STATUS OF α_1-ADRENOCEPTOR-ASSOCIATED PHOSPHOINOSITIDE PATHWAY IN HYPOTHYROID HEART.

BY

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Dedicated to

"People with dreams who reached out to touch them"

To My Parents

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List of Abbreviation

Adenosine triphosphate	ATP
Chloroethylclonidine	CEC
Congestive heart failure	CHF
Diacylglycerol	DAG
[^{3}H]-Dihydroalprenolol	[^{3}H]DHA
Dithiothreitol	DTT
Endothelial derived growth factor	EGF
Guanine-nucleotide binding protein	G protein
Hydrogen peroxide	H_2O_2
Hypochlorous acid	HOCl
Inositol 1-monophosphate	Ins(1)*P*
Inositol 1,4-bisphosphate	Ins(1,4)P_2
Inositol 1,4,5-trisphosphate	Ins(1,4,5)P_3
Krebs-Henseleit	K-H
Left ventricle	LV
Maximal developed pressure	P_{max}
Maximal rate of pressure development	+dP/dt
Maximal rate of pressure decline	-dP/dt
Methyl methanethiosulfonate	MMTS
Myosin heavy chain	MHC

Norepinephrine	NE
N-ethylamaleimide	NEM
para-nitrophenol	pNP
para-nitrophenyl phosphate	pNPP
p-Chloromercuriphenylsulfonic acid	p-CMPS
Platelet derived growth factor	PDGF
Protein kinase C	PK C
Phenylephrine	PE
Phosphatidylinositol	PtdIns
Phosphatidylinositol 4-phosphate	PtdIns4*P*
Phosphatidylinositol (4,5)-bisphosphate	PtdIns(4,5)P_2
Phospholipase C	PL C
Phospholipase D	PL D
[^{3}H]-Prazosin	[^{3}H]Pz
Propylthiouracil	PTU
Sarcolemma	SL
Sarcoplasmic reticulum	SR
Thin Layer Chromatography	TLC
Thyroxine	T_4
Triiodothyronine	T_3
Right ventricle	RV

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ABSTRACT

Sympathomimetic amines such as isoproterenol, epinephrine and norepinephrine are known to increase both the rate and strength of contraction of the heart by interacting primarily with ß-adrenoceptors. However, post-synaptically located α-adrenoceptors also mediate positive inotropism in the myocardium. These α-adrenoceptors have been shown to be of the α_1 subtype and they mediate the physiological response following interaction with catecholamines, although the responses are qualitatively different. Unlike ß-mediated response, the α_1-adrenoceptor mediated augmentation of the contractility occurs without an increase in cellular cyclic AMP. The response to the α_1-stimulation is mediated *via* Ca^{2+} mobilization from the intracellular stores and/or influx of extracelluar Ca^{2+} coupled to changes in phosphoinositide cycle.

In the SL membrane about 10% of the PtdIns pool is phosphorylated in two consecutive steps, catalyzed by two kinases (PtdIns kinase and PtdIns4*P* kinase) to yield PtdIns4P and PtdIns(4,5)P_2, respectively. Stimulation of SL α_1-adrenoceptor (via agonist binding) activates, possibly via a G protein, a phosphoinositide specific phospholipase C (PL C) which preferentially hydrolyzes PtdIns(4,5)P_2 yielding two intracellular messengers, intra-SL *sn*-1,2-diacylglycerol (DAG), which phosphorylate a series of proteins, and cytosolic inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), which releases Ca^{2+} from the SR. Any change in these activities will induce a change in the response of the heart to the α_1-adrenoceptor stimulation.

The α_1-adrenoceptor pathway (which is masked by ß-adrenergic response under physiological conditions) have been proposed to maintain myocardial responsiveness to

catecholamines under pathological conditions in which ß-adrenoceptors are impaired. Changes in the adrenoceptor densities have been reported in several pathological and clinical conditions including hypothyroidism and congestive heart failure. In this study we tested the hypothesis that stimulation of the α_1-receptors and subsequent changes in receptor associated phosphoinositide pathway may serve as a reserve mechanism to maintain the myocardial responsiveness to catecholamines, and may act as a secondary positive inotropic system in pathophysiological conditions of depressed ß-adrenergic activity.

In this study *in vitro* assay systems were utilized to detect the activities of three enzymes ofthe cardiac phosphoinositide pathway. We demonstrated that thyroid hormone upregulated the activity of PtdIns and PtdIns4*P* kinases, whereas low hormonal levels did not affect these enzymes in the hypothyroid rat hearts. On the other hand, thyroid hormone decreased the basal activity of both SL and cytosolic PL C and low levels resulted in a significant increase in the SL and cytosolic PL C (in both PTU-treated and thyroidectomized rats) of the myocardium. These enzymes were normalized with reversal of hypothyroidism. We further provided evidence for the increase in the phosphoinositide PL C activity in the hypothyroid rat heart parallel to the changes in the cardiac α_1/ß adrenoceptor ratio, and concluded that the α_1-adrenoceptor and its associated phosphoinositide pathway might play a compensatory role in the hypothyroid rats where the ß-adrenoceptor density and response are compromised.

The second model we used was moderate stage of congestive heart failure (8 weeks post-coronary ligation) in which we observed a marked depression in the activities of

PtdIns kinase, PtdIns4*P* kinase and PL C from the viable LV, where the decrease in PtdIns 4*P* was due to limitation of the phospholipid substrate. In contrast, in the RV the PtdIns kinase activities was increased, while the PtdIns4*P* kinase and PL C were not altered. These differences were attributed to the variation in the development of the disease. Overall, our results, which do not support the compensatory role of the α_1-adrenoceptors in CHF, emphasises the necessity of defining the compensatory role of the α_1-receptor and post-receptor mechanisms in each cardiac pathology associated with an enhanced α_1/ß receptor ratio.

I. INTRODUCTION

Cardiac performance is markedly increased by the sympathetic nervous system, where norepinephrine is known to serve as a neurotransmitter. The actions of norepinephrine on the myocardium are mediated through its interaction with the adrenoceptors which are located on the outer surface of the cardiomyocyte (sarcolemma, SL). Both ß and α-adrenoceptors are present in myocardial cells (Stiles *et al.*, 1984; Bruckner *et al.*, 1985; Sulakhe *et al.*, 1986) and serve as transducers linking norepinephrine to the second messenger signalling molecules with subsequent change in Ca^{2+} movements, thus modulating the myocardial contractility.

