EFFECTS OF FSH, INSULIN AND IGF-I ON RAT SERTOLI CELLS

EFFECTEN VAN FSH, INSULINE EN IGF-I OP SERTOLI CELLEN VAN DE RAT

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Voor mijn ouders
Voor Ruud

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Other papers related to this thesis

Grootegoed, J.A., Oonk, R.B., Jansen, R. and van der Molen, H.J. (1984) Sertoli cell-germ cell interactions and the hormonal control of spermatogenesis.
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Grootegoed, J.A., Oonk, R.B., Jansen, R. and van der Molen, H.J. (1985) Spermatogenic cells utilize metabolic intermediates from Sertoli cells.
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Grootegoed, J.A., Oonk, R.B., Toebosch, A.M.W. and Jansen, R. (1986) Extracellular factors that contribute to the development of spermatogenic cells.
In: Molecular and Cellular Endocrinology of the Testis. Eds. M. Stefanini, (Elsevier Amsterdam), pp 215-225.

Oonk, R.B. and Grootegoed, J.A. (1986) Insulin and insulin-like growth factor–I (IGF–I) receptors on rat Sertoli cells.
Annals of the New York Academy of Sciences, in press.

Grootegoed, J.A., Mackenbach, P. and Oonk, R.B. (1987) Sertoli cell-germ cell interactions in the regulation of spermatogenesis.
In: Proceedings VII Workshop on Development and Function of the Reproductive Organs, Turku, Finland, June 1987, (Raven Press New York), in press.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

The mammalian testis consists of two compartments, viz. the seminiferous tubules containing Sertoli cells and spermatogenic cells, and the interstitium containing mainly Leydig cells and macrophages. The tubules are lined by a layer of peritubular myoid cells and surrounded by endothelial cells, and blood vessels and nerves are present in the interstitium.

The seminiferous epithelium in the tubules has a specialized spatial organization, which is supported by the structure of the Sertoli cell. Sertoli (1865) was the first to describe the columnar shape of this "cellula ramificata" with its extensive cytoplasm in between the developing germ cells, the marked "gocciole di grasso" (lipid droplets), and the typical nucleus with the nucleolus. Specialized contacts between germ cells and Sertoli cells were demonstrated much later by electronmicroscopy. This morphological evidence contributed to the notion that the presence of Sertoli cells is essential for spermatogenesis (review: Russell, 1980).

From the anatomical organization of the tubules it has been inferred that Sertoli cells render not only mechanical support to the germ cells, but also may regulate spermatogenesis by controlling the biochemical environment in which the germ cells develop. Tight junctional complexes in between neighbouring Sertoli cells form a barrier (Dym and Fawcett, 1970), which isolates spermatogenic cells at and beyond the prophase of meiosis in an adluminal compartment, separated from the spermatogonia in the basal compartment (Fawcett, 1974, 1975) (Fig. 1.1). This so-called Sertoli cell barrier or "blood-testis barrier" excludes a variety of substances from entering the adluminal compartment. Using delicate micropuncture techniques it has been demonstrated that the composition of the intratubular fluid differs from the composition of blood plasma and testicular lymph with respect to the concentration of ions and the presence of different proteins. The Sertoli cell barrier may be important in maintaining an optimal environment for spermatogenesis, and Sertoli cells can contribute to the composition of the tubular fluid by active secretion of compounds (reviews: Setchell and Waites, 1975; Setchell, 1978).

Spermatogenesis starts with multiple divisions of differentiated spermatogonia (A, intermediate and B), which arise from undifferentiated spermatogonia A at the basement membrane lining the outside of the tubules. Subsequently, spermatogonia B develop via the last mitotic division into preleptotene spermatocytes, which go through S-phase (DNA replication) at the start of the meiotic prophase. In the meiotic prophase, homologous pairing of the chromosomes is initiated and completed during the leptotene, zygotene, pachytene and diplotene spermatocyte stages. The first meiotic division yields secondary spermatocytes, which rapidly go through the second meiotic division, without DNA synthesis. This results in the formation of haploid spermatids which contain half the amount of DNA present in diploid somatic cells in G_1. Without further cell divisions, the early round spermatids are then transformed via the late elongating spermatids into spermatozoa. The contacts between Sertoli cells and the spermatozoa are disconnected so that spermatozoa are released into the tubular lumen.

Spermatogonia enter meiosis at fixed intervals, spaced approximately 13 days in rats. The duration of the development from the undifferentiated spermatogonia stage to spermatozoa is also fixed, lasting approximately 60 days in rats. The different spermatogenic cell types are not randomly present in the seminiferous tubules, but occur as specific associations of the different gen-

erations of germ cells. In the rat fourteen of these associations, termed stages I to XIV of the cycle of the seminiferous epithelium have been described (Leblond and Clermont, 1952).

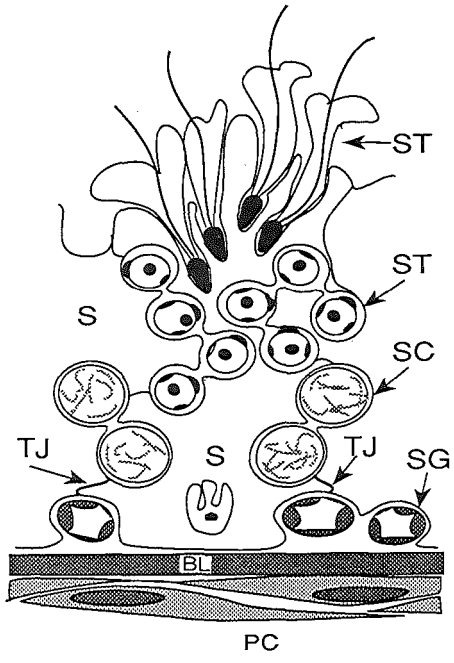

Figure 1.1. Schematic drawing of seminiferous epithelium illustrating the position of the cells in the tubulus. The lining of the tubulus is formed by the Sertoli cells (S) and peritubular cells (PC) which are separated by a basal lamina (BL). The spermatogenic cells are positioned in between the Sertoli cells. Tight junctions (TJ) between the neighbouring Sertoli cells divide the basal compartment containing the spermatogonia (SG) from the adluminal compartment containing the more advanced stages of the spermatogenic cell population, viz. the spermatocytes (SC) and round and elongating spermatids (ST).

Spermatogenesis in the rat is dependent on testosterone and the pituitary hormone FSH (follitropin; follicle stimulating hormone). The pituitary hormone LH (lutropin; luteinizing hormone) regulates the production of testosterone in interstitial Leydig cells, whereas FSH exerts direct effects on Sertoli cells. Much research, mainly involving restoration of gonadal function by gonadotropin and/or androgen treatment of adult rats at different times after hypophysectomy, has been performed to investigate the actions of the different hormones on spermatogenesis (reviews: Steinberger, 1971; Fritz, 1978). It was concluded that in adult rats FSH does not play a major role in maintenance of spermatogenesis, but is required for restoration of regressed spermatogenesis. Testosterone appears to be able to maintain complete spermatogenesis in adult rats, although at a slightly reduced quantitative level (Clermont and Harvey, 1967). The absence of FSH and testosterone, as caused by hypophysectomy of adult rats, resulted in enhanced degeneration of spermatocytes and spermatids, in particular at stage VII-VIII of the spermatogenic cycle (Russell and Clermont, 1977). Administration of FSH and LH in combination, starting at 12 h after hypophysectomy, maintained spermatogenesis at a quantitatively normal level (Russell and Clermont, 1977).

During the initiation of spermatogenesis, the role of FSH is more pronounced. In immature rats (32 days old, i.e. during the first wave of spermatogenesis) hypophysectomy also caused increased degeneration of different germ cell types, in particular those which were developmentally most advanced at that age (Russell et al., 1987). In these immature hypophysectomized rats, administration of both FSH and LH was required to reduce degeneration of spermatogenic cells, and this effect was not achieved by LH alone (Russell et al., 1987), indicating that testosterone alone, stimulated by LH, was not effective. Together with previous investigations reviewed by Steinberger (1971), this indicates the importance of FSH during the first wave of spermatogenesis in the developing testis. However, recent research on monkeys has indicated that in intact prepuberal animals administration of large amounts of testosterone resulted in a precocious initiation of spermatogenesis (Marshall et al., 1984). In this situation, FSH release by the pituitary

gland was completely or almost completely inhibited by the negative feedback regulation of testosterone on pituitary gonadotropin secretion. From this, it would appear that the contribution of FSH is not obligatory or that the role of FSH can be taken over by other factors.

At present, much research is addressing the question if other hormones or growth factors may play a regulatory role in testicular development. This work may lead to insight regarding to other factors in addition to FSH and/or testosterone which might be involved in the initiation and maintenance of spermatogenesis. Some of these factors could be produced by testicular cells, and exert so-called paracrine effects. The importance and the specificity of these effects has not yet been demonstrated.

Sertoli cells contain FSH receptors and are target cells for FSH action (reviews: Means et al., 1976; Means et al., 1980; Davies, 1981). Sertoli cells also possess androgen receptors (Mulder et al., 1976), whereas these steroid receptors may be absent in spermatogenic cells (Grootegoed et al., 1977). Hence, it is now generally assumed that the influence of pituitary hormones and androgens on spermatogenesis involves direct actions of these hormones on Sertoli cells, rather than on germ cells. Androgen action on Sertoli cells may be altered by the peritubular myoid cells, which possess androgen receptors and which have been reported to enhance androgen effects on Sertoli cells (Skinner and Fritz, 1985a, b).

Cultured Sertoli cells from immature rats can perform different metabolic and secretory activities, some of which can be influenced by hormones. It might be inferred that products secreted by Sertoli cells could be made available to, and could be essential for, spermatogenic cells at the luminal side of the Sertoli cell barrier, as these germ cells are completely dependent on supply of substrates and further support by Sertoli cells. The glucose metabolites pyruvate and lactate are essential energy substrates for isolated germ cells (Mita et al., 1982; Jutte, 1982), and chemicals that interfere with glucose metabolism might therefore be expected to induce testicular lesions. 5-Thio-D-glucose for example may cause sterility in mice and rats because this glucose derivative interferes with glucose metabolism (Zysk et al., 1975; Homm et al., 1977; Lobl and Porteus, 1978; Davies and Meanock, 1981).

1.2. Scope of this thesis

The response of Sertoli cells *in vivo* to FSH may be complex, as indicated by the important role played by FSH in the initiation and recovery of spermatogenesis (section 1.1). A large number of processes in Sertoli cells in culture can be stimulated by FSH. Some of these processes are typical for Sertoli cells, for example the secretion of androgen binding protein and a high aromatase activity, which converts testosterone to estradiol, but other processes involve general metabolism. The functional importance of androgen binding protein and aromatase activity with respect to spermatogenesis has not yet been elucidated. We have chosen to study glucose metabolism as a parameter in the present experiments for a number of reasons. As mentioned above, glucose metabolism is important for the supply of energy substrates to spermatogenic cells (section 1.1), and is relatively easy to measure *in vitro*. Insulin is known as one of the main regulatory hormones for glucose metabolism, and insulin-like growth factor I (IGF–I) can perform insulin-like actions. Hence, we have investigated insulin and IGF–I effects on glucose metabolism by Sertoli cells as compared with the effects of FSH.

In addition to rapid metabolic effects, the hormones insulin, IGF–I and FSH can influence growth and development. This is discussed in Chapter 2 in connection with the possible roles of other factors. In this respect, the studies presented in this thesis aimed to gain more insight in the possible importance of insulin and IGF–I as regulating hormones of immature Sertoli cell functions, in relation with the importance of FSH.

The presence of specific FSH receptors on Sertoli cells has been extensively documented (review: Means et al., 1980), but information was scarce about the presence of insulin and IGF–I receptors on Sertoli cells. To investigate whether insulin and IGF–I exert effects on Sertoli cells via spe-

cific receptors, insulin and IGF–I receptors on cultured immature Sertoli cells were quantitated and characterized (Chapter 4; appendix papers 1 and 2).

Furthermore, the relationship between the actions of insulin, IGF–I and FSH with respect to the development of the testis was evaluated. In this regard we have investigated the amount of insulin and IGF–I binding in membrane fractions from total testis tissue, using immature and adult rats (Chapter 4; appendix paper 2).

In addition, the effects of FSH, insulin and IGF–I on glucose transport, glycolysis, and CO_2 production from glucose were studied to analyze differences and similarities of the actions of these hormones on several aspects of glucose metabolism by cultured Sertoli cells. The results of these investigations are described in Chapter 5 and appendix papers 3, 4 and 5.

The possible roles of FSH, insulin and IGF–I in the regulation of metabolic activities of Sertoli cells and testis development are discussed in Chapter 6.

CHAPTER 2

HORMONES AND GROWTH FACTORS IN RELATION TO SPERMATOGENESIS

2.1 Introduction

- Hormone action

Hormones have been originally defined as internal secretions of the endocrine glands, being transported via the blood to their target cells. Classical hormones, defined as such, exert endocrine effects. Examples of endocrine organs are the pituitary gland, the gonads and the pancreas, which secrete, among other hormones, the trophic hormones FSH and LH, the steroid hormones testosterone and estradiol, and the peptide hormone insulin, respectively. The use of the term "hormone" has expanded with the recognition that multiple non-glandular tissues can produce the same hormones. Pro-opiomelanocortin and its derivatives, including ACTH, are produced in other tissues besides the pituitary gland.

Growth factors are also made by a variety of non-glandular tissues, and may not be transported to other tissues to exert their biological effects. Examples of growth factors are the insulin-like growth factors, epidermal growth factor, transforming growth factors, platelet derived growth factor, fibroblast growth factors and nerve growth factor. In the different tissues, where these growth factors are produced, they may act on the cell type of origin (autocrine effects), or they may act on other cell types within the same tissue (paracrine effects). However, a given hormone or growth factor may have different effects in different tissues, and its mode of action is not necessarily limited to either endocrine or paracrine action.

- Testicular development

Testicular differentiation of the gonads is determined by the Y chromosome (Ohno, 1985; Byskov, 1986). An important role in testicular differentiation has been assigned to a testis determining factor (TDF; also termed sex determining male antigen) which is encoded by on the Y chromosome. This putative factor is probably not the H-Y male antigen (McLaren et al., 1984). Recent studies indicated that the genes for TDF and H-Y antigen are located on different parts of the human Y chromosome (Simpson et al., 1987).

The initial stage of testicular differentiation of the fetal rat gonad involves the differentiation of precursor Sertoli cells at day 13 of fetal life, which aggregate into seminiferous cords enclosing the primordial germ cells (Jost, 1985). As soon as the gonads are differentiated into the direction of testes, the precursor Sertoli cells start to produce anti-Müllerian hormone (AMH) (Josso et al., 1977, 1980). AMH is a protein hormone which causes the regression of the Müllerian ducts, the primordia of the female reproductive tract. The action of AMH is necessary for complete virilization, which is effectuated mainly by androgens. The secretion of AMH is age-dependent. It is detectable mainly in fetal and neonatal testes, and AMH bioactivity rapidly decreases after birth to a low level. It has been described, however, that in adult Sertoli cells AMH synthesis is not totally absent (Josso et al., 1980).

Shortly after the formation of testicular cords, cytological differentiation of interstitial cells or Leydig cells and concomitant production of testosterone take place, even before LH can be detected (Byskov, 1986). Later in development, LH controls Leydig cell testosterone production. Testosterone primes masculine differentiation of the sex ducts (growth of the Wolffian ducts) and the secondary sex characteristics. The fetal rat pituitary releases measurable amounts of FSH and LH from 18-20 days of fetal life onwards. Testosterone level in the fetal rat testis reaches a peak at 19 days, but is low at

birth and during neonatal life (Setchell, 1978). FSH appeared to be necessary in the male fetal rat to stimulate the rate of Sertoli cell proliferation, which is maximal at day 20 of fetal life, and becomes very low at 15-20 days after birth (Orth, 1982; 1984).

FSH levels in the rat are low after birth until 15-20 days of age (Ketelslegers et al., 1978). In this respect, it is important that Sertoli cells produce inhibin, a hormonal factor produced by the male and female gonads, which exerts a specific negative feedback action on the secretion of FSH by the pituitary gland (De Jong, 1979). The interaction between FSH and inhibin may play a decisive role to determine testicular growth and development in the prepuberal rat. The prepuberal FSH levels may be regulated by inhibin production by the immature Sertoli cells, in addition to the feedback role exerted by testosterone. A role of the inhibin/FSH couple in the adult testis has not yet been elucidated.

The plasma FSH level increases rapidly at puberty from 20 to 40 days of age and declines to an adult plateau level between days 50 and 60 (De Jong and Sharpe, 1977; Ketelslegers et al., 1978). The plasma LH level increases from 30-55 days, and shortly after that the testosterone level rises sharply between 35 and 60 days of age. Thereafter both hormones remain at adult levels (De Jong and Sharpe, 1977;

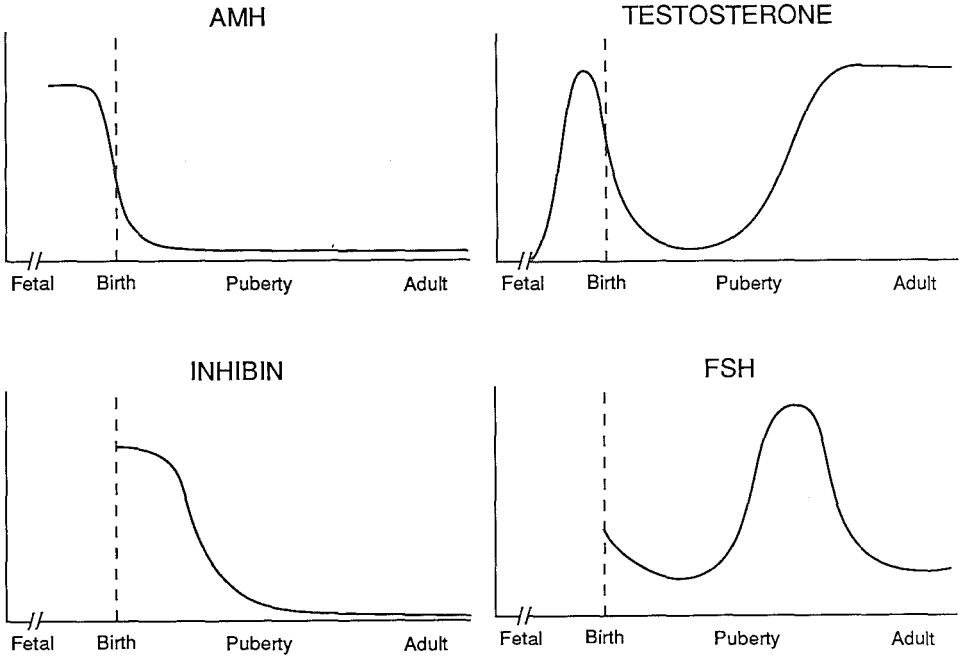

Figure 2.1. Relative levels of some hormones during male rat life. AMH levels represent bioactivity as described by Josso et al. (1977). Testosterone levels at fetal age represent testicular contents (Setchell, 1978) and after birth represent plasma levels (De Jong and Sharpe, 1977; Ketelslegers et al., 1978). Inhibin is represented by the amount secreted by Sertoli cells *in vitro* from rats at different ages (Ultee-van Gessel and De Jong, 1987). FSH levels represent plasma levels (De Jong and Sharpe, 1977; Ketelslegers et al., 1978).

Ketelslegers et al., 1978). A schematic representation of hormone levels during rat life is given in Fig. 2.1.

It is well appreciated that FSH is very important during the initiation of spermatogenesis. The first wave of spermatogenesis in rats occurs between birth and 60 days of age. FSH exerts multiple effects on its target cell, the Sertoli cell, and its action is most evident in the immature testis (Fritz, 1978). Marked effects of FSH *in vitro* on Sertoli cells of rats older than 30-40 days of age have not been shown. For example, FSH was found to stimulate protein kinase A-inhibitor activity (see section 2.2.4) in Sertoli cells from 9- and 16-day-old rats, but in Sertoli cells from 32-day-old rats FSH could only stimulate protein kinase A-inhibitor activity in the presence of the phosphodiesterase inhibitor MIX (Tash et al., 1981). This may indicate that Sertoli cells from older animals are less responsive to FSH. Indeed, maintenance of fully established spermatogenesis in the adult rat can be achieved by testosterone alone. However, for quantitative restoration of impaired spermatogenesis in for example hypophysectomized rats, also FSH is required.

It will be clear from the above, that testicular growth and spermatogenesis are regulated in a precise and coordinate fashion. Experiments with hypophysectomized rats have indicated the importance of FSH, LH and testosterone (see section 1.1). However, the precisé mechanisms by which these hormones regulate spermatogenesis are still not clear.

Several growth factors have been discovered which can regulate growth and differentiation of many tissues and cells. Among these, the existance of distinct growth factors, produced by Sertoli cells, has been postulated (Feig et al, 1980; Holmes et al., 1986). Furthermore, several growth factors, for which a testicular origin has not been demonstrated, were shown to influence a number of processes in the testis.

In this chapter, background information concerning a number of hormones and growth factors and their possible effects on the hormonal regulation of spermatogenesis will be presented. This chapter is intended to give a compendious, but by no means complete survey of some of the factors which might effect the hormonal regulation of testicular development. The discussion of the possible roles and the mechanisms of action of FSH, insulin and the IGFs, and a description of the receptors for these hormones will account for the main part of this chapter, as this is most relevant to the studies presented in this thesis. Furthermore, a number of other hormones and growth factors and their effects on Sertoli cells will be briefly discussed. Attention will be given to interactions of gonadotropins and growth factors, and cell-cell interactions within the testis, which may be involved in the regulation of spermatogenesis.

2.2 FSH

2.2.1 Functional aspects of FSH

FSH is a glycoprotein hormone of 38,000 Da. It consists of an α-subunit (16,000 Da) that is common to FSH, LH and thyroid stimulating hormone (TSH), and an FSH-specific β-subunit (21,000 Da) (Pierce and Parsons, 1981). FSH is secreted by the anterior pituitary gland, and exerts its actions in the testis and the ovary.

The involvement of FSH in spermatogenesis has been discussed briefly in section 1.1 and 2.1. The large amount of research performed to provide insight into the role of FSH in spermatogenesis has been surveyed in a number of reviews (Steinberger, 1971; Means et al., 1976; Fritz, 1978; Means et al., 1980; Davies, 1981). In summary, FSH appears to be the major factor in fetal life in controlling the expansion of the Sertoli cell population. In neonatal and prepuberal life the FSH/inhibin couple could be the main control system for testicular growth and development. Furthermore, the quantitative restoration of impaired spermatogenesis in hypophysectomized adult rats is supported most effectively by a combination of FSH and testosterone.

In the female, FSH acts to stimulate the development of ovarian follicles, which accounts for its name. Besides FSH, the hormones LH, estradiol and progesterone act together in coordination to regulate the

menstrual cycle, and thereby the regular production and release of a ripe ovum.

2.2.2 *FSH-receptors*

The initial event in FSH action is its binding to receptors in the testis and the ovary. In the testis, specific binding of ^{125}I-FSH to seminiferous tubules has been demonstrated (Bhalla and Reichert, 1974; Reichert and Bhalla, 1974), and this binding appeared to be limited to Sertoli cells (Thanki and Steinberger, 1978). Autoradiographically, localization of FSH binding sites on spermatogonia of rat testis has been reported (Orth and Christensen, 1978), but these data have not been confirmed by receptor binding studies. Ovarian FSH receptors have been demonstrated exclusively on granulosa cells (Richards et al., 1976; Ireland and Richards, 1978). Much effort has been put in the characterization of FSH interaction with its receptor by the group of Reichert (Reichert et al., 1983). These investigations have met numerous problems, for example the sparsity of FSH receptors and the instability of the isolated receptor substructures. Recently, however, the resolution of quaternary structural relationships of several FSH receptor subunits isolated from calf testis and from porcine granulosa cells has been reported (Shin and Ji, 1985; Smith et al., 1986). The elucidation of the amino acid sequence of the receptor protein and the nucleotide sequence of the gene coding for the receptor will have to await further purification of the FSH receptor protein.

2.2.3 *Effects of FSH on the target cells*

- Effects of FSH on Sertoli cells from immature rats in culture

The synthesis and release of several products by Sertoli cells, and a number of metabolic activities of Sertoli cells are stimulated by FSH. The actions of FSH on Sertoli cells (summarized in Table 2.1) are most evident using cells from immature testes. The effect of FSH *in vitro* on inhibin secretion was found to be maximal around 3 weeks of age (Ultee-van Gessel and De Jong, 1987). The stimulation by FSH of cAMP production (Davies, 1981) and aromatase activity (Dorrington and Armstrong, 1979) was highest by Sertoli cells, isolated from rats between 5-15 days of age, and declined with increasing age. In fact, the *in vitro* bioassay system to estimate FSH activity is based on the stimulation of Sertoli cell aromatase activity by FSH (Van Damme et al., 1979). Not only the age of the rats at the time of Sertoli cell isolation, but also the number of days in culture influenced the FSH effects on the secretion of ABP and transferrin (Rich et al., 1983; Perez-Infante et al., 1986). The observations indicate that FSH acted primarily on immature Sertoli cells and that the effects of FSH diminished during testis maturation. From the data in Table 2.1 it appears that FSH effects could often be mimicked by dbcAMP (section 2.2.4). For some parameters, also other stimulating factors have been listed.

- Effects of FSH on cultured granulosa cells

Homologies between granulosa cells and Sertoli cells have often been noted (Fritz, 1982). Both types of gonadal somatic cells influence the development of adjacent germinal cells, although the structural relationships in ovary and testis are different. Several biochemical similarities can be noted, particularly in immature granulosa and Sertoli cells. These include the secretion of inhibin, AMH, and several other products (Table 2.1), and the presence of specific receptors for FSH (section 2.2.3). Furthermore, both cell types can convert testosterone to estrogens via FSH-stimulated aromatase activity. Differences between the two cell types include receptors and responses to LH and hCG, *de novo* steroidogenesis and progesterone synthesis, which are specific for granulosa cells from follicles at later stages of development (Fritz, 1982). In view of the idea that granulosa cells and Sertoli cells share a common embryological origin and perform a number of similar biochemical actions, we will also discuss in this chapter hormone and growth factor effects on granulosa cells. Only a small number of data on paracrine actions of growth factors and hormones on Sertoli cells has been reported in detail, whereas these have been more extensively described for granulosa cells.

Table 2.1. Stimulatory effects of FSH on Sertoli cell products and activities.

Product/activity	Stimulated by	References
Androgen binding protein production	FSH, testosterone, insulin, retinol	Hansson et al., 1975 Fritz et al., 1976 Rommerts et al., 1978 Louis and Fritz, 1979 Karl and Griswold, 1980
Aromatase activity	FSH, dbcAMP	Dorrington et al., 1978 Rommerts et al., 1978
Glucose transport	FSH, dbcAMP Insulin, IGF–I	Hall and Mita, 1984 Mita et al., 1985 appendix paper 5
Glucose metabolism	FSH, dbcAMP, insulin	Grootegoed et al., 1983, 1986b
Inhibin production	FSH, dbcAMP	De Jong, 1979 Ultee-van Gessel et al.,1986, 1987 Bicsak et al., 1987
Lactate production	FSH, dbc AMP, insulin	Mita et al., 1982 Jutte et al., 1983 Oonk et al., 1985
Morphological changes	FSH, dbcAMP Ca^{2+}/EGTA	Tung et al., 1975 Welsh et al., 1980
Plasminogen activator synthesis	FSH, dbcAMP	Lacroix et al., 1977 Lacroix et al., 1981
Prostaglandin synthesis	FSH	Cooper and Carpenter, 1987
Protein synthesis	FSH, dbcAMP	Dorrington et al., 1975 Means et al., 1976 Wilson and Griswold, 1979 Wright et al., 1981 DePhilip and Kierszenbaum, 1982
Protein phosphorylation	FSH, Ca^{2+}	Spruill et al., 1983a, b
RNA polymerase I and II activity	FSH, testosterone	Means et al., 1976 Lamb et al., 1981
Transferrin secretion	FSH, testosterone, retinol, insulin, MSA	Skinner and Griswold, 1980, 1982, 1983

In early stages of follicular growth, FSH increased the number of FSH receptors on the granulosa cells. This effect was promoted by estradiol. At a later stage, FSH increased the number of LH receptors, and estradiol again promoted this FSH effect (Richards, 1979; Dorrington and Armstrong, 1979). *De novo* steroidogenesis by rat granulosa cells from preovulatory follicles was stimulated by FSH (Dorrington and Armstrong, 1979). FSH increased progesterone synthesis, and this FSH action was augmented by estradiol and testosterone (Dorrington and Armstrong, 1979).

Inhibin biosynthesis by granulosa cells is stimulated by FSH, thereby effectuating a feedback system (Bicsak et al., 1986; Zhiwen et al., 1987). FSH stimulated a number of other parameters in cultured rat granulosa cells, for example the release of plasminogen activator (Martinat and Combarnous, 1983), granulosa cell proliferation, DNA- and protein synthesis (Dorrington and Armstrong, 1979; Richards, 1979), and lactate production (Hillier et al., 1985). Furthermore, FSH induced marked morphological changes in cultured rat granulosa cells which involve microfilaments (Lawrence et al., 1979) (review: Hsueh et al., 1984).

2.2.4 Mechanism of FSH action

- Mechanisms of hormone action

In general, the initial interaction of a peptide hormone with its target cell is binding to its receptor at the cell surface. Several different systems have been described which can transduce the signal elicited by hormone binding to the cell interior. The systems involved in transmembrane signalling generally consist of a hormone-specific receptor, which is coupled via a transducer element to an effector. The effector leads to the production of a second messenger, which can exert different effects in the cell. The transducer elements or coupling proteins are guanine nucleotide regulatory proteins, named N- or G-proteins, which bind and hydrolyze GTP (Birnbaumer et al., 1985).

Adenylate cyclase is a common effector protein which catalyzes the formation of the second messenger cAMP. Receptors of the R_S type are linked via a stimulatory G_S-coupling factor to adenylate cyclase, and hormone action via R_S receptors will stimulate cAMP production. An experimental approach to identify the presence of G_S proteins involves the use of choleratoxin. Choleratoxin can catalyze the transfer of an ADP-ribosyl group from NAD to the G_S-protein (ADP-ribosylation), and this results in an increase of cAMP levels by activation of G_S and adenylate cyclase. R_i type receptors are linked via inhibitory G_i-coupling factors to adenylate cyclase, and hormone binding to R_i type receptors will result in decreased cAMP production (review: Birnbaumer et al., 1985).

The second messenger cAMP activates cAMP-dependent protein kinase A (PK-A). PK-A phosphorylates many proteins, and is thereby involved in multiple different cellular functions, such as cellular metabolism and protein synthesis. A general scheme for cAMP-regulating transducing systems is given in Figure 2.2.

In addition to cAMP, products of inositol phospholipid breakdown can act as intracellular second messengers. A number of agonists, including catecholamines (via α_1-adrenergic receptor), acetylcholine (via muscarinic cholinergic receptor), vasopressin and angiotensin are coupled via this system of phosphatidylinositide turnover to an increase of the intracellular calcium concentration and activation of protein kinase C (PK-C) (Berridge, 1984). The breakdown of inositol phospholipids is catalyzed by phospholipase C. The effect of hormone-receptor interaction on phospholipase C is mediated by an G_p-protein, which has not yet been characterized. Briefly, receptor-mediated phosphatidylinositide breakdown involves hydrolysis of phosphatidylinositol bisphosphate to inositol trisphosphate, which can increase the intracellular Ca^{2+} level, and to diacylglycerol, which activates PK-C and is a source of arachidonic acid. PK-C can also be activated directly by tumor-promoting phorbol esters (Nishizuka, 1984; Berridge, 1984). Using phorbol esters as a tool, it has been shown that PK-C can phosphorylate numerous proteins involved in many different cell functions. A general scheme of the phosphatidyl-

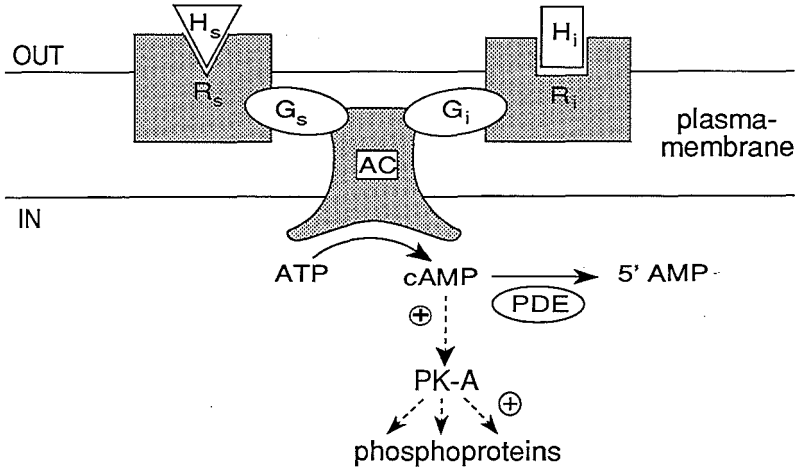

Figure 2.2. Schematic representation of the adenylate cyclase-coupled transducing systems. For explanation see text. AC: adenylate cyclase. G_s, G_i: stimulatory and inhibitory guanine nucleotide regulatory proteins. R_s, R_i: receptors coupled to G_s or G_i, respectively. H_s, H_i: hormones binding to R_s or R_i, respectively. PK-A: protein kinase A. PDE: cAMP-phosphodiesterase.

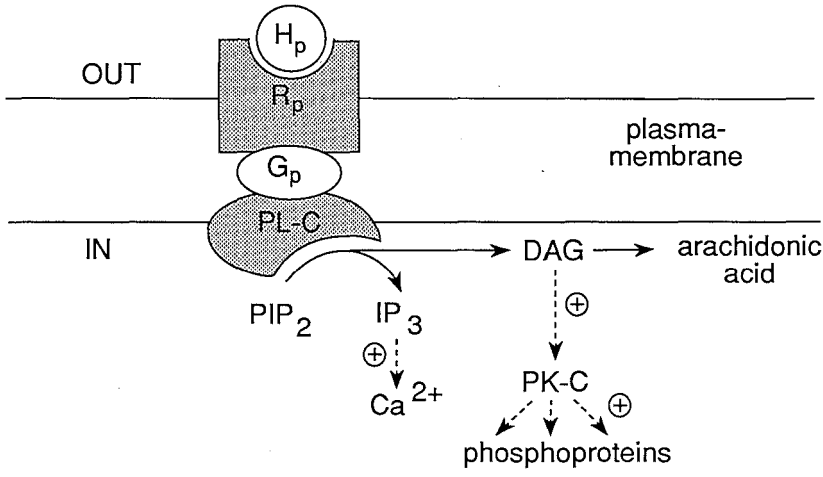

Figure 2.3. Schematic representation of the transducing system involving phosphatidylinositide breakdown. For explanation see text. H_p: hormone binding to R_p. R_p: receptor presumably coupled via G_p to PL-C. G_p: putative guanine nucleotide regulatory protein. PL-C: phospholipase C. PIP_2: phosphatidylinositol bisphosphate. IP_3: inositol trisphosphate. DAG: diacylglycerol. PK-C: protein kinase C.

inositol system is given in Figure 2.3.

Possible connections exist between the two systems of adenylate cyclase/cAMP and phospholipase C/phosphatidyl-inosi-tides, respectively. The protein kinases PK-A and PK-C may phosphorylate in part the same proteins. PK-C has been reported to phosphorylate the β-adrenergic receptor (Sibley et al., 1987) and may also act on G-proteins. It appears therefore that the two second messenger systems might be mutually interactive.

- Mechanism of FSH action

After binding of FSH to its receptors a series of biochemical events is triggered. Adenylate cyclase is activated, which results in increased cAMP production and stimulation of cAMP-dependent PK-A (Means et al., 1976; Hansson et al., 1983).

FSH elicits an elevation of cAMP concentrations in Sertoli cell cultures (Dorrington et al., 1975; Heindel et al., 1975; Oonk et al., 1985). As it appeared that cholera toxin also increased cAMP levels and mimicked FSH actions (Fritz et al., 1978), it can be concluded that FSH acts through an R_S receptor (see above). Receptors for glucagon and β-adrenergic agents (Eikvar et al., 1985; Heindel et al., 1981) are also of the R_S type, but adenosine receptors (Monaco and Conti, 1986) are R_i receptors. Both types of receptors (R_S and R_i) can be present in the same cell type, as in this case the Sertoli cells.

In concordance with the effect of FSH on cAMP production, cAMP analogs have been shown to elicit similar or even stronger stimulatory effects as FSH. This has been observed for the effect of dbcAMP on ABP formation (Fritz et al., 1976), estradiol secretion (Rommerts et al., 1978) and lactate production (Mita et al., 1982; Jutte et al., 1983; Oonk et al., 1985). Apart from stimulatory effects on cAMP and PK-A, FSH probably causes feedback inhibition of the activity of PK-A via stimulation of the activity of an inhibitor of protein kinase A (Tash et al., 1981).

- Desensitization of hormone responses

Desensitization or refractoriness to a hormone means that the cellular response to a hormone diminishes or ceases in course of time, despite the continuous presence of the hormone. Hormone-induced refractoriness implies that the subsequent response to a new stimulus with the same hormone is absent or attenuated. This is referred to as homologous desensitization. When exposure of a cell to a hormone induces attenuation of the response to another hormone operating through a different receptor, this is referred to as heterologous desensitization. The mechanisms involved in desensitization have been, and still are, extensively investigated.

Prolonged exposure of a cell to a hormone often induces hormone-mediated down-regulation of the receptor for this hormone, which is one mechanism of desensitization. In this process, the number of hormone receptors at the cell surface is diminished by internalization of the hormone-receptor complex. Subsequently, either the hormone-receptor complex is degraded, or the receptors are functionally regenerated within an intracellular compartment. Hormone removal from the cells induces termination of the refractory state. Hormone responsiveness is restored via synthesis of new receptor molecules, and/or via redistribution of receptors back to the plasma membrane. The exact mechanisms of receptor recycling are unknown (Kahn and Baird, 1978; Olefsky and Kao, 1982).

Inactivation of adenylate cyclase, leading to decreased cAMP levels, is another common mechanism of desensitization (Lefkowitz et al., 1980). Desensitization of adenylate cyclase may be effectuated via feedback regulation (Lefkowitz et al., 1980). Recently another mechanism leading to refractoriness has been reported (Sibley et al., 1987). When a hormone stimulates adenylate cyclase, cAMP levels rise, PK-A is activated and the receptor involved becomes phosphorylated and thereby refractory. This type of refractoriness has been demonstrated for the β-adrenergic receptor (Sibley et al., 1987).

-Desensitization of FSH responses

Desensitization of the responsiveness of Sertoli cells to FSH has been observed, which was related to down-regulation of the number of FSH binding sites (O'Shaugnessy, 1980). This loss of re-

ceptors is caused by the internalization and degradation of hormone-receptor complexes after prolonged incubation in the presence of FSH (Saez and Jaillard, 1986). Involvement of other mechanisms of desensitization also has been demonstrated in the following experiments. In immature rats, FSH was injected *in vivo* and 12-24 h later the effects of FSH *in vitro* on cAMP production by and on phosphodiesterase activity in testis tubular membranes were determined. It was observed that the effect of FSH *in vitro* on cAMP production was strongly reduced (O'Shaugnessy, 1980; Jahnsen et al., 1980), and that the *in vivo* FSH treatment had increased the activity of phosphodiesterase, the enzyme that catalyzes the breakdown of cAMP (Conti et al., 1983a). In cultured Sertoli cells from immature rats, similar effects were observed. After preincubation in the presence of FSH during 24 h, adenylate cyclase was desensitized to FSH, resulting in decreased cAMP levels in response to FSH stimulation (Verhoeven et al., 1980; Jahnsen et al., 1982). In these experiments, phosphodiesterase activity was increased by FSH (Conti et al., 1981; Verhoeven et al., 1981). It can be concluded that refractoriness of Sertoli cells to FSH is a result of decreased adenylate cyclase activity and an increased cAMP catabolism by phosphodiesterase (Conti et al., 1983b), in addition to the FSH-induced down-regulation of the number of FSH receptors. Whether the mechanism of receptor phosphorylation via PK-A activation (Sibley et al., 1987) might also apply to the desensitization mechanisms induced by FSH is unknown.

Besides cAMP, calcium ions might play a role as second messenger in the mechanism of action of FSH. Ca^{2+} release from intracellular stores can be promoted by inositol trisphosphate (see above), and Ca^{2+} influx into the cell from the external medium can be regulated via Ca^{2+} channels in the plasma membrane. The ubiquitous intracellular binding protein for Ca^{2+} is calmodulin. The presence of calmodulin in Sertoli cells has been demonstrated (Means et al., 1980; Welsh et al., 1980). Stimulation of ABP secretion could be effected by FSH, but also by EGTA, a Ca^{2+} chelator (Means et al., 1980; Welsh et al., 1980). Sertoli cell shape changes were induced by incubation of the cells in the presence of FSH or dbcAMP (Tung et al., 1975), and also during incubation in EGTA-containing medium in the absence of Ca^{2+} (Means et al., 1980; Welsh et al., 1980; appendix paper 5). These shape changes were reversible by removal of FSH or dbcAMP (Tung et al., 1975) or by addition of Ca^{2+} (Welsh et al., 1980; appendix paper 5). Microfilament proteins rather than microtubules are involved in the effects of FSH and Ca^{2+} on the shape of cultured Sertoli cells (Welsh et al., 1980). Furthermore, it has been reported that FSH and Ca^{2+} cause phosphorylation of vimentin, an intermediate filament protein (Spruill et al., 1983a, b). It must be noted, however, that these effects are only correlations and that no definitive proof has demonstrated that FSH employs Ca^{2+} as a second messenger.

Effects of FSH on adenylate cyclase and cAMP-dependent phosphodiesterase (Knecht et al., 1983; Conti et al., 1984) and on cell morphology (Lawrence et al., 1979) have also been observed in granulosa cells.

In conclusion, the mechanism of action of FSH involves adenylate cyclase and cAMP-dependent phosphodiesterase. Ca^{2+}/calmodulin could take part in the regulation of microfilaments and the process of protein secretion. The possible involvement of the recently discovered phosphoinositide breakdown in FSH action is not known. The presence of PK-C in Sertoli cells was demonstrated by Galdieri et al. (1986). Furthermore, it has been reported by Monaco and Conti (1987) that phorbol esters, which activate PK-C, inhibited FSH-induced cAMP production. It would be of interest to show if this inhibition involves phosphorylation of the FSH receptor. As described above, such a mechanism was observed for the β-adrenergic receptor (Sibley et al., 1987). Further investigation of the interrelationships between these different types of signal transducing systems will be needed to elucidate the mechanisms by which FSH controls Sertoli cell function.

2.3 Insulin and the IGFs

2.3.1 Functional aspects of insulin and the IGFs

Insulin is a small peptide hormone of 5,8 kDa, produced by the β-cells of the islets of Langerhans. It is synthesized as a large preprohormone. The pre-part is a signal peptide which is cleaved from the rest of the molecule during transport into the endoplasmic reticulum. Proinsulin is processed into insulin by specific proteases, which remove a connecting peptide (C-peptide), and insulin is stored in secretion granules. The A and B chain of insulin are linked by two disulfide bridges. Insulin is secreted in a pulsatile way by exocytosis into the bloodstream. Together with glucagon, which is produced by the α-cells of the pancreatic islets, it controls metabolism in various tissues. Insulin and glucagon are involved in maintaining glucose homeostasis. Insulin is considered to be an anabolic hormone, in that it stimulates the synthesis of protein, lipid and glycogen, and inhibits degradation of these compounds. Glucagon counteracts the metabolic actions of insulin by increasing catabolic processes like glycogenolysis and lipolysis, and it acts mainly in liver. The key target tissues for insulin are liver, skeletal muscle and adipose tissue.

Insulin deficiency by a complete or almost complete failure to produce insulin leads to type I diabetes, a severe illness in which glucose homeostasis is greatly disturbed, leading to a multiple pathology of nerves and vascular tissue, and kidney failure by changed membrane permeability. Type II diabetes does not involve pancreatic failure, but resistance to insulin action. The altered sensitivity of the tissues to insulin in type II diabetes may be caused by a reduction either in number or in sensitivity of insulin receptors. In rare pathological conditions, insulin resistance is caused by the presence of autoantibodies against the insulin receptor (section 2.3.3). Experimental diabetes in animals, not treated with insulin, often leads to impaired spermatogenesis. The consequences of the experimental diabetes can be limited and delayed by treatment with insulin. However, in men with type I diabetes who were under insulin treatment testicular dysfunction and spermatogenic disruption may occur (Handelsman et al.,1985a; Cameron et al., 1985).

Insulin is present in a broad range of extrapancreatic tissues in rats and humans at higher concentrations than those in plasma, and it has been suggested that part of this insulin may be synthesized extrapancreatically by these tissues themselves. However, this is not yet supported by substantial evidence (Rosenzweig et al., 1980).

The history of the insulin-like growth factors started with the observation by Salmon and Daughaday (1957) that a factor in serum from normal or from growth hormone treated hypophysectomized rats increased ^{35}S-sulfate incorporation into cartilage explants and that growth hormone itself or in combination with serum from untreated hypophysectomized rats had no effect. This growth hormone-dependent factor was termed "sulfation factor". Later studies with preparations of growth hormone-dependent plasma factors also demonstrated insulin-like and mitogenic actions. Daughaday et al. (1972) therefore proposed the more general term "somatomedin". In clinical studies the effects of somatomedins as mediators of growth hormone action on sulfate incorporation into cartilage were soon confirmed (Hall et al., 1971; Van den Brande and van Buul, 1975). Two different somatomedin species were recognized after purification and characterization, using as bioassay the incorporation of sulfate or thymidine into cartilage, viz. somatomedin A, a neutral fraction, and somatomedin C, a basic fraction (review: Van Wyk and Underwood, 1978).

From the diabetes branch of research a biologically active insulin-like peptide(s) was characterized. Using human serum, a large discrepancy was observed between the bioassay (stimulation of glucose uptake by rat fat pads) estimating insulin-like activity, and the insulin radioimmunoassay which estimated immunoreactive insulin. The portion of serum insulin-like activity that could not be neutralized with excess antibodies to insulin was initially termed non-suppressible insulin-like activity (Zapf et al., 1978) and

became later known as the insulin-like growth factors, IGFs. The primary structure of the IGFs has been elucidated by Rinderknecht and Humbel (1978a, b). The molecular weight of IGF–I is 7649. IGF–II is somewhat smaller, having a MW of 7471. They exhibit a striking homology with proinsulin. However, the C-domain which is removed during processing of proinsulin to insulin is present in the IGFs. The amino acid sequences of the A- and B-domains show differences, and immunologically IGF–I, IGF–II and insulin are distinct proteins (Froesch and Zapf, 1985).

From cultured media from a rat liver cell line a peptide fraction with multiplication stimulating activity was isolated (Dulak and Temin, 1973) and characterized (Marquardt et al., 1981).

All these peptides described above shared common properties and seemed to belong to a "somatomedin family" (Van Wyk and Underwood, 1978). After the primary structure of the different peptides was elucidated, it appeared that somatomedin C is identical with IGF–I (Spencer et al., 1983), somatomedin A consists of mainly IGF–II and small amounts of IGF–I (Spencer et al., 1983), and multiplication stimulating activity is the rat equivalent of IGF–II (Marquardt et al., 1981).

The liver has been thought to be the primary site of IGF synthesis and it has been calculated that IGF–I production by the liver could account for the total rat serum IGF–I levels (Schwander et al., 1983). In fetal mice and adult rats IGF–I was demonstrated in liver, but also in a number of extrahepatic tissues (D'Ercole et al., 1980a, 1984). Growth hormone administration to hypophysectomized rats caused IGF–I levels to increase in all tissues (D'Ercole et al., 1984). Evidence from a number of studies now indicate that IGFs are synthesized in many mammalian tissues besides the liver (D'Ercole et al., 1986; Brown et al., 1986; Lund et al., 1986; Han et al., 1987; Murphy et al., 1987).

IGF–I serum levels in humans, measured by radioimmunoassay, are relatively low at birth and increase gradually from infancy to adolescence until adult levels are reached; during puberty a sharpe transient increase occurs (Bala et al., 1981; Luna et al., 1983). With older age IGF–I concentrations fall, probably as a result of decreasing pituitary growth hormone secretion (Furlanetto and Cara, 1986). IGF–II production is less dependent on growth hormone than IGF–I (Hall and Sara, 1984), and also is not much influenced by puberal stage (Luna et al., 1983). Its precise physiological role in adults is not understood. In general, IGF–II is postulated to have a major role in embryonal and fetal growth (Underwood and D'Ercole, 1984).

