THE ROLE OF STEROL CARRIER PROTEIN 2 IN THE REGULATION OF LEYDIG CELL STEROIDOGENESIS

Free University Press is an imprint of
VU Boekhandel/Uitgeverij bv
De Boelelaan 1105
1081 HV Amsterdam
The Netherlands

Phone (0)20-444355
Telex 18191 vuboe nl

Isbn 90-6256-836-x
Nugi 742

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THE ROLE OF STEROL CARRIER PROTEIN 2 IN THE REGULATION OF LEYDIG CELL STEROIDOGENESIS

DE ROL VAN STEROL CARRIER PROTEIN 2 IN DE REGULATIE VAN LEYDIG CEL STEROIDOGENESE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. C.J. RIJNVOS
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN

DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 1 NOVEMBER 1989 OM 15.45 UUR

door

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GEBOREN TE UTRECHT

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ABBREVIATIONS AND TRIVIAL NAMES

AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
ATP	adenosine 5'-trisphosphate
BSA	bovine serum albumin
cAMP	adenosine cyclic-3',5'-monophosphate
cholesterol	5-cholestene-3β-ol
CSCC	cholesterol side chain cleavage
cyanoketone	2α-cyano-17β-hydroxy-4,4',17α-trimethyl-5-androstene-3-one
Da	Dalton, unit of molecular mass
DAG	diacylglycerol
dbcAMP	N^6-2'-O-dibutyryl adenosine cyclic-3',5'-monophosphate
EGF	epidermal growth factor
ER	endoplasmic reticulum
FABP	fatty acid binding protein
FSH	follicle stimulating hormone; follitropin
g	unit of gravity
h	hour(s)
IGF-II	insulin like growth factor II
IgG	immunoglobulin G
IL-1	interleukin-1
IP_3	inositol-1,4,5,-trisphosphate
LH	luteinizing hormone; lutropin
LHRH(-A)	luteinizing hormone releasing hormone (-agonist); luliberin
min	minute(s)
NADPH	reduced nicotinamide adenine dinucleotide phosphate
nsL-TP	nonspecific lipid transfer protein
PA	4β-phorbol-13-monoacetate
PK-A	cAMP-dependent protein kinase
PK-C	Ca^{2+}/phospholipid dependent protein kinase
PMA	4β-phorbol-12-myristate-13-acetate
pregnenolone	5-pregnene-3β-ol-20-one
SAP	sterol activator protein
SCC	side chain cleavage
SCP_2	sterol carrier protein 2
SDS-PAGE	polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate
SU-10603	7-chloro-3,4-dihydro-2(3-pyridyl)-1-(2H)-naphtalenone
testosterone	4-androstene-17β-ol-3-one
25-hydroxycholesterol	5-cholestene-3β,25-diol

CHAPTER 1. GENERAL INTRODUCTION

1.1. Aim of this study

The aim of the studies described in this thesis was to clarify the role of SCP_2 in the regulation of steroid production in rat Leydig cells.

The rate of steroid production in the adrenal, the ovary, the placenta and the testis is determined by the rate of conversion of cholesterol to pregnenolone. The complex of reactions and the localization of the enzymes involved in this so called cholesterol side chain cleavage are well known. The P-450_{scc} enzyme complex catalyzing the production of pregnenolone from cholesterol is located in the inner mitochondrial membrane of steroidogenic cells (Fig.1.1).

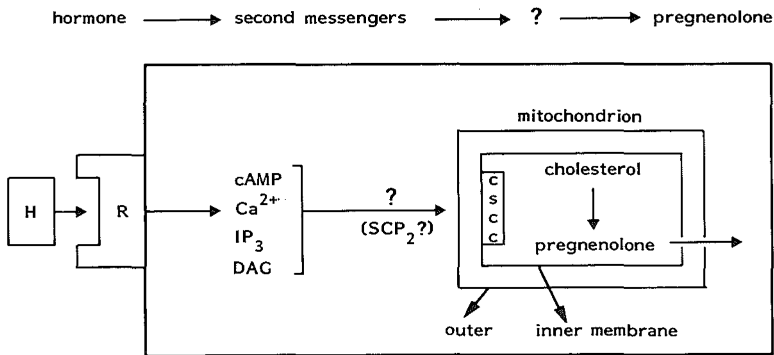


Fig.1.1. Simplified scheme for regulation of steroid production in steroidogenic cells. The hormone (H) binds to the receptor (R) on the plasma membrane. This binding enhances the production of intracellular second messengers such as cAMP, Ca^{2+}, IP_3 and DAG which via an as yet not clarified process results in enhancement of the rate of production of pregnenolone inside the mitochondria.

Several substances that regulate steroidogenesis, regulate the rate of pregnenolone formation via an intracellular transducing mechanism after binding to the plasma membrane of the cell. Different "second messengers" are involved in the transduction of the signal from the plasma membrane to other processes/messengers in the cell which are involved in regulation of steroid production. It is also known that protein synthesis and the transfer of cholesterol to the inner mitochondrial membrane are important in regulation of steroidogenesis. It is not known, however, if, and in which way the induction of second messenger systems in steroidogenic cells is linked with protein synthesis, cholesterol transfer and activation of the cholesterol side chain cleavage reaction inside the mitochondria. In this respect, sterol carrier protein 2 (SCP_2) is one of the possible proteins that could be involved in transduction of the signal from the second messengers to the CSCC enzyme complex.

1.2. Regulation of Leydig cell steroidogenesis

Biosynthesis of steroid hormones from cholesterol takes place in several steroidogenic tissues, the final products of the biosynthetic pathway being dependent on the particular enzymes present in the different tissues (for a review see Waterman and Simpson, 1985).

In the male a specific class of steroid hormones, the androgens, are responsible for the development and maintenance of spermatogenesis and for the primary and secondary sex characteristics. Androgen production takes place in the Leydig cells, which are present in the interstitial compartment of the testis. This steroid production is mainly under the control of the pituitary hormone lutropin (LH).

1.3. The rate-determining step in steroid hormone biosynthesis

The rate of production of steroid hormones is determined by the rate of conversion of cholesterol to pregnenolone (cholesterol side chain cleavage) (Fig.1.2).

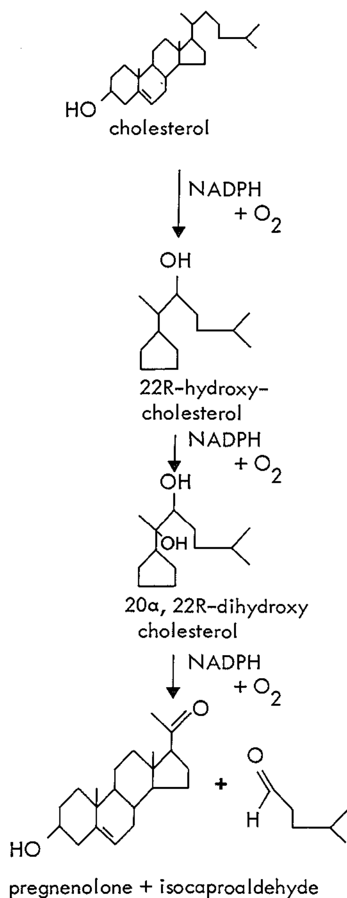


Fig.1.2. Mechanism of cholesterol side chain cleavage.

Cholesterol side chain cleavage is catalyzed by a specific cytochrome P-450 ($P\text{-}450_{scc}$) (for a review see: Simpson, 1979; Lambeth and Stevens, 1984-1985) which is located in the inner mitochondrial membrane (Privalle et al., 1983) and receives reducing equivalents from NADPH via a flavoprotein, adrenodoxin reductase, and an iron-sulfur protein, adrenodoxin (Fig.1.3).

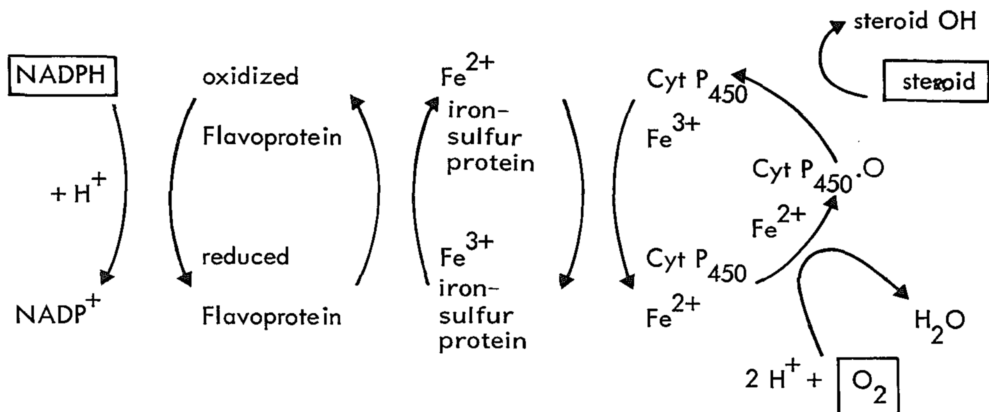


Fig.1.3. Mechanism of action of mitochondrial P-450 enzymes in steroidogenic cells.

1.4. Regulation of the rate-determining step
Transducing systems

The production of pregnenolone in steroidogenic cells is stimulated by peptide hormones, which bind to membrane receptors and activate transducing systems. Binding of LH to the membrane receptor of the Leydig cell results in an increase of intracel-

lular cyclic AMP and free calcium levels (Dufau et al., 1977; Themmen et al., 1985a,b; Sullivan and Cooke, 1986). In the Leydig cell steroid production can also be stimulated by compounds such as LHRH or the phorbol ester PMA. These activators utilize second messenger systems other than adenylate cyclase, such as the phosphatidylinositol cycle (Mukhopadhyay and Schumacher, 1985; Themmen et al., 1985a,b, 1986; Sullivan and Cooke, 1986).

Several reports indicate that other extracellular factors like factors in interstitial fluid (Sharpe and Cooper, 1984; Rommerts et al., 1986; Melsert and Rommerts, 1987) and growth factors, such as IGF II (Lin et al., 1986, 1988), EGF (Verhoeven and Cailleau, 1986) and IL-1 (Verhoeven et al., 1988), can also affect steroid production. Biological effects on steroid production of most of these factors have been demonstrated in vitro only after incubation periods of more than 24 h (Handelsman et al., 1985; Lin et al., 1986, 1988; Behnamed et al., 1987). Only albumin or substances associated with albumin in interstitial fluid appear to contribute to the acute stimulation of steroid production (Melsert et al., 1988).

The activation of different transducing systems in the Leydig cell probably results in phosphorylation and/or synthesis of specific proteins (Cooke et al., 1976; Bakker et al., 1983; Themmen et al., 1986). However, which of these proteins are involved in stimulation of cholesterol side chain cleavage and how they exert their actions is still not clear.

The stimulation of cholesterol side chain cleavage activity may be the result of the effect(s) of several mechanisms, such as

(1) stimulation of <u>synthesis</u> of the enzyme,
(2) <u>activation</u> of the enzyme by phosphorylation, phospholipids and proteins and
(3) regulation of the <u>availability of cholesterol</u> for the enzyme by the cytoskeleton and proteins (Fig.1.4)

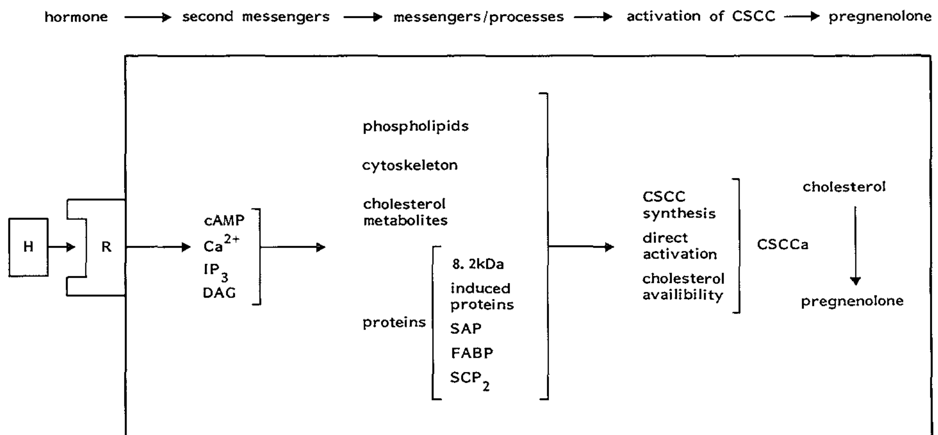


Fig.1.4. Possible transducing mechanisms involved in the stimulation of CSCC after incubation of the Leydig cell with hormone. For a complete explanation see text (1.5-1.13). Abbreviations used: H:hormone; R:receptor; IP_3:inositol trisphosphate; DAG:diacylglycerol; 8.2 kDa: protein with a molecular mass of 8.2 kDa; SAP:steroidogenesis activator protein; FABP: fatty acid binding protein; SCP_2:sterol carrier protein 2; CSCC:cholesterol side chain cleavage enzyme complex; CSCCa:active CSCC.

1.5. Regulation of the synthesis of P-450_{scc}

Levels of both P-450_{scc} and adrenodoxin can be increased by hormones. The increased levels, due to the effects of ACTH in adrenocortical cells, of FSH in granulosa cells, and of LH and hCG in Leydig cells, result from an increased transcription of mRNA species for both proteins. These effects can be mimicked by analogs of cyclic AMP suggesting that the effect of these hormones on synthesis is mediated by cyclic AMP (Waterman and Simpson, 1985). Stimulation of protein synthesis is not observed within twelve h of hormonal treatment. Therefore increased levels of P-450_{scc} can only (in part) account for long-term effects of hormonal stimulation on steroid production. The acute action of hormones on steroid production cannot be explained by regulation of synthesis of enzymes of the CSCC enzyme complex.

1.6. Activation of P-450_{scc} by phosphorylation

It has been demonstrated in vitro that purified P-450_{scc} can be selectively phosphorylated by a calcium ion activated, phospholipid-sensitive protein kinase (protein kinase C) preparation (Vilgrain et al., 1984). In addition, purified inner mitochondrial membrane preparations from adrenocortical cells have been found to contain protein kinase C activity. These findings, together with the effects of activators of protein kinase C activity (such as phorbol esters) on steroid production, have led to the suggestion that phosphorylation of cytochrome P-450_{scc} by an intramitochondrial protein kinase C could be important in regulation of steroidogenesis (Vilgrain et al., 1984). However, there is no evidence that such a regulation may be important in intact cells since it has not been demonstrated that phosphorylation of P-450_{scc} leads to activation of the enzyme.

1.7. Regulation of P-450$_{SCC}$ by phospholipids

An important role for the regulation of P-450$_{SCC}$ activity has been assigned to the poly-phosphorylated phospholipids from the phosphatidylinositol cycle. In vitro, stimulation of pregnenolone production was observed when poly-phosphorylated phosphatidylinositols were added to adrenal mitochondria (Farese et al., 1987). A comparable stimulation of CSCC was found upon addition of poly-phosphorylated phosphatidylinositols to purified cytochrome P-450$_{SCC}$ in a phospholipid vesicle (Kowluru et al., 1983). The relevance of these findings for regulation of P-450$_{SCC}$ in intact cells is questionable, particularly in view of the high poly-phosphorylated phosphatidylinositol concentrations which were required to elicit these effects. In mitochondria isolated from rat tumour Leydig cells, stimulating effects on steroid production could be obtained only at unphysiological high concentrations (100 μM) of phospho- lipids. Also, in these cells no clear-cut correlation between the level of phosphorylated phospholipids and CSCC could be observed under different experimental conditions (Terpstra et al., 1985).

In the adrenal gland, however, phospholipids are apparently required to optimize activity of P-450$_{SCC}$. In an aqueous environment, lipophilic cytochromes aggregate (Takagi et al., 1975) and interactions with lipids may modulate the state of aggregation which in turn may affect interactions of the cytochrome both with cholesterol and electron transfer proteins (Schwartz et al., 1982; Greinert et al., 1982).

1.8. Modulation of P-450$_{SCC}$ activity by cholesterol metabolites

In intact mitochondria from rat adrenals the side chain cleavage of exogenous cholesterol is inhibited by cholesterol sulfate (Lambeth et al., 1987). This inhibition appears to be specific since related compounds, including pregnenolone sulfate, were not effective. The inhibition is not exerted via cytochrome P-450$_{SCC}$ since neither the spin state of the hemoprotein nor the metabolism of 25-hydroxycholesterol were inhibited. The inhibition was abolished for 70% by sonic disruption of mitochondria. This indicates that cholesterol sulfate might inhibit an intramitochondrial cholesterol translocation system. The high content of cholesterol sulfate in the adrenal cortex suggests a possible regulatory role for this molecule (Lambeth et al., 1987; Xu and Lambeth, 1989).

During steroidogenesis in several tissues several oxygenated sterols are formed. Some of these oxygenated sterols have been found to inhibit cholesterol synthesis when added to cultures at concentrations in the range of 10^{-9} to 10^{-6} M (Kandutsch et al., 1978). The most potent inhibitor is 25-hydroxycholesterol which does affect both degradation and synthesis of HMG-CoA reductase in several types of cells (Taylor and Kandutsch, 1984; Saucier et al., 1985). The mechanism of action of cholesterol metabolites involves binding of the inhibitor to an oxysterol binding protein which may act as a receptor involved in the regulation of mRNA levels for HMG-CoA reductase (Kandutsch et al., 1984; Taylor et al., 1984, 1988). In rat Leydig cells _de novo_ cholesterol synthesis is most important for the supply of substrate for the regulation of CSCC (see 1.9.). This suggests a possible regulatory role for oxygenated sterols. Acute effects of oxygenated sterols on regulation of HMG-CoA reductase activity have been demonstrated thus far only in chinese hamster ovary cells (von Gunten and Sinensky, 1989).

1.9. Availability of cholesterol to cytochrome P-450_{SCC}

In mature rat Leydig cells, cholesterol esters present in cytosolic lipid droplets are not the source of cholesterol for side chain cleavage. In this respect the rat Leydig cell is different from the mouse Leydig cell (Freeman et al., 1987), the adrenal gland (Ohashi et al., 1982) and the ovary, which use cytosolic cholesterol esters as the source of cholesterol for steroidogenesis. In rat Leydig cells, cholesterol is derived mainly from de novo synthesis (Charreau et al., 1981; Van der Molen and Rommerts, 1981). The rate-limiting enzyme in the de novo synthesis of cholesterol is HMG-CoA reductase (Geelen et al., 1986) which is mainly localized in the endoplasmic reticulum (Skalnik et al., 1988; Reinhart et al., 1987) but is also found in the peroxisomes (Keller et al., 1985; Krisans et al., 1987; Thompson et al., 1987) and may also be present in the mitochondria in Leydig cells (Pignatarro et al., 1983). In the Leydig cell line MA-10 (obtained from mouse Leydig cell tumors) the primary store of cholesterol for steroidogenesis appears to reside in the plasma membrane (Freeman et al., 1987). In rat ovarian granulosa cells cholesterol appears to be the stored mainly intra- cellularly (Lange et al., 1988).

Irrespective of the origin of intracellular cholesterol, to act as a substrate for steroid production cholesterol must move to the P-450_{SCC} located on the inside of the inner mitochondrial membrane (Privalle et al., 1983). Intermembrane transfer of sterols and steroids is restricted by the rate of dissociation from the lipid phase to the aqueous phase. This process is very slow for the highly lipid soluble cholesterol (t ½= 1-2 hr (Fugler, et al., 1985), while it is several orders of magnitude faster after the C_{21}-C_{27} side chain has been removed. It appears likely, therefore, that for LH stimulated androgen production the required rapid transfer of cholesterol to P-450_{SCC} depends on more than simple membrane-water transfer, while for all other steroids in the pathway transfer by membrane-water diffusion may

be sufficiently rapid. Hence, it can be expected that stimulation of steroid production is accompanied by an alternative means of cholesterol transfer between Leydig cell membranes.

1.10. Involvement of the cytoskeleton

Cellular uptake and intracellular transport of various substances in the Leydig cell appear to involve the cytoskeleton (Crivello and Jefcoate, 1978; Murono et al., 1982). The cytoskeleton contains protein fibers of different diameter present in the cytoplasm and nucleoplasm (Chaly et al., 1977; Henderson and Weber, 1979; De Brabander, 1982). The most important structures are contractile proteins i.e. actin and myosin (microfilaments) and tubulin (microtubules). Agents, such as cytochalasins, DNase I and anti-actin antibodies, that interfere with microfilament elongation or organization markedly reduce hormone stimulated production of steroids in adrenocortical cells and testicular Leydig cells (Hall, 1984b). In granulosa cells, agents such as colchicine and nocodazole that depolymerize microtubules have a similar effect (Carnegie et al., 1987, 1988). In each case, the agent appears to act at a step in the steroidogenic pathway proximal to pregnenolone formation. This suggests that microfilaments and/or microtubules are involved in the regulation of the conversion of cholesterol to pregnenolone by an as yet unknown mechanism.

In response to either cyclic nucleotides or tropic hormones that increase cAMP levels, the shape of a number of mammalian cells changes (Hall et al., 1985; Sato et al., 1970; Lawrence et al., 1979; Albertini and Herman, 1984). In cultured granulosa cells, organelles which were dispersed throughout the cytoplasm in untreated cells became clustered in the peri-nuclear region 2-4 h after administration of hormone. This may facilitate movement of substrate among organelles and steroid synthesis. It has been suggested that cytochalasins, which also lead to changes of the

cell shape may inhibit steroidogenesis because they act too rapidly, thus interfering with events critical to steroidogenesis (Sato et al., 1986).

The results concerning association between changes in cell shape and enhancement of steroid production are ambiguous. For example, incubation of adrenal tumour Y-1 cells on poly(HEMA) induces rounding of the cells as well as increased production of steroid (Betz and Hall, 1987). The same authors showed that culturing cells on poly-lysine results in flattening of cells and a decreased steroid production. It has been suggested that in a flattened cell transport of cholesterol is reduced by geometrical problems associated with the internal distribution of mitochondria and perhaps other structures. In contrast Y-1 cells treated with proteases became rounded but did not produce increased amounts of steroid even in the presence of tropic hormones (Voorhees et al., 1984).

1.11. Evidence for the involvement of proteins

Inhibition of protein synthesis by cycloheximide or by puromycin, results in an inhibition of the lutropin stimulated steroidogenesis. This suggests that protein synthesis is necessary for the effect of LH on steroid production (Hall and Eik-Nes, 1962; Ferguson, 1963; Sakamoto et al., 1973; Cooke et al., 1975; Mendelson et al., 1975; Bakker et al., 1985). The rapid decrease in glucocorticoid production after addition of protein synthesis inhibitors to ACTH-stimulated adrenocortical cells, led Garren et al.(1965) to suggest that synthesis of a short-lived (i.e. labile) protein is required for steroido- genesis. Actinomycin-D, an inhibitor of DNA transcription, had no effect on acutely stimulated steroid output (Vernikos-Danellis and Hall, 1965), re-emphasizing the importance of the ribosomal site, e.g. translation of mRNA into proteins, in this effect. Using the same antibiotics evidence for a labile, rapidly turning over, protein

was also found in the Leydig cell (Cooke et al., 1976; Bakker et al., 1985). Recently, with adrenal cells, support was provided for the existence of a labile protein under conditions in which total protein synthesis is not inhibited. Incorporation of amino acid analogs (Krueger and Orme-Johnson, 1988) caused a rapid inhibition of steroid production, thus indicating the rapid production of a peptide that contained amino acid analogs and was therefore not functional.

1.12. Proteins that are possibly involved in regulation of steroid production

A possible involvement of protein factors in the regulation of steroid production is supported by the isolation of several proteins and peptides which can stimulate steroidogenesis in isolated mitochondria and/or whose occurrence is correlated with the steroidogenic capacity of cells.

Fatty acid binding protein

Fatty acid binding protein (FABP), a major protein in liver cytosol, accumulates concomitantly with cholesterol in adrenal mitochondria following inhibition of P-450_{SCC} (Conneely et al., 1984). This protein is also designated sterol carrier protein (SCP, Dempsey et al., 1981, 1985). It has been proposed that SCP is synthesized in liver and intestine and is secreted into the blood stream where it associates mainly with the high density lipoprotein fraction. ACTH stimulation of adrenal cells would then rapidly enhance uptake and movement of the protein-cholesterol complex to the inner mitochondrial membrane (Dempsey et al., 1986). The proposed role for this protein as cholesterol carrier has been disputed, due to a failure to observe cholesterol exchange activity (Scallen et al., 1985). However, FABP may induce changes in membrane-lipid content, fluidity and structure

which may contribute to activation of cholesterol side chain cleavage (see 1.7.).