Sympathomimetic amines such as epinephrine, norepinephrine and isoproterenol are known to increase both the rate and strength of contraction of the heart by interacting primarily with ß-adrenoceptors (Sulakhe *et al.*, 1986). However, there is evidence that α-adrenoceptors also mediate positive inotropism in the myocardium (Skomedal *et al.*, 1982). Post-synaptically located α-adrenoceptors have been found in the myocardium of various species (Benfey, 1980) including humans (Bruckner *et al.*, 1984). These α-adrenoceptors have been shown to be of the α_1 subtype and they mediate the physiological response following interaction with catecholamines (Brown *et al.*, 1985; Williams *et al.*, 1981; Buxton *et al.*, 1986). The mechanical effects associated with α_1-adrenoceptor stimulation are qualitatively different from those of ß-adrenoceptor stimulation. Unlike ß-mediated response, the α_1-adrenoceptor mediated augmentation

of contractility occurs without an increase in cellular cyclic AMP (Benfey, 1980). However, enhancement of contractility and prolongation of the plateau phase of the action potential (Giotti *et al.*, 1973) by α_1-stimulation suggest that the effects may be mediated through receptor-coupled increases in the intracellular concentration of Ca^{2+}. Indeed, the mechanism of action for α_1-adrenoceptors seems likely to be related to Ca^{2+} mobilization from the intracellular stores and/or influx of extracellular Ca^{2+} coupled to changes in phosphoinositide cycle (Fain and Garcia-Sainz 1980; Brown *et al.*, 1985).

Phosphoinositides [phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4*P*), and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2)] constitute 2-3% of the total cardiac SL phospholipids (Panagia *et al.*, 1981). In the SL membrane about 10% of the PtdIns pool is phosphorylated in two consecutive steps, catalyzed by two kinases (PtdIns kinase and PtdIns4*P* kinase) to yield PtdIns4P and PtdIns(4,5)P_2, respectively. The actions of these kinases are quickly equilibrated by phosphomonoesterases that remove the phosphate groups from the 5- and 4- positions of the inositol ring (Berridge, 1984). The presence of PtdIns4*P* and PtdIns(4,5)P_2 in heart was first shown in 1966 by Gaut and Huggins. Moreover the kinase activities were observed in many different tissues, including brain, liver and skeletal muscle (Rana *et al.*, 1990). However, their occurrence and role in cardiac membranes were neglected until recently when they were studied in dog (Quist *et al.*, 1989; Kasinathan *et al.*, 1989) and rabbit hearts (Wolf, 1990).

Stimulation of SL α_1-adrenoceptor (via agonist binding) activates a phosphoinositide-specific phospholipase C (PL C) (Meij *et al.*, 1989) possibly via a G

protein (Renard *et al.*, 1990; Meij *et al.*, 1991). PL C hydrolyzes PtdIns(4,5)P_2 yielding two intracellular messengers, intra-SL sn-1,2-diacylglycerol (DAG) and cytosolic inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] (Edes *et al.*, 1990; Meij and Panagia, 1992). DAG activates protein kinase C which in turn can phosphorylate a series of proteins. On the other hand, Ins(1,4,5)P_3 releases Ca^{2+} from the SR (Nosek *et al.*, 1986; Gilbert *et al.*, 1991) possibly by binding to an SR Ins(1,4,5)P_3 receptor, thus increasing the intracellular concentration of free Ca^{2+}. Any change in these activities will induce a change in the response of the heart to the α_1-adrenoceptor stimulation.

The α_1-adrenoceptor pathway has been proposed to maintain myocardial responsiveness to catecholamines under pathological conditions in which ß-adrenoceptors are impaired (Bruckner *et al.*, 1985; Osnes *et al.*, 1985; Homcy *et al.*, 1991). Changes in the adrenoceptor densities have been reported in several pathological and clinical conditions such as: congestive heart failure (Bristow *et al.*, 1982, Homcy *et al.*, 1991, Dixon *et al.*, 1991), hypothyroidism (McConnaughey *et al.*, 1979; Kunos *et al.*, 1980; Fox *et al.*, 1985), hypoxia (Heathers *et al.*, 1988; Kagiya *et al.*, 1991) and chronic treatment with ß-adrenergic antagonists (Mugge *et al.*, 1985). The increase in α_1-adrenoceptor density in these conditions was also associated with an enhanced responsiveness to α_1-adrenergic agonists. An increase in the positive inotropic effect of α_1-agonists was reported in the myocardium of hypothyroid animals (Nakashima *et al.*, 1971; 1973; Simpson and McNeil, 1980) and in congestive heart failure (Dixon *et al.*, 1991). However, little is known about the status of the functional activity of the α_1-adrenoceptor-associated signal transduction pathway (via phosphoinositide pathway) under these two conditions.

Given the enhanced relevance of the α-receptors under conditions of impaired ß-adrenoceptor function, we proposed to test the hypothesis that *stimulation of the α_1-receptors and subsequent changes in receptor-associated phosphoinositide pathway may serve as a reserve mechanism to maintain the myocardial responsiveness to catecholamines, and may act as a secondary positive inotropic system in pathophysiological conditions of depressed β-adrenergic activity*. The changes in the α_1-adrenoceptor-associated phosphoinositide pathway were studied in the hypothyroid condition and for comparison, some of the pathway's activities were also studied in a rat model of congestive heart failure following myocardial infarction.

II. REVIEW OF THE LITERATURE

A. Introduction

The sympathetic nervous system is the primary regulator of myocardial function. Sympathetic activation increases the release of endogenous catecholamines, which in turn exert their action by binding to the plasma membrane adrenoceptors. Due to vast difference of the effect of endogenous catecholamines on different tissues, Alquist (1948) proposed the division of these receptors into two general classes, termed α- and ß-adrenoceptors. Extensive studies of adrenoceptor pharmacology led to further division of each receptor family into two types. Lands *et al.* (1967) divided the ß-adrenoceptors into two classes, termed $ß_1$ and $ß_2$, based on the relative potencies to isoproterenol epinephrine, and norepinephrine. The difference in α-adrenoceptor-mediated response to different agonists and antagonists also pointed to the presence of two classes of α-adrenoceptros, termed α_1 and α_2 (Langer, 1974). Recently, with the advent of the DNA cloning and molecular biological techniques, each of α-adrenoceptor class was further subdivided into three subtypes, namely, α_{1A}, α_{1B} and α_{1C} for the α_1 receptor (Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990; Lomasney *et al.*, 1991) and α_{2A}, α_{2B} and α_{2C} for the α_2 receptors (Kobilka *et al.*, 1987; Zeng *et al.*, 1990; Lomasney *et al.*, 1990). In this review the α_1-adrenoceptor, its related second messenger systems and its changes during the different pathologies will be the focus of further discussion.

