REGULATION OF INHIBIN PRODUCTION IN THE RAT TESTIS

REGULATIE VAN INHIBINE PRODUCTIE IN DE TESTIS VAN DE RAT

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
prof.dr. P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 1 november 1995 om 13.45 uur.

door

INGRID ALWINE VAN DER KLEIJ-KLAIJ

geboren te Delft

PROMOTIECOMMISSIE

Promotores:	Prof. dr. F.H. de Jong
	Prof. dr. J.A. Grootegoed
Overige leden:	Prof. dr. S.W.J. Lamberts
	dr. D.G. de Rooij
	Prof. dr. E.J.J. van Zoelen

Dit proefschrift werd bewerkt binnen de Vakgroep Endocrinologie & Voortplanting van de Faculteit der Geneeskunde en Gezondheidswetenschappen, Erasmus Universiteit Rotterdam. De vakgroep maakt deel uit van de Onderzoekschool Pathofysiologie van Groei en Differentiatie.

Het beschreven project werd gesubsidieerd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO, Gebied Medische Wetenschappen)

CONTENTS

Abbreviations

ABP	androgen binding protein
ACTH	adrenocorticotropic hormone
AMH	anti-müllerian hormone
AP-2	activating protein 2
bFF	bovine follicular fluid
B/I ratio	bioactivity/immunoreactivity ratio
BSA	bovine serum albumin
cAMP	adenosine cyclic-3',5'-monophosphate
(c)DNA	(complementary) deoxyribonucleic acid
CRE	cAMP-responsive element
CREBP	CRE-binding protein
DAG	diacylglycerol
dbcAMP	N^6-2'-O-dibutyryl adenosine cyclic-3',5'-monophosphate
EDF	erythroid differentiation factor (=activin)
EGF	epidermal growth factor
FSH	follicle-stimulating hormone (follitropin)
G protein	GTP-binding protein
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
IGF	insulin-like growth factor
IP_3	inositol trisphosphate
kb	kilo base
kDa	kilo Dalton
LH	luteinizing hormone (lutropin)
LHRH	LH-releasing hormone
mRNA	messenger ribonucleic acid
pFF	porcine follicular fluid
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PMA	4β-phorbol-12-myristate-13-acetate
pMSG	pregnant mare serum gonadotrophin
RIA	radioimmunoassay
rSCCM	rat Sertoli cell-conditioned medium
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGP	sulphated glycoprotein
SSC	standard saline citrate
TGFβ	transforming growth factor β
VAD	vitamin A deficient

General introduction

1.1 Regulation of testis function

1.1.1 The mammalian testis

The testis is composed of two compartments: the seminiferous tubules, in which spermatogenesis takes place, and the interstitium, which is composed of blood vessels, nerve fibres, macrophages, endothelial cells, and also contains Leydig cells, which are the steroid-producing cells of the testis. The seminiferous tubules consist of spermatogenic epithelium formed by Sertoli cells and developing germ cells, surrounded by peritubular myoid cells. Sertoli cells are important for physical support of the developing germ cells. Furthermore, adjacent Sertoli cells form tight junctions, thus forming a barrier (the so-called Sertoli cell barrier or blood-testis barrier; Dym and Fawcett, 1970; Setchell et al., 1988), which provides the developing germ cells behind this barrier with a separate environment. Spermatogonia are found outside the blood-testis barrier, in the basal compartment, whereas leptotene spermatocytes start to migrate through the Sertoli cell barrier; the adluminal compartment contains more advanced spermatocytes and spermatids (Dym and Fawcett, 1970). In order to reach cells in the adluminal compartment, substances must pass through Sertoli cells, which thereby determine the composition of the spermatogenic micro-environment. Moreover, Sertoli cells produce a number of factors, which are important for germinal cells (see 1.1.2).

Two pituitary hormones are extremely important for the initiation and maintenance of spermatogenesis: follicle-stimulating hormone (FSH), one of the main regulators of Sertoli cell function (for a review see Means et al., 1976), and luteinizing hormone (LH), which regulates Leydig cell function. During the development of the testis, the physiological responses to FSH change. In the rat, from just before birth until about 15 days of age, FSH is important as a Sertoli cell mitogen (Orth; 1984). As the rat reaches the age of 15-20 days, the mitotic activity of Sertoli cells is greatly reduced, and the primary action of FSH is on maturation of Sertoli cells. During development, the synthesis of several protein products of Sertoli cells can be stimulated by FSH in culture and *in vivo* (for a review see Griswold, 1988). In adult rats, Sertoli cells appear relatively refractory to FSH, partly because the cells have an increased phospho-diesterase activity (Means, 1977); an important role in the maintenance of spermatogenesis of adult animals can be ascribed to testosterone (see e.g. Bartlett et al., 1989a). In response to FSH, Sertoli cells are thought to produce inhibin, a hormone which inhibits FSH secretion, thus forming a closed negative feedback loop with the pituitary gland (see section 1.3). In response to LH, Leydig cells produce testosterone, which inhibits LH production by the pituitary and is essential for the maintenance of spermatogenesis and the secondary sex characteristics.

1.1.2 Sertoli cells

The function of Sertoli cells in the testis concerns physical support of germ cells, the formation of the blood-testis barrier, and production of several factors. Sertoli cells synthesize and secrete a number of proteins thought to be essential in the maintenance and control of spermatogenesis, including transferrin (Skinner and Griswold, 1980), ceruloplasmin (Skinner and Griswold, 1983), sulfated glycoprotein 1 (SGP-1; Collard et al., 1988), sulfated glycoprotein 2 (SGP-2; Collard and Griswold, 1987), androgen binding protein (ABP; for a review see Tindall, 1985), and inhibin and activin (see section 1.3). Transferrin and ceruloplasmin are metal transport proteins, binding iron and copper ions, respectively. These proteins are needed to deliver iron and copper to spermatogenic cells, since the blood-testis barrier prevents access of serum transferrin and ceruloplasmin to the adluminal compartment. SGP-1 has been identified as the precursor for sulfatide/G_{M1} activator protein which is a necessary component in the degradation of glycosphingolipids (Collard et al., 1988), whereas the function of SGP-2 is still unknown; the latter protein is also produced by epididymal cells (Sylvester et al., 1984) and has been detected by immunofluorescence on mature spermatozoa. Effects of inhibin and activin will be discussed in section 1.3.3. Another important compound which is produced by Sertoli cells is lactate, which serves as an energy-yielding substrate for the developing germ cells (for a review, see Grootegoed and den Boer, 1989).

The production of Sertoli cell proteins and products such as lactate are regulated by a number of factors, including hormones (such as FSH and testosterone), growth factors (such as insulin-like growth factor 1), vitamins (vitamin A), and the presence of germ cells (see 1.1.4). In Chapter 6, the regulation of inhibin production in Sertoli cells by these factors will be summarized.

1.1.3 Spermatogenesis

Spermatogenesis is a continuous process, which leads to the production of spermatozoa. In each section of the seminiferous tubules in adult rat testes, 4 or 5 germ cell types are found in one of a series of well-defined associations, called the stages of the cycle of the seminiferous epithelium (Fig. 1.1). This cycle can be divided, on the basis of the morphology of spermatids (see below), into 14 stages (LeBlond and Clermont, 1952). In adult animals, each stage has a constant duration, and in the Wistar rat the complete cycle lasts 12.8 days (Hilscher et al., 1969). The production of spermatozoa starts with periodic divisions of undifferentiated type A spermatogonia. After a period of quiescence, most of the undifferentiated spermatogonia differentiate into type A_1 spermatogonia, whereas the remaining undifferentiated spermatogonia start to proliferate again to produce a new complement of differentiating spermatogonia in the next cycle. The type A_1 spermatogonia first

divide to give rise to 3 further generations of type A spermatogonia (called types A_2 to A_4). A mitotic division of A_4 spermatogonia leads to the formation of intermediate spermatogonia (In), which then divide to form type B spermatogonia. Type B spermatogonia divide and develop into primary spermatocytes, which go through the prophase of the first meiotic division, and are named preleptotene, leptotene, zygotene, pachytene, and diplotene spermatocytes. The first meiotic division results in 2 diploid secondary spermatocytes, which rapidly divide without DNA synthesis (second meiotic division) and yield haploid early round spermatids. The formation of spermatids is followed by a maturation process called spermiogenesis, which involves phases of acrosome development (steps 1-7), nuclear elongation and condensation (steps 8-15), and cytoplasma reduction (steps 16-19). Finally, the spermatids are released from the spermatogenic epithelium.

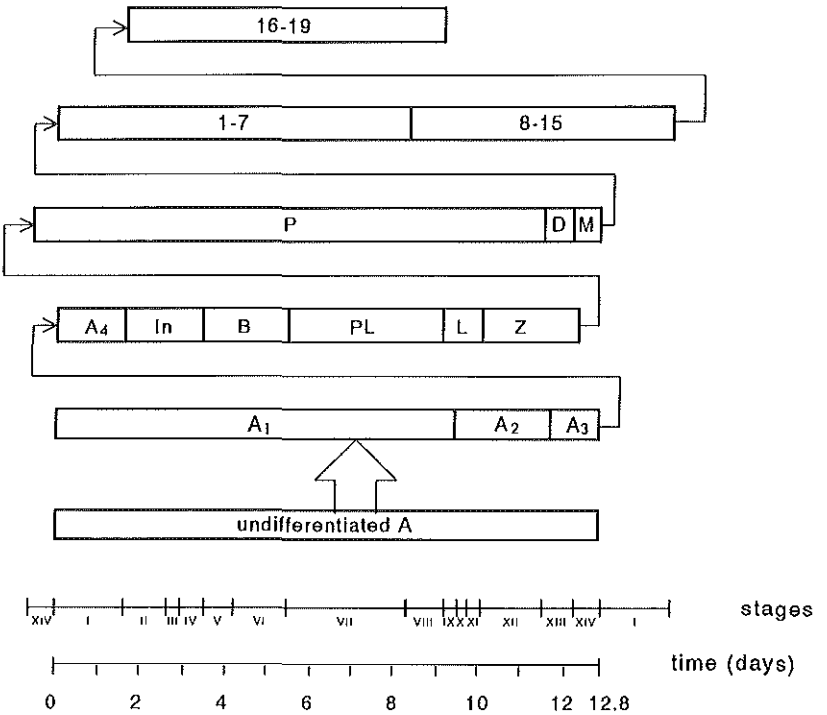

Fig. 1.1: Schematic representation of the 14 stages of the spermatogenic cycle of the Wistar rat (modified from Grootegoed, 1995) as defined by LeBlond and Clermont (1952). The stages are designated by Roman numerals. Abbreviations: A_{1-4} = type A spermatogonia, In = intermediate spermatogonia, B = type B spermatogonia, PL = preleptotene spermatocytes, L = leptotene spermatocytes, Z = zygotene spermatocytes, P = pachytene spermatocytes, D = diplotene spermatocytes, M = secondary spermatocytes undergoing meiotic division, 1-19 = spermatids at the successive steps of spermiogenesis.

1.1.4 Reinitiation of spermatogenesis in vitamin A deficient rats as a model for stage-dependent expression

Spermatogenesis is normally a process which is asynchronous in different parts of the seminiferous tubules, and hence provides a constant testicular output of sperm. Sertoli cells undergo functional changes (see below) in response to the requirements of each of the stages of the cycle of spermatogenesis. However, the simultaneous presence of all stages of the spermatogenic cycle in an entire testis complicates the biochemical determination of possible stage-dependent events. Therefore, manual dissection of rat testicular tubules has been used to separate portions of the tubules at different stages of the cycle of spermatogenesis (for a review, see Parvinen, 1982). Using this technique, stage-dependent production of, for example, ABP (Ritzen et al., 1982) and plasminogen activator (Lacroix et al., 1981) has been demonstrated. Alternatively, cross-sections of tubules were subjected to immunohistochemistry or *in situ* hybridization, and it was shown, for example, that transferrin mRNA was most abundant in Sertoli cells associated with stages XIII and XIV of the spermatogenic cycle, and that SGP-2 mRNA levels were highest at stages VII-VIII (Morales et al., 1987a).

Another approach to examine cyclic activities of Sertoli cells, is the use of stage-synchronized testes. When rats are subjected to a vitamin A deficient diet, degeneration and loss of germ cells takes place. The tubules of vitamin A-deficient rats only contain Sertoli cells, type A spermatogonia, and some spermatocytes (Morales et al., 1989; van Pelt and de Rooij, 1990). Retinol administration reinitiates a synchronized (Morales and Griswold, 1987b), but otherwise normal (Huang and Hembree, 1979) spermatogenesis. Instead of all 14 stages, only 3 to 4 closely related stages are present in the whole testis, and spermatozoa are produced during 2 to 3 days of every 12.8 days. Since the timing of the cycle remains intact, synchronized testes enriched in defined stages can be obtained simply by sacrificing the rats at various times after retinol replenishment. The advantage of this approach is the availability of large amounts of testicular tissue in defined stages, which allows characterization of the relationship between stage of the spermatogenic cycle and the levels of expression of low abundance proteins or mRNAs. Furthermore, when testes are studied at different time intervals after the re-start of spermatogenesis upon retinol administration, it is possible to study whether the appearance of a certain differentiated germ cell type is correlated with changes in expression of Sertoli cell products. The expression of transferrin and SGP-2 mRNAs in stage-synchronized rats was in agreement with the expression found with *in situ* hybridization at the different spermatogenic stages in normal rat testes (Morales et al., 1989). Furthermore, it was shown that the level of SGP-1 mRNA does not change significantly throughout the cycle. A possible disadvantage of this *in vivo* model in comparison with *in situ*

hybridization lies in difficulties in the interpretation of data, since mRNA isolated from synchronized testes originates from many cell types, and the mRNA encoding the protein studied may be expressed in more than one cell type. For example, the expression of the retinoic acid receptor α mRNA was not only demonstrated in Sertoli cells, but also in germ cells (Kim and Griswold, 1990).

1.2 Mechanisms of hormone action

Communication between cells is generally mediated through substances known as growth/differentiation factors, cytokines, hormones, and some other compounds including vitamin A and D. The mechanisms of action of these substances (ligands) involve the binding to receptors and activation of signaling pathways, resulting in changes in gene expression and/or cellular metabolism. A distinction can be made between intracellular receptors and plasma membrane-bound receptors. Small hydrophobic molecules, such as steroid and thyroid hormones or retinoids, readily enter the cell and bind to their respective receptors which are located in the cytoplasm or in the nucleus. Binding of the ligand results in activation of the receptor which then binds to a specific DNA sequence, named hormone-responsive element (for a review see Evans, 1988). In contrast, protein and peptide hormones are unable to traverse the cell membrane, and instead recognize and bind to receptors located on the surface of the cell. Thereby, the receptor serves as a communicative link between the outside and the inside of the cell. As a response to the binding of the ligand, protein and peptide hormone receptors stimulate signal transduction pathways by way of activating GTP-binding proteins (G proteins), or by activation of intrinsic receptor protein kinase activity (Fig. 1.2). The G protein-coupled receptors identified so far are characterized by having seven transmembrane segments connected by extracellular and intracellular loops (e.g. Parmentier et al., 1989). The transmembrane helices interact with each other to form a pocket, where the ligand binds to induce the conformational change responsible for activating the G protein. In contrast to most G protein-coupled receptors, gonadotrophin receptors contain a large extracellular domain that is involved in the binding of the large ligand molecule. After activation, the Gα subunit exchanges GDP for GTP and the heterotrimeric G protein dissociates into Gα and G$\beta\gamma$ subunits. Approximately 20 different α-subunits of active G proteins have been identified. These couple to many effectors including adenylyl cyclase, phospholipase C-β, responsible for the generation of the second messengers cAMP and diacylglycerol, which in turn activate the protein kinases A and C (PKA and PKC), respectively. In this section, mechanisms of protein hormone action will be discussed in more detail.

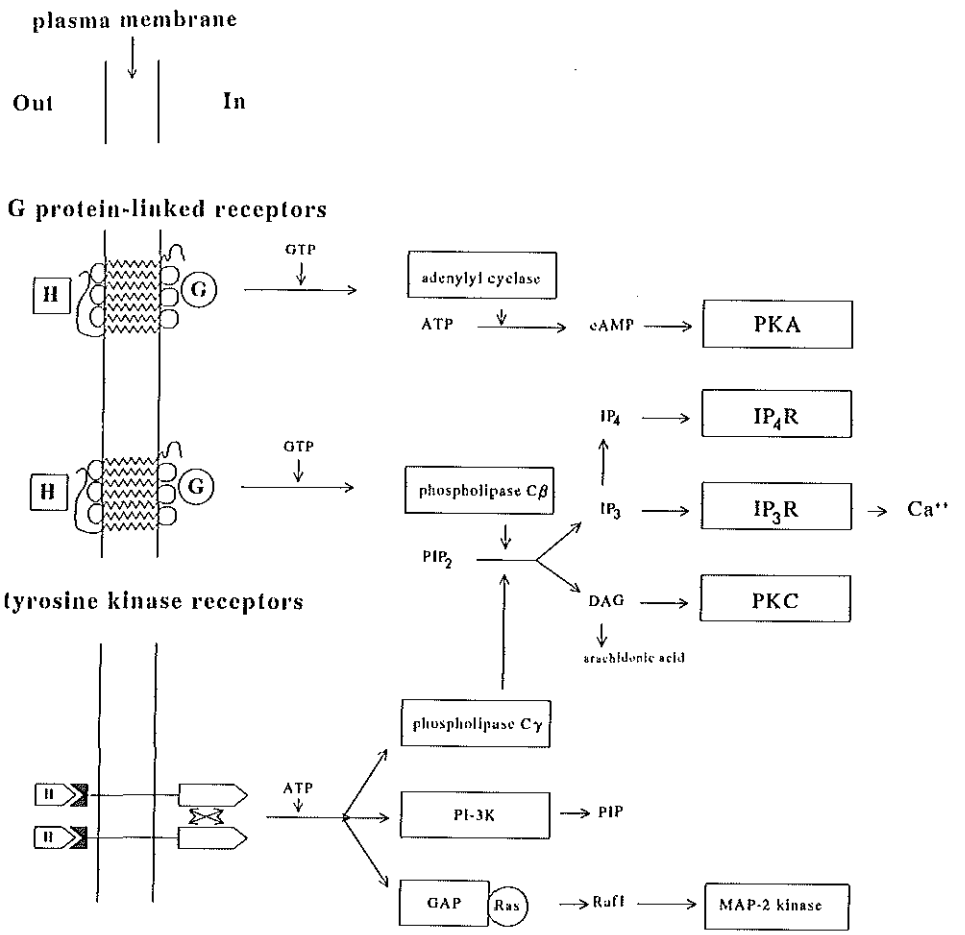

Fig. 1.2: Summary of the receptor-mediated pathways for stimulating the formation of cAMP, inositol trisphosphate (IP_3) and diacylglycerol (DAG). Many agonists bind to receptors with 7 transmembrane helices, which use a GTP-binding protein (G) to activate adenylyl cyclase or phospholipase C-β (PLC-β), whereas PLC-γ is stimulated by receptors with intrinsic tyrosine kinase activity. The latter type of receptors also activate other effectors such as phosphatidylinositol 3-OH kinase (PI-3K), which generates the putative messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and the GTPase-activating protein (GAP) that regulates Ras. For references, see the text. H, hormone; IP_3R, IP_3 receptor; PKC, protein kinase C; PKA, protein kinase A.

1.2.1 Protein kinase A

Many peptide and protein hormones such as adrenocorticotrophin (ACTH), thyroid- stimulating hormone (TSH), FSH and LH, exert (part of) their actions through activation of cAMP-dependent protein kinase (PKA; for reviews, see Birnbaumer et al., 1985; Meyer and Habener, 1993; Spaulding, 1993; Neer, 1995; and references therein). After binding of the hormone, the α-subunit of the G_s protein binds GTP and is released. The activated α_s-GTP associates with adenylyl cyclase, thereby activating this enzyme, which results in an increased conversion of ATP into cAMP. Binding of cAMP to the regulatory subunits (R) of protein kinase A holoenzymes liberates their catalytic subunits (C), which are then free to phosphorylate proteins. When the cAMP level falls, the R subunits regain their affinity for free C subunits and thus form inactive holoenzymes (R_2C_2). Prolonged exposure of cells to hormone leads to desensitization, which is a diminished response to the hormone despite its continuous presence. Desensitization is caused by downregulation of the number of hormone receptors, by an increased activity of phosphodiesterase which breaks down cAMP to adenosine monophosphate, by changes in the coupling of the G protein to the receptor, and/or by changes in the relative amounts of R- and C-subunits of protein kinase A (Øyen et al., 1988 and references therein).

A major nuclear target for PKA is the cAMP-responsive element binding (CREB) family of proteins, which bind to cAMP responsive elements (CREs) in the promoter regions of cAMP-responsive genes (for a review, see Roesler et al., 1988). Phosphorylation of CREB proteins alters their ability to form dimers and to interact with CREs.

A number of chemicals can applied on cultured cells to show whether action of a certain hormone is mediated through the protein kinase A pathway. For instance, forskolin activates adenylyl cyclase directly, whereas protein kinase A can be activated directly by analogs of cAMP such as dibutyryl cAMP and 8-bromo cAMP.

1.2.2 Protein kinase C

Another important signal transduction pathway which can be stimulated by G protein-linked hormone receptors, is the protein kinase C pathway (for reviews see Taylor and Marshall, 1992; Berridge, 1993). Activation of $G\alpha_q$ leads to stimulation of phospholipase C-β which hydrolyses the precursor phosphatidylinositol 4,5-bisphosphate (PIP_2). This reaction generates two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). While IP_3 binds to an IP_3-receptor (IP_3R) to mobilize stored calcium from the endoplasmic reticulum and to promote an influx of extracellular calcium, DAG activates protein kinase C (PKC). In addition,

DAG can serve as a precursor for the synthesis of arachidonic acid, which can be metabolized to eicosanoids such as prostaglandins and leukotrienes, which in their turn modulate phosphoinositide turnover or act as second messengers (Naor, 1991; Asoaka et al., 1992).

The PKC family consists of at least twelve members, all serine/threonine protein kinases (Dekker and Parker, 1994). A given set of these kinases is present in quiescent cells as cytosolic inactive enzymes, and these proteins are translocated to membranes when cells are stimulated. Each isoform has specific requirements for calcium, DAG and phosphatidylserine, and displays specific tissue expression. Some PKC isoforms are known to be the intracellular receptors for phorbol esters such as 4β-phorbol-12-myristate-13-acetate (PMA) Therefore, phorbol esters are often used as a tool to demonstrate involvement of PKC in the regulation of protein production. It should however be noted, that chronic activation of PKC by phorbol esters leads to cellular differentiation or proliferation, and downregulation of protein kinase C. Responses of specific mRNAs to stimulation of PKC should therefore be studied after a short time period (minutes to a few hours). A protein kinase C inhibitor used for the studies described in Chapter 3 is staurosporine (Tamaoki et al., 1986).

Functional effects of increased intracellular free calcium levels are exerted through calcium binding proteins (for a review, see Clapham, 1995), most importantly calmodulin (Veigl et al., 1985) which undergoes a conformational change after binding calcium, and subsequently interacts with enzymes dependent on calcium/calmodulin. Direct binding of calcium to calcium-dependent enzymes is also possible. The involvement of calcium in hormone action can be studied using calcium ionophores (for example A23187), which cause increased intracellular calcium levels by influx of extracellular calium. Calcium-channel blockers, or the use of calcium-depleted culture media, can prevent hormone-induced increase of intracellular free calcium.

The formation of DAG does not only occur through conversion of PIP_2. Calcium mobilizing agonists also induce rapid hydrolysis of phosphatidylcholine, generating DAG, and phosphatidic acid which itself could be a potential mediator (for a review see Billah and Anthes; 1990). Phospholipase C represents only one subfamily of the phospolipase family. Other phospholipases such as phospholipase A and D are now known to be also involved in the formation of DAG (for a review see Naor et al., 1991; Mobbs et al., 1991).

1.2.3 Receptors with kinase activity

Receptors with intrinsic protein kinase activity consist of a single membrane-spanning domain, an extracellular ligand-binding domain, and an intracellular protein kinase domain. Until now, two families of receptor kinases are distinguished: tyrosine kinases and serine/threonine kinases. Receptor tyrosine kinases, such as the receptors for platelet derived growth factor and epidermal growth factor (for reviews see Ullrich and Schlessinger, 1990; Schlessinger, 1993; Heldin, 1995), contain two kinase domains. Binding of the hormone to receptor tyrosine kinases induces dimerization of receptors, allowing the two receptors to phosphorylate each other on specific tyrosine residues, which provides docking sites responsible for interaction with different members of the SH2 family. This family consists of proteins which contain domains called *src* homology 2 and 3 (SH2 and SH3) which are found in many proteins concerned with signal transduction, such as GTPase-activating protein (GAP), phosphatidyl-inositol 3-OH kinase (PI-3K) and phospholipase C-γ. After phosphorylation on specific residues, phospholipase C-γ hydrolyses PIP_2 to yield IP_3 and DAG, thus stimulating the protein kinase C pathway.

The family of receptor serine/threonine kinases includes the TGFβ type I and II receptors, activin type I, II and IIB receptors, anti-müllerian hormone type II receptor, and Daf1 (for reviews see Lin and Lodish, 1993; Grootegoed et al., 1994; Massagué et al., 1994; Matthews, 1994; Heldin, 1995). These receptors have relatively small extracellular domains in comparison with the receptor tyrosine kinases; the sequences of these domains are very divergent. Type I receptors all have a characteristic region rich in glycine and serine residues (GS domain) in their cytoplasmic juxtamembrane domains. The C-terminal extensions of the type II receptors are serine/threonine rich, suggesting that they could be sites of autophosphorylation. Signaling by receptor serine/threonine kinases occurs by cooperation of type II receptors and type I receptors. A type II receptor shows a constitutive kinase activity and binds the ligand first. This complex then recruits a type I receptor, which can only bind ligand in the presence of a type II receptor. The type I receptor is subsequently phosphorylated by the type II receptor on serine residues in the GS domain. This phosphorylation presumably activates the type I receptor kinase that now can act on downstream components in the signal transduction pathway. Attisano et al. (1993) identified two type I receptors which both bind activin in the presence of activin type II receptor. One of these (ActR-I) mediates only transcriptional response to activin. The other type I receptor binds activin, but also TGFβ when associated with the TGFβ II receptor, indicating that some type I receptors can be shared by different members of the TGFβ/activin family. Recently, it was shown that inhibin binds to the activin type II receptor through its β-subunit, and it was suggested that this binding results in a dominant-negative inhibition of assembly of the heterodimeric (type II-type I) activin

receptor complex, since the α-subunit of inhibin is incapable of binding to activin type I receptor (Xu et al., 1995).

Concluding remarks on signal transduction pathways

The above mentioned signal transduction pathways interact in several ways. Firstly, hormones can activate more than one pathway. Gonadotrophin-releasing hormone (GnRH) and LH, for example, stimulate both adenylyl cyclase and phospholipase C (for review, see Stojilkovic et al., 1994; Saez, 1994). Furthermore, activation of one signal transduction pathway can inhibit another pathway. For example, PKC inhibits FSH-stimulated cAMP production in Sertoli cells (Nikula et al., 1990). Finally, transcription factors might be activated through two signal transduction pathways: activator protein 2 (AP-2) mediates effects of both PKC and PKA (Imagawa et al., 1987).

1.2.4 Mechanisms of FSH action

The first, and most important, second messenger which was described as a mediator of FSH action in Sertoli cells is cAMP (for a review see Davies, 1981). Although the attention in most studies on the mechanisms of FSH action is focussed on cAMP-mediated effects, effects of FSH on the phospholipase C pathway have also been reported. Coupling of FSH to its receptor on Sertoli cells causes a decrease in Na^+/Ca^{++} exchange, presumably through effects on membrane potential (Grasso et al., 1991), thus suppressing intracellular free Ca^{++}. However, the net effect of FSH is an increase in the intracellular Ca^{++} level by stimulation of various plasma membrane calcium channels (Grasso and Reichert, 1989; Gorczynska and Handelsman, 1991). FSH effects on Ca^{++} levels appear therefore mediated through influx rather than mobilization of intracellular stores.

Although FSH by itself has no effect on the turnover of inositol phosphates in Sertoli cells from 15-day-old (Monaco et al., 1988) and 18-day-old rats (Quirk & Reichert, 1988), it inhibits accumulation of inositol phosphates in immature Sertoli cells once phosphatidyl inositol turnover is stimulated by serum derived factors (Monaco et al., 1988). Furthermore, FSH can inhibit PKC activity in Sertoli cells for 50% in both the soluble and particulate fractions (Galdieri et al., 1986). In mature rats, FSH does not affect formation of inositol phosphates (Monaco et al., 1988).

Therefore, it is suggested that, depending on the maturation of the Sertoli cells, FSH can also affect the phospholipase C/ Ca^{++} pathway.

1.3 Inhibin

"Inhibin is a glycoprotein 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).

1.3.1 The inhibin-family and the detection of its members

Purification

Although the existence of a testicular regulator of pituitary function was already postulated in 1923 by Mottram and Cramer, and the name 'inhibin' was used for a hydrophilic factor exerting this action in 1932 by McCullagh, the structure of this protein was not elucidated until 1985. Important information in the search for inhibin was the demonstration of inhibin bioactivity in ovarian follicular fluid, which provided a rich source of inhibin (de Jong and Sharpe, 1976). Almost a decade later, inhibin was purified from bovine (Robertson et al., 1985; Fuduka et al., 1986) and porcine (Ling et al., 1985; Rivier et al., 1985; Miyamoto et al., 1985) follicular fluid (bFF and pFF respectively). Two porcine inhibins (A and B) with molecular masses of 32 kDa were isolated, and each was shown to be a dimer composed of a common α-chain (18 kDa) and one of two distinct, but highly homologous, β-chains (βA, 14.7 kDa and βB, 14 kDa). The subunits are held together by a disulphide bridge. A similar 32 kDa inhibin (Fuduka et al., 1986; Robertson et al., 1986) as well as a 58 kDa species consisting of a 44 kDa α-subunit and a 14 kDa βA-subunit (Robertson et al., 1985) were isolated from bFF. In Fig. 1.3, the structures of the inhibin subunit precursors and mature subunits are shown, together with the most prevalent combinations.

During the purification of inhibin from pFF, side-fractions which stimulated FSH production in cultured pituitary cells were detected, and it was shown that the factor which exhibited the FSH-stimulating activity of these fractions consisted of 2 inhibin βA-subunits (Ling et al., 1986b; Vale et al., 1986) or both a βA- and a βB-subunit (Ling et al., 1986a), linked by a disulphide bridge. These factors were called activin A and activin AB respectively. Activin B, a homodimer of two βB-subunits of inhibin, was isolated from pFF several years later (Nakamura et al., 1992).

Several molecular weight forms of inhibin peptides exist in follicular fluid, as became evident during the initial purification of inhibin (Robertson et al., 1985; Fuduka et al., 1986), and also using immunoprecipitation of ^{35}S-labelled proteins from cultured rat granulosa cells followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Bicsak et al., 1988), two-dimensional electrophoresis and Western blotting (Miyamoto et al., 1986), or SDS-PAGE followed by detection of inhibin bioactivity in gel slices (Grootenhuis et al., 1989). The use of immobilized antibodies directed

against the α-subunit of inhibin for immuno-purification of inhibin subsequently resulted in the detection and purification of αC (the C-terminal part of the inhibin α-subunit precursor; Knight et al., 1989), pro-αC (the pro-sequence of the N-terminal part of the inhibin α-subunit linked by a disulphide bond to the C-terminal part of the α-subunit; Robertson et al., 1989; Sugino et al., 1989), αN (the N-terminal part of the inhibin α-subunit; Robertson et al., 1989) and 105 kDa and 95 kDa inhibin, consisting of a 50 kDa βA-subunit coupled to respectively a 57 kDa or 43 kDa α-subunit (Sugino et al., 1992).

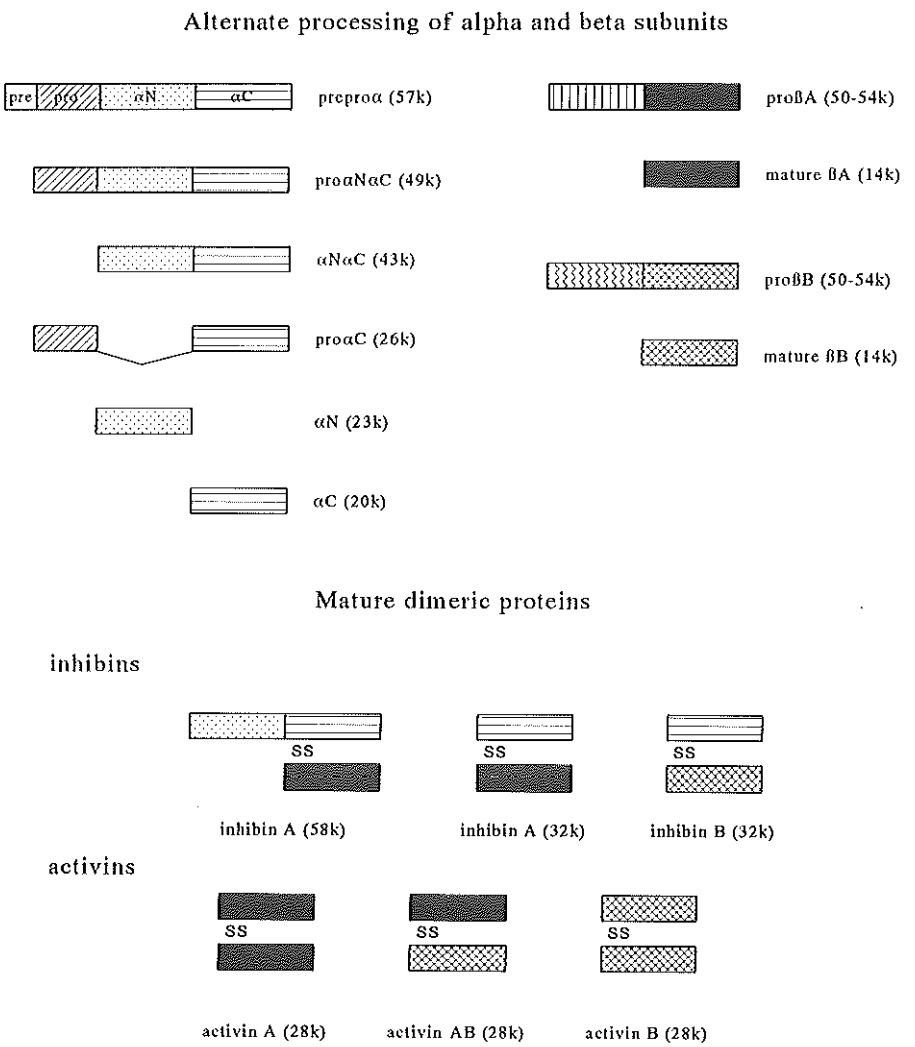

Fig. 1.3: Schematic representation of inhibin α- and β-subunit processing products and their combinations leading to the formation of mature inhibin and activin polypeptides.

Testicular inhibin was purified from ovine rete testis fluid by Bardin et al. (1987), who isolated two 30 kDa forms of inhibin, both consisting of an α- and a βA-subunit; one of these forms had a truncated α-subunit. Inhibin was also purified from rat Sertoli cell-conditioned medium (rSCCM) by Grootenhuis et al. (1990a); this 30 kDa inhibin was shown to consist of a combination of the α-subunit with the βB-subunit.

Gene structure

Using the N-terminal amino acid residues of the inhibin subunits to predict cDNA sequences, inhibin cDNAs from porcine (Mason et al., 1985), bovine (Forage et al., 1986), human (Mason et al., 1986), and rat ovaries (Esch et al., 1987a, Woodruff et al., 1987) and human placenta (Mayo et al., 1986) were cloned. The inhibin α-, βA-, and βB-subunits are encoded by separate genes and are synthesized as pre-pro-hormones. The βA- and βB-subunits showed a remarkable (approximately 70%) similarity in amino acid sequence, whereas the α-subunit amino acid sequence was less homologous with that of the β-subunits. The inhibin β-subunit genes are very conserved between species: the mature βA-subunits of human, porcine, bovine, and rat inhibin are identical, whereas the βB-subunit shows one amino acid difference between human and porcine inhibins and three amino acids difference between human and rat inhibin. The α-subunit is less conserved, but still shares more than 80% amino acid sequence-identity in the different species.

Homology studies identified inhibin as a member of the transforming growth factor β (TGFβ) superfamily; other members of this family of growth and differentiation factors are anti-müllerian hormone (AMH; Cate et al., 1986), the decapentaplegic gene product of *Drosophila* (Padgett et al., 1987), the *Xenopus* Vg-1 gene product (Weeks & Melton, 1987), and bone morphogenic proteins (Wozney et al., 1988). Later, erythroid differentiation factor (EDF) was shown to be identical to a homodimer of the βA-subunit of inhibin (Murata et al., 1988).

Elucidation of the genomic organization (Fig. 1.4; Table 1.1) of the inhibin-subunit genes revealed that all three genes contain only one intron which interrupts the region coding for the precursor portion of the respective subunits. An intron of 1.7 kb is present in the human (Stewart et al., 1986) and of 1.5 kb in the rat (Feng et al., 1989b; Albiston et al., 1990) α-subunit gene; it is located at a position corresponding to the encoded amino acid 91 in the precursor αN sequence. In the promoter region of this gene a CRE was found, together with AP-2 binding sites (Albiston et al., 1990; Pei et al., 1991), of which the function can be modulated by cAMP and phorbolesters (Comb et al., 1986; Imagawa et al., 1987), and a putative glucocorticoid responsive element (GRE; Albiston et al., 1990). One main mRNA species with a size of 1.6 kb is transcribed from the α-subunit gene, although occasionally a hybridizing band with a size of 3.1 kb is observed on Northern blots (Esch et al., 1987).

The rat βB-subunit gene contains an intron of 3 kb, which is located in the sequence coding for amino acid 154 (Feng et al., 1989b), whereas the intron in the human gene is located in the sequence coding for amino acid 169 and has a size of 2.5 kb (Mason et al., 1989). It was recently demonstrated, that two promoter regions are utilized for initiation of transcription of the βB-gene, resulting in two mRNAs of 4.2 and 3.5 kb, respectively (Feng et al., 1993a; Dykema & Mayo, 1994, Feng et al., 1995). The promoter region first reported (Feng et al., 1989a; Mason et al., 1989) is used to generate the 3.5 kb mRNA; the second promoter region is located 1050 bp upstream of the transcription start site reported for the 3.5 kb mRNA (Dykema & Mayo, 1994; Feng et al., 1995). In both rat βB-subunit promoters and in the human βB-subunit promoter, several GGGCGG repeats and their inverted sequences were found (Feng et al., 1989a; Mason et al., 1989), which are potential binding sites for the transcription factor SP1 (Dynan and Tjian, 1983). Genes which contain such sequences and lack TATA-boxes are usually involved in so-called housekeeping or in regulation of growth, and are constitutively active (Sehgal et al., 1988). The promoter that gives rise to the rat 4.2 kb βB-subunit mRNA contains two AP-2 binding sites (Dykema & Mayo, 1994), indicating that initiation of transcription is differentially regulated in the two βB-subunit gene promoters. Interestingly, the 4.2 kb βB-subunit mRNA contains three short open reading frames in the 5'-region, of which the function is still unknown. Possibly, these sequences affect translational efficiency or stability of the mRNA. Three CRE-core elements were identified in the promoter of the human βB-subunit gene, transcription from which is likely to result in the 3.5 kb mRNA (Mason et al., 1989). In contrast, in neither of the rat βB-subunit gene promoters the presence of a CRE was found.

In neither the α nor the βB-subunit gene a CAAT- or a TATA-box was found. Conversely, the human βA-subunit gene contains classical CAAT- and TATA-boxes. The rat βA-subunit gene has not yet been described; the human sequence contains an intron of 9-10 kb (Stewart et al., 1986; Tanimoto et al., 1991), which is located at a position corresponding to the amino acids 130 and 131. In the 5'-untranslated region of the gene, a CT repeat was found. Other possible regulatory elements were localized in the 3'-untranslated region of the gene, such as a putative AP-1 binding site, SV40 enhancer core elements, and CA repeats (Tanimoto et al., 1991). The human βA-subunit 3'-region is very homologous with the rat βA-subunit 3'-region, suggesting that these sequences indeed play a role in the regulation of this gene. Using a DNA fragment containing the putative AP-1 sequence in promoter studies, it was shown that this sequence acts as an activator element, dependent upon *c-jun* activity (Tanimoto et al., 1993). In the 3'-region of the βA-subunit gene, eight alternative polyadenylation signals are present, which account for the existence of βA-subunit mRNAs of 6.4, 4.9, 4.3, 3.0, and 2.0 kb (Tanimoto et al., 1993).

The α-subunit and βB-subunit genes have been assigned to human chromosome 2, and mouse chromosome 1, whereas the βA-subunit gene is located on human chromosome 7 and chromosome 13 of the mouse (Barton et al., 1989).

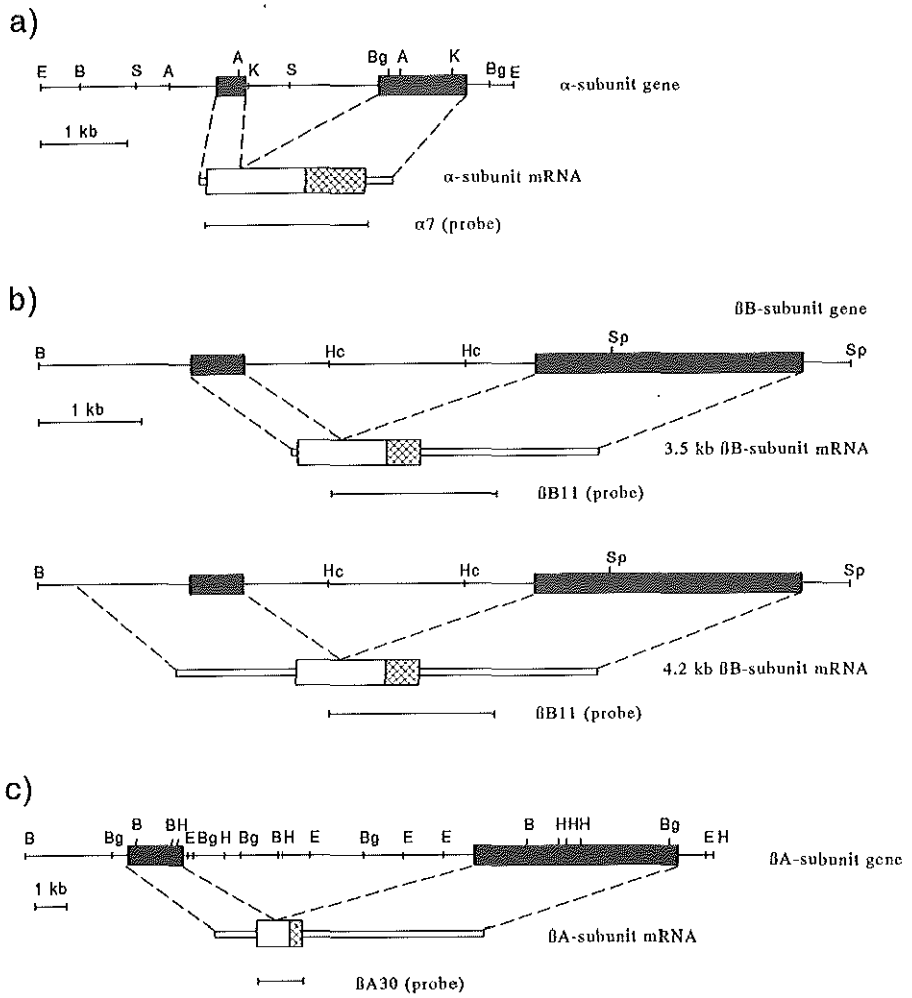

Fig. 1.4: The structure of the rat inhibin α-subunit (a) and βB-subunit genes (b), and of the human inhibin βA-subunit gene (c), are presented together with the structure of the corresponding mRNAs and the location of the cDNA probes used in the studies described in Chapters 2 to 5. Exons are indicated as closed boxes. Untranslated sequences of the mRNAs are represented as a thin open box. The coding region for the pro-sequences is indicated by a thick open box, and for the mature subunits by a shaded box. Restriction endonuclease sites indicated are: A, ApaI; B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; Hc, HincII; K, KpnI; S, SacI; Sp, SphI.