IGF–I and IGF–II in serum do not circulate freely but are bound to binding proteins of MW 150,000 in the adult and 40,000 in the fetus (D'Ercole et al., 1980b). These binding proteins may be necessary to temper the insulin-like activity and to prolong the half-life of IGFs. Other physiologically important effects of these proteins are not known (Froesch and Zapf, 1985).

In general, the synthesis of IGF–I in multiple tissues is regulated by the level of growth hormone, whereas IGF–II synthesis is not growth hormone dependent. In agromegaly and the pituitary type of dwarfism, the levels of growth hormone correspond with those of IGF–I. However, this correlation is not observed in certain other disease conditions. Under circumstances of malnutrition or diabetes the levels of IGF–I are very low and do not reflect growth hormone levels, but rather the nutritional state and the degree of metabolic control by insulin. Another discrepancy in IGF–I and growth hormone levels is seen in pygmies and Laron dwarfs who have normal to increased growth hormone levels, but very low IGF–I levels (review: Van Wyk and Underwood, 1978).

The somatomedin hypothesis, originally postulated by Salmon and Daughaday (1957), implies that the action of growth hormone is mediated by circulating peptides of the somatomedin family. A number of experiments support this notion. Treatment of hypopituitary Snell dwarf mice with a partially purified somatomedin preparation increased total body length and the weight of body and organs (Van Buul-Offers and Van den Brande, 1979). These results were confirmed using a biosynthetic IGF–I preparation identical to natural human IGF–I (Van

Buul-Offers et al., 1986). This confirmed and extended the observations of Schoenle et al. (1982), that in hypophysectomized rats purified IGF–I had the same potency as growth hormone to stimulate body weight and tibial epiphyseal width. A preliminary report indicated that also in intact rats without growth hormone deficiency administration of a biosynthetic IGF–I preparation increased body length and weight (Hizuka et al., 1986). Furthermore, in growth-arrested insulin-deficient diabetic rats, infusion of IGF–I, but not of growth hormone restored growth (Scheiwiller et al., 1986). One seemingly contradictory report was published, suggesting that the somatomedin hypothesis may be too restrictive. Recombinant human methionyl-IGF–I was tested in hypophysectomized rats, and it was concluded that IGF–I is a poor growth-promotor relative to growth hormone (Skottner et al., 1987). However, the small stimulatory effects of IGF–I observed by Skottner et al. (1987) were significant. Furthermore, evidence has been demonstrated that the action of growth hormone involved local production of IGF–I in cartilage (Schlechter et al., 1986). It can be concluded that IGF–I not only acts as a circulating growth hormone mediator, but that also local production of IGF–I is important for stimulation of growth of tissues.

2.3.2 Biological effects of insulin and the IGFs

The main actions of insulin are the regulation of the metabolism of glucose and other substrates, and their storage in liver, adipose tissue and skeletal muscle. IGFs have their major effects on the growth and differentiation of cells of mesodermal origin, and triggers the long-term anabolic reactions in these cells (cartilage, bone, muscle). Both insulin and IGFs can have a similar spectrum of activities. Typical insulin-like effects are stimulation of glucose uptake and utilization, amino acid transport, glycogen synthesis, inhibition of lipolysis and stimulation of a number of membrane-bound and cytosolic enzymes. These processes can be stimulated both by insulin and IGFs, although in general 10-100 times more IGF–I or IGF–II than insulin is required to elicit a similar response. Effects on protein and DNA-synthesis, and on replication and differentiation of cultured cells are preferentially stimulated by IGFs. Insulin shares the capacity to stimulate these processes, but is 50-100 times less potent than the IGFs in most systems. However, evidence has been reported that in a number of cell types, including hepatoma cells, the growth-stimulatory effect of insulin is mediated by binding of insulin to high-affinity insulin receptors (Koontz, 1984; review: Straus, 1984). In general, the IGFs have the strongest potency in growth-promoting effects, whereas insulin is more potent in metabolic effects, although insulin is required for normal growth in immature vertebrates (reviews: Kahn et al., 1981; Perdue, 1984; Straus, 1984; Froesch and Zapf, 1985).

While IGF–I and IGF–II have largely similar activities, their respective receptors are very different. In contrast, the receptors for insulin and IGF–I are very similar (section 2.3.3). The receptors for insulin, IGF–I and IGF–II are hormone-specific (section 2.3.3), but crossreactivity has been reported (Zapf and Froesch, 1978). Studies in adipocytes using anti-insulin receptor antibodies have suggested that the acute insulin-like effects of IGFs are mediated primarily through the insulin receptor and that the effects of insulin on DNA-synthesis are mediated by IGF–receptors (King et al., 1980; Kahn et al., 1981, 1984). Using monoclonal antibodies to the IGF–I receptor, evidence was obtained that in cultured fibroblasts insulin stimulated DNA-synthesis indeed through the IGF–I receptor (Van Wyk et al., 1985). Furthermore, the IGF–I receptor was shown to mediate the rapid effects of IGF–II on amino acid transport (Yu and Czech, 1984b).

It appears that due to the extensive crossreactivity of the ligands to these receptors it is difficult to assign particular biological reponses to a specific receptor type.

- Effects of insulin and IGFs on cultured Sertoli cells

The effects of insulin and IGFs on Sertoli cells in culture have been summarized in Table 2.2. For most effects low concentrations of IGF–I or IGF–II were required, versus high concentrations of insulin. However, the stimulation of lactate

production and glucose transport could be effectuated by equally low concentrations of insulin and IGF–I (Oonk et al., 1985; Oonk and Grootegoed, 1987a,b; appendix paper 5).

- Effects of insulin and IGF–I on cultured Leydig cells

Some Leydig cell functions are sensitive to insulin and IGF–I. Under conditions of experimental diabetes LH/hCG receptors in rat testes were reduced, and the administration of insulin restored the binding capacity for LH to normal values (Charreau et al., 1978). Recent observations indicated that insulin and IGF–I alone could stimulate Leydig cell steroidogenesis and the number of LH receptors, and that insulin/IGF–I and hCG had synergistic effects on these parameters. Furthermore, both insulin and IGF–I acted at nanomolar concentrations, indicating that the stimulatory effects of insulin and IGF–I are mediated via the insulin and IGF-I receptors, respectively (Lin et al., 1986; Bernier et al., 1986; Chatelain et al., 1986; Benahmed et al., 1987).

- Effects of insulin and IGF–I on cultured granulosa cells

It has been reported that insulin and IGF–I can augment FSH- and hCG-induced cAMP production, and can enhance FSH-stimulated estrogen production in cultured granulosa cells from rats and pigs (Adashi et al., 1985; Davoren et al., 1985; Veldhuis et al., 1985a). Recently, it was demonstrated that IGF–I and insulin increased basal and FSH-stimulated production of inhibin in rat (Bicsak et al., 1986; Zhiwen et al., 1987).

Table 2.2. Effects of insulin and IGF–I on Sertoli cells.

Activity	Stimulated by	References
Androgen binding protein production	Insulin	Karl and Griswold, 1980
Transferrin production	Insulin	Skinner and Griswold, 1982
	IGF–II	Skinner and Griswold, 1983
DNA- and protein synthesis	Insulin; IGF–I; IGF–II	Borland et al., 1984
Glucose transport	Insulin; IGF–I	Mita et al., 1985
	Insulin; IGF–I	appendix paper 5
Lactate production	Insulin	Jutte et al., 1983
	Insulin	Oonk et al., 1985
	Insulin; IGF–I; IGF–II	Borland et al., 1984
	Insulin; IGF–I	appendix paper 5

A difference between Sertoli cells and granulosa cells is found in the steroidogenic capacity and the presence of LH receptors in the latter cell type. These granulosa cell parameters could also be stimulated by insulin and IGF–I, and insulin/IGF–I acted synergistically with FSH, cAMP, hCG and estradiol on granulosa cells from rats and pigs (Adashi et al., 1985; Davoren et al., 1985; Veldhuis et al., 1983, 1985a; Baranao and Hammond, 1984).

In general, the actions of insulin could be attained with 10-100 fold higher concentrations than of IGF–I, indicating insulin action via IGF–I receptors. These *in vitro* findings suggest a modulating role of insulin and IGF–I in gonadotropin actions on granulosa cells (review: Adashi et al., 1985).

2.3.3 Receptors for insulin and the IGFs

Much research has been performed on the insulin binding characteristics, and the insulin receptor structure. Recently, also the structure and some properties of IGF–I and IGF–II receptors have been elucidated. This information will be given where appropriate, so that comparisons with the insulin receptor can be made.

Before the insulin receptor was cloned, it was only defined by its binding characteristics. The binding assays were performed with biologically active mono-iodinated insulin (Freychet et al., 1971). In the early seventies, insulin receptors have been demonstrated in a vast number of tissues in most vertebrate species (Posner et al., 1974) and in many cultured cell types (Gavin et al., 1973). Binding characteristics, as described by De Meyts (1976) include high affinity for insulin, rapid and reversible binding, and saturability. Most important, however, is specificity, to be determined by competition for maximal hormone binding by different ligands. The binding kinetics for the binding of insulin to its receptor are complex. This is reflected by Scatchard plots, which are typically curvilinear, indicating negative cooperativity between binding sites and/or heterogeneity of binding sites (De Meyts et al., 1973). The Scatchard plots for the binding of IGF–I and IGF–II to their respective receptors are linear, indicating a single class of non-interacting binding sites for both receptor types (De Meyts, 1976; Kahn et al., 1981).

A complication in performing binding studies is the occurrence of degradation of receptor-bound insulin (Gammeltoft and Gliemann, 1973) and a decrease in receptor concentration (Gavin et al., 1974), which are caused by internalization and subsequent degradation of the insulin-receptor complex (Kahn and Baird, 1978; Olefsky and Kao, 1982; Knutson et al., 1983). These complications arise particularly using whole cells, incubations at higher temperatures, and extended time courses. Receptor inactivation is inhibited by cycloheximide, resulting in an accumulation of insulin receptors at the cell surface (Knutson et al., 1983; Kadle et al., 1983). Besides degradation of the insulin-receptor complex upon binding, insulin receptors can be recycled to the plasma membrane after internalization (review: Houslay, 1985). It is not known if recycling occurs upon IGF–I binding to IGF–I receptors. The regulation of IGF–II receptors will be discussed below.

To elucidate the structure and functional properties of insulin receptors several approaches have been used. By binding to lectins and by biosynthetic labelling with radioactive sugars it has been demonstrated that the insulin receptor is a glycoprotein (Hedo et al., 1981a, b). The methods employed for receptor characterization and purification include affinity chromato-graphy using agarose linked insulin. More recently, photoaffinity labelling was applied using photoreactive insulin or the chemical crosslinker disuccinimidyl sube-rate to crosslink insulin to the receptor (review: Czech et al., 1984). In all of these studies insulin appeared to bind to a 125-135 kDa protein, which was termed the α-subunit. The subunit structure of the insulin receptor was determined using electrophoresis under reducing and non-reducing conditions (Pilch and Czech, 1979; Massague et al., 1980). Under reducing conditions a band of 135 kDa was observed, and a little labelling in the 90-95 kDa region. Under non-reducing conditions, the receptor migrated as a 300-350 kDa complex on gels, indicating that the receptor subunits are linked by disulfide bonds. From these studies it appeared that the insulin receptor is a heterotetramer,

composed of two insulin-binding α-subunits of 125-135 kDa and two smaller β-subunits of 90-95 kDa.

With the affinity crosslinking technique also the structure of the insulin-like growth factor receptors was studied. The structure of the IGF–I receptor appears to be very similar to that of the insulin receptor, whereas the IGF–II receptor is a completely different, single-chain polypeptide with a relative molecular mass of 220-260 kDa (Kasuga et al., 1981; Massague and Czech, 1982).

The discovery that proteins were phosphorylated on tyrosine (rather than on serine or threonine) by protein-tyrosine kinases (Eckhart et al., 1979) started investigations leading to the identification of a number of protein-tyrosine kinases which could be activated by growth factors. An example is the phosphorylation of tyrosine residues by the EGF-activated protein kinase, which later appeared to be the EGF receptor itself (Ushiro and Cohen, 1980). Later it was discovered that the insulin receptor and growth factor receptors, viz. platelet-derived growth factor, EGF and IGF–I, display intrinsic protein-tyrosine kinase activity, and that phosphorylation on tyrosine residues of the receptor protein occurs upon binding of the growth factor (review: Hunter and Cooper, 1985). The involvement of phosphorylation reactions with the binding of insulin to its receptor has been extensively studied. Kasuga et al. (1982a, b) were able to demonstrate that insulin stimulated ^{32}P-incorporation into the β-subunit of the insulin receptor, and that besides serine residues also tyrosine residues were phosphorylated. Subsequently, in a cell-free system it was observed that insulin stimulated ^{32}P-incorporation from γ-^{32}P-labelled ATP into the β-subunit of the receptor. This phosphorylation occurred exclusively on tyrosine residues (Kasuga et al., 1982c). Insulin not only stimulated tyrosine-phosphorylation of its own receptor, but also insulin binding resulted in an increased receptor-associated protein-tyrosine kinase activity towards other substrates *in vitro*, for example histones and caseins (Yu and Czech, 1984a). These results demonstrated that the β-subunit of the insulin receptor contains intrinsic protein-tyrosine kinase activity.

Similar observations have been reported for the IGF–I receptor, which appears to show not only structurally but also functionally extensive homology with the insulin receptor (Yu et al., 1986). In contrast, the IGF–II receptor does not contain protein-tyrosine kinase activity. Insulin appeared to effectuate a redistribution of the number of IGF–II receptors from a large intracellular pool to the plasma membrane (Oppenheimer et al., 1983; Oka et al., 1984). This latter effect of insulin resembles the insulin-induced membrane redistribution of the glucose transporter (Cushman and Wardzala, 1980; Suzuki and Kono, 1980) and the insulin-induced redistribution of transferrin receptors (Davis et al., 1986). It is not clear what the physiological role of the IGF–II receptor is. King et al. (1980) and Yu and Czech (1984b) showed that high concentrations of IGF–II mediated rapid metabolic effects through the insulin and the IGF–I receptor, respectively. It might be that DNA-synthesis, for example in fetal tissues, is regulated by low concentrations IGF–II via IGF–II receptors. In summary, in Table 2.3 some aspects of the receptors for insulin and the IGFs are compared, and Figure 2.4 shows schematically the different receptor structures.

Recently, the cloning of the insulin receptor gene has been performed (Ullrich et al., 1985). The single gene structure codes for a 1370 amino acid sequence of the receptor precursor. The data indicate that both receptor subunits are encoded by a single mRNA. The β-subunit contains a small transmembrane section and the α-subunit appears to be entirely extracellular, held in place by disulfide bonds to the extracellular domain of the β-subunit. The intracellular domain of the β-subunit contains a number of tyrosine and serine residues, which can be phosphorylated.

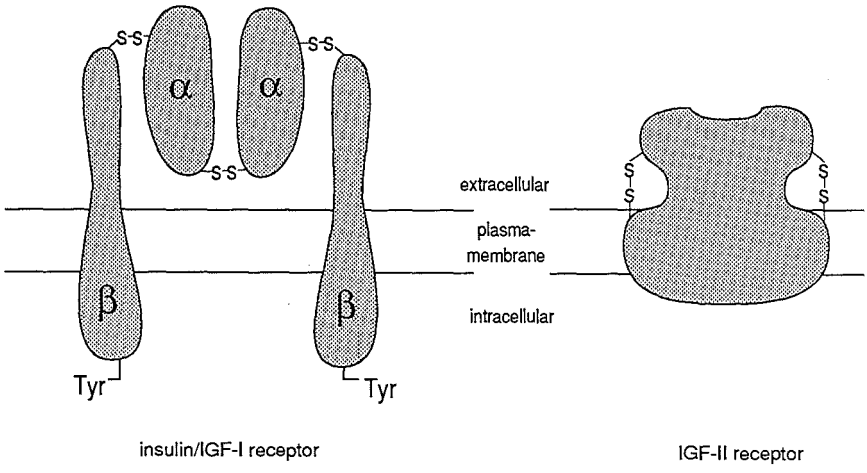

Figure 2.4. Schematic structure of the receptors for insulin and IGF–I, and for IGF–II. Tyr denotes protein-tyrosine kinase activity, when phosphorylated. For full details see text.

Table 2.3. Comparison of the receptors for insulin, IGF–I and IGF–II.

	Insulin	IGF–I	IGF–II
Relative binding potency	Insulin>IGF–I>IGF–II	IGF–I>IGF–II≥Insulin	IGF–II>IGF–I
M_r (non-reduced)	>300,000	>300,000	220,000
M_r (reduced)	130,000 (α)	130,000 (α)	260,000
	95,000 (β)	95,000 (β)	
Down-regulation by ligand	yes	yes	no
β-subunit-phosphorylation	yes	yes	no
Tyrosine kinase activity	yes	yes	no

Sequence homology of the cytosolic domain of the β-subunit appeared to exist to the cytosolic domain of the epidermal growth factor receptor and to the *src* family of oncogene products, all protein-tyrosine kinases (Hunter and Cooper, 1985). The possible importance of the receptor protein kinase activity in the mechanism of insulin action will be discussed in the next section.

- Receptors for insulin and IGFs on Sertoli cells

With the affinity crosslinking technique, specific binding of IGF–I and IGF–II to cultured Sertoli cells from immature rats has been demonstrated, whereas under the same experimental conditions insulin binding was virtually undetectable (Borland et al., 1984). A recent report mentioned that specific IGF–I binding, but no significant insulin binding to pig Sertoli cells could be demonstrated (Perrard-Sapori et al., 1987). In our experiments, the insulin binding to rat Sertoli cells has been identified and charac-

Sertoli cells has been identified and characterized (Oonk and Grootegoed, 1987a) and the IGF–I binding to rat Sertoli cells has been more extensively characterized and quantitated (Oonk and Grootegoed, 1987b).

- Receptors for insulin and IGF–I on Leydig cells

Binding studies with isolated Leydig cells from rats and pigs revealed specific receptors for IGF–I (Handelsman et al., 1985b; Lin et al., 1986; Chatelain et al., 1986) and for insulin (Lin et al., 1986). The number of IGF–I receptors on cultured porcine Leydig cells was reported to increase by incubation in the presence of hCG (Chatelain et al., 1986)

- Receptors for insulin and IGF–I on granulosa cells

Specific binding of insulin to insulin receptors on granulosa cells has been reported (Otani et al., 1985). Receptors for IGF–I could also be demonstrated (Baranao and Hammond, 1984; Veldhuis et al., 1985b). Treatment of rat granulosa cells with FSH augmented the number of IGF–I receptors (Adashi et al., 1986). Thus, both in Leydig cells (see above) and in granulosa cells gonadotropins can effect the IGF–I receptor number. The effect of FSH on the IGF–I receptor number on Sertoli cells is not known.

2.3.4 Mechanisms of insulin action

In the previous section we described that the insulin receptor contains intrinsic protein-tyrosine kinase activity. What remains to be ascertained are the functional consequences of the protein-tyrosine kinase activity and the identification of the key substrate proteins. Several protein species, e.g. histones and caseins, have been shown to be phosphorylated by the insulin-stimulated protein-tyrosine kinase activity of the receptor, but these proteins are not the physiological target of insulin action. Simpson and Hedo (1984) demonstrated that a polyclonal antiserum against the insulin receptor mimicked the effect of insulin on glucose transport but did not cause any alteration in the phosphorylation of the receptor β-subunit. These data might indicate that insulin receptor phosphorylation is not involved directly in mediating acute insulin action. However, in this respect the effect of vanadate ions should be mentioned (Shechter and Karlish, 1980). Vanadate mimics insulin stimulation of glucose uptake and glucose oxidation(Shechter and Karlish, 1980; Clark et al., 1985). Vanadate stimulates phosphorylation of the insulin receptor on tyrosine residues (Clark et al., 1985), which can be caused by the inhibiting effects of vanadate on a phosphatase, involved in the dephosphorylation of phosphorylated tyrosine residues (Shechter and Karlish, 1980). However, vanadate activated tyrosine phosphorylation also in purified insulin receptor preparations (Tamura et al., 1984), and it was concluded that this effect could not be explained by phosphatase inhibition. Recently, Tracey and Gresser (1986) reported that vanadate rapidly esterifies the phenol side chain of tyrosine, thereby possibly activating the protein-tyrosine kinase activity of the insulin receptor. The studies with vanadate suggest that protein-tyrosine kinase activity can be involved in glucose uptake and metabolism (Clark et al., 1985). Furthermore, considering the homology with a number of growth factor receptors which exhibit protein-tyrosine kinase activity, receptor protein-tyrosine kinase activity may intrinsically be associated with growth-promoting actions in the cell.

Farese et al. (1985) have demonstrated that phorbol esters can mimic some of the rapid metabolic actions of insulin, but antagonize the insulin stimulation of these processes. Furthermore, insulin increased diacylglycerol levels (Farese et al., 1985). PK-C, which is activated by diacylglycerol and phorbol esters, was recently observed to phosphorylate the insulin receptor directly on serine residues, thereby reducing its protein-tyrosine kinase activity (Bollag et al., 1986). It might be that some insulin stimulated cellular processes are controlled by changes in phospholipids (see below) and/or direct modification of insulin receptor phosphorylation by PK-C.

As it became clear that none of the until then established intracellular mediators (cAMP, cGMP and Ca^{2+}) could be directly involved as second messengers of insulin action, a search was initiated for a novel

mediator. Larner (1984) was the first to provide evidence that such a compound might exist. However, for many years these investigations were hampered by experimental problems and so far no group has been able to purify this substance completely and to define its structure (Czech, 1984). A kind of mediator function might be performed by the insulin-stimulated hydrolysis of a novel inositol-glycolipid (Saltiel et al., 1986), resulting in the formation of diacylglycerol and two carbohydrate substances, with chemical properties alike those described by Larner (1984) for the putative mediator substance. Saltiel et al. (1986) suggested that insulin may trigger a phospholipase C activity, thereby generating the mediator compounds.

One of the best described and most pronounced effects of insulin is that the rate of glucose transport and metabolism in several cell types is increased. Cushman and Wardzala (1980) and Suzuki and Kono (1980) have observed insulin-stimulated translocation of intracellular glucose carriers to the plasma membrane. According to Houslay (1985) this process may be triggered by the clustering and subsequent internalization of insulin receptors, following binding of insulin to its receptor, rather than by the protein-tyrosine kinase activity of the receptor. Upon insertion of glucose transporter molecules into the plasma membrane, the intrinsic activity of the glucose carriers may be regulated by insulin (Simpson et al., 1983). According to Houslay (1985) this second step might be regulated by G_{ins}, a specific guanine nucleotide regulatory protein of 25 kDa, through which insulin might exert some of its effects by regulating the activity of both cAMP-dependent and cAMP-independent protein kinases.

The mechanism of insulin action gets even further complicated, as it seems that through a variety of routes insulin can regulate various metabolic processes in the cell. Recently, using an *in vitro* system of isolated insulin receptors and purified enzymes, Sale et al. (1987) demonstrated that phosphofructokinase and a number of other glycolytic enzymes were phosphorylated on tyrosine residues by the insulin receptor kinase. This indicates that substrates with physiological functions may be targets for the insulin receptor kinase, although it remains to be demonstrated if enzyme phosphorylation on tyrosine residues also occurs *in vivo* and if enzyme activities can be regulated by this process.

In conclusion, the available evidence concerning the molecular mechanism of insulin action suggests that the situation is very complex. The pleiotypic nature of the insulin response suggests that insulin might exert its action via a so-called "multi-pathway" mechanism (Houslay, 1985).

2.4 Gonadal factors involved in spermatogenesis

2.4.1 Testosterone

Testosterone is a steroid hormone, synthesized from the steroidogenic precursor cholesterol. Cholesterol is intramitochondrially converted to pregnenolone by the cholesterol-side-chain-cleavage enzyme (C_{20-22}-lyase). Pregnenolone is converted via the multienzyme steroidogenic pathway into testosterone (Setchell, 1978). Testosterone is produced in testicular Leydig cells and in ovarian theca cells.

The general mechanism of steroid action involves diffusion of the steroid through the plasma membrane and the cytosol to the nucleus of the target cell. In the nucleus the steroid hormone binds to its receptor, thereby inducing a conformational change in the receptor structure to a receptor form that interacts with certain parts of the DNA. This leads to changes in the synthesis of specific mRNAs and proteins involved in the regulation of cell functions.

The action of testosterone on the seminiferous tubules may, in addition to Sertoli cells, involve the peritubular myoid cells, as both cell types contain androgen receptors and are sensitive to testosterone (Fritz, 1978; Skinner and Fritz, 1985a, b).

In section 1.1 and 2.1 the involvement of testosterone in male sexual development and spermatogenesis has been briefly discussed. Testosterone in the fetal rat appeared to control the development of the male testicular system from the Wolffian ducts and the differentiation of the urogenital system into male external genitalia. Testosterone has no effect on the regression of the Müllerian

ducts, which is effectuated by AMH.

Research conducted on the action of testosterone on spermatogenesis in the rat after hypophysectomy (reviews: Stein-berger, 1971; Fritz, 1978) has led to the suggestion that testosterone is mainly involved in maintenance of spermatogenesis. Testosterone could prevent regression of the seminiferous tubules which occurs after hypophysectomy of adult rats and support qualitatively normal spermatogenesis. FSH may be necessary to initiate spermatogenesis and to supplement quantitatively the effect of testosterone (Setchell, 1978).

In hypophysectomized adult monkeys testosterone alone could maintain the complete process of spermatogenesis, but as proliferation of type B spermatogonia appeared to be impaired in the absence of FSH, this maintenance was not quantitative (Marshall et al., 1986). In Macaque monkeys, testosterone alone was able to initiate precocious puberty concomitant with qualitatively normal spermatogenesis (Marshall et al., 1984). Matsumoto et al. (1986) reported that in normal men with selective FSH deficiency induced by hCG administration a quantitative reduction in sperm production was observed, although testosterone levels were normal or supranormal. FSH, but not testosterone replacement stimulated sperm production nearly back to control levels. Thus, the conclusions for the functional importance of testosterone and FSH in rats also seem to apply to primates and humans.

2.4.2 Inhibin

Inhibin is a glycoprotein hormone, which is secreted by the gonads. Inhibin is produced in the Sertoli cells of the testis and the granulosa cells of the ovary (De Jong, 1979). The main action of inhibin may be to regulate the peripheral concentrations of FSH, viz. to inhibit FSH secretion by the pituitary gland. FSH in turn increased inhibin secretion by cultured Sertoli cells and granulosa cells (Ultee-van Gessel et al., 1986; Ultee-van Gessel and De Jong, 1987; Bicsak et al., 1986, 1987). The secretion of inhibin by cultured Sertoli cells was age-dependent and decreased with the age of the animal (Ultee-van Gessel and De Jong, 1987). The possible physiological role of inhibin has been discussed in section 2.1. After many years of laborious investigations, during which the existence of this gonadal factor was often disputed, inhibin has been purified, among others from bovine follicular fluid (Robertson et al., 1985, 1986; Fukuda et al., 1986). The amino acid sequences of bovine, porcine and human inhibins recently have been elucidated by cloning and sequence analysis of cDNAs (Mason et al., 1985; Forage et al., 1986).

For bovine follicular fluid a relative molecular mass of 31-56 kDa (Robertson et al., 1985, 1986) and 65-68 kDa (Van Dijk et al., 1984, 1986) were reported. For human and porcine follicular fluids, inhibin was reported to have a relative molecular mass of 32 kDa (Mason et al., 1985). Inhibin appears to be composed of an α-subunit of 18 kDa and a β (β_A or β_B)-subunit of 14 kDa. At the N-terminal end of the α-subunit a pro-domain may be present, resulting in the different MWs observed. In inhibin α-β_A and α-β_B the α- and β-subunits are crosslinked by disulfide bridges. Sequence homology of the β-subunits of inhibin with TGF-β and the C-terminal part of AMH has been reported (Mason et al., 1985; Cate et al., 1986).

In cultured granulosa cells, stimulation of aromatase activity by FSH was counteracted by inhibin, whereas FSH-stimulated aromatase activity was further increased by very low concentrations of TGF-β. The actions of inhibin and TGF-β were antagonistic, suggesting opposite roles for inhibin and TGF-β in modulating the hormonal regulation of granulosa cell function (Ying et al., 1986b).

2.4.3 Activin

Recently, it has been described that heterodimers or homodimers of the two forms of the β-subunit of inhibin were co-purified with (α-β) inhibin. Both the β_A-β_A homodimer (Vale et al., 1986; Ling et al.,

1986a) and the β_A-β_B heterodimer (Ling et al., 1986b) stimulated the secretion of FSH by pituitary cells, and thus have opposite effects compared to inhibin. TGF-β (Ying et al., 1986a) has also been reported to stimulate FSH secretion by cultured pituitary cells. The β-β dimers have been termed activin, as opposed to inhibin (Ling et al., 1986a), and FSH-releasing protein (FRP) (Vale et al., 1986). The latter abbreviation is confusing in view of a granulosa cell protein referred to as follicle-regulatory protein (FRP) which has been purified to homogeneity (Ono et al., 1986). It would be of interest to study the possible physiological role of activin, especially in relation to possible paracrine gonadal effects of inhibin and TGF-β (see above).

2.4.4 Anti-Müllerian hormone

Anti-Müllerian hormone (AMH), also termed Müllerian inhibiting substance, is a glycoprotein, present from early fetal life in testicular tissue. Its functional importance in causing the regression of the Müllerian ducts has been discussed in section 2.1. The action of AMH is necessary for complete virilization, which cannot be completed by androgens alone.

The secretion of AMH by immature, cultured Sertoli cells seemed to be independent of FSH or testosterone modulation (Vigier et al., 1985). Recently, AMH was also detected in follicles, where it might function as an oocyte maturation inhibitor, being produced by mature granulosa cells (Takahashi et al., 1986). It has been shown, that AMH is a disulfide-bond linked homodimer of 140 kDa, under reducing conditions observable as a single 72 kDa protein (Josso et al., 1980). Recently the genes for bovine and human AMH have been isolated and the amino acid sequence deduced (Cate et al., 1986). AMH contains a highly conserved C-terminal domain that shows sequence homology with the β-chain of inhibin (section 2.4.1) and TGF-β (section 2.4.4).

2.4.5 Pro-opiomelanocortin and its derivatives

Pro-opiomelanocortin (POMC) is a precursor protein, initially detected in the pituitary gland. The POMC molecule contains the sequences for ACTH, α- and β-melanocyte stimulating hormone, β-endorphin and enkephalin. The POMC-derived peptides have also been detected in a variety of non-pituitary tissues. In the testis and the ovary immunoreactive POMC derivatives have been observed, and, recently, the presence of POMC mRNA in the testis (Chen et al., 1984) and the ovary (Chen et al., 1986) has been reported. In the testis, POMC is synthesized in Leydig cells (Chen et al., 1984) and the precursor protein may be processed into the component proteins within the testis. Boitani et al. (1986) showed that α-MSH stimulated the aromatase activity and cAMP production by Sertoli cells. Furthermore, β-endorphin, which is also produced in the testis, inhibited the FSH-induced Sertoli cell proliferation in fetal and neonatal rat testes (Orth, 1986; Boitani et al., 1985).

These findings imply that endogenous testicular POMC-derived peptides may modify Sertoli cell functions via peptide receptors on Sertoli cells. This could involve a paracrine interaction between Leydig cells and Sertoli cells.

2.4.6 Growth factors, produced by gonadal cells

Sertoli cell-specific growth factors, possibly distinct from other well-characterized growth factors, have been demonstrated in Sertoli cell culture medium (Table 2.4). A mitogenic peptide, termed seminiferous growth factor (SGF) was detected in prepuberal Sertoli cells and in tubules from newborn and adult mice, and subsequently partially purified from tubules from 2-4-week-old calves (Feig et al., 1980; Bellvé and Feig, 1984). The level of SGF was highest in tubules from newborn mice. The *in vitro* proliferation of prepuberal mouse Sertoli cells was promoted in a considerably stronger way by partially purified SGF than by FSH. Bellvé and Feig (1984) suggested a role for SGF in promoting testicular development.

Another interesting Sertoli cell-specific factor with mitogenic properties has been demonstrated, viz. rat Sertoli cell-secreted growth factor (SCSGF) (Lamb et al., 1986; Holmes et al., 1986). In contrast with SGF, it appeared that SCSGF secretion was higher at puberal than at prepuberal age (Lamb et al., 1986). Furthermore, the secretion of the factor by Sertoli cells was increased by FSH, insulin, testosterone and retinol. Although SCSGF competed with EGF binding to the EGF receptor, it was claimed that SCSGF is distinct from EGF, TGF-α, IGF–I and SGF. However, of all non-testicular growth factors, TGF-α was most similar to SCSGF (Holmes et al., 1986).

In rat testis, IGF–I immunoreactivity has been detected (D'Ercole et al., 1984; Handelsman et al., 1985b). The testicular content of extractable IGF–I was increased by growth hormone treatment of hypophysectomized rats (D'Ercole et al., 1984). Rat and pig Sertoli cells in culture secrete an IGF–I-like compound (Hall et al., 1983; Ritzén, 1983; Tres et al., 1986; Chatelain et al., 1986; Benahmed et al., 1987). The production of this IGF–I-like activity was increased by FSH and growth hormone (although only in the presence of the phosphodiesterase inhibitor MIX), and also by dbcAMP (Tres et al., 1986). The partial purification of an 8,000 Da peptide, exhibiting IGF–I-like activity, was reported (Smith et al., 1987). This IGF–I-peptide could be demonstrated after acid hydrolysis of a 25,000 Da peptide, which might represent a complex of the IGF–I-like factor with a binding protein (Smith et al., 1987).

In cultured pig granulosa cells also production of IGF–I-like activity was demonstrated (Hammond et al., 1985) and the IGF–I-like factor secretion was observed to be modulated by gonadotropins, estradiol and cAMP (Hsu and Hammond, 1987). IGF–I-like factor was also detected in rat ovary, and the ovarian content was increased by growth hormone (Davoren and Hsueh, 1986). At present it is not known, whether granulosa cell-specific mitotic factors exist.

2.5 Other growth factors

2.5.1 Transforming growth factors

Transforming growth factors elicit reversible uncontrolled growth of normal, non-neoplastic cells. TGFs can be defined by their ability to induce anchorage-independent growth (colony formation in soft agar) when added to cells normally requiring a solid substratum for proliferation, and to abolish contact inhibition. Two very different molecules have been identified which accomodate this definition.

- Transforming growth factor α

TGF-α is a single polypeptide of 5,600 Da, first isolated from murine sarcoma virus-transformed cell culture medium. Although TGF-α exhibits limited sequence homology with EGF (section 2.4.5),

Table 2.4. Comparison of the growth factors secreted by Sertoli cells

Factor	Molecular mass (Da)	Production affected by	References
Seminiferous growth factor	15,700	not determined	Feig et al., 1980 Bellvé and Feig, 1984
Sertoli cell secreted growth factor	2,000 and 8,000	FSH, testosterone, insulin, retinol	Holmes et al., 1986 Lamb et al., 1986
IGF–I-like activity	8,000	dbcAMP, FSH+MIX, GH+MIX	Smith et al., 1987 Tres et al., 1986

TGF-α and EGF equipotently interact with the EGF receptor. TGF-α production appeared to be linked to cellular transformation. However, TGF-α was demonstrated also in human placenta and in rat embryos. TGF-α alone has only weak transforming activity and probably acts in combination with TGF-β (review: Goustin et al., 1986). Antagonistic actions of TGF-α and TGF-β on FSH-stimulated granulosa cell aromatase activity have been reported (Adashi and Resnick, 1986). TGF-α has similar attenuating effects as EGF on FSH-stimulated aromatase activity in granulosa cells (Adashi et al, 1987).

- Transforming growth factor-β

TGF-β is a disulfide-bond linked homodimer of 25 kDa, which is synthesized by many different cells. TGF-β acts via a specific receptor molecule (500-600 kDa), which appears to have no protein-tyrosine kinase activity, unlike other types of growth factor receptors. The TGF-β receptor is found in several fibro-blast cell lines and a number of tumor-derived cell lines (Massagué and Like, 1985).

TGF-β is mitogenic for a number of fibroblast cell lines. However, it was shown for many other cells that TGF-β can inhibit cell proliferation. In this respect, it has been demonstrated that TGF-β is a bifunctional regulator of cell growth (Roberts et al., 1985).

It was mentioned in section 2.4.2, that the TGF-β subunits show marked similarities with the β-chain of inhibin, and that TGF-β is an inhibin antagonist, similar to the effect of activin. Likewise, TGF-β and inhibin had opposite effects on FSH-stimulated aromatase activity by granulosa cells (Ying et al., 1986b). The synergistic effects of TGF-β and FSH on granulosa cell aromatase activity were confirmed by Adashi and Resnick (1986). TGF-β was observed to enhance the inhibitory actions of EGF on cultured granulosa cells and TGF-β seemed to regulate this synergistic effect by increasing the amount of EGF receptors on the cells (Feng et al., 1986). The inhibitory effects of EGF on granulosa cells will be discussed in section 2.5.2.

Bifunctional effects of TGF-β have been demonstrated also in granulosa cells. In the presence of low concentrations of FSH, TGF-β exerted synergistic effects on the induction of LH-receptors (Knecht et al., 1986, 1987). However, when higher concentrations of FSH were used, TGF-β antagonized the FSH-effect (Knecht et al., 1986, 1987). These results may indicate that the effects of TGF-β on cells are determined by the type of cells on which it acts and on the presence of other growth factor and hormones acting on the cells. It is not known if these *in vitro* observations on isolated cells apply to the *in vivo* situation.

2.5.2 Epidermal growth factor

EGF has been isolated from mouse submaxillary glands, which are a rich source of EGF. EGF is a 6,000 Da single polypeptide chain with sequence homology to TGF-α (section 2.5.1). Besides stimulatory effects on newborn epidermal tissues, EGF has the ability to stimulate the growth of cultured cells (Goustin et al., 1986). The receptor for EGF is a 170 kDa membrane protein. The extracellular domain can bind both EGF and TGF-α. The intracellular domain, coupled to the ligand binding domain via a transmembrane region, exhibits protein-tyrosine kinase activity (Ushiro and Cohen, 1980). In fact, the EGF receptor was the first growth factor receptor of which the intrinsic protein-tyrosine kinase activity was described.

For the ovary it has been reported that granulosa cells possess EGF receptors (St.Arnaud et al., 1983). It has been demonstrated that EGF inhibited the gonadotropin-induced stimulation of steroid production by cultured granulosa cells and by cultured Leydig cells (Hsueh et al., 1981). Inhibitory actions of EGF on FSH-induced cAMP production and LH receptor

synthesis could be reinforced by TGF-β. TGF-β acted by increasing the number of EGF receptors on the granulosa cells (Feng et al., 1986). From *in vitro* studies it appears that several growth factors may be able to modulate gonadotropin effects on cultured ovarian cells. The physiological meaning of these *in vitro* effects is not known.

With respect to EGF action in the testis not many details are known. In cultured Sertoli cells, EGF stimulated lactate production and inhibited FSH-stimulated estradiol secretion (Mallea et al., 1986). Sertoli cells secrete a growth factor (SCSGF; section 2.4.6) that shows some similarities with TGF-α, and this factor blocked EGF binding to its receptor (Holmes et al., 1986). EGF might play an important role in male reproduction. Recent experiments indicated that in mice the extirpation of the submaxillar glands resulted in a very low serum EGF concentration and in impairment of spermatogenesis. FSH and testosterone levels were normal, but the numbers of mature sperm and of spermatids were decreased. EGF administration restored all defects to normal. These results are consistent with some possible role for EGF in spermatogenesis in mice (Tsutsumi et al., 1986). However, if this mechanism plays a role also in other animals needs to be investigated.

2.6 Cell-cell interactions in the testis

2.6.1 Sertoli cell-germ cell interactions

The complex process of spermatogenesis must be executed in a rigidly organized way, to result in the regular production of a large number of spermatozoa. To this end, the spermatogonia undergo multiple mitotic divisions necessary for extensive proliferation of germ cell number, and subsequently enter the spermatocyte stage. Meiotic divisions give then rise to the spermatids, and spermiogenesis leads to the development of the spermatozoa. Meanwhile, the differentiating germ cells undergo controlled movement from the basement membrane towards the lumen of the seminiferous tubule, where the spermatozoa are eventually released. Germ cells at different stages of development are associated in specific groups, and are intimately associated with Sertoli cells (Setchell, 1978). Fourteen of these associations of spermatogenesis (I-XIV) have been defined in the rat, termed stages of the spermatogenic cycle (Leblond and Clermont, 1952).

Sertoli cells provide physical and nutritional support to the differentiating spermatogenic cells. Some aspects of the mutual influence of both cell types have been investigated over the past years. It has become clear that a number of Sertoli cell functions change quantitatively dependent on the stage of the spermatogenic cycle. The number of FSH receptors and the responsiveness to FSH, the production of cAMP, the activity of adenylate cyclase and of cAMP-phosphodiesterase seem to be regulated in a stage-dependent fashion, as has been reviewed by Parvinen (1982). This may also be the case for the production of androgen binding protein and especially for plasminogen activator. Plasminogen activator activity from Sertoli cells showed a distinct peak in stages VII and VIII of the spermatogenic cycle. At these stages spermatogonia enter the meiotic prophase. A role for plasminogen activator in the transfer of preleptotene spermatocytes through the tight junctions has been suggested (Lacroix et al., 1981; Parvinen, 1982). At the same stage spermiation takes place, and plasminogen activator might also play a role in the disconnection of the Sertoli cell-elongated spermatid contact (Lacroix et al., 1981; Parvinen, 1982). The particular complement of germ cells present at certain stages may determine some of the quantitative changes of Sertoli cell activities. This may be illustrated by the observation that the effect of FSH on ABP production could be enhanced by co-culture with germ cells (Le Magueresse and Jégou, 1986). Only pachytene spermatocytes increased ABP production, whereas round spermatids had no effect (Galdieri et al., 1984). It has been suggested that cell-cell contact was necessary for this effect. However, Le Magueresse and Jégou (1986) have demonstrated that also the spent media of germ cells had similar effects, viz. an increase in FSH-stimulated ABP

production and a decrease in FSH-induced estradiol production.

Whereas Sertoli cell activities may be influenced by the germ cells, the germ cells may require Sertoli cell proteins. Sertoli cells secrete transferrin (Wright et al., 1981; Skinner and Griswold, 1980), and transferrin binding sites were present on Sertoli cells and on spermatogenic cells (Holmes et al., 1983; Sylvester and Griswold, 1984). Active uptake of iron from transferrin by spermatogenic cells was demonstrated by Toebosch et al. (1987), and may represent an important interaction.

Furthermore, germ cells may require metabolic products secreted by the Sertoli cells, such as lactate (see section 5.1). Spermatogenic cells in co-culture with Sertoli cells display prolonged viability as compared to the isolated germ cells (Tres and Kierszenbaum, 1983). In the presence of Sertoli cells, the spermatogenic cells can be maintained in culture for some time in medium containing glucose without added pyruvate and lactate (Jutte et al., 1982). This indicates that lactate is supplied to germ cells via glucose metabolism by Sertoli cells under these culture conditions, and it points to a possible local interaction of Sertoli cells and germ cells. Direct evidence that spermatogenic cells *in situ* in the spermatogenic epithelium can utilize metabolic intermediates that are produced by Sertoli cells, came from investigations by Grootegoed et al (1985a). It was shown that Sertoli cells convert leucine to α-ketoisocaproate via transamination. Spermatogenic cells did not produce α-ketoisocaproate from leucine, but they can reduce exogenous α-ketoisocaproate to α-hydroxyisocaproate. This reduction does not occur in Sertoli cells or in any other cell type. When fragments of spermatogenic epithelium were incubated with leucine, the hydroxy acid was produced, indicating an intercellular pathway of leucine catabolism which involves transport of α-ketoisocaproate from Sertoli cells into germ cells (Grootegoed et al., 1985a). Another indication of Sertoli cell-germ cell interaction is the conversion of fluoroacetate by Sertoli cells to fluorocitrate, which blocks the citric acid cycle. Fluoroacetate at sublethal doses inhibits spermatogenesis *in vivo*. However, *in vitro* fluoroacetate had no deleterious effect at all on the isolated spermatogenic cells, whereas fluorocitrate inhibited very efficiently ATP production by isolated germ cells. Apparently, the lethal synthesis of fluorocitrate from fluoroacetate is performed by Sertoli cells, and subsequently fluorocitrate is taken up by the germ cells (Grootegoed et al., 1985b). Although this lethal synthesis is not physiologically useful, these observations and the aforementioned ones indicate communication between Sertoli cells and germ cells.

The Sertoli cells, by setting the local environment, may support the progression of germ cells through spermatogenesis. Experiments by Russell et al. (1987) indicated that the most developmentally advanced germ cells in normal rats were most often involved in degeneration. According to Russell et al. (1987) these most advanced cell types could meet an environment which initially is not adequate. As the Sertoli cells mature, and all hormonal requirements are met, an appropriate environment will be established for further germ cell development.

2.6.2 Sertoli cell-peritubular cell interactions

The seminiferous tubules are surrounded by a layer of peritubular myoid cells and by other peritubular cells at the periphery of the tubules. A basal lamina is present between the peritubular myoid cells and the seminiferous epithelium, which is composed of collagen fibers and extracellular matrix components. It has been described that Sertoli cells and peritubular cells secreted different extracellular matrix components. Neither cell type in monoculture could synthesize all extracellular matrix components. Peritubular cells release immunocytochemically detectable fibronectin, which is not produced by Sertoli cells. On the other hand, Sertoli cells are immunocytochemically positive for laminin, in contrast to peritubular cells. Both cell types released type IV collagen (Tung and Fritz, 1984; Tung et al., 1984). Morphologically it has been observed that formation of a limiting membrane occurred when Sertoli cells were co-cultured with peritubular cells

(Tung and Fritz, 1980). With enzyme-linked immunosorbent assays and immunocytochemical techniques, evidence was obtained for cooperativity of peritubular cells and Sertoli cells in the deposition of the extracellular matrix components fibronectin and laminin, whereas collagen was deposited by both cell types. These results indicate that basal lamina formation can take place in co-cultures of Sertoli cells and peritubular cells (Skinner et al., 1985).

When maintained in culture, peritubular myoid cells require serum for survival, whereas in the presence of Sertoli cells the peritubular cells can survive for months in serum-free medium. Peritubular cells, in turn, can stimulate Sertoli cell activities for a prolonged period. This is indicated by an elevated secretion of ABP and transferrin in cocultures, as compared to Sertoli cell monocultures (Tung and Fritz, 1980; Hutson and Stocco, 1981; Holmes et al., 1984). These interactions were thought to require cell-cell contact.

However, certain cell-cell interactions between Sertoli cells and peritubular cells may not require direct intercellular contact. Peritubular cells produce a protein factor, which was named P–Mod–S, that stimulates transferrin and ABP production by Sertoli cells to the same extent as could be obtained by maximal hormonal stimulation (Skinner and Fritz, 1985a). Initial characterization revealed that P–Mod–S is a 70 kDa non-mitogenic protein (Skinner and Fritz, 1986). Androgen receptors are present in peritubular cells (Sar et al., 1975) and the production of P–Mod–S was increased by testosterone (Skinner and Fritz, 1985a, b). These results led to the hypothesis that androgens act at two levels on the Sertoli cells, viz. a direct action via Sertoli cell androgen receptors, and an indirect effect mediated via peritubular cell androgen receptors and P-Mod-S.

2.6.3 Sertoli cell-Leydig cell interactions

Spermatogenesis in mammals is dependent on intratesticular testosterone. Therefore it may be hypothesized that local regulatory mechanisms are required to control the level of testosterone within the testis (Sharpe et al., 1986). Testosterone production by the Leydig cells may be controlled directly, or the response to LH stimulation may be influenced, by factors which are produced locally, probably by the Sertoli cells.

Different experimental approaches have led to the evidence that Sertoli cells influence Leydig cell function. i) From 20-40 days of age in the rat plasma FSH levels increased concomitantly with an increase in LH receptor levels on Leydig cells (Ketelslegers et al., 1978). ii) Hypophysectomy of immature rats resulted in a rapid and almost complete loss of Leydig cell responsiveness to LH. Administration of FSH starting immediately after hypophysectomy prevented this loss of Leydig cell sensitivity completely (Van Beurden et al., 1976). iii) Morphological studies indicated that FSH administration to immature hypophysectomized rats induced increased interstitial fluid volume and hypertrophy of Leydig cells (Kerr and Sharpe, 1985). iv) In coculture of Leydig cells with Sertoli cells, addition of FSH stimulated the number of LH/hCG binding sites on Leydig cells and augmented the hCG-stimulated steroidogenesis by these Leydig cells (Tabone et al., 1984; Benahmed et al., 1985a; Perrard-Sapori et al., 1986, 1987). Also, addition of hCG to these co-cultures resulted in increased FSH-receptor numbers and a higher level of FSH-stimulated plasminogen activator activity by the Sertoli cells (Perrard-Sapori et al., 1986). These results indicate reciprocal interactions between the two cell types.

