METABOLISM, FLOW AND CONTRACTILITY OF THE *LANGENDORFF* HEART

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Ye mariners all as you pass by
call in and drink if you are dry
come spend me lads your money brisk
and pop your nose in a jug of this

(Traditioneel Engels volksliedje)

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5. Sephadex-induced reduction of coronary flow in the isolated rat heart: A model for ischemic heart disease.
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6. Effect of glucose on AMP-catabolite release during fatty acid perfusion in normal and ischemic rat hearts.
H. Stam & W. Breeman. Life Sciences, accepted for publication.

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GLOSSARY

Calcium	:	Calcium in any form: free, bound or ionized
Ca^{2+}	:	Ionized calcium
Sodium	:	Sodium in any form: free, bound or ionized
Na^{+}	:	Ionized sodium
Potassium	:	Potassium in any form: free, bound or ionized
K^{+}	:	Ionized potassium
CrP	:	Creatine phosphate
ATP	:	Adenosine-5'-triphosphate
GTP	:	Guanosine-5'-triphosphate
AMP	:	Adenosine-5'-monophosphate
cyclic AMP	:	Adenosine-3',5'-monophosphate
cyclic GMP	:	Guanosine-3',5'-monophosphate
P_i	:	Inorganic phosphate
FFA	:	Free fatty acids (not esterified)
CoA	:	Coenzyme A
Fatty acyl CoA	:	Fatty acid ester with CoA
NADH	:	Nicotinamide-adenine dinucleotide (reduced)
NAD^{+}	:	Nicotinamide-adenine dinucleotide (oxidized)
S.E.M.	:	Standard error of the mean
[ ]	:	Concentration

SUMMARY

This thesis reviews current literature and describes experimental studies on the regulation and modification of coronary flow and contractility in isolated rat hearts. In chapter I and introduction is given to the problems of fatty acid toxicity and myocardial function. Coronary flow rate and pump function of the myocardium are mainly determined by the contractile status of vascular smooth muscle cells and cardiac striated muscle cells, respectively. Therefore in chapters II and III morphological and (ultra)structural aspects of both types of cells have been described.
In chapters IV and V functional and metabolic aspects of coronary circulation and contractility are illustrated. In both vascular smooth and cardiac striated muscle cells:

(i) the intracellular calcium concentration is the main determinant of the contractile status of actomyosin,
(ii) contraction takes place after the action potential-induced calcium-influx through the plasmamembrane and calcium release from intracellular stores (sarcoplasmic reticulum, mitochondria),
(iii) relaxation is achieved after reduction of the cytoplasmic calcium level by calcium-pump systems in the plasmamembrane, sarcoplasmic reticulum and mitochondria,
(iv) calcium-ions trigger the coupling between the contraction-relaxation cycle with energy metabolism since glycogenolysis and lipolysis are both stimulated by calcium.

Cardiac striated muscle contraction and relaxation are energy-dependent processes. Under conditions of limited ATP production (anoxia, hypoxia, ischemia) contractile function impairs. Pharmacological, neurohumoral and metabolic control of intracellular calcium levels in smooth and striated muscle cells takes place via two mechanisms:

(i) directly, by alteration of calcium-transport in the plasmamembrane leading to increased or decreased calcium levels and

(ii) indirectly, by cyclic nucleotide (cyclic AMP and cyclic GMP)-dependent changes in calcium-binding and transport in sarcoplasmic reticulum and the sarcolemma. This process is mediated by cyclic nucleotide-dependent protein kinase(s), which alter(s) the phosphorylation state of specific membrane sites.

Perfusion experiments have been performed with hearts containing increased cytosolic levels of free fatty acids (FFA). Such hearts have been obtained from fasted, streptozotocin-diabetic and rapeseed oil fed rats. Also hearts from normal rats have been used by including FFA in the perfusion medium.
Our observations about the role of intracellular FFA in various membranous and contractile processes are visualized in fig. 1, in which the secondary actions of catecholamines and prostaglandins have been eliminated.

Fatty acids are the main substrates for energy metabolism in heart. After uptake by the sarcolemma they are probably stored as triglycerides in lipid-filled lysosomes or autophagosomes. By the action of membrane-bound (acid) lipase, triglyceride hydrolysis takes place (appendix paper 3). Inhibition of endogenous lipolytic activity by the lysosomal "inhibitor" chloroquine is associated with depressed contractility of isolated hearts which could be counteracted by the addition of fatty acids, by increasing the calcium concentration in the perfusion fluid or by increasing the cytosolic AMP levels (by adding catecholamines or glucagon). In hearts from rapeseed oil fed rats both the chloroquine-inhibition of lipolysis and contractility were lower than in hearts from control fed rats. These experiments indicate that "physiological" amounts of intracellular FFA are indispensible for the maintenance of contractile function. Probably fatty acids act as calcium-vehicles in the cytosol thereby promoting calcium-transport from the sites of release to the sites of contraction (appendix paper 4). Furthermore it is shown that fatty acid-mediated increased calcium fluxes promote the fusion (exocytosis) of catecholamine-

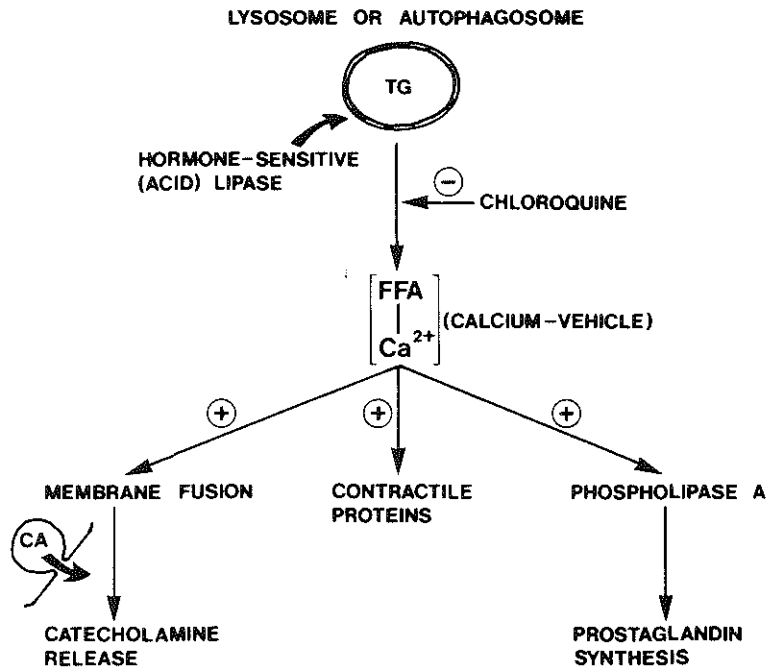

Fig. 1. The role of free fatty acids as calcium-vehicles in myocardial membrane and contractile processes.
TG = triglyceride, FFA = free fatty acids, CA = catecholamines. ⊕ = stimulatory action, ⊖ = inhibitory action.

filled storage granules with nerve-ending cell membranes and thus lead to enhanced catecholamine release (appendix paper 2). They may stimulate the activity of the calcium-dependent phospholipase A_2 leading to increased prostaglandin synthesis from membrane phospholipids.
Increased catecholamine "activity" and prostaglandin release, in association with enhanced coronary flow rates, have been observed in hearts from fasted and streptozotocin-diabetic rats (appendix paper 1). In hearts from normal rats, endogenous catecholamines,

released spontaneously during perfusion, proved to be involved in the maintenance of the coronary flow rate under normoxic conditions (appendix papers 1 and 2). The vasodilatory actions of (exogenous and endogenous) catecholamines and of prostaglandin-like substances are likely to be mediated by a rise in vascular smooth muscle cytosolic cyclic AMP levels. In contrast to vascular smooth muscle cells, which relax under conditions of enhanced cytoplasmic cyclic AMP levels, cardiac striated muscle cells show increased contractility following addition of norepinephrine, glucagon and prostaglandin E_1 (appendix paper 4).

The calcium-ionophoric properties of fatty acids and of certain prostaglandins are involved in coronary vasodilation, probably by stimulating calcium sequestration in coronary vascular smooth muscle cells. Therefore, the increased coronary flow rates in hearts from rapeseed oil fed rats may be related to the observed increased rates of basal (and hormone-sensitive) lipolytic activity. The expected increase of fatty acid release from the lipid infiltrated striated muscle cells may be responsible for vascular smooth muscle relaxation by the calcium-removing action of fatty acids as well.

Experiments were carried out to study the effects of fatty acid perfusion during normoxic and ischemic conditions. Embolization (Sephadex polysaccharide microspheres)-induced reduction of coronary flow was developed and characterized as an experimental model for myocardial ischemia (appendix paper 5). Reduced concentrations of creatine phosphate and ATP as well as cellular acidosis during anoxia, hypoxia and ischemia, are responsible for impairment of the energy-dependent calcium pumps (Ca^{2+}-ATPases) of sarcolemma, sarcoplasmic reticulum and possibly also of mitochondria, so that a hypercontracted "stone" heart may result. It is proposed that the release of ATP catabolites (adenosine, inosine and hypoxanthine) during oxygen (and/or substrate)-limited conditions may be used as a marker for coronary artery and ischemic heart diseases in man.

Perfusion with a fatty acid-albumin complex (at a relatively high fatty acid:albumin molar ratio, which is the main determinant of FFA uptake) in the perfusion buffer resulted in a depression of contractile function. The deleterious role of increa-

sed intracellular levels of fatty acids and their CoA and carnitine esters under normoxic and ischemic conditions have been described on the basis of their chaotropic action on membranes as detergents (see fig. 1 and appendix papers 2 and 6). Catecholamines, liberated from endogenous stores upon fatty acid perfusion may also participate in the decline of myocardial function. Therefore, mention is made of observations on detrimental actions of catecholamines upon cellular membranes and metabolism. It was found that fatty acid-induced impairment of contractility could be prevented by catecholamine-depletion indeed. Furthermore, the addition of glucose, by supplying glycerol-3-phosphate for triglyceride synthesis, which probably reduces the intracellular levels of FFA (and of the CoA- and carnitine ester derivatives), was found to

(i) decrease catecholamine release
(ii) counteract the depression of myocardial energetics and
(iii) increase contractile function,

all occurring during fatty acid-perfusion under fully oxygenated and oxygen-limited conditions.

Our results suggest an important role of fatty acids in myocardial function under normal and pathological conditions, a role in which their ionophoric properties are of central significance.

CHAPTER I

GENERAL INTRODUCTION AND AIM OF THIS THESIS

In all tissues blood flow is responsible for the supply of substrates and oxygen, and for the removal of catabolites. The maintenance and regulation of the circulation is dependent on the contractile activity of the myocardium. For its own energy demand and waste product removal the heart has a coronary circulatory system which perfuses the cardiac muscle tissue. It is obvious that myocardial contractility must be adaptable to the variation of energy demands of the body and that, in turn, there must be a close relationship between its own performance and the actual coronary drainage rate. For that reason myocardial contractile function as well as coronary circulation are most likely subject to a complex control system. Exogenous as well as endogenous metabolic, neural and humoral influences are involved in regulation. Knowledge of their properties is essential to understand the physiological mechanisms of control as well as for the evaluation of preventive and therapeutic measures in (coronary) heart disease. Since the main elements of which the coronary circulation and cardiac muscle consist are vascular smooth and cardiac striated muscle cells, detailed knowledge of the properties of these two types of muscle is mandatory.
Therefore attention is firstly paid to morphological, structural, and functional aspects of both the coronary circulation and the myocardial striated musculature.

In the last decades numerous reports about the effects, and in particular the toxic effects of free fatty acids (FFA) upon the myocardium under normal and pathological conditions have appeared. Yet, the biochemical mechanisms underlying these detrimental effects are still not fully understood. We therefore studied FFA-induced alterations of metabolism, coronary flow and contractility in a rather simple heart-preparation, the isolated rat heart, perfused retrogradely as first described by *Langendorff* in 1895. In this preparation the aorta is cannulated and the

coronary circulation is perfused under a certain perfusion pressure. The number of variables is limited and can be controlled rather easily.

Myocardial changes induced by FFA can be studied in several ways:

i) in hearts in which lipid accumulation has occurred as a consequence of altered carbohydrate- and lipid metabolism. For this purpose we studied hearts from experimental (streptozotocin-induced) diabetic rats, hearts from 48 hour fasted rats and hearts from rats put on a special lipid-rich diet (e.g. an erucic acid-rich rapeseed oil diet).
Hearts from these experimental groups of rats were perfused under normoxic conditions and parameters of myocardial function (coronary flow rate, contractility) and metabolism (high-energy phosphates, lipolysis and prostaglandin synthesis) as determined in tissue extracts and coronary effluent samples can be compared with experimental findings in perfused hearts from control rats, and

ii) in hearts from normal rats subjected to perfusion with fatty acid-containing buffers. Metabolic and functional alterations then can be studied again under fully oxygenated and oxygen (and/or substrate)-deprived conditions. To achieve the latter circumstances hearts were perfused with buffer equilibrated with gas-mixtures containing reduced amounts of oxygen (hypoxia, anoxia) or by severe reduction of the coronary flow rate (ischemia).

With the above-mentioned techniques information (available in full detail in the appendix papers 1 - 6) about the nature and origin of lipolytic activities in cardiac muscle cells is presented. This thesis discusses, in the light of the results obtained, and in reference to the available literature, fatty acid toxicity upon membranes, energy metabolism and contractile behaviour of the myocardium during oxygen-limited and ischemic conditions. In addition a model for myocardial ischemia has been developed for further study of the protective actions of glucose and catecholamine depletion.

The important role of endogenous FFA as calcium-

ionophores and intracellular calcium-vehicles appears throughout the present work. Free fatty acids induce the release of endogenous catecholamines and prostaglandin-like substances and play an important role in the regulation of myocardial function.

CHAPTER II

CORONARY CIRCULATION AND VASCULAR SMOOTH MUSCLE

2.1. Macroscopic and microscopic aspects

The blood supply of the myocardium arises from the coronary arteries and their branches which enter and disperse in the cardiac muscle tissue. The venous effluent is collected in small intramyocardial veins and is transported back to the right atrium. It is well established that the vessels of the venous arc of the coronary circulation contribute little to the total vascular resistance and although the venous calibre is an important determinant of fluid transport across the capillary wall, its structure(s) will not be discussed here.

In general, the muscular arterial wall is organized in three coats (tunics). The innermost intimal layer (*tunica intima*) consists of an endothelial lining and a subendothelial layer, containing strands of collagen and some elastic fibres. The middle coat (*tunica media*) consists of smooth muscle cells and elastic fibres, disposed concentrically. The middle layer is coated by the adventitial layer (*adventitia*) containing collagen, elastin and mucopolysaccharides (1). Large, so-called elastic, arteries are vessels like the aorta and pulmonary arteries, while smaller ones are called muscular arteries. On basis of diameter the following subdivision of arteries is made (2):

large arteries → small arteries → arterioles
→ metarterioles → capillaries

Arterioles (20-100 μm in diameter) have a relatively thick wall and its vascular musculature is richly innervated by nerves of the autonomic nervous system; almost exclusively sympathetic fibres. In general these nerves do not enter the smooth muscle cell containing medial layer. Endings of unmyelinated axons are located on the outer surface of the *elastica externa*, a network

of elastic fibres around the layers of smooth muscle cells. From the finest arterioles or metarterioles (10-20 μm) numerous capillaries (7-9 μm) arise. At the origin of the capillary network so-called precapillary sphincters are located. Anatomically we can say that the precapillary sphincter is the final smooth muscle cell of the (met)arteriolar distribution (3). The precapillary sphincters possess contractile activity and they are completely devoid of control by the autonomic nervous system, but their muscle tone is sensitive to local chemical influences (4).

The capillaries are narrow tubes, with walls composed of a single layer of endothelial cells. Outside the endothelial layer a basement-membrane is found, which plays a role as physical barrier to penetrating materials or as a supportive structure affording apparent rigidity to the capillaries. In the capillaries pericytes were demonstrated in close association with the capillary basement-membrane. Their branched cytoplasmic processes (side-arms) form a web in the endothelium and may have elastic capacity (5). Capillaries possess no known effector mechanism to control their calibre and hence undergo flow changes passively. The capillary wall permits water and solutes to pass through (permeability) either via interendothelial gaps or transcellularly (5). Capillary permeability can be influenced by several substances (histamine, bradykinin, see ref. 6).

2.2. Ultrastructural aspects

The contractile elements within the vascular smooth muscle cells are arranged in a multidirectional way which enables the cell to have local contraction. The plasmamembrane consists of two types of alternating areas. The first one is the plasmamembrane dense body with which bundles of myofilaments appear to merge. The other one contains numerous micropinocytotic vesicles (7). The surface vesicles are supposed to play a role in the calcium homeostatis during the contraction-relaxation cycle (see later). The presence of receptors for various natural and pharmacological substances has been observed (7).

In the cell several contractile proteins have been visualized (8) and ultrastructure of the contractile apparatus is compatible with a conventional sliding filament mechanism of contraction in which thick (myosin) and thin (actin) filaments slide relative to each other. The thin filaments are anchored to the above mentioned plasmamembrane dense bodies. The parallel distribution of myosin filaments and their length relative to the filaments of striated muscle may contribute, in addition to their lower myosin ATPase activity, to the relative high tension bearing capacity of smooth muscle. Intracellular organelles, involved in the contraction process and in energy metabolism, are characterized. Both the sarcoplasmic reticulum and the mitochondria are in close proximity to the plasmamembrane and the surface vesicles.

2.3. Activation and contraction of vascular smooth muscle

Electro-mechanical and pharmaco-mechanical coupling (9) activate a contractile mechanism based on a calcium-sensitive actomysin ATPase system in vascular smooth muscle (10). The primary event in smooth muscle contraction is a rise in the intracellular free calcium concentration (10,11). The participation of intracellular structures in the determination of the cytoplasmic calcium-ion concentration is presented in Fig. 2. Transport of extracellular calcium through the plasmamembrane and their vesicular excavates (surface vesicles), mobilization of membrane-bound calcium and release of calcium from internal storage sites (sarcoplasmic reticulum and mitochondria) determine the cytosolic calcium concentration which induces an interaction between actin and myosin leading to contraction (12). The regulation of filament interaction does probably not involve a troponin-like protein (such as has been described for cardiac striated muscle) but a myosin-linked calcium binding component (13). Agents which are known to modify calcium transport in membranes may influence the contractile state of vascular smooth muscle.

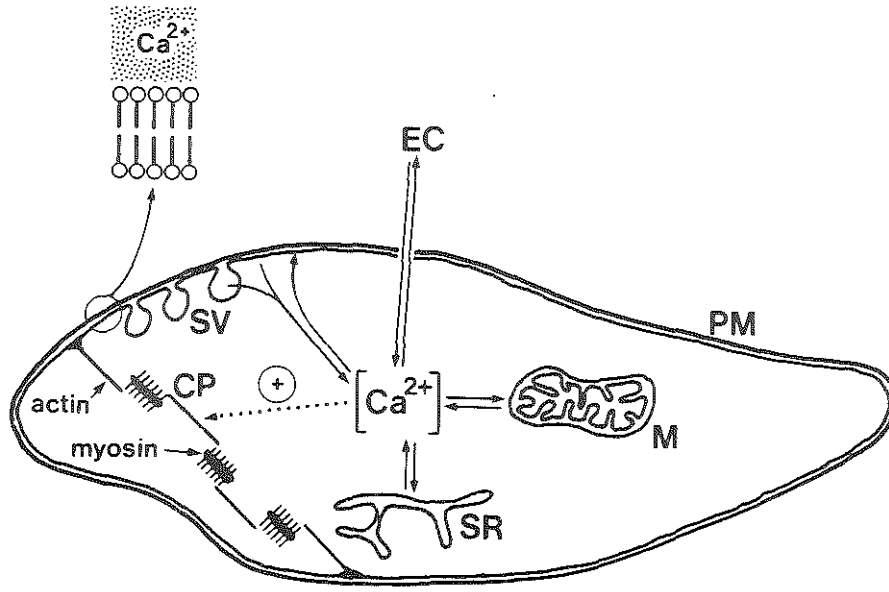

Fig. 2. Subcellular determinants of the intracellular free calcium concentration in vascular smooth muscle cells. EC = extracellular, PM = plasmamembrane, SV = surface vesicles, SR = sarcoplasmic reticulum, M = mitochondria, CP = contractile proteins.

2.4. Metabolism of vascular smooth muscle

Carbohydrates are the major though not immediate source of energy in vascular smooth muscle as indicated by a respiratory quotient of nearly one (see for a review ref. 14). They contain glycogen stored in storage granules. Phosphorylase is the rate-limiting enzyme in glycogen breakdown. Vascular smooth muscle is able to maintain a developed muscle tone for long periods under aerobic conditions. Glycolysis can be demonstrated under aerobic as well as anaerobic conditions (15,16), with lactate as main end-product. Finally vascular smooth muscle cells have an active lipid metabolism (i.e. fatty acid synthesis, phospholipid synthesis, cholesterol synthesis and lipolytic synthesis).

CHAPTER III

STRUCTURE AND FUNCTION OF CARDIAC STRIATED MUSCLE

3.1 Gross structural and microscopic aspects

The heart consists of pacemaker and conducting cells, striated contractile cells and a fibro-elastic matrix. Macroscopically the heart is divided in four pumping enteties: the right and left atria and left and right ventricles. Valves are situated between the cavities of the atria and ventricles, and between the ventricles and the pulmonary and aortic outflow tracts.

The experimental studies presented in this thesis were performed with rat hearts perfused according to *Langendorff* (17). In this preparation the heart is perfused retrogradely via the aorta at a certain perfusion pressure. Due to this pressure the valves in the aortic "outflow" tract close and the perfusion buffer is "forced" into the coronary circulation originating from sinuses above the mentioned mitral valves. Atrial tissue is removed and an atrio-ventricular block is made by cutting a bundle of stimulus conducting cells situated in the atrial septum: the *His* bundle (see appendix paper 1). The coronary effluent flows over the surface of the heart and can easily be collected. The structural aspects of pacemaker and conducting cells will not be discussed because in all experiments the hearts were electrically paced via electrodes placed on the right ventricle.

The left ventricle is the main determinant of the contractile activity of the *Langendorff* heart. Its wall consists of three layers. The inner layer is the endocardium, the outer the epicardium. Both layers consist mainly of connective tissue. In between the ventricular myocardium is situated, consisting of series of overlapping sheets of muscle bundles containing the myofibrils.

The heart is innervated by both sympathetic and parasympathetic nerve fibres. No specialized nerve endings have been identified

in cardiac muscle tissue. The nerves terminate in depressions of the cell membranes of the innervated cells (18). The cardiac striated muscle cells form a branched network, separated from each other by so-called intercalated discs. These cell junctions appear as densely staining transverse bands along the long axis of the myofibril.

3.2. Ultrastructural aspects

The myocytes (muscle cells) are bounded by the cell membrane (sarcolemma) which, in general, is similar to the plasmamembrane of other mammalian cells. The sarcolemma is surrounded by a rather indefinite layer, the basement-membrane, consisting of glycoproteins, which are probably involved in ion-exchange (19). It holds ions, such as calcium, likely in equilibrium with calcium-ions in the extracellular space. The muscle cell contains large numbers of myofibrils, which make up 47% of the cell volume (20). Mutual cohesion between myocytes is provided by the already mentioned intercalated discs. The *fascia adherens* (myofilament insertion region) is the predominant junctional component of the discs. Proteinaceous material between the two plasmamembranes at this junction is probably responsible for tight mutual "binding". Cytoplasmic myofibrils are strongly attached to the filamentous material adjacent to the *fascia adherens*. Other specialized parts of the intercalated discs, the *nexus*, represent sites of intimate contact between the sarcolemmas of two cardiac striated muscle cells. It is proposed that phospholipids form the material joining two cells at a *nexus* (21). Such sites have a low electrical resistance and ion-movements can take place between both cells, permitting the spread of depolarization upon electrical stimulation (22). A third group of closely related junctions situated in the intercalated disc are desmosomes which serve for the attachment of special cytoplasmic filaments (tonofilaments). The tonofilaments interconnect several desmosomes and may have a mechanical function.

From the sarcolemmal surface invaginations arise which

run mainly transversely across the cell. These transverse tubules (T-tubules or T-system) represent portions of the extracellular space carried into the interior of the cell. Sarcolemma and T-system act as a barrier permitting an intracellular environment which differs from that of the extracellular space. The maintenance of the chemical and charge differences between the intra- and extracellular space is mediated by ion-pumps and channels in these membranes. Inside the cardiac striated muscle cell there is a three dimensional network of fine tubules, the sarcoplasmic reticulum (or sarcotubular system). It forms cisternae with the transverse tubules, where they meet.

"Rough-surfaced" endoplasmic reticulum is present in small quantities, its tubules being continues with the "smooth-surfaced" sarcotubular system. It is considered to be involved in protein synthesis (23).

The contractile elements are subdivided into contractile units (sarcomeres) consisting of thick filaments (myosin) and thin filaments (actin). Situated at certain intervals along the actin are the regulatory proteins: troponin and tropomyosin. Contraction and relaxation of the myocardium has been visualized in terms of binding and release of calcium-ions from troponin (i.e. troponin C, which is the "calcium-receptor"), whereas tropomyosin transmits the calcium-induced conformational changes from troponin to the contractile proteins, leading to a sliding movement of actin and myosin and so to shortening of the sarcomere. The energy for the contraction-relaxation cycle is provided by hydrolysis of ATP by the (acto)myosin ATPase.

For the supply of energy for contraction and ion-transport the cell contains numerous mitochondria and storage granules for glycogen and lipid containing vesicles. Other organelles found in the myocardial muscle cell are lysosomes (24,25), Golgi-apparatus and specific granules which contain catecholamines (mainly norepinephrine, see refs. 26,27).

3.3. Activation and contraction of cardiac striated muscle

Four cellular organelles are involved in the process of calcium-mediated contraction: sarcolemma, sarcotubular system, mitochondria and the myofibrils. Pacemaker-induced electrical depolarization of the sarcolemmal T-system complex is associated with a calcium influx from superficial sites in the sarcolemma (the slow inward current of the action potential) and from intracellular organelles (sarcoplasmic reticulum and mitochondria), which is followed by contraction. This proces of excitation-contraction coupling has been subject to many excellent reviews (28-37).

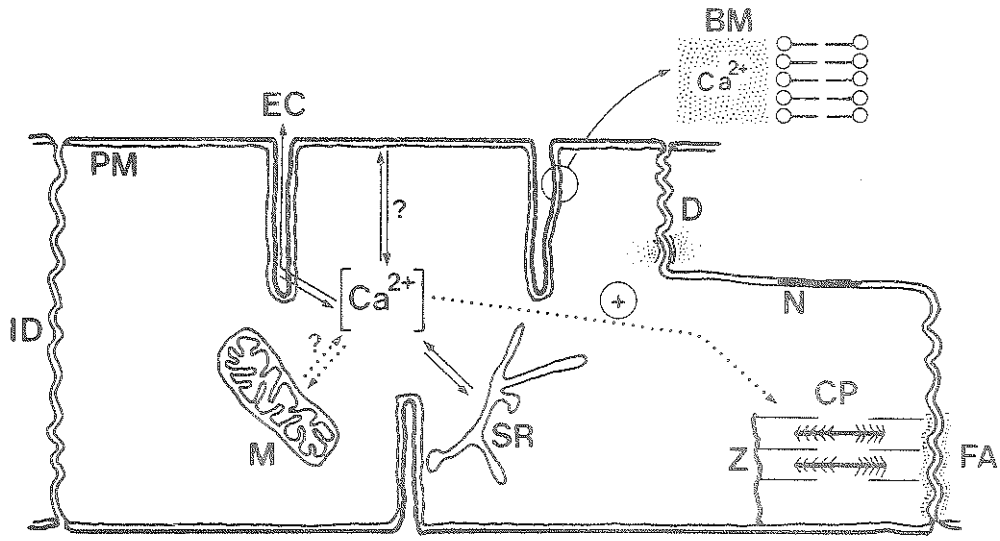

Fig. 3. Cellular organelles participating in intracellular calcium-homeostasis in cardiac striated muscle. EC = extracellular space, PM = plasmamembrane, BM = basement-membrane, ID = intercalated disc, D = desmosome, N = *nexus*, FA = *fascia adherens*, CP = contractile proteins, Z = Z-band, SR = sarcoplasmic reticulum, M = mitochondrion.

Fig. 3 illustrates the involvement of the already mentioned cellular components participating in the regulation of intracellular calcium concentration. The intracellular free calcium concentration is lowered by the activation of different "calcium pumping" mechanisms located at the sarcolemma, sarcoplasmic reticulum and probably also mitochondria (38). The "calcium pumps" are ATP-dependent and their activity leads to uptake and accumulation of calcium in sarcoplasmic reticulum and possibly in mitochondria, and to calcium extrusion into the intracellular space. Sarcolemmal calcium extrusion may, however, also take place through a carrier involving a Na^+-Ca^{2+} exchange mechanism (39). Cardiac sarcolemma contains Na^+,K^+-ATPase and adenylate cyclase (40,41). Both enzymes are involved in the regulation and modification of calcium movements across the plasmamembrane. Sarcoplasmic reticular membranes can bind and take up calcium, and a Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase, possibly involved in calcium transport has recently been isolated and purified by Levitsky *et al.* (42). Like the sarcolemma, sarcoplasmic reticulum might contain Na^+,K^+-ATPase and adenylate cyclase activities (43), and these enzymes are also involved in the calcium transport properties of the sarcotubular membranes. The contribution of mitochondrial calcium uptake in the contraction-relaxation process is still controversial.

3.4. Metabolism of myocardial muscle cells

Under normal (physiological) conditions myocardial metabolism is entirely aerobic and oxygen utilization is closely related to ATP synthesis. *In vivo* and under normoxic circumstances fatty acids and lactate are the main substrates for cardiac muscle cells (44,45). When the fatty acids are abundantly available, insulin is generally limited and glucose utilization inhibited by depressed glucose uptake and inhibition of several steps in the glycolytic pathway. The fatty acid:albumin molar ratio is the main determinant of fatty acid uptake. During

diabetes and fasting when the catecholamine:insulin ratio increases dramatically, endogenous and adipose tissue fatty acid mobilization is rapid and fatty acid uptake, oxidation and storage are increased (45). When the fatty acid:albumin molar ratio is high (>3), the rate of acetyl-CoA utilization is limiting fatty acid oxidation and metabolic intermediates like acetyl-CoA, acetyl-carnitine, fatty acyl-CoA and fatty acyl-carnitine accumulate in the myocardial tissue. Furthermore endogenous triglycerides, stored in lipid-droplets, can be used for energy metabolism, depending on the availability of exogenous fatty acids, hormones (catecholamines, insulin) and on the level of cardiac work.

Under fully oxygenated conditions isolated hearts can use many substrates to provide in their energy demands (ketone bodies, fatty acids, pyruvate, glucose, see refs. 46-48) although an important source of energy may be the oxidation of endogenously stored triglycerides (49). The endogenous triglyceride lipase activity is hormone-sensitive (50-53) and may be localized in the lysosomal or autophagic vacuolar membranes (see appendix paper 3). Direct hormonal control of metabolism is not possible in isolated heart. However, endogenous catecholamines, stored in the nerve-endings of adrenergic (orthosympathetic) nerve-endings may interfere under conditions when they are released. The contractile activity, by determining the requirement of high-energy phosphates and therefore the redox-state of the tissue, is the main control of energy metabolism.

Under oxygen-deprived conditions (i.e. hypoxia, anoxia and ischemia) fatty acid oxidation is reduced sharply, while the utilization of glucose and glycogen (from storage granules) accelerates markedly (54). These processes will lead to enhanced tissue levels of lactate, fatty acids, fatty acyl-CoA esters and fatty acyl-carnitine, and of ATP depletion since glycolytic ATP synthesis cannot meet ATP demands for contraction and osmotic work, when oxidative phosphorylation is inhibited.