Table 1.1: *Sequence features of the inhibin genes. For the inhibin α- and βB-subunit genes, the rat sequences are described. For the rat βA-subunit gene, no information is avaible as yet; therefore features of the human gene are presented.*

Regulatory element	inhibin gene			
	α-subunit	βB-subunit 3.5 kb	βB-subunit 4.2 kb	βA-subunit
CAAT box	± (CTCAAT)	-	-	+
TATA box	± (TAGAA)	-	-	+
CT repeat	-	-	-	+
SV40 enhancer core element	3	-	-	3
polyadenylation signal	1	2	2	8
ATTTA (destabilization)	-	1	1	5
AP-1 binding site	-	-	-	1
AP-2 binding site	6	3	-	-
CRE	1	-	-	-
CRE-core element	6	-	-	-
SP1 binding site	3	6	4	1
GRE	1	-	-	-
CA/GT stretch	1	-	-	1

Numbers indicate the number of consensus sequences present in the gene.
± indicates the presence of a sequence which does not correspond to the consensus-sequence, but could be used for initiation of transcription instead of a "classical" CAAT/TATA box.

FSH-suppressing factors other than inhibin

During the purification of inhibin, fractions were found which inhibited FSH secretion by cultured pituitary cells, but did not bind to antibodies against inhibin. In 1987, these active proteins were purified and identified as members of another protein family (Robertson et al., 1987; Ueno et al., 1987), called follistatins or FSH-suppressing proteins (FSPs). Their amino acid sequences were determined using cDNA-analysis (Esch et al., 1987b). Follistatin exerts its action through binding of activin, thus preventing activin action (Nakamura et al., 1990). Follistatin also binds to inhibin, presumably through the β-subunit; activin has 2 binding sites for follistatin whereas inhibin has 1 binding site (Shimonaka et al., 1991). Most likely, binding of

both subunits of activin to follistatin is needed in order to abolish activin activity, since a two-fold molar excess is needed for neutralization of activin activity (Shimonaka et al., 1991; Xiao et al., 1992). Presence of follistatin has been demonstrated in a number of tissues (for a review see Michel et al., 1993) including the testis (Shimasaki et al., 1989) and the pituitary gland (Kogawa et al., 1991); it can also be detected in human serum and follicular fluid (Krummen et al., 1993).

In Sertoli cells, the steroid 3α-hydroxy-4-pregnen-20-one is produced, which has been reported to selectively suppress FSH secretion *in vivo* (Wiebe and Wood, 1987) and *in vitro* (Wood and Wiebe, 1989) with a lowest effective dose of 10^{-16}M. These observations have not been confirmed by other authors.

Detection methods

Several techniques are available at present for the detection of mRNAs encoding the inhibin subunits, and of inhibin and inhibin-related proteins.

There are mainly three techniques for the detection of inhibin subunit mRNAs. Northern blotting is commonly used for the detection of levels and sizes of inhibin subunit mRNAs. For this technique, RNAs are isolated, separated on agarose gels and blotted on filters; these are hybridized with labelled cDNA probes for the inhibin subunits. In this way, sizes of mRNAs can be determined and expression can be measured in a quantitative way using densitometry. *S1*-nuclease analysis uses hybridization of RNAs to a labelled inhibin RNA probe in solution, followed by degradation of single-stranded unhybridized probe and RNAs by *S1*-nuclease. Hybrids of cDNA probes/mRNAs, which are protected against degradation, are thereafter separated on gel. This so-called RNase protection technique is more sensitive than Northern blotting, but does not allow measurement of the size of the mRNAs. Finally, *in situ* hybridization involves the hybridization of probes to histological sections of tissues, and analysis with light microscopy. This technique is used to localize the expression of mRNAs in specific cell types.

Bioassays were the first assays used for detection of bioactive inhibin (for a review, see de Jong, 1988, and references herein). The bioassay most widely used at this moment is based on suppression of FSH release by cultured pituitary cells (for example see de Jong et al., 1979). A drawback of this method is interference with other substances which also affect FSH secretion or production. In the pituitary cell bioassay, activin stimulates the release of FSH; the amount of activin required to cause a significant change in the net inhibin bioactivity measured is logarithmically related to the inhibin concentration (Krummen et al., 1992). Therefore, absolute levels of bioactive inhibin cannot be determined when biological fluids are used which contain both inhibin and activin. Another protein which might interfere in the bioassay is follistatin, which also suppresses the release of FSH.

Another bioassay, which is mostly applied for the detection of activin, but which can also detect inhibin by its activin-counteracting activity, uses the human erythroleukemic cell line K562. Activin causes haemopoietic differentiation of this cell line, and therefore hemoglobin synthesis can be used as a measure of the amount of activin present in the sample (Schwall and Lai, 1990). Both inhibin (Krummen et al., 1992) and follistatin (Krummen et al., 1993) neutralize the effects of activin in this assay in a linear fashion, with a maximal neutralization at a two-fold excess of inhibin or follistatin.

Immunodetection is widely used to quantitate levels of inhibin and inhibin-related molecules. It was first employed to solve problems caused by the relatively low sensitivity of bioassays, and to circumvent problems with activin-containing mixtures in the bioassay (see above). Radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and immunoradiometric assay (IRMA) are quantitative assays based on the binding of inhibin to antibodies. Conventionally, these assays were used with one antibody directed against inhibin. The main problem of these one-site assays is specificity, since many of the antibodies recognize the α-subunit of inhibin, and different inhibin α-subunit containing molecules have different affinities for a given anti-inhibin antiserum (Grootenhuis et al., 1989). For example, the cross-reactivity of antiserum 1989, which was used in the studies described in Chapters 4 and 5, with pro-αC is 288% (Robertson et al., 1989; Schneyer et al., 1990). Two-site assays using antibodies directed against the α-subunit and against the β-subunit of inhibin circumvent this problem. Groome (1991) used an ELISA with anti-α as catching antibody and anti-β as detection antiserum, whereas Betteridge and Craven (1991) used anti-β as catching antibody, and biotinylated anti-α as detection antiserum. Immunoreactive values obtained using these assays were lower than obtained in conventional RIA, and the ratio between bioactive and immunoreactive inhibin was more constant in different inhibin-containing test samples (Betteridge and Craven, 1991), indicating that this immunoassay measures dimeric inhibin. However, a cross-reactivity of 5% of activin was found when anti-β was used as catching antibody. Furthermore, binding of follistatin to inhibin possibly caused lower values of immunoreactivity than expected on basis of bioactivity (Krummen et al., 1993). A sensitive two-site ELISA was developed using antibodies against recombinant inhibin A and activin A (Baly et al., 1993). In this assay, no cross-reactivity of activin was found, while follistatin did not interfere with binding of the antibodies. Finally, Knight et al. (1991) developed an IRMA, which also is specific for dimeric inhibin, but measured low values of inhibin immunoreactivity compared with bioactivity. Recently these autors (Knight et al., 1994) showed that low values result from selective binding of the anti-β antibody to the oxidized form of the peptide, which is the minor form of inhibin in fresh test samples. The sensitivity of the assay was improved by including a

pre-assay oxidation step; levels of bioactive and immunoreactive inhibin were fully correlated using this assay (Knight et al., 1994).

Since biological fluids often contain several inhibin-related proteins, which can result in variable ratios between bioactive and immunoreactive inhibin, it is important to know which inhibin-related proteins are present. Therefore, an important tool for the study of inhibin-containing samples is Western blotting, since this technique visualizes the inhibin immunoreactive proteins after separation by SDS-PAGE. Finally, localization of inhibin subunit proteins in several cell types can be achieved by immunohistochemistry.

From the above, it is clear that in inhibin bioassays and in conventional immunoassays, follistatin, activin, and subunit monomers, can interfere with the measurement of inhibin. Therefore, a combination of methods for the measurement of inhibin is needed for a reliable estimation of the amount of dimeric inhibin present in an unknown sample. For the studies described in this thesis, Northern blotting, Western blotting, the pituitary cell bioassay, and an inhibin RIA were used, in order to obtain information about the relationship between inhibin mRNA levels and inhibin production.

1.3.2 Production and regulation of inhibin production in the ovary

Production of inhibin and activin in vivo

Inhibin is produced in ovarian granulosa cells (for a review see Woodruff and Mayo, 1990). Initial immunohistochemical localization studies of inhibin within the ovary revealed that intense α-subunit staining occurred in the inner follicular granulosa cell layers, whereas no staining was seen in the theca layer outside the basement membrane (Cuevas et al., 1987). However, expression of the inhibin α-subunit mRNA was later detected at a low level in theca cells and interstitial gland cells (Meunier et al., 1988b). Inhibin α-subunit was also detected immunohistochemically in the luteal cells of the rat corpus luteum (Cuevas et al., 1987; Merchenthaler et al., 1987).

In cyclic rats, newly recruited follicles (which have the potential to ovulate 4 days later) express the inhibin α-subunit mRNA at a low level on the morning of oestrus. Once these follicles have been recruited by the secondary FSH surge, the expression of the inhibin α-subunit mRNA increases, whereas the expression of the βA- and βB-subunit mRNA is initiated (Meunier et al., 1988b). The increase of inhibin subunit mRNA expression is accompanied by increased serum inhibin levels, which eventually lead to the decline of FSH to basal levels. Half of the recruited follicles become atretric, and in these follicles the β-subunit mRNAs are undetectable, whereas the α-subunit expression is decreased to a low level. In the developing follicles, the

expression of α- and β-subunit mRNAs increases, until the preovulatory gonadotrophin surge occurs. This gonadotrophin surge is responsible for a dramatically decreased expression of the inhibin β-subunit mRNAs and an increased expression of the α-subunit mRNA on the evening of pro-oestrus, followed by a sharp decline of inhibin α-subunit mRNA expression in preovulatory follicles immediately before ovulation. At the same time, exclusively βA-subunit mRNA and immunoreactivity were found in large tertiary follicles, indicating that these follicles might produce activin A. These observations were accompanied by decreased serum inhibin levels during the period from pro-oestrus to oestrus (Rivier et al., 1989b). It was therefore postulated that a decrease in inhibin production (and increased activin production) is responsible for the secondary FSH surge early on oestrus. Following ovulation and luteinization, inhibin subunit mRNA expression remains low. In the corpus luteum, α-subunit mRNA and protein were found, whereas β-subunit mRNA expression was undetectable (for a review, see Woodruff and Mayo, 1990).

In the ovaries of acyclic immature and old persistent oestrous rats, high levels of inhibin subunit mRNAs were found. In old persistent oestrous rats, healthy and cystic follicles expressed α-subunit mRNA in theca cells, granulosa cells and stroma, whereas βA-subunit mRNA expression was found only in granulosa cells of healthy follicles. In immature rats, βA-subunit mRNA was present only in large follicles, whereas α-subunit mRNA was also expressed in small antral follicles. Expression of α-subunit mRNA in theca interna cells increased with age (Jih et al., 1993).

When immature rats were sequentially treated with pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG), administration of PMSG, which mimics FSH action, led to a rapid increase of α, βA and βB-subunit mRNA expression in large preovulatory follicles, whereas hCG treatment (to induce ovulation) caused a decrease in the levels of the mRNAs (Meunier et al., 1989). A further indication for a role of FSH in the regulation of inhibin subunit mRNA expression in the ovary, is the observation that in unilaterally ovariectomized rats a higher hybridization of α- and βA-subunit mRNAs was found in the remaining ovary (D'Agostino et al., 1989).

Production of inhibin and activin by cultured granulosa cells

As a model for regulation of inhibin production in female rats, cultured granulosa cells from immature diethylstilbestrol (DES)-primed rats are widely used. In this model, it was shown that ovine FSH stimulates the production of both bioactive and immunoreactive inhibin in a dose-dependent manner (Bicsak et al., 1986; Zhiwen et al., 1987). FSH action could be mimicked by addition of forskolin, dibutyryl cyclic AMP, prostaglandin E2 and 8-bromo-cAMP (Bicsak et al., 1986; Suzuki et al., 1987), which all act through stimulation of protein kinase A (see section 1.2.1). Testosterone

had no effect on basal immunoreactive inhibin production, but decreased the ED50 of FSH, whereas cortisol increased the ED50 (Suzuki et al., 1987).

FSH stimulated the expression of α, βA and βB-subunit mRNAs in cultured granulosa cells 60-fold, 70-fold and 66-fold, respectively, whereas testosterone had no effect (Turner et al., 1989; LaPolt et al., 1990). However, stimulation of βA-subunit mRNA expression by forskolin showed a different dose-response curve compared to the α-subunit mRNA; more forskolin was needed to stimulate βA-subunit mRNA expression than to stimulate expression of α-subunit mRNA to a half-maximal level (LaPolt et al., 1990). This suggests that another mechanism is responsible for the increase of βA-subunit mRNA after stimulation with FSH, which is also indicated by the absence of CREs in the βA-subunit promoter (see section 1.3.1). A high dose of PMSG (160-500 mU/ml) suppressed the expression of inhibin α- and βA-subunit mRNAs (Michel et al., 1991). This observation might explain the biphasic regulation of the inhibin subunit mRNAs by FSH during the oestrous cycle (see above).

When granulosa cells were first incubated with FSH to induce LH receptors, inhibin production was stimulated by LH and hCG. GnRH inhibited FSH-stimulated inhibin production (Bicsak et al., 1986), whereas 12-O-tetradecanoylphorbol-13-acetate, when added to cultured granulosa cells for three days (which in other studies was shown to inhibit protein kinase C activity), also inhibited immunoreactive inhibin production (Suzuki et al., 1987). Effects of LH and GnRH were also found on the level of inhibin α- and βA-subunit mRNA expression (LaPolt et al., 1990).

IGF-1 and insulin (in a 100-fold higher concentration than IGF-1), vasoactive intestinal peptide (Bicsak et al., 1986), and platelet extract (Suzuki et al., 1987) stimulated basal- and FSH-stimulated inhibin production, whereas EGF inhibited basal and FSH-stimulated inhibin production (Bicsak et al., 1986; Zhiwen et al., 1987). Finally, FGF had no effect (Bicsak et al., 1986). EGF also inhibited inhibin α- and βA-subunit mRNA expression (LaPolt et al., 1990). IGF-1, however, had no effect on the inhibin subunit mRNAs, and therefore it was suggested that this factor exerts its action through posttranscriptional actions. When bFGF was used at a higher concentration than tested for effects on immunoreactive inhibin, it decreased basal and FSH-stimulated inhibin subunit mRNA expression (LaPolt et al., 1990).

Activin and TGFβ stimulated basal- and FSH-stimulated α- and βA-subunit mRNA expression and inhibin production (Zhiwen et al., 1988; LaPolt et al., 1989; Xiao & Findlay, 1991). It was shown that activin by itself had no effect on cAMP accumulation. However, in combination with FSH or forskolin, a further stimulation of cAMP levels by activin was found. Activin action was therefore suggested to be mediated partly through the protein kinase A pathway.

1.3.3 Production of inhibin by testicular cells

Following the initial description of inhibin as a testicular hormone (McCullagh, 1932), most cell types in the testis have been suggested as a source of inhibin. Most of these suggestions were based on circumstantial evidence from *in vivo* experiments. A number of authors reported increased levels of FSH after damage of specific germ cell populations. For example, spermatocytes and/or early spermatids were suggested as a source of inhibin since FSH levels were increased in busulfan-treated rats (Gomes et al., 1973) and in men with insufficient numbers of spermatids (Franchimont et al., 1972). Likewise, mature spermatids (Swerdloff et al., 1971) and residual bodies (Johnsen, 1964) were considered to be sources of inhibin. Furthermore, Sertoli cells and/or spermatogonia were indicated as inhibin-producing cells, because levels of FSH were increased in men after chemotherapy (van Thiel et al., 1972), in rats with Sertoli cell damage (Rich and de Kretser, 1977), and in Sertoli cell-only patients, the latter with a further increase after castration (Christiansen, 1975). In 1976, Steinberger and Steinberger demonstrated that cultured Sertoli cells secrete inhibin-like activity. This observation was confirmed and extended by Le Gac and de Kretser (1982), who showed that the secretion of inhibin by Sertoli cells could be stimulated by FSH. After these observations, cultured Sertoli cells have been used to study regulation of inhibin production by many authors. Cultured immature Sertoli cells produce 30 kDa inhibin (Grootenhuis et al., 1990a), but also the α-subunit-derived proteins pro-αC (Grootenhuis et al., 1990b, de Winter et al., 1992a), and αN (Hancock et al., 1992).

Using immunohistochemistry, Cuevas et al. (1987) concluded that Sertoli cells are the only cells in the testis which contain inhibin α-immunoreactive molecules. From that time onwards, the picture has become more complicated. Risbridger et al. (1989a) showed that Leydig cells from adult rats also express α-subunit mRNA and produce immunoreactive and bioactive inhibin in culture. Using *in situ* hybridisation, Roberts et al. (1989b) showed that adult Leydig cell express α-subunit mRNA, but no βB-subunit mRNA. However, βB-subunit immunostaining was found in these cells (Roberts et al., 1989b). Furthermore, these authors detected α-subunit mRNA in 12-day-old Leydig cell clusters and βA-subunit mRNA in isolated interstitial cells. Treatment with hCG stimulated α-subunit mRNA but not βA-subunit mRNA expression in these animals (Roberts et al., 1989b). Lee et al. (1989) reported production of activin by cultured pig Leydig cells, and the presence of βA and βB subunit mRNAs in a tumor Leydig cell line. Thereafter, Shaha et al (1989) showed βA-immunoreactivity in interstitial cells in neonatal rats. Finally, de Winter et al. (1992a) showed that cultured Leydig cells from 21-day-old and adult rats only express α-subunit mRNA and secrete α-subunit protein, whereas Leydig cell tumors can produce bioactive inhibin. Maddocks and Sharpe (1989) concluded from a study in which EDS was used to destroy Leydig cells, that these cells contribute little to the

intra-testicular and blood levels of immunoreactive inhibin in the adult rat.

Possibly, confusion about the production of inhibin/activin by Leydig cells has arisen from the fact that the various authors used different cell isolation methods and therefore obtained different populations of interstitial cells. Sertoli cells also produce activin (de Winter et al., 1993), and therefore, the βB-subunit immunoreactivity described in adult Leydig cells (Roberts et al., 1989b) might be produced by and transported from Sertoli cells to Leydig cells. Finally, a recent study showed that cultured peritubular myoid cells express βA-subunit mRNA and produce activin A (de Winter et al., 1994). These cells might be a contaminating cell population in interstitial cell preparations.

1.3.4 Production and regulation of inhibin production in non-gonadal tissues

The production of inhibin was first thought to be restricted to the gonads. However, when S1-nuclease analysis was used, the expression of inhibin subunit mRNAs was demonstrated in many other tissues by Meunier et al. (1988a). It was shown that besides ovary and testis, also placenta (βA), pituitary (α, βA, βB), adrenal (α, βA), bone marrow (βA), spleen (α), kidney (α), brain (α, βA) and spinal cord (α, βA) express inhibin subunit mRNAs.

Extra-gonadal expression of inhibin α-subunit mRNA was first shown in the ovine adrenal cortex (Crawford et al., 1987). The same authors showed that ACTH stimulated α-subunit expression. Dexamethasone, which inhibits the production of ACTH, decreased α-subunit mRNA expression *in vivo*. Immunoreactive inhibin was present in the zonae glomerulosa and reticularis of the rat adrenal cortex (Merchenthaler et al., 1991); after treatment with ACTH, inhibin α-subunit immunoreactive material also appeared in the medulla. All three inhibin subunit mRNAs and proteins were detected in human fetal and adult adrenal cortices (Spencer et al., 1992). In addition, in mid-gestation fetal and neonatal adrenals, presence of activin A was demonstrated. In cultured fetal adrenals the expression of inhibin α-subunit mRNA was stimulated by ACTH and dbcAMP (Voutilainen et al., 1991; Spencer et al., 1992), whereas βA-subunit mRNA expression was spontaneously induced by culture of the cells, and was further increased by long term stimulation with phorbol ester (Voutilainen et al., 1991) or ACTH (Spencer et al., 1992). Concurrently, a rise in levels of immunoreactive α-subunit secreted by fetal and adult adrenal cells was observed (Spencer et al., 1992). Epidermal growth factor, fibroblast growth factor TGF-β and activin A had no effect on inhibin subunit mRNA expression (Spencer et al., 1992).

In the pituitary gland, inhibin might play a role as an autocrine regulator, since inhibin α-, βA-, and βB-subunit mRNA expression and immunoreactivity were

localized in the cytoplasm of FSH and LH immunoreactive pituitary cells (Roberts et al., 1989a; Corrigan et al., 1991). In these cells, oestradiol seemed to regulate the expression of inhibin subunits, since ovariectomy increased the size and number of these cells, and the expression of the mRNAs, whereas oestrogen treatment of ovariectomized animals prevented these increases (Roberts et al., 1989a). In cultured rat anterior pituitary cells, the predominant inhibin subunit produced was the βB-subunit. Treatment with phorbol ester or forskolin enhanced the rate of production of this subunit. Activin A caused a 30% inhibition of βB-subunit production, whereas inhibin A or follistatin increased the production of βB-subunit (Bilezikjian et al., 1993).

In the brain, βA-immunoreactive material was localized in the nucleus of the solitary tract (Sawchenko et al., 1988), where it might be involved in the control of oxytocin secretion.

The human placenta is a source of bioactive and immunoreactive inhibin (McLachlan et al., 1987), with a bio/immuno ratio different from that found in human follicular fluid. Petraglia et al. (1987) showed that inhibin is produced in the cytotrophoblast. Furthermore, in cultured human trophoblasts, production of immunoreactive inhibin was stimulated by hCG, dbcAMP and forskolin (Petraglia et al., 1987). In the decidua (Petraglia et al., 1990), α and βB-subunit mRNA expression and immunoreactivity were found, whereas sparse βA-subunit immunoreactivity was detected with a distribution pattern that differed from that of α- and βB-subunit immunoreactivity. The expression of inhibin subunit mRNAs increases throughout gestation.

With the unexpected presence of activin A in culture medium from the phorbol ester-treated human monocytic leukemia cell line THP-1 (Eto et al., 1987), studies of inhibin production and action in hematopoietic cells were started. Normal human monocytes also produce activin (Erämaa et al., 1992b). Furthermore, inhibin βA-subunit mRNA is present in bone marrow (Meunier et al., 1988a); in bone marrow stromal cells from several species inhibin βA-subunit mRNA, and activin A bioactivity and immunoreactivity were found (Shao et al., 1992; Takahasi et al., 1992; Yamashita et al., 1992). The expression of βA-subunit mRNA and production of activin in bone marrow stromal cells was stimulated by interleukin 1α, tumor necrosis factor α, lipopolysaccharide, and phorbol ester.

In cultured fibrosarcoma cells, the expression of βA-subunit was stimulated by cAMP. Since no CRE was detected in the 5'-region of the human βA-subunit gene, the effect of cAMP was postulated to be mediated through an increased stability of βA-subunit mRNA (Tanimoto et al., 1992).

1.3.5 Effects of inhibin and activin

Expression of inhibin and activin subunits takes place in many tissues (see above), from early embryogenesis (Roberts et al., 1991; Albano et al., 1993; Roberts and Barth, 1994) through adulthood. The responses which are evoked by inhibin and activin are very specific for the type of the responding cell. In short, two types of actions of inhibin and activin can be distinguished. The first type encompasses actions on differentiated cells. Effects of inhibin and activin on hormone production were found not only in the pituitary gland, but also in testis and ovary (see below). Furthermore, activin stimulated, whereas inhibin decreased, the production of GnRH, progesterone and hCG in placental cells (Petraglia et al., 1989). Activin stimulates insulin secretion in rat pancreatic islets (Totsuka et al., 1988), and stimulates glycogenolysis in rat hepatocytes by an increase of phosphorylase activity (Mine et al., 1989). Furthermore, oxytocin secretion is stimulated by activin (Sawchenko et al., 1988). The second type of action of inhibin and activin is found in differentiating and proliferating cells. Activin promotes differentiation of erythroid cells (Eto et al., 1987; Yu et al., 1987) and inhibits proliferation of thymocytes, whereas inhibin stimulates their proliferation (Hedger et al., 1989). Neural survival was promoted by activin (Schubert et al., 1990), whereas neural cell differentiation was inhibited (Hashimoto et al., 1990). Furthermore, activin induces axial mesoderm and anterior structures in explants from *Xenopus* and *chicken* blastulae (van den Eijnden-van Raaij et al., 1990; Smith et al., 1990; Thomsen et al., 1990). Inhibin inhibited meiotic maturation of oocytes *in vitro* (O et al., 1989), whereas follistatin stimulated maturation division and activin had no effect. However, in another study (Itoh et al., 1990) activin stimulated meiotic maturation. In the following section, effects of inhibin and activin in the pituitary gland and gonads will be discussed in more detail.

A relatively new approach to answer the question which actions of inhibin and/or activin are of crucial importance in development and reproduction, is the use of inhibin-subunit knock-out mice (Matzuk et al., 1992). Mice homozygous for a deleted α-subunit gene initially developed normally, but ultimately mixed or incompletely differentiated gonadal stromal tumors (Matzuk et al., 1992) and adrenal cortical tumors (Matzuk et al., 1994) were found in nearly every animal. On basis of these findings, inhibin was postulated to act as a tumor suppressor. Alternatively, the large amounts of activin produced in the gonads of these animals (Matzuk et al., 1994) might have a tumor-promoting effect. Mice which lacked a functional βB-subunit gene (Vassalli et al., 1993) and therefore are expected to be deficient in activin B, activin AB and inhibin B, were viable and fertile. However, a defect in eyelid development was observed, and offspring from mutant females showed a high perinatal lethality, likely as a result of impaired milk let-down by the mother. Activin βA-deficient mice developed to term, but died within 24 h after birth (Matzuk et al., 1995a). They

lacked whiskers and lower incisors and had defects in their secondary palates, and, as a result of the latter defects, failed to suckle. Mice deficient in all activins and inhibins (Matzuk et al., 1995a), exhibited a combination of the defects found in activin-βA and activin-βB deficient mice, but they had no additive defects over those seen in individual activin-βA or -βB mutant mice. This indicates that individual activin homodimers are not functionally compensating for one another during development. Furthermore, the activin βAβB heterodimer does not have a unique function during embryogenesis. Unexpectedly, mice deficient in activin receptor type II showed a phenotype different from mice deficient in inhibin and activin (Matzuk et al., 1995b). Most of these animals developed into adults. Their FSH levels were suppressed, but spermatogenesis occurred although the testes were small. These data show that spermatogenesis can occur without signal transduction through activin receptor type II (Matzuk et al., 1995b).

Effects of inhibin and activin on the pituitary gland

Inhibin was originally defined as a regulator of FSH release by the pituitary gland. As such, many reports have been presented on the effect of inhibin on the pituitary gland (for a review, see de Jong, 1988); in this paragraph more recent literature on effects of inhibin and activin on the rat pituitary gland will be presented.

The effect of inhibin on FSH production is most likely mediated through a specific lowering of FSH β-subunit mRNA expression (Attardi et al., 1989). Recombinant human inhibin A, when injected into adult male rats, first caused a decrease in FSH β-subunit mRNA expression (Carroll et al., 1991). However, a rebound was observed after 10 h. Administration of anti-inhibin antiserum increased FSH levels in immature rats, but had no effect on FSH levels in adult male rats (Culler and Negro-Vilar, 1988; Rivier and Vale, 1989). However, when EDS was first applied to abolish Leydig cell function, a further increase of FSH levels was observed after injection with the anti-inhibin antiserum, suggesting that in adult rats a relatively small contribution to the regulation of FSH is made by inhibin, which is masked by Leydig cell function (Culler and Negro-Vilar, 1990).

Activin stimulates FSH β-subunit mRNA expression in cultured pituitary cells (Carroll et al., 1989), which results in an increased FSH secretion (Ling et al., 1986a,b; Vale et al., 1986; Schwall et al., 1988). At a low cell density, the stimulation of FSH secretion by activin is higher than at a high cell density (Kitaoka et al., 1989; Katayama et al., 1990), suggesting that the cells are already stimulated by autocrine or paracrine factors such as activin B (see 1.3.4). *In vivo*, similar to the effect of inhibin, the effect of activin on FSH levels in the male rat seems to be dependent on age, since recombinant human activin A caused slightly increased FSH levels in immature male rats and had no effect on FSH levels in adult male rats

(Carroll et al., 1991).

In the female rat, inhibin plays an important role in regulation of FSH in adulthood, since immunoneutralization of endogenous inhibin by injection with anti-inhibin antiserum resulted in elevated FSH levels from 20 days of age onwards (Rivier et al., 1986; Culler and Negro-Vilar, 1988). In cyclic rats treated with anti-inhibin antiserum, ovulation rate was increased (Rivier and Vale, 1989; Sander et al., 1991), likely because of increased FSH levels. Activin stimulated FSH synthesis and secretion in both immature (Schwall et al., 1989; Carroll et al., 1991) and adult (Carroll et al., 1991; Rivier and Vale, 1991) female rats.

The effect of inhibin on LH secretion is unclear, since some *in vitro* studies show a slightly decreased LH secretion after addition of inhibin (Farnworth et al., 1988a; Attardi et al., 1989), whereas others find no effect of inhibin on basal LH release (Fuduka et al., 1987; Campen and Vale, 1988b; Farnworth et al., 1988b; Jakubowiac et al., 1990). However, since inhibin does not affect LH secretion *in vivo* (DePaolo et al., 1991; Rivier et al., 1991a,b; Robertson et al., 1991), and does not change LH β-subunit mRNA expression (Mercer et al., 1987; Attardi et al., 1989; Carroll et al., 1989), it is unlikely that inhibin action *in vivo* is directed towards LH secretion.

In addition to the direct effect on FSH production, inhibin decreases the number of GnRH receptors on gonadotrophs in the rat (Wang et al., 1988), which might be the reason for suppression of GnRH-induced secretion of both FSH and LH (Fuduka et al., 1987; Campen and Vale, 1988; Farnworth et al., 1988b; Kotsuji et al., 1988). Apart from gonadotrophs, activin also affects somatotrophs and corticotrophs. In primary cultures of rat anterior pituitary cells, biosynthesis (Billestrup et al., 1990) and secretion of growth hormone was inhibited by activin A (Vale et al., 1986; Kitaoka et al., 1988; Bilezikjian et al., 1990). Furthermore, activin suppressed ACTH secretion by cultured pituitary cells (Vale et al., 1986). These studies indicate that activin can play a role in regulation of multiple pituitary cell types.

Effects of inhibin and activin in testis and ovary

The first effect of inhibin on testicular function which was reported (Franchimont et al., 1981) was an inhibition of [^{3}H]-thymidine incorporation in testes from 42-day-old rats. Later these studies were extended by the observation that an inhibin-containing fraction reduced numbers of types A_4, I_n and B spermatogonia in testes of adult mice and Chinese hamsters (van Dissel-Emiliani et al., 1989), whereas numbers of undifferentiated spermatogonia at stages IV-VII were increased. Furthermore, possible effects of inhibin and activin are suggested by binding of these hormones to defined cell types: inhibin binds to leptotene and pachytene spermatocytes and to spermatids, whereas inhibin binding to spermatogonia was low (Woodruff et al., 1992). Binding of activin to spermatogonia was also low. Leptotene and zygotene

spermatocytes did not bind activin, whereas late pachytene and diplotene spermatocytes and spermatids showed a high binding of activin (Woodruff et al., 1992). In accordance with binding of activin, activin receptor type II mRNA is highly expressed in stage XIII spermatocytes, during meiotic division, and in early round spermatids steps 1-3 (de Winter et al., 1992c). The mRNA encoding the activin receptor type IIB is highly expressed in types A1 and A2 spermatogonia (Kaipia et al., 1993).

Effects of inhibin and activin in the testis are not restricted to germ cells. In long term cultures, activin inhibited LH-stimulated testosterone production by Leydig cells, while inhibin stimulated testosterone production (Hsueh et al., 1987). The inhibitory effect caused by activin was most likely due to inhibition of LH-induced cAMP formation (Lin et al., 1989). In cultured Sertoli cells, activin A inhibited FSH-stimulated aromatase activity and androgen receptor expression (de Winter et al., 1994). Furthermore, transferrin and inhibin secretion were stimulated by activin A (de Winter et al., 1994). Finally, activin A caused reaggregation of Sertoli cells into seminiferous tubule-like structures in Sertoli cell/germ cell cocultures (Mather et al., 1990).

In granulosa cells, activin caused induction of the FSH receptor (Hasegawa et al., 1988). Furthermore, inhibin production and cAMP levels (LaPolt et al., 1989), and FSH-induced LH receptor expression and progesterone synthesis (Sugino et al., 1988) were stimulated in these cells by activin. Inhibin reversed the effects of activin. Activin furthermore stimulated testosterone production in theca cells (Hsueh et al., 1987). Both activin receptors type II and IIB are expressed in the ovary (Feng et al., 1993b)

Apart from inhibin and activin, also parts of the inhibin α-subunit were shown to affect gonadal function. The monomeric αC-subunit protein inhibits FSH binding *in vitro*, and the response to FSH was inhibited (Schneyer et al., 1991). The αN-subunit might play a role in ovulation, since immunization against the αN-subunit impaired fertility in sheep (Findlay et al., 1989), as a result of a decreased ovulation rate (Findlay et al., 1994).

1.4 Aim and scope of this thesis

The general aim of the experiments described in this thesis was to study the regulation of inhibin production by Sertoli cells, since these cells are the most important source of inhibin in the male rat. Mechanisms of hormone action were studied in cultured Sertoli cells from immature rats, whereas effects of factors such as increased FSH levels and stage of the spermatogenic cycle were studied *in vivo*. In

Chapters 2 and 3, cultured Sertoli cells from immature Wistar rats were used to determine whether activation of protein kinase A, protein kinase C, and/or an increase in intracellular calcium levels regulate inhibin production. In Chapter 4, rats with stage-synchronized spermatogenesis were used to study effects of stages of the cycle of the spermatogenic epithelium on inhibin mRNAs and inhibin levels. Effects of hemicastration on inhibin production and FSH levels in immature rats of various ages were studied in Chapter 5. Finally, in Chapter 6 an overview is presented of current knowledge on the regulation of inhibin production. Furthermore, parallels are drawn between regulation of inhibin production in granulosa cells and Sertoli cells, and a model is presented in which the regulation of inhibin production in Sertoli cells is summarized.

Regulation of inhibin α- and βB-subunit mRNA levels in rat Sertoli cells.

I.A. Klaij, A.M.W. Toebosch, A.P.N. Themmen, S. Shimasaki, F.H. de Jong and J.A. Grootegoed.

Molecular and Cellular Endocrinology (1990) 68, 45-52

Summary

The hormonal regulation of the expression of the inhibin α-subunit and βB-subunit genes was studied in cultured rat Sertoli cells. The α-subunit mRNA level increased during incubation of the cells in the presence of follicle-stimulating hormone (FSH), reaching maximal levels within 1.5 h. This stimulation was mimicked by addition of dibutyryl-cyclic AMP, indicating that FSH action on the α-subunit gene is exerted via cyclic AMP. Inhibition of translation by cycloheximide (CX) caused upregulation of the α-subunit mRNA, and did not block the effect of FSH on the level of this mRNA. In FSH-stimulated cells, the half-life of the α-subunit mRNA was 6 h, and this half-life was prolonged by inhibition of transcription using actinomycin D (AD). It is concluded that the effect of FSH on α-subunit mRNA expression represents a direct effect on the α-subunit gene, and that α-subunit mRNA levels are influenced by a short-lived mRNA destabilizing protein.

The levels of two βB-subunit mRNAs (4.2 kb and 3.5 kb) were not affected by FSH or dbcAMP. However, these mRNAs were also upregulated by CX treatment. Experiments using AD showed that the 4.2 kb mRNA is less stable than the 3.5 kb mRNA.

The differential regulation of the inhibin α- and βB-subunit mRNAs is discussed.

Introduction

In the testis, Sertoli cells are thought to play a crucial role in the initiation and maintenance of spermatogenesis. This role may include the production and secretion of a variety of proteins. One of these proteins is inhibin, a heterodimer consisting of an α-subunit and a β-subunit (de Kretser and Robertson, 1989), which preferentially suppresses the secretion of follicle-stimulating hormone (FSH) by the pituitary gland. Two different β-subunits exist, βA and βB, which are encoded by separate genes (Mason *et al.*, 1986; Esch *et al.*, 1987a).

Apart from its action in the pituitary gland, inhibin has been suggested to play a paracrine role as a growth factor in the ovary (Ying *et al.*, 1986; Hutchinson *et al.*, 1987), and recent studies indicate that inhibin may exert biological effects on testicular cell types (Hsueh *et al.*, 1987; van Dissel-Emiliani *et al.*, 1989).

In the past few years, studies using bioassays or radio-immunoassays (RIA) for the estimation of inhibin have been performed, to elucidate the respective roles of different hormones in the regulation of inhibin production (Franchimont *et al.*, 1986; Ultee-van Gessel *et al.*, 1986; Bicsak *et al.*, 1987; Morris *et al.*, 1988; Toebosch *et*

al., 1988). From these studies it has become evident that FSH is the principal regulator of inhibin production in immature Sertoli cells. Bicsak *et al.* (1987) have shown that the stimulation of inhibin production by FSH can be mimicked by factors which increase intracellular cAMP levels.

Studies at the level of transcription may yield further information on the mechanisms by which the production of inhibin subunits is regulated. Recently, FSH has been shown to increase the α-subunit mRNA level in cultured Sertoli cells (Toebosch *et al.*, 1988; Keinan *et al.*, 1989). In contrast, the expression of the βB-subunit mRNAs was not influenced.

The aim of the present experiments was to study in more detail the molecular mechanisms which regulate the levels of the mRNAs encoding the inhibin subunits in cultured Sertoli cells.

Materials and Methods

Materials

Collagenase (CLS-I) was obtained from Worthington (Freehold, NJ, U.S.A.). Eagle's minimum essential medium (MEM) and non-essential amino acids were from Gibco (Grand Island, NY, U.S.A.). DNase (DN25), bovine serum albumin fraction V (BSA) and dibutyryl cyclic adenosine monophosphate (dbcAMP) were from Sigma (St. Louis, MO, U.S.A.). Actinomycin D was obtained from Boehringer Mannheim (Mannheim, F.R.G.), and cycloheximide was from BDH Chemicals (Poole, U.K.). Ovine FSH (NIH-S13) was a gift from NIADDK (Bethesda, MD, U.S.A.). Fetal calf serum (FCS) was purchased from Gibco (Paisley, U.K.).

Sertoli cell culture

Sertoli cell preparations were isolated from testes of 21- to 23-day-old Wistar rats (substrain RI Amsterdam), essentially as described by Oonk *et al.*, (1985). After incubation of decapsulated testis tissue in phosphate-buffered saline (PBS, Dulbecco and Vogt, 1954), containing DNase (5 μg/ml), and collagenase (0.5 mg/ml) for 30 min at 37°C, the tubule fragments were washed by sedimentation at unit gravity in PBS containing DNase, and then incubated under the same conditions for another period of 30 min, but using 1 mg/ml collagenase. Subsequently, the tubule fragments were washed twice, dounced, and the remaining small cell clusters were washed 3 times in PBS containing DNase by centrifugation for 2 min at 100 x g. The resulting cell preparation was plated in 150 cm^2 culture flasks (Costar, Cambridge, U.K.) at a

density of 25-30 μg protein/cm^2, and cultured in MEM supplemented with non-essential amino acids, glutamine, antibiotics, and 1% FCS, at 37°C under an atmosphere of 5% CO_2 in air. After 48 h of culture the cells were shocked hypotonically for 2.5 min in 0.1 x MEM to remove spermatogenic cells (Galdieri *et al.*, 1981; Toebosch *et al.*, 1988). The culture was continued for 24 h in MEM without FCS. Subsequently the cells were washed once with MEM, and incubated in the presence or absence of FSH (500 ng/ml), dbcAMP (0.5 mM), actinomycin D (5 μg/ml) or cycloheximide (50 μg/ml) for the indicated time periods.

Estimation of protein and RNA synthesis

Sertoli cells were isolated and cultured as described above. The cell preparations were incubated for 30 min in the presence or absence of cycloheximide or actinomycin D. Subsequently, 5 μCi/ml [5-^{3}H]uridine (final specific radioactivity 185 GBq/mmol, Amersham International, Little Chalfont, U.K.) or 0.5 μCi/ml L-[1-^{14}C]leucine (final specific radioactivity 0.2 GBq/mmol) was added to the wells. The incubations were stopped after 6 h by cooling to 4°C, removal of the medium, and lysis of the cells by addition of 1% sodium dodecyl sulphate (SDS) (w/v) in water containing an excess unlabelled uridine or leucine. Cellular protein and RNA were precipitated with 10% (w/v) trichloroacetic acid for 10 min at 4°C. The precipitate was washed 2 times with 10% trichloroacetic acid to remove non-incorporated precursors, and was dissolved in 10 ml Ultima Gold scintillation fluid (Packard, Downers Grove, U.S.A.) for estimation of radioactivity.

RNA isolation and analysis

After removal of the culture medium, cultured Sertoli cells were frozen on solid CO_2/ethanol, and stored at -80°C. Total RNA was isolated using a guanidinium thiocyanate extraction procedure (Chirgwin *et al.*, 1979), followed by centrifugation through cesium chloride (Glisin *et al.*, 1974), phenol/chloroform extraction and ethanol precipitation. 40 μg of each RNA sample was glyoxylated, electrophoresed and blotted on Genescreen (NEN, Boston, MA, U.S.A.) by diffusion (Thomas, 1980). Filter were baked for 2 h at 80°C, stained with methylene blue, and subsequently prehybridized for 6 h at 42°C in hybridization solution containing 50% formamide, 9% w/v dextran sulphate, 10 x Denhardt's (1 x Denhardt's contains 0.02% w/v Ficoll, 0.02 w/v polyvinyl pyrrolidone, 0.02% w/v BSA), 5 x SSC (1 x SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10 mM sodium phosphate (pH 6.8), and 100 μg/ml denatured salmon sperm DNA.

