SIGNAL TRANSDUCTION IN CULTURED CARDIOMYOCYTES

Alpha$_1$-adrenergic and endothelin receptor mediated responses

SIGNAAL OVERDRACHT IN GEKWEEKTE HARTSPIERCELLEN

Alpha$_1$-adrenerge- en endotheline receptor gemedieerde responsen

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aan mijn ouders

voor Frans

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Chapter 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Already in ancient times the Greek were aware of the heart in the human body and they gave it the name *kardia*, which is still in use in words as cardiac, myocardial, tachycardia and bradycardia. In those times the importance of the heart was appraised by Aristotle (384-322 B.C.), who thought that the heart was the seat of the soul and centre of man. In 1628, however, the basis of the modern concepts of the circulation was laid by Harvey (1578-1657) who described the function of two ventricles of the heart and the whole-body blood circulation, and who gave the first description of heart failure (Harvey, 1628).

Nowadays, it is known that the function of the systemic circulation is to deliver blood to all organs of the body in order to provide them with sufficient supply of oxygen and metabolic substrates. The blood supply to the organs is accomplished primarily by the pump function of the heart. The rhythmic contractions of the heart underlying this pumping action are elicited by the electrical signals raised by the specialized cells of the atrium. Thus, the initiation of contraction is dictated by the cellular organization, subsequently leading to the complex process termed excitation-contraction coupling occurring within cardiomyocytes. It are the phasic inward and outward movements of Ca^{2+} across the sarcolemma (SL) and the sarcoplasmic reticulum (SR) and the phasic on and off interactions of Ca^{2+} with the troponin C component of the sarcomeres that determine the operation of the contraction-relaxation cycle. Various regulatory mechanisms control these Ca^{2+} movements and interactions, processes which occur all within the confines of the cell membranes of the cardiomyocytes.

The structure and functioning of the heart and its muscle cells

Structure

The rhythmic contractions of the heart, required to regularly pump the blood through the vessels, are dictated by the composition of the heart. The main part of the heart tissue (about 75 % on a volume basis) is made up of cardiomyocytes, which are held together by a collagen weave. The myocytes are interconnected by highly specialized parts of their surrounding membrane, termed the intercalated discs. These provide an intercellular linkage for the transfer of mechanical energy, for the functional contacts, as well as for the low-resistance electrical coupling to propagate electrical activity between adjacent heart muscle cells. The latter characteristic makes the myocardial muscle fibers different from other types of striated muscle. Other regions of the heart contain pacemaker and conducting cells, that respectively generate and propagate the heart's electrical activity. Furthermore, a rich capillary network and nonmyelinated nerves are present for respectively blood supply and autonomous sympathetic regulation.

The cardiac myocytes are surrounded by the SL which invaginates into the cell to form an extended tubular network, called T-tubules (Fig.1). This network is an extension of the extracellular space into the cellular interior and is closely coupled to the cell's SR membrane, which consists of an intracellular complex network of tubules. The exact nature and function of the coupling of these two membrane systems is still a matter of debate. Generally, the SR

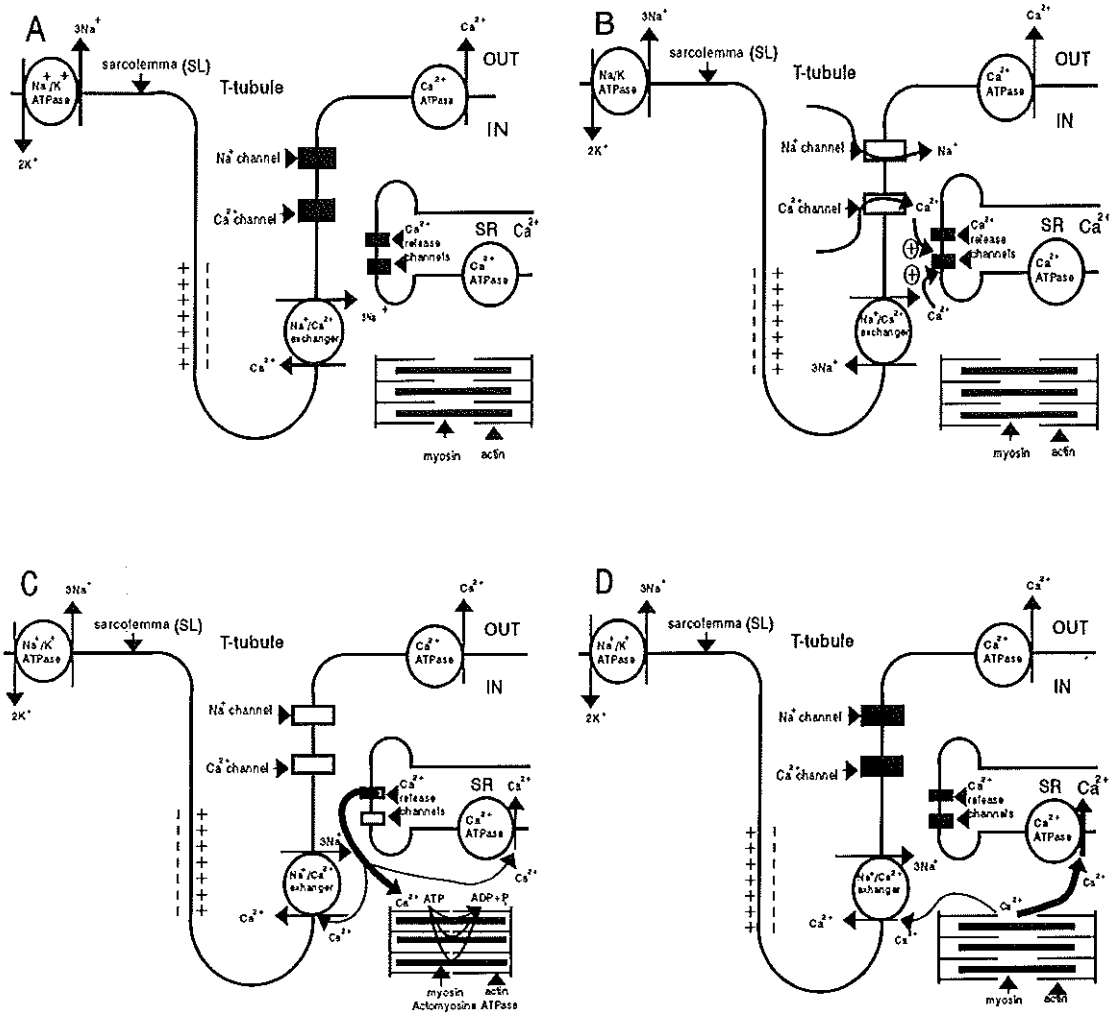

Figure 1 Schematic representation of the ion movements in the cardiomyocyte during contraction. *A*: processes proceeding in the resting cardiomyocyte; *B*: processes proceeding in the early contraction, before the rise of cytosolic Ca^{2+} ($[Ca^{2+}]_i$); *C*: processes associated with the rise in $[Ca^{2+}]_i$ thereby generating the relative displacement of the actin and myosin filaments; *D*: processes associated with a fall of $[Ca^{2+}]_i$ and relaxation. SR, sarcoplasmic reticulum. (Adapted from Barry and Bridge, 1993).

serves as a local intracellular Ca^{2+}-pool which plays a critical role in the excitation-contraction coupling of the muscle (Thomas *et al.*, 1992). The voltage-operated Ca^{2+}-selective channels in the SL, which, upon depolarization from the resting membrane potential, are conformationally changed to enable Ca^{2+} entry, reside in the T-tubular parts near the terminal cisternae of the SR, in which the ryanodine-sensitive Ca^{2+} release channels reside. Ca^{2+} ions entering through the Ca^{2+} channels of the SL initiate the release of Ca^{2+} ions from the SR through the Ca^{2+} release channels, a process termed Ca^{2+} induced Ca^{2+} release (Berridge, 1993; Clapham, 1995).

Besides T-tubules, SR and nuclei (often more than one), the cardiomyocytes contain parallel running bundles of structures, which are the myofilaments. These structures consist of serially repeated units (sarcomers) which are responsible for contractile activity. The cardiomyocytes are also rich of mitochondria, which are located near the myofilaments. In the mitochondria the oxidative phosphorylation takes place, resulting in the production of ATP, which is the fuel for the contractile activity of the myofilaments (Opie, 1991; Braunwald, 1992).

Electrical activity

Contration is triggered by the electrical activity of the heart, which is generated in the pacemaker cells localized in the sinoatrial node, and is subsequently propagated via the atrial tissue to the atrioventricular node, His bundle, left and right bundle branches and the Purkinje fibers. The frequency of the pacemaker discharges determines the heart rate, and the autonomic nervous system can modulate this discharge rate. Under conditions of exercise or stress, the sympathetic nervous system increases heart rate by the release of adrenergic hormones, epinephrine from the adrenals and norepinephrine from the nerve endings. These hormones act on the adrenergic receptors on the membranes of the pacemaker cells. At resting condition, when the activity of the parasympathetic system predominates, an inhibitory cholinergic substance, acetylcholine, is released which acts as a neuromodulator on the release of norepinephrine from the nerve endings, thereby attenuating heart rate (Opie, 1992).

Excitation-contraction coupling, relaxation and its modulation by neurohumoral factors

Myocardial contraction and relaxation is controlled by the process of excitation-contraction coupling. The contractile apparatus in the myocytes is composed of myosin and actin filaments. The latter contain also troponin-tropomyosin-complexes which regulate the formation and dissociation of crossbridges between the actin and myosin filaments. Upon formation of the crossbridges, actin moves relative to myosin, thereby generating force (Nayler, 1980). The action of the troponin-tropomyosin-complex is dependent upon the binding of Ca^{2+} to the troponin C component of the troponin complex. The electrical excitation wave causes the SL membrane potential of the myocytes to depolarize slightly, which consequently induces the opening of the voltage-gated Na^+ and Ca^{2+} channels and the reversed operation of the Na^+/Ca^{2+} exchanger (Lamers *et al.*, 1981) (Na^+ out, Ca^{2+} in) in the SL and leads to full depolarization (Fig. 1A). These small amounts of Ca^{2+} that cross the sarcolemma via the Ca^{2+} channels and the Na^+/Ca^{2+} exchanger (Fig. 1B) induce a much

larger release of Ca^{2+} ions from the SR (Barry and Bridge, 1993; Philipson, 1992) (Fig. 1C). The elevated free Ca^{2+} concentration in the cytosol stops the inhibitory action of the troponin-tropomyosin-complex on the actin-myosin binding, resulting in the formation of crossbridges. Subsequently, the voltage-gated Ca^{2+} channels start to close, and Ca^{2+} is removed from the myofilaments to initiate muscle relaxation and the SL membrane repolarizes by the reopening of the K^+ channels. The required lowering of the free Ca^{2+} concentration in the cytosol to resting values is accomplished by reuptake of Ca^{2+} ions into the SR via the operation of the SR Ca^{2+} pump and by extrusion of Ca^{2+} ions through the action of the Na^+/Ca^{2+} exchanger (Na^+ in, Ca^{2+} out) and the Ca^{2+} pump of the SL (Fig. 1D) (Barry and Bridge, 1993).

The processes of excitation-contraction coupling and subsequent relaxation can be modulated by various neurohumoral factors such as catecholamines, endothelin, angiotensin II and acetylcholine. The interaction of these neurohumoral factors with their specific receptors on the SL leads to activation of intracellular signalling cascade reactions of which the ultimate effects are the modulation of diastolic or systolic free Ca^{2+} levels or the modulation of the interactions of Ca^{2+} with the myofilaments. Additionally, the levels of cytosolic free Ca^{2+} can alter the activity of enzymes intimately involved in the signalling pathways (e.g. phospholipases C and A_2, protein kinase C-isoenzymes). Therefore, in the cardiomyocytes Ca^{2+} does not only critically determine the course of excitation-contraction coupling and relaxation processes, but has also an important function as second messenger in neurohumoral factor-induced cell signalling (Gruver *et al.*, 1992; McDonough *et al.*, 1994; Van Heugten *et al.*, 1994; Zhang *et al.*, 1993).

Heart disease and failure

Heart failure, i.e. dysfunction of the heart as a pump, has its origin in several primary heart diseases. Basically there are two mechanisms for heart failure:

A) Myocardial dysfunction caused by acute ischemia (ischemic heart disease), inflammatory origin (myocarditis) or unknown (often hereditary) factors (cardiomyopathy).

B) Myocardial dysfunction due to chronic exposure of the heart to pressure or volume overload.

Myocardial dysfunction due to acute ischemia, inflammation or cardiomyopathy

Ischemic heart disease comprises a number of clinical syndromes, e.g. angina pectoris, acute myocardial infarction, chronic post-ischemic heart failure and sudden ischemic cardiac death, which are mostly determined by a chronic history of atherosclerosis. Atherosclerotic lesions can be experimentally induced by various stimuli, ranging from high lipid diets to immunological reactions. Formation of atherosclerotic lesions is found to be associated in humans with the occurrence of thrombotic events, hypertension and high levels of serum cholesterol. The lesions induce the remodelling of the arterial wall, which leads to the reduction of the lumen and the impairment of the blood flow. While generally the ischemic heart develops in the presence of a chronic atherosclerotic background, the actual trigger of acute ischemia may be a sudden excessive increase in myocardial demand in the presence

of a flow-limiting coronary stenosis, or a thrombotic occlusion at the site of a coronary stenosis. The cardiac response to ischemic stimuli manifests clinically with angina pectoris, myocardial necrosis, acute cardiac failure followed by morphological adaptation, and cardiac arrhythmias (Maseri, 1995; Braunwald, 1992). For example, cardiac arrhythmias, a general term used for abnormalities in the heart's electrical activity, often have an ischemic origin. Ventricular arrhythmias are mostly based on the existence of an infarcted or ischemic zone responsible for automaticity, reentry (due to local conduction block and slow conduction) and triggered activity.

Acute myocardial ischemia develops if a reduction of coronary flow impairs the supply of oxygen and substrates to the myocardium to a level that is inadequate to meet the oxygen and substrate demands of the tissue. Then, the oxidative metabolism of fatty acids, normally the main source of ATP of the cardiomyocytes, becomes severely disturbed. The oxygen shortage will diminish the activities of mitochondrial β-oxidation, tricarboxylic acid cycle and respiratory chain, and will increase the rate of anaerobic glycolysis. These alterations in energy metabolism result in the accumulation of free fatty acids, and of metabolites such as lactate, acyl-CoA, acyl-carnitine (Van der Vusse *et al.*, 1992) and of protons. High concentrations of some of these metabolites are harmful: acyl-CoA inhibits mitochondrial ATP/ADP translocase and acyl-carnitine inhibits the SL Na^+/K^+ pump and has a membrane disruptive action (Lamers *et al.*, 1995). The concomitant reduction in the rate of phospholipid resynthesis and/or the accelerated hydrolysis of membrane phospholipids is also believed to play a role in the ischemia-induced damage of the SL membrane, which may lead to ventricular arrhythmias and cell necrosis (Opie, 1991; Van der Vusse *et al.*, 1992). Particularly, the accumulation of membrane-active lysophosphoglycerides is thought to be potentially arrhythmogenic (Corr *et al.*, 1979 and 1982).

Apart from the membrane damage caused by accumulation of free fatty acids, acyl-CoA, acyl-carnitine and lysophosphoglycerides, the other current theories to explain the development of irreversible cell damage during ischemia are: 1) mechanically induced membrane damage by abnormalities in cell volume regulation leading to intracellular edema, 2) the depletion of cellular ATP to critical levels, leading to rigor complexes and contracture, 3) the formation of oxygen free radicals which are highly toxic, and 4) the development of intracellular Ca^{2+} overload. Particularly, the loss of Ca^{2+} homeostasis, considered to be a major cause of cell damage, is initiated by the impaired rate of active Ca^{2+} removal from the cytosol due to the lack of ATP. This leads secondarily to further ATP depletion as a consequence of the increased ATP required by the mitochondria to buffer cytosolic Ca^{2+}. This all leads to inadequate maintenance of the ionic gradients, resulting in loss of the membrane integrity. Furthermore, the massive cytosolic Ca^{2+} accumulation activates phospholipases C-β (PLC-β) and A_2 (PLA_2), which raise the levels of lysophosphoglycerides and thereby provoke fatal arrhythmias (Opie, 1991).

Other heart diseases which are distinct from ischemic heart disease in their origin, may lead to impaired pump function as well. Myocarditis has been described during and following a wide variety of viral, bacterial, and protozoal diseases, but virtually any infectious agent may produce cardiac inflammation, which could precipitate cardiac dysfunction.

Cardiomyopathy confines a group of diseases of unknown etiology, in which the dominant feature is the involvement of the heart muscle itself. Characteristic for this group is that they are not the result of ischemic, hypertensive, congenital, valvular, or pericardial diseases

(Braunwald, 1992). Cardiomyopathy as well as cardiac inflammation, lead to impairment of heart function and may induce, like ischemic heart disease, the scala of morphological and metabolic adaptation processes which are described below.

Myocardial dysfunction due to chronic exposure to pressure- and volume overload

Chronic pressure overload of the heart occurs, for instance, in response to aortic stenosis or sustained severe hypertension, when left ventricular systolic pressure must increase to overcome the resistance to the ejection of the blood. Acutely, the heart will adapt to this situation by recruitment of inotropy using the Frank-Starling mechanism, which results in a higher rate of systolic pressure development in the left ventricle. However, due to left ventricular dilatation, wall stress is greatly increased, which makes the left ventricle vulnerable to failure. In later stages, the ventricular wall adapts by becoming thicker (cardiac hypertrophy) with or without left ventricular dilatation. Even the compensated form of cardiac adaptation to pressure overload may change chronically into left ventricular dilatation and heart failure (Opie, 1991). The mechanisms underlying these changes will be discussed in the next section of this chapter.

The initial event in chronic volume overload is, for instance, regurgitation of either the mitral valve or the aortic valve, a ventricular septum defect or infarction. Per heart stroke a greater volume of blood must be dealt with, which is the basis for the volume overload. The heart will attempt to compensate for the defect by increasing the chamber size without increase of the wall thickness. This must lead to a rise in wall tension, higher oxygen demands and, ultimately, heart failure.

Three stages in the reaction to chronic pressure or volume overload can be observed: 1) the initial hemodynamic load, which induces hypertrophy, 2) hypertrophy of the heart which compensates for the increased workload/cardiac mass ratio, and 3) a period in which stroke work per unit of cardiac mass decreases due to progressive impairment of the heart to fill normally and to generate force, a condition generally referred to as heart failure (Opie, 1991; Maseri, 1995).

Hypertrophy and heart failure

As discussed before, in response to chronic hemodynamic overload due to infarction, valvular insufficiency, hypertension, septal defect or cardiomyopathy, the heart (or a part of the heart) undergoes various adaptations. Those adaptations include changes in the shape and structure of the ventricles due to growth of the myocytes and due to changes in the metabolism of cardiomyocytes and nonmyocytes in the heart (Anversa *et al.*, 1986; Weber *et al.*, 1992 and 1994). The process of adaptation is characterized by an increase in the size and the phenotype of the cardiomyocytes and the simultaneous proliferation of nonmyocytes of the heart (Vliegen *et al.*, 1991). The proliferation and phenotypic changes of nonmyocytes, such as fibroblasts, vascular smooth muscle cells and endothelial cells and extracellular matrix formation, all take part in the 'remodelling' of the myocardium. Myocardial remodelling has detrimental effects on the mechanical properties of the heart such as increase in myocardial stiffness (Doering *et al.*, 1988; Katz, 1990; Weber *et al.*, 1992 and 1994). However, the major anatomic changes in the hypertrophic myocardium are brought about by

the increase of the size of the cardiomyocytes. An important characteristic of myocytes is that, shortly after birth, they loose their ability to divide (Zak, 1974; Ueno *et al.*, 1988). Consequently, if the number of functional myocytes is diminished by, for instance infarction, an excessive burden is put on the remaining viable myocytes. The latter will respond to this situation by an increase in their diameter and/or length, depending upon the extent, nature, and the duration of the workload (Anversa *et al.*, 1986 and 1990). In addition to the increase in size of the cardiomyocytes, other characteristic events occur such as reexpression of certain fetal isoproteins (Clark *et al.*, 1993). Although the newly synthesized contractile

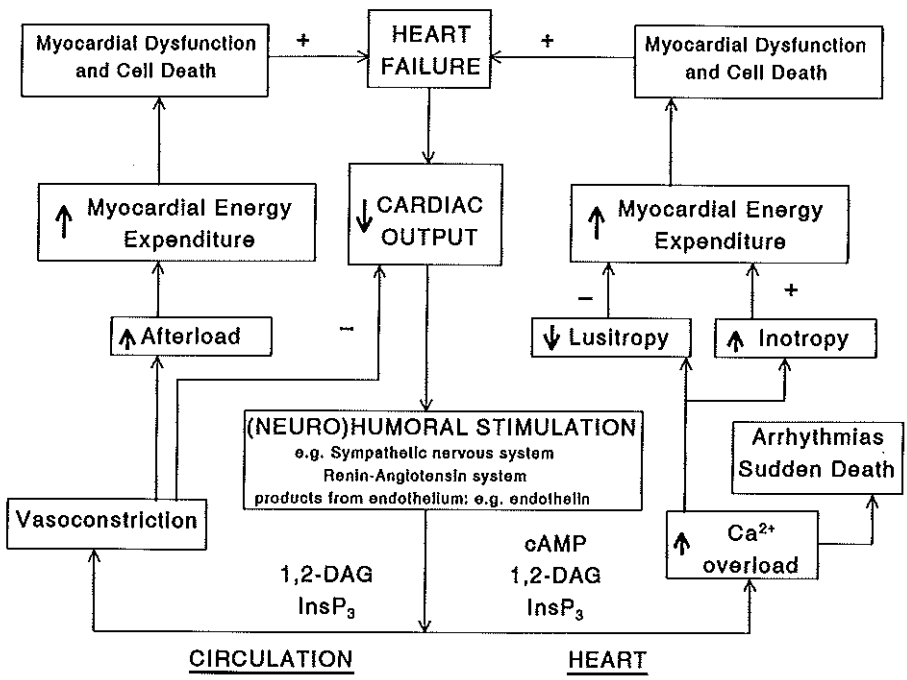

Figure 2 Schematic representation of the adaptive responses of the circulation and heart in the downward spiral of heart failure (adapted from Katz, 1990).

proteins possess a lower rate of Ca^{2+}-stimulated ATPase and thus are energy-sparing, the increased mass of contractile protein requires more ATP for its function. The increased consumption of energy by a larger contractile apparatus is not parallelled by an adequate increase in the number of mitochondria, which further exacerbates the energy deficit. Moreover, the supply of metabolic substrates and oxygen appears not optimal as the growth of the myocytes is not accompanied by a corresponding growth of the coronary capillaries (Katz, 1990). Especially in the subendocardial layers of the hypertrophic left ventricle the myocardial perfusion reserve is rapidly exhausted, leading to subendocardial ischemia during exercise (Hittinger *et al.*, 1990 and 1991; Vatner *et al.*, 1990).

Energy deprivation of the hypertrophied cardiomyocytes will also disturb the energy

demanding processes (e.g. SL and SR Ca^{2+} pump and SL Na^{+}/Ca^{2+} exchanger) leading to impairment of Ca^{2+} homeostasis (Bing *et al.*, 1991; Gwathmey *et al.*, 1987). Subsequently, left ventricular contraction and relaxation become compromised. Depressed contractility of the failing heart reduces the need for energy and is compensatory in terms of the energetics of individual myocardial cells. However, the ensuing left ventricular dilatation is harmful, and limits the reserve capacity of the heart during exercise.

Cardiac hypertrophy leading to impaired cardiac performance has, when sustained, detrimental long-term effects on the heart and the circulation, and thereby accelerates the spiral downwards leading to end-stage heart failure. The adjustments of the heart function and the peripheral circulation are initiated by increased formation of (neuro)humoral factors, such as those derived from the heart itself (ANF) and from the sympathetic system, the renin-angiotensin system and the endothelium. These neurohumoral factors by binding to their specific receptors in cells of the cardiovascular system, induce intracellular signalling cascade processes such as phosphatidylinositol (PtdIns)-cycle and adenylate cyclase mediated reactions. The left part of Figure 2 depicts the activation of the PtdIns-cycle in the vascular smooth muscle cells by neurohumoral stimulation. Neurohumoral stimulation is responsible for vasoconstriction, which is detrimental as it increases afterload further, leading to less cardiac output and higher energy demands of the failing heart (Katz, 1990). The right part of Figure 2 shows the second messenger responses (cyclic adenosine 3'5'-monophosphate (cAMP), 1,2-diacylglycerol (DAG) and inositoltrisphosphate ($InsP_3$)) elicited by neurohumoral stimulation of cardiomyocytes and likely responsible for the development of intracellular Ca^{2+} overload which can produce irreversible damages to the myocyte (Katz, 1990; Brown and Martinson, 1992; Endoh and Blinks, 1988).

Modulation of heart function: contractility by catecholamines and endothelin

In the normal heart the contractility is regulated by the sympathetic nervous system, which exerts its effects by the release of adrenaline and noradrenaline. These agonists act on the α- and β-adrenergic receptors to produce increases in force of contraction (positive inotropy), in the frequency of contraction (positive chronotropy) and acceleration of the relaxation and hence shortening of the duration of contraction (lusitropy) (Scholz *et al.*, 1986).

β-Adrenergic receptors

The G-protein-coupled receptors involved in regulation of cardiac contractility are predominantly receptors of the β-adrenergic type, which, upon stimulation, lead to the elevation of cAMP levels via the activation of adenylate cyclase (Endoh and Blinks, 1988; Brodde *et al.*, 1992). Accumulated cAMP, on its turn, will activate cAMP-dependent protein kinase, which induces phosphorylation of specific proteins in the SL, SR, and myofilaments. Due to phosphorylation of the Ca^{2+}-channel and the phospholamban component of the Ca^{2+}-ATPase in SR, Ca^{2+} transients have become larger. Thus, stimulation of the β-adrenergic receptor leads to a higher conductance of the voltage-gated slow Ca^{2+} channels, an accelerated Ca^{2+} reuptake through the SR, and a weakened Ca^{2+} binding to troponin C in the myofilaments. The end result of these altered intracellular Ca^{2+} handling reactions is

increased force of contraction associated with accelerated relaxation and shortening of contraction duration (Endoh and Blinks, 1988; Tsien, 1977; Demaille *et al.*, 1983). Therefore, at present, the intracellular signal transduction via adenylate cyclase seems well characterized. In the failing heart the density of β-adrenergic receptors is decreased, presumably as an adaptive response to the increased activity of the sympathetic nervous system during heart failure (Michel *et al.*, 1992). This loss of receptors can be seen as one of the phenotypic changes characteristic of the failing heart. Other reports deal with changes in the contents of G_s and G_i proteins in the failing heart (Brodde and Michel, 1992; Brodde *et al.*, 1995).

α-Adrenergic receptors

Besides the β-adrenergic receptor system, which causes a positive inotropic effect mediated by the adenylate cyclase pathway, the α-adrenergic receptor system in the heart may cause positive inotropic effects mediated by the PtdIns pathway.

The involvement of α-adrenergic receptors in the increase in contractility by catecholamines in rat ventricular strips was reported for the first time by Wenzel and Su in 1966, and was confirmed later by Govier who described an α-adrenergically mediated positive inotropic effect in guinea pig atria and ventricle (Wenzel and Su, 1966; Govier, 1967; Terzic *et al.*, 1993). Adrenergic receptors which mediate the positive inotropic response in heart are of the subclass α_1 (Bristow, *et al.*, 1988; Terzic *et al.*, 1993). In comparison to the β-adrenergic effect, the α_1-adrenergically mediated positive inotropy develops relatively slow, and is accompanied by lengthening instead of shortening of the duration of the contraction (Scholz *et al.*, 1986; Terzic *et al.*, 1993; Fedida, 1993). Furthermore, in contrast to the β-adrenergic effect, the signals, produced by activation of α_1-adrenergic G-protein-coupled receptors, are mediated by phospholipase C-β (PLC-β), which hydrolyses phosphatidylinositol-(4,5)-bisphosphate (PtdIns(4,5)P_2) into Ins(1,4,5)P_3 and 1,2-DAG (Brodde *et al.*, 1992; Terzic *et al.*, 1993). Stimulation of myocardial α-adrenergic receptors in the normal heart does not lead to a pronounced increase in heart rate, and the number of α-adrenergic receptors in the nonfailing human heart is rather small (Bristow *et al.*, 1988; Brodde *et al.*, 1992; Michel *et al.*, 1990). In contrast to the multiple reports on the decrease in the number of β-receptors, experimental reports on changes of the α_1-receptor density in the failing heart are controversial, varying from slightly increased (Bristow *et al.*, 1990 and 1988; Scholz *et al.*, 1986) to unchanged (Bristow *et al.*, 1990; Michel *et al.*, 1993,) and even to decreased density (Michel *et al.*, 1990). These controversial findings, in combination with the fact that a relatively small amount of α_1-adrenergic receptors is present in the human heart, indicate that the contribution to the positive inotropy of the α_1-adrenergic receptor may be minor (Bristow *et al.*, 1988; Brown *et al.*, 1994). It should, however, be noticed that one of the early reports on α_1-adrenergic receptors supposed that these represent a reserve mechanism serving under conditions of impaired β-adrenergic receptor-mediated responses (Scholz *et al.*, 1986). According to the conclusions of this latter review, stimulation of α_1-adrenergic receptors under physiological or pathological conditions could regulate the cardiac rhythm, conduction and force of contraction. Perhaps too much attention has been paid to the number of α_1- relative to β_1-adrenergic receptors, because it should be kept in mind that the α_1-adrenergic signalling pathway supplies second messengers that are distinct from that

of the β-adrenergic pathway. Therefore, more information is required concerning the change of activities of the G-protein-coupled PLC-β signalling system under various pathological conditions. For example, changes in G_q content of the hypertrophic failing heart have not been studied so far.

Endothelin

The force of contraction of the heart under normal physiological conditions has for a long time been assumed to be mainly dependent upon the degree of activation of the sympathetic nervous system. Cardiac contractility may, however, also directly or indirectly be affected by factors released by the coronary endothelium. The balanced release of vasodilator and vasoconstrictor agents from the endothelium of the large coronary arteries regulate coronary blood flow and, thereby, cardiac contractile function (Krämer *et al.*, 1992). More recently, paracrine factors, mostly derived from the microvascular endothelium, have been recognized to be involved in the direct modification of cardiac force by their positive inotropic action. One of these is a 21-amino acid vasoconstrictor peptide, called endothelin (ET), originally isolated and characterized from the supernatant of cultured porcine aortic endothelial cells (Yanagisawa *et al.*, 1988). ET has been found to mediate a potent positive inotropic effect in the mammalian heart (Krämer *et al.*, 1991 and 1992; Nayler, 1990; Ishikawa *et al.*, 1988). At least three members in the ET family have been recognized, encoded by three distinct genes, however only ET-1 was found to be expressed in vascular endothelial cells and in intact heart (Krämer *et al.*, 1992; Sakurai and Goto, 1993; Ito *et al.*, 1993; Yorikane *et al.*, 1994). ET-1 binds to the cardiac endothelin receptor ET_A with an affinity that is 100 times higher than its family member ET-3 (Sakurai and Goto, 1993; Vogelsang *et al.*, 1994), although this affinity might be species dependent (Nayler, 1990). The ET_A receptor was shown to be coupled to PLC-β activity (Prasad, 1991) by a pertussis toxin insensitive G-protein (Neer and Clapham, 1992). When examining the contractile response of isolated rat atria upon stimulation with ET-1, an elevation of inositolphosphates (InsP, $InsP_2$, and $InsP_3$) was observed (Vigne *et al.*, 1989). Similarly, ET-1 mediated enhanced levels of $Ins(1,4,5)P_3$ as well as 1,2-DAG, the other putative messengers resulting from phospholipase C-β catalyzed hydrolysis of $PtdIns(4,5)P_2$, were found in isolated perfused rat hearts (Prasad, 1991). More recently, also in human atrium activation of ET_A receptors resulted in an elevation of inositolphosphates (Sugden *et al.*, 1993). However, coupling of the ET_A receptor to other effector systems such as adenylate cyclase and the voltage-operated Ca^{2+} channel was observed as well (Kelly *et al.*, 1990; Vogelsang *et al.*, 1994; Lüscher and Wenzel, 1995). Although at present little is known about the factors that regulate the expression of the endothelins and their receptors in cardiac tissue *in vivo*, experiments in rat heart models have shown that the number of ET_A receptors had increased during workload (Brown *et al.*, 1995).

Induction of myocardial hypertrophy: possible role of norepinephrine and endothelin

Hormone-induced cardiac hypertrophy, including the characteristic changes in phenotype, are considered to be the primary result of α_1-adrenergic receptor stimulation (Clark *et al.*, 1993; Simpson and McGrath, 1983; Simpson 1988). This could suggest that α_1-adrenergic receptors

play a key role in the mechanism of development of cardiac hypertrophy.

The possible involvement of α_1-adrenergic receptors in the process of cardiac hypertrophy has been supported by results obtained in cultured neonatal and adult rat cardiomyocytes (Clark *et al.*, 1993; Simpson, 1983 and 1988). Also *in vivo* studies support the implication of catecholamines in the pathogenesis of left ventricular hypertrophy. In dogs, chronic infusion of subhypertensive doses of norepinephrine results in left ventricular hypertrophy (Laks *et al.*, 1969; Rossi *et al.*, 1985; Long *et al.*, 1991). Moreover, an increase in sympathetic activity of the heart, detected on the basis of electrophysiological signs or by measurement of the rate of noradrenaline turnover, was observed in several types of experimental hypertrophy and in hypertensive patients (Östman-Smith, 1981; Timaraco *et al.*, 1985). Particularly, α_1-adrenergic receptors were shown to be involved in the mechanism of hypertrophy in hypertensive patients as the treatment with the α_1-adrenergic antagonist prazosine led to a progressive decrease in left ventricular mass in the first 12 months (Leenen *et al.*, 1987).

An important role of ET in the pathophysiology of hypertrophy and heart failure is supported by reports of elevation of ET-1 levels in plasma of humans and certain experimental animal models (dogs) of heart failure (Margulies *et al.*, 1990), although in other studies on hypertensive patients plasma levels of ET-1 were found to be normal (Lüscher *et al.*, 1995). It should, however, be noticed that plasma levels of ET-1 are only indicative for an increased local formation, because the reported plasma levels (10^{-11}-10^{-12} M)(increased or not increased) are always far below the concentrations required for receptor activation. Moreover, the mechanisms of stimulation of paracrine and autocrine release of ET-1 in relation to other systems involved in hypertrophy are still unclear. It is suggested that ET-1 is committed to the renin-angiotensin system (RAS), and therefore participates in the development of hypertrophy and heart failure. The mechanism of the involvement of ET-1 in RAS awaits further investigation. In the RAS, the vasoconstrictory effects mediated by ET-1 may be beneficial in the early stages of heart failure by augmenting cardiac preload through venoconstriction and Na^+ retention and by increasing the vascular resistance to maintain perfusion pressure (Weber *et al.*, 1992). On the other hand, angiotensin II may be involved in *de novo* synthesis and release of ET-1 as shown in cultured neonatal rat cardiomyocytes (Ito *et al.*, 1993). A potential role of paracrine and/or autocrine release of ET-1 was suggested in the mechanism of angiotensin II-induced hypertrophy of the neonatal cardiomyocytes (Ito *et al.*, 1993).

The involvement of ET-1 in the induction of hypertrophy was also studied using experimental rat models. For example, pressure overload of the heart was generated by banding of the abdominal aorta of the rats, which resulted in hypertrophy of the left ventricle. The expression of prepro ET-1 mRNA in the left ventricle was significantly higher than in the left ventricle of sham-operated rats or the right ventricle (Yorikane *et al.*, 1993 and 1994; Ito *et al.*, 1994). In agreement with the latter finding, the ventricular ET-1 levels of pressure overloaded rat hearts were elevated in parallel with an increased density of ET-1 binding sites in the left ventricle (Arai *et al.*, 1995). The plasma levels of ET-1 were shown to be elevated in these aorta banded rats as well, but when the suprarenal abdominal aorta was clipped, which was used to increase the load to the myocardium, these ET-1 levels where found to be only transiently elevated (within 30 min) (Yorikane *et al.*, 1994; Ito *et al.*, 1994; Arai *et al.*, 1995). Induction of hypertrophy by aortic banding in rats could also be blocked by the specific ET_A receptor antagonist BQ 123 in aorta banded rats (Ito *et al.*, 1994). In a rat

model of chronic volume overload of the left ventricle induced by an aortovenocaval fistula, the levels of ET_A receptors and ET-1 mRNA were found to be increased in the hypertrophied ventricles, in parallel to an increase of ET-1 levels in the plasma. However, no change in [^{125}I]-ET-1 binding sites could be observed (Brown *et al.*, 1995). That ET-1 and the ET_A receptor are also involved in the development of hypoxia-induced right ventricular hypertrophy was demonstrated in a rat model with pulmonary hypertension, using the ET_A specific blocker BQ 123 (Bonvallet *et al.*, 1994).

Role of phospholipid hydrolysis in α_1-adrenergic and endothelin mediated cell signalling

Consistent with the hypothesis on their similar role in inducing myocardial hypertrophy, the α_1-adrenergic and ET_A receptors share the pathway of intracellular signal transduction. Upon agonist-receptor binding and receptor-G-protein coupling, the membrane associated phospholipase C-β (PLC-β) is activated which initiates hydrolysis of PtdIns(4,5)P_2 (Fig. 3)

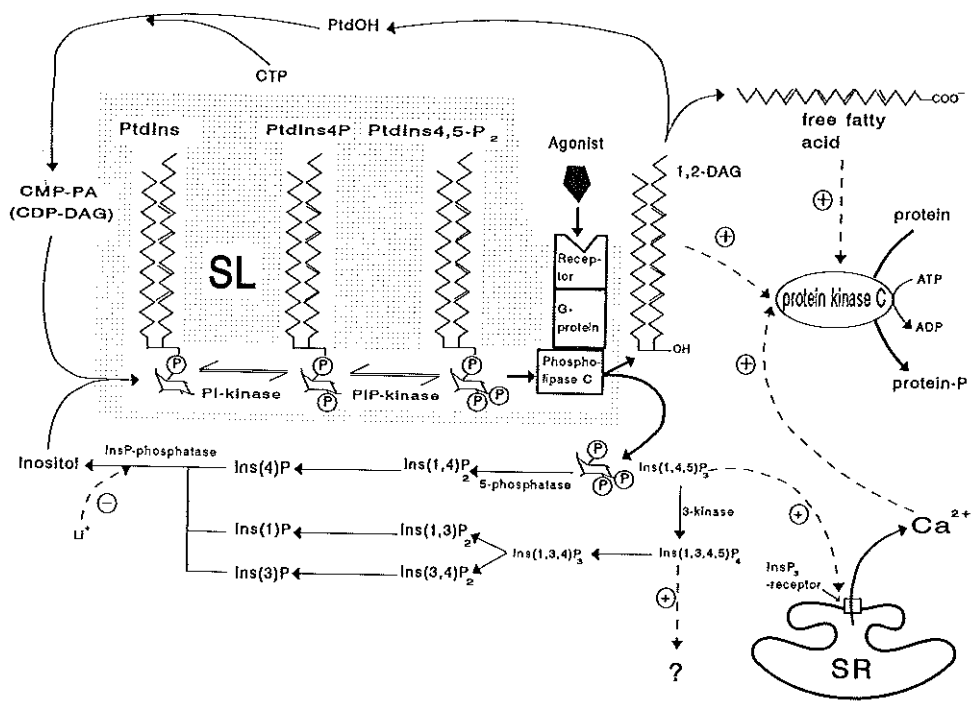

Figure 3 Schematic representation of the phosphatidyl inositol (PtdIns)-cycle. See text for explanation. SL, sarcolemma; SR, sarcoplasmic reticulum; 1,2-DAG, 1,2-diacylglycerol, CDP-DAG, cytidyldiphosphate-DAG; CMP-PA, cytidylmonophosphate-phosphatidic acid; CTP, cytidyltrisphosphate; PtdOH, phosphatidic acid; PI or PtdIns, phosphatidyl inositol; PIP or PtdIns(4)P, phosphatidyl inositol phosphate; InsP, inositol phosphate.

(Berridge and Irvine, 1989; de Chaffoy de Courcelles, 1989; Rana and Hokin, 1990). PtdIns(4,5)P_2 hydrolysis leads to the formation of two putative messengers, Ins(1,4,5)P_3 and 1,2-DAG. The Ins(1,4,5)P_3 effect is usually transient because it is rapidly degraded via the competitive pathways of direct dephosphorylation (5-phosphatase pathway) or phosphorylation followed by dephosphorylation (3-kinase pathway) (Fig. 3). PtdIns is resynthesized from 'recycled' free inositol and cytidyldiphosphate-DAG (CDP-DAG or CMP-PA). The stepwise phosphorylation of PtdIns by successively PtdIns kinase (PI kinase) and PtdIns4P- kinase (PIP kinase) closes the PtdIns-cycle (Fig. 3) (Rana and Hokin, 1990; Mesaeli *et al.*, 1992; Lamers *et al.*, 1992). The other compound generated during PtdIns(4,5)P_2 hydrolysis is 1,2-DAG, which is either converted to phosphatidic acid (PtdOH)and CDP-DAG to return to the PtdIns-cycle, or subsequently hydrolysed by DAG-lipases and nonspecific esterases into free fatty acids to be lost from the cycle (Berridge and Irvine, 1989; Nishizuka, 1992). PLC-β mediated hydrolysis of PtdIns(4,5)P_2 will accelerate the PtdIns-turnover, which can be traced only by almost complete blockade of Ins(1)P phosphatase with Li^+ ions (Fig. 3) (Berridge *et al.*, 1982; Rana and Hokin, 1990; Nahorski *et al.*, 1991). The putative messengers formed upon PtdIns-cycle activation, Ins(1,4,5)P_3 and 1,2-DAG, are thought to be involved in, respectively, the release of Ca^{2+} from the Ins(1,4,5)P_3 sensitive Ca^{2+} release stores of the SR and the activation of members of the protein kinase family (PKC) family (Brown and Martinson, 1992). There is now abundant evidence that the activation of certain PKC isoenzymes is involved in the initiation of myocardial hypertrophy. For instance, it has been shown that direct activation of PKC isoenzymes by phorbolesters induces hypertrophy and characteristic phenotypic changes in cultured cardiomyocytes (Steinberg *et al.*, 1995). At present it is largely unknown how the signalling proceeds downstream of PKC isoenzymes.

The involvement of phospholipids other than PtdIns(4,5)P_2 in cell signalling is complex, one should be aware that besides PLC-β transmembrane signalling other potential phospholipid signalling may be active in myocardium too. For instance, the phospholipase A_2 and D mediated systems but also the 3-phosphorylation of PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, the interactions of polyphospho-PtdIns with components of the cytoskeleton or the possibility of operation of the PtdIns-cycle in the nucleus may be involved in initiation of hypertrophy (Divecha and Irvine, 1995). In this thesis we focus on the characterization of the α_1-adrenergic and ET-1 stimulated PLC-β transmembrane signalling pathway in the cardiomyocyte and on the investigation of potential sites of the PtdsIns-cycle susceptible for modulation, such as participating enzymes (PLC-β, PKC), proteins (G-proteins) and the physicochemical state of the SL membrane bilayer. Evidently, the latter modulations (e.g. Ca^{2+} regulation of PLC-β and influence of chemical composition of membrane phospholipids on receptor-G-protein-PLC-β coupling) will have consequences for the ultimate biological responses such as the degree of hypertrophy and the phenotype of the cardiomyocytes.

Fatty acid composition of membrane phospholipids and heart function

Phospholipids, the major structural lipids of the myocardial cells, are an important substituent of the various cell membranes, including the SL. In the major phospholipid constituents, the free base group (e.g. of choline, ethanolamine) and the phosphate group possess charge,

which provides a polar head in combination to a non-polar tail, and thus allowing the formation of the lipid bilayer. Like in other bilayer surface membranes, multiple complex proteins are embedded in the SL, including receptors, PLC-β and G-proteins that may require specific "core-" phospholipids and a certain membrane fluidity for optimal activity (Opie, 1991). The fatty acid composition of the membrane phospholipids is in part a reflection of the diet (Tahin *et al.*, 1981, Stubbs and Smith 1984; Lamers *et al.*, 1987), although the composition has also been shown to be altered by certain metabolic and pathological (e.g. diabetes) effects. For example, the n-9, n-6 and n-3 polyunsaturated fatty acids (PUFA) compete for the fatty acid desaturation and elongation pathways, but compete also in the processes involving fatty acid incorporation in the membrane phospholipids during their *de novo* synthesis and normal turnover. The regulation of the fatty acid composition of membrane phospholipids is mediated by compartmentalized enzymes (desaturases and elongases) within the endoplasmic reticulum, in which *de novo* synthesis takes place, and within the mitochondria, SR or SL, in which reacylation systems regulate the fine adjustments. The *de novo* synthesis and the fine adjustments are of crucial importance for the physicochemical adaption of the SL bilayer to meet the optimal motional characteristics and environmental requirements for the resided proteins. The fatty acid composition of the core-phospholipids provide the optimal environment for the enzymes and ion channels, and the fatty acid composition of the bulk phospholipids determines the distribution of the distinct phospholipids in different stable domains. The latter phenomenon is, in association with the local sarcolemmal cholesterol content, of considerable importance for the dynamic characteristics of the membrane (Applegate and Glomset, 1991a and 1991b, Yechiel and Barenholz, 1985; Yechiel *et al.*, 1985). Moreover, by their evident role as being a substrate for various signalling pathways, certain small pools of specific membrane phospholipids (e.g. PtdIns(4,5)P_2) carry information for transmembrane signalling in addition to their important structural role. This information is released by the action of effector enzymes in signal transduction pathways such as phospholipase C, D, and A_2 (Van Bilsen and Van der Vusse, 1995). Obviously, dietary-, metabolically- and pathologically-induced changes of the fatty acid composition of the membrane phospholipids may modulate phospholipid signalling and so change the ultimate receptor-mediated biological responses.

Scope of this thesis

The role of the β-adrenergic receptors in the short-term modulation of the contractile force of the heart under normal physiological and pathological conditions is thoroughly investigated during the last two decades. However, as discussed above it is evident that the α_1-adrenergic receptors may become important when the heart grows to compensate for a chronically increased workload. The adaptive processes of the heart to compensate for the increased workload vary from rapid recruitment of positive inotropy to progressive development of hypertrophy. Like α_1-adrenergic receptors, the ET_A receptor may be involved in inotropic responses and induction of hypertrophy, and both, α_1-adrenergic agonists and ET-1, use the PtdIns-cycle to transduce their signal into the cell. The PtdIns-cycle in myocardium appears to be a common pathway for many other hormones, such as angiotensin II, bradykinin, muscarinic agonists, 5-hydroxy tryptamine, α-thrombin, and purinergic agonists, that control

metabolism, inotropy and hypertrophy in myocardium. A complete overview of these functions of the PtdIns-cycle in myocardium is presented in Chapter 2.

In this thesis we studied the specific PtdIns-cycle-related second messenger responses of α_1-adrenergic and ET_A receptor stimulation in an experimental model of cultured neonatal rat cardiomyocytes. As discussed before, intracellular Ca^{2+} ions do not only play a key role in the regulation of the rate and force of contraction but also in the regulation of enzymes (PLC-β and PKC isoenzymes) involved in the PtdIns signalling cascade. Therefore, we studied the influence of changes of the internal and external Ca^{2+} ion concentrations on the PLC-β mediated PtdIns-cycle activity upon stimulation with the α_1-adrenergic agonist phenylephrine (PHE) or ET-1 (Chapter 3).

The agonist dependency of the receptor-mediated InsP-isomer- and 1,2-DAG responses as well as protein phosphorylation was studied in Chapter 4. To gain a better understanding in the observed responses of the putative Ca^{2+} releasing messengers, the Ca^{2+} responses mediated by PHE and ET-1 were measured in detail using single cell imaging dual wavelength fura-2 fluorescence microscopy (Chapter 5).

It has long been known that the ingestion of dietary PUFAs have potential biological effects on the function of blood(cells), the cells of vasculature and the cardiomyocytes. However, the effects of PUFAs on the cellular level is still largely unclear, particularly in heart. Therefore, Chapter 6 presents an overview of the available data regarding the reported effects of altered dietary or exogenously added PUFAs on cardiac function. This brief review is followed by the presentation of our own work (Chapter 7) on the influence of PUFAs on the specific responses of the PHE and ET-1 stimulated PtdIns-cycle and on the (ir)regularity of spontaneous contractions of cultured neonatal rat cardiomyocytes.