8.2 KDa protein

Recently a small 8.2 kDa peptide was purified from bovine adrenal (fasciculata) cells (Yanagibashi et al., 1988). This peptide appears to be capable to stimulate synthesis of pregnenolone by isolated mitochondria in a concentration dependent fashion when cholesterol is added to the medium (maximal increase in steroid production 2-3-fold). This protein can also accelerate the transport of cholesterol from the outer to the inner membrane of mitochondria. It has been proposed that this protein is involved in the regulation of steroidogenesis in bovine fasciculata cells by stimulating the entry of cholesterol into the outer membrane of mitochondria and by altering the intramitochondrial distribution of cholesterol.

Induced proteins

Orme-Johnson and co-workers have reported the induction of a group of proteins in rat adrenals by ACTH which coincides with stimulation of steroid production (Krueger and Orme-Johnson, 1983; Pon et al., 1986; Pon and Orme-Johnson, 1986). Proteolytic polypeptide mapping suggests that the hormonally induced proteins designated i_a and i_b, are structurally similar to other proteins (p_a and p_b respectively) which are present only in unstimulated cells. Production of protein i_b (molecular weight app. 28,000) is sensitive to cycloheximide and the amount of i_b that is produced is correlated with steroid output. Protein i_b is not produced from p_b post-translationally, but it may be formed by a co-translational modification, apparently phosphorylation. All four proteins were also found in rat corpus luteum (Pon and Orme-Johnson, 1986) and Leydig cells (Pon et al., 1986; Stocco et al., 1988). The induced proteins i_a and i_b seem to be located primarily in the mitochondria (Stocco and Kilgore, 1989) which suggests that they also act there.

Sterol activator peptide

In 1983, Pedersen and Brownie reported the isolation of steroidogenesis activator peptide (SAP) which appears in rat adrenocortical cytosol in response to corticotropin administration to hypophysectomized rats. The production of SAP is sensitive to cycloheximide and the peptide is not present in adrenocortical cytosol from hypophysectomized rats. An apparently very similar peptide, isolated from the H-540 Leydig cell tumour, has been sequenced (Pedersen and Brownie, 1987) and has been shown to contain 30 amino acid residues (molecular weight 3200). When SAP was added to mitochondria from adrenals and H-540 Leydig cell tumors pregnenolone formation was increased 5-10-fold. Addition of this peptide also produced spectral changes characteristic of increased cholesterol binding to cytochrome $P\text{-}450_{scc}$ (Pedersen and Brownie, 1983). Homology exists between SAP and the C-terminal region of GRP78 (Glucose Related Polypeptide 78), a minor heat shock protein. It has been postulated that SAP is derived from GRP78 by Ca^{2+}-protease dependent proteolysis.

Sterol carrier protein 2

Another activator of mitochondrial steroidogenesis is sterol carrier protein 2 (SCP_2; molecular weight apr. 14,000). SCP_2, also designated nonspecific lipid transfer protein (nsL-TP), has been purified from rat, bovine and human liver (Bloj and Zilversmit, 1977; Poorthuis et al., 1981; Trzaskos and Gaylor, 1983; Van Amerongen et al., 1987a,b).

It has been shown that SCP_2 can stimulate the transfer of cholesterol as well as that of phospholipids between membranes in vitro (Bloj and Zilversmit, 1977; Poorthuis et al., 1981; Scallen et al., 1985) and that it may play a role in the intracellular transport or metabolism of phospholipids and cholesterol (Teerlink et al., 1984; Chanderbhan et al., 1982; Seltman et al., 1985).

Studies with adrenal mitochondria have shown that addition of purified SCP_2 or addition of cytosol containing SCP_2 can increase the amount of cholesterol at the level of the inner mitochondrial membrane (Vahouny et al., 1983, 1984) as well as pregnenolone production (3-fold). In contrast to SAP, SCP_2 does not affect directly the association of cholesterol with cytochrome $P\text{-}450_{scc}$ (Vahouny et al., 1983). It has been proposed that SCP_2 as a carrier protein binds and transfers cholesterol in a 1:1 molar complex from intracellular membranes to the outer mitochondrial membrane and from the outer mitochondrial membrane to the inner mitochondrial membrane where $P\text{-}450_{scc}$ is located (Vahouny et al., 1983, 1984; Fig. 1.5). The rate of steroid production would then depend on the amount of SCP_2 available for the transfer of cholesterol. However, there is no direct evidence that the formation of SCP_2 is affected by ACTH in the adrenal.

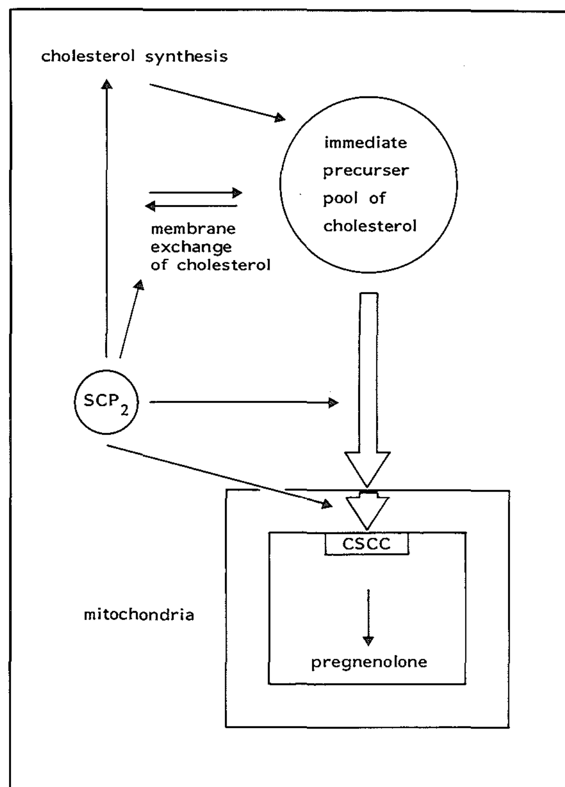


Fig.1.5. Possible role of sterol carrier protein 2 in regulation of steroidogenesis: SCP_2 might be involved in transport of cholesterol to the mitochondria and between the outer and inner mitochondrial membrane.

1.13. Studies described in this thesis

The possible role of SCP_2 in regulation of steroid production in rat Leydig cells was investigated, since the cellular amount of SCP_2 in steroidogenic cells could determine the rate of steroid production. Purified SCP_2, polyclonal antibodies raised against SCP_2 and an enzyme immunoassay to measure the amount of SCP_2 could be obtained from Wirtz and colleagues.

In the studies presented in this thesis we tried to answer the following questions:

- 1. **Is SCP_2 present in the Leydig cell ? (chapter 2).**
  SCP_2 levels were determined by an enzyme immunoassay in 105,000 x g supernatant (soluble) fractions of rat testes and in different isolated testicular cells.
- 2. **Is the level of SCP_2 regulated by hormones and does the amount of SCP_2 in the 105,000 x g (soluble) fraction of the cell regulate the rate of steroid production ? (chapter 2 and 3).**
  The effects of substances that regulate steroid production on the amount of SCP_2 in the 105,000 x g fractions of cells, after short term incubations in vitro, and testes, after long term incubations in vivo were investigated.
  SCP_2 levels were also determined in a "mutant" mouse tumour Leydig cell clone which has normal levels of second messengers and a functionally active cholesterol side chain cleavage enzyme complex but which shows a very poor steroidogenic response after incubation with hCG (chapter 6).
- 3. **Which cellular second messenger systems are involved in SCP_2 action and by which mechanism is the cellular level of SCP_2 controlled ? (chapter 3-6).**
  The cellular amount and distribution of SCP_2 were studied using enzyme immunoassay and immunocytochemistry. Kinetic aspects of hormone action and the role of calcium on SCP_2 levels in the soluble fraction of cells were investigated.

- 4. **Does SCP_2 influence steroid production ? (chapter 4 and 7).**
The effect of SCP_2 on the production of pregnenolone in isolated tumour Leydig cell mitochondria was investigated. The affinity of SCP_2 for sterol (25-hydroxycholesterol) was studied using equilibrium dialysis studies with purified SCP_2 **(chapter 7).**

CHAPTER 2

LOCALIZATION AND HORMONAL REGULATION OF THE NONSPECIFIC LIPID TRANSFER PROTEIN (STEROL CARRIER PROTEIN 2) IN THE RAT TESTIS

In testis tissue from mature rats the nonspecific lipid transfer protein (nsL-TP), also called sterol carrier protein 2 (SCP_2), is concentrated in the Leydig cells and cannot be detected in Sertoli cells or germinal cells. Conclusions were reached after cell fractionation studies with normal testis tissue and after selective destruction of Leydig cells or germinal cells in vivo.

The amount of SCP_2 in testis tissue increased 2-fold 48 h after two daily injections of human chorionic gonadotrophin (100 i.u., s.c.) and decreased 2-fold after plasma luteinizing hormone levels were suppressed to almost undetectable levels with silicone elastomer implants containing testosterone.

The specific localization in the Leydig cells and the luteinizing hormone-dependent cellular concentration of SCP_2 support the possibility that this protein could play a role in the regulation of steroidogenesis by regulating the availability of cholesterol for the P_{450} side chain cleavage enzyme in the mitochondria of Leydig cells.

after: Van Noort M, Rommerts FFG, Van Amerongen A and Wirtz KWA (1986) J. Endocrinol. 109, R13-R16.

INTRODUCTION

The rate-limiting step of steroidogenesis is the conversion of cholesterol to pregnenolone by the P-450 cholesterol side-chain cleavage (P-450_{scc}) enzyme (Stone and Hechter, 1954; Simpson, 1979; Lambeth and Stevens, 1984-1985), which occurs in the inner mitochondrial membrane (Privalle et al., 1983). Luteinizing hormone (LH) stimulates the production of pregnenolone in Leydig cells of the rat testis, after binding to membrane receptors and activation of different second messenger systems (Bakker et al., 1983; Themmen et al., 1985). Several mechanisms may be involved in the stimulation of cholesterol side chain cleavage activity, for example the stimulation of the synthesis of the P-450_{scc} enzyme by hormones (Waterman and Simpson, 1985), activation of the system in isolated mitochondria by a protein of 2.2 kD molecular weight (Pedersen and Brownie, 1983), activation by phosphorylation (Vilgrain et al., 1984), and regulation of the availability of cholesterol for the enzyme in the inner mitochondrial membrane (Chanderbhan et al., 1982).

Recent studies with adrenal mitochondria have shown that the nonspecific lipid transfer protein (nsL-TP), also called sterol carrier protein 2 (SCP_2), can stimulate (3-fold) cholesterol transfer from the outer membrane of the mitochondria to the inner membrane as well as pregnenolone production (3-4-fold) (Vahouny et al., 1984; Vahouny et al., 1985). This protein may therefore contribute to the regulation of steroidogenesis, although the hormonal regulation of the cellular amount of this protein has not been demonstrated (Vahouny et al., 1984-1985).

SCP_2 has been purified from rat and bovine liver (Bloj and Zilversmit, 1977; Noland et al., 1980; Poorthuis et al., 1981; Trzaskos and Gaylor, 1983). It has been shown to stimulate in vitro the transfer of cholesterol as well as that of phospholipids between membranes (Bloj and Zilversmit, 1977; Poorthuis et al., 1981; Scallen et al., 1985). It has been suggested that it may play a role in the intracellular transport or metabolism of

phospholipids and cholesterol (Teerlink et al., 1984; Chanderbhan et al., 1982; Seltman et al., 1985). Scallen et al. (1985) have argued strongly that the physiological role of SCP_2 is limited to various aspects of cholesterol metabolism including steroidogenesis.

To evaluate whether SCP_2 may be involved in the regulation of testicular steroidogenesis, we have investigated the cellular localization of SCP_2 and the hormonal regulation of the amount of this protein in rat testis tissue.

MATERIALS AND METHODS

Animals Male rats (Wistar substrain R-Amsterdam) were used when they were 22 days or 4-8 months old. Sterile rats, with germinal cell-depleted testes, were obtained after irradiation of pregnant rats at day 20 of gestation with a dose of 1.5 Gy X-rays (Beaumont, 1960). Leydig cell depleted testes were obtained 3 days after one intraperitoneal injection of ethanedimethylsulphonate (EDS) (75 mg/kg body weight) of a solution containing 30 mg/ml in DMSO:H_2O, 1:3 (Molenaar et al., 1985).

Human chorionic gonadotrophin (100 i.u.) was injected s.c. on 2 consecutive days. Other rats received a testosterone-filled capsule (5 cm, ϕ 5 mm) subcutaneously in the neck 2 days before isolation of the testis tissue.

Cell isolation and measurements Animals were killed by decapitation. Blood was collected and plasma stored at -20°C. Testes were removed immediately and decapsulated. Leydig cells (80% pure), Sertoli cells (95% pure) and germinal cells (95% pure) were isolated according to methods described previously by Rommerts et al.(1985), Oonk et al.(1985) and Grootegoed et al.(1982) respectively.

Tissue or isolated cells were disrupted by sonication (5 x 10 sec, amplitude 16 microns, frequence 60 Hz) and a 105,000 x g supernatant fraction was prepared as described previously (Bakker et al., 1981). Levels of SCP_2 were determined in supernatant after acid (pH 5.1) and heat treatment (5 min, 90°C) using an enzyme immunoassay (Teerlink et al., 1984). The amount of SCP_2 adsorbed to a plastic surface in the presence of a large excess of serum albumin was measured using a specific IgG antibody against SCP_2 from rat liver as described previously by Teerlink et al.(1984). The detection limit was 20 ng SCP_2/mg protein. Protein was determined according to Lowry et al.(1951). Plasma testosterone was measured according to procedures described by Verjans et al.(1973) and luteinizing hormone was measured as described by Welschen et al. (1975).

RESULTS AND DISCUSSION

Cellular localization of SCP_2 in the testis

Levels of SCP_2 were determined in isolated testicular cells and in testis tissues containing different amounts of Leydig cells. As shown in Table 2.1 the SCP_2 level in purified isolated Leydig cells was more than 10-fold higher than in testis tissue whereas in other testicular cells SCP_2 was not detectable.

Table 2.1. Localization of sterol carrier protein 2 (SCP_2) in fractions isolated from rat testis tissue. Values are means ± S.D. from three different cell preparations.

	SCP_2 (ng/mg) protein
Testis tissue	55 ± 7 (3)
Leydig cells	671 ± 27 (3)**
Dissected tubules	30 ± 3 (3)**
Sertoli cells	<20* (3)
Sertoli cells + germinal cells	<20* (3)

**P<0.01 compared with testis tissue.
* detection level

The higher (10-fold) level of SCP_2 in the Leydig cells corresponded with the purification factor of Leydig cells which is approximately 10. Some SCP_2 was detected in tubules isolated from the testis of an untreated rat, but this may be explained by the amount of contaminating Leydig cells present (Molenaar et al., 1986). Testicular tissues enriched or devoid of Leydig cells after in vivo treatments were also analyzed. Prenatal irradiation causes a 10-fold enrichment of Leydig cells in the testis when germ cells are destroyed (Molenaar et al., 1985). A comparable increase in the amount of SCP_2 was observed in these testes (Table 2.2).

Table 2.2. Localization of sterolcarrier protein$_2$ (SCP_2) in the mature rat testis after different treatments in vivo. Values are means ± S.D. of three different animals.

Treatment	SCP_2 (ng/mg) protein
None	55 ± 7 (3)
Prenatal irradiation	557 ± 25 (3)**
Ethanedimethylsulphonate	<25*

**P<0.01 compared with testis tissue
* detection level

On the other hand, when Leydig cells were specifically destroyed 3 days after the treatment of mature rats with the alkylating compound EDS (Molenaar et al. 1985), the amount of SCP_2 in the testis decreased to almost undetectable levels. Release of the protein from degenerating Leydig cells can explain the small amount of SCP_2 after EDS treatment although other cells in the interstitium, such as macrophages, may also contain some SCP_2 and also active secretion from Leydig cells cannot be excluded (Crain and Clark, 1985).

These results indicate that SCP_2 in the testis is mainly if not entirely concentrated in the Leydig cells and cannot be detected in germinal and Sertoli cells. The absence of SCP_2 from Sertoli cells which are active in lipid metabolism (Jutte et al., 1985) and which contain many lipid droplets (Kerr et al., 1984) makes it unlikely that SCP_2 facilitates the transfer of cholesterol or phosphoglycerids between membranes in tubular cells as was shown in vitro for isolated liver protein and suggested for liver cells (Bloj and Zilversmit, 1977; Poorthuis et al., 1981). The very low levels of this protein in certain types of tumour cells have led to similar conclusions (Crain et al., 1983; Van Heusden et al., 1985). In liver and Leydig cells similar amounts of SCP_2 were detected (Table 2.1 and Teerlink et al., 1984).

Hormonal regulation of SCP_2 in the testis

Stimulation of cholesterol transfer between mitochondrial membranes by SCP_2 in vitro was shown using adrenal fractions (Scallen et al., 1985). If SCP_2 is involved in cholesterol transfer in steroidogenic cells it may be expected that the levels of this protein are under hormonal control, if intracellular transport of cholesterol is a rate limiting step in steroidogenesis. To investigate the hormonal regulation of SCP_2 levels in Leydig cells by gonadotrophins, mature rats were injected with hCG or endogenous luteinizing hormone was suppressed by high testosterone levels. Treatment with hCG led to increased blood plasma levels of Leydig cell stimulating hormones from 150 μg/l (luteinizing hormone) to 400 μg/l (hCG). This treatment resulted in a 1.7-fold increase in the amount of testicular SCP_2 after 48 h (Fig. 2.1).

Subcutaneously implanted testosterone-filled capsules suppressed luteinizing hormone in plasma to almost undetectable levels (less than 25 μg/l) and caused a 2-fold decrease in the amount of SCP_2 (Fig.2.1). These results indicate that luteinizing hormone regulates the amount of SCP_2 in Leydig cells. This regulation could be a direct consequence of luteinizing hormone activation on the cell or an indirect effect caused by an luteinizing hormone induced requirement for cholesterol.

Since a high molecular weight form of SCP_2 is present in rat liver (Van der Krift et al., 1985), levels of SCP_2 may be regulated by precursor proteolysis as well as by de novo synthesis.

The results show that in the rat testis a lipid transfer protein is specifically localized and hormonally regulated in the Leydig cell which is the only steroidogenic cell in the testis. Since this protein has been shown to facilitate the transfer of cholesterol to the $P\text{-}450_{scc}$ enzyme (Vahouny et al., 1984), it may be important in the regulation of steroidogenesis in the Leydig cell. This study strongly suggests that nsL-TP/SCP_2 may have specific functions in cholesterol metabolism in Leydig cells, but the mechanism of action of this protein and the specific terminology remain to be clarified.

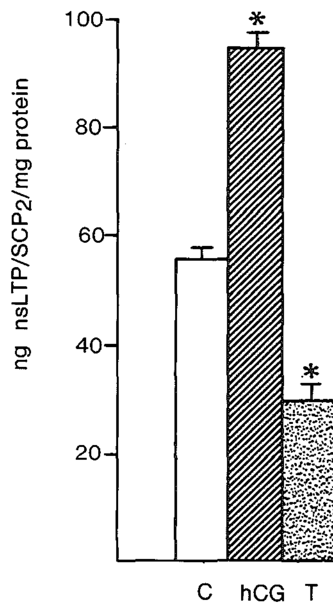


Fig.2.1. Levels of nonspecific lipid transfer protein/sterol carrier protein 2 (nsL-TP/SCP_2) in testis tissue of untreated rats (C; plasma luteinizing hormone 150 µg/l), hCG-injected rats (hCG; plasma hCG 400 µg/l) and rats treated with a high dose of testosterone (T; plasma luteinizing hormone < 25 µg/l).
*$P<0.05$ compared with control.
Values are means ± S.D. of three different animals.

CHAPTER 3

REGULATION OF STEROL CARRIER PROTEIN 2 (SCP_2) LEVELS IN THE SOLUBLE FRACTION OF RAT LEYDIG CELLS: KINETICS AND THE POSSIBLE ROLE OF CALCIUM INFLUX

The rate-determining step in steroidogenesis is the conversion of cholesterol to pregnenolone by the cholesterol side chain cleavage enzyme. The transport of substrate for this reaction may be facilitated by sterol carrier protein 2 (SCP_2). In rat testis tissue SCP_2 is specifically localized in the Leydig cells and tissue levels of SCP_2 are regulated by LH. The present study concerns short-term regulation of SCP_2 in isolated rat Leydig cells.

Levels of SCP_2 in the membrane-free supernatant are increased 2-fold already after 2 min incubation with LH and remain elevated for 24 h. The same response occurs with cells preincubated in the presence of cycloheximide for 4 h. SCP_2 levels are also 2-fold increased after incubation with dibutyryl cAMP or 4β-phorbol 12-myristate 13-acetate (PMA) while these compounds stimulate steroid production 5.5- and 2-fold respectively. LHRH, which can stimulate steroid production more than 3-fold does not influence SCP_2 levels, neither are SCP_2 levels altered when LH is added in the presence of the Ca^{2+}-channel blocker diltiazem or in the absence of extracellular Ca^{2+}. A restoration of the LH effect on SCP_2 levels was already obtained in the presence of 1 μM extracellular Ca^{2+}. These results suggest that Ca^{2+} influx through the plasma membrane may play an important role in the control of SCP_2 levels. In most of the experiments no correlation between steroid production and SCP_2 levels could be observed. The soluble amount of SCP_2 is probably not the rate-limiting factor in the control of cholesterol side chain cleavage activity but SCP_2 in the membrane-free supernatant may play a permissive role.

after: Van Noort M, Rommerts FFG, Van Amerongen A and Wirtz KWA (1988) Molec. Cell. Endocrinol. 56, 133-140.

INTRODUCTION

Steroidogenesis in Leydig cells of the testis is regulated by luteinizing hormone (LH). LH activates transducing systems which results in an increase of intracellular cyclic AMP and free calcium levels (Dufau et al., 1977; Themmen et al., 1985; Sullivan and Cooke, 1986). Steroidproduction can also be stimulated by compounds such as LHRH or the phorbol ester PMA. These activators utilize second messenger systems other than adenylate cyclase such as the phosphatidylinositol cycle and protein kinase C (Mukhopadhyay and Schumacher, 1985; Themmen et al., 1985, 1986a, 1986b; Sullivan and Cooke, 1986). The activation of these different transducing systems leads to phosphorylation and/or synthesis of specific proteins (Cooke et al., 1976; Bakker et al., 1983) and finally results in increased conversion of cholesterol into pregnenolone which is the rate-determining step of steroidogenesis (Simpson, 1979; Lambeth and Stevens, 1984-1985).

The activity of the cholesterol side chain cleavage may be controlled at different levels: (1) stimulation of the synthesis of the P_{450}-cholesterol side-chain cleavage (P-450_{scc}) enzyme (Waterman and Simpson, 1985), (2) activation of the enzyme by a steroidogenesis activator polypeptide (SAP, 2000-3000 MW) (Pedersen and Brownie, 1983, 1987), (3) activation of the enzyme by phosphorylation (Vilgrain et al., 1984) and (4) regulation of the available amount of cholesterol near the P_{450}-scc enzyme which is localized in the inner mitochondrial membrane (Privalle et al., 1983).

Studies with adrenal mitochondria have shown that sterol carrier protein 2 (SCP_2) added or in cytosol can increase the amount of cholesterol at the level of the inner membrane (Vahouny et al., 1983, 1984). SCP_2 may thus facilitate the transfer of cholesterol through aqueous compartments of the cell and also from the outer to the inner mitochondrial membrane. The amount of SCP_2 in the soluble fraction of the cell could therefore be rate-limiting for cholesterol transfer.

SCP_2, also designated nonspecific lipid transfer protein which has been purified from rat, bovine and human liver (Bloj and Zilversmit, 1977; Poorthuis et al., 1981; Trzaskos and Gaylor, 1983; Van Amerongen et al., 1987) can also catalyze the transfer of phospholipids between membranes (Bloj and Zilversmit, 1977; Poorthuis et al., 1983; Scallen et al., 1985; Megli et al.,1986). Recently we have shown that SCP_2 is specifically localized in the Leydig cells of rat testis (Van Noort et al., 1986) which is in accordance with a role in cholesterol metabolism. We have also shown that the amount of SCP_2 in soluble fractions of testicular tissue can be increased after treatment of rats with hCG for 48 h and decreased after lowering peripheral LH levels (Van Noort et al., 1986). However these in vivo studies were not suitable to study the mechanisms underlying this hormonal regulation.

In the present study we have investigated the regulation of soluble SCP_2 with particular reference to the role of Ca^{2+} and the relation with steroid production.