Probes for hybridization were labelled with ^{32}P by random oligonucleotide labelling (Feinberg and Vogelstein, 1983), denatured in boiling water for 5 min and added directly to the hybridization solution. Following overnight hybridization at 42°C the filters were washed to a final stringency of 0.1 x SSC, 0.1% SDS, 50°C. Filters were exposed to Amersham Hyperfilm-MP at -70°C for various lenghts of time, using an intensifying screen. Intensity of bands was estimated by scanning of the autoradiograms using a Bio-Rad video densitometer (model 620, Bio-Rad Laboratories, Richmond, CA, U.S.A.). The densitometric method was validated by hybridization of different known amounts of RNA and DNA with the cDNA probes. A linear relationship between amount of RNA and optical density was observed. Routinely, the loading of equal amounts of total RNA to the lanes was confirmed using an actin cDNA probe, or using staining of the gel with ethidium bromide.

The cDNA probes used were a 1.25 kb EcoRI-fragment encoding the α-subunit (α7/pUC18), and a 1.5 kb EcoRI-fragment encoding part of the βB-subunit (βB11/pUC18) of rat inhibin (Esch *et al.*, 1987a). Sizes of hybridizing mRNAs were a 1.6 kb species hybridizing with the α-cDNA probe, and two βB-subunit mRNAs (4.2 kb and 3.5 kb), as described by Toebosch *et al.* (1988). In addition, a very minor 3.5 kb mRNA hybridizing with the α-cDNA probe was found. The low expression of this mRNA did not allow quantitation with the densitometer.

Results

Time course of α-subunit mRNA stimulation by FSH and dbcAMP

Addition of FSH to immature rat Sertoli cells results in an increased expression of the inhibin α-subunit mRNA in a dose-dependent fashion (Toebosch *et al.*, 1988). In the present experiments, the time course of the increase in α-subunit mRNA levels after addition of a maximally stimulating dose of FSH was studied.

Fig. 2.1a shows a representative Northern blot, whereas Fig. 2.1b contains quantitative data, obtained in the experiment shown in Fig. 2.1a and two additional experiments by scanning of the Northern blots. A 4.5-fold stimulation of the α-subunit mRNA content was observed within 1.5 h ($P<0.005$, Bonferroni *t*-test), and no significant further change occurred during the following 22.5 h. The effects of 0.5 mM dbcAMP and 500 ng/ml FSH on the α-subunit mRNA were not significantly different (Fig. 2.1b). The expression of the two βB-subunit mRNAs was not affected by either FSH or dbcAMP (not shown).

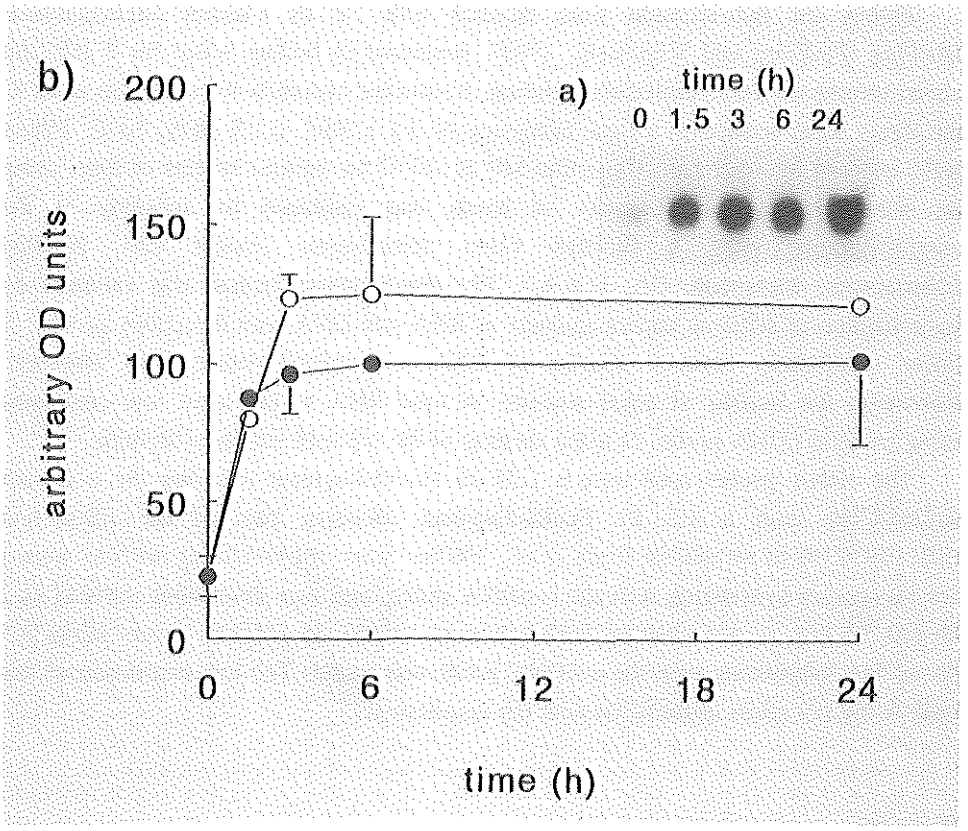

Fig. 2.1: Time course of the effect of FSH (●) and dbcAMP (○) on inhibin α-subunit mRNA expression in cultured Sertoli cells. FSH (500 ng/ml) or dbcAMP (0.5 mM) were added after 3 days of culture. RNA was extracted after different time intervals. Per lane, 40 μg of total RNA was electrophoresed, blotted and hybridized to the α-subunit cDNA probe. (a) A representative autoradiograph for FSH-stimulation is shown. (b) The relative amount of α-subunit mRNA at each time point was estimated by densitometric scanning of the autoradiographs, and expressed relatively to the optical density after 6 h of stimulation with FSH (mean ± SEM of three different experiments).

Inhibition of RNA and protein synthesis

The efficiency of the inhibition of RNA synthesis by actinomycin D (AD) and protein synthesis by cycloheximide (CX) was studied using the labelled precursors uridine and leucine. As shown in Table 2.1, AD inhibited incorporation of [^{3}H]uridine

into RNA 98 ± 1%, whereas CX inhibited leucine incorporation into protein 96 ± 2%. Addition of the inhibitors did not impair cell viability as indicated by a constant recovery of RNA from the cells and by the integrity of the mRNAs.

Table 2.1: ***Inhibition of synthesis of RNA and protein in cultured Sertoli cells by cycloheximide (CX) and actinomycin D (AD)***

The incorporation of [5-^{3}H]uridine and L-[1-^{14}C]leucine into RNA and protein of cultured Sertoli cells was estimated as described in Materials and Methods. Values represent the mean ± SEM of six incubations.

Inhibitor	Incorporation (dpm x 10^{-3}/well)	
	[^{3}H]uridine	[^{14}C]leucine
Control	36.4 ± 1.6	4.68 ± 0.69
CX (50 μg/ml)	-	0.15 ± 0.03
AD (5 μg/ml)	0.5 ± 0.02	-

Effect of inhibitors of transcription or translation on the stimulation of α-subunit mRNA by FSH

The requirement of ongoing RNA and protein synthesis for the stimulation of α-subunit mRNA by FSH was examined. Sertoli cells were preincubated for 30 min with AD or CX. Subsequently, the incubations were continued for 6 h in the presence of FSH, in the continuing presence of AD or CX. The results are presented in Fig. 2.2. The α-subunit mRNA level in non-stimulated cells (control) was not different from the α-subunit mRNA level in the presence of the transcription inhibitor AD. However, addition of AD completely abolished the stimulatory effect of FSH on α-subunit mRNA expression, affirming that ongoing transcription is necessary for the increased α-subunit mRNA level. The basal and FSH-stimulated levels of α-subunit mRNA were upregulated by inhibition of translation by CX (CX versus control, $P<0.005$; CX plus FSH versus CX, $P<0.05$; CX plus FSH versus FSH, $P<0.05$). It is discussed herein (see Discussion) that this upregulation can be caused by various factors. However, an important point is that inhibition of translation by CX did not block the FSH effect.

Effect of actinomycin D on α-subunit mRNA stability upon withdrawal of FSH

In Sertoli cells which had been preincubated for 24 h in the presence of FSH, the expression of the mRNA encoding the α-subunit markedly decreased during a subsequent 6 h of incubation in the absence of FSH in the medium. The time course of this decrease showed a half-life of the α-subunit mRNA of approximately 6 h (Fig. 2.3). Addition of AD stabilized the α-subunit mRNA during the 6 h incubation period in the absence of FSH ($P<0.001$ at 6 h; Fig. 2.3), as was the case in non-stimulated cells (Fig. 2.2).

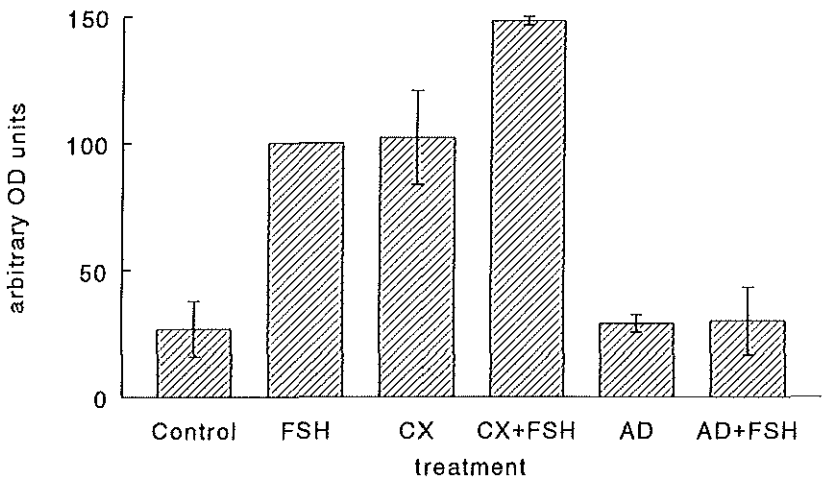

Fig. 2.2: Effect of actinomycin D (AD) and cycloheximide (CX) on FSH stimulation of α-subunit mRNA. After 3 days of culture, the Sertoli cells were preincubated for 30 min in the presence or absence of AD or CX, and subsequently incubated for 6 h with FSH (500 ng/ml) in the continuous presence of AD or CX. Per lane, 40 μg of total RNA was electrophoresed, blotted and hybridized to the α-subunit cDNA probe. The relative amount of α-subunit mRNA at each time point was estimated by densitometric scanning of the autoradiographs, and expressed relatively to the optical density after 6 h of stimulation with FSH in the absence of inhibitors (mean ± SEM of three different experiments).

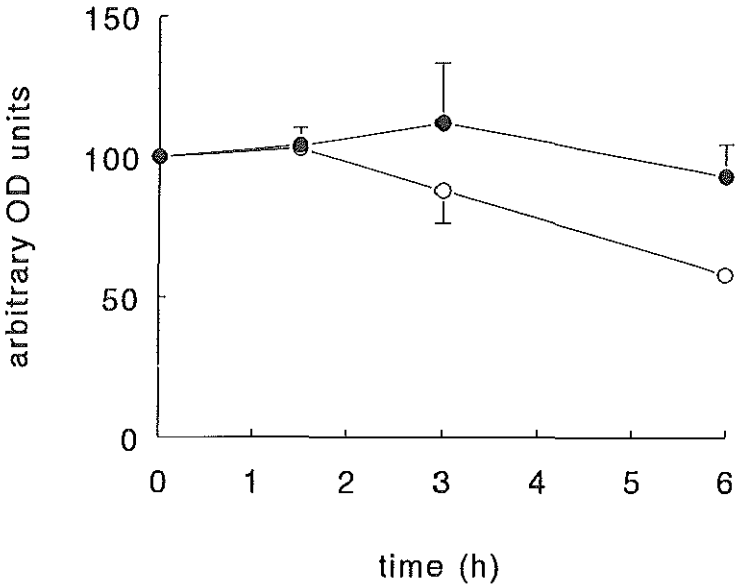

Fig. 2.3: Effect of actinomycin D (AD) on the stability of FSH-stimulated α-subunit mRNA levels. After three days of culture, the Sertoli cells were preincubated for 24 h in the presence of FSH (50 ng/ml)(t=0), and further incubated for different time periods in the absence of FSH, but in the absence (○) or presence (●) of AD. Per lane, 40 μg of total RNA was electrophoresed, blotted and hybridized to the α-subunit cDNA probe. The relative amount of α-subunit mRNA at each time point was estimated by densitometric scanning of the autoradiographs, and expressed relatively to the optical density after 24 h of stimulation with FSH (t=0) (mean ± SEM of three different experiments).

Effect of inhibitors of transcription or translation on the expression of the βB-subunit mRNAs

Inhibin βB-subunit mRNA expression in cultured Sertoli cells is not affected by FSH, insulin-like growth factor-I (IGF-I) or testosterone (Toebosch *et al.*, 1988). In the present experiments, the turnover and transcription of the two βB-subunit mRNAs were studied, using CX and AD. When the cells were cultured for 6 h in the presence of AD, a marked decrease in the expression of the 4.2 kb βB-mRNA was observed, while the 3.5 kb βB-subunit mRNA was decreased to a lesser extent (Fig. 2.4a and 2.4b). In the presence of CX the expression of both βB-subunit mRNAs was significantly increased (Fig. 2.4a), with a factor 2 ± 0.8 ($P<0.05$) after 1.5 h.

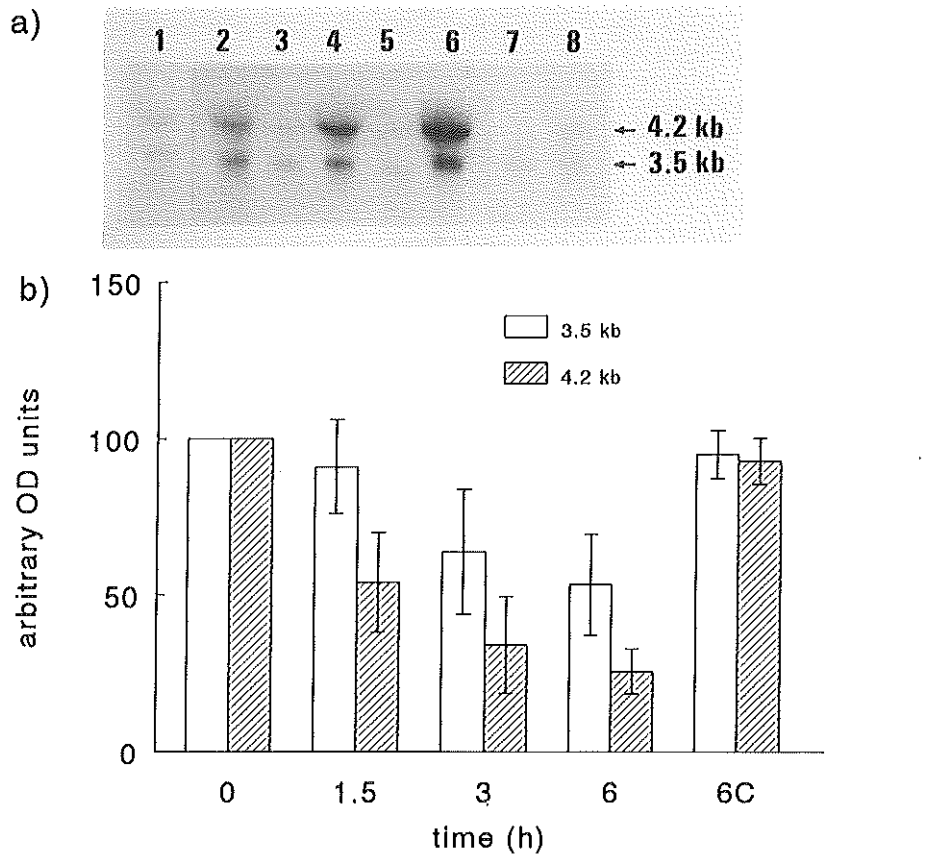

Fig. 2.4: Effect of actinomycin D (AD) and cycloheximide (CX) on the expression of 4.2 kb and 3.5 kb βB-subunit mRNAs. The cells were incubated for different time periods in the absence (C) or presence of AD or CX. Per lane 40 μg of total RNA was electrophoresed, blotted and hybridized to the βB-subunit cDNA probe. (a) A representative Northern blot is shown. Lanes: 1,8, Control; 2, CX 1.2 h; 4, CX 3 h; 6, CX 6 h; 3, AD 1.5 h; 5, AD 3 h; 7, AD 6 h. (b) Effect of AD: the relative amount of βB-subunit mRNA at each time point was estimated by densitometric scanning of the autoradiographs, and expressed relatively to the optical density at t=0 (mean ± SEM of four different experiments) (6C = Control at t=6 h).

Discussion

FSH is an important hormonal regulator of Sertoli cell function, which can stimulate the biosynthesis of a number of Sertoli cell proteins at the level of transcription, including the nuclear *c-fos* proto-oncogene (Hall *et al.*, 1988), androgen

binding protein (Joseph *et al.*, 1988), aromatase (Chan and Tan, 1987), the androgen receptor (Verhoeven and Cailleau, 1988; Blok *et al.*, 1989), and inhibin (Bicsak *et al.*, 1987; Toebosch *et al.*, 1988). FSH acts through activation of adenylyl cyclase, as indicated by a transient FSH-induced increase of the levels of intracellular cAMP in cultured Sertoli cells (Verhoeven *et al.*, 1980; Oonk *et al.*, 1985; Hall *et al.*, 1988), and by the stimulatory effects of cAMP derivatives which mimick FSH action.

In the present experiments, it was observed that FSH had a pronounced stimulatory effect on the expression of the α-subunit mRNA of inhibin within 1.5 h. This rapid FSH action was mimicked by dbcAMP, indicating that the cAMP response caused by FSH could mediate the increase in α-subunit mRNA expression. The promoter region of genes that are regulated via the cAMP-dependent protein kinase pathway contains a consensus sequence, the cAMP-responsive element (CRE) (Montminy *et al.*, 1986). Moreover, CRE binding proteins have been identified (CREBPs) which are involved in the effects of cAMP on gene expression (Hoeffler *et al.*, 1988; Yamamoto *et al.*, 1988). It is anticipated that sequence analysis of the promoter region of the α-subunit gene may reveal the presence of one or more CREs. However, transcriptional activation of the α-subunit gene may eventually prove to be a much more complex process, and may be modulated by other responsive elements and transcription factors. For example, the expression of *c-fos* and *junB* mRNA, which encode nuclear transcription factors, is increased in Sertoli cells by FSH (Smith *et al.*, 1989).

The protein synthesis inhibitor CX stalls mRNAs on polysomes (Pachter *et al.*, 1987). The experiments using this inhibitor show that stimulation of the expression of inhibin α-subunit mRNA by FSH does not require ongoing protein synthesis, and is therefore not mediated by hormone-induced synthesis of another, gene-regulatory, protein. However, the fold stimulation by FSH was decreased in the presence of CX. This may be explained by a CX-induced inhibition of the synthesis of transcription factors other than CREBP which could modulate the transcriptional activation of the α-subunit gene by FSH.

The observed upregulation of α-subunit mRNA expression by CX treatment does not seem to be a specific response of this gene. A similar effect of CX has been described for many other genes in different cell types (Lau and Nathans, 1987; Almendral *et al.*, 1988). Moreover, the expression of the 4.2 kb and 3.5 kb βB-subunit mRNAs increased during treatment of the Sertoli cells with CX. This CX-induced phenomenon may be explained by an inhibition of mRNA degradation, caused by CX-induced disappearance of a short-lived mRNA-destabilizing protein. It is also possible that inhibition of the synthesis of a short-lived transcriptional repressor protein results in an increased transcription rate. In Sertoli cells, CX has been shown to increase cAMP levels, in particular in the presence of FSH due to inhibition of

FSH-induced synthesis of phosphodiesterase (Verhoeven *et al.*, 1981). An effect of CX on cAMP levels may contribute to the upregulation of the inhibin α-subunit mRNAs by CX treatment. However, elevated cAMP levels cannot contribute to CX-induced upregulation of the inhibin βB-subunit mRNAs, since these mRNAs are not regulated by cAMP.

The half-life of the α-subunit mRNA (6 h) was estimated in FSH-stimulated cells after withdrawal of FSH. It should be noted that it is not certain whether removal of FSH by washing of the cells also results in a rapid loss of receptor-bound FSH and quenching of the FSH effect. Therefore, the actual half-life of the mRNA may even be shorter than the observed 6 h.

Treatment of the cells with AD, following the incubation with FSH, resulted in an increase of the half-life of the α-subunit mRNA. From this result, it is more clear that a short-lived mRNA-destabilizing protein may be involved in the degradation of the α-subunit mRNA. Such labile mRNA destabilizers have been identified in several cell types, controlling the turnover of different mRNAs (Brewer and Ross, 1989).

It was shown that, in the presence of AD, the βB-subunit mRNAs were not stable (Fig. 2.4a and 2.4b). The 4.2 kb βB-subunit mRNA had a shorter half-life than the 3.5 kb mRNA. The two different βB-mRNAs have not yet been cloned and sequenced; it is not known whether the different sizes represent differences in either the 3'- or 5'-untranslated regions or in the open reading frame of the mRNAs. Pending this information, it can be speculated that the difference in half-life of the two βB-subunit mRNAs may be caused by a different polyadenylation of the two mRNAs. Several polyadenylation signals (Tosi *et al.*, 1981) are present, approximately 450 bases apart, in the 3'-untranslated region of the βB-subunit cDNA (Esch *et al.*, 1987). Utilization of the first polyadenylation signal might result in the 3.5 kb mRNA, lacking a 3'-untranslated AU-rich sequence (AUUUA). It has been proposed that this sequence is a recognition site for a mRNA degradation pathway, which involves action of a labile protein (Shaw and Kamen, 1986). The larger 4.2 kb βB-subunit mRNA may contain the AUUUA consensus sequence and could therefore be more susceptible to degradation than the 3.5 kb βB-subunit mRNA.

In summary, the expression of the α-subunit and βB-subunit mRNAs is differentially regulated. Maximal expression of α-subunit mRNA requires a continuous stimulation with FSH, whereas the level of βB-subunit mRNAs does not seem to be subject to direct hormonal control. Furthermore, inhibition of transcription stabilizes α-subunit mRNA, but not the βB-subunit mRNAs. Besides regulation at the level of transcription, regulation of the stability of the α- and βB-subunit mRNAs seems to play an important role in determining the level of expression. It would be of interest to study whether the two βB-subunit mRNAs are regulated in a diverging manner.

Regulation of inhibin production does not only take place at the mRNA level.

Studies of the efficiency of translation of the different mRNAs will also be important to answer questions concerning rate-limiting steps in the production of inhibin.

Acknowledgements

This study was financially supported by the Dutch Foundation for Medical Research (Medigon). We gratefully acknowledge the gift of oFSH of the NIADDK.

Regulation of inhibin βB-subunit mRNA expression in rat Sertoli cells: consequences for the production of bioactive and immunoreactive inhibin.

Ingrid A. Klaij, Marianna A. Timmerman, Leen J. Blok,
J. Anton Grootegoed and Frank H. de Jong.

Molecular and Cellular Endocrinology (1992) 85, 237-246

Summary

In Sertoli cells from 21-day-old rats, the expression of the mRNA encoding the α-subunit of inhibin, and the production of immunoreactive inhibin are stimulated by FSH. In contrast, the amount of βB-subunit mRNA is not increased after FSH treatment of the cells, and the ratio between bioactive and immunoactive inhibin decreases after stimulation with FSH. These data suggest that the βB-subunit is the limiting factor in the production of bioactive inhibin. The aim of the present experiments was to investigate the effect of changes in the amount of βB-subunit mRNA on the production of bioactive and immunoreactive inhibin.

During early postnatal testicular development, the relative amounts of the 4.2 kb and 3.5 kb mRNAs encoding the βB-subunit of inhibin changed markedly. The meaning of this changing ratio between βB-subunit mRNAs is not clear, since both mRNAs are actively translated, as demonstrated by polysomal analysis. The total amount of βB-subunit mRNA correlated with the in vitro production of bioactive inhibin as published earlier.

Prolonged stimulation of cultured Sertoli cells from 14-day-old rats with 4β-phorbol-12-myristate-13-acetate (PMA), caused a decreased expression of the βB-subunit mRNAs, presumably by down-regulation of protein kinase C. A similar effect was obtained after addition of the calcium ionophore A23187. Concomitantly, a decreased production of bioactive inhibin was observed. Furthermore, Western blotting revealed, that secretion of the 32 kDa inhibin αβ-dimer was decreased, whereas secretion of the combination of the C-terminal part with the pro-region of the α-subunit was increased.

It is concluded that the level of the βB-subunit of inhibin is rate-limiting for the production of bioactive inhibin in cultured Sertoli cells, and that its expression can be influenced by modulation of protein kinase C, and/or intracellular calcium levels.

Introduction

In the testis, Sertoli cells produce the dimeric glycoprotein inhibin, which can suppress the production of FSH by the pituitary gland. One known regulator of inhibin production by Sertoli cells is FSH, as was demonstrated by the observation that the production of immunoreactive inhibin by cultured Sertoli cells could be stimulated by FSH (Bicsak *et al.*, 1987). However, only the expression of the mRNA encoding the α-subunit of inhibin was stimulated by FSH, presumably through an increase of cAMP, whereas the expression of the βB-subunit mRNAs was not affected by FSH or dbcAMP (Toebosch *et al.*, 1988; Klaij *et al.*, 1990). Furthermore, testicular βB-subunit mRNA levels were not

changed significantly in hypophysectomized rats (Krummen *et al.*, 1989), whereas the α-subunit mRNA level in the testes of these animals was decreased and could be restored to normal by injection of FSH (Krummen *et al.*, 1989; Keinan *et al.*, 1989).

When proteins from Sertoli cell conditioned medium were separated by SDS-polyacrylamide gelelectrophoresis (SDS-PAGE), it was shown that the secretion of bioactive inhibin was not increased significantly by FSH (Grootenhuis *et al.*, 1990b). Other authors found, possibly depending on the Sertoli cell culture system used, a stimulation of bioactive inhibin of 1.5 fold (Toebosch *et al.*, 1988) to 2-fold (Handelsman *et al.*, 1989).

In contrast, in all studies the inhibin immunoreactivity was elevated dramatically after stimulation with FSH (Bicsak *et al.*, 1987; Toebosch *et al.*, 1988; Risbridger *et al.*, 1989b; Grootenhuis *et al.*, 1990b), and thus the ratio between inhibin bioactivity and immunoreactivity was decreased under these conditions. These observations suggest that the expression of the βB-subunit gene may be limiting for the production of bioactive inhibin. Therefore, in the present study it was investigated whether a change in the expression of the inhibin βB-subunit mRNAs can affect the bioactivity and molecular species of the secreted inhibin and inhibin related proteins. Furthermore, we studied the polysomal distribution of the inhibin subunit mRNAs in order to ascertain if only one, or both, of the βB-subunit mRNAs are translated in Sertoli cells.

Materials and Methods

Materials

4β-phorbol-12-myristate-13-acetate (PMA), was from Sigma (St. Louis, MO, USA). The calcium ionophore A 23187 (A23187) was obtained from Boehringer (Mannheim, Germany). Staurosporine was purchased from Serva Feinbiochemica (Heidelberg, Germany).

Sertoli cell culture

Sertoli cell preparations were isolated from testes of 14-day-old Wistar rats essentially as described by Themmen *et al.* (1991). In short, 48 decapsulated testes were incubated for 20 min in a shaking waterbath at 37°C in 20 ml phosphate-buffered saline (PBS), containing DNase 1 (5μg/ml; DN25, Sigma), collagenase (1 mg/ml; CLS, Worthington, Freehold, NJ, USA), trypsin (1 mg/ml; TRL, Worthington) and hyaluronidase (1 mg/ml; I-S, Sigma). After four successive washes by sedimentation at unit gravity in 40 ml PBS/DNase, the tubular fragments were incubated in 20 ml PBS/DNAse containing 1

mg/ml collagenase and 1 mg/ml hyaluronidase. The fragments were washed four times, and dispersed using a dounce homogenizer (ten strokes). The cells were then washed four times with PBS/DNase, and once with Eagle's minimal essential medium (MEM; Gibco, Grand Island, NY, USA) by sedimentation at 100 x g for 2 min.

The resulting cell preparation was plated in 150 cm^2 culture flasks (Costar, Cambridge, UK) at a density of 25 - 30 μg protein/cm^2, and cultured in MEM supplemented with non-essential amino acids, glutamine, antibiotics, and 1% FCS, at 37°C under an atmosphere of 5% CO_2 in air. After 48 hours of culture the cells were shocked hypotonically for 2.5 min in 0.1xMEM to remove spermatogenic cells (Galdieri *et al.*, 1981; Toebosch *et al.*, 1988). The culture was continued for 24 h in MEM without FCS. Subsequently the cells were washed once with MEM, and incubated as described in the Results section.

RNA isolation and analysis

After removal of the culture medium, cultured Sertoli cells were frozen on solid CO_2/ethanol, and stored at -80°C. Testes of rats of various ages were snapfrozen in liquid nitrogen, and stored at -80°C. Total RNA was isolated using an acid guanidinium thiocyanate-phenol-chloroform extraction procedure as described by Chomczynski and Sacchi (1987). Of each sample, 40 μg of total RNA was denatured in formamide/formaldehyde at 55°C for 15 min, prior to electrophoresis on denaturing 1 % agarose/formaldehyde gels. After electrophoresis, RNA was blotted on Hybond N+ (Amersham, Buckinghamshire, UK) by diffusion. Filters were baked for 2 h at 80°C, and subsequently prehybridized for 2 h at 42°C in a hybridization solution containing 50% formamide, 9% w/v dextransulphate, 10xDenhardt's (1xDenhardt's contains 0.02% w/v Ficoll, 0.02% w/v polyvinyl pyrrolidone, 0.02% w/v BSA), 5xSSC (1xSSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10 mM sodium phosphate (pH 6.8), and 100 μg/ml denatured salmon sperm DNA.

Probes for hybridization were labelled with ^{32}P by random oligonucleotide labelling (Feinberg and Vogelstein, 1983), denatured in boiling water for 5 min and added directly to the hybridization solution. Following 48 h of hybridization at 42°C the filters were washed to a final stringency of 0.1xSSC, 0.1% SDS, 50°C. Amersham Hyperfilm-MP was exposed to the filters at -70°C for various lengths of time, using an intensifying screen.

Intensity of bands was measured by densitometric scanning as described previously (Klaij *et al.*, 1990); the presence of equal amounts of RNA was confirmed by staining of the gel with ethidium bromide. When RNA from cultured Sertoli cells was analysed, values were normalized against values obtained by hybridization with a 1 kb hamster β-actin cDNA probe as an internal standard.

The cDNA probes used were a 1.25 kb EcoRI-fragment encoding the α-subunit (α7/pUC18), and a 1.5 kb EcoRI-fragment encoding part of the βB-subunit (βB11/pUC18) of rat inhibin (Esch *et al.*, 1987a).

Analysis of polysomal distribution of inhibin mRNAs

Methods used were essentially as described by Kew *et al.* (1989). Testes were homogenized at 4°C in 1 ml HKM buffer (20 mM HEPES, 100 mM KCl, 20 mM $MgCl_2$) containing 0.5% Triton X-100, 3mM β-mercaptoethanol, 300 U/ml RNase-inhibitor (RNAsin, Promega, Madison, WI, USA), and 10 mM EGTA to inhibit RNases. After centrifugation for 15 min at 10.000 g to pellet nuclei, supernatants were applied on to a linear gradient of 10 - 50% (w/v) sucrose in HKM buffer. Gradients were centrifuged in a Beckman SW-40 rotor for 2 h at 36,000 rpm. To determine nonspecific association of transcripts with polysomal fractions, postnuclear supernatants were also prepared and separated using buffers in which $MgCl_2$ was replaced with 10 mM EDTA (HKE buffer). After centrifugation, gradients were collected in fractions of 1.5 ml. RNA in these fractions was precipitated by addition of 1 vol iso-propanol. Precipitates were resuspended in 0.1% SDS/10 mM Tris (pH 7.5)/1 mM EDTA, and extracted twice with phenol and once with chloroform. Finally each fraction was ethanol-precipitated, and dissolved in water. Equivalent percentages of total RNA from each fraction were then denatured and electrophoresed as described above.

Analysis of inhibin-forms

Media of Sertoli cell cultures were collected after 24 h of incubation. The samples were concentrated, using disposable cartridges with YM10 membranes (molecular weight cut-off at 10 kDa; Centriprep 10; Amicon, Lexington, MA, USA), and proteins were separated on 15%, 0.75 mm thick SDS-polyacrylamide gels as described by Laemmli (1970) using the Mini-Protean II system (Biorad, Richmond, CA, USA). Subsequently the separated proteins were blotted, using the same system, onto Problott (Applied Biosystems, Foster City, CA, USA) at 100V in 1h. Immunostaining was performed as described by van Laar *et al.* (1989), using an antiserum directed against the 22 N-terminal amino acid residues of the αC-subunit of 32 kDa bovine inhibin (Grootenhuis *et al.*, 1989), or an antibody against the 20 C-terminal amino acid residues of the pro-part of the rat inhibin α-subunit precursor (de Winter *et al.*, 1992a).

Inhibin bioassay

Inhibin bioactivity was determined in an in vitro bioassay, in which suppression of spontaneous FSH-release from cultured rat pituitary cells was estimated (Grootenhuis *et al.*, 1989). Before measurement, 0.01% BSA was added to the Sertoli cell culture media, which were subsequently exchanged and concentrated, using Centriprep 10 membranes, in order to remove PMA, A23187, or staurosporine, since these substances affect gonadotropin secretion by pituitary cells. In control experiments with media, to which these agents had been added, it was shown that concentrated media did not change FSH secretion. Charcoal-treated bovine follicular fluid preparation with an arbitrary potency of 1 U/μg protein was used as a standard in the bioassay (Grootenhuis *et al.*, 1989).

Statistical analysis

All data have been presented as means $\pm$ SEM (n=3-4). The significances of differences between results of various treatments were assessed using Student's *t*-tests. Differences were considered significant if $P<0.05$ (two-tailed).

Results

Developmental expression of inhibin subunit mRNAs

As shown in Fig. 3.1a, the relative amounts of the 4.2 kb and 3.5 kb βB-subunit mRNAs (Toebosch *et al.*, 1988) changed markedly during early postnatal testis development; a decrease of the 4.2 kb transcript was accompanied by an increase of the 3.5 kb species during the first 3 weeks of life. The total amount of βB-subunit mRNA decreased after the age of 14 days (Fig. 3.1), and did not change further after 28 days of age. The amount of 1.6 kb α-subunit mRNA per mg total RNA was relatively constant until the age of 21 days, and decreased only slightly afterwards. Optical density of hybridizing bands was not normalized against the β-actin signal, since the testicular concentration of this mRNA also changes with age (Slaughter *et al.*, 1987)

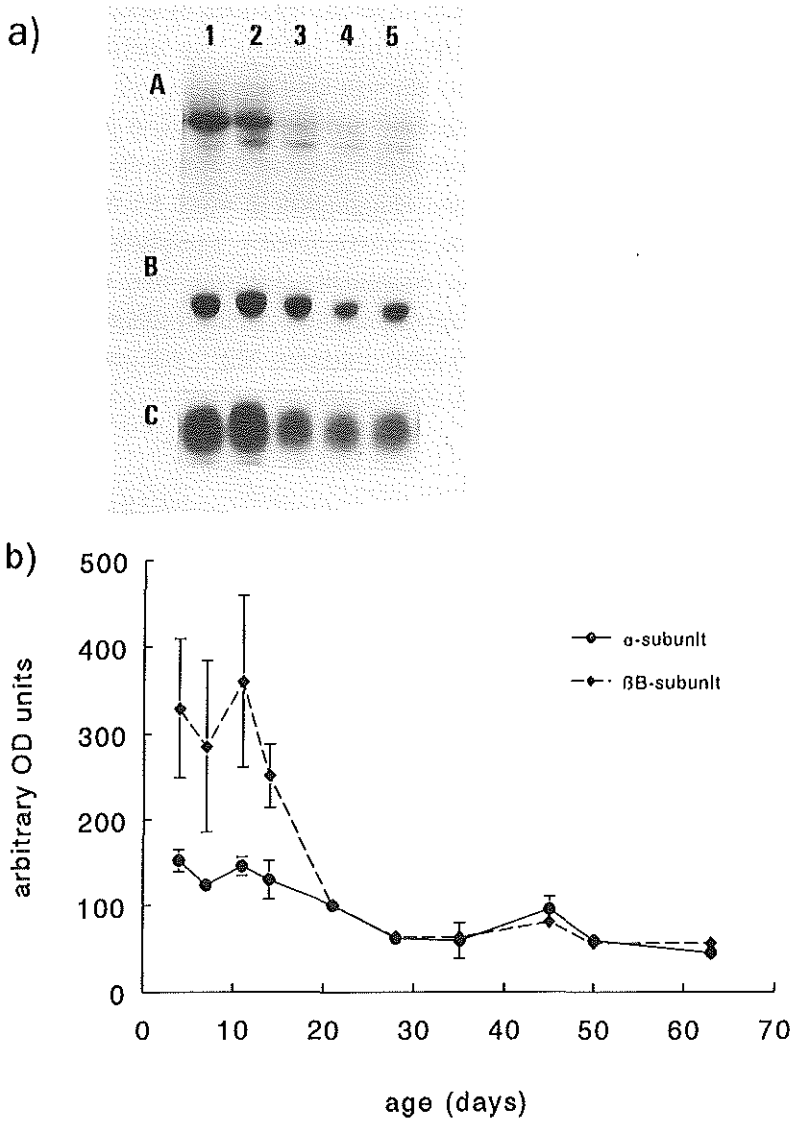

Fig. 3.1: Expression of inhibin α-subunit and βB-subunit mRNA in total RNA from testes of rats of different ages. (a) Per lane, 40 μg of total RNA was applied, and Northern blots were hybridized with probes for the inhibin βB-subunit (A), α-subunit (B) and actin (C). Numbers above the lanes indicate the age of the rats in weeks. (b) Relative OD values (day 21 = 100) of autoradiograms of Northern blots of testes from rats of various ages, hybridized with probes for the α- and βB-subunit of inhibin. Values represent means ± SEM of three RNA preparations, each extracted from pooled testes from 3 - 10 rats.

Distribution of inhibin subunit mRNAs in different fractions of sucrose gradients

In order to study whether both βB-subunit mRNAs are actively translated, centrifugation of postnuclear supernatants through continuous sucrose gradients was performed. In this way, polysomes, monosomes, and ribonucleoprotein fractions can be separated. Fig. 3.2a shows the OD_{260} profiles of a gradient in which the polysomes are intact in the presence of Mg^{2+} (HKM), and of a gradient in which the transcripts were dissociated from the ribosomes by addition of EDTA (HKE). Equivalent percentages of each fraction were applied on gel, and ethidiumbromide was used to confirm the intactness of RNA (not shown). Both βB-subunit mRNAs and the α-subunit mRNA were present in the polysomal fraction of the gradient containing Mg^{2+}, and could be detached from the ribosomes by addition of EDTA (Fig. 3.2b).

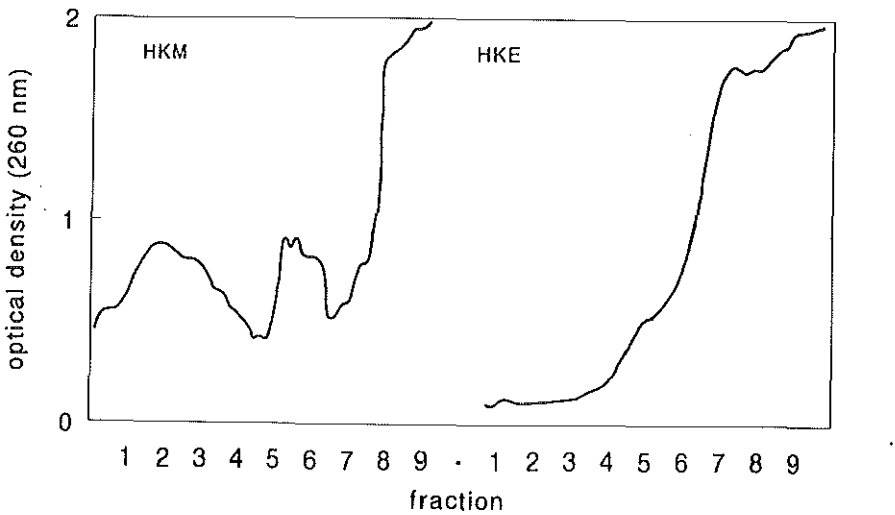

Fig. 3.2a: OD_{260nm} profiles of sucrose gradients of testicular polysomes. Intact polysomes (HKM) and dissociated polysomes (HKE) were isolated from 21-day-old rat testes. Fractions have been numbered from bottom (left) to top (right).

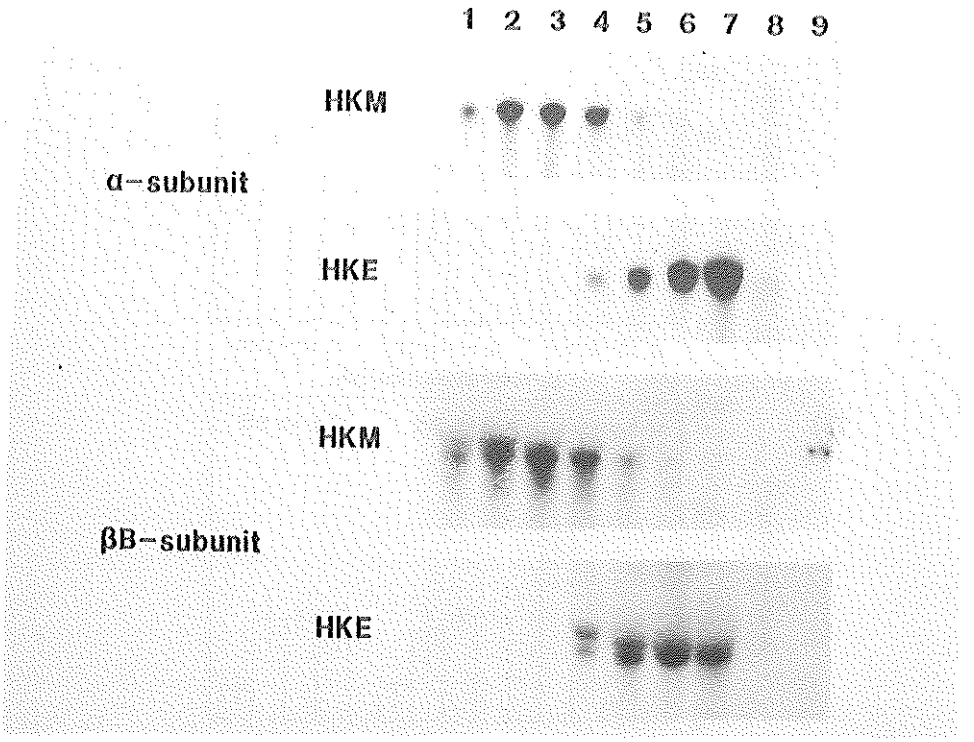

Fig. 3.2b: Polysomal distribution of inhibin α-subunit and βB-subunit mRNAs from rat testis. Fractions of sucrose gradients of polysomes were analyzed on Northern blot.