In vivo, Sertoli cells and Leydig cells are not in direct cell-to-cell contact. Therefore, it was hypothesized that stimulatory effects of Sertoli cells on Leydig cells and *vice versa* should be mediated by diffusable factors. Several of such factors have been observed since. In spent medium from Sertoli cell cultures "LHRH-like activity" was observed (Sharpe et al., 1981), which seemed to inhibit Leydig cell activities. However, these results were later considered artefactual (Cooke and Sullivan, 1985). In general, *in vitro* incubations of isolated Leydig cells with LHRH-analogues increased basal and LH-stimulated Leydig cell steroidogenesis (Themmen et al., 1986a, b). Other more promising protein factors from Sertoli cell culture medium acting on Leydig

cells, whose production could be stimulated by FSH, have been reported (Benahmed et al, 1985b; Verhoeven and Cailleau, 1985; Perrard-Sapori et al, 1987). Furthermore, testicular interstitial fluid, which consists of many serum factors and proteins, but may also contain products secreted by the seminiferous tubules, contained testis-specific factor(s) acting on Leydig cells (Sharpe and Cooper, 1984). All the reported bioactivities had stimulatory effects on basal and/or hCG-stimulated Leydig cell steroidogenesis and/or on the number of LH-receptors. On the other hand, some authors have described that seminiferous tubules from specific stages of the spermatogenic cycle, but also cultured Sertoli cells, produced factor(s) with inhibitory activity toward Leydig cell steroidogenesis (Syed et al., 1985; Benahmed et al., 1986). Apparently several different factors may be produced by Sertoli cells, most exerting stimulatory effects, but some displaying inhibitory effects on Leydig cells. This confusion can only be solved by identification of these compounds. Therefore, a major difficulty in the interpretation of the above mentioned results is that up to now none of these factors has been sufficiently purified and characterized. Preliminary characterization indicated that most of these factors are proteins.

The evidence obtained suggests an FSH-stimulated production of polypeptide factors by Sertoli cells with regulatory effects on LH-stimulated testosterone production by Leydig cells. Furthermore, Sertoli cell functions may be affected by Leydig cell products other than testosterone. This has been observed in co-culture systems (Perrard-Sapori et al., 1986). As has been described in section 2.4.5, POMC-derived peptides synthesized by Leydig cells may influence Sertoli cell proliferation and function (Boitani et al., 1985; Orth, 1986).

2.7 Concluding remarks

It is well known that male fertility is dependent on the pituitary hormones FSH and LH. The timing of testicular development and the maintenance of spermatogenesis during adulthood are controlled by the pattern of secretion of the gonadotropins LH and FSH by the pituitary gland. Regulation of gonadotropin secretion involves stimulation by hypothalamic GnRH and feedback inhibition by inhibin and testosterone. The gonadotropins are the primary controllers of testicular function, which is illustrated by the rapid cessation of normal testicular function when gonadotropin support is withdrawn. However, the control of spermatogenesis is much more complicated, as is suggested by the fact that the majority of infertile men have normal gonadotropin levels.

It gradually is appreciated that paracrine mechanisms may influence the actions of FSH and LH. In the testis, FSH and LH actions may require the correct functioning of local modulators. To this end local interactions between the various testicular cell types must occur successfully, in order to establish efficient spermatogenesis and continous production of testosterone. Several growth factors, circulating or locally produced, could play a role in these interactions.

The influences of growth factors and locally produced hormones on gonadotropin action may change during testis development. The physiological significance of Sertoli cell inhibin is most likely of largest importance in the regulation of FSH levels in prepuberal animals.With respect to IGF–I, it might be hypothesized that, in analogy to the observations on granulosa cells, circulating or testicular IGF–I could support FSH-stimulated Sertoli cell functions. Alternatively, it might be suggested that one of the FSH-stimulated paracrine functions may be the production of "supplemental growth factors" such as the IGF–I-like activity by Sertoli cells. Spermatogenic disturbances caused by diabetes suggest a role for insulin in the maintenance of optimal testicular cell functions. This does not necessarily involve an effect of insulin on glucose metabolism, although it is evident that spermatogenesis is extremely sensitive to disturbances of glucose metabolism.

It should be kept in mind that most of the experiments which indicate paracrine interactions between testicular cells were carried out with *in vitro* systems. As cell functions may change upon isolation and culture,

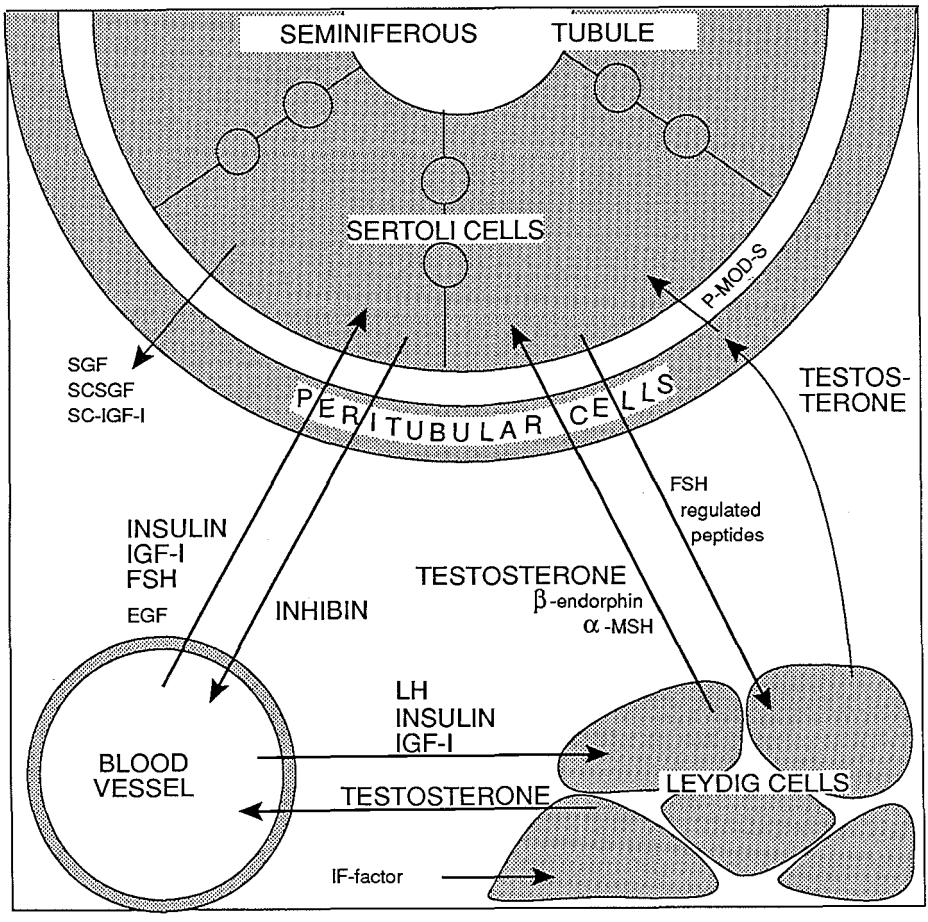

Figure 2.5. Diagrammatic representation of some endocrine and paracrine actions between testicular cells, discussed in this chapter. SGF: seminiferous growth factor. SCSGF: Sertoli cell-secreted growth factor. SC-IGF–I: Sertoli cell-IGF–I. P–Mod–S: peritubular protein, which modulates Sertoli cell function. IF–factor: interstitial fluid factor. See text for full details.

such results may not represent the *in vivo* situation. It will be necessary in the near future to undertake *in vivo* studies and to try to validate in this way the *in vitro* findings, but the complexity of the *in vivo* situation is an obstacle in the interpretation of results obtained using a whole animal.

Up to now, our shortage of knowledge of paracrine interactions between testicular cells, the relation with gonadotropic regulation of testis development, and last but certainly not least, the way in which testosterone exerts its effects, preclude the design of a detailed model of hormonal (inter)-actions involved in spermatogenesis. In figure 2.5 we have schematically indicated a number of possible endocrine and paracrine actions in the testis, based on the *in vitro* observations reported in this chapter.

CHAPTER 3

METHODS

3.1 Introduction

The use of isolated cell types can give useful information about the regulation of metabolism of a particular cell type. *In vivo* studies may suffer from the presence of different cell types. Moreover, the complexity of interactions between different cell types, such as present in the testis, greatly interferes with the determination of the regulation of a defined variable in one cell type. Studies *in vivo* may be meaningful, however, when an activity can be studied that is specifically restricted to one selected cell type. In Sertoli cells, glucose metabolism and many other biochemical activities certainly are not unique variables. Therefore, we have concluded that for our studies on Sertoli cells an *in vitro* system of purified Sertoli cells is required. It should be kept in mind, however, that several studies have shown that Sertoli cell function *in vitro* is influenced by the presence of germ cells, peritubular cells and Leydig cells (section 2.7). Hence, experiments performed *in vitro* with purified cells might not be the best reflection of Sertoli cell function *in vivo*.

Some general aspects of the methods which are of interest for the work presented in this thesis, and which are not described in detail in the appendix papers, will be presented in this chapter. Further details of the materials and methods used in particular experiments have been described in more detail in the appendix papers.

3.2 Isolation, incubation and purity of Sertoli cell preparations

3.2.1 Isolation procedures

The animals used were 3-week-old male Wistar-R rats (21-23 days old). At this age spermatogenesis has progressed up to the pachytene spermatocyte stage. The meiotic divisions have not yet started, and spermatids or more advanced germ cells are not present. For the experiments described in appendix papers 3 and 4 Sertoli cells were isolated from the testes of rats which had been irradiated *in utero* on day 20 of gestation (Beaumont, 1960). At this stage the germ cells are very sensitive to irradiation and as a result of this treatment sterile testes devoid of germ cells are obtained. It has been shown that these germ cell-depleted testes (also termed "Sertoli cell-enriched" testes) from immature rats showed FSH-responsiveness and ABP production equal to testes from normal intact rats, but the formation of the blood-testis barrier was delayed for ten days (review: Means et al., 1976).

For all other experiments Sertoli cells were isolated from 3-week-old intact rats, using the same isolation procedure. In these cell preparations a hypotonic shock treatment was applied after 2 days of culture to remove the remaining spermatogenic cells (appendix paper 1). It was found that both type of Sertoli cell preparations exhibited similar rates of lactate production and similar hormone responsiveness. The isolation procedure has been described in detail in appendix paper 3. An outline is given in Figure 3.1.

A Sertoli cell-membrane fraction was prepared after 3 days of incubation of the Sertoli cells. The membrane isolation procedure is described in appendix paper 1.

Membrane fractions from whole testis homogenates were prepared as described in appendix paper 2.

3.2.2 Incubation conditions

Isolation of the Sertoli cells was performed in Dulbecco's phosphate-buffered saline. The cells were incubated at 32°C in

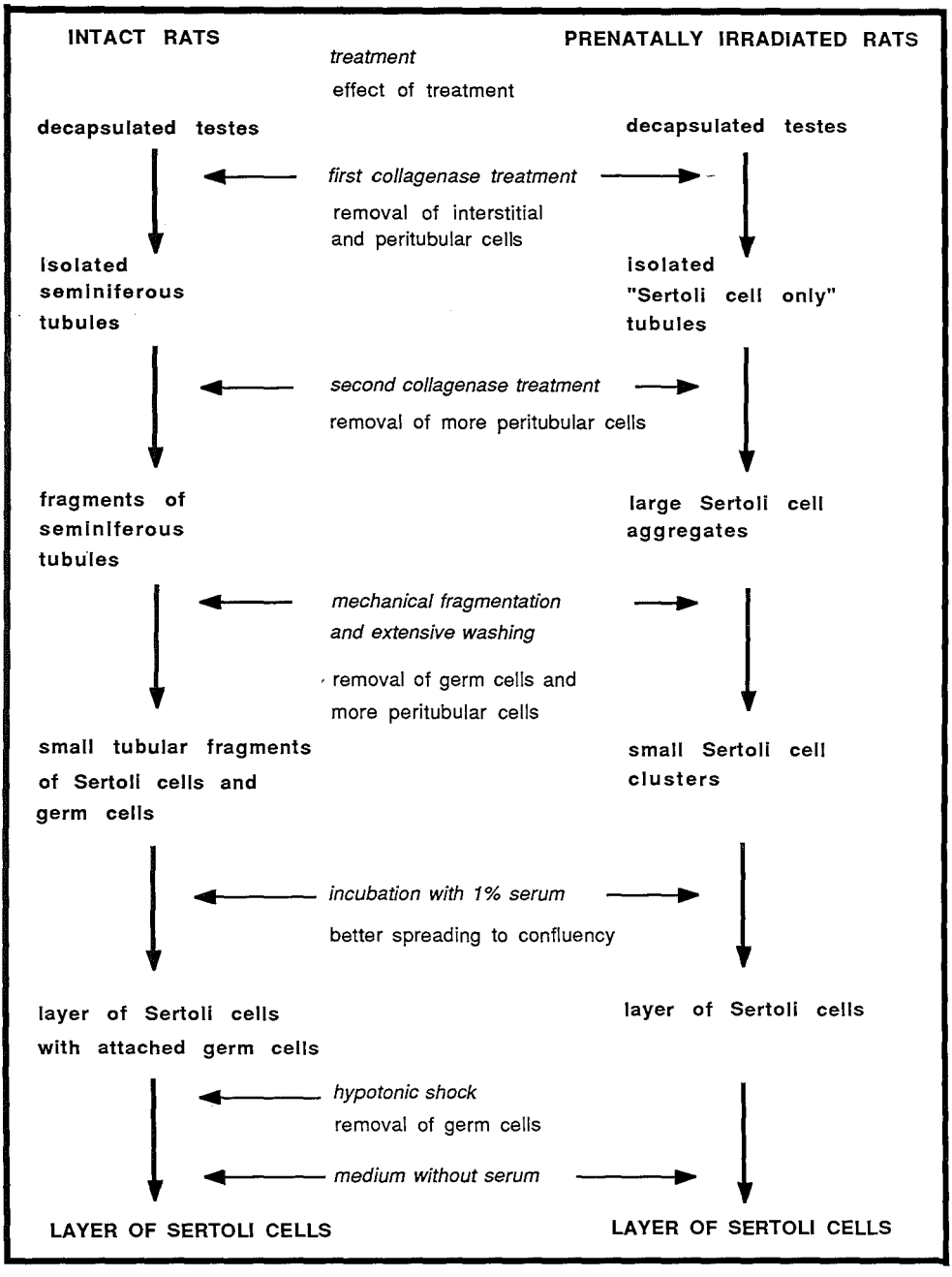

Figure 3.1. Outline of the isolation procedure for Sertoli cells from intact and prenatally irradiated rats.

an atmosphere of 5% CO_2 in air in Eagle's minimum essential medium (MEM), in the presence of 1% (v/v) fetal calf serum for the first two days of culture to promote spreading to confluency of the small tubule fragments. Sertoli cells isolated from intact rats were subsequently treated during 1 min with 10 times diluted MEM to remove the remaining spermatogenic cells. All cultures were incubated in MEM for one additional day in the absence of fetal calf serum to try to remove hormonal and growth factor activities present in serum. After three days of culture, Sertoli cells were used to estimate biochemical activities or to determine the binding of ^{125}I-labelled hormones. This period was considered necessary for several reasons. A layer of Sertoli cells was obtained after a few days in culture, when the tubule fragments had spread. These Sertoli cells represent a "stabilized" population of cells adapted to *in vitro* conditions. During the initial three days of incubation many spermatogenic cells already detached from the tubule fragments. Membrane receptor structures may be damaged by the enzyme treatment used during the isolation procedure, and restoration of receptors may require a relatively long incubation period. Furthermore, it had been observed that lactate production estimated directly after isolation of the Sertoli cells was high, and decreased to a constant level after a few days (Grootegoed et al., 1983).

3.2.3 Purity of the Sertoli cell preparations

The Sertoli preparations were used to conduct studies on the presence of receptors for insulin and IGF–I and on the effects of FSH, insulin and IGF–I on glucose metabolism. As these activities are not specific for Sertoli cells, the presence of other cell types should be limited as much as possible. The most persistent contaminating cells in Sertoli cell preparations are germ cells and peritubular cells. Germ cells can be avoided by using testes from prenatally irradiated rats, or they can be removed to a large extent by a hypo-osmotic shock procedure. Furthermore, germ cells are much less active in glucose metabolism than Sertoli cells (Grootegoed et al., 1983) and probably do not possess a considerable amount of receptors for insulin and IGF–I. However, it is very well possible that peritubular cells produce lactate from glucose and contain insulin- and IGF–I receptors. Peritubular cells also produce an IGF–I-like compound, which may interfere with the estimation of IGF–I receptors on Sertoli cells (Skinner and Fritz, 1986). Therefore, in our studies we used a prolonged, intensified treatment with collagenase, which minimizes as much as possible the contamination by peritubular cells, since collagenase breaks down the collagen in the basal lamina and thereby causes disconnection of peritubular cells from the tubules..

The purity of the Sertoli cell preparations was microscopically evaluated after fixation of a portion of the final cell preparation in ethanol-glacial acetic acid (3:1, v/v), application to microscope slides and drying. The fixed cell nuclei were examined with phase contrast microscopy after addition of a drop of acetic acid (45%, v/v). The morphology of the different nuclei can be well discerned in these preparations (appendix paper 3). Freshly isolated Sertoli cell preparations from irradiated rats contained at least 94% Sertoli cells, and freshly isolated Sertoli cell preparations from intact rats showed 70-75% Sertoli cells and 25-30% germ cells. In both preparations less than 1% peritubular myoid cells or other peritubular cells were observed.

- Fibronectin assay

It has been described that peritubular myoid cells produce fibronectin, an extracellular matrix component, and that this protein is not produced by Sertoli cells (Tung et al., 1984; section 2.7.2). An immunoassay directed against fibronectin was therefore performed to identify contaminating cells in a Sertoli cell preparation (Dr. J.M. Fentener van Vlissingen, according to her protocol). Sertoli cells (isolated as described in this chapter) and peritubular cells (isolated as described by Tung et al., 1984) were cultured on glass coverslips for three days. The immunoassay was performed at 20°C unless specified otherwise. The preparations were washed and fixed in phosphate buffer (pH 7.3) containing 4% (w/v) paraformaldehyde, 0.01% (w/v) saponine and 0.1% (w/v) BSA, washed 3x

with phosphate buffer and incubated with a rabbit-anti-fibronectin antibody overnight at 4°C, and again washed 3x with phosphate buffer. The second antibody, swine-anti-rabbit, was added for 30 min at 20°C, and the preparations were washed 3x with phosphate buffer followed by an incubation with a peroxidase-anti-peroxidase antibody for 45 min at 20°C, 3x washed with phosphate buffer and stained with diaminobenzidine. In fibronectin-positive cells a brown reaction product will precipitate. A counterstain for nuclei was performed with haematoxylin. The controls performed included control for endogenous peroxidase, use of normal rabbit serum instead of rabbit-anti-fibronectin, leaving out the first, the second or both antibodies, and preabsorption of rabbit-anti-fibronectin with excess fibronectin. All controls were negative. The results confirmed the conclusions of Tung et al. (1984) that Sertoli cells were negative and peritubular myoid cells positive for fibronectin. In a Sertoli cell preparation after 3 days of culture only very few fibronectin-positive cells could be detected (<1% of the cells, by Dr. J.M. Fentener van Vlissingen), confirming the observations on ethanol-acetic acid fixed nuclei in freshly isolated preparations. Tung et al. (1984) reported that the use of hyalu-ronidase, following trypsin and collagenase, could further improve the purity of the Sertoli cell preparation, reducing peritubular myoid cell contamination to 0.3%. However, our method of Sertoli cell isolation was adequate for the present experiments.

3.3 Biochemical parameters of cellular activities

The amount of lactate produced from glucose was measured enzymically, using lactate dehydrogenase and NAD^+, as described by Hohorst (1970) (appendix paper 3).

The conversion of D–[U–^{14}C]glucose to lipids was estimated as described in appendix paper 3.

The amount of cyclic AMP in cells and medium was determined using a cAMP-assay kit (appendix paper 3).

Protein synthesis was estimated from the L–[4,5–^{3}H]lysine incorporation into TCA precipitable material, as described in appendix paper 3.

The methods employed to determine the conversion of D–[5–^{3}H]glucose to ^3H_2O and the oxidation of D–[1–^{14}C]-glucose and D–[6–^{14}C]glucose to CO_2, the oxidation of [1–^{14}C]pyruvate and [2–^{14}C]-pyruvate to CO_2 and the oxidation of the amino acids L–[U–^{14}C]leucine, L–[1–^{14}C]-leucine and L–[U–^{14}C]glutamine to CO_2 have been described in appendix paper 4.

Estimation of the amount of glucose carriers was performed by determination of the number of D–glucose-inhibitable cytochalasin B binding sites. Cytochalasin B binds to the plasma membrane glucose carrier in intact cells and D-glucose can compete for this binding. The binding was estimated as follows. After the three day culture period (section 3.2.2), the Sertoli cells were washed two times with PBS and incubated in PBS for 30 min at 32°C to try to remove the glucose, present in MEM. Incubation was started in fresh PBS containing 250 nM [^{3}H]-CB in the absence or presence of 200 mM D-glucose. FSH (0.5 μg/ml) or insulin (5 μg/ml) were added at the start. Incubations were terminated at different times after addition of [^{3}H]-CB, to establish the time-course of binding. The displacement of bound [^{3}H]-CB was estimated using incubations with 10 nM [^{3}H]-CB plus increasing concentrations of unlabelled CB, in the presence and absence of 200 mM D-glucose. In some experiments also 10 μM cytochalasin E was added at the start. The incubations were terminated after 30 min by placing the culture vessels on ice and rapidly removing the incubation medium. Cells were dissolved in 1 M NaOH and cell-associated radioactivity was counted, and cellular protein was determined.

Glucose uptake was measured using 2-deoxy-D–[2,6–^{3}H]glucose (2-DOG) as described in appendix paper 5.

The amount of protein was determined according to Lowry et al. (1951) for cell cultures and according to Bradford (1976) for membrane preparations, using BSA as a standard.

3.4 Receptor assays

The binding of ^{125}I-insulin to cultured Sertoli cells was determined in order to establish whether the observed binding properties met the criteria set for true hormone-receptor interactions, as defined by De Meyts (1976). The method applied is described in appendix paper 1. The binding of ^{125}I-IGF–I to Sertoli cells was determined using the same method (appendix paper 2). Further analysis of the binding data is evaluated in Chapter 4.

The qualitative nature of the binding of ^{125}I-insulin to Sertoli cells was evaluated also by electrophoresis of proteins of a Sertoli cell-membrane fraction, in which the plasma membrane insulin receptors were affinity-labelled by covalent cross-linking of receptor-bound ^{125}I-insulin. The affinity-labelling and crosslinking conditions are described in appendix paper 1.

The binding of ^{125}I-insulin and ^{125}I-IGF–I to membrane fractions of testes and kidney homogenates from immature and adult rats was determined using a microfuge separation assay and is described in appendix paper 2.

CHAPTER 4

RECEPTOR BINDING STUDIES WITH INSULIN AND IGF–I

4.1 Introduction

The first step in hormone action is the binding of the hormone to a specific receptor. Sertoli cells have been shown to contain specific receptors for FSH (reviews: Means et al., 1980; Davies, 1981) (see section 2.2.2) and testosterone (Mulder et al., 1976; Sanborn et al., 1983). Sertoli cells in culture are sensitive to a number of other hormones, adenosine, and the vitamins A (retinol) and D_3, and in several cases specific receptors and binding proteins have been demonstrated. A survey is presented in Table 4.1.

Receptors for insulin have been demonstrated not only in the classical insulin targets liver (Freychet et al., 1972), fat (Kono and Barham, 1971) and muscle (Beguinot et al., 1985), but also in numerous other tissues and cell types, for example lymphocytes (Gavin et al., 1973). Besides insulin receptors, distinct receptors for IGF–I and IGF–II could be demonstrated on most cell types. Some cell types, for example H35 hepatoma cells (Koontz, 1984), may contain only receptors for insulin and not for IGF–I or IGF–II. In most cell types which exhibit sensitivity to physiological concentrations of insulin, receptors for insulin could be demonstrated. For Sertoli cells, low concentrations of insulin (Oonk et al., 1985) and IGF–I (Borland et al., 1984; Oonk and Grootegoed, 1987b) can effect metabolism of glucose, but thus far only a large amount of IGF–I and IGF–II binding, but no binding of insulin, to Sertoli cells has been observed (Borland et al., 1984). However, the quantitative estimation of the amount of receptors and the kinetics of binding were not studied sufficiently. In this respect, the aim of the present studies was to identify and characterize receptors for insulin (appendix paper 1) and IGF–I (appendix paper 2) on cultured Sertoli cells.

Table 4.1. Receptors in Sertoli cells

Receptor	References
FSH	Thanki and Steinberger, 1978; Means et al., 1980; Davies, 1981
Testosterone	Mulder et al., 1976; Sanborn et al., 1983
Estrogen	Nakhla et al., 1984
Glucagon	Eikvar et al., 1985
Insulin	Oonk and Grootegoed, 1987a
IGF–I	Oonk and Grootegoed, 1987b
IGF–I and IGF–II	Borland et al., 1984
EGF	Mallea et al., 1986
Opiate (α-MSH; β-endorphin)	Fabbri et al., 1985; Boitani et al., 1986; Orth, 1986
Isoproterenol (β-adrenergic)	Heindel et al., 1981; Kierszenbaum et al., 1985
1, 25-$(OH)_2$ Vitamin D_3	Levy et al., 1986
Retinol (vitamin A)	Galdieri and Monaco, 1983; Porter et al., 1985
Transferrin	Sylvester and Griswold, 1984
Adenosine	Monaco and Conti, 1986

Table 4.2. Effect of pH on the maximal specific binding of ^{125}I-insulin and ^{125}I-IGF–I

	pH					
	6.5	7.0	7.5	8.0	8.5	
^{125}I-Insulin bound*	26	18	53	100	79	(a)
^{125}I-IGF–I bound*	31	51	82	100	84	(b)

* % of maximal binding
(a) mean of duplicates, differing less than 6%
(b) mean of duplicates, differing less than 11%

The numbers of receptors for insulin and IGF–I per cell or tissue are often not constant, but may change during cell differentiation and tissue development (Sara et al., 1983; Beguinot et al., 1985; Shimizu et al., 1986). During development of the testis, insulin binding to total testis preparations appears to increase with age (Saucier et al., 1981). Therefore, we have compared the binding of insulin and IGF–I to membrane fractions isolated from testes from immature and adult rats (appendix paper 2).

4.2 Receptors for insulin and IGF–I on Sertoli cells

4.2.1 Binding studies with ^{125}I-insulin and ^{125}I-IGF–I

To investigate the presence of receptors for insulin and IGF–I and to characterize the observed binding precisely, procedures were followed in accordance with the criteria formulated by De Meyts (1976). The Sertoli cell preparations used were isolated from 3-week-old rats and preincubated during three days as described in appendix paper 1. The labelled hormone preparations used were mono-iodinated on tyrosine and, according to the specifications of the manufacturer, biologically active. Binding of ^{125}I-insulin and ^{125}I-IGF–I to Sertoli cells was optimal at pH 8. The optimal range of pH for insulin binding was markedly narrower than the pH range for IGF–I binding (Table 4.2). The optimal temperature was considered to be 22°C, because at higher temperatures the hormone may be degraded, and lower temperatures are even more unphysiological for the cells. During short incubations not exceeding 2 h, at a temperature of 22°C, hormone degradation was observed to be very low (Olefsky and Kao, 1982). Furthermore, 1.6% BSA was present in the incubation media to prevent degradation of hormones and to avoid non-specific adsorption of insulin and IGF–I to non-cellular surfaces.

The specificities of the binding of ^{125}I-insulin and ^{125}I-IGF–I are presented in appendix papers 1 and 2.

Scatchard plots of insulin binding (appendix paper 1) were nonlinear with upward concavity. This indicates that either the binding sites are non-homogeneous or site to site interactions are present. The binding data represented in a Scatchard plot do not permit to conclude whether the hormone-receptor interaction fits a multiple sites model or a cooperative model. For IGF–I binding the Scatchard plots (appendix paper 2) were linear, indicating a single homogeneous population of hormone binders, devoid of cooperative site to site interactions.

Another method of analyzing hormone-receptor binding data is to present the data in a Hill plot (De Meyts, 1976). This logit-log transformation of bound hormone (B) versus free hormone concentration (F), viz. the plot of the logit of B ($\log\{B/B_{max}-B\}$) versus $\log\{F\}$, has use

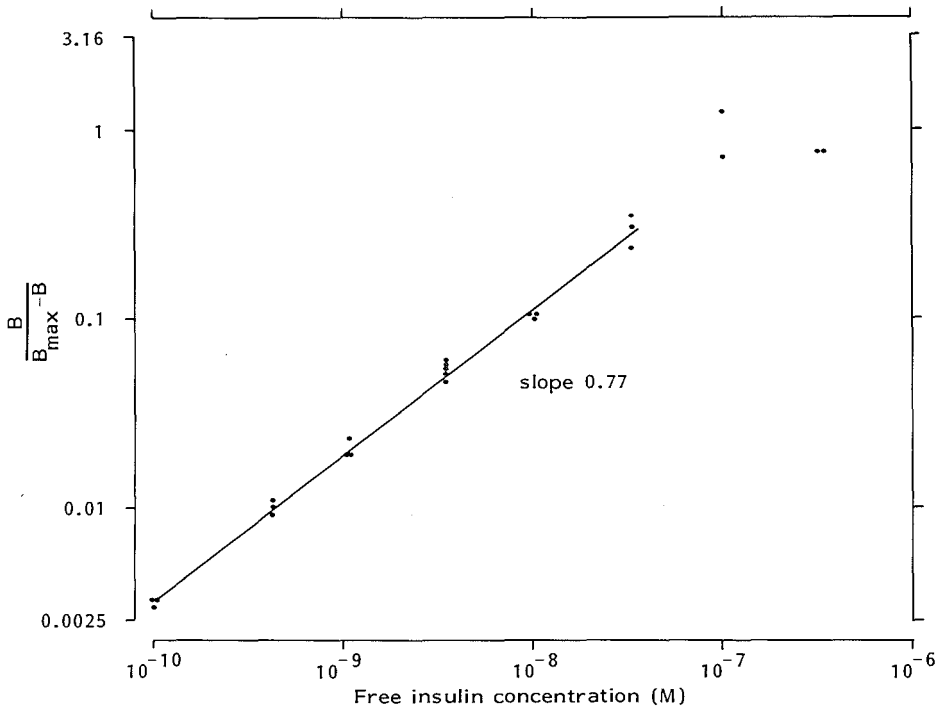

Figure 4.1. Hill plot for insulin binding to cultured Sertoli cells. The same data were used to construct the Scatchard plot shown in appendix paper 1. The Hill coefficient was 0.77 (< 1). B_{max}: maximal amount of hormone bound, and equals R_o for total binding (high plus low affinity). B: the amount of hormone specifically bound at a given hormone concentration.

ful linearizing properties, and the slope of the line thus obtained gives an indication about cooperative interactions. This slope is also called the Hill coefficient. A value equal to 1 represents a non-cooperative system. A value > 1 indicates positive cooperativity, as shown for example for the binding of oxygen to hemoglobin (De Meyts, 1976). A value < 1 is consistent either with heterogeneity of binding sites or with negative cooperativity. When the slope of the line is not equal to 1, the Hill transformation only describes accurately the middle portion of the binding curve, and the line calculated starts to deviate from linearity when the ratio bound hormone/free hormone (B/F) approaches zero. Figure 4.1 shows the Hill plot for insulin binding to Sertoli cells, and it can be seen that the Hill coefficient is 0.77. Figure 4.2 shows the Hill plot for IGF–I binding, and it appears that in this case the Hill coefficient is 0.98. This confirms the differences in binding of insulin and IGF–I to Sertoli cells described above.

De Meyts et al. (1973) have described a simple method to detect cooperative interactions in the binding of a polypeptide hormone to its receptors. To this end, the dissociation of labelled hormone from the receptors is studied after diluting the hormone-receptor complex which prevents reassociation of dissociated tracer hormone, and dissociation is estimated in the absence and presence of an excess of unlabelled hormone. In the absence of site to site interactions, the dissociation rates should be identical in both cases. De Meyts et al. (1973) showed a 10-fold increase in dissociation rate ofinsulin bound to lymphocytes by the addition of excess unlabelled insulin,

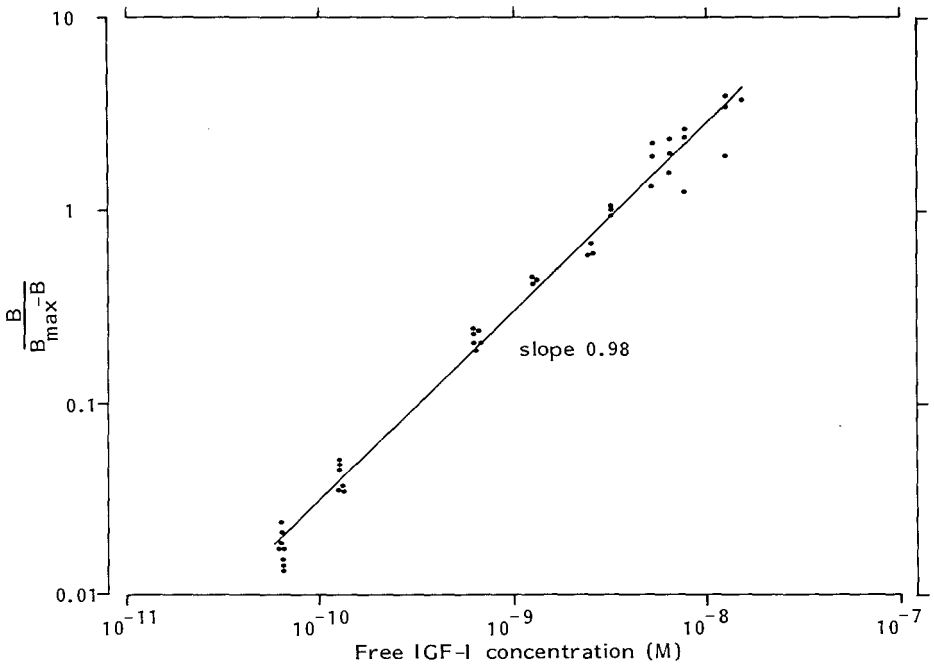

Figure 4.2. Hill plot for IGF–I binding to cultured Sertoli cells. The same data were used to construct the Scatchard plot shown in appendix paper 2. The Hill coefficient was about 1 (0.98). B_{max}: maximal amount of hormone bound, and equals R_0 for total binding. B: the amount of hormone specifically bound at a given hormone concentration.

suggesting that binding sites occupied by the unlabelled insulin lower the affinity of other sites for the hormone. This phenomenon was called negative cooperativity. The present results on Sertoli cells indicate a smaller (2-fold) increase in dissociation rate after dilution in the presence of unlabelled insulin (appendix paper 1). This indicates that in the Sertoli cell system the negatively cooperative site to site interactions were not very large, and we have analyzed the binding data for insulin observed in the Scatchard plot with a method fitting a two-site model for a heterogeneous receptor population.

From the results in appendix paper 2 it was concluded, that spermatogenic cells do not contain IGF–I receptors. The very small amount of specific binding of IGF–I to isolated spermatogenic cells observed may be explained by the presence of less than 1% Sertoli cells in the germ cell preparations.

In table 4.3 a comparison is given of the binding characteristics of insulin and IGF–I receptors on cultured Sertoli cells. From these data we conclude that a relatively low number of insulin receptors and a relatively high number of IGF–I receptors can be demonstrated on cultured Sertoli cells from immature rats. The two receptor populations exhibit different binding kinetics. The insulin receptors represent a heterogeneous receptor population of binding sites with different affinities for insulin, and the IGF–I receptors represent a homogeneous receptor population of binding sites with a similar affinity for IGF–I.

Table 4.3. Comparison of the binding characteristics of ^{125}I-insulin and ^{125}I-IGF–I to Sertoli cells.

	Insulin	IGF–I
pH optimum	8	8
Equilibrium association time (min)	60	120
Dissociation half-time (min)	13	22
Idem, + excess unlabelled hormone (min)	6	not determined
Competition:		
50% displacement of maximal binding by unlabelled hormone at	1.5 nM insulin 65.0 nM IGF–I	> 167 nM insulin 2 nM IGF–I
Scatchard plots:	curvilinear	linear
K_d (nM)	1.8 ± 0.3	3.5 ± 0.2
R_o (fmol/mg protein)	8.5 ± 1.0	2080 ± 140
r	0.87	0.91

For insulin, only the parameters of the high affinity binding site are given.

4.2.2 Affinity labelling studies with ^{125}I-insulin

In a series of pilot experiments, Sertoli cell membranes were isolated according to Borland et al. (1984) which yields a crude, nuclei-free membrane preparation. These membrane fractions were incubated with ^{125}I-insulin, and proteins were chemically crosslinked using disuccinimidylsuberate (DSS) as described in appendix paper 1. No specific binding of insulin to membrane proteins could be detected, but the low purity of the membrane fractions used might influence the binding of ^{125}I-insulin.

More purified plasmamembrane preparations from Sertoli cells were subsequently isolated using the procedure described in appendix paper 1. We have not evaluated the composition of this preparation using marker enzyme activities. Kew et al. (1986), however, have analyzed the composition of a Sertoli cell membrane preparation isolated with the same method, and they concluded that the membrane preparation consisted of mainly plasma membranes, but with a considerable contamination of endoplasmic reticulum fragments.

The membrane preparations obtained in this manner (appendix paper 1) were incubated with ^{125}I-insulin. After washing away the unbound radioactivity, bound ^{125}I-insulin was crosslinked to membrane proteins with DSS. Subsequently, the membrane proteins were separated by electrophoresis in polyacrylamide gels under reducing and non-reducing conditions, and the gels were sliced and counted (appendix paper 1). This procedure demonstrated covalent cross-linking of ^{125}I-insulin to proteins at the top of the gel under non-reducing conditions (M_r > 300 kDa) and to a protein of 130 kDa under reducing conditions (appendix paper 1). After incubation of the membrane preparations with ^{125}I-insulin in the presence of a 100-fold molar excess of unlabelled insulin, the radioactivity associated with these proteins was strongly reduced. It was concluded that the proteins > 300 kDa and 130 kDa represented the undissociated receptor structure and the α-subunit of the insulin receptor, respectively (see also section 2.3.3.). This affinity labelling of the insulin receptor was not carried out using a 100-fold molar excess of unlabelled IGF–I, which leaves open the possibility that also some binding of ^{125}I-insulin to IGF–I receptors was detected. However, the labelling of the observed proteins was achieved with a concentration of 4 nM ^{125}I-insulin, which virtually does not compete with ^{125}I-IGF–I binding to IGF–I

receptors (appendix paper 2).

4.3 Age-dependent testicular binding of ^{125}I-insulin and ^{125}I-IGF–I

We have compared the specific binding of ^{125}I-insulin and ^{125}I-IGF–I in testicular membrane fractions of immature (21-day-old) and adult (4-month-old) rats. From competition experiments in which displacement of maximally bound tracer ^{125}I-hormone by unlabelled hormone was estimated, it was observed that immature and adult testis membrane fractions showed specific binding of insulin and IGF–I (appendix paper 2). From the binding data, presented in Scatchard plots, the kinetic parameters indicating affinity and capacity of binding sites were calculated (appendix paper 2). It was found, that the affinities of the binding for insulin and for IGF-I in immature and adult testis were all in the nanomolar range. This was also observed for the membrane fractions from kidney, which was used for comparison. A larger number of experiments would be required to determine if the small differences observed in the K_ds are statistically mean-ingful.

The results obtained on maximal specific binding of tracer amounts of ^{125}I-insulin and ^{125}I-IGF–I are presented in appendix paper 2. The data, expressed as fmol/g wet weight, indicate that in immature testis the binding of IGF–I is relatively high and the binding of insulin is relatively low. The data for whole testis, as a matter of fact, will not represent Sertoli cells only. IGF–I binding to cultured Sertoli cells, however, appeared to be very high, and IGF–I binding to Sertoli cells might explain the total amount of IGF–I binding in immature testis. Such a comparison obviously meets a number of difficulties, as described in appendix paper 2. For a number of reasons, the binding observed to testis membrane fractions may be underestimated. One complicating factor might be the intratesticular presence of insulin and IGF–I. Rosenzweig et al. (1980) have demonstrated that intratesticular insulin levels in the adult rat are substantially higher than serum levels of insulin. For IGF–I it has been demonstrated that Sertoli cells (Tres et al., 1986; Smith et al., 1987) and peritubular cells (Skinner and Fritz, 1986; Tres et al., 1986) secrete IGF–I-like material. Thus, it cannot be excluded that the actual number of receptors for IGF–I and insulin might be somewhat higher as a result of receptor occupancy by endogenous hormone. It is also important to consider the possibility that Sertoli cells can undergo significant changes when cultured for a few days. Freshly isolated Sertoli cells from immature rats are unresponsive to glucagon (Eikvar et al., 1985) and do not bind β-adrenergic agonists (Kierszenbaum et al., 1985). However, after culture for a few days Sertoli cells may become responsive to glucagon (Eikvar et al., 1985) and β-adrenergic receptors have been detected (Kierszenbaum et al., 1985). Therefore, the possibility remains open that in our culture system the IGF–I receptor number on Sertoli cells was increased.

In adult testis similar amounts of binding were observed for insulin and IGF–I, indicating that between 21 days and adult age the amount of insulin binding per g testis weight increased and that of IGF–I decreased (appendix paper 2). The large difference in receptor numbers for insulin and IGF–I observed in immature testis tissue and in cultured Sertoli cells is not observed in the adult testis. Data on receptor numbers for the different cell types present *in situ* in the immature and adult testis are not available. Furthermore, the exact relation between receptor numbers for insulin and IGF–I and responsiveness to insulin and IGF–I is not known for testicular cells. It has been described by Ebina et al. (1985) that chinese hamster ovary cells, which expressed the cloned human insulin receptor, and contained a five times larger number of insulin receptors than normal chinese hamster ovary cells, were responsive to a 30 times lower concentration of insulin than the normal cells. Thus, in this cell system a positive correlation between receptor number and hormone responsiveness exists.

4.4 Conclusions

The results presented in this chapter indicate that Sertoli cells, isolated from immature rats and cultured for three days,

contain specific receptors with high affinity for insulin and for IGF–I. From the present data it appears that a relatively low number of insulin receptors and a much higher number of IGF–I receptors could be demonstrated on cultured Sertoli cells from immature rats. The insulin receptors showed the characteristics of a heterogeneous receptor population with high and low affinities for insulin, and the IGF–I receptors were characterized as a homogeneous receptor population with a single high affinity for IGF–I.

In testicular membrane fractions from 21-day-old rats also a relatively low amount of insulin binding and a relatively high amount of IGF–I binding was observed. In testicular membrane fractions from adult rats, however, the amount of insulin binding equalled the amount of IGF–I binding. Binding affinities of IGF–I and insulin binding to immature and adult testis were in the nanomolar range. Apparently, between 21 days and adult age the amount of insulin binding per g testis weight increased and that of IGF–I decreased. The present data may indicate that IGF–I is more important than insulin during testis development in immature rats, and that in the testicular tubules the Sertoli cells may be the principal target for IGF–I.

CHAPTER 5

HORMONE EFFECTS ON GLUCOSE TRANSPORT AND GLUCOSE METABOLISM

5.1 Introduction

Hormonal control of spermatogenesis probably involves actions of hormones on testicular somatic cells including Sertoli cells, whereby secretory products from Sertoli cells are important for germ cells (section 1.1). After formation of the blood-testis barrier (Dym and Fawcett, 1970), the germ cells at the luminal side of this barrier obtain nutrients and possibly other specific requirements from Sertoli cells.

Glucose metabolism by Sertoli cells may have an important supporting function in germ cell development. The energy balance of germ cells appears to be dependent on the glucose metabolites lactate and pyruvate. Isolated spermatocytes and spermatids can perform metabolic activities to a much larger extent in the presence of lactate as an energy-yielding substrate, than in the presence of glucose (Jutte et al., 1981; Grootegoed et al., 1984). Recently, it has been shown that glucose is in fact detrimental to isolated germ cells, causing a rapid depletion of ATP (Grootegoed et al., 1986a). This detrimental effect of glucose was not observed when spermatocytes were co-incubated with Sertoli cells in seminiferous tubule fragments (Jutte et al., 1982). This could be explained by the lactate production of Sertoli cells. Furthermore, in immature rats FSH was found to stimulate lactate and pyruvate production from glucose by Sertoli cells (Grootegoed et al., 1981; Mita et al., 1982; Jutte et al., 1982, 1983). Hence, the possibility that hormonal regulation of lactate production by Sertoli cells might be important for the energy supply of the spermatogenic cells appeared attractive. In this respect, not only FSH, but also insulin (and IGF–I) could play a role because these hormones are generally considered to exert regulatory effects on cellular glucose metabolism.

The studies described in this chapter involve the hormonal regulation of Sertoli cell metabolism. The metabolism of glucose, pyruvate and amino acids (section 5.2.1 and 5.2.2; appendix papers 3 and 4), and glucose transport (section 5.2.3 and appendix paper 5) have been investigated, to obtain information on the actions of FSH, insulin and IGF–I on different aspects of Sertoli cell metabolism.

5.2 Glucose conversion to lactate

Lactate is produced by cultured Sertoli cells at a high rate. This rate was shown to be highest immediately after isolation of the cells and to decline to a constant level after 2-3 days of incubation (Grootegoed et al., 1983). Furthermore, migration of Sertoli cells from tubular fragments to form a cell layer required two days of incubation (section 3.2.2). For these and other reasons we have decided to use a standard incubation period of 3 days of the Sertoli cell cultures before determining lactate production rates, hormone receptor numbers and hormone effects.

When lactate production at different times after renewal of the medium was measured, it appeared that during the first 3-9 h the production rate was high and almost constant (appendix paper 3). This was followed by a decline in production rate after 6-9 h of incubation. In fact, the net lactate production was virtually zero during the last 6 h of a 24 h incubation period in the presence of FSH- and insulin (appendix paper 3). The effects of FSH and insulin on lactate production reflect the overall rate of glycolysis, because the rate of ^3H_2O formation from [5-^{3}H]-labelled glucose showed comparable effects of FSH and

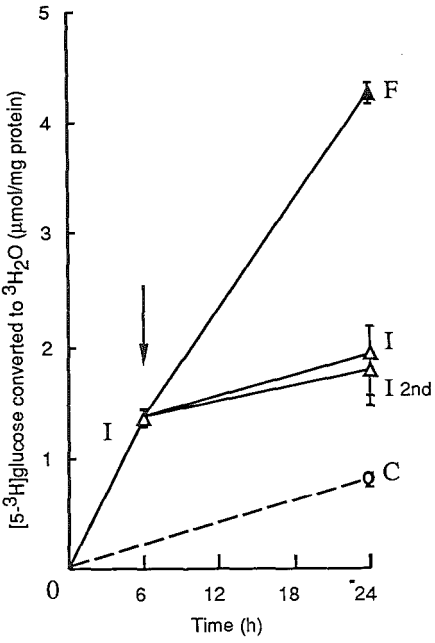

Figure 5.1. Time-course of the effect of FSH and insulin on glucose metabolism after incubation in the presence of insulin. The incubations were started in fresh medium in the absence (control, C, 0 – 0) or presence (Δ—Δ) of insulin, I (5 µg/ml). To the incubation medium FSH, F (0.5 µg/ml), or insulin, I 2nd (5 µg/ml), were added 6 h after the start of the incubations. After 24 h the incubations were terminated and the amount of ^3H_2O formed from [5-^{3}H]glucose was determined as described in appendix paper 4. The results represent mean ± S.D. of triplicate incubations.

insulin in time (Figure 5.1). It was discussed in appendix paper 3 that a loss of hormone stimulation through degradation of the hormones in the incubation medium could not explain the decline in lactate production rate. More importantly, desensitization of the cells for the homologous hormone could play a role (appendix paper 3). However, the decline in lactate production rate might also in part be caused by accumulation of inhibitory factors in medium and cells.

The accumulation of lactate also does not cause the inhibition of glycolysis described above. This was shown by the addition of 2 mM of L-lactate at the start of the incubations. In the presence of this extra amount of lactate, FSH and insulin effects on lactate production were observed, which followed the same time-course from 0-18 h of incubation. It would appear that after prolonged incubation a putative inhibitor of glycolysis is being produced that interferes with lactate production. However, we have no indications about the nature of this putative inhibitor of glycolysis, and, most importantly, the inhibition is not absolute. Addition of FSH to Sertoli cells after prolonged incubation in the presence of insulin caused a sharp increase in the lactate production rate (appendix paper 5), and in the rate of ^3H_2O production from [5-^{3}H]glucose (Figure 5.1). This indicates that the inhibition of glycolysis observed after incubation in the presence of insulin can be abolished or bypassed by FSH.