MATERIAL AND METHODS

Leydig cells from mature rat testis and the Leydig cell tumour H540 were isolated as described previously (Rommerts et al., 1985). Isolated cells were allowed to attach to Costar multiwell dishes in modified Eagle's medium with Earle's salts and non essential amino acids containing 100 μg streptomycin/ml, 0.6 μg fungizone/ml, 100 IU penicillin/ml and 1% (v/v) fetal calf serum (Gibco, Grand Island, NY, U.S.A.) (MEM/FCS). After 1 h incubation the floating cells were removed by washing, and the cells attached to the dishes were used for the experiments (approx. 1.5 x 10^6 Leydig cells). Incubations were carried out in 2 ml MEM/FCS at 32°C (mature Leydig cells) or 37°C (tumour Leydig cells) in air containing 5% CO_2. Inhibitors of pregnenolone metabolism, cyanoketone (5 μmol/l) and SU-10603 (19 μmol/l) were also added (Van der Vusse et al., 1974). MEM contained 1.7 mM Ca^{2+}. In some experiments modified Hepes-buffered Krebs Ringer with 0.2% (w/v) glucose, 0.1% (w/v) BSA, containing 1 μM calcium was used. The inclusion of ethanedioxy-bis-(ethylamine)-tetra-acetic acid (EGTA; 0.1 mmol/l) in the culture medium assured the virtual absence of any calcium

Ovine LH (NIH-LH-S20; 1.03 IU/mg) and [D-Ser-t-bu^6, des-Gly^{10}-NH_2] LHRH-ethylamide (HOE 766, an LHRH agonist; LHRH) were

kindly provided by the Endocrinological Study Section of the National Institute of Health (Bethesda, Maryland, U.S.A.) and Hoechst Pharma (Amsterdam, The Netherlands) respectively. Diltiazem, 4β-phorbol 12-myristate 13-acetate (PMA), 4β-phorbol 13-monoacetate (PA) (Sigma, St. Louis, MO, U.S.A.) and A23187 (Boehringer, Mannheim, F.R.G.) were used at the concentrations indicated. In some experiments pregnenolone production was measured in the presence of 22R-hydroxycholesterol (19μM). This 22R-hydroxycholesterol supported pregnenolone production reflects the endogenous $P\text{-}450_{scc}$ activity not limited by the supply of cholesterol (Brinkmann et al., 1984).

At the end of the incubations the media were collected for determination of pregnenolone by radioimmunoassay (Van der Vusse et al., 1975). For determination of SCP_2 the cells were washed 2 times with phosphate-buffered saline (PBS) containing the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF; Sigma, St. Louis, MO, U.S.A.). Cells were harvested in 500 μl PBS/PMSF, using a Costar disposable cell scraper. Harvested cells were disrupted by sonication (5 x 3 s, amplitude 16 μm, frequency 60 Hz) and a 105,000 x g supernatant fraction was prepared as described previously (Bakker et al., 1981). Levels of SCP_2 were determined in supernatant after heat treatment (5 min, 90°C) using an enzyme immunoassay (Teerlink et al., 1984). The amount of SCP_2, adsorbed to a plastic surface in the presence of a large excess of serum albumin was measured with help of a specific IgG antibody against SCP_2 from rat liver as described previously by Teerlink et al. (1984). Protein was determined according to Bradford (1976).

RESULTS

Regulation of SCP_2 levels by LH: kinetics

A maximal stimulatory dose of LH (1000 ng/ml) added to tumour Leydig cells caused a 2-fold increase in the level of SCP_2 in the soluble fraction of the cells between 1 and 2 min (Fig.3.1). This level remained the same from 2 min up to 24 h of incubation with LH. To investigate the possible role of a precursor protein in this LH-dependent process, cells were preincubated for 4 h with the protein synthesis inhibitor cycloheximide (110 μM). After removal of cycloheximide and addition of LH the changes in SCP_2 levels paralleled those in cells preincubated without cycloheximide.

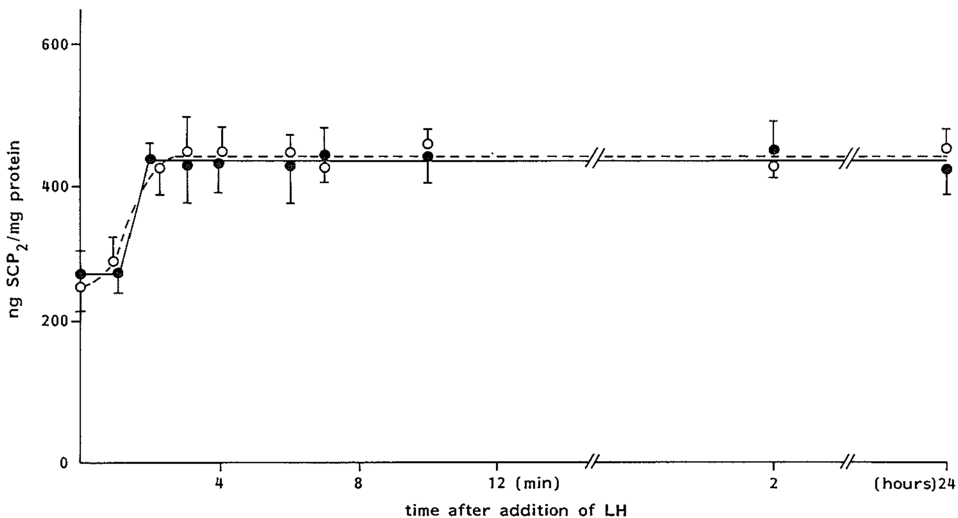


Fig.3.1. Effect of 1000 ng/ml LH on the level of sterol carrier protein 2 (SCP_2) in rat tumour Leydig cells after 4 hour preincubation with (●) or without (○) cycloheximide (110 μM).
During incubation no cycloheximide was added. Values are means ± S.D. (n=8) of 3 cell preparations.

Regulation of SCP_2 levels via different transducing systems

Since steroid production can be regulated by different pathways we have investigated how more or less selective stimulation of one of these systems influences cellular SCP_2 levels. For this purpose the effects of maximal stimulatory doses of LH (100 ng/ml for mature rat Leydig cells; or 1000 ng/ml for tumour Leydig cells), PMA (100 ng/ml), PA (100 ng/ml), dibutyryl cAMP (50 μM) or LHRH (40 nM) on steroid production and SCP_2 levels were compared. Most of the studies were carried out with tumour Leydig cells since large quantities of pure cells could

easily be obtained. Experiments were also carried out with Leydig cells isolated from mature rats. For investigation of the effects of LHRH the use of mature rat testis Leydig cells was obligatory because tumour Leydig cells have no receptors for LHRH. An incubation time of 30 min was chosen since at this time hormone effects on SCP_2 levels were steady (Fig.3.1) and effects on steroid production could also be measured accurately.

As shown in Fig.3.2, levels of SCP_2 in soluble fractions from mature rat Leydig cells were higher than in tumour Leydig cells. These SCP_2 levels were 2-fold increased after addition of LH, dibutyryl cAMP and PMA whereas LHRH and PA had no effect. There was no correlation between the effects on SCP_2 levels and steroid production. Steroid production was stimulated from 200 to 800% after addition of PMA, dibutyryl cAMP or LH (table 3.1) whereas stimulation of SCP_2 levels was always 2-fold. LHRH stimulated steroid production more than 3-fold but had no effect on SCP_2 levels. PA did not have an effect on steroid production.

Table 3.1. Effects of maximal stimulatory doses of LH, dibutyryl cAMP, PMA, PA and LHRH on steroid production by Leydig cells isolated from the H540 tumour tissue or mature rat testis.

additions	pregnenolone production (% of basal) tumour Leydig cells	 mature rat Leydig cells
LH (100-1000 ng/ml)	370 ± 170	840 ± 120
dbcAMP (500 μM)	550 ± 250	680 ± 90
PMA (100 ng/ml)	200 ± 40	260 ± 40
PA (100 ng/ml)	100 ± 10	100 ± 10
LHRH (40 nM)	N.E.	320 ± 80

Values are means ± s.d. of 4 cell preparations
N.E.: not examined

Basal pregnenolone production in tumour Leydig cells varied between 60 and 167 pmol/10^6 cells/30 min. LH-stimulated pregnenolone production varied between 179 and 645 pmol/10^6 cells/30 min. The basal pregnenolone production of Leydig cells from mature rat testis was 9 ± 3 pmol/10^6 cells/30 min.

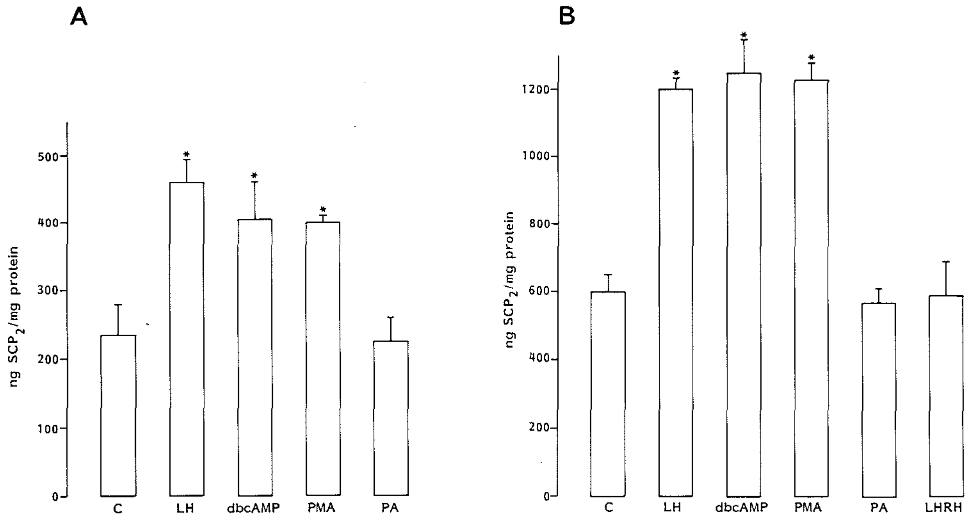


Fig.3.2. Level of sterol carrier protein 2 (SCP_2) in 105,000 x g supernatant fractions of tumour Leydig cells (A) and mature testis Leydig cells (B) after 30 min incubation with LH (1000 ng/ml (A); 100 ng/ml (B)), dbcAMP (500μM), PMA (100 ng/ml) and LHRH (40 nM (B)). Mean values ± S.D. of 8-10 observations from 3-4 cell preparations are shown. *P<0.01 when compared to controls (C).

<u>Role of calcium ions in regulation of SCP_2 levels</u>

Many hormones cause a change in intracellular calcium levels. Therefore the specific role of calcium was studied in more detail. This was carried out by artificially enhancing the intracellular free calcium concentration using the calcium ionophore A23187 (0.1 μM), blocking calcium channels with diltiazem (100 μM) or decreasing the extracellular calcium concentration.

Blocking calcium influx with diltiazem inhibited the LH-stimulated steroid production by 45% (table 3.2). Similarly incubation of cells in the absence of extracellular Ca^{2+} inhibited the LH-stimulated steroid production by 55%. This inhibition of steroid production appeared not to be caused by nonspecific inhibitory effects on mitochondria since steroid production in the presence of 22R-hydroxycholesterol was not affected by diltiazem or low extracellular calcium.

Effects on SCP_2 levels were not observed either when calcium influx was inhibited with diltiazem (Fig.3.3) or when calcium was omitted from the incubation medium (Fig.3.4). Again no correlation with steroid production could be observed since under both conditions LH stimulated steroid production although to a smaller degree. When extracellular calcium was 1 μM a 2-fold increase in SCP_2 by LH was observed (Fig.3.4). Treatment with the calcium ionophore A23187 also resulted in increased levels of SCP_2 and the amount of SCP_2 reached levels comparable to those obtained after incubation with LH (Fig.3.3).

condition	control	LH (1μg/ml)	22R-hydroxy-cholesterol (19 μM)
Ca^{2+} (0μM)	125 ± 30	307 ± 20	1288 ± 210
Ca^{2+} (1μM)	140 ± 23	613 ± 63	1647 ± 48
Ca^{2+} (1.7mM)	179 ± 39	677 ± 76	1668 ± 207
A23187 (0.1μM)	245 ± 44	609 ± 28	1567 ± 130
diltiazem (100μM)	136 ± 29	375 ± 33	1473 ± 155

Table 3.2. Pregnenolone production by 10^6 tumour Leydig cells: role of calcium
Values are means ± S.D. (n=6) of 2 different cell preparations

DISCUSSION

Regulation of SCP_2 levels in soluble fractions by compounds which stimulate steroid production was investigated in Leydig cells isolated from mature rat testis tissue and from the transplantable Leydig cell tumour H540 (Cooke et al., 1979). SCP_2 levels were 2-fold increased by LH, dibutyryl cAMP and PMA. SCP_2 levels were not changed by LHRH or when LH was added in the presence of the Ca^{2+}-channel blocker diltiazem or in the absence of extracellular Ca^{2+}. The regulation of SCP_2 levels probably occurs via Ca^{2+} and/or protein kinase C. In most of the experiments no correlation between SCP_2 levels and steroid production could be demonstrated.

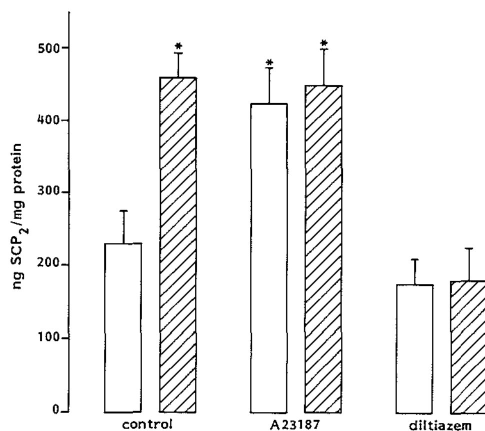


Fig.3.3. Effects of calcium ionophore A23187 (0.1 μM) and calcium channel blocker diltiazem (100 μM) on SCP_2 levels in tumour cells: basal (open bars) and LH-stimulated (1000 ng/ml: crosshatched bars); 105,000 x g supernatant fractions were isolated. Values are means ± S.D. (n=10) of 3 different cell preparations. *$P<0.01$ compared to basal levels.

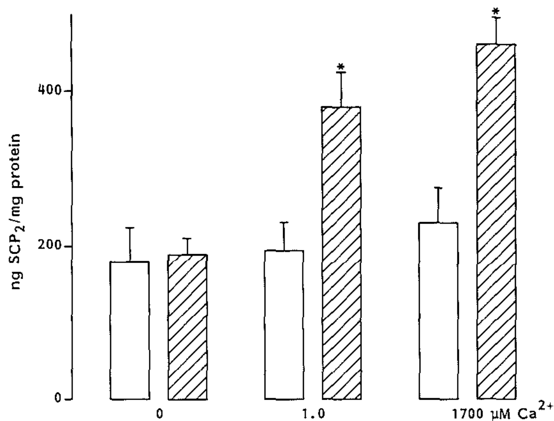


Fig.3.4. Effects of extracellular calcium concentration on SCP_2 levels in tumour cells: basal (open bars) and LH-stimulated (cross-hatched bars); 105,000 x g supernatant fractions were isolated. Cells were incubated for 30 min in MEM (1.7 mM Ca^{2+}) or Krebs Ringer EGTA (0.1 mM) with the indicated Ca^{2+} concentrations. Values are means ± S.D. (n=10) of 3 different cell preparations. *$P<0.01$ compared to basal levels.

After incubation of tumour Leydig cells with LH a 2-fold increase in SCP_2 levels was observed between 1 and 2 min which persisted for at least 24 h (Fig.3.1). The kinetics and magnitude of the effect were not influenced by preincubation of the cells with cycloheximide for 4 h. However, when the cells are preincubated and incubated in the presence of cycloheximide, LH causes

a transient increase in SCP_2. The level is increased 2- fold during the first 2 min and returns to basal levels during the next 2 min (Van Noort, et al., 1987; Van Amerongen et al., 1987). We could not find a satisfactory explanation for this transient effect of LH on SCP_2 levels in the presence of cycloheximide.

The rapid increase in the levels of SCP_2 could indicate post translational processing of a precursor for SCP_2. A high molecular weight protein 50-60 kDa, cross reacting with the antibody raised against purified rat liver SCP_2 has been described in rat liver tissue (Van der Krift et al., 1985) and has also been demonstrated in rat Leydig cells (not shown). A 14.4 kDa precursor has been described in adrenocortical cells (Trzeciak et al. 1987). Since the stimulated level of SCP_2 remained constant for 24 h of incubation, continuous posttranslational processing of a precursor for SCP_2 should be accompanied by continuous breakdown of SCP_2. This seems unlikely since Trzeciak et al. (1987) reported a half life of 32 h for SCP_2 (12.3 kDa) in adrenocortical cells and could not demonstrate a 14.4 kDa precursor in intact cells. Moreover the 50-60 kDa precursor seems neither unstable, since kinetics and the amount of SCP_2 were not influenced by preincubation with cycloheximide. The observed changes in soluble SCP_2 levels under influence of LH might therefore be due to an alteration in the intracellular distribution of the protein although it is difficult to envisage how cycloheximide can effect SCP_2 levels in this way. The amount of SCP_2 was determined in a 105,000 x g supernatant fraction of cells after heating. It was shown earlier that the high molecular weight protein (50-60 kDa) crossreacting with the antibody but not any SCP_2 is lost during this heating step (Teerlink et al., 1984). Using this procedure SCP_2-levels could be measured accurately in soluble fractions. However, due to these necessary purification steps nothing is known about the total cellular SCP_2 levels. In addition to the soluble fraction SCP_2 is also associated with other subcellular fractions (Chanderbahn et al., 1986; Van der Krift et al., 1984). It is unknown how much SCP_2 is

present in membraneous fractions. However, measurement of SCP_2 in these fractions requires a new assay procedure, since membranes interfere in the determination procedure used.

Whereas addition of LH to cells cultured in MEM/FCS resulted in a 2-fold increase in soluble SCP_2 levels, no regulation by LH occurred either when the Ca^{2+}-channel blocker diltiazem was present, or when extracellular calcium was absent from the incubation media. Incubation with the calcium ionophore A23187 resulted in an increased amount of SCP_2 comparable with that obtained by LH. These results indicate that calcium influx in the Leydig cells may play an important role in the control of SCP_2 levels. Calcium might exert its effect by interaction with calcium receptor proteins such as calmodulin, parvalbumin and troponin C (Heizman et al., 1986) thereby modulating the cytoskeleton of the cell leading to a modification in the intracellular distribution of SCP_2. The amount of SCP_2 was increased by LH, dibutyryl cyclic AMP and PMA but not changed by LHRH. Both LH and dibutyryl cAMP have been described to stimulate calcium channels whereas LHRH may stimulate intracellular calcium concentrations not via influx through the plasma membrane but by mobilization of calcium from intracellular stores (Themmen et al., 1986; Sullivan and Cooke 1986). PMA could regulate SCP_2 levels via protein kinase C.

A correlation between SCP_2 levels in the membrane-free supernatant and steroid production was not always observed. SCP_2 levels were 2-fold increased by LH, and dibutyryl cyclic AMP, but the steroidogenic response to these effectors was always more than this. LHRH, which stimulated steroid production more than 3-fold did not increase SCP_2 levels. LH stimulated steroid production in the presence of diltiazem or absence of extracellular calcium at least 1.5-fold, under these conditions SCP_2 levels were not increased. A relationship between SCP_2 levels and cholesterol metabolism could neither be demonstrated in liver tissue (Van Heusden et al., 1985). The rate-determining step in steroidogenesis may therefore be regulated by other factors such

as the cytoskeleton of the cell (Hall et al.1981), phosphorylation (Vilgrain et al. 1984) and/or synthesis of P-450_{scc} (Waterman and Simpson 1985), SAP (Pedersen and Brownie 1983, 1987) and 25-27000 MW proteins (Pon et al. 1986), although from our results we can not exclude a regulation by SCP_2 attached to membranous structures. The soluble amount of SCP_2 seems not to be rate-limiting for steroidogenesis. However when the amount of SCP_2 is decreased by fusion of adrenocortical cells with liposomes containing anti-SCP_2 antibody, ACTH-stimulated steroidogenesis is reduced (Chanderbhan et al., 1986). Moreover, the extremely low levels of SCP_2 in livers of infants with cerebro-hepato-renal (Zellweger) syndrome may contribute to hepatic dysfunction in various aspects of cholesterol metabolism (Van Amerongen et al., 1987).

Therefore, we suggest that SCP_2 in the membrane-free supernatant may be permissive for cholesterol metabolism in various cells. The level of SCP_2 in the membrane-free supernatant may be controlled by different hormones or factors which control Ca^{2+} fluxes or by factors which control protein kinase C activity in the cell. The molecular mechanisms for this proposed permissive action of SCP_2 are totally unknown and remain to be investigated.

CHAPTER 4

INTRACELLULAR REDISTRIBUTION OF SCP_2 IN LEYDIG CELLS AFTER HORMONAL STIMULATION MAY CONTRIBUTE TO INCREASED PREGNENOLONE PRODUCTION

Sterol carrier protein 2 (SCP_2) also designated nonspecific lipid transfer protein (nsL-TP), added to tumour Leydig cell mitochondria as a pure compound or in cytosolic preparations, stimulates pregnenolone production 2-3-fold. This stimulation can be abolished by addition of anti rat SCP_2 but not by preimmune IgG-antibodies. SCP_2- levels in the cytosol are increased in less than two min after addition of lutropin (LH). This increased SCP_2 level may contribute to stimulation of steroid production in intact cells. After hormonal stimulation the subcellular distribution of SCP_2 changes. A 2-fold increase of SCP_2- levels in the supernatant fraction and 4-fold decrease in extracts of the particulate fraction was observed 30 min after stimulation of tumour Leydig cells with LH and subsequent fractionation. This apparent shift of SCP_2 can be explained by an altered association with membranes or a true relocation of the protein from the particulate to the supernatant fractions under the influence of the hormone.

after: Van Noort M, Rommerts FFG, Van Amerongen A and Wirtz KWA (1988) Biochem. Biophys. Res. Commun. 154, 60-65.

INTRODUCTION

Sterol carrier protein 2 (SCP_2), also designated nonspecific lipid transfer protein (nsL-TP), accelerates the transfer of both phospholipid and cholesterol between membranes (Bloj and Zilversmit, 1977; Scallen et al., 1985; Van Amerongen et al., 1985). Addition of SCP_2 to isolated mitochondria of adrenocortical cells results in an enhanced conversion of cholesterol into pregnenolone (Chanderbhan et al., 1982). In the rat testis SCP_2 is specifically localized in the steroidogenic active Leydig cells (Van Noort et al., 1986). The amount of SCP_2 in soluble fractions of Leydig cells and in adrenocortical cells is regulated by hormones (Van Noort et al., 1986, 1988a; Trzeciak et al., 1987). This suggests that SCP_2 is one of the factors involved in the hormonal control of steroidogenesis. After addition of LH to rat tumour Leydig cells the level of SCP_2 in the cytosol is increased 2-fold within 2 min and this 2-fold higher level is maintained for at least 24 h (Van Noort et al., 1988a). This rapid increase in the level of SCP_2 in the supernatant could result from a hormone dependent post-translational processing of a precursor for SCP_2 (Trzeciak et al., 1987; Van der Krift et al., 1985; Van Amerongen et al., 1987). However maintenance of a constant 2-fold higher SCP_2 level for 24 h after the very rapid increase while the reported half life of SCP_2 is 32 h (Trzeciak et al., 1987), strongly indicates that other processes are involved. To investigate if SCP_2 is functionally active in Leydig cells and how the intracellular level may be regulated by hormones , we have determined whether SCP_2 can stimulate steroid production in isolated mitochondria and how SCP_2 is distributed between the soluble and the particulate fraction.