B. α_1-Adrenoceptor

1. Structure of α_1-adrenoceptor

The α_1-adrenoceptor have been classified as a member of a large family of plasma membrane receptors which includes α_2-, β_1- and β_2-adrenergic receptors, five muscarinic cholinergic receptor subtypes, angiotensin receptor and many others (up to 20 members) (Dohlman *et al.*, 1987; Lefkowitz *et al.*, 1988). These receptors share several structural features including: seven α-helical rods spanning the membrane, an extracellular amino-terminus, three extracellular and three intracellular loops, and an intracellular carboxy-terminus (Lefkowitz *et al.*, 1988). The extracellular amino terminal contain glycosylation sites (Benovic *et al.*, 1987; Repaske *et al.*, 1987; Samutz *et al.*, 1987), while the membrane spanning domain, which shows at least 40-45% sequence identity in different adrenergic receptors, appear to be involved in binding of the ligands (Lefkowitz and Caron, 1990). The third intracellular loop and carboxy-terminal region, which show greater heterogeneity of receptors, especially α-adrenoceptor (Lefkowitz and Caron, 1990), contain potential sites for phosphorylation (Lefkowitz *et al.*, 1988) and are involved in receptor coupling to the G proteins (Strader *et al.*, 1987; Kubo *et al.*, 1988; Cotecchia *et al.*, 1990).

2. Species difference of cardiac α_1-adrenoceptors

α_1-Adrenoceptors are found in almost all mammalian tissues including the myocardium. The first evidence for the existence of α_1-adrenoceptors in the myocardium was presented, in mid 1960s, by measuring in the presence of ß-blockers the α_1-mediated positive inotropic effect of hearts from different species (Wenzel and Su, 1966; Benfey *et al.*, 1967; Govier 1967) including human atrium (Schumann *et al.*, 1978) and ventricle (Bruckner *et al.*, 1984). These functional data were further supported by the demonstration of specific and high affinity binding of a tritiated α-adrenoceptor antagonist to membrane fractions derived from rat (Williams *et al.*, 1978; Guicheny *et al.*, 1978) and rabbit heart (Schummann *et al.*, 1979). More recently, the α-adrenoceptor binding was also demonstrated in isolated cardiomyocytes (Buxton *et al.*, 1986). In addition, using α_1-subtype selective radioligands ([^{3}H]prazosin, and [^{125}I]IBE 2254), the cardiac α-adrenoceptor binding sites were classified as α_1 type (Steinberg *et al.*, 1982; Mukherjiee *et al.*, 1983).

The density of α_1-adrenoceptors varies with species. Rat and rabbit heart showed higher density of α_1-adrenoceptor binding sites when compared to the canine myocardium (Mukherjiee *et al.*, 1983). These differences in α_1-adrenoceptor density were detected under similar circulating level of catecholamines (Mukherjiee *et al.*, 1983). Buxton and Brunton (1986), using [^{3}H]prazosin estimated that the adult rat ventricular cell possesses 13 α_1-receptors/μm^2 (8 X 10^4 α_1-adrenoceptors), while the estimated density of $ß_1$-adrenoceptor was 33 $ß_1$-receptors/μm^2 (Buxton and Brunton, 1985). Furthermore, Endoh

et al. (1991) showed that the ratio of α_1- to β_1-receptor was on average 5 fold larger in rats compared to rabbits. Despite the variation of the density of adrenoceptors in different species, no significant difference was found between the left and the right ventricle of the rat heart (Muntz *et al.*, 1985), while the ventricular tissue possesses higher α_1-adrenoceptor density than the atrium (Steinfath *et al.*, 1992a).

3. α_1-Adrenoceptor subtypes and their existence in the myocardium

Findings from several tissues suggest that there are pharmacologically distinct subtypes of α_1-adrenoceptors (Han *et al.*, 1987a; Minneman, 1988; Hanft *et al.*, 1989) which may activate different mechanisms of signal transduction (Crews *et al.*, 1988; Gross *et al.*, 1988; Minneman, 1988; Tsujimoto *et al.*, 1989). These subtypes, α_{1A} and α_{1B}, have been distinguished on the basis of their sensitivity toward selective antagonists. The α_{1A} shows higher affinity than α_{1B} for the antagonists 5 methyl-urapidil, WB-4101, and (+)-niguldipine or the novel prazosin derivative SZL-49 (Morrow *et al.*, 1985; Han *et al.*, 1987b; Gross *et al.*, 1988). Incontrast, α_{1B} is irreversibly alkylated by chloroethylclonidine (CEC) (Han *et al.*, 1987a; Minneman *et al.*, 1988). Therefore, the distribution of the α_1 subtypes in different tissues and different species could be further demonstrated using these specific antagonists. Recently, Eltze and Boer (1992) have demonstrated the differentiation between α_{1A}- and α_{1B}-adrenoceptors in various rat tissues by using an agonist, SDZ NVI 085, with higher specificity for the α_{1A}-receptor.

Gross *et al.* (1988) showed the presence of only low affinity sites (α_{1B}) for 5-

methyl-urapidil in the membranes from rat liver and spleen, while the other tissues had a combination of high and low affinity binding sites with different ratios. In the rat myocardium 20% of the α_1-adrenoceptors was found to correspond to α_{1A} and the remaining 80% of the binding sites to the α_{1B} subtype (Gross and Hanft, 1988; Gross *et al.*, 1988; Gross and Hanft, 1989). In comparison the membrane fraction derived from rabbit ventricles and pretreated with 10 μM CEC showed a decrease in the B_{max} of [^{3}H]prazosin binding to 37 % of the controls, suggesting that 68% of the α_1-adrenoceptors in rabbit myocardium belongs to the α_{1B}-subtype (Takanashi *et al.*, 1991). The presence of α_{1A} and α_{1B}-receptors was also demonstrated in canine myocardium (del Blazo *et al.*, 1990) by studying the effects of CEC and WB4101 on norepinephrine-induced changes in heart rate. Approximately 25% of the specific α_1-adrenoceptor binding sites labelled by [^{125}I]IBE 2254 were sensitive to CEC (del Blazo *et al.*, 1990).

