of secondary and tertiary follicles and then decreased in mature preovulatory follicles (Shimasaki *et al.*, 1989). The rat FSP mRNA was not detected in theca, stroma or interstitial cells, and some rat corpora lutea showed slight hybridization. Granulosa cells from preovulatory bovine follicles produce immunoreactive FSP *in vitro* (R. Klein, D. M. Robertson, L. Shukovski, J. K. Findlay & D. M. de Kretser, unpublished observations).

Control of ovarian production of inhibin-related peptides

FSH and PMSG increase the ovarian content (Cahill *et al.*, 1985) and ovarian vein secretion rate of inhibin bioactivity (Tsonis *et al.*, 1989) and immunoreactivity in sheep (McNeilly *et al.*, 1989;

Murray *et al.*, 1989a, b). There is indirect evidence that the increasing inhibin production by the dominant follicles in the follicular phase of sheep is driven by LH in the face of falling concentrations of FSH (Findlay *et al.*, 1990b). Inhibin secretion is pulsatile in sheep (McNeilly & Baird, 1989; Campbell *et al.*, 1989; Murray *et al.*, 1989a) and may be related to the LH pulses in the follicular phase (Murray *et al.*, 1989a), although Campbell *et al.* (1989) were unable to establish a direct relationship between LH pulses (exogenous or endogenous) and inhibin secretion in anoestrous ewes. Evidence from rat granulosa cells shows that low doses of LH can stimulate inhibin production, provided that the cells have been exposed to FSH, whereas luteinizing doses of LH will inhibit inhibin production (Zhiwen *et al.*, 1988). The fact that inhibin concentrations fall after the preovulatory surge of LH in ewes (Findlay *et al.*, 1990b) is consistent with the latter observation and the loss of β_A inhibin mRNA at this time (Rodgers *et al.*, 1989; Torney *et al.*, 1989; Luck *et al.*, 1990), and would allow the second rise in FSH to occur.

Both systemic and arterial infusions of epidermal growth factor (EGF) at the time of progesterone sponge withdrawal resulted in a significant reduction in inhibin secretion rate in the early and late follicular phase in ewes (Murray *et al.*, 1989b). Oestradiol (but not androstenedione) secretion rate was also reduced by EGF treatment, implying that the site of action of EGF on production of inhibin was the granulosa cells. This supports earlier work showing an inhibitory action of EGF on inhibin production by bovine granulosa cells *in vitro* (Franchimont *et al.*, 1986).

Earlier studies on production of bioactive inhibin by bovine granulosa cells suggested that androgen may regulate its secretion (Henderson & Franchimont, 1981, 1983). Luck *et al.* (1990) showed that treatment of bovine granulosa cells with 5 nM–5 μM testosterone, in the presence or absence of serum, had no effect on production of immunoreactive inhibin. Treatment with aminoglutethimide inhibited steroidogenesis but had no effect on inhibin secretion. It remains possible that the effects of testosterone on inhibin bioactivity observed by Henderson & Franchimont (1981, 1983) reflect changes in activin and FSP rather than inhibin production.

Although inhibin production by bovine (Henderson & Franchimont 1981, 1983; Luck *et al.*, 1990) and ovine (Campbell, 1989) granulosa cells can be demonstrated during the first 24 h *in vitro*, the quantities produced thereafter are severely reduced. Serum supplementation was recommended by Henderson & Franchimont (1981, 1983), but Campbell (1989) has shown that insulin will stimulate inhibin production by ovine granulosa cells. Insulin was included in the serum-free medium used by Luck *et al.* (1990). Nevertheless, a decline in inhibin production still occurred after the first 24 h, suggesting other factors were controlling the synthesis and secretion of inhibin. This may be related to the propensity of ruminant granulosa cells to luteinize spontaneously in culture and, concomitantly, turn off the expression of β_A inhibin mRNA (Rodgers *et al.*, 1989; Luck *et al.*, 1990).

We investigated the role of activin during the transition from preovulatory to luteinized granulosa cells (Shukovski & Findlay, 1990). Using differentiated bovine granulosa cells in a serum-free medium that included insulin, we showed that bovine activin ($\beta_A\beta_A$) had a time and dose-dependent (1–100 ng/ml; 0·04–4 nmol/l) inhibitory effect on progesterone and oxytocin production, markers of luteinization (Fig. 4). This is consistent with a role for activin as a local regulator capable of preventing the onset of luteinization. However, activin was unable consistently to prevent the fall in inhibin (or oestrogen) production by these cells over 72 h (L. Shukovski & J. K. Findlay, unpublished observations), suggesting that other factors stimulate the expression of the inhibin genes and/or the post-translational control of assembly of the α- and β_A-subunits *in vitro*. There are increasing numbers of local factors which have stimulatory or inhibitory actions on differentiation of rat granulosa cells (see Carson *et al.*, 1989; Findlay *et al.*, 1990c) which could be involved.

