MYOCARDIAL α_1-ADRENOCEPTORS AND PHOSPHOINOSITIDE CYCLE

α_1-Adrenoceptoren en Fosfoinositide Cyclus in de Hartspiercel

PROEFSCHRIFT

Ter verkrijging van de graad van doctor

aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. C.J. Rijnvos
en volgens het besluit van het College van Dekanen.

De openbare verdediging zal plaatsvinden op
woensdag 6 december 1989 om 13.45 uur.

door

Johanna Theodora Antoinette Meij
geboren te Nijmegen

universiteits
Eburon
DRUKKERIJ
1989

PROMOTIECOMMISSIE

PROMOTOR:	Prof. Dr. J.F. Koster
CO-PROMOTOR:	Dr. J.M.J. Lamers
OVERIGE LEDEN:	Prof. Dr. P.R. Saxena Prof. Dr. T.J.C. Ruigrok Prof. Dr. P.D. Verdouw

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Zoals iemand, die sterk is, van zijn lichaamskracht geniet en behagen vindt in oefeningen waarbij hij hij zijn spieren kan gebruiken, zo geniet de analyticus van de geestelijke werkzaamheid, waardoor ingewikkelde problemen worden opgelost, zelfs wanneer het de banaalste dingen betreft.

Edgar Allan Poe, *The murders in the Rue Morgue.*

Voor mijn vader, in gedachte, en mijn moeder

Table of Contents

Chapter I

General Introduction

In vertebrates the cardiovascular system has an important function. The blood that runs through it transports oxygen from the lungs and metabolites to the tissues and removes carbondioxyde and catabolites. Central in this system is the myocardium that keeps the blood circulating by its rhythmic contractions. The mass of the myocardium consists of striated muscle tissue. Each muscle fiber, surrounded by the sarcolemma, contains parallel running myofibrils, each of which is constituted of serially repeated units of filaments. These form the contractile apparatus. Between the filaments lie many mitochondria, the tubular network of the sarcoplasmic reticulum (SR) and other cell components. A myocardial muscle fiber differs structurally from other striated muscle fibers because it is divided - by so-called intercalated discs - into individual, bi- or mononucleate cells. Functionally, however, the myocardium may be considered a syncytium as the intercalated discs do not restrict the conduction of the electrical impulse [Katz *et al.* 1978].

Rhythmic contraction of the myocardium is achieved by the repeating association/dissociation of two interdigitating filaments, the proteins actin and myosin. This association/dissociation is linked with phasic changes in cytoplasmic calcium concentration, as depicted in Figure I.1. Calcium ions enter the cardiomyocyte due to depolarization of the sarcolemma through voltage-dependent calcium channels and by the operation of sodium/calcium exchange. These entering calcium ions trigger the release of a larger quantity of calcium ions out of the intracellular stores of the sarcoplasmic reticulum. Binding of calcium to the myofilaments allows the formation of crossbridges between actin and myosin and thus force is generated. When the voltage-dependent calcium and sodium channels are closed, the sarcolemma repolarizes by increase in the potassium current. The removal of calcium occurs by reversed sodium/calcium exchange, by ATP-driven calcium pumps present in the sarcolemma and the sarcoplasmic reticulum and possibly by the electron transport chain driven calcium

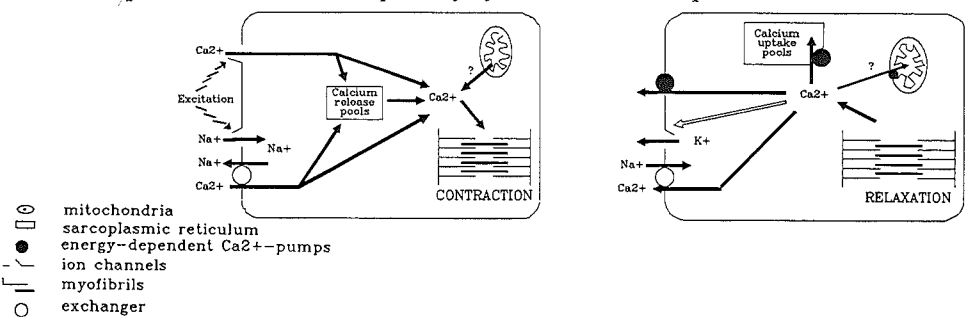

Figure I.1. Regulation of calcium movements in the myocardial cell

pump in the mitochondria [Hohnerjäger 1986]. As the force and duration of contraction are directly related to the magnitude and duration of the calcium signal the above-mentioned calcium movements determine the contraction/ relaxation cycle. Modulation of myocardial contraction and relaxation by extracellular signals like hormones, neurotransmitters and drugs, is therefore basically achieved through intracellular messengers influencing one or more of the calcium-transport systems [Chapman 1982].

I.1 Transduction of extracellular signals to the calcium-regulating systems

Generally, transduction of extracellular signals to the calcium-regulating mechanisms occurs through a cascade of reactions, the first of which is the binding of an agonist to a membrane receptor. The receptor is coupled to a membrane-bound system that generates a so-called "second messenger". This messenger transfers the signal to an intracellular location where possibly a third messenger is activated. The final messenger in the sequence often is a protein kinase that by phosphorylation affects a regulator protein. A number of messengers that occur in the myocardium are illustrated in Figure I.2.

Of a number of agents the pathway of action in the myocardium has been known for some time. Others have only partly been discovered. For instance (i) phospholipid methylation is believed to be a consequence of (ß-)adrenergic stimulation [Hirata and Axelrod 1980], however its role in further transduction of the signal is so far unknown; (ii) tyrosine kinases have recently been found to be directly activated by insulin, and factors like epidermal growth factor, but substrate proteins other than the receptor itself have not yet been identified [Denton 1986; Carpenter 1987].

The hormones adrenaline, glucagon, histamine, some prostaglandins and the sympathetic neurotransmitter noradrenaline act through stimulation or inhibition of cyclic AMP-levels. The effects of the increase in cyclic AMP level leading to increased intracellular calcium-concentrations in the myocardium are well known and have been reviewed [Tada and Katz 1982; Reuter 1983; Lamers 1985; Lamers 1987; Scholz 1986]. The parasympathetic neurotransmitter acetylcholine is thought to act through stimulation of the cyclic GMP-level [Doods *et al.* 1986]. The effects of cyclic GMP were often found to be the opposite of cyclic AMP effects but muscarinic cholinergic agonists only sometimes reverse cyclic AMP effects [Hartzell and Fischmeister 1986; Lindemann and Watanabe 1985]. However this may be due to a complex of adrenergic-cholinergic interactions at various levels [Doods *et al.* 1986].

A messenger function of calcium itself was first discovered in excitable cells, where it transfers the membrane depolarization signal to the secretory or contractile apparatus. Two calcium 'receptor' proteins have been identified: calmodulin (CaM) and troponin C [Rasmussen 1986a]. The many regulatory actions of the first of these two proteins in myocardial cells have been reviewed [Haiech and Demaille 1983; Lamers 1984; Lamers 1985]. However also in non-excitable cells calcium appeared to have the function of messenger for the receptors that were therefore called 'calcium mobilizing

Messenger nº	
1st:	*Extracellular* sarcolemma depolarization neurotransmitters, hormones pharmacological agents
2nd:	*Intracellular* Ca^{2+}, cyclic AMP, cyclic GMP, diglyceride (DG), inositoltrisphosphate (IP_3) N-methylated phosphatidylethanolamines (PMME,PDME) tyrosine kinases
3rd/4th:	cyclic AMP-dependent protein kinases (PKA) cyclic GMP-dependent protein kinase (PKG) cyclic GMP-activated cyclic nucleotide phosphodiesterase Ca^{2+} Ca^{2+}/phospholipid-dependent protein kinase (PKC) Ca^{2+}-calmodulin-dependent protein kinases: myosin light chain kinase phosphorylase *b* kinase,
	Target systems Ca^{2+}-pumping ATPases, Na^+/Ca^{2+} antiporter Ca^{2+}-channel, K^+-channel, sarcoplasmic reticular Ca^{2+} release channel phospholamban, calciductin troponin I, C-protein, Na^+/H^+ antiporter glycogen synthase *a/b* , glycogen phosphorylase *b/a*, myosin light chains
	Physiological effect

Figure I.2. Signal transducers in the myocardium

receptors'. A breakthrough in the search for the mechanism of these receptors was achieved after Michell (1975) noted the correlation between calcium mobilizing receptors and receptors coupled to the so-called 'phospholipid effect', the enhanced breakdown and resynthesis of phosphatidylinositol lipids.

Among the group of calcium-mobilizing receptors, however, there are some that are known to be present not just in non-excitable cells but also in excitable cells. So in

the latter cell type calcium has a dual messenger role. Reports that the α_1-adrenergic receptor was coupled to calcium mobilization in several cell types [Exton 1985] seem of particular interest with regard to their function in myocardial cells.

I.2 Alpha$_1$-adrenoceptors in the myocardium

Because of the predominance in the mammalian myocardium of adrenergic receptors of one (the ß-) type the simultaneous presence of α-adrenergic receptors was not revealed until specific ß-blocking agents became available [Benfey 1980]. By ligand binding techniques it has been established that the myocardial postsynaptic α-adrenoceptors, which mediate physiological responses, are of the α_1-type. The α_2-adrenoceptors are located presynaptically, where they mainly regulate neurotransmitter release. Details of α-adrenoceptor subclassification have been reviewed by Starke (1981).

Alpha$_1$-adrenoceptor stimulation in the myocardium of several mammalian species including humans [Brückner *et al.* 1984] leads -like ß-adrenergic stimulation- to inotropic effects triggered by enhanced slow inward calcium current. Closer investigation revealed that the electrophysiological effects of α_1-stimulation differ essentially from those of ß-stimulation. The development of the α_1-mediated inotropic effects is much slower; α_1-stimulation increases the contraction amplitude but - unlike ß-stimulation - does not reduce the 'time to peak tension' or the 'relaxation time'; α_1-stimulation activates glycogen phosphorylase only moderately but - like ß-stimulation - it inactivates glycogen synthase, activates phosphofructokinase and increases glucose uptake [Osnes *et al.* 1985]. However the predominance of $ß_1$-receptors masks these effects under 'normal' circumstances.

Lately it has become apparent that the contribution of α_1-adrenoceptors may be proportionally larger under various conditions of impaired ß-adrenergic influence such as hypothyroidism, diabetes, cholinergic stimulation, adenosine release and ischaemia [Brückner *et al.* 1985; Osnes *et al.* 1985; Lee 1989]. The relevance of α_1-adrenoceptors under these conditions is on one hand that they may act as a reserve mechanism for ß-receptors but also that they actually mediate the described arrhythmogenic effects of catecholamines during acute myocardial ischaemia and reperfusion [Sheridan *et al.* 1980; Sharma and Corr 1985; Culling *et al.* 1987; Thandroyen *et al.* 1987]. However, the reports that during ischaemia or postischaemic reperfusion the α_1-adrenergic receptor number increases [Mukherjee *et al.* 1980; Corr *et al.* 1981; Maisel *et al.* 1986] are contradicted by others [Broadley *et al.* 1985; Dillon *et al.* 1988].

I.3 The phosphoinositide cycle

Phosphatidylinositol (PI), of which in Figure I.3 the 1-stearoyl 2-arachidonoyl molecular species is shown, is a relatively minor lipid (10-15 % of total phospholipids) that occurs predominantly in the cytoplasmic leaflet of the lipid bilayer of cellular membranes [Gill

1985; Mayr 1988], including cardiac sarcolemma [Post *et al.* 1988]. In the plasma membrane two phosphorylated derivatives of PI are stepwise formed by ATP-requiring reactions. PI-kinase catalyzes the formation of phosphatidylinositol 4-phosphate (PI(4)P) and the subsequent action of PI(4)P-kinase results in the formation of phosphatidyl-inositol 4,5-bisphosphate ($PI(4,5)P_2$) (Figure I.4). The polyphosphoinositides PI(4)P and $PI(4,5)P_2$ account for only about 10 % of total PI lipids in membranes [Gill 1985; Mayr 1988].

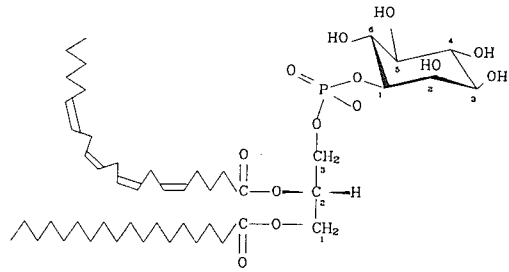

Figure I.3. The 1-stearoyl 2-arachidonoyl species of phosphatidylinositol

In 1953 Hokin and Hokin observed that certain hormones enhanced the phosphorylation of PI and phosphatidic acid. Later on it was found that this 'phospholipid effect' is secondary to the phosphodiesteratic cleavage of PI lipids by phospholipase C (PLase C) (Figure I.4) and that it precedes calcium mobilization. Contrary to the earliest assumptions the precursor molecule used by PLase C to generate second messengers is not PI. The finding that inositol 1,4,5-trisphosphate (IP_3) is the messenger responsible for the mobilization of calcium from intracellular stores was in itself not conclusive [Berridge 1983; Streb *et al.* 1983; Berridge and Irvine 1984]. But because of the combination of the facts that

(a) the polyphosphoinositides disappeared faster than PI,
(b) PI disappearance required energy and
(c) IP_3 appeared before inositol 1,4-bisphosphate ($I(1,4)P_2$ and inositol 1-monophosphate ($I(1)P_1$),

$PI(4,5)P_2$ seemed a likelier substrate for receptor-stimulated PLase C. The loss of PI can be explained as a result of its phosphorylation to $PI(4,5)P_2$ [Sekar and Hokin 1986]. The appearance of $I(1,4)P_2$ and $I(1)P_1$ can on one hand be seen as a consequence of the degradation of IP_3 by phosphatases. On the other hand the IP_3 induced rise in intracellular calcium enables PLase C to hydrolyze PI as well [Majerus *et al.* 1988]. The PI cycle is different from the other afore-mentioned signal transduction mechanisms in that primarily not one but (at least) two second messengers - IP_3 and diacylglycerol - are generated. Therefore the assumption that secondarily hydrolysis of PI takes place, would mean that then the emphasis of second messenger production is put on diacylglycerol (DG).

Although DG may be a precursor for other messengers like arachidonic and phosphatidic acid (Figure I.4), its main function is activation of protein kinase C (PKC)

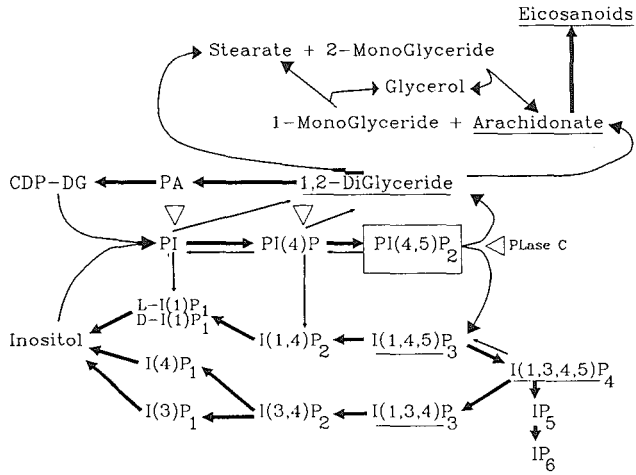

Figure I.4. The phosphoinositide cycle

[Nishizuka 1984]. Activated PKC firstly phosphorylates proteins that contribute to the final response. Secondly, it is also a modulator of several receptor mechanisms including the receptor-phosphatidylinositol pathway. The homologous and heterologous interactions of PKC in general have been extensively reviewed [Berridge 1986; Rasmussen 1986b; Berridge 1987]. Those in the myocardium will be detailed further below.

Calcium and PKC appear not to be the only third messengers activated in the PI pathway. It seems that IP_3 can be phosphorylated to inositol 1,3,4,5-tetrakisphosphate, which is proposed as the messenger responsible for initiating Ca^{2+} entry from the cell exterior [Berridge 1986; Irvine and Moor 1986; Nahorski and Batty 1986]. Its 5-monoesterase product inositol 1,3,4-trisphosphate is less potent than IP_3 on intracellular calcium stores [Mayr 1988]. The occurrence of inositol 1,3,4-phosphate in several tissues and the presence of a calcium- and calmodulin-dependent IP_3 3-kinase activity in many other tissues suggests that this may be a universal pathway [Ryu *et al.* 1987].

I.4 Regulation of the PI cycle

Important points of regulation of the PI cycle are the activities of PI-kinase and PI(4)P-kinase, which determine the availability of PLase C substrate. PI-kinase activity has been shown to be present in cellular membranes of various cell types, whereas PI(4)P-kinase activity has often been reported to be present in the plasma membrane only [Cockcroft *et al.* 1985; Hidalgo *et al.* 1986; Rawyler *et al.* 1987; Carrasco *et al.* 1988;

Stubbs *et al.* 1988]. However, papers reporting its localization in cardiac sarcoplasmic reticulum [Enyedi *et al.* 1984; Jakab *et al.* 1988] should not be ignored.

PI-kinase activity is - as usual for a kinase - Mg^{2+}- and ATP-dependent [Hidalgo *et al.* 1986; Knowles 1986] and is inhibited by mmolar Ca^{2+} [Rana and MacDonald 1986; Suga *et al.* 1986; Carrasco *et al.* 1988] and its product PI(4)P (0.5 nmol/mg protein) [Stubbs *et al.* 1988]. PI-kinase seems to be inactivated by adenosine and -related compounds [Doctrow and Lowenstein 1987] and has in one report been suggested to be inactivated by cyclic AMP [Lapetina 1986], but most other reports have shown activation by cyclic AMP-dependent phosphorylation [Enyedi *et al.* 1983; Sarkadi *et al.* 1983; Enyedi *et al.* 1984; De Chaffoy De Courcelles *et al.* 1986; Suga *et al.* 1986; Giraud *et al.* 1988; Jakab *et al.* 1988]. Also activation by Ca^{2+}/calmodulin-dependent kinase [Enyedi *et al.* 1984] and by PKC [Giraud *et al.* 1988] have been reported.

PI(4)P-kinase purified from rat brain has been described as a 100-110 kD protein stimulated by PS [Cochet and Chambaz 1986] however by others as a 46 kD protein [Van Dongen *et al.* 1986]. Generally PI(4)P-kinase activity is Mg^{2+}-dependent [Cochet and Chambaz 1986; Hidalgo *et al.* 1986; Lundberg *et al.* 1986] and requires nmolar Ca^{2+} [Carrasco *et al.* 1988]. The enzyme is also activated by GTP analogues [Urumow and Wieland 1986] and by its substrate PI(4)P [1 mM, Varsanyi *et al.* 1986; 0.5 nmol/mg protein, Stubbs *et al.* 1988] and inhibited by $PI(4,5)P_2$ (substrate/product ratio 10 to 2) [Lundberg *et al.* 1986; VanRooijen *et al.* 1986]. Furthermore PI(4)P-kinase is activated by cyclic AMP-dependent phosphorylation [Enyedi *et al.* 1983; Sarkadi *et al.* 1983; Enyedi *et al.* 1984; Jakab *et al.* 1988] and calcium/calmodulin-dependent kinase [Enyedi *et al.* 1984], but not by PKC [Cochet and Chambaz 1986; Giraud *et al.* 1988].

Another point of regulation of the PI cycle is PLase C activity. The papers describing the properties of a PLase C are mostly based on its PI hydrolyzing ability, which may be entirely different from its $PI(4,5)P_2$ hydrolyzing properties. Furthermore, of the PI-specific PLase C there seem to be several types with different properties: e.g. in bovine heart four isoforms were found [Low and Weglicki 1983]. In contrast no information is available on the properties of $PI(4,5)P_2$-specific PLase C in myocardium.

Recently the coupling of the receptor to PLase C has been shown to be a point of regulation. In several pathways of transmembrane signalling members of a family of guanine nucleotide-binding proteins (G proteins, previously called N proteins) serve as transducers [Graziano and Gilman 1987] and the receptor-PLase C pathway seems to be one of these [Cockcroft and Gomperts 1985; Smith *et al.* 1986; Cockcroft 1987]. Binding of GTP to a G protein (in the case of PLase C called G_P) activates PLase C probably by reducing its calcium requirement to a physiological level [Uhing *et al.* 1986]. On one hand the point of regulation may be G_P, on the other hand it may also be the receptor, because PKC is suggested to inhibit the PI cycle by phosphorylation of G_P [Orellana *et al.* 1987] but also of an α_1-adrenoceptor subunit [Bouvier *et al.* 1987 ;Leeb-Lundberg *et al.* 1987]. The activity of PKC itself is reported to be down-regulated by proteolysis by a plasma membrane-bound, Ca^{2+}-activated neutral protease (CANP or calpain) [Suzuki *et al.* 1987; Young *et al.* 1987; Kishimoto *et al.* 1989].

I.5 Possible interactions between the PI cycle and other messenger systems in the myocardium

The most obvious points of interaction between PI cycle derived messengers and other messenger systems are common protein kinase substrates. The sarcoplasmic reticular and sarcolemmal Ca^{2+}-regulator phospholamban has been shown to be a substrate of Ca^{2+}/calmodulin-dependent protein kinase, cyclic AMP-dependent protein kinase and PKC [Tada and Katz 1982; Haiech and Demaille 1983; Iwasa and Hosey 1984; Movsesian *et al.* 1984; Lamers 1985; Yuan and Sen 1986], although also a contrary report showing no effect of PKC exists [Presti *et al.* 1985b]. A specific sarcolemmal 15 kD protein is a common substrate for cyclic AMP-dependent protein kinase and PKC [Presti *et al.* 1985a, 1985b]. Also PKC has been shown to desensitize the cyclic AMP-coupled ß(2)-adrenergic receptor by phosphorylation of a subunit [Limas and Limas 1985; Bouvier *et al.* 1987]. Furthermore PKC activates the Na^+/H^+-antiporter which may increase intracellular calcium indirectly via the Na^+/Ca^{2+}-antiporter [Hathaway and March 1989].

Interaction between phospholipid N-methylation and the PI cycle has been suggested at the level of PKC [Ishizaka *et al.* 1984; Crews and Heiman 1985; Villalba *et al.* 1987], at the level of $PI(4,5)P_2$ breakdown [Pike and DeMeester 1988] perhaps also at the level of phospholipid biosynthesis [Henry *et al.* 1984].

The presence of PKC activity [Wise *et al.* 1982a,b] and of endogenous substrates for PKC [Presti *et al.* 1985b; Yuan and Sen 1986] in the myocardium indicate that a PI cycle coupled receptor may be present in the myocardium. And indeed, among the wide variety of calcium-mobilizing receptors that have been identified as being coupled to PI breakdown [Sekar and Hokin 1986; Mayr 1988] the muscarinic cholinergic and α_1-adrenergic receptor are present in the myocardium. The results of Brown *et al.* (1983; 1985) suggested that both these receptor types were coupled to enhanced $PI(4,5)P_2$ hydrolysis, but that the α_1-adrenoceptor was the most potent. However, muscarinic cholinergic stimulation was known to inhibit cyclic AMP formation and thus counteract ß-adrenergic effects, whereas α_1-adrenergic stimulation did not.

I.6 Phosphatidylethanolamine N-methylation

Within the membrane exists, in addition to rapid PI phosphorylation turnover, a stepwise conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC). The methylation of PE is one of the two pathways through which PC is synthesized. The other, major pathway involves the transfer of phosphocholine from CDP-choline to diacylglycerol, whereas the transmethylation pathway comprises the transfer in three steps of methyl groups from S-adenosylmethionine (AdoMet) to the amino moiety of a PE molecule catalyzed by two or more phospholipid N-methyltransferases (Figure I.5) [Mato and Alemany 1983].

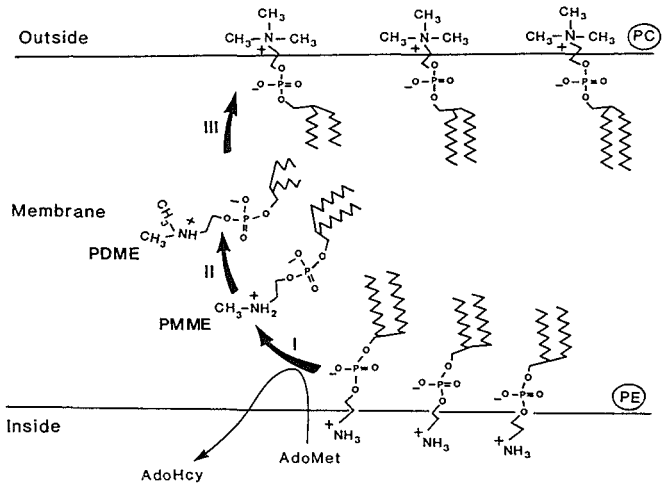

Figure I.5. Phosphatidylethanolamine N-methylation

It is generally thought that the methylated phospholipids are translocated from the cytoplasmic side of the membrane to the exterior surface, whereby the intermediate products phosphatidyl N-monomethyl ethanolamine (PMME) and phosphatidyl N,N-dimethyl ethanolamine (PDME) decrease membrane viscosity [Hirata and Axelrod 1980].

The liver is particularly rich in PE methyltransferase activity, probably to provide the necessary PC molecules for bile or lipoproteins. A co-ordinated mechanism to regulate the the PC level in the liver has been proposed [Mato and Alemany 1983]. This scheme is however not applicable to the many non-hepatic tissues in which PE methyltransferase activity also has been found [Vance and Ridgway 1988]. Indications that PE N-methylation may be involved in signal transduction are that inhibitors of PE N-methylation also inhibit the responses of cells to certain stimuli and that many cell stimuli (e.g. ß-adrenergic agonists) also activate PE N-methylation [Hirata and Axelrod 1980; Mato and Alemany 1983].

In the myocardium PE N-methylation has been characterized in sarcolemmal, mitochondrial and microsomal fractions [Panagia *et al.* 1984]. On the basis of kinetic parameters, pH profile and requirement for divalent cations the existence of three different catalytic sites has been demonstrated. Methyltransferase site I requires Mg^{2+}, is optimal at pH 8.0 and has an apparent K_m of 0.1 μM AdoMet; sites II and III have no cation requirement; site II has a pH optimum at pH 7.0 and a K_m of 3.6 μM AdoMet; site III has a pH optimum at pH 10.5 and a K_m of 119 μM AdoMet. Furthermore at each catalytic site one main product is formed: at site I PMME, at site II PDME and at site III PC [Panagia *et al.* 1984, 1985].

It has been shown *in vitro* that AdoMet-mediated PE N-methylation influences several membrane-related calcium-transport systems of the cardiomyocyte. Both the sarcoplasmic reticular and sarcolemmal Ca^{2+}-ATPase activities are activated by PE N-methylation [Panagia *et al.* 1987]. In the sarcolemma Na^+-dependent Ca^{2+}-uptake is inhibited upon methylation at sites II and III. Unaffected in methylated sarcolemmal membranes are however Na^+-induced Ca^{2+}-release and Na^+/K^+-ATPase activity [Panagia *et al.* 1987]. Furthermore changes in PE N-methylation activity have been identified in different experimental models of heart disease and methionine-induced PE N-methylation has been shown to produce a positive inotropic effect in isolated perfused rat heart [Panagia *et al.* 1988]. Although these findings suggest an important role for PE N-methylation in rat heart function, the exact mechanism is still unclear. In view of the reported changes in membrane viscosity [Hirata and Axelrod 1980] PE N-methylation may induce small changes in the specific phospholipid microdomains of membrane proteins such as enzymes and receptors, and thereby modify their function. This proposed way of action, however, could apply to PI turnover as well, because this process also induces small, local changes in the phospholipid environment of membrane proteins. Apart from the above-mentioned interactions between PE N-methylation and PI turnover no information is available about the direct interactions of these phospholipid signalling processes.

I.7 Possible involvement of polyunsaturated fatty acids in transmembrane signalling

The polyunsaturated fatty acid composition of membrane phospholipids reflects the composition of the diet, although not strictly [Takin *et al.* 1981; Stubbs and Smith 1984; Lands 1986]. The fatty acyl composition of membranes is regulated by enzymes within the endoplasmatic reticulum, the mitochondria, sarcoplasmic reticulum and sarcolemma. There is also competition between fatty acyl moieties for the incorporation into phospholipid molecules during *de novo* synthesis in the endoplasmic reticulum or during reacylation processes within the organelles and the sarcolemmal membrane. For example dietary n-6 polunsaturated fatty acids (PUFAs) are metabolically competitive with n-3 PUFAs [Fragiskos *et al.* 1986]. The oxidative desaturation of 18:2n-6 is inhibited by n-3 PUFAs. N-3 PUFAs of C20 and C22 chain length compete powerfully for the 5-desaturase with C20 n-6 fatty acids [Takin *et al.* 1981; Stubbs and Smith 1984]. Besides these competitive phenomena at the level of fatty acid synthesis, lysophospholipid formation followed by reacylation, transacylation and head group exchange all play an important role in the dietary fatty acid-induced adjustment of membrane fatty acyl group composition (Figure I.6). It has also been shown that in the myocardium adjustment of membrane fatty acyl incorporation, particularly in the n-3 PUFAs at the expense of 18:2n-6, occurs after chronic noradrenaline stress in rats [Montfoort *et al.* 1986]. It is not clear at present, whether this adjustment is caused by stimulation of myocardial α_1- or ß-adrenergic receptors.

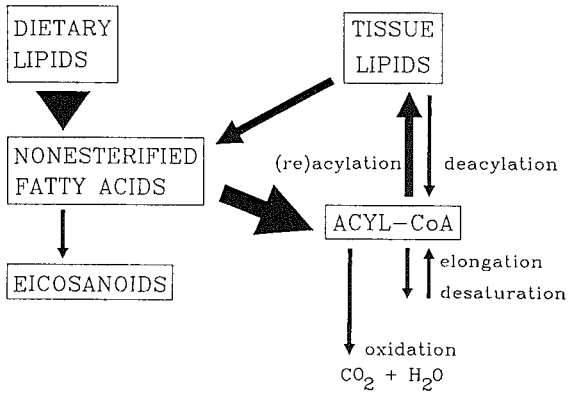

Figure I.6. The influence of dietary fatty acids on tissue fatty acyl group composition

All the consequences of extensive alterations in the phospholipid fatty acid composition for specific functions of the heart membrane, are not clear at present. It is generally assumed that the selectivities of the many enzymatic and transport functions of membrane depend mainly on the structural complexity of the protein molecules and that the phospholipids only have a supporting role. That this is a serious underestimation of the role of phospholipids is proven by the following facts. Firstly, the fluidity of the membrane core which probably affects agonist-receptor interaction and other membrane-linked processes, is partially determined by its PUFA composition [Farias *et al.* 1975]. Secondly, 20:4n-6 and 20:5n-3 generated from membrane phospholipids by (receptor-activated) phospholipase A_2, are enzymatically converted into eicosanoids (prostaglandins and leukotrienes). The major actions of eicosanoids on myocardial function are that coronary perfusion and the activity of blood-borne cells like platelets and leukocytes, are modified. A potent stimulus for local eicosanoid production and release is regional ischaemia and some eicosanoids appear to have close relations to pathophysiological events typical for myocardial ischaemia [Schrör 1985]. Thirdly, enzymes with a phospholipid substrate, especially the receptor-mediated PLase C, may have preference for a particular molecular species of phospholipid. Furthermore, in the case of PLase C, the PUFA composition of the diglyceride formed is critical for its potency to activate PKC [Takai *et al.* 1979; Kishimoto *et al.* 1980]. So, in conclusion, from a biochemical point of view it is apparent that phospholipid fatty acid composition can modulate certain processes.

Physiological consequences of altered myocardial phospholipid fatty acid composition have been reviewed recently [Lamers 1987]. With regard to its role in transmembrane signalling, however, reports of altered responses to catecholamines are

of main interest [Gudbjarnason *et al.* 1978; Crandall *et al.* 1982; Benediktsdòttir and Gudbjarnason 1986; Montfoort *et al.* 1986]. Most of these reports involve noradrenaline-mediated effects. That changes in phospholipid fatty acid composition also affect the response to α_1-adrenergic receptor stimulation has recently been reported by Reibel *et al.* (1988). However whether changes in PI turnover are implicated in the altered α_1-adrenergic response first had to be established.

I.8 The scope of this thesis

With regard to the present knowledge about α_1-adrenergic influences on heart function and changes seen in some pathophysiological states of the myocardium, as presented above, more detailed knowledge was required on the biochemical mechanism involved in the development of the cardiac α_1-adrenergic response. Based on the fact that this mechanism somehow involves changes in the intracellular calcium concentration and on the results of Uchida *et al.* (1982) and Brown *et al.* (1985) the possibility that α_1-adrenergic receptors are coupled to the PI cycle seemed very likely.

A lot of consideration and efforts were given to find and develop the most suitable model in which α_1-adrenoceptor mediated PI turnover in myocardial cells could be investigated. The considerations are detailed in Chapter II. The model finally chosen was that of cultured neonatal rat cardiomyocytes. A modified procedure was set up to yield an almost homogenous cardiomyocyte preparation. In this model it was attempted to characterize the biochemical response to α_1-adrenoceptor stimulation as enhancement of PI turnover (Chapter III).

The development of the α_1-adrenoceptor stimulated PI response and the possibility of (feedback) inhibition by PKC activation were studied next (Chapter IV). This study was carried out in view of reports that show an increased number of α_1-receptors in myocardial ischaemia. Intracellular messengers regulating the receptor activity were not taken into account in those reports [e.g. Corr *et al.* 1981]

The observation of phorbol ester modulation of α_1-adrenergic $PI(4,5)P_2$ hydrolysis brought us to investigate a cell free system, isolated microsomal membranes, in which the PLase C, endogenous PKC and substrate proteins for PKC could be detected and separately activated. The results of this study are described in Chapter V.

The phospholipid environment of the α_1-adrenergic receptor, the phosphoinositides, PLase C etc, may influence the PI-response. As an example the interactions between phospholipid methylation, a process that slightly changes the phospholipid environment, and the α_1-adrenergic receptor stimulated $PI(4,5)P_2$ breakdown were investigated (Chapter VI). Furthermore the phospholipid molecular species were changed by inducing incorporation of specific polyunsaturated fatty acids into the phospholipids of the cultured cells. The results of the study of α_1-adrenoceptor stimulated PI breakdown in these cells are described in
Chapter VII.

REFERENCES

1. BENEDIKTSDòTTIR VE, GUDBJARNASON S (1986) Arachidonic acid and docosahexaenoic acid content of sarcolemmal phospholipids in relation to ventricular fibrillation in rats. J Mol Cell Cardiol **18**: 90 (Abstr.)
2. BENFEY BG (1980) Cardiac α-adrenoceptors. Can J Physiol Pharmacol **58**: 1145-1158.
3. BERG I, GUSE AH, GERCKEN G (1989) Carbamoylcholine-induced accumulation of inositol mono-, bis-, tris- and tetrakisphosphates in isolated cardiac myocytes from adult rats Biochim Biophys Acta **1010**: 100-107.
4. BERRIDGE MJ (1983) Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. Biochem J **212**: 849-858.
5. BERRIDGE MJ (1986) Cell signalling through phospholipid metabolism. J Cell Sci Suppl **4**: 137-153.
6. BERRIDGE MJ (1987) Inositol trisphosphate and diacylglycerol: two interacting second messengers. Ann Rev Biochem **56**: 159-193.
7. BERRIDGE MJ, IRVINE RF (1984) Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature **312**: 315-321.
8. BOUVIER M, LEEB-LUNDBERG LMF, BENOVIC JL, CARON MG, LEFKOWITZ RJ (1987) Regulation of adrenergic receptor function by phosphorylation. II. Effects of agonist occupancy on phosphorylation of α_1- and $ß_2$-adrenergic receptors by protein kinase C and the cyclic AMP-dependent protein kinase. J Biol Chem **262**: 3106-3113.
9. BROADLEY KJ, CHESS-WILLIAMS RG, SHERIDAN DJ (1985) [^{3}H]Prazosin binding during ischaemia and reperfusion in the guinea pig Langendorff heart. Br J Pharmacol **86**: 759P
10. BROWN JH, BROWN SL (1984) Agonists differentiate muscarinic receptors that inhibit cyclic AMP formation from those that stimulate phosphoinositide metabolism. J Biol Chem **259**: 3777-3781.
11. BROWN JH, BUXTON IL, BRUNTON LL (1985) α_1-Adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. Circ Res **57**: 532-537.
12. BRÜCKNER R, MEYER W, MÜGGE A, SCHMITZ W, SCHOLZ H (1984) α-Adrenoceptor-mediated positive inotropic effect of phenylephrine in isolated human ventricular myocardium. Eur J Pharmacol **99**: 345-347.
13. BRÜCKNER R, MÜGGE A, SCHOLZ H (1985) Existence and functional role of alpha$_1$-adrenoceptors in the mammalian heart. J Mol Cell Cardiol **17**: 639-645.
14. CARPENTER G (1987) Receptors for epidermal growth factor and other polypeptide mitogens. Ann Rev Biochem **56**: 881-914.

15. CARRASCO MA, MAGENDZO K, JAIMOVICH E, HIDALGO C(1988) Calcium modulation of phosphoinositide kinases in transverse tubule vesicles from frog skeletal muscle. Arch Biochem Biophys **262**: 360-366.
16. CHAPMAN RA (1983) Control of cardiac contractility at the cellular level. Am J Physiol **245**: H535-552.
17. COCHET C, CHAMBAZ EM (1986) Catalytic properties of a purified phosphatidylinositol-4-phosphate kinase from rat brain. Biochem J **237**: 25-31.
18. COCKCROFT S (1987) Polyphosphoinositde phosphodiesterase: regulation by a novel guanine nucleotide binding protein G_p. Trends Biochem Sci **12**: 75-78.
19. COCKCROFT S, GOMPERTS BD (1985) Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature **314**: 534-536
20. COCKCROFT S, TAYLOR JA, JUDAH JD (1985) Subcellular localisation of inositol lipid kinases in rat liver. Biochim Biophys Acta **845**: 163-170.
21. CORR PB, SHAYMAN JA, KRAMER JB, KIPNIS RJ (1981) Increased α-adrenergic receptors in ischemic cat myocardium. J Clin Invest **67**: 1232-1235.
22. CRANDALL DL, GRIFFITH DR, BEITZ DC (1982) Protection against the cardiotoxic effect of isoproterenol-HCl by dietary polunsaturated fatty acids and exercise. Toxicology and Applied Pharmacology **62**: 152-157.
23. CULLING W, PENNY WJ, CUNLIFFE G, FLORES NA, SHERIDAN DJ (1987) Arrhythmogenic and electrophysiological effects of alpha adrenoceptor stimulation during myocardial ischaemia and reperfusion. J Mol Cell Cardiol **19**: 251-258.
24. DE CHAFFOY DE COURCELLES D, ROEVENS P, VAN BELLE H (1986) Agents that elevate platelet cAMP stimulate the formation of phosphatidylinositol-4-phosphate in intact human platelets. FEBS Lett **195**: 115-118.
25. DENTON RM (1986) Early events in insulin action. Adv Cycl Nucl Prot Phosph Res **20**: 293-341.
26. DILLON JS, GU XH, NAYLER WG (1988) $Alpha_1$ adrenoceptors in the ischaemic and reperfused myocardium. J Mol Cell Cardiol **20**: 725-735.
27. DOCTROW SR, LOWENSTEIN JM (1987) Inhibition of phosphatidylinositol kinase in vascular smooth muscle membranes by adenosine and related compounds. Biochem Pharmacol **34**: 2255-2262.
28. DOODS HN, DE JONGE A, VAN ZWIETEN PA (1986) Muscarine-receptoren in het cardiovasculaire systeem. Hart Bulletin **17**: 50-57.
29. ENYEDI á, FARAGó A, SARKADI B, GARDóS G (1984) Cyclic AMP-dependent protein kinase and Ca^{2+}-calmodulin stimulate the formation of polyphosphoinositides in a sarcoplasmic reticulum preparation of rabbit heart. FEBS Lett **176**: 235-238.

30. ENYEDI á, FARAGó A, SARKADI B, SZáSZ I, GARDóS G (1983) Cyclic AMP-dependent protein kinase stimulates the formation of polyphosphoinositides in the plasma membranes of different blood cells. FEBS Lett **161**: 158-162.
31. EXTON JH (1985) Mechanisms involved in α-adrenergic phenomena. Am J Physiol **248**: E633-647.
32. FARIAS RN, BLOJ B, MORERO RD, SINERIZ F, TRUCCO RE (1975) Regulation of allosteric membrane-bound enzymes through changes in membrane lipid composition. Biochim Biophys Acta **415**: 231-251.
33. FRAGISKOS B, CHAN AC, CHOI PC (1986) Competition of n-3 and n-6 polunsaturated fatty acids in the isolated perfused rat heart. Ann Nutr Metabol **30**: 331-334.
34. GILL DL (1985) Receptors coupled to calcium mobilization. Adv Cycl Nucl Prot Phosph Res **19**: 307-324.
35. GIRAUD F, GASCARD P, SULPICE JC (1988) Stimulation of polyphosphoinositide turnover upon activation of protein kinases in human erythrocytes. Biochim Biophys Acta **968**: 367-378.
36. GRAZIANO MP, GILMAN AG (1987) Guanine nucleotide-binding regulatory proteins: mediators of transmembrane signaling. Trends Pharmacol Sci **8**: 478-481.
37. GUDBJARNASON S, OSKARDOTTIR G, DOELL B, HALLGRIMSON J (1978) Myocardial membrane lipids in relation to cardiovascular disease. Adv Cardiol **25**:130-144.
38. HAIECH J, DEMAILLE JG (1983) Phosphorylation and the control of calcium fluxes. Phil Trans R Soc Lond B **302**: 91-98.
39. HARTZELL HC, FISCHMEISTER R (1986) Opposite effects of cyclic GMP and cyclic AMP in Ca^{2+} current in single heart cells. Nature **323**: 273-275
40. HATHAWAY DR, MARCH KL (1989) Molecular cardiology: New avenues for the diagnosis and treatment of cardiovascular disease. J Am Coll Cardiol **13**: 265-282.
41. HENRY SA, KLIG LS, LOEWY BS (1984) The genetic regulation and coordination of biosynthetic pathways in yeast; amino acid and phospholipid synthesis. Annu Rev Genet **18**: 207-231.
42. HIDALGO C, CARRASCO MA, MAGENDZO K, JAIMOVICH E (1986) Phosphorylation of phosphatidylinositol by transverse tubule vesicles and its possible role in excitation-contraction coupling. FEBS Lett **202**: 69-73.
43. HIRATA F, AXELROD J (1980) Phospholipid methylation and biological signal transmission. Science **209**; 1082-1090.
44. HOHNERJÄGER P (1986) Regulation of myocardial force of contraction by sarcolemmal ion channels, the sodium pump, and sodium-calcium exchange. In: *The regulation of heart function*, ed. H. Rupp, Thieme Inc., New York, pp 159-177.

