INHIBIN AND RELATED PROTEINS: PURIFICATION, DETECTION AND EFFECTS

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INHIBINE EN GERELATEERDE EIWITTEN: ZUIVERING, DETECTIE EN EFFECTEN

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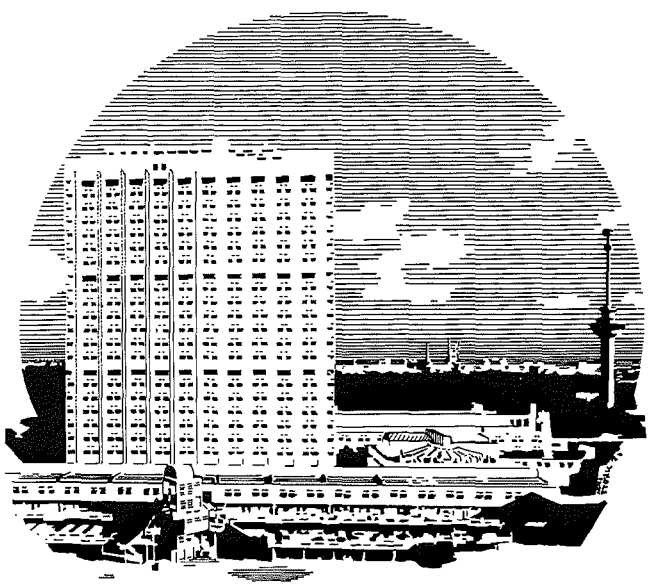

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CONTENTS

LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
bFF	bovine follicular fluid
B/I ratio(s)	bioactivity/immunoactivity ratio(s)
BMPs	bone morphogenetic proteins
bPcas	bovine plasma of ovariectomized cow
BSA	bovine serum albumin
cAMP	adenosine cyclic-3',5'-monophosphate
(c)DNA	(complementary) deoxyribonucleic acid
CrSCCM	control rat Sertoli cell conditioned medium
Dpp-C	decapentaplegic gene complex
EDF	erythroid differentiation factor
FCS	fetal calf serum
FF	follicular fluid
FR	fraction
FrSCCM	FSH-stimulated rat Sertoli cell conditioned medium
FSH	follicle stimulating hormone
FSP	FSH suppressing protein
g	gravity
GnRH	gonadotrophin releasing hormone
GnSIF	gonadotrophin surge inhibiting factor
hCG	human chorionic gonadotrophin
hFSH	human follicle stimulating hormone
kDa	kilo Dalton
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
MEM	minimal essential medium
MIS	Müllerian inhibiting substance
mRNA	messenger ribonucleic acid
MW(M)	molecular weight (marker)
oFF	ovine follicular fluid
oRTF	ovine rete testis fluid
PBS	phosphate-buffered saline
pFF	porcine follicular fluid
PMA	4β-phorbol-12-myristate-13-acetate
rIF	rat interstitial fluid
RIA(s)	radioimmunoassay(s)
RSA	relative specific activity
(r)SCCM	(rat) Sertoli cell conditioned medium
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
TGF-β	transforming growth factor-β
U	unit
v/v	volume/volume
w/v	weight/volume

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION AND SCOPE OF THE THESIS

The main functions of the gonads are the production of sperm or eggs and the production of hormones which influence sexual characteristics. The production of male and female germ cells is the result of a complex interplay between the brain, the pituitary gland, the gonads and the accessary reproductive organs. In the female, one or more oocytes are recruited periodically from a pool of oocytes already present at birth. Once recruited the oocyte itself does not increase greatly in size, but rapid growth occurs in the surrounding steroid producing layers of granulosa and thecal cells. In contrast, sperm production in the testis is a continuous process involving multiplication of the spermatogonial stem cells in most mammalian species, with the exception of seasonal breeders. The mammalian testis consists of two compartments, the seminiferous tubules containing Sertoli cells and spermatogenic cells and the interstitium containing the steroid producing Leydig cells.

Mottram and Cramer reported in 1923 that radiation-induced destruction of the seminiferous tubules of male rats caused hypertrophy and the appearance of "castration cells" in the pituitary gland. The administration of an aqueous testicular extract to gonadectomized rats was found by McCullagh (1932) to prevent the appearance of "castration cells" in the pituitary. The water soluble testicular principle responsible for this activity was termed "inhibin" and was considered to be distinct from the testicular factor "androtin" which was soluble in organic solvents and was later shown to be testosterone. We now know that the pituitary gland produces two gonadotrophic hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH) which are the main regulators of the gonads. In the testis LH is the principal regulator of testosterone production in Leydig cells, whereas FSH influences several Sertoli cell functions. The release of FSH and LH from the pituitary gland is stimulated by the hypothalamic decapeptide, gonadotrophin releasing hormone (GnRH). The secretion of FSH and LH is also regulated by negative feedback actions, exerted by hormones produced by the target glands. In castration experiments in male animals it was shown that increased LH serum levels can be lowered by the steroid

hormone testosterone. The increased FSH levels occurring after castration can only be partially suppressed by testosterone, whereas FSH serum levels return to precastration levels after administration of a steroid free aqueous extract of the testis. Inhibin was believed to be the active principle in these aqueous extracts. Inhibin is a glycoprotein hormone consisting of two dissimilar, disulphide-linked subunits, which inhibits pituitary gonadotrophin production and/or secretion, preferentially that of FSH (Burger and Igarashi, 1988). The existence of inhibin has been debated for a long time, but with the purification of inhibin from follicular fluid and cloning of genes encoding for the inhibin subunits, the existence of inhibin is now generally accepted.

The aims of the present study were the elucidation of the molecular structure of testicular inhibin, development of sensitive immunoassays for detection of inhibin and investigation of the role of inhibin in testicular physiology.

The determination of the molecular weight of inhibin, the presence of an FSH stimulatory activity in female and male gonadal fluids and the development of a radioimmunoassay for the inhibin α-subunit is described in Chapter 2.

In Chapter 3 the purification and characterization of inhibin from rat Sertoli cell conditioned medium is presented.

In Chapter 4 a 29 kDa inhibin α-protein without known biological activity is described. The release of this protein from Sertoli cells is increased under the influence of FSH.

In Chapter 5 the short-term stimulatory effect of Sertoli cell conditioned medium on Leydig cell steroidogenesis is described; this effect is shown to be due to factors different from inhibin.

In Chapter 6 the effect of the presence of a Sertoli cell tumor on the release of FSH and LH after stimulation with GnRH and on peripheral levels of testosterone and oestradiol were investigated.

Finally, in Chapter 7 the relative importance of inhibin and related proteins in several systems are considered and possible directions of future research on inhibin are discussed.

1.2 Purification and cloning of inhibin and related proteins

1.2.1 Purification of inhibin

After the introduction of the concept of inhibin by Mottram and Cramer (1923) and McCullagh (1932), more than half a century passed before significant progress on the purification of gonadal inhibin was reported. The purification of inhibin proved to be difficult for several reasons. The aberrant behaviour of the molecule when conventional purification techniques were used, resulted in poor recoveries and over- as well as under-estimation of the molecular weight caused by the interactions with other proteins and/or column material. Also, the use of different bioassays which were insensitive and susceptible to aspecific effects (see Chapter 1.3.1) has contributed to misinterpretations in the detection of inhibin. The different sources chosen for the purification of inhibin also caused confusion. Following the observation of de Jong and Sharpe (1976) that ovarian follicular fluid is the richest accessible source of inhibin, several groups have used this material for purification and elucidation of the molecular composition of inhibin. Information on molecular weight and subunit composition of inhibins obtained after purification of inhibin from female gonadal sources, are summarized in Table 1. Similar information about inhibin from male gonadal sources is listed in Table 2. This information will now be discussed, and reported forms of inhibin and related proteins are shown in Figure 1.

The purification of inhibin from ovarian follicular fluid is based on a selection of one of the different (120, 108, 88, 65, 55 and 32 kDa) molecular weight forms of proteins which show biological inhibin activity, and which have been detected after separation of follicular fluid proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Hasegawa et al., 1986; Miyamoto et al., 1986; Chapter 2).

Homogeneous preparations of inhibin purified from bovine follicular fluid (bFF) were first reported by Robertson et al. (1985) and Forage et al. (1986). SDS-PAGE of this preparation under non-reducing conditions revealed a 58 kDa protein, whereas after reduction two subunits of 44 and 14 kDa were found, which

were named the α- and β-subunit, respectively. Subsequently, it was shown that the inclusion of a 30% acetic acid precipitation step in the purification procedure of bFF inhibin gave rise to a 31 kDa inhibin form, consisting of two subunits of 18 and 14 kDa (Robertson et al., 1986a). The difference in the molecular weight of these two forms of inhibin is probably due to cleavage of the larger α-subunit, which is supported by the following observations: 1) Antibodies against the 58 kDa inhibin form also recognize the 31 kDa inhibin, 2) On the basis of the derived amino acid sequence from the bovine inhibin α-subunit gene (Forage et al., 1986) it appears that the N-terminal amino acids of the 44 and 18 kDa α-subunit are preceded by basic amino acids which are susceptable to proteolytic cleavage, and 3) Iodinated 58 kDa inhibin is clipped to a 31 kDa molecule after incubation in serum or acid treatment (McLachlan et al., 1986a).

Table 1. Molecular weights and subunit composition of inhibin from ovarian follicular fluid

Reference	Source	Molecular weight (kDa)	
		nonreduced	reduced
Fukuda et al. (1986)	bovine	32	α 20 β* 13
Robertson et al. (1985) Forage et al. (1986)	bovine bovine	58	α 44 β-A 14
Robertson et al. (1986)	bovine	31	α 18 β-A 14
Leversha et al. (1987)	ovine	30	α 20 β-A 15
Ling et al. (1985) Mason et al. (1985)	porcine porcine	32	α 18 β-A 14 β-B 14
Rivier et al. (1985)	porcine	32	α 18 β* 14
Miyamoto et al. (1985)	porcine	32	α 20 β* 13

* = β is used in case not enough N-terminal amino acids of the β-subunit have been determined for the discrimination between β-A and β-B

In molar terms the specific biological activity of 58 kDa inhibin is 2-3 times higher than that of the 31 kDa form, suggesting that the clipped region of the 44 kDa subunit contributes to the biological activity of the inhibin molecule (Robertson et al., 1986a). Fukuda et al. (1986) reported several molecular weight forms of inhibin in bFF, from which they purified a 32 kDa dimer.

Also from porcine follicular fluid (pFF) a 32 kDa inhibin dimer has been purified and sequenced (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985). Ling et al. (1985) reported the purification of two forms of 32 kDa inhibin from pFF. Amino acid sequence analysis revealed that both forms had identical N-terminal α-subunit amino acids but certain amino acids in the β-subunit were different (Mason et al., 1985). These two forms of inhibin are now known as inhibin A (αβ-A) and inhibin B (αβ-B).

Finally, Leversha et al. (1987) purified a 30 kDa αβ-A dimer from ovine follicular fluid (oFF).

Many investigators have used ovine rete testis fluid (oRTF), as starting material for the purification of male gonadal inhibin. Baker et al. (1982) reported molecular weight forms of inhibin between 60 and 90 kDa utilizing gel filtration chromatography, although under denaturing conditions on SDS-PAGE a 30 kDa bioactive inhibin form was isolated. Vaughan et al. (1989) reported the presence of several immunoreactive bands detected with antibodies raised against the 26 N-terminal amino acids of the α-chain of 32 kDa porcine inhibin after separation of oRTF proteins by SDS-PAGE. They purified a 32 kDa inhibin dimer, consisting of a 22 kDa α-subunit and 15 kDa β-subunit. N-terminal amino acid sequence analysis of the β-subunit revealed amino acids similar to those reported for the β-A subunit of rat, human, porcine and bovine inhibin, with one exception at position 17 where tyrosine was replaced by phenylalanine. Bardin et al. (1987) purified two forms of 32 kDa inhibin from oRTF, one of which was characterized by a truncated α-subunit: one was 15 amino acids shorter than the other. They also found the N-terminal amino acids of the β-A-subunit with the same amino acid change at position 17, reported by Vaughan et al. (1989).

Table 2. Molecular weight and subunit composition of inhibin from male gonadal sources

Reference	Source	Molecular weight (kDa)		
		determined by	nonreduced	reduced
Baker et al. (1982)	oRTF[a]	gel filtration	60-90	-
		SDS-PAGE	30	-
Vaughan et al. (1989)	oRTF[a]	SDS-PAGE	32	α 22
				β-A 15
Bardin et al. (1987)	oRTF[a]	SDS-PAGE	32	α 18
				β-A 16.5
Sharpe et al. (1988)	rat tubule medium	gel filtration	35	-
Bicsak et al. (1987)	rat SCCM[b]	gel filtration	32	-
Noguchi et al. (1987)	Cynomolgus monkey SCCM[b]	gel filtration	40	-
Grootenhuis et al. (1989) (Chapter 2)	rat testis	SDS-PAGE	32	-
Grootenhuis et al. (1990) (Chapter 3)	rat SCCM[b]	SDS-PAGE	32	α 18
				β-B 12

[a] = oRTF (ovine rete testis fluid)
[b] = SCCM (Sertoli cell conditioned medium)

Sharpe et al. (1988) have reported the detection of a 100 kDa immunoreactive peak of inhibin in rat testis extract after gel filtration chromatography, and found a 30-100 kDa peak in rat interstitial fluid (rIF) and a 35 kDa peak in rat seminiferous tubule conditioned medium. Bicsak et al. (1987) found a 32 kDa immunoreactive and bioactive form in conditioned medium from rat Sertoli cells (rSCCM), which had been stimulated with forskolin, an activator of adenylate cyclase. Noguchi et al. (1987) detected a 40 kDa bioactive inhibin form in culture medium from immature Cynomolgus monkey Sertoli cells, after gel filtration chromatography. They found that inhibin was completely retained on Concavanalin A-Sepharose and could be eluted with 0.2 M α-methylmannoside, indicating that this inhibin is a glycoprotein. We also found a 32 kDa inhibin form in rat testis extract and rSCCM after SDS-PAGE (Chapter 2) and isolated a 32 kDa $\alpha\beta$-B dimer

from rSCCM (Chapter 3).

1.2.2 Purification of activin and inhibin related proteins

During the purification of inhibin from pFF on a reversed phase column Ling et al. (1985) noticed that some fractions eluting before inhibin A and B stimulated FSH release from cultured pituitary cells. They purified this FSH releasing entity from pFF and found it to be a heterodimer of a β-A and a β-B subunit of inhibin (Ling et al., 1986a). At the same time Vale et al. (1986) reported the purification of another FSH releasing protein from pFF, which appeared to be a homodimer of β-A inhibin subunits. This observation was confirmed by Ling et al. (1986b). These proteins, now termed activin AB and activin A are potent stimulators (half-maximal effect with 4 ng/ml) of FSH release, but have no effect on LH release from pituitary cells in vitro. McLachlan et al. (1987) also reported the purification of activin A from bFF, although no information about purification strategy, molecular weight or amino acid sequence analysis were provided.

An intriguing aspect is that a polypeptide has been purified from the conditioned medium of a human monocytic leukemia cell line (THP) which has the same primary structure as activin (Eto et al., 1987). This polypeptide caused differentiation of Freund erythroleukemic cells and was named erythroid differentiation factor (EDF). The THP cell line was chosen for the large scale production of EDF/activin A, after screening several human cell lines for the induction of EDF with PMA (4β-phorbol-12-myristate-13-acetate). Tsuji et al. (1988) found that besides THP cells, K-562 cells, A-253 (carcinoma epidermoid), HEC-1-A (endometrial carcinoma), T24 (bladder carcinoma), SK-N-SH (neuroblastoma), HT-1080 (fibrosarcoma) and Detroit 551 (human skin) cells also secrete EDF activity upon PMA stimulation.

Miyamoto et al. (1986) showed very elegantly with monoclonal antibodies against the α- and β-subunit of inhibin on Western blots, that besides the isolated 32 kDa and 58 kDa bioactive inhibin forms, other inhibin molecules and fragments are present in pFF. Sugino et al. (1989) reported the purification from bFF of a 26 kDa pro-αC inhibin form. This form is composed of the

pro-sequence (amino acids 18-60) of the N-terminal part of inhibin α-subunit linked by disulphide bond(s) to the C-terminal part of the α-subunit (αC, amino acids 227-360) from the bovine inhibin α-subunit precursor (amino acids 1-360) (Fig.1). The presence of fragments of the inhibin α-subunit precursor has been confirmed by Robertson et al. (1989), who purified both the pro-αC subunit and the clipped region (αN-subunit, 61-226) from bFF. The αC subunit of inhibin has also been purified from bFF by Knight et al. (1989). In Chapter 4 evidence is provided that cultured rat Sertoli cells secrete the pro-αC subunit under the

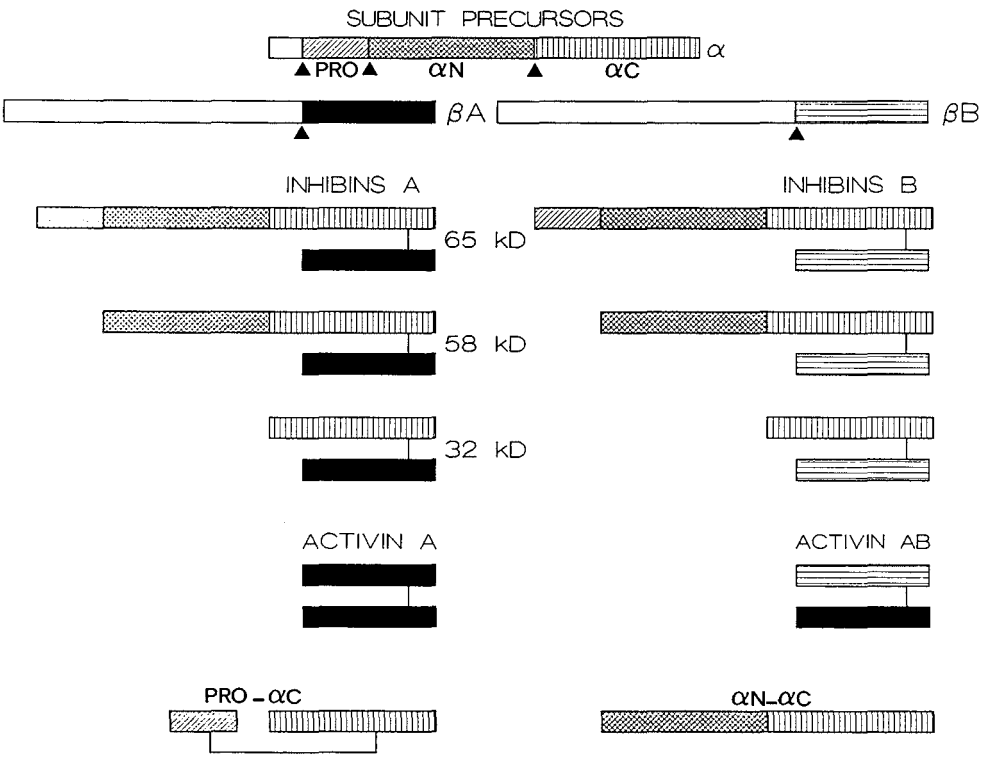

Figure 1. Schematic representation of the inhibin α, β-A and β-B subunit precursors and the derived inhibins, activins and α-subunit forms which have been purified from follicular fluid. The inhibin α-subunit precursor is made up of three parts, pro-αN-αC, these forms are preceded by proteolytic cleavage sites (indicated with ▲). The inhibin β-A and β-B subunit precursors are always processed to the 14 kDa form. The different α-subunit forms can combine with the β-A or β-B sununits, forming 65 kDa, 58 kDa and 32 kDa αβ-A or αβ-B inhibins. Also individual pro-αC and αN+C fragments are found. The β-A and β-B subunits can also form activins.

influence of FSH. No inhibin antagonistic or agonistic activities of these fragments on the FSH release from cultured pituitary cells have been found.

1.2.3 Other FSH release inhibiting factors, different from inhibin

Ueno et al. (1987) reported the purification of two follistatins, monomeric proteins with identical N-terminal amino acids and molecular weights of 32 and 35 kDa from pFF, which specifically inhibited FSH release from cultured pituitary cells. The half-maximal effective dose of the follistatins was 6-7 ng/ml, which corresponds to 1/3 of the potency of inhibin. Robertson et al. (1987) confirmed and extended these results, by purifying 3 monomeric proteins with molecular weights of 32, 35 and 39 kDa with identical N-terminal amino acids from bFF. They named these proteins FSH suppressing proteins (FSPs).

3α-Hydroxy-4-pregnen-20-one, a steroid isolated from the conditioned medium of cultured rat Sertoli cells, has been reported to inhibit FSH release <u>in vitro</u> specifically (lowest effective dose 10^{-16}M). FSH serum concentrations <u>in vivo</u> could also be suppressed by administration of this steroid to rats (Wiebe and Wood, 1987; Wood and Wiebe, 1989).

The purification of the proteins α-inhibin-92, α-inhibin-52, and β-inhibin-94 from seminal plasma (for review see Li and Ramasharma, 1987) will not be discussed here, because several groups found them to be inactive in the pituitary cell assay.

1.2.4 Cloning of the inhibin genes

Mason et al. (1985) reported the isolation of complementary deoxyribonucleic acid (cDNA) clones for the inhibin α,β-A, and β-B subunit genes from a porcine ovarian cDNA library, screened with probes designed on the basis of N-terminal amino acids of these subunits. These results have been confirmed and extended for porcine (Mayo et al., 1986; Mason et al., 1985), bovine (Forage et al., 1986 and 1987), human (Mason et al., 1986 and 1989; Stewart et al., 1986; Mayo et al., 1986; Keinan et al., 1989; Feng et al., 1989; Murata et al., 1988) and rat (Esch et

al., 1987; Woodruff et al., 1987) inhibin subunits. Table 3 summarizes information about the inhibin genes which have been sequenced and the sources used to make cDNA libraries.

Table 3. Deduced number of amino acids of cloned inhibin subunit genes and source of cDNA library

Species/Reference	deduced amino acids			library from
	α	β-A	β-B	
Human				
Mayo et al. (1986)	1-366	--	--	placenta
Mason et al. (1986)	20-366	1-426	55-407	ovary
Mason et al. (1989)	1-366	1-426	1-407	ovary
Stewart et al. (1986)	1-366	311-426	--	macrophages*
Keinan et al. (1989)	1-366	--	--	testis
Feng et al. (1989)	--	--	22-407	testis
Murata et al. (1988)	--	1-426	--	THP (monocytic cell line)
Bovine				
Forage et al. (1986)	1-360	258-425	--	granulosa cells
Forage et al. (1987)	1-360	1-425	--	granulosa cells
Porcine				
Mason et al. (1985)	1-364	1-424	59-407	ovary
Mayo et al. (1986)	1-364	--	--	ovary
Rat				
Esch et al. (1987)	1-366	1-424	129-407	ovary
Woodruff et al. (1987)	1-366	1-424	--	ovary

* cloned from a genomic library

The genomic organization of inhibin genes has been reported for human material (Forage et al., 1987; Mason et al., 1989; Stewart et al., 1986) and has been indicated for bovine material by Forage et al. (1986). The structure of the human genes is simple, each gene has one intron. In the human α-gene an intron of 2 kb has been found in the precursor α-N sequence between amino acids 90 and 91, the same position was found in the bovine gene. The introns in the human β-A and β-B genes are located between amino acids 130 and 131 and 92 and 93, respectively and are 10 kb and 2.8 kb long.

The inhibin α and β-B genes were mapped to human chromosome 2 and mouse chromosome 1. The β-A genes are located on human chromosome 7 and mouse chromosome 13 (Barton et al., 1989).

A high degree of homology is found between the translation products of the inhibin subunit genes in different species (rat, bovine, human, porcine) in the C-terminal end. The deduced amino acids of the α-subunit, β-A and β-B subunit genes have about 80, 100 and 85% homology respectively.

A closer look at the deduced amino acid sequences of inhibin subunits reveals that preceding the inhibin α-subunit molecules which have been purified (18 kDa αC, 26 kDa pro-αC and 25 kDa αN), pairs of basic amino acids are present, which are susceptable to proteolytic cleavage (Steiner et al., 1980).

The similar location of 7 cysteines in the αC-subunit and mature β-A and β-B subunits (both 9 cysteines) and the high degree of homology between the subunits suggests that the α, β-A and β-B inhibin genes have been derived from one single ancestral gene.

1.2.5 The inhibin super-family

Striking homologies have been reported between the deduced amino acid sequence of the C-terminal part of the inhibin subunits and of several factors which are involved in the regulation of growth and differentiation in other systems, such as transforming growth factor β (TGF-β) and Müllerian inhibiting substance (MIS). TGF-β has been found in many cell types and can stimulate or inhibit cell proliferation depending on the cell type and conditions used in the assay (Roberts et al., 1988). TGF-β is present in an inactive proform of 140 kDa which can be activated after acidification or removal of the sugar chain or its sialic acid residues (Miyazono and Heldin, 1989). Cloning of the human cDNA for TGF-β1 revealed 40, 38 and 28% homology in the C-terminal end with the inhibin β-A, β-B and α genes respectively (Derynck et al., 1985). TGF-β has been shown to stimulate FSH release from cultured rat pituitary cells (Ying et al., 1986a), although these results have not been confirmed (Vale et al., 1988).

MIS is secreted by the differentiating testis and causes the regression of the Müllerian duct. MIS has been purified as a 140 kDa dimer, composed of 2 identical subunits. Cloning of the human and bovine MIS genes (Cate et al., 1986) revealed that the C-terminal part of the MIS precursor has 7 cysteines in the same

positions as found in the inhibin subunits and that the C-terminal part it is preceded by basic amino acids. Pepinsky et al. (1988) showed that the C-terminal part can be cleaved off by plasmin, resulting in a bioactive 25 kDa dimeric protein.

Recently, genes coding for proteins involved in bone formation named bone morphogenetic proteins (BMPs) have been cloned (Wozny, 1988). Again the N-terminal amino acids of the 30 kDa dimeric bioactive protein have been found in the C-terminal part of the predicted BMP precursor.

In the predicted amino acid sequence of the translation product of the decapentaplegic gene complex (Dpp-C) of Drosophila, which contributes to the determination of the dorsal structures in the developing embryo, homology with the inhibin genes is found in the C-terminal part (Padgett et al., 1987).

The predicted product of the Xenopus Vg-1 gene, which codes for RNA synthesized during oogenesis and is inherited in the cleaving embryo throughout the blastula stage, appears to be involved in the induction of endoderm on the vegetal pole of the oocyte (Weeks and Melton, 1987).

The precursors of inhibin, TGF-β, MIS, BMPs, Dpp-C and Vg-1 have several features in common; they carry the smallest active unit at their C-terminal end preceded by basic amino acids which are susceptible to proteolytic enzymes, they are similar in size, have typical signal sequences at their N-terminal end, and have a similar distribution of cysteine residues. Also, all these factors are active in dimeric forms, which suggests an origin from distantly related genes. Much can be learned from a comparison of these factors, in terms of: regulation of transcription and translation, development of detection systems and receptor mediated second messenger pathways.

1.3 Detection of inhibin and related proteins

1.3.1 Bioassays

Initially, the detection of inhibin activity was dependent on <u>in vivo</u> methods, monitoring the biological consequences of the suppression of FSH by inhibin preparations. The inhibition of the human chorionic gonadotrophin (hCG)-induced increase in ovarian

or uterine weight, or the suppression of the FSH increase after castration have been used for the detection of inhibin (for a review see de Jong, 1988). Such in vivo bioassays are insensitive, have poor precision, require large quantities of inhibin and large numbers of test animals and are also susceptible to aspecific effects of FSH binding inhibitors (Sluss et al., 1987).

Dispersed pituitary cells have been widely used as assay for the isolation of hypothalamic releasing factors (Vale et al., 1972) and also offer the most reliable bioassay system for the detection of inhibin. Rat pituitary cells are sufficiently sensitive for the detection of inhibin in gonadal extracts, gonadal fluids and conditioned media from granulosa cells and Sertoli cells. However, for the detection of inhibin in serum, the more sensitive ovine pituitary cells have to be used (Tsonis et al., 1986). End-points in the pituitary cell assay are the suppression of basal or GnRH-stimulated FSH release (de Jong et al., 1979; Eddie et al., 1979) or the suppression of cellular FSH content (Scott et al., 1980). Significantly higher inhibin values are obtained when purified 32 kDa bFF inhibin is tested against a bFF standard, using the basal release procedure than with cell content or GnRH-stimulated release procedures (Robertson et al., 1986b).

The inhibin assay used in the studies presented in this thesis includes preincubation of isolated rat pituitary cells for 3 days. Subsequently medium is renewed, inhibin samples are added, cells are cultured for another 3 days period and medium is removed and assayed for FSH and LH immunoreactivity. Suppression of FSH release is indicative for inhibin although aspecific effects can influence FSH secretion. These aspecific effects can be detected by: 1) The simultaneous measurement of LH which should not be suppressed by inhibin, 2) The use of parallel line assay statistics to assess parallelism between sample and standard, and 3) Testing for cytoxic effects by evaluation of the pituitary cell morphology or testing ^{51}Cr-release from prelabelled pituitary cells (Robertson et al., 1982). To what extend factors such as activins, TGF-βs and follistatins interfere in the determination of bioactive inhibin is not known, but conceivable, since these factors are present in follicular

fluid. An FSH releasing activity of 25 kDa is present in SCCM (Chapter 2) and follistatin has been cloned from a testicular cDNA library (Shimasaki et al. 1988).

The effect of activin A on the secretion of FSH from dispersed pituitary cells is dependent on the cell-density of the pituitary cells: the higher the density, the smaller the stimulatory action of activin A on FSH release (Kitaoka et al., 1989). This effect of cell-density on the detection of activin could be caused by secretion of inhibin or activin by the pituitary cells themselves, since both α and β-B mRNAs and proteins were detected in the pituitary and pituitary cells, respectively (Meunier et al., 1988a; Roberts et al., 1989).

1.3.2 Immunological methods

Immunization against purified inhibin preparations has only resulted in low titer antibodies. This could be caused by the extensive homology in the structure of inhibin subunits in different species (see Chapter 1.2.4). Raising polyclonal antibodies in chicken is one way to circumvent these problems (Hasegawa et al., 1988a). Another approach is the use of synthetic fragments of the inhibin subunits as immunogens. Regions to be used as antigen can be chosen on the basis of hydrophilicity and chain flexibility analysis of the amino acid sequence of inhibin. However, this approach is complicated by the lack of knowledge of the positions of intra- and inter-subunit disulphide bridges and the three dimensional structure of the inhibin molecule.

Inhibin radioimmunoassays (RIAs), using polyclonal antibodies against purified inhibin preparations, have been reported by several groups. Initially McLachlan et al. (1986a) reported the development of a RIA, using rabbit antibodies against 58 kDa bFF inhibin, which was not sensitive enough to measure inhibin in serum and had only low cross-reactivities for human, rat and ovine inhibin. Subsequently, this RIA has been adapted for inhibin measurement in serum and could detect monkey, rat, ovine and human inhibin (McLachlan et al., 1987; Robertson et al., 1988; Fingscheidt et al., 1989). These improvements were obtained

through immunization with inhibin preparations of increasing purity. Other RIAs directed against 32 kDa bFF and pFF inhibin, were reported by Hamada et al. (1989), Taya et al. (1989), Hasegawa et al. (1986) and Suzuki et al. (1987).

Specificities of these RIAs were evaluated by calculating bioactivity/immunoactivity ratio's (B/I ratio's) throughout the purification of inhibin from bFF and pFF. McLachlan et al. (1986a) noticed that at the start of the purification of inhibin from bFF, more immunoactivity than bioactivity was detected, although in later stages in the purification B/I ratio's around 0.3-0.4 were observed. Hasegawa et al. (1986) found that the different molecular weight forms of inhibin could be detected with their RIA and had the same B/I ratio's. Recently Robertson et al. (1989) reported the purification of an inhibin subunit precursor (pro-αC) from bFF which cross-reacts in their RIA, and comprises 10-20% of the immunoreactivity found in bFF.

There are only a few reports on raising monoclonal antibodies against inhibin. Both Lee et al. (1986) and de Jong et al. (1987) reported monoclonals which were able to immunoneutralize inhibin bioactivity in vitro from follicular fluid. Both groups have not reported that these monoclonals could be used for the purification or detection of inhibin. Miyamoto and colleagues (1986) have raised monoclonal antibodies which specifically recognize the α and β-subunit of bFF inhibin forms.

Several groups have raised antibodies against synthetic fragments of inhibin subunits. Rivier and colleagues (1985) reported a low titer antibody (working dilution for RIA 1:3.000) against the 6 N-terminal amino acids of the porcine (p) αC-subunit with an additional Glycine and Tyrosine (called pαC(1-6)GlyTyr-OH). The titers of these antisera have been improved by using longer synthetic peptides like pαC(1-25)GlyTyr-OH (Rivier et al., 1986), pαC(1-26)GlyTyr-OH (Bicsak et al., 1986), pαC(1-29)Tyr-OH (Ying et al., 1987b) and many others (see Table 4). These antibodies have been raised in rabbits or sheep after coupling of the peptides to carrier proteins. Several of these authors reported that their antibodies could immunoneutralize inhibin bioactivity, indicating that the C-terminus of the αC-subunit is part of the

Table 4. Synthetic inhibin fragments which have been used to raise antibodies and some of their reported applications

Reference	immunogen	used for
	α-subunit peptides	
Rivier et al. (1985)	pαC(1-6)GlyTyr-OH	RIA(1:3.000), immunoneu.
Rivier et al. (1986)	pαC(1-25)GlyTyr-OH	RIA(1:300.000), immunoneu.
Bicsak et al. (1986) Rivier et al. (1988) Vaughan et al. (1989)	pαC(1-26)GlyTyr-OH	RIA(1:300.000), immunocyt., Western, immunopur.
Sharpe et al. (1988)	pαC(1-26)GlyTyr-OH	RIA(1:100.000)
Ying et al. (1987b)	pαC(1-29)Tyr-OH	RIA(1:30.000/100.000), immunoneu., immunopur.
Michel et al. (1989)	pαC(1-30)GlyTyr-OH	RIA(1:15.000)
Schanbacher (1988)	pαC(2-29)Tyr-OH	RIA(1:60.000)
Knight et al. (1989)	hαC(1-30)GlyTyr-OH	RIA(1:16.000/80.000), Western, immunopur.
Culler et al. (1988)	hαC(1-30)GlyTyr-OH	RIA(1:320.000), immunoneu.
Saito et al. (1989)	hαC(1-16)TyrLys-NH_2	RIA(1:200.000), Western, immunoneu., immunocyt.
	LysTyrGlyhαC(13-24)-NH_2	RIA(1:20.000)
	LysGlyGlyhαC(109-123)-NH_2	RIA(1:40.000)
Ying et al. (1987a)	cycCys^{6}Tyr^7 hαC(6-30)	RIA(1:80.000)
Grootenhuis et al. 1989 (Chapter 2)	bαC(1-22)Tyr-OH	RIA(1:200), Western
	β-subunit peptides	
Saito et al. (1989)	LysTyrGlyhβ-A(69-79)-NH_2	RIA(1:19.000)
	CysGlyGlyhβ-A(93-105)-NH_2	RIA(1:8.000), Western, immunoneu., immunocyt.
	CysGlyGlyhβ-B(93-104)-NH_2	RIA(1:17.000), immunoneu., immunocyt.
Vaughan et al. (1989)	cycpβ-A(81-113)	Western, immunopur.
Shaha et al. (1989)	hβ-B(80-112)	immunocyt.

p:porcine, h:human, b:bovine, cyc:cyclic, immunoneu.:immunoneutralization, immunopur.:immunopurification, immunocyt.:immunocytochemistry and Western: Western blot

bioactive centre. Only two groups (Vaughan et al., 1989 and Saito et al., 1989) have tested different regions of the inhibin molecule, and both groups found that the C-terminus of the αC-subunit is the most immunogenic.

Specificity of these RIAs has been analyzed in most of the cases by evaluating whether inhibin containing samples had parallel displacement curves with the synthetic standard.

We have observed (Chapter 2) that different molecular weight forms of inhibin in bFF had B/I ratio's which differ more than five-fold. Bicsak et al. (1987) have found that after gel filtration of rSCCM (which had been stimulated with forskolin), the bioactive 32 kDa inhibin peak was also detected with an antibody against pα(1-26)GlyTyr-OH.