Mechanism of action of inhibin

In-vivo administration of material with inhibin activity decreased the production of mRNA for the β-subunit of FSH in the pituitary gland of sheep (Mercer *et al.*, 1987) and cattle (Beard *et al.*, 1989),

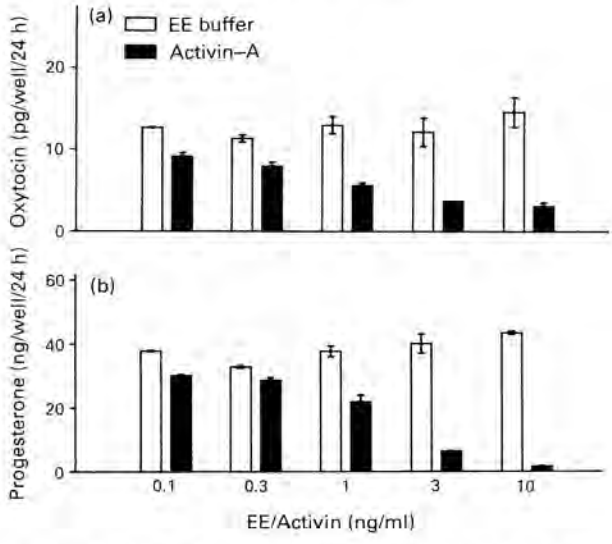

Fig. 4. The dose–response effects of bovine activin-A and control buffer (EE) on (a) oxytocin and (b) progesterone production by bovine granulosa cells on Day 4 in serum-free culture. (After Shukovski & Findlay, 1990.)

and also caused a decrease in circulating concentrations of FSH (sheep: see Findlay *et al.*, 1987; cattle: Beard *et al.*, 1989). That this action is due to M_r 31–32 000 inhibin has been demonstrated by the ability of purified bovine inhibin to suppress FSH secretion in sheep (Findlay *et al.*, 1987). FSH concentrations in ovariectomized ewes are suppressed by human recombinant inhibin in a dose-dependent manner (J. K. Findlay, B. Doughton, D. Poole, D. M. Robertson & R. G. Forage, unpublished observations). None of these purified preparations appeared to influence LH secretion in sheep.

Martin *et al.* (1988) have described a significant synergistic interaction between oestradiol and inhibin, given to ovariectomized ewes in the form of charcoal-treated bovine follicular fluid, on plasma concentrations of FSH. Progesterone had little effect. We have confirmed this synergistic interaction of oestradiol and inhibin in ovariectomized ewes, using a similar treatment protocol (J. K. Findlay, B. Doughton & D. Poole, unpublished observations). It now needs to be demonstrated that this interaction occurs between oestradiol and preparations of purified inhibin. A more difficult question concerns the relative importance of inhibin and oestradiol in controlling FSH secretion. Mann *et al.* (1989a) consider that, during the follicular phase, oestradiol is the more important feedback regulator of FSH. This is supported to some extent by the effects of immunization against inhibin when it has been shown that FSH concentrations increase but mainly in the late luteal phase, rather than in the follicular phase (Al-Obaidi *et al.*, 1987; Findlay *et al.*, 1989b; O'Shea *et al.*, 1989a; Mann *et al.*, 1989b). Nevertheless, our data showing increased oestradiol and inhibin concentrations in the follicular phase of the ewe (Findlay *et al.*, 1990b), together with the synergistic interaction of the two hormones on FSH (Martin *et al.*, 1988), suggest that further experimentation will be required to resolve the issue.

The fact that FSH concentrations can rise in response to active immunization of sheep against various inhibin immunogens (see Forage *et al.*, 1990) also accords with the physiological role of inhibin as a feedback regulator of FSH. The FSH response to active immunization has been variable, and is often small, and appears to depend on the type of immunogen. Passive immunization of ewes in the luteal phase against inhibin also led to increased concentrations of FSH (Mann *et al.*, 1989b). The FSH response to immunization against inhibin may depend on the degree to which its secretion is being controlled by oestradiol and inhibin at the time of measurement of FSH.

