Annexin V in the rat heart

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ANNEXIN V IN THE RAT HEART

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
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The truth is out there
The X-files

Aan mijn ouders
Voor Robert

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ANF	atrial natriuretic factor
BSA	bovine serum albumine
CFLC	cardiac fibroblast-like cells
CMC	cardiomyocytes
DG	diacylglycerol
ELISA	enzyme-linked immunosorbent assay
FA	fatty acids
FCS	foetal calf serum
FTTC	fluorescein isothiocyanate
HPLC	high-performance liquid chromatography
Ig	immunoglobulin
kDa	kiloDalton
LDH	lactate dehydrogenase
mRNA	messenger ribonucleic acid
Mw	molecular weight
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PE	phenylephrine
PI	phosphatidylinositol
PL	phospholipid
PLA ₂	phospholipase A ₂
PS	phosphatidylserine
RHEC	rat heart endothelial cell
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TG	triacylglycerol
WKY	Wistar Kyoto

Chapter 1

General introduction

1.1 GENERAL SCOPE

Annexins constitute a family of structurally related proteins with phospholipid and calcium-binding properties (Raynal and Pollard, 1994; Kaetzel and Dedman, 1995). At present thirteen different members of the family have been identified. Annexins are widely distributed. They have been detected in almost all tissues. In cardiac tissue annexin V seems to be one of the most predominant annexins (Doubell *et al.*, 1993; Pula *et al.*, 1990). The physiological significance of annexin V in the heart has not yet been elucidated. However, as the annexins are known to possess a diverse range of properties *in vitro*, multiple *in vivo* actions of the annexins in the heart can be envisaged. Members of the annexin family have been found to inhibit phospholipid hydrolyzing enzymes, like phospholipase A₂, in *in vitro* experiments (Ahn *et al.*, 1988; Davidson *et al.*, 1987). This suggests that they may play a role in the regulation of the phospholipid homeostasis *in vivo*. In the present thesis the possible role of annexin V in the regulation of the phospholipid homeostasis was investigated on artificial membranes and in energy-deprived cardiac myocytes. Annexins have also been thought to be implicated in growth and differentiation processes in different tissues and cell types. As such annexin V could be involved in the control of cardiac maturational or pathological growth. Therefore, attention has been paid to the annexin V localization and quantity during physiological and pathological growth of the heart, in order to get an indication whether annexin V might play a role in these processes.

The first sections of this chapter will give an overview on the annexin family, cardiac phospholipid homeostasis, the events occurring during cardiac energy-deprivation, such as ischemia and metabolic inhibition, and the possible role of annexins therein. In the last sections the possible relation of annexins with maturational and hypertrophic growth processes in the heart will be described. Finally the aims of the experimental part of this thesis will be presented.

1.2 THE ANNEXIN FAMILY

1.2.1 Biochemical characteristics of the annexins

In the last decade the annexins have been investigated in multiple areas of research, which resulted in a lot of different names for the same proteins (for review see Raynal and Pollard, 1994). An overview of the nomenclature of the annexins is given in table 1.1. At present thirteen different members of the family have been identified. The molecular weight of most annexins ranges from 35 to 40 kDa, except for annexin VI (68-76 kDa), annexin VII (47-52 kDa) and annexin XI (54 kDa).

The structural relationship of the annexins is based on the presence of a 4- or 8-fold repeated domain of 65-70 amino acid residues, each repeat containing a 17 amino acid consensus sequence, the so-called "endonexin fold" (Geisow *et al.*, 1986). Based on this structural relationship an evolutionary model was proposed in which the annexin family evolved by gene-duplication events from an ancestor gene encoding a single domain

Table 1.1. Nomenclature of the annexins

Annexin	Synonym	kDa
I	lipocortin I (lipomodulin, macrocortin, renocortin) p35 (EGF receptor substrate) calpactin II chromobindin 9	38.6
II	calpactin I heavy chain p36 (pp60src substrate) protein I lipocortin II chromobindin 8	38.5
III	lipocortin III placental anticoagulant protein (PAP)-III 35- α -calcimedlin calphobindin III	36.2
IV	inositol-1,2-cyclic-phosphate 2-hydrolase endonexin I protein II 32.5K-calelectrin chromobindin 4 placental anticoagulation protein (PAP)-II placental protein PP4-X 35- β -calcimedine lipocortin IV	35.7
V	placental anticoagulant protein (PAP)-I endonexin II inhibitor of blood coagulation 35K-calelectrin placental protein PP4 vascular anticoagulant (VAC) ancherin CII 35- τ -calcimedlin calphobindin I lipocortin V	35.8
VI	p68 67K-calelectrin protein III chromobindin 20 67-calcimedlin calphobindin II synhibin lipocortin VI	75.7
VII	synexin	50.2
VIII	vascular anticoagulant (VAC) β	36.7
IX	<i>Drosophila melanogaster</i> annexin	(32.9)
X	<i>Drosophila melangaster</i> annexin	35.6
XI	calcyclin-associated annexin (CAP)-50	53.9
XII	<i>Hydra vulgaris</i> annexin	35.0
XIII	intestine-specific annexin (ISA)	35.3

Molecular masses (expressed as kDa) are given according to the cDNA sequences from human [I (Wallner *et al.*, 1986), II (Huang *et al.*, 1986), III (Pepinsky *et al.*, 1988), IV (Grundmann *et al.*, 1988), V (Funakoshi *et al.*, 1987), VI (Crompton *et al.*, 1988; Sudhof *et al.*, 1988), VII (Burns *et al.*, 1989), VIII (Hauptmann *et al.*, 1989) and XIII (Wice and Gordon, 1992)], bovine [XI (Towle and Treadwell, 1992)], *Drosophila* [IX is a partial clone and X (Johnston *et al.*, 1990)], and *Hydra* [XII (Schlaepfer *et al.*, 1992)].

(Crompton *et al.*, 1988). This would explain why the various members of the annexin family share a high degree of homology of the internal repeats (Hauptmann *et al.*, 1989). Interspecies differences for any given annexin are small. For example, the core sequence of human and rat annexin V share 89% identity at the amino acid level (Pepinsky *et al.*, 1988). Poor homology has been found in the amino terminal tail for the different members of the annexin family.

The crystal structures of human (Huber *et al.*, 1990; Huber *et al.*, 1990; Lewit-Bentley *et al.*, 1992; Sopkova *et al.*, 1993), chicken (Bewley *et al.*, 1993) and rat (Concha *et al.*, 1993) annexin V have been determined, as well as that of human annexin I (Weng *et al.*, 1993). The proteins are composed of four structural domains corresponding to the four repeats. Each domain consists of five homologous amphiphilic α -helices organized in a right-handed superhelix. Domains I and IV, and II and III, respectively, form tight modules with approximate two-fold symmetry. The 4 superhelix domains are folded into an almost planar array with a convex and a concave face and a hole in its center with charged residues.

Recently, the membrane-bound structure of annexin V has been analyzed (Pigault *et al.*, 1994; Voges *et al.*, 1994). It was shown that the membrane binding is mediated by the convex surface of the molecule that contains the Ca^{2+} -binding sites. The binding sites become coplanar with the membrane and the annexin V molecule does not penetrate into the membrane.

The ability to bind calcium and phospholipids is the common denominator of all annexins. The calcium binding sites, three in number for annexin V, are located in domains I, II, and IV (Huber *et al.*, 1990). Recently, it was demonstrated that a Ca^{2+} -induced conformational change of rat annexin V leads to the formation of a fourth Ca^{2+} -binding site in domain III (Concha *et al.*, 1993). This reorganization also leads to the loss of the two calcium binding sites located in domains II and IV. Specific amino acid residues involved in the binding of calcium have been identified in annexin I, II and V. This model was recently disputed by Swairjo and associates (1994) who found that one annexin V molecule is able to bind 12 Ca^{2+} molecules. The intrinsic affinity of the annexins, with exception of annexin VI, for calcium is relatively low. However, in the presence of phospholipids the affinity for calcium increases dramatically (Schlaepfer *et al.*, 1987). The annexins bind to negatively charged, acidic phospholipids with very high affinity. They display highest affinity for phosphatidic acid, followed by phosphatidylserine and subsequently phosphatidylinositol. Binding to phosphatidylethanolamine is very weak and to phosphatidylcholine and sphingomyelin negligible. The calcium concentrations for half-maximal binding of the annexins to phosphatidic acid or phosphatidylserine are in the range of 0.2 to 16 μM and increase in the order annexin II, I, VI, IV, III and V (Blackwood and Ernst, 1990).

1.2.2 Localization of the annexins

Annexins can be found in many different tissues and cell types, but individual family members have distinct distributions. For example, annexin XIII has only been found in the

crypt epithelial cells and enterocytes of the intestine (Wice and Gordon, 1992), while annexin V has been detected in almost all tissues. Especially highly vascularized organs, tissues rich in endothelial cells, contain high annexin V concentrations. Annexin V, for example, has been found in human placenta (Ahn *et al.*, 1988; Bohn *et al.*, 1979; Buhl *et al.*, 1991; Funakoshi *et al.*, 1987a; Römisch and Heimbürger, 1990; Tait *et al.*, 1988), arteries of the umbilical cord (Reutelingsperger *et al.*, 1985), bovine aorta (Reutelingsperger *et al.*, 1988) and human and bovine lung (Almus *et al.*, 1993; Boustead *et al.*, 1988; Khanna *et al.*, 1990).

Intracellularly, annexins have been found to be associated with the plasma membrane (Iida *et al.*, 1992; Paffle *et al.*, 1988; Semich *et al.*, 1989) as well as to be localized in the cytoplasm (Koster *et al.*, 1993; Raynal *et al.*, 1992; Schaefer *et al.*, 1992; Sun *et al.*, 1992). Some annexins were also detectable in the nucleus (Sun *et al.*, 1992; Koster *et al.*, 1993). Although annexin V lacks a secretory signal sequence, it has been recovered from extracellular fluids like blood, amniotic fluid and seminal plasma (Christmas *et al.*, 1991; Flaherty *et al.*, 1990; Römisch *et al.*, 1992). In addition, Genge and co-workers (1991) found that annexins present in matrix vesicles from mineralizing tissues selectively partitioned into the lipophilic milieu under acidic conditions. They suggested that under acidic conditions annexins undergo a conformational change, which imparts a marked increase in the hydrophobicity of the proteins. However, whether the presence of the annexin V in the extracellular compartment is due to active secretion or to loss from dead cells is still largely unknown.

In heart tissue annexin V and VI appear to be the predominant annexins (Doubell *et al.*, 1993; Pula *et al.*, 1990). Immunohistochemical studies on rat heart tissue indicate that most annexin V is present in the non-myocytes (Doubell *et al.*, 1993; Giambanco *et al.*, 1991). Regarding the presence of annexin V in the myocytes conflicting results have been reported. Using westernblot analysis (using a polyclonal antibody raised against human recombinant annexin V) Van Bilsen and co-workers (1992) failed to detect annexin V in myocytes isolated from adult rat hearts. In contrast, Giambanco and colleagues (1991) and Doubell and associates (1993) using polyclonal antibodies raised against porcine lung and rat heart, respectively, were able to detect annexin V in rat cardiac myocytes. Moreover, annexin V was found to be either associated with the sarcolemma (Giambanco *et al.*, 1991; Luckcuck *et al.*, 1995) or to be confined to the cytoplasm (Doubell *et al.*, 1993). Annexin VI was found in myocytal as well as non-myocytal cells (Doubell *et al.*, 1993). In the myocytes annexin VI was located at the level of the sarcolemma (Iida *et al.*, 1992; Luckcuck *et al.*, 1995).

Skeletal muscle was found to contain mainly annexin V (Reutelingsperger *et al.*, 1994) and VI (Tagoe *et al.*, 1994). However, the annexin V content proved to be much higher than annexin VI levels (Giambanco *et al.*, 1993). Annexin V was diffusely distributed in skeletal muscle cells (Giambanco *et al.*, 1991). In contrast, annexin VI was reported to be localized in close vicinity of the sarcolemma and the transverse tubules (Iida *et al.*, 1992). Annexin VI has also been found in colocalization with the Ca^{2+} -ATPase of the sarcoplasmic reticulum, suggesting a regulatory role in the Ca^{2+} -release/uptake cycle in the sarcoplasmic

reticulum (Hazarika *et al.*, 1991).

1.2.3 Proposed functions of the annexins

Although many *in vitro* actions have been described for the annexins, their physiological significance is largely unknown. Putative functions of the annexins are: i) anticoagulant activity, ii) component of cellular structures, iii) ion-channel activity, iv) a role in vesicular transport (preceding putative functions will be discussed in the present paragraph in detail), v) anti-phospholipase activity (see paragraph 1.3.4 for a detailed report), and vi) a role in growth and differentiation associated processes (see paragraph 1.4.2 for a detailed discussion).

One of the proposed functions for annexins is the anticoagulant activity of these proteins (Reutelingsperger *et al.*, 1985). Annexin V has been found to be an effective inhibitor of the prothrombinase and tenase complex (Andree *et al.*, 1992; Almus *et al.*, Hauptman *et al.*, 1989; Rao *et al.*, 1992; Reutelingsperger *et al.*, 1988). In blood coagulation the negatively charged phospholipids form the catalytic surface to which the different coagulant complexes assemble. In *in vitro* clotting studies annexins have been shown to prevent the formation of these complexes by shielding off the negatively charged phospholipids.

It has also been proposed that annexins are a component of cellular and extracellular structures. Annexins not only bind to phospholipids but also to some structural proteins. Annexin V has been found to bind to collagen types I, II, III, V and X, to chondrocalcin (C-propeptide of type II collagen), to the cytoskeleton, and to F-actin (Giambanco *et al.*, 1990; Kirsch and Pfäffle, 1992; Mollenhauer and Von der Mark, 1983; Schlaepfer *et al.*, 1987; Wu *et al.*, 1991).

Some studies indicate that annexins might possess ion-channel activity. Annexin I, V, VI and VII have been found to form Ca^{2+} -selective, voltage-dependent ion channels in artificial phospholipid bilayers (Pollard *et al.*, 1988; Pollard *et al.*, 1992; Rojas *et al.*, 1990). In a recent study it was shown that overexpression of annexin VI in transgenic mice was associated with marked changes in cardiac Ca^{2+} transients (Genteski-Hamblin *et al.*, 1996). The disturbances in Ca^{2+} homeostasis were suggested to be due to annexin VI-mediated dysregulation of sarcolemmal ion pumps and/or exchangers. The structure of the annexin V molecule, with its central hydrophilic pore (see section 1.2.2), suggests an ion channel conduction pathway (Berendes *et al.*, 1993). Huber and colleagues (Huber *et al.*, 1992) proposed that the molecule forms a voltage-dependent Ca^{2+} -channel in phospholipid bilayers with key amino acid residues acting as selective filters and voltage sensors, thereby regulating the permeability of the channel pore (Demange *et al.*, 1994). As a consequence annexins might have a function in calcium transport. This hypothesis has been challenged by Goossens and colleagues (1995), who failed to show transport of Ca^{2+} by annexin V, using large unilamellar phospholipid vesicles.

Finally, it has been suggested that annexins play a role in vesicular transport (for review see Burgoyne and Clague, 1994). First, experimental observations showed that some

annexins not only bind to phospholipids, but also promote aggregation of phospholipid vesicles with biological membranes. Early studies indicated that annexin II might be involved in Ca^{2+} -dependent exocytosis (Burgoyne, 1988; Drust and Creutz, 1988). It was suggested that annexin II concentrates early endosomes (large vesicles formed by fusion of coated vesicles with other vesicles) at the plasma membrane and facilitates vesicle aggregation, fusion, and targeting. In addition, annexin I becomes phosphorylated in the multivesicular body (membranous structures containing numerous vesicles) and, hence, has been suggested to be involved in this aspect of vesicular transport (Futter *et al.*, 1993). Furthermore, annexin VI was proposed to be required in coated vesicle budding (Lin *et al.*, 1992). However, recent findings do not support the latter notion (Smythe *et al.*, 1994).

1.3 PHOSPHOLIPID HOMEOSTASIS IN THE HEART

1.3.1 The cardiac membrane phospholipids

All cells are surrounded by a plasma membrane which is predominantly composed of proteins, cholesterol and phospholipids. The phospholipid molecule (see figure 1.1) contains a hydrophilic headgroup and a hydrophobic tail attached to a glycerol backbone. This hydrophobic tail comprises two long-chain fatty acyl chains covalently bound to the first (sn-1) and second (sn-2) carbon atom of the glycerol backbone. An alcohol molecule is bound via an inorganic phosphate group to the third carbon atom (sn-3) of the glycerol backbone.

Figure 1.1. Schematic representation of the chemical structure of diacylphospholipids. R_1 and R_2 are fatty acyl chains; X is commonly choline, ethanolamine, serine, inositol, or glycerol. The specific sites of action of the various phospholipases are indicated by the arrows. Numbers refer to sn-1, sn-2 and sn-3 positions of the glycerol carbon-atoms.

This alcohol, which can be choline, ethanolamine, serine, inositol or glycerol, determines the nature of the hydrophilic headgroup. In the hydrophobic tail the number of carbon atoms and the number of unsaturated bonds in the fatty acyl moieties may vary. A phospholipid can also be present in the plasmalogen form, in which the hydrophobic tail at the sn-1 position is connected to the first carbon atom of the glycerol backbone by a vinyl ether linkage instead of an ester bond.

In cardiac tissue phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant phospholipids. Phosphatidylinositol (PI) and phosphatidylserine (PS) are present in relatively small amounts (Roquelin *et al.*, 1989). In cardiac myocytes the majority of the phospholipids is part of the intracellular membranes. Only a small amount, in the order of 5-10%, is located in the sarcolemma (Corr *et al.*, 1984). Membrane phospholipids form a bilayer with their hydrophobic tails in the inner and outer leaflet pointing to each other. The hydrophilic headgroups of both leaflets are located towards the cytosolic compartment and extracellular space, respectively. The distribution of the various phospholipid species over the inner and outer leaflet of the sarcolemma is asymmetric. The main species of the outer leaflet are PC and sphingomyelin, whereas the inner leaflet consists of PC, PE and PI. PS is almost exclusively present in the inner leaflet (Post *et al.*, 1988). Because the number of unsaturated carbon-carbon linkages in the fatty acid tails is lower in the choline phospholipids than in the amino phospholipids, this asymmetric distribution would create a lower degree of fluidity of the outer membrane than of the inner membrane layer. To compensate for this difference the outer leaflet contains more cholesterol than the inner leaflet (Fisher, 1976). The predominance of negatively charged headgroups of PI and PS in the inner leaflet causes a slight overall negative charge of the inner membrane surface.

1.3.2 Phospholipid turnover in the normal heart

Under normal circumstances phospholipids are continuously synthesized and degraded. This turnover is required to repair damaged phospholipid molecules and to make adjustments of the membrane to changes in (extra)cellular conditions. Several metabolic pathways of degradation and synthesis of membrane phospholipids are operative in the cardiac myocyte. Specific enzymes, phospholipases, are responsible for the hydrolytic degradation of phospholipids (figure 1.1). Phospholipase A₁ hydrolyses the sn-1 glycerol acylester bond, phospholipase A₂ breaks the sn-2 glycerol acylester bond, phospholipase C attacks the bond between the glycerol backbone and the phosphate group, whereas phospholipase D releases the alcoholic headgroup from the phosphate group. Lysophospholipases remove the fatty acyl moieties of lysophospholipids, which are formed by the hydrolytic degradation of phospholipids by phospholipase A₁ or A₂.

In cardiac tissue several phospholipases are present. However, the release of fatty acids from phospholipids is likely to be achieved by various forms of phospholipase A₂ (Van Bilsen and Van der Vusse, 1995). Until now, three different types of phospholipase A₂ have been detected in cardiac tissue, i.e., group II phospholipase A₂, high molecular weight

phospholipase A₂ and plasmalogen-specific phospholipase A₂. These enzymes display differences in molecular mass, substrate specificity and sensitivity for calcium ions.

Group II phospholipase A₂ has a molecular mass of 15 kDa and was found to be membrane-associated in rat spleen (Ishizaki *et al.*, 1989). Recently, Kriegsmann and co-workers (1993) demonstrated the presence of a membrane-associated phospholipase A₂ in cardiac myocytes using monoclonal antibodies against bee venom phospholipase A₂, which is closely related to the mammalian group I and II phospholipases A₂. Activation of the enzyme is Ca²⁺ dependent (millimolar range) (Ono *et al.*, 1988). Some substrate preference for PE exists compared to PC. This also holds for phospholipids with an ester linkage at the sn-1 position rather than a vinyl ether bond. Current evidence suggests that the activity of group II phospholipase A₂ is mainly regulated by transcriptional means, rather than by posttranslational modification (Van Bilsen and Van der Vusse, 1995).

A phospholipase A₂ which hydrolyzes diacylphospholipids and plasmalogens with a high specificity for sn-2-arachidonyl containing phospholipids was detected by Sharp and White (1993) in cardiac tissue. This phospholipase A₂ has a molecular weight of 80-110 kDa. Under normal conditions the enzyme is localized in the cytosol. Specific stimulation of the cell evokes a translocation of the enzyme from the cytosolic compartment to membrane structures (Clark *et al.*, 1991). This translocation requires a rise in intracellular Ca²⁺ levels (micromolar range). The activity of cytoplasmic phospholipase A₂ is most likely modulated by phosphorylation of the enzyme (Lin *et al.*, 1993).

In addition, a phospholipase A₂ with a preference for plasmalogens is present in the heart. Ford and Gross (1989) identified a phospholipase which was able to hydrolyze the fatty acid at the sn-2 position of plasmalogens. This plasmalogen-specific phospholipase has a molecular mass of circa 40 kDa and does not require Ca²⁺ for catalytic activity. The intracellular localization is both cytosolic and microsomal (Hazen *et al.*, 1992). Furthermore, the activity of the plasmalogen-specific phospholipase A₂ is dependent on the cellular ATP concentration (Hazen and Gross, 1991). Recent findings have shown that the catalytic subunit of this phospholipase A₂ is able to interact with four molecules of phosphofructokinase, thereby forming a 400 kDa protein complex (Hazen *et al.*, 1993 and 1994). The formation of this complex might play a role in the regulation of the activity of plasmalogen-selective phospholipase A₂.

Phospholipases other than phospholipase A₂ have also been identified in several (sub)cellular compartments of the cardiac myocyte. Phospholipase A₁ has been identified in the cytosol and bound to membranes (Arthur *et al.*, 1986; Franson *et al.*, 1978; Nalbony and Hostetler, 1985; Palmer *et al.*, 1981). Phospholipase C is predominantly present in the cytoplasmic compartment and in lysosomes (Franson *et al.*, 1986; Schwartz *et al.*, 1987; Wolf and Gross, 1985). Phospholipase D is also active in cardiac tissue (Lindmar *et al.*, 1988; Panagia *et al.*, 1991).

Resynthesis of phospholipids takes place via a variety of metabolic routes. Lysophospholipids can be reacylated, whereas *de novo* synthesis of the phospholipid molecule can occur with diacylglycerol as intermediate compound. Both pathways are energy-dependent and offer the opportunity to remodel the acyl groups of the phospholipid

molecules. The actual turnover rate of the total cardiac phospholipid pool is not yet known and may be different for each phospholipid species, cellular organel membrane, and cell type (Van der Vusse *et al.*, 1992).

1.3.3 Alterations in phospholipid homeostasis under energy-depriving conditions

Energy-deprivation occurs during conditions such as ischemia and metabolic inhibition. Cardiac ischemia is caused by complete or partial cessation of blood flow. As a consequence supply of oxygen and nutrients are insufficient as is the removal of waste products. Because of the oxygen shortage oxidative mitochondrial metabolism is impaired and NADH and FADH accumulate rapidly in the mitochondrial matrix, resulting in inhibition of fatty acid oxidation and of the citric acid cycle (Van der Vusse *et al.*, 1992). Initially, anaerobic glycolysis delivers energy for ATP regeneration. However, because of accumulation of glycolytic products (lactate, H^+ and NADH) in the cytosolic compartment regulatory enzymes of the glycolytic pathway become inhibited (Mochizuki and Neely, 1979; Rovetto *et al.*, 1975). Thereafter, the cellular ATP level rapidly decreases and its degradation products, like AMP and adenosine, start to accumulate. The fall in ATP levels in cardiac cells causes an inhibition of ATP-dependent processes, such as maintenance of transmembrane ionic gradients. Due to impaired transmembrane transport of ions intracellular Na^+ and Ca^{2+} levels rise. The increase of intracellular calcium activates Ca^{2+} -dependent enzymes, including phospholipases and proteases. Moreover, accumulation of degradation products, such as lactate and inorganic phosphate, leads to an increase of the osmolar load of the cardiac myocytes and, hence, to intracellular water retention.

As a consequence of these events membranes start to lose their function as semi-permeable barriers. Cellular membranes become disrupted and intracellular macromolecules, such as enzymes, are released into the interstitial and vascular compartment. Disruption of the sarcolemma is believed to be a critical event in the process of lethal cell damage (Jennings and Reimer, 1981; Post *et al.*, 1995). Both physical and chemical factors may be responsible for the occurrence of membrane disruption. The physical forces might be mechanical stress and/or osmotic load. Internal reorganisation, like multilammellar vesicle formation and aggregation of membrane proteins, or impaired anchoring of the sarcolemma to the cytoskeleton, might render the membrane more susceptible to physical forces. According to the chemical degradation theory the sarcolemma loses its integrity because of digestion of the phospholipid constituents of the sarcolemma. So, knowledge about the nature of the disturbances in phospholipid homeostasis during ischemia could help to elucidate which processes and factors play a crucial role in the development of irreversible cell damage.

Pioneering studies of Weglicki and co-workers (1973) have demonstrated that arachidonic acid, which is almost exclusively found at the sn-2 position of PC and PE, is released from the endogenous lipid stores after 30 minutes of ischemia and accumulates in the flow-deprived tissue. A number of other studies has confirmed that arachidonic acid, among other fatty acids, increases in the ischemic tissue after 20-45 min of ischemia (Chien

et al., 1984; Van Bilsen *et al.*, 1989). Other degradation products of phospholipids, such as lysophospholipids (Davies *et al.*, 1992; Van Bilsen *et al.*, 1989), diacylglycerols (Ford and Gross, 1989), inositolphosphates (Otani *et al.*, 1988), also accumulate in the ischemic heart. In addition, the total phospholipid pool was found to decline during long-term ischemia (Chiariello *et al.*, 1987; Chien *et al.*, 1979; Chien *et al.*, 1984; Chien *et al.*, 1985).

Net degradation of phospholipids might be caused by impaired phospholipid formation, enhanced phospholipid hydrolysis or a combination of both. Impaired phospholipid (re)synthesis might be related to changes in adenine nucleotide levels in the ischemic myocardium (Chien *et al.*, 1985). It is not likely that the decline in ATP is the cause of the depressed phospholipid (re)synthesis, as the affinity of fatty acylCoA synthetase for ATP is rather high. Accumulation of arachidonic acid already occurs before ATP levels drop below the affinity constant of ATP for the enzyme (Van Bilsen *et al.*, 1989). AMP and adenosine could inhibit fatty acylCoA synthetase activity, because the concentrations in the ischemic heart surpass the concentrations known to block the activity of fatty acylCoA synthetase *in vitro* (Van Bilsen *et al.*, 1989). In addition, the activity of lysophosphatidylcholine acyltransferase has been reported to be diminished in ischemic cardiac tissue (Das *et al.*, 1986; Otani *et al.*, 1986).

Enhanced phospholipid hydrolysis, leading to irreversible loss of cellular integrity in flow deprived myocardial tissue, may be caused by an uncontrolled increase in the activity of intracellular phospholipases. Experimental findings strongly suggest that phospholipase A₂ is involved in ischemia-induced acceleration of membrane phospholipid degradation (Prasad *et al.*, 1991; Van der Vusse *et al.*, 1982; Van der Vusse *et al.*, 1992). However, attempts to measure the effect of ischemia on cardiac phospholipase A₂ activity have led to conflicting results (Bentham *et al.*, 1987; Das *et al.*, 1986; Hazen *et al.*, 1991). Das and co-workers (1986) found that in membrane fractions of ischemic and reperfused pig myocardium the activity of phospholipase A₂ was enhanced. In contrast, Bentham and colleagues (1987) showed a decreased phospholipase A₂ activity in homogenates and subcellular fractions of ischemic rat hearts. Hazen and associates (1991) established that not the activity of the diacylphospholipase A₂ but the activity of plasmalogen-selective phospholipase A₂ was elevated in homogenates obtained from rabbit hearts within 2 min after onset of ischemia.

In addition to alterations in the activity of the phospholipases per se, changes in the microenvironment of the enzymes and their substrates might evoke enhanced phospholipid hydrolysis in ischemic cardiac tissue. For example, Ca²⁺ and H⁺ may play a role in ischemia-induced phospholipid degradation. In oxygen-deprived tissue both H⁺ and Ca²⁺ levels increase. Since low molecular weight phospholipase A₂ is Ca²⁺-dependent and lysosomal phospholipase A₂ has an acid pH optimum, they may be activated under these circumstances. Alternatively, enzyme activation can be caused by a chemical modification of the inactive enzyme form. Increased contact between membrane phospholipids and the enzyme might also enhance the rate of phospholipid hydrolysis. The increased contact can be due to a destabilization of the sarcolemma which occurs during ischemia, or higher concentration of phospholipases in the proximity of the membrane. As mentioned earlier, the formation of

a complex of plasmalogen-specific phospholipase A₂ with phosphofructokinase could facilitate the delivery of the enzyme to its substrate, i.e., the membrane phospholipids. It was demonstrated that phosphofructokinase protein and activity was translocated from the cytosol to the membrane compartment with a time course similar to the translocation of the calcium-independent phospholipase A₂ activity during ischemia (Hazen *et al.*, 1994). However as the activity of the latter enzyme is ATP dependent and in ischemia ATP levels drop steadily, a decrease rather than an increase of plasmalogen-specific phospholipase A₂ activity has to be anticipated.

Cardiac phospholipase A₂ activity may also be modulated by non-enzymatic proteins like annexins (Raynal and Pollard, 1994). Although annexins are able to inhibit cardiac phospholipase A₂ activity *in vitro*, a role of these proteins *in vivo* in ischemia-induced breakdown of phospholipids remains to be established (see paragraph 1.3.4).

In addition to ischemia-induced cellular damage, isolated myocytes, subjected to metabolic inhibition, are subjected to metabolic and morphological changes, ultimately leading to cell death (Piper, 1988). With severe depletion of cellular energy levels, cells undergo contracture, and initially elongated cells shorten into square cells. Absence of mechanical restraints allows squaring of isolated cells to be more severe than in the intact heart (Amstrong and Ganote, 1991). Mitochondria protrude from the lateral aspects of cells into small microblebs (Piper, 1988). These microblebs are not associated with disruptions of the lateral costamere junctions (Pardo *et al.*, 1983), which attach the plasma membrane to Z-bands (Ganote and Vander Heide, 1987; Terracio *et al.*, 1990). Continued depletion of energy results in a progressive development of osmotic fragility of cells. As a consequence, swelling of cells in hypo-osmotic media results in formation of large subsarcolemmal blebs and rupture of the sarcolemma (Vander Heide and Ganote, 1987). Subsarcolemmal blebs are associated with disruption of the membrane-cytoskeletal complex (Ganote and Vander Heide, 1987, 1988; Vander Heide and Ganote, 1987). Following development of osmotic fragility, cell membranes remain capable of excluding extracellular marker dyes for an additional time interval of 30 to 60 min in isotonic medium. When the cells are exposed to phospholipase A₂ inhibitors they remain viable for a prolonged period of time (Chien *et al.*, 1981; Buja *et al.*, 1985; Weglicki and Low, 1987). These findings suggest that phospholipase degradation of membranes is most likely involved in the sequence of events leading to irreversible cell injury.

Exposure of rat myocytes to metabolic inhibitors, such as iodoacetate, 2-deoxyglucose, cyanide, or combinations thereof, induces morphological and biochemical alterations resembling those in the ischemic myocardium (see above). Similarities are: i) depletion of high-energy phosphate pools (Chien *et al.*, 1985; Esumi *et al.*, 1991; Gunn *et al.*, 1985), ii) the development of ultrastructural changes (Amstrong and Ganote, 1992; Post *et al.*, 1993; Post *et al.*, 1995), including sarcolemmal discontinuities (Sen *et al.*, 1988), iii) activation of phospholipase activity (Gross *et al.*, 1985), iv) selective catabolism of sarcolemmal phospholipids (Miyazaki *et al.*, 1990) and release of unsaturated fatty acids, including arachidonic acid (Gunn *et al.*, 1985; Chien *et al.*, 1985), v) and loss of cytoplasmic proteins in the extracellular compartment (Chien *et al.*, 1985). As cell death induced by metabolic

inhibition and by ischemia show many similarities, the former technique has been applied by a variety of investigators to elucidate the mechanism of injury in energy-depleted cells in more detail.