In Chapter 4 evidence is provided that these antibodies recognize in addition to the bioactive 32 kDa αβ inhibin form, a 29 kDa inhibin α-subunit form in rSCCM, which could be the rat equivalent of pro-αC.

1.3.3 RNA detection

A number of approaches can be used for the detection of specific inhibin messenger ribonucleic acid (mRNA). The easiest way is to isolate total-RNA from a tissue, to spot it on filters, and to hybridize the filter bound mRNA with cDNA encoding for the mRNA. Northern blots can be made for the determination of the size of the mRNA and exclude aspecific interactions of the cDNA with ribosomal-RNA. The sensitivity of this technique is increased by analysis of mRNA, instead of total-RNA. With <u>in situ</u> hybridization, mRNAs can be detected in individual cells of tissue sections.

Very low levels of mRNA can be detected with the S1-nuclease technique. In this technique RNA is hybridized with an inhibin-probe in solution. Subsequently all single stranded RNA is digested with S1-nuclease, and hybridized probes are separated by electrophoresis.

1.3.4 Concluding remarks on the detection of inhibin and related proteins

Until recently, inhibin was estimated routinely with the pituitary cell bioassay, but it is now evident that the effect on FSH release measured with this technique reflects the combined effects of inhibin, activins and follistatins in a test sample. Detection of mRNAs in tissues will predict only the subunits of inhibin which may be formed. Also the currently available antibodies against inhibin or synthetic proteins are not able to discriminate between inhibin, inhibin α-subunit forms and activins. The analysis of samples on Western blots is one useful approach to investigate the existence of different forms of inhibin related proteins. This complex of difficulties indicates, that conclusions about the presence of inhibin in a tissue or cell type can only be made after the combination of results, obtained with different techniques.

1.4 Localization of inhibin and related proteins

1.4.1 Gonadal localization

Ovarian localization of inhibin and related proteins will be discussed briefly; the most important contributions are shown in Table 5. Testicular localization will be discussed more extensively; it has been summarized in Table 6.

After the demonstration that ovarian follicular fluid is a potent source of bioactive inhibin (de Jong and Sharpe, 1976), many studies have confirmed the presence of inhibin bioactivity in the ovary. The first report indicating that granulosa cells are the source of bioactive inhibin was from Erickson and Hsueh (1978). The production of inhibin-(subunits) by granulosa cells has been confirmed by bioassays, inhibin RIAs, detection of mRNA and immunocytochemical localization of inhibin subunits (see Table 5). Activins (both A and AB) have been purified from ovarian follicular fluid (see Chapter 1.2.2). Detection of FSH release stimulating proteins of 25 kDa after SDS-PAGE separation of ovarian homogenates is another approach to detect activin-like

Table 5. Ovarian localization of inhibin related proteins

Cell type / reference	inhibin related proteins				method
	$\alpha\beta$	α	β-A	β-B	
Granulosa cells					
Bicsak et al. (1986)	+				bioassay
Bicsak et al. (1988)		+			RIA, immunopr.
	+	+	+		Western
Cuevas et al. (1987)		+			immunocyt.
Erickson et al. (1978)	+				bioassay
Merchenthaler et al. (1987)		+			immunocyt.
Meunier et al. (1988b)		+	+	+	immunocyt., in situ
Rivier et al. (1989)		+	+	+	in situ
Torney et al. (1989)		+	+		in situ
Woodruff et al. (1988)		+	+		in situ
Woodruff et al. (1989)		+	+		in situ
Thecal cells					
Cuevas et al. (1987)		-			immunocyt.
Merchenthaler et al. (1987)		-			immunocyt.
Meunier et al. (1988b)		+	-	-	immunocyt., in situ
Rivier et al. (1988)		+			in situ
Torney et al. (1989)		+	-		in situ
Woodruff et al. (1988)		-	-		in situ
Interstitial cells					
Meunier et al. (1988b)		+	-	-	immunocyt., in situ
Rivier et al. (1988)		+	-	-	in situ
Woodruff et al. (1988)		-	-		in situ
Luteal cells/ corpus luteum					
Cuevas et al. (1987)		+			immunocyt.
Davis et al. (1986)		+			Northern
Davis et al. (1987)		+	+		Northern
Merchenthaler et al. (1987)		+			immunocyt.
Meunier et al. (1988b)		+	-	-	immunocyt., in situ
Rodgers et al. (1989)		-	-		Northern
Suzuki et al. (1987)	+				RIA
Torney et al. (1989)		-	-		in situ
Tsonis et al. (1987)	+				bioassay
Woodruff et al. (1988)		-	-		in situ
Woodruff et al. (1989)		-	-		in situ

immunocyt.: immunocytochemie, immunopr.: immunoprecipitation
in situ: in situ hybridization, Northern: Northern blot, Western: Western blot

(+) = detected, (-) = not detected, ( ) = not tested

activity (see Chapter 2). Bicsak et al. (1988) were not able to detect activin in rat granulosa cell conditioned medium using a protein antibody (cyclicβ-A(81-113)-OH on Western blots, although the same antibodies stained inhibin.

There are no reports on the localization of inhibin or activin-like bioactivity in thecal cells. Contradictory results on the localization of α-subunit in thecal cells have been obtained with <u>in situ</u> hybridization and immunocytochemistry.

Several authors, have shown the presence of inhibin α-subunits in luteinizing granulosa cells and corpora lutea, whereas others could not detect inhibin in this tissue. Studies with luteinizing granulosa cells <u>in vitro</u> (Tsonis et al., 1987; Suzuki et al., 1987) indicate that the secretion of inhibin is lower after several days of culture and becomes responsive to LH.

Table 6. Testicular localization of inhibin related proteins

Cell type / reference	inhibin related proteins					method
	$\beta\beta$	$\alpha\beta$	α	β-A	β-B	
Sertoli cells						
Cuevas et al. (1987)			+			immunocyt.
Feng et al. (1989)			+	+	+	Northern
Merchenthaler et al. (1987)			+			immunocyt.
Rivier et al. (1988)			+			immunocyt.
Saito et al. (1989)			+	+	+	immunocyt.
Shaha et al. (1989)			+	-	+	immunocyt.
Toebosch et al. (1988)			+	-	+	Northern
Ying et al. (1987b)			+			immunocyt., RIA
Leydig cells						
Merchenthaler et al. (1987)			-			immunocyt.
Lee et al. (1989)	+	-	-	+	+	bioassay, Northern
Risbridger et al. (1989a)	-	+	+			bioassay, Northern
Rivier et al. (1988)			-			immunocyt.
Saito et al. (1989)			-	-	-	immunocyt.
Shaha et al. (1989)			-	+	-	immunocyt.
Germ cells						
Merchenthaler et al. (1987)			-			immunocyt.
Rivier et al. (1988)			-			immunocyt.
Saito et al. (1989)			-	-	-	immunocyt.
Shaha et al. (1989)			-	+	-	immunocyt.

immunocyt.: immunocytochemie, Northern: Northern blot,

(+) = detected, (-) = not detected, ( ) = not tested

The presence of inhibin subunit mRNAs and the synthesis and secretion of inhibin-(subunits) by Sertoli cells will be discussed in more detail in Chapter 1.5. The localization of inhibin subunits in this cell type has been confirmed by in situ hybridization and immunocytochemistry (see Table 6).

No consensus exists on the presence of mRNAs for the inhibin subunits and the secretion of inhibin bioactivity by Leydig cells. Lee et al. (1989) have reported the presence of β-A and β-B inhibin subunit mRNAs in mouse tumor Leydig cell line (TM3) and detected activin-like bioactivity in the conditioned medium of pig Leydig cells. Risbridger et al. (1989a) reported the presence of inhibin α-subunit mRNA in adult rat Leydig cells, and detected inhibin-like bioactivity in the conditioned medium. Using immunohistochemistry Shaha et al. (1989) reported the presence of inhibin β-A immunoreactivity in Leydig cells.

The same authors also reported the immunocytochemical detection of the inhibin β-A subunit in nuclei of immature germ cells; this observation has not been confirmed by others.

1.4.2 Non-gonadal localization

The localization of inhibin subunits in non-gonadal tissues is summarized in Table 7 and only the most relevant contributions will be discussed here. With the sensitive S1-nuclease technique inhibin subunit mRNAs have been detected in the adrenal gland, bone marrow, central nervous sytem, pituitary gland, kidney, placenta, spinal cord and spleen of the rat (Meunier et al., 1988a).

The localization of inhibin α-subunit mRNA in the adrenal gland was first reported by Crawford et al. (1987), who showed that the amount of α-subunit mRNA in sheep adrenal cortex cells was increased after adrenocorticotrophic hormone (ACTH) stimulation.

The presence of inhibin β-A subunits in bone marrow cells has been confirmed with the purification of activin A from the conditioned medium of a human monocytic leukemia cell line (THP) by Eto et al. (1987).

In the central nervous system inhibin β-A subunits have been localized using immunocytochemistry in the efferent projections

of the nucleus of the solitary tract, including prominent projections to the paraventricular and supraoptic nuclei where oxytocin-containing magnocellular neurons are located (Sawchenko et al., 1988).

Table 7. Non-gonadal localization of inhibin related proteins

Organ / Cell type reference	inhibin related proteins					method
	$\beta\beta$	$\alpha\beta$	α	β-A	β-B	
Adrenal gland/cortex						
Crawford et al. (1987)			+			Northern
Meunier et al. (1988a)			+	+		S1-nuclease
Bone marrow						
Eto et al. (1987)	+					bioassay
Meunier et al. (1988a)					+	Northern
Central nervous system						
Meunier et al. (1988a)			+	+	+	S1-nuclease
Sawchenko et al. (1988)			-	+		immunocyt.
Pituitary gland/gonadotrophs						
Cuevas et al. (1987)			-			immunocyt.
Meunier et al. (1988a)			-	+	+	S1-nuclease
Roberts et al. (1989)			+	+	+	immunocyt.
Kidney						
Meunier et al. (1988a)			+	-	-	S1-nuclease
Placenta/cytotrophoblast						
Davis et al. (1986)			-		+	Northern
Davis et al. (1987)			+	+		Northern
Mayo et al. (1986)			+			cDNA
McLachlan et al. (1986b)		+				bioassay
Merchenthaler et al. (1987)			+			immunocyt.
Meunier et al. (1988a)			+	+	+	S1-nuclease
Petraglia et al. (1987)			+			immunocyt.
Spinal cord						
Meunier et al. (1988a)			+	+	-	S1-nuclease
Spleen						
Meunier et al. (1988a)			+	+	-	S1-nuclease

immunocyt.: immunocytochemie, Northern: Northern blot

(+) = detected, (-) = not detected, ( ) = not tested

The pituitary localization of mRNAs for inhibin subunits has been confirmed by the immunocytochemical detection of α- and β-B subunits of inhibin in the cytoplasm of the gonadotrophs and the nuclear detection of β-A subunit in all pituitary cells (Roberts et al., 1989).

The presence of inhibin subunit mRNAs in placental tissue has been confirmed by Davis et al. (1987) (α- and β-A mRNAs in human) and the inhibin α-subunit gene has been cloned from a human placental cDNA library (Mayo et al., 1986). In rat placenta no inhibin α-subunit mRNA could be detected by Davis et al. (1987). McLachlan et al. (1986b) detected inhibin-like bioactivity in human placental homogenates, but noticed a discrepancy between the amounts of immunoactivity and bioactivity.

1.5 Regulation of inhibin secretion by Sertoli cells

Since Steinberger and Steinberger (1976) showed that rat Sertoli cells secrete inhibin-like bioactivity, many investigators have confirmed this observation and reported on its possible endocrine (FSH and LH) and paracrine (products of surrounding cells) regulation. Recent contributions have been summarized in Table 8 and will be discussed here briefly.

Peritubular cells secrete under the influence of androgens a factor, PmodS, which stimulates the secretion of inhibin immunoactivity from Sertoli cells (Skinner et al., 1989).

There are only a few reports on the effects of spermatogenic cells on the inhibin production of Sertoli cells. However, both with immunocytochemistry and detection of mRNA in segments of seminiferous tubules, stage dependent expression of inhibin subunits have been observed (Merchenthaler et al., 1987; Rivier et al., 1988; Bhasin et al., 1989). Ultee-van Gessel et al. (1986) found that in cocultures of Sertoli cells and spermatogenic cells the secretion of FSH-stimulated bioactive inhibin was lower than in cocultures of Sertoli cells and thymocytes. The inhibition of FSH-induced secretion of inhibin immunoactivity by adenosine analogues is also a possible paracrine regulation system of inhibin secretion by germ cells (Conti et al., 1988).

Table 8. Effects of different factors on basal or FSH-stimulated secretion of inhibin-like activity from rat Sertoli cells in vitro

Factor / reference	detection method	effect of factor on basal	FSH-stimulated
FSH			
Bicsak et al. (1987)	RIA 2	+	
Conti et al. (1988)	RIA 2	+	
Handelsman et al. (1989)	RIA 1	+	
Le Gac et al. (1982)	bio	+	
Morris et al. (1988)	RIA 2	+	
Risbridger et al. (1989b)	bio	+/=*)	
	RIA 1	+	
Skinner et al. (1989)	RIA 1	+	
Toebosch et al. (1988)	bio/RIA 1	+	
Toebosch et al. (1989)	RIA 1	+	
Ultee-van Gessel et al. (1986)	bio	+	
Ultee-van Gessel et al. (1987)	bio	+	
Verhoeven et al. (1983)	bio	=	
Ying et al. (1987b)	RIA 2	+	
Grootenhuis et al. (1990) Chapter 4	bio	=	
	RIA 2	+	
Testosterone			
Bicsak et al. (1987)	RIA 2	=	=
Handelsman et al. (1989)	RIA 1	=	+
Morris et al. (1988)	RIA 2	=	=
Skinner et al. (1989)	RIA 2	=	=
Toebosch et al. (1989)	RIA 1	=	=
Ultee-van Gessel et al. (1986)	bio	-	-
Ultee-van Gessel et al. (1987)	bio	-	-
Verhoeven et al. (1983)	bio	+	
Insulin/IGF-I			
Handelsman et al. (1989)	RIA 1	+	=
Skinner et al. (1989)	RIA 2	=	+**)
Toebosch et al. (1988)	RIA 1/bio	=	=
Retinol			
Handelsman et al. (1989)	RIA 1	=	=
Skinner et al. (1989)	RIA 1	=	+**)
hCG/Prolactine			
Bicsak et al. (1987)	RIA 2	=	=
PmodS			
Skinner et al. (1989)	RIA 1	+	+
β-endorphin/adenosine			
Conti et al. (1988)	RIA 2	=	-
Morris et al. (1987)	RIA 2	=	-

Inhibin-like activity detected with the native inhibin RIA (RIA 1) or with antibodies against synthetic inhibin fragments (RIA 2) (see Chapter 1.3.2) or with bioassay (see Chapter 1.3.1)

(+) = stimulating effect, (-) = inhibiting effect, (=) = no effect, ( ) = not tested, *) with high doses of FSH, **) insulin + retinol together

The observed variable effects of FSH and testosterone on the inhibin secretion from Sertoli cells may reflect the difference between the detection methods used, since bioassays detect the bioactive αβ-B form of inhibin and RIAs detect both αβ-B dimers and separate α-subunits. Both Risbridger et al. (1989b) and Toebosch et al. (1988) observed that with increasing doses of FSH, the B/I ratio of secreted inhibin decreased. Risbridger et al. (1989b) found that the low B/I ratio of inhibin was due to increased secretion of a 27 kDa immunoactive inhibin form and observed no change in the secreted 30 kDa bioactive inhibin form. In Chapter 4 evidence is provided that the FSH-induced inhibin-like activity with a low B/I ratio, is due to increased secretion of a 29 kDa immunoreactive α-subunit. Since the 29 kDa protein could be reduced to a 20 kDa immunoreactive α-subunit, we postulate that the 29 kDa molecule is the rat equivalent of the pro-αC-subunit which has been purified from bFF (Sugino et al., 1989; Robertson et al., 1989). Other reasons for the variable effects of different factors on the release of inhibin from Sertoli cells could be: 1) The variable effects of FSH on the inhibin production of Sertoli cells from rats of different ages, since Ultee-van Gessel and de Jong (1987) have shown that the secretion of inhibin bioactivity could only be stimulated by FSH from 21-day-old Sertoli cells, 2) The different approaches which have been used for the elimination of interfering effects of FSH in the test sample and 3) The variable contamination of Sertoli cell preparations with peritubular cells and spermatogenic cells.

Recently the regulation of the expression of the mRNAs for the inhibin subunits have been studied in Sertoli cells. FSH, but not testosterone, caused an increased expression of the inhibin α-subunit, without any effect on the β-B subunit (Keinan et al., 1989; Klaij et al., 1990; Toebosch et al., 1988,1989). The effect of FSH on the expression of the inhibin α-subunit mRNA was already detectable after 1.5 hour and could be mimicked with dibutyryl-cAMP (Klaij et al., 1990).

1.6 Paracrine effects of inhibin and related proteins in the testis

Most of the research on effects of inhibin and activin involves endocrine regulation of FSH release from the pituitary gland. However, a small number of authors have investigated also the possible intratesticular (paracrine) role of inhibin and activin.

Paracrine regulation of Leydig cell steroidogenesis has been studied extensively, both with cocultures of Leydig cells and seminiferous tubules or Sertoli cells and with the addition of Sertoli cell conditioned medium (SCCM) to Leydig cells. Factor(s) present in SCCM can stimulate or inhibit basal and/or LH-stimulated steroidogenesis, but have not yet been purified (for review see Sharpe, 1984). In long term cultures of neonatal rat Leydig cells, Hsueh et al. (1987) reported a stimulatory effect of inhibin (32 kDa $\alpha\beta$-A purified from oRTF) on LH-stimulated testosterone production. Activin A inhibited both LH-stimulated and inhibin enhanced LH-stimulated testosterone production. Unfortunately this stimulatory effect of inhibin could not be confirmed using purified 32 kDa bFF inhibin (Findlay et al., 1987) or purified 32 kDa rat inhibin ($\alpha\beta$-B dimer, Chapter 5) in short term cultures, using basal or hCG-stimulated steroid production of adult and immature rat Leydig cells as response parameter. Recently Lin et al. (1989) performed detailed studies on the modulation of testosterone production of adult rat Leydig cells by inhibin and activin. After 48 hours, activin had no effect on basal testosterone production, but inhibited hCG-stimulated testosterone and cAMP formation. Also the conversion of pregnenolone to testosterone was inhibited by activin. The inhibitory effects of activin on Leydig cell function were reversed by concomitant addition of inhibin.

Franchimont et al. (1981) have reported that a partially purified inhibin preparation of oRTF may exert a direct inhibitory effect on the synthesis of DNA in spermatogenic cells of immature rats. Also de Jong et al. (1978) reported a reduction of pachytene spermatocytes after injection of bFF in immature rats. After intratesticular injection of a partially or highly purified inhibin preparation isolated from rSCCM, van Dissel-Emiliani et al. (1989) have found inhibitory effects on

differentiated spermatogonia in adult hamster and mice. Since the number of differentiated spermatogonia in the contra-lateral placebo-injected testis was not affected, an indirect effect mediated by the suppression of FSH was excluded. The elucidation of the mechanism by which inhibin affects differentiated spermatocytes awaits the development of in vitro culture systems for spermatogenic cells.

1.7 Other effects of inhibin and related proteins

After the purification of inhibin and related proteins and cloning of the inhibin genes from gonadal sources, the expression of inhibin subunit genes was reported in other organs. To elucidate the possible role of inhibin and activin in these organs, experiments were performed which will be discussed briefly.

Pituitary gland. Effects of inhibin and activin on the release of FSH have been discussed in the section on bioassays (Chapter 1.3.1). The specificity of the effect of inhibin in suppressing FSH, but not LH secretion under basal conditions is probably a reflection of the higher tonic release of FSH and the specific lowering of FSHβ-subunit mRNA (Attardi et al., 1989). Inhibin suppresses both GnRH-induced secretion of FSH and LH, which is probably caused by the down regulation of GnRH receptors on the gonadotrophs by inhibin (Wang et al., 1988). Increased plasma levels of inhibin-like immunoactivity in dogs with Sertoli cell tumors have been observed, together with the absence of a stimulatory effect of GnRH on release of LH and FSH (Chapter 6). This could have been caused by down regulation of GnRH receptors by the high plasma levels of inhibin.

Central nervous system. The administration of activin near the paraventricular nucleus caused the rapid release of oxytocin (Sawchenko et al., 1988) confirming the relevance of immunocytochemical detection of the inhibin β-A subunit in this region (Chapter 1.4.2).

Erythroid cells, thymocytes and fibroblasts. Since Eto et al. (1987) have purified activin A from a human leukemic cell line,

several authors have confirmed the role of activin and inhibin on the differentiation and proliferation of erythroid cells. Yu et al. (1987) have shown that prolonged exposure of a human erythroleukemic cell line (K562) to activin caused the cells to become terminally differentiated (induction of haemoglobin production) and limited their proliferation. Activin also potentiated the effect of erythropoietin on the proliferation and differentiation of erythroid progenitor cells in human bone marrow culture (Yu et al., 1987; Broxmeyer et al., 1988). These effects were reversed by inhibin. Recently, specific receptors for activin have been shown in several erythroleukemic cell lines (Campen and Vale, 1988; Hino et al., 1989; Kondo et al., 1989). The mechanism of action of activin involves the intracellular release of Ca^{2+} by stimulating hydrolysis of polyphosphoinositides (Shibata et al., 1987).

Lectin-induced proliferation of thymocytes was stimulated with inhibin and reversed by activin (Hedger et al., 1989). Depending on the test system used, activin A inhibited the stimulatory effect of serum on the proliferation of 3T3 fibroblasts (Kojima and Ogata, 1989) or increased proliferation of these cells by making them more competent for factors present in platelet poor plasma (Hedger et al., 1989; Kojima and Ogata, 1989).

Placenta. The cytotrophoblast layer of villi in the placenta secretes inhibin-like immunoactivity; this secretion is stimulated by hCG. The production of activin-like activity by these cells has not yet been established (Petraglia et al., 1987). Activin A increases the release of GnRH and progesterone and augments the release of hCG induced by GnRH from the cytotrophoblast. These effects of activin could be reversed by inhibin (Petraglia et al., 1989).

Pancreas and liver. Activin A stimulated insulin secretion from rat pancreatic islets and potentiated the effect of glucose-induced insulin release (Totsuka et al., 1988). Activin A also stimulated glycogenolysis in hepatocytes; the effects of activin A and glucagon on the glucose release were additive. Also in this system, activin A increased the intracellular Ca^{2+} concentration probably via activation of phosphatidylinositol turnover (Mine et

al., 1989).

Ovary. In the ovary several effects of inhibin and activin have been reported, in particular the regulation of FSH-induced estrogen production and the maturation of oocytes. Recently Sugino et al. (1988a) have shown the presence of activin receptors on granulosa cells, which were increased in number by FSH. Remarkably, activin induced FSH receptors on granulosa cells (Hasegawa et al., 1988b) and augmented the FSH-induced synthesis of LH receptors on granulosa cells (Sugino et al., 1988b). The FSH-induced conversion of androstenedione to estrogen was inhibited by inhibin (Ying et al., 1986b). However, Hutchinson et al. (1987) could not reproduce this effect of inhibin but reported a stimulatory effect of activin on the FSH-induced aromatase activity. Also, contradictory effects on the FSH-induced progesterone production have been reported. Hutchinson et al. (1987) reported an inhibitory effect of activin and no effect of inhibin, whereas Sugino et al. (1988b) reported an augmentation of the FSH-induced progesterone secretion by activin and an inhibitory effect of inhibin. LH-stimulated androstenedione production by thecal cells was stimulated by inhibin and inhibited by activin (Hsueh et al., 1987). Finally, activin also stimulated the secretion of inhibin-like immunoactivity (Sugino et al., 1988b).

The LH-induced maturation of oocytes (resumption of meiosis) in vitro was inhibited by TGF-β and inhibin together and not by inhibin and activin separately (Tsafriri et al., 1989). On the other hand, O et al. (1989) reported an inhibition of oocyte maturation by inhibin and found no effect of activin.

The relative importance of these effects of inhibin and activin is not yet clear, since contradictory results have been reported and several observations have not been confirmed by other groups. Also the physiological significance of these in vitro observations and the source of inhibin and activin for the versatile effects in non-gonadal systems have not been resolved.

However, since inhibin subunit mRNAs and proteins have been detected in a number of different organs, the reported effects can be caused by locally produced inhibin and activin.

1.8 REFERENCES

Attardi, B., H.S. Keeping, S.J. Winters, F. Kotsuji, R.A. Mauer, and P. Troen (1989). Rapid and profound suppression of messenger ribonucleic acid encoding follicle-stimulating hormoneβ by inhibin from primate Sertoli cells. Mol. Endocrinol. 3: 280-287.

Baker, H.W.G., L.W. Eddie, R.E. Higginson, B. Hudson, and H.D. Niall (1982). Studies on the purification of ovine inhibin. Ann. N.Y. Acad. Sci. 383: 329-342.

Bardin, C.W., P.L. Morris, C.-L. Chen, C. Shaha, J. Voglmayr, J. Rivier, J. Spiess, and W.W. Vale (1987). Testicular inhibin: structure and regulation by FSH, androgens and EGF. In: Inhibin-non-steroidal regulation of follicle stimulating hormone secretion, edited by H.G. Burger, J.K. Findlay, D.M. de Kretser, and M. Igarashi. New York: Raven Press, p. 179-190.

Barton, D.E., T.L. Yang-Feng, A.J. Mason, P.H. Seeburg, and U. Francke (1989). Mapping of genes for inhibin subunits α, β_A, and β_B on human and mouse chromosomes and studies of jsd mice. Genomics 5: 91-99.

Bhasin, S., L.A. Krummen, R.S. Swerdloff, B.S. Morelos, W.H. Kim, G.S. DiZerega, N. Ling, F. Esch, S. Shimasaki and J. Toppari (1989). Stage dependent expression of inhibin α and β-B subunits during the cycle of the rat seminiferous epithelium. Endocrinology 124: 987-991.

Bicsak, T.A., E.M. Tucker, S. Cappel, J. Vaughan, J. Rivier, W. Vale, and A.J W. Hsueh (1986). Hormonal regulation of granulosa cell inhibin biosynthesis. Endocrinology 119: 2711-2719.

Bicsak, T.A., W. Vale, J. Vaughan, E.M. Tucker, S. Cappel, and A.J.W. Hsueh (1987). Hormonal regulation of inhibin production by cultured Sertoli cells. Mol. Cell. Endocrinol. 49: 211-217.

Bicsak, T.A., S.B. Cajander, W. Vale, and A.J.W. Hsueh (1988). Inhibin: studies of stored and secreted forms by biosynthetic labeling and immunodetection in cultured rat granulosa cells. Endocrinology 122: 741-748.

Broxmeyer, H.E., L. Lu, S. Cooper, R.H. Schwall, A.J. Mason, and K. Nikolics (1988). Selective and indirect modulation of human multipotential and erythroid hematopoietic progenitor cell proliferation by recombinant human activin and inhibin. Proc. Natl. Acad. Sci. USA 85: 9052-9056.

Burger, H. and M. Igarashi (1988). Inhibin: Definition and nomenclature, including related substances. Endocrinology 122: 1701-1702.

Campen, C.A., and W. Vale (1988). Characterization of activin A binding sites on the human leukemia cell line K562. Biochem. Biophys. Res. Commun. 157: 844-849.

Cate, R.L., R.J. Mattaliano, C. Hession, R. Tizard, N.M. Farber, A. Cheung, E.G. Ninfa, A.Z. Frey, D.J. Gash, E.P. Chow, R.A. Fisher, J.M. Bertonis, G. Torres, B.P. Wallner, K.L. Ramachandran, R.C. Ragin, T.F. Manganaro, D.T. MacLaughlin, and P.K. Donahoe (1986). Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. Cell 45: 685-698.

Conti, M., M.D. Culler, and A. Negro-Villar (1988). Adenosine receptor-dependent modulation of inhibin secretion in

cultured immature rat Sertoli cells. Mol. Cell. Endocrinol. 59: 255-259.
Crawford, R.J., V.E. Hammond, B.A. Evans, J.P. Coghlan, J. Haralambidis, B. Hudson, J.D. Penschow, R.I. Richards, and G.W. Tregear (1987). α-Inhibin gene expression occurs in the ovine adrenal cortex, and is regulated by adrenocorticotropin. Mol. Endocrinol. 1: 699-706.
Cuevas, P., S.-Y., Ying, N. Ling, N. Ueno, F. Esch, and R. Guillemin (1987). Immunohistochemical detection of inhibin in the gonad. Biochem. Biophys. Res. Commun. 142: 23-30.
Culler, M.D., and A. Negro-Vilar (1988). Passive immunoneutralization of endogenous inhibin: sex-related differences in the role of inhibin during development. Mol. Cell. Endocrinol. 58: 263-273.
Davis, S.R., F. Dench, I. Nikolaidis, J.A. Clements, R.G. Forage, Z. Krozowski, and H.G. Burger (1986). Inhibin A-subunit gene expression in the ovaries of immature female rats is stimulated by pregnant mare serum gonadotrophin. Biochem. Biophys. Res. Commun. 138: 1191-1195.
Davis, S.R., Z. Krozowski, R.I. McLachlan, and H.G. Burger (1987). Inhibin gene expression in the human corpus luteum. J. Endocrinol. 115: 21-23.
de Jong, F.H. (1988). Inhibin. Physiol. Rev. 68: 555-607.
de Jong, F.H., and R.M. Sharpe (1976). Evidence for inhibin-like activity in bovine follicular fluid. Nature 263: 71-72.
de Jong, F.H., S.D. Smith, and H.J. van der Molen (1979). Bioassay of inhibin-like activity using pituitary cells in vitro. J. Endocrinol. 80: 91-102.
de Jong, F.H., A.J. Grootenhuis, H.J. Sander, J. Steenbergen, M.A. Timmerman, and S. van Dijk (1987). Comparison between inhibin from bovine follicular fluid and rat Sertoli cell culture medium. In Inhibin-non-steroidal regulation of follicle stimulating hormone secretion, edited by H.G. Burger, D.M. de Kretser, J.K. Findlay, M. Igarashi. New York: Raven Press, p. 35-46.
Derynck, R., J.A. Jarrett, E.Y. Chen, D.H. Eaton, J.R. Bell, R.K. Assoian, A.B. Roberts, M.B. Sporn, and D.V. Goeddel (1985). Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature 316: 701-705.
Eddie, L.W., H.W.G. Baker, R.E. Higginson, and B. Hudson (1979). A bioassay for inhibin using pituitary cell cultures. J. Endocrinol. 81: 49-60.
Erickson, G.F., and A.J.W. Hsueh (1978). Secretion of "inhibin" by rat granulosa cells in vitro. Endocrinology 103: 1960-1963.
Esch, F.S., S. Shimasaki, K. Cooksey, M. Mercado, A.J. Mason, S.-Y. Ying, N. Ueno, and N. Ling (1987). Complementary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins. Mol. Endocrinol. 1: 388-396.
Eto, Y., T. Tsuji, M. Takezawa, S. Takano, Y. Yokogawa, and H. Shibai (1987). Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. Biochem. Biophys. Res. Commun. 142: 1095-1103.
Feng, Z.-M., C.W. Bardin, and C.-L.C. Chen (1989). Characterization and regulation of testicular inhibin β-

subunit mRNA. Mol. Endocrinol. 3: 939-948.
Findlay, J.K., L.A. Hutchinson, and G.P. Risbridger (1987). Intragonadal regulation by inhibin-related peptides. In Inhibin-non-steroidal regulation of follicle stimulating hormone secretion, edited by H.G. Burger, D.M. de Kretser, J.K. Findlay, M. Igarashi, New York: Raven Press, p. 299-312.
Fingscheidt, U., G.F. Weinbauer, D.M. Robertson, D.M. de Kretser, and E. Nieschlag (1989). Radioimmunoassay of inhibin in the serum of male monkeys. J. Endocrinol. 122: 477-483.
Forage, R.G., J.M. Ring, R.W. Brown, B.V. McInerney, G.S. Cobon, R.P. Gregson, D.M. Robertson, F.J. Morgan, M.T.W. Hearn, J.K. Findlay, R.E.H. Wettenhall, H.G. Burger, and D.M. de Kretser (1986). Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proc. Natl. Acad. Sci. USA 83: 3091-3095.
Forage, R.G., R.W. Brown, J.M. Ring, A.G. Stewart, H.M. Milborrow, K.J. Oliver, B.T. Atrache, P.L. Devine, G.C. Hudson, N.H. Goss, P. tolstoshev, D.M. Robertson, B. Doughton, D.M. de Kretser, H.G. Burger, and J.K. Findlay (1987). The cloning and expression of inhibin genes: subunit use as a fecundity vaccine. In Inhibin-non-steroidal regulation of follicle stimulating hormone secretion, edited by H.G. Burger, D.M. de Kretser, J.K. Findlay, M. Igarashi, New York: Raven Press, p. 89-103.
Franchimont, P., F. Croze, A. Demoulin, R. Bologne, and J. Hustin (1981). Effect of inhibin on rat testicular desoxyribo-nucleic acid (DNA) synthesis in vivo and in vitro. Acta Endocrinol. 98: 312-320.
Fukuda, M., K. Miyamoto, Y. Hasegawa, M. Nomura, M. Igarashi, K. Kangawa, and H. Matsuo (1986). Isolation of bovine follicular fluid inhibin of about 32 kDa. Mol. Cell. Endocrinol. 44: 55-60.
Grootenhuis, A.J., J. Steenbergen, M.A. Timmerman, A.N.R.D. Dorsman, W.M.M. Schaaper, R.H. Meloen, and F.H. de Jong (1989). Inhibin and activin-like activity in fluids from male and female gonads: different molecular weight forms and bioactivity/immunoactivity ratios. J. Endocrinol. 122: 293-301.
Grootenhuis, A.J., Timmerman, M.A., Hordijk, P.L., and F.H. de Jong (1990). Inhibin in immature rat Sertoli cell conditioned medium: a 32 kDa $\alpha\beta$-B dimer. Mol. Cell. Endocrinol. 70: 109-116.
Hamada, T., G. Watanabe, T. Kokuho, K. Taya, S. Sasamoto, Y. Hasegawa, K. Miyamoto, and M. Igarashi (1989). Radioimmunoassay of inhibin in various mammals. J. Endocrinol. 122: 697-704.
Handelsman, D.J., J.A. Spaliviero, E. Kidston and D.M. Robertson (1989). Highly polarized secretion of inhibin by Sertoli cells in vitro. Endocrinology 125: 721-729.
Hasegawa, Y., K. Miyamoto, M. Fukuda, Y. Takahashi, and M. Igarashi (1986). Immunological study of ovarian inhibin. Endocrinol. Japon. 33: 645-654.
Hasegawa, Y., K. Miyamoto, S. Iwamura, and M. Igarashi (1988a). Changes in serum concentrations of inhibin in cyclic pigs J. Endocrinol. 118: 211-219.
Hasegawa, Y., K. Miyamoto, Y. Abe, T. Nakamura, H. Sugino, Y. Eto, H. Shibai, and M. Igarashi (1988b). Induction of follicle stimulating hormone receptor by erythroid

differentiation factor on rat granulosa cell. Biochem. Biophys. Res. Commun. 156: 668-674.
Hedger, M.P., A.E. Drummond, D.M. Robertson, G.P. Risbridger, and D.M. de Kretser (1989). Inhibin and activin regulate [^{3}H]thymidine uptake by rat thymocytes and 3T3 cells in vitro. Mol. Cell. Endocrinol. 61: 133-138.
Hino, M., A. Tojo, K. Miyazono, Y. Miura, S. Chiba, Y. Eto, H. Shibai, and F. Takaku (1989). Characterization of cellular receptors for erythroid differentiation factor on murine erythroleukemia cells. J. Biol. Chem. 264: 10309-10314.
Hsueh, A.J.W., K.D. Dahl, J. Vaughan, E. Tucker, J. Rivier, C. W. Bardin, and W. Vale (1987). Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. Proc. Natl. Acad. Sci. USA 84: 5082-5086.
Hutchinson, L.A., J.K. Findlay, F.L. de Vos, and D.M. Robertson (1987). Effects of bovine inhibin, transforming growth factor-β and bovine activin-A on granulosa cell differentiation. Biochem. Biophys. Res. Commun. 146: 1405-1412.
Keinan, D., M.B. Madigan, C.W. Bardin, and C.-L.C. Chen (1989). Expression and regulation of testicular inhibin α-subunit gene in vivo and in vitro. Mol. Endocrinol. 3: 29-35.
Kitaoka, M., K. Takano, I. Kojima, and E. Ogata (1989). A stimulatory effect of somatostatin: enhancement of activin A-mediated FSH secretion in rat pituitary cells. Biochem. Biophys. Res. Commun. 162: 958-962.
Klaij, I.A., A.M.W. Toebosch, A.P.N. Themmen, S. Shimasaki, F.H. de Jong, and J.A. Grootegoed (1990). Regulation of inhibin α- and β_B-subunit mRNA levels in rat Sertoli cells. Mol. Cell. Endocrinol. 68: 45-52.
Knight, P.G., A.J. Beard, J.H.M. Wrathall, and R.J. Castillo (1989). Evidence that the bovine ovary secretes large amounts of monomeric inhibin α subunit and its isolation from bovine follicular fluid. J. Mol. Endocrinol. 2: 189-200.
Kojima, I., and E. Ogata (1989). Dual effect of activin A on cell growth in Balb/c 3T3 cells. Biochem. Biophys. Res. Commun. 159: 1107-1113.
Kondo, S., M. Hashimoto, Y. Etoh, M. Murata, H. Shibai, and M. Muramatsu (1989). Identification of the two types of specific receptor for activin/EDF expressed on Friend leukemia and embryonal carcinoma cells. Biochem. Biophys. Res. Commun. 161: 1267-1272.
Lee, V.W.K., N.C. Kraft, R.C. Atkins, and H.G. Burger (1986). Monoclonal antibody to rat ovarian inhibin. J. Endocrinol.. 109: 379-383.
Lee, W., A.J. Mason, R. Schwall, E. Szonyi, and J.P. Mather (1989). Secretion of activin by interstitial cells in the testis. Science 243: 396-398.
Le Gac, F., and D.M. de Kretser (1982). Inhibin production by Sertoli cell cultures. Mol. Cell. Endocrinol. 28: 487-498.
Leversha, L.J., D.M. Robertson, F.L. de Vos, F.J. Morgan, M.T.W. Hearn, R.E.H. Wettenhall, J.K. Findlay, H.G. Burger, and D.M. de Kretser (1987). Isolation of inhibin from ovine follicular fluid. J. Endocrinol. 113: 213-221.
Li, C.H., and K. Ramasharma (1987). Inhibin. Ann. Rev. Pharmacol. Toxicol. 27: 1-21.
Lin, T., J.H. Calkins, P.L. Morris, W. Vale, and C.W. Bardin

(1989). Regulation of Leydig cell function in primary culture by inhibin and activin. Endocrinology 125: 2134-2140.