MATERIALS AND METHODS

Leydig cells from the Leydig cell tumour H540 were isolated as described previously (Rommerts et al., 1985). After isolation cells were incubated for 30 min with or without lutropin (LH,1000 ng/ml) in a shaking waterbath at 37°C in modified Eagle's medium with Earle's salts and non essential amino acids containing 100 μg streptomycin/ml, 0.6 μg fungizone/ml and 100 IU penicillin/ml. Subcellular fractions were prepared and treated as described previously (Van Amerongen et al., 1989). In this procedure fractions are extracted with high salt to remove SCP_2 from membranes. The amount of SCP_2 released is measured using an enzyme immunoassay (EIA) (Van Amerongen et al., 1989; Megli et al., 1986). Protein was determined according to Bradford (Bradford, 1976).
For isolation of mitochondria $150x10^6$ tumour Leydig cells were suspended in 7 ml buffer containing 10 mM Tris-Cl pH 7.4, 1 mM EDTA and 250 mM D-mannitol (Bakker et al., 1983; Whips and Halestrap, 1984) and homogenized using a Dounce-Wheaton glass homogenizer (clearance 0.025-0.03 mm, 10 strokes). After differential centrifugation mitochondria were suspended in buffer containing 125 mM sucrose, 25 mM Tris-Cl (pH 7.4), 5 mM $MgCl_2$, 60 mM KCl to a final concentration of 1 mg protein/ml.
Incubation was started by addition of 100 μl of the mitochondrial suspension to 400 μl buffer at 37°C, containing 5 mM DL-isocitrate. Incubation was stopped after 10 min by cooling on ice and by addition of 2 volumes ethylacetate (p.a.). Pregnenolone production was determined as described previously (Van der Vusse et al., 1974). Where indicated cytosolic preparations were preincubated with anti rat SCP_2 (50 μg) (Teerlink et al., 1984) or preimmune IgG antibody (150 μg) in a total volume of 200 μl for 18 h at 4°C. The mixtures were centrifuged at 10,000 x g for 20 min and aliquots of the supernatant were used for experiments.

RESULTS

The effect of SCP_2, pure or in cytosolic preparations, on pregnenolone synthesis by mitochondria isolated from tumour Leydig cells was investigated first. Addition of SCP_2 (0.7 μM) to a suspension of mitochondria from tumour Leydig cells resulted in a 2-fold stimulation of pregnenolone production from endogenous cholesterol (Table 4.1). The steroidogenic activity increased 1.5- to 3-fold depending on SCP_2-concentrations between 10^{-7} M and 10^{-5} M (data not shown). The stimulatory effect of SCP_2 was neutralized by pretreatment with anti rat SCP_2 IgG (anti-SCP_2).

The addition of this antibody to the mitochondria also inhibited pregnenolone production within the incubation period of 10 min. Addition of cytosolic preparations from Leydig tumour cells incubated with LH also stimulated the pregnenolone production 2-fold. This stimulatory effect was abolished by pretreatment with anti-SCP_2 whereas pretreatment with rat serum IgG (IgG) had no effect.

Table 4.1. Steroid production by isolated mitochondria.

Additions to mitochondria	pregnenolone/ 100 μg protein/min (pmol)
none	22.4 ± 2.7
cytosol	36.8 ± 1.4
cytosol + IgG	38.4 ± 1.4
cytosol + anti-SCP_2	21.6 ± 1.1
SCP_2	39.6 ± 1.6
SCP_2 + IgG	40.3 ± 2.3
SCP_2 + anti-SCP_2	12.8 ± 0.8
IgG	20.3 ± 1.6
anti-SCP_2	15.0 ± 2.1

Results are mean ± SD (6) of 3 different mitochondrial preparations. For further details see Methods.

Hormonal regulation of intracellular SCP_2 levels was studied in intact rat tumour (H540) Leydig cells (Cooke et al., 1979) . After incubation for 30 min, 1000 ng/ml LH stimulated pregnenolone production approximately 3-fold (basal: 90 ± 13 pmol/10^6 cells/30 min; stimulated: 275 ± 43 pmol/10^6 cells/30 min; mean ± SD, n=4). The subcellular distribution of SCP_2 in rat tumour Leydig cells was studied after fractionation of homogenates of cells by differential centrifugation in a 105,000 x g particulate membranous fraction (P) and a membrane-free supernatant (S) fraction. After exposure of cells to LH the amount of SCP_2 was decreased 4-fold in extracts of the particulate fraction (P) and increased 2-fold in the membrane-free supernatant fractions (S) (Fig.4.1). After incubation of cells the SCP_2 levels in the

cytosol from control cells : 238 ± 51 mg 105,000 x g protein and from cells incubated with LH : 406 ± 41 ng/mg 105,000 x g protein (mean ± SD, n=4), were similar to those earlier reported by us (Van Noort et al., 1986; Van Noort et al., 1988). The total cellular amount of protein and SCP_2 in extracts were not affected by hormonal stimulation.

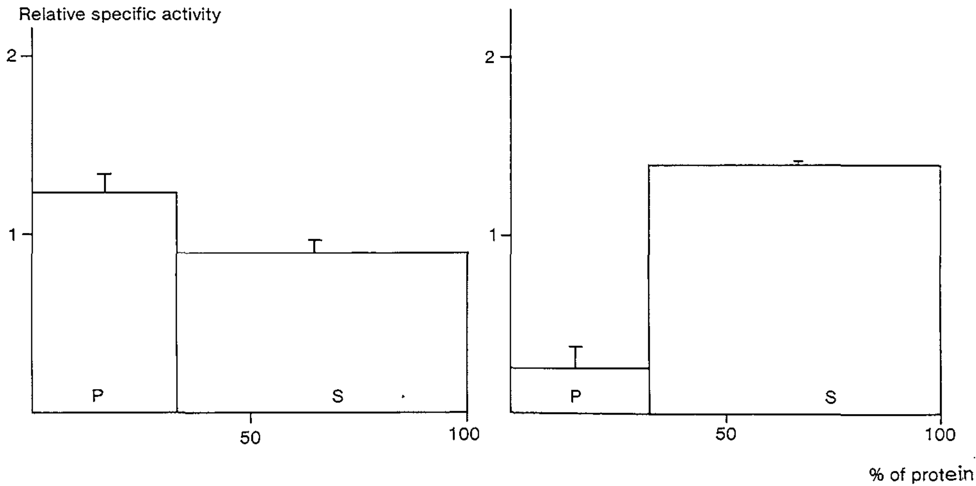


Fig.4.1. Effect of LH on the distribution of SCP_2 between the 105,000 x g particulate (P) and supernatant (S) fractions of isolated rat tumour cells. Cells were incubated for 30 min without LH (left panel) or with 1000 ng/ml LH (right panel). The total amount of SCP_2 was not changed after hormonal stimulation.

DISCUSSION

Sterol carrier protein 2 (SCP_2) also designated nonspecific lipid transfer protein (nsL-TP), has been purified from rat, bovine and human liver (Bloj and Zilversmit, 1977; Van Amerongen et al., 1987; Crain and Zilversmit, 1980; Noland et al., 1980; Poorthuis and Wirtz., 1983; Trzaskos and Gaylor, 1983). In vitro the protein stimulates the transfer and biosynthesis of cholesterol (Bloj and Zilversmit, 1977; Scallen et al., 1985) and accelerates the transfer of both phospholipid and cholesterol between membranes.

When added to mitochondria from adrenocortical cells (Chanderbhan et al., 1982) and tumour Leydig cells (Table 4.1), SCP_2 enhanced the conversion of cholesterol into pregnenolone. Antibodies to SCP_2 blocked the stimulatory effect of both pure rat liver SCP_2 and SCP_2 in cytosolic preparations. Anti-SCP_2 could even lower the basal production of pregnenolone in isolated mitochondria from Leydig cells (Table 4.1), presumably by binding of the antibody to SCP_2 located at the outer mitochondrial membrane. The basal production of pregnenolone production was not lowered when an antibody to SCP_2 was added together with cytosolic preparations. This may be caused by the presence of other stimulatory proteins or peptides in the cytosolic preparations. A 2 kDa peptide, sterol activator protein (SAP), has been isolated and this peptide can stimulate steroid production in isolated mitochondria from Leydig and adrenocortical cells (Pedersen and Brownie, 1983, 1987).

The rapid increase in SCP_2-levels in the cytosol after exposure of cells to LH may contribute to the stimulation of steroid production in intact cells because in isolated mitochondria, pregnenolone production can be regulated by SCP_2. Also, fusion of adrenocortical cells with liposomes containing anti-SCP_2 antibody reduced ACTH stimulated steroidogenesis (Chanderbhan et al., 1986). It is unlikely that SCP_2 is the labile intracellular protein postulated to mediate hormone action (Cooke et al., 1979;

Bakker et al., 1985) since the half life of this protein in adrenal cells is 32 h (Trzeciak et al., 1987). The half life in Leydig cells seems to be high also since pretreatment of cells with cycloheximide for 18 h did not significantly lower the amount of SCP_2 detected in the supernatant fraction of the cells (Van Noort et al., 1988a). In addition, it seems unlikely that the rapid 2-fold increase in the level of soluble SCP_2 between 1 and 2 min and the maintenance of this level for at least 24 h results from de novo synthesis or precursor proteolysis (Van Noort et al., 1988a). Since the half time of SCP_2 is 32 h (Trzeciak et al., 1987) this rapid increase can only be explained by assuming that 200 ng SCP_2/mg protein/min is produced within 2 min by fast processing of a precursor, immediately followed by a 1000-fold decrease in the production rate. Our data on SCP_2-levels in fractions of tumour Leydig cells after incubation with LH show that the rise of SCP_2 in supernatant fractions can better be explained by a relocation of extractable (loosely bound) protein from the particulate to the supernatant fractions (Fig.4.1). It is not certain if hormonal stimulation results in true redistribution of SCP_2 or in diminished binding of the protein to membranes to such extent that it can be released during homogenization. Whatever explanation is correct, the data show that LH can modify the interaction between SCP_2 and membranes. SCP_2 bound to rat liver mitochondria could be removed by washing with a high ionic strength buffer (Megli et al., 1986). This suggests that the interaction between SCP_2 and membranes is of an electrostatic nature. We have observed earlier that calcium- ions are important for increasing SCP_2-levels in Leydig cell supernatant fractions (Van Noort et al., 1988a). Rapid changes in the cellular Ca^{2+} concentrations that occur in Leydig cells after incubation with LH (Sullivan and Cooke, 1986) could play an important role in regulating the interaction between SCP_2 and membranes.

Hormone effects on subcellular redistribution of other proteins also have been demonstrated. For instance phorbol esters cause redistribution of protein kinase C (Nikula and Huhtaniemi, 1988; Munari-Silem et al., 1987) and it has been shown recently that translocation of this protein is coupled with activation (Munari-Silem et al., 1987). Hormones may thus influence the subcellular redistribution of various proteins or aggregates of proteins such as multi-enzyme complexes. More attention should be paid to this "topodynamic regulation" of proteins when studying mechanisms for regulation of cell function (Kaprelyants, 1988).

CHAPTER 5

THE LH INDUCED INCREASE OF SCP_2 IN THE MEMBRANE-FREE SUPERNATANT OF LEYDIG CELLS MAY REFLECT CHANGED INTERACTIONS WITH MEMBRANES

The distribution of sterol carrier protein 2 over the various subcellular fractions of Leydig cells was studied by enzyme immunoassay and immunocytochemistry. Using the enzyme immunoassay it was shown that the distribution of SCP_2 changes after incubation with lutropin (LH). After incubation of cells with LH, less SCP_2 was present in membranous fractions, including the mitochondrial fraction, and the amount of SCP_2 was increased in the membrane-free supernatant. In the lysosomal fraction SCP_2 levels remained constant. Immunocytochemical data showed co-localization of SCP_2 with the peroxisomal marker enzyme catalase.

INTRODUCTION

Sterol carrier protein 2 (SCP_2) also designated nonspecific lipid transfer protein (nsL-TP), has been purified from rat, bovine and human liver (Bloj and Zilversmit, 1977; Crain and Zilversmit, 1980; Noland et al., 1980; Poorthuis and Wirtz, 1983; Van Amerongen et al., 1987; Traszkos and Gaylor, 1983). The protein stimulates the biosynthesis of cholesterol in isolated cells and accelerates the transfer of both phospholipids and cholesterol between synthetic lipid membranes (Crain and Zilversmit, 1980; Scallen et al., 1985).

Addition of SCP_2 to mitochondria of Leydig and adrenocortical cells results in an enhanced production of pregnenolone (Van Noort et al., 1988b; Chanderbhan et al., 1982). Levels of SCP_2 in the soluble fraction of Leydig cells and in adrenocortical cells are hormonally controlled (Van Noort et al., 1986, 1988a; Trzeciak et al., 1987). This correlation suggests that SCP_2 is involved in regulation of steroid production. Support for this hypothesis is derived from the observation that fusion of adrenocortical cells with liposomes containing anti-SCP_2 antibody reduced ACTH-stimulated steroidogenesis (Vahouny et al.., 1983). Therefore, it has been proposed that SCP_2 binds and transports cholesterol from intracellular sources to the CSCC enzyme in the mitochondria, this transfer being rate-limiting for steroidogenesis. A correlation between the level of SCP_2 and the active metabolism of cholesterol in mitochondria of adrenal tissue has been postulated since the mitochondrial fraction from the rat adrenal gland contained about half of the total tissue SCP_2 (Chanderbhan et al., 1986). These observations suggest that SCP_2 may play a major role in the intra-mitochondrial transport of cholesterol.

In contrast with this observation Van Amerongen et al. (1989) reported recently that in adrenal tissue only 11% of total tissue SCP_2 is localized in the mitochondrial fraction. In this study the mitochondrial fraction appeared to contain the lowest levels

of SCP_2 of all the membrane fractions isolated and this does not correlate with active metabolism of cholesterol in steroid hormones.

In the Leydig cell we could not find a strict correlation between the rate of steroid production and SCP_2 levels in the soluble fraction (Van Noort et al., 1988a). We have therefore postulated that in the control of the activity of the CSCC enzyme the amount of SCP_2 in the membrane-free supernatant is probably not rate-limiting but that the presence of SCP_2 is obligatory and that the activity of SCP_2 may be a rate-limiting factor.

Moreover, changes in cytosolic SCP_2 levels appear to result from a changed distribution of SCP_2 over the membranous and soluble fractions under the influence of LH (Van Noort et al., 1988b) and these changes may be connected with the cholesterol transfer activities of SCP_2.

In this study the detailed subcellular distribution of SCP_2 in the rat tumour Leydig cell and the effects of LH on this distribution have been investigated in order to establish which changes in the subcellular distribution of SCP_2 may contribute most to regulation of steroidogenesis using an enzyme immunoassay on subcellular fractions of homogenized cells and enzyme immunocytochemistry on fixed cells.

MATERIALS AND METHODS

Preparation and characterization of subcellular fractions

Leydig cells were isolated from the Leydig cell tumour H540 as described previously (Rommerts et al., 1985). After isolation 10^8 cells were incubated for 30 min with or without 1 μg/ml ovine LH (NIH-LH-S22; 1.03 IU/mg). Incubations were carried out in a shaking waterbath at 37°C in 10 ml Eagle's medium with Earle's salts and nonessential amino acids containing 100 μg streptomycin/ml, 0.6 μg fungizone/ml and 100 IU penicillin/ml.

Cells were collected by centrifugation (150 x g), washed and suspended in 7 ml buffer containing 10 mM Tris-Cl pH 7.4, 1 mM EDTA and 250 mM D-mannitol. Cells were homogenized using a Dounce-Wheaton glass homogenizer (clearance 0.025-0.03 mm, 10 strokes). Subcellular fractions were prepared by differential

centrifugation and treated as described previously (Van Amerongen et al., 1989). In this procedure fractions are extracted with salt to remove SCP_2 from membranes (Megli et al., 1986). The amount of SCP_2 released was measured using an enzyme immunoassay (EIA; Van Amerongen et al., 1989; Teerlink et al., 1984). Protein was determined according to Bradford (1976) using bSA as a standard.

The following marker enzymes were used to characterize the purity of the subcellular fractions. Alkaline phosphatase was used as a plasma membrane marker (Evans, 1979); succinate dehydrogenase as a mitochondrial marker (Weiner, 1970); catalase as a peroxisomal marker (Holmes and Masters, 1970); β-N-acetyl-glucosaminidase as a lysosomal (Leaback and Walker, 1961) and NADH-cytochrome c reductase activity as a microsomal marker (Weiner, 1970).

Immunocytochemistry

After isolation, tumour Leydig cells were allowed to attach to culture dishes in MEM containing 1% (v/v) fetal calf serum (Gibco, Grand Island, NY, U.S.A.). After incubation for 1 hour the floating cells were removed by washing and the cells attached to the dishes were fixed. Fixation was performed during 1 hour using 0.5% glutaraldehyde, 2% paraformaldehyde and 1% acrolein in 0.1 M Na-cacodylate, pH 7.2. The fixed cells were washed 3 times 10 min with 0.1 M Na-cacodylate and harvested using a Costar disposable cell scraper. Gelatin blocks and sections were prepared according to Posthuma et al. (1987). We used only one layer of gelatin. Sections were incubated with a purified (Teerlink et al., 1984) anti-SCP_2 antibody solution (1:200) and for visualization with 100 nm gold particles conjugated to protein A. For double immunolabelling (Geuze et al., 1983), sections were also incubated with a catalase antibody (generous gift of dr. G.Posthuma, Dept. Cell Biology, Univ. Utrecht) and visualized using 10 nm gold particles conjugated to protein A.

RESULTS

Enzyme immunoassay

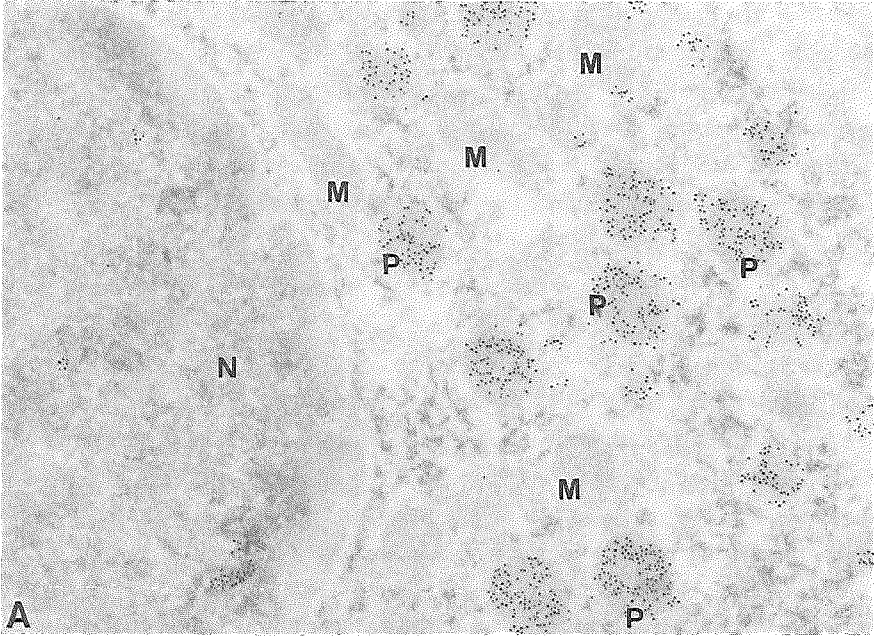
The subcellular distribution of SCP_2 was determined in rat tumour Leydig cells which were incubated in the presence or absence of LH. Homogenates of cells were fractionated by differential centrifugation in a nuclear (N), a mitochondrial (M), a lysosomal (L), a microsomal (P) and a membrane-free supernatant (S). The distribution of marker enzymes over the different subcellular fractions does not change after incubation with LH

(Fig.5.2). In contrast, the distribution of SCP_2 is changed after incubation with LH. The amount of SCP_2 in the 105,000 x g supernatant fraction is increased under the influence of LH, whereas the amount of SCP_2 in the mitochondrial and microsomal fractions is decreased under the influence of LH. The amount of SCP_2 in the lysosomal fraction is independent of LH.

Immunocytochemistry

Determination of the amount of SCP_2 in the different subcellular fractions by an enzyme immunoassay requires disruption of the cells. To determine the localization in intact cells, we have used immunocytochemistry. Fixed rat Leydig cells were incubated with antibodies for SCP_2 and the peroxisomal marker enzyme catalase. The electron micrograph (Fig.5.1) shows clearly that the vast majority of gold particles visualizing SCP_2 co-localize with the particles visualizing catalase. This indicates localization of SCP_2 in peroxisomes. Some of the gold particles visualizing SCP_2, were found in the mitochondria.

Fig.5.1. Distribution of gold particles with a diameter of 100 nm, visualizing SCP_2, and 10 nm, visualizing catalase, in mature rat testis (A) and in Leydig cells isolated from mature rats (B). Magnifications 80,000 x (A) and 25,000 x (B). Abbreviations used: P=peroxisome; M=mitochondrion; N=nucleus. →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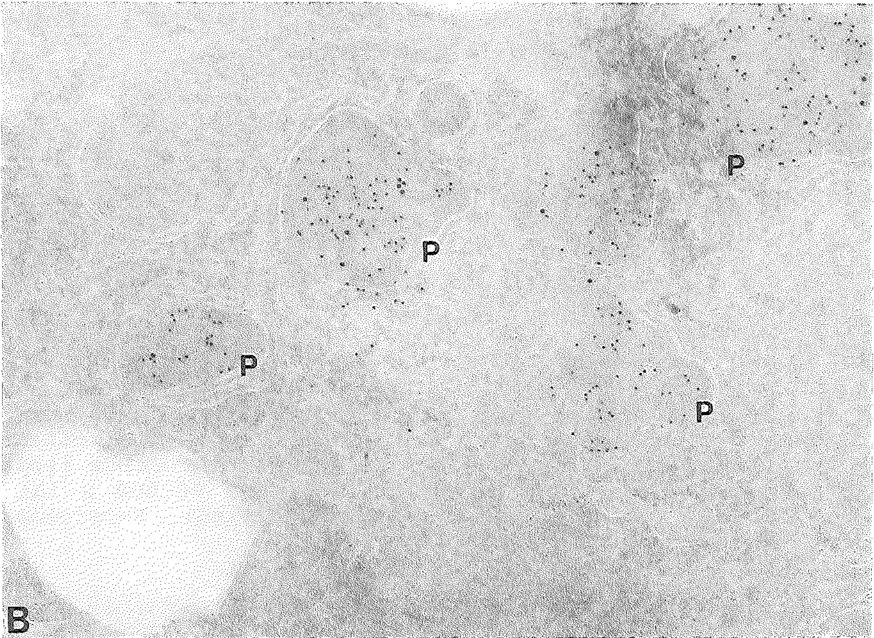
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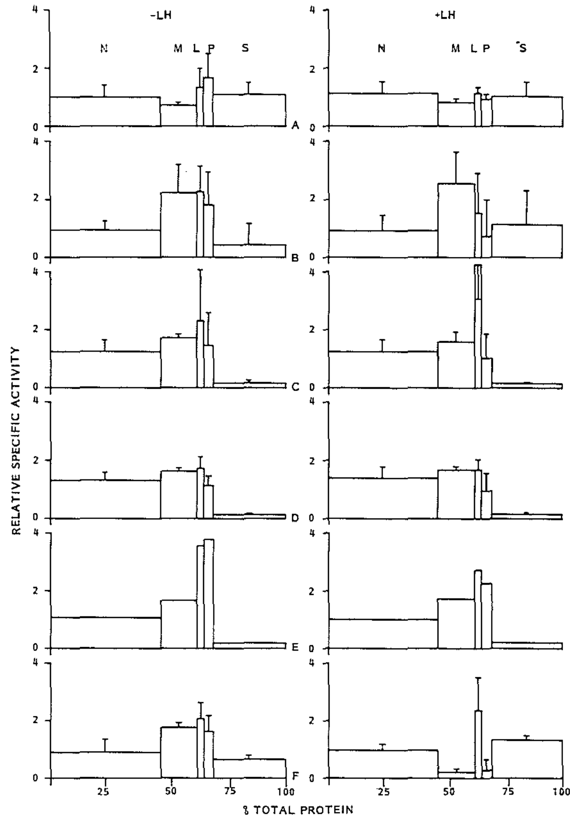


Fig.5.2. Subcellular distribution of alkaline phosphatase (panel A), succinate dehydrogenase (panel B), catalase (panel C), β-N- acetylglucosaminidase (panel D), NADH-cytochrome C reductase (panel E) and sterol carrier protein 2 (SCP_2) (panel F) in rat tumour Leydig cells incubated for 30 min. without (left panels) or with LH (1000 ng/ml; right panels). N=nuclear (1000 x g pellet), M=mitochondrial (10,000 x g pellet), L=lysosomal (20,000 x g pellet), P=microsomal (105,000 x g pellet), S=supernatant (105,000 x g supernatant) fraction.

DISCUSSION

In the present study we have determined the amount of SCP_2 in various subcellular fractions of rat tumour Leydig cells. According to the immunocytochemical data, the amount of SCP_2 localized in the mitochondria is very small. Also, the specificity of the labelling of mitochondria is doubtful because of the low dilution of antibody used in the experiment. This observation is surprising since the mitochondria are the proposed site of action of SCP_2 in steroidogenic cells (Vahouny et al., 1983, 1984; Van Noort et al., 1988b).