In the present thesis, impaired cardiac phospholipid homeostasis was also studied by metabolic inhibition with the use of iodoacetate and 2-deoxyglucose in isolated adult ventricular rat cardiomyocytes. Although extrapolation of results from an *in vitro* model to *in vivo* ischemia should be done with caution, the cultured adult myocardial cell model offers several important advantages. Compared with the intact heart, the cultured cells provide a more homogenous cell population, as no non-myocytal cells are present in the culture which could contribute to arachidonate release. Moreover, external conditions can be strictly controlled. More specific for the present thesis, quantitative recovery of the radiolabeled fatty acids is possible during the experiments. Adult cardiac myocytes were investigated rather than cultured neonatal cardiac cells since it was felt that studies dealing with adult cells provide more relevant information on the mechanism(s) underlying damage inflicted upon cardiac structures under energy-depriving conditions. However, it can not be denied that the use of isolated cardiac cells also has certain limitations. Potentially important influences of other cardiac cell types, as exerted in the intact heart, on the development of damage of the cardiac myocytes are removed. Additionally, isolated adult cardiac myocytes do not contract, and accordingly have a low metabolic rate, which may render them more resistant to pathological stimuli.

1.3.4 Inhibition of phospholipase activity by annexins

Anti-phospholipase activity of annexins was reported first for annexin I and later also for other annexins. Annexin I (Ahn *et al.*, 1988; Blackwell *et al.*, 1980; Chap *et al.*, 1988; Davidson *et al.*, 1987; Hayashi *et al.*, 1990; Hirata *et al.*, 1980; Pepinsky *et al.*, 1986), annexin II (Ahn *et al.*, 1988; Chap *et al.*, 1988; Davidson *et al.*, 1987), annexin III (Comera *et al.*, 1990), annexin IV (Ahn *et al.*, 1988; Chap *et al.*, 1988; Fauvel *et al.*, 1987), annexin V (Ahn *et al.*, 1988; Chap *et al.*, 1988; Comera *et al.*, 1990) as well as annexin VI (Ahn *et al.*, 1988; Comera *et al.*, 1990; Fauvel *et al.*, 1987) displayed the potency to inhibit phospholipase A₂ activity *in vitro*. However, the mechanism underlying the inhibitory effect is still a point of controversy. Specific interaction of annexins with the enzyme as well as depletion of the substrate by annexins bound to the phospholipids in the presence of Ca²⁺ have been proposed.

Evidence in favor of the 'substrate depletion' model is that annexins appear to inhibit *in vitro* different types of phospholipases (Bohn *et al.*, 1992; Machoczek *et al.*, 1989). In addition, when PC, which has a very poor affinity for annexins, was used as substrate the action of phospholipase A₂ was not inhibited (Blackwood *et al.*, 1990; Edwards *et al.*, 1991; Khanna *et al.*, 1990). Moreover, Ca²⁺-independent phospholipases were only inhibited by annexins in the presence of Ca²⁺ (Gassama-Diagne *et al.*, 1990). Furthermore, experiments, in which the phospholipid or annexin concentrations were varied, showed that phospholipase A₂ was only inhibited at low phospholipid over enzyme ratios (Aarsman *et al.*, 1987;

Davidson *et al.*, 1987). Finally, in support of the substrate depletion model, evidence against direct inhibition of the enzyme activity was provided by Ahn and co-workers (Ahn *et al.*, 1988). In mixtures of purified annexin I, II, IV, V or VI and pancreatic phospholipase A₂ they found only a weak association between the enzyme and the inhibitor ($K_d > 3 \times 10^{-5}$ M), which is insufficient to account for direct enzyme inhibition.

Evidence against the substrate depletion model has been presented by Hayashi and colleagues (1990), who characterized a monoclonal antibody against annexin I which abolished the phospholipase A₂-inhibitory activity of annexin I without changing its binding properties to phospholipids. In another study it was demonstrated that phospholipase A₂ prepared from human epidermis was inhibited by annexin I when PC, a phospholipid lacking affinity for annexins, was used as the sole substrate (Cartwright *et al.*, 1989). However, this study was in contradiction with data indicating that the presence of PE was required for the inhibition of human skin phospholipase A₂ by annexin I, II and V (Bastian *et al.*, 1993).

In conclusion, the majority of studies indicate that, annexins can be considered as Ca²⁺-dependent inhibitors of the hydrolytic action of phospholipases *in vitro*, due to their ability to bind to phospholipids in the presence of Ca²⁺, thus covering the substrates for the phospholipase A₂ enzyme (figure 1.2).

Extracellular space

Figure 1.2. Putative model of the inhibition of phospholipase A₂ by annexins. In this highly schematic representation, the membrane phospholipids are covered with annexin molecules, which prevents the phospholipases to reach and subsequently hydrolyse the membrane phospholipids.

1.4 GROWTH AND DIFFERENTIATION OF THE HEART

1.4.1 Normal cardiac growth and development

Growth of the embryonic heart is largely dependent on an increase in the number of myocytes. During this hyperplastic phase, cardiac myocytes become progressively more elongated and myofibrils start to align. The myofibrils eventually cluster at the cell periphery and attach to the cell membrane at electron dense regions. Sarcomeres are clearly identified with Z-lines, A-bands and I-bands, but M-lines are not yet present. In addition, T-tubules are still absent in the myocytes and the intercalated disk regions are only partially developed. Although all elements of the intercalated disk are present, the orientation is still parallel to the long axis of the cell and distributed over the entire cell surface (Bishop, 1990).

Shortly after birth the proliferation of cardiac myocytes ceases (Korecky and Rakusan, 1978). After cell division has stopped, growth is accomplished by enlargement of the cardiac myocytes (trophic phase) (Clubb and Bishop, 1984; Li *et al.*, 1996; Zak, 1974). This trophic phase is characterized by an increase in contractile protein content of individual cardiac myocytes. In this trophic period the adult structure of the cells develops rapidly. The myofibrils become longitudinally orientated and mitochondria, sarcoplasmic reticulum, T-tubules, nuclei, and other components acquire their adult configuration. Although the capacity of hyperplastic growth is most likely lost, myocytes may retain some capacity for DNA replication, resulting in polyploidy. In this respect it is of interest to note that in the adult rat heart, nearly 90% of the ventricular myocytes are binuclear (Anversa *et al.*, 1991; Bishop and Drummond, 1979; Clubb and Bishop, 1984). During cardiac growth, capillaries proliferate in proportion to the myocardial volume. Connective tissue and neural elements mature also along with the myocytes.

The factors that control the above mentioned different stages in myocardial growth and development are incompletely understood (Bishop, 1990). Fetal growth by hyperplasia is strongly governed by inherent factors (Bishop, 1990), and is only slightly influenced by external, environmental factors. Hemodynamic factors mainly dictate the stage in which growth is accomplished by enlargement of cells. As annexins have been shown to be involved in growth related processes in a variety of cell types (see 1.4.2), it can be envisaged that they also play a role in the growth and development of cardiac cells.

1.4.2 Role of annexins in growth and differentiation

Recent studies have demonstrated a role of several annexins in proliferation and differentiation related processes. First, annexin I and II were identified as *in vitro* target proteins of the epidermal growth factor receptor and pp60^{v-src} (Cheng and Chen, 1981; Erikson and Erikson, 1980; Fava and Cohen, 1984; Radke *et al.*, 1980; Pepinsky and Sinclair, 1986; Sawyer and Cohen, 1985) and the insulin receptor mediated signalling processes (Karasik *et al.*, 1988). Stimulation of these receptors is known to activate protein kinase activity, which can lead to cell cycle entrance and transformation. Annexin I

(Schlaepfer and Haigler, 1988; Varticovski *et al.*, 1988) and annexin II (Gould *et al.*, 1986; Summers and Creutz, 1985) were also found to be *in vitro* substrates for protein kinase C (PKC), a Ca^{2+} - and phospholipid-activated protein kinase with specificity for serine and threonine residues. PKC is also involved in mechanisms of cell transformation (Bazzi and Nelsestuen, 1993). Annexin V and VI are very poor substrates for PKC (Fauvel *et al.*, 1987; Kaplan *et al.*, 1988), but annexin V may play a role in preventing phosphorylation of annexin I by PKC (Schlaepfer *et al.*, 1992; Shibata *et al.*, 1992).

In addition, it was shown that annexin VI undergoes a growth-dependent phosphorylation (Moss *et al.*, 1992). The protein is not phosphorylated in quiescent fibroblasts or T-lymphoblasts, but both phosphoserine and phosphothreonine are detected several hours after induction of growth. Further, it was recently demonstrated that annexins are present in the nucleus of some cell types (Jindal *et al.*, 1991; Koster *et al.*, 1993; Mizutani *et al.*, 1992; Raynal *et al.*, 1992; Sun *et al.*, 1992). The physiological significance of this finding is unknown, but as annexin II was found to be involved in the activation of DNA polymerase α (Jindal *et al.*, 1991), annexins may play a role in cell replication.

Furthermore, synthesis of annexin II seems to be related to an advanced differentiation state (Schlaepfer and Haigler, 1990; Fox *et al.*, 1991). Annexin II mRNA levels increased during differentiation of the embryonic chicken lens epithelium (Talian and Zelenka, 1991). In addition, expression of annexin II was demonstrated to be upregulated during embryonic development of rat cerebellum (Burgoyne and Cambraydeakin, 1989) and chicken limb (Carter *et al.*, 1986). These observations, however, are in contrast with recent findings in *Xenopus*, where annexin II expression was found to be downregulated after early embryogenesis (Izant and Bryson, 1991). Other annexins, such as annexin I in the mammary gland (Horlick *et al.*, 1991) as well as annexin V in chick embryo (Hofmann *et al.*, 1992), have also been shown to be developmentally regulated. Moreover, annexin VI levels were found to change during maturing of B-lymphocytes (Clark *et al.*, 1991) and expression of the annexin VIII gene was demonstrated to be high in acute promyelocytic leukemia cells (Chang *et al.*, 1992; Sarkar *et al.*, 1993). Further, Schlaepfer and Haigler (1990) reported that annexin I and V expression changed as a function of growth status in human foreskin fibroblasts. Stimulation of proliferation was associated with higher levels of annexin I and V. In contrast, Giambanco *et al.* (1993) observed in a human-derived glioma cell line re-expression of annexin V when cell proliferation slowed down, suggesting that annexin V in glioma cells is mostly expressed in connection with cell differentiation.

Giambanco and co-workers (1993) were the first to study annexin V and VI protein levels in rat skeletal and cardiac muscle tissue during postnatal development. Using immunochemical techniques they found that in skeletal muscle annexin V levels decrease after postnatal day 1. In heart tissue annexin V and VI levels were nearly constant throughout postnatal development.

1.5 AIM OF THE STUDY AND OUTLINE OF THE THESIS

From the above overview it can be concluded that a diverse range of functions, such

as anti-phospholipase A_2 activity, anticoagulant activity, ion-channel activity, a role in exocytosis or in growth and differentiation related processes, has been ascribed to annexin V. In the present thesis the possible role of annexin V in the regulation of the phospholipid homeostasis of cardiac myocytes by phospholipase A_2 inhibition was investigated using, on the one hand, an experimental model with artificial phospholipid membranes and, on the other hand, energy-deprived myocytes. In addition, annexin V localization and quantity were studied during maturational and hypertrophic growth of the heart, in order to get an indication whether annexin V plays a role in these processes.

The first aim of the present thesis was to explore the reliability of a variety of immunochemical techniques that can be used to investigate the subcellular localization of annexin V in rat heart tissue and isolated cells. In chapter 2 the effects of the labeling technique and permeabilization conditions were tested to establish whether these analytical procedures have an effect on the subcellular localization of annexin V in the cardiac myocyte. Results were analyzed on the light, confocal scanning laser and electron-microscopic level.

The second aim, presented in chapter 3, was to assess which annexins are present in the rat heart and to determine the (sub)cellular localization and content of annexin V in rat cardiac tissue and the various cardiac cell types. At the start antibodies against human recombinant annexin V were used to detect rat heart annexin V. Although homology between the primary structure of human and rat annexin V is high, cross-reactivity of anti-human annexin V antibodies with rat annexin V was not found to be optimal. Therefore, it was decided to raise specific antibodies against rat annexin V. Annexin V protein was isolated and purified from rat heart tissue. The purified antigen was used for the production of polyclonal antibodies. The specific antibodies were used to detect annexin V in rat heart tissue and the various cell types of the rat heart by western blot analysis. A quantitative immunochemical assay (ELISA) was developed in order to assess the cellular content of annexin V in isolated cardiomyocytes, endothelial cells and fibroblast-like cells. The presence of annexin V mRNA in the different cell types of the rat heart was also investigated.

The proposed mechanism of phospholipase A_2 inhibition by annexins is through interacting with the substrate of the phospholipases. As the amounts of annexin V in the cardiac myocytes could be too low to cover the total membrane, the effect of partial covering of a phospholipid membrane with annexin V on phospholipase A_2 activity, under conditions mimicking the physiological circumstances as much as possible, is investigated as third aim in chapter 4. For these measurements ellipsometry was applied.

Under energy-deprived conditions the phospholipid homeostasis is disturbed. There is net degradation of phospholipids. The fourth aim of the present thesis is to study the possible role of annexin V, with its anti-phospholipase A_2 properties, in the phospholipid homeostasis under energy-deprived conditions (chapter 5). As a model system isolated adult cardiac myocytes were subjected to metabolic inhibition, as a result of which the cellular ATP pool becomes depleted. Lactate dehydrogenase (LDH) release was measured as a marker of loss of cell membrane integrity. In addition, morphological changes of the cells

during the experiments were monitored. Labeled arachidonic acid was incorporated in membrane phospholipids of the isolated adult cardiac myocytes prior to metabolic inhibition. The labeled arachidonic acid content in the fatty acid and phospholipid pool in the cardiac myocytes during metabolic inhibition was estimated and related to annexin V localization and LDH release.

The fifth aim of the present thesis is to explore whether in the heart annexin V content and localization change during maturational and hypertrophic growth. Preliminary findings of this line of research are presented in chapter 6. Annexin V content and localization were studied in the intact heart during perinatal development. Annexin V content in the different compartments of the adult and neonatal rat heart was determined as well. Moreover, the effect of hypertrophy on annexin V content and localization was investigated using hypertrophied adult rat hearts and neonatal cardiac myocytes. Cardiac hypertrophy was induced by coarctation of the abdominal aorta. Hypertrophic growth of the neonatal myocytes was stimulated with the α_1 -adrenergic agonist phenylephrine.

Finally a general discussion about the implications of the subcellular localization and quantity of annexin V in the normal and hypertrophic adult heart and during maturational cardiac growth, and the possible role of annexin V in cardiac phospholipid metabolism, is presented in chapter 7.

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Chapter 2

Exploration of immunohistochemical and cytochemical techniques to determine the subcellular localization of annexin V in the adult rat heart and isolated cardiac cells

SUMMARY

This chapter describes in detail our experience with immunochemical techniques to investigate the subcellular localization of annexin V in cardiac tissue, isolated adult cardiac myocytes, and cultured endothelial cells and fibroblasts. At the light microscopic level immunolabeling on cryosections of formaldehyde fixed rat heart tissue revealed annexin V staining of non-myocytal as well as myocytal cells. The subcellular localization in the non-myocytal cells was found to be cytoplasmic, while in the cardiac myocytes annexin V labeling was shown in close vicinity of the sarcolemma. These findings were confirmed by electron microscopic analysis of annexin V labeled cryosections of rat heart tissue. The subcellular localization of annexin V in isolated cells was found to be influenced by permeabilization of the cells. Treatment of intact fixed cardiac myocytes with 0.1% or 1% Triton X-100, or 0.1% saponin resulted in a cytoplasmic staining pattern, while no annexin V staining was observed at the cell boundaries. These results, obtained with the use of a confocal laser microscope, were in contrast with the sarcolemmal staining found after annexin V labeling of cryosections of isolated cardiac myocytes or cardiac tissue. Immunogold labeling of intact cardiac myocytes resulted also in annexin V labeling at the level of the sarcolemma. However, lack of penetration of the antibody-gold particle complex into the cell interior can not be excluded in this experimental approach. The results obtained at the light and electron microscopic level, when labeling was performed on cryosections of cardiac tissue or cells, were considered to be the most reliable. Hence, the latter technique was the method of choice for subsequent studies.

2.1 INTRODUCTION

For proper understanding of the functional significance of annexin V in cardiac cells, it is of paramount importance to elucidate the subcellular localization of this protein under normal and pathological conditions.

In vitro studies have shown that annexin V is able to attach to negatively charged phospholipids. Calcium ions are a prerequisite for this binding (Raynal and Pollard, 1994). As negatively charged phospholipids, such as phosphatidylserine, are predominantly present in the inner leaflet of the plasmalemma, annexin V is supposed to be attached to this particular membrane under normal, physiological conditions (Jans *et al.*, 1995). However, immunohistochemical data presented in literature are inconclusive. In the cardiac muscle cell, both plasmalemmal and cytosolic localizations have been reported (Doubell *et al.*, 1993; Giambanco *et al.*, 1991; Luckcuck *et al.*, 1995; Wang *et al.*, 1995). Because annexin V is attached to membrane phospholipids in a reversible fashion, it can not be excluded that during immunohistochemical assay procedures the subcellular localization of annexin V is inadvertently altered.

In this chapter attempts were made to identify possible pitfalls in a variety of techniques that are routinely applied to monitor the subcellular localization of annexins in tissue and isolated cells. Experiments were performed both on cardiac tissue sections and on isolated cells, originating from rat myocardium. Although the "golden standard" for immunohistochemical and cytochemical techniques for subcellular localization of annexin V does not exist, it was felt that

the chances of introducing artifacts are less when annexin V localization will be studied on cryosections of pre-fixed tissue specimens of the heart.

The tissue and cellular preparations were analyzed both on the light microscopic and electron microscopic level. Special attention was paid to ventricular myocytes. In addition endothelial cells and fibroblasts of cardiac origin were studied. Annexin V was detected with the use of an antibody specifically raised against rat heart annexin V (see chapter 3 for details). On the light microscopic level the antibody-annexin V complex was identified with fluorescence techniques. When the electron microscope was used, immunogold labeling was applied. In figure 2.1 the techniques studied in this chapter are schematically shown.

Figure 2.1. Schematical presentation of the techniques applied in this chapter. The paragraph numbers refer to the paragraphs in the method section.

The intrinsic advantage of cryosections of pre-fixed tissue specimens lies in the fact that the antigens (i.e., annexin V) are easily accessible for the specific antibodies (see for details par. 2.2.2). A potential disadvantage, however, might be that only a small number of annexin V molecules (those present at the surface of the cryosection) are available for interaction with the antibody. Theoretically in isolated intact cells all antibodies are available, when antibodies are able to reach the subcellular site of localization. To this end the plasmalemma has to be opened by permeabilizing agents to guarantee unimpeded access of the antibodies to the cell interior (par. 2.2.3). However, this procedure might introduce unwanted side effects such as intracellular transition of annexin V during the permeabilization procedure or incomplete access of the antibody to the most central parts of the cell, both leading to erroneous conclusions. The strength of this approach, however, is that the cellular preparation can be studied with the confocal laser microscope, which offers the opportunity to elucidate the subcellular localization of annexin V in more detail. Alternatively, immunolabeling can be performed on cryosections of isolated cells (par 2.2.4). As indicated above, the drawback of this technique is that only antigens at the surface of the cryosections are available for the antibody.

When the electron microscope is used, more detailed information can be obtained about the precise localization of annexin V in the cells under investigation. In this chapter immunogold labeling techniques were applied to cryosections of cardiac tissue and isolated cardiomyocytes (par 2.2.5 and 2.2.6, respectively). The isolated cardiomyocytes were studied after mild fixation and permeabilization of the sarcolemma to allow for entry of the antibodies.

The outcome of the present explorative study is that the results obtained with immunocytochemical techniques to identify the subcellular localization in isolated cells should be interpreted with caution. This study strongly suggest that artifacts can be easily introduced in studies on intact, isolated cardiomyocytes and cultured endothelial cells and fibroblasts, when permeabilizing agents are applied. At present, immunohistochemistry on cryosections of cardiac tissue is most likely the technique of choice to investigate the subcellular localization of annexin V in cells residing in the myocardium. The same holds for the immunocytochemical analysis of cryosections of isolated cardiomyocytes.

2.2 Methods

2.2.1 Antibodies

For annexin V detection in tissue and cells of rat cardiac origin a rabbit polyclonal antibody raised against rat heart annexin V was used. Prior to application the antibody was purified by immunoaffinity with the use of a Sepharose 4B column (Pharmacia, Uppsala, Sweden) with immobilized purified rat heart annexin V (see chapter 3, par. 2.3).

For light microscopic studies fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG antibodies (Dakopatts, Copenhagen, Denmark) were used for secondary detection. For electron microscopy either 10 nm gold conjugated anti-rabbit IgG (Janssen Pharmaceutica, Beerse, Belgium) or 8 nm gold conjugated protein A (provided by J. Lucocq, University of Dundee, Department of Anatomy and Physiology) was applied. All incubations with antibodies

were performed at room temperature unless indicated otherwise.

2.2.2 Immunofluorescence labeling on cryosections of rat heart tissue (light microscopy)

Hearts were removed from adult male Lewis rats of 12 weeks old under general pentobarbital anaesthesia (0.06 mg/g body weight i.p.). After thoracotomy the heart was rapidly excised, flushed with phosphate buffered saline (PBS) and fixed in 2% (v/v) paraformaldehyde in PBS at 4°C for 24 h. Thereafter the heart was cut into small fragments. These fragments were subjected to sucrose infiltration (2.3 M sucrose in PBS) until total immersion was achieved (4°C). Immersion with sucrose was considered to be complete when tissue fragments had sunk to the bottom of the vials used. Subsequently, tissue was frozen in isopentane, cooled in liquid nitrogen. Samples were stored at -80°C. Cryosections (5 µm) of the tissue fragments were cut on a cryostat by -20 °C. The cryosections were postfixed with methanol at -20°C for 2 min, followed by acetone fixation at -20°C for 30 s.

After air-drying for 15 min the sections were exposed to the above mentioned antibodies against annexin V (1:100 in PBS) for 2 h. In the second step, FITC-conjugated swine anti-rabbit IgG antibodies were added to the sections for 45 min. Sections were washed three times for 10 min with PBS between each step. For controls, the primary antibodies were replaced by pre-immune serum, or the anti-annexin V antibodies were pre-absorbed with rat heart annexin V before use. Sections were mounted in 1,4-diazobicyclo-[2,2,2]-octane (DABCO) (Sigma, St. Louis, MO, USA). Simultaneously sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) to visualize the nuclei.

2.2.3 Immunofluorescence labeling of intact cardiac cells; effect of the permeabilization procedure on cardiomyocytes

Cells were isolated and cultured from adult rat hearts as described in chapter 3 and by Linssen *et al.* (1990). The endothelial cell lines RHEC-50 (passage 12) and RHEC-116 (passage 8) and the fibroblast-like cell line CFLC-122 (passage 20) (Linssen *et al.*, 1993) were used in the present experiments. Isolated cardiac myocytes, endothelial (RHEC-50 and RHEC-116) and fibroblast-like cells (CFLC-122) were plated on glass coverslips in culture medium containing 40% M199 (Gibco, Life Technologies Ltd., Paisley, Scotland, UK), 40% RPMI1640 (Gibco), 20% FCS (Boehringer Mannheim GmbH, Mannheim, FRG), 2 mM L-glutamine (Sigma), 13 mM NaHCO₃, 10 mM HEPES, 50 µg/ml gentamicin, 20 U/ml heparin and approximately 40 µg/ml bovine brain extract, supplemented with 2.5 µg/ml fungizone. Endothelial and fibroblast-like cells were cultured for 1 day after passaging. Freshly isolated cardiac myocytes were allowed to attach for 4 h in M199 medium (Gibco) containing, 2 mM L-glutamine, 13 mM NaHCO₃, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 4% FCS.

Before fixation cells were washed with PBS. Endothelial and fibroblast-like cells were fixed in 3.5% (v/v) formaldehyde in PBS (nominally Ca²⁺ free) for 10 min, and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min. Cardiac myocytes were subjected to various permeabilization procedures. Cardiac myocytes were treated with either 0.1 % Triton X-100 for

10 min, 1% Triton X-100 for 10 min, or with 0.1% saponin for 20 min. Thereafter, the cells were incubated for 60 min with anti-annexin V antibodies (1:100 in PBS) and subsequently washed three times with PBS. This procedure was followed by an incubation with FITC-labeled swine anti-rabbit IgG antibodies (1:500 in PBS) for 60 min. Cells were mounted as described in paragraph 2.2.2.

Cells were studied with a Zeiss Axioskop (Carl Zeiss, Oberkochen, FRG). In addition, immunostained adult cardiac myocytes were studied with a Bio-Rad MRC-600 confocal scanning laser microscope (CSLM; Bio-Rad Laboratories, Richmond, CA, USA), equipped with a Crypton/Argon mixed gas laser (Ion Laser Technology, Salt Lake City, UT, USA) with a wavelength for the excitation of fluorescein isothiocyanate (FITC, 488 nm).

2.2.4 Immunofluorescence labeling on cryosections of isolated cardiac myocytes (light microscopy)

After isolation of the cardiomyocytes from adult rat hearts according to Linssen and co-workers (1990), adult cardiac myocytes were pelleted and fixed in 2% (v/v) paraformaldehyde in PBS at 4°C for 12 h. After fixation, cells were resuspended in a 10% (w/v) sucrose solution and incubated for 2 h. In the next step cells were embedded in a 4% (w/v) gelatin solution (10 min at 37°C) and subsequently placed on ice. Specimens were frozen in isopentane cooled in liquid nitrogen. Samples were stored at -80 °C.

Cryosections (5 µm) were cut by a cryostat at -20 °C and air-dried. The sections were incubated with the anti-annexin V antibodies (1:50 in PBS) at room temperature for 2 h, followed by 3 times 5 min washing in PBS and subsequently a 60 min incubation with FITC-labeled swine anti-rabbit antibodies (1:100 in PBS). Sections were mounted and counterstained as described in paragraph 2.2.2.

2.2.5 Immunogold labeling of cryosections of rat heart tissue for electron microscopy

For immunohistochemistry at the electron microscopic level, the heart of an adult male Wistar rat was excised after thoracotomy under general pentobarbital anaesthesia (0.06 mg/g body weight i.p.). The apex was removed and cut in small pieces, which were stored in fixative [0.2% (v/v) glutaraldehyde, 2% (v/v) formaldehyde in 0.1 M PBS, pH 7.4] at 4 °C for 48 h. Subsequently the tissue was cryoprotected according to a procedure modified after Tokuyasu (Tokuyasu, 1973; Griffiths *et al.*, 1984). Briefly, the glutaraldehyde/formaldehyde-fixed tissue pieces were rinsed in PBS and kept in a 1.0 M sucrose (in 0.1 M PBS) solution until total immersion was achieved. This step was followed by an incubation in 2.0 M sucrose until total immersion. Finally tissue was put into solution containing 2.3 M sucrose and polyvinylpyrrolidone (Sigma) which was also used as mounting medium. After mounting the tissue fragments were placed on the specimen holders, the samples were quickly frozen in liquid nitrogen and subsequently stored in this medium until cryosectioning.

Ultrathin cryosections were cut using a Reichert S cryotome with the cryochamber and knife temperature set at -110°C; cutting speed was about 1 mm/s. Sections were quickly removed

from the knife edge with a stainless steel wireloop filled with 2.3 M sucrose and transferred to carbon coated formvar grids. The grids were placed on 0.1 M PBS drops with the sections downwards.

Immediately after sectioning the grids were processed for immunogold labeling using the anti-annexin V antibody in three different dilutions [1:10, 1:20 and 1:50 in 5% (v/v) fetal calf serum (FCS) in 0.1 M PBS]. The antibody was visualized by incubation with 8 nm gold conjugated protein A (1:30). The labeling procedure was started by blocking with 10% (v/v) FCS in 0.1 M PBS for 20 min. This procedure was followed by 2 times 5 min washing with 0.1 M PBS and subsequently a 30 min incubation with the antibody against annexin V. The latter incubation step was omitted in control grids. Thereafter, sections were rinsed 3 times for 5 min with 0.1 M PBS and incubated with 8 nm gold conjugated protein A in 5% FCS in 0.1 M PBS for 30 min. Subsequently, sections were washed 3 times for 5 min with PBS followed by a 3 times 10 min rinse with distilled water. Finally, sections were counterstained and embedded using uranyl methylcellulose [0.3% (w/v) uranylacetate and 3% (w/v) methylcellulose in 0.1 M PBS]. This final step was carried out on ice and excess uranyl methylcellulose was removed with filtration paper. The sections were air-dried and examined in a Philips CM10 electron microscope using a 80 KeV electron beam.

2.2.6 Immunogold labeling of intact cardiac myocytes for electron microscopy

For immunoelectron cytochemistry, freshly isolated cardiac myocytes (Linssen *et al.*, 1990) were allowed to attach for 4 h onto glass coverslips, precoated with culture medium (96% M199, 2 mM L-glutamine, 13 mM NaHCO₃, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 4% FCS). Cells were fixed in periodate-lysine-paraformaldehyde fixative (0.01 M periodate, 0.075 M lysine, 2% paraformaldehyde) at 4°C for 3 h. Subsequently cells were permeabilized in buffer A (5 mM D-Glucose, 5.4 mM KCl, 137 mM NaCl, 0.4 mM KH₂PO₄, 0.3 mM Na₂HPO₄, 4.2 mM NaHCO₃, 5 mM Pipes (Piperazine-N,N'-bis[2-ethane-sulfonic acid]), 2 mM EGTA, 1 mM MgCl₂) containing 0.5% Triton X-100. The samples were then extensively washed in buffer A, and blocked with 5% normal goat serum in Tris Buffered Saline (TBS) (pH 7.2), containing 0.1% Bovine Serum Albumin (BSA). After a brief wash with TBS containing 0.1% BSA cardiac myocytes were incubated overnight with the anti-annexin V antibodies (1:1000) in TBS containing 0.1% BSA. After washing the samples were incubated in the presence of 10 nm gold conjugated anti-rabbit IgG for 3 h. After extensive washing with PBS, the preparations were fixed in PBS containing 1% glutaraldehyde during 10 min and post-fixed in 2% OsO₄ in veronalacetate-buffer (pH 7.4) during 30 min at 4°C. After washing with PBS, the preparations were dehydrated in ethanol and infiltrated in LX 112 (Sigma) for embedment. Ultrathin sections (< 0.15 µm) were examined by electron microscopy (CM100, Philips, Eindhoven, the Netherlands).

2.3 RESULTS

2.3.1 Immunofluorescence labeling of annexin V of cryosections of rat heart tissue

After immunolabeling cryosections of adult rat heart tissue were examined at the light microscopic level (see chapter 3 figure 3.5a; for method see paragraph 2.2.2). In the non-myocytal cells annexin V was found over the entire cell suggesting a cytoplasmic localization. The cytoplasm of the cardiac myocytes was found to be nearly devoid of annexin V staining. Immunostaining was observed in the vicinity of the cardiac cell boundaries. This could represent annexin V labeling either of the sarcolemma, or of the non-myocytal cells present in the spaces in between cardiac myocytes. Control experiments, in which the primary antibodies were replaced by IgG-purified pre-immune serum, or in which the anti-annexin V antibodies were pre-absorbed with purified rat heart annexin V were also performed. In these control experiments immunostaining of cardiac myocytes and non-myocytes was not detectable (photographs not shown).

2.3.2 Immunofluorescence labeling of annexin V of intact isolated cardiac cells

After fixation, isolated cardiac myocytes and cultured endothelial cells and fibroblast-like cells were opened by permeabilization with Triton X-100 (0.1%) for 10 min (for method see paragraph 2.2.3). Findings showed that in the endothelial cells annexin V was most likely localized in the cytoplasm (figure 2.2a), as the intensity of the immunofluorescence staining correlated with the thickness of the cytoplasm. In the nuclei of the endothelial cells annexin V staining seemed to be absent. A deviating picture was observed when isolated cardiac fibroblast-like cells were investigated (figure 2.2b). In general, these cells show an uniform labeling. This might indicate either a plasmalemmal localization of annexin V or a cytoplasmic localization, or both. In case the cytoplasm is stained, the nuclei are most likely also annexin V positive. The staining of isolated cardiomyocytes did not follow a specific, reproducible pattern (figure 2.2c). Some cells are rather uniformly stained, whereas others show staining at the cell boundaries. As the present technique does not provide conclusive information on the subcellular localization of annexin V in isolated cardiomyocytes, this study was extended to the application of the confocal scanning microscope (see next paragraph). In addition, the effect of various permeabilization techniques on the subcellular localization of annexin V was investigated.

