CROSS-TALK BETWEEN PHOSPHOLIPASES
Intracellular signalling involved in cardiac (mal)adaptation

'CROSS-TALK' TUSSEN FOSFOLIPASES
Intracellulaire signalen betrokken bij cardiale (mal)adaptatie

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voor Peter

CONTENTS

Chapter 1

GENERAL INTRODUCTION

The contractile activity of the muscular walls of the heart propels blood throughout the body, delivering nutrients to and removing wastes from each organ. The heart also provides for the transport of hormones, neurotransmitters, and other messengers between various regions of the body. These transport functions are made possible by the rhythmic pumping action of the heart, which can be viewed simply as a hollow muscular pump provided with valves that moves blood "around in a circle continuously" [1, 2].
The existence of the heart was already known by the Greeks, who gave it the name 'kardia', which nowadays still can be found in terms like cardiac en tachycardia. Aristotle thought that the heart was the centre of man and contained the soul. During the Roman times the word 'kardia' was modified to 'cor', which is still in clinical use, in words like 'cor pulmonale'. The old word 'herton' is also derived from cor and leads us via the medieval word 'heorte' to 'heart' in our language. The basis of the modern concepts of circulation were laid by Harvey in the seventeenth century, describing the function of the heart as pump of blood of the circulation; he also described the heart as composed of two ventricles [3].

STRUCTURE AND FUNCTION OF THE MYOCARDIUM

Heart as a pumping organ

The mammalian heart is divided into four chambers, the right and left atria and the right and left ventricles. Between the cavities of the atria and the ventricles lie the atrioventricular valves. Blood returning from the lungs enters the left atrium via the mitral valve, passes into the left ventricle, and is then ejected via the aortic valve into the aorta. Venous blood returning from the body is collected in the right atrium, passes via the tricuspid valve into the right ventricle and is then pumped via the pulmonary valve into the lungs to become saturated with oxygen. The valves prevent the backflow of blood from the aorta to the ventricle, the atrium and the veins and are opened and closed by pressure differences between the heart chambers and the blood vessels [3].
The blood supply of the heart itself arises from the right and left coronary arteries and their branches, which originate from the aorta and course over the epicardial surface of the heart. The venous effluent from the myocardial tissue is collected in small intramyocardial veins that carry venous blood to larger veins that parallel the coronary arteries on the epicardial surface of the heart. Finally, the venous effluent of the heart enters the coronary sinus, which empties in the right atrium.

Several cell types are present in the myocardium, representing functionally specialized striated muscle cells, vascular smooth muscle cells, endothelial cellls, fibroblasts etc. Most important are the working myocardial cells of the atria and ventricle, which are specialized for contraction, the Purkinje fibres rapidly conduct the electrical impulse through the heart, and the nodal cells of the sinoatrial and the atrioventricular node are respectively responsible for pacemaker activity and slow atrioventricular conduction.
In summary, the myocardium consists of three types of muscle cells:

* small muscle cells, found in the sinoatrial node and atrioventricular nodes, weakly contractile, autorhythmic and with slow conduction between cells.
* large myocardial cells, found in the ventricular endocardium, weakly contractile, but specialized in fast conduction and spreading of the excitation.
* intermediate-sized myocardial cells, strongly contractile and representing the working cells in the heart.

The walls of the left ventricle are thick and muscular, called working myocardium. The heart also contains a pericardium, a fibrous fluid-filled flexible bag [1, 3].

Electrical activity

A heartbeat consists of rhythmic contraction and relaxation of the whole muscle mass. Contraction of each cell is preceeded by an action potential. The electrical activity is initiated in

the pacemaker region in the heart, and electrical activity spreads from one cell to the other, because cells are electrically coupled via membrane junctions, also called intercalated disks. The pacemaker region, also called sinoatrial node, is present in the right atrium. Electrical activity generated in the sinoatrial node will rapidly spread through the atria and reach the atrioventricular node, which is slowly conducting, to ensure a delay between excitation of the atria and the ventricles. When the signal leaves the atrioventricular node, the signal is conducted through the bundle of His, the bundle branches and conducting fibres consisting of Purkinje cells to establish excitation-contraction coupling in the cells of the working myocardium [2, 3].

The heart rate is determined by the initial rate of pacemaker activity, and controlled by the autonomic nervous system. One branch, the sympathetic or adrenergic nervous system, releases catecholamines, like epinephrine and norepinephrine that increase heart rate. The other branch, the parasympathetic nervous system, releases the inhibitory acetylcholine, so that the heart rate can be balanced. During exercise or stress the heart rate can increase by sympathetic stimulation, and during rest or sleep the activity will go down under influence of the parasympathetic system [3]. Catecholamines not only stimulate heart rhythm, but also increase the force of contraction, the rate of relaxation of the heart, and on the long-term, stimulate hypertrophic growth (see later).

STRUCTURE AND FUNCTION OF THE WORKING MYOCARDIAL CELL

The cells of the working myocardium contain large numbers of contractile proteins that are organized in myofibrils and arranged in a regular array of thick and thin filaments [2, 3] (Figure 1). Muscle contraction results from sliding of thin filaments along the thick filaments by formation of cross-bridges, thereby using energy in the form of ATP. A large fraction of the cell volume is also occupied by mitochondria, which consume oxygen and oxidize substrates, thereby regenerating ATP from the ADP and P_i formed by cross-bridge cycling. Together, contractile filaments and mitochondria occupy almost 85% of the myocardial cell volume. Other membrane structures include the sarcolemma (SL), which surrounds the cell and plays a central role in controlling many of the critical electrical and chemical processes in the myocardial cells. Extensions of the extracellular space are brought into the central regions of the cell by the transverse tubular or T-system of the SL, which opens freely to the extracellular space. The SL harbours proteins such as Na^+/K^+-pump, Na^+/Ca^{2+}-exchanger, Na^+, Ca^{2+} and K^+-channels that are involved e.g. in action potential generation, allowing Na^+ and Ca^{2+} to diffuse rapidly into the myocardial cell. The SL harbours also specific receptors for hormones, neurotransmitters and growth factors (e.g. α_1- and β-adrenergic agonists, endothelin-1 (ET-1), angiotensin II (Ang II), transforming growth factor-β, insulin-like growth factor-I, and many more), GTP-binding proteins (G_s, G_i and G_q), several effector enzymes such as phospholipases (PLA_2, PLC and PLD), adenylyl cyclase and translocated protein kinase C (PKC) isozymes.

In addition to the SL and the membranes of the T-system, which separates extracellular and intracellular spaces, intracellular membranes divide the cell interior into separate regions, one of which is the sarcoplasmic reticulum (SR). This is a specialized form of the endoplasmic reticulum. It lacks ribosomes which makes it smooth in appearance and it plays a major role in controlling the free Ca^{2+} concentration in the cytosol. It consists of two regions, a network of intracellular tubules, the sarcotubular network, surrounding bundles of contractile proteins, whereas other parts of the SR flatten to form specialized structures, the subsarcolemmal cisternae, where they come in contact with the SL and T-system. In contrast to the great diversity of SL functions, the SR has two major functions. The first is to release activated Ca^{2+} that is associated with the subsarcolemmal cisternae (Ca^{2+} triggered Ca^{2+} release mechanism). The second function is the active reuptake of Ca^{2+} that allows muscle to relax. This Ca^{2+} movement is driven by a densely packed array of Ca^{2+} pump ATPase proteins embedded in the membrane of the sarcotubular network. The latter proteins span the bilayer and so contact the aqueous spaces on either side of the bilayer.

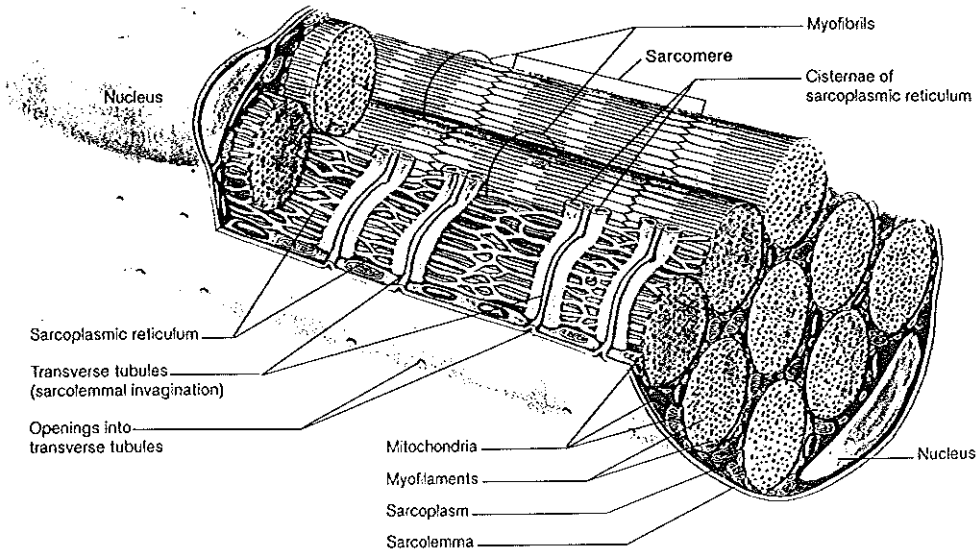


Fig. 1. Ultrastructure of the myocardial cell. Contractile proteins are arranged in a regular array of thick and thin myofilaments (seen in cross section at the right). The sarcoplasmic reticulum (SR), a membrane network that surrounds the contractile proteins, consists of the sarcotubular network at the centre of the sarcomere, and the cisternae which are in close connection to the transverse tubular system (T-tubules) and the sarcolemma (SL). The T-tubules are lined by a membrane that is continuous with the sarcolemma, so that the lumen of the T-tubules carries the extracellular space toward the centre of the myocardial cell. Mitochondria are shown in the central sarcomere and in cross section at the right, as well as the nucleus. (From [4] Zubay G. Biochemistry, 3rd edition, WCB Publishers, Dubuque IA, USA, 1993).

Cardiac SR contains also a regulatory protein phospholamban, a target protein for cyclic AMP and Ca^{2+}-calmodulin-dependent protein kinases (resp. PKA and CaMPK), that by modulating Ca^{2+} pump ATPase plays a key role in regulating the positive ino- and lusitropic responses of myocardium to β-adrenergic agonists.

Like all other cell types, the cardiomyocyte maintains its integrity and responds to external stimuli through signals (hormones, neurotransmitters, cytokines or growth factors) that activate plasma membrane receptors (or nuclear receptors, e.g. those for thyroid hormone). These in turn use signalling proteins (in the usual sequence for e.g serpentine receptors: GTP binding proteins - activated effector enzymes - second messengers - activated protein kinases - activated transcription factors) to transfer the message into the nucleus, which modifies gene expression. Delineation of the receptors, the signalling proteins and where and how gene expression (regulating e.g. cell growth and the level of Ca^{2+} handling proteins) is altered is of prime importance. The processes involved in ET-1, α_1-adrenergic agonist and AngII mediated signalling in the heart are studied in this thesis.

In particular, the receptor-mediated signalling pathways leading to the activation of PKC isoenzymes are investigated. PKC isozymes located in the cytosol are activated via stimulation of multiple SL receptors linked to PLC- and PLD-mediated signalling pathways. Phosphorylation of target proteins by PKC isozymes depends on their location at the time of action. After PKC is activated, it translocates to other cellular compartments such as the SL, myofibrils, SR or the nucleus. This compartmentalisation is determined by the architecture and intracellular localization of anchoring

proteins (e.g Receptor for Activated C Kinase (RACK)). Direct involvement of PKC activation in the signal transduction pathway that leads to (aspects of) myocardial hypertrophy was demonstrated in transfection studies employing constitutively activated PKC isozymes [5-8]. Downstream signalling from activated PKC towards gene regulation can involve direct phosphorylation of nuclear proteins (transcription factors, histone, RNA- and DNA polymerases). At present there is also evidence that stimulation of the majority of the PKC isoenzymes by phorbolester can mimic ischemic preconditioning (see later) and that specific PKC inhibitors can block this protection [33, 34, 37]. This result has led to the hypothesis that PKC is an intracellular signal mediating the preconditioning response.

STRUCTURE AND FUNCTION OF MYOCARDIAL MEMBRANES

The basic structure of all biological membranes is the lipid bilayer which contains a hydrophobic core that is essentially impermeable to charged molecules. Charged "head-groups" of phospholipid molecules line the two surfaces of this hydrophobic core of fatty acid chains, allowing the bilayer to interact with the aqueous media on two sides of the membrane. The membrane bilayer is mainly made up of phospholipid molecules, but most membranes also contain cholesterol molecules that are deeply imbedded in the core of the bilayer and play an important role in the dynamic characteristics of the membrane. The abundant glycerolipid class of phospholipid molecules is made up of a polar (charged) "head-group" and one or two hydrophobic fatty acid "tails" which are generally esterified to glycerol that makes up the "backbone" of the phospholipids (see Table 1) [1, 3].

In recent years it has become clear that membrane phospholipids represent more than simply the building blocks of an inert barrier. The bilayer phospholipids also serve as substrate for phospholipases (PLA_2, PLC-β and PLD) for the (indirect) formation of important cellular messengers.

Table 1 Nomenclature and distribution of the major cardiac SL membrane phospholipids*

Substituent on the phosphate	Name of phospholipid	Abbreviation	mol % of total
Choline	Phosphatidylcholine	PtdChol	45.9
Ethanolamine	Phosphatidylethanolamine	PtdEtn	30.9
Inositol	Phosphatidylinositol	PtdIns	9.0
Choline	Sphingomyelin	Sph	9.0
Serine	Phosphatidylserine	PtdSer	4.0
Hydrogen	Phosphatidic acid	PtdOH	<0.9
Inositolphosphate	Phosphatidylinositol (4,5)bisphosphate	$PtdIns(4,5)P_2$	<0.2

* From [9].

One of these, arachidonic acid (20:4n-6), represents the source of the prostaglandins, thromboxanes, and leukotrienes, a family of 20-carbon fatty acid derivatives that can generate a variety of biological responses [10]. These substances are produced by enzymatic modification of 20:4n-6 after it is cleaved from its glycerol backbone by phospholipase A_2 (PLA_2). Two additional intracellular messengers can be formed from membrane phospholipids when another phospholipase, phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2)-specific PLC-β is activated. Two second messengers, inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and 1,2-diacylglycerol (1,2-DAG), each of which controls an intracellular signal transduction pathway, are formed. Moreover, recent evidence suggest that PLD may also be involved in intracellular signal transduction pathways. PLD preferentially hydrolyses phosphatidylcholine (PtdChol) and cleaves the phosphodiesterase bond distal to the glycerol backbone [11, 12] resulting in the formation of phosphatidic acid (PtdOH) and choline. PtdOH is hydrolysed by PtdOH hydrolase, to produce 1,2-DAG. Both are known to have biological responses. For instance, several PKC isozymes require Ca^{2+} and 1,2-DAG for activation [13, 14]. PtdOH has been shown to stimulate PLC which may be a positive feed-back mechanism for maintaining activity of PKC [15, 16]. The molecular species of PtdOH and 1,2-DAG formed by PLD and/or PLC from their major substrate phospholipids, PtdChol and PtdIns(4,5)P_2, respectively, are likely to be different in structure. The major fatty acids present in the PtdIns pool are 18:0 and 20:4n6 while in PtdChol the major species are 16:0, 20:4n6 and 18:0 (table 2).
In this thesis we also address the important issue whether 1,2-DAG might exert its activating action on PKC isozymes by an increase of its total level or by altered competition between particular molecular species.

Table 2. Major fatty acids as found in two different phospholipids (PtdChol and PtdIns) of neonatal rat cardiomyocytes*

Numerical symbol	Structure	(Trivial) name	mol % of total PtdChol and PtdIns	
16:0	$CH_3.(CH_2)_{14}.COOH$	Palmitic acid	25.2	4.1
18:0	$CH_3.(CH_2)_{16}.COOH$	Stearic acid	22.8	41.9
18:1n-9	$CH_3.(CH_2)_7.(CH=CH).(CH_2)_7.COOH$	Oleic acid	12.6	2.4
18:2n-6	$CH_3.(CH_2)_4.(CH=CH.CH_2)_2.(CH_2)_6.COOH$	Linoleic acid	2.5	0.5
20:4n-6	$CH_3.(CH_2)_4.(CH=CH.CH_2)_4.(CH_2)_2.COOH$	Arachidonic acid	26.3	40.2
22:6n-3	$CH_3.(CH_2).(CH=CH.CH_2)_6.(CH_2).COOH$	Docosahexaenoic acid	4.1	1.5

*From [9]

Therefore, we studied the molecular species composition of PtdChol and PtdIns (after PLC treatment) and 1,2-DAG before and during agonist stimulation of cardiomyocytes. There was lack of information on this matter, as previous studies in myocardium were mostly limited to analysis of total fatty acid composition of phospholipid classes (Table 2).

Up to this point, the description of membrane structure has focused on the phospholipid bilayer, which accounts for the barrier function of the membrane and on some of the phospholipids

serving as substrates for formation of second messenger molecules. However, membrane proteins, some of which contain covalently bound lipid (phosphatidyl inositol anchor) or carbohydrate (glycoproteins), represent a highly diverse group of structural and functional entities that can make up more than half the weight of membranes. These proteins which, as listed before, include antigens, receptors (for instance ET_A and ET_B specific for endothelins, α_1-adrenergic and AT-1 and -2 receptors specific for AngII, tyrosine kinase and cytokine receptors, integrins, enzymes such as PLA_2, PLC and PLD, adenylyl cyclase, GTP binding proteins, docking proteins for PKC, ionchannels (for Na^+, K^+ and Ca^{2+}), ion exchangers (Na^+/Ca^{2+} and Na^+/H^+) and pumps (Ca^{2+} pumps in SL and SR and Na^+/K^+ pump in SL), serve many of the key biological functions of cardiac SL and SR.

MYOCARDIAL EXCITATION-CONTRACTION COUPLING

By definition, excitation-contraction coupling encompasses the sequence of steps that begins when an action potential depolarizes the plasma membrane, and ends with binding of Ca^{2+} to troponin C on the thin filaments (systole), the Ca^{2+} receptor of the cardiac contractile proteins. In the last two decades, the concept of excitation-contraction coupling has broadened and now also includes the proces of removal of Ca^{2+} from the contractile protein, leading to relaxation of the heart (diastole) [1, 3]. The Ca^{2+} fluxes that initiate contraction involve passive, downhill movements of this ion through membrane channels and by exchanger proteins (in SL and SR). This results from the fact that the level of ionized Ca^{2+} in extracellular fluid is about 1 mM and even higher in the intracellular SR stores, whereas the Ca^{2+} concentration needed to saturate troponin C is about 100-500 fold less, all being considerably higher than the Ca^{2+} concentration in the cytosol of the resting cardiomyocyte (0.1 μM).

Contractile proteins

The cardiomyocyte is the muscle cell acting as contractile unit in the heart. The essential components for contraction in the cardiomyocytes are the thick filaments composed of myosin heavy chains (MHC's) which can form cross-bridges with the thin filaments composed of actin. The troponins and tropomyosin all have regulatory functions. Contraction will only occur when sufficient ATP and Ca^{2+} are present. Each thick filament is composed of about 300 individual myosin molecules, each ending in a myosin head. The thin filaments contain two helical chains of actin units, each carried on a backbone of tropomyosin, with troponin C/I/T complexes placed at intervals (at each seventh actin molecule). Troponin-C is the component to which the essential Ca^{2+} can bind to remove the inhibitory effect of troponin I on the interaction between actin and myosin heads. Troponin-T links the troponin complex to tropomyosin.

Muscle contraction results when the thin filaments move along the thick myosin filaments by formation of crossbridges of myosin heads with troponin-complexes. The crossbridges undergo cyclical changes. ATP is hydrolysed during each cycle and the interactions are tightly controlled by the changing level of cytosolic free Ca^{2+} concentration [1, 3].

Regulation of contraction

The intracellular regulator of muscle contraction is Ca^{2+}, of which the cytoplasmic concentration fluctuates during one cardiac cycle. Ca^{2+} ions move across the SL through the voltage-dependent Ca^{2+} channels and reversed action of the Na^+/Ca^{2+} exchanger. The incoming Ca^{2+} ions are signals to release a greater amount of Ca^{2+} from the intracellular stores of the SR [17]. Ca^{2+} is needed for actin-myosin interaction and thus contraction of the muscle. When the Ca-influx channels close again, the Ca^{2+} is removed from the cytosol by Na^+/Ca^{2+} exchange and by ATP dependent Ca^{2+} pumps in the sarcolemma and sarcoplasmic reticulum. Mitochondria can also actively pump and passively release Ca^{2+} ions, but the rate is too slow to follow the heart cycle [3]. Most likely the latter process has an important Ca^{2+} buffering function when the myocyte is threatened by Ca^{2+}

overload, but normally it maintains intramitochondrial Ca^{2+} level for optimal activity of several Ca^{2+} dependent dehydrogenases [18].
The actual duration of a contraction/relaxation cycle is dependent on the strenght of the Ca^{2+} signal as determined by the net Ca^{2+} movements (see Figure 2). Rhythmic contraction is achieved by association/dissociation cycles of Ca^{2+} ions with troponin C. Binding of Ca^{2+} to troponin C allows the formation of cross bridges between actin and myosin and while actin moves relative to myosin the cells contract and blood is ejected from the ventricle [19]. Relaxation is achieved by the removal of Ca^{2+} from the contractile protein by action of the Ca^{2+} pump in the sarcoplasmic reticulum.

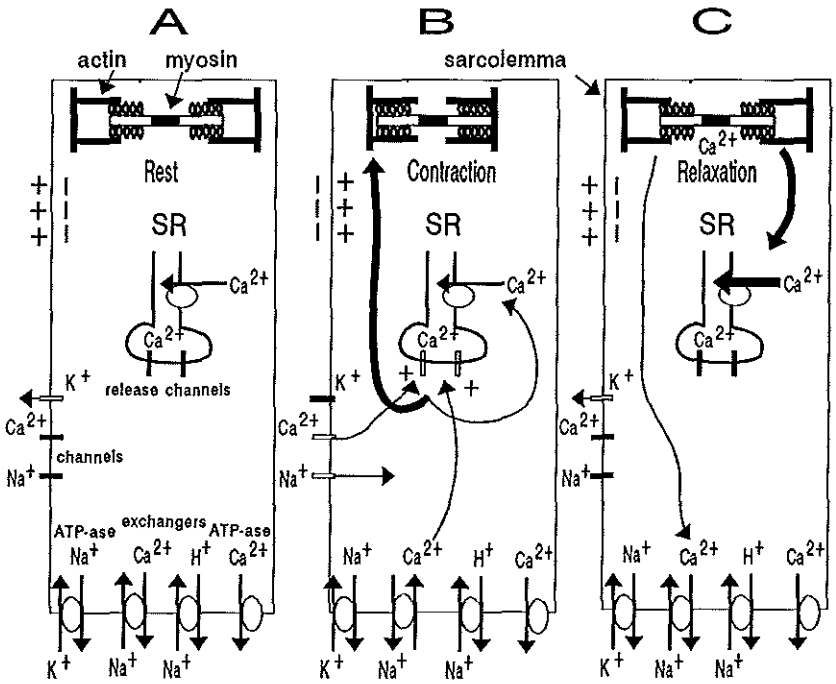


Fig. 2. Schematic representation of the ion movements and myofilament action during contraction-relaxation cycle in the cardiomyocyte.
A) The resting cardiomyocyte; closed Ca^{2+} and Na^+ channels, low intracellular Ca^{2+} level mostly due to active Ca^{2+} transport by SR, but also by sarcolemmal Na^+/Ca^{2+} exchange and Ca^{2+} pump. **B)** Excitation-contraction coupling; Na^+ entry through the opened Na^+ channels, Ca^{2+} entry through opened Ca^{2+} channels and reversed Na^+/Ca^{2+} exchanger action (due to the transient Na^+ influx and reversed membrane potential). This Ca^{2+} triggers the passive release of Ca^{2+} from the SR through specific release channels. Increased intracellular Ca^{2+} initiates cross-bridge cycling between thick and thin filaments. **C)** Relaxation; Na^+ and Ca^{2+} channels are closed, membrane potential restores by action of Na^+/K^+ ATPase and K^+ efflux through opened K^+ channels in SL, and the Ca^{2+} pump in SR and sarcolemmal Na^+/Ca^{2+} exchange and Ca^{2+} pump reduce intracellular Ca^{2+} levels so that actin-myosin complexes become dissociated, leading to relaxation. The stoichiometry of Na^+/K^+ ATPase, Na^+/Ca^{2+} exchanger, Na^+/H^+ exchanger and Ca^{2+} pump is respectively $3Na^+$ for $2K^+$, $3Na^+$ for $1Ca^{2+}$, 1 Na^+ for 1 H^+ and $1Ca^{2+}$/ATP. (Adapted from Barry and Bridge [17], 1993).

MYOCARDIAL PATHOPHYSIOLOGY

Balanced supply of oxygen and substrates is necessary to provide for the energy required for intracellular environment, and supply/demand imbalance causes derangements of cellular energy metabolism. The regulation of intracellular free Ca^{2+} concentration is also a crucial event, which normally controls the contractile activity of the heart. Disturbances in Ca^{2+} handling may lead to contractile dysfunction. However, it is becoming more and more clear, that the receptor-mediated regulation of cardiac contraction, rhythm, metabolism, growth and phenotype (see later) is important as well, particularly for the initial causes of irreversible ischemic damage, late development of cardiac hypertrophy and failure and even the protection by ischemic preconditioning.

Congestive heart failure is primarily the result of chronic hemodynamic overload of the heart post-infarction, by hypertension or valvular disease. Congestive heart failure develops slowly and is characterized by mechanical stress of viable myocardial cells. Compensation at the myocardial level includes cellular hypertrophy (e.g. more sarcomeres per cell are formed). However, this process is also accompanied by several maladaptive changes in the expression of contractile proteins and of proteins involved in the regulation of intracellular free Ca^{2+}, some of which underlie the transition to systolic and diastolic dysfunction [20]. The adaptive growth of ventricular myocardial mass is based upon the enlargement of individual cardiomyocytes (cellular hypertrophy). Apart from the mechanical stress, several neurohumoral stimuli have been implicated in the regulation of growth of cardiomyocytes, including AngII, ET-1, noradrenaline, growth factors (TGF-β, thyroid hormone) and interleukin-like agents such as cardiotrophin-1. The work described in this thesis focuses on the manner by in which cardiac myocytes sense signals and respond to signals, such as ET-1, α_1-adrenergic agonists and Ang II, that ensue from enhanced demand for cardiac output (chronically overloaded myocardium) or from acutely occurring imbalance of oxygen demand and supply (preconditioned myocardium).

Acute myocardial ischemia exists when the reduction of coronary flow is so severe that the supply of oxygen to the myocardium is insufficient to meet the oxygen demands of the tissue. Moreover the reduction of coronary flow also causes less supply of substrate and accumulation of (toxic) metabolic end products. Myocardial ischemia is by definition initially reversible. Prolonged ischemia causes irreversible changes, with development of myocardial infarction.

On the other hand, repeated brief episodes of ischemia and reperfusion can protect the myocardium from infarction (infarctsize reduction) induced by a subsequent longer episode of ischemia, the phenomenon called ischemic preconditioning [21]. The protection is likely based upon action of para- and autocrinically released stimuli (depending on the species; adenosine, bradykinin, noradrenaline, acetylcholine, Ang II and ET-1) which, similar to when they stimulate myocardial cell growth, activate intracellular signalling pathways via specific membrane receptors.

Acute myocardial ischemia leading to infarction

Acute myocardial ischemia results from an imbalance between oxygen supply and oxygen demand leading to decreased ATP levels that cannot maintain intracellular movements of Ca^{2+} and the maximum activity of Ca^{2+} activated actomyosin ATPase. If there is coronary artery disease already, exercise induced increase in the oxygen demand could precipitate angina. When supply is decreased abruptly caused by coronary artery spasms or when a thrombus starts to occlude the coronary artery, angina at rest may be precipitated. A sustained imbalance of oxygen supply and demand could lead to irreversible cell injury ending in cell death called myocardial infarction. The current theories for development of irreversible ischemic damage are mutually related 1) massive Ca^{2+} overload of the myocardial cell which causes generalized cellular energy depletion, excessive Ca^{2+} overloading of mitochondria (amorphous dense bodies), hypercontraction of the myofibrils, activation of phospholipases and proteins [22]; 2) formation and destructive action of oxygen free radicals, most likely during the reperfusion phase [3]; 3) metabolically induced membrane damage and accumulation of lipid intermediates (free fatty acids, lysophospholipids, acyl CoA and

acylcarnitine) inside the ischemic cells [23]; 4) extra- and intracellular acidosis, as the anaerobic metabolism during ischemia causes accumulation of lactate and H^+ ions (the latter from breakdown of ATP) followed by increased H^+ extrusion from the myocardial cells through the Na^+/H^+ exchanger (Figure 2). This reduces the Na^+ gradient, thereby contributing via the Na^+/Ca^{2+} exchanger to the development of Ca^{2+} overload [24, 25].

Limitation of infarct-size

Exposure of the myocardium to brief periods of ischemia can result in marked protection (infarctsize reduction) during subsequent prolonged episodes of ischemic stress. Thus, one or more brief abrupt total coronary artery occlusion and reperfusion sequences reduce myocardial infarct-size produced by a subsequent prolonged coronary artery occlusion [27, 30]. Other stimuli (for instance ventricular pacing, activation of K^+_{ATP} channels, stimulation by various agonists or phorbolester) have also been shown to mimic the protected state of the preconditioned myocardium during subsequent prolonged coronary artery occlusion [28, 34].

Depending on the species, the activation of various receptors, such as those specific for adenosine [26, 29], bradykinin [30, 31], α_1-adrenergic agonists [32, 33], ET-1 [34] and AngII [35] has been proposed to be involved in the adaptive protection process called ischemic preconditioning. Since understanding of the mechanism of ischemic preconditioning provides a potential therapeutic option in humans, it is important to resolve the postulated receptor-mediated intracellular signalling processes involved. A signal transduction pathway that is common to all afore-mentioned receptor stimuli is that mediated by PLC-β (and possibly also PLD). This explains why the activation of the PKC isoenzymes, as being responsible for the intracellular events leading to the protective adaptation, has received wide attention. Indeed, there is now abundant evidence that stimulation of most of the isoenzymes of PKC by phorbolester can mimic ischemic preconditioning and that inhibition by specific PKC blockers can prevent the protection [33, 35, 37]. For instance, it has been shown that ET-1 reduces infarct size through activation of PKC and opening of the ATP dependent K^+ channels (K^+_{ATP} channels) in isolated rat hearts [36].

At present, there is little evidence for the putative target-proteins of translocated/activated PKC. The suggested candidates are the K^+_{ATP} channel [37], ecto-5'-nucleotidase [38] and the Na^+/H^+ exchanger [39, 40, 41] (see Figure 2). With respect to the latter target protein, it is interesting to note that preconditioning reduces the acidification occurring during a prolonged period of ischemia. Therefore, PKC activation might indirectly, by increasing Na^+/H^+ exchange, be responsible for the decreased acidification observed in preconditioned hearts [42]. On the other hand, the excess of protons produced during ischemia stimulates Na^+/H^+ exchange even in the absence of a transsarcolemmal pH gradient. The resulting Na^+ gain can contribute to deleterious Ca^{2+} overload via Na^+/Ca^{2+} exchange during ischemia and/or during the initial phase of reperfusion [reviewed in 44]. In this view the postulated PKC-mediated activation of Na^+/H^+ exchanger would not be beneficial but detrimental for the heart.

Hypertrophy and congestive heart failure

Besides coronary artery disease and its threat of oxygen inadequacy, the other major type of problem the heart can be confronted with is an excess mechanical load. Previously, the etiology of heart failure was primarily the result of uncontrolled chronic hypertension, valvular heart disease (rheumatic, usual mitral stenosis) or certain cardiomyopathies [43, 45]. Today, the most common cause of heart failure is left ventricular dysfunction due to infarction. The remaining viable cardiac myocytes may respond to signals from injured or dying myocytes. These signals may be both mechanical and chemical, and may give the cell nucleus the message to alter its phenotype (hypertrophic growth and specific changes in gene-expression). Thus, cellular hypertrophy is among the earliest adaptive responses to myocardial injury. In a sense, the viable cardiac myocytes in the infarcted heart try to adapt to a new environment by synthesizing new proteins and make more sarcomeres in order to maintain cardiac performance. Although in its earliest stage, hypertrophy

of the myocardium is adaptive, the accompanying changes in phenotype may become maladaptive. A positive adaptation is clearly the induction of atrial natriuretic factor (ANF). Maladapative are for example, the down regulation of the β-adrenergic receptor and the Ca^{2+} pump of SR, associated with depressed systolic and diastolic function, respectively. Moreover, hypertrophy, even as an initial response to injury, may be the first step to development of myocardial contractile dysfunction. Hypertrophy causes also an imbalance between energy expenditure and energy products, perhaps as a consequence of increased distance between capillaries and relative underperfusion of the subendocardium [46]. Thus, the ultimate compensation of hypertrophied myocardial cells is a reduced contractile state, as usually observed in patients suffering from congestive heart failure. Stimuli like AngII, ET-1, cardiotrophin-1, basic fibroblast growth factor, insulin-like growth factor-I and α_1-adrenergic agonists induce hypertrophy in *in vivo* and *in vitro* models. Possibly the Na^+/H^+ exchanger is also involved [47, 48]. ET-1 and AngII have definitely been shown to play a role involved in the development of hypertrophy caused by myocardial overload in *in vivo* models. The ventricle growth was prevented by administration of antagonists of the ET_A or AT-1 specific receptors and the pharmacologic treatment improved long-term survival in heart failure [49-53]. Although other signalling pathways contribute as well (see below), ET_A, α_1-adrenoceptor and AT-1 receptors (all seven-transmembrane spanning domain or serpentine receptors) are coupled by Gq to PLC and possibly PLD, ultimately leading to 1,2-DAG formation, intracellular Ca^{2+} release and PKC activation. The final response of these signalling molecules is stimulation of overall protein synthesis and reprogramming of gene expression [54-57]. Particularly, the isozymes of PKC are believed to be important in inducing cell growth and the adjustment of gene expression. For instance, the tumor-promotor phorbol 12-myristate 13-acetate (PMA), a direct activator of PKC, induces hypertrophy in the model of serum-free cultured neonatal rat cardiomyocytes [55, 58].

TRANSSARCOLEMMAL, CYTOSOLIC AND NUCLEAR SIGNALLING PATHWAYS, AND CROSS-TALK BETWEEN THESE PATHWAYS IN MYOCARDIUM

Signal transduction in cardiac myocytes elicited by various stimuli shown to be involved in the adaptive responses such as hypertrophy and ischemic preconditioning has been a major research topic for over the last decade. A few years ago the main topic was the signalling by seven-transmembrane spanning domain (serpentine) receptors coupled to G-proteins. More recently, a second class of receptors has been implicated in hypertrophy induction, namely (growth factor) receptors that harbour intrinsic tyrosine kinase activity [59]. Very recently it became clear that yet another class of receptors can be associated with hypertrophy, namely the cytokine receptors that produce signals through receptor-associated tyrosine kinases. Finally, it is likely that mechanical load will directly stimulate integrin-receptors by altered binding to surrounding extracellular matrix proteins and increase of internal cytoskeletal tension [60].

Another aspect that has received much attention recently, is the cross-talk between the various intracellular signalling pathways. Serpentine- and growth factor-transduction pathways as well as cytokine-receptor-activated signal transduction pathways may cross-talk via the mitogen activated protein kinase (MAPK) cascade pathways. MAPK signalling seems to be the converging point to which signal transduction elicited by AngII, ET-1, α_1-adrenergic agonist and basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), and cardiotrophin-1 is routed, all inducing hypertrophy. Cross-talk may also exist upstream of the MAPK cascade pathways as PLC-β and PLD may interact with each other through activation of PKC as first proposed by Shukla *et al.* and Kiss [61, 62] based upon reports on non-myocardial cells. The latter potential cross-talk in cardiomyocytes forms a major study objective of the present thesis. Activation of the G-protein G_i results in decreased activity of adenylyl cyclase, and the subsequent reduction in the activity of cyclic AMP dependent protein kinase A (PKA) may serve to relieve the inhibition of PKC activated c-Raf (MAPKKK), which is another example of cross-talk upstream of MAPK in the MAPK cascade

pathway.

Below, we will describe the protein kinase cascade pathways employed by serpentine-, growth factor- and cytokine-receptors, followed by a description of signalling by specific agonists supposed to be involved in adaptive responses such as hypertrophy and ischemic preconditioning. A simplified overview of these signalling pathways is presented in Figure 3.

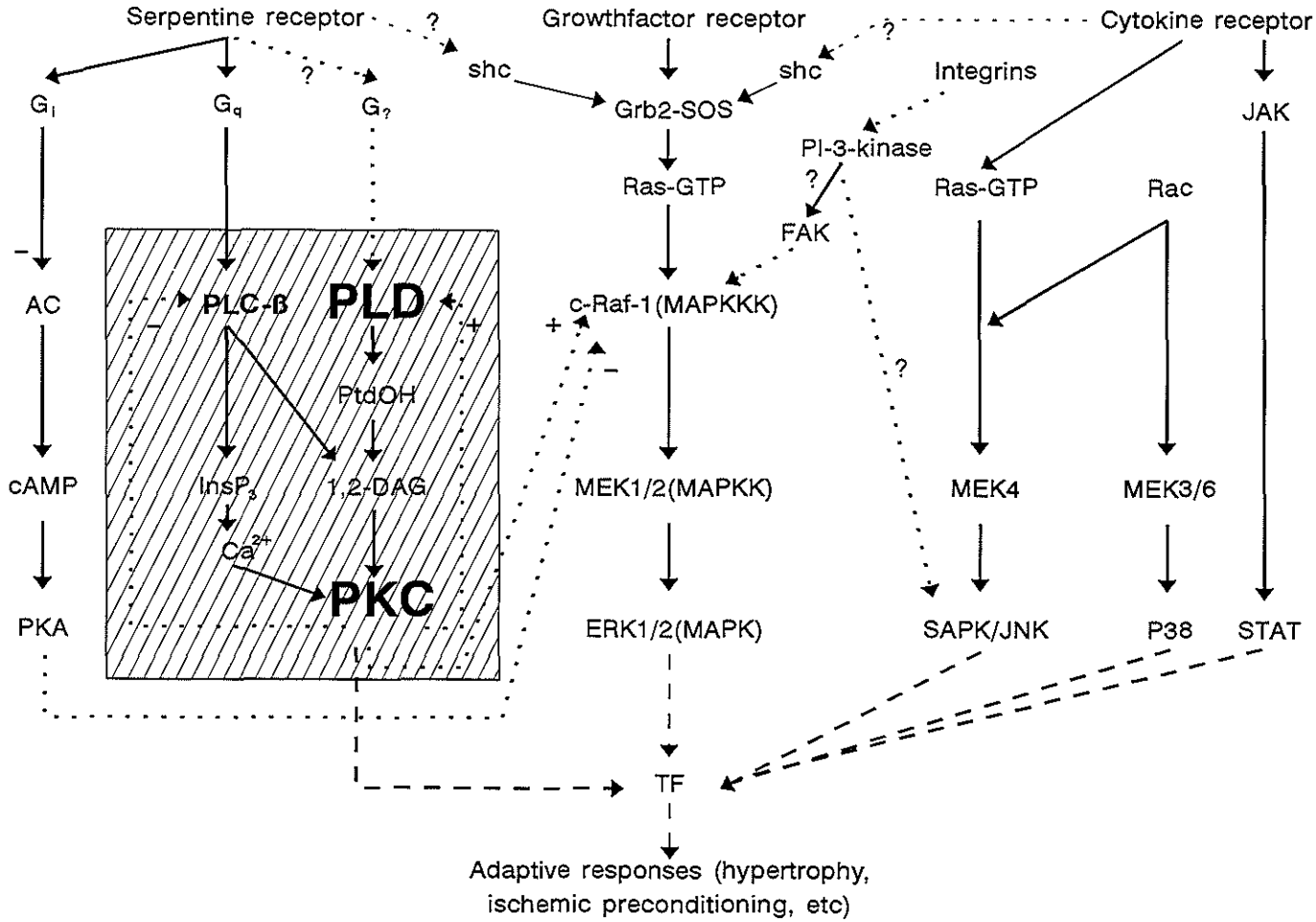
Serpentine receptors and the phosphoinositide pathway

Several seven-transmembrane-domain containing receptors have been detected in cardiac myocytes. These include the PLC-β-coupled receptors for endogenous mediators of hypertrophic growth and ischemic preconditioning such as AngII, ET-1, and α_1-adrenergic agonists [56]. Associated with these receptors are heterotrimeric GTP-binding proteins, of which the G_α subunit can bind GDP or GTP. Upon occupation, the receptor catalyses exchange of GDP for GTP on G_α. This exchange activates the G_α protein and causes its dissociation from $G_{\beta\gamma}$. Both the G_α-GTP complex and $G_{\beta\gamma}$ [64] can regulate effector molecules. Signalling by G-proteins is transient because of the intrinsic GTPase of the G_α subunit.

Two classes of G-proteins are activated: G_q, which activates PLC-β, and G_i, which inhibits adenylyl cyclase [13]. G_i activation through the serpentine receptor results in decreased cAMP levels and decreased PKA activity. This change may serve to reduce the inhibition of c-Raf activation caused by phosphorylation by PKA. Activation of G_q, which is essential for α_1-adrenergic agonist induced hypertrophy and maybe ischemic preconditioning, was shown to lead to activation of the PLC β-isozyme which then hydrolyses PtdIns(4,5)P_2 giving rise to production of inositol (1,4,5)-trisphosphate (Ins(1,4,5)P_3) and 1,2-DAG. In general, Ins(1,4,5)P_3 releases Ca^{2+} from intracellular stores. However, in cardiomyocytes this mechanism seems to be ablated by rapid phosphorylation and dephosphorylation of Ins(1,4,5)P_3 [65]. PLD has also been suggested to be involved in development of ischemic preconditioning and hypertrophy [16]. Preconditioning effects have been shown to enhance PLD activity [66, 67]. It was also shown that PLD is involved in the hypertrophic response to exposure to mechanical stretch and/or AngII of cultured cardiomyocytes [68].

On the other hand, the production of 1,2-DAG serves an important role in signalling since it activates PKC. The PKC isotypes in their turn are able to cross-talk with the MAPK pathway by activation of c-Raf (MAPKKK) [63, 69]. PKC stimulation through α_1-adrenergic receptor, AngII receptor and ET-1 receptor stimulation may additionally increase intracellular pH (alkalinization), enhancing Na^+/H^+ exchanger activity, and may subsequently activate Na^+/Ca^{2+} exchange, thereby increasing the influx of Ca^{2+} [70]. The Na^+/H^+ exchanger has been postulated to be one of the PKC target proteins. Cellular acidosis, which occurs during ischemia could, therefore, be prevented by PKC activation and thereby limit infarct size. Some studies have suggested that PKC-induced alkalinization is also involved in the hypertrophic growth response [47, 48]. However, during cardiac hypertrophy stimulation of the Na^+/H^+ exchange is impaired, so that ET-1 and AngII are unable

Fig. 3. Simplified schematic overview of signal transduction pathways activated by serpentine-, growth factor- and cytokine receptors, the hatched area illustrating the pathways that were under investigation in this thesis. Activation of serpentine receptors leads to inhibition of adenylate cyclase (AC) through the Gi GTP-binding protein, and a decrease in the activity of protein kinase A (PKA). This releases the inhibition that is brought about by raf phosphorylation. Through Gq the same receptors activate phospholipase C (PLC) which lead to PKC activation, raf activation and MAPK activation. PLD is possibly also activated by a G-protein, but sofar only a positive feedback mechanism via PLC is known to activate PLD, also giving rise to PKC activation. Growth factor receptors directly activate the MAPK pathway. Cytokine receptors activate transcription regulation through STAT, but also through SAPK/JNK. They are thought to cross-talk to the MAPK pathway by a cytoplasmic adaptor protein Shc that might be involved in serpentine receptor signalling as well. Arrows denote stimulatory pathways and when "-" is added it represents an inhibitory step. Dashed arrows indicate possible cross-talk between PLC and PLD, the MAPK pathway and the extracellular matrix (ECM) connected integrins through PI-3-kinase and FAK, serpentine receptors, and cytokine receptors [Adapted from 63].


Serpentine receptor
?
Growthfactor receptor
?
Cytokine receptor
shc
shc
Integrins
G_i
G_q
$G_?$
Grb2-SOS
JAK
PI-3-kinase
Ras-GTP
?
Ras-GTP
Rac
FAK
AC
PLC-β
PLD
c-Raf-1(MAPKKK)
PtdOH
?
cAMP
$InsP_3$
1,2-DAG
MEK1/2(MAPKK)
MEK4
MEK3/6
Ca^{2+}
PKC
PKA
ERK1/2(MAPK)
SAPK/JNK
P38
STAT
TF
Adaptive responses (hypertrophy,
ischemic preconditioning, etc)

to cause intracellular alkalinization like in control cells [48].

At present, it is not known by which mechanism(s) endogenous stimuli such as AngII, ET-1 and α_1-adrenergic agonist activate PLD. Studies in other tissues than myocardium have provided evidence that PLD activation, like that of PLC-β, is mediated by a serpentine receptor coupled G-protein [71, 72, 96, 97]. However, other studies have reported that phorbolesters are effective in inducing PtdChol hydrolysis through PLD activity, which suggest that PKC isozymes link PLC-β to PLD [73, 74]. Indeed, over-expression of PKC-β in rat fibroblasts by cDNA transfection greatly enhanced PLD activation in response to phorbolester, ET-1 and α-thrombin [75]. It was demonstrated earlier, that phorbolester inhibits the α_1-adrenoceptor as well as ET-1 stimulated PLC-β activity in cardiomyocytes [76-78]. Therefore, receptor-mediated PLC-ß may cross-talk with PLD via activation of specific PKC isozymes.

The PKC family of Ser/Thr kinases consists of at least 12 members that can be divided into three groups: classical cPKC isozymes, requiring Ca^{2+} and 1,2-DAG for activation; novel nPKCs, requiring only 1,2-DAG; and atypical aPKCs, for which the activation mechanism is still obscure [14]. The presence of cPKC-α, nPKC-ε, nPKC-δ, and aPKC-ζ has been well documented in neonatal rat cardiac myocytes [13,14]. In contrast, only PKC-ε, together with a low amount of PKC-δ is present in adult cardiac myocytes. The presence of PKC-β in cardiac myocytes remains controversial. Translocation of PKC from the cytosol to a particulate (membrane) fraction is judged to reflect activation, as was the case for nPKC-δ and nPKC-ε after stimulation with ET-1 or phenylephrine (PHE) [75,77]. Direct involvement of PKC activation in the signal transduction pathway that leads to hypertrophy was shown in transfection studies employing constitutively activated PKC isoforms [8]. Downstream signalling from activated PKC towards regulation of gene transcription can either involve direct phosphorylation of nuclear proteins (transcription factors, histone, RNA- and DNA-polymerases) or occur indirectly via crosstalk to the MAPK pathway through phosphorylation and activation of c-Raf.

Tyrosine kinase receptors and the MAP kinase pathway

Growthfactor protein tyrosinekinase receptors also activate the MAPK cascade pathway (reviewed in [13, 79]), see Figure 3. These receptors homodimerize upon binding of the growth factor (such as transforming growth factor-β and insulin-like growth factor-I), followed by autophosphorylation of an intracellular tyrosine residue serving as a docking site for adaptor proteins. The Growth factor Receptor-Binding protein Grb-2 is bound to the receptor through its Src Homology (SH2) domain, which is selective for phosphotyrosine-containing sequences. Another domain of Grb2, the SH3 domain, binds to SOS (Son Of Sevenless). In this way, SOS, a Guanine nucleotide Exchange Factor (GEF), is brought into the vicinity of membrane-bound Ras, catalyzing the exchange of GDP for GTP on Ras. Membrane-bound Ras-GTP now binds to and activates raf (MAPKKK), the first enzyme of the MAP kinase pathway. This raf-kinase is a substrate for PKC (stimulation) as well as for PKA (inhibition), indicating that this is the enzyme that integrates multiple signals into one downstream phosphorylation event.