The model

We used cultured neonatal rat cardiomyocytes as an experimental model for the purposes mentioned above. Primary cultures were prepared from 1-2 day old Wistar rats by stepwise trypsinization (Yagev *et al.*, 1984), followed by preplating to increase the relative cardiomyocyte number to about 90 % (Blondel *et al.*, 1971). The use of neonatal cardiomyocytes in culture offered us several important advantages: i) biochemical responses can be ascribed most likely to cardiomyocytes only as cultures were 90 % pure. This experimental advantage we consider of major importance because the intact myocardium is composed of multiple cell types such as endothelial, vascular smooth muscle, connective tissue cells and cardiomyocytes. In all these cells the components of the PtdIns-cycle are very active. ii) When seeded in a confluent monolayer the spontaneous contractions reappear, which allows measurement of the effects of signalling pathways on rate and rhythm of these contractions. iii) The neonatal cardiomyocytes can be cultured on materials such as plastics and glass, which provide the opportunity to measure the intracellular signalling processes (changes in intracellular free Ca^{2+}) in microscopes, spectrophotometers, etc., and iv) α_1-adrenergic agonists and ET-1 induce -besides positive inotropy- hypertrophy of neonatal rat cardiomyocytes in culture (Simpson and McGrath, 1983; Simpson *et al.*, 1991; Ito *et al.*, 1993). Thus the model provides us an excellent biological preparation to study the α_1-adrenergic and ET-1 mediated signal transduction through and beyond the PtdIns

pathway.

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Chapter 2

SIGNAL TRANSDUCTION BY THE PHOSPHATIDYLINOSITOL CYCLE IN MYOCARDIUM

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SUMMARY

Diverse and distinct hormonal stimuli arriving at the cardiomyocyte engage specific surface receptors to initiate hydrolysis of inositol phospholipids by phospholipase C whereby information flows from changes in intracellular levels of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate, 1,2-diacylglycerol and Ca^{2+} to the specific phosphorylation of cellular proteins by various protein kinases such as the protein kinase C family, Ca^{2+}-calmodulin-dependent kinase and mitogen activated kinases. The phosphorylation products are potential regulators of the inotropic and chronotropic state, hypertrophic growth and ischemic preconditioning of the myocardium. This review summarizes the current state of knowledge concerning the phosphatidylinositol cycle and its potential role in mediating various functional responses in myocardium. The multiplicity of receptortypes, G-proteins, phospholipases C and protein kinases raises fundamental questions about the mechanisms that assure the precision and timing of the myocardial response to hormonal stimuli.

INTRODUCTION

In myocardium, signals that control metabolism, the rate and force of contraction, as well as the size and differentiation of cardiomyocytes are initiated at the cell surface by the interaction of hormones, neurotransmitters and para- or autocrine factors with their specific receptors. Depending on the agonist, signals are propagated via GTP-binding protein (G-protein)- or non-G-protein-linked receptors.

Receptors are coupled, via G-proteins or otherwise, to effectors such as adenylate cyclase, guanylate cyclase, ionchannels, phospholipase C-β (PLC-ß) and -γ, phospholipase D (PLD) and A_2 (PLA_2), and tyrosine kinases. Modulation of the activity of these effectors changes the levels of second messengers: cyclic AMP (cAMP), cyclic GMP, Ca^{2+}, inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$), 1,2-diacylglycerol (1,2-DAG) and arachidonic acid. The most common mechanism by which these second messengers function is via direct or indirect activation of protein kinases such as cAMP- (cAMP-PK), cyclic GMP- or Ca^{2+}-calmodulin-dependent protein kinases (CaM-PK), Ca^{2+}-dependent and Ca^{2+}-independent DAG-stimulated protein kinases C (PKC), and/or mitogen-activated protein kinases (MAPK and MAPKK) which then phosphorylate specific proteins ultimately leading to functional responses.

In this paper we review the current knowledge on receptor-mediated signalling via PLC-ß and -γ and the role(s) of this messenger system with the emphasis on myocardium. A review in this field after those by Brown and Jones (1986), Brown and Martinson (1992), De Chaffoy de Courcelles and Lamers *et al.* (1993a) appears timely since evidence is accumulating that activation of specific members of the PKC isozyme family, CaM-PK and MAPK(K) modulate inotropism, chronotropism, hypertrophy, gene expression and ischemic preconditioning (Morgan and Baker, 1991; Chien *et al.*,1991; Komuro and Yazaki, 1993; Sadoshima and Izumo, 1993a and b; Gruver *et al.*, 1992; and Bogoyevitch *et al.*, 1994).

HISTORICAL BACKGROUND

The first of the experiments that ultimately led to the present understanding of the ubiquitous PLC-mediated signalling pathway were carried out by Hokin and Hokin (1958) who demonstrated that acetylcholine stimulated $^{32}P_i$ incorporation into the inositol lipid fraction in slices of pancreas and brain. Several years later it was shown that PLC catalyzed the receptor-mediated inositol lipid hydrolysis and the link with Ca^{2+} signalling was postulated (Michell, 1975). A decade later Ins(1,4,5)P_3 was identified as the Ca^{2+} mobilizing second messenger and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) was shown to be the preferred substrate for PLC *in vivo* (Streb *et al.*, 1983). At that time PKC was discovered and its normal and pathological control by 1,2-DAG and phorbolester tumour promotors was investigated (Castagna *et al.*, 1982). Activation of protein kinase C by 1,2-DAG and Ca^{2+} represents the second limb of the inositol lipid signalling pathway.

In the past ten years many of the molecular details of this signalling pathway have been elucidated and an equally impressive development is the unexpected recognition that this pathway regulates a large array of cellular processes, including metabolism, secretion, muscle contraction, neural activity, fertilization, cell hypertrophy and proliferation (Berridge M, 1993; Nishizuka, 1992). The diversity of this regulation became better understood when it was discovered that certain growth factors directly stimulate a non-G-protein linked PLC-γ activated by tyrosine phosphorylation (Rhee and Choi, 1992; Fantl *et al.*, 1993).

TRANSMEMBRANE SIGNALLING

Receptors

The PLC-ß-coupled receptors are good representatives of G-protein linked receptors that generally show a high degree of homology and contain seven transmembrane-spanning regions. These receptors contain multiple potential N-glycosylation sites in the extracellular NH_2-terminal domain together with cysteine residues in the four extracellular domains that are thought to be sites for disulfide bridge formation. In myocardium, PLC-ß-coupled receptors have now been identified for α_1-adrenergic and muscarinic agonists, angiotensin II (AngII), endothelin (ET-1), serotonin (5-HT), κ-opioids and α-thrombin (Chien *et al.*, 1991; Brown *et al.*, 1992; Lamers *et al.*, 1993a). Several genes identified in this receptor class heve been termed "orphan receptors" (Mills and Duggan, 1993). Recently an "orphan" receptor has been identified that is expressed in the heart, but the ligand has yet to be identified (Okazaki, *et al.*, 1993).

Cardiac α_1-adrenergic receptors belong to a family of closely related isoforms (Terzic *et al.*, 1993). Pharmacological evidence suggests the existence of two different isoforms, α_{1A} and α_{1B}. Indeed, two different genes for the α_1-adrenoceptor heve been identified, but more members of this class exist. In mammalian heart, α_{1A}- as well as α_{1B}-adrenoceptors are expressed and it has been shown that the α_{1A}-adrenoceptor mediates activation of the PtdIns cycle (Knowlton *et al.*, 1993; Terzic *et al.*, 1993).

AngII receptors can be subdivided into two major classes, AT_1 and AT_2 (Timmermans *et al.*, 1993) that are both expressed in the myocardium (Sechi *et al.*, 1992) but the function of the AT_2 receptor is not established. Studies with cultured rat ventricular myocytes indicated

that AngII, by stimulation of the AT_1 receptor, not only activates the PtdIns cycle, but also activates PLD, PLA_2 and adenylate cyclase in these cells (Sadoshima, 1993b).

ET comprises a family of three peptides (ET-1, -2 and -3) and has not only been shown to be synthesized and excreted by vascular endothelial cells, but also to be produced in a multitude of nonvascular tissues including cardiomyocytes (Sakurai, *et al.*, 1992; Suzuki *et al.*, 1993). Two subtypes of ET receptors have now been identified; ET_A, that preferentially recognizes ET-1, and ET_B that has equipotent affinity for all three ET isoforms, and both receptors are able to activate the PtdIns cycle. Both receptor subtypes are present in the heart (Irons *et al.*, 1993), but because ET-1 is a much more potent activator of the PtdIns cycle than ET-3 in neonatal atrial cells (Irons *et al.*, 1993), it is likely that only the ET_A receptor, coupled to PLC-ß, is functionally important.

Through cloning, 5 muscarinic receptor-subtypes have been identified, $m_{1\text{-}5}$ (Bonner, 1989). Not all muscarinic receptors are linked to the same signalling pathway: m_2 and m_4 inhibit adenylate cyclase and weakly activate PLC-ß while m_1, m_3, and m_5 activate PLC-ß, PLA_2 and PLD. Only the m_2 receptor subtype is present in mammalian heart and was shown to be coupled to the PtdIns cycle (Jones *et al.*, 1987; McMorn *et al.*, 1993).

Two 5-HT receptor subtypes (HT_1 and HT_2) have been pharmacologically characterized of which only the 5-HT_2 receptor subtype is coupled to PLC. In cultured cardiomyocytes it was shown that 5-HT stimulates PtdIns(4,5)P_2 hydrolysis, independent of α_1-adrenergic stimulation suggesting the presence of HT_2 receptors in myocardium (Hamamori *et al.*, 1990).

α-Thrombin, a protease involved in coagulation, also possesses a receptor on various celltypes including the cardiomyocyte (Chien *et al.*, 1990) and the α-thrombin-receptor mRNA was also detected in these cells (Glembotski *et al.*, 1993). The α-thrombin receptor is slightly different from other members of the G-protein-linked family, as activation of the receptor occurs after cleavage of the N-terminal extracellular domain by α-thrombin. This produces a peptide that activates the remaining receptor structure leading to activation of PLC-ß (Steinberg *et al.*, 1991).

Besides the agonists mentioned above that have been the subject of many studies in the heart, other receptors were also shown to be coupled to PLC-ß, but are still less well-studied in myocardium. These include e.g. the P_2-purinoceptors and κ-opioid receptors (Brown and Martinson, 1992).

A multitude of non-G-protein linked receptors with inherent tyrosine kinase activity, likely coupled to PLC-γ, have been described and include many receptors for growth factors such as platelet derived growth factor, epidermal growth factor, insulin-like growth factor, fibroblast growth factor (FGF) and transforming growth factor, all exerting an influence on the heart (Long *et al.*, 1991; Fuller *et al.*, 1992). Besides containing a cytoplasmic tyrosine kinase domain that contains a 5 amino acid long signal molecule binding stretch that is tyrosine-phosphorylatable, these tyrosine kinases show no extended homology in the extracellular domains.

G-proteins

In general, G-proteins are heterotrimeric and are composed of α-, β- and γ-subunits. G-proteins are membrane- and receptor-associated but not membrane-spanning proteins. On agonist binding, the receptor catalyzes exchange of GDP for GTP on the G_α subunit, which activates the protein and causes its dissociation from the receptor and from the $G_{\beta\gamma}$-complex.

Both the G_α-GTP complex and $G_{\beta\gamma}$ are now thought to regulate effector molecules such as PLC-ß. Signalling by G-proteins is transient because of the intrinsic GTPase of the G_α subunit. Besides controlling interaction of G_α with the receptor, $G_{\beta\gamma}$ may also directly regulate the activity of effectors, one of which is cytoplasmic PLC, presumably PLCß$_2$ (Iniguez-Lluhi *et al.*, 1993).

About 20 isoforms of the α-subunit (all about 42 kDa) have been identified together with 4 isoforms of the β- and 6 isoforms of the γ-subunit. As several isoforms of each subunit are expressed in a cell-type specific manner this limits the amount of different G-proteins (Iniguez-Lluhi *et al.*, 1993). This also lays a rational base for the multitude of effectors that can be stimulated by G-proteins in different celltypes. On the basis of amino acid homology, the 20 G_α-subunits are classified in 5 groups: G_s, G_i, G_o, G_q and G_{12} and these groups seem to interact with different effectors. Only the α-subunits from the G_q group interact with and activate PLC-ß (Lee *et al.*, 1992). So far, four members have been identified in this G_q family: $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$ and $G_{\alpha 16}$ of which $G_{\alpha q}$ and $G_{\alpha 11}$ have been detected in heart together with $G_{\beta 1}$, $G_{\beta 2}$ and $G_{\gamma 2}$ (Neer and Clapham, 1992). Furthermore, a rat and bovine cardiac $G_{\alpha h(7)}$ was described that is coupled to the α_1-adrenergic receptor, but no comparison with the G_q group of α-subunits was made (Baek *et al.*, 1993).

The G_q family has been characterized by the absence of residues critical for ADP-ribosylation and inactivation by pertussis toxin. Indeed, most of the studies showing receptor-mediated PtdIns(4,5)P_2 hydrolysis in the heart have reported its insensitivity to inhibition by pertussis toxin (Neer and Clapham, 1992; Brown and Martinson, 1992).

Phospholipase C

Three PLC classes (PLC-β, -γ and -δ) can be discerned that contain X and Y regions with high homology (60% and 40% respectively) (Rhee and Choi, 1992). In PLC-γ the region between X and Y contains three domains (SH2 and SH3) that have homology to the non-catalytic region occurring in a family of tyrosine kinases. This PLC-γ class is activated by receptor-associated tyrosine kinase interaction with the SH domains and subsequent phosphorylation of the enzyme (Fantl *et al.*, 1993). In general, three PLC-β-, two PLC-γ- and two PLC-δ-isozymes have been recognized up till now and only isozymes of the PLC-β class are activated by interaction with the G_q-class-GTP complex (Jhon *et al.*, 1993). PLC-$\beta_{1\text{-}3}$ can be activated by $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$ and $G_{\alpha 16}$ but only PLC-β_3 showed some G_α subunit specificity (Jhon *et al*, 1993). As PLC-β_1 is more active than PLC-β_3, and PLC-β_2 is only weakly activated by G-proteins this suggests different interactions of α-subunits with PLC-β isozymes. In heart, the most abundant PLC isozyme is PLC-γ, followed by PLC-β and PLC-δ (Neer and Clapham, 1992). Up till now not much information exists on the subclasses of PLC-ß in the myocardium, but PLC-ß$_2$ seems to be absent while PLC-ß$_1$ and -ß$_3$ expression is lower compared to other tissues (Jhon *et al.*, 1993).

G-protein linked PLC in isolated cardiac membrane preparations was shown to be Ca^{2+} dependent (Renard and Poggioli, 1990). Recently we could confirm the Ca^{2+}-dependence of ET-1 and α_1-adrenergic stimulated PLC in intact cardiomyocytes (Van Heugten *et al.*, 1994). Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) may thus play a regulatory role in the receptor-mediated PtdIns turnover particularly when $[Ca^{2+}]_i$ increases due to Ca^{2+} mobilization. The rate of transmembrane signalling by PLC-ß can also be influenced by changes in the fatty acid composition of the membrane bilayer. We have observed that the presence of high

amounts of linoleic or eicosapentanoic acid in the cardiomyocyte membrane decreases the rate of α_1-adrenergic stimulated PtdIns(4,5)P_2 hydrolysis (Lamers *et al.*, 1992).

SECOND MESSENGERS

It is now generally recognized that Ins(1,4,5)P_3 is the second messenger that is able to release Ca^{2+} from non-mitochondrial pools thus promoting the Ca^{2+}-induced influx of external Ca^{2+}, possibly in conjunction with Ins(1,3,4,5)P_4 (Berridge, 1993).

Termination of the InsP_n arm of the PtdIns pathway involves several dephosphorylation reactions (Nahorski *et al.*, 1991). Dephosphorylation of Ins(1,4,5)P_3 results in the formation of biologically inactive products Ins(1,4)P_2, Ins(4)P and free inositol. The 3-kinase product of Ins(1,4,5)P_3, i.e Ins(1,3,4,5)P_4, is degraded to the biologically inactive forms Ins(1,3,4)P_3 which is dephosphorylated to Ins(1,3)P_2 or Ins(3,4)P_2, thereafter to Ins(1)P or Ins(3)P and ultimately to free inositol. Proof of the extremely fast resynthesis of inositol lipids during maximal stimulation with ET-1 of cultured cardiomyocytes, in the presence of the monophosphatase blocker Li^+, came from our observation that the free inositol level decreased dramatically, while the levels of PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 remained relatively constant (Van Heugten *et al.*, 1993).

Normally, Ins(1,4,5)P_3 metabolism in intact cells, including cardiomyocytes, was studied upon incorporation of [^{3}H]inositol into InsP_n. Apart from artefacts introduced by differences in extraction procedures, problems in studying Ins(1,4,5)P_3 metabolism arise from the fact that the dephosphorylation reactions are extremely rapid (Woodcock *et al.*, 1992; Woodcock and Anderson, 1993). The latter problem is partially circumvented by the addition of Li^+ that inhibits the Ins(1,3,4)P_3 and Ins(1,4)P_2 1-phosphatase and InsP monophosphatase activities (Nahorski *et al.*, 1991). The whole heart, or atrial and ventricular tissues examined in the earliest studies, not only contained cardiomyocytes but vascular smooth muscle cells, fibroblasts, vascular endothelial cells, and nerve endings as well, which all might contribute to the formation [^{3}H]inositol-labelled InsP_n. More recent studies that used almost homogeneous preparations of cultured or freshly isolated cardiomyocytes have most conclusively shown the coupling of the PtdIns(4,5)P_2 hydrolysis to α_1-adrenergic agonist- (Steinberg *et al.*, 1989; Kohl *et al.*, 1990; Terzic *et al.*, 1993; Woodcock *et al.*, 1992; Meij and Lamers, 1989), ET-1- (Shubeita *et al.*, 1990; McDonough *et al.*, 1993; Van Heugten *et al.*, 1993), AngII- (Abdellatif *et al.*, 1991), muscarinic agonist- (Brown and Martinson, 1992), α-thrombin-, 5-HT- (Neer and Clapham, 1992) and κ-opioidreceptors (Ventura *et al.*, 1991). When measured by high performance liquid chromatography, the Ins(1,4,5)P_3 rise is transient and returns to basal values within minutes. Besides inositol phosphatases, the heart contains Ins(1,4,5)P_3 3-kinase (Woodcock *et al.*, 1992). Using α_1-adrenergic stimulated left atria (Kohl *et al.*, 1990) or ventricular strips from adult hearts (Renard and Poggioli, 1987) dephosphorylation products of Ins(1,3,4,5)P_4 were detected. Likewise, in cultured neonatal rat cardiomyocytes we found that stimulation with phenylephrine and in particular with ET-1 led to elevation of Ins(1,3,4)P_3 and Ins(1,4)P_2 levels while the biologically active products were only slightly increased (unpublished results). This may indicate that the other limb of the PtdIns pathway, PKC activation by 1,2-DAG, is functionally more important in myocardium.

In neonatal ventricular myocytes 1,2-DAG level has not been shown to increase as early

as Ins(1,4,5)P_3 but stays elevated for at least 60 minutes (Shubeita *et al.*, 1990; Bordoni *et al.*, 1991; Sei *et al.*, 1991; McDonough *et al.*, 1993). 1,2-DAG is converted to phosphatidic acid by the action of DAG kinase and is subsequently reincorporated into the inositol lipids. It can also be hydrolyzed by DAG lipase and presumably by nonspecific esterases to free fatty acids and glycerol (Nishizuka, 1992). This can explain the fact that, due to the high PtdIns turnover rate during hormonal stimulation and its metabolism as described above, 1,2-DAG rapidly reaches a plateau level. Receptor-stimulated translocation (and concomitant activation) of PKC from the cytosol to the sarcolemma (SL) or subcellular sites (e.g. myofilaments and nucleus) also reflected the elevation of 1,2-DAG levels (Mochly-Rosen *et al.*, 1990; Allo *et al.*, 1991; Kaku *et al.*, 1991; Shubeita *et al.*, 1992; Talosi and Kranias, 1992; Bogoyevitch *et al.*, 1993). However, the presence of various isoforms of PKC and their possible differential activation by external stimuli serving to mediate distinct physiological functions in heart have only recently been recognized (Mochly-Rosen *et al.*, 1990; Brown and Martinson, 1992; Bogoyevitch *et al.*, 1993 and 1994; Terzic *et al.*, 1993; Sadoshima *et al.*, 1993b; Lamers *et al.*, 1993a). The isoforms of PKC may differ in their extent of activation by certain molecular species of 1,2-DAG (Allen and Katz, 1991). In this regard it is important to mention that we found that the generally assumed 1-stearoyl and 2-arachidonyl fatty acid composition on the glycerol backbone of PtdIns(4,5)P_2 is not characteristic for myocardium (Lamers *et al.*, 1993b). The "cross-talk" between PLC (substrate PtdIns(4,5)P_2) and PLD (substrate phosphatidylcholine) via activation of PKC may be the mechanism by which the cardiomyocyte can maintain increased levels of 1,2-DAG for a prolonged time and it can also be expected to lead to changes in the molecular species of 1,2-DAG (Nishizuka, 1992; Lamers *et al.*, 1993a).

SECOND MESSENGER RESPONSES

Ins(1,4,5)P_3 receptor

Several studies have provided evidence that Ins(1,4,5)P_3 can induce Ca^{2+} release from the SR, although the effect appears to be modest as compared to the ryanodine-sensitive Ca^{2+}-induced Ca^{2+} release (Kentish *et al.*, 1990; Zhu and Nosek, 1991; Molina-Viamonte *et al.*, 1991; Fabiato, 1992). The presence of the Ins(1,4,5)P_3 receptor (IP_3R) in heart has been indicated from analyses using whole heart tissue (Furuichii *et al.*, 1990); Marks *et al.*, 1990; Migneri *et al.*, 1990). Recently, Kijima *et al* (1993) and Verma *et al* (1992) definitely showed the presence of IP_3R separate from ryanodine receptors (RYR) in ventricular cardiomyocytes by immunolocalization and autoradiography, respectively. It was found that the density of IP_3R in ventricular myocytes was much lower than that of RYR (Verma *et al.*, 1992). Specific Ins(1,4,5)P_3 binding activity was found to be enriched in a subcellular fraction containing intercalated discs and was nearly absent from the longitudinal and junctional SR while specific ryanodine binding activity was highest in junctional SR (Kijima *et al.*, 1993). These results suggest a possible role of Ins(1,4,5)P_3 in Ca^{2+} entry through the intercalated discs and/or intercellular signalling.

The four cloned IP_3R genes bear partial sequence homology to the skeletal muscle RYR_1 and cardiac RYR_2 genes, particularly in their C-terminal membrane spanning regions (Mikoshiba, 1993; Berridge, 1993). The IP_3R as well as the RYR receptor appear to have 4-

fold symmetry compatible with a tetrameric structure of the Ca^{2+} channel. Each subunit in this IP_3R tetramer binds one $Ins(1,4,5)P_3$ molecule non-cooperatively on a site localized in the cytoplasmic N-terminal tail (Berridge, 1993) but there is still no understanding how $Ins(1,4,5)P_3$ acts to open the individual channels.

The considerable structural and molecular homology shared by RYR- and IP_3R receptor-Ca^{2+} channel proteins is mirrored in many functional similarities, the most striking of which is the common sensitivity to Ca^{2+} displayed by a Ca^{2+}-induced Ca^{2+} release (Berridge, 1993; Fabiato, 1992). However, in contrast to the RYR, the IP_3R Ca^{2+} channel is critically dependent on the filling inside the SR with Ca^{2+} and generates cyclic releases of Ca^{2+} of much smaller rate and amplitude.

Intracellular free Ca^{2+} and H^+

The increase of $[Ca^{2+}]_i$ due to stimulation of the PtdIns cycle has been studied using Ca^{2+}sensitive fluorescent indicators or aequorine and were performed on either adult or immature isolated myocytes, maintained in suspension or attached to coverslips. Eckel *et al.* (1991) showed that the α_1-adrenergic response in quiescent adult ventricular myocytes results in mobilization of the intracellular Ca^{2+} stores and similar conclusions had been drawn by Iwakura *et al.* (1990). The amount of external Ca^{2+} (Ca^{2+}_e) and Na^+ affects the magnitude of α-adrenergic stimulation of systolic Ca^{2+} suggesting the involvement of the Na^+/Ca^{2+} exchanger (Capogrossi *et al.*, 1991 and Iwakura *et al.*, 1990). However, in experiments performed to investigate the influence of α-adrenergic stimulation on electrically driven adult ventricular myocytes under moderate $[Ca^{2+}]_e$ it was demonstrated that neither the Ca^{2+} current nor Ca^{2+} transients were significantly altered (Terzic *et al.*, 1992).

Like α-adrenergic agonists, ET-1 was reported to induce a concentration dependent rise of $[Ca^{2+}]_i$ in neonatal rat atrial cells (Vigne *et al.*, 1990). The increase of $[Ca^{2+}]_i$ resulted from the mobilization of caffeine- and ryanodine-insensitive intracellular stores but also from Ca^{2+} entry through voltage dependent L-type Ca^{2+} channels. However, it was also shown that ET-1 enhanced Ca^{2+} entry through voltage dependent T-type Ca^{2+} channels in cultured neonatal rat ventricular myocytes (Furukawa *et al.*, 1992). Taken together, the effects of ET-1 on Ca^{2+} transients are variable depending on the species and/or maturity of the myocyte preparations (Kohmoto *et al.*, 1993).

Stimulation with AngII has been found to rapidly (within 60 sec) decrease $[Ca^{2+}]_i$ transients in chick embryo ventricular myocytes (Kohmoto *et al.*, 1993), although in cultured neonatal rat ventricular myocytes the AngII stimulation caused an steady state as well as transient $[Ca^{2+}]_i$ rise (Allen *et al.*, 1988), and the initial Ca^{2+}_i increase was derived predominantly from the SR (Kem *et al.*, 1991).

α-Adrenergic agonist-induced pH_i increase has been shown to occur in perfused rat heart, isolated adult ventricular myocytes in suspension and on coverslips using fluorescent pH-sensitive probes and partition of [2-^{14}C]5,5'-dimethyloxazolidine-2,4-dione (Terzic *et al.*, 1993). Furthermore, α_1-adrenergic and purinergic stimulation accelerated the recovery from acidosis induced by a NH_4Cl pre-pulse (Pucéat *et al.*, 1993). ET-1 as well as AngII induced a small decrease in pH_i in cultured chick embryo- and neonatal rat ventricular myocytes, whereas these agonists induced a pH_i increase in adult rabbit myocytes (Kohmoto *et al.*, 1993). The hypothesis that the Na^+/H^+ exchanger is involved, is primarily based upon the findings that specific blockers of the Na^+/H^+ exchanger were able to abolish or prevent pH_i

increase after α_1-adrenergic stimulation (Iwakura *et al.*, 1990; Fuller *et al.*, 1991; Terzic *et al.*, 1993). Terzic *et al.* (1993) proposed that PKC-induced modification of the Na^+/H^+ exchanger turnover rate is responsible for cellular alkalinization, by either a change in affinity of the allosteric site for H^+ and/or an increase in the maximal rate of ionic exchange of the antiport. The shift to alkaline pH was also induced by phorbolester and prevented by PKC inhibitors H-7, sphingosine and staurosporine in ventricular myocytes in suspension (Iwakura *et al.*, 1990; Gambassi et al. 1992) but not in ventricular myocytes attached to glass coverslips (Pucéat *et al.*, 1993). Alternatively, Na^+/H^+ exchanger activation by CaM-PK has been proposed as the mechanism of pH_i increase (Iwakura *et al.*, 1990), but evidence against this hypothesis has been presented too (Pucéat *et al.*, 1993).

Protein phosphorylation

At present there is little information available on *in vivo* phosphorylation by PKC and CaM-PK of cardiac substrate proteins. A 15 kDa substrate protein for PKC (Presti *et al.*, 1985) as well as cAMP-PK (Lamers and Stinis, 1982) was first identified in isolated cardiac SL vesicles *in vitro*. Later, this 15 kDa protein was shown to be phosphorylated in rat hearts stimulated by α_1-adrenergic agonist *in vivo* (Lindemann, 1986). More recent findings provided evidence that PKC activation *in vivo* is associated with increased phosphorylation of the 15 kDa protein (Talosi and Kranias, 1992; Hartmann and Schrader, 1992) but not with phospholamban (PLB).

AngII and phorbolester TPA (12-O-tetradecanoylphorbol-13-acetate) were shown to enhance phosphorylation of the same set of proteins (32 and 83 kDa) in intact neonatal rat cardiomyocytes (Dösemeci *et al.*, 1988). The 83 kDa protein was suggested to be the widespread occurring PKC substrate protein ("87 kDa") of which the cellular localization is unknown. The 32 kDa protein is likely to be the myofilament protein troponin I (TnI).

Apart of the afore-mentioned 15, 32 and 83 kDa proteins, a number of other cardiac proteins have been shown to be substrates for PKC *in vitro* in subcellular fractions enriched in SL, SR and myofibrils. These include TnI (30 kDa), TnT (40 kDa) (Noland and Kuo, 1991 and 1993), myosin light chain-2 (MLC-2) (20 kDa) (Clement *et al.*, 1992), and PLB (24 kDa) (Movsesian *et al.*, 1984). Several of these proteins (PLB, MLC-2) appear to be good substrates for CaMPK as well (Clement *et al.*, 1993; Venema *et al.*, 1993; Movsesian *et al.*, 1984). It was also shown that CaM-PK but not PKC or cAMP-PK can phosphorylate the C-terminal domain of the rabbit cardiac Na^+/H^+-antiporter *in vitro* (Fliegel *et al.*, 1992). In some of the *in vitro* studies the consequences of phosphorylation for the protein or organelle function have been investigated. Stimulation of cardiac SR Ca^{2+} uptake by activation of cAMP-PK, CaM-PK and PKC has been shown (Movsesian *et al.*, 1984). However, using digitonin-permeabilized cultured ventricular myocytes, it was found that PKC activation by phorbolester inhibits SR Ca^{2+} uptake (Rogers *et al.*, 1990). Recently, an unique Ca^{2+}-CaM-dependent phosphorylation site of cardiac RYR was identified which, when phosphorylated, activated the Ca^{2+} release channel (Witcher *et al.*, 1991). Phosphorylation of TnI and TnT by PKC in reconstituted actomyosin resulted in decreased Ca^{2+}-stimulated Mg^{2+}-ATPase activity and this was reversed by dephosphorylation (Noland and Kuo, 1991 and 1993). The addition of Ca^{2+}-CaM-dependent MLC-kinase to chemically skinned heart cells results in an increased Ca^{2+}-sensitivity of force development and actomyosin Ca^{2+}-stimulated Mg^{2+}-ATPase activity (Clement *et al.*, 1993). In the latter experiments PKC-induced phosphorylation of MLC-2

together with phosphorylation of TnI and TnT induced a large increase in Ca^{2+} sensitivity of the filaments.

Recently it was shown that stimulation with ET-1, α_1-adrenergic agonist, acidic FGF (aFGF) and phorbolester activates two MAPK (42- and 44-kDa proteins) in cultured cardiomyocytes that belong to a family of threonine-kinases (Bogoyevitch *et al.*, 1994). The activation results from phosphorylation of tyrosine and threonine residues within MAPK. A MAPK activator, MAPKK, was also shown to be stimulated by ET-1 and aFGF in cardiac myocytes and PKC-α, -δ and -ϵ are not involved in this activation (Bogoyevitch *et al.*, 1994). One possible activator of MAPKK is PKC-ζ that has been shown to be activated by the putative second messenger PtdIns(3,4,5)P_3, which is formed by the action of PtdIns-3 kinase that is frequently associated with receptor tyrosine kinases (Bogoyevitch et al., 1994). Downstream target proteins for MAPK activity in cardiac myocytes have not been investigated yet.

(PATHO)PHYSIOLOGICAL IMPLICATIONS

Ino- and chronotropism

Agonists that stimulate myocardial receptors coupled to the PtdIns cycle are frequently shown to be positive inotropic agents. In particular, this property was described for α_1-adrenergic agonists (Terzic *et al.*, 1993) and similar results were obtained with ET-1 (Kohmoto *et al.*, 1993), AngII (Ikenouchi *et al.*, 1992), α-thrombin and purinergic agonists (Brown and Martinson, 1992). Stimulation of cardiac m_2 receptors not only activated the PtdIns cycle but also G-protein linked K^+ channels which explains the net negative inotropic effect (McMorn *et al.*, 1993). The role of PtdIns cycle-linked second messengers in mediating the positive inotropic effect is, however, not yet fully clarified. As discussed before, no doubt exists about the presence of Ins(1,4,5)P_3 sensitive Ca^{2+} channels in myocardium and the occurrence of a significant elevation of Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ due to stimulation of receptor coupled PLCß seen in many studies (Brown and Martinson and, 1992; Terzic *et al.*, 1993). However, it is now believed that the changes of $[Ca^{2+}]_i$ are too transient to explain the sustained positive inotropic responses. That activated PKC alone modulates contractility is also unlikely as phorbolesters usually produce negative inotropic effects (e.g. Capogrossi *et al.*, 1990; Kohmoto *et al.*, 1993). Recently, the attractive hypothesis has been forwarded that agonists enhance myofilament responsiveness to Ca^{2+} by stimulating the PtdIns cycle (Terzic *et al.*, 1993). PKC and/or CaM-PK-dependent phosphorylation of regulatory myofilament proteins (MLC-2, TnI, etc.) and/or direct effects of cytosolic alkalinization on myofilament function could represent the mechanism(s) involved in Ca^{2+}-sensitization. Evidence for this theory is mainly derived from studies on the α_1-adrenergic agonist effect on cardiac preparations (Brown and Martinson, 1992; Terzic et al., 1993) Apparently, the inotropic effect depends on the net effects of protein phosphorylation, the change in $[H^+]_i$, the size of intracellular Ca^{2+} transient and other unknown factors.

Several agonists, including α_1-adrenergic agonists (Terzic *et al.*,1993), AngII (Allen *et al.*, 1988) and α-thrombin (Steinberg *et al.*, 1991) that are able to stimulate receptor-mediated PtdIns(4,5)P_2 hydrolysis, can enhance the automaticity and prolong the repolarization of ventricular cardiomyocytes and isolated Purkinje fibers. In this regard it is important to notice

that e.g. α_1-adrenergic agonists do not alter the pacemaker rate of sinoatrial cells and as a result do not change the nodal rhythm of the heart. Whether second messengers of the PtdIns cycle contribute to the automaticity response to α_1-adrenergic agonists, AngII and α-thrombin remains to be determined.

Hypertrophic cellgrowth

Chronic hemodynamic overload of the heart because of infarction, hypertension or either volume or pressure overload ultimately leads to hypertrophy of the left ventricular wall. Many agonists that activate the PtdIns cycle and induce hypertrophy were identified using *in vitro* models that employ cultured cardiomyocytes. These include α_1-adrenergic agonists (Mochly-Rosen *et al.*, 1990; Knowlton *et al.*, 1993), AngII (Sadoshima and Izumo, 1993a and b), ET-1 (Shubeita *et al.*, 1990, and Suzuki *et al.*, 1990) and α-thrombin (Glembotski *et al.*, 1993). Recent evidence suggests that stretch-induced activation of the PtdIns cycle and hypertrophy is mediated by release of AngII from the cardiomyocytes and stimulation of the PtdIns cycle (Komura and Yazaki, 1993; Sadoshima *et al.*, 1993c). Furthermore, ET-1 is locally generated and released by cardiomyocytes after stimulation with AngII (Ito *et al.*, 1993) implicating that stretch-induced induction of hypertrophy is brought about by an autocrine mechanism.

Activation of PKC is sufficient to give several of the hypertrophic responses in cardiomyocytes (Allo *et al.*, 1991; Shubeita *et al.*, 1992) and this was elegantly confirmed by PKC transfection studies (Shubeita *et al.*, 1992, Kariya *et al.*, 1992). However, activation of PKC is not the only factor involved in induction of hypertrophy. Sei *et al.* (1991) showed that inhibition of PKC activity resulted in a 75% decline in hypertrophic response after α_1-adrenergic stimulation. On the other hand, inhibition of CaM-PK completely blocked hypertrophy development, in agreement with the requirement for Ca^{2+} of the hypertrophy process. Conflicting results were reported concerning the role of $Ca^{2+}{}_e$ in stimulation of protein synthesis by ET-1. In one study, a Ca^{2+} blocker had no effect (Ito *et al.*, 1991), while the group of Suzuki *et al* (1991) showed that the same Ca^{2+} channel blocker partly inhibited ET-1 induced increase in protein synthesis (Suzuki *et al.*, 1990 and 1991). On the other hand, it was shown that an increase in $[Ca^{2+}]_i$ leads to a temporal stimulation of transcription of MLC-2, skeletal α-actin and troponin genes (Ito *et al.*, 1991) and the AngII induced induction of the transcription factor c-fos by PKC activation also requires Ca^{2+} (Sadoshima, 1993b). The involvement of Ca^{2+}/CaM regulated processes in induction of cell growth is further substantiated by a study in which overexpression of CaM led to induction of hypertrophy as well as hyperplasia (Gruver *et al.*, 1992). Other kinases that might be involved in hypertrophy are the MAPK(K). These MAPK(K) are activated by α_1-adrenergic agonist and ET-1 stimulation of cardiomyocytes (Bogoyevitch *et al.*, 1993b and 1994). Coordinated expression of several genes takes place as a result of α_1-adrenergic agonist, ET-1 and AngII stimulation (Shubeita *et al.*, 1990; Chien *et al.*, 1991; Ito *et al.*, 1991; McDonough *et al.*, 1993; Sadoshima and Izumo, 1993a). Early and transient responses involve transcription of socalled immediate early genes (c-fos, c-jun and EGR-1) encoding known or putative transcription factors. The c-fos/c-jun heterodimer binds to AP-1 DNA consensus sequences resulting in activation of transcription of genes containing these elements, e.g. in the ANF gene (Chien *et al.*, 1991). The expression of EGR-1 seems of prime importance for development of hypertrophy as blocking the translation of the ERG-1 mRNA by an antisense oligonucleotide completely inhibits ET-1 induced stimulation of protein synthesis, an indicator

of hypertrophy (Neyses *et al.*, 1991). At a later stage of hypertrophy, protein synthesis increases probably as a result of induction or enhancement of transcription of several late genes (MLC-2, ANF, skeletal α-actin, ß-myosin heavy chain (ß-MHC)) (Chien *et al.*, 1991). In contrast to α_1-adrenergic stimulation, ET-1 not only increased transcription of the embryonic MHC β-isozyme but also of the adult α-form (Wang *et al.*, 1992). This suggests that differences in signal transduction pathways leading to hypertrophy exist between α_1- and ET-1. It has also been shown that exposure of cardiomyocytes to ET-1 leads to partial homologous desensitization of the PtdIns cycle activity, a phenomenon not present during α_1-adrenergic stimulation (Van Heugten *et al.*, 1993, and McDonough *et al.*, 1993). Whether this is the underlying mechanism that is responsible for the difference MHC isozyme expression as described above and the rapid decrease of ET-1 induced ANF mRNA levels remains to be determined (McDonough *et al.*, 1993).

AngII, like ET-1, provokes PLC activity that is rapidly desensitized (Abdellatif *et al.*, 1991). As research on the hypertrophic effect of AngII is relatively young, studies on expression of contractile and embryonic genes are not yet available and conclusions regarding the role of desensitization in regulation of transcription can not be drawn.

A fourth receptor that is coupled to induction of hypertrophy through activation of the PtdIns cycle is the α-thrombin receptor (Chien *et al.*, 1990, and Glembotski *et al.*, 1993). Stimulation of cardiomyocytes with α-thrombin leads to morphological and genetical changes reminiscent of hypertrophy; cells are enlarged and contain highly organized contractile units while ANF expression is induced in a dose-dependent manner. The signal transduction pathway involves both PKC and tyrosine kinase activation but further details are unknown.

We have to be aware of the fact that activation of receptor-tyrosine kinases can also lead to increased PtdIns cycle activity through phosphorylation and activation of the PLC γ-isozyme. (Fantl *et al.*, 1993). At present the only evidence for the involvement of PLC-γ in induction of hypertrophy comes from FGF-induced activation of tyrosine kinases and MAP kinases (Bogoyevitch *et al.*, 1994) and FGF-initiated growth of cardiomyocytes (Parker *et al.*, 1990).

Ischemic preconditioning

Many studies have investigated the possible mechanisms involved in the infarctsize limiting effect of preconditioning. Some of the most likely mechanisms for this protective effect are discussed in recent reviews (Lawson and Downey, 1993; Cohen and Downey, 1993; Parratt, 1994) and involve the release of endogenous substances like adenosine, noradrenaline, bradykinin and prostacyclin from the myocardium. It is now believed that the activation and translocation of PKC during the preconditioning stimulus accounts for the ability of the cardiomyocytes to "remember" the ischemic episode thereby increasing the tolerance during the subsequent prolonged ischemic period. PKC stays associated with the membrane for about an hour after ischemia and can phosphorylate proteins early in a second ischemic period thus mediating protection. The role of PKC in mediating protection was confirmed in studies where inhibitors of PKC blocked protection while pretreatment of the heart with phorbolester mimicked preconditioning. Furthermore, it was found that muscarinic m_2- and α_1-adrenergic agonists are equipotent with adenosine in their ability to precondition the myocardium (Cohen and Downey, 1993; Parratt, 1993). However, at present no data on changes of PtdIns cycle and PKC activation and translocation are available in the

preconditioned myocardium to support the PKC hypothesis.

PERSPECTIVES

In spite of the fact that several important advances have been made in understanding the receptor-mediated signalling through the PtdIns cycle in the heart, we must concede that our knowledge of the receptor-specific signaltransduction cascades is still very limited and is hampered by the variability in responses observed at different developmental stages and/or in different species. At many steps, G-protein- and PLC function, Ins(1,4,5)P_3- and 1,2-DAG metabolism, CaM-PK-, PKC- and MAPK(K) actions, the receptor-mediated signalling may diverge. The composition of the regulatory G proteins that are interacting with specific receptors is largely unknown. The distinct modes of activation, together with the intracellular translocation of the various PKC species implies their specialized functions in the myocardial response to hormones. For some growth factors the pathway responsible for the release of Ins(1,4,5)P_3 and 1,2-DAG begins with tyrosine receptors linked with PLC-*γ*, a pathway which needs to be elucidated. In addition, the identity and functions of the multiple targetproteins of the various protein kinases have to be unravelled. Moreover, one should pay attention to the "cross-talk" of the PtdIns cycle with other signalling pathways, e.g. PLD as this leads to a long-lasting increase of 1,2-DAG levels with different fatty acid composition. Further exploration of the networks of receptor-specific signalling via PtdIns cycle such as described in this review is important for our understanding of the regulation of myocardial function.

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Chapter 3

CALCIUM AND THE ENDOTHELIN-1 AND α_1-ADRENERGIC STIMULATED PHOSPHATIDYL INOSITOL CYCLE IN CULTURED RAT CARDIOMYOCYTES

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ABSTRACT

Cultured neonatal rat cardiac myocytes have been utilized as a model for the study of the effect of variations in cytoplasmic free Ca^{2+} on the activity of phospholipase C, a key enzyme in agonist-stimulated signal transduction through the phosphoinositide pathway. Cells prelabelled with [^{3}H]inositol were exposed to various agents in an attempt to modulate the cytoplasmic free Ca^{2+} concentration and the formation of [^{3}H]inositolphosphates (15-30 min) in the presence of Li^+ was taken as a measure for phospholipase C activity. Not the basal but the endothelin-1 (10^{-8} M) induced [^{3}H]inositolphosphate production (15 min) was stimulated 1.54- and 1.43- fold by A23187 (10 μM external Ca^{2+}) and 50 mM K^+ (1.3 mM external Ca^{2+}) treatment of cells respectively. The phenylephrine (10^{-4} M) induced response was also stimulated (1.35-fold) by A23187, however it was 43 % inhibited by high K^+. Ouabain (10 μM) treatment of cells did not affect either basal or agonist stimulated phosphoinositide turnover. On the other hand, total removal of external free Ca^{2+} by addition of 50 μM ethylene glycol bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid strongly inhibited (75 %) the endothelin-1 induced but not the basal phospholipase C activity. Endothelin-1 binding to its receptor was shown not to be inhibited by the absence of external Ca^{2+} while resynthesis of [^{3}H]phosphatidylinositol 4,5-bisphosphate was not rate-limiting under this condition. The lack of external Ca^{2+} eventually resulted in total standstill of the ET-1 induced PtdIns turnover after 30 min. Although not always as predicted, effects on basal and agonist-activated phospholipase C were observed too when cells were treated with low Ca^{2+} medium, Ca^{2+} entry blocker nifedipine (1 μM) or Ca^{2+}-channel agonist Bay K8644 (1 μM) but most of these effects were only seen after 90 min incubation. Fluorometric (fura-2) measurements showed that total removal of external free Ca^{2+} for a short period decreased, while short exposure to high K^+ increased cytoplasmic free Ca^{2+} but neither Ca^{2+} free buffer or nifedipine nor Bay K8644 had any effect. Furthermore, in saponin-permeabilized cardiomyocytes we could demonstrate that basal as well as GTPγS (30 μM) stimulated phospholipase C activity was strongly activated by free Ca^{2+} in the concentration range of 0.1 to 10 μM. We conclude that in the intact cardiomyocyte the signalling pathway through phospholipase C/phosphatidylinositol 4,5-bisphosphate, stimulated by agonist-receptor interaction that activates GTP-binding proteins as does GTPγS, is likely to be a Ca^{2+} dependent process.

INTRODUCTION

In heart, signal transduction through the phosphatidylinositol (PtdIns) pathway plays an important role in regulation of inotropy and the development of hypertrophy (Brown, *et al.*, 1992; Morgan and Baker, 1991). Receptors for e.g. endothelin-1 (ET-1), angiotensin II and phenylephrine (PHE), were shown to be coupled to this signal transduction pathway. Activation of phospholipase C (PLC) by agonist binding to the GTP-binding protein (G-protein) coupled receptors results in generation of the second messengers 1,2-diacylglycerol ((1,2)DAG) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) by the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$). $Ins(1,4,5)P_3$ releases Ca^{2+} from internal stores while this increase in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}_{free}]_i$) together

with elevated (1,2)DAG levels activates protein kinase C, a key enzyme in regulation of cellular functions as e.g. in the transcriptional control during the development of hypertrophy (Morgan and Baker, 1991).

In various cell types it was shown that Ca^{2+} has profound effects on PLC activation either directly or by potentiating the receptor-mediated response (Abdel-latif, 1986; Cockcroft and Thomas, 1992). The direct effects of Ca^{2+} occur both in the physiological micromolar range as well as at high millimolar levels which are of less relevance for the intact cell. Potentiation of receptor-mediated stimulation of PLC by Ca^{2+} occurs at micromolar levels (Abdel-latif, 1986; Cockcroft and Thomas, 1992). The myocardial studies available used two different approaches to show the Ca^{2+} dependence of PLC: isolated membrane preparations (Schwerz, *et al.*, 1987; Schwerz and Halverson, 1989; Edes and Kranias, 1990; Renard and Poggioli, 1990) and saponin-permeabilized cells (McDonough, *et al.*, 1988; Jones and Brown, 1988). In three of these studies the reported membrane-bound PLC activity was found to require Ca^{2+} in the absence of GTP analog, however these Ca^{2+} levels were well above the normal cytoplasmic range (Schwertz, *et al.*, 1987; Schwertz and Halverson, 1989; Edes and Kranias, 1990). Other investigations demonstrated that only during stimulation of PLC by G-protein the Ca^{2+} sensitivity of the enzyme was in the physiological range (Renard and Poggioli, 1990; McDonough, *et al.*, 1988; Jones and Brown, 1988). Therefore, it became important to obtain answers to the following questions for intact cardiomyocytes: *(i)* does an increase of cytoplasmic free Ca^{2+} stimulate basal PLC activity? and *(ii)* does the elevation of $[Ca^{2+}_{free}]_i$ have a feedforward stimulatory role in the receptor-mediated activation of PLC?