CHAPTER IV

REGULATION AND MODIFICATION OF CORONARY FLOW

4.1. INTRODUCTION

The circulation of the myocardium is influenced by mechanical, neural and metabolic factors. Coronary flow is dependent on the ("driving") perfusion pressure and on the vascular resistance. The latter is mainly determined by the muscle tone of coronary resistance vessels (diameter <100 µm) but also by a cyclic extravascular compression of the coronary arteries by the contracting myocardial muscle during systole (55,56). Early observations of Driscoll *et al.* (57) indicated a correlation between coronary flow and perfusion pressure in the isolated, nonworking and fibrillating heart. However, an autoregulatory mechanism which implies that steady-state flow remains constant despite changes in perfusion pressure has been observed (55,58). Isolated, perfused rat hearts do not show this phenomenon (appendix paper 1): changes in perfusion pressure were always followed by changes in coronary flow rate. Before discussing the metabolic and neural influences on coronary circulation we will review current knowledge about the determination of smooth muscle contractility.

4.2. Intracellular determinants of vascular smooth muscle tone

As already mentioned in chapter III, the muscle tone of vascular smooth muscle is responsible for coronary resistance and the contractile state of vascular smooth muscle is primary determined by the intracellular concentration of calcium-ions (see fig. 1). Smooth muscle depends largely upon intracellular supplies of calcium, since they can function for a long time in the absence of extracellular calcium (59). Since smooth

muscle cells are excitable, the action potential, by increasing calcium permeability may play a direct role in mediating contraction. It will be clear that pharmacological modification of calcium-influx during the action potential directly will determine coronary tone (e.g. acetylcholine and other parasympathicomimetics). The energy metabolism is coupled to contraction since enhanced calcium levels provoke to conversion of phosphorylase b → phosphorylase a and thus initiate glycogenolysis. Relaxation of vascular smooth muscle is achieved by the calcium-pump activity of the plasmamembrane, sarcoplasmic reticulum and mitochondria. The calcium-pumps can be stimulated by a mechanism involving cyclic AMP which is formed from ATP through the action of membrane bound or soluble adenylate cyclase (60,61). It is proposed that cyclic AMP, by facilitating phosphorylation of protein or lipoprotein (by a cyclic AMP-dependent protein kinase) increases the calcium binding to "binding-sites" (and subsequent transport) in smooth muscle cells (60,62,63). The resulting decrease in free intracellular calcium levels causes relaxation. The involvement of a cyclic AMP stimulation of microsomal and plasmamembrane Na^+,K^+-ATPase, followed by a decreased Na^+-Ca^{2+} exchange as relaxatory mechanism has been proposed by Limas and Cohn (64). A rise in intracellular cyclic AMP is not only mediated by increased adenylate cyclase activity but can also be achieved by inhibition of cyclic AMP phosphodiesterase (e.g. by methylxanthines).

If relaxation is caused by an increased level of cyclic AMP it seems reasonable to assume that decreased levels may be associated with contraction enhancement (65). Indeed it was noticed that drug-induced aortic vasoconstriction was associated with a decreased cyclic AMP level (66). Since also changes in vascular smooth muscle cyclic GMP levels during the contraction-relaxation cycle are observed, this nucleotide may also be involved in the regulation of the intracellular calcium level. Moreover cyclic GMP has been proposed to stimulate calcium release from microsomes in various types of smooth muscle (67). Drug-induced activation of membrane-bound or cytosolic (soluble) guanylate cyclase, followed by rises in cyclic GMP were associated with smooth muscle contraction (68). However, (i) the fact that

guanylate cyclase activity is stimulated by calcium-ions, (ii) the observation that cytoplasmic cyclic GMP increases under conditions when smooth muscle cells do neither contract nor relax and, (iii) the finding of drug-induced smooth muscle contraction without a change in the level of cyclic GMP (69) seem to rule out any direct regulatory action of cyclic GMP in the smooth muscle contraction-relaxation cycle. It may be conceivable that cyclic GMP plays a role as feedback signal to speed up the calcium removal by decreasing the influx of calcium (68).

The cyclic nucleotide modification of vascular smooth muscle function is presented in fig. 4.

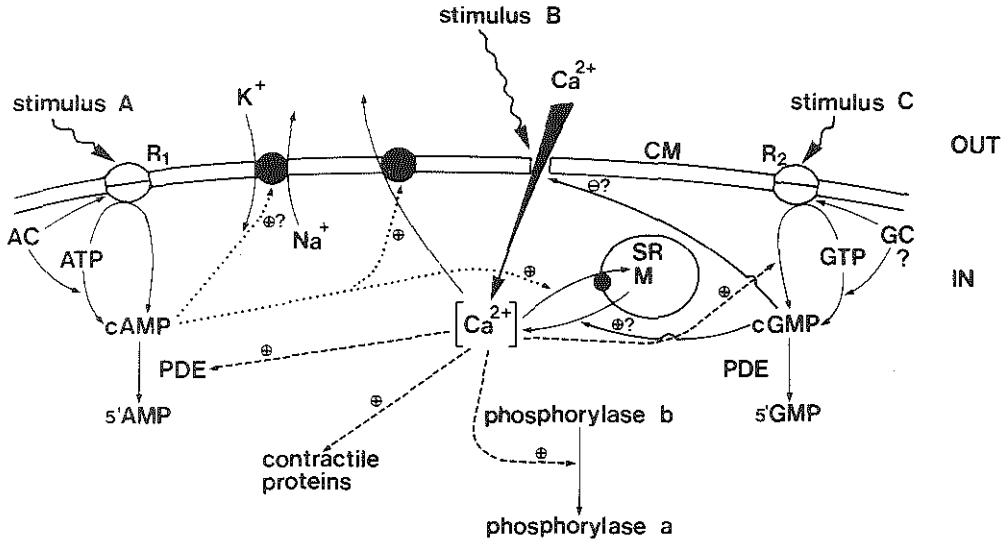

Fig. 4. The role of cyclic nucleotides in vascular smooth muscle function. CM = cell membrane, R_1 + R_2 = hormone (metabolite)-receptors, AC + GC = membrane-bound and soluble adenylate and guanylate cyclase, SR = sarcoplasmic reticulum, M = mitochondrion, PDE = phosphodiesterase, ⊕ = stimulation, ⊖ = inhibition. The black dots represent active "pump" systems.

In the next paragraphs a number of metabolic and neural influences on coronary flow of isolated hearts will be discussed on the basis of this (hypothetical) scheme.

4.3. Relation between cardiac metabolism and coronary flow

Although extra-cardiac mechanisms play a role in the modification of coronary flow, the primary regulatory mechanism(s), responsible for the adjustment of coronary resistance reside(s) within the heart. The metabolic demands of vascular smooth muscle cells are negligible when compared to the high energy demands of contracting cardiac striated muscle cells. Therefore, smooth muscle can maintain contractile function under long periods of hypoxia, while striated muscle will loose its contracting and relaxing capacity. Alterations in cardiac striated muscle metabolism, however, modulate smooth muscle "activity" and numerous intrinsic factors have been suggested as mediators (for a review see ref. 55). Lactate, CO_2, K^+, P_i, osmolality, low pO_2 and adenine nucleotides have all been proposed to play a role in the modification of coronary flow under various experimental conditions, but most fail to fulfil physiological criteria. Substantial evidence has accumulated to show that increased cardiac activity leads to enhanced coronary flow (70,71) and the relation between these two processes has been attributed to increased cardiac levels of cyclic AMP (72), since phosphodiesterase inhibitors potentiate this metabolically induced coronary dilatation during hyperactivity (72). In the next sections some metabolites which may be involved in coronary flow regulation are discussed.

a. Adenosine

During cardiac hyperactivity (associated with increased oxygen demand), whether induced by pacing at high rates or due to catecholamines, adenosine is released from the isolated rat heart

(73-75). Furthermore adenosine formation is enhanced in association with vascular dilatation following hypoxia and anoxia (73, appendix paper 5), reactive hyperemia (coronary vasodilation occurring after short periods of interruption of flow; see refs. 76,77) and ischemia (appendix papers 5 and 6, 78). Adenosine is formed by enzymatic hydrolysis of 5'-AMP through the action of 5'-nucleotidase which is a plasmamembrane-bound enzyme (79). A rise in intracellular AMP levels, indeed, is likely to occur during the conditions mentioned above, while changes in ATP and ADP levels (inhibitory) and AMP (stimulatory) determine 5'-nucleotidase activity (80).

The mechanism by which adenosine exerts its vasodilatory action is still unknown. Adenosine-receptors have been proposed to mediate the vasodilatory capacity of adenosine (81-83). Olsson *et al.* (81,82) first observed adenosine receptors on the coronary myocyte surface while Schrader *et al.* (83) presented evidence for adenosine-receptors both on coronary myocytes and atrial muscle cells. Adenosine-stimulation of adenylate cyclase has been reported for ventricular muscle (84,85), and if the same mechanism occurs in vascular smooth muscle it may well be that the increased cytoplasmic cyclic AMP levels are the mediators of adenosine-induced coronary vasodilation (adenosine may then act as stimulus A, see ref. 3). Only limited experimental evidence is available which does not support a role for cyclic AMP in the adenosine-induced coronary relaxation (86). Schrader *et al.* (87) observed adenosine-inhibition of action potential-linked calcium-ion influx in atrial muscle, also leading to decreased intracellular calcium levels ("negative" stimulus B in fig. 4).

b. Prostaglandins

Endogenous prostaglandins, synthetized from essential fatty acids in phospholipids (as indicated in fig. 5) have been implicated in the regulation of coronary flow (appendix paper 1). In the isolated dog and rat heart they are probably not involved in the maintenance of coronary flow during normoxic conditions

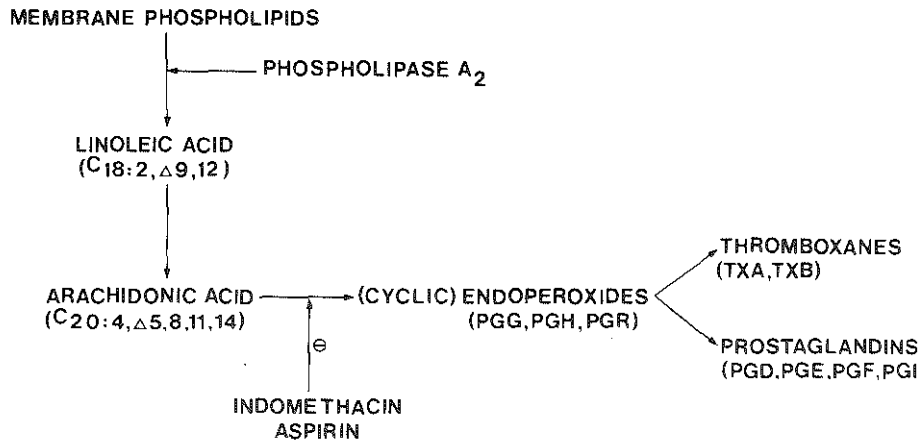

Fig. 5. Prostaglandin synthesis from unsaturated fatty acids.
Θ : inhibitory action.

whereas they seem to do in guinea pig hearts (88,89). For rabbit hearts contradictory results have been reported (90,91). Like for adenosine, release of various prostaglandins have been reported during hypoxia, anoxia, ischemia, reactive hyperemia and in response to added catecholamines together with the vasodilation observed under these pathological conditions (90,92-94). Furthermore it has been shown by De Deckere *et al.* (95) that prostacyclin (PGI_2) forms the major contribution to the prostaglandins released from rat and rabbit heart during anoxia. This finding is important since PGI_2 has been found to possess strong anti-aggregatory and vasodilatory properties and, thus, may be a self-protective substance for the myocardium (96,97). The vasodilatory [PGE_1, PGE_2 (?), PGG_2, PGH_2, PGH_3 and PGI_2] and vasoconstrictor [PGA_2, PGD_2, PGE_2 (?), $PGF_{2\alpha}$, TXA_2 and TXA_3] properties of prostaglandins, thromboxanes and their endoperoxide precursors upon the coronary vascular system (98,99) seem to have changes in intracellular cyclic AMP and cyclic GMP levels as determinants. A membrane receptor for prostaglandin E_1 in liver plasmamembranes has been characterized and localized (100). However, about prostaglandin-receptors in smooth muscle and

cardiac striated muscle plasmamembranes no information is available. In smooth muscle cells of various origin a vasodilator prostaglandin-induced increase in cyclic AMP levels has been reported (72,101-104), while vasoconstrictor-prostaglandin-induced increase in cyclic GMP has been mentioned (102). Although these findings indeed may infer a role of cyclic nucleotides in the vasoactive actions of prostaglandins other explanations can be opposed.

Firstly, prostaglandins, particularly PGE_1, can bind calcium-ions and may act as calcium-ionophores (105,106) thereby directly stimulating the efflux of calcium from smooth muscle cells and secondly by inhibition of Na^+-activated ATPase as a result of increased calcium binding. The latter is observed for rat brain synaptosomal Na^+-stimulated ATPase (107).

During fasting and experimental (streptozotocin) diabetes the release of prostaglandin-like substances is enhanced in association with increased coronary flow rates (appendix paper 1). However, endogenous catecholamines are involved in this process (see later).

c. Fatty acids

When rat hearts are perfused with medium and long chain fatty acids vasodilation is observed (104, appendix papers 2 and 6). As shown by Hülsmann (104) this fatty acid-mediated relaxation of coronary smooth vasculature is neither mediated by a release of adenosine from the hearts, nor by stimulation of adenylate cyclase (increase in cyclic AMP) nor by activation of the adrenergic system although under some conditions fatty acid-induced release of endogenous catecholamines is observed (appendix paper 2).

Lipophilic fatty acids (and their CoA and carnitine esters), like for example prostaglandin E_1 (105,106), may possess ionophoric properties (104,108) concerning calcium binding and transport through membranes. This could lead to calcium sequestration from vascular smooth muscle cells and result in vasodilation.

On the other hand, fatty acid-induced inhibition of myocardial plasmamembrane Na^+,K^+-ATPase, as reported by Lamers and Hülsmann (109) might be involved, since it may lead to decreased extra(myocardial)cellular and smooth muscle calcium levels.

4.4. Neurotransmitter-mediated modification of coronary flow

Nerve endings from both divisions of the autonomic nervous system (sympathetic and parasympathetic) innervate coronary vessels. Stimulation of these autonomic nerves influences myocardial performance and metabolism. In isolated hearts a direct neural influence is absent. However, under various experimental conditions release of neurotransmitter substances, stored in nerve-ending vesicles can take place thereby mimicking the effects of direct nerve stimulation. Sympathetic neurotransmitters (catecholamines) are also synthesized in the adrenal glands and can be released in the circulation. Circulating neurotransmitters have the same influence as transmitters released upon nerve stimulation.

a. Parasympathetic (cholinergic) neurotransmitters

Acetylcholine is the neurotransmitter in parasympathetic nerve endings. Acetylcholine is capable to "activate" several types of membrane-receptors: nicotinic-receptors present in skeletal muscle and muscarinic-receptors present in myocardial striated and smooth muscles. It has been shown that stimulation of parasympathetic (vagal) nerves results in coronary vasodilation in the *in situ* dog heart (110). This observation might be related to the acetylcholine-induced increase in smooth muscle cyclic GMP levels (111). For isolated hearts little is known about cholinergic control of coronary flow. However, isolated, perfused mammalian hearts (cat, rabbit, guinea-pig)

do not release acetylcholine, neither spontaneously, nor during vagal stimulation (112) and for this reason it seems reasonable to state that cholinergic neurotransmitters do not participate in the maintenance of coronary flow in isolated hearts under normoxic conditions. About the role of acetylcholine on coronary flow during pathological conditions (anoxia, hypoxia, ischemia) no information is available.

b. Sympathetic (adrenergic) neurotransmitters

The myocardium and coronary vasculature receive a plentiful sympathetic nerve supply. Epinephrine and in particular norepinephrine are neurotransmitter substances in the nerve-endings of the adrenergic system. Together they are called catecholamines, while other synthetic agents like isoproterenol, phenylephrine and oxyphedrine behave like catecholamine-agonists. Their effects are mediated via "activation" of several receptors (113). These receptors are designated α, β_1, and β_2 (114, see for a review ref. 115). α-Receptors are not present in arterioles. They are mainly present in the larger coronary arteries (116). α-Receptors are specifically activated by phenylephrine and can be blocked by phentolamine, phenoxy-benzamine and dibozane. β_2-Receptors exist in large and small arteries of the coronary circulation although some disagreement concerning the latter is present. Isoproterenol is a specific β-receptor agonist while propranolol is a specific β-blocking agent. The relative potencies of some catecholamines for α-receptor stimulation are: epinephrine $\approx$ norepinephrine >> isoproterenol, while for β-receptors this is: isoproterenol > epinephrine > norepinephrine (113).

The overall coronary vascular response to catecholamines is vasodilation (117), and comprised of direct vasoconstriction via α-adrenoceptors (118) and vasodilation mediated by β-adrenoceptors, probably of the β_2-type (116,118). Mohrman and Feigl (56), however, oppose that vasodilation after sympathetic stimulation is a result of an α-receptor mediated constrictor mechanism overruled by a metabolic vasodilation, which may be

related to chatecholamine-induced release of adenosine (74). Catecholamines exert their actions via the cyclic nucleotide system. This would imply that α-receptor stimulation leads to a decrease in intracellular levels of cyclic AMP (65) while the opposite occurs during β-receptor stimulation. However, the contraction of coronary arterioles associated with α-receptor stimulation appeared not to be mediated by changes in cyclic AMP levels while, indeed, relaxation associated with β-receptor stimulation was mediated by an increase in cytosolic cyclic AMP levels (65,119).

In isolated, perfused rat hearts released endogenous catecholamines are involved in the maintenance of coronary flow under normoxic conditions (appendix papers 1 and 2), since after catecholamine-depletion by reserpin pretreatment or preperfusion with tyramine, a lower coronary flow rate is observed. During fasting and streptozotocin-diabetes the increased flow rates are probably determined by a direct vasodilatory action of released catecholamines and by a catecholamine-induced release of vasodilatory prostaglandin-like substances (appendix paper 1). Increased catecholamine contents in tissue-slices of hearts from fasted rats have been reported (120) while Chaudurni and Shipp (121) observed increased cyclic AMP levels in hearts of diabetic rats. Furthermore, catecholamine-induced release of prostaglandins from the heart is well established (93,122).

A large part of nerve-ending catecholamines is stored in so-called adrenergic vesicles. The lipid composition of this vesicle membrane reveals a relatively high concentration of the membrane-fusion promoting lyso-phosphatidylcholine (123,124). Calcium-ions are the trigger for fusion of adrenergic vesicles and plasmamembranes resulting in exocytosis and catecholamine-release while removal of calcium from the nerve endings will be responsible for the budding of, to form granules (124). Catecholamine-secretion from isolated hearts, indeed, proved to be stimulated by perfusion with Ca^{2+},Mg^{2+}-ionophore X-537A, long chain fatty acids and high concentrations of prostaglandin E_1 (appendix paper 2). The calcium-ionophoric properties of fatty acids and prostaglandins may have triggered catecholamine-

Table I

THE EFFECT OF DIETARY FATS ON CORONARY FLOW OF ISOLATED RAT HEARTS

All rats were from the Wistar strain and weighed 200-345 g. The control diet consisted of laboratory pellets, supplemented with a standard vitamin mixture. The hearts were perfused according to *Langendorff* (17) at the indicated perfusion pressures (Pp) and heart rates (HR). The perfusion buffer contained glucose as substrate. Coronary flow is expressed as ml.min.$^{-1}$ (a), ml.min.$^{-1}$ g dry weight (b) and ml.min.$^{-1}$ g wet weight (c). SSO = sunflowerseed oil, PO = palmoil, RSO = rapeseed oil. All results are given in mean values ± S.E.M., the number of experiments is given in parenthesis and significance was determined according to Students t-test. $P>0.05$ was considered to be not significant (NS).

Author(s)	Dietary fat	Duration	Pp (mmHg)	HR (beats/min)	Coronary flow: Control		Fat fed
Reid *et al.* (125)	32% beef lard 6% olive oil 2% cholesterol (65 cal% fat)	3-4 weeks	60	200	7.9 ± 0.2^{a} (4)	—NS—	6.8 ± 0.7^{a} (4)
De Deckere & Ten Hoor (126)	50 cal% SSO	3 days	90	360	98 ± 6^{b} (10)	—NS—	114 ± 6^{b} (10)
	50 cal% PO	"	"	"	62 ± 7^{b} (7)	—NS—	76 ± 3^{b} (7)
	50 cal% SSO	"	50	"	56 ± 4^{b} (10)	-P<0.01—	69 ± 3^{b} (10)
	50 cal% PO	"	"	"	42 ± 3^{b} (7)	— NS —	46 ± 3^{b} (7)
Stam & Hülsmann (unpublished observations)	40 cal% RSO	"	73	300	6.32 ± 0.09^{b} (10)	-P<0.001-	7.34 ± 0.16^{c} (10)
	40 cal% SSO	"	"	"	6.28 ± 0.12^{c} (10)	— NS —	6.20 ± 0.13^{c} (6)

release from nerve-endings of the adrenergic nervous system. The released catecholamines, however, are not involved in the medium and long chain fatty acid-induced coronary vasodilation since reserpin-mediated depletion of endogenous catecholamines did not alter the coronary vasodilatory response during fatty acid perfusion (104).

4.5. Dietary fat-mediated modification of coronary flow

The results of some investigations concerning the effects of changes in dietary fat content upon coronary flow rates in isolated hearts, perfused under normoxic conditions, are summarized in table I.

Diets containing 40 cal% of rapeseed oil (with high amounts of erucic acid, $C_{22:1}$ $\Delta 13$) increased basal coronary flow rates in isolated rat hearts, while for diets containing sunflowerseed oil (with high amounts of linoleic acid, $C_{18:2}$ $\Delta 9,12$) at 40 cal% no increase was found, in contrast to 50 cal%, for which De Deckere and Ten Hoor (126) found that higher flow rates were also obtained. In spite of the fact that linoleic acid is a precursor of prostaglandins and thromboxanes of both the 1 and 2 series, the increased flow rates after high linoleic acid containing diets are not mediated by increased prostaglandin formation and release (127). About the role of prostaglandins in the rapeseed oil-mediated increased flow rates nothing is known.

Short term feeding (3-6 days) of rats with dietary erucic acid causes gross triglyceride accumulation and infiltration in the heart which disappears after prolonged feeding (128). Evidence has been presented that the oxidation of long chain fatty acids is suppressed by erucic acid (129). The increased levels of intracellular long chain fatty acids may promote calcium loss from vascular smooth muscle, resulting in vasodilation. They may also be involved in the release of endogenous catecholamines from adrenergic vesicles, leading to an "overall" vasodilation and to enhanced lipolytic activity (as judged from the increased glycerol release from hearts of rapeseed oil fed rats, appendix paper 3).

Since adenosine release from hearts of rapeseed oil fed rats was not significantly different from adenosine release from hearts from sunflowerseed oil fed and control fed rats, adenosine is not involved in the observed coronary vasodilation.

Some other aspects of erucic acid feeding are discussed in chapter V.

4.6. Conclusions

Regulation and modification of coronary flow in isolated hearts under various experimental conditions is mediated by changes in the intracellular calcium-ion concentration. It is proposed that metabolic and neurohumoral modification of cytoplasmic calcium levels takes place via direct actions of calcium-binding and transporting agents (ionophores) and indirectly via the cyclic nucleotides (cyclic AMP and possibly cyclic GMP) mediated binding of calcium to intracellular membranes. Catecholamines and adenosine act probably via the indirect mechanism, fatty acids directly on basis of their Ca^{2+}-ionophoric properties and prostaglandins may affect smooth muscle calcium levels in both ways. Dietary fats, finally may act on smooth muscle calcium by modification of smooth muscle cell metabolism (lipolysis) leading to enhanced intracellular fatty acid levels.

CHAPTER V

REGULATION AND MODIFICATION OF MYOCARDIAL CONTRACTILITY

5.1. Introduction

The performance of the heart is dependent on contractile behaviour of the myocardium. The fundamental structural and functional unit of contraction in the cardiac striated cell is the sarcomere: the basic repeating unit in the longitudinally oriented myofibrils. The sarcomere is composed of different kinds of contractile proteins, which can slide relatively to each other (30) and give the sarcomere a characteristic banded pattern. The degree of overlap between these thick (myosin) and thin (actin) filaments in each sarcomere determines to a considerable extent the contractile force or actively developed tension of heart muscle. Generally applicable to both cardiac and skeletal muscle is that the developed force of contraction is a function of the initial muscle length, the length-tension relation, while the velocity at which the muscle shortens is inversely related with the developed force, the force-velocity relation (130). The contractile function of the intact heart is modified predominantly by changes in muscle fibre length (the Frank-Starling principle), the frequency of contraction as well as the parasympathetic and, particularly, sympathetic tone. Numerous factors including the concentration of H^+ and other ions, drugs, tissue oxygen, metabolite concentrations and temperature can influence the actual contractile state of the myocardium.

When the metabolic demands of the contracting myocardium are beyond the capacity of the coronary circulation, for example due to coronary atherosclerosis or other conditions which lead to underperfusion of the tissue, the contractile behaviour of the "ischemic" area alters dramatically and may lead to myocardial infarction. Many investigations have been presented dealing with metabolic intermediates in cardiac muscle in different types of heart failure (ischemia, hypoxia,

hypertrophy, cardiomyopathies, etc.) during the search of the biochemical abnormalities involved in the deterioration of contractile function and during the search for biochemical markers suitable for reliable quantitation and characterization of tissue-damage (54,131-137).

5.2. Intracellular regulation of calcium-ion "activity"

Because the heart is functionally a syncitium all myocardial muscle cells contract during the cardiac cycle and, in contrast with skeletal muscle, contractility cannot be modulated by varying the number of active (contracting) cells. Each cardiac cell must be capable of a versatility of contractile states which may be the result of changes in action potential, the amount of calcium released for activation of contraction, or of the contractile proteins themselves. As already mentioned in chapter III (paragraph 3.3) calcium is thought to be the link between the excitory event taking place at the sarcolemma (action potential) and the contraction process that occurs at the sarcomere (29-39). However, the amount of calcium-ions penetrating the cardiac striated muscle cell during the slow inward current of the action potential (whether relaying from the pacemaker cells or via external stimuli), which amounts about 10 μmoles.kg wet weight^{-1}, is not sufficient to achieve 10% activation of contractile force (31,138). It is proposed that the remainder of the required calcium-ions is derived from intracellular "sinks", like phospholipid components in the sarcolemma (associated with the Na^+,K^+-ATPase, see refs. 37,139), and the sarcoplasmic reticulum. This calcium release from cellular "sinks" in response to the electrogenic influx of small quantities of calcium is called "the calcium-induced calcium release". Mitochondria may release calcium-ions in exchange for sodium (38,140) although mitochondrial involvement in the contraction-relaxation cycle is disputed (141). Furthermore calcium can enter the cell at the sarcolemmal level where bidirectional exchange of calcium

with cations such as Na^+, K^+, H^+ and possibly Mg^{2+} is proposed although the exact mechanisms are not understood (36).

Relaxation is a consequence of decreased cytosolic calcium levels. A number of mechanisms are involved in the sequestration of calcium-ions from the cytoplasm. Firstly, sarcolemma and sarcoplasmic reticulum possess a stimulated Mg^{2+}-dependent ATPase which may be responsible for calcium binding and transport (uptake, efflux). Secondly, mitochondria can accumulate calcium linked to respiration (142,143). Thirdly, the already mentioned bidirectional exchange systems may be involved. The Na^+,K^+-ATPase, mainly localized at the sarcolemma, plays a central role in some of the calcium-exchange systems. For instance, inhibition of the enzyme by glycosides or, possibly, cyclic AMP (144) gives rise to increased intracellular sodium concentration and may be followed by a Na^+-Ca^{2+} exchange at the sarcolemma (145), or calcium release from the mitochondria. The resulted increased cytoplasmic calcium levels alter the contractile state of the myocardium.

Other enzymes, involved in the regulation of myocardial contractility, are adenylate and guanylate cyclase, since oscillations of cyclic AMP and cyclic GMP during one cardiac contraction cycle have been observed (146,147). It indicates a participiation of cyclic nucleotides under normal physiological conditions. It is suggested by Brooker (147) that oscillations in cyclic AMP are caused by transient stimulation of adenylate cyclase during the action potential while it also may reflect fluctuating calcium levels through activation of cyclic AMP phosphodiesterase (148) or as mentioned by Tada *et al.* (41) through feedback inhibition of adenylate cyclase.

Adenylate cyclase is localized in sarcolemma and in microsomal fractions (43,149) and its activity is responsible for cyclic AMP formation. Together with cyclic AMP phosphodiesterase activity it determines the actual intracellular concentration of cyclic AMP (150). This cyclic nucleotide in turn can stimulate specific cyclic AMP-dependent protein kinases involved in phosphorylation of specific sites of the sarcoplasmic reticulum leading to augmented calcium uptake (139). A sarcoplasmic reticulum protein (phospholamban),

capable of incorporation of phosphate in the presence of cyclic AMP has been isolated (43). This cyclic AMP-protein kinase in the sarcoplasmic reticulum seems to be functionally coupled to glycogen metabolism since from dog heart sarcoplasmic reticulum a fraction has been isolated by Entman and co-workers (151,152), containing both the calcium modulating system and enzymes of glycogenolysis (adenylate cyclase, protein kinase, phosphorylase kinase, phosphorylase and debranching enzyme). The phosphorylating action of the cyclic AMP-protein kinase system may not only be restricted to the sarcoplasmic reticulum. Cyclic AMP has been shown to cause elongation of the action potential, leading to increased calcium influx and enhanced contractility (153) while on the other hand cyclic AMP stimulation of sarcolemmal calcium pump activity may contribute in the reduction of the relaxation time (36). Finally, cyclic AMP appears to be involved in phosphorylation of the inhibitory subunit of troponin in association with increased contractility (153-155).

In heart tissue particulate and soluble guanylate cyclase is present (156) while also other enzymes involved in the intracellular cyclic GMP homeostasis and actions (cyclic GMP-dependent protein kinase and cyclic GMP phosphodiesterase) have been described (see for a review ref. 157). It is proposed that soluble guanylate cyclase originates from the particular (membrane bound) fraction which has been identified in plasmamembranes, endoplasmatic reticulum, mitochondrial membranes and in the cell nucleus. In contrast with adenylate cyclase, the enzyme is rather insensitive to hormonal agents (except cholinergic agents) and its activity is regulated by calcium-ions. The observation of George *et al.* (158) that cardiac contractility was depressed in association with an acetylcholine-induced rise in cyclic GMP levels *plus* the observed cyclic GMP oscillations during the cardiac cycle (146,147) speak in favour of a cyclic GMP modulation of intracellular calcium levels, for instance by stimulating mitochondrial calcium uptake (61,159). However, the same argument which disputes a cyclic GMP role in vascular smooth muscle is appropriate here, namely the inverse relation between calcium and cyclic GMP, for intracellular calcium regulates guanylate

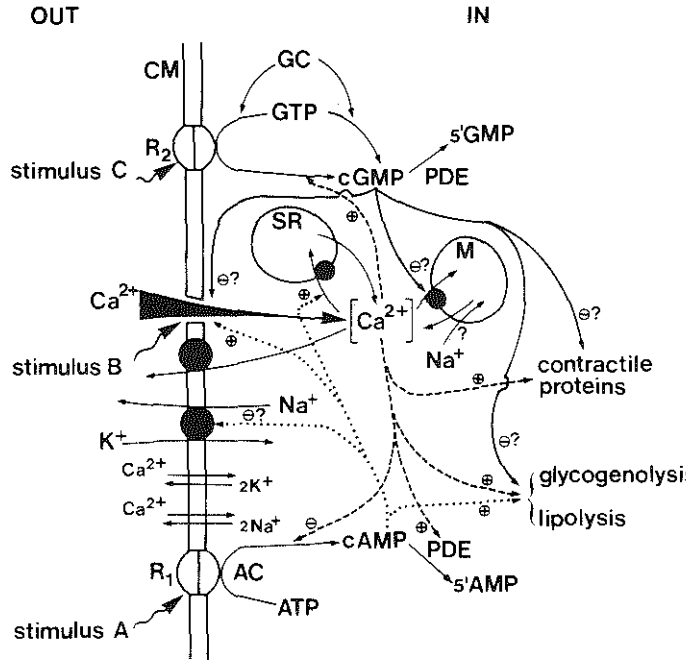

Fig. 6. The role of cyclic nucleotides in intracellular calcium homeostasis in cardiac striated muscle. CM = cell membrane, R_1 + R_2 = hormone- (metabolite) receptors, AC = adenylate cyclase, GC = particulate and soluble guanylate cyclase, SR = sarcoplasmic reticulum, M = mitochondrion, PDE = phosphodiesterase, ⊕ = stimulation, ⊖ = inhibition. The black dots represent active "pump" systems.

cyclase activity.