Several MAP kinase cascades have now been identified, all involving a three-enzyme module. The basic components of the MAPK cascade are the following: 1) The MAPK Kinase Kinase MAPKKK (c-Raf) is also referred to as MEKK (i.e., MAPK/ERK Kinase Kinase). This is a Ser/Thr protein kinase that becomes activated by interaction with ras-GTP and probably requires additional tyrosine-phosphorylation for full activation. 2) MAPK kinase (MAPKK), the second component of the module, is also referred to as MEK (MAPK/ERK kinase). MAPKK is phosphorylated by MAPKKK on Ser-XXX-Ser/Thr, where phosphorylation of both sites is required for full activation. MAPKK is able to phosphorylate Ser/Thr as well as Tyr residues. 3) The third component of the module gives its name to the cascade: MAPK (or ERK) becomes phosphorylated by MAPKK, again requiring dual phosphorylation for activation. MAPK itself is a Ser/Thr kinase.

Several cytosolic substrates have been described for MAPK. The cytosolic protein PHAS-I (Phosphorylated Heat- and Acid-Stable protein) is a target for phosphorylation by MAPK, and

complex formation between PHAS-I and eukaryotic Initiation Factor (eIF)4E protein is decreased upon PHAS-I phosphorylation. In this way, mRNA Cap-binding protein eIF-4E becomes available to initiate complex formation between ribosomes and mRNA, stimulating protein synthesis. Another way in which the MAPK pathway can regulate protein synthesis is by phosphorylation and activation of $p70^{S6K}$ [80, 81]. This S6 kinase, in its turn, phosphorylates the S6 protein in the 40S small ribosomal subunit, thereby stimulating the rate of protein synthesis. Phosphorylation of SOS, raf, and MEK by MAPK (ERK) possibly reflects feedback regulation. Further feedback regulation is brought about by MAPK-induced phosphorylation of Protein Phosphatase 2C (PP2C) [82], resulting in decreased protein phosphatase activity and prolongation of tyrosine kinase signal transduction. Here, another point of cross-talk between serpentine- and growth factor receptors exists; activation of PKC leads to phosphorylation and inactivation of protein phosphatase PP2A [83], suggesting that tyrosine signalling can be prolonged by activation of PKC.

Upon activation, MAPK also partly translocates to the nucleus. As a result, several phosphorylation processes can be initiated that mediate transcriptional regulation. One of the nuclear substrates for MAPK is $p62^{TCF}$ (Ternary Complex Factor, or Elk-1) [84]. Other transcription factors that are phosphorylated and activated are c-myc (by ERK2 and not by ERK1) and c-jun, together with RNA polymerase II. Activation of the MAPK pathway can stimulate protein and RNA synthesis in general but can also lead to the activation of specific genes.

Some observations directly implicate the MAPK (ERK) pathway in the development of hypertrophy. Microinjection of constitutively active ras in myocytes is accompanied by morphological changes that are characteristic for hypertrophy and leads to increased ANF expression [85]. Furthermore, transfection with constitutively activated MEK increased the ANF promoter activity. Cotransfection with wild-type (wt)-ERK2 led to enhanced stimulation of this promoter [86] as well as to activated β-MHC, skeletal α-actin, and c-fos promoter. The MAPK pathway is also known to be activated during ischemia/reperfusion. Phosphorylation of another member of the MAPK superfamily p38 MAPK correlates with the preconditioning's protection and its activation through ischemia/reperfusion is a possible important step in the signalling cascade of ischemic preconditioning [87].

Another member of the MAPK superfamily present in cardiomyocytes is the p54 Stress-Activated Protein Kinase (SAPK). Although this pathway is not activated by tyrosine kinase receptors, it might also play a role, through as yet unidentified cross-talk mechanisms [88] in the hypertrophy-inducing AngII pathway [89]. The SAP kinase, also known as JNK, i.e. c-Jun NH_2-terminal Kinase, activates c-jun through phosphorylation of amino-terminal serine residues. Although in cardiomyocytes, upstream activation signals for SAPK activation have only received limited attention, it is likely that MEK4 and ras-GTP are involved, stimulated by the cytokine receptor.

Cytokine receptors and the JAK/STAT pathway

Receptors for the cytokine family all share a β-receptor unit (gp130) in their signal transduction [90]. The specific receptor for the hypertrophic cytokine-like protein CardioTrophin-1 (CT-1) is the Leukaemia Inhibitory Factor (LIF) receptor. Upon dimerization with the cytokine-specific receptor complex (CT-1*LIF), gp130 transduces the cytokine signal to the cytosol. Although gp130 does not harbour intrinsic tyrosine kinase activity, dimerization leads to activation of gp130-associated tyrosine kinases. These Janus-Associated Kinase (JAKs), in their turn, directly phosphorylate and activate the dormant cytosolic transcription factors STAT (i.e. Signal Transducer and Activator of Transcription) that, upon translocation to the nucleus, can homodimerize or heterodimerize into Sis Inducing Factor (SIF) complexes that regulate sis-inducing element (SIE) containing target genes.The cytokine receptor also stimulates Ras-GTP and MEK4 and then SAPK/JNK, which activates transcription. Also Rac is thought to be involved in activating p38 kinase and possible interferes with the Ras-GTP and MEK4 pathway.

The signal transduction pathway initiated by cytokine receptors can, as was the case for serpentine receptors, crosstalk with the MAPK pathway and might therefore also be involved in hypertrophy

Chapter 2

REGULATION AND FUNCTIONAL SIGNIFICANCE OF PHOSPHOLIPASE D IN MYOCARDIUM

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and AngII. To confirm the role for PKC isozymes in the two pathophysiological processes, we tried to measure their translocation, not only in a primary culture of cardiomyocytes, but also in homogenates of left ventricular biopsies taken from the porcine heart *in vivo*. We have first reviewed and thereafter experimentally addressed the following specific issues and/or questions:

1) What is known about PLD in general and about its specific functions in myocardium? (This topic is reviewed in Chapter 2).
2) What are the effects of PHE, ET-1 and AngII on PLC-β and PLD in cardiomyocytes maintained in serum-free conditions, with emphasis on the dual phospholipase stimulation and how are these related to stimulation of the rate of protein synthesis? Furthermore, how do the agonist effects compare to the effects of direct stimulation of PKC by phorbolester? What are the responses of PKC isozymes during agonist-induced receptor stimulation of the cardiomyocytes with emphasis on their cellular redistribution? Is this redistribution involved in the cross-talk between PLC-β and PLD? (Chapter 3).
3) As PLC-β and PLD use respectively PtdIns(4,5)P_2 and PtdChol as substrates 1,2-DAG formed is expected to be different. Does 1,2-DAG exert its activating action on PKC isozymes by its measured total level or by altered competition between particular molecular species. Therefore, the molecular species as well as the level of 1,2-DAG are measured before and after stimulation of the cardiomyocytes maintained in serum-free conditions with PHE, ET-1 and PMA (Chapter 4).
4) The Na^+/H^+ exchanger is likely to be one of the candidate target proteins for PKC isozymes and its activation has been suggested to be involved in the induction of hypertrophic growth as well as in ischemic preconditioning. We use single cell imaging fluorescence microscopy to measure intracellular changes of pH before and during ET-1 stimulation (Chapter 5).
5) Does PKC play a pivotal role in the mediation of ischemic preconditioning? (Chapter 6 reviews this matter).
6) Can cellular PKC redistribution be demonstrated in whole homogenates of left ventricular tissue taken from hearts preconditioned by a brief period of coronary artery occlusion in open-thorax anaesthetized pigs? (Chapter 7)

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and ischemic preconditioning. However, the mechanism of this cross-talk is largely unclear and is hypothesized to involve an adaptor protein that activates Ras. Cross-talk may also occur through PI-3-kinase and Focal Adhesion Kinase (FAK) through integrins [91, 92].

MODELS USED FOR STUDYING INTRACELLULAR SIGNALLING IN MYOCARDIAL HYPERTROPHY AND ISCHEMIC PRECONDITIONING

From the foregoing review on post-receptor signalling cascades involved in myocardial hypertrophy and ischemic preconditioning it follows that *in vivo* many pathways at the same time with different extent of stimulation and with different interactions (cross-talk) operate in concert. However, the study of such complex interactions is facilitated by the use of primary cultures of cardiomyocytes as *in vitro* model for the study of the hypertrophic response (increased protein/DNA ratio [63], increased cell size or generation of individual contractile proteins into sarcomeric units [93]), which appears to be essential for identification of the various types of stimuli likely involved *in vivo* and molecular dissection of the multiple intracellular signalling pathways.
The classic ischemic preconditioning phenomenon refers to the limitation of infarct size by one or more brief preceding cycles of ischemia and reperfusion in *in vivo* models. Most of the neuro-endo-, para- and autocrine mediators have been identified (e.g. adenosine, acetylcholine, catecholamines, AngII, ET-1, bradykinin) on the basis of the observation that the protective effect can be blocked by administration of specific receptor antagonists or that administration of receptor agonists over a time period corresponding to the period of preconditioning ischemia in non-preconditioned heart, mimics the protective effect. Which mediators and to what extent they contribute to the preconditioning response may vary between species. The individual preconditioning stimuli, similar to all those involved in hypertrophy, are balanced *in vivo*. Again, to be able to find evidence for cross-talk between the signalling-pathways and between signals and their cellular responses, isolated or cultured cardiomyocytes, are suitable models. However, it is not clear how the endpoint of infarct size reduction (e.g. revealed by triphenyltetrazoliumchloride staining) in the *in vivo* situation can be translated in the model of isolated or cultured cardiomyocytes [94]. For example, isolated rabbit cardiomyocytes have been subjected to hypoxia and lack of glucose substrate to study subsequent morphological alterations or indicator uptake on the single cell level as end-points. However, great caution is requested when extrapolating findings from simulated ischemia and increased cell survival to true ischemia and reduction of infarct size [95]. Therefore, in the present thesis we have limited our studies on ischemic preconditioning to analysis of the translocation of PKC isozymes in biopsies taken from the control (non-ischemic zone) and preconditioned (10 min coronary artery occlusion and 7.5 min reperfusion) myocardium of the *in situ* open-thorax model of anaesthetized pig. This protocol was previously used to demonstrate the occurrence of infarct size reduction during a subsequent episode of ischemia. Nevertheless, it is plausible that the signalling pathways of PLC-β and PLD investigated in cultured cardiomyocytes are not only operative *in vivo* to induce hypertrophy but also in the protective effect of ischemic preconditioning.

AIM OF THE THESIS

The major objective of the work described in this thesis was to provide evidence for cross-talk between receptor-mediated PLC-β and PLD and subsequent PKC activation, as these signalling processes are involved in hypertrophy and ischemic preconditioning. In most of the experiments primary cell cultures of beating neonatal rat cardiomyocytes were used. The cells were cultured under serum-free conditions to obtain a maximal hypertrophic response (increase of protein synthesis and protein/DNA ratio). In our search for a role of PLC-β and PLD, several putative mediators of hypertrophic and ischemic preconditioning were used: an α_1-adrenergic agonist (PHE), ET-1

ABSTRACT

There is now clear evidence that receptor-dependent phospholipase D is present in myocardium. This novel signal transduction pathway provides an alternative source of 1,2-diacylglycerol, which activates isoforms of protein kinase C. The members of the protein kinase C family respond differently to various combinations of Ca^{2+}, phosphatidylserine, molecular species of 1,2-diacylglycerol and other membrane phospholipid metabolites including free fatty acid. Protein kinase C isozymes are responsible for phosphorylation of specific cardiac substrate proteins that may be involved in regulation of cardiac contractility, hypertrophic growth, gene expression, ischemic preconditioning and electrophysiological changes. The initial product of phospholipase D, phosphatidic acid, may also have a second messenger role. As in other tissues, the question how the activity of phospholipase D is controlled by agonists in myocardium is controversial. Agonists, such as endothelin-1, atrial natriuretic factor and angiotensin II that are shown to activate phospholipase D, also potently stimulate phospholipase C-β in myocardium. PMA stimulation of protein kinase C inactivates phospholipase C and strongly activates phospholipase D and this is probably a major mechanism by which agonists that promote phosphatidylinositol-4,5-bis-phosphate hydrolysis secondary activate phosphatidylcholine-hydrolysis. On the other hand, one group has postulated that formation of phosphatidic acid secondary activates phosphatidylinositol-4,5-bis-phosphate hydrolysis in cardiomyocytes. Whether GTP-binding proteins directly control phospholipase D is not clearly established in myocardium. Phospholipase D activation may also be mediated by an increase in cytosolic free Ca^{2+} or by tyrosine-phosphorylation.

INTRODUCTION

The signal transduction pathway initiated by phospholipase C-β (PLC-β) has been recognized as a major route in myocardium by which stimuli, such as α_1-adrenergic agonists, endothelin-1 (ET-1), angiotensin II (AngII), purinergic and muscarinic agonists, opioids and thrombin induce various functional and pathological responses: positive inotropy, automaticity, ischemic preconditioning and hypertrophy [1, 2]. For example, development of hypertrophy by increased workload of the myocardium due to hypertension, valve-insufficiency or after infarction is thought to be partially dependent on the actions of locally formed noradrenaline, ET-1 and AngII [3]. As a result of interaction of these auto- and/or paracrinic factors with specific membrane receptors, PLC-β is activated in the cardiac sarcolemma via specific GTP binding proteins, which induces the intracellular formation of the second messengers inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$), inositol-1,3,4,5-tetrakis-phosphate ($Ins(1,3,4,5)P_4$) and 1,2-diacylglycerol (1,2-DAG). By actions of Ca^{2+}-calmodulin- and 1,2-DAG-dependent protein kinases, specific proteins become phosphorylated which subsequently transduce the hypertrophic signal to the cellular nucleus. The final result is the stimulation of the overall protein synthesis and reprogramming of gene expression [3-5]. In particular the isozymes of protein kinase C (PKC) are believed to be important in inducing cell-growth and the adjustment of gene expression. Indeed, a potent activator of PKC, phorbolester (PMA), induces hypertrophy in a model of cultured cardiomyocytes. In the same model the α_1-adrenergic agonist phenylephrine (PHE), AngII, ET-1, thrombin and purinergic agonists induce hypertrophy by PKC dependent pathways. Substrate proteins of PKC, such as Raf and Ras, activate on their turn the mitogen activated protein kinases (MAP-kinase and MAPK-kinase), which directly or indirectly activate transcription factors in the cardiomyocyte nucleus [6, 7].

Phospholipase D (PLD) may be another important source of the PKC-activator 1,2-DAG in myocardium. It was shown that some of the above mentioned stimuli also induce PLD-mediated hydrolysis of phosphatidylcholine (PtdChol) into phosphatidic acid (PtdOH) [8-10]. PtdOH is converted into 1,2-DAG by the enzyme PtdOH-hydrolase. The PLD pathway can therefore give rise to two products, with either known (1,2-DAG) or potential (PtdOH) second messenger function.

In this article, we review the current knowledge on the receptor-mediated signalling by PLD in myocardium and the most likely mechanism(s) of PLD activation. It is already shown in

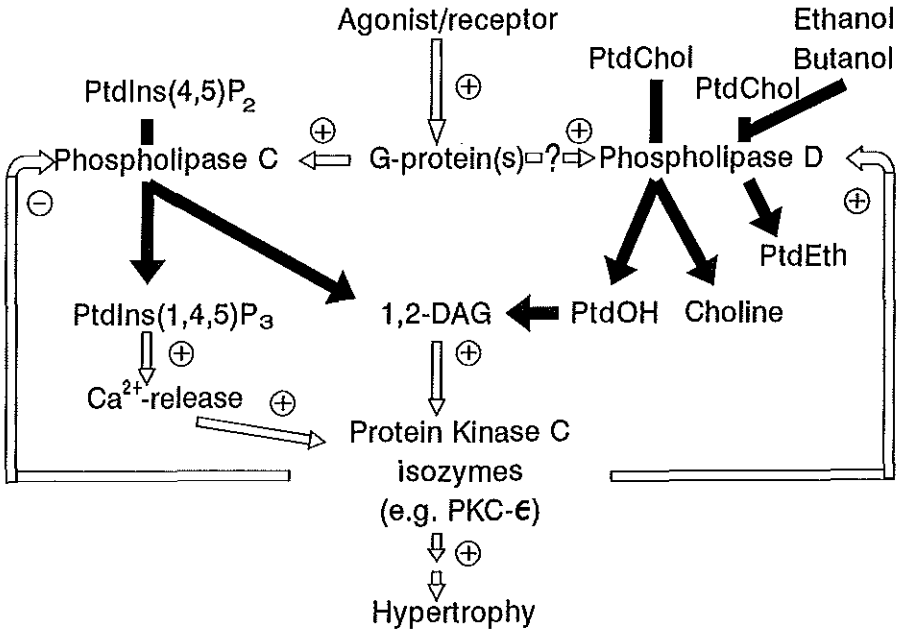

Fig. 1. PLC and PLD signalling pathway after receptor stimulation in myocardium.

other tissues that the PKC activator PMA markedly stimulates PLD. Furthermore, we demonstrated earlier that PMA inhibits the α_1-adrenoceptor as well as ET-1 stimulated PLC-β [11-13]. Therefore, PKC may function as a switch which reduces the rate of phosphatidylinositol-(4,5)bisphosphate-hydrolysis (PtdIns(4,5)P_2-hydrolysis) catalyzed by PLC-β and stimulates the rate of PtdChol hydrolysis by PLD [9, 10]. Through this "cross-talk" mechanism as illustrated in Fig. 1, the cardiomyocyte may be continuously supplied with 1,2-DAG, because the concentration in the cell of PtdChol is generally about 100 times higher than the PtdIns(4,5)P_2 concentration [9, 10, 14]. This continuous production of 1,2-DAG could be of major importance for the maintenance of activation of specific PKC isoenzymes involved in the development of e.g. myocardial hypertrophy. The emphasis in this article has been placed on the PLD action in myocardium during prolonged agonist-receptor stimulation. Readers interested in the topic of PLD in general, not specifically devoted to the myocardium, are referred to recent reviews elsewhere [9, 15-18].

Phospholipases in general

Over the last decade, studies on phospholipid turnover have begun to dominate the field of second messenger research. It is realized more and more that phospholipids contain "information" in addition to their known structural role in membrane function. The "information" stored in phospholipid molecules can be released by the action of several types of phospholipases as illustrated in Fig. 2. In general, the group of phospholipases consists of acylhydrolases such as phospholipase A_1 (PLA_1), PLA_2 and phosphodiesterases such as PLC and PLD.

PLA_1 is not known to have an important function in signal transduction and that is in contrast to PLA_2. PLA_2 hydrolyzes the ester bond in the *sn-2* position of the phospholipid-structure, releasing polyunsaturated fatty acids from this position with the formation of a lysophospholipid [19]. The

main function of PLA_2 is to produce arachidonic acid (AA), which can be further metabolized to eicosanoids [20]. PLC is capable of hydrolysing the glycerophosphate ester in a variety of phospholipids resulting in the formation of 1,2-DAG, which activates PKC, and the formation of a phosphorylated base. When PLC acts on inositol containing phospholipids, two of the products are the second messengers $Ins(1,4,5)P_3$ and its phosphorylation product $Ins(1,3,4,5)P_4$, that can mobilize Ca^{2+} from intracellular stores. PLD cleaves on the other side of the phosphoryl linkage to form phosphatidic acid (PtdOH) and the free base mostly from PtdChol but also from phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer) and phosphatidylinositol (PtdIns). PtdOH can subsequently be hydrolysed by PtdOH-hydrolase to 1,2-DAG, which activates PKC isozymes.

The first evidence for the existence of a receptor-coupled PLD activity in mammalian systems was obtained in studies on rat brain some 20 years ago [21]. Much later, Lindmar *et al.* [22] showed that muscarinic receptors, stimulated by carbachol, were coupled to PLD in the perfused chicken

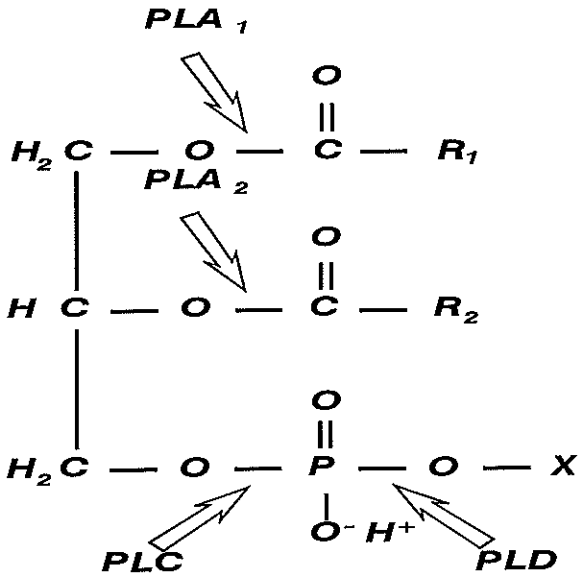

Fig. 2. Hydrolysis of glycerophosphatides by phospholipase A_1, A_2, C or D (X = choline, ethanolamine, inositol, etc.). R_1 and R_2 are hydrocarbon chains of long chain fatty acids.

heart. More recently, Panagia *et al.* [23] demonstrated by using isolated subcellular membranes from rat ventricular myocardium that an active PLD is indeed bound to the sarcolemmal membranes. The reason for the relatively late discovery of the existence of receptor-coupled PLD was the established route of agonist-dependent 1,2-DAG production by hydrolysis of $PtdIns(4,5)P_2$ by PLC as well as the fact that the increase of observed PtdOH in many cells following receptor stimulation was generally thought to result from the rapid action of DAG-kinase on 1,2-DAG [24]. However, later it was reported by several other laboratories that the formation of 1,2-DAG was dissociated in time from generation of inositolphosphates [25-27], often to the extent that 1,2-DAG is formed in the complete absence of $Ins(1,4,5)P_3$ accumulation [28, 29].

IDENTIFICATION OF THE PLD PATHWAY IN MYOCARDIUM

PtdOH production from (prelabelled) PtdChol

One useful methodology for measuring PLD activity after receptor stimulation is determining the increase of PtdOH formed from PtdChol hydrolysis by PLD. The second messenger PtdOH is converted into 1,2-DAG by the activity of PtdOH-hydrolase. PtdOH can be separated from other phospholipids by thin layer chromatography (TLC) and quantified by photodensitometry. Recently it was shown that PtdOH increased in response to noradrenaline and ET-1 in adult rabbit ventricular myocytes [30]. In this study it was also demonstrated that the PLD product, PtdOH, stimulated the production of inositol phosphates. As exogenous PtdOH activated PLC, it was assumed that this second messenger is the activator of PLC-β following PLD activation. Another possibility is that PtdOH is produced from 1,2-DAG via phosphorylation when PLC-β is stimulated by an agonist. Newly formed PtdOH could then function as a positive feedback mechanism for PLC-β via PtdIns(4,5)P_2. PLD is also said to produce PtdOH which serves as an alternative pathway by which agonists could activate PLC-β-mediated cleavage of PtdIns(4,5)P_2 [31]. The coupling function of PtdOH in this article is in contrast to most other articles, where it is believed that PtdOH originates mainly from PLD action and will subsequently be transformed to 1,2-DAG.

Another suitable method for identifying PLD activity is prelabelling cells with [^{32}P]PO_4^{3-}, separating cell extracts on TLC and scraping off the [^{32}P]PtdOH spots followed by the assay of inorganic phosphate and counting of the radioactivity by liquid scintillation. PLD was shown to be activated by norepinephrine in rat aorta, as the amount of [^{32}P]PtdOH increased [32]. Also cellular PtdChol can be prelabelled with [^{32}P] under conditions where ATP is not labelled. This can be achieved by prelabelling of the cells with [^{32}P]-2-lysoPtdChol, which can easily enter the cells and becomes rapidly acylated into [^{32}P]PtdChol. The latter methodology has only been applied in non-myocardial studies.

AngII was shown to activate PLD via the AT_1-receptor present in cardiomyocytes (33). Cardiomyocytes prelabelled with [^{3}H]myristic acid showed a rapid increase in [^{3}H]PtdOH within minutes and the [^{3}H]PtdOH accumulation persisted for more than 30 min, indicating that it was derived from [^{3}H]myristoyl-PtdChol. [^{3}H]PtdOH could, however, also be produced by PtdChol-hydrolysis catalyzed by PtdChol-specific PLC and subsequent phosphorylation of 1,2-DAG catalyzed by DAG kinase. Both reactions could also explain the observed early [^{3}H]1,2-DAG response. However, when a DAG kinase inhibitor was used there was still an accumulation of [^{3}H]PtdOH. The latter result proved that PLD was responsible for AngII stimulated [^{3}H]PtdOH production in cardiomyocytes [33]. Recently, atrial natriuretic factor (ANF) was shown to stimulate PtdChol-specific-PLD as well as -PLC activity in heart muscle plasma membranes [34].

In rat cardiac fibroblasts it was shown that AngII induced rapid PtdOH formation via AT_1-receptors, which was a sustained response for over 2 h. PtdOH itself is thought to act as a second messenger inducing Ca^{2+}-mobilization. PtdOH has also been proposed to facilitate the influx of Ca^{2+} through the plasma membrane [35]. PtdOH might, therefore, have an until sofar underestimated second messenger action on gene expression and cell growth in cardiac cells [36]. Recently we observed a transient upregulation of proto-oncogenes and a late upregulation of the TGF-β gene in rat cardiac fibroblasts after stimulation by AngII which could be transmitted through PLD activation [37]. However, AngII is a potent activator of PLC-β in these cells (C.A.M. van Kesteren, personal communications), again suggesting that this pathway is the initial trigger for nuclear events.

1,2-DAG production and changes in molecular species

As mentioned before, 1,2-DAG can be produced in cells after receptor stimulation by the activity of several types of phospholipases. The first is the PtdIns (PtdIns, PtdIns(4)P and PtdIns(4,5)P_2)-specific PLC pathway, the second the PtdChol-specific PLC pathway and the third the PtdChol-specific PLD pathway which is followed by the conversion of PtdOH into 1,2-DAG by PtdOH-hydrolase. It is difficult to discriminate between these pathways on the basis of 1,2-DAG production that is

initiated after agonist stimulation. Both PLC and PLD were shown to be Ca^{2+}-dependent [38-43], with similar characteristics excluding the possibility of blocking the elevation of $[Ca^{2+}]_i$ to differentiate between PLC and PLD as a source for 1,2-DAG.
Thus, the clearest manner to identify the source of receptor stimulated 1,2-DAG or PtdOH production is to analyze their fatty acid compositions. Several non-myocardial studies indicate that 1,2-DAG formed in the early transient phase of receptor-stimulation predominantly contains fatty acids present in the PtdIns(4,5)P_2 pool (stearate, arachidonate), whereas the later phase contains more saturated fatty acids typically found in PtdChol [44, 45].

Choline production from [^{3}H]choline-labelled phosphatidylcholine

A great number of cell types have been demonstrated to produce choline-containing metabolites from endogenous PtdChol after stimulation by agonists. There are, however, many differences in product kinetics and profiles among different cell types and the agonist used for stimulation [16]. PLD activity can be detected by measuring the formation of free choline in the extracellular buffer or perfusion medium. One could also measure the decrease in PtdChol, but the problem in either case is to establish whether the increase of choline production and decrease of PtdChol was brought about by PLD and/or PtdChol specific-PLC. Therefore, the transphosphatidylation reaction that will be described below, has been generally accepted as the most useful method to confirm the occurrence of PLD activity since this reaction, where H_2O in the PtdChol hydrolysis reaction can be replaced by an alcohol such as butanol or ethanol, is specific for PLD. We measured choline formation in cultured cardiomyocytes prelabelled with [^{3}H]choline followed by a short incubation in an unlabelled choline-containing medium. In these cardiac myocytes, [^{3}H]choline production increased above control cells between 20 and 40 min after ET-1- and between 10 and 20 min after phorbolester (PMA) stimulation (unpublished results). The late responses suggest that PLD is involved in the hydrolysis of PtdChol. This was further investigated by in parallel studying the transphosphatidylation reaction of exogenously added ethanol into phosphatidylethanol (PtdEthanol). The results indicate that at least in myocardium PLD is more likely to be responsible for the hydrolysis of PtdChol than PLC-β.

In a recent review [46] it was reported that maximal stimulation of PtdEthanol formation by the addition of excessive amounts of ethanol resulted in almost complete inhibition of 1,2-DAG production from PtdChol. This suggests that 1,2-DAG was produced by PLD and not by PLC. Thus, PLC activity on PtdChol only seems of minor importance.

Transphosphatidylation reaction

PLD has the unique property to catalyze a transphosphatidylation reaction in phospholipids. Therefore the transphosphatidylation method is a very convenient method to distinguish PLD- from PLC-mediated PtdChol hydrolysis after receptor stimulation. PtdEthanol is formed when cells are stimulated with specific agonists in the presence of exogenous alcohol in a concentration varying from 0.1-1%. The alcoholgroup of ethanol can be transferred to the phosphatidylgroup of a phospholipid substrate where upon PtdEthanol is formed. This compound will accumulate in the cell, as it is a poor substrate for PtdOH-hydrolase, thus making PtdEthanol accumulation a suitable marker for PLD activity [47].

To perform the transphosphatidylation reaction, cells are prelabelled with an isotope which preferentially incorporates in PtdChol. Saturated fatty acids like [^{14}C]palmitic acid or [^{3}H]myristic acid can be used for this purpose. In cardiomyocytes we could thus show that ET-1, PMA as well as AngII stimulate PtdEthanol formation with a similar time-course as was observed in experiments recording [^{3}H]choline production. AngII stimulation was the weakest inducer of PtdEthanol accumulation (unpublished results).

Table 1. 'Receptor-coupled' phospholipase D in myocardium

Model	Stimulus	Methodology	References
Isolated perfused myocardium	Ischemic stress	PtdEthanol/PtdOH	Moraru *et al.*, 1992 [65]
Cultured cardiomyocytes	ET-1/PMA	PtdEthanol/PtdChol	Lamers *et al.*, 1995 [29]
	Ang II	PtdOH/PtdEthanol	Sadoshima & Izumo, 1993 [33]
	ET-1/noradrenaline	PtdOH/PtdButanol	Hongping *et al.*, 1994 [30]
	Mechanical stretch	PtdOH/PtdEthanol	Sadoshima & Izumo, 1993 [55]
Cardiac sarcolemma/membrane	Oleate	PtdOH/PtdGlycerol	Panagia *et al.*, 1991 [23]
	ANF	PtdOH/PtdEth./PtdChol	Baldini *et al.*, 1994 [34]
	Basal	PtdEthanol/PtdOH	Wang *et al.*, 1991 [48]

PLD activity in cell free preparations

Mammalian PLD activity exists in both membrane-bound and cytosolic forms, indicating either the occurrence of strictly localized distinct isozymes or of activation-related translocation of the enzyme from the cytosol to the (plasma)membrane [17]. Activation by translocation of PLD is unlikely to occur as evidence has been provided for different characteristics of the soluble and the membrane-associated form. The membrane-bound form exhibits specificity for PtdChol, whereas the cytosolic form hydrolyses PtdChol as well as PtdEtn and PtdIns and has different action requirements, as was also shown in myocardial tissue [48]. The most extensively characterized PLD is the microsomal-bound enzyme of brain, largely through the work of Chalifour and Kanfer [49]. A remarkable observation is that in cell free preparations PLD activity can only be detected in the presence of surfactants such as oleate [50].

Panagia *et al.* performed experiments with myocardial membranes [23]. Subcellular distribution studies indicated that PLD is only present in a particulate form: in sarcolemma-, sarcoplasmic reticulum-, and mitochondrial-membranes. PLD is able to catalyze a transphosphatidylation reaction in membranes and PLD is suggested to be associated with PtdOH-hydrolase, to act in a coordinated manner.

In intact cells, one has the advantage of working with a functionally intact system where the PLD activity can be monitored under physiological conditions. With the cell free extracts, the assay conditions may be optimal for enzyme activity (e.g. exogenous phospholipid substrate in its physiological form (micellar or lysosomal), pH, presence of detergents, cofactors, ionic strength etc.), but then conditions may be quite different from the actual intracellular conditions [9]. Furthermore, since there are more forms of PLD within the myocardium, it is uncertain whether

the relevant PLD, i.e. the enzyme that is stimulated by agonists, is being studied using cell-free extracts.

An alternative approach that was frequently chosen to assess the enzymatic characteristics of the membrane-bound form of PLD, is to study its regulation in permeabilized cells [51]. However, distinction between soluble and membrane-bound forms cannot be made with certainty as the release of cytosolic-enzymes from the cell after permeabilization may not be complete. On the other hand, it is hypothesized on basis of results of experiments measuring GTPγS (Guanosine-5'-O-(3-thio-triphosphate)) stimulation of PLD in permeabilized cells, that cytosolic factors might be recruited in a Ca^{2+} and/or G-protein-dependent manner and that these factors play a major role in obtaining the full PLD response [52].

MECHANISMS OF PHOSPHOLIPASE D ACTIVATION

In many cell types PLD activation appears to be receptor-linked and most of the agonists that cause PtdChol-hydrolysis also induce PtdIns(4,5)P_2-breakdown. The general picture that is emerging from all those studies is that the early phase of 1,2-DAG production is probably derived from the hydrolysis of PtdIns(4,5)P_2 by PLC-ß and the second late phase is derived from PtdOH, which itself is generated by PLD [16]. Therefore, it was postulated that receptor-linked activation of PLD may involve multiple factors derived from the PtdIns signalling cascade including Ca^{2+}, 1,2-DAG, PKC, G-protein and tyrosine kinases. Until now, the question whether PtdIns(4,5)P_2-hydrolysis per se is sufficient for PLD-activation or if it only has modulatory effects on receptor-mediated PLD activation, has not been addressed. The concerted action of PLC and PLD can be nicely determined by experiments in which PtdChol and PtdIns(4,5)P_2 are double-labelled with [^{32}P] in the phosphoryl-moiety and [^{3}H] in the fatty acid-moiety (or [^{14}C] in the glycerol-moiety and [^{3}H] in the fatty acid-moiety). After stimulation by agonist the[^{32}P]/[^{3}H] or [^{14}C]/[^{3}H] ratios of PtdChol, PtdIns(4,5)P_2, PtdOH and 1,2-DAG should delineate the relative contribution of PLC and PLD in synthesis of 1,2-DAG, either directly or through PtdOH. In several studies on tissues other than myocardium, it has been shown that PtdIns(4,5)P_2-hydrolysis is not essential for PtdChol-hydrolysis but only has a modulatory function.

Free Ca^{2+}

Ca^{2+} ionophores and chelators have been widely used to study the Ca^{2+} dependence of PLD activation in intact tissues, but research in myocardium has been lagging behind, as is also true for the effects of Ca^{2+} on PLD in cell-free preparations [18]. Anyhow, from those investigations follows that receptor-mediated PLD activity is obligatory dependent on Ca^{2+}, indicating that Ca^{2+} in addition to active accessory proteins such as GTP-binding proteins or PKC, may also act directly at the level of PLD protein.

On the other hand it is well known, that PMA activation of PLD in intact cells does not require Ca^{2+} [17, 18]. These observations might be consistent with the demonstration in cell-free preparations of Ca^{2+} dependent- and independent forms of PLD, but are no definitive proof yet [17]. The effects of agonists, that stimulate the PtdIns(4,5)P_2-hydrolysis, on Ca^{2+} transients are variably depending on the species and/or making of cardiomyocyte preparations [2]. For example, we have observed only small and delayed (> 6 min) $[Ca^{2+}]_i$ elevations in rat neonatal cardiomyocytes after stimulation with α_1-adrenergic agonist or ET-1 [53]. Recently we observed stimulation of PLD by the measurement of [^{14}C]palmitoyl-PtdEthanol formation in the same model [29]. Therefore, the possibility that PLD in myocardium is activated as a consequence of Ca^{2+} mobilization induced by the PtdIns cycle activation seems unlikely. Moreover, the major PKC isoform translocated and activated by ET-1 stimulation of these cells is the ε-form, which is Ca^{2+} independent [29].

GTP-binding proteins

A role for G-proteins in receptor-linked PLD activation, analogous to the coupling of receptors to PLC-catalyzed PtdIns(4,5)P_2-hydrolysis, has mainly been based on the observation that non-hydrolysable GTP-analogues, such as GTPγS, activate PLD in permeabilized cells and cell-free preparations from many cells, although this aspect was not studied in cardiomyocytes [16, 18]. A possible indication of a link between the activation of PLC-ß and PLD is the sensitivity of receptor-mediated PLD activation to pertussis toxin. But this is no definitive proof for a direct coupling of PLD to G-proteins. It should be noted that PLC-β activity is not sensitive to pertussis toxin, because it is coupled to G_q. The unequivocal proof for GTP-binding protein regulation of receptor-mediated PtdChol-hydrolysis must await appropriate reconstitution studies as were done with PtdIns(4,5)P_2 specific PLC [54]. One possible explanation forwarded for the receptor-coupled activation of both PLD and PLC is that a single receptor GTP-binding protein complex couples both effector enzymes and that coupling is perhaps regulated by PKC [18], but this is highly unlikely due to the fact that PLC-β G_q is pertussis toxin insensitive, in contrast to the pertussis toxin inhibition of PLD activity.

Protein kinase C

Phorbolesters (e.g. PMA) appear to be universally effective in inducing PtdChol-hydrolysis by PLD [16]. Recently, we could definitively show this to be true for PLD present in cultured rat neonatal cardiomyocytes as well [29]. Down-regulation of PKC by prolonged exposure to PMA usually blocks PLD activation by agonists such as ET-1, carbachol and vasopressin. Moreover, over-expression of PKC-β in rat fibroblasts by cDNA transfection greatly enhances PLD activation in response to PMA, ET and α-thrombin [17]. It should, however, be noticed that the activation of PLD by phorbolester has in many cases been shown to be insensitive to PKC inhibitors such as staurosporine or Ro 31-8220 (reviewed in [18]).

The Ca^{2+} independent PKC-ε was found by us to be the major isoform that translocates from the cytosol to a membrane-containing fraction in cardiomyocytes after either PMA or ET-1 stimulation of cardiomyocytes [29]. Therefore it is attractive to assume that the rapid activation of PKC-ε as a result of a rise in 1,2-DAG due to PtdIns(4,5)P_2 hydrolysis is involved in PLD stimulation. A more rapid rise of 1,2-DAG due to ET-1 than after α_1-adrenergic agonist stimulation of cardiomyocytes was observed [28]. An even more rapid and stronger rise in 1,2-DAG was seen with AngII stimulation of this cardiomyocyte preparation [5]. Indeed, Sadoshima *et al.* demonstrated that AngII strongly activates PLD by measuring [^{3}H]myristoyl-PtdEthanol formation and [^{3}H]myristoyl-PtdOH formation [33]. These authors also showed that mechanical stretch activates PLC as well as PLD, although on basis of their data no comparison between time-courses of PLC and PLD activation by stretch can be made [55].

Tyrosine-phosphorylation

Generally, most growth factors promote PtdChol hydrolysis and this is thought to be mainly due to activation of PKC as a result of PtdIns(4,5)P_2 breakdown [20]. However, in some cases, PtdChol-hydrolysis occurs in the absence of PtdIns(4,5)P_2 hydrolysis, implying another mechanism that does not involve PtdIns(4,5)P_2-derived 1,2-DAG. The action of growth factors often involves intrinsic-tyrosine kinase activity of the growth factor receptors. In fibroblasts it was shown to be possible to stimulate PLD activity by receptor-linked tyrosine kinase activity with the agonists platelet-derived-growth factor (PDGF) and epidermal growth factor (EGF) in the absence of apparent stimulation of PtdIns(4,5)P_2 hydrolysis [47]. However, one should be aware that PLC-γ can be phosphorylated and thus activated by tyrosine kinase activity of growth factor receptors and that this PLC subtype is not distinguishable from PLC-β.

Using an inhibitor of phosphotyrosine dephosphorylation, pervanadate, evidence was provided that tyrosine phosphorylation is involved in the activation of PLD [17].

Since $p21^{ras}$ protein participates in the signalling cascades elicited by growth factors, it is possible that not only tyrosine phosphorylation but also this G-protein is involved in PLD activation

[20, 47]. Cardiomyocyte stretch rapidly activates a plethora of second messenger pathways, including tyrosine kinases, $p21^{ras}$, PKC, PLC and PLD [55]. Precise kinetic analysis of each pathway is necessary to determine time-dependent and hierarchical relationships of activation of each pathway, but it has yet not been carried out. Anyhow, the initial results raise the possibility that tyrosine phosphorylation or $p21^{ras}$ is involved in the activation of PLD during cardiomyocyte stretch leading to hypertrophic growth [55].

FUNCTIONAL SIGNIFICANCE OF AGONIST-INDUCED PHOSPHOLIPASE D

Myocardial hypertrophy and gene expression

An obvious function of PtdChol-derived 1,2-DAG is to induce prolonged activation of PKC, because PKC provides a positive feedback signal to PLD. In contrast, PKC down-regulates PLC (see Fig. 1). Sustained elevation of 1,2-DAG is a prerequisite for long-term cell responses such as cell growth and differentiation. The hypertrophic response of cardiomyocytes in response to mechanical overload *in vivo* and *in vitro* closely resembles the mitogenic response of other cell types to growth factor stimulation. Mechanical overload also plays a critical role in determining cardiac muscle phenotypes. Recently, many laboratories have begun a systematic analysis to identify biochemical pathways by which the mechanical load is transduced into extra- and intracellular signals regulating hypertrophy and gene expression [2, 4, 5, 33, 56, 57]. Using an *in vivo* model of stretch-induced hypertrophy Sadoshima and Izumo [55] demonstrate that mechanical stress activates PLD. How cell stretch leads to activation of PLD is, at present, unclear. The most likely possibility is that mechanical stress releases growth factor(s), such as AngII, which activates its receptor and subsequent second messenger cascades such as PLC and PLD, but also PLA_2, $p21^{ras}$ and tyrosine kinases.

In line with an important role of PLD is the accumulating evidence that suggests that PKC is involved in the mechanism of development of myocardial cell hypertrophy [4, 56-58]. The latter suggests that prolonged PKC activation may represent a common signalling event in the activation of cardiac gene expression and subsequent protein synthesis during development of cardiac hypertrophy. When cultured cardiomyocytes were used as hypertrophy model, activated PKC isoenzymes were found to translocate from cytosol to the membrane and cytoskeletal fraction in response to a variety of stimuli, such as α_1-adrenergic agonists, ET-1, AngII and thrombin [29, 59-62]. From these studies it became also clear that after activation, each PKC isoenzyme may have its specific location and substrates for phosphorylation to regulate hypertrophy and specific gene expression. The members of the PKC isoenzyme family probably respond differently to various combinations of Ca^{2+}, 1,2-DAG-species varying in fatty acid composition and membrane phospholipid metabolites including free fatty acids [63]. This may be one of the underlying reasons for the observed agonist-dependent characteristics of the hypertrophic and gene reprogramming responses.

Ischemia and ischemic preconditioning

Brief periods of myocardial ischemia trigger an adaptive response that protects the heart against injury from a subsequent prolonged period of ischemia and reperfusion, a phenomenon known as "ischemic preconditioning" [64].

In perfused rat heart, prelabelled with [^{14}C]arachidonic acid, ischemia (30 min)-reperfusion (30 min) induced a significant increase in the amount of radio label incorporated into PtdOH and 1,2-DAG [65]. In experiments where oleate was added to the perfusate to further stimulate PLD, an improved functional recovery of the ischemic heart during reperfusion was found. The fact that oleate stimulates PLD in perfused rat heart was already demonstrated before by Lindmar *et al.* [22]. The mechanisms by which PLD is activated during ischemia-reperfusion and by which PLD activation protects the cardiomyocyte from the ischemic-reperfusion injury are not obvious. Myocardial ischemia has been shown to enhance adrenergic neural traffic, to release catecholamines from nerve terminals and to increase myocardial responsiveness to α- and β-adrenergic stimulation.

The release of ET is increased during hypoxia and myocardial infarction leads to increased plasma levels of ET [66]. Therefore, it is possible that α_1-adrenergic or ET receptors are responsible for PLD activation during ischemia. However, in cultured cardiomyocytes we showed that ET-1 or phenylephrine stimulation during prolonged hypoxia and subsequent reoxygenation gave rise to increased rather than decreased damage [67], suggesting that a short period of ischemia is important for development of the protective effect.

However, the PLD activation observed by Moraru *et al.* [65] could be the underlying cause of ischemic preconditioning.

Experiments with specific agonists and antagonists have indicated that brief ischemic-stress induced release of adenosine, acetylcholine, noradrenaline and AngII is involved in ischemic preconditioning depending on the species [68]. As all these stimuli activate PLC and/or PLD it is now believed that the activation and translocation of PKC during preconditioning accounts for the ability of the cardiomyocyte to "remember" the ischemic episode thereby increasing the tolerance during subsequent prolonged ischemic periods.

Recently Mitchell *et al.* [69], showed in rat heart that the Ca^{2+}-independent PKC isoforms PKC-δ and PKC-ε were translocated with transient ischemic stimulation. Since the initial PLC response usually is accompanied by increases in Ca^{2+} and 1,2-DAG and followed by a prolonged increase of 1,2-DAG with no rise in Ca^{2+}, the observed translocation of PKC-δ and PKC-ε is more likely due to the secondary PLD activation. The gradual decay of protection takes hours after the ischemic event which is more in agreement with the involvement of PLD than PLC.

Positive inotropy

Perfusion medium containing PLD (from *Streptomyces chromofuscus*) was shown to increase peak force development in rabbit papillary muscles [70]. This positive inotropic effect of exogenous PLD was ascribed to a specific increase of PtdOH in the sarcolemma that produced an increase in net anionic charge of the membrane. The results were in agreement with a previous study of the same group where it was shown that PLD addition induces a large increase in sarcolemmal Ca^{2+} binding [71]. Furthermore, Philipson and Nishimoto reported that PLD addition stimulated Na^+/Ca^{2+} exchange in cardiac sarcolemmal vesicles [72]. There is no study available showing that receptor-mediated stimulation of the endogenous PLD and positive inotropy occurs at the same time. Therefore, studies using exogenous PLD should be interpreted carefully with respect to their physiological relevance.

However, there is now evidence for ET-1 receptor coupled PLD activity in isolated adult and neonatal rat ventricular myocytes [29, 30]. The time course and dose-dependency of PtdOH accumulation in ET-1 stimulated cells are equal to that of the developing positive inotropy in adult rabbit ventricular myocardium, indicating that receptor-mediated PLD is involved in the mechanism of the positive inotropic effect of ET-1 [30].