A significant stimulatory effect on lactate production of FSH, insulin and IGF–I could not be detected at incubation times shorter than 60 min (appendix paper 5), although it was observed that FSH could stimulate cAMP production within 10 min (appendix paper 3). We observed the fastest rate of lactate production when Sertoli cells were incubated with FSH, insulin or IGF–I for 3-9 h (appendix paper 3). The duration of FSH-stimulated lactate production is at variance with FSH-stimulated cAMP production, which declines after 1 h (appendix paper 3). It might be suggested, that either the short burst in cAMP level triggers a longer lasting intracellular event, or that, in addition to adenylate cyclase, other transducing systems are involved in the mechanism of FSH action. The latter was reported for the action of LH on steroidogenesis (Themmen et al., 1985). Enzyme synthesis appears not to be involved, because it was observed that effects of FSH and insulin on lactate production were independent of protein synthesis (appendix paper 3). Results, presented in appendix paper 5, indicate that FSH and insulin actions on glucose metabolism by Sertoli cells are not dependent on extracellular calcium. Our results indicate, that the time-course and maximal stimulatory effect of FSH and in-

sulin on lactate production were virtually identical, despite the different mechanisms of action. Sale et al. (1987) showed tyrosine-phosphorylation of glycolytic enzymes by the insulin receptor, using the isolated proteins (see section 5.4.2). An interesting possibility for the mechanism of action of FSH and insulin on lactate production would be glycolytic enzyme activation by phosphorylation. If such a mechanism can occur in cells has not yet been established.

The stimulatory effects of FSH and insulin on lactate production were transient ant followed by a decrease in hormone stimulation. We have studied if Sertoli cells could be stimulated long-term by FSH and insulin, using lactate production as a parameter of hormone action. To prevent the desensitizing and inhibitory effects of prolonged incubation in the presence of hormones, we have applied an incubation scheme of alternating presence and absence of hormones. After preincubation, Sertoli cells were incubated for periods of 6 h in the presence of hormones, alternated with 18 h periods in fresh medium without hormone additions. An unexpected response was observed. During the first 6 h period, FSH and insulin stimulated lactate production 3-fold, as has been observed in other experiments. However, in all subsequent 6 h periods the control lactate production was as high as the hormone-stimulated production (appendix paper 5). The results suggest that lactate production was stimulated by daily renewal of the medium, after prolonged incubation.

It has been described that various cell types respond to "stress" factors with increased glucose uptake and lactate production. Such stress factors may include mechanical stresses like agitation or deformation by pressure (Kodícek, 1986) and heat shock, addition of arsenite and viral infection (Warren et al., 1986). Furthermore, in eukaryotic cells, the synthesis of a number of proteins is induced by glucose depletion. The synthesis of these proteins, termed glucose-regulated stress proteins, was also evoked by low pH treatment or 2-mercaptoethanol (Whelan and Hightower, 1985). In our experiments, it was observed that different concentrations of 2-mercaptoethanol enhanced basal as well as FSH-stimulated lactate production (Table 5.1), indicating that this compound may also be a stress factor for cultured Sertoli cells. It should be noticed that in addition to a supposed stress effect, 2-mercaptoethanol might influence the hexose carrier directly. 2-DOG transport in myoblasts was inhibited by covalent crosslinking of sulfhydryl groups (D'Amore and Lo, 1986b). 2-Mercaptoethanol may exert a protecting action on the sulfhydryl groups of the hexose carrier. A similar effect may possibly contribute to the observed stimulatory effect of 2-mercaptoethanol on lactate production by Sertoli cells. Furthermore, Hutson (1984) reported effects of changes in osmolarity and pH of the medium on lactate production by Sertoli cells. Apparently, stress factors may induce lactate release from Sertoli cells. It cannot be excluded, although it is difficult to explain, that renewal of the medium

Table 5.1. Effect of 2-mercaptoethanol on lactate production.
After 3 days of incubation Sertoli cells were incubated during 6 h in the presence and absence of FSH (0.5 μg/ml), in combination with different concentrations of 2-mercaptoethanol (2-ME). The results represent duplicate incubations.

	nmol lactate/mg protein per h			
	2-ME concentration (mM)			
Incubation condition	0	0.05	0.5	5
Control	113; 129	137; 155	227; 293	388; 454
+ FSH	332; 228	337; 369	420; 450	488; 550

at day 4 and later is a stress factor for cultured Sertoli cells, inducing increased lactate production. The results indicate, that when Sertoli cell metabolism is studied in an *in vitro* system, care has to be taken to avoid possible stress factors.

5.2.1 Time-course of the FSH effect on lactate production

Many different effects of FSH on cultured Sertoli cells from immature rats have been reported. Because large differences in the time-courses of the different FSH effects were observed, the time-course of a number of these FSH effects will be discussed below and compared with our observations on the time-course of FSH-stimulated lactate production (Figure 5.2).

The rate of ABP production by Sertoli cells from 3-week-old rats in culture was not maintained at a constant level, but decreased with time from the first day of incubation onwards. This decrease could be partly prevented only when FSH was present in the culture medium from the first day of incubation (Fritz et al., 1976; Rommerts et al., 1978). Co-culture with peritubular cells can markedly improve some long-term functions of Sertoli cells, such as the rate of ABP production (Tung and Fritz, 1980).

Transferrin production by Sertoli cells was increased by FSH maximally between 4 and 6 days of incubation when FSH had been continuously present from the first day of incubation, and after 6 days the stimulatory effect declined (Skinner and Griswold, 1982).

Aromatase activity in immature rat Sertoli cells was at a low constant level under basal conditions, but stimulation of aromatase activity was initiated and maintained in the continuous presence of FSH

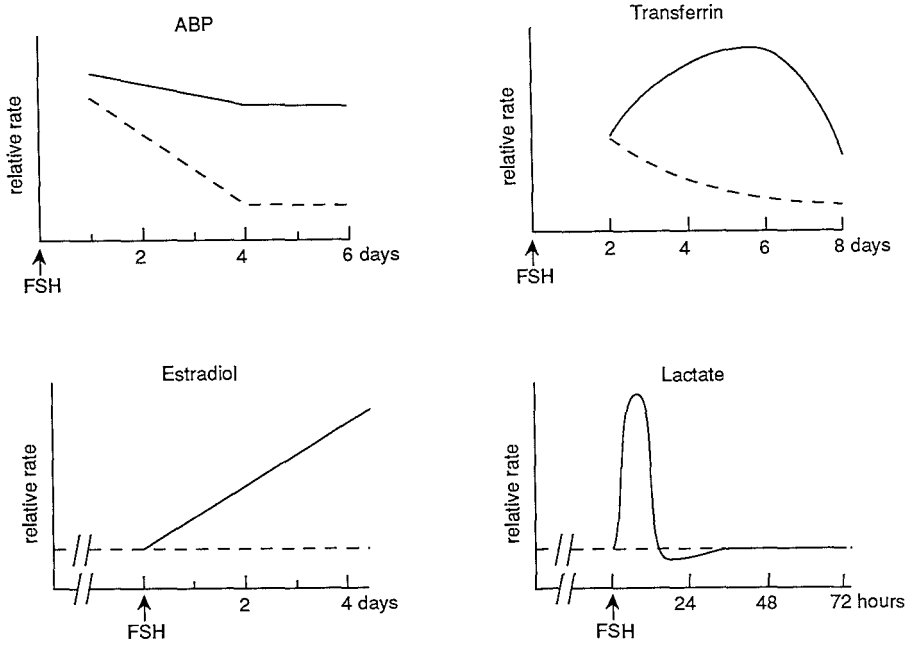

Figure 5.2. Schematic representation of the different time-courses of the production rates of ABP, transferrin, estradiol and lactate. The rate of ABP production and estradiol production were adapted from Rommerts et al. (1978). The rate of transferrin production was adapted from Skinner and Griswold (1982). The rate of lactate production was adapted from Oonk et al. (1985) and from appendix paper 5.

for several days (Rommerts et al., 1978; Dorrington et al., 1978). This could also be achieved when FSH was not added directly from the first day of incubation but from the fifth day of incubation (Dorrington and Armstrong, 1979). A difference between ABP and transferrin production, on the one hand, and estradiol and lactate production (see below) on the other hand, is that the former products required the presence of FSH from the start of the incubation. It can be suggested that after isolation some cell properties diminish or are lost, which are essential for the stimulation of ABP and transferrin production, but not for estradiol and lactate production.

FSH furthermore stimulated amino acid incorporation into proteins (Dorrington et al., 1975; Means et al., 1976) and influenced the synthesis of several proteins (Wilson and Griswold, 1979; DePhilip and Kierszenbaum, 1982). These effects all required prolonged (> 6 h) presence of FSH and could be suppressed by inhibition of protein synthesis with cycloheximide.

The time-course for FSH-stimulation of lactate production was markedly different from most of the above mentioned FSH-stimulated processes. When Sertoli cells were preincubated in the absence of hormones for several days, and subsequently FSH was added, lactate production was stimulated within 60 min. This rapid increase in lactate production was not dependent on the duration of preincubation in the absence of hormones, and was followed by a decline to, and sometimes below, basal levels of lactate production after 9-24 h of incubation (appendix paper 3). The effect of FSH on lactate production rate was very similar to that of insulin, concerning time-course, desensitization, and independence of protein synthesis and extracellular Ca^{2+}. The most marked difference observed was the level of cAMP, which was increased by FSH but not by insulin (appendix paper 3). A discrepancy exists between the time-course of FSH-stimulated cAMP production and all other effects of FSH, including FSH-stimulated lactate production, as discussed in section 5.2.

5.3 Metabolism of radiolabelled substrates

The metabolism of glucose by cultured Sertoli cells has been shown to result mainly in lactate production. Robinson and Fritz (1981) found that of the total amount of [U-^{14}C]glucose utilized almost 96% was converted to anions, mainly lactate, less than 3% was converted to CO_2 and the remainder was incorporated into lipids and glycogen. Our results indicate that 1.2-1.6% of [U-^{14}C]glucose was converted to lipids (appendix paper 3) and 1-6% was converted to CO_2 (appendix paper 4). This type of metabolism performed by the Sertoli cells, viz. the conversion of glucose to lactate in the presence of oxygen, is similar to the glucose metabolism as observed for cultured proliferating normal and tumour cells (Lanks et al., 1986).

Apparently, Sertoli cells do not need the maximal amounts of energy from glucose under basal and FSH-stimulated conditions. When oxidation rates of several substrates are compared with respect to their possible energy yield in the form of ATP, it becomes clear that Sertoli cells can also produce much ATP from the oxidation of fatty acids and amino acids (appendix paper 4; Jutte et al., 1985). This agrees with observations (Jutte et al., 1982), that Sertoli cells survive for at least 24 h in the absence of glucose. The hormonal regulation of amino acid and fatty acid metabolism is for the greater part unknown. Glutamine oxidation was slightly enhanced by dbcAMP (appendix paper 4). FSH and insulin had no stimulatory effects on leucine oxidation (Figure 5.3). It is not known, whether the oxidation of fatty acids by Sertoli cells is subject to hormonal control (Jutte et al., 1985). The conversion of glucose to lactate under aerobic conditions may not be of vital importance to the Sertoli cells themselves, but might serve the purpose of producing the energy-yielding substrate lactate, which can be used by the spermatogenic cells.

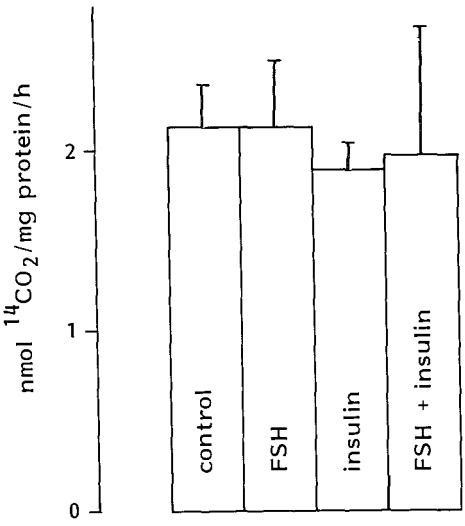

Figure 5.3. Effects of FSH and insulin on leucine oxidation by cultured Sertoli cells. Production of $^{14}CO_2$ from 0.4 mM L-[U-^{14}C]leucine was estimated during 6 h in the presence or absence of FSH (0.5 µg/ml) and/or insulin (5 µg/ml) as described in appendix paper 4. The results represent mean ± S.D. of triplicate incubations.

5.4 Glucose uptake

5.4.1 Cytochalasin B binding to Sertoli cells

The fungal metabolite cytochalasin B (CB) affects motility related cell functions by inhibition of contractile microfilaments (Wessells et al., 1971). It also inhibits sugar transport in several different cell types (Kletzien and Perdue, 1973). Also in Sertoli cells, 2-deoxyglucose uptake was inhibited for approximately 90% by CB (appendix paper 5). In human erythrocytes and in Ehrlich ascites tumour cells CB was observed to bind to three types of CB-binding proteins (Jung and Rampal, 1977; Cuppoletti et al., 1981). The binding of CB to one of these binding sites was competitively inhibited by D-glucose, indicating that this site may represent the glucose transporter. Another part of the CB-binding was sensitive to displacement by cytochalasin E (CE), and this part may be associated with cytoskeletal proteins. This method of determining D-glucose inhibitable cytochalasin B-binding sites has been used as a measure of glucose carriers also in several other cell types, such as adipocytes (Cushman and Wardzala, 1980; Suzuki and Kono, 1980) and cultured muscle cells (Klip et al., 1982).

The objective of our cytochalasin B binding studies was to obtain information about the number of glucose carriers present on Sertoli cells and their affinity for glucose and the possible regulation of number and/or affinity by FSH and insulin. In cultured Sertoli cells we observed CB-binding reaching equilibrium between 10 and 30 min of incubation. No stimulation by FSH or insulin was found. CB-binding was displaced by high concentrations of CB, and part of this binding was sensitive to CE. However, D-glucose hardly decreased the binding of CB, and meaningful Scatchard analysis was impossible.

Our observations are compatible with studies on rat hepatoma cells (Plagemann et al., 1977) and normal chick embryo fibroblasts (Salter and Weber, 1979). In these studies no inhibition of CB binding by D-glucose was observed, although the authors reported inhibitory effects of CB on hexose transport (Plagemann et al., 1977; Salter and Weber, 1979). For various reasons, we decided not to continue work on cytochalasin B binding as a tool to measure glucose carriers in Sertoli cells.

5.4.2 2-Deoxyglucose uptake studies

Further studies concerning glucose transport in Sertoli cells were performed using the non-metabolizable glucose analogue 2-deoxyglucose (2-DOG). Olefsky (1978) has evaluated in detail the use of this compound for uptake studies. Short-term incubations of cultured Sertoli cells in the presence of [^{3}H]-2-DOG and in the presence or absence of added hormones yielded the following results. Uptake of 2-DOG was inhibited by cytochalasin B, but not by cytochalasin E. D-glucose appeared to be a strong competitor for 2-DOG uptake, while D-galactose was much less effective. D-

galactose can also be transported by the hexose carrier but with a lower affinity than D-glucose (Klip et al., 1982; D'Amore and Lo, 1986a). Under conditions where transport was rate-limiting, the kinetic constants were determined. It appeared that insulin and IGF–I increased the V_{max} for 2-DOG uptake to 130% and that FSH had no effect. The K_m for 2-DOG uptake was not influenced by any of the hormones. These results are presented in detail in appendix paper 5.

In most systems in which hexose transport was investigated, conditions were established in which the transport of 2-DOG was the rate-limiting step (Kletzien and Perdue, 1973; Olefsky, 1978; Wieringa et al., 1981; Klip et al., 1982; D'Amore and Lo, 1986a). A problem one has to face is that high concentrations of 2-DOG (1-10 mM) caused a rapid decline in ATP levels in adipocytes (Wieringa et al., 1985). Thus, provided that conditions are established in which transport and not hexokinase activity is rate-limiting and the cells can maintain high ATP levels, 2-DOG is a convenient glucose analogue to estimate the glucose transporter step.

Our results (appendix paper 5) are in agreement with the results in adipocytes, which showed an insulin-induced increase in V_{max} of 2-DOG uptake (Olefsky, 1978; Wieringa et al., 1982).

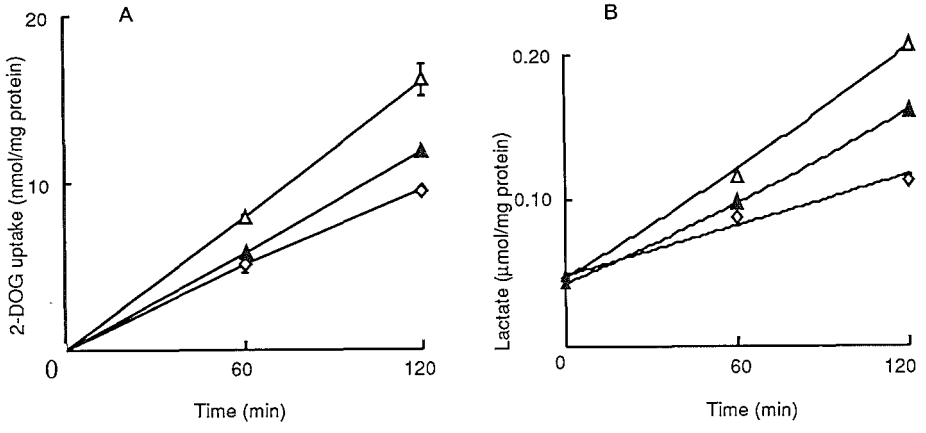

Figure 5.4. A) Effects of FSH and insulin on 2-deoxyglucose uptake in the presence of D-glucose. Sertoli cells were washed 3 times with PBS, followed by a 60 min incubation in PBS, containing 0.5 mM D-glucose. After this 60 min period, hexose uptake measurements were started by addition of 50 µM [^{3}H]-2-DOG. Control (◇—◇). 0.5 µg/ml FSH, (▲—▲) or 5 µg/ml insulin, (Δ—Δ) were added simultaneously with the radioactive 2-DOG. After 60 and 120 min the incubations were terminated and hexose uptake determined as described in appendix paper 5. The results represent mean ± S.D. of triplicate incubations. B) Effects of FSH and insulin on lactate production from 0.5 mM D-glucose. Incubations were carried out under the same conditions as described under A). FSH and insulin were added after 60 min of incubation in PBS, containing 0.5 mM D-glucose. This was termed t = 0. After 60 and 120 min the incubations were terminated and the amount of lactate determined as described in appendix paper 5. The results represent the mean of duplicate incubations differing not more than 12%.

In Sertoli cells, 3-O-methylglucose (3-OMG) transport has been investigated (Hall and Mita, 1984; Mita et al., 1985). It has been discussed in appendix paper 5 that the authors measured stimulatory effects of FSH, insulin and IGF–I on the long-term, intracellular accumulation of 3-OMG, but that these results probably do not reflect initial uptake rates of 3-OMG (see above).

It has been observed by Foley et al. (1980) that, in adipocytes, the uptake of trace amounts radiolabelled 2-DOG was accelerated when the cells had been preincubated with a high concentration of glucose. This acceleration was only provoked by sugars which, like D-glucose, are metabolized via the glycolytic pathway. In this respect, our results, that FSH did not stimulate glucose transport, might indicate that FSH does not stimulate glucose uptake directly, like insulin and IGF–I, but might alter the concentration of glycolytic intermediates by affecting the activities of enzymes in the glycolytic pathway. This is suggested, because FSH stimulated lactate production, and in these incubations 5.6 mM D-glucose was present. We have studied the possibility of indirect effects of the concentration of glycolytic intermediates by estimating the effects of FSH and insulin during incubation of Sertoli cells for 2 h in the presence of 0.5 mM D-glucose.

Under these conditions, 2-DOG uptake reached approximately 70% of its maximal value, as was expected due to competition of D-glucose and 2-DOG for the same carrier (appendix paper 5). FSH appeared to stimulate 2-DOG uptake slightly ($P < 0.01$) under these circumstances (Figure 5.4a), although to a lesser extent than insulin. When lactate production from 0.5 mM D-glucose was studied, similar stimulatory effects were observed for FSH and insulin (Figure 5.4b). These results may indicate that FSH stimulates glucose transport by Sertoli cells *in vitro* via a process which involves glycolytic intermediates, rather than via direct activation of the glucose carrier, by increasing its V_{max} for glucose.

The observed stimulatory effects of insulin and IGF–I on lactate production by Sertoli cells were much larger than the effects of these hormones on the rate of glucose transport. Moreover, the effect of FSH on lactate production was pronounced, while FSH did not stimulate glucose transport at all. This may suggest that glycolytic enzyme activities also are stimulated by these hormones. The mechanism of activation of glycolytic enzymes by hormones is not known. Recently, the phosphorylation of glycolytic enzymes on tyrosine residues by the insulin receptor kinase has been reported (Sale et al., 1987), using a cell-free *in vitro* system of isolated and purified proteins. The highest amount of phosphotyrosine was detected on phosphofructokinase (Sale et al., 1987). It has yet to be determined if in intact cell systems tyrosine phosphorylation of glycolytic enzymes alters their enzymatic properties and if this process is involved in the stimulation of the rate of glycolysis by insulin, and by other hormones.

5.5. Conclusions

1. Lactate production by Sertoli cells can be stimulated, to the same extent and following the same time course, by FSH, insulin and IGF–I. The time-course of the FSH effect on lactate production is markedly different from other, tropic effects of FSH. A number of stress factors may also induce increased lactate production.

2. The metabolism of glucose by Sertoli cells is probably not of vital importance for energy production in the Sertoli cells, but is directed mainly to conversion of glucose to lactate and pyruvate.

3. The maximal velocity of 2-DOG uptake by Sertoli cells can be increased by insulin and IGF–I, but not by FSH. The hormones did not change the K_m for 2-DOG uptake.

4. The rate of glucose conversion to lactate by Sertoli cells is not only rate-limited by glucose influx via the glucose carrier. FSH may enhance lactate production via hormone stimulation of enzyme activities in the glycolytic pathway. Insulin and IGF–I on the other hand seem to affect both glucose transport and glycolytic enzyme activities.

CHAPTER 6

GENERAL DISCUSSION

6.1 Introduction

In adult rat testes, developing germ cells rapidly degenerate when FSH and testosterone levels are lowered by hypophysectomy. In immature rats, hypophysectomy causes germ cell degeneration and an arrest of testis development. Besides FSH and testosterone, several other hormones and growth factors may be involved in the development and modulation of testicular functions (Chapter 2). Furthermore, some locally produced undefined factors may be involved in cell-cell interactions between the different cell types.

Sertoli cells are target cells for different hormones and can be considered as mediators in the action of hormones on spermatogenesis. *In vitro* glucose metabolism could be stimulated by FSH. Based on these observations, it has been postulated that the efffect of FSH on spermatogenesis might be mediated by the effects on lactate production in Sertoli cells. In general, however, insulin is one of the important regulators of glucose metabolism, whereas IGF–I can also regulate glucose metabolism in an insulin-like way. IGF–I, however, is mainly involved in growth and development. Hence, the studies described in this thesis were directed to identify and characterize the receptors for insulin and IGF–I on cultured Sertoli cells from immature rats. Furthermore, we were interested on the one hand in different aspects of the regulation of glucose metabolism in cultured Sertoli cells by FSH, insulin and IGF–I, and on the other hand in developmental aspects of the regulation of insulin and IGF–I receptor numbers.

In section 6.2 we will discuss hormone effects on Sertoli cell glucose metabolism and the possible role of glucose metabolism for spermatogenesis. In this respect, the effects of interference with glucose metabolism on spermatogenesis will also be considered. The involvement of insulin and IGF–I and their receptors with respect to growth and development in general and in gonadal development and puberty in particular, will be discussed in section 6.3. In section 6.4 possible functions of insulin and IGF–I with respect to the hormonal regulation of spermatogenesis will be discussed.

6.2 Regulation of glucose metabolism by FSH, insulin and IGF–I

Cultured Sertoli cells can convert glucose to lactate at a high rate (Chapter 5). Most of the lactate is released into the incubation medium. It has been discussed in Chapter 5 and section 2.7.1 that lactate from Sertoli cells may support the ATP requirements of the spermatogenic cells. However, it has never been proven that germ cells *in situ* require exogenous lactate, although spermatocytes co-incubated with Sertoli cells in isolated tubular fragments could survive in medium without added lactate, apparently because they were supplied with lactate from the Sertoli cells (Jutte et al., 1982). Furthermore, reduction of α-ketoisocaproate to α-hydroxyisocaproate by germ cells (Grootegoed, 1985a) might occur concomitantly with the oxidation of lactate to pyruvate, thus providing an NADH-reoxidizing system for the spermatogenic cells (Grootegoed et al., 1985a, b). This may be an indication that lactate is used as an energy substrate by the spermatogenic cells *in situ*.

Regarding the amount and concentration of lactate, which may be needed by the spermatogenic cells, it appears that isolated spermatids and spermatocytes can maintain

high ATP levels in the presence of 3-6 mM L-lactate. Certainly, the lactate concentration in the spermatogenic microenvironment should not exceed a certain limit, because lactate in high concentrations may be toxic, as has been shown for neurons in the brain. Nevertheless, brain tissue converts an appreciable fraction of total glucose to lactate. Cohen (1985) postulated that a certain excess of lactate is deliberately maintained to ensure permanent availability of energy substrates in every part of the brain. This hypothesis might also apply for the spermatogenic epithelium, which resembles brain tissue in that both brain tissue and the spermatogenic epithelium are critically dependent on energy metabolism. It is attractive to propose a buffer function for lactate also in the testis, providing the presence of a readily available energy substrate for the spermatogenic cells.

A number of observations has indicated that spermatogenesis *in vivo* is very sensitive to disturbances in energy metabolism. Interference with glucose metabolism, causing a decrease in ATP content of spermatogenic cells, may induce interruption of spermatogenesis. In this respect, detrimental effects on spermatogenesis of some toxic agents and of diabetes will be discussed in section 6.2.1 and 6.2.2.

With respect to lactate production by Sertoli cells *in vitro*, the following observations appear relevant:

- Sertoli cells *in vitro* increase lactate production upon a variety of stimuli. These stimuli include FSH (Jutte et al., 1983), glucagon (Eikvar et al., 1984), insulin (Oonk et al., 1985) and EGF (Mallea et al., 1986).
- Insulin and glucagon, however, are known to have opposite effects on glucose metabolism in their target tissues, and FSH stimulates and EGF inhibits Sertoli cell aromatase activity (Dorrington et al., 1978; Mallea et al., 1986).
- Moreover, incubation temperature, medium osmolarity and medium pH (Hutson, 1984), 2-mercaptoethanol and repeated medium renewal (Chapter 5) were found to have pronounced effects on lactate production by Sertoli cells.

We have interpreted these results as an indication that a broad spectrum of factors is likely to increase lactate production, because these factors might impose "stress" upon the cells. Hormonal stimulation of cellular activities could be such a stress factor. However, factors which increase lactate production by Sertoli cells *in vitro* may not be involved in such an effect on Sertoli cells *in vivo*. Nevertheless, lactate production may be a useful parameter to study hormone effects on Sertoli cells.

The results presented in Chapter 5 indicate that FSH had insulin-like stimulatory effects on lactate production. FSH stimulation of lactate production followed a typical time-course, compared with other actions of FSH. The initial step in glucose metabolism, viz. glucose uptake, was not a primary target of FSH, whereas it could be stimulated by insulin.

Even if FSH can stimulate glucose metabolism by Sertoli cells *in vitro*, it may be suggested that regulation of glucose metabolism by FSH may not be an important function of FSH *in vivo*. This may also be inferred from the diminishing influence of FSH during maturation of the testis (Fritz, 1978), which renders an important role for FSH in glucose metabolism by adult testes less plausible. Spermatogenic cells also in adult testes may still require lactate as energy-yielding substrate. In fact, it is most likely that, in accordance with its function in many other tissues, insulin is the main regulator of glucose metabolism in testicular tissue. However, on cultured Sertoli cells and in testicular membrane fractions from immature rats the amount of insulin receptors was found to be very low, in contrast to the number of IGF–I receptors (Chapter 4). Lactate production and the maximal rate of glucose transport by cultured Sertoli cells could be stimulated by low doses of insulin and also by IGF–I (Chapter 5). The results presented in this thesis suggest that in immature rats glucose transport and glucose metabolism in testicular tubules can be regulated by insulin via the high affinity insulin receptors, and also by IGF–I, exerting insulin-like effects via IGF–I receptors. It has been reported, that in chinese hamster ovary cells a relatively high insulin sensitivity is observed when the insulin receptor number is high, and *vice versa* (Ebina et al., 1985). In this

respect, it may be suggested that, as the amount of high affinity insulin receptors appeared to increase with age, the sensitivity of the testis for insulin may become more prominent with increasing age.

6.2.1 Effects on spermatogenesis of some chemicals which may interfere with glucose metabolism

In the previous section we discussed the importance of glucose metabolism with respect to the maintenance of the ATP content of spermatogenic cells. Inhibition of glucose metabolism, caused for example by certain chemicals or by diabetes, may interfere with spermatogenesis.

Gossypol, a compound isolated from the seeds of the cotton plant, has been considered as a potential male anti-fertility agent, as it effectively inhibits sperm function and also spermatogenesis (Prasad, 1984). However, its applicability is limited by toxic side effects (Prasad, 1984). The mechanism of action of gossypol *in vivo* has not yet been elucidated. There are indications that gossypol interferes with cellular energy metabolism. Reyes et al. (1984) reported that gossypol acts as an uncoupler of oxidative phosphorylation, decreasing the efficiency of mitochondrial ATP production. Spermatogenic cells may produce ATP at a close to maximal rate (Grootegoed et al., 1984) and thus may be more susceptible to the effects of gossypol, than cells with a large reserve capacity of mitochondrial oxidative phosphorylation.

A similar reasoning could be applied in the case of the effect of the rat poison fluoroacetate, which is converted via lethal synthesis to the aconitase inhibitor fluorocitrate (section 2.7.1). Thus, this agent, which at low doses inhibits spermatogenesis, also interferes with mitochondrial ATP production.

5-Thio-D-glucopyranose (thioglucose), an analogue of D-glucose, fed to mice and rats induced complete loss of sperm production by degeneration of all germ cells except spermatogonia (Zysk et al., 1975; Homm et al., 1977; Lobl and Porteus, 1978). The sterility induced was sometimes irreversible. Thioglucose inhibited active, Na^+-dependent D-glucose uptake (Whistler and Lake, 1972), but did not affect facilitated diffusion through the glucose carrier, estimated as deoxyglucose uptake (Davies and Meanock, 1981). The mechanism of action of thioglucose is not clear, but it is likely that it decreases glucose metabolism, which might explain its antispermatogenic action. Thioglucose has been considered for use as a male antifertility agent. However, in rats and mice thioglucose induces hyperglycemia (Lobl and Porteus, 1978). This effect of thioglucose seems compatible with the observed inhibition of D-glucose uptake. The hyperglycemic (diabetogenic) effect of thioglucose rendered it unacceptable as an antifertility drug.

In general, the *in vivo* effects of these drugs seem to support the *in vitro* observations, indicating a very high susceptibility of spermatogenic cells to disturbances of energy metabolism.

6.2.2 Diabetes and testicular function

Diabetes mellitus often results in disturbances in male reproductive function (Rodriguez-Rigau, 1980). Studies on the testicular function of diabetic men gave divergent results in the past, probably because the severity of diabetes and the degree of diabetic control by insulin treatment varied greatly between different patients (review: Rodriguez-Rigau, 1980).

Since the discovery that the drug streptozotocin induced hyperglycemia by causing degeneration of pancreatic islets in rats and dogs (Rakieten et al., 1963; Junod et al., 1967) this drug has been used in numerous studies to render laboratory animals diabetic. Streptozotocin, however, also causes anti-leukemic effects (Evans et al., 1965). Nevertheless, experimental diabetes induced in laboratory animals has the advantage to study the effects of diabetes not treated with insulin. It appears, however, that the reaction of animals to streptozotocin is not uniform. Not all animals develop the same degree of diabetes, and also the detrimental effects on spermatogenesis observed are different from one animal to another (see below).

A study, using rats that had received streptozotocin but had not developed hy-

perglycemia, showed that no significant changes in testicular histology had occurred (Anderson and Thliveris, 1986). Only streptozotocin-diabetic rats, after three months of diabetes, had significantly decreased tubule diameters and an increased testicular vascularization (Anderson and Thliveris, 1986). This decrease could be prevented by insulin treatment. In previous studies it had been observed that in streptozotocin-diabetic rats the effects on tubule diameter and sloughing of germinal epithelium were not observed for all tubules (Oksanen, 1975). Some tubules showed severe atrophy, whereas others showed the full complement of spermatogenic cells, although the numbers of germ cells were diminished (Oksanen, 1975). When rats were made streptozotocin-diabetic at prepuberal age, the complete spermatogenic cycle could be identified after five months of diabetes, although the number of spermatozoa produced was severely reduced (Paz et al., 1978). This indicates a quantitative, rather than a qualitative effect of streptozotocin-diabetes on spermatogenesis. In adult streptozotocin-diabetic rats the numbers of interstitial cells, and FSH, LH and testosterone levels were decreased (Paz et al., 1978; Murray et al., 1981), indicating concomitant effects at the hypothalamic-pituitary level and at the testicular level.

Recent studies in diabetic men have shown that testicular volume and sperm production were reduced, and plasma gonadotropin levels were elevated significantly, when compared with an appropriate control group of age-matched normal men (Handelsman et al., 1985a). Testosterone levels were not different in the two groups (Handelsman et al., 1985a). Morphologically, disrupted spermatogenesis and degenerative changes in apical Sertoli cell cytoplasm have been observed (Cameron et al., 1985).

From the studies on experimental diabetic animals and on insulin-treated diabetic men, it appears that testicular dysfunction may result from several different effects, caused by the diabetic condition. The hypothalamic-pituitary-gonadal axis may be disturbed by impaired GnRH secretion due to chronic hyperglycemia (Handelsman et al., 1985a). The abnormalities seen in testicular tissue may in part result from the insulin deficiency, as insulin may be required to maintain proper Sertoli cell and Leydig cell functions, acting directly on these cells or indirectly via functional alterations at the hypothalamic and pituitary level. The disturbances in carbohydrate metabolism caused by diabetes may affect the testis on different levels. First, reduced intracellular availability of glucose and pyruvate caused by insulin deficiency may interfere with the energy metabolism of testicular cells. Second, the hyperglycemia caused by diabetes may have detrimental effects on cell replication and viability, as has been reported for cultured endothelial cells (Lorenzi et al., 1985).

In summary, diabetes may result in testicular dysfunction by affecting the levels of the hormones, required for spermatogenesis, and by causing disturbances in carbohydrate metabolism which may have detrimental effects on testicular cells. Insulin deficiency and hyperglycemia may both play an important role in these effects.

6.3 Role of IGF–I and insulin in growth, puberty and testicular development

In this section the present results on insulin and IGF–I binding to Sertoli cells and to testicular membranes will be discussed in the context of literature on insulin/IGF–I hormone levels and the presence of receptors for insulin/IGF–I during fetal growth and development, and during puberty. A large number of IGF–I receptors were present on Sertoli cells and on testicular membrane fractions from immature rats, and IGF–I appeared to influence cultured Sertoli cells. IGF–I may influence the immature testis, promoting growth and development and also exerting metabolic effects. In this respect, one may wonder whether FSH exerts a specific effect in the immature testis. One could ask, which processes are triggered and promoted by FSH, but not by IGF–I? The effects of age and puberty on FSH levels and FSH receptor levels will be considered.

- Levels of FSH and FSH receptors at different ages

In the testis, gonadotropin receptors show a developmental pattern. Receptors for FSH and LH could be detected first at 15-17 days of gestation (Warren et al., 1984). FSH binding in fetal rats increased by day 20-21, which is also the time period of maximal Sertoli cell proliferation (Orth, 1982). The studies by Orth (1984), estimating DNA synthesis by fetal Sertoli cells from intact, decapitated, or anti-FSH injected rat fetuses, strongly suggested that FSH has a central role in regulating Sertoli cell proliferation in fetal rat testes. Fetal FSH- and LH-levels in rats have not been reported. In the first two weeks of postnatal life plasma levels of FSH are low, but show a transient increase with a peak between days 20 and 40, i.e. during the first wave of spermatogenesis (Thanki and Steinberger, 1978; De Jong and Sharpe, 1977; Ketelslegers et al., 1978). The peak in FSH level does not correlate with the peak in testis FSH receptors (see below), but it strongly correlates with an increase in LH receptors and precedes the puberal rise in testosterone (De Jong and Sharpe, 1977; Ketelslegers et al., 1978). This suggests that in addition to the actions of FSH on testicular tubules, also the maturation of Leydig cells may be partially under the control of FSH. After birth, the concentration of LH receptors in total testis (expressed per mg protein) increased up to 60 days of age, but the concentration of FSH receptors (expressed per g tissue or per mg protein) showed a peak between 10 and 20 days of age, thereafter declining to a constant low level. The affinity of FSH binding did not change with age (Thanki and Steinberger, 1978; Ketelslegers et al., 1978). The total number of FSH receptors per testis increased 6- to 8-fold after 20 days of age up to 90 days. Sertoli cell numbers did not increase more than two times after 20 days of age (De Jong and Sharpe, 1977), which means that the FSH receptor numbers per Sertoli cell increase. However, the response of Sertoli cells to FSH and the effect of FSH on spermatogenesis seem to decrease with age. The changed responsiveness of the testis to FSH cannot be attributed to changes in the affinity of the FSH receptors. It is not clear therefore how the high FSH receptor concentration on adult Sertoli cells is related to the relatively low sensitivity of the adult Sertoli cells and the spermatogenic process to FSH. However, it is known that in older rats the activity of adenylate cyclase (section 2.2.4) may be decreased and that phosphodiesterase activity is increased (Means et al., 1980). Therefore, care must be taken in drawing conclusions with respect to the physiological importance of changes in hormone receptor numbers with age, as besides changes in receptor number, changes in sensitivity to the hormone may be caused by other factors.

- Regulation of FSH levels in immature rats

The possible role of inhibin and testosterone in the regulation of FSH levels in the immature male rat have been discussed in section 2.1. Several observations support the importance of inhibin, especially in immature rats.

- Possible role of FSH at adult age

It has been discussed above that in adult hypophysectomized rats FSH is required to restore regressed spermatogenesis and to supplement quantitatively the effect of testosterone in the maintenance of spermatogenesis. In seasonal breeders, FSH probably is necessary for the reinitiation of spermatogenesis and testicular growth. In other animal species, which are not seasonal breeders, FSH still might play a role in the quantitative maintenance of spermatogenesis. However, in general it seems that testosterone levels are adequate to maintain spermatogenesis in the adult male. It would be interesting to suppress FSH production or action completely and selectively to elucidate a possible long-term function for FSH at adult age.

- Role of insulin in growth regulation

Insulin appears to be essential for normal growth and development, in addition to its known effects on metabolic regulation (Straus, 1984). Abnormalities in insulin production or in the sensitivity to insulin are accompanied by abnormalities in growth (see below). The neutralization of insulin by insulin antibodies caused severe growth

retardation in chick embryos, whereas insulin administration accelerated chick embryo growth (De Pablo et al., 1985). Two lines of evidence from pathological conditions indicate the importance of insulin as growth-promoting hormone in humans *in vivo*. Diabetes in human infants, either by insulin deficiency or by extreme resistance to insulin action, is characterized by retarded fetal growth, small muscle mass, and impaired deposition of fat tissue. These infants usually fail to thrive and grow (Straus, 1984). In contrast, neonates born to poorly controlled diabetic mothers often have excessive size and weight, which has been attributed to the stimulation of fetal insulin production by the transplacental passage of excessive glucose, and the subsequent action of insulin as a growth factor (Straus, 1984). The actions of insulin in these children may be divided in metabolic effects of insulin interacting with insulin receptors, for example on increased fat deposition and protein and glycogen synthesis, and growth effects via IGF–I receptors resulting in excessive bone and organ growth (Straus, 1984). It has not yet been determined in these patients to what extent the growth-promoting effects of insulin are mediated by direct action of insulin, or by regulatory effects on IGF–I release and IGF–I action (see below).

- Insulin may exert growth promoting action via IGF–I receptors

Insulin binding sites could be detected in early stages of embryogenesis in chicken and mouse (Hendricks et al., 1984; Rosenblum et al., 1986). In human fetal liver and brain also insulin receptors were present (Sara et al., 1983). Insulin binding in human and rat liver increased with fetal age (Sara et al., 1983; Blázquez et al., 1986), and in rat liver reached a maximum in late fetal and early neonatal life (Blázquez et al., 1986). Insulin appears to be essential for fetal growth (see above), but in early life the insulin receptor number is low, and experiments inducing growth of fetal tissues in culture required high doses of insulin. It is suggested, therefore, that insulin acts as a growth modulator via receptors for IGF–I. High doses of insulin could stimulate growth of the bones in fetal rat paws *in vitro* (Cooke and Nicoll, 1984). However, *in vivo* such high concentrations of insulin are not present.

Embryonic chick cartilage could grow *in vitro* in the absence of serum by production of its own IGF–I-like peptide. After this peptide was immunoneutralized by IGF–I antibody, addition of high concentrations of insulin mimicked the IGF-like effect (Burch et al., 1986). Insulin-dependent diabetes and also malnutrition are often accompanied by decreased serum IGF–I levels. In Chapter 2 we have discussed that growth hormone is the principal regulator of IGF–I production. Maes et al. (1986) have suggested from *in vivo* experiments with insulin-dependent diabetic or malnourished rats, that in these conditions insulin is required to restore growth hormone-regulated IGF–I levels, indicating indirect growth promoting action of insulin. Two conclusions can be drawn from these *in vitro* and *in vivo* experiments, viz. that high concentrations of insulin may replace somatomedin and induce growth responses, and further that insulin influences the effect of growth hormone on local somatomedin production.

- Relative importance of insulin, IGF–I and IGF–II in fetal development.

Early in human gestation receptors for IGF–I and IGF–II are found in fetal brain and liver (Sara et al., 1983). In rats, IGF–II levels in fetal serum were higher than in adult serum (Daughaday et al., 1982). However, it has been reported that serum IGF–I levels were considerably lower in fetal rats than in adult rats (Sara et al., 1980; Daughaday et al., 1982) and almost undetectable in human fetal serum (Sara et al., 1983). Since fetal development is a condition characterized by very rapid growth, the observed low IGF–I serum levels in humans at birth seemed puzzling. Sara et al. (1986), however, have demonstrated the existence of a variant fetal brain somatomedin, a truncated form of IGF–I, which crossreacts with fetal brain IGF–I receptors. Besides the possible presence of such a variant IGF–I in serum, another explanation may be feasible. It has been shown that a number of fetal human tissues contain high IGF–I levels which could not

have accumulated from plasma (D'Ercole et al., 1986). The presence of mRNAs for IGF–I and IGF–II in connective tissues of several human fetal organs (Han et al., 1987) shows that IGFs can be produced in tissues. The amount of mRNA for IGF–II was much larger than that for IGF–I (Han et al., 1987). Serum IGF–I or IGF–II levels are not indicative for IGF activity in the tissues. IGF–II is thought to have its most pronounced role in fetal growth. It is also produced by cultured embryonal cells (Nagarajan et al., 1985). IGF–II mRNAs were shown to be present to a much greater extent in fetal rat tissues than in adult rat tissues (Brown et al., 1986; Lund et al., 1986). Differences in the abundance of IGF–I mRNAs with respect to age were less marked, although the levels were consistently higher in fetal tissues (Lund et al., 1986). In adult rats, mRNA for IGF–I appeared to be present in all tissues examined, albeit at different levels, whereas IGF–II mRNAs were only detectable in a few adult rat tissues (Murphy et al., 1987). Although in several non-fetal tissues IGF–II receptors can be estimated, a function for IGF–II in full-grown tissues has not yet been elucidated. For insulin binding a peak value was observed at the time of birth in several organs in rat (kidney, muscle, liver, brain) and insulin binding declined towards adult age (Saucier et al., 1981; Blázquez et al., 1986). It was discussed above (see p. 69) that receptor number may not be equivalent to response. On the other hand, the presence of (mRNAs for) IGF–I and IGF–II in human fetal tissues (D'Ercole et al., 1986; Han et al., 1987) together with the presence of IGF–I and IGF–II receptors in the human fetus (Sara et al., 1983) suggests functional importance for IGF–I and IGF–II from early stages of fetal growth. The differences in levels of IGF–I and IGF–II may indicate that IGF–II is the most important regulator of fetal growth in the earliest stages, whereas IGF–I is also important in postnatal life. Insulin plays a major role in regulation of metabolism throughout life, but also influences the regulation of IGF–I production.

- Possible role of IGF–I in puberty

In humans the serum IGF–I levels increase in correlation with the stage of puberal development, whereas there is no good correlation with growth rate (Luna et al., 1983; Furlanetto and Cara, 1986). Serum IGF–I levels in the male correlated well with the increase in testosterone at puberty (Caufriez and Copinschi, 1986), which suggests a functional relationship between the regulation of gonadal function at hypothalamic-pituitary level and the circulating IGF–I levels. Despite this correlation, Handelsman et al. (1987) demonstrated that in rats the puberal rise in IGF–I was increased rather than prevented by prepuberal castration. It was proposed by Handelsman et al. (1987) that the peripuberal IGF–I surge in the rat is not caused by gonadal secretion of IGF–I, as has been inferred by Hall et al. (1983), or induction of IGF–I production by testicular steroids, but is programmed at hypothalamic-pituitary level in the pre- or perinatal period, and occurs independent of the presence of gonads. Growth hormone deficiency in children is often accompanied by retardation of sexual maturation, and in addition to growth, often the progression of puberal development is seen during growth hormone therapy. The actions of growth hormone on the development of puberty may be mediated by IGF–I (Underwood et al., 1986).

6.4 IGF–I and IGF–II in the testis; paracrine factors

In chick embryonal tissues developmental regulation of the numbers of insulin and IGF receptors has been reported (Bassas et al., 1985, 1987). A strong correlation between IGF-binding capacity and growth rate of eye lens epithelial cells was observed, while insulin binding was very low. In the differentiated state of the cells, the insulin binding was higher and IGF–I binding lower (Bassas et al., 1987). Such a developmental pattern for insulin binding has also been observed in other systems. In differentiating cell lines a developmental shift in receptor numbers for insulin and IGF–I was reported similar to that observed in fetal development. Undifferentiated, rapidly proliferating myoblasts and preadipocytes possess low numbers of

insulin receptors but high numbers of IGF–I and IGF–II receptors. During differentiation to myotubes and adipocytes, respectively, insulin binding increased and IGF binding decreased (Beguinot et al., 1985; Shimizu et al., 1986).

In the present experiments (Chapter 4), the age-related differences observed on the increase in insulin binding and the decrease in IGF–I binding in testis tissue between 21 days of age and the adult age resemble the developmental changes described above. This can be interpreted to indicate that IGF–I plays a more important role than insulin in testicular development. Concentrations of IGF–I and IGF–II, and mRNAs for IGF–I and IGF–II in multiple tissues showed a developmental regulation (see above). Some IGF–I immunoreactivity was present in the testis of adult rats (Underwood et al., 1986), and the amount of IGF–I and IGF–II mRNA in adult rat testes was very low (Murphy et al., 1987). No information is available on the testicular content of IGFs and their mRNAs in prepuberal rats. However, effects of IGF–I and IGF–II, and a large amount of binding of IGF–I and IGF–II have been observed using isolated Sertoli cells (Skinner and Griswold, 1983; Borland et al., 1984; Oonk and Grootegoed, 1987b). Furthermore, the secretion *in vitro* of a somatomedin-like compound by Sertoli cells from immature rats, but also by seminiferous tubules from adult rats has been reported (Ritzén, 1983; Hall et al., 1983). Tres et al. (1986), using a monoclonal antibody to IGF–I, observed immunofluorescence on Sertoli cells and peritubular cells, isolated from immature rats. Recently, the partial purification of an IGF–I-like peptide from the culture medium of immature rat Sertoli cells was reported (Smith et al., 1987), which appears to be the rat equivalent of human IGF–I. It would be very interesting to investigate if mRNA levels for the IGFs are more abundant in immature testes than in adult testes.

In summary, the results presented in this thesis indicate that the main role of insulin in the testis may be the regulation of glucose homeostasis, like in the rest of the body, and that, especially in immature rats, IGF–I may be an important factor in testis development.

It has been discussed in this chapter that growth hormone regulates local IGF–I production in different tissues, thereby inducing growth responses in those tissues. Testicular development before puberty, however, is seemingly independent of growth of other tissues and bones. A suggestion for regulation of testicular IGF–I production could be that gonadotropins, in particular FSH acting on Sertoli cells, stimulate IGF–I production during testis development. The IGF–I-like factor from Sertoli cells (Tres et al., 1986) might be one of the postulated FSH-regulated peptides, discussed in section 2.6.3.