45. HOKIN LE (1985) Receptors and phosphoinositide-generated second messengers. Ann Rev Biochem **54**: 205-235.
46. HOKIN MR, HOKIN LE (1953) Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreas slices. J Biol Chem **203**: 967-977
47. IRVINE RF, MOOR RM (1986) Microinjection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external calcium. Biochem J **240**: 917-920.
48. IWASA Y, HOSEY MM (1984) Phosphorylation of cardiac sarcolemma proteins by the calcium-activated phospholipid-dependent protein kinase. J Biol Chem **259**: 534-540.
49. JAKAB G, RAPUNDALO ST, SOLARO RJ, KRANIAS EG (1988) Phosphorylation of phospholipids in isolated guinea pig hearts stimulated with isoprenaline. Biochem J **251**: 189-194.
50. KATZ AM, GOODHART PJ, GOODHART HL (1978) Physiology of the heart. Raven Press, New York, 379 pp.
51. KISHIMOTO A, MIKAWA K, HASHIMOTO K, YASUDA I, TANAKA S, TOMINAGA M, KURODA T, NISHIZUKA Y (1989) Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J Biol Chem **264**: 4088-4092.
52. KISHIMOTO A, TAKAI Y, MORI T, KIKKAWA U, NISHIZUKA Y (1980) Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. J Biol Chem **255**:2273-2276.
53. KNOWLES AF (1986) Endogenous phosphorylation of proteins and phosphatidylinositol in the plasma membrane of a human astrocytoma. Arch Biochem Biophys **249**: 76-87.
54. LAMERS JMJ (1984) Calmoduline in de functie van hart- en vaatspier. Hart Bulletin **15**: 203-209.
55. LAMERS JMJ (1985) Calcium transport systems in cardiac sarcolemma and their regulation by the second messengers cyclic AMP and calcium-calmodulin. Gen Physiol Biophys **4**: 143-154.
56. LAMERS JMJ (1987) Cardiac sarcolemmal calcium transport systems and their modulation by the second messengers cyclic AMP, calcium and phosphoinositide products. In: *Sarcolemmal biochemistry Vol. II*, ed. A.M. Kidwai, CRC Press, Boca Raton, pp 67-98.
57. LAMERS JMJ, DE JONGE-STINIS JT, HÜLSMANN WC, VERDOUW PD (1986) Reduced *in vitro* ^{32}P incorporation into phospholamban-like protein of sarcolemma due to myocardial ischaemia in anaesthetizes pigs. J Mol Cell Cardiol **18**: 115-125.
58. LANDS WEM (1986) Renewed questions about polunsaturated fatty acids. Nutr Rev **44**: 189-195.

59. LAPETINA EG (1986) Incorporation of synthetic 1,2-diacylglycerol into platelet phosphatidylinositol is increased by cyclic AMP. FEBS Lett **195**: 111-114

60. LEE HR (1989) α_1-Adrenergic receptors in heart failure. Heart Failure **5**: 62-70.

61. LEEB-LUNDBERG LMF, COTECCHIA S, DEBLASI A, CARON MG, LEFKOWITZ RJ (1987) Regulation of adrenergic receptor function by phosphorylation.I. Agonist-promoted desensitization and phosphorylation of α_1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT_1 MF-2 smooth muscle cells. J Biol Chem **262**: 3098-3105.

62. LIMAS CJ, LIMAS C (1985) Phorbol ester- and diacylglycerol-mediated desensitization of cardiac ß-adrenergic receptors. Circ Res **57**: 443-449.

63. LINDEMANN JP, WATANABE AM (1985) Muscarinic cholinergic inhibition of ß-adrenergic stimulation of phospholamban phosphorylation and Ca^{2+} transport in guinea pig ventricles. J Biol Chem **260**: 13122-13129.

64. LOW MG, WEGLICKI WB (1983) Resolution of myocardial phospholipase C into several forms with distinct properties. Biochem J **215**: 325-334.

65. LUNDBERG GA, JERGIL B, SUNDLER R (1986) Phosphatidylinositol-4-phosphate kinase from rat brain. Eur J Biochem **161**: 257-262.

66. MAISEL AS, MOTULSKY HJ, INSEL PA (1986) Receptor traffic in the myocardium: comparison of α- and ß-adrenergic receptors. J Am Coll Cardiol **7 [Suppl 2]**: 80A.

67. MAISEL AS, MOTULSKY HJ, INSEL PA (1987) Life cycles of cardiac α_1- and ß-adrenergic receptors. Biochem Pharmacol **36**: 1-6.

68. MAJERUS PW (1988) The production of phosphoinositide-derived messenger molecules. The Harvey Lectures, Series **82**: 145-155.

69. MAJERUS PW, CONNOLLY TM, BANSAL VS, INHORN RC, ROSS TS, LIPS DL (1988) Inositol phosphates: synthesis and degradation. J Biol Chem **263**: 3051-3054.

70. MAJERUS PW, CONNOLLY TM, DECKMYN H, ROSS TS, BROSS, TE, ISHII H, BANSAL VS, WILSON DB (1986) The metabolism of phosphoinositide-derived messenger molecules. Science **234**: 1517-1526.

71. MATO JM, ALEMANY S (1983) What is the function of phospholipid N-methylation ? Biochem J **213**: 1-10.

72. MAYR GW (1988) Inositol phosphates: structural components, regulators and signal transducers of the cell - a review. In: *Topics in biochemistry*, ed. by Boehringer Mannheim, 18 pp.

73. MICHELL B (1986) A second messenger function for inositol tetrakisphosphate. Nature **324**: 613.

74. MICHELL RH (1975) Inositol phospholipids and cell surface receptor function. Biochim Biophys Acta **415**: 81-147.

75. MONTFOORT A, VAN DER WERF L, HARTOG JM, HUGENHOLTZ PG, VERDOUW PD, HÜLSMANN WC, LAMERS JMJ (1986) The influence of fish oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidylethanolamine. Basic Res Cardiol **81**: 289-302.

76. MORAD M, CLEEMANN L (1987) Role of Ca^{2+} channel in development of tension in heart muscle. J Mol Cell Cardiol **19**: 527-553.

77. MOVSESIAN MA, NISHIKAWA M, ADELSTEIN RS (1984) Phosphorylation of phospholamban by calcium-activated phospholipid-dependent protein kinase. J Biol Chem **259**: 8029-8032.

78. MUKHERJEE A, HOGAN M, MCCOY K, BUJA LM, WILLERSON JT (1980) Influence of experimental myocardial ischemia on alpha$_1$ adrenergic receptors. Circulation **61-62 [Suppl III]**: 149.

79. NAHORSKI SR, BATTY I (1986) Inositol tetrakisphosphate: recent developments in phosphoinositide metabolism and receptor function. Trends Pharmacol Sci **7**: 83-85

80. NISHIZUKA Y (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature **308**: 693-697.

81. ORELLANA S, SOLSKI PA, BROWN JH (1987) Guanosine 5'-O-(thiotriphosphate)-dependent inositol trisphosphate formation in membranes is inhibited by phorbol ester and protein kinase C. J Biol Chem **262**: 1638-1643.

82. OSNES JB, AASS H, SKOMEDAL T (1985) On adrenergic regulation of heart function: role of myocardial α-adrenoceptors. In: *α-Adrenoceptor blockers in cardiovascular disease*, eds. H. Refsum and O.D. Mjo/s, Churchill Livingstone, New York, pp 69-102.

83. PANAGIA V, GANGULY PK, DHALLA NS (1984) Characterization of sarcolemmal phospholipid methylation. Biochim Biophys Acta **792**: 245-253.

84. PANAGIA V, GANGULY PK, OKUMURA K, DHALLA NS (1985) Subcellular localization of PE N-methylation activity in rat heart. J Mol Cell Cardiol **17**: 1151-1159.

85. PANAGIA V, MAKINO N, GANGULY PK, DHALLA NS (1987) Inhibition of Na^+-Ca^{2+} exchange in heart sarcolemmal vesicles by PE N-methylation. Eur J Biochem **166**: 597-603.

86. POST JA, LANGER GA, OP DEN KAMP JAF, VERKLEIJ AJ (1988) Phospholipid asymmetry in cardiac sarcolemma - Analysis of intact-cells and gas-dissected membranes. Biochim Biophys Acta **943**: 256-266.

87. PRESTI CF, JONES LR, LINDEMANN JP (1985a) Isoproterenol-induced phosphorylation of a 15-kilodalton sarcolemmal protein in intact myocardium. J Biol Chem **260**: 3860-3867.

88. PRESTI CF, SCOTT BT, JONES LR (1985b) Identification of an endogenous protein kinase C activity and its intrinsic 15-kilodalton substrate in purified canine cardiac sarcolemmal vesicles. J Biol Chem **260**: 13879-13889.

89. RANA RS, MACDONALD MJ (1986) Phosphatidylinositol kinase in rat pancreatic islets: subcellular distribution and sensitivity to calcium. Horm Metabol Res **18**: 659-662.

90. RASMUSSEN H (1986a) The calcium messenger system (First of two parts). N Engl J Med **314**: 1094-1101.

91. RASMUSSEN H (1986b) The calcium messenger system (Second of two parts). N Engl J Med **314**: 1164-1170.

92. RAWYLER AJ, ROELOFSEN B, WIRTZ KWA, OP DEN KAMP JAF (1987) (poly)Phosphoinositide phosphorylation is a marker for plasma membrane in Friend erythroleukemic cells. FEBS Lett **148**: 140-144.

93. REIBEL DK, HOLAHAN MA, HOCK CE (1988) Effects of dietary fish oil on cardiac responsiveness to adrenoceptor stimulation. Am J Physiol **254**: H494-499.

94. REUTER H (1983) Calcium channel modulation by neurotransmitters and drugs. Nature **301**: 569.

95. RYU SH, LEE SY, LEE KY, RHEE SG (1987) Catalytic properties of inositol trisphosphate kinase: activation by Ca^{2+} and calmodulin. FASEB J **1**: 388-393.

96. SARKADI B, ENYEDI á, FARAGó, MÉSZáROS G, KREMMER T, GARDóS G (1983) Cyclic AMP-dependent protein kinase stimulates formation of polyphosphoinositides in lymfocyte plasma membrane. FEBS Lett **152**: 195-198.

97. SCHOLZ H (1986) Positive inotropic agents: Different mechanisms of action In: *Cardiac glycosides 1785-1985*, eds. E.Erdmann, K. Greeff, J.C. Skou, Steinkopff Verlag Darmstadt, pp 181-188.

98. SCHRÖR K (1985) Editorial in: *Prostaglandins and other eicosanoids in the cardiovascular system* Karger, Basel, pp 1-6.

99. SEKAR MC, HOKIN LE (1986) The role of phosphoinositides in signal transduction. J Membrane Biol **189**: 193-210.

100. SHARMA AD, CORR PB (1985) α-Adrenergic mediated effects of catecholamines in the ischaemic and reperfused heart. In: *α-Adrenoceptor blockers in cardiovascular disease*, eds. H. Refsum and O.D. Mjo/s, Churchill Livingstone, New York, pp 69-102.

101. SHERIDAN DJ, PENKOSKE AA, SOBEL BE, CORR PB (1980) Alpha adrenergic-mediated contributions to dysrhythmias during myocardial ischaemia and reperfusion in cats. J Clin Invest **65**: 161-171.

102. SKOMEDAL T, AASS H, OSNES JB (1986) The interaction between calcium and the cyclic nucleotide systems with the heart as a model. The Aford Benzon Symp. 22. (Drug action), 1-12.

103. SMITH CD, COX CC, SNYDERMAN R (1986) Receptor-coupled activation of phosphoinositide-specific phospholipase C by an N protein. Science **232**: 97-100.

104. STARKE K (1981) α-Adrenoceptor subclassification. Rev Physiol Biochem Pharmacol **88**: 199-236.

105. STREB H, IRVINE RF, BERRIDGE MJ, SCHULZ I (1983) Release of calcium from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5-triphosphate. Nature **306**: 67-69.

106. STUBBS CD, SMITH AD (1984) The modification of mammalian polunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta **779**: 89-137.

107. STUBBS EB, KELLEHER JA, SUN GY (1988) Phosphatidylinositol kinase, phosphatidylinositol-4-phosphate kinase and diacylglycerol kinase activities in rat brain subcellular fractions. Biochim Biophys Acta **958**: 247-254.

108. SUGA K, KAMBAYASHI J, KAWASAKI T, SAKON M, MORI T (1986) Phosphorylation of phosphoinositides in human platelets. Thrombosis Res **44**: 155-163.

109. SUZUKI K, IMALOH S, EMORI Y, KAWASAKI H, MINAMI Y, OHNO S (1987) Calcium-activated neutral protease and its endogenous inhibitor. Activation at the cell membrane and biological function. FEBS Lett **220**: 271-277.

110. TAKAI Y, KISHIMOTO A, KIKKAWA U, MORI T, NISHIZUKA Y (1979) Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated phospholipid dependent protein-kinase system. Biochem Biophys Res Commun **91**:1218-1224.

111. TAKIN QS, BLUM M, CARAFOLI (1981) The fatty acid composition of subcellular membranes of rat liver, heart and brain: diet-induced modifications. Eur J Biochem **121**: 5-13.

112. THANDROYEN FT, FLINT NS, WORTHINGTON MG, OPIE LH (1987) Arrhythmogenic action of α_1-adrenoceptor stimulation in normoxic rat ventricular myocardium: influence of nisoldipine, reduced extracellular Ca^{2+} and ryanodine. J Mol Cell Cardiol **19**: 841-851.

113. UCHIDA T, BHATNAGAR GM, LAKATTA EG, FILBURN CR (1982) Alpha-adrenergic stimulation of $^{32}PO_4$ labelling of phosphatidylinositol (PI) and phosphaditic acid (PA) in cultured rat heart cells. Fed Proc **41**: 1523 (Abstr).

114. UHING RJ, PRPIC V, JIANG H, EXTON JH (1986) Hormone-stimulated polyphosphoinositide breakdown in rat liver plasma membranes. Roles of guanine nucleotides and calcium. J Biol Chem **261**: 2140-2146.

115. URUMOW T, WIELAND OH (1986) Stimulation of phosphatidylinositol 4-phosphate phosphorylation in human placenta membranes by GTPγS. FEBS Lett **207**: 253-257.

116. VANCE DE, RIDGWAY ND (1988) The methylation of phosphatidylethanolamine. Prog Lip Res **27**: 61-79.

117. VAN DONGEN CJ, KOK JW, SCHRAMA LH, OESTREICHER AB, GISPEN WH (1986) Immunochemical characterization of phosphatidylinositol 4-kinase from rat brain. Biochem J **233**: 859-864.

118. VAN ROOIJEN LAA, ROSSOWSKA M, BAZAN NG (1986) Inhibition of phosphatidylinositol-4-phosphate kinase by its product phosphatidylinositol-4,5-bisphosphate. Biochem Biophys Res Commun **126**: 150-155.

119. VARSANYI M, MESSER M, BRANDT NR, HEILMEYER LMG (1986) Phosphatidylinositol 4,5-bisphosphate formation in rabbit skeletal and heart muscle membranes. Biochem Biophys Res Commun **138**: 1395-1404.

120. WISE BC, RAYNOR RL, KUO JF (1982a) Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. I Purification and general properties. J Biol Chem **257**: 8481-8488.

121. WISE BC, GLASS DB, CHOU CHJ, RAYNOR RL, KATOH N, SCHATZMANN RC, TURNER RS, KIBLER RF, KUO JF (1982b) Phospholipid-sensitive Ca^{2+}-dependent protein kinase from heart. II Substrate specificity and inhibition by various agents. J Biol Chem **257**: 8489-8495.

122. YOUNG S, PARKER PJ, ULLRICH A, STABEL S (1987) Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem J **244**: 775-779.

123. YUAN S, SEN AK (1986) Characterization of the membrane-bound protein kinase C and its substrate proteins in canine cardiac sarcolemma. Biochim Biophys Acta **886**: 152-161.

Chapter II

Heart Cell Culture

II.1 Models for the biochemical investigation of the myocardium

One of the main purposes of our investigations was to study α_1-adrenoceptor mediated phosphatidylinositol turnover in rat cardiomyocytes. For accurate analysis of this process in the cardiac muscle cell the following three conditions had to be fulfilled. Firstly, any contribution of non-myocytes (e.g. vascular smooth muscle cells, fibroblasts, endothelial cells) must be negligible. Secondly, the presence and operation of specific receptors must be comparable to the *in vivo* status. Finally, the accessibility of the cardiomyocyte for adrenergic agonists and of the myocyte (poly)phosphoinositide pool for radiolabelling with [^{32}P-]PO_4 or [^{3}H-]*myo*inositol must be adequate.

The Langendorff model of isolated perfused rat heart is often used for biochemical studies of the myocyte but this model has some drawbacks with respect to the aforementioned conditions: in the adult rat heart the number of cells of other types than myocytes is 75-80% [Claycomb and Palazzo 1980]. Furthermore, most of the non-myocytes provide a barrier between the perfusion fluid and the cardiomyocytes. The latter argument also goes against the use of bathed pieces of myocardial tissue, whereas freshly isolated heart cells appear to be unstable with regard to their membrane properties [Jacobson and Piper 1986].

A possibility that meets the requirements of cell homogeneity, stability and accessibility is the application of heart cell culture [Wollenberger 1985]. At present, there are three models of cultured isolated cardiomyocytes: two models of adult heart cells and one of immature heart cells. In all three models the cells are separated by proteolytic digestion of heart tissue as described by Harary and Farley (1960). These authors were the first to present a culture model for beating heart cells which were obtained from newborn rats. The neonatal rat cardiomyocytes have a rounded, unorganized shape just after isolation but as soon as they are attached to the substratum they start to form pseudopod-like extensions and to flatten. Within 48 hours of culture they are well differentiated [Moses and Kasten 1979]. Then beating can be observed but single cells do not always show rhythmic contractions. Only if the cells are grown to a confluent monolayer does the whole cell population beat in synchrony [Cedergren and Harary 1964a].

A problem encountered with isolated adult rat cardiomyocytes is their subsequent intolerance to physiological concentrations of calcium. More recently this problem appears to have been surmounted by many investigators. Farmer *et al.* (1983) provided a review. The first model of adult rat heart cell culture, described by Jacobson in 1977, contains many cardiomyocytes that keep beating spontaneously although only until all the cells - after some days - have gradually changed from the initial rod-like shape to a spheroidal shape and finally have become flattened. This model provides a long-term culture system.

Jacobson and Piper (1986) call it - for obvious reasons - the *redifferentiated* model. Opposite this they set the second type of adult rat heart cell culture which they call the *rapid-attachment* model, because the isolated cardiomyocytes are induced to adhere rapidly to the substratum [Piper *et al.* 1985c]. The cells in this model show no spontaneous contractions and remain stable for up to one week, thereby decreasing in number. If these cells are cultured for more than one week they start showing the same morphological changes as cells of the redifferentiated model [Jacobson and Piper 1986; Bugaisky and Zak 1989].

1.1 Heart cell cultures compared with in vivo cardiomyocytes

Regarding the characteristics superficially, a culture of adult rat cardiomyocytes may seem to approach *in vivo* cardiomyocytes more accurately. However, although cultured neonatal rat cardiomyocytes may have an external shape that differs from adult rat cardiomyocytes *in vivo*, they do not differ very much from adult rat cardiomyocytes in culture. Only their ultrastructure is different because it is still under construction, as is witnessed by the presence of different developmental stages of mitochondria, of myofilaments parallelled by polysomes [Cedergren and Harary 1964a and b] and of rudimentary T-tubules [Moses and Kasten 1979]. On the other hand, the presence of intercalated discs can be observed between neighbouring cells [Cedergren and Harary 1964b; Moses and Kasten, 1979] and the fact that cells connected by these specialized membrane-contacts beat synchronously, suggests that functional gap junctions are also present. Furthermore both the redifferentiated and the rapid-attachment model show discrepancies too.

Cells in the redifferentiated model are externally different from cardiomyocytes *in vivo* [Jacobson 1977; Claycomb 1985] and ultrastructurally some differences exist as well. During their de-differentiation the cultured adult rat cells lose most of their T-system, which in some preparations is reconstructed during the re-differentiation period [Moses and Claycomb 1982] but in others remains completely absent [Nag and Cheng 1981]. Moses & Claycomb (1982) furthermore report the presence of 'residual bodies' or 'lipofuscin granules' in their adult rat cultured cardiomyocytes. For this phenomenon they can find no equivalent *in vivo* other than in the **aged** myocardium. The occurrence of these granules may therefore be a symptom of deterioration of the cells and - as the authors themselves suggest - be a consequence of proteolytic damage during dissociation. A third dissimilarity is the fact that cultured adult rat myocytes again start DNA replication, a feature absent in cardiomyocytes *in vivo* after neonatal development [Claycomb and Bradshaw 1983; Nag and Cheng 1986b]. A fourth point is that long-term cultured adult rat ventricular cells contain predominantly myosin isozyme V_3 which is the embryonic type [Nag and Cheng 1986a].

Cells in the rapid-attachment model do not have the differences mentioned above for the redifferentiated model and are externally and ultrastructurally almost analogous to

in vivo cardiomyocytes. However the cells in this model are at rest and do not contract unless electrically stimulated. Therefore their electrophysiology and metabolic activity differ from those of beating *in vivo* cardiomyocytes, but appear to be comparable to cardiomyocytes in diastolic rest [Jacobson and Piper 1986]. This means that their metabolic activity is about 10 to 20% of beating cardiomyocytes in an animal at rest and anaesthesized and about 2% of cells in a heart under maximal work load and metabolic stimulation provided e.g. by catecholamines [Kammermeier and Rose 1988]. The fact that adult rat cardiomyocytes are usually cultured dispersed, causes an ultrastructural difference from *in vivo* adult rat cardiomyocytes in both adult models. The cells contain neither intercalated discs nor gap junctions unless the isolation from other cardiomyocytes has not been complete. The lack of cell-to-cell interactions may explain the absence in single cultured cardiomyocytes of irreversible damage that can be induced in isolated (perfused) myocardium by the calcium and oxygen paradox [McDonough and Spitzer 1983; Piper *et al.* 1984; Schwartz *et al.* 1984; Piper *et al.* 1985a; Piper *et al.* 1985b; Stern *et al.* 1985]. In the model of neonatal rat cardiomyocytes, where cells are usually grown to a confluent monolayer, damage by the oxygen paradox has been reported [Van der Laarse *et al.* 1985; Vemuri *et al.* 1985]. The calcium paradox can be indeed observed in this type of cell [Acosta *et al.* 1983] although - based on findings with perfused hearts of young (up to 11 days old) rats - calcium overload and enzyme leakage seem to be bad indicators of irreversible cell damage in neonatal rat cardiomyocytes [Elz and Nayler 1987].

In summary, once in culture, cells in the redifferentiated model are not very distinct from neonatal rat cardiomyocytes. The rapid-attachment model of **non-contracting** adult rat cardiomyocytes seems disadvantageous for the investigation of intracellular reactions which are at the basis of **contractile** changes following α_1-adrenergic stimulation. Additionally, the preparation of neonatal rat cardiomyocytes offers the most practical advantages as, in comparison, isolated adult rat cardiomyocytes remain vulnerable. Firstly, they are far more intolerant to treatments like sedimentation, stirring and triturating. Secondly, any chemical factor unsuspectedly present in the gaseous, fluid or solid culture materials disturbs them [Jacobson 1985]. Furthermore, as Jacobson and Piper (1986) state clearly: "experience with cultured adult cardiomyocytes is in a nascent state". Therefore, no adult rat preparation has been fully defined as to its electro-physiological and metabolic properties, whereas substantial information about cultured neonatal rat cardiomyocytes exists.

1.2 Properties of cultured neonatal rat cardiomyocytes

One of the conditions mentioned at the beginning of this chapter concerning the analysis of α_1-adrenoceptor mediated phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) breakdown has not sufficiently been settled yet. It should be ascertained that the cardiomyocytes in the used culture contain adrenergic receptors and that those are functionally the same as in adult rat cardiomyocytes. The work of Ettaiche *et al.* (1985), based on frequency responses, indicated that neonatal rat cardiomyocytes posses (i) functional $ß_1$- and $ß_2$-

adrenoceptors, the response to ß-agonists being mainly mediated by the $ß_1$-type and (ii) functional α_1-adrenoceptors, with less chronotropic influence than ß-adrenoceptors. There were no functionally detectable α_2-adrenoceptors. The findings of Variot *et al.* (1987) indicated the absence of functional muscarinic receptors and any possible cyclic-AMP/cyclic-GMP antagonism in these cells. The results of Marvin *et al.* (1984), investigating the influence of neurons of various origin in coculture with ventricular muscle cells, confirm these findings.

In Table II.1 an overview of α_1-adrenoceptor radioligand binding studies in rat myocardium is given. The K_D and B_{max} of cultured neonatal rat cardiomyocytes correspond very well with those of other preparations.

The metabolic properties of cultured neonatal rat cardiomyocytes have been extensively studied. Fatty acid metabolism was investigated by the group of Harary in the 1960's. The response of cultured heart cells to serum in the growth medium has been reported by Frelin *et al.* (1974) and Grynberg *et al.* (1986). Glucose metabolism and amino

Table II.1. *α_1-Adrenoceptor binding characteristics in rat myocardium*[a]

Radioligand[e]	Preparation[f]	K_D[b]	B_{max}[c]	Reference
[^{3}H-]DHE	adult m	1.60	33	Williams 1981
[^{3}H-]prazosin	"	0.10	36	" "
"	"	0.087	N.R.[g]	Stiles 1983
"	"	5.84	167	Mukherjee 1983
"	"	0.16	111	Colucci 1984
[^{3}H-]DHE	"	0.92	43	Wei 1979
[^{125}I-]IBE2254	"	N.R.	88	Bristow 1988
[^{3}H]prazosin	"	0.076	70	Buxton 1986
"	adult ic	0.084	70	" "
"	adult ic m	0.087	88	" "
"	adult ph	0.41	88	Edwards 1988
"	adult ph h	1.10	43	" "
"	adult ph SL	0.10	300	" "
"	slices	0.55	4.5[d]	Muntz 1985
"	adult ic	0.156	77	Skomedal 1984
[^{125}I-]HEAT	fetal m	0.24	108	Schaffer 1986
[^{125}I-]IBE2254	neonatal cc	0.324	60	Kupfer 1982
[^{125}I-]HEAT	neonatal m	0.23	91	Schaffer 1986
[^{125}I-]HEAT	6-day old m	0.15	112	" "
	25-day old m	0.23	80	" "
"	42-day old m	0.24	57	" "
"	adult m	0.21	53	" "
"	2 year old m	0.23	50	" "

[a]Expressed in [b]nM; [c]fmol/mg protein; [d]grains per mm^2
[e]DHE = dihydroergocryptine; IBE 2254 = I-HEAT = I-2-(ß-hydroxyphenyl) ethylaminomethyl tetralone
[f]m = membrane; ic = isolated cells; ph = perfused heart;
h = homogenate; SL = sarcolemma; cc = cultured cells;
[g]N.R. = not recorded.

acid metabolism have been described by Frelin *et al.* (1976, 1980). The glucose uptake of cultured cardiomyocytes was 10 to 20 times higher than had been reported for *in vivo* cells and led at first to excretion of lactate into the medium, which was later utilized again. The essential amino acids provided by the medium were also extensively used by the cultured cells, whereas glycine, proline and high amounts of alanine, all synthesized *de novo*, were released. In both these metabolic aspects cultured cardiomyocytes do not behave like *in vivo* cells and therefore should not be considered a homeostatic system.

Phospholipid headgroup composition and phospholipid fatty acid composition of cultured cells have been described by Rogers (1974) and the latter also by Grynberg *et al.* (1986), and only minor differences from *in vivo* rat heart were noted. Phospholipid headgroup composition of only the sarcolemma and the distribution of the phospholipid classes between the outer and inner layer of the sarcolemmal lipid bilayer have recently been described by Post *et al.* (1988a, 1988b). Compared with adult rat heart sarcolemma [Singal *et al.* 1980], sarcolemma from cultured neonatal rat cardiomyocytes contained (in %-age of total) more phosphatidylcholine. Furthermore, all of PI and PS, both negatively charged phospholipids, and most of PE appeared to be located in the inner layer (cytoplasmic side) of the sarcolemma.

Also recently, by employing *in situ* hybridization, cultured neonatal and isolated adult rat cardiomyocytes have been shown identical in the expression of the mRNA for collagen type IV, a component of the basement membrane. The mRNAs for collagen type I an III, both major constituants of the heart extracellular matrix, were abundant in fibroblasts but not present in cardiomyocytes [Eghbali *et al.* 1989].

II.2 Preparation of cultured neonatal rat ventricular myocytes

2.1 Methods to reduce the number of non-myocytes

The neonatal rat heart contains by volume 80% myocytes and 20% non-muscle cells, mostly fibroblasts and also endothelial, smooth muscle and blood cells. Neonatal rat cardiomyocytes divide once or twice and have a proliferative cycle of 60 hr, whereas fibroblasts proliferate incessantly every 20 hr. Therefore precautions have to be taken to avoid that fibroblasts overgrow the myocytes. In our study the following methods were applied to reduce the number of non-muscle cells in the cardiomyocyte culture. Firstly, after incision of the rat body only the lower, ventricular part of the protruding heart was taken. This part is comparatively richer in muscle cells. Secondly, the first two trypsinization suspensions were discarded as those contain mainly cells from the blood vessels and endothelium. Thirdly, the cell suspension was replated twice. The replating method is based on the fact that fibroblasts adhere much more rapidly to the substratum than myocytes do [Blondel *et al.* 1971]. Fourthly, cells were plated in such a high density that there is not enough space for fibroblasts to proliferate. The maximum growth density of the cells is about 1.8×10^6 cells per 35 mm (diameter) dish so 1800 cells/mm^2 [Yagev *et al.* 1984]. Millart and Seraydarian (1986) report an average of 419 cells/mm^2 as high density but

they must have mixed up the diameter with radius in their calculations.

The success of this procedure was checked microscopically. The periodic acid-Schiff (PAS) staining method was applied to distinguish myocytes. The proportion of myocytes, visible as cells containing stained glycogen granules, increased after each replating step.

A more detailed preparation procedure is given below.

2.2 *Special materials*

- Ham F10 culture medium, powder; Special without Ca^{2+}, Mg^{2+}, phosphates, inositol (Gibco, Scotland, UK)
- Horse serum (Boehringer, Mannheim, FRG)
- Fetal Calf Serum (Boehringer, Mannheim, FRG)
- streptomycin-penicillin (Boehringer, Mannheim, FRG)
- trypsin type III (Sigma, USA)
- 60 ml syringe
- MILLEX-GV sterilising filter unit (0.22 μm, SLGV 025 BS, Millipore, Molsheim, France)
- sterilized serum bottles
- sterilized Erlenmeyer flask with screw cap (Wheaton, USA)
- sterilized glass petridish (35 mm diam.)
- sterilized 25 ml incubation flask with two side arms, jacketed and containing a magnetic bar attached to the stopper (US patent #3.572.651, Wheaton, USA)
- sterilized nylon gauze
- sterilized funnel or pair of teflon rings to hold nylon gauze
- sterile plastic 10 ml pipettes ("Stripettes", Costar, Cambridge MA, USA)
- sterile plastic conical centrifuge tubes + caps, 50 ml (Greiner, Nürtingen, FRG)
- sterile plastic petridishes 35/10 mm (Greiner, Nürtingen, FRG)
- sterile plastic culture flasks, 250 ml (Costar, Cambridge MA, USA)
- a small forceps and a small pair of scissors
- a large pair of scissors
- 2 to 4 day old rats (Wistar), 20-40 animals each time

2.3 *Preparation of complete growth medium*

The Ham F10 Special powder was dissolved in distilled or Millipore filtered water (9.46 g/l). To this were added 144.28 mg/l $CaCl_2$ (anhydr.), 83 mg/l KH_2PO_4 (anhydr.), 152.8 mg/l $MgSO_4.7H_2O$, 195.8 mg/l $Na_2HPO_4.2H_2O$, 0.541 mg/l myo-inositol, 0.715 mg/l D-CaPantothenate, 200 mg/l streptomycin, 20.000 Units/l penicillin. This was stirred for some time to get a homogeneous solution and finally 1200 mg/l $NaHCO_3$ was added.

After stirring again the Ham's solution was sterilized over a combination of 0.22 μm + 0.60 μm Millipore filters into sterilized serum bottles. For small amounts a 60

ml syringe and a Millex-V 0.22 μm filter were used. The Ham's solution was stored at -20 °C until first use. Before use the Ham's, Horse Serum (HS) and Fetal Calf Serum (FCS) were thawed and complete growth medium was constituted out of 80% Ham's, 10% HS and 10% FCS. This was kept at 0-4°C for maximally 2-3 months.

2.4 Preparation of trypsinization medium

Most proteolytic enzymes are inhibited by Ca^{2+}, Mg^{2+} and proteins. The trypsinization medium was therefore prepared freshly before each isolation without serum as follows. Ham F10 Special powder (946 mg/100 ml) was dissolved in distilled and Millipore filtered water. Added were 8.3 mg/100 ml KH_2PO_4 (anhydr.), 19.58 mg/100 ml $Na_2HPO_4.2H_2O$, 0.054 mg/100 ml myo-inositol, 0.072 mg/100 ml D-CaPantothenate and 120 mg/100 ml $NaHCO_3$. A few ml of this special medium was used for mincing and washing the hearts. The rest was used to dissolve 0.1% (w/v) trypsin (± 100 mg) later. The 60 ml syringe and a Millipore filter unit were applied to sterilize and transfer the trypsin solution into a sterilized Erlenmeyer.

2.5 Isolation procedure

The whole isolation procedure was carried out under sterile conditions in a flow cabinet. The working space in the flow cabinet was covered with aluminum foil. Some paper tissue was put on top of that in the corner used for decapitating the rats. The incubation flask, standing on a magnetic stirrer, was connected to a thermostated bath outside the flow cabinet and the temperature was set to 32-35°C. The small forceps and scissors were put in a 25 ml glass beaker filled with 70% ethanol. The syringe was filled with a few ml of the Ca^{2+}/Mg^{2+}-free medium and emptied through a sterile filter unit into the sterilized petridish. The dish was kept covered most of the time.

About 10 rats were killed by decapitation with the large scissors and then put on the tissue. One by one, each body was sterilized by submerging the dead animal, held by the tail, in 70% ethanol in a 50 ml beaker. The body was put on the aluminum foil so the skin could be clasped dorsally and the skin ventrally stretched. The small scissors were used to make a small cut in the chest just below where the heart should be. When the heart protruded from the cut, the ventricular part was taken off with the small forceps and put in the petridish. When the hearts of all 10 decapitated rats had been taken the next group of 10 was killed, and so on until all hearts had been collected.

Next the hearts were rinsed with a few ml of sterilized Ca^{2+}/Mg^{2+}-free medium and cut to small fragments (1 to 2 mm^3) with scissors. The syringe was used to sterilize the trypsinization medium which was subsequently kept in a sterilized Erlenmeyer flask. A few drops were added to the minced hearts upon which the fragments started to clot thus making further mincing possible. The tissue fragments were incubated in about 10 ml trypsinization medium in the incubation flask (32-35°C) at a stirring velocity of 100-200 rpm. After 10-15 min stirring was stopped and the fragments were allowed to settle

before the supernatant fluid was poured off and fresh trypsinization medium was added to the fragments.

The first and second trypsinization fractions were discarded because these contain mainly cell debris, red blood cells, pericardial and endothelial cells. The next trypsinization fractions were poured into a sterile conical tube that already contained a few ml complete growth medium to inactivate the trypsin. Thus the stepwise trypsinization prevented that already dissociated cells were further affected by the proteolysis.

After having collected all the supernatants from the successive trypsinization periods, the cells were pelleted by centrifugation at 300 x g for 15 minutes. The supernatant was replaced by complete growth medium and the cell pellet was resuspended. The contents of all the conical tubes were poured over a sterile gauze to exclude lumps into one sterile culture flask. The flask was allowed to stay in the incubator (37°C, 5% CO_2, 90% humidity) for 30 minutes. Then it was taken out and shaken to loosen the unattached cells. The suspension was transferred to another culture flask and allowed to stay in the incubator for 1.5 to 2 hours. Most of the non-muscular cells were by then attached to the culture flask, while gently shaking resulted in a pure myoblast suspension. A sample was taken from this suspension for counting - in a haemocytometer - round, smooth and birefringent cells. Complete growth medium was added until the suspension had a concentration of about 9 x 10^5 cells/ml. Finally seeding was carried out by pipetting 2ml suspension into each plastic petridish. Cells were distributed evenly over the bottom by pushing the dishes back-and-forth and left-to-right, avoiding whirling.

After 24 to 36 hours in the incubator (37°C, 5% CO_2, 90% humidity) a confluent monolayer of beating cardiomyocytes had come about. The medium was changed every 48 hours after seeding. Because medium components and serum factors induce a change in cell metabolism, experiments were done no sooner than 12 and no later than 36 hours after a change of medium, when the cells were in a steady state.

II.3 Experimental use of neonatal rat cardiomyocytes

In the following chapters our studies on phospholipid signalling processes are described that were carried out with cultures of neonatal rat cardiomyocytes prepared as detailed above. Various pretreatments of the myocytes in serum-containing or serum-free medium had to be worked out in order to study (i) the α_1-adrenoceptor stimulated $PI(4,5)P_2$ breakdown itself, (ii) in a cell-free system, the enzymes involved and (iii) the influence of alterations of the phospholipid bilayer on the transduction process.

Prelabelling of the (poly)phosphoinositide pool for the study of the rate of $PI(4,5)P_2$ hydrolysis, was performed by incubating the cells in a modified Krebs-Henseleit buffer containing ^{3}H-myoinositol for 2 hours. The period of 2 hours appeared sufficient for prelabelling, which probably means that inositol diffuses unlimitedly across the myocardial sarcolemmal membrane. In several experiments longer periods (up to 24 hours) of preincubation with ^{3}H-myoinositol were used but the time course of inositol phosphate formation found was similar. At any rate this indicated that steady state labelling of

polyphospho-inositides was not essential to estimate the rate of $PI(4,5)P_2$ breakdown.

The cultured myocytes were also homogenized for the preparation of a sarcolemma-enriched membrane fraction. The main purpose was to study the characterization of the endogenous GTP-sensitive $PI(4,5)P_2$-PLase C and the endogenous PKC and its substrate proteins. Prior to this study a few trials were done with a sarcolemma preparation that was isolated by a high-velocity stream of nitrogen, as described by Langer *et al.* (1978). Samples of this sarcolemma-enriched membrane preparation were kindly supplied by Dr. J.A. Post and coworkers, who have characterized the phospholipid bilayer properties [Post *et al.* 1988a,b]. However, for the purpose of studying $PI(4,5)P_2$-PLase C and PKC, this preparation was not suitable, because of the lability of both membrane-bound enzymes. Furthermore, the yield was so low that the commercially available ^{3}H-$PI(4,5)P_2$ did not have a specific radioactivity that was high enough to estimate the $PI(4,5)P_2$-PLase C activity accurately in small quantities of membrane fragments.

For the measurement of the rate of PE N-methylation no long-term prelabelling of the intracellular S-adenosyl-methionine pool with ^{3}H-methionine was required. The pools of methionine and S-adenosyl-methionine in the cell rapidly equilibrate with extracellular ^{3}H-methionine and the rates of PE N-methylation appeared to be so fast that actually the experiments were done at or close to steady-state labelling of ^{3}H-methylated PE-lipids. For measurement of the initial rate of PE N-methylation (within 30 sec after addition of ^{3}H-methionine) the used setup appeared not convenient because of the time necessary for taking the dish with cells to and from the incubator and for rinsing the cells before stopping the reaction.

In the final series of experiments the cells were exposed to different albumin-bound PUFAs in order to accomplish significant incorporation of these PUFAs into the cellular phospholipids. For this purpose cells were preincubated for 24 hours with growth medium prepared with foetal calf serum to which previously 20:5n-3 or 18:2n-6 had been added. No trials were done to reach changes in membrane PUFAs by replacing or supplementing whole lipoprotein particles because in culture the uptake of fatty acids from these sources has been shown to be much slower.

REFERENCES

1. ACOSTA D, RAMOS K, GOLDMAN CP (1983) Cellular injury of primary cultures of rat myocytes incubated in calcium-free medium followed by recovery in calcium. In Vitro **19**: 141-144.
2. BLONDEL B, ROIJEN I, CHENEVAL JP (1971) Heart cells in culture: A simple method for increasing the proportion of myoblasts. Experientia **27**: 356-358.
3. BRISTOW MR, SANDOVAL AB, GILBERT EM, DEISHER T, MINOBE W, RASMUSSEN R (1988) Myocardial α- and ß-adrenergic receptors in heart failure: Is cardiac-derived norepinephrine the regulatory signal? Eur Heart J **9 Suppl H**:35-40.
4. BUGAISKY LB, ZAK R (1989) Differentiation of adult cardiac myocytes in cell culture. Circ Res **64**: 493-500.
5. BUXTON ILO, BRUNTON LL (1986) α-adrenergic receptors on rat ventricular myocytes: characteristics and linkage to cAMP metabolism. Am J Physiol **251**: H307-313
6. CEDERGREN B, HARARY I (1964a) In vitro studies on single beating rat heart cells. VI Electron microscopic studies of single cells. J Ultrastruct Res **11**: 428-442.
7. CEDERGREN B, HARARY I (1964b) In vitro studies on single beating rat heart cells. VII Ultrastructure of the beating cell layer J Ultrastruct Res **11**: 443-454.
8. CLAYCOMB WC (1985) Long-term culture and characterization of the adult ventricular and atrial muscle cell. Basic Res Cardiol **80 Suppl 2**: 171-174.
9. CLAYCOMB WC, BRADSHAW HD (1983) Acquisition of multiple nuclei and the activity of DNA polymerase alpha and reinitiation of DNA replication in terminally differentiated adult cardiac muscle cells in culture. Dev Biol **99**: 331-337.
10. CLAYCOMB WC, PALAZZO MC (1980) Culture of the terminally differentiated adult cardiac muscle cell: A light and scanning electron microscope study. Dev Biol **80**: 466-482.
11. COLUCCI WS, GIMBRONE MA, ALEXANDER RW (1984) Regulation of myocardial and vascular α-adrenergic receptor affinity. Effects of guanine nucleotides, cations, estrogen, and catecholamine depletion. Circ Res **55**: 78-88.
12. EDWARDS SJ, RATTIGAN S, COLQUHOUN EQ, WOODCOCK EA, CLARK MG (1988) Characterization of α_1-adrenergic receptors in perfused rat heart. J Mol Cell Cardiol **20**: 1025-1034.
13. EGHBALI M, BLUMENFELD OO, SEIFTER S, BUTTRICK PM, LEINWAND LA, ROBINSON TF, ZERN MA, GIAMBRONE MA (1989) Localization of types I, III and IV collagen mRNAs in rat heart cells by *in situ* hybridization. J Mol Cell Cardiol **21**: 103-113.
14. ELZ JS, NAYLER WG (1987) Quantification of calcium paradox in neonatal rat hearts. Am J Physiol **253**: H1358-1364.
15. ETTAICHE M, ATHIAS P, VARIOT M, KLEPPING J (1985) Characterization

of postjunctional adrenoceptor subtypes in isolated rat myocardial cells in culture. Can J Physiol Pharmacol **63**: 1221-1227.