Ling, N., S.-Y. Ying, N. Ueno, F. Esch, L. Denoroy, and R. Guillemin (1985). Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid. Proc. Natl. Acad. Sci. USA 82: 7217-7221.

Ling, N., S.-Y. Ying, N. Ueno, S. Shimasaki, F. Esch, M. Hotta, and R. Guillemin (1986a). Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature 321: 779-782.

Ling, N., S.-Y. Ying, N. Ueno, S. Shimasaki, F. Esch, M. Hotta and R. Guillemin (1986b). A homodimer of the β-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. Biochem. Biophys. Res. Commun. 138: 1129-1137.

Mason, A.J., J.S. Hayflick, N. Ling, F. Esch, N. Ueno, S.-Y. Ying, R. Guillemin, H. Niall, and P.H. Seeburg (1985). Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. Nature 318: 659-663.

Mason, A.J., H.D. Niall, and P.H. Seeburg (1986). Structure of two human ovarian inhibins. Biochem. Biophys. Res. Commun. 135: 957-964.

Mason, A.J., L.M. Berkemeier, C.H. Schmelzer, and R.H. Schwall (1989). Activin B: precursor sequences, genomic structure and in vitro activities. Mol. Endocrinol. 3: 1352-1358.

Mayo, K.E., G.M. Cerelli, J. Spiess, J. Rivier, M.G. Rosenfeld, R.M. Evans, and W. Vale (1986). Inhibin A-subunit cDNAs from porcine ovary and human placenta. Proc. Natl. Acad. Sci. USA 83: 5849-5853.

McCullagh, D.R. (1932). Dual endocrine activity of the testes. Science 76: 19-20.

McLachlan, R.I., D.M. Robertson, H.G. Burger, and D.M. de Kretser (1986a). The radioimmunoassay of bovine and human follicular fluid and serum inhibin. Mol. Cell. Endocrinol. 46: 175-185.

McLachlan, R.I., D.L. Healy, D.M. Robertson, H.G. Burger, and D.M. de Kretser (1986b). The human placenta: a novel source of inhibin. Biochem. Biophys. Res. Commun. 140: 485-490.

McLachlan, R.I., D.M. Robertson, D.L. Healy, H.G. Burger, and D.M. de Kretser (1987). Circulating immunoreactive inhibin levels during the normal human menstrual cycle. J. Clin. Endocrinol. Metab. 65: 954-961.

Merchenthaler, I., M.D. Culler, P. Petrusz, and A. Negro-Vilar (1987). Immunocytochemical localization of inhibin in rat and human reproductive tissues. Mol. Cell. Endocrinol. 54: 239-243.

Meunier, H., C. Rivier, R.M. Evans, and W. Vale (1988a). Gonadal and extragonadal expression of inhibin α, βA, and βB subunits in various tissues predicts diverse functions. Proc. Natl. Acad. Sci. USA 85: 247-251.

Meunier, H., S.B. Cajander, V.J. Roberts, C. Rivier, P.E. Sawchenko, A.J.W. Hsueh, and W. Vale (1988b). Rapid changes in the expression of inhibin α-, βA-, and βB-subunits in ovarian cell types during the rat estrous cycle. Mol. Endocrinol. 2: 1352-1363.

Michel, U., H. Jarry, M. Metten, and W. Wuttke (1989). Inhibin production by porcine granulosa and luteal cells: development and biological validation of a RIA. Acta Endocrinol. 120: 511-518.

Mine, T., I. Kojima, and E. Ogata (1989). Stimulation of glucose production by activin-A in isolated rat hepatocytes. Endocrinology 125: 586-591.

Miyamoto, K., Y. Hasegawa, M. Fukuda, M. Nomura, M. Igarashi, K. Kangawa, and H. Matsuo (1985). Isolation of porcine follicular fluid inhibin of 32K daltons. Biochem. Biophys. Res. Commun. 129: 396-403.

Miyamoto, K., Y. Hasegawa, M. Fukuda, and M. Igarashi (1986). Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32K inhibin. Biochem. Biophys. Res. Commun. 136: 1103-1109.

Miyazono, K., and C.-H. Heldin (1989). Role for carbohydrate structures in TGF-β1 latency. Nature 338: 158-160.

Morris, P.L., W.W. Vale, and C.W. Bardin (1987). β-endorphin regulation of FSH-stimulated inhibin production is a component of a short loop system in testis. Biochem. Biophys. Res. Commun. 148: 1513-1519.

Morris, P.L., W.W. Vale, S. Cappel, and C.W. Bardin (1988). Inhibin production by primary Sertoli cell-enriched cultures: regulation by follicle-stimulating hormone, androgens, and epidermal growth factor. Endocrinology 122: 717-725.

Mottram, J.C., and W. Cramer (1923). On the general effects of exposure to radium on metabolism and tumour growth in the rat and the special effects on testis and pituitary. Quart. J. Exp. Physiol. 13: 209-229.

Murata, M., Y. Eto, H. Shibai, M. Sakai, and M. Muramatsu (1988). Erythroid differentiation factor is encoded by the same mRNA as that of the inhibin β_A chain. Proc. Natl. Acad. Sci. USA, 85: 2434-2438.

Noguchi, K., H.S. Keeping, S.J. Winters, H. Saito, H. Oshima, and P. Troen (1987). Identification of inhibin secreted by Cynomolgus monkey Sertoli cell cultures. J. Clin. Endocrinol.in. Metab. 64: 783-788.

O., W.-S., D.M. Robertson, and D.M. de Kretser (1989). Inhibin as an oocyte meiotic inhibitor. Mol. Cell. Endocrinol. 62: 307-311.

Padgett, R.W., R.D. St. Johnston, and W.M. Gelbart (1987). A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-β family. Nature 325: 81-84.

Pepinsky, R.B., L.K. Sinclair, E.P. Chow, R.J. Mattaliano, T.F. Manganaro, P.K. Donahoe, and R.L. Cate (1988). Proteolytic processing of Müllerian inhibiting substance produces a transforming growth factor-β-like fragment. J. Biol. Chem. 263: 18961-18964.

Petraglia, F., P. Sawchenko, A.T.W. Lim, J. Rivier, and W. Vale (1987). Localization, secretion, and action of inhibin in human placenta. Science 237: 187-189.

Petraglia, F., J. Vaughan, and W. Vale (1989). Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone from cultured human placental cells. Proc. Natl. Acad. Sci. USA 86: 5114-5117.

Risbridger, G.P., J. Clements, D.M. Robertson, A.E. Drummond, J. Muir, H.G. Burger, and D.M. de Kretser (1989a). Immuno- and bioactive inhibin and inhibin α-subunit expression in rat Leydig cell cultures. Mol. Cell. Endocrinol. 66: 119-122.

Risbridger, G.P., A. Hancock, D.M. Robertson, Y. Hodgson, and D.M. de Kretser (1989b). Follitropin (FSH) stimulation of inhibin biological and immunological activities by seminiferous tubules and Sertoli cell cultures from immature rats. Mol. Cell. Endocrinol. 67: 1-9.

Rivier, J., J. Spiess, R. McClintock, J. Vaughan, and W. Vale (1985). Purification and partial characterization of inhibin from porcine follicular fluid. Biochem. Biophys. Res. Commun. 133: 120-127.

Rivier, C., J. Rivier, and W. Vale (1986). Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat. Science 234: 205-208.

Rivier, C., S. Cajander, J. Vaughan, A.J.W. Hsueh and W. Vale (1988). Age-dependent changes in physiological action, content, and immunostaining of inhibin in male rats. Endocrinology 123: 120-126.

Rivier, C., V. Roberts, and W. Vale (1989). Possible role of luteinizing hormone and follicle-stimulating hormone in modulating inhibin secretion and expression during the estrous cycle of the rat. Endocrinology 125: 876-882.

Roberts, A.B., K.C. Flanders, P. Kondaiah, N.L. Thompson, E. Van Obberghen-Schilling, L. Wakefield, P. Rossi, B. de Crombrugghe, U. Heine, and M.B. Sporn (1988). Transforming growth factor β: biochemistry and roles in embryogenesis, tissue repair and remodeling, and carcinogenesis. Rec. Progr. Horm. Res. 44: 157-193.

Roberts, V., H. Meunier, J. Vaughan, J. Rivier, C. Rivier, W. Vale, and P. Sawchenko (1989). Production and regulation of inhibin subunits in pituitary gonadotropes. Endocrinology 124: 552-554.

Robertson, D.M., C.L. Au, and D.M. de Kretser (1982). The use of ^{51}Cr for assessing cytotoxicity in an in vitro bioassay for inhibin. Mol. Cell. Endocrinol. 26: 119-127.

Robertson, D.M., L.M. Foulds, L. Leversha, F.J. Morgan, M.T.W. Hearn, H.G. Burger, R.E.H. Wettenhall, and D.M. de Kretser (1985). Isolation of inhibin from bovine follicular fluid. Biochem. Biophys. Res. Commun. 126: 220-226.

Robertson, D.M., F.L. de Vos, L.M. Foulds, R.I. McLachlan, H.G. Burger, F.J. Morgan, M.T.W. Hearn, and D.M. de Kretser (1986a). Isolation of a 31 kDa form of inhibin from bovine follicular fluid. Mol. Cell. Endocrinol. 44: 271-277.

Robertson, D.M., M.S. Giacometti, and D.M. de Kretser (1986b). The effects of inhibin purified from bovine follicular fluid in several in vitro pituitary cell culture systems. Mol. Cell. Endocrinol. 46: 29-36.

Robertson, D.M., R. Klein, F.L. de Vos, R.I. McLachlan, R.E.H. Wettenhall, M.T.W. Hearn, H.G. Burger, and D.M. de Kretser (1987). The isolation of polypeptides with FSH suppressing activity from bovine follicular fluid which are structurally different to inhibin. Biochem. Biophys. Res. Commun. 149: 744-749.

Robertson, D.M., S. Hayward, D. Irby, J. Jacobsen, L. Clarke, R.I. McLachlan, and D.M. de Kretser (1988). Radioimmunoassay of rat serum inhibin: changes after PMSG stimulation and

gonadectomy. Mol. Cell. Endocrinol. 58: 1-8.
Robertson, D.M., M. Giacometti, L.M. Foulds, J. Lahnstein, N.H. Goss, M.T.W. Hearn, and D.M. de Kretser (1989). Isolation of inhibin α-subunit precursor proteins from bovine follicular fluid. Endocrinology 125: 2141-2149.

Rodgers, R.J., S.J. Stuchbery and J.K. Findlay (1989). Inhibin mRNAs in ovine and bovine ovarian follicles and corpora lutea throughout the estrous cycle and gestation. Mol. Cell. Endocrinol. 62: 95-101.
Saito, S., P.C. Roche, D.J. McCormick, and R.J. Ryan (1989). Synthetic peptide segments of inhibin α- and β-subunits: preparation and characterization of polyclonal antibodies. Endocrinology 125: 898-905.
Sawchenko, P.E., P.M. Plotsky, S.W. Pfeiffer, E.T. Cunningham, J. Vaughan, J. Rivier, and W. Vale (1988). Inhibin β in central neural pathways involved in the control of oxytocin secretion. Nature 334: 615-617.
Schanbacher, B. (1988). Radioimmunoassay of inhibin: serum responses to unilateral and bilateral orchidectomy. Endocrinology 123: 2323-2330.
Scott, R.S., H.G. Burger, and H. Quigg (1980). A simple and rapid in vitro bioassay for inhibin. Endocrinology 107: 1536-1542.
Shaha, C., P.L. Morris, C.-L.C. Chen, W. Vale, and C.W. Bardin (1989). Immunostainable inhibin subunits are in multiple types of testicular cells. Endocrinology 125: 1941-1950.
Sharpe, R.M. (1984). Intratesticular factors controlling testicular function. Biol. Reprod. 30: 29-49.
Sharpe, R.M., I.A. Swanston, I. Cooper, C.G. Tsonis, and A.S. McNeilly (1988). Factors affecting the secretion of immunoactive inhibin into testicular interstitial fluid in rats. J. Endocrinol.. 119: 315-326.
Shibata, H., E. Ogata, Y. Etoh, H. Shibai, and Ib. Kojima (1987). Erythroid differentiation factor stimulates hydrolysis of polyphosphoinositide in friend erythroleukemia cells. Biochem. Biophys. Res. Commun. 146: 187-193.
Shimasaki, S., M. Koga, F. Esch, K. Cooksey, M. Mercado, A. Koba, N. Ueno, S.-Y. Ying, N. Ling, and R. Guillemin (1988). Primary structure of the human follistatin precursor and its genomic organization. Proc. Natl. Acad. Sci. USA 85: 4218-4222.
Skinner, M.K., R.I. McLachlan, and W.J. Bremner (1989). Stimulation of Sertoli cell inhibin secretion by the testicular paracrine factor PModS. Mol. Cell. Endocrinol. 66: 239-249.
Sluss, P.M., A.L. Schneyer, M.A. Franke, and L.E. Reichert, Jr. (1987). Porcine follicular fluid contains both follicle-stimulating hormone agonist and antagonist activities. Endocrinology 120: 1477-1481.
Steinberger, A., and E. Steinberger (1976). Secretion of an FSH-inhibiting factor by cultured Sertoli cells. Endocrinology 99: 918-921.
Steiner, D.F., P.S. Quinn, S.J. Chan, J. Marsh, and H.S. Tager. (1980). Processing mechanisms in the biosynthesis of proteins. Ann. N.Y. Acad. Sci. 343: 1-16.
Stewart, A.G., H.M. Milborrow, J.M. Ring, C.E. Crowther, and R.G. Forage (1986). Human inhibin genes. Genomic

characterisation and sequencing. FEBS Lett. 206: 329-334.
Sugino, H., T. Nakamura, Y. Hasagawa, K. Miyamoto, M. Igarashi, Y. Eto, H. Shibai, and K. Titani (1988a). Identification of a specific receptor for erythroid differentiation factor on follicular granulosa cell. J. Biol. Chem. 263: 15249-15252.
Sugino, H., T. Nakamura, Y. Hasagawa, K. Miyamoto, Y. Abe, M. Igarashi, Y. Eto, H. Shibai, and K. Titani (1988b). Erythroid differentiation factor can modulate follicular granulosa cell functions. Biochem. Biophys. Res. Commun. 153: 281-288.
Sugino, K., T. Nakamura, K. Takio, K. Titani, K. Miyamoto, Y. Hasegawa, M. Igarashi, and H. Sugino (1989). Inhibin alpha-subunit monomer is present in bovine follicular fluid. Biochem. Biophys. Res. Commun. 159: 1323-1329.
Suzuki, T., K. Miyamoto, Y. Hasegawa, Y. Abe, M. Ui, Y. Ibuki, and M. Igarashi (1987). Regulation of inhibin production by rat granulosa cells. Mol. Cell. Endocrinol. 54: 185-195.
Taya, K., H. Komura, G. Watanabe, and S. Sasamoto (1989). Peripheral blood levels of immunoreactive inhibin during pseudopregnancy, pregnancy and lactation in the rat. J. Endocrinol. 121: 545-552.
Toebosch, A.M.W., D.M. Robertson, J. Trapman, P. Klaassen, R.A. de Paus, F.H. de Jong, and J.A. Grootegoed (1988). Effects of FSH and IGF-I on immature rat Sertoli cells: inhibin α- and β-subunit mRNA levels and inhibin secretion. Mol. Cell. Endocrinol. 55: 101-105.
Toebosch, A.M.W., D.M. Robertson, I.A. Klaij, F.H. de Jong, and J.A. Grootegoed (1989). Effects of FSH and testosterone on highly purified rat Sertoli cells: inhibin α-subunit mRNA expression and inhibin secretion are enhanced by FSH but not by testosterone. J. Endocrinol. 122: 757-762.
Torney, A.H., Y.M. Hodgson, R. Forage, and D.M. de Kretser (1989). Cellular localization of inhibin mRNA in the bovine ovary by in-situ hybridization. J. Reprod. Fert. 86: 391-399.
Totsuka, Y., M. Tabuchi, I. Kojima, H. Shibai, and E. Ogata (1988). A novel action of activin A: stimulation of insulin secretion in rat pancreatic islets. Biochem. Biophys. Res. Commun. 156: 335-339.
Tsafriri, A., W. Vale, and A.J.W. Hsueh (1989). Effects of transforming growth factors and inhibin-related proteins on rat preovulatory Graafian follicles in vitro. Endocrinology 125: 1857-1862.
Tsonis, C.G., A.S. McNeilly, and D.T. Baird (1986). Measurement of exogenous and endogenous inhibin in sheep serum using a new and extremely sensitive bioassay for inhibin based on inhibition of ovine pituitary FSH secretion in vitro. J. Endocrinol. 110: 341-352.
Tsonis, C.G., S.G. Hillier, and D.T. Baird (1987). Production of inhibin bioactivity by human granulosa-lutein cells: stimulation by LH and testosterone in vitro. J. Endocrinol. 112: R11-R14.
Tsuji, T., Y. Eto, S. Takano, M. Takezawa, Y. Yokogawa, and H. Shibai (1988). Discovery of a human erythroid differentiation factor (EDF) and its large-scale production. Biotechnol. Bioengin. 31: 675-681.
Ueno, N., N. Ling, S.-Y. Ying, F. Esch, S. Shimasaki, and R.

Guillemin (1987). Isolation and partial characterization of follistatin: a single-chain M_r 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. Proc. Natl. Acad. Sci. USA 84: 8282-8286.

Ultee-van Gessel, A.M., F.G. Leemborg, F.H. de Jong, and H.J. van der Molen (1986). In vitro secretion of inhibin-like activity by Sertoli cells from normal and prenatally irradiated immature rats. J. Endocrinol. 109: 411-418.

Ultee-van Gessel, A.M., and F.H. de Jong (1987). Inhibin-like activity in Sertoli cell culture media and testicular homogenates from rats of various ages. J. Endocrinol. 113: 103-110.

Vale, W., G. Grant, M. Amoss, R. Blackwell, and R. Guillemin (1972). Culture of enzymatically dispersed anterior pituitary cells: functional validation of a method. Endocrinology 91: 562-572.

Vale, W., J. Rivier, J. Vaughan, R. McClintock, A. Corrigan, W. Woo, D. Karr, and J. Spiess (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature 321: 776-779.

Vale, W., C. Rivier, A. Hsueh, C. Campen, H. Meunier, T. Bicsak, J. Vaughan, A. Corrigan, W. Bardin, P. Sawchenko, F. Petraglia, J. Yu, P. Plotsky, J. Spiess, and J. Rivier (1988). Chemical and biological characterization of the inhibin family of protein hormones. Rec. Progr. Horm. Res. 44: 1-34.

Van Dissel-Emiliani, F.M.F., A.J. Grootenhuis, F.H. de Jong and D.G. de Rooij (1989). Inhibin suppresses spermatogenesis in testes of adult mice and Chinese hamsters. Endocrinology 125: 1898-1903.

Vaughan, J.M., J. Rivier, A.Z. Corrigan, R. McClintock, C.A. Campen, D. Jolley, J.K. Vogelmayr, C.W. Bardin, C. Rivier, and W. Vale (1989). Detection and purification of inhibin using antisera generated against synthetic peptide fragments. In: Methods in Enzymology, vol. 168 (Hormone Action, Part K: Neuroendocrine Peptides), edited by P.M. Conn. Orlando, Fl.: Academic Press, p. 588-617.

Verhoeven, G., and P. Franchimont (1983). Regulation of inhibin secretion by Sertoli cell-enriched cultures. Acta Endocrinol. 102: 136-143.

Wang, Q.F., P.G. Farnworth, J.K. Findlay, and H.G. Burger (1988). Effect of purified 31K bovine inhibin on the specific binding of gonadotropin-releasing hormone to rat anterior pituitary cells in culture. Endocrinology 123: 2161-2166.

Weeks, D.L., and D.A. Melton (1987). A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-β. Cell 51: 861-867.

Wiebe, J.P., and P.H. Wood (1987). Selective suppression of follicle-stimulating hormone by 3α-hydroxy-4-pregnen-20-one, a steroid found in Sertoli cells. Endocrinology 120: 2259-2264.

Wood, P.H., and J.P. Wiebe (1989). Selective suppression of follicle-stimulating hormone secretion in anterior pituitary cells by the gonadal steroid 3α-hydroxy-4-pregnen-20-one. Endocrinology 125: 41-48.

Woodruff, T.K., H. Meunier, P.B.C. Jones, A.J.W. Hsueh, and K.E. Mayo (1987). Rat inhibin: molecular cloning of α- and β-subunit complementary deoxyribonucleic acids and expression

in the ovary. Mol. Endocrinol. 1: 561-568.
Woodruff, T.K., J. D'Agostino, N.B. Schwartz, and K.E. Mayo (1988). Dynamic changes in inhibin messenger RNAs in rat ovarian follicles during the reproductive cycle. Science 239: 1296-1299.
Woodruff, T.K., J. D'Agostino, N.B. Schwartz, and K.E. Mayo (1989). Decreased inhibin gene expression in preovulatory follicles requires primary gonadotropin surges. Endocrinology 124: 2193-2199.
Wozney, J.M., V. Rosen, A.J. Celeste, L.M. Mitsock, M.J. Whitters, R.W. Kriz, R.M. Hewick, and E.A. Wang (1988). Novel regulators of bone formation: molecular clones and activities. Science 242:1528-1533.
Ying, S.-Y., A. Becker, A. Baird, N. Ling, N. Ueno, F. Esch, and R. Guillemin (1986a). Type beta transforming growth factor (TGF-β) is a potent stimulator of the basal secretion of follicle stimulating hormone (FSH) in a pituitary monolayer system. Biochem. Biophys. Res. Commun. 135: 950-956.
Ying, S.-Y., A. Becker, N. Ling, N. Ueno, and R. Guillemin (1986b). Inhibin and beta type transforming growth factor (TGF-β) have opposite modulating effects on the follicle stimulating hormone (FSH)-induced aromatase activity of cultured rat granulosa cells. Biochem. Biophys. Res. Commun. 136: 969-975.
Ying, S.-Y., A. Becker, G. Swanson, P. Tan, D. Wadleigh, J. Czvik, N. Ling, N. Ueno, F. Esch, T.-C. Chiang, R. Hu, M.-H. Dong, K. Sato, T. Munegumi, and R. Guillemin (1987a). Reciprocal relationship between the secretion of follicle stimulating hormone (FSH) and production of inhibin in the rat. In: Inhibin-non-steroidal regulation of follicle stimulating hormone secretion, edited by H.G. Burger, D.M. de Kretser, J.K. Findlay, M. Igarashi. New York: Raven Press, p. 135-148.
Ying, S.-Y., J. Czvik, A. Becker, N. Ling, N. Ueno, and R. Guillemin (1987b). Secretion of follicle-stimulating hormone and production of inhibin are reciprocally related. Proc. Natl. Acad. Sci. USA 84: 4631-4635.
Yu, J., L. Shao, V. Lemas, A.L. Yu, J. Vaughan, J. Rivier, and W. Vale (1987). Importance of FSH-releasing protein and inhibin in erythrodifferentiation. Nature 330: 765-767.

CHAPTER 2

INHIBIN AND ACTIVIN-LIKE ACTIVITY IN FLUIDS FROM MALE AND FEMALE GONADS: DIFFERENT MOLECULAR WEIGHT FORMS AND BIOACTIVITY/IMMUNOACTIVITY RATIOS

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Inhibin and activin-like activity in fluids from male and female gonads: different molecular weight forms and bioactivity/immunoactivity ratios

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ABSTRACT

The existence of various molecular weight forms of inhibin in ovarian follicular fluid has been reported earlier, while there is no information on the form of inhibin in testicular tissue. Inhibin bioactivity was therefore estimated in eluates of slices, obtained after SDS-PAGE of rat testicular and ovarian homogenates, rat Sertoli cell-conditioned medium (rSCCM) and bovine ovarian follicular fluid (bFF).

The only form of inhibin detected in testes from 22-day-old rats and in rSCCM was a 30 kDa protein. In rat ovarian extracts, larger forms of inhibin were also found as well as the predominant 30 kDa form. An activin-like activity was found in the 25 kDa SDS-PAGE eluates of both rSCCM and ovarian homogenates, which caused a dose-dependent increase of FSH release from cultured pituitary cells.

Activin-like activity and several forms of inhibin were found in bFF after SDS-PAGE. After purification of inhibin from bFF using dye affinity, anion-exchange, lentil lectin affinity chromatography and a subsequent reversed phase chromatography step, two pools of inhibin activity were obtained. These were separated by SDS-PAGE revealing 30 and 58 kDa inhibin forms. The immunoactivity of these forms of inhibin was then estimated using antibodies against the 22 N-terminal amino acids of the α subunit of 30 kDa bovine inhibin. It appeared that the two molecular weight forms of inhibin had bioactivity/immunoactivity ratios which differed more than five-fold. This indicates that results of radioimmunoassays of inhibin of ovarian origin, using peptide antisera, should be interpreted with caution.

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INTRODUCTION

Inhibin is a glycoprotein of gonadal origin, consisting of two dissimilar disulphide-linked subunits (α and β), which inhibits pituitary gonadotrophin production and/or secretion, particularly that of follicle-stimulating hormone (FSH) (Burger, 1988).

Inhibin has been isolated from bovine (Fukuda, Miyamoto, Hasegawa *et al.* 1986; Robertson, de Vos, Foulds *et al.* 1986), porcine (Ling, Ying, Ueno *et al.* 1985; Miyamoto, Hasegawa, Fukuda *et al.* 1985) and ovine (Leversha, Robertson, de Vos *et al.* 1987) ovarian follicular fluid. Ling *et al.* (1985) reported the existence of two forms of 32 kDa porcine inhibin which differed with respect to their β subunits, while their α subunits were identical. These results were confirmed and extended by Mason, Hayflick, Ling *et al.* (1985) who reported the cloning of one α-subunit and two β-subunit genes. On the basis of the amino acid sequence derived from the cDNA data, several potential proteolytic cleavage sites were found in the α chain which could explain some of the reported differences in the molecular weight of inhibin (de Jong & Robertson, 1985). This was confirmed by the isolation from bovine follicular fluid of a 58 kDa inhibin with an extended α-subunit chain (Robertson, Foulds, Leversha *et al.* 1985).

Testicular inhibin has not been purified to homogeneity, although progress in the isolation of inhibin from rat Sertoli cell-conditioned medium (rSCCM) has been reported (de Jong, Grootenhuis, Sander *et al.* 1987). Several molecular weight forms of

testicular inhibin have been reported: a 60–90 kDa form was detected after gel filtration, while results of electrophoresis indicated a 30 kDa form of inhibin in ovine rete testis fluid (Baker, Eddie, Higginson *et al.* 1982).

Until recently, inhibin was quantified by bioassay methods using dispersed pituitary cells (for review see de Jong, 1988). Using this type of assay, Robertson *et al.* (1986) reported that 58 kDa inhibin suppressed FSH secretion more effectively than 32 kDa inhibin, when expressed on a molar basis. Since then, several radioimmunoassay systems have been developed using monoclonal (Lee, Kraft, Atkins & Burger, 1986) or polyclonal (McLachlan, Robertson, Burger & de Kretser, 1986; Robertson, Tsonis, McLachlan *et al.* 1988) antibodies raised against purified inhibin preparations, or antibodies against peptides from the N-terminal part of the α chain of 30 kDa porcine inhibin (Rivier, Rivier & Vale, 1986; Schanbacher, 1988; Sharpe, Swanston, Cooper *et al.* 1988).

In these anti-peptide immunoassays, inhibin standards and female or male inhibin-containing preparations displaced the labelled peptide in parallel, but it is not clear from these data whether peptide-based radioimmunoassays can recognize the different molecular weight forms of inhibin. Furthermore, Robertson *et al.* (1988) reported different bioactivity/immunoactivity ratios for inhibin in sera from men and from women, while van Dijk, Steenbergen, Gielen & de Jong (1986) found that inhibin from ovarian follicular fluid was immunoneutralized more effectively than testicular inhibin.

The aim of the present study was to investigate which forms of inhibin are present in ovarian and testicular preparations and to estimate the relative bioactivity and immunoactivity of these inhibins.

MATERIALS AND METHODS

Inhibin preparations

Bovine, porcine and ovine follicular fluid (bFF, pFF and oFF respectively) were aspirated from ovaries collected at a local slaughterhouse. Protein concentrations in follicular fluids were 65 g/l. Inhibin was purified from bFF using immobilized Procion Red A (Red A column; Amicon, Lexington, MA, U.S.A.), Sephadex G-25, Mono Q anion-exchange, lentil lectin affinity columns and finally reversed phase chromatography on a C1/C8 column (all from Pharmacia, Uppsala, Sweden) using a gradient of 0–50% (v/v) acetonitrile containing 0·1% (v/v) trifluoroacetic acid (de Jong *et al.* 1987). Ovaries from pro-oestrous rats, testes from 22-day-old rats and Sertoli cells from 22-day-old rat testes were homogenized in a Tris–HCl buffer (20 mmol/l; pH 7·5) containing EDTA tetrasodium salt (1 mmol/l), phenylmethylsulphonyl-fluoride (0·6 mmol/l), leupeptine (1 mmol/l; all from Sigma, St Louis, MO, U.S.A.) and bacitracin (0·5 mmol/l; Janssen, Beerse, Belgium). Homogenates were centrifuged at 100 000 *g* for 1 h at 4 °C.

Sertoli cells were isolated from 22-day-old rats by a double collagenase digestion method as described by Oonk, Grootegoed & van der Molen (1985), plated in 150 cm^2 plastic culture flasks (Costar, Cambridge, MA, U.S.A.) in 20 ml medium (Eagle's Minimal Essential Medium; Gibco, Grand Island, NY, U.S.A.), containing non-essential amino acids (Gibco), fungizone (600 ng/ml; Squibb, Rijswijk, The Netherlands), streptomycin (100 μg/ml; Specia, Paris, France) and penicillin (100 IU/ml; Gist-Brocades, Delft, The Netherlands) and 1% (v/v) fetal calf serum (FCS; Gibco) in a humidified incubator in an atmosphere of 5% CO_2 in air at 37 °C. The next day, attached cells were washed and new medium (20 ml) without FCS was added. The rSCCM was collected every 3 or 4 days for 4–5 weeks. Inhibin was purified from rSCCM by diafiltration, Mono Q anion-exchange and reversed phase chromatography as described by de Jong *et al.* (1987).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Samples were incubated with sample buffer for 1 h at 37 °C and separated by SDS-PAGE (Laemmli, 1970) on gels of 10 or 15% (w/v) acrylamide, containing 0·8% (w/v) bisacrylamide. After separation, 2 mm slices were cut and proteins eluted from the slices overnight at 4 °C in Tris–HCl (20 mmol/l; pH 7·5) containing 1% SDS and precipitated by the addition of methanol. All experiments involving SDS-PAGE separation and elution were repeated at least three times. The results of one representative experiment are shown.

Methanol precipitation of proteins

Proteins in eluates from gel slices and other inhibin-containing samples were precipitated by the addition of 5 volumes of methanol (samples containing less than 500 μg protein/ml were supplemented with this amount of bovine serum albumin (BSA; Sigma)), incubated for 30 min at −80 °C and centrifuged for 30 min at 13 000 *g* at 4 °C. The methanol-containing layer was decanted and the remaining proteins were dried in air and redissolved in the appropriate buffer.

Bioassay of inhibin activity

Inhibin bioactivity was measured using cultures of dispersed pituitary cells (de Jong, Smith & van der Molen, 1979). Samples were assayed in triplicate at two dose levels. The suppression of FSH release was

compared with that caused by the addition of three triplicate doses of a standard preparation of charcoal-treated bFF with an arbitrary potency of 1 unit/μg protein. Inhibin potencies were calculated using statistics for assay parallelism (Finney, 1964). Concentrations of FSH and luteinizing hormone (LH) in the culture media of the pituitary cells were determined by radioimmunoassay, using the antibodies and dilutions described by Welschen, Osman, Dullaart *et al.* (1975). Sample (100 μl), antibody (50 μl) and label (50 μl) were mixed and incubated overnight at room temperature. Separation of free and bound hormone was facilitated by adding goat anti-rabbit serum (50 μl; 1:30 dilution in phosphate-buffered saline (PBS; 10 mmol/l; pH 7·4) containing NaCl (150 mmol/l) and 0·5% (w/v) BSA). Thirty minutes later, 1 ml PBS containing 5% (w/v) polyethylene glycol (M_r 20 000) and 0·1% (v/v) triton X-100 was added and, after 30 min at room temperature, the tubes were centrifuged for 30 min at 6000 *g*, the supernatant was decanted and the residue counted. All results are expressed in terms of NIADDK-rat FSH-RP-1 and NIADDK-rat LH-RP-1. Intra- and interassay coefficients of variation were 11·5 and 5·1% for FSH and 16·0 and 14·1% for LH ($n=50$).

Production of antiserum

The N-terminal 22 amino acid sequence of the α subunit of 30 kDa bovine inhibin (Forage, Ring, Brown *et al.* 1986) with an additional tyrosine at the C-terminus (bIα(1-22)Tyr) was synthesized (Fournier, Wang & Felix, 1988) and coupled to keyhole limpet haemocyanin with glutaraldehyde (Pfaff, Mussgay, Böhm *et al.* 1982).

Three-month-old castrated rabbits were injected at two sites intradermally and at two sites intramuscularly with a total of 2 mg conjugate dissolved in 1 ml PBS and mixed with an equal volume of complete Freund's adjuvant. After 6 weeks the rabbits were bled every 2 weeks and sera were examined for their capacity to bind an iodinated peptide, with the amino acid sequence of the first 32 amino acids of the α subunit of 32 kDa porcine inhibin, to which an extra tyrosine had been added at the N-terminal end (Tyr-pIα(1-32), Peninsula, Merseyside, U.K.).