Cyclic GMP phosphorylation of cardiac troponin inhibitory subunit (TN-I) has been observed (160,161) but it is yet not clear whether this action of the cyclic GMP-protein kinase system is related with the depressive actions of cyclic GMP. Recently it was demonstrated immunocytochemically by Ong and Steiner (162) that binding of cyclic GMP fluorescent antibody was observed in the A zone of the sarcomere while cyclic AMP-binding antibody was recovered in the area of sarcoplasmic reticulum and sarcolemma in both skeletal and cardiac muscle. The presence of cyclic

GMP-binding antibody in the A band suggests a role for cyclic GMP in the regulation of myosin function.

The various above mentioned mechanisms which may be involved in the regulation and modification of the intracellular calcium-ion concentration in cardiac muscle, in relation with contractile function and metabolism, are summarized in fig. 6. In the subsequent paragraphs, neurohumoral, metabolic and pharmacological effects on the calcium-ion activity are discussed, while reference is made to this figure.

5.3. Changes in myosin as determinant of contractility

Hydrolysis of ATP by myosin ATPase activity determines the velocity of the contractile machinery of cardiac muscle, and it has been suggested by Katz (133) that changes in contractility in the failing heart are associated with changes in the rate of ATP hydrolysis by the myofibrils. Decreased myosin ATPase and altered myocardial contractility have been observed in endocrinopathies (hyper- and hypothyreoidism, adrenalectomy), during ageing and in heart failure due to chronic hemodynamic overload (hypertension, aortic constriction, pulmonary stenosis, see refs. 133,135,163). Furthermore, significant changes in myosin and its ATPase activity have been observed after administration of catecholamines and during myocardial ischemia (164,165).

5.4. Metabolic control of contractile function

Intracellular energy metabolism in myocardial muscle may be divided in three stages:

(i) energy production by oxidation of substrates coupled to ATP synthesis,

(ii) "storage" of high-energy phosphate-bonds in creatinephosphate and

(iii) utilization of chemical energy for mechanical work and ion-pumps (ATPases).

It has been long apparent that ATP is necessary for the chemical reactions which form the basis of cardiac contractile function. During contraction myosin cross-bridges interact with the thin actin filaments which are pulled towards the center of the sarcomere. Relaxation takes place during dissociation of the actomyosin complex. However, it is still not clear whether ATP is involved in the contractory events or in the relaxation process. It is proposed that dissociation of the actomyosin complex only proceeds when ATP is present and, if not, muscle stiffness and inextensibility (rigor, "stone heart") may ensue (166). Nevertheless, it is obvious that under conditions when energy production is limited, contractile function is impaired. However, since decline in contractility and tissue high-energy phosphates were not found to be associated, other factors, directly or indirectly related with cellular energy metabolism, have to be involved, while the imbalance between intracellular compartments of adenine-nucleotides needs further study (167-169).

a. Hypoxia, anoxia and ischemia.

The delivery of oxygen to myocardial cells is crucial for normal function of the heart. With increased cardiac work, oxygen demand increases proportionally and it is met by an autoregulatory increase in coronary flow rate and increased oxygen extraction. ATP synthesis during oxidative metabolism appears to be strictly controlled by ATP utilization, while mitochondrial respiration and citric acid cycle activity are controlled by the cytoplasmic phosphate potential $\{[ATP]/([ADP] \times [Pi])\}$ and the mitochondrial NAD oxidation-reduction state (170). During acute ischemia, hypoxia and anoxia energy metabolism alters drastically. Some of the alterations occurring in isolated rat hearts are discussed in appendix papers 5 and 6. In that work reduction of coronary flow was achieved by microsphere-embolization of the coronary circulation. In this model for ischemia the vascular response (vasodilation of the non-

obstructed vessels) is retained. Reduced ATP levels observed during ischemia, hypoxia (and anoxia) were correlated with depressed contractility although "factors other than tissue levels of creatine phosphate and ATP were rate-limiting". An important factor may be acidosis, produced by metabolic alterations during substrate- and oxygen limited conditions (171). Both proton generation and reduced levels of high-energy phosphates may influence several calcium-mediated events in the contraction-relaxation cycle:

(i) inactivation of the slow calcium-ion channels (slow inward calcium influx during the action potential) by reduced membrane phosphorylation when ATP levels are decreased (172),

(ii) reduced interaction between the contractile proteins or failure of actin and myosin filaments to dissociate when ATP is lacking (166),

(iii) proton competition with calcium for activation sites at the troponin molecules leading to interruption (or reduction) of actin-myosin interaction (173),

(iv) reduced calcium efflux due to inactivation of calcium "pumps" in sarcolemma, sarcoplasmic reticulum and mitochondria at low ATP levels,

(v) proton inhibition of calcium accumulation in sarcoplasmic reticulum as shown for skeletal muscle (174) and

(vi) proton inhibition of actomyosin ATPase (Prof. P. Harris, personal communication).

The observed decrease in Na^+,K^+-ATPase activity (175) and increased lysosomal fragility (176) during ischemia may also be hazardous to the myocardial tissue.

In early stages, loss of contractility due to the above mentioned events is probably fully reversible. However, sustained ischemia will lead to irreversible damage of myocardial cells (infarction). During the onset of ischemia catecholamine release from endogenous stores takes place leading to enhanced cytosolic cyclic AMP levels (177). It is proposed that cyclic AMP is involved in arrhythmogenic action occurring in response to

administered catecholamines (178) and during ischemia (179, 180). The metabolic response to increased intracellular cyclic AMP during ischemia are (i) stimulation of prostaglandin synthesis and release (93) (ii) enhanced lipolysis, which in combination with decreased carnitine levels and respiratory-chain activity, lead to increased levels of long chain fatty acids and their CoA and carnitine esters (181) and (iii) enhanced glyco(geno)lysis resulting in depletion of glycogen stores (182,183).

b. Prostaglandins

Besides the already mentioned effect on coronary flow, prostaglandins have marked effects on cardiac contractility and on lipid and carbohydrate metabolism (see for a review ref. 184), while they are supposed to play a modulatory role in neuro-(muscular)transmission (94,122). Generally spoken, most prostaglandins increase cardiac contractility in a dose dependent manner. The positive inotropic effects are mediated by cyclic AMP since prostaglandin-induced increased in adenylate cyclase activity (185) and enhanced cyclic AMP levels have been observed (186). On the other hand prostaglandin-induced increased cellular membrane permeability to calcium has been observed in frog hearts (187). It has been proposed by Vergroesen and De Boer (188) that prostaglandins were antagonizing the depressive actions of potassium-ions rather than facilitating calcium uptake. Prostaglandin-mediated increase in contractile function is coupled to increased glucose and fatty acid oxidation (189) and increased phosphorylase activity has been reported (187). Furthermore, prostaglandin E_1 proved to inhibit myocardial lipolysis at high doses while increased lipolysis occurred at low doses (190, 191).

The modulatory action of prostaglandins (especially the E, A and F series) upon adrenergic neurotransmission and/or response to exogenous catecholamines has recently been reviewed by Westfall (122). In numerous investigations in various species

and preparations, prostaglandins have been reported to inhibit, to stimulate or to possess no influence upon the overflow of catecholamines and/or effector cell response to nerve stimulation. Our studies proved that, in isolated, perfused norepinephrine-loaded rat hearts, high concentrations of prostaglandin E_1 induced a sudden release of catecholamines (+ metabolites) in the coronary effluent (appendix paper 2). This observation can be explained by the proposed calcium-ionophoric properties of prostaglandins (104,105,124,187, see also chapter IV, paragraph 4.4.b).
Prostaglandin E_1 and I_2 possess membrane stabilizing properties (lysosomes, red blood cells) and inhibit platelet aggregation (192-194). Together with the already mentioned vasodilatory properties of prostaglandins, these effects may be involved in the preservation of ischemic tissue as judged from reduced myocardial enzyme release (195), rhythm disturbances (196) while it has been proposed by Riemersma *et al.* (197) that prostaglandin-mediated inhibition of lipolysis and concomittantly reduced myocardial free fatty acid extraction and oxygen consumption, are involved in the beneficial role of prostaglandins during ischemia.

c. Fatty acids

Endogenous triglycerides form a major source of ATP *in vivo* as well as in isolated, perfused hearts during normoxia. Even with exogenous glucose present the perfused heart still metabolizes fatty acids (derived from endogenous triglycerides) to a large extent (49,148). Some aspects of fatty acid metabolism in the myocardium are presented in fig. 7. Lipoprotein lipase localized in the endothelial cell membrane, is involved in the removal (hydrolysis) of exogenous triglycerides from lipoproteins (chylomicrons and very low density lipoproteins). Subsequently, fatty acids penetrate the cell, are bound to a fatty acid binding protein (Z-protein) or as observed by Gloster and Harris (199) to myoglobin. They are activated to fatty acyl-CoA esters

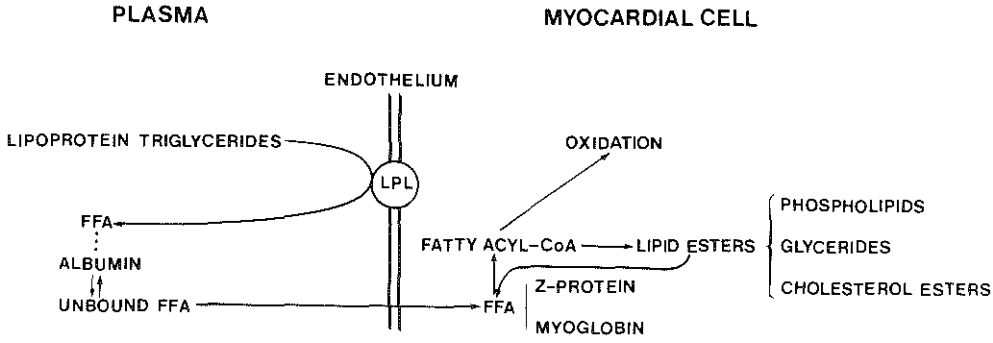

Fig. 7. Utilization of free fatty acids in the myocardium. FFA = free fatty acids, LPL = lipoprotein lipase, fatty acyl-CoA = fatty acyl coenzyme A.

(200), then converted to glycerol esters or carnitine esters. The latter may penetrate the mitochondrial inner membrane for oxidation of the fatty acyl moiety during β-oxidation. It has been proposed by Zierler (201) that plasma (or perfusate) free fatty acids are esterified to triglycerides prior to hydrolysis and oxidation. Hydrolysis of endogenous triglycerides may be of lysosomal origin and is hormone-sensitive (appendix paper 3) since the lysosomal-"inhibitor" chloroquine totally inhibited basal and hormone-stimulated lipolytic activity as determined by glycerol release from the heart. It was observed that chloroquine-inhibition of lipolysis was associated with a depressed contractile state of the isolated heart (see fig. 8) which could be restored by the addition of octanoate or excess calcium to the perfusion fluid.

On comparison of hearts from control fed and rapeseed-oil fed rats, we observed a relation between endogenous lipolytic activity and chloroquine-induced inhibition of glycerol release associated with depression of contractile function (appendix paper 4). It is therefore concluded that endogenous fatty acids may be involved in the maintenance of the contractile status in heart. Since the intracellular availability of calcium-ions is the main determinant of contractility under normal conditions,

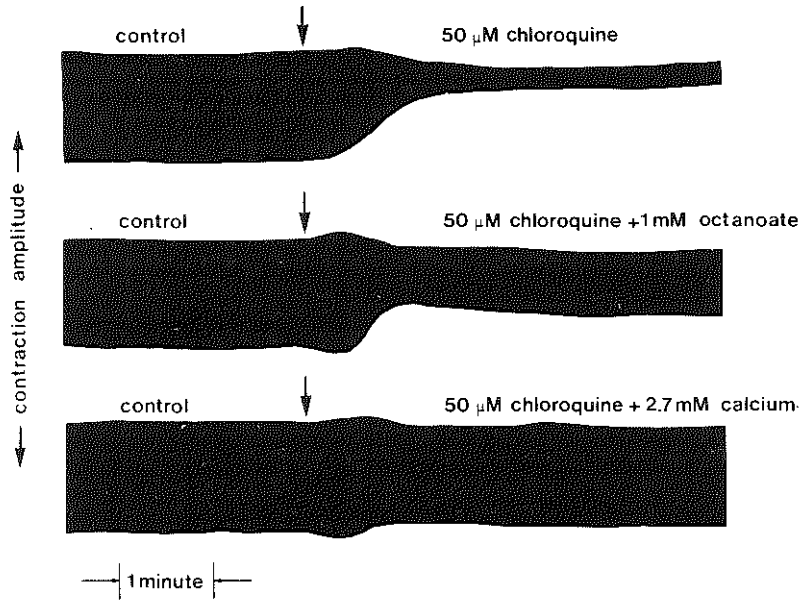

Fig. 8. The effect of octanoate and excess calcium upon chloroquine-depressed contractility in isolated rat heart. Rat hearts were perfused retrogradely with a modified Tyrode buffer at a pressure of 10 kPa, at a rate of 300 beats/min. Contractility was recorded as apex displacement. After control perfusion ($[Ca^{2+}]$ = 1.35 mM) additions were made as indicated.

we propose that fatty acids, by serving as calcium-vehicles, increase the intracellular availability of calcium for the contractile machinery. Indeed, under conditions when intracellular "free" fatty acid levels are reduced by chloroquine, administration of octanoate, palmitate, prostaglandin E_1 or extra calcium restored contractile function. Also, under conditions of enhanced intracellular cyclic AMP (norepinephrine, glucagon, prostaglandin E_1) and thereby increased availability of calcium, chloroquine-depression of contractility partially disappears. Furthermore, as demonstrated in fig. 9, the addition of the antibiotic Ca^{2+},Mg^{2+}-ionophore X-537A during chloroquine

perfusion completely restored contractility. This action is not solely mediated by the X-537A-induced release of endogenous catecholamines (202) since in catecholamine-depleted hearts also a reestablishment of contractile behaviour is observed. The calcium-vehicle or ionophoric role of fatty acids explains the positive inotropic effect of fatty acids upon contractility of isolated hearts, perfused at low external calcium levels as first observed by Hülsmann (104). Furthermore, the role of fatty acids in the determination of contractility has been observed by De Boer *et al.* (203) who reported a stimulatory effect of fatty acids upon contractions of frog hearts made hypodynamic by monoiodoacetic acid or high potassium levels in the perfusion fluid.

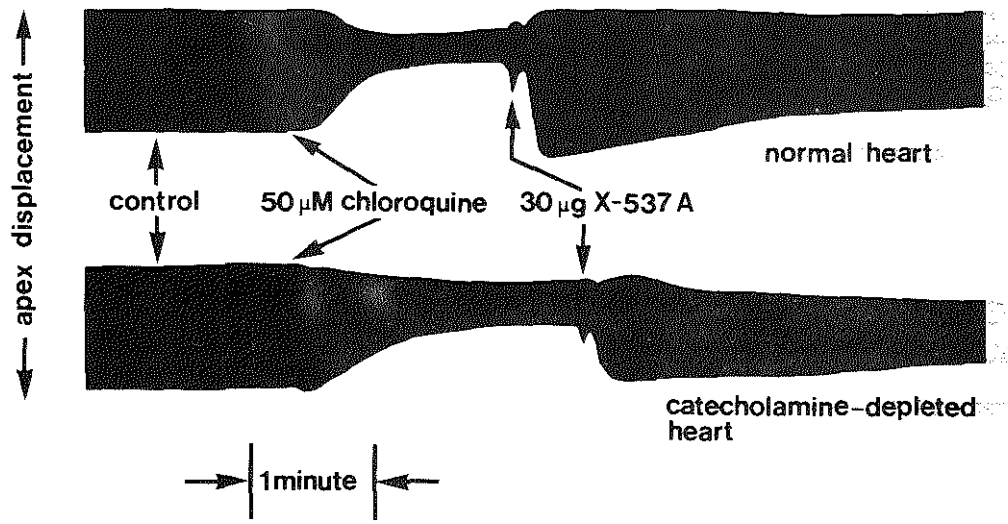

Fig. 9. The effect of ionophore X-537A upon chloroquine-depressed contractility in control and catecholamine-depleted rat hearts. See legend of fig. 8. X-537A was dissolved in 50% (^{w}/v) ethanol and injected in the aortic canula. Catecholamine-depletion was achieved by preperfusion (15 min) with tyramine (6.5 µg/min) followed by a 15 min wash-out perfusion.

In contrast to "physiological" levels of fatty acids which as we propose are necessary for the maintenance of contractile function, high intracellular levels of fatty acids (and their CoA and carnitine ester derivatives) are harmful for the myocardium. The intracellular concentration of fatty acids (+ esters) is determined by the uptake (which is mainly dependent on their molar ratio with serum or perfusate albumin; it is stimulated by catecholamines, see ref. 204) by the rate of mitochondrial β-oxidation and by the balance between esterification and (hormone-sensitive) lipolysis. Under oxygen-limited conditions (anoxia, hypoxia and ischemia) indeed elevated endogenous free fatty acid levels (and their CoA and carnitine esters) is observed (205). The deleterious effects of high free fatty acid levels are mediated by their membrane-detergent properties, by their calcium-ionophoric properties and their inhibiting capacity of various enzymes involved in energy metabolism (see table II). The actions of a high molar fatty acid: albumin ratio in the perfusion buffer upon metabolism and function in normal and ischemic rat hearts are discussed in appendix papers 2 and 6. It was noticed that both depletion of endogenous catecholamines and the addition of 11 mM glucose during fatty acid perfusion partially restored the depressive actions upon contractility and energy metabolism (as determined by release of AMP-catabolites from the heart). The protective action of glucose can be explained by its ability to supply glycerol-3-phosphate for triglyceride formation and/or by an increased glycolytic ATP synthesis. These protective actions may form at least part of the basis of the glucose-insulin-potassium therapy, advocated by Sodi-Pallares *et al.* (218) and Opie (219) in the treatment of ischemia and other forms of coronary heart disease. Besides glucose, anti-lipophilic drugs (220,221) have been proposed to protect the myocardium from the free fatty acid "toxicity", while also carnitine receives much attention in this field since it is hypothesized by Shug *et al.* (181) to reduce the adenine nucleotide translocator inhibition by long chain fatty acids.

Table II

EFFECTS OF (HIGH INTRACELLULAR LEVELS OF) FREE FATTY ACIDS (+ CoA ESTERS) UPON MYOCARDIAL FUNCTION AND METABOLISM

	Action	Effect	Reference(s)
I	Ionophoric	- Enhanced contractility at low external calcium levels	104
		- Release of endogenous catecholamines	appendix paper 2
		- Stimulation of contractility during inhibition of lipolysis	" " 4
		- Altered mitochondrial proton and cation permeability → uncoupling of oxidative phosphorylation	108,143,206
II	Membrane detergent	- Destabilization of lysosomal membranes → loss of degradative enzymes	207,208
		- Swelling and labilization of mitochondria, directly or via lipolysis	206,209
		- Alteration of plasmamembrane permeability for macromolecules → enzyme release	210
III	Enzymes	- Inhibition of Na^+,K^+-ATPase	109
		- Inhibition of the adenine nucleotide translocator	206,211, appendix paper 6
		- Inhibition of key enzymes of glycolysis and citric acid cycle	212-214, appendix paper 6
		- Inhibition of di- and tricarboxylate carrier systems	215-217

5.5. Cholinergic and adrenergic modulation of contractility

Atria and junctional tissue are richly innervated by parasympathetic (cholinergic) and sympathetic (adrenergic) fibres. The latter predominate in ventricles (222). The myocardial response on stimulation of parasympathetic and sympathetic fibres is mediated

by acetylcholine and (nor)epinephrine respectively and can be mimicked by exogenous perfusion with these transmitters. The suppression of cardiac contractility by acetylcholine was associated with the rise in ventricular muscle cyclic GMP and with decreased cyclic AMP levels (111,158) and could be imitated by the exogenous addition of cyclic GMP-derivatives (223) while dibutyryl-cyclic GMP could reverse both the positive ionotropic and glycogen phosphorylase activating actions of isoproterenol (224).

Adrenergic stimulation of cardiac contractility is associated with increased myocardial cyclic AMP levels (153) and concomittant decrease in cyclic GMP content (225). Together with the observed phasic changes of both cyclic AMP and cyclic GMP during the contraction-relaxation cycle (146,147) it has led to the "Yin Yang" hypothesis for both cyclic nucleotides in heart and other organs (226). However, the correlation between inotropic actions of acetylcholine (+ derivatives) and catecholamines and myocardial tissue levels of cyclic GMP and cyclic AMP, respectively, has been disputed (227,228). Without neglecting the importance of cholinergic actions we will not further discuss acetylcholine-mediated affection of myocardial metabolism and function.

As hypothesized by Robinson *et al.* (65) catecholamine actions via α- and β-adrenergic receptors can modify the activity of adenylate cyclase and result in changes in the rate of cyclic AMP formation. Indeed, in addition to the well established positive inotropic effects evoked by β-adrenoceptor stimulation, inotropic effects (although contradictory) have been reported for myocardial α-adrenoceptor stimulation (229, 230). Since, however, β-receptor density is larger than α-receptor density in the myocardium, β-adrenergic effects are predominant (229).

Both cardiac smooth muscle and striated muscle cells possess specific catecholamine-binding sites which are mainly located in plasmamembranes. They show (i) reversible catecholamine-binding, (ii) high affinity and saturation kinetics and (iii) stereo specificity and thus fulfil the criteria for true receptors (for a review see ref. 231). β-Receptor stimulation

and adenylate cyclase activity seem to be related although Hu and Venter (232) did not observe changes in cardiac cellular cyclic AMP levels upon stimulation of the β-receptors with isoproterenol which was bound to polymers whereas stimulation of cat papillary muscle contractility continued. The catalytic unit of adenylate cyclase may be coupled not only to β-receptors but also to receptors for glucagon and histamine. In other words, probably two or more different receptors are coupled to the same adenyl cyclase (233,234).

The receptor-adenylate cyclase interaction starts after release of catecholamines from endogenous stores (adrenergic vesicles) in the postsynaptic space by exocytosis of the storage vesicles; a process probably mediated by increased calcium influx (124), while acetylcholine, nicotinic acid, ionophore X-537A, cyclic nucleotides, phosphodiesterase inhibitors and inhibition of oxidative phosphorylation, which causes loss of Ca^{++} from mitochondria, also lead to increased catecholamine liberation (124,140,202). As discussed in appendix paper 2 and chapter IV, paragraphs 4b, 4c and 4d, fatty acids and prostaglandin E_1 may also promote catecholamine release from nerve-ending adrenergic vesicles. Receptor-adenylate cyclase interactions at the adrenergic neuroeffector junction cease after catecholamine inactivation which occurs by at least three different mechanisms:

(i) enzymatic destruction by catechol-O-methyl transferase (COMT) located in the post-synaptic membranes,

(ii) overflow in the circulation and

(iii) reuptake by the nerve-terminal.

Reuptake of secreted catecholamines is an active transport, and believed to be the chief mechanism of neurotransmitter inactivation (235). After reuptake, catecholamines are restored in vesicles or metabolized by mitochondrial monoamine oxidase (MAO).

The inotropic and metabolic response upon β-adrenoceptor stimulation are mediated by cyclic AMP-dependent protein kinases (see paragraph 5.2). Increased contractility is coupled to increased metabolic rates. Catecholamines induce glycogenolysis and stimulate endogenous lipolysis (see appendix papers

3 and 6). However, the elevation of cyclic AMP in the myocardial muscle cell may not be necessarily sufficient for complete control of physiological and metabolic responses to catecholamines. Besides cyclic AMP, allosteric effectors and inhibitors may influence the overall response to catecholamines, while calcium-ions are also involved in the determination of the rate of glycogenolysis and lipolysis (236) both in the absence or presence of cyclic AMP (237). It is possible that fatty acids by serving as calcium-vehicles may be involved in these actions since Ca^{2+}-stimulation of protein kinase is well established (238), as well as Ca^{2+}-stimulation of various lipolytic enzymes. The close relation between enhanced contractility and metabolism is possibly accounted for by phosphorylase kinase, a calcium-dependent kinase involved in glycogenolysis and in phosphorylation of the inhibitory subunit of troponin (TN-I, see ref. 239).

Since the observations of Moore and Ruska (240) and Parker (241) concerning budding, vacuolization and expansion of vascular smooth muscle cell membranes which are situated in the vicinity of adrenergic nerve terminals, many reports appeared about catecholamine-induced damage of myocardial plasmamembranes, resulting in increased permeability for macromolecules (horse-radish peroxidase, see ref. 242), enzyme release (243-245) and platelet aggregation (246). The detrimental effects of circulating and/or released endogenous catecholamines may be responsible for the induction of myocardial necrosis as reported by Rona *et al.* (247). Explanations for these catecholamine effects are still hard to give, but some interesting observations have been done:

(i) catecholamines may alter membrane fluidity (248) and hereby the activity of membrane-bound enzymes (144,249)

(ii) catecholamines may affect membrane phospholipid metabolism (250) and

(iii) oxidation products of catecholamines interfere with "calcium pump"-systems of the microsomal membranes and may lead to calcium overload and cellular disfunction (251)

(iv) catecholamine, by stimulating endogenous lipolysis, may increase the detergent (soaps and activated fatty acids) concentration.

Moreover, studies of Waldenström *et al.* (252) indicated that massive release of endogenous catecholamines induced by perfusion with tyramine, caused a many-fold increase of the loss of creatine kinase from isolated perfused rat hearts. Hence, catecholamine stores in isolated, denervated hearts are large enough (a value of $\pm 10^{-6}$ M in heart homogenates can be estimated, see ref. 253) to affect membranous and metabolic processes when they are released. Catecholamine-induced alteration of membrane structure and function may be amplified by the observation that the fatty acid products of lipolysis will in its turn again stimulate catecholamine release (appendix paper 2). Catecholamines (and other hormones, such as glucagon) may, however, not only modify sarcolemma but also intracellular membranes via phosphorylation by the cyclic AMP-protein kinase system and thereby affect enzyme activities associated with these membranes. Some actions of the cyclic AMP-protein kinase system upon microsomal and lysosomal membranes are discussed in appendix paper 3.

5.6. Dietary fat and contractile status

Since cardiac membranes are highly involved in fundamental cellular processes like contractility, osmotic regulation and various energy producing metabolic pathways, the fatty acid composition of cardiac phospholipids may be very important in the determination of myocardial function. Membrane-phospholipid fatty acid composition is influenced by nutritional (dietary), hormonal, pharmacological and environmental means and since dietary fat and serum lipids are considered as risk factors in cardiovascular diseases in man, attention has been paid to the relation between dietary fat and cardiac contractility.

In studies of Gudbjarnason and Hallgrimson (254) it

was shown that increasing amounts of various polyunsaturated fatty acids in cardiac lipids were able to protect development of myocardial necrosis in rats following overstimulation with isoproterenol while, on the other hand, rapeseed-oil feeding is associated with cardiopathogenic lesions (255). Erucic acid-rich diets induce gross fat accumulation in the myocardium (128, 255) after 3-6 days of feeding, which may be caused by (i) increased uptake of erucic acid (256, see however ref. 257), (ii) erucyl carnitine inhibition of palmitate oxidation (129,258) and (iii) decreased β-oxidation of erucate (259). Intracellular levels of free fatty acids are increased (appendix paper 3), while also changes in overall myocardial but, in particular, mitochondrial phospholipid composition and cholesterol content have been observed (260-262). As a result of these changes in membrane phospholipids mitochondrial function may be impaired (259,263,264) and the activity of adenylate cyclase reduced (265). In table III the effects of a four day diet of rats with 40 cal% rapeseed oil upon the energy charge of their isolated, perfused hearts is presented. The results are compared with values from perfused hearts of rats fed with 40 cal% sunflower-seed oil and hearts from rats fed control laboratory pellets. The reduced energy charge in hearts from rapeseed oil fed rats may be a consequence of moderate uncoupling by intracellular fatty acids of mitochondrial oxidative phosphorylation (209,215, 266) or inhibition of the adenine nucleotide translocator (211). The inhibition of mitochondrial function may be responsible for the decrease in contractility of hearts from rapeseed oil (267) and beef lard fed rats (125). Another contribution may be the hormonal imbalance of animals fed diets rich in (very) long chain fatty acids, which could lead to myocardial lesions. Enlargement of thyreoid and adrenal glands have been observed (268,269) while Hülsmann (270) recently demonstrated enhanced levels of corticosterone and testosterone and concluded that long chain fatty acid rich diets provoke abnormal stress reactions.
Decreased contractility, probably associated with impaired mitochondrial function has also been observed in hearts from essential fatty acid-deficient rats (271,272).

Table III

EFFECT OF DIETARY FAT UPON ENERGY CHARGE OF RAT MYOCARDIUM

Three groups of Wistar rats (180-220 g) were fed control pellets (a), 40 cal% rapeseed oil (b) or 40 cal% sunflowerseed oil (c). After four days the animals were anesthetised and the hearts excised and perfused retrogradely, with a modified Tyrode buffer at a pressure of 10 kPa and at a rate of 300 beats/min. After 30 min perfusion the hearts were clamped with tongues precooled in liquid nitrogen. In the tissue extracts adenine nucleotides were determined spectrofotometrically according to standard analytical procedures. The energetic state of the tissue is expressed as energy charge $\{([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])\}$. Results are given in means $\pm$ S.E.M. of n experiments and statistical analysis was performed with the unpaired t-test (two-tailed). $P>0.05$ was considered to be not significant (NS).

Diet	n	Energy charge		
Control (pellets)	8	0.870 ± 0.008	$P<0.025$	-
Rapeseed oil	3	0.829 ± 0.007	NS	NS
Sunflowerseed oil	3	0.850 ± 0.012	NS	-

5.6. Discussion

The importance of the availability of calcium ions in the detemination of contractile function of the heart in relation to energy metabolism has been reviewed. From the presented experiments it has been proved that endogenous free fatty acids play an important role in the maintenance of contractile function by serving as calcium-vehicles, promoting transport of calcium from the sites of release to the sites of contraction. Modulation of the contractile status of the myocardium can take place via alterations in calcium-influx through cellular membranes either directly (prostaglandins, ionophores), via the intracellular cyclic nucleotide-protein kinase system (acetylcholine, catecholamines, prostaglandins) or via interventions in

energy metabolism (anoxia, hypoxia, ischemia and dietary fats). Intracellular free fatty acids (and their CoA and/or carnitine esters) may be involved in all three mentioned mechanisms;

(i) by acting as calcium-ionophores and by increasing calcium transport through the sarcolemma,

(ii) by releasing endogenously stored catecholamines and

(iii) by affecting mitochondrial oxidative phosphorylation (uncoupling and inhibition of the adenine translocator).

The importance of free fatty acids in cell function implies their strict metabolic control. It is generally believed now (201) that plasma free fatty acids, taken up from the circulation, are esterified, probably by sarcoplasmic reticulum, transported as triglycerides and stored in "lipid droplets". Similar "droplets" have been isolated from beef heart, while electronmicroscopy revealed that these particles were surrounded by a unit membrane (273). This also holds for the lipid containing droplets from hearts of rats fed rapeseed oil, which when stained for acid phosphatase were found to contain reaction product in their periphery (W.C. Hülsmann, unpublished). Therefore it is likely that the lipid particles are lipid-filled lysosomes or autophagic vacuoles. Indeed, both basal and norepinephrine-stimulated lipolysis are inhibited when lysosomal-"activity" was eliminated by the lysosomal inhibitor chloroquine. Acid lipase can therefore be considered as the enzyme responsible for intracellular triglyceride hydrolysis and be involved in the supply of fatty acids for energy metabolism and in intracellular calcium-transport.