Effect of PMA and staurosporine on inhibin subunit mRNA expression

A possible role of the protein kinase C pathway in the regulation of the inhibin production was studied in cultured Sertoli cells, using the phorbol ester PMA, and the protein kinase C inhibitor staurosporine. After a 24 h preincubation period, the cells were incubated for 24 h in the presence of different concentrations of PMA or staurosporine. This incubation period was chosen, since some of the treatments suppressed the amount of inhibin bioactivity below the detection limit of the bioassay when cells were incubated for shorter time periods. As a control, the solvent of these agents, dimethylsulphoxide (DMSO), was added. Figs. 3.3a and 3.3b show the effects of PMA and staurosporine on the mRNA levels of the inhibin α- and βB-subunit. PMA caused a dose-dependent decrease of the amount of both βB-subunit mRNAs (4.2 kb mRNA: 10^{-8} M, $P<0.05$, 10^{-7} M, $P<0.025$; 3.5 kb mRNA, 10^{-7} M, $P<0.05$), whereas no effect on the α-subunit mRNA level was found. Staurosporine at a concentration of 10^{-7} M also caused a significant decrease of both βB-subunit mRNAs (4.2 kb mRNA $P<0.01$, 3.5 kb mRNA $P<0.005$). At this concentration, the level of α-subunit mRNA was significantly increased ($P<0.01$).

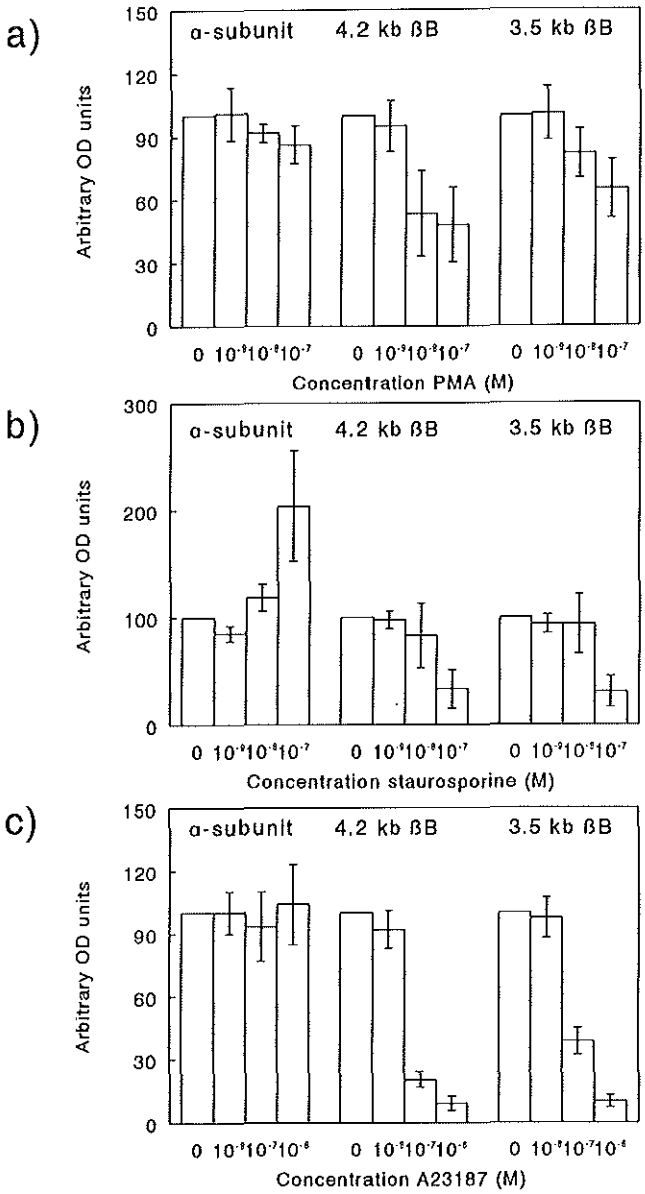

Fig. 3.3: Effect of PMA (a), staurosporine (b) and A23187 (c) on inhibin α- and βB-subunit mRNA expression in cultured Sertoli cells. Sertoli cells were incubated for 24 h in the presence of different concentrations of PMA or staurosporine. The inhibin-subunit mRNA levels were assessed by Northern blot analysis, using 40 μg of total RNA per lane. The inhibin subunit mRNA bands were scanned, and the resulting OD values were expressed relative to the control value (100%). Values represent means ± SEM of three to four separate experiments.

Involvement of calcium in the regulation of the inhibin subunit mRNA expression

The calcium ionophore A23187 was used to determine whether a rise in intracellular calcium can affect the inhibin subunit mRNAs. Sertoli cells were cultured for 24 h in the presence of various concentrations of A23187. A23187 at concentrations ranging from 10^{-8}-10^{-6} M caused a dose-dependent decrease of the βB-subunit mRNAs (Fig. 3.3c; 10^{-7} M and 10^{-6} M, $P<0.0005$). At 10^{-5} M A23187, the expression of the 3.5 kb βB-subunit mRNA was dramatically elevated, whereas an additional smaller band hybridizing with the βB-probe was found (Fig. 3.4), and expression of α-subunit mRNA and actin was decreased. Furthermore, the morphology of the cells was altered at this concentration of A23187, and attachment to the flask was decreased.

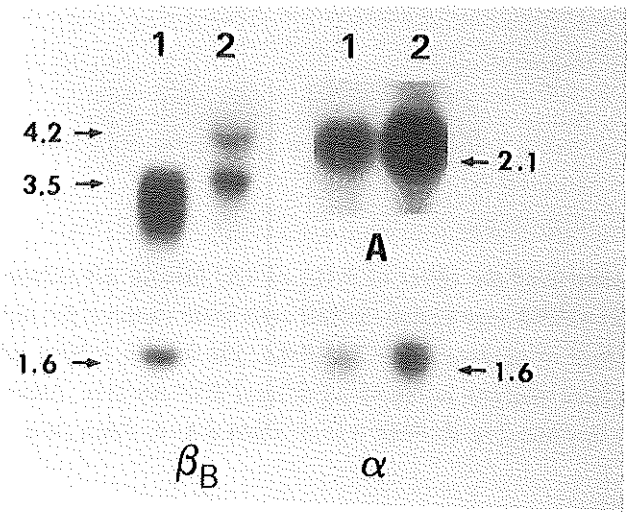

Fig. 3.4: Effect of 10^{-5}M A23187 on inhibin α- and βB-subunit mRNA expression in cultured Sertoli cells. The inhibin subunit (βB and α) and actin (A) mRNA levels were assessed by Northern blot analysis. Lanes:1, 10^{-5}M A23187; 2, control.

Time course of the effect of PMA, Staurosporine and A23187 on the expression of βB-subunit mRNAs

Cells were cultured for different periods of time in the presence of 10^{-7} M PMA, 10^{-7} M Staurosporine, or 10^{-7} M A23187. The expression of the βB-subunit mRNAs was initially increased after incubation with PMA for 2 h (Fig. 3.5), and thereafter decreased. Staurosporine had a similar effect on the βB-subunit mRNA expression, whereas the expression of the α-subunit mRNA was gradually increased with time by staurosporine (not shown). A23187 caused a time-dependent decrease of the βB-subunit mRNAs, wherease the α-subunit mRNA was not influenced (not shown).

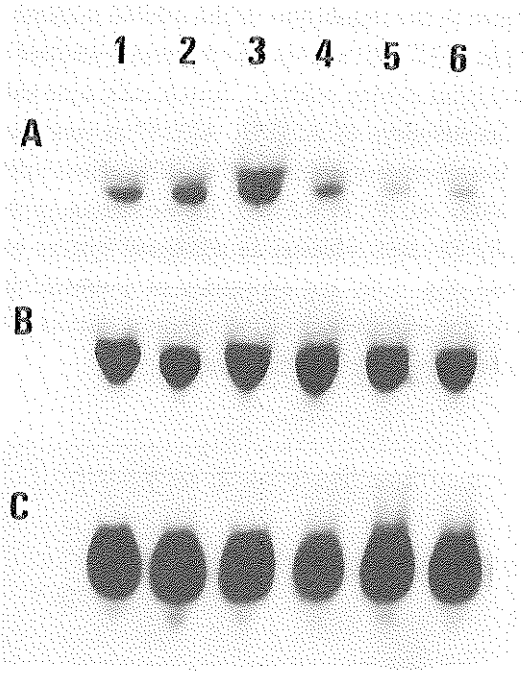

Fig. 3.5: Time-course of the effect of PMA on (A) inhibin βB-subunit (B) inhibin α-subunit (C) actin mRNA expression. Sertoli cells were cultured for different periods of time in the presence or absence of 10-7 M PMA. The inhibin subunit mRNA levels were assessed by Northern analysis, using 40 μg of total RNA per lane. Lanes: 1, control 0 h; 2, control 24 h; 3, PMA 2 h; 4, PMA 4 h; 5, PMA 8 h; 6, PMA 24 h.

Effect of PMA, Staurosporine, and A23187 on inhibin bioactivity and immunoactivity

Media from the cultures described above were concentrated and used for determination of inhibin bioactivity and immunoactivity. PMA, staurosporine, and A23187 caused a decrease in the amount of bioactivity present in the media (Table 3.1). When the media of Sertoli cells cultured in the presence of PMA or A23187 were subjected to Western blotting with an antiserum against the 22 N-terminal amino acid residues of the inhibin α-subunit, an increased amount of a protein with a molecular weight of 26 kDa, was observed (Fig. 3.6), whereas the amount of the 32 kDa αβ-inhibin dimer was decreased. Development of a parallel blot with an antiserum against the pro-part of the α-subunit indicated an increase of the same 26 kDa moiety.

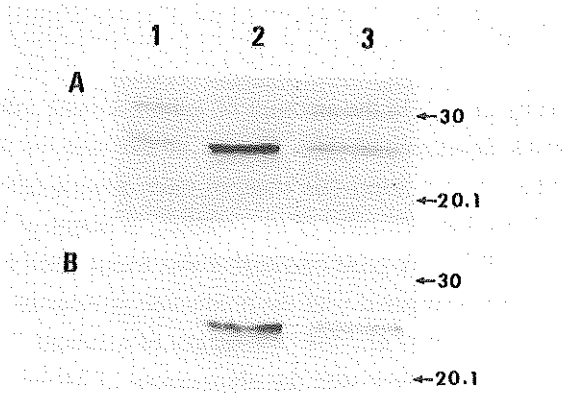

Fig. 3.6: Effect of PMA and A23187 on inhibin α-subunit containing proteins detected by Western blotting. Sertoli cells were cultured for 24 h. (A) Western blot of proteins detected with an antiserum directed against the αC-part of inhibin. (B) Western blot of proteins detected with an antiserum directed against the pro-part of the inhibin α-subunit. Lanes: 1, Control; 2, A21387 (10^{-7} M); 3, PMA (10^{-7} M).

Table 3.1: ***Inhibin bioactivity in the media of Sertoli cells cultured for 24 h in the presence of different concentrations of PMA, Staurosporine or A23187.***

Addition	Bioactivity (U/ml)
DMSO (Control)	21.1 ± 0.8
PMA 10^{-9}M	15.0 ± 0.6[a]
PMA 10^{-8}M	10.3 ± 1.5[a]
PMA 10^{-7}M	10.7 ± 2.7[c]
Staurosporine 10^{-9}M	17.0 ± 0.6[b]
Staurosporine 10^{-8}M	13.0 ± 2.1[c]
Staurosporine 10^{-7}M	15.6 ± 2.2[d]
A23187 10^{-8}M	3.0 ± 3.0[a]
A23187 10^{-7}M	2.7 ± 1.5[a]
A23187 10^{-6}M	2.2 ± 2.0[a]

[a] $p<0.005$, [b] $p<0.01$, [c] $p<0.025$, [d] $p<0.05$, values represent means ± SEM of three incubations.

Discussion

The most strinking change in the amounts of inhibin subunit mRNAs which occurs during postnatal testis development, is a change in the ratio of the two βB-subunit mRNAs. Other studies did not detect this changed ratio, since a different technique was used (S1-nuclease protection; Meunier *et al.*, 1988a), or testes of older animals were analysed (Feng *et al.*, 1989a). A good correlation was found when the total of both βB-subunit mRNA levels was compared with the production of bioactive inhibin by cultured Sertoli cells (Ultee-van Gessel *et al.*, 1987). Changes of α-subunit mRNA expression at the various ages were in agreement with the observations of Meunier *et al.* (1988a) and Keinan *et al.* (1988), and are correlated with the amount of immunoreactive inhibin found in testes of rats of various ages (Rivier *et al.*, 1988). The interpretation of the data is complicated by the fact that Leydig cells also express inhibin α-subunit mRNA (Risbridger *et al.*, 1989a; de Winter *et al.*, 1992a). However, the contribution of these cells to the intratesticular level of α-subunit mRNA seems relatively small (de Winter *et al.*, 1992a). Furthermore, Maddocks and Sharpe (1989) suggested that the production of inhibin by Leydig cells *in vivo* is negligible, and de Winter *et al.* (1992a) could not show production of bioactive inhibin by Leydig cells.

It remains unclear, which factor causes the switch to a smaller βB-subunit mRNA with increasing age. Also the functional significance of the occurrence of the two βB mRNAs which are both actively translated on ribosomes (Fig. 3.2b) remains unknown. These RNAs could differ in polyadenylation, but it is also possible that they code for different proteins. The genomic organization of the rat βB-subunit gene shows the presence of a 3 kb intron in the 5' end of the coding region (Feng *et al.*, 1989b). Since the βB cDNA probe used herein only contains approximately 60 bases which are located in the first exon, and more than 1.4 kb located in the second exon, it is possible that this probe hybridizes with mRNAs originating from two different first exons, spliced to the same second exon. Such mRNAs would code for different proteins, as also has been demonstrated for TGFβ1, which is another member of the TGFβ-family (Kondaiah *et al.*, 1988).

Prolonged stimulation of protein kinase C by PMA suppresses βB-subunit mRNA expression, after an initial increase. Most likely, the suppressive effect after 24 h is caused by an inhibition of protein kinase C, since longterm stimulation with PMA leads to a downregulation of this kinase (Hepler *et al.*, 1988, and review by Nishizuka, 1988). This also explains, why the protein kinase C inhibitor staurosporine had similar effects on the βB-subunit mRNA expression after 24 h, as compared to the effect of PMA. However, the reason for the initial stimulation of βB-subunit mRNA expression by

staurosporine is not clear, since staurosporine interacts with the catalytic moiety of protein kinase C (Nakadate *et al.*, 1988; Wolf and Baggiolini, 1988). The increased α-subunit mRNA expression at 10^{-7} M staurosporine could be explained by the fact that staurosporine at higher doses also affects other protein kinases (Rüegg and Burgess, 1989). Finally, addition of the Ca^{2+}-ionophore A23187 also suppressed the expression of the βB-subunit mRNAs. Although protein kinase C and Ca^{2+} mobilization are generally synergistic, not all protein kinase C subspecies are calcium-dependent (for a review, see Nishizuka, 1988), and hormones can affect intracellular calcium levels without affecting protein kinase C (for a review, see Nemere and Norman, 1991). This could explain the diverging short-term effects of PMA and A23187 on the βB-subunit mRNA expression. Although 10^{-5} M A23187 has been used in other studies, we think data using this dose of A23187 should be interpreted with caution in view of our findings of a diverging size of βB-subunit mRNA and an altered morphology of the cells.

A decreased βB-subunit mRNA expression was always accompanied by a decrease in bioactive inhibin. However, there was no direct correlation between the decrease of βB-subunit expression and bioactivity, indicating that effects at the translational level may also play a role in the suppression of bioactivity. Alternatively, differences might be explained on basis of the fact that the bioactivity in the media results from a 24 h accumulation process, whereas mRNA levels only reflect the situation at the end of this period. The decreased bioactivity in the media parallelled the increase at the amount of 26 kDa immunoreactivity detected on Western blot using antibodies against the C-terminal and pro-regions of the inhibin α-subunit. This indicates that the 26 kDa entity consists of an αC molecule linked to the pro-region of the α-subunit of inhibin (pro-αC). The pro-αC molecule has been isolated from follicular fluid by Sugino *et al.* (1989) and Robertson *et al.* (1989), and has been shown to be produced by Sertoli cells (de Winter *et al.*, 1992a).

The increased production of pro-αC after decreased expression of βB-subunit mRNA suggests, that the βB-subunit is a limiting factor in the production of bioactive inhibin. Since the pro-αC molecule is 2.9-fold more immunoreactive with the antibodies against the α-chain of inhibin than the intact inhibin molecule (Robertson *et al.*, 1989) and a decreased production of bioactive inhibin is accompanied by an increased production of pro-αC, our results are in contrast with observations of Gonzales et al (1989a,b), who found a decreased inhibin immunoreactivity after treatment with PMA of cultured adult rat seminiferous tubules. A possible explanation for this discrepancy could be that cultured tubules of mature rats show less down-regulation of protein kinase C by PMA than immature Sertoli cells.

The question remains which physiological factor is responsible for the regulation of βB-subunit mRNA expression in Sertoli cells. Interestingly, in cultured rat granulosa cells, FSH not only increases the expression of the α- and βA-subunit mRNAs, but also

the expression of βB-subunit mRNAs (Turner *et al.*, 1989). In these granulosa cells, only the 4.2 kB βB-subunit mRNA is present. This suggests that in those cells also the protein kinase C pathway is affected by FSH. Indeed, Pennybacker and Herman (1991) have shown that FSH influences *c-fos* mRNA expression in granulosa cells via protein kinase C.

In conclusion, we have shown that the expression of the βB-subunit mRNAs is differentially regulated *in vivo*. Furthermore, our results indicate that both βB-subunit mRNAs are actively translated. Finally a decreased expression of βB-subunit mRNAs in cultured Sertoli cells leads to a decreased production of bioactive inhibin, and an increased production of a molecule which appears to be a combination of the pro- and C-terminal parts of the inhibin α-subunit.

Acknowledgements

This work was supported financially by the Dutch Science Foundation (N.W.O.) through GB-MW (Medical Sciences). We would like to thank Dr. S. Shimasaki, and Dr N. Ling for their generous gift of the inhibin cDNA probes, and the NIADDK for the materials used for the estimation of FSH in the inhibin bioassay.

Expression of inhibin subunit mRNAs and inhibin levels in the testes of rats with stage-synchronized spermatogenesis

I.A. Klaij, A.M.M. van Pelt*, M.A. Timmerman, L.J. Blok, D.G. de Rooij* and F.H. de Jong

*Department of Endocrinology and Reproduction, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, The Netherlands. *Department of Cell Biology, Medical School, State University of Utrecht, The Netherlands*

Journal of Endocrinology (1994) 141, 131-141

Abstract

Inhibin α- and βB-subunit mRNA expression, and levels of bioactive and immunoreactive inhibin were studied in rat testes, synchronized for the stage of the cycle of the seminiferous epithelium by treating vitamin A-deficient rats with vitamin A. Measurement of inhibin subunit mRNA expression and inhibin levels was started directly after the start of vitamin A treatment, and continued for 65 days.

Inhibin subunit mRNA expression, and testicular bioactive and immunoreactive inhibin levels increased after the start of vitamin A treatment, reaching maximum values after 9 days, when B spermatogonia and preleptotene spermatocytes had appeared in the stage-synchronized testes. The ratio between βB- and α-subunit mRNA expression was high at that time-point, whereas the ratio between bioactive and immunoreactive inhibin remained low. These data suggest a relatively high production of activin at that moment, and this may play a role in the development of B spermatogonia into preleptotene spermatocytes during the initiation of spermatogenesis.

Stage-dependency was demonstrated for inhibin subunit mRNA expression, and for the levels of bioactive and immunoreactive inhibin, in rats with complete spermatogenesis. Inhibin α-subunit mRNA expression was relatively high at stages V and XIII of the spermatogenic cycle, whereas βB-subunit mRNA expression was high at stage XIII but not at stage V. This resulted in a high βB/α subunit mRNA ratio at stage XIII. Since it has been shown that expression of the activin receptor is high at stages XIII-I, locally formed activin might play a role in the regulation of meiosis. Bioactive and immunoreactive inhibin were highly correlated during the cycle, with maximum levels at stages XIV-I.

It was concluded that the production of inhibin, and possibly activin, is dependent on the stage of the cycle of the seminiferous epithelium; these growth factors might play a paracrine role in the differentiation of spermatogenic cells.

Introduction

Sertoli cells produce a variety of peptide growth factors which may be involved in the initiation and maintenance of spermatogenesis. One of these factors is inhibin, a heterodimeric glycoprotein consisting of an α- and a β-subunit, which initially has been characterized by its ability to suppress follicle-stimulating hormone (FSH) production by the pituitary gland (for review see de Jong, 1988). Recently it has become evident that the role of inhibin, and of the homodimer of two β-subunits,

activin, is not limited to regulation of FSH production, but can also involve regulation of cell proliferation and differentiation. Activin can affect differentiation of haemopoietic cells (Eto *et al.* 1987), induces mesoderm formation in Xenopus embryos (van den Eijnden-van Raaij *et al.* 1990), and stimulates proliferation of spermatogonia in the testis (Mather *et al.* 1990). The presence of activin receptor II mRNA has been demonstrated in defined populations of pachytene spermatocytes and round spermatids (de Winter *et al.* 1992b), whereas activin receptor IIB mRNA is present in spermatogonia (Kaipia *et al.* 1993). Inhibin can counteract the effects of activin in a number of systems, and has been shown to suppress the number of differentiated spermatogonia in testes of mice and Chinese hamsters (van Dissel-Emiliani *et al.* 1989).

Sertoli cells are in close contact with a population of developing spermatogenic cells, the composition and characteristics of which vary with the stage of the cycle of the seminiferous epithelium (LeBlond and Clermont, 1952; Parvinen, 1982). Interactions with spermatogenic cells could therefore affect Sertoli cells in a stage-specific manner. Indeed, stage-dependent production of many factors, such as transferrin (Morales *et al.* 1989), androgen-binding protein (Ritzen *et al.* 1982) and plasminogen activator (Lacroix *et al.* 1981) by Sertoli cells has been demonstrated. Using immunohistochemistry on testes of adult rats, Merchenthaler *et al.* (1987) showed that cross-sections of some tubules contained inhibin-immunoreactive material, whereas other tubules did not. After this initial observation, other authors (Bhasin *et al.* 1989; Kaipia *et al.* 1991) also found that the expression of inhibin subunit mRNAs is stage-dependent by studying dissected seminiferous tubules.

In order to investigate the effects of these stage-dependent changes in the expression of inhibin subunit mRNA on levels of bioactive and immunoreactive inhibin, we have used the model of the "synchronized testis", which can be obtained by administration of vitamin A after induction of vitamin A-deficiency. In the vitamin A-deficient (VAD) situation, spermatogenesis comes to a halt at the level of A spermatogonia whereas, after readministration of vitamin A, spermatogenesis starts in a synchronized manner (Morales *et al.* 1987; van Pelt and de Rooij, 1990). Measurements of the expression of inhibin subunit mRNAs and the intratesticular levels of bioactive and immunoreactive inhibin were started directly after the start of vitamin A treatment, and continued until 65 days after vitamin A readministration. In this way, it was possible to study whether the appearance of a certain differentiated germ cell type is associated with an alteration in inhibin levels (early after vitamin A readministration), and to measure bioactive and immunoreactive inhibin concentrations at different stages of the cycle of the seminiferous epithelium.

Materials and methods

Animals

Pregnant Wistar rats (18 - 20 days postcoitum) were fed a VAD diet (Teklad Trucking, Madison, WI, USA). Male rats born to these females received the same diet until, at the age of 9 - 11 weeks, body weights decreased. At this time, when VAD was achieved, each animal was treated once with 5 mg retinol acetate (Sigma, St. Louis, MO, USA) i.p., followed by a normal vitamin A-containing diet (Hope Farms, Woerden, The Netherlands) (van Pelt and de Rooij, 1990). Groups of three to eight animals were killed and their testes were removed on various days after the treatment with retinol acetate.

Experimental design

Two experiments were performed. In experiment 1, stage-specific events within one cycle of completed synchronized spermatogenesis were studied in samples from rats killed daily between days 50 and 65 after vitamin A administration. Furthermore, rats were killed with intervals of 6 or 7 days between days 30 and 50 after vitamin A resubstitution in order to study events at stages VII and XIV. Experiment 2 was designed to study changes in inhibin during the early phase of reinitiation of spermatogenesis following vitamin A resubstitution (days 1 to 15), and to evaluate possible changes that occur when more advanced cell types appear in the spermatogenic epithelium (weekly samples between days 19 and 65). Data for testicular parameters, obtained in both experiments, were combined. In the second experiment, serum was collected for hormone estimations. As a control, 18-week-old rats which had received a normal diet were used, since it has been demonstrated that serum levels of FSH, luteinizing hormone (LH) and immunoreactive inhibin (Brown and Chakraborty, 1991), and testicular expression of inhibin α-subunit (Keinan *et al.* 1989; Klaij *et al.* 1992) and βB-subunit (Feng *et al.* 1989a; Klaij *et al.* 1992) mRNA do not change considerably between 9 and 18 weeks of age. By using 18-week-old rats, a direct comparison between controls and animals with repopulated testes was possible.

One testis of each rat was fixed in Bouin's fluid and used for determination of the synchronization of the seminiferous epithelium by routine histological procedures. The spermatogenic stages present in the epithelium were determined in each animal. Material from rats with atrophic testes was not included in this study. A number of animals was studied in more detail: the epithelial stage of 200 or more tubular cross-sections was determined, and the mid-point of synchronization was calculated (van

Beek and Meistrich, 1990). The other testis was weighed, frozen in liquid nitrogen, stored at - 80°C, and used for RNA isolation and hormone measurements.

RNA isolation and analysis

RNA was isolated from half of a testis from each rat, using an acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Chomczynski and Sacchi, 1987). Of each sample, 40 μg total RNA was subjected to Northern blotting (Klaij *et al.* 1992). Amersham Hyperfilm-MP (Amersham International plc, Amersham, Bucks, U.K.) was exposed to the filters at -80°C for various lengths of time, using an intensifying screen. The intensity of hybridizing bands was measured by densitometric scanning as described previously (Klaij *et al.* 1990); the values of the hybridization signals of the 4.2 and 3.5 kb inhibin βB-subunit mRNAs were added. Hybridization with an actin cDNA probe was performed as a control for equal amounts of mRNA in all lanes, and hybridization signals were normalized against this control, since actin expression does not change throughout the spermatogenic cycle (Bhasin *et al.* 1989).

In order to be able to compare expression of mRNAs on different blots, on each blot a sample of 40 μg from a pool of total testis RNA of 21-day-old rats was included, and the intensities of the hybridizing bands were related to the intensity of this standard.

The inhibin cDNA probes used were a 1.25 kb EcoRI fragment encoding the α-subunit (α7/pUC18), and a 1.5 kb EcoRI fragment encoding part of the βB-subunit (βB11/pUC18) of rat inhibin (Esch *et al.* 1987a).

Inhibin measurements

Half of a testis from each rat was homogenized in a 5 ml Teflon-glass homogenizer in 2 ml phosphate-buffered saline (PBS). Homogenates were centrifuged at 100,000 g for 1 h at 4°C and supernatants collected. A volume of 50 μl was used for the measurement of testosterone (see below), and the remaining supernatant was incubated with an equal volume of dextran-coated charcoal suspension (1% Norit, 0.1 % dextran T300 in PBS, pH 7.0) at 4°C for 30 min, in order to remove the steroids. Thereafter, charcoal was removed by centrifugation at 1500 g for 10 min and cytosols were collected, sterilized by filtration through a 0.2 μm filter (Schleicher and Schuell, Dassel, Germany) and stored at -20°C until estimation of inhibin bioactivity and immunoreactivity.

Levels of immunoreactive inhibin in testis homogenates and serum were measured by radioimmunoassay as described by Robertson *et al.* (1988), using an antiserum against purified 32 kDa bovine follicular fluid (bFF) inhibin and iodinated 32 kDa bFF

inhibin. These materials were kindly provided by Dr. G Bialy (NICHHD, Bethesda, MD, USA). All samples were measured in one assay. The intra-assay coefficient of variation amounted to 17.5%. The amount of bioactive inhibin was determined by an *in vitro* bioassay in which suppression of spontaneous FSH release from cultured rat pituitary cells was estimated (Grootenhuis *et al.* 1989). A charcoal-treated bFF preparation with an arbitrary potency of 1 U/μg protein was used as a standard in both bio- and immunoassays for inhibin (Grootenhuis *et al.* 1989).

Measurement of FSH, LH and testosterone

FSH and LH were measured by radioimmunoassay as described by Grootenhuis *et al.* (1989), using the antibodies developed by Welschen *et al.* (1975). All results are expressed in terms of NIADDK-rat FSH-RP-3 and NIADDK-rat LH-RP-2. Testosterone was measured by radioimmunoassay as described by Verjans *et al.* (1973). All samples for each hormone were measured in one assay. Intra-assay variation was 5.1% for FSH, 14.1% for LH and 3% for testosterone.

Statistical analysis

All data are presented as means ± SEM (n=3-8 rats). Statistical significances of differences between VAD rats and control rats were assessed using the Student's t-test. Differences were considered significant if $P<0.05$. Correlation between data was determined using regression analysis.

Results

Testis weights and synchronization

Testis weights were decreased in VAD rats as compared with levels in 9-week old control rats (0.50 ± 0.38 g and 1.52 ± 0.13 g respectively; $P<0.05$). After vitamin A administration, testis weights increased to approximately 85% of that in control rats 50 days after the start of treatment (Fig. 4.1).

Stages of the spermatogenic cycle and the most advanced cell types which were present in the testes of groups of rats at different time-points are shown in Table 4.1. The midpoint of synchronization has been indicated in rats up to 50 days after vitamin A administration. Stages were estimated by identification of spermatogonia and spermatocytes up to 30 days after treatment, since spermatids were not yet present in the testes. Spermatogenesis was complete from 50 days onwards. Although the number

of stages within each rat was limited, synchronization between rats from 50 days after vitamin A administration was not optimal. Testes of a number of rats from the older groups were therefore studied in more detail; stage-frequency was determined and midpoints of synchrony were calculated (Table 4.2).

Table 4.1: ***Stages of the cycle of the seminiferous epithelium, and most advanced germinal cell types in stage-synchronized rat testes after various periods of vitamin A readministration.*** *Mid-points of synchronization in rats up to 50 days after treatment are shown in parentheses.*

Day	Stages present	Most advanced cell type
9	(VI)	preleptotene spermatocyte
15	(XI-XII)	pachytene spermatocyte
19	(XIV)	pachytene spermatocyte
26	(VII)	pachytene spermatocyte
30	XII-II (I)	step 2 spermatid
32	I-V (II)	step 5 spermatid
37	VI-X (VII)	step 10 spermatid
39	VIII-XIV (IX)	step 14 spermatid
43	XIV-IV (I)	step 17 spermatid
45	I-VI (III)	step 16 spermatid
50	VI-XIV	step 19 spermatid
52	VII-X	
53	X-IV	
54	VII-II	
55	VII-IV	
56	XII-VI	
57	XIV-VIII	
58	XIV-VII	
59	IV-X	
60	V-I	
61	VI-XIV	
62	VII-I	
64	VII-XIV	
65	VIII-I	

Table 4.2: Relative frequency of stages of the spermatogenic cycle in individual rats with synchronized spermatogenesis at various times after vitamin A administration. Hormonal data for these animals are shown in Figures 3c,d and 5c,d. Midpoints of synchronization have been underlined. Control data are derived from Parvinen (1982).

Time (days)	Percentage of tubules													
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
55	37.3	20.9	4.0	8.4	0.9	2.2	-	-	0.5	-	-	-	9.3	11.6
58	6.7	28.7	24.4	18.2	8.6	4.8	2.9	1.9	1.0	0.9	-	-	-	1.0
59	5.0	6.0	7.8	9.7	6.5	30.9	16.6	0.9	0.5	1.8	1.8	0.5	3.2	8.8
59	0.5	0.9	4.5	17.7	14.9	22.6	24.9	10.9	-	-	0.4	-	-	-
60	-	-	1.9	4.8	18.4	35.8	29.0	7.2	-	-	-	-	-	-
61	-	-	-	2.4	2.4	7.3	36.6	33.2	11.2	4.9	1.0	-	1.0	-
50	-	-	0.4	0.4	0.8	12.3	24.7	43.4	12.3	0.9	-	-	0.4	0.4
64	1.8	2.7	0.5	1.3	-	-	12.6	25.2	17.1	13.5	5.0	1.8	2.7	2.3
64	0.5	0.9	1.4	0.5	1.4	1.8	8.8	25.9	13.9	17.1	12.9	4.2	4.2	4.2
64	0.9	0.5	0.5	0.5	0.5	0.5	2.8	25.9	14.8	18.0	14.3	3.7	6.5	2.3
53	4.9	3.1	0.5	0.5	-	-	0.9	2.2	4.5	6.7	13.5	4.0	37.0	21.5
control	12.1	8.1	2.1	4.5	5.1	9.2	21.8	7.4	2.5	2.5	2.5	11.2	6.1	4.9

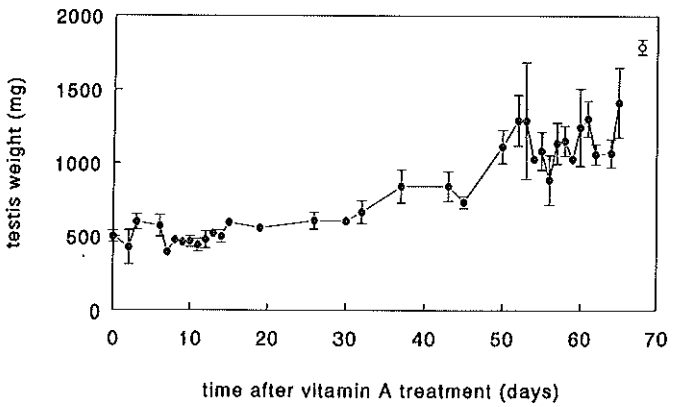

Fig. 4.1: Testis weights in stage-synchronized rats (●) and in 18-week-old control rats (○). Vitamin A-deficient rats were treated with retinol acetate as described in Materials and Methods, and killed at different times following vitamin A administration. One testis of each animal was weighed. Data represent means ± SEM of three to eight testes. For points without SEM bars, the values fell within the symbols.

Serum hormone levels

FSH (20.3 ± 2.9 ng/ml) and LH (1.1 ± 0.6 ng/ml) levels were significantly elevated in VAD rats when compared with those in 18-week-old control animals (10.0 ± 0.1 ng/ml and <0.1 ng/ml; $P<0.01$ and $P<0.05$ respectively). As shown in Fig. 4.2a and b, vitamin A administration resulted in a temporary dramatic increase in gonadotrophin levels within 2 days. Subsequently, levels returned within 2 days to the concentrations found before the start of vitamin A administration. Thereafter, FSH and LH levels gradually decreased, and reached control levels after 58 days of vitamin A administration.

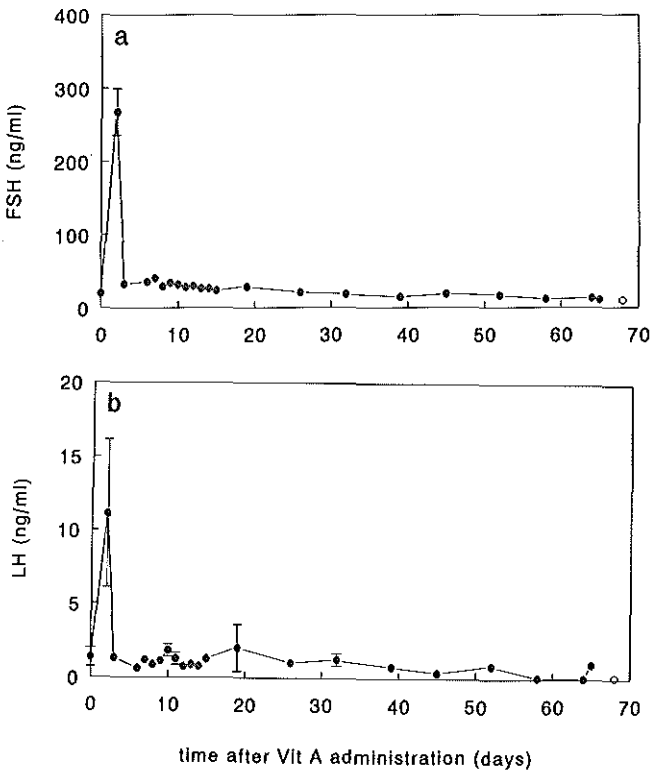

Fig. 4.2: Serum levels of (a) FSH, (b) LH in stage-synchronized rats (●) and in 18-week-old control rats (○). Vitamin A- deficient rats were treated with retinol acetate as described in Materials and Methods, and killed at different times following vitamin A administration. Data represent means ± SEM of three to eight rats. For points without SEM bars, the values fell within the symbols.

In VAD rats, plasma testosterone levels were not significantly different from those in controls (Fig. 4.2c). Vitamin A treatment resulted in increased testosterone levels, with a maximum at 10 days after the commencement of vitamin A administration and testosterone levels fluctuated thereafter. Plasma testosterone levels were highly correlated with testicular testosterone ($r=0.81$, $n=74$, $P<0.0005$).

Plasma levels of immunoreactive inhibin were increased in VAD rats, when compared with plasma levels in control rats ($P<0.0005$). Following vitamin A administration, a decrease in immunoreactive inhibin levels was observed, followed by an increase, with a maximum at 11 days after commencement of vitamin A administration. Thereafter, levels decreased to a more constant level (Fig. 4.2d).

No correlation was found between the levels of gonadotrophins and immunoreactive inhibin or gonadotrophin and testosterone levels in each sample.

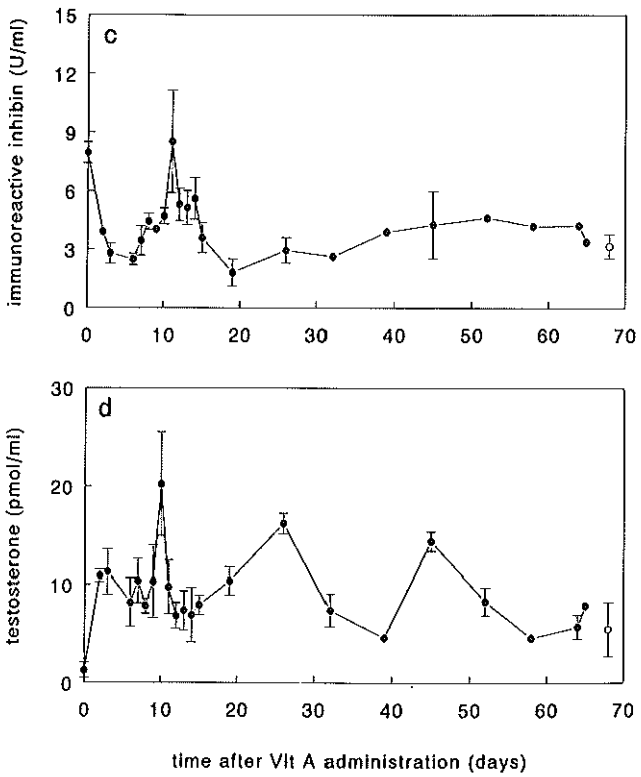

Fig. 4.2: Serum levels of (c) testosterone and (d) immunoreactive inhibin in stage-synchronized rats (●) and in 18-week-old control rats (○). Vitamin A- deficient rats were treated with retinol acetate as described in Materials and Methods, and killed at different times following vitamin A administration. Data represent means ± SEM of three to eight rats. For points without SEM bars, the values fell within the symbols.

Inhibin subunit mRNA expression

Inhibin α-subunit mRNA levels (Fig. 4.3a), and βB-subunit mRNA levels (Fig. 4.3b) were decreased in the testes of VAD rats, when compared with those in the testes of adult control animals ($P<0.0005$). Vitamin A treatment resulted in an increased expression of both subunit mRNAs with a maximum after 9 days. Thereafter, levels decreased for 15 days and then remained more constant until, at 50 days, complete spermatogenesis was present. From day 50 onwards, fluctuating levels of inhibin α- and βB-subunit mRNA expression were found, with maximal levels of inhibin α-subunit expression at 53 days (approximate average midpoint of synchronization stage XIII) and 59 days (approximate average midpoint of synchronization stage V) after vitamin A treatment, and maximal levels of βB-subunit mRNA expression at 53 days (stage XIII).

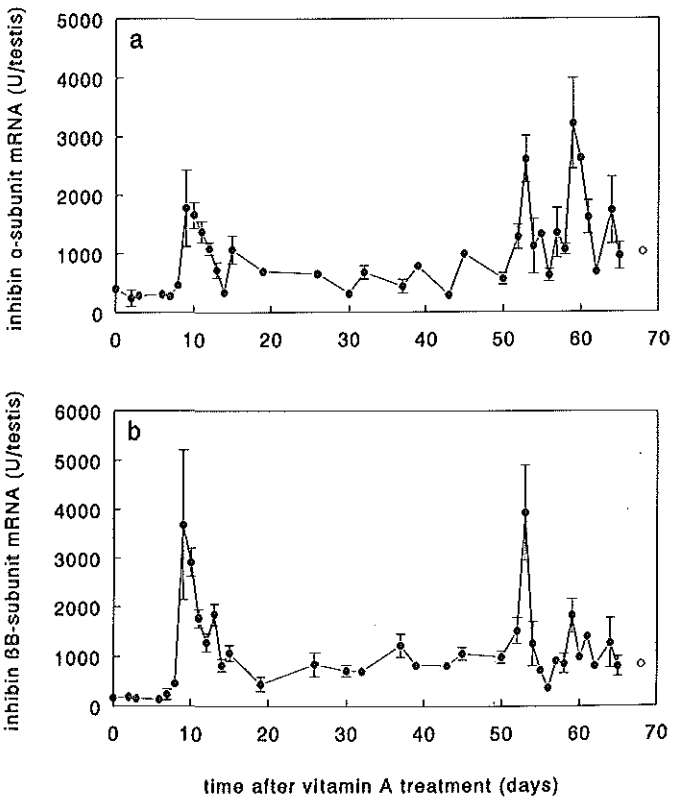

Fig. 4.3: Testicular inhibin (a) α-subunit and (b) βB-subunit mRNA content in stage-synchronized rats (●) and 18-week-old control rats (○). RNA was isolated from testes from rats killed at different time-points after vitamin A administration, and subjected to Northern blotting. Data were normalized against actin mRNA, and represent means ± SEM of three to eight rats. For points without SEM bars, the values fell within the symbols.

Because there was no complete synchrony of spermatogenesis between individual rats, inhibin subunit mRNA levels in testes from individual rats between 50 and 65 days after vitamin A repletion with different midpoints of synchronization (Table 4.2) are shown in Fig. 4.3c and d. The expression of α-subunit mRNA was highest at stages V and XIII. The lowest expression was observed at stages VIII-IX (Fig. 4.3c). βB-subunit mRNA expression was high only at stage XIII. A minimal expression of βB-subunit mRNA was found at stages III and IX (Fig. 4.3d).

Figure 4.4a shows the ratio between βB-subunit and α-subunit mRNA expression. Periodically increased values were observed at 9 and 13 days after vitamin A administration. A pulsatile pattern was observed between 20 and 50 days after treatment.