Inhibin radioimmunoassay

The bFF standard or inhibin preparation (100 μl) was diluted with assay buffer (PBS containing 1% BSA). Inhibin antiserum (50 μl; no. 55–10; diluted 1:200 in assay buffer without BSA and containing a total of 1·5% (v/v) normal rabbit serum) was added and the tubes were incubated for 6 h at room temperature. Iodinated Tyr-pIα(1–32) (in 50 μl assay buffer containing 5000 c.p.m.) was added and the tubes were further incubated for 24 h at room temperature. Free and bound hormone were separated as described for the gonadotrophin estimations.

Dilution of several fractions obtained at different stages of the purification of inhibin from bFF, oFF, pFF and rSCCM, but not plasma of an ovariectomized cow (bP_{cas}), gave displacement curves in the radioimmunoassay which paralleled that of unlabelled bFF standard. Results for bFF, bP_{cas} and an inhibin-containing fraction after Red A chromatography are shown in Fig. 1. The sensitivity of the assay (90% B/B_0) was 0·49 ± 0·19 U (mean ± S.D.; $n=5$). The interassay coefficient of variation of repeated measurements of multiple dilutions of a pool of Red A-purified inhibin was 14% ($n=5$). The intra-assay coefficient of variation was 1·7%.

Iodination procedure

For the iodination, 2·5 μg rat LH and FSH tracer I-6 (NIADDK, Bethesda, MD, U.S.A.) or synthetic Tyr-pIα(1–32) dissolved in 25 μl PBS, 0·3 μCi $Na^{125}I$ (Amersham International plc, Amersham, Bucks, U.K.) and 10 mg Protag (Baker, Phillipsburg, NJ, U.S.A.; dissolved in 1 ml H_3BO_3 (10 mmol/l; pH 8·2 with NaOH) containing 0·9% (w/v) NaCl) were mixed. The reaction was terminated after 5 min by the addition of 25 μl KI (1 mol/l) and the reaction mixture was chromatographed on Sephadex G-25 (FSH and LH) or on a small Red A column (Tyr-pIα(1–32)) using the buffers described by Jansen, Steenbergen, de Jong & van der Molen (1981).

RESULTS

Separation of the various molecular weight forms of inhibin was performed by SDS-PAGE. The separated proteins were eluted from the gel and precipitated with methanol to remove SDS. When concentrated rSCCM was coated with 1% (w/v) SDS for 1 h at 37 °C and precipitated with methanol, 62 ± 5·7% (mean ± S.D.; $n=3$) of the bioactivity was recovered. The method was further evaluated by separating 1 mg protein from bFF and from bP_{cas} on SDS-PAGE and estimating inhibin activity in the methanol-precipitated eluates in the bioassay. Results for the 20–40 kDa range of the gel are shown in Fig. 2. Addition of eluates from the slices on which bP_{cas} was run did not affect FSH release from the pituitary cells. In contrast, the proteins eluted from the bFF gel could stimulate (25 kDa) or inhibit (30 kDa) the release of FSH from the cells, indicating the presence of both activin-like and inhibin activity in these eluates. Finally, proteins from rSCCM were concentrated, separated on SDS-PAGE and precipitated with methanol. After these treatments, 56·8 ± 12·9% (mean ± S.D., from three different pools of rSCCM) of

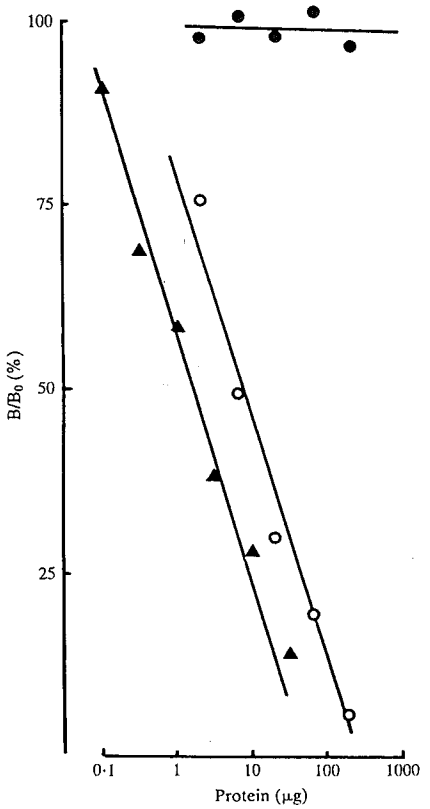

FIGURE 1. Displacement of ^{125}I-labelled (N-terminal 32 amino acid sequence of the α subunit of 30 kDa porcine inhibin with an additional tyrosine at the N-terminus (Tyr-pIα(1–32)) from anti-peptide antiserum by bovine follicular fluid (bFF; ○), a partially purified bFF preparation (Red A fraction; ▲) and by serum from an ovariectomized cow (●).

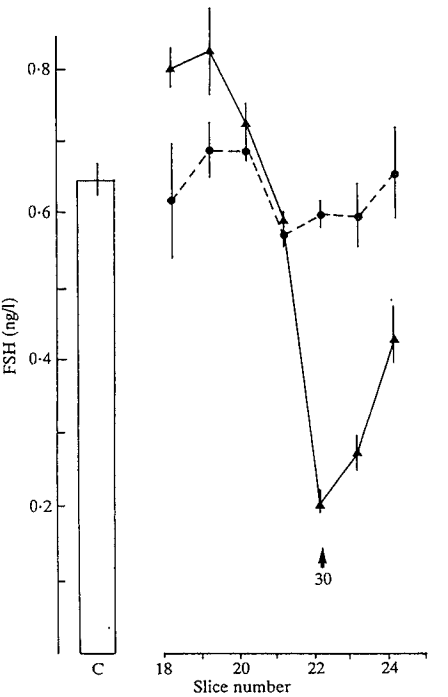

FIGURE 2. Release of FSH from control cultured rat pituitary cells (C) and after addition of methanol-precipitated eluates of slices obtained after SDS-PAGE from bovine follicular fluid (▲) or plasma of an ovariectomized cow (●). The position of the 30 kDa molecular weight marker is indicated by the arrow. Values are means ± S.E.M., $n = 3$.

the inhibin activity was recovered. Proteins eluted from gels after SDS-PAGE did not significantly affect LH release from pituitary cells (data not shown).

Testes of 22-day-old rats were homogenized in a buffer containing protease inhibitors. After separation of proteins in this homogenate by SDS-PAGE, suppression of the FSH release by pituitary cells was elicited by the eluates of the slices containing 30 kDa proteins (Fig. 3*a*). This suppression was dose-dependent, and paralleled that caused by the addition of a standard bFF preparation to the cells (data not shown). In concentrated rSCCM (Fig. 3*b*) and in the bioactive fractions after Mono Q anion-exchange (Fig. 3*c*) and after subsequent reversed phase chromatography (Fig. 3*d*), a similar 30 kDa form of inhibin was found. The suppression of FSH release by proteins with molecular weights around 60–70 kDa in rSCCM (Fig. 3*b*) was not dose-dependent. When proteins of 25 kDa from rSCCM, isolated after SDS-PAGE (slice 23 in Fig. 3*b*) were tested with higher doses (equivalent to 18 and 54 μl unconcentrated rSCCM/0·5 ml pituitary cell incubation medium) in the pituitary cell assay, a dose-dependent increase in FSH secretion was observed (Fig. 4). Proteins from the 30 kDa slice gave a dose-dependent suppression of the FSH release with an equivalent of 4 and 12 μl rSCCM, which was parallel with the bFF standard curve. No FSH release-stimulating activity was detected when higher doses of the eluates of the 25 kDa slices of Mono Q or reversed phase inhibin-

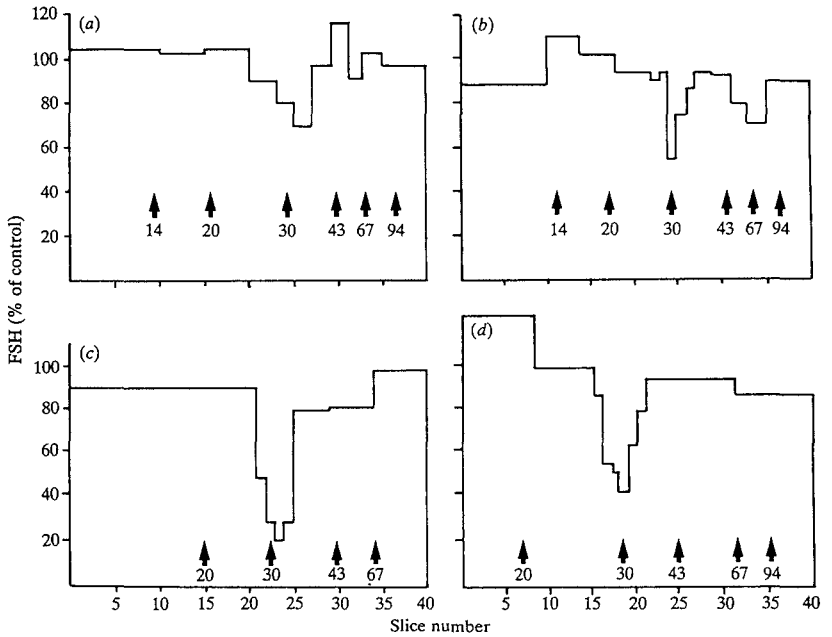

FIGURE 3. Suppression of FSH release (expressed as percentage of control) from cultured rat pituitary cells after addition of eluates of gel slices, obtained after SDS-PAGE of (*a*) homogenates of 22-day-old rat testes, (*b*) rat Sertoli cell-conditioned medium (rSCCM) and fractions of rSCCM after (*c*) purification by Mono Q anion exchange and (*d*) subsequent reversed phase chromatography. The positions of molecular weight markers (kDa) are indicated by arrows.

containing preparations were added to the pituitary cells (Fig. 3*c*,*d*).

The predominant inhibin form (86% of total activity recovered from the gel) in rat ovarian homogenates (Fig. 5) had an apparent molecular weight of 30 kDa after SDS-PAGE, while the larger forms (>40 kDa) were less abundant (14% of total recovered activity). After addition of eluates of the slice containing 25 kDa proteins, an activin-like stimulation of FSH release was found.

When bFF proteins were separated by SDS-PAGE, suppression of FSH release was detected after addition of a range of proteins to the pituitary cells (Fig. 6*a*). With a 23-fold purified fraction of bFF obtained after chromatography on a Red A column and desalting on Sephadex G-25, molecules in the range of 32–45, 58 and 97 kDa had inhibin-like activity (Fig. 6*b*). The eluates of the 25 kDa slices of both native bFF and bFF after purification on a Red A column stimulated FSH release from pituitary cells in a dose-dependent manner (data not shown). Finally, two inhibin-containing samples from a reversed phase column were analysed. The column was loaded with an inhibin preparation from bFF which was prepurified on Red A, Sephadex G-25, Mono Q and lentil lectin Sepharose. In the pool of 25–30% acetonitrile eluates (Fig. 6*c*), a 30–40 kDa FSH-suppressing zone was found, while in the pool of 30–40% acetonitrile eluates (Fig. 6*d*) a 30 kDa and a 58 kDa protein which suppressed FSH release were found.

With the peptide radioimmunoassay, the immunoactivities of several inhibin-containing fractions obtained during the purification of inhibin from bFF were measured. Although the inhibin bioactivity increased from 1 (bFF) to 950 (reversed phase column), the relative specific activity of the peptide radioimmunoassay only increased from 1 to 30. To provide further insight into the differences in bioactivity and immunoactivity, inhibin immunoactivity was also estimated in the two inhibin-containing fractions from the reversed phase column separated on SDS-PAGE (Fig. 7). The 30 kDa form of inhibin had a bioactivity/immunoactivity ratio of 2·5 and the

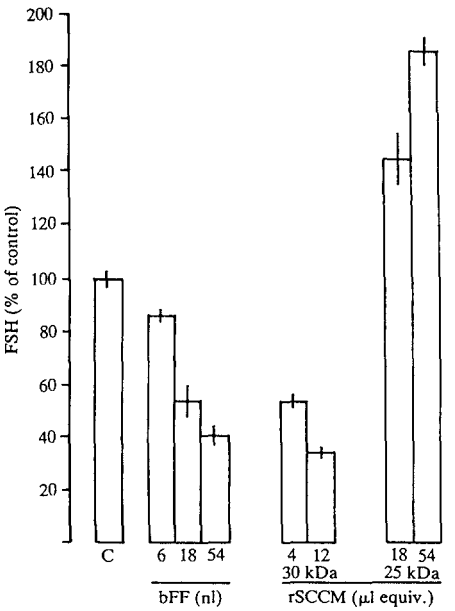

FIGURE 4. Effects of addition of 25 kDa activin-like proteins and 30 kDa inhibin-like proteins, eluted from a 15% SDS-polyacrylamide gel after electrophoresis of rat Sertoli cell-conditioned medium (rSCCM), on the release of FSH from rat pituitary cells expressed as a percentage of control. In addition, suppression of FSH release by a standard preparation of bovine follicular fluid (bFF) is shown. Values are means ± S.E.M., $n = 3$.

58 kDa form a bioactivity/immunoactivity ratio of 0·5.

DISCUSSION

The results from the experiments described here indicate that only a 30 kDa form of inhibin is present in rat testes, while several molecular weight forms of inhibin were found in rat ovarian homogenates and bFF. Furthermore, in both male and female gonadal fluids, an activin-like activity was present.

The superior resolution of SDS-PAGE under protein-denaturing conditions was used for the separation of inhibin-containing samples, followed by diffusion elution instead of electro-elution to facilitate the handling of many samples. Methanol precipitation was effective in separating proteins from SDS, since inhibin-free samples precipitated in a similar way did not cause non-specific effects in the pituitary

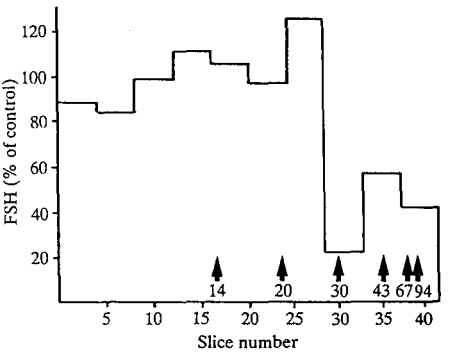

FIGURE 5. Suppression of FSH release (expressed as a percentage of control) from cultured rat pituitary cells after addition of gel slices, obtained after SDS-PAGE of pro-oestrous rat ovarian homogenate. The positions of molecular weight markers (kDa) are indicated by arrows.

cell assay. The recovery of inhibin in the bioassay after methanol precipitation (62%) contrasts with the increase in bioactivity (143%) found by Robertson *et al.* (1986). The reason for this difference remains unclear.

In 22-day-old rat testes only a 30 kDa form of inhibin was found. This contrasts with the reports by Au, Robertson & de Kretser (1983) and Sharpe *et al.* (1988) in which larger forms were found (50–60 kDa and 100 kDa respectively after gel filtration). These differences are likely to have been caused by differences in the separation technique used, as is demonstrated by the results of Baker *et al.* (1982). Since high molecular weight forms of inhibin were found in ovarian homogenates using the same technique, it is postulated that rat testicular inhibin is present as a protein with an apparent molecular weight of 30 kDa.

The results presented here are the first indications of the presence of activin-like activity in rSCCM. In rat testes, only the mRNA of the α and β-B subunits of inhibin were found by Toebosch, Robertson, Trapman *et al.* (1988), while Meunier, Rivier, Evans & Vale (1988) reported very small amounts of mRNA for the β-A subunit in the presence of predominantly α and β-B messages. The reported activin molecules (Ling, Ying, Ueno *et al.* 1986*a*; Vale, Rivier, Vaughan *et al.* 1986) are homodimers of β-A or heterodimers of β-A and β-B subunits. The observation of production of activin-like activity by rat Sertoli cells indicates the possibility of a new β-B homodimer of activin. Alternatively, the activity may be due to a transforming growth factor-β (TGF-β)-like molecule (also 25 kDa) which has been reported to be secreted by

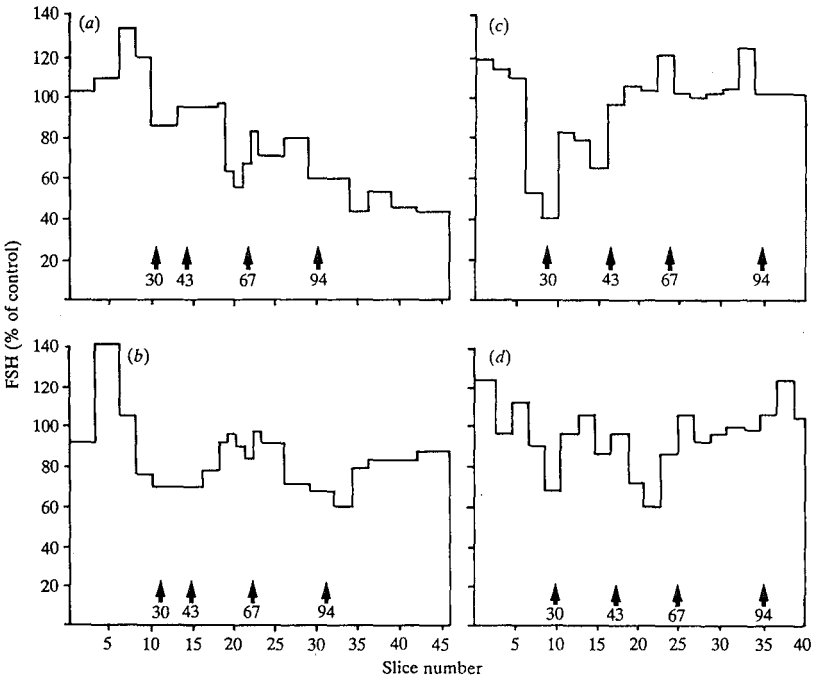

FIGURE 6. Release of FSH (expressed as percentage of control) from cultured rat pituitary cells after addition of eluates of gel slices, obtained after SDS-PAGE of (*a*) bovine follicular fluid (bFF), (*b*) a fraction obtained after Red A chromatography of bFF and (*c*) a pool of 25–30% acetonitrile or (*d*) 30–40% acetonitrile after reversed phase chromatography with a partially purified bFF preparation. See text for details of previous purification steps. The positions of molecular weight markers (kDa) are indicated by arrows.

Sertoli cells (Benahmed, Cochet, Keramidas *et al.* 1988). TGF-β can stimulate FSH release from cultured pituitary cells (Ling, Ying, Ueno *et al.* 1986*b*). Because of the loading capacity of the SDS-PAGE, insufficient testes extract could be separated for the detection of activin-like activity in this material.

In rat ovarian extract, larger forms of inhibin were also found, while activin-like activity was more abundant than in rSCCM. As a consequence, the amount of inhibin activity in an ovarian homogenate will be underestimated in a pituitary cell bioassay of inhibin.

Several molecular forms of inhibin were found in bFF. These were apparently co-purified during the various steps of the purification procedure and separated only after reversed phase chromatography. The rather broad range of molecular weights associated with inhibin activity in bFF may be caused by the loading of large amounts of protein on the gel; this problem was largely solved with the more purified preparations obtained after Red A or reversed phase chromatography. In both acetonitrile pools after reversed phase chromatography, a 30 kDa inhibin was present, as was reported by Ling *et al.* (1985), who suggested that these are αβ-A and αβ-B dimers.

The increase in bioactivity/immunoactivity ratio during the purification may have been caused by loss of activin during the purification, while further changes may reflect preferential retention of one or other sub-form of inhibin. The differences between bioactivity/immunoactivity ratios for the 30 and 58 kDa forms of inhibin remain unexplained, especially since it might be expected that the part of the molecule which was used as a peptide-immunogen would be more exposed in the lower molecular weight form. In addition, McLachlan *et al.* (1986) and Robertson *et al.* (1988) reported large differences in bioactivity/immunoactivity ratios in the purification

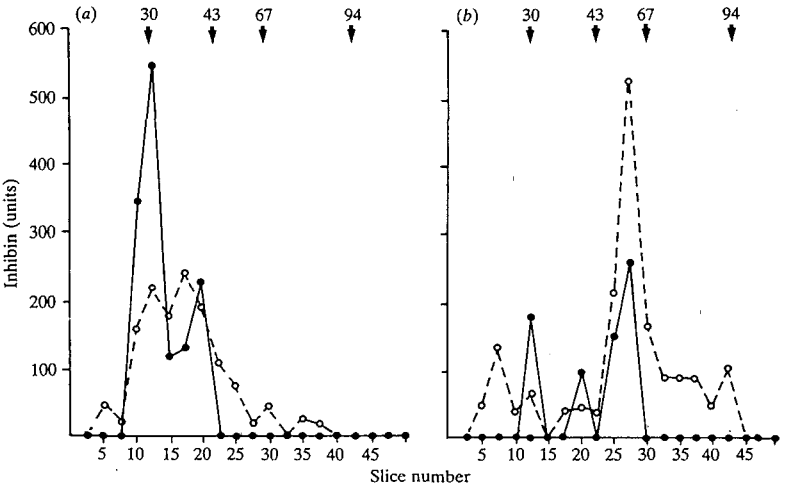

FIGURE 7. Immunoactive (○) and bioactive (●) inhibin in eluates of gel slices after SDS-PAGE separation of proteins in a (*a*) 25–30% and (*b*) 30–40% pool of acetonitrile after reversed phase chromatography of a partially purified bFF preparation (determined in the same eluates as used for the bioactivity shown in Fig. 6*c* and *d*). Mobility of molecular weight markers (kDa) is indicated by arrows.

of bFF, although both 30 and 58 kDa forms of inhibin had the same bioactivity/immunoactivity ratios.

This difference in bioactivity/immunoactivity ratios emphasizes the importance of investigating which forms of inhibin are present in the circulation in female and male animals, and indicates that results of radioimmunoassays of inhibin, especially when inhibin of ovarian origin is estimated using peptide antisera, should be interpreted with caution.

ACKNOWLEDGEMENTS

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REFERENCES

Au, C. L., Robertson, D. M. & de Kretser, D. M. (1983). *In vitro* bioassay of inhibin in testes of normal and cryptorchid rats. *Endocrinology* **112**, 239–244.

Baker, H. W. G., Eddie, L. W., Higginson, R. E., Hudson, R. & Niall, H. D. (1982). Studies on the purification of ovine inhibin. *Annals of the New York Academy of Sciences* **383**, 329–342.

Benahmed, M., Cochet, C., Keramidas, M., Chauvin, M. A. & Morera, A. M. (1988). Evidence for FSH dependent secretion of a receptor reactive transforming growth factor β-like material by immature Sertoli cells in primary culture. *Biochemical and Biophysical Research Communications* **154**, 1222–1231.

Burger, H. G. (1988). Commentary: Inhibin: definition and nomenclature, including related substances. *Journal of Endocrinology* **117**, 159–160.

van Dijk, S., Steenbergen, J., Gielen, J. Th. & de Jong, F. H. (1986). Sexual dimorphism in immunoneutralization of bioactivity of rat and ovine inhibin. *Journal of Endocrinology* **111**, 255–261.

Finney, D. J. (1964). Parallel line assays. In *Statistical Methods in Biological Assay*, edn 2, pp. 101–138. London: Griffin.

Forage, R. G., Ring, J. M., Brown, R. W., McInerney, B. V., Cobon, G. S., Gregson, R. P., Robertson, D. M., Morgan, F. J., Hearn, M. T. W., Findlay, J. K., Wettenhall, R. E. H., Burger, H. G. & de Kretser, D. M. (1986). Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. *Proceedings of the National Academy of Sciences of the U.S.A.* **83**, 3091–3095.

Fournier, A., Wang, C. & Felix, A. M. (1988). Applications of BOP reagent in solid phase synthesis. *International Journal of Proteins and Peptide Research* **31**, 86–97.

Fukuda, M., Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K. & Matsuo, H. (1986). Isolation of bovine follicular fluid inhibin of about 32 kDa. *Molecular and Cellular Endocrinology* **44**, 55–60.

Jansen, E. H. J. M., Steenbergen, J., de Jong, F. H. & van der Molen, H. J. (1981). The use of affinity matrices in the purification of inhibin from bovine follicular fluid. *Molecular and Cellular Endocrinology* **21**, 109–117.

de Jong, F. H. (1988). Inhibin. *Physiological Reviews* **68**, 555–607.

de Jong, F. H., Grootenhuis, A. J., Sander, H. J., Steenbergen, J., Timmerman, M. A. & van Dijk, S. (1987). Comparison between inhibin from bovine follicular fluid and rat Sertoli cell conditioned medium. In *Inhibin-Non-Steroidal Regulation of Follicle Stimulating Hormone Secretion*. Eds H. G. Burger, D. M. de Kretser, J. K. Findlay & M. Igarashi. New York: Raven Press.

de Jong, F. H. & Robertson, D. M. (1985). Inhibin: 1985 update on action and purification. *Molecular and Cellular Endocrinology* **42**, 95–103.

de Jong, F. H., Smith, S. D. & van der Molen, H. J. (1979). Bioassay of inhibin like activity using pituitary cells *in vitro*. *Journal of Endocrinology* **80**, 91–102.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lee, V. W. K., Kraft, N. C., Atkins, R. C. & Burger, H. G. (1986). Monoclonal antibody to rat ovarian inhibin. *Journal of Endocrinology* **109**, 379–383.

Leversha, L. J., Robertson, D. M., de Vos, F. L., Morgan, F. J., Hearn, M. T. W., Wettenhall, R. E. H., Findlay, J. K., Burger, H. G. & de Kretser, D. M. (1987). Isolation of inhibin from ovine follicular fluid. *Journal of Endocrinology* **113**, 213–221.

Ling, N., Ying, S.-Y., Ueno, N., Esch, F., Denoroy, L. & Guillemin, R. (1985). Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid. *Proceedings of the National Academy of Sciences of the U.S.A.* **82**, 7217–7221.

Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. & Guillemin, R. (1986*a*). Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. *Nature* **321**, 779–782.

Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. & Guillemin, R. (1986*b*). A homodimer of the β-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. *Biochemical and Biophysical Research Communications* **138**, 1129–1137.

McLachlan, R. I., Robertson, D. M., Burger, H. G. & de Kretser, D. M. (1986). The radioimmunoassay of bovine and human follicular fluid and serum inhibin. *Molecular and Cellular Endocrinology* **46**, 175–185.

Mason, A. J., Hayflick, J. S., Ling, N., Esch, F., Ueno, N., Ying, S.-Y., Guillemin, R., Niall, H. & Seeburg, P. H. (1985). Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. *Nature* **318**, 659–663.

Meunier, H., Rivier, C., Evans, R. M. & Vale, W. (1988). Gonadal and extragonadal expression of inhibin α, βA, and βB subunits in various tissues predicts diverse functions. *Proceedings of the National Academy of Sciences of the U.S.A.* **85**, 247–251.

Miyamoto, K., Hasegawa, Y., Fukuda, M., Nomura, M., Igarashi, M., Kangawa, K. & Matsuo, H. (1985). Isolation of porcine follicular fluid inhibin of 32K Daltons. *Biochemical and Biophysical Research Communications* **129**, 396–403.

Oonk, R. B., Grootegoed, J. A. & van der Molen, H. J. (1985). Comparison of the effects of insulin and follitropin on glucose metabolism by Sertoli cells of immature rats. *Molecular and Cellular Endocrinology* **42**, 39–48.

Pfaff, E., Mussgay, M., Böhm, H. O., Schulz, G. E. & Schaller, H. (1982). Antibodies against a preselected peptide recognize and neutralize foot and mouth-disease virus. *EMBO Journal* **1**, 869–874.

Rivier, C., Rivier, J. & Vale, W. (1986). Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat. *Science* **234**, 205–208.

Robertson, D. M., Foulds, L. M., Leversha, L., Morgan, F. J., Hearn, M. T. W., Burger, H. G., Wettenhall, R. E. H. & de Kretser, D. (1985). Isolation of inhibin from bovine follicular fluid. *Biochemical and Biophysical Research Communications* **126**, 220–226.

Robertson, D. M., Tsonis, C. G., McLachlan, R. I., Handelsman, D. J., Leask, R., Baird, D. T., McNeilly, A. S., Hayward, S., Healy, D. L., Findlay, J. K., Burger, H. G. & de Kretser, D. M. (1988). Comparison of inhibin immunological and *in vitro* biological activities in human serum. *Journal of Clinical Endocrinology and Metabolism* **67**, 438–443.

Robertson, D. M., de Vos, F. L., Foulds, L. M., McLachlan, R. I., Burger, H. G., Morgan, F. J., Hearn, M. T. W. & de Kretser, D. M. (1986). Isolation of a 31 kDa form of inhibin from bovine follicular fluid. *Molecular and Cellular Endocrinology* **44**, 271–277.

Schanbacher, B. (1988). Radioimmunoassay of inhibin: serum responses to unilateral and bilateral orchidectomy. *Endocrinology* **123**, 2323–2330.

Sharpe, R. M., Swanston, I. A., Cooper, I., Tsonis, C. G. & McNeilly, A. S. (1988). Factors affecting the secretion of immunoactive inhibin into testicular interstitial fluid in rats. *Journal of Endocrinology* **119**, 315–326.

Toebosch, A. M. W., Robertson, D. M., Trapman, J., Kluassen, P., de Paus, R. A., de Jong, F. H. & Grootegoed, J. A. (1988). Effects of FSH and IGF-I on immature rat Sertoli cells: inhibin α- and β-subunit mRNA levels and inhibin secretion. *Molecular and Cellular Endocrinology* **55**, 101–105.

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D. & Spiess, J. (1986). Purification and characterization of a FSH releasing protein from porcine ovarian follicular fluid. *Nature* **321**, 776–779.

Welschen, R., Osman, P., Dullaart, J., de Greef, W. J., Uilenbroek, J. Th. J. & de Jong, F. H. (1975). Levels of follicle-stimulating hormone, luteinizing hormone, oestradiol-17β and progesterone, and follicular growth in the pseudopregnant rat. *Journal of Endocrinology* **64**, 34–47.

CHAPTER 3

INHIBIN IN IMMATURE RAT SERTOLI CELL CONDITIONED MEDIUM: A 32 KDA $\alpha\beta$-B DIMER

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Inhibin in immature rat Sertoli cell conditioned medium: a 32 kDa $\alpha\beta$-B dimer

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Key words: Inhibin; Sertoli cell; Testis; Purification

Summary

Conditioned medium of cultured Sertoli cells from 21-day-old rats was used as starting material for the isolation of inhibin. Inhibin activity was monitored by the dose dependent suppression of the follicle-stimulating hormone release of cultured rat pituitary cells. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of the highly purified inhibin preparation revealed a 32 kDa protein after silver staining, which could be separated in subunits of 18 kDa and 12 kDa after reduction. Western blot analysis with an antibody recognizing the 22 N-terminal amino acids of the α-subunit of 32 kDa bovine inhibin confirmed the presence of a 32 kDa inhibin molecule under non-reducing conditions, whereas an 18 kDa α-subunit was found after reduction. An antibody recognizing the β-A subunit of inhibin did not yield a signal after Western blotting. N-terminal amino acid sequence analysis of two highly purified preparations of inhibin obtained using different methods yielded the sequence predicted for a 32 kDa $\alpha\beta$-B dimer on basis of cDNA nucleotide sequence. This result is in agreement with the large excess of β-B over β-A mRNA in the rat testis.

Introduction

Inhibin is a gonadal glyprotein hormone consisting of two dissimilar disulphide-linked subunits, which inhibits pituitary gonadotrophin production and/or secretion, preferentially that of follicle stimulating hormone (FSH) (Burger and Igarashi, 1988). The isolation of inhibin has been hampered by the hydrophobic nature of the molecule, the use of different assay systems with the potential for detection of non-specific effects and the presence of different molecular weight forms of inhibin (de Jong, 1988). Inhibin is present in several gonadal fluids; the highest concentration of inhibin is found in ovarian follicular fluid (de Jong and Sharpe, 1976). Inhibin with a molecular mass of 32 kDa has been purified from both bovine (Fukuda et al., 1986; Robertson et al., 1986), ovine (Leversha et al., 1987) and porcine (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985) follicular fluid (bFF, oFF and pFF respectively). Upon reduction of 32 kDa inhibin two subunits (α- and β-subunit) of 18 and 14 kDa, respectively, were found. Ling et al. (1985) isolated two forms of inhibin from pFF. These

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forms were named inhibin A and B and differed in the N-terminal amino acid sequences of their β-subunits, which are now termed β-A and β-B. Subsequently, genes coding for the α, β-A and β-B-subunits have been cloned from human, porcine, bovine and rat cDNA libraries (for reviews see de Kretser and Robertson, 1989 and Vale et al., 1988).

Relatively little is known about the structure of testicular inhibin. Bardin et al. (1987) reported the purification of two forms of inhibin from ovine rete testis fluid, both composed of an αβ-A dimer but one with a truncated α-subunit. Partial characterization of inhibin from Sertoli cell conditioned medium has been reported for rat (Bicsak et al., 1987; de Jong et al., 1987) and cynomolgus monkey (Noguchi et al., 1987). Here, we report the isolation, subunit composition and N-terminal amino acid sequence of a 32 kDa inhibin isolated from rat Sertoli cell conditioned medium.

Materials and methods

Bioassay for inhibin

Inhibin-like bioactivity was determined using a bioassay detecting the suppression of spontaneous FSH release from incubated pituitary cells (Grootenhuis et al., 1989). Briefly, after a preincubation of pituitary cells for 3 days, media were changed and inhibin containing samples were added at two or three dose levels in triplicate. After a second 3-day culture period the concentration of FSH in the incubation medium was determined. A bFF preparation with an arbitrary potency of 1 U/μg protein was used as standard. The International Research Standard for Inhibin (86/890, Waites et al., 1987) has a relative specific activity of 60 ± 10 U/μg (mean ± SEM, $n = 5$) expressed in units of this bFF standard. Inhibin potencies were calculated using statistics for assay parallelism (Finney, 1964).

Sertoli cell culture

Sertoli cells were isolated from 21-day-old Wistar rats using a double collagenase digestion method as described by Oonk et al. (1985) and Grootenhuis et al. (1989). Briefly, approximately 3×10^6 Sertoli cells (8 mg protein) were plated in 150 cm^2 plastic flasks (Costar, Cambridge, MA, U.S.A.) in 1% fetal calf serum (FCS; Gibco, Grand Island, NY, U.S.A.) in minimal essential medium (MEM, Gibco). On the next day cells were washed and further cultured at 37°C in MEM, supplemented with antibiotics. Rat Sertoli cell conditioned medium (rSSCM) was collected every 3 or 4 days for 4–5 weeks and stored at −20°C.

Purification

Two pools of 5 l rSCCM were concentrated to a volume of 220 ml each under nitrogen pressure at 4°C using a filtration unit (Amicon, Lexington, MA, U.S.A.) using a hollowfibre (HIP10-43; Amicon) with a molecular weight cut-off at 10 kDa. The concentrate was repeatedly exchanged with buffer A of the Mono Q column (see below). Two different column sequences were used for the purification of inhibin, in both methods batches of 40 ml concentrated rSCCM were applied to a Mono Q anion-exchange column (HR 5/5) attached to a standard FPLC system with a GP-250 controller (Pharmacia, Uppsala, Sweden). After the OD_{280nm} profile reached baseline, retained proteins were eluted using a 30 ml linear gradient of 0–600 mmol/l NaCl in buffer A (Tris-HCl buffer; 20 mmol/l, pH 9.2) at 1 ml/min and 1 ml/fraction. The fractions containing the peak of bioactivity were pooled and purified according to one of the methods described below. Lentil Lectin-Sepharose (Pharmacia) chromatography of hollowfibre-concentrated rSCCM was performed according to de Jong et al. (1987).

Method 1. The pooled fractions, containing inhibin bioactivity from the Mono Q columns, were separated in five runs on a C1/C8 reversed phase column (HR 5/20, Pharmacia) using a 30 ml gradient of 0–50% (v/v) acetonitrile containing 0.1% (v/v) trifluoracetic acid (TFA) at 1 ml/min and 1 ml/fraction (de Jong et al., 1987) using an FPLC system. Bioactive fractions were lyophilized and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels (see below). After separation, 2 mm slices were cut and proteins were eluted and methanol precipitated as described by Grootenhuis et al. (1989).