2.3.3 Immunofluorescence labeling of annexin V on intact cardiac myocytes examined by confocal scanning laser microscopy: effect of permeabilization

To investigate whether the type of permeabilization technique of the cardiac myocytes influences the immunolocalization of annexin V, intact isolated cardiac myocytes were permeabilized after fixation in three different ways (0.1% or 1% Triton X-100, or 0.1% saponin) (for method see paragraph 2.2.3). Confocal scanning laser microscopy was used to obtain information on the intracellular annexin V localization. By confocal scanning laser microscopy

Figure 2.2. Evaluation of subcellular localization of annexin V in isolated, cultured rat heart cells as assessed by immunofluorescence microscopy. The rat heart endothelial cells RHEC-50 (a), the fibroblast-like cells CFLC-122 (b), and cardiac myocytes (c) were formaldehyde fixed and permeabilized with 0.1% Triton X-100 for 10 min (for details see paragraph 2.2.3). The preparations were first incubated with anti-rat heart annexin V antibodies and secondly with FITC labeled anti-rabbit antibodies. In the endothelial cells, the cell interior is most likely the site of staining (a). The nuclei seem to be devoid of annexin V staining. The fibroblast-like cells (b) appear to be uniformly stained. Substantial cell-to-cell differences were observed when in isolated cardiac myocytes the localization of annexin V was studied with immunofluorescence microscopy (c). Bars represent 10 µm. For colour illustrations see p. 148 .

Figure 2.3. Evaluation of the effect of permeabilization on the annexin V localization of adult cardiac myocytes as assessed with confocal scanning laser microscopy. After formaldehyde fixation cells were permeabilized with 0.1% Triton X-100 for 10 min (a), 1% Triton X-100 for 10 min (b) or 0.1 % saponin for 20 min (c) (for details see paragraph 2.2.3). Cells were first labeled with anti-rat heart annexin V and secondly with FITC conjugated anti-rabbit antibodies. After permeabilization with 0.1% (a) or 1% (b) Triton X-100 a striated cytoplasmic localization is observed. After permeabilization with saponin (c) a cytoplasmic granular pattern is found. No specific sarcolemmal localization could be observed. Bars represent 20 μm for figure 2.4a and 10 μm for figure 2.4b and c. For colour illustrations see p. 148 .

optical sections of the intact cardiac cells are obtained. In these experiments 15-25 optical sections of 0.8 μm each per cardiac myocyte were analyzed. Results of all permeabilization methods indicated full penetration of the antibodies, as immunostaining was also found in the centre of the cardiac myocytes. However, the subcellular distribution of antigenic sites was found to be dependent on the type of permeabilization procedure applied. After permeabilization with 0.1% or 1% Triton X-100 an intracellular labeling, which followed a striated pattern, was observed (figure 2.3a and 2.3b). Using saponin as a permeant annexin V was also found to be present in the cell interior. Interestingly, staining was now concentrated in small granular structures (figure 2.3c). No annexin V labeling was found at the level of the sarcolemma, irrespective of the type of permeabilization treatment used.

2.3.4 Immunofluorescence labeling of annexin V on sections of isolated cardiac myocytes

To avoid possible permeabilization artefacts (see paragraph 2.3.3) labeling was performed on sections of pre-fixed cardiac myocytes and not on intact, permeabilized cardiomyocytes. Results (figure 2.4) showed an annexin V immunolocalization in close vicinity of the sarcolemma. Moreover, staining was also observed inside the cardiomyocyte. The intensity of the cytoplasmic staining, however, varied from cell to cell (photographs not shown).

Figure 2.4. Immunofluorescence labeling of annexin V on cryosections of isolated adult cardiac myocytes. Isolated cardiac myocytes were fixed in paraformaldehyde followed by sucrose infiltration. Subsequently cells were embedded in gelatine and frozen in isopentane (for details see paragraph 2.2.4). Labeling took place on cryosections of the cardiac myocytes. First the cells were labeled with anti-rat heart annexin V antibodies and thereafter with FITC conjugated anti-rabbit antibodies. White colouring indicates the presence of annexin V staining. Annexin V staining was found at level of the sarcolemma (arrow) and to some extent in the cell interior. Bar represents 20 μm .

2.3.5 Immunogold labeling of annexin V on cryosections of rat heart tissue examined by electron microscopy

To determine the subcellular localization of annexin V in more detail, ultra-thin cryosections of fixed adult rat heart tissue were labeled with anti-annexin V antibodies, followed by immunogold labeling (for method see paragraph 2.2.5). The advantage of electron microscopy over light microscopy is that with electron microscopy (sub)cellular structures can be more easily distinguished. Using this preparation technique cellular structures were well preserved, even the sarcolemma could be distinguished. Examination of the sections of rat ventricular tissue revealed that the gold particles on the cryosections were present in close vicinity of the sarcolemma, including the intercalated disc (see chapter 3 figure 3.6 shows labeling of the intercalated disc). Gold particles were also seen at the level of the membrane of the transverse tubules (not shown in picture 3.6). Transverse tubules were identified as vesicular structures in the vicinity of the Z-lines. In other subcellular structures of the cardiomyocyte, such as myofibrils, mitochondria, and nuclei, no label was detected. In control experiments, in which the anti-annexin V antibody had been omitted from the procedure, immunogold labeling could not be seen.

2.3.6 Immunogold labeling of intact isolated adult cardiac myocytes examined by electron microscopy

In this pre-embedment labeling procedure intact pre-fixed cardiac myocytes were permeabilized with 0.5% Triton X-100 to open the cells for the anti-annexin V antibodies and anti-rabbit-IgG-conjugated gold particles. In the isolated adult cardiac myocytes annexin V was detected by the immunogold labeling method. After embedment of the intact cardiac myocytes ultra-thin sections were cut for subsequent electron microscopic examination (for method see paragraph 2.2.6). Subcellular structures, such as mitochondria, were found to be heavily damaged. In addition, the sarcolemma was not visible. Gold particles were predominantly present at the cell boundary (figure 2.5). Only a few gold particles were observed intracellularly.

2.4 DISCUSSION

For the proper understanding of the functional significance of proteins in general and of annexin V in particular, the cellular and subcellular localization should be known with a high degree of certainty. In addition to biochemical techniques, such as tissue fractionation, histochemical and cytochemical techniques have been developed to elucidate the (sub)cellular localization of the protein(s) of interest. Commonly, the protein under investigation is made visible with the use of a specific antibody. A variety of techniques is at hand to subsequently identify the antibody-antigen complex, such as fluorescence tags, enzymatic techniques, gold labeling, etc. The (sub)cellular localization of the protein can be established with both the light microscope and the electron microscope. In the latter case a higher resolution is obtained allowing a more exact determination of the subcellular localization.

Although immunohisto/cytochemistry has been applied with great success in the

Figure 2.5. Evaluation of subcellular localization of annexin V in isolated adult rat cardiomyocytes as assessed by immunoelectron microscopy. Isolated cardiomyocytes were periodate-lysine-paraformaldehyde fixed, permeabilized with 0.5% Triton X-100, labeled and subsequently sectioned as described in paragraph 2.2.6. Cells were incubated with anti-rat heart annexin V antibodies and thereafter with gold labeled anti-rabbit antibodies. Gold particles are present in close vicinity of the sarcolemma (arrow). Inside the cell gold-particles are only occasionally observed. The bar represents 0.74 μm .

identification of a host of cellular proteins, some proteins, such as annexin V, require special attention. The determination of the subcellular site of residence might be sensitive to inadvertent side-effects inherent to the histochemical technique used. This is mainly caused by the fact that annexin V is attached to membrane phospholipids in a highly reversible manner. Biochemical studies have shown that annexin V is attached to negatively charged phospholipids, such as phosphatidylserine, in a Ca^{2+} - mediated fashion (Andree *et al.*, 1990; Blackwood and Ernst, 1990; Schlaepfer *et al.*, 1987). Each step in the overall immunohistochemical technique that interferes with the binding of annexin V to membrane phospholipids might lead to erroneous results. For this reason, in the present chapter a variety of commonly used immunohistochemical and immunocytochemical techniques has been evaluated to rate their applicability in determining the subcellular localization of annexin V in preparations of both cardiac tissue and isolated cardiac cells.

Although no "golden standard" exists with respect to immunohistochemical determination

of the subcellular localization of annexin V in cardiac cells using the light microscope, it is felt that the technique described in section 2.2.2 provides the most reliable results. In this procedure, cryosections were made of pre-fixed specimens of cardiac tissue. The localization of the antigen in the tissue sections was elucidated with the use of an antibody specifically raised against rat heart annexin V (see for details chapter 3). The outcome of this technique revealed that annexin V staining of cardiomyocytes mainly occurred at the boundaries of this particular cell type. Non-cardiomyocytes, present around the coronary vessels and in the spaces in between the cardiac muscle cells, show a predominant cytoplasmic staining. Due to the limited spatial resolution of the light microscope, the precise localization of annexin V at the edges of the cardiomyocytes can not be determined. In addition to a sarcolemmal localization, the staining may be caused by annexin V present in cellular structures localized in the interstitial space (Anversa *et al.*, 1978). This possibility has been explored in full detail in chapter 3 with the use of double labeling techniques to identify the presence of non-myocytal material in close vicinity of the staining found at the edges of the cardiomyocytes. As will be discussed in the next chapter it is highly unlikely that the staining at the level of the boundaries of the cardiomyocytes is of non-myocytal origin.

The application of the electron microscope allows for a more precise subcellular localization of annexin V due to its high spatial resolution. Therefore, subcellular structures, such as mitochondria, sarcoplasmic reticulum and myofibrils, can be distinguished. In section 2.2.5 the immunogold labeling technique, making use of an electron microscopic analysis of cryosections of rat ventricular tissue, has been explored. Prior to cryosectioning tissue specimens were subjected to a fixation procedure commonly used for the subcellular localization of proteins. The gold labeling technique revealed that annexin V was present in close vicinity of the sarcolemma, including intercalated discs and the T-tubular system. In this respect the electron microscopic findings support the observations made in the light microscopical studies. A drawback of the electron microscope as a tool for studying the subcellular localization of a protein is the fact that the whole procedure is very time consuming and relatively expensive.

An advantage of the use of cryosections is that the antibody has direct access to the antigen as cellular membranes are cut and can not act as barrier for the antibodies to intrude the cell interior. A potential drawback is that only a limited number of antigens is available for interaction with the antibodies, i.e., those antigens that are present close to the surface of the cryosection. However, when isolated cells are used, the antigens of the entire cell are theoretically available for interaction with the intruding antibodies, when the cellular membrane is opened by permeabilizing agents. Following this approach, whole cell immunolabeling was initially evaluated with the use of the traditional light microscope. The staining of the cultured rat heart endothelial and fibroblast-like cells, and freshly isolated cardiomyocytes after permeabilization of the cell membrane with 0.1% Triton X-100 was investigated. As all three cell types were positively stained the conclusion could be derived that both cardiac endothelial cells, fibroblasts and cardiomyocytes contain annexin V. Combined with the observations made on cryosections of cardiac tissue, these findings strongly suggest that the non-myocytal staining of cryosections is related to the presence of annexin V in the endothelial cells surrounding the (micro)vessels and the fibroblasts localized in the interstitial compartment. The fact that staining

of the endothelial cells was most intense in those areas of the permeabilized cells where the cytoplasmic volume was maximal points towards a cytoplasmic rather than a plasmalemmal localization of annexin V. This notion is in line with the observation made on cryosections of cardiac tissue.

However, the technique applied does not allow for detailed conclusions about the subcellular localization of annexin V in cardiomyocytes. To do so, the sophisticated confocal laser microscopic technique might be of help. When isolated cardiomyocytes were permeabilized with 0.1% Triton X-100, a compound used to dissolve cellular membranes, the distribution of the staining in an optical section obtained with the confocal microscope suggested an intracellular localization of annexin V. More precisely, a typical striated staining pattern was observed. Comparable findings were obtained when the Triton X-100 concentration was increased to 1%. It is of interest to note that Selbert *et al.* (1996) found a similar pattern of annexin VI immunostaining in skeletal muscle. On the basis of mathematical considerations, the authors concluded that the observed pattern suggests an annexin V localization in the T-tubular system. When saponin, a compound specifically dissolving cholesterol present in cellular membranes, was used annexin V staining was found to be concentrated in small granular structures, the identity of which remains unknown. In all three cases no staining could be observed at the level of the sarcolemma. The latter findings are at variance with the localization of annexin V observed in cryosections of rat myocardium. Although no definitive explanation can be offered for this apparent discrepancy, treatment of isolated cells with compounds such as Triton X-100 or even with the less aggressive permeant saponin (Bourne, 1983), may affect the interaction between annexin V and the sarcolemma and, hence, affect the intracellular localization of this protein.

When the use of isolated cardiomyocytes is required for resolving a specific scientific question, the problems associated with permeabilization of the cells can be circumvented by preparing cryosections of pre-fixed cells. These cryosections are subsequently immunolabeled. The present data (see section 2.3.4) indicate that this procedure leads to an immunostaining of annexin V both in close vicinity of the sarcolemma and in the cell interior. The relative intensity of staining of these cellular structures was found to vary from cell to cell. The cytoplasmic staining, if present, followed a pattern that may coincide with the T-tubular system, localized in between the myofibrils of the cardiomyocyte.

Permeabilization of cardiomyocytes prior to immunolabeling was also studied in preparations made for the electron microscope. Although the gold label was mainly observed at the cell boundaries, no sarcolemma could be distinguished. This might be due to the dissolving action of Triton X-100. As it cannot be excluded that the dense packing of the myofibrils inside the cardiomyocytes has prevented the second antibody, which bears a golden microsphere of 10 nm with a negatively charged, hydrophobic surface (Smith, 1980), from penetrating the interior of the cell, the interpretation of the latter findings should be done with caution.

Two other groups of scientists have recently reported the localization of annexin V in isolated cardiomyocytes. Their experimental findings, however, showed an apparent discrepancy. Wang *et al.* (1995) reported that immunoreactivity for annexin V in cultured adult atrial cardiomyocytes appeared as striations along myofibrils, showing an overlap with the immune product for α -actinin, a marker protein for the Z-line. In addition, neither sarcolemma nor

intercalated discs showed immunoreactivity. In contrast, Luckcuck *et al.* (1995) have described that in adult ventricular cardiomyocytes immunoreactivity for annexin V was detected at the intercalated discs and the sarcolemma, and more faintly, in a cross-striated pattern within the cells. In the latter study cardiomyocytes were permeabilized with 1% Triton X-100 and as stated in this chapter this permeant may affect the binding characteristics of annexin V with the sarcolemma and, thus, its immunolocalization. It should be noted that the findings of Luckcuck *et al.* (1995) in isolated cardiomyocytes were in agreement with the results of immunolabeling of transverse sections from porcine left ventricle (Luckcuck *et al.*, 1995). As no immunolabeling on sections of the isolated cardiac myocytes was performed by Luckcuck *et al.* (1995), comparison with the present findings is difficult. In the study of Wang and associates (1995), isolated adult atrial cardiomyocytes were fixed with acetone/methanol (1:1) at -20 °C for 15 min, whereafter immunolabeling was performed. Although the cells were not permeabilized with a non-ionic detergent, the organic solvents acetone and methanol do not only fix the cells but also permeabilize them by dissolving the lipids composing the sarcolemma. Hence, it can not be excluded that annexin V associated with the sarcolemma and intercalated disc was lost during acetone/methanol fixation. The authors claim that this was not the case in their study because they have observed a similar annexin V immunoreactive striation pattern in paraformaldehyde fixed rat ventricular tissue. It is also possible that the above mentioned discrepancy is due to differences in localization of annexin V in atrial versus ventricular myocytes.

The contrasting findings described above might also be due to the specificity of antisera used in the experiments or a tissue-specific or species-specific expression of annexin V isoforms. It should be noted that Doubell and colleagues (1993) used antibodies specific for rat heart annexin V and, thus, avoided this possible cause of artefacts. In our study also antibodies specific for rat heart annexin V were used. However, the present results are still in contrast with the findings of Doubell *et al.* (1993), which might implicate that other factors, such as the type of fixation method employed, are the cause of the discrepancy in immunolocalization of annexin V in cardiac muscle cells. Doubell and coworkers fixed the rat hearts in a rather strong fixative, namely Bouin's solution, consisting of picric acid, acidic acid and formaline, for 24 h, whereafter tissue was embedded in paraffine. In contrast, in the present study rat heart tissue for light microscopy was fixed in paraformaldehyde for 24 h and subsequently frozen.

In summary, the present findings indicate that cryosectioning of cardiac tissue and isolated cardiomyocytes prior to immunolabeling of annexin V provides most likely a reliable pattern of immunostaining of this protein in cardiac myocytes. Permeabilization of the cellular membrane with Triton X-100 or saponin may lead in erroneous results, especially when proteins with a putative membrane associated localization, such as annexin V, are the subject of study.

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Chapter 3

Annexin V in the adult rat heart: isolation, cellular localization and quantitation

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Annexin V in the adult rat heart: Isolation, localization and quantitation

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SUMMARY

Annexins belong to a family of proteins with calcium- and phospholipid-binding properties. The present study was performed to identify which members, in addition to annexin V and VI (Doubell *et al.*, 1993), of the annexin family are present in the rat heart and to determine the cellular distribution and protein content of the most prominent annexin, i.e., annexin V, in rat cardiac tissue, in isolated ventricular myocytes, cultured endothelial cells and fibroblast-like cells. The presence of a variety of members of the annexin family, including annexin I plus II, III, IV, V and VI, in rat cardiac tissue was established with western blot analysis. As cross-reactivity between anti-human annexin V antibodies and rat heart annexin V in enzyme-linked immunosorbent assay (ELISA) technique and western blot turned out to be low, annexin V was purified from rat cardiac tissue and polyclonal antibodies were raised against rat heart annexin V. Western blot analysis with these antibodies revealed that annexin V is present in both cardiomyocytes and non-myocytal cells of the heart. These results were confirmed by immunohistochemical analysis of rat heart tissue sections. Subcellular localization of annexin V in non-myocytal cells seemed to be cytoplasmatic, whereas the localization in the cardiac myocytes was found to be in close vicinity of the sarcolemma. Northern blot analysis demonstrated that all cell types investigated showed expression of annexin V. Annexin V mRNA levels were highest in the fibroblast-like cells, followed by endothelial cells, and a relatively weak signal was observed in cardiomyocytes. By means of a sandwich-type ELISA with antibodies specific for rat heart annexin V, annexin V content in intact adult rat heart, isolated myocytes, cultured cardiac endothelial cells and fibroblast-like cells was found to be 0.70, 0.17, 1.63 and 3.84 $\mu\text{g}/\text{mg}$ total protein, respectively. The differences in quantity and localization of annexin V in myocytes and non-myocytes may reflect differences in function of annexin V in the various cell types.

3.1 INTRODUCTION

Annexins constitute a family of structurally related calcium- and phospholipid-binding proteins (Crompton and Dedman, 1990; Raynal and Pollard, 1994). The annexins exhibit a variety of functions (for an extensive review see Raynal and Pollard, 1994). The participation in exocytosis (Ali *et al.*, 1989), anti-coagulation (Reutelingsperger *et al.*, 1985), calcium-channel activity (Burns *et al.*, 1989), and involvement in anti-inflammatory processes (Huang *et al.*, 1986) have been proposed for the different members of the family. In addition, annexins have been reported to inhibit phospholipase A₂ activity *in vitro* (Davidson *et al.*, 1987). Inhibition of the latter enzyme would be of particular interest to cardiac tissue (Van Bilsen *et al.*, 1992) since phospholipid breakdown has been implicated as the major cause for irreversible damage to cardiac tissue following ischemia and reperfusion. Understanding the regulatory role of the annexins in cardiac tissue requires insight into which types of annexins are present in cardiac tissue and into their (sub)cellular distribution within the heart.

Annexins have been identified in a variety of tissues. In heart tissue annexin V appears to be the most abundant of all annexins (Pula *et al.*, 1990). Immunohistochemical studies on rat

heart tissue indicated the prominent presence of annexin V in non-myocytes (Doubell *et al.*, 1993; Giambanco *et al.*, 1991; Luckcuck *et al.*, 1995). Conflicting findings have been reported regarding the presence of annexin V in cardiac myocytes. Low myocytal levels of annexin V were found by Giambanco and associates (Giambanco *et al.*, 1991) and Doubell and co-workers (Doubell *et al.*, 1993). In contrast Van Bilsen and colleagues (Van Bilsen *et al.*, 1992) were unable to detect the protein in cardiomyocytes isolated from adult rat hearts, using antibodies against human recombinant-annexin V (Mauer-Fogy *et al.*, 1988). Immunohistochemical studies on rat heart tissue indicated the prominent presence of annexin V in the cytoplasm of non-myocytes (Doubell *et al.*, 1993; Giambanco *et al.*, 1991). Regarding the subcellular localization of annexin V in cardiac myocytes also conflicting results have been reported. Annexin V was found to be either associated with the sarcolemma (Giambanco *et al.*, 1991; Luckcuck *et al.*, 1995) or predominantly confined to the cytoplasmic space (Doubell *et al.*, 1993). At present no satisfactory explanation for the discordant findings can be offered.

The aim of the present study was to delineate which members of the annexin family are present in the adult rat heart. Furthermore, the localization and content of annexin V in various cell types present in rat heart has been studied in more detail. Western and northern blot analysis were performed using intact rat hearts, isolated cardiomyocytes and stable endothelial and fibroblast-like cell lines of adult rat heart origin. For quantitation of annexin V in tissue and cellular preparations a sensitive sandwich-type enzyme-linked immunosorbent assay (ELISA) was developed with antibodies against purified rat heart annexin V, since cross-reactivity of anti-human recombinant-annexin V with rat heart annexin V was found to be too low to detect the amount of annexin V in rat heart tissue.

3.2 METHODS

3.2.1 Tissue handling and fixation for morphological studies

Hearts were removed from male adult Lewis rats of 12 weeks old under general pentobarbital anaesthesia. For immunohistochemistry at the light microscopical level, the heart was rapidly excised, flushed with phosphate buffered saline (PBS) and fixed in 2% (v/v) formaldehyde in PBS for 24 h at 4°C. After sucrose (2.3 M) infiltration until total immersion was achieved (4°C), tissue was frozen in isopentane followed by liquid nitrogen. Samples were stored at -80°C (see for details chapter 2).

For immunohistochemistry at the electron microscopical level, the heart of a male Wistar rat was excised under general pentobarbital anaesthesia. The apex was removed and cut in small fractions, which were stored for 48 h in a fixative, composed of 0.2% (v/v) glutaraldehyde, 2% (v/v) formaldehyde in 0.1 M PBS, pH 7.4. Subsequently the tissue was cryoprotected according to a modified Tokuyasu procedure (Tokuyasu, 1973; Griffiths *et al.*, 1984). Briefly, after fixation the tissue was rinsed in PBS and kept in a 1.0 M sucrose (in 0.1 M PBS) solution until total immersion was achieved. This step was followed by a storage in 2.0 M sucrose until total immersion. Immersion with sucrose was considered to be complete when tissue fragments had sunk to the bottom of the vials used. Finally tissue was put into a 2.3 M sucrose solution with

polyvinylpyrrolidone (PVP) which was also used as mounting medium. After mounting the tissue fragments were placed on the specimen holders, the samples were quickly frozen in liquid nitrogen and stored in this medium until cryosectioning.

For western blot analysis and ELISA the hearts of male adult Lewis rats (12 weeks old) were rapidly flushed with ice-cold phosphate buffered saline (PBS) to remove blood from the tissue, which was subsequently homogenized (15% homogenate, w/v) in SET buffer (250 mM sucrose, 2 mM EDTA, 50 mM Tris-HCl, pH 7.2).

3.2.2 Isolation of cells

Cells were isolated from adult rat hearts. In short, after perfusion of the heart with a collagenase solution, the ventricles were used for the isolation of myocytes. Freshly isolated myocytes were used for the experiments. The perfusate was collected and subsequently used for the isolation of endothelial cells and fibroblast-like cells, as described in detail before by Linssen *et al.* (1990). From the endothelial cultures two continuous cell lines (RHEC-50, RHEC-116) have been established and checked for the presence of Von Willebrand factor and internalization of Dil-Ac-LDL, and were stained with RECA-1, a specific antibody directed against endothelial cell surface antigen (Linssen *et al.*, 1993). A cardiac fibroblast-like cell line (CFLC-122) was obtained by subculturing the fibroblast-like cells. These cells were also checked for the above-mentioned endothelial cell markers. The results were negative indicating the absence of endothelial cells in this cellular preparation. All cell lines used were of Lewis rat heart origin, except for RHEC-116 which was obtained from a Wistar rat. Unless otherwise indicated the endothelial cell lines RHEC-50 and RHEC-116 and the fibroblast-like cell line CFLC-122 were used in the present experiments.

3.2.3 Isolation and purification of rat cardiac annexin V, and preparation of antibodies against rat cardiac annexin V

Approximately two hundred rat hearts (200 g of heart tissue) were homogenized in buffer A [50 mM Tris (pH 7.9), 1 mM CaCl_2 , 5 mM benzamidin, 100 $\mu\text{g/ml}$ Soy-bean Trypsin Inhibitor (STI)]. The homogenate was centrifuged at 17,600 g for 1 h, and the supernatant was saved and checked for the presence of annexin V with negative result. The pellet, containing proteins that bind to the membrane fraction in a calcium-dependent manner, was washed twice in buffer A. After centrifugation the final pellet was suspended in buffer B [15 mM HEPES (pH 7.5), 4 mM EDTA, 1 mM EGTA] to release calcium-dependent phospholipid-binding proteins. The suspension was subsequently centrifuged for 40 min at 28,300 g. The supernatant was dialyzed against 50 mM Tris (pH 7.8, buffer C). The dialyzed extract (extract I) was loaded onto a column of Q-sepharose (anion-exchanger, HR 26/10, 53 ml gel, Pharmacia, Uppsala, Sweden) equilibrated with buffer C. After washing with buffer C, the bound proteins were eluted with a linear gradient from 0.0 to 0.5 M NaCl in buffer C. Fractions were tested by western blot analysis with antibodies raised against human recombinant annexin V (Maurer-Fogy *et al.*, 1988) and positive fractions were pooled and applied to a Mono Q column (anion-exchanger, HR 5/5, 1 ml

gel, Pharmacia, Uppsala, Sweden), equilibrated with buffer C. Proteins were eluted with a linear gradient from 0.0 to 0.5 M NaCl. Fractions were analyzed and positive fractions were pooled and applied to cation-exchange chromatography with a Mono S column (HR 5/5, 1 ml gel, Pharmacia, Uppsala, Sweden), that was equilibrated with buffer D (25 mM Na-acetate, pH 5.2). The protein peak that eluted from the Mono S with a linear NaCl gradient in buffer D, appeared to be homogeneous for annexin V as determined by Coomassie Blue staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by western blotting. Polyclonal antibodies to annexin V, isolated and purified from adult rat heart, were raised in rabbits using standard techniques. After bleeding of the rabbits the blood was centrifuged (30 min, 3000 g) and the obtained serum was applied to a Protein A column (8 ml gel, Pharmacia, Uppsala, Sweden) for IgG purification. Subsequently antibodies were immunoaffinity purified, using a sepharose 4B column (Pharmacia, Uppsala, Sweden) with immobilized purified rat heart annexin V.

3.2.4 Other antibodies used in the study

Other antibodies used in this study were polyclonal antibodies raised against human placental annexin I/II, III, IV and VI (Römisch *et al.*, 1990) (generously provided by Dr J. Römisch, Research Laboratories, Beringwerke AG, Marburg, Germany). The antibodies used against annexin I and II did not discriminate between the two annexins and are therefore denoted as antibodies against annexin I/II. Also used in this study were immunoaffinity purified antibodies to human recombinant (r) annexin V and annexin VIII (Hauptman *et al.*, 1989; Maurer-Fogy *et al.*, 1988). In addition, twenty-seven monoclonal mouse antibodies to human r-annexin V (Van Heerde *et al.*, 1994) were tested. For secondary detection alkaline phosphatase conjugated antibodies raised against rabbit IgG, peroxidase conjugated rabbit anti-mouse antibodies and peroxidase conjugated goat anti-rabbit antibodies were used.

Mouse monoclonal antibodies against human vimentin (RV203) (generously provided by Prof. Dr. F.C.S. Ramaekers, Department of Molecular Cell Biology and Genetics, Maastricht University, Maastricht, the Netherlands) and mouse monoclonal antibodies directed against rat endothelial cell surface antigen (RECA-1) (generously provided by Dr. A.M. Duijvestijn, Department of Immunology, Maastricht University, Maastricht, the Netherlands) were used.

For secondary detection fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG antibodies and Texas-red-conjugated goat anti-mouse antibodies were used. For electron microscopy a 8 nm gold conjugated protein A (Janssen Pharmaceutica, Beerse, Belgium) was used. All experiments with antibodies were performed at room temperature unless indicated otherwise.

3.2.5 Western blot analysis

Isolated cardiomyocytes, cardiac endothelial and fibroblast-like cells were lysed in PBS (pH 7.8), containing 1% Triton X-100, 1% SDS, 1 mM EDTA, and 1 mM phenyl methyl sulfonyl fluoride (PMSF). The destruction of DNA was accomplished by ultrasonification. To the lysed

cell preparations, rat heart homogenate and extract I (see section 2.2.3) SDS (2% final concentration) and β -mercaptoethanol (2.5 mM final concentration) were added. One-dimensional SDS-PAGE was performed according to Laemmli (Laemmli, 1970) on 10% gels. Twenty μ g of protein per lane was loaded onto the polyacrylamide gels. After separation by SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose (Towbin *et al.*, 1979). Immunoaffinity purified antibodies against rat heart annexin V (see above) were used at a dilution of 1:5000 for detection of annexin V in rat heart homogenates and cell lysates. The blot was exposed to the antibody overnight. Extract I was analyzed by western blotting with the use of antibodies against human annexin I/II, III, IV, V, VI and VIII, respectively (antibodies were diluted by at least 1:5000). For secondary detection alkaline phosphatase-conjugated antibodies raised against rabbit IgG were used at a dilution of 1:3000. Antigen-antibody complexes were visualized by addition of Nitro Blue Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl-Phosphate to the bound alkaline phosphatase.

3.2.6 Immunofluorescence microscopy on cryosections of rat heart tissue

Cryosections (5 μ m) of the formaldehyde fixed rat heart tissue were postfixed with methanol at -20°C for 2 min followed by acetone fixation at -20°C for 30 s. After air-drying for 15 min the sections were exposed to immunoaffinity purified antibodies raised against rat heart annexin V (1:100 in PBS) for 2 h. In the second step, FITC-conjugated swine anti-rabbit IgG antibodies were added to the sections for 45 min. Sections were washed three times for 10 min with PBS between each step. For controls, the primary antibodies were replaced by pre-immune serum, or the anti-annexin V antibodies were pre-absorbed with the rat heart annexin V before use. This preparative technique has been described in full detail in chapter 2 (section 2.2.5).

For double-labeling studies a mixture of mouse monoclonal antibodies directed against rat endothelial cell surface antigen (RECA-1), a marker for endothelial cells, and a mouse monoclonal antibody (RV203) against human vimentin, a marker for cells of mesenchymal origin, such as endothelial cells and fibroblasts, were used. These antibodies were subsequently made visible with Texas-red-conjugated goat anti-mouse antibodies. For controls, the antibodies to annexin V, RECA-1 and vimentin were replaced by pre-immune serum.

3.2.7 Immunogold labeling on cryosections of rat heart tissue for electron microscopy

Ultrathin cryosections were cut using a Reichert S cryotome with cryochamber and knife temperature set at -110°C ; cutting speed was about 1 mm/s. Sections were quickly removed from the knife edge with a stainless steel wireloop filled with 2.3 M sucrose and transferred to carbon coated formvar grids. The grids were placed on 0.1 M PBS drops with the sections downwards. Immediately after sectioning the grids were processed for immunogold labeling using the rabbit polyclonal, affinity purified, antibody against rat heart annexin V (see 3.2.3) in three different dilutions [1:10, 1:20 and 1:50 in 5% (v/v) fetal calf serum (FCS) in 0.1 M PBS]. This antibody was visualized by incubation with a 8 nm gold conjugated protein A (1:30). The labeling procedure was started by blocking with 10% (v/v) FCS in 0.1 M PBS for 20 min. This procedure

was followed by 2 times 5 min washing with 0.1 M PBS and subsequently a 30 min incubation with the antibody against annexin V. The latter incubation step was omitted in control sections. Thereafter, sections were rinsed 3 times for 5 min with 0.1 M PBS and incubated 30 min with 8 nm gold conjugated protein A in 5% FCS in 0.1 M PBS. Subsequently, sections were washed 3 times for 5 min with PBS followed by a 3 times 10 min rinse with distilled water. Finally, sections were counterstained and embedded using uranyl methylcellulose [0.3% (w/v) uranylacetate and 3% (w/v) methylcellulose in 0.1 M PBS]. This final step was carried out on ice. Excess uranyl methylcellulose was removed with filtration paper. The sections were air-dried and examined in a Philips CM10 electron microscope using a 80 KeV electron beam. This technique has been described in full detail in chapter 2 (section 2.2.8).