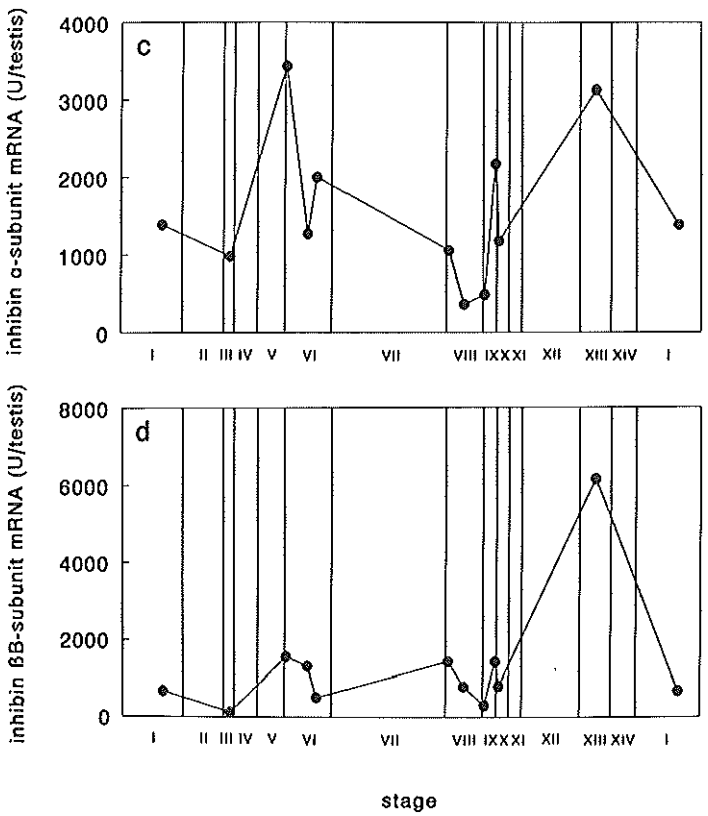

Fig. 4.3: Testicular inhibin (c) α-subunit and (d) βB-subunit mRNA content in stage-synchronized rats. RNA was isolated from testes from rats killed at different time-points after vitamin A administration, and subjected to Northern blotting. Data were normalized against actin mRNA. The mid-point of synchronization in individual rats (described in Table 4.2) is plotted against the corresponding level of α-subunit or βB-subunit mRNA expression respectively. The stages of the spermatogenic cycle are delineated by the vertical lines and are proportional to their duration in the cycle.

Testicular immunoreactive and bioactive inhibin content

The amounts of bioactive inhibin (Fig. 4.5a) and immunoreactive inhibin (Fig. 4.5b) per testis were markedly decreased in the testes from VAD rats, when compared with amounts in adult control animals ($P<0.0005$). Vitamin A treatment led to an increase of bioactive and immunoreactive inhibin, with a maximum after 12 days which was followed by a decrease up to 19 days. During the period between 1 and 19 days, a significant correlation was found between testicular levels of bio- and immunoreactive inhibin (Fig. 4.6; $r=0.65$, $n=52$, $P<0.005$). Thereafter, bioactive inhibin levels increased until 50 days after treatment, whereas immunoreactive inhibin levels fluctuated.

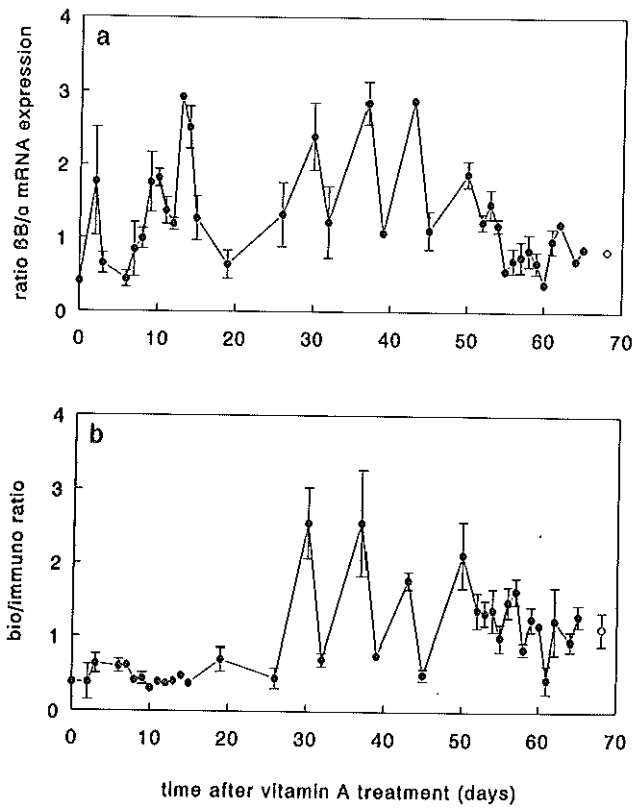

Fig. 4.4: Ratios between (a) inhibin βB-subunit and α-subunit mRNA expression and (b) bioactive and immunoreactive inhibin in rat testes from stage-synchronized rats (●) and 18-week-old control rats (○). Data represent means ± SEM of three to eight rats. For points without SEM bars, the values fell within the symbols.

Fluctuations of both immunoreactive and bioactive inhibin were found from 50 days onwards after the start of vitamin A administration. Also, during this period, a significant correlation existed between bio- and immunoreactive levels of testicular inhibin (Fig. 4.6; $r=0.69$, $n=59$, $P<0.0005$). However, the slope of the regression line during this period differed significantly from that obtained during the period between days 1 and 19 after vitamin A readministration. This resulted in ratios between bioactive and immunoreactive inhibin (B/I ratios) of 0.46 $\pm$ 0.03 during the period between day 1 and day 19, and 1.37 $\pm$ 0.10 after day 50. The B/I ratio (Fig. 4.4b) during the experiment showed a similar pattern when compared with the ratio between βB-subunit and α-subunit mRNA expression (Fig. 4.4a), except during the first 15 days after vitamin A administration, when a constant B/I ratio was observed.

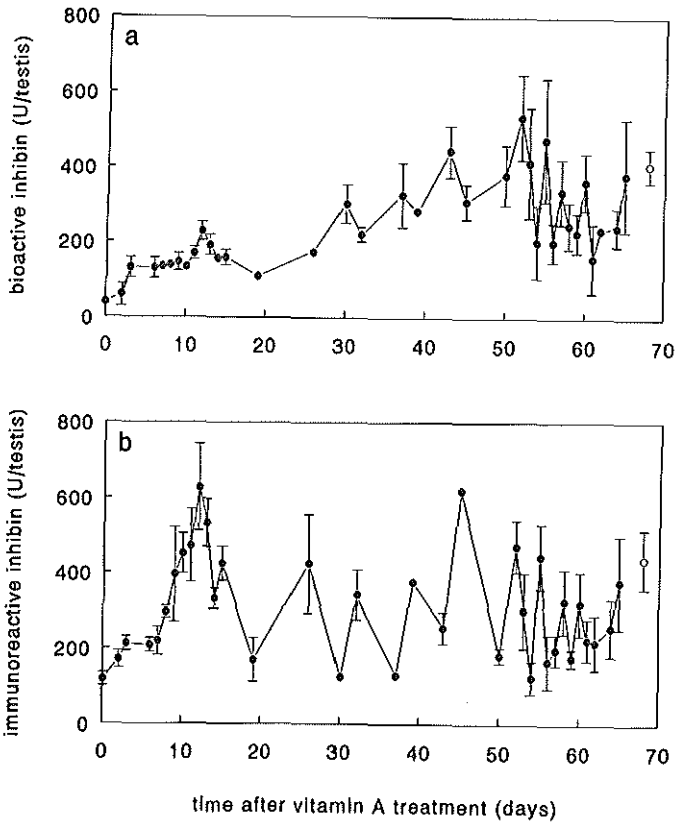

Fig. 4.5: Testicular (a) bioactive and (b) immunoreactive inhibin content in stage-synchronized rats (●) and 18-week-old control rats (○) at different time-points after vitamin A administration. Data represent means ± SEM of three to eight rats. For points without SEM bars, the values fell within the symbols.

The B/I ratio in rats after day 15 of vitamin A administration correlated significantly with the ratio between the expression of the mRNAs for the βB- and α-subunits of inhibin (r=0.24, n=65, P<0.025). When levels of inhibin in precisely staged testes were studied in more detail, it was found that both bio- and immunoreactive levels of inhibin were high at stage I, and low at stages VI-IX (Fig. 4.5c and d).

From day 50 onwards, when spermatogenesis was complete, expression of α-subunit and βB-subunit mRNA were correlated with testicular levels of immunoreactive inhibin (r=0.21, P<0.05 and r=0.33, P<0.01 respectively) and bioactive inhibin (r=0.30, P<0.01 and r=0.30, P<0.01 respectively).

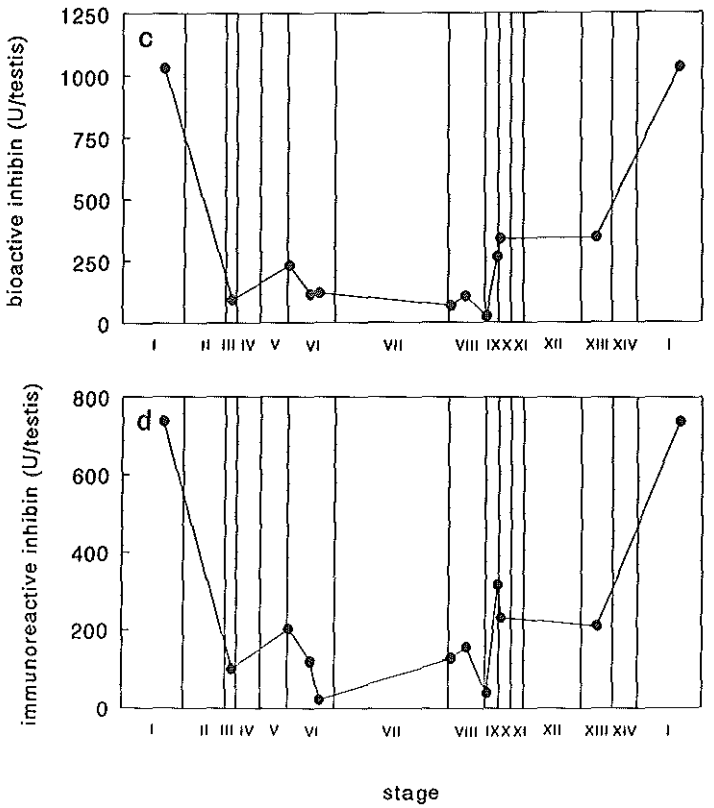

Fig. 4.5: Testicular (c) bioactive and (d) immunoreactive inhibin content in stage-synchronized rats. The mid-point of synchronization in individual rats (described in Table 4.2) is plotted against the corresponding level of bioactive or immunoreactive inhibin respectively. The stages of the spermatogenic cycle are delineated by the vertical lines and are proportional to their duration in the cycle.

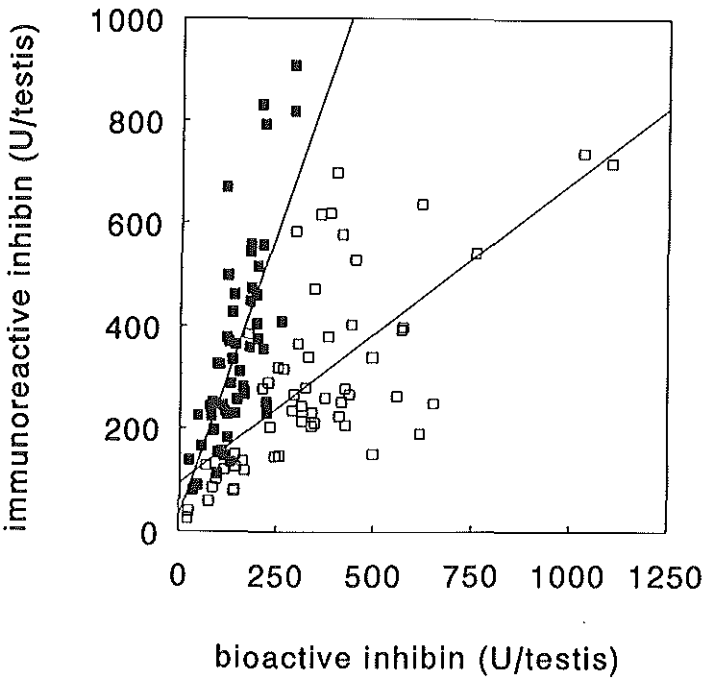

Fig. 4.6: Correlation between bioactive inhibin and immunoreactive inhibin in testes of rats with vitamin A-synchronized spermatogenesis. The closed symbols represent data from rats between 1 and 20 days after vitamin A treatment, whereas the open symbols are data from rats with more than 50 days of vitamin A supplementation.

Discussion

Endocrine changes in VAD rats

Vitamin A deficiency resulted in elevated serum FSH and LH levels as described previously (Rich *et al.* 1977, 1979; Huang *et al.* 1983, Bartlett *et al.* 1989). These authors used control groups of the same chronological age compared with the vitamin A deficient animals; in these control groups no age-dependent changes of LH, FSH or testosterone levels were observed. The increased levels of gonadotrophins have possibly resulted from impaired testicular function. Testicular inhibin subunit mRNA expression and bioactive and immunoreactive inhibin levels, when expressed per total testis, were decreased. However, as a result of the decreased testis weights in VAD animals, inhibin α-subunit mRNA expression and inhibin immunoreactivity were

increased, when expressed per mg testis tissue. These increased concentrations may have been caused by the relative enrichment of Sertoli and Leydig cells, and additionally by stimulation of α-subunit mRNA expression in Leydig cells (de Winter *et al.* 1992c) by the increased levels of LH, or by FSH stimulation of Sertoli cell expression of this subunit (Klaij *et al.* 1990). Finally, peripheral levels of immunoreactive inhibin were increased in VAD rats. This increase may have been caused by the secretion of a relatively large amount of inhibin from the testes with impaired spermatogenesis and a defective blood-testis barrier (Huang *et al.* 1988); Maddocks *et al.* (1992) have shown that the route of secretion of inhibin is changed from basal to apical during development as a result of the formation of the blood-testis barrier.

Day 1 to 19 after vitamin A administration

Vitamin A administration resulted in an immediate short-lived increase in peripheral levels of LH and FSH, which may have been caused by a partial release of the increased stores of gonadotrophins in the pituitary gland in VAD rats (Bartlett *et al.* 1989). It is unlikely that these increased levels of both LH and FSH are caused by increased testicular secretion of activin, since no significant increase of the ratio between βB- and α-subunit mRNA expression was found at this time. The low levels of testosterone in the VAD rats increased more than fivefold during the first 2 days after addition of vitamin A to the diet, and remained in the normal range for the rest of the study.

The drop in peripheral levels of immunoreactive inhibin preceded the changes in expression of inhibin subunit mRNAs in the testis, but coincided with increased testicular levels of immunoreactive and bioactive inhibin. This combination of data indicates that the secretory pattern of inhibin changed during the first few days after vitamin A treatment, probably as a result of the increased effectiveness of the blood-testis barrier (Huang *et al.* 1988). The increase of testicular and peripheral inhibin levels around 10 days after the start of vitamin A treatment follows the simultaneous large increase of expression of inhibin α- and βB-subunit mRNAs in the testis at day 9. It is highly unlikely that this increase was caused by the high gonadotrophin levels 7 days previously, since the effect of FSH on inhibin α-subunit mRNA expression is very rapid, and no effects of FSH on βB-subunit mRNA expression have been found (Klaij *et al.* 1990). A more likely explanation for the increase of inhibin subunit mRNA levels might be that Sertoli cell function, which is impaired in the VAD animals, recovers after vitamin A administration or that autocrine mechanisms evoked by the appearance of B spermatogonia or preleptotene spermatocytes in the testes at this point in time play a role. The reason for the subsequent decrease of the expression

of the inhibin subunit mRNAs remains unclear; the appearance of pachytene spermatocytes (Kaipia *et al.* 1991; Allenby *et al.*1991) might be involved.

The increase in the expression of the inhibin βB-subunit mRNA between days 9 and 15 was larger than that of the α-subunit mRNA, resulting in an increased ratio of βB over α expression. At the same time, a decrease of the B/I ratio occurred, indicating an increased production of activin during this period since activin counteracts inhibin action in the bioassay for inhibin (Ling *et al.* 1986). These increased levels of activin might be involved in the stimulation of early spermatogenesis (Mather *et al.* 1990; Woodruff *et al.* 1992). Alternatively, since the inhibin RIA is specific for α-subunits of inhibin (Robertson *et al.* 1989), inhibin α-subunits produced by Leydig cells (Risbridger *et al.* 1989a; de Winter *et al.* 1992a) might be part of the explanation for the low B/I ratio after 9 days after vitamin A administration, although the contribution of Leydig cell inhibin α-subunit to the intratesticular and blood levels in the adult rat is limited (Maddocks and Sharpe, 1989; de Kretser *et al.* 1989).

Finally, follistatin mRNA has been detected in the testis (Shimasaki *et al.* 1989). Therefore, a low B/I ratio may also have been caused by a vitamin A induced decrease of the levels of follistatin in the testis. So far, the cellular localization of follistatin has not been resolved. However, de Jong *et al.* (1993) showed that a human teratoma cell line expresses the follistatin mRNA; this expression was suppressed after addition of retinoic acid to the cultured cells.

Day 20 - day 50 after vitamin A administration

A limited number of measurements were performed between 20 and 50 days after vitamin A treatment, in order to study whether stage-specific events at stages VII and XIV change when the epithelium becomes filled with more advanced cell types. During this period, fluctuations in α-subunit mRNA level were observed, whereas βB-subunit mRNA expression was relatively stable. A high expression of α-subunit mRNA coincided with a high level of immunoreactive inhibin, whereas low levels of α-subunit mRNA resulted in a low testicular inhibin immunoreactivity. This might be expected, because a large part of the inhibin immunoreactivity is probably pro-αC which cross-reacts for 300% in the inhibin RIA (Robertson *et al.* 1989). In contrast, the level of bioactive inhibin was relatively stable. Therefore, the expression of βB-subunit mRNA appears to be limiting for the production of bioactive inhibin during this period. Changes in the ratio between βB-subunit and α-subunit mRNA expression and B/I ratio occurred synchronously and in parallel during this period, indicating that the ratio between βB-subunit and α-subunit mRNA expression is likely to determine which inhibin-like molecule is formed, assuming that the translation rates of the

subunit mRNAs do not change.

Immunoreactive inhibin levels in testes from rats killed at 26 (stage VII) and 30 days (stage I) and testicular bioactive inhibin levels in rats killed at 30 days diverged from levels expected on the basis of data obtained from rats with a corresponding mid-point of synchrony and complete spermatogenesis (see Fig. 5, and below). However, B/I ratios at these time-points were in agreement with βB/α- subunit mRNA ratios. These data suggest that final stage-dependency of expression of inhibin subunits starts with the presence of spermatids.

Day 50 - 65 after vitamin A administration

During this period, the expression of the inhibin subunit mRNAs in the testis is dependent on the stage of the cycle of the seminiferous epithelium. A maximal expression of α-subunit mRNA was found in stages V (59 days after vitamin A readministration) and XIII (53 days after vitamin A readministration) of the spermatogenic cycle, whereas expression was low in stages VIII-IX. The pattern of βB-subunit mRNA expression was in agreement with that found by *in situ* hybridization by Kaipia *et al.* (1992), and was essentially similar to that of α-subunit mRNA expression, with the exception of stage V. The ratio between α-subunit and βB-subunit mRNA expression therefore changes throughout the spermatogenic cycle. This changing ratio will cause changes in the relative production of inhibin, pro-αC (Klaij *et al.*, 1992), and activin by Sertoli cells, thus leading to secretion of different inhibin-like products at different stages.

The present data on inhibin subunit mRNA expression confirm the results of Bhasin *et al.* (1989), and are essentially in accordance with those of Kaipia *et al.* (1991) who found a maximal expression of α-subunit mRNA in stage XIV-IV and low levels in stage VI-VII. The main difference between the present and published results concerns the high expression of α-subunit mRNA at stage V in the stage-synchronized rats, which was not found when dissected tubules were studied (Kaipia *et al.*, 1991). This discrepancy might be explained by differences in standardization in these studies, or by the presence of mRNA from Leydig cells, which also contain α-subunit mRNAs, in our studies.

Testicular levels of bioactive and immunoreactive inhibin were correlated throughout the cycle. The highest levels of testicular inhibin were found at stage I, whereas the concentration of inhibin was low at stages VI-IX. Patterns of immunoreactive inhibin are in accordance with levels found by Gonzales *et al.* (1989c), who measured inhibin immunoreactivity in dissected tubules.

A significant correlation was observed between inhibin subunit mRNA expression and levels of bioactive or immunoreactive inhibin after day 50. However, the patterns of inhibin immuno- or bioactivity did not exactly match the patterns of inhibin subunit

mRNA expression. This may be caused by the fact that testicular inhibin content depends on both production and transport from the testis. Moreover, the ratio between βB-subunit and α-subunit mRNA probably determines which inhibin-related proteins are produced by the Sertoli cell: in the presence of a fixed large amount of α-subunit, increasing levels of β-subunit production will first cause an increase in bioactivity, whereas even higher levels of β-subunit production may cause production of activin, which will counteract inhibin in the bioassay (Ling *et al.* 1986).

In testes with complete spermatogenesis after 50 days of vitamin A administration, the βB/α-subunit mRNA ratio was low at stages I-VI and increased at stages VII-XIII, indicating the possibility of a relatively high production of activin at the latter stages. The mRNA encoding the activin receptor type IIB is highly expressed in A1 and A2 spermatogonia at stages IX-XI of the spermatogenic cycle (Kaipia *et al.* 1993). Locally produced activin might therefore act as a spermatogonial growth factor. Furthermore, expression of activin receptor type II mRNA has been demonstrated in mid- and late- pachytene spermatocytes and in round spermatids (de Winter *et al.* 1992b). Results of *in situ* hybridization indicate that this activin receptor mRNA expression was localized in secondary spermatocytes and spermatids at stages XIII-I (Kaipia *et al.* 1992; de Winter *et al.* 1992b), and a high binding of activin and inhibin was found in late pachytene and secondary spermatocytes (Woodruff *et al.* 1992). Activin might therefore play a role in the regulation of meiotic divisions. Finally, van Dissel-Emiliani *et al.* (1989) have shown that the numbers of intermediate and B spermatogonia can be suppressed by intratesticular injection of bioactive inhibin. The increase of inhibin concentration at stage V may contribute to regulation of spermatogonial divisions at this stage, thus preventing excessive cell numbers.

It is concluded that inhibin mRNA expression and bio- and immunoreactive inhibin levels in the testis show a pattern which depends on the stage of the spermatogenic cycle. This pattern gives rise to the production of different inhibin-related molecules at those stages of the spermatogenic cycle at which the effects of these molecules on the differentiation of spermatogenic cells have been postulated.

Acknowledgements

This work was supported financially by the Dutch Science Foundation (NWO) through GB-MW (Medical Sciences). The authors would like to thank Dr. S Shimasaki and Dr. N. Ling for the gift of the inhibin cDNA probes, the NIDDK for the materials used in the FSH and LH immunoradioassay, the NICHHD for the materials used in the inhibin radioimmunoassay, and Dr. J.A. Grootegoed for his critical review of this manuscript.

Testicular and serum levels of inhibin and expression of inhibin subunit mRNAs are differentially affected by hemicastration in rats of various ages

I.A. Klaij, M.A. Timmerman, P. Kramer, H.M.A. Meijs-Roelofs and F.H. de Jong

Journal of Endocrinology (1994) 141, 143-151

Abstract

Age-related short-term effects of hemicastration on testicular weight, serum FSH, immunoreactive inhibin, LH and testosterone, testicular levels of inhibin subunit mRNA expression, and bioactive and immunoreactive inhibin were studied in rats of 8, 15 and 22 days of age. Hemicastration led to an increased weight of the remaining testis after 24 h in 8- and 15-day-old rats, but not in 22-day-old rats. Serum FSH levels were elevated in all hemicastrated rats after 8 h. However, serum immunoreactive inhibin levels were decreased only after 72 h in 8-day-old rats and after 24 h in 15- and 22-day-old rats. Inhibin α-subunit mRNA expression was increased in the testes of hemicastrated rats of 8 and 15 days of age, whereas inhibin βB-subunit mRNA expression was elevated in the testes of 15-day-old rats but not in those of 8- and 22-day-old rats. The increase in α-subunit mRNA content per testis was caused by an increased concentration and increased testicular weight, whereas the increase in βB-subunit mRNA in the remaining testis parallelled the increased testicular weight, indicating that different mechanisms play a role in the regulation of these mRNAs. In 22-day-old rats, a transiently decreased expression of inhibin βB-subunit mRNA was observed 8 h after hemicastration. The increased inhibin α- and βB-subunit mRNA expression in 8- and 15-day-old rats did not result in increased testicular bioactive and immunoreactive inhibin content of the remaining testis, whereas in 22-day-old rats an increased immunoreactive inhibin content of the remaining testis was observed. These data indicate that efficiency of translation, post-translational modifications or transport from the testis play an important role in determining the final testicular content of inhibin.

In conclusion, the response of the remaining testis and the role of inhibin in the regulation of the pituitary-testis axis after unilateral castration depend on the age at which the animals are hemiorchidectomized.

Introduction

The testicular glycoprotein hormone inhibin was first identified by its ability to inhibit the secretion of follicle-stimulating hormone (FSH) by the pituitary gland (for review see de Jong, 1988). Under physiological circumstances in male rats this mechanism appears to be effective only in immature animals, since after castration an acute increase in FSH secretion occurred only in rats of 15 to 35 days of age, whereas in older rats only minor effects on FSH levels were found (Hermans *et al.* 1980; Rivier *et al.* 1989a). Furthermore, passive immunization against inhibin caused increased

levels of FSH in prepubertal, but not in adult rats (Culler and Negro-Vilar, 1988; Rivier *et al.* 1988). On the other hand, FSH was shown to stimulate production of inhibin by cultured Sertoli cells from immature rats (Bicsak *et al.* 1987), and daily administration of FSH to newborn rats stimulated testicular bioactive inhibin levels (Ultee-van Gessel *et al.* 1988). Finally, decreased serum FSH levels after hypophysectomy caused a larger decrease in inhibin α-subunit mRNA expression in immature rats than in mature rats (Krummen *et al.* 1989). Based on this evidence, it appears that regulation of FSH by inhibin and regulation of inhibin by FSH are only tightly interlinked during the prepubertal period.

Compensatory hypertrophy of the remaining testis 20 days after hemicastration of immature rats has been described (Moger, 1977; Cunningham *et al.* 1978; Hochereau-de Reviers and Courot, 1978; Putra and Blackshaw, 1982); this hypertrophy was associated with increased FSH levels (Brown *et al.* 1991). In addition, hemicastration of neonatal rats results in an increased amount of bioactive inhibin in the remaining testis after 1 to 6 weeks (Ultee-van Gessel and de Jong, 1987). In the present experiments, short-term effects of decreased serum inhibin levels on FSH and of elevated FSH levels on inhibin production were studied, using the model of hemicastrated immature rats of different ages. Furthermore, the possibility that regulation of the expression of inhibin subunit mRNAs contributes to the regulation of inhibin production *in vivo* was studied by measuring inhibin mRNA levels in the remaining testis.

Materials and Methods

Experimental Procedure

To investigate the age-dependent effects of hemicastration on testicular inhibin content, Wistar rats (substrain RI) were hemicastrated at 8, 15 and 22 days after birth. The testes which were removed at the time of hemicastration were snap-frozen and used to generate the data on testicular mRNA and hormone levels at time zero. Groups of six to ten hemicastrated rats were killed by decapitation 8, 24 or 72 h after surgery, along with equal numbers of age- and body weight-matched sham-operated animals. Testes were snap-frozen in liquid nitrogen and stored at -80°C until further processing. Five testes from 8- to 11-day-old rats were pooled for the isolation of RNA; and two pools of two and a half testes were used for the measurement of inhibin and testosterone. Testes from 15- to 18-day-old and 22- to 25-day-old rats were halved, and two to six halves were pooled for RNA isolation. The other hemitestes were used for hormone assay. Sera were collected and stored at -20°C until

hormone assays were carried out.

RNA isolation and analysis

RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure described by Chomczynski and Sacchi (1987). The yield of total RNA per mg tissue was not significantly different between groups, and amounted to 3.17 ± 0.08 μg RNA/mg testicular tissue (mean ± SEM; n=57). Of each sample, 40 μg total RNA was subjected to Northern blotting, and hybridized with inhibin subunit cDNA probes as described earlier (Klaij *et al.* 1992). The cDNA probes used were a 1.25 kb EcoRI fragment encoding the α-subunit (α7/pUC18), and a 1.5 kb EcoRI fragment encoding part of the βB-subunit (βB11/pUC18) of rat inhibin (Esch *et al.* 1987a).

Intensity of hybridizing bands was measured by densitometric scanning as described previously (Klaij *et al.* 1990). As a control for equal amounts of RNA, gels were stained with ethidium bromide to visualize ribosomal RNA bands. Hybridizing signals were not normalized against actin, since the expression of this mRNA in the testis changes with age (Slaughter *et al.* 1987).

In order to compare the expression of RNAs on different blots, a sample of 40 μg from a pool of total testicular RNA from 21-day-old rats was included on each blot, and the intensity of the hybridizing bands was related to the intensity of this standard.

Inhibin measurements

Testicular tissue was homogenized in a 5 ml Teflon-glass homogenizer in 1 ml phosphate-buffered saline (PBS). Homogenates were centrifuged at 100,000 g for 1 h at 4 °C in a Beckmann L5-65 ultra-centrifuge using an SW60 rotor, and supernatants were collected. A volume of 50 μl was collected for the measurement of testosterone, and the remaining supernatant was incubated with an equal volume of dextran-coated charcoal suspension (1% Norit, 0.1 % dextran T300 in PBS, pH 7.0) at 4 °C for 30 min in order to remove the steroids. Thereafter, charcoal was removed by centrifugation at 1500 g for 10 min and cytosols were collected, sterilized by filtration through a 0.2 μm filter (Schleicher and Schuell, Dassel, Germany) and stored at −20°C until estimation of inhibin bioactivity and immunoreactivity.

Levels of immunoactive inhibin in testis homogenates and serum were measured by radioimmunoassay as described by Robertson *et al.* (1988), using an antiserum against purified 32 kDa bovine follicular fluid (bFF) inhibin and iodinated 32 kDa bFF inhibin. These materials were kindly made available by Dr. G. Bialy (NICHHD, Bethesda, MD, USA). A charcoal-treated bFF preparation with an

arbitrary potency of 1 U/μg protein was used as a standard (Grootenhuis *et al.* 1989). Testicular and serum immunoreactive inhibin was measured in two separate assays; the intra-assay variation coefficient amounted to 24.6 % for testicular immunoreactive inhibin and 18.3 % for serum immunoreactive inhibin.

The amount of bioactive inhibin was determined by an *in vitro* bioassay in which suppression of spontaneous FSH release from cultured rat pituitary cells was estimated (Grootenhuis *et al.* 1989). The bFF preparation described above was used as a standard. The precision index (λ) of these assays amounted to 0.145 $\pm$ 0.006 (mean $\pm$ SEM, n=5).

Measurement of FSH, LH and testosterone

FSH and luteinizing hormone (LH) were measured by radioimmunoassay (Grootenhuis *et al.* 1989), using the antibodies developed by Welschen *et al.* (1975). All results are expressed in terms of NIADDK-rat FSH-RP-3 and NIADDK-rat LH-RP-2. Testosterone was measured by radioimmunoassay, using the antiserum described by Verjans *et al.* (1973). All samples for each hormone were measured in one assay. Intra-assay coefficients of variation were 17.9% for FSH, 11.4% for LH and 3.5% for testosterone.

Statistical analysis

All data are presented as means $\pm$ SEM, except for results of Northern blotting of mRNA from 8-day-old hemicastrated rats which are single measurements of a pool of five testes. Data were analysed for each age group by two-way analysis of variance (ANOVA), with time and hemicastration as variables. When the effect of hemicastration was significant, significances of differences between control and hemicastrated rats at each time-point were assessed using the Student's t-test. Differences were considered significant if $P<0.05$. Correlation between different parameters was determined using regression analysis.

Results

Body and testis weights

Body weights increased in an age-dependent manner throughout the experiment and were not affected by hemicastration at any age (not shown). In control and hemicastrated animals, testis weights increased with time and were correlated with

body weights (controls: $r=0.93$, d.f.$=64$, $P<0.0005$; hemicastrated rats: $r=0.94$, d.f.$=64$, $P<0.0005$).

In hemicastrated rats of 8 and 15 days of age, a significant increase in the weight of the remaining testis occurred 24 h ($P<0.0005$ and $P<0.01$ respectively) and 72 h ($P<0.01$ and $P<0.005$ respectively) after hemicastration, when compared with testis weights in control rats at the same time-points (Fig. 5.1). In contrast, no difference was found between the weights of the testes from 22-day-old hemicastrated and control animals.

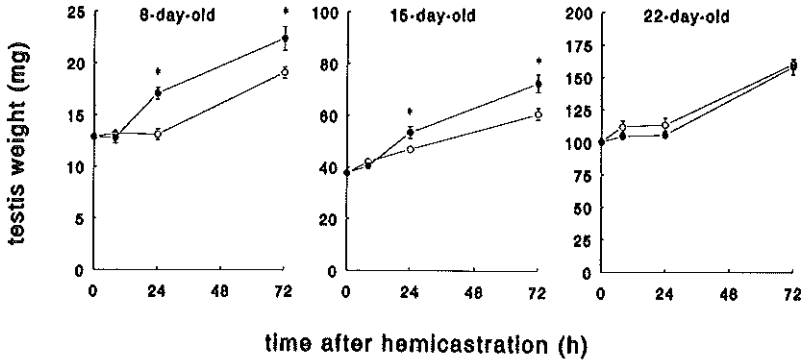

Fig. 5.1: Effect of hemicastration on testis weight. Rats were hemicastrated (●) at the age of 8, 15 or 22 days, and killed after 8, 24 or 72 h. Age-matched sham-operated animals (○) were used as controls. Values are means ± SEM of six to ten observations. The values fell within the symbols for points without SEM bars.
** $P<0.05$ compared with controls (ANOVA and Student's t-test).*

Serum hormone levels

FSH levels remained constant with age in control animals (Fig. 5.2a), and were significantly elevated from 8 h after operation in hemicastrated rats of all ages when compared with control levels. However, the relative increase and the time-course of the increase differed between the three age groups.

Immunoreactive inhibin levels in serum were constant in control rats of 8-11 days of age, increased in rats from 15 days of age onwards, and decreased in rats from 22 days of age onwards (Fig. 5.2b). A significant decrease in immunoreactive inhibin was only observed in 8-day-old hemicastrated animals at 72 h after hemicastration ($P<0.0005$), whereas in 15- and 22-day-old rats significant decreases were found after

24 ($P<0.025$ and $P<0.005$ respectively) and 72 h ($P<0.005$ and $P<0.05$ respectively).

LH levels in control rats decreased with age from 2.4 ± 0.1 ng/ml in 8-day-old rats to 0.5 ± 0.1 ng/ml in 22-day-old rats. Hemicastration had no effect on LH levels (data not shown).

Testosterone levels were not measured in 8-day-old rats, since the amount of serum from these rats was limited. Testosterone levels in 15- and 22-day-old hemicastrated rats were not significantly different from levels in control rats (data not shown).

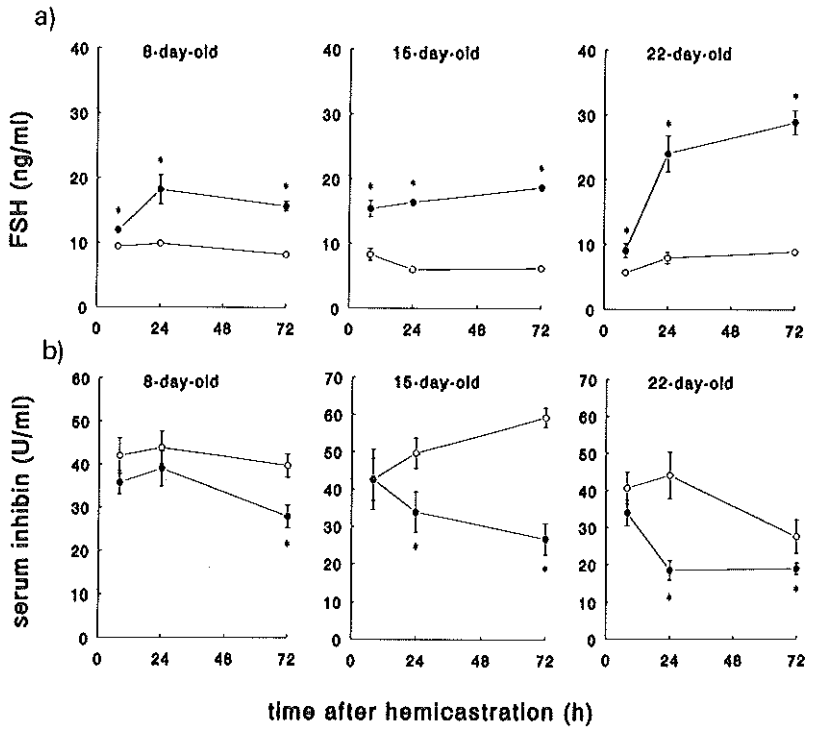

Fig. 5.2: Effect of hemicastration on serum hormone levels. Rats were hemicastrated (●) at the age of 8, 15 or 22 days, and killed after 8, 24 or 72 h. Age-matched sham-operated animals (○) were used as controls. (a) FSH or (b) immunoreactive inhibin were measured in serum by radioimmunoassay. Values are means ± SEM of six to ten observations. The values fell within the symbols for points without SEM bars.
** $P<0.05$ compared with controls (ANOVA and Student's t-test)*

Inhibin subunit mRNA levels

Inhibin α-subunit mRNA levels (Fig. 5.3a) increased with time in control animals, when expressed as arbitrary units per testis. This increase was due to increasing testis weight, since the expression of α-subunit mRNA per mg testis decreased with time. Compared with its expression in testes from intact rats, α-subunit mRNA expression was significantly increased in hemicastrated rats of 8 and 15 days of age, from 24 h and 8 h onwards respectively. In contrast, no significant effect of hemicastration was observed in 22-day-old rats.

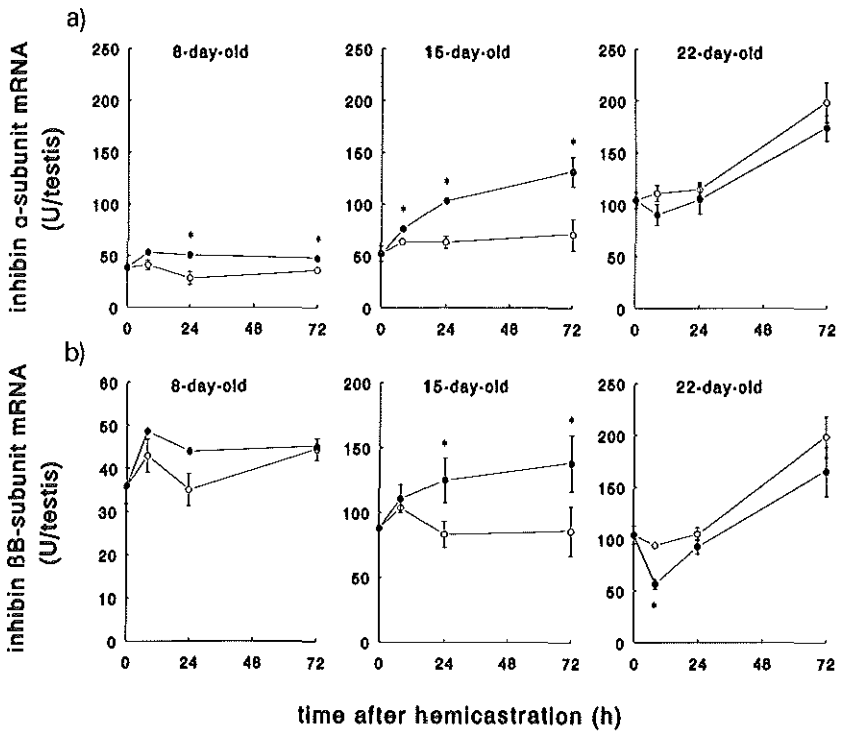

*Fig. 5.3: Effect of hemicastration on (a) inhibin α-subunit, and (b) inhibin βB-subunit mRNA expression in the testis. Rats were hemicastrated (●) at the age of 8, 15 or 22 days, and killed after 8, 24 or 72 h. Age-matched sham-operated animals (○) were used as controls. Testicular RNA was subjected to Northern blotting, and hybridizing bands were quantitated by densitometric scanning and expressed relative to the expression in a pool of testes from 21-day-old rats. Hybridization signals for the 4.2 kb and 3.5 kb βB-subunit mRNAs were added. Values are means $\pm$ SEM of three to six pools of testis from two to six animals each, except for 8-day-old hemicastrated rats where one pool of testes from five rats was used. For other points without SEM bars, the values fell within the symbols. * $P<0.05$ compared with controls (ANOVA and Student's t-test)*

Combined levels of the 4.2 kb and 3.5 kb βB-subunit mRNAs are presented in Fig. 5.3b. Total inhibin βB-subunit mRNA expression per testis was constant in control rats of 8-11 days of age, the expression in 15-day-old rats was twice as high, and from 22 days onwards βB-subunit expression increased further (Fig. 5.3b). No significant difference existed between βB-subunit mRNA expression in intact and hemicastrated rats of 8 days of age, whereas a significant increase was found in hemicastrated rats of 15 days after 24 and 72 h ($P<0.05$). In 22-day-old rats, a significant decrease in βB-subunit mRNA expression was found at 8 h after hemicastration. The ratio between 4.2 kb and 3.5 kb βB-subunit mRNA decreased with age from 2.20 ± 0.37 in 8-day-old rats to 0.46 ± 0.03 in 25-day-old rats. Hemicastration had no effect on the ratio in 8- and 15-day-old rats, but in 22-day-old hemicastrated rats the ratio was significantly increased when compared with the ratio in controls (Table 5.1).

Table 5.1: ***Ratio between 4.2 kb and 3.5 kb βB-subunit mRNA expression in testes from control and hemicastrated rats of different ages at various times after surgery.*** *Values are presented as means ± SEM of three to six pools of testis from two to six animals each, except for 8-day-old hemicastrated rats where one pool of testes from five rats was used.*

age (days)	time after surgery (h)	Ratio	
		control rats	hemicastrated rats
8	0	2.20 ± 0.37	2.20 ± 0.37
	8	1.59 ± 0.16	1.49
	24	2.72 ± 0.09	2.27
	72	2.06 ± 0.12	2.07
15	0	0.73 ± 0.06	0.73 ± 0.06
	8	0.79 ± 0.05	0.77 ± 0.02
	24	0.85 ± 0.09	0.83 ± 0.01
	72	0.78 ± 0.05	0.67 ± 0.02
22	0	0.64 ± 0.03	0.64 ± 0.03
	8	0.52 ± 0.01	0.63 ± 0.03 *
	24	0.48 ± 0.04	0.61 ± 0.06
	72	0.47 ± 0.03	0.54 ± 0.00 *

* $P<0.05$ compared with control rats (ANOVA and Student's t-test).