Future research should be directed to elucidate the exact roles of insulin and IGF–I in testicular function. Synergistic or antagonistic effects of different factors, and the identity of factors which play a role in cell-cell interactions should be determined. Most importantly, the physiological significance and biochemical mechanisms of paracrine interactions between the different testicular cell types should be clarified.

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SUMMARY

In the seminiferous epithelium of the mammalian testes, Sertoli cells are intimately associated with the spermatogenic cells. Sertoli cells are the target cells for FSH and testosterone, the two main hormones regulating spermatogenesis. Spermatogenic cell development may be supported by compounds secreted by Sertoli cells. FSH stimulates glucose metabolism and lactate production by Sertoli cells *in vitro*, and lactate is an essential energy-yielding substrate for spermatogenic cells. Insulin is known as an important hormone regulating glucose metabolism. Insulin-like growth factor I is predominantly involved in growth and development, but can also perform insulin-like metabolic actions. The aim of the experiments described in this thesis was to identify and to characterize the receptors for insulin and IGF–I on cultured Sertoli cells from immature rats, to obtain insight in the importance of insulin and IGF–I, in connection with FSH effects, as possible modulators of Sertoli cell functions and testis development. In Chapter 1 some aspects of spermatogenesis and the scope of this thesis have been described.

Testicular development in immature animals and the maintenance of spermatogenesis during adulthood are under hormonal control, among others via the secretion of FSH and LH by the pituitary gland. In addition to the primary endocrine control of spermatogenesis by the gonadotropins and the feedback regulation of pituitary gonadotropin secretion by inhibin and testosterone, other hormones and growth factors might be involved in the more precise regulation of testicular function. Other factors locally produced by testicular cells are probably also involved in paracrine cell-cell interactions between the different cell types. In Chapter 2 known information is given on receptors, the mechanisms of action and the possible functions of FSH, insulin and IGF–I, and on a number of other hormones and growth factors and testicular cell-cell paracrine interactions.

Chapter 3 gives a short summary of the methods used in the present studies, and an evaluation of the purity of the Sertoli cell preparations.

In Chapter 4 and Appendix papers 1 and 2 experiments are described that show the presence of insulin and IGF–I receptors on cultured Sertoli cells from immature rats. It appeared from binding studies with ^{125}I-iodinated hormones that a relatively low number of high affinity insulin receptors was present on these Sertoli cells, whereas IGF–I receptors were abundant. Specific binding of insulin to the insulin receptor was also demonstrated using covalent crosslinking of receptor-bound ^{125}I-labelled insulin.

In immature rats, binding of insulin to total testis membrane fractions was relatively low and binding of IGF–I was relatively high. In adult rat testes, the amount of insulin binding was comparable to the amount of IGF–I binding. It appears that the number of insulin and IGF–I receptors change during the development of rat testes. The present results suggest that IGF–I might be more important than insulin during testicular development, and that Sertoli cells might be the principal target for IGF–I in the testicular tubules.

Effects of FSH, insulin and IGF–I on glucose transport and metabolism by cultured Sertoli cells are described in Chapter 5 and Appendix papers 3, 4 and 5. FSH, insulin and IGF–I had comparable effects on lactate production by cultured Sertoli cells. The metabolic effect of FSH followed a time-course which is quite different from the time-course of other tropic actions of FSH. Sertoli cells convert glucose mainly to pyruvate and lactate, and amino acids and fatty acids can be utilized as energy substrates. It was concluded that glucose metabolism is probably not of vital importance for Sertoli cells. FSH had no stimulatory effects on 2-deoxyglucose uptake, whereas insulin and IGF–I increased the maximal velocity of hexose transport. From the discrepancies between the hormone effects on lactate production and on glucose transport it is concluded that the rate of glucose transport is not the sole

determining factor for the rate of lactate production, but that FSH, insulin and IGF–I may influence also enzyme activities in the glycolytic pathway.

Data on disturbances of spermatogenesis, possibly caused in part by interference with glucose metabolism, are discussed to illustrate the importance of energy metabolism for appropriate testicular function (Chapter 6).

The regulation of FSH levels during testicular development, and the role of insulin and IGF–I in relation with receptor numbers for insulin and IGF–I during fetal growth and testicular development is described in Chapter 6.

From the results presented in this thesis, it is concluded (Chapter 6) that in testicular tissue insulin may be the main regulator of glucose transport and metabolism, acting via high affinity receptors for insulin. IGF–I may be an important testicular paracrine factor, regulating many different activities, including glucose metabolism, by Sertoli cells via a large number of receptors on Sertoli cells. Furthermore, IGF–I possibly plays an important role during the development of the testis in immature rats.

SAMENVATTING

In de testikels (testes) van zoogdieren bevinden zich de zaaddragende buisjes (de tubuli seminiferi) en het tussenliggend weefsel (het interstitium). In de zaaddragende buisjes vindt de ontwikkeling van de mannelijke zaadcellen (de spermatogenese) plaats in nauw kontakt met de steuncellen, de Sertoli cellen. In het interstitium bevinden zich verschillende typen cellen, en tevens de capillaire bloedvaten die hormonen en voedingsstoffen aanvoeren. Het luteïniserend hormoon, LH, afkomstig uit de hypofyse (het hersenaanhangsel), stimuleert de Leydig cell in het interstitium om testosteron te maken. Testosteron is een steroïd hormoon, en het bepaalt de ontwikkeling van de mannelijke geslachtskenmerken. De aanwezigheid van het follikel-stimulerend hormoon, FSH, uit de hypofyse en van testosteron is noodzakelijk voor de ontwikkeling van spermatozoa. Sertoli cellen zijn de doelwitcellen voor FSH en testosteron; dat wil zeggen, Sertoli cellen hebben receptoren voor deze hormonen en kunnen daarom reageren op de aanwezigheid van FSH en testosteron. De regulering van de spermatogenese vindt waarschijnlijk niet plaats door rechtstreekse effecten van FSH en testosteron op de zich ontwikkelende spermatozoa, de germinale cellen, maar indirect via effecten van deze hormonen op de Sertoli cellen. De ontwikkeling van de spermatozoa kan tevens worden beïnvloed door verbindingen, die uitgescheiden worden door de Sertoli cellen. Gekweekte Sertoli cellen zijn in staat tot verschillende stofwisselingsactiviteiten, waaronder de afbraak van glucose. Afbraakprodukten van glucose, zoals onder andere lactaat, zijn mogelijk van belang als energiebron voor de germinale cellen. FSH bleek de produktie van lactaat uit glucose door gekweekte Sertoli cellen te kunnen stimuleren. Het glucosemetabolisme wordt echter in belangrijke mate gereguleerd door insuline, terwijl insuline-achtige groeifactor I (IGF–I, ook wel somatomedine C genoemd), een groeifactor, die voornamelijk betrokken is bij de groei en ontwikkeling van weefsels, ook insuline-achtige effecten op het glucosemetabolisme heeft. De experimenten beschreven in dit proefschrift waren erop gericht om een beter inzicht te krijgen in het belang van insuline en IGF–I, in relatie met FSH, als mogelijke beïnvloeders van Sertoli celfunkties en de ontwikkeling van de testis. Hiertoe is de aanwezigheid van receptoren voor insuline en voor IGF–I op gekweekte Sertoli cellen van jonge ratten onderzocht en zijn deze receptoren nader gekarakteriseerd. In Hoofdstuk 1 zijn de spermatogenese en de regulatie hiervan door hormonen in het kort beschreven, evenals het doel van dit proefschrift.

De ontwikkeling van de testis in jonge dieren, en het in stand houden van de ontwikkeling van spermatozoa in volwassen dieren worden beïnvloed door hypofysehormonen, onder andere door FSH en LH. Naast de regulering van de FSH en LH afgifte door de hypofyse door middel van de terugkoppelende werking van inhibine (uit Sertoli cellen) en van testosteron (uit Leydig cellen), de zogenoemde endocriene regulering, zouden ook andere hormonen en groeifactoren betrokken kunnen zijn bij de nauwkeurige regulering van het functioneren van de testis. Voorts zouden factoren, die ter plaatse door cellen in de testis geproduceerd worden, mogelijk betrokken kunnen zijn bij zogenoemde paracriene cel-cel interacties tussen de verschillende celtypen in de testikel. In Hoofdstuk 2 wordt enige achtergrondinformatie gegeven over de receptoren, de mechanismen van werking en de mogelijke functies van FSH, insuline en IGF–I, terwijl tevens een aantal andere hormonen en groeifactoren en paracriene cel-cel interacties in de testis worden besproken.

Hoofdstuk 3 geeft een korte samenvatting van de gebruikte methoden en tevens een beschrijving van de gebruikte Sertoli cel-preparaten.

In Hoofdstuk 4 en bijgevoegde artikelen 1 en 2 worden experimenten beschreven die de aanwezigheid van receptoren voor insuline en IGF–I aantonen op gekweekte Sertoli cellen van jonge ratten. Het bleek uit bindingsstudies met - met radioactief jodium gemerkte - hormonen, dat een naar verhou-

ding gering aantal receptoren met hoge affiniteit voor insuline aanwezig was op de Sertoli cellen van jonge ratten, terwijl een groot aantal IGF–I receptoren aanwezig was. Specifieke binding van insuline aan de insuline receptor kon ook worden aangetoond met een andere techniek, waarbij het gemerkte, reeds aan de receptor gebonden insuline met een bepaalde verbinding wordt gekoppeld (covalente cross-linking) aan de receptor.

In jonge ratten bleek de binding van insuline aan celmembraan-fracties van de gehele testis naar verhouding laag te zijn en de binding van IGF–I naar verhouding hoog. In volwassen ratten was de binding van insuline aan testis membraanfracties even hoog als de binding van IGF–I. De resultaten wijzen op een verandering van de aantallen insuline en IGF–I receptoren in de rattetestis gedurende de ontwikkeling. IGF–I is mogelijk belangrijker dan insuline gedurende de ontwikkeling van de testis in de jonge rat, en Sertoli cellen zijn mogelijk het voornaamste doelwit voor IGF–I in de zaadbuisjes van de testis.

Effecten van FSH, insuline en IGF–I op het transport en het metabolisme van glucose door gekweekte Sertoli cellen worden beschreven in Hoofdstuk 5 en bijgevoegde artikelen 3, 4 en 5. FSH, insuline en IGF–I hadden vergelijkbare effecten op de lactaatproductie door gekweekte Sertoli cellen. Het effect van FSH op de lactaatproductie toonde echter een tijdsverloop dat sterk verschilde van het tijdsverloop van andere, langdurige effecten van FSH. Sertoli cellen zetten glucose voornamelijk om in lactaat, waarbij ATP geproduceerd wordt. Uit waarnemingen, dat Sertoli cellen ook aminozuren en vetzuren kunnen verbruiken als energiebron, is geconcludeerd, dat het glucosemetabolisme waarschijnlijk niet van vitaal belang is als energiebron voor de Sertoli cellen zelf. FSH had geen stimulerende effecten op de opname van 2-deoxyglucose, als maat voor glucosetransport, terwijl insuline en IGF–I de maximale snelheid van de glucoseopname verhoogden. Uit de verschillen tussen de hormoon effecten op de lactaatproductie en op het glucosetransport werd geconcludeerd dat de snelheid van de lactaatproductie niet uitsluitend bepaald wordt door de snelheid van het glucosetransport, maar dat FSH, insuline en IGF–I ook effecten kunnen hebben op enzymactiviteiten in de glycolyse (de reacties betrokken bij de omzetting van glucose naar lactaat).

Literatuurgegevens over verstoringen van de spermatogenese die mogelijk voor een deel veroorzaakt worden door verstoringen in het glucosemetabolisme, worden besproken om het belang van het glucosemetabolisme als energiebron voor het optimaal functioneren van de testis te illustreren (Hoofdstuk 6).

De veranderingen in de FSH niveaus gedurende de ontwikkeling van de testis, en de rol van insuline en IGF–I in verband met de aantallen receptoren voor insuline en IGF–I gedurende foetale groei en testis ontwikkeling worden beschreven in Hoofdstuk 6.

Uit de resultaten beschreven in dit proefschrift werd geconcludeerd (Hoofdstuk 6) dat in testisweefsel insuline waarschijnlijk de voornaamste regulator is bij het transport en het metabolisme van glucose, waarbij insuline kan werken via interactie met receptoren met een hoge affiniteit voor insuline. IGF–I kan via een groot aantal receptoren op Sertoli cellen vele verschillende functies, waaronder glucosemetabolisme, van Sertoli cellen reguleren. Verder speelt IGF–I mogelijk een belangrijke rol bij de ontwikkeling van de testis in jonge ratten.

ABBREVIATIONS

ABP	androgen binding protein
ACTH	adrenocorticotropic hormone
AMH	anti-Müllerian hormone
α-MSH	α-melanocyte stimulating hormone
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
CB	cytochalasin B
CE	cytochalasin E
cAMP	adenosine cyclic-3':5'-monophosphate
(c)DNA	(complementary) deoxyribonucleic acid
cGMP	guanosine cyclic-3':5'-monophosphate
dbcAMP	N^6-2'-O-dibutyryl adenosine cyclic-3':5'-monophosphate
2-DOG	2-deoxyglucose
DSS	disuccinimidylsuberate
EGF	epidermal growth factor
EGTA	ethyleenglycol-bis (β-aminoethylether)-N,N'-tetraacetic acid
FSH	follicle stimulating hormone; follitropin
GnRH	gonadotropin releasing hormone
GTP	guanine-5'-triphosphate
hCG	human chorionic gonadotropin
IGF	insulin-like growth factor
(k)Da	(kilo)Dalton
K_d	dissociation constant
K_m	Michaelis constant
LH	luteinizing hormone; lutropin
LHRH	LH-releasing hormone
M	mol/liter
MEM	Eagle's minimum essential medium
MIX	3-isobutyl-methylxanthine
M_r	relative molecular mass
(m)RNA	(messenger) ribonucleic acid
MW	molecular weight
n	number of determinations
NAD(H)	(reduced) nicotinamide adenine dinucleotide
3-OMG	3-O-methylglucose
PBS	phosphate buffered saline
PK-A	protein kinase A
PK-C	protein kinase C
POMC	pro-opiomelanocortin
R_0	concentration of binding sites
S.D.	standard deviation
TGF	transforming growth factor
V_{max}	maximal rate
v/v	volume/volume
w/v	weight/volume

TRIVIAL NAMES

Cholesterol	5-cholestene-3β-ol
Estradiol	1,3,5,(10)-oestratriene-3,17β-diol
Testosterone	17β-hydroxy-4-androstene-3-one

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CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 21 januari 1955 geboren te Lichtenvoorde. In Amsterdam behaalde zij in 1973 het diploma Gymnasium β aan het Hervormd Lyceum Zuid. In hetzelfde jaar werd begonnen met de studie biologie aan de Universiteit van Amsterdam, waar in juni 1977 het kandidaats-examen B4 werd behaald. Hierna werd de studie voortgezet met het hoofdvak biochemie (Prof. Dr. K. van Dam) en de bijvakken electronenmicroscopie (Prof. Dr. N. Nanninga) en microbiologie (Prof. Dr. D.W. Tempest). In deze periode werd tevens de onderwijsbevoegdheid voor biologie behaald. Het doctoraal examen werd in april 1982 behaald. In de periode van mei 1982 tot oktober 1982 heeft zij als informatrice land- en tuinbouwonderwijs gewerkt op de land- en tuinbouwtentoonstelling "Floriade" te Amsterdam.

Het in dit proefschrift beschreven werk werd 1 oktober 1982 gestart op de afdeling Biochemie II (Chemische Endocrinologie), alwaar zij was aangesteld als wetenschappelijk assistent in tijdelijke dienst. Vanaf 1 oktober 1987 zal zij als postdoctoraal onderzoekster werkzaam zijn bij Dr. JoAnne S. Richards, Associate Professor, op het Department of Cell Biology van het Baylor College of Medicine te Houston.

APPENDIX PAPERS

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Identification of insulin receptors on rat Sertoli cells

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Key words: Sertoli cell; Insulin receptor; IGF-I; Testis

Summary

The binding of insulin to rat Sertoli cells was investigated to establish if effects of insulin on Sertoli cells can be mediated via insulin receptors. Sertoli cells were isolated from the testes of 3-week-old rats, and preincubated for 3 days in the absence of hormones. Binding of ^{125}I-porcine insulin to the Sertoli cells was 75–80% specific and this binding was time- and pH-dependent and reversible. Scatchard analysis of the binding data resulted in curvilinear plots with a high affinity binding of $K_d = 1.8 \times 10^{-9}$ M. Porcine and bovine insulin competed equally well for ^{125}I-porcine insulin binding. Porcine proinsulin was 10–50 times less potent, corresponding to its lower biological activity. Insulin-like growth factor-I (IGF-I) was 30–40 times less potent, indicating low affinity binding of IGF-I to the insulin receptor. Lutropin which was used as a control gave no competition with the ^{125}I-insulin binding.

Affinity labelling of Sertoli cell membrane proteins with ^{125}I-insulin using the cross-linking agent disuccinimidylsuberate revealed binding of insulin to (a) protein(s) of $M_r > 300\,000$ or $M_r = 130\,000$ after electrophoresis under non-reducing or reducing conditions, respectively. Affinity labelling with ^{125}I-insulin was largely prevented by unlabelled insulin. It is concluded that the protein of M_r 130 000 may represent the α-subunit of the insulin receptor.

The presence of insulin receptors as well as IGF-I receptors on cultured rat Sertoli cells may suggest that insulin and IGF-I have specific functions in regulating the maturation and activities of Sertoli cells during the initiation and maintenance of spermatogenesis.

Introduction

Glucose metabolism by Sertoli cells isolated from the testes of immature rats results in a net production of lactate (Robinson and Fritz, 1981; Jutte et al., 1982). This is of interest, because spermatogenic cells require exogenous lactate as an energy-yielding substrate (Jutte et al., 1981; Mita and Hall, 1982; Grootegoed et al., 1984, 1986). Several authors have reported stimulatory effects of high concentrations of insulin on glucose transport, glycolysis and lactate production by Sertoli cells isolated from immature rats (Jutte et al., 1983; Borland et al., 1984; Mita et al., 1985). Other stimulatory effects of supraphysiological doses of insulin on Sertoli cells that have been reported include production of androgen binding protein (Karl and Griswold, 1980), incorporation of $^{32}P_i$ into RNA (Griswold and Merryweather, 1982), transferrin secretion (Skinner

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and Griswold, 1982) and DNA synthesis (Borland et al., 1984). It was observed, however, that most of these in vitro effects could also be obtained by low doses of insulin-like growth factors (IGF-I and IGF-II) (Skinner and Griswold, 1983; Borland et al., 1984; Mita et al., 1985). This could be explained, because it has been reported that insulin receptors were hardly detectable on cultured Sertoli cells, whereas IGF-I and IGF-II receptors were abundant (Borland et al., 1984).

Insulin can bind to IGF-I receptors, although with a much lower affinity than IGF-I (Kahn et al., 1981). It has been suggested, therefore, that the effects of insulin on Sertoli cells were mediated via binding of insulin to IGF-I receptors (Borland et al., 1984; Mita et al., 1985). We have observed, however, that the stimulatory effect of insulin on lactate production by Sertoli cells from immature rats was rapid and dose-dependent, and half-maximal stimulation was obtained at a low dose of insulin (50 ng/ml) (Oonk et al., 1985). Furthermore, in total rat testis tissue high affinity binding of insulin has been demonstrated, but the localization of insulin binding among the various testicular cell types was not specified (Saucier et al., 1981).

In the present experiments, we have estimated in vitro the number and affinity of binding sites for insulin on cultured Sertoli cells isolated from immature rats.

Materials and methods

Materials

Porcine insulin and mono-^{125}I-(Tyr-A_{14})-porcine insulin were purchased from Novo Industri, Copenhagen, Denmark. Collagenase (CLS – I) was purchased from Worthington, Freehold, NJ, U.S.A. Bovine serum albumin (fraction V) (BSA) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma, St. Louis, MO, U.S.A. Lactate dehydrogenase from pig heart, NAD^+-free acid grade I and dibutyryl cAMP were purchased from Boehringer Mannheim, Mannheim, F.R.G. Insulin-like growth factor-I (IGF-I/Somatomedin C) was purchased from Amersham International, Amersham, U.K. Follitropin (NIH-ovine-FSH-S13) and lutropin (NIH-ovine-LH-S18) were gifts from The Endocrinological Study Section of the NIH, Bethesda, MD, U.S.A. Disuccinimidylsuberate (DSS) was purchased from Pierce Chemical Company, Rockford, IL, U.S.A. All other chemicals were of analytical grade.

Isolation and incubation of Sertoli cells

Sertoli cells were isolated from the testes of 3-week-old rats (Wistar, substrain R-1 Amsterdam). In one series of experiments 1- and 2-week-old rats were used as indicated in the text. The rats were killed by cervical dislocation and the Sertoli cells were isolated and incubated as described previously at 32°C under an atmosphere of 5% CO_2 in air (Oonk et al., 1985). The composition of the cell preparations was evaluated using the method described by Oonk et al. (1985). The initial preparations of small clusters of cells contained approximately 70–75% Sertoli cells, 25–30% spermatogenic cells, and less than 1% peritubular cells. During incubation the Sertoli cells migrate and form a layer of flattened cells. Many spermatogenic cells are released during this process, but after 2 days of incubation a substantial number of spermatogenic cells was still present. To remove the remaining spermatogenic cells, a modification of the hypo-osmotic shock procedure described by Galdieri et al. (1981) was applied. After 2 days of incubation in Eagle's minimum essential medium (MEM, Gibco, Grand Islands, NY, U.S.A.) plus supplements (Oonk et al., 1985) containing 1% (v/v) fetal calf serum, the cells were exposed for 1 min to a 1:9 (v/v) mixture of medium and water. Subsequently, the cells were washed twice with undiluted medium without serum to remove the detached cells. Using phase-contrast microscopy it was observed that the hypotonic treatment caused the swelling and detachment of most of the spermatogenic cells, and that Sertoli cell attachment was not influenced. Vacuolization of Sertoli cells was seen, but this effect was reversed within a few hours. Following the removal of the spermatogenic cells, the Sertoli cells were incubated for 24 h in medium without serum. Subsequently, lactate production was estimated during incubation for 6 h in the absence and presence of follitropin and insulin, as described previously (Oonk et al., 1985), or binding of ^{125}I-insulin was estimated as described below.

Isolation of a Sertoli cell membrane fraction

The membrane isolation procedure was adapted from a procedure to isolate a plasma membrane fraction from spermatogenic cells (Millette et al., 1980). The Sertoli cells were preincubated for 3 days as described above in 75 cm^2 flasks (30–40 mg protein per experiment). The attached cells were washed twice in Tris-buffered saline (TBS; 0.16 M NaCl, 3 mM $MgCl_2$, 5 mM KCl in 10 mM Tris-HCl, pH 7.4), at 4°C. The cells were scraped from the flasks using a policeman, collected in TBS, and centrifuged for 2 min at $200 \times g$. Cells were swollen during 5 min in $0.1 \times$ TBS, and homogenised with 5 strokes in a glass Dounce homogeniser with glass pestle (Wheaton Scientific, Millville, NJ, U.S.A., No. 357542, clearance 0.025–0.075 mm). One-tenth volume of $10 \times$ TBS was added to restore isotonicity. From this homogenate plasma membranes were isolated by centrifugation on discontinuous sucrose gradients exactly as described by Millette et al., 1980.

Measurement of ^{125}I-insulin binding to Sertoli cells

All experiments on insulin binding were performed using Sertoli cells which had been preincubated for 3 days as described above in 4 cm^2 culture wells (approximately 200 μg protein/well). Binding of ^{125}I-insulin to Sertoli cells was measured using a modification of the method described by Raizada et al. (1980) as follows. The attached cells were washed twice with phosphate-buffered saline (PBS, Dulbecco and Vogt, 1954) and subsequently incubated for 60 min at 22°C in 1 ml PBS containing 1.6% (w/v) bovine serum albumin (BSA) and 0.1 nM ^{125}I-insulin (approximately 100 000 cpm). Where indicated, the incubations were carried out for different time periods and at a different pH. Non-specific binding was determined by incubation in the presence of 1.67 μM unlabelled insulin. Specific binding represents total binding minus non-specific binding. The incubations were terminated by rinsing the culture wells 5 times with PBS containing 0.8% BSA at 4°C. The cells were dissolved in 0.5 ml 2 M NaOH and the solution was transferred to tubes. The culture dishes were rinsed with 0.5 ml water which was combined with the corresponding samples. The radioactivity was estimated using an LKB 1280 γ-counter at an efficiency of 80%.

Affinity-labelling conditions

The Sertoli cell membrane fractions were washed once with 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 1 mM PMSF and pelleted for 15 min at $60\,000 \times g$. The conditions for affinity labelling were adapted from Massague et al. (1980). The membrane fractions were incubated for 70 min at 10°C in PBS containing 1% BSA and 5 nM ^{125}I-insulin, in the absence and presence of 0.4 μM unlabelled insulin. The incubations were terminated by 10-fold dilution in PBS (4°C) and centrifugation for 15 min at $60\,000 \times g$. The pellets were resuspended in PBS containing 0.25 mM disuccinimidylsuberate (DSS) (Pilch and Czech, 1979) and incubated for 15 min at 0°C. The cross-linking reaction was quenched by addition of excess cold Tris-HCl buffer and the membranes were pelleted at $60\,000 \times g$ for 15 min. The pellets were resuspended in 100 μl sample buffer with or without 50 mM dithiothreitol and boiled for 3 min. The proteins were separated by electrophoresis according to Laemmli (1970) using 5% polyacrylamide slab gels. Following electrophoresis, the gels were fixed, stained with Coomassie blue, destained and each lane was sliced in 2 mm slices. The gel slices were transferred to plastic tubes and counted in an LKB 1280 γ-counter at an efficiency of 80%. To estimate molecular weight values, high MW markers (Bio-Rad Laboratories, Richmond, CA, U.S.A.) were used: myosin (200 000), β-galactosidase (116 000), phosphorylase *b* (92 500), bovine serum albumin (66 000) and ovalbumin (43 000).

Estimation of lactate and cellular protein

The amount of lactate in the spent incubation media was determined enzymically, as described by Hohorst (1970). The amount of cellular protein was determined as described by Lowry et al. (1951), using BSA as a standard.

Statistical procedures

The significance of differences between the results of different experimental treatments was assessed using Student's *t*-test. Differences were considered to be statistically significant when P was < 0.01 (two-tailed).

Results

Effect of hypotonic treatment on lactate production

After the hypotonic treatment to remove spermatogenic cells, as described in Materials and methods, Sertoli cells were incubated for 24 h in medium without serum. Subsequently, the medium was renewed and lactate production was estimated during incubation for 6 h in the absence and presence of follitropin (0.5 μg/ml) and insulin (5 μg/ml). The amounts of lactate produced were 100 ± 6 (control), 294 ± 16 (follitropin-stimulated) and 328 ± 15 (insulin-stimulated) nmol/mg protein per h (mean $\pm$ SD; $n = 4$).

These results indicate that the basal and hormone-stimulated lactate production by Sertoli cells, purified using the hypotonic treatment were comparable with previously published observations on Sertoli cells from irradiated rats which were not subjected to hypotonic treatment (Oonk et al., 1985). Therefore this treatment was used routinely as it improved the purity of the Sertoli cell preparations.

Insulin binding

Binding of ^{125}I-insulin to Sertoli cells

The binding of insulin to Sertoli cells was time-dependent and reached a maximum after 60 min at 22°C (Fig. 1). At equilibrium 1.44 ± 0.40 fmol/mg protein ($n = 9$) of the total amount of ^{125}I-insulin added (100 pmol per incubation) was bound, and 75–80% of the total binding represented specific binding.

The optimum pH for specific binding of insulin to Sertoli cells was pH 8. Hormone binding was lower at pH 7.5 and pH 8.5 (Fig. 2). Further experiments on insulin binding were routinely performed for 60 min at pH 8.

After reaching equilibrium binding and subsequent dilution of the tracer, the ^{125}I-insulin dissociated from the Sertoli cells in a time-dependent fashion (Fig. 3). In the absence of unlabelled insulin most of the ^{125}I-insulin had dissociated after 2 h ($t_{1/2}$ of 13 min). The rate of dissociation of ^{125}I-insulin was enhanced by the presence of unlabelled insulin in the dilution medium ($t_{1/2}$ of 6 min) (Fig. 3). These results indicate that binding of ^{125}I-insulin to Sertoli cells is rapidly reversible.

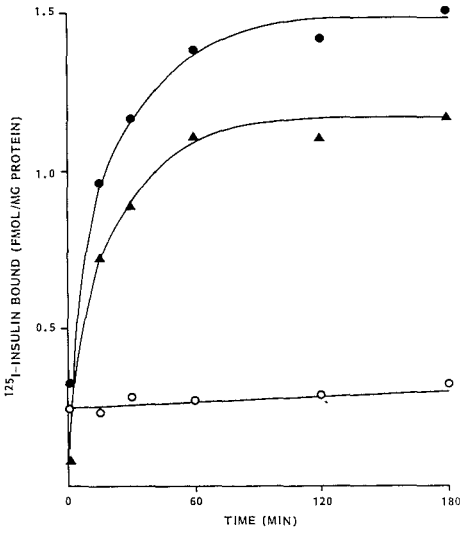

Fig. 1. Time-course of ^{125}I-insulin binding. Sertoli cells were incubated for the indicated time periods at 22°C in the presence of 0.1 nM ^{125}I-insulin. Non-specific binding was estimated as described in Materials and methods. The results are expressed as fmol ^{125}I-insulin bound/mg protein (mean of duplicate values, which differed by less than 4%). Specific binding (▲———▲) represents total (●———●) minus non-specific (○———○) binding.

At higher concentrations of insulin the specific binding of ^{125}I-insulin to the Sertoli cells reached a maximum, whereas the aspecific binding was further increased (Fig. 4). This results shows that ^{125}I-insulin binding to Sertoli cells is saturable.

The specificity of ^{125}I-insulin binding was investigated by estimating the maximal ^{125}I-insulin binding to Sertoli cells and the competition with this binding of different concentrations of several unlabelled peptide hormones (Fig. 5). Porcine and bovine insulin were equally effective in their ability to displace ^{125}I-insulin binding ($ID_{50} = 1.5$ nM) *. Porcine proinsulin was 10–50 times less potent, which corresponds to its low insulin-like activity in classical insulin target cells (De Meyts,

* ID_{50} = concentration of unlabelled ligand required to decrease maximal ^{125}I-insulin binding by 50%.

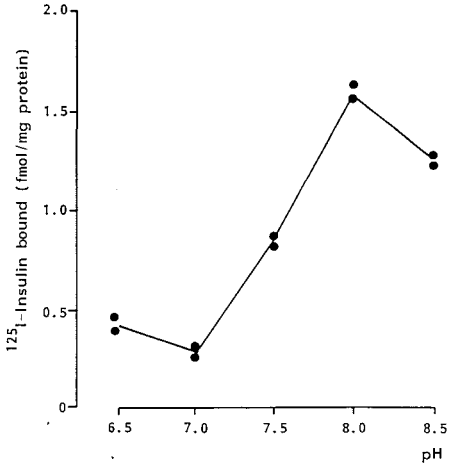

Fig. 2. Effect of pH on ^{125}I-insulin binding. Sertoli cells were incubated for 60 min at 22°C in PBS at the indicated pH values, in the presence of 0.1 nM ^{125}I-insulin and 1.6% BSA. The results represent specific binding (duplicate incubations).

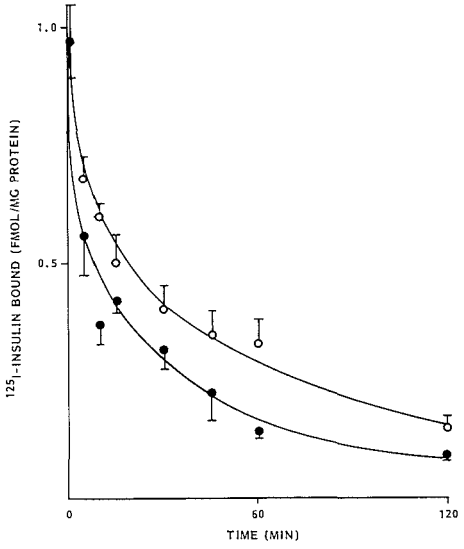

Fig. 3. Dissociation of ^{125}I-insulin binding. Sertoli cells were incubated in the presence of 0.1 nM ^{125}I-insulin for 60 min at 22°C. Subsequently, the cells were washed to remove unbound radioactivity, and the incubations were continued for the indicated time periods at 22°C in PBS containing 1.6% BSA, either in the presence (●———●) or absence (○———○) of 1.67 μM unlabelled insulin. The results are expressed as fmol ^{125}I-insulin bound/mg protein and represent total binding (mean ± SD of triplicate incubations).

1976). IGF-I displaced 50% of the bound ^{125}I-insulin at a 40-fold higher concentration than insulin, indicating that IGF-I was about 2–3% as potent as unlabelled insulin. In contrast, the gonadotrophic hormone lutropin at a high concentration had no effect on the maximal ^{125}I-insulin binding (Fig. 5).

Data on the inhibition of ^{125}I-insulin binding by different concentrations of unlabelled insulin are presented in Fig. 6A and 6B. In Fig. 6B these data are plotted as femtomoles of ^{125}I-insulin bound per mg of protein (B) versus B/F (F = concentration of unbound insulin) (Scatchard, 1949). Curvilinear plots were obtained which were resolved graphically as described by Rosenthal (1967) into high and low affinity components (Fig. 6B, broken lines). Linear regression analysis gave estimates for the K_d and high affinity binding capacity of $1.8 \pm 0.3 \times 10^{-8}$ M and 8.5 ± 1.0 fmol/mg protein, respectively. Analysis of the low affinity binding indicated a K_d of $1.5 \pm 0.2 \times 10^{-7}$ M and a binding capacity of 215 ± 17 fmol/mg protein.

TABLE 1

EFFECT OF INSULIN ON LACTATE PRODUCTION

Sertoli cells were incubated for 3 days at 32°C in the absence of added hormones and subsequently, after medium renewal, for 6 h at 32°C in the presence and absence of insulin. Net lactate production during the 6 h incubation period was expressed as nmol/mg protein per h (mean ± SD; number of incubations of three experiments between brackets).

	Net lactate production (nmol/mg protein per h)	
	Sertoli cells from 1-week-old rats	Sertoli cells from 2-week-old rats
Control	201 ± 20 (16)	170 ± 19 (19)
Insulin, 50 ng/ml	284 ± 13 * (8)	330 ± 20 * (11)
Insulin, 5 μg/ml	345 ± 15 * (8)	374 ± 25 * (11)

* $P < 0.01$ compared to control (Student's t-test).

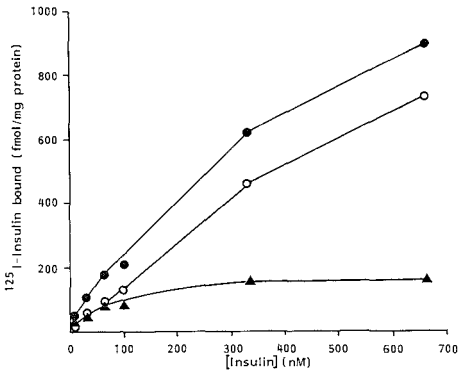

Fig. 4. Binding of ^{125}I-insulin at different insulin concentrations. Sertoli cells were incubated for 60 min at 22°C in the presence of 0.1 nM ^{125}I-insulin and different concentrations of unlabelled insulin. Specific binding was determined as described in Materials and methods (mean of duplicate incubations, which differed by less than 15%). The specific binding (▲——▲) represents total (●——●) minus non-specific (○——○) binding.

Affinity cross-linking of ^{125}I-insulin to Sertoli cell membranes

After polyacrylamide gel electrophoresis under non-reducing conditions of membrane proteins which were affinity-labelled with ^{125}I-insulin most radioactivity was associated with high molecular weight proteins near the top of the gel (Fig. 7A). In the presence of dithiothreitol, however, such high molecular weight peaks were absent, and a major peak was found at M_r 130000 (Fig. 7B). Unlabelled insulin strongly competed with ^{125}I-insulin during the affinity labelling of the insulin-binding membrane proteins (Fig. 7A and B).

Effect of insulin on lactate production by Sertoli cells from 1- and 2-week-old rats

A significant stimulation ($P < 0.01$) of lactate production by insulin was observed for Sertoli cells from 1-week-old and 2-week-old rats, also at a low dose of insulin (50 ng/ml; 8.3 nM) (Table 1). The results in Fig. 8 show that lactate production by Sertoli cells from 2-week-old rats was

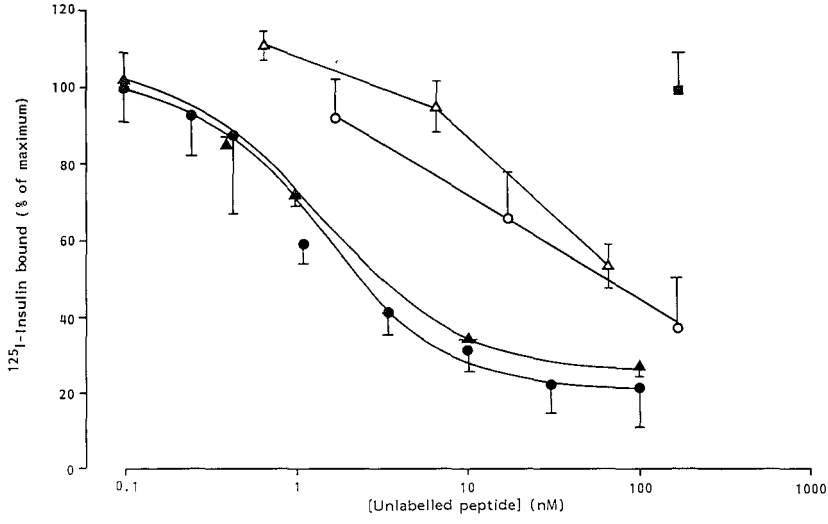

Fig. 5. Specificity of binding of ^{125}I-insulin. Sertoli cells were incubated for 60 min at 22°C in the presence of 0.1 nM ^{125}I-insulin and different concentrations of porcine insulin (●——●), bovine insulin (▲——▲), porcine proinsulin (○——○), insulin-like growth factor-I (IGF-I, △——△) and lutropin (□) as described in Materials and methods. Binding of ^{125}I-insulin is expressed as percentage of the binding in the absence of any other peptide. The data were corrected for non-specific binding (mean ± SD of 2–8 incubations).

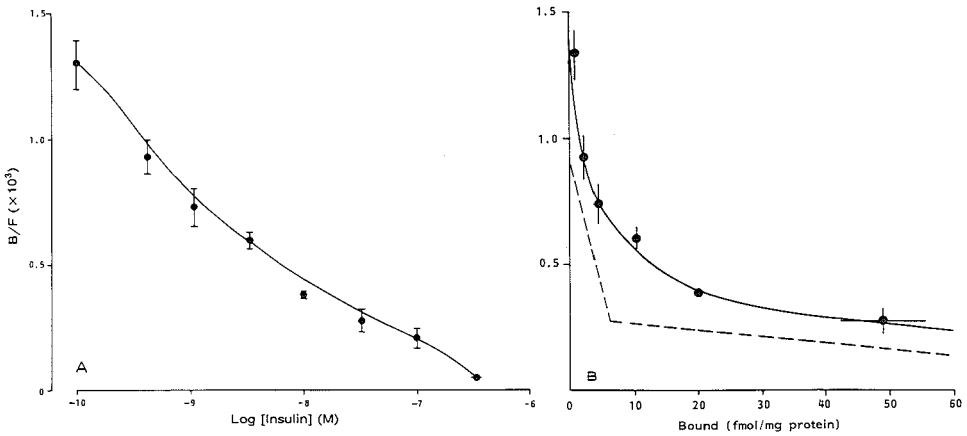

Fig. 6. Displacement of ^{125}I-insulin binding by unlabelled insulin. Sertoli cells were incubated for 60 min at 22°C in the presence of ^{125}I-insulin and different concentrations of unlabelled insulin. Specific binding expressed as the ratio bound/free (B/F) insulin was plotted as a function of unlabelled insulin concentration (*A*) and as a function of the amount of hormone bound (*B*). The curve in Fig. 6*B* was resolved into two components shown by the dotted lines, which were subjected to linear regression analysis.

increased by insulin in a dose-dependent way, and that half-maximal stimulation was obtained at an insulin concentration of 10 ng/ml (1.6 nM).

TABLE 2

EFFECT OF PREINCUBATION IN THE PRESENCE OF INSULIN ON LACTATE PRODUCTION

Sertoli cells from 3-week-old rats were incubated for 3 days at 32°C in the presence and absence of insulin (5 μg/ml). Subsequently, the medium was renewed and the cells were incubated for 6 h at 32°C in the presence of follitropin (0.5 μg/ml), insulin (5 μg/ml) and dibutyryl cAMP (dbcAMP, 0.5 mM), and in the absence of hormones (control). Net lactate production during the 6 h period was estimated and expressed as nmol/mg protein per h (mean ± SD of triplicate incubations).

	Net lactate production (nmol/mg protein per h)	
	Preincubation in the absence of insulin	Preincubation in the presence of insulin
Incubation		
Control	109 ± 3	76 ± 3
Follitropin	338 ± 8 *	321 ± 11 *
Insulin	293 ± 14 *	132 ± 6 *,**
dbcAMP	354 ± 10 *	337 ± 11 *

* $P < 0.01$ compared to control.

** $P < 0.01$ compared to Sertoli cells treated with insulin after preincubation in the absence of insulin (Student's *t*-test).

Effect of preincubation in the presence of insulin

Lactate production by Sertoli cells from 3-week-old rats was stimulated approximately 3-fold by insulin, follitropin and dibutyryl cAMP during incubation for 6 h following a 3-day preincubation period in the absence of added hormones. However, when insulin had been present during the preincubation period, the effect of insulin was greatly reduced, whereas the stimulatory effects of follitropin and dibutyryl cAMP were the same (Table 2).

Discussion

The present results indicate that the binding of ^{125}I-insulin to rat Sertoli cells in vitro is specific, rapid, saturable, reversible and competed for by low hormone concentrations. These properties meet the principal criteria for a true hormone-receptor interaction (De Meyts, 1976).

The differences in competition for ^{125}I-insulin binding by insulin, proinsulin, IGF-I and lutropin indicate a specificity profile of insulin binding to the receptor that is comparable with that described for typical insulin target cells, such as adipocytes (Gammeltoft and Gliemann, 1973) and for other cell types including lymphocytes (Gavin

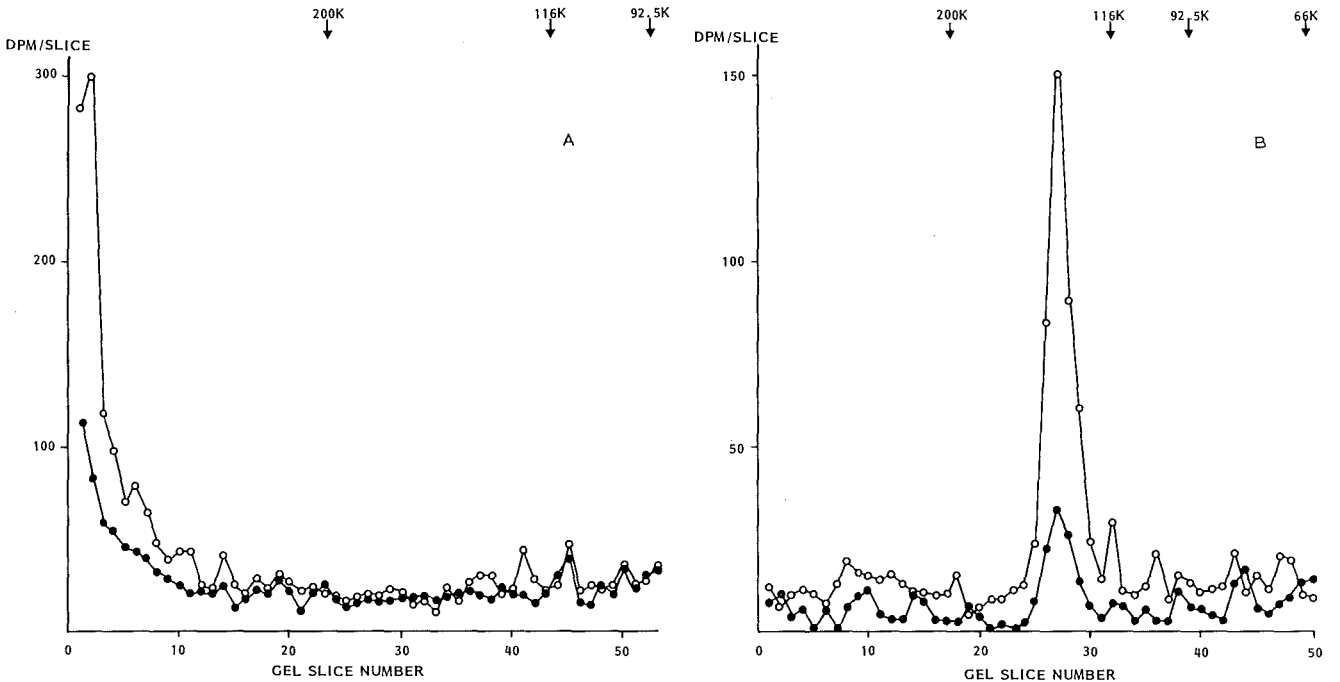

Fig. 7. Affinity labelling of membrane proteins with ^{125}I-insulin. A membrane fraction from Sertoli cells was incubated with 5 nM ^{125}I-insulin in the presence (●——●) and absence (○——○) of 0.4 μM unlabelled insulin, and treated with 0.25 mM disuccinimidylsuberate, as described in Materials and methods. Electrophoresis was performed in the absence (*A*) or presence (*B*) of 50 mM dithiothreitol. The position of the molecular weight markers after electrophoresis is indicated.

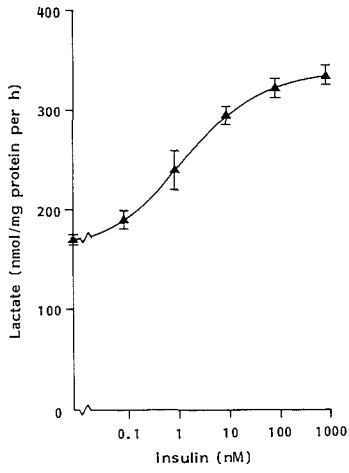

Fig. 8. Dose-response curve of the effect of insulin on lactate production by Sertoli cells from 2-week-old rats. These Sertoli cells were incubated as described in the legend to Table 1. Net lactate production during the 6 h incubation period at 32°C was expressed as nmol/mg protein per h (mean ± SD of triplicate incubations).

(Gavin et al., 1973), mouse brain cells (Van Schravendijk et al., 1984) and bovine endothelial cells (Bar and Boes, 1984). Furthermore, the failure of IGF-I to block ^{125}I-insulin binding rules out the possibility that ^{125}I-insulin is bound exclusively to IGF-I receptors and not at all to insulin receptors. The sharp pH optimum we observed is comparable with that found for insulin binding to lymphocytes (Gavin et al., 1973) and fibroblasts (Raizada et al., 1980).

Non-linear Scatchard plots were obtained, consistent with results reported in several other systems. The typical curvilinear shape could be explained for by the presence of a single class of binding sites displaying negative cooperativity, but it is also possible that there are two or more classes of binding sites with different affinities for insulin (De Meyts, 1976). Dissociation of bound insulin was enhanced by the presence of unlabelled insulin in the dilution medium, which could indicate that the curved Scatchard plot is due to negative cooperativity (De Meyts et al., 1973). Nevertheless, we have analysed the curve in terms of a two-site model with different affinities, using the graphical method of Rosenthal (1967). This method offers a convenient calculation of affinities and binding capacities, which makes it possible to compare the present results with results reported for other cell types. The calculated high affinity constant ($K_d = 1.8 \times 10^{-9}$ M) is in accordance with values reported for insulin binding to granulosa cells (Otani et al., 1985) as well as for the typical insulin target cells (Gammeltoft and Gliemann, 1973; Gavin et al., 1973; Kahn et al., 1981). The interpretation of the low affinity component of binding ($K_d = 1.5 \times 10^{-7}$ M) requires some caution, as our incubation conditions could have resulted in a cellular uptake of insulin (Kahn and Baird, 1978; Olefsky and Kao, 1982). Moreover, significant low affinity binding of insulin to IGF-I receptors could occur at higher concentrations of insulin (Bar and Boes, 1984; Rosenfeld et al., 1985).