3.2.8 Isolation and northern blot analysis of mRNA

Total RNA was isolated from freshly isolated myocytes, cultured fibroblast-like cells and endothelial cells, and from intact rat heart ventricular tissue, according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Total RNA (10 µg per lane) was size fractionated by agarose gel electrophoresis (1% gel containing 3% formaldehyde) and transferred to positively charged Nylon membranes (Boehringer Mannheim) by capillary transfer using 20 x SSC (3 M NaCl, 0.3 M sodiumcitrate, pH 7.0). After cross-linking the RNA was stained with methylene blue to visualize the ribosomal RNA bands and to check for possible differences in loading and/or transfer. A Hind III fragment (0.8 kilobases) of a rat annexin V cDNA probe (generous gift from Dr. J. Browning from Biogen Research Inc., Cambridge, Massachusetts, U.S.A.) was labeled with ^{32}P by random priming and allowed to hybridize at 60°C overnight. Next day the membranes were washed at the appropriate stringency and exposed to X-ray film (FUJI RX).

3.2.9 Measurement of annexin V levels with an enzyme-linked immunosorbent assay (ELISA) with anti-human r-annexin V antibodies

The annexin V ELISA of the sandwich-type was performed in 96-well plates pre-coated (2 h, 37°C) with 0.25 µg/well polyclonal human r-annexin V diluted in 0.1 M carbonate buffer (pH 9.6). The wells were washed five times with PBT (PBS containing 0.05% Tween 20 plus 0.1% BSA, pH 7.4). All other steps were performed in 100 µl PBT. Next, the samples, standards and blanks were incubated in duplicate for 90 min at room temperature. Afterwards, the wells were washed three times with PBT. Monoclonal antibody to human r-annexin V (0.5 µg/well diluted in PBT) was incubated for 1 h at room temperature. After washing with PBT, peroxidase conjugated rabbit anti-mouse antibodies (1:200 diluted in PBT) were incubated during 1 h at room temperature. Alternatively, biotinylated polyclonal antibody to human r-annexin V was used as detector. After washing avidin-horseradish peroxidase (HRP) solution was added to each well. Unbound peroxidase-conjugated rabbit anti-mouse antibodies or avidin-HRP complexes were washed away and peroxidase activity was developed with 45 mM *ortho*-phenylenediamine and 8 mM H_2O_2 dissolved in 0.5 M citrate buffer (pH 5.0). The reaction was terminated with 4

NH_4SO_4 . The absorbance was measured at 492 nm with a Titertek Multiscan MK II.

For preparation of the samples cells were lysed in PBS (pH 7.2) containing 1% Triton X-100 and 10 mM EDTA. Tissue homogenates were prepared in the same buffer. Homogenates were frozen and thawed four times and centrifuged for 2 min at 10,000 g. For protein measurements cells were diluted in PBS. The amount of protein was assessed according to Lowry and colleagues (Lowry *et al.*, 1951).

3.2.10 Measurement of annexin V in a phospholipid binding assay

The annexin V phospholipid binding assay was performed in 96-well plates. The inner 60 wells were coated with 4 μg phosphatidylserine (PS) per well from a stock solution of 80 μg PS/ml ethanol. The ethanol was evaporated with N_2 . The coated wells were blocked with buffer X (50 mM Tris-HCl, pH 7.8, 100 mM NaCl and 5 mM CaCl_2) supplemented with 10% FCS for 30 min at room temperature. Samples were diluted in buffer X plus 0.1% BSA and 50 μl was added to each well. Subsequently 50 μl of a buffer containing 50 mM Tris-HCl, pH 7.8, 5 mM CaCl_2 and 1 M NaCl was added to each well and the samples were incubated overnight at 4°C. All samples, standards and blanks were incubated in duplicate. After washing with buffer Y (50 mM HEPES-NaOH, pH 7.8, 100 mM NaCl and 5 mM CaCl_2), the wells were incubated with buffer Y, plus 3.5% formaldehyde for 30 min at room temperature. A subsequent washing step with buffer Y was performed to remove the free formaldehyde. Polyclonal antibodies to human r-annexin V were diluted in buffer X containing 0.1% BSA were incubated for 1 h at room temperature. After washing with buffer X, containing 0.05% Tween 20, peroxidase labeled goat anti-rabbit IgG (1:1500 diluted in buffer X, containing 0.05% Tween 20) was added and incubated for 1 h. Unbound peroxidase-conjugated goat anti-mouse was washed away and peroxidase activity was developed as described above. The absorbance was measured with an Titertek multiscan MK II.

For preparation of the samples cells were lysed in 10 mM Tris, 10 mM EDTA, 5 mM STI pH 7.9. Samples were frozen and thawed four times and centrifuged for 2 min at 10,000 g. For protein measurements cells were diluted in PBS.

3.2.11 Measurement of annexin V with an enzyme-linked immunosorbent assay (ELISA) with anti-rat heart annexin V antibodies

A sandwich-type ELISA was performed on extracts from rat heart tissue, isolated cardiac myocytes, cardiac endothelial and fibroblast-like cells. Microtiter plates (Becton Dickinson, Oxnard, U.S.A.) were coated with 600 ng immunoaffinity purified antibodies against rat heart annexin V in 75 μl 0.1 M carbonate buffer (pH 9.6) at 37°C for 2 h. Thereafter, the plates were washed five times with PBT (PBS containing 0.05% Tween 20 plus 0.1% BSA, pH 7.4). All other steps were performed in 50 μl PBT. Homogenate samples were incubated in the coated wells overnight at 4°C. Blank values were assessed by incubating 50 μl PBT in the IgG coated wells. All samples, standards and blanks were determined in duplicate. The next day the wells were washed 5 times and per well 400 ng biotinylated-anti-rat heart-annexin V was added and

incubated for 90 min. After washing 50 μ l of an avidin-HRP solution was added to the wells and the final mixture was incubated for 1 h. Excess avidin-HRP complex was removed by multiple washing with PBT and peroxidase activity was measured as described before. Absorbance was measured at 492 nm with a Titertek Multiskan MK II. Preparation of the samples and protein measurements took place as described in section 3.2.9.

3.3 RESULTS

3.3.1 The presence of various members of the annexin family in the rat heart

Rat heart tissue was investigated for the presence of members of the annexin family by western blotting. Figure 3.1 illustrates that in addition to annexin V the annexins I/II, III, IV and VI were expressed in the cardiac tissue. In contrast, annexin VIII could not be detected in the rat heart.

Figure 3.1. Western blot analysis of a Ca^{2+} dependent phospholipid binding enriched protein fraction of rat heart. Each lane contained equal amounts of proteins (20 μ g). After blotting the lanes were cut and treated separately with antibodies against human annexins. Lane 1: anti-annexin I/II, lane 2: anti-annexin III, lane 3: anti-annexin IV, lane 4: anti-annexin V, lane 5: anti-annexin VI and lane 6: anti-annexin VIII.

3.3.2 Sandwich ELISA technique with monoclonal and polyclonal antibodies against human r-annexin V

A sandwich ELISA was developed with the use of polyclonal and monoclonal antibodies raised against human r-annexin V. The polyclonal antibodies raised against human r-annexin V were coated on the plates and served as catcher. First, the monoclonal antibodies raised to human r-annexin V were tested as detector (see 3.2.4). Of the twenty-seven monoclonal antibodies tested only six showed a positive signal with human r-annexin V. In figure 3.2a an example of a typical calibration curve (monoclonal WAC2A) with human r-annexin V is given. Using this monoclonal antibody as detector the detection limit (calculated as two times the blank value) for human annexin V was 1.5 ng/ml. Annexin V content in HUVEC cells and human fibroblasts amounted to 1.5 μg and 3.5 $\mu\text{g}/\text{mg}$ protein, respectively. In samples of rat cardiac endothelial

Figure 3.2. Calibration curves of the sandwich enzyme-linked immunosorbent assays for annexin V. Figure 3.2a shows the indicated amounts of human r-annexin V assayed in the ELISA in which anti-human r-annexin V polyclonal antibodies were used as catcher and monoclonal antibodies raised against human r-annexin V as detector (for details see paragraph 3.2.9.). Figure 3.2b shows the indicated amounts of human r-annexin V as assayed in the ELISA technique with PS as catcher and polyclonal antibodies raised against human r-annexin V as detector (for details see paragraph 3.2.10.). In figure 3.2c a typical calibration curve of the ELISA technique with PS as catcher for rat heart annexin V is given (For details see paragraph 3.2.10.). Figure 3.2d shows the indicated amounts of purified rat heart annexin V tested in the ELISA with anti-rat heart annexin V antibodies as catcher and detector (for detailed description see paragraph 3.2.11.).

cells (RHEC-50 and RHEC-116) annexin V was not detectable. Similar results were obtained when other monoclonal antibodies against human r-annexin V were tested. Second, polyclonal biotinylated human r-annexin V antibodies were tested as detector with 10-50 ng of purified rat heart annexin V per well and showed a cross-reactivity of maximally 30%. As such these antibodies were not usable in the sandwich ELISA for the detection of rat heart annexin V. In a next step to obtain a sensitive ELISA system phosphatidylserine (PS) was tested as catcher (see 3.3.3).

3.3.3 ELISA technique with phosphatidylserine (PS) as catcher

An ELISA with PS coated on the plate as binding agent for annexins and polyclonal antibodies raised against human r-annexin V as detector was tested. An example of a typical calibration curve for human r-annexin V is given in figure 3.2b. Moreover, a typical calibration curve for rat heart annexin V is shown in figure 3.2c (for isolation of rat heart annexin V see section 3.3.4). The detection limit was 1 ng/ml with human r-annexin V. In samples of HUVEC cells annexin V content averaged to 2 µg/mg protein. The detection limit with rat heart annexin V was 5.5 ng/ml. In cell preparations originating from rat tissue no annexin V could be detected.

The fact that no annexin V could be detected in rat samples by the tests described in 3.3.2 and 3.3.2 is likely to be due to low cross-reactivity between the anti-human annexin V antibodies and the rat heart annexin V antigen. Accordingly it was decided to raise antibodies against purified rat heart annexin V by immunizing rabbits.

3.3.4 Isolation and purification of cardiac annexin V

A successful attempt was made to isolate and purify annexin V from an enriched protein fraction obtained from adult rat cardiac tissue. The protein fraction concerned binds to phospholipid membranes in a calcium dependent manner. This material was fractionated by chromatography on a Q-Sepharose column. The fractions eluting from the column were analyzed by western blotting using antibodies directed against human recombinant annexin V (Maurer-Fogy *et al.*, 1988). Annexin V antigen appeared in the fractions that eluted with 0.24-0.26 M NaCl. The annexin V containing fractions were pooled and applied to a Mono Q column. The bound proteins were released from the column by a linear gradient of NaCl (figure 3.3a). The eluted fractions were analyzed by SDS-PAGE. Those fractions containing a 32 kDa band were pooled, dialyzed and subsequently loaded on a Mono S column. Under the conditions described potentially contaminating proteins did not bind to the column. The 32 kDa band, which did bind, eluted from the column as a single peak upon application of a linear gradient of NaCl (figure 3.3b). This peak was analyzed by SDS-PAGE, isoelectric focusing (IEF) and western blotting and appeared to be homogeneous for annexin V as a 32 kDa protein with an isoelectric point of 4.8. The purification procedure yielded 5 µg of purified annexin V per gram of heart tissue.

Figure 3.3. Purification of annexin V from rat heart homogenate. Ion-exchange chromatography on Mono Q and Mono S. The fractions of Q-Sepharose chromatography, that contained annexin V antigen, were applied to Mono Q. The bound proteins were released by a linear gradient of NaCl (a). The Mono Q derived fractions, that contained a 32 kDa protein, were submitted to Mono S chromatography using a linear gradient of NaCl (b).

Figure 3.4. Western blot analysis of cardiac tissue and different cardiac cell types. Samples were prepared as described in the method section. Samples (20 μ g protein/lane) were submitted to SDS-gel electrophoresis and western blot analysis using a polyclonal antibody to rat heart annexin V. Lane 2: rat heart homogenate; lanes 3 and 4: cardiac myocytes; lane 5: fibroblast-like cells (CFLC-122); lanes 6 and 7: endothelial cells (RHEC-50 and RHEC-116). Purified rat heart annexin V (1 μ g) was run as reference (lane 1).

3.3.5 Annexin V in the adult rat heart and a variety of cardiac cell types (western blotting)

The purified rat heart annexin V was used to raise antibodies in rabbits. These antibodies were employed in a western blot analysis of total rat cardiac tissue and isolated rat cardiac myocytes, endothelial and fibroblast-like cells. Figure 3.4 illustrates that all samples investigated contained annexin V, albeit at various levels. The fibroblast-like cells contained the highest amount of annexin V per mg cellular protein as compared to endothelial cells and cardiomyocytes. The latter contained the lowest amount of annexin V.

3.3.6 Immunofluorescence labeling on cryosections of rat heart tissue

In cryosections of rat ventricular tissue, anti-rat heart annexin V stained endothelial cells, fibroblasts and myocytes (figure 3.5a). Staining of fibroblasts and endothelial cells was much more intense than of cardiac myocytes. In control experiments, in which the primary antibodies were replaced by IgG-purified pre-immune serum or the anti-annexin V antibodies were pre-absorbed with pure rat heart annexin V, immunostaining of endothelial cells, fibroblasts, and cardiac myocytes was absent (findings not shown).

Figure 3.5. Distribution of annexin V in rat heart and co-localization with vimentin and RECA-1 as assessed by immunofluorescence microscopy. Sections of rat heart were prepared as described in Methods. In brief, after fixation in paraformaldehyde tissue was infiltrated with sucrose, frozen in isopentane, and sectioned. For the distribution the sections were incubated with anti-rat heart annexin V antibodies and subsequently with FITC conjugated anti-rabbit antibodies (a). For the co-localization the sections were first incubated with rabbit anti-rat heart annexin V and mouse anti-vimentin and anti-RECA-1 antibodies and thereafter with FITC labeled anti-rabbit and Texas-Red labeled anti-mouse antibodies (b). Annexin V localization in non-myocytal cells is demonstrated by the yellow colouring, while annexin V staining of the sarcolemma of the cardiac myocytes is demonstrated by the green colouring. Bars represent 20 μ m. For colour illustrations see p. 149.

Double labeling experiments with a mixture of anti-RECA-1 (a marker of endothelial cells), anti-vimentin (a marker of cells of mesenchymal origin, such as endothelial cells and fibroblasts) and anti-rat heart annexin V showed that RECA-1 (red colouring), vimentin (red colouring) and annexin V (green colouring) colocalized (resulting in yellow colouring) in the endothelial cells and fibroblasts (figure 3.5b). This yellow colouring was uniformly distributed over the non-myocytal cells, indicating a cytoplasmic annexin V localization. In the RECA-1 and vimentin negative myocytes a weak green staining of annexin V was observed in close vicinity of the sarcolemma. In control experiments for the double labeling study, in which the antibodies to annexin V, RECA-1 and vimentin were replaced by pre-immune serum, immunostaining was absent (findings not shown).

3.3.7 Immunoelectron microscopy on cryosections of rat heart tissue

To determine the subcellular localization of annexin V in more detail, ultra-thin cryosections of fixed adult rat heart tissue were labeled with anti-rat heart annexin V, followed by immunogold labeling. Examination of the cardiac myocytes revealed that the gold particles were seen in close vicinity of the intercalated disc and sarcolemma (figure 3.6). In other subcellular structures of the cardiomyocyte such as myofibrils, mitochondria, and nuclei, no label was detected. No immunogold labeling was seen in control experiments, in which the anti-annexin V antibody was omitted from the procedure.

3.3.8 ELISA technique with antibodies raised against rat heart annexin V

With antibodies raised against rat heart annexin V a specific sandwich ELISA for the quantitation of rat heart annexin V was developed. In figure 3.2d an example of a typical calibration curve is shown. The intra- and interassay variation coefficients were 3.6% and 4.7%, respectively. The detection limit of the assay was 0.5 ng/ml. To evaluate whether annexin V binding to the catching antibody was influenced by other proteins the recovery was determined. Therefore known amounts of purified rat heart annexin V were added into a tissue homogenate (spiking). The ELISA recovered at least 90% of the added annexin V.

3.3.9 Content of annexin V in the adult rat heart and a variety of cardiac cell types

The rat heart annexin V content in adult rat heart tissue amounted to 120 $\mu\text{g/g}$ wet tissue (0.70 $\mu\text{g/mg}$ total protein). The annexin V content was also determined in different rat heart cell types. In isolated adult myocytes, cultured cardiac endothelial cells (RHEC-50 and RHEC-116) and fibroblast-like cells annexin V content was found to be 0.17, 0.99, 1.63, and 3.84 $\mu\text{g/mg}$ total protein, respectively. The results are shown in table 3.1.

Based on a study by Anversa and co-workers (1980), 78% of the total heart volume consists of cardiac myocytes, 3.2% of endothelial cells, and 1.9% of fibroblasts. Assuming that protein content of all cells is the same, it can be calculated that 1 mg of total heart protein contains $0.78 \times 0.17 = 0.13$ μg annexin V from cardiac myocytes, $0.032 \times 1.33 = 0.042$ μg annexin V from

Figure 3.6. Evaluation of the subcellular localization of annexin V in rat ventricular tissue as assessed by immunoelectron microscopy. Cardiac tissue was fixed in glutaraldehyde/formaldehyde, cryoprotected by sucrose infiltration and quickly frozen in liquid nitrogen as indicated in paragraph 3.2.1. Sections were labeled with anti-rat heart annexin V and thereafter with gold labeled protein A. Gold particles are present at the level of the intercalated disk (arrows). In other subcellular structures of the cardiomyocyte, such as myofibrils and mitochondria, hardly any label could be detected. Bars represent 0.32 μm .

Table 3.1

Tissue, cell type	annexin V content ($\mu\text{g}/\text{mg}$ protein)
Heart homogenate	0.70 ± 0.06
CMC	0.17 ± 0.08
RHEC-50	0.99 ± 0.25
RHEC-116	1.63 ± 0.34
CFLC-122	3.84 ± 1.19

Data represents means $\pm$ S.D. for $n=3$, except for CMC: $n=6$. CMC (cardiomyocytes), RHEC-50 (rat heart endothelial cell line 50), RHEC-116 (rat heart endothelial cell line 116) and CFLC-122 (cardiac fibroblast cell line 122).

endothelial cells and $0.019 \times 3.84 = 0.073 \mu\text{g}$ annexin V from fibroblast-like cells, making a total of $0.25 \mu\text{g}$ annexin V/mg total protein in total heart tissue. The calculated value is 2.8 times lower than the value directly measured in a total heart homogenate.

3.3.10 Annexin V mRNA in isolated cells of the heart

Northern blot analysis of annexin V mRNA from isolated cardiac myocytes and cultured endothelial (RHEC-116) and fibroblast-like cells (CFLC-122) revealed that all three cell types contained a single band of 1.6 kb, that hybridized with the rat annexin V cDNA probe (figure 3.7). The intensities of the bands indicated that fibroblast-like cells contained more annexin V mRNA than endothelial cells, which in turn contained far more mRNA of annexin V than cardiac myocytes. It should be noted that a similar ratio was measured for annexin V protein levels (see section 3.3.9.).

Figure 3.7. Expression of rat heart annexin V mRNA in cardiac tissue and different cardiac cell types. Hybridization was carried out with the 0.8 kb Hind III fragment of a rat heart annexin V cDNA probe. Lanes contain $10 \mu\text{g}$ of total RNA. Lane 1: rat heart, lane 2: endothelial cells (RHEC-116), lane 3: fibroblasts-like cells (CFLC-122), lane 4: cardiac myocytes (CMC).

3.4 DISCUSSION

In this study the cellular distribution and subcellular localization of annexin V in the adult rat heart was examined by a combination of immunochemical techniques. The present findings demonstrate that annexin V is expressed in endothelial cells, fibroblast-like cells, and myocytes of the rat heart, albeit at substantially different levels. In addition it was shown, by immunolabeling of sections of rat heart tissue, evaluated by light and electron microscopy, that the subcellular localization differs with the cell types studied. In the mesenchymal-derived endothelial cells and fibroblast-like cells annexin V seems to be localized throughout the cytoplasmic compartment, whereas in the mesodermal-derived cardiac myocytes its localization is most likely confined to the plasma membrane.

Annexin V appears to be the most prominent representative of the annexin family in cardiac tissue (Pula *et al.*, 1990). The present study indicates that also other members of the annexin family are present in rat cardiac tissue. Annexin I/II, III, IV, V, as well as VI, were detected by western blotting. In contrast, annexin VIII could not be detected in the adult rat heart using antibodies raised against human annexin VIII. This finding most likely indicates that annexin VIII is absent in rat heart, although it can not be excluded that the negative result is caused by lack of cross-reactivity between rat annexin VIII and antibodies against the human antigen.

In order to quantify the level of annexin V in total cardiac tissue and in the different cardiac cell types an enzyme linked immunosorbent assay (ELISA) of the sandwich type using antibodies against human annexin V was developed initially. Catcher in this assay were polyclonal antibodies raised against human r-annexin V and as detector served monoclonal antibodies to human r-annexin V. In cells of human origin annexin V could be measured with this ELISA, but in samples of rat heart annexin V could not be detected. These results indicated that cross-reactivity between rat and human was far from optimal.

Accordingly, an alternative approach was developed, i.e., an assay in which phosphatidylserine served as catcher for annexin V. In this assay the polyclonal antibody against human r-annexin V was used as detector. Again, with this ELISA it was possible to detect human annexin V. Annexin V levels measured in HUVEC-cells were comparable with the levels measured with the anti-human r-annexin V sandwich ELISA. However, for the detection of rat annexin V the sensitivity was too low and thus the detection limit too high. Annexin V could not be detected in samples of rat heart. As a consequence the decision was taken to develop an assay to measure rat heart annexin V with antibodies raised against this specific antigen. For this purpose annexin V was isolated and purified from adult rat cardiac tissue. The yield of annexin V protein was 5 µg/g wet weight. The results of the ELISA showed that the total amount of annexin V is equal to 120 µg/g wet weight, implying that the recovery of annexin V at the end of the purification procedure is approximately 4%. The purified protein was used for immunization of rabbits and a specific immunoaffinity purified polyclonal antibody to rat heart annexin V was obtained. These antibodies were used to develop a sandwich ELISA specific for rat heart annexin V. To the best of our knowledge, our assay system is the first developed ELISA for annexin V using immunoaffinity purified antibodies against rat heart annexin V.

In cardiac tissue the content of annexin V amounted to 120 µg/g wet weight, which is below the value (approximately 180 µg/g) reported by Giambanco and co-workers (Giambanco *et al.*, 1993) using an antibody raised against porcine lung annexin V. The findings of the ELISA technique with respect to the content of annexin V in isolated cardiac cells were consistent with the data of the western blot analysis. Annexin V was found to be most abundantly present in the fibroblast-like cells, followed by the endothelial cells, whereas relatively small amounts could be detected in the cardiac myocytes. The estimation of the total annexin V content per mg cardiac protein, based on the values measured in the different cell types, resulted in an approximately three fold lower value than the level of annexin V measured in a total heart homogenate. A possible explanation could be that the content in the cultured endothelial cell lines and fibroblast-like cell line are different from the annexin V content of these cell types *in situ*. This notion is supported by the finding that the annexin V content differs between the two endothelial cell lines

studied. Downregulation of the expression of annexin V in those cell types during culturing might have occurred to a different extent.

Northern blot analysis revealed that the mRNA of rat annexin V is present in all cell types of the rat heart. The mRNA level was found to be highest in the fibroblast-like cells, followed by the endothelial cells and lowest in the cardiac myocytes. As indicated above western blot analysis and ELISA showed the same trend with respect to protein content. The co-existence of annexin V mRNA and protein in all cell types of the rat heart studied indicates that each of the cell types has the capacity to synthesize annexin V.

Recently, several groups have investigated the cellular distribution of annexin V in rat heart (Doubell *et al.*, 1993; Giambanco *et al.*, 1991). In line with these studies, annexin V was found in endothelial cells, fibroblast-like cells and cardiac myocytes by western blot analysis. In previous experiments, using antibodies raised against r-human annexin V, we were unable to detect annexin V in rat cardiac myocytes by western blot analysis (Van Bilsen *et al.*, 1992). The most likely explanation for these conflicting observations is the fact that the antibody used in the present study is raised against rat heart annexin V and, hence, displays a higher reactivity against the rat antigen which is present in low amounts in the cardiomyocyte. Results of the ELISA with anti-human annexin V antibodies also showed that cross-reactivity between human and rat annexin V is too limited to detect low amounts of annexin V in rat tissue (see above). The present findings of the western blot analysis were confirmed by the immunolight and electronmicroscopical observations on sections of intact rat heart. These findings indicate that annexin V immunoreactivity was localized in the endothelium, the fibroblasts as well as the cardiac myocytes.

The exact subcellular localization of annexin V in the cardiac myocyte is still a matter of debate. Both a cytoplasmic (Doubell *et al.*, 1993) and a sarcolemmal localization (Giambanco *et al.*, 1991) have been reported for this type of annexin. Interestingly, Doubell and co-workers (Doubell *et al.*, 1993) claimed that the cytoplasmic localization of annexin V truly reflects the *in vivo* situation, because they used antibodies raised against rat heart annexin V. Giambanco and colleagues (Giambanco *et al.*, 1991) applied antibodies that were supposedly less specific, as they were raised against porcine lung annexin V. Nevertheless, despite the use of antibodies raised against rat heart annexin V and the application of a reliable immunohistochemical technique (post-embedding labeling on rat heart tissue sections examined on the light and electronmicroscopical level; for technical details see chapter 2) in the present study we consistently observed a predominant sarcolemmal localization of annexin V in the cardiac myocytes.

In contrast to the cardiac myocytes the subcellular localization of annexin V in non-myocytal cells was observed to be cytoplasmic rather than plasmalemmal. The difference in subcellular localization and concentration of annexin V between myocytes and non-myocytes suggests a difference in the physiological role of annexin V in these particular cell types. The localization throughout the cytoplasm in the fibroblast-like and endothelial cells points to a possible association of annexin V with intracellular membranes or elements of the cytoskeleton. It has been reported that annexin V is able to bind to F-actin at high Ca^{2+} concentrations *in vitro* (Schlaepfer *et al.*, 1992). Sun *et al.* (1992 and 1993) found that in cultured A549 cells annexin

V was associated with both the cytoplasm and nucleoli, and suggested a model in which annexin V promotes the transport of ribosomal proteins between these compartments. Koster *et al.* (1993) reported that annexin V, in agreement with the present findings in rat heart fibroblast-like cells, was located throughout the cytoplasm in cultured chick-embryo fibroblasts. The high level of annexin V in the fibroblast-like and endothelial cells suggests an important cellular role of this protein in these cell types. For example, annexin V could play a role in blood coagulation by secreting annexin V from endothelial cells in case of vascular damage (Van Reycher *et al.*, 1993). As fibroblasts are known to release a variety of substances, annexin V could play a role in the transport of vesicles within these cells. Another possibility is that annexin V plays a structural role in the cytoskeleton of endothelial cells and fibroblasts (Koster *et al.*, 1993).

Although the precise function of annexin V present in the vicinity of, or bound to, the sarcolemma of cardiac myocytes is not yet clear, its localization might suggest that annexin V in the cardiac myocyte is involved in membrane-associated processes, such as exocytosis and the regulation of membrane phospholipid turnover. Doubell *et al.* (1993) have isolated annexin V out of atrial natriuretic factor granula of the atria of the rat heart. Annexin V could be involved in the binding of these granula to the sarcolemma of atrial cardiac myocytes. However, as ventricular myocytes from normal hearts do not contain atrial natriuretic factor this hypothesis does not hold for these types of cells.

In the heart there is a continuous turnover of membrane lipids. Enhanced hydrolysis of phospholipids can be harmful for the heart (Van der Vusse *et al.*, 1992). Van Bilsen *et al.* (Van Bilsen *et al.*, 1992) reported that under *in vitro* conditions annexin V is able to inhibit the activity of endogenous cardiac phospholipases in a Ca^{2+} -dependent manner, similar to what others have found for exogenously added phospholipases (Raynal and Pollard, 1994; Davidson *et al.*, 1990). In this thesis annexin V was found to be present at the sarcolemma which might suggest that annexin V is involved in the phospholipid homeostasis of the cardiac cell membrane by inhibiting the phospholipid hydrolysing enzyme phospholipase A_2 . The mechanism by which annexin V influences phospholipase A_2 activity is incompletely understood. Studies by Davidson *et al.* (1987) support the idea that the inhibitory effect of annexin V on phospholipase activity is not the result of an interaction with the enzyme itself. Instead, the Ca^{2+} -dependent binding of annexin V to the membrane surface diminishes the accessibility of the phospholipid substrate for the phospholipase enzyme (the so-called "substrate depletion model"). Accordingly, it could be speculated that this mechanism is also operative at the sarcolemma.

The phospholipid content of isolated myocytes amounts to 175 nmol/mg protein (Dormans-Linssen, 1993). Assuming that the sarcolemmal phospholipids comprise 5% of the cellular phospholipid content (Van der Vusse *et al.*, 1992) it can be estimated that the inner leaflet of the sarcolemma contains approximately 6.6 nmol of phospholipids per mg cellular protein. In table 3.1 of this chapter it is shown that adult myocytes contain 0.17 μg (that is 5.3 pmol) of annexin V per mg cellular protein. Hence, the molar ratio of annexin V over inner leaflet phospholipids is in the order of 1/1250 in case all annexin V is bound to the sarcolemma of the cardiac myocyte. In *in vitro* systems more favourable ratios (1/250) of annexins over phospholipids are required to achieve "substrate depletion" (Van Bilsen *et al.*, 1992; Davidson *et al.*, 1990). In addition, biophysical analysis of the interaction of annexin V with artificial bilayers has shown that only

in the order of 40 phospholipid molecules are masked by one molecule of annexin V (Andree *et al.*, 1990). Based on these arguments it seems questionable whether "substrate depletion" is a factor of importance in controlling sarcolemmal phospholipid homeostasis if the assumption is correct that annexin V plays a crucial role in this process. In this respect, it is noteworthy, that in a recent study (McKanna *et al.*, 1992) it was shown that reperfusion of ischemic kidney resulted in a marked induction of another member of the annexin family (annexin I), which has also been implicated in the regulation of phospholipase activity (Davidson *et al.*, 1990), suggesting the other members of the annexin family could also play a regulatory role in phospholipid homeostasis.

So, at this moment the biological significance of the presence of annexin V at the sarcolemma (membrane-cytoskeleton interaction, calcium homeostasis) of the cardiac myocyte is still a matter of debate. In order to further delineate the biological significance of annexin V in the heart *in vitro* studies have to be conducted to investigate in detail the potential effect of annexin V on phospholipase A₂-mediated phospholipid hydrolysis. Special attention should be paid to the determination of the ratio of annexin V over phospholipids needed to achieve inhibition of phospholipid breakdown. In addition it is of importance to determine possible changes in the cellular content and subcellular localization of annexin V in relation to alterations in phospholipid homeostasis in response to pathophysiological stimuli.

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Chapter 4

Partial coverage of phospholipid membranes with annexin V may completely inhibit their degradation by phospholipase A₂

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Partial coverage of phospholipid model membranes with annexin V may completely inhibit their degradation by phospholipase A₂

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SUMMARY

Phospholipase A₂-mediated hydrolysis of membrane phospholipids was measured by ellipsometry, and the inhibition of this process by annexin V was studied. Physiological conditions were mimicked by the choices of membrane composition, type of phospholipase A₂, rate of phospholipid hydrolysis, and degree of coverage of the membrane surface with annexin V. Planar membranes, consisting of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (PC/PE/PS; 54/33/13, on molar basis), were degraded by pancreatic phospholipase A₂, and the rate of hydrolysis was limited to about 0.7% per minute. The influence of graded coverage of the membrane with annexin V was studied.

The degree of phospholipase A₂ inhibition was nonlinearly related to the amount of membrane-bound annexin V, and binding of only 12% and 54% of full membrane coverage resulted in, respectively, 50% and 93% inhibition. These findings indicate that the inhibition of phospholipase A₂-mediated hydrolysis by annexin V cannot be simply explained by shielding of phospholipid substrates from the enzyme. Moreover, the present results leave room for a role of endogenous annexin V in regulating phospholipid turnover in the plasma membrane of parenchymal cells such as cardiomyocytes.

4.1 INTRODUCTION

Under normal conditions the membrane phospholipids of cardiomyocytes are subjected to a continuously balanced degradation and resynthesis cycle. However, under pathophysiological circumstances, like ischemia/reperfusion, there is a net degradation of phospholipids (Chien *et al.*, 1984; Van der Vusse *et al.*, 1992; Weglicki *et al.*, 1973). The mechanisms underlying the net decline of the membrane phospholipid pool under pathophysiological circumstances are incompletely understood. It is suggested that the phospholipid degradation rate, although enhanced, is controlled by intrinsic factors during ischemia/reperfusion (Van der Vusse *et al.*, 1992). One of these factors might be the binding to the membrane of annexin V, a member of a family of calcium and phospholipid binding proteins (for recent reviews of the annexins see [Raynal and Pollard, 1994; Van Heerde *et al.*, 1995]). Annexin V was found to be present at the sarcolemma of the cardiac myocyte (Jans *et al.*, 1995). The protein binds to membranes containing negatively charged phospholipids (Andree *et al.*, 1990; Tait *et al.*, 1988), such as phosphatidylserine (PS), which under normal conditions are predominantly located in the inner leaflet of the cardiac cellular membrane (Post *et al.*, 1995).