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Chapter 3

CROSS-TALK BETWEEN RECEPTOR-MEDIATED PHOSPHOLIPASE C-ß AND D VIA PROTEIN KINASE C AS INTRACELLULAR SIGNAL POSSIBLY LEADING TO HYPERTROPHY IN SERUM-FREE CULTURED CARDIOMYOCYTES

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ABSTRACT

Phospholipase C-ß (PLC-ß) signalling via protein kinase C (PKC) has been recognized as a major route by which stimuli such as α_1-adrenergic agonists, endothelin-1 (ET-1) and angiotensin II (AngII) induce hypertrophy of myocytes. The goal of this study was to evaluate the role of phospholipase D (PLD) in contributing to the formation of the PKC activator 1,2-diacylglycerol (1,2-DAG) and to study the mechanism(s) of PLD activation by agonists. Stimulation of serum-free cultured neonatal rat cardiomyocytes with ET-1 (10^{-8} M), phenylephrine (PHE, 10^{-5} M) or AngII (10^{-7} M) resulted in a rapid (0-10 min) activation of PLC-ß to an extent (ET-1 > PHE > AngII) that correlated with the magnitude of stimulation of protein synthesis ([^{3}H]leucine incorporation into protein) measured after 24 h. Phorbol 12-myristate 13-acetate (PMA, 10^{-6}M) and ET-1 were equipotent in stimulating protein synthesis. ET-1 and PMA, but not PHE and AngII stimulated [^{3}H]choline formation from labelled PtdChol after a lag-phase of about 10 min. That this [^{3}H]choline formation was due to the action of PLD was confirmed by measurement of phosphatidylgroup-transfer from cellular [^{14}C]palmitoyl-phosphatidylcholine to exogenous ethanol. ET-1 and PHE, to much lesser extent, produced a rapid (0-5 min) translocation of PKC-ε immunoreactivity from the cytosol to the membrane fraction, whereas no intracellular redistribution of PKC-α, -δ and -ζ immunoreactivities was observed. PMA caused translocation of PKC-α, PKC-ε as well as PKC-δ. Cellular redistribution of PKC activity measured by [^{32}P]-incorporation into histone III-S was not observed with ET-1 and PHE but only with PMA stimulation. Down-regulation of PKC isozymes by 24 h pretreatment of cells with PMA or blockade of PKC by chelerythrine (10^{-4} M) inhibited ET-1 and PMA stimulated [^{3}H]choline production. Staurosporine (10^{-6} M) had, however, no effect. In conclusion, the results indicate that in serum-free cultured cardiomyocytes, ET-1 initially activates PLC-ß and after a lag-phase PLD, whereas PHE and AngII activate only PLC-ß. PLC-ß stimulated by ET-1, may cross-talk with PLD via translocation of PKC-ε. These signals are possibly linked to the hypertrophic response.

INTRODUCTION

The signal transduction pathway initiated by receptor-mediated phospholipase C-ß (PLC-ß) has been recognized as a major route in heart by which stimuli such as α_1-adrenergic agonists, endothelin-1 (ET-1), angiotensin-II (AngII), purinergic agonists, opioids and thrombin produce hypertrophic growth of myocytes [1-3]. PLC-ß is a GTP-binding protein (G-protein)-coupled effector-enzyme that produces the second messengers inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and 1,2-diacylglycerol (1,2-DAG) from phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$). Subsequently, by the action of Ca^{2+}-calmodulin and 1,2-DAG dependent protein kinases, specific proteins become phosphorylated, which then transduce the growth signal to the nucleus [4]. The final result is stimulation of overall protein synthesis and reprogramming of gene expression [1, 2, 5, 6]. In particular, certain isozymes of protein kinase C (PKC) have been shown to be involved in the induction of hypertrophy and adjustment of gene expression after stimulation with the agonists, as mentioned above. Transfection of cardiomyocytes with constitutively-activated PKC produces several responses equal to those in hypertrophy [7-9]. Furthermore, phorbol 12-myristate 13-acetate (PMA), a potent activator of PKC, induces hypertrophy in the model of serum-free cultured neonatal rat cardiomyocytes [2, 4].

Up to now, phospholipase D (PLD) has obtained poor attention as a potential contributor to the formation of PKC-activating 1,2-DAG in myocardium induced by the afore mentioned hypertrophic stimuli. However, it was previously shown that several of the PLC-ß stimulating agonists, such as α_1-adrenergic agonists, ET-1, AngII and muscarinic agonists, atrial natriuretic factor [10-14], also stimulate PLD mediated hydrolysis of phosphatidylcholine (PtdChol) into phosphatidic acid (PtdOH). PtdOH can rapidly be converted into 1,2-DAG by the enzyme PtdOH hydrolase. At present, it is not known by which mechanism(s) the hypertrophic stimuli activate PLD. Non-myocardial studies have provided evidence that PLD activation, like that of PLC-β, is mediated by a receptor

coupled G-protein [15, 16]. However, other studies reported that phorbolesters are very effective in inducing PtdChol hydrolysis through PLD activity (reviewed in [17, 18]). Overexpression of PKC-β in rat fibroblasts by cDNA transfection greatly enhances PLD activation in response to PMA, ET-1 and α-thrombin [19]. We demonstrated earlier, that PMA inhibits the α_1-adrenoceptor as well as ET-1 stimulated PLC-β activity in cardiomyocytes [20-22]. Therefore, receptor-mediated PLC-ß may cross-talk with PLD via activation of specific PKC isozymes. Indeed, several non-myocardial studies (e.g. [23, 24]) indicate that the 1,2-DAG formed in an early transient phase of receptor stimulation predominantly contains fatty acids derived from the PtdIns(4,5)P_2 pool, whereas in a later phase it contains fatty acids more typically of those normally seen in PtdChol.

The aim of the present study was to investigate whether α_1-adrenergic agonists, ET-1 and/or AngII, known to evoke hypertrophy, activate PLD in addition to PLC-β, in serum-free cultured neonatal cardiomyocytes. A possible role of the translocation and/or activation of the major PKC isoforms PKC-α ,-ε, -δ and -ζ in the cross-talk between PLC-β and PLD activity is studied by immuno blot analysis and by [^{32}P]-incorporation into histone III-S. To further delineate the role of PKC in the cross-talk, we tested the influence of down-regulation of PKC or direct inhibition of PKC on the agonist-stimulated PLD activation.

MATERIALS AND METHODS

Reagents

Phenylephrine (PHE), Sar^1 AngII and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (St. Louis MO, USA) and ET-1 from Boehringer Mannheim (Germany). Culture dishes (4-well Multidish) were from Nunc (Roskilde, DK), while the culture media DMEM and M199 were both obtained from Gibco (UK); fetal calf serum, horse serum, penicillin/streptomycin and trypsin were obtained from Boehringer Mannheim. [1-^{14}C]Palmitic acid (57 mCi/mmol) was from Dupont NEN products (Boston MA, USA). High performance thin-layer chromatography plates (HPTLC) were obtained from Merck (Darmstadt, Germany). [^{3}H]Leucine (54 Ci/mmol), (Methyl[^{3}H])choline chloride (75 Ci/mmol), myo-[2-^{3}H]inositol (17.5 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham International PLC (Amersham, UK). Dowex 50W-X8 (100-200 mesh, H^+-form) and Dowex AG 1-X8 (200-400 mesh, formate form) were respectively from Fluka AG (Buchs, Switzerland) and Bio-Rad Laboratories (Richmond CA, USA). Chelerythrine was obtained from Biomol, SanverTech (Breda, The Netherlands). Staurosporine was obtained from Sigma. The scintillation cocktail Instagel-plus was from Canberra Packard Benelux N.V./S.A. (Groningen, The Netherlands). Rabbit polyclonal antibodies for PKC-α, -ε, -δ and -ζ and their competing immunizing peptides (to prove specificity) were purchased from Santa Cruz Biotechnology (Santa Cruz CA, USA). The ε-peptide was from Pierce Chemical Company (Rockford IL, USA). Chemiluminescence images are obtained by applying the Supersignal™ CL-HRP Substrate (Pierce) with the imager system GS-363 from Bio-Rad. Prestained SDS-PAGE standard proteins (Broad Range) were obtained from Bio-Rad Laboratories.

Cultured cardiomyocytes

Primary cultures of neonatal ventricular myocytes were prepared from hearts from 1-2-day old Wistar rats, as described previously [25], using a single preplating step (surface area of approximately 10 cm^2 per rat heart) to further increase cardiomyocyte to non-cardiomyocyte ratio. The cultures contain 90-95% myocytes, as routinely assessed by microscopic observations of beating cells and by usage of the periodic acid Schiff (PAS) staining method to distinguish myocytes from non-myocytes. The proportion of myocytes, visible as cells containing stained glycogen granules is strongly increased after preplating.

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 h.

Cultured non-myocytes

Non-myocytes (mostly fibroblasts) were separated from cardiomyocytes by a 75 min preplating step. After this step, fibroblasts are attached to the culture flasks and the cardiomyocytes (still in suspension) are removed and seeded into their final culture plates, which are then used for the experiments. The fibroblasts are cultured in the flasks until confluency and then passaged and seeded at a density of 7.5×10^4 cells/cm^2 in 1.8 cm^2 wells until confluency is reached. 24 h after seeding, the growth medium is changed to serum-free medium [26].

Both cardiomyocytes and fibroblasts were kept in 5% CO_2 at 37°C in complete growth medium consisting of DMEM/M199 (4:1), 25 mM HEPES supplemented with 5% fetal calf serum and 5% horse serum, 100 U penicillin/ml and 100 µg streptomycin/ml for the first 24 h. Growth medium was renewed 24 h after seeding with complete serum-free growth medium, and every 48 h thereafter. Experiments were routinely performed at 5-6 days after plating of the cells.

Protein synthesis

Cardiomyocytes were incubated with 2 µCi [^{3}H]leucine/ml for 24 h in complete serum-free growth medium. The concentrations of the agonists used were ET-1 (10^{-8} M), PHE (10^{-5} M) , Sar^1 AngII (10^{-7} M) and PMA (10^{-6}M). Incubations were terminated after 24 h by rapidly washing the cells with ice-cold 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), followed by three successive wash and precipitation steps (30 min, 4°C) with 250 µl 10% trichloric acetic acid. After lysis of the cells in 1 M NaOH (500 µl) at 4°C overnight, the protein fractions were collected and counted by liquid scintillation counting.

Total [^{3}H]inositol phosphates

Cardiomyocytes were labelled with 2 µCi myo[2-^{3}H]inositol/ml for 48 h in complete serum-free growth medium. After washing and pre-incubation (15 min, 37°C) with incubation buffer (see protein synthesis, except for the inclusion of 10 mM LiCl), cells were stimulated with various agonists. The concentrations of the agonists used were again ET-1 (10^{-8}M), PHE (10^{-5}M), AngII (10^{-7} M) and PMA (10^{-6} M). Incubations were terminated after 0, 10 and 40 min by rapidly washing with ice-cold buffer followed by two successive extractions with 250 µl 4% $HClO_4$ after which lipids were extracted twice with 250 µl ice-cold methanol/HCl (100:1 v/v). The perchloric acid extract was rapidly neutralized by addition of 100 µl 2 M KOH/1 M K_2CO_3. The water-soluble [^{3}H]inositol phosphates in the perchloric acid extract were quantified by Dowex column chromatography on Dowex AG 1-X8 as originally described [27]. The amounts of total [^{3}H]inositolphosphates ([^{3}H]InsP_n) were expressed as percentage of the total cellular amount of [2-^{3}H]inositol labelled products defined as the sum of [^{3}H]inositol-containing water-soluble intermediates plus [^{3}H]inositol-containing lipids.

[^{3}H]choline formation

Cardiomyocytes or fibroblasts were labelled with 2 µCi (methyl[^{3}H])choline/ml for 24-48 h in complete serum-free growth medium. After extensively washing three times with radioactive-free medium, twice the amount of volume as during labelling, and thereafter pre-incubation (15 min, 37°C) with incubation buffer, cells were stimulated with various agonists (concentrations as described above) in the presence of unlabelled choline chloride (25 µM). Incubations were terminated after 0, 10 and 40 min by collecting the incubation buffer, keeping the cells on ice and washing them with ice-cold buffer. By subsequent extracting with methanol, the total amount of [^{3}H]choline incorporated into the cellular lipids and the intracellular free [^{3}H]choline was measured. The incubation buffer was used to determine the free [^{3}H]choline released by the cells during the incubation. By means of Dowex column chromatography (Dowex 50W-X8) [28] the amount of free [^{3}H]choline was quantified and could be expressed as percentage of the total cellular amount of [^{3}H]choline labelled intermediates defined as the sum of [^{3}H]choline containing water-soluble products plus [^{3}H]choline containing lipids. A representative elution pattern of separation of cell extracts (not incubation buffer) isolated from control and 40 min ET-1, PHE and PMA stimulated cardiomyocytes is shown in Figure 1.

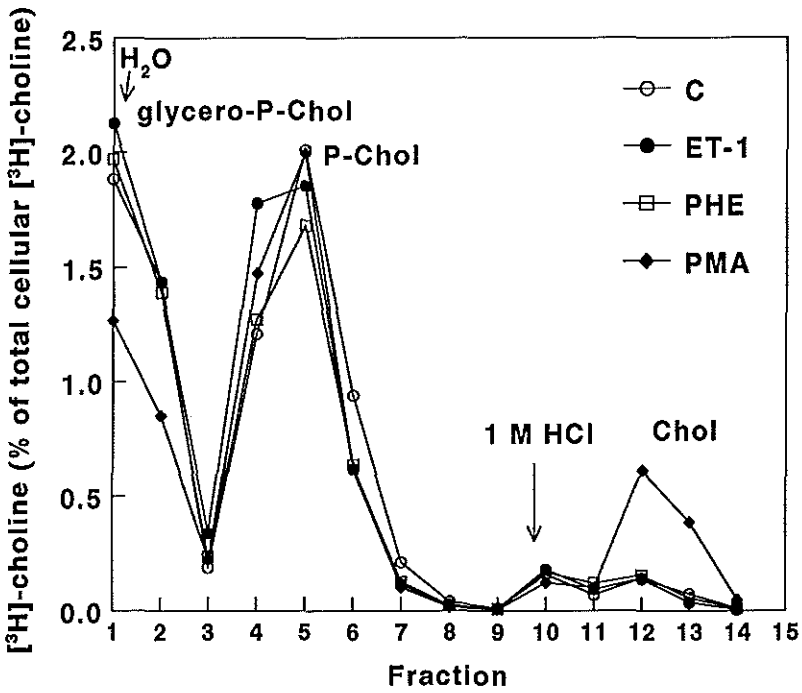

Fig. 1. Elution patterns (Dowex 50W-X8) representing the separation of water-soluble [^{3}H]choline containing intermediates (glycerophosphocholine (Glycero-P-Chol), phosphocholine (P-Chol) and choline (Chol)) extracted from serum-free cultured and 48 h [^{3}H]choline labelled cardiomyocytes that were either unstimulated or stimulated with ET-1 (10^{-6} M), PHE (10^{-5} M) or PMA (10^{-6} M) for 40 min. The levels are expressed as percentage of the total [^{3}H]choline incorporated in water-soluble [^{3}H]choline containing intermediates and [^{3}H]choline containing lipids.

[^{14}C]palmitoyl-phosphatidylethanol formation

Cardiomyocytes were labelled with 1 µCi [^{14}C]palmitic acid/ml for 48 h in complete serum-free growth medium containing 4.2 mg/ml fatty acid-free albumin. After washing and pre-incubation (15 min, 37°C) with incubation buffer containing 21.0 mg/ml fatty acid-free albumin, the cells were stimulated with various agonists (for the concentrations see above) and 1% (v/v) ethanol was added to quantify the PLD-catalysed [^{14}C]palmitoyl-phosphatidylethanol ([^{14}C]palmitoyl-PtdEth) formation. After incubation cellular lipids were extracted by two successive extractions with 250 µl methanol/HCl (100:1 v/v). Phase separation was performed by adding 500 µl chloroform and 250 µl 2.5 M HCl. The organic phase was collected and the waterphase reextracted with 500 µl chloroform/methanol/HCl (0.6 M) (3:48:47 v/v/v). The organic lipid extract was collected and used for separation of [^{14}C]palmitoyl-PtdEth on HPTLC plates with an analytically pure PtdEth as reference for mobility, and chloroform/methanol/acetic acid/water (75:46:3:1 v/v/v/v) as mobile phase [16]. The analysis of the radioactive spots was performed on the CS-screen of the Molecular Imager (Bio-Rad), and the amount of [^{14}C]palmitoyl-PtdEth was determined as percentage of the total cellular amount of [^{14}C]palmitate-containing lipids.

PKC inhibition and ET-1 stimulated PLD activity

Incubation conditions of cardiomyocytes in these experiments were the same as those used in the experiments on [^{3}H]choline formation (see before), except that the pre-incubation step was prolonged to 30 min with or without PKC-inhibitor. The PKC inhibitors used were staurosporine (1 µM) and

chelerythrine (100 µM). Moreover, some wells obtained PMA (1 µM) 24-48 h before the incubation in order to down-regulate PKC. The agonists used for 40 min stimulation were ET-1 (10^{-9} M) and PMA (10^{-6} M). Samples were analysed for [^{3}H]choline released in the incubation medium as described above.

Immunoreactivity and phosphorylation activity of PKC isozymes in subcellular fractions towards histone III-S

Immunoreactivity measurements by Western blotting were carried out, essentially as described [29]. Immuno blots were prepared from samples taken from all different time points and analysed for the PKC-α, -ε, -δ and -ζ distribution in cytosol and membrane fraction, after incubation of cultured cardiomyocytes with PHE, ET-1 or PMA (concentrations as above). The cell lysis, cell fractionation, measurement of protein content and immuno blot analysis of subcellular fractions were performed as previously described [30]. The analysis of the bands was performed on the CH-screen of the Molecular Imager. PKC-ε was always located at 90-96 kDa, PKC-α at 80-85 kDa, PKC-δ at about 74 kDa and PKC-ζ at about 78 kDa (see also Fig. 4). The immunoreactivities were inhibited by the addition of the polypeptide, against which the specific antibody was raised [31]. Prior to the (immunore)activity measurements, the membrane fraction was solubilized in 0.5 mM EGTA, 2 mM EDTA, 2 mM PMSF, 10 mM ß-mercaptoethanol, 5 mg/ml leupeptin and 1.0% (v/v) Triton X-100 in 20 mM Tris-HCl (pH 7.5) and the insoluble material was removed by centrifugation. The immunoreactivities were expressed as percentage of the reactivity measured at time zero.

Total PKC activity was assayed in the cytosolic and membrane fraction in 100 µl reaction medium containing 5 mM $MgCl_2$, 10 µM ß-mercaptoethanol, 2.5 mg/ml bovine serum albumin, 200 nM okadaic acid, 0.5 mg/ml histone III-S, 10 µM [γ-^{32}P]ATP (50-100 ct/min/pmol), 20 mM Tris-HCl (pH 7.5) with or without 2.5 mM $CaCl_2$, and an ultrasonified mixture of 0.16 mg/ml phosphatidylserine (PtdSer) and 0.04 mg/ml 1,2-DAG. When the phosphorylation of the ε-peptide was measured, the reaction mixture did not contain Ca^{2+} and, instead of 0.5 mg/ml histone III-S, 30 µM ε-peptide. The assay mixture was pre-incubated at 30°C for 2 min and the reactions were started with the cardiomyocyte subfractions and stopped after 5 min by the addition of 200 µl ice-cold 25% trichloric acetic acid plus 20 µl 10 mg/ml bovine serum albumin. Thereafter, the mixture was Millipore filtrated. The filters were washed four times with ice-cold 10% trichloric acid and counted by liquid scintillation. Ca^{2+} plus 1,2-DAG stimulated activities are expressed as pmol incorporated [^{32}P]/mg protein/min.

Statistics

The statistical significance of any of the observed effects was evaluated by the Students t-test and significance was set at probability less than 0.05.

RESULTS

Stimulation of PLC-ß and protein synthesis

We have shown earlier that stimulation of serum-cultured neonatal rat myocytes with either ET-1 or PHE results in rapid activation of PLC-β in a dose-dependent manner [22]. In another report, we demonstrated that development of hypertrophy (measured as an increase in protein/DNA ratio), can only be shown by stimulating serum-free cultured cells with these agonists and AngII. Because serum addition by itself produces hypertrophy in these cells, agonists could not further stimulate this hypertrophic growth process in serum-cultured cardiomyocytes [5, 6]. Therefore, prior to this study, we checked if the potent and rapid (within minutes) effects of these agonists on PLC-ß were still present in the serum-free cultured cardiomyocytes. Figure 2 demonstrates that ET-1 (10^{-9} M) and PHE (10^{-5} M) gave the strongest stimulation of [^{3}H]InsP$_n$ formation (as measure of PLC-ß activity), remaining almost linear during the period of measurement (40 min). Stimulation of [^{3}H]InsP$_n$ is, respectively, 10.1-fold for ET-1 and 4.3-fold for PHE. AngII only gave a small stimulation (1.8-fold) of PLC-ß activity, which is in accordance with a previous report showing rapid desensitization of the PLC-ß response [32]. As expected, phorbolester PMA (10^{-6} M) had no effect on PLC-ß.

The extent of stimulation of PLC-ß activity by ET-1, PHE and AngII correlated with the magnitude of stimulation of [^{3}H]leucine incorporation (as measure of protein synthesis)(see Table 1). Phorbolester PMA and ET-1 were equipotent in stimulating the rate of protein synthesis (respectively 182 and 174% from control).

Table 1. The rate of protein synthesis measured by [^{3}H]-leucine incorporation in serum-free cultured cardiomyocytes stimulated by various agonists for 24 h.

	[^{3}H]-leucine incorporation (% of control)
ET-1	182.0±10.0*
PHE	147.0± 8.0*
AngII	123.0± 5.0*
PMA	174.0±10.0*

Concentrations used for the incubations were ET-1 (10^{-8} M), PHE (10^{-5} M), Sar^1 AngII (10^{-7} M) and PMA (10^{-6} M) and the number of experiments carried out, is 12. *: $p < 0.05$ compared to the control (100%).

Stimulation of PLD

Cardiomyocytes were labelled with [^{3}H]choline for 24-48 h and thereafter stimulated with agonists. Initially, we not only measured free [^{3}H]choline, but also the intermediates [^{3}H]phosphocholine and [^{3}H]glycerophosphocholine in the cell extracts (compare the elution pattern shown in Fig. 1). No increase of the levels of water-soluble [^{3}H]choline containing metabolites was found with either ET-1 or PMA in the cell extracts (upper part of Table 2). Only after 40 min of PMA stimulation is there a significant rise in the percentage of cellular free [^{3}H]choline, which indicates stimulation of PLD activity. However, the major fraction of [^{3}H]choline formed appeared to be released in the incubation buffer, and in this fraction a significant stimulation was observed with ET-1 as well as PMA (respectively 2.06 and 2.67% of total cellular [^{3}H]choline compared to control 1.64%), which indicates that PLD is activated by these agonists (lower part of Table 2). Moreover, it was found to be necessary to prevent re-utilization of formed [^{3}H]choline by the cells by addition of 25 µM unlabelled choline in the incubation buffer. It is interesting to note that the most potent activator of PLD, PMA even induced a reduction of [^{3}H]phosphocholine and [^{3}H]glycerophosphocholine content of cell extracts. The latter effect is likely caused by a decrease in the specific radioactivity of [^{3}H]PtdChol, due to its accelerated breakdown by PMA and its resynthesis from the exogenous excess of unlabelled choline. The time-course of [^{3}H]choline in the incubation buffer after stimulation of cardiomyocytes with ET-1 and PMA was subsequently measured (Fig. 3).

A noteworthy finding was that there was already release of [^{3}H]choline under control conditions. After stimulation of the cells with ET-1 and particularly with PMA, [^{3}H]choline first became detectable after a lag phase of about 10 min. In another series of experiments we comparatively studied the effects of ET-1, PHE, AngII and PMA (Table 3). In contrast to the stimulations of PLD observed with ET-1 and PMA, only small and non-significant increases of [^{3}H]choline release were observed with PHE and AngII.

ET-1 stimulation of [^{3}H]choline formation may not be a property of the cardiomyocytes, but also of the contaminating non-cardiomyocytes (mostly fibroblasts). This is unlikely, based upon the

fact that the culture contains 90-95% cardiomyocytes as assessed by microscopical observations of cells beating and by the use of the periodic acid Schiff (PAS) glycogen staining (results not shown). To exclude a possible contribution of fibroblasts, we also tested the agonists in a homogenous preparation of cardiac fibroblasts isolated in parallel with the cardiomyocytes. ET-1, PHE and AngII had no effect on PLD up to 40 minutes of stimulation (Table 4). But, as in the cardiomyocytes, PMA is a strong stimulator of PLD activity, being significant after 10 minutes. These results demonstrate that the ET-1 and PMA effects on PLD are properties of the cardiomyocytes and not of the small number of contaminating fibroblasts.
Increased choline formation may not be a definite proof for occurrence of PLD. PLD has a unique ability to catalyse the transfer of the phosphatidyl group from PtdChol to exogenous primary alcohols. This reaction was used to distinguish PLD-mediated [^{3}H]choline-formation from other pathways

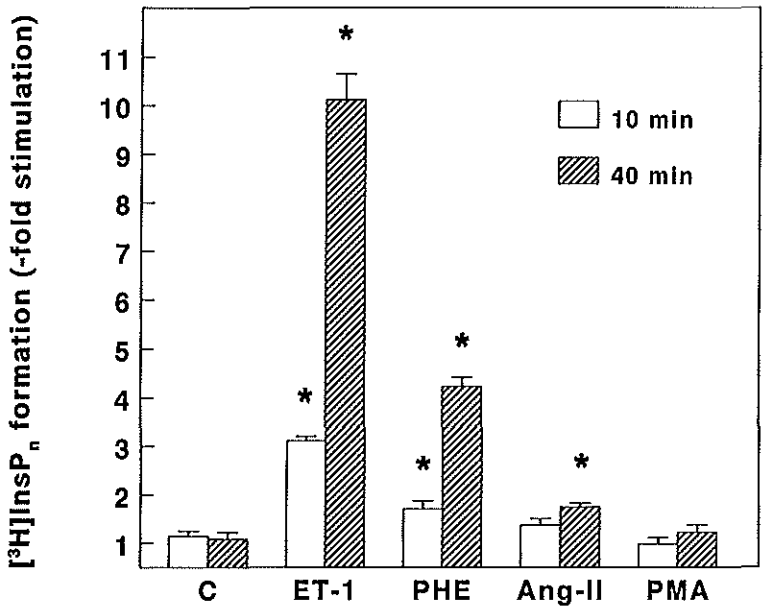

Fig. 2. Cellular [^{3}H]$InsP_n$ levels after stimulation of serum-free cultured cardiomyocytes with various agonists. Cardiomyocytes labelled for 48 h with [^{3}H]inositol were stimulated with ET-1 (10^{-8} M), PHE (10^{-5} M), AngII (10^{-7} M) or PMA (10^{-6} M) for the times indicated. Total [^{3}H]inositolphosphate ([^{3}H]$InsP_n$) levels were determined in cell extracts as described in the Materials and Methods section. The [^{3}H]$InsP_n$ levels are expressed as -fold stimulation (± SEM) above corresponding time controls. * :$p < 0.05$.

of PtdChol hydrolysis. Cardiomyocytes were labelled with [^{14}C]palmitic acid which has previously been shown to be a major fatty acid component of PtdChol [33].
Subsequently, cells were stimulated by the agonists in the presence of exogenous ethanol (1.0% v/v). The results on [^{14}C]palmitoyl-PtdEth degradation/formation are shown in Table 3. In control-, PHE- and AngII-stimulated cardiomyocytes, [^{14}C]palmitoyl-PtdEth, present from the beginning of the incubation, was slowly degraded (from 76.1 to 46.3% of the content at zero time). This initial [^{14}C]palmitoyl-PtdEth was formed during the labelling period from the ethanol (0.05% v/v), used

as necessary solvent of the [^{14}C]palmitic acid added. The observed degradation may represent the basal turnover of PtdChol as observed by the formation of [^{3}H]choline in control cardiomyocytes (compare Fig. 3). Whether this breakdown is caused by action of PLA_1, PLA_2, PLC-ß or PLD cannot be derived from these data. The rate of degradation of [^{14}C]palmitoyl-PtdEth in the absence or presence of PHE or AngII was similar, which indicates that PLD is not stimulated by these agonists (Table 3) . However, as also expected from the results on [^{3}H]choline formation, ET-1 as well as PMA significantly increased the [^{3}H]PtdEth formation (at 40 min respectively 120% and 289.8% from time zero compared to 46.1% from time zero in the control).

Translocation (activation) of PKC-α, -ε, -δ and -ζ (immunore)activity

Bogoyevitch *et al.* [34] and Disatnik *et al.* [35] showed the presence of α-, -β ,δ-, ε- and ζ-PKCs in cultured neonatal rat heart cells. It was also demonstrated that norepinephrine and endothelin trigger the translocation of PKC-ε and PKC-δ from the cytosol to the membrane in neonatal and adult rat heart cells [29, 36]. They observed no translocation of PKC-ζ with either agonist, and only partial translocation of PKC-α after ET-1 stimulation. In order to study the possible role of PKC translocation (activation) in the mechanism of receptor-mediated PLD activation, we followed the cellular redistribution of PKC-α, -ε, -δ and -ζ (cytosol v membrane fraction) under the same incubation conditions as used in the assay of PLC-β and D activities. PKC-α,-ε, -δ and -ζ immunoreactivities were detected and measured on Western blots by chemiluminescence imaging. Prior to these experiments, the rabbit polyclonal antibodies were proven to react specifically with

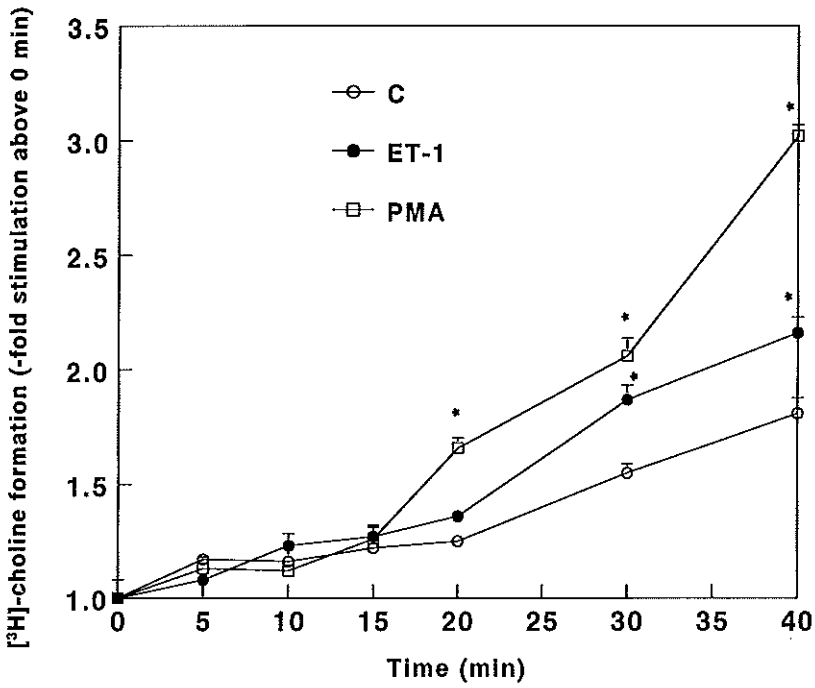

Fig. 3. [^{3}H]Choline released in the incubation medium of serum-free cultured cardiomyocytes stimulated with ET-1 or PMA. Cardiomyocytes were labelled with [^{3}H]choline for 48 h, and were either unstimulated or stimulated with ET-1 (10^{-8} M) or PMA (10^{-6} M) for the indicated times. Unlabelled choline (25 µM) was added to the incubation medium to prevent reutilization of released [^{3}H]choline by the cells. The [^{3}H]choline levels were determined in the incubation medium as described in Materials and Methods. [^{3}H]choline formation is expressed as percentage ± SEM of control levels at time zero. *: $p < 0.05$ compared to corresponding control values.

Table 2. Levels of the water-soluble [^{3}H]choline containing metabolites in cell extract and incubation buffer of serum-free cultured cardiomyocytes stimulated with ET-1 and PMA.

	Cell extract (% of total cellular [^{3}H]-choline), n=8					
	[^{3}H]choline		[^{3}H]phosphocholine		[^{3}H]glycero-P-choline	
	10 min	40 min	10 min	40 min	10 min	40 min
Control	0.87±0.08	0.61±0.05	2.06±0.09	2.31±0.25	2.46±0.28	3.26±0.19
ET-1	0.84±0.10	0.78±0.08	2.40±0.33	2.24±0.18	2.76±0.32	3.23±0.19
PMA	0.94±0.04	1.14±0.06*	2.52±0.32	1.66±0.11*	2.65±0.20	2.55±0.11*

	Incubation buffer (% of total cellular [^{3}H]-choline), n=4					
	[^{3}H]choline		[^{3}H]phosphocholine		[^{3}H]glycero-P-choline	
	10 min	40 min	10 min	40 min	10 min	40 min
Control	1.22±0.03	1.64±0.08	0.45±0.03	0.44±0.05	0.48±0.05	0.69±0.02
ET-1	1.32±0.09	2.06±0.10*	0.31±0.02	0.45±0.02	0.40±0.05	0.62±0.06
PMA	1.19±0.04	2.67±0.06*	0.43±0.06	0.46±0.12	0.54±0.09	0.66±0.17

The number of experiments were performed with ET-1 (10^{-8} M) and PMA (10^{-6} M). For the measurements of [^{3}H]choline containing products in the cell extracts and those in the incubation buffer we carried out respectively eight and four experiments. *: $p < 0.05$ compared to the control value at the corresponding timepoint.

Table 3. Time course of release of [^{3}H]choline in the incubation buffer and the level of [^{14}C]PtdEth in the cell extract of serum-free cultured cardiomyocytes after stimulation with various agonists

	[^{3}H]choline (% of the control at 0 min)			[^{14}C]PtdEth (% of the control at 0 min)	
	10 min	20 min	40 min	10 min	40 min
Control	101.9±4.6	120.1±5.6	151.7±6.8	76.1± 6.0	46.3± 6.1
ET-1	107.4±6.9	128.7±7.1	182.1±6.6*	105.8±25.1	120.0± 6.5*
PHE	99.9±3.8	119.5±4.1	163.4±4.1	84.7±4.3	44.5± 5.4
AngII	96.5±2.6	131.9±4.6	165.2±9.2	101.5± 8.6	53.1± 4.4
PMA	103.2±2.9	156.2±5.6*	219.6±7.1*	175.4±11.1*	289.8±53.9*

The number of [^{3}H]choline and [^{14}C]phosphatidylethanol experiments amounted respectively twelve and four. The agonist concentrations used were ET-1 (10^{-9} M), PHE (10^{-5} M), AngII (10^{-7} M) and PMA (10^{-6} M). The ethanol concentration used in the [^{14}C]-phosphatidylethanol experiments was 1.0% v/v). The control values at zero time were set at 100 %. *: $p < 0.05$ compared to the control value at the corresponding timepoint.

Table 4. Time course of release of [^{3}H]choline in the incubation buffer of serum-free cultured fibroblasts after stimulation with various agonists.

	[^{3}H]choline (% of the control at 0 min)	
	10 min	40 min
Control	120.4±4.3	158.9±2.1
ET-1	119.5±4.3	162.7±2.3
PHE	122.4±2.6	151.1±7.3
AngII	127.9±3.5	170.9±1.9
PMA	166.5±2.4*	378.9±2.7*

The number of experiments amounted eight. The agonist concentrations used were ET-1 (10^{-8} M), PHE (10^{-5} M), AngII (10^{-7} M) and PMA (10^{-6} M). The control values at zero time were set at 100%. *: $p < 0.05$ compared to the control value at the corresponding timepoint.

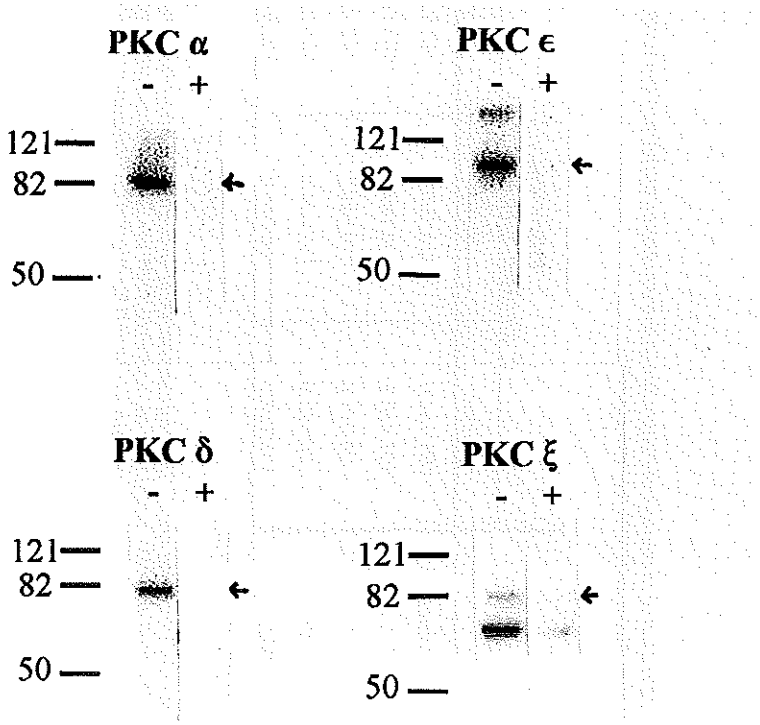

Fig. 4. Immuno blot analysis of a total protein extract (10 µg/well) from freshly isolated neonatal rat heart, performed with PKC-specific antibodies that were either not (-) or (+) were preabsorbed with 0.5 µg of the corresponding antigen peptide. Chemiluminescence images are visualised by applying the Supersignal™ CL-HRP Substrate System GS-363 and the CL-screen of the Molecular Imager. Standard proteins (MW 121, 82 and 50 kDa) were from Bio-Rad (Richmond CA, USA). The arrows indicate the mobility of the PKC isotype.

the rat heart PKC-α, -ε, -δ and -ζ by inhibition of the reaction by the respective polypeptides, against which the antibodies were raised (Fig. 4).
Notice that Figure 4 shows immunoreactivity patterns of a total protein extract from freshly isolated neonatal rat heart, and that these patterns are not necessarily the same in the cell fractions obtained from cultured cardiomyocytes. Clerk *et al.* [37] and Bogoyevitch *et al.* [36], who were also using rat heart fractions, suggested that the immunoreactive 68 kDa band is proteolytically degraded PKC-ζ, and the 78 kDa band is a cross-reaction with PKC-α.
However, in the cytosol and membrane fraction of the cultured neonatal rat cardiomyocytes, the 78 kDa was always a major- and the 68 kDa a minor band. Therefore, we considered the 78 kDa to be intact PKC-ζ and unlikely to be a cross-reaction with PKC-α.

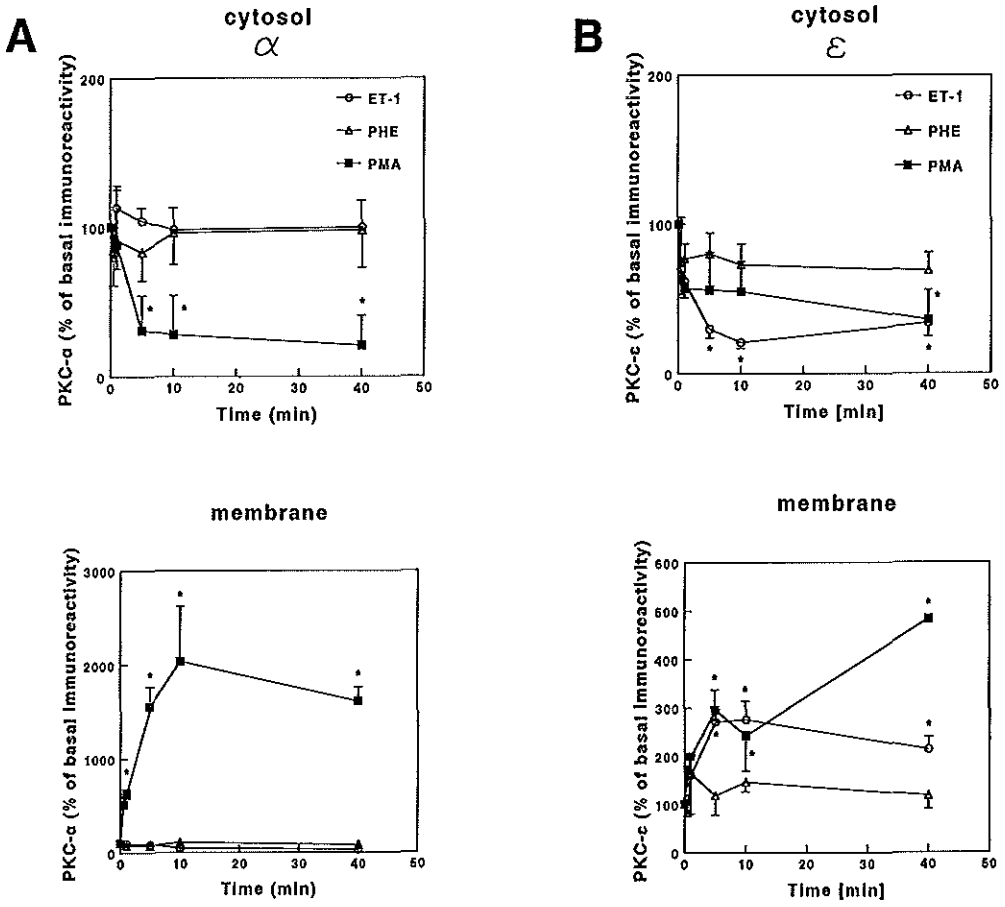

Fig. 5. Intracellular redistribution of PKC-α (A) and PKC-ε (B) immunoreactivities in cultured neonatal rat cardiomyocytes after stimulation with PHE (10^{-5} M), ET-1 (10^{-8} M) or PMA (10^{-7} M). Cell lysis, cell fractionation, protein content determinations and immuno blot analysis of cytosol and membrane fractions were performed as described in the Materials and Methods section. Data represent the mean (± SEM) percentages from the immunoreactivity measured at time zero. Number of experiments for PKC-α and ε were: ET-1 (n=8), PHE (n=8) and PMA (n=4). *: $p < 0.05$.

The results in Figure 5A demonstrate that neither PHE nor ET-1 affected the relative cellular distribution of PKC-α in the cytosol and membrane fraction. In sharp contrast, PMA stimulation of the cardiomyocytes caused rapid (within minutes) removal of PKC-α from the cytosol and its appearance in the membrane fraction. Similar experiments were carried out for PKC-ε. ET-1 as well as PMA caused a rapid (within 5-10 min) removal of PKC-ε from the cytosol and its appearance in the membrane fraction (Fig. 5B). However, PHE only had minor effects on PKC-ε distribution. Results on PKC-δ and PKC-ζ are shown in Fig. 6A and B, although, in this case, only measurements on PMA stimulation were carried out for PKC-δ. These isotypes did not show translocation during stimulation of the cardiomyocytes with ET-1 (10^{-8} M) or PHE (10^{-5} M). PKC-δ slightly increased

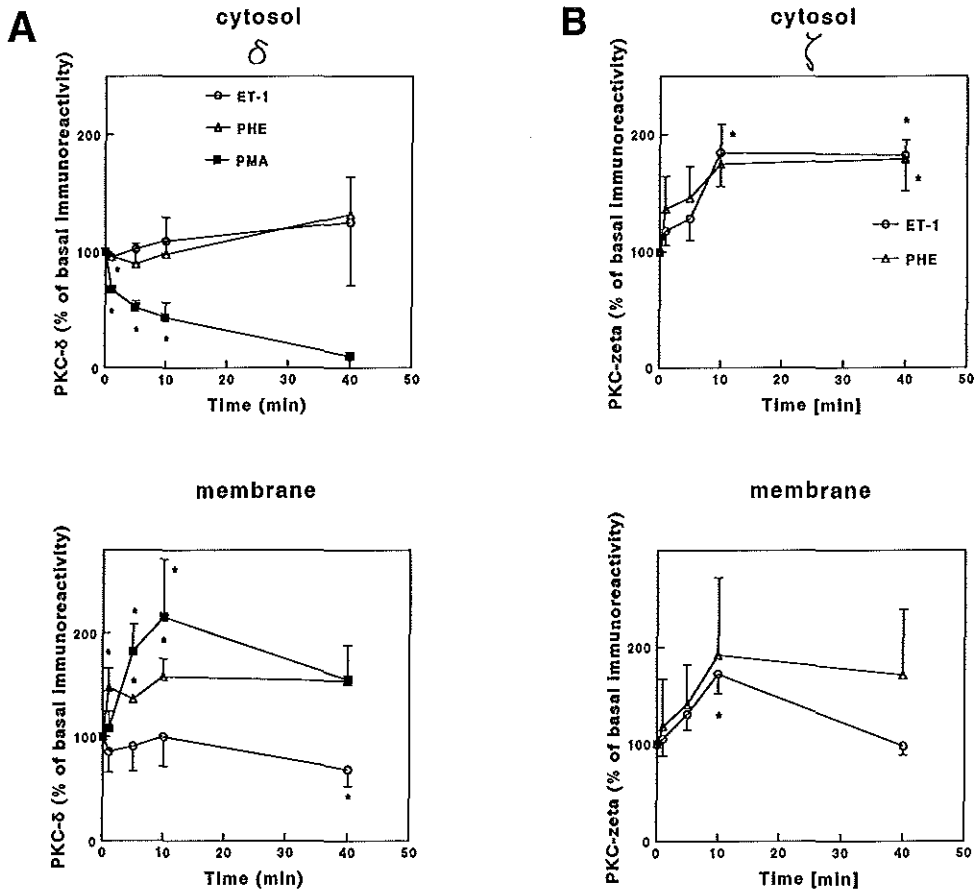

Fig. 6. Intracellular redistribution of PKC-δ (A) and PKC-ζ (B) immunoreactivities in cultured neonatal rat cardiomyocytes after stimulation with PHE (10^{-5} M), ET-1 (10^{-8} M) or PMA (10^{-7} M). Cell lysis, cell fractionation, protein content determinations and immuno blot analysis of cytosol and membrane fractions were performed as described in the Materials and Methods section and figure 5. Data represent the mean (± SEM) percentages from the immunoreactivity measured at time zero. Number of experiments for PKC-δ and ζ amounted 4. *: $p < 0.05$.

in the membrane fraction after PHE stimulation, but no parallel decrease in the cytosolic fraction was found. PMA stimulation, however, showed a rapid disappearance from the cytosol and appearance in the membrane fraction of PKC-δ. PKC-ζ showed a time-dependent increase in both cytosolic and membrane fraction after ET-1 or PHE stimulation, which may indicate that it is translocated from the nuclear (plus myofibrillar) fraction. The latter fraction is always discarded because its inhomogeneity did not allow accurate measurements of the low immunoreactivities present.

The process of translocation of PKCs has been generally thought to be equivalent with enzyme activation. Therefore, we also measured the total PKC activity by histone III-S phosphorylation in the absence and presence of the activators Ca^{2+}, 1,2-DAG plus PtdSer. PMA, but not ET-1 and PHE, caused a rapid disappearance of histone III-S activity from the cytosol, accompanied by its appearance in the membrane fraction (Fig. 7). The latter results are in accordance with the PKC-α, but not the PKC-ε immunoreactivity patterns. This discrepancy may be due to the general observation that histone III-S is a suitable substrate for PKC-α, but less for PKC-ε. Therefore, we measured the total PKC activity with a more favourable substrate for PKC-ε: ε-peptide (30 μM). However, very low 1,2-DAG plus PtdSer-dependent activities were found in cytosol and membranes using this substrate (results not shown).

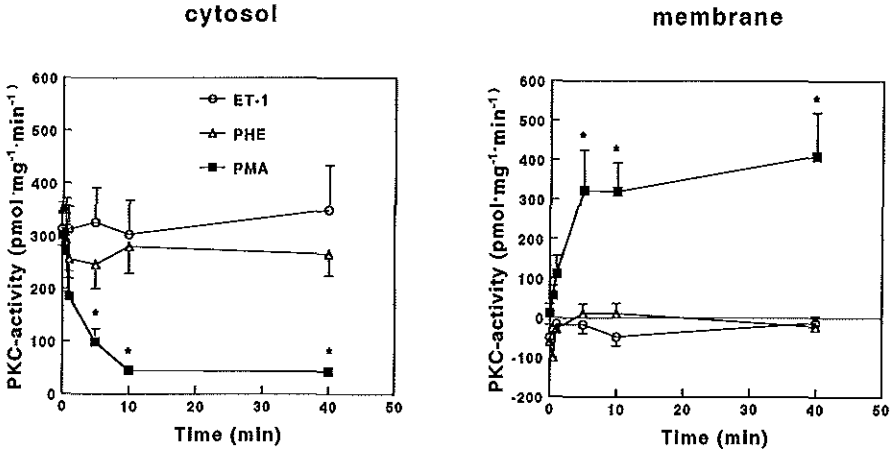

Fig. 7. Rate of Ca^{2+} plus 1,2-DAG stimulated [^{32}P]-incorporation into histone III-S in cytosolic and membrane fractions obtained from serum-free cultured neonatal rat cardiomyocytes after stimulation with PHE (10^{-5} M), ET-1 (10^{-8} M) and PMA (10^{-7} M) for the indicated times. Cell lysis, cell fractionation, protein content determinations and immuno blot analysis of cytosol and membrane fractions were performed as described in the Materials and Methods section. Activities, expressed as pmol [^{32}P]/mg protein/min, represent the mean ± SEM, n=5 for all agonists tested. *: $p < 0.05$.