The $[Ca^{2+}_{free}]_i$ rises from 100 to several hundred nM during each cardiac cycle, so contractile-associated Ca^{2+} transients could normally induce PtdIns(4,5)P_2 hydrolysis. Mechanical loading of cultured cardiomyocytes results in the activation of PtdIns(4,5)P_2 hydrolysis and this effect may be caused by alterations of the $[Ca^{2+}_{free}]_i$ (Komura, *et al.*, 1990). After ET-1 stimulation of cardiomyocytes Ca^{2+} was transiently increased (Vigne, *et al.*, 1990; Hirata, *et al.*, 1989) suggesting that PLC fluctuates accordingly. Another implication of regulation of PLC by Ca^{2+} is of pathophysiological relevance as during myocardial ischemia-reperfusion the development of intracellular Ca^{2+} overload might cause activation of PLC. The product of PLC activation Ins(1,4,5)P_3 on its turn might induce release of Ca^{2+} from the sarcoplasmic reticulum thereby aggravating the Ca^{2+}-overload which is believed to be causally related to myocardial injury (Lee and Allen, 1991). Indeed, several reports have shown that basal and α_1-adrenergic agonist-induced PtdIns(4,5)P_2 hydrolysis is increased during myocardial hypoxia or ischemia-reperfusion (Otani, *et al.*, 1989; Mouton, *et al.*, 1991; Heathers, *et al.*, 1989).

We used intact cultured neonatal rat ventricular myocytes as a model system to study the effect of variations in $[Ca^{2+}_{free}]_i$ on the activity of PLC. It was shown that agonist (ET-1 or PHE)-induced generation of inositol phosphates ($InsP_n$) is modulated by protocols (depolarization by high K^+ concentration or Ca^{2+} chelation by ethylene glycol bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA)) that were demonstrated by fura 2 fluorescence measurements to respectively raise or lower cytosolic levels of Ca^{2+} in intact cultured neonatal rat ventricular cardiomyocytes. Ca^{2+} ionophore A23187 in the presence of micromolar Ca^{2+} was also shown to stimulate agonist-induced $InsP_n$ accumulation. Furthermore, in saponin-permeabilized cardiomyocytes Ca^{2+} concentrations of 0.1 to 10 μM were shown to strongly stimulate basal as well as G-protein activated phospholipase C

activity. The cytoplasmic free Ca^{2+} concentration may play a regulatory role in the receptor-mediated PtdIns turnover particularly when Ca^{2+} increases due to the Ca^{2+} mobilizing action of Ins(1,4,5)P_3.

MATERIALS AND METHODS

Reagents

Culture dishes (4 well multidish) were obtained from Nunc (Roskilde, DK). Cell culture medium Ham F10 was obtained from Gibco (UK), while fetal calf serum, horse serum and penicillin/streptomycin was from Boehringer Mannheim (Germany) as was Ca^{2+} ionophore A23187. Trypsin (type III) was from Sigma (St. Louis, USA) as were PHE and phosphoinositide standards. ET-1 was obtained from Peninsula Laboratories (Belmont CA, USA). Fura 2-AM was from Molecular Probes (Eugene, OR, ASU) while ionomycin was from Calbiochem (USA) Bay K1040 (nifedipine) was from Bayer (Leverkusen, Germany), Bay K8644 was obtained from Pharmuka Laboratories (Gennevilliers, France) while g-strophanthin (ouabain) was from MERCK (Darmstadt, Germany). *Myo*-[2-^{3}H]inositol (17.5 Ci/mMol) was from Amersham International PLC (Amersham, UK) and Dowex AG 1-X8 (200-400 mesh, formate form) was from Bio-Rad Laboratories (Richmond CA, USA) while En^{3}Hance was from NEN (Boston, USA).

Cell culture

Primary cultures of neonatal ventricular myocytes were prepared from 1-2 day old Wistar rats as described before (Yagev, *et al.*, 1984) using preplating (Blondel, *et al.*, 1971) to further increase cardiomyocyte to non-myocyte ratio. Cardiomyocytes were seeded in 1.9 cm² wells at 150 to 175 x 10^3 cells/cm² giving a confluent monolayer of spontaneously contracting cells after 24 hours. The cells were maintained at 37°C and 5% CO_2 in complete growth medium consisting of Ham F10 supplemented with 10% fetal calf serum, 10% horse serum, 100 U penicillin/ml and 100 μg streptomycin/ml. Growth medium was renewed 24 hours after seeding and every 48 hours thereafter. Experiments were routinely performed 5 or 6 days after plating of the cells.

Measurement of water-soluble inositolphosphates and inositol-containing lipids

Cardiomyocytes were labelled with 2 μCi *myo*-[2-^{3}H]inositol/ml for 24 hours in complete growth medium containing 3 μM inositol. Prior to performing the experiments, the cells were washed with incubation buffer (130 mM NaCl; 4.7 mM KCl; 1.3 mM $CaCl_2$; 0.44 mM NaH_2PO_4; 1.1 mM $MgSO_4$; 20 mM $NaHCO_3$; 11 mM glucose; 20 mM HEPES; pH 7.4, 37°C and aerated with 5% CO_2). Thereafter, the cells were incubated as described in the legends to the figures. Incubations were terminated by rapidly washing the cells with ice-cold buffer followed by two successive extractions with ice-cold 4% (w/v) $HClO_4$. Lipids were subsequently extracted by incubation with ice-cold methanol:12 M HCl (100:1). The $HClO_4$ extract, containing water-soluble products, was neutralized by addition of a solution of 2 M KOH and 1 M K_2CO_3. Lipids were extracted from the methanol:HCl fraction by phase-separation after addition of 1 volume of chloroform and 0.5 volumes of 2.5 M HCl. The resulting organic (lower) phase was reextracted once with 1 volume of

chloroform:methanol:0.6 M HCl (3:48:47 v/v/v) and used for lipid analysis as described below. The total cellular amount of *myo*-[2-^{3}H]inositol-containing compounds (defined as the sum of water-soluble inositol-containing products together with inositol-containing lipids) was constant during the experiments. The [^{3}H]inositol phosphates ([^{3}H]InsP$_n$) were separated from [^{3}H]inositol and [^{3}H]glycero-phosphoinositol by chromatography on Dowex AG 1-X8 as originally described by Berridge, *et al.* (1982). [^{3}H]Inositol and [^{3}H]glycero-phosphoinositol were eluted with water and 5 mM Borax in 30 mM CHOONa respectively. Total [^{3}H]InsP$_n$ was subsequently eluted with 1.0 M $CHOONH_4$ in 0.1 M HCOOH.

The [^{3}H]inositol-containing phospholipids PtdIns, phosphatidylinositol 4-monophosphate (PtdIns(4)P) and PtdIns(4,5)P_2 in the organic phase were analyzed by thin layer chromatography on Silica gel 60 plates (MERCK) in chloroform:acetone:methanol:acetic acid:water (40:15:13:12:8 v/v/v/v/v) as described (Jolles, *et al.*, 1981) and were visualized by fluorography after spraying with En^{3}Hance. Quantification of the separate inositol-containing lipids was carried out by scraping the spots off the plates and counting the scrapings in scintillation cocktail.

Inositol phosphate production in permeabilized cardiomyocytes

Cardiomyocytes were isolated and cultured as described above. After labelling with 2 μCi *myo*-[2-^{3}H]inositol/ml for 24 hours the cells were washed with phosphate buffered saline (37°C) and subsequently permeabilized for 5 min with intracellular buffer (20 mM HEPES; 10 mM NaCl; 110 mM KCl; 1 mM KH_2PO_4;4 mM $MgCl_2$; 1 mM EGTA; 3 mM ATP; 8 mM creatine phosphate; 6 U creatine kinase/ml; pH 7.0) with 100 μg saponin/ml. Hereafter, the cells were washed 3 three times with intracellular buffer lacking saponin and fresh intracellular buffer containing 10 mM LiCl with Ca^{2+} and GTPγS as required was added. Permeabilized cells were incubated for 15 min at 37°C, buffer was collected and cells were extracted with PCA and methanol/HCl as described above. As >60% of InsPn was seen to be present in the buffer (not shown), both buffer and PCA extract of the cells were analyzed by Dowex AG 1-X8 chromatography as described above.

Measurement of intracellular free Ca^{2+} concentration

Cardiomyocytes were grown on glass cover slips essentially as described above. After washing with incubation buffer (see above) the cells were loaded for 60 min at 37^{0}C with 2 μM fura-2 AM in the presence of 2.5 mM probenicid. Subsequently, the cover slip was washed with incubation buffer and inserted in a holder tightly fitting into a quartz cuvette containing 2 ml incubation buffer. The cuvette was placed in a Perkin- Elmer LS-3B fluorescence spectrometer with a thermostated (37°C) cuvette holder. Fluorescence was continuously recorded at 510 nm using excitation wavelengths of 340 and 380 nm. The ratio of the fluorescence elicited at 340 nm ($F_{340\ nm}$) and 380 nm ($F_{380\ nm}$) was determined and taken as measure of the cytoplasmic free Ca^{2+} level. Calibration of the fluorescence signal was performed after each assay by addition of 2.5 μM ionomycin to obtain maximal ratio that ranged from 1.26 to 6.44. Hereafter, 10 mM EGTA was added to obtain the ratio at minimal Ca^{2+} saturation of the fluorescent dye (i.e. 0.93 $\pm$ 0.02, n=15). Maximal ratio values were variable, therefore absolute Ca^{2+} concentrations could not be determined. Before starting the treatment, the cells were incubated for 15 min in buffer. During both periods $F_{340\ nm}$ and $F_{380\ nm}$ were determined several times and averaged.

Statistical analysis

Data were evaluated for statistical significance by one-way or multivariate analysis of variance where applicable.

RESULTS

Effects of agents that modulate cytoplasmic free Ca^{2+} on basal PLC activity

To determine whether PtdIns(4,5)P_2 hydrolysis in unstimulated neonatal rat ventricular myocytes can be activated by an increase in $[Ca^{2+}_{free}]_i$, cells prelabelled with [^{3}H]inositol were exposed to the Ca^{2+} ionophore A23187 at different Ca^{2+} concentrations in the presence of LiCl (Table 1). Li^+ (10 mM) was routinely added in order to block inositol mono- and bisphosphatase and to take the accumulation of total [^{3}H]InsP_n (after incubation for 15 min) as a measure for PLC activity (van Heugten, *et al.*, 1992; Meij and Lamers, 1989; Lamers, *et al.*, 1992). Increasing the extracellular Ca^{2+} (10-100 μM) well above physiological intracellular levels in the presence of Ca^{2+} ionophore A23187 did not result in activation of InsP_n accumulation above basal values (Table 1). The possibility that the $[Ca^{2+}_{free}]_i$ was already optimal for PLC activity was investigated by total removal of external free [Ca^{2+}] through the addition of 50 μM EGTA to Ca^{2+} free medium. Even this drastic treatment of the cells did not significantly affect the basal InsP_n production (Table 1).

Table 1. Effect of agents modulating $[Ca^{2+}_{free}]_i$ on basal PLC activity in rat neonatal cardiomyocytes.

Condition	[^{3}H]InsP_n levels	n
1.3 mM Ca^{2+}	1.03 ± 0.07	13
0 μM Ca^{2+}, 1 μM A23187	0.90 ± 0.06	6
1 μM Ca^{2+}, 1 μM A23187	1.10 ± 0.09	12
10 μM Ca^{2+}, 1 μM A23187	1.08 ± 0.08	11
100 μM Ca^{2+}, 1 μM A23187	1.10 ± 0.08	13
0 μM Ca^{2+}, 50 μM EGTA	0.91 ± 0.02	4

Cardiomyocytes were prelabelled for 24 hours with *myo*-[2-^{3}H]inositol. Cells were washed with Ca^{2+} free incubation buffer and incubated for 15 min in the presence of 10mM LiCl with buffer containing 1.3 mM Ca^{2+}, with buffer containing 1 μM Ca^{2+} ionophore A23187 and different Ca^{2+} concentrations as indicated, or with 50 μM EGTA in Ca^{2+} free buffer. Extraction and quantification of [^{3}H]InsP_n levels by Dowex chromatography were performed as described in Materials and Methods. [^{3}H]InsP_n levels are expressed as percentage of total cellular [2-^{3}H]inositol (defined as the sum of water-soluble inositol-containing products together with inositol-containing lipids). n refers to the number of experiments (mean ± S.E.).

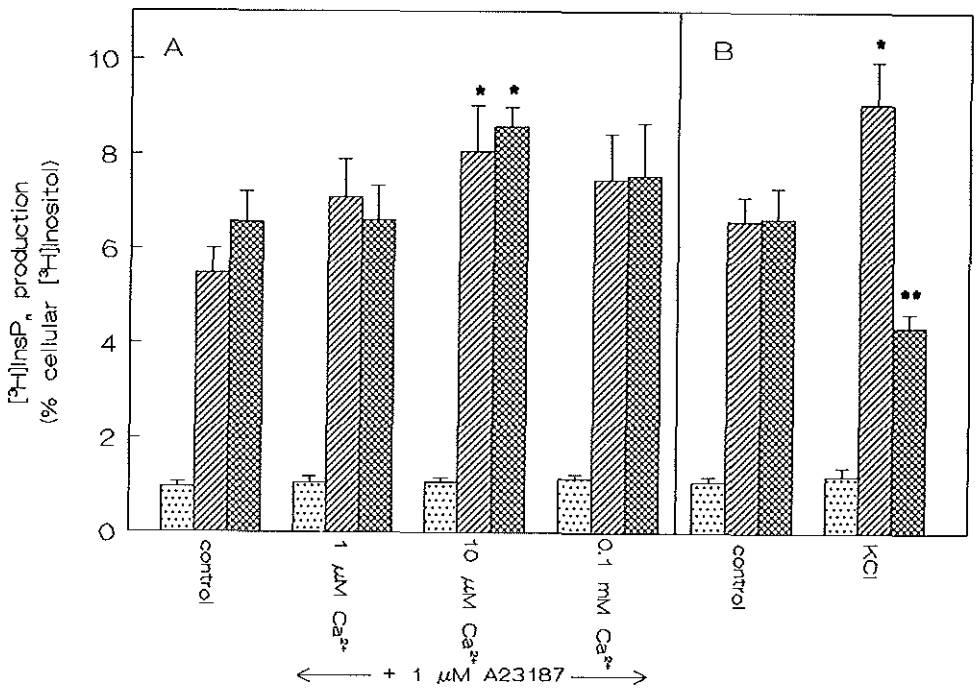

Figure 1. Effect of agents modulating $[Ca^{2+}_{free}]_i$ on basal or ET-1- and PHE-stimulated PLC activity. **A)** After labelling for 24 hours with *myo*-[2-^{3}H]inositol, cells were washed with Ca^{2+} free buffer and incubated for 15 min in the presence of 10mM LiCl with buffer containing 1.3 mM Ca^{2+} ("control") or with buffer containing 1 μM Ca^{2+} ionophore A23187 and various Ca^{2+} concentrations as indicated. Subsequently cells were stimulated as described below; **B)** After labelling for 24 hours with *myo*-[2-^{3}H]inositol, cells were washed with normal buffer and subsequently incubated for 15 min in the presence of 10 mM LiCl in incubation buffer with 4.7 mM KCl ("control") or with 50 mM KCl ("KCl"). Subsequently cells were stimulated for 15 min by addition of buffer (dotted bars), of ET-1 to 10^{-8} M (hatched bars) or of PHE to 10^{-5} M (cross-hatched bars). Cells were extracted and $InsP_n$ levels were determined as described in Materials and Methods. [^{3}H]$InsP_n$ levels are expressed as percentage of total cellular [2-^{3}H]inositol (defined as the sum of water-soluble inositol-containing products together with inositol-containing lipids). Data are expressed as mean ± S.E. (n=8 for buffer and n=12 for agonist stimulations). *, P<0.05 and **, P<0.005 *versus* control stimulation with the same agonist.

Effects of agents that increase cytoplasmic free Ca^{2+} on agonist-stimulated PLC activity

As it was reported (Schwertz and Halverson, 1989; Edes and Kranias, 1990; Renard and Pogglioli, 1990) that activation of the G-protein(s) coupled to PLC can increase Ca^{2+} sensitivity of PtdIns(4,5)P_2 hydrolysis, we incubated the cardiomyocytes with ET-1, a hormone known to activate phosphoinositide turnover in these cells (van Heugten, *et al.*, 1992). The Ca^{2+} sensitivity of PtdIns turnover might be dependent on the agonist employed through the involvement of different G-protein or PLC isozymes so we performed parallel experiments in

which cells were incubated with the α_1-agonist PHE (Meij and Lamers, 1989; Lamers, *et al.*, 1992). Previously we demonstrated that the ET-1 and PHE stimulated [^{3}H]InsP$_n$ production is almost linear during the first 30 min of incubation of the cardiomyocytes (van Heugten, *et al.*, 1992; Meij and Lamers, 1989). ET-1 as well as PHE-evoked PtdIns(4,5)P$_2$ hydrolysis (15 min) could indeed be significantly stimulated by increasing [Ca^{2+}_{free}]$_i$ by using Ca^{2+}-ionophore A23187 in combination with 10μM external Ca^{2+} (Fig 1A) which is well above the normal intracellular level. ET-1 induced InsP$_n$ accumulation was 1.54-fold increased while the PHE-coupled InsP$_n$ production was 1.34-fold stimulated at 10 μM external Ca^{2+}. No significant stimulation was seen at 100 μM external Ca^{2+}.

Depolarization of cardiomyocytes with high extracellular K^+ is known to induce an increase in [Ca^{2+}_{free}]$_i$ (Shue, *et al.*, 1986). Treatment of unstimulated cardiomyocytes with high K^+ medium did not have a significant effect on PLC activity (Fig 1B). In contrast, the ET-1 induced InsP$_n$ accumulation was 1.43-fold stimulated by treatment with 50 mM K^+ while, unexpectedly, the PHE-evoked PtdIns turnover was 43% inhibited.

Inhibition of the Na^+/K^+ ATPase by ouabain is also known to increase [Ca^{2+}_{free}]$_i$ in cultured rat neonatal cardiomyocytes (Hallaq, *et al.*, 1989). For this purpose the cells were preincubated for 10 min with 0, 0.1 and 10 μM ouabain, the latter two concentrations inhibiting the activity of Na^+/K^+ ATPase with 10 and 40% respectively (Xie , *et al.*, 1989). Hereafter the cells were incubated for 15 min in the presence of 10 mM Li^+ to measure PLC activity. [^{3}H]InsP$_n$ productions (in % of cellular [^{3}H]inositol) were 0.91 ± 0.07, 0.94 ± 0.07 and 0.98 ± 0.06 (n=7) in the absence of agonist, 3.89 ± 0.07, 3.88 ± 0.43 and 3.54 ± 0.44 (n=12) in the presence of 10^{-8} M ET-1 and 5.02 ± 0.25, 4.71 ± 0.44 and 4.30 ± 0.30 (n=12) in the presence of 10^{-4} M PHE for 0, 0.1 and 10 μM ouabain respectively. Thus, neither basal nor agonist-induced PtdIns(4,5)P$_2$ hydrolysis was significantly altered by partially blocking Na^+/K^+-ATPase.

Effect of EGTA treatment of cells on ET-1 receptor-stimulated PLC activity

To determine whether agonist-induced activation of PLC requires any Ca^{2+} at all we incubated cardiomyocytes with ET-1 in buffer containing 1.3 mM Ca^{2+} or in Ca^{2+} free buffer with 50 μM EGTA. Part of the experiment was designed to evaluate the influence of EGTA on ET-1 receptor binding making use of the fact that ET-1 is a stable binder to its receptor (Bolger, *et al.*, 1990). The presence of EGTA during the preincubation period where ET-1 but not Li^+ was present only led to a minor but not significant effect on binding of ET-1 to the receptor as judged by subsequent [^{3}H]InsP$_n$ accumulation during the incubation period where ET-1 (and EGTA) were removed and Li^+ was added to monitor PtdIns turnover (Fig 2A). However, EGTA being present during the incubation period of the experiment where ET-1 receptors were already occupied resulted in 75% inhibition of InsP$_n$ accumulation above the basal level that was not affected itself (Fig 2B). The possibility that resynthesis of PtdIns(4,5)P$_2$ through PtdIns 4- and 5-kinase was severely inhibited by EGTA leading to PtdIns(4,5)P$_2$ depletion was investigated by quantification of the cellular levels of the inositol-containing lipids of the experiment depicted in Fig 2. As shown in Table 2, the cellular [^{3}H]PtdIns, [^{3}H]PtdIns(4)P and [^{3}H]PtdIns(4,5)P$_2$ levels were even significantly higher after inhibition of [^{3}H]InsP$_n$ accumulation by EGTA as compared to the levels remaining after ET-1 induced PtdIns turnover suggesting that the resynthesis of PtdIns(4,5)P$_2$ was not

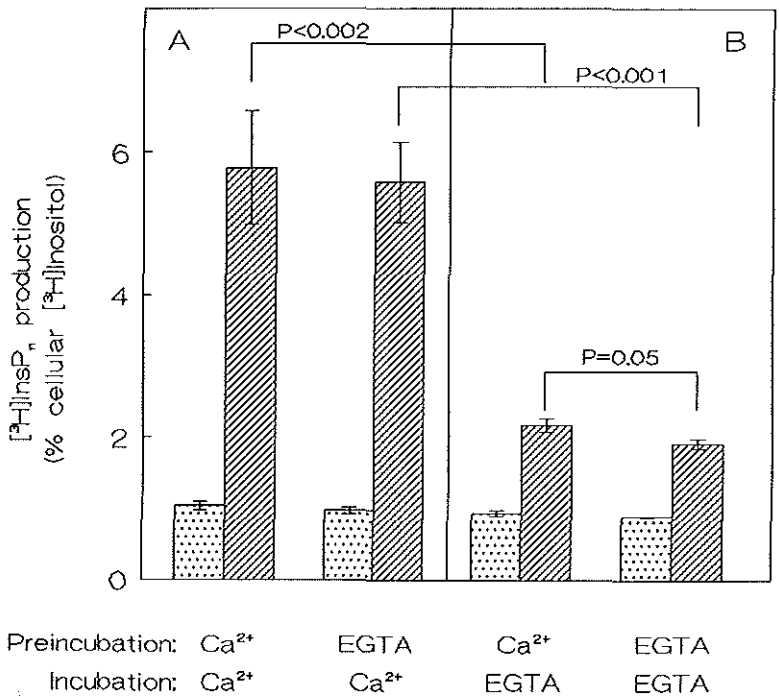

Figure 2. Effect of EGTA treatment on the basal and ET-1-stimulated PLC activity in cardiomyocytes. Cardiomyocytes, labelled for 24 hours with *myo*-[2-^{3}H]inositol, were washed with Ca^{2+}-free buffer and subsequently preincubated for 15 min with (hatched bars) or without (dotted bars) 10^{-8} M ET-1 in buffer containing either 1.3 mM Ca^{2+} or 50 μM EGTA (in Ca^{2+}-free buffer) as indicated. Following this preincubation period Ca^{2+}, EGTA and unbound ET-1 were removed by washing with Ca^{2+}-free buffer. Hereafter PtdIns turnover was monitored by incubation for 15 min in the presence of 10 mM LiCl in 1.3 mM Ca^{2+} buffer or in Ca^{2+}-free buffer containing 50 μM EGTA as indicated in the Figure. Accumulated [^{3}H]$InsP_n$ was quantified as described in Materials and Methods. [^{3}H]$InsP_n$ levels are expressed as mean percentage ± range/2 and ± S.E. of the total amount of [2-^{3}H]inositol labelled products, defined as in Figure 1, for buffer (n=2) and ET-1 (n=6) experiments respectively. Significant differences are indicated in the Figure.

rate-limiting. The only [^{3}H]inositol-containing lipid not affected by EGTA was [^{3}H]lyso-PtdIns that is not an intermediate of the PtdIns cycle. EGTA did not inhibit the ET-1 coupled PtdIns(4,5)P_2 hydrolysis by simply slowing down PtdIns turnover, as is shown in Fig 3. Even after preincubation with EGTA for 15 min, addition of ET-1 led to significant production of [^{3}H]$InsP_n$ during the first 30 min of stimulation. However, after this initial increase further [^{3}H]$InsP_n$ accumulation was totally blocked (Fig 3) although [^{3}H]PtdIns(4,5)P_2 levels were not decreased (data not shown). As will be shown later, the velocity of ET-1 induced [^{3}H]$InsP_n$ accumulation at normal extracellular Ca^{2+} (1.3 mM) only starts diminishing after about 60 min.

Table 2. Changes in concentration of [^{3}H]inositol-containing lipids after incubation of ET-1 preincubated cardiomyocytes with normal Ca^{2+} or EGTA in Ca^{2+} free buffer.

	[^{3}H]Inositol-phospholipid content		
	1.3 mM Ca^{2+}	50 μM EGTA	P
PtdIns	69.5 ± 0.4	73.4 ± 0.4	<0.001
lysoPtdIns	2.83 ± 0.11	2.80 ± 0.17	>0.5
PtdIns(4)P	2.59 ± 0.05	2.99 ± 0.07	<0.002
PtdIns(4,5)P_2	2.36 ± 0.09	2.66 ± 0.04	<0.02

Cardiomyocytes were treated and stimulated exactly as described in the legend to Fig 2. After extraction of water-soluble products, lipids were extracted with methanol:12 M HCl (100:1, v/v) and analyzed as described in Materials and Methods. No significant differences in [^{3}H]inositol-containing lipid content were detected between preincubation with ET-1 (10^{-8} M) in the presence of Ca^{2+} or in the presence of 50 μM EGTA in Ca^{2+} free buffer. Therefore, [^{3}H]Inositol-phospholipid content after incubation with 10 mM Li^+ and 1.3 mM Ca^{2+} is taken from preincubation with Ca^{2+} together with data from preincubation with EGTA (ie Fig 2A) while the phospholipid levels after incubation in the presence of 10 mM Li^+ and 50 μM EGTA in Ca^{2+} free buffer are accumulated data of preincubation with Ca^{2+} and with EGTA (Fig 2B). [^{3}H]Phosphoinositide levels are expressed as percentage of total cellular [^{3}H]inositol defined as in the legends to Figure 1 (mean ± S.E., n=12). Statistical significance is indicated by the P values.

Effects of low Ca^{2+}, Ca^{2+} antagonist and agonist on basal and receptor-mediated PLC activity

The foregoing results on PLC were obtained incubating cells with Ca^{2+}-ionophore A23187, high K^+ buffer and EGTA, treatments that are expected to produce dramatic changes in $[Ca^{2+}_{free}]_i$ levels close to or in the non-physiological range. We asked ourselves whether or not the physiological range of $[Ca^{2+}_{free}]_i$ is optimal for PLC activity in cardiomyocytes. Therefore, we also analyzed the effect of decreasing normal Ca^{2+} influx on basal as well as agonist-induced [^{3}H]InsP_n accumulation. The effect of these protocols could also be studied over a much longer period (up to 90 min). ET-1 evoked InsP_n accumulation was not significantly affected by low Ca^{2+} medium. Only 90 min of incubation of cardiomyocytes in low Ca^{2+} medium resulted in stimulation of basal PLC activity which finding was unexpected (Fig 4A). Treatment of cardiomyocytes with the dihydropyridine Ca^{2+}-channel blocker nifedipine (1 μM) reduced ET-1 induced [^{3}H]InsP_n accumulation (30 min) by 33% and did not have a significant effect on basal PtdIns turnover (Fig 4B). After 60 min no significant effect of nifedipine on PtdIns turnover prevailed. The influence of either low Ca^{2+} buffer or nifedipine on G-protein coupled PLC activity was not agonist dependent as data similar to those illustrated in Fig 4 were obtained after stimulation of the cells with 10^{-4} M PHE (data not shown).

Fig 5 shows that the ET-1 induced PtdIns turnover could be stimulated by increasing Ca^{2+} influx through the use of the Ca^{2+}-channel opener Bay K8644 but only after 90 min of incubation. Basal PtdIns turnover was seen to be increased by the use of Bay K8644 resulting in a doubling of InsP_n accumulation after 90 min of incubation.

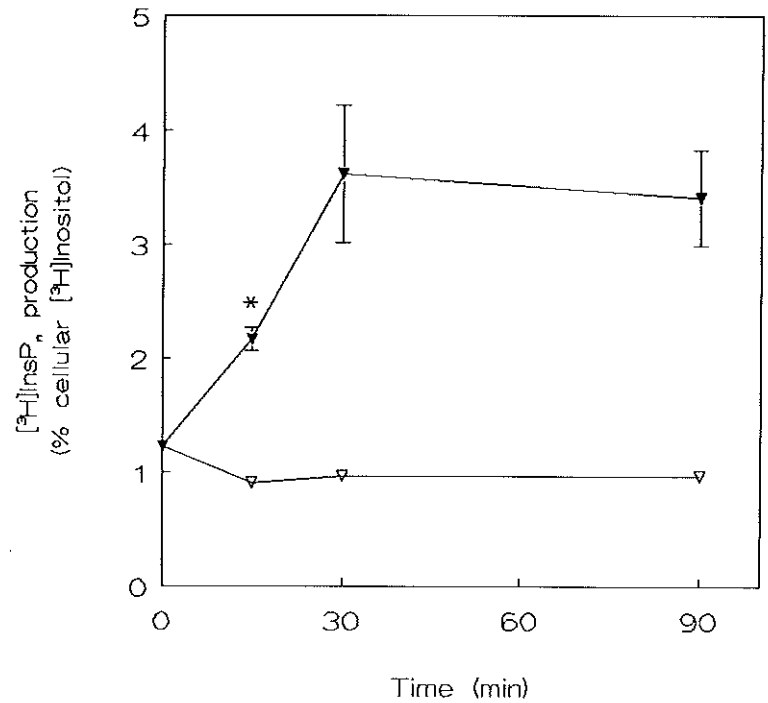

Figure 3. Time course of $[^3H]InsP_n$ accumulation after stimulation of cardiomyocytes with ET-1 in the presence of EGTA in Ca^{2+}-free buffer. Cells labelled with *myo*-[2-^{3}H]inositol were washed with Ca^{2+}-free buffer and incubated for 15 min in Ca^{2+}-free buffer containing 50 μM EGTA and 10 mM LiCl. Hereafter, buffer (open symbols) or ET-1 was added to 10^{-8} M (closed symbols) and incubation was continued for the periods indicated. Extraction and quantification of $[^3H]InsP_n$ was performed as described in Materials and Methods. $[^3H]InsP_n$ levels are expressed as percentage of the total amount of [2-^{3}H]inositol labelled products as defined in Figure 1 (mean ± range/2 for buffer, n=2 and mean ± S.E., n=6 for ET-1). *, P<0.001 , ET-1 (15 min, n=6) *versus* 0 min (n=6).

Fluorometric measurement of changes in intracellular free Ca^{2+} concentration of cultured cardiomyocytes

To determine whether the protocols that were described above indeed had effect on $[Ca^{2+}_{free}]_i$, we set up a fluorometric assay on cardiomyocytes grown on glass cover slips using fura-2 AM. Due to the fact that cardiomyocytes became detached from the glass cover slip after prolonged incubation (60 min of loading with fura-2 AM and 40 to 50 min incubation in the fluorometer), no extended incubations could be performed to assess long-term effects on $[Ca^{2+}_{free}]_i$. As expected, table 3 shows that the treatments that had the largest effect on agonist-induced $InsP_n$ accumulation also produced significant alterations in $[Ca^{2+}_{free}]_i$ as judged by the ratio of fluorescence elicited at 340 nm ($F_{340\ nm}$) and 380 nm ($F_{380\ nm}$). Addition of 50 μM EGTA, shown to decrease ET-1 induced $InsP_n$ accumulation (Figs 2 and 3) also significantly decreased $[Ca^{2+}_{free}]_i$, while a depolarization with 50 mM KCl that stimulated ET-1 induced and inhibited PHE mediated PLC activity (Fig 1) led to an increase of $[Ca^{2+}_{free}]_i$.

However, the presence of nifedipine that led to a significant decrease in ET-1 and PHE coupled $InsP_n$ accumulation after 30 min (Fig 4B) did not show an effect on $[Ca^{2+}_{free}]_i$. Although low Ca^{2+} medium did not change ET-1 and PHE coupled $InsP_n$ production (Fig 4A), a significant decrease in $[Ca^{2+}_{free}]_i$ was observed, but not of the same magnitude as after addition of EGTA. Bay K8644, not affecting agonist coupled $InsP_n$ production during the first 30 min (Fig 5), did not influence $[Ca^{2+}_{free}]_i$ either.

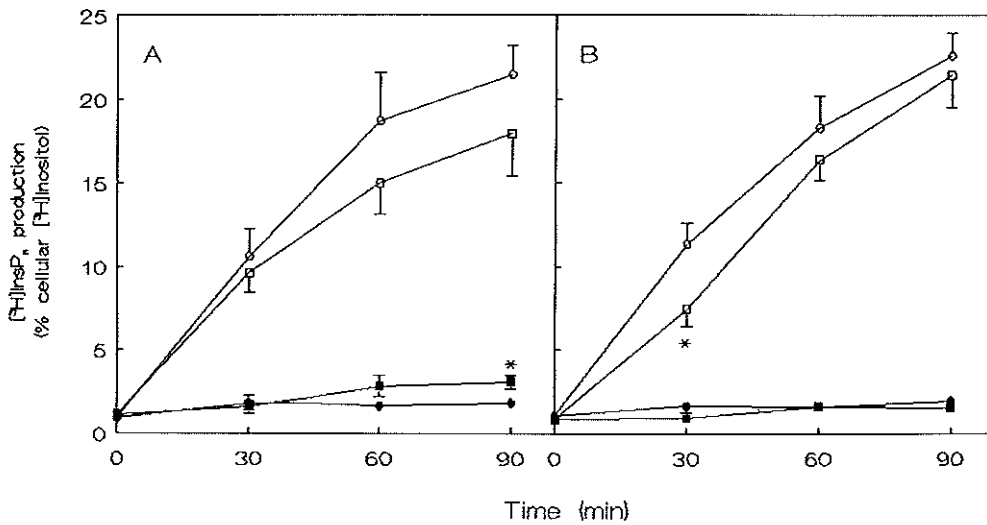

Figure 4. Effect of low Ca^{2+} or slow Ca^{2+}-channel blockade by nifedipine on the time-course of $[^3H]InsP_n$ production after stimulation of cardiomyocytes with ET-1. Cardiomyocytes were prelabelled with *myo*-[2-^{3}H]inositol for 24 hours. **A.** The cells were washed in Ca^{2+} free buffer and incubated for 15 min with 10 mM LiCl containing buffer with 1.3 mM Ca^{2+} (○, ●) or without Ca^{2+} (□, ■). Cells were then stimulated for the periods indicated by addition of buffer (closed symbols) or of ET-1 to 10^{-8} M (open symbols). **B.** Cells were washed and incubated for 15 min in normal buffer containing 10 mM LiCl with (□, ■) or without (○, ●) 1 μM nifedipine and were subsequently stimulated for the periods indicated by addition of buffer (closed symbols) or of ET-1 to 10^{-8} M (open symbols). After incubation, the cells were extracted and $[^3H]InsP_n$ was quantified as described in Materials and Methods. $[^3H]InsP_n$ content is expressed as percentage of the total cellular content of [2-^{3}H]inositol defined as in the legends to Figure 1. Results are mean ± S.E. (A: n=3 for buffer and n=6 for ET-1; B: n=4 for buffer and n=9 for ET-1). *, $P<0.05$ *versus* similar incubation in the presence of Ca^{2+} (A) or in the absence of nifedipine (B).

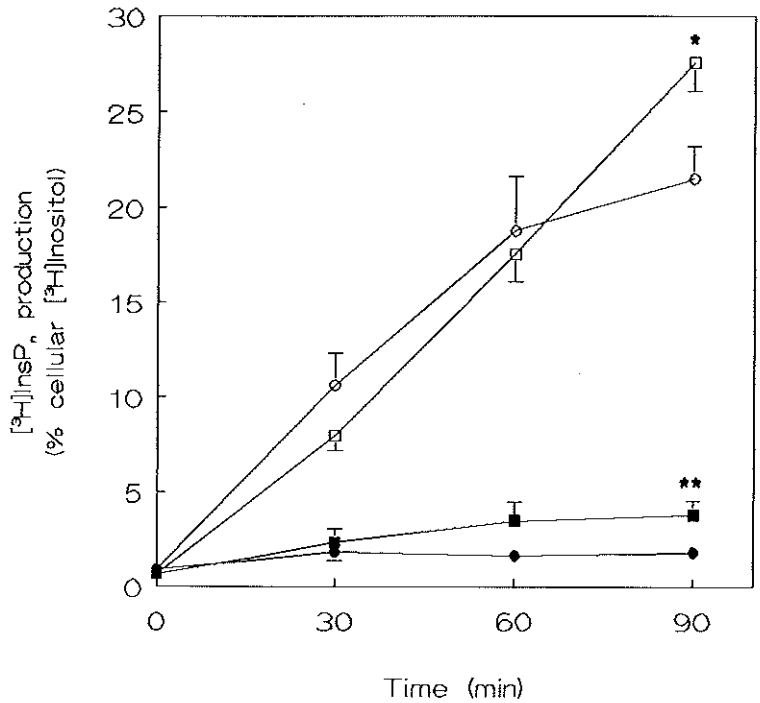

Figure 5. Influence of Ca^{2+}-channel opener Bay K8644 on the time course of $[^3H]InsP_n$ accumulation after stimulation of cardiomyocytes with ET-1. Cardiomyocytes were prelabelled for 24 hours with *myo*-[2-^{3}H]inositol. Thereafter, the cells were incubated with incubation buffer containing 10 mM LiCl with (□,■) or without (○,●) 1 μM Bay K8644 for 15 min before addition of buffer (closed symbols) or ET-1 to 10^{-8} M (open symbols) for the periods indicated. Extraction and quantification of $InsP_n$ was performed as described in Materials and Methods. $[^3H]InsP_n$ content is expressed as percentage of the total cellular content of [2-^{3}H]inositol as defined in legends to Fig 1. Results are mean ± S.E. (n=6 for ET-1 and n=3 for buffer). *P<0.02 and **P<0.05 *versus* similar incubation in the absence of Bay K8644.

Effect of intracellular free Ca^{2+} concentration on basal and GTPγS stimulated $InsP_n$ accumulation in permeabilized cardiomyocytes

The effect of free Ca^{2+} on the PLC activity was studied more directly using saponin-permeabilized cardiomyocytes as described by McDonough, *et al.* (1988). Addition of an ATP regenerating system was essential to stabilize the cells during permeabilization. The results (Fig 6) show that after permeabilization of cardiomyocytes the basal and GTPγS (30 μM) stimulated PLC activity (15 min of incubation) was strongly activated by increasing $[Ca^{2+}_{free}]_i$ from 0.1 to 10 μM and even slighly more by increasing Ca^{2+} to 192 μM. Relative stimulation of PLC by GTPγS was largest in the nanomolar range of Ca^{2+}.

Table 3. Fluorometric measurement of $[Ca^{2+}_{free}]_i$ concentration of cultured cardiomyocytes.

Cell treatment	$F_{340\ nm}/F_{380\ nm}$-ratio Before	After	P (n)
Low Ca^{2+}	1.17 ± 0.04	1.03 ± 0.04	<0.05 (6)
50 μM EGTA (no Ca^{2+})	1.03 ± 0.04	0.93 ± 0.01	<0.05 (6)
50 mM KCl	1.14 ± 0.05	1.41 ± 0.06	<0.02 (4)
1 μM Bay K8644	1.21 ± 0.02	1.30 ± 0.05	NS (6)
1 μM Nifedipine	1.16 ± 0.04	1.16 ± 0.06	NS (4)

Cardiomyocytes were grown on glass cover slips and loaded for 60 min at 37°C with 2 μM fura-2 AM in the presence of 2.5 mM probenicid. The cover slip was inserted in a holder tightly fitting into a quartz cuvette containing 2 ml incubation buffer. Fluorescence was recorded at 510 nm using excitation wavelengths of 340 and 380 nm. The ratio of the fluorescence elicited at 340 nm ($F_{340\ nm}$) and 380 nm ($F_{380\ nm}$) can be taken as a measure of the $[Ca^{2+}_{free}]_i$. After each assay calibration of the fluorescence signal was performed by addition of 2.5 μM ionomycin to obtain maximal ratio followed by 10 mM EGTA to obtain the ratio at zero $[Ca^{2+}_{free}]_i$ (i.e 0.93 ± 0.02, n=15). Cells were incubated for 15 min in buffer ("Before") followed by 20 min incubation with various additions ("After") as indicated in the Table. Fluorescence ratios represent $[Ca^{2+}_{free}]_i$ during relaxation. Control cardiomyocytes contracted irregularly (8 to 15 times/min) which activity was completely blocked by low Ca^{2+} with or without 50 μM EGTA, 50 mM KCl and 1 μM nifedipine. Peak $F_{340\ nm}/F_{380\ nm}$ ratio during contraction in control was 1.45 ± 0.09, n=8 (P<0.02 vs 1.19 ± 0.02, n=8 during relaxation). Data are expressed as means ± SEM from 4 different cellbatches, n refers to the number of experiments while P values represent statistical significance between ratio before and after treatment; NS, not significant.

DISCUSSION

We studied the effect of agents modulating $[Ca^{2+}_{free}]_i$ on basal as well as agonist-dependent PLC activity that is involved in the signal transduction through the PtdIns cycle in cardiomyocytes. In a variety of cell types it was shown that Ca^{2+} ionophore A23187 (Wakelam, 1983; Monaco, 1987; Godfrey and Putney, 1984) and depolarization by high K^+ (Gurwitz and Sokolowski, 1987; Haberman and Laux, 1986) activated PtdIns(4,5)P_2 breakdown by PLC. In saponin-permeabilized chick heart cells basal PLC activity was also shown to be Ca^{2+} dependent (McDonough, *et al.*, 1988, Jones and Brown, 1988). In our study using intact rat cardiomyocytes basal activity of PLC (30 min) could not be stimulated by either ionophore A23187 (external Ca^{2+} 1-100 μM) (Table 1) or high K^+ concentration (external Ca^{2+} 1.3 mM) (Fig 1). Using fura-2 fluorescence it was shown that high Ca^{2+} induced increased $[Ca^{2+}_{free}]_i$ (Table 3). As A23187 is fluorescent no fura-2 measurements could be performed for this protocol. The basal $InsP_n$ production as well as $[Ca^{2+}_{free}]_i$ was also not affected after 30 min of incubation with Bay K8644 but unexpectedly, an increase in $InsP_n$ production was seen after 90 min incubation. On the other hand, total removal of external free Ca^{2+} through addition of 50 μM EGTA to Ca^{2+} free medium, Ca^{2+} free medium or nifedipine (1 μM) did not significantly affect the basal $InsP_n$ production (30 min) either (Table 1). Using fura-2 fluorescence it was shown that low external Ca^{2+} with or without

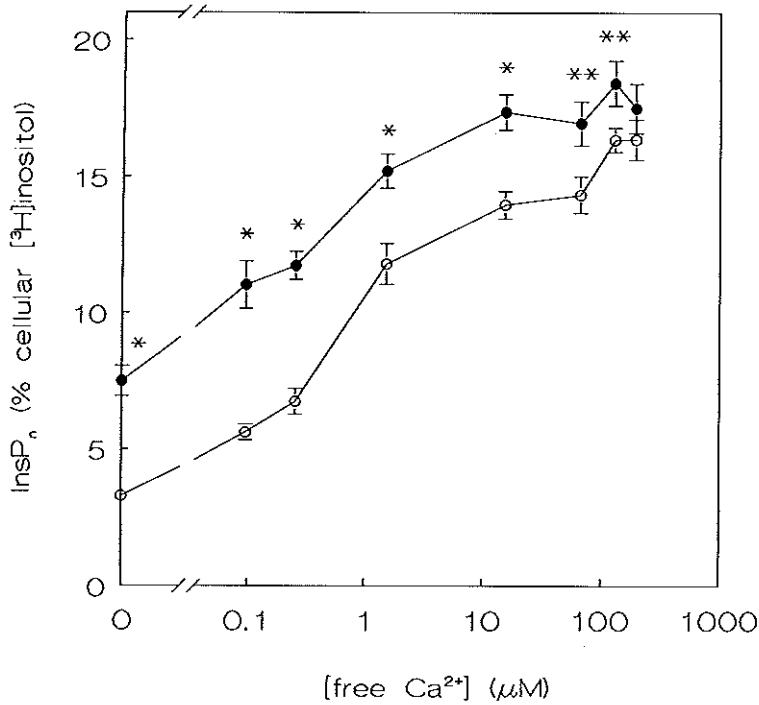

Figure 6. Effect of $[Ca^{2+}_{free}]_i$ on basal and GTPγS stimulated $InsP_n$ accumulation in permeabilized cardiomyocytes. Cardiomyocytes, prelabelled with *myo*-[2-^{3}H]inositol were washed with buffer and permeabilized for 5 min with 100 μg saponin/ml intracellular buffer as described in the experimental section. Hereafter, cells were washed with buffer without saponin and intracellular buffer was added. In addition to 10 mM LiCl this buffer contained Ca^{2+} as required with (●) or without (○) 30 μM GTPγS. Free Ca^{2+} in the buffer was calculated using the SPECS computer program described by Fabiato (1988). After 15 min incubation at 37^{0}C the buffer was collected and the cells were extracted with perchloric acid and methanol/HCl as described in the experimental section. [^{3}H]$InsP_n$ content is the sum of inositolphosphates in buffer and perchloric acid extract and is expressed as percentage of the total cellular content of [2-^{3}H]inositol defined as in the legends to Figure 1. Results are mean ± S.E. , n=12. *P<0.005 and **P<0.05 *versus* incubation with the same Ca^{2+} concentration in the absence of GTPγS.

EGTA decreased $[Ca^{2+}_{free}]_i$. Taken together these short-term effects suggest that changes in $[Ca^{2+}_{free}]_i$ do not affect basal PLC activity in intact cardiomyocytes. This conclusion is however not supported by experiments using permeabilized cardiomyocytes (Fig 6) where basal PLC activity was seen to be strongly Ca^{2+} dependent in the range of 0.1 to 10 μM. It is possible that due to saponin-permeabilization the cells were depleted of PLC regulating factors. Although saponin treatment abolishes receptor activation of PLC, it is possible that some purinergic receptor activity remains.

It was reported that activation of PLC by G-proteins in rat heart membrane fragments lowers the concentration of Ca^{2+} necessary for optimal activity of PLC (Renard and Pogglioli, 1990;

Jones and Brown, 1988). Likewise, we observed that relative stimulation of PLC by GTPγS was largest in the nanomolar range of Ca^{2+} in saponin-permeabilized rat cardiomyocytes (Fig 6). Activation of G-proteins by either ET-1 or PHE in intact cardiomyocytes indeed revealed Ca^{2+}-sensitivity of PLC activity. Using Ca^{2+} ionophore A23187 at different Ca^{2+} concentrations only showed significant stimulation of $[^3H]InsP_n$ accumulation at 10 μM external Ca^{2+} resulting in 54 and 34% increases in PLC activity induced by ET-1 and PHE respectively. Whether PLC or G-proteins were activated by Ca^{2+} can not be deduced from these experiments. The absence of stimulation by A23187 at 100 μM external Ca^{2+} in intact cardiomyocytes (Fig 1) might arise from a possible cell necrotic effect of the combined addition of 100μM Ca^{2+} and ionophore A23187 as high Ca^{2+} alone did not show an inhibitory effect at 100 μM and higher using permeabilized cells (Fig 6). On the other hand, secondary effects of Ca^{2+} overload (e.g. increases in intracellular Na^+ or K^+ concentrations) might also be detrimental for PLC activity.

The Ca^{2+}-channel agonist Bay K8644 induced a slight increase in PLC activity, but only after 90 min incubation with agonist (Fig 5). Sei *et al.* (1991) also showed that Bay K8644 had no effect on PHE-induced PtdIns turnover during 60 min of incubation of neonatal rat ventricular myocytes.

Although the Na^+/K^+ ATPase inhibitor ouabain (0.05-5 μM) was reported to have positive inotropic activity and to increase $[Ca^{2+}_{free}]_i$ in cultured rat neonatal cardiomyocytes (Hallaq, *et al.*, 1989), the receptor-mediated PtdIns response was not affected as established in the present study. Using the same model system prolonged exposure to ouabain although at higher level (1 mM), was shown to increase Na^+ and to decrease K^+ and Mg^{2+} concentrations in the cytoplasm together with a decrease in ATP content (Morris, *et al.*, 1989). The complexity of cellular responses to ouabain makes it difficult to evaluate the $InsP_n$ accumulation data in the light of elevated $[Ca^{2+}_{free}]_i$.

Besides increasing $[Ca^{2+}_{free}]_i$ (Table 3), depolarization of the membrane by high K^+ concentrations is known to change G-protein expression after prolonged exposure (Foster, *et al.*, 1990). Incubation of cardiomyocytes in high K^+ buffer stimulated ET-1 induced PtdIns cycle activity at the same time inhibiting PHE-coupled $[^3H]InsP_n$ accumulation (Fig 1). These results, together with stimulation of $[^3H]InsP_n$ accumulation by A23187 at 10 μM Ca^{2+} suggest that the $[Ca^{2+}_{free}]_i$ was not the only factor involved in high K^+-induced changes in PtdIns cycle activity at least in the case of PHE. Either G-protein activity or membrane electrical depolarization might be additional factors regulating the PtdIns pathway.