Antiphospholipase activity *in vitro* of the annexins was first reported for annexin I and later also for other annexins (Raynal and Pollard, 1995). The inhibitory mechanism proposed involves masking of substrate (phospholipids) by annexins (Davidson *et al.*, 1987). As such, annexins are thought to be Ca²⁺-dependent non-specific inhibitors of phospholipases. This notion suggests that they may be involved in the phospholipid homeostasis in the heart. However, the estimated molar ratio of annexin V over the phospholipids of the inner leaflet, which is in the order of 1:1250, does not support a role for annexin V in the cardiac myocyte by simply shielding off phospholipids (Jans *et al.*, 1995).

The aim of the present study was to investigate the potency of annexin V to inhibit phospholipase A₂-mediated hydrolysis of phospholipids under physiologically relevant conditions. Features of the chosen experimental conditions are: (i) Planar membrane surfaces were used and these are more comparable with the sarcolemma than vesicles. For instance, they allow formation of annexin clusters, while such clustering is hampered by high surface curvature in phospholipid vesicles (Andree *et al.*, 1992). (ii) The phospholipid composition of the artificial membrane was PC/PE/PS at molar ratios of 54/33/13, which grossly resembles the composition of the inner leaflet of the sarcolemma of the cardiac myocyte (Post *et al.*, 1995). (iii) In ischemia-induced membrane damage group II phospholipase A₂ is thought to be of importance (Prasad *et al.*, 1991). The pancreatic phospholipase A₂ used in the present study is structurally and functionally related to secretable (group II) phospholipase A₂ (Dennis, 1994). These enzymes have low affinity for PC membranes and need anionic lipids for optimal activity. (iv) The phospholipase A₂ activity used was low, i.e., yielding about 0.7% phospholipid hydrolysis per minute, which is in the same range as the maximal phospholipase activity measured in cardiac tissue (Van der Vusse *et al.*, 1989). (v) The membrane was only partly covered by annexin V which is most likely the case in the cardiac myocyte.

A new ellipsometric technique for simultaneous *in situ* monitoring of the binding of annexin V to planar phospholipid membranes, as well as the rate of membrane degradation by phospholipase A₂ was used (Speijer *et al.*, 1996). This technique allows accurate control of the degree of coverage of the phospholipid surface with annexin V and continuous measurement of the mole fractions of degradation products in the membrane.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Dioleoyl-phosphatidylcholine (PC) and dioleoyl-phosphatidylserine (PS) were obtained from Avanti Polar Lipids, Alabaster, Alabama, USA. Dioleoyl-phosphatidylethanolamine (PE) and porcine pancreatic phospholipase A₂ were purchased from Sigma, St. Louis, USA. Recombinant annexin V was prepared as described (Maurer-Fogy *et al.*, 1988). Silicon wafers (1-0-0, type n, phosphorus doped, thickness 500 μm) were obtained from Aurel GmbH, Landsberg, Germany. All chemicals used were of the highest grade available.

4.2.2 Planar bilayers

Planar bilayers on silicon discs were prepared by adsorption of small unilamellar vesicles as described (Giesen *et al.*, 1991). Briefly, vesicles were prepared by mixing PC, PE and PS in a molar ratio of 54/33/13, drying of the lipids under a stream of nitrogen, and sonication of the lipid dispersions (1 mM phospholipids) in Hepes buffer (50 mM, pH 7.4, 100 mM NaCl) for 10 minutes on ice at 7.5 μm peak to peak amplitude. Vesicles were added at a final concentration of 20 μM phospholipid to a vessel containing a rotating hydrophilic silicon disc. Adsorption of vesicles to the disc results in the formation of a continuous planar bilayer (Giesen *et al.*, 1995).

After formation of a continuous planar membrane surface on the disc (2 cm^2), non-bound vesicles were removed. The membrane-coated disc was transferred, avoiding exposure to air, to the ellipsometer cuvette filled with 5 ml of Hepes buffer containing 1 mM CaCl_2 . In all experiments the discs were rotated at a constant angular velocity of 78 rad/s. Phospholipid concentrations were determined by phosphate analysis (Buttcher *et al.*, 1961).

4.2.3 Ellipsometry

Changes in the adsorbed surface mass were measured directly on the rotating disc by ellipsometry at 21°C (Cuyppers *et al.*, 1983; Willems *et al.*, 1993). Light from a He-Ne laser passes a polarising prism and is reflected by the rotating silicon disc, mounted in the ellipsometer cuvette. After reflection the laser beam passes a second prism, the analyzer, before reaching a photodiode. Adsorption of biomolecules to the silicon disc results in changes of the polarization state of the reflected light. The positions of the polarizer and analyzer are automatically adjusted by a computer program such that the light intensity reaching the photodiode is minimized (null-ellipsometry). From the polarizer and analyzer positions the amount of adsorbed phospholipid mass can be calculated with a precision of 3-5 ng/cm^2 , that is, about 1% of the mass of an adsorbed planar bilayer (445 ng/cm^2 , see results).

4.2.4 Phospholipase A_2 -dependent phospholipid hydrolysis

After preparation of a planar bilayer on a silicon disc and measurement of a baseline level of surface mass, hydrolysis of membrane phospholipids was started by addition of phospholipase A_2 (0.3 to 30 ng/ml). Subsequently, the cuvette was continuously infused at 5 ml/min with the same concentrations of phospholipase A_2 . This procedure was performed in order to minimize depletion of phospholipase A_2 from the bulk phase, for instance due to adsorption of phospholipase A_2 to the cuvette walls. Also, the hydrolysis products appearing in the buffer were thus removed.

4.2.5 Annexin V binding to planar membranes and subsequent phospholipase A_2 -dependent phospholipid hydrolysis

Annexin V binding was started by addition of annexin V at final concentrations ranging from 0.1 to 1 $\mu\text{g}/\text{ml}$. Adsorption was interrupted by removal of annexin V from the bulk phase by flushing the cuvette with 50 ml buffer containing 1 mM CaCl_2 . This procedure takes advantage of irreversible binding of annexin V to planar membranes, probably caused by formation of annexin V clusters on the surface (Andree *et al.*, 1992). It results in partial coverage of the membrane with annexin V. Throughout this report, partial coverage will be expressed as percentage of the maximal annexin V binding capacity. This is not necessarily the same as real physical covering of the membrane. A maximally annexin-covered membrane may still have considerable open space between annexin molecules (see Discussion). When a stable level of adsorbed annexin V surface mass was recorded, phospholipid hydrolysis was started by addition

of phospholipase A₂ (1 ng/ml), and the cuvette was continuously infused with phospholipase A₂ (1 ng/ml) at 5 ml/min. After 2000 s, EDTA (2 mM final concentration) was added to remove annexin V from the membrane and to inhibit phospholipase A₂ activity.

4.3 RESULTS

5.3.1 Phospholipid hydrolysis of planar bilayers by phospholipase A₂

Exposure of rotating hydrophilic silicon discs to phospholipid vesicles composed of PC/PE/PS (54/33/13, mol/mol/mol) resulted in adsorption of vesicles and formation of a stable membrane, as shown by the initial part of the curves in figure 4.1, i.e., prior to addition of phospholipase A₂.

Figure 4.1. Phospholipid hydrolysis of PC/PE/PS (54/33/13, mol/mol/mol) membranes by Phospholipase A₂ (PLA₂). PLA₂ was added (arrow) at concentrations as indicated in the figure.

The adsorbed lipid mass was 445 ± 15 ng/cm² (mean $\pm$ S.D.) in the various experiments. For clarity, the initial surface mass was normalized to 100% for each experiment. After recording the baseline level of lipid mass, phospholipid hydrolysis was started by addition of porcine pancreatic phospholipase A₂ (varying from 0.3 to 30 ng/ml). The observed desorption rates, reflecting phospholipid hydrolysis and release of hydrolytic products from the membrane,

Figure 4.2. Effect of annexin V surface coverage on phospholipase A_2 -dependent phospholipid hydrolysis. Membranes were partially covered with annexin V. The degree of coverage is indicated in the figure. Phospholipid hydrolysis was started after 500 s by addition of phospholipase A_2 (PLA_2) (1 ng/ml). After 2500 s, EDTA was added to remove annexin V from the membrane and to inhibit phospholipase A_2 activity. Solid curves present averages and dashed lines indicate the standard errors of the mean.

increased with the phospholipase A_2 concentration used. Desorption rates ranged from about 0.5% per minute with 0.3 ng/ml phospholipase A_2 to about 4% per minute with 30 ng/ml phospholipase A_2 . Product desorption rates were not linear with the phospholipase A_2 concentration, as indicated by a plot of the initial rates against phospholipase A_2 concentration (figure 1, inset). Phospholipid hydrolysis rates in (patho)physiological systems are maximally 1% per minute, as observed in anoxic rat cardiac tissue (Van der Vusse *et al.*, 1989). Therefore, we

decided to study the effect of annexin V coverage on membrane phospholipid hydrolysis by 1 ng/ml phospholipase A₂.

4.3.2 Phospholipid hydrolysis of membranes partially covered with annexin V

PC/PE/PS membranes were partially covered with annexin V by addition of annexin V (0.1 to 1 $\mu\text{g/ml}$) to the cuvette, monitoring of annexin V binding, and flushing of the cuvette with buffer when the desired amount of protein had adsorbed. Maximal annexin V coverage observed in the presence of 1 mM Ca²⁺ was about 190 ng/cm², which is in agreement with previous results (Andree *et al.*, 1990). As shown in Figure 4.2, membranes were prepared with 0, 5, 12, 27, 54 and 100% of maximal annexin V adsorption. Hydrolysis of membrane phospholipids was started by addition of phospholipase A₂ (1 ng/ml). At the end of the experiments EDTA was added, resulting in instantaneous desorption of annexin V and complete inhibition of phospholipase A₂ activity. In figure 4.2, the amount of immediately desorbing annexin V, after addition of EDTA, was about equal to the amount of initially adsorbed annexin V, because either the degraded fraction of the membrane or the amount of adsorbed annexin V was small. The resulting partial

Figure 4.3. Phospholipid hydrolysis rates as function of annexin V surface coverage.

The mean phospholipid hydrolysis rate of all individual experiments, summarized in figure 4.2, are plotted. Degradation rates are expressed as percentage of the original amount of phospholipid adsorbed on the rotating disc.

annexin V adsorptions to the membrane (with the number of experiments) were: 0 (n=10), 9 (n=4), 23 (n=3), 50 (n=3), 100 (n=2) and 190 (n=1) ng/cm². Standard errors of the mean are presented for the four lower coverages with annexin V. It is apparent from figure 4.2 that increased surface coverage with annexin V resulted in decreased phospholipid hydrolysis rates.

Figure 4.3 more directly illustrates the relation between observed mean phospholipid hydrolysis rates and annexin V surface coverage. Hydrolysis rates were calculated from the adsorbed phospholipid surface mass, measured before addition of annexin V, minus the amount after 2000 s of phospholipase A₂ action, as inferred from the remaining phospholipid mass after annexin V desorption and phospholipase A₂ inhibition by addition of EDTA. Already at low surface coverages significant inhibition of the phospholipid hydrolysis rate was observed. For instance, about 22% inhibition of phospholipase A₂ activity occurred when the membrane was covered for only 5% with annexin V, 50% inhibition for only 12% coverage and almost complete inhibition for only 50% coverage.

4.4 DISCUSSION

In the present study we examined the effect of annexin V on phospholipase A₂-dependent degradation of planar model membranes. Ellipsometry was used to measure membrane coverage with annexin V and phospholipase A₂-dependent phospholipid hydrolysis at the same time. Previously, we have shown that the degradation curves reflect desorption of degradation products, while unaffected phospholipids remain adsorbed (Speijer *et al.*, 1996). Adsorption of phospholipase A₂ to the membrane cannot be observed, because even when the phospholipase A₂ adsorption rate would be transport-limited, it would still be less than a few percent of product desorption rates (Speijer *et al.*, 1996). Thus, even with the highest phospholipase A₂ concentration used, desorption rates adequately reflect true desorption of phospholipid hydrolysis products.

4.4.1 Choice of the phospholipid composition of the membrane

Whereas the outer leaflet of the sarcolemma mainly consists of PC and sphingomyelin, the inner leaflet mainly contains PC, PE, PS and a small fraction of phosphatidylinositol (PI). The composition used in the present study, of PC/PE/PS at molar ratios of 54/33/13, roughly resembles the composition of the sarcolemmal inner leaflet. It should be noted, however, that the artificial membrane did not contain naturally occurring membrane proteins as does the sarcolemma, and thus on this point is far from physiological. Possible consequences of this are discussed below.

4.4.2 Choice for pancreatic phospholipase A₂ as model enzyme

Three different phospholipases A₂ have been detected in cardiac tissue: group II phospholipase A₂, high molecular weight phospholipase A₂, and plasmalogen-specific phospholipase A₂ (Van Bilsen *et al.*, 1995). In ischemia-induced membrane damage of the heart,

group II phospholipase A₂ is suggested to be of major importance (Prasad *et al.*, 1991). Additional evidence for this role has been provided by experiments showing that other fatty acids than arachidonic acid are also found as hydrolytic products (Van der Vusse *et al.*, 1989). Because high molecular weight phospholipase A₂ is very specific for sn-2-arachidonyl-containing-phospholipids, it thus seems less important for ischemia-induced phospholipid hydrolysis. In addition, mainly lysophospholipids are found as phospholipid degradation products (Van der Vusse *et al.*, 1989), and this rules out the plasmalogen-specific phospholipase A₂ as a likely candidate. Since purified heart-specific group II phospholipase A₂ was not available in sufficient quantity, pancreatic phospholipase A₂ was used in the present study because of its structural and functional resemblance to group II phospholipase A₂ (Dennis, 1994), making it a fair alternative for the cardiac enzyme. The Ca²⁺-requirement of pancreatic phospholipase A₂ (and of heart-specific group II phospholipase A₂) for optimal activity largely exceeds normal intracellular levels (Dennis, 1994). However, ischemic conditions may result in elevated intracellular Ca²⁺ concentrations (Steenbergen *et al.*, 1987). Furthermore, it has been proposed that in close vicinity to the sarcolemma Ca²⁺ concentrations are higher than in the cytoplasm, and could be in the range of the Ca²⁺ concentration used in the present study (1 mM) (Borgers *et al.*, 1984).

4.4.3 Choice of the phospholipase A₂ concentration

The average phospholipid hydrolysis rate of 0.7% per minute, observed for a phospholipase A₂ concentration of 1 ng/ml, is in the range of degradation rates observed in (patho)physiological systems. For instance, it was shown that endogeneous hydrolytic activity in rat cardiac tissue, deliberately damaged by freezing and thawing prior to storage under anoxic conditions, is about 1% phospholipid breakdown per minute (Speijer *et al.*, 1996). This may indicate the maximal phospholipase activity at membranes of the cardiac myocyte.

4.4.4 Possible mechanisms for phospholipase A₂ inhibition by annexin V

The mechanism of phospholipase inhibition by annexins is still controversial. Specific protein-protein interactions between annexins and phospholipase A₂ have been proposed, but evidence for such interactions is not conclusive (Raynal and Pollard, 1994). It was observed that by increasing the phospholipid to annexin ratio, inhibition of phospholipase A₂ was abolished (Aarsman *et al.*, 1987; Davidson *et al.*, 1987). This led to the so-called substrate depletion model, with annexin V masking the substrate phospholipids (Davidson *et al.*, 1987). It should be noted that in most experiments the actual coverage of the phospholipid substrates with annexins was not precisely known. Our results indicate that simple masking of the membrane phospholipids is not sufficient to describe the observed inhibition. Furthermore, for higher (20-30 ng/ml) phospholipase A₂ concentrations, substantial phospholipid hydrolysis was observed even when the membranes were maximally covered with annexin V (results not shown). Obviously, these membranes still provide binding sites for phospholipase A₂, in agreement with the observation that coagulation factors Xa, Va and prothrombin may still bind to such maximally covered

membranes (Andree *et al.*, 1992). Annexin V may form two-dimensional clusters on the phospholipid membrane (Andree *et al.*, 1992), and thereby could decrease the lateral mobility of membrane-bound phospholipase A₂. It is not clear, however, how such a mechanism could operate efficiently for low surface coverages with annexin V.

Annexin V binds to PS with higher affinity than to PC and PE (Andree *et al.*, 1990, Blackwood and Ernst, 1990), and this could cause clustering of PS. Indeed, experimental evidence for annexin-induced clustering of anionic phospholipids such as phosphatidic acid (Bazzi and Nelsestuen, 1991) and phosphatidylglycerol (Bazzi and Nelsestuen, 1991; Junker and Creutz, 1993) has been provided. The binding affinity of pancreatic phospholipase A₂ for PC membranes is low and is markedly enhanced by the incorporation of anionic phospholipids, like PS (Jain and Berg, 1989). Therefore, clustering of PS by annexin V could explain more than proportional inhibition of phospholipase A₂. However, two observations are incompatible with such a mechanism. First, the enzymatic activity of the complex of coagulation factors Xa and Va, assembled on the surface of PS-containing membranes, also strictly requires the presence of PS and this activity is inhibited by annexin V in (linear) proportion to membrane coverage (Andree *et al.*, 1992). Second, phospholipid hydrolysis of PS-containing membranes by phospholipase A₂ from snake venom is also completely inhibited by half-maximal annexin V surface coverage (results not shown), although hydrolytic activity of this enzyme is not dependent on the presence of PS (Speijer *et al.*, 1996). Therefore, complete inhibition of phospholipase A₂ by annexin V cannot be explained by annexin V-induced clustering of PS.

Annexin V could interfere with the influence of phospholipid hydrolysis products on phospholipase A₂ activity. Although the precise role of hydrolysis products in phospholipase A₂ action is not completely elucidated (Burack and Biltonen, 1994), their accumulation in the membrane may greatly reduce the activity of phospholipase A₂ by product inhibition or substrate dilution (Kupferberg *et al.*, 1981; Lathrop and Biltonen, 1992; Smith *et al.*, 1972). Although in our experimental set-up the hydrolysis products desorb from the membrane, some accumulation is inevitable. From a product desorption rate of 0.7% per minute, as observed with 1 ng/ml phospholipase A₂, a product mole fraction in the membrane of about 0.03 can be calculated (Speijer *et al.*, 1996). A similar value can be estimated from the remaining desorption after inhibition of phospholipase A₂ by EDTA (Figure 4.2, lower curve). Product accumulation in our experiments is modest, and the non-linear dependence of the phospholipase A₂ activities as function of phospholipase A₂ concentration (Figure 4.1, inset) would suggest strong product inhibition. Annexin V could increase this effect by inhibiting desorption of the products from the membrane, for instance by steric hindrance. However, no effect of annexin V binding was observed on the product desorption rates from PS/PE/PC membranes containing 10 mole % of lysoPC and oleic acid (result not shown). It has been reported that annexins reduce the lateral mobility of phospholipids (Gilmanshin *et al.*, 1994; Junker and Creutz, 1993; Meers *et al.*, 1991), even of lipid components with low affinity for annexins such as PC (Gilmanshin *et al.*, 1994; Meers *et al.*, 1991). In the latter studies it was concluded that fluid-fluid phase separation occurred upon annexin binding to PG/PC membranes. Reduced lateral mobility of PC may thus be the result of a decrease in free space in which the lipid can diffuse. Similarly, annexin V may

decrease the rate of lateral diffusion of phospholipid hydrolysis products away from their production site, thereby promoting local product inhibition. This effect could be enhanced by simultaneous reduction of the lateral mobility of phospholipase A₂ molecules by annexin V clusters. Indeed, strong local product inhibition of phospholipase A₂, even at early stages of phospholipid hydrolysis and at low overall product concentrations in the membrane has been suggested (Burack and Biltonen, 1994). Thus, apart from masking of the substrate, annexins may have an additional inhibitory effect on phospholipase A₂ activity by increasing product inhibition.

4.4.5 Is annexin V a candidate for regulating cellular phospholipase A₂ in vivo?

The adsorbed mass of phospholipids on silicon discs is 445 ng/cm², that is about 0.55 nmol phospholipids per cm². We observed 50% inhibition of phospholipase A₂ activity at a coverage of the phospholipid membrane with 23 ng/cm² of annexin V (12% of the maximal annexin V coverage), that is 0.65 pmol/cm². Hence, the molar annexin V/phospholipid ratio is 1/850 for a bilayer and 1/425 for a monolayer. This ratio is about three times the estimated annexin V over sarcolemmal phospholipid ratio of 1/1250 in the rat cardiac myocyte (Jans *et al.*, 1995). However, a major part of the sarcolemma consists of proteins which, by bulging from the membrane, could make parts of the membrane inaccessible for membrane-directed enzymes. Also, cytoskeletal proteins are partially covering the inner phospholipid bilayer. Thus, the ratio of annexin V over freely accessible phospholipid may be considerably higher than 1/1250. Together with our finding that low phospholipid coverages by annexin V are sufficient for substantial phospholipase A₂ inhibition, this suggests that annexin V could be of biological importance in the regulation of phospholipid degradation at the sarcolemma of the cardiac myocyte.

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Chapter 5

Phospholipid degradation in energy-deprived cardiac myocytes: does annexin V play a role?

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Phospholipid degradation in energy-deprived cardiac myocytes: does annexin V play a role?

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SUMMARY

This study explored whether annexin V, a protein with established phospholipase A₂ inhibiting properties, plays a role in the degradation of membrane phospholipids of adult cardiac myocytes during metabolic inhibition (20 mM 2-deoxyglucose and 1 mM iodo-acetic acid). Experiments were carried out on [¹⁴C]- arachidonic acid prelabeled isolated cardiac myocytes, which were subsequently subjected to metabolic inhibition for up to 240 min. Under control conditions, annexin V was found to be localized predominantly at the sarcolemma. After 120 min of metabolic inhibition, the release of lactate dehydrogenase (LDH) was still comparable with control cells, while morphological changes were already visible. After 240 min of metabolic inhibition, LDH release was significantly elevated compared to control cells incubated for the same period of time (35% versus 20% of total cellular activity). All myocytes had lost their typical elongated shape and sarcolemmal "blebs" had been formed. In metabolically inhibited cells, annexin V localization seemed to be more pronounced at the level of the sarcolemma compared to controls, whereas membrane phospholipid hydrolysis occurred at a significantly elevated rate, as evidenced by a significantly enhanced accumulation of labeled arachidonic acid within the cells. The present findings are not in favor of the hypothesis that the increase in net degradation of phospholipids in energy-deprived cardiac myocytes is caused by a loss of annexin V from the sarcolemma, which would increase the vulnerability of the sarcolemma to phospholipase A₂ activity.

5.1 INTRODUCTION

Phospholipids are the main constituents of cardiac membranes and play a pivotal role in preservation of cellular integrity. Under normal conditions, the membrane phospholipid pool is subjected to a continuous turnover process, in which resynthesis keeps up with the hydrolytic degradation of phospholipids (Van der Vusse *et al.*, 1992). Under energy-depriving conditions, such as ischemia and metabolic inhibition, a net breakdown of phospholipids was found to occur (Chien *et al.*, 1984; Chien *et al.*, 1985; Das *et al.*, 1986; Van Bilsen *et al.*, 1989; Van der Vusse *et al.*, 1982). Net degradation of phospholipids, in these circumstances, most likely plays an important role in the development of irreversible cell damage (Van der Vusse *et al.*, 1990). This apparent imbalance of cardiac phospholipid homeostasis can be caused by elevated degradation or impaired (re)synthesis of phospholipids or a combination of both (Van Bilsen *et al.*, 1989; Lochner *et al.*, 1989).

Uncontrolled activity of phospholipid hydrolyzing enzymes, such as phospholipase A₂, is one of the possible causes of enhanced breakdown of phospholipids (Chien *et al.*, 1984; Das *et al.*, 1986; Van Bilsen *et al.*, 1989; Van Bilsen and Van der Vusse, 1995; Van der Vusse *et al.*, 1982). A variety of factors and mechanisms are thought to be responsible for the regulation of enzymes involved in degradation of phospholipids, but the nature of these regulating factors and mechanisms is as yet incompletely understood (Van der Vusse *et al.*, 1992).

Because annexin V, a Ca²⁺ dependent phospholipid binding protein (Van Heerde *et al.*, 1995), is known to inhibit phospholipid hydrolysis *in vitro* (Ahn *et al.*, 1988; Chap *et al.*, 1988; Comera

et al., 1990; Raynal and Pollard, 1994), including myocardial phospholipid degradation (Van Bilsen *et al.*, 1992), a regulatory role of this member of the annexin family in cardiac phospholipid homeostasis has been proposed. In normal cardiac myocytes, annexin V is predominantly localized in close vicinity to the sarcolemma (Giambanco *et al.*, 1991; Jans *et al.*, 1995; Luckcuck *et al.*, 1995). As the sarcolemma has been suggested to be the main site of phospholipid degradation in energy-depleted cardiac cells (Chien *et al.*, 1985; Miyazaki *et al.*, 1990), the loss of annexin V from the sarcolemma might be an important cause of enhanced hydrolytic degradation of membrane phospholipids.

Therefore, the aim of the present study was to investigate annexin V localization in the cardiac cell under energy-depriving conditions, and to determine whether there is a relationship between the rate of degradation of the cellular phospholipid pool and loss of cellular integrity, on the one hand, and a possible change in annexin V localization, on the other. It was hypothesized that under energy-depriving conditions loss of annexin V from the sarcolemma renders the membrane more susceptible for phospholipid hydrolysis. To test this hypothesis, [^{14}C]-arachidonic acid (AA) was incorporated into the endogenous lipid pools of cardiac myocytes, isolated from adult rat heart, and the cells were subsequently subjected to metabolic inhibition. As shown by Chien *et al.* (1985), Gunn *et al.* (1985) and Post *et al.* (1993) this experimental approach has provided useful insights into the mechanism of cell damage induced under energy-depriving conditions. Annexin V localization was analysed immunocytochemically at the light microscopical level. The rate of phospholipid degradation was estimated by measuring the release of radioactive AA from the pre-labeled membrane phospholipid pool. Loss of cellular integrity was assessed by monitoring the release of the cytosolic enzyme lactate dehydrogenase into the incubation medium.

5.2 METHODS

5.2.1 Isolation of cells

Adult cardiac myocytes (CMC) were isolated according to the following protocol. This protocol is a combination of two earlier published isolation procedures (Fisher *et al.*, 1991; Linssen *et al.*, 1990). In brief, hearts were removed from 12 week old male adult Lewis rats under pentobarbital anesthesia. The hearts were subsequently placed in ice-cold modified Krebs Ringer buffer (MKR) containing: 115 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 10 mM NaHCO_3 , 10 mM 2-(4-(2-Hydroxy Ethyl)-1-Piper-aziny)-Ethanesulfonic acid (HEPES), 11 mM glucose, 4 mM taurine; pH was set at 7.4. After cannulation of the aorta, the heart was perfused in a retrograde fashion with a coronary flow of 2 ml/min with MKR which was gassed with 95% O_2 plus 5% CO_2 . An 0.8 μm filter was placed in the system to remove blood cells from the solution. After a washing period of about 5 min, the heart was perfused in a recirculating manner with 40 ml MKR containing 15 mM 2,3-butanedione monoxime (BDM) (Sigma Chemical Co., St. Louis, MO, USA), 0.7% bovine serum albumin (BSA, essentially fatty acid free; Sigma) and 0.07% collagenase (type I, Gibco, Life Technologies Ltd., Paisley, Scotland, UK). After 2 min CaCl_2 concentration was elevated to 0.1 mM and 1 min later to 0.2 mM.

Coronary flow was increased gradually from 2 to 20 ml/min, over a period of 10 to 20 min. When the tissue was softened enough, the heart was removed from the apparatus and blood vessels, atria and fibrous tissue were trimmed from the heart. Thereafter, the ventricles were opened and digested in 30 ml MKR containing 7.5 mM BDM, 1.35% BSA, 0.035% collagenase type I and 0.2 mM CaCl_2 and were gently stirred for 5 min. The tissue was then chopped into small pieces and incubated in MKR medium containing 0.2 mM CaCl_2 and 2% BSA for 5 min. In four steps, each lasting about 1 min, the CaCl_2 concentration was elevated from 0.2 mM CaCl_2 to 1.8 mM CaCl_2 . The cell suspension was subsequently filtered through a 200 μm nylon sieve. Thereafter cells were centrifuged at 17 x g for 40 s. The pellet was resuspended in MKR containing 2% BSA plus 1.8 mM CaCl_2 and the cells were allowed to settle over 3 min. The pellet consisted predominantly of cardiomyocytes. The cells were washed once more by centrifugation at 17 x g for 40 s, to remove any remaining collagenase activity. Finally, the cells were resuspended in CMC culture medium [M199, 2 mM L-glutamine, 13 mM NaHCO_3 , 10 mM HEPES, 100 U/ml penicillin (Gibco), 100 $\mu\text{g}/\text{ml}$ streptomycin (Gibco), 4% fetal calf serum (batch 60045402 from Boehringer Mannheim GmbH, Mannheim, FRG) and 2% BSA]. All steps were performed at 37°C under gassing with 95% O_2 and 5% CO_2 . The yield of the present isolation procedure was found to be about $8\text{--}10 \times 10^6$ cardiac myocytes per heart with 70–80% rod shaped cells.

5.2.2 Labeling of cellular lipids with [^{14}C]-arachidonic acid

An 20 μl aliquot (1.0 μCi) of a stock solution of 50 $\mu\text{Ci}/\text{ml}$ [^{14}C]-arachidonic acid (AA) (Amersham International, Buckinghamshire, UK) was transferred to a glass tube. After evaporation of the solvent (ethanol) the [^{14}C]-AA was redissolved in 200 μl 1,2-propanediol. Subsequently the [^{14}C]-AA was diluted in 9.8 ml CMC-medium to 2 μM (0.1 $\mu\text{Ci}/\text{ml}$).

Immediately after the final step of the isolation procedure, 5 ml CMC-medium containing 0.5 μCi [^{14}C]-AA and 2% BSA was administered to 5 ml of a suspension of $2\text{--}4 \times 10^6$ cells, in order to allow incorporation of [^{14}C]-AA into the cellular lipid pools. BSA was present to enhance cellular viability of cardiac myocytes in suspension. Incubations were performed for 60 min at 37°C and were gently shaken. To remove [^{14}C]-AA not taken up by the cells after 60 min of incubation, the cell suspension was extensively washed with CMC-medium containing 1% BSA. Cells used to study cellular integrity and cells used to monitor annexin V localization were treated in the same way but without the addition of labeled AA.

In a subsequent step, cardiac myocytes were allowed to attach to Falcon dishes. However, as cardiac myocytes do not attach to Falcon dishes in the presence of BSA, it was necessary to gradually decrease the BSA concentration. Accordingly, cell suspensions were kept in CMC-medium plus 1% BSA for 30 min, then in CMC-medium plus 0.5% BSA for 15 min and finally in CMC-medium without BSA for 15 min at 37°C while gentle shaken. Thereafter, the cells ($\pm 10^5$ cells/plate) were allowed to attach to 3.5 cm diameter Falcon dishes in BSA-free CMC-medium. After 10 min the dishes were washed with Hanks' Balanced Salt Solution (HBSS) to get rid of unattached cells. All media were pregassed with 5% CO_2 and 95% O_2 . Fifteen minutes later, the cardiac myocytes attached to the dishes were washed again with HBSS and metabolic inhibition was started ($t=0$ min) (see below).

5.2.3 Metabolic inhibition

Cells were incubated under normoxic conditions in HBSS (2 ml per dish) containing: 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 0.8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 mM Na_2HPO_4 , 0.4 mM KH_2PO_4 . Control cells were incubated in HBSS with the addition of 5 mM glucose. In order to subject cardiac myocytes to metabolic inhibition, glucose was replaced by 20 mM 2-deoxyglucose (DG) (Sigma) and 1 mM iodo-acetic acid (IAA) (Sigma) according to Post *et al.*, 1993. All incubations were performed at 37°C.

5.2.4 Assessment of cellular damage

Integrity of the myocytes was checked both biochemically, by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH), and morphologically. Supernatants and cardiac myocytes on the dishes were collected separately after 0, 120 and 240 min of incubation. The supernatants were centrifuged to remove cell debris. The cells were removed from the plates with HBSS containing 0.1% Triton-X100 and collected in Eppendorf tubes. After addition of 2% BSA, to preserve LDH activity during storage, samples were rapidly transferred to Eppendorf tubes and stored at -80°C until analysis. LDH was assessed spectrophotometrically according to Bergmeyer and Bernt (1974) with the use of a Cobas Bio auto-analyser. All measurements were done in duplicate. The relative release of LDH was determined by comparing the LDH activities of the supernatants with the LDH activities of the total cell homogenates. Before incubation (t=0 min) and after 120 and 240 min of incubation cells were immediately photographed using a light microscope and examined for morphological changes, such as hypercontracture and rounding up of cells, and sarcolemmal bleb formation. In general, quantitative estimations of the number of rod-shaped cardiac myocytes were made by counting six randomly taken photographs, displaying in total about 300 cells.