16. FARMER BB, MANCINA M, WILLIAMS ES, WATANABE AM (1983) Minireview. Isolation of calcium tolerant myocytes from adult rat hearts: Review of the literature and description of a method. Life Sci **33**: 1-18.
17. FRELIN C (1980) Amino acid metabolism by new-born rat heart cells in monolayer cultures. J Mol Cell Cardiol **12**: 479-491.
18. FRELIN C, PADIEU P (1976) Pleiotypic response of rat heart cells in culture to serum stimulation. Biochimie **58**: 953-959.
19. FRELIN C, PINSON A, MOALIC JM, PADIEU P (1974) Energy metabolism of beating rat heart cell cultures. II.- Glucose metabolism. Biochimie **56**: 1597-1602.
20. GRYNBERG A, ATHIAS P, DEGOIS M (1986) Effect of change in environment on cultured myocardial cells investigated in a standardized medium. In Vitro **22**: 44-50.
21. HARARY I, FARLEY B (1960) In vitro studies of single isolated beating heart cells. Science **131**: 1674-1675.
22. JACOBSON SL (1977) Culture of spontaneously contracting myocardial cells from adult rats. Cell Struct and Funct **2**: 1-9.
23. JACOBSON SL, BANFALVI M, SCHWARZFELD TA (1985) Long-term primary cultures of adult human and rat cardiomyocytes. Basic Res Cardiol **80 Suppl 2**: 79-82.
24. JACOBSON SL, PIPER HM (1986) Review Article. Cell cultures of adult cardiomyocytes as models of the myocardium. J Mol Cell Cardiol **18**: 661-678.
25. KAMMERMEIER H, ROSE H (1988) Are isolated cardiomyocytes a suitable experimental model in all lines of investigation in basic cardiology ? Basic Res Cardiol **83**: 343-349.
26. KUPFER LE, ROBINSON RB, BILEZIKIAN JB (1982) Identification of α_1-adrenergic receptors in cultured rat myocardial cells with a new iodinated α_1-adrenergic antagonist, [^{125}I]IBE 2254. Circ Res **51**: 250-254.
27. LANGER GA, FRANK JS, PHILIPSON KD (1978) Preparation of sarcolemmal membrane from myocardial tissue culture monolayer by high-velocity gas dissection. Science **200**: 1388-1391.
28. MARVIN WJ, ATKINS DL, CHITTICK VL, LUND DD, HERMSMEYER K (1984) In vitro adrenergic and cholinergic innervation of the developing rat myocyte. Circ Res **55**: 49-58.
29. MCDONOUGH KH, SPITZER JJ (1983) Effects of hypoxia and reoxygenation on adult rat heart cell metabolism. Proc Soc Exp Biol Med **173**: 519-526.
30. MILLART H, SERAYDARIAN MW (1986) Influence of plating density on individual cell growth, cell division and differentiation of neonatal rat heart primary cultures. Tissue Cell **18**: 209-218.
31. MOSES RL, CLAYCOMB WC (1982) Ultrastructure of terminally differentiated adult rat cardiac muscle cells in culture. Am J Anat **164**:113-131.

32. MOSES RL, KASTEN FH (1979) T-tubes in cultured mammalian myocardial cells. Cell Tissue Res **203**: 173-180.
33. MUKHERJEE A, HAGHANI Z, BRADY J, BUSH L, MCBRIDE W, BUJA LM, WILLERSON JT (1983) Differences in myocardial α- and ß-adrenergic receptor numbers in different species. Am J Physiol **245**: H957-961.
34. MUNTZ KH, GARCIA C, HAGLER HK (1985) α_1-Receptor localization in rat heart and kidney using autoradiography. Am J Physiol **249**: H512-519.
35. NAG AC, CHENG M (1986) Biochemical evidence for cellular dedifferentiation in adult rat cardiac muscle cells in culture: expression of myosin isozymes. Biochem Biophys Res Commun **137**: 855-862.
36. NAG AC, CHENG M (1986) DNA synthesis of adult mammalian cardiac muscle cells in long-term culture. Tissue Cell **18**: 491-497.
37. PIPER HM, SCHWARTZ P, SPAHR R, HÜTTER JF, SPIECKERMANN PG (1984) Absence of reoxygenation damage in isolated heart cells after anoxic injury. Pflügers Arch **401**: 71-76.
38. PIPER HM, SCHWARTZ P, SPAHR R, HÜTTER JF, SPIECKERMANN PG (1985a) Anoxic injury of adult cardiac myocytes. Basic Res Cardiol **80 Suppl 1**: 37-41.
39. PIPER HM, SPAHR R, HÜTTER JF, SPIECKERMANN PG (1985b) The calcium and the oxygen paradox: non-existent on the cellular level. Basic Res Cardiol **80 Suppl 2**: 159-163.
40. PIPER HM, SPAHR R, PROBST I, SPIECKERMANN PG (1985c) Substrates for the attachment of adult cardiac myocytes in culture. Basic Res Cardiol **80 Suppl 2**: 175-180.
41. POST JA, LANGER GA, OP DEN KAMP JAF, VERKLEIJ AL (1988a) Phospholipid asymmetry in cardiac sarcolemma - Analysis of intact-cells and gas-dissected membranes. Biochim Biophys Acta **943**: 256-266.
42. POST JA, VERKLEIJ AL, ROELOFSEN B, OP DEN KAMP JAF (1988b) Plasmalogen content and distribution in the sarcolemma of cultured neonatal rat myocytes. FEBS Lett **240**: 78-82.
43. ROGERS CG (1974) Fatty acids and phospholipids of adult rat and newborn rat hearts and of cultured, beating neonatal rat myocardial cells. Lipids **9**: 541-547.
44. SCHAFFER W, WILLIAMS RS (1986) Age-dependent changes in expression of alpha$_1$ adrenergic receptors in rat myocardium. Biochem Biophys Res Commun **136**: 387-391.
45. SCHWARTZ P, PIPER HM, SPAHR R, SPIECKERMANN PG (1984) Ultrastructure of cultured adult myocardial cells during anoxia and reoxygenation. Am J Pathol **115**: 349-361.
46. SINGAL PK, NIJJAR MS, DHALLA KS, ANAND-SRIVASTAVA MB, DHALLA NS (1980) Structure-function relationship in heart sarcolemma. In: *Adv Myocardiol Vol 1*, eds M. Tajuddin, P.K. Das, M. Tariq, N.S. Dhalla, University Park Press, Baltimore (USA), pp 33-42.

47. SKOMEDAL T, AASS H, OSNES JB (1984) Specific binding of [^{3}H]prazosin to myocardial cells isolated from adult rats. Biochem Pharmacol **33**: 1897-1906.
48. STERN MD, CHIEN AM, CAPOGROSSI MC, PELTO DJ, LAKATTA EG (1985) Direct observation of the "oxygen paradox" in single rat ventricular myocytes. Circ Res **56**: 899-903.
49. STILES GL, HOFFMAN BB, HUBBARD M, CARON MG, LEFKOWITZ RJ (1983) Guanine nucleotides and alpha$_1$ adrenergic receptors in the heart. Biochem Pharmacol **32**: 69-71.
50. VAN DER LAARSE A, ALTONA JC, BLOYS VAN TRESLONG CHF (1985) Anoxia in neonatal rat heart cell cultures. Basic Res Cardiol **80 Suppl 1**: 137-142.
51. VARIOT N, ETTAICHE M, ATHIAS P (1987) Physiological effects of carbamoylcholine, cyclic GMP and related agents on isolated rat ventricular myocytes. Arch Int Pharmacodyn **287**: 258-271.
52. VEMURI R, YAGEV S, HELLER M, PINSON A (1985) Studies on oxygen and volume restrictions in cultured cardiac cells I A model for ischemia and anoxia with a new approach. In Vitro **21**: 521-525.
53. WEI JW, SULAKHE PV (1979) Regional and subcellular distribution of ß and α adrenergic receptors in the myocardium of different species. J Gen Pharmacol **10**: 263-267.
54. WILLIAMS RS, DUKES DF, LEFKOWITZ RJ (1981) Subtype specifity of α_1-adrenergic receptors in rat heart. J Cardiovasc Pharmacol **3**: 522-531.
55. WOLLENBERGER A (1985) Isolated heart cells as a model of the myocardium. Basic Res Cardiol **80 Suppl 2**: 9-14.
56. YAGEV S, HELLER M, PINSON A (1984) Changes in cytoplasmic and lysosomal enzyme activities in cultured rat heart cells: the relationship to cell differentiation and cell population in culture. In Vitro **20**: 893-898.

Chapter III

Alpha-1-adrenergic Stimulation of Phosphoinositide Breakdown in Cultured Neonatal Rat Ventricular Myocytes

Johanna T.A. Meij and Jos M.J. Lamers (1989) Mol Cell Biochem **88**: 73-75.

Summary

The regulation of and the intracellular events following α_1-adrenergic receptor stimulation in the myocardium still remain to be disclosed. The effect of α_1-adrenergic stimulation on phosphoinositide breakdown was studied in cultured neonatal rat ventricular myocytes. Phenylephrine (30 μM) stimulated inositolphosphates formation, but only in the presence of 10 mM LiCl this could be measured. The increase was antagonized by prazosin (1 μM) but not by propranolol (1 μM). The variability in proportional distribution of the three inositolphosphates is discussed.

Introduction

In ventricular myocardium both α_1- and ß-adrenergic agonists provoke an increase of slow inward calcium current and enhance the contractile force [1,2]. However qualitative and quantitative differences between the responses of the heart to α_1-agonists and ß-agonists are prominent. The well-known mechanism underlying ß-adrenoceptor stimulation - activation of adenylate cyclase followed by cyclic AMP-dependent protein phosphorylation - does not seem to apply to α_1-adrenoceptors.

In isolated rat ventricles [3], papillary muscle [4] and adult cardiomyocytes [5] stimulation of α_1-adrenoceptors was reported to enhance phosphoinositide breakdown. Although in these preparations the PI cycle of other cell types could have contributed, myocardial α_1-adrenergic receptors may be coupled to a phospholipase C which upon activation hydrolyses PIP_2 into IP_3 and DG. IP_3 is thought to mobilize Ca^{2}_{+} from intracellular stores [6,7] and DG is an activator of PKC [8].

In our study of α_1-adrenergic stimulation we used primary cultures of neonatal rat ventricular myocytes. Our preparation contained a near to homogeneous population of spontaneously beating cardiac muscle cells. The activity of PI-phospholipase C was measured as the acculmulation of IP_3 and its breakdown products IP_2 and IP_1. Dephosphorylation of IP_1 was blocked by LiCl.

Experimental procedures

Primary heart cell culture

Cardiac cells were isolated from ventricles of 2-4 day old Wistar rats by stepwise trypsinization accordong to Yagev *et al.* (1984) [9]. A near to homogeneous suspension of cardiomyocytes was obtained by two successive periods of 30 and 90 min preplating. Finally 1.8 x 10^6 myoblasts were seeded per Petridish (Greiner,TC 35/10), which after 24 to 26 h incubation (37°C, 5% CO_2, 95% hum.) resulted in a confluent monolayer of beating cardiomyocytes. Every 48 h the growth medium (Ham F-10 (Gibco), supplemented with 10% FCS, 10% HS, 2 x 10^5 U/l penicillin and 0.2 g/l streptomycin (Boehringer); and 135 mg/l $CaCl_2.2H_2O$) was changed. Experiments were done on the third or fifth day after plating.

Cell incubation

After decanting the growth medium cardiomyocytes were rinsed twice with 1 ml incubation buffer (130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20mM $NaHCO_3$, 0.44 mM NaH_2PO_4, 1.1 mM $MgCl_2$, 0.2% D-glucose, 37°C, gassed, pH 7.4) followed by preincubation in 0.5 ml 6.7μCi [2-^{3}H-]myo-inositol/ml buffer for 2 h. After this period the cells were rinsed twice with 0.5 ml buffer and incubated in buffer, 10 mM LiCl and other additions as indicated, at a final volume of 1 ml.
Incubations were terminated by rinsing with 0.5 ml ice-cold buffer immediately followed by addition of 0.5 ml methanol/13 M HCl (100:1 v/v).

Assay of [^{3}H-]inositolphosphates

Cells were scraped and kept overnight at -20°C in methanol/ chloroform/ 13 M HCL (200:100:1 v/v). Phase separation was obtained in chloroform/methanol/2.5 M HCl (2:2:1 v/v). After rinsing the organic phase once with 1 ml synthetic upper phase (chloroform/methanol/0.6 M HCl, 3:48:47 v/v) the combined upper phases were diluted with water and applied to 1 ml Dowex 1X8 anion exchanger (formate form). Inositolphosphates were separated according to Berridge *et al.* (1982) [10]. One volume of eluate was mixed with two volumes of Instagel (Packard) and counted for radioactivity by liquid scintillation (Tricarb 2660 from Packard).

Statistical analysis

Data were evaluated for statistical significance by ANOVA and the Bonferroni t-test for multiple comparisons. $P < 0.05$ was considered significant.

Results

As is shown in Table III.1 the addition of 30 μM PhE to ^{3}H-inositol labelled cardiomyocytes caused after 30 minutes an increase in ^{3}H-inositolphosphates accumulation only in the presence of Li^+. This increase was completely blocked by the specific α_1-adrenergic antagonist prazosin. The ß-adrenergic antagonist propranolol had no effect on the PhE-stimulated inositolphosphates formation.

Table III.1. *Receptor specificity of phenylephrine-stimulated [^{3}H-] inositol phosphates formation in neonatal rat cardiomyocytes*

Addition(s)	[^{3}H-]inositolphosphates (% of control ± S.E.M.)
None	93 ± 20
Li^+	100
Phenylephrine	116 ± 14
Li^+, phenylephrine	433 ± 83*
Li^+, phenylephrine, prazosin	117 ± 11
Li^+, phenylephrine, propranolol	356 ± 53*

Data are means ± S.E.M. of at least 6 samples. Control value (100%) is 322 ± 35 d.p.m./dish (n=9). The concentrations used were 10 mM LiCl, 30 μM phenylephrine, 1 μM prazosin and 1 μM propranolol. Incubation time was 30 minutes. *$P < 0.05$.

In two experiments we examined the proportional distribution of the three inositolphosphates. This was found to vary very much between the experiments: IP_1 43.0% - 59.8%; IP_2 33.2% - 30.3%; IP_3 23.8% - 9.9%. In two other experiments we examined the same parameter after shorter incubation periods. Again a large variation: 1 min. IP_1 61.2% - 49.6%; IP_2 25.4% - 34.0%; IP_3 13.4% - 16.4%; 6 min. IP_1 63.1% - 61.0%; IP_2 24.3% - 26.9%; IP_3 12.6% - 12.1%. Although at each time point most of the label was present in IP_1, in every experiment the IP_1 level was seen rising while the radioactivity in IP_3 and IP_2 was constant.

Discussion

Our findings show that myocardial α_1-adrenergic receptors are coupled to the phosphoinositol cycle. This is in agreement with other reports [3,4,5].
The fact that we were not able to show any rise in the level of IP_3 shortly (1 min) after stimulation could be interpreted as an indication that not PIP_2 but PI is the substrate for agonist stimulated phospholipase C in myocardial cells. On the other hand the activity

of the IP_3- and IP_2-phosphatases may be that high that the IP_3- and IP_2-peaks are too transient to be measured directly. The IP_1-phosphatase certainly must be very active as can be derived from the impossibility to measure inositolphosphates formation in the absence of Li^+ (Table III.1). This monovalent cation acts as an inhibitor of this phosphatase [10]. The continuous formation of IP_1 should in this case be seen in consequence of IP_3-formation and -dephosphorylation.
How this could agree with the generally supposed second messenger action of IP_3 is not easily answered. During agonist-receptor binding there could exist a constant flow of IP_3 from its formation site to the calcium storage site. The IP_3-phosphatase may even be involved in the action of IP_3 on the calcium stores. But this is mere speculation and will stay this until some intracellular IP_3-binding site has been identified.
Of more importance as a second messenger (especially in heart muscle) may be the other PIP_2-breakdown product, which was not measured in our experiments: the diglyceride. It is supposed that DG activates PKC which has several protein substrate sites in the myocardium e.g. phospholamban [11]. Whether this phosphorylation actually occurs under α_1-adrenergic stimulation is at present not known. However it would indicate a common target of α_1- and ß-adrenergic stimulation.

REFERENCES

1. BRÜCKNER R, MÜGGE A, SCHOLZ H :Existence and functional role of alpha$_1$-adrenoceptors in the mammalian heart. J Mol Cell Cardiol **17**: 639-645, 1985.
2. OSNES JB, AASS H, SKOMEDAL T: On adrenergic regulation of heart function: role of myocardial α-adrenoceptors. In H Refsum and OD Mjo/s (eds.) *α-Adrenoceptor Blockers In Cardiovascular Disease.* Churchill Livingstone, Edinburgh, London, Melbourne and New York, 1985, pp 69-102.
3. BROWN JH, BUXTON IL, BRUNTON LL: α_1-Adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. Circ Res **57**: 532-537, 1985.
4. POGGIOLI J, SULPICE JC, VASSORT G: Inositol phosphate production following α_1-adrenergic, muscarinic or electrical stimulation in isolated rat heart. FEBS Lett **206**: 292-298, 1986.
5. SCHMITZ W, SCHOLZ H, SCHOLZ J, STEINFATH M, LOHSE M,PUURUNEN J, SCHWABE U: Pertussis toxin does not inhibit the α_1-adrenoceptor-mediated effect on inositol phosphate production in the heart. Eur J Pharmacol **134**: 377-378, 1987.
6. HIRATA M, SUEMATSU E, HASHIMOTO T, HAMACHI T, KOGA T: Release of Ca^{2+} from a non-mitochondrial store site in peritoneal macrophages treated woth saponin by inositol 1,4,5-trisphosphate. Biochem J **223**: 229-236, 1984.
7. VOLPE P, SALVIATI G, DIVIRGILIO F, POZZAN T: Inositol 1,4,5-trisphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle. Nature **316**: 347-349, 1985.
8. BERRIDGE MJ: Inositoltrisphosphate and diacylglycerol as second messengers. Biochem J **220**: 345-360, 1984.
9. YAGEV S, HELLER M, PINSON A: Changes in cytoplasmic and lysosomal enzyme activities in cultured rat heart cells: the relationship to cell differentiation and cell population in culture. In Vitro **20**: 893-898, 1984.
10. BERRIDGE MJ, DOWNES CP, HANLEY MR: Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. Biochem J **206**: 587-7595,1982.
11. MOVSESIAN MA, NISHIKAWA M, ADELSTEIN RS: Phosphorylation of phospholamban by calcium-activated, phospholipid-dependent protein kinase. J Biol Chem **259**: 8029-8032, 1983.

Chapter IV

Phorbolester Inhibits α_1-Adrenoceptor Mediated Phosphoinositide Breakdown in Cardiomyocytes

Johanna T.A. Meij and Jos M.J. Lamers (1989) J Mol Cell Cardiol **21**: 661-668.

Abstract

The regulation of and the intracellular events following α_1-adrenergic receptor stimulation of myocardium are not completely understood. The α_1-adrenergic stimulation of phosphoinositide breakdown was examined in a culture of neonatal rat ventricular myocytes and the influence of a protein kinase C activator, phorbol 12-myristate 13-acetate, on this process was studied. Inositolphosphate accumulation was stimulated by phenylephrine (EC_{50} 5 μM) in the presence of 10 mM LiCl. The increase was antagonized by prazosin (10^{-6} M) but not by propranolol (10^{-6} M). The rate of inositolphosphate accumulation after prolonged α_1-adrenoceptor stimulation decreased without clear evidence of depletion of the membrane phosphatidylinositolbisphosphate pool. Phorbol ester treatment (IC_{50} 10^{-8} M) led to a dose-dependent inactivation of α_1-adrenoceptor stimulated phosphoinositide breakdown. These findings provide evidence that protein kinase C plays a role in the regulation of α_1-adrenoceptor sensitivity.

Introduction

Both α_1- and ß-adrenergic agonists increase the force of contraction in ventricular myocardium. Although both types of agonists evoke an increase of slow inward calcium current the mechanical and electrophysiological responses of the heart to α_1-agonists differ qualitatively and quantitatively from those to ß-agonists [Brückner et al. 1985; Osnes et al. 1985]. Metabolically there are differences as well: α_1-adrenergic stimulation activates glycogen phosphorylase only moderately compared with ß-stimulation [Osnes et al. 1985]. In contrast to the comparatively well-established role of cyclic AMP-dependent protein phosphorylation in the mediation of the ß-adrenergic effects, the cellular mechanisms underlying the α_1-adrenergic effects in the myocardium are largely unknown. In a number of rat heart models stimulation of α_1-adrenoreceptors has been found to enhance phosphoinositide breakdown, e.g. isolated adult ventricular cells [Brown et al.. 1985], isolated papillary muscle [Poggioli et al. 1986], isolated ventricles [Schmitz et al. 1987] and cultured heart cells [Uchida et al. 1982]. The general concept is that receptor stimulation activates phospholipase C to hydrolyse phosphatidylinositol-4,5-bisphosphate (PIP_2)[1] into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). IP_3 may act by mobilizing Ca^{2+} from intracellular stores [Hirata et al. 1984; Volpe et al. 1985] and DG by activating protein kinase C (PKC) [Berridge, 1984]. PKC is also identical to the intracellular "receptor" of tumor-promoting phorbol esters [Castagna et al. 1982] and these have become tools

[1]Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol bisphosphate; IP, inositolphosphate(s); IP_1, inositolmonophosphate; IP_2, inositolbisphosphate; IP_3, inositoltrisphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PhE, phenylephrine; PS, phosphatidylserine.

for investigating the role of PKC.

Generally, the effect of phorbol ester treatment appear to be inhibition of (norepinephrine) activation of phosphoinositide metabolism [Cotecchia *et al.* 1985; Corvera *et al.* 1986] and Ca^{2+}-efflux [Cooper *et al.* 1985], accompanied by a decrease of α_1-adrenoceptor agonist affinity [Cooper *et al.* 1985; Cotecchia *et al.* 1985]. Further reports suggest that through PKC-dependent phosphorylation the α_1- and $ß_2$-adrenergic receptors are desensitized [Bouvier *et al.* 1987; Leeb-Lundberg *et al.* 1987].

To examine the effect of PKC-activation on α_1-adrenoceptor mediated PI-breakdown we used a purified culture of neonatal rat ventricular myocytes. This culture has two advantages over other rat heart models: it contains a near to homogeneous population of cardiac muscle cells and these myocytes have had time to recover fully from the proteolytic treatment used to isolate them. First we characterized the α_1-adrenergic PI metabolism in these cells. Next we examined the effect of phorbol 12-myristate 13-acetate (PMA) on the α_1-adrenoceptor mediated PI breakdown.

Materials and methods

Chemicals

Growth medium: Nutrient mixture Ham F10 (Gibco, Scotland) supplemented with 10% fetal calf serum, 10% horse serum, 200000 units/L penicillin, 0.2 g/L streptomycin (all from Boehringer Mannheim) and 135 mg/L $CaCl_2.2H_2O$; Petri dishes (TC 35/10) were from Greiner, trypsin (type III) was from Sigma as were DL-propranolol, phosphoinositide standards (P-6023) and phorbol 12-myristate 13-acetate; [2-^{3}H-]myoinositol was from Amersham International PLC (Amersham); phenylephrine HCl was from Brocades (Delft); prazosin HCl from Pfizer (New York); Dowex 1X8 (100-200 mesh formate form) from BioRad Labs (California); HPTLC plates (HP-KF, 200 μ) were from Whatman (Clifton, N.J.); Instagel was from Packard (Groningen) and En^{3}Hance from NEN (Boston). All other chemicals were of analytical grade.

Primary heart cell culture

Cardiomyocytes were isolated from the ventricles of 2 to 4-day old Wistar rats by trypsinization according to Yagev *et al.* (1984), We applied a modified enrichment method (Blondel *et al.* 1971) of two successive periods of 30 and 90 min preplating. Of the final suspension containing 9 x 10^5 myoblasts/ml growth medium, 2 ml were seeded per Petri dish. After 24 to 26 h incubation (37°C, 5% CO_2, 95% humidity) this resulted in a confluent monolayer of beating cardiomyocytes. The growth medium was changed every 48 h after seeding. Experiments were done on the third or fifth day after plating under the same incubation conditions as already mentioned.

Cell incubation

Cardiomyocytes were rinsed twice with 1 ml incubation buffer (130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.44 mM NaH_2PO_4, 1.1 mM $MgCl_2$, 0.2% D-glucose, 37°C, equilibrated with 5% CO_2/95 % O_2, pH 7.4) followed by addition of 0.5 ml [2-^{3}H-]myoinositol in buffer. To study ^{3}H-inositolphosphate formation 6.7 μCi/-ml of [2-^{3}H-]myoinositol was added but to study the (possible) change of ^{3}H-phosphoinositides after prolonged incubation 15 μCi/ml of [2-^{3}H-]myoinositol was added. In both cases the dishes were preincubated for 2 h, rinsed twice with 0.5 ml buffer and incubated in buffer, 10 mM LiCl and other additions, as indicated, at a final volume of 1 ml. Incubations were terminated, at the indicated times, by rinsing with 0.5 ml cold buffer followed by addition of 0.5 ml cold methanol/13 M HCl (100:1 v/v). The cells were scraped using a rubber policeman and the suspension was transferred to a glass tube. The dish was rinsed once more with 0.5 ml of the acidified methanol, which was also transferred to the glass tube. Then 0.5 ml chloroform was added and the extract was kept at - 20°C overnight. The phases were separated by adding 0.5 ml 2.5 N HCl and 0.5 ml chloroform and centrifugation (10 min, 1770 x g).

Assay of [^{3}H]-inositolphosphates

The aqueous (upper) phase was transferred to another tube and the organic (lower) and intermediate phases were washed with 1 ml synthetic upper phase (chloroform/methanol/0.6 M HCl 3:48:47 v/v), followed by centrifugation (10 min, 1770 x g). The upper phases were combined and diluted 5 times with distilled water. The inositolphosphates were separated by anion exchange using 1 ml Dowex 1X8 as originally described by Berridge *et al.* (1982). Briefly free inositol and glycerophosphoinositides were eluted with 20 ml distilled water and 10 ml 5 mM disodiumtetraborate in 30 mM Na-formate respectively. IP_1, IP_2 and IP_3 were eluted either separately with increasing concentrations of 10 ml ammoniumformate (0.2 M, 0.5 M and 1 M, respectively) in 0.1 M formic acid [Bijsterbosch *et al.* 1985] or all together with 10 ml 1 M ammoniumformate in 0.1 M formic acid. One volume of the eluate was mixed with two volumes Instagel and counted for radioactivity by liquid scintillation (Tri-carb 2660 from Packard).

Assay of [^{3}H]-phosphoinositides

The organic (lower) phases of 4 to 5 similarly treated samples were combined and evaporated to dryness with N_2, redissolved in methanol/chloroform/water (25:75:2 v/v) and spotted on HPTLC plates - previously impregnated with 1% potassiumoxalate in methanol/water (2:3 v/v) and activated (15 min, 110°C). The plates were developed in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8 v/v) as described by Jolles *et al.* (1981). After spraying the plates with En^{3}Hance the spots were visualized by autoradiography (Kodak SB 100) and identified by comparison with simultaneously run standard phosphoinositides. The scraped spots were sonicated in 5 ml Instagel and counted

for radioactivity by liquid scintillation (Tricarb 2660 from Packard).

Statistical analysis

Data were evaluated for statistical significance by analysis of variance and the Bonferroni t-test for multiple comparisons. $P < 0.05$ was considered significant.

Results

Phosphoinositide breakdown

Addition of 30 μM phenylephrine (PhE) to ^{3}H-inositol-labelled cardiomyocytes caused an increase in ^{3}H-IP formation only in the presence of Li^+, as is shown in Table IV.1. The experiments in which IP_1, IP_2 and IP_3 were eluted separately, demonstrated that most of the ^{3}H-label was present in IP_1. This is consistent with the observations of others [Brown *et al.* 1985; Schmitz *et al.* 1987; Steinberg *et al.* 1987]. We always found that the increase in ^{3}H-IP_3 was low. The mean (± S.E.M.) factor of stimulation by PhE of ^{3}H-IP_3 at 6 min was 1.1 ± 0.1, at 30 min 1.8 ± 0.5 and at 60 min 2.6 ± 1.4. This was not at all a reflection of the relative stimulation of total inositolphosphates. In all other experiments we therefore measured the total ^{3}H-IP formation.

The increase of IP-formation was completely blocked by the specific α_1-adrenergic antagonist prazosin. The ß-adrenergic antagonist propranolol had no effect on PhE-stimulated IP-formation (Table IV.1). The results shown in Table IV.1 were those after 30 min but those after 15 min (not shown) were identical.

To find out whether PhE-stimulation resulted in a continuous IP-formation the time-course of ^{3}H-IP accumulation was measured. The results are illustrated in Figure IV.1. Stimulation periods of up to 30 min resulted in a time-dependent increase in ^{3}H-

TABLE IV.1. Receptorspecificity of phenylephrine-stimulated[^{3}H]-inositol phosphate formation in neonatal rat cardiomyocytes.

Addition(s):	[^{3}H]-inositolphosphates (% of control ± S.E.M.)
None	93 ± 20
Li^+ (control)	100
Phenylephrine	116 ± 14
Li^+, phenylephrine	433 ± 83*
Li^+, phenylephrine, prazosin	117 ± 11
Li^+, propranolol	100 ± 15
Li^+, phenylephrine, propranolol	356 ± 53*

Data are means ± S.E.M. of at least six samples. Control value (100%) is 322 ± 35 d.p.m./dish (n=9). The concentrations used were 10 mM LiCl, 30 μM phenylephrine, 1 μM prazosin and 1 μM propranolol. Incubation time was 30 min.
*Significantly different from control ($P \leq 0.05$).

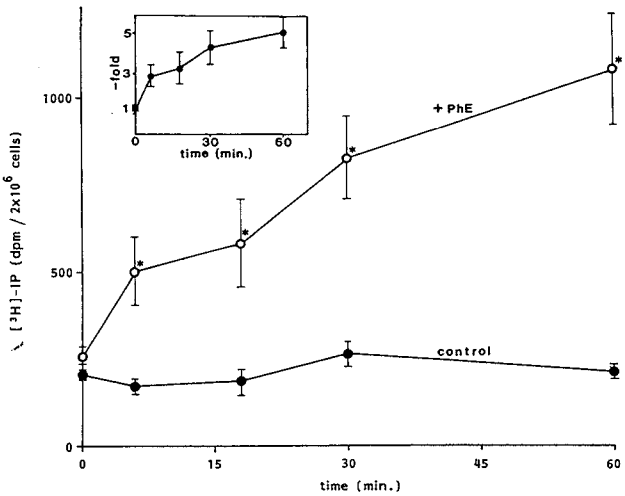

Figure IV.1. Time course of PhE-stimulated ^{3}H-IP formation in neonatal rat ventricular myocytes. ^{3}H-Inositol labelled cardiomyocytes in medium with 10 mM LiCl were incubated with (o) or without (o) 30 μM PhE for the indicated times. Determinations were as described in Materials and Methods. Data are the means $\pm$ S.E.M. of duplicates from up to 5 experiments. *Significant increase from control (P 0.05).
Insert. Time course of the relative Phe-induced stimulation of ^{3}H-IP-formation.
" -fold" expresses the factor of stimulation by PhE of the ^{3}H-IP-level at each time point. Data are the means $\pm$ S.E.M. of duplicates from up to 5 experiments.

IP accumulation. Between 30 and 60 min the ^{3}H-IP accumulation tended to plateau. This tendency was more pronounced if the relative stimulation was regarded (Fig. IV.1 insert). It was not due to submaximal labelling of the ^{3}H-phosphoinositide pool because a similar time-course was obtained with cells labelled overnight (unpubl. obs.). To examine whether the attenuation of ^{3}H-IP formation was due to depletion of the ^{3}H-PIP_2 pool we separated the (poly)-phosphoinositides and visualized them by autoradiography. Except for the PI-spots the latter was only possible after combining extracts of several (4 to 5) dishes and using a higher concentration of ^{3}H-inositol. Figure IV.2 shows the pattern of ^{3}H-phospholipids and the position of the phosphoinositide-standards. No difference in amount of ^{3}H-PIP_2 or other ^{3}H-PI-lipids between unstimulated and stimulated cells is visible. Counting of TLC-scrapings did not reveal significant differences, due to prolonged incubation or stimulation, in the total amount of ^{3}H-phosphoinositides (Table IV.2). The increases in total d.p.m. incorporated into PI lipids after 6 min incubation and the decreases after 30 and 60 min incubation of both stimulated and unstimulated cells are not statistically significant. The distribution of label among the three phosphoinositides changed slightly in the PhE-stimulated cells. The percentage of label present in PIP_2 of stimulated cells increased with time and was significantly different from that of unstimulated cells after 30 and 60 minutes. However, in absolute d.p.m. this increase was compensated by the not significant decrease in the total d.p.m. of ^{3}H-phosphoinositides. These results indicate that up to 60 min incubation with PhE no depletion of labelled phospholipase C substrate

Table IV.2 *[^{3}H]-Inositol incorporated in PI-lipids*

Incubation time (min)	% dpm/dish of total PI	PIP	PIP_2	total dpm PI-lipids/dish
		stimulated		
0	91.33 ± 1.42	4.50 ± 1.74	4.17 ± 0.40°	147343 ± 18230
6	91.39 ± 1.48	4.14 ± 1.71	4.47 ± 0.54	179895 ± 31847
30	89.28 ± 1.06	5.34 ± 1.38	5.33 ± 0.45*	119705 ± 9017
60	88.46 ± 1.64	5.23 ± 1.62	6.32 ± 0.46+°	114856 ± 13005
		unstimulated		
6	91.20 ± 1.55	4.69 ± 1.68	4.11 ± 0.13	170348 ± 27811
30	91.84 ± 1.12	4.31 ± 1.06	3.85 ± 0.12*	163900 ± 34076
60	90.77 ± 2.15	4.85 ± 2.09	4.38 ± 0.22+	166648 ± 38264

Data are mean (percentage) ± S.E.M. from 3 experiments.
*+° Significantly different from each other ($P < 0.05$).

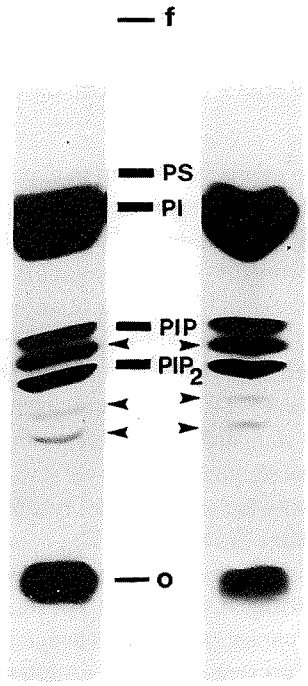

Figure IV.2. Autoradiograph of ^{3}H-labelled phospholipids separated by thin layer chromatography.
^{3}H-labelled cardiomyocytes in medium with 10 mM LiCl were incubated with (left lane) or without (right lane) 30 μM PhE for 30 minutes. Lipid extraction and t.l.c. were as described in Materials and Methods. Position of standards (middle lane) as detected by iodine vapour. Indicated (arrow) spots represent lyso(poly)phosphoinositides. **o** = origin; **f** = front.

occurs. Whether this is also applicable to the total (poly)PI-pool can only be answered by measurement of the unlabelled PIP_2-concentration.

We also tested the effect of different concentrations of PhE on ^{3}H-IP formation. As is illustrated in Figure IV.3, PhE evoked a concentration-dependent activation of phospholipase C. At concentrations over 30 μM the activity seems at a maximum. Under 3 μM the initial rate of ^{3}H-IP-formation is very low (no IP accumulation after 6 min). In the dose-response plot of the 30 min incubation experiments an EC_{50} of 5 μM PhE was found. However the PhE concentration at which a half-maximal response is obtained differed depending on time of incubation (EC_{50} = 15 μM in the 6 min incubation experiments).

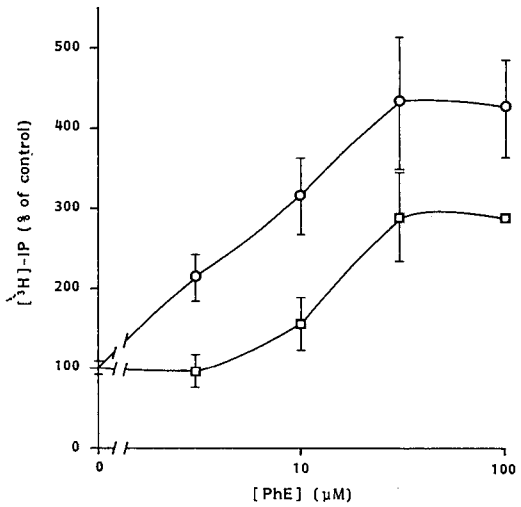

Figure IV.3. Dose-dependence of PhE-stimulated ^{3}H-IP formation in neonatal rat cardiomyocytes. ^{3}H-inositol labelled cardiomyocytes in medium with 10 mM LiCl were incubated for 6 min ( ) or for 30 min (o) with PhE at the indicated doses. Determinations were as described in Materials and Methods. Data are the means (± S.E.M.) of duplicates or triplicates (% of control) from up to 3 separate experiments. Control values, obtained with 10 mM LiCl only, are 168±23 d.p.m./dish (n=4) for 6 min and 269±33 d.p.m./dish (n=5) for 30 min.

Effect of phorbolester

To study the effect of PKC on phospholipase C-activation by PhE we added different concentrations of the tumor-promoter PMA to the myocytes and incubated them (10 mM LiCl) with or without 30 μM PhE for 30 min. The effect of PMA on the activated and basal ^{3}H-IP-accumulation is illustrated in Figure IV.4. PMA progressively reduced PhE-stimulated ^{3}H-IP-accumulation at the tested concentrations although inhibition was not complete even at 1 μM PMA.

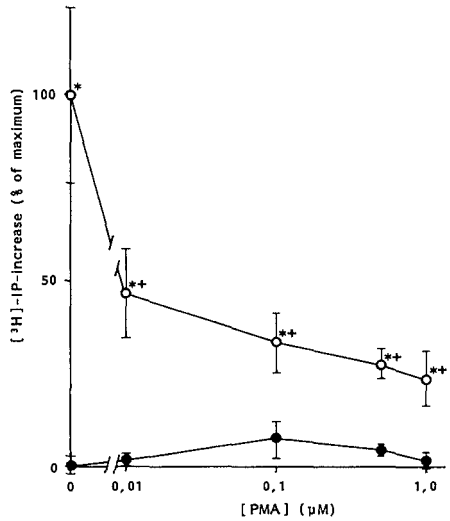

Figure IV.4. Effect of PMA on ^{3}H-IP phosphate formation in neonatal rat cardiomyocytes. ^{3}H-inositol labelled cardiomyocytes in medium with 10 mM LiCl were incubated with PMA (0-1 μM) and with (o) or without (o) 30 μM PhE for 30 min. Determinations were as described in Materials and Methods. Data are the means (± S.E.M.) of duplicates from 4-5 experiments expressed as percentage of maximum increase (0 μM PMA, 30 μM PhE). *Significant increase from control (P 0.01) $^+$Significant decrease from maximum.

Discussion

Our finding that α_1-adrenergic receptors in cardiomyocytes are coupled to phosphoinositide breakdown agrees with the reports of others [Uchida *et al.* 1982; Brown *et al.* 1985; Otani *et al.* 1986; Steinberg *et al.* 1987; Woodcock *et al.* 1987a,b and c; Scholz *et al.* 1988]. In this respect neonatal rat ventricular myocytes prove to be an excellent model. We preferred this model for reasons already mentioned (see Introduction), although it may be that the α_1-adrenoceptor response qualitatively differs from that of isolated adult cardiomyocytes because minor morphological differences exist: not rod-shaped, still rudimentary T-tubules present(Moses and Kasten, 1979; Simpson and Savion, 1982). None the less, in the present study it became apparent that inositolmonophosphatase is also very active in the neonatal cell type. Without inhibiting this enzyme by Li^+ no ^{3}H-IP-formation due to α_1-adrenoceptor stimulation could be measured [*cf.* Brown *et al.* 1985].

Alpha$_1$-adrenoceptor stimulated ^{3}H-IP-formation almost plateaued after 30 min. Apparently the rate of IP-formation, which reflects the activity of phospholipase C, gradually decreased. Brown *et al.* (1985) observed in similar experiments with adult rat cardiomyocytes a ^{3}H-IP increase which was linear for at least 40 min. The decrease observed in the present study is not caused by depletion of PI-lipids, as neither the total ^{3}H-PI-pool nor the ^{3}H-PIP$_2$-pool decreased significantly after 30 min PhE stimulation. The attenuation

of the α_1-adrenergic effect may therefore have another cause. Fratelli and De Blase (1987) showed evidence for agonist-induced α_1-receptor sequestration in smooth muscle. Also it has been suggested that the adrenergic receptors are desensitized by protein kinase activation [Cooper *et al.* 1985; Cotecchia *et al.* 1985; Colucci *et al.* 1986; Garcia-Sainz *et al.* 1986]. Indeed it has been shown that the α_1-adrenoceptor is a substrate for PKC-dependent phosphorylation [Leeb-Lundberg *et al.* 1985, 1987]. PKC-induced phosphorylation of the α_1-adrenoceptor could be a cause of the slowing down of PhE-stimulated IP-formation. DG formation is also stimulated and DG in turn activates PKC. But there is no proof that in our system PKC is activated. PKC-activation through another PI cycle coupled receptor in neonatal rat cardiomyocytes has recently been reported. Dösemeci *et al.* (1988) showed that angiotensin II activated PKC through stimulation of phosphoinositide breakdown. Furthermore they showed that this resulted in a protein phosphorylation pattern similar to that induced by direct activation of PKC by a phorbol ester. We showed that the α_1-adrenergic response spontaneously decreased after prolonged stimulation of cells and also that the PKC-activator PMA markedly reduced the α_1-adrenergic response (Fig 3). So if we assume a parallel between α_1-adrenergic and PMA-induced effects on the level of PKC, then PKC-induced phosphorylation of an α_1-adrenoceptor subunit could explain our results.

The effects of phorbol esters on unstimulated cardiac functions in perfused rat heart [Yuan *et al.* 1987] and in neonatal rat cardiomyocytes [Dösemeci *et al.* 1988] have recently been reported. Phorbol ester induced a negative inotropic effect and activated PKC in both systems in a concentration range similar to the one effective in the present study (10-100 nM). This negative inotropic effect of phorbol ester is in contradiction with our assumption that PKC-activation is a consequence of α_1-adrenoceptor stimulation.

Apart from an attenuation after prolonged PhE-stimulation (30-60 min) the rate of IP-formation within the first minutes of PhE-stimulation is particularly dependent on the used agonist-dose. Figure IV.2 shows that stimulation with 30 μM PhE or a higher dose immediately gives a maximal rate of IP-formation. The rate of IP-formation at doses less than 30 μM PhE is much lower. Hardly any formation of IP has occurred within the first 6 min at 3 μM PhE. The EC_{50} value found in the 30 min incubation series is in accordance with K_D values found for phenylephrine competing for radioligand binding sites in cultured heart cells [Kupfer *et al.* 1982; Muntz *et al.* 1985]. Why a different EC_{50} of PhE is found for the 6 min incubation series cannot be explained on the basis of the present results. At any rate the incubation time dependence of the EC_{50} cannot be explained by the observed attenuation of the α_1-response after prolonged incubation (Fig. 1). The latter would either give an unaltered EC_{50} or a higher EC_{50} for the longer incubation period. So far there does not only appear to be a down-regulation at optimal α_1-agonist concentration but also a sensitization of the receptor which is dose-dependent.

Acknowledgements

The authors wish to thank Prof. A. Pinson (The Hebrew University Hadassah Medical

School, Jerusalem) for his advice in setting up the heart cell culture and Ms. A.C. Hanson for excellent secretarial assistance.