Regulation of PtdIns cycle activity by changes in $[Ca^{2+}_{free}]_i$ as described above suggests that the signal transduction pathway through PLC and $PtdIns(4,5)P_2$ might be absolutely dependent on the presence of Ca^{2+} in intact cells as was also detected for permeabilized cardiomyocytes (Fig 6). Indeed, total removal of extracellular free Ca^{2+} by including 50 μM EGTA in the Ca^{2+} free incubation buffer markedly inhibited the ET-1 induced $InsP_n$ accumulation (Fig 3). Under these conditions the fluorescence ratio was decreased to that detected at zero intracellular free Ca^{2+} (see also the legends to Table 3). ET-1 binding to its receptor was shown not to be inhibited by EGTA (Fig 2) while resynthesis of $PtdIns(4,5)P_2$ was not rate-limiting in the absence of external Ca^{2+} (Table 2). The latter finding is in agreement with our previous observation that rat heart sarcolemmal PtdIns 4- and 5-kinases are Ca^{2+} independent enzymes which are inhibited by micromolar Ca^{2+} concentrations (Mesaeli, *et al.*, 1992). The absence of free Ca^{2+} in the cell eventually results in the total

standstill of ET-1 induced PtdIns turnover. Whether the Ca^{2+} that remained bound to membrane phospholipids or G-proteins/PLC is responsible for the initial but attenuated activity of PLC remains to be elucidated. The simple withdrawal of extracellular Ca^{2+} did not significantly affect [^{3}H]InsP_n accumulation during a 90 min incubation with ET-1 or PHE (Fig 4A) suggesting that the residual Ca^{2+} that was present under these conditions (Table 3) was sufficient to give normal PLC activation by the agonists. Ca^{2+}-influx inhibition by addition of the Ca^{2+}-channel blocker nifedipine (1 μM) led to significant inhibition of ET-1 and PHE-induced PtdIns cycle activity (33 and 30% respectively (Fig 4B) after 15 min preincubation and 30 min stimulation in the presence of nifedipine. However, incubation of the cells with nifedipine for 20 min did not result in a change in $[Ca^{2+}_{free}]_i$ (Table 3). Prolonged incubation with nifedipine led to complete recovery of PLC activity (Fig 4B). Although the incubations were carried out in the dark, instability of the dihydropyridine might be the cause of the late reversal of the nifedipine inhibition. On the other hand, Wetzel *et al.* (1991) demonstrated that neonatal rat ventricular myocytes are relatively deficient in slow Ca^{2+}-channel activity when compared to adult cardiomyocytes.

At this point it is important to stress the fact that fura-2 measurements were performed on unstimulated cells to determine whether the experimental protocols were having any effect on $[Ca^{2+}_{free}]_i$, largely explaining the effects on basal PLC activity. However, we have to be cautious in interpreting the results from the experiments where cardiomyocytes were stimulated with ET-1, as stimulation with this agonist might lead to increased $[Ca^{2+}_{free}]_i$, as described before (Vigne, *et al.*, 1990).

In conclusion, the present study shows that the signalling pathway through PLC/PtdIns(4,5)P_2 activated by agonist-receptor interaction is likely to be a Ca^{2+} dependent process in the intact cardiomyocyte as was the case for permeabilized cardiomyocytes. On the other hand, basal PLC activity in intact cardiomyocytes seems not to be sensitive to changes in the $[Ca^{2+}_{free}]_i$, although after saponin-treatment of the cardiomyocytes the basal PLC activiy also became Ca^{2+}-dependent. The activation of PLC by an increase in $[Ca^{2+}_{free}]_i$ level may follow upon Ins(1,4,5)P_3-induced release of Ca^{2+} from the sarcoplasmic reticulum. Ca^{2+} potentiation of agonist-mediated PLC activation may also play a role in aggravating cellular Ca^{2+} overload developing during myocardial ischemia-reperfusion (Lee and Allen, 1991; Otani, *et al.*, 1989; Mouton, *et al.*, 1991; Heathers, *et al.*, 1989).

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Chapter 4

DISTINCT α_1-ADRENERGIC AGONIST- AND ENDOTHELIN-1-EVOKED PHOSPHOINOSITIDE CYCLE RESPONSES IN CULTURED NEONATAL RAT CARDIOMYOCYTES

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ABSTRACT

Previously it was shown by us and others in cultured neonatal rat cardiomyocytes that the desensitization of the phenylephrine (PHE)- and endothelin-1 (ET-1)-mediated response of phospholipase C (PLC) was receptor-specific. The aim of this study was to characterize receptor-dependent specificities downstream of PLC. PHE (10^{-4} M) as well as ET-1 (10^{-8} M) stimulated the total [^{3}H]inositolphosphate ([^{3}H]InsP_n, predominantly [^{3}H]Ins(4)P) formation to about the same extent whereas Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 did not increase. Yet, ET-1 but not PHE stimulated Ins(1,3,4)P_3 and Ins(3,4)P_n formation. Activation of PLC in saponin-permeabilized cells by GTPγS- and Ca^{2+} gave predominantly the formation of Ins(1,4)P_2. The PHE- and ET-1-mediated increase of [^{3}H]1,2-diacylglycerol was significant after respectively 16 and 8 min. PHE but not ET-1 stimulated phosphorylation of a 30 kDa protein which was likely of myofibrillar origin. It is concluded that receptor-dependent specificities exist not only at the level of PLC but also downstream.

INTRODUCTION

In heart inotropy, long term induction of hypertrophy and concomitant reprogramming of gene expression have been shown to be partially mediated by agonists such as α_1-adrenergic hormone and endothelin-1 (ET-1), that transduce their signals after receptor-GTP-binding protein (G-protein) coupling to the activation of phospholipase C-ß (PLC-ß)(1-3). PLC-ß on its turn hydrolyses phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), thereby producing the second messengers 1,2-diacylglycerol ((1,2)DAG), inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4). (1,2)DAG is known to activate members of the protein kinase C (PKC) family by binding to the regulatory domain of the enzymes resulting in translocation to plasmamembrane and intracellular sites such as cytoskeletal elements (4-7). The other product of PLC-ß action, Ins(1,4,5)P_3, is known to be involved in the release of Ca^{2+} from the non-mitochondrial Ca^{2+} pools thereby promoting the Ca^{2+}-induced influx of external Ca^{2+}, possibly in conjunction with Ins(1,3,4,5)P_4 (1-3). (1,2)DAG metabolism concerns either conversion to phosphatidic acid mediated by (1,2)DAG kinase and subsequent reincorporation into the phospholipids, or hydrolysis by (1,2)DAG lipase and presumably by nonspecific esterases to free fatty acids and glycerol (4). Ins(1,4,5)P_3 metabolism involves two metabolic pathways as well: successive dephosphorylation reactions finally resulting in free Ins, and phosphorylation by 3-kinase to Ins(1,3,4,5)P_4 also followed by dephosphorylation reactions (8,9). It is becoming more and more clear that the diversity of receptors linked with this signalling pathway and the heterogeneity of the downstream elements such as the G-proteins, PLC, and PKC, determine the receptor-specific responses of the signalling pathway to a large extent.

The first evidence for occurrence of receptor-dependent specificities in PtdIns cycle signalling in myocardium was previously obtained by us and others in studying the time-course and mutual influences of desensitization of the PHE- and ET-1-evoked total inositolphosphate (InsP_n) responses (10,11). In order to characterize receptor-dependent specificities downstream of PLC, in this study we followed the formation in time of second messengers and some of their products and the ultimate ^{32}P incorporation into cellular proteins upon

stimulation of cardiomyocytes by PHE and ET-1.

MATERIALS AND METHODS

Reagents

PHE was obtained from Sigma (St. Louis MO, USA) and ET-1 was from Peninsula Laboratories (Belmont CA, USA). Culture dishes (4 well Multidish) were from Nunc (Roskilde, DK), while culture medium HAM F10 was from Gibco (UK); fetal calf serum, horse serum and penicillin/streptomycin were obtained from Boehringer Mannheim (Germany). Trypsin (type III) was from Sigma (St. Louis MO, USA). *Myo*-[2-^{3}H]inositol (17-19 Ci/mmol), [2-^{3}H]glycerol (1 Ci/mmol) and $^{32}P_i$ (carrier free) were obtained from Amersham International plc (Amersham, UK). En^{3}Hance was from Dupont, NEN products (Boston, USA) and scintillation cocktail Flo-scint IV was from Canberra Packard Benelux N.V./S.A. (The Netherlands).

Cell culture

Primary cultures of neonatal ventricular myocytes were prepared from 1-2 day old Wistar rats as described before (10,11) using preplating (13) to further increase cardiomyocyte to non-cardiomyocyte ratio. Minor modifications were introduced, replacing the previously used double preplating step for a single preplating step on an increased surface (10 cm^2/rat heart). Cardiomyocytes were seeded in 1.8 cm^2 wells at 150 to 175x10^3 cells/cm^2 giving a confluent monolayer of spontaneously contracting cells after 24 hrs. The cells were kept at 37°C and 5% CO_2 in complete growth medium consisting of Ham F10 supplemented with 10% fetal calf serum, 10% horse serum, 100 U penicillin/ml and 100 μg streptomycin/ml. Growth medium was renewed 24 hrs after seeding and every 48 hrs thereafter. Experiments were performed 5 to 6 days after plating of the cells.

Inositolphoshate analysis

Cardiomyocytes were labelled with 10 μCi *myo*-[2-^{3}H]Ins/ml for 48 hrs in Ins free complete growth medium. After washing and preincubation (15 minutes, 37°C) with incubation buffer (130 mM NaCl; 4.7 mM KCl; 1.3 mM $CaCl_2$; 0.44 mM NaH_2PO_4; 1.1 mM $MgSO_4$; 20 mM $NaHCO_3$; 0.2% glucose; 10 mM HEPES; pH 7.4, 37°C) the cells were stimulated with agonist in the presence of 10 mM LiCl. Incubations were stopped by rapidly washing the cells with ice-cold buffer. Water-soluble [^{3}H]Ins products were extracted by two subsequent extractions with $HClO_4$. The pooled $HClO_4$ fractions were neutralized with 2 M KOH; 1 M K_2CO_3. The remainder, water-insoluble products, was subsequently extracted with ice-cold CH_3OH:HCl (100:1 v/v) for 5 min at 4°C. The [^{3}H]InsP_n isomers were separated by HPLC using a Partisil 10 SAX column (Alltech, Deerfield, IL, USA) using gradient elution (2.0 M NH_4COOH/H_3PO_4, pH 3.7) (14) and an on-line ^{3}H detector (Radiometric, Flow-one beta A515 (Canberra Packard Benelux N.V./S.A.) and the amounts were expressed as % of total cellular [^{3}H]Ins (defined as the sum of water-soluble plus -insoluble [^{3}H]Ins containing products).

Permeabilization of cardiomyocytes prelabelled for 48 hrs with *myo*-[2-^{3}H]Ins, was performed by treatment with 100 μg saponin/ml in intracellular buffer (20 mM HEPES; 10 mM NaCl; 110 mM KCl; 1 mM KH_2PO_4; 4 mM $MgCl_2$; 1 mM EGTA; 3 mM ATP; 8 mM creatine phosphate; 6 U creatine kinase/ml; pH 7.0) for 5 min, at 37°C (15). The cells were rinsed

three times with the latter buffer before incubation in fresh buffer containing 10 mM LiCl, Ca^{2+} (0 and 96 nM [Ca^{2+}_{free}]) and/or 30 μM GTPγS for 15 min at 37°C, whereafter the buffer was collected for estimation of water-soluble [^{3}H]InsP_n by HPLC. The remainder of [^{3}H]Ins products were extracted with ice-cold $HClO_4$ and CH_3OH:HCl (100:1 v/v) respectively. The $HClO_4$ extract was immediately neutralized. To be able to compare amounts of water-soluble [^{3}H]InsP_n found in buffer and $HClO_4$ extract, the collected buffer was acidified with $HClO_4$ as well, followed by neutralization with 2 M KOH and 1 M K_2CO_3.

1,2-Diacylglycerol analysis

Cardiomyocytes were labelled with 2 μCi [2-^{3}H]glycerol/ml for 48 hrs in complete growth medium. The cells were rinsed, preincubated and stimulated as described above. After termination of the incubation the [^{3}H]glycerol containing lipids were extracted by two successive extractions with ice-cold CH_3OH:HCl (100:1 v/v). Water-soluble products were separated from lipids by phase-separation after addition of 1 volume of $CHCl_3$ and 0.5 volumes of 2.5 M HCl. The resulting organic phase was re-extracted once with 1 volume of $CHCl_3$:CH_3OH:0.6 M HCl (3:48:47 v/v/v). The [^{3}H]glycerol containing lipids were dried under N_2-gas followed by resuspension in $CHCl_3$:CH_3OH:H_2O (75:25:2 v/v/v). The analysis of the [^{3}H]glycerol containing (1,2)DAG was performed by thin layer chromatography as described before (16). A few modifications were introduced as follows; Silica gel 60 plates (MERCK, Darmstadt, Germany) were impregnated with 1% boric acid in CH_3OH and activated for 1.5-2 hrs at 110°C and $CHCl_3$:CH_3COCH_3 (96:4 v/v) was used as solvent system. The [^{3}H]glycerol lipids were fluorographically visualized after spraying with En^{3}Hance and subsequently scraped from the plates and counted by liquid scintillation. The amounts of [^{3}H](1,2)DAG were expressed as % of total cellular [^{3}H]glycerol (defined as the sum of water soluble plus water-insoluble [^{3}H]glycerol containing products).

Protein phosphorylation

After washing the cells with P_i-free complete growth medium these were labelled with $^{32}P_i$ (100 μCi/ml, carrier free) in the same medium for 2 hrs. Thereafter, the cells were rinsed with incubation buffer and preincubated for 15 min at 37°C before stimulation with agonists. After removal of the medium, the cells were lysed in 150 μl Laemmli sample buffer and labelled proteins in 35 μl aliquots were separated on 10-20% gradient SDS PAAGE, and visualized by autoradiography as described (17).

Statistics

Data were evaluated for statistical significance by the Students t-test and significance was set at probability of less then 0.05.

RESULTS

Inositolphosphate isomers

Earlier we have shown that stimulation of neonatal rat ventricular myocytes with either PHE or ET-1 results in the activation of the PtdIns-cycle in a dose dependent manner with both agonists, respectively at 10^{-4} M and 10^{-8} M, having almost the same maximal effect on the

formation of total [^{3}H]InsP$_n$ separated by chromatography on Dowex (10). In this study we separated the specific [^{3}H]InsP$_n$ isomers by HPLC. The biologically active Ins(1,4,5)P$_3$ was found not to be elevated above control levels (Fig. 1) while Ins(1,3,4,5)P$_4$ was not detectable (not shown) even up to 30 min of stimulation by either agonist. Stimulation with PHE resulted in a moderate increase of Ins(1,4)P$_2$, setting off at 4 min after agonist addition and accomplishing a level of 0.23% after 30 min, which was significantly higher than control (Fig. 1). Yet, ET-1 evoked an immediate significant increase of Ins(1,4)P$_2$, resulting after 30 min

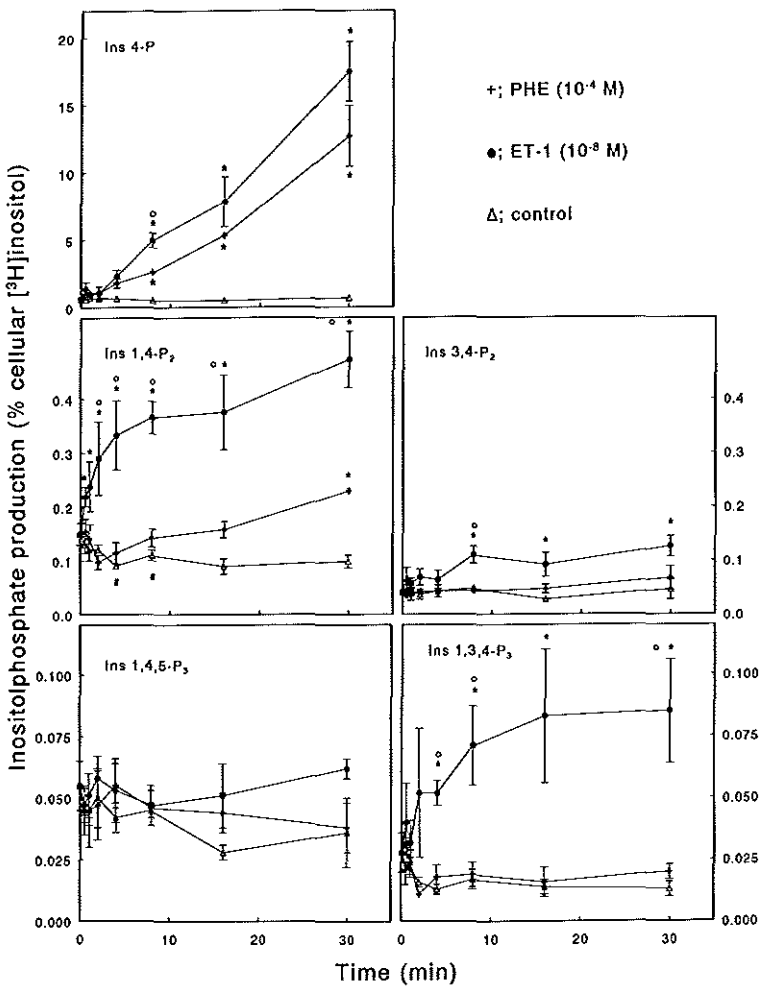

Figure 1 Time course of InsP$_n$ isomer formation in [^{3}H]Ins prelabelled cardiomyocytes after stimulation with PHE, ET-1 or vehicle. The results shown are the mean (± SEM) of 5-8 experiments performed with different cell batches. *, p< 0.05 compared to control; #, p<0.05 versus 0 min control; °, p<0.05 ET-1 versus PHE.

in a higher level (0.47 %) comparing to PHE. On the other hand, PHE and ET-1 stimulation resulted in a similar bulk production of Ins(4)P (12.7 % vs 17.5 % respectively, $p>0.05$) after 30 min. Only upon ET-1 stimulation, rapid increases of dephosphorylation products of Ins(1,3,4,5)P_4, i.e. Ins(1,3,4)P_3 and Ins(3,4)P_2, were measured, which were significant compared to control already after about 5 min of agonist stimulation, resulting in levels of 0.09% and 0.13% respectively after 30 min (Fig. 1). The lack of elevation of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 may indicate the occurrence of very small and local changes of the second messengers and/or extremely high activity of the 5-phosphatase. To circumvent the action of the latter enzyme in another experiment the [^{3}H]Ins-labelled cells were permeabilized by saponin and subsequently PLC was activated by GTPγS (and Ca^{2+}) as described (15). Assuming that the formed Ins(1,4,5)P_3 leaks immediately out of the permeable cells, it would therefore become less accessible to the 5-phosphatase and 3-kinase action as derived from another report (18). When PLC was activated with GTPγS and Ca^{2+}, the bulk of $InsP_n$ found in the incubation buffer was, however, Ins(1,4)P_2 (8%) (Table 1), while neither Ins(1,4,5)P_3 nor Ins(1,3,4)P_3 were detectable (not shown). As expected, Ins(4)P was the predominant $InsP_n$ component found in the cell extract (Table 1).

Table 1 Levels of $InsP_n$ isomers in the incubationbuffer and the acid extract of saponin permeabilized cardiomyocytes after stimulation by GTPγS and/or Ca^{2+}. The values represent the mean (± range/2) of duplicate measurements in one cell batch.

Stimuli		Inositolphosphate production (% of cellular [^{3}H]Inositol)			
		Incubationbuffer		Cell extract	
GTPγS	*[Ca^{2+}_{free}]*	*Ins(4)P*	*Ins(1,4)P_2*	*Ins(4)P*	*Ins(1,4)P_2*
-	-	1.20(0.41)	0.92(0.12)	1.71(0.65)	0.23(0.02)
30 μM	-	3.43(1.46)	5.22(0.51)	2.50(0.24)	1.20(0.05)
-	96 nM	1.31(0.52)	2.13(0.62)	3.04(0.28)	0.98(0.00)
30 μM	96 nM	1.53(0.05)	7.98(0.91)	4.42(0.13)	1.67(0.17)

1,2-Diacylglycerol

The formation of (1,2)DAG was measured up till 16 minutes, a period of stimulation with time when the formation of $InsP_n$ still occurred at a linear rate (Fig. 1). After 16 min stimulation with PHE (10^{-4} M) (1,2)DAG levels raised significantly above control levels. ET-1 also evoked significant increase of the (1,2)DAG level above control that was reached after 8 min. Like the Ins(1,4,5)P_3 levels (Fig. 1), the control (1,2)DAG levels behaved irregularly after vehicle addition (Fig. 2). This may indicate that slight movements of the incubation buffer on top of the cells produces mechanical stretch which previously has been shown to lead to activation of the PtdIns cycle (19, 20).

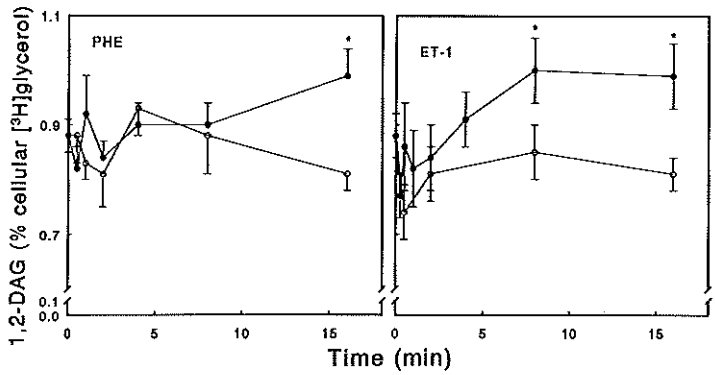

Figure 2 Time course of [^{3}H](1,2)DAG production of [^{3}H]glycerol prelabelled cardiomyocytes after stimulation (●) with PHE (10^{-4} M) and ET-1 (10^{-8} M) or after vehicle addition (○). The results shown are the mean (± SEM) of 6-8 experiments performed with different cell batches. *, $p<0.05$ compared to control.

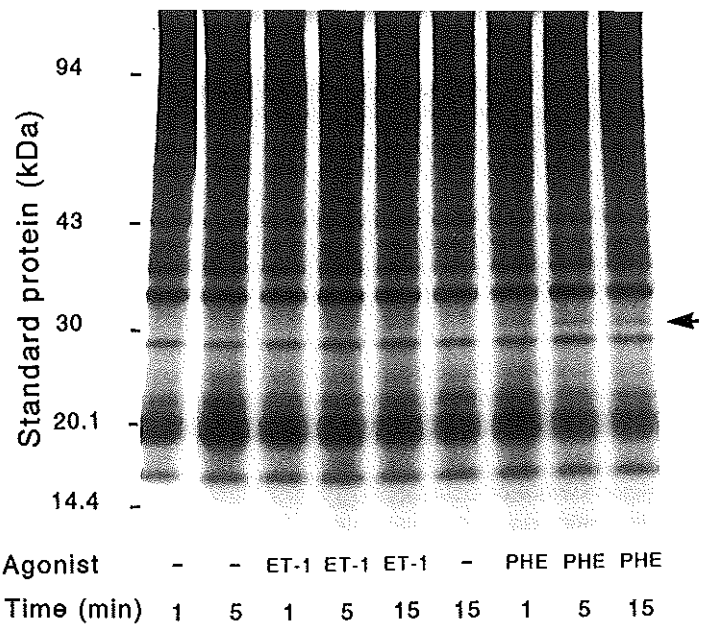

Figure 3 Autoradiogram of proteins labelled upon stimulation of $^{32}P_i$ prelabelled cardiomyocytes with PHE (10^{-5} M) or ET-1 (10^{-8} M). The experiment shown is representative for experiments carried out with 5 separate cell batches. The arrow indicates phosphorylation of a 30 kDa protein.

Protein phosphorylation

To further analyze receptor-dependent specificity downstream of PLC we investigated the protein phosphorylation pattern mediated by PHE (10^{-5} M) or ET-1 (10^{-8} M) in cardiomyocytes prelabelled with $^{32}P_i$. Stimulation of the cardiomyocytes with PHE resulted in rapid (1 min) phosphorylation of a 30 kDa protein, that became more pronounced after 5 and 15 min. This in contrast to ET-1 stimulation that did not evoke phosphorylation of this protein.

DISCUSSION

In previous work we observed that the PLC activation is homologously desensitized after stimulation with ET-1 but not with PHE. Furthermore, the rate of desensitization of the $InsP_n$ response of the cardiomyocytes after addition of PHE was very slow compared to ET-1 (10,11). It was suggested that either the targets for regulation of the rate of desensitization are the receptors or that PHE and ET-1 use different isoforms of PLC-ß and/or G-protein. Therefore it was of interest to further characterize the receptor-mediated responses more downstream of PLC.

The second messenger, Ins(1,4,5)P_3, was not elevated above control (Fig. 1) while Ins(1,3,4,5)P_4 was not detectable suggesting that Ca^{2+} mobilization by these mediators is of limited or strictly compartmentalized importance in the spontaneously contracting cardiomyocytes. Looking at the receptor-dependent temporal differences in the appearance of (1,2)DAG (Fig. 2), the question arises whether this (1,2)DAG is solely originating from PtdIns(4,5)P_2 or from cross-talk with other (1,2)DAG producing signalling pathways such as phospholipase D (PLD) (2,3,21). The alliance of this two signalling pathways might be established by a PKC isoenzyme, possibly being dependent on the receptor activated (1,2)DAG release, or agonist-receptor coupled activation of $\beta\gamma$ subunits derived from G-proteins (22). Alternatively, the temporal differences in (1,2)DAG production could have its origin in the kinetics of different PLC-ß isoenzymes and/or PLD.

The receptor-dependent differences in the Ins(1,4,5)P_3 metabolism can be interpreted on other levels. If the PLC isoenzymes involved in transducing either the ET-1 or the PHE signal are not identical, the possibility of different kinetics or competition of the 5-phosphatase and the 3-kinase pathway can be supposed. In general, experiments to measure the metabolic destiny of Ins(1,4,5)P_3 performed with cell homogenates or permeabilized cells generally indicated that the 5-phosphatase pathway is predominant (23-25). Moreover, we should regard the consequences of different subcellular location of 5-phosphatase and 3-kinase on the Ins(1,4,5)P_3 metabolism (25). For instance, in liver and intestinal epithelial cells the bulk of the 5-phosphatase is restricted to the plasma membrane, while the 3-kinase is cytosolic and therefore its access to Ins(1,4,5)P_3 would not seem to be restricted. This would agree with our experiment performed with GTPγS stimulated G-protein-PLC in saponin-permeabilized cardiomyocytes showing no Ins(1,4,5)P_3 but predominantly the 5-phosphatase product Ins(1,4)P_2 in the extracellular buffer, while 3-kinase products were absent. Furthermore, the regulation of 5-phosphatase and 3-kinase activity may be critical. Receptor-induced increase of intracellular $[Ca^{2+}_{free}]$ can increase the V_{max} of the 3-kinase (25,26), while the 5-phosphatase is insensitive to Ca^{2+}. Compartmentalization of the processes could create microenvironments that would come up to these requirements.

The receptor-specific protein phosphorylation may also be interpreted by the intervention of other signalling pathways, e.g. cross-talk to the ß-adrenergic signal transduction pathway. The 30 kDa protein that is phosphorylated upon PHE but not ET-1 stimulation (Fig. 3) appeared not to be membrane-bound or of cytoplasmic origin (results not shown) suggesting its association with the cytoskeleton or myofilaments. In fact, this protein may be identical to troponin I (30 kDa) which has been shown to be substrate for PKC *in vitro* in isolated myofibrils (27).

In conclusion, the results demonstrate that after maximal stimulation of PLC in cardiomyocytes with ET-1 or PHE several differences are present downstream of PLC. Distinct receptor-dependent responses can be noted concerning Ins(1,4,5)P_3 metabolism, temporal formation of (1,2)DAG and protein phosphorylation.

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ALPHA-ADRENERGIC AGONIST AND ENDOTHELIN-1 INDUCED INTRACELLULAR Ca^{2+} RESPONSE IN THE PRESENCE OF A Ca^{2+} ENTRY BLOCKER IN CULTURED RAT VENTRICULAR MYOCYTES.

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ABSTRACT

Previously we demonstrated that stimulation of cultured neonatal rat ventricular myocytes by either α_1-adrenergic agonist or endothelin-1 resulted in a rapid formation of total inositolphosphates, although the levels of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate did not rise significantly. The aim of this study was to examine whether stimulation by α_1-adrenergic agonist and endothelin-1 could still elicit phosphatidylinositol cycle mediated intracellular Ca^{2+} mobilization in these cells. The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by single cell imaging dual wavelength fluorescence microscopy in fura-2-loaded cardiomyocytes. The interference of agonist induced $[Ca^{2+}]_i$ responses by the beat to beat variation of $[Ca^{2+}]_i$ was prevented by arresting the cells with the Ca^{2+} entry blocker diltiazem (10 μM). The $[Ca^{2+}]_i$ response (expressed as % of baseline ratio of fluorescence intensities of fura-2 at 340 nm and 380 nm excitation wavelength), induced by phenylephrine (10^{-4} M) and endothelin-1 (10^{-8} M) was small, up to 20 % of baseline after 9-20 min. In contrast, Ca^{2+}-influx induced by incubation in Na^+-free buffer caused a steep increase of $[Ca^{2+}]_i$ up to 150 % of baseline after 30 s.

Analysis of single cells following stimulation with phenylephrine or endothelin-1 showed heterogeneity with respect to a rise in $[Ca^{2+}]_i$. However, if rapid Ca^{2+}-influx was induced by incubation in Na^+-free buffer, $[Ca^{2+}]_i$ responses in individual myocytes occurred homogeneously.

It is concluded that the α_1-adrenergic agonist and endothelin-1 induced $[Ca^{2+}]_i$ responses are delayed in time, small and quite heterogeneous among cells. The findings are in agreement with earlier observations which revealed no detectable overall increase of the Ca^{2+} releasing inositolphosphates under these conditions and suggest that other second messengers such as 1,2-diacylglycerol are involved in the agonist mediated Ca^{2+} signals.

INTRODUCTION

In cardiomyocytes, Ca^{2+} homeostasis is strictly controlled: besides the transient elevation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) per beat, $[Ca^{2+}]_i$ responds to several hormones, neurotransmitters and agonists, giving rise to inotropic, chronotropic, lusitropic and hypertrophic effects [1,2].

After excitation of the cardiac sarcolemma, $[Ca^{2+}]_i$ is initially increased by Ca^{2+}-influx through L-type Ca^{2+} channels and partially through the Na^+-Ca^{2+} antiporter [3]. This small increase of $[Ca^{2+}]_i$ triggers the release of large amounts of Ca^{2+} from the sarcoplasmic reticulum (SR) through the ryanodine-sensitive Ca^{2+} release channels. The increased $[Ca^{2+}]_i$ returns to resting levels to promote relaxation by the combined action of the sarcolemmal Na^+-Ca^{2+} antiporter, the uptake of Ca^{2+} by the SR Ca^{2+} pump, and the extrusion of Ca^{2+} by the sarcolemmal Ca^{2+} pump [4].

Ca^{2+} transients and changes of end-diastolic and peak systolic $[Ca^{2+}]_i$ also play an important role as second messengers in cell signalling likely mediated by adenylate cyclase and phospholipase A_2, C, and D and G-protein regulated Ca^{2+} channels. Agonists such as α_1-adrenergic agonist phenylephrine (PHE) and endothelin-1 (ET-1), which transduce their signals partially by G-protein-coupled phospholipase C-β (PLCβ), induce positive inotropy and

on the long term hypertrophy and concomitant reprogramming of gene expression [5-7]. PLC-β hydrolyses phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), thereby producing the second messengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) and 1,2-diacylglycerol ((1,2)DAG) [2,8-12]. The second messenger Ins(1,4,5)P_3 is known to be involved in the mobilization of Ca^{2+} from intracellular Ca^{2+} pools [5,13], while (1,2)DAG possibly in conjunction with Ca^{2+} activates members of the protein kinase C isoenzyme family [14]. Previously we have shown that stimulation of neonatal rat ventricular myocytes with PHE or ET-1 resulted in the activation of the phosphatidylinositol (PtdIns) cycle in a dose-dependent manner [15]. The activity of the PtdIns cycle was measured by recording the formation of the total inositolphosphates ($InsP_n$). However, when examining the formation of the second messengers Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 upon stimulation with PHE or ET-1, no significant elevations were present [16]. These results suggested that either a) these second messengers had very short lifetime, b) the intracellular Ca^{2+}-mobilizing action of these compounds was strictly compartmentalized, c) the overall increase of the Ca^{2+} releasing inositolphosphates was not detectable due to cellular heterogeneity of the response [10,11,17,18]. On the other hand, evidence was provided that stimulation of rat atrial myocytes and isolated rabbit papillary muscles with α_1-adrenergic agonist or ET-1 induces a rise of $[Ca^{2+}]_i$ [11,18,19]. Earlier, we observed a late (8-16 min) rise of (1,2)DAG in PHE and ET-1 stimulated neonatal rat ventricular myocytes [16], and based upon some other studies [20-22] it is not unlikely that activation of protein kinase C is involved in PHE and ET-1 induced rise of $[Ca^{2+}]_i$ in cardiomyocytes.

Hence, it was important to characterize the Ca^{2+}-responses in time after stimulation with PHE (10^{-4} M) or ET-1 (10^{-8} M) in our model of cultured neonatal rat ventricular myocytes. These experiments were performed in a recently developed two-compartment culture dish using single cell imaging fluorescence microscopy with fura-2 as Ca^{2+} indicator which allowed evaluation of the treatment with or without agonist simultaneously in the same culture [23]. The interference of agonist induced $[Ca^{2+}]_i$ responses by beat to beat fluctuations of $[Ca^{2+}]_i$ was prevented by arresting the Ca^{2+}-influx with the L-type Ca^{2+} channel blocker, diltiazem. To validate the small and heterogeneous $[Ca^{2+}]_i$ responses following stimulation with agonists under the condition of L-type Ca^{2+} channel blockade, we measured the rapid and large elevation of $[Ca^{2+}]_i$ that should occur as a result of a net Ca^{2+}-influx induced by incubation of the cardiomyocytes in Na^+ free medium [24].

MATERIALS AND METHODS

Reagents

Phenylephrine (PHE) and probenicid were obtained from Sigma (St. Louis, MO, USA) and ET-1 was from Peninsula Laboratories (Belmont, CA, USA). Ham's F10, fetal calf serum and horse serum were purchased from Flow Laboratories (Irvine, UK). Collagenase (type 1) was from Worthington (Freehold, NJ, USA). Tissue culture dishes, Ø 35 mm, were purchased from Becton and Dickinson (Oxnard, CA, USA). Fura-2 acetoxymethylester (Fura-2 AM) was obtained from Molecular Probes (Eugene, OR, USA). *Myo*-[2-^{3}H]inositol (17-19 Ci/mmol) was obtained from Amersham International (Amersham, UK) and Dowex AG 1-X8 (200-400 Mesh, formate form) was from Bio-Rad Laboratories (Richmond, CA, USA).

Cell culture

Hearts were dissected from two-day-old Wistar rats and transferred to buffer containing 137 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 5.5 mM glucose, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 0.5 mM phenol red, pH 7.4. Ventricles of ten neonatal rat harts were minced and subsequently dissociated by three times 15 min incubation with collagenase at 37°C, as described [25]. After centrifugation (10 min, 50 g) and suspension in complete growth medium (Ham's F10, supplemented with 10 % fetal calf serum and 10 % horse serum), the cardiomyocyte to noncardiomyocyte ratio was further increased by preplating [26]. The myocytes were either seeded on circular glass cover slips (Ø 25 mm) in tissue culture dishes (Ø 35 mm) (used for fura-2 fluorescence imaging) or were seeded in 1.8 cm^2 wells at 150 to 175x10^3 cells/cm^2 giving a confluent monolayer of spontaneously contracting cells after 24 h. Growth medium was renewed after 4 h and every 48 h thereafter. Experiments were performed 4 to 5 days after plating of the cells.

Measurements of $[Ca^{2+}]_i$

Myocytes grown on circular glass coverslips were rinsed with HEPES buffer containing 125 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 1 Mm KH_2PO_4, 10 Mm $NaHCO_3$, 10 mM HEPES, 5 mM glucose and 2.5 mM probenicid. After loading the cells with 2 μM fura-2/AM in HEPES-buffer for 40 min at 37 °C, the cells were rinsed twice with HEPES-buffer. Then the glass coverslip, holding the myocytes, was mounted in a two-compartment incubation dish, to have the opportunity to expose one cell culture to two treatments simultaneously and to circumvent differences between individual cultures, as described [23]. At this point diltiazem (10^{-5} M) was added to the 500 μl HEPES-buffer in each compartment to stop the contractions in diastole. The dish was placed in a temperature controlled micro-incubator [27], positioned on the stage of a dual-wavelength fluorescence microscope equipped with a *x/y*-table fitted with two digital precision sliding gauges (Mitutoyo, UK) for precise positioning of the culture dish. For further details of the type and set up of the fluorescence microscope, the video camera and software the reader is referred to Draijer et al.[28]. At indicated timepoints an image pair of 340 nm and 380 nm excitation wavelength was made, which was analyzed off-line, resulting after dividing the 340 nm image by the 380 nm image on a pixel-by-pixel base, in a ratio image ($R=F_{340\ nm}/F_{380\ nm}$).

The ratio R of measured fluorescence intensities can be converted to $[Ca^{2+}]_i$ values [29], but the common problem with the calibration of fura-2 was met [30]. Therefore, no attempts were made to convert the ratio values to $[Ca^{2+}]$. Instead, we expressed Ca^{2+} responses as the percentage of change in R as compared to the baseline value. After a period of 15 min, during which the myocytes were left to stabilize at 34 °C for 15 min, PHE (10^{-4} M), ET-1 (10^{-8} M) or vehicle was added. Every minute, an imagepair $F_{340\ nm}$ and $F_{380\ nm}$ was recorded for 20-25 min. The submaximal temperature was maintained during the continuation of the experiment to conserve proper stability of the preparation.

Separate series of experiments were performed with cultures in which Ca^{2+}-influx was stimulated. To this purpose the myocytes in culture were first equilibrated with HEPES-buffer supplemented with 10^{-5} M diltiazem for 30 min at 37 °C. Then the medium of one compartment was exchanged with Na^+-free HEPES-buffer (HEPES-buffer in which Na^+ was substituted by choline on a molar basis [31] supplemented with 10^{-5} M diltiazem). Every 17 s, an imagepair $F_{340\ nm}$ and $F_{380\ nm}$ was recorded for a period of 30 min. The R-value of

individual myocytes as well as the mean of these R-values were expressed as percentage change as compared to t=0.

Measurements of total [^{3}H]InsP$_n$

Cardiomyocytes were labelled with 2 μCi *myo*-[2-^{3}H]Ins/ml for 48 h in complete growth medium. After washing and preincubation with or without 10^{-5} M diltiazem (15 min, 37 °C) in incubation buffer (130 mM NaCl; 4.7 mM KCl; 1.3 mM $CaCl_2$; 0.44 mM NaH_2PO_4; 1.1 mM $MgSO_4$; 20 mM $NaHCO_3$; 11 mM glucose; 10 mM LiCl; 10 mM HEPES; pH 7.4, 37 °C) the cells were stimulated with PHE (10^{-4} M). Incubations were stopped by rapidly washing the cells with ice-cold buffer. Water-soluble [^{3}H]Ins containing products were extracted by two subsequent 5 min extractions with 4% $HClO_4$ at 4°C. The pooled $HClO_4$ fractions were neutralized with 2 M KOH and 1 M K_2CO_3. The remainder, containing water-insoluble products, was subsequently extracted from the cells with ice-cold CH_3OH:HCl (100:1 v/v) for 5 min at 4 °C. The [^{3}H]InsP$_n$ were separated from [^{3}H]Ins and [^{3}H]glycerol-P-Ins by chromatography on Dowex AG 1-X8 as described previously [8].

Statistics

Data were evaluated for statistical significance by the Students t-test and significance was set at probability of less than 0.05. However, in the experiments that were performed in the two-compartment incubation dishes the statistical evaluation was limited due to obliged incidental asynchronism between agonist and control-induced $[Ca^{2+}]_i$ measurements.

Table 1 Effect of L-type Ca^{2+}-channel blocker diltiazem (10 μM) on the [^{3}H]InsP$_n$ formation in neonatal rat cardiomyocytes after stimulation with PHE (100 μM)

	[^{3}H]InsP$_n$ (in % of total incorporated [^{3}H]Ins)			
	0 min	4 min	8 min	16 min
Control	2.13 ± 0.03	4.44 ± 0.14	6.28 ± 0.19	12.08 ± 0.60
10 μM diltiazem	1.86 ± 0.20	3.93 ± 0.12 *	5.13 ± 0.22 **	11.59 ± 1.66

The formation of total [^{3}H]InsP$_n$ was measured after stimulation of *myo*-[2-^{3}H]Ins prelabelled neonatal rat cardiomyocytes with PHE (100 μM) in the absence or presence of 10 μM diltiazem during the indicated periods of time. Values represent [^{3}H]InsP$_n$ as percentage of total incorporated [^{3}H]Ins expressed as mean values ± SEM of four experiments; *, $p<0.05$ and **, $p<0.01$ versus control at corresponding times. In the absence of PHE diltiazem had no significant effect on the [^{3}H]InsP$_n$ formation. These values varied between 1.86 % (at 0 min) and 2.84 % (at 16 min) of total incorporated [^{3}H]Ins.

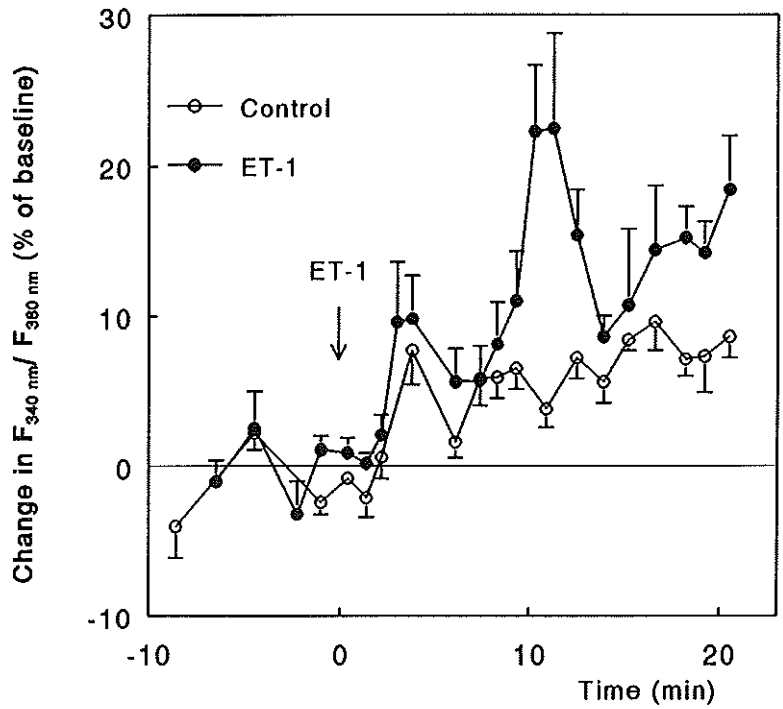

Figure 1 Time-dependent changes of ratio values ($R=F_{340\ nm}/F_{380\ nm}$) reflecting the intracellular Ca^{2+} concentration of 18 individual cardiomyocytes from five different cell batches measured by fura-2 fluorescence after stimulation with 10^{-8} M endothelin (ET-1), expressed as percentage change of baseline value (recorded at time close to zero). The time-dependent ratio value per cell was averaged for all 18 cells (± SEM): $p< 0.05$ at 12.5, 18.2, 19.2, and 20.5 min of ET-1 (●) versus control (○). As can be seen the ET-1 induced $[Ca^{2+}]_i$ response of relevance at points 10.9 and 11.2 min could not statistically be compared with a control value due to asynchronism (see methods).

RESULTS

Ca^{2+} entry blockade and [^{3}H]InsP$_n$ formation

Normally, neonatal cardiomyocytes in culture at 34 °C contract synchronously but irregularly (50 to 60 beats/min) in clusters of cells. The Ca^{2+} transients observed in these cells mask the small effects of PHE and ET-1. Therefore, the fluorescence measurements were performed in the presence of the L-type Ca^{2+} channel blocker diltiazem (10^{-5} M) which arrested Ca^{2+}-influx, Ca^{2+} transient and contractile activity of the cells. To exclude the possibility that diltiazem had a major effect on agonist-induced phospholipase C activation, we prelabelled myocytes with *myo*-[2-^{3}H]Ins for 48 h. Then the myocytes were stimulated with PHE (10^{-4} M) in the presence or absence of diltiazem (10^{-5} M). Phospholipase C activity measured by [^{3}H]-InsP$_n$ production of intact cells was only slightly affected in the early phase of PHE stimulation (11.5 % and 18.3 % after 4 and 8 min of stimulation, respectively) (Table 1).

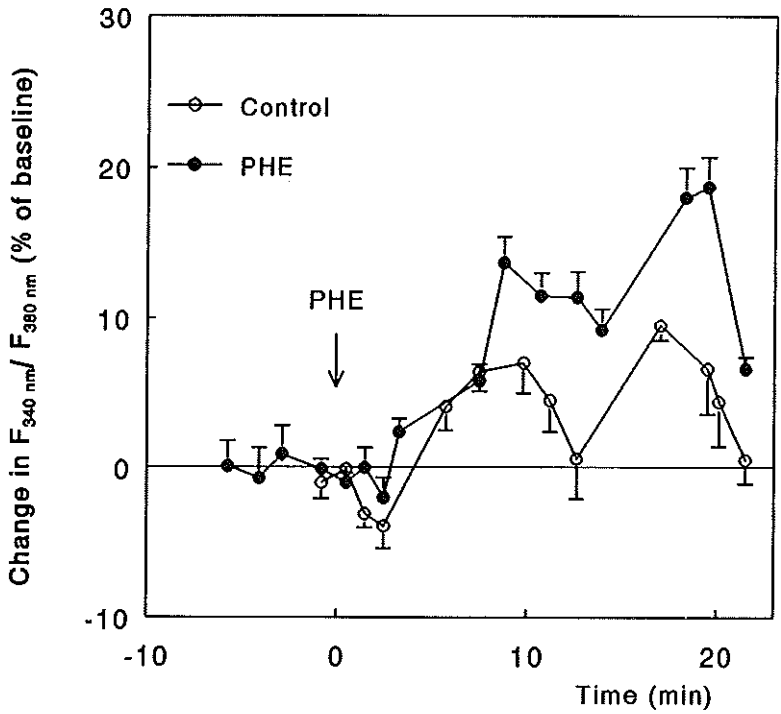

Figure 2 Time-dependent changes of ratio values ($R=F_{340\ nm}/F_{380\ nm}$) reflecting $[Ca^{2+}]_i$ of 12 individual cardiomyocytes from three different cell batches measured by fura-2 fluorescence after stimulation with 10^{-4} M phenylephrine (PHE), expressed as percentage change of baseline value (recorded at time close to zero). The time-dependent ratio value per cell was averaged for all 12 cells (± SEM): $p<0.05$ at t=12.6, 19.5, and 21.5 min of PHE (●) versus control (○). As can be seen the PHE induced $[Ca^{2+}]_i$ response of relevance at points 8.75, 10.7, 13.9 and 18.3 min could not statistically be compared with a control value due to asynchronism (see methods).

In agreement with this we previously demonstrated that L-type Ca^{2+} channel blocker nifidipine (1 μM) had a slight inhibitory effect on ET-1 induced [^{3}H]-InsP_n formation [8].