5.2.5 Analysis of ^{14}C -arachidonic acid in the cellular lipid fraction

The incubation medium of the dishes was carefully removed and centrifuged (100 x g for 1 min) to collect floating cells, which were subsequently dissolved in 1 ml ice-cold methanol. The remaining cells were scraped from the plate with 1 ml ice-cold methanol. Both cellular methanol extracts were combined and lipids were extracted with 6 ml chloroform/methanol (2:1, by vol) according to Folch *et al.* (1957). The lipid classes were separated by thin layer chromatography (TLC) using plates coated with silicagel (Kieselgel 60, Merck, Darmstadt, FRG). After application of the lipid extracts to the plates, the plates were predeveloped with chloroform/methanol/acetic acid/water (10:10:1:1, by vol) until the solvent front had moved 1 cm from the origin. After drying the plates were further developed with petroleum ether/diethyl-ether/acetic acid (120:25:1.5, by vol). TLC was performed in a sealed glass jar at room temperature. After the TLC step, the plates were dried at room temperature. To determine the position of the different radiolabeled lipid pools the lanes were scanned with an automatic TLC-linear analyser (Berthold, Tracemaster 20, type LB2821, Wildbad, FRG). Thereafter, the spots

were scraped from the plate and counted in a liquid scintillation counter (Beckmann, type LS3801, Fullerton, California, USA).

5.2.6 Immunofluorescence cytochemistry

After 0, 120 and 240 min of incubation, cells attached to the Falcon dishes were fixed with 2% paraformaldehyde in phosphate buffered saline (PBS). Cells were flushed from the dishes with 2% paraformaldehyde in PBS and collected. The cardiac myocytes were stored overnight at 4°C in tubes containing 2% paraformaldehyde in PBS. The next day cells were transferred to 10% sucrose in PBS (4°C). After 2 h the cells were embedded in 4% agar and quickly frozen in isopentane cooled on liquid nitrogen. The cellular preparations were stored at -80°C until use. Cryosections (4 µm) of the adult cardiac myocytes were cut with a cryotome and air-dried for 15 min. Sections were first incubated with polyclonal antibodies raised against rat heart annexin V (1:100 in PBS) for 2 h (Jans *et al.*, 1995). Sections were washed three times for 10 min with PBS. In the second step, fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG antibodies (Dakopatts, Copenhagen, Denmark) were added for 45 min after which sections were washed again (see above). Thereafter sections were mounted in 1,4-diazobicyclo-[2,2,2]-octane (DABCO). Sections were examined with a fluorescence microscope.

5.2.7 Enzyme-linked immunosorbent assay (ELISA) of annexin V

To assess the possible release of annexin V from the cardiac myocytes into the surrounding medium, the supernatants were analysed for the presence of annexin V with a specific and sensitive ELISA. Details of this technique have been described in chapter 3.

5.2.8 Statistics

The data are presented as mean values $\pm$ SD. Differences between groups were tested with the non-parametric Mann-Whitney U test for independent samples. Alterations in the distribution of [¹⁴C]-AA over the phospholipid pool and the fatty acid fraction were analyzed with the non-parametric Sign-test for dependent samples in a pairwise fashion for each badge of isolated cells. P values less than 0.05 were considered to be statistically significant.

5.3 RESULTS

5.3.1 Cellular morphology, and release of LDH and annexin V

At the start of the experiment, about 80% of the cardiac myocytes were rod-shaped. After 120 min of incubation about 60% of the control and 20-30% of the metabolically inhibited cardiac myocytes displayed the typical elongated form. Some cells were contracted, and others showed "macroblebs" (Piper *et al.*, 1985). After 240 min of incubation 40-60% of the control cells still possessed their elongated shape. However, after 240 min of metabolic inhibition nearly 100%

of the myocytes had lost their typical elongated shape, and had changed from rod shaped to rounded cells. In addition, "macroblebs" extruded from the sarcolemma, indicating severe damage of the membrane. The morphological appearance of cardiac myocytes at the end of a typical experiment, is shown in figures 5.1a (240 min of control incubation) and 5.1b (240 min metabolic inhibition).

LDH release from cardiac myocytes after 0, 120 and 240 min of control incubation or metabolic inhibition is shown in table 5.1. At the onset of the experiment ($t=0$ min), LDH was found to be already present in the supernatant fraction (8.7% of the total LDH activity), because samples were taken 15 min after the last rinse. During the first 120 min of control incubation and metabolic inhibition, there was a further release of LDH. At this point, LDH release of metabolically inhibited cells was not significantly different from corresponding control cells. However, LDH release after 240 min metabolic inhibition differed significantly from control cells incubated for the same period of time, and also from metabolically inhibited cells incubated for 120 min. After 240 min of metabolic inhibition, cells had lost 35% of the LDH activity, while control cells incubated for the same period of time had released only 20% LDH, indicating more cells had lost their integrity during metabolic inhibition than under control conditions.

Table 5.1 LDH release from control and metabolically inhibited cardiac myocytes

Incubation time (min)	LDH release (%)
0	8.7 ± 5.2
Control	
120	18.6 ± 8.4^a
240	20.0 ± 7.3^a
Metabolic inhibition	
120	23.6 ± 9.0^a
240	$35.0 \pm 10.9^{a,b,c}$

Data represents mean $\pm$ SD for $n=18$ per group. Differences between groups were tested with the non-parametric Mann-Whitney U test; $p < 0.05$ were considered to be statistically significant. Superscripts indicate: ^a significantly different from 0 min, ^b significantly different from corresponding control incubation, ^c significantly different from 120 min metabolic inhibition.

After 120 and 240 min of metabolic inhibition, no annexin V protein could be detected in the supernatants of the cardiac myocytes ($n=4$), indicating that, in contrast to the cytosolic LDH, annexin V is not released from the damaged cardiac myocytes into the surrounding medium. This observation is in line with the finding that the total cellular content of annexin V, measured in

a subset of experiments ($n=4$) with a sensitive ELISA, did not change during the course of metabolic inhibition (data not shown).

Figure 5.1. Morphological features of isolated adult cardiac myocytes.

Figure 5.1a shows a typical example of cells incubated for 240 min under control conditions (HBSS buffer with 5 mM glucose). Figure 5.1b displays cardiac myocytes, incubated in the presence of 20 mM 2-deoxyglucose and 1 mM iodo-acetic acid for 240 min (metabolic inhibition). Note the rounding up of cells and the appearance of sarcolemmal blebs. Bar represents 20 μ m.

5.3.2 Localization of annexin V in control and metabolically inhibited cells

At the onset of the experiment, annexin V immunoreactivity was demonstrated in rod-shaped, contracted, and rounded cells mainly at the level of the sarcolemma of the cardiac myocyte. Immunostaining of parts of the sarcolemma was found to be more pronounced in contracted cells compared to rod-shaped cells, while in rounded cells immunostaining of the whole sarcolemma was more intense. This difference in staining intensity was also found in rod-shaped, contracted, and rounded cells after 120 and 240 min control incubation and after 120 min and 240 min of metabolic inhibition, the only difference being the number of these cells that were contracted or rounded up after the various incubation conditions. Macroblebs formed at the sarcolemma were strongly positive for annexin V. The qualitative findings of immunostaining of cells subjected to the various incubation conditions are shown in figures 5.2a (240 min control incubation) and 5.2b (240 min of metabolic inhibition).

Figure 5.2. Immunocytochemical detection of annexin V in isolated adult cardiac myocytes. Figure 5.2a represents cells incubated under control conditions for 240 min (HBSS buffer with 5 mM glucose). Figure 5.2b shows cardiac myocytes exposed to 240 min of metabolic inhibition (HBSS buffer supplemented with 20 mM 2-deoxyglucose and 1 mM iodo-acetic acid). Note the increase in intensity of immunofluorescence at the level of the sarcolemma of the rounded up cells. Bar represents 10 μ m.

5.3.3 Distribution of [14 C]-arachidonic acid over the cellular fatty acid, triacylglycerol, diacylglycerol and phospholipid pools

Prelabeling of adult cardiac myocytes with [14 C]-arachidonic acid (AA) resulted in the incorporation of 2 to 4% of the added label in the cellular lipid pool. Due to the batch to batch variation in the total amount of [14 C]-AA incorporated in the cardiac myocytes, it was preferred to present [14 C]-AA incorporation in the various lipid pools as a percentage of the total amount of label in the cells (table 5.2). At the end of the prelabeling period, the incorporation of label in the diacylglycerol and triacylglycerol pool was low (0-2% of the total cellular activity). About 3-10% of the label could be detected in the unmetabolized fatty acid pool. The majority of [14 C]-AA was incorporated in the cellular phospholipid pool (85-90%).

After plating of the cells on dishes, the experiments were started 15 min later ($t=0$ min). The distribution of [14 C]-label over fatty acids, triacylglycerols, diacylglycerols and phospholipids in both control and metabolically inhibited cardiac myocytes is shown in table 5.2. At $t=0$ min, the distribution of label was comparable with that observed at the end of the prelabeling period (see above). During the first 120 min, a significant increase of [14 C]-AA could be detected in the cellular (non-esterified) fatty acid pool under both conditions. However, the increase of labeled AA was significantly higher in the metabolically inhibited cells than in control cardiac myocytes.

After 240 min of metabolic inhibition, the difference in the percentage of labeled AA in the cellular fatty acid pool between control cells and metabolically inhibited cells was even more pronounced.

The increase of [^{14}C]-AA in the fatty acid pool was accompanied by a small, but significant decline of radioactivity in the cellular phospholipid pool. After 120 min of metabolic inhibition a significant decrease (-2.3%) of label was measured in the phospholipid pool compared to the situation at the onset of the experiment ($t=0$ min), while no significant change was observed after 120 min control incubation. After 240 min incubation, [^{14}C]-AA in the phospholipid pool of control cells showed a minor, but significant decrease as compared to the situation at the onset of the experiment ($t=0$ min). However, after 240 min of metabolic inhibition the loss of label from the cellular phospholipid pool (-3.6%) was more pronounced than after 240 min control conditions (-0.6%). No significant changes in the label of the triacylglycerol and diacylglycerol pools of metabolically inhibited and control cells could be detected.

Table 5.2 Distribution of [^{14}C]-AA over the various lipid pools (% of total amount of label) in control and metabolically inhibited cardiac myocytes.

Incubation time (min)	Lipid fractions			
	PL	FA	DG	TG
0	90.6 $\pm$ 3.9	6.2 $\pm$ 3.1	3.0 $\pm$ 0.9	0.7 $\pm$ 0.4
Control				
120	90.3 $\pm$ 3.0	7.2 $\pm$ 2.4 ^a	1.8 $\pm$ 0.6	0.7 $\pm$ 0.4
240	90.0 $\pm$ 4.5 ^a	7.2 $\pm$ 3.6 ^a	2.0 $\pm$ 1.3	0.9 $\pm$ 1.0
Metabolic inhibition				
120	88.3 $\pm$ 2.6 ^{a,b}	8.6 $\pm$ 2.1 ^{a,b}	2.0 $\pm$ 0.6	1.0 $\pm$ 1.3
240	87.0 $\pm$ 2.1 ^{a,b,c}	10.4 $\pm$ 1.5 ^{a,b,c}	1.9 $\pm$ 1.1	0.7 $\pm$ 0.6

AA, arachidonic acid; PL, phospholipids; FA, fatty acids; DG, diacylglycerols; TG, triacylglycerols. Data represent means $\pm$ SD for $n=8$ pairs of experiments. Differences between groups were tested with the non-parametric Sign-test in a pairwise fashion; p values < 0.05 were considered to be statistically significant. Superscripts indicate: ^a significantly different from 0 min, ^b significantly different from corresponding control incubation, ^c significantly different from 120 min metabolic inhibition.

5.4 DISCUSSION

In this study, the possible relationship between the cellular localization of annexin V, on the one hand, and loss of cellular integrity and hydrolysis of membrane phospholipids, on the other, was investigated during energy depletion of cardiac myocytes. The present findings indicate that during energy depletion caused by metabolic inhibition, loss of cellular integrity is accompanied by an accelerated degradation of membrane phospholipids. The observation that the sarcolemmal staining for annexin V remained intense, or became even more intense, in damaged cells, does not favor the notion that enhanced membrane phospholipid degradation is caused by a loss of the potentially protective annexin V from the sarcolemma.

Metabolic inhibition of isolated cardiac myocytes resulted in loss of normal morphology, indicating damage of cellular structures, most likely due to energy deprivation (Post *et al.*, 1995). The cardiac myocytes lost their typical elongated shape and sarcolemmal alterations resulted in the formation of membranous blebs. After 240 min of metabolic inhibition all cells had lost their normal appearance. In contrast, a substantial number of cells incubated under control conditions maintained their normal rod shaped form. It is of interest to note that after 240 min of metabolic inhibition about 35% of the total cellular LDH activity was released into the incubation medium. Taking into account that all cells lost their normal morphological appearance, the conclusion has to be drawn that loss of the typical rod shaped appearance itself is not indicative of disruption of the sarcolemma and loss of cellular integrity, but rather presents a stage occurring prior to membrane disruption.

Metabolic inhibition resulted in a significant net degradation of the membrane phospholipid pool. This conclusion is based on two, mutually related, observations. First, the percentage of radioactivity in the cellular (non-esterified) fatty acid pool steadily increased during the course of energy deprivation. Second, the percentage of label in the phospholipid pool showed a relatively small, but significant decline. The increased of label in the cellular fatty acid pool is most likely due to release of radioactive AA from membrane phospholipids as the amount of label in the diacylglycerol and triacylglycerol pool did not change. It should be noted that also in control cells, phospholipid degradation and concomitant accumulation of label in the fatty acid fraction occurred to some extent. The rate of these alterations was, however, significantly lower than in metabolically inhibited cells.

Net hydrolysis of membrane phospholipids and accumulation of radiolabeled AA, as observed in the present experimental set-up, supports the notion that energy depletion impairs normal phospholipid homeostasis. Earlier studies on neonatal cardiac myocytes revealed that metabolic inhibition accelerated the release of labeled AA from the cellular phospholipid pool (Chien *et al.*, 1985; Gunn *et al.*, 1985). Hypoxia exerted a similar effect on neonatal cardiac muscle cells (Kawaguchi *et al.*, 1991) and adult ventricular myocytes (Hagve *et al.*, 1990; Härtel *et al.*, 1991). Phospholipid degradation and accumulation of AA, a fatty acid predominantly incorporated in the cellular phospholipid pool (Van der Vusse *et al.*, 1992), was also readily observed in intact dog and rat hearts subjected to ischemia (Chien *et al.*, 1984; Das *et al.*, 1986; Prinzen *et al.*, 1984; Van Bilsen *et al.*, 1989; Van der Vusse *et al.*, 1982; Weglicki *et al.*, 1973). Because all cellular membranes, including the sarcolemma, mitochondrial and sarcoplasmic

reticular membranes, are constituted of AA-containing phospholipids, some uncertainty might exist about the precise cellular site(s) of phospholipid degradation. However, studies performed by Miyazaki and colleagues (1990) suggest that the sarcolemma is the main site of phospholipid hydrolysis under energy-depriving conditions.

The mechanisms underlying net degradation of membrane phospholipids, caused by an imbalance between phospholipid (re)synthesis and hydrolysis, in cardiac tissue and isolated cardiac myocytes are incompletely understood. Both lack of ATP, required for phospholipid (re)synthesis, and accumulation of AMP, a well known inhibitor of acyl CoA synthetase, the enzyme catalyzing the formation of arachidonoyl CoA, have been proposed as causative factors (Van Bilsen *et al.*, 1989; Van der Vusse *et al.*, 1992). Moreover, Lochner and coworkers (1989) have shown that the activity of enzymes involved in *de novo* phospholipid synthesis is depressed in ischemic rat hearts. Finally, Das and colleagues (1986) observed a significant decline in the activity of lysophospholipid acyltransferase, a key enzyme in the reacylation pathways of phospholipids, in the flow deprived heart. On the other hand, enhanced phospholipase A₂ activity may contribute to the net degradation of membrane phospholipids (Van der Vusse *et al.*, 1992).

Hazen and colleagues (1991) observed a rapid increase in plasmalogen-specific phospholipase A₂ activity in the ischemic rabbit heart. This suggests that this enzyme may play an important role in enhanced degradation of membrane lipids under unfavorable conditions. Earlier studies performed by Bentham and colleagues (1987) and Grynberg and coworkers (1988), on intact rat hearts and isolated cardiac myocytes, respectively, indicated a decline, rather than an increase, in phospholipase A₂ activity under oxygen-restricted conditions. As in these studies, the activity of the enzyme was measured in cell free preparations, extrapolation to the intact organ or cardiac myocyte should be done with care. Prasad and colleagues (1991), however, making use of an antibody against group II phospholipase A₂, presented convincing data that the latter enzyme is involved in membrane phospholipid degradation in rat heart under pathological conditions. Moreover, Weglicki and co-workers (1984) have hypothesized that membrane phospholipids damaged by the peroxidative action of oxygen free radicals are more avidly attacked by cellular phospholipase A₂. Finally, Ca²⁺-sensitive phospholipases A₂ activation due to increased intracellular levels of Ca²⁺ cannot be excluded (Borgers and Piper, 1986).

Recent studies have provided circumstantial evidence that non-enzymatic proteins, such as various members of the annexin family, are potent inhibitors of phospholipase A₂ (Raynal and Pollard, 1994). Annexin V is relatively abundantly present in rat cardiac tissue (Doubell *et al.*, 1993). Its subcellular localization was found to be predominant at the sarcolemmal membrane (Giambanco *et al.*, 1991; Jans *et al.*, 1995; Luckcuck *et al.*, 1995).

Van Bilsen and colleagues (1992) showed that human recombinant annexin V significantly depressed phospholipid degradation when added to a crude homogenate of rat cardiac tissue. Based on these observations, it was hypothesized that enhanced membrane phospholipid degradation and concomitant accumulation of AA may be (partly) caused by an intracellular redistribution of annexin V, either due to a shift of the protein from the sarcolemma to cytoplasmic sites, or by a release into the extracellular compartment.

The present study excludes the possibility that annexin V is lost from energy-deprived cardiac cells, as was recently suggested by Kaneko and coworkers (1994), in the Langendorff

perfused rat heart model. Moreover, the immunocytochemical data indicate that a shift of annexin V from the sarcolemma to intracellular sites is unlikely. The staining of annexin V remained predominantly associated with the sarcolemma. The more intense staining observed in some metabolically inhibited cells might indicate an enhanced deposition of annexin V at the plasma membrane. Hot spots of annexin V are possibly caused by the presence of lipid domains in the sarcolemma, as a consequence of lateral phase separation of the membrane lipids by enhanced intracellular Ca^{2+} levels (Post *et al.*, 1993). In this respect, it is of interest to note that macroblebs extruding from the sarcolemma were particularly annexin V positive. However, it should not be overlooked that the transition of the cell shape from elongated to rounded caused more visual intensification of the immunological staining.

It should be mentioned that the present staining technique using cryosections of cardiac myocytes, does not allow for discrimination between the inner and outer leaflet of the sarcolemma. Hence, part of the annexin V might be attached to the outer leaflet of the membrane of the energy-depleted cells. As annexin V shows a high affinity for negatively charged phospholipids, binding to the outer leaflet of the sarcolemma can only occur when negatively charged phospholipids, such as phosphatidylethanolamine and phosphatidylserine, are present in the outer leaflet. Indeed, recent findings of Post and coworkers (1993) revealed that a shift of plasmalemmal phospholipids takes place under pathological conditions, giving rise to an enhanced content of phosphatidylethanolamine in the outer leaflet. However, only phosphatidylethanolamine asymmetry was found to be lost, and thus, phosphatidylserine remained exclusively restricted to the inner leaflet of the sarcolemma (for review see Post *et al.*, 1995). As studies with artificial membranes have shown that annexin V binding to phosphatidylethanolamine is phosphatidylserine dependent (Stuart *et al.*, 1995), annexin V binding to the outer leaflet of the sarcolemma in the present study seems unlikely. Hence, annexin V is presumed to remain inside the energy-deprived cardiac myocytes. This notion is supported by earlier observations that the intracellular Ca^{2+} concentration readily increases in energy-deprived cardiac myocytes (Borgers and Piper, 1986). As discussed above, enhanced Ca^{2+} levels will increase the binding affinity of annexin V for membrane structures, provided that enough binding sites for annexin V are present (Andree *et al.*, 1990).

Increased hydrolysis of membrane phospholipids in energy-deprived cardiac myocytes, despite persistent sarcolemmal localization of annexin V, might be caused by the earlier reported incomplete coverage by annexin V of the inner leaflet of the sarcolemma (Jans *et al.*, 1995) in combination with enhanced phospholipase A_2 activity, for instance, due to increased intracellular Ca^{2+} levels. However, the present findings do not exclude the possibility that the increase in labeled AA in energy-deprived cells is caused by impaired re-incorporation of AA in the membrane phospholipid pool. As no data are available indicating a role of annexin V in the latter process, it is unknown whether annexin V may exert a negative effect on the re-acylation of phospholipids in energy-deprived cells.

In conclusion, the present data do not favor the notion that the increase in net degradation of phospholipids in energy-deprived cardiac myocytes is caused by a loss of annexin V from the sarcolemma, rendering the plasma membrane more vulnerable for phospholipase A_2 activity.

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Chapter 6

Differential expression and localization of annexin V in cardiac myocytes during growth and hypertrophy

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SUMMARY

Recently it was shown that annexin V is the most prominent member of the annexin family in the adult heart (Jans *et al.*, 1995). Amongst others, annexin V has been suggested to play a role in developmental processes. The aim of the present study was to explore whether in the heart annexin V content and localization change during maturational and hypertrophic growth, in order to obtain indications that annexin V is involved in cardiac growth processes. First, in the intact rat heart annexin V content and localization were studied during perinatal development. It was clearly demonstrated that annexin V content in total heart transiently increased in the first week after birth, from 0.79 ± 0.06 $\mu\text{g}/\text{mg}$ protein at 1 day before birth to a peak value of 1.24 ± 0.08 $\mu\text{g}/\text{mg}$ protein 6 days after birth, whereafter annexin V protein levels declined to a value of 0.70 ± 0.06 $\mu\text{g}/\text{mg}$ protein at 84 days after birth ($p < 0.05$). Differences in annexin V content were also observed between myocytes isolated from neonatal and adult hearts [0.81 ± 0.09 and 0.17 ± 0.08 $\mu\text{g}/\text{mg}$ protein, respectively ($p < 0.05$)]. Moreover, during cardiac maturational growth the subcellular localization of annexin V might change from a cytoplasmic to a more prominent sarcolemmal localization. Second, *in vivo* hypertrophy induced by aortic coarctation resulted in a marked degree of hypertrophy (22% increase in ventricular weight), but was not associated with a change in annexin V localization or content. The quantitative results obtained with intact hypertrophic rat hearts are supported by findings in neonatal ventricular myocytes, in which hypertrophy was induced by phenylephrine (10^{-5} M). In the latter model no changes in annexin V content could be observed either. In conclusion, the marked alterations in annexin V content during the maturational growth in the heart suggest a possible involvement of this protein in this process. In contrast, the absence of changes in annexin V content and localization in hypertrophied hearts compared to age matched control hearts suggests that annexin V does not play a crucial role in the maintenance of the hypertrophic phenotype of the cardiac muscle cell. This notion is supported by observations in phenylephrine-induced hypertrophied neonatal cardiomyocytes.

6.1 INTRODUCTION

The annexins constitute a family of calcium- and phospholipid-binding proteins (Raynal and Pollard, 1994). Annexins have been shown to be widely distributed in animal tissues. In cardiac tissue annexin V appears to be most abundant of all annexins (Giambanco *et al.*, 1991; Jans *et al.*, 1995). Although a variety of functions has been proposed for the different members of the annexin family their precise biological significance is still a matter of speculation (Liemann and Lewit-Bentley, 1995). Circumstantial evidence indicates that several members of the annexin family, including annexin V, are involved in cellular growth and differentiation. This notion is based on the observation that annexins are also present in the nucleus of a variety of cells (Altieri *et al.*, 1996; Jindal *et al.*, 1991; Koster *et al.*, 1993; Mizutani *et al.*, 1992; Raynal *et al.*, 1992; Sun *et al.*, 1992) which suggests a role for these proteins in gene expression. Indeed, annexin II was identified as a primer recognition protein which was able to activate DNA polymerase (Jindal *et al.*, 1991). For annexin V and several other annexins, it was shown that

the level of expression depended on the differentiation state of the cells under investigation (Chiang *et al.*, 1993; Giambanco *et al.*, 1993a; Masaki *et al.*, 1994; Moss *et al.*, 1992; Schlaepfer and Haigler, 1990; Theobald *et al.*, 1994). Little is known, however, about the potential role of annexin V in cell proliferation and differentiation of the heart, and more specifically, of the cardiac myocytes.

During embryonic development, cardiac growth is largely dependent on an increase of the number of cardiac myocytes (proliferative phase). However, within the first weeks of postnatal development, radical changes in cardiac growth take place. Namely, cell division ceases and further growth of the heart is accomplished by the enlargement of the cardiac myocytes (trophic phase) (Clubb and Bishop, 1984; Korecky and Rakusan, 1978; Li *et al.*, 1996). During this postnatal period the cardiac myocyte undergoes a major genetic switch during which many cardiac genes are either activated or silenced (Argentin *et al.*, 1994). In the event that annexin V plays a role in cardiac maturational growth it is feasible that changes in cellular content and/or localization of annexin V would occur during this transition phase. The same holds in case annexin V should be involved in another adaptive process, i.e., cardiac hypertrophy which is also associated with marked changes in the phenotype of cardiac myocytes (Van Bilsen and Chien, 1993).

Accordingly, the present study was designed to investigate changes in the cardiac annexin V protein expression during both maturational growth and hypertrophy, using various experimental models. Firstly, annexin V content and (sub)cellular localization was investigated during the peri-natal development of the heart. Secondly, hypertrophied adult rat heart and hypertrophied neonatal ventricular myocytes were examined. Cardiac hypertrophy was induced by coarctation of the abdominal aorta. Hypertrophic growth of the neonatal myocytes was obtained by stimulation with the α_1 -adrenergic agonist phenylephrine (Knowlton *et al.*, 1991; Shubeita *et al.*, 1992).

6.2 METHODS

6.2.1 Tissue handling

Experiments were performed on Wistar Kyoto (WKY) rats of either sex. To obtain fetal hearts, pregnant rats were sacrificed at day 21 of pregnancy (day -1). Hearts were removed from the fetus or neonates after decapitation at the day of birth, and at day 1, 2, and 6 after birth. Hearts were also obtained from rats at day 21, 70 and 84 after birth. The latter animals were anesthetized with pentobarbital (0.06 mg/g body weight, i.p.) prior to the thoracotomy. All procedures have been approved by the Institutional Animal Care and Use Committee of the Maastricht University.

For the determination of the tissue content of annexin V with ELISA the hearts were rapidly rinsed with ice-cold phosphate buffered saline (PBS), subsequently frozen in liquid nitrogen, and stored at -80°C until use. For immunohistochemistry the hearts were rapidly excised, washed in PBS and fixed in 2% formaldehyde. Thereafter the hearts were incubated in 5% sucrose for 1 h and in 2% sucrose overnight. The next day tissue was frozen in isopentane cooled in liquid

nitrogen.

6.2.2 Hypertrophy of the adult rat heart

Male Lewis rats, 6-7 weeks of age, were anesthetized with pentobarbital (0.06 mg/g, i.p.). The abdominal aorta was exposed through a midline abdominal incision. In the experimental group a ligature was tightened supra-renally around the abdominal aorta (Snoeckx *et al.*, 1990). In sham-operated rats the abdomen was closed after exposure of the aorta. Seven weeks after surgery the animals were sacrificed. The hearts were excised and ventricles and atria were separated. Ventricles were weighed and ventricle weight was used as an indicator of cardiac hypertrophy. Ventricular tissue samples were prepared for immunohistochemistry or for ELISA (as described below).

6.2.3 Isolation of adult and neonatal rat ventricular myocytes

Myocytes from hearts of adult rats (age ~ 84 days) were isolated as described in full detail before (Linssen *et al.*, 1990). In short, after perfusion of the heart with a collagenase solution, the ventricles were used for the isolation of myocytes. Freshly isolated myocytes were used for the determination of annexin V protein content by ELISA.

Neonatal cells were isolated and cultured basically according to Iwaki and co-workers (1990). Neonatal rats (1-3 days old) were decapitated, the heart was excised, atrial tissue was removed and ventricular tissue was minced. The minced tissue was incubated in a spinner flask at 37°C in a 0.5% collagenase/0.5% pancreatin (Gibco, Life Technologies Ltd. Paisley, Scotland, UK) solution in buffer A (116 mM NaCl, 18.3 mM HEPES, 1.0 mM NaH₂PO₄, 5.5 mM glucose, 5.4 mM KCl, 0.83 mM MgSO₄, pH 7.35). After 6 min the enzyme solution was decanted. Fresh enzyme solution was added and the tissue remains were incubated while stirring at 37°C for 20 min. This procedure was repeated four more times. The supernatant of the first digestion step was discarded because it contained mainly damaged cells and blood cells. The supernatants of the last four digestion steps were collected separately. After the addition of newborn calf serum (NCS) (Sera-Lab Ltd., Crawley Down, UK) they were centrifuged at 395 x g for 6 min at room temperature (Sigma 4K10, Osterode, Germany). The pellets obtained were resuspended in NCS and pooled. The pooled cells were centrifuged at 395 x g for 6 min at room temperature. Cells were washed once with buffer A (395 x g, 6 min, room temperature) and resuspended in buffer A. Next cardiac myocytes were separated from other cell types by centrifugation in a discontinuous percoll gradient (Sigma, St. Louis, MO, USA) (top percoll is 41% percoll in buffer A, and the bottom percoll is 59% percoll in buffer A) at 1580 x g for 30 min at room temperature. Under these conditions cardiac myocytes were found in the interphase between the top and bottom percoll. These cells were collected, washed by centrifugation once in buffer A (395 x g, 6 min, room temperature), and the final pellet was resuspended in plating medium [68% Dulbecco's modified Eagle's medium (DMEM) (Gibco), 17% M199 (Gibco), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 10% horse serum (Gibco), 5% NCS].

6.2.4 Culturing of neonatal cardiomyocytes for induction of hypertrophy

After isolation the neonatal cardiomyocytes were plated on 1% gelatine (type B, Sigma) precoated Falcon dishes at a density of 40,000 cells/cm² and placed in a stove humidified with 95% air plus 5% CO₂ at 37°C. Cell cultures were provided with fresh maintenance medium (80% DMEM, 20% M199, 100 U/ml penicillin, 100 µg/ml streptomycin) after 24 h. Subsequently, the cells were stimulated with 10⁻⁵ M phenylephrine (PE). Control cells were kept on maintenance medium. Two days after the start of stimulation cells were harvested for annexin V content analysis by ELISA.

6.2.5 Antibodies

Antibodies used in this study were immunoaffinity purified rabbit polyclonal antibodies raised against rat heart annexin V (Jans *et al.*, 1995). For secondary detection fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG antibodies (Dakopatts, Glostrup, Denmark) were used. All histochemical analyses with antibodies were performed at room temperature unless indicated otherwise.

6.2.6 Immunofluorescence histochemistry

Cryosections (4 µm) of hearts from rats aged 1, 6 or 84 days and of hypertrophied rat hearts were fixed with methanol at -20°C for 2 min followed by acetone fixation at -20°C for 30 s. After air-drying for 15 min sections were exposed overnight to immunoaffinity purified antibodies against rat heart annexin V (1:20 in 1% BSA dissolved in PBS). Sections were washed 3 times for 10 min with PBS between each incubation step. In the second step, FITC-conjugated swine anti-rabbit IgG antibodies were exposed to the sections for 45 min. For controls, the primary antibodies were replaced by IgG-purified pre-immune serum. Sections were mounted in 1,4-diazobicyclo-[2,2,2]-octane (DABCO). Simultaneously sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) to visualize the nuclei.

6.2.7 Enzyme-linked immunosorbent assay (ELISA)

Homogenates of cardiac tissue (5% w/v) were prepared in PBS, containing 1% Triton X-100 and 10 mM EDTA (pH7.2), for ELISA. The same solution was used to prepare cellular lysates of adult and neonatal myocytes. ELISA for annexin V was performed as described earlier (Jans *et al.*, 1995). Protein content was measured according to Lowry and colleagues (1951).

6.2.8 Statistical analysis

Data are represented as mean values ± standard deviation (S.D.). Differences between groups were tested for significance using the Mann-Whitney U test. Critical values of p less than 0.05 were considered to be statistically significant.

6.3 RESULTS

6.3.1 Quantity and immunolocalization of annexin V in pre- and post-natal rat hearts

In order to delineate a possible role of annexin V in the maturational growth of the heart, annexin V protein content was measured in rat heart tissue from pre-natal day 1 to post-natal day 84 with an ELISA technique. The cardiac annexin V content was found to increase transiently in the first week after birth to a maximum of 1.24 ± 0.08 $\mu\text{g}/\text{mg}$ protein in six days old rats (table 6.1, section A). The content of annexin V in the myocardium steadily decreased thereafter, reaching a level of 0.70 ± 0.06 $\mu\text{g}/\text{mg}$ protein in twelve weeks old rats.