Stimulation of glucose metabolism by insulin via insulin receptors has been demonstrated for a number of different cell types, including mouse fibroblasts (Raizada et al., 1980), rat brain cells (Clarke et al., 1984), rabbit colon epithelial cells (Pillion et al., 1985), bovine adipocytes (Vernon et al., 1985) and, as mentioned above, porcine granulosa cells (Otani et al., 1985). In this respect, the present results indicate that a similar effect of insulin can be observed using cultured rat Sertoli cells. However, it has been observed that in many of the other cell types the K_d values for biological effects were much lower than the K_d for insulin binding, whereas in Sertoli cells the ED_{50} for insulin stimulation of lactate production (1.6–8.3 nM) and the K_d for insulin binding (1.8 nM) were found to be within the same range. A difference between Sertoli cells and typical insulin target cells may concern the relatively small number of high affinity receptors on Sertoli cells. It has been observed that Chinese hamster ovary (CHO) cells contained a 5 times lower number of insulin receptors than CHO-HIR3 cells which had been transfected with insulin receptor-cDNA. The ED_{50} of the stimulation of glucose uptake by the CHO cells was 30 times higher than that for the transfected CHO-HIR3 cells, but the K_ds for insulin binding by the different cells were the same (Ebina et al., 1985). These observations indicate that the

number of occupied receptors that is required for a biological response may be relatively high when the number of receptors present is relatively low.

In the present experiments ^{125}I-insulin was covalently attached using a cross-linking agent to high molecular weight components of a Sertoli cell membrane fraction. The labelling of these compounds was performed at a ^{125}I-insulin concentration of 4 nM, which is around the K_d of the insulin receptor, and was largely inhibited by a 100-fold higher concentration of unlabelled insulin. The non-displaceable ^{125}I-insulin binding could represent low affinity binding to the IGF-I receptor (Beguinot et al., 1985; Jialal et al., 1985). It has been demonstrated that insulin at high concentrations can displace ^{125}I-IGF-I binding to the IGF-I receptor, and that IGF-I at high concentrations can displace ^{125}I-insulin binding to the insulin receptor (Kasuga et al., 1981a; Rosenfeld et al., 1985). We have observed M_rs of $> 300\,000$ or of 130 000 after electrophoresis under non-reducing or reducing conditions, respectively, of the affinity-labelled Sertoli cell membranes. Massague et al. (1980) demonstrated that in the non-reduced state the insulin receptor is a tetrameric complex ($\alpha_2\beta_2$) with an M_r of more than 300 000. On reduction of disulfide bridges the separate α-(M_r 130 000) and β-(M_r 95 000) subunits are formed. Cross-linking of ^{125}I-insulin with the receptor then reveals the insulin binding α-subunit. Cross-linking studies with IGF-I receptors have indicated that IGF-I receptors and insulin receptors are very similar in size (Kasuga et al., 1981a). However, from the present experiments it is concluded that the M_r 130 000 labelled compound from Sertoli cells may represent the α-subunit of the insulin receptor. Evidence for the presence of large numbers of receptors for IGF-I and IGF-II on plasma membranes of Sertoli cells from 13-day-old rats has been reported by Borland et al. (1984). In the same study, insulin receptors were hardly detectable and also no stimulatory effect of low doses of insulin on lactate production could be observed. The present results show that glucose metabolism by Sertoli cells of 1- and 2-week-old rats could also be stimulated by physiological concentrations of insulin. The discrepancy between the results in the literature and the present results therefore does not reflect that we have used 3-week-old rats for the insulin binding studies as compared to the 13-day-old rats used by Borland et al. (1984). Another difference in experimental design concerns the preincubation conditions. Borland et al. (1984) have added 10 μg insulin per ml incubation medium during the first 2 days of the preincubation, whereas in our experiments no hormones were added to the preincubation medium. We have previously shown (Oonk et al., 1985) that the response of Sertoli cells to insulin with respect to lactate production was lost after 18 h of preincubation in the presence of insulin. The present results indicate that a similar loss of response was observed when the Sertoli cells were preincubated for a longer period in the presence of insulin. This effect might have been caused by down-regulation of the number of insulin receptors on the cell surface (Gavin et al., 1974; Kasuga et al., 1981b) and could explain the discrepancy described above.

At present, the role of insulin and insulin-like growth factors and their respective receptors in the development of the testis and in the maintenance of spermatogenesis is not clear. Using total testis tissue it has been found that high affinity binding of insulin increased with age (Saucier et al., 1981). For two other cell systems, viz. preadipocytes developing into adipocytes (Shimizu and Roth, 1985) and myoblast cells developing into myotubes (Beguinot et al., 1985), it has been shown that the undifferentiated cells possessed a low number of insulin receptors and a high number of IGF-I and II receptors, whereas during differentiation the number of insulin receptors increased and the number of IGF receptors decreased. A similar differential development of the two receptor systems was observed in chick embryos (Bassas et al., 1985), where in the course of embryogenesis IGF-I receptor numbers decreased and insulin receptor numbers increased. In this respect it was suggested that IGFs may be important in particular at early stages of development and differentiation. Similarly, the action of IGFs on Sertoli cells may play an important role in testicular development.

However, it cannot be excluded that insulin also may play a physiological role, in particular with respect to the maintenance of spermatogenesis. In this respect, it is of interest that impairment of spermatogenesis is often observed in experi-

mental diabetes. However, it is not clearly established that effects of diabetes on spermatogenesis are due to the overall pathology of diabetes or caused by the absence of insulin itself (Oksanen, 1975; Paz et al., 1978; Anderson and Thliveris, 1986).

In conclusion, insulin and IGF-I may act on cultured rat Sertoli cells via specific receptor systems. The effects of insulin and IGF-I on different biochemical activities of Sertoli cells are currently under investigation.

Acknowledgement

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INSULIN-LIKE GROWTH FACTOR–I (IGF–I) RECEPTORS ON SERTOLI CELLS FROM IMMATURE RATS AND AGE-DEPENDENT TESTICULAR BINDING OF IGF–I AND INSULIN.

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Summary

Insulin-like growth factor–I (IGF–I) binding to cultured Sertoli cells from immature rats was quantitatively evaluated. The binding of ^{125}I-IGF–I to the Sertoli cells was specific, time- and pH- dependent and reversible. Scatchard analysis yielded a K_d of 3.5×10^{-9} M and a binding capacity of 2080 fmol/mg protein. Competition with IGF–I resulted in a half-maximal displacement by 2 nM IGF–I, whereas insulin up to a concentration of 100 nM gave virtually no displacement of IGF–I binding. Similarly, the gonadotropic hormones follitropin and lutropin did not compete with ^{125}I-IGF–I binding. In previous studies, it was shown that cultured Sertoli cells from immature rats bind insulin with a K_d of 1.8×10^{-9} M and a binding capacity of 8.5 fmol/mg protein.

The binding of IGF–I and insulin to a total testis membrane fraction was studied using testes from immature and adult rats. In testis from 21-day-old rats, the maximal specific binding was relatively high for IGF–I (871 ± 50 fmol/g wet weight) and relatively low for insulin (118 ± 11 fmol/g wet weight). In adult testis, the maximal specific binding of IGF–I was 324 ± 40 fmol/g wet weight and that of insulin was 330 ± 17 fmol/g wet weight. The binding of IGF–I and insulin expressed as fmol bound per testis was increased 6-fold and 45-fold, respectively, during testis development from 21-days of age up to the adult age.

It is discussed that the numbers of receptors for IGF–I and insulin in testis may be developmentally regulated, and that IGF–I may be more important than insulin with respect to testis development and Sertoli cell maturation in the immature rat.

Keywords: Sertoli cells, testis, IGF–I, insulin, spermatogenesis.

Introduction

Under defined incubation conditions Sertoli cells from immature rats perform glycolysis at a high rate, which results in accumulation of lactate in the incubation medium (Robinson and Fritz, 1981; Jutte et al., 1982). Exogenous lactate may be an essential energy substrate for spermatogenic cells (Jutte et al., 1981; Mita et al., 1982; Grootegoed et al., 1984, 1986b), and effects of hormones on glucose metabolism by Sertoli cells might have an influence on the efficiency of spermatogenesis.

Glucose transport and lactate production by cultured Sertoli cells are stimulated by insulin-like growth factor–I (IGF–I) via IGF–I receptors (Borland et al., 1984; Mita et al., 1985). We have reported previously that glucose metabolism by cultured Sertoli cells also was stimulated by low, physiological concentrations of insulin (Oonk et al.,

1985). This rapid metabolic effect of insulin probably was mediated via insulin receptors, in view of the presence of high affinity binding sites for insulin on Sertoli cells (Oonk and Grootegoed, 1987). Therefore, glycolysis and other biochemical activities by Sertoli cells may be targets for both IGF–I and insulin. With respect to this, the relative importance of IGF–I and insulin has not been established.

IGF–I binding to receptors on Sertoli cells has been demonstrated using affinity labelling techniques (Borland et al., 1984). However, the binding of IGF–I has not been further characterized. The aim of the present experiments was to study the effect of IGF–I on glucose metabolism by cultured rat Sertoli cells, and to characterize the binding of IGF–I in this system.

Developmental regulation of the number of IGF–I and insulin receptors has been described for different tissues and cell types (Sara et al., 1983; Beguinot et al., 1985; Shimizu et al., 1986), and it has been reported that insulin binding in total testis preparations increased during testis development (Saucier et al., 1981). Therefore, we have also measured the binding of IGF–I and insulin to total testis membrane fractions from immature and adult rat testes.

Materials and Methods

Materials

The recombinant analogue (Thr^{59}) of human insulin-like growth factor–I (IGF–I or somatomedin C) and (3–[^{125}I]iodotyrosyl)-(Thr^{59})-IGF–I were purchased from Amersham International, Amersham, U.K.. Porcine insulin and mono-^{125}I-(Tyr A14)-porcine insulin were purchased from Novo Industri, Copenhagen, Denmark. Follitropin (NIH-ovine-FSH-S13) and lutropin (NIH-ovine-LH-S18) were gifts from The Endocrinological Study Section of the National Institutes of Health, Bethesda, MD, U.S.A.. Collagenase (CLS–1) was purchased from Worthington, Freehold, NJ, U.S.A.. Bovine serum albumin (fraction V) (BSA) was purchased from Sigma, St. Louis, MO. U.S.A.. All chemicals were of the highest purity available.

Isolation and incubation of Sertoli cells

Sertoli cells were isolated from testes from 3-week-old rats (Wistar, substrain R–1 Amsterdam). The Sertoli cells were isolated using a collagenase-digestion method, and incubated in Eagle's minimum essential medium (MEM; Gibco, Grand Islands, NY, U.S.A.), supplemented with L-glutamine and antibiotics, at 32°C under an atmosphere of 5% CO_2 in air, as described previously (Oonk et al., 1985). After two days of incubation in medium containing 1% (v/v) fetal calf serum, the cells were treated with a hypo-osmotic shock to remove the spermatogenic cells (Oonk and Grootegoed, 1987). Following this treatment, the Sertoli cells were incubated for 24 h in medium without serum. Subsequently, the binding of ^{125}I-IGF–I was estimated as described below, or the medium was renewed and the cells were incubated for 6 h (in the absence and presence of IGF–I) to estimate lactate production (Oonk et al., 1985).

Binding of ^{125}I-IGF–I to Sertoli cells

The experiments on IGF–I binding were performed using a confluent layer of Sertoli cells, attached to the bottom of 4 cm^2 culture wells (approximately 200 μg protein per well), after incubation for three days as described above. The binding of ^{125}I-IGF–I was estimated under the same conditions as used previously to evaluate insulin binding (Oonk and Grootegoed, 1987). The cells were incubated for 120 min at 22°C in phosphate-buffered saline (PBS), pH 8, containing 1.6% (w/v) BSA and 0.065 nM ^{125}I-IGF–I (approximately 50,000 c.p.m.). Where indicated, different time periods or different pHs were used. Non-specific binding was determined by incubation in the presence of the ^{125}I-labelled IGF–I and 65 nM unlabelled IGF–I. The incubations were terminated by rinsing the culture wells with ice-cold PBS containing 0.8% BSA to remove unbound radioactivity. The cells were dissolved in 1 M NaOH and cell-bound radioactivity was determined using an LKB 1280 γ–counter. Cellular protein was

determined according to Lowry et al. (1951), using BSA as a standard.

Specific binding represents total minus non-specific binding. The non-specific binding of IGF–I accounted for 11-17% of total binding.

Preparation of membrane fractions

For the preparation of a membrane fraction from rat testis and kidney homogenates the method described by Saucier et al. (1981) was used. Testes and kidneys were taken from 21-day-old and adult (4-month-old) rats. Decapsulated testes and kidneys were kept at 0°C and weighed rapidly. Testis weights represent mean wet weight of 20 testes (3-week-old rats) or 6 testes (adult rats) per preparation and kidney weights represent mean wet weight of 10 kidneys (3-week-old rats) or 4 kidneys (adult rats) per preparation. Testes and kidneys were finely minced using surgical scissors and homogenized in 5 volumes ice-cold 0.3 M sucrose, containing 25 mM Tris-HCl (pH 7.6), with 5 up-and-down strokes of a mechanically driven Teflon/glass homogenizer. The homogenate was centrifuged for 20 min at 900 x *g* and the pellet was discarded. The supernatant was centrifuged for 60 min at 100,000 x *g* in a Beckman L5-65B ultracentrifuge. The 100,000 x *g* pellet was resuspended in 25 mM Tris-HCl buffer (pH 7.6), containing 0.12 M NaCl, 2.5 mM KCl and 6 mM $MgSO_4$, to a protein concentration of 4-10 mg/ml. The membrane suspension was rapidly frozen and kept in liquid N_2 until use.

Binding assay

Portions of the membrane preparations were incubated with 0.65 nM ^{125}I-IGF–I or 0.83 nM ^{125}I-insulin in a total volume of 200 µl. Non-specific binding was estimated by adding 65 nM unlabelled IGF–I or 833 nM unlabelled insulin. The incubation buffer, which was also used for the preparation of the dilutions of the labelled and unlabelled hormones, was a 25 mM Tris-HCl buffer (pH 7.6), containing 0.5% (w/v) BSA. An estimated amount of 0.2 mg membrane protein was added to each incubation, but the exact amount of protein was determined at the end of the incubations. Incubations were carried out in Eppendorf vessels at 0°C for 4 h. At the end of the incubations, a portion of 150 µl was taken from each tube and layered on top of 200 µl 0.3 M sucrose in microfuge tubes, which were inserted in larger tubes and centrifuged at 15,000 x *g* for 10 min at 4°C. The microfuge tubes were frozen in liquid nitrogen and the lower end of the tubes containing the membrane pellet was cut off with a hot wire. The radioactivity in each tube end was determined using a γ-counter, and the pellets were dissolved in 1 M NaOH to estimate the protein content according to Bradford (1976), using BSA as a standard. The separation of bound and free radioactivity was very efficient, as the bottom of tubes without membranes contained less than 0.08% of the added radioactivity. The linear Scatchard plots for IGF–I binding were analyzed using linear regression analysis. Scatchard plots for insulin binding were curvilinear, and were analyzed into a high and a low affinity component, according to Rosenthal (1967).

The membrane fractions may contain proteolytic enzyme activity. Degradation of IGF–I and insulin during the incubations at 0°C was determined by precipitation with 10% (w/v) trichloroacetic acid, as described by Donner (1980). Under the present incubation conditions (4 h at 0°C), the same amounts of ^{125}I-IGF–I and ^{125}I-insulin were degraded, which amounted to 2.2 ± 1.7% in the membrane fractions from testes and 7.8 ± 4.8% in the membrane fractions from kidneys.

Non-specific binding of ^{125}I-IGF–I, in the presence of 65 nM unlabelled IGF–I, was 6.8% in testicular and 9.4% in kidney membrane fractions. Non-specific binding of ^{125}I-insulin, in the presence of 833 nM unlabelled insulin, amounted to 2.1% in testicular and 3.4% in kidney membrane fractions.

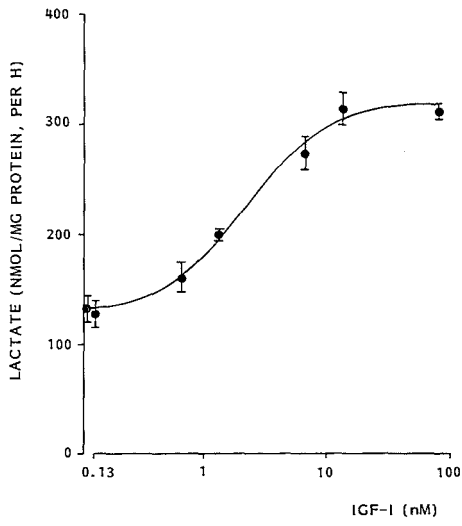

Figure 1. Dose-response curve of the effect of IGF–I on lactate production. Sertoli cells were isolated and preincubated as described in Materials and Methods, and incubated for 6 h at 32°C in the presence of different concentrations of IGF–I. Net lactate production during this 6 h-incubation period was expressed as nmol/mg protein per h (mean ± S.D. of triplicate incubations).

Results

IGF–I binding to Sertoli cells

After a 3-day preincubation period in the absence of added hormones, lactate production by Sertoli cells was stimulated by IGF–I in a dose-dependent way, and the half-maximal effect was obtained at a concentration of 2 nM (Fig. 1). This stimulation was estimated after 6 h, but results from other experiments indicate that stimulation occurred within 30 min.

Specific binding of ^{125}I-IGF–I to Sertoli cells was maximal at pH 7.5-8.5, with a sharp decline at more acidic or alkaline pH (not shown). The specific binding reached a maximum after 2 to 3 h (Fig. 2a), and was reversible (Fig. 2b).

Figure 3 illustrates competition by IGF–I and insulin for tracer IGF–I binding to the cultured Sertoli cells. Half-maximal displacement of tracer was observed at 2 nM IGF–I, whereas insulin was a much less effective competitor. Insulin at a concentration of 167 nM caused a displacement of only 30% of maximal tracer IGF–I binding. The gonadotropic hormones lutropin and follitropin did not compete with IGF–I binding (Fig. 3).

Scatchard plots of IGF–I binding were linear ($r = 0.91$) and indicated a K_d of 3.5 ± 0.2 nM. The receptor concentration calculated was equivalent to 2080 ± 140 fmol/mg protein (Fig. 4).

IGF–I binding was also determined in preparations of isolated spermatogenic cells. Spermatocytes and spermatids were isolated and incubated for 18 h as described by Grootegoed et al. (1984; 1986a). After this preincubation, the cells contained transferrin receptors, as concluded from studies on active iron transport (Toebosch et al., 1987). However, the specific binding of IGF–I estimated in these preparations was very low. The maximal specific binding of IGF–I to pachytene spermatocytes was 1.3 fmol/mg protein and to round spermatids 0.4 fmol/mg protein.

IGF–I and insulin binding to membrane fractions of total testis

Hormone binding was analyzed using membrane fractions from testes and kidneys. Data on insulin binding in adult rat testis and kidney membrane fractions have been reported (Saucier et al., 1981), and in the present experiments the kidneys were included to make a comparison with testes.

The specific binding of ^{125}I-IGF–I and ^{125}I-insulin to membrane fractions from testes and kidneys reached a maximum after 4 h of incubation at 0°C, and did not increase further up to 20 h of incubation (not shown). Therefore, all the incubations were performed during 4 h at 0°C.

In membrane fractions from testes of 21-day-old and adult rats, specific binding of IGF–I (Fig. 5) and insulin (Fig. 6) was observed. The displacement characteristics for IGF–I binding appeared to be similar in the two preparations. However, with respect to insulin binding a difference was observed, in that insulin binding to the testis

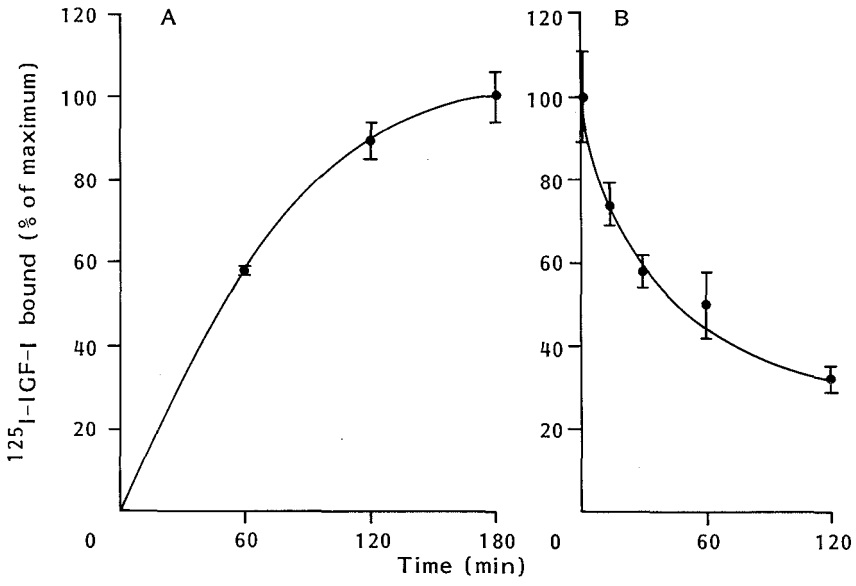

Figure 2. Association and dissociation of ^{125}I-IGF–I binding to Sertoli cells. A) Time-course of ^{125}I-IGF–I binding. Sertoli cells were incubated at 22°C in the presence of ^{125}I-IGF–I for the indicated time periods, as described in Materials and Methods, and specific binding was determined. The results are expressed as the percentage of maximal radioactivity specifically bound at each time (mean ± S.D. of triplicate incubations). ^{125}I-IGF–I bound was 427 ± 12 fmol/mg protein after 180 min. B) Time-course of ^{125}I-IGF–I dissociation. Sertoli cells were incubated at 22°C in the presence of ^{125}I-IGF–I for 120 min. The cells were then washed and incubated with 1 ml PBS containing 0.8% BSA in the presence of 65 nM unlabelled IGF–I for the indicated time periods. The results are expressed as percentage of ^{125}I-IGF–I bound at zero time (334 ± 35 fmol IGF–I/mg protein) (mean ± S.D. of triplicate incubations).

membrane fractions from 21-day-old rats was displaced only by a five times higher concentration of insulin, and that IGF–I seemed to be a more effective competitor (Fig. 6A), as compared to the data for the adult testis membrane fractions (Fig 6B). Such age differences in competition behaviour were not observed using membrane fractions from kidneys. The displacement curves obtained using kidney membrane fractions, indicating highly specific binding for IGF–I and insulin, are not shown.

Scatchard analysis of IGF–I and insulin binding to the membrane fractions from immature and adult rat testes resulted in straight lines for IGF–I binding (Fig. 7) and curvilinear plots for insulin binding (Fig. 8). The Scatchard plots for kidney membrane fractions are not shown. The binding parameters calculated from the present data are given in Table 1. For insulin binding, only the high affinity component was analyzed, as the available data precluded precise resolution of the binding parameters for the low affinity sites, and in addition insulin may bind with low affinity to IGF–I receptors. The binding constants for IGF–I and for high affinity insulin binding were all below 6 nM (Table 1). The total number of binding sites for IGF–I and insulin per testis and per kidney increased with age (Table 1).

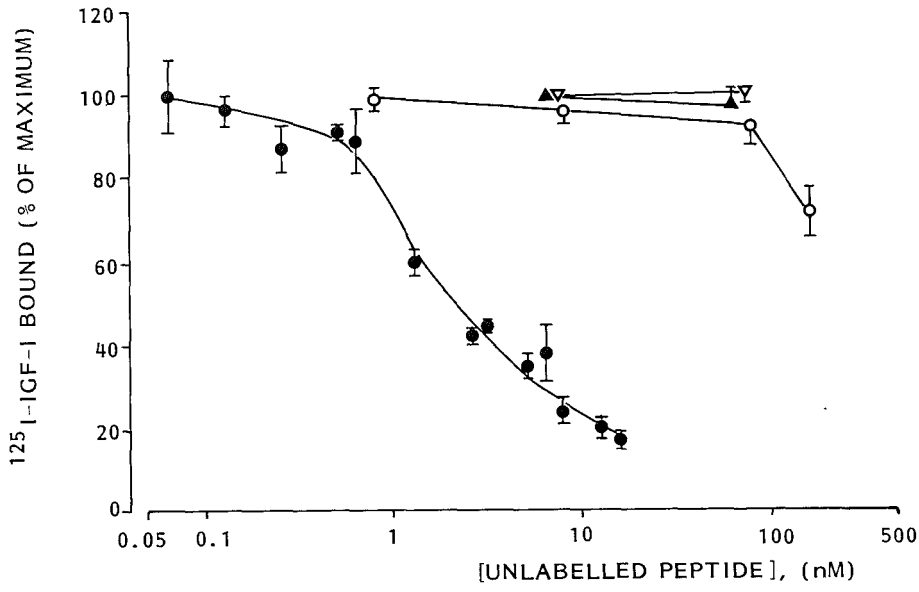

Figure 3. Specificity of binding of ^{125}I-IGF–I. Sertoli cells were incubated for 120 min at 22° C in the presence of 0.065 nM ^{125}I-IGF–I and different concentrations of unlabelled IGF–I (●—●), porcine insulin (o—o), follitropin (▲–▲) and lutropin (▽–▽), as described in Materials and Methods. Binding of ^{125}I-IGF–I is expressed as percentage of the binding in the absence of any other added hormone. The data were corrected for non-specific binding (mean ± S.D. of 3-6 incubations).

Table 2 shows the maximal specific binding of IGF–I and insulin at low hormone concentrations (0.65 nM and 0.83 nM, respectively) to the different membrane fractions. Binding data, expressed as maximal specific binding per whole testis or kidney, showed an increase with age (Table 2). However, testis weights (decapsulated, wet weight) and kidney weights (wet weight) were much higher in the adult animals (Table 2). Therefore, binding was also expressed as fmol bound/g wet weight, and it appeared that IGF–I binding was relatively high and insulin binding was relatively low in testes from 21-day-old rats, as compared to adult testes and immature and adult kidneys (Table 2). The IGF–I binding decreased and the insulin binding increased during testis development.

Discussion

The present results indicate that Sertoli cells may be an important testicular target for IGF–I. Lactate production by cultured Sertoli cells from 21-day-old rats was markedly stimulated by IGF–I, with a half-maximal effective concentration of 2 nM. Furthermore, binding studies showed specific IGF–I binding to high affinity IGF–I receptors. The calculated number of IGF–I receptors on the cultured Sertoli cells (2080 ± 140 fmol/mg protein) is very large, compared to the number of high affinity insulin receptors (8.5 ± 1.0 fmol/mg protein), which was observed in a previous study (Oonk and Grootegoed, 1987). The presence of IGF–I receptors on Sertoli cells has also been observed using an affinity labelling technique (Borland et al., 1984), but in these

studies the receptors were not further quantitated and characterized.

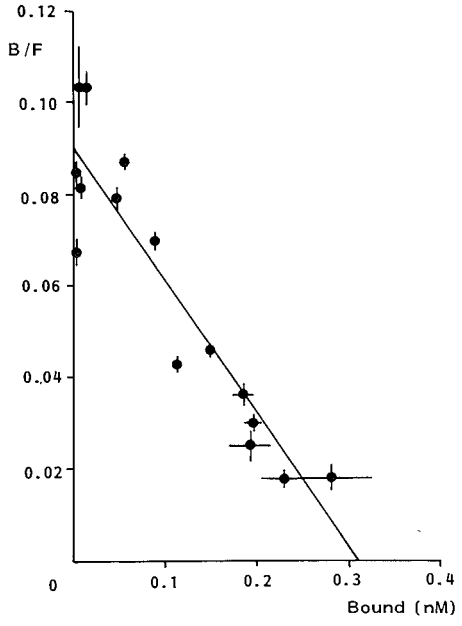

Figure 4. Scatchard analysis of ^{125}I-IGF–I binding to Sertoli cells. ^{125}I-IGF–I binding was measured as described in Materials and Methods, in the presence of increasing concentrations of unlabelled IGF–I (0-15 nM). Specific binding is expressed as the ratio bound/free (B/F) and plotted as a function of the amount of hormone bound (mean ± S.D. of triplicate incubations). The correlation coefficient of the line calculated by linear regression analysis was 0.91.

Receptors for IGF–I and insulin are structurally very similar (Kasuga et al., 1981; Nissley et al., 1985). Most cells possess IGF–I and insulin receptors, and IGF–I and insulin can interact with both receptor types. At first, it has been proposed that long-term effects of IGF–I and insulin (for example DNA- and protein synthesis, cell growth) were mediated exclusively via IGF–I receptors, and that rapid metabolic effects of IGF–I and insulin (for example glucose transport and metabolism) were mediated predominantly via insulin receptors (Kahn et al., 1981). However, this generalization may not hold, because it has been reported that insulin can promote cell growth after binding to specific high affinity insulin receptors (Koontz, 1984), and the IGF–I-receptors may be the principal receptors involved in distinct rapid metabolic effects of IGF–I (Yu and Czech, 1984).

In cultured Sertoli cells, a number of effects are induced by low concentrations of IGF–I, but also by very high concentrations of insulin, for example stimulatory effects on DNA- and protein synthesis (Borland et al., 1984). This may indicate insulin action via IGF–I receptors. This is in agreement with the present observations on the marked abundance of IGF–I receptors on cultured Sertoli cells, as compared to a low number of high affinity insulin receptors (Oonk and Grootegoed, 1987). However, under defined incubation conditions rapid metabolic effects of insulin on Sertoli cells are mediated most likely via the high affinity insulin receptors (Oonk et al., 1985; Oonk and Grootegoed, 1987).

There are indications that IGF–I might play a role in gonadal function and differentiation. In the testis, the presence of IGF–I receptors has been demonstrated for Sertoli cells (Borland et al., 1984; present results), and for Leydig cells (Handelsman et al., 1985; Lin et al., 1986; Chatelain, 1986). Recently, Lin et al. (1986) and Bernier et al. (1986) showed responsiveness of Leydig cell steroidogenesis to IGF–I. In the ovary, the presence of receptors for IGF–I on granulosa cells has been observed (Baranao and Hammond, 1984; Veldhuis et al., 1985). IGF–I stimulates granulosa cell proliferation and differentiation, and synergizes with follitropin (Adashi et al., 1985). Human chorionic gonadotropin (hCG) enhances IGF–I binding to Leydig cells (Chatelain et al., 1986) and follitropin enhances IGF–I binding to granulosa cells (Adashi et al., 1986), suggesting that acquisition of IGF–I responsiveness might be important in the development of Leydig cells and granulosa cells. With respect to this, it is of interest to establish if IGF–I can act synergistically with follitropin on Sertoli cells, and if follitropin affects IGF–I binding to Sertoli cells.

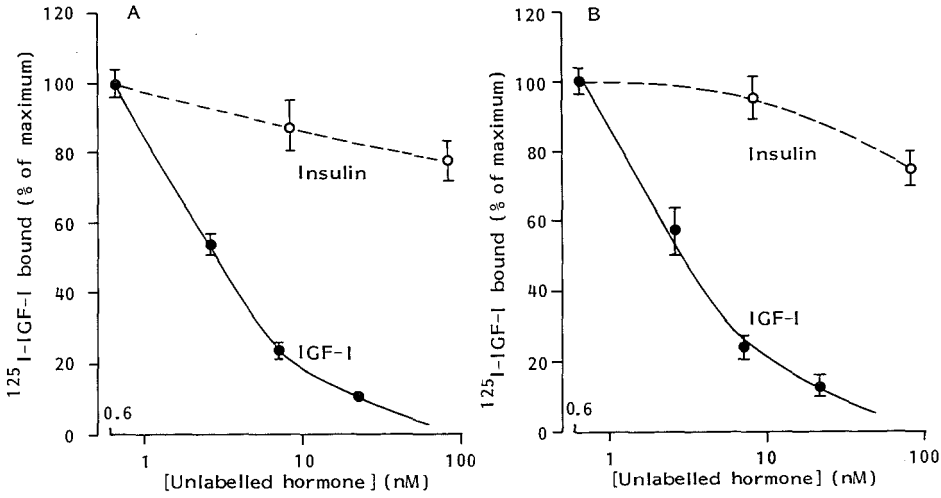

Figure 5. Specificity of binding of ^{125}I-IGF–I to testis membrane fractions from (A) 21-day-old rats and (B) adult rats. Specific binding of ^{125}I-IGF–I was determined, as described in Materials and Methods, in the absence and presence of different concentrations of unlabelled IGF–I (●–●) and insulin (o--o). Binding of ^{125}I-IGF–I is expressed as percentage of the binding in the presence of 0.65 nM ^{125}I-IGF–I. Each point represents the mean ± S.D. of 2-3 experiments with duplicate incubations.

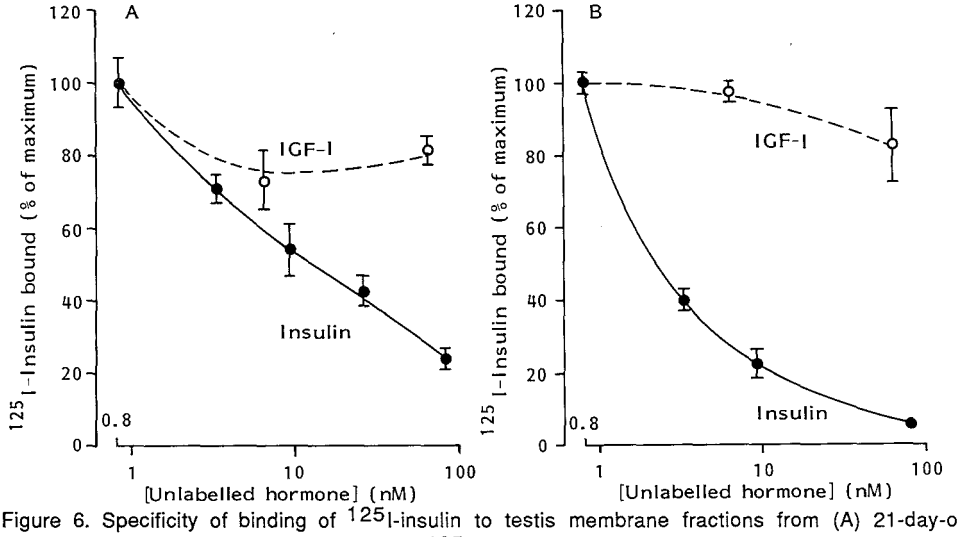

Figure 6. Specificity of binding of ^{125}I-insulin to testis membrane fractions from (A) 21-day-old rats and (B) adult rats. Specific binding of ^{125}I-insulin was determined, as described in Materials and Methods, in the absence and presence of different concentrations of unlabelled insulin (●–●) and IGF–I (o--o). Binding of ^{125}I-insulin is expressed as percentage of the binding in the presence of 0.83 nM ^{125}I-insulin. Each point represents the mean ± S.D. of 2-3 experiments with duplicate incubations.

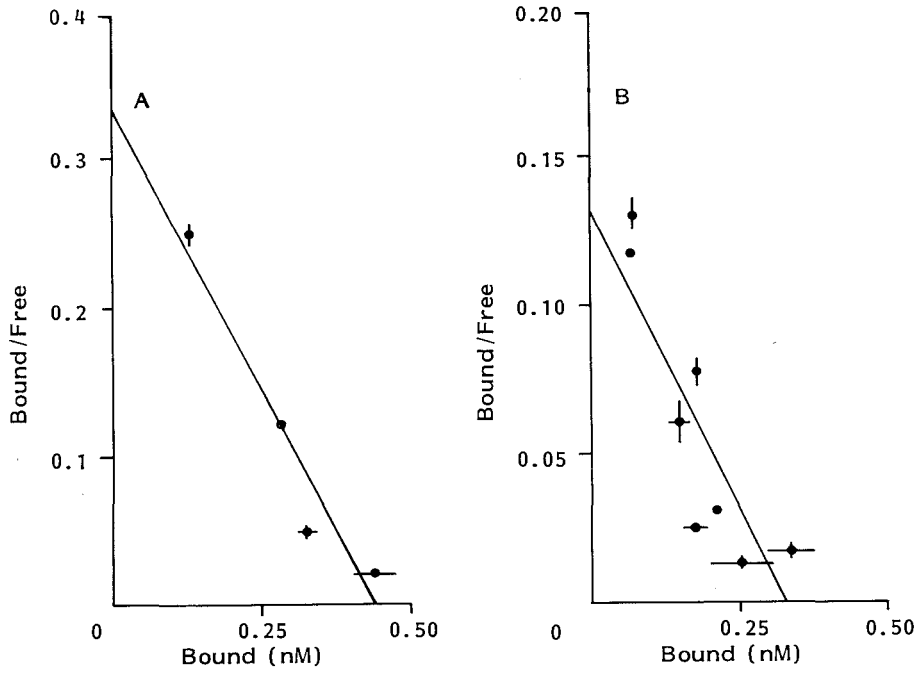

Figure 7. Scatchard plots of ^{125}I-IGF–I binding to testis membrane fractions from (A) 21-day-old rats and (B) adult rats. Specific binding was expressed as the ratio bound/free hormone (B/F) and plotted as a function of hormone bound. The data are from a representative experiment. The compiled data from this serie of experiments on the binding kinetics are given in Table 1.

The present results on hormone binding to total tissue membrane fractions may represent a quantitative estimate of the specific binding of IGF–I and insulin to whole testes and kidneys (Saucier et al., 1981). The present results indicate that, expressed per g wet weight, IGF–I binding in testis is decreased, and insulin binding in testis is increased during testis growth. However, the binding of IGF–I and insulin per testis and per kidney is increased with age. This effect was most marked for insulin, also because insulin binding in the immature testis was very low. Concomitantly, in 21-day-old testis the competition by insulin for maximal tracer insulin binding was not very strong, but it has not been studied if this is a consequence of a low number of high affinity insulin binding sites.

Spermatogenic cells quantitatively constitute most of the adult testis with fully established spermatogenesis. However, the spermatogenic cells may not possess IGF–I or insulin receptors. From results obtained using an immunocytochemical method, it has been suggested that spermatogenic cells display binding sites for IGF–I (Tres et al., 1986). In the present experiments, however, virtually no specific binding of IGF–I to purified late pachytene spermatocytes and round spermatids was observed, under conditions that allowed for transferrin receptor-mediated iron uptake (Toebosch et al. 1987). The very low amount of IGF–I binding estimated for the spermatogenic cell preparations can be explained by less than 1% contamination with Sertoli cells in these preparations. Testicular cell types other than spermatogenic cells include Sertoli cells, Leydig cells and peritubular myoid cells. In cultured adult Leydig cells IGF–I receptors have been demonstrated (Lin et al., 1986). However, Sertoli cells may contain much

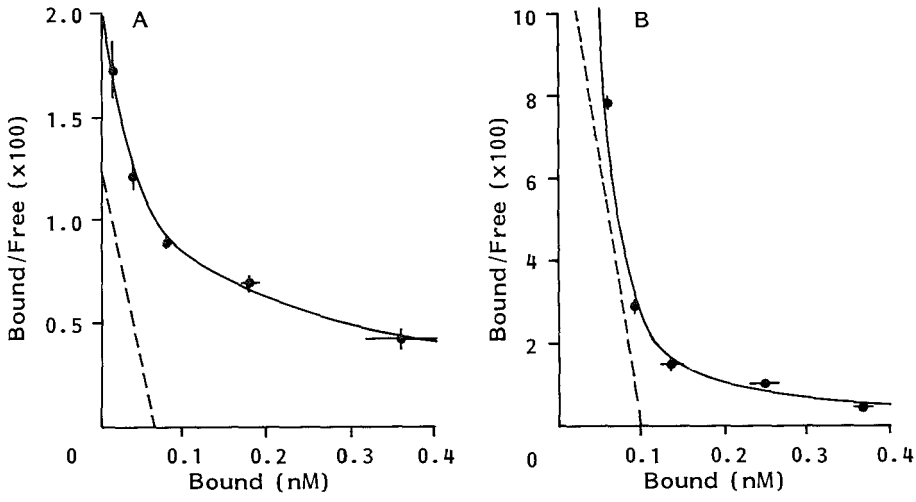

Figure 8. Scatchard plots of ^{125}I-insulin binding to testis membrane fractions from (A) 21-day-old rats and (B) adult rats. Specific binding was expressed as the ratio bound/free hormone (B/F) and plotted as a function of hormone bound. The data are from a representative experiment. The compiled data from this serie of experiments on the binding kinetics are given in Table 1.

Table 1. Affinity constants (K_d) and the number of binding sites (R_o) in testes and kidneys from 21-day-old and adult rats.

		K_d (nM)	R_o (fmol/organ)	r *
IGF-I binding	Testis, 21 days	1.4 ± 0.2	49 ± 6	-0.96
	Testis, adult	2.5 ± 0.2	389 ± 60	-0.81
	Kidney, 21 days	2.9 ± 0.2	61 ± 4	-0.95
	Kidney, adult	4.5 ± 0.4	651 ± 52	-0.96
Insulin binding	Testis, 21 days	4.9 ± 0.6	7 ± 1	-0.87
	Testis, adult	0.8 ± 0.1	161 ± 21	-0.95
	Kidney, 21 days	5.3 ± 0.4	70 ± 5	-0.88
	Kidney, adult	4.2 ± 0.3	308 ± 24	-0.99

The data are determined from Scatchard plots as shown in Figs. 7 and 8. For insulin, the two-site model was used, and the binding parameters for the high affinity site are shown. *r, correlation coefficient for the line, calculated with linear regression analysis. The values shown are the mean ± S.D. of two or three different experiments with duplicate incubations.

Table 2. Maximal specific binding of ^{125}I-IGF–I and of ^{125}I-insulin to testes and kidneys from 21-day-old and adult rats.
A).

	Maximal specific binding of IGF–I		
	fmol/organ	organ wet weight (g)	fmol/g wet weight
Testis, 21 days	83 ± 2	0.087	871 ± 50
Testis, adult	473 ± 20	1.33	324 ± 40
Kidney, 21 days	87 ± 8	0.21	403 ± 35
Kidney,adult	817 ± 16	1.50	544 ± 11

B).

	Maximal specific binding of insulin		
	fmol/organ	organ wet weight (g)	fmol/g wet weight
Testis, 21 days	10 ± 1	0.087	118 ± 11
Testis, adult	446 ± 17	1.33	330 ± 17
Kidney, 21 days	82 ± 10	0.21	383 ± 47
Kidney, adult	625 ± 10	1.50	417 ± 6

The maximal specific binding in the presence of 0.65 nM ^{125}I-IGF–I (A) and 0.83 nM ^{125}I-insulin (B) to testis and kidney membrane fractions was determined as described in Materials and Methods. The values represent the mean ± S.D. of 2-3 experiments with duplicate incubations.

more IGF–I receptors, because the amount of IGF–I receptors on cultured Sertoli cells from immature rats was 2080 fmol/mg protein. It can be calculated that 870 fmol IGF–I bound per g wet weight corresponds to approximately 10 fmol IGF–I bound per mg protein (100 mg protein/g wet weight, Mills et al., 1977). In testis from 21-day-old rats about 10% of the cells are Sertoli cells (De Jong and Sharpe, 1977). Therefore, IGF–I binding to Sertoli cells may explain the total amount of IGF–I binding to immature testis. However, it cannot be excluded that the number of IGF–I binding sites on Sertoli cells is increased during culture. Moreover, the total testis IGF–I binding may be too low an estimate of the actual number of testicular IGF–I binding sites due to occupancy of receptors by endogenous hormone and receptor loss during preparation of the membrane fractions.

It has been described, that in myoblast-myotube (Beguinot et al., 1985) and in preadipocyte-adipocyte (Shimizu et al., 1986) cell systems insulin binding increased and IGF–I binding decreased concomitant with cell differentiation. A developmental shift in receptor numbers has also been reported for chicken embryo brain tissue, where an early dominance of IGF–I receptors over insulin receptors was observed during embryogenesis (Bassas et al., 1985). Furthermore, studies on ontogenic development of receptors in human fetuses indicate that IGF–I and specific IGF–I receptors were active from early development, whereas insulin and specific insulin receptors might play a more important role later in fetal development (Sara et al., 1983, 1986). In this respect, the testis may show some similarity with the systems described above. During postnatal growth of the testis, the number of receptors for IGF–I and insulin may undergo a similar developmental switch.

Experimental evidence indicates that somatomedins (IGFs) originate not only in the liver, but in many different tissues, in-

cluding the gonads. IGF–I activity in rat testis was first reported by Ritzén (1983). IGF–I immunoreactivity has been extracted from rat testes (D'Ercole et al., 1984; Handelsman et al., 1985), and this activity was enhanced by growth hormone treatment (D'Ercole et al., 1984). Cultured Sertoli cells from immature rats accumulate endogenous IGF–I immunoreactivity (Tres et al., 1986), and recently the partial purification and characterization of IGF–I-like activity from Sertoli cell culture medium was reported (Chatelain et al., 1986; Smith et al., 1987). Furthermore, it has been reported that peritubular myoid cells, which may be present in different amounts in the various Sertoli cell preparations, also secrete IGF–I (Skinner and Fritz, 1986; Tres et al., 1986). The role of testicular IGF–I is unknown, but may involve autocrine and paracrine effects on Sertoli cells and Leydig cells. In the ovary, production of IGF–I by granulosa cells has been reported (Hammond et al., 1985), and recent data indicate regulation of granulosa cell IGF–I production by growth hormone (Davoren and Hsueh, 1986), gonadotropins and estradiol (Hsu and Hammond, 1987).

In conclusion, cultured Sertoli cells from immature rats contain a very large number of high affinity IGF–I receptors. In agreement with this, low doses of IGF–I stimulate glucose metabolism and other biochemical activities in cultured Sertoli cells. The effects of IGF–I on Sertoli cells may play an important role in testicular development.

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Comparison of the effects of insulin and follitropin on glucose metabolism by Sertoli cells from immature rats

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Summary

Sertoli cells were isolated from the testes of 3-week-old sterile rats (prenatally irradiated) and incubated for 3 days in the absence of added hormones. Subsequently the effects of follitropin and insulin on glucose metabolism were investigated using this in vitro system.

A marked stimulation of net lactate production by either follitropin or insulin was observed within 3 h after addition of the hormones. This response was not inhibited in the presence of the protein synthesis inhibitor cycloheximide. Production of cAMP by the Sertoli cells was markedly enhanced by follitropin, but not at all by insulin. The addition of 0.5 mM dibutyryl cAMP to the incubation medium also resulted in a rapid increase of the rate of lactate production by the Sertoli cells. The stimulation of lactate production by follitropin and insulin was dose-dependent (ED_{50} of approx. 10 ng NIH-FSH-S13/ml and of approx. 50 ng insulin/ml). It is suggested that the observed effects of insulin on Sertoli cells are mediated via insulin receptors, rather than via receptors for insulin-like growth factors. Within 18 h after addition of either follitropin or insulin the cells became refractory with respect to lactate production to the homologous hormone, whereas the cells could still respond to the heterologous hormone. It is concluded, that follitropin and insulin, acting via different mechanisms, exert similar rapid stimulatory effects on glucose metabolism by Sertoli cells from immature rats in vitro. These effects are not dependent on de novo protein synthesis and may differ from long-term trophic effects of follitropin, insulin, and/or insulin-like growth factors.

In the spermatogenic epithelium from mammalian testes, Sertoli cells may provide a micro-environment for development and differentiation of spermatogenic cells. Spermatogenesis is regulated by at least 2 hormones, viz. follitropin and testosterone. Follitropin appears to be involved mainly in the initiation of spermatogenesis and the concomitant maturation of Sertoli cells in testes from immature rats, whereas testosterone is important to maintain spermatogenesis in adult rats (Fritz, 1978). In the spermatogenic epithelium, Sertoli cells rather than the spermatogenic cells appear to be target cells for follitropin and testosterone (Fritz, 1978). In this regard it has been shown, that follitropin exerts pronounced stimulatory effects on the production of proteins

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and other compounds by Sertoli cells from immature rats in vitro, including androgen binding protein (Louis and Fritz, 1979), plasminogen activator (Lacroix et al., 1977), transferrin (Skinner and Griswold, 1982), and myoinositol (Robinson and Fritz, 1979). Similarly, Sertoli cells from immature rats in vitro are responsive to insulin and/or insulin-like growth factors. Transferrin secretion is enhanced during incubation in the presence of a high dose of insulin (5 μg/ml) (Skinner and Griswold, 1982), but it has been suggested that insulin might act on Sertoli cells via receptors for insulin-like growth factors (Skinner and Griswold, 1983).

Rat Sertoli cells in vitro utilize glucose at a high rate and produce lactate (Robinson and Fritz, 1981; Jutte et al., 1981, 1982). Net lactate production by Sertoli cells could be important, because it has been shown that exogenous lactate rather than glucose is an essential energy substrate for spermatocytes and spermatids (Jutte et al., 1981, 1982; Grootegoed et al., 1984). Stimulatory effects of follitropin, insulin, and/or IGF-I on glucose metabolism and lactate production by Sertoli cells in vitro have been reported (Grootegoed et al., 1981; Mita et al., 1982; Jutte et al., 1982, 1983; Le Gac et al., 1983; Borland et al., 1984). The effect of insulin on glucose metabolism might be caused by binding of insulin to receptors for insulin-like growth factors (Borland et al., 1984), similar to the effects of insulin on transferrin secretion. In fact, it has been suggested that rat Sertoli cells lack insulin receptors (Borland et al., 1984).