Average $[Ca^{2+}]_i$ responses

Using the Ca^{2+} indicator fura-2 we measured the ratio of fluorescence intensities at 340 nm ($F_{340\ nm}$) and 380 nm ($F_{380\ nm}$ excitation wavelength)($R=F_{340nm}/F_{380nm}$) before and after addition of either agonist. Forced by the uncertainties in the calibration of intracellular fura-2, we expressed the changes in $[Ca^{2+}]_i$ in terms of changes in fura-2 fluorescence ratios (Fig. 1). The $[Ca^{2+}]_i$ level at t=0 was taken as a baseline value to calculate the percentual increase. At first instance, we calculated the average R-value of all cells in the field of view which revealed hardly any increase in $[Ca^{2+}]_i$. However, when we analyzed individual cells, chosen at random in the image of t=0, and then recalculated the average R-value of those cells, ET-1 stimulation was found to result in a moderate increase of $[Ca^{2+}]_i$, which became significantly elevated, up to 20% of baseline, between 10 and 13 min and again after 18 min (Fig. 1). Taking the control response into account the biphasic pattern of the $[Ca^{2+}]_i$ response is

evident. Calculation of the Δ % change was, due to the obliged incidental asynchronism between agonist and control-induced $[Ca^{2+}]_i$ measurements (see method section), restricted to a few critical time-points. For instance, at 12.5 min the Δ % towards control was 8.2 ± 3.3, at 15.2 min 2.3 ± 5.1 and at 20.5 min 9.8 ± 3.9, which data confirm the biphasic pattern. A moderate $[Ca^{2+}]_i$ response was found as well after addition of PHE (10^{-4} M), significantly setting off at about 9 min, however lacking a biphasic pattern (Fig. 2). Calculation of the Δ % change towards control gave for instance the following data: at 12.6 min the Δ % was 10.1 ± 3.2, at 19.5 min 12.1 ± 3.6 and 21.5 min 6.1 ± 1.8. The $[Ca^{2+}]_i$ response, observed after addition of vehicle (the control side of the two compartment dish) was always irregular and showed an elevation of the baseline value as well. We observed this phenomenon earlier in experiments which were performed to study the agonist mediated production of the $InsP_n$ isomers and (1,2)DAG [16]. We assume that slight movements of the incubation buffer due to addition of vehicle create mechanical stretch,

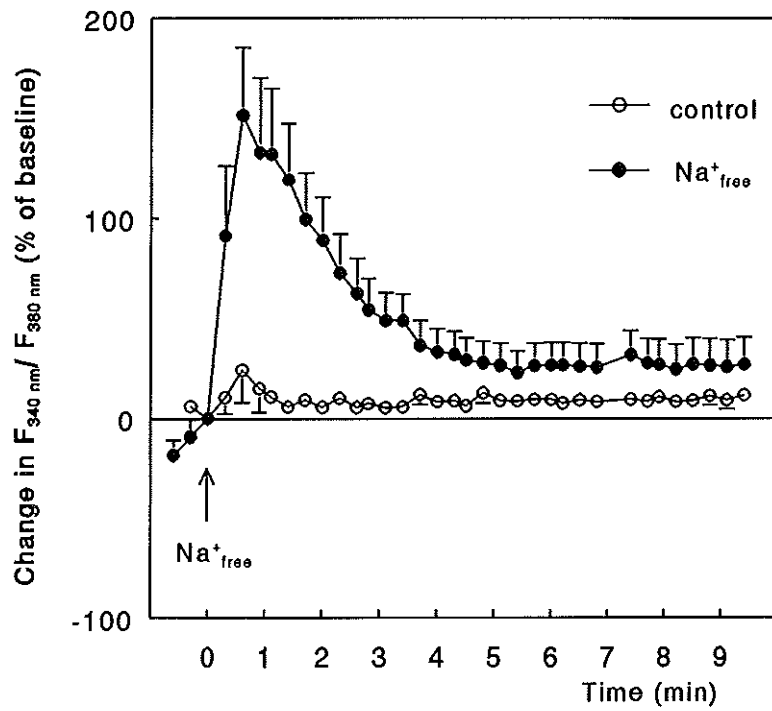

Figure 3 Time-dependent changes of ratio values ($R=F_{340\ nm}/F_{380\ nm}$) reflecting $[Ca^{2+}]_i$ of 20 individual cardiomyocytes from three different cell batches measured by fura-2 fluorescence during incubation of the cells with Na^+-free buffer (●), or during incubation with Na^+ containing buffer (○), expressed as percentage change of baseline value (recorded at time close to zero). The time-dependent ratio value per cell was averaged for all 20 cells (± SEM). Significant differences ($p<0.05$) of treatment (●) versus control (○) were found for all points between 0.3 and 3.7 min.

which has been demonstrated to activate the PtdIns cycle [32,33]. Intracellular signalling induced by stretch may also include intracellular Ca^{2+}-release, or Ca^{2+}-influx through channels which normally carry Na^{+}-ions [34].

In order to improve the validation of the minor $[Ca^{2+}]_i$ elevations in time and magnitude after stimulation with PHE or ET-1 under the condition of Ca^{2+}-entry blockade we compared these elevations to changes of $[Ca^{2+}]_i$ due to massive Ca^{2+}-influx caused by Na^{+}-free diltiazem-containing incubation medium as was found by others [24]. Na^{+}-free buffer causes the Na^{+}-Ca^{2+} antiporter to reverse, leading to Na^{+}-efflux coupled to Ca^{2+}-influx, the latter which subsequently causes Ca^{2+}-induced Ca^{2+} release from the SR. Immediately following replacement of the medium to Na^{+}-free buffer, $[Ca^{2+}]_i$ increased considerably to reach peak levels of 150 % of baseline fura-2 fluorescence ratio within 30 s (Fig. 3). Subsequently, $[Ca^{2+}]_i$ declined to a basal steady state level after 5 min which was still higher than before Na^{+}-free incubation by 26.5 %. Also in the control experiments, $[Ca^{2+}]_i$ was elevated slightly in the first 30 s and returned to basal values after 1 min. As expected, even in the presence of 10 μM diltiazem, cultured cardiomyocytes are able to undergo an elevation of $[Ca^{2+}]_i$.

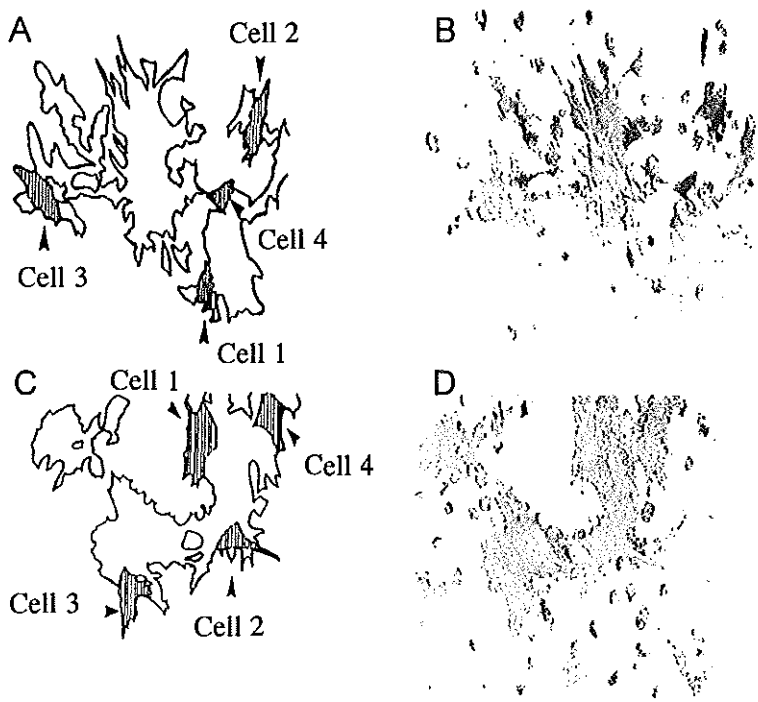

Figure 4 "Negative video" representation of the ratio image obtained in fura-2 loaded cardiomyocytes recorded 11 min after stimulation with ET-1 (10^{-8} M) (panel B) , or PHE (10^{-4} M) (panel D) after subtraction of the ratio images obtained at baseline (recorded at a time close to zero). Panels B and D reflect the net $[Ca^{2+}]_i$ elevations due to ET-1 or PHE stimulation, respectively. Darkened areas indicate higher intracellular Ca^{2+}. The schematic drawings in panels A and C identify the individual cells which were studied, the $[Ca^{2+}]_i$ responses of which are shown in Figures 5 and 6.

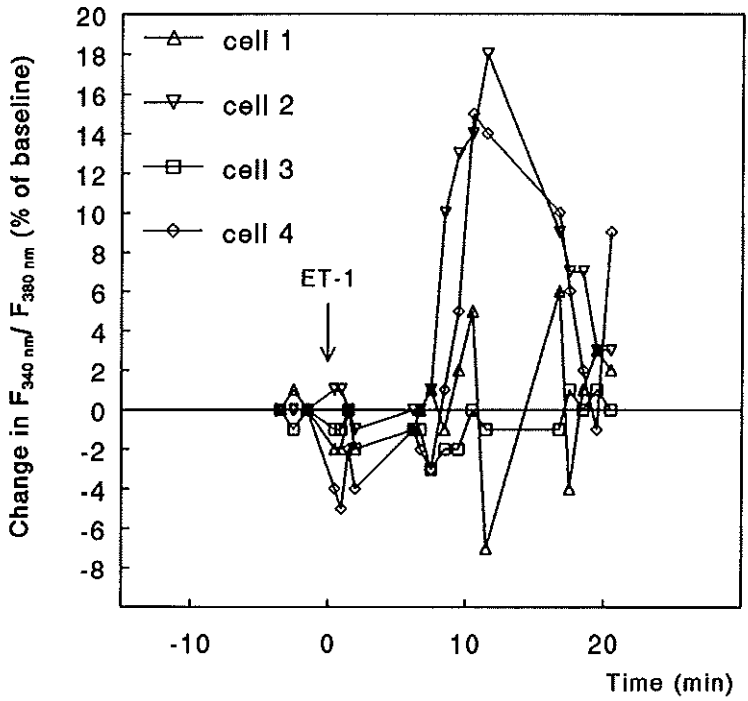

Figure 5 Time dependent changes in ratio values ($R=F_{340\ nm}/F_{380\ nm}$), reflecting $[Ca^{2+}]_i$ of four individual cardiomyocytes as are depicted in Fig 4A (cells 1, 2, 3 and 4) after stimulation with 10^{-8} M endothelin-1 (ET-1), expressed as percentage change of baseline value.

$[Ca^{2+}]_i$-responses of single cardiomyocytes

Mean $[Ca^{2+}]_i$ responses of cardiomyocytes stimulated by PHE or ET-1 were small. However, if the $[Ca^{2+}]_i$ responses of individual cells were studied, responses appeared to be heterogeneous among myocytes in the field of view. This is clearly illustrated by Fig. 4, which shows the "negative video" representation (panels B and D) of the ratio-image recorded at 11 min after addition of ET-1 or PHE, respectively, after subtraction of the ratio-image recorded before addition of these agonists. Dark areas represent myocytes with elevated $[Ca^{2+}]_i$. The changes in ratio values expressed as a percentage of baseline observed in individual cells (nr. 1-4) stimulated with ET-1 are depicted in Fig. 5. Following stimulation with ET-1, the net responses revealed a maximal R-value of 18 % in cell nr. 2, 15 % in cell nr. 4, 7 % in cell nr. 2 and 6 % in cell nr. 1, occurring at different times (Fig. 5). The net responses after stimulation with PHE revealed a peak R-value of 11 % in cell nr.4, 7 % in cell nr. 1 and 5 % in cells nr. 2 and 3 (Fig. 6). In Fig. 4, the left panels A and C depict the localization of the cells which were analyzed individually. The heterogeneity of the Ca^{2+} responses evoked by ET-1 and PHE is spatial, temporal and quantal. If myocytes were exposed to Na^+-free buffer, the resulting peak cellular $[Ca^{2+}]_i$ responses are uniform in time, in magnitude and in all cells, although the magnitude of the steady state $[Ca^{2+}]_i$ levels, to which these levels

returned after 5 min of exposure to Na^+-free medium revealed a slight cell-dependency (results not shown).

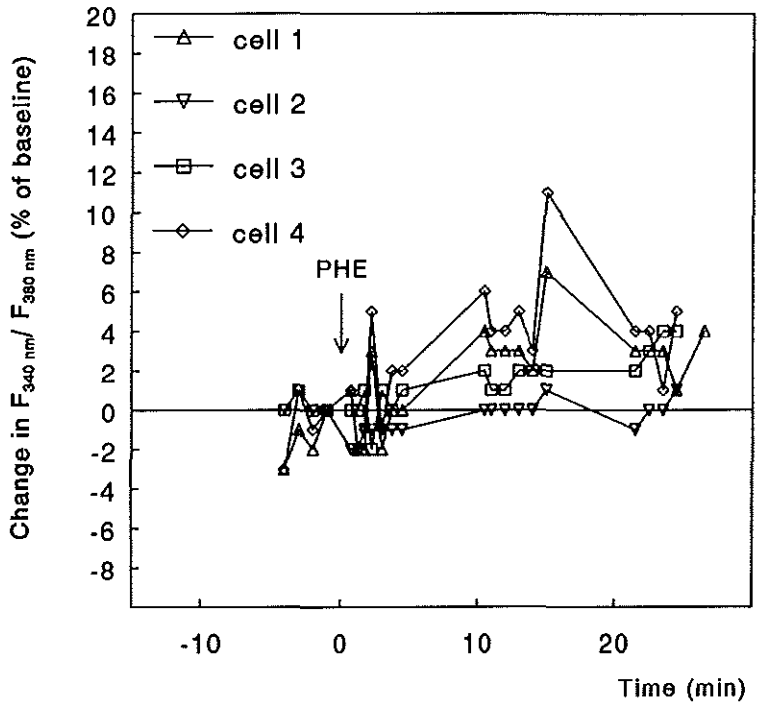

Figure 6 Time-dependent changes in ratio values ($R=F_{340\ nm}/F_{380\ nm}$), reflecting $[Ca^{2+}]_i$ of four individual cardiomyocytes as are depicted in fig 4C (cells 1, 2, 3 and 4) after stimulation with 10^{-4} M phenylephrine (PHE), expressed as percentage change of baseline value.

DISCUSSION

Previously, we measured the formation of total [^{3}H]InsP_n and showed that stimulation of neonatal rat ventricular myocytes with either PHE or ET-1 resulted in a rapid (within seconds) and a strong activation of the PtdIns-cycle in a dose-dependent manner [15]. Subsequently, when we studied the formation of second messengers upon stimulation with PHE (10^{-4} M) or ET-1 (10^{-8} M) no significant elevation of putative $[Ca^{2+}]_i$ increasing compounds, i.e. Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4, was detected [16]. Therefore it is suggested that in the model of cultured neonatal rat ventricular myocytes, the Ca^{2+}-mobilizing function, implied for these compounds, was limited or strictly compartmentalized [5-7]. After PHE or ET-1 stimulation, we observed, however, a late rise (8-16 min) of (1,2)DAG. Although an intracellular Ca^{2+} mobilizing role for (1,2)DAG is not generally assumed, there are a number of studies that have shown that activation of protein kinase C in ventricular myocytes can produce a rise in $[Ca^{2+}]_i$ or Ca^{2+} currents [20-22]. Moreover, we and others demonstrated recently that ET-1 (and α_1-adrenergic) stimulation of ventricular myocytes produces a late activation of

phospholipase D which leads to intracellular formation of phosphatidic acid and (1,2)DAG [35,36].

On the other hand, we have provided evidence for feedforward activation of phospholipase C by elevation of $[Ca^{2+}]_i$ during agonist-induced activation. This was proven e.g. by exposure of intact cardiomyocytes to various exogenous agents, such as Ca^{2+} ionophore A23187 or ethylene glycol bis(β-aminoethyl) N,N,N',N'-tetraacetic acid (EGTA) [8]. In saponin-permeabilized cells, we showed indeed that phospholipase C activity itself was Ca^{2+} dependent by adding Ca^{2+} and GTPγS to the incubation medium. Therefore, we were interested to know whether PHE (10^{-4} M) or ET-1 (10^{-8} M) could evoke a $[Ca^{2+}]_i$ response, to obtain more knowledge of the position of intracellular Ca^{2+} in the integrated pattern of cell signalling mediated by α_1-adrenergic agonists or endothelins.

In the present study, we examined the PHE- and ET-1-induced $[Ca^{2+}]_i$ responses by single cell imaging fluorescence microscopy with fura-2 as Ca^{2+} indicator. Because subtle $[Ca^{2+}]_i$ rises after either agonist were hardly detectable on top of the beat to beat $[Ca^{2+}]_i$ transients, we added the L-type Ca^{2+} channel blocker diltiazem which suppressed the Ca^{2+} transients completely. The use of diltiazem was preferred to other Ca^{2+} entry blockers such as nifidipine, which is light sensitive, or verapamil which has antagonistic properties against α_1-adrenergic receptors [37,38]. Diltiazem (10^{-5} M) had only a slight inhibitory effect on PHE-induced phospholipase C activity, which is in agreement with the previously observed effects of the Ca^{2+} entry blocker nifidipine on ET-1 (10^{-8} M) stimulated phospholipase C activity [8].

Stimulation of the cultured cardiomyocytes with PHE (10^{-4} M) or ET-1 (10^{-8} M) in the presence of diltiazem (10^{-5} M) revealed delayed and small elevations of $[Ca^{2+}]_i$, apparently different in pattern (a monophasic- and biphasic response, respectively) setting off at 8-9 min after agonist addition (Figs. 1 and 2). Earlier, we did not detect any elevation of putative $[Ca^{2+}]_i$ increasing compounds (Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4) although high activity of the PtdIns turnover was demonstrated by detection of phosphorylation and dephosphorylation products of Ins(1,4,5)P_3 [16]. This would suggest that the agonist-mediated $[Ca^{2+}]_i$ increase in single cells is a result of a cascade of Ca^{2+}-induced Ca^{2+}-releases, perhaps initially induced by small rises of Ca^{2+} releasing Ins(1,4,5)P_3 concentrations confined to microdomains below the plasma membrane [39], to subsarcolemmal 'clefts' [40], or to a reduced number of cells in which intracellular Ca^{2+}-responses were elicited as observed with single cell Ca^{2+} imaging. The minor Ins(1,4,5)P_3 elevations may not be detectable with current methods, leaving the cause of an increase of $[Ca^{2+}]_i$ following agonist stimulation to be elucidated. Up to now no immunocytochemical studies have been published which have used antibodies against Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 to determine intracellular and intercellular heterogeneity in responses in myocardial cells. In view of the late onset of the PHE and ET-1 induced $[Ca^{2+}]_i$ increases as well as the distinction in pattern, it can be assumed that the previously observed (1,2)DAG responses are more likely involved [16]. In this respect, it is interesting that in the previous study on PHE and ET-1 induced cellular responses we found distinct second messenger (inositolphosphate isomers and (1,2)DAG) responses [16]. More recently, we observed that protein kinase C-ϵ is translocated by stimulation of cardiomyocytes by ET-1. This isoenzyme of protein kinase C does not require $[Ca^{2+}]_i$ elevations for its activation [35]. Additionally, Clerk and colleagues (1994) demonstrated proportional differences in protein kinase C-ϵ translocation after either ET-1 or PHE stimulation of neonatal ventricular myocytes [41], while others demonstrated in

cardiomyocytes that ET-1 stimulates T-type Ca^{2+} channels and that the effect is likely mediated by protein kinase C [22]. The involvement of protein kinase C-isoenzymes in the α_1-adrenergic agonist or ET-1 mediated $[Ca^{2+}]_i$-increases was demonstrated in cultured adult ventricular myocytes using activators and inhibitors of protein kinase C [42,43]. As far as the involvement of the change in Na^+/H^+ exchange in the agonist-induced $[Ca^{2+}]_i$ responses is concerned, no distinction can be made between the effect of PHE and ET-1. Both agonists were inducing an alkalinization, which is believed to contribute to the $[Ca^{2+}]_i$ rise by its influence on the Ca^{2+} influx via Na^+/Ca^{2+} exchange.

The Ca^{2+}-responses in individual myocytes within the field of view appeared to be heterogeneous in time, magnitude and among cells (Figs. 5 and 6). One aspect of this phenomenon has been described for populations of enzymatically isolated adult cardiomyocytes which revealed different subcellular patterns of $[Ca^{2+}]_i$ associated with different types of cellular appearance and behaviour [44]. Differences in basal $[Ca^{2+}]_i$ might be reflected in the level of SR Ca^{2+} content and consequently in different Ca^{2+} release patterns and/or subcellular positive feedback [45]. However, in the present study there was no relationship between the baseline $[Ca^{2+}]_i$ level and the degree of change of $[Ca^{2+}]_i$ on single myocyte level (results not shown). Another aspect contributing to the heterogeneity of cellular responses may be the developmental state and changes through differentiation and maturation of cardiomyocytes in culture. The expression of α_1-adrenoceptor subtypes, i.e. α_{1A} and α_{1B}, is suggested to vary with culture time, representing a potential modulatory role for α_1-adrenoceptor subtype-mediated positive chronotropy in neonatal rat cardiomyocytes [46]. Furthermore it is shown that different ionic mechanisms may be responsible for α_1-adrenoceptor activation during the different stages of development, although already after 3 days of culture the receptor has changed from coupling to L-type voltage-gated Ca^{2+} channel to coupling to the PtdIns cycle pathway [47]. The heterogeneity in formation of gap junctions in myocytes in culture might cause heterogeneity in $[Ca^{2+}]_i$ responses as well, depending obviously on cell density but also on intracellular pH and $[Ca^{2+}]$ [48].

Very recently, during the preparation of this manuscript, a report appeared describing noradrenaline-induced Ca^{2+} responses in quiescent single rat neonatal cardiomyocytes in combination with measurements of $InsP_3$ formation although no distinction was made between the IP_3 isomers. Considerable heterogeneity in Ca^{2+} responses among cells was observed in parallel with a large increase of $InsP_3$ [49]. In our previous study we observed no significant increase of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 after PHE stimulation, but if we used ET-1 we found a 4 fold increase of Ins(1,3,4)P_3 after 8 min [16]. Therefore the lack of discrimination between $InsP_3$ isomers makes comparison with that recent study rather difficult [49].

In conclusion, $[Ca^{2+}]_i$ responses induced by the α_1-adrenergic agonist and ET-1 in cardiomyocytes arrested by diltiazem are delayed in time, small and vary among cells. Under the conditions chosen, a dramatic and homogeneous increase of $[Ca^{2+}]_i$ may occur by allowing Ca^{2+}-influx using Na^+-free medium. The results are in agreement with earlier observations of our group which showed no detectable increase of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 after stimulation with α_1-adrenergic agonist or ET-1 in the same cell preparation and suggest that other second messengers such as (1,2)DAG are involved in agonist mediated Ca^{2+} signals.

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Chapter 6

POLYUNSATURATED FATTY ACIDS AND SIGNALLING VIA PHOSPHOLIPASE C-β AND A_2 IN MYOCARDIUM

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ABSTRACT

Dietary n-6 and n-3 polyunsaturated fatty acids (PUFAs) have potent biological effects on the blood(cells), the vasculature and the myocardium. In the epidemiological studies in which the benefit from the regular ingestion of n-3 PUFAs was reported, the responsible mechanisms remain obscure. A great deal of the PUFA-effect can be explained by the known interference with the eicosanoid metabolism. Many processes, believed to be involved in atherogenesis such as adhesion and infiltration of bloodcells (in)to the vasculature, platelet aggregation, secretion of endothelium-derived factors and mitogenic responses of vascular smooth muscle cells are partially mediated by receptor-activated phospholipases C-β and A_2. As PUFAs take part at many steps of the signalling pathways, the latter could represent important action sites to beneficially interfere with atherogenesis. In this brief review, we have discussed the results of studies on the influence of alteration of PUFA composition of the membrane phospholipids or of exogenously administered non-esterified PUFAs on phospholipid signalling. For convenience, we have mainly focused our discussion on those studies available on the myocardium. By changing the PUFA composition of the phospholipids, the endogenous substrates for the membrane-associated phospholipase C-β and A_2 are changed. This is accompanied by changes in their hydrolytic action on these substrates resulting in altered products (the molecular species of 1,2-diacylglycerols and the non-esterified PUFAs) which on their turn evoke changes in events downstream of the signalling cascades: activation of distinct protein kinase C isoenzymes, formation of distinct eicosanoids and non-esterified PUFA effects on Ca^{2+}channels. It has also become more clear that the membrane physicochemical properties, in terms of fluidity and cholesterol content of the bilayer, might undergo changes due to altered PUFA incorporation into the membrane phospholipids. The latter effects could have consequences for the receptor functioning, receptor-GTP-binding protein coupling, GTP-binding protein-phospholipase C-β or A_2 coupling as well. It should be noted that most of these studies have been carried out with cardiomyocytes isolated from hearts of animals on PUFA diet or incubation of cultured cardiomyocytes with non-esterified PUFAs in the presence of albumin. Studies need to be performed to prove that the PUFA-diet induced modulations of the phospholipid signalling reactions do occur *in vivo* and that these effects are involved in the mechanism of beneficial effects of dietary PUFAs on the process of atherosclerosis.

INTRODUCTION

Dietary polyunsaturated fatty acid (PUFA) intake influences the fatty acyl composition of cardiac cell membranes especially by altering the degree of polyunsaturation. The effects of dietary PUFA on serum lipoprotein levels and membrane phospholipid fatty acyl composition and its (patho)physiological and biochemical consequences have extensively been studied in the cardiovascular system, however the mechanisms underlying the beneficial effects of PUFAs, of either the n-6 or n-3 family, are largely unclear. For instance, epidemiologic studies by Bang and colleagues (1976) under Greenland Eskimo's, by Kawaga and colleagues under Japanese fishermen, and by Kromhout and colleagues under Dutch men in the city of Zutphen have supported an inverse correlation between the consumption of fish and the mortality of coronary heart disease [1-4]. The epidemiological evidence has led to

a great number of experimental and clinical studies that revealed beneficial effects on serum lipoprotein levels and anti-aggregatory, anti-inflammatory, anti-hypertensive and anti-atheromatous effects of dietary n-3 PUFA-enriched marine oils, results with which have been dealt in several recent reviews [5-11]. It is known for a long time that different types of eicosanoids are formed from eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) as compared to arachidonic acid (20:4n-6). Eicosanoids have multiple effects related to the regulation of e.g. bloodpressure, capillary permeability, inflammatory reactions and platelet function. Therefore, the general finding that eicosanoids derived from n-3 fatty acids are less potent agonists than the corresponding compounds derived from 20:4n-6 provides partially explanations for the mechanisms of the afore mentioned effects of n-3 dietary PUFAs on the pathophysiology of atherosclerosis [5-11].

The intracellular signalling pathway, called the phosphatidylinositol-cycle (PtdIns-cycle), involves the receptor-mediated activation of GTP-binding protein- (G-protein) associated phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2)-specific phospholipase C-β (PLC-β) and tyrosine phosphorylation-regulated PLC-γ. Activation of PLC-β and PLC-γ results in the formation of the putative Ca^{2+} releasing compound inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and the activator of the protein kinase C (PKC) isoenzymes, 1,2-diacylglycerol (1,2-DAG) [12,13]. The signalling pathway of the PtdIns-cycle links various stimuli such as hormone- and growth factor- and mechanical stretch to their final responses at the transcriptional level through formation of the PKC and Ca^{2+}-calmodulin-dependent PKC activators: 1,2-DAG, Ca^{2+}, and Ca^{2+}-calmodulin. Many processes, believed to be involved in atherogenesis, such as expression of leucocyte adhesion molecules, adhesion and infiltration of blood cells to the vasculature, platelet aggregation [14], mechanical stress effects on the endothelium [15], secretion of endothelium-derived factors [16] and mitogenic responses of vascular smooth muscle cells [5,17] are partially mediated by activation of the PtdIns-cycle. This signalling mechanism may provide an additional target for n-3 fatty acids to interfere with the atherosclerotic process. The PtdIns-cycle activity could be affected by the modification of the fatty acyl composition of the membrane phospholipids at several steps of the signalling cascade. As examples, the incorporation of n-3 and n-6 PUFAs in the membrane phospholipids has been shown to inhibit the α_1-adrenergic receptor mediated production of total inositolphosphates ($InsP_n$) in rat cardiomyocytes [18], to partially block thrombin stimulation of platelets in rabbits [19], to affect α_1- and β-adrenoceptor binding characteristics in heart [20], to influence the regulatory characteristics of G-protein activating protein in neoplastic cells [21], and to modulate the PKC activity in lymphocytes [22].

Many of the biological effects of n-3 and n-6 PUFAs are ascribed to their known effects on the eicosanoid metabolism and the substrates for cyclooxygenase and lipoxygenase, 20:4n-6, 20:5n-3 and 22:6n-3, are believed to be produced by stimulation of the receptor-coupled PLA_2. Less attention, however, has been given to the possible effects of alterations in membrane phospholipid fatty acyl composition by dietary PUFA on the PLA_2 signalling pathway. Not only the eicosanoids formed from 20:4n-6, 20:5n-3 and 22:6n-3 by cyclooxygenase and lipoxygenase action are different, but also the activity of the PLA_2, producing the PUFA substrates, could be influenced by the environmental core-phospholipids or fluidity of the membrane and the molecular species of the phospholipid substrate. Other factors are the competition that occurs between the n-3 and n-6 PUFAs at the active site of cyclooxygenase and lipoxygenase and that n-3 as compared to n-6 PUFAs are rather poor

Table 1. Metabolic pathways for desaturation and elongation of n-3 and n-6 PUFAs

N-6 family	enzymes	N-3 family
18:2		18:3
↓	⇐ Δ6-desaturase ⇒	↓
18:3		18:4
↓	⇐ elongase ⇒	↓
20:3		20:4
↓	⇐ Δ5-desaturase ⇒	↓
20:4		20:5
↓	⇐ elongase ⇒	↓
22:4		22:5
↓	⇐ Δ4-desaturase ⇒	↓
22:5		22:6

Mammalian cells cannot transform n-3 and n-6 PUFAs into one another, but n-3 and n-6 PUFAs share common enzymes for some of their metabolic pathways. N-3 PUFAs usually have higher affinity for the elongation and desaturation enzymes than do n-6 PUFAs [7].

substrates for the eicosanoid forming key-enzymes [5,7].

Dietary n-6 and n-3 PUFAs are also metabolically competitive. As shown in Table 1, mammalian cells cannot transform the essential n-3 and n-6 fatty acids into one another, although n-3 and n-6 PUFA metabolism share common enzymes for some of their metabolic pathways (Table 1). N-3 PUFAs usually have higher affinity than the n-6 PUFAs for the elongation and desaturation enzymes [7]. The Δ-6 desaturase is often the rate-limiting enzyme in the elongation and desaturation process. In addition to mixing with the large pool of tissue derived non-esterified PUFAs, their subsequent desaturation and elongation, incorporation into the membrane phospholipids, conversion into eicosanoids, oxidative breakdown, storage in tissue triglyceride and incorporation into circulating lipoproteins are all subjected to competition between members of the two PUFA families. The enzymes that regulate the degree of unsaturation of membrane phospholipids such as the Δ6-, Δ5- and Δ4-desaturases (Table 1), are the principal regulatory enzymes in the further desaturation of the essential n-6 and n-3 PUFAs. The expression and so the activities of these enzymes are modified by the content of PUFAs in the diet [23].

The consequences of changing the fatty acyl composition in terms of membrane lipid dynamics and protein- or cellular function await further definition. In view of reported beneficial effects of dietary PUFAs on atherogenesis, thrombosis, vascular reactivity and myocardial ischemia, more insight is needed into the effects of PUFAs on the specific cellular functions in the cardiovascular system. Therefore, in this brief review we have focused the discussion onto the reported effects of increased PUFA incorporation into the cardiac membrane phospholipids by dietary means or otherwise, to the consequences for the trans-

membrane signalling by PLC-β and PLA_2. In addition, attention is given to the possible effects of PUFA on membrane protein dynamics and bilayer function. The multiple sites at which PUFAs could interfere with PLA_2, C-β and D signalling ultimately leading to an altered biological response are schematically presented in Figure 1.

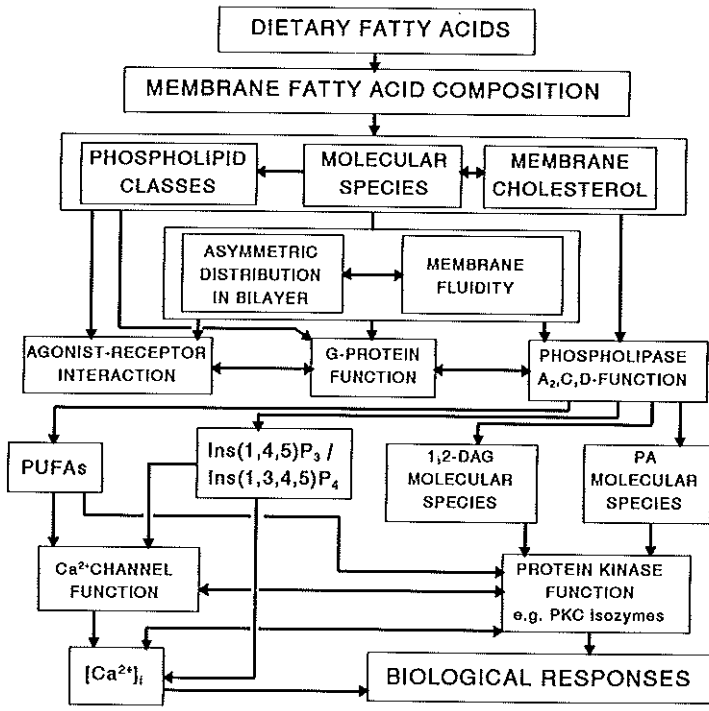

Figure 1 Schematic presentation of the transmembrane signalling via phospholipase A_2, C and D, interactions and possible action sites of the modification in the fatty acid profile of the membrane phospholipids, ultimately affecting the biological responses in cardiomyocytes. See the text for explanation.

INCORPORATION OF (DIETARY) PUFA INTO HEART PHOSPHOLIPIDS

The natural mode of modifying the membrane phospholipids is by altering the n-6 and n-3 PUFA content of the diet. Changes of the n-6 and n-3 PUFA composition of the membrane phospholipids reflect the composition of the diet, although not strictly [24-27]. The alliance is distorted by metabolic effects like the competition between n-6 and n-3 PUFA for desaturation and elongation (Table 1). The regulation of the fatty acyl group composition of membranes is mediated by compartmentalized enzymes. In the endoplasmic reticulum the novo synthesis of the n-9 PUFAs takes place, while in the sarcoplasmic reticulum, mitochondria, and sarcolemma the fine adjustments of PUFA content of membrane phospholipids occur via rapid deacylation-reacylation processes. By enzymatic desaturation of n-3, n-6 and n-9 PUFAs and their mixing with the cellular pool of non-esterified fatty acids

the overall polyunsaturation degree of the membrane bilayer is adapted to maintain its optimal motional characteristics and to meet the environmental requirements of the embedded proteins. Furthermore, the biosynthetic processes such as the acylation of phospholipids, desaturation and elongation of PUFAs are subjected to competition between n-6 and n-3 PUFAs leading to a balanced incorporation into the phospholipid molecules. For instance, when marine oil-enriched diets were taken, an increase in 20:5n-3 and 22:6n-3 acid was seen in the phospholipids of the heart [24,25]. However, when no n-3 PUFAs but 18:2n-6 was present in the diet, apart from an increased incorporation of 18:2n-6, more of docosapentaenoic acid (22:5n-6) is formed by elongation and desaturation of 18:2n-6, resulting in an elevation of its level, revealing a reciprocal correlation between the levels of n-6 and n-3 PUFAs [25].

The fatty acid composition of newly synthesized phospholipids can also be modified by elongases and desaturases at their site of action. This is suggesting that these enzymes, responsible for the fine adjustments, play an important role in the adaptation of membrane fatty acyl group composition to environmental influences such as changes in the lipid profile of the diet or the culture medium of cardiomyocytes [25]. Although the overall contents of the different phospholipid classes are mostly unchanged, a dynamic distribution of the different molecular species of the phospholipids over the lipid bilayer is demanded by the enzymatic requirement of the substrates, because the corresponding membrane-bound- or cytosol-localized enzymes should be able to reach the appropriate bilayer leaflet for membrane function [18,25]. While the composition and orientation of phospholipid molecular species including their fatty acyl composition likely influence the process of phospholipid signalling (receptor-G-protein-phospholipase coupling), it is the latter process that on its turn may have consequences for changes in the molecular species and distribution of the phospholipid classes over the lipid bilayer [18]. Moreover, e.g. the rate of conversion of phosphatidylethanolamine into phosphatidylcholine (by phospholipid methylation) and PtdIns into PtdIns(4,5)P_2 (by PtdIns kinases) may be indirectly stimulated by α_1- and β-adrenergic receptor stimulation of the methylating or phosphorylating membrane-associated enzymes.

As said before, phospholipids do contain "information" in addition to their important structural role in membrane function. The "information" stored in phospholipid molecules can be released mediated by receptor-G-protein- and receptor-non-G-protein-coupled activation of phospholipases A_2, C and D. Therefore, the hydrolysis of phospholipids is a major event in transmembrane signalling. Thus the signaltransduction by phospholipases, the fatty acyl composition of membrane phospholipids and phospholipid class distribution could be mutually influencing (Fig. 1).

The influence of different dietary fatty acids on fatty acid composition of myocardial phospholipids has been studied in an variety of experimental models. Many of these studies involve the search for the influence of diets providing amounts of n-6 PUFA (e.g. 18:2n-6 in soybean- or sunflower-oil) and n-3 PUFA (e.g. 20:5n-3 and 22:6n-3 in marine oils) in abundance to the normal situation, and were performed with rats [24-30]. The mode of stimulation of incorporation of the different PUFAs into the cardiac membrane phospholipids varied from feeding the animals with the diet for a period of time and subsequent investigation of biopsy material from the heart [27,30,31] to first feeding the animals, and subsequently collecting their sera to grow primary rat cardiomyocyte cultures on [32] to perfusion or incubation of cardiomyocytes with fatty acid enriched medium [18, 33-39]. When

Table 2. Effects of n-3 and n-6 PUFA treatment on physicochemical characteristics (A) and signalling function (B) of the sarcolemma of neonatal rat cardiomyocytes

	Intact cells		Permeabilized cells	
	n-3 PUFA	n-6 PUFA	n-3 PUFA	n-6 PUFA
A)				
PUFA content of phospholipid	↑	↑	↑	↑
Membrane fluidity	↓	N.S.	-	-
Cholesterol/phospholipid ratio	↑	N.S.	-	-
Double bond index	↑	N.S.	↑	N.S.
Resistance to saponin permeabilization	↑	N.S.	-	-
B)				
PLC-β activity mediated by:				
α_1-adrenoceptor	↓	N.S.	-	-
Endothelin-1 receptor	↑	N.S.	-	-
GTPγS	-	-	↓	↓

N.S., -, ↓, and ↑ stands for a not significant, a not measured, a decreasing and an increasing effect, respectively.
The changes described in this Table are based upon results as described in [39].

studying the intact heart it is impossible to determine the chemical composition of lipids of the cardiomyocytes separately from the other cells, such as endothelial cells, smooth muscle cells or fibroblasts. But, as the fatty acyl composition of isolated or cultured cardiomyocytes appears to be as stable as that of the whole heart [33,40], many investigators have been using the model of primary cultures enriched in cardiomyocytes which are placed in controlled extracellular environments such as superfusion or incubation medium with a long enough viability to allow the effective and rapid fatty acid incorporation that can normally be achieved *in vivo* [18,33-39].

The consequences of the alterations in the phospholipid fatty acid composition in terms of membrane lipid dynamics and protein- or cellular function of the heart membrane is still in the phase of speculation. It is, however, certain that the selectivity of many enzymatic and transport functions of the plasmamembrane requires the optimal lipid environment of each integral and peripheral membrane protein. The rotational and lateral diffusion of the membrane proteins must be determined by the membrane fluidity which is directly dependent on e.g. the physical properties of the phospholipid acyl chains such as the degree of unsaturation and length of the acyl chain. The molecular species and asymmetric distribution of the phospholipids in the membrane bilayer and the molecular species of core-phospholipids of each functional membrane protein as well as the amount of membrane

cholesterol can all potentially be changed by alterations in the lipids in the diet or in the incubation medium of isolated cells. Therefore, it is interesting to note that the cholesterol over phospholipid ratio of neonatal rat ventricular myocytes exposed to 20:5n-3 enriched culture medium for 5 days, increased 1.7 times (Table 2 and ref. 39). In this study the overall PUFA composition of the cellular phospholipids was drastically changed: 23 mole % 20:5n-3 incorporated (versus 0.5 mole % 20:5n-3 in control-treated cells) mainly at the expense of 18:2n-6 and 20:4n-3, which results confirmed our previous observations [18]. Accordingly, the double bond index, defined as the mean number of double bonds per mole of fatty acid, was markedly increased in 20:5n-3 pretreated cardiomyocytes (2.33 versus 1.76 in control-treated cells), whereas in linoleic acid (18:2n-6) pretreated cardiomyocytes only a slight, non-significant, increase was found (Table 2 and ref. 39). Unexpectedly, the membrane fluidity, assessed by measuring the fluorescence steady state anisotropy (r_{ss}) of the plasma membrane specific fluorophore TMA-DPH (1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene, p-toluenesulfonate), decreased 10 % in the 20:5n-3 pretreated cardiomyocytes as compared to control-treated cells (Table 2). Apparently, in ventricular myocytes the increase of membrane cholesterol compensates more than the fluidizing effect of the marked increase in the degree of unsaturation of the fatty acids. It is also interesting to note that we observed an increased resistance of the 20:5n-3 treated cells to permeabilization by saponin (Table 2 and ref. 39). As saponin is known to destabilize the plasma membrane of cells by complexation of cholesterol, the higher stability due to the 20:5n-3 treatment can be ascribed to the observed increase of membrane cholesterol content. In rat smooth muscle cells it was reported that no differences in cholesterol content were found after 12 h of incubation with 20:5n-3- or 22:6n-3-bovine serum albumin complexes while 8 and 9.5 mole %, respectively, of these particular fatty acids were incorporated in the phospholipids, at the expense of the n-6 PUFAs [41]. It should be noticed that in the smooth muscle cells from the control fed rats the relative incorporation of 18:2n-6 and 20:4n-6 was only 5 and 3.4 mole % respectively, in comparison to the 17 and 18 mole %, respectively, that we found in the control treated rat neonatal cardiomyocytes [39]. However, in contrast with our data obtained in rat neonatal cardiomyocytes, the increased incorporation of 20:5n-3 and 22:6n-3 in the smooth muscle cell phospholipids, enhanced the mechanism that regulates the cholesterol homeostasis, that is plasma membrane cholesterol desorption from the cell membrane bilayer. It was hypothesized that the cholesterol efflux from the plasma membrane is determined by the physicochemical membrane properties, that on their turn depend on the membrane phospholipid fatty acid profile [41]. It was suggested that the PUFAs incorporated in the phospholipids altered the transbilayer differences in fluidity reversing the membrane transbilayer cholesterol distribution through specific pools or domains [41].

DIETARY PUFA AND THE SIGNALLING CASCADE OF THE RECEPTOR-MEDIATED PHOSPHOLIPASES

The PLA_2 signalling pathway is usually thought to be influenced by PUFAs mainly at the level of the pattern of eicosanoids formed. It can not be excluded that PUFAs are effective upstream of lipoxygenase and cyclooxygenase: e.g. by alterations of molecular species of the PLA_2 substrates, the core-phospholipids and the membrane fluidity and by competition

Table 3. Studies available dealing with dietary n-3 and n-6 PUFA effects on membrane-bound phospholipase C-β and A_2 of myocardial cells

Model	Phospholipase	Growth medium supplement	Dietary oil	Ref.
Cultured rat ventricular myocytes	PLC-β	22:6n-3	-	[38]
Cultured rat ventricular myocytes	PLC-β	18:2n-6 or 20:5n-3	-	[18]
Isolated rat left atrial cardiomyocytes	PLC-β	-	fish oil	[30]
			safflower oil	
			coconut oil	
			olive oil	
			palm oil	
Cultured rat ventricular myocytes	PLA_2	20:5n-3 or 22:6n-3		[42]
Cultured rat ventricular myocytes	PLA_2	20:5n-3 or 22:6n-3		[34]
Homogenates of Atlantic salmon hearts	PLA_2	-	sunflower oil	[79]
			linseed oil	
			fish oil	

between n-6 and n-3 PUFAs for cyclooxygenase as well as lipoxygenase. By their incorporation into the membrane phospholipids and by mixing with the cellular non-esterified fatty acid pool dietary PUFAs can also potentially affect various steps of the signalling pathway initiated by receptor-mediated PLC-β activation. In Table 3, a list of the few existing studies on dietary PUFA effect on the PLC-β and A_2 activity in myocardium is presented. Although several studies have focused on the effects of PUFA-treatment on the functioning of particularly receptors, G-proteins, PLC-β, PLA_2, second messengers $Ins(1,4,5)P_3$ and 1,2-DAG, and PKC isoenzymes, the specific steps affected and the overall consequences of PUFA-treatment on the signalling cascade has still to be unravelled. A number of these studies are discussed below [18,30,34,38,42].

Receptors

There is some evidence that changes of the fatty acid composition of the phospholipids may alter the agonist-receptor binding characteristics. It is reported, that, when the fatty acid composition of certain phospholipid classes like phosphatidylcholine and phosphatidylethanolamine of myocardium are changed by dietary means or pharmacologically after repeated administration of hydrocortisone and/or epinephrine [20,31], these alterations paralleled decreases in affinity of the α_1-adrenergic receptors and coincided with changes in the number of β-adrenergic binding sites [20]. However, these results should be interpreted with care because the observed effects on α_1-adrenoceptor-binding characteristics are more likely caused by chronic stress-induced receptor desensitization. Next to the differential mechanisms responsible for α_1- and β-adrenoceptor regulation, it was speculated that because adrenergic receptors span the entire thickness of the membrane bilayer a change in membrane phospholipid acylchains could result in an increase in bilayer volume associated with a conformational change in the membrane receptor. In our laboratory the influence of n-3 and n-6 PUFA treatment on the PtdIns-cycle function was studied in intact cultured neonatal rat cardiomyocytes either stimulated by α_1-adrenergic agonist or endothelin-1 [18,39]. The $InsP_n$ production after α_1-adrenergic agonist stimulation of intact cardiomyocytes was inhibited but it was increased after endothelin-1 stimulation (Table 2 and refs. 18 and 39). To circumvent agonist-receptor-interaction, in separate experiments, saponin-permeabilized PUFA treated cardiomyocytes were stimulated with GTPγS. Direct stimulation of the G-protein-coupled PLC-β with GTPγS in permeabilized cells was reduced in n-3 PUFA treated cells as compared to control treated cells. These results indicate that at least the n-3 PUFA treatment-induced modifications of the phospholipids in the sarcolemma affects transmembrane signalling by PLC-β at the level of the agonist-receptor interaction.

GTP-binding-proteins

The next in line of the cascade of cell signalling is the function of heterotrimeric G-proteins and the low-molecular-mass G-proteins or the Ras-related superfamily of G-proteins [43]. The heterotrimeric G-proteins consist of a GTP-binding α-subunit and a regulatory $\beta\gamma$ heterodimer. They are localized to the inner surface of the plasma membrane and are, in general, coupled to members of the transmembrane receptors that share the seven transmembrane spanning topological structure. Low-molecular-mass G-proteins can hydrolyse GTP to GDP. The inactive GDP-bound protein is conformationally activated to obtain the GTP-bound state, which is regulated by guanine nucleotide exchange factors. The intrinsic GTPase activity of

the low-molecular-mass G-proteins can in turn be regulated by GTPase-activating proteins. The modifications of G-proteins have been shown to be related to their association with cell membranes, either through direct interaction with the lipid bilayer or by facilitating the interaction with other proteins such as receptor proteins, specific interacting accessory $\beta\gamma$ complexes, exchange factors or guanine nucleotide dissociation factors [43]. For example, besides the covalent modification of the G-proteins by isoprenylation, carboxyl methylation, palmitoylation and myristoylation, one could suggest that the membrane phospholipid fatty acid composition, imposing the membrane fluidity and phospholipid core, determines the anchoring and or mobility of the different G-protein subunits: the α-monomer and the $\beta\gamma$-heterodimer. However, at present there are only a few myocardial studies available that report specifically on the effects of dietary PUFAs on G-protein functioning. Evidence has been obtained in polymorphonuclear leucocytes that 20:4n-6 directly modulates the G-protein-mediated signals [44]. Another study on atrial cells from the bullfrog showed that 20:4n-6 modulates the G_k-protein-mediated inwardly rectifying K^+ current via its products formed by cyclooxygenase and lipoxygenase [45]. In the latter study, blockade of the cyclooxygenase resulted in enhancement of the rate of G_k activation hence mediated by the 20:4n-6 metabolites formed by lipoxygenase, while blockade of the lipoxygenase pathway resulted in the opposite effect on G_k activation [45]. It was reported that in adipocytes the G_i-protein, that couples inhibitory receptors to adenylate cyclase, was effected specifically by free 20:5n-3, resulting in the inhibition of adenylate cyclase activity, while neither 22:6n-3 nor 20:4n-6 did so. This conclusion was drawn based upon the observations that the inhibitory effect of 20:5n-3 on adenylate cyclase activity was GTP-dependent and non-competitive with β-adrenergic agonist isoprenaline. The inhibitory effect of 20:5n-3 on G_i was eliminated following by treatment of either intact adipocytes or isolated adipocyte plasma membranes with pertussis toxin, which is known to induce ADP-ribosylation of the G_i and thereby to inactivate G_i activity [21].