Recently, with the development of molecular cloning techniques the presence of different α_1-adrenoceptor subtypes detected by pharmacological studies has been confirmed. Lefkowitz's group has isolated cDNAs for three subtypes of α_1-adrenoceptors from different species. α_{1A}-Adrenoceptor cDNA was cloned from rat cerebral cortex (Lomasney *et al.*, 1991), α_{1B}-adrenoceptor cDNA was cloned from hamster (Cotecchia *et al.*, 1988), and the cDNA of the third subtype called α_{1C} was from bovine brain (Schwinn *et al.*, 1990). The newly discovered α_{1C}-receptor subtype appeared to have pharmacological properties which are intermediate between α_{1A} and α_{1B} (Schwinn *et al.*, 1991). The inhibition of α_{1C}-adrenoceptor by CEC is only partial (as opposed to a complete inhibition of α_{1B}); in addition methoxamine, a week agonist for α_{1B} and a

stronger agonist for α_{1A}, activates almost fully the α_{1C}-adrenoceptor subtype. The northern blots of different rat and rabbit tissues (by hybridization of labelled probes from α_{1A}, α_{1B} or α_{1C}) showed that α_{1C} is not present in any of the rat tissues, while it is only present in rabbit liver (Schwinn *et al.*, 1991). Furthermore, α_{1A} and α_{1B} are present in different tissues from rat and rabbit, although, with different densities (Lomasney *et al.*, 1991; Schwinn *et al.*, 1991). Han and Minneman (1991) have reported the persistence of low-affinity sites for niguldipine after CEC pretreatment which are distinct from the α_1 subtypes so far discussed. Perez *et al.* (1991) have cloned yet another α_1 subtype using solution phase library screening and they called the new α_1-adrenoceptor α_{1D}. The existence of many α_1-adrenergic receptor subtypes could perhaps explain the vast responses observed by stimulation of the α_1-adrenoceptor. Although some differences in subtypes could be attributed to species or even tissue differences, only time can resolve the details of α_1-adrenoceptors and their importance.

4. Role of G protein in α_1-adrenoceptor function

Guanine nucleotide binding protein are heterotrimeric ($\alpha, ß, \gamma$) proteins involved in transduction of the stimuli from the receptor to an intracellular effector (Gilman, 1987; Birnbaumer *et al.*, 1990). Upon activation by GDP/GTP-exchange, the α-GTP-complex dissociates from the $ß\gamma$ complex and then interacts with effector proteins. The hydrolysis of GTP by GTPase activity of α subunit results in the inactivation of the α sububit and the formation of the heterotrimer again. The involvement of a G protein in any receptor

action is studied by bacterial toxin-mediated ADP ribosylation of the α subunit or using GTP or its nonhydrolyzable analogues GTP γS and Gpp(NH)P.

The involvement of G protein in the α_1-adrenoceptor signalling pathway was demonstrated by the ability of GTP and its non-hydrolyzable analogues to stimulate the breakdown of phosphoinositides (Cockcroft and Gomperts, 1985; Gonzales and Crews, 1985; Burch *et al.*, 1987; Jones *et al.*, 1987). In addition, GTP has been shown to reduce the agonist binding affinity to cardiac α_1-adrenoceptors (Colucci *et al.*, 1984; Buxton and Brunton 1986; Gross *et al.*, 1988; Han *et al.*, 1989). The reports on the effect of pertussis toxin on the α_1-adrenoceptor activated G protein have been controversial, which is probably due to the presence of more than one type of G protein associated with the different α_1-adrenoceptor subtypes (Nichols and Ruffolo, 1988). Multiple G proteins could be supported by the existence of more than one α_1-receptor subtype and also the different signal transduction pathways which convey the messages from the α_1-adrenoceptors. Nichols *et al.* (1989) demonstrated the presence of both pertussis toxin sensitive and insensitive G proteins associated with the α_1-adrenoceptor in rat vascular system. However, studies on myocardial tissues failed to show the presence of α_1-associated pertussis toxin sensitive G proteins (Bohm *et al.*, 1987; Schmitz *et al.*, 1987; Jones *et al.*, 1988).

Recently, a cholera and pertussis toxin insensitive G protein associated with α_1-adrenoceptor has been purified from rat liver membranes stimulated with (-)-epinephrine (Im and Graham, 1990). The isolated G protein called G_h had an apparent molecular weight of 74 kDa, and copurified with the α_1-adrenergic receptor following ternary

complex formation (Im *et al.*, 1990). G_h appears to differ, by its molecular mass and chromatographic behaviour, from the other pertussis toxin-insensitive G proteins including the G_q family which is described as the regulator for the isozyme $ß_1$ of phospholipase C (Im and Graham, 1990; Im *et al.*, 1990; Blank *et al.*, 1991; Martin *et al.*, 1991; Berstein *et al.*, 1992).

5. Second messenger pathways of the α_1-adrenergic receptors

It is clear that many of the cellular effects of α_1-adrenoceptor stimulation are caused by an increase in the cytosolic free Ca^{2+}. Such an increase could be caused by release of Ca^{2+} from intracellular organelles and/or influx from the extracellular space. To date the involvement of several mechanisms have been demonstrated in the α_1-adrenoceptor-mediated response. These mechanisms may involve Ins(1,4,5)P_3 (Berridge and Irvine, 1989), diacylglycerol (Bogoyevitch *et al.*, 1993), phospholipase A_2 (Burch *et al.*, 1986), or a direct regulation of some ion channels (Fedida *et al.*, 1990) or antiporter such as Na^+/H^+ exchanger (Wallert and Frohlich, 1992). The α_1-adrenoceptor agonist dose not affect the basal levels of cAMP and cGMP (Osnes and Oye, 1975; Brodde *et al.*, 1978). However, cAMP levels were reported to decrease when the α_1-adrenoceptor activation was preceded by a ß-adrenoceptor stimulation (Watanabe *et al.*, 1977; Buxton and Brunton 1985), an effect which was attributed to the stimulation of the cAMP-phosphodiesterase activity by the α_1-adrenoceptor.