Method 2. From a second batch of rSSCM the bioactive fractions from the Mono Q runs were also pooled. One-third of this pool peak of bioac-

tivity (an equivalent of 1200 ml rSCCM) was applied to a Bakerbond wide-pore C18 column (Baker Chemicals, Phillipsburg, NJ, U.S.A.) using a liquid chromatography system from Waters Associates (Milford, MA, U.S.A.), which consisted of a M-720 system controller, a M-6000A solvent delivery system, an M-441 UV/Vis absorbance detector, on-line equipped with a Shimadzu C-R3A chromatopac data module. The sample was fractionated using a 70 ml linear gradient of 20–48% acetonitrile in phosphate buffer (10 mmol/l; pH 6.8) at 1 ml/min, 1 ml/fraction and the protein profile was recorded at 214 nm. The bioactive fractions were pooled, dried by rotary evaporation in vacuo, applied on a C18 aquapore RP-300 column connected to a microbore 130A Separation System (Applied Biosystem, Foster City, CA, U.S.A.) and eluted with a 50 min linear gradient from 18–48% acetonitrile in 0.1% TFA at 200 µl/min and 400 µl/fraction. Proteins in the eluate were detected at 210 nm.

N-terminal amino acid sequence analysis

The amino acid sequence determination was performed on an Applied Biosystems model 470A protein sequencer, on-line equipped with a model 120A PTH analyzer.

SDS-PAGE

Proteins in the fractions of the last purification step were separated by SDS-PAGE (Laemmli, 1970) on gels of 15% (w/v) acrylamide and 0.8% (w/v) bisacrylamide, without or with prior reduction (5 min 100°C, 1% (v/v) β-mercaptoethanol).

Isoelectrofocussing

Isoelectrofocussing was performed on gels with a pH gradient of 3–9 using the Phast gel system (Pharmacia) according to manufacturer's application notes. For calibration an isoelectrofocussing kit (Pharmacia) was used.

Silver staining and Western blot analysis

For silver staining, gels were stained with Page Blue 83 (Bakker et al., 1987), incubated 1 h with 10% glutaraldehyde, washed overnight with distilled water and stained according to the method of Wray et al. (1981).

For Western blot analysis, separated proteins were electroblotted onto nitrocellulose and incubated with an antiserum against the 22 N-terminal amino acids of the α-subunit of bovine 32 kDa inhibin, which was described earlier by Grootenhuis et al. (1989).

Results

Sertoli cell conditioned medium

Immature rat Sertoli cells cultured in medium without serum at 37°C produce appreciable amounts of bioactive inhibin (Fig. 1). After an initial significant rise of the inhibin concentration in the medium in the second week, large amounts of bioactive inhibin are produced up to the fifth week. The relative specific activity (RSA) of the two pools of rat Sertoli cell conditioned (rSCCM) medium were 20.6 and 17.1 when compared with the bFF standard (see Table 1). Recovery of inhibin bioactivity after concentration of the pooled media was 121% and 95% for the first and second pool, respectively.

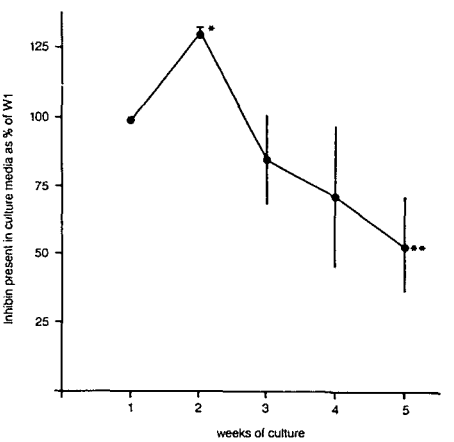

Fig. 1. Time-dependent production of bioactive inhibin by Sertoli cells, derived from 21-day-old rats cultured at 37°C. Media were changed twice a week for 5 weeks. Media from 1 week were pooled and assayed together. Values are obtained from three different Sertoli cell preparations and are expressed as percentage of the value for week 1 (means ± SEM; $*P < 0.05$ compared with week 1, $**P < 0.05$ compared with week 2 (paired Student's *t*-test)).

TABLE 1

RELATIVE SPECIFIC ACTIVITY (RSA) AND RECOVERY OF INHIBIN AFTER SEVERAL STEPS DURING ITS PURIFICATION FROM RAT SERTOLI CELL CONDITIONED MEDIUM (rSCCM)

The amount of rSCCM used in the purification with two different methods is indicated, and fractions (fr) used for the following purification steps are listed. Protein content was determined using: [a]Bradford, [b]OD_{280nm}, or [c]amino acid analysis.

Preparation	Total units ($\times 10^3$)	RSA (U/mg) ($\times 10^3$)	Recovery at each step (%)	Purification factor
Method 1				
rSCCM (4100 ml)	1,340	20.6 [a]	100	1
> 10 kDa	1,620	34.7 [a]	121	1.7
Mono Q fr 9+10	983	88.2 [b]	61	4.3
C1/C8 fr 5, 6, 7	189	121 [b]	28	5.9
SDS-PAGE 29–32 kDa proteins	123	16,400 [c]	65	800
Method 2				
rSCCM (1200 ml)	452	17.1 [a]	100	1
> 10 kDa	429	21.5 [a]	95	1.26
Mono Q fr 10, 11, 12	174	41.3 [b]	40	2.5
C18/pH 6.8 fr 29–40	43	114 [b]	17	6.7
C18 0.1% TFA fr 18	1,156	1,300 [c]	5.3	76

Purification of rat Sertoli cell inhibin

Batches of approximately 13 mg of Amicon concentrated rSCCM protein were chromatographed on Mono Q anion exchange columns. Inhibin bioactivity eluted between 140 and 260 mmol/l NaCl (fraction 8–13, Fig. 2). The recovery of applied inhibin bioactivity in the pooled bioactive fractions from the Mono Q columns was 61% and 40% for the first and the second pool of rSCCM, respectively. We also used Lentil Lectin-Sepharose chromatography of Amicon concentrated rSCCM. From this column $94 \pm 7.6\%$ (mean $\pm$ SEM, $n = 5$) of the recovered bioactivity could be eluted with 0.2 M α-methylmannoside, with no apparent increase in RSA.

For *method 1*, pooled bioactive fractions from the Mono Q columns with an RSA of 88.2 were separated in five runs on a C1/C8 column, using a gradient of acetonitrile containing 0.1% TFA. Inhibin bioactivity was found in fractions 5, 6 and 7 with an RSA of 425. However, after lyophilization RSA dropped to 121, indicating a 72% loss of bioactivity during this procedure. This material (1.56 mg) was separated by SDS-PAGE, eluted, methanol precipitated and analyzed for inhibin

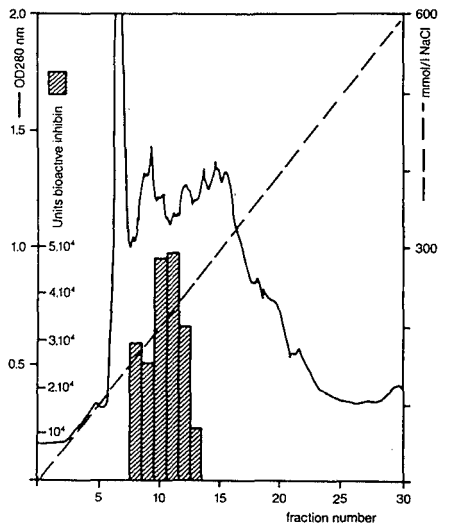

Fig. 2. Anion-exchange chromatography of concentrated rSCCM on a Mono Q column. Proteins (———, OD_{280nm}) were eluted with a lineair gradient of 0–600 mmol/l NaCl (— — —) in a 20 mmol/l Tris-HCl buffer pH 9.2. The hatched area represents inhibin bioactivity.

bioactivity. In eluates of slices 18 and 19, containing proteins of 29–33 kDa, 65% of the applied bioactivity was recovered. On basis of amino acid sequence analysis, 7.5 μg protein was present in these eluates, resulting in a 800-fold purified inhibin preparation with an RSA of 16.4×10^6.

For *method 2*, bioactive fractions from a second pool of rSCCM separated on Mono Q columns, were combined. Proteins of this pool were separated on a C18 column in a phosphate buffer at pH 6.8. Inhibin bioactivity was found in fractions 25–40, with a recovery of 25% of the applied bioactivity (Fig. 3). Fractions 29–40, containing the peak of inhibin activity, were pooled, partly lyophilized and subsequently loaded onto an RP-300 C18 column. Proteins were eluted with a gradient of acetonitrile containing 0.1% TFA. All eluted inhibin bioactivity was found in fraction 18 (Fig. 4) although most of the activity was lost (5% recovery). This eluate was used for amino acid sequencing.

One-third of the Mono Q pool was purified in parallel. Inhibin bioactivity was again found in fraction 18 from the C18 column developed with the TFA buffer. The RSA of this inhibin preparation was 1.3×10^3. This indicates a 76-fold purification factor compared with rSCCM, with a yield of 0.035%. This preparation was also used for amino acid sequencing.

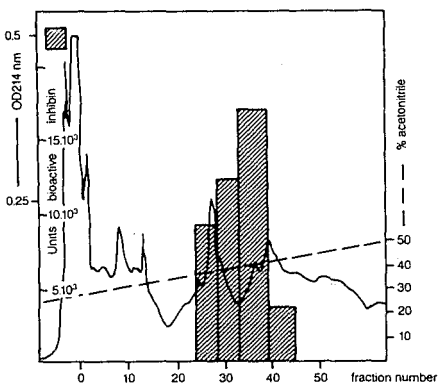

Fig. 3. Reversed-phase chromatography of the inhibin containing fractions from the Mono Q column (Fig. 2) on a C18 column. Proteins (———, OD_{214nm}) were eluted with a lineair gradient of acetonitrile (— — —) in a phosphate buffer (10 mmol/l; pH 6.8). The hatched area represents inhibin bioactivity.

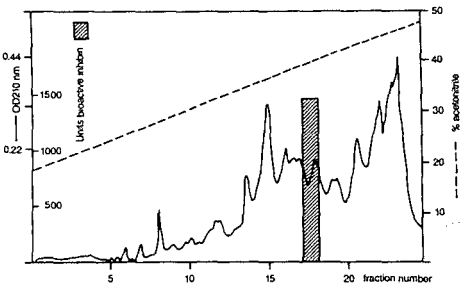

Fig. 4. Reversed-phase chromatography of the inhibin containing fractions (29–40) from the C18 column in Fig. 3 on a C18 column developed with a gradient of acetonitrile (— — —) in a buffer containing 0.1% TFA. Proteins (———, OD_{210nm}) and inhibin bioactivity (hatched area) are indicated.

Analysis of purified inhibin

SDS-PAGE. Fractions of the latter column were analyzed by SDS-PAGE. Part of the gel was silver stained, whereas another part was used for Western blot analysis using an antibody against the 22 N-terminal amino acids of the α-subunit of 32 kDa bovine inhibin. The appearance of immunoreactive inhibin α-subunit (Fig. 5) coincided with the presence of bioactive inhibin in fraction 18. Under non-reducing conditions a 32.5 kDa immunoreactive band was found. After reduction the apparent size of the immunoreactive protein was 19 kDa. The same bioactive fraction was also analyzed (with or without prior reduction) on Western blots with an antibody raised against the N-terminal amino acids 5–24 of the β-A subunit of 32 kDa bovine inhibin. No immunoreactive bands were detected, although with bFF a 14 kDa band emerged after reduction (results not shown). Furthermore, no staining was observed when the blots were incubated with preimmune serum (data not shown). Silver staining of these samples revealed that in the bioactive fraction besides a 31.7 kDa band two bands of 49 kDa en 21 kDa were present. After reduction of these samples the 31.7 kDa band disappeared and 18.4 kDa and 11.7 kDa bands emerged. Isoelectrofocussing and silver

staining of this sample revealed after silver staining one prominent protein with an apparent isoelectric point of 4.8 (results not shown).

Amino acid analysis. Purified fractions obtained after both methods 1 and 2 were subjected to N-terminal amino acid sequence analysis without further derivation. Most of the cycles revealed 2–4 amino acids. Table 2 lists the common amino acids which were found in the inhibin preparations obtained after both purification methods and the amino acids as predicted from rat cDNA sequences (Esch et al., 1987; Woodruff et al., 1987). In all cycles, with the exception of cycle 9, the amino acids found were consistent with the presence of an $\alpha\beta$-B inhibin dimer in rSCCM, while the amino acids specific for the β-A sequence were not detected. Glycine could not be detected with both methods in cycle 1 and 6, due to residual glycine from the SDS-PAGE buffer. Cysteines were not identified in cycle 4, 11 and 12 because no prior derivation was performed.

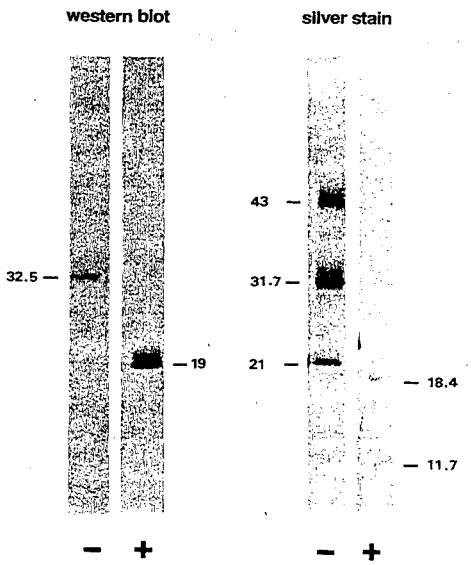

Fig. 5. Western blot analysis with an antiserum against the 22 N-terminal amino acids of the bovine inhibin α-subunit and silver staining of fraction 18 of Fig. 4. Fractions were analyzed by SDS-PAGE on 15% gels, with (+) or without (−) prior reduction with β-mercaptoethanol. Indicated molecular weights are based on the positions of molecular weight markers, which have not been indicated in this figure.

TABLE 2

AMINO ACID SEQUENCE ANALYSIS OF INHIBIN PREPARATIONS PURIFIED ACCORDING TO TWO METHODS

The N-terminal amino acid sequence of rat 30 kDa inhibin, derived from cDNA sequences (Esch et al., 1987 and Woodruff et al., 1987), is also presented for comparison.

Cycle	Amino acids detected with method 1 and 2	Predicted amino acids		
		α	β-A	β-B
1	S	S	G	G
2	Q, A, L	A	L	L
3	P, E	P	E	E
4	S	S	C	C
5	D, M	M	D	D
6	P	P	G	G
7	W, R	W	K	R
8	P, T	P	V	T
9	K, L	W	N	S
10	S, L	S	I	L
11	P	P	C	C
12	A	A	C	C
13	A	A	K	R
14	L, Q	L	K	Q
15	R, Q	R	Q	Q
16	L	L	F	F
17	F, L	L	F	F
18	Q	Q	V	I

Discussion

Although rSCCM is a rich source of inhibin, with an RSA of 17–21 compared with bFF, it is difficult to obtain sufficient material for purification procedures. This was the reason to use the double collagenase method for the isolation of Sertoli cells, instead of the isolation method used to obtain highly purified Sertoli cells, which gives lower yields (Toebosch et al., 1989). Although in this rSCCM some peritubular cell derived proteins will be present, the report that the peritubular cell derived factor PmodS stimulates inhibin production (Skinner et al., 1989), makes it also attractive to use the former method. Up to the fifth week of culture appreciable amounts of bioactive inhibin were produced after an initial rise in the second week.

The Amicon hollowfibre concentration system with a molecular weight cut-off at 10 kDa was a convenient way to concentrate/exchange large pools of rSCCM in a short period. Inhibin bioactivity was never detected in the < 10 kDa fractions, also not after concentration of this fraction using a membrane with a cut-off at 1 kDa and testing equivalents up to 6 ml 1–10 kDa rSCCM per 0.5 ml pituitary culture medium (data not shown). After concentration several purification steps have been explored. The Mono Q anion-exchange column resulted in inhibin preparations with high yields and a reasonable increase of RSA. When reversed-phase chromatography was performed directly on Amicon concentrated rSCCM, FSH-releasing activity eluting in front of inhibin was observed. After the Mono Q column this FSH-releasing activity was lost (results not shown).

In the first purification method after the Mono Q column a C1/C8 column was used, followed by SDS-PAGE. Amino acid sequence analysis revealed a heterogeneous preparation. With the second purification method, two reversed-phase chromatography steps at neutral and acid pH were used. With this method a low RSA was obtained compared with the result of the first method. Western blot analysis of the inhibin preparation purified with method 2 revealed that only 15 U of bioactive inhibin resulted in a signal which was stronger than 1000 U starting rSCCM. This indicates that bioactivity is less stable than immunoactivity, resulting in an underestimation of the RSA of the purified inhibin using method 2.

A 32 kDa protein with inhibin bioactivity was purified from rSCCM. This protein is composed of a 18.4 kDa α-subunit and 11.7 kDa β-B subunit linked by disulphide bond(s). The molecular weight of this purified inhibin preparation confirms the observation that in rat testis homogenates and rSCCM directly separated by SDS-PAGE only one form of 32 kDa bioactive inhibin was found (Grootenhuis et al., 1989). This makes it likely that the 32 kDa $\alpha\beta$-B inhibin molecule is the predominant form of inhibin in the rat testis, although it cannot be excluded completely that other forms of inhibin have been lost during the purification procedure. Also Bicsak et al. (1987) reported a 30 kDa inhibin form in rSCCM chromatographed under denaturing conditions on a Superose-12 gel permeation column. A similar molecular weight form of inhibin (32 kDa) was found in the conditioned medium of rat granulosa cells (Bicsak et al., 1986). Molecular weight estimations of rat testicular preparations using gel permeation chromatography under non-denaturing conditions revealed large differences (30–100 kDa) in molecular weight (Au et al., 1983; Noguchi et al., 1987; Sharpe et al., 1988), probably due to hydrophobic interactions. The high molecular weight form of inhibin, found after Lentil Lectin-Sepharose chromatography of purified rSCCM, which we reported earlier (de Jong et al., 1987), was probably due to a silver stain artefact at 65 kDa.

The molecular weight of inhibin and its subunits after reduction agrees well with the reports on purified 32 kDa ovarian inhibin (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985; Fukuda et al., 1986; Robertson et al., 1986; Leverha et al., 1987). The amino acid sequence which was obtained after combination of the data for the two inhibin preparations shows that rat Sertoli cell inhibin is an $\alpha\beta$-B dimer. Additional evidence was obtained using an antiserum recognizing the β-A subunit of inhibin; using this antiserum no immunoreactive bands were detected on the Western blot. This agrees with the presence of mRNAs for the α- and β-B subunits of inhibin in rat Sertoli cells and the low or undetectable levels of β-A mRNA (Toebosch et al., 1988; Feng et al., 1989; Klaij et al., 1990). The only other reports on the characterization of male inhibin revealed a 30 kDa $\alpha\beta$-A dimer in ovine testicular lymph (Bardin et al., 1987; Vaughan et al., 1989).

The retention of inhibin bioactivity on Lentil Lectin-Sepharose indicates that rat inhibin is a glycoprotein as was reported for inhibin from other species. The isoelectric point of 32 kDa rat inhibin is similar to that reported by van Dijk et al. (1985) for bFF inhibin purified on a chromatofocussing column.

It is concluded that rat testicular inhibin is a 32 kDa $\alpha\beta$-B dimer. This contrasts sharply with the situation in follicular fluid where both $\alpha\beta$-A and $\alpha\beta$-B dimers were found with a number of molecular weight forms of inhibin (Ling et al., 1985; Robertson et al., 1985; Miyamoto et al., 1986; Grootenhuis et al., 1989).

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References

Au, C.L., Robertson, D.M. and de Kretser, D.M. (1983) Endocrinology 112, 239–244.

Bakker, G.H., Hoogerbrugge, J.W., Rommerts, F.F.G. and van der Molen, H.J. (1981) Biochem. J. 198, 339–346.

Bardin, C.W., Morris, P.L., Chen, C.-L., Shaha, C., Voglmayr, J., Rivier, J., Spiess, J. and Vale W.W. (1987) in Inhibin-Non-Steroidal Regulation of Follicle Stimulating Hormone Secretion (Burger, H.G., Findlay, J.K., de Kretser, D.M. and Igarashi, M., eds.), pp. 179–190, Raven Press, New York.

Bicsak, T.A., Tucker, E.M., Cappel, S., Vaughan, J., Rivier, J., Vale, W. and Hsueh, A.J.W. (1986) Endocrinology 119, 2711–2719.

Bicsak, T.A., Vale, W., Vaughan, J., Tucker, E.M., Cappel, S. and Hsueh, A.J.W (1987) Mol. Cell. Endocrinol. 49, 211–217.

Burger, H.G. and Igarashi, M. (1988) Acta Endocrinol. 118, 159–160.

Esch, F.S., Shimasaki, S., Cooksey, K., Mercado, M., Mason, A.J., Ying, S.-Y., Ueno, N. and Ling, N. (1987) Mol. Endocrinol. 1, 388–396.

Feng, Z.-M., Bardin, C.W. and Chen, C.-L.C. (1989) Mol. Endocrinol. 3, 939–948.

Finney, D.J. (1964) in Statistical Methods in Biological Assay, 2nd edn., pp. 101–138, Griffin, London.

Fukuda, M., Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K. and Matsuo, H. (1986) Mol. Cell. Endocrinol. 44, 55–60.

Grootenhuis, A.J., Steenbergen, J., Timmerman, M.A., Dorsman, A.N.R.D., Schaaper, W.M.M., Meloen, R.H. and de Jong, F.H. (1989) J. Endocrinol. 122, 293–301.

de Jong, F.H. (1988) Physiol. Rev. 68, 555–607.

de Jong, F.H. and Sharpe, R.M. (1976) Nature 263, 71–72.

de Jong, F.H., Grootenhuis, A.J., Sander, H.J., Steenbergen, J., Timmerman, M.A. and van Dijk, S. (1987) in Inhibin-Non-Steroidal Regulation of Follicle Stimulating Hormone Secretion (Burger, H.G., Findlay, J.K., de Kretser, D.M. and Igarashi, M., eds.), pp. 35–46, Raven Press, New York.

de Kretser, D.M. and Robertson, D.M. (1989) Biol. Reprod. 40, 33–47.

Klaij, I.A., Toebosch, A.M.W., Themmen, A.P.N., Shimasaki, S., de Jong, F.H. and Grootegoed, J.A. (1990) Mol. Cell. Endocrinol. 68, 45–52.

Laemmli, U.K. (1970) Nature 227, 680–685

Leversha, L.J., Robertson, D.M., de Vos, F.L., Morgan, F.J., Hearn, M.T.W., Wettenhall, R.E.H., Findlay, J.K., Burger, H.G. and de Kretser, D.M. (1987) J. Endocrinol. 113, 213–221.

Ling, N., Ying, S.-Y., Ueno, N., Esch, F., Denoroy, L. and Guillemin, R. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 7217–7221.

Miyamoto, K., Hasegawa, Y., Fukuda, M., Nomura, M., Igarashi, M., Kangawa, K. and Matsuo, H. (1985) Biochem. Biophys. Res. Commun. 129, 396–403.

Miyamoto, K., Hasegawa, Y., Fukuda, M. and Igarashi, M. (1986) Biochem. Biophys. Res. Commun. 136, 1103–1109.

Noguchi, K., Keeping, H.S., Winters, S.J., Saito, H., Oshima, H. and Troen, P. (1987) J. Clin. Endocrinol. Metab. 64, 783–788.

Oonk, R.B., Grootegoed, J.A. and van der Molen, H.J. (1985) Mol. Cell. Endocrinol. 42, 39–48.

Rivier, J., Spiess, J., McClintock, R., Vaughan, J. and Vale, W. (1985) Biochem. Biophys. Res. Commun. 133, 120–127.

Robertson, D.M., Foulds, L.M., Leversha, L., Morgan, F.J., Hearn, M.T.W., Burger, H.G., Wettenhall, R.E.H. and de Kretser, D.M. (1985) Biochem. Biophys. Res. Commun. 126, 220–226.

Robertson, D.M., de Vos, F.L., Foulds, L.M., McLachlan, R.I., Burger, H.G., Morgan, F.J., Hearn, M.T.W. and de Kretser, D.M. (1986) Mol. Cell. Endocrinol. 44, 271–277.

Sharpe, R.M., Swanston, I.A., Cooper, I., Tsonis, C.G. and McNeilly, A.S. (1988) J. Endocrinol. 119, 315–326.

Skinner, M.K., McLachlan, R.I. and Bremner, W.J. (1989) Mol. Cell. Endocrinol. 66, 239–249.

Toebosch, A.M.W., Robertson, D.M., Trapman, J., Klaassen, P., de Paus, R.A., de Jong, F.H. and Grootegoed, J.A. (1988) Mol. Cell. Endocrinol. 55, 101–105.

Toebosch, A.M.W., Robertson, D.M., Klaij, I.A., de Jong, F.H. and Grootegoed, J.A. (1989) J. Endocrinol. 122, 757–762.

Vale, W., Rivier, C., Hsueh, A., Campen, C., Meunier, H., Bicsak, T., Vaughan, J., Corrigan, A., Bardin, W., Sawchenko, P., Petraglia, F., Yu, J., Plotsky, P., Spiess, J. and Rivier, J. (1988) Recent Prog. Horm. Res. 44, 1–30.

van Dijk, S., Steenbergen, C., de Jong, F.H. and van der Molen, H.J. (1985) Mol. Cell. Endocrinol. 42, 245–251.

Vaughan, J.M., Rivier, J., Corrigan, A.Z., McClintock, R., Campen, C.A., Jolley, D., Voglmayr, J.K., Bardin, C.W., Rivier, C. and Vale, W. (1989) Methods Enzymol. 168, 588–618.

Waites, G.M.H., Bialy, G., Gordon, W.L., Findlay, J.K., de Jong, F.H., Robertson, D.M., Schwartz, N.B. and Storring, P.L. (1987) in Inhibin-Non-Steroidal Regulation of Follicle Stimulating Hormone Secretion (Burger, H.G., Findlay, J.K., de Kretser, D.M. and Igarashi, M., eds.), pp. 281–287, Raven Press, New York.

Woodruff, T.K., Meunier, H., Jones, P.B.C., Hsueh, A.J.W. and Mayo, K.E. (1987) Mol. Endocrinol. 1, 561–568.

Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. 118, 197–203.

CHAPTER 4

FSH-STIMULATED SECRETION OF A 29 KDA INHIBIN α-SUBUNIT COMPLEX, BUT NOT OF 32 KDA BIOACTIVE INHIBIN, FROM CULTURED IMMATURE RAT SERTOLI CELLS

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submitted for publication

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SUMMARY. The medium of cultured Sertoli cells from immature rat testes contains 29 and 32 kDa proteins, which are recognized by an antiserum against the 22 N-terminal aminoacids of the inhibin α-subunit. These proteins were detected by immunoprecipitation of labelled proteins after a pulse-chase incubation of Sertoli cells with ^{35}S-methionine, and by Western blotting. The amount of the 32 kDa protein was not affected by the addition of FSH to the culture medium of the Sertoli cells, whereas FSH induced a large increase of the amount of the 29 kDa protein. Finally, the 29 and 32 kDa proteins in the medium from control and FSH-stimulated Sertoli cells were separated by SDS-polyacrylamide electrophoresis, and inhibin bio- and immunoactivity were determined in eluates of the slices of the gel. Equal amounts of bioactivity were found in control and FSH-stimulated samples at 32 kDa, while the amount of immunoactivity at 29 kDa was increased; no bioactivity was detected in the eluates of these slices.

It is concluded that FSH stimulates the secretion of a 29 kDa inhibin-like protein, which does not contain inhibin bioactivity. This indicates that results of experiments, in which antibodies against N-terminal peptides of the inhibin α-subunit are used to detect inhibin, do not necessarily reflect the amount of bioactive inhibin produced.

INTRODUCTION

Inhibin is a gonadal glycoprotein which preferentially suppresses follicle stimulating hormone (FSH) secretion from the pituitary gland. It is a dimer of an α-subunit and one of two highly homologous β-subunits (β-A or β-B), joined by disulphide bond(s) (for reviews see de Jong, 1988; de Kretser & Robertson, 1989; Ying, 1988). Recently, dimers of β-subunits were isolated from ovarian follicular fluid. Since these proteins stimulate FSH release from cultured pituitary cells, they were called activins (Ling, Ying, Ueno et al. 1986; Vale, Rivier, Vaughan et al. 1986). Finally the presence of loose inhibin α-subunits of 18 kDa and 26 kDa in follicular fluid were described (Knight, Beard, Wrathall et al. 1989; Sugino, Nakamura, Takio et al. 1989; Robertson, Giacometti, Foulds et al. 1989); the biological

significance of the presence of these subunits is not clear.

Detection of inhibin was initially dependent on bioassays, but with purification and elucidation of the molecular structure of inhibin sensitive radioimmunoassays have been developed using antisera against the native protein (McLachlan, Robertson, Burger et al. 1986; Robertson, Tsonis, McLachlan et al. 1988) or against the synthetic peptides with the amino acid sequence of the N-terminal part of the α-chain of 32 kDa inhibin of porcine (Ying, Czvik, Becker et al. 1987; Bicsak, Vale, Vaughan et al. 1987), bovine (Grootenhuis, Steenbergen, Timmerman et al. 1989) or human (Knight et al. 1989) origin. The evaluation of the specificity of these immunoassays has been of particular concern, because several molecular weight forms of inhibin showed large differences in the ratio between bioactivity and immunoactivity determined using antibodies against native inhibin (McLachlan et al. 1986) or against an inhibin derived synthetic peptide (Grootenhuis et al. 1989).

Sertoli cells are the source of bioactive inhibin in the testis (Le Gac & de Kretser, 1982; Ultee-van Gessel, Leemborg, de Jong & van der Molen, 1986; Grootenhuis et al. 1989). The secretion of inhibin by Sertoli cells can be stimulated by FSH, although the magnitude of the effect depends on the conditions of the Sertoli cell culture and the method used for the determination of inhibin. Toebosch, Robertson, Trapman et al. (1988) found that after FSH stimulation of highly purified Sertoli cells the bioactivity/immunoactivity ratio (B/I ratio) of secreted inhibin decreased.

In this paper we describe that Sertoli cells from immature rat testes secrete bioactive inhibin with an apparent molecular weight of 32 kDa and an additional α-subunit immunoreactive band with a molecular weight of 29 kDa. Under influence of FSH the inhibin 29 kDa α-subunit immunoreactivity is increased, while the amount of inhibin bioactivity does not change.

Results of immunoprecipitation of ^{35}S-labelled proteins, followed by autoradiography, indicate that the FSH-induced secretion of the 29 kDa protein can already be detected at 2 h after the end of the labelling period.

MATERIALS AND METHODS

Isolation and culture of Sertoli cells. Sertoli cells were isolated from testes of 21-day-old Wistar rats and cultured in 150 cm^2 plastic flasks (Costar, Cambridge, MA, U.S.A.) in Minimal Essential Medium (MEM, Gibco, Grand Island, NY, U.S.A.) containing 1% (v/v) fetal calf serum (FCS, Gibco) in a humidified incubator in an atmosphere of 5% CO_2 in air at 37°C (Grootenhuis et al. 1989). After 24 h the Sertoli cells were washed and cultured further in 20 ml MEM without FCS, with or without 500 ng ovine FSH/ml (NIH S16; a gift from NIH, U.S.A.). Media were renewed twice weekly for 4 weeks. Rat Sertoli cell conditioned media (rSCCM) from control cultures (CrSCCM) or FSH-stimulated cultures (FrSCCM) collected during the first 2 weeks and the second period of 2 weeks were pooled. Subsequently, the pools were concentrated 25-fold and exchanged with a Tris-HCl buffer (20 mmol/l; pH 7.9) using YM10 membranes (molecular weight cut-off at 10 kDa; Amicon, Lexington, MA, U.S.A.).
In the pulse-chase labelling experiments, Sertoli cells were cultured in 2 ml MEM containing 1% FCS, in 6 cm Petri dishes (Greiner, Solingen, F.R.G.) for 72 h. Subsequently, a hypotonic shock was applied, in order to remove spermatogenic cells (Toebosch, Robertson, Klaij et al., 1989). During a subsequent period of 24 h, the cells were cultured in the presence or absence of 500 ng ovine FSH/ml, followed by the labelling with ^{35}S-methionine (see below).

Labelling of secreted Sertoli cell proteins with ^{35}S-methionine. Media were replaced with methionine-free MEM for 30 min. Subsequently, cells were cultured for a period of 60 min in the presence of 100 μCi ^{35}S-methionine ("pulse" medium; S.A. 37 TBq/mmol, Amersham, Amersham, U.K.), followed by replacement of the medium by MEM containing 100 μM methionine ("chase medium") and further culture of the cells for various periods. FSH was added to all media of cells, which had also been pre-incubated in the presence of the hormone. After the chase period media were collected and centrifuged at 8000 x g for 5 min. Supernatants were stored at -20°C until immunoprecipitation.
Proteins were immunoprecipitated by addition of a rabbit antiserum against the 22 N-terminal amino acids of the α-subunit of 32 kDa bovine inhibin (Grootenhuis et al., 1989) or against a preparation of partially purified bovine follicular fluid inhibin (de Jong et al., 1987). After overnight incubation at 4°C 50 μl of protein A Sepharose suspension (Pharmacia, Uppsala, Sweden) was added, followed by 30 min of continuous rotation of the tubes at 4°C. Tubes were subsequently centrifuged for 20 min at 8000 x g, supernatants were discarded and pellets washed 3 times with 1 ml phosphate buffered saline (PBS; 10 mmol/l, pH 7.0, containing 150 mmol/l NaCl), containing 1 mM EDTA, 0.05 % sodium dodecylsulphate (SDS) and 1% Triton X-100 and twice with 1 ml PBS, 10-fold diluted with distilled water. Pellets were then taken up in 20 μl sample buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), boiled for 3 min and loaded on SDS-gels.

SDS-PAGE. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15%, 1.5 mm thick gels as described by Laemmli (Laemmli, 1970), with or without prior reduction (5 min 100°C, 1% (v/v) β-mercaptoethanol). The gels were either used for Western blotting or were cut in 2 mm slices. Proteins were eluted from the slices overnight at 4°C in Tris-HCl (20 mmol/l; pH 7.5) containing 1% SDS, and methanol precipitated (Grootenhuis et al. 1989). In parallel lanes the molecular weight markers phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa)

(Pharmacia, Sweden) were separated for molecular weight determination.

Western blotting. After separation of the samples on 15% SDS-PAGE, proteins were blotted on nitrocellulose using a transfer apparatus (Biorad-Trans-Blot cell; Biorad, Richmond, CA, U.S.A.) in a Tris (16.5 mmol/l)-glycine (pH 8.3) buffer containing 20% (v/v) methanol overnight at a constant voltage of 50 V. The nitrocellulose was incubated in PBS with 3% bovine serum albumin (BSA; Sigma, St. Louis, MO, U.S.A.) for 1 hour, followed by a 1 hour incubation with a 1:200 dilution of a rabbit antiserum raised against the 22 N-terminal amino acids of the α-subunit of bovine 32 kDa inhibin as described previously (Grootenhuis et al. 1989). The antiserum was diluted in PBS containing 0.1% Tween 20 (PBS/Tween). The blots were subsequently washed 4 times for 10 min with PBS/Tween, incubated for 1 hour with goat anti rabbit horseradish peroxidase (Biorad, Richmond, CA, U.S.A., 1:2000 diluted in PBS/Tween), washed 3 times for 10 min with PBS/Tween and developed. The development reagent used was Tris-HCl buffer (20 mmol/l;pH 7.5) containing 500 mmol/l NaCl, hydrogen peroxide (0.015% v/v) and 0.05% (w/v) 4-chloro-1-naphthol. The specificity of the Western blot procedure was confirmed by the absence of bands after incubation with pre-immune rabbit serum (results not shown).

Bioassay of inhibin activity. Inhibin bioactivity was determined using an in vitro bioassay detecting the suppression of spontaneous FSH release from cultured rat pituitary cells. A bovine follicular fluid preparation with an arbitrary potency of 1 U/μg protein was used as a standard (Grootenhuis et al. 1989). The International Research Standard of Inhibin (86/690) has a relative specific activity of 60 ± 10 U/μg (mean ± s.e.m., n=5) when expressed in units of this bFF standard.