CHAPTER VI

FINAL NOTES AND SPECULATIONS

* Isolated rat hearts, perfused retrogradely under controlled conditions are useful objects in studies of myocardial metabolism, and of the regulation and modification of coronary circulation, while an estimation of contractile function from isotonic contractions of the heart can be made by registration of the apex displacement in the vertical direction.

The question arises now whether control of coronary flow rates and contractile behaviour in isolated, hemoglobin-free perfused hearts are comparable with the *in vivo* regulation?

Myocardial capillary density is rather uniform and capillary perfusion is maintained by the diameter of resistance vessels and the tonus of precapillary sphincters. When we compare various systems which control the contractile state of arterial (and arteriolar) and sphincter smooth muscle cells, it appears that local metabolic influences overrule the neurohormonal control mechanisms. Metabolic control appears to operate in isolated hearts although autoregulation during pressure-flow studies cannot be observed. In non-working *Langendorff* hearts mechanical expansion of coronary vessels probably overrules any other mechanism while metabolic demands of the hearts are low and also saturated at low perfusion pressure. The intactness of intrinsic control (hypoxia, anoxia), however, makes it more reliable to extrapolate findings concerning flow regulation in isolated hearts to the *in vivo* heart.

Coronary circulation appears to be not homogenous. A close examination of studies from Steenbergen *et al.* (274) with NADH surface fluorescence of hemoglobin-free, perfused, working rat hearts, reveals that even under normoxic conditions (pO_2>600 mm Hg) a heterogenous tissue perfusion pattern exists which aggravates during hypoxia and ischemia. Thus, not all capillaries are perfused under control conditions. This may explain the apparent latency of myocardial lipoprotein lipase

release from endothelial plasmamembranes upon perfusion with heparin. In fact, under conditions when it is likely to have a more homogenous perfusion of myocardial tissue (high perfusion pressure and heart rate, and norepinephrine in the perfusion fluid), heparin-perfusion could provoke almost complete release of lipoprotein lipase from the isolated heart (appendix paper 3).

In contrast to apparent similarity in control of coronary circulation in isolated, perfused hearts and the *in vivo* beating heart, the regulation and modification of contractility in the *in vivo* myocardium is much more complex. Especially, the absence of direct neurohumoral regulation in isolated hearts (although endogenous neurotransmitter substances may still be of interest) makes it hard to extrapolate findings in the isolated, non-working heart to the *in vivo* cardiovascular system with its mutual interrelationships, is absent in isolated hearts. However, the function of isolated as well as *in vivo* hearts is directly dependent on proper energy metabolism; therefore findings, in this field, in isolated hearts may also be important in the *in vivo* heart.

** Coronary vascular and cardiac striated muscle cells are the functional "units" in circulatory and contractile behaviour. A review of both types of cells leads to the impression that they possess opposite properties. For instance:

(i) fatty acids relax vascular smooth muscle and increase contractile status of striated muscle at low calcium levels (104),

(ii) agents which increase intracellular cyclic AMP levels (catecholamines, prostaglandins) induce increased contraction of cardiac striated muscle cells while they relax vascular smooth muscle and

(iii) adenosine induces substantial coronary vasodilation, while no or little effect is observed upon contractile behaviour of the striated muscle cell.

Since the contractile state of both types of muscle cells is

mediated by the intracellular calcium-ion concentration, the regulation of calcium-homeostasis may be different in both types of cells. Apparently, like in the first example, fatty acids induce a one-way shift of calcium from the smooth muscle to the cardiac striated muscle and this process fits well in the proposed calcium-vehicle function of fatty acids in contracting myocardial muscle cells. Fatty acid-induced liberation of endogenous catecholamines and subsequent elevation of cyclic AMP is not involved in the fatty acid-mediated coronary vasodilation since the same observation was done in catecholamine depleted hearts (appendix paper 2).
Cyclic AMP is involved in microsomal calcium-binding in both types of cells. However, nothing is known about a cyclic AMP-stimulated calcium-influx in smooth muscle as is generally accepted for striated muscle. A suitable hypothesis may be that in smooth muscle only relaxing (calcium-binding) actions of cyclic AMP are present while in striated muscle both contracting (increased calcium-influx) and relaxing actions of cyclic AMP are involved. This assumption explains the relaxing action of cyclic AMP in skinned cardiac muscle cells (275).

*** The role of endogenous catecholamines in the function of hearts, must not be neglected. During perfusion experiments with electrically paced hearts, there will be a continuous overflow of catecholamines which is, at least, involved in the maintenance of coronary flow. Their role in membrane damage, enzyme release and metabolic "disorders", especially under stressful conditions (diabetes, starvation, long chain fatty acid-rich feeding) warrant further investigations.

**** Increased intracellular levels of free fatty acids (their CoA and/or carnitine esters) may be involved in the onset of arrhythmias via inhibition of Na^+,K^+-ATPase as shown by Lamers and Hülsmann (109). Since chloroquine both inhibits basal and catecholamine-increased lipolysis, and reveals anti-arrhythmic properties, we hypothesize that inhibition of endo-

genous (overstimulated) lipolysis is the mechanism of action of certain anti-arrhythmic drugs. Indeed lidocaine, a well-established anti-arrhythmic drug, proved to inhibit basal and norepinephrine-stimulated glycerol release from the perfused rat heart (unpublished observations).

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SAMENVATTING

Dit proefschrift geeft een literatuuroverzicht, en beschrijft experimentele studies met betrekking tot de regulatie en modificatie van de coronaire doorstroming en contractiliteit van geïsoleerde ratteharten.
Hoofdstuk I geeft een inleiding omtrent het probleem van de vetzuur-toxiciteit en hartfunktie. De doorstromingssnelheid van de coronair vaten en de pomp-funktie van het hart worden hoofdzakelijk bepaald door de contractie-toestand van, respectievelijk, de gladde spiercellen in de vaatwand en de dwarsgestreepte cellen van de hartspier. Hoofdstukken II en III beschrijven dan ook de morfologische en (ultra)structurele aspecten van beide celtypen. In hoofdstukken IV en V worden funktionele en stofwisselingsaspecten van de coronaire circulatie en de contractiliteit toegelicht. In zowel de gladde spiercellen van de vaatwand als de dwarsgestreepte spiercellen van het hart:

(i) wordt de contractie-toestand hoofdzakelijk bepaald door de intracellulaire calcium-concentratie,

(ii) vindt de contractie plaats na calcium-influx door het plasmamembraan gedurende de actie-potentiaal, en door het vrijkomen van intracellulair opgeslagen calcium (in het sarcoplasmatisch reticulum en mitochondriën),

(iii) treedt relaxatie op nadat specifieke calcium-pomp systemen in het plasmamembraan, het sarcoplasmatisch reticulum en de mitochondriën de cytoplasmatische calcium-concentratie hebben verlaagd en,

(iv) vormen calcium-ionen de schakel tussen de contractie-relaxatie cyclus en de energie-stofwisseling aangezien zowel glycogenolyse (glycogeen-afbraak) en lipolyse (vet-afbraak) gestimuleerd worden door calcium

De contractie en relaxatie van de dwarsgestreepte hartspiercellen zijn energie-afhankelijke processen. Een verslechtering van de contractie-kracht treedt dan ook op wanneer de productie van ATP is geremd (anoxie, hypoxie en ischemie).

Farmacologische, neurohumorale en metabole regulatie van de intracellulaire calcium-"spiegel" in gladde en dwarsgestreepte spiercellen kan plaats vinden op twee manieren:

(i) direkt, door een wijziging in de calcium-influx door het plasmamembraan hetgeen verhoogde of verlaagde calcium niveaus in het cytoplasma tot gevolg heeft en

(ii) indirekt, door veranderingen in de cyclische nucleotiden (cyclisch AMP en cyclisch GMP) afhankelijke binding en transport van calcium door het sarcoplasmatisch reticulum en het sarcolemma. Dit proces wordt gereguleerd door cyclische nucleotiden-afhankelijke eiwit kinasen door middel van de fosforylering van specifieke plaatsen op het membraan.

Er zijn perfusie-experimenten uitgevoerd met harten die verhoogde cytoplasmatische vrije vetzuur (VVZ) spiegels bevatten.
Deze harten zijn verkregen uit gevaste, streptozotocine-diabetische en raapolie gevoede ratten. Tevens zijn harten uit normale ratten doorstroomd met vetzuur-bevattende buffers.
Onze waarnemingen met betrekking tot de rol van intracellulaire VVZ in diverse membraan- en contractiele processen zijn weergegeven in fig. 10, waarin de secundaire werking van catecholamines en prostaglandines is weggelaten
Vetzuren zijn een belangrijk substraat voor de energie-stofwisseling van het hart. Na opname door het sarcolemma worden ze waarschijnlijk opgeslagen als triglyceriden in met lipide-gevulde lysosomen of autofagosomen. Door de activiteit van een membraan-gebonden (zure) lipase vindt de hydrolyse van triglyceriden plaats (appendix artikel 3). Remming van deze endogene lipolytische activiteit door de lysosomale "remmer" chloroquine gaat gepaard met een achteruitgang van de contractiekracht van geïsoleerde ratteharten die kan worden opgeheven door de toevoeging van vetzuren, door een verhoging van de calcium-concentratie in de doorstromingsvloeistof of door een verhoging van de cytoplasmatische cyclisch AMP concentratie (door toediening van catecholamines en glucagon). In harten van raapolie gevoede ratten waren zowel de chloroquine-remming van de lipolyse als die van de contractiliteit lager dan in harten van

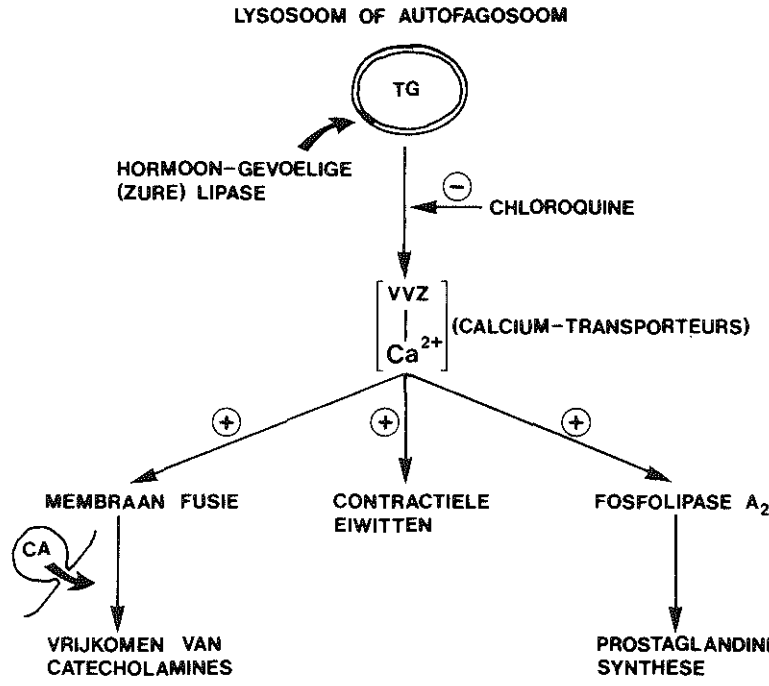

Fig. 10. De rol van vrije vetzuren als calcium-transporteurs in membraan- en contractiele processen van het hart. TG = triglyceride, VVZ = vrije vetzuren, CA = catecholamines. ⊕ = stimulerende werking, ⊖ = remmende werking.

normaal gevoede ratten. Deze proeven duiden erop dat "fysiologische" hoeveelheden VVZ onontbeerlijk zijn voor de instandhouding van de contractiliteit. Vermoedelijk zijn vetzuren werkzaam als calcium-transporteurs in het cytoplasma en bevorderen hierdoor het transport van calcium-ionen van die plaatsen in de cel waar ze vrijkomen naar de contractiele eiwitten (appendix artikel 4). Verder is gebleken dat de onder invloed van vetzuren toegenomen calcium-flux door membranen de fusie (exocytose) bevordert tussen catecholamines bevattende synaptische blaasjes en de membraan van het zenuwceluiteinde, waardoor het vrijkomen van catecholamines wordt gestimuleerd (appendix artikel 2). Vetzuren verhogen mogelijk

ook de activiteit van het calcium-afhankelijke fosfolipase A_2 hetgeen aanleiding is tot de vorming van prostaglandines uit membraan-fosfolipiden.
Een verhoogde catecholamine-"aktiviteit" en het vrijkomen van meer prostaglandines, gepaard gaande met een toename in de coronaire doorstroming, werd waargenomen in harten van gevaste en streptozotocine-diabetische ratten (appendix artikel 1). Endogene catecholamines die vrijkomen tijdens de perfusie bleken betrokken te zijn bij de instandhouding van de coronaire doorstroming onder normoxische omstandigheden (appendix artikelen 1 en 2). Deze vaat-verwijdende werking van (exogene en endogene) catecholamines, maar ook die van prostaglandine-achtige verbindingen wordt waarschijnlijk veroorzaakt door een toename van het cyclisch AMP niveau in het cytoplasma van de gladde spiercellen van de coronair vaten. In tegenstelling echter tot deze gladde spiercellen, die relaxeren bij verhoogde cytoplasmatisch cyclisch AMP spiegels, neemt de contractiliteit van de dwarsgestreepte hartspiercellen toe na de toediening van norepinephrine, glucagon en prostaglandine E_1 (appendix artikel 4). Met hun calcium-ionofore eigenschappen zijn vetzuren en sommige prostaglandines betrokken in de coronaire vaatverwijding omdat ze de beschikbare hoeveelheid calcium in de gladde spiercellen verlagen. De toegenomen coronaire doorstromingssnelheid in harten van raapolie gevoede ratten staat daarom mogelijk in verband met de waargenomen verhoging van de basale (en hormoon-gevoelige) lipolytische activiteit. De te verwachten toename in vetzuurproductie en uitscheiding door de vervette dwarsgestreepte hartspiercellen is vermoedelijk verantwoordelijk voor de relaxatie van de gladde vaatmusculatuur door dezelfde calcium-verwijderende werking.

Dit proefschrift beschrijft experimenten die zijn uitgevoerd om de effecten van vetzuur-perfusies onder normoxische en ischemische condities te bestuderen. De verlaging van de coronaire doorstroming door embolisatie van de coronair arteriën (met behulp van zeer kleine polysaccharide Sephadex partikels) werd gebruikt en gekarakteriseerd als een experimenteel model voor myocardiale ischemie (appendix artikel 5). De afname in creatinefosfaat en ATP, en intracellulaire acidose, die optreden tijdens

perioden van anoxie, hypoxie en ischemie zijn verantwoordelijk voor de verlaagde activiteit van de energie-afhankelijke calcium-pomp systemen (Ca^{2+}-ATPases) van het sarcolemma, het sarcoplasmatisch reticulum en van de mitochondriën, hetgeen mogelijk een verstard ("stenen") hart tot gevolg heeft. De productie en uitscheiding van ATP-catabolieten (adenosine, inosine en hypoxanthine) die plaats vindt gedurende zuurstof (en/of substraat)-arme omstandigheden kan mogelijk gebruikt worden als een aanwijzing voor hart- en vaatziekten bij de mens.

De doorstroming van ratteharten met een vetzuur-bevattende buffer (in een relatief hoge molaire vetzuur:albumine ratio, hetgeen met name bepalend is voor de vetzuur opname door het hart) leidt tot een verlaging van de contractiliteit. De schadelijke effecten van verhoogde cytoplasmatische spiegels van vetzuren en hun CoA en carnitine esters, zowel onder normoxische als ischemische condities, zijn beschreven op basis van hun chaotrope rol als membraandetergentia (zie fig. 10 en appendix artikelen 2 en 6). Ook catecholamines, onder invloed van vetzuren vrijgekomen uit de zenuwuiteinden spelen mogelijk een rol in de achteruitgang van de hartfunctie. In dit kader zijn observaties omtrent de nadelige werking van catecholamines op cellulaire membranen en stofwisseling besproken. Gevonden werd dat de afname in contractiliteit onder invloed van vetzuren inderdaad kon worden "voorkomen" wanneer de endogene catecholamines werden gedepleteerd. Ook de toevoeging van glucose, dat door te voorzien in glycerol-3-fosfaat, de triglyceride synthese stimuleert en de intracellulaire VVZ (en CoA en carnitine ester) spiegels verlaagt, voorkwam (gedeeltelijk)

(i) het vrijkomen van catecholamines,
(ii) de achteruitgang van de myocardiale energie-huishouding en
(iii) de afname in contractiliteit

die optraden gedurende vetzuur-perfusie onder zuurstof-rijke en zuurstof-arme condities.

Onze resultaten duiden op een belangrijke rol van vetzuren in de functie van het hart onder normale en pathologische omstandigheden, een rol waarin hun ionofore eigenschappen van wezenlijk belang zijn.

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APPENDIX

Paper 1. Effect of fasting and streptozotocin-diabetes on the coronary flow in isolated rat hearts: A possible role of endogenous catecholamines and prostaglandins

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Effect of fasting and streptozotocin-diabetes on the coronary flow in isolated rat hearts: A possible role of endogenous catecholamines and prostaglandins*)

Der Einfluß des Fastens und von Streptozotocin-Diabetes auf die koronare Durchströmung des isolierten Rattenherzens: die Rolle von endogenen Katecholaminen und Prostaglandinen

H. Stam and *W. C. Hülsmann*

With 3 figures and 2 tables

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Summary

The coronary flow rate of retrogradely perfused hearts from fasted (group I) and streptozotocin-diabetic rats (group II) is increased when compared with the flow rate of control, fed animals (group III). The enhanced coronary flow is absent when hearts of groups I and II are perfused in the presence of indomethacin (1 μg/ml) in the perfusion fluid and the lowest flow rates are observed after depletion of the endogenous catecholamines by reserpin. Hearts from groups I and II showed a marked increase in prostaglandin-release which was counteracted both in the presence of indomethacin (1 μg/ml) and by reserpin-pretreatment. The results suggest that the increased coronary flow rates in hearts from fasted and streptozotocin-diabetic rats are mediated by an effect of released endogenous catecholamines on coronary vascular smooth muscle *and* by a catecholamine-induced release of vasodilatory, prostaglandin-like substances.

Metabolic regulation and pharmacological modification of the coronary flow in isolated, perfused heart preparations, under different experimental conditions, have well been documented (1–7). Studies of *Rubio* and *Berne* (1, 5) dealt with the vasodilatory properties of the nucleoside adenosine released during myocardial hyperactivity, hypoxia and ischemia. *Giles* et al. (2, see also 3) recently reviewed the evidence for the vasodilatory effect of catecholamines and *Hülsmann* (6) did this for the vasodilatory properties of medium- and long-chain fatty acids. In numerous reports prostaglandins have been proposed as mediators or modulators of the coronary vasodilation (8–10), especially under hypoxic and ischemic conditions (11–14). That prostaglandins not only play a role under oxygen-

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limited conditions is pointed out by *Schrör* et al. (15–17), who presented evidence that in guinea-pig hearts endogenous prostaglandin-like substances are possibly involved for the maintenance of the coronary vascular resistance since indomethacin, a potent inhibitor of prostaglandin synthesis (18), significantly increased the coronary vascular pressure under optimal perfusion conditions.

Recently *Markelonis* and *Garbus* (19) reviewed some mechanisms serving as stimuli evoking prostaglandin biosynthesis and release. Mediators of stress (catecholamines) were proposed to stimulate prostaglandin synthesis since infusion of norepinephrine in the isolated, perfused rabbit heart caused an efflux of prostaglandin E_2 (20). Similar observations were done for the dog spleen (21) and rabbit kidney (22), while *Broadley* (23) mentioned the release of a coronary vasodilator metabolite from the guinea-pig isolated heart stimulated by catecholamines, histamine and electrical pacing, possibly a prostaglandin-like substance.

The important role of endogenous catecholamines as a determinating factor of the coronary flow follows from an observation of *Krebs* and *Schrör* (24) that depletion of endogenous myocardial catecholamine stores after reserpin-pretreatment leads to a significant decrease in coronary vascular resistance in isolated, perfused, paced guinea-pig hearts. The significance of catecholamines in spontaneously beating, perfused rat hearts was pointed out by *Schaffer* et al. (25) whose data suggested a clear link between the vasodilatory effect of the calcium ionophore X-537 A and the release of norepinephrine from endogenous stores, since reserpin pretreatment und the β-adrenergic blocking agent propranolol abolished the effect of X-537 A.

The study presented here deals with the observation that the coronary flow in isolated, perfused hearts from fasted and streptozotocin-diabetic rats is markedly higher than the flow in control, fed animals. The possible roles of endogenous catecholamines and prostaglandins as responsible factors for this enhanced flow rate have been studied.

Methods and materials

Animals

Three groups of male Wistar rats (200-250 g) were used. The first group (I) of rats was fasted for 48 hours but had free access to water. The second group (II) of rats was made diabetic by an intravenous (tailvein) injection of streptozotocin (50 mg/kg body weight) (26), and the third group (III) consisted of normally fed animals (controls). The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg body weight) and subsequently heparinized intravenously with 500 I.U. Depletion of endogenous catecholamines was induced by an intraperitoneal injection of reserpin (10 mg/kg body weight) 6 hours before sacrifice (25).

Perfusion procedure

The hearts were perfused at 37 °C and pH 7.4 with a modified tyrode solution (27) equilibrated with 95 % O_2 + 5 % CO_2, according to the *Langendorff* procedure. All non-ventricular tissue was removed. Isolated hearts were paced at 300 pulses/minute after a total atrio-ventricular block was made by cutting the bundle of *His*. After a thirty minute control perfusion at a pressure

of 100 cm H_2O the perfusion pressure was varied from 40 to 80 mm Hg as measured in the coronary inflow tract, using a Statham P 23 Gb pressure transducer. The coronary flow rate was measured by collecting the fluid dripping from the heart during a fixed period of time of by drop counting. After the experiment the hearts were blotted dry and weighed.

Indomethacin was neutralized with equimolar amounts of sodium hydroxide and added to the perfusion medium in a final concentration of 1 μg/ml.

Extraction of prostaglandins from the effluents

After the control perfusion period, the coronary effluents were collected for thirty minutes under N_2 gas at 0 °C, acidified with HCl to pH 3.0 and extracted twice with ethyl acetate. The combined extracts were evaporated to dryness *in vacuo* at 50–60 °C using a rotary evaporator (Rotavap, Büchi, Germany). The dried extracts were dissolved in saline (500 μl) and stored at –20 °C. Bioassay of effluent prostaglandins took place within 48 hours after the extraction.

Bioassay of the extracted prostaglandins

Prostaglandins recovered from the extraction procedure were detected and assayed on a rat stomach strip and a rat colon, superfused at 37 °C at pH 7.4 with a Krebs buffer solution (21). The changes in length of the assay tissues were detected by a Harvard isotonic heart/smooth muscle transducer and recorded on a Rikadenki multi-pen recorder. The contractions of the tissues after addition of 100 μl of the effluent extract were compared with contractions occurring after the addition of the same volume of a known prostaglandin E_2 solution to the superfusion fluid.

Materials

Prostaglandin E_2 was a gift of Dr. *J. E. Pike* from the Upjohn Company (Kalamazoo, U.S.A.). Heparin was purchased from Organon (Oss, The Netherlands), reagents (all of analytical grade) and reserpin from Merck (Darmstadt, Germany) and streptozotocin from Calbiochem (Luzern, Switzerland).

Statistical analysis

Most results are given in mean values ± standard error of the mean. n is the number of observations. Significance was calculated with *Student's* t-test. $P > 0.05$ was considered to be not significant.

Results

During the perfusion of hearts, isolated from diabetic rats for other purposes, it was noticed that the coronary flow rate had increased compared with the flow rate in control hearts. Since at least a number of metabolic alterations that occur in diabetes may also be observed in the fasted non-diseased state, also hearts from fasted rats were tested.

The relation between the coronary flow and perfusion pressure in perfused *Langendorff* hearts from streptozotocin-diabetic, fasted and control hearts is presented in figure 1. The flow rate in hearts from diabetic and fasted rats was significantly higher than the coronary flow in control hearts. From earlier studies it appeared that endogenous catecholamines (25) and prostaglandin-like substances (15) might be involved in the maintenance of coronary flow in isolated perfused hearts. Therefore we studied the effects of indomethacin (a potent inhibitor of prostaglandin

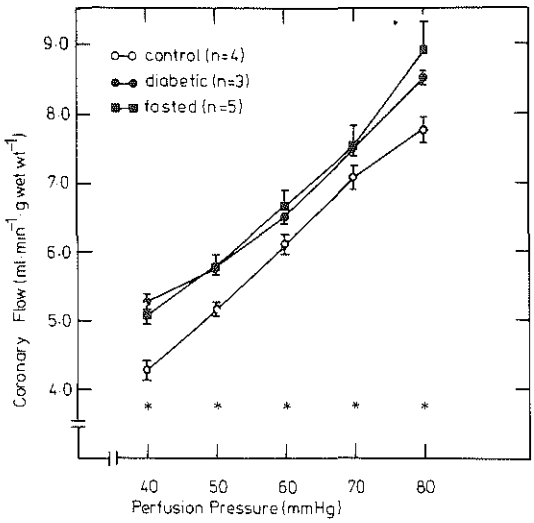

Fig. 1. Relation between the coronary flow and the perfusion pressure in isolated, perfused hearts of fasted, streptozotocin-diabetic and control rats. Each point represents the mean ± S.E. of the indicated number of experiments. * $P < 0.05$ for ○–○ versus ●–● and ■–■

synthesis) and reserpin-pretreatment (inducing depletion of endogenous catecholamine stores) on the coronary flow in hearts from all groups. The pressure-flow relations of hearts from fasted and diabetic rats during a

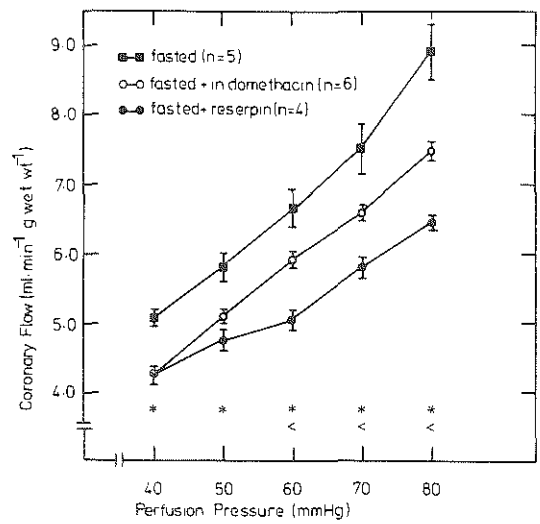

Fig. 2. The effects of indomethacin (1 μg/ml perfusate) and reserpine-pretreatment on the pressure-flow relation in isolated, perfused hearts of fasted rats. Each point represents the mean ± S.E. of the indicated number of experiments. * $P < 0.05$ for ■–■ versus ○–○ and ●–●, < $P < 0.05$ for ○–○ versus ●–●

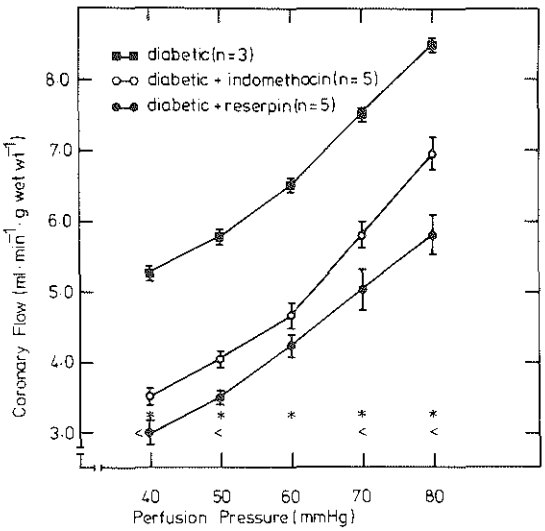

Fig. 3. The effects of indomethacin (1 μg/ml perfusate) and reserpin-pretreatment on the pressure-flow relation in isolated, perfused hearts of streptozotocin-diabetic rats. Each point represents the mean ± S.E. of the indicated number of experiments. * $P < 0.05$ for ■–■ versus ○–○ and ●–●, < $P < 0.05$ for ○–○ versus ●–●

standard perfusion in the presence of indomethacin (1 μg/ml) and after reserpin-pretreatment is presented in figures 2 and 3. In both groups, perfusion in the presence of indomethacin significantly reduced the coronary flow rates and an additional reduction appeared after pretreatment of the rats with reserpin, which, in fasted hearts, was more pronounced at higher perfusion pressures.

Table 1 demonstrates the above mentioned findings for perfusions at a pressure of 80 mm Hg, with additional figures concerning the effects of indomethacin and reserpin in hearts from control, fed rats. Control hearts showed no influence of added indomethacin on the flow rate, indicating that endogenous prostaglandins are not involved in controls hearts. On the other hand, in hearts from fasted and diabetic hearts indomethacin caused a reduction in flow. Reserpin-pretreatment induced a further decrease in coronary flow in all groups of animals.

With indomethacin flow rates of hearts from fasted rats reached the same level as in control hearts, perfused with or without indomethacin. Also, the final flow rates after pretreatment with reserpin were not significantly different in hearts from the fasted and control groups. However, in hearts from streptozotocin-diabetic rats, indomethacin and reserpin caused a fall in coronary flow which was significantly different from flows in control hearts under both conditions. The inhibitory effect of indomethacin on the enhanced flow rates in fasted and diabetic hearts indicated a possible role of prostaglandins as a responsible factor for this increase in coronary flow. Therefore we determined the release of prostaglandin-like substances from hearts of all groups. The results are

Table 1. Effect of indomethacin and reserpin-pretreatment on the coronary flow of control, fasted and streptozotocin-diabetic rat hearts perfused at a perfusion pressure of 80 mm Hg.

Experimental	Coronary flow		
	Fasted	Control	Diabetic
Standard perfusion	8.93 ± 0.40 ($n = 5$)	$P < 0.05$ — 7.75 ± 0.22 ($n = 4$)	$P < 0.05$ — 8.45 ± 0.11 ($n = 3$)
	$P < 0.005$	NS	$P < 0.005$
— indomethacin (1 µg/ml)	7.49 ± 0.10 ($n = 6$)	NS — 7.77 ± 0.11 ($n = 4$)	$P < 0.025$ — 6.92 ± 0.24 ($n = 5$)
	$P < 0.001$	$P < 0.001$	$P < 0.001$
+ reserpin pretreatment	6.44 ± 0.11 ($n = 4$)	NS — 6.66 ± 0.03 ($n = 5$)	$P < 0.05$ — 5.82 ± 0.32 ($n = 5$)

Mean values are indicated $\pm$ S.E.; n = number of experiments; coronary flow is expressed as ml · min^{-1} · g wet wt^{-1}; NS = not significant ($P > 0.05$).

presented in table 2. Release of prostaglandin-like substances was enhanced in hearts from fasted and diabetic rats, and this increase was not only counteracted when the hearts were perfused in the presence of indomethacin but also after reserpin-pretreatment. This indicates that endogenous catecholamines are involved in the regulation of prostaglandin biosynthesis.

Table 2. Effects of indomethacin and reserpin-pretreatment on the release of prostaglandin-like substances from hearts of fasted, streptozotocin-diabetic and control rats.

Experimental	Release of prostaglandin-like substances (ng · 30 min^{-1} · g wet wt^{-1}) *)
Control	3.2
Fasted	13.4
Fasted — indomethacin	3.8
Fasted + reserpin	5.8
Diabetic	14.0
Diabetic + indomethacin	3.7
Diabetic + reserpin	4.9

*) Prostaglandin-like substances expressed as prostaglandin E_2 activity.

Discussion

In recent years investigations on the mode of action of prostaglandins and catecholamines on the coronary vascular system have been subjects of many studies [see for references *Dempsey* and *Cooper* (3) and *Nakano* (28)]. Most of the studies presented, however, dealt with interactions occurring during hypoxia, ischemia or other pathological conditions. About the role of prostaglandins and that of circulating and endogenous catecholamines in the regulation of coronary flow, under normoxic conditions, much uncertainty exists.