One or a combination of the above mechanisms could lead to α_1-adrenoceptor

mediated action such as: changes in cardiac inotropy and chronotropy (Shibata *et al.*, 1980; Benfey, 1987; Endoh, 1986; 1991), atrial natriuretic peptide secretion (Ruskoaho *et al.*, 1985; Matsubara *et al.*, 1988), arrhythmogenity (Corr *et al.*, 1989) and cellular hypertrophy (Karliner *et al.*, 1990). However, whether the α_1-adrenoceptor mediated hypertrophy is mediated via the above mechanisms or another pathway, involving the activation of proto-oncogenes, is still under investigation (Simpson *et al.*, 1986). Mechanisms related to the α_1-adrenoceptor response are discussed individually in the following sections.

a. Phosphoinositide pathway

i. Inositol phosphates

α_1-Adrenergic receptor activation increases inositol phospholipid turnover in almost every tissue studied so far (for review see Minneman, 1988; Ruffolo *et al.*, 1991). In 1966 the first evidence for the involvement of phosphoinositide metabolism in the adrenergic system was provided by a norepinephrine induced increase in ^{32}P-incorporation in the phosphoinositide fraction of cardiac phospholipids after *in vivo* Na^+-orthophosphate administration (Gaut and Huggins, 1966). Later, Brown *et al.* (1985) for the first time demonstrated the epinephrine-induced (rapid and prolonged) increase in the [^{3}H]inositol monophosphate (Ins*P*) formation in rat ventricular cardiomyocytes. This effect was maximal with 30μM norepinephrine (EC_{50} of 1 μM) and was antagonized by prazosin. Subsequently, this observation was generalized for the myocardial tissue of

different species as embryonic chick heart cells (Brown and Jones, 1986), perfused rat hearts (Woodcock *et al.*, 1987), rat ventricles (Poggioli *et al.*, 1986), cultured rat myocardial cells (Steinberg *et al.*, 1987), rat papillary muscles (Otani *et al.*, 1988), rat atria (Scholz *et al.*, 1988; Kohl *et al.*, 1990), canine cardiomyocytes (Heathers *et al.*, 1989) and right ventricular slices from rat, rabbit and dog hearts (Endoh *et al.*, 1991). Endoh *et al.* (1991) showed a differential accumulation of [^{3}H]-inositol monophosphate (Ins*P*) in different species, which they attributed to diverse α_1-adrenoceptor density. However, these authors' observation that in dog ventricular slices [^{3}H]Ins*P* was not accumulated by epinephrine, once again suggests the importance of the α_1-subtypes and their different post-receptor pathways in inducing the physiological response to adrenergic agonists. The existence of multiple subtypes of the α_1-adrenoceptors has been clearly demonstrated (See above, section 3). The physiological responses, second messengers, and coupling mechanism activated by binding of the agonists to different subtypes of the α_1-receptor have been the focus of attention of many laboratories. The discovery of specific antagonists sheds some light on the second messenger pathway involved with the different α_1-subtype. Michel *et al.* (1990) have demonstrated that blocking the α_{1A} subtype in the rat cerebral cortex, by 5-methylurapidil, did not affect the noradrenaline-stimulation of the Ins(1,4,5)P_3 generation, whereas chloroethylclonidine (α_{1B} specific antagonist) completely blocked this response. This observation was also supported in other tissues including rat liver, vas deferens, spleen and myocardium (Minneman, 1988; Hanft and Gross, 1989; Terman *et al.*, 1990). Thus the conclusion that the α_{1B} adrenoceptor subtype utilizes the phosphoinositide pathway for production of intracellular signal, while

the α_{1A}-subtype will result in the increase in intracellular Ca^{2+} via the activation of a membrane ionic channel.

It is now accepted that agonist binding to α_1-adrenoceptors stimulates a cell membrane-associated PL C which hydrolyzes intra-membranal phosphatidylinositol (4,5)bisphosphate (PtdIns(4,5)P_2) with the production of cytosolic inositol (1,4,5)-trisphosphate (Ins(1,4,5)P_3). Poggioli *et al.* (1986) have shown that the α_1-adrenoceptor stimulation is associated with a decrease in PtdIns(4,5)P_2 and a concomitant increase in Ins(1,4,5)P_3, which reaches maximum levels at 30s. This was further supported by inhibiting the phenylephrine-induced [^{3}H]-inositol phosphates formation with neomycin (an antibiotic which specifically binds to PtdIns(4,5)P_2 to prevent its further metabolism) (Otani *et al.*, 1988). To resolve the individual isomers of inositol phosphates formed following α_1-adrenoceptor occupation, Steinberg *et al.* (1989) used a high-pressure liquid chromatography (HPLC) method and demonstrated that in cultured rat ventricular myocytes norepinephrine resulted in rapid and transient increase in the Ins(1,4,5)P_3 levels followed by a slower sustained Ins(1,3,4)P_3, inositol (1,4)bisphosphate (Ins(1,4)P_2) and Ins$P$ formation. However, in their procedure they did not measure the inositol (1,3,4,5)tetrakisphosphate [Ins(1,3,4,5)P_4]. This is the product of Ins(1,4,5)P_3 phosphorylation (Kohl *et al.*, 1990), and its dephosphorylation would result in the formation of Ins(1,3,4)P_3.

ii. Diacylglycerol

In addition to Ins(1,4,5)P_3, the α_1-adrenoceptor mediated PL C activation yields

yet another second messenger, the intramembranal *sn*,1-2diacylglycerol (DAG) (Majerus *et al.*, 1986; Nishizuka, 1992). The content of DAG in the rat ventricular tissue following stimulation with norepinephrine showed a significant increase over 10-60 min time, with a maximal response of 80% increase over controls at 60 min (Okumura *et al.*, 1988). Furthermore, this increase was blocked by prazosin and phentolamine but not by propranolol. DAG can activate protein kinase C (PKC) (Berridge, 1984; Nishizuka, 1984) which results in translocation of this enzyme from the cytosol to the sarcolemma (Henrich and Simpson, 1988; Mochly-Rosen *et al.*, 1990; Otani *et al.*, 1992; Talosi and Kranias 1992). So far ten subtypes of PK C have been identified in different mammalian tissues (Nishizuka 1992; Azzi *et al.*, 1992), three of which (δ-, ϵ- and Σ-isozymes of PK C) have been detected in the myocardial tissue (Ono *et al.*, 1988). Immunofluorescence technique resolved the translocation of a specific isozymes of PKC to specific sites inside the cell (membrane, myofilaments and nucleus) (Mochly-Rosen *et al.*, 1990). Moreover, the α_1-adrenoceptor agonist, epinephrine, induced ϵ-isozyme of PKC to translocate to the sarcolemma of both neonatal and adult cardiomyocytes (Bogoyevitch *et al.*, 1993). It has been shown that one of the fatty acyl chains of *sn*-1,2-DAG must be unsaturated for optimal activation of PK C (Nishizuka, 1988; Bell and Burns, 1991; Allen and Katz, 1991).