REFERENCES

1. BERRIDGE MJ (1984) Inositoltrisphosphate and diacylglycerol as second messengers. Biochem J **220**: 345-360.
2. BERRIDGE MJ, DOWNES CP, HANLEY MR (1982) Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. Biochem J **206**: 587-595.
3. BIJSTERBOSCH MK, MEADE CJ, TURNER GA, KLAUS GGB (1985) B lymphocyte receptors and polyphosphoinositide degradation. Cell **41**: 999-1006.
4. BLONDEL B, ROIJEN I, CHENEVAL JP (1971) Heart cells in culture: A simple method for increasing the proportion of myoblasts. Experientia **27**: 356-358.
5. BOUVIER M, LEEB-LUNDBERG LMF, BENOVIC JL, CARON MG, LEFKOWITZ RJ (1987) Regulation of adrenergic receptor function by phosphorylation II Effects of agonist occupancy on phosphorylation of α_1- and β_2-adrenergic receptors by protein kinase C and the cyclic AMP-dependent protein kinase. J Biol Chem **262**: 3106-3112.
6. BROWN JH, BUXTON IL, BRUNTON LL (1985) α_1-Adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. Circ Res **57**: 532-537.
7. BRüCKNER R, MüGGE A, SCHOLZ H (1985) Existence and functional role of alpha$_1$-adrenoceptors in the mammalian heart. J Mol Cell Cardiol **17**: 639-645.
8. CASTAGNA M, TAKAI Y, KAIBUCHI K, SANO K, KIKKAWA U, NISHIZUKA Y (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem **257**: 7847-7851.
9. COLUCCI WS, GIMBRONE MA, ALEXANDER RW (1986) Phorbol diester modulates α-adrenergic receptor-coupled calcium efflux and α-adrenergic receptor number in cultured vascular smooth muscle cells. Circ Res **58**: 393-398.
10. COOPER RH, COLL KE, WILLIAMSON JR (1985) Differential effects of phorbol ester on phenylephrine and vasopressin-induced Ca^{2+} mobilization in isolated hepatocytes. J Biol Chem **260**: 3281-3288.
11. CORVERA S, SCHWARTZ KR, GRAHAM RM, GARCIA-SAINZ JA (1986) Phorbol esters inhibit α_1-adrenergic effects and decrease the affinity of liver cell α_1-adrenergic receptors for (-)-epinephrine. J Biol Chem **261**: 520-526.
12. COTECCHIA S, LEEB-LUNDBERG LMF, HAGEN PE, LEFKOWITZ RJ, CARON MG (1985) Phorbol ester effects on α_1-adrenoceptor binding and phosphatidylinositol metabolism in cultured vascular smooth muscle cells. Life Sci **37**: 2389-2398.
13. DÖSEMECI A, DHALLAN RS, COHEN NM, LEDERER WJ, ROGERS TB (1988) Phorbol ester increases calcium current and simulates the effects of angiotensin II on cultured neonatal rat heart myocytes. Circ Res **62**: 347-357.
14. FRATELLI M, DEBLASI A (1987) Agonist-induced α_1-adrenergic receptor changes.

Evidence for receptor sequestration. FEBS Lett **212**: 149-153.

15. GARCIA-SAINZ JA, HERNANDEZ-SOTOMAYOR SMT, TUSSIE-LUNA MI (1986) Homologous and heterologous desensitization of one of the pathways of the α_1-adrenergic action. Effects of epinephrine, vasopressin, angiotensin II and phorbol 12-myristate 13-acetate. Biochim Biophys Acta **887**: 73-79.
16. HIRATA M, SUEMATSU E, HASHIMOTO T, HAMACHI T, KOGA T (1984) Release of Ca^{2+} from a non-mitochondrial store site in peritoneal macrophages treated with saponin by inositol 1,4,5-trisphosphate. Biochem J **223**: 229-236.
17. JOLLES J, ZWIERS H, DEKKER A, WIRTZ KA, GISPEN WH (1981) Corticotropin-(1-24)-tetracosapeptide affects protein phosphorylation and polyphosphoinositide metabolism in rat brain. Biochem J **194**: 283-291.
18. KUPFER LE, ROBINSON RB, BILEZIKIAN JP (1982) Identification of α_1-adrenergic receptors in cultured rat myocardial cells with a new iodinated α_1-adrenergic antagonist [^{125}I-]IBE 2254. Circ Res **51**: 250-254.
19. LEEB-LUNDBERG LMF, COTECCHIA S, DEBLASI A, CARON MG, LEFKOWITZ RJ (1987) Regulation of adrenergic receptor function by phosphorylation I Agonist-promoted desensitization and phosphorylation of α_1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT_1 MF-2 smooth muscle cells. J Biol Chem **262**: 3098-3105.
20. LEEB-LUNDBERG LMF, COTECCHIA S, LOMASNEY JW, DEBERNARDIS JF, LEFKOWITZ RJ, CARON MG (1985) Phorbol esters promote α_1-adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. Proc Natl Acad Sci USA **82**: 5651-5655.
21. MOSES RL, KASTEN FH (1979) T-Tubes in cultured mammalian myocardial cells. Cell Tiss Res **203**: 173-180.
22. MUNTZ KA, GARCIA C, HAYLER HW (1985) $_1$-receptor localization in rat heart and kidney using autoradiography. Am J Physiol **249**: H512-519.
23. OSNES JB, AASS H, SKOMEDAL T (1985) On adrenergic regulation of heart function: role of myocardial α-adrenoceptors. In: *α-Adrenoceptor blockers in cardiovascular disease*, edited by H Refsum, OD Mjo/s. Edinburgh, London, Melbourne and New York, Churchill Livingstone, pp 69-102.
24. OTANI H, OTANI H, DAS DK (1986) Evidence that phosphoinositide response is mediated by α_1-adrenoceptor stimulation, but not linked with excitation-contraction coupling in cardiac muscle. Biochem Biophys Res Commun **136**: 863-869.
25. POGGIOLI J, SULPICE JC, VASSORT G (1986) Inositol phosphate production following α_1-adrenergic, muscarinic or electrical stimulation in isolated rat heart. FEBS Lett **206**: 292-298.
26. SCHMITZ W, SCHOLZ H, SCHOLZ J, STEINFATH M, LOHSE M, PUURUNEN J, SCHWABE U (1987) Pertussis toxin does not inhibit the α_1-adrenoceptor-mediated effect on inositol phosphate production in the heart. Eur J Pharmacol **134**: 377-378.

27. SCHOLZ J, SCHAEFER B, SCHMITZ W, SCHOLZ H, STEINFATH M, LOHSE M, SCHWABE U, PUURUNEN J (1988) *Alpha*-1 adrenoceptor mediated positive inotropic effect and inositol trisphosphate increase in mammalian heart. J Pharmacol Exp Ther **245**: 327-335.
28. SIMPSON P, SAVION S (1982) Differentiation of rat myocytes in single cell cultures with and without proliferating nonmyocardial cells. Circ Res **50**: 101-116.
29. STEINBERG SF, CHOW YK, ROBINSON RB, BILEZIKIAN JB (1987) A pertussis toxin substrate regulates α_1-adrenergic-dependent phosphatidylinositol hydrolysis in cultured rat myocytes. Endocrinology **12**: 1889-1895.
30. UCHIDA T, BHATNAGAR GM, LAKKATTA EG, FILBURN CR (1982) Alpha-adrenergic stimulation of $^{32}PO_4$ labelling of phosphatidylinositol (PI) and phosphatidic acid (PA) in cultured rat heart cells (Abstr) Fed Proc **41**: 1523.
31. VOLPE P, SALVIATI G, DIVIRGILIO F, POZZAN T (1985) Inositol 1,4,5-trisphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle. Nature **316**: 347-349.
32. WOODCOCK EA, MCLEOD JK, SMITH IA, CLARK MG (1987a) Study of receptor-stimulated phosphatidylinositol hydrolysis in intact perfused rat hearts. Clin Exp Pharmacol Physiol **14**: 209-213.
33. WOODCOCK EA, SMITH IA, WALLACE CA, WHITE LBS (1987b) Evidence for a lack of inositol-(1,4,5)*tris*phosphate kinase activity in norepinephrine-perfused rat hearts. Biochem Biophys Res Commun **148**: 68-77.
34. WOODCOCK EA, WHITE LBS, SMITH IA, MCLEOD JK (1987c) Stimulation of phosphatidylinositol metabolism in isolated, perfused rat heart. Circ Res **61**: 625-631.
35. YAGEV S, HELLER M, PINSON A (1984) Changes in cytoplasmic and lysosomal enzyme activities in cultured rat heart cells: the relationship to cell differentiation and cell population in culture. In Vitro **20**: 893-898.
36. YUAN S, SUNAHARA TA, SEN AK (1987) Tumor-promoting phorbol esters inhibit cardiac functions and induce redistribution of protein kinase C in perfused beating rat heart. Circ Res **61**: 372-378.

Chapter V

Phorbol Ester and the Activities of Phosphatidylinositol 4,5-Bisphosphate Specific Phospholipase C and Protein Kinase C in Microsomes Prepared from Cultured Cardiomyocytes

Johanna T.A. Meij, Karel Bezstarosti and Jos M.J. Lamers (1989) Submitted for publication.

Abstract

Microsomes were prepared from cultured neonatal rat cardiomyocytes. Incubation of microsomes in buffer containing 5 μM $CaCl_2$, 9 mM cholate and 100 nM [^{3}H]-PI(4,5)P_2 resulted in the formation of ^{3}H-IP_3. GTPτS (125 μM) stimulated the production of ^{3}H-IP_3. Microsomes prepared from phorbol ester-treated (100 nM phorbol 12-myristate 13-acetate, PMA) cardiomyocytes showed decreased activities of basal as well as GTPτS-stimulated [^{3}H-]PI(4,5)P_2 hydrolysis. In the microsomes a 15 kD protein was demonstrated to be the major substrate phosphorylated by intrinsic protein kinase C, which was activated by 0.5 mM Ca^{2+}. Addition of phorbol ester (100 nM PMA) enhanced the ^{32}P-incorporation into the 15 kD protein. Protein kinase C, purified from rat brain, in the presence of Ca^{2+}, diglyceride, and phosphatidylserine did not change the phosphorylation pattern any further. In conclusion, it was shown that phorbol ester pretreatment of neonatal rat cardiomyocytes reduces microsomal GTPτS stimulated PI(4,5)P_2-specific phospholipase C and that in cardiomyocyte microsomes phorbol ester activates protein kinase C-induced 15 kD protein phosphorylation. The results indicate that phorbol ester may down-regulate α_1-adrenoceptor mediated PI(4,5)P_2 hydrolysis by activation of protein kinase C-induced 15 kD protein phosphorylation.

Introduction

The mammalian ventricular myocardium contains two types of receptors: ß- and α_1-adrenoceptors [Osnes 1985]. Although the $ß_1$-adrenoceptors by far outnumber the α_1-adrenoceptors, the latter type has lately become a focus of attention because of alterations in the number of α_1-type adrenoceptors in ischaemic myocardium [Lee 1988]. The responses of the heart to α_1-adrenergic stimulation are much different from those to $ß_1$-adrenergic stimulation. Therefore it seemed likely that the cellular mechanisms underlying these responses were also different.

In contrast to the comparatively long-established role of cyclic AMP-dependent protein phosphorylation in the mediation of the ß-adrenergic effects, the mechanism transducing the α_1-adrenoceptor signal in the myocardium has only recently been discovered. It has become apparent from several studies [Brown et al. 1985; Poggioli et al. 1986; Steinberg et al. 1987; Woodcock et al. 1987; Meij and Lamers 1989a,b] that myocardial α_1-adrenergic receptors are coupled to phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) breakdown. The hydrolysis of this sarcolemmal phospholipid by a specific phospholipase C (PI(4,5)P_2-PLase C) yields two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) [Berridge 1984]. IP_3 may act by mobilizing calcium from intracellular stores [Hirata et al. 1984; Volpe et al. 1985] and DG by activating protein kinase C (PKC) [Berridge 1984]. PKC appears also identical to the

intracellular "receptor" of tumor-promoting phorbol esters [Castagna *et al.* 1982] and these have become tools for investigating the role of PKC.

Generally, the effects of phorbol ester treatment appear to be inhibition of (norepinephrine) activation of phosphoinositide metabolism [Cotecchia *et al.* 1985] and Ca^{2+}-efflux [Colucci *et al.* 1986], accompanied by a decrease of α_1-adrenoceptor agonist affinity [Cotecchia *et al.* 1985]. Further reports suggest that through PKC-dependent phosphorylation the α_1- and $ß_2$-adrenergic receptors are desensitized [Bouvier *et al.* 1987; Leeb-Lundberg *et al.* 1987].

Previously we characterized the myocardial α_1-adrenoceptor mediated $PI(4,5)P_2$ breakdown and examined the effect of phorbol ester on this process using cultured neonatal rat ventricular myocytes [Meij and Lamers 1989b]. We found that pretreatment of these cells with phorbol 12-myristate 13-acetate (PMA, 0.01 to 1 μM) led to a dose-dependent inactivation of α_1-adrenoceptor stimulated $PI(4,5)P_2$ breakdown. This finding suggested that PKC is involved in the down-regulation of α_1-adrenoceptor responsiveness. Phosphorylation by PKC of a receptor subunit [Leeb-Lundberg *et al.* 1987] or a GTP-binding protein coupling the receptor to $PI(4,5)P_2$-PLase C [Orellana *et al.* 1987] may be involved.

Therefore it became interesting to study the action of PMA on membrane-bound enzyme systems involved in the transduction of the α_1-signal. In the present study we measured the $PI(4,5)P_2$-PLase C activity in microsomes from cultured neonatal rat cardiomyocytes and its sensitivity to prior treatment of the cardiomyocytes with PMA. Also the PMA effects on the PKC intrinsic to the microsomes, were studied whereby attention was focussed on its specific substrate protein(s).

Materials and methods

Materials

Growth medium: Nutrient mixture Ham F10 (Gibco, Scotland) supplemented with 10% fetal calf serum, 10% horse serum, 200000 units/L penicillin, 0.2 g/L streptomycin (all from Boehringer Mannheim) and 135 mg/L $CaCl_2.2H_2O$; Petri dishes (TC 35/10) were from Greiner; trypsin (type III) was from Sigma and so were the phosphoinositide standards, phosphatidylserine (PS), diolein (DG), phorbol 12-myristate 13-acetate (PMA) and the catalytic subunit of cyclic AMP-dependent protein kinase (CSU); phosphatidylinositol 4,5-bisphosphate [inositol-2-^{3}H(N)] (4.4 Ci/mmol) was from DuPont NEN (Boston, USA); [γ-^{32}P]ATP was purchased from Amersham (Amersham, UK); Dowex 1X8 (100-200 mesh formate form) was from BioRad Labs (California, USA); HPTLC plates (HP-KF, 200 μ) were from Whatman (Clifton NJ, USA); Instagel was from Packard (Groningen, The Netherlands). All other chemicals were of analytical grade. Protein kinase C (PKC), purified from rat brain (Snoek *et al.* 1988), was a kind gift of Dr. G.T. Snoek, Centre for Biomembranes and Lipid Enzymology, State University of Utrecht, Utrecht, The Netherlands.

Primary heart cell culture

Cardiac cells were isolated from ventricles of 2-4 day old Wistar rats by stepwise trypsinization as described before [Meij and Lamers 1989b]. A near to homogeneous suspension of cardiomyocytes was obtained by two successive periods of 30 and 90 min preplating. Finally 1.8 x 10^6 myoblasts were seeded per petridish, which after 24 to 26 h incubation (37°C, 5% CO_2, 95% humidity) resulted in a confluent monolayer of beating cardiomyocytes. Every 48 h the growth medium was changed. Pretreatment of cells and the isolation of microsomes were done on the third day after plating.

Pretreatment of the cardiomyocytes

In the experiments in which the cardiomyocytes were pretreated with PMA the following method was employed. After decanting the growth medium cardiomyocytes were rinsed twice with 1 ml incubation buffer (130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20mM $NaHCO_3$, 0.44 mM NaH_2PO_4, 1.1 mM $MgCl_2$, 11.1 mM D-glucose, 37°C, gassed, pH 7.4) followed by preincubation in 1 ml buffer with or without 100 nM PMA for 30 min as previously described [Meij and Lamers 1989b]. Pretreatment was stopped by decanting the buffer and rinsing the cells, followed by the isolation of microsomes.

Preparation of microsomal fraction

After decanting either the growth medium or the PMA containing buffer the cells were rinsed twice with 1 ml 0.9% NaCl. The cells were scraped from the culture dishes in homogenization buffer containing 20 mM 2-morpholinoethanesulfonic acid (MES pH 6.5) and 5 mM DL-dithiothreitol (DTT). Cells were homogenized at 4°C in a Polytron (PT 10/35, Kinematica, Kriens, Switzerland) by two short bursts at position 4 followed by a 10 sec burst at position 6. Further homogenization was done in a tissue grinder with teflon pestle (Braun, Melsungen, FRG). Small aliquots were taken from the homogenate and the remaining volume was centrifuged for 10 min at 5000 x g_{av} (Sorvall RC-5, SS 34 rotor, Dupont Instruments, Newtown CT, USA). The pellet was discarded and the supernatant was centrifuged for 60 min at 100000 x g_{av} (Spinco L50, TY 65 rotor, Beckman Instruments, Mijdrecht, The Netherlands). The resulting supernatant was discarded, the pellet resuspended in 0.1 M MES (pH 6.5) and rapidly frozen in liquid nitrogen and kept at -80°C. Protein measurements were done according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Phospholipase C assay

Essentially, the method described by Jackowski *et al.* (1987) was applied. The commercially obtained solution of [^{3}H-]PI(4,5)P_2 was transferred in 25 μl portions to small acid-washed glass vials with a teflon-sealed screw cap. Prior to each experiment the solvent of one

portion was evaporated under nitrogen and the ^{3}H-PI(4,5)P_2 redissolved in 15 μl 240 mM sodium cholate, overnight at 4°C. Shortly before incubation an equal volume of water was added and the solution held in a sonication bath for 30 sec, after which the solution was mixed with the other reaction ingredients.

[^{3}H-]PI(4,5)P_2 breakdown was measured in a reaction mixture containing 0.1 M MES (pH 6.5), 5 μM $CaCl_2$, 15 μg microsomal protein, 100 nM [^{3}H-]PI(4,5)P_2, and 9 mM sodium cholate in a final volume of 40 μl. The samples were incubated at 37°C for the indicated times. The reaction was stopped by cooling on ice immediately followed by the addition of 144 μl $CHCl_3/CH_3OH/HCl$ (1:2:0.2 v/v). Phases were separated by adding 48 μl 2 M KCl and 48 μl $CHCl_3$ and microcentrifugation. The inositol phosphates were separated from the upper phase by anion exchange on Dowex 1X8 columns and quantitated by liquid scintillation counting as described before [Meij and Lamers 1989b].

To check the chemical composition of the dissolved [^{3}H-]PI(4,5)P_2, in some experiments the lower phases were dried under nitrogen and redissolved in $CHCl_3$. Phosphoinositides were separated on high performance thin layer chromatography (HPTLC) plates as previously described [Meij and Lamers 1989b] and identified by co-chromatographed standards. The spots were scraped, mixed with Instagel and counted for liquid scintillation.

Phosphorylation of microsomal proteins

Proteins were phosphorylated in a reaction mixture containing 50 mM 3-[N-morpholino]propanesulfonic acid (MOPS, pH 7.5), 4 mM $MgCl_2$, 4 mM NaF, 0.5 mM $CaCl_2$ or ethyleneglycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 10 μg microsomal protein and additions of PKC, DG, PS, PMA, CSU as indicated, in a final volume of 50 μl. The PKC, purified from rat brain, was kept at 4°C in a stock solution containing 20 mM Tris, 2 mM EGTA, 5 mM ethylenedinitrilotetraacetic acid (EDTA), 10% glycerol, 1 mM dithioerythrit, 100 μg/ml leupeptin, 1 IU/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF) and diluted before use. The correct amount from a stock chloroform solution of PS was, where appropriate, mixed with the correct amount from a stock chloroform solution of DG, evaporated under N_2 followed by redissolving in water by sonication (3 times 10 sec). PMA was added from a stock solution in dimethylsulfoxide (DMSO) to the sonicated solution of PS.

After 3 min preincubation at 30°C, incubation was started by the addition of 10 μM [^{32}P-]ATP (10 μCi). The reaction was terminated after 2 min by the addition of 3% (w/v) sodium dodecyl sulfate (SDS), 6% (v/v) glycerol, 1% (v/v) ß-mercaptoethanol. Samples were boiled for 10 min and separated on 15% SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) and analyzed as previously described [Lamers and Stinis 1980].

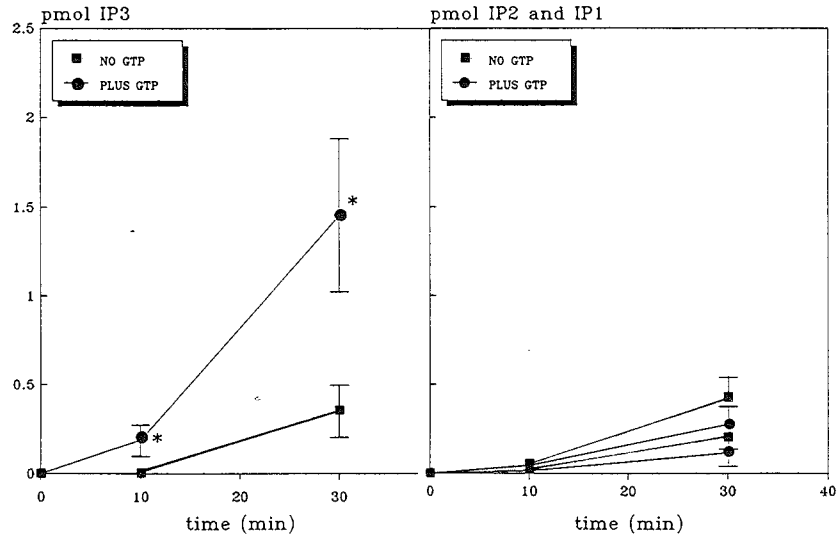

Figure V.1. Time-course of GTPγS-stimulated inositol phosphate formation in microsomes prepared from cultured neonatal rat cardiomyocytes. Panel A: The formation of IP_3, panel B:The formation of IP_2 and IP_1. Microsomes were incubated with (●) or without (■) 125 μM GTPγS, as described under **Materials and Methods**, for the indicated times. Data are the means ± S.E.M. of 13 experiments. Significantly different from the unstimulated value at the same time ($P < 0.05$).

Results

Hydrolysis of [^{3}H-]PI(4,5)P_2 in cardiomyocyte microsomes

Microsomes were prepared from cultured neonatal rat cardiomyocytes by ultracentrifugation of a 5000 x g_{av} supernatant. The yield was about 10 to 20 μg protein per mg homogenate protein. The microsomes were incubated with [^{3}H-]PI(4,5)P_2, in the presence of 5 μM $CaCl_2$ and 9 mM cholate as detergent. The time course of the formation of [^{3}H-]inositol phosphates is illustrated in Figure V.1. There was no major inositol phosphate formed as e.g. after 30 min incubation almost equal amounts of ^{3}H-IP_3 and ^{3}H-IP_2 had been produced. This pattern changed dramatically when 125 μM of the nonhydrolyzable GTP analogue GTPγS was included in the reaction mixture. GTPγS markedly stimulated the formation of ^{3}H-IP_3, whereas even some reduction was found in the formation of ^{3}H-IP_2 and ^{3}H-IP_1. The effect of GTPγS on PI(4,5)P_2 hydrolysis was not constant as the stimulation factor varied considerably between single experiments (1.6 to 14.7). Nonetheless, on the average the GTPγS-stimulated IP_3 formation differed significantly from control.

In some experiments the chemical identity of the [^{3}H-]PI(4,5)P_2 was checked by separation of the organic extracts on HPTLC plates. No [^{3}H-]PI(4,5)P_2 derivatives (e.g. [^{3}H-]PI(4)P or [^{3}H-]PI) were found either before or after incubation with cardiomyocyte

microsomes for 30 min. This result indicates that the ^{3}H-IP$_2$ and ^{3}H-IP$_1$ are produced by PLase C activity on ^{3}H-PI(4,5)P$_2$.

The effect of phorbol ester pretreatment of cardiomyocytes on microsomal [^{3}H-]PI(4,5)P$_2$ breakdown

Previously we demonstrated that during 30 minutes incubation of cultured neonatal rat cardiomyocytes with 0.01 to 1 μM PMA the α_1-adrenoceptor stimulated formation of inositol phosphates was inhibited [Meij and Lamers 1989b]. Therefore in the present study the effect of PMA pretreatment of cardiomyocytes on the activity of the microsomal PI(4,5)P$_2$-PLase C was tested. Four separate experiments have been carried out. In these experiments GTPτS stimulated ^{3}H-IP$_3$ formation and even ^{3}H-IP$_2$ and ^{3}H-IP$_1$ formation, but none of these changes were statistically significant (Figure V.2). However, pretreatment of the cells with 0.1 μM PMA led to a significant decrease in basal as well as GTPτS-stimulated ^{3}H-IP$_3$ formation in the microsomes (Figure V.2A). Also the basal and the GTPτS-reduced formations of ^{3}H-IP$_2$ and ^{3}H-IP$_1$ were further reduced by PMA, but none of these decreases were significant (Figure V.2B).

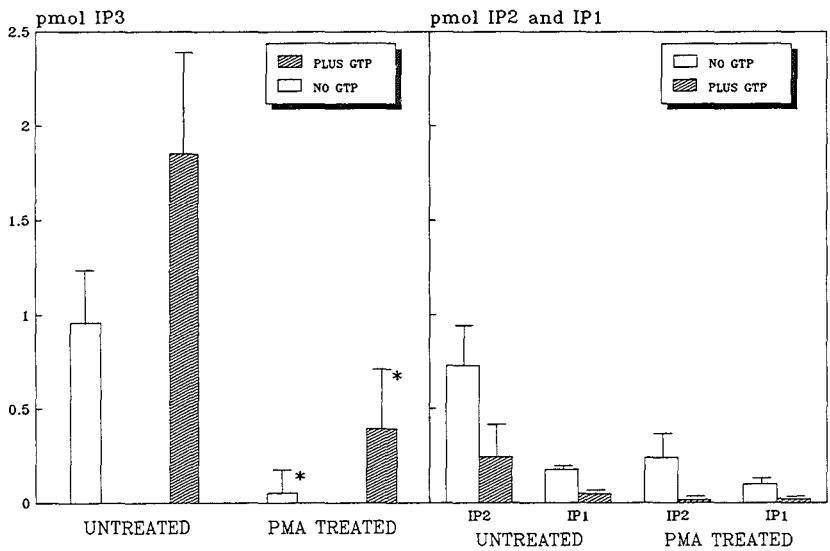

Figure V.2. Effect of PMA on inositol phosphate formation in microsomes prepared from cultured neonatal rat cardiomyocytes. Panel A: the formation of IP$_3$; panel B: the formation of IP$_2$ and IP$_1$. Microsomes prepared from either untreated (right bars) or PMA-treated (left bars) cardiomyocytes were incubated with or without 125 μM GTPτS, as described under **Materials and Methods**, for 30 min. Data are the means ± S.E.M. of 4 experiments. *Significantly different from microsomes of untreated cells.

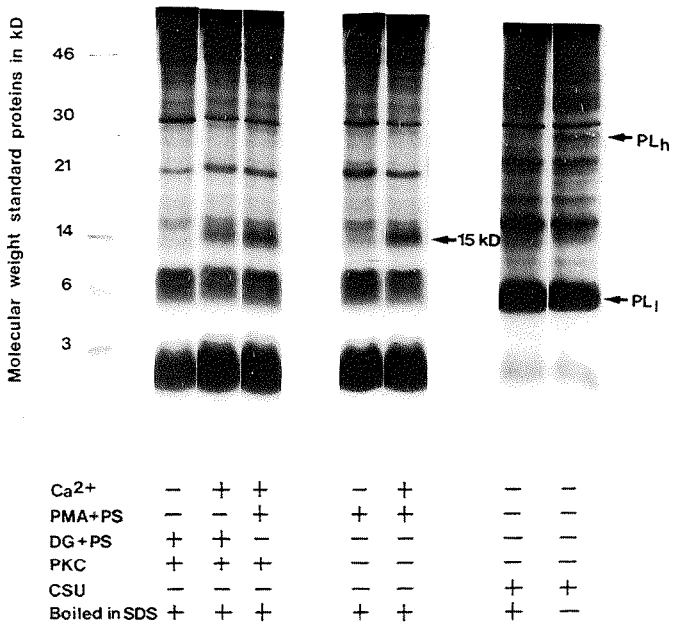

Figure V.3. Phosphorylation pattern (^{14}C- and ^{32}P autoradiogram) of microsomes prepared from cultured neonatal rat cardiomyocytes induced by protein kinase C (PKC) and catalytic subunit of cyclic AMP-dependent protein kinase (CSU). Proteins were phosphorylated as described under **Materials and Methods**. The concentrations of the respective additions were 10μg/ml PKC, 10 μg/ml DG, 100 μg/ml PS, 0.1 μM PMA, 1000 U/ml CSU. Rainbow [^{14}C-]labelled protein standards, shown in the most left slot, were obtained from Amersham Int plc, Amersham UK. The sample most right ws not boiled in SDS to identify the high molecular weight form (about 27 kD) of phospholamban.

Phosphorylation of microsomal substrate proteins by protein kinase C and catalytic subunit of cyclic AMP dependent protein kinase

One of the established receptors for phorbol ester (PMA) is the Ca^{2+}- and DG-stimulated, phospholipid-dependent protein kinase, generally called protein kinase C (PKC). To see whether the action of PMA on the cardiomyocytes was followed by PKC-mediated phosphorylation of specific substrate proteins in the membranes, the cardiomyocyte microsomes were incubated with Ca^{2+}, PMA, PS and τ-^{32}P-ATP. The autoradiogram of SDS-PAGE-separated ^{32}P-proteins is illustrated in Figure V.3. Several ^{32}P-labelled proteins were present in the microsomes. However, ^{32}P-incorporation was dependent on Ca^{2+} in only one protein (15 kD) (compare lanes 1 and 2, 4 and 5). The phosphorylation of this protein was not further increased by addition of exogenous, purified rat brain PKC (compare lanes 3 and 5). Also addition of DG and PS appeared not required to activate the phosphorylation of the 15 kD protein by endogenous PKC (not shown). A most interesting result was that PMA (100 nM) enhanced the PKC-induced phosphorylation of the 15 kD protein (compare lanes 2 and 3). Previously we [Lamers and Stinis 1982] and others [Manalan and Jones 1982; Presti *et al.* 1985b] demonstrated that the 15 kD protein is specifically localized in cardiac sarcolemma in contrast to phospholamban (high molecular weight form PL_h = 27 kD and low molecular weight form PL_l = about 5.5 kD), which is found in both cardiac sarcolemma and sarcoplasmic reticulum [Lamers and Stinis 1980; Rinaldi *et al.* 1982]. Phospholamban has been shown to be a substrate for cyclic AMP-, Ca^{2+}-calmodulin- and cyclic GMP-dependent protein kinases [Tada *et al.* 1979; LePeuch *et al.* 1979; Raeymakers *et al.* 1988] and PKC as well [Iwasa and Hosey, 1984; Movsesian *et al.* 1984]. However the results in Figure V.3 show that neither endogenous nor the exogenous PKC were able to stimulate ^{32}P-incorporation into proteins having mobilities in the range of 27 or 5.5 kD (lanes 2, 3 and 5). Catalytic subunit of cyclic AMP-dependent protein kinase was therefore applied to prove the presence of phospholamban in the cardiomyocyte microsomes. The ^{32}P-phospholamban was identified by its characteristic mobility change (27 to 5.5 kD) upon boiling in SDS (compare lanes 6 and 7) [Lamers and Stinis 1980]

Discussion

Hydrolysis of [^{3}H-]PI(4,5)P_2 in cardiomyocyte microsomes

Our results show the formation of labelled inositol phosphates from [^{3}H-]PI(4,5)P_2 by a microsomal fraction prepared from cultured neonatal rat cardiomyocytes. The non-hydrolyzable GTP-analogue GTPτS enhanced the formation of ^{3}H-IP_3, whereas the formation of ^{3}H-IP_2 and ^{3}H-IP_1 remained about constant (Figure V.1). These findings indicate that a PI(4,5)P_2-PLase C is present in neonatal rat cardiomyocytes and that this enzyme is activated by a GTP-binding protein (G protein).

Previously we found that α_1-adrenergic stimulation of cultured neonatal rat cardiomyocytes increased the formation of inositol phosphates [Meij and Lamers 1989a,b]. In these intact cells the formation of IP_1 predominated, which we attributed to the high rate of inositol phosphate breakdown by inositolphosphatases. This explanation was supported by the fact that without addition of the IP_1-phosphatase inhibitor Li^+ in intact cells no inositol phosphate formation could be detected at all. The inositolphosphatases are presumably soluble and therefore separated from the microsomes during their isolation, as no requirement for Li^+ became manifest using the latter fraction.

Still, the possibility remains that the α_1-adrenoceptor coupled PLase C activity found in the intact cells is not related to the GTPτS-stimulated, $PI(4,5)P_2$-PLase C found in the microsomes of the present study. In the first place, the former could have a specificity for phosphoinositides in general, whereas the microsomal enzyme is clearly $PI(4,5)P_2$-specific. The probability that ^3H-IP_2 and ^3H-IP_1 produced by the microsomes have been generated by the direct breakdown of ^3H-PI(4)P or ^3H-PI is very unlikely, considering the impossibility to detect these ^3H-phosphoinositides even after 30 min incubation. Secondly, both PLases C could be $PI(4,5)P_2$-specific but the latter may be coupled to another receptor than the α_1-adrenoceptor.

It is not unlikely that cardiomyocytes contain more than one $PI(4,5)P_2$-PLase C, because with respect to the PI-PLase C also several types with distinct properties have been reported: e.g. in bovine heart four isoforms were found [Low and Weglicki 1983]. In contrast to the lack of information about the properties of $PI(4,5)P_2$-PLase C there exist numerous reports about the presence of several PI-PLase C forms in a wide variety of cell types. These are mostly purified from cytosolic fractions [Low and Weglicki 1983; Rebecchi and Rosen, 1987; Ryu *et al.* 1987], although Katan and Parker (1987) have purified one PI-PLase C activity from the particulate fraction of bovine brain and Lee *et al.* (1987) have found PI-PLase C molecules in bovine brain that were distributed between soluble and particulate fractions. Furthermore, comparison of the sequences of cDNAs encoding for the three immunologically unrelated enzymes isolated from bovine brain cytosol has revealed a low overall homology [Suh *et al.* 1988]. Up until now it is still unclear how the functions of PI-PLases C are related to cellular signal transduction.

In the present study we demonstrated that GTPτS stimulated the formation of ^3H-IP_3 (Figure V.1). The involvement of a G protein, G_P, in the coupling of $PI(4,5)P_2$-PLase C to receptors was suggested some years ago after the first evidence for a role of a regulatory GTP-binding protein in $PI(4,5)P_2$ breakdown had been produced by studies with permeabilized cells [Cockcroft and Gomperts 1985]. The possibility exists to characterize e.g. G_s and G_i - G proteins involved in receptor-adenylate cyclase coupling - by cholera toxin- and pertussis toxin-induced ADP-ribosylation, respectively. However, G_P appeared not to be affected by either of these toxins, which has hampered its identification [Michell and Kirk 1986]. Nevertheless, in some cell types the inhibition of receptor mediated $PI(4,5)P_2$ breakdown by pertussis toxin (PT) has been reported [Graziano and Gilman 1987]. PT is known to inactivate G_i, a 41 kD GTP binding protein involved in the inhibition of adenylate cyclase and subsequently of cyclic AMP formation. With regard to myocardial

α_1-adrenoceptor mediated PI(4,5)P_2 breakdown the inhibitory action of PT is a controversial issue [Böhm *et al.* 1987; Steinberg *et al.* 1987]. The possibility that PT pretreatment inhibits PI(4,5)P_2 breakdown does not directly prove that the substrate is G_P. Recently it has been shown that cyclic AMP mediated the inhibition of GTPτS-stimulated PLase C activity [Yada *et al.* 1989]. Therefore the different "α_1"-adrenergic agonists used by the pro-PT-inhibition-group (noradrenaline) and contra-PT-inhibition-group (phenylephrine combined with propranolol) could explain the difference in results. The PT pretreatment could allow cyclic AMP to be increased by noradrenaline to a much higher level and to subsequently inhibit α_1-adrenergic receptor-G_P-mediated PI(4,5)P_2 breakdown.

The effect of phorbol ester pretreatment of cardiomyocytes on microsomal [^{3}H-]PI(4,5)P_2 breakdown

Our previous studies have shown that the phorbol ester PMA (0.01 to 1.0 μM) inhibited the α_1-adrenoceptor stimulated formation of [^{3}H-]inositol phosphates in [^{3}H-]myoinositol prelabelled neonatal rat cardiomyocytes [Meij and Lamers 1989b]. Our present results show that the formation of ^{3}H-inositol phosphates from exogenous ^{3}H-PI(4,5)P_2 in microsomes prepared from 0.1 μM PMA pretreated cells is lower. Similar results have been reported before in other systems [Orellana *et al.* 1987; Geny *et al.* 1988; Rittenhouse *et al.* 1988; Stutchfield and Cockcroft 1988]. Our previous data led us to propose that PKC may be the mediator of the PMA-induced inhibition of PI(4,5)P_2 breakdown. Orellana *et al.* (1987) reported that treatment of microsomes with PKC elicited effects similar to those in microsomes of PMA-pretreated cells, so in this respect our present results support our earlier proposal.

Based on other reports [Bouvier *et al.* 1988; Leeb-Lundberg *et al.* 1988] we suggested that PKC-mediated phosphorylation of an α_1-adrenergic receptor subunit and subsequent desensitization could be at the basis of the inhibition of PI(4,5)P_2 breakdown. Our present results partially confirm this suggestion. PMA had no effect on PI(4,5)P_2 breakdown in intact nonstimulated cells [Meij and Lamers 1989b], whereas the basal PI(4,5)P_2 breakdown in microsomes from PMA treated cells is lower compared to that of untreated cells. On the other hand, the time-course of inositol phosphate formation in intact nonstimulated cells showed that even after 60 min incubation the level was unchanged [Meij and Lamers 1989b], whereas the time-course of basal inositol phosphate formation in microsomes displays a rise after 10 min (Figure V.1). It seems that, under the used incubations conditions, in microsomes the basal PI(4,5)P_2-PLase C is slightly activated, whereas in intact nonstimulated cells the PLase C remains inactive. The finding that PMA pretreatment of cells reduces the basal as well as the GTPτS-stimulated activity of PLase C in microsomes could indicate that the final target of PMA is PLase C itself and not G_P as Orellana *et al.* (1987) have suggested. Our allegation is supported by the findings of Geny *et al.* (1988) and Stutchfield and Cockcroft (1988) which show inhibition by PMA of GTPτS-as well as of Ca^{2+}-stimulated PLase C activity. Thus, the basal PLase C activity in our study could be due to the presence of calcium.

Phosphorylation of microsomal substrate proteins by protein kinase C and catalytic subunit of cyclic AMP dependent protein kinase

The cardiomyocyte microsomes contained one specific substrate protein (15 kD) for the endogenous protein kinase C. Addition of exogenous PKC, purified from rat brain did not affect the phosphorylation pattern. This 15 kD protein was shown to be a specific sarcolemmal protein in dog and rat myocardium by us [Lamers and Stinis 1982] and others [Manalan and Jones 1982]. In the presence of alamethicin it can act also as a substrate protein for cyclic AMP-dependent protein kinase. The present observation that phorbol ester increased the ^{32}P-incorporation from γ-^{32}P-ATP induced by endogenous PKC, confirms recent work of Presti *et al.* (1985b), Yuan and Sen (1986) and Vetter *et al.* (1989). Moreover Presti *et al.* (1985b) demonstrated that the stimulation by phorbol ester of the 15 kD phosphorylation was most marked when assayed in the absence of Ca^{2+}. This would mean that the intrinsic Ca^{2+}-dependent PKC can exist in another conformation with different (Ca^{2+}-independent) properties or that another Ca^{2+}-independent isoform of PKC is present in the cardiac sarcolemma. Clearcut evidence for this has already been obtained for rat brain tissue [Malviya *et al.* 1986], whereas Schaap *et al.* (1989) showed the expression of the Ca^{2+}-independent PKC-ϵ in rat brain, heart and lung tissue.

A rapid increase of ^{32}P-incorporation into the 15 kD protein, in positive correlation with the increase in the maximal rate of developed tension was demonstrated upon ß-adrenergic [Presti *et al.* 1985a] as well as upon α_1-adrenergic stimulation [Lindemann 1986]. It is likely that these effects on 15 kD phosphorylation in intact myocardium are caused by the actions of cyclic AMP-dependent protein kinase and PKC, respectively. At present there is no study showing effects of phorbol ester on 15 kD phosphorylation in intact myocardium, but functional changes in the myocardium induced by phorbol ester have been reported. Limas and Limas (1985) demonstrated that phorbol ester induced desensitization of ß-receptors in myocytes isolated from adult rats, whereas Yuan *et al.* (1987) have shown that phorbol esters, while activating PKC, induce negative inotropy and chronotropy in perfused rat heart. All together this indicates that the phosphorylation of the 15 kD protein does not play a role in the α_1- or ß-adrenergic inotropic response, but has a general action in down-regulating the adrenoceptor responsitiveness or the transduction of the adrenergic signal through G proteins.

In conclusion, the experiments described in this report identify phorbol ester effects on the PI(4,5)P_2-PLase C and on PKC, both endogenously present in cardiomyocyte microsomes. *In vitro* the microsomal target protein for PKC was shown to be a 15 kD protein. Also in view of our previous observations [Meij and Lamers 1989b], it may be hypothesized that phorbol ester down-regulates the α_1-adrenoceptor mediated PI(4,5)P_2 hydrolysis by activation of PKC-induced 15-kD-protein phosphorylation which affects adrenoceptor function possibly through alteration of the transducing mechanism.