In the isolated, perfused rabbit heart *Block* et al. (11) did not observe indomethacin-induced alterations in the coronary flow while *Schrör* et al. (16) noticed an increase in coronary vascular pressure caused by indomethacin in isolated guinea-pig hearts under conditions of constant perfusion rate. During normoxic perfusions we only observed a reduction in coronary flow by indomethacin in hearts from fasted and streptozotocin-diabetic rats while in control hearts there was no diminution in flow rate. This indicates that in hearts from control, fed, rats prostaglandin-like substances are not involved in the maintenance of the coronary flow, while, on the other hand, they are in hearts from fasted and diabetic rats.

The involvement of endogenous catecholamines in the regulation of the coronary flow under control conditions in spontaneously beating or electrically stimulated hearts is far from clear.

Significant norepinephrine release was found to occur from isolated guinea-pig hearts under conditions of electrical stimulation (16). For rats, less is known about the release of catecholamines from endogenous nerve-ending storage granules, but that the stored amounts are very well capable to induce a net coronary vasodilation has been observed by *Schaffer* et al. (25). The ionophore X-537 A induced a markable increase in coronary flow in spontaneously beating, perfused working heart preparations which was absent after depletion of endogenous catecholamines with reserpin. As *Schaffer* et al. (25) suggested, the enhanced flow is probably caused by the facilitated release of catecholamines from endogenous stores induced by the ionophore.

Our finding that reserpin-pretreatment led to a significant drop in coronary flow rate in electrically driven, perfused, hearts from control, fed, as well as fasted and diabetic rats, indicates that catecholamines, likely to be released from the endogenous granule stores, serve as a supplementary factor in the regulation of the coronary flow under normoxic conditions.

The possibility that free fatty acids, which possess coronary vasodilatory properties (6), were involved in the increased flow of fasted and diabetic hearts was also investigated (not presented).

The release of free fatty acids under normoxic conditions from hearts of all three groups was found to be very low and since only minor differences were found, it was concluded that fatty acids do not contribute significantly to the increased coronary flow rate in hearts from fasted and diabetic rats.

. Our results concerning the effects of indomethacin and reserpin-pretreatment during normoxic conditions in hearts from control, fed, rats are in clear contrast with the results of *Schrör* et al. (16) and those of *Krebs* and *Schrör* (24) in isolated guinea-pig hearts, perfused at a constant flow rate.

The indomethacin effect resembles that observed in isolated rabbit hearts (11, 13). As the experiments with indomethacin indicated, prostaglandin-like substances probably were involved in the regulation of vascular smooth muscle tone in hearts from fasted and diabetic rats while no influence was present in control hearts. The role of endogenous catecholamines was evident in all groups of hearts. Therefore we tested the effect of indomethacin as inhibitor of the prostaglandin biosynthesis, and depletion of endogenous catecholamines upon the release of prostaglandin-like substances from hearts of all groups. From the literature only little is known about the release of prostaglandins from isolated, perfused rat hearts under normoxic conditions. We observed that prostaglandin release from one heart during control conditions is rather low and in the lowest range of the bioassay-sensitivity. When effluents of four hearts were pooled clearly measurable amounts could be extracted and assayed (see table 2).

The release of prostaglandin-like substances from hearts from fasted and diabetic rats is about four times higher than the release from control hearts. This confirms the effect of indomethacin on coronary flow rates in the perfusion experiments. Both indomethacin and pretreatment with reserpin abolished this enhanced release of prostaglandin-like substances, indicating the involvement of endogenous catecholamines in the biosynthesis and release of prostaglandins in hearts from fasted and diabetic rats. The regulatory role of catecholamines in the prostaglandin synthesis has already been described by *Junstadt* and *Wennmalm* (20) who observed an increased prostaglandin release from the rabbit heart evoked by an infusion with norepinephrine.

Coronary flow rates and cardiac or arterial smooth muscle levels of cyclic AMP are very well related. *Hülsmann* (6) detected a significant rise in adenylcyclase activity of cultured rabbit aorta smooth muscle cells incubated with prostaglandin E_1 in a concentration that fairly increased the coronary flow rate in retrogradely perfused rat hearts. These observations are confirmed by *Sen* et al. (29) who clearly demonstrated a good correlation between myocardial cyclic AMP levels and the coronary flow rate in the presence of prostaglandin E_2 and/or exogenous norepinephrine. The influence of prostaglandins and endogenous catecholamines on the coronary flow might have a common denominator in cyclic AMP as a dilatory substance for vascular smooth muscle cells. About the level and release of catecholamines from endogenous stores in hearts from fasted and diabetic rats little is known.

Under conditions of stress, however, the rate of synthesis of norepinephrine is increased (30) and the availability of catecholamines at the receptor site is enhanced (31). On the basis of the experiments presented we propose that under fasting and "acute", streptozotocin-induced, diabetic conditions also an increase in catecholamine release from endogenous

stores takes place. The released catecholamines are directly involved in a (cyclic AMP-mediated) vasodilatory action on the coronary vascular smooth muscle and stimulate prostaglandin synthesis and release, also resulting in coronary vasodilation. An increase in prostaglandin synthesis in diabetes mellitus is described by *Halushka* et al. (32), who observed an enhanced platelet aggregation in relation with a stimulated prostaglandin synthesis. Since it is generally accepted that prostaglandin synthesis is limited by the availability of essential fatty acids, which are stored in (phospho)glycerides, it is possible that a cyclic AMP or bradykinin-mediated increase of (phospho)glycerolipid hydrolysis (33) is the basis of increased prostaglandin synthesis, which ultimately causes removal of Ca^{++} from the cytosol of smooth muscle, so that vasodilation ensues.

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Zusammenfassung

Die koronare Durchströmung der retrograd perfundierten Herzen der gehungerten (Gruppe I) und Streptozotocin-diabetischen Ratten (Gruppe II) ist erhöht im Vergleich mit der koronaren Durchströmung von Kontrolltieren (Gruppe III). Zunahme der Durchströmung ist nicht vorhanden, wenn mit Indomethacin (1 µg/ml) Herzen aus Gruppen I und II perfundiert werden. Die niedrigste Durchströmungsgeschwindigkeit wird gemessen, wenn die Tiere mit Reserpin vorbehandelt werden. Herzen aus Gruppen I und II zeigten eine bedeutende Zunahme der Freisetzung von Prostaglandinen, welche gehemmt wurde, wenn Indomethacin hinzugefügt wurde (1 µg/ml) oder nach Vorbehandlung mit Reserpin. Die Ergebnisse suggerieren, daß die erhöhte koronare Durchströmung des Herzens gehungerter oder Streptozotocin-diabetischer Ratten veranstaltet wird von dem Effekt freigesetzter, endogener Katecholamine auf glatten, konoraren Gefäßmuskeln und von einer katecholamin-induzierten Freisetzung gefäßerweiternder, prostaglandinartiger Substanzen.

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Paper 2. The role of endogenous catecholamines in the depressive effects of free fatty acids on isolated, perfused rat hearts.

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Basic Res. Cardiol. 73, 1978, 208-219

Errata

p. 208 r. 6 v.o.	stres, lees: stores
p. 209 r. 13	ref. 18, lees: ref. 13
p. 213 r. 5 v.o.	ref. 49, lees: ref. 32
p. 213 r. 21 v.o.	(CrP/ATP ratio, lees: (CrP)/ATP ratio
p. 213 r. 20 en 21 v.o.	energy charge ($\frac{1}{2}$ [ADP] + [ATP]/ [AMP] + [ADP] + [ATP]), moet zijn {($\frac{1}{2}$ [ADP] + [ATP])/([AMP] + [ADP] + [ATP])}

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The role of endogenous catecholamines in the depressive effects of free fatty acids on isolated, perfused rat hearts*)

Die Rolle von endogenen Katecholaminen in der depressiven Wirkung von freien Fettsäuren auf isolierte, perfundierte Rattenherzen

H. Stam and *W. C. Hülsmann*

With 4 figures and 1 table

(Received July 5, 1977)

Summary

Langendorff perfusion of hearts with 0.5 mM palmitate complexed with albumin in a molar ratio of six showed a depression of contractility to about 50 % of control values. The coronary flow rate was enhanced and no arrhythmias occured. This decrease in contractility during fatty acid perfusion was irreversible since reperfusion with control medium containing 11 mM glucose as substrate did not lead to a recovery in contractile behaviour, while coronary flow rates reached values equal to, or below control levels. The deleterious effects of palmitate were partly overcome by adding 11 mM glucose to the fatty acid containing medium. No significant change in energy charge of ventricular tissue was observed after a thirty minute recirculating perfusion with palmitate when compared with the energy charge of hearts perfused with palmitate plus 11 mM glucose.

In hearts, depleted from their endogenous catecholamines by preperfusion with tyramine, the fatty acid induced decrease in contractility was significantly less compared with control hearts during perfusion with palmitate. Reperfusion of tyramine-pretreated hearts with control medium was followed by a slow recovery of contractility. Hearts, preloaded with [^{3}H] – norepinephrine showed a sudden release of radioactivity in the perfusate upon introduction of 0.5 mM palmitate, while no release occurred upon introduction of 0.5 mM palmitate in the presence of 11 mM glucose. These findings suggest that catecholamines released from endogenous stres may play an important role in the depressive actions of fatty acids. The role of the intracellular levels of fatty acids and their possible ionophoric role in the release of catecholamines is discussed in relation to the deterioration of myocardial function. Prostaglandin E_1, of which ionophoric properties have also been shown, added instead of fatty acids, was found to have a similar effect.

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In the last two decades many investigations have been presented about the effects of free fatty acids on the electrophysiology, hemodynamics and metabolism of the mammalian heart under normal and pathological conditions (1–13). Fatty acids possess deleterious actions upon the myocardium which depend on the chain-length and the concentration applied (5, 13), on the molar ratio with albumin (6) and on the combination with other substrates (4, 6, 8). Fatty acids may play a role in the incidence of arrhythmias, especially under hypoxic conditions (8), during ischemia (1), in the reperfusion period following ischemia (1), and during reoxygenation after hypoxia (8). Recently, studies from this laboratory proved the coronary vasodilatory actions of medium and long-chained fatty acids which may further deteriorate myocardial function during ischemia or at insufficient perfusion pressures (18), as also is observed in isolated, perfused livers (14). The mechanism(s) by which free fatty acids can depress cardiac performance and influence the electrophysiological properties are not easy to explain, since high intracellular levels of free fatty acids, as well as their activated, strongly detergent, coenzyme A ester derivatives, have many effects on myocardial energy metabolism as well as on cellular and intracellular membrane structures and enzymes (14–22).

It may be of interest to note that fatty acids, but also fatty acyl-CoA and fatty acylcarnitine inhibited the cardiac $(Na^+ + K^-)$-stimulated Mg^{2+}-ATPase (20, 22), and increased the cation permeability of mitochondrial membranes (19, 21). These ionophoric properties explain the stimulatory effects of fatty acids upon contractility and coronary flow of isolated, perfused rat hearts at low Ca^{2+} levels in the perfusion medium (13). Protection of the depressing actions of (activated) fatty acids by glucose is probably due to esterification with glycerol-3-phosphate.

This also holds for the fatty acid induced enzyme loss from damaged, ischemic rat heart cells (23). The cellular concentration of free fatty acids is determined by the net uptake, possibly influenced by endogenous catecholamines (24, 25), β-oxidation, esterification, and lipolysis in the heart. Catecholamines, liberated from endogenous stores during anoxia and ischemia are able to alter the membrane permeability for macromolecules, since acetylcholine- and anoxia induced release of myocardial enzymes could be markedly reduced by depletion of the endogenous catecholamines (26–28). Nerve-endings of (ortho-)sympathetic nerves contain two compartments of catecholamines: a granular store (synaptic vesicles) and an extragranular cytoplasmatic store (29). Calcium ions are thought to play a key role in the release of catecholamines from both stores (30).

Agents, known to promote calcium-ion transport across the plasma membranes induce catecholamine release from endogenous stores (30).

The present study was undertaken to find a link between the negative inotropic effect of free fatty acids and the role of released endogenous catecholamines in isolated, perfused rat hearts. It reemphasizes the ionophoric properties of lipophilic fatty acids.

Methods and Materials

Animals

Male Wistar rats (200–250 g) were fed *ad libitum*, anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg body weight) and intra-

venously heparinized with 500 I.U. If depletion of endogenous catecholamines took place with reserpin, the drug was injected intraperitoneally (10 mg/kg body weight) six hours before sacrifice.

Perfusion procedure

The hearts were quickly excised, cannulated and connected to the perfusion apparatus for retrograde perfusion *(Langendorff)* as described earlier (31, 32). Contractility was measured as apex displacement and registered as described previously (31).

Apex displacement after 30 min perfusion was taken as control. Coronary flow rates were measured by timed collection. After the experiments the hearts were cut open, blotted dry thoroughly and weighed. The standard perfusion medium (SPM) consisted of a modified *Tyrode* solution (13, 31), pH 7.4, containing 11 mM glucose and/or 0.5 mM potassium palmitate complexed with fatty acid free bovine serum albumin (BSA) (in a molar ration of 6:1). The perfusion fluids were equilibrated with 95 % O_2 + 5 % CO_2, and foaming of the albumin containing solutions was prevented by a silicone defoamer (Antifoam A).

The palmitate-albumin complex was prepared by adding recristallized potassium palmitate in water of 80° C to an albumin solution under vigorous stirring. Before use, the solution was passed through a washed, 1.2 μ Millipore membrane filter (Sartorius, Göttingen, Germany).

Perfusion of the hearts occurred in three periods. All experiments were started with an initial recovery period of 30 min (period I) in which perfusion took place with SPM containing 11 mM glucose (31, 32). It was followed by a 30 min recirculating perfusion (period II) with 75 ml of SPM containing 0.5 mM palmitate (complexed to 0.08 mM BSA) ± 11 mM glucose or, in control experiments, SPM containing 11 mM glucose ± 0.08 mM BSA. Subsequently perfusion took place, again for 30 min (period III), with SPM containing 11 mM glucose.

Depletion of endogenous catecholamines was induced by an infusion of tyramine (6.5 μ/min) in the aortic cannula during the first 15 min of period I (34). Control experiments proved that tyramine washout during perfusion was complete at the end of the period I.

Radioactive experiments

After a 15 min equilibration perfusion, the transmitter stores of the heart were labelled in a 15 min perfusion with glucose-medium, containing 5.10^{-8} M DL-[7-^{3}H] norepinephrine ([^{3}H]NE).

The specific activity of the [^{3}H]NE was 11 Ci/mmol. Efflux of [^{3}H]-labelled compounds was followed by collection of effluents during perfusion with control medium for 10 min. Subsequently the [^{3}H]NE-labelled hearts were exposed to SPM either containing a) 11 mM glucose + 0.08 mM BSA, or b) 0.5 mM palmitate + 0.08 mM BSA, or c) 11 mM glucose, 0.5 palmitate + 0.08 mM BSA, or d) 11 mM glucose + 10^{-6} M prostaglandin E_1 (PGE_1). During these non-recirculating perfusions effluent samples were collected for 20 min and 2 ml of all samples was added to 10 ml Instagel. Radioactivity was determined by liquid scintillation counting.

Analyses

At the end of perfusion period I beating hearts were freeze-clamped between aluminium blocks at the temperature of liquid nitrogen (35) and extracted as previously described (33).

The tissue extracts were determined for creatine phosphate, ATP, ADP and AMP spectrophotometrically (36).

Materials

Heparin was purchased from Organon (Oss, The Netherlands), reagents (all of analytical grade), reserpin and tyramine from Merck (Darmstadt, Germany). Fatty acid-free bovine serum albumin was obtained from Fluka (Bucks, Switzerland), sodium pentobarbital from Abbott (Saint-Remy sur Avre, France) and Antifoam A from Dow Corning (Michigan, USA). The enzymes and cofactors for the determination of creatine phosphate, ATP, ADP and AMP were obtained from Boehringer and Sons (Mannheim, Germany), DL-[7-^{3}H] Norepinephrine hydrochloride from The Radiochemical Centre (Amersham, England) and Instagel from Packard Instrument Company (Downers Grove, Illinois, USA).

Statistics

Results are presented in mean values ± standard error of the mean (S. E. M.) n is the number of observations. Values of P were calculated with *Student's* t-test (two-tailed). $P > 0.05$ was considered to be not significant.

Results

Changes in ventricular contractility and coronary flow during fatty acid perfusion: Protective action of glucose.

Control experiments were carried out to test the effect of a recirculating perfusion with 75 ml of SPM containing 11 mM glucose and 0.08 mM BSA. As figure 1 illustrates, only minor changes in apex displacement were observed during period II and this slight decrease in contractility was irreversible, in view of the lack in recovery during perfusion without albumin (period III). The coronary flow rate increased from 6.67 ± 0.14 at the end of the equilibration period to 7.99 ± 0.42 ml. $min^{-1} \cdot g$ wet wt^{-1}

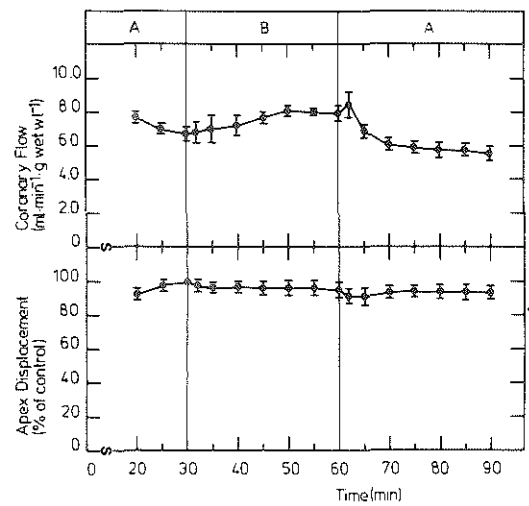

Fig. 1. Changes in coronary flow and apex displacement in isolated rat hearts during successive 30 min perfusions with SPM containing 11 mM glucose (A) or 11 mM glucose + 0.08 mM BSA (B). Each point represents the mean value ± S. E. M. of 4 experiments.

after the second period of perfusion. This increase in coronary flow was overcome during continued perfusion with SPM + 11 mM glucose. The final coronary flow rate was 5.54 ± 0.22 ml · min^{-1} · g wet wt^{-1}.

The consequences of recirculating SPM + 0,5 mM palmitate in a molar ratio with BSA of 6:1 and the protective effect of glucose are presented in figure 2. Perfusion with palmitate induced a marked increase in coronary flow rate from 6.58 ± 0.16 to 9.42 ± 0.81 · min^{-1} · g wet wt^{-1}. When 11 mM glucose was present in the palmitate containing SPM, the final coronary flow rates at the end of perfusion period II were identical. However, with glucose present, the rate of increase in coronary flow is significantly higher ($P < 0.05$ for the first three observations in period II). During continued perfusion with SPM + 11 mM glucose (period III) the flow rate decreased to control levels or less, with a significantly lower rate in hearts previously subjected to palmitate perfusion in the absence of 11 mM glucose (period II).

Ventricular contractility decreased to about 50% of the control value when 0.5 mM palmitate was the sole substrate. When glucose was present during fatty acid perfusion contractility decreased only to 80% of the control value. No recovery in contractile behaviour was observed in both groups of hearts during perfusion with SPM + 11 mM glucose (period III). During all experiments carried out under the conditions described above, no irregularities in ventricular contractions were observed.

The depressive effects of palmitate upon cardiac function were also overcome by 5 mM glycerol, whereas pyruvate (5 mM), lactate (5 mM),

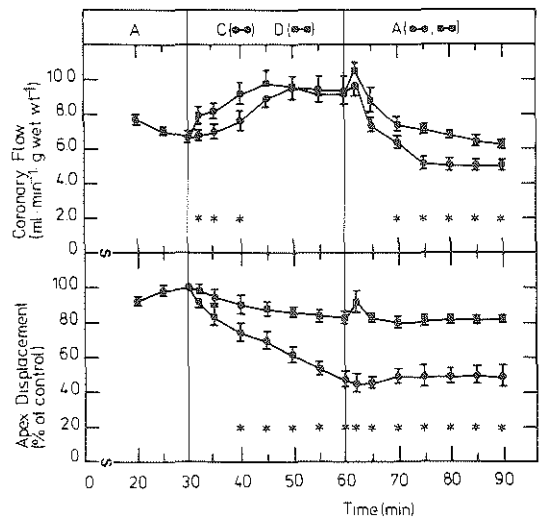

Fig. 2. Changes in coronary flow and apex displacement in isolated rat hearts, during successive 30 min perfusions with SPM containing 11 mM glucose [A, A (●-●, ■-■)], 0.5 mM palmitate + 0.08 mM BSA [C (●-●)] and 0.5 mM palmitate, 0.08 mM BSA and 11mM glucose [D (■-■)].Each point represents the mean value ± S. E. M. of 6–9 experiments. * $P < 0.05$ for ●-● versus ■-■.

Table 1. The effect of fatty acid perfusion on the energetic state of isolated, perfused rat hearts; effect of glucose.

Substrates	n	Energy charge $\frac{1/2\,[ADP] + [ATP]}{[AMP] + [ADP] + [ATP]}$	CrP/ATP
0.5 mM palmitate (0.08 mM BSA)	3	0.85 ± 0.03	1.53 ± 0.04
		NS	$P < 0.01$
0.05 mM palmitate (0.08 mM BSA) + 11 mM glucose	3	0.87 ± 0.03	1.79 ± 0.04

Mean values are indicated ± S. E. M., n = number of observations, NS = not significant ($P > 0.05$).

fumarate (5 mM) and carnitine (1 mM) were ineffective or even induced more severe depression of contractility (not shown).

Energetic state of fatty acid perfused hearts

The changes in tissue levels of creatine phosphate, ATP, ADP and AMP were investigated in hearts perfused with 0.5 mM palmitate ± 11 mM glucose. The results are presented in table 1. The energetic state of the ventricular tissue is expressed as energy charge ($^1/_2$ [ADP] + [ATP] / [AMP] + [ADP] + [ATP]) and as the creatine phosphate (CrP/ATP ratio. No significant difference was observed in energy charge of hearts after 30 min. palmitate perfusion in the presence or absence of 11 mM glucose. The creatine phosphate levels, however, were decreased 15 % after palmitate perfusion in the absence of glucose.

Effect of palmitate on cardiac function in hearts depleted of endogenous catecholamines

To investigate the possible role of endogenous catecholamines during fatty acid perfusion, hearts were depleted of their catecholamine stores by preperfusion with tyramine as described in the methods and materials section. The results are illustrated in figure 3. After equilibrium perfusion, the coronary flow rates of hearts preperfused with tyramine reached significantly lower values when compared with the control hearts ($P < 0.01$ for flow rates after 30 min). That catecholamines, liberated from endogenous stores play a role in the maintenance of coronary flow under normoxic conditions has been described earlier (49). The coronary flow rates in hearts preperfused with tyramine showed no significant difference in response on fatty acid perfusion when compared with control hearts, while also the same flow levels were reached during period III. Contractility, however, was less depressed in catecholamine depleted hearts. Moreover,

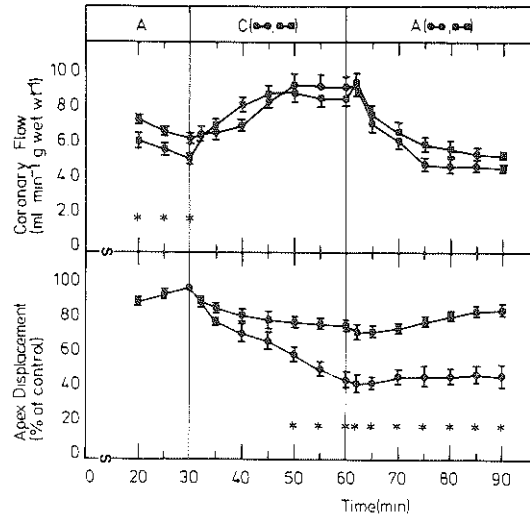

Fig. 3. Changes in coronary flow and apex displacement during successive 30 min perfusions with SPM containing 11 mM glucose (A) and 0.5 mM palmitate + 0.08 mM BSA (C) in control hearts (●–●) and in hearts depletedfrom endogenous catecholamines by preperfusion with tyramine (■–■). Each point represents the mean value ± S. E. M. of 6–9 experiments. * $P < 0.05$ for ●–● versus ■–■.

in hearts preperfused with tyramine a slow, but significant recovery was observed during period III ($P < 0.01$ between the contractility of preperfused hearts after 60 and 90 min). Hearts, isolated from rats depleted of endogenous catecholamines by reserpin, also showed a reduced depressive action of palmitate perfusion (not shown).

Experiments with [^{3}H]norepinephrine-loaded hearts

The experiments with tyramine-preperfused hearts suggested that endogenous catecholamines were involved in the depressive action of fatty acids. Therefore we studied the effect of palmitate on the release of radioactivitity from hearts preloaded with [^{3}H]norepinephrine ([^{3}H]NE), as is presented in figure 4. [^{3}H] NE preloaded hearts showed a sudden release of label in the perfusate-effluent upon introduction of 0.5 mM palmitate which was prevented when 11 mM glucose was added to the fatty acid containing perfusion fluid. In control perfusions with 11 mM glucose and 0.08 mM BSA no release of radioactivity from [^{3}H]NE loaded hearts was detected. For comparison, the release of label was studied upon introduction of prostaglandin E_1 (PGE_1). PGE_1 gave rise to an even more pronounced and longer-lasting release.

Discussion

The deleterious effects of high levels of free fatty acids have been subject to many investigations (1–24) and the protective action of glucose is well known. About the actual mechanism by which fatty acids and their

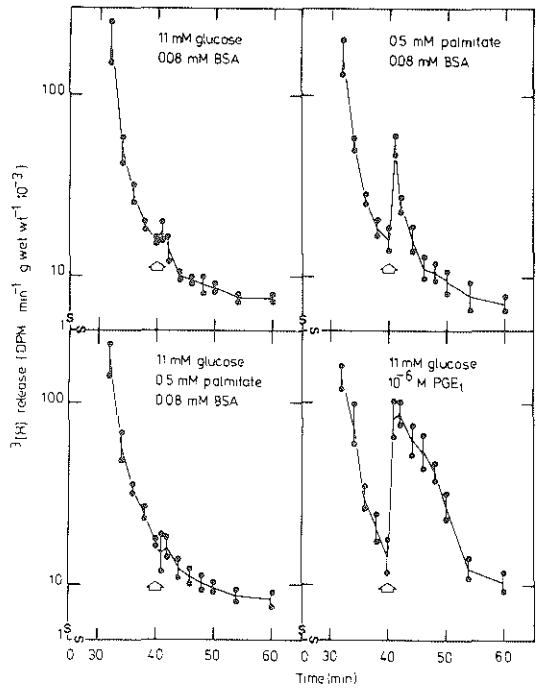

Fig. 4. Release of radioactivity in the coronary effluent of isolated rat hearts preloaded with [^{3}H]norepinephrine. Two separate experiments (●) are presented. The arrow indicated introduction of SPM containing the mentioned constituents.

acyl-CoA esters exert their actions, however, no clearcut explanation exists. In this paper we confirmed the detrimental effect of a high free fatty acid: albumin ratio on myocardial contractility under normoxic conditions (5, 6) and the vasodilatory action as described earlier (13). Nevertheless, no irregularities in ventricular contractions, as mentioned by *Opie* (5) and *Willebrands* et al. (6, 8) were observed. Possibly, because the vulnerability of arrhythmias is suppressed by electrical stimulation of the hearts in our experiments. The intracellular levels of free fatty acids and their coenzyme A esters are supposed to play a major role in the depression of metabolism and contractility, and prevention of intracellular accumulation of free fatty acids by stimulating triglyceride synthesis is an important way to protect deterioration. Conversely, the myocardial lipase activity, part of which may be hormone-sensitive (37) can affect the fatty acid levels in the cell, while glucose and glycerol may cause a decrease by supplying glycerol-3-phosphate for triglyceride formation (38). Our study proved that the energy charge of the hearts after a 30 min fatty acid perfusion was not decreased and was independent of the protective presence of glucose. The decrease in creatine phosphate levels in the absence of glucose may reflect a slightly depressed rate of net ATP synthesis, whether

due to depressed glycolysis or oxidative phosphorylation leading to decreased creatine phosphate synthesis (40, 41).

Our observation that the deleterious effects of palmitate upon the contractility were irreverible illustrates the membrane damaging properties of high levels of lipophilic fatty acids.

Studies of *Mathur* and *Mokler* (25) indicated that endogenous catecholamines play an important role in the fatty acid metabolism and for that reason we studied the effects of fatty acid perfusion in rat hearts depleted of their catecholamine stores by preperfusion with tyramine. We observed a marked reduction of the negative inotropic action of palmitate, while continued perfusion with fatty acid free medium resulted in a slow recovery of contractile behaviour. The vasodilatory response to palmitate was identical in control and catecholamine-depleted hearts. This confirms earlier observations that catecholamines were not involved in the vasoactive properties of fatty acids (13).

Catecholamines can interfere with the fatty acid metabolism by mediating their transport through the cellular membrane, whether directly or through increased cytosolic metabolism, or by stimulating the hormone-sensitive lipase. Furthermore it is known that catecholamines released from endogenous stores alter the membrane permeability for macromolecules (27, 28).

The secretion of catecholamines from nerve-endings of sympathetic neurons occurring during myocardial ischemia and hypoxia (26), is suggested to be mediated by Ca^{2+} ions (29, 30), and can be enhanced by ionophores (42).

That lipophilic fatty acids, but also prostaglandin E_1, may possess ionophoric properties has been demonstrated before (13, 19, 21, 43). In hearts preloaded with [^{3}H]NE, introduction of a high intracellular level of free fatty acids (and of acyl-CoA esters, since fatty acid activation is not limiting for metabolism) resulted in a sudden release of radioactivity from the heart, representing the release of norepinephrine or its metabolites. The release of the neurotransmitter or its metabolites induced by fatty acids may be explained by their Ca^{2+} ionophoric properties. It is likely that, in states of impaired energy metabolism (ischemia, anoxia) when increased neurotransmitter release is observed (26), intracellular fatty acid levels are increased, since fatty acid oxidation will be hampered and lipolysis is increased because of stress.

Upon introduction of prostaglandin E_1, from which the ionophoric properties have been described (43), liberation of radioactivity also occurred, which strengthens the idea that the lipophilic fatty acids (or their acyl-CoA esters), have ionophoric properties. That the released, catecholamines did not induce a positive inotropic effect may be due to the action of long chain fatty acids on membranous processes.

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Zusammenfassung

Die Kontraktilität des retrograd perfundierten Rattenherzens wird um 50% herabgesetzt, wenn 0,5 mM Palmitinsäure, komplexiert mit 0,08 mM Albumin, im Perfusionsmedium vorhanden ist. Die Fettsäureperfusion erhöht die Koronardurchströmungsgeschwindigkeit, während keine Rhythmusstörungen beobachtet wurden. Die fettsäureinduzierte Kontraktilitätssenkung war nicht umkehrbar, weil nachträgliche Durchströmung mit 11 mM Glukose anstatt Fettsäure die Kontraktilität nicht verbesserte. Wenn Glukose während der Fettsäuredurchströmung zu gleicher Zeit hinzugefügt wurde, war die schädliche Wirkung der Fettsäure beschränkt. Nach halbstündiger Durchströmung mit Palmitinsäure als einzigem Brennstoff war der Gehalt energiereicher Adeninnukleotide unverändert, obwohl eine geringe Herabsetzung des Kreatinphosphatgehaltes beobachtet wurde. Wenn Herzen während 15 Minuten mit Tyramine präperfundiert wurden, um die endogenen Katecholamine zu entfernen, hatte Palmitatdurchströmung nur einen kleinen Effekt auf die Kontraktilität, während die geringe Herabsetzung reversibel war, wenn eine nachträgliche Durchströmung mit Glukose erfolgte. Diese Beobachtung machte uns auf die Möglichkeit aufmerksam, daß Fettsäure die endogenen Katecholamine mobilisieren. Wenn Herzen mit [^{3}H]-Norepinephrin in der frühen Perfusionszeit aufgeladen wurden, stellte sich heraus, daß nachher die Radioaktivität auf asymptotische Weise aus dem Herzen freigemacht wurde. Wenn Palmitinsäure (mit Albumin im 6:1-Molar-Verhältnis) angeboten wurde, wurde plötzlich viel mehr Radioaktivität freigemacht, was nicht geschieht, wenn zu gleicher Zeit Glukose angeboten wurde. Diese Befunde deuten darauf, daß freie Fettsäuren endogene Katecholamine mobilisieren können. Es ist möglich, daß die ionophoren Eigenschaften von Fettsäure dabei eine wesentliche Rolle spielen, weil wir auch fanden, daß Prostaglandin E_1 (dessen Rolle als ionophore Verbindung bewiesen wurde) den gleichen Effekt zeigte wie Fettsäure, während bakterielle Ionophore, wie X-537 A, in der Literatur auch Katecholamine mobilisierende Eigenschaften zeigten.