PKC inhibition and ET-1 stimulated PLD

Cardiomyocytes were labelled with [^{3}H]choline for 24-48 h and in some experiments PKC was down-regulated by adding PMA (1 μM) during this period [34, 38]. Cells were then pretreated,

either with or without PKC-inhibitor, for 30 min. Subsequently, cardiomyocytes were either unstimulated or stimulated with ET-1 or PMA for 40 min and the [^{3}H]choline released in the incubation buffer was measured. When no PKC inhibitor or no PKC down-regulation was applied, both ET-1 (115% of control) and PMA (168% of control) stimulated [^{3}H]choline formation significantly (Fig. 8).
The PKC inhibitor chelerythrine (10^{-4} M) completely inhibited the ET-1- and PMA stimulated [^{3}H]choline formation although, peculiarly, the inhibition was always larger than the agonist-induced increase. However, the PKC inhibitor staurosporine (10^{-6} M) had no inhibitory effect. Prior down-regulation of PKC by PMA pretreatment led to complete inhibition of ET-1 and PMA induced PLD response.

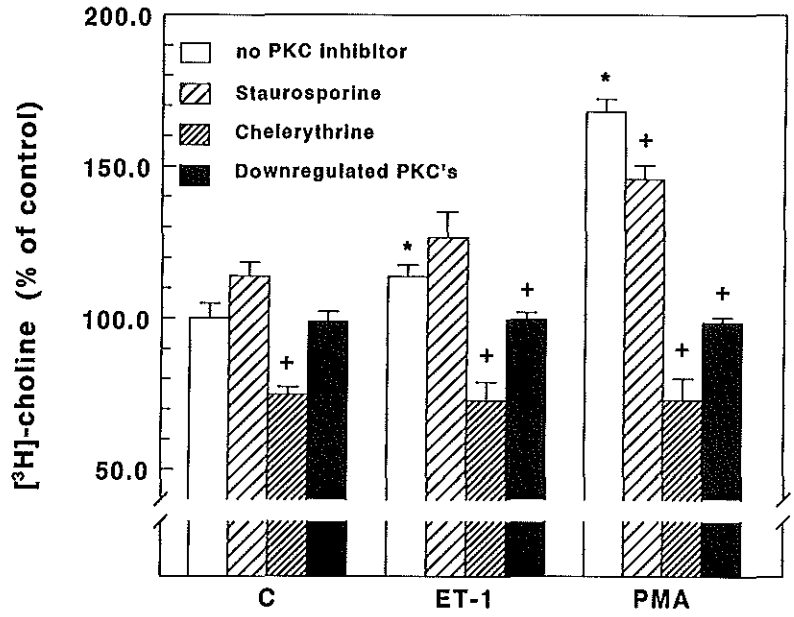

Fig. 8. [^{3}H]Choline released in the incubation medium of serum-free cultured cardiomyocytes stimulated with or without ET-1 or PMA after inhibition of PKC or down-regulation of PKC. Cardiomyocytes were labelled with [^{3}H]choline for 24-48 h, preincubated for 30 min with or without PKC inhibitor and thereafter stimulated for 40 min. In another series the cells were during the labelling step at the same time pretreated with PMA (1 µM) to down-regulate PKC. [^{3}H]Choline levels were determined in incubation medium as described in the Materials and Methods section. [^{3}H]choline formation is expressed as percentage ± SEM of control levels at time zero, n=8. * $p < 0.05$ compared to non-inhibited control. +: $p < 0.05$ compared to non-inhibited condition under the same stimulus.

DISCUSSION

The results of the present study demonstrate that ET-1 rapidly (0-10 min) and potently stimulates PLC-ß and, after a lag-phase of about 10 minutes, PLD in serum-free cultured rat neonatal cardiomyocytes. PHE and AngII only stimulate PLC-ß, with less potency compared to ET-1. In this model ET-1 (and to lesser extent PHE and AngII) and the PKC activator PMA were equipotent

in stimulating protein synthesis (24 h), a process associated with the occurring hypertrophy. The extent of PLC-ß activation by the physiologically acting agonists correlated with the magnitude of stimulation of protein synthesis (ET-1 > PHE > AngII). The results suggest that the direct PKC activator 1,2-DAG, formed by ET-1-mediated stimulation of PLC-ß, and subsequently PLD, takes part in the transduction of the ET-1 signal to the ultimate hypertrophic response. Furthermore, the present experiments provide evidence for the hypothesis that the translocation (activation) of PKC-ε might mediate the "cross-talk" between PLC-ß and PLD.

In general, the agonists known to stimulate PLD also promote PtdIns(4,5)P_2 hydrolysis in their target cells. Thus, a typical response pattern of these agonists is a biphasic increase in 1,2-DAG, with an initial rapid, transient peak, due to receptor-mediated PLC-ß activation, followed by a more slowly developing, but prolonged 1,2-DAG accumulation due to PLD activation [24]. Analysis of the molecular species of 1,2-DAG of e.g. thrombin stimulated IIC9 fibroblasts indicated that they were initially formed from PLC-ß catalysed breakdown of PtdIns(4,5)P_2 and then from PLD catalysed breakdown of PtdChol [23]. The present results on ET-1 induced activation of PLC-ß, shortly followed by activation of PLD, are consistent with those common findings in other target cells. The other less potent PLC-ß activators in the serum-free cultured cardiomyocytes, PHE and AngII, did not significantly stimulate [^{3}H]choline and [^{14}C]palmitoyl-PtdEth formation. However, that AngII did not cause PLD activation is in contrast with the activation observed by Sadoshima and Izumo [11, 39]. ET-1 and the most potent activator of PKC as well as PLD, PMA, appears also to be most effective in increasing protein synthesis and protein/DNA ratio which are both markers for hypertrophic growth [5, 6]. Therefore, an attractive hypothesis is that by secondary activation of PLD via its cross-talk with PLC-ß, the cardiomyocyte is assured of a continuous supply of 1,2-DAG derived from PtdChol which is present in the sarcolemma in at least 100-fold excess compared to PtdIns(4,5)P_2. The participation of PKC isozymes in the cardiac hypertrophic response has also been inferred from transfection experiments of cardiac myocytes with plasmids encoding PKCs rendered constitutively active by large N-terminal deletions or small pseudosubstrate mutations (reviewed in [4]). Activation of PKC is known to initiate a series of intracellular events (e.g. activation of mitogen-activated protein kinase (MAPK) cascade) that couples it to the hypertrophic response. Likewise ET-1 and PHE have previously been shown to cause translocation of PKC isozymes leading to the activation of the MAPK cascade [29]. The latter authors reported that ET-1 caused a rapid translocation of PKC-ε and -δ, but not of PKC-α and -ζ in cardiomyocytes cultured serum-free from 24 h before exposing the cells to agonists. PHE caused translocation of PKC-ε, and possibly of PKC-δ as well. The proportion of PKC-ε lost from the soluble fraction was somewhat less with PHE compared to ET-1, which is in agreement with the present work. However, in the present study, no ET-1 and PHE induced translocation of PKC-δ was found, which is not in agreement with the results of Clerk *et al.* [29], showing translocation of PKC-δ after ET-1 stimulation although the EC_{50} (11-15 nM) for ET-1 was about 10-fold higher than that for PKC-ε. However, we found a rapid translocation of PKC-δ after PMA stimulation. Moreover, in the present study, PKC-ζ shows a time-dependent increase in both cytosolic and membrane fraction after ET-1 or PHE stimulation, which was also not found by Clerk *et al.* [29].

The reason for the discrepancy of the findings on PKC-δ and -ζ with those of Clerk *et al.* [29] is not clear, however it should be noted that our cardiomyocytes are cultured serum-free for 4 days before performing the experiments to optimize hypertrophic response after ET-1 stimulation, whereas Clerk *et al.* [29] withdrew serum for only 24 h. Moreover, Clerk *et al.* [29] used 10% horse serum instead of 5% used by us until 1 day after the isolation of the cardiomyocytes. In this context, it should also be noted that we found different PLC-β response patterns in serum-free cultured cardiomycoytes compared to our previous study [22] in serum-cultured cardiomyocytes.

The small PLC-ß response to PHE and AngII may also be the reason that we did not observe any PLD activation under this condition. Ye *et al.* [40] already showed in isolated adult rabbit ventricular myocytes that both noradrenaline (EC_{50} = 3 x 10^{-8} M) and endothelin-1 (EC_{50} = 5.3 x 10^{-9} M) stimulated PtdOH formation (as measure of PLD activity) and that, indeed, the maximum

response to ET-1 (10^{-7} M) was about two-fold higher than to PHE (10^{-6} M). In the latter study, the transphosphatidylation of exogenous butanol was used to prove the action of PLD and, with this assay, the ET-response was three-fold larger than that of noradrenaline. An essential difference with the present work is, however, that these authors observed immediate activation of PLC-ß as well as PLD. They propose that the product of PLD, PtdOH, may directly stimulate PLC-ß. However, the presently observed time-courses of [^{3}H]inositolphosphate and [^{3}H]choline formation are not consistent with this hypothesis. On the contrary, our results indicate that the product(s) of PLC-ß action initiate PLD.

Other studies propose that PLD is directly regulated by G-proteins [15, 24, 41]. Guanine nucleotide analogues, such as GTPγS, are potent activators of PLD when added to permeabilized cells or directly to membranes [15]. It has recently been discovered that the ADP-ribosylating factor (ARF), a small G-protein with a well established role in the vesicular transport, and G-proteins of the Rho family also can act as regulators of PLD [41]. An interesting feature of ARF-regulated PLD is the requirement of PtdIns(4,5)P_2, but this effect has only been observed *in vitro*. One study on intact rat and chicken atria showed that G-protein activator AlF_4^- evoked a parallel rise of PLD and PLC-ß activity [42]. The phorbolester 4ß-phorbol-12ß,13α-dibutyrate (PBD) enhanced PLD, and this effect was blocked by prior down-regulation of PKC. Obviously, these studies do not delineate the mechanisms by which PLD is regulated by agonists. Until the PLD's are purified and reconstituted with pure G-proteins and other factors, the molecular mechanism will remain uncertain [24].

PKC isozymes control PLD, as has been shown previously by many studies and the results of the present investigation in cardiomyocytes confirm this [18, 24]. PKC-inhibition by chelerythrine, or down-regulation of PKC by prolonged phorbolester treatment, blocked the activation of PLD by ET-1 and PMA (Fig. 8). In this respect, it should be noted that Bogoyevitch *et al.* [34] were first to demonstrate that prolonged exposure of cultured rat neonatal cardiomyocytes to a TPA concentration of 1 µM caused almost complete down-regulation of the major PKC isozymes (α, δ, ε). Taking these results together with the presently observed rapid (0-5 min) translocation of PKC-ε from the cytosol to the membranes (after stimulation of cardiomyocytes with ET-1), one may conclude that the translocation (activation) of PKC-ε is a factor involved in the cross-talk between PLC-ß and PLD. Not consistent with this possible action of PKC-ε is our finding that staurosporine had no effect on ET-1 stimulated PLD and only partially inhibited PMA stimulated PLD. However, overall the use of staurosporine as PKC inhibitor led to conflicting data concerning PLD [24]. For instance, Périanin *et al.* [43] found stimulation of PLD (as non-specific effect of staurosporine) in human polymorphonuclear leucocytes by staurosporine concentrations that decreased PKC activity in both soluble and particulate cell fractions. Chelerythrine not only completely inhibited ET-1 and PMA stimulated PLD, but also partially inhibited the basal release of [^{3}H]choline. This may indicate that PKC is partially involved in the basal PLD activity as well. However, the results on PLD activity after down-regulation of PKC are not in agreement with this hypothesis, because no decrease of basal PLD activity was found under this condition.

In the present study, only a clear ET-1 induced translocation (an indication for activation) of PKC-ε (but not PKC-α, -δ and -ζ) was shown by measurements of immunoreactivity.

In spite of the observed translocation of PKC-ε immunoreactivity no translocation of phosphorylating activity of total PKC towards histone III-S was found. We found low activities towards peptide ε in our assay, which is not in agreement with the results obtained by Clerk *et al.* [37], who found higher activities than those found using the histone III-S substrate. However, the latter authors measured activity in total neonatal rat ventricle, activity which may partially be derived from PKC present in fibroblasts, smooth muscle cells and endothelial cells. Furthermore, TPA (12-O-tetradecanoylphorbol-13-acetate) was used instead of 1,2-DAG, which is a stronger activator than the physiologically active 1,2-DAG.

So far, only the α- and ß-isoforms of PKC have been directly shown to regulate PLD activity (recently reviewed in [18]). The fact that we did not find definite proof for ET-1-induced phosphorylation of external substrates by PKC-ε does not exclude its involvement in PLD activation. Available

evidence suggests that besides phosphorylating also non-phosphorylating mechanisms may be involved [18, 24, 44].
In conclusion, the results indicate that in serum-free cultured cardiomyocytes, ET-1 initially activates PLC-ß and after a lag-phase PLD, whereas PHE and AngII activate only PLC-ß. PLC-ß, stimulated by ET-1, may cross-talk with PLD via translocation of PKC-ε. These signals might be linked to the ultimate hypertrophic response.

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Chapter 4

PHOSPHOLIPID SOURCE AND MOLECULAR SPECIES COMPOSITION OF 1,2-DIACYLGLYCEROL IN AGONIST STIMULATED RAT CARDIOMYOCYTES

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ABSTRACT

The aim was to investigate the consequences of simultaneous stimulation of phospholipase C and D by agonists for the molecular species composition of 1,2-diacylglycerol and phospholipids in cardiomyocytes. Serum-free cultured rat neonatal cardiomyocytes were stimulated by endothelin-1, phenylephrine or phorbolester. The molecular species of 1,2-diacylglycerol (in mol %) and those derived from phosphatidylcholine and phosphatidylinositol were analyzed by high performance liquid chromatography and their absolute total concentration (nmol/dish) by gas-liquid chromatography. Phospholipids were labelled with [^{14}C]glycerol or double labelled with [^{14}C]16:0 and [^{3}H]20:4n6 for measurements of respectively, the amount of or relative rate of label incorporation into 1,2-diacylglycerol. The major molecular species of 1,2-diacylglycerol in unstimulated cells was found to be 18:0/20:4 (57 mol %). The same species was observed predominantly in phosphatidylinositol (73 mol % compared to 11 mol % in phosphatidylcholine). A significant decrease (about 10 mol %) was found for the 18:0/20:4 species of 1,2-diacylglycerol during stimulation (10-40 min) with endothelin-1 or phorbolester, but not phenylephrine. The results of double labelling experiments were consistent with the latter finding: the ratio [^{3}H]20:4 over [^{14}C]16:0 in 1,2-diacylglycerol decreased from 1.70 in control to 1.40 during 10 min endothelin-1 or phorbolester stimulation, but not phenylephrine. The [^{14}C]glycerol incorporation into 1,2-diacylglycerol remained relatively constant under agonist stimulated conditions as did the total concentration of 1,2-diacylglycerol. 1,2-Diacylglycerol present in unstimulated cardiomyocytes is likely derived from phosphatidylinositol. During stimulation with endothelin-1 and phorbolester, but not phenylephrine, phosphatidylcholine becomes an increasingly important source for 1,2-diacylglycerol due to sustained activation of phospholipase D. The 1,2-diacylglycerol level remains relatively constant during agonist stimulation which strongly indicates that particular molecular species of 1,2-diacylglycerol more than its total concentration determine the activation of protein kinase C isoenzymes.

INTRODUCTION

Many stimuli such as the α_1-adrenergic agonist phenylephrine (PHE) and endothelin-1 (ET-1) have been shown to be positive inotropic and to induce hypertrophy and changes in phenotype in myocardium [1-3]. Studies have revealed that these agonists stimulate GTP-binding protein-coupled receptors linked to phospholipase C-β (PLC-β) leading to hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) into inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and the lipid second messenger 1,2-diacylglycerol (1,2-DAG). More recently, PLC-γ interacting with tyrosine phosphorylated receptors has been proposed for angiotensin II to be an important downstream effector as well [4,5]. At present, it is generally assumed that activation by 1,2-DAG (and Ca^{2+}) of distinct protein kinase C (PKC)-isozymes results in activation of various sets of downstream targets such as raf-kinase, MAP-kinase and myofibrillar component proteins such as troponin I, C and C-protein which lead to the (patho)physiological responses [2,6,7].

Previously, we reported that PtdIns and PtdIns(4,5)P_2 in cultured neonatal rat cardiomyocytes have a fatty acid composition that is very different from phosphatidylserine (PtdSer) and phosphatidylcholine (PtdChol) [8,9]. Thus the molecular species of 1,2-DAG formed by PLC-β, PLC-γ and/or PLD from these phospholipids will not be the same. 1,2-DAG, which is considered to be the major lipid activator of PKC might exert this action by its increased total level or by altered competition between particular molecular species [10]. However, at present there is no information on the molecular species composition of 1,2-DAG in cardiomyocytes before and after agonist stimulation.

Recently, others [11,12] and we obtained evidence that during ET-1 stimulation of serum-free cultured rat cardiomyocytes the PKC activator 1,2-DAG might not only be derived from receptor-dependent hydrolysis of (PtdIns(4,5)P_2) through immediate action of PLC-β detectable by measurement of [^{3}H]inositolphosphates ([^{3}H]InsP_n) but after a lag-phase of about 5-10 min from PtdChol by the action of phospholipase D (PLD) the latter detectable by [^{3}H]choline formation

[11]. We could confirm this action of PLD by showing in parallel occurrence of [^{14}C]phosphatidylgroup transfer from endogenous [^{14}C]16:0-labelled PtdChol to exogenous ethanol. [^{3}H]choline formation appeared not to be due to action of PtdChol-directed PLC, because phospho-[^{3}H]choline was not found to be increased. However, the action of PC-PLC cannot totally be excluded on basis of the unaffected phosphocholine concentration, because phosphocholine is likely degraded to choline. No phosphocholine phosphatase inhibitors are presently available to block this pathway. The previous results indicated that 1,2-DAG production from PtdChol-specific PLD is slow in onset and sustained, in contrast to the rapid and more transient production of 1,2-DAG from PtdIns(4,5)P_2-specific PLC-β [11]. On the other hand, phosphatidic acid (PtdOH), the first product of PLD action is implicated as an intracellular mediator of several cellular processes, such as mitogenesis and actin assembly. This product can be rapidly converted to 1,2-DAG by PtdOH phosphohydrolyse or to lyso-PtdOH by phospholipase A_2. We also obtained evidence that during ET-1 stimulation cross-talk between PLC-β and PLD occurs through PKC-ε activation. We proved activation of both PLC and PLD by ET-1, but not by PHE or phorbol 12-myristate 13-acetate (PMA). PHE only stimulates PLC-β and PMA, as expected only PLD. PMA, but not PHE, induced a cellular translocation/activation of PKC-ε, -δ as well as -α [12], confirming the results of Bogoyevitch et al. [13] and Clerk et al. [14].

Studies in cell-types other than cardiomyocytes (e.g. fibroblasts) indicate that 1,2-DAG formed in the early (15 sec-1 min) transient phase of receptor-stimulation predominantly shows fatty acids present in PtdIns pool (stearic acid 18:0, arachidonic acid 20:4n6) whereas the later phase shows more saturated fatty acids such as palmitic acid 16:0 typically found in PtdChol ([15,16] and reviews [17,18]). However, Hermans et al. [19] showed in pancreatic acini that in the later phase 1,2-DAG remains derived from both PtdIns and from PtdChol.

Therefore, the aim of the present study was to investigate the changes in the molecular species of 1,2-DAG and PtdChol and PtdIns of neonatal rat cardiomyocytes that are stimulated by ET-1, PHE or PMA, possibly more relevant for activation of PKC than the absolute amount of 1,2-DAG. High performance liquid chromatography (HPLC) was used for separation of the different molecular species in free 1,2-DAG and 1,2-DAG obtained from PtdChol and PtdIns exogenously treated by PLC purified from respectively C. *Welchii* and B. *Cereus*. Moreover, the relative incorporation of [^{14}C]16:0 (a 10-fold higher percentage of 16:0 is present in PtdChol compared to PtdIns) (compare also [8]) and [^{3}H]20:4n6 (a 2-fold higher percentage of 20:4 is present in PtdIns compared to PtdChol) (compare also [8]) into 1,2-DAG was followed during agonist stimulation.

MATERIAL AND METHODS

Materials

Integrid$_{TM}$ culture dishes (154 cm^2) and 20 cm^2 dishes were from Falcon (Becton-Dickinson, Oxnard, Ca, USA) and culture dishes (4 well Multidish, 1.8 cm^2/well) were from Nunc (Roskilde, DK), while the culture media DMEM and M199 were both obtained from Gibco (UK); fetal calf serum, horse serum, penicillin/streptomycin, trypsin and ET-1 were obtained from Boehringer Mannheim (Germany). [1-^{14}C]palmitic acid (16:0) (57mCi/mmol) and [^{3}H]arachidonic acid (20:4n6) (210 Ci/mmol) were from Dupont NEN products (Boston MA, USA). [U-^{14}C]glycerol (156 mCi/mmol) was from Amersham International PLC (Amersham, UK). PMA, PHE, DPH (diphenyl-1,3,5 hexatrien), difluorescein, phospholipase C (Perfingens, C. *Welchii*), phospholipase C (B. *Cereus*, PtdIns-specific), DNBC (3,5-dinitrobenzoylchloride), BF_3 and the internal standard 21:0 were all obtained from Sigma (St. Louis MO, USA). High performance thin-layer chromatography (HPTLC) plates and thin layer chromatography (TLC) plates (Kieselgel 60) were obtained from Merck (Darmstadt, Germany). Instagel-plus was from Canberra Packard Benelux N.V./S.A. (Groningen, The Netherlands).

Culture of neonatal rat ventricular myocytes

Primary cultures of neonatal ventricular myocytes were prepared from hearts from 1-2 day old Wistar rats as described before [20] using a single preplating step (surface area of approximately 10 cm^2 per rat heart) to further increase cardiomyocyte to non-cardiomyocyte ratio. The cultures contain 90-95 % myocytes as routinely assessed by microscopical observations of cells beating and by usage of the periodic acid Schiff (PAS) staining method to distinguish myocytes from non-myocytes. Cardiomyocytes were seeded in 1.8, 20 or 154 cm^2 wells/dishes (for respectively labelling experiments, GLC and HPLC analysis) at 150 to 175 x 10^3 cells/cm^2 giving a confluent monolayer of spontaneously contracting cells after 24 hrs. Cardiomyocytes were kept in 5% CO_2 at 37°C in complete growth medium consisting of DMEM/M199 (4:1), 25 mM HEPES supplemented with 5% fetal calf serum and 5% horse serum, 100 U penicillin/ml and 100 µg streptomycin/ml for the first 24 hrs. Growth medium was renewed 24 hrs after seeding with serum-free growth medium and every 48 hours thereafter. Experiments were routinely performed at 5 to 6 days after plating of the cells.

Molecular species of free 1,2-DAG and 1,2-DAG derived from PtdIns and PtdChol

Cardiomyocytes were either used as control or stimulated with ET-1 (10^{-8} M), PHE (10^{-5} M) or PMA (10^{-6} M). Lipids (from 2 to 3 154 cm^2 culture dishes) were extracted by a modified method of Bligh and Dyer [21] (using chloroform containing 50 mg/l butylated hydroxytoluene). Throughout the procedure samples were kept under N_2 and if required overnight, at -20°C. 1,2-DAG was separated from other lipids by thin layer chromatography (TLC) using hexane/diethylether/formic acid (30:70:2 v/v/v) as solvent. The phospholipids remaining at the origin were scraped off again and three times reextracted in chloroform/methanol (2:1) and then separated by two-dimensional TLC according to Bütikofer et al. [22] with as running solvents for the first dimension chloroform/methanol/NH_4OH/H_2O (90:74:12:8 v/v/v/v) and for the second dimension chloroform/methanol/acetone/acetic acid/H_2O (80:30:30:24:16 v/v/v/v/v). Plates were sprayed with 1 % K-oxalate in MeOH/H_2O (2:3 v/v), dried and activated at 90°C for 1 hour before separation. Spots were visualized by spraying with DPH (0.03 % in chloroform), scraped off and lipids were extracted from the silica by the Arvidson method [23]. The phospholipids were subsequently dispersed by sonication in 2 ml of either 30 mM K_2HPO_4, 30 mM boric acid (pH 7.0; for PtdOH), or 30 mM Tris, 30 mM boric acid (pH 7.4; for PtdIns), or 50 mM Tris, 5mM $CaCl_2$, 30 mM boric acid (pH 7.4; for PtdChol). PLC from B. *cereus* (P7147, for PtdOH (12 U) and P8804, PtdIns-specific (0.2 U)) and phospholipase C from C. *Welchii* (P4039, for PtdChol (5 U)) was added followed by 5 ml diethylether. The mixtures were incubated under argon overnight in a shaking water bath (37°C). After completion of the PLC-catalyzed formation of 1,2-DAG, which was routinely checked by subjecting an aliquot of the ether phase to one-dimensional TLC using diethylether/hexane (3:2, v/v) as a running solvent, the ether phase was collected. The lower phase was extracted again with diethylether and combined ether phases were blown to dryness with nitrogen and subsequently dried under vacuum overnight. After addition of 25 mg of DNBC the mixture was again dried overnight under vacuum. Derivatization of the 1,2-DAGs was achieved by addition of 1 ml of dry pyridine and subsequent incubation for 15 min at 64°C. The extract was chilled on ice for 15 s and 3 ml of ice-cold water was added together with 2 ml of hexane. The water phase was extracted twice with 2 ml of hexane and the combined hexane phases were dried under nitrogen. The extract was redissolved in 2 ml hexane and successively washed with 2 ml NaCl (1 M) and 2 ml water. The 1,2-DAG subclass was separated by HPTLC using hexane/diethylether (7:3, v/v) as a running solvent. The spots were visualized by dichlorofluorescein (0.001% in 2 mM NaOH) and extracted with diethylether. The extract was then washed with distilled water in order to remove the water soluble degradation products. After evaporation of the ether phase the extract was dissolved in 100 µl of acetonitrile/isopropanol (8:2, v/v) and subjected to reverse-phase HPLC using an ODS Hypersil column (5 µm C18, 200 x 2.1 mm, Hewlett Packard, Böblingen, Germany). The column was eluted with acetonitrile/isopropanol (8:2, v/v) at a flow rate of 0.25 ml/min. The elution profile of the single DNBC-diacylglycerols was monitored at 254 nm by means of a Hewlett Packard 1050 UV detector. Molecular species analysis was performed essentially as described. [24]. The molecular species of the derivatized 1,2-DAGs were identified on basis of the retention times obtained with standard dinitrobenzoyl-diacylglycerols and/or the relative retention times reported by Takamura et al [25]. An example of an HPLC separation of PtdChol is shown in Figure 1.

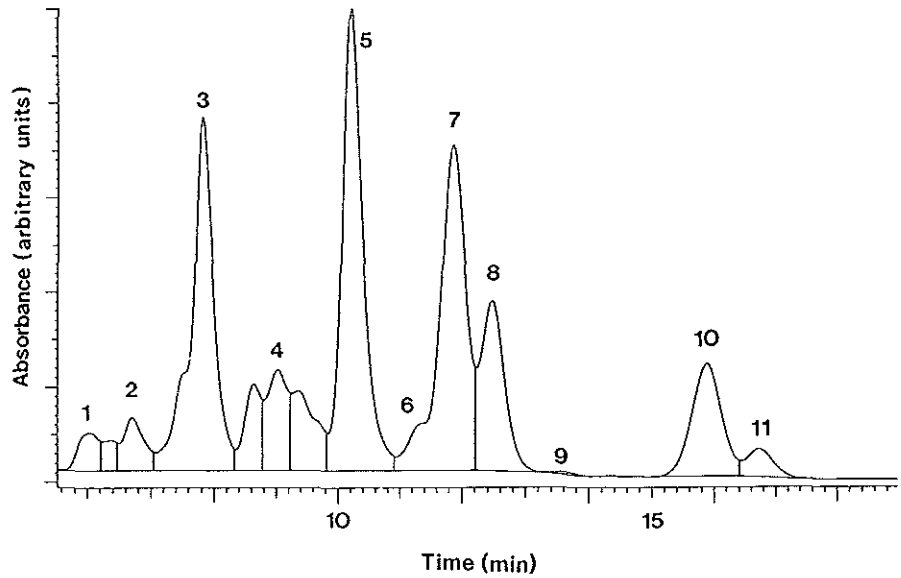

Fig. 1. HPLC separation pattern of DNBC-1,2-DAGs representative for PLC-treated PtdChol that were extracted from cardiomyocytes stimulated for 40 min by ET-1. The identity of numbered peaks is: **1)** 18:1/22:6n3; **2)** 16:0/22:6n3; **3)** 16:0/20:4n6; **4)** 16:0/18:2n6; **5)** 18:0/20:4n6; **6)** 18:0/22:5n6; **7)** 18:1/18:1; **8)** 16:0/18:1 + 18:0/18:2n6; **9)** 16:0/16:0; **10)** 18:0/18:1; **11)** 18:0/16:0. The DNBC-1,2-DAGs were measured at 254 nm.

Fatty acid composition of PtdIns and PtdChol

For gas liquid chromatography (GLC) analysis phospholipids were transmethyl esterified using BF_3 as originally described by Morrison and Smith [26] and as used by us in other studies [8,27]. For the gas chromatographic separation of the fatty acid methylesters a CP9000 capillary column chromatograph (Chrompack, Middelburg, The Netherlands), equipped with a CP-Sil 88 coated fused silica capillary column (Chrompack, WCOT 50 m x 0.25 mm, 0.2 µm film), was used. The separated peaks were identified and quantified on the basis of the retention times as compared to those of known amounts standards [27]. The absolute total concentrations (nmol/dish) of 1,2-DAG, PtdChol and PtdIns were also measured by GLC.

Double label experiments

Cardiomyocytes were labelled with both [^{14}C]16:0 (0.5 µCi/ml) and [^{3}H]20:4n6 (0.1 µCi/ml) for 24 hours in complete serum-free growth medium supplemented with 0.04 % fat-free Bovine Serum Albumin (BSA). Cells were extensively washed (three times) with radioactive-free medium (containing 0.2 % fat-free BSA), twice the amount of volume as during labelling. Cells were then preincubated (15 min, 37°C) in medium 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 $NaHCO_3$; 0.2% glucose; 10 mM HEPES; pH 7.4, 37°C and stimulated with either ET-1, PHE or PMA. After termination lipids were extracted as described before [28]. TLC plates were pretreated by impregnation with 1 % boric acid in CH_3OH and activated. $CHCl_3$:CH_3COCH_3 (94:4 v/v) was used as solvent system. The labelled lipids were visualized by means of autoradiography and the 1,2-DAG spots were scraped off and counted by liquid scintillation counting.

1,2-DAG level by [^{3}H]glycerol incorporation

Cardiomyocytes were labelled with 2μCi (U-[^{14}C])glycerol/ml for 24 hours in complete serum-free growth medium. Cells were extensively washed (three times) with radioactive-free medium, twice the amount of volume as during labelling. Cells were then preincubated (15 min, 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) and stimulated with either ET-1, PHE or PMA. After incubation the [^{14}C]glycerol containing lipids were extracted, TLC separated and the 1,2-DAG counted as described before [28].

Statistics

The statistical significance of any of the observed effects was evaluated by the Students t-test and significance was set at probability less than 0.05.

RESULTS

Molecular species of free 1,2-DAG and 1,2-DAG derived from PtdIns and PtdChol in control and agonist stimulated cardiomyocytes

PtdIns(4,5)P_2, of which small amounts are endogenously formed from PtdIns by action of PtdIns kinases, and the bulk of phospholipids PtdChol, are the endogenous substrates of respectively PLC-β and PLD. Therefore, we treated PtdIns and PtdChol extracted from cardiomyocytes in vitro with PLC purified from C *Welchii* (for PtdChol) and from B. *cereus* (specific for PtdIns) to obtain their constitutive 1,2-DAG molecular species. Although the PtdIns(4,5)P_2 content in cardiomyocytes is too low for accurate determination of its molecular species, it is the direct substrate for PLC-β [9]. Table 1 shows the mol % of the major molecular species of PtdIns and PtdChol of cells unstimulated and stimulated by ET-1, PHE, and PMA during 40 min. No significant agonist-dependent differences were found over this time period. The 18:1/18:1 (26 mol %) appeared to be a major molecular species in PtdChol, whereas PtdIns was mainly composed of 18:0/20:4n6 (73 mol %).

In order to obtain an estimate for the absolute amounts, we measured the total concentration of fatty acid methylesters obtained from PtdChol, PtdIns and 1,2-DAG in control and ET-1 stimulated cardiomyocytes by GLC. No significant changes due to ET-1 stimulation in the absolute concentration (nmol/dish) of phospholipid and 1,2-DAG occurred (Table 2).

The concentration of 1,2-DAG in the cardiomyocytes is very low comparing to that of the phospholipids independent of whether the cells were stimulated by agonists or not (Table 2). Therefore, in order to measure the molecular species composition, it was necessary to combine at least two to three 154 cm^2 culture dishes for accurate HPLC analysis. The results are shown in Table 3. Firstly, it can be noted that 18:0/20:4n6 is the major 1,2-DAG molecular species (about 57 mol %) present in unstimulated control cardiomyocytes. This indicates that under control conditions basal turnover of PtdIns and PLC-β activity contributes mostly to the 1,2-DAG pool in cardiomyocytes. When the cardiomyocytes were stimulated with ET-1 and PMA for 10 min, a decrease of about 10 mol % was found in the relative content of 18:0/20:4n6. In ET-1 stimulated cells this decrease was only accompanied by a significant increase in relative content of 18:0/18:1, and in PMA stimulated cells in the relative content of 18:1/18:1. Previously, we reported that PHE is a strong stimulator of PLC-β, however in comparison to ET-1 and PMA it had only small effects on PLD [12]. In agreement with the latter finding is that 10 min PHE stimulation did not alter the molecular species composition of 1,2-DAG (Table 3). When the cardiomyocytes were stimulated with ET-1, PHE or PMA for 40 min, the mol % of 18:0/20:4 decreased not further. The comparison of Table 1 and 3 may raise the suggestion that the tendency to the non-significant decrease of 18:0/20:4 in PtdChol accounts for the reduction of 18:0/20:4 in 1,2-DAG. This can, however, almost be excluded on basis of the relative and absolute 1,2-DAG content (Table 2). The total concentration of PtdChol and PtdIns is respectively 100- and 10-fold higher than that of free 1,2-DAG (Table 2). We have not measured

Table 1. Molecular species composition (mol %) of DNBC-1,2-DAG derived from PtdChol and PtdIns of control and agonist stimulated myocytes.

	PtdChol, 40 min				PtdIns, 40 min			
Molecular species	Control n=6	ET-1 n=6	PHE n=6	PMA n=6	control n=6	ET-1 n=6	PHE n=5	PMA n=5
18:1/22:6	0.8±0.4	1.1±0.4	0.9±0.4	1.0±0.3	-	-	-	-
16:0/22:6	2.0±0.6	1.9±0.4	1.5±0.5	1.4±0.6	-	-	-	-
16:0/20:4	6.5±1.1	11.1±2.2	11.2±1.9	10.0±3.1	7.4±1.9	9.2±3.2	6.7±1.4	8.5±3.5
16:0/18:2	7.4±1.5	9.2±0.6	8.4±1.3	6.8±1.8	5.7±1.9	4.5±1.7	5.1±1.6	4.7±2.7
18:0/20:4	10.6±3.1	16.4±3.2	16.1±2.2	14.5±4.8	73.4±3.3	68.6±3.0	78.0±3.2	65.3±9.7
18:0/22:5	6.6±2.2	3.5±0.9	3.5±0.9	2.0±0.9	-	-	-	-
18:1/18:1	25.6±4.4	27.9±2.7	25.9±2.2	21.1±5.7	-	-	-	-
16:0/18:1+ 18:0/18:2	14.2±1.9	12.2±2.6	12.6±1.3	12.3±3.5	11.0±2.7	11.9±3.8	8.0±3.7	9.5±5.4
16:0/16:0	4.9±4.5	1.1±0.9	0.8±0.7	0.2±0.1	2.4±0.3	2.6±1.2	2.1±0.9	11.9±8.8
18:0/18:1	10.3±4.9	8.9±3.2	9.0±2.7	7.3±3.0	-	-	-	-
18:0/16:0	6.3±3.6	2.6±0.6	5.2±3.7	1.9±0.7	-	-	-	-

The experiments were performed with serum free cultured cardiomyocytes unstimulated or stimulated with ET-1 (10^{-8}M), PHE (10^{-5}M) or PMA (10^{-6}M), with different cell batches. Results are shown as means ± SEM (mol %), n=number of experiments. The molecular species composition at 0 and 10 min were also measured but not found to be significantly different from the values measured at 40 min at the $p<0.05$ level.

Table 2. Absolute concentration (nmol/dish) of PtdChol, PtdIns and 1,2-DAG of control and ET-1stimulated cardiomyocytes.

	1,2-DAG	PtdChol	PtdIns
Control	6.3±1.9	531.0±86.0	62.0±15.0
ET-1 40 min	9.1±1.6	623.0±61.0	52.0±8.0

The experiments were performed in serum free cultured cells either in control cells or cells 40 min stimulated with ET-1 (10^{-8}M). The experiments were carried out with 3 different cell batches, for both PtdChol and PtdIns and 6 for 1,2-DAG. Results are shown as mean ± SEM (nmol).

the molecular species composition of 1,2-DAG earlier than 10 min to trace the early action of PLC-β as shown in other cell types [15-18]. On the other hand, the unusual high initial 18:0/20:4 content (Table 3) of 1,2-DAG in unstimulated cardiomyocytes complicates detection of the relative increase of this molecular species.

PtdOH is the first product formed by PLD-catalyzed PtdChol degradation. Therefore, we also tried to determine its containing 1,2-DAG molecular species. Unlike 1,2-DAG, the PtdOH content was too low for accurate analysis on HPLC. Its fatty acid composition could, however, be determined on GLC and was found to have closest resemblance to that of PtdChol (Table 4).
On the other hand, 1,2-DAG and PtdOH are generally believed to be interconvertible through the action of PtdOH-hydrolase and 1,2-DAG kinase [12,29,30], but no evidence for this proposal was obtained.

Fatty acid composition of PtdIns and PtdChol

Small, but non-significant, changes due to agonist stimulation in the 1,2-DAG molecular species composition of PtdChol and PtdIns were observed (Table 1). For this reason the fatty acid composition was also determined by GLC. On the basis of the HPLC results on one hand a slightly different pattern of total fatty acid composition of PtdChol and PtdIns should have been expected on GLC, but more importantly, the results on GLC prove once again that no changes of fatty acid patterns of PtdChol and PtdIns occur during agonist stimulation. From the data on fatty acid composition can also be derived that 16:0 is a major component of PtdChol (25 mol %) in contrast to its minimal presence in PtdIns (4 mol %). On the other hand, PtdIns contains 2-fold more (mol %) of the major component 20:4n6 than PtdChol. This information was used for the design of the double label experiments (see below).

Incorporation of [^{14}C]16:0 and [^{3}H]20:4 into 1,2-DAG

ET-1 and PMA stimulation of cardiomyocytes caused a small, but significant decrease in mol % of 18:0/20:4n6 in 1,2-DAG after 10 min stimulation, which was accompanied by an increase of respectively mol % 18:0/18:1 and 18:1/18:1 (Table 3). Experiments were designed based upon the analyzed fatty acid composition of PtdChol and PtdIns (Table 4), not showing significant changes between 40 min control and ET-1 stimulation, to provide additional proof for the shift from PLC-β induced hydrolysis of PtdIns to PLD induced hydrolysis of PtdChol as became apparent by HPLC analysis of 1,2-DAG. For this purpose, cardiomyocytes were labelled with [^{14}C]16:0 and [^{3}H]20:4. Subsequently, the cardiomyocytes were stimulated by agonists and the relative

incorporation of [^{14}C] and [^{3}H] into free 1,2-DAG was counted. After 10 and 40 min stimulation with either ET-1 or PMA a significant decrease of the ratio [^{3}H]20:4/[^{14}C]16:0 was found (Figure 2). The ratio changed from 1.70 to 1.40 for both ET-1 and PMA after 10 min and from 1.70 to 1.56 for PMA and from 1.70 to 1.39 for ET-1 after 40 min. The absolute incorporation of [^{3}H]20:4 and [^{14}C]16:0 into 1,2-DAG both increased respectively 9 % and 32 % after 10 min ET-1 and with 16 % and 40 % after 10 min PMA stimulation, which is likely due to agonist-induced increase of 1,2-DAG content (see also Figure 3). In agreement with the results on molecular species composition, there were no significant changes in labelling ratio of 1,2-DAG in control incubated cardiomyocytes and those stimulated by PHE.

1,2-DAG concentration ([^{14}C]glycerol incorporation)

Previously, we demonstrated by [^{3}H]glycerol labelling of serum-grown cardiomyocytes, that the [^{3}H]1,2-DAG level raised not more than 1.2-fold after 15 min stimulation of cells by ET-1 and PHE [28]. We repeated these measurements for serum-free cultured cells, except that this time [^{14}C]glycerol was used. Again, only a small rise in the [^{14}C]1,2-DAG after ET-1 stimulation was observed, which disappeared after 40 min stimulation (Figure 3). These results are consistent with the GLC analysis (Table 2). PMA stimulation did also not result in a clear increase of [^{14}C]1,2-DAG. These results together with the previous [12] indicate that relative changes in concentration of particular molecular species of 1,2-DAG, have more importance in the activation of PKC isoenzymes than its overall concentration.

DISCUSSION

The results of this study provide for the first time evidence that during ET-1- and PMA-, but not PHE-stimulation of cardiomyocytes, PtdChol slowly becomes a significantly contributing source for formation of 1,2-DAG through sustained activation of PLD. This finding is consistent with our previous data on $InsP_n$- formation (PLC-β activity measurements) and choline-formation (PLD activity measurements) during agonist stimulation [12]. Although the rate of formation of labelled $InsP_n$ and choline does not have to parallel that of 1,2-DAG when PLC-β and PLD are activated, the fact that PtdChol becomes a significant source of 1,2-DAG proves that a part of 1,2-DAG arises from the dephosphorylation of PLD-generated PtdOH by PtdOH phosphohydrolase [31]. Another, though unexpected finding in this study is that the molecular species composition of 1,2-DAG in unstimulated cells closely resembles that of PtdIns. Furthermore, no significant elevation of the total concentration of 1,2-DAG was observed during prolonged ET-1 and PMA stimulation, for ET-1 measured by both [^{3}H]glycerol incorporation and GLC of fatty acid methylesters. It was previously shown by Western blotting that under these conditions (ET-1 and PMA stimulation) several PKC isozymes (such as ε, α and δ) become translocated/activated [12-14]. Therefore, the question was becoming increasingly important whether elevation of the total 1,2-DAG content and/or change in particular molecular species of 1,2-DAG determines the activation of PKC-isozymes. On the other hand, it cannot be excluded that 1,2-DAG formed from TAG and phospholipids is distinctly compartmentalized, that elevated levels of second messenger molecular species of 1,2-DAG cannot be detected on top of the pool(s) of 1,2-DAG derived from basal TAG and phospholipid turnover. In one experiment we also analyzed the fatty acid composition of TAG (Table 4), which showed a pattern different from what would be expected on basis of the molecular species composition of 1,2-DAG in unstimulated cells.

In contrast to some other reports, neither amounts of 1-alk-1-enyl-2-acyl-glycerophospholipids nor of 1-alkyl-2-acyl-glycerophospholipids in PtdChol were found, although they can easily be separated from 1,2-DAG during the preparation of DNBC-derivatives [19]. Post et al. [32] could detect small amounts of alkyl-acyl-glycero-P-choline in serum grown rat neonatal cardiomyocytes. In the present study, however, the neonatal cardiomyocytes were kept in serum-free medium for

Table 3. Molecular species composition (mol %) of DNBC-1,2-DAG of control and agonist stimulated myocytes.

	1,2-DAG, 10 min				1,2-DAG, 40 min			
Molecular species	Control	ET-1	PHE	PMA	control	ET-1	PHE	PMA
18:1/20:4	4.1±1.1	4.3±1.2	3.0±0.8	3.3±0.8	3.8±1.5	2.2±1.0	3.4±1.2	3.5±0.9
16:0/20:4	1.1±0.6	0.5±0.4	0.6±0.1	0.5±0.1	0.6±0.2	2.5±2.0	1.3±0.9	0.8±0.2
18:0/22:6	7.9±2.2	11.3±5.5	11.9±5.5	11.2±3.7	13.3±5.4	12.0±3.2	14.2±4.4	10.8±2.0
18:0/20:4	57.5±2.9	46.2±2.9*	56.1±2.1	47.3±2.7*	60.7±1.2	42.8±5.6*	48.7±6.6	43.3±5.6*
18:1/18:1	11.2±0.5	10.5±4.1	7.9±4.5	17.5±1.8*	5.4±2.9	14.5±4.3	9.7±4.4	12.4±5.8
16:0/18:1+ 18:0/18:2	10.7±1.7	10.8±2.7	10.7±3.8	12.7±1.6	7.3±3.2	15.6±3.3	12.6±1.3	15.3±4.1
18:0/18:1	2.8±0.3	5.3±0.7*	3.6±1.5	3.7±0.3	4.3±1.5	7.2±1.2	3.4±0.4	4.8±0.5
others	4.7±1.9	11.0±6.3	6.2±2.0	8.0±2.7	3.8±1.5	6.2±2.3	6.4±1.2	9.1±2.7

The experiments were performed with serum free cultured cardiomyocytes unstimulated or stimulated with ET-1 (10^{-8}M), PHE (10^{-5}M) or PMA (10^{-6}M), with different cell batches. Results are shown as means ± SEM (mol %), n=5. The molecular species composition of 1,2-DAG was also measured at time zero, but not found to be significantly different from that measured in control cardiomyocytes at 10 and 40 min. Values marked with * are significantly different from the corresponding control value.

Table 4. Fatty acid composition (mol %) of PtdChol, PtdIns and PtdOH and TAG of control and ET-1 stimulated cardiomyocytes.