Phospholipase C-β

Several non-myocardial studies support that dietary PUFA affects the receptor-mediated activation of PLC-β. It is likely that these effects of dietary PUFA contribute to the mechanism responsible for the vasodilatory, anti-thrombotic, anti-inflammatory and anti-atheromatous effects of dietary PUFAs. For example, it was shown that thrombin-stimulated $InsP_n$ production of platelets was reduced in rabbits fed with a 20:5n-3 enriched diet for five weeks, whereas the thromboxane (TXA) production was not affected [19]. A n-3 PUFA rich diet suppressed low density lipoprotein (LDL) or angiotensine II induced $Ins(1,4,5)P_3$ production in smooth muscle cells from rat aorta [46,47]. The suppression of LDL mediated $Ins(1,4,5)P_3$ production after n-3 PUFA rich diet was shown to be partly due to restrained TXA_2 formation. However, in the angiotensine II-stimulated cells the diminished $Ins(1,4,5)P_3$ formation could not be restored by TXA_2 receptor-antagonists or inhibitors of the TXA_2 synthesis. The latter would demonstrate that the dietary PUFAs do exert their effect on PLC-β not by modifying PLA_2 or cyclooxygenase activity.

It is unclear whether the dietary PUFA-induced activation of phospholipid composition has its effect at the level of agonist-receptor interaction, receptor-G-protein- or G-protein-PLC-β coupling. We found that the α_1-adrenergically stimulated PLC-β in neonatal rat ventricular myocytes was reduced in n-3 PUFA pretreated cells while the endothelin-1 stimulated PLC-β

was enhanced (Table 2) [18,39]. More recently, it was also found in left atrial myocytes isolated from rats fed n-3 and n-6 PUFA enriched oils for eight weeks, the total release of $InsP_n$ was reduced in the presence or absence of norepinephrine [30].

Phospholipase A_2

There is also much evidence that the receptor-mediated PLA_2 is involved in the mechanisms that contributes in vasodilatory, anti-thrombotic, anti-inflammatory and anti-atheromatous effects of dietary PUFAs by alteration of the available substrate profile for the cyclooxygenase and the lipoxygenase. PLA_2 catalyses the hydrolysis of unsaturated fatty acids esterified at the *sn-2* position of the membrane phospholipids. Subsequently these PUFAs formed serve as a precursor for the eicosanoid pathway [48]. The eicosanoids produced from 20:4n-6 and 20:5n-3 are different compounds (Fig. 2). In general, the eicosanoids formed with 20:5n-3 as a precursor, are poor agonists in comparison to their 20:4n-6 counterparts. Moreover, 20:4n-6 and 20:5n-3 will compete for the cyclooxygenase and lipoxygenase (Fig. 2). These effects of dietary PUFA downstream of PLA_2 do not exclude dietary PUFA effects exerted on PLA_2 itself. Although the mechanism of activation of the PLA_2 isotype involved is largely unclear,

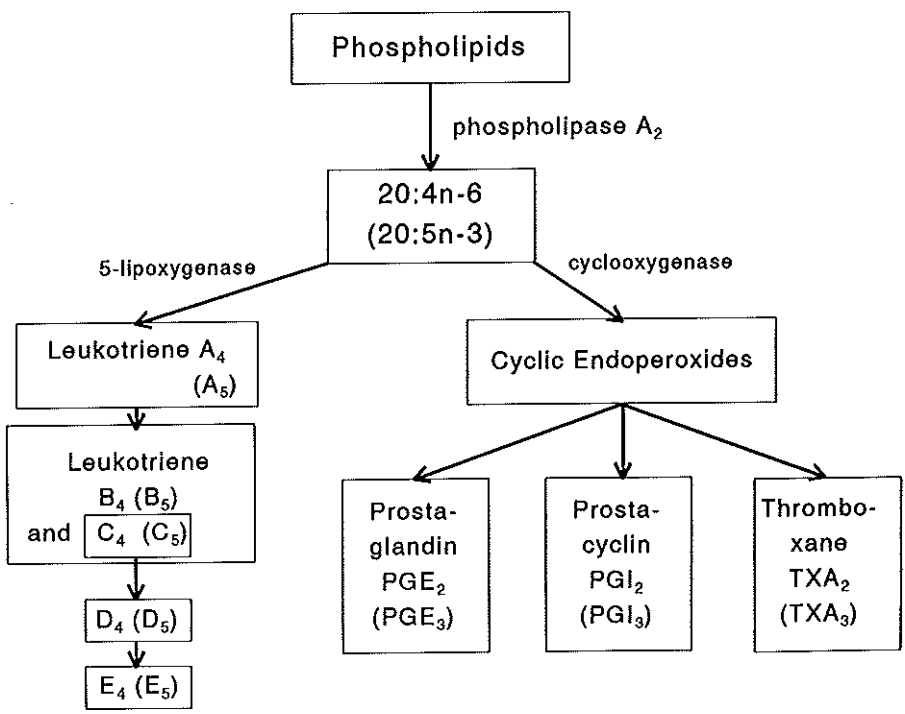

Figure 2 Schematic presentation of the involvement of phospholipase A_2 in the production of eicosanoids from 20:4n-6 and 20:5n-3. Eicosanoids formed with 20:5n-3 as a precursor are given in parentheses. Depending on the precursor molecule, the eicosanoids will contain different numbers of double bonds and display different biological activity [7]. In general, the 20:5n-3 derived eicosanoids are weaker agonists in comparison to the analogous compounds derived from 20:4n-6.

it was recently reported that the mechanical stretch induced release of 20:4n-6 in myocardial cells is mediated by an angiotensin II-stimulated PLA_2 that is G-protein-coupled [45,49]. Presently, little information is available on the characteristics of myocardial phospholipase(s) of the reactive type A_2. Up till now, several low-molecular weight PLA_2 forms were found, e.g. a membrane-bound enzyme in cultivated cardiomyocytes [50], and a Ca^{2+}-dependent enzyme in human brain [51]. Recently, in a human monocytic cell line a high-molecular weight PLA_2 was also found, which is referred to as a cytosolic PLA_2 and having properties that meet the characteristics for a regulatory role in the generation of 20:4n-6 derived eicosanoids [52]. Upon activation the cytosolic PLA_2 may translocate to the membrane. The activity of the membrane-bound PLA_2 is regulated by free Ca^{2+} concentration [48], but Ca^{2+}-independent membrane-bound PLA_2 forms have been found as well [43].

Poor attention has been given to the possibility that the modification of the membrane phospholipid fatty acid composition modulates the membrane-bound PLA_2 activity. A study on the catalytic activity of pancreatic PLA showed that small changes in the interfacial lipid architecture, such as unsaturation of fatty acids or lipid composition, modulates the intensity of the hydrophobic interactions at the active site of the enzyme, and hence regulates the over-all catalytic potency of the enzyme [54,55]. In n-6 PUFA treated ventricular myocytes the activity of alkaline PLA, which is mostly on account of PLA_2 activity, was found to be 34 % higher than the activity in n-3 PUFA treated cells. In these studies it was concluded that the increase of PLA (the sum of A_1 and A_2) activity of n-6 PUFA treated cells was caused by alteration of the fatty acid composition of the membrane phospholipids [42]. That alteration of the fatty acid incorporation into the membrane phospholipids may influence the PLA-activity was also demonstrated by the observation that PLA (the sum of A_1 and A_2) activity was higher in 22:6n-3- than in 20:5n-3-treated cells [34].

PLC-β and PLA_2 -derived (second) messengers

Upon PLC-*β* catalysed hydrolysis of PtdIns(4,5)P_2 two messengers are formed: Ins(1,4,5)P_3, which is generally known to release Ca^{2+} from intracellular stores [13] and 1,2-DAG which is known to activate members of the PKC family, some of which are also dependent on Ca^{2+} [56,57].

In addition to the overall suppression of the rat left atrial PtdIns-cycle activity, caused by dietary n-3 or n-6 PUFA, Woodcock and colleagues [30] showed qualitative differences in the production profiles of the inositolphosphate isomers. In the left atria of n-6 compared to n-3 PUFA fed rats, more accumulation of the putative Ca^{2+} releasing component Ins(1,4,5)P_3 was found [30]. In another more recent study of this group it was reported that feeding rats with n-3 PUFA the rise of the reperfusion induced Ins(1,4,5)P_3 level in perfused hearts was inhibited, while under normoxic conditions basal- or norepinephrine stimulated total accumulation of $InsP_n$ were unaffected [58]. It was hypothesized that Ins(1,4,5)P_3 induced Ca^{2+} release may predispose the heart tissue to increased resting diastolic and/or systolic Ca^{2+} levels [58], although from other work it appeared that the heart may be relatively insensitive to Ins(1,4,5)P_3 in terms of Ca^{2+} release [59].

Diacylglycerol must have the 1,2-*sn*-configuration to act as activator of PKC and the various fatty acyl moieties are determining the kinetic characteristics (K_a and maximal activation) of the activation of PKC [60]. This implies that the molecular species of 1,2-DAG

may be of importance for its potency to activate PKC. As in other tissues, in myocardium the *sn*-2 position of the PtdIns is mainly occupied by 20:4n-6, which is thought to play an significant role in signal transduction processes [61] as for the latter can be referred to the examples mentioned in this paper: the influence of 20:4n-6 on G-protein function or activation of PKC-isozymes [44]. It is, however, important to mention that we demonstrated that the generally assumed 1-stearoyl, 2-arachidonyl-glycerol structure of PtdIns(4,5)P_2 appears not to be characteristic for rat and pig myocardium [62]. The agonist induced release of 20:4n-6 from the *sn*-2 position of phospholipids can proceed via activation of PLA_2 or via the activation of PLC-β, PLD and the subsequent action of DAG-lipase. Apart from its origin, 20:4n-6 formation is physiologically relevant, since it the main precursor of the biologically active eicosanoids [63]. Therefore, influencing all the pathways involving 20:4n-6 metabolites by changing the membrane phospholipid fatty acid composition must have certain consequences. For instance, in cultured ventricular myocytes treated with 22:6n-3, a more persistent activation of membrane-bound PKC was found after α_1-adrenergic stimulation [38]. It was suggested that this persistent activation is the result of longlasting action of 22:6n-3-containing 1,2-DAG being a relatively poor substrate for DAG-kinase. Therefore molecular species of 1,2-DAG may be of importance for its metabolic fate, that is either phosphorylation to phosphatidic acid by DAG-kinase or hydrolysis by DAG-lipase.

In many tissues, but especially in the heart, Ca^{2+} is playing a very important role in modulation of metabolic routes, muscle mobility and a multiplicity of membrane-linked processes [64,65]. Activation of the PtdIns-cycle in myocardium is leading to increases of intracellular Ca^{2+} ($[Ca^{2+}]_i$) but on its turn the activity of PLC-β is modulated by $[Ca^{2+}]_i$ [66]. Therefore the modulation of $[Ca^{2+}]_i$ by fatty acids can be of importance for efficient feedforward activity of PLC-β. It has been reported that the protection against the harmful effects of ouabain on neonatal rat cardiac myocytes by 20:5n-3 was associated with the prevention of increases of $[Ca^{2+}]_i$ to toxic levels [67]. The same group subsequently showed that in 20:5n-3- or 22:6n-3-treated myocytes the steady state Ca^{2+} influx following exposure to ouabain was reduced [37]. The authors do not ascribe these effects on ouabain-induced Ca^{2+} influx to the physiological changes into the cell membrane following the incorporation of n-3 fatty acids in the phospholipids, but to direct effects of non-esterified n-3 PUFA on Ca^{2+} channels. Because similar responses were obtained using cells aft2er a 3-5 day fatty acid incubation or acute addition to the bathing medium of the fatty acids, it was concluded, that this effect could be exerted directly or indirectly via the metabolites of the fatty acids. Furthermore, the $[Ca^{2+}]_i$-increasing effect by the L-type Ca^{2+} channel agonist BAY K 8644 was abolished after simultaneous exposure of cardiomyocytes to 22:6n-3. It was suggested that the action of 22:6n-3 on the dihydropyridine L-type Ca^{2+} channel was highly specifically directed at the dihydropyridine binding site of the Ca^{2+} channel, possibly as a consequence of the intrinsic structural conformation partially determined by the degree of unsaturation of the fatty acid incorporation in the core-phospholipids [37]. Additionally, the specific protein-lipid or lipid-lipid alliance between L-type Ca^{2+} channels in cardiac membranes had possibly been altered [68]. In contrast to the foregoing, direct effects of long chain fatty acids on myocardial $[Ca^{2+}]_i$ has been found in pathologic reactions evoked by myocardial ischemia [69]. The disturbance of the membrane function, followed by irreversible cardiac damage is postulated as a consequence of increase of the tissue levels of non-esterified fatty acids due to ischemia-induced blockade of β-oxidation, leading to increased Ca^{2+} permeability of the

sarcolemma [70] and to increased voltage dependent Ca^{2+} currents, I_{Ca} [69]. These effects could not be ascribed to the activation of PKC because inhibition of this kinase with staurosporin did not prevent the increase in I_{Ca} [69]. Direct effects of n-3 PUFAs on Ca^{2+} currents were also suggested in normoxic neonatal cardiac myocytes, for β-adrenergic mediated arrhythmias were prevented by addition of 20:5n-3, but revived when the PUFA was trapped by fatty acid-free bovine serum albumin [36]. It is not surprising to see that dietary n-3 PUFAs can also affect the function of intracellular membranes like the sarcoplasmic reticulum either due to changes in the phospholipid fatty acyl chains. Feeding rats with n-3 PUFA rich menhaden oil resulted in an increase of the n-3 to n-6 PUFA ratio in the total phospholipid formation of ventricular myocytes. $[Ca^{2+}]_i$ would be affected as well because a 30 % decrease in oxalate-facilitated ATP dependent Ca^{2+} uptake and a concomitantly impaired Ca^{2+}-ATPase activity was found [71]. Therefore, changes in fatty acid composition of the membrane can influence Ca^{2+} signalling either by influencing receptor-mediated transmembrane signalling processes or by influencing Ca^{2+} pumps and carriers that are initially involved in the excitation-contraction coupling.

Protein kinase C isoenzymes

Activation of PKC-isoenzymes by non-esterified fatty acids is generally found only to be exerted by the cis-unsaturated ones. Saturated and *trans*-unsaturated fatty acids were found to be inactive [44]. It is found that depending on the PKC-isoenzyme, the activation by unsaturated fatty acids is independent of 1,2-DAG and phosphatidylserine (PKC-α, β, γ and ζ), or Ca^{2+} (PKC-γ and ϵ) or the activation was synergistically enhanced by Ca^{2+} (PKC-α and β). Also, differences in potencies of stimulation of PKC by the various fatty acid types have been observed. While in the absence of other lipid activators PKC was activated by non-esterified 18:2n-6 and 20:4n-6, in the presence of phosphatidylserine- and 1,2-DAG PKC activity was inhibited by these PUFAs [44,72,73]. Moreover, 22:6n-3 did not activate PKC in the absence of phosphatidylserine and 1,2-DAG but did strongly inhibit the activity in the presence of these lipid-activators [44]. In myocardium the PKC isoenzymes are expressed in an age dependent manner [74]. For example, in neonatal ventricular myocytes the PKC isoforms α, δ, ϵ and ζ are present while only the Ca^{2+} insensitive isoforms PKC-δ and ϵ are present in isolated adult ventricular myocytes. It is hypothesized that the individual isoforms of PKC become active in a distinct manner to transduce signals from the cell surface to the responsive elements of the nuclear DNA [74]. The responses of different isoforms might be modulated by their distinct affinities for various types of non-esterified fatty acids but also for various molecular species of 1,2-DAG. In many cell types, including neutrophils, hepatocytes and fibroblasts, the 1,2-DAG response after exposure to a stimulus is frequently biphasic. As 1,2-DAG often primarily originates from $PtdIns(4,5)P_2$ hydrolysis catalysed by PLC-β, and later by (PKC-mediated) activation of PLD [75], the overall molecular species composition of 1,2-DAG, and hence the PKC-substrate, could be crucial for the sequential distinct activation and action of PKC-isoenzymes. Moreover, it is becoming evident that PLC-β and PLD usually act in concert on the maintenance of increased 1,2-DAG levels to activate PKC isoenzymes [75]. This pattern of PLC-β and PLD activation has also been found in myocardium by us [76,77]. Many studies have shown the important role of non-esterified PUFAs, incorporated PUFAs in 1,2-DAG in activating PKC, although hardly any study was performed on heart. PKC is an enzyme which is believed to be involved in a variety of cellular responses such as

hypertrophic and proliferative growth and changes in gene expression [73]. Indirect evidence for the modulation of PKC-activity by non-esterified PUFAs was found in cardiac myocytes by studying purinergically induced Ca^{2+} responses. Transient ATP-stimulated elevations in $[Ca^{2+}]_i$ was found to be higher after incubation with non-esterified 20:4n-6 and 18:2n-6 as well as 22:6n-3. The PUFA-mediated increases of ATP-induced $[Ca^{2+}]_i$ transients were significantly reduced by the addition of PKC-inhibitors staurosporin or sphingosine [63]. Purinergic receptors are coupled to PLC-β [78], and therefore it is likely that the PUFA effect on Ca^{2+} responses is mediated by PUFA-action on 1,2-DAG activated PKC.

CONCLUDING REMARKS

Although from epidemiological observations there is a significant amount of evidence suggesting that PUFAs, particular of the n-3 type, are important in preventing the development of atherosclerosis, inflammatory, hypertensive, ischemic and thrombolytic complications, the mechanism underlying these possible beneficial effects at the cellular level are still poorly understood. A great deal of PUFA effects can be explained by their known interference with the eicosanoid metabolism. As PUFAs take part in many steps of the receptor-mediated phospholipid signalling path via PLA_2 and PLC-β, the latter are another possible action site for PUFAs to interfere with the afore-mentioned pathological responses. In trying to elucidate the part played by the phospholipid signalling several studies have been devoted to the effects of dietary PUFAs on the mechanism of phospholipid signalling of the cardiomyocyte. Indeed, changes in incorporation of PUFAs in the plasmamembrane phospholipids of cardiomyocytes have been shown to affect receptor-mediated PLA_2 and PLC-β signalling on many steps of the way. By changing the fatty acyl composition of the phospholipids, the endogenous substrates for the membrane associated PLC-β and A_2 are changed, and subsequently also their products which results in altered events downstream of these signalling enzymes: activation of distinct PKC isoenzymes, regulation by non-esterified PUFAs of Ca^{2+} channels, and changed rate of the formation of distinct eicosanoids. Additionally, it has become clear that the membrane dynamic characteristics in terms of membrane fluidity and cholesterol concentration can be modified upon altered n-3 and n-6 PUFA incorporation in the phospholipids. This could likely have consequences for the receptor function, G-proteins and the activity of the signal transducing phospholipases as well. It should be noticed that most of these studies have been carried out with cardiomyocytes isolated from hearts of animals on PUFA diet or incubation of cultured cardiomyocytes with non-esterified PUFAs in the presence of albumin. *In vivo* studies need to performed to prove that the PUFA diet-induced modulation of the phospholipid signalling reactions do occur and are indeed involved in the beneficial effects of dietary PUFAs on atherogenesis.

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EICOSAPENTAENOIC ACID INCORPORATION IN MEMBRANE PHOSPHOLIPIDS MODULATES RECEPTOR-MEDIATED PHOSPHOLIPASE C AND MEMBRANE FLUIDITY IN RAT VENTRICULAR MYOCYTES IN CULTURE

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ABSTRACT

The influence of increased incorporation of 18:2n-6 and 20:5n-3 in membrane phospholipids on receptor-mediated phospholipase C-β activity in cultured rat ventricular myocytes was investigated. For this purpose cells were grown for 4 days in control, 18:0/18:1n-9, 18:2n-6 and 20:5n-3 enriched media and subsequently assayed for the basal and phenylephrine or endothelin-1 induced total inositol phosphate formation. The various fatty acid treatments resulted in the expected alterations of fatty acid composition of membrane phospholipids. In 18:2n-6 treated cells the incorporation of this 18:2n-6 in the phospholipids increased from 17.1 mole % in control cells to 38.9 mole %. In 20:5n-3 treated cells, incorporation of 20:5n-3 and 22:5n-3 in the phospholipids increased from 0.5 and 2.7 mole % in control cells to 23.2 and 9.7 mole %, respectively. When 20:5n-3 treated cells were stimulated with phenylephrine or endothelin-1, the inositolphosphate production decreased by 33.2 % and increased by 43.4 %, respectively, as compared to cells grown in control medium. No effects were seen in 18:2n-6 treated cells. When 18:0/18:1n-9 treated cells were stimulated with endothelin-1, inositolphosphate formation increased by 26.4 %, whereas phenylephrine stimulated inositolphosphate formation was not affected. In saponin-permeabilized cells, that were pretreated with 20:5n-3, the formation of total inositolphosphates after stimulation with GTPγS, in the presence of Ca^{2+}, was inhibited 19.3 %. This is suggesting that the 20:5n-3 effect on intact cardiomyocytes could be exerted either on the level of agonist-receptor, receptor-GTP-binding-protein coupling or GTP-binding-protein-PLC-β interaction. Investigation of the time-course of saponin-induced permeabilization of the cardiomyocytes, measured by the release of lactate dehydrogenase, unmasked a slight decrease in the rate of permeabilization by 20:5n-3 pretreatment indicating a protective effect. This led us to measure the cholesterol/phospholipid molar ratio, the double bond index of membrane phospholipids, and the membrane fluidity, the latter by using a diphenylhexatriene probe. In 20:5n-3 pretreated cells we observed a strong increase in the cholesterol/phospholipid molar ratio (from 0.23 to 0.39), a marked increase in the double bond index (from 1.76 to 2.33), and a slight decrease in fluidity (rotational mobility r_{ss} of the diphenylhexatriene probe increased from 0.196 to 0.217). Thus, treatment of cardiomyocytes for 4 days with 20:5n-3, but not with 18:2n-6, causes alterations of receptor-mediated phospholipase C-β activity. A causal relationship may exist between the 20:5n-3 induced alterations of the physicochemical properties in the bilayer and of the agonist-stimulated phosphatidylinositol-cycle activity.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) have structural roles in phospholipids of all cell membranes in the body, influencing membrane viscosity and permeability and, associated herewith, the function of membrane proteins. Moreover, different types of eicosanoids are formed from the fatty acids arachidonic acid (20:4n-6) and eicosapentaenoic acid (20:5n-3). In general, eicosanoids derived from 20:5n-3 are weak agonists compared to the corresponding compounds derived from arachidonic acid (20:4n-6) (Sassen *et al.*, 1994, Leaf, 1994; Harris, 1989). PUFAs also have important functions in modulating triglyceride and cholesterol metabolism. Epidemiological studies have reported favourable effects in

prevention of cardiovascular disease by the regular ingestion of fish, containing n-3 PUFAs, although the responsible mechanism remains obscure (Bang, *et al.*, 1976; Kagawa *et al.*, 1982; Kromhout *et al.*, 1985; Lamers *et al.*, 1987; Leaf, 1994). Nevertheless, many experimental studies have provided evidence that n-3 PUFAs have diverse and often potent actions on e.g. platelet-, endothelial cell- and vascular smooth muscle function, which may explain many of their salutary effects on cardiovascular diseases (Goodnight, 1991).

The phosphatidylinositol- (PtdIns-) cycle is a signalling pathway involved in receptor-mediated biological responses such as platelet aggregation, smooth muscle contraction, and formation of relaxing and contracting factors by endothelial cells. A common mechanism of n-3 and n-6 PUFAs in these biological responses may be by interference with the functioning of the PtdIns cycle. Previously, we reported that cultured cardiomyocytes, which were pretreated with 18:2n-6 or 20:5n-3 showed less phospholipase C-β activity in response to α_1-adrenoceptor stimulation (Lamers *et al.*, 1992). Recently, this was confirmed by Woodcock *et al.* (1995), who showed that feeding rats with n-3- and n-6 PUFAs caused a depression of total release of inositol phosphates ($InsP_n$) in left atrial tissue in the presence or absence of norepinephrine. In accordance, Reibel *et al.* (1988) demonstrated that n-6 and n-3 PUFAs attenuated the α_1-adrenergic-mediated positive inotropy in perfused rat hearts. Furthermore, it was shown by Kang and Leaf (1995) that dietary n-6 or n-3 PUFAs could prevent and terminate isoproterenol-induced arrhythmias.

Modification of the fatty acyl composition of the membrane phospholipids can influence many steps of the PtdIns signalling cascade. It has been shown, for example, that incorporation of n-3 and n-6 PUFAs in the membrane phospholipids was associated with a decreased affinity of the α_1-adrenoceptors for their antagonist ligand [^{3}H]prazosin in heart muscle (Skúladóttir *et al.*, 1993). In isolated atrial cells, G_k-protein-kinetics involved in the stimulation of inwardly rectifying K^+ current by muscarinic receptor agonist were demonstrated to be modulated by 20:4n-6-derived metabolites (Scherer and Breitwieser, 1990). Furthermore, the function of protein kinase C (PKC)-isozymes may be affected by either free PUFAs or by alteration of the molecular species of 1,2-diacylglycerol ((1,2)DAG) due to changed fatty acid composition of the phospholipid source (Graber *et al.*, 1994; Bordoni *et al.*, 1992).

Obviously, we need to learn more about the effects of incorporation of n-3 and n-6 PUFA in membrane phospholipids on structure and function of the sarcolemma, in terms of the various steps of the transmembrane signalling by the PtdIns cycle, and the physicochemical properties such as fluidity of the bilayer, cholesterol content and resistance to permeabilizing agents. In the present study we used primary cultures of spontaneously beating, neonatal rat ventricular myocytes to investigate the influence of addition of n-6 and n-3 PUFAs to the culture medium on their spontaneous contractile activity and on the receptor-mediated activation of PLC-β. After permeabilization of the cells with saponin, we examined the effect of n-3 and n-6 PUFAs at PLC-β on the level of its GTP-binding-protein (G-protein) and Ca^{2+} activation. As 20:5n-3 pretreatment of the cardiomyocytes appeared to decrease the rate of cell permeabilization by saponin, we decided to measure also the membrane cholesterol contents and membrane fluidity.

MATERIALS AND METHODS

Materials

Phenylephrine (PHE) was obtained from Sigma (St. Louis, MO, USA) and endothelin-1 (ET-1) was from Peninsula Laboratories (Belmont, CA, USA). The 4-well Multidish as well as single culture dishes (Ø 35 mm) were from Nunc (Roskilde, DK), the culture medium Ham F10 was from Gibco (Paisley, UK). Fetal calf serum, horse serum and penicillin/streptomycin were obtained from Boehringer (Mannheim, Germany). Trypsin (type III) was from Sigma. *Myo*-[2-^{3}H]Ins (17-19 Ci/mmol) was obtained from Amersham International (Amersham, UK). TMA-DPH (1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate) was obtained from Molecular Probes (Eugene, OR, USA). A_2C (2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)-octanoate) as well as the free fatty acids oleic acid (cis-9-octadecenoic acid, 18:1n-9), stearic acid (octadecanoic acid, 18:0), linoleic acid (cis-9,12-octadecadienoic acid, 18:2n-6) and cis-5,8,11,14,17-eicosapentaenoic (20:5n-3) acid were from Sigma. Dowex AG 1-X8 (100-200 mesh formate form) was from BioRad Laboratories (Richmond, CA, USA). Free cholesterol was determined using a kit (Boehringer-Mannheim). All other chemicals were of analytical grade.

Cell culture

Primary cultures of neonatal ventricular myocytes were prepared from two-day-old Wistar rats as described before (Van Heugten *et al.*,1994). For measurement of contraction frequency, cardiomyocytes were seeded on circular glass cover slips (Ø 25 mm) at 150 to 175x10^3 cells/cm^2. For measurements of phospholipase C activity or lactate dehydrogenase (LDH) release myocytes were seeded in wells with 1.8 cm^2 surface at 150 to 175x10^3 cells/cm^2. For the determination of cholesterol content and total phospholipids the cardiomyocytes were seeded (150 to 175x10^3 cells/cm^2) in tissue culture dishes with 8 cm^2 surface, and for measurement of membrane fluidity on rectangular glass coverslips in similar tissue culture dishes (Ø 35 mm, 8 cm^2) at a density of 4x10^4 cells/cm^2. Cell cultures were grown at 37°C and 95% air and 5% CO_2 in complete growth medium consisting of Ham F10 supplemented with 10% (v/v) fetal calf serum, 10% (v/v) horse serum, 100 U penicillin/ml and 100 μg streptomycin/ml.

The cultures were exposed to the following conditioned growth media 24 h after seeding: a) growth medium supplemented with the vehicle (0.3 % (v/v) pure ethanol); b) growth medium supplemented with 18:0 and 18:1n-9 (107 μM each); c) growth medium supplemented with 18:2n-6 (214 μM); and d) growth medium supplemented with 20:5n-3 (214 μM), as we described before (Lamers *et al.*, 1992). After two days the cultures were renewed with their concurring enriched medium. Treatment with fatty acids did not result in cell density- or hypertrophic- changes as was tested by assessment of the protein content and protein over DNA ratio, respectively. For instance, protein contents (mg) per mg DNA in the treated cells were 43.1 ± 1.7 (Control), 45.5 ± 0.7 (18:0/18:1n-9), 42.6 ± 2.6 (18:2n-6) and 43.8 ± 1.4 (20:5n-3). All experiments were performed 4 to 5 days after plating the cells.

Separation of the phospholipids and quantification of the fatty acid methyl esters

To examine the incorporation of supplemented fatty acids in the phospholipid fraction after 4 to 5 days, the cardiomyocytes were rinsed with cold buffer, scraped and extracted with

organic solvents as described previously (Lamers *et al.*, 1992). Briefly, the phospholipid fractions were separated from the triglyceride, diacylglycerol, cholesterol and unesterified fatty acid fractions by thin layer chromatography. To separate the fatty acid methyl esters, a CP9000 capillary column chromatograph (Chrompack, Middelburg, The Netherlands), equipped with a CP-Sil 88 coated fused silica capillary column, was used (Lamers *et al.*, 1992).

Spontaneous contractions

After 4 days the culture grown on a circular glass coverslip was rinsed once with incubation buffer I, containing 125 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 10 mM $NaHCO_3$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), and 5 mM glucose (pH 7.4, 37°C), and subsequently mounted on a teflon culture dish (Ince *et al.*, 1985). The dish was placed in a temperature controlled micro-incubator (Ince *et al.*, 1983) and the contractions of the cardiomyocytes were visualized as described earlier (Le *et al.*, 1993). The contracting cells were made visible on a videomonitor, which was equipped with an interactive system to convert the time-dependent grey levels in a particular cursor-defined area on the screen into a time-dependent voltage. This so-called contraction amplitude signal was counted in a heart rate counter to obtain the contraction frequency signal expressed as beats per min. The irregularity of beating equalled the variation coefficient of the contraction frequency.

Inositolphosphate production in intact and saponin-permeabilized cardiomyocytes

Cardiomyocytes were labelled with 2 μCi *myo*-[2-^{3}H]Ins/ml for 48 h in conditioned growth medium. After washing and preincubation (15 min, 37°C) in incubation buffer II, containing 130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 0.44 mM NaH_2PO_4, 1.1 mM $MgSO_4$, 20 mM $NaHCO_3$, 11.1 mM glucose, and 10 mM HEPES (pH 7.4, 37°C) the cells were stimulated with 5 μM PHE or 5 nM ET-1 in the presence of 10 mM LiCl. The incubations were stopped by rapidly washing the cells with ice-cold buffer. Water-soluble [^{3}H]Ins products were extracted by two subsequent extractions with 660 mM $HClO_4$. The pooled $HClO_4$ fractions were neutralized with 2 M KOH and 1 M K_2CO_3. The remainder, i.e. water-insoluble products, was subsequently extracted with ice-cold CH_3OH:HCl (100:1 v/v) for 5 min at 4°C. The [^{3}H]inositol phosphates ([^{3}H]InsP_n) were separated from [^{3}H]Ins and [^{3}H]glycerophosphoinositol by chromatography on Dowex AG 1-X8 as described previously (Van Heugten *et al.*, 1994). The *myo*-[2-^{3}H]Ins prelabelled cells were rinsed with phosphate-buffered saline, at 37°C, and subsequently treated with 100 μg saponin/ml in intracellular buffer (10 mM NaCl; 110 mM KCl; 1 mM KH_2PO_4; 4 mM $MgCl_2$; ethylene glycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tertraacetic acid (EGTA); 3 mM ATP; 8 mM creatine phosphate; 6 U/ml creatine kinase; 20 mM HEPES; pH 7.0) for 5 min, at 37°C. Thereafter, the myocytes were rinsed three times with intracellular buffer followed by incubation for 15 min at 37°C in intracellular buffer containing 10 mM LiCl, and about 96 nM free Ca^{2+} ions (estimated according to SPECS, a software program based on the formulas described by Fabiato, 1988) with or without 30 μM GTPγS. After incubation, the collected buffer and the cells were extracted for measurement of [^{3}H]InsP_n formation as previously described (Van Heugten *et al.*, 1994).

Rate of permeabilization induced by saponin

The cardiomyocytes cultured with fatty acid enriched medium in 1.8 cm^2 wells, were washed to remove the small amounts of LDH released upon exchanging the growth medium for incubation buffer, and preincubated with intracellular buffer. After a 5 min preincubation period a sample was taken to measure the LDH release in the absence of saponin. Then the buffer was removed and replaced by intracellular buffer containing 100 μg/ml saponin. Samples of the incubation buffer were taken after 1, 2, 5, and 10 min, and stored on ice for determination of LDH release. Thereafter, the remainder of the buffer was immediately removed and a solution of 1 % Triton in intracellular buffer was added to release the rest of cellular LDH to determine the total LDH content of the cells. The LDH activity was assayed spectrophotometrically in potassium phosphate buffer (95 mM, pH 7.0), containing 7.6 mM sodium pyruvate and 0.2 mM NADH, and was expressed as % of total cellular LDH.

Membrane fluidity

Membrane fluidity was assessed by measuring the fluorescence steady-state anisotropy (r_{ss}) of the plasma membrane specific fluorophore 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulphonate (TMA-DPH) according to Sumbilla and Lakowicz (1983) and modified by Bastiaanse *et al.*, (1994). The cardiomyocytes grown on glass coverslips were washed twice and preincubated with incubation buffer I for 30 min at 37°C. Thereafter the cells were loaded with 10 μM TMA-DPH for 30 min at 37°C. Then the glass coverslips were placed in a cuvette containing 2.5 ml buffer I, and after a period of 10 min the fluorescence steady-state anisotropy (r_{ss}) was measured according to the following formula: $r_{ss}=(I_{vv}-I_{vh}*G)/(I_{vv}+2I_{vh}*G)$ where I_{vv} and I_{vh} represent the fluorescence intensities parallel and perpendicular to the excitation plane (when set vertically), respectively. G is the correction factor for the difference in the monochromator's transmission efficiency for vertically and horizontally polarized light, and equals I_{hv}/I_{hh}. The measurements were performed on a spectrofluorometer (Perkin-Elmer LS-3), provided with a polarization accessory (Perkin-Elmer 5212-3269), at 37°C.

To validate the procedure, separate series of experiments were conducted before and after the membrane fluidity had been changed by addition of the "fluidizer" 2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)-octanoate (A_2C, 20 μM). After measuring r_{ss} in the absence of A_2C the glass coverslips were replaced to their original preincubation loading dish to which 20 μM A_2C was added and subsequently incubated for 30 min at 37°C. Immediately thereafter, the r_{ss} was measured again. To ensure a maximal fluorescent signal the fluidity measurements were always carried out in cells seeded at an optimal density ($4x10^4$ cells/cm^2) which was not influenced by any fatty acid treatment.

Free cholesterol and total phospholipids

The myocytes cultured in fatty acid enriched medium grown in 8 cm^2 dishes were rinsed twice with a physiological salt solution. Then the cells were scraped in 2 ml methanol and transferred to a glass tube (fat free) containing 0.75 ml physiological salt solution. After addition of 1 ml $CHCl_3$, the mixture was vigorously stirred, whereafter the supernatant was separated from the pellet by centrifugation (5 min, 3000 rpm). The supernatant was stored on ice, and the pellet was washed with 1.9 ml $CHCl_3$:methanol:H_2O (5:10:4 v/v/v). Again the supernatant was separated from the pellet by centrifugation and added to the supernatant

of the prior step. The pellet was discarded. Subsequently 1.5 ml $CHCl_3$ and 1.5 ml H_2O were added to the supernatant fraction, and after vigorously mixing and centrifugation, the upper phase was removed from the lower phase. The latter was dried with a little scoop of Na_2SO_4, and was stored at -20 °C for 12 h. Then the solution was transferred to a new glass tube, and the Na_2SO_4 pellet was washed with 1 ml $CHCl_3$. The wash fluid was combined with the solution. Subsequently the organic phase was evaporated and the residue dissolved in 200 µl peroxide-free 2-propanol. Cholesterol was determined enzymatically using a the kit (Boehringer, Mannheim, Germany). In order to determine the total phospholipid concentration, a sample of cell extract was destructed with H_2SO_4:$HClO_4$ (1:1 v/v) at 210°C for 5 h, until a clear solution was obtained. In this solution, the phosphate concentration was quantified according to Bartlett (1959), using a 1 % instead of a 0.22% ammoniumheptamolybdaat solution.

Statistics

Data are presented as mean values ± S.E.M. unless stated otherwise. Student's t-test was used for statistical comparison with corresponding control values and analysis of variance was performed followed by the Student Newman Keul's test when appropriate. A p-value less than 0.05 was considered to indicate statistical significance.

RESULTS

Irregularity in frequency of spontaneous contractions

A previous study (Hallaq *et al.*, 1992) using the same model of cultured ventricular myocytes has shown that incubation of the cells for 3-5 days with 5 µM 20:5n-3 prevented the arrhythmogenic effect of 0.1 mM ouabain. No effect was seen when the cells were pretreated with 5 µM 20:4n-6. In our hands, however, confluent monolayers of control cells in confluency always exhibit spontaneously irregular contractions. Fig. 1A shows typical recordings from the control ventricular myocytes and those treated for 3 days with 18:0/18:1n-9, 18:2n-6 and 20:5n-3 rich medium. Pretreatment of cells with 18:2n-6 as well as with 20:5n-3 improved the regularity of the spontaneous contractions significantly in comparison to control pretreatment (Fig. 1B). This effect, however, was not significant compared to 18:0/18:1n-9. So it appears that the fatty acid pretreatment, independently of the degree of polyunsaturation, induces a regular pattern of spontaneous contractions. Because in the model used the cardiomyocytes are grown in a confluent monolayer, resulting in the spontaneous contractions, the particular area of monitoring by video could hardly be placed on a single cell, which would be necessary to assess shortening in velocity. Therefore, no exact measurements of the shortening velocity were performed.

Phospholipid fatty acid composition

Incubation of myocytes with media enriched with 18:0/18:1n-9, 18:2n-6 and 20:5n-3 changed the fatty acid composition of the phospholipids (Table 1). Incubation with an 18:0/18:1n-9 supplemented medium caused a markedly increased incorporation of 18:0 and 18:1n-9 fatty acids and a reduction in 16:0, 18:1n-7, 18:2n-6 and 20:4n-6 contents. As expected, the sum of PUFAs decreased from 47.0 to 38.5 mole %. Treatment with 18:2n-6

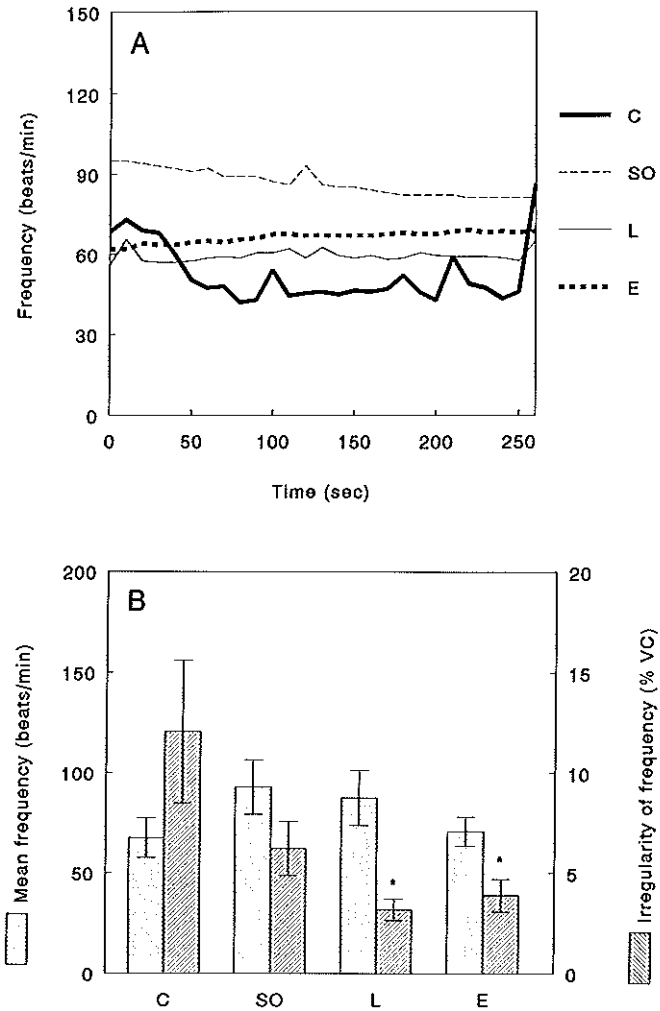

Figure 1 Frequency of spontaneous beating and regularity of beating frequency of ventricular myocytes grown in control medium (C) or in 18:0/18:1- (SO), 18:2n-6- (L) and 20:5n-3- (E) enriched medium. Cellular contractions were recorded using a video technique, and were counted to obtain contraction frequency and irregularity of frequency. The upper panel A shows a representative experiment illustrating the frequency versus time for cardiomyocytes subjected to the various fatty acid treatments. The lower panel B shows the mean frequency and irregularity of frequency, measured over a period of 5 min (% VC: variation coefficient) representing the mean data of 4 different cell batches. Bars represent mean values ± S.E.M. *: $p<0.05$ versus control cells.

caused a dramatic increase of 18:2n-6 from 17.1 to 38.9 mole %, associated with a decrease of 18:1n-9 and 20:4n-6. Furthermore, as we expected, the sum of PUFAs increased from 47.0 to 56.5 mole %. Treatment with 20:5n-3 led to the increased incorporation of this fatty acid (from 0.5 to 23.2 mole %) and its elongation product 22:5n-3 (from 2.7 to 9.7 mole %),

but a decreased incorporation of 22:6n-3. The increased incorporation of n-3 PUFAs occurred mainly at the expense of n-6 fatty acids as the sum of PUFAs remained almost constant. The double bond index, DBI (mean number of double bonds per mole fatty acid) is a parameter that is indicative for possible changes in the fluidity of the membrane bilayer. As can be seen from Table 1, particularly the treatment with 20:5n-3 caused a considerable increase of DBI from 1.76 (control cells) to 2.33. Compared to treatment with 18:0/18:1n-9 and 18:2n-6, the 20:5n-3 treated cells had much higher DBI. Moreover, in 18:2n-6 treated cells, a slight decrease from 1.76 to 1.60 was observed.

Table 1. Fatty acid composition (mole %) of the total phospholipid fraction of ventricular myocytes grown in control medium or in 18:0/18:1n-9- (SO), 18:2n-6- (L) or 20:5n-3- (E) enriched medium. Values represent mean values ± S.E.M.

Fatty acids	Control	SO	L	E
	(n=3)	(n=3)	(n=3)	(n=4)
16:0	13.83 ± 1.27	7.51 ± 0.25	12.55 ± 1.11[d]	14.80 ± 0.84[d]
18:0	23.31 ± 0.56	28.24 ± 0.51	21.08 ± 0.94[d]	23.03 ± 1.12[d]
18:1n-7	3.09 ± 0.06	1.68 ± 0.10	2.04 ± 0.07	2.59 ± 0.05
18:1n-9	9.61 ± 0.45	22.32 ± 0.52	5.25 ± 0.04	7.00 ± 0.22
18:2n-6	17.10 ± 1.43	14.43 ± 0.93[d]	38.87 ± 0.88	6.08 ± 0.48
20:4n-6	18.21 ± 1.16	15.39 ± 0.65[d]	11.46 ± 1.11	7.63 ± 0.58
20:5n-3	0.48 ± 0.07	1.12 ± 0.55[d]	0.20 ± 0.05	23.22 ± 1.31
22:5n-3	2.69 ± 0.27	2.33 ± 0.68[d]	1.53 ± 0.18	9.72 ± 0.44
22:6n-3	3.71 ± 0.34	2.38 ± 0.26	1.67 ± 0.14	1.33 ± 0.07
others[a]	7.97 ± 0.06	4.59 ± 0.83	5.36 ± 0.22	4.60 ± 0.14
DBI[b]	1.76 ± 0.08	1.56 ± 0.07	1.60 ± 0.04	2.33 ± 0.05[e]
ΣPUFA[c]	47.05 ± 2.03	38.48 ± 0.80	56.50 ± 0.53	50.01 ± 0.64

[a] Only the major fatty acids and the sum of minor fatty acids, termed "others" (mainly 16:0dma, 18:0dma, 18:3n-3, 20:3n-6, 22:0, 24:0 and 22:4n-6) are listed. Dma stands for dimethylacetal.
[b] DBI stands for double bond index (mean number of double bonds per mole fatty acid)
[c] ΣPUFA is the sum of all PUFA.
[d] Values, not differing significantly from control values. All other values were significantly different from control.
[e] $p < 0.05$ in comparison to C, SO, and L-treatments

Agonist-induced [^{3}H]InsP$_n$ response in intact cardiomyocytes

Earlier we have shown that stimulation of *myo*[2-^{3}H]Ins labelled neonatal rat ventricular myocytes with either PHE or ET-1 results in activation of PLC-β in a dose-dependent manner, both agonists reaching about the same maximal effect on the formation of total [^{3}H]InsP$_n$ (Van Heugten *et al.*, 1993). To investigate the influence of an altered fatty acid composition of the plasma membrane phospholipids on the agonist-induced PLC activation, the

cardiomyocytes grown in the control medium and media enriched with 18:0/18:1n-9, 18:2n-6 and 20:5n-3, were stimulated for 15 min with submaximal concentrations of PHE (5 μM) or ET-1 (5 nM). The formation of [^{3}H]InsP$_n$ upon PHE and ET-1 stimulation, in cardiomyocytes that were cultured in control medium, amounted 11 % and 9.5 %, respectively, of total incorporated [^{3}H]Ins, which was a 2.9 and 2.5 fold increase, respectively, compared to the basal [^{3}H]InsP$_n$ production (3.8 %) (Fig. 2). Pretreatment of cardiomyocytes with 18:0/18:1n-9 or 18:2n-6 enriched medium had no influence on the PHE stimulated [^{3}H]InsP$_n$ formation. To our surprise, however, basal [^{3}H]InsP$_n$ formation in the 18:0/18:1n-9 treated cells increased to 6.1 % and the ET-1 stimulated [^{3}H]InsP$_n$ formation to 11.6 %. PHE stimulation of cardiomyocytes preexposed to 20:5n-3-rich medium resulted in a significantly less [^{3}H]InsP$_n$ formation of 7.3 % of total incorporated [^{3}H]Ins compared to control cardiomyocytes, whereas stimulation with ET-1 of 20:5n-3 treated cardiomyocytes caused a significantly higher [^{3}H]InsP$_n$ formation (13.5 % of total incorporated [^{3}H]Ins).