REFERENCES

1. BERRIDGE MJ (1984) Inositoltrisphosphate and diacylglycerol as second messengers. Biochem J **220**: 345-360.
2. BOUVIER M, LEEB-LUNDBERG LMF, BENOVIC JL, CARON MG, LEFKOWITZ RJ (1987) Regulation of adrenergic receptor function by phosphorylation. II Effects of agonist occupancy on phosphorylation of α_1- and $ß_2$-adrenergic receptors by protein kinase C and the cyclic AMP-dependent protein kinase. J Biol Chem **262**: 3106-3112.
3. BÖHM M, SCHMITZ W, SCHOLZ H (1987) Evidence against a role of a pertussis toxin-sensitive guanine nucleotide-binding protein in the alpha$_1$-adrenoceptor-mediated positive inotropic effect in the heart. Naunyn-Schmiedebergs Arch Pharmacol **335**: 476-479.
4. BROWN JH, BUXTON IL, BRUNTON LL (1985) α_1-Adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. Circ Res **57**: 532-537.
5. CASTAGNA M, TAKAI Y, KAIBUCHI K, SANO K, KIKKAWA U, NISHIZUKA Y (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem **257**: 7847-7851.
6. COCKCROFT S, GOMPERTS BD (1985) Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature **314**: 534-536.
7. COLUCCI WS, GIMBRONE MA, ALEXANDER RW (1986) Phorbol diester modulates α-adrenergic receptor-coupled calcium efflux and α-adrenergic receptor number in cultured vascular smooth muscle cells. Circ Res **58**: 393-398.
8. COTECCHIA S, LEEB-LUNDBERG LMF, HAGEN PE, LEFKOWITZ RJ, CARON MG (1985) Phorbol ester effects on α_1-adrenoceptor binding and phosphatidylinositol metabolism in cultured vascular smooth muscle cells. Life Sci **37**: 2389-2398.
9. GENY B, STUTCHFIELD J, COCKCROFT S (1988) Phorbol ester inhibits polyphosphoinositide phosphodiesterase activity stimulated by either Ca^{2+}, fluoride of GTP analogues in HL60 membranes and in permeabilized HL60 cells. Cellular Signalling, in press.
10. GRAZIANO MP, GILMAN AG (1987) Guanine nucleotide-binding regulatory proteins: mediators of transmembrane signalling. Trends Pharmacol Sci **8**: 478-481.
11. HIRATA M, SUEMATSU E, HASHIMOTO T, HAMACHI T, KOGA T (1984) Release of Ca^{2+} from a non-mitochondrial store site in peritoneal macrophages treated with saponin by inositol 1,4,5-trisphosphate. Biochem J **223**: 229-236.

12. IWASA Y, HOSEY MM (1984) Phosphorylation of cardiac sarcolemma proteins by the calcium-activated phospholipid-dependent protein kinase. J Biol Chem **259**: 534-540.
13. JACKOWSKI S, RETTENMEIER CW, SHERR CJ, ROCK CO (1987) Phosphatidylinositol 4,5 bisphosphate phospholipase C assay. Biotechnology Update **2**: 4-5.
14. KATAN M, PARKER PJ (1987) Purification of phosphoinositide-specific phospholipase C from a particulate fraction of bovine brain. Eur J Biochem **168**: 413-418.
15. LAMERS JMJ, STINIS JT (1980) Phosphorylation of low molecular weight proteins in purified preparations of rat heart sarcolemma and sarcoplasmic reticulum. Biochim Biophys Acta **624**: 443-459.
16. LAMERS JMJ, STINIS JT (1982) Alamethicin effects on phosphorylation of cardiac sarcolemmal fraction predominantly consisting of right-side out vesicles. In: *Adv Stud Heart Metabolism*, eds. C.M. Caldarera, P. Harris, CLUEB, Bologna (Italy), pp 41-47.
17. LEE HR (1988) α_1-Adrenergic receptors in heart failure. Heart Failure **5**: 62-70.
18. LEE KY, RYU SH, SUH PG, CHOI WC, RHEE SG (1987) Phospholipase C associated with particulate fractions of bovine brain. Proc Natl Acad Sci USA **84**: 5540-5554.
19. LEEB-LUNDBERG LMF, COTECCHIA S, DEBLASI A, CARON MG, LEFKOWITZ RJ (1987) Regulation of adrenergic receptor function by phosphorylation. I Agonist-promoted desensitization and phosphorylation of α_1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT_1 MF-2 smooth muscle cells. J Biol Chem **262**: 3098-3105.
20. LEPEUCH CJ, HAIECH J, DEMAILLE JG (1979) Concerted regulation of cardiac sarcoplasmic reticulum calcium transport by cyclic adenosine monophosphate dependent and calcium-calmodulin-dependent phosphorylations. Biochemistry **18**: 5150-5157.
21. LIMAS CJ, LIMAS C (1985) Phorbol ester- and diacylglycerol-mediated desensitization of cardiac ß-adrenergic receptors. Circ Res **57**: 443-449.
22. LINDEMANN JP (1986) α-Adrenergic stimulation of sarcolemmal protein phosphorylation and slow responses in intact myocardium. J Biol Chem **261**: 4860-4867.
23. LOW MG, WEGLICKI WB (1983) Resolution of myocardial phospholipase C into several forms with distinct properties. Biochem J **215**: 325-334.
24. LOWRY OH, ROSENBROUGH NJ, FARE AL, RANDALL RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem **193**: 265-275.
25. MALVIYA AN, LOUIS JC, ZWILLER J (1986) Separation from protein kinase C - a calcium-independent TPA-activated phosphorylating system. FEBS Lett **199**: 213-216.

26. MANALAN AS, JONES LR (1982) Characterization of the intrinsic cAMP-dependent protein kinase activity and endogenous substrates in highly purified cardiac sarcolemmal vesicles. J Biol Chem **257**: 10052-10062.
27. MEIJ JTA, LAMERS JMJ (1989a) Alpha-1-adrenergic stimulation of phosphoinositide breakdown in cultured neonatal rat ventricular myocytes. Mol Cell Biochem **88**: 73-75.
28. MEIJ JTA, LAMERS JMJ (1989b) Phorbolester inhibits α_1-adrenoceptor mediated phosphoinositide breakdown in cardiomyocytes. J Mol Cell Cardiol **21**: 661-668.
29. MICHELL B, KIRK C (1986) G-protein control of inositol phosphate hydrolysis. Nature **323**: 112-113.
30. MOVSESIAN MA, NISHIKAWA M, ADELSTEIN RS (1984) Phosphorylation of phospholamban by calcium-activated, phospholipid-dependent protein kinase. Stimulation of cardiac sarcoplasmic reticulum calcium uptake. J Biol Chem **259**: 8029-8032.
31. ORELLANA S, SOLSKI PA, BROWN JH (1987) Guanosine 5'-*O*-(thiotriphosphate)-dependent inositol trisphosphate formation in membranes is inhibited by phorbol ester and protein kinase C. J Biol Chem **262**: 1638-1643.
32. OSNES JB, AASS H, SKOMEDAL T (1985) On adrenergic regulation of heart function: role of myocardial α-adrenoceptors. In: *α-Adrenoceptor blockers in cardiovascular disease*, edited by H Refsum, OD Mjo/s. Edinburgh, London, Melbourne and New York, Churchill Livingstone, pp 69-102.
33. POGGIOLI J, SULPICE JC, VASSORT G (1986) Inositol phosphate production following α_1-adrenergic, muscarinic or electrical stimulation in isolated rat heart. FEBS Lett **206**: 292-298.
34. PRESTI CF, JONES LR, LINDEMANN JP (1985a) Isoproterenol-induced phosphorylation of a 15-kilodalton sarcolemmal protein in intact myocardium. J Biol Chem **260**: 3860-3867.
35. PRESTI CF, SCOTT BT, JONES LR (1985b) Identification of an endogenous protein kinase C activity and its intrinsic 15-kilodalton substrate in purified canine cardiac sarcolemmal vesicles. J Biol Chem **260**: 13879-13889.
36. RAEYMAKERS L, HOFMANN F, CASTEELS R (1988) Cyclic GMP-dependent protein kinase phosphorylates phospholamban in isolated sarcoplasmic reticulum from cardiac and smooth muscle. Biochem J **252**: 269-273.
37. REBECCHI MJ, ROSEN OM (1987) Purification of a phosphoinositide-specific phospholipase C from bovine brain. J Biol Chem **262**: 12511-12518.
38. RINALDI ML, CAPONY JP, DEMAILLE JG (1982) The cyclic AMP-dependent modulation of cardiac sarcolemmal slow calcium channels. J Mol Cell Cardiol **14**: 279-289.
39. RITTENHOUSE SE, BANGA HS, SASSON JP, KING WG, TARVER AP (1988) Regulation of platelet phospholipase C. Phil Trans Roy Soc Lond B **320**: 299-312.

40. RYU SH, SUH PG, CHO KS, LEE KY, RHEE SG (1987) Bovine brain cytosol contains three immunologically distinct forms of inositol phospholipid-specific phospholipase C. Proc Natl Acad Sci USA **84**: 6649-6653.
41. SCHAAP D, PARKER PJ, BRISTOL A, KRIZ R, KNOPF J (1989) Unique substrate specificity and regulatory properties of PKC-ϵ: a rationale for diversity. FEBS Lett **243**: 351-357.
42. SNOEK GT, FEIJEN A, HAGE WJ, VANROTTERDAM W, DELAAT SW (1988) The role of hydrophobic interactions in the phospholipid-dependent activation of protein kinase C. Biochem J **255**: 629-637.
43. STEINBERG SF, CHOW YK, ROBINSON RB, BILEZIKIAN JP (1987) A pertussis toxin substrate regulates α_1-adrenergic dependent phosphatidylinositol hydrolysis in cultured rat myocytes. Endocrinology **120**: 1889-1895.
44. STUTCHFIELD J, COCKCROFT S (1988) Effects of phorbol ester on IP_3 production and secretion in permeabilized HL60 cells. Biochem Soc Trans **16**: 994-995.
45. SUH PG, RYU SH, MOON KH, SUH HW, RHEE SG (1988) Cloning and sequence of multiple forms of phospholipase C. Cell **54**: 161-169.
46. TADA M, KIRCHBERGER MA, KATZ AM (1975) Phosphorylation of a 22,000-dalton component of the cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. J Biol Chem **250**: 2640-2647.
47. VETTER R, HAASE H, WILL-SHAHAB L (1989) Cardiac sarcolemma vesicles: ATP-dependent Ca^{2+} transport and inhibition of protein kinase reactions by the cardiotonic drug amrinone. In: *Proc Int Symp Heart Disease*, ed. I.S. Anand, Kluwer Academic Publishers, Norwell MA (USA),in press.
48. VOLPE P, SALVIATI G, DIVIRGILIO F, POZZAN T (1985) Inositol 1,4,5-trisphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle. Nature **316**: 347-349.
49. WOODCOCK EA, WHITE LBS, SMITH IA, MCLEOD JK (1987) Stimulation of phosphatidylinositol metabolism in isolated, perfused rat heart. Circ Res **61**: 625-631.
50. YADA Y, NAGAO S, OKANO Y, NOZAWA Y (1989) Inhibition by cyclic AMP of guanine nucleotide-induced activation of phosphoinositide-specific phospholipase C in human platelets. FEBS Lett **242**: 368-372.
51. YUAN S, SEN AK (1986) Characterization of the membrane-bound protein kinase C and its substrate proteins in canine cardiac sarcolemma Biochim Biophys Acta **886**: 152-161.
52. YUAN S, SUNAHARA TA, SEN AK (1987) Tumor-promoting phorbol esters inhibit cardiac functions and induce redistribution of protein kinase C in perfused beating rat heart. Circ Res **61**: 372-378.

Chapter VI

Discrete Interactions Between Phosphatidylethanolamine-N-methylation and Phosphatidylinositolbisphosphate Hydrolysis in Rat Myocardium

Johanna T.A. Meij, Giovanni Paolillo, Karel Bezstarosti, Pieter D. Verdouw, Vincenzo Panagia and Jos M.J. Lamers (1989) Mol Cell Biochem, in press.

Abstract

Bothphosphatidylethanolamine(PE)-N-methylationandphosphatidyl-inositolbisphosphate(PI-bisphosphate) breakdown potentially modify the microdomains in the sarcolemmal lipid bilayer. In this study the possibility of a mutual interaction between the enzymes responsible for these phospholipid reactions is examined. In sarcolemma purified from rat heart, prior hydrolysis of PI lipids by exogenous specific phospholipase C inhibited (to 75, 59 and 78% of control for sites I, II and III, respectively) the PE-N-methyltransferase system. In cultured rat cardiomyocytes the addition of L-methionine, a precursor for the methyldonor S-adenosylmethionine, stimulated PE-N-methylation in a concentration (0.2- 300 μM)-dependent manner. Methionine (50 μM) decreased the basal rate of PI-bisphosphate hydrolysis (to 72% of control), but had no effect on the phenylephrine-stimulated PI-bisphosphate hydrolysis. Maximal activation of the PI-bisphosphate breakdown by 30 μM phenylephrine did not affect the rate of PE-N-methylation in the presence of exogenous methionine (50 μM). These findings support the existence of interactions, although discrete, between the enzymes involved in the PE-N-methylation and PI turnover.

Introduction

Phospholipids play an important role in maintaining membrane structure and function. The phospholipid composition of biomembranes is adjusted by a complicated network of biosynthetic and degradative reactions. Little is known about the interactions between those reactions. Studies on receptor mediated stimulation of phospholipid metabolism have centered around two pathways, phosphatidylethanolamine (PE)-N-methylation and phosphatidylinositol (PI) bisphosphate formation and breakdown. The PI turnover is affected by α_1-adrenergic and muscarinic receptor stimulation [1,2], whereas at present the proposed ß-adrenergic stimulation of PE-N-methylation has not been definitely clarified in myocardium [3,4]. It has been shown, however, that the rate of PE-N-methylation in the isolated rat heart is increased by perfusing with exogenous methionine [5]. Both alterations in PI turnover and PE-N-methylation modify the localized phospholipid domains in the bilayer [6,7] which may result in changes of membrane protein functions. Perfusing the myocardium with methionine, in order to stimulate PE-N-methylation, increases the sarcolemmal and sarcoplasmic reticular Ca^{2+} pumping ATPase activities [8]. Varsanyi et al. [7] have shown that the sarcoplasmic reticular Ca^{2+} transport ATPase is activated by PI-bisphosphate. Cardiac sarcolemmal Ca^2+ pump activity is depressed after treatment of the membranes with exogenous PI-specific phospholipase C [10]. Furthermore micromolar concentrations of guanosine-5'-O-(thiotriphosphate) (GTP-τ-S), stimulating endogenous PI-bisphosphate-specific phospholipase C, also inhibits Ca^2+ pump activity of rat heart

sarcolemmal vesicles [11]. Therefore a linkage between phospholipid N-methylation and PI turnover is plausible because both lipid conversions do affect the Ca^{2+} pump activity [8-11]. Some studies indeed point to interactions between PE-N-methylation and PI cycle [12-14].

In the present experiments we studied a possible relationship between S-adenosylmethionine-stimulated PE-N-methylation and breakdown of PI lipid by exogenous PI-specific phospholipase C in purified sarcolemmal vesicles. Furthermore the interaction between methionine-stimulated PE-N-methylation and basal and α_1-adrenergic stimulated PI breakdown was investigated in cultured neonatal rat cardiomyocytes.

Materials and methods

Materials

S-adenosyl-L-(methyl-[^{3}H])-methionine (specific activity , 80.6 Ci/mmol) was purchased from New England Nuclear, Mississauga, Canada. L-[^{3}H-methyl]methionine (specific activity 70 C_i/mmol) and [2-^{3}H-]-myoinositol (specific activity 9 Ci/mmol) were from Amersham International, Amersham, UK. S-adenosyl-L-methionine was from RBI, Natick, USA. Standards of phosphatidyl-N-monomethylethanolamine (PMME) and phosphatidyl-N,N,-dimethylethanolamine (PDME) were obtained from Calbiochem-Behring, San Diego, USA. Silica 60 F-254 thin layer chromatography plates were obtained from E. Merck, Darmstadt, FRG. Phenylephrine HCl was from Brocades , Delft, The Netherlands. Dowex 1X8 (100-200 mesh formate form) from Biorad Labs, Richmond, USA. The growth medium : Nutrient mixture Ham F10 (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum, 10% horse serum, 200000 U/ml penicillin, 0.2 g/ml streptomycin which ingredients were all from Boehringer (Mannheim, FRG). Furthermore 135 mg/ml $CaCl_2.2H_2O$ was added to this growth medium. Petri dishes (TC 35/10) were from Greiner. Trypsin (type III) was from Sigma, St Louis, USA. Instagel was from Packard, Groningen, The Netherlands. All other chemicals were obtained from either Merck, Boehringer and Sigma Chemicals. PI-specific phospholipase C was isolated from *Staphylococcus aureus* as described [15]. The enzyme was suspended in 50 mM Tris-acetate, 3 mM NaN_3 (pH 7.4) at a concentration of 0.145 mg/ml and stored at -20°C.

Experiments carried out with sarcolemma

Sarcolemmal membranes were prepared from rat myocardium as described previously [16]. Ouabain-sensitive Na^+/K^+-ATPase (homogenate and sarcolemmal activities were 1.7 ± 0.3 and 13.1 ± 1.1 μmol $P_i.h^{-1}.mg^{-1}$, respectively) and 5'-nucleotidase (homogenate and sarcolemmal activities amounted to 19.2 ± 1.9 and 90.1 ± 5.5 nmol $P_i.min^{-1}.mg^{-1}$, respectively) were routinely used as sarcolemmal marker enzymes and were measured according to the procedures outlined in detail elsewhere [17,18]. In other work it was demonstrated that these sarcolemmal membrane fragments have a receptor-mediated

modulation pattern of adenylate cyclase which is expected for sarcolemma of cardiac origin [19]. The sarcolemmal membranes (1 mg of protein) were incubated with 25 μl of 0.145 mg/ml PI-specific phospholipase C, equivalent to 3.6 μg catalytic protein/ml, in a medium containing 0.25 M sucrose, 10 mM Tris HCl (pH 7.4) for 30 min at 37°C. The samples were cooled at 0°C and centrifuged at 100000 x g for 60 min. The pellet was washed twice with the sucrose-Tris buffer and resuspended in the same buffer. Control membranes were treated in the same manner except that phospholipase was not added. Phospholipid composition of untreated and phospholipase C-treated sarcolemma was analyzed to check the selective depletion of the PI pool. Extraction of phospholipids, separation on thin-layer plates and the quantitative estimation of phosphorus in the perchloric acid digested lipid spots has been described [20]. Phospholipid methyltransferase was assayed by measuring the incorporation of [^{3}H]-methyl groups into membrane phospholipids in the presence of S-adenosyl-L-(methyl-[^{3}H])-methionine ([^{3}H]-AdoMet) as described [21]. Three different assay conditions were found to be optimal for testing three catalytic sites in the methylation reaction as well as to be typical for the synthesis of PMME, PDME and phosphatidylcholine (PC), the major N-methylated lipid products at sites I,II and III, respectively [21]. After incubation, the methylated phospholipids were extracted with chloroform/methanol/2 N HCl (6:3:1, by volume). The detailed procedure for the measurement of individual N-methylated phospholipids has been reported before [21]. The PI-specific phospholipase preparation was examined for endogenous protease activity by testing its ability to solubilize a fibrin clot (Boehringer Mannheim test kit). Protein content was determined by the method of Lowry *et al.* [22] with bovine serum albumin (fraction V) as standard.

Experiments carried out with primary heart cell culture

Cardiomyocytes were isolated from ventricles of 2-4 day old rats by trypsinization according to Yagev *et al.* [23] but the enrichment method was modified by two successive periods of 30 and 90 min of preplating [24]. After 24-26 h incubation (37°C, 5% CO_2, 95% humidity) this resulted in a confluent monolayer of beating cardiomyocytes. Experiments on PE-N-methylation and PI-bisphosphate hydrolysis were done on the third day after plating. At least one hour before the experiment the growth medium above the cells was replaced by a medium containing 130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.44 mM NaH_2PO_4, 1.1 mM $MgCl_2$, 0.2% D-glucose (pH 7.4).

For measurement of the incorporation of [^{3}H-]methyl groups into phospholipids of the cardiomyocytes, each dish containing about 1 mg cell protein, was incubated with 0-300 μM L-[^{3}H]-methyl]methionine in 120 mM NaCl, 10 mM LiCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.44 mM NaH_2PO_4, 1.1 mM $MgCl_2$, 0.2% D-glucose (pH 7.4) at 37°C for the indicated times. In some experiments [^{3}H-]methyl incorporation (1 min reaction) was measured in control and 30 μM phenylephrine stimulated cardiomyocytes. Prior to incubation for estimation of the rate of PE-N-methylation the cells were preconditioned to the medium without [^{3}H-]methionine for at least 1 h. Incubations were

terminated by adding 0.5 ml cold methanol. The cells were then scraped from the dishes using a rubber policeman transferring them to organic extraction tubes. N-Methylated phospholipid products were extracted by chloroform/methanol/2 N HCl mixture, fractionated and quantitated as described [21].

To study [^{3}H-]PI-bisphosphate breakdown cardiomyocytes were incubated at 37°C in 6.7 μCi/ml [2-^{3}H-]myoinositol in normal incubation medium (total 0.5 ml per dish) for 30 min. The PI-bisphosphate breakdown was maximally stimulated by adding 30 μM phenylephrine [24]. Prior to this incubation the cells were incubated for 1 h in the same medium without [2-^{3}H-]myoinositol except that various amounts (50 and 300 μM) of methionine were present. Incubations were terminated by transferring the dish contents with 0.5 ml cold methanol/13 M HCl (100:1, by volume) to an organic extraction tube. Another 0.5 ml of acidified methanol was used to wash the dish and finally 1 ml chloroform and 0.5 ml 2.5 N HCl were added to obtain phase separation. After centrifugation (10 min, 1770 x g) the labelled inositol monophosphate (IP_1), inositol bisphosphate (IP_2) and inositol trisphosphate (IP_3) were separated in the aqueous upper phase by anion exchange using 1 ml Dowex 1X8 per dish as described previously [24].

Statistical analysis

Statistical analysis was performed by the Student's t test for both paired and unpaired observations and a probability of <0.05 was considered statistically significant.

Results

Isolated sarcolemmal membranes

Treatment of rat heart sarcolemma with PI-specific phospholipase C induced an appreciable (77% of the untreated membranes, see also ref 20) hydrolysis of PI, whereas the content of all other phospholipid classes remained constant (Table VI.1). The breakdown of PI-monophosphate and PI-bisphosphate could not be followed because of the presence of extremely low quantities of these phosphorylated PI intermediates in the sarcolemma, but it is likely that their hydrolysis was also catalyzed by the phospholipase C preparation used.

Table VI.2 shows the phospholipid methyltransferase activities for catalytic sites I, II and III in untreated and phospholipase C-treated sarcolemmal membranes. Pretreatment of sarcolemma with phospholipase C inhibited each catalytic site although the strongest reduction was seen at site II (41% compared to 25 and 22% at sites I and III respectively). It should be noted that in both untreated and phospholipase C- treated membranes PMME, PDME and PC were the major lipid products formed under site I, II and III conditions, respectively (results not shown). It could not be excluded *a priori* that the methyltransferase inhibition observed in this study could not be due to protease contamination of the phospholipase C. Endogenous protease activity of the phospholipase

Table VI.1. *Phospholipid composition of untreated and PI-specific phospholipase C-treated sarcolemma*

Class	nmol lipid-P/mg protein					
	untreated			treated		
Phosphatidylcholine	81.2	±	4.4	85.2	±	6.7
Lysophosphatidylcholine	5.8	±	0.6	6.1	±	0.8
Phosphatidylethanolamine	64.9	±	6.6	68.5	±	5.3
Phosphatidylserine	9.6	±	1.3	8.0	±	0.7
Phosphatidylinositol	6.6	±	0.6	1.5	±	0.2*
Diphosphatidylglycerol	8.4	±	1.1	9.1	±	0.9
Sphingomyelin	11.5	±	0.7	12.7	±	1.2
Phosphatidic acid	3.0	±	0.2	3.4	±	0.4
Others	2.6	±	0.2	2.8	±	0.2

Sarcolemma isolated from rat heart was incubated with PI-specific phospholipase C at 37°C for 30 min and subsequently centrifuged for washing the membrane pellet. Thereafter the membranes were analyzed for their phospholipid composition as described in Methods. The values represent the mean ± S.E.M. of three experiments. * denotes significantly different from the untreated membranes ($P < 0.05$).

Table VI.2. *The effect of PI pool depletion on the rates of the PE-N-methyltransferase catalytic sites I, II and III in cardiac sarcolemma*

Catalytic site	[^{3}H]-methyl group incorporation into N-methylated phospholipids (pmol/mg/30 min)			
	untreated	treated		(%)
I	0.41 ± 0.03	0.31 ± 0.02*	75	± 5
II	4.9 ± 0.6	2.9 ± 0.3*	59	± 10
III	93.8 ± 7.1	74.1 ± 4.5*	78	± 6

Membranes were analyzed for intrinsic PE-N-methyltransferase I, II and III activities following incubation at 37°C for 30 min without (untreated) or with (treated) PI-specific phospholipase C. For further details see Methods. Values are means ± S.E.M. of 5 experiments and represent the total [^{3}H-]methyl incorporation into N-methylated phospholipids (PMME + PDME + PC). * denotes significantly different from the untreated membranes.

C preparation was tested by incubating it with fibrin clot. In the enzyme concentration used in this study, we could not detect any proteolytic activity (detection limits were 0.025 with trypsin) on the phospholipase C preparation.

Cultured cardiomyocytes

The rate of incorporation of [^{3}H-]methyl groups from L-[^{3}H-methyl]-methionine into phospholipids was first examined at various concentrations of L-methionine after a 30 min incubation period (Fig. VI.1). The rate became almost maximal at 300 μM of L-methionine, a result similar to that found with the Langendorff perfused rat heart [5]. In subsequent experiments it was demonstrated that after 30 min incubation the levels of [^{3}H-]methyl incorporation into phospholipids were not representative for the initial rates. In fact the data shown in Fig. VI.1 represent steady state values that were reached after an incubation lasting approximately 2 min. An example of the time course of total [^{3}H]-methyl incorporation at 25 μM L-methionine is shown in Fig. VI.2. It was not possible to measure accurately the initial rate of incorporation between 0 and 30 sec because of the inconvenience in handling the cultured cells attached to the dish.

Because of the high total [^{3}H]-methyl incorporation rate it was of interest to know whether the time course of incorporation into PMME, PDME or PC was different. The

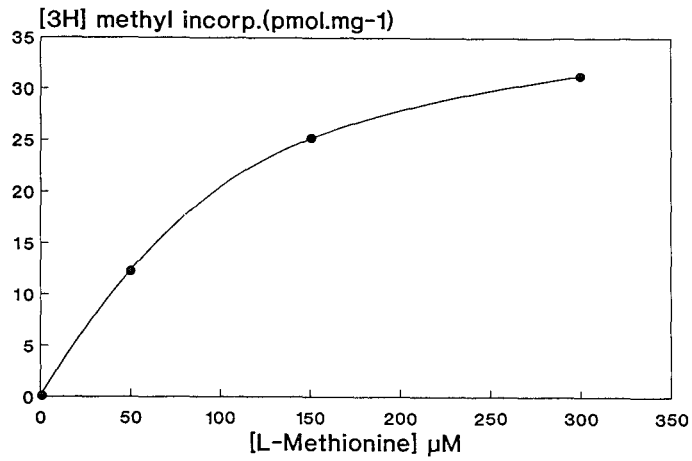

Figure VI.1. Total [^{3}H] methyl incorporation into the phospholipids of cultured cardiomyocytes as a function of the [^{3}H]-L-methionine concentration. The cells were incubated at 37°C for 30 min. Each point represents the average of duplicate measurements in a typical experiment.

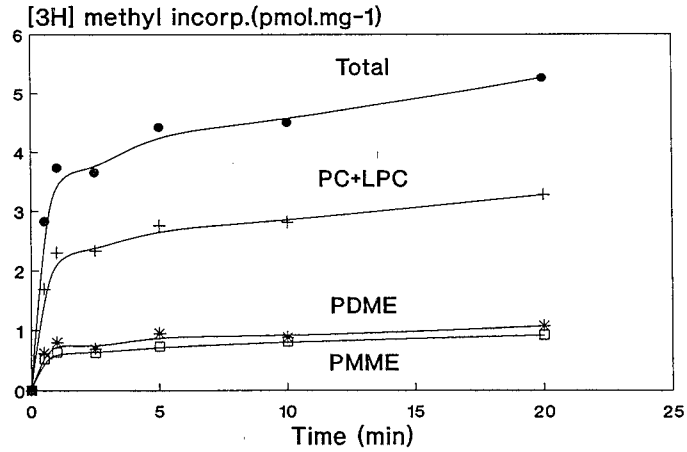

Figure VI.2. Time-course of formation of total and intermediate [^{3}H] methyl labelled PE phospholipids during incubation of cultured cardiomyocytes with 25 μM L-methionine. The cells were incubated at 37°C. Each point represents the average of duplicate measurements in a typical experiment.

thin layer chromatographical separated ^{3}H-labelled PC and lysoPC spots were taken together and considered to be representing the PE-N-methylation into PC [25]. Fig. VI.2 shows that none of the reaction sites I, II and III had a specific time-course. The same was true at higher concentrations of L-methionine (not shown).

The possibility of a mutual interaction between PE-N-methylation and PI-bisphosphate hydrolysis was first investigated by studying the effect of preincubation of the cells with 50 and 300 μM L-methionine on both the basal and phenylephrine-stimulated PI-bisphosphate breakdown. During preincubation with methionine the PI pool was also labelled by adding [2-^{3}H-]myoinositol to the medium. Previously we demonstrated that the labelling of PI-bisphosphate was sufficient to obtain an almost linear rate of inositolphosphate formation over a 30 min incubation period [26]. The formation of inositolphosphates was taken as the sum of IP_1, IP_2 and IP_3 because in cardiomyocytes a rapid dephosphorylation of IP_3 and IP_2 occurs [24,26]. Li^+ ions were present during the incubation at least to prevent dephosphorylation of IP_1 [24,26]. Fig. VI.3 shows the effects of preincubation of cells with L-methionine on basal and phenylephrine-stimulated formation of inositoltriphosphates. The basal rate of PI-bisphosphate hydrolysis was already significantly inhibited at 50 μM L-methionine (28%), whereas no effect was seen on the α_1-adrenergic stimulated (30 μM phenylephrine) reaction. A 6-fold higher concentration of L-methionine did not further reduce the basal rate of PI-bisphosphate hydrolysis and there was still no effect on the stimulated response.

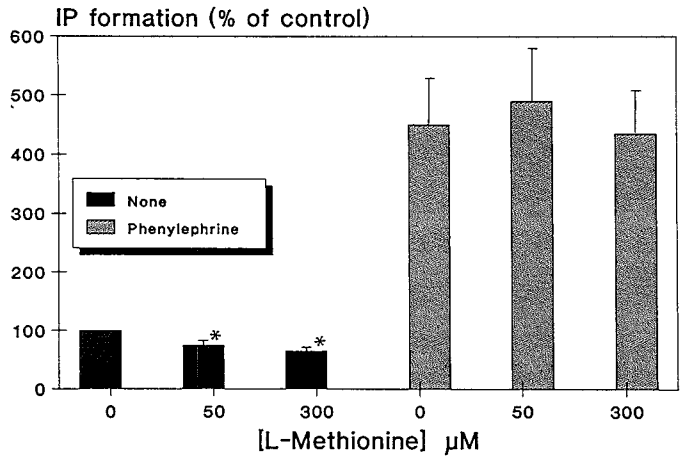

Figure VI.3. The effect of two concentrations of methionine on the rate of PI-bisphosphate hydrolysis in basal and 30 μM phenylephrine-stimulated cultured cardiomyocytes. The columns represent total inositolphosphate (IP_1, IP_2 and IP_3) formation expressed as % from that found when the cells were incubated in medium without phenylephrine and L-methionine. Values represent the mean ± S.E.M. of 4 separate experiments. * $P < 0.05$ versus 0 μM L-methionine.

To further examine a possible interaction between PE-N-methylation and PI-

Table VI.3. *[^{3}H]-Methyl incorporation from L-[^{3}H-methyl]methionine into PE-N-methylated lipids of control and phenylephrine(30 μM)-stimulated cultured cardiomyocytes*

	[^{3}H]-methyl incorporated (pmol.mg^{-1}.min^{-1})			
	PMME	PDME	PC	Total
Control	0.54 ± 0.06	0.44 ± 0.13	1.48 ± 0.42	2.49 ± 0.58
PhE	0.51 ± 0.14	0.51 ± 0.14	1.58 ± 0.32	2.69 ± 0.51

Cultured cardiomyocytes were preincubated at 37°C for 10 min with and without phenylephrine (PhE, 30 μM) followed by an 1 min incubation with 50 μM L-[^{3}H-methyl]methionine in order to estimate the rate of PE-N-methylation. Total N-methylation represents the sum of PMME, PDME and PC (see Table IV.2 for further explanation). The values represent the mean ± S.E.M. of 3 experiments.

bisphosphate hydrolysis the effect of phenylephrine on the rate of PE-N-methylation was analyzed. As can be seen from Table VI.3 there was no effect of phenylephrine on any methylation site.

Discussion

PE-N-methylation is carried out by a membrane-bound methyltransferase system. The rapid turnover of the methylated PE intermediates has been implicated in a large number of receptor-mediated events in various cell types [3,7,12,27]. However, there is no direct information about these responses in the myocardium [4]. The process is stimulated in the isolated perfused heart by exogenous methionine [4,5,8)] and in heart biopsies taken after treating animals with catecholamine [4]. PI is an acidic phospholipid whose turnover is also stimulated by a variety of receptors [28]. In the myocardium the PI turnover has been shown to be activated by e.g. α_1-adrenergic agonists [1,2,24,26]. Both PE-N-methylation and phosphorylated PI metabolites affect active Ca^{2+} transporting systems (e.g. the sarcolemmal and sarcoplasmic reticulum Ca^{2+} pumps, see refs 8-11). Recently it has been shown that the Ca^{2+} pump of rat heart sarcolemma is inhibited when the endogenous phospholipase C is activated by GTP-τ-S [11] or when the membranes are treated with exogenous PI-specific phospholipase C [10]. Therefore we studied possible interactions between these two lipid pathways.

Isolated sarcolemmal membranes were depleted of PI by treatment with an exogenous PI-specific phospholipase C. The low concentrations of PI-monophosphate and PI-bisphosphate within the sarcolemma made it impossible to verify their hydrolysis, but the latter reaction is likely catalyzed by the polyphospho-PI-unspecific phospholipase C used [29,30]. Moreover during the *in vitro* incubation the polyphospho-PI pool is presumably attacked by phosphatases. Depletion of the PI pool in the sarcolemma preparation resulted in inhibition of the methyltransferase reaction sites I, II and III (Table VI.2). The inhibition can not be caused by accumulated inositolphosphates (IP_1, IP_2 and IP_3) because the membranes were washed prior to estimation of PE-N-methylation. Under the conditions employed for PI hydrolysis none of the other phospholipid class contents were affected. PI is involved in anchoring a diverse group of membrane proteins which may include methyltransferase(s) [31]. Therefore the inhibition of PE-N-methylation by PI depletion may be due to a loss of enzyme activity during washing of the membrane fragments. We have not performed systematically activity measurements in the supernatant to exclude this possible event. In one experiment we could detect a low site II activity in the supernatant obtained after phospholipase C treatment of the membranes. In cultured cardiomyocytes depletion of the PI pool after receptor mediated PI-bisphosphate hydrolysis does not even occur after 1 h of maximal stimulation [26]. Hence the latter system is more convenient to investigate possible interactions between PE-N-methylation and PI-bisphosphate hydrolysis.

Initially the effect of exogenous L-methionine on PE-N-methylation was characterized in cultured neonatal cardiomyocytes (Figs. VI.1 and VI.2). At each of the

sites (I, II and III) the initial rate of PE-N-methylation was extremely fast and reached a plateau value after about 2 min. The level of this plateau could be increased by raising the exogenous L-methionine concentrations to 300 μM. The amount of [^{3}H-]methyl incorporation from exogenous L-[^{3}H-methyl]methionine into PMME, PDME and PC measured after 20 min incubation was one tenth of that found in the Langendorff perfused rat heart [5]. The basal rate of PI-bisphosphate hydrolysis was significantly reduced by 50 μM L-methionine, whereas no effect was seen on the α_1-adrenergic stimulated rate (Fig. VI.3). A higher concentration of L-methionine (300 μM) did not further increase the inhibition of basal rate of PI-bisphosphate hydrolysis. These findings do not exclude a relationship between PE-N-methylation and PI-bisphosphate hydrolysis.

The isolated rat heart sarcolemma showed an inhibitory effect of PI (including PI-monophosphate and PI-bisphosphate) depletion on the PE-N-methyltransferase(s). We therefore expected to see a similar effect in the cultured cardiomyocytes. However, α_1-adrenergic stimulation of PI-bisphosphate breakdown did not affect the initial rate of [^{3}H-]methyl incorporation from L-[^{3}H-methyl]methionine into PMME, PDME and PC (Table VI.3). Some studies reported an interaction between PE-N-methylation and PI turnover in mast cells and basophils [12,13]. In contrast with the decreased PE-N-methyltransferase activity found in isolated sarcolemma upon PI pool depletion, these studies showed a potentiation of the PE-N-methylation reaction by increased PI-bisphosphate breakdown. One of the proposed mechanisms is the involvement of protein kinase C that is stimulated by the product diglyceride formed during PI-bisphosphate hydrolysis. In agreement with this hypothesis Villalba *et al.* [14] demonstrated that a partially purified rat liver PE-N-methyltransferase can be phosphorylated and thereby activated by rat brain protein kinase C. Previously we obtained evidence that protein kinase C is activated during α_1-adrenergic agonist stimulation of the PI turnover [26]. It may therefore be possible that protein kinase C activation has masked the PI-bisphosphate breakdown-induced decrease in methylation which was expected to occur after α_1-adrenergic stimulation of the cardiomyocytes. On the other hand we found that phorbol 12-myristate 13-acetate which is a potent activator of protein kinase C [32], did not affect the initial rate of PE-N-methylation in cardiomyocytes (unpublished results). This would indicate that, unlike liver, PE-N-methylation of neonatal myocardium is insensitive to the protein kinase C-dependent phosphorylation of membrane protein(s). Thus other factors like a different intramembranous organization of the two phospholipid pathways at various stages of postnatal development may explain the diverse interactions of PI breakdown and PE-N-methylation.

In conclusion, the present results obtained with isolated sarcolemma and cultured cardiomyocytes support the existence of discrete but significant interactions between the PE-N-methylation and PI-bisphosphate systems of which the mechanism(s) are unkown.

Acknowledgements

This work was supported by grants from the NATO, Belgium and Medical Research Council, Canada. We like to thank Dr. M.G. Low, Columbia University, U.S.A, for generously supporting us with the PI-specific phospholipase C preparation.

REFERENCES

1. BROWN JH, BUXTON IL, BRUNTON LL: α_1-Adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. Circ Res **57**: 532-537, 1985.
2. POGGIOLI J, SULPICE JC, VASSORT G: Inositol phosphate production following α_1-adrenergic, muscarinic or electrical stimulation in isolated rat heart. FEBS Lett **206**: 292-298, 1986.
3. HIRATA F, STRITTMATTER WJ, AXELROD J: ß-Adrenergic receptor agonists increase phospholipid methylation, membrane fluidity and ß-adrenergic receptor-adenylate cyclase coupling. Proc Natl Acad Sci USA **76**: 368-372, 1979.
4. TAIRA Y, PANAGIA V, SHAH KR, DHALLA NS: In vivo stimulation of cardiac phospholipid N-methylation by isoprotenerol. J Mol Cell Cardiol **20 (Suppl III)**: 524, 1988.
5. PANAGIA V, GUPTA MP, GANGULY PK, DHALLA NS: Methionine-induced positive inotropic effect in rat heart: Possible role of phospholipid N-methylation. Circ Res **62**: 51-55, 1988.
6. MAJERUS PW, CONNOLLY TM, DECKMIN H, ROSS TS, BROSS TE, ISHII H, BANSEL VS, WILSON DB: The metabolism of phosphoinositide-derived messenger molecules. Science **234**: 1519-1526, 1986.
7. MATO JM, ALEMANY S: What is the function of phospholipid N-methylation? Biochem J **213**: 1-10, 1983.
8. GUPTA MP, PANAGIA V, DHALLA NS: Phospholipid N-methylation-dependent alteration of cardiac contractile function by L-methionine. J Pharm Exp Ther **245**: 664-672, 1988.
9. VARSANYI M, TOLLE H-G, HEILMEYER LMG, DAWSON RMC, IRVINE RF: Activation of sarcoplasmic reticular Ca^{2+} transport ATPase by phosphorylation of an associated phosphatidylinositol. EMBO J **2**: 1543-1548, 1983.
10. PIERCE GN, PANAGIA V, LOW MG: Cardiac sarcolemmal calcium pump activity is depressed after treatment with phosphatidylinositol-specific phospholipase C. J Mol Cell Cardiol **20 (suppl IV)**: S39, 1988.
11. KUO TH: Guanine nucleotide-, and inositol trisphosphate-induced inhibition of the Ca^{2}+ pump in rat heart sarcolemmal vesicles. Biochem Biophys Res Commun **152**: 1111-1116, 1988.
12. CREWS FT, HEIMAN AS: Interaction of phospholipid methylation and phosphatidylinositol metabolism in stimulation of secretion. In: LA Horrocks *et al.* (eds) *Phospholipids in the nervous system. Volume 2 Physiological roles*, Raven Press, New York, 1985, pp 87-98.
13. ISHIZAKA T, STREK AR, SULLIVAN TJ: Relationship between phospholipid methylation and PI turnover induced by bridging of IgE receptors in mast cells and basophils. Fed Proc **43**: 1625, 1984.

14. VILLALBA M, PAJARES MA, RENART MF, MATO JM: Protein kinase C catalyses the phosphorylation and activation of rat liver phospholipid methyltransferase. Biochem J **241**: 911-916, 1987.
15. LOW MG: Phosphatidylinositol-specific phospholipase C from Staphylococcus aureus. In: JM Lowenstein (ed) *Methods in Enzymology, volume 71*, Academic Press, New York, 1981, pp 741-746.
16. DHALLA NS, ANAND-SRIVASTAVA MB, TUANA BS, KHANDELWAL RL: Solubilization of a calcium-dependent adenosine triphosphatase from rat heart sarcolemma. J Mol Cell Cardiol **13**: 413-423, 1981.
17. LAMERS JMJ, HEYLIGER, CE, PANAGIA V, DHALLA NS: Properties of 5'-nucleotidase in rat heart sarcolemma. Biochim Biophys Acta **742**: 568-575, 1983.
18. PANAGIA V, LAMERS JMJ, SINGAL PK, DHALLA NS: Ca^{2+}- and Mg^{2+}-dependent ATPase activities in deoxycholate-treated rat heart sarcolemma. Int J Biochem **14**: 387-397, 1982.
19. ANAND-SRIVASTAVA MB: Regulation of adenylate cyclase by adenosine and other agonists in rat myocardial sarcolemma. Arch Biochem Biophys **243**: 439-446, 1985.
20. PANAGIA V, MICHIEL DF, DHALLA KS, NIJJAR MS, DHALLA NS: Role of phosphatidylinositol in basal adenylate cyclase activity in rat heart sarcolemma. Biochim Biophys Acta **676**: 395-400, 1981.
21. PANAGIA V, GANGULY PK, OKUMURA K, DHALLA NS: Subcellular localization of phosphatidylethanolamine N-methylation activity in rat heart. J Mol Cell Cardiol **17**: 1151-1159, 1985.
22. LOWRY OH, ROSEBROUGH MJ, FARR AL, RANDALL AJ: Protein measurement with the Folin phenol reagent. J Biol Chem **193**: 265-289, 1951.
23. YAGEV S, HELLER M, PINSON A: Changes in cytoplasmic and lysosomal enzyme activities in cultured rat heart cells: the relationship to cell differentiation and cell population in culture. In Vitro **20**: 893-898, 1984.
24. MEIJ JTA, LAMERS JMJ: Alpha-1-adrenergic stimulation of phosphoinositide breakdown in cultured neonatal rat ventricular myocytes. Mol Cell Biochem **88**: 73-75, 1989.
25. PANAGIA V, OKUMURA K, SHAH KR, DHALLA NS: Modification of sarcolemmal phosphatidylethanolamine N-methylation during heart hypertrophy. AmJ physiol **253**: H8-H15, 1987.
26. MEIJ JTA, LAMERS JMJ: Phorbolester inhibits α_1-adrenoceptor mediated phosphoinositide breakdown in cardiomyocytes. J Mol Cell Cardiol **21**: 661-668, 1989.
27. HIRATA F, AXELROD J: Phospholipid methylation and biological signal transmission. Science **209**: 1082-1090, 1980.
28. BERRIDGE MJ: Inositoltriphosphate and diacylglycerol as second messengers. Biochem J **220**: 345-360, 1984.