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Paper 3. Intracellular origin of hormone-sensitive lipolysis in the rat

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Erratum p. 57 Table I, sub. a, The adipocytes were incubated at 37°C in Krebs-Ringer bicarbonate buffer containing 3% bovine serum albumin (BSA), ...

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INTRACELLULAR ORIGIN OF HORMONE-SENSITIVE LIPOLYSIS IN THE RAT

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SUMMARY

The hormone-stimulated hydrolysis of endogenous triglycerides in heart and adipose tissue was found to be inhibited by chloroquine, which is known to accumulate in lysosomes and to inhibit the lysosomal degradation of protein and cholesterolesters.

When the triglyceride depôt in heart cells was increased by feeding rats a diet enriched in erucic acid for three days prior to in vitro perfusion of the heart, the spontaneous and the norepinephrine stimulated rates of lipolysis were both found to be increased. Both were inhibited by chloroquine. Analysis of trioleoylglycerol hydrolysis in heart homogenates after in vitro heparin perfusion revealed the virtual absence of neutral lipase in contrast to an acid lipase activity. The results of this study suggest that lipolytic activity of lysosomal origin is the main source of hormone-sensitive endogenous triacylglycerol hydrolysis.

Catecholamine activation of lipolysis in adipose tissue has been well documented[1,2]. The enzyme has been partially purified[3]. Adipose tissue also contains lipoprotein lipase, which may be removed by heparin treatment. In heart, catecholamine stimulated lipolysis is also known[4,5], as well as the presence of (heparin-releasable) lipoprotein lipase. In heart homogenates Björntorp and Furman[6] distinguished two lipases: a "tissue lipase" with a pH optimum of 6.8 and lipoprotein lipase with pH optimum of 8.5. In liver, two triacylglycerol hydrolases may also be distinguished: a lysosomal lipase, which has been purified[7], and a heparin-releasable enzyme. The heparin-releasable enzymes of extrahepatic and hepatic tissues are different[8,9,10], but it is not certain whether the "tissue lipases" are different. It has been shown by Teng and Kaplan[7] that the purified enzyme has a pH optimum of 4.0, but that highly stimulatory cardiolipin shifts the pH optimum to 4.5. Hence the lipid environment of the membrane-bound enzyme codetermines the pH optimum. All "tissue lipase systems" may be stimulated by cyclic AMP, as judged by the catecholamine stimulation of endogenous lipid hydrolysis, which was mentioned above. Guder et al.[11] reported glucagon stimulation of ketogenesis in liver and found also that the hormone increased the lability of acid lipase in in vitro perfused livers of starved rats. Therefore, it is possible that cyclic AMP stimulates the lysosomal action. Ashford and Porter[12]

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demonstrated a lysosomal enzyme in autophagosomes after perfusion of liver with glucagon, while Deter and De Duve[13] reported that glucagon not only increased the formation of autophagic vacuoles, but also the fragility and osmotic sensitivity of lysosomes.

A suitable tool to identify the involvement of lysosomes in degradative processes seems to be (pre)treatment of tissues with chloroquine, a weakly basic drug, which mainly accumulates in lysosomes and binds to the lysosomal membranes[14,15]. It has been used to demonstrate lysosomal protein degradation [16,17] and cholesterolester hydrolysis[18]. The present paper describes experiments with chloroquine on heart and adipocytes, it discusses determinations of lipolytic activities in heart and liver, from which the conclusion is drawn that "tissue lipases" of heart, adipose tissue and liver are all attached to intracellular (lysosomal?) membranes. In sonicated homogenates of heart the enzyme itself is not sensitive to chloroquine, while in the intact organ full sensitivity is obtained.

METHODS

For the heart perfusion experiments male Wistar rats were fed with normal rat pellets or put on a 3-day diet containing 40% of the total calories as rapeseed oil. After pentobarbital anaesthesia (70 mg/kg body weight), the hearts were quickly excised and perfused retrogradely, as described earlier[19]. The hearts were electrically paced at a rate of 300 beats/min. The mean coronary flow rate in the presence of norepinephrine was 9.0 ± 1.3 ml/min^{-1}. g wet wt^{-1}.

Glycerol and lactate, released from the heart of both groups of rats during control (15 min) perfusion and during continuous infusion of norepinephrine (final concentration 10^{-7} M) were determined fluorometrically[20]. Careful calibration of fluorescence was carried out in each experiment by adding a known amount of NADH. Chloroquine diphosphate was dissolved in saline and injected intraperitoneally (75 mg/kg body weight) two hours prior to perfusion of the hearts[21]. Preperfusions, with 50 µM chloroquine, lasted 30 min to ensure sufficient "wash-in" of the drug.

The release of lipoprotein lipase activity from the hearts was followed during perfusion with Tyrode-buffer containing 1% (w/v) fatty-acid-free bovine serum albumin, 5 I.U. heparin/ml and 5.10^{-7} M norepinephrine. Five successive 20 ml effluent fractions were collected in precooled glycerol[22] (final concentration 20% v/v) and lipase activity was estimated immediately.

Adipocytes were isolated from epididymal fat pads with collagenase according to Rodbell[23].

Lipolytic activities at pH 8.2 were tested with 100 µl 20% (w/v) glycerol containing perfusates or 10% (w/v) heart homogenates (a Polytron PT10 homogenizer was used at 4000 rev./min) in perfusion medium in a final volume of 250 µl by incubation with Intralipid (Vitrum, Sweden), 0.5% Intralipid (containing 0.5% w/v soybean oil and 0.03% egg phospholipid) was made radioactive by the manufacturer by the addition of glyceroltri[9,10(n)-^{3}H]oleate. 1.9 ml of this Intralipid was mixed with 0.5 ml 10% non-labeled, commercially available, Intralipid to give a triglyceride solution of about 28 mM (spec.act. 0.35 µCi/µmole). 40 µl of this substrate was used in each test. The final concentrations were: 40 mM Tris-HCl, 2.5% w/v bovine serum albumin, 0.8 mM $CaCl_2$, 0.15 M NaCl, 1.9 mM KCl, 8 mM $NaHCO_3$, 0.1 mM NaH_2PO_4, 0.4 mM $MgCl_2$, 4.4 mM glucose, 4.5 mM triglyceride, when indciated 8 µg pure apolipoprotein C_{II}/ml, and perfusate or heart homogenate.

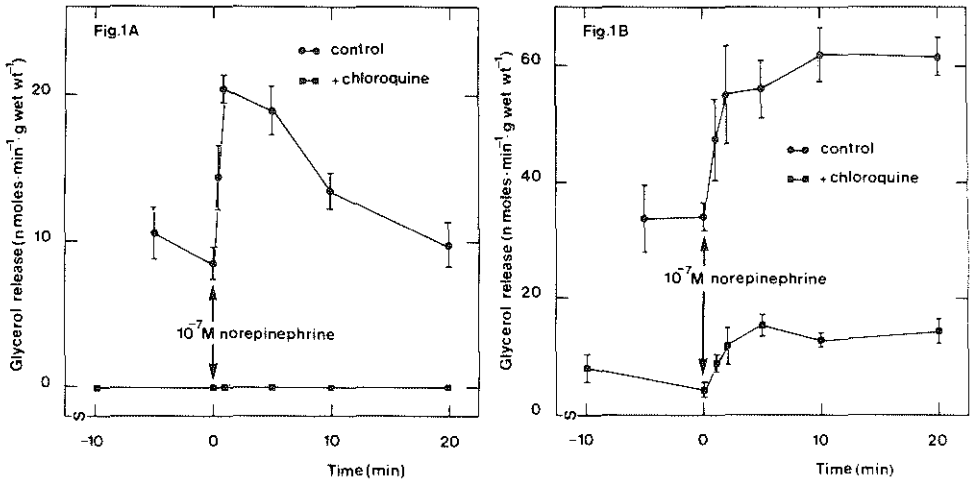

Fig. 1 A+B. Effect of 50 µM chloroquine on the glycerol release from isolated, perfused hearts of normal fed and rapeseed-oil fed rats during control perfusion and in the presence of 10^{-7} M norepinephrine. The results are the mean ± S.E.M. of four experiments.

Final pH 8.2. After 30 min at 37°C, the reactions were stopped with chloroform/methanol/heptane, followed by mixing with borate buffer of pH 10.5 to extract the oleate into the aqueous phase, exactly as described[24]. After centrifugation, 1 ml was counted in a liquid scintillation counter.

Lipolytic activity at a final pH of 5.2 was tested with 100 µl W/v heart homogenate (as above) in 250 µl final volume. Other additions (final concentrations) were 50 mM glyceroltri[1-^{14}C]oleate (spec.act. 0.03 µCi/µmole), 1.5% W/v gum acacia and 125 mM cacodylate buffer pH 5.2. These additions were sonicated (3 x 1 min at 21 kHz per ml emulsion). The enzyme preparation was also sonicated in this case (1 min/ml at 21 kHz). In 3 experiments the effect of 50 µM chloroquine was also tested as mentioned in the text. After incubation for 30 min at 37°C the reaction was stopped and counting performed as mentioned above.

Lipolytic activities were expressed as nmoles fatty-acid released per min (mU) per g wet wt. Results are given as mean ± S.E.M.

RESULTS

In vitro perfusions of rat hearts with chloroquine

From Fig. 1A it can be seen that in hearts from normal fed rats the glycerol production rate is about 10 nmoles/min/g wet wt., which is doubled immediately following the addition of 0.1 µM norepinephrine. Both the spontaneous and the hormone-stimulated lipolysis are completely inhibited by adding chloroquine (50 µM) to the perfusion medium. The effect of chloroquine was found to be fully reversible (not shown). The rate of lipolysis can be increased 3-fold

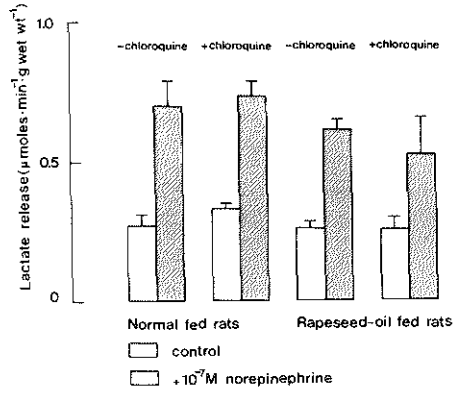

Fig. 2. Effect of chloroquine (50 μM) on basal and norepinephrine-stimulated lactate release from isolated, perfused hearts of normal fed and rapeseed-oil fed rats. Control values represent the lactate levels at the end of the pre-perfusion period. Norepinephrine-stimulated values represent the lactate levels 5 min after the infusion of the hormone. The results are the mean ± S.E.M. of four experiments.

(Fig. 1B) by increasing the triglyceride content of the heart by feeding the animals for 3 days with a diet rich in erucic acid[25] (40 cal%). Apparently the rate of lipolysis is affected by the lipid store of the organ. Again both the spontaneous and the norepinephrine-stimulated rates of lipolysis were strongly although not completely inhibited by chloroquine. Chloroquine does not interfere with glycogenolysis as judged by the absence of an effect on the rate of lactate production (Fig. 2). Therefore it may be concluded that the effect of chloroquine on lipolysis is specific.

Effect of chloroquine on lipolysis in adipocytes

Norepinephrine-stimulated lipolysis in adipocytes, is also highly sensitive to chloroquine inhibition (Table I). Doubling the amount of chloroquine or using 1 μM norepinephrine instead of 0.2 μM did not influence the results. Dibutyryl cyclic AMP-stimulated lipolysis was also inhibited by chloroquine (not shown).

Relative contributions of triacylglycerol hydrolases in heart and liver

It has been noted by a number of authors, including our group[26] that in vitro perfusion of rat heart with heparin only partially removes lipoprotein lipase. If a proper coronary flow rate is maintained (by millipore filtration of the albumin and heparin containing perfusion medium prior to use; electrical stimulation of

TABLE I

EFFECT OF CHLOROQUINE ON LIPOLYSIS IN ADIPOCYTES[a]

Additions	n[b]	% of norepinephrine-stimulated activity
none	5	3.0 ± 1.1
25 μM chloroquine	5	3.1 ± 1.6
0.2 μM norepinephrine	5	100
0.2 μM norepinephrine +25 μM chloroquine	5	32.9 ± 9.7

[a] The adipocytes were incubated at 37°C in Krebs-Ringer bicarbonate buffer, equilibrated with a gas mixture of 95% O_2 and 5% CO_2 (v/v) to give a final pH of 7.4. The reactions were stopped after 15 min by cooling, followed by centrifugation. In the infranatant glycerol was determined as described under Methods.

[b] n = number of experiments.

the heart and a sufficiently high perfusion pressure (100 cm H_2O)) 89% of the lipoprotein lipase is removed in 11 min of perfusion with 5 I.U. heparin/ml (Table II). The residual trioleoylglycerol hydrolase activity in the heart homogenate, measured at pH 8.2, can be characterized as lipoprotein lipase, since the activity may be stimulated by apolipoprotein C_{II}, while the non-stimulated activity is inhibited by the lipoprotein lipase inhibitor protamine sulfate[9]. We tested for a hormone-sensitive lipase in the homogenate by including 0.5 μM norepinephrine in the perfusion medium, but no significant trioleoylglycerol hydrolase activity can be found at pH 8.2 except lipoprotein lipase. The trioleoylglycerol hydrolase activity at pH 5.2 (broad pH optimum between 5 and 6.5 - data not shown) is much higher. This (acid) lipase may be of lysosomal origin since a lysosomal lipase does exist in heart, as found by others and as judged by the chloroquine inhibition observed in Fig. 1. The lipase activity at pH 5.2, as measured in sonicated homogenates, was not sensitive to 50 μM chloroquine (Table II; comparison of paired experiments revealed no inhibition).

DISCUSSION

In liver most of the neutral (or alkaline) trioleoylglycerol hydrolase activity is removed by heparin perfusion[27], provided the liver is perfused at a sufficiently high flow rate. The small residual activity (measured at pH 8.5) is completely due to heparin-releasable lipase, since the residual activity is

TABLE II

DIFFERENTIATION OF MYOCARDIAL LIPOLYTIC ACTIVITY

	apo C_{II}	n[c]	mU/g wet wt
heparin-releasable LPL[a] in perfusate	+	9	483 ± 132
non-releasable LPL in heart	+	9	60.4 ± 4.0
non-releasable LPL in heart	-	9	18.4 ± 2.1
non-releasable LPL in heart + 50 µg/ml protamine sulfate	-	9	9.0 ± 0.9
acid lipase[b]	-	9	103.4 ± 5.8
acid lipase + 50 µM chloroquine	-	3	91.0 ± 3.6

[a] Lipoprotein lipase (LPL) is defined as apolipoprotein C_{II} (apo C_{II}) dependent trioleoylglycerol hydrolase activity and measured at pH 8.2, as described under Methods.

[b] Acid lipase was measured in the absence of apo C_{II} with trioleoylglycerol at pH 5.2, as described under Methods.

[c] n = number of experiments.

inhibited by an antibody against the heparin-releasable enzyme. Therefore there is no other neutral (alkaline) triacylglycerol hydrolase. There is, however, an acid lipase which has been highly purified[7]. In heart, a similar situation exists: besides heparin-releasable lipase there is only an (acid) tissue lipase (Table II). This agrees with the observations of Björntorp and Furman[6], who found two lipases, and observed a similarity between the tissue lipase and the adipose tissue lipase. The similarities may now be extended. The tissue lipase activities are hormone-sensitive and they are inhibited by chloroquine, a known inhibitor of lysosomal activity. Therefore we ask whether the hormone-sensitive lipase in all tissues is embedded in the membrane of lysosomes and/or autophagic vacuoles. This question may help to resolve the mechanism of hormone-sensitivity. Whether activation is due to cyclic-AMP-dependent membrane phosphorylation or enzyme phosphorylation cannot yet be concluded[3]. The highly stimulatory effect of negatively charged phospholipids on the purified lysosomal lipase from liver[7], raises the possibility that membrane phosphorylation can influence the regulatory membrane environment of the enzyme, which may also be influenced by the weakly basic inhibitor chloroquine.

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Paper 4. The relation between fatty acid mobilization and contractility in the isolated, perfused rat heart

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Erratum p. 612 Table II, sub b, not significant vs control

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THE RELATION BETWEEN FATTY ACID MOBILIZATION AND CONTRACTILITY IN THE ISOLATED, PERFUSED RAT HEART

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SUMMARY

The contractility of hearts from normal fed rats is decreased by 70% during perfusion with 50 μM chloroquine, which is a potent inhibitor of endogenous lipolysis. In triacylglycerol-rich hearts, obtained by feeding rats rapeseed-oil, chloroquine depresses lipolysis much less, while contractility was found to be inhibited only 30%. In both groups of hearts the effect of chloroquine was decreased by adding fatty acids, prostaglandin E_1, the Ca^{2+}/Mg^{2+} ionophore X-537A or more Ca^{2+} to the perfusion fluid. Norepinephrine and glucagon also stimulate chloroquine-depressed hearts. The conclusion is therefore reached that fatty acids act as Ca^{2+}-vehicles in heart cells and that chloroquine, by inhibiting lipolysis, decreases Ca^{2+}-transport by lowering unesterified fatty acid levels.

Alteration of the intracellular "free" ionic calcium concentration controls the contractile state of cardiac striated and vascular smooth muscle[1]. Therefore pharmacological, metabolic or hormonal modification of the inotropic state of these tissues may be caused by altered calcium transport through or binding to membranes such as the cell membrane, the sarcoplasmic reticulum and the inner mitochondrial membrane[1]. The Ca^{2+}-ionophoric properties of fatty acids have been concluded from earlier experiments carried out in our laboratory[2]. Medium- and long-chain fatty acids were found to have a positive inotropic effect when in vitro perfusions were carried out at low Ca^{2+}-concentrations and to increase the coronary flow rate.

The availability of ionic calcium to the contractile proteins may not only be determined by membrane processes involved in calcium uptake and release to the cytoplasm, but also by (intracellular) substances which influence the calcium transport from the sites of release to the sites of contraction. It may be questioned then if endogenous fatty acids, as products of intracellular lipolysis, are also involved in the contractile behaviour of the heart.

In a previous publication we[3] have shown that the weakly basic chloroquine which selectively accumulates in the lysosomes[4,5,6] and inhibits lysosomal degradative processes[4,7,8], inhibits basal- and hormone-stimulated lipolysis of isolated, perfused rat hearts from normal fed and rapeseed-oil fed rats.

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Inhibition in control hearts is complete, while in hearts from rapeseed-oil fed rats still some lipolysis was observed. It was noticed in the same study that the chloroquine-induced inhibition of lipolysis was accompanied by a depression of the contractility and that this depression was markedly less in hearts from rapeseed-oil fed rats. The present paper discusses the mechanism of chloroquine-induced reduction of myocardial contractility and suggests that fatty acids may act as calcium-vehicles during the excitation-contraction cycle.

METHODS

Male Wistar rats (200-300 g) were fed a standard laboratory chow (Purina pellets) or put on a 3-4 day diet containing 40% of the calories (40 cal%) as rapeseed-oil (RSO), containing 38% $^{w}/_{v}$ erucic acid. The animals were anesthetized by an intraperitoneal injection of pentobarbital (70 mg/kg body weight), the hearts were quickly excised and perfused retrogradely as described previously[9]. Potassium palmitate (0.5 mM) was dissolved in distilled water of 70-80°C and added dropwise to a fatty acid-free bovine serum albumin (BSA) solution in a molar ratio of 1:1. Before use the fatty acid-containing BSA solution was passed through a, washed, 1.2 μ Millipore membrane filter. Glucagon and norepinephrine were infused at 10^{-7} M final concentration in the aortic canula. Contractility was revealed as apex displacement[10] and the coronary flow rate was determined. After a 15-20 min preperfusion the hearts showed a constant contraction amplitude and coronary flow rate. The values for contractility and coronary flow rate at the end of this stabilization period were taken as controls and changes in both parameters occurring during the subsequent perfusion were expressed in % of control. Basal coronary flow rates in hearts from rapeseed-oil fed rats differed significantly from flow rates in hearts from normal fed rats (7.34 ± 0.16 (n=10) vs 6.32 ± 0.09 (n=20), $P<0.001$)[11]. In the collected coronary effluents glycerol was determined fluorometrically[3,12]. The results are presented as mean values ± standard error of the mean (S.E.M.). n is the number of observations and significance was calculated with Students t-test (two tailed) $P>0.05$ was considered to be not significant.

RESULTS

Table I presents the depressive action of chloroquine upon the contractility of hearts from normal fed, and rapeseed-oil fed rats. Rapeseed-oil feeding leads to an increase of the endogenous triglyceride levels and lipolytic activities of the heart[3,13] and postheparin serum[14]. The, concentration-dependent, chloroquine-induced reduction of the contraction amplitude in hearts from rapeseed-oil fed rats was only about 30% while a 70% decrease was observed in hearts from normal fed rats. From Table II it can be observed that the detrimental effect of chloroquine could (partially) be overcome by the addition of octanoate, palmitate, prostaglandin E_1 or more Ca^{2+} (2.7 mM final concentration) to the perfusion medium. The effects of chloroquine and of the additions made during perfusion reached a steady state within 2-3 minutes and were completely reversible during subsequent control perfusion. Octanoate, palmitate, prostaglandin E_1

TABLE I

THE EFFECT OF CHLOROQUINE UPON CONTRACTILITY OF HEARTS FROM NORMAL FED, AND RAPESEED-OIL FED RATS

Perfusion	Cardiac contractility (% of control)			
	normal	(n)	rapeseed-oil	(n)
control	100		100	
" + 20 µM chloroquine	69.5 ± 2.4	(5)	n.d.[a]	
" + 50 µM chloroquine	30.5 ± 3.9[b]	(6)	70.7 ± 3.3[b]	(4)

[a] n.d. = not determined.

[b] $P<0.001$ for hearts from normal fed rats *vs* hearts from rapeseed-oil fed rats.

or excess Ca^{2+} were not able to overcome the chloroquine-inhibition of endogenous lipolysis, as measured by the glycerol release into the perfusate (not shown). The stimulatory effect of 1 mM octanoate upon chloroquine depressed contractility was not due to enhanced formation of acetyl-CoA since 5 mM 3-hydroxybutyrate, added to the chloroquine containing perfusion medium instead, did not have any effect (not presented).

A complete restoration of the depressed contractility was reached by injection of 10 µl of 3 mg/ml Ca^{2+}/Mg^{2+} ionophore X-537A, in 50% ethanol (w/v) in the aortic canula. 10 µl of 50% ethanol (w/v) instead was without effect. These findings suggest that chloroquine limits the Ca^{2+} availability to the contractile apparatus of the cells.

It has been shown before[3] that chloroquine does not alter hormone-stimulated glycogenolysis in heart. The addition of 10^{-7} M norepinephrine or 10^{-7} M glucagon leads to an increase of both the basal and chloroquine-depressed contractile states in hearts of normal fed, and rapeseed-oil fed rats (Table III). The further addition of octanoate proved to be without significant stimulatory effect.

DISCUSSION

In a previous communication from this laboratory we reported the chloroquine-induced inhibition of basal- and norepinephrine-stimulated lipolytic activity in rat hearts and in adipocytes[3]. The inhibition of lipolysis was complete in hearts from rats fed control food and 75% in hearts from rapeseed-oil fed rats. Since the weakly basic chloroquine is selectively accumulated by the lysosomes[4], and thereby increases the intralysosomal pH[17], it is concluded that endogenous lipolysis might be of lysosomal origin.

TABLE II

THE EFFECTS OF FATTY ACIDS, PROSTAGLANDIN E_1, EXCESS CALCIUM, AND IONOPHORE X-537A, UPON BASAL AND CHLOROQUINE-DEPRESSED CONTRACTILITY IN HEARTS FROM NORMAL FED, AND RAPESEED-OIL FED RATS

Perfusion	Cardiac contractility (% of control)			
	normal	(n)	rapeseed-oil	(n)
control	100		100	
" + 50 µM chloroquine	30.5 ± 3.9	(6)	70.6 ± 3.3	(4)
" + 1 mM octanoate	105.6 ± 4.6[b]	(5)	n.d.[a]	
" + 50 µM chloroquine + 1 mM octanoate	65.7 ± 2.8[c]	(5)	80.6 ± 2.0[d]	(4)
" + 0.5 mM palmitate	94.2 ± 0.8	(3)	n.d.[a]	
" + 50 µM chloroquine + 0.5 mM palmitate	54.8 ± 1.4	(3)	n.d.[a]	
" + 10^{-7} M prostaglandin E_1	112.9 ± 1.6	(5)	n.d.[a]	
" + 50 µM chloroquine + 10^{-7} M prostaglandin E_1	55.7 ± 4.0[c]	(6)	83.2 ± 3.2[d]	(4)
" + 2.7 mM calcium	127.3 ± 4.4	(5)	n.d.[a]	
" + 50 µM chloroquine + 2.7 mM calcium	89.1 ± 2.6[c]	(4)	90.7 ± 2.3[e]	(4)
" + 50 µM chloroquine + 30 µg X-537A	94.0-102.4	(2)	n.d.[a]	

[a] n.d. = not determined

[b] not significant

[c] P 0.001 vs control + 50 µM chloroquine

[d] P 0.05 vs control + 50 µM chloroquine

[e] P 0.005 vs control + 50 µM chloroquine

As judged from the glycerol release, chloroquine induces a severe reduction of the long-chain fatty acid formation in hearts from control fed rats. It also will reduce fatty acid levels in hearts from rapeseed-oil fed rats, which have been found to be increased[18].

The present work demonstrates that chloroquine depresses the contractile state of hearts from control rats more severely when compared with hearts from rapeseed-oil fed rats. At all experimental conditions chloroquine inhibited glycerol release. Since chloroquine probably is not acting upon energy metabolism (no stimulation of nucleoside release from the hearts was observed; not shown) and does not influence hormone-sensitivity, it may be assumed that the intracellular fatty acid levels are related to the decrease in contractility. Increase of the intracellular fatty acid concentration, by the addition of 1 mM octanoate or 0.5 mM palmitate indeed restores the decreased contractile state to a large extent in both groups of hearts. The stimulatory effect of octanoate

TABLE III

THE EFFECTS OF NOREPINEPHRINE AND GLUCAGON (PLUS ADDITIONAL OCTANOATE) UPON CHLOROQUINE-DEPRESSED CONTRACTILITY IN HEARTS FROM NORMAL FED RATS

Perfusion	Cardiac contractility (% of control) (n)
control	100
" + 50 µM chloroquine	30.5 ± 3.9 (6)
" + 10^{-7} M norepinephrine	120.4 ± 1.1 (7)
" + 50 µM chloroquine + 10^{-7} M norepinephrine	71.8 ± 4.8 (5)
" + 50 µM chloroquine + 10^{-7} M norepinephrine + 1 mM octanoate	81.9 ± 2.2[a] (3)
" + 10^{-7} M glucagon	141.4 ± 2.7 (5)
" + 50 µM chloroquine + 10^{-7} M glucagon	102.3 ± 4.9 (3)
" + 50 µM chloroquine + 10^{-7} M glucagon + 1 mM octanoate	109.0 ± 6.1[a] (3)

[a] not significant *vs* control + 50 µM chloroquine + 10^{-7} M norepinephrine resp. glucagon.

in hearts from normal fed rats is more prominent than in hearts from rapeseed-oil fed rats. Also by increasing the availability of Ca^{2+} to the contractile apparatus (by the addition of prostaglandin E_1, of which the ionophoric properties have been shown[19,20], the Ca^{2+}/Mg^{2+} ionophore X-537A or by increasing the calcium concentration in the perfusion fluid) an increase of the contraction amplitude is observed in both groups of hearts, in the presence of chloroquine. Since, however, the recovery of the chloroquine-induced decrease in contractility is not completely overcome by these additions, the drug may have an additional, inhibitory action, which is possibly related to membrane phosphorylation. The norepinephrine- and glucagon-induced stimulation of myocardial contractility is probably mediated by cyclic AMP-dependent protein kinase activation, leading to increased phosphorylation of the cell membrane and sarcoplasmic reticulum[21]. Indeed, under conditions of elevated intracellular cyclic-AMP levels the chloroquine-induced decrease in contractility is less marked. Fatty acids do not activate the myocardial adenylcyclase system[2], but may still be involved in protein kinase activation if they serve as Ca^{2+}-vehicles in the cells, since Ca^{2+}-stimulation of protein kinase is well documented. Therefore, it may be concluded that endogenous fatty acids possibly serve as Ca^{2+}-vehicles through membranes and as Ca^{2+}-shuttles between intracellular compartments, such as the sites of Ca^{2+}-storage and contractile proteins. This hypothesis implies that endogenous

lipolysis might be involved in the modulation (or regulation) of myocardial contractility.

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Paper 5. Sephadex-induced reduction of coronary flow in the isolated rat heart: A model for ischemic heart disease

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J. Mol. Cell. Cardiol. 9, 1977, 633-650

Journal of Molecular and Cellular Cardiology (1977) 9, 633–650

Sephadex-induced Reduction of Coronary Flow in the Isolated Rat Heart: A Model for Ischemic Heart Disease*

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H. STAM AND J. W. DE JONG. Sephadex-induced Reduction of Coronary Flow in the Isolated Rat Heart: A Model for Ischemic Heart Disease. *Journal of Molecular and Cellular Cardiology* (1977) 9, 633–650. Embolization of the coronary arteries with a polysaccharide (Sephadex) microsphere suspension caused a reduction in coronary flow of isolated perfused rat hearts. Obstruction by the spheres occurred in arterioles at the sub-epicardial layers of the ventricles, resulting in a whole-heart ischemia. Flow reduction was proportional to the amount of microspheres injected and was followed by an increase in flow. The increase in coronary flow after the initial decrease is probably due to vasodilation of the non-obstructed vessels. During the first 10-min period of this Sephadex-induced ischemia (with 0.70 mg of microspheres) the energy charge, ([ATP] $+\frac{1}{2}$ [ADP])/([ATP] + [ADP] + [AMP]), dropped from 0.87 $\pm$ 0.01 to 0.77 $\pm$ 0.01 ($\bar{x} \pm$ S.E., $P < 0.005$) and creatine phosphate content dropped from 34.9 $\pm$ 1.45 to 13.5 $\pm$ 0.63 ($P < 0.001$) μmol. g myocardial protein^{-1}. In the effluents of the ischemic hearts total nucleoside (adenosine, inosine, hypoxanthine) and lactate release increased indicating severe oxygen deprivation during ischemia. Mechanical performance measured as apex displacement, correlates well with left ventricular systolic pressure. Under ischemic conditions a rapid rise in contractility during the first minute (to 129% of control values, $P < 0.005$) was followed by a sharp decrease to 40%. The results of the experiments with ischemic hearts were compared with those from hearts made hypoxic by lowering the oxygen percentage in the gas-mixture bubbling through the perfusion medium (60%, 30% and 0% oxygen). With 30% oxygen, no significant differences were measured between ischemic and hypoxic hearts with respect to contractility (apex displacement), energy charge and creatine phosphate content, whereas total nucleoside and lactate release were in the same order of magnitude. The model described here could be useful in studies of ischemia in which a low coronary flow accompanying hypoxia is essential, and of consequences of the vascular response to embolization-induced ischemia.

KEY WORDS: Creatine phosphate; ATP; ADP; AMP; Adenosine; Inosine; Hypoxanthine; Lactate; Coronary flow; Apex displacement; Sephadex-induced ischemia; Hypoxia.