Table 6.1 Annexin V content in (A) cardiac tissue as a function of age and in (B) neonatal and adult cardiomyocytes.

	Annexin V content ($\mu\text{g}/\text{mg}$ protein)	n
A. Cardiac tissue		
Age (days)		
-1	0.79 ± 0.06	5
0	$1.18 \pm 0.15^*$	6
2	$1.10 \pm 0.08^*$	6
6	$1.24 \pm 0.08^*$	6
21	0.85 ± 0.10	5
70	0.78 ± 0.15	5
84	0.70 ± 0.06	3
B. Isolated cardiomyocytes		
Cultured neonatal cardiomyocytes	0.81 ± 0.09	4
Adult cardiac myocytes	$0.17 \pm 0.08^*$	6

Data presented as means $\pm$ SD. The amount of annexin V was measured with an ELISA technique. In section A asterisk indicates significantly different ($p < 0.05$) from values measured at day 1 before birth (-1). In section B asterisk indicates significantly different from neonatal cardiomyocytes.

As total cardiac tissue contains a variety of different cell types, including endothelial cells, fibroblasts and cardiac muscle cells, in a subset of experiments, the content of annexin V was measured in purified cardiomyocyte preparations. To this end, neonatal cardiomyocytes were cultured for 48 h and their annexin V content was compared with that of cardiomyocytes isolated

from hearts of adult rats (age 84 days). Data shown in table 6.1, section B, clearly indicates that the annexin V content in cardiomyocytes declines with age.

Immunohistochemical analysis revealed that in hearts obtained at day 1 after birth (figure 6.1a), annexin V immunoreactivity seemed to be spread out over the entire cardiomyocyte, as in the majority of cells the fluorescence signal did not show a specific subcellular localization. In the cardiac myocytes of hearts of 6 days old rats (figure 6.1b), some cells showed a typical sarcolemmal staining, while in other cells the signal was found to be distributed more evenly over the cardiomyocyte. In the hearts of 12 weeks old rats (figure 6.1c) the staining of annexin V was found to be prominent at the level of the sarcolemma. Annexin V immunoreactivity could not be demonstrated in the nucleus of the cardiac myocytes, irrespective of the stage of development. In control experiments, in which the primary antibodies were replaced by IgG-purified pre-immune serum, immunostaining was not detectable (pictures not shown).

Figure 6.1. Immunolocalization of annexin V in postnatal rat hearts: (a) 1 day, (b) 6 days, (c) 84 days of age. In hearts of 1 day old rats in the majority of cardiomyocytes annexin V seems to be spread out over the cells, no specific subcellular localization is shown, while in hearts of 84 days old rats annexin V immunostaining is found mainly at the level of the sarcolemma. In the hearts of 6 days old rats some cells show a sarcolemmal staining, while in other cells the signal is found to be spread out over the cells. Arrows point to sarcolemma. Sections were first incubated with anti-rat heart annexin V and thereafter with FITC-conjugated anti-rabbit antibodies. Bars represent 7 μ m for panel a, b and c.

6.3.2 Quantity of annexin V in the atria and ventricles of the rat heart

In a subset of hearts, the annexin V content was determined in different cardiac compartments, i.e., the left and right atria and ventricles, of neonatal [3-4 days postnatal, i.e., during the switch from hyperplastic to hypertrophic growth of cardiomyocytes (Li *et al.*, 1996)] and adult (84 days) rat hearts (table 6.2). In neonatal hearts it was found that the ventricles contained higher levels of annexin V than the atria. In contrast, in adult heart the annexin V content measured in the right and left ventricles was about half the amount measured in both the right and left atria.

6.3.3 Quantity and localization of annexin V in the hypertrophied rat heart

Next we explored the possibility whether hypertrophy is associated with changes in annexin V content and/or localization. To this end annexin V content and immunolocalization were measured in ventricles of sham-operated and abdominal aorta-banded animals. After 7 weeks the ventricular weights amounted to 0.88 ± 0.02 g and 1.13 ± 0.07 g ($p < 0.05$) for the sham group and aorta-banded group, respectively. This finding indicates a significant degree of hypertrophy, i.e., a 22% increase in ventricular weight. Furthermore, the size of cardiac myocytes was significantly larger in the hypertrophied hearts compared to control hearts (figure 6.2).

At the immunohistochemical level annexin V localization showed no differences in pattern between hypertrophied and control hearts (figure 6.2a and 6.2b). In both groups annexin V staining was predominantly present in close vicinity of the sarcolemma. No cytoplasmic or nuclear staining was observed. Moreover using a sensitive ELISA, annexin V levels in the ventricles of hypertrophied hearts were found not to be significantly different from control hearts when expressed per mg protein (table 6.3, section A).

Figure 6.2. Immunolocalization of annexin V in control hearts (a) and hypertrophic rat hearts (b). In both groups annexin V immunolocalization is detected in close vicinity of the sarcolemma of the cardiac myocytes. Arrows point to sarcolemma. Sections of the ventricles were first incubated with anti-rat heart annexin V antibodies and subsequently with FITC-conjugated anti-rabbit antibodies. Note that on both photographs the cardiomyocytes are sectioned transversely. Bars represent 10 μ m. For colour illustrations see p. 149.

Table 6.2 Annexin V content in the different compartments of the neonatal and adult rat heart.

Tissue	Annexin V content ($\mu\text{g}/\text{mg}$ protein)	n
Neonatal (3-4 days):		
atria	0.47 ± 0.09	6
ventricles	1.05 ± 0.12^a	4
Adult (84 days):		
right atrium	1.22 ± 0.54	4
left atrium	1.09 ± 0.09	5
right ventricle	0.50 ± 0.05^b	5
left ventricle	0.47 ± 0.06^b	5

Data presented as means $\pm$ SD. ^a Indicates significantly different ($p < 0.05$) from values measured in the corresponding atria in the neonatal hearts and ^b indicates significantly different ($p < 0.05$) from values measured in the corresponding right atria of adult hearts. Annexin V was measured with an ELISA.

Table 6.3 Content of annexin V in (A) hypertrophic adult rat ventricles and in (B) phenylephrine stimulated neonatal cardiac myocytes.

	Annexin V ($\mu\text{g}/\text{mg}$ protein)	n
A. Adult rat ventricles		
Sham-operated	0.63 ± 0.11	6
Aorta-banded	0.72 ± 0.11	10
B. Neonatal rat cardiomyocytes		
Absence of PE	0.81 ± 0.09	4
Presence of PE	0.93 ± 0.10	4

Data presented as means $\pm$ SD. Annexin V was measured with an ELISA (A) in ventricles of sham-operated (control) and aorta-banded (hypertrophy) rats and (B) in neonatal rat cardiomyocytes, cultured in the absence or presence of phenylephrine (PE, 10^{-5} M) for 48 h. Significant differences were not observed.

6.3.4 Annexin V content of neonatal ventricular myocytes after phenylephrine stimulation

As a second model to investigate the possible role of annexin V in cardiac hypertrophy, neonatal ventricular myocytes were stimulated with the α_1 -adrenergic agonist phenylephrine (PE, 10^{-5} M) (Van Bilsen and Chien, 1993). Forty eight hours after PE stimulation cell volume had increased significantly (not shown). As compared to non-stimulated cells the protein content per dish ($\sim 3 \times 10^5$ cells) of PE-stimulated cells had increased 1.9 ± 0.6 fold. As shown in table 6.3, section B, PE stimulation did not result in a significant change in the cellular content of annexin V, when expressed per mg protein.

6.4 DISCUSSION

Although *in vitro* studies have provided some clues about the biological function(s) of annexins, their *in vivo* role in the physiological context remains to be established. Annexins have been implicated in such apparently diverse processes as anti-coagulation, signal transduction, and exocytosis (Burgoyne and Clague, 1994). In a previous study it was shown that annexin V is the most abundant member of the annexin family in cardiac tissue and that in the adult cardiac muscle cell it is localized at the level of the sarcolemma (Jans *et al.*, 1995). Several observations indicate that annexins, among which annexin V, may also play a role in cellular growth and differentiation (Fernandez *et al.*, 1994; Giambanco *et al.*, 1993a; Giambanco *et al.*, 1993b; Hofmann *et al.*, 1992; Schlaepfer and Haigler, 1990). In a first attempt to explore whether annexin V is involved in cardiomyocyte growth and differentiation, immunological techniques were applied to determine whether alterations in cardiac muscle cell phenotype are associated with changes in cellular content and/or localization of annexin V.

6.4.1 Cardiomyocyte differentiation and annexin V

In the hearts of adult rats annexin V is mainly localized at the level of the sarcolemma. This pattern was less clear when hearts of neonatal rats were studied. The fluorescence signal was found to be more spread out over the cardiomyocyte, which might indicate a less distinct sarcolemmal localization of annexin V in cardiomyocytes of young age. However, because neonatal cardiomyocytes are substantially smaller in size than adult cardiac muscle cells the conclusion that annexin V in the former cell type is predominantly localized in the cytoplasm should be drawn with caution. It is of interest to note that in hearts of 6 days old rats, both cells with a clear-cut sarcolemmal staining and cells with a more diffusely localized fluorescence signal were found. Furthermore, in the first week after birth a transient increase in annexin V content of the heart was demonstrated. In theory this change in annexin V expression could be due to a rise in annexin V content in either cardiac muscle cells or non-muscle cells. However, in isolated neonatal ventricular myocytes annexin V content was found to be appreciably higher than in adult ventricular myocytes. Accordingly, the observations with the isolated cells support the idea that the postnatal change in annexin V content in the intact rat heart is, at least partially, due to changes in annexin V level in the cardiac myocytes.

Interestingly, the postnatal changes in annexin V content (and probably also in localization) coincide with the postnatal switch from hyperplastic to (maturational) trophic growth of cardiac muscle cells (Clubb and Bishop, 1984; Korecky and Rakusan, 1978; Li *et al.*, 1996). Before birth and in the first week after birth growth of the heart is accomplished by the increase in number of cells by cell division. After this hyperplastic phase, further growth of the heart can only be accomplished by enlargement of cardiac myocytes (trophic phase). During this switch important changes in cardiac gene expression take place, among which the down-regulation of ANF expression in the ventricular compartment (Argentin *et al.*, 1994).

In a variety of cell types the expression of annexin V has been shown to be dependent on the differentiation state of the cells under investigation (Giambanco *et al.*, 1993a; Schlaepfer and Haigler, 1990). In addition, a role for annexins in gene expression was suggested as several annexins, among which annexin V, were also located in the nucleus of various cell types (Altieri *et al.*, 1996; Jindal *et al.*, 1991; Koster *et al.*, 1993; Mizutani *et al.*, 1992; Raynal *et al.*, 1992; Sun *et al.*, 1992). It should be noted, however, that in the present study annexin V could not be detected in the nucleus of the cardiac myocyte, neither during hyperplastic growth nor during trophic growth. Other data which favor a role of annexins in growth and differentiation are based on the identification of annexin I and II as target proteins of receptor protein tyrosine kinases, that are involved in cell differentiation (Cheng and Chen, 1981; Pepinsky and Sinclair, 1986; Sawyer and Cohen, 1985). Hofmann and co-workers (1992) proposed that annexin V might be involved in somite development as it was transiently upregulated in somites during chick embryo development. Furthermore, sequence analysis of the 5'-untranslated region of human annexin V revealed cis-regulatory elements, which are known to be regulated by oncogenic and growth-related stimuli (Fernandez *et al.*, 1994).

About the cause or physiological meaning of developmental or differentiation-dependent alterations in subcellular localization or protein content of annexin V one can only speculate. In a recent study it was shown that overexpression of annexin VI in transgenic mice was associated with marked changes in cardiac Ca^{2+} transients (Genteski-Hambin *et al.*, 1996). The alterations in Ca^{2+} homeostasis were suggested to be due to annexin VI-mediated dysregulation of sarcolemmal ion pumps and/or ion exchangers. Annexin V has also been proposed to play a role in ion homeostasis. In fact it has been suggested that annexin V itself possesses ion channel activity (Demange *et al.*, 1994). In this respect it is noteworthy that during postnatal development there is a marked change in cardiac Ca^{2+} handling (Vornamen, 1996). In adult cardiac myocytes the transient rise in cytosolic Ca^{2+} during contraction is largely dependent on the well-developed sarcoplasmic reticulum, whereas in neonatal myocytes the sarcoplasmic reticulum is still poorly developed (Imanaka-Yoshida *et al.*, 1996). Therefore, at this stage the contribution of the sarcolemmal Ca^{2+} channel and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger to cellular Ca^{2+} handling are relatively important. Alternatively, as *in vitro* studies have shown that annexin V acts as a high-affinity inhibitor of protein kinase C (Raynal *et al.*, 1993; Schlaepfer *et al.*, 1992), a role of annexin V in PKC-mediated processes can be envisaged.

Annexin V has also been implicated in vesicle transport and exocytosis (Burgoyne and Clague, 1994), in general, and it has been questioned whether annexin V is involved in ANF secretion from cardiac cells, in particular. This functional link between ANF and annexin V was

first suggested by the investigations of Doubell and coworkers (1993) who isolated annexin V from heart tissue by using the binding capacity of annexin V to ANF-vesicles. In addition to that, these investigators observed an increased annexin V immunoreactivity in the ANF expressing cells, i.e., the atrial cells of the adult heart. Indeed our ELISA data also showed that in the adult rat the annexin V level in the atria is higher than in the ventricles. However, to clarify the significance of annexin V for exocytosis of proteins like ANF further research is required. In this respect it is noteworthy that we were not able to observe granular localization of annexin V in hypertrophic hearts, thereby questioning the association of annexin V with ANF vesicles under *in vivo* conditions.

6.4.2 Hypertrophy and annexin V

In addition to normal cardiac growth, cardiac hypertrophy is also characterized by a switch in muscle cell phenotype, as evidenced by the re-expression of a panel of fetal genes (Van Bilsen and Chien, 1993). Hypertrophy is an adaptive response of the heart to a chronic increase in hemodynamic load. In the present study hypertrophic growth of the adult rat heart was induced by constriction of the abdominal aorta. Despite the development of a marked degree of hypertrophy, this change in cell phenotype was not associated with marked changes in either annexin V localization or annexin V protein content. However, it should be mentioned that no samples were taken during the early stages of the development of hypertrophy. Hence, it can not be excluded that annexin V content or localization are transiently altered in the early phase of hypertrophy. Again the results with the isolated cells corroborate the quantitative total heart observations, i.e., induction of hypertrophy by stimulating the neonatal cardiac myocytes with the α_1 -adrenergic agonist phenylephrine did not lead to alterations in annexin V content. Hence, the assembled data do not favor the notion that changes in annexin V localization or content are required to maintain the hypertrophic phenotype.

6.4.3 Conclusions

In conclusion, annexin V was found to be transiently upregulated in the first week after birth. In addition, annexin V immunohistochemical analysis suggests a change in annexin V localization in the cardiac muscle cell during maturational growth of the heart. These results might indicate that annexin V plays a role during maturational growth, especially around birth, and as such might have different functions during cardiac development. As no changes in annexin V localization or content were demonstrated in hypertrophied adult cardiac myocytes, alterations in annexin V content or localization are probably not playing an essential role in the maintenance of the hypertrophic phenotype of cardiac myocytes.

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Chapter 7

General discussion

7.1 INTRODUCTION

The annexins form a multigene family of proteins that share structural and functional features. They contain a conserved amino acid sequence, initially termed the endonexin loop (Geisow *et al.*, 1986), which is repeated four or eight fold in the C-terminal region. These conserved repeats form the basis for the shared biological property of annexins to bind to phospholipids in a Ca^{2+} -dependent manner (Huber *et al.*, 1990), whereas specific functions of each annexin are determined by their type-specific N-terminal regions. Annexins have been discovered in the kingdoms of plants and animals. Within the latter they occur in widely separated phyla (Crompton *et al.*, 1988; Johnston *et al.*, 1990; Robitzki *et al.*, 1990; Schlaepfer *et al.*, 1992; Wallner *et al.*, 1986). In mammals almost all cell types express several annexin proteins, which together make up in some cell types more than 1% of total cellular protein (Raynal and Pollard, 1994). In cardiomyocytes a substantially lower content of annexins was observed. To date 13 different annexins have been found in animals and their primary sequences have been fully elucidated. These sequences show that the different annexins share 40-60% structural homology. Within the phylum of vertebrates the individual annexins are highly conserved between the different species. This has led to the hypothesis that the family has evolved from gene duplications of an ancestral gene encompassing the endonexin loop motif (Crompton *et al.*, 1988). Diversification of the family may then have occurred under the selection pressure to diversify on the theme of Ca^{2+} -dependent phospholipid binding.

Such evolutionary pressure has likely led to the appearance of annexin V. This member of the family is the most ubiquitous one, which is present in every tissue of the organism. The large list of synonyms for annexin V reminisces of this and reflects a widespread interest in annexin V and its potency to be active in diverse biological systems (Crompton and Dedman, 1990; Raynal and Pollard, 1994; Van Heerde *et al.*, 1995). The first report concerning annexin V was by Bohn, who isolated it from human placenta and termed it placental protein 4 (PP4) (Bohn, 1979). Later reports described annexin V in conjunction with a biological activity such as a Ca^{2+} -dependent chromaffin granule binding protein possibly involved in exocytosis [chromobindin 5 (Creutz *et al.*, 1983) and 35k calelectrin (Südhof *et al.*, 1984)] and as a collagen binding protein of the chondrocyte (anchurin CII) (Mollenhauer and von der Mark, 1983). Annexin V was isolated from the human umbilical cord artery by virtue of its anticoagulant activity (Vascular Anticoagulant α) (Reutelingsperger *et al.*, 1985). Other synonyms include calphobindin I (Iwasaki *et al.*, 1987), placental anticoagulant protein I (PAP-I) (Funakoshi *et al.*, 1987), endonexin II (Kaplan *et al.*, 1988), lipocortin V (Pepinsky *et al.*, 1988), and 35 g-calcimedlin (Kaetzel *et al.*, 1989).

After cloning of the cDNA of human annexin V it was successfully expressed in bacterial systems (Iwasaki *et al.*, 1987; Kaplan *et al.*, 1988 Maurer-Fogy *et al.*, 1988). This recombinant source catalysed numerous investigations exposing biological, biochemical and physicochemical properties of annexin V, including its *in vitro* anti-phospholipase, anticoagulant, anti-kinase, Ca^{2+} -channelling and phospholipid binding activity, and its free and phospholipid-bound tertiary structure (for recent reviews see Raynal and Pollard, 1994; Swairjo and Seaton, 1994; Van Heerde *et al.*, 1995). Interpretation of these accumulated data from a (patho)physiological

perspective has so far led to the postulation of a number of *in vivo* functions, that equals or even exceeds the number of discovered *in vitro* biological activities. The phospholipid binding property of annexin V appears central in most considerations and is regarded as the key activity elaborated by the organism to serve the as yet unknown role(s).

In the present thesis we investigated the role of annexin V in the rat heart from the point of view that annexin V regulates phospholipid homeostasis by its potential to inhibit phospholipid hydrolyzing enzymes like phospholipase A₂ (Reutelingsperger *et al.*, 1988; Schlaepfer *et al.*, 1987; Van Bilsen *et al.*, 1992). Regarding the pathophysiological implications of phospholipid hydrolysis during cardiac ischemia, annexin V embraces the potency to act as a protective factor in this pathology by virtue of its phospholipase inhibiting properties.

The first approach to shed light on the (patho)physiological significance of rat heart annexin V comprised experiments to elucidate its localization in and expression by the rat heart tissue (chapter 3). Since annexin V expression appears to be associated with cell growth and differentiation (Schlaepfer and Haigler, 1990), localization and expression of rat heart annexin V was studied as a function of maturational and hypertrophic cardiac growth (chapter 6). From *in vitro* data a model was inferred that explained the phospholipase inhibitory activity of annexins by a shielding phenomenon in which annexins bind to the phospholipids and block thereby access of the enzyme to its substrate (Davidson *et al.*, 1987). If that would be the case for annexin V then the ratio annexin V: phospholipids in rat heart cells would have major consequences for the importance of annexin V as a regulator of phospholipase activity. In order to be able to understand the significance of phospholipase inhibition by annexin V in the rat heart cells the ability of annexin V to inhibit phospholipases was studied as a function of phospholipid surface coverage by annexin V (chapter 4). Moreover, annexin V localization was investigated in cardiomyocytes during phospholipid degradation induced by metabolic inhibition (chapter 5).

7.2 THE CONTENTS OF ANNEXIN V IN RAT HEART

As described knowledge about the level of protein expression would offer insight in the (patho)physiological significance of rat heart annexin V. Before starting the investigations of this thesis little was known about the content of annexin V in the rat heart. By Western blotting it was demonstrated that rat heart contains annexin V using antibodies against the endonexin loop motif (Kaetzel *et al.*, 1989) and against rat heart annexin V (Doubell *et al.*, 1993). Van Bilsen and coworkers showed that rat heart annexin V was predominantly present in non-myocytal cells (Van Bilsen *et al.*, 1992) and was not present in cardiac myocytes. However, in their study they used antibodies against human annexin V. Further studies of our laboratory demonstrated that a low cross-reactivity exists between antibodies against human annexin V and rat heart annexin V. Doubell and co-workers (Doubell *et al.*, 1993) were able to detect annexin V in rat cardiac myocytes by using antibodies that were raised against rat heart annexin V. Altogether this shows that in spite of the high degree of conservation of annexin V primary structure between the various mammalian species (Pepinsky *et al.*, 1988) antibodies against annexin V of one species not necessarily recognize annexin V of another species. Therefore we purified rat heart annexin V and raised antibodies against it in order to analyse rat heart for annexin V localization and

contents (chapter 2 and 3).

To serve the purpose of measuring annexin V contents of rat heart we developed a highly sensitive and selective sandwich ELISA on basis of immunopurified antibodies against rat heart annexin V (chapter 3). Using this ELISA we established that adult rat heart tissue contains about 700 ng annexin V per mg protein. In order to assess whether differential expression of annexin V exists we isolated and cultured the various cell types of the rat heart. This resulted in the selection of spontaneously immortalized cell lines of fibroblasts and endothelial cells. Such spontaneous immortalization did never happen with cardiac myocytes. Therefore for each measurement freshly isolated ventricular myocytes were used. Analyzing these rat heart cells by ELISA showed that annexin V, indeed, is differentially expressed by the various cell types. The fibroblast contains the highest level (3840 ng annexin V/mg protein) and the myocytes the lowest (170 ng annexin V/mg protein). In between is the endothelial cell, which contains 1000 - 1700 ng annexin V/mg protein, depending on the cell line (chapter 3). We are herewith the first to quantitate annexin V in cardiac myocytes. Quantitation of annexin V in human fibroblasts and endothelial cells has been reported by others. In those cases annexin V levels were also found to be higher in fibroblasts as compared to endothelial cells (Reutelingsperger *et al.*, 1994; Römisch *et al.*, 1992).

Although our *in vitro* experiments revealed differential expression of annexin V it is very well possible that this is caused by the culture conditions. However, analyzing the organ we showed that in the rat heart regional differences exist for annexin V levels. The atria of the rat heart contain about 1100 - 1200 ng annexin V/mg protein whereas the ventricles comprise significantly less amounts of annexin V (450-500 ng/mg protein, chapter 6). This demonstrates that *in vivo* zonation of annexin V does exist in the rat heart. Assuming that the atrium and ventricle resemble relatively in composition of the various cell types, the difference in annexin V contents should reflect differences in expression levels for the same cell type, e.g. it is possible that the myocyte within the atrium expresses higher levels of annexin V as compared to the ventricular myocyte. In agreement with this others have shown by qualitative analysis that annexin V immunoreactivity is higher in atrial than ventricular myocytes (Doubell *et al.*, 1993). Remarkably, the neonatal heart is opposite to the adult heart as far as relative annexin V levels in atrium and ventricle are concerned. The functional significance of these observations is uncertain. A possible role of annexin V may be in the regulation of the secretion of atrial natriuretic factor (ANF) from the heart, as was suggested by Doubell and associates (1993). This proposal is supported by present results as the distribution of annexin V over the neonatal and adult cardiac compartments resembles the distribution, reported by others, of ANF (Argentin *et al.*, 1994; Zeller *et al.*, 1987). Whether a direct link exists between these proteins remains to be established, but is rather tantalizing since *in vitro* it has been shown that annexin V binds to ANF vesicles, although the calcium concentrations used in their study were not comparable to the intracellular situation (Doubell *et al.*, 1993).

Differential expression of annexin V by the various cardiac cell types could be due to differences in transcription rate and/or protein turnover. That cells may regulate annexin V levels by the latter mechanism has been shown for fibroblasts by pulse chase experiments, which revealed that under constant synthesis rates different rates of annexin V degradation occur as a

function of cell growth (Schlaepfer and Haigler, 1990). Regulation at the transcription level may also occur since it has been found that the rat annexin V gene contains two structurally different promoters (Imai and Koshaka, 1995). One of the promoters contains features typical for housekeeping genes. The other, however, contains regulatory elements like a TATA box, a glucorticoid-responsive element and two AP-1 sites, indicating that transcription of this annexin V gene can be upregulated under certain cellular and/or environmental conditions.

In addition to quantitation of annexin V in the adult rat heart, we investigated annexin V contents of the developing heart (chapter 6). Around day 4 after birth myocytes undergo a phenotypical switch from proliferating cells to (maturational) trophic growing cells (Clubb and Bishop, 1984; Korecky and Rakusan, 1978; Li *et al.*, 1996; Zak, 1974). This switch could have effects on annexin V expression since it was shown that a relationship exists between cell growth and annexin V levels in fibroblasts (Barwicz and Walker, 1996; Schlaepfer and Haigler, 1990) and neuronal cells (Schlaepfer and Haigler, 1990). The physiological importance of such a relationship is emphasized by the findings that i) c-fos, a proto-oncogene active in cell growth and differentiation, enhances annexin V gene transcription in fibroblasts *in vitro* (Brasemann *et al.*, 1992) and ii) the rat heart annexin V gene promoter comprises AP-1 elements, which serve as DNA binding sites for fos/jun hetero dimers (Imai and Koshaka, 1995). Measuring annexin V in the developing heart revealed a transient but significant elevation of annexin V levels in the first week after birth (chapter 6). To investigate whether the observed change of annexin V expression is associated with the phenotypical switch of the myocytes, we measured annexin V in ventricular myocytes isolated from postnatal hearts of 3 days old and from adult hearts of 84 days old by ELISA. The neonatal ventricular myocyte contains about 5 times more annexin V than the adult ventricular myocytes suggesting that the expression of this protein might be involved in the process by which cardiac growth from hyperplasia of myocytes ceases and cardiac maturational growth from trophy of myocytes starts. Remarkably this relationship is opposite to the situation in fibroblasts and neuronal cells, where annexin V levels were lowest in dividing cells (Schlaepfer and Haigler, 1990).

Finally, we investigated annexin V contents under pathological hypertrophic conditions. Such hypertrophy is also characterized by a phenotypic switch of the enlarged cardiac myocytes (Van Bilsen and Chien, 1993). Hypertrophic growth was induced *in vivo* in adult rat heart by constriction of the abdominal aorta and *in vitro* by stimulating neonatal cells with the alpha-1-adrenergic agonist phenylephrine. In the hypertrophied heart annexin V content was found not to alter, despite a marked degree of hypertrophy. In the hypertrophied cultured neonatal myocytes no change in annexin V content could be observed either (chapter 6). These findings imply that alteration of annexin V expression is not required for the maintenance of hypertrophic phenotype in the heart. However, a temporary change in annexin V expression can not be excluded since the quantity of annexin V was not examined during earlier stages of the development of hypertrophy. Accordingly, a possible role of annexin V during the onset of the hypertrophying process still can be conceived. In this context it is of interest to study annexin V expression during the early phase of cardiac hypertrophy.

In conclusion, the work presented shows that annexin V was differentially expressed in the various cell types of the rat heart, the atria and the ventricles and during rat heart maturation in

the cardiac myocyte. Moreover, the annexin V content of the myocyte is not constant, but seems to depend on environmental conditions suggesting regulated transcription/translation in myocytes. In this respect the annexin V gene does not behave like a housekeeping gene.

7.3 LOCALIZATION OF RAT HEART ANNEXIN V

In addition to knowledge about the contents of annexin V, detailed information about the localization of annexin V would help to understand the (patho)physiological significance of annexin V in the heart. In literature conflicting data have been published concerning the intracellular localization of annexin V in the different cell types of the heart and especially its presence in cardiac myocytes. Whereas some investigators reported the presence of annexin V in cardiac myocytes at the level of the sarcolemma (Giambanco *et al.*, 1991; Luckcuck *et al.*, 1995), others detected annexin V in the cytoplasm of cardiac myocytes (Doubell *et al.*, 1993; Wang *et al.*, 1995). It should be noted that the determination of the subcellular localization of annexin V is faced with a variety of difficulties.

In the present thesis it was shown that apart from the use of a specific antibody (the immunoaffinity purified antibody against rat heart annexin V), there are various aspects of importance which could influence the determination of annexin V localization. Annexin V is a protein which binds reversibly to membrane phospholipids, thus each interference with this binding could lead to erroneous results. In chapter 2 it was found that the use of Triton X-100 or saponin to permeabilize the cells, most likely causes the loss of the sarcolemma, and as such the proteins bound to the sarcolemma are no longer detectable. To avoid these types of artefacts labeling on (cryo)sections can be used. In this labeling assay permeabilization of cells is not necessary as the labeling takes place on sections of cells, i.e., the antibodies do not have to penetrate an intact cell. Labeling on sections can be used both, at the light and electron microscopical level and to the best of our knowledge this is the most reliable method for determination of proteins localized at the plasmalemma, such as annexin V.

In the various cardiac cell types a cytoplasmic as well as a sarcolemmal localization was detected. In sections of cardiac tissue annexin V staining of endothelial and fibroblast-like cells was found to be cytoplasmic. This subcellular localization of annexin V strongly suggests that most likely annexin V has an intracellular function. During the past decade a pile of information about the biological activity of annexins has been gathered, however, without solving the enigma of the (patho)physiological significance of the annexins. The bulk of the information about annexins stems from *in vitro* studies and points to roles in processes such as vesicle traffic (Burgoyne and Clague, 1994), signal transduction of Protein Kinase C (Dubois *et al.*, 1996), anticoagulation (Van Heerde *et al.*, 1995) and inflammation (Raynal and Pollard, 1994).

Immunostaining on sections of cardiac tissue on the light and electron microscopical level showed that in the cardiac myocyte annexin V was present in close vicinity of the sarcolemma, which includes the intercalated disks and the T-tubular system. No annexin V was found in other parts/organelles of the cells. It should be noted that the present staining techniques do not allow for discrimination between inner and outer leaflet of the sarcolemma. Hence, it cannot be excluded that part of the annexin V is attached to the outer leaflet of the sarcolemma. Binding

of annexin V to the sarcolemma depends on both the phospholipid composition and Ca^{2+} concentration. Together with the Ca^{2+} level the fraction of phosphatidylserine (PS) in the membrane determines the number of annexin V binding sites (Andree *et al.*, 1990; Stuart *et al.*, 1995; Tait and Gibson, 1992). As in viable cardiac myocytes phosphatidylserine is exclusively restricted to the inner leaflet of the sarcolemma (for review see Post *et al.*, 1995), annexin V binding to the outer leaflet of the sarcolemma seems not likely.

As annexin V was found to be localized at the level of the sarcolemma a possible involvement in membrane associated processes, such as ion-channel activity, membrane-cytoskeleton interaction, exocytosis, and sarcolemmal phospholipid turnover can be envisaged.

Annexin V was also found to be differently located in the cardiac myocyte during maturational growth. In cardiac myocytes of neonatal hearts annexin V was demonstrated to be predominantly cytoplasmic, whereas in the myocytes of adult hearts annexin V was found at the level of the sarcolemma (see above). These data indicate that within a cardiac cell type translocation of annexin V can occur in addition to changes in levels of protein expression. A translocation of annexin V has been reported in human fibroblasts (Raynal *et al.*, 1996). Incubation of the fibroblasts with the calcium ionophore A23187 increased intracellular Ca^{2+} concentration, resulting in a relocation of annexin V to the nuclear envelope. The meaning of the different location of annexin V during cardiac maturational growth is not yet clear. However, the change in annexin V localization during maturational growth points, in addition to the change in annexin V levels, to a possible involvement of annexin V in the physiological growth process.

Annexin V localization was also studied under pathological circumstances, i.e., hypertrophy and energy deprivation. In the hypertrophied heart annexin V localization was found at the level of the sarcolemma of the cardiac myocyte. In the present thesis it was also shown that under energy-deprived conditions annexin V was persistently present in close vicinity of the sarcolemma. So, under both circumstances annexin V localization was found to be basically the same as in the normal cardiac myocyte.