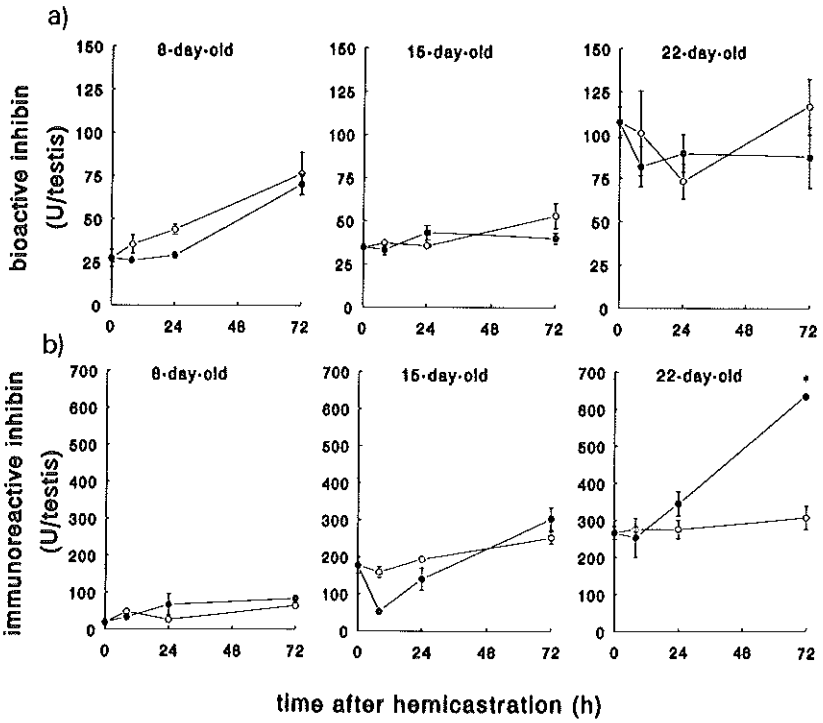

*Fig. 5.4: Effect of hemicastration on (a) testicular bioactive and (b) immunoreactive inhibin. Rats were hemicastrated (●) at the age of 8, 15 or 22 days, and killed after 8, 24 or 72 h. Age-matched sham-operated animals (○) were used as controls. Values are means ± SEM of six to ten observations. The values fell within the symbols for points without SEM bars. * $P<0.05$ compared with controls (ANOVA and Student's t-test)*

Testicular hormone contents

Testicular bioactive inhibin content in control rats increased with time in rats of 8-11 days of age, and was constant in 15- to 18-day-old rats and 22- to 25-day-old rats (Fig. 5.4a). No significant effect of hemicastration on bioactive inhibin content was found.

Testicular immunoreactive inhibin content increased with age until 22 days in control animals (Fig. 5.4b). No significant difference was observed between immunoreactive inhibin contents in control and hemicastrated rats of 8 days and 15

days of age. The testicular content of immunoreactive inhibin increased time-dependently after hemicastration in 22-day-old hemicastrated rats, but was only significantly higher than in control rats after 72 h ($P<0.0005$). Testicular levels of bio- and immunoreactive inhibin were correlated in testes from control ($r=0.589$, d.f.$=37$, $P<0.0005$) and hemicastrated ($r=0.628$, d.f.$=22$, $P<0.005$) rats.

Testicular testosterone levels in control and hemicastrated rats were not significantly different at any time-point studied (data not shown).

Discussion

FSH levels and testicular hypertrophy

Changes in FSH levels with age in control rats are in agreement with those reported previously (Culler and Negro-Vilar, 1988; Ackland and Schwartz, 1991). The time-course of the increase of FSH in 15-day-old hemicastrated rats confirms the data of Brown and Chakraborty (1991).

The present results show that the testicular hypertrophy which has been observed by other authors at 20 days after hemicastration of prepubertal rats (Moger, 1977; Cunningham *et al.* 1978; Hochereau-de Reviers and Courot, 1978; Putra and Blackshaw, 1982; Brown and Chakraborty, 1991), is evident after 24 h in rats hemicastrated at the ages of 8 and 15 days. In contrast, no increase in testis weight was observed after hemicastration in 22-day-old rats up to 72 h after hemicastration.

Testicular hypertrophy has been associated with changes in serum FSH (Orth, 1982, 1984a), and has been attributed to two phenomena: an increase in the number of Sertoli cells per testis (Cunningham *et al.* 1978; Orth *et al.* 1984b), or an increase in the number of germ cells per Sertoli cell (Putra and Blackshaw, 1982). An increased number of Sertoli cells was only observed in rats hemicastrated when younger than 20 days (Cunningham *et al.* 1978; Putra and Blackshaw, 1982); Sertoli cell division stops in normal rats at around 15-18 days of age (Steinberger and Steinberger, 1971; Orth, 1982; van Haaster *et al.* 1992). Apparently, FSH is able to stimulate the existing proliferation of Sertoli cells but cannot prolong the period during which proliferation takes place. The present study shows that FSH levels were significantly increased in hemicastrated rats of 8, 15 and 22 days of age after 8 h, but this resulted in a rapid increase of testis weight in 8- and 15-day-old rats only. The increased testis weight after 20 days described by others in rats hemicastrated at 20-25 days of age (Moger, 1977; Putra and Blackshaw, 1982) might therefore be caused by a mechanism which differs from the stimulation of Sertoli cell proliferation by FSH, e.g. by the stimulation of germ cell numbers in older rats. The role of inhibin and activin in this

process remains unclear, since, in the present experiments, an increased amount of testicular immunoreactive but not bioactive inhibin was observed in 22-day-old hemicastrated rats.

Inhibin subunit mRNA expression

Age-dependent changes in the expression of inhibin α-subunit mRNAs in intact rats are in agreement with previous observations (Meunier *et al.* 1988a; Keinan *et al.* 1989; Klaij *et al.* 1992): the concentration of inhibin α-subunit mRNA decreased with age but, together with an increase in testis weight, this resulted in an increasing testicular content. The expression of α-subunit mRNA per mg testis increased after hemicastration in 8- and 15-day-old rats. This is most probably caused by the increased FSH levels, since FSH has been shown to stimulate inhibin α-subunit mRNA expression in vitro (Toebosch *et al.* 1988; Keinan *et al.* 1989) by an increase in cyclic AMP (Klaij *et al.* 1990). In contrast, increased FSH levels did not cause an increased inhibin α-subunit mRNA expression in 22-day-old hemicastrated rats.

Levels of inhibin βB-subunit mRNA expression are in agreement with data obtained in a previous study (Klaij *et al.* 1992), in which a change in the ratio between the expression of 4.2 kb and 3.5 kb βB-subunit mRNA during testicular development was also observed. In contrast to the increased concentration of inhibin α-subunit mRNA after hemicastration, βB-subunit mRNA expression per mg testis did not change in 8- and 15-day-old rats after hemicastration. This is in agreement with the fact that FSH does not affect βB-subunit mRNA expression in cultured Sertoli cells (Toebosch *et al.* 1988; Klaij *et al.* 1990). The increase in testicular βB-subunit mRNA content can be explained on the basis of increased testis weight, indicating that inhibin α- and βB-subunit mRNA expression are differentially regulated *in vivo*. The transient decrease in βB-subunit mRNA expression in 22-day-old hemicastrated rats might have been caused by an effect of FSH on intracellular calcium levels (Grasso *et al.* 1991); increased calcium levels suppress βB-subunit mRNA expression in cultured Sertoli cells (Klaij *et al.* 1992). Conversely, Feng *et al.* (1989a) have shown that hypophysectomy results in increased inhibin βB-subunit mRNA concentration and content, possibly by decreasing FSH levels.

The functional difference between the two βB-subunit mRNAs is not known. Although only one βB-subunit gene exists in the rat (Feng *et al.*, 1989b), the two βB-subunit mRNAs originate from initiation of transcription at alternative transcription initiation sites (Feng and Chen, 1993), and it is not clear whether this results in different open reading frames in the two mRNAs. Further investigation of the mRNAs is necessary to determine which differences exist between the two βB-subunit mRNAs and whether translation of the mRNAs results in different protein products. The ratio between the 4.2 kb and 3.5 kb βB-subunit mRNA changes with age, and may be

indicative of the state of maturation of the Sertoli cells. The increased FSH levels after hemicastration in 22-day-old rats might delay maturation, as suggested by the relative increase in the ratio after hemicastration in rats of this age.

Bioactive and immunoreactive inhibin in testes and serum

The increased content of inhibin α- and βB-subunit mRNA expression in 8- and 15-day-old hemicastrated rats was expected to cause increased testicular levels of bioactive and immunoreactive inhibin. Surprisingly, the testicular bioactive inhibin content was unchanged in 8- and 15-day-old rats. Immunoreactive inhibin was unchanged in 8-day-old hemicastrated rats, and did not change systematically in 15-day-old rats. The reason for these observations might be found in an increased secretion of inhibin from the testis, for plasma inhibin levels were decreased to a lower extent than would be expected on the basis of the initial short biological half-life of inhibin (Robertson *et al.* 1988; Woodruff *et al.* 1993). The blood-testis barrier has not yet been formed in 8- or 15-day-old rats (Setchell *et al.* 1988; Russell *et al.* 1989), and the secretion of inhibin into the circulation is therefore not restricted at these ages. An increased amount of testicular immunoreactive but not bioactive inhibin was observed in 22-day-old hemicastrated rats; this increased amount was not caused by a higher level of α-subunit mRNA expression. A possible explanation might be an increased translation rate of α-subunit mRNAs. Furthermore, altered post-translational modification of inhibin might take place in hemicastrated rats and might cause altered protein stability, as has been described for glycosylation of LH after castration (Keel and Grotjan, 1985), where discrepancies have also been described between gonadotrophin mRNA levels and protein levels in castrated rats at certain ages (Pakarinen and Huhtaniemi, 1992). The same explanations might be applicable for the observation of decreased ratios between bioactive and immunoreactive inhibin (B/I ratios) in the testes of 15- to 22-day-old rats as compared with B/I ratios in 8-day-old rats.

The decrease in serum inhibin levels does not precede the increase in FSH levels. This does not agree with the theory that increased levels of FSH in hemicastrated animals are caused by decreased levels of inhibin. However, it is not known whether the immunoreactive inhibin which is measured in the serum also is bioactive, since the RIA used is specific for the α-subunit of inhibin (Robertson *et al.* 1989, Schneyer *et al.* 1990) and α-subunit containing proteins different from bioactive inhibin can be secreted by the testis (de Winter *et al.* 1992a). Furthermore, a small decrease in bioactive inhibin levels might be sufficient to cause increased secretion of FSH from the pituitary.

It is concluded from this study that the effects of hemicastration on different

testicular functions are related to the age at which the animals are hemicastrated. Short-term hypertrophy and effects on inhibin subunit mRNA expression occurred only in hemicastrated rats of 8 and 15 days of age. Testicular bioactive and immunoreactive inhibin content were not affected at these ages, probably because of increased transport from the testis.

Acknowledgements

This study was supported financially by the Dutch Science Foundation (NWO) through GB-MW (Medical Sciences). The authors would like to thank Dr. S. Shimasaki and Dr. N. Ling for the inhibin cDNA probes, the NIDDK for the materials used in the FSH and LH radioimmunoassays and the NICHHD for the materials used in the inhibin radioimmunoassay.

General discussion

6.1 Introduction

The aim of the studies described in this thesis was to gain further insight in the regulation of inhibin production by the testis, in particular by Sertoli cells. In Chapters 2 and 3, cultured Sertoli cells isolated from immature rats were used for experiments which yielded information on mechanisms of hormone action responsible for the regulation of inhibin production. *In vivo* studies were performed in Chapters 4 and 5, in order to determine whether changes in FSH levels, and/or the stage of the spermatogenic cycle play a physiological role in the regulation of inhibin. In the following paragraphs, regulation of inhibin production in the testis (section 6.2) will be discussed, and suggestions for future studies on the regulation of inhibin production will be presented (section 6.3).

6.2 Regulation of inhibin production in the testis

In this section, regulation of testicular inhibin production *in vivo* will be discussed. Subsequentially, effects of several hormones and other factors on inhibin production by cultured Sertoli cells from immature rats and by other testicular cells will be described.

6.2.1 *In vivo* regulation of testicular inhibin

Development

Inhibin bioactivity has been detected in testes of 1-day-old rats (Au et al., 1986; Ultee-van Gessel et al., 1987). The concentration of testicular bioactive inhibin, and the amount of bioactive inhibin produced by cultured Sertoli cells, decrease with age (Ultee van Gessel et al., 1987). However, the total testicular bioactive inhibin content increases with age as a result of the increase in testis weight. Therefore, it was postulated that in immature animals most of the inhibin produced is secreted into the circulation (Ultee-van Gessel et al., 1987). Immunoreactive inhibin in plasma is highest in 8- to 15-day-old rats and thereafter decreases with age. The concentration of immunoreactive testicular inhibin per wet weight also declines with age, whereas the amount per testis increases until 90 days of age (Rivier et al., 1988). Concomitantly, the expression of the α-subunit mRNA per testis increases with age as a result of increased testicular weight; the concentration of this mRNA slightly decreases from 21 days onwards (Meunier et al., 1988a; Keinan et al., 1989; Chapter 3). For the βB-subunit, the ratio between the amounts of the 4.2 kb and 3.5 kb mRNAs decreases during postnatal testis development (Chapter 3), whereas the sum of the concentrations of these mRNAs decreases between 14 and 28 days of life and thereafter does not change (Chapter 3). The βA-subunit mRNA is hardly expressed in testicular tissue; at

21 days of age the expression of this mRNA was undetectable by S1-nuclease analysis (Meunier et al., 1988a), whereas a faint 6.5 kb hybridizing band was detected using Northern blot analysis of total RNA of testes from 20-day-old rats (Feng et al., 1989a). This can be explained by the fact that this mRNA is produced by peritubular myoid cells (de Winter et al., 1994).

FSH, LH and testosterone

The first indication that pituitary hormones regulate the production of inhibin *in vivo* stems from a study by Au et al. (1985), who showed that the testicular content of bioactive inhibin was decreased in hypophysectomized rats. Later it was shown, that α-subunit mRNA expression was dramatically decreased after hypophysectomy of immature and mature animals (Krummen et al., 1989, 1990; Keinan et al., 1989). Furthermore, it was shown that FSH, but not testosterone, could restore the expression of the α-subunit mRNA in the hypophysectomized animals (Krummen et al., 1989). Hypophysectomy did not affect testicular βB-subunit mRNA content in mature animals (Krummen et al., 1989). In contrast, the concentration of testicular βB-subunit mRNA was increased in immature hypophysectomized animals (Krummen et al., 1989, 1990; Feng et al., 1989b). However, when βB-subunit mRNA expression was calculated per testis, an increased (Feng et al., 1989b), unchanged (Krummen et al., 1989), or decreased βB-subunit mRNA expression (Krummen et al., 1990) was found.

Interestingly, administration of LH to the hypophysectomized rats restored α-subunit mRNA expression in mature, but not in immature, animals (Krummen et al., 1990). This increase can probably be attributed to increased expression of inhibin α-subunit in Leydig cells, since it has been shown that *in vivo* administration of hCG or LH to rats increased immunoreactive inhibin levels (McLachlan et al., 1988; Drummond et al., 1989; Maddocks and Sharpe, 1990), and that the presence of Leydig cells, which express the inhibin α-subunit (Risbridger et al., 1989a; de Winter et al., 1992), is necessary for this increase (Drummond et al., 1989; Maddocks and Sharpe, 1990).

Effect of hemicastration

Short-term effects of hemicastration were presented in Chapter 4. The effect of elevated FSH levels on inhibin production depends on the age of the rats at the time of hemicastration. Immunoreactive and bioactive inhibin content were unchanged in 8-day-old and 15-day-old hemicastrated rats, although inhibin α- and βB-subunit mRNA expression was increased by hemicastration in these animals. Immunoreactive inhibin content was increased in 21-day-old hemicastrated rats, whereas no effect of hemicastration on α-subunit mRNA expression was found. Since changes in inhibin α-

subunit and βB-subunit mRNA expression did not coincide with changes in bioactive or immunoreactive inhibin levels, it was concluded that posttranscriptional events, such as translation and transport play an important role in determining the final testicular concentration of inhibin.

Effects of GnRH-antagonist and testosterone

When mature rats were treated with a GnRH antagonist (Perheentupa et al., 1993), serum FSH levels were suppressed, whereas testicular inhibin α-subunit, βB-subunit and βA-subunit mRNA concentrations were not significantly decreased. However, treatment with a combination of GnRH antagonist and testosterone caused a dramatic increase in the expression of βA-subunit mRNA and an increase in the expression of α-subunit mRNA as compared to antagonist treated animals, while serum FSH levels were unchanged and pituitary FSH was increased as compared with the antagonist treated group. Serum immunoreactive inhibin levels were unchanged in these rats. In immature rats, again no significant effect of the GnRH antagonist on the expression of the inhibin subunit mRNAs was found, whereas only the βA-subunit mRNA expression was increased by cotreatment with testosterone. Interestingly, immunoreactive serum inhibin levels and serum and pituitary FSH levels in the antagonist treated animals were significantly decreased, whereas testosterone treatment increased serum inhibin levels and pituitary FSH levels, but not serum FSH levels, to control values. A possible explanation for the pronounced effect of testosterone on βA-subunit mRNA in adult and immature rats might be a stimulation of βA-subunit expression in peritubular cells.

Effect of cryptorchidism

In experimentally cryptorchid testes, an increased inhibin production was found three days after the operation (Gonzales et al., 1989d), which was followed by a decreased inhibin production, which might be caused by impaired Sertoli cell function as a result of prolonged exposure to the increased testicular temperature or the absence of germ cells. Also in cultured Sertoli cells, a higher production of inhibin was observed at 37°C than at 32°C (Ultee-van Gessel et al., 1986). In a pilot experiment (data not shown), an increased production of bioactive and immunoreactive inhibin was observed at 37°C when compared to that at 32 °C. This was not associated with an increased expression of the inhibin subunit mRNAs. Therefore, it is likely that the effect of temperature on cultured cells results from a higher translation rate at 37 °C, or changes in processing.

Stage-specific expression of inhibin subunits and effect of germinal cells

Sertoli cells express the mRNAs encoding the inhibin subunits in a stage-specific

manner, as was also shown for a number of other mRNAs and proteins (see section 1.1.4). After the initial observation by Merchenthaler et al. (1987), that some cross-sections of tubules contain more immunoreactive inhibin-like material than others, it was shown that α-subunit mRNA and the βB-subunit mRNA are expressed highest at stages XIII-I (Bhasin et al., 1989; Kaipia et al., 1991; Kaipia et al., 1992). We found an expression of the βB-subunit similar to the expression described by these authors and an additional high expression of the α-subunit mRNA at stage V in stage-synchronized rats (Chapter 4). The inhibin βA-subunit mRNA is expressed in Sertoli cells during a very limited part of the spermatogenic cycle, highest at stages IX-XI (Kaipia et al., 1992), which may contribute to the fact that we were not able to detect this mRNA in whole testis extracts (Chapter 3). In Chapter 4, it was shown that the expression of the inhibin subunit mRNAs results in highest levels of bioactive and immunoreactive inhibin at stage I (in accordance with Gonzales et al., 1989d), whereas the testicular concentration of inhibin is low at stages VI-IX.

When methoxy acetic acid (MAA) was used to deplete pachytene, diplotene and secundary spermatocytes from the testes, the ensuing absence of late spermatids *in vivo* was associated with decreased plasma inhibin levels and a decreased production of immunoreactive inhibin, as measured in subsequently cultured tubules (Allenby et al., 1991). The absence of pachytene spermatocytes led to an increased immunoreactive inhibin production, although it was thought that MAA itself also had a stimulatory effect. However, when rats were irradiated to create a 'window' in spermatogenesis (Kaipia et al., 1991), a similar increase in α-subunit mRNA expression was observed when pachytene and round spermatocytes were absent from the testis. Finally, also in stage-synchronized testes (Chapter 4), the appearance of pachytene spermatocytes might be responsible for decreased inhibin levels from day 15 after vitamin A-induced reinitiation of spermatogenesis onwards.

These data indicate that spermatogenic cells, which were postulated at the early days of the inhibin concept as a source of inhibin (see section 1.3.3), regulate the production of inhibin in the testis.

6.2.2 Regulation of inhibin production by cultured Sertoli cells

FSH

FSH is one of the main regulators of Sertoli cell function in immature rats. Treatment of cultured Sertoli cells with FSH caused a dose-dependent increase of immunoreactive inhibin in the spent medium (Ying et al., 1987), and this action of FSH could be mimicked by factors which increase intracellular cAMP levels, such as cholera toxin, forskolin, or dbcAMP (Bicsak et al., 1987). It is less clear if FSH can stimulate bioactive inhibin production by Sertoli cells. A number of authors showed

that FSH can stimulate production of bioactive inhibin by Sertoli cells (Le Gac and de Kretser, 1982; Ultee-van Gessel et al., 1986; Toebosch et al., 1988; Risbridger et al., 1989b). On the other hand, high levels of FSH inhibit the production of bioactive inhibin (Risbridger et al., 1989b). Grootenhuis et al. (1990) showed that FSH does not stimulate the production of 32 kDa inhibin; instead the secretion of 29 kDa proαC was stimulated. In all studies in which bioactive and immunoreactive inhibin were measured, FSH decreased the ratio between bioactive inhibin and immunoreactive inhibin (Toebosch et al., 1988; Risbridger et al., 1989b; Janecki et al., 1990). The conflicting results might be caused by the fact that these authors used different culture systems and Sertoli cells from animals of different ages, which might result in differences in the stimulation of βB-subunit mRNA expression by FSH (see below).

Toebosch et al. (1988) showed that FSH stimulated the expression of inhibin α-subunit mRNA, whereas the βB-subunit mRNA level was not changed. Similarly, dbcAMP stimulated the expression of α-subunit mRNA, but not the expression of βB-subunit mRNA (Chapter 2; Pineau et al., 1990; Dykema and Mayo, 1994). These results are in accordance with the reported cAMP-responsive element (CRE) in the promoter of the α-subunit gene and the absence of this element in the rat βB-subunit gene. Blok et al. (1992) and Najmabadi et al. (1993a) showed, using nuclear run-on assay, that stimulation of the expression of the α-subunit gene by FSH results from an increased transcription rate of this gene; the stability of α-subunit mRNA was also increased by cAMP (Najmabadi et al., 1993a). In contrast to our observations showing lack of an effect of dbcAMP on βB-subunit mRNA expression (Chapter 2), Najmabadi et al. (1993b) showed that the transcription of the βB-subunit gene was stimulated by 8-bromo-cAMP. The reason for this discrepancy might again be the different age of the cultured Sertoli cells used. This might cause differences in the amount of protein kinase C in these cells, which decreases with age (Galdieri et al., 1986), and which was shown to affect βB-subunit mRNA expression (Chapter 3). In cultured human Sertoli cells from fetal testes, dbcAMP stimulated the expression of both the α- and βB-subunit mRNAs (Erämaa et al., 1992a). This can be explained on basis of the observation that, in contrast to the rat promoter, the promoter of the human βB-gene contains three putative CREs (Mason et al., 1989).

Increased intracellular Ca^{++} levels, and inhibition of protein kinase C caused a decreased expression of βB-subunit mRNAs (Chapter 3). Therefore, FSH might stimulate expression of inhibin α- and βB-subunit mRNAs by stimulating the protein kinase A pathway, and inhibit expression of the βB-subunit mRNAs by increasing intracellular Ca^{++} levels and inhibiting protein kinase C (see section 1.2.4).

The age-dependent decrease in the expression of βB-subunit mRNAs (Chapter 3) coincides with a decreased activity of protein kinase C in Sertoli cells during progression of Sertoli cell differentiation (Galdieri et al., 1986). The promoter of the

rat βB-subunit gene which is responsible for the formation of the 4.2 kb βB-subunit mRNA contains two AP-2 elements, of which the function can be modulated by cAMP and phorbol esters (Imagawa et al., 1987), whereas the promoter responsible for the 3.5 kb mRNA does not contain these elements, suggesting that protein kinase C regulates only the expression of the 4.2 kb βB-subunit mRNA, which might lead to a decreasing ratio between the expression of the 4.2 kb βB-subunit mRNA and the 3.5 kb βB-subunit mRNA during development.

Steroids

The effects of steroids on inhibin production are still ambiguous. Steinberger (1981) preincubated Sertoli cells from immature rats for 2 days with testosterone, and found an increased production of bioactive inhibin in the spent medium of cells cultured for the following three days. In 7-day-cultured Sertoli cells from 19-day-old rats (Verhoeven and Franchimont, 1983), bioactive inhibin levels were stimulated by testosterone and dihydrotestosterone. In contrast to these observations, bioactive inhibin production was inhibited when immature Sertoli cells were preincubated for 8 h with testosterone and cultured for 24 h (Ultee-van Gessel et al., 1986). Morris et al. (1988) showed that testosterone inhibited basal, and that androstenedione inhibited basal and FSH-stimulated immunoreactive inhibin production in immature Sertoli cells cultured for three days. Finally, Bicsak et al. (1987), Toebosch et al. (1989) and Skinner et al. (1989) found no effect of androgens on immunoreactive inhibin production or inhibin subunit mRNA expression in cultured immature rat Sertoli cells.

A possible explanation for the variable effects of testosterone on inhibin production might be interaction of factors from peritubular myoid cells. Since longterm cultures of different Sertoli cell preparations contain variable amounts of peritubular myoid cells, which produce factors in response to testosterone, it is possible that effects of testosterone are mediated indirectly through these cells. One of these factors is pModS, which stimulates the production of immunoreactive inhibin (Skinner et al., 1989).

Spermatogenic cells

Effects of spermatogenic cells on inhibin production were not only demonstrated *in vivo* (see section 6.2.1). Spermatocytes and spermatids significantly suppressed the production of bioactive inhibin by cultured immature Sertoli cells (Ultee-van Gessel et al., 1986). In contrast, early spermatids stimulated the production of basal and dbcAMP-stimulated immunoreactive inhibin, whereas pachytene spermatocytes only stimulated the latter (Pineau et al., 1990). This stimulatory effect of pachytene spermatocytes is in contrast with the increased inhibin production *in vivo* when pachytene spermatycytes are absent from the testis (see above). However, the

dbcAMP-stimulated immunoreactive inhibin production was also stimulated by liver epithelial cells as a control, indicating that a non-specific effect might play a role. Using spent media from early spermatids, also a stimulation of bioactive inhibin production was observed. Concomittantly, the α-subunit mRNA was increased, whereas βB-subunit mRNA expression was not measured (Pineau et al., 1990).

Other factors

The secretion of immunoreactive inhibin was inhibited by adenosine agonists, most likely by attenuation of adenylyl cyclase activity (Conti et al., 1988). FSH-induced inhibin production was also inhibited by β-endorphin, which is produced by LH-stimulated Leydig cells, probably by inhibition of FSH-receptor coupling to adenylyl cyclase (Morris et al., 1987).

Insulin reduced basal and FSH-stimulated inhibin immunoreactivity produced by long term cultured seminiferous tubules from adult rats (Gonzales et al., 1989a); this effect could be mimicked by the phorbol ester PMA. Using cultured Sertoli cells from 14-day-old rats (Chapter 3), we found an increased amount of immunoreactive inhibin after treatment of the cells with PMA; this increase was due to an increase of the production of pro-αC. The reason for these diverging results is unclear.

EGF increased the amount of immunoreactive inhibin in cultured tubules from adult rats, an effect which was counteracted by insulin or PMA (Gonzales et al., 1989b). Also in Sertoli cells from immature rats, EGF stimulated the amount of immunoreactive inhibin (1.5 fold; Morris et al., 1988); this stimulation was additive with FSH stimulation, in agreement with the different mechanisms of hormone action.

Activin stimulated the amount of basal and FSH-stimulated immunoreactive inhibin in cultured Sertoli cells from 21-day-old rats (de Winter et al., 1994). Since Sertoli cells also produce activin, this might represent an autocrine regulatory mechanism.

In summary

In Fig. 6.1, a model is presented for regulation of inhibin production by Sertoli cells. Although effects of FSH on inhibin production have been thoroughly studied, many questions remain. It is very well possible that apart from effects at the level of mRNA expression; FSH exerts its effects through increased translation or glycosylation, the latter might change the stability of the protein. The roles of other factors in the regulation of inhibin production have been indicated as far as their mechanisms of action have been established. For some factors, only the end-result on inhibin production is known.

Subunit assembly determines whether inhibin or activin is the final product of α/βB expression in Sertoli cells. It is, at present, not known which factor(s)

determine(s) whether α- and β-subunits or β- and β-subunits combine, although the pro-region of the βA-subunit was shown to be of crucial importance for the formation of activin A (Gray and Mason; 1990).

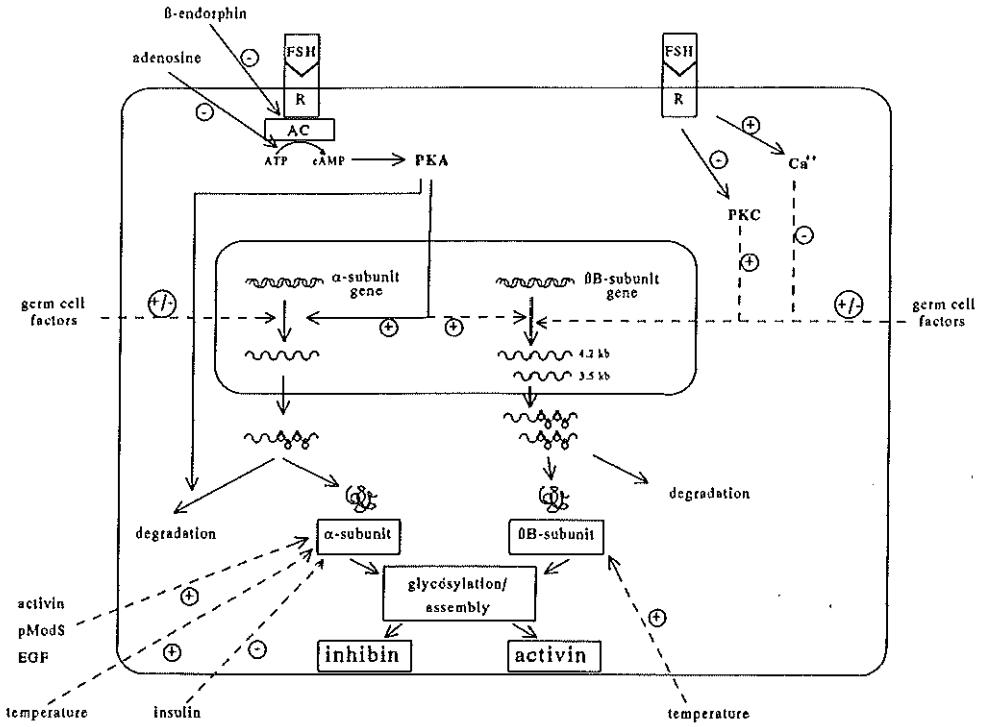

Fig. 6.1: Regulation of inhibin production in Sertoli cells.
Some of the pathways have not been elucidated in detail as yet, and are indicated with (-----). Effects of factors which influence mRNA expression indicated with (----), have been suggested at the level of transcription, although it is also possible that these factors act through effects on mRNA processing and/or stability. (-) inhibitory effect, (+) stimulatory effect.

6.2.3 Regulation of inhibin subunit production by Leydig cells

Production of inhibin subunits by Leydig cells changes with age, as discussed in section 1.3.3. In short, isolated interstitial cells from prenatal and neonatal rats produce βA-subunits, and Leydig cell clusters in these rats produce α-subunits, whereas Leydig cells from adult rats produce only α-subunits. Production of immunoreactive inhibin was stimulated by LH in cultured Leydig cells from immature (de Winter et al., 1992a) and adult rats (Risbridger et al., 1989b; de Winter et al. 1992a). Inhibin and testosterone showed a similar dose-response for LH in these cells (Simpson et al., 1991). However, LH stimulated testosterone production predominantly through cAMP, whereas inhibin was stimulated through calcium dependent mechanisms, since LH stimulation of inhibin was decreased in cells cultured in calcium-depleted medium, whereas the calcium channel-blocking agents diltiazem and verapamil blocked the stimulation of inhibin and had no effect on testosterone production.

In Leydig cell tumors, which can express α-, βA- and βB-subunits, cAMP increased inhibin α-subunit mRNA expression and caused a strongly decreased expression of βA-subunit mRNA (Chen et al., 1993). Since the promoter of the βA-subunit gene does not contain a CRE, it is likely that the effect of cAMP on βA-subunit mRNA is caused by an effect on the stability of this mRNA.

6.2.4 Regulation of inhibin subunit mRNA expression in peritubular myoid cells

Recently, production of activin A by cultured peritubular cells was demonstrated (de Winter et al., 1994). In freshly isolated cells, expression of inhibin α-subunit, βA- and βB-subunit mRNAs was found. Culture of these cells caused decreased levels of α-subunit and βB-subunit mRNAs (possibly by a diminished contribution by Sertoli cells), whereas the expression of βA-subunit was increased to a large extent. The expression of βA-subunit mRNA in the cultured cells was inhibited by 5 x 10-4 M dbcAMP, and stimulated by SCCM. The cultured cells produced activin A, whereas the presence of 20 kD αC was also demonstrated in the spent medium of these cells.

6.3 Future studies on the regulation of inhibin production

From the studies described in this thesis, one thing becomes clear: interpretation of data concerning the regulation of inhibin production is often very complicated, partly due to the existence of several inhibin-related molecules. For an unambiguous interpretation, a combination of different assays should be applied to the samples. However, the sensitivity of these assays is relatively low, and the amount of serum or tissue is often too limited to perform all of these assays. Alternatively, sandwich

immunoassays which are specific for one member of the inhibin-family have recently been developed; these assays might be advantageous in solving the problems. In the paragraphs below, suggestions for further studies on the regulation of inhibin production will be presented.

6.3.1 A comparison between regulation of inhibin production in Sertoli cells and granulosa cells.

The properties of Sertoli cells and granulosa cells change with age, with the stage of the cycle of spermatogenesis and during the oestrous cycle. Although it is not clear to what extent differentiated granulosa cells resemble Sertoli cells at certain points in their development, an attempt is made in this paragraph to compare what is known about regulation in these cells with analogous function in the male and female gonad.

At the level of inhibin subunit mRNA expression, two striking differences exist between cultured Sertoli cells from 21-day old rats and cultured granulosa cells: 1) in Sertoli cells the βA-subunit mRNA is not expressed at a detectable level, whereas in granulosa cells this is the most prominent β-subunit encoding mRNA, 2) in Sertoli cells both the 4.2 kb and the 3.5 kb βB-subunit mRNAs are expressed, whereas in granulosa cells only the 4.2 kb βB-subunit mRNA is expressed. This illustrates that differences must exist between regulation of inhibin subunit mRNA expression in Sertoli cells and granulosa cells. Most studies on granulosa cell inhibin subunit mRNA expression were restricted to the expression of βA-subunit mRNA, which makes a direct comparison of the regulation of βB-subunit mRNA expression between the two cell types impossible. The available data, however, show that FSH stimulates the expression of βB-subunit mRNA only in granulosa cells, and not in Sertoli cells. In contrast to inhibition of protein kinase C by FSH in Sertoli cells, a stimulation of protein kinase C activity was demonstrated in granulosa cells (Pennybacker and Herman; 1991). This might explain the different effects of FSH on Sertoli and granulosa cells, with the result that inhibin α-subunit and β-subunit mRNA expression is coordinately regulated by FSH in granulosa cells, whereas a differential regulation occurs in Sertoli cells.

Also for other factors, divergent effects were found in granulosa cells and Sertoli cells; insulin increased basal and FSH-induced immunoreactive inhibin production in granulosa cells (Suzuki et al., 1987), whereas in Sertoli cells the production of immunoreactive inhibin was decreased (Gonzales et al., 1989a). Furthermore, EGF increased the production of immunoreactive inhibin in isolated seminiferous tubules from adult rats (Gonzales et al., 1989b), but caused a decrease in inhibin secretion in rat granulosa cells (Suzuki et al., 1987; Zhiwen et al., 1987).

Finally, activin stimulated immunoreactive inhibin production in both Sertoli cells (de Winter et al., 1994) and granulosa cells (LaPolt et al., 1989; Xiao and Findlay,

1991).

The mechanism of action of these hormones in the regulation of inhibin production are not known as yet. Therefore, when effects diverge in Sertoli cells and granulosa cells, it is not clear whether similar signal transduction pathways act differently on regulation of inhibin production, or whether similar hormones act through different signal transduction pathways. It would be of interest to study mechanisms of action of the above mentioned factors in regulation of inhibin production in more detail.

6.3.2 Other factors which might regulate inhibin production

Although many factors were shown to affect inhibin production in cultured granulosa (see section 1.3.2) and Sertoli cells, and the mechanisms of action of many of these factors still remain to be solved, the question remains which factors other than FSH are of physiological importance for the regulation of inhibin production in the male rat, especially for the stage-dependent regulation. One possible candidate is nerve growth factor (NGF), which is produced by germ cells in a stage-specific manner (Ayer-le Lievre et al., 1988). Since the receptor for this factor is also stage-specifically expressed in Sertoli cells (Persson et al., 1990), NGF might be responsible for the stage-specific expression of the inhibin-subunits.

Other hormones which might be of physiological importance in the regulation of inhibin production are glucocorticoids. Glucocorticoid receptors are present in the rat testis, in Sertoli cells and peritubular myoid cells. The concentration of these receptors shows an age-dependent decrease (Levy et al., 1989). Effects of glucocorticoids on Sertoli cells have been described scarcely. In bovine Sertoli cells, dexamethasone inhibited the production of plasminogen activator (Jenkins and Ellison, 1986). In a pilot experiment, dexamethasone was shown to transiently decrease expression of the βB-subunit mRNAs in cultured Sertoli cells from immature rats (not shown).

Another hormone which might affect inhibin production is thyroid hormone. Triiodothyronine (T_3) stimulated IGF-1 production by Sertoli cells (Palmero et al., 1990), and receptors for T_3 are only found during a very limited time period in Sertoli cells (Janini et al., 1990). In immature hypothyroid rats, a retardation of morphological differentiation of Sertoli cells was induced, leading to an increase in the number of Sertoli cells in the adult rats. Since in these rats FSH levels were continuously decreased and inhibin levels were increased from 35 days of age (Kirby et al., 1992; van Haaster et al., 1992), it would be of interest to study the regulation of inhibin production by T_3 in more detail.

6.3.3 Concluding remarks on future studies

The studies described in this thesis were focussed on the regulation of inhibin α- and βB-subunit mRNAs in Sertoli cells. Since expression of βA-subunit mRNA was below the limit of detection in total testicular RNA used the experiments described in Chapter 3, levels of this mRNA were not measured in the experiments described in Chapters 4 and 5. It might be of interest to subject the samples used in these studies to hybridisation with a βA-subunit cDNA probe, in order so see whether βA-subunit mRNA expression changes to a measurable level under these experimental conditions.

Recently, a putative inhibin βC-subunit gene was cloned from a liver cDNA library (Hötten et al., 1995). Experiments should be performed to study whether this new inhibin subunit can form heterodimers with the other inhibin subunits, which biological effects such possible dimers might have, and whether the mRNA encoding this subunit is expressed in the testis.

The characterization of regulatory elements in the promoters and other sequences of the inhibin genes might be facilitated by the use of transfection of primary Sertoli cell cultures (Rossi et al., 1990). Using such methods, it should be possible to study the factors responsible for the age-related changes in the expression of the βB-subunit mRNAs.

Finally, elucidation of the mechanism which determines whether inhibin or activin molecules are produced is crucial for finding the answer to the question how inhibin production is regulated. Although quantitation of mRNA expression and translation efficiency might lead to part of the answer, studies on subunit assembly and processing will be necessary.

References

Ackland, J.F. and Schwartz, N.B. (1991). Changes in serum immunoreactive inhibin and follicle-stimulating hormone during gonadal development in male and female rats. Biol. Reprod. 45, 295-300.

D'Agostino, J.B., Woodruff, T.K., Mayo, K.E. and Schwartz, N.B. (1989). Unilateral ovariectomy increases inhibin messenger ribonucleic acid levels in newly recruited follicles. Endocrinology 124, 310-317.

Albano, R.M., Groome, N. and Smith, J.C. (1993). Activins are expressed in preimplantation mouse embryos and in ES and EC cells and are regulated on their differentiation. Development 117, 711-723.

Albiston, A.L., Lock, P., Burger, H.G. and Krozowski, Z.S. (1990). Cloning and characterization of the rat α-inhibin gene. Mol. Cell. Endocrinol. 68, 121-128.

Allenby, G., Foster, P.M.D. and Sharpe, R.M. (1991). Evidence that secretion of immunoactive inhibin by seminiferous tubules from the adult rat testis is regulated by specific germ cell types: Correlation between *in vivo* and *in vitro* studies. Endocrinology 128, 467-476.

Almendral, J.M., Sommer, D., MacDonald-Bravo, H., Burckhardt, J., Perera, J. and Bravo, R. (1988) Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. 8, 2140-2148.

Asaoka, Y., Nakamura, S.-I., Yoshida, K. and Nishizuka, Y. (1992). Protein kinase C, calcium and phospholipid degradation. Trends Biochem. Sci. 17, 414-417.

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

Attisano, L., Cárcamo, J., Ventura, F., Weis, F.M.B., Massagué, J. and Wrana, J.L. (1993). Identification of human activin and TGFβ type I receptors that form heterodimeric kinase complexes with type II receptors. Cell 75, 671-680.

Au, C.L., Robertson, D.M. and de Kretser, D.M. (1985). Effects of hypophysectomy and subsequent FSH and testosterone treatment on inhibin production by adult rat testes. J. Endocrinol. 105, 1-6.

Au, C.L., Robertson, D.M. and de Kretser, D.M. (1986). Measurement of inhibin and an index of inhibin production by rat testes during postnatal development. Biol. Reprod. 35, 37-43.

Ayer-LeLievre, C., Olson, L., Ebendal, T., Hallböök, F. and Persson, H. (1988). Nerve growth factor mRNA and protein in the testis and epididymis of mouse and rat. Proc. Natl. Acad. Sci. USA 85, 2628-2632.

Baly, D.L., Allison, D.E., Krummen, L.A., Woodruff, T.K., Soules, M.R., Chen, S.A., Fendly, B.M., Bald, L.N., Mather, J.P. and Lucas, C. (1993). Development of a specific and sensitive two-site enzyme-linked immunosorbent assay for measurement of inhibin-A in serum. Endocrinology 132, 2099-2108.

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

Bartlett, J.M.S., Weinbauer, G.F. and Nieschlag, E. (1989a). Differential effects of FSH and testosterone on the maintenance of spermatogenesis in the adult hypophysectomized rat. J. Endocrinol. 121, 49-58.

Bartlett, J.M.S., Weinbauer, G.F. and Nieschlag, E. (1989b). Quantitative analysis of germ cell numbers and relationship to intratesticular testosterone following vitamin A-induced synchronization of spermatogenesis in the rat. J. Endocrinol. 123, 403-412.

Barton, D.E., Yang-Feng, T.L., Mason, A.J., Seeburg, P.H. and Francke, U. (1989). Mapping of genes

for inhibin subunits α, βA, and βB on human and mouse chromosomes and studies of *jsd* mice. Genomics 5, 91-99.

van Beek, M.E.A.B. and Meistrich, M.L. (1990). A method for quantifying synchrony in testes of rats treated with vitamin A deprivation and readministration. Biol. Reprod. 42, 424-431.

Berridge, M.J. (1993). Inositol trisphosphate and calcium signalling. Nature 361, 315-325.

Betteridge, A. and Craven, R.P. (1991). A two-site enzyme-linked immunosorbent assay for inhibin. Biol. Reprod. 45, 748-754.