1. Introduction

Lack of oxygen during hypoxia and ischemia is responsible for the alteration of myocardial high-energy phosphate content while cardiac function is strongly impaired [*10*, *18*, *29*]. In studies with isolated perfused heart preparations, hypoxia was produced by lowering the oxygen tension of the perfusate without any decrease in coronary flow. Ischemia was produced by ligation of the main branch of the left

* This investigation was supported by the Dutch Heart Foundation.

coronary artery [5], by placing a one-way valve in the aortic outflow tract preventing retrograde perfusion of the coronary arteries during diastole [27, 31], by lowering of the perfusion pressure [14] or, *in vivo*, by constricting the abdominal aorta [7].

The reduced coronary flow under these ischemic conditions impairs the delivery of oxygen and energy substrates and prevents an adequate removal of metabolic products. During hypoxia as well as ischemia, metabolic breakdown products of ATP, such as adenosine, inosine and hypoxanthine, are released by the isolated perfused heart [3, 4, 16] and *in vivo* [7]. The increased anaerobic glycolytic flux enhances lactate production and release [31].

In the past, several investigators reported the use of embolization of the coronary arteries with microspheres of several kinds and sizes in order to produce a cardiogenic shock [1, 19, 33, 37]. Other studies dealt with the coronary vascular response after occlusion of a coronary artery or coronary embolization with spheres [13, 25]. The reduction in coronary flow after embolization and some circulatory, biochemical and pathological consequences were studied by Bing *et al.* [6, 34].

The abovementioned studies were performed in closed- or open-chested animals (dogs, calves), while Monroe *et al.* [26] first studied embolization in the isolated, supported dog heart.

In the study presented here, rat hearts were perfused retrogradely and reduction of coronary flow was induced by embolization of the coronary arteries with Sephadex G-25 microspheres. The ischemic state induced by embolization was characterized and the alterations in cardiac function, high-energy phosphate content of the myocardial tissue and release of nucleosides and lactate from the heart following embolization were compared with results obtained under hypoxia. The observed changes in cardiac function, high-energy phosphate content, nucleoside and lactate release after Sephadex addition indicate that ischemia induced by embolization of the coronary arteries may be useful to create the combination of hypoxia and decreased coronary flow, as is observed in ischemic heart disease.

2. Materials and Methods

Male Wistar rats (220 to 250 g) were fed *ad libitum*, anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg. kg^{-1} body weight, Abbott, Saint-Remy sur Avre, France). Prior to the cannulation procedure they were heparinized with 500 iu Thromboliquine (Organon, Oss, The Netherlands). The hearts were perfused retrogradely at 37°C and pH 7.4 with a modified Tyrode solution [24], equilibrated with 95% O_2 + 5% CO_2, according to the Langendorff technique. The hearts were paced at 300 pulses. min^{-1}, 2 ms duration and 0.2 mA. The perfusion pressure was 100 cm H_2O. Contractility was measured as apex displacement (AD), using a Hewlett-Packard transducer (7-DCDT-100), fitted with a core of tampon steel (1.3 g), which was hooked to the apex of the heart [41].

Left ventricular pressure was measured by inserting a 19-gauge needle through the apex of the heart into the left ventricle, connected with a Statham pressure transducer (model P23Gb). Pressure and displacement were recorded simultaneously on a SE Oscillograph 3006/DL. Hypoxia was induced by substituting the oxygen in the medium by nitrogen. Final oxygen percentages were 60, 30 and 0%.

Reduction of coronary flow was induced by injecting Sephadex G-25 *fine* microspheres (particle size 20 to 80 μm), suspended in oxygenated perfusion medium, into the lumen of the cannula. Hypoxia and embolization took place after 30 min of control perfusion.

At regular intervals, effluent was collected in liquid nitrogen and stored at −20°C. At the end of the perfusion beating rats hearts were clamped between aluminium blocks at the temperature of liquid nitrogen [*40*] and extracted as described by De Jong [*9*]. The myocardial tissue extracts were stored at −20°C. All determinations in extracts and effluents were carried out within 48 h after clamping the heart. Creatine phosphate, ATP, ADP and AMP were determined according to standard enzymatical procedures [*21*] on a Gilford 2400 recording spectrophotometer. Values for AMP were corrected for AMP-like material in the NADH (Boehringer, Mannheim). Adenosine, inosine and hypoxanthine were estimated with an Aminco DW-2, double-beam spectrophotometer. Lactate was determined enzymatically on an AutoAnalyzer II (Technicon, Tarry Town, New York) according to Apstein *et al.* [*2*]. Protein was measured by the biuret method [*15*]. All chemicals were analytical grade.

For microscopic examination whole hearts were rapidly frozen in isopentane cooled to −150°C with liquid nitrogen [*23*], 5 min after embolization with 0.70 mg of Sephadex microspheres. Sections, from different parts of the myocardium, at a thickness of 6 to 10 μm were cut on a cryostat-microtome. The cross-sections were stained with hematoxilin-eosin (HE). For histological demonstration of glycogen in the tissue staining was carried out with the periodic acid-Schiff (PAS) reaction. Due to the fixation and staining technique the Sephadex microspheres were subjected to shrinkage. Results are given in mean values ± S.E., and significance was calculated with Student's *t*-test. $P>0.05$ was considered to be not significant.

3. Results

Apex displacement as a marker for contractility

Measurement of the apex displacement of the beating heart can be regarded as the registration of isotonic contraction [*24*]. Apex displacement, so, is a parameter for contractility. Figure 1 illustrates the similarity of the changes in the apex displacement and left ventricular systolic pressure (LVSP) during anoxia and embolization-induced reduction of coronary flow (ischemia: see next section). Immediately after

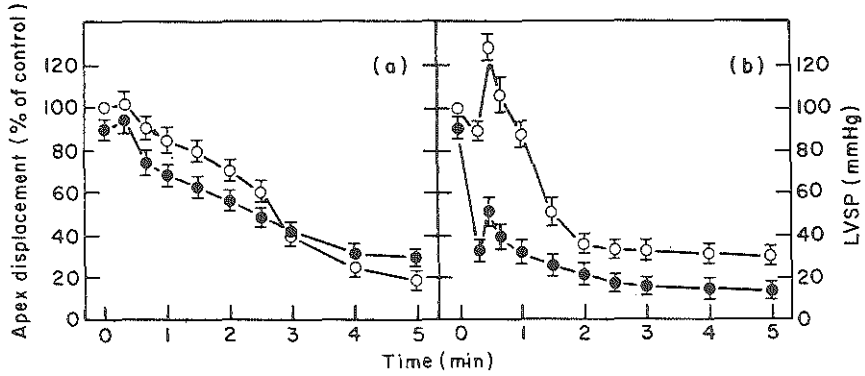

FIGURE 1. Changes in apex displacement and left ventricular systolic pressure (LVSP) of isolated, perfused rat hearts during (a) anoxia (0% oxygen) and (b) ischemia induced by Sephadex. Anoxia and ischemia started at time zero after a 30-min equilibration period ($\bar{x} \pm$ s.e., $n=3$). (○—○) Apex displacement; (●—●) LVSP.

injection of 0.70 mg Sephadex microspheres. LVSP decreases sharply from about 90 to 32 mm Hg ($P<0.001$) followed by an increase to 50 mmHg ($P<0.05$) after which a further decrease occurred. After 5 min of ischemia LVSP reached 18 mmHg. End-diastolic pressure under this condition remained constant at a value of 10 mmHg (not shown). Apex displacement showed a rather similar change when compared with the LVSP as ischemia occurs: there was a decrease to 89% ($P<0.005$) followed by a rise to 129% of the control value ($P<0.001$) and a rapid decrease to about 30% of control after 5 min of ischemia. During anoxia, the increase in apex displacement and LVSP after the first minute is less marked and both parameters decrease subsequently to 20 to 30% of control. The end-diastolic pressure increased from 10 mmHg at time zero to 17 mmHg after 5 min (not shown). The good correlation between the changes in LVSP and apex displacement under ischemic ($r = 0.72$, $P<0.01$) and anoxic ($r = 0.98$, $P<0.001$) circumstances is an indication that the isotonic contraction can be used as a parameter for the contractility of the heart.

Changes in coronary flow and ventricular contractility following embolization with Sephadex microspheres

The consequences of coronary embolization with Sephadex G-25 polysaccharide microspheres upon coronary flow and ventricular contractility are presented in Figures 2 and 3. After a 30-min control perfusion, different amounts of microspheres were injected into the cannula, causing embolization of the coronary system.

Immediately after the injection, coronary flow dropped, proportional to the amount of microspheres used. After a fast initial decrease, coronary flow increased.

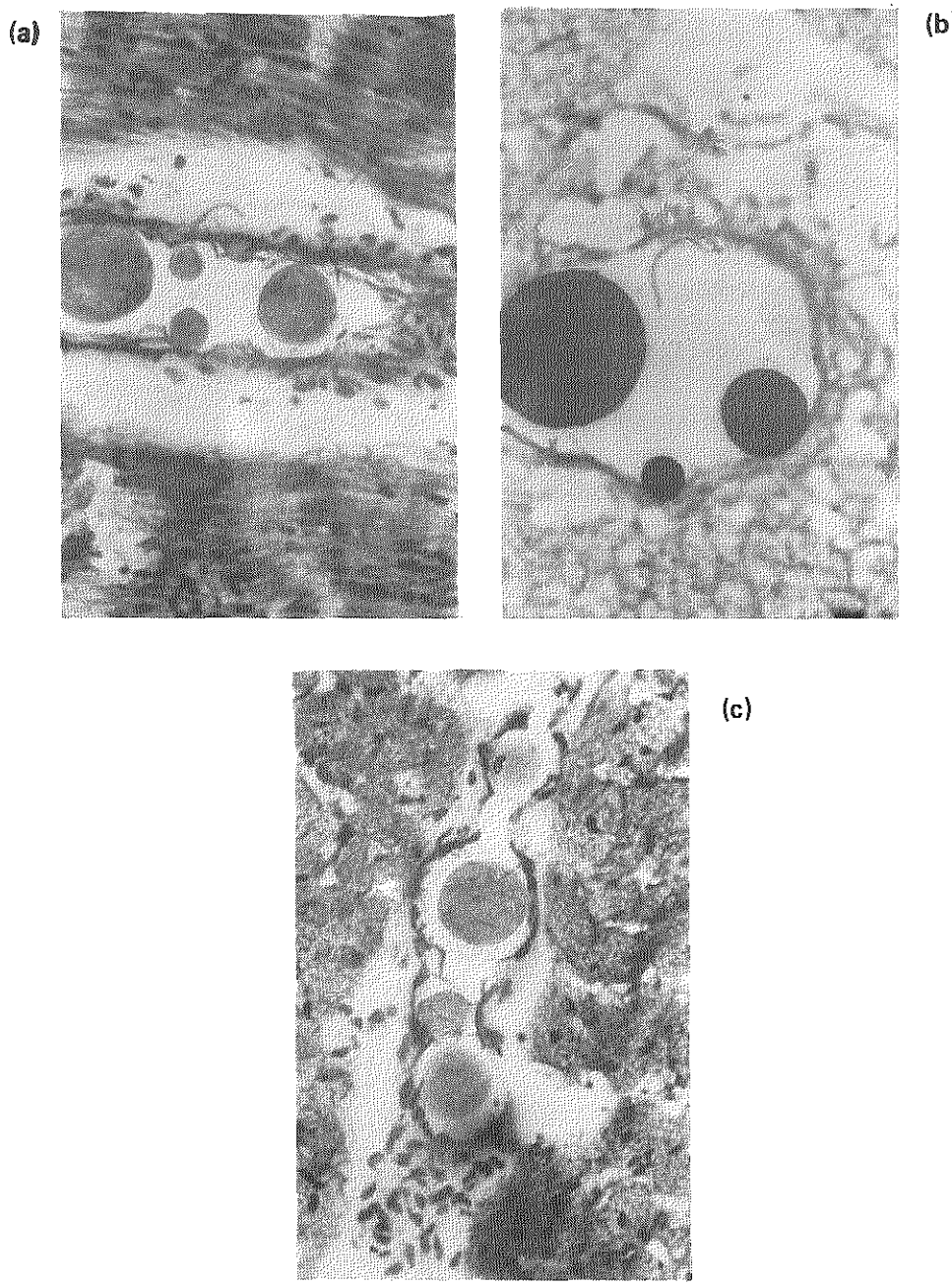

PLATE I. (a), (b), (c). Sephadex microspheres (20 to 80 μm) trapped in arteries of sub-epicardial tissue. The longitudinal section (c) shows spheres dislocating the arteriolar wall. (a) HE × 280; (b) PAS × 350; (c) HE × 245.

[*facing page* 636]

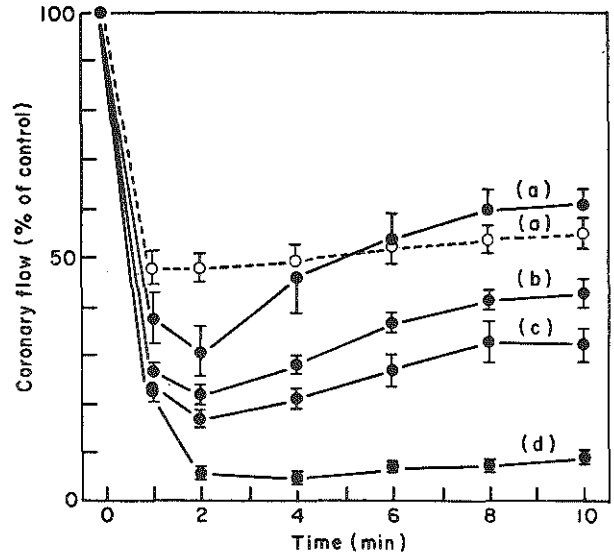

FIGURE 2. The effect of different amounts of Sephadex microspheres on coronary flow of isolated, perfused rat hearts in the absence (——) and presence (- - - -) of 100 μM adenosine. Microspheres were injected at time zero after a 30 min equilibration period ($\bar{x} \pm$ S.E., n=4–8). (a) 0.14 mg; (b) 0.35 mg, (c) 0.70 mg; (d) 1.40 mg.

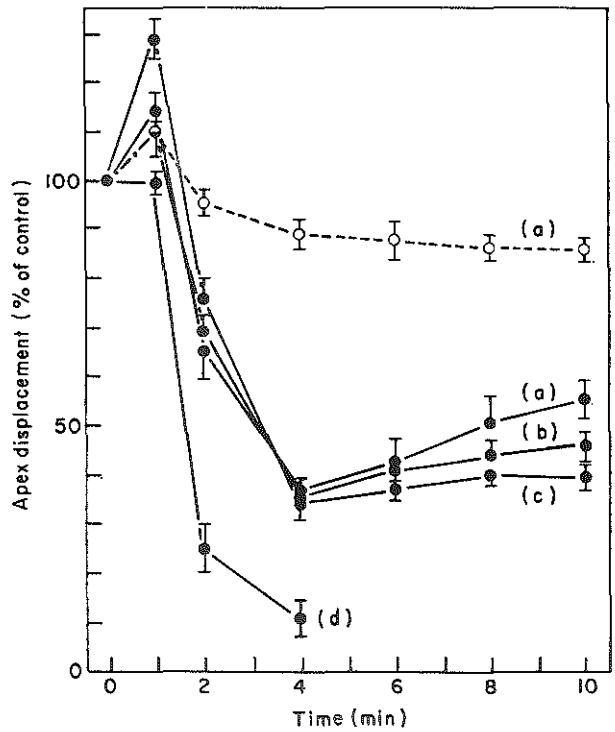

FIGURE 3. The effect of different amounts of Sephadex microspheres on apex displacement of isolated, perfused rat hearts in the absence (——) and presence (- - - -) of 100 μM adenosine (see legend to Figure 2). (a) 0.14 mg; (b) 0.35 mg; (c) 0.70 mg; (d) 1.40 mg.

Ten min after inserting the microspheres, coronary flow rate reached a constant level. Coronary flow 10 min after the onset of embolization-induced ischemia was higher than that observed after 2 min of ischemia. These differences were statistically significant when 0.14, 0.35 and 0.70 mg of microspheres were injected ($P<0.05$, $P<0.025$ and $P<0.025$, respectively).

Perfusion in the presence of 100 μM adenosine in the perfusion fluid caused a severe increase in coronary flow (6.40 ± 0.61 during control in the absence, and 22.15 ± 0.27 ml. min^{-1}. g wet wt^{-1} in the presence of adenosine). With adenosine present, embolization with 0.14 mg Sephadex microspheres led to a decrease in coronary flow of about 50% of control (10.1 ± 0.50 ml. min^{-1}. g wet wt^{-1} which was not followed by the increase occurring in the absence of adenosine.

Figure 3 illustrates the changes in ventricular contractility following embolization with microspheres. Injection of 0.14, 0.35 and 0.70 mg of Sephadex G-25 is followed by a significant increase in contractile behaviour, responding to the dose of microspheres injected ($P<0.001$). No increase was detected when embolization with 1.40 mg of microspheres took place. After the initial rise in contractility, a sharp decrease occurred, followed by an enhancement in contractility, in a dose-dependent manner. When 0.14 and 0.35 mg of microspheres were used, left ventricular contractility, 10 min after injection of the spheres was significantly higher than contractility occurring after 4 min ($P<0.05$). For 0.70 mg of Sephadex, contractility (10 min after injection) did not differ significantly from contractility after 4 min. With 0.35 and 0.70 mg of microspheres ventricular contractions became irregular 10 min after injection. Embolization with 1.40 mg of microspheres caused a rapid deterioration in contractility and irregular contractions of the heart were seen after 4 min.

Embolization with 0.14 mg of microspheres in the presence of 100 μM adenosine in the perfusion fluid also resulted in an increase in contractility to about 110% of control ($P<0.01$). This increase is followed by a slow decrease to a constant level of about 85% of control.

Microscopic findings

Sephadex microspheres stain light-red with hematoxilin-eosin and deep-purple with the periodic acid-Schiff reaction. Examination of cross-sections from the heart indicated that clusters of spheres were present in the arteries and larger arterioles in the sub-epicardial and supra-myocardial layer of especially the left ventricle (Plate 1). As Plate 1 (c) indicates, the spheres distend the arteries they obstruct. No spheres were seen in the deeper parts of the myocardium and the endocardium. In cross-sections containing obstructed arterioles and arteries also few vessels with, apparently, the same size were observed which did not contain spheres. Preparations from the distal part of the myocardium showed less microspheres and no spheres

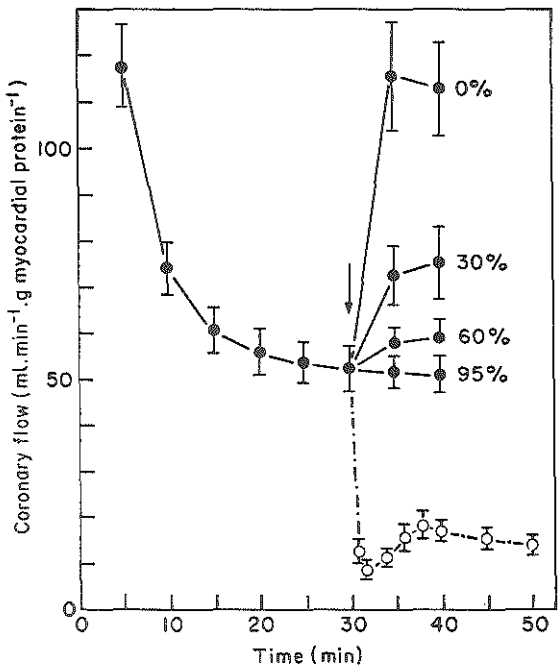

FIGURE 4. The effect of different degrees of hypoxia (——) and Sephadex-induced ischemia (– . –) on coronary flow of isolated perfused, rat hearts. The arrow indicates the induction of hypoxia and ischemia ($\bar{x} \pm$ S.E., n=4–8).

could be observed in sections cut from the apex of the heart. All sections were examined for glycogen distribution. No difference in purple-red staining could be observed between the different cross-sections and between epi-, myo- and endocardial tissue, indicating a homogeneous distribution of the tissue glycogen present after embolization.

The reduction of coronary flow induced by injection with 0.70 mg of Sephadex microspheres was used as a model for ischemia and metabolic and hemodynamic alterations were compared with changes during different degrees of hypoxia. The results are presented in the following sections.

Changes in coronary flow and contractility during control, hypoxia and Sephadex-induced ischemia

In Figures 4 and 5 the apex displacement and coronary flow under different hypoxic and ischemic conditions are presented. During the 30 min equilibration perfusion, coronary flow dropped from the high value (118 ml. min^{-1}. g myocardial $protein^{-1}$) 5 min after opening the thorax, to about 52 ml. min^{-1}. g myocardial $protein^{-1}$.

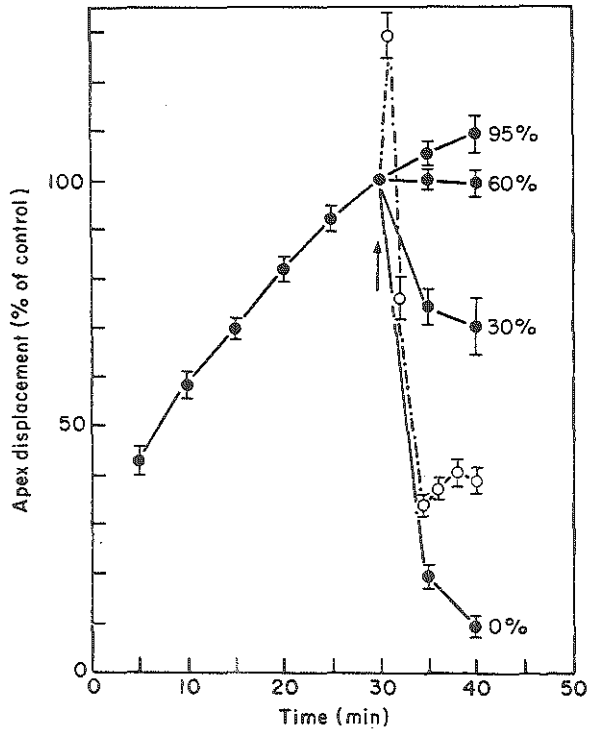

FIGURE 5. The effect of hypoxia (——) and ischemia (– . –) on apex displacement of isolated, perfused rat hearts (see legend to Figure 4).

An inverse relationship between oxygen percentage in the gas mixture and coronary flow was observed (see Figure 4). Injection of Sephadex into the cannula caused a rapid reduction in flow (80 to 90%, $P<0.001$). A 70% reduction in flow was observed after 10 min. In contrast with the coronary flow, apex displacement of the heart 5 min after opening the thorax was small (see Figure 5). Apex displacement after 30 min was taken as 100%, although contractility was only constant after 40 to 50 min. Apex displacement decreased with the severity of hypoxia, while during the first minute of ischemia an increase of contractility by about 30% ($P<0.001$) was observed, followed by a rapid deterioration.

Energetic state of the hypoxic and ischemic hearts

The changes in myocardial tissue levels of creatine phosphate, ATP, ADP and AMP were determined in control hearts, in hearts submitted to different degrees of hypoxia, and in hearts with embolization-induced ischemia. The results are presented in Figures 6 and 7. In hypoxic hearts the level of creatine phosphate rapidly declines, dependent on the oxygen percentage of the Tyrode solution ($P<0.005$ for

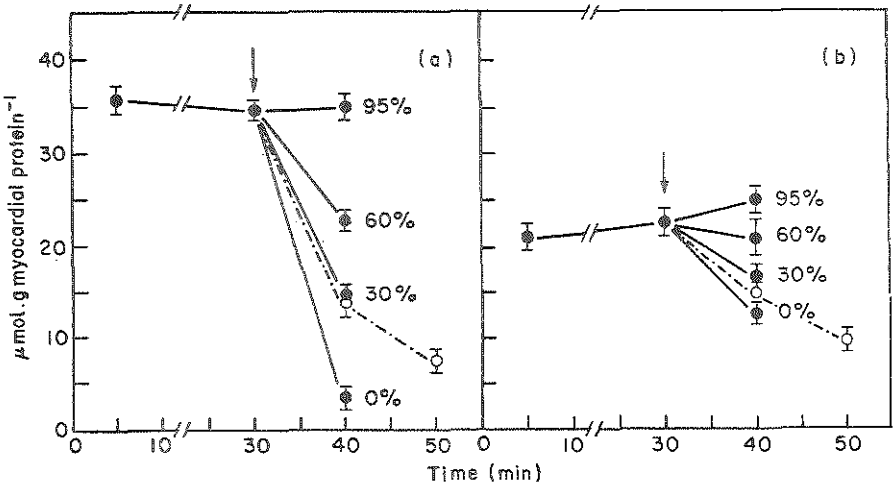

FIGURE 6. Creatine-phosphate (a) and ATP (b) content in hypoxic (——) and ischemic (– . –) rat hearts (see legend to Figure 4).

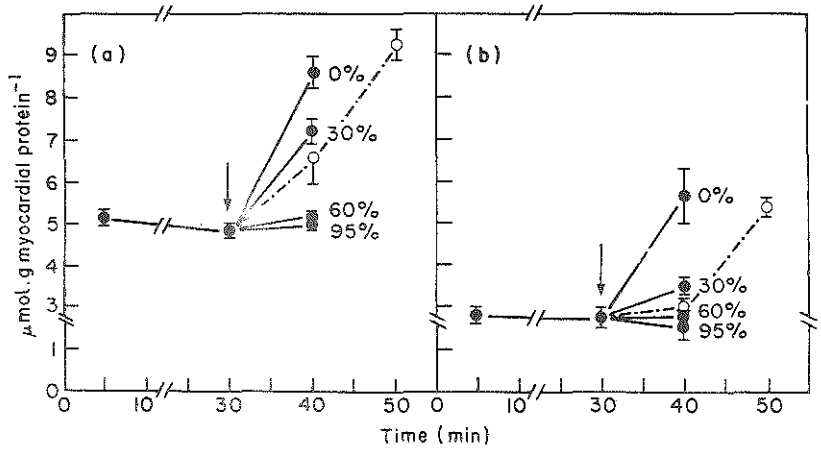

FIGURE 7. ADP (a) and AMP (b) contents in hypoxic (——) and ischemic (– . –) rat hearts (see legend to Figure 4).

all degrees of hypoxia compared with the control value). The decrease in tissue levels of ATP is less rapid than creatine phosphate breakdown, but still significant ($P<0.025$ for all degrees of hypoxia).

The decrease in tissue ATP levels is accompanied by a rise in ADP and AMP contents during the different degrees of hypoxia ($P<0.05$ for ADP and $P<0.01$ for AMP). The energy charge of the myocardial tissue, ([ATP] + ½ [ADP])/([ATP] + [ADP] + [AMP]), decreased from 0.87 ± 0.01 with fully oxygenized medium to 0.66 ± 0.22 under anoxic conditions ($P<0.005$). During the first 10 min of

ischemia, myocardial creatine phosphate levels dropped about 60%: after 20 min only 20% was left ($P<0.005$). Also the ATP breakdown during ischemia is significant: 45% after 10 min and 60% after 20 min ($P<0.005$ in both cases). The catabolism of ATP is reflected in the increase of ADP and AMP levels ($P<0.01$ for ADP and $P<0.001$ for AMP after 20 min of ischemia). Energy charge dropped from 0.87 ± 0.01 to 0.77 ± 0.01 ($P<0.005$) early during ischemia and finally to 0.61 ± 0.03 ($P<0.005$), demonstrating the low energetic state of the myocardial tissue.

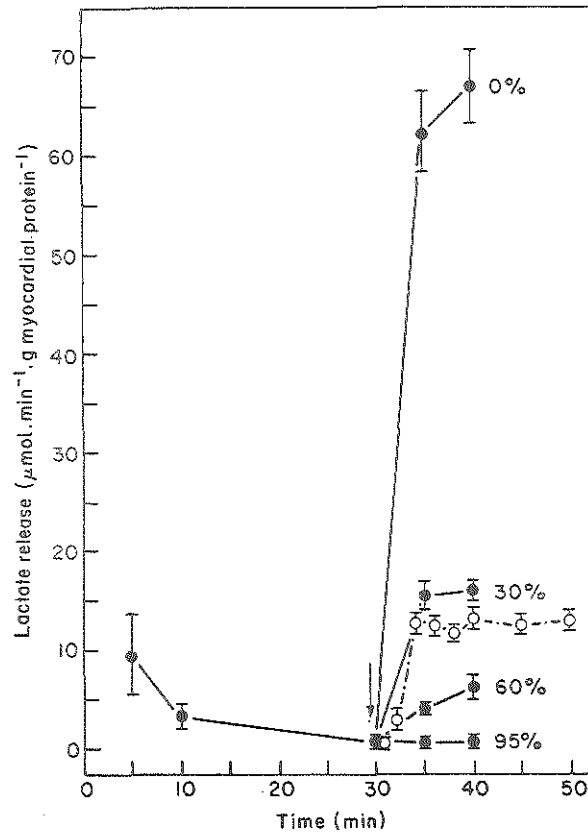

FIGURE 8. Lactate release from isolated perfused rat hearts during hypoxia (——) and ischemia (– . –) (see legend to Figure 4).

The impaired energy metabolism during ischemia as well as hypoxia is reflected in the rate of lactate release from the heart. As Figure 8 indicates, the lower the oxygen tension during experimental hypoxia, the higher the lactate release. This release increased rapidly during the first minutes of perfusion and reached an equilibrium after about 2 min. During ischemia, lactate release could be found

2 min after the injection of the Sephadex particles and reached a constant level after about 4 min. Figure 8 demonstrates that the lactate release during ischemia was somewhat lower than the release during perfusion with medium saturated with 30% oxygen ($P<0.05$ after 10 min).

Purine nucleoside release from the heart under hypoxic and ischemic conditions

The release of AMP breakdown products from the perfused rat heart during ischemia and different degrees of hypoxia is demonstrated in Figure 9. During hypoxia, adenosine, inosine and hypoxanthine were released from the heart at higher rates when the oxygen supply in the perfusion medium was lower. Especially during severe hypoxia nucleoside release was high, which was most apparent in the rapid inosine release. During ischemia, no adenosine was released in the first 10 min, whereas inosine and hypoxanthine did appear progressively in the effluent. The intramyocardial concentration of adenosine after 10 min of ischemia was 1.21 ± 0.23 μmol.g myocardial protein^{-1}. The low release of adenosine in the first 10 min was overcome in the second 10-min period of ischemia, when it was quickly released.

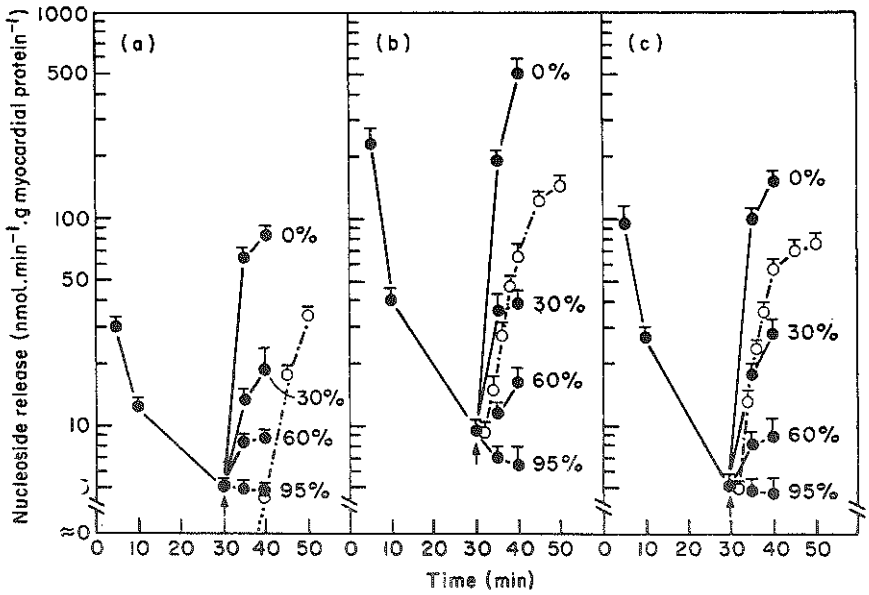

FIGURE 9. Semilogarithmic plot of adenosine, inosine and hypoxanthine release from isolated, perfused rat hearts during hypoxia (——) and ischemia (– . –) (see legend to Figure 4). (a) Adenosine; (b) inosine; (c) hypoxanthine.