	PtdChol, 40 min		PtdIns, 40 min		PtdOH, 40 min	TAG, 40 min
Fatty acid	Control	ET-1	control	ET-1	ET-1	ET-1
16:0	25.2±0.9	25.4±0.8	4.1±0.8	5.3±0.7	18.9±2.9	19.4
18:0	22.8±0.9	22.1±0.7	41.9±2.6	40.7±0.5	25.1±1.8	19.2
18:1n7/n9	12.6±0.5	12.3±0.6	2.4±0.8	4.1±0.4	8.7±1.4	15.9
18:2n6	2.5±0.1	2.6±0.2	0.5±0.1	1.3±0.5	-	9.2
20:3n6	1.1±0.1	1.1±0.1	1.8±0.1	1.6±0.6	7.6±1.4	2.1
20:4n6	26.3±0.7	26.8±1.0	40.2±2.9	38.8±2.4	13.4±1.5	11.3
22:4n6	1.1±0.2	1.0±0.1	1.0±0.3	0.8±0.1	0.9±0.2	5.3
22:5n3	1.8±0.1	1.8±0.1	1.5±0.4	1.1±0.2	1.5±0.6	3.9
22:6n3	4.1±0.3	3.9±0.2	1.5±0.7	1.0±0.2	2.2±0.6	3.7
others	2.5±0.3	3.0±0.6	5.1±1.5	5.3±0.8	5.3±0.8	10

The experiments were performed in serum free cultured cells either in control or cells stimulated with ET-1 (10^{-8}M). Three experiments with different cell batches were carried out. In one experiment TAG was also measured. Results are shown as mean ± SEM (mol %). The fatty acid composition of PtdChol, PtdIns, PtdOH was also measured at time zero, but not found to be different from that measured at 40 min. Differences present between 40 min control and ET-1 stimulation for either PtdIns or PtdChol were shown not to be significant at the $p < 0.05$ level.

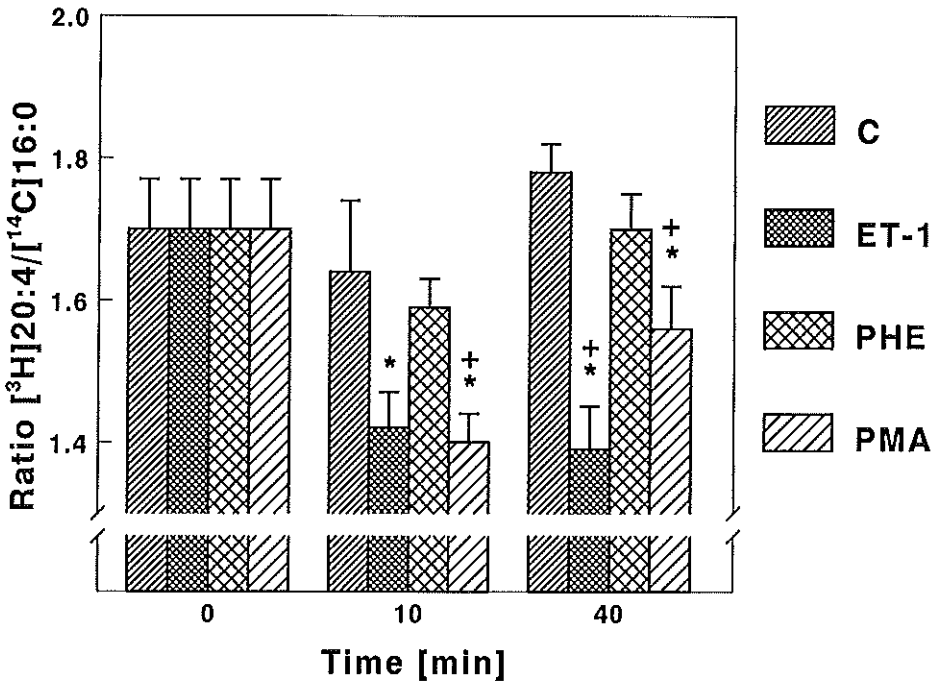

Fig. 2. Incorporation of [^{3}H]20:4 relative to [^{14}C]16:0 in 1,2-DAG in control and agonist stimulated cardiomyocytes. Data are presented as means ± SEM of three separate experiments with triplicate measurements for each condition. Datapoints marked with * are significantly different from zero time and those with + are significantly different from the corresponding control ($p < 0.05$).

a period of 48 hours prior to the experiments. The reason for choosing these culture conditions is that, in contrast to in the presence of serum, PHE, ET-1 and PMA induce hypertrophy. Serum addition in itself produces hypertrophy of cardiomyocytes, because it contains growth factors and fatty acids which may also influence phospholipid turnover. Hermans et al. [19] found 1-alk-1-enyl-2-acyl in PtdChol of these species in pancreatic acini using the methodology for 1,2-DAG separation and identification as used in this study. Thus, it appears that the analysis used must have been sensitive enough to detect these species if they were present, and that other factors (such as long-term presence of serum) likely are the cause for the absence of alkyl-acyl or alkenyl-acyl species.

We are not the first to show subtle time-dependent changes in the endogenous 1,2-DAG molecular species with only certain polyunsaturated fatty acid containing species showing significant agonist-stimulated decrease [15,17,19]. Studies of α-thrombin-stimulated IIC9 chinese hamster embryo fibroblasts [15], bombesin-stimulated Swiss 3T3 mouse fibroblasts [17] and bradykinin-stimulated human fibroblasts [16] support the idea that PtdIns(4,5)P_2 could be the source of 1,2-DAG at early stimulation times (15 sec-1 min), but PtdChol at all times. The changes in 1,2-DAG species observed in the afore mentioned cell types and in the present study demonstrate not only that there may be differences in phospholipase activity or phospholipid substrate with time of agonist stimulation, but also differences in cellular responses (e.g. activation/translocation of PKC isoenzymes). Although until now there is limited information concerning the specificity of molecular 1,2-DAG species in

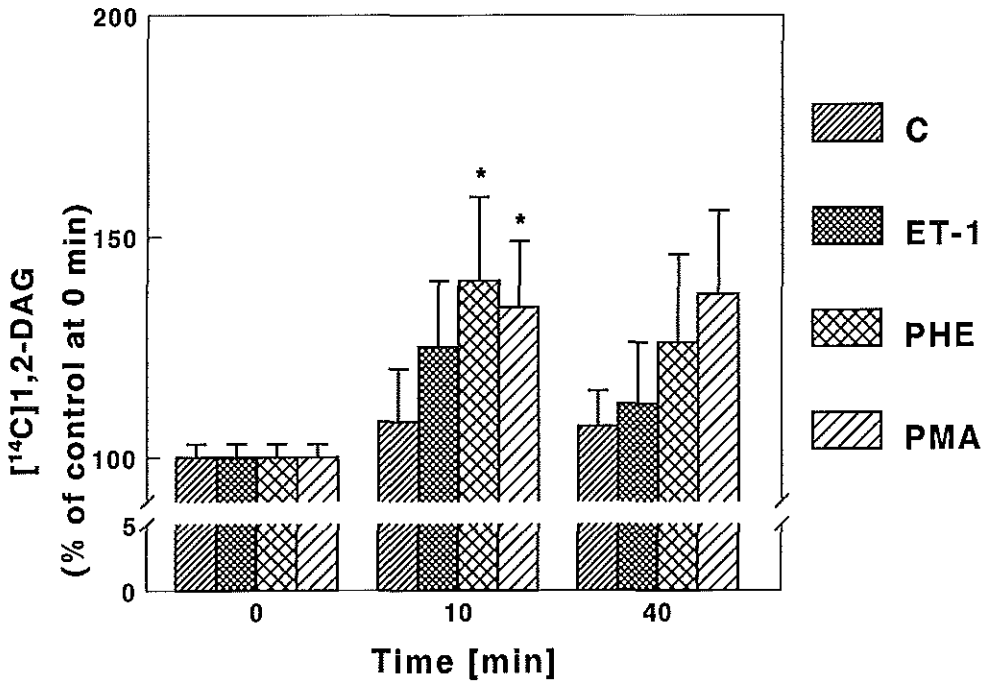

Fig. 3. [^{14}C]glycerol content of 1,2-DAG in control and agonist stimulated cardiomyocytes. The [^{14}C]glycerol content was standardized on basis of the total [^{14}C]glycerol incorporation (in water-soluble and -insoluble fraction) to correct for variation in number of cells extracted as done before [28]. Results are presented as means ± SEM of 4 experiments. Points marked with * are significantly different from the corresponding control ($p < 0.05$).

activating individual PKC isoforms, it was hypothesized by Pettitt and Wakelam [17] that distinct changes in 1,2-DAG molecular species patterns may relate to a distinct pattern of differential activation of PKC isoforms and protein phosphorylation. In this view it is important to note that it was shown by us and others [12-14] that during ET-1 stimulation of rat neonatal cardiomyocytes PKC-ε becomes rapidly translocated/activated, whereas no translocation/activation was found during PHE stimulation. Thus, the present results which also show no effect of PHE on 1,2-DAG molecular species are indicating to a critical role of the pattern of changes in 1,2-DAG molecular species for PKC isoenzyme activation.

The double label experiments designed to find support for the conclusions from the data on molecular species composition of 1,2-DAG demonstrated indeed that less [^{14}C]20:4n6 compared to [^{3}H]16:0 is incorporated into 1,2-DAG during ET-1 and PMA, but not PHE stimulation. These results are consistent with increased contribution of PtdChol to formation of 1,2-DAG after prolonged stimulation, because 6-fold more 16:0 is found to be present in PtdChol comparing to PtdIns and 1.5-fold less of 20:4n6 is present in PtdChol comparing to PtdIns (Table 4). Moreover, PMA was definitely proven to uniquely activate PLD [12]. On the other hand, PHE only stimulates PLC-β [12], which is in accordance with the finding of no changes in the [^{14}C]20:4n6/[^{3}H]16:0 incorporation ratio.

In the [^{14}C]glycerol labelling experiments, for the estimation of concentration changes in 1,2-DAG during agonist stimulation, only small elevations of 1,2-DAG in the cells during ET-1 and PMA

stimulation were found. Also this finding provides evidence that the increase of cellular 1,2-DAG level is less important in the transduction of receptor-mediated signals to PKC. However, it should be noted that 1,2-DAG may have functions apart from stimulating PKC isoenzymes. For example 1,2-DAG can become a source for free 20:4n6 which is the main substrate for cyclo- and lipoxygenase to form respectively prostaglandins and leukotrienes [33].

From the results it can be concluded that 1,2-DAG present in unstimulated rat neonatal cardiomyocytes is likely derived from PtdIns(4,5)P_2. During ET-1 and PMA, but not PHE stimulation, PtdChol becomes an increasingly important source for 1,2-DAG due to only early PLC-β and sustained PLD activation [12]. Unlike that of 1,2-DAG, the molecular species composition of PtdChol and PtdIns remained constant during agonist stimulation. The 1,2-DAG level only slightly rises during agonist stimulation, whereas PKC isoenzymes have been shown to become translocated/activated under these conditions. The latter result provides strong evidence that particular molecular species of 1,2-DAG, more than its overall concentration, determine the activation of PKC isoenzymes.

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Chapter 5

LIMITED ALPHA$_1$-ADRENERGIC AND ENDOTHELIN-1 INDUCED INTRACELLULAR ALKALINIZATION RESPONSES IN SERUM-FREE CULTURED RAT CARDIOMYOCYTES

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ABSTRACT

Background: Endothelin-1 (ET-1) and α_1-adrenergic agonist phenylephrine (PHE) to a much lesser extent have been shown to activate phospholipase C and D, and to induce translocation of protein kinase C-ε in serum-free cultured rat neonatal cardiomyocytes. There is also evidence that Na^+/H^+ exchanger activation can occur as a consequence of protein kinase C-induced phosphorylation. *Objectives:* The purpose of the present study was to investigate the alkalinization responses potentially evoked by ET-1, PHE and phorbolester in cultured neonatal rat cardiomyocytes. *Methods:* We employed ratio imaging of fluorescent ion indicator 2',7'-bis(carboxyethyl)-5',6'-carboxyfluorescein (BCECF) to measure changes in intracellular pH (pH_i) during agonist stimulation and during direct protein kinase C activation by phorbolester (PMA). *Results:* Application of 10^{-8}M ET-1 and 10^{-5}M PHE caused pH_i to rise slowly to reach a small but significant maximal alkalinization after about 35 min of respectively 7.16±0.05 and after 25 min of 7.13±0.04 above the baseline of 7.10±0.00. Exposure of cells to 10^{-6}M PMA was followed by a small and insignificant alkalinization. The presence of a potentially highly active Na^+/H^+ exchanger was demonstrated by rapid recovery from acidosis in cardiomyocytes challenged by a 20 mM NH_4Cl pulse. *Conclusion:*We conclude that only limited α_1-adrenergic and ET-1 induced intracellular alkalinization responses occur in serum-free cultured rat cardiomyocytes and that protein kinase C does not play a major role in this.

INTRODUCTION

The Na^+/H^+ exchanger plays a key role in regulating cardiac intracellular pH (pH_i) [1,2]. Cardiac muscle is continuously exposed to acid challenges under various physiological (e.g. contractile and metabolic activities) and pathophysiological (e.g. hypoxia, ischemia, mechanical overload and neurohumoral stimulation) conditions. Forward exchange (one external Na^+ in for one internal H^+ out) mediates pH-recovery from intracellular acidosis. However, at least in cardiac muscle the exchanger is believed to be minimally active at neutral pH (the physiological pH_i) despite a strongly inward directing Na^+ gradient that could drive pH_i to more alkaline levels [3]. The minimal activity of the exchanger can be attributed to the allosteric regulation of the Na^+/H^+ exchanger by internal protons [4]. However, external stimuli such as α_1-adrenergic and purinergic agonists [3,5,6,7], ET-1 [8,9] thrombin and angiotensin II (AngII) [9,10] have been suggested to modulate the cardiac Na^+/H^+ exchange activity at neutral pH. Also in tissues other than myocardium it has been shown that a wide variety of stimuli (phorbolesters, growth factors, hormones, neurohormones, etc) can activate the Na^+/H^+ exchanger activity and that this activity leads to cytosolic alkalinization which persists as long as the signals exist, inducing various cellular responses [1,2,11]. In myocardium the positive inotropic effects of α_1-adrenergic agonist, ET-1 and AngII are partially explained as a consequence of intracellular alkalinization and resultant myofibrillar sensitization to Ca^{2+} [5,6,8,10]. On the other hand, studies have demonstrated that the Na^+/H^+ antiporter inhibitor amiloride can block the ventricular remodeling (e.g. cardiac hypertrophy) processes [12,13]. These studies suggest that the Na^+/H^+ exchanger has an important role in the chronic long-term growth response in non-infarcted regions of infarcted hearts. The emerging evidence that Na^+/H^+ exchanger activation does not only occur as a result from proton generation, but also as consequence of receptor-linked mechanism via phosphorylation reactions (Ca^{2+}-calmodulin-dependent protein kinase, protein kinase C isozymes and/or MAP kinases) would be consistent with a hypothesis that the agonist-induced hypertrophy is partially mediated by cellular alkalinization [1,2].

In the last decade numerous reports have appeared demonstrating that single agonists such as PHE, ET-1, AngII and PMA induce hypertrophy *in vitro* using serum-free cultured rat cardiomyocytes (see reviews [14-16]). The criteria range from true hypertrophy (increased cell size, protein content and development of a well-ordered contractile apparatus) to re-expression of atrial natriuretic factor (ANF). Single rat cardiomyocytes have also been used to show increased pH_i by the same agonists

and some reports provide evidence that protein kinase C is involved [3-9, 17-19]. The latter studies exclusively used isolated (collagenase/trypsin-dissociated) myocytes in suspension. Recently, we reported that ET-1, and PHE to a much lesser extent, produced activation of phospholipase C and indirectly phospholipase D, as well as a rapid intracellular translocation/activation of PKC-ε which was on the long-term (24h) followed by hypertrophic responses in serum-free cultured rat cardiomyocytes [20]. The phospholipase D activator and direct protein kinase C activator PMA was shown to induce rapid translocation of protein kinase C-ε, as well as -α and -δ, and hypertrophy. Moreover, in a follow-up study [21] we obtained evidence that particular molecular species of 1,2-diacylglycerol rather than its absolute concentration determines the translocation/activation of PKC-ε. Therefore, the purpose of the present study was to reinvestigate the α_1-adrenergic and ET-1-induced intracellular alkalinization response in serum-free cultured rat cardiomyocytes and compare these with that evoked by phorbolester all compared to control. pH_i in cardiomyocytes can be measured using digital imaging fluorescence microscopy and the fluorescent ion pH indicator 2',7'-bis(carboxyethyl)-5',6'-carboxyfluorescein (BCECF) [22].

MATERIALS AND METHODS

Reagents

Phenylephrine (PHE), phorbol 12-myristate 13-acetate (PMA), probenicid and nigericin were obtained from Sigma (St. Louis MO, USA) and endothelin-1 (ET-1) from Boehringer Mannheim (Germany). Ham's F10, fetal calf serum and horse serum were purchased from Flow Laboratories (Irvine, UK). The culture media DMEM and M199 were obtained from Gibco (UK); penicillin/streptomycin was obtained from Boehringer Mannheim (Germany). Collagenase (type 1) was from Worthington (Freehold, NJ, USA). Tissue culture dishes, 60 mm and 35 mm diameter, were obtained from Becton-Dickinson (Oxnard, CA, USA). BCECF and BCECF-AM were both obtained from Molecular Probes (Eugene, OR, USA). Chelerythrine was obtained from Biomol (SanverTech, Breda, The Netherlands).

Cell culture

Cardiomyocyte cultures were prepared using the method described before [23] and cultured serum-free during the last 24 h before the experiments began. Briefly, hearts of 2-day old Wistar rats were dissected and transferred to a solution containing 137 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 0.4 mM KH_2P0_4, 5.5 mM glucose, 20 mM HEPES, 0.5 mg/ml phenol red, pH 7.4. The ventricles were minced into small fragments and dissociated using collagenase during two periods of 20 min in a shaking water bath at 37°C. After centrifugation at 50 x g for 15 min, the cells were suspended in a culture medium consisting of Ham's F-10 medium supplemented with 10% fetal calf serum and 10% horse serum. The cells were plated in 60 mm culture dishes in a density of 4.2×10^6 cells per dish. Myocytes were separated from nonmuscle cells using a selective adhesion technique [24]. After 45 min the medium, enriched in myocytes, was transferred to three 35 mm culture dishes, which contained 25 mm round glass coverslips. The myocyte cultures were kept in a humidified incubator (37°C) with an atmosphere of 95% air and 5% CO_2. Culture medium was changed after 3 h and 48 h. One day before performing the experiments, culture medium was replaced by serum-free medium DMEM/M199 (4:1, supplemented with penicillin/streptomycin). Three days after cell isolation, the monolayers of spontaneously beating myocytes were used for the experiment.

Digital imaging fluorescence microscopy

Intracellular pH (pH_i) was measured by digital imaging fluorescence microscopy, using the fluorescent ion indicator 2',7'-bis(carboxyethyl)-5',6'-carboxyfluorescein (BCECF). The cardiomyocytes were loaded with BCECF by incubation with 1 μM BCECF/AM in 1 ml HBSS (125 mM NaCl; 5 mM KCl; 1 mM $MgSO_4$; 1 mM KH_2PO_4; 2.5 mM $CaCl_2$; 10 mM $NaHCO_3$; 10 mM HEPES; 5 mM glucose and 2.5 mM probenicid, pH 7.1) for 30 min at 37°C. Then, the glass coverslip was rinsed three times with HBSS and mounted in the special culture dish to be used in the microscope [25].

The culture dish was mounted in a temperature controlled micro-incubator in a dual-wavelength fluorescence microscope equipped with a computer controlled X/Y-table fitted for precise positioning of the culture dish. The set-up consists of an inverted microscope (Leitz Diavert, Wetzlar, Germany) equipped with a 20x fluorite objective (Nikon, Tokyo, Japan) and a mercury light source (HBO-100, Osram, Montgomery, NY, USA). A filterwheel (Sutter Instruments, Novato, CA, USA) regulates the selection of excitation filters (440 nm and 490 nm). Emission fluorescence is led through a high-pass filter (530 nm for BCECF), and is imaged by a high-sensitivity silicon intensified target camera (Hamamatsu C2400-08, Herrsching, Germany). The resulting video image is digitised by a frame-grabber board (Imaging Technologies Inc., Woburn, MA, USA) in a PC-AT 486 computer. Spatial resolution of the images is 256x256 pixels, with an 8 bits intensity resolution. The images were processed using dedicated image processing software (TIM®, Difa, Breda, The Netherlands). After the subtraction of the background fluorescence, the 440 nm image was divided by the 490 nm image on a pixel by pixel basis to yield a ratio image. Using a template, made by setting a certain treshhold level, the extracellular space (the spaces between the cells) is given the pixel value zero (in the 440 nm image) or the pixel value 1 (in the 490 nm image). For quantitative analysis of the ratio images the software allows the analysis of individual cells as well as overall analysis, excluding the pixels with a value of zero. In that way only "cellular pixels" are taken into account. Statistical parameters (mean, median, standard deviation) were calculated by the software and used to calculate intracellular pH.

Callbration of BCECF

The fluorescence of BCECF was calibrated *in situ* in BCECF-loaded cardiomyocytes as described previously [26]. BCECF calibration buffer contained 140 mM KCl, 3.8 mM NaCl, 1.2 mM $MgSO_4$, 20 mM HEPES (for pH_e 6.8-7.8) or 20 mM MOPS (for pH_e 6.1-6.8) adjusted to various pH, and 10 µM of the K^+-H^+-ionophore nigericin. The resulting calibration curve was used to calculate pH_i (Fig. 1).

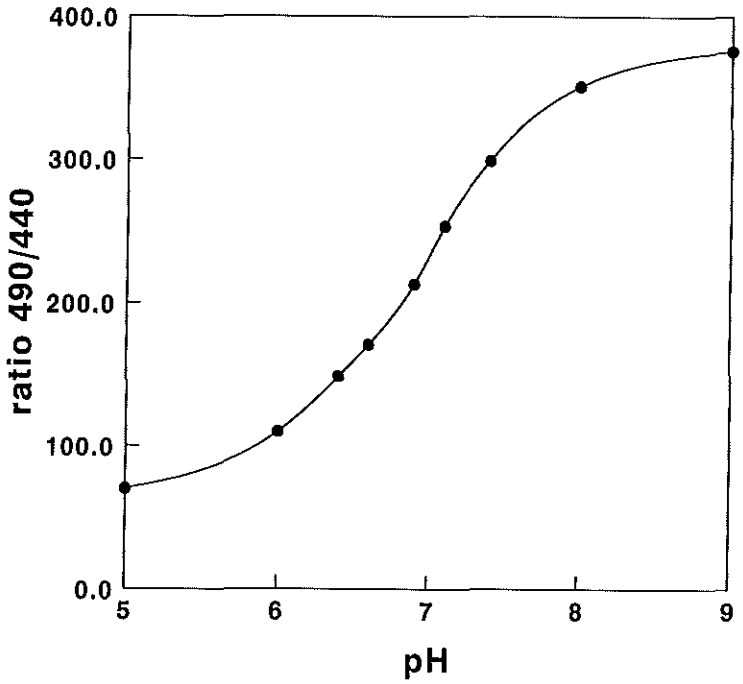

Fig. 1. Calibration curve of the emission ratios using the ratio image of two images acquired at 490 nm and 440 nm excitation wavelenghts (f490/f440) measured in cardiac cells exposed to solutions of varying pH_e containing 10µM K^+-H^+-ionophore nigericin.

Experimental protocol

After the loading protocol, the incubation dish with the BCECF-loaded myocyte culture on the coverslip was inserted in the micro-incubator on the stage of the fluorescence microscope and the culture halves were equilibrated with HBSS for 30 min at 37°C before starting the measurements. During this period, suitable fields of view were selected and their positions stored in the XY table-controller of the computer. Then, control buffer (HBSS pH 7.1) or agonist was added (ET-1 10^{-8} M, PHE 10^{-5} M or PMA 10^{-6} M) and measurements were performed during 40 minutes incubation and pH_i was measured. To test the ability of cardiomyocytes to recover from a NH_4Cl pulse, changes in pH_i were measured by replacement of HBSS medium by low Na^+ HBSS medium (Na^+ 105 mM)containing 20 mM NH_4Cl allowing the cells to incubate for about 20 min whereafter the medium was changed again to normal HBSS at pH 7.1 for another 20 minutes. In two experiments BCECF-loaded cells were pretreated with the PKC-inhibitor chelerythrine (10^{-4}M), whereafter they were stimulated with PMA. Off-line, the ratio images of BCECF were calculated and statistics were performed of all "cellular pixels" within one image.

Statistics

Values are expressed as mean ± standard deviation unless stated otherwise. Statistical analysis was performed with the use of ANOVA, followed by the Bonferroni t-test when appropriate. Differences were considered significant at $P < 0.05$.

RESULTS

Recovery of intracellular pH_i after a NH_4Cl pulse

Before studying the effect of agonists, we tested the ability of cardiomyocytes to recover from a NH_4Cl pulse to unmask the H^+ regulated activity of the Na^+/H^+ exchanger. In response to this standard protocol and as shown previously by numerous other reports [3-6, 8-10, 19,27,28], the abrupt exposure to NH_4Cl caused a transient small increase of pH_i as basic NH_3 rapidly enters the cardiomyocyte (Fig. 2). The pH then fell as charged NH_4^+ entered the cell via K^+ channels and became dissociated [9,10,28]. Upon washout of extracellular NH_4Cl (after 20 min), intracellular acidosis was maximized as internal NH_3 left the cell causing the intracellular retention of H^+. Thereafter, the cardiomyocytes recovered in about 20 minutes from this acidosis depending predominantly on forward Na^+/H^+ exchange [19].

The alkalinization response to the agonist of cardiomyocytes measured on single cell level, in contrast to the acidotic response to a NH_4Cl pulse, appeared to be very heterogeneous. Therefore, all agonist effects were statistically processed in the overall images.

Effects of different agonists on pH_i

The overall basal pH_i of the serum-free cultured rat neonatal cardiomyocytes incubated in HEPES-buffered (pH=7.1) medium was 7.10±0.00 (Fig. 3, see also Fig. 2).

Intracellular pH remained stable without noticeable 'run-down' for a period of 40 min. The application of 10^{-8}M ET-1 produced a small but significant alkalinization reaching a maximal average value of 7.16 ±0.05 (Fig. 3) above baseline after 35 min. An almost similar response was observed after applying 10^{-5}M PHE (maximal average value of 7.13±0.04 at 25 min). The effect of 10^{-6}M PMA was, however, appreciably less, not significant over the period of 40 min (maximal average value 7.13±0.05 at 35 min).

We also performed two experiments with PMA (10^{-6}M) stimulation in the presence of the PKC inhibitor chelerythrine (10^{-4}M). This PKC inhibitor tended to detach part of the cells from the coverslip, which interfered with the pH_i measurement. Nevertheless, we found a similar response pattern comparing with that of PMA alone (results not shown). PLD activation (indirectly through PKC) by PMA has been shown in earlier experiments to be inhibited completely by chelerythrine. Also basal release of [^{3}H]choline (as measure for PLD activation) was inhibited as shown in earlier experiments [20]. However, cells used for pH_i measurements were cultured in a small density

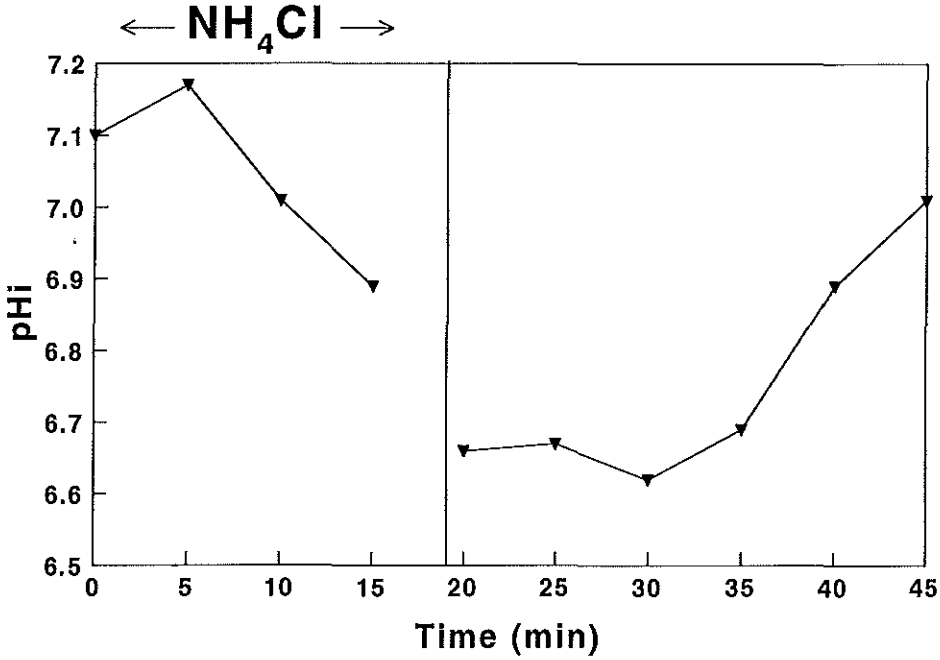

Fig. 2. Recovery of intracellular pH_i of cultured rat neonatal cardiomyocytes from a 20 min exposure to 20 mM NH_4Cl. NH_4Cl caused a rapid increase in pH_i followed by recovery and acidosis. NH_4Cl removal after 20 min caused a further transient cytosolic acidification followed by recovery. Measurement of pH_i was performed 5 min before washout and rapidly after washout, because of technical difficulties to measure pH_i at the moment of washout.

and on glass instead of plastic as we used in earlier experiments (chapter 3), which might have an influence on the strength of adherence of the cells.

DISCUSSION

Our finding that both α_1-adrenergic agonist PHE and ET-1 significantly increase pH_i in serum-free cultured rat neonatal cardiomyocytes is consistent with previous reports about such experiments performed in acutely isolated collagenase-dissociated rat cardiomyocytes [3-9,17-19]. In the present study the maximal alkalinization responses induced by agonist are relatively slow in onset and limited in size in comparison to those generally described in suspensions of rat cardiac myocytes. For example, Ito *et al.* [9] and Krämer *et al.* [8] showed that ET-1 (10^{-8}M) caused a significant increase of pH_i of about 0.10 units, which was already reached within a few minutes compared to control rat ventricular myocytes. Similarly, Puceat *et al.* [3] found a maximal increase of pH_i of about 0.10 unit already reached after 5 minutes. In several studies it was also investigated whether the agonists could abbreviate the time the cells need to recover from an acid load induced by a NH_4Cl pulse protocol [3,6,9,10]. Indeed, exposure to either ET-1 or PHE enhanced the rate of recovery from intracellular acidification which can be ascribed to forward Na^+/H^+ exchange. The rate of exchange of external Na^+ ions for internal protons is low at normal steady state pH close to neutral, but it is maximally stimulated by acidosis. Its activity is modified by an intrinsic H^+ modifier site giving rise to the steep activation of exchange at a lowered intracellular pH [1,2]. This allows the exchanger

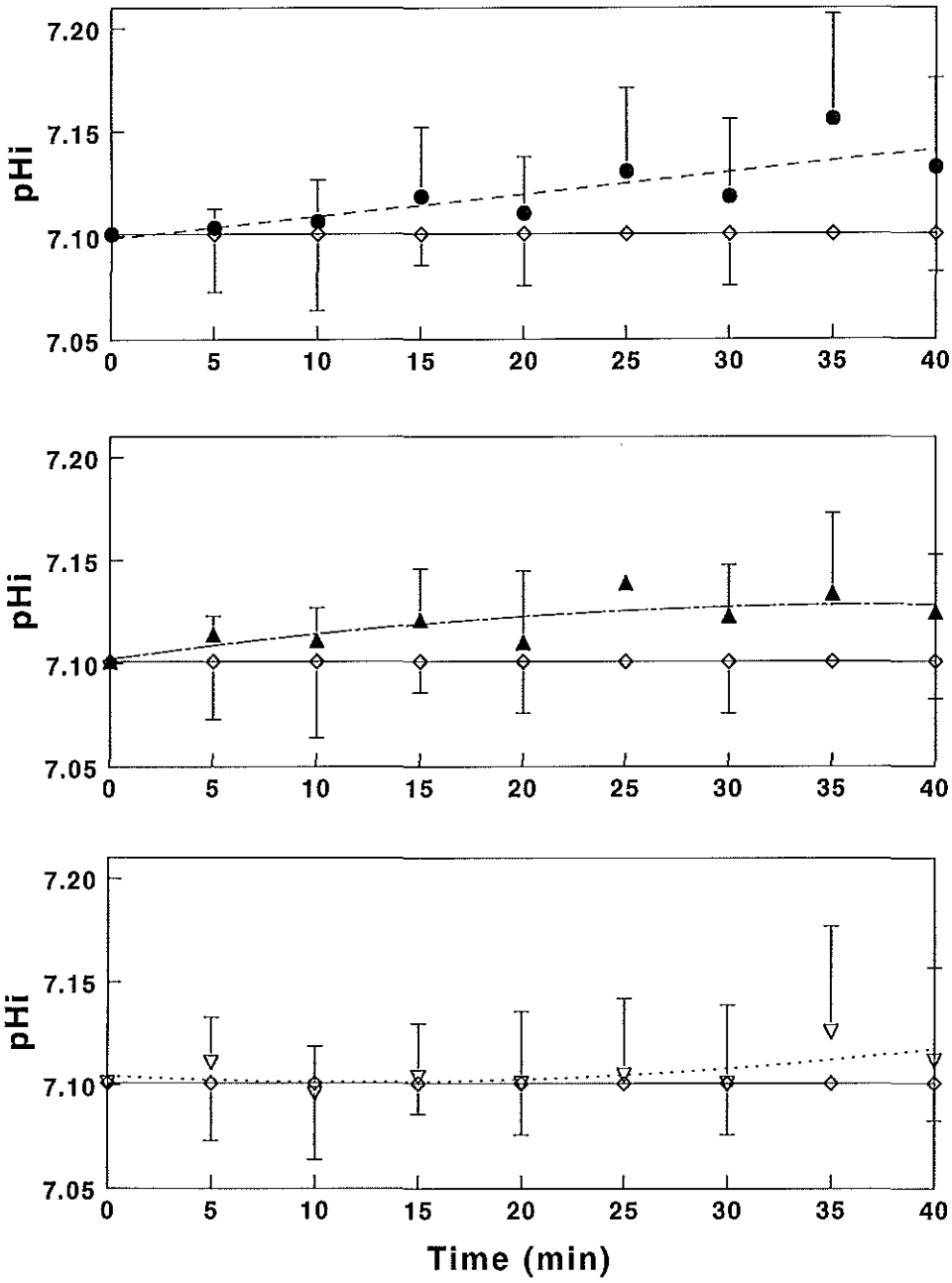

Fig. 3. Average effects of ● ET-1 (10^{-8}M), ▲ PHE (10^{-5}M) and ▽ PMA (10^{-6}M) and ◇ control on pH_i of cultured rat neonatal cardiomyocytes. Points are expressed as means ± SD. On average n=4 for control measurements, n=6 for ET-1, n=4 for PHE and n=5 for PMA. Both ET-1 and PHE produced a small but significant alkalinization above baseline after 35 min (ANOVA and Bonferroni t-test).

protein to adjust its activity to changes in intracellular pH. This high rate of exchanger activity, although occurring after a lag phase of about 10 min, has also been observed in the present study in serum-free cultured rat neonatal cardiomyocytes challenged by a NH_4Cl pulse. The changes in pH we obtained from cells exposed to a NH_4Cl pulse are similar to those in reported previously except that some acidosis develops already before the NH_4Cl washout [9,10,19,28]. This is likely due to K^+ channels that are highly permeable to NH_4^+ [29-31].
Extrinsically, agonists such as PHE and ET-1 rapidly modulate the Na^+/H^+ exchanger activity by phosphorylation, thereby stimulating the Na^+/H^+ exchanger and shifting pH_i curve to more alkaline values [2]. Despite the broad knowledge about different signaling cascades (such as PLC-β, small GTPases of the Rho family, myosin light chain kinase and p42/p44 MAPK pathway) that influence the Na^+/H^+ exchanger, it is not yet known which kinase actually phosphorylates the Na^+/H^+ exchanger at its serine residues [1]. However, phosphorylation is not the only mechanism by which Na^+/H^+ exchange is regulated (compare [32]).
Whether a phosphorylation-independent mechanism is involved in the alkalinization responses of PHE and ET-1, may be concluded from the comparison of previously observed effects on PLC-β, PLD and PKC-ε in the same model [20]. ET-1 (10^{-8}M) strongly stimulated PLC-β and PLD and induced translocation of PKC-ε. However, no evidence for PKC-ε activation (phosphorylation of histone IIIS or peptide-ε) was found. Likewise, PHE (10^{-5}M) strongly stimulated PLC-β but had relatively minor effects on PLD and PKC-ε, -α and -δ. These results support the view that IP_3-mediated Ca^{2+} response of the phosphoinositide pathway is in some manner involved in the activation of the Na^+/H^+ exchanger. This is even more likely because direct stimulation of PLD and PKC-ε by PMA (10^{-6}M) as we showed previously [20] did not have a significant effect on pH_i in the present study (Fig. 3). The latter observation is consistent with the results with collagenase-dissociated rat ventricular myocytes obtained by Puceat *et al.* [3] and MacLeod and Harding [19], but not with those of Ito *et al.* [9], who found an effect of the phorbolester TPA (12-O-tetradecanoylphorbol-13-acetate, 80 nM) comparable to the effects of 10 nM ET-1 and AngII. It should be noticed that we previously showed only very small and cell-heterogeneous Ca^{2+} responses when serum-cultured rat neonatal cardiomyocytes were stimulated by ET-1 or PHE [33]. This findings suggest that Ca^{2+} is also not involved in the receptor-mediated alkalinization response.
Protein kinase C-ε involvement in the ET-1 and PHE induced alkalinization response is also unlikely based upon the fact that PHE did only slightly translocate PKC-ε.
From the present study we conclude that only limited α_1-adrenergic and ET-1 induced alkalinization responses occur in serum-free cultured rat cardiomyocytes and that protein kinase C (isozymes) is most likely not involved. Previously, we observed in the same model hypertrophic responses (increased [^{3}H]leucine incorporation and increased protein/DNA ratio) induced by both agonists as well as by PMA (first detectable after 6h) [34,35]. Alkalinization might only be a permissive factor in this growth effect.

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Chapter 6

DOES PROTEIN KINASE C PLAY A PIVOTAL ROLE IN THE MECHANISMS OF ISCHEMIC PRECONDITIONING?

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ABSTRACT

This communication reviews the evidence for the pivotal role of protein kinase C in ischemic myocardial preconditioning. It is believed that several intracellular signalling pathways via receptor-coupled phospholipase C and its "cross-talk" with phospholipase D converge to activation of protein kinase C isotypes, which is followed by phosphorylation of until now (a number of) unknown target proteins, which produce the protective state of ischemic preconditioning.
After briefly introducing the general biochemical properties of protein kinase C, its isotypes and the limitations of the methodology used to investigate the role of protein kinase C, studies are discussed in which pharmacological inhibition and activation and (immunore)activity and/or isotypes measurements of protein kinase C isotypes were applied to assess the role of activation of protein kinase C in ischemic myocardial preconditioning.
It is concluded that definitive proof for the involvement of protein kinase C in preconditioning requires future studies which must focus on the isotype(s) of protein kinase C that are activated, the duration of action, cellular translocation sites and the identity and stability of (covalently bound phosphate) of phosphorylated substrate proteins.

INTRODUCTION

Ischemic preconditioning is not an organ specific phenomenon, as it does not only occur in the heart [1,2], but also in kidney [3], liver [4] and skeletal muscle [5], while the brain is protected against the consequence of a new ischemic event at 24 hours after the preconditioning stimulus is applied [6]. Furthermore, brief ischemia in organs other than the heart may also limit irreversible damage produced by a subsequent coronary artery occlusion. Thus, in rats a 15-minute occlusion of the mesenteric artery with 10 minutes of reperfusion prior to a 60-minute coronary artery occlusion limited myocardial infarct size by 40% [7]. Since ganglion blockade abolished myocardial protection by mesenteric artery occlusion-reperfusion but not by brief coronary artery occlusion-reperfusion, the mechanism of protection by brief ischemia-reperfusion in other organs appears to differ from that by brief myocardial ischemia-reperfusion [7].

Initially, ischemic preconditioning studies focussed on time characteristics and the search for extracellular endogenous and exogenous factors that either mimicked or inhibited the phenomenon. It proved that protection occurred during two distinct episodes: a classical preconditioning period (CPP) that lasted 2 to 3 hours after the preconditioning stimulus was applied [1,2], and a second window of protection (SWOP) between 24 and 72 hours [8-10]. The mechanisms of protection for these two windows are most likely not the same. Rapidly produced endogenous factors may activate intracellular pathways during CPP, while the slower process of induction of heat-shock proteins may be involved during the SWOP. Initially, attention focussed on activation of adenosine A_1-receptors [11,12] or K^+_{ATP} channel opening [13,14], as the mechanisms for protection during CPP. More recently activation of protein kinase C has received wide attention [15]. Prior to reviewing the role of protein kinase C, we review the current state of knowledge of the molecular mechanism(s) of ischemic preconditioning, introduce the generally known biochemical properties of protein kinase C and discuss the limitations of the techniques used to investigate the potential role of protein kinase C. Finally, the evidence that activation of protein kinase C and the intracellular signalling pathways leading to its activation play a pivotal role in the mechanism of ischemic preconditioning is summarized. However, not all studies support a role for protein kinase C in preconditioning and this issue remains therefore controversial at the present time.

Most studies on ischemic preconditioning used infarct size as endpoint, but other endpoints such as recovery of contractile function, and protection against reperfusion-induced ventricular arrhythmias have also been used. Because these other endpoints require different experimental conditions (i.e. shorter duration of the sustained occlusions) we have limited ourselves to studies

which used infarct size as endpoint. For this same reason we have excluded studies on ischemic preconditioning in other organs.

Current state of knowledge of the molecular mechanism(s) of ischemic preconditioning

Activation of receptors by exogenously administered stimuli such as adenosine [11,12], bradykinin [16,17], noradrenaline [18,19], acetylcholine [20,21], endothelin-1 [22] or opiates [23] mimic myocardial protection by ischemic preconditioning. Intracellular signalling by these stimuli, via GTP-binding-protein-linked receptors and phospholipase C and possibly phospholipase D (see later) [24,25], leads to activation of one or more isotypes of the protein kinase C family which then phosphorylate putative target proteins [15,26]. Possible target proteins are those that regulate opening of K^+_{ATP} channels [13,14,27], activate ecto-5'-nucleotidase [28] (during CPP) or modulate transcriptional regulation of the expression of heat shock proteins [29,30] (during SWOP). For instance, K^+_{ATP} channels are opened when an ischemic preconditioning stimulus is applied, while blockade of K^+_{ATP} channels prevents ischemic preconditioning [13,14]. It is likely that modulation of K^+_{ATP} channels in the mitochondria, sarcoplasmic reticulum or the nucleus are involved in the mechanism of protection as blockade of the action potential shortening by dofetilide does not abolish protection by ischemic preconditioning [31]. Since protein kinase C can be activated via various receptors linked to phospholipase C- and possibly phospholipase D-mediated signalling pathways, these receptors may act synergistically [15]. Opening of K^+_{ATP} channels by pharmacological substances lowers the threshold for ischemic preconditioning [32], which is consistent with the hypothesis that K^+_{ATP} channels are target proteins for protein kinase C. Kitakaze et al [28] reported that ischemic preconditioning increased ecto-5'-nucleotidase activity and that activation of protein kinase C increases ecto-5'-nucleotidase activity in isolated rat cardiomyocytes, supporting the candidacy of ecto-5'-nucleotidase as another target protein of protein kinase C.

General biochemical properties of protein kinase C

In general, the conformation of protein kinases consists of two regions which are connected by a region functioning as a hinge. The protein substrate fits into the groove between the two regions and interacts with a catalytic domain and cofactors interacting with the regulatory domain [33]. The specificity of protein kinases such as cyclic AMP dependent protein kinase, Ca^{2+}-calmodulin dependent protein kinase (CaM-PK) and protein kinase C for their substrate proteins is determined by both the primary sequence of these proteins around their phosphorylation site and the capacity of these sites to interact with the catalytic domain of the protein kinase. Generally, protein kinases are inactivated by the interaction between a pseudo substrate region in the protein kinase's primary sequence and the active site [33]. This restraint is removed during activation by changes in protein kinase conformation due to interaction of second messengers (e.g. cyclic AMP, 1,2-diacylglycerol (1,2-DAG) and Ca^{2+}) with the protein kinase's regulatory sites and by competition between the protein substrates and the pseudo substrate site, all present at the N-terminal region of the primary structure of the protein kinases (Fig. 1). Indeed, activation of most protein Serine/Threonine (Ser/Thr) kinases is preceded by receptor activation followed by synthesis or release of low-molecular-weight protein kinase effectors or second messengers.

Protein kinases can be divided in several types. Protein kinase A is a cyclic AMP dependent protein-Ser/Thr kinase, while protein kinase C is a group of protein-Ser/Thr kinases which are phosphatidylserine (PtdSer-), 1,2-DAG- and/or Ca^{2+}-dependent. Recently, protein kinase D was discovered in COS cells to be dependent on 1,2-DAG and phorbol esters, but information on this enzyme in myocardium is not yet available [35]. In myocardial cells protein kinase C regulates various processes, including myocardial contraction, ion transport, energy metabolism, gene expression and hypertrophic growth [25,36,37]. The role of protein kinase C in growth and proliferation has been implied by its identification as a high-affinity intracellular receptor for tumor-promoting phorbol esters which directly activate most protein kinase C isotypes in a relatively unspecific

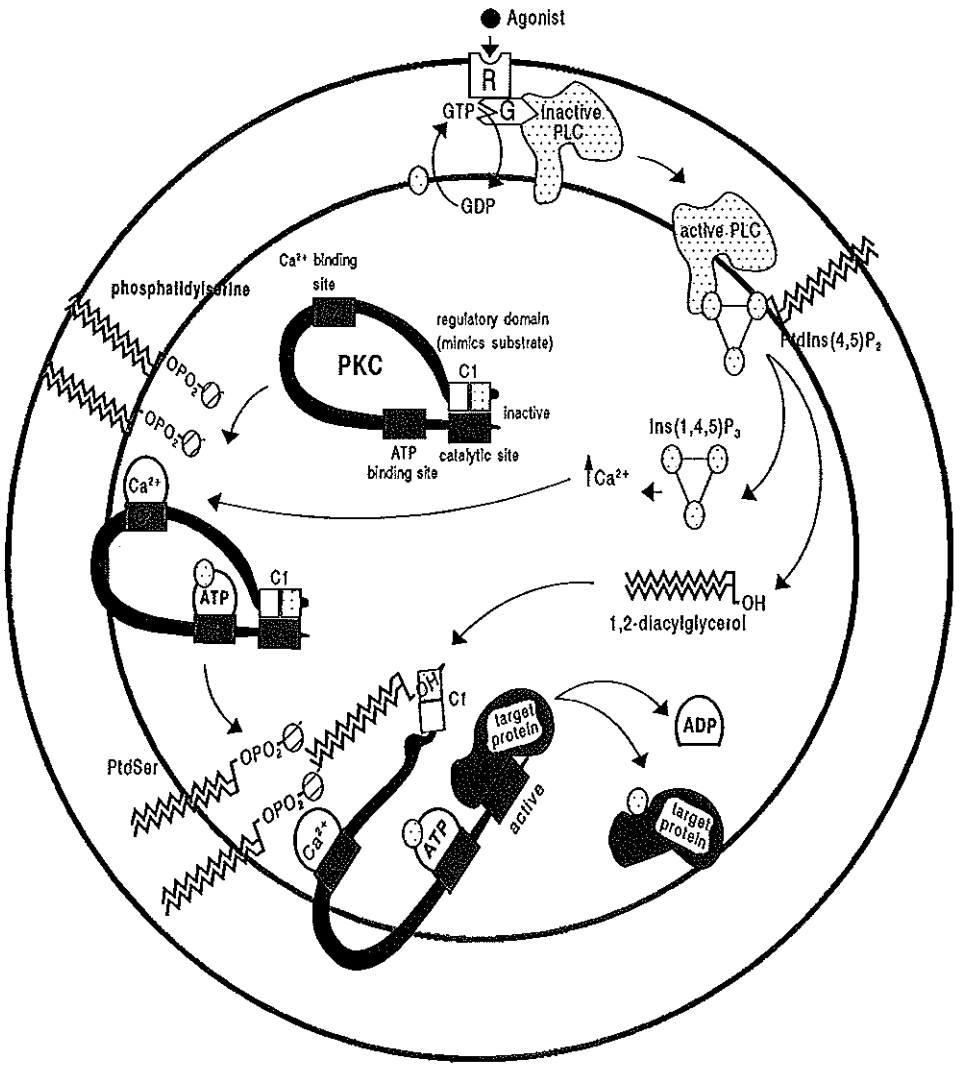
manner. Phosphorylation of target proteins by protein kinase C isotypes depends on their intracellular location at the time of action. This compartmentalization may be caused by the architecture and intracellular localization of anchor proteins e.g. the so-called receptors for activated C kinase (RACK) [38,39]. Therefore, after protein kinase C is activated it translocates to other cellular compartments such as the sarcolemma where it exerts its principal action (Fig. 1). However, several other compartments such as mitochondria, myofibrils, sarcoplasmic reticulum and the perinuclear zone also possess specific receptor sites for protein kinase C isotypes [40].