29. SMITH CD, COX CC, SNYDERMAN R: Receptor-coupled activation of phosphoinositide specific phospholipase C by an N protein. Science **232**: 97-100, 1986.

30. ORELLANA S, SOLSKI PA, BROWN JH: Guanosine 5'-*O*-(thiotriphosphate)-dependent inositol trisphosphate formation in membranes is inhibited by phorbol ester and protein kinase C. J Biol Chem **262**: 1638-1643, 1987.

31. LOW MG: Biochemistry of the glycosyl-phosphatidylinositol membrane protein anchors. Biochem J **244**: 1-13, 1987.

32. CASTAGNA M, TAKAI Y, KAIBUCHI K, SANO K, KIKKAWA U, NISHIZUKA Y: Direct acivation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem **257**: 7847-7851, 1982.

Chapter VII

Alterations in Polyunsaturated Fatty Acid Composition of Cardiac Membrane Phospholipids and α_1-Adrenoceptor Mediated Phosphatidylinositol Turnover

Johanna T.A. Meij, Alessandra Bordoni, Dirk H.W. Dekkers, Carlo Guarnieri and Jos M.J. Lamers (1989) Cardiovasc Res, in press.

Abstract

Polyunsaturated fatty acids are involved in several steps of the α_1-adrenoceptor mediated phosphatidylinositol turnover. Rat neonatal ventricular myocytes were incubated for 24 h in culture media supplemented with linoleic acid (18:2n-6) or eicosapentaenoic acid (20:5n-3) to change the polyunsaturated fatty acid composition of their membrane phospholipids. In 18:2n-6 treated cells the 18:2n-6 content of the total phospholipid fraction rose from 45 to 68 nmol.mg protein^{-1} and in 20:5n-3 treated cells the 20:5n-3 and docosapentaenoic acid (22:5n-3) contents increased from 1.5 to 12.5 and 5.1 to 14.7 nmol.mg protein^{-1} respectively. The major n-3 fatty acid 22:6n-3 (11.4 nmol.mg protein^{-1}) did not change after 20:5n-3 treatment of the cells. Although the phosphatidylinositol fraction showed changes in 18:2n-6, 20:5n-3 and 22:5n-3 content parallel with those in the total phospholipid fractions, none were significant. In the phosphatidylinositol fraction 22:5n-3 (0.4 nmol.mg protein^{-1}) appeared to be the major n-3 fatty acid. The fatty acid treated cardiomyocytes were prelabelled with [^{3}H-]inositol to estimate the rate of phosphatidylinositol 4,5-bisphosphate turnover. There were no differences in the rate of [^{3}H-]-inositolphosphate formation between control, 18:2n-6 and 20:5n-3 treated cells. Prolonged α_1-adrenergic stimulation of control, 18:2n-6 and 20:5n-3 treated cells did not change the polyunsaturated fatty acid composition of the total phospholipid and phosphatidylinositol fractions. It is concluded that the α_1-adrenoceptor mediated phosphatidylinositol turnover rate is not affected by the changes in polyunsaturated fatty acid composition of membrane phospholipids and that prolonged α_1-adrenergic stimulation does not lead to significant depletion of any specific or total polyunsaturated fatty acids in the phosphatidylinositol lipids.

Introduction

The mammalian myocardium contains two types of adrenergic receptors, the ß-type and the α_1-type, in about equal amounts [1]. Intracellularly the ß-adrenergic effects are mediated by an increase in cyclic AMP followed by phosphorylation of specific target proteins. Analogous to many other tissues the primary effect of myocardial α_1-adrenoceptor stimulation is an increased conversion of sarcolemmal phosphatidylinositol 4,5-bisphosphate in inositol 1,4,5-trisphosphate and diacylglycerol by a specific phospholipase C [2,3]. Both these substances have been reported to act as second messengers. Inositol 1,4,5-trisphosphate may act by mobilizing Ca^{2+} from intracellular stores [4] and DG by activating Ca^{2+}- and phospholipid dependent protein kinase [5]. Although this signal pathway bifurcates, a synergistic relationship between the two limbs has been demonstrated for the regulation of a whole variety of cellular processes [6].

Except for the calcium-mobilizing action of inositol 1,4,5-trisphosphate, the general relationship between inositol lipids and Ca^{2+} signalling is still not fully understood. In contrast to this the function of diacylglycerol as a protein kinase C activator is by now well-established. It has been shown both in vivo and in vitro that one of the fatty acid chains of diacylglycerol must be unsaturated for optimal protein kinase C activation [7,8]. Diacylglycerol is further metabolized by diacylglycerol lipase [9] and monoacylglycerol lipase thereby functioning as a source of arachidonic acid for eicosanoid production [10]. The reports that inositol phospholipids in mammalian tissues are rich in arachidonic acid (20:4n-6) at the *sn*-2-position [11] support both functional conditions of diacylglycerol.

Several matters require investigation as to whether a different fatty acid composition of phosphatidylinositol 4,5-bisphosphate influences the of phospholipase C activity within the membrane. Information is required on the fatty acid composition of phosphatidylinositol in myocardium [12] because of its role as precursor for the second messenger diacylglycerol. Another point of interest is whether the polyunsaturated fatty acid composition alters after prolonged stimulation of the phosphatidylinositol turnover as previously we [13] and others [14] have shown that chronic norepinephrine stress in rats produced an increase in relative content of the polyunsaturated n-3 fatty acid 22:6 and a decrease in 18:2n-6 in the myocardial total phospholipid as well as in the phosphatidylcholine and phosphatidylethanolamine fractions.

Reibel *et al.* [15] reported that fish oil feeding in rats resulted in changes in the fatty acid composition of myocardial membrane phospholipids. They demonstrated that dietary fish oil induced alteration of the α_1- but not of the ß-adrenoceptor mediated changes in cardiac inotropy. Gudbjarnason *et al.* [14] reported an increased sensitivity to catecholamine induced damage to the myocardium upon cod liver oil feeding of rats. Fish oil contains large amounts of polyunsaturated 20:5n-3 and docosahexaenoic acid (22:6n-3) and in both former reports most dramatic increase was found in these fatty acid components of the cellular total phospholipid fraction [13-16]. However, the alterations specifically occurring in the small phosphatidylinositol fraction are yet unknown. The question arises whether the difference in the α_1-adrenergic response after incorporation of those polyunsaturated fatty acids into heart membranes is caused by alterations in the rate of phosphatidylinositol 4,5-bisphosphate breakdown by the specific phospholipase C or by an altered diacylglycerol activation pattern of protein kinase C. Reibel *et al.* [15] showed at least that fish oil induced changes in prostaglandin production were not involved.

Recently it was demonstrated that the fatty acid composition of cultured cardiomyocytes can be modified by addition of fatty acids to the culture medium [17]. Using these primary cultures of beating, neonatal rat ventricular myocytes we have investigated the rate of α_1-adrenoceptor mediated phosphatidylinositol turnover after preincubating the cells with 18:2n-6 or 20:5n-6 -rich culture medium. The total phospholipid as well as the phosphoinositide pool have been examined for their polyunsaturated fatty acid composition. Furthermore the fatty acid composition has been

analysed before and after α_1-adrenergic stimulation to see whether the membrane total phospholipids and the phosphatidylinositol fraction are changed with respect to specific polyunsaturated fatty acids.

Materials and methods

Chemicals

Growth medium: Nutrient mixture Ham F10 (Gibco, Scotland) supplemented with 10% v/v fetal calf serum, 10% v/v horse serum, 200000 units.litre^{-1} penicillin, 0.2 g.litre^{-1} streptomycin (all from Boehringer Mannheim) and 135 mg.litre^{-1} $CaCl_2.2H_2O$; Petri dishes (TC 35/10) were from Greiner (Nürtingen, FRG) ; trypsin (type III) was from Sigma as were the 18:2n-6 and 20:5n-3, and boron trifluoride/methanol; standard fatty acid methylesters and the internal standard 21:0 for the transmethylation reaction were from Alltech Ass., Inc. (Deerfield USA); [2-^{3}H-]myoinositol was from Amersham International PLC (Amersham, U.K.); [dipalmitoyl-1-^{14}C]-phosphatidylcholine, [L-α-myoinositol-2-^{3}H(N)]-phosphatidylinositol and [U-^{14}C-]palmitic acid were from NEN (Boston, USA); phenylephrine HCl was from Brocades (Delft, The Netherlands); High performance thin layer chromatography plates (HP-KF, 200 micron) were from Whatman (Clifton, U.S.A.), phosphoinositide standard from Sigma (St Louis, U.S.A.), Dowex 1X8 (100-200 mesh formate form) from BioRad Labs (California, U.S.A.) and Instagel was from Packard (Groningen, The Netherlands). All other chemicals were of analytical grade.

Primary heart cell culture

Cardiomyocytes were isolated from the ventricles of 2-4 days old Wistar rats by trypsinization and grown according to Yagev *et al.* [18]. We applied a modified enrichment method of two successive periods of 30 and 90 min plating [19,20]. Of the final suspension containing 9 x 10^8 myoblasts.litre^{-1} , 2 ml were seeded per Petri dish. After 24-26 h incubation (37°C, 5% CO_2, 95% humidity) this resulted in a confluent monolayer of beating cardiomyocytes. Experiments were started 48 h after seeding when the growth medium was replaced by conditioned medium.

Cell incubation

At the beginning of each experiment the batch of dishes was divided into three sets, each set receiving one of the following conditioned media (inositol-free growth medium): 1) control medium; 2) 18:2n-6 rich medium which was supplemented with 55 μmol.litre^{-1} free 18:2n-6; 3) 20:5n-3 rich medium which was supplemented with 43 μmol.litre^{-1} free 20:5n-3. Because the fatty acids were dissolved in ethanol when added to the fetal calf serum during preparation of each medium, control medium contained

an equal concentration (0.3% v/v) of pure ethanol. Furthermore every medium was supplemented with either 88 nmol.litre^{-1} unlabelled myoinositol or - to study [^{3}H-]inositolphosphate formation - 88 nmol.litre^{-1} [2-^{3}H-]-myoinositol (1.67 mCi.litre^{-1}). The dishes were then incubated with these media for 24 h (37°C, 5% CO_2, 95% humidity).

Cell stimulation

After the 24 h lasting preincubation of the cardiomyocytes in the conditioned culture media they were rinsed twice with 1 ml buffer (130 mmol.litre^{-1} NaCl, 4.7 mmol.litre^{-1} KCl, 1.3 mmol.litre^{-1} $CaCl_2$, 20 mmol.litre^{-1} $NaHCO_3$, 0.44 mmol.litre^{-1} NaH_2PO_4, 1.1 mmol.litre^{-1} $MgCl_2$, 0.2% w/v D-glucose, pH 7.4, equilibrated at 37°C with 5% CO_2 at 95% humidity) and equilibrated in this buffer for 30 min. Then LiCl was added to a final concentration of 10 mmol.litre^{-1}. After another 10 min of preincubation 30 μmol.litre^{-1} phenylephrine or buffer was added to a final volume of 1 ml. Incubations were terminated after 30 min by rinsing with 0.5 ml cold buffer followed by addition of 0.5 ml cold methanol for the separation of the total phospholipid fraction according to Bligh and Dyer [21]. The cells were scraped using a rubber policeman and suspensions of 2 dishes were collected in one glass tube. The dishes were rinsed once more with 0.5 ml of cold methanol, which was transferred to the same glass tube. Then 0.5 ml chloroform per number of dishes was added and the extract kept at -20°C overnight. The phases were separated by adding 0.5 ml of 0.9% w/v NaCl and 0.5 ml chloroform per dish of cells and centrifuged (10 min, 1770 x g). For separation of the phosphatidylinositol lipids extracts from 4-6 dishes of cells were collected with cold methanol/ 12 mol.litre^{-1} HCl and to obtain two phases 2.5 mmol.litre^{-1} HCl instead of NaCl was added as described [20]. Trace amounts of labelled phosphatidylinositol (13000 dpm per extraction) were added to record its recovery during extraction and thin layer separation. Similarly for the estimation of the recovery of total phospholipid we added trace amounts of labelled phosphatidylcholine (19000 dpm per extraction). The organic (lower) phases obtained from the extracted cells were evaporated to dryness with N_2 and redissolved in a small volume of organic solvent for further separation of total phospholipids or phosphoinositides (see later).

Following the Bligh and Dyer [21] extraction method, 0.75 ml of each conditioned medium was extracted with 2.0 ml methanol/chloroform (1:1 v/v) to check the fatty acid composition of the nonestrified fatty acid fraction and of the total lipid fraction. In the latter case no separation was performed as the fatty acids of triglycerides, phospholipids and cholesterolester altogether were transmethylated. To estimate the recovery of fatty acids in the nonesterified fatty acid fraction trace amounts of labelled palmitic acid (80000 cpm per extraction) were added.

Separation of phospholipids and quantitation of fatty acid methylesters

Phospholipids and nonesterified fatty acids were separated from other lipids by thin layer chromatography using hexane/diethylether/acetic acid (60:40:1 v/v) as solvent system. The phospholipid spot remaining at the origin was scraped off. The nonesterified fatty acid spot (R_f about 0.25) was localized using parallel running standards and scanning for ^{14}C-counts of the added tracer [U-^{14}C]-palmitic acid. For separation of the phosphoinositides the dried organic phases were redissolved in methanol/chloroform/water (25:75:2 v/v) and spotted on high performance thin layer chromatography (HPTLC) plates previously impregnated with 1% potassiumoxalate in methanol/water (2:3 v/v) and activated (15 min, 110°C). The plates were developed in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8 v/v) as described [22]. The phosphatidylinositol spot was visualized with iodine vapour and identified by comparison with cochromatographed standard and by scanning for ^{3}H-counts of the added tracer ^{3}H-phosphatidylinositol. In both solvent systems 0.02% w/v butylated hydroxytoluene was included to avoid oxidation of fatty acids.

After extraction of the scraped spots (total phospholipids, phosphatidylinositol or nonesterified fatty acids) 21:0 fatty acid was added (40, 10 and 10 nmol, respectively) as an internal standard for the transmethylesterification reaction and the extracts were dried under N_2. The phospholipids were transmethylesterified using boron trifluoride/methanol and gaschromatographed to determine their fatty acid composition as previously described [13]. For the gaschromatographic separation of the fatty acid methylesters a Pye Unicam 102 equipped with a CP-Sil 88 (WCOT) capillary column (Chrompack, Delft, The Netherlands) and carrier gas N_2 were used. The separated peaks were identified on the basis of their retention times as compared to standards.

Assay of [^{3}H-]inositolphosphates

For studying the rate of phosphatidylinositol 4,5-bisphosphate hydrolysis the [2-^{3}H-]myoinositol prelabelled cells (see before) were used and as initial step for the separation of water soluble ^{3}H-inositolphosphates a lipid extraction as described for phosphatidylinositol lipids was applied. The lower phase, containing the phospholipids (including the ^{3}H-labelled phosphoinositides) was discarded, although in a few experiments these extracts were further separated by HPTLC for tracing the ^{3}H-phosphatidylinositol 4,5-bisphosphate spot to analyse its fatty acid pattern.

The upper phase was diluted 5 times with distilled water. The inositolphosphates were separated by anion exchange using 1 ml Dowex 1X8 based on the method described by Berridge *et al.* [23]. Briefly, free inositol and glycerophosphoinositides were eluted with 20 ml distilled water and 10 ml 5 mmol.litre^{-1} disodiumtetraborate in 30 mmol.litre^{-1} Na-formate respectively. Inositolmonophosphate, inositolbisphosphate and inositoltrisphosphate were eluted altogether with 10 ml 1 mol.litre^{-1} ammoniumformate in 0.1 mol.litre^{-1} formic acid. One volume of the eluate was mixed with two volumes Instagel and counted for radioactivity by liquid scintillation (Tri-carb 2660 from Packard, Groningen, The Netherlands).

Statistical analysis

All data are expressed as means ± S.E.M.. Statistical analyses for multigroup comparisons were done by analysis of variance and Bonferroni t-test. Comparisons between stimulated and unstimulated cells were done using Student's t-test. $P \leq 0.05$ was considered significant.

Results

Total and phosphatidylinositol lipids of unstimulated cells

The respective fatty acid additions to the culture medium resulted in relatively higher 18:2-n-6 or 20:5n-3 contents. Table VII.1 shows the total and nonesterified fatty acid content in μmol.litre^{-1} of the three culture media. The supplementation of the media with nonesterified fatty acids only moderately changed the total fatty acid content. The latter pool of fatty acids is mainly comprised of triglyceride, cholesterolester and phospholipid present in the lipoproteins of the added sera. It is, however, important to note that the nonesterified fatty acid pool (Table VII.1), constituting 2 to 3 % of the total fatty acid pool, has been found to be the major source of lipid for the cultured cells [24]. As can be seen in the nonesterified fatty acids a dramatic change occurred in 18:2n-6 or 20:5n-3 contents.

The data of Table VII.2 present the fatty acid contents in nmol.mg protein^{-1} of the total phospholipid fraction of unstimulated cells. No change in total fatty acid content occurred after either treatment of cells. As expected the 18:2n-6 treated cells showed a significant increase in 18:2n-6 content in comparison with control and 20:5n-5 treated cells. The increase in 18:2n-6 content was not accompanied by an increase in the 18:2n-6 derived arachidonic acid (20:4n-6). Rather the latter fatty acid had the tendency to decrease.

The 20:5n-3 treated cells showed a significant increase in 20:5n-3 and docosapentaenoic acid (22:5n-3) contents compared with control and 18:2n-6 treated cells (Table VII.2). The content in the major n-3 fatty acid 22:6n-3 did not change after 20:5n-3 treatment of the cells. Consequently, the n-3/n-6 ratio was significantly higher in 20:5n-3 treated than in control and 18:2n-6 treated cells. Apparently elongation of 20:5n-3 has occurred due to 20:5n-3 treatment of the cardiomyocytes, because only small and almost similar amounts of 22:5-n-3 were present in the control, 18:2n-6- and 20:5n-3-rich medium (Table VII.1).

Table VII.1. *Total and nonesterified fatty acid composition of conditioned media*

Fatty acid	Concentration (μmol.litre^{-1})[a]					
	control medium		18:2n-6 rich medium		20:5n-3 rich medium	
	Total	NEFA	Total	NEFA	Total	NEFA
16:0	220.9	9.8	218.5	9.3	227.2	9.6
18:0	226.3	5.9	193.4	5.1	216.4	5.7
18:1	221.1	7.5	218.5	7.4	239.0	8.1
18:2n-6	410.4	5.9	484.1	47.4	398.7	5.8
20:4n-6	19.4	0.2	22.6	0.3	22.1	0.3
20:5n-3	n.d.[c]	n.d.	n.d.	n.d.	37.4	45.8
22:4n-6	n.d.	n.d.	n.d.	n.d.	1.5	n.d.
22:5n-3	5.2	n.d.	5.3	n.d.	5.8	n.d.
22:6n-3	6.5	n.d.	7.5	n.d.	7.7	n.d.
Σ PUFA[b]	441.5	6.2	519.5	47.7	473.2	51.9

[a]Values represent concentrations of fatty acids in the total lipid fraction (phospholipids, triglycerides, cholesterolesters and nonesterified fatty acids) and in the nonesterified fatty acid (NEFA) fraction, extracted from the conditioned media.
[b]Σ PUFA represents the sum of all polyunsaturated fatty acids.
[c]n.d. means "not detectable".

There was no significant decrease in 18:2n-6 and 20:4n-6 contents due to 20:5n-3 treatment of the cells (Table VII.2) like it is usually found in myocardium after dietary 20:5n-3 treatment [12-16]. Despite the relative increase in polyunsaturated 18:2n-6 and 20:5n-3 as well as 22:5n-3 contents in the total phospholipid fraction of 18:2n-6, respectively 20:5n-3 treated cells, there was no significant change of the sum of all polunsaturated fatty acids (Σ PUFA) nor of the saturated over unsaturated fatty acid ratio (Sat/unsat) compared to control treated cells (Table VII.2).

The fatty acid content of phosphatidylinositol fraction was at many points different from that of total phospholipid fraction. E.g in control treated cells the phosphatidylinositol fraction contained more mol % of 18:0 (34.2 *vs* 21.5) and less of 18:2n-6 (7.8 *vs* 13.8), 20:4n-6 (10.1 *vs* 14.7), 22:4-n-6 (not-detectable *vs* 1.6) and 22:6n-3 (0.5 *vs* 3.2) than the total phospholipid fraction, which gives it a much higher saturated over unsaturated fatty acid ratio (Tables VII.2 and VII.3). At any rate its relative 20:4n-6 content is surely not higher than that of the total phospholipid fraction. Calculated from the total fatty acid contents the phosphatidylinositol fraction represents about 6 mol % of total phospholipids (Tables VII.2 and VII.3).

As a consequence of 18:2n-6 or 20:5n-3 treatment the phosphatidylinositol fraction showed, like the total phospholipid fraction, increases in 18:2n-6 and 20:5n-3 as well as 22:5n-3 contents, respectively (Table VII.3). However, unlike the total phospholipid

Table VII.2. *Total phospholipid fatty acid composition of unstimulated cardiomyocytes preincubated with media mainly varying in 18:2n-6 and 20:5n-3 contents*

Fatty acid[b]	Content (nmol.mg protein^{-1})[a] control medium		18:2n-6 rich medium		20:5n-3 rich medium	
16:0	[6]5.7	(2.8)	66.3	(2.8)	62.7	(3.7)
18:0	74.5	(3.8)	73.1	(5.3)	73.2	(5.4)
18:1	47.9	(3.5)	41.7	(2.4)	42.9	(2.5)
18:2n-6	45.4	(4.1)	67.6	(6.4)$^{+}$	42.6	(3.2)
20:4n-6	51.0	(4.8)	43.3	(4.5)	48.0	(5.4)
20:5n-3	1.5	(0.4)	0.9	(0.3)	12.5	(1.5)*
22:4n-6	5.6	(1.2)	5.6	(1.1)	4.0	(1.1)
22:5n-3	5.1	(1.1)	4.1	(0.8)	14.7	(1.4)*
22:6n-3	11.2	(1.3)	8.9	(1.4)	11.4	(2.2)
Others[b]	38.9	(16.2)	35.2	(13.8)	33.9	(13.4)
Σ PUFA[c]	122.4	(14.3)	133.5	(16.0)	138.3	(17.5)
Sat/unsat[d]	0.82	(0.06)	0.94	(0.11)	0.74	(0.04)
N-3/n-6[e]	0.20	(0.02)	0.14	(0.02)	0.45	(0.03)*

[a]Values represent the mean (S.E.M.) of 7 determinations of the fatty acid content of the total phospholipid fraction in each group of fatty acid treated cardiomyocytes.
[b]Only major fatty acids are listed. The sum of minor fatty acids is listed and termed "others". E.g. "others" for phospholipids of control cells represents the sum (nmol.mg protein^{-1}) of 14:0, 2.0 (1.0); 16:0 dimethylacetal, 6.7 (1.5); 18:0 dimethylacetal, 2.9 (1.4); 16:1, 14.3 (4.8); 18:3n-3, 2.6 (1.4); 22:0, 2.5 (1.3); 22:1, 7.4 (4.4); 20:5n-6, 0.1 (0.1); 24:0, 0.4 (0.2) and 24:1, 0.2 (0.2).
[c]Σ PUFA means the sum of all polyunsaturated fatty acids.
[d]Sat/unsat is the ratio of the total nmol of saturated fatty acids over the total nmol of unsaturated fatty acids.
[e]N-3/n-6 is the ratio of the total nmol of n-3 fatty acids over the total nmol of n-6 fatty acids.
$^{*}P \leq 0.01$ *vs* control and 18:2n-6 treated cells; $^{+}P \leq 0.05$ *vs* control and $P \leq 0.01$ *vs* 20:5n-3 treated cells.

fractions none of these changes were significant. What is interesting is that the elongated derivative 22:5n-3 constitutes the main part of the n-3 fatty acids in the phosphatidylinositol fraction, even after the 20:5n-3 treatment of the cells.

Phosphatidylinositol turnover rate in fatty acid treated cells

The cardiomyocytes pretreated with control medium, 18:2n-6 and 20:5n-3 rich medium were tested for the basal and α_1-adrenoceptor stimulated phosphatidylinositol 4,5-bisphosphate breakdown. The illustrated ^{3}H-inositolphosphate production (Fig VII.1) represents the sum of ^{3}H-inositol mono-, bis- and trisphosphate formation. Previously we demonstrated that the major inositolphosphate formed is the monophosphate due to rapid breakdown of tris- and bisphosphate by phosphatases during incubation [20]. No significant differences in basal or in phenylephrine (30 μmol.litre^{-1}) stimulated inositolphosphate formation between the three groups of fatty acid treated cells were observed (Fig VII.1). The inositolphosphate formation was measured over a time period

Table VII.3. *Phosphatidylinositol fatty acid composition of unstimulated cardiomyocytes preincubated with media mainly varying in 18:2n-6 and 20:5n-3 contents*

Fatty acid[b]	Content (nmol.mg protein^{-1})[a]					
	control medium		18:2n-6 rich medium		20:5n-3 rich medium	
16:0	4.8	(0.9)	5.6	(0.6)	5.2	(0.7)
18:0	6.4	(0.9)	5.2	(0.4)	5.9	(0.4)
18:1	2.7	(0.4)	2.5	(0.4)	2.7	(0.7)
18:2n-6	1.5	(0.3)	1.8	(0.4)	1.2	(0.2)
20:4n-6	1.9	(0.4)	1.7	(0.1)	1.5	(0.4)
20:5n-3	n.d.[f]		n.d.		0.2	(0.1)
22:4n-6	n.d.		n.d.		0.1	(0.1)
22:5n-3	0.4	(0.2)	0.5	(0.2)	0.7	(0.2)
22:6n-3	0.1	(0.1)	n.d.		n.d.	
Others[b]	1.0	(0.5)	1.5	(0.4)	1.3	(0.4)
Σ PUFA[c]	3.8	(0.5)	4.00	(0.5)	3.8	(0.7)
Sat/unsat[d]	1.80	(0.12)	1.89	(0.33)	1.7	(0.24)
N-3/n-6[e]	0.14	(0.05)	0.15	(0.08)	0.33	(0.09)

[a]Values represent the mean (S.E.M.) of 6 determinations of the fatty acid content of the phosphatidylinositol fraction in each group of fatty acid treated cardiomyocytes.
[b]Only major fatty acids are listed. The sum of minor fatty acids is listed and termed "others". E.g. "others" for phosphatidylinositol of control cells represents the sum (nmol.mg protein^{-1}) of 14:0, 0.4 (0.3); 16:0 dimethylacetal, 0.2 (0.2); 22:1, 0.1 (0.1); 24:0, 0.1 (0.1) and 24:1, 0.2 (0.1).
[c]Σ PUFA means the sum of all polyunsaturated fatty acids.
[d]Sat/unsat is the ratio of the total nmol of saturated fatty acids over the total nmol of unsaturated fatty acids.
[e]N-3/n-6 is the ratio of the total nmol of n-3 fatty acids over the total nmol of n-6 fatty acids.
[f]n.d. means "not detectable".

of 30 min. Earlier it was demonstrated by us that the rate of inositolphosphate formation decreases after prolonged (30-60 min) α_1-adrenergic stimulation [25] due to downregulation of the receptor mediated phosphatidylinositol 4,5-bisphosphate hydrolysis. Therefore in the present work also some experiments with shorter time periods (6 min) were carried out. However, no effect of 18:2n-6 or 20:5n-3 treatment of the cells was seen either (not shown).

Effect of prolonged α_1-adrenergic stimulation on fatty acid composition of cellular phospholipids

After 30 min of maximal phosphatidylinositol turnover rate, induced by phenylephrine (30 μmol.litre^{-1}), the total phospholipid polyunsatured fatty acid contents of control, 18:2n-6 and 20:5n-3 treated cells did not change (Tables VII.2 and VII.4). The sum of

Table VII.4. *Total phospholipid fatty acid composition of α_1-adrenoceptor stimulated cardiomyocytes preincubated with media mainly varying in 18:2n-6 and 20:5n-3 contents*

Fatty acid[b]	Content (nmol.mg protein^{-1})[a] control medium		18:2n-6 rich medium		20:5n-3 rich medium	
16:0	65.9	(3.9)	73.6	(6.7)	66.4	(3.9)
18:0	83.2	(4.3)	77.7	(5.9)	75.3	(4.2)
18:1	48.5	(3.9)	43.0	(3.0)	49.4	(3.3)
18:2n-6	43.5	(3.6)	66.9	(9.8)$^{+}$	42.8	(4.3)
20:4n-6	53.7	(5.8)	39.7	(3.0)	44.1	(4.0)
20:5n-3	0.8	(0.4)	0.2	(0.2)	10.7	(1.8)*
22:4n-6	7.6	(0.8)	2.6	(1.3)	6.3	(1.4)
22:5n-3	4.9	(1.2)	6.4	(1.3)	13.3	(1.7)*
22:6n-3	11.2	(2.4)	6.9	(0.9)	9.4	(1.3)
Others[b]	28.1	(8.8)	29.9	(9.7)	28.9	(10.7)
Σ PUFA[c]	124.6	(15.3)	126.9	(17.8)	131.2	(16.6)
Sat/unsat[d]	0.88	(0.05)	0.92	(0.08)	0.82	(0.06)
N-3/n-6[e]	0.18	(0.03)	0.17	(0.04)	0.40	(0.04)*

[a]Values represent the mean (S.E.M.) of 6 determinations of the fatty acid content of the total phospholipid fraction in each group of fatty acid treated cardiomyocytes.
[b]Only major fatty acids are listed. The sum of minor fatty acids is listed and termed "others" (See legend to Table VII.2 for further specification).
[c]Σ PUFA means the sum of all polyunsaturated fatty acids.
[d]Sat/unsat is the ratio of the total nmol of saturated fatty acids over the total nmol of unsaturated fatty acids.
[e]N-3/n-6 is the ratio of the total nmol of n-3 fatty acids over the total nmol of n-6 fatty acids.
$^{*}P \leq 0.01$ *vs* control and 18:2n-6 treated cells; $^{+}P \leq 0.05$ *vs* control and $P \leq 0.01$ *vs* 20:5n-3 treated cells.

all polyunsaturated fatty acids also remained constant after either treatment. Also no significant change was seen in contents of any specific component or the sum of polyunsaturated fatty acids of the phosphatidylinositol lipid fraction after α_1-adrenoceptor mediated phosphatidylinositol 4,5-bisphosphate hydrolysis over a 30 min incubation period (compare Tables VII.3 and VII.5). Attempts were made to measure the fatty acid content of phosphatidylinositol-4,5-bisphosphate with and without α_1-adrenergic stimulation by extracting the separated phospholipid from the high performance thin layer chromatography (HPTLC) plates. However no accurate fatty acid analysis could be undertaken because phosphatidylinositol 4,5-bisphosphate represents a too small fraction of the membrane phospholipid pool and some impurities appeared to have mobilities on HPTLC which were close to that of phosphatidylinositol 4,5-bisphosphate.

Table VII.5. *Phosphatidylinositol fatty acid composition of α_1-adrenoceptor stimulated cardiomyocytes preincubated with media mainly varying in 18:2n-6 and 20:5n-3 contents*

	Content (nmol.mg protein^{-1})[a]					
Fatty acid[b]	control medium		18:2n-6 rich medium		20:5n-3 rich medium	
16:0	5.0	(0.6)	6.1	(0.7)	4.7	(0.6)
18:0	6.4	(0.8)	5.5	(0.9)	6.5	(0.5)
18:1	2.3	(0.3)	2.8	(0.7)	2.3	(0.3)
18:2n-6	1.3	(0.1)	1.3	(0.2)	1.6	(0.4)
20:4n-6	1.9	(0.4)	1.6	(0.5)	1.7	(0.4)
20:5n-3	n.d.[f]		n.d.		0.1	(0.1)
22:4n-6	n.d.		n.d.		n.d.	
22:5n-3	0.4	(0.2)	0.3	(0.1)	0.4	(0.1)
22:6n-3	0.2	(0.1)	0.1	(0.1)	0.1	(0.1)
Others[b]	1.1	(0.4)	1.0	(0.4)	1.3	(0.5)
Σ PUFA[c]	3.9	(0.6)	3.3	(0.5)	4.4	(1.0)
Sat/unsat[d]	2.15	(0.39)	1.96	(0.23)	2.06	(0.40)
N-3/n-6[e]	0.13	(0.10)	0.11	(0.04)	0.31	(0.10)

[a]Values represent the mean (S.E.M.) of 6 determinations of the fatty acid content of the phosphatidylinositol fraction in each group of fatty acid treated cardiomyocytes.
[b]Only major fatty acids are listed. The sum of minor fatty acids is listed and termed "others" (See legend to Table VII.3 for further specification).
[c]Σ PUFA means the sum of all polyunsaturated fatty acids.
[d]Sat/unsat is the ratio of the total nmol of saturated fatty acids over the total nmol of unsaturated fatty acids.
[e]N-3/n-6 is the ratio of the total nmol of n-3 fatty acids over the total nmol of n-6 fatty acids.
[f]n.d. means "not detectable"

Discussion

Polyunsaturated fatty acids are involved in several steps in the α_1-adrenoceptor mediated phosphatidylinositol turnover. These fatty acids are present: 1) in the phospholipid bilayer in which the α_1-adrenergic receptor and phospholipase C are embedded; 2) in the substrate phosphatidyl-4,5-bisphosphate for the phospholipase C; 3) in diacylglycerol, the natural activator of protein kinase C; 4) as free 20:4n-6 and 20:5n-3 which both are substrates for cyclooxygenase in the prostaglandin synthesis. Some recent reports indicated that a diet enriched in n-3 polyunsaturated fatty acids produces changes in the response of the heart to catecholamines, particularly in the α_1-type [13-15]. Stimulation of α_1-adrenergic receptors has been shown to increase the rate of phosphatidylinositol turnover in myocardium which results in formation of inositol trisphosphate and diacylglycerol. The latter intermediates most probably are the intracellular messengers producing the biological effects of the catecholamine [3,15,20,25]. Therefore the purpose of the present study was to obtain a homogeneous culture of cardiomyocytes with a varying polyunsaturated fatty acid profile of their membrane phospholipids to study the influence on the α_1-adrenoceptor mediated phosphatidylinositol turnover. An almost

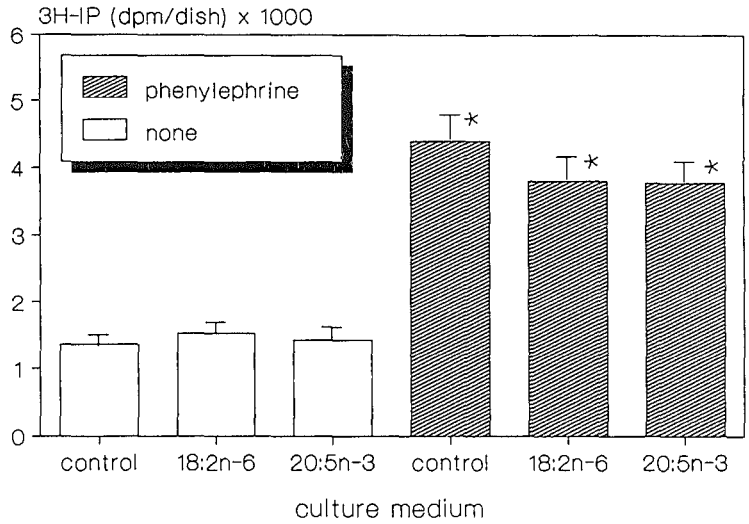

Figure VII.1. The effect of preincubation of cardiomyocytes with media mainly varying in 18:2n-6 and 20:5n-3 contents on the rate of basal and α_1-adrenoceptor mediated phosphatidylinositol-4,5-bisphosphate breakdown. The incubation time was 30 min and the phenylephrine concentration was 30 μmol.litre^{-1}. The columns represent the mean (± S.E.M.) total [^{3}H]-inositolphosphate formation ([^{3}H-]inositol mono-, bis- and trisphosphate) of 5 experiments. $^*P \leq 0.05$ *vs* corresponding basal [^{3}H]-inositolphosphate formation.

homogeneous culture of cardiomyocytes was used to avoid the problem of contribution by other cell types with active phosphatidylinositol turnover like e.g. the smooth muscle cell.

As a consequence of supplementation of the medium with fatty acids the cellular phospholipid fatty acid composition changed (Tables VII.2 and VII.3). More dramatic changes were found by Hasin *et al.* [28], but in this study the sera offered to the cells were first delipidated to less than 2% of the original lipid content followed by addition of nonesterified fatty acids. In addition rather long culture periods (12-16 days) were used. Delipidation may be less suitable as essential factors for optimal growth are lost and if cells are aged in culture the outgrowth of other cell types like fibroblasts and endothelioid cells becomes more likely [18,25]. In the study of Grynberg *et al.* [17], who composed synthetic media of nonesterified fatty acids complexed to albumin in the absence of sera, somewhat larger changes in the n-3/n-6 ratios than in the present work were seen (control 0.16, n-6 fatty acid rich medium 0.05 and n-3 fatty acid rich medium 0.71). However, in the former study the main n-3 fatty acid offered to the cells was 18:3n-3, which is very well incorporated into the phospholipids however hardly desaturated to 20:5n-3 [17]. The present work was carried out in the light of the results of Reibel *et al.* [15], who found changes in 20:5n-3, 22:5n-3 and 22:6n-3. The changes in 20:5-n-3 and 22:5n-3 presently observed are, although less, in similar direction. It

should be noted that, because of the absence of 22:6n-3 in the medium, in the present study no change in 22:6n-3 content in the myocyte phospholipids was found. It has been reported before that in many types of cultured cells a medium too rich in nonesterified fatty acid leads to accumulation of triglyceride within the cells [24]. The accumulation becomes visible as cytoplasmic inclusions [24,29]. This kept us from adding higher concentrations of nonesterified fatty acids to the medium to induce more dramatic changes in the fatty acid composition of the phospholipids. But, as can be calculated from Table VII.1, in our experiments the total nonesterified fatty acid pool in the media used, varied between 30 and 75 μmol.litre^{-1}, which is lower than the albumin concentration (approximately 125 μmol.litre^{-1}). This should prevent fatty acid toxicity because previously we demonstrated that cardiac sarcolemmal membrane integrity (Ca^{2+}- and Na^{+}-permeability; Na^{+}/Ca^{2+}-antiporter, Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase activities) become affected when the nonesterified fatty acid over albumin ratio surpasses 5 [30,31].

It is unlikely that the increased concentration of nonesterified polyunsaturated fatty acids during the 24 h pretreatment of the myocytes activated protein kinase C and that, upon this, further activation due to Ca^{2+}-dependent proteolytic cleavage occurred. The simultaneous presence of high intracellular concentrations of an unsaturated fatty acid (18:1n-9, 18:2n-6 and 20:4n-6 >50 μmol.litre^{-1}), of diglyceride (3 μmol.litre^{-1}) and of Ca^{2+} (>500 μmol.litre^{-1}) are required [32-35]. The situation is certainly not like that under the 24 h pretreatment conditions employed in this study. Moreover our previous finding that phorbol ester activation of protein kinase C induced a marked inactivation of α_1-adrenoceptor mediated phosphatidylinositol 4,5-bisphosphate breakdown [25] does not comply with the present observation that the phosphatidylinositol 4,5-bisphosphate breakdown is unaffected by fatty acid treatment of the cells (Figure VII.1).

As can be derived from the changes in the total phospholipid fatty acid content induced by 18:2n-6 and 20:5n-3 there was no preferential incorporation (Table VII.2). This was unlike the results obtained by Reibel *et al.* [15] who fed rats cornoil (18:2n-6-enriched) or fishoil (enriched in 20:5n-3 and 22:6n-3) diets: the myocardial phospholipid fatty acid composition was affected more by the fishoil diet than by the cornoil diet. The cultured cardiomyocytes appear to elongate 20:5n-3 to 22:5n-3 analogous to the *in vivo* study [15].

The present study also shows the fatty acid content of the cellular phosphatidylinositol fraction of the membrane phospholipids. Since cellular phosphatidylinositol is believed to be in rapid metabolic equilibrium with phosphatidylinositol 4,5-bisphosphate, it is expected that these lipids share the same fatty acid profile. The reason why measurements of the fatty acid content of phosphatidylinositol 4,5-bisphosphate has not been undertaken is that it represents less than 0.1% of the total phospholipids in the cell. Our results on phosphatidylinositol fatty acid composition show that 20:4n-6 is not enriched in this lipid fraction. This is a remarkable finding because several authors have put forward that the *sn*-2 position of the cellular phosphoinositides is usually 20:4n-6 because of their function to release this

fatty acid in the form of diacylglycerol after stimulation of the phosphatidylinositol turnover [35-37]. However, during hormonal stimulation there may be a preferential degradation of phosphatidylinositol 4,5-bisphosphate species containing 20:4n-6 or the phosphatidylinositol kinase may have specificity for phosphatidylinositol species containing 20:4n-6. Convincing evidence in isolated hepatocytes for the latter proposal has been presented recently [26]. Previously we have shown that there is no change in total amount of (labelled) phosphoinositides due to 30 minutes of α_1-adrenergic stimulation of phosphatidylinositol turnover [25]. The present results confirm this finding as there is no change in total phospholipid or phosphatidylinositol fatty acid contents after prolonged phenylephrine stimulation of the cardiomyocytes. A change in phosphatidylinositol fatty acid composition during stimulation would be likely to occur because the breakdown of polyphosphoinositides should be compensated by phosphorylation of phosphatidylinositols which in turn are replaced by freshly synthesized molecules. Therefore it was interesting to find out whether the content of 20:4n-6 or another polyunsaturated fatty acid was selectively depleted after long term stimulation of phosphatidylinositol turnover. In the present study no significant depletion of any of the polyunsaturated fatty acids occurred in α_1-adrenergic stimulated cardiomyocytes, not even in the total phospholipid fatty acids. Previously, we [13] and others [14] have shown that chronic norepinephrine stress in rats resulted in an increase in the 22:6n-3 content and a decrease in the 18:2n-6 content of the total phospholipid fraction. This effect may be purely ß-adrenergic, but that should be tested using a ß-adrenergic agonist in the cultured cardiomyocytes.

The rate of basal and α_1-adrenoceptor mediated phosphatidylinositol turnover was not affected by the changes either in the total phospholipid or in the phosphatidylinositol fatty acid composition, induced by preincubation of the cardiomyocytes with different polyunsaturated fatty acids (Fig VII.1). Although the changes in fatty acid composition (including the n-3/n-6 ratios) were not exactly similar to those induced in intact rat heart, our results suggest that inotropic changes as found by Reibel *et al.* [15] do not result from changes in the rate of phosphatidylinositol turnover. It is more likely that these inotropic changes are generated at the level of diacylglycerol activation of protein kinase C or at the level of the inositol trisphosphate modulation of transmembrane Ca^{2+} fluxes.