Inhibin radioimmunoassay. Inhibin immunoactivity was determined with the same antiserum as used for the Western blots, and the bFF inhibin standard as in the bioassay (Grootenhuis et al. 1989).

Autoradiography. After SDS-PAGE of ^{35}S-labelled proteins, gels were fixed in water-acetic acid-methanol (3-2-5 v/v/v), stained with Coomassie brilliant blue, destained, incubated for 30 min in Amplify (Amersham) and dried on a Biorad gel dryer. Subsequently, the gel was autoradiographed at -80°C, using Hyperfilm MP (Amersham) for periods up to 5 weeks.

RESULTS

Immunoprecipitation of ^{35}S-labelled proteins in FrSCCM with the antiserum against the N-terminal 22 amino acids of the α-subunit of bovine 32 kDa inhibin and with the antiserum against native bovine inhibin after 24 h of chasing yielded essentially similar results: FSH-stimulated Sertoli cells secrete a newly synthesized inhibin-related protein with an apparent molecular weight of 29 kDa. Furthermore, the media contained a precipitated band at 90 kDa, and some higher molecular weight material (Fig.1). The 90 kDa band was also found if the immunoprecipitation was performed with pre-immuneserum, and is probably due to the presence of transferrin, one of the most

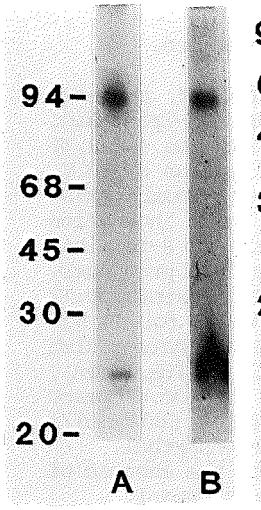

Figure 1.

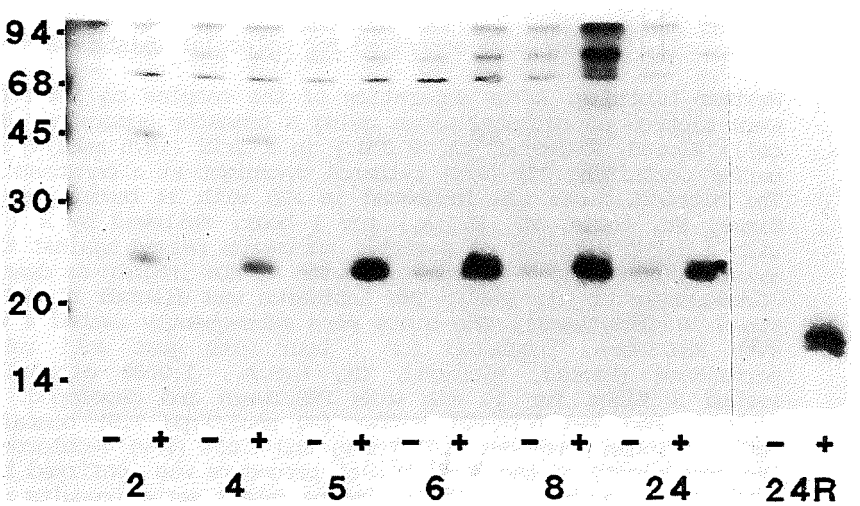

Figure 2.

Figure 1. Autoradiogram of ^{35}S-methione-containing proteins, secreted by FSH-stimulated Sertoli cells, which were immunoprecipitated using an antiserum against the N-terminal 22 aminoacids of the α-subunit of bovine 32 kDa inhibin (A) or against native bovine inhibin (B).
Figure 2. Autoradiogram of ^{35}S-methionine-containing proteins, immunoprecipitated from medium of control (-) and FSH-stimulated (+) Sertoli cells, collected at various times (h) after the start of the "chase"-period (see Materials and Methods for details). The last two lanes contained reduced (R) proteins, obtained after mercaptoethanol treatment of the immunoprecipitate, obtained after 24 h of chase.

abundant proteins present in Sertoli cell secretions.

In further experiments, the anti-peptide antiserum was used for immunoprecipitation. Results of a time-course experiment in the absence or presence of FSH are shown in Fig.2. Immuno-precipitation after 2 and 4 h of chase showed the appearance of the 29 kD protein in the medium of FSH-stimulated cells, which was stable between 5 and 24 h. In contrast, the small amount of immunoprecipitated 32 kDa protein, presumably inhibin, was not affected by FSH. Reduction of the immunoprecipitated proteins yieded a 18 kDa protein presumably the inhibin α-subunit (Fig.2. last two lanes).

In order to obtain sufficient material for determination of immuno- and bioactivity, experiments were performed with pooled CrSCCM and FrSCCM, collected over a 2 weeks period. In the concentrated CrSCCM both bioactive and immunoactive inhibin was determined, resulting in a B/I ratio of 3.4 ± 0.2 (mean ± s.e.m.

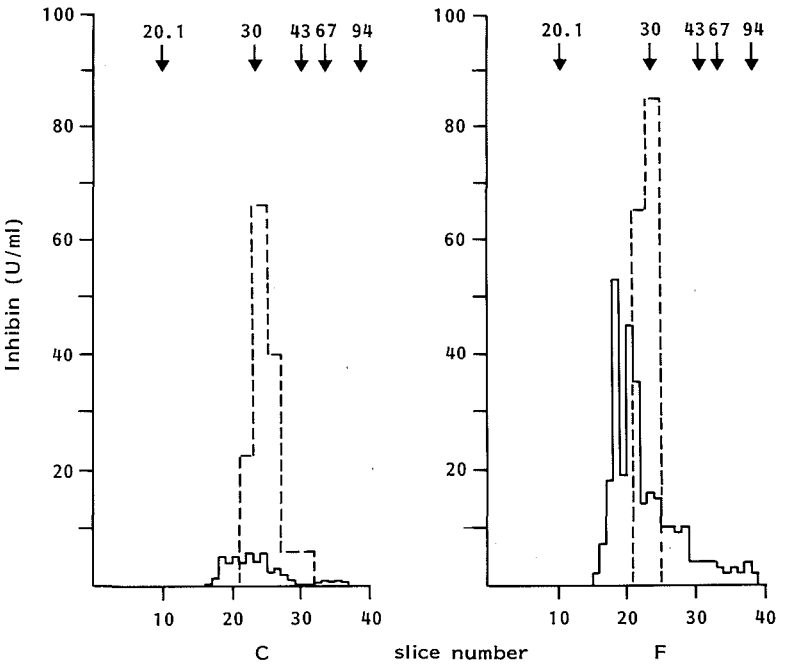

Figure 3. SDS-PAGE of control (C) and FSH (F) stimulated rat Sertoli cell conditioned medium. Eluted proteins from gel slices were analyzed for inhibin bioactivity (-----) and inhibin α-subunit immunoactivity (———) using an antiserum against the 22 N-terminal amino acids of the α-subunit of 32 kDa bovine inhibin. Positions of molecular weight markers (mwm) have been indicated.

of 4 determinations). For the determination of the molecular weight of bioactive and immunoactive inhibin concentrated media from the first 2 week pool of CrSCCM and FrSCCM were separated by SDS-PAGE. After slicing of the gel, a bioactive inhibin with an apparent molecular weight of 32 kDa was found in CrSCCM (Fig.3). Inhibin α-subunit immunoreactivity was found between 25 and 35 kDa (total amount was 48 U/ml SCCM) and around 32 kDa 303 U/ml SCCM bioactive inhibin was detected. After the same procedure, 300 U/ml SCCM of 32 kDa bioactive inhibin was found in FrSCCM. The inhibin α-subunit immunoreactivity was increased in FrSCCM up to 294 U/ml SCCM, with a peak at 28-29 kDa (Fig.3). Estimation of immunoreactive FSH in the eluates of the gel slices indicated that it was present at an apparent molecular weight of 38 kDa, indicating that the added FSH cannot interfere in the inhibin bioassay. Essentially the same results were found with the second pool of CrSCCM and FrSCCM and with a pool of SCCM

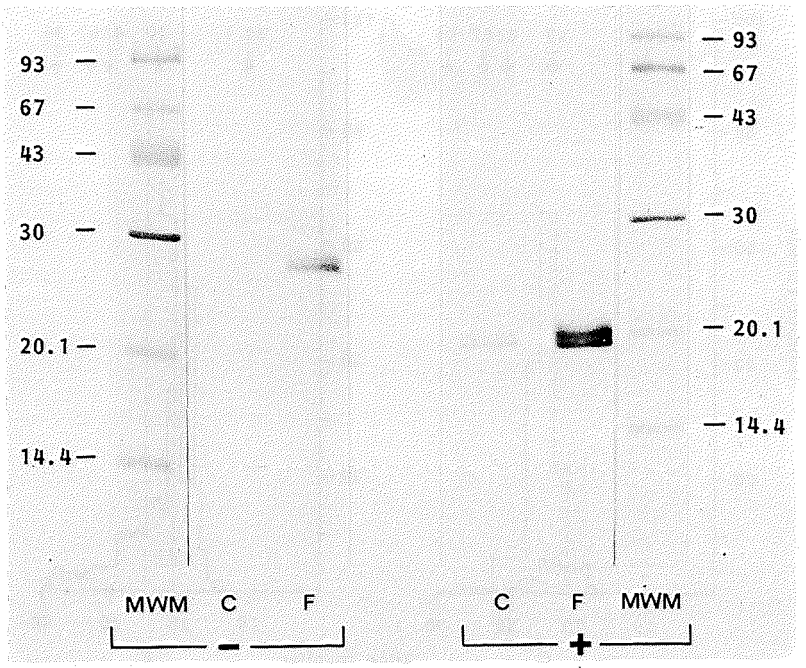

Figure 4. Detection of inhibin α-subunits on Western blots with (+) or without (-) prior reduction with β-mercaptoethanol after separation of control (C) and FSH (F) stimulated rat Sertoli cell conditioned medium by 15% SDS-PAGE using an antiserum against the 22 N-terminal amino acids of the α-subunit of 32 kDa bovine inhibin. Positions of molecular weight markers (mwm) have been indicated.

stimulated with both FSH and testosterone (results not shown). The proteins of the slices containing 28-29 kDa inhibin α-subunit immunoreactivity did not affect basal and inhibin reduced FSH release from cultured pituitary cells (data not shown).

Results of Western blots of the concentrated media are shown in Fig.4. Two dominant immunoreactive bands of 33.6 ± 1.4 kDa (mean ± s.e.m., n=3 independent blots) and 28 ± 1.7 kDa (mean ± s.e.m., n=3) were found in CrSCCM. In FrSCCM the 33.6 kDa band had the same intensity as in CrSCCM but the two smaller bands (29.6 ± 0.7 kDa (mean ± s.e.m., n=2) and 28.4 ± 0.9 kDa (mean ± s.e.m., n=2) were much more prominent than in CrSCCM. After reduction of both CrSCCM and FrSCCM two prominent bands at 20.5 ± 0.7 kDa (mean ± s.e.m., n=2) and 19.5 ± 0.8 kDa (mean ± s.e.m., n=3) and a faint 40 kDa band were found.

DISCUSSION

The results of three different methods, i.e. immunoprecipitation, Western blotting, and separation of proteins by SDS-PAGE, followed by immunoassay or bioassay of inhibin, all indicate that two proteins with inhibin-like immunoactivity are present in the medium of cultured Sertoli cells from the testes of immature rats. One of these proteins is found at an apparent molecular weight of 32 kDa, and has both inhibin-bioactivity and immunoactivity. This confirms earlier data of Grootenhuis et al. (1989, 1990) on the characterization of the molecular weight of inhibin in rat testis homogenates and CrSCCM, and of Risbridger et al. (1989), who showed the presence of immunoactive 30 kDa inhibin in a HPLC fraction of culture medium of seminiferous tubules, which also contained inhibin bioactivity. The other protein is located at 29 kDa, interacts with antisera against the 1-22 amino acids of the α-subunit of 32 kDa bovine inhibin and against a preparation of native bovine follicular fluid inhibin, and has no inhibin bioactivity after elution from SDS-PAGE. This immunoreactivity was also observed very recently by Risbridger et al. (1989), who did not estimate bioactivity of inhibin in the eluates obtained after SDS-PAGE. However, these authors found inhibin bioactivity in the HPLC fractions which contained the 29 kDa protein, and ascribed the immuno- and bioactivity to the same molecule.

Addition of FSH to the culture medium of Sertoli cells caused a large increase of the immunoactivity in the medium, collected after 24 h and in pooled media collected during a 2 week period. This observation confirms earlier data of Ying et al. (1987), Bicsak et al. (1987) and Morris, Vale, Cappel et al. (1988), who also used anti-peptide antisera, and of Toebosch et al. (1989) and Risbridger et al. (1989), who used antisera against native bovine inhibin. After SDS-PAGE fractionation of the proteins secreted by the stimulated Sertoli cells, increased secretion of a protein with an apparent molecular weight of 29 kDa was observed, whereas no clear increase of the 32 kDa protein was found (Fig. 3 and 4). Comparable results were obtained after 24 h (data not shown) and in the medium collected at 2-3 days intervals during 2 weeks, whereas the pulse-chase experiment also

provided similar results. Estimation of the bioactivity and immunoactivity was only performed in the SDS-PAGE fractions of the media collected during 2 weeks, because only in these media sufficient material was present to yield reliable results. Again, the peak of the bioactivity was detected in the 32 kDa slice of the gel, and the total amount of inhibin bioactivity was not different from that in the slices of the gel on which CrSCCM was electrophorized. This observation confirms earlier data of Ulteevan Gessel et al. (1986), who did not observe increased inhibin bioactivity in the medium of FSH-stimulated Sertoli cells cultured at 37°C, but contrasts with the results of Le Gac and de Kretser (1982), Toebosch et al. (1988) and Handelman et al. (1989), who did find increased inhibin bioactivity after culturing Sertoli cells with FSH at 37°C. Risbridger et al. (1989) also observed increased release of inhibin bioactivity after addition of FSH to seminiferous tubule fractions in culture at 34°C. All of these authors observed decreasing bio- over immunoactivity ratios after addition of FSH, indicating that immunoactivity increased to a larger extent than bioactivity.

The reason for the discrepancy between the present results - no increase of bioactivity after FSH - and the above mentioned data remains unclear. Differences in culture temperature and cell purity may have played a role. Another possibility is that the inhibin bioassay, based on the suppression of intracellular FSH, as used by most of the above-mentioned authors, leads to results which differ from those based on suppression of the release of FSH, as used in this study. Finally, the bioactivity of the 29 kDa protein may have been lost during the procedure of electrophoresis, elution from the gel slices and methanol precipitation. However, this is not very likely, because the overall recovery of inhibin-bioactivity after this procedure amounted to 60% of that in the material applied to the gel.

The molecular composition of the FSH-induced 29 kDa α-subunit immunoreactivity is not yet known. The presence of a 20 kDa inhibin α-subunit immunoreactive band on Western blots after reduction indicates that it may consist of the inhibin α-subunit linked to another peptide with disulphide bond(s). It is possible that the FSH induced 29 kDa inhibin α-subunit is the same

molecule as described by Sugino et al. (1989) who purified a 26 kDa inhibin α-subunit from porcine follicular fluid, which is composed of fragment 18-60 of the inhibin α-subunit precursor linked by disulphide bond(s) to fragment 227-360.

The increase of the amount of the 29 kDa immunoreactive material agrees with the observation that after FSH stimulation of immature rat Sertoli cells only the mRNA for the inhibin α-subunit is increased, but not for the β-B subunit (Toebosch et al. 1988, Klaij et al. 1990).

It is concluded that results obtained with radioimmunoassays detecting α-subunits of inhibin do not necessarily reflect the amount of bioactive inhibin. The biological significance of the FSH-stimulated production of the 29 kDa inhibin α-subunit complex is as yet unclear.

REFERENCES

Bicsak, T.A., Vale, W., Vaughan, J., Tucker, E.M., Cappel, S. and Hsueh, A.J.W. (1987). Mol. and Cell. Endocrinol. 49, 211-217.

de Jong, F.H., Grootenhuis, A.J., Sander, H.J., Steenbergen, J., Timmerman, M.A. and van Dijk, S. (1987). In Inhibin-non-steroidal regulation of follicle stimulating hormone secretion. (Burger, H.G., de Kretser, D.M., Findlay, J.K. and Igarashi, M., eds.) pp. 35-46, Raven Press, New York.

de Jong, F.H. (1988). Physiol. Reviews 68, 555-607.

de Kretser, D.M. and Robertson, D.M. (1989). Biol. Reprod. 40, 33-47.

Grootenhuis, A.J., Steenbergen, J., Timmerman, M.A., Dorsman, A.N.R.D., Schaaper, W.M., Meloen, R.H. and de Jong, F.H. (1989). J. Endocrinol. 121, 293-301.

Grootenhuis, A.J., Timmerman, M.A., Hordijk, P.L. and de Jong, F.H. (1990). Mol. Cell. Endocrinol., in press.

Handelsman, D.J., Spaliviero, J.A., Kiston, E. and Robertson, D.M. (1989). Endocrinology 125, 721-729.

Klaij, I.A., Toebosch, A.M.W., Themmen, A.P.N., Shimasaki, S., de Jong, F.H. and Grootegoed, J.A. (1990). Mol. Cell. Endocrinol. 68, 45-52.

Knight, P.G., Beard, A.J., Wrathall, J.H.M. and Castillo, R.J. (1989). J. Mol. Endocrinol. 2, 189-200.

Laemmli, U.K. (1970). Nature 227, 680-685.

Le Gac, F. and de Kretser, D.M. (1982). Mol. Cell. Endocrinol. 28, 487-498.

Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. and Guillemin, R. (1986). Nature 321, 779-782.

McLachlan, R.I., Robertson, D.M., Burger, H.G. and de Kretser, D.M. (1986). Mol. Cell. Endocrinol. 46, 175-185.

Morris, P.L., Vale, W.W., Cappel, S. and Bardin, C.W. (1988). Endocrinology 122, 717-725.

Risbridger, G.P., Hancock, A., Robertson, D.M., Hodgson, Y. and de Kretser, D.M. (1989). Mol. Cell. Endocrinol. 67, 1-9.

Robertson, D.M., Tsonis, C.G., McLachlan, R.I., Handelsman, R.J., Leask, R., Baird, D.T., McNeilly, A.S., Hayward, S., Healy, D.L., Findlay, J.K., Burger, H.G. and de Kretser, D.M. (1988). J. Clin. Endocrinol. Metab. 67, 438-443.

Robertson, D.M., Giacometti, M., Foulds, L.M., Lahnstein, J., Goss, N.H., Hearn, M.T.W. and de Kretser D.M. (1989). Endocrinology 125, 1-9.

Sugino, K., Nakamura, T., Takio, K., Titani, K., Miyamoto, K., Hasegawa, Y., Igarashi, M. and Sugino, H. (1989). Biochem. Biophys. Res. Commun. 159, 1323-1329.

Toebosch, A.M.W., Robertson, D.M., Trapman, J., Klaassen, P., de Paus, R.A., de Jong, F.H. and Grootegoed, J.A. (1988). Mol. Cell. Endocrinol. 55, 101-105.

Toebosch, A.M.W., Robertson, D.M., Klaij, I.A., de Jong, F.H. and Grootegoed, J.A. (1989). J. Endocrinol. 122, 757-762.

Ultee-van Gessel, A.M., Leemborg, F.G., de Jong, F.H. and van der Molen H.J. (1986). J. Endocrinol. 109, 411-418.

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D. and Spiess, J. (1986). Nature 321, 776-779.

Ying, S.-Y. (1988). Front. Neuroendocrinol. 10, 167-184.

Ying, S.-Y., Czvik, J., Becker, A., Ling, N., Ueno, N. and Guillemin, R. (1987). Proc. Nat. Acad. Sci. USA 84, 4631-4635.

CHAPTER 5

SHORT-TERM STIMULATORY EFFECT OF SERTOLI CELL CONDITIONED MEDIUM ON LEYDIG CELL STEROIDOGENESIS IS NOT MEDIATED BY INHIBIN

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SUMMARY. Addition of concentrated rat Sertoli cell conditioned medium (rSCCM) to isolated Leydig cells from immature rats stimulated steroid production more than 13-fold within 4 h. LH-stimulated steroidogenesis was not enhanced by addition of rSCCM. The biological activity of the concentrated rSCCM was higher after incubation of Sertoli cells with FSH, whereas FSH alone did not stimulate steroid production. This effect of rSCCM was not due to inhibin, since highly purified 32 kDa rat inhibin in doses, equivalent to those present in rSCCM, had no effect on steroidogenesis during the 4 h incubation period. Furthermore, inhibin could be separated from the Leydig cell stimulating factor by anion-exchange chromatography. These results indicate a short-term paracrine control of Leydig cell steroidogenesis by Sertoli cell derived factors, which differ from inhibin.

INTRODUCTION

Luteinizing hormone (LH) is the main regulator of Leydig cell steroidogenesis. However, there is abundant evidence, both from in vivo and in vitro experiments, that locally produced factors can modulate Leydig cell activity and that these factors are produced by cells in the seminiferous tubuli and by Sertoli cells in particular [1,2]. In hypophysectomized immature rats, in vivo administration of follicle-stimulating hormone (FSH) can induce Leydig cell hypertrophy and stimulate steroidogenic activity [3-5]. It is likely that this effect is mediated by Sertoli cells, since these are the only testicular cells containing FSH receptors [6]. Seminiferous tubule damage induced by different procedures (antifertility compounds [7], hydroxyurea, fetal irradiation [8] or cryptorchidism [9]) causes hypertrophy of Leydig cells. Factors which can stimulate or inhibit basal and/or LH stimulated Leydig cell steroidogenesis in vitro have been shown to be present in Sertoli cell conditioned medium (SCCM) [10-13], in medium from cultured seminiferous tubules [13,14] and

in testicular interstitial fluid [15,16]. Most of these authors reported relatively small effects after long-term incubations (up to 48 h) of Leydig cells with the added materials.

There is only little information on the molecular composition of the Leydig cell modulating factor(s) secreted by Sertoli cells. It has been shown that the active compound from rat interstitial fluid is likely to be rat serum albumin [16,17]. Possible Leydig cell modulating factors in SCCM are inhibin and activin, gonadal hormones composed of two subunits ($\alpha\beta$ and $\beta\beta$ respectively), which have been purified on basis of their suppressive and stimulatory effects on FSH release from cultured rat pituitary cells, respectively [18]. It has been shown that Sertoli cells secrete inhibin [19-21], and a 32 kDa $\alpha\beta$-B inhibin dimer has been purified from rat SCCM [22]. Inhibin has been reported to stimulate basal and human chorionic gonadotrophin (hCG)-induced testosterone production by cultured immature and neonatal rat Leydig cells respectively in long-term cultures; activin can counteract these effects [23,24].

The purpose of the present study was to investigate whether inhibin is also one of the components in SCCM which can influence Leydig cell steroid production during short-term incubations.

MATERIALS AND METHODS

Sertoli cell conditioned medium. Sertoli cells were isolated from the testes of 21-day-old Wistar rats and were cultured for 1 day in plastic 150 cm^2 flasks (Costar, Cambridge, MA, U.S.A.) in Eagle's minimal essential medium (MEM; Gibco, Grand Island, NY, USA) containing 1% (v/v) fetal calf serum (FCS, Gibco) as described [22]. On the following day the attached Sertoli cells were washed and cultured further in 20 ml MEM without FCS or other additions in the presence or absence of human FSH (hFSH; 25 mU/ml, Metrodin, Serono, Geneva, Switzerland). According to the manifacturer, this preparation contains less than 1 U of LH per 75 U of FSH. Media were renewed after 3 or 4 days periods and cultures were terminated after 4-5 weeks. Collected control and hFSH-stimulated rat SCCM (CrSCCM and FrSCCM) were stored at -20°C. After thawing media were pooled, concentrated 50-fold by diafiltration, exchanged with 5 ml MEM using disposable cartridges with YM10 membranes (molecular weight cut-off at 10 kDa; Amicon, Lexington, MA, USA) and added to the incubation medium of Leydig cells (see below). Protein concentrations in the 50-fold concentrated CrSCCM and FrSCCM, were 1.42 and 2.18 mg/ml, respectively, as measured using the protein assay kit purchased from Biorad (München, F.R.G.). As a preliminary characterization of the Leydig cell stimulating activity CrSCCM was chromatographed on a Mono Q anion exchange chromatography column (Pharmacia, Uppsala, Sweden), using the buffers described previously [22].

Bioactive and immunoactive inhibin were measured in CrSCCM [25], using a bovine follicular fluid (bFF) preparation with an arbitrary potency of 1 U/μg

protein as a standard. One unit of this standard reduces FSH release from rat pituitary cells in vitro to 57.6 ± 2.5% (mean ± S.D.; n=10) of the control secretion. The International Research Standard for Inhibin (86/690) has a relative specific activity of 60 ± 10 U/μg (mean ± s.e.m., n=5) expressed in units of this bFF standard [22]. Inhibin with a molecular weight of 32 kDa was purified from a pool of CrSCCM by Amicon YM10 diafiltration, Mono Q anion-exchange, c1/c8 reversed phase chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis as described previously [22]. The relative specific activity of this material was 16.4×10^6 U/mg protein [22].

Leydig cell incubations. Leydig cells were isolated from testes obtained from 21-22-day-old Wistar rats as described [26]. The Leydig cells (0.5×10^5 per well) attached to the surface of 96-well plates (Costar) during a 1 h incubation in MEM with 0.1% bovine serum albumin (Sigma, St. Louis, MO, U.S.A.). After washing, the cells were incubated in a total volume of 200 μl medium without BSA, in the presence of inhibitors of pregnenolone (3β-hydroxy-5-pregnen-20-one) metabolism (5 μM cyanoketone and 20 μM SU-10603) for 4 h at 32°C in 5% CO_2/air [27]. Various amounts (0.4-40 μl) of 50-fold concentrated CrSSCM and 32 kDa inhibin were added in the absence or presence of ovine LH (final concentration 20 ng/200 μl; NIH-LH-S-18). Pregnenolone in culture media was measured by radioimmunoassay [27].

RESULTS

During the 4 h incubation, pregnenolone production by incubated immature Leydig cells was stimulated in a dose dependent fashion after addition of CrSCCM (Fig.1). Various amounts of highly purified 32 kDa inhibin had no effect. Since 1 U of purified 32 kDa inhibin reduces pituitary FSH release by 57.6%, the amount of inhibin added to the Leydig cells with the highest dose (400 U/well) is rather large. In the presence of a maximally stimulating dose of LH, CrSCCM (Fig. 1) or 32 kDa inhibin had no further effect (not shown). With three different Sertoli and Leydig cell preparations the stimulation of steroid production with 20 μl of 50-fold concentrated CrSCCM was 21 ± 5.8 fold (mean ± s.e.m.).

Subsequently, the effect of hFSH on the release of the Leydig cell stimulating activity from Sertoli cells was studied. CrSCCM from the first collection period (day 1-3 after attachment of Sertoli cells) stimulated basal Leydig cell steroid production in a dose-dependent manner (Fig. 2). Media from hFSH-stimulated Sertoli cells (FrSCCM) were 1.5-3 times more potent than CrSCCM. Human FSH itself, in a concentration present in the highest dose of FrSCCM had no significant effect on Leydig cell steroid production (Student's t-test; Fig. 2).

The secretion of the Leydig cell stimulating factor and the

effect of FSH on its secretion diminished with increasing culture period of the Sertoli cells. CrSCCM collected during the first two weeks (4 collections) stimulated basal steroid production and FSH enhanced the biological activity. Thereafter, no effects of FSH on the steroid stimulatory activity was found. In contrast immunoreactive inhibin-like activity was still produced; its production could still be stimulated by addition of FSH (Table 1).

Finally, the fractions of CrSCCM, obtained after chromatography on a Mono Q anion-exchange column, were tested for Leydig cell stimulating activity. The bulk of the activity was eluted from the column in fractions 4, 5 and 6 at NaCl concentrations between 25 and 75 mmol/L, whereas the inhibin bioactivity was eluted in fractions 8 to 11, at a salt concentration between 100 and 180 mmol/L.

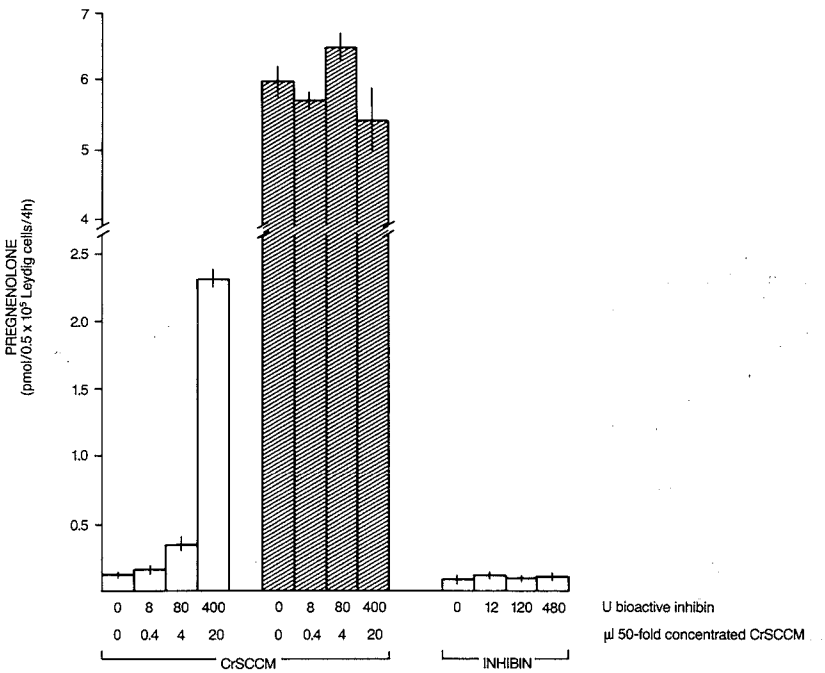

Figure 1. Effects of increasing doses of control rat Sertoli cell conditioned medium (CrSCCM) or highly purified 32 kDa inhibin (inhibin) on pregnenolone production in 4 h by immature Leydig cells incubated without (open bars) or with LH (hatched bars). Sertoli cell media were 50-fold concentrated and exchanged, and 0.4-20 µl of concentrated CrSCCM was added per 200 µl. Values are means ± s.e.m. from triplicate incubations.

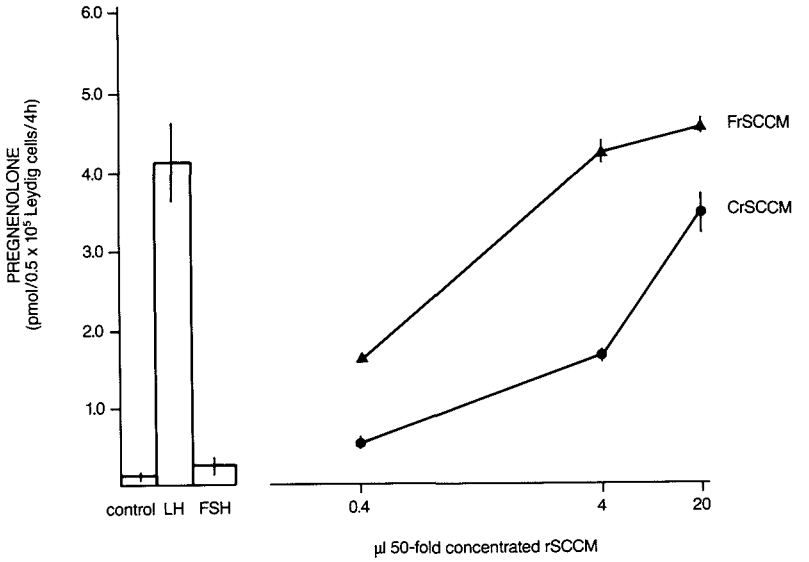

Figure 2. Effects of a 4 hour incubation with increasing concentrations of control and FSH (25 mU/ml) stimulated rat Sertoli cell conditioned medium (CrSCCM and FrSCCM respectively) collected during the first 3 days of culture on pregnenolone production by immature Leydig cells. The effects of 100 ng/ml LH and the highest dose of FSH (125 mU/ml) on pregnenolone production are also shown. Values are means ± s.e.m. from triplicate incubations.

Table 1. Time dependent and FSH-induced secretion of Leydig cell steroidogenesis stimulating factor and inhibin-like immuno-reactivity by cultured immature rat Sertoli cells. Media from Sertoli cells cultured in the absence (CrSCCM) or presence of FSH (FrSCCM) were collected during a period of $3\frac{1}{2}$ weeks (7 half-weekly collections), concentrated 50-fold and tested in a dose of 20 µl per 200 µl medium for their effects on basal immature Leydig cell pregnenolone production. Results are expressed as stimulation factor, compared with basal production. In these concentrated media the amount of inhibin-like immunoreactivity was also determined (expressed as % of CrSCCM (period 1); 100% = 60 U/ml unconcentrated media).

Collection period	Stimulation factor of Leydig cell steroidogenesis after addition of		Relative amount of inhibin-like immuno-activity present in	
	CrSCCM	FrSCCM	CrSCCM	FrSCCM
1	16.2	20.6	100%	156%
2	7.0	18.9	99%	225%
3	6.4	28.8	87%	280%
4	12.1	19.5	82%	144%
5	2.4	2.8	41%	136%
6	1.8	1.9	24%	243%
7	1.7	2.0	37%	195%

DISCUSSION

The results of the present study show that immature rat Sertoli cells in culture secrete a factor or factors stimulating basal steroidogenesis in Leydig cells within 4 h. The amount of this Leydig cell steroidogenesis stimulating activity in the medium of cultured Sertoli cells is increased after incubation of Sertoli cells with FSH. The stimulation of Leydig cell steroidogenesis is not due to the presence of inhibin in the medium.

In most of the earlier reports on Leydig cell stimulating factors produced by testicular cells, Leydig cells were incubated for periods of 5 to 48 h with spent media of tubuli or Sertoli cells and rather small effects were observed. In these studies Leydig cell steroid production may have been influenced by exhaustion of nutrients in the culture media. To avoid these problems, rSCCM was concentrated and exchanged in the present study. The maximal effect of rSCCM on Leydig cell steroidogenesis was much larger than reported by others [10,11,13] and comparable with the response to a maximally active dose of LH. We have not observed any additional effect of rSCCM on LH-stimulated steroid production of immature Leydig cells.

Although the medium was derived from cultured Sertoli cells, the origin of the stimulatory activity has not yet been determined unequivocally. The Sertoli cell preparations were not completely pure, as peritubular and germinal cells were also present. The fact that FSH can stimulate the secretion of this factor, however, is a strong indication that the factor is a Sertoli cell product, since only Sertoli cells have FSH receptors. These characteristics indicate that this factor may be a mediator of the effects of FSH on Leydig cells in vivo.

The nature of the biologically active factor(s) is not known, although it has been shown that Leydig cell stimulating activity is heat sensitive, and associated with a compound with a molecular mass larger than 10 kDa [10,13]. We also found that the Leydig cell stimulating activity is retained by a membrane with a molecular weight cut off at 10 kDa; the activity could be separated from inhibin by anion exchange chromatography. Inhibin has been reported to increase hCG-induced steroid production from neonatal rat Leydig cells [23], to stimulate basal but not hCG-

induced steroid production from immature rat Leydig cells [24] and to have no effect on basal or hCG-stimulated steroid production from mature rat Leydig cells [28]. On basis of these reports and the present observations we postulate that inhibin may act as a trophic regulator of Leydig cell function and is not involved in short term regulation of steroid production.

The physiological importance and identity of these factors have not yet been established. However, these results suggest short-term paracrine control of Leydig cell steroidogenesis by Sertoli cell derived factors.