Protein kinase C isotypes

The protein kinase C family can be divided into three distinct subfamilies on basis of their structure and catalytic and regulatory properties (Fig. 1). Classical protein kinase C isotypes (cPKC's) such as protein kinase C-α, -β_1, -β_2 and -γ are activated by Ca^{2+}, PtdSer and 1,2-DAG or phorbol esters such as phorbol-12-myristate-13-acetate (PMA). Novel protein kinase C isotypes (nPKC's) such as protein kinase C-δ, -ε, -η, -θ and -μ are Ca^{2+} independent and only need PtdSer and 1,2-DAG (or PMA) to become activated. Atypical protein kinase C isotypes (aPKC's) are protein kinase C-ζ, -ι and -λ which are also Ca^{2+}independent and only require PtdSer to become activated (Fig. 1). At present, the still growing number of isotypes can be discriminated by immunoblot or immunohistofluorescence analysis. Most investigators use histone III-S as substrate and γ-^{32}P-labelled ATP as phosphate donor to measure protein kinase C activity, which reflects the activity of some of the isotypes present in the cellular fraction. Histone III-S is a poor substrate for some nPKC's (δ, ε and η) compared to the other isotypes (α, β_1, β_2, γ) [41]. Moreover, techniques such as hydroxylapatite high-pressure-liquid-chromatography can be used to determine the activity of protein kinase C isotypes after separation [42]. Measurement of the mRNA concentration using specific cDNA probes on Northern blotting can also be used for the detection of protein kinase C isotypes [43], but mRNA levels offer only limited information because these do not always reflect the functional activities of the isotypes.

In a preliminary study it was found that α, ß, ε and ζ are the most prominent isotypes in the rat heart [44]. Similar observations have been made in cultured neonatal rat cardiomyocytes [45] and adult rat cardiomyocytes [46]. However, in a more recent study in adult rat ventricular myocytes, protein kinase C-ε was abundantly present and protein kinase C-α could not be detected [47,48], while in another study protein kinase C- α, -δ, -ε, -η, -θ, -ζ, -λ and -ι were detected in adult rat heart [49]. In the canine heart the presence of protein kinase C-α, -β_1, -β_2, -γ, -δ, -ε, -ι, -θ and

Fig. 1. Isotypes of protein kinase C, the functional domains in their primary structure, and receptor-coupled phospholipase C-mediated signal transduction leading to protein kinase C activation. The various protein kinase C isotypes share some sequence homology and have all a common ATP-binding site (C3) and catalytic site (C4). Only protein kinase C-α, -β_1, -β_2 and -γ have a Ca^{2+} binding site (C2) and 1,2-diacylglycerol (1,2-DAG)-binding site (C1). In the inactivated state the isotypes of protein kinase C are folded so that an endogenous "pseudo substrate" region on the N-terminal part of the protein occupies the catalytic site (C-terminal part). When agonists (see text) bind to their specific receptors linked to phospholipase C in the cardiac sarcolemma, receptor activation is followed by phospholipase C catalyzed hydrolysis of PtdIns(4,5)P_2 to form inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3). Ins(1,4,5)P_3 releases Ca^{2+} from the Ins(1,4,5)P_3 receptor-sensitive Ca^{2+} storage sites in the cardiac sarcoplasmic reticulum. PtdIns(4,5)P_2 hydrolysis also forms 1,2-diacylglycerol (1,2-DAG), which increases the affinity of some isotypes for Ca^{2+}. When the intracellular free Ca^{2+} concentration increases, some isotypes become more tightly associated with membranes containing the negatively charged head groups of PtdSer, allowing 1,2-DAG to reach its binding site (C1) on the protein kinase C. The 1,2-DAG-protein kinase C complex approaches the membrane by placing the kinase in a pocket of negatively-charged head groups of PtdSer in which Ca^{2+} remains attracted. When this occurs, the conformation of protein kinase C changes, exposing the unoccupied catalytic site, thereby allowing the kinase to phosphorylate cellular proteins. There is also evidence that specific binding proteins (e.g. MARCKS and RACK) determine the cellular translocation process of protein kinase C isotypes. The membrane-bound protein kinase C-DAG-(Ca^{2+})-membrane complex only slowly dissociates and this property is the basis for the commonly used "translocation assay" for assessment of PKC activation. Figure adapted from Zeisel et al [34], with permission of the FASEB Journal.



PKC isotype	Domain structure
$\alpha, \beta_1, \beta_2, \gamma$	C1
$\delta, \epsilon, \eta, \theta, \mu$	C1
ζ, ι, λ	C1

-ζ has been described [50]. Thus, the species and the type of assays determine the pattern of protein kinase C isotypes. Furthermore, the affinity and the specificity of the antibodies used to detect the protein kinase C isotypes determine the abundancy of detection but not the absolute concentration of the isotype *in vivo*. In the pig, using rabbit polyclonal antibodies, we have demonstrated the presence of protein kinase C-α and -ε, while the isotypes -δ and -ζ were barely detectable. Other isotypes were not studied [51]. Myocardial homogenates do not only contain homogenized myocytes but also homogenized fibroblasts, smooth muscle cells and endothelial cells. Consequently, the protein kinase C isotypes of these cells in these homogenates are measured all together. So far, in only one study immunohistochemistry was used to detect the isotypes in situ [48]. In that study it was concluded that protein kinase C-δ is probably the most important isoform involved in preconditioning in the rat heart [48].

Protein kinase C in coupling phospholipase C- to phospholipase D-activation

Endogenous ligands such as adenosine A_1 -, α_1-adrenergic- and muscarinic agonists, bradykinin, angiotensin II, endothelin-1 or opiates stimulate, via the receptor-G-protein coupled to phospholipase C, the intracellular signalling pathway. [25,48,52-56]. Phospholipase C catalyzes hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns $(4,5)P_2$) which leads to formation of the second messengers inositol-1,4,5-trisphosphate (Ins$(1,4,5)P_3$) and 1,2-DAG (Fig. 1) [25]. These messengers, directly or indirectly, activate Ca^{2+}-independent and/or Ca^{2+}-dependent protein kinase C isotypes and calmodulin-dependent protein kinase (CaM-PK). The activated protein kinase C isotypes and/or CaM-PK phosphorylate specific target proteins which may be responsible for CPP. Phospholipase D, another enzyme of which a stimulation leads to 1,2-DAG formation, can be strongly stimulated by phorbol esters [36,45,51,57,58]. It uses phosphatidylcholine (PtdChol) as substrate and its activation leads initially to formation of phosphatidic acid (PtdOH) and choline [59]. PtdOH is rapidly hydrolysed to 1,2-DAG and inorganic phosphate (P_i) by PtdOH hydrolase. The 1,2-DAG formed by phospholipase D potentially contributes to activation of protein kinase C isotypes [51]. Stimuli such as noradrenaline, angiotensin II and endothelin-1 stimulate both phospholipase C and phospholipase D [45,58,60]. In fact, protein kinase C has been proposed to function as a switch which reduces the rate of PtdIns$(4,5)P_2$ hydrolysis catalyzed by phospholipase C and stimulates the rate of PtdChol hydrolysis catalyzed by phospholipase D [57,58,59]. Through this "cross talk" mechanism between phospholipase C and D, the cardiomyocytes may be continuously supplied with 1,2-DAG after receptor stimulation, because the cellular concentration of PtdChol is about 100 times higher than that of PtdIns$(4,5)P_2$.The continuous production of 1,2-DAG could be of importance for maintenance of activation of the protein kinase C isotypes involved in ischemic preconditioning.

Evidence for a role of protein kinase C in ischemic preconditioning

Inhibition of protein kinase C (Table 1)

Selective inhibition of protein kinase C activation by administration of inhibitors prior to or after applying the cardioprotective stimulus is one approach to investigate the role of protein kinase C in ischemic preconditioning. Depending on the inhibitor, protein kinase C function can be blocked at its catalytic or regulatory sites (Fig. 1). It is also possible to downregulate protein kinase C activity by prolonged (1-2 days) stimulation with phorbol ester [61]. This last approach has not been used in ischemic preconditioning, but is of interest in view of the development of tolerance to ischemic preconditioning when a very large number of brief occlusion-reperfusion sequences are applied[62].

The most prominent drugs used to inhibit protein kinase activation or translocation are staurosporine [17,48,52,63], 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) [27,64], chelerythrine [53,65-67], calphostin-C [68], polymyxin B [17,52,55,56,64,66], bisindolylmaleimide [28,69] and colchicine [63]. Staurosporine, H-7, and polymyxin B are non-specific inhibitors of protein kinase C compared to cyclic AMP-dependent protein kinase, CaM-PK or protein-Tyr kinases [70]. All

non-selective inhibitors act on the catalytic domain of protein kinase C, which contains a high degree of sequence homology with other protein kinases. The more specific inhibitor calphostin-C [71] acts on the regulatory domain (Fig. 1). Chelerythrine, another specific inhibitor [67] interacts with the catalytic domain but also competes with the classically used protein substrates of protein kinase C [57]. Furthermore, polymyxin B directly blocks K^+_{ATP} channels, one of the possible target proteins of protein kinase C and is therefore inappropriate to investigate the role of protein kinase C in ischemic preconditioning [72,73]. Moreover, it is unknown whether protein kinase C inhibitors are equipotent for all enzyme isotypes. It is quite feasible that the degree of inhibition depends on both the isotype [60,74-76] and species. Table 1 shows that polymyxin B [66], staurosporine [48] and chelerythrine [48] abolished the protective effect in isolated rat hearts and calphostin-C [68] and chelerythrine [53] in the *in vivo* rat model. Polymyxin B [17,52,55,56] and staurosporine [17] abolished protection in *in vitro* and polymyxin B [52], staurosporine [52,63], chelerythrine [67] and colchicine [63] in *in vivo* rabbit model. In dogs, the data are different as Przyklenk et al [64] observed that polymyxin B and H-7 did not abolish cardioprotection by ischemic preconditioning. In a preliminary study in pigs, staurosporine and bisindolylmaleimide limited infarct size [69]. These studies suggest a different role for protein kinase C in rats and rabbits than in dogs and pigs. The different results in rat, rabbit and pig could be related to species-dependent differences in myocardial expression, in the task performed and the site of translocation of the various isotypes [38,39,48,64,77]. Moreover, the various isozymes might be activated differently by Ca^{2+}, 1,2-DAG and free fatty acids [24,25,36,37].

Reviewing the studies using pharmacological blockade one is tempted to conclude that the protein kinase C family is involved in the mechanism of ischemic preconditioning in rat and rabbit. However, none of the studies investigated whether blockade of protein kinase C actually occurred in the *in vivo* model. *In vitro* assays of enzyme activity will not provide conclusive answers also because these have to be performed in subcellular fractions in the absence of the inhibitor due to the isolation procedure and in the presence of optimal amounts of 1,2-DAG and/or Ca^{2+}. The ideal experimental design would be to study the enzyme activity *in vivo* by measuring phosphorylation degrees of one or more of the unknown specific target proteins.

Activation of protein kinase C (Table 2)

Activation of protein kinase C by administration of phorbol esters, such as 12-O-tetradecanoyl phorbol-13-acetate (TPA) and PMA [27,66] or 1,2-DAG analogues such as 1-stearoyl-2-arachidonoyl glycerol (SAG) [48], 1,2-dioctanoyl *sn*-glycerol (DOG) [65] and oleyl acetyl-glycerol (OAG) [64] prior to a sustained coronary artery occlusion has been a second approach to investigate the role of protein kinase C in ischemic preconditioning. The advantage of using phorbol esters over 1,2-DAG as activating substances is that they are not metabolized and produce prolonged protein kinase C activation. Protein kinase C translocation takes place after phorbol ester (or the 1,2-DAG analog) is bound to the enzyme's regulatory domain whereby it obtains not only an increased affinity for acidic membrane phospholipids (PtdSer) but also an increased activity (Fig. 1).

PMA and several 1,2-DAG analogs mimic preconditioning in the rat [53] and rabbit [52,63], but PMA failed to limit infarct size in pigs [69]. Przyklenk et al [64] measured protein kinase C translocation after administration of PMA in dogs. Although these studies lack information regarding activation (translocation) of the isotype(s) in relation to the protective effect, the results with activators suggest a role for protein kinase C in ischemic preconditioning in rat and rabbit. Furthermore, the route of administration and the dose used may be different for the large animal studies and the *in vitro* and *vivo* studies of small animals. For instance, Vogt et al [69] used intramyocardial administration of PMA (1 µM) to activate protein kinase C but failed to mimic the protective effect of ischemic preconditioning. However, the dose could have been too high because Cohen et al [78] found that 0.2 nM PMA was protective, while 2 nM PMA was damaging in the isolated rabbit heart. It is feasible that in that study the low dose of PMA activated an isotype that is protective, while at the higher dose an isotype was activated that was damaging [78].

Table 1: Protein kinase C inhibitors and protection by ischemic preconditioning.

Model	Species	CP-Stimulus x(I+R) (min)	Prolonged-Stimulus I(R) (min)	Protein kinase C inhibitor	Evaluation (ISL/FR)	Result	Reference
Isolated heart	Rat	3x(5I+5R)	30I(120R)	Polymyxin B, before CP-stim	ISL	Abolishes	[66]
		3x(5I+5R)	30I(120R)	Chelerythrine, before CP-stim	ISL	Abolishes	
		1x(2I+10R)	20I(40R)	Staurosporine, before CP-stim	FR	Abolishes	[48]
		Phenylephrine+10R	20I(40R)	Staurosporine, before CP-stim	FR	Abolishes	
		1x(2I+10R)	20I(40R)	Chelerythrine, before CP-stim	FR	Abolishes	
		Phenylephrine+10R	20I(40R)	Chelerythrine, before CP-stim	FR	Abolishes	
	Rabbit	1x(5I+10R)	30I(120R)	Staurosporine, before and after CP-stim	ISL	Failed to abolish	[17]
		1x(5I+10R)	30I(120R)	Staurosporine, after CP-stim	ISL	Abolishes	
		Bradykinin	30I(120R)	Staurosporine, after CP-stim	ISL	Abolishes	
		Bradykinin	30I(120R)	Polymyxin B, 50 min starting 5 min before CP-stim	ISL	Abolishes	
		1x(5I+10R)	30I(180R)	Polymyxin B, 5 min after CP-stim	ISL	Abolishes	[52]
		Phenylephrine	30I(120R)	Polymyxin B, before and after CP-stim	ISL	Abolishes	[55]
		Angiotensin II	30I(120R)	Polymyxin B, 50 min starting 5min before CP-stim	ISL	Abolishes	[56]
In Vivo	Rat	1x(5I+10R)	45I(150R)	Chelerythrine, after CP-stim	ISL	Abolishes	[53]
		3x(3I+5R)	90I(240R)	Calphostin C, before and after CP-stim	ISL	Abolishes	[68]
	Rabbit	1x(5I+10R)	30I(180R)	Staurosporine, 5 min after CP-stim	ISL	Abolishes	[52]
		1x(5I+10R)	30I(180R)	Polymyxin B, 5 min after CP-stim	ISL	Abolishes	
		1x(5I+10R)	30I(180R)	Chelerythrine, 8 min after CP-stim	ISL	Abolishes	[67]
		1x(5I+10R)	30I(180R)	Staurosporine, before CP-stim	ISL	Attenuates	[63]
		1x(5I+10R)	30I(180R)	Colchicine, 30 min before CP-stim	ISL	Abolishes	

Table 1: Continuation

Model	Species	CP-Stimulus x(I+R) (min)	Prolonged-Stimulus I(R) (min)	Protein kinase C inhibitor	Evaluation (ISL/FR)	Result	Reference
In Vivo	Dog	4x(5I+10R)	60I(240R)	H-7 (IV), before, during and after CP-stim	ISL	Failed to abolish	[64]
		4x(5I+10R)	60I(240R)	H-7 (IC), before, during and after CP-stim	ISL	Failed to abolish	
		4x(5I+10R)	60I(240R)	Polymyxin B, before, during and after CP-stim	ISL	Failed to abolish	
		4x(5I+5R)	90I(360R)	Polymyxin B, 5 min before and during CP-stim	ISL	Abolishes	[28]
		4x(5I+5R)	90I(360R)	GF109203X, 5 min before and during CP-stim	ISL	Abolishes	
		Methoxamine	90I(360R)	Polymyxin B, 5 min before and during CP-stim	ISL	Abolishes	
		Methoxamine	90I(360R)	GF109203X, 5 min before and during CP-stim	ISL	Abolishes	
	Pig	2x(10I+30R)	60I(120R)	Staurosporine	ISL	Failed to abolish	[69]
		BIS	60I(120R)	Bisindolylmaleimide	ISL	Mimics	

I=ischemia; R=reperfusion; CP=cardioprotective; BIS=bisindolylmaleimide; H-7=1-(5-isoquinolinesulfonyl)-2-methylpiperazine; ISL=infarct size limitation; FR=functional recovery.

Measurements of protein kinase C-translocation (activation) (Table 3)

Translocation of protein kinase C from the cytosol to the membrane has been investigated employing: *(1)* immunoblot analysis using protein kinase C isotype-specific antibodies of SDS-electrophoretically separated subcellular fractions isolated from myocardial homogenates [44,51,69]; *(2)* immunohistofluorescence detection (with confocal microscopy) of protein kinase C isotypes in sections of myocardial tissue [48,64]; *(3)* assay of total protein kinase C activity in subcellular fractions isolated from myocardial homogenates by measurement of Ca^{2+}- and/or 1,2-DAG-dependent ^{32}P incorporation from γ-^{32}P-labelled ATP into histone III-S or a protein kinase C isoenzyme-specific substrate protein such as peptide-ε [51,54,64,79] or other peptides [28,64]. All three methods have their limitations. For instance, in protein phosphorylation or immunoreactivity assays, cardiac biopsies are usually rapidly frozen in liquid N_2 followed by preparation of particulate fractions from the homogenates. It can not be excluded, however, that redistribution or (in)activation occurs during isolation of the subcellular fractions. In the subfractions both the basal rate and the maximum rate of histone III-S (or other substrate protein) phosphorylation are measured in the presence of Ca^{2+}, PtdSer and 1,2-DAG. The results of these measurements only reflect the total protein kinase C activities in the subcellular fractions. Moreover, histone III-S is a relatively poor substrate for some nPKC's (δ, ε and η) compared to the cPKC's [41]. Therefore, measurements of the rate of ^{32}P incorporation into the synthetic protein kinase C-ε-specific substrate peptide-ε may provide the required information about the ε isotype [51]. It should also be noted that mixed micelles of Ca^{2+}-1,2-DAG-PtdSer embedded in Triton-X-100 micelles, used to activate protein kinase C in the ^{32}P incorporation assays, only mimic the cellular membrane environment of protein kinase C in the intact cell. It is unknown whether myocardium is preconditioned homogenously or heterogeneously. In the latter case, the sampling site of the biopsy may pose a restriction. Because protein kinase C assayed in subcellular fractions isolated from homogenates of whole myocardial tissue represents a mixture of activities of myocytes, fibroblasts, smooth muscle cells and endothelial cells, immunohistofluorescence measurements must therefore be performed to provide information on the cell type involved in protein kinase C translocation/activation. Measurements of protein kinase C isotype activity by immunohistofluorescence must, however, be interpreted with caution, because the specific antibodies are not always capable to distinguish active from inactive protein kinase C isotypes.

Weinbrenner et al, using Western blotting, showed in rats a rapid translocation of the Ca^{2+}-dependent protein kinase C isotype α and the Ca^{2+}-independent isotypes (δ, ε and ζ) to the sarcolemma after brief ischemia and increased expression of the Ca^{2+}-independent protein kinases C-δ and -ε in the cytosol after prolonged ischemia [24]. Mitchell et al, using immunohistofluorescence, showed in rat hearts that protein kinase C-δ translocated from the cytosol to the sarcolemma after both brief ischemia and α_1-adrenergic stimulation [48]. Brief ischemia also caused translocation of protein kinase C-ε from the cytosol to the nuclear region. Measurements of other protein kinase C-isotypes (α, β_1, ζ and η) did not provide evidence for occurrence of translocation after brief ischemia or α_1-adrenergic stimulation [48]. These results provide the first evidence that (at least in the rat) specific protein kinase C isotypes are involved in ischemic preconditioning.

Przyklenk et al used a probe consisting of the protein kinase C inhibitor bisindolylmaleimide conjugated to fluorescein that selectively binds to active protein kinase C and observed no difference in the total amount and the cellular distribution of protein kinase C fluorescence with preconditioning in dogs [64]. The advantage of this method over immunofluorescence is that it distinguishes between active and inactive protein kinase C. In their study Przyklenk et al also obtained quantitative information on the changes in the amount and subcellular distribution of protein kinase C by measuring the rate of ^{32}P incorporation into the threonine group of a protein kinase C-specific peptide, which was not further specified [64]. A small rise in protein kinase C activity was found in the membrane fraction isolated from biopsies obtained after 10 min of ischemia compared to those isolated after four sequences of 5 min occlusion-reperfusion or no intervention [64]. However, no difference in protein kinase C activity between matched groups of controls and 'ischemic preconditioned'

Table 2: Protein kinase C activators and myocardial infarct size.

Model	Species	CP-Stimulus	Prolonged-Stimulus I(R) (min)	Evaluation (ISL/FR)	Result	Reference
Isolated heart	Rat	SAG	20I(40R)	FR	Mimics	[48]
	Rabbit	PMA	30I(180R)	ISL	Mimics	[52]
		OAG	30I(180R)	ISL	Mimics	
		PMA	30I(120R)	ISL	Mimics	[63]
In Vivo	Rat	DOG	45I(150R)	ISL	Mimics	[53]
	Rabbit	PMA	30I(180R)	ISL	Mimics	[63]
	Pig	PMA	None	ISL	Failed to mimic	[69]

I=ischemia; R=reperfusion; CP=cardioprotective; SAG=1-stearoyl-2-arachidonoyl glycerol; PMA=phorbol-12-myristate-13-acetate; OAG=oleyl acetyl-glycerol; DOG=1,2-dioctanoyl *sn*-glycerol; ISL=infarct size limitation; FR=functional recovery.

Table 3: Protein kinase C translocation/activation and protection by ischemic preconditioning.

Model	Species	CP-Stimulus x(I+R) (min)	Prolonged-Stimulus I(R) (min)	PKC assay	Result	Refe-rence
Isolated heart	Rat	1x(2I)	none	Immunohistofluorescence	PKC-δ to sarcolemma, ε to nucleus, no ζ, α, β_1	[48]
		Phenylephrine	none	Immunohistofluorescence	PKC-δ to sarcolemma, ζ to nucleus, no ε, α, β_1, η	
In Vivo	Dog	4x(5I+10R)	None	Fluorescent to binding by confocal microscopy	No PKC translocation	[64]
		4x(5I+10R)	None	Activity by protein phosphorylation	No PKC activation	
		None	10I	Activity by protein phosphorylation	PKC activation vs Con	
		4x(5I+10R)	10I	Activity by protein phosphorylation	PKC activation vs Con	
		PMA	None	Activity by protein phosphorylation	PKC activation	
	Dog	4x(5I+5R)	None	Activity by protein phosphorylation	PKC activation	[28]
	Pig	1x(10I+7.5R)	None	Immunoreactivity on Western blot	PKC-ε translocation	[58]
		1x(10I+7.5R)	None	Activity by protein phosphorylation	PKC-ε translocation	
	Pig	2x(10+30)	None	Activity by protein phosphorylation	PKC activation	[69]
		PMA	None	Activity by protein phosphorylation	PKC activation	

I=ischemia; R=reperfusion; CP=cardioprotective; PMA=phorbol-12-myristate-13-acetate; PKC= protein kinase C

dogs could be measured at time points comparable to the onset of the long occlusion or at 10 min into sustained ischemia [64]. Using the same protein kinase C analysis, Vogt et al found a modest (10 to 20%) redistribution of protein kinase C from the cytosol to the membrane fraction in pig hearts subjected to 10 min of ischemia [69]. In contrast to the studies by Przyklenk et al [64], Kitakaze et al [28] observed in the same canine model a marked translocation of Ca^{2+}- and lipid-dependent protein kinase C activity in cytosol and membrane fractions isolated from preconditioned epi- as well as endomyocardium. These authors ascribed their positive findings to the time of measurements (5 min after the preconditioning stimulus against 10 min by Przyklenk et al [64]).

We studied translocation of protein kinase C enzyme activity by ^{32}P incorporation into histone III-S and ε-peptide and immunoreactivity of a number of protein kinase C isoforms (α, ε, δ and ζ) of cytosolic and membrane fractions isolated from biopsies of porcine myocardium preconditioned by a 10-minute coronary artery occlusion and 7.5-minutes of reperfusion [51,80]. Ca^{2+} and 1,2-DAG-stimulated protein kinase C activity with histone III-S as substrate was higher in the cytosolic and particulate fractions isolated from the preconditioned myocardium than from the control region. Significant Ca^{2+}-independent, 1,2-DAG-stimulated phosphorylation of ε-peptide was found in the cytosolic fractions, but not in the particulate fractions. However, no significant increase of 1,2-DAG-stimulated phosphorylation of ε-peptide in the cytosolic fraction from the preconditioned myocardium was observed. These were rather unexpected findings in view of our protein kinase C-ε immunoreactivity measurements (see below). The cytosolic and particulate fractions were also examined by immunoblot analysis using rabbit polyclonal antibodies specific for protein kinase C-α, -δ, -ε and -ζ isotypes [51]. This analysis revealed significant levels of expression of the Ca^{2+}-independent isotype protein kinase C-ε, the abundant presence of protein kinase C-α, while protein kinase C-δ and -ζ were barely undetectable. The immunoreactivity data also indicate that neither the (Ca^{2+} and 1,2-DAG)-stimulated histone III-S and peptide-ε kinase activities of the cytosolic nor those of the particulate fractions reflect the relative immunoreactivities in the corresponding fractions. In contrast to the ^{32}P-incorporation data, the immunofluorescence data suggested that the total amount and subcellular distribution of protein kinase C-α and -ε was not altered in the preconditioned region compared to the non-ischemic region of the left circumflex coronary artery. Therefore, by using immunofluorescence we were unable to detect the occurrence of ischemia-induced expression of protein kinase C or ischemia-induced translocation of protein kinase C from the cytosolic to the particulate fraction of the protein kinase C isotype -α, -δ, -ε or ζ. On the other hand, binding proteins, such as myristoylated-alanine-rich-C-kinase-substrate (MARCKS) and RACK may determine whether activated protein kinase C isotypes translocate and are providing another mechanism for functional specificity to specific intracellular locations. Thus, in the *in vitro* phosphorylation assays of the subcellular fractions, different amounts of MARCKS- or RACK-bound protein kinase C isotypes can alter the protein kinase C activity measured [81]. This could cause the discrepancy between the activity assays and Western blotting. Nevertheless, our results on ^{32}P incorporation demonstrate an increase in cytosolic and membrane-bound protein kinase C activities due to brief ischemia and supports a role for protein kinase C in ischemic preconditioning in pigs [51].

CONCLUDING REMARKS

At present MARCKS is the only endogenous target protein for protein kinase C, that has been shown to be phosphorylated in preconditioned rabbit myocardium. However, the former is believed to be an intracellular location site rather than a protein factor intimately involved in the protective response [82]. Irrespective of the target protein(s) we are dealing with, its (their) covalently bound phosphates must be relatively stable during the 2 to 3 hours in which the cardioprotection is present (CPP). Furthermore, the precise time point that protein kinase C is maximally translocated (activated)

during ischemia or reperfusion (preconditioning stimulus) is unknown and consequently also the time point at which the enzyme reaches the target proteins for catalyzing their phosphorylation. It is quite feasible that protein kinase C is removed from its translocation site or proteolytically degraded after it has performed its action and thereafter it is not longer detectable by immunoreactivity or activity measurements. It is therefore mandatory to determine the time course of translocation/activation and subsequent relocalization/ inactivation or proteolytic degradation of the protein kinase C isotype and the time course(s) of phosphorylation and dephosphorylation or proteolytic degradation of the target protein(s). Because the time course of weaning of the protective effect of the CPP is roughly known, the time course of dephosphorylation/inactivation of the target protein could be correlated to the time course of weaning of protection. Candidate target proteins of protein kinase C involved in the CPP are e.g. the K^+_{ATP} channel [13,14] and/or the ecto-5'-nucleotidase [28], but experimental evidence for phosphate incorporation into these proteins or regulating proteins is lacking. If the K^+_{ATP} channel or the ecto-5'-nucleotides are target proteins the most likely translocation site for the protein kinase C isotype(s) involved in the CPP is the sarcolemma.

Protein kinase C is involved in the agonist-receptor interaction induced changes in gene expression of many cells [25,36,37,74,75,41,83]. Taking into account the time required for inducing heat shock/stress proteins [29,30] the former may only play a role in SWOP. Therefore, a transcription factor involved in the regulation of expression of heat shock/stress proteins could be another potential target protein of protein kinase C. If true, the nucleus may be the translocation site for the protein kinase C isotype(s).

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Chapter 7

EXPLORATION OF THE POSSIBLE ROLES OF PHOSPHOLIPASE D AND PROTEIN KINASE C IN THE MECHANISM OF ISCHEMIC PRECONDITIONING IN THE MYOCARDIUM

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INTRODUCTION

Brief periods of acute myocardial ischemia protect the heart against subsequent episodes of prolonged ischemia[1]. This endogenous phenomenon, first described by Murry *et al.*,[2] is known as ischemic preconditioning and has been demonstrated in a wide variety of species such as the rat [3], dog [4], rabbit [5] and pig [6,7]. For a number of reasons, the occurrence of ischemic preconditioning in the clinical setting has not yet been demonstrated convincingly, but evidence is accumulating that it occurs in situations such as angina preceding an acute myocardial infarction and percutaneous transluminal coronary angioplasty [8-10]. In addition to limiting infarct size caused by prolonged ischemia [3-7], preconditioning improves the recovery of function after reperfusion and reduces ventricular arrhythmias during ischemia and/or after reperfusion [3,11,12]. The protective state afforded by ischemic preconditioning lasts for about 2 hours [13,14]. In addition to brief episodes of total coronary occlusion, several other stimuli can mimic ischemic preconditioning as recently reviewed by Kloner *et al.* [15]. Partial coronary artery stenosis without reperfusion, hypoxia, stretch, catecholamines, rapid pacing and certain pharmacological interventions are such preconditioning-like stimuli [7,10,15].

Extensive studies have been undertaken to elucidate the mechanism(s) by which transient ischemia protects the myocardium. Liu *et al.* [16] took the first important step towards revealing the possible mechanism(s) of ischemic preconditioning, by describing the role of adenosine in mediating the phenomenon. Since then, results obtained by experiments with specific agonists and antagonists have shown that activation of K^+_{ATP} channels [17], α_1-adrenergic [18,19] and muscarinic [20,21] agonists, bradykinin [22,23], angiotensin II [24], A_1 adenosine agonists [16,25,26] and protein kinase C (PKC) activators [19,27-29] might be other endogenous mediators of preconditioning. At present, a unifying hypothesis for preconditioning proposes that these endogenous ligands such as A_1 adenosine-, α_1-adrenergic- and muscarinic agonists, bradykinin and angiotensin II initiate an intracellular pathway by acting via G-protein-coupled receptors, which leads to activation of phospholipase C-ß [15,19,24,27-30]. Subsequently, modulation of the latter enzyme changes levels of the second messengers inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and 1,2-diacylglycerol (1,2-DAG) [30]. The mechanism by which these messengers act is by direct or indirect activation of the Ca^{2+}-dependent and/or Ca^{2+}-independent PKC isoenzymes and Ca^{2+}-calmodulin-dependent protein kinase (CaM-PK). Activated PKC isoforms and/or CaM-PK then phosphorylate specific proteins that ultimately lead to the cardioprotective effect. The rate of dephosphorylation of these specific phosphoproteins by phosphoprotein phosphatases likely determines the period in which the protective state is maintained. A recent report describes that in superfused human right atrial trabeculae undergoing simulated ischemia the mechanism(s) of preconditioning may indeed act via PKC and rely on the activation of the K^+_{ATP} channel as the final effector [31]. This supports the evidence, obtained by whole cell patch-clamp or whole cell voltage-clamp of isolated rabbit ventricular myocytes, that the ATP-sensitive K^+ current is activated by a PKC-mediated phosphorylation. The potentiation of the K^+_{ATP} current by PKC provides an explicit basis for current paradigms of ischemic preconditioning [32,33].

In this report we will first briefly review the evidence derived from experiments using specific activators and blockers and enzymatic measurements that PKC plays a key role in the intracellular mechanism of preconditioning. The latter may be valid for all species despite the variability of the endogenous extracellular triggers. We will also present evidence derived from studies using an *in situ* porcine model that supports the PKC hypothesis for preconditioning. Until now the possible phospholipid sources of the PKC activator 1,2-DAG during preconditioning have been scarcely explored. For several of the postulated endogenous mediators of cardioprotection such as α_1-adrenergic-, A_1 adenosine and muscarinic agonists and angiotensin II it has been shown that in myocardium these stimuli activate not only phospholipase C-ß but also phospholipase D (PLD) [34-39]. Furthermore, Moraru *et al.* [40] provided direct evidence of membrane PLD stimulation induced by reversible ischemia in the isolated perfused rat heart. Considerable emphasis has

been put on the fact that administration of the potent PKC activator phorbol myristate acetate (PMA), mimics preconditioning-like protection [15,19,27-29,33]. This finding may indicate the involvement of PLD in ischemic preconditioning.

Translocation of PKC: a possible key event during ischemic preconditioning

Most studies assessing the role of PKC in ischemic preconditioning have used an indirect pharmacological approach. For instance, several groups of investigators attempted to block the cardioprotective effect of a brief antecedent ischemia episode by administration of PKC inhibitors such as polymyxin B [19,23,24,27,41,42], staurosporine [23,27,29,43], 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) [33,41], chelerythrine [28,31,42,44], and calphostin-C [45,46] before the onset of the prolonged period of ischemia. However, H-7, staurosporine and polymyxin B are not very selective for PKC compared with cyclic AMP-dependent protein kinase, CaM-PK or tyrosine kinase [47]. In contrast, chelerythrine [48] and calphostin C [49] are more specific inhibitors of PKC with high potencies. For instance, calphostin C acts on the regulatory domain of PKC, which is distinct from other protein kinases and calphostin C therefore induces a more specific inhibition than other PKC inhibitors. PKC inhibitors, such as staurosporine and H-7, acting on the catalytic domain of PKC, carry a high degree of sequence homology with other protein kinases and thus lack a high specificity for PKC. In addition, it is unknown whether these inhibitors effectively block all PKC isoenzymes. Molecular cloning techniques have revealed that PKC exists in at least 10 isoforms with distinct substrate proteins [50-53]. These isoforms may have different sensitivities towards the PKC inhibitors. In this respect it is important to note that Przyklenk *et al.* [41] found that neither H-7 nor polymyxin B attenuated preconditioning in an *in situ* canine model, which contrasts the results of the numerous reports [19,23,24,27-29,42,44-46] in which it was shown that PKC inhibitors abolished the protection by ischemic preconditioning. In contrast, the PKC inhibitors H-7 and polymyxin B produced the opposite effect: they further limited infarct size, suggesting inhibition of PKC protected the canine heart during sustained ischemia [41]. Differences in PKC isoform expression may provide an explanation for the disparity among dog, rabbit, rat and pig models. Myocardial expression of PKC isoforms is still poorly characterized, and it is quite feasible that not all PKC isoforms are expressed in the different species. For example, the Ca^{2+}-dependent PKC-α is the most abundant form of PKC in bovine heart [54], whereas in adult rat ventricular myocytes, the Ca^{2+}-independent isoform PKC-ε was abundantly present and PKC-α could not be detected [29,55]. These differences in isoform expression may be important because (*i*) the isoforms differ in their extent of activation by Ca^{2+}, 1,2-DAG and phorbolesters, (*ii*) distinct isoforms of PKC are thought to translocate to distinct intracellular sites (e.g., sarcolemma, sarcoplasmatic reticulum, myofibrils and the perinuclear region) and phosphorylate distinct proteins [41,56] and (*iii*) different isoforms of PKC may mediate different cellular functions.

The second approach used to evaluate the role of PKC in preconditioning is to activate PKC by phorbolester (e.g., PMA) [33,42], or other 1,2-DAG analogs such as oleyl acetylglycerol (OAG) [41], 1,2-dioctanoyl *sn*-glycerol (DOG) [31] and 1-stearoyl-2-arachidonoyl glycerol (SAG) [29]. Unlike 1,2-DAG, which is rapidly degraded, phorbolesters are not metabolized and are associated with sustained PKC activation and pathological changes [29]. Occupancy of the regulatory site of PKC by 1,2-DAG analogs induces intracellular PKC translocation. This partition mechanism is characterized by increased affinity of PKC for acidic membrane phospholipids, such as phosphatidylserine and increased activation. Thus, regulator-induced translocation and activation of PKC may be inseparable events, since occupation of the regulatory domain in PKC removes an N-terminal inhibitory (pseudosubstrate) sequence from its close juxtaposition to the catalytic site [29]. Binding of 1,2-DAG to the conventional Ca^{2+}-dependent cPKC's (PKC-α, -ß and -γ) increases their affinity for Ca^{2+} [50-53]. Recently, the nPKC's (PKC-δ, -ε, -η, and -θ), which are directly activated by 1,2-DAG without the need of Ca^{2+}, were discovered [50-53]. Several studies have demonstrated that activation of PKC by PMA or other 1,2-DAG analogs mimics preconditioning [28,29,31]. Such findings suggest that the mechanism of preconditioning in the rat and rabbit involves activation

of PKC.

A third strategy to examine the role of PKC in preconditioning is by directly measuring the translocation of PKC in response to repeated brief ischemia. The translocation from cytosolic to organellar compartments is believed to be a marker for activation of PKC. Translocation can be measured by direct visualization of PKC by (*i*) immunofluorescence staining of dissected myocardium, (*ii*) quantification of the distribution of PKC activity in subcellular fractions (isolated from myocardial homogenates) detected by measurement of Ca^{2+}- and/or 1,2-DAG-dependent ^{32}P incorporation from γ-^{32}P-labelled ATP into histone III-S or PKC isoenzyme-specific substrate peptide and (*iii*) immunoblot analysis using PKC isoenzyme-specific antibodies tested on subcellular fractions isolated from myocardial homogenates. At present, in only a few studies has one of these last approaches been used to investigate the role of PKC in ischemic preconditioning. Weinbrenner *et al.* [57] showed that α, ß, ϵ and ζ are the prominent isoforms of PKC in the rat heart which, except for the ß-form, is similar to what has been observed in cultured neonatal [38] and adult rat cardiomyocytes [58]. Weinbrenner *et al.* [57] also showed that a brief period of ischemia, caused a rapid translocation of the Ca^{2+}-independent subtypes (δ, ϵ and ζ) and the Ca^{2+} -dependent subtype α to the membrane. Prolonged ischemia led to the induction of Ca^{2+}-independent forms of PKC-δ and -ϵ in the cytosol. Immunohistofluore-scence studies of Mitchell *et al.* [29] showed translocation of the PKC-δ isoform to the sarcolemma following transient ischemia or α_1-adrenergic stimulation. In addition, transient ischemia resulted in PKC-ϵ translocation to the nucleus [29]. These results demonstrate activation of PKC with both transient ischemia and α_1-adrenergic agonist stimulation and suggest that the PKC-δ and -ϵ isoforms may be involved in ischemic preconditioning in the rat.

Immunohistofluorescence detection does not distinguish between active and inactive PKC. Przyklenk *et al.* [41] used a probe that binds to activated PKC (i.e., a bisindolylmaleimide PKC inhibitor yielded fluorescent through conjugation to fluorescein) and could not show a difference in the amount or distribution of PKC fluorescence before and after preconditioning in the canine model by using this new and sensitive technique [41]. To obtain quantitative information regarding the amount and subcellular distribution of PKC in control versus preconditioned myocardium, additional experiments were performed in which the incorporation of ^{32}P from γ-^{32}P-labelled ATP into the threonine group of PKC-specific peptide was measured [41]. Subtle, but significant increases in PKC activity in the particulate fraction in all dogs after 10 min of sustained occlusion compared with those sacrificed immediately after 5 min episodes of preconditioning ischemia or no intervention appeared [41]. However, there was no trend towards a difference in the amount of distributed PKC between control and preconditioned groups at any time point [41]. Using the same method of PKC analysis Vogt *et al.* [59] described a modest 10 to 20% redistribution of PKC from the cytosol to the particulate fraction in *in vivo* pig hearts subjected to 10 min of sustained coronary occlusion [59].

Does PKC play a role in ischemic preconditioning in porcine myocardium?

Although there is now considerable evidence that PKC activation may be involved in the mechanism leading to ischemic preconditioning, its role is not yet entirely clear as there is also conflicting evidence that inhibition of PKC may be beneficial in pig [59]. Using an *in situ* porcine model, we tested the hypothesis that translocation of PKC enzyme activity towards PKC-specific substrates (histone III-S and ϵ-peptide) and immunoreactivity of some major PKC isoforms (PKC-α, -ϵ, -δ and -ζ) from the cytosol to the membrane fraction (a rough particulate and sarcoplasmatic reticulum), could play a role in ischemic preconditioning. Experiments were performed with four open-chest anesthetized pigs in which the proximal left anterior descending coronary artery (LADCA) was occluded for 10 min followed by reperfusion, a standard protocol to induce ischemic preconditioning [7]. Needle biopsies from the LADCA (preconditioned) perfused bed and from the normally perfused left circumflex coronary artery (LCXCA) bed were taken at 15 min reperfusion, a time point at which the postischemic myocardium is preconditioned [7]. The homogenized biopsies

Table 1. PKC activity in cytosolic and particulate fractions isolated from homogenized biopsies excised at 15 min reperfusion from the LADCA-(preconditioned) and LCXCA-(control) perfused beds of the left ventricle from anesthetized open-chest pigs. *a*

	Histone III-S*b*		Peptide-ε*b*	
	Basal	Ca^{2+} and 1,2-DAG Stimulated	Basal	1,2-DAG Stimulated
LADCA				
Cytosolic	95 ± 11	232 ± 30	117 ± 11	36 ± 9
Particulate	56 ± 6	38 ± 8*c*	51 ± 9	-4 ± 5
LCXCA				
Cytosolic	86 ± 8	170 ± 5	111 ± 13	36 ± 8
Particulate	63 ± 4	8 ± 3	38 ± 3	-3 ± 1

a Pigs underwent a 10 min occlusion of the LADCA [6,7]. After 15 min reperfusion, transmural needle biopsies (1-3 mg) were taken from the LADCA-(preconditioned) and LCXCA-(control) perfused beds of the left ventricle, and immediately frozen in liquid nitrogen. Frozen tissue samples were homogenized at -80°C in 150 µl buffer containing 5 mM EGTA, 2 mM EDTA, 100 mM NaF, 200 µM PMSF, 2 µM leupeptine, 5 mM dithiothreitol in 25 mM TrisHCl (pH 7.4) using a dismembrator (Micro Dismembrator of Braun). After thawing the samples were centrifuged for 30 min at 48000 g in a JA 20 rotor (Beckman centrifuge J-2-21). The obtained supernatants (cytosolic fractions) were stored overnight at -80°C. The sediments were resuspended in homogenizing buffer and also stored overnight at -80°C. Before the PKC assay, the particulate fraction was solubilized in 0.5 mM EGTA, 2 mM EDTA, 2 mM PMSF, 10 mM ß-mercaptoethanol, 0.5% (w/v) leupeptine, 0.3% (v/v) Triton X-100 in 20 mM TrisHCl (pH 7.5). PKC was assayed in both the cytosolic and particulate fractions in 100 µl reaction medium containing 5 mM $MgCl_2$, 10 µM ß-mercaptoethanol, 0.25% (w/v) bovine serum albumin, 200 nM okadaic acid, 0.05% (w/v) histone III-S, 10 µM γ-^{32}-ATP (50-100 cpm/pmol), 20 mM TrisHCl (pH 7.5) with 2.5 mM $CaCl_2$ and an ultrasonified mixture of 0.016% (w/v) phosphatidylserine (PtdSer) and with or without 0.004% (w/v) 1,2-DAG. When the phosphorylation of the ε-peptide was measured, the reaction mixture did not contain Ca^{2+} but instead of histone III-S 30 µM ε-peptide. The assay mixture was preincubated at 30°C for 2 min and the reactions were started with fractionated tissue sample and stopped after 5 min by the addition of ice-cold 200 µl 25 % (w/v) trichloric acetic acid (TCA) plus 20 µl 1% (w/v) bovine serum albumin. Thereafter the mixture is millipore filtrated, the filters are four times washed with ice-cold 10% TCA and counted by liquid scintillation. *b* Activities are expressed as pmol incorporated ^{32}P. mg protein $^{-1}$. min $^{-1}$. *c* $P < 0.05$ versus the particulate fraction from the LCXCA (control) bed.

were centrifuged to obtain a supernatant (the cytosolic fraction) and a sediment (the particulate fraction). PKC activity was assayed in both fractions using γ-^{32}P-labelled ATP and histone III-S as peptidic substrate. As shown in Table 1, Ca^{2+} and 1,2-DAG stimulated PKC activity with histone III-S as substrate was significantly higher in the cytosolic as well as particulate fractions isolated from the LADCA-perfused myocardium (preconditioned) than from the LCXCA (control) region of the left ventricle, whereas the basal activities were the same. The peptide-ε was also used because it was previously shown that histone III-S is very poorly phosphorylated by PKC-ε [60], which in our hands turned out to be one of the major PKC isoforms in porcine myocardium (Fig. 1). We observed significant 1,2-DAG-stimulated activities towards the ε-peptide in the cytosolic

fractions, but none in the particulate fraction.
Based upon our PKC-ε immunoreactivity measurements (Fig. 1) this was an unexpected finding.