REFERENCES

1. OSNES JB, AASS H, SKOMEDAL T. Role of adrenergic receptors in myocardium. Eur Heart J 1989, in press
2. BERRIDGE MJ, IRVINE RF. Inositoltrisphosphate: a novel second messenger in cellular signal transduction. Nature 1984; **312**: 315-321.
3. BROWN JH, BUXTON IL, BRUNTON LL. α_1-Adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. Circ Res 1985; **57**: 532-537
4. HIRATA M, SUEMATSU E, HASHIMOTO T, HAMACHI T, KOGA T. Release of Ca^{2+} from a non-mitochondrial store site in peritoneal macrophages treated with saponin by inositol 1,4,5-trisphosphate. Biochem J 1984; **223**: 229-236.
5. NISHIZUKA Y. The role of protein kinase-C in cell surface signal transduction and tumour promotion. Nature 1984; **308**: 693-698.
6. BERRIDGE MJ. Inositol trisphosphate and diacylglycerol: two interacting second messengers. Ann Rev Biochem 1988; **56**: 159-193.
7. KISHIMOTO A, TAKAI Y, MORI T, KIKKAWA U, NISHIZUKA Y. Activation of calcium- and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. J Biol Chem 1980; **255**: 2273-2276.
8. TAKAI Y, KISHIMOTO A, KIKKAWA U, MORI T, NISHIZUKA Y. Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated phospholipid dependent protein-kinase system. Biochem Biophys Res Comm 1979; **91**: 1218-1224.
9. BELL RL, KENNERLY DA, STANFORD N, MAJERUS PW. Diglyceride lipase-pathway for arachidonate release from human platelets. Proc Natl Acad Sci USA 1979; **76**: 3238-3241.
10. PRESCOTT SM, MAJERUS PW. Characterization of 1,2-diacylglycerol hydrolysis in human platelets - Demonstration of an arachidonyl-monoacylglycerol intermediate. J Biol Chem 1973; **258**: 764-769.
11. HOLUB BJ. The cellular forms and functions of the inositol phospholipids and their metabolic derivatives. Nutr Rev 1987; **45**: 65-71.
12. LAMERS JMJ,HARTOG JM, VERDOUW PD, HULSMANN WC. Dietary fatty acids and myocardial function. Bas Res Cardiol 1987; **82** Suppl1: 209-221.
13. MONTFOORT A, VAN DER WERF L, HARTOG JM, HUGENHOLTZ PG, VERDOUW PD, HüLSMANN WC, LAMERS JMJ. The influence of fish oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidylethanolamine. Bas Res Cardiol 1986; **81**: 289-302.

14. GUDBJARNASON S, OSKARDOTTIR G, DOELL B, HALLGRIMSON J. Myocardial membrane lipids in relation to cardiovascular disease. Adv Cardiol 1978; **25**: 130-144
15. REIBEL DK, HOLAHAN MA, HOCK CE. Effects of dietary fish oil on cardiac responsivenes to adrenoceptor stimulation. Am J Physiol 1988; **254**: H494-H499.
16. HARTOG JM, VERDOUW PD, KLOMPE M, LAMERS JMJ. Dietary mackerel oil in pigs: effects on plasma lipids, cardiac sarcolemmal phospholipids and cardiovascular parameters. J Nutr 1987; **117**: 1371-1378.
17. GRYNBERG A, FANTINI E, ATHIAS P, DEGOIS M, GUENOT L, COURTOIS M, KHATAMI S. Modification of the n-6/n-3 fatty acid ratio in the phospholipids of rat ventricular myocytes in culture by the use of synthetic media: functional and biochemical consequences in normoxic and hypoxic conditions. J Mol Cell Cardiol 1988; **20**: 863-874.
18. YAGEV S, HELLER M, PINSON A. Changes in cytoplasmic and lysosomal enzyme activities in cultured rat heart cells: the relationship to cell differentiation and cell population in culture. In Vitro 1984; **20**: 893-898.
19. BLONDEL B, ROIJEN I, CHENEVAL JP. Heart cells in culture: A simple method for increasing the proportion of myoblasts. Experientia 1971; **27**: 356-358.
20. MEIJ JTA, LAMERS JMJ. Alpha-1-adrenergic stimulation of phosphoinositide breakdown in cultured neonatal rat ventricular myocytes. Mol Cell Biochem 1989; **88**: 73-75.
21. BLIGH EG, DYER WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; **37**: 911-918.
22. JOLLES J, ZWIERS H, DEKKER A, WIRTZ KA, GISPEN WH. Corticotropin-(1-24)-tetracosapeptide affects protein phosphorylation and polyphosphoinositide metabolism in rat brain. Biochem J 1981; **194**: 283-291.
23. BERRIDGE MJ, DOWNES CP, HANLEY MR. Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. Biochem J 1982; **206**: 587-595.
24. SPECTOR AA, MATHUR SN, KADUCE TL, HYMAN BT. Lipid nutrition and metabolism of cultured mammalian cells. Prog Lipid Res 1981; **19**: 155-186.
25. MEIJ JTA, LAMERS JMJ. Phorbolester inhibits α_1-adrenoceptor mediated phophoinositide breakdown in cardiomyocytes. J Mol Cell Cardiol 1989; **21**: 661-668.
26. AUGERT G, BLACKMORE, EXTON JH. Changes in the concentration and fatty acid composition of phosphoinositides induced by hormones in hepatocytes. J Biol Chem 1989; **264**: 2574-2580.
27. ROGERS CG. Fatty acids and phospholipids of adult and newborn rat hearts and of cultured, beating neonatal rat myocardial cells. Lipids 1974; **9**: 541-547.
28. HASIN Y, SAPOZNIKOV D, STEIN O, STEIN Y. Effect of fatty acid composition of rat heart myocytes on their electrical activity. J Mol Cell Cardiol 1982; **14**: 163-171.

29. MACKENZIE CG, MACKENZIE JB, REISS OK. Increase of in cell lipid and cytoplasmic particles in mammalian cells cultured at reduced pH. J Lipid Res 1967; **8**: 642-645.
30. LAMERS JMJ, HÜLSMANN WC. Inhibition of ($Na^+ + K^+$) stimulated ATPase of heart by fatty acids. J Mol Cell Cardiol 1977; **9**: 343-346.
31. LAMERS JMJ, STINIS HT, MONTFOORT A, HÜLSMANN WC. The effect of lipid intermediates on Ca^{2+} and Na^+ permability and ($Na^+ + K+$)-ATPase of cardiac sarcolemma. Biochim Biophys Acta 1984; **774**: 127-137.
32. MCPHAIL LC, CLAYTON CC, SNYDERMAN R. A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase. Science 1984; **224**: 622-624
33. VERKEST V, MCARTHUR M, HAMILTON S. Fatty acid activation of protein kinase C: dependence on diacylglycerol. Biochem Biophys Res Commun 1988; **152**: 825-829.
34. RANDO RR. Regulation of protein kinase C activity by lipids. FASEB J 1988; **2**: 2348-2355.
35. BERRIDGE MJ. Review article : Inositol trisphosphate and diacylglycerol as second messengers. Biochem J 1984; **220**: 345-360.
36. MARCUS AJ. The role of lipids in platelet function with particular reference to the arachidonic pathway. J Lipid Res 1978; **19**: 794-826.
37. BAKER RR, THOMPSON W. Positional distribution of fatty acids in phosphatidic acid, phosphoinositides, phosphatidylcholine and phosphatidylethanolamine in rat brains *in vivo*. Biochim Biophys Acta 1972; **270**: 489-503

Chapter VIII

General Discussion

In the studies described in this thesis cultured neonatal rat cardiomyocytes were used as a model to investigate the myocardial α_1-adrenoceptor mediated phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) breakdown. After this process had been studied in cultured cells, a more detailed study of $PI(4,5)P_2$-specific phospholipase C ($PI(4,5)P_2$-PLase C) and protein kinase C (PKC) was carried out in microsomes prepared from the cultured cells. Some advantages of the cultured neonatal rat cardiomyocytes over other available models, which are detailed in Chapter II, were exploited further. The cardiomyocyte culture permitted the introduction into the medium of physiological substances that induced certain changes the cellular membrane lipids. The effects of increased rate of PE N-methylation and of altered phospholipid fatty acid composition on $PI(4,5)P_2$ breakdown and *vice versa* were studied.

VIII.1 Transduction of the α_1-adrenoceptor signal

1.1 Alpha$_1$-adrenoceptor mediated PI(4,5)P$_2$ breakdown

Our finding that mammalian myocardial α_1-adrenergic receptors are coupled to the phosphoinositide cycle, as described in Chapter III, has been confirmed by the reports of others, although different approaches and a wide variety of models were employed [Uchida *et al.* 1982; Brown *et al.* 1985; Otani *et al.* 1986; Poggioli *et al.* 1986; Steinberg *et al.* 1987; Woodcock *et al.* 1987a,b and c; Okumura *et al.* 1988; Otani *et al.* 1988; Scholz *et al.* 1988]. For instance, Okumura *et al.* (1988) have demonstrated that α_1-adrenergic stimulation results in DG formation. Not surprisingly, the characteristics of $PI(4,5)P_2$ breakdown that have been reported are also varying. A decrease in $PI(4,5)P_2$ concomitant with an increase in IP_3 upon α_1-adrenergic stimulation has been reported after 15 sec in isolated heart [Poggioli *et al.* 1986], and after 30 sec in bathed papillary muscle [Otani *et al.* 1988] and left auricles [Scholz *et al.* 1988]. In perfused rat heart, stimulated for 15 sec, mainly IP_1 was found [Woodcock 1987b]. Longer stimulation periods produced predominantly IP_1 in all models. In our studies it was difficult to employ stimulation periods of less than one minute without creating changes in the temperature and pH during incubation. However, finding mainly IP_1 as the inositolphosphate formed after 1 to 30 min of α_1-adrenoceptor stimulation does agree with the afore-mentioned reports. As discussed in Chapter III and IV, IP_1 is probably the product of rapid IP_3-dephosphorylation. The fact that upon stimulation of the $PI(4,5)P_2$-specific PLase C in microsomes prepared from cultured neonatal rat cardiomyocytes, mainly IP_3 is found does not directly prove this point because a PLase C with properties different from the one in intact cells may be acting (Chapter V).

Finally, it should be mentioned that recently also in human ventricular tissue an α_1-adrenoceptor mediated increase in IP_3 and its association with the positive inotropic response have been shown [Kohl *et al.* 1989].

1.2 The involvement of GTP-binding proteins

2a G proteins involved in $PI(4,5)P_2$ breakdown

In Chapter V we described the presence of $PI(4,5)P_2$-PLase C activity in microsomes prepared from the cultured neonatal rat cardiomyocytes and furthermore that the enzyme was stimulated by GTPτS. This indicates that a GTP-binding protein is involved in the coupling of $PI(4,5)P_2$-PLase C to the receptor(s). The involvement of a G protein in the coupling of $PI(4,5)P_2$-PLase C to receptors, G_P, was implied some years ago when Litosch *et al.* (1985) demonstrated that, in membranes prepared from blowfly salivary glands, the stimulatory effect on $PI(4,5)P_2$ breakdown of 5-hydroxytryptamine depended on the addition of guanine nucleotides. The ability of GTP and its non-hydrolyzable analogues to stimulate breakdown of endogenous as well as exogenous, labelled phosphoinositides has been subsequently demonstrated in other systems [Cockcroft and Gomperts 1985; Gonzales and Crews 1985; Burch *et al.* 1986; Jackowski *et al.* 1986; Melin *et al.* 1986; Plantavid *et al.* 1986; Smith *et al.* 1986; Uhing *et al.* 1986; Orellana *et al.* 1987; Roth 1987 ; Behl *et al.* 1988; Jones *et al.* 1988; Bergers *et al.* 1989].

The most intensively studied G proteins are those involved in the dual regulation of adenylate cyclase activity and light-stimulated cyclic GMP hydrolysis in retinal rods and cones [Graziano and Gilman 1987]. All four classes of G proteins described (G_s, G_i, G_o and G_t) display a common, heterotrimeric structure consisting of an α, ß and τ subunit. The inactive state of an individual G protein is the trimer with GDP tightly bound to the α subunit. Upon the formation of an agonist-receptor complex interaction with the appropriate G protein is achieved. From the agonist-receptor-G protein complex GDP is dissociated which permits the binding of GTP to the α subunit. It is believed that subsequently the GTP-activated α subunit dissociates from the complex and modulates the appropriate effector. The intrinsic GTPase activity of the α subunit puts an end to the activated state.

About the identity of the putative G protein that is involved in the regulation of $PI(4,5)P_2$-PLase C activity, only one report is available at present. A novel GTP-binding, PI-PLase-C-activating 27 kD polypeptide has been identified in calf thymus [Wang *et al.* 1988]. That multiple pathways may be involved had already been suggested by the controversial effects of pertussis toxin [Lo and Hughes 1987], but recently the possibility for a cell to possess multiple G proteins that carry out the activation of $PI(4,5)P_2$-PLase C, but couple selectively to different receptors has been reported [Ashkenazi *et al.* 1989].

A new level of complexity in the actions of G proteins is suggested by experiments indicative of direct coupling of G proteins to ion channels. In the atrial myocardium a G protein - meanwhile named G_K - couples the muscarinic cholinergic receptor to a

specific potassium channel which, however, is not present in the ventricular muscle [Noma 1986]. Moreover, it has been demonstrated in ventricular myocytes and cardiac sarcolemmal vesicles that a G protein, probably activated G_s or its α subunit, directly regulates a calcium channel [Yatani *et al.* 1987]. Thus the cardiac calcium channels are dually regulated: directly by G_s and by second messengers generated by the target enzymes of G proteins.

2b G proteins in cardiovascular disease

Aside from the biochemical and physiological importance of G proteins, a growing body of data implicate alterations of these proteins in a variety of pathophysiological conditions including cardiovascular diseases. So far, the investigations have been restricted to the identifiable G proteins. At first, studies in animals with pressure- or volume-overload induced cardiac hypertrophy indicated that cardiac levels of G_s were decreased [Hammond *et al.* 1988; Longabaugh *et al.* 1988]. More recently reports have appeared describing alterations of G protein levels in failing human heart. Toxin labelling (ADP-ribosylation) of the α subunit of G_i was increased in hearts of patients with idiopathic dilated cardiomyopathy [Feldman *et al.* 1988]. Furthermore, this increase was proven to be due to an increased steady state level of mRNA encoding an αG_i subspecies. The αG_s mRNA level had also increased although normal levels of the protein were present [Feldman *et al.* 1989]. In another study G_s has been shown to be implicated in heart failure. Toxin labelling of lymfocyte G_{ss} which has been shown to correlate with cardiac G_{ss} was decreased by 80% in patients suffering from congestive heart failure [Horn *et al.* 1988]. Some care should be taken with the interpretation of this kind of data, because toxin labelling merely gives information about the availability of α-subunit for ADP-ribosylation but not about absolute levels of G protein [Insel and Ransnäs 1988]. Nevertheless, the data may indicate a mechanism for the reduction of $ß_1$-adrenoceptor responsiveness in the failing heart.

In contrast to the reduction of $ß_1$-adrenoceptor responsiveness, in experimental animal models of ischaemia and hypoxia α_1-adrenoceptor responsiveness has been shown to be increased [Sheridan *et al.* 1980; Sharma and Corr 1985], concomitant with an increase in α_1-adrenoceptor density [Corr *et al.* 1981; Heathers *et al.* 1987]. In models of ß-receptor blockade the α_1-adrenoceptor density has been reported to be increased without increased responsiveness [Mügge *et al.* 1985; Steinhaus *et al.* 1989]. However, as recently has been reported, in ischaemia and hypoxia also an enhanced production of IP_3 has been found [Otani *et al.* 1988; Heathers *et al.* 1989]. A report on α_1-adrenoceptors in human heart failure has also recently appeared. Vago *et al.* (1989) reported that in patients with idiopathic dilated cardiomyopathy cardiac α_1-adrenoceptors were increased in density and, in contrast to normal subjects, functionally coupled to a GTP-binding protein.

Because of the reduction of $ß_1$-adrenoceptor density α_1-adrenoceptors already compose a greater portion of the total adrenergic receptor population in failing heart. Additional down-regulation of the $ß_1$-receptor transduction mechanism and up-regulation

of α_1-adrenoceptor density and α_1-mediated IP_3 formation, would increase the importance of α_1-adrenoceptors even further. It will be interesting to find out in the future whether changes in G_P levels are implicated in increased α_1-responsiveness. Furthermore, taking for instance into account that pressure overload induces an increase in myocardial cardiac α_1-adrenoceptors long before onset of hypertrophy [Tamai *et al.* 1989] and thus before the above-reported decrease in G_s levels [Longabaugh 1988], it will be interesting to see if cross-talk between α_1- and β_1-adrenoceptor mechanisms is responsible for any of the observed changes in G protein levels.

1.3 The down-regulation of α_1-adrenoceptor mediated PI(4,5)P_2 breakdown

In Chapter IV the point was stressed that in our study after prolonged α_1-adrenergic stimulation (over 30 min) there is an attenuation of the formation of inositolphosphates. A similar result has been obtained by Otani *et al.* (1988), whereas Brown *et al.* (1985) and Steinberg *et al.* (1987) report an inositolphosphate formation that is linear up to 40 min stimulation in isolated adult rat cardiomyocytes and cultured neonatal rat cardiomyocytes, respectively. At present, we have no suitable explanation for this discrepancy. In our study we have been able to exclude any factors, like depletion of the PI(4,5)P_2 pool, that could have been the cause of the attenuation observed by us, as is amply discussed in Chapter IV.

Down-regulation of cardiac α_1-adrenergic receptors after prolonged stimulation has been reported before [Gengo *et al.* 1988]. After chronic (6-day) infusion of phenylephrine the B_{max} for [^{3}H-]prazosin decreased by 39%, but this loss of α_1-adrenergic receptors was not associated with attenuated inotropic responses. Also in a smooth muscle cell line α_1-adrenoceptor mediated PI(4,5)P_2 breakdown was reported to be attenuated after prolonged stimulation. This was associated with a reduction of the number of α_1-adrenoceptors and, moreover, with the phosphorylation of the α_1-adrenergic ligand binding subunit [Leeb-Lundberg *et al.* 1987]. Because phorbol ester had some similar effects the proposal was made that "a classical feedback loop" may have operated there, whereby diacylglycerol, generated from α_1-adrenoceptor stimulated PI(4,5)P_2 breakdown, activates PKC. PKC subsequently phosphorylates and desensitizes the α_1-adrenergic receptor, thereby attenuating the response [Sibley *et al.* 1987].

In our study in neonatal rat cardiomyocytes phorbol ester attenuated the α_1-adrenoceptor stimulated PI(4,5)P_2 breakdown. Therefore we proposed PKC-induced phosphorylation of an α_1-adrenoceptor subunit as an explanation for our results after prolonged α_1-adrenergic stimulation, although, as we stated, it had not been proven yet that PKC was activated in our system (Chapter IV). But, recently, it has been reported that PKC indeed is activated by α_1-adrenergic as well as by phorbol ester stimulation in cultured neonatal rat cardiomyocytes [Henrich and Simpson 1988]. The PKC activation by phorbol ester was much more pronounced and persistent than that by α_1-adrenoceptor stimulation, so proper caution should be taken before deducing from phorbol ester effects those of e.g. α_1-adrenoceptors.

In microsomes prepared from phorbol ester pretreated cardiomyocytes the basal as well as the GTPγS-stimulated $PI(4,5)P_2$-PLase C activity were decreased (Chapter V). As under these conditions the enzyme is not coupled to any receptor, down-regulation of $PI(4,5)P_2$ breakdown can not be attributed to phosphorylation of the receptor. Nor can it be attributed to phosphorylation of G_P, because the basal (in the presence 5 μM Ca^{2+}) $PI(4,5)P_2$ breakdown was reduced too. It is therefore likely that either the $PI(4,5)P_2$-PLase C itself or another regulatory protein (e.g. the 15 kD protein) is the PKC-substrate that mediates the down-regulation of $PI(4,5)P_2$ breakdown.

1.4 The activity of protein kinase C

The first report on protein kinase C described it as a soluble proenzyme from rat brain that could be activated by incubation with a Ca^{2+}-proteinase also purified from rat brain [Inoue *et al.* 1977]. What was called the proenzyme later turned out to be the Ca^{2+}/DG-activated, phospholipid-dependent enzyme we now know as PKC, whereas the proteolytically activated form, previously called PKM, is Ca^{2+}- and phospholipid-*in*dependent [Nishizuka 1988]. Over the past few years it has been shown that Ca^{2+}-dependent neutral proteases (calpains or CANPs) are cystein proteases preferring the membrane-bound, activated form of PKC as a substrate and subsequently releasing the catalytic fragment (PKM) into the cytosol. The physiological role of this process is still not clear [Murray *et al.* 1986], especially since the structure and functions of CANPs have appeared to be rather complex [Suzuki *et al.* 1987].

Phorbol esters have been shown to mimic DG effects in the activation of PKC [Castagna *et al.* 1982]. Long-term exposure of cells to phorbol ester leads to the loss or down-regulation of PKC [e.g. Henrich and Simpson 1988], which has been shown to be due to an increased rate of degradation by CANPs [Young *et al.* 1989]. At the last count 7 subspecies of PKC have been identified, which can be divided into two groups [Nishizuka 1988]. These PKC subspecies are differentially distributed in various cell types and possibly respond differently to activators *in vivo* [Kosaka 1988; Schaap *et al.* 1989]. Furthermore they have been shown to be differentially susceptible to CANPs [Kishimoto *et al.* 1989]. Thus the diverse functions attributed to PKC may well involve activation of different subspecies.

In addition to the positive forward action of PKC in the α_1-adrenoceptor mediated response, many reports indicate that PKC also provides negative feedback control over various steps of the cell-signalling processes e.g. receptor down-regulation [Leeb-Lundberg *et al.* 1987] and down-regulation of receptor-mediated $PI(4,5)P_2$ hydrolysis (Chapters IV and V). Since the catalytic fragment has been shown *in vitro* to have similar substrates as PKC and since the hydrophobic regulatory fragment contains putative DNA-binding amino acid sequences, it is unclear whether PKC proteolysis actually plays a role in cellular function or is just an intermediary step in the degradation of PKC [Murray *et al.* 1988]

In several of our experiments activation of PKC has been one of the aims. However there can be no doubt about which of the two PKC-forms has been activated. Stimulation of α_1-adrenoceptors and treatment with PMA for only 30 min (Chapters IV and V) can not be considered 'long-term' activation. Therefore the down-regulation of PKC by CANPs in these studies is negligible. In the studies described in Chapter VII, the purpose of the 24 h phase of preincubation with fatty acid-enriched media was certainly not activation of PKC. However, it should be noted that nonesterified fatty acids (NEFAs), especially unsaturated ones, are able to activate PKC [Berridge 1984; McPhail *et al.* 1984; Morimoto *et al.* 1988; Rando 1988; Verkest *et al.* 1988]. From these reports it has become clear that the simultaneous presence of high intracellular concentrations of unsaturated fatty acids, of diglyceride and of Ca^{2+} are required. The intracellular conditions in the cardiomyocytes in our study were certainly not like that. Furthermore, the above-mentioned reports tested the activation of PKC *in vitro*, whereas in our study increased amounts of NEFAs were offered to intact cardiomyocytes. There is still some controversy about the mechanism of cardiomyocyte fatty acid uptake, but the evidence is strong that it is a carrier-mediated process [Stremmel 1988]. It is therefore unlikely that in our study the intracellular concentration of NEFAs reflected the extracellular concentration at any moment during the preincubation period.

1.5 The possible roles of 15 kD protein phosphorylation

Activation of endogenous PKC in cardiomyocyte microsomes induced the incorporation of ^{32}P from γ-^{32}P-ATP into a protein with an estimated molecular weight of 15 kD (Chapter VI). The phosphorylation by PKC of a 15 kD protein has been reported before in cardiac sarcolemma [Presti *et al.* (1985b), Yuan and Sen (1986) and Vetter *et al.* (1989)], in skeletal muscle sarcolemma [Walaas *et al.* 1988], but also in plasma membranes of hepatocytes [Williamson *et al.* 1985] and polymorphonuclear leukocytes [Morimoto *et al.* 1988]. Based on the coincidence of 15 kD protein phosphorylation and maximum developed tension upon ß-adrenergic as well as upon α_1-adrenergic stimulation, Lindemann (1986) postulated that the 15 kD protein could be involved in the mediation of the increase in slow inward Ca^{2+} current. However, this is contradicted by the following facts: (i) as mentioned above, 15 kD protein is not restricted to contractile cells, (ii) the effect of PMA on the myocardial contractile state is induction of negative inotropy, both in perfused rat heart [Yuan *et al.* 1987] and cultured neonatal rat cardiomyocytes [Dösemeci *et al.* 1988], whereas (iii) incubation of microsomes or sarcolemma with PMA enhanced the phosphorylation of the 15 kD protein (Chapter V). Because, on the other hand, PMA treatment of cardiomyocytes reduced the α_1-adrenoceptor mediated $PI(4,5)P_2$ breakdown in intact cells as well as in microsomes it seems more likely that the phosphorylation of the 15 kD protein is involved in the down-regulation of receptor mediated responses. Since the protein has been shown to be a substrate of cyclic AMP dependent protein kinase as well as PKC this would mean

that the 15 kD protein mediates the homologous desensitization of adrenoceptors in general.

1.6 Second messengers involved in the myocardial α_1-adrenergic inotropic response

In the preceding part of this thesis a few pieces of the mechanism transducing the α_1-adrenoceptor signal in myocardial cells have been studied and discussed. From this and from the current state of the literature it is possible to put all the pieces together to a more complete picture.

Interaction of a ligand ((nor)adrenaline) with the α_1-adrenergic receptor turns a specific G protein, G_P, into an activated state. The activated G_P again activates a specific PLase C which cleaves $PI(4,5)P_2$ into IP_3 and DG. It has been suggested that IP_3 could be involved in excitation-contraction coupling - even as a replacement for the 'Ca^{2+}-induced Ca^{2+}-release' (see Chapter I) [Ochs 1986]- because it has been found that IP_3 is released upon electrical stimulation of myocardial cells [Poggioli *et al.* 1986]. However, in the cardiomyocytes used in this thesis, which were beating spontaneously and synchronously, no increase in basal inositol phosphate formation has been observed. Furthermore, the contractile effect of α_1-adrenoceptor stimulation on the myocardium is superimposed on the basal contraction.

Evidence that IP_3 is able to induce release of calcium from cardiac sarcoplasmic reticulum is scarce [Hirata *et al.* 1984; Volpe *et al.* 1985]. In fact, little is known of the nature and organization of the Ca^{2+} organelles responsive to IP_3. The opposite effects on calcium uptake of GTP in permeabilized smooth muscle cells versus isolated vesicles, suggested the presence of a second, IP_3-*in*sensitive Ca^{2+}-pool, communicating with the IP_3-sensitive pool through GTP-activated Ca^{2+}-translocation [Mullaney *et al.* 1988]. Based on these and other data Irvine [1988] has proposed a scheme which also includes the actions of the product of IP_3-3-kinase, inositol (1,3,4,5)-tetrakisphosphate as a factor promoting the transfer into the IP_3-sensitive Ca^{2+}-pool both from the intracellular IP_3-*in*sensitive Ca^{2+}-pool and indirectly from extracellular Ca^{2+}-sources. Recent data strongly suggest that the rough endoplasmic reticulum (RER) is both the Ca^{2+}-regulating organelle and the major site of functional IP_3-receptors [Ghosh *et al.* 1989]. There is, however, no evidence that an analogous organization of Ca^{2+} organelles is present in cardiac muscle cells. Recently, the presence of an IP_3-3-kinase in isolated adult rat cardiomyocytes, actively synthesizing inositol (1,3,4,5)-tetrakisphosphate after muscarinic stimulation, has been reported [Berg *et al.* 1989]. Furthermore the putative existence of two separate Ca^{2+}-release pools could simplify the distinction between Ca^{2+}-induced and superimposed IP_3-induced Ca^{2+}-release.

The other branch of the $PI(4,5)P_2$-derived messenger system starts with DG, which acts - probably in concert with the IP_3-induced Ca^{2+}-increase - primarily as the physiological activator of PKC. Secondarily, polyunsaturated fatty acids like arachidonic acid (20:4n-6), which are usually present at the *sn*-2 position, could be released from DG by DG-lipase activity.

Arachidonic acid is a substrate for the cyclooxygenase and lipoxygenase reactions producing several types of eicosanoids. The major prostaglandin released from intact hearts is prostacyclin (PGI_2), mainly derived from the vasculature, *i.e.* the endothelium and to a less extent smooth muscle cells. It is not clear whether cardiac myocytes produce PGI_2 [Needleman *et al.* 1986]. Thromboxane A_2 (TXA_2) is a major product of the eicosanoid pathway in platelets, but some may also be released from ischaemic myocardium [Coker and Parratt 1981]. Leukotrienes are mostly synthesized by leukocytes [Simmett and Peskar 1986], in addition to the ischaemic myocardial tissue [Barst and Mullane 1985; Evers *et al.* 1985]. Therefore, in contrast to nonischaemic conditions, local release of eicosanoids during the early phases of the ischaemic process may significantly modify myocardial and coronary function. As yet there is no evidence that eicosanoid production is increased after α_1-adrenergic stimulation of the myocardium and contributes to the ultimate functional response. Moreover, 20:4n-6 is known as activator of PKC [Morimoto *et al.* 1988], so that is probably its only contribution to the positive inotropic effect.

The positive forward actions of PKC may involve enhancement of myofibrillar responsiveness to calcium [Endoh and Blinks 1988], activation of glycogen phosphorylase [Osnes 1985], activation of adenylate cyclase [Yoshimasa *et al.* 1988], activation of PE N-methyltransferases [Villalba *et al.* 198], reduction of the voltage-activated transient outward K^+ current [Fedida *et al.* 1989] and stimulation of protein synthesis [Meidell *et al.* 1987]. Synergistic with the IP_3- and IP_4-induced Ca^{2+}-increase, the positive forward actions of PKC elicit a positive inotropic effect.

Furthermore PKC exerts a negative feedback action on the signal transduction process, possibly involving receptor down-regulation [Leeb-Lundberg *et al.* 1987], down-regulation of receptor mediated $PI(4,5)P_2$ hydrolysis (Chapters IV and V), stimulation of IP_3 phosphatase [Molina y Vedia and Lapetina 1986] and reduction of intracellular calcium by activation of the Ca^{2+}-transport ATPase and the Na^+/Ca^{2+}-antiporter [Furukawa *et al.* 1988]. These actions terminate the acute α_1-effects.

The activated PKC is cleaved by CANPs into two fragments. At present the role of the catalytic fragment in the α_1-adrenergic response is unknown. IP_3 is metabolized and the resulting inositol used to synthesize new PI molecules. The hydrolyzed $PI(4,5)P_2$ molecules are replaced by phosphorylation of PI and PI(4)P, which itself may have a regulatory function [Varsanyi *et al.* 1983]. The recently discovered polyphosphoinositides, PI(3)P, $PI(3,4)P_2$, and $PI(3,4,5)P_3$, are products of the PI-3'-kinase that is stimulated by receptors with intrinsic tyrosine kinase activity and were shown not to be involved in the mediation of the α_1-adrenoceptor signal [Auger *et al.* 1989].

VIII.2 The chemical composition of membrane phospholipids and transduction of the α_1-adrenoceptor signal

The cardiac sarcolemma is the site of many receptors, enzymes and ion channels, and in an integrated way these membrane processes have a paramount function in

controlling the beat-to-beat regulation of the heart as dictated by membrane de- and repolarization and by a variety of hormones and stimuli. It has been hypothesized several times that modulations of the molecular species of cardiac membrane phospholipids affect the function of membrane-associated processes. However there exists only poor evidence for it in the field of cardiac membrane research [McMurchie 1988]. Alterations in the membrane physicochemical properties influence the conformation of membrane-bound enzymes, the rate of lateral or rotational diffusion of enzyme proteins in the membrane, and vertical positioning of proteins in the lipid bilayer [McMurchie 1988]. In Chapter VI and VII of this thesis two different approaches of modification of the molecular species of cardiac sarcolemmal phospholipids have been studied with respect to their influence on the transmembrane conduction of the α_1-adrenoceptor signal.

2.1 Interactions with PE N-methylation

Previously it was reported that both PE N-methylation and the polyphosphoinositides affected similar Ca^{2+} transport systems in the myocardium. For example perfusing the myocardium with methionine, stimulating PE N-methylation, increases sarcolemmal and sarcoplasmic Ca^{2+}-pumping ATPase activities [Panagia *et al.* 1987] Varsanyi *et al.* (1983) have shown that the sarcoplasmic reticular Ca^{2+} transport ATPase is activated by polyphosphoinositides. Furthermore, Ca^{2+}-pumping activity is depressed after depletion of polyphosphoinositides by treatment of the sarcolemma with PI-PLase C [Pierce *et al.* 1989]. In accordance with their effects on similar Ca^{2+} transport systems, both α_1-adrenoceptor mediated $PI(4,5)P_2$ breakdown and methionine-stimulated PE N-methylation had been reported to be implicated in the induction of a positive inotropic effect by affecting Ca^{2+} movements, in isolated perfused rat heart [Woodcock *et al.* 1987; Panagia *et al.* 1988]. It therefore seemed interesting to investigate whether these two processes were interacting or if they determined each others rates. The results obtained using isolated rat cardiac sarcolemma and those using cultured neonatal rat cardiomyocytes were rather different, but, in a way, so were the methods employed (Chapter VI). Treatment of cardiac sarcolemma with an exogenous, bacterial PI-PLase C resulted in (i) depletion of the polyphosphoinositide pool and (ii) inhibition of PE N-methyltransferase activities (sites I, II and III). α_1-Adrenoceptor stimulation of intact cardiomyocytes did not result in inhibition of PE N-methyltransferase activities, but - as was shown before (Chapter IV) - the polyphosphoinositide pool is not depleted under these conditions. Bacterial PI-PLases C have been shown also to release plasma membrane PI-glycan-anchored enzymes [Low 1987]. Therefore the inhibition of PE N-methylation observed in sarcolemma may have been due to loss of PI-glycan-anchored methyltransferases, but then, obviously, α_1-adrenoceptor coupled PLase C does not play a role in the release of membrane-anchored enzymes.

Methionine-stimulated PE N-methylation did not affect α_1-adrenoceptor stimulated $PI(4,5)P_2$ breakdown in intact cardiomyocytes, but, on the other hand, PE N-methylation

did reduce the basal level of $PI(4,5)P_2$ breakdown. This "basal" level represents the total of ^{3}H-inositol phosphates formed during incubation without α_1-agonist and is constant, independent of time of incubation (Chapter IV). The effect of PE N-methylation can only be explained if it is assumed that the basal level represents an equilibrium between basal $PI(4,5)P_2$-PLase C activity and inositolphosphate dephosphorylation that is not completely inhibited by Li^+. The α_1-adrenoceptor activated G_p-$PI(4,5)P_2$-PLase C complex is apparently not as susceptible to PE N-methylation as the "free-floating" $PI(4,5)P_2$-PLase C is. These assumptions make it all the more likely that PE N-methylation-induced alterations in membrane physicochemical properties are involved in modifying the "basal" level of ^{3}H-inositolphosphates.

2.2 Interrelation with PUFA composition of membrane phospholipids

A probably more drastic modification of membrane functions was expected to be achieved by changing the membrane phospholipid polyunsaturated fatty acid (PUFA) content. Polyunsaturated fatty acids are involved in several steps in the α_1-adrenoceptor mediated PI turnover. These fatty acids are present in: 1) in the phospholipid bilayer in which the α_1-adrenergic receptor and PLase C are embedded; 2) in the substrate $PI(4,5)P_2$ for PLase C; 3) in DG, the activator of PKC. It has been reported, for instance, that diet-induced alterations of sarcolemmal phospholipid fatty acid composition produced changes in cardiac contractile functions and catecholamine responsiveness [McMurchie 1988]. Whereas several reports on the effects of diet on cardiac ß-adrenoceptor function are available and have been reviewed [McMurchie 1988], only one about α_1-adrenoceptor function has recently appeared [Reibel *et al.* 1988]. In the latter study PUFAs, derived from fish oil (20:5n-3 and 22:6n-3), were added to control diets. It was demonstrated that dietary n-3 PUFAs induced alteration of the α_1- but not the ß-adrenoceptor mediated inotropic effects on the myocardium. Since the transduction of the α_1-signal involves, among other things, a family of phospholipids, namely PI-lipids, the possibility existed that the reported changes in α_1-adrenoceptor function were due to changes in transmembrane signalling.

Also some other unresolved questions brought us to investigate the PUFA composition of cardiac membrane phospholipids in relation to α_1-adrenoceptor mediated $PI(4,5)P_2$ hydrolysis. Firstly there was the point of the relatively high presence of PUFA, in particular 20:4n-6, in PI lipids [Holub 1987], which had not been studied in myocardium yet. Secondly, we [Montfoort *et al.* 1986] and others [Gudbjarnason *et al.* 1978] had shown that chronic norepinephrine stress in rats produced a dramatic increase in 22:6n-3 and decrease in 18:2n-6 content in the major myocardial phospholipid fractions phosphatidylcholine and phosphatidylethanolamine.

As described in this thesis (Chapter VII), the model of cultured neonatal rat cardiomyocytes easily permitted the induction of changes in cardiomyocyte phospholipid PUFA content by enrichment of the growth medium with 18:2n-6 or 20:5n-3. In control treated cardiomyocytes the PI fraction had, compared to the total phospholipid fraction,

a low total PUFA as well as 20:4n-6 content. The 18:2n-6 or 20:5n-3 pretreatment of cardiomyocytes significantly changed the PUFA pattern of total phospholipids, although the changes were not as dramatic as in the studies of others [Gudbjarnason *et al.* 1978; Montfoort *et al.* 1986; Reibel *et al.* 1988]. A remarkable finding was that in the PI PUFA pattern no significant changes had occurred (Chapter VII). The alterations in total phospholipid PUFA content influenced neither the basal nor the α_1-adrenoceptor stimulated PI(4,5)P_2 breakdown.

Alpha$_1$-adrenoceptor stimulated PI(4,5)P_2 breakdown affected neither the total phospholipid PUFA composition nor the PI PUFA composition, indicating that neither PI(4,5)P_2-PLase C nor PI- and PI(4)P-kinase have a preference for certain molecular species of PI-lipid. Therefore it seems likely that the previously observed effects on membrane PUFAs after chronic norepinephrine stress in rats are purely ß-adrenergic. However, to definitely prove this point, the effects of ß-adrenergic agonist-stimulation on PUFA content of phospholipids from cardiomyocytes should be measured.

2.3 Conclusion

Taking together the results of Chapter VI and VII it seems that the membrane-associated proteins involved in the transduction of the α_1-adrenergic signal are less prone to changes in the molecular species of membrane phospholipids than, on basis of the literature, was expected. It is certainly surprising that the very local and minor change in membrane composition that PE N-methylation elicits, seems more effective than the overall change in phospholipid PUFA composition induced by PUFA-enriched culture medium.

REFERENCES

1. ASHKENAZI A, PERALTA EG, WINSLOW JW, RAMACHANDRAN J, CAPON DJ (1989) Functionally different G proteins selectively couple different receptors to PI hydrolysis in the same cell. Cell **56**: 487-493.
2. AUGER KR, SERUNIAN LA, SOLTOFF SP, LIBBY P, CANTLEY LC (1989) PDGF-Dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell **57**: 167-175.
3. BARST S, MULLANE K (1985) The release of a leukotriene D_4 like substance following myocardial infarction in rabbits. Eur J Pharmacol **114**: 383-387.
4. BEHL B, SOMMERMEYER H, GOPPELT-STRÜBE M, RESCH K (1988) Phospholipase C in rabbit thymocytes: subcellular distribution and influences of calcium and GTPτS on the substrate dependence of cytosolic and plasma-membrane-associated phospholipase C. Biochim Biophys Acta **971**: 179-188.
5. BERG I, GUSE AH, GERCKEN G (1989) Carbamoylcholine-induced accumulation of inositol mono-, bis-, tris- and tetrakisphosphates in isolated cardiac myocytes from adult rats. Biochim Biophys Acta **1010**: 100-107.
6. BERGERS M, LENDI S, MIER PD (1989) Phosphatidylinositol 4,5 bisphosphate phospholipase C activity in particulate preparations from rat brain. Lipids **24**: 13-16.
7. BERRIDGE MJ (1984) Inositol trisphosphate and diacylglycerol as second messengers. Biochem J **220**: 345-360.
8. BURCH RM, LUINI A, AXELROD J (1986) Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to α_1-adrenergic stimulation in FRTL5 thyroid cells. Proc Natl Acad Sci USA **83**: 7201-7205.
9. BROWN JH, BUXTON IL, BRUNTON LL (1985) α_1-Adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. Circ Res **57**: 532-537.
10. CASTAGNA M, TAKAI Y, KAIBUCHI K, SANO K, KIKKAWA U, NISHIZUKA Y (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem **257**: 7847-7851.
11. COCKCROFT S, GOMPERTS BD (1985) Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature **314**: 534-536
12. COKER SJ, PARRATT JR (1981) Prostacyclin-induced changes in coronary blood flow and oxygen handling in the normal and acutely ischemic canine myocardium. Basic Res Cardiol **76**: 457-462.
13. CORR PB, SHAYMAN JA, KRAMER JB, KIPNIS RJ (1981) Increased α-adrenergic receptors in ischemic cat myocardium. J Clin Invest **67**: 1232-1235.

14. DÖSEMECI A, DHALLAN RS, COHEN NM, LEDERER WJ, ROGERS TB (1988) Phorbol ester increases calcium current and simulates the effects of angiotensin II on cultured neonatal rat heart myocytes. Circ Res **62**: 347-357.
15. ENDOH M, BLINKS JR (1988)Actions of sympathomimetic amines on the Ca^{2+} transients and contractions of rabbit myocardium: reciprocal changes in myofibrillar responsiveness to Ca^{2+} mediated through α- and ß-adrenoceptors. Circ Res **62**: 247-265.
16. EVERS AS, MURPHEE S, SAFFIZ JE, JAKSCHIK BA, NEEDLEMAN P (1985) Effects of endogenously produced leukotrienes, thromboxane and prostaglandins on coronary vascular resistance in rabbit myocardial infarction. J Clin Invest **75**: 992-999.
17. FEDIDA D, SHIMONI Y, GILES WR (1989) A novel effect of norepinephrine on cardiac cells is mediated by α_1-adrenoceptors. Am J Physiol **256**: H1500-1504.
18. FELDMAN AM, CATES AE, BRISTOW MR, VAN DOP C (1989) Altered expression of α-subunits of G proteins in failing human hearts. J Mol Cell Cardiol **21**: 359-365.
19. FELDMAN AM, CATES AE, VEAZEY WB, HERSHBERGER RE, BRISTOW MR, BAUGHMAN KL, BAUMGARTNER WA, VAN DOP C (1988) Increase of the 40,000-mol wt pertussis toxin substrate (G protein) in the failing human heart. J Clin Invest **82**: 189-197.
20. FURUKAWA KI, TAWADA Y, SHIKEGAWA M (1989) Protein kinase C activation stimulates plasma membrane Ca^{2+} pump in vascular smooth muscle cells. J Biol Chem **264**: 4844-4849.
21. GENGO PJ, BOWLING N, WYSS VL, HAYES JS (1988) Effects of prolonged phenylephrine infusion on cardiac adrenoceptors and calcium channels. J Pharmacol Exp Ther **244**: 100-105.
22. GHOSH TK, MULLANEY JM, TARAZI F, GILL DL (1989) GTP-Activated communication between distinct inositol 1,4,5-trisphosphate-sensitive and -insensitive calcium pools. Nature **340**: 236-239.
23. GONZALES RA, CREWS FT (1985) Guanine nucleotides stimulate production of inositol phosphate in rat cortical membranes. Biochem J **232**: 799-804.
24. GRAZIANO MP, GILMAN AG (1987) Guanine nucleotide-binding regulatory proteins: mediators of transmembrane signaling. Trends Pharmacol Sci **8**: 478-481.
25. GUDBJARNASON S, OSKARDOTTIR G, DOELL B, HALLGRIMSON J (1978) Myocardial membrane lipids in relation to cardiovascular disease. Adv Cardiol **25**: 130-144
26. HAMMOND HK, RANSNÄS LA, WHITE FC, BLOOR CM, INSEL PA (1988) Myocardial ß-receptor number, pharmacological responsiveness, and G_s in volume overload hypertrophy in pigs (Abstr.). Circulation **78 Suppl II**: 163.