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REFERENCES

1. Risbridger G.P. and de Kretser D.M.: Paracrine regulation of the testis. In The Testis (Edited by H. Burger and D. de Kretser). Raven Press, New York, (1989), pp. 255-268.
2. Sharpe R.M.: Intratesticular factors controlling testicular function. Biol. Reprod. **30** (1984) 29-49.
3. Odell W.D. and Swerdloff R.S.: Etiologies of sexual maturation: a model system based on sexually maturing rat. Rec. Prog. Horm. Res. **32** (1976) 245-288.
4. Van Beurden W.M.O., Roodnat B., de Jong F.H., Mulder E. and Van der Molen H.J.: Hormonal regulation of LH stimulation of testosterone production in isolated Leydig cells of immature rats: the effect of hypophysectomy, FSH and estradiol-17β. Steroids **28** (1976) 847-865.
5. Teerds K.J., Closset J., Rommerts F.F.G., de Rooij D.G., Stocco D.M., Colenbrander B., Wensing C.J.G. and Hennen G.: Effects of pure FSH and LH preparations on the number and function of Leydig cells in immature hypophysectomized rats. J. Endocrinol. **120** (1989) 97-106.
6. Thanki K.H. and Steinberger A.: Effect of age and hypophysectomy on FSH binding by rat testes. Andrologia **10** (1978) 195-202.
7. Aoki A. and Fawcett D.W.: Is there a local feedback from the seminiferous tubules affecting activity of the Leydig cells? Biol. Reprod. **19** (1978) 144-158.
8. Rich K.A., Kerr J.B. and de Kretser D.M.: Evidence for Leydig cell dysfunction in rats with seminiferous tubule damage. Mol. Cell. Endocrinol. **13** (1979) 123-135.
9. Risbridger G.P., Kerr J.B. and de Kretser D.M.: Evaluation of Leydig cell function and gonadotropin binding in unilateral and bilateral cryptorchidism: evidence for local control of Leydig cell function by the seminiferous tubule. Biol. Reprod. **24** (1981) 534-540.
10. Verhoeven G. and Cailleau J.: A factor in spent media from Sertoli cell-enriched cultures that stimulates steroidogenesis in Leydig cells. Mol. Cell. Endocrinol. **40**

(1985) 57-68.
11. Benahmed M., Grenot C., Tabone E., Sanchez P. and Morera A.M.: FSH regulates cultured Leydig cell function via Sertoli cell proteins: an in vitro study. Biochem. Biophys. Res. Commun. **132** (1985) 729-734.
12. Benahmed M., Morera A.M. and Chauvin M.A.: Evidence for a Sertoli cell, FSH-suppressible inhibiting factor(s) of testicular steroidogenic activity. Biochem. Biophys. Res. Commun. **139** (1986) 169-178.
13. Papadopoulos V., Kamtchouing P., Drosdowsky M.A., Hochereau de Reviers M.T. and Carreau S.: Adult rat Sertoli cells secrete a factor or factors which modulate Leydig cell function. J. Endocrinol. **114** (1987) 459-467.
14. Syed V., Khan S.A. and Ritzen E.M.: Stage-specific inhibition of interstitial cell testosterone secretion by rat seminiferous tubules in vitro. Mol. Cell. Endocrinol. **40** (1985) 257-264.
15. Sharpe R.M. and Cooper I.: Intratesticular secretion of a factor(s) with major stimulatory effects on Leydig cell testosterone secretion in vitro. Mol. Cell. Endocrinol. 37 (1984) 159-168.
16. Melsert R., Hoogerbrugge J.W. and Rommerts F.F.G.: The albumin fraction of rat testicular fluid stimulates steroid production by isolated Leydig cells. Mol. Cell. Endocrinol. **59** (1988) 221-231.
17. Melsert R., Hoogerbrugge J.W. and Rommerts, F.F.G.: Albumin, a biologically active protein acting on Leydig cells. Mol. Cell. Endocrinol. **64** (1989) 35-44.
18. De Jong F.H.: Inhibin. Physiol.Rev. **68** (1988) 555-607.
19. Steinberger A. and Steinberger E.: Secretion of an FSH-inhibiting factor by cultured Sertoli cells. Endocrinology 99 (1976) 918-921.
20. Ultee-van Gessel A.M., Leemborg F.G., de Jong F.H. and van der Molen H.J.: In-vitro secretion of inhibin-like activity by Sertoli cells from normal and prenatally irradiated immature rats. J. Endocr. **109** (1986) 411-418.
21. Bicsak T.A., Vale W., Vaughan J., Tucker E.M., Cappel S. and Hsueh A.J.W.: Hormonal regulation of inhibin production by cultured Sertoli cells. Mol. Cell. Endocrinol. 49 (1987) 211-217.
22. Grootenhuis A.J., Timmerman M.A., Hordijk P.L. and de Jong F.H.: Inhibin in immature rat Sertoli cell conditioned medium: a 32 kDa $\alpha\beta$-B dimer. Mol. Cell. Endocrinol. (1990) in press.
23. Hsueh A.J.W., Dahl K.D., Vaughan J., Tucker E., Rivier J., Bardin C.W. and Vale W.: Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. Proc. Natl. Acad. Sci. U.S.A. **84** (1987) 5082-5086.
24. Morris P.L., Vale W.W., Vaughan J. and Bardin W.: Introgonadal modulators of immature Leydig cell function: inhibin-related peptides. In Programme and abstracts of 10th Annual Testis Workshop Baltimore, M.D., U.S.A. (1988) poster 33.
25. Grootenhuis A.J., Steenbergen J., Timmerman M.A., Dorsman A.N.R.D., Schaaper W.M.M., Meloen R.H. and de Jong F.H.: Inhibin and activin-like activity in fluids from male and

female gonads: different molecular weight forms and bioactivity/immunoactivity ratios. J. Endocrinol. **122** (1989) 293-301.
26. Rommerts F.F.G., Molenaar R. and Van der Molen H.J.: Preparation of isolated Leydig cells. Methods Enzymol. **109** (1985) 275-288.
27. van der Vusse G.J., Kalkman M.L. and van der Molen H.J.: 3β-Hydroxy steroid dehydrogenase in rat testis tissue. Inter- an subcellular localization and inhibition of cyanoketone and nagarse. Biochim. Biophys. Acta **348** (1974) 404-414.
28. Lin T., Harrington Calkins J., Morris P.L., Vale W. and Bardin C.W.: Regulation of Leydig cell function in primary culture by inhibin and activin. Endocrinology **125** (1989) 2134-2140.

CHAPTER 6

INHIBIN, GONADOTROPHINS AND SEX STEROIDS IN DOGS WITH SERTOLI CELL TUMORS

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INHIBIN, GONADOTROPHINS AND SEX STEROIDS IN DOGS WITH SERTOLI CELL TUMORS

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SUMMARY. Inhibin bioactivity and mRNA for inhibin subunits were measured in 4 dog Sertoli cell tumors and in testes of 5 normal control dogs. The tumors contained increased levels of inhibin ($p<0.05$) and mRNA for the α- and βB-subunits when compared with controls, whereas the mRNA for the βA-subunit was not detected in tumors or control testes. The inhibin bioactivity was associated with a 32 kDa molecule in both Sertoli cell tumors and normal dog testes; no higher molecular weight forms were found after SDS-polyacrylamide gel electrophoresis.

Peripheral levels of immunoassayable inhibin in Sertoli cell tumor dogs were higher than those in the controls ($p=0.01$). These increased inhibin concentrations are likely to be the cause of the suppressed peripheral levels of FSH ($p<0.02$). However, peripheral levels of LH ($p<0.02$) and testosterone ($p<0.01$) were also suppressed in the dogs with Sertoli cell tumors, whereas the concentrations of oestradiol in peripheral plasma of both groups did not differ. Finally, i.v. injection of the LHRH-agonist Buserelin caused a significant increase of LH and testosterone in the control dogs, but not in the dogs with Sertoli cell tumors.

It is concluded that secretory products from the Sertoli cell tumors suppressed pituitary gonadotrophin secretion in dogs with Sertoli cell tumours. It is unlikely that testosterone or oestradiol play a role in this respect. FSH may be suppressed by the high levels of inhibin in tumor bearing dogs, but it remains unclear if inhibin or another Sertoli cell product is responsible for the unresponsiveness of the pituitary gland to LHRH and the suppression of LH.

INTRODUCTION

Testicular tumors are rare and represent about 1% of all cancers in men. The annual incidence of testicular tumors is 6 per 100.000 males. The majority of testicular neoplasms (95%) is derived from the germ cells; the remaining 5% are mainly tumors of the Sertoli cells and the Leydig cells (Matsumoto, 1988).

Although rare, testicular tumors are more common in dogs than in men. They represent 5-15% of all neoplasms in male dogs (Theilen & Madewell, 1979) and have an estimated incidence of 67.8 per 100.000 (Dorn, Taylor, Schneider, Hibbard & Klauber,

1968). Sertoli cell tumors make up one third of all testicular neoplasms in the dog (Dorn et al. 1968). Cryptorchism is an important risk factor: the incidence of Sertoli cell tumors in cryptorchid testes is 23 times higher than that in scrotal testes (Hayes & Pendergrass, 1976).

About 20% of the Sertoli cell tumors in dogs are associated with a feminization syndrome: bilateral symmetric alopecia, gynecomastia, pendulous prepuce, atrophy of the penile sheath and the contralateral testis, and attraction of other male dogs (Lipowitz, Schwartz, Wilson & Ebert, 1973). Blood dyscrasias (anemia, leukopenia and pancytopenia) have been reported in a limited number of cases (Edwards, 1981; Sherding, Wilson & Kociba, 1981; Morgan, 1982).

Feminization and blood dyscrasias are believed to be caused by increased secretion of oestrogens by the tumor, but determinations of estrogen concentrations in peripheral blood of dogs with Sertoli cell tumors have yielded conflicting results. Increased levels of oestradiol were found in 3 dogs with Sertoli cell tumors (Comhaire, Mattheeuws & Vermeulen, 1974), but only one of these dogs showed signs of feminization. In a study of 6 dogs with Sertoli cell tumors, feminization and blood dyscrasias, increased levels of oestrogen were found in only 3 dogs (Morgan, 1982), whereas the remaining 3 dogs had normal levels. A report on 5 dogs with Sertoli cell tumors and feminization describes normal levels of oestrogen in 4 dogs and an increased level in 1 dog, in which the tumor was necrotic (Siegel, Forchielli, Dorfman, Brodey & Prier, 1967).

Another hormone produced by Sertoli cells is inhibin, a glycoprotein hormone involved in regulation of the pituitary-testis axis, which has been defined by its preferential suppressive action on the synthesis and release of FSH (see for review: de Jong, 1988). Recently, it has been shown in women that inhibin can be used as a marker for the detection of primary and recurrent granulosa cell tumors (LappÖhn, Burger, Bouma, Bangah, Krans & De Bruijn, 1989) and for granulosa cell development after ovarian hyperstimulation for <u>in vitro</u> fertilization (McLachlan, Robertson, Healy, de Kretser & Burger, 1986).

The aim of the present study was to determine whether plasma inhibin levels could be a marker of dog Sertoli cell tumors and

to study the consequences of the increased levels of inhibin on pituitary-testicular function. Therefore we determined levels of mRNAs of inhibin subunits and inhibin bioactivity in the tumors, and evaluated the response of LH, FSH, inhibin, oestradiol and testosterone in peripheral blood before and after stimulation with the LH-releasing hormone (LHRH)-agonist buserelin, both in normal dogs and dogs with Sertoli cell tumors.

MATERIALS AND METHODS

Dogs. Testicular tumors were found in 5 cryptorchid dogs of various breeds and ages (Table 1A). All dogs showed signs of feminization; one dog also had thrombopenia (99 x 10^9/l; reference values 150-400x10^9/l). In three dogs both testes were located in the abdomen; one dog had an inguinal testis and a scrotal testis; one dog had only one testis which was located in the abdomen. The other testis of this dog had been removed at an earlier occasion.

Treatment consisted of castration. The testes were examined histopathologically. Sertoli cell tumors were found in all enlarged testes; testicular atrophy was diagnosed in all contralateral testes. One dog also had small fields of seminoma in the atrophied contralateral testis (Table 1A, dog no.5). Five dogs of comparable age without testicular abnormalities or feminization were used as controls (Table 1B).

LHRH test. Buserelin acetate (Receptal^R, Hoechst, Frankfurt, F.R.G.) was given intravenously in a dose of 0.5 µg/kg bodyweight. Bloodsamples were taken shortly before and at 5, 10, 15, 20, 30 and 60 minutes after injection. Blood was collected in tubes with EDTA-di-potassium as an anticoagulant and centrifuged at 5000 g for 10 min. The plasma was pipetted into polystyrene tubes and stored in a refrigerator at -20°C until analysis.

Inhibin-like bioactivity. Testicular homogenates were made from 1 g testis or tumor tissue in 2 ml phosphate-buffered saline (10 mmol/l; pH 7.4, containing 150 mmol/l NaCl) with a 5 ml Teflon-glass homogenizer (Braun, Melsungen, F.R.G.). Homogenates were centrifuged at 1500 g for 10 min.

Inhibin-like bioactivity was determined using an <u>in vitro</u> bioassay detecting the suppression of spontaneous FSH release from cultured rat pituitary cells (Grootenhuis, Steenbergen, Timmerman, Dorsman, Schaaper, Meloen & de Jong, 1989). A bovine follicular fluid (bFF) preparation with an arbitrary potency of 1 U/µg protein was used as standard. The International Research Standard of Inhibin (86/690) has a relative specific activity of 60 ± 10 U/µg (mean ± s.e.m., n=5) when expressed in units of this bFF standard.

The molecular weight form of bioactive inhibin was determined after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of testis homogenates, followed by slicing of the gel in 2 mm slices, and elution and methanol precipitation of the proteins from the slices (Grootenhuis et al. 1989).

Inhibin radioimmunoassay. Plasma inhibin-like immunoactivity was measured using an antiserum against purified 32 K bFF inhibin (#1989) and iodinated 32 K bFF inhibin as tracer, obtained from Dr. D.M. Robertson (Dept, of Anatomy, Monash University, Melbourne, Australia) and described by Robertson, Giacometti, Foulds, Lahnstein, Goss, Hearn & de Kretser (1989). Control and tumor testis homogenates and plasma from a tumor bearing dog were assayed at

multiple dose levels in the inhibin radioimmunoassay (Fig. 1) and in the in vitro bioassay, using the bFF inhibin standard. In both assays, parallel dose-response curves were obtained. The sensitivity of the immunoassay (90% B/Bo) was 0.65 U of this bFF standard. Inhibin could not be detected in plasma of castrated dogs. All plasma inhibin levels were estimated in one assay. The intra-assay coefficient of variation was 17.5%.

RNA isolation and analysis. Total RNA was isolated from Sertoli cell tumors and control testes as described previously (Klaij, Toebosch, Themmen, Shimasaki, de Jong & Grootegoed, 1990). Subsequently, electrophoresis of 40 µg of these RNA samples was performed, together with RNA from bovine and rat granulosa cells as positive controls. After blotting, hybridization was performed with probes for the inhibin subunits and actin as described (Klaij et al. 1990). The optical densities of all bands on the autoradiograms of the blots were determined by scanning using a videodensitometer (Bio-Rad Laboratories, Richmond, CA, USA), as described before (Klaij et al. 1990). Values were corrected for amount of RNA using actin hybridization as a standard.

FSH and LH radioimmunoassay. LH concentrations were determined by a heterologous RIA as described by Nett, Akbar, Phemister, Holst, Reichert & Niswender (1975). The rabbit anti-ovine LH GDN#15, radioiodinated NIAMDD-bLH-4 and canine pituitary standard LER1685-1 were used in this assay. Plasma FSH was determined using a rat FSH-RIA (Welschen, Osman, Dullaart, de Greef, Uilenbroek & de Jong, 1975); results have been expressed in terms of NIADDK-rat FSH-RP-1 standard. Serial dilutions of castrated dog plasma resulted in curves parallel with that for the rat FSH standard after logit-log transformation (data not shown). Inter- and intra-assay coefficients of variation for these assays were 16.0 and 14.1% for LH and 11.5 and 5.1% for FSH.

Testosterone and oestradiol radioimmunoassay. Concentrations of testosterone and oestradiol were estimated in duplicate plasma samples by previously validated RIA methods (Dieleman, Kruip, Fontijne, de Jong & van der Weyden, 1983). The intra- and interassay coefficients of variation were 10 and 14%, and 8 and 9.6%, respectively. Sensitivities were 0.4 and 0.04 nmol/l, respectively.

Statistical analysis. Significances of differences between mean values obtained in the two groups of dogs or at various times after buserelin injection was estimated using unpaired or paired two-tailed Student's t-tests. Because of the abnormal distribution of plasma inhibin concentrations in the tumor bearing dogs, these results were log-transformed before applying Student's t-test. Differences were considered significant when $P<0.05$.

RESULTS

Testicular inhibin

Because of non-successful attempts to bring the tumors into culture, sufficient material for the estimation of biological activity was only left from 4 tumors, and for the estimation of mRNAs from 3 tumors.

The concentration of inhibin-like bioactivity in homogenates of dog Sertoli cell tumors was significantly higher than that

Table 1. Breed, age and clinical diagnoses of Sertoli cell tumor dogs and control animals

	Breed	Age	Clinical diagnosis
A. Sertoli cell tumor dogs			
1.	Crossbred	10 yrs	Feminization, bilateral cryptorchidism, Sertoli cell tumor in abdominal testis
2	Partridge dog	6 yrs	Feminization, monorchid/ cryptorchidism, Sertoli cell tumor in abdominal testis
3	Boxer	8 yrs	Feminization, unilateral cryptorchism, Sertoli cell tumor in inguinal testis
4	German shepherd	6 yrs	Feminization, thrombopenia, bilateral cryptorchism, Sertoli cell tumor in abdominal testis
5	Dachshund	7 yrs	Feminization, bilateral cryptorchism, Sertoli cell tumor in abdominal testis
B. Control dogs			
6	German pointer	8 yrs	Unilateral perineal hernia, enlarged prostate
7	Weimaranian pointer	7 yrs	Bilateral perineal hernia, enlarged prostate
8	Chow chow	12 yrs	Unilateral perineal hernia, enlarged prostate
9	German sherperd	10 yrs	Bilateral perineal hernia, enlarged prostate
10	German sherperd	7 yrs	Unilateral perineal hernia, enlarged prostate

found in testes of control dogs (Table 2). Addition of these homogenates to medium of cultured pituitary cells did not significantly affect LH release (data not shown). The molecular weight with which this inhibin-like bioactivity was associated, was estimated after separation of proteins in the homogenates of two control testes and two Sertoli cell tumors by SDS-PAGE. With all four homogenates dose-dependent suppression of FSH release was found after addition of eluates from slices containing proteins with a molecular weight around 32 K. No FSH releasing activity was detected with eluates containing proteins with a molecular weight around 25 K. Results obtained with one testis with a Sertoli cell tumor are shown in Fig.2.

Using parallel Northern blots of RNA isolated from control testes or testes with Sertoli cell tumors, hybridization with the

Table 2. Basal hormonal parameters in 5 dogs with Sertoli cell tumors and in 5 control dogs

	Testis	plasma				
	Bioactive inhibin (U/mg protein)	imm.active inhibin (kU/l)	LH (μg/l)	FSH (μg/l)	testos-terone (nmol/l)	oestra-diol (nmol/l)
with tumor						
1	-	690	0.9	42	1.4	0.04
2	69	1090	0.2	45	0.76	0.26
3	64	100	0.5	74	0.24	0.15
4	53	6000	0.3	87	1.9	0.59
5	152	80	0.7	55	0.48	0.66
mean	84.5	1590	0.52	61	0.96	0.34
s.e.m.	22.7	1118	0.13	8.6	0.31	0.12
without tumor						
mean	29.4	15.2	5.9	164	5.7	0.30
s.e.m.	4.3	3.3	1.8	33	0.97	0.07
P	<0.05	0.01	<0.02	<0.02	<0.01	-

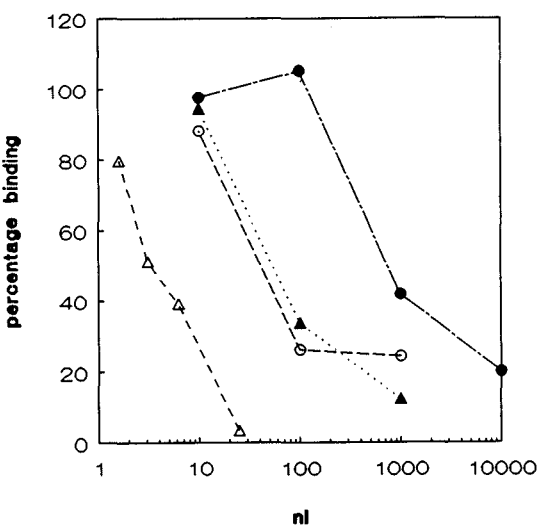

Figure 1. Effect of various doses of bovine follicular fluid (standard, △), Sertoli cell tumor homogenate (▲), control testis homogenate (o) or tumor bearing dog plasma (●) on binding (B/B_0) of radioiodinated inhibin to anti-bovine inhibin antiserum. The plasma volume in all samples was made up to 50 μl with castrate dog plasma. The points shown are means of duplicate values.

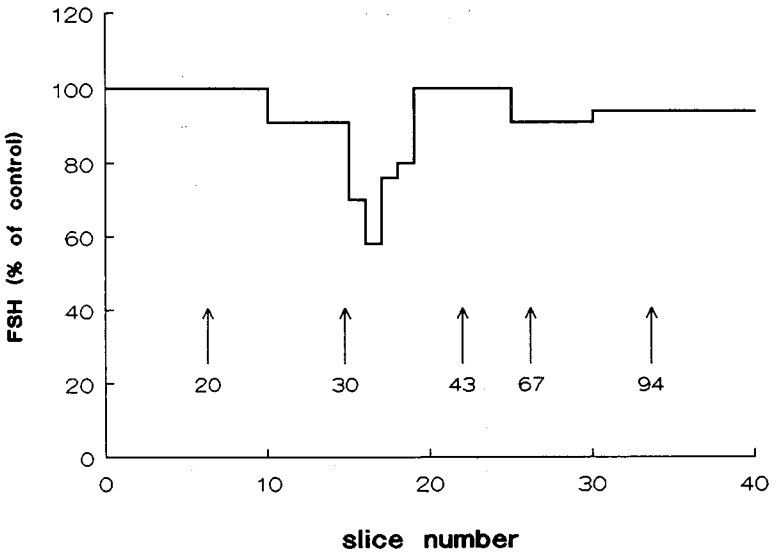

Figure 2. Suppression of FSH release (expressed as percentage of control) from cultured rat pituitary cells after addition of eluates of slices, obtained after SDS-PAGE of testicular homogenates of a dog with a Sertoli cell tumor. The positions of molecular weight markers (K) have been indicated with arrows.

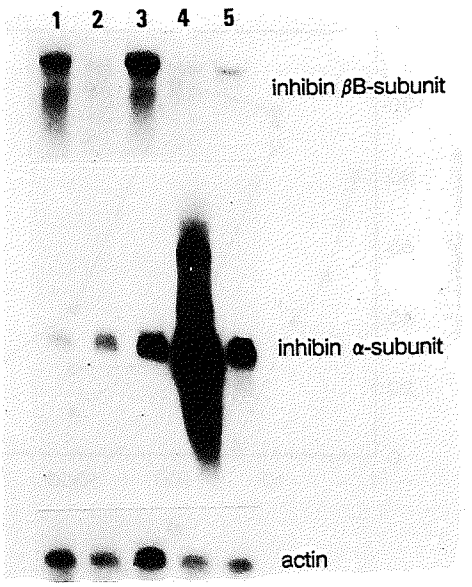

Figure 3. Expression of inhibin subunit mRNAs and actin mRNA in Sertoli cells tumors and granulosa cells. Per lane, 40 μg of total RNA was electrophorized, blotted and hybridized to cDNA probes for inhibin subunits and actin. Lanes 1, 2 and 3: tumors 3, 2 and 4. Lanes 4 and 5: bovine and rat granulosa cells.

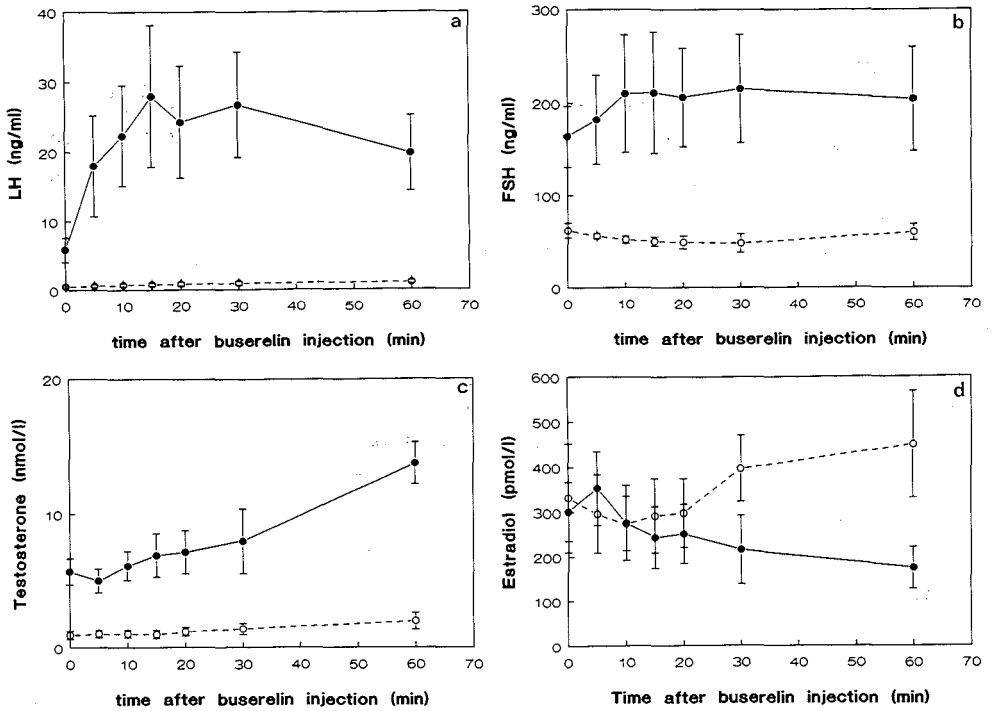

Figure 4. Plasma concentrations of LH (A), FSH (B), testosterone (C) and oestradiol (D) in control dogs (●——●) and dogs with Sertoli cell tumors (o——o) at various times after i.v. administration of 0.5 μg Buserelin kg b.w. (means ± sem, n=5).

probes for the α- and βB-subunits was found (Fig. 3). Hybridization with the βA-probe yielded negative results with testicular RNA, whereas incubation of both bovine and rat granulosa cell RNA in control lanes with this probe showed intense hybridization (data not shown). The sizes of the hybridizing dog testicular mRNAs were in agreement with those found in rat Sertoli cells (Klaij et al. 1990): an α-subunit mRNA of 1.6 kb, and βB-subunit mRNAs of 4.2 and 3.5 kb.

The levels of the inhibin α- and βB-subunit mRNAs in the Sertoli cell tumors showed a considerable variation (Fig. 3). While the relative optical densities of the α-subunit in the control testes was 1.5 ± 0.3 and those of the βB-subunit were too low for quantitation, tumors 2, 3 and 4 showed α-densities of 3.5, 1.9 and 7.9, respectively, while values for the β-B hybridization were 1.56 and 1.34, 5.3 and 5.8, and 5.1 and 4.0,

respectively for the 4.2 and 3.5 kb mRNAs.

Plasma hormones

In the plasma of dogs with Sertoli cell tumors significantly more inhibin-like immunoactivity was found than in plasma of control dogs (Table 2). Plasma levels of FSH, LH and testosterone in dogs with Sertoli cell tumors were significantly lower than in controls (Table 2). Oestradiol levels were not different between the two groups of dogs.

LHRH-tests

The effects of increased plasma levels of inhibin-like immunoactivity on pituitary responsiveness to LHRH were studied using the LHRH-agonist buserelin. In control dogs 30 and 60 min after buserelin injection LH plasma levels were significantly higher than before treatment (Fig. 4A, both $P<0.05$). In these dogs plasma testosterone levels were significantly higher 60 min after buserelin treatment (Fig. 4C, $P<0.01$) No significant effects of buserelin administration on plasma FSH levels (Fig. 4B) and oestradiol levels (Fig. 4D) were found. In dogs with Sertoli cell tumors no significant effects of buserelin on plasma levels of LH, FSH, testosterone and oestradiol were found (Fig. 4A-D). Peripheral levels of immunoassayable inhibin at 60 min after buserelin treatment were not significantly different from basal concentrations in control and tumor-bearing dogs (data not shown).

DISCUSSION

Dog Sertoli cell tumors contain increased concentrations of mRNA for the inhibin α- and especially βB-subunits when compared with control testes. This indicates separate mechanisms of the regulation of the expression of the subunits in the testis, as described earlier for the expression of inhibin α-and βB subunits in Sertoli cells from immature rats (Klaij et al. 1990). Furthermore, the observation that the tumor Sertoli cell overgrew the other testicular cell types may have contributed to the relatively larger increase of βB-subunit mRNA as compared to α-subunit mRNA: from the studies of Risbridger, Clements,

Robertson, Drummond, Muir, Burger & de Kretser (1989) it appears that the Leydig cell compartment of the testis may express the inhibin α-subunit.

Bioactivity of the inhibin in the dog Sertoli cell tumors and in the control testes was associated with a 32 k protein, as was the case in rat testes (Grootenhuis et al. 1989) and in conditioned medium of cultured Sertoli cells from immature rats (Grootenhuis, Timmerman, Hordijk & de Jong, 1990). This contrasts sharply with the situation in ovarian follicular fluid where many different molecular weight forms of inhibin can be detected (Miyamoto, Hasegawa, Fukuda & Igarashi, 1986; Grootenhuis et al. 1989). The reason for this differential splitting of the inhibin subunits in ovary and testis remains unclear, although it might be suggested that α-βA combinations, as found predominantly in the ovary, could be processed in a way which differs from that for the α-βB combination produced in Sertoli cells (Grootenhuis et al. 1990).

The increased testicular levels of inhibin bioactivity are reflected in increased peripheral levels of immunoassayable inhibin. It is not clear if all of the immunoreactivity reflects bioactive α-βB inhibin, or if it also includes a proform of the α-subunit, pro-αC, which is also detected with this antiserum (Robertson et al. 1989). The increased peripheral levels of immunoassayable inhibin might be used as a marker for the presence of a Sertoli cell tumor in males, just like the situation in females, where inhibin has been considered as a marker for granulosa cell tumors (Lappöhn et al. 1989). The specificity for this type of tumor remains questionable, however, because of the above-mentioned possibility of secretion of immunoassayable inhibin by Leydig cells in the testis (Risbridger et al. 1989) and by theca cells in the ovary (Meunier, Cajander, Roberts, Rivier, Sawchenko, Hsueh & Vale, 1988; Rivier, Roberts & Vale, 1989).

Peripheral levels of another alleged Sertoli cell tumor product, oestradiol, were only increased in 2 out of 5 dogs. The levels of oestradiol did not correlate directly with the degree of feminization of these animals, as discussed earlier by Siegel et al. (1967). The feminization in these animals is possibly due to the increased oestradiol over testosterone ratio rather than

to the absolute level of oestradiol.

In the dogs with Sertoli cell tumors, significantly suppressed levels of FSH, LH and testosterone were found. The combination of suppressed LH and testosterone makes it unlikely, that the Sertoli cell tumors exerted a primary inhibiting effect on the production and secretion of testosterone in the testicular Leydig cells, and indicates a primary effect of secretory products from the tumor at the level of the hypothalamus or pituitary gland. Effects of inhibin on hypothalamic release of LHRH are controversial (for review: see de Jong, 1988), and could not be analyzed in this study. Long-term in vivo effects of inhibin on the secretion of LH and FSH have not been reported, whereas short-term in vivo experiments showed selective suppression of FSH levels in acutely ovariectomized rats and ewes (Findlay, Robertson & Clarke, 1987; Ying, Czvik, Becker, Ling, Ueno & Guillemin, 1987). However, results of in vitro studies with high concentrations of pure inhibin indicate that basal release of both LH and FSH can be suppressed by inhibin (Farnworth, Robertson, de Kretser & Burger, 1988) and that inhibin can suppress the number of pituitary receptors for LHRH (Wang, Farnworth, Findlay & Burger, 1988). This latter effect might be the reason for the extremely low levels of LH detected in the Sertoli cell tumor bearing dogs in the present study, although effects of other secretory products, such as the postulated gonadotrophin surge inhibiting factor (GnSIF, Danforth, Sinosich, Anderson, Cheng, Bardin & Hodgen, 1987) cannot be ruled out at the present time.

Finally, a number of extra-pituitary effects of inhibin has been described. It is not clear if the hematological changes in Sertoli cell tumor dogs should be ascribed to inhibin. However, the fact that thrombopenia occurred in the animal with the highest circulating levels of immunoassayable inhibin, suggests that further investigation of inhibin and its effects in the model of dogs with Sertoli cell tumors might lead to unexpected new data on the pluripotent actions of inhibin.

REFERENCES

Comhaire F., Mattheeuws D. & Vermeulen A. (1974). Testosterone and oestradiol in dogs with testicular tumors. Acta

Endocrinologica 77, 408-416.
Danforth D.R., Sinosich M.J., Anderson T.L., Cheng C.Y., Bardin C.W. & Hodgen G.D. (1987). Identification of gonadotrophin surge-inhibiting factor (GnSIF) in follicular fluid and its differentiation from inhibin. Biology of Reproduction 37, 1075-1082.
Dieleman S.J., Kruip Th.A.M., Fontijne P., de Jong W.H.R. & van der Weyden G.C. (1983). Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone. Journal of Endocrinology 97, 31-42.
Dorn C.R., Taylor D.O.N., Schneider R., Hibbard H.H. & Klauber M.R. (1968). Survey of animal neoplasms in Alameda and Contra Costa Counties, California. II Cancer morbidity in dogs and cats from Alameda county. Journal of the National Cancer Institute 40, 307-318.
Edwards D.F. (1981). Bone marrow hypoplasia in a feminized dog with a Sertoli cell tumor. Journal of the American Veterinary Medical Association, 178, 494-496.
Farnworth P.G., Robertson D.M., de Kretser D.M. & Burger H.G. (1988). Effects of 31 kilodalton bovine inhibin on follicle stimulating hormone and luteinizing hormone in rat pituitary cells in vitro: actions under basal conditions. Endocrinology 122, 207-213.
Findlay J.K., Robertson D.M. & Clarke I.J. (1987). Influence of dose and route of administration of bovine follicular fluid and the suppressive effect of purified bovine inhibin (M_r 31 000) on plasma FSH concentrations in ovariectomized ewes. Journal of Reproduction and Fertility 80, 455-461.
Grootenhuis A.J., Steenbergen J., Timmerman M.A., Dorsman A.N.R.D.,Schaaper W.M.M., Meloen R.H. & de Jong F.H. (1989). Inhibin and activin-like activity in fluids from male and female gonads: different molecular weight forms and bioactivity/ immunoactivity ratios. Journal of Endocrinology 122, 293-301.
Grootenhuis A.J., Timmerman M.A., Hordijk P.L. & de Jong F.H. (1990). Inhibin in immature rat Sertoli cell conditioned medium: a 32 kDa $\alpha\beta$-B dimer. Molecular and Cellular Endocrinology 70, 109-116.
Hayes H.M. & Pendergrass T.W. (1976). Canine testicular tumors: epidemiologic features of 410 dogs. International Journal of Cancer 18, 482-487.
de Jong F.H. (1988). Inhibin. Physiological Reviews 68, 555-607.
Klaij I.A., Toebosch A.M.W., Themmen A.P.N., Shimasaki S., de Jong F.H. & Grootegoed J.A. (1990) Regulation of inhibin α- and β_B-subunit mRNA levels in rat Sertoli cells. Molecular and Cellular Endocrinology 68, 45-52.
Lappöhn R.E., Burger H.G., Bouma J., Bangah M., Krans M. & De Bruijn H.W.A. (1989). Inhibin as a marker for granulosa-cell tumors. New England Journal of Medicine 321, 790-793.
Lipowitz A.J., Schwartz A., Wilson G.P. and Ebert J.W. (1973). Testicular neoplasms and concomitant clinical changes in the dog. Journal of the Americal Veterinary Medical Association 163, 1364-1368.
Matsumoto A.M. (1988) The testis. In Textbook of medicine, pp. 1404-1421. Eds J.B. Wijngaarden & L.H. Smith Jr. Philadelphia: WB Saunders.

McLachlan R.I., Robertson D.M., Healy D.L., de Kretser D.M. and Burger H.G. (1986). Plasma inhibin levels during gonadotrophin-induced ovarian hyperstimulation for IVF: a new index of follicular function? Lancet **1**, 1233-1234.

Meunier H., Cajander S.B., Roberts V.J., Rivier C., Sawchenko P.E., Hsueh A.J.W. & Vale W. (1988). Rapid changes in the expression of inhibin α-, βA-, and βB-subunits in ovarian cell types during the rat estrous cycle. Molecular Endocrinology **2**, 1352-1363.