In this respect we have studied the effects of insulin on glucose metabolism by Sertoli cells from immature rats in vitro. The results show that low doses of insulin exert a rapid stimulatory effect on glucose metabolism and we have compared this effect with the effect of follitropin.

Materials and methods

Materials

Collagenase (CLS-I) was purchased from Worthington, Freehold, NJ, U.S.A. Follitropin (NIH-ovine-FSH-S13) was a gift from NIH, Bethesda, MD, U.S.A. Bovine insulin (26.8 U/mg) was purchased from Sigma, St. Louis, MO, U.S.A. Cycloheximide, dibutyryl cAMP, lactate dehydrogenase from pig heart and NAD^+-free acid grade I, were purchased from Boehringer Mannheim, Mannheim, F.R.G. D-[U-^{14}C]glucose (260 Ci/mol) and the cyclic AMP-assay kit were purchased from Amersham International, Amersham, U.K. L-[4,5-^{3}H]lysine (87 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A. All other chemicals were of analytical grade.

Isolation and incubation of Sertoli cells

In the present experiments we have used the testes of 3-week-old Wistar rats to isolate Sertoli cells. These rats had become sterile after irradiation in utero on day 20 of gestation with 150 Rad (Beaumont, 1960). Sertoli cell preparation from the sterile rats do not contain germ cells, which could interfere with the assay of glucose metabolism. The rats (mean body weight 44.5 ± 4.0 g) were killed by inhalation of CO_2 and the testes were removed quickly, decapsulated and weighed (mean wet weight of one testis 50.7 ± 5.7 mg). Subsequently, 10–12 testes were incubated in 20 ml Dulbecco's phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954) with 0.5 mg collagenase/ml, for 30 min at 32°C in a 100 ml Erlenmeyer flask in a shaking water bath (120 cycles/min). During this incubation the testes became dissociated into tubules. Blood vessels and a few small tissue clumps were removed with a Pasteur's pipette. The tubules were transferred to a 25 ml measuring cylinder and allowed to sediment at unit gravity for 2 min and washed once with 25 ml PBS. The tubules were subsequently incubated for another period of 30 min with collagenase under the same conditions as described above. As a result of the prolonged collagenase treatment the tubular wall dissociated from the Sertoli cells. The tubular fragments (Sertoli cell clusters) were washed (sedimentation for 2 min at $1 \times g$) 3 times with PBS and once with Eagle's minimum essential medium (MEM, Gibco, Grand Islands, NY, U.S.A.), supplemented with non-essential amino acids, glutamine, fungizone (625 μg/l), streptomycin (100 mg/l) and penicillin (10^5 IU/l). The Sertoli cell clusters were fragmented further by mechanical agitation using a Dounce homogenizer (10–15 strokes). The final preparation of small Sertoli cell clusters was washed 4–6 times with MEM (sedimentation for 2 min at $100 \times g$), and

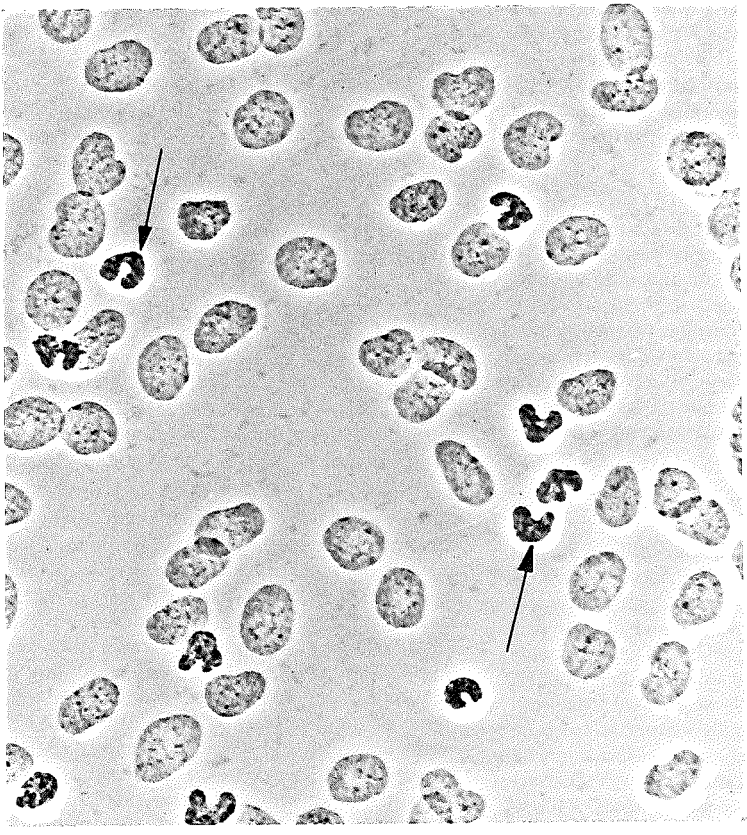


Fig. 1. Phase-contrast micrograph of a freshly isolated Sertoli cell preparation. The testes of 3-week-old rats were treated with collagenase for 15 min rather than 2 times 30 min to be able to show the presence of peritubular cell nuclei (arrow), next to the Sertoli cell nuclei. The cells were fixed in ethanol/acetic acid (3:1, v/v) and viewed in 45% (v/v) acetic acid.

the final pellet was resuspended in MEM (1:10, v/v). Portions of 0.1 ml were transferred to plastic cultures dishes (150–200 μg protein per well with a growth area of 4 cm^2) and incubated at 32°C under an atmosphere of 5% CO_2 in air.

To evaluate the purity and composition of the Sertoli cell preparations a portion of the final cell pellet was fixed in a mixture of ethanol and acetic acid (3:1, v/v). The fixed cells were applied to microscope slides, dried and examined with phase-contrast microscopy after addition of a drop of acetic acid (45%, v/v). The preparation contained at least 94% Sertoli cells (Fig. 1). During incubation the Sertoli cell clusters became attached to the plastic surface and formed a confluent layer within 2 or 3 days after plating. As described by Tung et al. (1984) contaminating peritubular cells may be present underneath the Sertoli cells and it is not absolutely certain that the number of peritubular cells does not increase during incubation. The following incubation scheme was used routinely. After incubation for 48 h in 2

ml MEM supplemented with 1% (v/v) fetal calf serum, the cells were washed twice and incubated for 1 day in 1 ml MEM without serum. After this 3-day incubation period in the absence of hormones, the cells were washed with MEM. Further incubations were performed in 1 ml MEM under the conditions described in the Results section.

Estimation of lactate

After incubation of Sertoli cells for time periods and in the presence of compounds as described in the Results section, the incubation medium was removed from the cells and kept at 4°C until lactate was determined. Lactate was determined enzymically, using lactate dehydrogenase and NAD^+, as described by Hohorst (1970). The cells were dissolved in 1 ml 1 M NaOH for determination of the amount of cellular protein (Lowry et al., 1951).

Estimation of the conversion of D-[U-^{14}C]glucose into lipids

Sertoli cells were incubated in medium supplemented with 1 μCi D-[U-^{14}C]glucose (5.6 mM glucose). The incubations were terminated by removal of the incubation medium and addition of 1 ml ice-cold 0.9% (w/v) NaCl. The cells were scraped from the surface using a rubber policeman, and the culture wells were rinsed twice with 0.9% (w/v) NaCl. The lipids were extracted from the cells by mixing the cell/saline suspension with 3 volumes of chloroform/ether (3 : 1, v/v). Extraction of the aqueous phase was repeated 3 times. The combined organic phases were evaporated in scintillation vials, the residue was dissolved in 10 ml Picofluor-TM15 (Packard Instruments, Downers Grove, IL, U.S.A.) and the radioactivity was counted using a Packard Tricarb B3255 liquid scintillation counter.

Estimation of cAMP

Sertoli cells were incubated under conditions as described in the Results section. The incubations were terminated by acid precipitation with perchloric acid (final concentration 5%, v/v). After centrifugation for 5 min at 5000 × *g*, the supernatants were neutralized with KOH. Cyclic AMP was estimated in the neutralized supernatant using a cAMP-assay kit.

Estimation of protein synthesis

Sertoli cells were incubated in medium containing 5 μCi L-[4,5-^{3}H]lysine (0.4 mM lysine), in the absence or presence of cycloheximide (50 μg/ml). The incubations were terminated by cooling to 4°C and addition of sodium dodecylsulphate (final concentration 1%, w/v) and unlabelled lysine (final concentration 2 mM). The cellular macromolecules were precipitated with 20% (w/v) trichloroacetic acid on 0.2 μm filters (Sartorius, Göttingen, F.R.G.). The precipitate was washed twice with 10% (w/v) trichloroacetic acid and once with 70% (v/v) ethanol. The filters were dried in scintillation vials, and subsequently dissolved in 10 ml Filtercount-TM (Packard) for estimation of the amount of radioactivity.

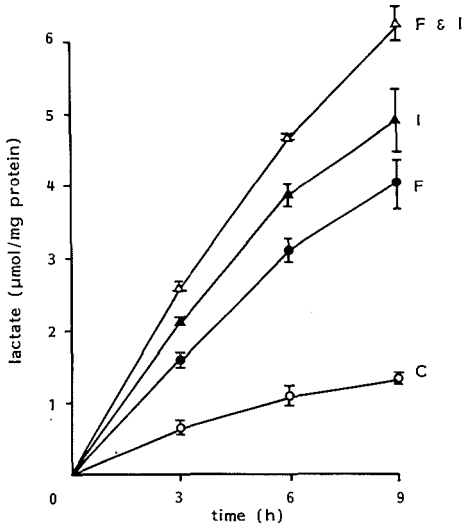
Results

The present experiments were performed after incubation of Sertoli cells for 2 days in medium containing 1% fetal calf serum, followed by incubation for 1 day in medium without serum.

After this 3-day incubation period in the absence of added hormones, a rapid increase of the rate of net lactate production was observed within 3 h after addition of either follitropin or insulin (Fig. 2). Control and hormone-stimulated rates of lactate production showed an almost linear time course up to 6 h, followed by a slight decline in production rate from 6 to 9 h of incubation (Fig. 2; see also below). In the presence of both hormones, however, the effects of follitropin and insulin were not additive (Fig. 2). This may indicate that insulin does not act exclusively on peritubular cells which could be present in the Sertoli cell preparation (see Materials and methods). Rather, the rate of lactate production by the Sertoli cells can be increased by follitropin, but also by insulin.

The stimulation of lactate production by Sertoli cells through addition of follitropin and insulin was dose-dependent (Fig. 3). Half maximal stimulation was obtained at approximately 10 ng follitropin/ml or approximately 50 ng insulin/ml. The time course of stimulation of lactate production by insulin was the same at 5 μg/ml, 0.5 μg/ml or 0.05 μg/ml (not shown).

During prolonged incubation without medium



renewal in the presence of follitropin or insulin, net lactate production was not significantly different from zero from 18 to 24 h of incubation (Table 1A). Inhibitory factors could accumulate in the incubation medium, causing inhibition of glucose metabolism. This is indicated by the decline in lactate production rates from 6 to 9 h of incubation either in the absence or presence of hormones (Fig. 2). In addition, the cells may have become refractory to the hormones. To study the latter possibility, fresh incubation medium was added after 18 h. The results of this series of experiments (Fig. 4) show that net lactate production by Sertoli

Fig. 2. Time course of the effects of follitropin and insulin on lactate production. Net lactate production was estimated after 3, 6 and 9 h of incubation either in the absence or presence of added hormones. The results represent means ± SD of triplicate incubations. Control (C) ○———○, 0.5 µg/ml follitropin (F) ●———●, 5 µg/ml insulin (I) ▲———▲, follitropin and insulin (F & I) △———△.

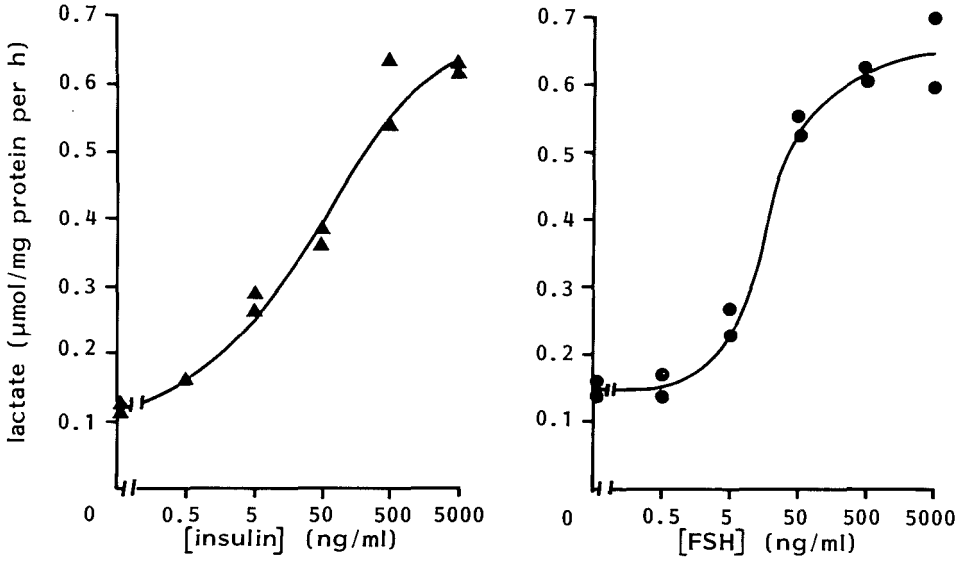


Fig. 3. Dose-response curves of the effects of follitropin and insulin on lactate production. Sertoli cells were incubated for 6 h in the presence of different concentrations of either insulin of follitropin. Net lactate production during this incubation period was expressed as µmol/mg protein per h. The results represent single values of duplicate incubations.

TABLE 1

EFFECTS OF FOLLITROPIN AND INSULIN ON LACTATE PRODUCTION (A) AND CONVERSION OF D-[U-^{14}C]GLUCOSE INTO LIPIDS (B) DURING PROLONGED INCUBATION

A	Net lactate production		
	0–18 h (a)	0–24 h (b)	b − a
Control	1.40 ± 0.16	1.74 ± 0.12	0.34 ± 0.20
Follitropin	3.93 ± 0.64	4.06 ± 0.54	0.13 ± 0.84
Insulin	3.40 ± 0.39	3.29 ± 0.26	−0.11 ± 0.47
B	^{14}C-incorporation into lipids		
	0–18 (a)	0–24 (b)	(mean of b) − (mean of a)
Control	16.8; 18.5	26.7; 29.9	10.7
Follitropin	33.8; 36.7	48.2; 57.1	17.4
Insulin	33.9; 42.0	68.7; 70.1	31.4

Net lactate production (A) and the amount of radioactivity from D-[U-^{14}C]glucose incorporated into lipids (B) were estimated during an incubation period of either 18 h or 24 h in the absence or presence of hormones (following 3 days of preincubation in the absence of hormones). Hormones added were either follitropin (0.5 μg/ml) or insulin (5 μg/ml). Net lactate production was expressed as μmol/mg protein, and the results represent means ± SD of quadruplicate incubations. ^{14}C-incorporation into lipids was expressed as nmol/mg protein, and the results represent duplicate incubations.

cells which had been incubated for 18 h in the presence of follitropin or insulin was very low during incubation from 18 to 24 h in fresh medium with or without the hormones. In fact, lactate production was inhibited as compared to the cells which had been incubated for 18 h without hormones. Hence, the results indicate that under the present incubation conditions Sertoli cells can become refractory (with respect to lactate production) to follitropin and insulin (Fig. 4). When the cells were incubated from 0 to 18 h in the presence of follitropin, and subsequently from 18 to 24 h in the presence of insulin, lactate production was stimulated by insulin (Fig. 4). Similarly, stimulation of lactate production by follitropin was observed, when the cells were incubated from 0 to 18 h in the presence of insulin, and subsequently from 18 to 24 h in the presence of follitropin (Fig. 4). These results indicate, that the cells had become refractory to the homologous hormone, rather than to the heterologous hormone.

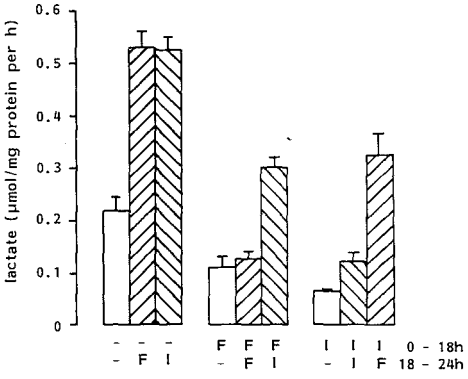


Fig. 4. Effect of prolonged incubation in the presence of follitropin and insulin on lactate production. Sertoli cells were preincubated from 0 to 18 h in the absence (−) or presence of hormones (follitropin, F, 0.5 μg/ml or insulin, I, 5 μg/ml). Subsequently, the medium was removed and fresh medium was added. From 18 to 24 h, the incubations were continued either in the absence (−) or presence of hormones (F, 0.5 μg/ml or I, 5 μg/ml). Net lactate production was estimated from 18 to 24 h of incubation and expressed as μmol/mg protein per h. The results represent means ± SD of quadruplicate incubations.

The amount of D-[U-^{14}C]glucose converted into lipids was also enhanced by follitropin and insulin. In contrast to the observations concerning lactate production this conversion was increased rather than decreased during prolonged incubation (24 h) in the presence of follitropin or insulin (Table 1B).

Net cyclic AMP production by Sertoli cells, estimated after incubation in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (MIX), was increased 25-fold within 10

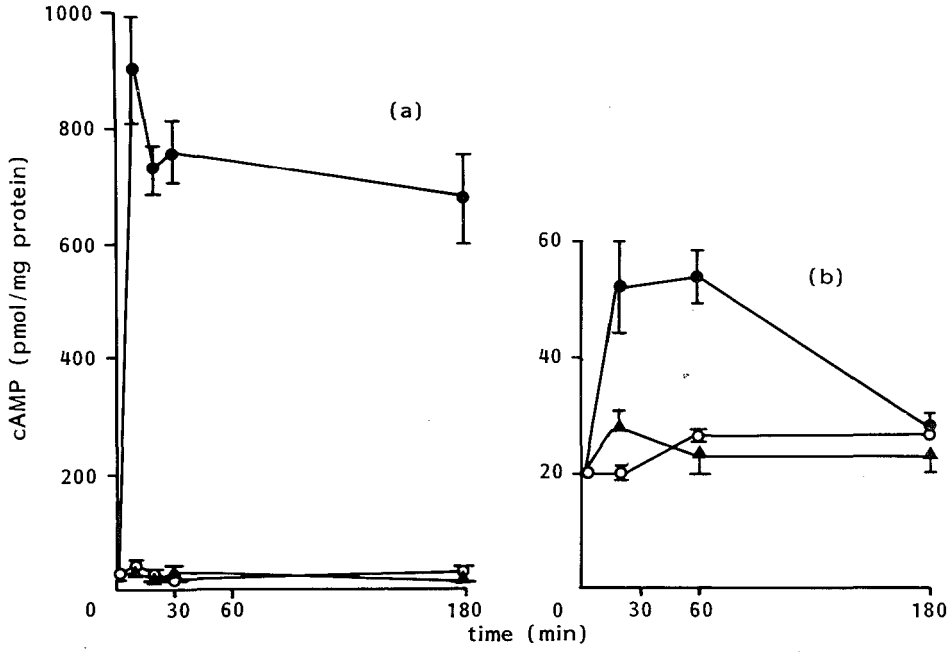


Fig. 5. Time course of the effect of follitropin and insulin on cyclic AMP production. Sertoli cells were incubated for different time periods either in the absence (○——○) or in the presence of hormones (0.5 μg/ml of follitropin (●——●) or 5 μg/ml of insulin (▲——▲)). The amount of cyclic AMP in medium plus cells was estimated after incubation either (*a*) in the presence of 0.25 mM isobutylmethylxanthine (MIX), or (*b*) in the absence of MIX. The results represent means ± SD of triplicate incubations.

min after addition of follitropin (Fig. 5a). In the absence of MIX, the response to follitropin was only 2-fold and transient (Fig. 5b). There was no effect of insulin on the cAMP production by the cells either in the absence or presence of MIX (Fig. 5). Under the present incubation conditions, the rate of lactate production by Sertoli cells was increased 5-fold by dibutyryl cAMP. During incubation for 6 h in the absence or presence of 0.5 mM dibutyryl cAMP, the rates of net lactate production were 0.18 ± 0.01 μmol/mg protein per h and 0.90 ± 0.02 μmol/mg protein per h respectively (means ± SD, $n = 3$).

To test whether or not the rapid stimulatory effects of follitropin and insulin on lactate production by Sertoli cells are dependent on de novo protein synthesis, the effect of cycloheximide was investigated. The concentration of cycloheximide used was 50 μg/ml, which inhibited the incorporation of ^{3}H-labelled lysine into acid-precipitable material by 94–98%. Cycloheximide was added 10 min before the hormones, and was present during the whole 6 h incubation period. The results presented in Fig. 6 show that there was no inhibitory effect of cycloheximide on the control and follitropin- or insulin-stimulated rates of lactate production. In fact, the stimulatory effect of insulin on lactate production was augmented by cycloheximide (Fig. 6).

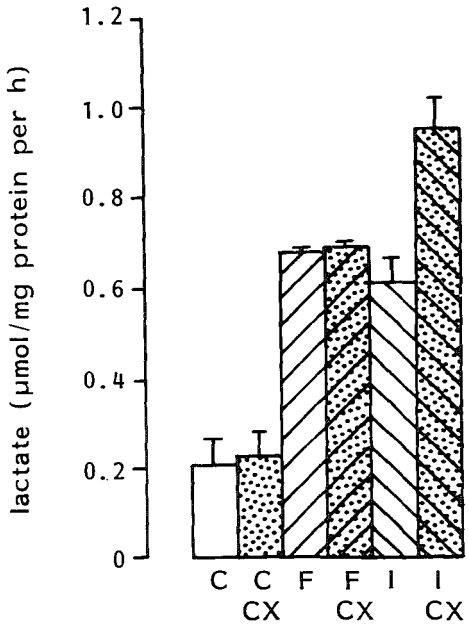


Fig. 6. Effects of follitropin and insulin on lactate production in the presence of cycloheximide. Sertoli cells were incubated either in the absence (c) or in the presence of hormones (0.5 µg/ml of follitropin, F or 5 µg/ml of insulin, I). Cycloheximide (CX, 50 µg/ml) was added 10 min before addition of the hormones. The incubations were terminated 6 h after addition of the hormones and net lactate production was estimated and expressed as µmol/mg protein per h. The results represent means plus range of duplicate incubations. Similar results were obtained in 4 experiments.

Discussion

The present results show rapid stimulatory effects (within 3 h) of follitropin or insulin on the rate of lactate production by rat Sertoli cells in vitro, after 3 days of preincubation of the Sertoli cells in the absence of added hormones. Inhibition of protein synthesis by cycloheximide did not exert an inhibitory effect on the basal and follitropin- or insulin-stimulated rates of lactate production. Hence, it can be concluded that the rapid stimulatory effects of follitropin and insulin on lactate production do not require de novo protein synthesis. Rather, these effects could reflect modulation of the activity of enzymes involved in carbohydrate metabolism, as well as regulation of glucose transport. Our results are in contradiction with the results of Mita et al. (1982), who have observed a stimulatory effect of follitropin on lactate production by Sertoli cells which seemed to be dependent on de novo RNA and protein synthesis. This discrepancy cannot be explained. In the present experiments, cycloheximide actually increased the stimulatory effect of insulin on lactate production. This could reflect, that cycloheximide causes an accumulation of insulin receptors at the cell surface, as has been described recently for cultured mouse fibroblasts (Kadle et al., 1983). Inhibition of the synthesis of proteins involved in insulin receptor turnover (Kadle et al., 1983), or lengthening of the receptor half-life by blocking a step in receptor inactivation (Knutson et al., 1983) by cycloheximide could be involved in such an accumulation of insulin receptors.

After 18 h of incubation in the presence of either follitropin or insulin, the Sertoli cells had become refractory to the homologous hormone. Sertoli cells may become refractory to follitropin, because exposure to follitropin results within several hours in inactivation of adenylate cyclase and an increased rate of cAMP catabolism mediated by follitropin stimulation of phosphodiesterase activity (Verhoeven et al., 1980, 1981; Conti et al., 1981, 1983; Attramadal et al., 1984). In agreement with these observations the present results show that, in the absence of MIX, the cAMP level reached a peak within 20 min after follitropin addition and returned to control level within 3 h. Furthermore, it was observed that the stimulatory effect of dbcAMP (0.5 mM) on lactate production was maintained during several days of incubation in the continuous presence of dbcAMP (not shown). Hence, the refractoriness to follitropin of lactate production by Sertoli cells under the present incubation conditions might involve refractoriness to follitropin of net cAMP production.

Apart from inactivation of adenylate cyclase or activation of phosphodiesterase, cells may become refractory because prolonged exposure to the hormones may result in receptor down-regulation. Such a mechanism has been described for the insulin receptor (Gavin et al., 1974; Knutson et al.,

1983), and may be involved in the observed refractoriness of lactate production by Sertoli cells to insulin and follitropin.

The production of androgen binding protein and transferrin by Sertoli cells in vitro can also be stimulated by follitropin and insulin. However, to elicit a clear stimulatory response of follitropin and/or insulin on the production of androgen binding protein or transferrin, these hormones should be added at the onset of the incubation and should be present continuously during several days (Fritz et al., 1976; Louis and Fritz, 1979; Karl and Griswold, 1980; Skinner and Griswold, 1982). These effects seem to reflect trophic hormone action, and are clearly different from the present observations which may reflect rapid metabolic effects. Apparently, trophic hormone action can be obtained, although changes in adenylate cyclase and phosphodiesterase activities, and receptor down-regulation seem to favour refractoriness.

Robinson and Fritz (1981) have reported the absence of stimulatory effects of follitropin and insulin on glucose metabolism (conversion to CO_2 and lactate) by Sertoli cells, but a high basal rate of glucose metabolism. Robinson and Fritz (1981) have preincubated the Sertoli cells for 48 h in the presence of hormones, and subsequently the effect of hormones was estimated by measuring the metabolism of radioactively labelled glucose during incubation for several hours in the absence of hormones. Our present results show that a prolonged preincubation in the presence of follitropin or insulin can densensitize the cells with respect to hormone effects on lactate production. Moreover, rapid stimulatory effects of follitropin and insulin on lactate production which are not dependent on de novo protein synthesis may not be maintained after removal of the hormone. However, different time periods of exposure to hormones cannot explain that the basal rate of glucose metabolism in the experiments reported by Robinson and Fritz (1981) was very high and in fact comparable to the maximal hormone-stimulated rate as observed in the present experiments. One could suggest, that the basal rate of glucose metabolism is influenced by some unknown factor(s) which may differ among different laboratories. This suggestion, however, needs to be verified in further experiments. The absence of hormone effects on glucose conversion to CO_2 and lactate, as reported by Robinson and Fritz (1981), was accompanied by a small but significant stimulation by insulin of the conversion of glucose into lipids. The latter is in agreement with our results (Table 1B), which indicate the absence of desensitizing effects (within 24 h) on the follitropin- or insulin-stimulated conversion of [U-^{14}C]glucose into lipids. Possibly, the effect of hormones on lipid production falls into the category of long-term, trophic effects.

Insulin effects on Sertoli cells may involve low affinity binding of insulin to receptors for insulin-like growth factors (IGF). Skinner and Griswold (1983) reported that transferrin secretion (by Sertoli cells from 3-week-old rats) was maximally stimulated by either 833 nM insulin or 10 nM multiplication stimulating activity (MSA). Similarly, Borland et al. (1984) described recently, that micromolar concentrations of insulin were required to obtain a small stimulatory effect on lactate production and DNA and protein synthesis by Sertoli cells from 2-week-old rats, whereas IGF-I or IGF-II were active in the nanomolar concentration range. In the present experiments on lactate production by Sertoli cells from 3-week-old rats, however, half maximal stimulation was observed at an insulin concentration of approximately 8 nM ($ED_{50} = 50$ ng/ml) and saturation was obtained at 80 nM (500 ng/ml). According to Rosenzweig et al. (1980) a concentration of 8 nM in tissues is within the physiological range. Hence, the present effects on lactate production seem to reflect binding of insulin to insulin receptors. Borland et al. (1984) observed that the Sertoli cells (from 2-week-old rats) which were used in their experiments, did not contain a significant number of high-affinity receptors for insulin, whereas receptors for IGF-I and IGF-II were abundant. The discrepancy between the results of Borland et al. (1984) and the present results could be explained if high-affinity receptors for insulin appear on Sertoli cells during prepubertal development from 2 to 3 weeks of age. It has been reported that high-affinity binding of insulin in rats testis increases during testis development (Saucier et al., 1981; Mueller et al., 1982). Preliminary observations indicate, however, that under the present incubation conditions lactate production by Sertoli cells from 2-week-old rats can also be stimulated

by low doses of insulin (5–50 ng/ml). Possibly, in the experiments by Borland et al. (1984) the response to insulin may have been lost because the Sertoli cells were preincubated for 2 days in the presence of 10 μg/ml insulin.

In conclusion, the rapid effects of insulin on glucose metabolism by Sertoli cells as observed in our experiments may well be mediated via insulin receptors. Furthermore, it appears that follitropin may exert rapid insulin-like effects. Sertoli cells in vivo could be target cells for both follitropin and insulin. This notion involves that impairment of spermatogenesis during experimental diabetes in rats (Oksanen, 1975; Paz et al., 1978) might be caused by the absence of insulin per se, rather than being a consequence of the systemic pathology associated with diabetes. In this regard it appears to be important to evaluate and to compare the acute or short-term effects of insulin and follitropin on metabolic activities of Sertoli cells in vivo, as well as the late or long-term effects of these hormones on the functional maturation of Sertoli cells during testis development.

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Metabolism of radiolabelled energy-yielding substrates by rat Sertoli cells

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Summary. The rates of metabolism *in vitro* of ^{3}H- or ^{14}C-labelled glucose, pyruvate, glutamine and leucine by Sertoli cells from immature rats were estimated. The overall rate of glucose utilization exceeded by far the rates of oxidation of pyruvate (derived from glucose) via the citric acid cycle and glucose metabolism via the oxidative branch of the pentose phosphate pathway. This pattern of glucose metabolism was not markedly altered after stimulation of glucose metabolism by FSH. The rate of oxidation of exogenous pyruvate indicated that the energy yield from glucose metabolism by Sertoli cells could be dependent on the extracellular concentrations of pyruvate and lactate. There is no evidence that a high rate of aerobic glycolysis is of vital importance for Sertoli cells. In medium containing glucose and all amino acids, ^{14}C-labelled glutamine and leucine were converted to $^{14}CO_2$ at considerable rates. It was calculated that the oxidation of glutamine and leucine in addition to glucose and fatty acids can yield much of the required energy of Sertoli cells.

Introduction

Sertoli cells from immature rats *in vitro* metabolize glucose via glycolysis at a high rate, which results in net production of lactate (Jutte, Grootegoed, Rommerts & van der Molen, 1981; Robinson & Fritz, 1981). The rate of lactate production is increased by FSH, an important regulator of spermatogenesis in immature rats (Jutte *et al.*, 1982; Mita, Price & Hall, 1982; Jutte, Jansen, Grootegoed, Rommerts & van der Molen, 1983). Exogenous lactate is essential to support ATP production by isolated rat spermatogenic cells; ATP is rapidly dephosphorylated when round spermatids are exposed to glucose in the absence of exogenous lactate (Grootegoed, Jansen & van der Molen, 1986). Glucose metabolism and lactate production by Sertoli cells probably play an important role to maintain the ATP content of the spermatogenic cells *in vivo*. In this respect, it is relevant to study the metabolism of energy-yielding substrates by Sertoli cells.

Using radiolabelled substrates, it should be possible to obtain information on the regulation of the different pathways which could be involved in the metabolism of these substrates. It has been reported, however, that FSH did not affect glucose metabolism by Sertoli cells when the metabolism of ^{3}H- or ^{14}C-labelled glucose was studied (Robinson & Fritz, 1981). This could indicate that FSH exerts an effect primarily on the mobilization of endogenous pools (glycogen). The activity of glycogen phosphorylase in Sertoli cells is stimulated by FSH (Slaughter & Means, 1983).

We have therefore investigated the metabolism *in vitro* of radiolabelled glucose, pyruvate, glutamine and leucine by Sertoli cells from rats.

Materials and Methods

Materials. The following radiolabelled compounds were purchased from Amersham International PLC, Amersham, Bucks, U.K.: D-[1-^{14}C]glucose (sp. act. 50–60 Ci/mol); D-[6-^{14}C]glucose (sp. act.

50–60 Ci/mol); D-[5-^{3}H]glucose (sp. act. 10–20 Ci/mol); L-[U-^{14}C]glutamine (sp. act. 40 Ci/mol); L-[U-^{14}C]leucine (sp. act. 330 Ci/mol); and L-[1-^{14}C]leucine (sp. act. 55 Ci/mol). Radiolabelled pyruvate ([1-^{14}C]pyruvate (sp. act. 5–20 Ci/mol) and [2-^{14}C]pyruvate (sp. act. 15–20 Ci/mol)) were purchased from New England Nuclear, Boston, MA, U.S.A.

Collagenase (code CLS) was obtained from Worthington Biochemical Corporation, Freehold, NJ, U.S.A. Follicle-stimulating hormone (NIH-FSH-S13) was a gift from the Endocrinology Study Section, National Institutes of Health, Bethesda, MD, U.S.A. Dibutyryl cAMP (dbcAMP) (N^6,2′-0-dibutyryladenosine 3′:5′-cyclic monophosphate) was from Boehringer Mannheim B.V., Mannheim, F.R.G. Phenazine methosulphate (*N*-methyldibenzopyrazine methyl sulphate salt) was purchased from Sigma Chemical Co., St Louis, MO, U.S.A.

Eagle's minimum essential medium (MEM; with Earle's salts, without glutamine) was obtained from Gibco Europe B.V., Hoofddorp, The Netherlands. The medium was supplemented with penicillin (10^5 units/l), streptomycin (100 mg/l) and fungizone (1 mg/l). L-Glutamine (final concentration 2 mM) was dissolved and added to the medium shortly before use.

Isolation and incubation of Sertoli cells. Sertoli cells were isolated from 3- or 4-week-old Wistar rats. The rats had been irradiated (1·5 Gy) *in utero* at Day 19 of gestation (Beaumont, 1960), and the testicular tubules of these animals contained Sertoli cells but no developing germ cells. The rats were killed by cervical dislocation and the testes were collected and decapsulated at room temperature. The decapsulated testes were treated with collagenase (10 mg per 6–10 testes) in 20 ml phosphate-buffered saline (PBS) (Dulbecco & Vogt, 1954), supplemented with 5·6 mM-glucose. This incubation was performed in a siliconized Erlenmeyer flask (100 ml) placed in a Dubnoff-type shaking water bath (120 cycles-min), for 30 min at 32°C. During the collagenase treatment large tubule fragments were released from the tissue. These tubules were washed twice in PBS by sedimentation for 2 min at unit gravity, and subsequently incubated for another 30 min under the same conditions in the presence of collagenase. At the end of this second collagenase treatment, the cells from the tubular wall had become dissociated almost completely from the Sertoli cells. The Sertoli cell clusters were separated from most of the tubular wall cells by repeated sedimentation at unit gravity for 2 min in PBS (five times) and were washed once in MEM. The removal of the tubular wall was observed using Nomarski differential-interference contrast optics. In addition, the Sertoli cell preparations were fixed in ethanol–glacial acetic acid (3:1, v/v) and applied to microscope slides. After evaporation of the fixative, a drop of acetic acid (40%) was added, and the nuclei were observed by phase-contrast optics. In these preparations, the different morphology of the nuclei of tubular wall cells and Sertoli cells can be recognized (Oonk, Grootegoed & van der Molen, 1985). The purity of the Sertoli cell preparations was greater than 94%. The Sertoli cells were incubated in plastic flasks (25 cm^2 growth area; 0·6–1·2 mg protein per flask) in 4 ml MEM, under an atmosphere of 5% CO_2 in air at 32°C. FSH (0·5 μg/ml) or dbcAMP (0·5 mM) were added at the start of the incubations.

Estimation of the metabolism of radiolabelled substrates. During incubation for 18 h, the Sertoli cell clusters had become firmly attached to the growth area of the flasks. The attached cells were washed with PBS and the incubations were continued for 2–4 h in 2 ml PBS, in the presence of glucose (1 μCi ^{3}H- or ^{14}C-labelled glucose; 5·6 mM) or pyruvate (0·1 μCi ^{14}C-labelled pyruvate; 0·01–1·0 mM). FSH or dbcAMP were added also during this incubation. The incubations in the presence of the labelled compounds were carried out in closed flasks, under air at 32°C.

The metabolism of ^{14}C-labelled L-glutamine and L-leucine was estimated during incubation of Sertoli cells (from 4-week-old sterile rats) in 2 ml MEM, which contained 5·6 mM-glucose, 2 mM-L-glutamine and 0·4 mM-L-leucine, in the presence of L-[U-^{14}C]glutamine, L-[U-^{14}C]leucine, or L-[1-^{14}C]leucine (0·1 μCi), in closed flasks under an atmosphere of 5% CO_2 in air.

At the end of the incubations with ^{14}C-labelled substrates, the reactions were terminated and CO_2 was chased from the medium by injection of 0·2 ml of 1 M-HCl into the incubation medium and injection of 50 μl of 6 M-NaOH into a small vessel, which contained a piece of filter paper and

was placed in the neck of the flasks. The $^{14}CO_2$ was trapped by the NaOH during further incubation for 60 min at 32°C. Subsequently, the filter paper was transferred to a liquid scintillation vial containing 0·2 ml water. After addition of 0·5 ml Carbo-Sorb II and 10 ml Pico-fluor 15 (Packard Instrument Company Inc., Downers, Grove, IL, U.S.A.) the amount of radioactivity (d.p.m.) was estimated using an Isocap-300 liquid scintillation counter (Searle Analytic, Des Plains, IL, U.S.A.).

The amount of ^3H_2O formed from [5-^{3}H]glucose was estimated by mixing 1 ml incubation medium with 0·1 ml 0·5 M-HCl and 0·1 ml of 0·05% merthiolate solution. A portion of this mixture (0·2 ml) was equilibrated with 1 ml of water for 48 h at 55°C. The amount of radioactivity in the 1 ml water was counted after addition of 10 ml Picofluor.

All incubations with radioactively labelled substrates were also carried out in the absence of cells, and the data were corrected for these blanks. The production of $^{14}CO_2$ or ^3H_2O was expressed as nmol substrate converted, calculated as follows: (d.p.m. in $^{14}CO_2$ or ^3H_2O/d.p.m. per ml medium) × (nmol substrate/ml medium). The outcome of this calculation was expressed as nmol substrate converted to ^3H_2O or $^{14}CO_2$/h per mg protein. The amount of protein per flask was measured at the end of the incubations in each individual flask by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard.

The ^{3}H from [5-^{3}H]glucose is lost to water in glycolysis, and the rate of formation of ^3H_2O represents the combined rates of flow of glucose through glycolysis and the pentose phosphate pathway. The ^{14}C from [1-^{14}C]glucose and [6-^{14}C]glucose is incorporated into $^{14}CO_2$ via the citric acid cycle and the oxidative branch of the pentose phosphate pathway. The amount of glucose that was metabolized via the pentose phosphate pathway was calculated as described by Ashcroft, Weerasinghe, Bassett & Randle (1972) by the equation of Katz & Wood (1963).

Results

Metabolism of glucose

The overall rate of glucose metabolism (formation of ^3H_2O) and the rate of glucose oxidation (formation of $^{14}CO_2$) by the isolated Sertoli cells were increased by FSH or dbcAMP (Table 1). Under all incubation conditions, the overall rate of glucose utilization was much higher than the rate of glucose oxidation (Table 1).

Table 1. Effects of FSH and dbcAMP on the metabolism of ^{3}H- or ^{14}C-labelled glucose by Sertoli cells from 3- or 4-week-old rats

	Substrate converted to ^3H_2O or $^{14}CO_2$ (nmol/h per mg protein)			Glucose metabolized *via* the pentose phosphate pathway (nmol/h per mg protein)
	D-[5-^{3}H]Glucose	D-[1-^{14}C]Glucose	D-[6-^{14}C]Glucose	
Sertoli cells from 3-week-old rats				
Control	52; 53	3·4 ± 0·2	1·5 ± 0·2	0·6
FSH	201; 204	8·8 ± 0·6	4·4 ± 0·7	1·5
dbcAMP	310; 347	12·6 ± 1·3	7·5 ± 0·8	1·8
Sertoli cells from 4-week-old rats				
Control	157; 167	2·8 ± 0·6	1·6 ± 0·7	0·4
FSH	260; 267	14·9 ± 1·1	9·6 ± 0·9	1·9
PMS	132; 144	112·5 ± 13·1	6·2 ± 0·6	80·2

The Sertoli cells were incubated for 18 h in MEM, in the absence or presence of FSH or dbcAMP. Subsequently, the cells were incubated for 4 h in PBS containing ^{3}H- or ^{14}C-labelled glucose (5·6 mM-glucose), in the continued absence or presence of FSH and dbcAMP. Phenazine methosulphate (PMS; 0·1 mM) was added at the start of the incubations with labelled glucose. The results represent duplicate or triplicate incubations (single values or mean ± s.d.).

The unstimulated overall rate of glucose metabolism (formation of ^3H_2O) by Sertoli cells from 3-week-old rats was lower than the rate of metabolism by cells from 4-week-old rats (Table 1). The results presented in Table 1 are from single experiments, because the rats used for the different experiments were not of exactly the same age (range 21–23 or 27–29 days of age). Using four different cell preparations obtained from 3-week-old rats, the rates of ^3H_2O production from [5-^{3}H]glucose in the absence or presence of FSH were, respectively, 55·4 ± 7·6 and 194·0 ± 53·1 nmol/h per mg protein (mean ± s.d.) ($P < 0{\cdot}005$, t test). The ratio of stimulated/control was 3·5 ± 0·6.

The rate of oxidation of [1-^{14}C]glucose by the isolated Sertoli cells was higher than the oxidation rate of [6-^{14}C]glucose (Table 1). This indicates that glucose was metabolized via the oxidative branch of the pentose phosphate pathway. The rate of this metabolism was increased after addition of FSH or dbcAMP (Table 1). Under the different incubation conditions, however, the pentose phosphate pathway did not account for more than 1·2% of the overall rate of glucose metabolism. When the artificial electron acceptor phenazine methosulphate (PMS) was added to the incubation medium, there was a very high rate of oxidation of carbon-1 of glucose (Table 1).

The present experiments were performed using a glucose concentration of 5·6 mM, which represents saturation conditions (Robinson & Fritz, 1981). The rate of formation of ^3H_2O from [5-^{3}H]glucose was constant during incubation for 4 h (Fig. 1). The amount of $^{14}CO_2$ produced from [6-^{14}C]glucose during 2–4 h of incubation, however, was ~4–8 times higher than during 0–2 h (Fig. 1). This increase was only ~1–2-fold when the PBS (containing [6-^{14}C]glucose) was replaced at $t = 2$ h by fresh PBS (containing [6-^{14}C]glucose) (not shown).

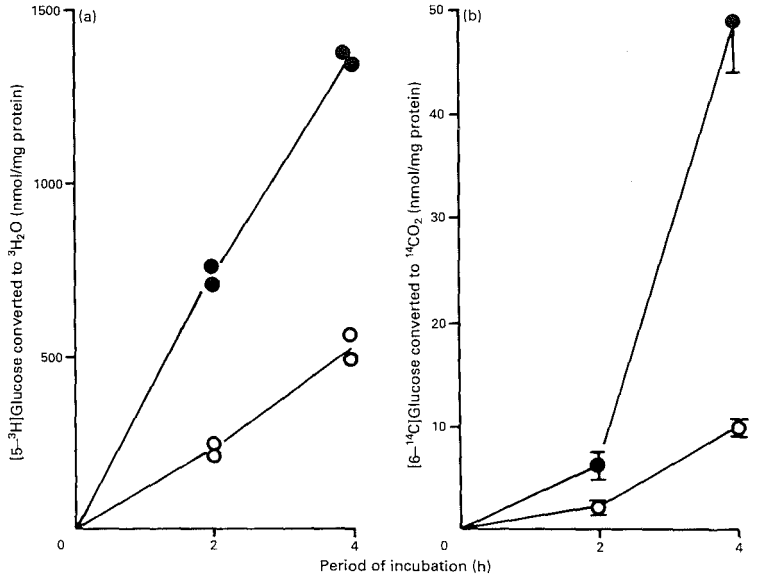

Fig. 1. Metabolism of ^{3}H- or ^{14}C-labelled glucose by Sertoli cells from 4-week-old rats, incubated for 18 h in MEM, in the absence (○) or presence (●) of 0·5 mM-dbcAMP. Subsequently, the cells were incubated for 2 or 4 h in PBS, containing D-[5-^{3}H]glucose or D-[6-^{14}C]glucose (5·6 mM-glucose), in the continued absence or presence of dbcAMP. The results represent duplicate or triplicate incubations (single values or mean ± s.d.).

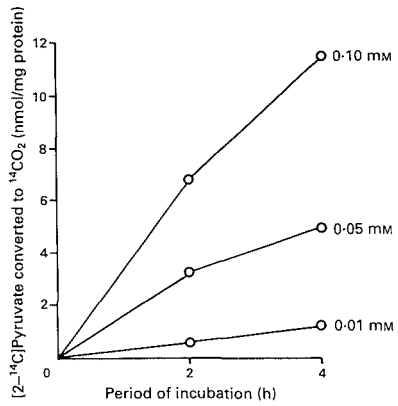

Fig. 2. Oxidation of low concentrations of exogenous ^{14}C-labelled pyruvate by Sertoli cells from 4-week-old rats. The cells were incubated for 18 h in MEM, and then for 2 or 4 h in PBS, containing 5·6 mM-glucose and 0·01, 0·05 or 0·10 mM-[2-^{14}C]pyruvate. The results represent duplicate incubations (mean).

Incorporation of ^{14}C from exogenous [2-^{14}C]pyruvate into $^{14}CO_2$ was observed at very low concentrations of exogenous pyruvate, during incubation of Sertoli cells in the presence of 5·6 mM-glucose (Fig. 2).

Metabolism of exogenous pyruvate

The addition of FSH to the incubation medium resulted in a relatively small increase (less than 2-fold) of the rates of $^{14}CO_2$ formation from [1-^{14}C]pyruvate or [2-^{14}C]pyruvate (Table 2). Carbon-1 of pyruvate is directly incorporated into $^{14}CO_2$ in the pyruvate dehydrogenase reaction, whereas carbon-2 of pyruvate is at first incorporated into pools of citric acid cycle intermediates. Consequently, the rate of $^{14}CO_2$ formation from [1-^{14}C]pyruvate was higher than that from [2-^{14}C]pyruvate (Table 2).

The effect of FSH on pyruvate oxidation (Table 2) was estimated using medium without glucose, and a stimulatory effect of FSH on the production of endogenous pyruvate (from glucose) could not have interfered with the rate of oxidation of exogenous pyruvate. In the same cell

Table 2. Effect of FSH on the oxidation of exogenous ^{14}C-labelled pyruvate by Sertoli cells from 3-week-old rats

	Substrate converted to $^{14}CO_2$ or ^3H_2O (nmol/h per mg protein)		
	[1-^{14}C]Pyruvate	[2-^{14}C]Pyruvate	D-[5-^{3}H]Glucose
Control	32·8 ± 4·4	11·3 ± 0·5	63; 65
FSH	55·3 ± 1·8	17·3 ± 0·8	252; 252

The Sertoli cells were incubated for 18 h in MEM, in the absence or presence of FSH, then for 4 h in PBS containing ^{14}C-labelled pyruvate (1 mM-sodium pyruvate) or ^{3}H-labelled glucose (5·6 mM-glucose), in the continued absence or presence of FSH. The results represent duplicate or triplicate incubations (single values or mean ± s.d.).

preparation, the overall rate of glucose utilization was stimulated 4-fold by FSH (Table 2). Under these incubation conditions, the rate of [1-^{14}C]pyruvate oxidation was 33 nmol/h per mg protein (Table 2). However, the overall rate of pyruvate consumption was 118 ± 5 nmol/h per mg protein (mean ± s.d., quadruplicate incubations; estimated from the amount of pyruvate in the spent incubation medium, as described by Jutte *et al.*, 1983). In addition, exogenous pyruvate was converted to lactate (85 ± 4 nmol/h per mg protein). Hence, the rate of pyruvate consumption minus the rate of lactate production was 118 − 85 = 33 nmol/h per mg protein, and equalled the rate of oxidation of ^{14}C-labelled pyruvate.