27. HEATHERS GP, EVERS AS, CORR PB (1989) Enhanced inositol trisphosphate response to α_1-adrenergic stimulation in cardiac myocytes exposed to hypoxia. J Clin Invest **83**: 1409-1413.
28. HEATHERS GP, YAMADA KA, KANTER EM, CORR PB (1987) Long-chain acylcarnitines mediate the hypoxia-induced increase in α_1-adrenergic receptors on adult canine myocytes. Circ Res **61**: 735-746.
29. HENRICH CJ, SIMPSON PC (1988) Differential acute and chronic response of protein kinase C in cultured neonatal rat heart myocytes to α_1-adrenergic and phorbolester stimulation. J Mol Cell Cardiol **20**: 1081-1085.
30. HIRATA M, SUEMATSU E, HASHIMOTO T, HAMACHI T, KOGA T (1984) Release of Ca^{2+} from a non-mitochondrial store site in peritoneal macrophages treated with saponin by inositol 1,4,5-trisphosphate. Biochem J **223**: 229-236.
31. HOLUB BJ (1987) The cellular forms and functions of the inositol phospholipids and their metabolic derivatives. Nutr Rev **45**: 65-71.
32. HORN EM, CORWIN SJ, STEINBERG SF, CHOW YK, NEUBERG GW, CANNON PJ, POWERS ER, BILEZIKIAN JP (1988) Reduced lymphocyte stimulatory guanine nucleotide regulatory protein and ß-adrenergic receptors in congestive heart failure and reversal with angiotensin converting enzyme inhibitor therapy. Circulation **78**: 1373-1379.
33. INSEL PA, RANSNÄS LA (1988) G proteins and cardiovascular disease (Editorial). Circulation **78**: 1511-1513.
34. INOUE M, KISHIMOTO A, TAKAI Y, NISHIZUKA Y (1977) Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. 2. Proenzyme and its activation by calcium-dependent protease from rat brain. J Biol Chem **252**: 7610-7616.
35. IRVINE RF (1988) How do inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate regulate intracellular Ca^{2+}? Biochem Soc Trans **17**: 6-9.
36. JACKOWSKI S, RETTENMEIER CW, SHERR CJ, ROCK CO (1986) A guanine nucleotide-dependent phosphatidylinositol 4,5-bisphosphate phospholipase C in cells transformed by the v-*fms* and v-*fes* oncogenes. J Biol Chem **261**: 4978-4985.
37. JONES LG, GOLDSTEIN D, BROWN JH (1988) Guanine nucleotide-dependent inositol trisphosphate formation in chick heart cells. Circ Res **62**: 299-305.
38. KISHIMOTO A, MIKAWA K, HASHIMOTO K, YASUDA I, TANAKA S, TOMINAGA M, KURODA T, NISHIZUKA Y (1989) Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). J Biol Chem **264**: 4088-4092.
39. KOHL C, SCHMITZ W, SCHOLZ H, SCHOLZ J, TóTH M, DÖRING V, KALMáR P (1989) Evidence for α_1-adrenoceptor-mediated increase of inositol trisphosphate in the human heart. J Cardiovasc Pharmacol **13**: 324-327.

40. KOSAKA Y, OGITA K, ASE K, NOMURA H, KIKKAWA U, NISHIZUKA Y (1988) The heterogeneity of protein kinase C in various rat tissues. Biochem Biophys Res Comm **151**: 973-981.
41. LEEB-LUNDBERG LMF, COTECCHIA S, DEBLASI A, CARON MG, LEFKOWITZ RJ (1987) Regulation of adrenergic receptor function by phosphorylation. I Agonist-promoted desensitization and phosphorylation of α_1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT$_1$ MF-2 smooth muscle cells. J Biol Chem **262**:3098-3105.
42. LINDEMANN JP (1986) α-Adrenergic stimulation of sarcolemmal protein phosphorylation and slow responses in intact myocardium. J Biol Chem **261**: 4860-4867.
43. LITOSCH I, WALLIS C, FAIN JN (1985) 5-Hydroxytryptamine stimulates inositol phosphate production in a cell-free system from blowfly salivary glands. J Biol Chem **260**: 5464-5471.
44. LO WWY, HUGHES J (1987) Receptor-phosphoinositidase C coupling. Multiple G-proteins? FEBS Lett **224**: 1-3.
45. LONGABAUGH JP, VATNER DE, VATNER SF, HOMCY CJ (1988) Decreased stimulatory guanosine triphosphate binding protein in dogs with pressure-overload left ventricular failure. J Clin Invest **81**: 420-424.
46. LOW MG (1987) Biochemistry of the glycosyl-phosphatidylinositol membrane protein anchors. Biochem. J. **244**: 1-13.
47. LUETJE CW, TIETJE KM, CHRISTIAN JL, NATHANSON NM (1988) Differential tissue expression and developmental regulation of guanine nucleotide binding regulatory proteins and their messenger RNAs in rat heart. J Biol Chem **263**: 13357-13365.
48. MCMURCHIE EJ (1988) Dietary lipids and the regulation of membrane fluidity and function. In: *Physiological regulation of membrane fluidity*, Alan R. Liss Inc., pp 189-237.
49. MCPHAIL LC, CLAYTON CC, SNYDERMAN R (1984) A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase. Science **224**:622-624
50. MEIDELL RS, SEN A, HENDERSON SA, SLAHETKA MF, CHIEN KR (1986) α_1-Adrenergic stimulation of rat myocardial cells increases protein synthesis. Am J Physiol **251**: H1076-1084.
51. MELIN PM, SUNDLER R, JERGIL B (1986) Phospholipase C in rat liver membranes. Phosphoinositide specificity and regulation by guanine nucleotides and calcium. FEBS Lett **198**: 85-88.
52. MOLINA Y VEDIA LM, LAPETINA EG (1986) Phorbol 12,13-dibutyrate and 1-oleyl-2-acetyldiacylglycerol stimulate inositol trisphosphate dephosphorylation in human platelets. J Biol Chem **261**: 10493-10495.
53. MONTFOORT A, VAN DER WERF L, HARTOG JM, HUGENHOLTZ PG, VERDOUW PD, HüLSMANN WC, LAMERS JMJ (1986) The influence of

fish oil diet and norepinephrine treatment on fatty acid composition of rat heart phospholipids and the positional fatty acid distribution in phosphatidylethanolamine. Bas Res Cardiol **81**: 289-302.

54. MORIMOTO YM, NOBORI K, EDASHIGE K, YAMAMOTO M, KOBAYASHI S, UTSUMI K (1988) Activation of protein kinase C by fatty acids and its dependency on Ca^{2+} and phospholipid. Cell Struct Funct **13**: 45-49.
55. MÜGGE A, REUPCKE C, SCHOLZ H (1985) Increased myocardial α_1-adrenoceptor density in rats chronically treated with propranolol. Eur J Pharmacol **112**: 249-252.
56. MULLANEY JM, YU M, GHOSH TK, GILL DL (1988) Calcium-entry into the inositol 1,4,5-trisphosphate-releasable calcium pool is mediated by a GTP-regulatory mechanism. Proc Natl Acad Sci USA **85**: 2499-2503.
57. MURRAY AW, FOURNIER A, HARDY SJ (1987) Proteolytic activation of protein kinase C: a physiological reaction? Trends Biochem Sci **12**: 53-54
58. NEEDLEMAN P, TURK J, JAKSCHIK BA, MORRISON AR, LEFKOWITH JB (1986) Arachidonic acid metabolism. Ann Rev Biochem **55**: 69-102.
59. NISHIZUKA Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature **334**: 661-665.
60. NOMA A (1986) GTP-Binding proteins couple cardiac muscarinic receptors to potassium channels. Trends Neurol Sci **9**: 142-143.
61. OCHS RS (1986) Inositol trisphosphate and muscle. Trends Biochem Sci **11**: 388-389.
62. OKUMURA K, KAWAI T, HASHIMOTO H, ITO T, OGAWA K, SATAKE T (1988) Sustained diacylglycerol formation in norepinephrine-stimulated rat heart is associated with α_1-adrenergic receptor. J Cardiovasc Pharmacol **11**: 651-656.
63. ORELLANA S, SOLSKI PA, BROWN JH (1987) Guanosine 5'-*O*-(thiotriphosphate)-dependent inositol trisphosphate formation in membranes is inhibited by phorbol ester and protein kinase C. J. Biol. Chem. **262**: 1638-1643.
64. OSNES JB, AASS H, SKOMEDAL T (1985) On adrenergic regulation of heart function: role of myocardial α-adrenoceptors. In: *α-Adrenoceptor blockers in cardiovascular disease*, eds. H Refsum, OD Mjo/s. Edinburgh, London, Melbourne and New York, Churchill Livingstone, pp 69-102.
65. OTANI H, OTANI H, DAS DK (1986) Evidence that phosphoinositide response is mediated by α_1-adrenoceptor stimulation, but not linked with excitation-contraction coupling in cardiac muscle. Biochem Biophys Res Commun **136**: 863-869.
66. OTANI H, PRASAD MR, ENGELMAN RM, OTANI H, CORDIS GA, DAS DK (1988) Enhanced phosphodiesteratic breakdown and turnover of phosphoinositides during reperfusion of ischemic rat heart. Circ Res **63**: 930-936.

67. PANAGIA V, MAKINO N, GANGULY PK, DHALLA NS (1987) Inhibition of Na^+-Ca^{2+} exchange in heart sarcolemmal vesicles by PE N-methylation. Eur J Biochem **166**: 597-603.

68. PANAGIA V, GUPTA MP, GANGULY PK, DHALLA NS (1988) Methionine-induced positive inotropic effect in rat heart : Possible role of phospholipid N-methylation. Circ. Res. **62**: 51-55.

69. PIERCE GN, PANAGIA V, LOW MG (1989) Cardiac sarcolemmal calcium pump activity is depressed after treatment with phosphatidylinositol-specific phospholipase C. Mol Cell Biochem, in press.

70. PLANTAVID M, ROSSIGNOL L, CHAP H, DOUSTE-BLAZY L (1986) Studies of endogenous polyphosphoinositide hydrolysis in human platelet membranes. Evidence that polyphosphoinositides remain inaccessible to phosphodiesterase in the native membrane. Biochim Biophys Acta **875**: 147-156.

71. POGGIOLI J, SULPICE JC, VASSORT G (1986) Inositol phosphate production following α_1-adrenergic, muscarinic or electrical stimulation in isolated rat heart. FEBS Lett **206**: 292-298.

72. PRESTI CF, SCOTT BT, JONES LR (1985) Identification of an endogenous protein kinase C activity and its intrinsic 15-kilodalton substrate in purified canine cardiac sarcolemmal vesicles. J Biol Chem **260**: 13879-13889.

73. RANDO RR (1987) Regulation of protein kinase C activity by lipids. FASEB J **2**: 2348-2355.

74. REIBEL DK, HOLAHAN MA, HOCK CE (1988) Effects of dietary fish oil on cardiac responsiveness to adrenoceptor stimulation. Am J Physiol **254**: H494-499.

75. ROTH BL (1987) Modulation of phosphatidylinositol-4,5-bisphosphate hydrolysis in rat aorta by guanine nucleotides, calcium and magnesium. Life Sci **41**: 629-634.

76. SCHAAP D, PARKER PJ, BRISTOL A, KRIZ R, KNOPF J (1989) Unique substrate specificity and regulatory properties of PKC-ϵ: a rationale for diversity. FEBS Lett **243**: 351-357.

77. SCHOLZ J, SCHAEFER B, SCHMITZ W, SCHOLZ H, STEINFATH M, LOHSE M, SCHWABE U, PUURUNEN J (1988) *Alpha*-1 adrenoceptor mediated positive inotropic effect and inositol trisphosphate increase in mammalian heart. J Pharmacol Exp Ther **245**: 327-335.

78. SHARMA AD, CORR PB (1985) α-Adrenergic mediated effects of catecholamines in the ischaemic and reperfused heart. In: *α-Adrenoceptor blockers in cardiovascular disease*, eds. H. Refsum and O.D. Mjo/s, Churchill Livingstone, New York, pp 69-102.

79. SHERIDAN DJ, PENKOSKE AA, SOBEL BE, CORR PB (1980) Alpha adrenergic-mediated contributions to dysrhythmias during myocardial ischaemia and reperfusion in cats. J Clin Invest **65**: 161-171.

80. SIBLEY DR, BENOVIC JL, CARON MG, LEFKOWITZ RJ (1987) Regulation of transmembrane signaling by receptor phosphorylation. Cell **48**: 913-922.
81. SIMMET T, PESKAR BA (1986) Eicosanoids and the coronary circulation. Rev Physiol Biochem Pharmacol **104**: 1-64.
82. SMITH CD, COX CC, SNYDERMAN R (1986) Receptor-coupled activation of phosphoinositide-specific phospholipase C by an N protein. Science **232**: 97-100.
83. STEINBERG SF, CHOW YK, ROBINSON RB, BILEZIKIAN JP (1987) A pertussis toxin substrate regulates α_1-adrenergic dependent phosphatidylinositol hydrolysis in cultured rat myocytes. Endocrinology **120**: 1889-1895.
84. STEINKRAUS V, NOSE M, SCHOLZ H, THORMÄHLEN K (1989) Time course and extent of α_1-adrenoceptor density changes in rat heart after ß-adrenoceptor blockade. Br J Pharmacol **96**: 441-449.
85. STREMMEL W (1988) Fatty acid uptake by isolated rat heart myocytes represents a carrier-mediated transport process. J Clin Invest **81**: 844-852.
86. SUZUKI K, IMAJOH Y, KAWASAKI H, MINAMI Y, OHNO S (1987) Calcium-activated neutral protease and its endogenous inhibitor. Activation at the cell membrane and biological function. Cell Struct Funct **220**: 271-277.
87. TAMAI J, HORI M, KAGIYA T, IWAKURA K, IWAI K, KITABAKE A, WATANABE Y, YOSHIDA H, INOUE M, KAMADA T (1989) Role of α_1-adrenoceptor activity in progression of cardiac hypertrophy in guinea pig hearts with pressure overload. Cardiovasc Res **23**: 315-322.
88. UCHIDA T, BHATNAGAR GM, LAKATTA EG, FILBURN CR (1982) Alpha-adrenergic stimulation of $^{32}PO_4$ labelling of phosphatidylinositol (PI) and phosphaditic acid (PA) in cultured rat heart cells. Fed Proc **41**: 1523 (Abstr).
89. UHING RJ, PRPIC V, JIANG H, EXTON JH (1986) Hormone-stimulated polyphosphoinositide breakdown in rat liver plasma membranes. J Biol Chem **261**: 2140-2146.
90. VAGO T, BEVILACQUA M, NORBIATO G, BALDI G, CHEBAT E, BERTORA P, BAROLDI G, ACCINNI R (1989) Identification of α_1-adrenergic receptors on sarcolemma from normal subjects and patients with idiopathic dilated cardiomyopathy: characteristics and linkage to GTP-binding protein. Circ Res **64**: 474-481.
91. VARSANYI M, TOLLE H-G, HEILMEYER LMG, DAWSON RMC, IRVINE RF (1983) Activation of sarcoplasmic reticular Ca^{2+} transport ATPase by phosphorylation of an associated phosphatidylinositol. EMBO J. **2**: 1543-1548.
92. VERKEST V, MCARTHUR M, HAMILTON S (1988) Fatty acid activation of protein kinase C: dependence on diacylglycerol. Biochem Biophys Res Commun **152**: 825-829.
93. VETTER R, HAASE H, WILL-SHAHAB L (1989) Cardiac sarcolemma vesicles: ATP-dependent Ca^{2+} transport and inhibition of protein kinase

reactions by the cardiotonic drug amrinone. In: *Proc Int Symp Heart Disease*, eds I.S. Anand, Kluwer Academic Publishers, Norwell MA (USA), in press.

94. VILLALBA M, PAJARES MA, RENART MF, MATO JM (1987) Protein kinase C catalyses the phosphorylation and activation of rat liver phospholipid methyltransferase. Biochem. J. **241**: 911-916.
95. VOLPE P, SALVIATI G, DIVIRGILIO F, POZZAN T (1985) Inositol 1,4,5-trisphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle. Nature **316**: 347-349.
96. WALAAS SI, HORN RS, ALBERT KA, ADLER A, WALAAS O (1988) Phosphorylation of multiple sites in a 15000 dalton proteolipid from rat skeletal muscle sarcolemma, catalyzed by adenosine 3',5'-monophosphate-dependent and calcium phospholipid-dependent protein kinases. Biochim Biophys Acta **968**: 127-137.
97. WANG P, TOYOSHIMA S, OSAWA T (1988) Properties of a novel GTP-binding protein which is associated with soluble phosphoinositides-specific phospholipase C. J Biochem **103**: 137-142.
98. WILLIAMSON JR, COOPER RH, JOSEPH SK, THOMAS AP (1985) Inositol trisphosphate and diacylglycerol as intracellular 2nd messengers in liver. Am J Physiol **248**: C203-216.
99. WOODCOCK EA, MCLEOD JK, SMITH IA, CLARK MG (1987a) Study of receptor-stimulated phosphatidylinositol hydrolysis in intact perfused rat hearts. Clin Exp Pharmacol Physiol **14**: 209-213.
100. WOODCOCK EA, SMITH IA, WALLACE CA, WHITE LBS (1987b) Evidence for a lack of inositol-(1,4,5)*tris*phosphate kinase activity in norepinephrine-perfused rat hearts. Biochem Biophys Res Commun **148**: 68-77.
101. WOODCOCK EA, WHITE LBS, SMITH IA, MCLEOD JK (1987c) Stimulation of phosphatidylinositol metabolism in isolated, perfused rat heart. Circ Res **61**: 625-631.
102. YATANI A, CODINA J, IMOTO Y, REEVES JP, BIRNBAUMER L, BROWN AM (1987) A G protein directly regulates mammalian cardiac calcium channels. Science **238**: 1288-1292.
103. YOSHIMASI T, SIBLEY DR, BOUVIER M, LEFKOWITZ RJ, CARON MG (1987) Cross-talk between cellular signalling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. Nature **327**: 67-70.
104. YOUNG S, PARKER PJ, ULLRICH A, STABEL S (1987) Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem J **244**: 775-779.
105. YUAN S, SEN AK (1986) Characterization of the membrane-bound protein kinase C and its substrate proteins in canine cardiac sarcolemma. Biochim Biophys Acta **886**: 152-161.

106. YUAN S, SUNAHARA TA, SEN AK (1987) Tumor-promoting phorbol esters inhibit cardiac functions and induce redistribution of protein kinase C in perfused beating rat heart. Circ Res **61**: 372-378.

Summary

The sympathetic regulation of the heart function appears not only to be mediated by ß-adrenoceptor-stimulated cyclic AMP action, but also by α_1-adrenoceptors. The current knowledge about α_1-adrenergic influences on heart function and about adrenoceptor changes seen in some pathophysiological states of the myocardium (Chapter I) demonstrates that more detailed information was required on the biochemical mechanism involved in the development of the cardiac α_1-adrenergic response.

The regulation of heart function in general, and by α_1-adrenoceptors in particular, involves changes in the intracellular calcium concentration. The receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) generates (at least) two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG), of which IP_3 is able to mobilize calcium from intracellular stores. Therefore it seemed likely that myocardial α_1-adrenoceptors were coupled to the phosphoinositide cycle (Chapter I).

Cultured cardiomyocytes were considered to be the most suitable model to study myocardial α_1-adrenoceptor mediated $PI(4,5)P_2$ breakdown, because, firstly, any contribution of non-myocytes could be eliminated, secondly, the presence and operation of specific receptors proved to be comparable to the *in vivo* state and, thirdly, there was ample accessibility of the cardiomyocyte for adrenergic agonists, [^{3}H-]-myoinositol etc. Out of the three available models of cultured cardiomyocytes: (1) adult rat redifferentiated cardiomyocytes, (2) adult rat rapid-attachment cardiomyocytes and (3) neonatal rat cardiomyocytes, the latter model was chosen on grounds of its adequate morphological, electrophysiological and pharmacological similarity with *in vivo* cardiomyocytes and its practical advantages. Therefore a procedure was set up to yield a near to homogeneous preparation of neonatal rat cardiomyocytes (Chapter II).

To study α_1-adrenoceptor mediated $PI(4,5)P_2$ breakdown, the cardiomyocytes were preincubated with [^{3}H-]myoinositol to label the membrane phosphoinositide pool and stimulated with the α_1-agonist phenylephrine (30 μM). This resulted in the accumulation of ^{3}H-inositolphosphates, however, only in the presence of the inositol monophosphatase inhibitor Li^+ (10 mM). Furthermore, ^{3}H-inositol monophosphate (^{3}H-IP_1) was predominant, whereas the level of labelled IP_3, the putative second messenger, was low. Apparently, the activities of the myocardial inositolphosphatases are high and IP3 is only transiently present in the cardiomyocyte (Chapter III).

The α_1-adrenergic receptor specificity (Chapter III and IV) as well as the time-course and the dose-response curve (Chapter IV) of phenylephrine induced $PI(4,5)P_2$ breakdown were determined. The rate of ^{3}H-inositolphosphate formation decreased after prolonged α_1-adrenoceptor stimulation without evidence of depletion of the membrane ^{3}H-$PI(4,5)P_2$ pool. Desensitization of the α_1-adrenergic receptor, possibly

through feedback inhibition by DG-activated protein kinase C (PKC), could explain this phenomenon. Direct activation of PKC by phorbol 12-myristate 13-acetate (PMA) treatment of cardiomyocytes led to a dose-dependent inactivation (IC_{50} 10^{-8} M PMA) of α_1-adrenoceptor stimulated $PI(4,5)P_2$ breakdown. These findings provide evidence that PKC plays a role in the regulation of α_1-adrenoceptor sensitivity.

This hypothesis was further investigated in a cell free system, isolated microsomal membranes, in which the $PI(4,5)P_2$-specific phospholipase C ($PI(4,5)P_2$-PLase C), endogenous PKC and substrate proteins for PKC could be detected and separately activated (Chapter V). Incubation of microsomes prepared from cultured neonatal rat cardiomyocytes, in buffer containing 5 μM $CaCl_2$, 9 mM cholate and 100 nM ^{3}H-$PI(4,5)P_2$ resulted in the formation of ^{3}H-IP_3. GTPτS (125 μM) stimulated the production of ^{3}H-IP_3. Microsomes prepared from phorbol ester-pretreated (100 nM PMA) cardiomyocytes, showed decreased activities of basal as well as GTPτS-stimulated ^{3}H-$PI(4,5)P_2$ hydrolysis.

In the microsomes a 15 kD protein was demonstrated to be the major substrate phosphorylated by intrinsic PKC, which was activated by 0.5 mM Ca^{2+}. Addition of phorbol ester (100 nM PMA) enhanced the ^{32}P incorporation into the 15 kD protein. PKC, purified from rat brain, in the presence of Ca^{2+}, phosphatidylserine and DG, did not change the phosphorylation pattern any further (Chapter V).

Thus three effects of phorbol ester were described: PMA-treatment of neonatal rat cardiomyocytes inactivated the α_1-adrenoceptor mediated $PI(4,5)P_2$ breakdown, PMA-pretreatment of neonatal rat cardiomyocytes reduced microsomal GTPτS-stimulated $PI(4,5)P_2$-PLase C activity and in cardiomyocyte microsomes PMA activated PKC-induced 15 kD protein phosphorylation. These results indicate that phorbol ester may down-regulate α_1-adrenoceptor mediated $PI(4,5)P_2$ hydrolysis by activation of PKC-induced 15 kD protein phosphorylation.

The phospholipid environment of the α_1-adrenergic receptor, the phosphoinositides, $PI(4,5)P_2$-PLase C *etc.*, may influence the rate of the phosphoinositide cycle. In this context, the mutual interactions between phosphatidylethanolamine (PE) N-methylation, a process that slightly changes the phospholipid environment, and the α_1-adrenergic receptor stimulated $PI(4,5)P_2$ breakdown were investigated (Chapter VI). In sarcolemma purified from rat heart, prior hydrolysis of phosphoinositides by exogenous PI-specific PLase C inhibited (to 75, 59 and 78% of control for catalytic sites I, II and III, respectively) the PE-N-methyltransferase system. In cultured rat cardiomyocytes the addition of L-methionine, a precursor for the methyldonor S-adenosylmethionine, stimulated PE-N-methylation in a concentration-dependent (0.2 - 300 μM) manner. Methionine (50 μM) decreased the basal rate of $PI(4,5)P_2$ hydrolysis (to 72% of control), but had no effect on the phenylephrine-stimulated $PI(4,5)P_2$ hydrolysis. Maximal activation of the $PI(4,5)P_2$ breakdown by 30 μM phenylephrine did not affect the rate of PE-N-methylation in the presence of exogenous methionine (50

μM). These findings support the existence of interactions, although discrete, between the enzymes involved in the PE N-methylation and phosphoinositide turnover.

Also in the context of changing the phospholipid environment, the phospholipid molecular species were changed by inducing incorporation of specific polyunsaturated fatty acids (PUFAs) into the phospholipids of cultured neonatal rat cardiomyocytes (Chapter VII). Special attention was paid to the PUFAs of vegetable (linoleic acid) and marine (eisosapentaenoic acid) origin because of the growing interest in theit actions on the cardiovascular system. PUFAs are also involved in certain steps of the α_1-adrenoceptor mediated phosphoinositide turnover e.g. through the $PI(4,5)P_2$ and DG molecules.

Neonatal rat cardiomyocytes were incubated for 24 h in culture media supplemented with linoleic acid (18:2n-6) or eicosapentaenoic acid (20:5n-3) to change the PUFA composition of their membrane phospholipids. In 18:2n-6 treated cells the 18:2n-6 content of the total phospholipid fraction rose from 45 to 68 nmol/mg protein and in 20:5n-3 treated cells the 20:5n-3 and docosapentaenoic acid (22:5n-3) contents increased from 1.5 to 12.5 and 5.1 to 14.7 nmol/mg protein, respectively. The major n-3 fatty acid 22:6n-3 (11.4 nmol/mg protein) did not change after 20:5n-3 treatment of the cells. Although the phosphatidylinositol (PI) fraction showed changes parallel with those in the total phospholipid fractions, none were significant. In the PI fraction 22:5n-3 (0.4 nmol/mg protein) appeared to be the major n-3 fatty acid.

The fatty acid treated cardiomyocytes were prelabelled with [^{3}H-]-myoinositol to estimate the rate of $PI(4,5)P_2$ turnover. There were no differences in the rate of ^{3}H-inositolphosphate formation between control, 18:2n-6 and 20:5n-3 treated cells. Prolonged α_1-adrenergic stimulation of control, 18:2n-6 and 20:5n-3 treated cells did not change the PUFA composition of the total phospholipid and PI fractions. It is concluded that the rate of α_1-adrenoceptor mediated phosphoinositide turnover is not affected by the changes in PUFA composition of membrane phospholipids and that prolonged α_1-adrenergic stimulation does not lead to significant depletion of any specific PUFA or of total PUFAs in the phosphoinositides.

Finally, from the combined discussion of the experimental studies and the latest developments reported in the literature, a more complete picture can be drawn of how the myocardial α_1-adrenergic response is brought about (Chapter VIII).

Samenvatting

Lange tijd heeft men verondersteld dat het sympathische zenuwstelsel de hartfunctie beïnvloedde via de interactie van noradrenaline met ß-adrenerge receptoren op de hartcelmembraan (sarcolemma), intracellulair gevolgd door de werking van de 'second messenger' cyclisch AMP. Met de later ter beschikking gekomen ß-receptor-blokkerende stoffen werd aangetoond dat ook een interactie met α_1-adrenerge receptoren kan bijdragen. De huidige kennis van de α_1-adrenerge beïnvloeding van de hartfunctie en van de veranderingen in adrenoceptoren, die worden gezien in sommige pathofysiologische toestanden van het myocard (Hoofdstuk I), toont aan dat meer gedetailleerde informatie vereist is omtrent het biochemische mechanisme dat betrokken is bij de totstandkoming van de α_1-adrenerge respons in het hart.

De regulatie van de hartfunctie in het algemeen, en door α_1-adrenoceptoren in het bijzonder, gebeurt o.a. door veranderingen in de intracellulaire calcium concentratie. De receptor-gestimuleerde hydrolyse van fosfatidylinositol 4,5-bisfosfaat ($PI(4,5)P_2$) leidt tot de vorming van (minstens) twee 'second messengers', inositol 1,4,5-trisfosfaat (IP_3) en diacylglycerol (DG), waarvan IP_3 calcium uit intracellulaire opslagplaatsen kan vrij maken. Mede daarom leek het aannemelijk dat α_1-adrenoceptoren in de hartspier gekoppeld waren aan de fosfatidylinositol (PI) cyclus (Hoofdstuk I).

Gekweekte cardiomyocyten werden het meest geschikte model geacht om de α_1-adrenoceptor geleide $PI(4,5)P_2$ hydrolyse te bestuderen, omdat, ten eerste, elke bijdrage van niet-spiercellen uitgesloten kon worden, ten tweede, de aanwezigheid en werkzaamheid van specifieke receptoren vergelijkbaar bleken met die die aanwezig zijn in *in vivo* en, ten derde, er ruime toegankelijkheid van de cardiomyocyt was voor adrenerge agonisten, [^{3}H-]myoinositol etc. Van de drie beschikbare vormen van gekweekte cardiomyocyten: (1) geredifferentieerde cardiomyocyten uit de adulte rat, (2) snel-aanhechtende cardiomyocyten uit de adulte rat, en (3) cardiomyocyten uit de neonatale rat, werd de laatste vorm gekozen op grond van een bevredigende morfologische, electrofysiologische en farmacologische gelijkenis met *in vivo* functionerende cardiomyocyten en ook om de praktische voordelen. Een procedure werd ontworpen om een vrijwel homogeen preparaat van neonatale rat cardiomyocyten te verkrijgen (Hoofdstuk II).

Om de α_1-adrenoceptor geleide $PI(4,5)P_2$ hydrolyse te onderzoeken werden de cardiomyocyten vóórgeïncubeerd met [^{3}H-]myoinositol om de membraan PI-pool te labelen, en daarna gestimuleerd met de α_1-agonist phenylephrine (30 μM). Dit resulteerde in de ophoping van ^{3}H-inositolfosfaten, doch slechts in aanwezigheid van de inositolmonofosfatase-remmer Li^+ (10 mM). Bovendien overheerste 3H-inositolmonofosfaat (^{3}H-IP_1), terwijl het niveau van gelabeld IP_3, de veronderstelde 'second messenger', laag was. Blijkbaar zijn in de hartspier de activiteiten van de

inositolfosfatasen hoog en is de aanwezigheid van IP_3 in de cel van voorbijgaande aard (Hoofdstuk III).

De α_1-adrenerge receptor specificiteit (Hoofdstukken III en IV) zowel als het tijdsverloop en de dosis-respons-curve (Hoofdstuk IV) van de phenylephrine-geïnduceerde $PI(4,5)P_2$ hydrolyse werden vastgelegd. De vormingssnelheid van ^{3}H-inositolfosfaat nam af na langere α_1-adrenoceptor stimulatie, zonder dat er aanwijzingen waren dat de membraan ^{3}H-$PI(4,5)P_2$-pool uitgeput was geraakt. Desensitizering van de α_1-adrenerge receptor, mogelijk via teruggekoppelde remming door DG-geactiveerd eiwitkinase C (PKC), zou dit verschijnsel kunnen verklaren. Directe activering van PKC, door behandeling van cellen met phorbol 12-myristaat 13-acetaat (PMA), gaf een dosis-afhankelijke remming (IC_{50} 10^{-8} M) van de α_1-adrenoceptor-gestimuleerde $PI(4,5)P_2$ hydrolyse. Deze bevindingen dragen het bewijs aan dat PKC een rol speelt in de regulatie van de α_1-adrenoceptor gevoeligheid.

Deze veronderstelling werd nader onderzocht in een cel-vrij systeem, nl. geïsoleerde microsomale membranen, waarin de $PI(4,5)P_2$-specifieke phospholipase C ($PI(4,5)P_2$-PLase C), het endogeen PKC en de substraateiwitten van PKC, apart geactiveerd en aangetoond konden worden (Hoofdstuk V). Incubatie van microsomen die bereid waren uit gekweekte neonatale rat cardiomyocyten, in een buffer met 5 μM $CaCl_2$, 9 mM cholaat an 100 nM ^{3}H-$PI(4,5)P_2$ resulteerde in de vorming van ^{3}H-IP_3. GTPτS (125 μM) stimuleerde de produktie van ^{3}H-IP_3. Microsomen die bereid waren uit phorbolester-voorbehandelde (100 nM PMA) cardiomyocyten, vertoonden een verminderde basale en GTPτS-gestimuleerde ^{3}H-$PI(4,5)P_2$-PLase C activiteit.

In de microsomen werd een 15kD-eiwit aangetoond als het belangrijkste substraat voor fosforylering door het intrinsieke PKC, die door 0.5 mM Ca^{2+} geactiveerd werd. Toevoeging van phorbolester (100 nM PMA) verhoogde de ^{32}P-incorporatie in het 15kD-eiwit. Uit rattehersenen gezuiverd PKC, in aanwezigheid van Ca^{2+}, phosphatidylserine en DG, gaf geen verdere wijzigingen in het fosforyleringspatroon (Hoofdstuk V).

Er zijn dus drie effecten van phorbolester beschreven: PMA-behandeling van neonatale rat cardiomyocyten inactiveerde de α_1-adrenoceptor gestimuleerde $PI(4,5)P_2$ hydrolyse, PMA-voorbehandeling van neonatale rat cardiomyocyten verminderde de microsomale GTPτS-gestimuleerde $PI(4,5)P_2$-PLase-C-activiteit, en in cardiomyocyt-microsomen activeerde PMA de PKC-geïnduceerde fosforylering van het 15kD-eiwit. Deze resultaten wijzen erop dat phorbolester de α_1-adrenoceptor geleide $PI(4,5)P_2$-hydrolyse zou kunnen uitschakelen door activering van PKC-geïnduceerde fosforylering van het 15kD-eiwit.

Het fosfolipide milieu van de α_1-adrenoceptor, van de PI-lipiden, van $PI(4,5)P_2$-PLase C *etc.*, zou de snelheid van de PI cyclus kunnen beïnvloeden. In dit verband zijn de wederzijdse interacties tussen phosphatidylethanolamine(PE)-N-methylering, een proces dat het fosfolipide milieu lichtelijk wijzigt, en de α_1-adrenoceptor gestimuleerde

$PI(4,5)P_2$-hydrolyse onderzocht (Hoofdstuk VI). In sarcolemma dat uit rattehart gezuiverd was, trad na hydrolyse van PI-lipiden door exogeen PI-specifiek PLase C, remming op van de catalytic sites I, II en III van het PE-N-methyltransferase systeem tot respectievelijk 75, 59 en 78% van de contrôle-waarden . In gekweekte neonatale rat cardiomyocyten stimuleerde de toevoeging van L-methionine, een precursor voor de methyldonor S-adenosylmethionine, de PE-N-methylering op een concentratie-afhankelijke (0.2 tot 300 μM) wijze. Methionine (50 μM) verlaagde de basale snelheid van de $PI(4,5)P_2$-hydrolyse tot 72% van de contrôle-waarde, maar had geen effect op de phenylephrine-gestimuleerde $PI(4,5)P_2$-hydrolyse. Maximale activering van de $PI(4,5)P_2$-hydrolyse met 30 μM phenylephrine had geen invloed op de snelheid van de PE-N-methylering in aanwezigheid van exogeen methionine (50 μM). Deze bevindingen bevestigen het bestaan van interacties, zij het van beperkte aard, tussen de enzymen betrokken bij PE-N-methylering en de PI cyclus.

Eveneens in verband met wijziging van het fosfolipide milieu, werden de moleculaire species van de fosfolipiden veranderd door incorporatie van specifieke meervoudig onverzadigde vetzuren (PUFAs) in de fosfolipiden van gekweekte neonatale rat cardiomyocyten (Hoofdstuk VII). Daarbij werd niet alleen gedacht aan het bekende linolzuur (van plantaardige oorsprong) maar ook aan de meer recentelijk in de belangstelling gekomen PUFAs die voorkomen in visoliën, met name eicosapentaeenzuur. PUFAs zijn bovendien in bepaalde stappen van de α_1-adrenoceptor geleide PI cyclus gewikkeld, bijv. via de $PI(4,5)P_2$- en DG-moleculen.

Neonatale rat cardiomyocyten werden 24 uur geïncubeerd in cultuurmedia aangevuld met extra linolzuur (18:2n-6) of eicosapentaeenzuur (20:5n-3) om de PUFA-samensteling van de membraanfosfolipiden te veranderen. In 18:2n-6 behandelde cellen steeg het 18:2n-6 gehalte van de totale fosfolipide fractie van 45 naar 68 nmol/mg eiwit en in 20:5n-3 behandelde cellen stegen de 20:5n-3 en docosapentaeenzuur (22:5n-3) gehaltes respectievelijk van 1,5 naar 12,5 en van 5,1 naar 14,7 nmol/mg eiwit. Het voornaamste n-3 vetzuur 22:6n-3 (11,4 nmol/mg eiwit) bleef gelijk. De veranderingen in de PI-fractie liepen weliswaar parallel aan die in de totale fosfolipide fractie, maar waren geen van alle significant. In de PI-fractie was overigens 22:5n-3 (0,4 nmol/mg eiwit) het voornaamste n-3 vetzuur.

De vetzuur-behandelde cardiomyocyten werden voorgelabeld met [^{3}H-]myoinositol om de snelheid van $PI(4,5)P_2$-hydrolyse te bepalen. Er waren geen verschillen in de snelheid van ^{3}H-inositolfosfaatvorming tussen contrôle, 18:2n-6 en 20:5n-3 behandelde cellen. Langere α_1-adrenerge stimulatie van contrôle, 18:2n-6 en 20:5n-3 behandelde cellen gaf geen wijzigingen in de PUFA-samenstelling van de totale fosfolipide of de PI-fractie. Hieruit volgt dat de snelhied van α_1-adrenoceptor gestimuleerde PI-omzetting niet wordt beïnvloed door veranderingen in de PUFA-samenstelling van

membraanfosfolipiden en dat langdurige α_1-adrenerge stimulatie niet leidt tot uitputting van enig specifiek meervoudig onverzadigd vetzuur of van de totale PUFAs in de PI-lipiden.

Uiteindelijk kan uit de gecombineerde bespreking van de experimentele studies en uit de laatste ontwikkelingen die in de literatuur zijn vermeld, een nog vollediger beeld worden gevormd van de wijze waarop de α_1-adrenerge respons van het hart tot stand komt (Hoofdstuk VIII).

Ten slotte

Dit proefschrift zou niet af zijn geweest en ook nooit af zijn gekomen als er een aantal personen ontbroken hadden. Ik zou hen hier daarom willen vernoemen en bedanken.
Allereerst is daar mijn directe begeleider en co-promotor Dr. J.M.J. Lamers. Jos, je wist altijd een uitgang te vinden als ik eens vast zat in de wirwar van proeven en resultaten. Ook wist je me meermalen een ingang te geven bij andere wetenschappers, niet in de laatste plaats door de culinaire prestaties van Tilly en jullie gastvrijheid - met een zachte "g".
Dan mijn promotor Prof. Hans Koster en de overige leden van de "kleine commissie", de Profs. Ruigrok, Saxena en Verdouw, die het manuscript hebben willen doorwerken en voor de snelle zakelijke afwikkeling zorg hebben gedragen.
De mensen van het lab van Dr. J.W. de Jong, met name Liz Keijzer en Bob Schoutsen, die mij introduceerden in de geheimen van de hartcelkweek.
Prof. Arié Pinson from Jerusalem, thanks to whom heart cell culture holds no secrets for me any more.
Dhr. Lansbergen en zijn medewerkers van het CPB, en Kees Schoonderwoerd, die ervoor zorgden dat mijn bestellingen van zwangere ratten goed aankwamen.
De "bewoners" van kamer 1018, die mij hun flow-kast en incubator ter beschikking stelden.
Dr. Alessandra Bordoni from Bologna with whom I had an instructive and pleasant time, also outside the lab, during her stay in Holland.
Dr. Vincenzo Panagia from Winnipeg, with whom I had a fruitful and friendly cooperation, which hopefully will be continued in the future.
Cécile Hanson, Jan en Lieke de Boer, en Ton van der Kraaij, die mij op gezette tijden hun computer ter beschikking hebben gesteld.
Mijn (oud)labgenoten - in volgorde van opkomst - Hanny, Janjaap, John, Dick, Karel, Bob en Netty, en al de andere mensen van de afdeling Biochemie I, die me altijd met raad en daad en praat bij hebben gestaan.
En *last* maar zeker niet *least*, mijn ouders, vanwege de vanzelfsprekendheid waarmee ze mij de ruimte en de kracht gaven om de wereld van de wetenschap in te gaan.

Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 25 mei 1958 te Nijmegen. In 1976 behaalde zij het diploma Gymnasium ß aan het Dominicus College te Nijmegen. In dat zelfde jaar werd begonnen met de studie Biologie aan de Katholieke Universiteit Nijmegen (Fac. der Wiskunde en Natuurwetenschappen), waar zij in juni 1985 het kandidaatsexamen B1g (keuzebijvak Geologie) behaalde. Hierna werd de studie voortgezet met als hoofdvak Ontwikkelingsbiologie der Dieren bij de afdeling Zoölogie I (Prof. J.M. Denucé), waar zij in de periodes januari '81 - maart '81 en januari '82 - maart '82 tevens werkzaam was als student-assistent bij het practicum Zoölogie der Ongewervelde Dieren/Embryologie. Na het bewerken van een bijvak aan het Biologisch Lab. van de K.N.O.-kliniek van het St-Radboudziekenhuis te Nijmegen (Dr. W. Kuijpers) en bij de afdeling Chemische Cytologie (wijlen Prof. Ch.M.A. Kuyper), werd in mei 1985 het doctoraaldiploma behaald. Na een kort verblijf in Australië trad zij in januari 1986 in dienst van de Erasmus Universiteit Rotterdam, waar zij tot januari 1990 is aangesteld als assistent-in-opleiding bij de afdeling Biochemie I (Prof. J.F. Koster). In deze periode leverde zij ook een bijdrage aan het Biochemie-onderwijs voor Geneeskundestudenten.