Miyamoto K., Hasegawa Y., Fukuda M. & Igarashi M. (1986). Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32K inhibin. Biochemical and Biophysical Research Communications **136**, 1103-1109.

Morgan R.V. (1982). Blood dyscrasias associated with testicular tumors in the dog. Journal of the American Animal Hospital Association **18**, 970-975.

Nett T.M., Akbar A.M., Phemister R.D., Holst P.A., Reichert L.E. & Niswender G.D. (1975). Levels of luteinizing hormone, oestradiol and progesterone in serum during the estrous cycle and pregnancy in the beagle bitch. Proceedings of the Society for Experimental Biology and Medicine **148**, 134-139.

Risbridger G.P., Clements J., Robertson D.M., Drummond A.E., Muir J., Burger H.G. & de Kretser D.M. (1989). Immuno- and bioactive inhibin and inhibin α-subunit expression in rat Leydig cell cultures. Molecular and Cellular Endocrinology **66**, 119-122.

Rivier C., Roberts V. and Vale W. (1989). Possible role of luteinizing hormone and follicle-stimulating hormone in modulating inhibin secretion and expression during the estrous cycle of the rat. Endocrinology **125**, 876-882.

Robertson D.M., Giacometti M., Foulds L.M., Lahnstein J., Goss N.H., Hearn M.T.W. & de Kretser D.M. (1989). Isolation of inhibin α-subunit precursor proteins from bovine follicular fluid. Endocrinology **125**, 2141-2149.

Sherding R.G., Wilson G.P. & Kociba G.J. (1981). Bone marrow hypoplasia in eight dogs with Sertoli cell tumor. Journal of the American Veterinary Medical Association **178**, 497-501.

Siegel E.T., Forchielli E., Dorfman R.I., Brodey R.S. & Prier J.E. (1967). An oestrogen study in the feminized dog with testicular neoplasia. Endocrinology **80**, 272-277.

Theilen G.H. & Madewell B.R. (1979). Tumors of the urogenital tract. In Veterinary Cancer Medicine, pp. 375-381. Eds G.H. Theilen & B.R. Madewell. Philadelphia: Lea & Febiger.

Wang Q.F., Farnworth P.G., Findlay J.K. & Burger H.G. (1988). Effect of purified 31K bovine inhibin on the specific binding of gonadotrophin-releasing hormone to rat anterior pituitary cells in culture. Endocrinology **123**, 2161-2166.

Welschen R., Osman P., Dullaart J., de Greef W.J., Uilenbroek J.Th.J. & de Jong F.H. (1975). Levels of follicle-stimulating hormone, luteinizing hormone, oestradiol-17β and progesterone, and follicular growth in the pseudopregnant rat. Journal of Endocrinology **64**, 37-47.

Ying S.-Y., Czvik J., Becker A., Ling N., Ueno N. & Guillemin R. (1987). Secretion of follicle-stimulating hormone and production of inhibin are reciprocally related. Proceedings of the National Academy of Sciences, USA **84**, 4631-4635.

CHAPTER 7

GENERAL DISCUSSION

7.1 Introduction

At the start of the studies described in this thesis in October 1985 several bioassays were available for the detection of inhibin activity and different molecular weight forms of inhibin had been obtained as partially purified preparations from follicular fluid. The working definition of inhibin at that time was: "Inhibin is presumed to be a protein produced in the gonads under FSH control and is thought to interact at the pituitary level to control FSH secretion by a receptor mechanism" (de Jong and Robertson, 1985).

In December 1985 the purification of two dimeric inhibin forms (consisting of combinations of an α-subunit and one of the β-subunits β-A or β-B) from pFF and the cloning of the three genes encoding for these subunits of inhibin were reported (Mason et al., 1985) and inhibin was redefined as "a glycoprotein hormone consisting of two dissimilar, disulphide-linked subunits, which inhibits pituitary gonadotrophin production and/or secretion, preferentially that of FSH" (Burger and Igarashi, 1988). The characterization of inhibin was followed by the purification of two proteins which stimulated the release of FSH from pituitary cells in vitro. These proteins are homodimers (β-A β-A) or heterodimers (β-A β-B) of the inhibin subunits, and were called activin A and activin AB (Ling et al., 1986; Vale et al., 1986).

The aims of the studies described in this thesis were to elucidate possible differences between inhibin from female and male animals, to characterize inhibin from rSCCM, to develop RIAs for inhibin estimation and to explore the possible role of inhibin in reproductive physiology.

In the following sections the characterization and purification of inhibin and related proteins (section 7.2) and the problems experienced in the detection of these proteins (section 7.3) will be discussed. Also, arguments pro and contra the significance of inhibin in the regulation of FSH are presented (section 7.4) and the possible paracrine role of inhibin in the testes (section 7.5) is considered. Finally, the significance of the non-gonadal effects of inhibin and related proteins is discussed (section 7.6) and suggestions for future research on inhibin and related proteins are presented (section 7.7).

7.2 Purification of inhibin and related proteins

The reported differences between inhibin from female and male animals were investigated using SDS-PAGE separation of inhibin-containing samples from ovary and testes. Bioactivity and immunoactivity of the different inhibin forms were recovered after separation of SDS and proteins by methanol precipitation of the protein. With this separation system we have found that inhibin bioactivity in bFF and rat ovaries is associated with proteins with several molecular weights, and that inhibin in rat testis extracts and rSCCM occurs only in a 32 kDa form (Chapter 2). In Chapter 3 it was shown that inhibin in rSCCM is composed of a 32 kDa $\alpha\beta$-B dimer. This is in contrast with the predominance of $\alpha\beta$-A dimers in follicular fluid (see Chapter 1.2.1). Since there are no reports on biological differences between inhibin $\alpha\beta$-A and $\alpha\beta$-B, the reported differences in immunoneutralization of inhibin using antiserum against bFF inhibin from female and male animals (van Dijk et al., 1986), could have been caused by the existence of different inhibin forms in female and male animals. This is supported by the differences in B/I ratios between the different forms of inhibin in bFF (Chapter 2). The presence of inhibin α-subunit forms in follicular fluid, which are recognized by inhibin RIAs, also could have contributed to the observed differences in B/I ratios of the different inhibin forms.

In Chapter 4 it was described that the FSH-induced release of inhibin immunoactivity is probably due to increased secretion of a precursor form of the α-subunit (pro-αC) without inhibin bioactivity.

7.3 Detection of inhibin and related proteins

For the estimation of inhibin bioactivity, the specific suppression of FSH release from cultured rat pituitary cells has been widely used. Through the identification of other proteins influencing FSH release, such as activin A and AB, follistatins (monomeric proteins which also suppress FSH release specifically) and probably TGF-β, it has become clear that an overall effect of these proteins on FSH release is measured. This indicates, that

bioassays for inhibin do not have an absolute specificity. On the other hand, none of the available RIAs discriminates between bioactive inhibin ($\alpha\beta$ dimers) and individual α-subunits and RIAs for activins and follistatins are not yet available. Also, the inhibin α-subunit RIA using an antibody raised against a peptide derived from the N-terminal sequence of the α-subunit of 32 kDa bovine inhibin, recognized both bioactive 32 kDa ($\alpha\beta$) inhibin and a smaller 29 kDa inhibin form without bioactivity. The latter probably is a precursor form (pro-αC) of the α-subunit (Chapter 4). A combination of SDS-PAGE separation of proteins followed by Western blot analysis and detection of bioactivity, as described in Chapter 4, should be performed to guarantee the most reliable assay of the detected forms of inhibin and related proteins.

7.4 Significance of inhibin in the regulation of FSH

Research on inhibin was stimulated by the interest in the endocrine control of FSH release. To support the hypothesis that FSH and inhibin are involved in a classical endocrine closed-loop feedback system, it is necessary to show that inhibin suppresses FSH secretion and that FSH in turn stimulates inhibin secretion both *in vitro* and *in vivo*. The *in vitro* regulation of inhibin production from Sertoli cells has been discussed (Chapter 1.5) but direct evidence for *in vivo* control of inhibin production has been hampered by inadequate detection techniques and the lack of purified inhibin.

In the past, the role of inhibin in modulating FSH secretion *in vivo* has been studied by injecting charcoal-extracted follicular fluid, which was presumed to contain only inhibin as a FSH suppressing factor. Results of these studies indicated that follicular fluid can specifically suppress serum FSH levels in male rats and in female rats after the second week of life (for review see de Jong, 1988). Since not only inhibin but also activin and follistatin are present in follicular fluid these studies should be repeated, however, with pure inhibin. Pure porcine 32 kDa inhibin suppressed the post-castration rise of serum FSH in female rats (Ying et al., 1987). Also 32 kDa bovine inhibin was biologically active *in vivo* to suppress the elevated serum FSH levels of ovariectomized ewes. A hypothalamic site of

action was excluded by the use in these experiments of hypothalamic-pituitary disconnected ewes (Findlay et al., 1987).

Inhibin secreting tumors can be used also as a model for the study of the *in vivo* effects of inhibin. In Chapter 6 it was described that dog Sertoli cell tumors contain more 32 kDa inhibin bioactivity and more inhibin α and β-B mRNAs than testes of control dogs. In such dogs with Sertoli cell tumors both plasma FSH and LH were lowered, plasma inhibin-like immunoactivity was increased and the pituitary did not respond to GnRH. We have suggested that inhibin could have decreased the number of pituitary receptors for GnRH, as has also been shown in *in vitro* experiments by Wang et al. (1988).

Another approach to study the regulation of inhibin production is to measure the modulation of inhibin messengers or proteins after various types of treatments. FSH-induced release of inhibin-like bioactivity from the ovary (both in women and sheep) during the follicular phase has been described using the sensitive bioassay for inhibin based on sheep pituitary cells *in vitro* (Tsonis et al., 1988a; Tsonis et al., 1988b). Since these results were confirmed with inhibin RIAs, it is highly likely that inhibin is measured and not follistatin.

The effects of passive or active immunization against inhibin might also give evidence for a possible role of inhibin in the control of FSH release. After the injection of antisera against the N-terminal amino acids of the α-chain of 32 kDa porcine inhibin, increased serum FSH levels were found in female rats older than 10 days (Rivier and Vale, 1987). Increased FSH serum levels were found after injection of this antiserum in male rats aged 10-24 days but not in older animals, indicating that only in immature male rats inhibin exerts a physiological role in the control of FSH release (Rivier et al., 1988). Increased peripheral levels of FSH and numbers of corpora lutea have been reported in sheep after immunization against the recombinant αC-subunit of bovine inhibin (Forage et al., 1987; Findlay et al., 1989b). This contrasts with the results obtained after immunization against the recombinant αN-subunit of bovine inhibin, where similar numbers of corpora lutea per ewe were found as in the control group, although the pregnancy rate was decreased (Findlay et al., 1989a).

However, there are several reports that serum inhibin and FSH levels are not correlated. De Kretser et al. (1989) demonstrated that serum inhibin immunoreactivity did not correlate with the high serum FSH levels in men with testicular disorders. Also, in men with low sperm counts and increased serum FSH levels due to chemotherapy-induced testicular damage, normal or even increased serum inhibin-like immunoreactivity was observed (Tsatsoulis et al., 1988). Possible explanations for these discrepancies between peripheral levels of FSH and inhibin are that the detected serum inhibin levels did not represent bioactive inhibin or that FSH is controlled both by inhibin and testosterone. This dual control of serum FSH is also apparent from studies in rats after heat treatment of the testes, which affects Sertoli cell function and presumably inhibin secretion, and causes a rise in FSH serum levels to 50% of the castration levels. Only heat treatment combined with administration of ethane dimethane sulphonate, a compound which specifically destroys Leydig cells, raised FSH serum levels into the castration range (O'Leary et al., 1986). These studies indicate that peripheral FSH levels are not solely regulated by inhibin, but that Leydig cells products also play a role.

7.5 Paracrine effects of inhibin in the testes

Apart from an endocrine role of inhibin in the regulation of FSH secretion, several lines of evidence point to a possible paracrine role of inhibin in the gonads. It is known that inhibin, like several other Sertoli cell products, is secreted bidirectionally (Handelsman et al., 1989), i.e. via the base of the Sertoli cell into the testicular interstitial fluid and via the apex of the Sertoli cell into the lumen of tubuli. Maddocks and Sharpe (1989) calculated that 30 times more inhibin-like immunoreactivity is secreted into the lumen of tubuli than into the interstitial fluid and that inhibin secreted into the tubular fluid may be reabsorbed into the peripheral blood in the rete testis. Also in rats a stage-dependent expression of the inhibin α and β-B subunit genes has been reported by Bhasin et al. (1989). The amount of inhibin secreted into the interstitial fluid might influence the number of differentiating

spermatogonia, since intratesticularly injected inhibin, which probably increases inhibin levels in the interstitial fluid, suppresses the number of differentiating spermatogonia at particular stages of the cycle (van Dissel-Emiliani et al., 1989).

We have investigated also the possible role of inhibin in the short-term regulation of steroid production by Leydig cells. In Chapter 5 evidence was provided that the factor(s) present in SCCM enhancing basal steroid production of Leydig cells are not 32 kDa $\alpha\beta$-B inhibin.

7.6 Effects of inhibin and activin not related with reproduction

The non-gonadal expression of inhibin subunit genes (Meunier et al., 1988) and the reported effects of inhibin and activin in non-gonadal tissues (see Chapter 1.7) indicate, that inhibin and activin could be involved in the regulation of other processes than the control of reproduction. As yet not many in vivo data are available to support these in vitro observations, but with the availability of recombinant activin A, more insight into these questions may be expected to emerge in the near future. For instance, infusion of recombinant activin A into immature female rats caused a marked increase in serum FSH levels and, in addition, induced an increase in circulating concentration of red blood cells and hemoglobin (Schwall et al., 1989).

7.7 Future research on inhibin and related proteins

Since inhibin and related proteins appear to be important in reproductive physiology and an increasing number of diverse effects has been described, future research should first resolve problems of specificity involved in the detection of inhibin and related proteins. Hence, specific RIAs for the different forms of inhibin, activin and related proteins should be developed. Sandwich assays with catching and detecting antibodies directed against different subunits in the case of inhibin, or monoclonal antibodies against epitopes formed by $\alpha\beta$ dimers (in case of inhibin) or $\beta\beta$ dimers (in case of activin) should be evaluated.

Purified preparations of the various forms of inhibin, activin

and related proteins are needed to raise and characterize these antibodies. Affinity chromatography using immobilized antibodies in combination with SDS-PAGE purification of inhibin and related proteins from follicular fluid appear to be the most logical method to obtain these proteins. Also recombinant subunits or fragments can be used, although similar purification problems as in the purification of inhibin and activin can be expected. In parallel bioactivities have to be determined since secondary modifications (glycosylation, sulphation, acetylation) could influence bioactivity without any effect on immunoactivity. For this purpose samples should be separated according to molecular weight or hydrophobicity, since inhibin, activin and related proteins have different molecular weights and can be separated on C18 reversed phase columns. When such proteins, antibodies and highly specific RIAs become available, the possible roles of inhibin, activin, and related proteins in reproduction should be reevaluated.

With specific antibodies for activin the observed 25 kDa FSH releasing entity secreted by Sertoli cells (Chapter 2) can be analyzed. Also the secretion products of different types of testicular tumors should be examined with the available antibodies, to determine if they can be used as markers for these types of tumors.

The 29 kDa inhibin-like immunoreactive molecule which is secreted by Sertoli cells under the influence of FSH, should be purified and characterized. If this molecule is the pro-αC subunit precursor of inhibin, it is likely that large amounts of αN-subunit are secreted also by Sertoli cells after FSH stimulation. Paracrine effects of the pro-αC and αN-subunits should be studied in <u>in vitro</u> systems with testicular cells. For the elucidation of the possible endocrine effects of the pro-αC and αN-subunit precursors, it should be established if these forms are found in peripheral blood. Also experiments have to be performed on the regulation of the concentration of these molecules. Finally, antibodies against the pro-, αN- and αC-part of the inhibin precursor can be used for the detection and passive immunoneutralization of these molecules.

The impaired fertility in sheep after immunization with the αN part, but not after immunization with the αC part of the inhibin

α-subunit precursor, indicates a possible role for the αN-subunit precursor in transport and/or implantation of the embryo. The possible role of inhibin and its subunit precursors in early embryogenesis could be evaluated by analysis of embryonic mRNA and secreted proteins.

The role of activin in different organs could also be studied by means of transgenic mice. Effects of high levels of activin on spermatogenesis could be studied, using a gene construct with the inhibin β-A or β-B subunit gene and a promoter of a gene preferentially transcribed during spermatogenesis, for instance the protamine promoter.

The results present in this thesis, and the ensuing discussion, hopefully have made it clear, that the available information offers only the start of further studies on the many and versatile effects of inhibin, activin, and related proteins.

7.8 REFERENCES

Bhasin, S., Krummen, L.A., Swerdloff, R.S., Morelos, B.S., Kim, W.H., diZerega, G.S., Ling, N., Esch, F., Shimasaki, S. and Toppari J. (1989). Stage dependent expression of inhibin α and β-B subunits during the cycle of the rat seminiferous epithelium. Endocrinology 124: 987-991.

Burger, H.G. and Igarashi, M. (1988). Inhibin: definition and nomenclature, including related substances. J. Endocrinol. 117: 159-160.

De Jong, F.H. (1988). Inhibin. Physiol. Rev. 68: 555-607.

De Jong, F.H. and Robertson, D.M. (1985). Inhibin: 1985 update on action and purification. Mol. Cell. Endocrinol. 42: 95-103.

De Kretser, D.M. McLachlan, R.I., Robertson, D.M. and Burger H.G. (1989). Serum inhibin levels in normal men and men with testicular disorders. J. Endocrinol. 120: 517-523.

Findlay, J.K., Robertson, D.M. and Clarke I.J. (1987). Influence of dose and route of administration of bovine follicular fluid and the suppressive effect of purified bovine inhibin (M_r31000) on plasma FSH concentrations in ovariectomized ewes. J. Reprod. Fert. 80: 455-461.

Findlay, J.K., Doughton B., Robertson, D.M. and Forage, R.G. (1989b). Effects of immunization against recombinant bovine inhibin α subunit on circulating concentrations of gonadotrophins in ewes. J. Endocrinol. 120: 59-65.

Findlay, J.K., Tsonis, C.G., Doughton, B., Brown, R.W., Bertram, K.C., Braid, G.H., Hudson, G.C., Tierney, M.L., Goss, N.H. and Forage, R.G. (1989a). Immunisation against the amino-terminal peptide (α_N) of the alpha $_{43}$ subunit of inhibin impairs fertility in sheep. Endocrinology 124: 3122-3124.

Forage, R.G., Brown, R.W., Oliver, K.J., Atrache, B.T., Devine, P.L., Hudson, G.C., Goss, N.H., Bertram, K.C., Tolstohev, P.,

Robertson, D.M., de Kretser, D.M., Doughton, B., Burger, H.G. and Findlay J.K. (1987). Immunization against an inhibin subunit produced by recombinant DNA techniques results in increased ovulation rate in sheep. J. Endocrinol. 114: R1-R4.

Handelsman, D.J., Spaliviero, J.A., Kidston, E. and Robertson, D.M. (1989). Highly polarized secretion of inhibin by Sertoli cells in vitro. Endocrinology 125: 721-729.

Maddocks, S. and Sharpe, R.M. (1989). The route of secretion of inhibin from the rat testis. J. Endocrinol. 120: R5-R8.

Mason, A.J., Hayflick, J.S., Ling, N., Esch, F., Ueno, N., Ying, S.-Y., Guillemin, R., Niall, H. and Seeburg, P.H. (1985). Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. Nature 318: 659-663.

Meunier, H., Rivier, C., Evans, R.M. and Vale, W. (1988). Gonadal and extragonadal expression of inhibin α, βA, and βB subunits in various tissues predicts diverse functions. Proc. Natl. Acad. Sci. USA 85: 247-251.

Ling, N., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. and Guillemin, R. (1986). Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature 321: 779-782.

O'Leary, P., Jackson, A.E., Averill, S. and de Kretser, D.M. (1986). The effects of ethane dimethane sulphonate (EDS) on bilaterally cryptorchid rat testes. Mol. Cell. Endocrinol. 45: 183-190.

Rivier, C and Vale, W. (1987). Inhibin: measurement and role in the immature female rat. Endocrinology 120: 1688-1690.

Rivier, C., Cajander, S., Vaughan, J., Hsueh, A.J.W. and Vale, W. (1988). Age-dependent changes in physiological action, content, and immunostaining of inhibin in male rats. Endocrinology 123: 120-126.

Schwall, R., Schmelzer, C.H., Matsuyama, E. and Mason, A.J. (1989). Multiple actions of recombinant activin-A in vivo. Endocrinology 125: 1420-1423.

Tsatsoulis, A., Shalet, S.M., Robertson, W.R., Morris, I.D., Burger, H.G. and de Kretser, D.M. (1988). Plasma inhibin levels in men with chemotherapy-induced severe damage to the seminiferous epithelium. Clin. Endocrinol. 29: 659-665.

Tsonis, C.G., McNeilly, A.S. and Baird, D.T. (1988b). Inhibin secretion by the sheep ovary during the luteal and follicular phases of the oestrous cycle and following stimulation with FSH. J. Endocrinol. 117: 283-291.

Tsonis, C.G., Messinis, I.E., Templeton, A.A., McNeilly, A.S., and Baird, D.T. (1988a). Gonadotropic stimulation of inhibin secretion by the human ovary during the follicular and early luteal phase of the cycle. J. Clin. Endocinol. Metab. 66: 915-921.

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D. and Spiess J. (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature 321: 776-779.

Van Dijk, S., Steenbergen, J., Gielen, J.Th. and de Jong, F.H. (1986). Sexual dimorphism in immunoneutralization of bioactivity of rat and ovine inhibin. J. Endocrinol. 111: 255-261.

Van Dissel-Emiliani, F.M.F., Grootenhuis, A.J., de Jong, F.H. and

de Rooij, D.G. (1989). Inhibin reduces spermatogonial numbers in testes of adult mice and Chinese hamsters. Endocrinology 125: 1898-1903.

Wang, Q.F., P.G. Farnworth, J.K. Findlay, and H.G. Burger (1988). Effect of purified 31 K bovine inhibin on the specific binding of gonadotrophin-releasing hormone to rat anterior pituitary cells in culture. Endocrinology 123: 2161-2166.

Ying, S.-Y., Czvik, J., Becker, A., Ling, N., Ueno, N. and Guillemin, R. (1987). Secretion of follicle-stimulating hormone and production of inhibin are reciprocally related. Proc. Natl. Acad. Sci. USA 84: 4631-4635.

SUMMARY

The mammalian testis consists of two compartments, the seminiferous tubules where spermatogenic cells and Sertoli cells are in close contact, and the interstitium where the steroid producing Leydig cells are located. The main regulators of the gonads are the pituitary hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH). The release of FSH and LH from the pituitary gland is stimulated by the hypothalamic decapeptide gonadotrophin releasing hormone (GnRH) and inhibited by gonadal steroids. However, the negative feedback regulation of FSH release cannot be explained solely on basis of steroid hormones, and already in 1932 McCullagh has suggested that FSH release is suppressed also by another gonadal factor named "inhibin".

Chapter 1 of this thesis comprises an overview of the recent literature on the purification of gonadal factors modifying pituitary FSH release, such as, inhibin, activin (a factor which stimulates FSH release from pituitary cells) and a monomeric FSH release inhibiting factor, follistatin.

Also cloning of the inhibin subunit genes, the complications in the detection of inhibin and related proteins, localization and reported (non)-gonadal effects of inhibin, activin and related proteins are discussed.

The present studies were initiated to elucidate the presumed differences between inhibin from female and male animals, to determine the structure of rat testicular inhibin, to develop inhibin RIAs and to explore the effects of inhibin in reproductive physiology.

In **Chapter 2** it is described that inhibin from rat ovarian extracts and bovine follicular fluid (bFF) is associated with several molecular weight forms and that only a single 32 kDa form of inhibin is found in rat testis extracts and rat Sertoli cell conditioned medium (rSCCM). In bFF and rSCCM also a factor of 25 kDa is found which stimulates FSH release from cultured pituitary cells. Antibodies against the N-terminal amino acids of the α-subunit of 32 kDa bovine inhibin were used to develop a RIA detecting inhibin (subunits).

In **Chapter** 3 the results of 3 years work on the purification of inhibin from rSCCM are described, which can be summarized in the statement that "inhibin from rSCCM is a 32 kDa $\alpha\beta$-B dimer".

Chapter 4 describes the results of studies on the hormonal regulation of inhibin secretion from cultured rat Sertoli cells. FSH stimulated the release of a 29 kDa immunoreactive inhibin form from Sertoli cells, whereas the amount of 32 kDa bioactive inhibin was not affected. It was concluded that results of inhibin RIAs do not necessarily reflect inhibin bioactivity, but that also precursor forms of the inhibin α-subunit can be detected.

Chapter 5 describes the results of studies to elucidate the nature of the factors present in rSCCM which stimulate Leydig cell steroidogenesis. Experiments with purified rat 32 kDa $\alpha\beta$-B inhibin and rSCCM revealed that these factor(s) differ from inhibin.

In **Chapter 6** the results of studies with normal dogs and dogs with Sertoli cell tumors are discussed. Sertoli cell tumors contain more bioactive inhibin and mRNA for the inhibin α and β-B subunits than testes of control dogs. The increased levels of plasma inhibin immunoactivity in dogs with Sertoli cell tumors are probably the reason for the decreased plasma FSH levels in these dogs, as compared to those in the control group. Since dogs with Sertoli cell tumors also show lower plasma LH levels and a lack of pituitary responsiveness towards GnRH, we have suggested that the increased plasma levels of inhibin might have caused a decrease in the number of pituitary GnRH receptors.

In **Chapter** 7 the results presented in the previous chapters are discussed. Arguments pro and contra the role of inhibin in the endocrine regulation of pituitary FSH release are discussed. Also some <u>in vivo</u> data are discussed which indicate a role of inhibin and related proteins on other organs, and some possibilities for future research of inhibin are presented.

SAMENVATTING

De testikel (zaadbal of testis) van zoogdieren bestaat uit een groot aantal zaadbuisjes en het daartussenliggende interstitiële weefsel. De twee belangrijkste produkten van de testikels zijn de zaadcellen (sperma) en een verbinding die verantwoordelijk is voor de mannelijke geslachtskenmerken (o.a. haargroei en potentie): het mannelijke geslachtshormoon testosteron. De wand van de zaadbuisjes wordt o.a. gevormd door Sertoli cellen, die als verzorg(st)ercellen fungeren voor de delende zaadcellen. Door het interstitium lopen de bloedvaten voor de gehele testis en hier bevinden zich ook de testosteron-producerende Leydig cellen.

De vorming van testosteron en zaadcellen gebeurt niet vanzelf, maar wordt beïnvloed door een aanhangsel van de hersenen, de hypofyse. De hypofyse scheidt twee hormonen uit die de functie van geslachtsorganen (gonaden) reguleren. De naamgeving van deze twee hypofysehormonen berust op de werking van deze hormonen op de vrouwelijke geslachtsklier, het ovarium: het follikel-stimulerend hormoon (FSH) regelt de follikel-/eicelrijping en het luteinizerend hormoon (LH) stimuleert de eisprong (ovulatie) en de luteinisatie van het follikelweefsel (cellen rondom de eicel) tot corpus luteum, dat het zwangerschapshormoon progesteron uitscheidt. In de testes stimuleert LH de produktie van testosteron in de Leydig cel. FSH stimuleert de Sertoli cel tot het maken van diverse stoffen die tezamen met testosteron belangrijk zijn voor het opstarten en onderhouden van de zaadcelvorming (spermatogenese).

Ook de afgifte van de hypofysehormonen FSH en LH vindt niet spontaan plaats, er zijn stoffen die de afgifte van LH en FSH stimuleren of remmen. In een hersendeel, de hypothalamus, wordt het gonadotrofine releasing (afgifte)-hormoon GnRH gemaakt dat de afgifte van FSH en LH door de hypofyse stimuleert. De afgifte van FSH en LH vindt niet continu plaats, maar wordt geremd door substanties die onder invloed van FSH en LH in de gonaden worden geproduceerd. Na castratie gaat het niveau van FSH en LH in het bloed omhoog, door het wegvallen van deze produkten uit de gonaden. Indien testosteron, het belangrijkste steroïdhormoon in de testis, wordt ingespoten in gecastreerde dieren, daalt de concentratie van LH tot het niveau van vóór de castratie. De

concentratie van FSH gaat ook wel omlaag maar bereikt niet de concentratie die voor de castratie aanwezig was. Als echter in een gecastreerd dier een eiwitmengsel dat geïsoleerd is uit de testis, wordt ingespoten, daalt de concentratie FSH tot het niveau dat aanwezig was vóór de castratie, terwijl de concentratie van LH niet verandert. Het eiwit dat onder invloed van FSH in de gonaden wordt gemaakt en ervoor zorgt dat de secretie van FSH door de hypofyse wordt geremd (geïnhibeerd), is het hormoon inhibine.

Voor het verkrijgen van een beter inzicht in de rol van inhibine in de regulatie van FSH, is het noodzakelijk om inhibine te zuiveren en de structuur op te helderen. Hiervoor is het nodig dat inhibine betrouwbaar en snel aangetoond kan worden. Het effect van ingespoten inhibine in de gecastreerde rat op de afgifte van FSH door de hypofyse en dus op de concentratie FSH in het bloed, is één van de gebruikte detectiesystemen. Als echter hypofysecellen in kweek worden gebracht en het inhibine daaraan wordt toegevoegd, kan het specifieke effect van inhibine op de FSH-afgifte van deze hypophysecellen met veel minder inhibine en met een veel kleiner gebruik van proefdieren bestudeerd worden.

Het onderzoek, beschreven in dit proefschrift, had de volgende doelstellingen;

1. Opheldering van de waargenomen verschillen tussen inhibine uit ovaria en testikels, en de zuivering van inhibine uit het kweekmedium van Sertoli cellen (SCCM);
2. De ontwikkeling van gevoeliger bepalingsmethoden die alleen inhibine herkennen: de zogenaamde radioimmunoassays (RIA's);
3. Het onderzoeken van de mogelijke effecten van inhibine op de Leydig cellen en het bestuderen van de effecten van hoge inhibine bloedconcentraties in honden met Sertoli cel tumoren.

In **Hoofdstuk 2** zijn experimenten beschreven waaruit blijkt dat het inhibine in het ovarium in verschillende vormen voorkomt (65000, 58000 en 32000 Dalton) terwijl in de testikels alleen een 32000-Dalton vorm van inhibine aantoonbaar is. Daarnaast is beschreven hoe een RIA voor inhibine werd ontwikkeld, door antilichamen te gebruiken tegen fragmenten van de α-subeenheid van inhibine.

Uit de in **Hoofdstuk 3** beschreven resultaten blijkt dat het

testiculaire inhibine, dat wij hebben gezuiverd uit SCCM, bestaat uit twee eiwitketens, α en β-B. Deze twee subeenheden vormen tezamen het biologisch actieve 32000-Dalton inhibine.

Uit de in **Hoofdstuk 4** beschreven resultaten blijkt echter dat de antilichamen opgewekt tegen fragmenten van de α-subunit, naast het biologisch actieve 32000-Dalton-$\alpha\beta$-B inhibine, ook losse α-subeenheden herkennen. Daar FSH de afgifte van deze losse α-subeenheden in Sertoli cellen kan stimuleren, is waarschijnlijk de met RIA's gemeten toename van inhibine α-subeenheden soms ten onrechte geïnterpreteerd als een toename van biologisch actief inhibine.

Mogelijke effecten van inhibine op processen in de testikels zijn beschreven in **Hoofdstuk 5**. In het SCCM zijn faktoren aanwezig die de vorming van steroïden (o.a. testosteron) door Leydig cellen stimuleren. In de literatuur is gesuggereerd dat deze factor mogelijk inhibine zou kunnen zijn. Op grond van experimenten met SCCM en "zuiver" 32000-Dalton $\alpha\beta$-B inhibine is echter gebleken dat de factor die de steroïdproduktie van Leydig cellen in kortdurende experimenten stimuleert, geen inhibine is.

Hoofdstuk 6 beschrijft de resultaten van het effect van hoge concentraties inhibine in het bloed van honden met Sertoli celtumoren op de hypofyse functie. Omdat zowel FSH als LH in het bloed verlaagd waren en de hypofyse niet reageerde op injectie van GnRH, hebben we geconcludeerd dat inhibine waarschijnlijk het aantal receptoren (ontvangststations) voor GnRH op hypofyse cellen heeft verlaagd.

In **Hoofdstuk 7** zijn de resultaten van de vorige hoofdstukken besproken, waarbij speciaal aandacht is besteed aan de problemen met de kwantitatieve bepaling van inhibine en aan argumenten voor en tegen een rol van inhibine bij de regulatie van FSH afgifte.

PAPERS RELATED TO THIS THESIS

F.F.G. Rommerts, A.J. Grootenhuis, J.W. Hoogerbrugge and H.J. van der Molen (1985). Ethane dimethane sulphonate (EDS) specifically inhibits LH stimulated steroidogenesis in Leydig cells isolated from mature rats but not in cells from immature rats. Molecular and Cellular Endocrinology 42, 105-111.

F.H. de Jong, A.J. Grootenhuis, H.J. Sander, J. Steenbergen, M.A. Timmerman and S. van Dijk (1987). Comparison between inhibin from bovine follicular fluid and rat Sertoli cell culture medium. In: Inhibin-non-steroidal regulation of follicle stimulating hormone secretion (Eds. H.G. Burger, D.M. de Kretser, J.K. Findlay, M. Igarashi) Serono Symposia, Raven Press, New York, pp 35-46.

A.M.W. Toebosch, D.M. Robertson, J. Trapman, P. Klaassen, A.J. Grootenhuis, R.A. de Paus, F.H. de Jong and J.A. Grootegoed (1988). FSH stimulates inhibin α-, but not inhibin β-subunit mRNA levels in rat Sertoli cells. In: Development and function of the reproductive organs vol II. (Eds. M. Parvinen, I. Huhtaniemi, I. and L.J. Pelliniemi) Ares-Serono Symposia, Rome, pp 225-230.

F.H. de Jong, A.J. Grootenhuis, I.A. Klaij, J.M.W. Toebosch, A.M. Ultee-van Gessel, S. Shimasaki and J.A. Grootegoed (1989). Regulation of inhibin production in rat Sertoli cells. In: Perspectives in Andrology (Ed. M. Serio) Raven Press, New York, pp 235-242.

F.M.F. van Dissel-Emiliani, A.J. Grootenhuis, F.H. de Jong and D.G. de Rooij (1989). Inhibin reduces spermatogonial numbers in testes of adult mice and Chinese hamsters. Endocrinology 125, 1898-1903.

F.H. de Jong, A.J. Grootenhuis, I.A. Klaij and W.M.O. van Beurden (1990). Inhibin and related peptides: localization, regulation and effects. In: Circulating regulatory factors and neuroendocrine functions (Ed. J. Porter) Plenum Press, New York, in press.

NAWOORD

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 16 april 1960 te Hollandia in het voormalige Nederlands Nieuw-Guinea. Aan het Vitus College te Bussum werd in 1978 het diploma VWO-B behaald. In hetzelfde jaar werd begonnen met de studie biologie aan de Vrije Universiteit te Amsterdam. Het doctoraal examen werd afgelegd op 16 oktober 1985 met het hoofdvak Endocrinologie (Prof.Dr. J. Joosse te Amsterdam en Prof.Dr. W.T. Schrader te Houston (USA)) en als bijvakken Histologie (Prof.Dr. H.H. de Boer te Amsterdam) en Chemische Endocrinologie (Prof.Dr. H.J. van der Molen te Rotterdam). Vanaf 15 oktober 1985 was de schrijver werkzaam als wetenschappelijk onderzoeker op de afdeling Biochemie II (Chemische Endocrinologie) aan de Faculteit der Geneeskunde van de Erasmus Universiteit te Rotterdam, alwaar het onderzoek beschreven in dit proefschrift werd uitgevoerd. Vanaf 1 januari 1990 is hij werkzaam bij Organon International te Oss binnen de afdeling Endocrinological Research Development Laboratories (ERDL) en betrokken bij het onderzoek naar immuuncontraceptie.