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 βB subunits during the cycle of the rat seminiferous epithelium. Endocrinology 124, 987-991.

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

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

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

Bilezikjian, L.M., Corrigan, A.Z. and Vale, W. (1990). Activin-A modulates growth hormone secretion from cultures of rat anterior pituitary cells. Endocrinology 126, 2369-2376.

Bilezikjian, L.M., Vaughan, J.M. and Vale, W.W. (1993). Characterization and the regulation of inhibin/activin subunit proteins of cultured rat anterior pituitary cells. Endocrinology 133, 2545-2553.

Billah, M.M. and Anthes, J.C. (1990). The regulation and cellular functions of phosphatidylcholine hydrolysis. Biochem. J. 269, 281-291.

Billestrup, N., González-Manchón, C., Potter, E. and Vale, W. (1990). Inhibition of somatotroph growth and growth hormone biosynthesis by activin *in vitro*. Mol. Endocrinol. 4, 356-362.

Birnbaumer, L., Codina, J., Mattera, R., Cerione, R.A., Hildebrandt, J.D., Sunyer, T., Rojas, F.J., Caron, M.G., Lefkowitz, R.J. and Iyengar, R. (1985). Structural basis of adenylate stimulation and inhibition by distinct guanine nucleotide regulatory proteins. In: Molecular mechanisms of transmembrane signalling. P. Cohen and M.D. Houslay eds. pp 131-182. Elsevier Amsterdam.

Blok, L.J., Hoogerbrugge, J.W., Themmen, A.P.N., Baarends, W.M., Post, M. and Grootegoed, J.A. (1992). Transient down-regulation of androgen receptor messenger ribonucleic acid (mRNA) expression in Sertoli cells by follicle-stimulating hormone is followed by up-regulation of androgen receptor mRNA and protein. Endocrinology 131, 1343-1349.

Blok, L.J., Mackenbach, P., Trapman, J., Themmen, A.P.N., Brinkmann, A.O. and Grootegoed, J.A. (1989). Follicle-stimulating hormone regulates androgen receptor mRNA in Sertoli cells. Mol. Cell. Endocrinol. 63, 267-271.

Brewer, G. and Ross, J. (1989). Regulation of *c-myc* mRNA stability *in vitro* by a labile destabilizer with an essential nucleic acid component. Mol. Cell. Biol. 9, 1996-2006.

Brown, J.L. and Chakraborty, P.K. (1991). Comparison of compensatory pituitary and testicular responses to hemicastration between prepubertal and mature rats. J. Androl. 12, 119-125.

Brown, J.L., Dahl, K.D. and Chakraborty, P.K. (1991). Effects of follicular fluid administration on serum bioactive and immunoactive FSH concentrations and compensatory testicular hypertrophy in hemicastrated prepubertal rats. J. Endocrinol. 130, 207-212.

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

Campen, C.A. and Vale, W. (1988). Interaction between purified ovine inhibin and steroids on the release

of gonadotropins from cultured rat pituitary cells. Endocrinology 123, 1320-1328.

Carroll, R.S., Corrigan, A.Z., Gharib, S.D., Vale, W. and Chin, W.W. (1989). Inhibin, activin, and follistatin: regulation of follicle-stimulating hormone messenger ribonucleic acid levels. Mol. Endocrinol. 3, 1669-1676.

Carroll, R.S., Kowash, P.M., Lofgren, J.A., Schwall, R.H. and Chin, W.W. (1991). *in vivo* regulation of FSH synthesis by inhibin and activin. Endocrinology 129, 3299-3304.

Cate, R.L., Mattaliano, R.J., Hession, C., Tizard, R., Farber, N.M., Cheung, A., Ninfa, E.G., Frey, A.Z., Gash, D.J., Chow, E.P., Fisher, R.A., Bertonis, J.M., Torres, G., Wallner, B.P., Ramachandran, K.L., Ragin, R.C., Manganaro, T.F., MacLaughlin, D.T. and Donahoe, P.K. (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.

Chen, C.-L.C., Pignataro, O.P. and Feng, Z.-M. (1993). Inhibin/activin subunits and activin receptor are co-expressed in Leydig tumor cells. Mol. Cell. Endocrinol. 94, 137-143.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979). Isolation of active ribonucleic acids from sources enriched in ribonuclease. Biochemistry 18, 5294-5299.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-159.

Christiansen, P. (1975). Urinary gonadotrophins in the Sertoli cell-only syndrome. Acta Endocrinol. 78, 180-191.

Clapham, D.E. (1995). Calcium signaling. Cell 80, 259-268.

Collard, M.W. and Griswold, M.D. (1987). Biosynthesis and molecular cloning of sulfated glycoprotein 2 secreted by rat Sertoli cells. Biochemistry 26, 3297-3303.

Collard, M.W., Sylvester, S.R., Tsuruta, J.K. and Griswold, M.D. (1988). Biosynthesis and molecular cloning of sulfated glycoprotein 1 secreted by rat Sertoli cells: sequence similarity with the 70-kilodalton precursor to sulfatide/G_{M1} activator. Biochemistry 27, 4557-4564.

Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E. and Goodman, H.M. (1986). A cyclic AMP- and phorbol ester-inducible DNA element. Nature 323, 353-356.

Conti, M., Culler, M.D. and Negro-Vilar, A. (1988). Adenosine receptor-dependent modulation of inhibin secretion in cultured immature rat Sertoli cells. Mol. Cell. Endocrinol. 59, 255-259.

Corrigan, A.Z., Bilezikjian, L.M., Carroll, R.S., Bald, L.N., Schmelzer, C.H., Fendly, B.M., Mason, A.J., Chin, W.W., Schwall, R.H. and Vale, W. (1991). Evidence for an autocrine role of activin B within rat anterior pituitary cultures. Endocrinology 128, 1682-1684.

Crawford, R.J., Hammond, V.E., Evans, B.A., Coghlan, J.P., Haralambidis, J., Hudson, B., Penschow, J.D., Richards, R.I. and Tregear, G.W. (1987). α-inhibin gene expression occurs in the ovine adrenal cortex, and is regulated by adrenocorticotropin. Mol. Endocrinol. 1, 699-706.

Cuevas, P., Ying, S.-Y., Ling, N., Ueno, N., Esch, F. and Guillemin, R. (1987). Immunohistochemical detection of inhibin in the gonad. Biochem. Biophys. Res. Commun. 142, 23-30.

Culler, M.D. and Negro-Vilar, A. (1988). Passive immunoneutralization of endogenous inhibin: sex-related differences in the role of inhibin during development. Mol. Cell. Endocrinol. 58, 263-273.

Culler, M.D. and Negro-Vilar, A. (1990). Destruction of testicular Leydig cells reveals a role of endogenous inhibin in regulating follicle-stimulating hormone secretion in the adult male rat. Mol. Cell. Endocrinol. 70, 89-98.

Cunningham, G.R., Tindall, D.J., Huckins, C. and Means, A.R. (1978). Mechanisms for the testicular hypertrophy which follows hemicastration. Endocrinology 102, 16-23.

Davies, A.G. (1981). Role of FSH in the control of testicular function. Arch. Androl. 7, 97-108.

Dekker, L.V. and Parker, P.J. (1994). Protein kinase C - a question of specificity. Trends Biochem. Sci.

19, 73-77.

De Paolo, L.V., Shimonaka, M., Schwall, R.H. and Ling, N. (1991). *In vivo* comparison of the follicle-stimulating hormone-suppressing activity of follistatin and inhibin in ovariectomized rats. Endocrinology 128, 668-674.

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

Drummond, A.E., Risbridger, G.P. and de Kretser, D.M. (1989). The involvement of Leydig cells in the regulation of inhibin secretion by the testis. Endocrinology 125, 510-515.

Dulbecco, R. and Vogt, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis virus. J. Exp. Med. 99, 167-182.

Dykema, J.C. and Mayo, K.E. (1994). Two messenger ribonucleic acids encoding the common βB-chain of inhibin and activin have distinct 5'-initiation sites and are differentially regulated in rat granulosa cells. Endocrinology 135, 702-711.

Dym, M. and Fawcett, D.W. (1970). The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biol. Reprod. 3, 308-326.

Dynan, W.S. and Tjian, R. (1983). The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell 35, 79-87.

Erämaa, M., Heikinheimo, K. and Voutilainen, R. (1992a). Developmental and cyclic adenosine 3',5'monophosphate-dependent regulation of inhibin subunit messenger ribonucleic acids in human fetal testes. J. Clin. Endocrinol. Metab. 75, 806-811.

Erämaa, M., Hurme, M., Stenman, U.-H. and Ritvos, O. (1992b). Activin A/erythroid differentiation factor is induced during human monocyte activation. J. Exp. Med. 176, 1449-1452.

Esch, F.S., Shimasaki, S., Cooksey, K., Mercado, M., Mason, A.J., Ying, S.-Y., Ueno, N. and Ling, N. (1987a). Complementary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins. Mol. Endocrinol. 1, 388-396.

Esch, F.S., Shimasaki, S., Mercado, M., Cooksey, K., Ling, N., Ying, S., Ueno, N. and Guillemin, R. (1987b). Structural characterization of follistatin: a novel follicle-stimulating hormone release-inhibiting polypeptide from the gonad. Mol. Endocrinol. 1, 849-855.

Eto, Y., Tsuji, T., Takezawa, M., Takano, S., Yokogawa, Y. and Shibai, H. (1987). Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. Biochem. Biophys. Res. Commun. 142, 1095-1103.

Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. Science 240, 889-895.

van den Eijnden-van Raaij, A.J.M., van Zoelen, E.J.J., van Nimmen, K., Koster, C.H., Snoek, G.T., Durston, A.J. and Huylebroek, D. (1990). Activin-like factor from a *Xenopus leavis* cell line responsible for mesoderm induction. Nature 345, 732-734.

Farnworth, P.G., Robertson, D.M., de Kretser, D.M. and Burger, H.G. (1988a). Effect 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.

Farnworth, P.G., Robertson, D.M., de Kretser, D.M. and Burger, H.G. (1988b). Effects of 31 kDa bovine inhibin on FSH and LH in rat pituitary cells *in vitro*: Antagonism of gonadotrophin-releasing hormone agonists. J. Endocrinol. 119, 233-241.

Feinberg, A.P. and Vogelstein, B. (1983). A technique of radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6-13.

Feng, Z.-M., Bardin, C.W. and Chen, C.-L.C. (1989a). Characterization and regulation of testicular inhibin β-subunit mRNA. Mol. Endocrinol. 3, 939-948.

Feng, Z.-M., Li, Y.-P. and Chen, C.-L.C. (1989b). Analysis of the 5'-flanking regions of rat inhibin α- and βB-subunit genes suggests two different regulatory mechanisms. Mol. Endocrinol. 3, 1914-1925.

Feng, Z.-M. and Chen, C.-L.C. (1993a). Two heterogeneous-sized inhibin/activin βB-subunits are transcribed from alternative initiation sites. Program of the 75th annual meeting of the Endocrine Society, Las Vegas, abstract 1752.

Feng, Z.-M., Madigan, M.B., and Chen, C.-L.C. (1993b). Expression of type II activin receptor genes in the male and female reproductive tissues of the rats. Endocrinology 132, 2593-2600.

Feng, Z.-M., Wu, A.Z. and Chen, C.C.-L. (1995). Characterization and regulation of two testicular inhibin/activin βB-subunit messenger ribonucleic acids that are transcribed from alternate initiation sites. Endocrinology 136, 947-955.

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. (1989). Immunisation against the amino-terminal peptide (αN) of the alpha 43 subunit of human inhibin impairs fertility in sheep. Endocrinology 124. 3122-3124.

Findlay, J.K., Russell, D.L., Doughton, B., Tsonis, C.G., Borchers, C. and Forage, R.G. (1994). Effect of active immunization against the amino-terminal peptide (alpha N) of the alpha 43 kDa subunit of inhibin (alpha 43) on fertility of ewes. Reprod. Fert. Dev. 6, 265-267.

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. and de Kretser, D.M. (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.

Franchimont, P., Millet, D., Vendrely, E., Letawe, J., Legros, J.J. and Netter, A. (1972). Relationship between spermatogenesis and serum gonadotropin levels in azoospermia and oligospermia. J. Clin. Endocrinol. Metab. 34, 1003-1008.

Franchimont, P., Croze, F., Demoulin, A., Bologne, R. and Hustin, J. (1981). Effect of inhibin on rat testicular desoxyribonucleic acid (DNA) synthesis *in vivo* and *in vitro*. Acta Endocrinol. 98, 312-320.

Franchimont, P., Hazee-Hagelstein, M.T., Charlet-Renard, C. and Jaspar, J.M. (1986) Effect of mouse epidermal growth factor on DNA and protein synthesis, progesterone and inhibin production by bovine granulosa cells in culture. Acta Endocrinol. 111, 122-127.

Fuduka, M., Miyamoto, K., Hasegawa, Y., Nomura, M., Igarashi, M., Kangawa, K. and Matsuo, H. (1986). Isolation of bovine follicular fluid inhibin of about 32 kDa. Mol. Cell. Endocrinol. 44, 55-60.

Fuduka, M., Miyamoto, K., Hasegawa, Y., Ibuki, Y. and Igarashi, M. (1987). Action mechanism of inhibin *in vitro* - cycloheximide mimics inhibin actions on pituitary cells. Mol. Cell. Endocrinol. 51, 41-50.

Galdieri, M., Ziparo, E., Palombi, F., Russo, M.A. and Stefanini, M. (1981). Pure Sertoli cell cultures: A new model for the study of somatic-germ cell interactions. J. Androl. 2, 249-254.

Galdieri, M., Caporale, C. and Adamo. S. (1986). Calcium-, phospholipid-dependent protein kinase activity of cultured rat Sertoli cells and its modifications by vitamin A. Mol. Cell. Endocrinol. 48, 213-220.

Glisin, V., Crkvenjakov, P. and Byus, C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry 13, 2633-2637.

Gomes, W.R., Hall, R.W., Jain, S.K. and Boots, L.R. (1973). Serum gonadotropin and testosterone levels during loss and recovery of spermatogenesis in rats. Endocrinology 93, 800-809.

Gonzales, G.F., Risbridger, G.P. and de Kretser, D.M. (1989a). The effect of insulin on inhibin production in isolated seminiferous tubule segments from adult rats cultured *in vitro*. Mol. Cell. Endocrinol. 61, 209-216.

Gonzales, G.F., Risbridger, G.P. and de Kretser, D.M. (1989b). Epidermal growth factor increases inhibin

synthesis by isolated segments of rat seminiferous tubules. J. Endocrinol. 123, 213-219.

Gonzales, G.F., Risbridger, G.P. Hodgson, Y.H., Pöllänen, P. and de Kretser, D.M. (1989c). Stage-specific inhibin secretion by rat seminiferous tubules. Reprod. Fertil. Dev. 1, 275-279.

Gonzales, G.F., Risbridger, G.P. and de Kretser, D.M. (1989d). *In vivo* and *in vitro* production of inhibin by cryptorchid testes from adult rats. Endocrinology 124, 1661-1668.

Gorczynska, E. and Handelsman, D.J. (1991). The role of calcium in follicle-stimulating hormone signal transduction in Sertoli cells. J. Biol. Chem. 266, 23739-23744.

Grasso, P. and Reichert, L.E. jr (1989). Follicle-stimulating hormone receptor-mediated uptake of 45 Ca^{++} by proteoliposomes and cultured rat Sertoli cells: evidence for involvement of voltage-activited and voltage-independent calcium channels. Endocrinology 125, 3029-3036.

Grasso, P., Joseph, M.P. and Reichert, L.E. jr. (1991). A new role for follicle-stimulating hormone in the regulation of calcium flux in Sertoli cells: inhibition of Na^+/Ca^{++} exchange. Endocrinology 128, 158-164.

Gray, A.M. and Mason, A.J. (1990). Requirement for activin A and transforming growth factor-β1 pro-regions in homodimer assembly. Science 247, 1328-1330.

Griswold, M.D. (1988). Protein secretions of Sertoli cells. Int. Rev. Cytol. 110, 133-156.

Groome, N. (1991). Ultrasensitive two-site assays for inhibin-A and activin-A using monoclonal antibodies raised to synthetic peptides. J. Imm. Meth. 145, 65-69.

Grootegoed, J.A. and den Boer, P.J. (1989). Energy metabolism of spermatids: A review. In: Cellular and Molecular Events in Spermatogenesis as targets for Fertility Regulation, pp. 193-215. Eds. D.W. Hamilton and G.H.M. Waites, Cambridge University Press.

Grootegoed, J.A. (1994). Welcome to the family: the anti-müllerian hormone receptor. Mol. Cell. Endocrinol. 100, 29-34.

Grootegoed, J.A. (1995). In: Scientific Essentials of Reproductive Medicine. Eds. Hillier, Kitchener and Neilson. W.B. Saunders, in press.

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). 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 de Jong, F.H. (1990a). Inhibin in immature rat Sertoli cell conditioned medium: a 32 kDa $\alpha\beta$B dimer. Mol. Cell. Endocrinol. 70, 109-116.

Grootenhuis, A.J., van Beurden, W.M.O., Timmerman, M.A. and de Jong, F.H. (1990b). FSH-stimulated secretion of a 29 kDa inhibin α-subunit complex, but not of 32 kDa bioactive inhibin, from cultured immature Sertoli cells. Mol. Cell. Endocrinol. 74, 125-132.

van Haaster, L.H., de Jong, F.H., Docter, R. and de Rooij, D.G. (1992). The effect of hypothyroidism on Sertoli cell proliferation and differentiation and hormone levels during testicular development in the rat. Endocrinology 131, 1574-1576.

Hall, S.H., Joseph, D.R., French, F.S. and Conti, M. (1988). Follicle-stimulating hormone induces transient expression of the protooncogene *c-fos* in primary Sertoli cell cultures. Mol. Endocrinol. 2, 55-61.

Hancock, A.D., Robertson, D.M. and de Kretser, D.M. (1992). Inhibin and inhibin α-chain precursors are produced by immature rat Sertoli cells in culture. Biol. Reprod. 46, 155-161.

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.

Hasegawa, Y., Miyamoto, K., Abe, Y., Nakamura, T., Sugino, H., Eto, Y., Shibai, H. and Igarashi, M. (1988). Induction of follicle stimulating hormone receptor by erythroid differentiation factor on rat

granulosa cell. Biochem. Biophys. Res. Commun. 156, 668-674.

Hashimoto, M., Kondo, S., Sakurai, T., Etoh, Y., Shibai, H. and Muramatsu, M. (1990). Activin/EDF as an inhibitor of neural differentiation. Biochem. Biophys. Res. Commun. 173, 193-200.

Heckert, L.L. and Griswold, M.D. (1991). Expression of follicle-stimulating hormone receptor mRNA in rat testes and Sertoli cells. Mol. Endocrinol. 5, 670-677.

Hedger, M.P., Drummond, A.E., Robertson, D.M., Risbridger, G.P. and de Kretser, D.M. (1989). Inhibin and activin regulate [^{3}H]thymidine uptake by rat thymocytes and 3T3 cells *in vitro*. Mol. Cell. Endocrinol. 61, 133-138.

Heldin, C.-H. (1995). Dimerization of cell surface receptor in signal transduction. Cell 80, 213-223.

Hepler, J.R., Earp, H.S. and Harden, T.K. (1988). Long-term phorbol ester treatment down-regulates protein kinase C and sensitizes the phosphoinositide signaling pathway to hormone and growth factor stimulation. J. Biol. Chem. 263, 7610-7619.

Hermans, W.P., van Leeuwen, E.C.M., Debets, M.H.M. and de Jong, F.H. (1980). Involvement of inhibin in the regulation of follicle stimulating hormone concentrations in prepubertal and adult, male and female rats. J. Endocrinol. 86, 79-92.

Hilscher, B., Hilscher, W. and Maurer, W. (1969). Autoradiographische Untersuchungen uber den Modus der Proliferation und regeneration des Samenepithels der Wistarratte. Z. Zellforsch. 94, 593-604.

Hochereau-de Reviers, M.T. and Courot, M. (1978). Sertoli cells and development of seminiferous epithelium. Annales de Biologie Animale, Biochimie et Biophysique 18, 573-583.

Hoeffler, J.P., Meyer, T.E., Yun, Y., Jameson, J.L. and Habener, J.F. (1988). Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental DNA. Science 242, 1430-1433.

Hötten, G., Neidhardt, H., Schneider, C. and Pohl, J. (1995). Cloning of a new member of the TGF-β family: a putative new activin βC chain. Biochem. Biophys. Res. Commun. 206, 608-613.

Hsueh, A.J.W., Dahl, K.D., Vaughan, J., Tucker, E., Rivier, J., Bardin, C.W. and Vale, W. (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.

Huang, H.F.S. and Hembree, W.C. (1979). Spermatogenic response to vitamin A in vitamin A deficient rats. Biol. Reprod. 21, 891-904.

Huang, H.F.S., Dyrenfurth, I. and Hembree, W.C. (1983). Endocrine changes associated with germ cell loss during vitamin A deficiency and vitamin A-induced recovery of spermatogenesis. Endocrinology 112, 1163-1171.

Huang, H.F.S., Yang, C.S., Meyerhofer, M., Gould, S. and Boccabella, A.V. (1988). Disruption of sustentacular (Sertoli) cell tight junctions and regression of spermatogenesis in vitamin-A-deficient rats. Acta Anat. 133, 10-15.

Hunter, T. (1995). Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80, 225-236.

Hutchinson, L.A., Findlay, J.K., de Vos, F.L., and Robertson, D.M. (1987). Effects of bovine inhibin, transforming growth factor-β and bovine activin-A on granulosa cell differentiation. Biochem. Biophys. Res. Commun. 146, 1405-1412.

Imagawa, M., Chiu, R. and Karin, M. (1987). Transcriptional factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell 51, 251-261.

Itoh, M., Igarashi, M., Yamada, K., Hasegawa, Y., Seki, M., Eto, Y. and Shibai, H. (1990). Activin stimulates meiotic maturation of the rat oocyte *in vitro*. Biochem. Biophys. Res. Commun. 166, 1479-1484.

Jakubowiak, A., Janecki, A. and Steinberger, A. (1990). Action kinetics of inhibin in superfused pituitary cells depend on gonadotropin-releasing hormone treatment. Endocrinology 127, 211-217.

Janecki, A., Jakubowiak, A. and Steinberger, A. (1990). Vectorial secretion of inhibin by immature rat Sertoli cells *in vitro*: reexamination of previous results. Endocrinology 127, 1896-1903.

Janini, E.A., Ulisse, S., Piersanti, D., Carosa, E., Muzi, P., Lazar, J. and D'Armiento, M. (1993). Early thyroid hormone treatment in rats increases testis size and germ cell number. Endocrinology 132, 2726-2728.

Jenkins, N. and Ellison, J.D. (1986). Corticosteroids suppress plasminogen activation in the bovine Sertoli cell. J. Endocrinol. 108, R1-R3.

Jih, M.H., Lu, J.K.H., Wan, Y.-J.Y. and Wu, T.-C. (1993). Inhibin subunit gene expression and distribution in the ovaries of immature, young adult, middle-aged, and old female rats. Endocrinology 132, 319-326.

Johnsen, S.G. (1964). Studies on the testicular-hypophyseal feed-back mechanism in man. Acta Endocrinol. Suppl. 90, 99-124.

de Jong, F.H. (1988). Inhibin. Phys. Rev. 68, 555-607.

de Jong, F.H. and Sharpe, R.M. (1976). Evidence for inhibin-like activity in bovine follicular fluid. Nature 263, 71-72.

de Jong, F.H. and Sharpe, R.M. (1977). Gonadotrophins, testosterone and spermatogenesis in neonatally irradiated male rats: evidence for a role of the Sertoli cell in follicle-stimulating hormone feedback. J. Endocrinol. 80, 91-102.

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

de Jong, F.H., de Winter, J.P., Wesseling, J.G., Timmerman, M.A., van Genesen, S., van den Eijnden-van Raaij, A.J.M. and van Zoelen, E.J.J. (1993). Inhibin subunits, follistatin and activin receptors in the human teratocarcinoma cell line tera-2. Biochem. Biophys. Res. Commun. 192, 1334-1339.

Joseph, D.R., Hall, S.H., Conti, M. and French, F.S. (1988). The gene structure of rat androgen-binding protein: Identification of potential regulatory deoxyribonucleic acid elements of a follicle-stimulating hormone-regulated protein. Mol. Endocrinol. 2, 3-13.

Kaipia, A. Parvinen, M. Shimasaki, S., Ling, N. and Toppari, J. (1991). Stage-specific cellular regulation of inhibin α-subunit mRNA expression in the rat seminiferous epithelium. Mol. Cell. Endocrinol. 82, 165-173.

Kaipia, A., Penttilä, T.-L., Shimasaki, S., Ling, N., Parvinen, M. and Toppari, J. (1992). Expression of inhibin βA and βB, follistatin and activin-A receptor messenger ribonucleic acids in the rat seminiferous epithelium. Endocrinology 132, 2703-2710.

Kaipia, A., Parvinen, M. and Toppari, J. (1993). Localization of activin receptor (act-IIB$_2$) mRNA in the rat seminiferous epithelium. Endocrinology 132, 477-479.

Katayama, T., Shiota, K. and Takahasi, M. (1990). Activin A increases the number of follicle-stimulating hormone cells in anterior pituitary cultures. Mol. Cell. Endocrinol. 69, 179-185.

Keel, B.A. and Grotjan, H.E., Jr. (1985). Characterization of rat pituitary luteinizing hormone charge microheterogeneity in male and female rats using chromatofocusing: effects of castration. Endocrinology 117, 354-360.

Keinan, D., Madigan, M.B., Bardin, C.W. and Chen, C.-L.C. (1989). Expression and regulation of testicular inhibin α-subunit gene *in vivo* and *in vitro*. Mol. Endocrinol. 3, 29-35.

Kew, D., Jin, D.F., Kim, F., Laddis, T. and Kilpatrick, D.L. (1989). Translational status of proenkephalin mRNA in the rat reproductive system. Mol. Endocrinol. 3, 1191-1196.

Kim, K.H. and Griswold, M.D. (1990). The regulation of retinoic acid receptor mRNA levels during spermatogenesis. Mol. Endocrinol. 4, 1679-1688.

Kirby, J.D., Jetton, A.E., Cooke, P.S., Hess, R.A., Bunick, D., Ackland, J.F., Turek, F.W. and

Schwartz, N.B. (1992). Developmental hormonal profiles accompanying the neonatal hypothyroidism-induced increase in adult testicular size and sperm production in the rat. Endocrinology 131, 559-565.

Kitoaka, M., Kojima, I. and Ogata, E. (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., Toebosch, A.M.W., Themmen, A.P.N., Shimasaki, S., de Jong, F.H. and Grootegoed, J.A. (1990). Regulation of inhibin α- and βB-subunit mRNA levels in rat Sertoli cells. Mol. Cell. Endocrinol. 68, 45-52.

Klaij, I.A., Timmerman, M.A., Blok, L.J., Grootegoed, J.A. and de Jong, F.H. (1992). Regulation of inhibin βB-subunit mRNA expression in rat Sertoli cells: consequences for the production of bioactive and immunoreactive inhibin. Mol. Cell. Endocrinol. 85, 237-246.

Knight, P.G., Beard, A.J., Wrathall, J.H.M. and Castillo, R.J. (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.

Knight, P.G., Groome, N.P. and Beard, A.J. (1991). Development of a two-site immunoradiometric assay for dimeric inhibin using antibodies raised against chemically synthesized fragments of the α and β subunit. J. Endocrinol. 129, R9-R12.

Knight, P.G. and Muttukrishna, S. (1994). Measurement of dimeric inhibin using a modified two-site immunoradiometric assay specific for oxidized (Met O) inhibin. J. Endocrinol. 141, 417-425.

Kogawa, K., Nakamura, T., Sugino, K., Takio, K., Titani, K. and Sugino, H. (1991). Activin-binding protein is present in pituitary. Endocrinology 128, 1434-1440.

Kondaiah, P., van Obberghen-Schilling, E., Ludwig, R.L., Dhar, R., Sporn, M.B. and Roberts, A.B. (1988) J. Biol. Chem. 263, 18313-18317.

Kotsuji, F., Winters, S.J., Keeping, H.S., Attardi, B., Oshima, H. and Troen, P. (1988). Effects of inhibin from primate Sertoli cells on follicle-stimulating hormone and luteinizing hormone release by perifused rat pituitary cells. Endocrinology 122, 2796-2802.

de Kretser, D.M., O'Leary, P.C., Irby, D.C. and Risbridger, G.P. (1989). Inhibin secretion is influenced by Leydig cells: evidence from studies using the cytotoxin ethane dimethane sulphonate (EDS). Int. J. Androl. 12, 273-280.

de Kretser, D.M. and Robertson, D.M. (1989). The isolation and physiology of inhibin and related proteins. Biol. Reprod. 40, 33-47.

Krummen, L.A., Toppari, J., Kim, W.H., Morelos, B.S., Ahmad, N., Swerdloff, R.S., Ling, N., Shimasaki, S., Esch, F. and Bhasin, S. (1989). Regulation of testicular inhibin subunit messenger ribonucleic acid levels *in vivo*: Effects of hypophysectomy and selective follicle-stimulating hormone replacement. Endocrinology 125, 1630-1637.

Krummen, L.A., Morelos, B.S. and Bhasin, S. (1990). The role of luteinizing hormone in regulation of testicular inhibin α and βB subunit messenger RNAs in immature and adult animals. Endocrinology 127, 1097-1104.

Krummen, L.A., Gibson, U., Stocks, D., Mann, E., Wong, W.L., Schwall, R. and Mather, J.P. (1992). Use of the rat pituitary FSH release and the K562 hemoglobin production assays for quantitation of biologically active inhibin and activin in complete fluids: studies of inhibin and activin interaction. Posterbook of the 7th European workshop on Molecular and Cellular Endocrinology of the Testis (Elmau, Germany), poster 74.

Krummen, L.A., Woodruff, T.K., DeGuzman, G., Cox, E.T., Baly, D.L., Mann, E., Garg, S., Wong, W.-L., Cossum, P. and Mather, J.P. (1993). Identification and characterization of binding proteins for inhibin and activin in human serum and follicular fluids. Endocrinology 132, 431-443.

van Laar, J.H., Voorhorst-Ogink, M.M., Zegers, N.D., Boersma, W.J.A., Claassen, E., van der Korput, J.A.G.M., Ruizeveld de Winter, J.A., van der Kwast, Th.H., Mulder, E., Trapman, J. and Brinkmann, A.O. (1989). Characterization of polyclonal antibodies against the N-terminal domain of the human androgen receptor. Mol. Cell. Endocrinol. 67, 29-38.

Lacroix, M., Parvinen, M. and Fritz, I.B. (1981). Localization of testicular plasminogen activator in discrete portions (stages VII and VIII) of the seminiferous tubule. Biol. Reprod. 25, 143-146.

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

LaPolt, P.S., Soto, D., Su, J.-G., Campen, C.A., Vaughan, J., Vale, W. and Hsueh, A.J.W. (1989). Activin stimulation of inhibin secretion and messenger RNA levels in cultured granulosa cells. Mol. Endocrinol. 3, 1666-1673.

LaPolt, P.S., Piquette, G.N., Soto, D., Sincich, C. and Hsueh, A.J.W. (1990). Regulation of inhibin subunit messenger ribonucleic acid levels by gonadotropins, growth factors, and gonadotropin-releasing hormone in cultured granulosa cells. Endocrinology 127, 823-831.

Lau, L.F. and Nathans, D. (1987). Expression of a set of growth-related immediare early genes in BALB/c 3T3 cells: Coordinate regulation with *c-fos* or *c-myc*. Proc. Natl. Acad. Sci. USA 84, 1182-1186.

Leblond, C.P. and Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium of the rat. Ann. N.Y. Acad. Sci. 55, 548-594.

Lee, W., Mason, A.J., Schwall, R., Szonyi, E. and Mather, J.P. (1989). Secretion of activin by interstitial cells in the testis. Science 243, 396-398.

Le Gac, F. and de Kretser, D.M. (1982). Inhibin production by Sertoli cell cultures. Mol. Cell. Endocrinol. 28, 487-498.

Levy, F.O., Ree, A.H., Eikvar, L., Govindan, M.V., Jahnsen, T. and Hansson, V. (1989). Glucocorticoid receptors and glucocorticoid effects in rat Sertoli cells. Endocrinology 124, 430-436.

Lin, T., Calkins, J.H., Morris, P.L., Vale, W. and Bardin, C.W. (1989). Regulation of Leydig cell function in primary culture by inhibin and activin. Endocrinology 125, 2134-2140.

Lin, H.Y. and Lodish, H.F. (1993). Receptors for the TGFβ superfamily: Multiple polypeptides and serine/threonine kinases. Trends Cell Biol. 3, 14-19.

Ling, N., Ying, S.-Y., Ueno, N., Esch, F., Denoroy, L. and Guillemin, R. (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., Ying, S.-Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. and Guillemin, R. (1986a). 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. and Guillemin, R. (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.

Maddocks, S. and Sharpe, R.M. (1989). Assessment of the contribution of Leydig cells to the secretion of inhibin by the rat testis. Mol. Cell. Endocrinol. 67, 113-118.

Maddocks, S. and Sharpe, R.M. (1990). The effects of sexual maturation and altered steroid synthesis on the production and route of secretion of inhibin-α from the rat testis. Endocrinology 126, 1541-1550.

Maddocks, S., Kerr, J.B., Allenby, G. and Sharpe, R.M. (1992). Evaluation of the role of germ cells in regulating the route of secretion of immunoactive inhibin from the rat testis. J. Endocrinol. 132, 439-448.

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.

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

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

Massagué, J., Attisano, L. and Wrana, J.L. (1994). The TGF-β family and its composite receptors. Trends Cell Biol.4, 172-178.

Mather, J.P., Attie, K.M., Woodruff, T.K., Rice, G.C. and Phillips, D.M. (1990). Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. Endocrinology 127, 3206-3214.

Mathews, L.S. (1994). Activin receptors and cellular signaling by the receptor serine kinase family. Endocrine Rev. 15, 310-325.

Matzuk, M.M., Finegold, M.J., Su, J.-G.J., Hsueh, A.J.W. and Bradley, A. (1992). α-inhibin is a tumour-suppressor gene with gonadal specificity in mice. Nature 360, 313-319.

Matzuk, M.M., Finegold, M.J., Mather, J.P., Krummen, L., Lu, H. and Bradley, A. (1994). Development of cancer cachexia-like syndrome and adrenal tumors in inhibin-deficient mice. Proc. Natl. Acad. Sci. USA 91, 8817-8821.

Matzuk, M.M., Kumar, T.R., Vassalli, A., Bickenbach, J.R., Roop, D.R., Jaenisch, R. and Bradley, A. (1995a). Functional analysis of activins during mammalian development. Nature 374, 354-356.

Matzuk, M.M., Kumar, T.R. and Bradley, A. (1995b). Different phenotypes for mice deficient in either activins or activin receptor type II. Nature 374, 356-360.

Matzuk, M.M., Lu, N., Vogel, H., Sellheyer, K., Roop, D.R. and Bradley, A. (1995c). Multiple defects and perinatal death in mice deficient in follistatin. Nature 374, 360-363.

Mayo, K.E., Cerelli, G.M., Spiess, J., Rivier, J., Rosenfeld, M.G., Evans, R.M. and Vale, W. (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 testis. Science 76, 19-20.

McLachlan, R.I., Healy, D.L., Robertson, D.M., Burger, H.G. and de Kretser, D.M. (1987). The human placenta: A novel source of inhibin. Biochem. Biophys. Res. Commun. 140, 485-490.

Means, A.R., Fakunding, J.L., Huckins, C., Tindall, D.J. and Vitale, R. (1976). Follicle-stimulating hormone, the Sertoli cell, and spermatogenesis. Recent progress in hormone research 32, 477-527.

Means, A.R. (1977). Mechanisms of action of follicle-stimulating hormone (FSH). In: Johnston, A.D. (ed.) The Testis. Academic Press, New York, vol. IV, 163-188.

Mercer, J.E., Clements, J.A., Funder, J.W. and Clarke, I.J. (1987). Rapid and specific lowering of pituitary FSHβ mRNA levels by inhibin. Mol. Cell. Endocrinol. 53, 251-254.

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

Merchenthaler, I., Culler, M.D. and Negro-Vilar, A. (1991). Localization of inhibin alpha-subunit immunoreactivity in the rat adrenal cortex. J. Neuroendocrinol. 3, 425-428.

Meunier, H., Rivier, C., Evans, R.M. and Vale, W. (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., Cajander, S.B., Roberts, V.J., Rivier, C., Sawchenko, P.E., Hsueh, A.J.W. and Vale, W. (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.

Meunier, H., Roberts, V.J., Sawchenko, P.E., Cajander, S.B., Hsueh, A.J.W. and Vale, W. (1989).

Periovulatory changes in the expression of inhibin α-, βA-, and βB-subunits in hormonally induced immature female rats. Mol. Endocrinol. 3, 2026-2069.

Meyer, T.E. and Habener, J.F. (1993). Cyclic adenosine 3'-5'-monophosphate response element binding protein (CREB) and related transcription-activating deoxyribonucleic acid-binding proteins. Endocrine Rev. 14, 269-290.

Michel, U., Krozowski, Z., McMaster, J., Jinghua, Y. and Findlay, J.K. (1991). The biphasic modulation of inhibin mRNA levels and secretion by PMSG in rat granulosa cells *in vitro*. Reprod. Fert. Dev. 3, 215-226.

Michel, U., Farnworth, P. and Findlay, J.K. (1993). Follistatins: more than follicle-stimulating hormone suppressing proteins. Mol. Cell. Endocrinol. 91, 1-11.

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

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

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

Mobbs, C.V., Kaplitt, M., Kow, L.-M. and Pfaff, D.W. (1991). PLC-α: a common mediator of the action of estrogen and other hormones? Mol. Cell. Endocrinol. 80, C187-C191.

Moger, W.H. (1977). Endocrine responses of the prepubertal rat to hemiorchidectomy. Biology of Reproduction 17, 661-667.

Monaco, L., and Conti, M. (1987). Inhibition by phorbol esters and other tumor promoters of the response of the Sertoli cell to FSH: evidence for dual site of action. Mol. Cell. Endocrinol. 49, 227-236.

Monaco, L., Adamo, S. and Conti, M. (1988). Follicle-stimulating hormone modulation of phosphoinositide turnover in the immature Sertoli cell in culture. Endocrinology 123, 2032-2039.

Montminy, M.R., Sevarino, K.A., Wagner, J.A., Mandel, G. and Goodman, R.H. (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA 83, 6682-6686.

Morales, C., Hugly, S. and Griswold, M.D. (1987a). Stage-dependent levels of specific mRNA transcripts in Sertoli cells. Biol. Reprod. 36, 1035-1046.

Morales, C. and Griswold, M.D. (1987b). Retinol-induced stage synchronization in seminiferous tubules of the rat. Endocrinology 121, 432-434.

Morales, C.R., Alcivar, A.A., Hecht, N.B. and Griswold, M.D. (1989). Specific mRNAs in Sertoli and germinal cells of testes from stage synchronized rats. Mol. Endocrinol. 3, 725-733.

Morris, P.L., Vale, W.W. and Bardin, C.W. (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., Vale, W.W., Cappel, S. and Bardin, C.W. (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 Cramer, W. (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., Eto, Y., Shibai, H., Sakai, M. and Muramatsu, M. (1988). Erythroid differentiation factor is encoded by the same mRNA as that of the inhibin βA chain. Proc. Natl. Acad. Sci. USA, 85, 2434-

2438.

Najmabadi, H., Rosenberg, L.A., Yuan, Q.X., Bhatia, N., Albiston, A.L., Burger, H. and Bhasin, S. (1993a). Transcriptional and posttranscriptional regulation of inhibin α-subunit gene expression in rat Sertoli cells by 8-bromo-3',5'-cyclic-adenosine monophosphate. Mol. Endocrinol. 7, 469-476.

Najmabadi, H., Rosenberg, L.A., Yuan, Q.X., Reyaz, G. and Bhasin, S. (1993b). Transcriptional regulation of inhibin βB messenger ribonucleic acid levels in TM.4 or primary rat Sertoli cells by 8-bromo-cyclic adenosine monophosphate. Mol. Endocrinol. 7, 561-569.

Nakadate, T., Jeng, A.Y. and Blumberg, P.M. (1988). Comparison of protein kinase C functional assays to clarify mechanisms of inhibitor action. Biochem. Pharm. 37, 1541-1545.

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K. and Sugino, H. (1990). Activin-binding protein from rat ovary is follistatin. Science 247, 836-838.

Nakamura, T., Asashima, M., Eto, Y., Takio, K., Uchiyama, H., Moriya, N., Ariizumi, T., Yashiro, T., Sugino, K., Titani, K. and Sugino, H. (1992). Isolation and characterization of native activin B. J. Biol. Chem. 267, 16385-16389.

Naor, Z. (1991). Is arachidonic acid a second messenger in signal transduction? Mol. Cell. Endocrinol. 80, C181-C186.

Neer, E.J. (1995). Heterotrimeric G proteins: Organizers of transmembrane signaling. Cell 80, 249-257.

Nemere, I., and Norman, A.W. (1991). Steroid hormone actions at the plasma membrane: induced calcium uptake and exocytotic events. Mol. Cell. Endocrinol. 80, C165-C169.

Nikula, H., Vihko, K. and Huhtaniemi, I. (1990). Protein kinase C and G_i-protein mediated modulation of cAMP production in different stages of the rat seminiferous epithelium, Mol. Cell. Endocrinol. 70, 247-253.

Nishizuka, Y. (1988). The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334, 661-665.

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

Oonk, R.B., Grootegoed, J.A. and van der Molen, H.J. (1985). Comparison of the effects of insulin and follitropin on glucose metabolism by Sertoli cells from immature rats. Mol. Cell. Endocrinol. 42, 39-48.

Orth, J.M. (1982). Proliferation of Sertoli cells in fetal and postnatal rats: A quantitative autoradiographic study. Anat. Rec, 203, 485-492.

Orth, J.M. (1984). The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. Endocrinology 115, 1248-1255.

Orth, J.M., Higginbotham, C.A. and Salisbury, R.L. (1984). Hemicastration causes and testosterone prevents enhanced uptake of [^{3}H]thymidine by Sertoli cells in testes of immature rats. Biol. Reprod. 30, 263-270.

Øyen, O., Eskild, W., Beebe, S.J., Hansson, V. and Jahnsen, T. (1988). Biphasic response to 3', 5'-cyclic adenosine monophosphate (cAMP) at the messenger ribonucleic acid level for a regulatory subunit of cAMP-dependent protein kinase. Mol. Endocrinol. 2, 1070-1076.

Pachter, J.S., Yen, T.J. and Cleveland, D.W. (1987). Autoregulation of tubulin expression is achieved through specific degradation of polysomal tubulin mRNAs. Cell 51, 283-292.

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

Pakarinen, P. and Huhtaniemi, I. (1992). Age-related discrepancies between serum and pituitary gonadotrophin, and pituitary gonadotrophin subunit mRNA responses to castration and testosterone replacement in male rats. J. Endocrinol. 135, 507-515.

Palmero, S., Prati, M., Barreca, A., Minuto, F., Giordano, G. and Fugassa, E. (1990). Thyroid hormone stimulates the production of insulin-like growth factor 1 (IGF-1) by immature rat Sertoli cells. Mol. Cell. Endocrinol. 68, 61-65.