Immunization against a synthetic peptide fragment of the α-subunit of inhibin decreased basal (O'Shea *et al.*, 1989b) and GnRH-stimulated LH release (Wrathall *et al.*, 1990), whereas basal LH was increased in ewes immunized against a monoclonal antibody purified fraction of pig follicular fluid (O'Shea *et al.*, 1989b). These are the only inhibin immunization studies which have demonstrated any effects on LH secretion (see Forage *et al.*, 1990). Whether or not they represent a direct action of inhibin on pituitary LH release or indirect effects via FSH stimulating ovarian steroid production is not known. In-vitro studies using sheep pituitary cells have shown that preparations of pig inhibin enchance GnRH-induced LH release (Huang & Miller, 1984), whereas with rat pituitary cells *in vitro* inhibin is equipotent in inhibiting GnRH-induced FSH and LH release (Farnworth *et al.*, 1988). One is led to conclude, in the absence of other data, that there may be species differences with respect to the role of inhibin on LH release.

Local action and physiological role of alpha N peptide

We addressed the question of the role of αN by immunizing sheep against bovine αN. This resulted in impaired fertility, reflected in significantly fewer lambs born per ewe mated (Findlay *et al.*, 1989a, c). This decrease in fertility could not be attributed to changes in circulating concentrations of inhibin (the antisera do not recognize inhibin of M_r 32 000), progesterone, or basal or GnRH-stimulated LH or FSH (there was a significant increase in basal FSH concentrations in the luteal phase of immunized ewes which could not explain the results) during the ovarian cycle. Nor could it be attributed to a decrease in the number of corpora lutea per ewe, indeed, immunization with a different αN fusion protein resulted in an increase in the number of corpora lutea but a decrease in the number of lambs born was still observed (Findlay *et al.*, 1989c). We then examined whether or not the ewes were ovulating. In control ewes, immunized against adjuvants alone, the egg recovery rate, based on the number of corpora lutea on the ovary, was 76% and the fertilization rate was 73%. The majority of eggs were at the 8-cell stage (range 4–16 cells). In ewes immunized against αN, the egg recovery rate was only 17%, and of those 7 eggs, 2 were fertilized (29%) and were at the 4-cell and 16-cell stage of development, similar to eggs from control ewes. In addition, the corpora lutea on ovaries of immunized ewes had the appearance of luteinized unruptured follicles. Overall, the data are consistent with a failure of ovulation in immunized ewes. We suggest that αN is acting locally to facilitate the process of ovulation.

Practical aspects

If the major action of inhibin is to act as a negative feedback regulator of FSH, it follows that attenuation of its biological activity through immunization should lead to an increase in circulating concentrations of FSH and a consequent increase in ovulation rate which in turn should lead to increased litter size. These effects have all been observed in sheep immunized against inhibin and increases in ovulation rate have also been observed in cattle (see Forage *et al.*, 1990). Two groups have now demonstrated that a single immunization of ewes against either a synthetic peptide conjugate or monoclonal antibody-purified bovine inhibin (O'Shea *et al.*, 1989b) or recombinant bovine α-inhibin fusion protein (Tsonis *et al.*, 1989) was sufficient to produce a 1·8–2·2-fold increase in ovulation rate, and a 1·6-fold increase in litter size (Tsonis *et al.*, 1989). Immunization against inhibin does not appear to change the length of the oestrous cycle, the expression of oestrus or seasonal anoestrous patterns, indicating that the method is unlikely to be useful for inducing out-of-season breeding of sheep. Another practical application now under investigation is the use of inhibin vaccines, either alone or in combination with other methods, in superovulation regimens. O'Shea *et al.* (1984) have shown that it is possible to advance puberty in Merino ewe lambs by immunization against inhibin. We have demonstrated a similar effect of immunizing Merino ewe lambs against the bovine α-inhibin fusion protein on onset of puberty (T. O'Shea, B. M. Bindon, C. G. Tsonis, R. G. Forage & J. K. Findlay, unpublished observations).

The application of this technology to cattle is not as advanced as in the sheep (see Forage *et al.*, 1990). It has proved difficult to obtain a consistent immune response of cows to inhibin, with the most promising immunogen being an immunoaffinity purified preparation of sheep follicular fluid, which caused 7/7 cows to respond with a mean ovulation of 11·6 compared to a control rate of 1·0 (Bindon *et al.*, 1988).

Nevertheless, the results demonstrated that immunization against inhibin has potential commercial applications to increase productivity. To achieve the potential benefits of this technology, modifications to existing management practices and good animal husbandry will be necessary.

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