The results of the present study show that in intact fixed Leydig cells antibodies raised against SCP_2 associate mainly with protein(s) present in the peroxisomes. This has been observed earlier in liver and adrenal tissue (Van der Krift et al., 1985; Keller et al., 1987; Krisans et al., 1987; Tsuneoka et al., 1988). Unfortunately, there is no consensus in the literature on the nature of the peroxisomal "SCP_2" protein that is detected in the immunocytochemical experiments. Localization of SCP_2 with a molecular mass of approximately 14 kDa in the matrix of peroxisomes of liver tissue has been reported recently (Tsuneoka et al., 1988). However, analysis of isolated peroxisomes by other investigators has indicated that SCP_2 is absent from this organelle (Van der Krift et al., 1985) or present in very low amounts (Krisans et al., 1987; Van Amerongen et al., 1987; Keller et al., 1987). A protein of higher molecular mass (58 kDa; Van der Krift et al., 1985; Van Amerongen et al., 1987), which is also present in Leydig cells (not shown), might be responsible for the immunological response. It has been suggested that aggregation of SCP_2 leads to the formation of the 58 kDa protein (Chanderbhan et al., 1986), but a relationship between SCP_2 and the 58 kDa protein has as yet not been shown. Thusfar the possibility that an amino acid sequence in a protein which is not related to SCP_2 is crossreacting with the antibody raised against SCP_2 can not be ruled out also.

Since the nature of the protein that is detected using immunocytochemistry is not known, we also determined the subcellular distribution of 14 kDa SCP_2 using a specific enzyme immunoassay (Teerlink et al., 1984) after homogenization and subcellular fractionation of Leydig cells.
In contrast with the immunocytochemical data it is shown that most SCP_2 is present in other subcellular fractions than the peroxisomal fraction. Moreover, while the immunocytochemical data for liver, adrenal and Leydig cells all show the same cellular localization for SCP_2, the subcellular distribution of SCP_2 after homogenization of cells and measurement by an enzyme immunoassay appears to be tissue specific (Van Amerongen et al., 1989; this study). In liver tissue the bulk (66%) of SCP_2 is present in the cytosol, whereas in the adrenal gland the supernatant contains 19% of total tissue SCP_2. The amount of SCP_2 in the cytosol of Leydig cells depends on hormonal stimulation and varies between 25%, in cells not incubated with LH, to 40% in cells incubated with LH. Several attempts have been made to explain the discrepancy between the localization of SCP_2 observed after immunocytochemistry on fixed cells and enzyme immunoassay on cellular fractions after disruption of cells. One possibility is that during homogenization of cells, SCP_2 is released from peroxisomes and subsequently becomes associated with different membranes depending on their lipid and protein composition. This could then result in a tissue specific distribution of SCP_2. However, if this is indeed the case, it is difficult to envisage how the distribution of SCP_2 is altered within 2 min of incubation with LH (Van Noort et al., 1988a).
It may be possible that the predominant peroxisomal localization of SCP_2 which we observed using immunocytochemistry resulted from the loss of antigenicity of SCP_2 in other subcellular fractions embedded in the sections (Van Amerongen et al., 1989). Thusfar the relationship between the peroxisomal protein and SCP_2 in other subcellular fractions has not been demonstrated in Leydig cells and other cells.

Since addition of 14 kDa SCP_2 to mitochondria of the Leydig cell leads to an increased mitochondrial pregnenolone production, we have concentrated on measuring this protein using a specific enzyme immunoassay. In previous experiments we have observed a rise of SCP_2 in supernatant fractions after hormonal stimulation (Van Noort et al., 1986, 1988a). Earlier this rise was explained by a relocation of extractable (loosely bound) SCP_2 from the particulate to the supernatant fractions (Van Noort et al., 1988b). The present experiments indicate that all membranous fractions except the lysosomal fraction contribute to this relocation (Fig.5.2).

It is not clear whether hormonal stimulation results in true redistribution of SCP_2 or in diminished binding of the protein to membranes to such extent that it can be released during homogenization. SCP_2 bound to rat liver mitochondria could be removed by washing with a high ionic strength buffer (Megli et al., 1986), which suggests that the interaction between SCP_2 and membranes is of an electrostatic nature. As described earlier calcium- ions are important for increasing SCP_2-levels in Leydig cell supernatant fractions (Van Noort et al., 1988a). Hence, rapid changes in the cellular Ca^{2+} concentrations that occur in Leydig cells after incubation with LH (Sullivan and Cooke, 1986) might play an important role in regulating the interaction between SCP_2 and membranes. After hormonal stimulation, the amount of SCP_2 in the mitochondrial fraction is low. This apparent release of SCP_2 from mitochondrial membranes after incubation of cells with LH, might reflect a weakened interaction of SCP_2 with membranes. This may be related to the possible function of SCP_2 to transfer cholesterol from intracellular stores to the mitochondria which is in accordance with the observed capacity of SCP_2 to stimulate cholesterol metabolism in isolated mitochondria of steroidogenic cells (Vahouny et al., 1983, 1984; Van Noort et al., 1988b).

CHAPTER 6

THE INTRACELLULAR DISTRIBUTION OF SCP_2 IN TWO SUBCLONES OF THE MA-10 MOUSE LEYDIG TUMOR CELL LINE AND THE CORRELATION WITH STEROID PRODUCTION

Cells of a subclone of the MA-10 mouse Leydig tumor cell line (clone LP) produce an abnormally low amount of steroids after hormonal stimulation (8 ng progesterone/10^6 cells/h.) compared to the parent MA-10 cells. This is probably due to a defect in the hormonal regulation of the CSCC enzyme complex (CSCC). Sterol carrier protein 2 (SCP_2) may be important in regulating the availability of cholesterol at the level of CSCC. Alterations in the intracellular distribution of SCP_2 may be correlated with the cholesterol transfer activity of SCP_2. In the present study it was tested whether the distribution of SCP_2 in clone LP was different from that in clone HP, a subclone of which steroid production is increased 80-fold in the presence of hCG (from 4.6 to 370 ng progesterone/10^6 cells/h.). An enzyme immunoassay was used to compare the intracellular distribution of SCP_2 over membranes (12,000 x g pellet) and cytosol (12,000 x g supernatant) in both clones. In clone HP, SCP_2 was present both in the 12,000 x g supernatant (150 ng/mg protein) and pellet fraction (120 ng/mg protein). After incubation with hCG (100 ng/ml, 3 h), the amount of SCP_2 was increased to 240 ng/mg protein in the supernatant fraction and decreased to 90 ng/mg protein in the pellet fraction. However in cells of clone LP, SCP_2 could not be detected in the pellet fraction of the cells incubated in the presence or absence of hCG. This impaired association of SCP_2 with membranes could indicate an abnormality in SCP_2 or an abnormality in the membranes and this may represent (part of) the lesion in steroid production in clone MA-10 LP.

INTRODUCTION

The rate-determining step in the biosynthesis of steroids in endocrine tissues is the side chain cleavage of cholesterol to form pregnenolone (Stone and Hechter, 1954; for review see:Simpson, 1979, Lambeth and Stevens 1984/1985). This reaction is catalyzed by the CSCC enzyme complex. The activity of this complex can be regulated by trophic hormones in all steroidogenic tissues. In the Leydig cell of the testis, lutropin (LH) or human chorionic gonadotrophin (hCG) regulate steroidogenesis through activation of different transducing systems e.g. intracellular cyclic AMP and free calcium (Themmen et al., 1985; Sullivan and Cooke, 1986). The activation of these different transducing systems leads to phosphorylation and/or synthesis of specific proteins (Cooke et al., 1976; Bakker et al., 1983) which in turn may activate the CSCC enzyme complex directly or regulate the available amount of cholesterol near the P-450_{scc} enzyme which is located at the inner mitochondrial membrane (Privalle et al., 1983).

In the past few years, several of these proteins and peptides have been isolated like steroidogenesis activator protein (SAP, Pedersen and Brownie, 1983, 1987) and sterol carrier protein 2 (SCP_2). It has been implied that these proteins regulate the amount of cholesterol available at the inner mitochondrial membrane, but their relative importance in regulating CSCC activity remains unknown.

Sterol carrier protein 2, also designated nonspecific lipid transfer protein (nsL-TP), accelerates the transfer of both phospholipid and cholesterol between synthetic membranes which are in contact with each other (Bloj and Zilversmit, 1977; Scallen et al., 1985; Van Amerongen et al., 1985). Using adrenal lipid droplets it has been shown that SCP_2 improves the transfer of cholesterol to mitochondria (Scallen et al., 1985; Chanderbhan et al., 1982). In the testis, SCP_2 is localized in the

steroidogenic active Leydig cells (Van Noort et al., 1986). Addition of SCP_2 to mitochondria isolated from adrenocortical and Leydig cells results in an enhanced production of pregnenolone (Chanderbhan et al., 1982; Van Noort et al., 1988b). It has also been demonstrated that treatment of rat Leydig cells with LH results in a 2-fold increase of SCP_2 in the cytosol within 2 min (Van Noort et al., 1988a) due to a changed subcellular distribution of SCP_2 (Van Noort et al., 1988b). In rat Leydig cells (Van Noort et al., 1988a) the amount of SCP_2 in the cytosolic fraction was not proportional to the rate of steroidogenesis. In adrenocortical cells hormonal stimulation of the amount of SCP_2 has not been demonstrated (Chanderbhan et al., 1986). However, when adrenocortical cells are fused with liposomes containing anti-SCP_2 antibody, ACTH-stimulated steroidogenesis is reduced (Chanderbhan et al., 1986). Therefore, it was postulated that not the precise amount of SCP_2 but rather the transfer capacity of SCP_2 is important in regulation of steroidogenesis (Van Noort et al., 1988).

Recently, two subclones of the MA-10 mouse Leydig tumor cell line have been isolated (Kilgore and Stocco, 1989). After exposure to hCG cells of one clone (MA-10 HP) produce a high amount of steroids (400 ng steroids/10^6 cells/h) while cells of the other clone (MA-10 LP) produce less than 14 ng steroids/10^6 cells/h. It was found that the low steroid production in the MA-10 LP cell line is caused by a lesion in the events that link the production of cAMP to the activation of CSCC. This genetic variant of the MA-10 cell line (Ascoli, 1981) may provide us with a model to assess the relative importance of SCP_2 and/or other proteins in regulation of steroidogenesis. In the present study we have investigated the effect of hCG on the subcellular distribution of SCP_2 in clones MA-10 HP and MA-10 LP.

MATERIALS AND METHODS

The MA-10 cells used in these experiments were subcloned from MA-10 cells generously provided by Dr. Mario Ascoli of The Population Council, Rockefeller University (Kilgore and Stocco, 1989 ; Ascoli, 1981). After subcloning 2 clones were selected, one with a high progesterone production (HP) and one with a very low progesterone production (LP) when incubated in the presence of hCG (the National Hormone and Pituitary Program, NICHD, NIH, USA) (Kilgore and Stocco, 1989). Cells were maintained in Corning T-flasks in modified Waymouth's MB752/1 medium (WMB) containing 15% (v/v) horse serum (HS) (GIBCO, Grand Island, N.Y.) and subcultured using standard techniques as described by Ascoli (1981). For experiments cells were plated out in 12 or 24 well plates (Corning) and grown for 24 h in medium plus serum. Subsequently cells were washed twice with Dulbecco's phosphate buffered saline containing calcium and magnesium (PBS; GIBCO, Grand Island, N.Y.) and thereafter incubated for 3 h in WMB lacking serum and containing 100 ng/ml hCG or no hormone. Progesterone production was measured by radioimmunoassay (Resko et al., 1974). Trypsin dispersed cells were counted using a Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida) and protein was measured as described by Sedmack and Grossberg (1977). Cells were homogenized by sonication in buffer containing 250 mM mannitol, 10 mM Tris (pH 7.4), 0.1 mM EDTA and centrifuged at 12,000 x g for 15 min. The pellet and supernatant fractions were lyophilized before measurement of SCP_2. Levels of SCP_2 in both the pellet and supernatant fractions were determined by use of an enzyme immunoassay as described by Teerlink et al. (1984) with modifications of Van Amerongen et al. (1989). In this procedure all fractions are treated with 125 mM KCl, 10 mM β-mercaptoethanol and 0.1 mM EDTA to extract SCP_2 from membranes. SCP_2 was measured in the extracts.

RESULTS AND DISCUSSION

SCP_2 is present in both the membranous (12k pellet) and supernatant (12k sup) fractions of clone MA-10 HP (Table 6.1). HCG stimulates steroid production in this clone (Table 6.2). After hormonal stimulation the amount of SCP_2 increases in the supernatant fraction and decreases in the membranous fraction. Similar observations have been made after exposure of Leydig cells isolated from the rat tumour Leydig cell line H540 to LH (Van Noort et al., 1988b). These results may reflect an altered

association of SCP_2 in cellular membranes after stimulation of steroidogenesis. In clone MA-10 LP, hCG stimulation does not lead to an increase in steroid production (Table 6.2). In this clone the pattern observed for the distribution of SCP_2 is strikingly different from the pattern in MA-10 HP cells (Table 6.1). While whole cell samples of clone MA-10 LP appear to contain more SCP_2 than did cells from clone MA-10 HP, we failed to detect SCP_2 in the membranous fraction. Moreover, the amount of SCP_2 in the supernatant fraction of cells is not influenced by incubation with hCG.

Table 6.1. Cellular distribution of SCP_2 in clones MA-10 HP and MA-10 LP after incubation with hCG.

	control	hCG
Clone MA-10 HP		
whole cells	10.9 ± 1.2	11.2 ± 2.2
12k pellet	119.6 ± 9.1	88.1 ± 1.5
12k sup	148.6 ± 19.5	237.0 ± 15.0
Clone MA-10 LP		
whole cells	19.7 ± 3.0	19.1 ± 1.3
12k pellet	N.D.	N.D.
12k sup	227.0 ± 10.5	227.2 ± 18.3

Data of 3 different experiments are expressed as means ± SD of the amount of SCP_2 present in whole cells (ng/10^6 cells) and in the 12,000 x g (12k) pellet and supernatant fractions (ng/mg protein); N.D.=not detectable.

Table 6.2. Progesterone production of clones MA-10 HP and LP.

Clone	Treatment	Progesterone ng/10^6 cells/h
MA-10 HP	control	4.60 ± 0.04
	hCG	371.00 ± 45.90
MA-10 LP	control	1.50 ± 0.07
	hCG	8.40 ± 1.70

Data are expressed as means ± S.D. of 3 different experiments.

The absence of SCP2 in the 12,000 x g pellet fraction of clone MA-10 LP, which contains membranes and mitochondria, may be connected with the lesion in steroid production in clone MA-10 LP. This aberrant distribution of SCP_2 might be due to an abnormal lipid and/or protein composition of the membranes or to an alteration in the molecular structure of SCP_2 itself. As an alternative explanation it should be considered that SCP_2 may be very tightly associated with membranes and can not be extracted using KCl, β-mercaptoethanol and EDTA. This seems unlikely since the total amount of SCP_2 in the 12k supernatant fraction of clone MA-10 LP equals the amount measured in the supernatant fraction of clone MA-10 HP after hormonal stimulation. Whatever explanation is correct, the present results show that the presence or absence of immunoreactive SCP_2 in the 12,000 x g pellet fraction that contains mitochondria correlates with the steroidogenic activity of subclones of the MA-10 Leydig cells.

Association of SCP_2 with membranes might be critically important for formation of the three dimensional structure of SCP_2 that is required for the action of SCP_2. It has been suggested that formation of a dimer of SCP_2 (Pastuzyn et al., 1987), which forms a bridge between two membranes (Van Amerongen et al., 1985) is important for transfer of cholesterol. The formation of this dimer could be influenced by the orientation of SCP_2 in membranes. However, this remains only speculative as long as the precise mode of action of the protein is not known.

If the lipid composition of the membranes is abnormal in clone MA-10 LP other processes involving cholesterol transport to the CSCC enzyme complex might be influenced. In this respect an evaluation of the composition of membrane lipids and membrane proteins will be important. As an alternative explanation it must be considered that immunoreactive SCP_2 in the supernatant fraction of MA-10 LP, may not represent biologically active SCP_2 and that the altered distribution of SCP_2 over membranous and supernatant fractions may reflect modification of the protein

structure of SCP_2. Therefore it is also important to investigate and compare the physical characteristics of SCP_2 in both clones.

In future experiments the biological properties of SCP_2 in mitochondrial side chain cleavage of cholesterol will be tested by comparing the effects of supernatant fractions and purified SCP_2 from clone LP or HP on steroid production of mitochondria isolated from clone HP.

CHAPTER 7

INTERACTION BETWEEN SCP_2 AND 25-HYDROXYCHOLESTEROL

Associations between 25-hydroxycholesterol and sterol carrier protein 2 (SCP_2) were investigated using equilibrium dialysis. Pure SCP_2 did not bind 25-hydroxycholesterol. Association between SCP_2 and 25-hydroxycholesterol occurred in the presence of proteins or peptides with positive charges such as bovine serum albumin (bSA) or poly-L-lysine (pL). In the presence of the negatively charged poly-L-aspartic acid (pA) there were no interactions between SCP_2 and 25-hydroxycholesterol. Very small amounts of bSA (0.07 nM) enabled interactions between SCP_2 and 25-hydroxycholesterol at concentrations of 3.6 nM and 1 nM respectively and as a result 8 nM of 25-hydroxycholesterol accumulated inside the dialysis bags. Increasing amounts of bSA or pL or SCP_2 did not affect the accumulation of 25-hydroxycholesterol. Accumulation of 25-hydroxycholesterol did not occur when Ca^{2+} was omitted from the dialysis buffer.

It is postulated that interaction between SCP_2 and 25-hydroxycholesterol is mediated by positive charges on peptides or membrane phospholipids and Ca^{2+}.

INTRODUCTION

Addition of SCP_2 to mitochondria isolated from Leydig cells and adrenal cortex tissue results in a 3-fold increasing pregnenolone production suggesting that SCP_2 may be involved in the regulation of cholesterol side chain cleavage activity (Van Noort et al., 1988b; Vahouny et al., 1984, 1985). SCP_2 may play a similar role in the conversion of cholesterol to bile-acids in the endoplasmic reticulum of liver cells (Lidström-Olsson and Wikvall, 1986). This process also requires side chain cleavage of cholesterol, although for bile-acid formation cholesterol is cleaved at C_{24} instead of C_{20}.

The mechanism of action of SCP_2 remains unclear. In vitro SCP_2 accelerates the transfer of a great variety of lipids including phospholipids, glycosphingolipids and cholesterol between synthetic membranes and is therefore also referred to as nonspecific lipid transfer protein (nsL-TP) (Van Amerongen et al., 1985). Thusfar in steroidogenic cells there are no indications that SCP_2 is active in the transfer of lipids other than cholesterol (Scallen et al., 1985).

The designation sterol carrier protein 2 suggests that cholesterol is carried between separated membranes. It has been proposed that SCP_2 carries cholesterol in a 1:1 molar soluble complex from intracellular membranes to the outer mitochondrial membrane and from the outer mitochondrial membrane to the inner mitochondrial membrane and that this transfer is rate-limiting for steroidogenesis (Vahouny et al., 1983, 1984). However, actual binding of cholesterol to SCP_2 has never been shown. SCP_2 is unable to transfer cholesterol between two separate monolayers. Moreover, it has been shown that SCP_2 only transfers cholesterol from mitochondrial membranes when lipid vesicles are in contact with the mitochondria (Van Amerongen et al., 1989).

The primary structure of bovine SCP_2 (Westerman and Wirtz, 1985) and rat SCP_2 (Pastuszyn et al., 1987) indicates that the single cysteine residue in the protein molecule is exposed at the

surface, which may result in the formation of SCP_2 protein dimers or in interactions of SCP_2 with other proteins. This may partially explain why SCP_2 has a great tendency to aggregate (Van Amerongen et al., 1985). The cysteine residue or the conformation of the protein in this area is probably important for the function of SCP_2 since chemical modification of this amino acid residue inactivates the lipid transfer activities of both rat and bovine SCP_2 (Poorthuis et al., 1981; Van Amerongen et al., 1985). These observations have led to the suggestion that SCP_2 enables transfer of cholesterol by bringing membrane interfaces together as a transient dimer.

In the postulated model, the protein mediated contact between vesicles enables lipids to move along the protein "bridge" and to redistribute between membranes. The proposed association between SCP_2 and membranes may enhance the affinity of the protein for cholesterol.

Using equilibrium dialysis at 4°C, we have investigated whether the affinity of SCP_2 for sterols can be modulated when SCP_2 interacts with other substances (proteins). Due to difficulties in dissolving cholesterol, dialysis experiments with cholesterol were not possible and 25-hydroxycholesterol has been used instead.

MATERIALS AND METHODS

Equilibrium dialysis

Binding of 25-hydroxycholesterol to purified rat SCP_2 was determined by equilibrium dialysis according to Fritz et al. (1976). SCP_2 (3.6 nM, unless otherwise indicated) in 0.5-1 ml 0.9% NaCl or 0.1 M NaPi-buffer present in a dialysis bag, was equilibrated against a solution (40 ml 0.9% NaCl or NaPi-buffer) of [26,27-^{3}H]- 25-hydroxy cholesterol (1 nM). Similar experiments were carried out with different proteins and peptides: bovine serum albumin (bSA), hemoglobin, calmodulin, poly-L-lysine and poly-L-aspartic acid (Sigma).

Two kinds of dialysis tubing were used: Visking size 9-36/32'' (cut off 10 kDa; Medicell International LTD) was used in most experiments and where indicated, Spectrapore 3 (cut off 3.5

kDa) was used. [26,27-^{3}H]-25-Hydroxycholesterol was obtained from Du Pont and stored in benzene: ethanol (9:1) at 4°C.

The 25-hydroxycholesterol solution was prepared as follows: 5 µl (5 µCi, 57 pmol) of labelled and 57 pmol of unlabelled 25-hydroxycholesterol in 5 ml 70% ethanol were dried under a stream of nitrogen, subsequently dissolved in 100 µl ethanol and added to 40 ml of saline or a Na-phosphate buffer (dialysis medium). Dialysis bags containing different mixtures of protein in 0.5 ml saline or buffer were placed in the solution. Equilibrium was reached in 16-48 h at 4°C. The binding of 25-hydroxycholesterol to the protein(s) was calculated from the accumulated [^{3}H]-label in the dialysis bag relative to the amount of [^{3}H]-label in the dialysis medium.

Determination of SCP_2

The amount of SCP_2 in the dialysis bag was determined by an enzyme immunoassay (EIA), according to Teerlink et al. (1984). Protein was determined according to Bradford (1976) using bSA as a standard.

RESULTS

25-Hydroxycholesterol was equally distributed over the solution outside and inside the dialysis bags after 16 h of dialysis at 4°C when the dialysis bags did not contain proteins. Of the label 70% was recovered, 30% was bound to the teflon stirring bar, the glass beaker and the dialysis bags. Thus the "free" (available for dialysis) concentration of 25-hydroxycholesterol was approximately 0.7 nM.

25-Hydroxycholesterol did not accumulate inside the dialysis bag when pure SCP_2 (50 ng/ml) was present (Fig.7.1). Significant ac cumulation was also not observed when pure hemoglobin (1mg/ml), calmodulin (1 mg/ml) or poly-L-lysine (pL, 1 mg/ml) were added. In the presence of bovine serum albumin (bSA, 1 mg/ml), 3 nM 25-hy-droxycholesterol accumulated. When 50 ng/ml SCP_2 was added in combination with 1 mg/ml hemoglobin (not shown), calmodulin (not shown), pL or bSA, an additional accumulation of 8 nM 25-hydroxycholesterol was found (Fig.7.1).

It appears that association between SCP_2 and 25-hydroxycholesterol occurs only in the presence of positive charges on

proteins (Fig.7.1). Poly-L-aspartic acid (pA, 1 mg/ml), which at neutral pH is negatively charged, did not induce accumulation of 25-hydroxy-cholesterol inside the dialysis bag containing SCP_2.