Metabolism of glutamine and leucine

The present measurements of the rates of oxidation of glucose and pyruvate were carried out during incubation of Sertoli cells for 4 h in a simple defined medium (phosphate-buffered saline). The saline was supplemented with glucose and/or pyruvate, but did not contain amino acids or other substrates which might be used by Sertoli cells as energy-yielding substrates.

Glutamine oxidation to $^{14}CO_2$ represents conversion of glutamine to glutamate and then to α-ketoglutarate, which is followed by oxidation of α-ketoglutarate via the citric acid cycle.

L-[U-^{14}C]Glutamine was converted to $^{14}CO_2$ at a considerable rate during incubation of Sertoli cells in Eagle's minimum essential medium (6·5 ± 0·2 nmol/h per mg protein) and this rate was stimulated by dbcAMP (11·2 ± 0·5 nmol/h per mg protein). The rate of glutamine oxidation to $^{14}CO_2$ was constant for 3 days after isolation of the Sertoli cells (6·8 ± 0·2 and 11·5 ± 0·5 nmol/h per mg protein, in the absence and presence of dbcAMP respectively; mean ± s.d. for triplicate incubations).

Leucine metabolism involves the formation of 4-methyl-2-oxopentanoate through transamination. The ^{14}C from L-[1-^{14}C]leucine is incorporated into $^{14}CO_2$ when 4-methyl-2-oxopentanoate is converted through oxidative decarboxylation to isovaleryl-CoA, which then can be converted to acetoacetate and acetyl-CoA followed by complete oxidation via the citric acid cycle.

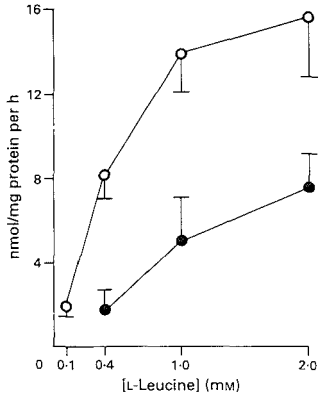

Fig. 3. Conversion of leucine to 4-methyl-2-oxopentanoate and CO_2 by Sertoli cells from 4-week-old rats, incubated for 48 h in MEM. Subsequently, the cells were incubated for 24 h in MEM, containing different concentrations of L-[1-^{14}C]leucine. The amount of 4-methyl-2-oxopentanoate in the spent incubation medium (●) was estimated as described by Taylor & Jenkins (1966). CO_2 production (○) represents the conversion of 4-methyl-2-oxopentanoate to isovaleryl-CoA. The results represent triplicate incubations (mean ± s.d.).

In the present experiments, $^{14}CO_2$ was produced from L-[U-^{14}C]leucine and L-[1-^{14}C]leucine at a ratio of 0·24–0·28 (rates of 3·6 ± 0·8 and 13·0 ± 0·9 nmol/h per mg protein respectively). Similar rates were observed in the presence of dbcAMP (2·8 ± 0·2 and 11·6 ± 1·4 nmol/h per mg protein; mean ± s.d. of triplicate incubations). At different leucine concentrations, 4-methyl-2-oxopentanoate derived from leucine was either converted to isovaleryl-CoA (70–85%) or released from the cells (15–30%) (Fig. 3).

Round spermatids from rats were isolated as described elsewhere (Grootegoed *et al.*, 1984) and incubated for 2 h in Eagle's minimum essential medium (2 mM-L-glutamine, 0·4 mM-L-leucine). The rates of conversion of L-[U-^{14}C]glutamine and L-[U-^{14}C]leucine to $^{14}CO_2$ were 0·32 ± 0·02 nmol and 12 ± 1 pmol/h per 4 × 10^6 cells respectively (means ± s.d. for triplicate incubations). This equals ~0·5 nmol glutamine and 0·02 nmol leucine/h per mg protein. For comparison, the rate of L-[U-^{14}C]lactate (3 mM-L-lactate) oxidation under these conditions was 12–16 nmol/h per 4 × 10^6 cells (Grootegoed *et al.*, 1984).

Discussion

In the present experiments, the overall rate of glucose metabolism by rat Sertoli cells was increased during incubation in the presence of FSH or dbcAMP. The dbcAMP-stimulated rate of ^3H_2O formation from ^{3}H-labelled glucose (300–350 nmol/h per mg protein for Sertoli cells from 3-week-old sterile rats) was almost as high as the rate observed by Robinson & Fritz (1981) for Sertoli cells from 3-week-old intact rats during incubation in the absence of dbcAMP (~450 nmol/h per mg protein). In the experiments reported by Robinson & Fritz (1981) the Sertoli cells metabolized glucose at a high and possibly maximum rate also in the absence of dbcAMP or FSH, but it is not clear which factor(s) might have initiated or maintained such a high rate. We have shown elsewhere (Oonk *et al.*, 1985) that FSH exerts rapid insulin-like effects on glucose metabolism by Sertoli cells independent of de-novo protein synthesis. The rapid effects of FSH are possibly mimicked by extracellular factors which are not ready catalogued and explicitly controlled.

For the present experiments we have used Sertoli cells from sterile animals to eliminate completely spermatogenic cells. In other experiments we have isolated Sertoli cells from intact rats and found rates of basal and FSH-stimulated lactate production which were in agreement with the present data (results not shown). Furthermore, the basal rate of overall glucose metabolism (^3H_2O production from ^{3}H-labelled glucose) by the isolated Sertoli cells was relatively high using 4-week-old rats as compared to 3-week-old rats. This is in agreement with our previous results (Jutte *et al.*, 1983) on lactate production by Sertoli cells from rats of different ages.

The energy-yield from glucose under the present incubation conditions is limited because only a small amount of the endogenous pyruvate produced from glucose is oxidized via the citric acid cycle (Robinson & Fritz, 1981; present results). Rather, pyruvate is converted to lactate and both pyruvate and lactate are released from the cells (Grootegoed, Oonk, Jansen & van der Molen, 1985b; Jutte *et al.*, 1983). In the present experiments, it was observed that Sertoli cells oxidized exogenous pyruvate at very low concentrations during incubation in the presence of glucose. This could explain that the rate of formation of $^{14}CO_2$ from ^{14}C-labelled glucose was markedly increased during 2–4 h of incubation, when the incubation medium was not renewed at $t = 2$ h, because the concentration of ^{14}C-labelled pyruvate in the medium will be higher during 2–4 h of incubation than during 0–2 h of incubation. In other words, the energy yield from glucose metabolism by Sertoli cells *in situ* is probably dependent on the steady-state extracellular pyruvate and lactate concentrations.

Exogenous pyruvate was converted to lactate, presumably until the pyruvate/lactate ratio had reached equilibrium. This conversion is an NADH-dependent reduction, and the extra reducing equivalents which are expended are most probably generated through mitochondrial metabolism. The rates of oxidation of [1-^{14}C]pyruvate and [2-^{14}C]pyruvate were stimulated by FSH. It is not

clear, however, whether this stimulation reflects an increased ATP utilization or could involve a more direct effect on the activity of the pyruvate dehydrogenase complex.

The rate of the oxidative branch of the pentose phosphate pathway is determined by the rate of NADPH oxidation. Under incubation conditions, the pentose phosphate pathway in Sertoli cells is not operating at its maximum rate. This is illustrated by the pronounced effect of phenazine methosulphate (Robinson & Fritz, 1981; present results). Hormonal signals may accelerate NADPH oxidation, as illustrated by the effects of FSH and dbcAMP on the metabolism of glucose via the pentose phosphate pathway. This effect may be related to the stimulatory effect of FSH on fatty acid biosynthesis (Oonk *et al.*, 1985). The reducing power required in this process is furnished by NADPH.

The present results indicate that effects of FSH on lactate production by Sertoli cells do not reflect mobilization of endogenous storage pools (glycogen). Rather, FSH stimulates the metabolism of exogenous (radiolabelled) glucose. This is indicated also by observations from Hall & Mita (1984) that the effect of FSH involves an increased rate of glucose transport across the plasma membrane of Sertoli cells. This is not to say, however, that exogenous glucose is the obligatory energy-yielding substrate of Sertoli cells. The observations of Jutte, Eikvar, Levy & Hansson (1985) have indicated that Sertoli cells (from 19-day-old rats) produce $^{14}CO_2$ from [1-^{14}C]palmitate at a rate of about 10 nmol/h per mg protein (calculated from the results of Jutte *et al.*, 1985) which could result in a maximum rate of ATP production in the order of 15 nmol/min per mg protein. This represents the rate of palmitate oxidation during incubation in the absence of hormones; it has not been reported that hormones or dbcAMP can stimulate palmitate oxidation by Sertoli cells. It can be calculated that an overall rate of glucose metabolism of 50–350 nmol/h per mg protein, and complete oxidation via the citric acid cycle of 1·5–7·5 nmol/h per mg protein (data from Table 1 for Sertoli cells from 3-week-old rats; basal and dbcAMP-stimulated rates) may result in about 2·5–15 nmol ATP/min per mg protein. The present results therefore support the conclusion from Jutte *et al.* (1985) that Sertoli cells from immature rats *in vitro* could extract much energy from lipids as compared to glucose.

It is well known that glutamine is a major energy-yielding substrate for proliferating normal and transformed cells (Zielke, Ozand, Tildon, Sevdalian & Cornblath, 1978; Reitzer, Wice & Kennell, 1979). Furthermore, for a number of rat tissues, including the testis, there appears to be a correlation between the activity of mitochondrial NAD(P)$^+$-dependent malic enzyme (which may function to convert glutamine-derived malate to pyruvate) and a high rate of cell renewal (Nagel, Dauchy & Sauer, 1980). The present results indicate that in the spermatogenic epithelium glutamine oxidation may be carried out by the non-proliferating Sertoli cells rather than by spermatogenic cells (round spermatids), although we have not measured glutamine oxidation by actively dividing spermatogonia. Complete oxidation of 6–12 nmol glutamine/h per mg protein by Sertoli cells (see 'Results') could result in the production of about 2–5 nmol ATP/min per mg protein. Therefore, in the presence of glucose and all amino acids a significant amount of energy is derived from glutamine. The present small stimulatory effect of dbcAMP on glutamine oxidation by Sertoli cells may reflect that hormonal signals directly or indirectly stimulate mitochondrial metabolism, as discussed above with respect to the effect of FSH on pyruvate oxidation.

In rat spermatogenic epithelium, the enzyme branched-chain amino acid aminotransferase, that catalyses the conversion of the branched-chain amino acids (valine, leucine, isoleucine) to the corresponding branched-chain α-oxo acids, is confined to Sertoli cells (Grootegoed, Jutte, Jansen & van der Molen, 1983). Sertoli cells, but not spermatocytes and spermatids, can produce 4-methyl-2-oxopentanoate from leucine and this compound is either released from the cells or converted to isovaleryl-CoA. We have described elsewhere (Grootegoed *et al.*, 1985a) that the released 4-methyl-2-oxopentanoate is utilized by spermatocytes and spermatids in a non-energy-yielding pathway. Further oxidation of isovaleryl-CoA in different tissues is not always complete (Buse, Jursinic & Reid, 1975). A ratio of $^{14}CO_2$ produced from L-[U-^{14}C]leucine to that from L-[1-^{14}C]leucine of 0·6 has been considered compatible with complete oxidation of the leucine molecule

in muscle tissue (Odessey & Goldberg, 1972). In the present experiments, a ratio of 0·24–0·28 was observed, and it is not certain what portion of the metabolic products of leucine is oxidized by Sertoli cells. Complete oxidation of 10–14 nmol isovaleryl-CoA/h per mg protein (see 'Results') could result in a maximum ATP production in the order of 5 nmol/min per mg protein. Moreover, Sertoli cells may extract energy from all three branched-chain amino acids (Grootegoed *et al.*, 1983). The formation of isovaleryl-CoA is rate-limiting for leucine oxidation (Buse *et al.*, 1975) and may not be subject to direct hormonal regulation in Sertoli cells, in view of the absence of an effect of dbcAMP under the present incubation conditions. Moreover, the pyruvate dehydrogenase complex which could be a target for hormonal signals does not participate in the further oxidation of isovaleryl-CoA.

In conclusion, the present results show that energy metabolism by Sertoli cells *in vitro* can involve a number of different substrates and pathways. For Sertoli cells *in vivo*, the relative importance of the different pathways is likely to be determined by unknown factors including substrate concentrations. For example, the present results indicate that the energy yield of glucose metabolism could be dependent on extracellular pyruvate and lactate concentrations. Furthermore, it is known that the utilization of glucose by cultured cells (proliferating) can be regulated by glutamine, and *vice versa* (Zielke *et al.*, 1978).

During incubation of Sertoli cells in Eagle's minimum essential medium, FSH and insulin exert rapid and pronounced stimulatory effects on aerobic lactate production (Oonk *et al.*, 1985). These effects could involve an increased rate of carrier-mediated glucose transport (Hall & Mita, 1984), but may not be driven by the cellular needs for ATP. A strong connection between ATP turnover and glycolysis in tumour cells has been described (Racker, Johnson & Blackwell, 1983). The contribution of glucose metabolism to maintain a high energy charge in Sertoli cells, however, may be relatively small compared to the energy yield from fatty acids (Jutte *et al.*, 1985) and amino acids (present results). It is therefore possible that in Sertoli cells the rate of glycolysis is not strictly controlled by the rate of ATP turnover. In view of these arguments, it is suggested that a high rate of aerobic glycolysis is probably not of vital importance for Sertoli cells.

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DIFFERENTIAL EFFECTS OF FOLLICLE STIMULATING HORMONE, INSULIN AND INSULIN-LIKE GROWTH FACTOR-I ON LACTATE PRODUCTION AND HEXOSE UPTAKE BY RAT SERTOLI CELLS

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Abstract

The present paper describes results on the stimulatory effects of follicle-stimulating hormone (FSH), insulin, and insulin-like growth factor–I (IGF–I) on lactate production and hexose uptake by Sertoli cells from immature rats. The time-course and the maximal stimulatory effects of FSH, insulin and IGF–I on lactate production were virtually identical. The action of FSH on lactate production was different from those of insulin and IGF–I, as indicated by observations that, when Sertoli cells were incubated in the presence of a combination of submaximal doses of FSH and insulin, or submaximal doses of FSH and IGF–I, additive effects were observed. However, the stimulatory effects of FSH and insulin were not dependent on extracellular calcium.

2-Deoxy-D-glucose (2-DOG), an analogue of D-glucose, was used to investigate the hexose transport system of Sertoli cells. Uptake of 2-DOG was linear in time and virtually all of the intracellular 2-DOG appeared to be in the phosphorylated form up to 30 min of incubation. 2-DOG uptake was inhibited by cytochalasin B. D-glucose, but not D-galactose, appeared to be an effective competitor of 2-DOG uptake. The K_m of 2-DOG uptake was not influenced by FSH, insulin and IGF–I. FSH had no effect on the V_{max} of 2-DOG uptake, whereas insulin and IGF–I caused a 30% stimulation of the V_{max}. From this, it would appear that FSH, insulin and IGF–I stimulate lactate production, but that only insulin and IGF-I stimulate hexose transport. For this reason, it was concluded that the rate of lactate production by Sertoli cells is not determined by glucose influx only, but involves also activation of glycolytic enzymes. FSH might principally stimulate glycolytic enzyme activities, whereas the present results indicate that insulin and IGF–I stimulate hexose uptake and glycolytic enzyme activities in Sertoli cells.

Running head: Glucose uptake by Sertoli cells

Introduction

Cultured Sertoli cells from immature rats convert glucose to lactate under defined incubation conditions (Robinson and Fritz, 1981; Jutte et al., 1982). Lactate production by Sertoli cells can be increased by follicle stimulating hormone (FSH) (Mita et al., 1982; Jutte et al., 1983), insulin (Borland et al., 1984; Oonk et al., 1985), insulin-like growth factor–I (IGF–I) (Borland et al., 1984; Oonk and Grootegoed, 1987), glucagon (Eikvar et al., 1984) and epidermal growth factor (EGF) (Mallea et al., 1986). The effect of FSH differed from the insulin effect with respect to the stimulation of cAMP levels (Oonk et al., 1985). However, the mechanism of action of FSH on lactate production is similar to that of insulin with respect to an independence of *de novo* protein synthesis. From the literature, it is known that the mechanism of insulin action on cells does not involve extracellular Ca^{2+} (Klip et al., 1984; Cheung et al., 1987). In this regard we have investigated if the effect of FSH and insulin on lactate production was dependent on extracellular Ca^{2+}

concentrations.

It has been reported previously, that the mechanism of the FSH effect on glucose transport by Sertoli cells was similar to those of insulin and IGF–I. The hormones increased the affinity of 3-O-methylglucose (3-OMG) transport (Hall and Mita, 1984; Mita et al., 1985). However, there are several disadvantages of the use of 3-OMG, particularly in systems with cells that are not in suspension (Olefsky, 1978; Klip et al., 1982). The rapid deviation of linearity of 3-OMG uptake makes it difficult to determine initial rates of 3-OMG uptake (Olefsky, 1978). The primary effect of insulin on 3-OMG uptake reflects an increase in maximal velocity by insulin (Toyoda et al., 1987), compatible with the translocation hypothesis postulated by Cushman and Wardzala (1980) and by Suzuki and Kono (1980). For these reasons we have reinvestigated the hexose uptake system of Sertoli cells, using 2-DOG as glucose analogue. With this analogue conditions can be relatively easily established in which transport of 2-DOG is rate limiting. However, a problem one has to face is that high concentrations of 2-DOG (1-10mM) caused a rapid decline in ATP levels in adipocytes (Wieringa et al., 1985). The influences of FSH, insulin and IGF–I on hexose transport were investigated and compared with the effects of FSH, insulin and IGF–I on lactate production by Sertoli cells.

Materials and Methods

Materials

Follicle stimulating hormone (NIH-ovine-FSH-S13) was a gift from the Endocrinology Study Section of the National Institutes of Health, Bethesda, MD, U.S.A.. Porcine insulin was purchased form Novo Industri, Copenhagen, Denmark. The recombinant analogue (Thr^{59}) of human insulin-like growth factor–I, 2-deoxy-D-[2,6-^{3}H]glucose (46.8 Ci/mmol) and [U-^{14}C]sucrose (560 mCi/mmol) were purchased from Amersham International, Amersham, U.K.. Collagenase (CLS-1) was from Worthington, Freehold, NJ, U.S.A. Lactate dehydrogenase from pig heart, NAD^+-free acid grade I and ATP disodium salt were obtained from Boehringer Mannheim, Mannheim, F.R.G.. The luciferin/luciferase mixture (Lumit PM) was from Lumac, Meise, Belgium. The L-(+)-lactic acid standard solution (0.40 mg/ml), 2-deoxy-D-glucose and bovine serum albumin fraction V were from Sigma, St. Louis, MO, U.S.A.. Cytochalasin B and cytochalasin E were purchased from Aldrich, Brussels, Belgium.

All other chemicals were obtained from commercial sources and were of the highest purity available.

Methods

Isolation and incubation of Sertoli cells

Sertoli cells were isolated from testes from 3-week-old rats (Wistar, substrain R-1 Amsterdam). The Sertoli cells were isolated using a collagenase digestion method, and incubated in Eagle's minimum essential medium (MEM; Gibco, Grand Islands, NY, U.S.A.), supplemented with L-glutamine and antibiotics, at 32°C under an atmosphere of 5% CO_2 in air, as described previously (Oonk et al., 1985). After two days of incubation in medium containing 1% (v/v) fetal calf serum, the cells were treated with a hypo-osmotic shock to remove the spermatogenic cells (Oonk and Grootegoed, 1987a). Following this treatment, the Sertoli cells were incubated for 24 h in medium without serum. Subsequently, lactate production and hexose uptake were estimated.

Measurement of lactate production

After incubation of Sertoli cells at 32°C as described in the Results section, the incubation medium was removed from the cells and kept at 4°C until lactate was determined. The amount of lactate in the incubation medium was estimated with the enzymatic method described by Hohorst (1970), using a Kontron fluorimeter (Kontron Instruments, Zürich, Switzerland) at an excitation wave length of 340 nm and an emission wave length of 455 nm. Cellular protein was determined with the method described by Lowry et al. (1951), using bovine serum albumin as standard.

Measurement of hexose uptake

All incubations were carried out at 32°C. The medium in the wells (4 cm^2 wells, containing 150-200 µg protein) was aspirated and the cells were washed three times with glucose-free phosphate-buffered saline (PBS). The incubations were continued in glucose-free PBS and after 20 min hexose uptake was initiated by addition of 50 µM 2-deoxy-D-[^{3}H]glucose. Hormones or cytochalasins were added 10 min before the start of the uptake measurements, in concentrations described in the Results section. The incubations were terminated after 10 min by aspiration of the medium and a single wash with ice-cold glucose-free PBS. Tracer amounts of [^{14}C]sucrose were present to calculate the amount of extracellular trapped radioactivity. The cells were dissolved in 1 M NaOH and the radioactivity of half of this solution was counted in 10 ml Picofluor 15 (Packard Instrument Company Inc., Downers Grove, IL, U.S.A.) using a double label (^{3}H/^{14}C) program in an Isocap-300 liquid scintillation counter (Searle Analytic, Des Plains, IL, U.S.A.). The other half of the solution was used to determine the cellular protein content (Lowry et al., 1951). Where indicated in the Results section, different incubation times or different 2-DOG concentrations were used. The cellular ATP content after incubation in the presence of different concentrations of 2-DOG was determined as described by Grootegoed et al. (1984).

Measurement of intracellular free hexose and phosphorylated hexose

The method used is a modification of the method described by D'Amore and Lo (1986). After termination of a hexose uptake experiment as described above, the internalized substrate was released by disruption of the cells with 750 µl 10% trichloroacetic acid per incubation well. Aliquots of 500 µl trichloroacetic acid solubilized material were extracted twice with 1 ml H_2O-saturated diethylether to remove the trichloroacetic acid.

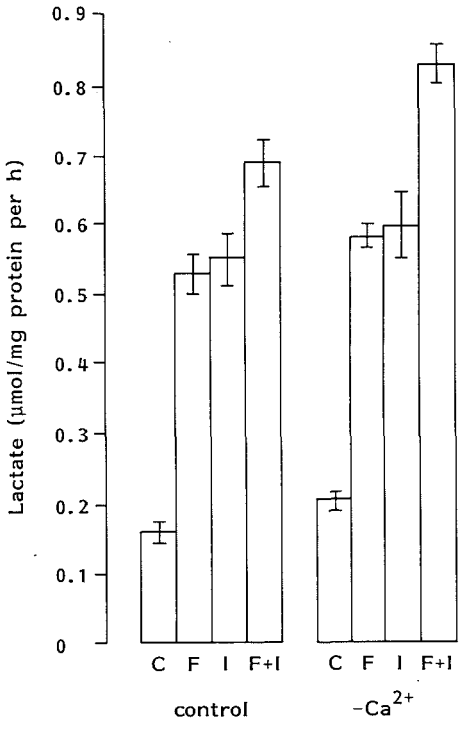

Figure 1. Effects of FSH and insulin on lactate production in the presence and absence of extracellular Ca^{2+}. Sertoli cells were incubated either in the absence (C) or in the presence of hormones (0.5 µg/ml of FSH, F or 5 µg/ml of insulin, I). In the control incubations (left) normal incubation medium containing 1.5 mM Ca^{2+} was present. -Ca^{2+}: Ca^{2+} was absent from the incubation medium, and 1 mM EGTA, a calcium chelator, was present. After 6 h the incubations were terminated and net lactate production was determined and expressed as µmol/mg protein per h. The results represent mean ± S.D. of triplicate incubations. Similar results were obtained in 3 experiments.

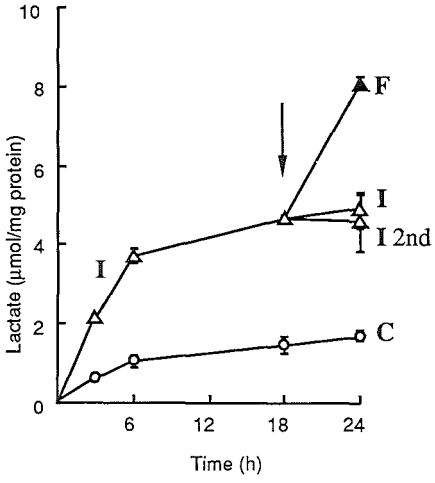

Figure 2. Time-course of the effects of FSH and insulin on lactate production after prolonged incubation in the presence of insulin. The incubations were started in fresh medium in the absence (0 – 0) or presence (Δ—Δ) of insulin, I (5 µg/ml). To the incubation medium FSH, F (0.5 µg/ml), or insulin, I 2nd (5 µg/ml), were added 18 h after the start of the incubation. After 24 h the incubations were terminated and net lactate production was determined and expressed as µmol/mg protein. The results represent mean ± S.D. of triplicate incubations. Similar results were obtained in 4 experiments.

Aliquots of 200 µl of the H_2O-fraction were then loaded onto a 0.7 x 10 cm column of Dowex 2 (anion exchange resin, 200-400 mesh, Cl^- form) (Fluka AG, Buchs, Switzerland). The free 2-DOG and the tracer amount of sucrose were first eluted with 3 ml H_2O. The hexose monophosphate which was retained on the column was subsequently eluted with 3 ml of 0.1 M HCl plus 0.1 M NaCl. The eluates were collected in scintillation vials, partly evaporated under an air stream of 60°C and the radioactivity was counted in 10 ml Picofluor 15, as described above.

Results

Lactate production by Sertoli cells

The experiments on lactate production and hexose uptake were performed using Sertoli cells which had been incubated for three days, as described in Materials and Methods. After this incubation period, effects of FSH and insulin on lactate production during a 6 h incubation were estimated in the absence and presence of extracellular Ca^{2+}. The Ca^{2+}-free medium was supplemented with 1 mM EGTA. The cells responded to the depletion of extracellular Ca^{2+} with a marked change of their morphology. The cells rounded up and showed vacuoles within a few minutes after Ca^{2+}-depletion. Replacement of the Ca^{2+}-free medium after 6 h with normal Ca^{2+}-containing incubation medium resulted in a normal morphology of the cells within a few hours. In the presence as well as in the absence of Ca^{2+} there was a 3-fold increase of net lactate production during the 6 h incubation in the presence of maximal effective concentrations of FSH and insulin. The effects of FSH and insulin were not additive during incubation in the presence of both hormones (Fig. 1).

Lactate production from 18-24 h of incubation during a 24 h incubation was very low under basal conditions (Fig. 2). In the presence of a maximal effective concentration of insulin lactate production rate during this incubation period was reduced to zero. After 24 h of incubation in the presence of insulin the amount of lactate in the spent medium was not significantly different from that after 18 h (Fig. 2). This could represent a reduced rate of glycolysis and/or a high rate of lactate oxidation from 18-24 h. However, data on the formation of ^3H_2O from D-[5-^{3}H]glucose (not shown) indicate that the rate of glycolysis had become very low. Readdition of insulin, without renewal of the medium, after 18 h did not result in a further increase of lactate (Fig. 2), indicating that the loss of the insulin effect was not due to degradation of insulin. Addition of FSH, without renewal of the medium, to the Sertoli cell cultures after 18 h of incubation in the presence of

Table 1. Lactate production during long-term incubations: 6 h incubation periods following daily washings.

6 h incubation period:	Net lactate production			
	1st period	2nd period	3rd period	4th period
Control	70 ± 3	312 ± 8*	404 ± 20*	313 ± 26*
FSH	265 ± 12	216 ± 5	240 ± 10	279 ± 11
Insulin	318 ± 7	316 ± 19	412 ± 17	326 ± 17

After preincubation Sertoli cells were incubated for 6 h in the presence or absence of FSH (0.5 μg/ml) or insulin (5 μg/ml), followed by washing (renewal of the medium) and incubation for 18 h in the absence of hormones, again followed by washing. This was repeated on four subsequent days. The table shows the lactate production in the 6 h periods of the subsequent incubation days, expressed as nmol lactate/mg protein per h (mean ± S.D. of three incubations).
* $P < 0.01$, significantly higher than during the first period.

insulin, however, resulted in a marked increase of the rate of lactate production (Fig. 2).

Repeated renewal of the medium after long-term incubations in the absence of hormones might cause an increase of lactate production (Table 1). Due to possible complications in long-term incubations, it was decided to perform short-term incubations to study initial rates of lactate production and 2-deoxyglucose uptake.

FSH, insulin and IGF–I stimulated lactate production within 60 min of incubation (Fig. 3). All three hormones caused a 2.5-fold stimulation within 180 min of incubation. After the first 10-30 min of incubation no significant stimulatory effect of hormones on the production of lactate could be observed (not shown). In separate experiments, the amounts of lactate in the cells and in the medium were analyzed separately, and the results indicated that no intracellular accumulation of lactate occurred during this first 30 min.

Dose-response curves were obtained for the effects of FSH, insulin and IGF–I on lactate production by Sertoli cells during a 2 h incubation period (not shown). The half-maximal stimulatory concentrations were 10-50 ng FSH/ml, 10-50 ng insulin/ml and 5-10 ng IGF–I/ml, respectively. Subsequently, lactate production was determined during a 2 h incubation period in the presence of submaximal doses of the hormones, and combinations of hormones. It was observed, that the effect of FSH was additive with the effects of insulin and IGF–I (Fig. 4).

2-Deoxyglucose transport

Hexose transport was studied using the uptake of the non-metabolizable glucose analogue 2-deoxyglucose (2-DOG). The uptake of 2-DOG is represented by the intracellular concentrations of 2-DOG and its phosphorylated product, 2-DOG-6-phosphate (2-DOG-6-P), and reflects both transport and hexokinase activity. The data in Figure 5 illustrate that at all time points 2-DOG was incorporated mainly into the 2-DOG-6-P pool. Up to 20 min of incubation intracellular 2-DOG was not detected. These results indicate that the rate-limiting step for 2-DOG uptake by Sertoli cells is hexose transport, rather than hexokinase activity.
Cytochalasin B is an inhibitor of glucose carriers (Kletzien and Perdue, 1973), and can be used to assess a glucose carrier mediated process. In the present experiments, the total uptake of 2-DOG was strongly inhibited by 1 μM cytochalasin B, whereas cytochalasin E, which
does not bind to the glucose carrier (Cuppoletti et al., 1981), did not inhibit 2-DOG uptake (Table 2).

The specificity of the hexose uptake system was studied by estimation of the competition of 2-DOG uptake by D-glucose and D-galactose. The results indicate that D-

glucose was a very effective competitor, whereas D-galactose hardly interfered with the transporter activity (Fig. 6).

Subsequently, the uptake of radiolabelled 2-DOG at different concentrations of 2-DOG was estimated during 10 min incubations (Fig. 7). No effect of the concentrations of 2-DOG tested, up to 1 mM, was observed on the ATP levels of the Sertoli cells (not shown). The K_m and V_{max} of 2-DOG uptake were calculated from Lineweaver-Burk plots (Fig. 7B), and are presented in Table 3. FSH and insulin did not influence the K_m of 2-DOG

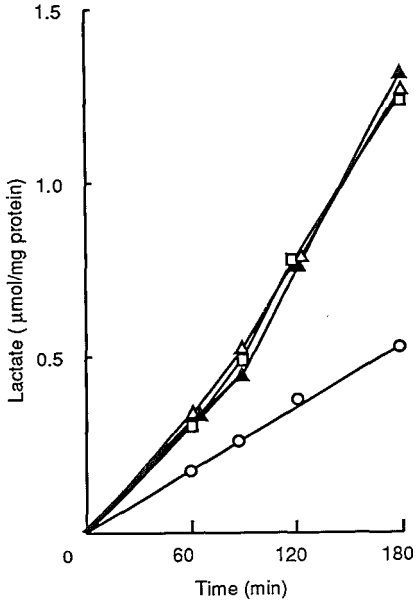

Figure 3. Time-course of the short-term effects of FSH, insulin and IGF–I on lactate production. Net lactate production was determined after the indicated incubation times either in the absence or presence of added hormones. The results represent the mean of duplicate incubations, which differed not more than 11%. Similar results were obtained in 3 experiments. 0–0, Control. ▲–▲, FSH (0.5 µg/ml). Δ–Δ, Insulin (5 µg/ml). □–□, IGF–I (100 ng/ml).

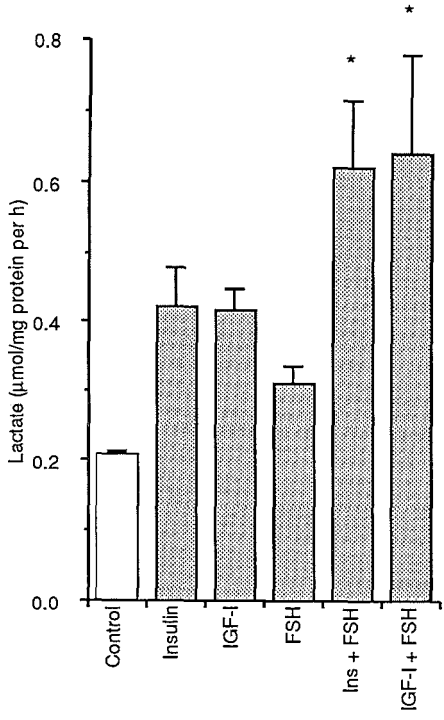

Figure 4. Effect of submaximal hormone concentrations on lactate production. Sertoli cells were incubated for 2 h in the absence (control) or presence of hormones. The concentrations used were 50 ng/ml of FSH, 500 ng/ml of insulin and 10 ng/ml of IGF–I. Net lactate production during this period was expressed as µmol/mg protein per h. The results represent mean ± S.D. of triplicate incubations. Incubation in the presence of combinations of these concentrations of FSH and insulin, and of FSH and IGF–I resulted in a significantly higher lactate production than observed in the presence of the individual hormones (*, $P < 0.05$).

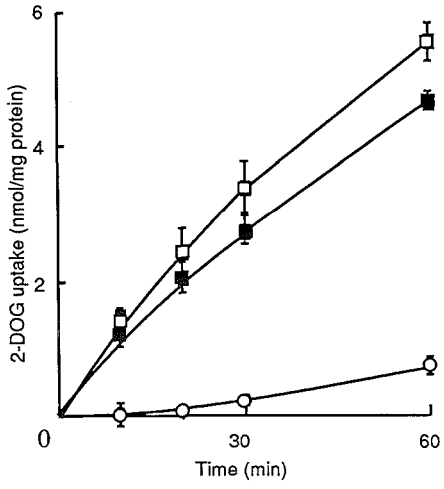

Figure 5. Time-course of 2-DOG uptake by Sertoli cells. Intracellular accumulation of 2-DOG-phosphate and free 2-DOG. The uptake of 50 μM 2-[^{3}H]DOG was carried out as described in Materials and Methods. The incubations were terminated after 10, 20, 30 and 60 min. The internalized sugars were released by immediate addition of 10% trichloroacetic acid. The amount of phosphorylated (■–■) and free 2-DOG (0–0) was then determined as described in Materials and Methods. The total amount of intracellular hexose (□–□) was determined in separate incubations. The results are expressed as nmol 2-DOG uptake/mg protein and represent mean ± S.D. of triplicate incubations.

uptake. Insulin increased the V_{max} of 2-DOG uptake by approximately 30%. In contrast, FSH did not show an effect on V_{max}. The insulin concentration used in this series of experiments was high (5 μg/ml), so that insulin could have exerted its effect on the V_{max} of 2-DOG uptake via both the insulin and IGF–I receptors. In another series of experiments it was found that IGF–I also increased the V_{max} of 2-DOG uptake by 31%, without an effect on K_m (not shown).

Discussion

The mechanisms of action of FSH and insulin are generally believed to be different. FSH, but not insulin, increased the level of cAMP in Sertoli cells (Oonk et al., 1985), indicating that FSH acts via the adenylate cyclase system. The mechanism of action of insulin is very complex and may include the intrinsic protein-tyrosine kinase activity of the insulin receptor (Kasuga et al., 1982). The present results show that the stimulatory effects of FSH and insulin on lactate production by cultured Sertoli cells during a 6 h incubation period are not dependent on the presence of extracellular Ca^{2+}. It is still disputed to what extent insulin effects on hexose transport require intra- and extracellular Ca^{2+} ions. Klip et al. (1984) and Cheung et al. (1987) reported that intra- and extracellular Ca^{2+} was not required for the mediation of the insulin effect on hexose transport in L6-muscle cells and in cardiac myocytes, respectively. Involvement of intracellular Ca^{2+} concentrations in insulin action on hexose transport in adipocytes was, however, reported by Pershadsingh et al. (1987). This discrepancy might be due to problems inherent to the determination of changes in intracellular Ca^{2+} concentrations or to tissue differences. The absence of effects of changes in extracellular Ca^{2+} concentrations on the FSH and insulin effects on lactate production by Sertoli cells indicates another similarity in FSH and insulin action in Sertoli cells, in addition to the previously reported independence of FSH and insulin action of *de novo* protein synthesis (Oonk et al., 1985).

The time-courses of actions of FSH, insulin and IGF–I on lactate production during 3 h of incubation with the hormones were identical. The similarities in kinetics were somewhat surprising in view of the different mechanism of action of FSH as compared to insulin and IGF–I (Oonk et al., 1985). Combination of the maximal effective amounts of FSH and insulin gave no additive stimulatory effect on lactate production. However, when submaximal stimulatory concentrations were used, it appeared that FSH in combination

Table 2. Effect of cytochalasin B on 2-deoxyglucose uptake by Sertoli cells in vitro.

Incubation	2-DOG uptake (% of control value)	
	Exp. 1	Exp. 2
2-DOG	100 ± 15	100 ± 9
2-DOG + 1 μM cytochalasin B	7 ± 7*	16 ± 1*
2-DOG + 1 μM cytochalasin E	92 ± 23	82 ± 8

* $P < 0.01$, significantly different from control.

Exp. 1 was performed for 5 min in the presence of 50 μM [^{3}H]-2-DOG. Control value was 258 ± 38 pmol 2-DOG uptake/mg protein.

Exp. 2 was performed for 30 min in the presence of 200 μM [^{3}H]-2-DOG. Control value was 5.3 ± 0.5 nmol 2-DOG uptake/mg protein.

The experiments were performed in duplicate. Mean ± range/2 are shown.

with insulin or IGF–I significantly enhanced lactate production, as compared to the effect of each hormone alone. This is in agreement with the different mechanisms of action of FSH versus insulin and IGF–I. Furthermore, when Sertoli cells had lost the response to insulin after prolonged incubation in the presence of insulin, and the rate of glycolysis had become very low, FSH could markedly stimulate lactate production. This heterologous desensitization, described earlier by Oonk et al. (1985), also indicates that FSH acted via a mechanism which is not affected by insulin.

As insulin was present in a high concentration in the present incubations it may have acted via the insulin receptor and via the IGF–I receptor. Both types of receptors are present on cultured Sertoli cells from immature rats, but the number of IGF–I receptors is much higher than the number of insulin receptors (Borland et al., 1984; Oonk and Grootegoed, 1987a, b). Apparently, after 6 h of incubation in the presence of a high concentration of insulin, the receptors for insulin and also for IGF–I are desensitized to a fresh large amount of insulin. The observed inhibition of the rate of glycolysis under these conditions can be bypassed by FSH.

Long-term incubations with renewal of medium enhanced the rate of lactate production by control cells to a similar extent as addition of FSH and insulin. This increase in lactate production might be

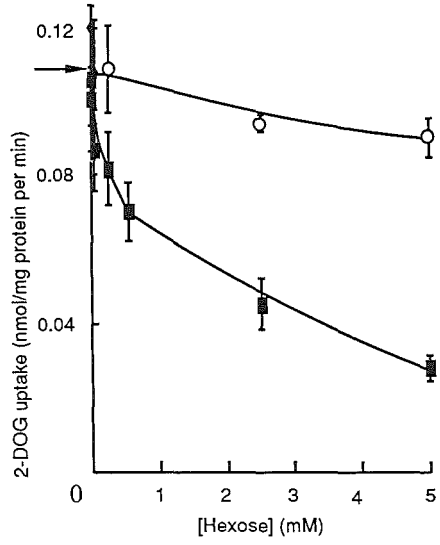

Figure 6. Competition of D-glucose and D-galactose with 2-DOG uptake. The uptake of 50 μM 2-[^{3}H]DOG in the absence (→) or presence of different concentrations of D-glucose (■–■) and D-galactose (0–0) was determined as described in Materials and Methods. The competing sugars were added to the cells at the same time as the labelled 2-DOG. The results are expressed as nmol 2-DOG uptake/mg protein per min and represent mean ± S.D. of triplicate incubations.

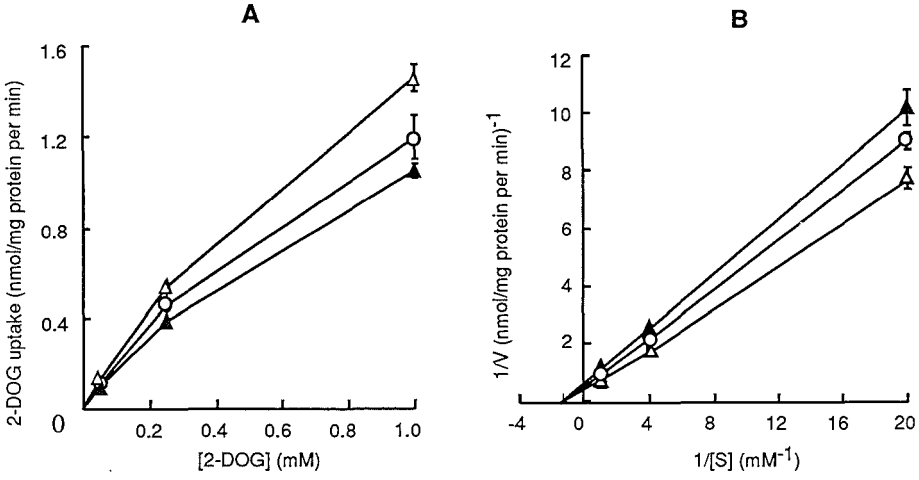

Figure 7. Kinetics of 2-DOG uptake by Sertoli cells. The uptake measurements were performed as described in Materials and Methods during 10 min incubations in the absence or presence of hormones. The hormones were added 10 min before the labelled 2-DOG. 0–0, Control. ▲–▲, 0.5 μg/ml of FSH. Δ–Δ, 5 μg/ml of insulin. A. The concentration of 2-DOG varied in the range described in the abscissa. The ordinate indicates the uptake rate of 2-DOG in nmol/mg protein per min. B. Double reciprocal plot of the uptake rate (V) versus 2-DOG concentration (S). The results represent mean ± S.D. of triplicate incubations.

similar to the acceleration of glucose metabolism and glucose transport which is observed in cells exposed to "stress factors". Possible stress factors are mechanical stress (Kodícek, 1986), heat shock and cellular transformation by viruses (Warren et al., 1986). Recently, two groups independently demonstrated that viral transformation of rodent fibroblasts not only increased glucose transport, but also elevated the levels of the glucose transporter mRNA (Flier et al., 1987; Birnbaum et al., 1987).

The present studies on hexose transport in Sertoli cells were performed using the non-metabolizable glucose analogue 2-deoxyglucose (2-DOG). Olefsky (1978) has evaluated in detail the use of this compound for uptake studies. 2-DOG is transported with an affinity in the same order as that for D-glucose, and 2-DOG is rapidly phosphorylated by hexokinase but not further metabolized. Thus the accumulation of 2-deoxyglucose-6-phosphate (2-DOG-6-P) in the cell can be used as a measure for hexose transport. Hence, using incubation conditions under which transport is rate-limiting for hexose uptake, 2-DOG is a reliable glucose analogue to estimate hexose transport.

It has been reported that high concentrations of 2-DOG (1-10 mM) can cause a rapid decline in ATP levels in adipocytes (Wieringa et al., 1985), concomitant with an increase in the rate of dephosphorylation of 2-DOG-6-P and the efflux of 2-DOG. In the present experiments, we have used concentrations of 2-DOG up to 1 mM, and under these conditions normal cellular ATP levels were observed. During short-term incubations using low 2-DOG concentrations, it was shown that transport and not hexokinase activity was rate-limiting.

Cytochalasin B, a microfilament inhibitor, inhibits hexose transport by binding to the hexose carrier (Kletzien and Perdue, 1973), whereas another cytoskeleton inhibitor, cytochalasin E, does not interact with the hexose carrier (Cupoletti et al., 1981). In the present experiments cytochalasin B, but not cytochalasin E,

Table 3. Kinetic constants for the uptake of 2-deoxy-D-[^{3}H]glucose by Sertoli cells.

	K_m (mM)	V_{max} (nmol/mg protein per min)
Control	1.15 ± 0.23	2.57 ± 0.31
FSH (0.5 μg/ml)	1.33 ± 0.12	2.45 ± 0.14
Insulin (5 μg/ml)	1.34 ± 0.15	3.41 ± 0.24*

* $P < 0.02$ compared to control. The data were calculated from Lineweaver-Burk plots (Fig. 7). The correlation coefficients of the three lines were 0.999. The results represent mean ± S.D. of three incubations.

inhibited 2-DOG uptake by Sertoli cells, indicating that 2-DOG uptake is a hexose carrier mediated process.

The competition of D-glucose and D-galactose with 2-DOG uptake was also estimated. D-galactose can be transported by the hexose carrier, but the affinity of hexokinase for D-galactose is very low. We observed that Sertoli cells did not convert D-galactose to lactate to a measurable extent (not shown), indicating that galactose is not efficiently converted to a glycolytic intermediate. The uptake of 2-DOG by Sertoli cells could be inhibited only 12% by D-galactose, as compared to 73% by D-glucose. These results are in agreement with results on the competition by different sugars of 2-DOG uptake by L6 muscle cells (Klip et al., 1982; D'Amore and Lo, 1986). D-galactose apparently interacts only weakly with the hexose carriers of the Sertoli cells, while D-glucose was a very effective competitor of 2-DOG uptake.

In contrast to our results, it has been reported by Hall and Mita (1984) that FSH can stimulate glucose transport in Sertoli cells, using 3-O-methylglucose (3-OMG) to determine glucose transport. These authors determined 3-OMG transport at different 3-OMG concentrations, and concluded that FSH decreased the K_m of 3-OMG transport without an effect on V_{max}. However, a main objection against this conclusion is that not the initial uptake rates were not measured, but steady state levels after 60-90min of incubation. Therefore, the reported K_m/V_{max} values do not represent the actual affinity and maximal velocity of the hexose transporter. Rather, the results represent intracellular accumulation of 3-OMG, and the stimulation by FSH of the intracellular amount of 3-OMG. Similar experiments yielding similar stimulatory effects of insulin and IGF–I on 3-OMG accumulation by Sertoli cells have been reported (Mita et al., 1985). Moreover, Olefsky (1978) considered 3-OMG less useful than 2-DOG for transport studies, because the influx of 3-OMG rapidly becomes non-linear. Klip et al. (1982) showed this to be valid especially in systems of attached cell layers. 3-OMG is transported across the plasma membrane, but not phosphorylated or metabolized. Only initial rates yield information on the transport of glucose across the plasma membrane into the cell. A very fast method of uptake estimation is required to determine initial rates of 3-OMG uptake. Such a method can be applied for cells in suspension, like adipocytes, but is difficult to apply on cell layers attached to culture wells, such as cultured Sertoli cells.

Effects of insulin on 3-OMG transport in adipocytes reflect mainly changes in V_{max} (Martz et al., 1986; Toyoda et al., 1987), although a conflicting observation, that insulin would influence primarily the K_m of 3-OMG transport, was reported (Whitesell and Abumrad, 1985). The present results, using 2-DOG to measure hexose uptake, also showed an insulin-induced increase in V_{max}. This is in accordance with the results on insulin stimulation of 2-DOG uptake in adipocytes (Olefsky, 1978; Wieringa et al., 1982). The maximal velocity of 2-DOG uptake was increased by insulin and IGF–I, but not by FSH. The hormones did not influence the affinity of 2-DOG uptake. In bovine granulosa cells, insulin stimulated

initial uptake rates of 3-OMG, whereas FSH had no significant stimulatory effect on 3-OMG uptake (Allen et al., 1981), which agrees with our results on 2-DOG uptake in Sertoli cells.

The effects of insulin and IGF–I on lactate production were quantitatively much larger than those on hexose transport. However, the stimulation of hexose transport was estimated at much shorter time intervals after hormone addition than the stimulation of lactate production. Furthermore, FSH did not stimulate hexose transport. Hence, we have concluded that FSH may enhance lactate production via effects on enzymes of the glycolytic pathway. Insulin and IGF–I probably affect both glucose transport and glycolytic enzyme activities.

It is not known in which way FSH, insulin and IGF–I might influence glycolytic enzyme activities. Using a cell-free system of several glycolytic enzymes and the purified insulin receptor kinase, it was reported that insulin can stimulate the phosphorylation of the glycolytic enzymes on tyrosine residues (Sale et al., 1987). However, it remains to be determined if tyrosine phosphorylation of glycolytic enzymes also occurs in intact cells, and if the activities of the cellular enzymes can be regulated in this way.

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