In conclusion, a cytoplasmic annexin V localization was found in the non-myocytal cells and the neonatal ventricular myocytes. Sarcolemmal annexin V localization was shown for the adult ventricular myocyte. This localization was found not to change upon hypertrophic or energy depriving conditions. The differential location of annexin V within the cardiac myocyte points to possibilities of translocation of annexin V. Accordingly, in addition to changes in annexin V content the phenotypical switch of myocytes occurring within the first week after birth also seems to be associated with an intracellular redistribution.

7.4 IS ANNEXIN V A REGULATOR OF CARDIAC PHOSPHOLIPASE A_2 ACTIVITY?

Under normal conditions the membrane phospholipid pool in cardiac cells is subjected to continuous turnover (Van der Vusse *et al.*, 1992). The velocities of phospholipid degradation and synthesis are in equilibrium under these circumstances. Under pathological circumstances, however, phospholipid homeostasis can be disturbed and a net degradation of phospholipids can occur (Chien *et al.*, 1984; Das *et al.*, 1986; Van Bilsen *et al.*, 1989; Van der Vusse *et al.*, 1982).

Elevated activity of phospholipid hydrolyzing enzymes, such as phospholipase A₂, is one of the possible causes for the net breakdown of phospholipids (Chien *et al.*, 1984; Das *et al.*, 1986; Van Bilsen *et al.*, 1989; Van der Vusse *et al.*, 1982). As annexin V is known to be a phospholipase A₂ inhibitor *in vitro* (Ahn *et al.*, 1988; Davidson *et al.*, 1987; Van Bilsen *et al.*, 1992), annexin V might play a role in the regulation of the phospholipid homeostasis *in vivo*.

The mechanism underlying annexin V-mediated inhibition of phospholipase A₂ activity is thought to be Ca²⁺ dependent binding of annexin V to the phospholipid membrane surface. In this way the accessibility of the phospholipid substrate for the phospholipase enzyme is supposed to be diminished (the so-called "substrate depletion" model) (Davidson *et al.*, 1987). The annexin V found at the level of the sarcolemma of adult cardiac myocytes could play a role in the regulation of the hydrolytic activity of endogenous phospholipase A₂ on membrane phospholipids by this mechanism if enough annexin V is attached to the sarcolemma.

The present results showed that 0.17 µg annexin V/ mg protein was present in cardiac myocytes and that annexin V was localized at the level of the sarcolemma of the cardiac myocyte (chapter 3). It was calculated that the molar ratio of annexin V over the phospholipids in the inner leaflet of the sarcolemma is in the order of 1/1250 (chapter 3). This ratio was thought to be too low to allow "substrate depletion" to occur sufficiently and thus, according to this calculation, a role of annexin V in the regulation of the cardiac phospholipid homeostasis was considered to be not very likely.

However, the question arises, whether it is possible that a partial coverage of the sarcolemma already interferes with phospholipase A₂ activity. For this purpose we developed an assay on the ellipsometer in which the potency of annexin V to inhibit phospholipase A₂-mediated hydrolysis of phospholipids was investigated under, as much as possible, physiologically relevant conditions. The ellipsometrical data demonstrated still a 50% inhibition of phospholipase A₂ activity at a coverage of the phospholipid membrane of 12% of the maximal annexin V coverage. At 12% of the maximal annexin V coverage, the molar ratio of annexin V over a phospholipid monolayer amounts to 1/425 (chapter 4). This ratio is still about three times the calculated annexin V/sarcolemma phospholipids ratio of 1/1250 in the cardiac myocyte. However, as part of the sarcolemma is covered by other proteins present in the sarcolemma or by the cytoskeletal proteins, the assumption seems justified that less annexin V is needed for the coverage of phospholipids in a biological membrane. In other words, the ratio of annexin V over freely accessible phospholipids may be considerably higher than 1/1250. Therefore, in combination with our finding that relatively low phospholipid coverages by annexin V are sufficient for substantial phospholipase A₂ inhibition, this suggests that in the cardiac myocyte sufficient amounts of annexin V could be present at the level of the sarcolemma to allow effective interference with endogenous phospholipase A₂ activity.

In addition we can conclude that the so-called substrate depletion model, with annexin V masking the substrate phospholipids (Davidson *et al.*, 1987) does not explain the relatively high degree of inhibition of phospholipase A₂ by partial annexin V surface coverage. We suggest the existence of an additional inhibitory effect on phospholipase A₂ activity. This inhibitory effect is proposed to be caused by a decreased rate of lateral diffusion of phospholipid hydrolysis products away from the product site by annexin V. In this manner, local product inhibition of

phospholipase A₂, a mechanism which was suggested by Burack and Biltonen (1994), might occur. This effect could be enhanced by simultaneous reduction of the lateral mobility of the phospholipase A₂ protein molecules.

Under circumstances in which net degradation of phospholipids occurs, i.e., energy deprivation, the inhibitory effect of annexin V on phospholipase A₂ activity might be of special interest. Loss of annexin V from the sarcolemma might be an important cause of enhanced hydrolytic degradation of sarcolemmal phospholipids. This hypothesis was tested in an experimental set-up in which isolated adult cardiac myocytes were subjected to metabolic inhibition in order to induce net membrane phospholipid hydrolysis (chapter 5). Labeled arachidonic acid was incorporated in membrane phospholipids prior to metabolic inhibition to be able to estimate phospholipid degradation during metabolic inhibition. Metabolic inhibition was chosen to mimic ischemic conditions because in neonatal cardiac myocytes the metabolic inhibition model was shown to result in depletion of cellular energy-stores, significant cellular damage in a relatively short time interval, and accelerated release of prelabeled arachidonic acid (AA) from the cellular phospholipid pool (Chien *et al.*, 1985; Gunn *et al.*, 1985), indicating net degradation of membrane phospholipids. In this thesis metabolic inhibition of isolated adult cardiac myocytes resulted in loss of the normal morphology, indicating damage of cellular structures, due to energy deprivation (Post *et al.*, 1995). Moreover, after 240 min of metabolic inhibition about 35% of the total lactate dehydrogenase (LDH) activity was released into the incubation medium. Metabolic inhibition resulted also in a slight but significant net degradation of the membrane phospholipids, as was shown by a steady increase of the percentage of radioactivity in the cellular (non-esterified) fatty acid pool and a small but significant decline of the percentage of label in the phospholipid pool (chapter 5).

The present study shows that under energy-depriving conditions annexin V remained persistently present at the level of the sarcolemma. It was also found that, in contrast to LDH, annexin V was not released from the cardiac myocyte. Therefore, it is rather unlikely that a loss of annexin V from the sarcolemma can be held responsible for the enhanced phospholipid hydrolysis, as readily occurs in the energy-deprived cardiac myocyte.

The question whether annexin V is of importance as a regulator of cardiac phospholipase activity can not be answered to date. However, its localisation at the sarcolemma and the amounts present argue in favour of such a role. Theoretically, a mechanism occurring at the sarcolemma explaining a role of annexin V during phospholipid degradation under energy-deprived conditions could be a shift of annexin V along the sarcolemma to phospholipid domains. These domains are thought to originate from lateral phase separation of membrane phospholipids (Post and Langer, 1992) due to enhanced intracellular Ca²⁺ levels inside the cell in close vicinity of the sarcolemma under energy-deprived conditions (Borgers *et al.*, 1984). The fact that net degradation of phospholipids occurs in energy deprived cells while annexin V remains present at the sarcolemma might point towards insufficient coverage of the sarcolemma by annexin V. Hence, it would be interesting to see what happens when in these cells over- or underexpression of annexin V has been induced.

7.5 FUTURE PERSPECTIVES

Although detailed knowledge has been obtained about the cellular distribution and content of annexin V in rat myocardial tissue a variety of questions remains to be answered. Below some examples will be discussed without attempting to provide an exhaustive list of studies that could be performed to elucidate the functional significance of annexin V in the cardiac context in more detail.

The role of annexin V in cardiac growth and development is still incompletely understood. The intriguing observation that the cardiac content of annexin V is maximal during the first week after birth strongly suggests that annexin V plays a role in the transition of hyperplastic to maturational trophic growth of cardiac cells. With the use of electron-microscopic techniques the alterations in intracellular annexin V localization should be clarified. The present study also showed that the content and localization do not change when the cardiac myocyte has reached the hypertrophied phase. This was observed both in neonatal cells stimulated by phenylephrine and in the adult rat heart in which hypertrophy was evoked by banding of the aorta. As it cannot be excluded that annexin V plays a role during the onset of the hypertrophic response, studies should be directed to the initial, transition phase from normal to hypertrophied cardiomyocyte. Both the cellular content and a possible shift in the subcellular localization should be subject of research.

It is of interest to note that two different types of cardiac hypertrophy can be recognized: one called physiological hypertrophy resulting in a hemodynamically stable (fully compensated) state, the other called pathological hypertrophy which will eventually lead to cardiac decompensation and concomitant pump failure. The above mentioned studies could be extended to both types of hypertrophy. As model for physiological hypertrophy the T_3 -treated rat is commonly used (Folkow *et al.*, 1992), whereas aorta banding readily results in pathological hypertrophy (Snoeckx *et al.*, 1990).

In the present study it was shown that annexin V is able to inhibit phospholipase A_2 even when the phospholipid bilayer is incompletely covered by the annexin V molecules. As test enzyme pancreatic group I phospholipase A_2 was used. Evidence is accumulating (Van Bilsen and Van der Vusse, 1995) that cardiac cells contain at least three other types of phospholipase A_2 , which are supposed to play important roles in cellular functioning under normal and pathological conditions. The presence of a group II phospholipase A_2 , a plasmalogen-selective Ca^{2+} -independent phospholipase A_2 and a cytosolic arachidonic acid-selective phospholipase in cardiac tissue has now been firmly established. Information about the possible interference of annexin V with these three types of phospholipase A_2 is absent. This issue could be addressed with the use of the ellipsometer technique if sufficient amounts of recombinant phospholipase A_2 have been produced. In this respect it is worth mentioning that the cardiac group II phospholipase A_2 is recently cloned in the laboratory of Physiology of CARIM and that this enzyme is currently produced with the use of recombinant DNA techniques.

It was observed that in metabolically inhibited cardiomyocytes the localization of annexin V did not substantially change. This observation is not in favor of the notion that loss of annexin V from the sarcolemma is the ultimate trigger for enhanced phospholipid degradation in energy-

depleted cells. It should be realized, however, that this observation does not exclude a more subtle role of annexin V in the induction of cellular damage. Moreover, it is unclear whether annexin V shows the same behavior in ischemic/reperfused cardiomyocytes as in metabolically inhibited cells. Studies can also be performed on isolated adult cardiomyocytes in which the annexin V level is up- or downregulated by pharmacological interventions, such as treatment with dexamethasone, a synthetic glucocorticoid, which was found to induce overexpression of annexin V in U-937 cells (Solito *et al.*, 1991). As an alternative, molecular biological techniques can be used to reach this goal. In this respect it is noteworthy that recent advances in molecular biology have demonstrated the possible application of recombinant replication-deficient adenovirus as vehicle to transfect adult cardiomyocytes with high efficiency.

It could be envisaged that generation of mice with overexpression of annexin V in cardiac structures or with the protein knocked-out, might provide a more direct answer to the question whether annexin V is involved in ischemia/reperfusion-induced degradation of membrane phospholipids. Recent developments in molecular biology allow for cardiac specific manipulation of gene expression (Fässler *et al.*, 1995; Kilby *et al.*, 1993), while the tetracycline-transactivator system allows for inducible annexin V expression. With the use of the latter techniques the annexin V gene can be activated when the heart is fully developed, circumventing the possible adverse effects of high levels of annexin V during embryogenesis. It is of interest to note that recently an isolated working mouse heart model has been developed and implemented at the laboratory of Physiology of CARIM, enabling the studies on the effect of ischemia/reperfusion using intact hearts from (transgenic) mice.

Since annexins lack a secretory sequence they are supposed to be intracellular proteins and act consequently. This concept fits very well to the proposed annexin V functions of the cardiac myocyte in the present thesis. However, some annexins, including annexin V, have reported extracellular localizations (Christmas *et al.*, 1991; Flaherty *et al.*, 1990; Römisch *et al.*, 1992). In addition, annexin V was shown to be present in the vascular compartment after ischemia/reperfusion injury of the heart (Römisch *et al.*, 1992). The circulating amounts of annexin V might be released from cells present in the vessel walls. In the present thesis cultured cardiac endothelial cells were shown to contain high levels of annexin V. Under normal conditions a functional role of annexin V in the vascular space is uncertain as outer leaflets of membranes containing negatively charged phospholipids, *i.e.*, docking sites for annexin V, are virtually absent. However, in the ischemic/reperfused heart the content of the negatively charged phospholipids may increase in the membrane leaflets facing the vascular space making a role of circulating annexin under such conditions feasible. Further experiments are required to explore this issue in more detail.

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SUMMARY

The annexins constitute a family of calcium- and phospholipid-binding proteins. One member of the family annexin V showed to have many different *in vitro* functions. However, the *in vivo* functional significance of annexin V is still incompletely understood. In this thesis our research was directed to annexin V in the cardiac setting.

In the present thesis the possible role of annexin V in the regulation of the phospholipid homeostasis of cardiac myocytes by phospholipase A₂ inhibition was investigated using, on the one hand, an experimental model with artificial phospholipid membranes and, on the other hand, energy-deprived myocytes. In addition, annexin V localization and quantity were studied during maturational and hypertrophic growth of the heart, in order to get an indication whether annexin V could play a role in these processes.

A general introduction of the thesis is provided in chapter 1. An introduction to the various members of the annexin family, cardiac phospholipid homeostasis and cardiac maturational growth is given. In addition, a short survey on the possible functions of annexin V in cardiac phospholipid metabolism and cardiac maturational growth and hypertrophy is provided in this chapter.

Chapter 2 describes in detail our experience with labeling techniques and permeabilization conditions on the subcellular localization of annexin V in the various cardiac cell types. Results were analyzed on the light, confocal scanning laser and electron microscopical level.

The subcellular localization of annexin V in isolated cells was shown to be influenced by permeabilization of the cells. In addition, immunogold labeling of intact cardiac myocytes was regarded to be less reliable as lack of penetration of the antibody-gold particle complex into the cell interior could not be excluded in this experimental approach. The results obtained at the light and electron microscopic level, when labeling was performed on cryosections of cardiac tissue or cells, were considered to be the most trustworthy.

In chapter 3 the presence of a variety of members of the annexin family, including annexin I plus II, III, IV, V and VI, in rat cardiac tissue was established with western blot analysis. Annexin V was purified from rat cardiac tissue and polyclonal antibodies were raised against rat heart annexin V. Western blot analysis with these antibodies revealed that annexin V is present in both cardiomyocytes and non-myocytal cells of the heart. These results were confirmed by immunohistochemical analysis of rat heart tissue sections. Subcellular localization of annexin V in non-myocytal cells seemed to be cytoplasmatic, whereas the localization in the cardiac myocytes was found to be in close vicinity of the sarcolemma. Northern blot analysis demonstrated that all cell types investigated showed expression of annexin V. Annexin V mRNA levels were highest in the fibroblast-like cells, followed by endothelial cells, and a relatively weak signal was observed in cardiomyocytes. By means of a newly developed sandwich-type ELISA with antibodies specific for rat heart annexin V, annexin V content in intact adult rat heart, isolated myocytes, cultured cardiac endothelial cells and fibroblast-like cells was found to be 0.70, 0.17, 1.63 and 3.84 µg/mg total protein, respectively.

The mechanism of inhibiting low molecular weight phospholipase A₂ activity by annexin V is investigated with use of an ellipsometer in chapter 4. The generally proposed mechanism of

phospholipase A₂ inhibition by annexins is through shielding of the phospholipid membranes, the substrate of the phospholipases. In chapter 3 it was shown that although annexin V was present at the level of the sarcolemma the amounts of annexin V in the cardiac myocytes are too low to cover the total membrane and as such a role of annexin V in cardiac phospholipid homeostasis seems questionable. In chapter 4, however, it was shown that partial covering of a phospholipid membrane with annexin V still inhibited phospholipase A₂ activity substantially. In other words, the degree of phospholipase A₂ inhibition was nonlinearly related to the amount of membrane-bound annexin V. These findings indicate that the inhibition of phospholipase A₂-mediated hydrolysis by annexin V cannot be simply explained by shielding of phospholipid substrates from the enzyme. Moreover, the present results leave room for a role of endogenous annexin V in regulating phospholipid turnover on the sarcolemma of the cardiac myocytes.

Under energy-deprived conditions the phospholipid homeostasis is disturbed. There is net degradation of phospholipids. In chapter 5 we studied the possible role of annexin V, with its anti-phospholipase A₂ properties, in the degradation of membrane phospholipids of adult cardiac myocytes during metabolic inhibition (20 mM 2-deoxyglucose and 1 mM iodo-acetic acid). After 240 min of metabolic inhibition, lactate dehydrogenase (LDH) release was significantly elevated compared to control cells incubated for the same period of time. In metabolically inhibited cells, annexin V localization seemed to be more pronounced at the level of the sarcolemma compared to controls, whereas membrane phospholipid hydrolysis occurred at a significantly elevated rate, as evidenced by a significantly enhanced accumulation of labeled arachidonic acid within the cells. The findings of chapter 5 are not in favor of the hypothesis that the increase in net degradation of phospholipids in energy-deprived cardiac myocytes is caused by a loss of annexin V from the sarcolemma, which would increase the vulnerability of the sarcolemma towards phospholipase A₂ activity.

Amongst others, annexin V has been suggested to play a role in developmental processes. In chapter 6 it was investigated whether in the heart annexin V content and localization change during maturational and hypertrophic growth, in order to obtain indications that annexin V is involved in cardiac growth processes. It was demonstrated that during perinatal development annexin V content in total heart transiently increased in the first week after birth, to a peak value 6 days after birth, whereafter annexin V protein levels declined again. Differences in annexin V content were also observed between myocytes isolated from neonatal and adult hearts. Moreover, during cardiac maturational growth the subcellular localization of annexin V might change from a cytoplasmic to a more prominent sarcolemmal localization. In addition, *in vivo* hypertrophy induced by aortic coarctation was not found to be associated with a change in annexin V localization or content. The quantitative results obtained with intact hypertrophic rat hearts were supported by findings in neonatal ventricular myocytes, in which hypertrophy was induced by phenylephrine (10^{-5} M). In conclusion, the marked alterations in annexin V content during the maturational growth in the heart suggest a possible involvement of this protein in this process. In contrast, the absence of changes in annexin V content and localization in hypertrophied hearts/myocytes compared to appropriate control hearts/myocytes suggests that annexin V does not play a crucial role in the maintenance of the hypertrophic phenotype of the cardiac muscle cell.

A general discussion of the studies presented in this thesis is provided in chapter 7. The main conclusions from the research described in the present thesis are the following:

- 1) Annexin V is present in the adult cardiac myocyte at the level of the sarcolemma in relatively small amounts.
- 2) Partial coverage of phospholipid membranes with annexin V leads to a substantial inhibition of phospholipase A₂ activity.
- 3) Besides the substrate depletion model another mechanism has to be active to explain the phospholipase A₂ inhibiting capacity of annexin V.
- 4) During energy-depriving conditions annexin V stays persistently present at the level of the sarcolemma and as such elevated phospholipid degradation under these conditions can not be explained by a substantial loss of annexin V from the sarcolemma.
- 5) As changes in annexin V localization and content in the first week after birth coincide with the switch from maturational hyperplastic to hypertrophic growth annexin V might be involved in the process of maturational growth.

SAMENVATTING

Alle cellen en hun organellen worden omgeven door een membraan. Eén van de bouwstenen van een membraan zijn fosfolipiden. Deze lipiden worden voortdurend afgebroken en opgebouwd. Dit proces, de fosfolipiden homeostase, is onder normale omstandigheden in balans. Onder pathologische omstandigheden, bijvoorbeeld één waarbij een tekort aan zuurstof optreedt, is de fosfolipiden homeostase zodanig verstoord dat dit resulteert in een netto afbraak van membraanfosfolipiden. Beschadiging van de celmembraan kan uiteindelijk tot celdood leiden. De verstoring van de balans van de fosfolipiden homeostase kan veroorzaakt worden door, óf een verhoogde afbraak van fosfolipiden, óf door een verminderde resynthese van fosfolipiden. Welke mechanismen en factoren een rol spelen in de regulatie van de fosfolipiden homeostase is nog onvolledig opgehelderd. De annexinen, een familie van eiwitten die calcium-afhankelijk aan fosfolipiden kunnen binden, zijn een mogelijke factor van betekenis in de regulatie van de fosfolipiden huishouding, omdat de annexinen het enzym remmen dat de fosfolipiden afbreekt, namelijk fosfolipase A_2 .

In dit proefschrift is onderzocht of een lid van de annexine familie, annexin V, mogelijk een rol speelt in de regulatie van de fosfolipide homeostase van de cardiomyocyt door remming van het fosfolipase A_2 enzym. Daarbij kunnen andere mogelijke functies van annexine V in het hart niet worden uitgesloten. Ook werd onderzocht of de cellulaire lokalisatie en hoeveelheid van annexine V veranderden gedurende de ontwikkelings-groei van het hart en na hypertrofe groei (groei zonder deling). Dit onderzoek is uitgevoerd om inzicht te krijgen of annexine V mogelijk een rol speelt in deze processen.

Enkele algemene aspecten van de fosfolipiden huishouding, de verschillende leden van de annexinen familie en de processen geassocieerd met cardiale groei worden beschreven in hoofdstuk 1. Tevens wordt een overzicht gegeven van annexine V en zijn fosfolipase remmende werking en annexine V in verband met groei en ontwikkeling.

In hoofdstuk 2 worden een aantal technieken, die mogelijk geschikt zijn om de lokalisatie van annexine V op hartweefsel en cellen aan te tonen, met elkaar vergeleken. De methode, waarbij gebruik gemaakt wordt van antilichamen tegen het te lokaliseren eiwit (anti-rattenhart annexine V antilichamen) en van antilichamen met een herkenbaar label zoals een fluorescerend of goud bolletje gericht tegen de anti-rattenhart annexine V antilichamen, wordt immunohistochemie genoemd. Met behulp van, respectievelijk, een fluorescentie- of een elektronenmicroscop, kan het label zichtbaar gemaakt worden en zo de lokalisatie van het eiwit in de cel of het weefsel bepaald. Om het eiwit bereikbaar voor de antilichamen te maken kan men de cellen doorlaatbaar maken met detergentia zoals Triton X-100 of saponine. In hoofdstuk 2 wordt aangetoond dat het gebruik van detergentia niet aan te raden is, omdat deze de immunolokalisatie lijken te veranderen. Deze veranderingen in immunolokalisatie worden mogelijk veroorzaakt door de zware beschadigingen van de celstructuren die tijdens deze procedure optreden. Ook wordt immunolabeling van intacte volwassen cardiomyocyten, waarbij een goud bolletje gekoppeld is aan een antilichaam, als minder betrouwbaar beschouwd. De oorzaak hiervan is dat met deze methode, waarbij goudbolletjes alleen aan de hartspiercelmembraan gevonden werden, niet duidelijk is of het complex in staat is volledig tot de kern van de cel te penetreren. De meest

betrouwbare resultaten worden verkregen door kleuring op dunne ingevroren hartweefsel- of celplakjes (coupes).

In hoofdstuk 3 is allereerst bepaald welke leden van de annexinen familie aanwezig zijn in het hart met behulp van Western blot analyse. Van de geteste eiwitten bleek annexine I en II, III, IV, V en VI aanwezig. Annexine V was zeer prominent aanwezig en daarom is dit lid van de familie verder onderzocht. Omdat al onze testen op het rattenhart werden uitgevoerd werd eerst een antilichaam verkregen dat specifiek gericht is tegen rattenhart annexine V. Hiertoe werd annexine V gezuiverd uit rattenhartjes en vervolgens werd een konijn met het gezuiverde eiwit geïmmuniseerd om tot antilichaam productie te komen. De antilichamen, die specifiek gericht zijn tegen rattenhart annexine V, werden gebruikt om aan te tonen in wel celtype van het hart en waar in deze celtypes annexine V aanwezig is. Western blot analyse en immunohistochemie op coupes van rattenhart weefsel laten zien dat annexine V aanwezig is in alle celtypes van het rattenhart, zowel de hartspiercellen als ook de endotheelcellen en de fibroblasten. In volwassen hartspiercellen is annexine V gelokaliseerd ter hoogte van het celmembraan, het sarcolemma, in tegenstelling tot de andere celtypen van het hart waar een cytoplasmatische kleuring van annexine V werd gevonden. Aantoning van annexine V op mRNA niveau (Northern blot analyse), waaruit afgeleid kan worden of annexine V ook in deze cel types wordt gesynthetiseerd, laat eveneens expressie zien in alle celtypes van het hart. De hoogste mRNA expressie werd gevonden in de fibroblast-achtige cellen en de laagste waarden in de hartspiercellen met tussenliggende waarden in endotheelcellen. De annexine V eiwit niveau's werden ook gekwantificeerd. Hiervoor werd een ELISA methode van het sandwich type ontwikkeld met antilichamen specifiek gericht tegen rattenhart annexine V. De annexine V inhoud in rattenhartweefsel, geïsoleerde hartspiercellen, gekweekte endotheelcellen en fibroblasten bedroeg respectievelijk, 0.70, 0.17, 1.63 en 3.84 $\mu\text{g}/\text{mg}$ totaal eiwit.

Aangezien annexine V ter hoogte van het sarcolemma van de hartspiercel gevonden wordt, is een rol voor annexine V in de fosfolipiden huishouding van het hartspiercelmembraan mogelijk. Als mogelijk mechanisme voor de remmende werking van annexine V op de fosfolipase A_2 activiteit is afscherming van de membraanfosfolipiden, het substraat voor fosfolipase A_2 , (het zogenaamde substraat-depletie model) voorgesteld. De hoeveelheid annexine V, die aangetoond kan worden in de hartspiercel, wijst erop dat dit niet voldoende is om de gehele binnenzijde van het sarcolemma af te screenen. In hoofdstuk 4 werd met behulp van kunstmatige membranen (ellipsometrie), onder condities die zoveel als mogelijk lijken op de omstandigheden in de hartspiercellen, onderzocht of gedeeltelijke bedekking van een fosfolipidenmembraan door annexine V gevolgen heeft voor de hydrolytische activiteit van fosfolipase A_2 . Er werd aangetoond dat een gedeeltelijke bedekking van het fosfolipide membraan de fosfolipase A_2 activiteit reeds aanzienlijk remt. Deze gegevens impliceren dat de remmende werking van annexine V op de activiteit van fosfolipase A_2 niet alleen door afscherming van het substraat (de membraanfosfolipiden) verklaard kan worden. Naast het substraat-depletie model is dus nog een ander, onbekend proces aanwezig. Verder toonden de resultaten in hoofdstuk 4 aan dat een rol voor annexine V in de fosfolipiden huishouding van het sarcolemma mogelijk is, zelfs bij een onvolledige bedekking van het sarcolemma.

Onder omstandigheden waarbij een zuurstof tekort optreedt, zoals bij een cardiale ischemie,

is de fosfolipiden huishouding verstoord. Er treedt netto afbraak van fosfolipiden op. In hoofdstuk 5 hebben we de mogelijke rol van annexine V in de afbraak van membraanfosfolipiden van geïsoleerde volwassen hartspiercellen bestudeerd onder energie-gedepriveerde omstandigheden door middel van metabole inhibitie. Dit experimenteel model leidt tot uitputting van de energie-voorraad van de cel, een situatie die ook in een ischemische cel optreedt. Na 120 en 240 minuten metabole inhibitie zijn de mate van celschade, de fosfolipide afbraak en de annexine V lokalisatie bepaald. Omdat schade aan hartspiercellen leidt tot het weglekken van lactaat dehydrogenase (LDH) uit de cel, werd de activiteit van dit enzym bepaald in de hartspiercellen en de bovenstaande vloeistof. Na 240 minuten metabole inhibitie was het LDH-verlies uit de hartspiercellen significant verhoogd in vergelijking met controle cellen. In metabool geremde cellen bleek annexine V sterker aanwezig aan het sarcolemma vergeleken met controle cellen. De fosfolipide afbraak was significant versneld, wat aangetoond werd door een verhoogd gehalte van veresterd arachidonzuur. Vrij arachidonzuur ontstaat door een fosfolipase A₂ gemedieerde hydrolyse van fosfolipiden. Deze resultaten laten zien dat de verhoogde netto fosfolipiden afbraak onder energie-gedepriveerde omstandigheden waarschijnlijk niet veroorzaakt worden door verlies van annexine V van het sarcolemma, hetgeen de gevoeligheid van de sarcolemmale fosfolipiden voor fosfolipase A₂ zou verhogen.

Een ander functie, die voor annexine V is voorgesteld, is een rol in groeiprocessen. In hoofdstuk 6 werd bestudeerd of de annexine V hoeveelheid en lokalisatie veranderen gedurende de groei in het ontwikkelende hart en hypertrofie (groei door volume vergroting van cellen, dus zonder celdeling) van het rattenhart. Deze gegevens geven mogelijk een aanwijzing van de betrokkenheid van annexine V in groeiprocessen van het hart. De annexine V inhoud en lokalisatie werd bestudeerd in rattenharten van vlak voor de geboorte tot de volwassen leeftijd van 12 weken. Opvallend was dat de hoeveelheid annexine V in het rattenhart steeg vlak naar de geboorte, waarna het weer daalde tot het niveau van voor de geboorte. De annexine V lokalisatie leek in de hartspiercellen van 2 tot 3 dagen oude ratten cytoplasmatisch, terwijl in het volwassen hart de lokalisatie ter hoogte van het sarcolemma was. Uit de literatuur is bekend dat in de eerste week na de geboorte ook een belangrijke verandering in het groeiproces optreedt. Het hart gaat namelijk over van groei door deling naar groei door volume vergroting van cellen. Deze verandering gaat tevens gepaard met een verhoging of verlaging van de cellulaire inhoud van een aantal specifieke eiwitten. Aangezien de tijdelijke verhoging van de annexine V inhoud samen lijkt te vallen met deze verandering in groei karakteristieken, impliceren deze resultaten dat annexine V mogelijk betrokken is bij de omslag in het groeiproces van het hart in de eerste week na de geboorte. Tevens is bestudeerd of hypertrofie van het hart geassocieerd is met een verandering in annexine V lokalisatie en/of hoeveelheid. Hypertrofie werd in rattenharten geïnduceerd door afbinding van de aorta (aorta coarctatie) en in geïsoleerde volwassen hartspiercellen door behandeling met fenylefrine. In de hypertrofe rattenharten werd geen verandering in de lokalisatie en hoeveelheid annexine V gevonden. Onveranderde annexine V hoeveelheden werden ook gevonden in de geïsoleerde neonatale hartspiercellen die hypertroof waren gemaakt door fenylefrine. Dit suggereert dat annexine V niet betrokken is bij het instandhouden van het hypertrofe fenotype. Over de rol van annexine V in de ontwikkeling van hypertrofie kan evenwel nog geen uitspraak gedaan worden.

In hoofdstuk 7 worden de resultaten zoals beschreven in dit proefschrift samengevat en in een breder kader geplaatst. De belangrijkste conclusies van dit proefschrift zijn:

- 1) Annexine V is aanwezig in de hartspiercel aan het sarcolemma in relatief kleine hoeveelheden.
- 2) Gedeeltelijke bedekking van een fosfolipidenmembraan met annexine V remt substantieel de fosfolipase A₂ activiteit.
- 3) Naast het substraat depletie model moet er nog een ander mechanisme aanwezig zijn om het fosfolipase A₂ remmende vermogen van annexine V te verklaren.
- 4) Gedurende deprivatie van energie blijft annexine V gebonden aan het sarcolemma, en dientengevolge kan de verhoogde fosfolipiden afbraak onder deze omstandigheden niet verklaard worden door een aanzienlijk verlies van annexine V van het celmembraan.
- 5) Daar de veranderingen in de hoeveelheid en lokalisatie van annexine V samenvallen met de verandering van hyperplastische naar hypertrofe groei, kan annexine V mogelijk bij het proces van de groei van het hart gedurende de ontwikkeling betrokken zijn.

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ABSTRACTS

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COLOUR ILLUSTRATIONS

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Figure 3.5 a and b, p. 69

Figure 6.2 a and b, p. 116

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 13 april 1968 te Heerlen. Na het behalen van het VWO diploma aan het Romboutscollege te Brunssum in juni 1986, werd in hetzelfde jaar begonnen met de studie Gezondheidswetenschappen, afstudeerrichting Biologische Gezondheidskunde, aan de Universiteit Maastricht. Na de afstudeerstage via het Erasmus studenten-uitwisselingsproject bij de Vakgroep Endocrinologie aan de Katholieke Universiteit Leuven (België) en een stage bij de Vakgroep Pathologie aan de Universiteit Maastricht, behaalde zij in september 1991 het doctoraal examen. Van september 1991 tot december 1995 was zij werkzaam als Onderzoeker in Opleiding bij de vakgroep Fysiologie aan de Universiteit Maastricht, alwaar zij de werkzaamheden zoals beschreven in dit proefschrift uitvoerde.

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