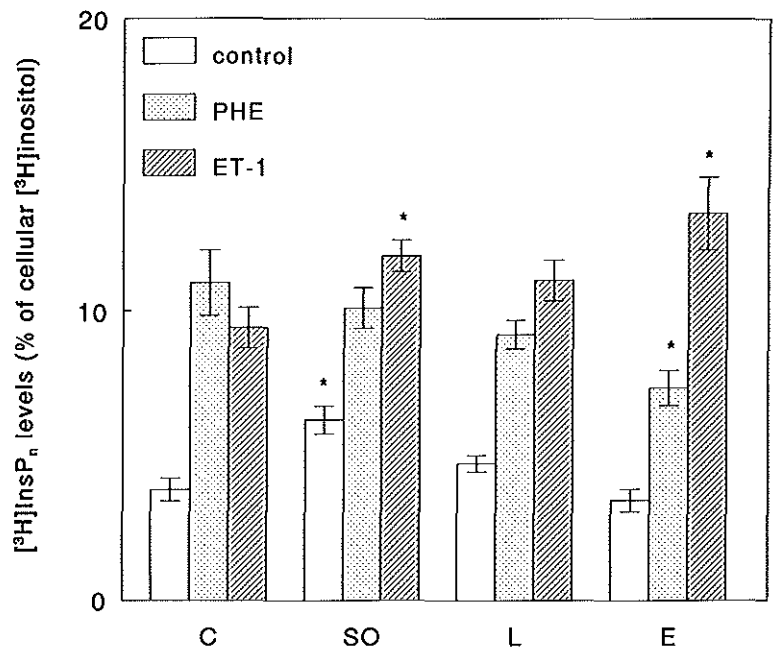

Figure 2 Agonist stimulated [^{3}H]InsP$_n$ production in intact ventricular myocytes grown in control medium (C) or 18:0/18:1- (SO), 18:2n-6- (L) and 20:5n-3- (E) enriched medium. Cardiomyocytes preincubated with *myo*-[2-^{3}H]Ins were stimulated with either phenylephrine (PHE; 5 μM) or endothelin-1 (ET-1; 5 nM) for 15 min at 37 °C. The cells were extracted and the [^{3}H]InsP$_n$ formed was separated from the other [^{3}H]Ins-labelled metabolites as described in Methods. Values are expressed as % of total incorporated [^{3}H]Ins and represent the mean values ± S.E.M. of 5 and 4 experiments, with PHE and ET-1, respectively. *: $p<0.05$, when comparing agonist mediated [^{3}H]InsP$_n$ responses in cells cultured in fatty acid enriched medium to the corresponding [^{3}H]InsP$_n$ responses in cells pretreated with control medium.

Table 2. GTPγS stimulated total [^{3}H]inositol phosphate ([^{3}H]InsP$_n$) production in the presence of Ca^{2+} in saponin permeabilized ventricular myocytes grown in control medium (C) or in 18:0/18:1n-9- (SO), 18:2n-6- (L), and 20:5n-3- (E) enriched medium.

	[^{3}H]InsP$_n$ production (% of total cellular [^{3}H]Ins)			
	C	SO	L	E
Incubation buffer only				
- GTPγS	1.13 ± 0.08	0.87 ± 0.07*	0.86 ± 0.07*	1.01 ± 0.08
+ GTPγS	3.10 ± 0.17	2.96 ± 0.23	2.46 ± 0.10*	2.52 ± 0.20*
Buffer plus cell extract				
- GTPγS	2.23 ± 0.10	1.81 ± 0.18	2.19 ± 0.32	2.11 ± 0.16
+ GTPγS	5.19 ± 0.31	4.78 ± 0.36	4.53 ± 0.24	4.19 ± 0.29*

Values are expressed as % of total incorporated [^{3}H]Ins and represent the mean value ± S.E.M. of 8 measurements. *: $p < 0.05$ versus control

Ca^{2+} and GTPγS-induced [^{3}H]InsP$_n$ response in saponin-permeabilized cardiomyocytes

The finding that the agonist-induced [^{3}H]InsP$_n$ formation depends upon the fatty acid composition of membrane phospholipids may be explained by changes at the level of agonist-receptor binding, receptor-G-protein coupling and/or G-protein-PLC-β interaction. To study whether the G-protein-PLC-β interaction is affected by changes in the fatty acid composition of membrane phospholipids, the *myo*[2-^{3}H]inositol labelled cardiomyocytes (after exposure to the different fatty acids) were permeabilized by saponin and then stimulated for 15 min with non-hydrolysable GTPγS in the presence of Ca^{2+} or not stimulated. After permeabilization about half of the [^{3}H]InsP$_n$ formed was released in the incubation buffer. The basal [^{3}H]InsP$_n$ production (in buffer plus cell extract) of the permeabilized cardiomyocytes was not affected by any of the fatty acid treatments (Table 2). GTPγS stimulation, in the presence of Ca^{2+}, of the permeabilized cardiomyocytes preexposed to 20:5n-3 media resulted in significantly less (4.2 %) [^{3}H]InsP$_n$ formation compared to control cells (5.2 %).

Resistance to saponin-induced permeabilization

As can be seen in Table 2 the fatty acid treatment dependent changes in [^{3}H]InsP$_n$ formation released in the buffer do not completely reflect the fatty acid dependent changes in [^{3}H]InsP$_n$ in the buffer plus cell extract, suggesting possible fatty acid treatment dependent differences in the permeabilization. The data illustrated in Figure 3 show that there was a lagphase of LDH release up to 1 min of exposure to saponin unaffected by either fatty acid treatment. We observed at 2 min a significant decrease only by 20:5n-3 treatment and at 5 min a small but not significant decrease only by 20:5n-3 treatment. Figure 3, however, demonstrates that 5-10 min of treatment with saponin resulted in almost complete (70-80 % of total LDH released) depletion of LDH which was independent of the fatty acid supplementation. Therefore, at a time (2 min) of partial permeabilization, the cardiomyocytes grown in 20:5n-3 rich medium,

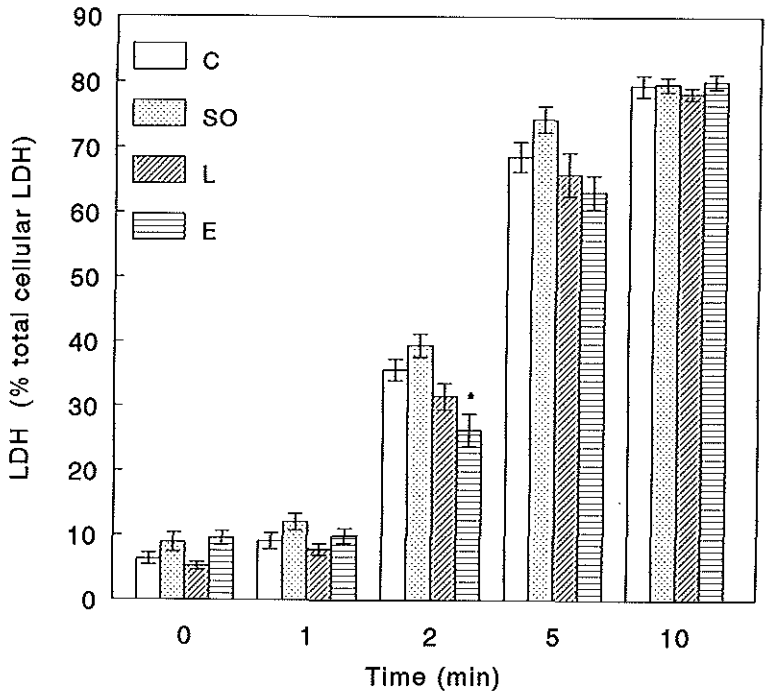

Figure 3 The rate of permeabilization by saponin (100 μg/ml) as assessed by release of lactate dehydrogenase (LDH) from ventricular myocytes grown in control medium (C) or in 18:0/18:1- (SO), 18:2n-6- (L) and 20:5n-3- (E) enriched medium. LDH activity was assayed spectrophotometrically. Values are expressed as % of total cellular LDH, and represent mean values ± S.E.M., in triplicate, of 3 different cell batches. *: $p<0.05$ versus control.

revealed significantly less LDH release, that is 26% as compared to 36% in cells grown in control medium. A similar tendency of a delayed LDH release was observed in the 18:2n-6 treated cells.

Cholesterol content and membrane fluidity

Saponin is believed to act mainly upon the cholesterol component of the sarcolemma (Glauert *et al.*, 1962; Dourmashkin *et al.*, 1962). Given the results of 20:5n-3 induced changes in cellular resistance to saponin-permeabilization, we determined the cholesterol content of whole cell extracts. Cholesterol is expressed either as nmol/mg protein or as nmol/nmol phospholipid (Fig.4). Exposure of cardiomyocytes to 18:0/18:1n-9 and 18:2n-6 resulted in a slight increase in cellular cholesterol content of 35 and 40 nmol/mg protein respectively as compared to 28 nmol/mg protein in control cells (Fig. 4). This cholesterol content was in the range as was found for control cells in another report on the same model (Bastiaanse et al., 1994 and 1995). Cholesterol expressed per nmol phospholipid, however, did not change by these fatty acid treatments (Fig. 4). This is due to the fact that phospholipid content (in nmol/mg protein) also increased independently of the type of fatty acid treatment (see legends to Fig. 4). It was most remarkable to observe that 20:5n-3 pretreatment produced a 3-fold increase in cellular cholesterol content expressed per mg protein and a 1.7 fold increase in cholesterol expressed per nmol phospholipid. The increase in cholesterol content

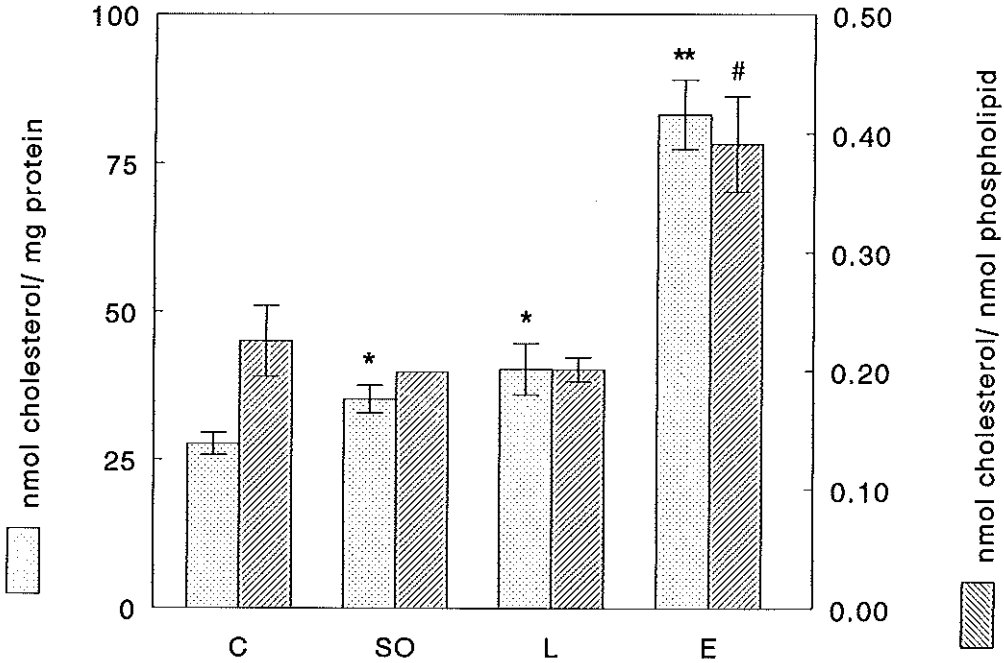

Figure 4 Cholesterol content of ventricular myocytes grown in control medium (C) or in 18:0/18:1- (SO), 18:2n-6- (L) and 20:5n-3- (E) enriched medium. Cholesterol was determined enzymatically in cellular extracts, and the total phospholipids in the extracts were determined by assessment of the phosphate concentration as described in Methods. Cholesterol is expressed either as nmol/mg protein or as nmol/nmol phospholipid. Phospholipid content (nmol/mg protein) did also change however independently of the fatty acid treatment: C, 134 ± 13, SO, 176 ± 15, L, 192 ± 18 and E, 217 ± 27 ($p<0.05$, fatty acid treatment versus control). Values represent mean values ± S.E.M. of 7-8 determinations. *: $p<0.02$ versus control; **: $p<0.001$ versus C, SO, and L; #: $p<0.005$ versus C, SO and L.

per mg protein was much more dramatic comparing those seen with 18:0/18:1n-9 (1.2 fold) and 18:2n-6 (1.4 fold) treatment.

Because of the large changes observed in membrane-cholesterol and DBI due to 20:5n-3 treatment of the cardiomyocytes, the membrane fluidity was assessed by measuring the fluorescence steady state anisotropy (r_{ss}) of TMA-DPH. Indeed, only the r_{ss} of cardiomyocytes grown in 20:5n-3 was significantly ($p<0.02$) affected, that is increased by 8% as compared to control cultures (Fig. 5A). Apparently, the bilayer "stiffening" effect of an adaptively increased cholesterol content overcompensates the fluidizing effect of increased degree of polyunsaturation of fatty acids in the phospholipids. To validate the method of fluidity measurement we tested in a separate series of experiments whether the r_{ss} value was influenced by the membrane mobility agent A_2C (Fig. 5B). Before and after exposure to 20 μM A_2C for 30 min, the r_{ss} of control cardiomyocytes was measured to reveal an increase of fluidity, i.e. a significant ($p< 0.05$) decrease of r_{ss} by 15.4%.

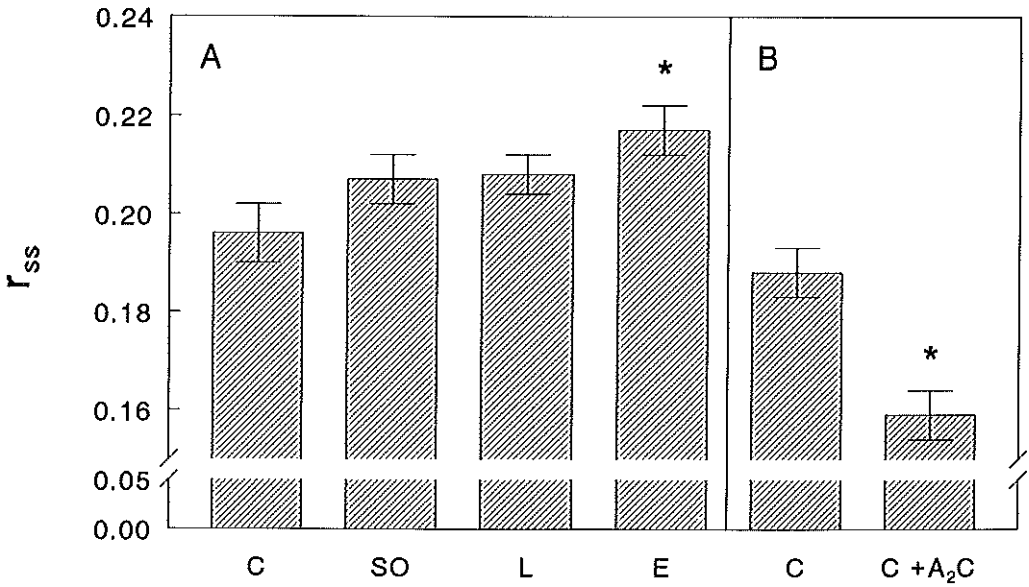

Figure 5 Membrane fluidity of ventricular myocytes grown in control medium or in 18:0/18:1- (SO), 18:2n-6- (L) and 20:5n-3- (E) enriched medium. Panel A: Ventricular myocytes grown in control medium (C) or in 18:0/18:1- (SO), 18:2n-6- (L) and 20:5n-3- (E) enriched medium were loaded with the fluorophore diphenylhexatriene, TMA-DPH for 30 min, at 37 °C, whereafter the fluorescence steady-state anisotropy r_{ss} of the fluorophore TMA-DPH was assessed as described in detail in Methods. Panel B: To validate the experimental method we measured r_{ss} before and after addition of the membrane mobility agent A_2C to TMA-DPH-loaded ventricular myocytes. Values are expressed as mean values ± S.E.M. of 3 experiments. *: $p<0.05$ versus control.

DISCUSSION

The aim of this study was to investigate the influence of increased n-6 and n-3 PUFA incorporation in the phospholipids of the cardiomyocyte membrane on the PHE and ET-1 stimulated PtdIns cycle, as compared to cells grown in unsupplemented growth medium. Moreover, in comparison, similar experiments were performed with saturated/monosaturated fatty acid pretreated cells. The mixture of the supplemented free PUFAs with the concentrated horse and calf sera, the latter which contain an excess of fatty acid-poor albumin molecules, ensured that the toxicity of a high concentration of non-esterified fatty acids was prevented. This was also based upon our earlier studies in which we showed in *in vitro* experiments that cardiac sarcolemmal membrane kept its integrity if the non-esterified fatty acid to albumin molar ratio did not exceed 5 (Lamers *et al.*, 1984). Moreover, in the present study, the control cells usually exhibited irregularities in their contraction frequency, whereas treatment with n-6 and n-3 PUFAs improved significantly the regularity of spontaneous contractions. A tendency to such improvement was also seen after treatment with 18:0/18:1n-9.

Although the free fatty acid enriched incubation medium was always carefully washed

away, followed by a period of stabilization before agonist stimulation of the cardiomyocytes, we can not exclude the additional influence of an altered composition of the non-esterified fatty acid pool which is performed by the normal turnover of cellular phospholipids. In neonatal cardiomyocytes, exogenous free n-3 PUFAs were found to exert their effect directly on Ca^{2+}-channels, as was assessed by measurements of the steady-state Ca^{2+} influx (Hallaq *et al.*,1992) and of the cell contractions mediated by β-adrenergic stimulation of the cells (Kang and Leaf, 1995). The β-adrenergic agonist induced arrhythmias could be prevented by exogenous non-esterified 20:5n-3, but revived when the fatty acid was washed away with fatty acid-free albumin (Kang and Leaf, 1995).

The various fatty acid treatments of the cardiomyocytes resulted in the expected alteration of fatty acid composition of the membrane phospholipids. In 18:2n-6 treated cells, incorporation of 18:2n-6 increased from 17.1 (control cells) to 38.9 mole %, while in the 20:5n-3 treated cells, incorporation of 20:5n-3- and its elongation product 22:5n-3 increased from 0.5 and 2.7 mole % (control cells), respectively, to 23.2 and 9.7 mole %, respectively. However, only in the 20:5n-3 treated cells a marked increase in double bond index to 2.33 was observed, in comparison to values of 1.76 in control-, 1.60 in 18:2n-6- and 1.56 in 18:0/18:1n-9- treated cells. As far as the PtdIns cycle is concerned, most remarkable effects were observed in the 20:5n-3 treated ventricular myocytes. In those myocytes, the PHE and ET-1 stimulated PLC-β response measured in intact cells was decreased (7.3 % of total incorporated [^{3}H]Ins) and increased (13.5 % of total incorporated [^{3}H]Ins), respectively, compared to ventricular myocytes grown in control medium (respectively 11 and 9.5 % of total incorporated [^{3}H]Ins). In 18:2n-6 treated cells, no significant effect was observed on receptor-mediated PLC-β activity. Also treatment with 18:0/18:1n-9 led to a slight increase of the ET-1 stimulated PLC-β-response. Earlier we have shown that the effects of 20:5n-3 on agonist-induced [^{3}H]InsP_n formation can not be ascribed to the alterations in the relative [^{3}H]Ins incorporation in phosphorylated PtdIns (Lamers *et al.*, 1992). Similar results were found by Bordoni *et al.* (1990) when cells were grown in 22:6n-3 enriched medium. The results, obtained with the saponin permeabilized cardiomyocytes, suggest that the effects of 20:5n-3 on receptor-mediated PLC-β of intact cardiomyocytes could be exerted either at the level agonist-receptor, receptor-G-protein coupling or G-protein-PLC-β interaction. In view of these findings the observations of Skúladóttir *et al.* (1993) in adult cardiomyocytes are interesting. Although in the latter study the changes of phospholipid fatty acid profile were obtained pharmacologically by repeated administration of hydrocortisone and/or epinephrine, the enhanced incorporation of 18:2n-6, 20:4n-6 and 22:6n-3 in phosphatidylcholine and phosphatidylethanolamine paralleled the decreases in affinity of the α_1-adrenoceptors and coincided with downregulation of the number of β-adrenergic binding sites. Next to the fact that the mechanisms responsible for α_1- and β-adrenoceptor regulation are different, it was speculated by Skúladóttir *et al.* (1993) that because adrenoceptors span the entire thickness of the membrane bilayer a change in membrane phospholipid acyl chains could result in an increase in bilayer volume associated with a conformational change in the membrane receptor.

Although the results of the present study indicate that the level of agonist-receptor is an important step in the impoundment of alterations in the phospholipid fatty acid profile on the PtdIns-cycle, the effects are certainly not restricted to this level. The effect of polyunsaturated fatty acid incorporation in the phospholipids on the membrane dynamic characteristics, such

as fluidity, membrane-cholesterol in correlation to integrity might influence the process of activation and the maintenance in the PtdIns-cycle on one hand. On the other hand, the induced alterations in the molecular species of PtdIns(4,5)P_2 will likely be of importance as well.

The direct stimulation of the G-proteins of saponin-permeabilized cardiomyocytes with GTPγS showed an attenuation of total [^{3}H]InsP$_n$ accumulation in 20:5n-3 treated cells as was measured in the cell extract plus incubation buffer. When the results on [^{3}H]InsP$_n$ formation in the buffer and cell extract were compared to those in the buffer only, slight discrepancies show up concerning the effects of various the fatty acid treatments. Thus, differences in permeabilization by saponin of the fatty acid treated cardiomyocytes could have occurred. However, investigation of the time-course of saponin-induced permeabilization of cardiomyocytes, measured by the release of LDH, unmasked a membrane protecting effect of 20:5n-3 pretreatment of the cardiomyocytes. The membrane permeabilization by saponin is generally believed to be caused by complexation with cholesterol in the plasma membrane, leading to instability of the bilayer (Glauert *et al.*, 1962; Dourmashkin *et al.*, 1962). The interrelationship between membrane stability, fluidity and cholesterol content of the bilayer and DBI of the acyl groups in the phospholipids could be the basis of the unexpected finding of a membrane protective effect of 20:5n-3 treatment of cardiomyocytes towards the permeabilizing agent saponin. The massive increase of cholesterol induced by 20:5n-3 treatment may initially protect the membrane for the cholesterol clustering, destabilizing and subsequent permeabilizing action of saponin.

In addition, 20:5n-3-treatment induced alterations in the plasma membrane fluidity and chemical composition, e.g. molecular species of phospholipids and/or cholesterol content, could mediate changes in the PtdIns-cycle activity. In line with this, the reduction of norepinephrine induced PtdIns-cycle activity was found to be most dramatic in n-3 PUFAs-treated atrial cells when comparing rats fed with n-6 PUFAs, mono unsaturated or saturated fatty acids (Woodcock *et al.*, 1995).

Only 20:5n-3 treatment resulted in a marked increase (1.7 fold) in cholesterol content expressed as cholesterol over phospholipid molar ratio. When cholesterol content was expressed per mg of protein, a slight increase in nmol cholesterol per mg cellular protein was observed in 18:0/18:1n-9 and in 18:2n-6 treated cells, but in the 20:5n-3-treated cells the increase (3.0 fold) was by far larger. In sarcolemma isolated from hearts of pigs fed a dietary supplement of 20:5n-3/22:6n-3, an increase of the cholesterol/phospholipid ratio from 0.38 to 0.64 was found by us as well, though the phospholipid/protein ratio decreased from 0.94 to 0.69 (Lamers *et al.*,1987). However, in rat smooth muscle cells incubated with 20:5n-3 or 22:6n-3 bovine serum albumin complexes for 12 h, cholesterol content hardly changed (Dusserre *et al.*, 1995). But in the latter study only 8 % and 9.5 %, respectively, of these fatty acids were incorporated in the phospholipids, at the expense of n-6 PUFAs but also at the expense of 18:0 and 18:1n-9 fatty acid incorporation. It should be noticed that in this model the incorporation of 18:2n-6 and 20:4n-6 into the smooth muscle cell membrane phospholipids of the control fed animals was only 5 % and 3.4 % respectively, in comparison to the 17 % and 18 %, respectively, which we have found in neonatal cardiomyocytes.

The changes in cholesterol content led us to measure the membrane fluidity of the treated cardiomyocytes. Surprisingly, a decreased fluidity was found in the cells grown in 20:5n-3 enriched medium (Fig. 5). This finding suggests that the fluidizing effect of a high double

bond index is overcompensated by a "stiffening" effect of increased cholesterol content. In agreement with this, the opposite effect was presented in a study on the same model of cultured neonatal cardiomyocytes (Bastiaanse *et al.*, 1995). In this study it was suggested the increase in sarcolemmal fluidity that was observed after metabolic inhibition was to be caused by a decrease in sarcolemmal free cholesterol content.

In conclusion, in neonatal ventricular myocytes, the activity of the PtdIns-cycle is affected by 20:5n-3 treatment of the cardiomyocytes and this effect could be exerted either on the level of agonist-receptor, receptor-G-protein coupling or G-protein-PLC-β interaction. We hypothesize that a causal relationship may exist between the 20:5n-3 induced alteration of the physicochemical properties in the bilayer and the agonist-dependent effects on the PtdIns-cycle activity.

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Chapter 8

GENERAL DISCUSSION

GENERAL DISCUSSION

The purpose of this thesis was to characterize the short-term PtdIns-cycle responses elicited by the α_1-adrenergic agonist PHE and ET-1 in cultured neonatal rat cardiomyocytes. Previously, both agonists were shown to produce an immediate positive inotropic effect and after several hours cell enlargement. Although the etiology of hypertrophy is likely multifactorial, α_1-adrenergic and endothelin receptors may belong to the major factors involved in the hypertrophic response *in vivo*. In general, agonists that couple to PLC-β were shown by us and others to cause hypertrophy in this model of cultured cardiomyocytes (Shubeita *et al.*, 1990; Suzuki *et al.*, 1991; Mochly-Rosen *et al.*, 1990; Knowlton *et al.*, 1993; Van Heugten *et al.*, 1995; Sugden *et al.*, 1995). Thus, hypertrophy may be induced through the pathway operating via G_q, PLC-β, PtdIns(4,5)P_2, Ins(1,4,5)P_3 and 1,2-DAG and through PKC-linked pathways. Direct activation of PKC with the phorbolester PMA or by transfection of cDNA constructs encoding constitutively active PKC induced hypertrophy of cultured neonatal cardiomyocytes (Sugden *et al.*, 1995). It was, therefore, of interest to compare the PtdIns-cycle related responses of an α_1-adrenergic agonist (PHE) and ET-1 in other to investigate whether agonist-dependent distinctions do exist. We also studied the effect of modification of the internal and external Ca^{2+} concentration and of the fatty acid composition of the membrane phospholipids on the activation of PLC-β by both agonists.

PtdIns-cycle related responses

Inositolphosphate isomers

The [^{3}H]inositolphosphate isomers which accumulated upon stimulation of [^{3}H]inositol prelabelled cardiomyocytes by PHE and ET-1 in the presence of the inositol-monophosphate phosphatase-inhibitor Li^+, revealed some interesting agonist-dependent differences (Chapter 4): After PHE stimulation the [^{3}H]inositolphosphate isomers formed suggest the operation of the 5-phosphatase pathway as a gradual accumulation of Ins(1,4)P_2 and Ins(4)P was observed, whereas no elevation of [^{3}H]inositolphosphate isomers associated with the 3-kinase pathway was detected. In contrast, ET-1 stimulation evoked an [^{3}H]inositolphosphate isomer response involving the 5-phosphatase- as well as the 3-kinase pathway with a production rate which was much higher than after stimulation with PHE. On the other hand, the bulk amount of [^{3}H]inositol was found to be incorporated in the Ins(4)P fraction which was independent of the type of agonist. The latter was expected because Li^+ acts predominantly by inhibition at the level of inositolmonophosphate hydrolysis. The different patterns of [^{3}H]inositolphosphate isomer formation induced by PHE and ET-1 provide evidence that the activity of the enzymes of the 5-phosphates and 3-kinase pathway are also distinctly influenced by receptor stimulation.

A remarkable similarity in the responses induced by PHE and ET-1 was the absence of any increase of [^{3}H]inositol incorporation into the putative Ca^{2+}-releasing compound Ins(1,4,5)P_3 and its direct phosphorylation product Ins(1,3,4,5)P_4. Therefore it is likely that in these cardiomyocytes the enzymes involved in the inositolphosphate degradation are particularly active. In a similar model of cultured rat cardiomyocytes, Woodcock *et al.*(1991 and 1992) could not observe a [^{3}H]Ins(1,3,4,5)P_4 accumulation after stimulation of the cells with α_1-adrenergic agonist norepinephrine either, but they did obsereve a rapid accumulation

of [^{3}H]Ins(1,4,5)P_3. The authors demonstrated that the degree of [^{3}H]Ins(1,4,5)P_3 accumulation depended on the age of the animals and culturing procedure as will be discussed later (Woodcock *et al.*, 1991 and 1992). With respect to a possible role of agonist-stimulated Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 elevation in the Ca^{2+} response, it is also important to notice that Ins(1,4,5)P_3 was reported to be either active (Nosek, *et al.*, 1986), inactive (Movsesian *et al.*, 1985) or weakly active with regard to its ability to release of Ca^{2+} from Ca^{2+} stores of cardiac SR (Kentish *et al.*, 1991). These controversial findings strengthen our conclusion that Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulations are of minor importance for the small Ca^{2+} response we observed following stimulation with α_1-adrenergic agonist and ET-1. As far as the α_1-adrenergic response is concerned, the same conclusion was drawn by Schmitz *et al.*(1991). Based upon our observation it is more likely that the agonist-induced elevations of 1,2-DAG levels (Chapter 4) are causally related to the observed small Ca^{2+} responses (Chapter 5).

1,2-Diacylglycerols

Like the inositolphosphate isomers, the various molecular species of 1,2-DAG are known to be rapidly metabolized by DAG lipases and/or kinases. Under conditions similar to those chosen to measure [^{3}H]inositolphosphate isomer responses, the cardiomyocytes showed significant total 1,2-DAG responses. After stimulation with PHE, the 1,2-DAG level became only elevated after 16 min, whereas stimulation with ET-1 caused an increase in the 1,2-DAG level already after 8 min (Chapter 4). As we postulated for the activity of inositolphosphate isomer degrading enzymes, the activities of DAG lipases and kinases could be distinctly influenced by the receptors involved. We can not deduce the origin of the 1,2-DAG formation from these experiments, however, the agonist-dependent time course of 1,2-DAG-formation may indicate the contribution of pathways other than PLC-β such as the activation of phospholipase D (PLD) (Thompson *et al.*, 1993; Boarder, 1994; Eskildsen-Helmond *et al.*, 1995). Thus, 1,2-DAG may also be produced by PLD-mediated hydrolysis of membrane phosphatidylcholine (PtdChol) which also lead to formation of PtdOH (Fig. 1). PtdOH is subsequently hydrolysed by a PtdOH phosphohydrolase (PPH) that may be innate in PLD (Sugden *et al.*, 1995). Indeed, recently we obtained evidence that ET-1 and PMA, but not PHE, markedly stimulated PLD activity in cultured neonatal cardiomyocytes (Lamers *et al.*, 1995). Production of 1,2-DAG from PLD may maintain elevated 1,2-DAG concentrations over a longer period following initial activation of PLC-β because PLD is activated by PKC (Fig. 1).

Cytosolic free Ca^{2+}

To monitor the intracellular Ca^{2+} ($[Ca^{2+}]_i$) changes, after stimulation with α_1-adrenergic agonist PHE or ET-1, a fluorescent Ca^{2+} probe, fura-2 AM was used in a dual-wavelength fluorescence microscope setup. The results of these experiments are presented in Chapter 5.

When we measured the ratio- (R-) value averaged over all myocytes in the field of view, we detected only marginal Ca^{2+} responses following stimulation with the two agonists. Only if Ca^{2+} responses of randomly chosen *individual* cardiomyocytes were analyzed, an agonist-dependent delayed monophasic and biphasic Ca^{2+} responses were observed in a number of cells after stimulation with PHE and ET-1, respectively. Obviously, the ability of the

cardiomyocytes to elicit a (synchronous) Ca^{2+} response upon stimulation with PHE or ET-1 was quite variable, which resulted in flattening of the *average* Ca^{2+} response. Apart from the fact that the presence of some nonmyocytes (mainly fibroblasts) might have disturbed the average Ca^{2+} response as well, variability between cardiomyocytes in the culture can be caused by a number of factors as proposed by others. For instance, it was shown that enzymatic dissociation of myocytes caused differences in subcellular patterns of $[Ca^{2+}]_i$ between individual cells affecting their cellular appearance and behaviour (quiescence, spontaneous contraction or hypercontraction) (Wier *et al.*, 1987). Also differences in basal $[Ca^{2+}]_i$, which is reflected by the different Ca^{2+} content of the SR, influence Ca^{2+} release patterns (Lipp and Niggli, 1994) as is discussed in Chapter 5. Another aspect which may introduce variability is the developmental state of the culture, which will be discussed later.

Protein kinase C activation and phosphorylation of protein substrates

In Chapter 4 it is described that stimulation of cardiomyocytes with PHE resulted in the rapid phosphorylation of a 30 kDa cytoskeleton or myofibrils-associated protein, whereas no protein was found to be detectably phosphorylated after stimulation with ET-1. On the other hand, the detection of ^{32}P-labelled phosphoproteins in whole homogenates of cardiomyocytes following separation on SDS gelelectrophoresis appeared to be hampered by the dominating presence of the non-specific phosphorylated proteins. Therefore, our results do not exclude the phosphorylation of several specific proteins after PHE and ET-1 stimulation. Based upon the observed translocation of PKC isoenzymes, it is likely that specific protein phosphorylation had occurred.

The assay of PKC is not easy: the translocation of classical- (c-) and novel- (n-) PKC's from the soluble to a particulate fraction is normally taken as being equivalent with activation (Sugden *et al.*,1995). PKC activity in particulate and soluble fraction is measured by the phosphorylation of suitable (model) substrates. For cPKCs, a suitable substrate is histon III S. For cPKCs or nPKCs, the commercially available peptide-ϵ (modelled on the PKC-ϵ pseudosubstrate) is suitable. We have used an oligopeptide sequence from the epidermal growth factor (EGF)-receptor and glycogen synthase (GS) receptor for measuring the activity of PKC after stimulation of cardiomyocytes with PHE, ET-1 or the phorbolester PMA, as was described by Shubeita *et al.* (1992). PKC assays were performed on agonist stimulated cells which were permeabilized by saponin and further incubated in presence of [γ-^{32}P]-ATP in the presence or absence of Ca^{2+} (100 nM). After stimulation of the cells with PMA, but not after stimulation with PHE or ET-1, we observed a marked Ca^{2+}-independent stimulation of ^{32}P incorporation into the exogenously added EGF receptor. These PKC assay methods, however, cannot discriminate unambiguously between the PKC isoforms. In order to do this, we and many others are now using Western blot analysis with polyclonal antisera raised against unique peptide sequences in the individual PKC isoforms or with monoclonal antibodies. PKC immunoblot analysis of the cytosolic and particulate fractions isolated from ET-1 or PHE stimulated cardiomyocytes, revealed a translocation of PKC-ϵ (Lamers *et al.*, 1995). Upon stimulation with ET-1 we found that PKC-ϵ was translocated within 1 min, whereas myocytes stimulated with PHE revealed only a modest disappearance of PKC-ϵ from the cytosol and PKC-ϵ could not convincingly be detected in the membrane fraction. The ϵ-PKC is one of the nPKCs that is Ca^{2+}-independent. Thus its translocation caused by stimulation with PHE and ET-1 is consistent with the observed minor and delayed elevation

of $[Ca^{2+}]_i$ and sustained (1,2)DAG accumulation. Indeed, no translocation of the major Ca^{2+}-dependent isoform PKC-α was observed after stimulation by either ET-1 (Lamers *et al.*, 1995) or PHE (unpublished observations). As expected, stimulation of the cardiomyocytes with PMA, however, resulted in an immediate translocation of PKC ϵ as well as PKC-α. Thus, using the Western blot approach, differences between isoforms, in terms of their susceptibility to translocate following exposure of cardiomyocytes to PHE or ET-1, were identified. However, it should be kept in mind that the proportions of inactive PKC isoforms present in the particulate fraction differs between the isoforms (Rybin and Steinberg, 1994) and may also be dependent upon extraction conditions. Furthermore, in the absence of standards during immunoblotting, the relative levels of expression of the different isoforms cannot simply be calculated from the densities of the immunoreactive bands on the blot because of differences in the affinity of the antibodies for the PKC isoforms. Nevertheless, the results on ^{32}P-incorporation into proteins of the cell homogenate and translocation of PKC immunoreactivity suggest that PKC isoenzymes other than PKC-α and PKC-ϵ are involved in PHE induced phosphorylation of the 30 kDa protein. The 30 kDa protein may be identical with the myofibrillar compound troponin I which is involved in the regulation of the Ca^{2+} affinity of troponin C (Wattanapermpool *et al.*, 1995). There is evidence that upon α_1-adrenergic stimulation of myocardium the Ca^{2+} sensitivity of the myofibrils is increased which could explain the positive inotropic effect due to PKC activation without the necessity of a large Ins(1,4,5)P_3 induced Ca^{2+} response (Terzic *et al.*, 1993).

The expression of the specific PKC isoenzymes, and their agonist-dependent translocation is still in a very early phase of investigation, and appears to be species and age dependent (Pucéat *et al.*, 1994; Rybin and Steinberg, 1994; Steinberg *et al.*, 1995; Disatnik *et al.*, 1994). In a study in which immunofluorescence techniques were used, six PKC isoenzymes, α, δ, ϵ, ζ but also βI and βII, were shown to be present in neonatal rat cardiomyocytes. Moreover, PKC-α and PKC-ζ were translocated from the cytoplasm to the perinuclear region after a 2-min exposure to norepinephrine (Disatnik *et al.*, 1994). Already 11 subspecies of PKC have been identified in mammalian tissues (Nishizuka, 1992; Harrington and Ware, 1995), of which some are likely to be expressed simultaneously in heart (Steinberg *et al.*, 1995). However, the incongruity in the reports on the presence, location, activation and translocation of PKC isoenzymes should make us aware of the complexity in regulation and location of these messengers.

Consequences of PKC activation in cardiomyocytes

PKCs may regulate many cardiac functions: energy metabolism, contractile state, cellular Ca^{2+} handling, hypertrophic growth, changes of phenotype and ischemic preconditioning (Sugden *et al.*, 1995). Different PKCs may influence a single biological event and the ultimate response depends on the overall integrated effect of these phosphorylations. One of the biological responses to activation of PKC that has been identified in the model of cultured cardiomyocytes is the hypertrophic growth concomitant with the characteristic alterations in specific gene transcriptions (reexpression of ANF, β-myosin heavy chain, skeletal muscle α-actin and upregulation of the constitutive genes encoding myofibrillar proteins, myosin light chain-2, cardiac muscle α-actin). Direct activation of PKC with phorbolesters (Henrich and Simpson, 1988; Dunmon *et al.*, 1990; Allo *et al.*, 1991 and 1992) or by transfection of cDNA constructs encoding constitutively active PKC (Shubeita *et al.*, 1992; Kariya *et al.*, 1991;

Decock *et al.*, 1994) produces hypertrophy in cultured cardiomyocytes. Mitogen activated kinases (MAPK) and MAPK kinase (MAPKK) are reported to be strongly activated by ET-1 and PHE (Bogoyevitch *et al.*, 1993 and 1994), which occurred in parallel with hypertrophy of cardiomyocytes. Because of this, it can be proposed that activation of MAPK and MAPKK may be one of the signalling events downstream of PKC activation in these cells (Fig. 1). At present, the mechanism by which the activation of the MAPK-cascade might lead to hypertrophy and an altered phenotype is not clear.

PHE and ET-1 receptor mediated activation of PLC-β: role of $[Ca^{2+}]_i$ and membrane phospholipids

In various cell types it was shown that $[Ca^{2+}]_i$ has profound effects on PLC-β activation either directly or by potentiating the receptor-mediated response (Abdel-latif, 1986; Cockcroft and Tomas, 1992). Chapter 3 describes the influence of changes in $[Ca^{2+}]_i$ on the PHE and ET-1 receptor mediated activation of PLC-β in cultured cardiomyocytes. For this purpose, PLC-β activity was monitored by measurement of the formation of total [^{3}H]-inositolphosphates ([^{3}H]InsP_n) in the presence of Li^+. Changes in cardiomyocyte $[Ca^{2+}]_i$ were induced by several means: addition of high $[Ca^{2+}]_e$, Ca^{2+} ionophores, Na^+/K^+-ATPase inhibitor (ouabain) and high $[K^+]_e$. Under some of the experimental conditions the actual changes in $[Ca^{2+}]_i$ were verified using the fluorescent Ca^{2+} probe fura-2. Basal PLC-β activity was shown to be unaffected by either increased or decreased $[Ca^{2+}]_i$. However, changes of $[Ca^{2+}]_i$ did influence the receptor-stimulated PLC-β activity. PHE as well as ET-1 evoked PLC-β activity was increased in the presence of high external Ca^{2+} concentration and Ca^{2+}-ionophore A23187. Depolarization of the SL of the cardiomyocytes with a high $[K^+]_e$, known to result in an increase of $[Ca^{2+}]_i$, increased ET-1-mediated PLC-β activity but unexpectedly reduced PHE- mediated PLC-β activity. On the other hand, as expected, total removal of external Ca^{2+} by addition of EGTA strongly inhibited receptor-mediated PLC-β activity. Several steps in the receptor-mediated transmembrane signalling cascade, e.g. receptor-G-protein coupling and G-protein interaction with PLC-β, are likely subjected to regulation by $[Ca^{2+}]_i$. The α_1-adrenergic receptor as well as the ET-1 receptor are characterized by having seven membrane-spanning domains connected by extracellular and intracellular loops (Berridge, 1993). The intracellular loops, for example, could provide sites for various actions such as phosphorylation or binding of modulating agents, e.g. Ca^{2+} (induced by high $[K^+]_e$), which could be causally related to the unexpected observation of an agonist dependence of high $[K^+]_e$ effects in receptor-mediated PLC-β (Chapter 3). In addition, the function of G-proteins, i.e. dissociation and association of its α- and $\beta\gamma$-subunits, or the putative role of the $\beta\gamma$ subunit in activation of PLC-β are likely to be influenced by $[Ca^{2+}]_i$ (Clapham, 1995). In Chapter 3 we report that GTPγS stimulated PLC-β activity in saponin-permeabilized cardiomyocytes was strongly activated by Ca^{2+} ions in the concentration range of 0.1 to 10 μM. To which degree PLC-β itself or the receptor-G-protein coupled PLC-β is activated by Ca^{2+} cannot be derived from these results. However, we conclude that the cytoplasmic free Ca^{2+} concentration plays a feedback activating role during the receptor-mediated PLC-β activation only when $[Ca^{2+}]_i$ increases due to action of Ins(1,4,5)P_3.

Membrane phospholipids

In Chapter 6 we reviewed the literature describing the effects of a change of the fatty acid composition of the membrane phospholipids by dietary means or otherwise on various functions of the intact heart (e.g. α_1- and β-adrenergically stimulation of contractility, slow Ca^{2+}-channel current, and β-adrenoceptor induced arrhythmias). We studied the effects of a changed polyunsaturated fatty acid (PUFA) composition of the membrane phospholipids on receptor stimulated PLC-β activity in an almost homogeneous preparation of cardiomyocytes (Chapter 7). A remarkable finding in this study was that cultured cardiomyocytes pretreated for 3-4 days with the PUFA 20:5n-3 revealed the most dramatic effects on agonist stimulated PLC-β activity when compared to cells not treated or treated with equimolar amounts of 18:0/18:1n-9 or the PUFA 18:2n-6. Moreover, receptor-mediated activity of PLC-β in 20:5n-3 pretreated cells was observed to be agonist dependent. Stimulation with ET-1 resulted in an increased PLC-β activity in comparison to cells treated with control-medium (without addition of fatty acid), whereas stimulation with PHE resulted in a decreased PLC-β activity. The mechanism of action of membrane 20:5n-3 on specific functional components of the signalling cascade underlying these agonist dependent differences is at present not clear. When the role of the receptor on PLC-β activation was circumvented by means of direct activation of PLC-β by GTPγS in saponin-permeabilized cells, the 20:5n-3- but not the 18:0/18:1n-9 or 18:2n-6 treated cells, revealed a reduced accumulation of [^{3}H]InsP$_n$ in comparison to the control treated cells. This finding implicates that agonist dependent effects of altered 20:5n-3 incorporation into the membrane phospholipids occur not only at the level of the agonist-receptor but also at the level of receptor-G-protein or G-protein-PLC-β coupling. As far as the receptor function is concerned, the PUFA effects may matter the number of binding sites or the affinity for the ligand, provided that α_1-adrenergic and ET-1 receptors are coupled to the same isotype of PLC-β.

On the basis of the results obtained in Chapter 6 and 7, we argue that the effect of modification of PUFA composition of the membrane phospholipids on receptor-mediated PLC-β can not be restricted to just one aspect. As we demonstrated before, the PUFAs are incorporated in membrane phospholipids, particularly in the bulk phospholipids PtdChol and PtdEth but also in PtdIns, PtdIns(4)P and Ptd(4,5)P$_2$ (Lamers *et al.*, 1992 and 1993, Chapter 6). Apart from changes in 1) the dynamic properties of the membrane, such as fluidity, 2) the bilayer distribution of phospholipids or 3) the total cholesterol content of the membrane, the affinity of the multiple enzymes involved in the PtdIns-cycle for the altered phospholipid substrates may be different. For example, PtdIns-kinase and PtdInsP-kinase may preferentially phosphorylate molecular species of phosphoinositides which contain particular PUFAs. Similarly, PLC-β itself can be affected by changes of the molecular species of PtdIns(4,5)P$_2$ or of the core phospholipids, for which evidence was obtained when the saponin-permeabilized 20:5n-3 treated cardiomyocytes were stimulated with GTPγS (Chapter 7). Changes in the molecular species of the PLC-β hydrolysis product 1,2-DAG are likely to be reflected in its ability to activate PKC isoenzymes, and this will have consequences for the events occurring downstream to PKC (see Chapter 6). The physicochemical and dynamic properties of the SL, such as its fluidity and cholesterol content, were also shown to be affected particularly by 20:5n-3 treatment of cardiomyocytes (Chapter 7). It further complicates the interpretation of the observed effect of 20:5n-3 treatment on receptor-mediated PLC-β activity. From our data it followed that in 20:5n-3 treated cells a marked

increase of membrane cholesterol content parallelled a slight decrease in membrane fluidity. Apparently, the fluidizing effect of a high degree of polyunsaturation of the fatty acids incorporated in the phospholipids overcompensated the 'stiffening' effects of increased membrane cholesterol content. In computer modeling studies and in monolayer studies, it has been demonstrated that on the one hand the fatty acid composition of DAG-moiety of the membrane phospholipids determines thermodynamically the stability of the acyl chain packing in the membrane bilayer because this packing is mostly favoured by the presence of PUFAs on the *sn*-2 position (Applegate and Glomset, 1991a and 1991b). This would explain the fact that phospholipids with a particular fatty acid composition are located in different stable domains. On the other hand, in a study in which synthesized monolayers were used, it was shown that cholesterol can preferentially form stable complexes with certain membrane phospholipids (Bittman *et al.*, 1994). Regarding possible changes in membrane acyl packing or the formation of stable domains it is interesting to note that we observed a resistance of 20:5n-3 treated cells to saponin permeabilization (Chapter 7). Saponin is considered to disturb membrane integrity, by clustering membrane cholesterol. Thus, by induction of an increase of membrane cholesterol content, 20:5n-3 treatment protects the membrane against the destabilizing action of saponin.

The relevance of the observed agonist-induced second messenger responses for the *in vivo* situation

In neonatal rat cardiomyocytes the putative Ca^{2+} releasing messengers ($Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$) were found to be unaffected upon either stimulation with PHE or ET-1, whereas 1,2-DAG levels were found to be increased after 8 to 16 min of stimulation. The question may arise: "What is the relevance of the PHE or ET-1 induced second messenger responses for the induction of positive inotropy and late hypertrophy in the *in vivo* situation?"