TABLE 1. Correlations between metabolic and functional changes in isolated perfused rat hearts during Sephadex-induced ischemia and different degrees of hypoxia

		Correlation coefficient (r)			
		vs Energy charge	vs Creatine phosphate	vs Adenosine release	vs Lactate release
Coronary flow	hypoxia*	—	—	0.94	—
	ischemia†	—	—	−0.16‡	—
Apex displacement	hypoxia*	0.98	0.94	—	−0.98
	ischemia†	0.95	0.97	—	−0.94

* 10 min after subjecting rat hearts to different degrees of hypoxia all parameters shown were determined and correlated to each other.

† Perfused rat hearts were made ischemic by injecting Sephadex microspheres into the coronary circulation system. At regular intervals during ischemia the parameters shown were determined and correlated.

‡ Not significant, all other correlations $P < 0.001$.

In Table 1, some correlations between metabolic and functional changes during ischemia and hypoxia are presented. During ischemia as well as during hypoxia, high-energy phosphate content, energy charge and lactate release correlate well with contractility. Coronary flow and adenosine release correlate significantly during hypoxia, while no correlation is found during ischemia.

4. Discussion

Pressure development in the left ventricle is often used as a parameter for the mechanical performance of the heart [*17, 20*]. In this study, the isotonic contraction, apex displacement was taken as marker for cardiac function. Evidence is presented that the apex displacement correlates well with the LVSP under ischemic and anoxic conditions. Measurement of the apex displacement is simple and the trauma caused by connecting the tampon steel to the apex of the heart seems less compared to the insertion of a needle or left atrial appendectomy prior to balloon catheterization [*35*]. The investigations presented here describe a model for the induction of ischemia by embolization of the coronary arteries by Sephadex microspheres with a particle size of 20 to 80 μm. The reduction of coronary flow caused by embolization is dependent on the amount of microspheres used. This implicates that the higher the amount of microspheres injected the greater the number of vessels obstructed by the spheres.

Obstruction occurs at the level of the coronary arteries and larger arterioles in the sub-epicardial layers of, especially, the left ventricle. So, epicardial flow, the major contributor of total coronary flow probably is affected especially by embolization.

The presence of non-obstructed arteries indicates that flow reduction is not uniform. The arterial muscular wall is well developed and these vessels play an important role in the circulatory mechanism. Vasomotion is controlled by the many sympathic fibres ending in the wall musculature and by vasoactive metabolites. In the isolated, perfused heart preparation, autonomic influences can be excluded. A second regulatory possibility is found in the precapillary sphincters, a small muscular cuff at the origin of capillaries and met-arterioles, the tone of which regulates fluid entrance to the capillary bed. The sphincter muscle-tone is sensitive to local chemical influences.

Cardiac metabolic activity and coronary flow rate are very well related and metabolites have been suggested to mediate coronary flow during different physiological and pathophysiological states. Berne and Rubio [*3*] reported the potent vasodilator adenosine as possible mediator of coronary blood flow.

The increase in coronary flow following the initial decrease after injection of the microspheres is absent in the presence of adenosine. It may be concluded that this increase is caused by coronary dilation of the non-obstructed vessels [*13*]. The coronary vascular response is less marked when more arterioles are obstructed after injection of a larger amount of microspheres. It is emphasized that coronary flow in embolized hearts, in the presence of adenosine, is still about three times higher than flow in embolized (ischemic) hearts perfused without adenosine. Obviously, in the presence of exogenous adenosine a greater number of capillaries is open for perfusion. Also ventricular contractility recovered from the low level 4 min after different degrees of embolization, probably by a partial re-establishment of energy metabolism due to the increase in coronary flow rates.

Several models for experimental ischemia in isolated heart preparations have been described [*5–7, 14, 27, 31, 39*]. The reduction of retrograde aortic perfusion as developed by Neely *et al.* [*17*] is often used in studies of biochemical and hemodynamic changes during the acute phase of whole heart ischemia. In this model, however, the reduction of coronary flow is an all or nothing effect in response to closure of the aortic outflow tract and the consequences of coronary vasodilation, occurring after the onset of ischemia cannot be studied in this preparation.

Embolization with microspheres reduces epicardial flow in a dose-dependent manner and the vascular response (vasodilation) to ischemia is only absent when an "overdose" of microspheres is used. With 0.70 mg of Sephadex microspheres not all the arterioles are obstructed. The glycogen, still present after 5 min of ischemia is distributed homogeneously over the myocardial tissue.

If embolization would cause local ischemia, differences in tissue glycogen staining between ischemic and non-ischemic tissue would be expected. Since this was not the case it can be concluded that, although flow reduction induced by microsphere embolization seemed not uniform, a whole heart ischemia is induced after injection of 0.70 mg of microspheres.

A substantial number of our experiments was carried out in which coronary

flow was reduced to 70% of control. Rovetto *et al.* [*31*] reduced the flow through isolated hearts to 90% using an one-way valve in the aortic outflow tract and of De Jong *et al.* [*8*] with a 74% reduction of the flow through the anterior descending coronary artery in open-chest pigs.

The changes in myocardial function and metabolism during ischemia (embolization with 0.70 mg of microspheres) were compared with changes of metabolism and function in rat hearts subjected to different degrees of hypoxia.

The increase in contractility (apex displacement) immediately after insertion of the microspheres is probably due to increased myocardial levels of adenosine 3', 5'-cyclic monophosphate mediated by catecholamines [*11, 30, 36*]. When 1.40 mg of microspheres are injected contractility remains at a 100% level for 1 min before a rapid deterioration takes place. The absence of an increase seen when an "overdose" of microspheres was used is probably caused by a fast improvement of energy metabolism, overruling the proposed catecholamine effect. The rise in contractility during hypoxia is far less marked which does not implicate any physiological importance. The abrupt interruption of coronary flow by Sephadex is more likely to give rise to increased cAMP levels than the more gradual induction of hypoxia. As can be seen from Figures 4 to 9 the anoxic period during part of the operation required for the cannulation of the aorta, and mounting of the heart in the perfusion apparatus, is responsible for the differences in mechanical and biochemical parameters between 5 and 30 min of the equilibration period. After this period the heart is assumed to have recovered from anoxia during surgery. The metabolic effects of the impaired coronary flow after Sephadex injection are drastic. Myocardial creatine phosphate and ATP levels decline and the increase in ADP and AMP contents are a reflection of this ATP-breakdown. The lactate release from ischemic heart indicates a severe degree of hypoxia accompanying ischemia. The decline in lactate release in working rat hearts after about 12 min of ischemia mentioned by Neely *et al.* [*27*], indicating a restriction of glycolytic flux, was not seen during our ischemic conditions where a steady-state lactate release was observed. This may be a consequence of the increased coronary flow after about 10 min of ischemia. The importance of coronary flow rates on the glycolytic flux has been reported in a recent study of Neely *et al.* [*28*]. Inhibition of the glucose utilization occurs at lower flow rates. The 70% reduction of flow in non-working hearts (this study) did not have an effect on the enhanced glycolysis.

The high rate of ATP breakdown is manifested in the release of purine nucleosides. AMP is degraded to adenosine by 5'-nucleotidase, localized in the cell membrane [*4*]. Adenosine present in the interstitial fluid gives rise to vasodilation of the coronary arterioles [*32*]. No adenosine is released during the first period of ischemia although it is formed intracellularly. The low coronary flow and the high rate of adenosine deamination [*9*] to inosine probably are responsible for this lack in adenosine release. Furthermore, no adenosine deaminase activity could be found in the effluents (not shown). We were able to demonstrate appreciable amounts

of intramyocardial adenosine after 10 min of ischemia. Adenosine deamination could be insufficient and as a result adenosine leaks out of the heart. Under hypoxic conditions an increase in coronary flow and the fall of contractility is seen to be dependent on the amount of oxygen in the perfusate. Creatine phosphate and ATP were also broken down at a rate dependent on the oxygen supply. This rapid catabolism elevated ADP and AMP levels, and is reflected in the release of adenosine, inosine and hypoxanthine.

The clear correlation between adenosine release and coronary flow during different degrees of hypoxia indicates the vasodilatory effect of this nucleoside (Table 1) [*4*]. The higher glycolytic rate is responsible for lactate release from the heart and also is dependent on the oxygen supply. The lower rate of lactate release during ischemia compared with hypoxia (30% oxygen) and anoxia (0% oxygen) illustrates the lower glucose supply and the inadequate catabolite removal due to the lower coronary flow.

The mechanical performance during perfusion with 60% oxygen and 30% oxygen parallels the decreasing myocardial ATP-levels (see Figures 6 and 7). During anoxia (0% oxygen), the fall in apex displacement is more severe than ATP breakdown, indicating that factors other than tissue levels of creatine phosphate and ATP are rate limiting under severe hypoxic conditions [*12*]. The clear negative correlation between lactate release and the apex displacement during hypoxia as well as during ischemia (Table 1) may be an indication that lactate, by decreasing the intracellular pH, plays a role in the deterioration of contractility. However, Lai and Scheuer [*20*] concluded that a fall in intracellular pH was not responsible for early mechanical damage during hypoxia (20% oxygen). Certainly the effects of high lactate concentrations on cardiac function could be important since Marrannes *et al.* [*22*] and Wissner [*39*] reported some effects of lactate upon the excitability of cardiac Purkinje fibres during metabolic acidosis.

The irregular contractions of the heart after 10 min of ischemia and anoxia could thus be caused by lactate. However, hydrogen-calcium competition concerning actine-myosine interaction [*18*, *19*] and calcium release from intracellular stores [*38*] have been proposed as factors responsible for the deterioration of cardiac contractility during hypoxia and ischemia.

Comparing the consequences of Sephadex-induced ischemia and hypoxia, it can be concluded that changes in metabolism and function induced by hypoxia (30% oxygen) and embolization-induced ischemia are in the same order of magnitude for myocardial energy charge, creatine-phosphate content and the release of lactate, inosine and hypoxanthine. Adenosine release is present during hypoxia, but absent in the first ischemic period. In this model for acute, whole heart ischemia the vascular response to the impaired metabolism is retained. In conclusion, a model for ischemic heart disease can be obtained with Sephadex-induced reduction of coronary flow.

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Paper 6. Effect of glucose on AMP-catabolite release during fatty acid perfusion in normal and ischemic rat hearts

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EFFECT OF GLUCOSE ON AMP-CATABOLITE RELEASE DURING FATTY ACID PERFUSION IN NORMAL AND ISCHEMIC RAT HEARTS

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Summary

Isolated rat hearts were perfused retrogradely with a modified, oxygenated Tyrode solution containing 0.5 mM palmitate (complexed to albumin in a molar ratio of 6:1) with or without 11 mM glucose. Fatty acid perfusion induced a decrease in contractile behaviour which was partly counteracted by glucose. The energy charge $\{([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])\}$ of the tissue was not altered although a significant drop was observed in creatine phosphate/ATP ratio in the absence of glucose. The release of AMP-catabolites, adenosine, inosine and hypoxanthine, occurring during fatty acid perfusion was reduced by glucose. In the absence of glucose fatty acids still induce lactate release indicating an enhanced glycogenolysis. In ischemic hearts the fatty acid-induced decrease in mechanical performance was significantly more severe when glucose was absent, while the glucose protection could also be observed in the energy charge of the ischemic tissue and the release of AMP-catabolites in the coronary effluent. The results suggest that loss of adenosine, inosine and hypoxanthine might contribute to the detrimental actions of a high fatty acid/albumin ratio upon the myocardium and confirms the protective action of glucose.

Although fatty acids are the main substrate for the myocardium (1), many investigations have been presented concerning the deleterious effects of free fatty acids and their CoA esters upon cardiac structure (2), function (3-7) and metabolism (3-8) in normal and ischemic hearts. Fatty acid-induced suppression of myocardial function and metabolism appeared to be dependent on chain-lenght, concentration, their molar ratio to albumin and the presence of other substrates (i.e. glucose). Free fatty acid (FFA) uptake is mainly regulated by their molar ratio to albumin (9) while, apart from FFA uptake, β-oxidation rate and the balance between esterification and lipolysis determine the intracellular fatty acid (+ fatty acyl CoA and fatty acyl carnitine) concentration. Of particular interest are the adenine nucleotide translocase inhibition (10) and oxidative phosphorylation uncoupling (11) properties of high intracellular levels of fatty acids and their CoA esters.

These actions lead to a decrease of the cytoplasmatic ATP/ADP ratio. The decrease in cytoplasmatic ATP levels may be buffered by the creatine kinase reaction. A decrease in creatine phosphate/ATP ratios during perfusion with a high acid/albumin ratio has been presented (8). The enhanced cytoplasmatic ADP concentration leads to an increased AMP formation which may initiate glycolysis (12). AMP may be deaminated (by AMP-deaminase) to IMP although this reaction has recently been proven to be inhibited by long-chain fatty acyl CoA (13). Another part of the molecule may be dephosphorylated by 5'-nucleotidase (14).

Effects of fatty acids upon glucose uptake, glycolysis and glycogen metabolism have been described. Low molar fatty acid-albumin ratios (0.6:1 and 1.2:1) inhibited glucose uptake and oxidation and abolished glycogenolysis (15). Higher molar ratios (3:1) stimulated glycogenolysis and lactate release from the heart (16). Effects of fatty acid perfusion upon the energic state of myocardial tissue are not well documented.

In this paper we describe the effects of a high fatty acid/albumin ratio (6:1), when it is likely to have an increased intracellular fatty acid concentration, upon lactate and nucleotide metabolism of normal and ischemic rat hearts. Ischemia was induced by the introduction 0.70 mg of an inert polysaccharide-microsphere suspension (Sephadex) in the coronary circulation (17). The results presented here indicate that the fatty acid-induced glycogenolysis and release of AMP breakdown products (adenosine, inosine and hypoxanthine), which could partially be inhibited by glucose, is additional evidence of the deleterious effects of free fatty acids upon myocardial function in normal and ischemic hearts.

Methods

Male Wistar rats (200-300 g) which had free access to water and laboratory chow, were anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg body weight). Prior to the cannulation procedure the animals were intravenously heparinized with 500 I.U. tromboliquine. After the cannulation of the aorta the hearts were excised and perfused retrogradely at a perfusion pressure of 100 cm H_2O (10 kPa) as described previously (8, 17, 18) and paced at a rate of 300 beats/min. Contractile behaviour was recorded as apex displacement, which correlates well with the left ventricular systolic pressure (17), and coronary flow was determined by timed collection. Preparation and contents of the perfusion buffers was identical as reported elsewhere (8, 17).

After a stabilization perfusion period of 30 min with glucose-containing perfusion buffer the hearts were perfused with a modified Tyrode buffer containing 0.5 mM potassium palmitate complexed to fatty acid free bovine serum albumin (BSA) in a molar ratio of 6:1 with or without 11 mM glucose. After the subsequent 10 min perfusion, when the coronary flow rate and apex displacement has reached equilibrium, 0.70 mg of polysaccharide-microspheres (Sephadex G-25, particle size 20-80 µm) suspended in the proper buffer was injected in the aortic cannula. Ischemic perfusion lasted 20 min. At the end of each perfusion period (30, 40 and 60 min after starting perfusion) the hearts were freeze-clamped (19) and extracted (20). The determination of creatine phosphate, ATP, ADP and AMP in the myocardial tissue extracts as well as lactate, adenosine, inosine and hypoxanthine in the coronary effluents occurred exactly as described before (17). The energetic state of the tissue was expressed as energy charge (21), $\{([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])\}$, and the creatine phosphate/ATP ratio.

Values of coronary flow and apex displacement were expressed as per cent of control (30 min value). All data are given in mean values $\pm$ S.E.M., and statistical analysis was performed with the unpaired t-test. $P > 0.05$ was considered to be not significant. n is the number of observations.

Results

The consequences of fatty acid perfusion and microsphere-induced myocardial ischemia upon coronary flow and apex displacement are presented in Fig.1. Perfusion with 0.5 mM palmitate (in a molar ratio of 6:1 with BSA) is followed by a 30% increase in coronary flow ($p < 0.001$). This enhancement of flow is independent of the presence of 11 mM glucose during fatty acid perfusion ($p < 0.001$). Immediately after injection of the polysaccharide-microsphere suspension coronary flow rates fall to about 15% of control in both groups of hearts, but increased gradually to about 20%. Furthermore, the flow response after the induction of ischemia in the fatty acid perfused hearts is independent of the presence of 11 mM glucose in the perfusion fluid.
Perfusion with a high molar palmitate/BSA ratio leads to a 33% decrease in apex displacement. However, with 11 mM glucose present during fatty acid perfusion only a 13% decrease in this contractility index was seen ($p < 0.001$).

FIG.1

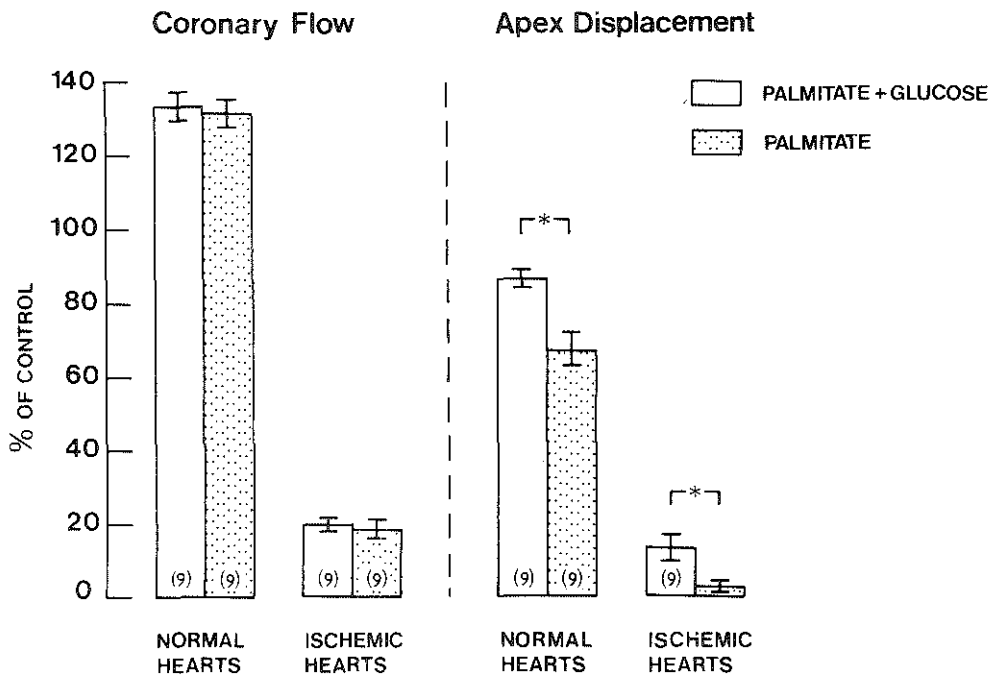

FIG.1. The effect of 0.5 mM palmitate (complexed to BSA in a molar ratio of 6:1) ± 11 mM glucose upon coronary flow and apex displacement in normal and ischemic rat hearts. The number of observations is show in parenthesis at the bottom of each bar. * = $p < 0.05$.

Apex displacement also decreases severely after induction of ischemia (10 min) and this decrease in contractility is again less ($p < 0.001$) when 11 mM glucose is present during palmitate perfusion. Irregular contractions are observed after about 10 min of ischemia when glucose is absent and only after 15 min when glucose was present (not shown).

The energetic state of the myocardial tissue during fatty acid perfusion and after 20 min of microsphere-induced reduction of coronary flow is presented in Fig. 2. The high-energy potential of the myocardial cells can be expressed as the energy charge $\{([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])\}$ (21) and as the creatine phosphate (CrP)/ATP ratio.

The energy charge of rat hearts after control perfusion with Tyrode buffer containing 11 mM glucose amounts 0.87 ± 0.01 (n=8) and the CrP/ATP ratio is 1.74 ± 0.04 (n=8). Perfusion with a high molar ratio of palmitate/BSA with or without 11 mM glucose does not influence the energy charge of the tissue, but a drop of the CrP/ATP ratio has been observed in the absence of glucose during palmitate perfusion ($p < 0.001$). After a 20 min period of ischemia a fall in energy charge is observed in both groups of hearts ($p < 0.001$ in both cases) with a lower energy charge in hearts perfused with palmitate as the sole substrate ($p < 0.05$). The CrP/ATP ratio decreases during the 20 min ischemic period and this decrease is not significantly different for both groups of hearts.

FIG.2

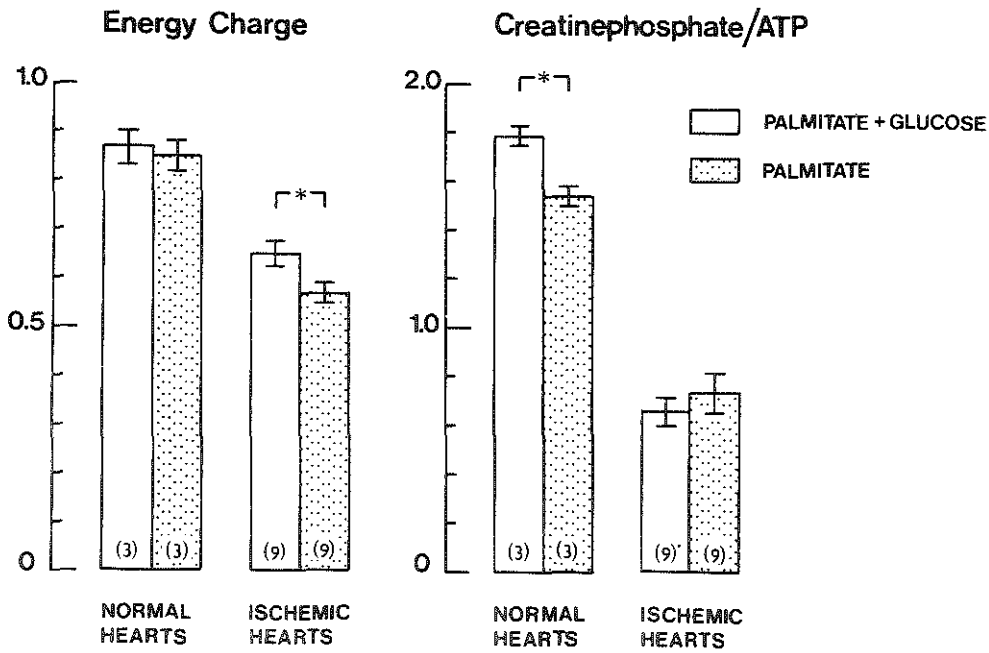

FIG.2. Changes in energy charge and CrP/ATP ratio of rat myocardial tissue during perfusion with 0.5 mM palmitate (complexed to BSA in a molar ratio of 6:1) ± 11 mM glucose and during ischemia. The number of observations is shown in parenthesis at the bottom of each bar. * = $p < 0.05$.

As presented in Fig.3 palmitate perfusion leads to an increased lactate release in the coronary effluent.
Switching from the control glucose containing perfusion medium to the palmitate containing buffer (without glucose) is followed by lactate production and release, indicating glycogenolysis. With glucose present a more severe lactate extrusion from the hearts occurs ($p < 0.05$ after 10 min of palmitate perfusion), suggesting limited availability of glycogen. Upon insertion of the polysaccharide microspheres, flow reduction causes a sharp drop in the lactate release, while during prolonged ischemia an increased lactate release is observed, which is higher during palmitate perfusion in the presence of 11 mM glucose ($p < 0.02$ after 10 min and $p < 0.05$ after 20 min of ischemia).

FIG.3

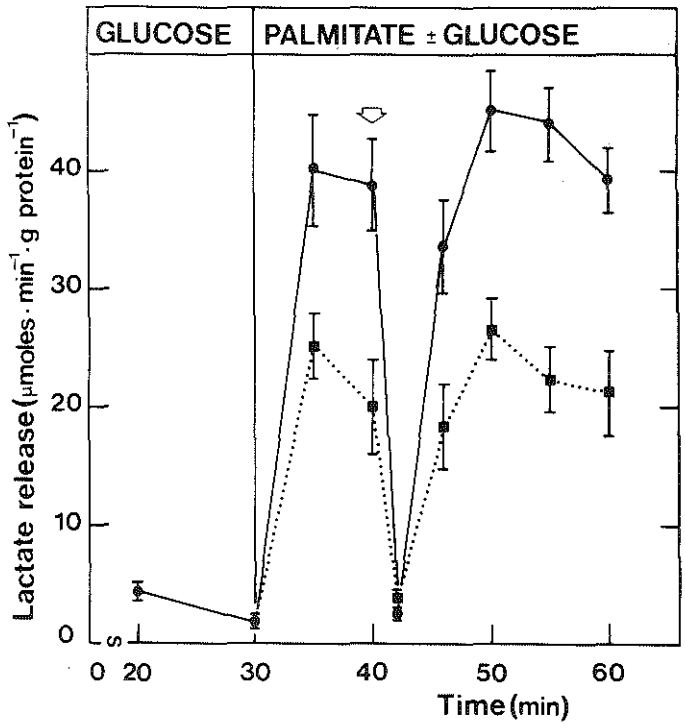

FIG.3. The effect of palmitate perfusion in the presence (●—●) or absence (■··■) of 11 mM glucose, and ischemia upon the lactate release from isolated rat hearts. Ischemia was introduced at t=40 min (see arrow). The results are the mean ± S.E.M. (n=9).

Fig.4 demonstrates that palmitate perfusion leads to an enhanced release of the AMP-breakdown products adenosine, inosine and hypoxanthine which was less when 11 mM glucose was present ($p < 0.005$ for all three AMP-catabolites after 10 min of palmitate perfusion).
During ischemia a progressive release of AMP-catabolites is observed while the presence of 11 mM glucose reduces the ischemia-induced release of adenosine, inosine and hypoxanthine ($p < 0.025$ for adenosine, $p < 0.025$ for inosine and $p < 0.01$ for hypoxanthine at the end of the ischemic period).

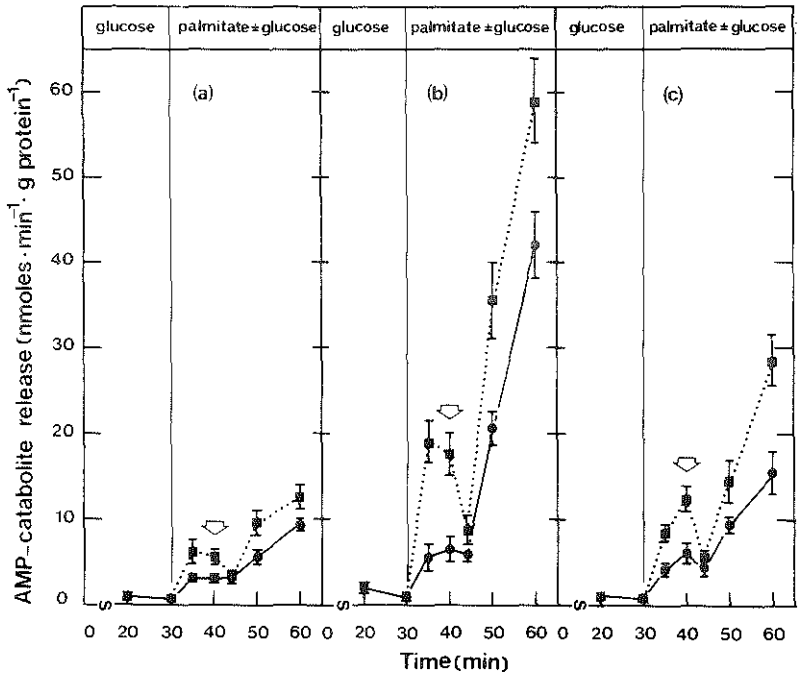

FIG.4. Release of the AMP-catabolites adenosine (a), inosine (b) and hypoxanthine (c) from isolated rat hearts in the presence (●—●) or absence (■··■) of 11 mM glucose and during ischemia. Ischemia was introduced at t=40 min (see arrow). The results are the mean ± S.E.M. (n=9).

Discussion

Fatty acids and their CoA esters exert many detrimental effects upon myocardial metabolism and thereby, upon myocardial function. In particular during oxygen deprived circumstances, i.e. during ischemia, when the fatty acid oxidation is hampered (22) and the intracellular levels of the membrane detergent fatty acids, fatty acyl CoA esters and fatty acyl carnitine (23) are likely to increase. Under these conditions uncoupling of oxidative phosphorylation (11) and inhibition of the adenine nucleotide translocator (10) may occur. Fatty acid-induced alterations in myocardial energy metabolism were studied in normal and ischemic rat hearts subjected to a high molar ratio of palmitate/albumin, in order to achieve a high intracellular fatty acid level, and attention was paid to the effect of glucose upon these fatty acid-induced changes in metabolism and function. The palmitate-induced release of AMP breakdown products adenosine, inosine and hypoxanthine during normoxic perfusion is not reflected in a decreased energy charge $\{([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])\}$ of the myocardial tissue, while the decreased CrP levels may

present a lowered CrP synthesis (24) combined with its ATP buffering function (25). By the action of the cytoplasmatic adenylate kinase reaction, a slight change in ATP concentration is amplified manyfold as an increase in the AMP level. AMP can be deaminated by AMP deaminase, a process which is inhibited by long-chain fatty acyl CoA esters (13) or dephosphorylated by 5'-nucleotidase to adenosine followed by further catabolism to inosine and hypoxanthine. The slight enhanced AMP levels during fatty acid perfusion (and subsequent activation) may initiate glycolysis (12). However, fatty acid-induced release of endogenous catecholamines may also be involved (8). Lactate release during fatty acid perfusion in the absence of glucose indeed indicates glycogenolysis. This observation is in contrast with findings during perfusion of rat hearts with low molar fatty acid: albumin ratios (15) when palmitate perfusion abolished glycogenolysis. By changes in NAD/NADH and CoA/acetyl CoA ratios (26), as a consequence of fatty acid oxidation, or by another action of fatty acids on inactivation of pyruvate dehydrogenase, lactate accumulation and release from the heart may be observed (16).
The palmitate-induced release of AMP-catabolites is reduced by the addition of 11 mM glucose during perfusion. The protective action is probably due to the formation of glycerol-3-phosphate which, in turn, by increasing triglyceride formation (27), reduced intracellular levels of fatty acids and their CoA esters. Since the CrP/ATP ratio rises, the inhibition of the adenine nucleotide translocator and uncoupling of oxidative phosphorylation are partly overcome. It has been shown before that most of the endogenous free fatty acids are indeed converted to triglycerides in the presence of glucose (28). With glucose present, fatty acids induce a marked lactate release. This has been mentioned before (16) and is related to a reduced pyruvate dehydrogenase action.
Perfusion with fatty acids leads to vasodilatation (29). It has been shown before that the vasoactive adenosine was not involved in this fatty acid mediated coronary vasodilatation. However, these studies were performed with a lower molar fatty acid/albumin ratio in the perfusion medium. From the experiments described here, it may be inferred that release of endogenously formed adenosine upon fatty acid perfusion, might contribute to the observed vasodilatation. Since the counteraction of adenosine release by glucose is not coupled to a reduction in coronary flow rates this hypothesis is probably incorrect. Furthermore, the actual concentration of adenosine (about 0.1 µM) in the coronary effluent is probably too low to induce the observed increase in coronary flow.
The glucose protection of AMP-catabolite release and myocardial energy metabolism leads to a marked protection of the fatty acid-induced decrease in contractile function. The Sephadex-induced reduction in coronary flow, by injecting 0.70 mg of these polysaccharide microspheres, is more severe during fatty acid perfusion when compared with the previously published values (17). Obviously, under conditions of fatty acid-induced vasodilatation more arterioles are reached by the microspheres resulting in a higher reduction of coronary flow. The microsphere-induced decrease in coronary flow rates were not significantly different in the two groups of hearts. During ischemia the energy charge drops, glyco(geno)lysis is enhanced, resulting in a high lactate release, and the rise in tissue AMP levels results in the release of adenosine, inosine and hypoxanthine. With glucose present, the higher glycolytic ATP formation (as concluded from a higher lactate release) may be responsibel for the significantly less decreased energy charge of the tissue and a less severe deterioration of the contractile function. The release of the AMP-catabolites is protected by glucose indeed. Our results indicate that under conditions of high intracellular levels of fatty acids (i.e. ischemia, anoxia), glycogen depletion and loss of AMP breakdown products may accelerate deterioration of myocardial function.

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