Activation of PKC results in the phosphorylation of a series of cellular proteins with various physiological responses including metabolic changes, secretion, contraction, proliferation and differentiation (Nishizuka, 1988). Phorbol ester-mediated PK C activation in the myocardium inhibits the activation of phosphoinositide metabolism

(Cotecchia *et al.*, 1985; Meij and Lamers, 1989), Ca^{2+} efflux (Colucci *et al.*, 1984) and a decrease in the α_1-adrenoceptor agonist affinity (Cotecchia *et al.*, 1985; Meij *et al.*, 1991b). In other reports, PK C activation did not change the myofilament sensitivity to Ca^{2+} (Capogrossi *et al.*, 1990). However, a decrease in diastolic and resting Ca^{2+} was observed (Capogrossi *et al.*, 1990; Uglesity *et al.*, 1987).

b. Phospholipase A_2

In numerous tissues, α_1-adrenoceptor stimulation has been reported to activate phospholipase A_2 (PL A_2) (Slivka and Insel, 1988; Weiss and Insel, 1991; for review see Axelrod *et al.*, 1988; Insel *et al.*, 1991). The activation of PL A_2 releases the fatty acid from the *sn*-2 position of the membrane phospholipids to form lysophospholipid. One of the major fatty acid released by PL A_2 activation is arachidonic acid (Smith, 1989). Arachidonic acid can activate PK C in a manner different from DAG (Khan *et al.*, 1992; for review see Bell and Burns, 1991), and can be further metabolized to produce prostaglandins, epoxides and leukotrienes. The involvement of arachidonic acid and its metabolites in the α_1-adrenoceptor mediated effect has been reported in myocardial tissues (Molderings and Schumann, 1987; Kurachi *et al.*, 1989; 1992). In addition, the fatty acid released by PL A_2 can stimulate yet another phospholipase known as phospholipase D (PL D). Fatty acid-induced stimulation of PL D has been recently demonstrated in sarcolemma and sarcoplasmic reticulum of rat myocardium (Dai *et al.*, 1993). The stimulation of PL D by activated PK C has also been reported in different tissues (Martinson *et al.*, 1990; Conricode *et al.*, 1992). Data are now available to

support the receptor-mediated PL D stimulation and one of these receptors may be the α_1-adrenoceptor (for review see Thompson *et al.*, 1991).

6. Consequences of α_1-adrenoceptor stimulation

a. Positive inotropic effect of α_1-adrenoceptor stimulation

Catecholamine-mediated positive inotropic effect have long been attributed to the stimulation of ß-adrenoceptors (Alquist, 1948; Moran 1963; Furchgott, 1970; Nickerson , 1973). However, the heart inotropic response to α_1 agonist in the presence of a ß-adrenoceptor antagonist has demonstrated the α_1-adrenoceptor role in cardiac positive inotropism. Wenzel and Su (1966) were the first to report a positive inotropic effect of phenylephrine (α_1-adrenoceptor agonist) in rat ventricular stripes. This observation was also reported in the rabbit (Benfey and Varma, 1967) and guinea pig atria (Govier, 1967). Thereafter, the α_1-adrenoceptor mediated positive inotropy has been reported in different myocardial preparations from almost all the species studied so far (Wagner and Brodde, 1978; Shibata *et al.*, 1980; Skomedal *et al.*, 1982; Terzic and Vogel, 1991; Fedida and Bouchard, 1992; Gambassi *et al.*, 1992) including human atrial and ventricular tissue (Schumann *et al.*, 1978; Bruckner *et al.*, 1984). In rat cardiac tissue Skomedal *et al.* (1988, 1990) have estimated that about 75% of the inotropic response is mediated through ß-adrenoceptor stimulation with the remaining 25% of the response being via α_1-adrenoceptor. The α_1-adrenoceptor-mediated positive inotropy was competitively inhibited by prazosin (Skomedal *et al.*, 1980). The α_1-adrenoceptor mediated response

in cardiac preparations exhibits a complex (biphasic, triphasic) time-course including a negative and a positive inotropic component (Govier, 1967; Skomedal *et al.*, 1982; Osnes *et al.*, 1985; Toshe *et al.*, 1987; Otani *et al.*, 1988; Ertl *et al.*, 1991). Otani *et al.*, (1988) observed a triphasic inotropic response in the rat papillary muscle following α_1-adrenoceptor stimulation. However, the exact molecular mechanism(s) involved in production of these responses are not yet clear. The involvement of Ins(1,4,5)P_3, PK C, Ca^{2+} channels and ion transporters have been suggested and all are currently under investigation.

The positive inotropic effect following the activation of α_1-adrenoceptors varies in magnitude from one species to another. While rat and rabbit myocardium have shown large increases in the developed force (Scholz *et al.*, 1986), dog and guinea pig myocardium showed smaller responses (Benfey, 1980; Hescheler *et al.*, 1988). This species difference could be attributed to either the differential densities of α_1-adrenoceptor (Mukerjee *et al.*, 1983; Endoh *et al.*, 1991; Steinfath *et al.*, 1992a), or the distribution of the α_1-adrenoceptor subtypes in these species. Blocking the α_{1B}-adrenoceptor subtype by alkylating agent CEC inhibited the positive inotropic response of the rabbit papillary muscle to α_1-adrenoceptor agonist (Takanashi *et al.*, 1991). In addition, WB-4101 (the α_{1A}-antagonist) was also able to inhibit, although to a much smaller extent, the phenylephrine-induced positive inotropic response (Endoh *et al.*, 1992). In rat papillary muscle (Rokosh and Sulakhe, 1991) and isolated cells (Gambassi *et al.*, 1991) the α_1-adrenoceptor-mediated positive inotropic effect was inhibited by SZL-49 and WB-4101, while CEC failed to induce any change. In contrast to these

studies Hanft and Gross (1989) have demonstrated that in rat right ventricles 23% of the [^{3}H]-prazosin binding sites had high affinity for 5-methyl-urapidil compared to 58% in vas deferens (an observation in agreement with the affinities for WB-4101 and phentolamine). In addition, these authors showed a greater depression in the α_1-adrenoceptor-mediated response in vas deferens compared to that in myocardium. Recently Endoh *et al.* (1992) have shown a time and concentration dependent [^{3}H]Ins*P* accumulation following stimulation by epinephrine in both rat and rabbit ventricular slices, a response which was parallel with the increase in contractile force. Furthermore, the positive inotropy in rat heart was not affected by the Ca^{2+} channel blocker nitrendipine (Hanft and Gross, 1989). As discussed below, α_1-adrenoceptor stimulation also can cause prolongation of action potential in different species including rabbit (Endoh *et al.*, 1992) and dog (Rosen *et al.*, 1977; Priori and Corr, 1990).