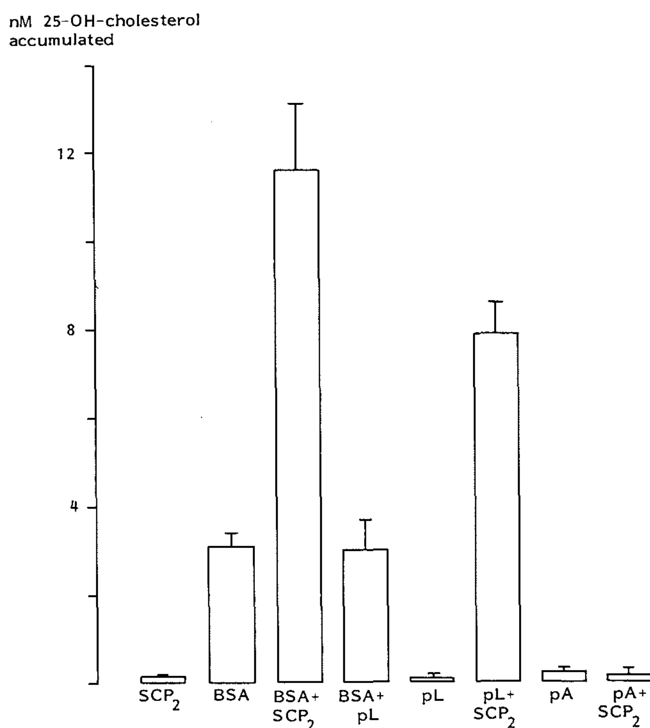


Fig.7.1. Accumulation of 25-hydroxycholesterol inside dialysis bags containing SCP_2 (50 ng/ml; 3.6 nM) and/or 1 mg/ml bovine serum albumin (bSA), poly-L-lysine (pL) and poly-L-aspartic acid (pA). Accumulation is expressed as the increase of [^{3}H]-25-hydroxycholesterol in the dialysis bags over steady state levels (0.7 nM). Values represent the means ± S.D. of three experiments. For further details see Materials and Methods.

As the concentration of SCP_2 (50 ng/ml) was kept constant and increasing amounts of bSA from 0.5 μg/ml to 5 mg/ml were added, an increasing accumulation of 25-hydroxycholesterol was observed (Table 7.1). When values were corrected for association between 25-hydroxycholesterol and pure bSA, it was shown that the SCP_2 dependent accumulation of 25-hydroxycholesterol was 8 nM for all combinations of 50 ng/ml SCP_2 (3.6 nM) and concentrations of bSA equal to or greater than 5 μg/ml (approximately 0.07 nM). Increased amounts of SCP_2 (not shown) did not alter the amount of 25-hydroxycholesterol that accumulated inside the dialysis bags. With poly-L-lysine similar results were obtained: for accumulation of 25-hydroxycholesterol a mixture of 3.6 nM SCP_2 and minimally 0.07 nM poly-L-lysine was required. Moreover, no further increase in accumulation of 25-hydroxycholesterol was observed at increasing poly-L-lysine concentrations (data not shown).

Proteins inside the dialysis bag		25OH-cholesterol accumulated (nM) not corrected	corrected
SCP_2 + bSA	0.5 μg/ml	1.1	1.1
	5	8.3	8.2
	50	8.5	8.1
	100	9.1	8.1
	1 mg/ml	10.6	7.9
	10	13.2	8.2
	100	15.4	8.1

Table 7.1. Accumulation of 25-hydroxycholesterol in dialysis bags containing 50 ng/ml sterol carrier protein 2 (SCP_2, MW 14kD) and different amounts of bovine serum albumin (bSA, MW app. 65kD) estimated by equilibrium dialysis at a steady state concentration of 0.75 nM 25-hydroxycholesterol. Where indicated, values were corrected for binding of 25-hydroxycholesterol to bSA and represent the means of three different experiments. The S.D. of the increase in ^3H-label in three experiments was less then 13%.

The effect of bSA on the association between 25-hydroxycholesterol and SCP_2 might be partially explained by a stabilizing effect of bSA on dissolved SCP_2. BSA might prevent denaturation of SCP_2 or binding of SCP_2 to dialysis bags. Therefore, the accumulation of $[^3H]$-25-hydroxycholesterol inside dialysis bags and the amount of (immunoreactive) SCP_2 were measured after incubating pure SCP_2 or a mixture of bSA and SCP_2 for 58 h. In addition, the amounts of 25-hydroxycholesterol and SCP_2 were measured in dialysis bags 26 h after addition of bSA to (32 h.) preincubated pure SCP_2 or 26 h after addition of SCP_2 to preincubated bSA (Fig.7.2). In those two dialysis bags the accumulation of 25-hydroxycholesterol was equal to the accumulation in the dialysis bag which contained a mixture of SCP_2 and bSA for the total dialysis period of 58 h. However, while 50 ng/ml SCP_2 was added to the dialysis bags, only 5 ng/ml SCP_2 (10%) could be measured by enzyme immunoassay at the end of the dialysis period in all dialysis bags.

Attempts to identify a complex between SCP_2, an other protein and 25-hydroxycholesterol, by steady-state gel electrophoresis (Ritzen et al., 1974) at a pH of 9.5 were not successful. Since the electric field may disrupt weak interactions between proteins and/or 25-hydroxycholesterol, we also tried 10-30% sucrose-gradient centrifugation to demonstrate a complex. To prevent dissociation of 25-hydroxycholesterol from SCP_2-protein complexes, the gradient contained 1 nM 25-hydroxycholesterol. Recovery of the labelled 25-hydroxycholesterol in the gradient was very low (<10%) due to attachment to plastic and only comigration of 25-hydroxycholesterol with bSA in the gradient could be shown.

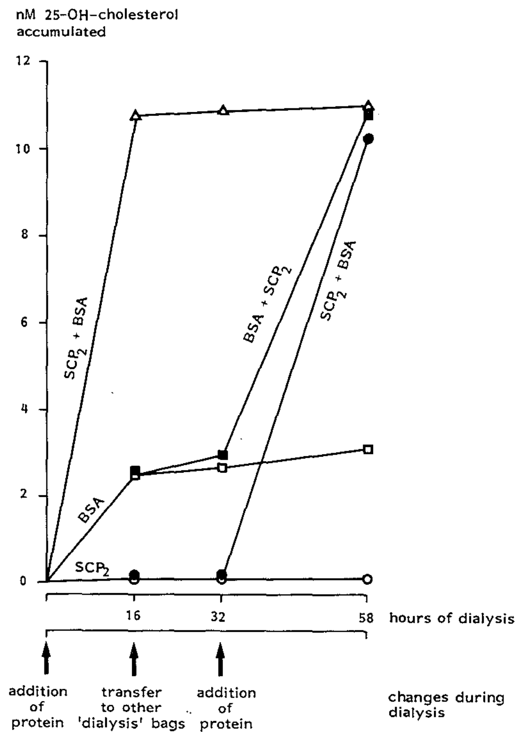


Fig.7.2. Accumulation of 25-hydroxycholesterol in dialysis bags containing SCP_2, bSA or a mixture of both proteins. Solutions containing 50 ng/ml SCP_2, 1 mg/ml bSA, or a combination of both proteins were dialyzed for 16 h (t=16 h) and subsequently transferred to new dialysis bags. After another 16 h of dialysis (t=32 h), SCP_2 was added to the dialysis bag containing only bSA and bSA was added to the dialysis bag containing only SCP_2. Dialysis was continued for 26 h more (t=58 h). Accumulation is expressed as the increase of $[^3H]$-25-hydroxycholesterol in the dialysis bags over steady state levels (0.76 nM).

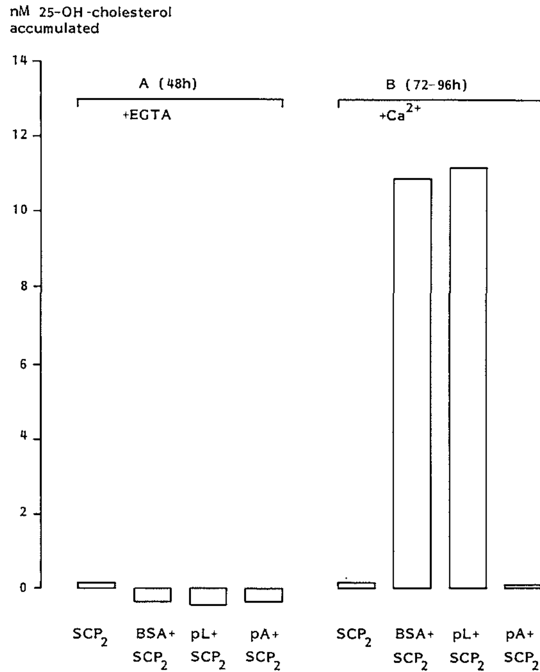


Fig.7.3. The influence of calcium on accumulation of 25-hydroxycholesterol.
The accumulation of 25-hydroxycholesterol was measured in dialysis bags containing 50 ng/ml sterol carrier protein 2 (SCP_2) and 1 mg/ml of either bovine serum albumin (bSA) or poly-L-lysine (pL) or poly-L-aspartic acid (pA). These protein mixtures were dialyzed for 48 h against a 0.1 M NaPi-buffer containing 5 mM EGTA (panel A). After 48 h $CaCl_2$ was added until a final Ca^{2+} concentration of 1 mM was reached. Accumulation of 25-hydroxycholesterol was measured at t=72-96 h (panel B) after a continuation of the dialysis for 24-48 h. Accumulation is expressed as the increase of $[^3H]$-25-hydroxycholesterol in the dialysis bags over steady-state levels (1.3 nM) after correction for accumulation with bSA alone. Values represent the means of 2 experiments.

The influence of calcium ions on the sequestration of 25-hydroxycholesterol by SCP_2 in the presence of bSA, poly-L-lysine or poly-L-aspartic acid was also studied since we suggested earlier that cellular Ca^{2+} concentrations could be important for the interaction between SCP_2 and membranous fractions (Van Noort et al., 1988b). Accumulation of 25-hydroxycholesterol inside the dialysis bags was constant at 0.1- 1 mM $CaCl_2$ in the dialysis buffer. However, accumulation of 25-hydroxycholesterol was absent after addition of 5 mM of the Ca^{2+} chelator EGTA to the dialysis buffer. Binding was completely restored 24 h after addition of $CaCl_2$ to this EGTA containing buffer until the final concentration of Ca^{2+} was 1 mM (Fig.7.3).

DISCUSSION

Using equilibrium dialysis experiments we have not observed a direct binding of 25-hydroxycholesterol to pure SCP_2 (Fig.7.1). Lack of direct binding to SCP_2 was also reported for cholesterol (Van Amerongen et al., 1989). The transfer of cholesterol between vesicles and mitochondria was enhanced by SCP_2 only when both particles could interact directly with each other.

Since pure SCP_2 does not bind (25-hydroxy) cholesterol it seems unlikely that this protein acts as a classical transport protein.

In the presence of proteins or peptides with positively charged groups an interaction between SCP_2 and 25-hydroxycholesterol could be demonstrated. The apparent discrepancy between the SCP_2 dependent accumulation of 25-hydroxycholesterol which is constant in time (Fig.7.2) and the 10% recovery of SCP_2 as measured by enzyme immunoassay is as yet unresolved. Formation of a complex between SCP_2, another protein and 25-hydroxycholesterol, could lead to shielding of the antigenic site of SCP_2. This may result in the observed very low detection of the protein in the enzyme immunoassay at the end of the dialysis experiment. However, we failed to demonstrate a complex between SCP_2, 25-hydroxycholesterol and other proteins.

It was proposed earlier that SCP_2 may function by linking two membranes into a transient ternary complex, thereby facilitating inter membrane lipid transfer (Van Amerongen et al., 1985, 1989; Crain and Zilversmit, 1980; Megli et al., 1986). This hypothesis starts from the assumption that SCP_2 interacts with membrane components. Formation of aggregates may enable SCP_2 to associate with 25-hydroxycholesterol. Addition of bSA to purified SCP_2 may induce similar changes so that binding sites for hydroxycholesterol are exposed.

Our data show that Ca^{2+} is required for the associations between 25-hydroxycholesterol, SCP_2 and bSA or poly-L-lysine (Fig.7.3). Indications for an important role of ions can also be

derived from other studies. Megli et al. (1986) suggested that the interaction between SCP_2 and membranes of liver mitochondria is of an electrostatic nature. We have shown earlier that the association between SCP_2 and membranes of the Leydig cell is modified by hormones, probably via Ca^{2+} (Van Noort et al., 1988a, 1988b). Cellular Ca^{2+} could thus be important in the regulation of the functional properties of SCP_2 by regulating the association between SCP_2 and membrane proteins and the association with sterol.

Strikingly, other proteins associated with membrane phospholipids, like protein kinase C, synexin and calpactin, also depend on Ca^{2+} for both membrane association and action (Klee, 1988).

In conclusion, the data show that SCP_2 does not bind cholesterol as a carrier protein. Earlier it was shown that regulation of the soluble amount of SCP_2 in steroidogenic cells by hormones is not predominant in the regulation of the rate of steroid production (Van Noort et al., 1988a). Instead, we postulate that hormones may modulate the interaction between SCP_2 and positive charges in proteins or phospholipid membranes and sterols (probably mediated by Ca^{2+}). As a result of these modifications a dimer of SCP_2 can be formed through which cholesterol can be transferred between membranes. In addition, regulation of the affinity of SCP_2 for cholesterol could also determine the rate of cholesterol transfer.

CHAPTER 8. GENERAL DISCUSSION

Sterol carrier protein 2: a protein with a permissive role in the regulation of Leydig cell steroidogenesis

The studies described in this thesis were carried out to establish the cellular localization and the possible function of SCP_2 in the regulation of steroidogenesis in the testis.

At the start of this study in 1985, SCP_2 was thought to transfer cholesterol in a 1:1 molar complex from cellular membranes to the outer mitochondrial membrane and from the outer mitochondrial membrane to the inner mitochondrial membrane (Vahouny et al., 1983, 1985) (Fig.8.1) where the rate-determining step in steroidogenesis, cleavage of the side chain of cholesterol to yield pregnenolone, takes place. In this model, the amount of SCP_2 in the cytosol and in the mitochondrial matrix of steroidogenic cells could determine the rate of steroid production. In order to explore a possible role of SCP_2 in the testis, we initially studied the amount of SCP_2 in 105,000 x g supernatant (i.e. soluble) fractions of tissue or isolated cells. Levels of SCP_2 were measured by way of a specific enzyme immunoassay using an affinity purified antibody against SCP_2 as described in detail by Teerlink et al. (1984). To determine the amount of SCP_2 (Mr approximately 14 kDa.) in soluble fractions, the fractions were heated for 5 min at 90°C to remove high molecular weight material (50-60 kDa) that cross reacted with the antibody. Admixture of samples with bSA prior to heating prevented the loss of SCP_2 (Teerlink et al., 1984).

While it was found that SCP_2 was localized in the Leydig cells and that LH regulated the amount of SCP_2 in the soluble fraction (chapter 2), a strict correlation between the amount of SCP_2 and the rate of steroidogenesis could not be demonstrated (chapter 3). Later studies did not show direct binding of sterol to SCP_2 (chapter 7), these observations are not consistent with SCP_2 being a carrier protein as was the prevailing model in 1985. This chapter will summarize and discuss new observations on the

localization, regulation and the functional activity of SCP_2 in Leydig cells and the development of new models on the role of SCP_2 in the regulation of steroid production.

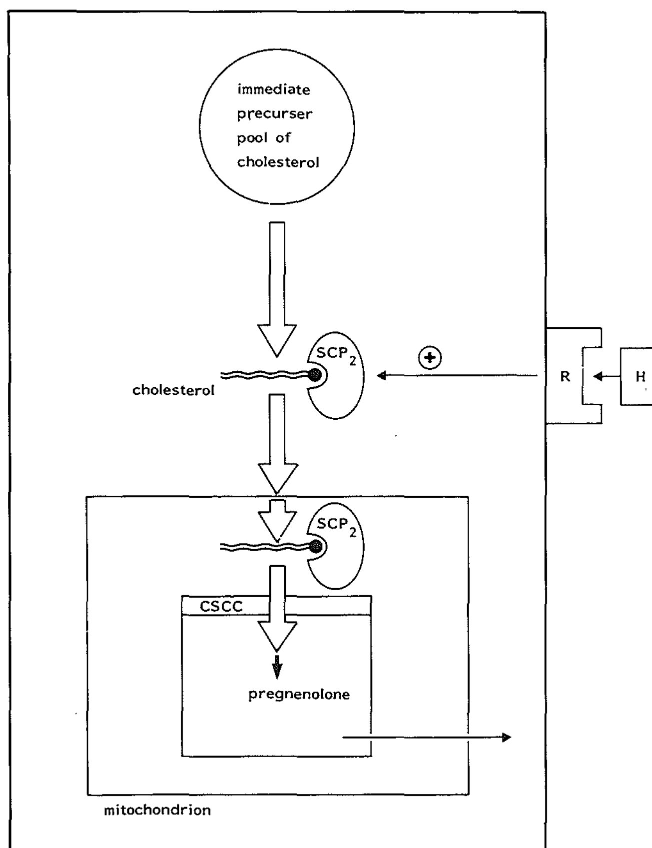


Fig.8.1. Hypothetical model on the role of sterol carrier protein 2 (SCP_2) in the regulation of steroidogenesis as derived in 1985 from studies with adrenal tissues: soluble SCP_2 binds cholesterol and transfers cholesterol in a 1:1 molar complex through the aqueous compartments of the cell to the inner mitochondrial membrane.
Abbreviations used: H=hormone; R=receptor; CSCC=cholesterol side chain cleavage enzyme complex.

8.1. Localization of SCP_2 in the rat testis

Testis tissue contains an amount of SCP_2 which is 3-fold lower than in adrenal tissue and 10-fold lower than in liver tissue (Teerlink et al., 1984) on a mg protein basis. We found that the amount of SCP_2 in isolated Leydig cells is more than 10-fold higher than the amount in testis tissue (chapter 2). This corresponded with the 10-fold purification factor of Leydig cells from testis tissue which was measured earlier using the Leydig cell marker enzyme esterase (Molenaar et al., 1985). In isolated Sertoli and germinal cells SCP_2 could not be detected. Also, when Leydig cells were destroyed 3 days after treatment of mature rats with the alkylating compound EDS, the amount of SCP_2 in the testis decreased to almost undetectable levels. From these results it was concluded that SCP_2 in the testis is mainly if not entirely localized in the Leydig cells. This is in accordance with a role of SCP_2 in regulating cholesterol metabolism in the testis since Leydig cells very actively produce steroids from cholesterol.

8.2. Regulation of SCP_2 levels in the Leydig cell

The rate of steroidogenesis depends on the presence of trophic hormones as well as on intracellular transport of cholesterol (see: Lambeth and Stevens, 1985) and since SCP_2 may enhance cholesterol transfer in steroidogenic cells, levels of this protein may be under hormonal control. We showed that the amount of SCP_2 in rat testis tissue increased 2-fold 48 h after two daily injections of human chorionic gonadotrophin (100 I.U., s.c.) and decreased 2-fold after plasma luteinizing hormone levels were suppressed to almost undetectable levels with silicone elastomer implants containing testosterone (chapter 2). These results indicated that luteinizing hormone regulates the amount of SCP_2 in Leydig cells. The amount of SCP_2 could be

regulated by _de novo_ protein synthesis. Since a high molecular weight protein cross-reacting with the antibody raised against SCP_2 was found in rat liver (Van der Krift et al., 1985), levels of SCP_2 might also be regulated by precursor proteolysis. Moreover, the regulation of the amount of SCP_2 could be mediated by a direct effect of LH on the turnover of SCP_2 or it could represent an indirect effect of LH caused by a depletion of cholesterol after stimulation of steroid production.

8.3. Identification of intracellular mediators that are involved in regulation of SCP_2 levels in the cytosol

Hormonal regulation of SCP_2 levels was investigated in isolated rat Leydig cells (chapter 3). Steroid production in the rat Leydig cell can be regulated by different second messenger systems (Themmen et al., 1985, 1986; chapter 1 of this thesis) and we have investigated how more or less selective stimulation of one of these systems influences cellular SCP_2 levels. For this purpose the effects were compared of maximally stimulatory doses of LH, the phorbol ester PMA and its ineffective analogue PA, dibutyryl cAMP and LHRH on steroid production and SCP_2 levels. SCP_2 levels in supernatant fractions were increased 2-fold after incubation of rat Leydig cells, isolated from testis or tumour tissue, with LH, PMA and dibutyryl cAMP. LHRH, which stimulates steroid production in testicular Leydig cells, did not increase SCP_2 levels. Thus, an increased rate of steroid production is not always accompanied by an increase in the amount of SCP_2 in the cytosol of the Leydig cell. Therefore, it is most likely that the amount of SCP_2 in the cytosol of the Leydig cell is regulated via specific second messenger system(s) that are activated by LH or PMA.

Since many hormones cause a rise in intracellular calcium levels, the role of calcium in the regulation of SCP_2 levels was studied in more detail (chapter 3). This was carried out by arti-

ficially enhancing the intracellular free calcium concentration using the calcium ionophore A23187, or by preventing increases in the intracellular calcium concentration either by blocking calcium channels in the plasma membrane with diltiazem or decreasing the extracellular calcium concentration. After incubation of the cells with ionophore, SCP_2 levels were 2-fold increased. Omission of Ca^{2+} from the incubation medium, or blocking Ca^{2+} channels in the plasma membrane with diltiazem abolished the LH-induced increase in SCP_2-levels. It was concluded that a rise in the intracellular concentration of Ca^{2+} by influx of Ca^{2+} through the plasma membrane of Leydig cells could be related to the observed 2-fold increase in the amount of SCP_2 in the cytosol. Activation of protein kinase C by PMA also leads to an increase in the amount of SCP_2 in the cytosol. Since a correlation between activation of PK-C and an increase in intracellular Ca^{2+} levels has not been shown, the PMA-mediated activation of PK-C may represent a distinct pathway in the control of SCP_2 levels in the cell.

8.4. The relationship between soluble SCP_2 levels and steroidogenesis in the Leydig cell

In most cases the amount of SCP_2 in the soluble fraction of the Leydig cell was increased by substances that stimulate steroid production. This positive correlation is in accordance with the proposal that a SCP_2-cholesterol complex is involved in regulation of the rate of steroidogenesis (Vahouny et al., 1983, 1984). However, the following observations show that there is no strict correlation between the amount of SCP_2 in the 105,000 x g membrane-free supernatant and the rate of steroid production (chapter 3): 1. Production of steroids was stimulated 2-8-fold after addition of PMA, dibutyryl cAMP or LH, whereas SCP_2 levels were only increased 2-fold; 2. LHRH, which stimulated steroid production more than 3-fold did not increase SCP_2 levels; 3. LH

stimulated steroid production in the presence of diltiazem or in the absence of extra-cellular calcium was at least 1.5-fold, whereas under these conditions SCP_2 levels were not increased.

Hence, our results did not support the current hypothesis that SCP_2 there is a linear relationship bewteen the transfer of cholesterol by equimolar binding to SCP_2 and the amount of SCP_2 in the soluble fraction of the cell (Vahouny et al., 1983, 1985). A relationship between soluble SCP_2 levels and cholesterol metabolism could also not be demonstrated in liver tissue (Van Heusden et al., 1985). Other observations in adrenal cells that SCP_2 levels were not increased even after 6 h of incubation with ACTH (Chanderbhan et al., 1986), also argue against an important role of the level of SCP_2 in the control of steroid production.

We conclude that the amount of SCP_2 in cells is normally sufficient to allow for different rates of cholesterol metabolism and steroidogenesis. However, when the amount of SCP_2 inside adrenocortical cells is decreased by fusion with liposomes containing anti-SCP_2 antibody, ACTH-stimulated steroidogenesis is reduced (Chanderbhan et al., 1986). Moreover, the extremely low levels of SCP_2 in livers of infants with cerebro-hepato-renal (Zellweger) syndrome may contribute to hepatic dysfunction in various aspects of cholesterol metabolism (Van Amerongen et al., 1987).

Therefore, it will be important to determine the lowest amount of SCP_2 necessary for maintaining steroid production in steroidogenic cells.

8.5. Regulation of the amount of SCP_2: from Specifically Cleaved Precursor to Subcellularly Changed Protein

8.5.1. A Specifically Cleaved Precursor

The amount of SCP_2 in the 105,000 x g supernatant fraction of rat Leydig cells was increased rapidly after incubation of the cells with LH, dibutyryl cAMP and PMA (chapter 3). Since the increase in SCP_2 levels already occurs 2 min after addition of LH (chapter 3), it is unlikely that the amount of SCP_2 is regulated by de novo synthesis. This was confirmed later by Trzeciak et al.(1987) who reported a half life of 32 h for adrenocortical SCP_2.

The rapid increase in the levels of SCP_2 (14 kDa) may have resulted from post-translational processing of a precursor for SCP_2. Evidence for the presence of a 50-60 kDa precursor of SCP_2 was obtained by immunoblotting of membrane-free cytosol from rat liver tissue (Van der Krift et al., 1985), rat adrenal tissue (Chanderbhan et al., 1986) and rat Leydig cells (Fig.8.2). Immunocytochemistry of liver and adrenal cells revealed that this high molecular weight protein may be present in large amounts in peroxisomes (Van der Krift et al., 1985; Keller and Krisans, 1987; Van Amerongen et al., 1987).