The cytosolic and particulate fractions were also examined by immunoblot analysis using rabbit polyclonal antibodies specific for PKC-α, -δ, -ε and -ζ isotypes. This analysis revealed significant levels of expression of the Ca^{2+}-independent isotype PKC-ε, which was detected as a band of 90-97 kDa molecular mass (Fig. 1B), while PKC-α was also abundantly present (Fig. 1A). The appearance of both bands could be blocked by addition of PKC-isopeptide demonstrating the specificity of detection. PKC-δ and -ζ were virtually undetectable using the rabbit polyclonal antibodies. The latter results, however, do not prove that PKC-δ and -ζ isoenzymes are not present in porcine myocardium, because it is possible that the polyclonal rabbit antibodies are not suitable for Western blot detection of PKC-δ and -ζ. The immunoreactivity expressed as chemiluminescence counts, determined by applying the ECL™ Western Blotting Kit and BioRad's Imaging Screen-CH in the Molecular Phosphor Imaging System, revealed no differences between the biopsies of the LADCA(preconditioned) and LCXCA (control) beds of the left ventricle (results not shown). These results indicate that neither the (Ca^{2+} and 1,2-DAG)-stimulated histone III-S kinase activities of the cytosolic nor those of the particulate fractions are reflected by relative changes in immunoreactivities (Fig. 1). Moreover, in the particulate fraction we found relatively more PKC-ε

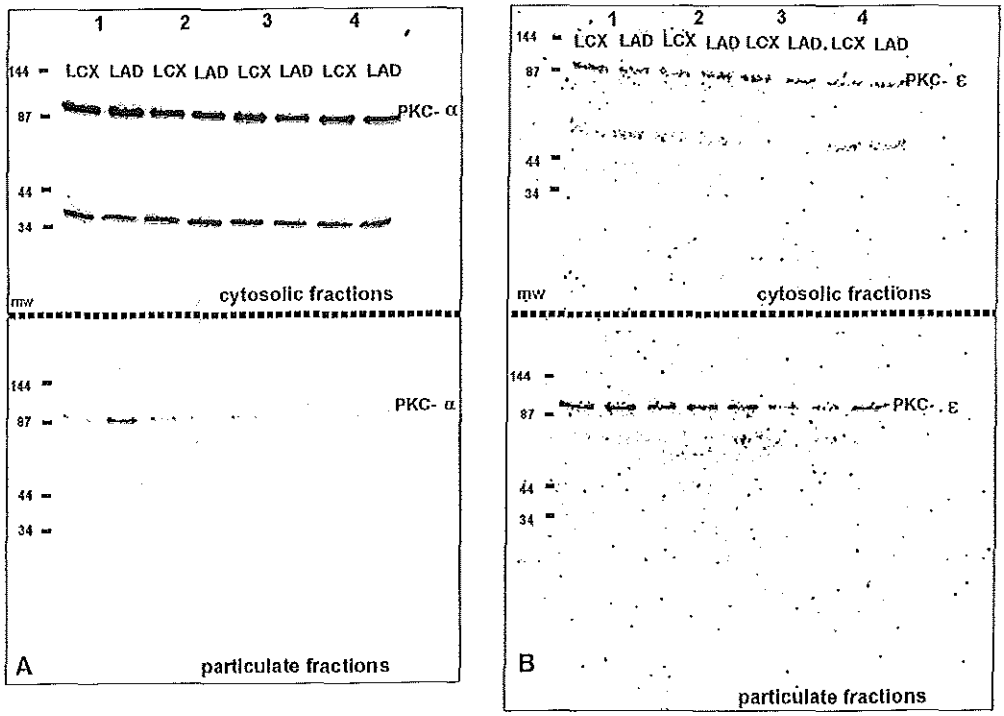

Fig. 1. Immunoreactivity images of Western blots on which subcellular fractions isolated from biopsies taken from the LADCA and LCXCA beds of pigs were separated.
([a] For details of the experimental protocol and the isolation procedure of cytosolic and particulate fractions from myocardial needle biopsies, see Table 1.). The immunoblot analysis is carried out on the same needle biopsies of which results are shown in Table 1. Parts A and B show, respectively, the PKC-α and PKC-ε obtained with cytosolic and particulate fractions from the LCX and the LAD beds. Immunoblotting was carried out essentially as described [61]. Rabbit polyclonal antibodies for PKC-α, -δ, -ε and -ζ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Chemiluminescence images of the blots are shown, obtained by applying the ECL™ Western Blotting Kit and BioRad's Imaging Screen-CH in the Molecular Phosphor Imaging System (GS-525).

immunoreactivity than in the cytosolic fraction (respectively, 903 ± 104 and 529 ± 38 chemiluminescence counts in biopsies taken from the LCXCA bed), whereas the particulate PKC activity determined with ε-peptide as substrate was not detectable (Table 1). The immunofluorescence results suggest that the amount or subcellular distribution of PKC-α and -ε was not altered in the LADCA (preconditioned) region compared to the LCXCA (control region). However, it could be argued that this detection method is not suitable for distinguishing between active and inactive PKC isoenzymes. Nevertheless, using histone III-S as substrate for determining total (Ca^{2+} and 1,2-DAG)-dependent PKC activity, increased activity was found in the cytosolic as well as in the particulate fractions isolated from the LADCA (preconditioned) compared with the LCXCA (control) bed of the ventricle. Therefore, by using these methods we were unable to discriminate between the occurrence of ischemia-induced expression of PKC isoenzymes or ischemia-induced translocation from the cytosolic to the particulate fraction. The results demonstrate activation of cytosolic and membrane-bound PKC with transient ischemia in the LADCA bed compared with the normally perfused LCXCA bed and suggest that PKC might be involved in preconditioning in the pig. However, it should be kept in mind that the presented PKC data were obtained at only one time point. In order to prove that intracellular redistribution and activation of PKC is an important mechanism for the reduction of infarct size the endogenous target proteins for PKC should be known. Comparing the time courses of phosphorylation of these target proteins on their serine or threonine residues by the PKC isoenzymes and their subsequent dephosphorylation with the gradual appearance and decay of protection during transient ischemia followed by reperfusion, is essential before a potential role of PKC as intracellular mediator of ischemic preconditioning can be accepted.

Exploration of the possible role of PLD in PKC-mediated preconditioning

The PKC hypothesis for ischemic preconditioning suggests that endogenous ligands such as A_1 adenosine-, α_1-adrenergic and muscarinic agonists, bradykinin and angiotensin II initiate an intracellular pathway resulting in stimulating PLC-β via G-coupled receptors ultimately leading to 1,2-DAG activation of Ca^{2+}-dependent and Ca^{2+}-independent PKC-isoenzymes. Many of the postulated endogenous ligands can also activate PLD. Lindmar *et al.* [62] were the first to show that muscarinic receptors, stimulated by carbachol, were coupled to PLD in the perfused chicken heart. Examining isolated subcellular membranes from rat ventricular myocardium, Panagia *et al.* [63] showed indeed that an active PLD is bound to the sarcolemmal membranes. However, a drawback of the PLD analysis in cell-free preparations is that PLD activity can only be detected in the presence of surfactants such as oleate [64] but not by stimulation with agonists. Angiotensin II activates PLD via the AT_1-receptor present in intact rat cardiomyocytes [65]. Cardiomyocytes, prelabelled with [^{3}H]myristic acid showed a rapid increase in [^{3}H]phosphatidic acid ([^{3}H]PtdOH) within minutes after stimulation by angiotensin II and the [^{3}H]PtdOH accumulation persisted thereafter for more than 30 minutes, suggesting that the [^{3}H]PtdOH was derived from [^{3}H]myristoyl-phosphatidylcholine ([^{3}H]myristoyl-PtdChol). However, [^{3}H]PtdOH could also be produced by PtdChol-hydrolysis catalyzed by PtdChol-specific PLC and subsequent phosphorylation of 1,2-DAG catalyzed by DAG kinase. Both reactions could also explain the early [^{3}H]1,2-DAG response. However, in the presence of a diacylglycerol kinase inhibitor, accumulation of [^{3}H]PtdOH persisted. The latter finding proved that PLD must be responsible for angiotensin II stimulated [^{3}H]PtdOH production in the cultured cardiomyocytes [65]. The PtdOH formed can also be separated from other phospholipids by thin layer chromatography (TLC) and the stained spot quantified by photodensitometry. This method was recently employed by Hongping *et al.* [66], who examined the response of adult rabbit ventricular myocytes to stimulation by noradrenaline and endothelin-1. In the latter study it was also demonstrated that exogenously added PtdOH stimulated the production of inositolphosphates ($InsP_n$). Because the presence of exogenous PtdOH activated PLC-ß, it was assumed by these authors that this second messenger is the activator of PLC-ß following PLD activation [66]. It is possible that PtdOH is produced from 1,2-DAG via phosphorylation when PLC-β is stimulated by muscarinic agonists, endothelin-1, angiotensin II or α_1-adrenergic agonists.

Newly formed PtdOH could then function as a positive feedback mechanism for PLC-β via increased PtdIns(4,5)P_2 hydrolysis. PLD most likely also produces PtdOH, which serves as an alternative pathway by which agonists could activate PLC-β-mediated cleavage of PtdIns(4,5)P_2 [67]. However, the coupling function of PtdOH between PLD and PLC-β, postulated in this study, is in contrast to most other reports, where PtdOH is believed to primarily originate from PLD action and will subsequently be transformed to 1,2-DAG. PLD activity can also be detected by measuring the formation of free choline in the extracellular medium, using incubation of cardiac tissue or isolated and cultured cardiomyocytes, or the medium by which the heart is perfused. We measured choline formation in cultured cardiomyocytes, of which endogenous PtdChol was first prelabelled with [^{3}H]choline followed by a short incubation in an unlabelled choline-containing medium. In these cardiac myocytes, [^{3}H]choline production increased above control cells between 20 and 40 min after endothelin-1 and increased markedly between 10 and 20 min after PMA stimulation (results not shown). These late responses suggest that PLD is involved in the hydrolysis of PtdChol and that direct PKC stimulation by PMA can lead to PLD activation.

Table 2. Activation of PLC-ß and PLD by agonists in cultured rat cardiomyocytes *a*

	[^{14}C]PtdEth Formation*(b)*		[^{3}H]InsP$_n$ Formation*(c)*	
	10 min	40 min	10 min	40 min
Control	0.053±0.003	0.037±0.012	4.34±0.41	4.09±0.53
PHE (10^{-5}M)	0.041±0.001	0.042±0.004	6.63±0.67*d*	16.07±0.76*d*
ET-1 (10^{-8}M)	0.115±0.003*d*	0.221±0.079*d*	11.96±0.39*d*	38.45±2.06*d*
Ang II (10^{-7}M)	0.067±0.017	0.058±0.000*d*	5.21±0.52	6.62±0.30*d*
PMA (10^{-6}M)	0.272±0.115*d*	0.528±0.065*d*	3.71±0.51	4.64±0.63

a Neonatal rat ventricular myocytes were cultured as previously described [38,73]. Briefly, ventricles from newborn, 2-day-old Wistar strain rats were minced and myocytes were isolated by 8 subsequent trypsinization steps at 30°C. Non myocytes were removed by differential preplating and myocytes were seeded at 150.000 cells/cm^2. Cells were grown in DMEM:M199 (4:1) supplemented with 5% fetal calf and 5% horse serum. After 24 hrs, medium was changed to serum-free DMEM:M199, which was changed every 48 hrs thereafter. Experiments were routinely performed 5-7 days after isolation of the cells. PLD activities were determined by measuring the production of [^{14}C]PtdEth in the presence of 0.5% ethanol in cardiomyocytes that were prelabelled for 48 h with [^{14}C]palmitic acid. After the incubation with agonists the cellular lipids were extracted and [^{4} C]PtdEth was separated from other phospholipids by TLC. PLC-ß activities were determined by measuring the production of [^{3}H]InsP$_n$ in cardiomyocytes that were prelabelled for 48 h with [^{3}H]Inositol. The [^{3}H]InsP$_n$ were separated from [^{3}H]Inositol and [^{3}H]glycerophosphoinositol by columnchromatography on a Dowex AG 1-X8 as previously described [74]. The values represent the mean ± SEM and are expressed as % of the total incorporated [^{14}C]palmitic acid*(b)* or [^{3}H]Inositol *(c)*. *d* $P < 0.05$ versus control (respectively n=2*(b)* and n=8*(c)*). PHE=phenylephrine, ET-1=endothelin-1, Ang II=angiotensin II, PMA=phorbolester.

These results are consistent with our measurements using the PLD-catalyzed transphosphatidylation assay (see below and in Table 2).

In general, the reason for the relatively late discovery of the existence of receptor-coupled PLD was the established route of agonist-dependent 1,2-DAG production by hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) by PLC-ß as well as the fact that the observed

increase of PtdOH in many cells following receptor stimulation was thought to result from the rapid action of DAG-kinase on 1,2-DAG [68]. However, it has been reported that the formation of 1,2-DAG was dissociated in time from the generation of $InsP_n$ [69], often to the extent that 1,2-DAG is formed in the complete absence of $Ins(1,4,5)P_3$ accumulation [30,39,70]. PLC-ß hydrolyzes the glycerophosphate ester in $PtdIns(4,5)P_2$ to form 1,2-DAG and $Ins(1,4,5)P_3$. PLD cleaves on the other side of the phosphoryl linkage to form PtdOH and the free base mostly from PtdChol and subsequently PtdOH is hydrolyzed by PtdOH-hydrolase to 1,2-DAG. The concentration in the cell of PtdChol is usually about 100 times higher than $PtdIns(4,5)P_2$ concentration. The continuous production of 1,2-DAG could be of major importance for the maintenance of activation of specific PKC isoenzymes involved in the mechanism of ischemic preconditioning. Furthermore, several nonmyocardial studies (see for instance ref. 71) indicate that 1,2-DAG formed in the early transient phase of receptor stimulation predominantly contains fatty acids present in the $PtdIns(4,5)P_2$ pool (stearate, 18:0 and arachidonate, 20:4n-6), whereas in the later phase of receptor stimulation it contains more saturated fatty acids typically found in PtdChol [71]. In line with these findings it can be expected that distinction in the molecular species of 1,2-DAG is responsible for the time-dependent differences in pattern of activation of PKC isoenzymes, which are often seen after stimulation with different agonists [30,37-39,72]. Recently, we observed a difference in fatty-acid composition, mainly in the 16:0 and 20:4n-6 of the 1,2-DAG formed comparing 10 and 40 min stimulation by endothelin-1 of cardiomyocytes (results not shown). These results indicate that after a 10 min stimulation 1,2-DAG originates mainly from $PtdIns(4,5)P_2$ and after 40 min from PtdChol.

The PLD-catalyzed transphosphatidylation assay (the formation of phosphatidylalcohol through a base exchange reaction of PtdChol with an alcohol such as ethanol or butanol) can be used to unmask the operation of the PLD pathway [39,66,72]. Accordingly, we determined the rate of [^{14}C]PtdEth formation in the presence of 0.5% ethanol in cardiomyocytes prelabelled with [^{14}C]palmitic acid. The [^{14}C]PtdEth formation was, respectively, 5.9-, 1.6- and 14.2-fold increased by 40 min of stimulation with, respectively, 10^{-8} M endothelin-1, 10^{-7} M angiotensin II and 10^{-6} M PMA (Table 2). α_1-Adrenergic stimulation, however, had no effect on PLD. These results were in agreement with those obtained by measuring [^{3}H]choline production from [^{3}H]choline prelabelled cardiomyocytes (see above). On the other hand, phenylephrine, endothelin-1 and angiotensin II markedly stimulated (respectively, 3.9-, 9.4- and 1.6-fold) PLC-ß in these cells (Table 2). As expected, PMA had no significant effect on $InsP_n$ production. Unpublished results from our own laboratory showed also that the activation of PLD does not begin earlier than 10 min after agonist addition. In contrast, we found that the agonist-induced activation of PLC-ß is very fast [70,74,75]. Also taking into account the strong activation of PLD by PMA, it is likely that the activation of PLD is a secondary response to PLC-ß.

Until now, the question whether $PtdIns(4,5)P_2$ hydrolysis per se is sufficient for PLD activation or if it only has modulatory effects on receptor-coupled PLD activation, has not been addressed. The unequivocal proof for GTP-binding protein regulation of receptor mediated PtdChol-hydrolysis must await appropriate reconstitution studies as were done with $PtdIns(4,5)P_2$ specific PLC [39]. One possible explanation forwarded for the receptor-coupled activation of both PLD and PLC is that a single receptor GTP-binding protein complex couples both effector enzymes and that this coupling is perhaps regulated by PKC [76]. However, this is highly unlikely due to the fact that PLC-β-G_q is pertussis toxin insensitive, in contrast to the pertussis toxin inhibition of PLD activity.

Because PMA appeared to be very effective in inducing PtdChol-hydrolysis by PLD in cardiomyocytes, it became interesting to comparatively investigate the translocation of PKC isoenzymes to the membranes during agonist and PMA stimulation in these cells. Therefore, we measured the translocation of two PKC isotypes α and ϵ that were readily detectable in the cardiomyocytes, using immunoblot analysis as was shown by Clerk *et al.* [77]. Endothelin-1 caused a very rapid and sustained disappearance of PKC-ϵ but not of PKC-α from the cytosol (Fig. 2).

Since the PKC-ε levels in the membrane fraction reaches almost the detection level (results not shown), the translocation of PKC-ε to the membrane was not always visible. PMA (10^{-7} M), however, caused a rapid, sustained and clearly detectable translocation of PKC-α as well as -ε. It is important to note that minimal proportions of PKC-α and -ε are associated with the membrane fraction in the cardiomyocytes under unstimulated conditions (Fig. 2).

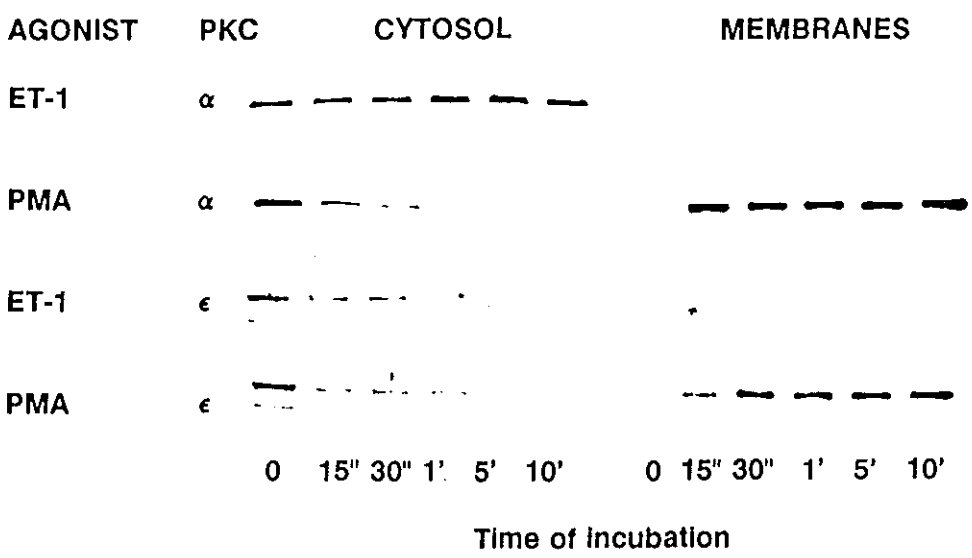

Fig. 2. Immunoblots of the time course of distribution of PKC-α and PKC-ε between cytosol and membrane fraction after exposure of cardiomyocytes to endothelin-1 (10^{-8} M) (ET-1) and PMA (10^{-7} M). Cultured cardiomyocytes were incubated with either PMA or endothelin-1 under the conditions given in Table 2. The cell lysis, cell fractionation and immunoblot analysis of subcellular fractions was performed as previously described [38].

Mitchell *et al.* [29], showed in rat heart that the PKC-δ and PKC-ε were translocated through transient ischemic stimulation. Since the initial PLC-ß response usually is accompanied by increases in Ca^{2+} and 1,2-DAG and followed by a prolonged increase of 1,2-DAG with no rise in Ca^{2+}, the observed translocation of PKC-δ and PKC-ε is more likely due to the secondary PLD activation. The gradual decay of protection takes hours after the ischemic event, which is more in agreement with the involvement of PLD than PLC in the mechanism of ischemic preconditioning. The question remains, however, whether PLD indeed is activated by ischemia. In perfused rat heart, prelabelled with [^{14}C]arachidonic acid, ischemia (30 min)-reperfusion (30 min) induced a significant increase in the amount of [^{14}C]arachidonic acid incorporated into PtdOH and 1,2-DAG [40]. Recently, it was shown that brief episodes of ischemia induced increased endothelin-1 secretion in the perfused rat heart [78]. Thus on the basis of these findings endothelin-1 can be added to the list of potential endogenous mediators of cardioprotection such as α_1-adrenergic-, A_1 adenosine and muscarinic

agonists and angiotensin II. A role for G-proteins in the PLD activation independent of PLC-ß and PKC activation has been suggested but is not generally assumed (Fig. 3) [79].

It seems more likely that the PLD activation by the agonists, endogenously formed during ischemia, is initiated by G-coupled-PLC-ß derived 1,2-DAG that activates PKC isoenzymes (Fig. 3). By switching to PtdChol as source for 1,2-DAG the cardiomyocyte is able to maintain increased levels of 1,2-DAG for a prolonged period of time, which is expected to lead to changes in the molecular species of 1,2-DAG required for a distinct pattern of activation of PKC isoenzymes.

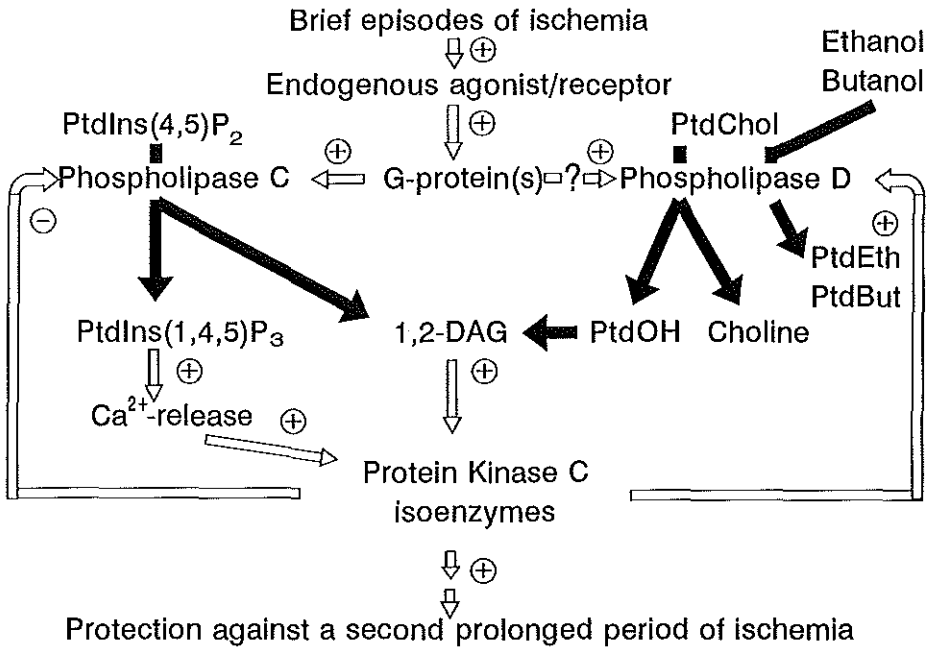

Fig. 3. Interrelationships between PLC-ß and PLD pathways that are possibly involved in the mechanism of ischemic preconditioning. Open and closed arrows refer to, respectively, stimulatory (+) or inhibitory actions (-) and chemical reactions. See text for further explanation.

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Chapter 8

GENERAL DISCUSSION

GENERAL DISCUSSION

One of the major aims of the work described in this thesis was to investigate the possible existence of cross-talk between receptor-mediated PLC-β and PLD signalling pathways in cardiomyocytes. Moreover, the potential involvement of PKC isozyme translocation/activation in this cross-talk was investigated. More detailed knowledge of the mechanism of activation of PLC-β, PLD and PKC in myocardium was needed, because these signalling systems are likely involved in the induction of various myocardial adaptive/protective responses such as compensatory hypertrophy and ischemic preconditioning. A preparation of beating neonatal rat cardiomyocytes maintained in serum-free culture, virtually free from other celltypes (endothelial cells, fibroblasts, smooth muscle cells) present in the heart, was used as model for evoking a maximal increase of protein synthesis and to study the signalling processes involved. The induction of hypertrophy and ischemic preconditioning appears to have many stimuli in common, among them are α_1-adrenergic agonists, ET-1, AngII and PMA which were, therefore, tested in this neonatal rat cardiomyocyte preparation.

To obtain evidence for the cross-talk mechanism, PLC-β and PLD activities were followed after agonist stimulation over a prolonged period of time (up to 40 min) (Chapter 3). Since both membrane-bound enzymes ultimately produce the second messenger 1,2-DAG, but from different phospholipid sources, analysis of molecular species composition of the total cellular 1,2-DAG pool was carried out in order to estimate time-course and hormone-dependence of the contribution of both pathways to this 1,2-DAG pool. To this aim we analysed the molecular species of cellular 1,2-DAG on HPLC and of its phospholipid sources PtdIns(4,5)P_2 (PLC-β) and PtdChol (PLD) after *in vitro* cleavage by a purified PLC preparation. It was demonstrated that both ET-1 and PHE (and as expected, not PMA) stimulate PLC-β activity and that only ET-1 (and PMA) stimulate PLD activity. By analysis of the molecular species of 1,2-DAG we obtained evidence that the 1,2-DAG present in unstimulated cardiomyocytes most likely is derived from PtdIns and PtdIns(4,5)P_2 due to basal activity of PLC (Chapter 4). During stimulation with ET-1 and PMA, but not PHE, PtdChol became an increasingly important source for 1,2-DAG due to sustained activation of PLD. Unexpectedly, the total 1,2-DAG concentration remained relatively constant during agonist stimulation which strongly indicates that particular molecular species of 1,2-DAG rather than its total concentration determine the activation of PKC isoenzymes.

To study the possible involvement of PKC in the cross-talk between PLC-β and PLD signalling pathways, we followed the translocation from the isozymes from the cytosol to the total membrane fraction and performed PKC activity measurements in cardiomyocytes. ET-1 (and PHE to a minimal extent) produced a rapid (0-5 min) translocation of PKC-ε immunoreactivity from the cytosol to the membrane fraction, whereas no intracellular redistribution of PKC-α, -δ and -ζ immunoreactivities was observed as judged by western blotting (Chapter 3). PMA caused translocation of PKC-α, PKC-ε as well as PKC-δ. Surprisingly, cellular redistribution of the enzymatic activity of PKC was not observed with ET-1 and PHE, although it was definitely shown to be present with PMA stimulation. Down-regulation of PKC isozymes by 24 h pretreatment with PMA or acute blockade of PKC by chelerythrine inhibited ET-1 and PMA stimulated PLD activity, providing evidence that PKC is involved in the cross-talk of PLD with PLC-β. The results indicate that in cardiomyocytes maintained in serum-free culture, ET-1 initially activates PLC-ß and after a brief lag-phase PLD, whereas PHE and Ang II activate only PLC-ß. PLC-ß stimulated by ET-1 may induce PLD activation via stimulation of the translocation of PKC-ε. At present, it is not clear how PKC-ε induces activation of PLD. The fact that we did not observe an increase of total membrane-bound PKC activity, points to a direct PKC-ε redistribution mediated effect. In agreement with this proposal, recently, it was shown in other celltypes that activation of PLD did not require ATP (as discussed below), phosphorylation activity was absent and PKC activity could not be blocked by kinase inhibitors. Thus, the activation might involve direct protein-protein interaction between PKC and PLD.
Development of hypertrophy in cardiomyocytes was measured by an increase of the rate of protein

synthesis. Previously, we have demonstrated that the increase of [^{3}H]leucine incorporation after 24 h was accompanied by increase in protein/DNA mass ratio [1]. ET-1 and PMA were both strong stimulators of protein synthesis (about 1.8-fold compared to control), whereas α_1-adrenergic agonist PHE and AngII stimulated protein synthesis 1.5-fold and 1.2-fold, respectively. Combined with the previously observed neurohormone-dependent stimulation patterns of PLC and PLD, these data indicate that the increase of protein synthesis is causally related to changes in PLD activity. In this thesis we also provided evidence for PKC involvement in the mechanism of ischemic preconditioning based on a study using an *in situ* porcine model (Chapter 7). The results, combined with the abundant evidence in the literature on endogenous triggers (such as adenosine, α1-adrenergic agonists, ET-1, AngII) and on the role of PKC involvement, suggest that receptor-mediated PLC-β and PLD may act upstream of PKC. For several of the postulated endogenous triggers of ischemic preconditioning such as α_1-adrenergic-, purinergic and muscarinic agonists, adenosine, AngII and ET-1, it has been shown that these stimuli in myocardium, can activate not only PLC-ß but also PLD [2]. Furthermore, a strong argument for a role of PLC-β and/or PLD in ischemic preconditioning is the finding is that *in vivo* administration of PMA mimics the protective effect.

The Na^+/H^+ exchanger is believed to be one of the candidate target proteins for PKC isozymes [3]. Activation of the Na^+/H^+ exchanger has also been hypothesized to be involved in induction of hypertrophy [4] as well as ischemic preconditioning [5]. Therefore, we applied single cell imaging fluorescence microscopy to measure intracellular changes of pH after stimulating cardiomyocytes with agonists that mimic ischemic preconditioning or induce hypertrophy. Application of ET-1 and PHE caused pH_i to rise slowly to reach a small but significant maximal alkalinization after about 0.5 h (Chapter 5). However, exposure of the cells to PMA (10^{-6}M) did not result in a significant effect on pH_i. We conclude that only limited α_1-adrenergic and ET-1 induced intracellular alkalinization responses occur in rat cardiomyocytes maintained in serum-free culture and that probably PKC is not involved in the change of Na^+/H^+ exchanger activity.

Mechanisms of PLD activation and its general action(s)

PLD is widely distributed in mammalian cells and is shown to be regulated by a variety of hormones, growth factors and other extracellular signals. Its major substrate in cells is PtdChol, which is primarily hydrolysed to phosphatidic acid (PtdOH) and choline. Over the last few years PLD has been extensively studied in many different species by several groups [6-9]. PLD is stimulated by PKC activation in most mammalian cells, but there is also abundant evidence that it can be activated by members of the small G-protein family, such as ARF (ADP ribosylation factor) and Rho as described below. Since Rho is involved in the formation of focal adhesions and actin stress fibres, there might be a role for PLD in the control of the actin cytoskeleton [8]. In this respect it is interesting to note that microtubuli have already for a long time been known to play an important role in hypertrophy as was also recently reviewed [10]. It was suggested that the presence of the microtubular component of the cytoskeleton may increase the load on the cardiomyocyte and therefore perpetuate the stimulus to hypertrophy, ultimately leading to cardiac failure [11]. Therefore, Rho-induced stimulation of PLD might be an additional signalling pathway involved in induction of hypertrophy. ARF is now recognized to play a role in vesicle trafficking in Golgi and has been implicated in the fusion of microsomal vesicles and endosomes, the assembly of nuclear membranes, and the formation of clathrin-coated vesicles. Therefore there might be an important role for ARF-stimulated PLD in the regulation of vesicular transport in Golgi as described by Ktistakis *et al.* [12].
PLD may exert it biological effects by several mechanisms [8]. The first is by changing the properties of cellular membranes by altering their lipid composition. A second mechanism is by generating PtdOH, which is a potent activator of many proteins in vitro [13]. A third mechanism of biological action of PLD arises from the fact that PtdOH is rapidly converted to 1,2-DAG, which activates PKC isozymes [14] as discussed for the myocardium before. A fourth potential consequence of PLD activation is the generation of lysophosphatidic acid (LPA) through the action of phospholipase A_2 on PtdOH, which is a major signal produced by activated platelets [15] and may also be active

in other cell types. Because basal levels of choline in cells are already high, this compound does probably not have an important signalling function, but it might serve as a substrate in neurons [16]. LPA has also been shown to utilize a $G_{\alpha 13}$-coupled receptor to activate Rho. This could then serve as a feedforward activating system for PLD. $G_{\alpha 13}$ could also be a direct activator of PLD [88].

Many advances in PLD enzymology have been made recently, but all possible isoenzymes of PLD need to be identified and their structure and function relationships and regulatory properties need to be defined [8]. Very recently, some mammalian isoforms of PLD have been cloned, which include humanPLD_{1a}, humanPLD_{1b}, humanPLD_2 and ratPLD_2 and ratPLD_1[17-21]. Very recently, it was also discovered that interaction of PKC with PLD (ratPLD_1) occurs separate from the sites at which RhoA and ARF interact. Interaction of PKC with PLD must involve the regulatory domain (an N-terminal site) of PKC. The activation of PLD by PKC was proven not to require ATP, phosphorylation was absent and it was not blocked by inhibitors of the catalytic activity of PKC. Thus, the *in vitro* activation of PLD appears to occur by a protein-protein interaction that results in an activating conformational change of PLD [22]. This would be in agreement with what we found in our PKC (immunore)activity (phosphorylation versus immunoreactivity) measurements. We did not observe any changes in phosphorylation activities due to neurohormonal stimulation, indicating that direct interaction of PKC with PLD might occur in our cells as well.

Is myocardial PLD solely activated by PKC? Do G-proteins and/or Ca^{2+} play additional roles?

As we showed in Chapter 3 and 4, the cross-talk between PLC-β and PLD signalling pathways may contribute importantly to the formation of the second messenger 1,2-DAG in myocardium. For example, it was demonstrated that the PLC-β-stimulating agonist ET-1 also stimulates PLD. This does not exclude that additional, more direct mechanisms, such as ET_A-receptor-G-protein coupling, are involved in the activation of PLD. In some non-myocardial studies this has been proven [7, 23, 24]. Phospholipases of the C type are activated by the receptor through the α and βγ subunits of heterotrimeric G-proteins as well as by receptor-associated and non-receptor-associated tyrosine kinases [25, 26]. Evidence for the involvement of G-proteins in PLD activity was obtained by others using poorly hydrolysable analogues of GTP, such as guanosine 5'-(γ-thio)-triphosphate (GTPγS) in isolated plasma membranes and permeabilised cells [27]. As discussed before, it is now accepted that these G-proteins are members of the ARF [28, 29], G_{13} [88], Rho subfamilies [8, 13, 30] or of small G-proteins. The responsiveness of PLD to activation by the small G-proteins Rho and ARF varies in different tissues and subcellular fractions [12, 13, 30, 31], which might indicate that different PLD isozymes coexist. There is also evidence that PKC, ARF and Rho interact synergistically in the activation of PLD [9, 19, 32, 33].

To specifically study the involvement of G-proteins in PLD activity in neonatal rat cardiomyocytes, we performed an additional series of experiments of which the results are shown in Figure 1. Like several other previous reports [reviewed in 8], we could demonstrate that PLD activity induced by GTPγS was Ca^{2+} dependent in permeabilized cardiomyocytes. It is interesting to note that a Ca^{2+} dependent pattern as well was found earlier by us for PLC-β in these cells [34] (Figure 1A). However, the K_a for Ca^{2+} of PLD appears to be much higher than that of PLC-β. Moreover, it is unlikely that cytoplasmic Ca^{2+} provides the cross-talk between PLC-β activation and PLD, because previously we could detect only marginal Ca^{2+} responses following stimulation of cardiomyocytes with ET-1 and PHE [35]. A fixed concentration of 1.5 μM $[Ca^{2+}]_i$ was chosen in the results of experiments shown in Figure 1B in which PLD activity was measured in the presence or absence of GTPγS (30 μM) following 40 min incubation minus or plus PMA (10^{-6}M). The results provide evidence that even in the presence of maximal PKC activation by PMA, GTPγS induced an identical stimulation of PLD activity to that observed in Fig. 1A. We, therefore, conclude that the PKC induced effect on PLD activity is additional and not synergistic with G-protein activation. It is concluded that G-proteins may additionally contribute in the activation of PLD by ET-1 in

myocardium. Clearly, experiments are required to test whether G-protein is activated during ET-1 stimulation of intact cells and to identify the actual G-protein(s) involved in cardiomyocytes.

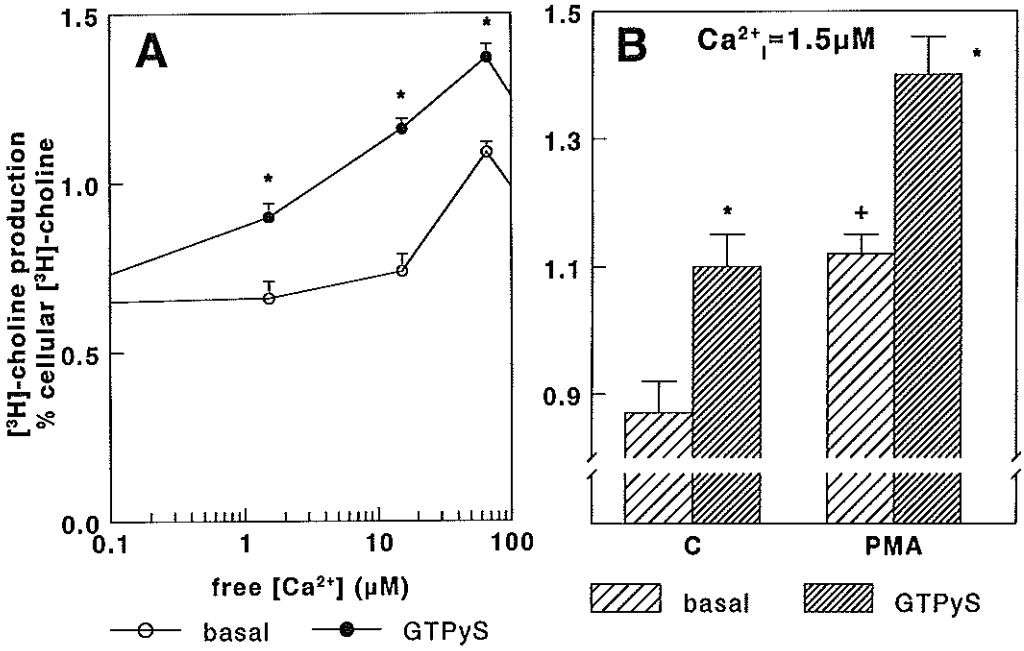


Fig. 1. **A)** Effect of GTPγS (30 μM) on PLD activity (measured by the rate of [^{3}H]choline production) in permeabilised control cardiomyocytes and its Ca^{2+}_i dependency. Mean results of 4 separate experiments are shown. * $p < 0.05$ compared to basal. **B)** Effect of GTPγS (30 μM) on PLD activity at fixed Ca^{2+} concentration (1.5 μM) with or without PMA (10^{-6} M). Mean results (±SEM) of 4 experiments were shown. * * $p < 0.05$ compared to basal. Cardiomyocytes were isolated as described in chapter 3. Permeabilization of cells by saponin was described in detail by Van Heugten *et al.* [36]. After labelling with [^{3}H]choline (2μCi/ml) for 24 hours in medium containing 5% horse serum, the cells were thoroughly washed with phosphate buffered saline (PBS, 37°C) and subsequently permeabilized for 2.5 min with an intracellular buffer (20 mM HEPES; 10 mM NaCl; 110 mM KCl; 1 mM KH_2PO_4; 4 mM $MgCl_2$; 1mM EGTA; 3mM ATP; 8 mM creatine phosphate; 6 U creatine kinase/ml; pH 7.0) containing 25 μM choline chloride and 80 μg saponin/ml. Hereafter, cells were washed 3 times with intracellular buffer lacking saponin and fresh intracellular buffer with Ca^{2+} (1.52 μM) and GTPγS (30 μM) was added. Permeabilized cells were incubated for 40 min at 37°C, intracellular buffer was collected and cells were extracted with methanol as described (Chapter 3). The intracellular buffer fraction was separated on DOWEX 50W (100-200 Mesh, X8; H^+ form) to isolate the formed [^{3}H]choline.

Are the activation of PLD and the second messenger(s) generated involved in the induction of cardiac hypertrophy?

Hypertrophy of the heart is caused by hemodynamic overload, for instance, by post-infarction, by hypertension, aortic stenosis and valvular insufficiency. It has become clear that the induction of hypertrophy is a multifactorial process, involving multiple signalling pathways. PKC seems, however, to play a pivotal role [37]. Possible involvement of PKC in the induction of hypertrophy and altered gene expression was shown by transfection studies employing constitutively activated PKC isoforms (-α, -ε, -ζ, -β) in cardiomyocytes [38, 39].

In our experiments with cultured cardiomyocytes *in vitro* we have shown that PHE, although to

a lesser extent than with ET-1, induces hypertrophy. However, during PHE stimulation the initial translocation of PKC-ε was neglectible, indicating that PKC-ε is not involved in the late hypertrophic response as was proposed by others [6]. However, since more than 11 isozymes of PKC are known, it remains possible that PKC isozymes other than PKC-ε become activated by PHE and that these isotypes are involved in induction of hypertrophy.

Sofar we have only studied four of the PKC isozymes (PKC-α, -ε, -δ, -ζ) known to be the major ones described to be expressed in rat neonatal cardiomyocytes [40, 41]. Not only PKC activation (by either PLC-β or PLD activity), but also other second messengers formed by PLD are suggested to play a role in the development of hypertrophy. Hydrolysis of PtdChol produces PtdOH, which in turn can produce 1,2-DAG, but it may also act independently as second messenger involved in proto-oncogene expression, cell proliferation and protein synthesis (Chapter 2). PtdOH has been reported to stimulate Ca^{2+} influx, which may play a permissive role in the development of cardiac hypertrophy, because it is known to serve as a cofactor for several enzymatic processes required for cell growth [reviewed in 42]. Another possible property of PtdOH is induction of phosphorylation of an (unknown) cardiac cytosolic protein, independent of PKC or Ca^{2+}, which would indicate that PtdOH may stimulate a novel protein kinase (possibly a tyrosine kinase) or serve as cofactor for this kinase [reviewed in 42]. PtdOH is also shown to be a putative physiological PKC-ζ activator [43].

PtdOH increases Ins(1,4,5)P_3 levels leading to hypertrophy [44]. In addition to the direct actions of PtdOH, PtdOH has been reported to stimulate the activity of MAP kinase and synthesis of DNA and protein. Considering these pleiotropic actions PtdOH could, therefore, influence the hypertrophy process [42].

Another molecule that can be formed from PtdOH is lysophosphatidic acid (LPA), by the action of phospholipase A_2. LPA stimulates mitogenesis and growth [reviewed in 13] and is now recognized as an important second messenger. However, the role of LPA in myocardial signalling has not been studied sofar.

A few months ago, it was experimentally proven in transgenic mice that the G-protein G_q is a critical molecule in the initiation of myocardial hypertrophy [45]. In those experiments a specific peptide ($G\alpha_q$) was designed that interacts dominantly negative with the intracellular domains of agonist-occupied receptors and transgenic mice with myocardial-targeted overexpression of the peptide were created. The results showed that cardiac G_q-coupled receptors indeed play a critical role in triggering pressure-induced ventricular hypertrophy. Targeting the receptor-G-protein interface may point the way to the development of therapies that may have potential advantage over traditional receptor antagonists, because an entire class of receptor signals (those coupled to this particular G protein) will potentially be blocked.

PKC activation by increased 1,2-DAG concentration and/or particular molecular species

PKC plays a pivotal role in many signalling processes in myocardium. PKC is activated by 1,2-DAG, which forms the trigger for translocation of the enzyme from the cytosol to the membrane fraction. Based upon our results, not mere the level of total 1,2-DAG, but the molecular species composition of 1,2-DAG determines PKC isozyme activation/translocation (Chapter 4). The question is which particular molecular species (or particular fatty acid(s)) is mostly responsible for this activation. Research on this important subject is scarce and sofar, we could not find studies dealing specifically with this matter in rat cardiomyocytes. The described function of 1,2-DAG is to activate PKC by increasing the affinity of PKC for its membrane binding sites, as well as to increase the activity of the enzyme once it partitions into the membrane. Structural modification of *sn*-1,2-DAG could potentially alter the activation pattern of PKC as we hypothesized in Chapter 4. This may eventually lead to growth and gene expression responses. Also the effects of free fatty acids on PKC activation *in vitro* have been studied. The results show that saturated and *trans*-unsaturated free fatty acids are incapable of activating classical PKC, whereas *cis*-unsaturated fatty acids such as 18:1ω9,

18:2ω6, 20:4ω6 and 18:3ω3 greatly enhance the phosphorylating capacity of cPKC *in vitro* [46-49]. However, the effects of arachidonic acid (20:4ω6), a eicosapentanoic acid (20:5ω3) or docosahexanoic acid (22:6ω3) at the sn_2-position of 1,2-DAG on PKC activation has not been studied yet. There are some results about effects of pure molecular species added to lipid vesicles (e.g. 18:0/20:4, 18:0/20:5 and 18:0/22:6) on PKC activation *in vitro* measured by histone phosphorylation [50]. Comparison between the species showed that 18:0/22:6 was significantly more potent than other molecular species in activating PKC, and that both 18:0/22:6 (1.8 times more potent at 0.5 mol% in vesicles compared to the others) and 18:0/20:4 were better activators than 18:0/20:5 and 18:1/18:1 in different concentrations used [50]. This may be true for the *in vitro* situation (pure molecular species added to vesicles), but no reports have been published sofar in whole cells or *in vivo* situations where other regulatory factors of PKC are playing a role too.

Classic and delayed ischemic preconditioning: involvement of PLC-β and PLD, PKC and stress activated protein kinases?

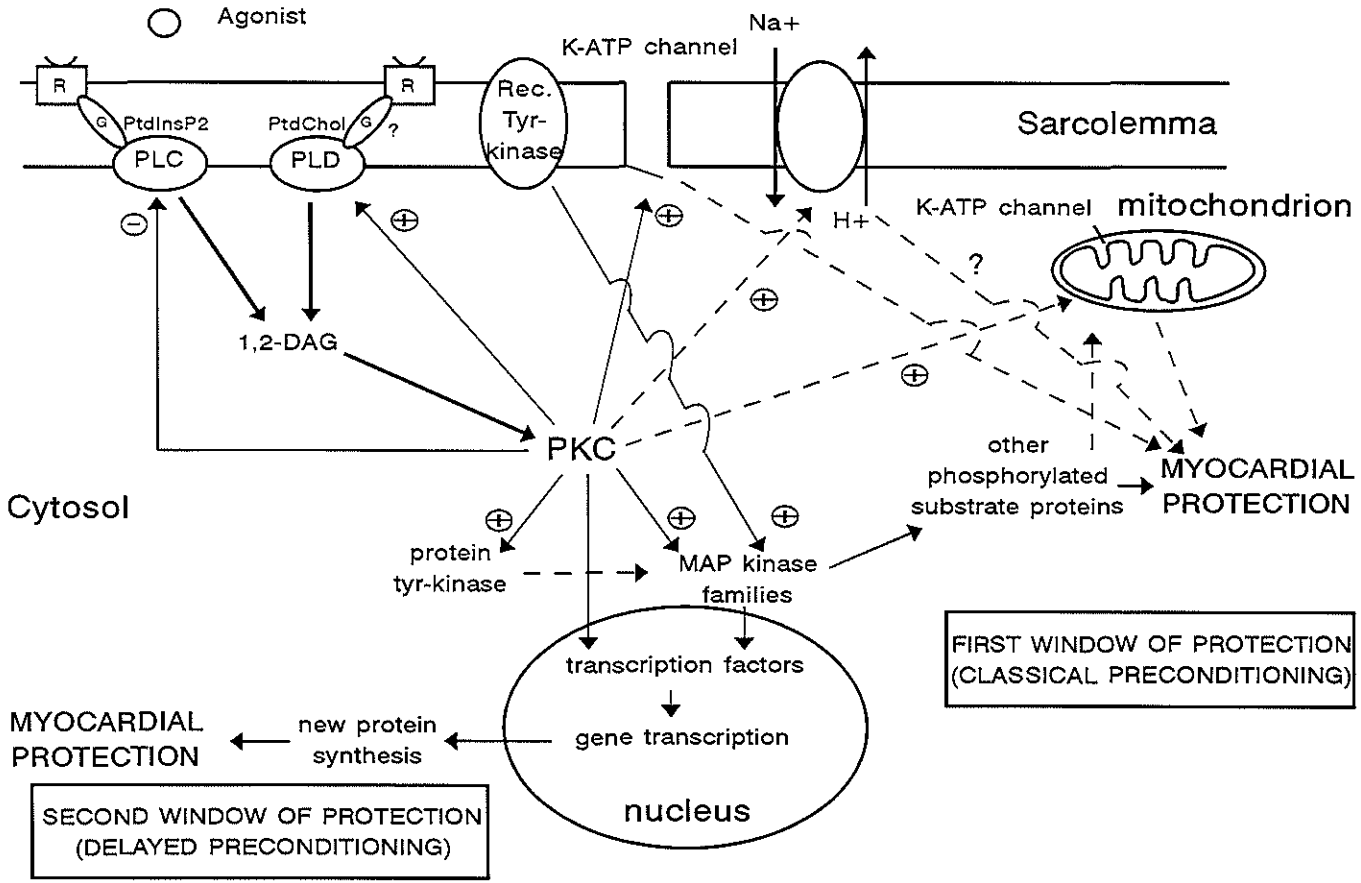
Classically, the stimulus for ischemic preconditioning is a critical brief reduction in blood flow, and the end-point is infarct size limitation [51]. As recently reviewed by Yellon *et al.* [52], it appears that there is a bimodal distribution of protection; the initial phase described by Murry *et al.* [51] lasts around one to three hours, depending on species and model (first window, see Fig 2), whereas a delayed preconditioning or "second window of protection" exists between 24 and 72 h following the initial ischemic insult. The mechanisms of protection for these two windows are most likely different, although activation of PKC appears to be a crucial intermediate step in both situations since pharmacological inhibition of PKC abolishes classic as well as delayed preconditioning [52] (see Fig. 2). Our experiments on PKC translocation/activation during ischemic preconditioning, as described in chapter 7, were performed in porcine hearts that were preconditioned *in situ* with a 10 min coronary artery (left anterior descending, LADCA) occlusion followed by reperfusion. Transmural biopsies were taken from the LADCA and left circumflex coronary artery (LCXCA)-perfused beds of the left ventricle, the latter samples representing the control area. The infarct size limiting effect (classic: usually determined 2h after a 1h LADCA occlusion) of this protocol of ischemic preconditioning was demonstrated before [53, 54]. Note, that no delayed preconditioning (second window of protection) has been reported in this porcine model. We observed activation of cytosolic and membrane-bound PKC with transient ischemia in the LADCA bed compared with the normally perfused LCXCA bed indicating that PKC activation might be associated with classic preconditioning (first window of protection) in the pig (Chapter 7). Vogt *et al.* [55] described a modest 10 to 20 % redistribution of PKC activity from cytosol to the particulate fraction in *in vivo* pig hearts subjected to 10 min of coronary artery occlusion which result partially agrees with our findings. Valhaus *et al.* [56] failed to demonstrate prevention of classic ischemic preconditioning in anaesthetized pigs using staurosporine as PKC inhibitor. However, the use of staurosporine as PKC inhibitor led to conflicting data concerning PKC-induced PLD activation [27]. Likewise, in our studies in rat cardiomyocytes on ET-1 and PMA-induced activation of PLD we observed no inhibitory, but rather a stimulatory effect of staurosporine, whereas the more selective antagonist of PKC chelerythrine completely inhibited activation of PLD (Chapter 3). At present, there is no study available that describes the potential endogenous trigger(s) of preconditioning in the pig. A great number of endogenous triggers have been identified in other species which are released and/or operative during preconditioning ischemia, including adenosine [57], acetylcholine [58, 59], catecholamines [60], AngII [61], bradykinin [62], ET-1 [63] and opioids [64]. Which triggers and to what extent they contribute to the preconditioning effect may vary between species. For example, in the rat heart adenosine was not found to be involved in ischemic preconditioning, but it appeared to be very important in rabbit [57], dog [65], pig [66] and human [67] myocardium. In rat heart α_1-adrenergic receptors seemed more important [60]. Moreover, it was shown in human atrial trabeculae that α_1-adrenergic receptors and PKC mediate, in part, the creatine kinase

preservation conferred by ischemic preconditioning, but that α_1-adrenergic preconditioning does not replicate the protection of ischemic preconditioning [68].