The α_1-adrenoceptor stimulation-induced increases in intracellular Ca^{2+} concentration and thus the positive inotropy, are dependent on both the release of Ca^{2+} from intracellular stores and/or the influx of Ca^{2+} from extracellular space. The Ins(1,4,5)P_3 produced by α_1-adrenoceptor stimulation is the major stimuli for Ca^{2+} release from sarco(endo)plasmic reticulum (Putney, 1987). However, the increase in Ca^{2+} influx might occur as a result of direct activation of plasma membrane (sarcolemma) associated Ca^{2+} channels (Brown and Birnbaumer, 1988; Meldolesi and Pozzan, 1987) or via membrane antiporters (Gilbert *et al.*, 1991). Direct measurement using whole-cell path clamp technique showed no increase in L-type Ca^{2+} channel under α_1-adrenoceptor stimulation of rabbit, guinea pig (Hescheler *et al.*, 1988), feline

(Hartmann *et al.*, 1988) or rat ventricular or atrial cells (Apkon and Nerbonne, 1988; Ertle *et al.*, 1991; Jahnel *et al.*, 1992; Fedida and Bouchard, 1992). On the other hand, reports obtained from frog ventricular cells (Alvarez *et al.*, 1987), canine ventricular and Purkinje cells (Tseng and Boyden, 1989) and neonatal rat ventricular myocytes (Liu *et al.*, 1992) showed an increase in T-type Ca^{2+} current after α_1-adrenoceptor stimulation. However, the T-type Ca^{2+} current have been recently shown to be transiently increased by increasing the intracellular Ca^{2+} concentration in canine Purkinje cells (Tseng and Boyden, 1991). Therefore, it is apparent that α_1-adrenoceptor agonist has a small direct effect on the Ca^{2+} current.

b. Effect of α_1-adrenoceptor stimulation on myocardial action potential duration

Prolongation of action potential is one of the responses which have been observed in isolated ventricular myocytes after catecholamine or synthetic α_1-adrenoceptor agonists (Apkon and Nerborne, 1988; Fedida *et al.*, 1989; Ravens *et al.*, 1989; Vogel and Terzic, 1989). However, once again this response showed species difference. The α_1-adrenoceptor stimulation of myocardial preparations from sheep and dog Purkinje fibres (Giotti *et al.*, 1973; Rosen *et al.*, 1977), rabbit atria and ventricles (Miura and Inui, 1984; Fedida *et al.*, 1989), dog ventricular myocytes (Priori and Corr, 1990) and bovine ventricles (Bruckner *et al.*, 1984) showed an increase in action potential duration, while the action potential duration of guinea pig ventricles was either not affected (Ledda *et al.*, 1980; Hescheler *et al.*, 1988) or slightly depressed. In the rat, which is very sensitive to α_1-stimulation, the prolongation of action potential was more pronounced in the atria

as compared to the ventricles (Ertle *et al.*, 1991). Furthermore, α_1 agonist-induced positive chronotropic effect [pithed rat heart (Flavahan and McGarth, 1981) and isolated atria (Tung *et al.*, 1985)] or no effect (Wagner and Reinhardt, 1974) have been reported. The prolongation of action potential could be achieved by either an increase in the inward Ca^{2+} current or the inhibition of repolarization of the membrane potential achieved by inhibition of the outward movement of K^+ (Fedida *et al.*, 1990). As described above the Ca^{2+} current is not altered significantly by stimulation of α_1-adrenoceptors.

In cardiomyocytes there are different K^+ currents, a transient outward current (I_{to}), two inward rectifying K^+ currents [background current (I_{k1}) and a muscarinic-activated ($I_{k\ Ach}$)] flux have been identified, some of which are affected by α_1-adrenergic receptors. α_1-Adrenoceptor agonists decrease these K^+ currents in cardiomyocytes from rat (Apkon and Nerbonne, 1988; Raven *et al.*, 1989; Toshe *et al.*, 1990; Ertle *et al.*, 1991; Fedida and Bouchard, 1992) and rabbit hearts (Fedida *et al.*, 1989; 1990). Fedida *et al.* (1989) suggesting that the decrease in I_{to} could explain the α_1-adrenoceptor induced increase in action potential duration in the rabbit myocytes. Inositol phosphates, PK C and a pertussis sensitive G-protein appeared not to be involved in transducing the α_1-adrenoceptor-mediated inhibition of I_{to} (Braun *et al.*, 1990; Toshe *et al.*, 1990). Furthermore, stimulation of both α_1-adrenoceptor subtypes, α_{1A} and α_{1B}, was found to contribute to the phenylephrine-induced reduction of I_{to} in isolated rat myocytes (Wang *et al.*, 1991). The difference between changes in action potential duration of guinea pig ventricular myocytes and other species under α_1-adrenoceptor stimulation was attributed to the absence of I_{to} channels in guinea pig ventricular myocytes (Toshe *et al.*, 1992).

After blocking the I_{to} by 4-aminopyridine, α_1-adrenoceptor agonists decreased I_{k1} in rabbit ventricular myocytes (Fedida *et al.*, 1991) and $I_{k\ Ach}$ mammalian atrial myocytes (Braun *et al.*, 1992), an effect which was insensitive to pertussis toxin and did not involve the activation of PK C (Fedida *et al.*, 1991; Braun *et al.*, 1992). Furthermore, α_1-adrenoceptor agonists reduced I_k in rat cardiomyocytes (Ravens *et al.*, 1989; Toshe *et al.*, 1990; Jahnel *et al.*, 1991) and Purkinje fibres (Shah *et al.*, 1988). In guinea pig ventricular myocytes, however, phenylephrine (10-30 μM) increased the I_k (the slowly activating currentI_{ks}), an effect which was reproduced by PK C activators and blocked by PK C inhibitors (Toshe *et al.*, 1987, 1992). Furthermore, $I_{k\ Ach}$ was activated by α_1-adrenoceptor stimulation in guinea pig atria (Kurachi *et al.*, 1989). This response was prevented by lipoxygenase inhibitors (nordihyroguaiaretic acid and AA 861) but was not affected by the cycloxygenase inhibitor, indomethacin (for review see Kurachi *et al.*, 1992).