Parmentier, M., Libert, F., Maenhaut, C., Lefort, A., Gerard, C., Perret, J., Van Sande, J., Dumont, J.E. and Vassart, G. (1989). Molecular cloning of the thyrotropin receptor. Science 246, 1620-1622.

Parvinen, M. (1982). Regulation of the seminiferous epithelium. Endocrine Reviews 3, 404-417.

Pei, L., Dodson, R., Schoderbek, W.E., Maurer, R.A. and Mayo, K. (1991). Regulation of the α-inhibin gene by cyclic adenosine 3',5'-monophosphate after transfection into rat granulosa cells. Mol. Endocrinol. 5, 521-534.

van Pelt, A.M.M. and de Rooij, D.G. (1990). The origin of the synchronization of the seminiferous epithelium in vitamin A-deficient rats after vitamin A replacement. Biol. Reprod. 42, 677-682.

Pennybacker, M. and Herman, B. (1991). Follicle-stimulating hormone increases *c-fos* mRNA levels in rat granulosa cells via a protein kinase C-dependent mechanism. Mol. Cell. Endocrinol. 80, 11-20.

Perheentupa, A., Bergendahl, M., de Jong, F.H. and Huhtaniemi, I. (1993). Differential regulation of FSH and inhibin gene expression and synthesis by testosterone in immature and mature male rats. J. Endocrinol. 137, 69-79.

Persson, H., Ayer-Le Lievre, C., Söder, O., Villar, M.J., Metsis, M., Olson, L., Ritzen, M. and Hökfelt, T. (1990). Expression of β-nerve growth factor receptor mRNA in Sertoli cells downregulated by testosterone. Science 247, 704-707.

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

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

Petraglia, F., Calza, L., Garuti, G.C., Abrate, M., Giardino, L., Genazzani, A.R., Vale, W. and Meunier, H. (1990). Presence and synthesis of inhibin subunits in human decidua. J. Clin. Endocrinol. Metab. 71, 487-492.

Pineau, C., Sharpe, R.M., Saunders, P.T.K., Gérard, N. and Jégou, B. (1990) Regulation of Sertoli cell inhibin production and of inhibin α-subunit mRNA levels by specific germ cell types. Mol. Cell. Endocrinol. 72, 13-22.

Putra, D.K.H. and Blackshaw, A.W. (1982). Morphometric studies of compensatory testicular hypertrophy in the rat after hemicastration. Aust. J. Biol. Sci. 35, 287-293.

Quirk, S.M. and Reichert, L.E. Jr. (1988). Regulation of the phosphoinositide pathway in cultured Sertoli cells from immature rats: effects of follicle-stimulating hormone and fluoride. Endocrinology 123, 230-237.

Rich, K.A. and de Kretser, D.M. (1977). Effect of differing degrees of destruction of the rat seminiferous epithelium on levels of serum follicle stimulating hormone and androgen binding protein. Endocrinology 101, 959-969.

Rich, K.A., Kerr, J.B. and de Kretser, D.M. (1979). Evidence for Leydig cell dysfunction in rats with seminiferous tubule damage. Mol. Cell. Endocrinol. 13, 123-135.

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

Risbridger, G.P., Hancock, A., Robertson, D.M., Hodgson, Y. and de Kretser, D.M. (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.

Ritzen, E.M., Boitani, C., Parvinen, M., French, F.S. and Feldman, M. (1982). Stage-dependent secretion of ABP by rat seminiferous tubules. Mol. Cell. Endocrinol. 25, 25-33.

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

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.

Rivier, C., Meunier, H., Roberts, V. and Vale, W. (1989a). Possible involvement of inhibin in altered follicle-stimulating hormone (FSH) secretion during dissociated luteinizing hormone (LH) and FSH release: Unilateral castration and experimental cryptorchidism. Biol. Reprod. 41, 967-981.

Rivier, C., Roberts, V. and Vale, W. (1989b). 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.

Rivier, C. and Vale, W. (1989). Immunoneutralization of endogenous inhibin modifies hormone secretion and ovulation rate in the rat. Endocrinology 125, 152-157.

Rivier, C., Schwall, R., Mason, A., Burton, L., Vaughan, J. and Vale, W. (1991a). Effect of recombinant inhibin on luteinizing hormone and follicle-stimulating hormone secretion in the rat. Endocrinology 128, 1548-1554.

Rivier, C., Corrigan, A. and Vale, W. (1991b). Effect of recombinant inhibin on gonadotropin secretion by the male rat. Endocrinology 129, 2155-2159.

Rivier, C. and Vale, W. (1991). Effect of recombinant activin-A on gonadotropin secretion in the female rat. Endocrinology 129, 2463-2465.

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

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

Roberts, V., Meunier, H., Sawchenko, P.E. and Vale, W. (1989b). Differential production and regulation of inhibin subunits in rat testicular cell types. Endocrinology 125, 2350-2359.

Roberts, V.J., Sawchenko, P.E. and Vale, W. (1991). Expression of inhibin/activin subunit messenger ribonucleic acids during rat embryogenesis. Endocrinology 128, 3122-3129.

Roberts, V.J. and Barth, S.L. (1994). Expression of messenger ribonucleic acids encoding the inhibin/activin system during mid- and late-gestation rat embryogenesis. Endocrinology 134, 914-923.

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). Isolation of inhibin from bovine follicular fluid. 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). Isolation of a 31 kDa form of inhibin from bovine follicular fluid. Mol. Cell. Endocrinol. 44, 271-277.

Robertson, D.M., Klein, R., de Vos, F.L., McLachlan, R.I., Wettenhall, R.E.H., Hearn, M.T.W., Burger, H.G. and de Kretser, D.M. (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., Hayward, S., Irby, D., Jacobsen, J., Clarke, L., McLachlan, R.I. and de Kretser, D.M. (1988). Radioimmunoassay of rat serum inhibin: changes after PMSG stimulation and gonadectomy. Mol. Cell. Endocrinol. 58, 1-8.

Robertson, D.M., Giacometti, M., Foulds, L.M., Lahnstein, J., Goss, N.H., Hearn, M.T.H. and de

Kretser, D.M. (1989). Isolation of inhibin α-subunit precursor proteins from bovine follicular fluid. Endocrinology 125, 2141-2149.

Robertson, D.M., Foulds, L.M., Prisk, M. and Hedger, M.P. (1992). Inhibin/activin β-subunit monomer: isolation and characterization. Endocrinology 130, 1680-1687.

Roesler, W.J., Vandenbark, G.R. and Hanson, R.W. (1988). Cyclic AMP and the induction of eukaryotic gene transcription. J. Biol. Chem. 263, 9063-9066.

Rossi, P., Grimaldi, P., Blasi, F., Geremia, R. and Verde, P. (1990). Follicle-stimulating hormone and cyclic AMP induce transcription from the human urokinase promoter in primary cultures of mouse Sertoli cells. Mol. Endocrinol. 4, 940-946.

Rüegg, U.T. and Burgess, G.M. (1989). Staurosporine, H-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol. Sci. 10, 218-220.

Russell, L.D., Bartke, A. and Goh, J.C. (1989). Postnatal development of the Sertoli cell barrier, tubular lumen, and cytoskeleton of Sertoli and myoid cells in the rat, and their relationship to tubular fluid secretion and flow. Am. J. Anat. 184, 179-189.

Saez, J.M. (1994). Leydig cells: endocrine, paracrine, and autocrine regulation. Endocrine Rev. 15, 574-626.

Sander, H.J., Kramer, P., van Leeuwen, E.C.M., van Capellen, W.A., Meijs-Roelofs, H.M.A. and de Jong, F.H. (1991). Ovulation rate, follicle population and FSH levels in cyclic rats after administration of an inhibin-neutralizing antiserum. J. Endocrinol. 130, 297-303.

Sawchenko, P.E., Plotsky, P.M., Pfeiffer, S.W., Cunningham, E.T.Jr., Vaughan, J., Rivier, J. and Vale, W. (1988). Inhibin β in central neural pathways involved in the control of oxytocin secretion. Nature 334, 615-617.

Schlessinger, J. (1993). How receptor tyrosine kinases activate *Ras*. Trends Biochem. Sci. 18, 273-275.

Schneyer, A.L., Mason, A.J., Burton, L.E., Ziegner, J.R. and Crowley, W.F.jr. (1990). Immunoreactive inhibin α subunit in human serum: implications for radioimmunoassay. J. Clin. Endocrinol. Metab. 70, 1208-1212.

Schneyer, A.L., Sluss, P.M., Whitcomb, R.W., Martin, K.A., Sprengel, R. and Crowley, W.F.Jr. (1991). Precursors of α-inhibin modulate follicle-stimulating hormone receptor binding and biological activity. Endocrinology 129, 1987-1999.

Schubert, D., Kimura, H., LaCorbiere, M., Vaughan, J., Karr, D. and Fischer, W.H. (1990). Activin is a nerve cell survival molecule. Nature 344, 868-870.

Schulte-Merker, S., Smith, J.C. and Dale, L. (1994). Effects of truncated activin and FGF receptors and of follistatin on the inducing activities of BVg1 and activin: does activin play a role in mesoderm induction. EMBO J. 13, 3533-3541.

Schwall, R.H., Nikolics, K., Szonyi, E., Gorman, C. and Mason, A.J. (1988). Recombinant expression and characterization of human activin A. Mol. Endocrinol. 2, 1237-1242.

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

Schwall, R. and Lai, C. (1990). Activin assay. In: Barnes, D., Mather, J.P. and Sato, G.H. (eds) Methods in Enzymology, vol 198, part C. Academic Press, New York. pp 340-346.

Schwall, R.H., Robbins, K., Jardieu, P., Chang, L., Lai, C. and Terrell, T.G. (1993). Activin induces cell death in hepatocytes *in vivo* and *in vitro*. Hepatology 18, 347-356.

Sehgal, A., Patil, N. and Chao, M. (1988). A constitutive promoter directs expression of the nerve growth factor receptor gene. Mol. Cell. Biol. 8, 3160-3167.

Setchell, B.P., Pöllänen, P. and Zupp, J. (1988). Development of the blood-testis barrier and changes in vascular permeability at puberty in rats. Int. J. Androl. 11, 225-233.

Shaha, C., Morris, P.L., Chen, C.-L.C., Vale, W. and Bardin, C.W. (1989). Immunostainable inhibin subunits are in multiple types of testicular cells. Endocrinology 125, 1941-1950.

Shao, L., Frigon, N.L., Sehy, D.W., Yu, A.L., Lofgren, J., Schwall, R. and Yu, J. (1992). Regulation of production of activin A in human marrow stromal cells and monocytes. Exp. Hematol. 20, 1235-1242.

Shaw, G. and Kamen, R. (1986). A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46, 659-667.

Shibata, H., Ogata, E., Etoh, Y., Shibai, H. and Kojima, I. (1987). Erythroid differentiation factor stimulates hydrolysis of polyphosphoinositide in friend erythroleukemia cells. Biochem. Biophys. Res. Commun. 146, 187-193.

Shimasaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba, A., Ueno, N., Ying, S.-Y., Ling, N. and Guillemin, R. (1988). Primary structure of the human follistatin precursor and its genomic organization. Proc. Natl. Acad. Sci. USA 85, 4218-4222.

Shimasaki, S., Koga, M., Buscaglia, M.L., Simmons, D.M., Bicsak, T.A. and Ling, N. (1989). Follistatin gene expression in the ovary and extragonadal tissues. Mol. Endocrinol. 3, 651-659.

Shimonaka, M., Inouye, S., Shimasaki, S. and Ling, N. (1991). Follistatin binds to both activin and inhibin through the common beta-subunit. Endocrinology 128, 3313-3315.

Shyu, A.-B., Greenberg, M.E. and Belasco, J.G. (1989). The *c-fos* transcript is targeted for rapid decay by two distinct mRNA degradation pathways. Genes Dev. 3, 60-72.

Simpson, B.J.B., Risbridger, G.P., Hedger, M.P. and de Kretser, D.M. (1991). The role of calcium in luteinizing hormone/human chorionic gonadotrophin stimulation of Leydig cell immunoreactive inhibin secretion *in vitro*. Mol. Cell. Endocrinol. 75, 49-56.

Skinner, M.K. and Griswold, M.D. (1980). Sertoli cells synthesize and secrete transferrin-like protein. J. Biol. Chem. 255, 9523-9525.

Skinner, M.K. and Griswold, M.D. (1983). Sertoli cells synthesize a ceruloplasmin-like protein. Biol. Reprod. 28, 1225-1229.

Skinner, M.K., McLachlan, R.I. and Bremner, W.J. (1989). Stimulation of Sertoli cell inhibin secretion by the testicular paracrine factor PModS. Mol. Cell. Endocrinol. 66, 239-249.

Slaughter, G.L., Needleman, D.S. and Means, A.R. (1987). Developmental regulation of calmodulin, actin, and tubulin RNAs during rat testis differentiation. Biol. Reprod. 37, 1259-1270.

Smith, E.P., Hall, S.H., Monaco, L., French, F.S. Wilson, E.M. and Conti, M. (1989). A rat Sertoli cell factor similar to basic fibroblast growth factor increases *c-fos* messenger ribonucleic acid in cultured Sertoli cells. Mol. Endocrinol. 3, 954-961.

Smith, J.C., Price, B.M.J., van Nimmen, K. and Huylebroeck, D. (1990). Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin A. Nature 345, 729-731.

Spaulding, S.W. (1993). The way in which hormones change cyclic adenosine 3', 5'-monophosphate-dependent protein kinase subunits, and how such changes affect cell behaviour. Endocrine Rev. 14, 632-649.

Spencer, S.J., Rabinovici, J. and Jaffe, R.B. (1990). Human recombinant activin-A inhibits proliferation of human fetal adrenal cells *in vitro*. J. Clin. Endocrinol. Metab. 71, 1678-1680.

Spencer, S.J., Rabinovici, J., Mesiano, S., Goldsmith, P. and Jaffe, R.B. (1992). Activin and inhibin in the human adrenal gland; Regulation and differential effects in fetal and adult cells. J. Clin. Invest. 90, 142-149.

Steinberger, A. and Steinberger, E. (1971). Replication pattern of Sertoli cells in maturing rat testis *in vivo* and in organ culture. Biol. Reprod. 4, 84-87.

Steinberger, A. and Steinberger, E. (1976). Secretion of an FSH-inhibiting factor by cultured Sertoli cells.

Endocrinology 99, 918-921.

Steinberger, A. (1981). Regulation of inhibin secretion in the testis. In: Intragonadal regulation of reproduction (eds. P. Franchimont and C.P. Channing) pp. 283-298. Academic Press, London.

Stewart, A.G., Milborrow, H.M., Ring, J.M., Crowther, C.E. and Forage, R.G. (1986). Human inhibin genes: Genomic characterisation and sequencing. FEBS Lett. 206, 329-334.

Stojilkovic, S.S., Reinhart, J. and Catt, K.J. (1994). Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. Endocrine Rev. 15, 462-498.

Sugino, H., Nakamura, T., Hasegawa, Y., Miyamoto, K., Abe, Y., Igarashi, M., Eto, Y., Shibai, H. and Titani, K. (1988). Erythroid differentiation factor can modulate follicular granulosa cell functions. Biochem. Biophys. Res. Commun. 153, 281-288.

Sugino, K., Nakamura, T., Takio, K., Titani, K., Miyamoto, K., Hasegawa, Y., Igarashi, M. and Sugino, H. (1989). Inhibin alpha-subunit monomer is present in bovine follicular fluid. Biochem. Biophys. Res. Commun. 159, 1323-1329.

Sugino, K., Nakamura, T., Takio, K., Miyamoto, K., Hasegawa, Y., Igarashi, M., Titani, K. and Sugino, H. (1992). Purification and characterization of high molecular weight forms of inhibin from bovine follicular fluid. Endocrinology 130, 789-796.

Suzuki, T., Miyamoto, K., Hasegawa, Y., Abe, Y., Ui, M., Ibuki, Y. and Igarashi, M. (1987). Regulation of inhibin production by rat granulosa cells. Mol. Cell. Endocrinol. 54, 185-195.

Swerdloff, R.S., Walsh, P.C., Jacobs, H.S. and Odell, W.D. (1971). Serum LH and FSH during sexual maturation in the male rat: effect of castration and cryptorchidism. Endocrinology 88, 120-128.

Sylvester, S.R., Skinner, M.K., and Griswold, M.D. (1984). A sulfated glycoprotein synthesized by Sertoli cells and by epididymal cells is a component of the sperm membrane. Biol. Reprod. 31, 1087-1101.

Takahashi, S., Uchimaru, K., Harigaya, K.-I., Asano, S. and Yamashita, T. (1992). Tumor necrosis factor and interleukin-1 induce activin A gene expression in a human bone marrow stromal cell line. Biochem. Biophys. Res. Commun. 188, 310-317.

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{++}dependent protein kinase. Biochem. Biophys. Res. Comm. 135, 397-402.

Tanimoto, K., Handa, S.-I., Ueno, N., Murakami, K. and Fukamizu, A. (1991). Structure and sequence analysis of the human activin βA subunit gene. J. DNA Seq. 2, 103-110.

Tanimoto, K., Tamura, K., Ueno, N., Usuki, S., Murakami, K. and Fukamizu, A. (1992). Regulation of activin βA mRNA level by cAMP. Biochem. Biophys. Res. Commun. 182, 773-778.

Tanimoto, K., Murakami, K. and Fukamizu, A. (1993). Possible roles of the 3'-flanking sequences of the human activin βA-subunit gene in its expression. Arch. Biochem. Biophys. 302, 409-416.

Taylor, C.W. and Marshall, I.C.B. (1992). Calcium and inositol 1,4,5-trisphosphate receptors: a complex relationship. Trends. Biochem. Sci. 17, 403-407.

Themmen, A.P.N., Blok, L.J., Post, M., Baarends, W.M., Hoogerbrugge, J.W., Parmentier, M., Vassart, G. and Grootegoed, J.A. (1991). Follitropin receptor down-regulation involves a cAMP-dependent post-transcriptional decrease of receptor mRNA expression. Mol. Cell. Endocrinol. 78, R7-R13.

van Thiel, D.H., Sherins, R.J., Myers, G.H. and de Vita, V.T. (1972). Evidence for a specific seminiferous tubular factor affecting follicle-stimulating hormone secretion in man. J. Clin. Invest. 51, 1009-1019.

Thomas, P.S. (1980). Hybridisation of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA 77, 5201-5205.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D.A. (1990). Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. Cell 63, 485-493.

Tindall, D., Rowley, D.R., Murthy, L., Lipshultz, L.I. and Chang, C.H. (1985). Structure and biochemistry of the Sertoli cell. Int. Rev. Cytol. 94, 127-149.

Toebosch, A.M.W., Robertson, D.M., Trapman, J., Klaassen, P., de Paus, R.A., de Jong, F.H. and 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. 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). Effects of FSH and testosterone on highly purified 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., Robertson, D.M. and de Kretser, D.M. (1992). Characterization of inhibin and related proteins in bovine fetal testicular and ovarian extracts: evidence for the presence of inhibin subunit products and FSH-suppressing protein. J. Endocrinol. 133, 111-120.

Tosi, M. Young, R.A., Hagenbüchle, O. and Schibler, U. (1981). Multiple polyadenylation sites in a mouse α-amylase gene. Nucleic Acids Res. 9, 2313-2323.

Totsuka, Y., Tabuchi, M., Kojima, I., Shibai, H. and Ogata, E. (1988). A novel action of activin A: stimulation of insulin secretion in rat pancreatic islets. Biochem. Biophys. Res. Commun. 156, 335-339.

Turner, I.M., Saunders, P.T.K., Shimasaki, S. and Hillier, S.G. (1989). Regulation of inhibin subunit gene expression by FSH and estradiol in cultured rat granulosa cells. Endocrinology 125, 2790-2792.

Ueno, N., Ling, N., Ying, S.-Y., Esch, F., Shimasaki, S. and Guillemin, R. (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.

Ullrich, A. and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell 61, 203-212.

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

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

Ultee-van Gessel, A.M., Timmerman, M.A. and de Jong, F.H. (1988). Effects of treatment of neonatal rats with highly purified FSH alone and in combination with LH on testicular function and endogenous hormone levels at various ages. J. Endocrinol. 116, 413-420.

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.

Vassalli, A., Matzuk, M.M., Gardner, H.A.R., Lee, K.-F. and Jaenisch, R. (1994). Activin/inhibin βB subunit gene disruption leads to defects in eyelid development and female reproduction. Genes Dev. 8, 414-427.

Veigl, M.L., Vanaman, T.C. and Sedwick, W.D. (1984). Calcium and calmodulin in cell growth and transformation. Biochim. Biophys. Acta 738, 21-48.

Verhoeven, G., Cailleau, J. and de Moor, P. (1980). Desensitization of cultured rat Sertoli cells by follicle-stimulating hormone and by L-isoproterenol. Mol. Cell. Endocrinol. 20, 113-116.

Verhoeven, G., Cailleau, J. and de Moor, P. (1981). Hormonal control of phosphodiesterase activity in cultured rat Sertoli cells. Mol. Cell. Endocrinol. 24, 41-51.

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

Verhoeven, G. and Cailleau, J. (1988). Follicle-stimulating hormone and androgens increase the

concentration of the androgen receptor in Sertoli cells. Endocrinology 122, 1541-1550.

Verjans, H.L., Cooke, B.A., de Jong, F.H., de Jong, C.M.M. and van der Molen, H.J. (1973). Evaluation of a radioimmunoassay for testosterone estimation. J. Steroid Biochem. 4, 665-676.

Voutilainen, R., Erämaa, M. and Ritvos, O. (1991). Hormonally regulated inhibin gene expression in human fetal and adult adrenals. J. Clin. Endocrinol. Metab. 73, 1026-1030.

Wang, Q.F., Farnworth, P.G., Findlay, J.K. and Burger, H.G. (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.

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

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

de Winter, J.P., Timmerman, M.A., Vanderstichele, H.M.J., Klaij, I.A., Grootenhuis, A.J., Rommerts, F.F.G. and de Jong, F.H. (1992a). Testicular Leydig cells *in vitro* secrete only inhibin α-subunits, whereas Leydig cell tumors can secrete bioactive inhibin. Mol. Cell. Endocrinol. 83, 105-115.

de Winter, J.P., Themmen, A.P.N., Hoogerbrugge, J.W., Klaij, I.A., Grootegoed, J.A. and de Jong, F.H. (1992b). Activin receptor mRNA expression in rat testicular cell types. Mol. Cell. Endocrinol. 83, R1-R8.

de Winter, J.P., van de Kant, H.J.G., Hoogerbrugge, J.W., de Rooij, D.G., Themmen, A.P.N., Grootegoed, J.A. and de Jong, F.H. (1992c). Activin receptor mRNA expression in germ cells of the male rat. Posterbook of the 7th European workshop on Molecular and Cellular Endocrinology of the Testis (Elmau, Germany), poster 75.

de Winter, J.P., Vanderstichele, H.M.J., Timmerman, M.A., Blok, L.J., Themmen, A.P.N. and de Jong, F.H. (1993). Activin is produced by rat Sertoli cells *in vitro* and can act as an autocrine regulator of Sertoli cell function. Endocrinology 132, 975-982.

de Winter, J.P., Vanderstichele, H.M.J., Verhoeven, G., Timmerman, M.A., Wesseling, J.G. and de Jong, F.H. (1994). Peritubular myoid cells from immature rat testes secrete activin-A and express activin receptor type II *in vitro*. Endocrinology 135, 759-767.

Wolf, M. and Baggiolini, M. (1988). The protein kinase inhibitor staurosporine, like phorbol esters induces the association of protein kinase C with membranes. Biochem. Biophys. Res. Comm. 154, 1273-1279.

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

Woodruff, T.K., Meunier, H., Jones, P.B.C., Hsueh, A.J.W. and Mayo, K.E. (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, and Mayo, K.E. (1990). Regulation of inhibin synthesis in the rat ovary. Ann. Rev. Physiol. 52, 807-821.

Woodruff, T.K., Borree, J., Attie, K.M., Cox, E.T., Rice, G.C. and Mather, J.P. (1992). Stage-specific binding of inhibin and activin to subpopulations of rat germ cells. Endocrinology 130, 871-881.

Woodruff, T.K., Krummen, L.A., Chen, S., DeGuzman, G., Lyon, R., Baly, D.L., Allison, D.E., Garg, S., Wong, W.L., Hebert, N., Mather, J.P. and Cossum, P. (1993). Pharmacokinetic profile of recombinant human (rh) inhibin A and activin A in the immature rat. I. Serum profile of rh-inhibin A and rh-activin A in the immature female rat. Endocrinology 132, 715-724.

Wozney, J.M., Rosen, V., Celeste, A.J., Misock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M. and Wang, E.A. (1988). Novel regulators of bone formation: Molecular clones and activities. Science 242, 1528-1533.

Xiao, S. and Findlay, J.K. (1991). Interactions between activin and follicle-stimulating hormone-suppressing protein and their mechanisms of action on cultured rat granulosa cells. Mol. Cell. Endocrinol. 79, 99-107.

Xiao, S., Farnworth, P.G. and Findlay, J.K. (1992). Interaction between activin and follicle-stimulating hormone-suppressing protein/follistatin in the regulation of basal inhibin production by cultured rat granulosa cells. Endocrinology 131, 2365-2370.

Xu, J., McKeehan, K., Matsuzaki, K. and McKeehan, W.L. (1995). Inhibin antagonizes inhibition of liver cell growth by activin by a dominant-negative mechanism. J. Biol. Chem. 270, 6308-6313.

Yamamoto, K.K., Gonzalez, G.A. Biggs III, W.H. and Montminy, M.R. (1988). Phosphorylation-induced and transcriptional efficacy of nuclear factor CREB. Nature 334, 494-495.

Yamashita, T., Takahashi, S. and Ogata, E. (1992). Expression of activin A/erythroid differentiation factor in murine bone marrow stromal cells. Blood 79, 304-307.

Ying, S.-Y., Becker, A., Ling, N., Ueno, N. and Guillemin, R. (1986). 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., 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.

Yu, J., Shao, L., Lemas, V., Yu, A.L., Vaughan, J., Rivier, J. and Vale, W. (1987). Importance of FSH-releasing protein and inhibin in erythrodifferentiation. Nature 330, 765-767.

Zhiwen, Z., Herington, A.C., Carson, R.S., Findlay, J.K. and Burger, H.G. (1987). Direct inhibition of rat granulosa cell inhibin production by epidermal growth factor *in vitro*. Mol. Cell. Endocrinol. 54, 213-220.

Zhiwen, Z., Findlay, J.K., Carson, R.S., Herington, A.C. and Burger, H.G. (1988). Transforming growth factor β enhances basal and FSH-stimulated inhibin production by rat granulosa cells *in vitro*. Mol. Cell. Endocrinol. 58, 161-166.

Summary

In 1988, inhibin was defined as a glycoprotein consisting of two dissimilar, disulphide linked subunits, named α, and βA or βB, which inhibits pituitary gonadotrophin production and/or secretion, preferentially that of FSH. Since then, many other effects have been reported of inhibin and its counterpart activin, which is a dimer of the inhibin β-subunits. The main site of inhibin production in the male rat are Sertoli cells, which play a crucial role in the development of germ cells in the testis. The aim of the experiments described in this thesis was to study the regulation of inhibin production in the testis, in particular in Sertoli cells. These experiments were performed *in vitro* (using cultured Sertoli cells) or *in vivo*, and concerned mechanisms of hormone action and effects of factors on inhibin production, respectively.

In Chapter 1, background information is presented on spermatogenesis, mechanisms of hormone action, inhibin, inhibin effects and regulation of inhibin production.

In cultured Sertoli cells from immature (21-day-old) rats, FSH stimulated the expression of inhibin α-subunit mRNA, but not of inhibin βB-subunit mRNA. The stimulatory effect on α-subunit gene expression was mimicked by addition of dbcAMP, indicating that FSH action on the α-subunit gene is exerted through the protein kinase A pathway (Chapter 2). In Chapter 5, *in vivo* effects of elevated FSH levels on inhibin production are described. When 8-, 15-, and 22-day-old male rats were hemicastrated, elevated serum FSH levels resulted in increased total testicular inhibin α-subunit mRNA expression in 8-, and 15-day-old rats only. Furthermore, βB-subunit mRNA expression was unchanged in 8-day-old rats, increased in 15-day-old rats and decreased in 22-day-old rats. These results indicate that the response of the testis depends on the age of the animal. Since increased inhibin subunit mRNA levels did not result in an increased testicular content of bioactive or immunoreactive inhibin, it is thought that efficiency of translation, post-translational modifications, and/or transport from the testis play an important role in determining the final testicular content of inhibin.

A half-life of 6 h was found for the α-subunit mRNA in Chapter 2. This half-life was prolonged by inhibition of transcription by actinomycin D, indicating that a short-lived mRNA-destabilizing protein may influence the stability of the α-subunit mRNA.

The involvement of protein kinase C and increased Ca^{++} levels in regulation of inhibin production by cultured Sertoli cells was studied in Chapter 3. Inhibition of protein kinase C by prolonged exposure to the phorbol ester PMA or the protein kinase C inhibitor staurosporine resulted in decreased βB-subunit mRNA expression, whereas the expression of the α-subunit mRNA was unaffected. Increased intracellular

Ca^{++} levels, caused by addition of the calcium ionophore A23187, also resulted in decreased βB-subunit mRNA levels. Concomitantly, the production of bioactive inhibin was inhibited, and the production of the immunoreactive inhibin-related molecule pro-αC was increased, indicating that the level of βB-subunit mRNA is rate-limiting for the production of bioactive inhibin by cultured Sertoli cells.

In Chapter 4, effects of the stage of the cycle of the seminiferous epithelium on inhibin production were demonstrated, using a model of stage-synchronized rats. Stage-dependency was found for inhibin subunit mRNA expression and for the testicular levels of bioactive and immunoreactive inhibin in rats with complete spermatogenesis. The level of inhibin α-subunit mRNA expression was relatively high at stages V and XIII, whereas the expression of βB-subunit mRNA was high at stage XIII only. As a result, the ratio between βB-subunit mRNA and α-subunit mRNA changed throughout the cycle, and it is therefore likely that the relative production of inhibin and activin varies. A high ratio between βB- and α-subunit mRNAs at stage XIII might result in production of activin, which might play a role in the final phase of meiosis. Bioactive and immunoreactive inhibin levels were highly correlated throughout the spermatogenic cycle, with maximum levels at stages XIV-I.

Current knowledge on regulation of inhibin production is reviewed in Chapter 6. Furthermore, some suggestions for future studies on regulation of inhibin production are presented.

Samenvatting

Inhibine is gedefinieerd als een glycoproteïne dat de productie en/of secretie van gonadotropinen door de hypofyse onderdrukt, vooral van FSH, en dat bestaat uit twee verschillende subeenheden, α en βA of α en βB, die verbonden zijn door een disulfidebrug. Er zijn echter ook veel andere effecten van inhibine beschreven. Combinatie van twee inhibine β-subeenheden leidt tot de vorming van activine, een hormoon dat in de meeste gevallen een werking heeft die tegengesteld is aan die van inhibine. Het voornaamste celtype waar inhibine gemaakt wordt in de mannelijke rat is de Sertoli cel. Sertoli cellen bevinden zich in de zaadbal of testis en spelen een onmisbare rol bij de ontwikkeling van zaadcellen. Het doel van de experimenten die in dit proefschrift beschreven zijn, was het bestuderen van de regulatie van de productie van inhibine in de testis, in het bijzonder in Sertoli cellen van onvolwassen ratten.

Experimenten betreffende effecten van hormonen en hun werkingsmechanismen werden in gekweekte Sertoli cellen (*in vitro*) uitgevoerd, terwijl effecten van andere factoren in experimenteel behandelde ratten (*in vivo*) werden bestudeerd.

In Hoofdstuk 1 wordt achtergrondinformatie gegeven over spermatogenese, mechanismen van hormoonwerking, inhibine, effecten van inhibine en regulatie van inhibine productie.

FSH stimuleerde de expressie van het inhibine α-subeenheid mRNA, maar niet van het inhibine βB-subeenheid mRNA, in gekweekte Sertoli cellen afkomstig van ratten van 21 dagen. Het stimulerende effect van FSH op de expressie van het gen dat codeert voor de α-subeenheid kon worden nagebootst door toevoeging van dbcAMP. Dit is een aanwijzing dat het effect van FSH op het α-subeenheid gen tot stand komt via proteïne kinase A (Hoofdstuk 2). In Hoofdstuk 5 worden *in vivo* effecten van verhoogde FSH niveaus op inhibine productie beschreven. Wanneer ratten met een leeftijd van 8, 15, of 22 dagen werden onderworpen aan hemicastratie, resulteerden verhoogde niveaus van FSH in het serum alleen in een verhoogde expressie van het inhibine α-subeenheid mRNA in ratten van 8 en 15 dagen oud. Verder was de expressie van het βB-subeenheid mRNA onveranderd in 8 dagen oude ratten, verhoogd in 15 dagen oude ratten, en verlaagd in 22 dagen oude ratten. Deze resultaten geven aan dat de reactie van de testis op veranderingen in FSH afhangt van de leeftijd van het dier. Omdat verhoogde niveaus van inhibine mRNAs niet resulteerden in een verhoogde inhoud van bioactief of immunoreactief inhibine van de testis, spelen efficiëntie van translatie, posttranslationele modificaties, en/of transport vanuit de testis, kennelijk ook een belangrijke rol in het bepalen van de uiteindelijke hoeveelheid inhibine in de testis.

De halfwaardetijd van het α-subeenheid mRNA in gekweekte Sertoli cellen bedroeg 6 uur (Hoofdstuk 2). Deze halfwaardetijd kon worden verlengd door remming

van transcriptie met behulp van actinomycine D. Dit suggereert dat een mRNA-destabiliserend eiwit met een korte levensduur de stabiliteit van het α-subeenheid mRNA beïnvloedt.

In de experimenten beschreven in Hoofdstuk 3 werd onderzocht of proteïne kinase C en/of verhoogde intracellulaire Ca^{++} niveaus betrokken zijn bij regulatie van inhibine productie in gekweekte Sertoli cellen. Remming van proteïne kinase C door langdurige blootstelling van de cellen aan de phorbolester PMA, of aan de proteïne kinase C remmer staurosporine, had een verlaging van de expressie van het βB-subeenheid mRNA tot gevolg, terwijl de expressie van het α-subeenheid mRNA onveranderd bleef. Verhoogde Ca^{++} niveaus door toevoeging van de calcium ionofoor A23187 leidden ook tot verlaagde βB-subeenheid mRNA niveaus. Tegelijkertijd werd de productie van bioactief inhibine geremd, en werd de productie van pro-αC, een molekuul dat verwant is aan inhibine en zeer immunoreactief is, gestimuleerd. Dit geeft aan dat het niveau van het βB-subeenheid mRNA beperkend is voor de productie van bioactief inhibine door gekweekte Sertoli cellen.

Effecten van het stadium van de spermatogene cyclus op inhibine productie werden bestudeerd met behulp van een model dat gebruik maakt van ratten waarvan de spermatogene cyclus gesynchroniseerd is (Hoofdstuk 4). Expressie van inhibine subeenheid mRNAs en de testiculaire niveaus van bioactief en immunoreactief inhibine bleken afhankelijk te zijn van het stadium van de spermatogene cyclus in ratten met een complete spermatogenese. Het niveau van inhibine α-subeenheid mRNA expressie was relatief hoog in de stadia V en XIII, terwijl de expressie van βB-subeenheid mRNA alleen verhoogd was in stadium XIII. Daaruit voortvloeiend verandert de verhouding tussen βB-subeenheid mRNA en α-subeenheid mRNA gedurende de cyclus van de spermatogenese, en daarom is het waarschijnlijk dat de relatieve productie van inhibine en activine varieert. Een hoge verhouding tussen βB- en α-eenheden in stadium XIII zou kunnen uitmonden in productie van activine, dat een rol zou kunnen spelen in de laatste fase van meiose. Niveaus van bioactief en immunoreactief inhibine waren nauw gecorreleerd tijdens de spermatogene cyclus, met maximum waarden in stadium XIV-I.

Een overzicht van de huidige kennis van regulatie van inhibine productie wordt gegeven in Hoofdstuk 6. Tevens worden enkele mogelijkheden voor verder onderzoek naar de regulatie van inhibine productie geopperd.

Papers related to this thesis

A.M.W. Toebosch, 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.

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, 235-242.

A.J. Grootenhuis, F.J. van Sluijs, I.A. Klaij, J. Steenbergen, M.A. Timmerman, M.M. Bevers, S.J. Dieleman and F.H. de Jong (1990). Inhibin, gonadotrophins and sex steroids in dogs with Sertoli cell tumours. J. Endocrinol. 127, 235-242.

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 (Eds. J. Porter and D. Jezová) Plenum Press, New York, 271-293.

J.P. de Winter, M.A. Timmerman, H.M.J. Vanderstichele, I.A. Klaij, A.J. Grootenhuis, F.F.G. Rommerts and F.H. de Jong (1992). Testicular Leydig cells in vitro secrete only inhibin α-subunits, whereas Leydig cell tumors can secrete bioactive inhibin. Mol. Cell. Endocrinol. 83, 105-115.

J.P. de Winter, A.P.N. Themmen, J.W. Hoogerbrugge, I.A. Klaij, J.A. Grootegoed and F.H. de Jong (1992). Activin receptor mRNA expression in rat testicular cell types. Mol. Cell. Endocrinol. 83, R1-R8.

F.H. de Jong, J.P. de Winter, A.J. Grootenhuis, I.A. Klaij, R.H. Meloen, F.F.G. Rommerts and J.A. Grootegoed (1993). Regulation of inhibin and inhibin-subunit production in testicular cells. In: Current Topics in Andrology (Eds. H. Oshima and H.G. Burger), Tokoh-sha Co Ltd, Tokyo, Japan, 53-58.

F.H. de Jong, J.P. de Winter, I.A. Klaij, A.P.N. Themmen and J.G. Wesseling (1994). Inhibin, activin and activin receptors in the testis: Localization, effects and the activin paradox. Frontiers in Endocrinology 3, 233-244.

A.Kooistra, A.J.M. van den Eijnden-van Raaij, I.A. Klaij, J.C. Romijn and F.H. Schröder (1995). Stromal inhibition of prostatic epithelial cell proliferation not mediated by transforming growth factor β. Brit. J. Cancer 72, 427-435.

Curriculum vitae

Ingrid van der Kleij-Klaij werd geboren in 1963 te Delft. In 1981 behaalde zij het VWO diploma aan het Christelijk Lyceum Delft te Rijswijk, en begon zij met de studie Biologie aan de Rijksuniversiteit Leiden. Het kandidaatsexamen (B1) werd in 1984 afgelegd. Het doctoraalexamen, dat in 1987 werd afgelegd, omvatte het hoofdvak Moleculaire Plantkunde (Botanisch Laboratorium, Leiden) en het bijvak Immunologie (Primatencentrum TNO, Rijswijk). In augustus 1987 begon de schrijfster van dit proefschrift aan het onderzoek dat hierin beschreven is. Het onderzoek voor en het schrijven van dit proefschrift werden na de geboorte van haar dochter in 1991 part-time voortgezet en nogmaals onderbroken door de zwangerschap en geboorte van haar zoon. Momenteel is zij part-time werkzaam als vertaler van octrooien op het gebied van DNA-technologie.

Dankwoord

Tenslotte wil ik iedereen die mij heeft geholpen bij het tot stand komen van dit proefschrift hartelijk bedanken.

Mijn twee promoters wil ik bedanken voor de goede begeleiding: Frank, ik heb veel van je geleerd en je hebt zeker de laatste jaren ontzettend veel geduld met me gehad. Opmerkelijk was je mateloze enthousiasme wanneer iets onverwachts ontdekt werd ("interessant"). Anton, vooral bedankt voor de fijne begeleiding in het begin (ook voor het "brainstormen" 's morgens vroeg, wat soms wel eens wat uitliep); ik vind het jammer dat we de laatste tijd niet meer zoveel van gedachten hebben kunnen wisselen. Samen waren jullie voor mij de perfecte combinatie ondanks (dankzij?) het feit dat jullie vaak van mening verschillen en ik denk nog steeds dat jullie door samenwerking tot grote dingen kunnen komen.

De leden van de leescommissie wil ik bedanken voor hun bereidheid om het manuscript te beoordelen.

Alle medewerkers van Biochemie II en E & V, in het bijzonder alle (ex)bewoners van het spermatogenese-lab en alle (ex)inhiberianen, bedankt voor de gezelligheid tijdens of na het werk, praktische hulp en samenwerking, en de leerzame discussies tijdens werkbesprekingen en literatuurbesprekingen.

Marianna, hartelijk bedankt dat je mijn paranimf wilt zijn. Ik hoop dat jij net zoveel plezier aan onze samenwerking hebt beleeft als ik. Ik vind het leuk dat je nu doorgaat met experimenten op het moleculair genetische vlak.

De medebewoners van het promohok wil ik ook graag bedanken: Piet en Roel voor de ellenlange gesprekken, al dan niet over spermatogenese. Zonder hen had ik me niet zo snel eigen kunnen maken met de endocrinologie van de testis die voor mij toen volledig onbekend was. Leen, je hebt altijd voor me klaar gestaan om een helpende hand toe te reiken. Zonder jou waren alle hybridisaties gestopt tijdens mijn zwangerschap en had het schrijven van dit proefschrift nog veel langer op zich laten wachten. Lizzy, vooral bedankt voor de gezelligheid tijdens ons gezamelijke congres in St. Odile en de peptalks toen wij beiden thuis probeerden onze proefschriften af te maken.

Arijan, bedankt voor onze gesprekken over gegoochel met kolommen en inhibine-literatuur.

Axel, bedankt voor het meelezen en meedenken, ook al vond je me vaak eigenwijs.

Ria, Marjolein, Rosemarie, Petra, Cobie, Franny, Lizzy, Johan en Marianna wil ik bedanken voor ontspanning tijdens de lunch.

Pim, Ria, Marja, Petra, Rosemarie en Peter, bedankt voor het uitvoeren van

allerlei uiteenlopende werkzaamheden die nodig zijn om de afdeling draaiende te houden.

Ans van Pelt en Dick de Rooij, bedankt voor de vruchtbare samenwerking, die uiteindelijk geleid heeft tot Hoofdstuk 4.

Piet Kramer en Jiet Meijs-Roelofs, bedankt voor de fijne samenwerking bij het "Pjiet"-experiment.

Het is nu nauwelijks voor te stellen dat, toen ik begon te werken bij Biochemie II, er bij die vakgroep niet met moleculair genetische technieken werd gewerkt. In die tijd kon ik altijd op de tiende verdieping bij de groep van Jan Trapman terecht voor labellen, plasmiden isoleren etc. Bedankt hiervoor.

Mijn ouders wil ik bedanken voor hun voortdurende hulp en stimulans. Mama, zonder jouw bereidwilligheid om op Femke en Ruben te passen zou dit proefschrift zijn geëindigd bij Hoofdstuk 3.

Tenslotte Clemens, aan de ene kant ondanks jou (als ik je niet ontmoet had, had ik ongetwijfeld veel meer tijd en energie in het onderzoek gestopt), maar ook dankzij jou (je stuurde me altijd weer naar m'n werkkamertje als ik daar 's avonds geen zin in had) is het schrijven van dit proefschrift uiteindelijk tot een goed einde gekomen.