Yellon *et al.* [52] emphasized in their recent review that triggers (neuroendocrine, para- and autocrine) must be distinguished from mediators of preconditioning. The trigger is operative during the preconditioning episode and the mediator during sustained ischemia. PKC could be the mediator assuming that it remains maximally activated during part of the latter episode. Based upon our results on PLD activation in cultured neonatal rat cardiomyocytes by agonists such as ET-1, which were reported to be operative during preconditioning ischemia [63, 69], we hypothesized that PLD activation is involved in the mechanism of preconditioning (Chapter 6 and 7). Since then, a number of reports have appeared that show that repeated brief episodes of ischemia and reperfusion exert beneficial effects on the rat and rabbit heart by triggering the activation of PLD [70-72]. Either the transphosphatidylation assay [72], generation of 1,2-DAG and PtdOH [71] or assay of PLD in cardiac homogenates /dispersed myocytes [70] were used to demonstrate activation of PLD during preconditioning. Moreover, Cohen *et al.* [70] showed that a potent activator of PLD, oleate, mimicked preconditioning and an inhibitor of PLD, propranolol, blocked ischemic preconditioning. Up till now, all the data on PLD have been obtained in rat and rabbit heart. Thus, for the pig no data on the role of PLD are available. A distinction should also be made between PLD as a trigger or a mediator of preconditioning. From our experiments on neonatal rat heart myocytes it follows that PLD is activated by PKC-ε whose the translocation is induced by 1,2-DAG formed by receptor-mediated PLC-β (Chapter 3). Therefore, PLD activation likely is an event downstream of PKC, and therefore by definition a mediator during sustained ischemia. Similar to the investigation of hypertrophy and altered gene expression, the mechanisms of classic [72-75] and perhaps also delayed preconditioning [52, 76] may in addition to PKC, involve tyrosine kinase- and MAP kinase signalling cascades.

The initial evidence was that transient ischemia rapidly increases total MAP kinase activity in rat heart [72]. This was followed by other studies showing that ischemia and reactive oxygen species activate p38 kinase and JNK (SAPK) [77, 78]. However, the activation of PKC and activation of tyrosine kinase and MAP kinase signalling cascades may all be epiphenomena, and may not be associated at all with preconditioning. Nevertheless, many abstracts in the last year meeting of the American Heart Association (nrs 391, 1396-1399, 1739, 1745,1746, 3207, 3860, 3861) reported results that implicated a role for tyrosine kinase and stress-activated kinase in classic and delayed preconditioning (Fig. 2). There is general lack of evidence concerning activity changes in the putative cytoprotective proteins, serving as substrates for the afore-mentioned kinases (protein phosphorylation), or about a possible effect on their synthesis by a pre- or post-translational mechanism regulated by these kinases (protein expression). Furthermore, whether tyrosine kinases and/or stress-activated kinases are activated in series, parallel with PKC activation and/or as consequence of PKC activation remains to be established. Similar to the cross-talk between PLC-β and PLD (Chapter 3), there are multiple links between PKC, tyrosine kinase and stress-activated protein kinase (see the simplified review in Fig.3 in Chapter 1). To resolve the steps of this network that are critical to the triggering and mediation of the preconditioning effect, will be an enormous task. Most of the studies are limited to pharmacological interventions or to measurement of a specific kinase translocation/activation at one or two time-points during the transient ischemia/reperfusion period or sustained ischemic period. However, to unravel the logistics of the interactions of these complex signalling system for the triggering and mediation of preconditioning, the time-course of each reaction step in the signalling cascade involved over the whole period from a brief preconditioning ischemia, reperfusion, and sustained ischemia, need to be analysed.

Fig. 2. Simplified representation of cellular events thought to occur after a preconditioning stimulus. Dashed arrows represent pathways for which only suggestive evidence was obtained. Solid arrows represent pathways established with some experimental evidence (adapted from Yellon *et al.* [52]).


Agonist
K-ATP channel
Na+
R
R
G
PtdInsP2
PtdChol
G
?
PLC
PLD
Rec.
Tyr-
kinase
Sarcolemma
K-ATP channel
mitochondrion
H+
?
1,2-DAG
PKC
Cytosol
other
phosphorylated
substrate proteins
MYOCARDIAL
PROTECTION
protein
tyr-kinase
MAP kinase
families
FIRST WINDOW OF PROTECTION
(CLASSICAL PRECONDITIONING)
transcription factors
gene transcription
nucleus
new protein
synthesis
MYOCARDIAL
PROTECTION
SECOND WINDOW OF PROTECTION
(DELAYED PRECONDITIONING)

Classic ischemic preconditioning: involvement of the Na^+/H^+ exchanger?

All known classic preconditioning stimuli appear to converge in PKC. However, the target proteins, distal mechanisms and fundamental effectors of the cardioprotection remain unclear. A potential role of the Na^+/H^+ exchanger in mediating classic preconditioning has been proposed by a number of investigators [reviewed by 79]. Steenbergen *et al.* [80] were the first to demonstrate that $[H^+]_i$, $[Na^+]_i$ and $[Ca^{2+}]_i$ rose to a lesser extent during ischemia in preconditioned hearts than in unpreconditioned hearts. That myocardial acidosis during sustained ischemia is less severe in preconditioned hearts was also shown by others [81, 82] who attributed this finding to glycogen depletion during transient ischemia which limits the amount of substrate available for anaerobic glycolysis. Furthermore, Steenbergen *et al.* [80] hypothesized that one of the beneficial effects of preconditioning is the slowing down of anaerobic glycolysis, resulting in less acidosis, less Na^+/H^+ exchange and less Na^+/Ca^{2+} exchange than in untreated hearts. In accordance with this proposal, data from several independent laboratories which used specific Na^+/H^+ exchanger inhibitors, showing that Na^+/Ca^{2+} exchange is implicated in ischemic injury [reviewed in 83]. Thus, early termination of the injurious H^+ accumulation that would lead to Ca^{2+} overload, remains an attractive mechanism as recently reviewed by Fröhlich and Karmazyn [79]. Miura *et al.* [84] and Bugge and Ytrehus [5] showed that Na^+/H^+ exchange inhibitors limited infarct size in rabbit and rat hearts respectively. However, Miura *et al.* [84] reported a lack of PKC involvement in the protective effect of Na^+/H^+ exchange inhibition by HOE 642 compared to the apparent requirement for PKC activation for preconditioning, providing evidence for dissimilar mechanisms. In agreement with the latter study, is the observation that at concentrations which effectively inhibited Na^+/H^+ exchanger inhibition, HOE 642 failed to reverse the preconditioning effect [85]. It appears, therefore, that further studies are necessary in order to unravel the role of PKC activation and Na^+/H^+ exchanger inhibition in ischemic preconditioning, since their beneficial effects appear to be partially additive [5, 86]. At this point experimental work on the alkalinization responses of ET-1, α_1-adrenergic agonist PHE without such a response of phorbolester in cultured cardiomyocytes reported in this thesis (Chapter 5) should be taken into account. Firstly, PMA did not significantly affect pH in these cells, which indicates that direct PKC activation does not cause an increase in Na^+/H^+ exchanger activity. Secondly, ET-1 and to a slight extent PHE induced PKC-ε translocation as well as a limited alkalinization of the cells. Thus, activation of the Na^+/H^+ exchanger by these triggers of preconditioning is difficult to reconcile with a potential beneficial effect on the heart. On the contrary, the resulting Na^+ gain is believed to contribute to deleterious Ca^{2+} overload via Na^+/Ca^{2+} exchange during ischemia and/or during the initial phase of reperfusion [83]. In this respect it is interesting to note that Rehring *et al.* [87] tried to find an explanation for α_1-adrenergic preservation of myocardial pH during ischemia. Neither of the two cPKC isoforms tested (PKC-α and -β) were likely to be involved in the α-adrenergic agonist and exogenous 1,2-DAG induced anti-acidotic effects. These authors hypothesized that the Ca^{2+} mobilization by the $Ins(1,4,5)P_3$ limb of PLC-β activation is required for attenuation of ischemic acidosis, because they observed lack of translocation of the Ca^{2+} independent PKC-ε form by α_1-adrenergic agonist. However, our group showed previously in cultured cardiomyocytes that the $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ accumulation are of minor importance in the marginal Ca^{2+} response evoked by α_1-adrenergic agonist [34].

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SUMMARY

The experimental work described in this thesis provides evidence for the occurrence of cross-talk between receptor-mediated PLC-β and PLD signalling pathways in rat cardiomyocytes and the possible involvement of PKC isozyme translocation/activation. It increases our insight in the mechanism(s) of stepwise activation of PLC-β, PLD and PKC in myocardium which is necessary, because these signalling systems have been shown to be involved in several myocardial adaptive responses such as hypertrophy and ischemic preconditioning. Indeed in the mean time a recent study on transgenic mice has convincingly demonstrated that Gq (receptor-G-protein interface that leads to PLC-β activation) is a critical molecule in the initiation of high pressure-induced hypertrophy in myocardium.

A preparation of serum-free cultured, beating rat cardiomyocytes, almost free from other celltypes (endothelial cells, fibroblasts, smooth muscle cells) present in the heart, is used as model to evoke a maximal response of protein synthesis and to study the signalling processes involved. The hypertrophic and ischemic preconditioning responses have many stimuli in common, among them are α_1-adrenergic agonists, ET-1 and AngII. Therefore, these agonists are tested in the cardiomyocyte preparation.

Over the past few years the presence of active PLD in myocardium was fairly well established (reviewed in chapter 2). The enzyme potentially contributes to the cellular pool of 1,2-DAG, which activates isotypes of PKC. The members of the PKC isozyme family may respond differently to the varying mixtures of Ca^{2+}, PtdSer, (molecular species of) 1,2-DAG and other membrane phospholipid metabolites including free fatty acids. Apart from activation of PLD, PKC isozymes are responsible for phosphorylation of specific cardiac substrate proteins involved in regulation of cardiac excitation-contraction coupling, sarcomere function, hypertrophic cell growth, gene expression, ischemic preconditioning and electric activity. However, a major problem is that most of the target proteins of PKC responsible for these effects are unknown.

The question how the activity of PLD is controlled by agonists in myocardium has remained controversial for a long time. Agonists, such as ET-1, ANF and AngII that were shown to activate PLD, also potently stimulate PLC-β in myocardium. Direct stimulation of PKC by PMA inactivates receptor-mediated PLC-β and potently activates PLD. Thus, PKC stimulation of PLD may be a major mechanism by which agonists, such as ET-1 and AngII, that promote PtdIns-4,5-P_2 hydrolysis, secondary activate PtdChol-hydrolysis (chapter 3). On the other hand, the initial product of PLD action PtdOH was postulated by others to secondary activate PtdIns-4,5-P_2 hydrolysis in cardiomyocytes. Whether direct receptor-G-protein coupling also is involved in myocardial PLD activation is not yet clear. We obtained preliminary evidence for involvement of GTP-binding proteins as well in activating PLD (chapter 8). Furthermore, PLD activation may also be mediated by an increase in cytosolic free Ca^{2+} or by tyrosine-phosphorylation.

In chapter 3 and 4 we study the contribution of PLD to the total cellular formation of PKC activator 1,2-DAG and the mechanism(s) involved in PLD activation by agonists. Serum-free cultured neonatal rat cardiomyocytes are stimulated by ET-1 (10^{-8} M), PHE (10^{-5} M) or Ang II (10^{-7} M) which results in an immediate (0-10 min) activation of PLC-ß to an extent (ET-1 > PHE > Ang II) that correlates with the magnitude of stimulation of protein synthesis ([^{3}H]leucine incorporation into total protein) measured after 24 h. Although not affecting PLC-β activity, PMA (10^{-6} M) is equipotent with ET-1 in stimulating PLD activity and protein synthesis. Similar to the rate of protein synthesis, the protein/DNA mass ratio increases after ET-1 and PMA stimulation, which confirms the existence of hypertrophic cell growth. ET-1 and PMA, but not PHE and Ang II, stimulate [^{3}H]choline formation from [^{3}H]PtdChol after a lag-phase of about 10 min. This delayed [^{3}H]choline formation is due to the action of PLD as confirmed by occurrence of [^{14}C]Ptd-group-transfer from cellular [^{14}C]palmitoyl-PtdChol to exogenous ethanol.

To obtain evidence for the involvement of PKC in PLD activation, cardiomyocytes are stimulated with agonists and the PKC isotype translocation is followed from the cytosol to the

membrane by measuring both immunoreactivity and kinase activity. ET-1 and to a much lesser extent PHE, produces rapid (0-5 min) translocation of PKC-ε immunoreactivity from the cytosol to the membrane, whereas no intracellular redistribution of PKC-α, -δ and -ζ immunoreactivities is observed. As expected, PMA causes translocation of PKC-α, PKC-ε as well as PKC-δ. Cellular redistribution of PKC activity, measured by [^{32}P]-incorporation from [γ-^{32}P]-ATP into histone III-S is not detectable with ET-1 and PHE, but it is definitely present during PMA stimulation. Recently, it was demonstrated by other groups that direct protein-protein interaction of PKC and PLD may occur as well, which could be the explanation for discrepancy between immunoreactivity and kinase activity data.
Down-regulation of PKC isozymes by 24 h pretreatment of cells with PMA or blockade of PKC by chelerythrine (10^{-4} M) inhibits ET-1 and PMA stimulated [^{3}H]choline production, which may indicate that PKC is indeed involved in PLD activation. Another PKC inhibitor, staurosporine (10^{-6} M) has, however, no effect. This PKC inhibitor is known to be rather unspecific and also appears to stimulate PLD. The results show that in serum-free cultured cardiomyocytes, ET-1 initially activates PLC-ß and after a lag-phase PLD. PHE and Ang II activate only PLC-ß. PLC-ß stimulated by ET-1, may cross-talk with PLD by the induction of PKC-ε translocation.

PKC is shown to be activated by 1,2-DAG, which can be derived from PtdIns (PLC-β activity), but also from PtdChol (PLD activity). In chapter 4 we investigate the consequences of simultaneous stimulation of PLC-β and PLD by agonists for the molecular species composition of 1,2-DAG and for that of phospholipids in cardiomyocytes. Again, serum-free cultured cardiomyocytes were stimulated by ET-1, PHE or PMA. The molecular species composition of 1,2-DAG (in mol %) and that isolated from PtdChol and PtdIns (in mol % after treatment with exogenous PLC) are analysed by HPLC. Their total concentration (nmol/dish) is determined by GLC. Phospholipids are also labelled with [^{14}C]glycerol or double labelled with [^{14}C]16:0 and [^{3}H]20:4n6 for measurement of the relative incorporation of labelled fatty acids into 1,2-DAG. The major molecular species of 1,2-DAG in unstimulated cells is found to be 18:0/20:4 (57 mol %). This molecular species appears also to be the predominant species of PtdIns (73 mol % compared to 11 mol % present in PtdChol). A significant decrease (about 10 mol %) is found for the 18:0/20:4 species of 1,2-DAG during stimulation (10-40 min) with ET-1 or PMA, and these results are in line with the results on PLC-β and PLD activation and PKC-ε translocation. In double label experiments we applied [^{14}C]16:0 as a reference fatty acid for PLD comparing to PLC-β activity, based upon the observed large difference in 16:0 contents of PtdIns (25 mol %) compared to PtdChol (4 mol %). [^{3}H]20:4 is used as reference fatty acid for PLC-β activity, because of its abundant presence in PtdIns. The results of the double label experiments are consistent with the HPLC analysis: the incorporation ratio [^{3}H]20:4/[^{14}C]16:0 in 1,2-DAG decreased from 1.70 in control to 1.40 during 10 min ET-1 or PMA stimulation, and not in the presence of PHE. Thus, the results demonstrate that during prolonged agonist stimulation relatively less 20:4 becomes incorporated in the total cellular pool of free 1,2-DAG. The [^{14}C]glycerol incorporation into total cellular 1,2-DAG remained relatively constant during agonist stimulation as did the concentration of 1,2-DAG measured by GLC. The results demonstrate that 1,2-DAG, present in unstimulated cardiomyocytes, is mainly derived from PtdIns. During stimulation with ET-1 and PMA, but not PHE, PtdChol becomes an increasingly important source for 1,2-DAG due to sustained activation of PLD. Most remarkably, the total 1,2-DAG concentration remained constant during agonist stimulation which provides evidence that particular molecular species of 1,2-DAG more than its total concentration determine the activation of PKC isoenzymes.

Because it was previously hypothesized that changes of intracellular pH (pH_i) plays a role in the mechanism of both ischemic preconditioning and hypertrophy, we employed ratio imaging of fluorescent ion indicator 2',7'-bis(carboxyethyl)-5',6'-carboxyfluorescein (BCECF) for measuring intracellular pH (pH_i) during stimulation by agonists of cardiomyocytes (chapter 5). Addition of 10^{-8}M ET-1 and 10^{-5}M PHE to cardiomyocytes causes pH_i to rise slowly to reach a small but significant maximal intracellular alkalinization after about 35 min. Exposure of the cells to 10^{-6} M PMA does, however, not significantly affect pH_i. On the other hand, the presence of potentially high activity

of Na^+/H^+ exchanger is demonstrated by the observed rapid recovery from intracellular acidosis in cardiomyocytes challenged by a 20 mM NH_4Cl pulse. It is concluded that only limited α_1-adrenergic and ET-1 induced intracellular alkalinization responses occur in serum-free cultured rat cardiomyocytes and that PKC-ε translocation likely is not involved.

The studies in literature that have tried to prove the involvement of PKC in ischemic myocardial preconditioning are reviewed in chapter 6. It was shown by us and others that signalling pathways via receptor-coupled PLC-β and its "cross-talk" with PLD converge to activation of PKC isotypes, followed by phosphorylation of until now unknown target proteins, ultimately producing the ischemic preconditioning response. We describe the general biochemical properties of PKC, the known isotypes and the limitations of the methodologies available to investigate the role of PKC in preconditioning. Pharmacological inhibition or activation and (immunore)activity and/or isotypes measurements of PKC isotypes were applied to assess their role in ischemic myocardial preconditioning. It is concluded from these results, that more definitive proof for the involvement of PKC in preconditioning requires future studies focussing on the different isotype(s) of PKC that are activated, the molecular species of 1,2-DAG involved, the duration of PKC action, its cellular translocation sites and the identity and stability of (covalently bound phosphate) of phosphorylated substrate proteins. Moreover, there is lack of studies on the preconditioned porcine heart as most of the previous studies in this field deal with either rat or rabbit heart.

In chapter 7 we experimentally address the question whether PKC plays a role in ischemic preconditioning of the porcine heart *in situ* by enzymatic and immunoreactivity measurements. In homogenate fractions we observe 1,2-DAG-stimulated activities towards the ε-peptide, the preferred substrate for PKC-ε, in the cytosolic fraction and not in the particulate fraction. Histone III-S phosphorylation is significantly higher in the cytosolic as well as particulate fraction. Immunoreactivity levels of PKC-α and -ε measured are not different in the particulate or cytosolic fraction. However, it should be noted that the immunological essay does not distinguish between active and inactive PKC isoenzymes. Finally, for several of the postulated endogenous mediators of ischemic preconditioning such as α_1-adrenergic-, A_1 adenosine and muscarinic agonists, ET-1 and AngII it is shown that in myocardium these stimuli (dependent on animal species) activate at least PLC-ß, and one of them (ET-1) also PLD, which is proven in chapter 3 for neonatal rat cardiomyocytes. It means that the 1,2-DAG which is activating PKC is not necessarily derived from PtdIns, but also from PtdChol.

SAMENVATTING

Het werk beschreven in dit proefschrift levert experimenteel bewijs voor het optreden van communicatie tussen twee receptor-gestuurde enzymen die zich in het celmembraan van ratten hartspiercellen bevinden en die betrokken zijn bij de overdracht van signalen van buiten naar binnen de hartspiercel. Deze enzymen, het receptor-gestuurde fosfolipase C-β (PLC) en het fosfolipase D (PLD) produceren beiden het signaal molecuul 1,2-diacylglycerol (1,2-DAG), medeactivator van het enzym eiwit kinase C (PKC) aanwezig in het celsap. Tijdens de activering verhuist het PKC naar het celmembraan en activeert op haar beurt weer andere eiwitten. Het resulteert uiteindelijk in een stijging van de aanmaak van praktisch alle eiwitten in de spiercel. Daardoor groeit de spiercel en hypertrofieert ten gevolge hiervan. Dit proefschrift geeft meer inzicht in de mechanismen van stapsgewijze activering van PLC, PLD en PKC. Dit is van belang omdat hypertrofie een aanpassingsproces van het hart is, die optreedt als gevolg van hart- en vaatziekten, zoals het acute hartinfarct, een hoge bloeddruk en kleplijden. De hypertrofie van de hartspiercellen gaat vooraf aan hartfalen. Bovendien lijken de enzymen PLC, PLD en PKC ook betrokken bij het mechanisme van een (experimenteel) fenomeen dat ischemische preconditionering wordt genoemd en ongeveer tien jaar gelden werd ontdekt. Namelijk, een korte periode van afsluiting van de kransslagader (ischemie) kan beschermt het hart tegen een volgende, langere en beschadigende periode van kransslagader afsluiting.

Wij hebben de enzymen PLC, PLD en PKC onderzocht in een model van kloppende rattenhartspiercellen die worden gekweekt in een speciaal groeimedium. Het laatste om een maximale opwekking van hypertrofe groei na te kunnen bootsen. Serum wordt in het groeimedium weggelaten, omdat dit veel groeifactoren bevat die al bij voorbaat hypertrofie veroorzaken. Uit eerder werk van ons laboratorium en dat van anderen is gebleken, dat neurohumorale stoffen zoals endotheline-1 (ET-1), angiotensine II (AngII) en de α_1-adrenerge agonisten zoals het fenylefrine (PHE), hypertrofie opwekken in de hartspiercelkweek. De invloed van deze verbindingen op de signaaloverdracht in hartspiercellen is vergelijkenderwijs bestudeerd zoals beschreven.

De afgelopen jaren was er al uitgebreid onderzoek gedaan om aan te tonen dat het enzym PLD aanwezig is in het hart (overzicht in hoofdstuk 2). Dit enzym vormt, evenals PLC, het 1,2-DAG. Er zijn verschillende types PKC, die geactiveerd worden door 1,2-DAG, en men denkt dat ze ieder een eigen functie hebben in de cel en op verschillende manier geactiveerd worden. Bijvoorbeeld, de concentratie van calcium in de cel, de soorten vetzuurstaarten gebonden in 1,2-DAG en het fosfatidylserine (dit fungeert ook als bouwsteen van het celmembraan) en vrije vetzuren zijn bij de PKC activering betrokken. De PKC's voegen gewoonlijk een fosfaatgroep toe aan eiwitten in de spiercel, die op DNA niveau de expressie van andere eiwitten kunnen veranderen. Dat verklaart het optreden van hypertrofe groei en verandering van het fenotype in een chronisch overbelast hart en heeft misschien ook te maken met het mechanisme van ischemische preconditionering.

Het aanvankelijke produkt van de PLD werking, het fosfatidaat, heeft mogelijk zelf ook een signaalfunctie, maar dit is nog onvoldoende onderzocht. De vraagstelling bij de experimenten van dit proefschrift was vooral hoe de werking van PLD wordt geregeld door neurohumorale stimuli, zoals het ET-1, ANF (atrial natriuretic factor) en AngII. We hebben aangetoond dat het ET-1 het PLD activeert en voorafgaand daaraan het PLC-β. Een lichaamsvreemde stof die in staat is PLD sterk te activeren, maar PLC-β juist remt is de tumor promotor PMA. PMA staat bekend om zijn eigenschap praktisch alle in de cel aanwezige PKC's direct te stimuleren. Alles wijst erop dat het ET-1 via PLC de afbraak van het fosfatidylinositol-4,5-bifosfaat (bouwsteen van het celmembraan) stimuleert, en pas later via PLD de afbraak van het fosfatidylcholine (bouwsteen van het celmembraan). De activering van PKC door de werking van PLC geeft aanleiding tot activering van het PLD (verklaart de communicatie tussen PLC en PLD) (hoofdstuk 3). Directe activering van het PLD is ook mogelijk, bijvoorbeeld via een membraan receptor gekoppeld aan een G-eiwit. Ook voor het laatst genoemde mechanisme hebben we experimentele aanwijzingen verkregen en Ca^{2+} en tyrosine fosforylering spelen vermoedelijk hierbij een rol (zie hoofdstuk 8).

De bovenstaande hypothese van PLC activering die leidt tot PLD activering hebben we in hoofdstuk 3 en 4 bestudeerd in hartspiercellen. Voor dit doel hebben we de spiercellen gestimuleerd met ET-1 (concentratie 10^{-9} M), PHE (10^{-5} M) of AngII (10^{-7} M) hetgeen resulteerde in activering van PLC en PLD. De mate van PLC en PLD stimulering door de verschillende stoffen komt overeen met de mate van stijging van de eiwitaanmaak (hypertrofie), die pas na 24 uur optreedt. PMA (10^{-6} M) blijkt even sterk als ET-1 de eiwitaanmaak te stimuleren. Het DNA gehlate van de cellen blijft nagenoeg constant, het aantal cellen verandert immers niet. De gewichtsverhouding van eiwit over DNA neemt inderdaad vooral na ET-1 en PMA stimulatie toe, hetgeen betekent dat er sprake is van hypertrofie. Immers, als de hoeveelheid DNA zou zijn toegenomen, dan zou er sprake geweest zijn van celdeling en hypertrofie is slechts een vergroting van het volume van al bestaande cellen.
De activiteit van het PLD is gemeten aan de hand van vorming van radioactief choline uit radioactief gemerkt fosfatidylcholine. Deze choline vorming werd pas zichtbaar na een aanloopfase van rond 10 minuten. Het PLD kan ook via een andere weg aangetoond worden, namelijk door meting van fosfatidylethanol dat gevormd wordt uit alcohol toegevoegd aan het incubatiemedium.

Om de rol van het PKC bij de activering van PLD te bepalen hebben we hartspiercellen gestimuleerd met genoemde stimuli en de verhuizing van de PKC isoenzymen van het celsap naar de celmembraan gemeten. ET-1, maar in veel mindere mate PHE, veroorzaken een snelle verhuizing (0-5 min) van de PKC-ε vorm. Dit geldt niet voor de PKC-α, -δ en -ζ vormen. Zoals verwacht, de tumorpromotor PMA veroorzaakt een verhuizing van zowel PKC-α, -ε als -δ. De PKC activiteit gemeten door middel van fosfaatgroep inbouw in het eiwit histon IIIS verhuist niet, behalve bij PMA stimulatie. Bij langdurige PMA stimulering wordt het PKC geleidelijk aan inactief. PKC's kunnen ook geblokkeerd worden door toevoeging van remmers, zoals chelerythrine. Beide ingrepen voorkomen de stimulering van PLD door ET-1 en PMA, hetgeen betekent dat PKC erbij betrokken is. Dus, het ET-1 stimuleert allereerst PLC via de ET_A receptor en het gevormde 1,2-DAG veroorzaakt PKC-ε verhuizing naar het membraan. Hier activeert het PKC-ε het PLD. Met andere woorden, het PKC-ε verzorgt de communicatie tussen PLC en PLD.

Uit het voorgaande volgt dat het 1,2-DAG, aanvankelijk gevormd uit het fosfatidylinositol-bisfosfaat, later voornamelijk afkomstig is uit fosfatidylcholine. We zijn dit nagegaan door de vetzuren aanwezig in het 1,2-DAG te analyseren voor en na stimulering van cellen met het ET-1 of PHE (hoofdstuk 4). De verschillende combinaties van vetzuren in het 1,2-DAG (in mol %) en die aanwezig in fosfatidylcholine en fosfatidylinositol worden geanalyseerd met een scheidingsmethode voor intacte diacylglycerolen ("high performance liquid chromatography") en voor de vetzuren na afsplitsing ("gas-liquid chromatography"). In andere experimenten zijn de fosfolipiden eerst radioactief gemerkt met koolstof 14 bevattend glycerol en palmitinezuur (16:0) en tritium bevattend arachidonzuur (20:4). Vervolgens wordt in het door de hartspiercellen gevormde 1,2-DAG de aanwezigheid van deze radioactieve gemerkte stoffen bepaald. De meest voorkomende vetzuur combinatie die wordt aangetroffen in 1,2-DAG van niet-gestimuleerde cellen is het 18:0/20:4 (57 mol %). Deze vetzuur combinatie blijkt voornamelijk aanwezig te zijn in het fosfatidylinositol (73 mol % vergeleken met 11 mol % in fosfatidylcholine). Een lichte afname (± 10 mol %) in 18:0/20:4 treedt op in 1,2-DAG na stimulering van cellen met ET-1 of PMA en, zoals verwacht op basis van de PLC en PLD analyses, niet met PHE. De resultaten van de radioactieve experimenten zijn hiermee in overeenstemming: de verhouding getritieerd 20:4 over koolstof 14 gemerkt 16:0 in 1,2-DAG neemt af van 1.70 in niet-gestimuleerde cellen tot 1.40 tijdens 10 min ET-1 of PMA stimulering, en niet tijdens PHE stimulering. De inbouw van koolstof 14 gemerkt glycerol in het 1,2-DAG blijft constant na stimulering met agonist, evenals de concentratie van het 1,2-DAG. Samenvattend, deze resultaten laten zien dat 1,2-DAG aanwezig in niet-gestimuleerde cellen vrijwel alleen afkomstig is van fosfatidylinositol. Tijdens stimulering met ET-1 en PMA, maar niet met PHE wordt fosfatidylcholine in toenemende mate een bron voor het 1,2-DAG door activiteit van het PLD. Een ander belangrijk resultaat is dat de 1,2-DAG concentratie relatief constant blijft tijdens ET-1 stimulering. Dus blijkbaar zijn de soorten vetzuren in 1,2-DAG, meer dan haar concentratie bepalend zijn voor de activering van

het PKC-ε.

Omdat in de literatuur de verandering van de intracellulaire zuurgraad (pH_i) een rol werd toebedacht in het mechanisme van hypertrofe groei en het fenomeen ischemische preconditionering, hebben we in ons kweekmodel ook hier aandacht aan besteed. Men beweerde namelijk dat het PKC de Na^+/H^+ uitwisselaar in het celmembraan stimuleert. Door middel van imaging van een fluorescerende H^+ indicator wordt de pH_i-verandering in beeld gebracht tijdens stimulatie van de cellen met ET-1, PHE of PMA (hoofdstuk 5). ET-1 en PHE veroorzaken inderdaad een lichte stijging van de pH_i maximaal na ongeveer 35 min. PMA heeft geen effect. De Na^+/H^+ uitwisselings carrier in het hartspiercelmembraan reguleert de pH_i. Deze blijkt een hoge activiteit te bezitten. Dit is door ons aangetoond door de cellen kort bloot te stellen aan een hoeveelheid NH_4Cl. De verzuring die dan optreed verdwijnt daarna weer snel. Onze conclusie is dat PHE en ET-1 een licht alkaliniserende werking hebben op de cellen en dat PKC activering hiervoor niet verantwoordelijk kan zijn.

De uitgebreide literatuur over de sleutelrol van het PKC in het mechanisme van het fenomeen ischemische preconditionering is samengevat en kritisch beschouwd in hoofdstuk 6. Dit hoofdstuk geeft een algemeen overzicht van de biochemische eigenschappen van het PKC en de verschillende isoenzymen die kunnen voorkomen. Effecten van bekende remmers van het PKC op de ischemische preconditionering en omgekeerd nabootsing van het fenomeen door activering van PKC, zijn veelvuldig gehanteerd als experimenteel bewijs voor de sleutelrol van het PKC. Echter, activiteitsmetingen van PKC ontbreken in de meeste studies. Tevens hebben we in dit overzichtsartikel voor het eerst onder de aandacht gebracht dat niet alleen het PLC, maar ook het PLD, verantwoordelijk moet zijn voor het door korte ischemie gevormde 1,2-DAG. Immers, in dit proefschrift is voor één van de gepostuleerde endogene triggers van ischemische preconditionering, het ET-1, het optreden van activering van zowel PLC-β als PLD aangetoond. Er is dus meer behoefte aan studies met nadruk op activiteitsmetingen van PKC, PLC en PLD tijdens ischemische preconditionering. Bovendien, moet meer onderzoek verricht worden aan de doeleiwitten van het PKC die verantwoordelijk moeten zijn voor het beschermende effect. Ook moet de vraag worden beantwoord hoe stabiel het fosfaat is dat in deze doeleiwitten wordt ingebouwd, gezien de tijdelijkheid van het fenomeen.

In hoofdstuk 7 beschrijven we enkele experimenten in onder narcose gebrachte varkens waarbij PKC activiteit in ischemisch gepreconditioneerd hart immunologisch en enzymatisch wordt gemeten. De resultaten van deze experimenten ondersteunen de hypothese dat PKC bij het ontstaan van preconditionering betrokken is. Aan de hand van een specifiek substraat voor het PKC-ε, het zogenaamde ε-eiwit, vinden we namelijk een toegenomen PKC activiteit in het celsap, maar niet in de membraanfractie. Met histon III-S als algemeen substraat , is de PKC activiteit zowel in celsap als membraanfractie verhoogd. Immunologische analyse van PKC-α en -ε leveren geen verschillen op. Het is mogelijk dat de laatste analyse niet toereikend is om tussen actief en niet-actief PKC onderscheid te maken.

SAMMENFATNING

Hovedmålet for denne afhandling er at bevise en mulig kommunikation mellem to enzymer i plasmamembranen omkring hjerteceller som er involveret i signal overførsel fra udenfor til indeni cellen. Disse enzymer, fosfolipaseC-β og fosfolipase D former begge to signalmolekylet 1,2-diacylglycerol, som aktiverer proteinkinase C i cellevæsken. Ved denne aktivering flytter proteinkinase C sig fra cellevæsken til cellens plasmamembran og kan aktivere andre proteiner, som endelig resulterer i en forandret produktion af cellens proteiner. Disse signaloverførsels-veje er muligvis involveret i opståen af adaptions processer som hypertrofi (forstørret hjerte) og iskemisk prekonditionering (korte perioder af forsnævring af hjertets kranspulsåre som kan beskytte hjertet til en næste længere periode af kranspulsåre forsnævring).

Hjertemuskelceller fra nyfødte rotteunger, kultiveret til en kontraherende lag hjerteceller i en petriskål, er kultiveret i vækst medium uden serum, for at få en god udvikling af hypertrofi. Serum består af mange vækstfaktorer som på forhånd kan bevirke hypertrofi med mulig engagement af andre faktorer, som hormoner/ vækstfaktorer vanskeliggører undersøgelser. Litteratur har vist at nogle stoffer kan bevirke hypertrofi og iskemisk prekonditioning. Mellem disse befinder sig muglige stoffer som fenylefrin, endothelin-1 og angiotensin II, som vi har også brugt i vores experimenter.

Vi har vist at enzymerne fosfolipase C og D virkelig kommunikerer via aktivering af forskellige former af proteinkinase C. Signalstoffet 1,2-diacylglycerol aktiverer proteinkinase C og bliver produceret af både fosfolipase C og D. Det er ikke niveauet af 1,2-diacylglycerol som er mest vigtigt i aktiveringen af proteinkinase C, men det er kombinationen af fedtsyrer i 1,2-diacylglycerol som gør det. Spørgsmålet om hvilke kombinationer af fedtsyrer og hvilke former af proteinkinase C bliver aktiveret forbliver endnu ubesvaret.

Signaloverførsels-veje som bevirker hypertrofi og iskemisk prekonditionering viser sig at være meget komplicerede og forskellige stimuli bevirker ikke altid det samme reaktion. *Summa Sumarum:* I denne afhandling er der svaret på enkelte af spørgsmålene i hypertrofiens og iskemisk preconditionerings gåden, men der er stadig masser af spørgsmål til masser af forskning i mange år endnu.

ABBREVIATIONS

1,2-DAG	1,2-Diacylglycerol
AA	Arachidonic acid
AC	Adenylate cyclase
ADP	Adenosine 5'-diphosphate
ANF	Atrial natriuretic factor
Ang II	Angiotensin II
aPKC	Atypical protein kinase C
ARF	ADP-ribosylating factor
ATP	Adenosine 5'-triphosphate
BCECF	2',7'-bis(carboxyethyl)-5',6'-carboxyfluorescein
BIS	Bisindolylmaleimide
CaM-PK	Ca^{2+}-calmodulin dependent protein kinase
cAMP	Adenosine 3'5'-monophosphate
CP	Cardioprotective
cPKC	Classical protein kinase C
CPP	Classical preconditioning period
c-raf	MAPKKK
CT	Cardiotropin
DNBC	3,5-dinitrobenzoylchloride
DOG	1,2-dioctanoyl sn-glycerol
DPH	Diphenyl-1,3,5-hexatriën
EGF	Epidermal growth factor
EGTA	Ethylene glycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetra-acetic acid
eIF	Eukaryotic initiation factor
ERK	Extracellularly regulated kinase (MAPK)
ET-1	Endothelin-1
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FR	Functional recovery
GC	Gas liquid chromatography
GDP	Guanosine 5'-diphosphate
GEF	Guanine nucleotide exchange factor
GLC	Gas liquid chromatography
G-protein	GTP-binding protein
Grb	Growth factor binding protein
GTP	Guanosine 5'-triphosphate
GTPγS	Guanosine-5'-O(3-thio-triphosphate)
H-7	1-(5-isoquinolinesulfonyl)-2-methylpiperazine
HPLC	High performance liquid chromatography
HPTLC	High performance thin-layer chromatography
I	Ischemia
IGF	Insulin-like growth factor
Ins(1,4,5)P_3	Inositol 1,4,5-trisphosphate
Ins (1,3,4,5)P_4	Inositol 1,3,4,5-tetrakisphosphate
ISL	Infarct size limitation
JAK	Janus associated factor
JNK	C-jun NH_2 terminal kinase
LADCA	Left anterior descending coronary artery
LCXCA	Left circumflex coronary artery
LIF	Leukaemia inhibitory factor
MAPK	Mitogen-activated protein kinase (ERK)

MAPKK	Mitogen activated protein kinase-kinase
MAPKKK	MAPK kinase kinase (c-raf)
MARCKS	Myristoylated-alanine-rich-C-kinase substrate
MEK	MAPK/ERK kinase
MEKK	MAPK/ERK kinase kinase
MHC	Myosin heavy chain
nPKC	Novel protein kinase C
OAG	Oleyl-acetyl glycerol
PAS	Periodic acid Schiff
PBD	4β-phorbol-12β,13α dibutyrate
PDGF	Platelet derived growth factor
PHAS	Phosphorylated heat and acid stable protein
PHE	Phenylephrine
PKA	cAMP dependent protein kinase
PKC	Protein kinase C
$PLA_{1,2}$	Phospholipase $A_{1,2}$
PLC-β	Phospholipase C-ß
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PtdChol/-Eth/-Etn/-Ins/-Ser	Phosphatidylcholine/-ethanol/-ethanolamine/-inositol/-serine
$PtdIns(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate
PtdOH	Phosphatidic acid
R	Reperfusion
RACK	Receptor for activated C-kinase
SAG	1-stearyl-2-arachidonoyl glycerol
SAPK	Stress activated protein kinase
SH	Src homology domain
SIE	Sis inducing element
SIF	Sis inducing factor
SL	Sarcolemma
Sos	Son of sevenless
Sph	Sphingomyelin
SR	Sarcoplasmic Reticulum
STAT	Signal transducer and activator of transcription
SWOP	Second window of protection
TAG	Triacylglycerol
TCA	Trichloric acetic acid
TCF	Ternary complex factor
TGF-β	Transforming growth factor-β
TLC	Thin layer chromatography
TPA	12-O-tetradecanoylphorbol-13-acetate

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Abstracts

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* **Y.E.G. Eskildsen-Helmond**, K. Bezstarosti, D.H.W. Dekkers, B. Engelmann, J.M.J. Lamers. Is receptor-mediated phospholipase D indirectly linked to phospholipase C in cardiomyocytes? Proc. of the XVIII Annual Meeting, International Society for Heart Research North American Section on Cellular Signaling in the Cardiovascular System, June 9-13, 1996, Chicago, USA.
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Dan 'last but certainly not least', mijn man, mijn beste vriend en maatje, Peter, we hebben samen heel wat doorgemaakt en je hebt heel wat met mij te doen gehad, soms een dip, soms heel veel enthousiasme, je wist me altijd weer op te beuren en me door dik en dun te steunen. Ik kijk er naar uit om weer meer tijd samen door te kunnen brengen, want ik de laatste intensieve maanden alleen in Nederland en jij in Denemarken was niet altijd even leuk en makkelijk, maar het was ook een extra stok achter de deur. Ik kijk uit naar onze 'nieuwe' toekomst samen, wij redden het samen wel, dat hebben we al bewezen!

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Yvonne

CURRICULUM VITAE

Yvonne Elisabeth Geertruida Eskildsen-Helmond, werd geboren te Rotterdam op 14 Oktober 1967. In 1987 behaalde zij haar diploma VWO aan de openbare scholengemeenschap J.C. de Glopper te Capelle a/d Yssel. Hierna begon ze aan haar studie Biologie aan de Rijksuniversiteit Utrecht, waarin zij de specialisatie-richting Medische Gerichte Biologie volgde. Tijdens haar studie volgde zij een cursus Didactiek en gaf 3 maanden Biologie les als stagiaire aan de bovenbouw HAVO/VWO op het Gregorius college te Utrecht. De studie werd afgesloten in 1993 met de volgende doctoraal stages:

*Bij de Vakgroep Moleculaire Celbiologie, Rijksuniversiteit Utrecht, Projectgroep Electronenmicroscopische structuuranalyse (begeleiders: Prof. Dr. A.J. Verkleij, Ing. C. Schneijdenberg) bestudeerde zij de fatty-acid overload als een verklaring voor de primaire oorzaak van ischemische beschadiging (een biochemisch en morfologisch onderzoek aan hartspiercellen).

*Bij de Vakgroep Haematologie, faculteit Geneeskunde, Academisch Ziekenhuis Utrecht (begeleiders: Prof. Dr. J.J. Sixma, Ir. H. van Zanten) bestudeerde zij de extracellulaire matrix van gladde spiercellen in atherosclerose en restenose onder flow-condities (een morfologisch en immunohistochemisch onderzoek).

Aansluitend aan haar studie was zij van September 1993 tot September 1997 werkzaam als onderzoeker in opleiding aangesteld door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO, Gebied Medische Wetenschappen) aan de Erasmus Universiteit te Rotterdam. Daar volgde zij AIO/OIO onderwijs, begeleidde geneeskunde studenten en verrichtte, onder leiding van Prof. Dr. J.M.J. Lamers, het in dit proefschrift beschreven onderzoek, waarvan een gedeelte tevens is uitgevoerd aan het 'Physiologisches Institut der Universität München', in Duitsland bij Dr. B. Engelmann en in het Academisch Ziekenhuis Leiden bij Prof. Dr. A. van der Laarse. Na haar promotie verhuist zij naar Denemarken.

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Yvonne Elisabeth Geertruida Eskildsen-Helmond, was born in Rotterdam on the 14th of October 1967. She completed her high school education (VWO) in 1987 at the J.C. de Glopper college in Capelle a/d Yssel and entered the M.S. program in Biology (Medical Biology direction) at the University of Utrecht. During her study she attended a Didactics course with three months practical training at the Gregorius college in Utrecht. She finished her studies in 1993 with the following subjects:

*At the Department of Molecular Cell Biology, University of Utrecht, Projectgroup Electronmicroscopy and Structure analysis (supervisors: Prof. Dr. A.J. Verkleij, Ing. C. Schneijdenberg) she studied the fatty-acid overload as an explanation for the primary cause of ischaemic damage (biochemical and morphological research in cardiomyocytes).

*At the Department of Haematology, faculty of Medicine and Health Sciences, University Hospital Utrecht (supervisors: Prof. Dr. J.J. Sixma, Ir. H. van Zanten) she studied the extracellular matrix of smooth muscle cells in atherosclerosis and restenosis under flow-conditions (morphological and immunohistochemical research).

After her study she worked from September 1993 until September 1997 as PhD-student, supported by the Netherlands Organisation for Scientific Research (NWO, Medical Sciences) at the Erasmus University Rotterdam. She attended PhD courses, taught medical students and worked, under supervision of Prof. Dr. J.M.J. Lamers, on the research as described in this thesis. Part of it was performed at the 'Physiologisches Institut der Universität München', in Germany with Dr. B. Engelmann and at the University Hospital Leiden with Prof. Dr. A. van der Laarse. After her defending she will emigrate to Denmark.
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