There may be a precursor-product relationship between the amount of SCP_2 measured in the supernatant and the presence of the high molecular weight protein in peroxisomes. This can be inferred from observations that SCP_2 was barely measurable in the supernatant fraction of the Morris hepatoma 7777 cells where the number of peroxisomes is greatly reduced (Teerlink et al., 1984); as well as in liver tissue from Zellweger patients, which do not have detectable amounts of hepatic peroxisomes (Van Amerongen et al., 1987). Considering the proposed primary defect in this cerebro-hepato-renal syndrome, i.e. the defective assembly of peroxisomes, it is tempting to speculate that the presence of the

50-60 kDa protein in peroxisomes precedes the occurrence of 14 kDa SCP_2 (Fig.8.3).

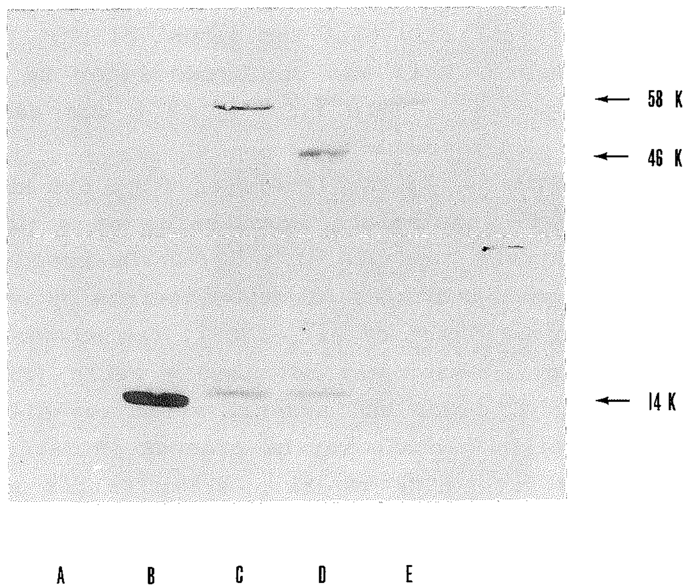


Fig.8.2. Immunoblotting with an affinity purified anti-SCP_2 antibody of a reducing SDS-polyacrylamide gel to which 105,000 x g supernatant proteins isolated from 10^7 Sertoli cells according to Oonk et al. (1985) (lane **A**), 10^7 hepatocytes (lane **C**), 10^7 tumour Leydig cells (lane **D**) and interstitial cells from 21 day old rats (lane **E**) isolated according to Molenaar and Rommerts (1985) and 50 ng of pure bovine SCP_2 (lane **B**) (Teerlink et al., 1984) were applied. Proteins cross reacting with the antibody were visualized using ^{125}I-protein A and autoradiography.

8.5.2. The soluble amount of SCP_2 is not regulated by proteolysis of a precursor

To investigate a possible relationship between the 50-60 kDa protein and 14 kDa SCP_2, cellular proteins were radioactively labelled by incubation of Leydig cells with a combination of the ^{35}S labelled amino acids methionine and leucine for 4 h (the half life of SCP_2 of 32 h in adrenal cells was reported one year after the completion of these experiments). Part of the cells were incubated with LH for 30 min. The labelled proteins were separated by SDS-polyacrylamide gel electrophoresis and subsequently analyzed by autoradiography. If LH-induced proteolysis of a precursor protein and formation of SCP_2 would occur, this should be revealed by a higher degree of labelling of a protein with a molecular weight corresponding to that of SCP_2 (14 kDa). However, such a precursor-protein relationship could not be demonstrated.

Moreover, after incubation of tumour Leydig cells with LH, a 2-fold increase in (14 kDa) SCP_2 levels was observed between 1 and 2 min and this 2-fold increased level persisted for at least 24 h. Since the kinetics and magnitude of the effect were not influenced by preincubation of the cells with cycloheximide for 4-18 h (chapter 3), the half life of SCP_2 and the half life of the possible precursor protein must be more than 10 h. Assuming a half life of SCP_2 in Leydig cells of 20 h, the rapid increase of the amount of SCP_2 in the 105,000 x g supernatant could be explained by proteolysis of a relatively stable precursor protein only by assuming that 200 ng SCP_2/mg protein/min is produced within 2 min by fast processing of the precursor, immediately followed by a 1000-fold decrease in the processing rate (chapter 3).

From these data we concluded that the 2-fold increase in SCP_2 levels in the 105,000 x g supernatant fraction of Leydig cells incubated with LH, PMA or dibutyryl cAMP did not result from either de novo synthesis of SCP_2 or from proteolysis of a precursor of SCP_2.

8.5.3. A Subcellularly Changed Protein

It has been shown in adrenal and liver tissue that SCP_2 is not only present in the 105,000 x g soluble fraction, but that SCP_2 is also associated with membranous fractions (Chanderbahn et al., 1986; Van der Krift et al., 1984). The observed changes in SCP_2
levels in soluble fractions of the Leydig cells under the influence of LH, might be influenced by alterations in the intracellular distribution of SCP_2.

The effect of LH on the subcellular localization of SCP_2

We demonstrated that the 2-fold increase of SCP_2 levels in supernatant fractions of Leydig cells, that were incubated with LH and subsequently disrupted, can be explained by a relocation of SCP_2 from the 105,000 x g particulate to the supernatant fraction (chapter 4).

It is not certain if hormonal stimulation of Leydig cells results in true intracellular redistribution of SCP_2 or diminished binding of the protein to membranes to such an extent that it can be released during homogenization. Whatever explanation is correct, the data show that LH can modify the interaction between SCP_2 and membranes. To determine the exact origin of SCP_2 in the supernatant fraction, we studied the distribution of SCP_2 over various subcellular fractions.

8.6. Subcellular localization of SCP_2 in the rat Leydig cell

Measurement of SCP_2 in the membranous fractions of the Leydig cell, required an adaptation of the enzyme immunoassay since membranes can interfere (Teerlink et al., 1984). SCP_2 bound to rat liver mitochondria could be removed by washing with a high ionic strength buffer (Megli et al., 1986). Membranous fractions were therefore treated with KCl, β-mercaptoethanol and EDTA to release SCP_2 (Van Amerongen et al., 1989) and SCP_2 was measured in the extracts. The subcellular distribution of SCP_2 was also studied using immunocytochemistry (chapter 5).

Immunocytochemistry

Using immunocytochemistry we have shown that in the rat Leydig cell antibodies raised against SCP_2 associated mainly with protein(s) present in the peroxisomes (chapter 5). This was observed earlier in liver and adrenal tissue (Van der Krift et al., 1985; Keller et al., 1987; Krisans et al., 1987; Tsuneoka et al., 1988; Van Amerongen et al., 1989). Unfortunately there is no consensus in the literature on the nature and biological function of the peroxisomal protein that cross-reacts with the antibody raised against SCP_2. It could be either 14 kDa SCP_2 (Tsuneoka et al., 1988), the 50-60 kDa protein (Van Amerongen et al., 1987; Van der Krift et al., 1985; Keller, 1987), or a totally unrelated protein. Morris et al. (1988) have postulated a peroxisomal leader sequence in the cytosolic SCP_2 with a molecular mass of 2 kDa, which is cleaved off at the peroxisomal membrane.

The relationship between SCP_2 and the 50-60 kDa protein is as yet uncertain. It was shown earlier that the 50-60 kDa protein is not a precursor of SCP_2 (chapters 3 and 4). Data rather indicate that the reverse process occurs and that SCP_2 may be a precursor for the 50-60 kDa protein. Formation of 50-60 kDa aggregates of SCP_2 when preparing samples for gel electrophoresis, has been reported by Chanderbhan et al. (1986). This 50-60

kDa protein has also been observed after gel chromatography (Crain and Clarke, 1985; Teerlink et al., 1984). It is known that SCP_2 has a tendency to aggregate and losses of the protein during purification have always been attributed to aggregation (Van Amerongen et al., 1985; Bloj et al., 1978). Assuming that the protein that is immunocytochemically labelled in the peroxisomes with the anti-SCP_2 antibody represents 14 kDa SCP_2 or aggregates, the function of this SCP_2 (or aggregates) in the peroxisomes of steroidogenic cells is not clear. Since in liver cells it has been demonstrated that the rate-determining enzyme in cholesterol synthesis, HMG-CoA reductase, is localized in peroxisomes as well as in the endoplasmic reticulum (Keller and Krisans, 1987), SCP_2 could be functional in peroxisomal cholesterol metabolism. A possible function of peroxisomal SCP_2 as a buffer for SCP_2 localized in other parts of the cell should also be considered.

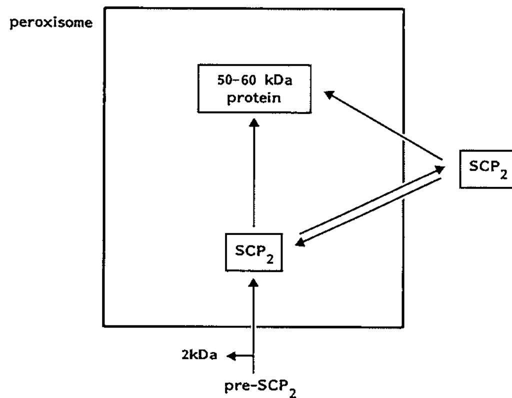


Fig.8.3. Different explanations on the nature of the high amount of material that is cross-reacting with the anti-SCP_2 antibody in the peroxisomes of different cell types. This material may be :

1. 50-60 kDa aggregate of SCP_2 which is a functional protein.
2. SCP_2 localized in peroxisomes by specific cleavage of a peroxisomal leader sequence at the peroxisomal membrane.
3. A totally unrelated protein.

The function of this material may be:

a. Regulation of peroxisomal lipid metabolism.
b. Buffering the amount of SCP_2 functioning in other compartments of the cell (for example in mitochondrial steroidogenesis).

SCP_2 in membranous fractions

Although the immunocytochemical data showed that SCP_2-like material is present mainly in the peroxisomes, the measurement of SCP_2 by enzyme immunoassay showed that the highest amount of SCP_2 is present in other subcellular fractions, such as the 105,000x g supernatant fraction, of Leydig cells (chapter 5) and of adrenal and liver tissue (Van Amerongen et al., 1989).
Several attempts have been made to explain this discrepancy. It was suggested that the observed peroxisomal localization in the immunocytochemical experiment may have resulted from the in-ability of the antibodies to interact with extra peroxisomal SCP_2 embedded in sections (Van Amerongen et al., 1989). Alternatively, it could be speculated that massive amounts of SCP_2 are released from peroxisomes during homogenization and subsequently associate with other membranes. The association with particular membranes could be dependent on the lipid and/or protein composition of the membranes. This could explain why the subcellular distribution of SCP_2 differs in Leydig (chapter 5), liver and adrenal cells (Van Amerongen et al., 1989) when measured by way of the enzyme immunoassay. However, it is difficult to envisage that the observed increased SCP_2 level in the soluble fraction of Leydig cells within 2 min of incubation with LH (chapter 4), occurs by a change in the lipid/protein composition of Leydig cell membranes. The amount of SCP_2 in the different subcellular fractions of the Leydig cell before and after incubation of isolated cells with LH is reported in chapter 5. It was found that the 2-fold increase of SCP_2 levels in supernatant fractions of Leydig cells, which were incubated with LH and subsequently disrupted, can be explained by a relocation of SCP_2 from almost all of the membranous fractions to the supernatant fraction. Among the membranous fractions only the lysosomal fraction (peroxisomes?) retained all SCP_2 (chapter 5).

Whatever the exact origin of SCP_2 may be, SCP_2 is probably involved in cholesterol transfer to the mitochondria in steroidogenic cells (chapter 4). The observed changes in SCP_2 levels in the soluble fraction of Leydig cells may be (partly) derived from changes in the subcellular distribution of SCP_2 under the influence of stimulation of steroid production.

Effects of hormones, or substances mimicking the effect of hormones, on the subcellular redistribution of proteins other than SCP_2 have been demonstrated previously. For instance, phorbol esters cause an apparent redistribution of protein kinase C (Nikula and Huhtaniemi, 1988; Munari-Silem et al., 1987) and it has been shown recently that translocation of this kinase is coupled with activation (Munari-Silem et al., 1987). Hormones may thus influence the subcellular redistribution of proteins or aggregates of proteins such as multi-enzyme complexes.

In this respect, the presence of radioactively labelled proteins with molecular weights of 14,27 and 30 kDa in the 10,000 x g supernatant fraction of sonicated immature Leydig cells after treatment of the cells with LH, LHRH, PMA and PL-C, has been interpreted as an effect of these different stimuli on protein synthesis (Themmen et al., 1986). However, it is not clear to what extent protein redistribution has contributed to the intensity of these protein bands.

8.7. Effect of SCP_2 on steroid production of isolated Leydig cell mitochondria

Steroid production by isolated adrenocortical mitochondria could be stimulated by SCP_2 in a dose-dependent fashion (Vahouny et al., 1982-1984). Using mitochondria isolated from Leydig cells we observed an increase in steroid production after addition of SCP_2 (chapter 4), but this increase did not depend on the amount of SCP_2 added.

It was shown that mitochondria that after incubation with an anti-SCP_2 antibody showed a lower capacity to synthesize steroids (chapter 4). It appears unlikely that the antibody penetrated the outer mitochondrial membrane and subsequently bound to SCP_2 located in between the outer and the inner mitochondrial membrane. Therefore, we concluded that the inhibition of mitochondrial pregnenolone synthesis is probably due to binding of the antibody to SCP_2 located on the outer mitochondrial membrane. In rat liver microsomes (Seltman et al., 1985) where SCP_2 enhances 7α-hydroxylation of cholesterol, the rate-limiting step in bile-acid formation a similar observation was made. Microsomes immunotitrated with anti-SCP_2 antibody exhibited considerably less capacity to synthesize 7α-hydroxycholesterol from cholesterol.
From these data it can be concluded that SCP_2 probably functions at the outer mitochondrial membrane and improves the transfer of cholesterol from other intracellular membranes to the outer mitochondrial membrane. During the preparation of the mitochondria SCP_2 could be disrupted from the mitochondria if we assume that SCP_2 is loosely attached to membranes and acts at membrane interfaces. Addition of SCP_2 may restore the minimal amount of SCP_2 needed for production of pregnenolone by isolated mitochondria. It is not clear if, and how much SCP_2 is required to maintain steroidogenesis and bile-acid formation in the mitochondrial fraction of steroidogenic cells and the microsomal fraction of liver cells respectively.

8.8. Interaction with membranes may be important for the action of SCP_2; study of a "mutant" mouse Leydig cell

An indication that association of SCP_2 with membranes in the cell might be important for its actions, is provided by a study on the subcellular distribution of SCP_2 in the mouse Leydig tumour subclone MA-10 LP (chapter 6). MA-10 LP produces very low amounts of steroids in the presence of hCG (Kilgore and Stocco, 1989). It was demonstrated that this low steroid production is caused by abnormalities in the mechanism which couples the effect of hCG-induced cAMP formation to the activity of the CSCC enzyme (Kilgore and Stocco, 1989). In contrast to the intracellular distribution of SCP_2 in Leydig cells from the rat tumour H540 and the mouse tumour MA-10 HP, SCP_2 could not be detected in the pellet fraction (which contained mostly plasma membranes and mitochondria) of cells from clone MA-10 LP . The amount of SCP_2 in the supernatant fraction of cells incubated in the absence or presence of hCG was similar in this clone. The impaired association of SCP_2 with membranes of MA-10 LP cells, could indicate an abnormality in SCP_2 or in the membranes and could represent (part of) the lesion in steroid production in clone MA-10 LP.

8.9. SCP_2 is not a Sterol Carrier Protein

The designation "sterol carrier protein 2" suggests that cholesterol is carried by this protein membranes. It has been proposed that SCP_2 carries cholesterol in a 1:1 molar soluble complex to the inner mitochondrial membrane and that this transfer is rate-limiting for steroidogenesis in the adrenal (Vahouny et al., 1983, 1984).

In contrast to this model for regulation of adrenal steroidogenesis, we found in the Leydig cell that regulation of the amount of SCP_2 in cytosol and membranes is not correlated with regulation of the rate of steroid production. It was found that the mitochondrial fraction of steroidogenically active Leydig cells contains only a low amount of SCP_2 (chapter 5).

In vitro SCP_2 accelerates the transfer of a great variety of lipids including phospholipids, glycosphingolipids and cholesterol between synthetic membranes and SCP_2 is therefore also referred to as "nonspecific lipid transfer protein" (nsL-TP) (Van Amerongen et al., 1985). It was proposed that SCP_2 may function as a carrier of phosphatidylcholine (Nichols, 1987), but binding of lipids other than cholesterol to the protein was disputed by Chanderbhan et al. (1982) and Scallen et al. (1985).
However, actual binding of cholesterol to SCP_2 has never been demonstrated. Using lipid monolayers and vesicles, lack of direct binding of cholesterol to SCP_2 was observed. The transfer of cholesterol was greatly enhanced by SCP_2 (Van Amerongen et al., 1989) only when vesicles and monolayer were in close proximity to each other. Furthermore, SCP_2 only transferred cholesterol from mitochondrial membranes to lipid vesicles when the lipid vesicles were in close proximity to the mitochondria.

It was proposed that SCP_2 may function by linking two membranes into a transient ternary complex, thereby facilitating intermembrane lipid transfer (Van Amerongen et al., 1985,; Crain and Zilversmit, 1980; Megli et al., 1986).

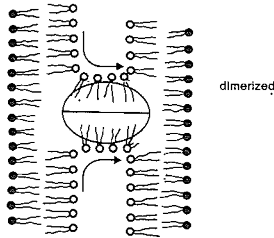


Fig.8.4. SCP_2-dimer bringing membrane interfaces together: the non-polar tail of the lipids may move along the protein bridge".

The primary structure of bovine SCP_2 (Westerman and Wirtz, 1985) and rat SCP_2 (Pastuszyn et al., 1987) indicates that the single cysteine residue in the protein molecule is exposed at the surface. This residue or the conformation of the protein in this area is important for activity since chemical modification of the residue abolishes the effect of both rat and bovine SCP_2 on lipid transfer between synthetic membranes (Poorthuis et al., 1981; Van Amerongen et al., 1985). Therefore it has been postulated that SCP_2 is instrumental in bringing membrane interfaces together either through the formation of disulfide bonds (Van Amerongen et al., 1985) or through the formation of a dimer enclosing a hydrophobic pocket that can sequester cholesterol (Pastuszyn et al., 1987; Van Amerongen et al., 1988). SCP_2-mediated contact between membranes may enable lipids to redistribute between membranes, the non-polar tail of the lipids moving along the protein "bridge" (Fig.8.4).

We have investigated the affinity of SCP_2 for cholesterol using equilibrium dialysis at 4°C (chapter 7 of this thesis). 25-

Hydroxycholesterol was used rather than cholesterol since the latter did not penetrate dialysis bags. It was shown that for association between SCP_2 and 25-hydroxycholesterol the presence of other proteins with positive charges is essential. A stochiometric relationship between the amounts of SCP_2, other protein and 25-hydroxycholesterol was not observed. It was also found that calcium ions are important for the interaction between SCP_2, proteins and 25-hydroxycholesterol.

We propose that electrostatic interactions between SCP_2 and positive charges on peptides, possibly mediated by Ca^{2+}, are necessary for the interaction between SCP_2 and 25-hydroxycholesterol. As observed earlier for cholesterol conversion into steroids in intact cells, there was no correlation between the amount of SCP_2 and the interaction of SCP_2 with 25-hydroxycholesterol.
Since pure SCP_2 does not bind 25-hydroxycholesterol, it seems unlikely that SCP_2 binds cholesterol in a 1:1 molar complex and acts as a classical transport protein as suggested by Vahouny et al. (1983, 1984). SCP_2 is not a "Sterol Carrier Protein".

8.10. A model for the role of SCP_2 in regulation of steroid production: a permissive action

From the data on the interaction of SCP_2 with sterol (chapter 7) and the effect of LH on the intracellular distribution of SCP_2 in the Leydig cell (chapter 2-6), it appears that (electrostatic) interactions between SCP_2 and proteins/lipids in membranes are important for the action of SCP_2. Changes in (cellular) Ca^{2+} concentrations may modify in the Leydig cell the electrostatic interaction of SCP_2 with membranes and in equilibrium dialysis the interaction of SCP_2 with other proteins and 25-hydroxycholesterol.

We propose that a certain association of SCP_2 with cellular membranes may be critically important for formation of the specific three dimensional structure of the protein which is responsible for the action of the protein. We postulate that interaction between SCP_2 and positive charges on peptides, or membrane phospholipids, which can be modulated by Ca^{2+} and possibly by PK-C, favors the formation of a dimer of SCP_2. If this dimer contains a hydrophobic pocket which can enclose the non-polar tail of sterols, SCP_2 may function as a protein bridge between membranes (Fig.8.5) for the transfer of cholesterol, the flow of cholesterol being directed by a cholesterol consuming process such as the formation of steroids.

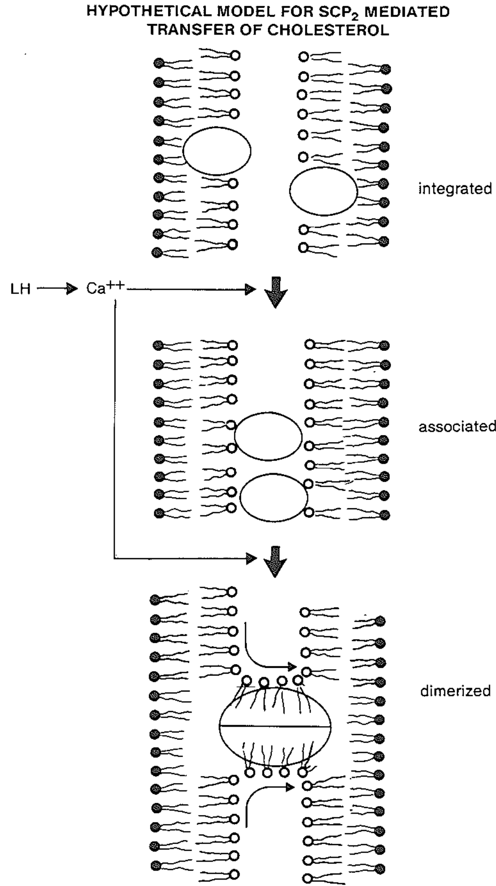


Fig.8.5. New model for SCP_2 mediated transfer of cholesterol and the regulation of this process by LH in the rat Leydig cell: The interaction of SCP_2 and positive charges on (proteins in) membranes is modulated by LH-induced Ca^{2+} fluxes. A certain interaction with membranes and Ca^{2+} favors the formation of a dimer of SCP_2. A dimer of SCP_2 may function as a protein-"bridge" between membranes for transfer of cholesterol.

In this model SCP_2 does not regulate the rate of steroid production but only enables a rapid establishment of an equilibrium of cholesterol inside the cell. The rate determining step in steroidogenesis may thus reside in the cytoskeleton of the cell involved in transfer of cholesterol (Hall et al. 1984), or in phosphorylation (Vilgrain et al., 1984) and/or synthesis of $P\text{-}450_{scc}$ (Waterman and Simpson, 1985), or in activators of CSCC such as SAP (Pedersen and Brownie, 1983, 1987), the 8.2 kDa protein (Yanagibashi et al., 1988) and app. 28 kDa proteins (Pon et al., 1986) (Fig.8.6).

Fig.8.6. Activation of CSCC in the rat Leydig cell: a model for the coupling of different cellular second messenger systems with the activity of P-450_{SCC}.