This question can be addressed from two points of view:

Firstly, the $Ins(1,4,5)P_3$ limb of the PtdIns cycle is of less or no importance following receptor-mediated PLC-β action in transducing the message. Generally, the PtdIns-cycle is a signalling system of which the $Ins(1,4,5)P_3$ mediated Ca^{2+} release is a central event in the development of the biological responses in many tissue. In the heart, Ca^{2+} is essential to the generation of electrical activity in the pacemaker cells, conduction in the conducting cells, and the processes of excitation-contraction coupling and relaxation in the cardiomyocytes. Interference of the precisely controlled Ca^{2+} fluxes by receptor-mediated Ca^{2+} signalling could therefore be undesirable. Ca^{2+} may not be a "safe" second messenger for mediating hormonal signals in pacemaker cells, conducting cells and cardiomyocytes, and its sudden cellular mobilization is prevented by relatively fast operation of the enzyme systems involved in phosphate degradation. The other product of PLC-β's action, 1,2-DAG, plays a major role in activating PKCs, which are responsible for the phosphorylation of target proteins, such as components of the contractile system (sensitization of the myofilaments), ion channels and transcription factors (Fig. 1). Moreover, these PKCs may provide cross-talk with other messenger systems, such as PLD, tyrosine kinase or MAPK systems, to propagate the agonist-induced signal (Fig. 1). In turn, cross-talk with other signalling pathways such as PLD are of importance in maintaining the necessary levels of DAG for continued PKC activation

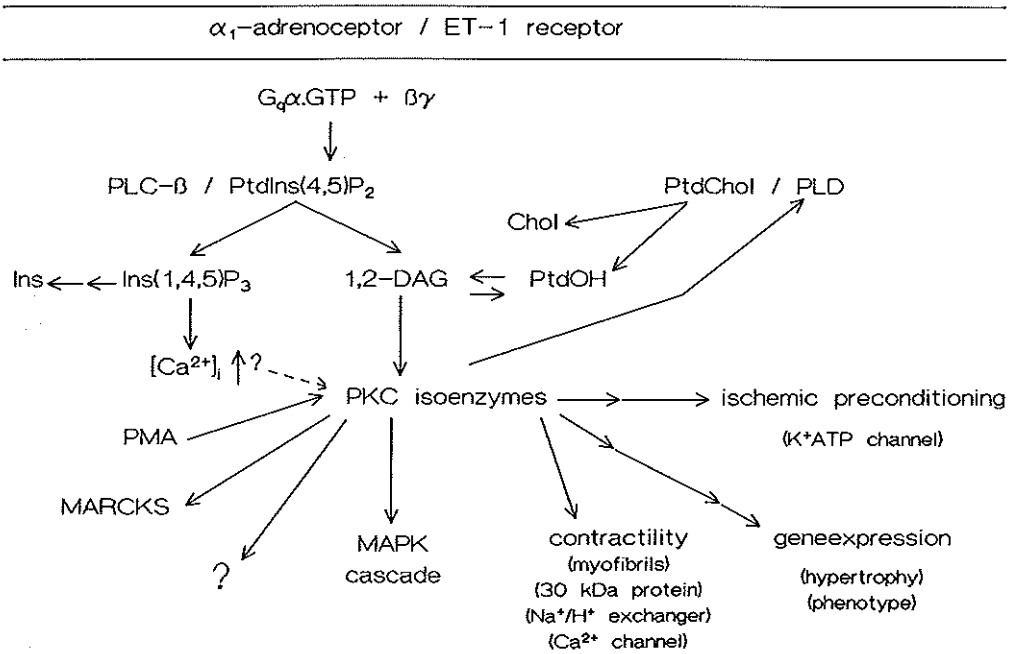

Figure 1 Signalling pathway mediated through phospholipid hydrolysis. Binding of α_1-adrenergic agonists or ET-1 to their membrane receptor results in G_q (α_q-GTP-$\beta\gamma$) dissociation and α_q-GTP loading. α_q-GTP activates the membrane-bound enzyme PLC-β which stimulates hydrolysis of membrane-bound PtdIns(4,5)P_2 to produce the messengers: hydrophilic Ins(1,4,5)P_3 and hydrophobic 1,2-DAG. Ins(1,4,5)P_3 regulates intracellular Ca^{2+} movements, but is probably of limited importance in this regard in cardiac myocytes. 1,2-DAG activates classical (cPKCs) and novel (nPKCs) PKCs in a process that involves translocation of soluble PKC to the membrane. c- and n- PKCs can be directly activated by the phorbolester PMA. PKCs directly phosphorylate or indirectly bring about phosphorylation of a number of proteins (30 kDa protein, MAPK, myristoylated alanine-rich C kinase substrate (MARCKS), Na^+/Ca^{2+} exchanger protein, K^+_{ATP} channel protein, etc.) which couple PKCs to the ultimate biological responses: positive inotropy, hypertrophy, altered gene expression or ischemic preconditioning. 1,2-DAG is rapidly phosphorylated to PtdOH by DAG kinase to terminate its activity and is subsequently salvaged for phospholipid synthesis. 1,2-DAG can also be produced by PLD-mediated hydrolysis of membrane PtdChol to produce PtdOH. In this reaction PtdOH is formed, and subsequently hydrolysed by a PtdOH phosphohydrolase (PPH) activity that may be intrinsic to the PLD enzyme. Production of 1,2-DAG from PLD/PtdChol may maintain 1,2-DAG concentrations over a prolonged period because PLD is activated by PMA and PKC. Modified after Sugden and Bogoyevitch (1995). (Nishizuka, 1992; Nakamura *et al.*, 1993). However, in our experiments slight amounts of Ca^{2+} are released in cardiomyocytes upon receptor stimulation, although somewhat delayed. These slight amounts of Ca^{2+} released are likely regulatory to the signalling processes, such as feed-forward activation of PLC-β (Chapter 3; Clapham, 1995).

Secondly, the locality of the signalling processes may be of major importance, which would vouch for the presence and the integrity in function of the different (agonist-dependent) signalling pathways (Chapter 4). This issue comprises a major problem in the elucidation of signalling processes in general, and is likely to be a future challenge in this area. Concerning the PtdIns-signalling route activated by α_1-adrenergic agonists and ET-1, the creation of very local environments could explain why the Ca^{2+} releasing components Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were not detectably elevated. In the conception of a microenvironment messenger concentrations (of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 but also of Ca^{2+}) are elevated to levels which may locally activate channel proteins, protein kinases etc. (Clapham, 1995). In that way undesired interference with the precisely controlled whole cell Ca^{2+} fluxes involved in action potential generation, and conduction, excitation-contraction coupling and relaxation are prevented. Regarding microenvironments, in neonatal rat cardiomyocytes the localization of three different exchangeable Ca^{2+} compartments was reported: 1) a slow exchangeable mitochondrial compartment; 2) a compartment with an intermediate rate, most likely the SR; and 3) a fast compartment, resided in the subsarcolemmal space between SR and SL (Post and Langer, 1992; Post *et al.*, 1993). The local intracellular Ca^{2+} concentration could activate Ca^{2+}-dependent protein kinases (Ca^{2+}-calmodulin-dependent protein kinase and Ca^{2+} dependent PKCs) which would in turn activate Ca^{2+} release channels. Moreover, Ca^{2+} itself could modulate the activity of the Ca^{2+} release channels by direct binding to these channels (Zang *et al.*, 1993; Clapham, 1995). Also the excitation-contraction coupled Ca^{2+} accumulation in the heart is suggested to be under "local control". The whole cell Ca^{2+} transients are explained in terms of synchronous recruitment of single, stereotypic unitary local Ca^{2+} transients, controlled by single L-type Ca^{2+} channels in the sarcolemma (López-López *et al.*, 1995). Furthermore, the subcellular distribution of the RYR receptor and the IP_3R in the SR meets the concept of locality (Kijima *et al.*, 1993). The distinct location of these two Ca^{2+} releasing channels would ensure the functional integrity of signalling systems which employ Ins(1,4,5)P_3 as messenger. It has been demonstrated immunocytochemically and at the level of the electron microscope that in rat ventricular cardiomyocytes the Ins(1,4,5)P_3 receptor was specifically located at the intercalated discs and not in the SR (Kijima *et al.*, 1993). The RYRs, however, were observed as transverse bands along the entire length of the ventricular cardiomyocyte and corresponded with the I-bands closely localized to the terminal cisternae of the SR (Kijima *et al.*, 1993). The authors of the latter report postulated the role of the Ins(1,4,5)P_3 receptor in the mediation of Ca^{2+} entry through the intercalated discs and/or intercellular signalling between cardiomyocytes.

"Cross-talk" between different signalling pathways must include the concept of locality as well. The existence of different α_1-adrenergic and ET-1 receptor subtypes, the presence of 20 α- and at least 4 β- and six γ-subunits of G-proteins and selectivity of the signal transduction for the different PLC subtypes might depend on compartmentalization of the different sets of subtypes and/or on selective protein-protein interactions. Specific interactions could be accomplished by differences in affinity or efficacy of interactions and could be modulated by local regulation of the relative number of each receptor subtype or G-protein subunit (Iniguez-Liuhi *et al.*, 1993; Neer, 1995). In this regard, the locality concept concerns the G-protein as well. The prior belief that the role of $\beta\gamma$-subunits is only to chaperone the α-subunit between the active and inactive state, is more and more set aside since $\beta\gamma$ dimers were found to activate ion-channels, adenylate cyclase, PLA_2, PtdIns 3-kinase and the PLC-β

mediated PtdIns signalling cascade (Boyer *et al.*, 1994; Iniguez-Liuhi *et al.*, 1993). Even the MAPK pathway is reported to be activated by $\beta\gamma$-dimers through *ras* (Neer, 1995).

Similarly, the functional integrity of the PKCs can be explained by the locality concept. The members of the PKC family show different requirements of phospholipid metabolites. Moreover, the fatty acid composition of 1,2-DAG may vary depending on whether they are derived from PtdIns or PtdChol, which may be of importance for the activation of the different PKCs (Chapter 6). Most likely, the various PKCs respond differently to Ca^{2+}, molecular species of 1,2-DAG and other phospholipid degradation products, and thereby produce distinct activation patterns with respect to the extent, duration, and intracellular localization (Nakamura *et al.*, 1993).

The matter of locality is probably one of the most challenging issues in the area of signal transduction and awaits further investigation to bridge the "grey" area of transmembrane events and more downstream up to the level of the nucleus, and beyond.

The relevance of the used model for agonist-induced biological responses *in vivo*

In the experiments described in this thesis cultured neonatal rat cardiomyocytes were used, which have offered us the advantage of a fairly pure cell culture (more than 90% cardiomyocytes) grown on glass and plastic, which revealed spontaneous contractions. Moreover, neonatal rat cardiomyocytes in culture possess the PtdIns-cycle coupled α_1-adrenergic or ET-1 receptors that upon stimulation lead to a hypertrophic response, including the transient expression of immediate-early genes, the expression of the late genes, the reexpression of some fetal proteins and late down-regulation of the Ca^{2+} ATPase of the SR, sharing many features observed *in vivo*. However, a number of observations with neonatal rat cardiomyocytes in culture do not allow simple extrapolation to 1) adult rat cardiomyocytes, 2) cardiomyocytes of other species, and 3) to the *in vivo* situation.

Although after birth cardiomyocytes loose their capacity to proliferate, *in vitro* ventricular myocytes from 2 days old rats continue to differentiate during 15 days after mitogen withdrawal. In the absence of hemodynamic and neurohumoral influences, the cellular differentiation was shown to result in the formation of cross-striations, increased proportion of the M-subunit of creatine kinase, a stable expression of α-cardiac actin and myosin heavy chain mRNAs and downregulation of skeletal α-actin mRNA (Ueno *et al.*, 1988). Serum, as it was also present in the culture medium used in our experiments, contains a number of stimuli such as catecholamines, which act as potent growth agonists exerting multiple effects. For example, skeletal α-actin mRNA expression is an important indicator of differentiation of cardiomyocytes but in the case of myocytes in culture, of de-differentiation. Serum can exert striking effects on the expression of skeletal α-actin mRNA formation, such as inhibition of skeletal α-actin mRNA when it is normally expressed and induction when it is normally silent, depending on the state of differentiation (Ueno *et al.*, 1988). Moreover, it was reported that cellular cholesterol content increased with age of the cultured cardiomyocytes (Shmeeda *et al.*, 1995). This is relevant in particular to our experiments in which the contraction frequency was studied after long-term PUFA treatment of the cardiomyocytes. The increase of cellular cholesterol reported by Shmeeda *et al.* (1995) was concurrent with a decrease in the cells' beating rate, which could be restored by removal of the excess of serum cholesterol by

treatment with small unilamellar vesicles of egg phosphatidylcholine (Shmeeda *et al.*, 1995). Accordingly, it is of importance to standardize the experiments on cultured neonatal cardiomyocytes particularly with regard to the age of the cultured cells. We have carried out our experiments always after 5-6 days.

Apart from variation in culture time, a variety of dissimilarities are found between isolated myocytes and intact heart preparations, between myocytes from neonatal and adult rats, as well as between cardiomyocytes of different mammalian species.

For example, the expression and density of α_1-adrenergic receptors showed differences between parts of the heart but also between species. The density of α_1-adrenergic receptors in atrial tissue is much lower than in ventricular tissue of rabbit, pig, calf and human (Steinfath *et al.*, 1992). Moreover, the density of α_1-adrenergic receptors in rat ventricular tissue appears to be exceptionally high, 5 to 8 fold higher than in ventricular tissue of rabbit, pig, calf and human. In cultured neonatal rat ventricular myocytes the α_1-adrenergic receptor subclasses 1A and 1B are expressed, of which α_{1A} was demonstrated not only to mediate the PtdIns(4,5)P_2 hydrolysis but also the induction of the immediate-early genes and the increase in myocardial cell size (Knowlton *et al.*, 1993; Kimura *et al.*, 1994). In rabbit ventricular myocytes it was reported that the α_{1B}-subtype mediated the positive inotropy and PtdIns hydrolysis (Suzuki *et al.*, 1990; Endoh *et al.*, 1992). The existence of the different subtypes was shown to vary with culture time and species. At day 3 after isolation, neonatal rat cardiomyocytes possessed the subtypes α_{1A} and α_{1B} in a ratio of 35:65, of which the α_{1B}-subtype appeared to play an important role in the norepinephrine-induced positive chronotropic response. At the seventh day after isolation, 74% of the α_1-adrenergic receptors belong to the α_{1A}-subtype and 26% to the α_{1B}-subtype (Kimura *et al.*, 1994). Furthermore, in the presence of the β-adrenergic receptor blocker propranolol, the α_1-adrenergic agonist PHE was shown to increase the L-type channel Ca^{2+} current in neonatal but not in young (1-2 months old) rat cardiomyocytes, suggesting that the α_1-adrenergic receptor may have switched from coupling to the L-type voltage gated Ca^{2+} channel to coupling the PtdIns hydrolysis pathway (Liu *et al.*, 1994; Kimura *et al.*, 1994).

Not only differences in receptor density, subtype or coupling to channel or messenger systems, but also differences in the messenger systems such as the PtdIns turnover pathway were observed between cultured neonatal myocytes and intact neonatal heart tissue. These differences may reflect the differences in developmental stage due to de-differentiation of the cardiomyocytes maintained in culture *ex vivo*. It was reported that cell isolation and cell culture may induce changes in the nature of the pathway. Particularly in cultured neonatal cardiomyocytes it was reported that a remnant of the Ins(1,4,5)P_3 kinase pathway had remained, as was also found in a number of different heart preparations, e.g. isolated perfused rat heart and isolated guinea-pig heart (Woodcock *et al.*, 1991 and 1992). This remnant part of the pathway in cultured neonatal cardiomyocytes was suggested to be selectively deleted during maturation and differentiation of cardiomyocytes *in situ* (Woodcock *et al.*, 1992). The pathway in intact neonatal heart was similar to that in adult heart (Woodcock *et al.*, 1992 and 1987).

In addition, methodological problems with regard to isolation- or extraction procedures may give rise to ambiguous results. For example, enzymatic isolation of rat heart cells was reported to be associated with differences in cytoplasmic subcellular Ca^{2+} patterns of the cardiomyocytes (Wier *et al.*, 1987). The isolated population of cardiomyocytes appeared to

be heterogeneous containing quiescent cells, spontaneously contracting cells, and hypercontracted cells. This heterogeneity is likely to have consequences for the signal transduction pathways and Ca^{2+} responses. Extraction procedures were mentioned to produce artifacts. For example acidic chloroform/methanol extraction of intact heart tissue may result in the formation of methyl-phosphoryl-inositol 1,4,5-trisphosphate, which could erroneously be identified as Ins(1,3,4)P_3 (Woodcock *et al.*, 1992 and 1993).

Another major difference between rat neonatal and adult cardiomyocytes on the one hand and cardiomyocytes of various species on the other hand is the expression, function and regulation of the PKC isoenzymes. Rat neonatal and adult cardiomyocytes both express PKC α, δ, and ϵ. PKC ζ is expressed in neonatal cardiomyocytes but only very weak in adult cardiomyocytes (Pucéat *et al.*, 1994). PKC α immunoreactivity was low in extracts from adult hearts, whereas PKC α was completely absent in extracts from isolated adult ventricular myocytes, and it was suggested that in the adult heart PKC α resides in nonmyocyte elements. PKC δ was detected in greater abundance in fetal and neonatal myocardium than in adult myocardium. The PKC ζ isoform was most abundant in fetal heart extracts compared to cardiac tissue of older animals (Rybin and Steinberg, 1994).

Conclusion

In this thesis we have shown that stimulation of neonatal rat cardiomyocytes with PHE and ET-1, ultimately leading to a "hypertrophic"-like response in these cells, activate the PtdIns-cycle and downstream signalling processes in a distinct manner depending on the agonist used. With both stimuli, Ins(1,4,5)P_3-levels remained low either by rapid direct dephosphorylation (PHE) or phosphorylation to Ins(1,3,4,5)P_4 and subsequent dephosphorylation (ET-1). Ins(1,4,5)P_3 appears to be less important as second messenger in these cells, in contrast to 1,2-DAG that was increased either rapidly (ET-1) or after some lagphase (PHE). Cellular Ca^{2+} responses and protein kinase C activation by the agonists and consequent protein phosphorylation were also agonist-dependent. Membrane bilayer dynamics and its fysicochemical properties influenced the activity of the recptor-mediated signalling pathways as well, as was observed after the change of the n-3 polyunsaturated fatty acid content of the membrane phospholipids. The data show that the signal transduction leading to hypertrophy is complex and the multiple stimuli that are involved *in vivo* likely do not all lead to exactly the same responses.

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LIST OF ABBREVIATIONS

AA	arachidonic acid (20:4n-6)
A_2C	2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octylcyclopropyl)-octanoate
ADP	adenosine 5'-diphosphate
ANF	atrial natriuretic factor
AngII	angiotensin II
$AT_{1 \text{ or } 2}$	angiotensin receptor 1 or 2
ATP	adenosine 5'-triphosphate
$[Ca^{2+}_{free}]_i$	cytoplasmic free Ca^{2+} concentration
CaM-PK	Ca^{2+}-calmodulin-dependent protein kinase
cAMP	cyclic adenosine 3'5'-monophosphate
cAMP-PK	cAMP-dependent protein kinase
CDP-DAG	cytidyldiphosphate-diacylglycerol
cGMP	cyclic guanosine 3'5'-monophosphate
cGMP-PK	cGMP-dependent protein kinase
CMP-PA	cytidylmonophosphate-phosphatidic acid
cPKC	classical protein kinase C's
CTP	cytidyltriphosphate
1,2-DAG	1,2-diacylglycerol
DBI	double bond index
DHA	docosahexaenoic acid (22:6n-3)
DPA	docosapentaenoic acid (22:5n-3)
EGTA	ethylene glycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetra-acetic acid
EGF	epidermal growth factor
EPA	eicosapentaenoic acid (20:5n-3)
ET_A	cardiac endothelin-1 receptor
ET-1	endothelin-1
FGF	fibroblast growth factor
$F_{340 \text{ nm or } 380 \text{ nm}}$	fluorescence intensity at 340 nm or 380 nm
Fura-2 AM	fura-2 acetoxymethylester
GLC	gas-liquid chromatography
G-protein	guanosine 5'-triphosphate-binding protein
GS	glycogen synthetase
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
Lyso-PtdIns	lysophosphatidylinositol
Ins	inositol
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid
HPLC	high performance liquid chromatography
5-HT	serotonin
$InsP_n$	total inositolphosphates
$Ins(1,4,5)P_3$	inositol(1,4,5)trisphosphate
$Ins(1,3,4,5)P_4$	inositol(1,3,4,5)tetrakisphosphate
IP_3R	$Ins(1,4,5)P_3$ receptor in the SR
LDH	lactate dehydrogenase
MAPK	mitogen-activated protein kinase
MAPKK	MAPK-kinase
MHC	myosin heavy chain
MLC	myosin light chain
mRNA	messenger ribonucleic acid
nPKC	novel protein kinase C's
PHE	phenylephrine
PLC	phospholipase C
PLD	phospholipase D
PLA_2	phospholipase A_2
PMA	phorbol 12-myristate 13-acetate
PtdIns	phosphatidylinositol
PtdIns(4)P	phosphatidylinositol 4-monophosphate
$PtdIns(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PtdOH	phosphatidic acid
PUFAs	polyunsaturated fatty acids
R	ratio image, resulting after dividing the 340 nm image ($F_{340 \text{ nm}}$) by the 380 image ($F_{380 \text{ nm}}$)
r_{ss}	fluorescence steady state anisotropy
RYR	ryanodine receptor in the SR
SL	sarcolemma
SR	sarcoplasmic reticulum
TLC	thin layer chromatography
TMA-DPH	1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulphonate
TnI	troponin-I
TnT	troponin-T
TPA	12-O-tetradecanoylphorbol-13-acetate

SUMMARY

The adrenergic nervous system plays an important role in the acute positive inotropic, chronotropic and late hypertrophic response of the myocardium to a cardiac workoverload. In the normal situation, the adrenergic inotropic and chronotropic response is predominantly the result of stimulation of β-adrenergic receptors. However, under pathological conditions the contribution of the α_1-adrenergic receptors increases, particularly with regard the role of catecholamines in the development of myocardial hypertrophy. Hypertrophy is paralleled by phenotypic changes such as e.g. respectively down- and up regulation of the sarcoplasmic reticulum (SR) Ca^{2+} ATPase pump and atrial natriuretic factor expression. Indeed, it is known for a long time that the β-adrenergic receptors of the heart, which are coupled to the G_s-protein-adenylate cyclase-cAMP pathway, are decreased in number under conditions of chronically increased workload. The number of α_1-adrenergic receptors is reported to be increased or to be unchanged under these conditions. α_1-Adrenergic receptors are coupled to the G_q-protein-phospholipase C-β (PLC-β) mediated phosphatidylinositol (PtdIns) cycle. In this signalling pathway, PLC-β catalyses the hydrolysis of the membrane compound phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) yielding three putative messengers: inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$) and 1,2-diacylglycerol (1,2-DAG). $Ins(1,4,5)P_3$ has been shown to release Ca^{2+} ions from intracellular Ca^{2+} stores (e.g. the SR in cardiomyocytes), while the role of $Ins(1,3,4,5)P_4$ in the mobilization of Ca^{2+} ions from these stores in myocytes is largely unknown. 1,2-DAG has been shown to activate protein kinase C (PKC) isoenzymes, sometimes in conjunction with Ca^{2+}. Activation of PKC leads to phosphorylation and activation or inactivation of various functional proteins of the contractile apparatus, ion channels in the sarcolemma, and transcription factors. Recently it has become clear that factors other than catecholamines play a role in the induction of myocardial hypertrophy. One of these factors is endothelin-1 (ET-1), a peptide which is locally secreted by the coronary endothelial cells. In the cardiomyocyte, ET-1 can activate the specific ET_A receptor which, similarly to the α_1-adrenergic receptor, is coupled to the G_q-protein-PLC-β mediated PtdIns cycle.

At the start of our investigation the signalling mechanism via the PtdIns-cycle in cardiac tissue was not completely understood. Moreover, α_1-adrenergic receptors as well as ET-1 receptors, linked to the PtdIns cycle were involved in the induction of myocardial hypertrophy which ran parallel to phenotypic alterations of the heart. This explains our interest to comparatively study the α_1-adrenergic and ET-1 receptor-activated PtdIns-cycle in heart cells.

Phospholipids, as they are present in the sarcolemma of the cardiomyocytes, have many functions. They play a structural role in the bilayer, thereby facilitating the optimal motional conditions for activity of the multiple complex proteins embedded in the sarcolemma. Sarcolemmal phospholipids, such as the minor component $PtdIns(4,5)P_2$, are also substrates for hydrolytic enzymes, such as PLC-β. In this manner these are important for the transduction of signals from the exterior to the interior of the cell. Earlier, it has been shown that modulation of the polyunsaturated fatty acid composition of the membrane phospholipids by dietary means affected the α_1-adrenergic receptor mediated positive inotropic responses in heart.

To study the receptor-mediated PtdIns cycle and the effects of polyunsaturated fatty acids thereupon, spontaneous beating neonatal rat cardiomyocytes in culture were utilized. Earlier,

we and others had shown that prolonged stimulation (24-48 h) of neonatal cardiomyocytes with the α_1-adrenergic agonist phenylephrine (PHE) and ET-1, results in hypertrophy accompanied by the characteristic phenotypic changes. The cultures of 90 % pure myocytes could be cultured on plastics and glass, and therefore, we could study the particular cardiomyocytic Ins(1,4,5)P_3, 1,2-DAG and phosphorylation responses by means of prelabelling of the cells with [^{3}H]inositol, [^{3}H]glycerol and $^{32}P_i$, respectively. Changes in intracellular free Ca^{2+} concentration were assessed fluorometrically in microscopes and spectrofluorometer using fura-2 as a calcium probe.

The activity of PLC-β following stimulation with PHE or ET-1 is regulated by cytosolic Ca^{2+}, as we observed in experiments in which we changed the external and internal Ca^{2+} concentrations (Chapter 3). Total removal of external free Ca^{2+} by addition of EGTA strongly inhibited the ET-1 induced but not the basal PLC-β activity. High external Ca^{2+} in the presence of calcium ionophore revealed an elevation of ET-1 and PHE stimulated PLC-β activity, but not of basal activity. When cytosolic Ca^{2+} was increased by high external K^+ concentration stimulation of the cardiomyocytes with ET-1 induced an elevated [^{3}H]InsP_n production but, unexpectedly, [^{3}H]InsP_n production after stimulation with PHE was inhibited. Nevertheless, in saponin-permeabilized cardiomyocytes we could definitively demonstrate that basal as well as GTPγS stimulated phospholipase C activity was activated by free Ca^{2+} in the physiologically relevant concentration range of 0.1-10 μM. It was concluded that the cytoplasmic free Ca^{2+} concentration plays a feedback activating role during the receptor-mediated PLC-β activation. In this phase of the investigation the question remained whether Ca^{2+} actually increased upon agonist stimulation.

To address this question whether Ca^{2+} rises or not, the responses of the putative Ca^{2+} releasing messengers produced upon stimulation with either agonist were studied. Most remarkable in these experiments is the observation that Ins(1,4,5)P_3 was not detectably elevated after either stimulation with PHE or ET-1 in the presence of monophosphatase inhibitor Li^+, nor was its direct phosphorylation product Ins(1,3,4,5)P_4. That PLC-β had been active after stimulation with either agonist was proven by the abundant accumulation of [^{3}H]inositolmono(4)phosphate. It appeared that in neonatal cardiomyocytes Ins(1,4,5)P_3 was either rapidly dephosphorylated by 5-phosphatase (after stimulation with PHE) or directly phosphorylated by the 3-kinase and subsequently dephosphorylated (after stimulation with ET-1). We could not explain that after stimulation of the cardiomyocytes with ET-1 the inositol follows a metabolic route which slightly differed from that observed after stimulation with PHE. However, in general, these data demonstrate that the biologically active inositolphosphates are rapidly dephosphorylated to attenuate the agonist-induced Ca^{2+} mobilizations which may disturb the normal Ca^{2+} transients intimately involved in to excitation-contraction coupling.

In contrast to Ins(1,4,5)P_3, the other product of PLC-β activity, 1,2-DAG, did increase upon stimulation with PHE or ET-1, although the time course of the 1,2-DAG response appeared to be dependent of the agonist used. We concluded from these experiments that in neonatal cardiomyocytes 1,2-DAG is likely to play an important role in transducing the signal activation of PKC isozymes.

Not all PKC isozymes need Ca^{2+} for their activation, and thus it was of interest to investigate whether activation with PHE or ET-1 leads to a Ca^{2+} response. The Ca^{2+} responses, upon stimulation with PHE or ET-1, were shown to be small and heterogeneous among cells of the same culture. It appeared that the agonist induced Ca^{2+} responses could only be measured when the Ca^{2+} transients, elicited by the spontaneous contractions, were

blocked by a Ca^{2+} channel blocker (diltiazem). The observed average Ca^{2+} responses were small and distinct in character, respectively monophasic and biphasic after stimulation with PHE and ET-1. Possible blockade of intracellular Ca^{2+} mobilization by the Ca^{2+} antagonist was excluded, because in the presence of diltiazem, a decrease of the external Na^{+} concentration resulted in a rapid and large Ca^{2+} response. From these experiments we concluded that the PHE and ET-1 mediated biological response (e.g. hypertrophy) in neonatal cardiomyocytes in culture are not determined by the increase of cytosolic Ca^{2+} concentration.

PKC is generally considered to play a key role in the induction of hypertrophy. The PKC activity was assessed by protein phosphorylation, but this method does not discriminate between the various PKC isozymes. In these assays, stimulation with either agonist did not reveal significant changes in PKC activity, in contrast to the marked activity found after stimulation with phorbolester (PMA). With immunoanalysis (Western blotting) we could assess the involvement of different PKC isozymes. Stimulation of the cardiomyocytes with ET-1 revealed translocation of the Ca^{2+}-independent PKC-ϵ and not of the Ca^{2+}-dependent PKC-α. Stimulation with PMA evoked translocation of both isozymes. These results agree with the concept that 1,2-DAG, instead of Ca^{2+}, plays the major role in the transduction of the PHE and ET-1 elicited biological signal. Moreover, the activation of the PtdIns-cycle revealed many agonist-dependent differences. Protein phosphorylation after stimulation with either agonist in whole cell homogenates showed also distinction in pattern. Stimulation of the cardiomyocytes with PHE but not ET-1 led to phosphorylation of a 30 kDa protein, which is likely a part of the contractile apparatus. In summary, we conclude that upon stimulation with PHE and ET-1 the activation of the PtdIns cycle may be the initial onset of signalling leading to hypertrophy and phenotypic changes of cultured cardiomyocytes.

Finally, we have investigated the influence of altered fatty acid incorporation in the membrane phospholipids on the activity of agonist activated PLC-β. In these experiments we pretreated the cells with polyunsaturated fatty acids (18:2n-6 or 20:5n-3) in comparison to monounsaturated and saturated fatty acids (18:1:n-9 and 18:0, respectively). The most remarkable results were obtained from cells pretreated with 20:5n-3. Stimulation of 20:5n-3 pretreated cells with PHE resulted in an inhibition of the PLC-β activity (as was measured by accumulation of total [^{3}H]inositolphosphates ([^{3}H]InsP$_n$)), whereas stimulation with ET-1 evoked an increased PLC-β activity. Direct stimulation of PLC-β by GTPγS in saponin-permeabilized cardiomyocytes resulted in a reduced accumulation of [^{3}H]InsP$_n$ only in the 20:5n-3 pretreated cells. Thus, increased incorporation of 20:5n-3 into the membrane phospholipids may affect the transmembrane signal transduction pathway on the level of receptor, G-protein or PLC-β. The effect of the changed membrane phospholipid fatty acid composition is likely not only restricted to functional but also structural aspects. The 20:5n-3 pretreated cells appeared to be initially resistant to the permeabilizing effect of saponin. Earlier the saponin-mediated membrane destabilizing effect was reported to be due to clustering of membrane cholesterol. Consistent with this view is our finding that the cholesterol/phospholipid ratio of 20:5n-3 treated cells was 1.7 fold increased. Moreover, this increase in membrane cholesterol balanced the high unsaturation of the membrane phospholipids, as we observed a slight decrease in membrane fluidity of the 20:5n-3 pretreated cells. The latter data indicate that the changes in membrane dynamic and physicochemical properties are related to the alterations in the PHE and ET-1 induced PLC-β response.

SAMENVATTING

Het adrenerge systeem draagt in belangrijke mate bij aan de acute positief-inotrope, en positief-chronotrope respons, en later optredende hart-hypertrofie bij een overbelasting van het hart. Onder normale omstandigheden is de adrenerge inotrope en chronotrope respons voornamelijk het gevolg van de stimulatie van β-adrenerge receptoren. Echter onder pathologische omstandigheden neemt de bijdrage van de α_1-adrenerge receptoren toe, met name in de rol van de catecholamines in de ontwikkeling van hypertrofie van het hart. Dit gaat gepaard met fenotypische veranderingen zoals bijvoorbeeld verlaagde expressie van de Ca^{2+} ATP-ase pomp in het sarcoplasmatisch reticulum (SR) en verhoogde expressie van de atriale natriuretische factor. Het was al ruime tijd bekend dat de β-adrenerge receptoren in het hart, die gekoppeld zijn aan het G_s-eiwit-adenylaatcyclase-cAMP systeem, in aantal afnemen onder omstandigheden waarin het hart bloot staat aan chronische belasting. Onder dergelijke omstandigheden blijft het aantal α_1-adrenerge receptoren in het hart gelijk of neemt zelfs toe. In tegenstelling tot het β-adrenerge systeem, zijn de α_1-adrenerge receptoren via het G_q-eiwit en het fosfolipase C-β (PLC-β) gekoppeld aan de fosfatidylinositol- (PtdIns-) cyclus. Na receptor activatie katalyseert PLC-β de hydrolyse van de membraan-gebonden component fosfatidylinositol 4,5-bisfosfaat. Hierdoor ontstaan drie potentiële boodschapper moleculen: inositol 1,4,5-trisfosfaat ($Ins(1,4,5)P_3$), inositol 1,3,4,5-tetrakisfosfaat ($Ins(1,3,4,5)P_4$) en 1,2-diacylglycerol (1,2-DAG). Van $Ins(1,4,5)P_3$ is aangetoond dat het Ca^{2+} ionen kan vrijmaken uit de intracellulaire Ca^{2+} opslagplaatsen, zoals het SR in hartspiercellen. De rol van het $Ins(1,3,4,5)P_4$ in het mobiliseren van Ca^{2+} ionen in de hartspiercel is vooralsnog grotendeels onbekend. Van 1,2-DAG is aangetoond dat het eiwit-kinase-C-(PKC-)isozymen kan activeren, al dan niet in samenwerking met Ca^{2+}. De geactiveerde PKC-isozymen veroorzaken op hun beurt weer fosforylering, en daarmee activatie of inactivatie van verscheidene functionele eiwitten van het contractiele apparaat, ionkanalen in het membraan en transcriptie factoren. Recent werd aangetoond dat ook andere factoren dan catecholaminen betrokken zijn in het opwekken van hypertrofie. Eén daarvan is het endotheline-1 (ET-1), een peptide dat door de coronaire endotheelcellen wordt uitgescheiden. In de hartspiercel kan het ET-1, zoals het α_1-adrenerge systeem, de PtdIns-cyclus activeren via een specifieke receptor (de ET_A receptor) die gekoppeld is aan het G_q-eiwit-PLC-β.

In het begin van het onderzoek, dat is beschreven in dit proefschrift was het signaaloverdracht-systeem via de PtdIns-cyclus in het hart nog gedeeltelijk onbekend. Daarnaast zijn de α_1-adrenerge- en de ET-1 receptoren beiden verbonden aan de PtdIns-cyclus en betrokken bij de opwekking van hypertofie in het hart en daarmee geassocieerde fenotypische veranderingen. Dit gaf reden tot het bestuderen en het vergelijken van de α_1-adrenerge en ET-1-receptor-gekoppelde PtdIns-cyclus in het hart.

Fosfolipiden, aanwezig in de plasmamembraan van hartspiercellen, hebben vele functies. Ze spelen een rol in de handhaving van de structuur van de bilaag, waarbij ze optimale omstandigheden creëren voor de activiteit van eiwitten in de plasmamembraan. Een aantal fosfolipiden, zoals $PtdIns(4,5)P_2$, is bovendien substraat voor hydrolytische enzymen zoals PLC-β. Op deze manier zijn fosfolipiden van belang bij de overdracht van signalen van buiten naar binnen de cel. Reeds eerder was aangetoond dat een verandering van de meervoudig onverzadigde vetzuursamenstelling van de fosfolipiden in de membraan door middel van een dieet effect had op de α_1-adrenerge positief-inotrope respons van het hart.

Om de receptor-geactiveerde PtdIns-cyclus en de invloed van meervoudig onverzadigde

vetzuren te bestuderen, werden primaire kweken van spontaan kloppende neonatale rattehart- cellen gebruikt. Al eerder was aangetoond door onderzoekers uit ons laboratorium, maar ook door anderen, dat na een langdurige (24-48 uur) stimulatie van deze cellen met de α_1-adrenerge agonist fenylefrine (PHE) of ET-1, hypertrofie en de daarmee gepaard gaande fenotypische veranderingen opgewekt konden worden. Deze primaire kweken bestonden voor meer dan 90 % uit hartspiercellen die bovendien konden worden gekweekt op glas en plastic. Dit maakte het mogelijk om specifiek in de hartspiercellen de Ins(1,4,5)P_3-, 1,2-DAG- en fosforylerings responsen te bestuderen, door de cellen op te laden met respectievelijk [^{3}H]inositol, [^{3}H] glycerol en $^{32}P_i$. De veranderingen in de cytoplasmatische Ca^{2+} concentratie werden fluorimetrisch bepaald in een videomicroscoop en spectrofotometer, waarbij fura-2 werd gebruikt om het Ca^{2+} zichtbaar te maken.

De activiteit van PLC-β als gevolg van stimulatie met PHE of ET-1 wordt gereguleerd door cytoplasmatisch Ca^{2+}, zoals we hebben waargenomen in de experimenten waarin we de Ca^{2+} concentratie buiten en binnen de cel hebben gevarieerd (hoofdstuk 3). Als de Ca^{2+} ionen buiten de cellen werden weggevangen door toevoeging van EGTA, vonden we dat de ET-1 gestimuleerde PLC-β activiteit sterk geremd was, terwijl de basale PLC-β activiteit niet veranderde. Het aanbieden van een hoge concentratie Ca^{2+} in de aanwezigheid van een calcium ionofoor zorgde ervoor dat de door ET-1 en de PHE gestimuleerde PLC-β activiteit toenam werd terwijl ook hier de basale activiteit niet veranderde. Als we door het aanbieden van hoge concentraties K^+ de cytoplasmatische Ca^{2+} concentratie verhoogden, bleek de door ET-1 gestimuleerde vorming van alle [^{3}H]inositolfosfaten ([^{3}H]InsP_n) verhoogd te zijn terwijl onverwacht de [^{3}H]InsP_n ophoping na stimulatie met PHE verlaagd was. In neonatale hartspiercellen, gepermeabiliseerd met saponine, werd de basale PLC-β activiteit, maar ook de activiteit na directe stimulatie met GTPγS verhoogd door het aanbieden van Ca^{2+} ionen in de fysiologisch relevante concentratie van 0.1 tot 10 μM. We concludeerden dan ook dat de cytoplasmatische Ca^{2+} concentratie een terugkoppelende, activerende rol in de receptor-gestimuleerde PLC-β activiteit speelde. Nu bleef echter de vraag of de cytoplasmatische Ca^{2+} concentratie ook daadwerkelijk verhoogd werd na stimulatie met de eerder genoemde agonisten.

Om de vraag te beantwoorden of het cytoplasmatisch Ca^{2+} zou stijgen hebben we eerst de responsen van de potentiële Ca^{2+}-vrijmakende boodschapper moleculen na stimulatie met de agonisten bestudeerd. Het meest opmerkelijke resultaat van deze experimenten is dat Ins(1,4,5)P_3, dat verondersteld wordt Ca^{2+} ionen vrij te maken uit de opslag plaatsen, niet aantoonbaar verhoogd werd door stimulatie met PHE of ET-1 in de aanwezigheid van de monofosfatase remmer Li^+. Evenmin konden we het directe fosforyleringsproduct van Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 aantonen. Dat PLC-β wel degelijk actief was geweest na stimulatie met PHE of ET-1 bleek uit de enorme toename van [^{3}H]inositolmono(4)fosfaat. Blijkbaar werd in neonatale hartspiercellen het gevormde Ins(1,4,5)P_3 ofwel zeer snel gedefosforyleerd door het 5-fosfatase (na stimulatie met PHE) ofwel eerst gefosforyleerd door 3-kinase en daarna gedefosforyleerd (na stimulatie met ET-1). Voor het feit dat na stimulatie van hartspiercellen met ET-1 het metabolisme van Ins(1,4,5)P_3 via een ander pad verloopt dan na stimulatie met PHE is nog geen verklaring gevonden. Maar in zijn algemeenheid wijzen de resultaten erop dat in de hartspier een zeer snelle defosforylering van de biologisch actieve inositolfosfaten optreedt om de door agonisten-geïnduceerde Ca^{2+}-mobilisaties af te zwakken, die mogelijk de normale slag-op-slag Ca^{2+}-oscillaties gedurende de excitatie-

contractiekoppeling kunnen verstoren.

Daarentegen, steeg het andere PLC-β produkt, 1,2-DAG, wel in concentratie na stimulatie met PHE of ET-1, hoewel het tijdsverloop van de stijging verschilde tussen PHE en ET-1. Wij concludeerden hieruit dat in hartspiercellen de α_1-adrenerge en ET-1 gemedieerde signaaloverdracht voornamelijk verloopt via activatie van PKC-isozymen.

Omdat niet alle PKC isozymen Ca^{2+} ionen nodig hebben voor activatie gingen we na of stimulatie met PHE of ET-1 leidde tot een Ca^{2+} respons. De Ca^{2+} responsen na stimulatie met agonist waren maar klein en zeer heterogeen tussen de cellen uit eenzelfde kweek. Het bleek dat een agonist geïnduceerde Ca^{2+}-respons slechts meetbaar was als de slag-op-slag Ca^{2+}-oscillaties, behorende bij de spontane contracties, stilgelegd werden door het gebruik van een Ca^{2+}-antagonist (diltiazem). De op die manier gemeten gemiddelde Ca^{2+} respons was klein en vertoonde een monofasisch en bifasisch karakter, respectievelijk na PHE en ET-1 stimulatie. Mogelijke blokkade van Ca^{2+}-mobilisatie in de cel door het gebruik van een Ca^{2+}-antagonist werd uitgesloten omdat in de aanwezigheid van diltiazem een verlaging van de extracellulaire Na^{+}-concentratie een zeer grote intracellulaire Ca^{2+}-respons veroorzaakte. Deze resultaten wijzen erop dat de door PHE en ET-1 geïnduceerde biologische respons (bijvoorbeeld hypertrofie) in dit hartspiermodel niet direct bepaald worden door een verhoging van de cytoplasmatische Ca^{2+}-concentratie.

PKC lijkt een sleutelrol te spelen in de inductie van hypertrofie. De activiteit van PKC werd bepaald door meting van eiwitfosforylering, maar met deze methode kon geen onderscheid worden gemaakt tussen de verschillende PKC-isozymen. De assays lieten na stimulatie met PHE of ET-1 geen significante verandering in PKC-activiteit zien, in tegenstelling tot de grote PKC-activiteit die werd gemeten na stimulatie met een forbolester (PMA). Door het gebruik van antilichamen gericht tegen specifieke stukken van de PKC-isozymen (Westernblotting), konden we wel de betrokkenheid van de verschillende PKC isozymen bepalen. Stimulatie van de hartspiercellen met ET-1 liet een translocatie zien (wat meestal staat voor activatie) van het Ca^{2+}-onafhankelijke PKC-ϵ maar niet van het Ca^{2+}-afhankelijke PKC-α. Stimulatie met PMA resulteerde in translocatie van beide isozymen. Deze resultaten steunen het concept dat 1,2-DAG, in plaats van Ca^{2+}, de hoofdrol speelt in de overdracht van de door PHE- en ET-1- gemediëerde biologische respons. Bovendien bestaan er agonist-afhankelijke verschillen in het activerings-patroon van de PtdIns-cyclus. Dit laatste werd nog eens duidelijk toen de eiwit-fosforylering in totale celhomogenaten werd bestudeerd. Na stimulatie met PHE, maar niet met ET-1, werd een 30 kDa eiwit gefosforyleerd dat vermoedelijk onderdeel is van het contractiele systeem. Samenvattend kan uit de bovenstaande data geconcludeerd worden dat na stimulatie met PHE of ET-1 de activatie van de PtdIns-cyclus de initiële aanzet is van het signaal dat tot hypertrofie en fenotypische veranderingen in gekweekte hartspiercellen leidt.

Tenslotte werd de invloed van een veranderde vetzuurincorporatie in de membraanfosfolipiden op de door agonist geactiveerde PLC-β activiteit bestudeerd. Daartoe werden de hartspiercellen verrijkt met meervoudig onverzadigde vetzuren (18:2n-6 of 20:5n-3) ter vergelijking met monoonverzadigde (18:1n-9) en verzadigde (18:0) vetzuren. De meest opmerkelijke resultaten werden verkregen bij cellen die verrijkt waren met 20:5n-3. Stimulatie van deze 20:5n-3 behandelde cellen met PHE leidde tot een verlaging van de PLC-β activiteit (gemeten door ophoping van [^{3}H]InsP$_n$), terwijl stimulatie met ET-1 juist een verhoging van de PLC-β activiteit te zien gaf. Door de receptor-G-eiwit-interactie te omzeilen door directe stimulatie van PLC-β met GTPγS in met saponine gepermeabiliseerde cellen, werd alleen in

de 20:5n-3 behandelde cellen een verminderde ophoping van [^{3}H]InsP_n gevonden. Een verrijking van de membraanfosfolipiden met 20:5n-3 zou invloed hebben op de transmembraan-signaaloverdracht op verschillende niveaus zoals het niveau van de receptor, het G-eiwit of het PLC-β. Het effect van de veranderde vetzuursamenstelling van de membraanfosfolipiden is waarschijnlijk niet alleen beperkt tot functionele maar betreft ook structurele aspecten. De met 20:5n-3 verrijkte cellen bleken initieel beter bestand tegen de permeabiliserende werking van saponine. Al eerder was bewezen dat het membraan-destabiliserende effect van saponine wordt veroorzaakt door het "clusteren" van het cholesterol in de plasmamembraan. In overeenstemming met dit idee is onze bevinding dat de cholesterol/fosfolipide ratio in de 20:5n-3 behandelde cellen 1,7 maal verhoogd was. Deze stijging was blijkbaar in balans met het meervoudig onverzadigde karakter van de membraanfosfolipiden, aangezien we een verlaging van de membraanvloeibaarheid in de 20:5n-3 behandelde cellen maten. Laatst genoemde resultaten wijzen op een mogelijk verband tussen veranderingen van dynamische en fysicochemische eigenschappen van de membraan en de waargenomen effecten van 20:5n-3 op de PLC-β-response die is geïnduceerd door PHE en ET-1.

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CURRICULUM VITAE

Jet de Jonge werd geboren te Haarlem op 29 april 1966. Achttien jaar later behaalde zij haar diploma VWO-B aan het Christelijk Atheneum Adriaen Pauw te Heemstede. Een jaar "parkeer"-studie aan de Hogere Laboratoriumschool Rijnland te Leiderdorp volgde, terwijl in datzelfde jaar aan de Rijksuniversiteit Leiden het tentamen scheikunde werd behaald als aanvulling op het zeven-vakken-pakket van het VWO-B. In 1989 begon zij met de studie biologie aan de Rijksuniversiteit Leiden, waarin zij de specialisatie-richting medische biologie koos. In september 1989 vertrok zij voor haar eerste doctoraal stage naar de Verenigde Staten van Amerika waar zij, naast het "beagelen", een onderzoek verrichtte in het farmacologisch "Cancer Research"-laboratorium van Dr. Julie J. Sando aan de Universiteit van Virginia. Onder leiding van Dr. R. Frankis werden de verschillen in de transcriptie-factoren van de 5'flanking regulatoire sequentie van het IL-2-gen in een "sensitive" en "resistant" EL-4 cellijn bestudeerd. Weer terug in Nederland deed zij haar tweede doctoraal stage aan de Rijksuniversiteit in het laboratorium van Dr. J.W. Kijne van de vakgroep Moleculaire Plantkunde. Hierbij werd o.l.v. Dr. P.J. Hoedemaeker geprobeerd om de opbrengst van recombinant lectine uit *E. Coli* te verbeteren. Vanaf september 1991 werkte zij als onderzoeker in opleiding aangesteld door de Nederlandse organisatie voor Wetenschappelijk Onderzoek bij de vakgroep Biochemie aan de Erasmus Universiteit Rotterdam. Daar volgde zij AIO/OIO-onderwijs, begeleidde geneeskunde-studenten en een laboratorium-stagiaire en verrichtte, onder leiding van Prof. Dr. J.M.J. Lamers, het onderzoek dat beschreven staat in dit proefschrift.