A. Transfer of cholesterol to the outer mitochondrial membrane may be regulated by:

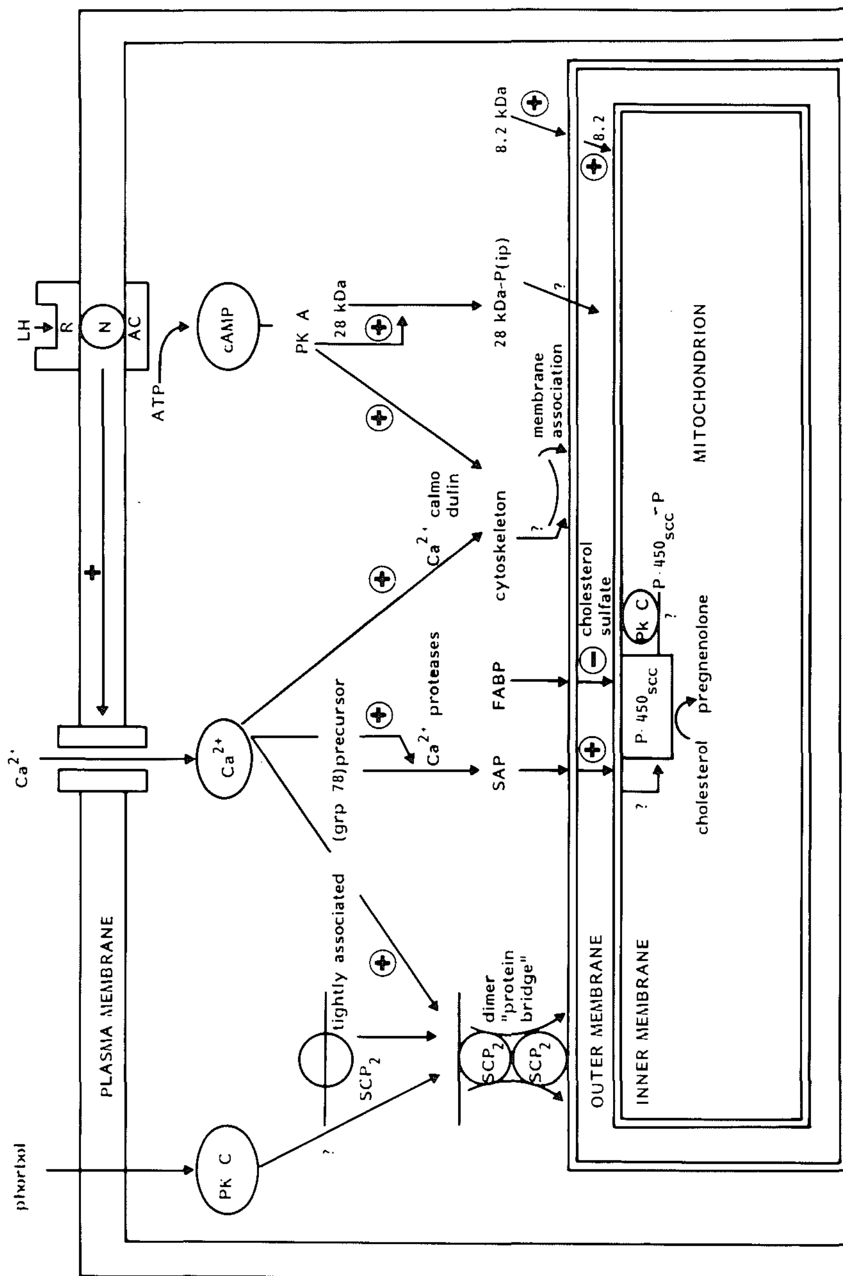
1. The **cytoskeleton** which regulates the interaction between cellular membranes and which in turn is regulated by a complex of reactions involving phosphorylation and the action of calmodulin.
2. Formation of a permissive protein bridge between cellular membranes consisting of a **SCP_2-dimer**, which regulated by Ca^{2+} fluxes and possibly by the action of PK-C. The transfer efficiency but not the amount of SCP_2 is important in regulation of the activity of P-450_{SCC}; the flow of cholesterol through this bridge is directed by the consumption of cholesterol in the mitochondria.
3. A **8.2 kDa protein.**

B. Transfer of cholesterol to the inner mitochondrial membrane may be regulated by:

1. The amount of **SAP**, which is rapidly regulated by cleavage of a large precursor protein (GRP-78) by a Ca^{2+}-dependent protease and rapid degradation of SAP itself.
2. The amount of the **8.2 kDa protein.**
3. The amount of **cholesterol sulphate** in between the mitochondrial membranes.

The action of **FABP** may change the lipid environment and increase the activity of P-450_{SCC}. Phosphorylation of P-450_{SCC} by intramitochondrial PK-C and proteins of approx 28 kDa Mr (28 kDa) by PK-A (28 kDa-P (ip)) may also contribute to activation of CSCC.

For further details see chapter 1.



LH
R
N
AC
ATP
cAMP
PK A
28 kDa
28 kDa-P(ip)
8.2 kDa
8.2
?
Ca^{2+}
Ca^{2+}
PLASMA MEMBRANE
(grp 78)precursor
Ca^{2+} proteases
SAP
FABP
Ca^{2+}, calmo dulin
cytoskeleton
membrane association
cholesterol sulfate
Pk C
P-450scc
P-450scc~P
cholesterol
pregnenolone
MITOCHONDRION
OUTER MEMBRANE
INNER MEMBRANE
phorbol
PK C
SCP_2
tightly associated
dimer "protein bridge"
SCP_2
SCP_2

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SUMMARY

In the male a specific class of steroid hormones, the androgens, are essential for the development and maintenance of germinal cells and male sex characteristics. Androgen production takes place in the Leydig cells, which are present in the interstitial compartment of the testis. The rate of steroid production in the testis is determined by the rate of conversion of cholesterol to pregnenolone which is catalyzed by the CSCC enzyme complex, that is located at the inner mitochondrial membrane of the Leydig cell.

The production of androgens is mainly under the control of the pituitary hormone luteinizing hormone releasing hormone (LH), which regulates the production of steroids through the activity of CSCC. LH regulates the rate of production of pregnenolone formation after binding to the plasma membrane of the cell via an intracellular transducing mechanism. Different "second messengers", such as cAMP and Ca^{2+}, are involved in the primary transduction of the signal. Other processes in the cell such as protein synthesis and the rate of transfer of cholesterol to the inner mitochondrial membrane are also involved in regulation of steroid production.

As presented in chapter 1, spontaneous intermembrane transfer of cholesterol is slow and it can therefore be expected that the acute stimulation of steroid production involves a facilitated transfer of cholesterol in Leydig cells. In search for the processes which link second messengers with activation of CSCC several proteins have been implicated among which is sterol carrier protein 2 (SCP_2) .
From studies in the rat adrenal it has been concluded that SCP_2 acts as carrier protein and transfers cholesterol in a 1:1 molar complex from intracellular membranes to the outer mitochondrial membrane and from the outer mitochondrial membrane to the inner mitochondrial membrane viz. to CSCC. The amount of soluble SCP_2

in steroidogenic cells could thus be a rate-limiting factor for the supply of cholesterol.

To obtain a better understanding of the role of SCP_2 in testicular steroidogenesis the cellular localization and hormonal regulation of soluble SCP_2 in the rat testis in vivo have been investigated (**chapter** 2). It was found that SCP_2 is concentrated in the Leydig cells and that the amount of SCP_2 in the soluble fraction is influenced by circulating LH levels. The specific localization of SCP_2 in the Leydig cells and the LH-dependent concentration in the soluble fraction, support the possibility that SCP_2 plays a role in the regulation of steroidogenesis in the Leydig cell by regulating the availability of cholesterol for the CSCC. This was further substantiated by studies described in **chapter** 4 which showed that SCP_2 added to tumour Leydig cell mitochondria, stimulates pregnenolone production 2-to 3-fold. This stimulatory effect was abolished by addition of a polyclonal anti-SCP_2 antibody. Addition of this antibody also decreased the mitochondrial pregnenolone production, presumably by binding of the antibody to SCP_2 located at the outer mitochondrial membrane.

The regulation of soluble SCP_2 levels has been further studied using isolated Leydig cells (**chapter** 3). It was shown that Ca^{2+} and possibly protein kinase C are the signal transducing systems which are involved in the short-term regulation of soluble SCP_2 levels by LH.

Since a high molecular weight protein (50-60 kDa) is present in Leydig cells that cross-reacts with anti-SCP_2-antibodies on a Western blot (**chapter** 8) it has been assumed that the amount of soluble SCP_2 (14 kDa) is regulated by proteolytic cleavage of a high molecular weight precursor. However, kinetics of the LH-dependent regulation of SCP_2 levels (**chapter** 3) do not support this assumption, nor a regulation of SCP_2 levels by de novo synthesis. Possible relations between SCP_2 and the 50-60 kDa protein are discussed in **chapter** 8.

In **chapters** 4 and 5 it is shown that the rise of SCP_2 in supernatant fractions can be explained by a relocation of

(loosely bound) SCP_2 from all particulate fractions (except the lysosomal fraction) to the supernatant fraction. It is not certain whether hormonal stimulation results in a true intracellular redistribution of SCP_2 or in an altered binding of the protein to membranes to such extent that it can be released during homogenization. Whatever explanation is correct, the data show that LH can modify the interaction between SCP_2 and membranes. Since Ca^{2+} is important in the LH-induced regulation of soluble SCP_2 levels it is postulated that the association of SCP_2 with membranes is modulated by Ca^{2+}.

Chapter 6 presents results on the subcellular distribution of SCP_2 in mouse tumor Leydig cells. Cells of a subclone of the MA-10 cell line (clone LP) produce low amounts of steroids after hormonal stimulation and have an impaired association of SCP_2 with membranes. It was suggested that the impaired association of SCP_2 with membranes represents (part of) the abnormalities in the production of steroids in clone LP.

Determination of the amount of SCP_2 in the different subcellular fractions by an enzyme immunoassay requires disruption of the cells. To determine the localization in intact cells, we have used immunocytochemistry (**chapter** 5). This technique showed co-localization of SCP_2 and the peroxisomal marker enzyme catalase, which suggests that SCP_2 is localized in the peroxisomes. The apparent discrepancy on the localization of SCP_2 between data obtained by the use of immunocytochemistry and enzyme immunoassay are discussed in **chapter 5 and 8**.

The amount of soluble SCP_2 and the rate of steroidogenesis are not correlated (**chapter** 3). Moreover, steroidogenically active Leydig cells also appear to contain a low level of SCP_2 in the mitochondria (**chapter 5**). From these results it was concluded that the amount of SCP_2 is not a rate-limiting factor in the regulation of the rate of steroid production.

To investigate the affinity of SCP_2 for cholesterol we used equilibrium dialysis studies. In these studies 25-hydroxycholesterol was used rather than cholesterol since the latter did not

penetrate the dialysis tubing. The affinity of SCP_2 for the sterol 25-hydroxycholesterol is described in **chapter 7**. It was shown that pure SCP_2 does not bind 25-hydroxycholesterol. Association between SCP_2 and 25-hydroxycholesterol occured only in the presence of other proteins but not when Ca^{2+} was omitted from the dialysis buffer. It is postulated that interaction between SCP_2 and (25-hydroxy)cholesterol is modulated by positive charges on proteins and Ca^{2+}.

In **chapter 8** different concepts are discussed which have been developed during the course of this study on the possible role of SCP_2 in regulation of Leydig cell steroidogenesis. Initially, it was proposed that the rate of steroid production was dependent on the amount of soluble SCP_2. However, the data obtained support the hypothesis that the LH-dependent regulation of steroid production depends more on regulation of the activity of SCP_2, the latter being influenced by interactions with membranes.

It is proposed that a specific association of SCP_2 in the membranes results in formation of a dimer of SCP_2. Such a dimer might function as a protein "bridge" between membranes. The non-polar tail of cholesterol may move along this protein bridge, the flow of cholesterol being directed by a gradient of cholesterol which is formed by the formation of steroids. Therefore we assume that a minimal amount of SCP_2 is important for the regulation of Leydig cell steroidogenesis.

SAMENVATTING

In het mannelijk geslachtsorgaan, de testikel of zaadbal, worden steroid hormonen (androgenen) gevormd. Androgenen zijn belangrijk voor de rijping van de zaadcellen en de ontwikkeling en in stand houding van de mannelijke geslachtskenmerken. De produktie van androgenen in de testikel vindt plaats in de cellen van Leydig. De snelheid waarmee androgenen gevormd worden is afhankelijk van de snelheid waarmee cholesterol wordt omgezet in pregnenolon. Deze omzetting wordt gekatalyseerd door het cholesterol-zijketen splitsend enzym complex dat in de Leydig cellen gelegen is in de binnenmembraan van de mitochondriën.

De produktie van steroid hormonen in de Leydig cel wordt voornamelijk gereguleerd door het eiwit hormoon LH. LH wordt gemaakt in de hypofyse (hersenaanhangsel) en kan de aktiviteit van het cholesterol-zijketen splitsend enzym complex in de Leydig cel reguleren. Na binding van LH aan de plasma membraan (buitenkant) van de Leydig cel, wordt binnen in de cel door zogenaamde "tweede boodschappers", zoals cAMP en calcium-ionen, een signaal doorgegeven aan andere boodschappers of processen in de cel die betrokken zijn bij de regulatie van steroid produktie. De synthese van eiwitten en de snelheid waarmee cholesterol naar de binnen membraan van de mitochondriën wordt getransporteerd, spelen ook een rol in de regulatie van steroid produktie.

In **hoofdstuk** 1 wordt beschreven dat cholesterol spontaan slechts met een kleine snelheid kan worden uitgewisseld tussen membranen in de cel. Het lijkt dus geoorloofd aan te nemen dat de snelle aktivering van steroid produktie gepaard gaat met een aktieve overdracht van cholesterol naar het cholesterol-zijketen splitsend enzym complex. In **hoofdstuk** 1 worden verschillende processen beschreven die bij een aktief cholesterol transport een rol kunnen spelen. Het eiwit "sterol carrier protein 2" (SCP_2; vrij vertaald: "dragereiwit van cholesterol") maakt hier deel van uit.

Gebaseerd op studies met bijnierweefsel is voor de rol van SCP_2 in steroid producerende cellen de volgende hypothese gefor-

muleerd. Na binding van cholesterol aan SCP_2 (in een 1:1 verhouding) kan cholesterol, dat normaal slecht in water oplost, door de waterige fase (verder aangeduid als supernatans) van de cel getransporteerd worden. Transport vindt plaats van membranen in de cel naar de buiten membraan van de mitochondriën en hiervandaan naar de binnen membraan van de mitochondriën waar zich het cholesterol-zijketen splitsend enzym complex bevindt. De snelheid waarmee steroiden worden gevormd zou dus mede bepaald kunnen worden door de hoeveelheid SCP_2 molekulen in de supernatans van de cel.

Om de rol van SCP_2 in de regulatie van steroid produktie in de testis beter te begrijpen is de hoeveelheid SCP_2 gemeten m.b.v. een enzym immunoassay in supernatantia van ratte testes en hieruit geisoleerde cellen. Door een voorbehandeling van de supernatantia wordt alleen SCP_2 (14 kDa) gemeten. Andere eiwitten die een kruisreaktie vertonen met het in de enzym immunoassay gebruikte antilichaam worden verwijderd. In **hoofdstuk** 2 wordt beschreven dat SCP_2 alleen meetbaar is in Leydig cellen en niet in andere testiculaire cellen. Tevens wordt de hoeveelheid SCP_2 in de testis gereguleerd door de hoeveelheid LH in de bloedsomloop. De specifieke lokalisatie van SCP_2 in de Leydig cel en de LH-afhankelijke regulatie van de hoeveelheid SCP_2 in het supernatant, duiden er op dat SCP_2 een rol kan spelen bij de regulatie van steroid produktie in de Leydig cel. Dit wordt bevestigd door de waarneming dat de produktie van pregnenolon door mitochondriën uit Leydig cellen toeneemt met een faktor 2-3 na toevoeging van SCP_2 (**hoofdstuk** 4). Het stimulerende effekt van SCP_2 op de pregnenolon produktie kan worden opgeheven door antilichamen tegen SCP_2 toe te voegen. De pregnenolon produktie is dan zelfs lager, mogelijk door binding van antilichamen aan SCP_2 dat geassocieerd is met de buitenmembraan van mitochondriën.

Gebruik makend van geisoleerde Leydig cellen, is onderzocht welke "tweede boodschappers" betrokken zijn bij de LH-afhankelijke regulering van de hoeveelheid SCP_2 in het supernatant van de Leydig cel (**hoofdstuk** 3). Calcium-ionen en mogelijk ook

protein kinase C blijken betrokken te zijn bij een snelle regulering van de hoeveelheid SCP_2 in het supernatant. De synthese van nieuwe SCP_2 molekulen (**de novo** eiwitsynthese) zou kunnen leiden tot een toename van de hoeveelheid SCP_2. Synthese van eiwitten neemt echter uren in beslag, terwijl de hoeveelheid SCP_2 in het supernatans van Leydig cellen reeds na 2 minuten is toegenomen o.i.v. LH (**hoofdstuk** 3). Aangezien een eiwit met een hoger molekuulgewicht (50-60 kDa) dan SCP_2 (14 kDa) op een Western blot reageert met een antilichaam dat is opgewekt tegen gezuiverd SCP_2, zou de hoeveelheid SCP_2 gereguleerd kunnen worden door afsplitsing van SCP_2 uit dit grotere molekuul (proteolyse van een precursor). De kinetiek van de LH-afhankelijke toename van de hoeveelheid SCP_2 in het supernatant maakt ook regulatie door proteolyse van een precursor onwaarschijnlijk. Mogelijke relaties tussen SCP_2 en het 50-60 kDa eiwit worden besproken in **hoofdstuk 8**.

Uit de resultaten in **hoofdstuk 4** blijkt dat de LH-afhankelijke toename van de hoeveelheid SCP_2 in het supernatant van de Leydig cel verklaard kan worden door een herverdeling van SCP_2 binnen de cel. Onder invloed van LH verhuist SCP_2 van membranen naar het supernatant. Na opsplitsing van "membranen" in verschillende frakties d.m.v. centrifugatie, blijkt dat de hoeveelheid SCP_2 afneemtin vrijwel alle membraanfrakties in de cel. De enige uitzondering vormt de lysosomale fraktie (**hoofdstuk** 5). Gekonkludeerd kan worden dat LH, mogelijk via Ca^{2+} en protein kinase C, de interaktie tussen SCP_2 en membranen kan veranderen. In een mutant van de muize tumor Leydig cel MA-10, kloon LP, is een verstoorde associatie van SCP_2 met membranen (**hoofdstuk** 6) mogelijk (deels) een oorzaak van de lage steroid produktie wanneer hormoon wordt toegevoegd.

Omdat voor meting van de hoeveelheid SCP_2 de cellen niet intakt blijven, is ook gebruik gemaakt van immunocytochemie op gefixeerde (intakte) cellen om de lokalisatie van SCP_2 in de Leydig cel vast te stellen. Het antilichaam tegen SCP_2 blijkt dan hoofdzakelijk in de peroxisomen van de Leydig cel gebonden te

worden. Of 14 kDa SCP_2 in de peroxisomen gelokaliseerd is, is niet zeker omdat bij deze behandeling het antilichaam ook andere eiwitten kan herkennen (**hoofdstuk** 5 **en** 8).

De snelheid van steroid produktie blijkt niet overeen te komen met de hoeveelheid SCP_2 in het supernatant (**hoofdstuk** 3) of in de membranen (**hoofdstuk** 5) van de Leydig cel. Kennelijk bepaalt de hoeveelheid SCP_2 niet de snelheid waarmee steroiden gevormd worden. Tevens is waargenomen dat SCP_2 25-hydroxycholesterol niet kan binden (**hoofdstuk** 7). Gebruik makend van een evenwichtsdialyse is bepaald dat de associatie van SCP_2 en 25-hydroxycholesterol slechts plaatsvindt als tevens andere eiwitten en calcium ionen aanwezig zijn.

Op grond van de resultaten beschreven in dit proefschrift, is de opvatting over de rol van SCP_2 in de regulatie van steroid produktie in de Leydig cel veranderd. Bij de aanvang van dit onderzoek werd verondersteld dat een LH-afhankelijke regulatie van de <u>hoeveelheid</u> SCP_2 in het supernatans bij zou kunnen dragen aan de regulatie van de snelheid van steroid produktie. Op dit moment veronderstellen wij dat de <u>aktiviteit</u> van SCP_2 wordt gereguleerd door LH via de vorming van een SCP_2-dimeer. Deze dimeer zou een "eiwit brug" kunnen slaan tussen membranen. De apolaire staart van cholesterol kan zich dan over deze brug bewegen, waardoor cholesterol tussen membranen overgedragen kan worden. De richting waarin het cholesterol zich verplaatst, wordt dan bepaald door een gradiënt van cholesterol in de cel die ontstaat door het verbruik van cholesterol bij de vorming van steroiden in de mitochondriën. Een kleine hoeveelheid SCP_2 is dan noodzakelijk voor het handhaven van een evenwicht van cholesterol binnen de cel.

DANKWOORD

Nu de resultaten van de proeven op schrift zijn gesteld in dit proefschrift, wil ik iedereen bedanken die hieraan een bijdrage heeft geleverd. Met name:

Mijn co-promotor, Focko Rommerts, die aan de opzet en uitvoering van dit onderzoek de belangrijkste bijdrage heeft geleverd. Focko, ik dank je voor de enthousiaste begeleiding, de gouden snoepjes, de nodige vrijheid, de vele suggesties en ook voor de snelle korrektie rondes bij het onstaan van dit proefschrift.

Mijn promotor, Henk van der Molen, voor de mij geboden mogelijkheid om aan de Fakulteit der Geneeskunde te promoveren na vier keer te zijn uitgeloot voor de desbetreffende studie. Henk, helaas was je er te weinig om mijn onderzoek op de voet te kunnen volgen. Bedankt voor de vaak subtiele wijzigingen die je aanbracht in de engelse tekst van mijn proefschrift.

De leden van de promotiecommissie, prof.dr. Hülsmann, prof.dr. Brownie en prof.dr. Wirtz voor hun bereidheid het manuscript zo snel te beoordelen.
I thank you, Alistair, for the thorough reading of my manuscript.

Zonder de medewerking van Karel Wirtz en Aart van Amerongen was dit onderzoek niet mogelijk geweest. Juliie beiden bedank ik voor een uiterst plezierige samenwerking (ook nu, Aart) die leidde tot de koppeling van twee vakgebieden (vandaar de 2 in SCP_2).

Mijn beide paranimfen, Gea Dreteler en Lizzy van Haren wil ik bedanken voor de gezelligheid tijdens respektievelijk het begin en einde van mijn promotietijd. Lizzy, door alle kleine korrekties die jij aanbracht in figuren en tekst, heb je mij veel tijd bespaard.

Voor de hulp die ik kreeg bij de praktische uitvoering van dit onderzoek ben ik Willeke van Laar, Ruud Jansen en Paul Reuvers veel dank verschuldigd. Dat het een "seriously complicated protein" was, hebben jullie gemerkt.

De (overige) leden van "KLM", Jos Hoogerbrugge (door jouw geduld kwam ik over mijn angst voor ratten heen), Roel Melsert (de ellenlange "labbabbels" en jouw collegialiteit zal ik niet snel vergeten), Ria Dijkhof (bedankt Ria, voor de interesse en de ria's) en Axel Themmen (wie deed er nu ook al weer het denkwerk?) bedank ik voor de goede sfeer.

Douglas Stocco, I thank you for the many stimulating discussions, for the warm welcome in Lubbock and for the pleasant collaboration which resulted in chapter 6 of this thesis. I thank Michael Kilgore for all the MA-10 samples and data.

Katja Teerds, dat jij tijd hebt vrijgemaakt om voor mij de titelpagina en de EM-opnames van dit proefschrift te verzorgen, heb ik zeer gewaardeerd. Bedankt ook voor alle raad bij de opmaak van het manuscript, alle dropjes en de voortdurende samenwerking.

Alle Biochemie II'ers bedank ik voor de gezelligheid en de uitvoering van de vele "kleine" werkzaamheden (zoals reparaties: Pim, typen en de regelklusjes: Marja en Rosemarie, medium bereiden: Marianna etc., etc.) die mijn onderzoek draaiende hielden.

Mijn ouders dank ik voor de gelegenheid te kunnen studeren. Pa en ma, jullie voortdurende belangstelling en steun is tijdens mijn hele opleiding een grote stimulans geweest.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 20 augustus 1959 geboren te Utrecht. Na het doorlopen van het gymnasium, werd in 1977 het VWO-B diploma behaald aan het Revius Lyceum te Doorn. In 1977 werd begonnen met de studie biologie aan de Vrije Universiteit te Amsterdam alwaar in 1980 het kandidaatsexamen B1 werd behaald. In 1985 werd het doktoraalexamen behaald met als hoofdvak biochemie (Prof.dr. G.S.P. Groot en Prof.dr. R.J. Planta) en als bijvakken molekulaire genetica (Prof.dr. H.J.J. Nijkamp) en microbiële fysiologie (Prof.dr. A.H. Stouthamer). Tevens werd de bevoegdheid tot het geven van onderwijs in de biologie verkregen. Vanaf april 1985 tot oktober 1988 was zij werkzaam als wetenschappelijk assistente op de afdeling Biochemie II van de Faculteit der Geneeskunde van de Erasmus Universiteit te Rotterdam, alwaar het in dit proefschrift beschreven werk werd verricht. Sinds februari 1989 is zij als wetenschappelijk medewerkster verbonden aan het Centraal Diergeneeskundig Instituut te Lelystad.