c. Effect of α_1-adrenoceptor on sarcolemmal ion exchangers

α_1-Adrenoceptor agonists also produce an intracellular alkalinization in different myocardial preparations including perfused hearts (Fullre *et al.*, 1991), atria (Terzic *et al.*, 1991), isolated ventricular myocytes (Astarie *et al.*, 1991; Gambassi *et al.*, 1992; Puceat *et al.*, 1993) and Purkinje fibres (Breen and Pressler , 1988). Prazosin abolished this alkalinization (Terzic *et al.*, 1992; Wallert and Fohlich, 1992). The α_1-adrenoceptor mediated alkalinization was blocked by selective inhibitors of Na^+/H^+ exchange (Iwakura *et al.*, 1990; Terzic *et al.*, 1991; 1992; Gambassi *et al.*, 1992), and replacement

of extracellular Na^+ with *N*-methylglucamine (Wallert and Frolich, 1992). Furthermore, α_1-adrenoceptor agonists enhanced intracellular pH recovery from acidosis under conditions were the recovery primarily depends on Na^+/H^+ exchange (Terzic *et al.*, 1992; Puceat *et al.*, 1993). The stimulation of Na^+/H^+ exchange by phorbol ester (Frelin *et al.*, 1988) and blocking of α_1-adrenoceptor mediated alkalinization by PK C inhibitor (Sharma and Sheu, 1987; Breen and Pressler, 1988; Iwakura *et al.*, 1990) suggest the role of PK C in regulation of cardiac Na^+/H^+ exchanger. Other reports have shown the inhibition of α_1-mediated alkalinization by blocking the Ca^{2+}-calmodulin-dependent protein kinase using W7 (Iwakure *et al.*, 1990; Wallert and Frohlich, 1992). However, Puceat *et al.*, (1993) failed to confirm this results in rat ventricular myocytes. Thus the mechanism for regulation of Na^+/H^+ exchange by α_1-adrenoceptor is not yet clear.

In addition to the alkalinization, the activation of Na^+/H^+ antiporter by α_1-agonists should result in an increase of the intracellular Na^+ concentration. However, this effect may not be observed due to concomitant increase of the Na^+/K^+ pump activity under α_1-adrenoceptor stimulation (Zaza *et al.*, 1990; Wilde and Kleber, 1991). Indeed, Terzic *et al.* (1991) showed an increase in intracellular Na^+ concentration when the α_1-adrenoceptor agonist was added in the presence of ouabain, an inhibitor of Na^+/K^+ pump. The increase in intracellular Na^+ can increase the Ca^{2+} concentration via the Na^+/Ca^{2+} exchanger (Mullins, 1979). The role of the Na^+/Ca^{2+} exchanger in increasing the intracellular concentration of Ca^{2+} was suggested by Iwakura *et al.* (1990) and Jahnel *et al.* (1991, 1992) based on their observation that α_1-adrenoceptor agonist

increased diastolic intracellular Ca^{2+} in quiescent and electrically stimulated rat heart cells. In addition, Pierce and Panagia (1989) have demonstrated increase in the rat cardiac sarcolemmal Na^{+}/Ca^{2+} exchanger after hydrolysis of membrane-associated phosphatidylinositol. These authors suggested that phosphatidylinositol may be an inhibitor of sarcolemmal Na^{+}/Ca^{2+} exchanger or that this phospholipid may anchor an inhibitory protein to the membrane. Further studies are essential in order to draw any conclusion on the effect of α_1-adrenoceptor on the sarcolemmal Na^{+}/Ca^{2+} exchanger.

C. *Phosphoinositide Pathway*

Although phosphoinositides comprise less than 10% of the total cellular phospholipids, their metabolism have attracted the attention of many investigators in the last decade. To date, the hydrolysis of the phosphoinositides has been linked to the stimulation of various receptors such as α_1-adrenergic (Brown *et al.*, 1985; Endoh *et al.*, 1991), muscarinic (Brown *et al.*, 1985), angiotensin II (Baker and Singer, 1988), and endothelin receptors (Vigne *et al.*, 1989), thus inducing many different cellular responses.

The occurrence of inositol as a constituent of membrane phospholipids was first reported by Anderson and Roberts (1930) in avian tubercle bacillus; a decade later Folch and Wooley (1942) described a brain phospholipid containing inositol which they later called phosphoinositide. Phosphoinositides are synthesized in the cell through several enzymatic reactions. The living cell can obtain its supply of *myo*-inositol from different sources: uptake from the medium, *de novo* synthesis and/or recycling from degraded

inositol-containing compounds. The cellular *myo*-inositol is transferred by a *myo*-inositol 3-phosphatidyltransferase to a CDP-diacylglycerol to synthesize phosphatidylinositol (PtdIns). This takes place in the endo(sarco)plasmic reticulum as described for brain (Venuti *et al.*, 1988), liver (Paulus *et al.*, 1960; Brophy *et al.*, 1978) and heart (Wolf, 1990). Quantitatively, PtdIns is the major membranal phosphoinositide and it is involved in different pathways. Glycosylated PtdIns are important in anchoring many membrane associated proteins (Low, 1989) and can be hydrolysed by an internal phospholipase (C or D) (Low and Prasad, 1988), resulting in a soluble glycosyl-inositolphosphate which is proposed to play a second messenger role (Low and Saltiel, 1988). PtdIns also serves as a precursor for the synthesis of polyphosphoinositides which are involved in signal transduction.

The newly synthesized PtdIns is transferred from the site of production to that of phosphorylation via a PtdIns transfer protein (Schermoly *et al.*, 1983; Helmkamp, 1985; Wolf, 1990). The PtdIns, then, undergoes sequential phosphorylation (FIGURE 1) to produce phosphatidylinositol 4-phosphate (PtdIns4*P*) and PtdIns(4,5)P_2, regulated by two membrane-bound enzymes, PtdIns 4-kinase and PtdIns4*P* 5-Kinase, respectively (Quist *et al.*, 1989; Wolf, 1990). Enzyme-catalyzed equilibria are established rapidly because the phosphorylation reactions can be reversed by phosphomonoesterases that remove the phosphate groups from the 5- and 4- position (Berridge, 1984). Recently, another PtdIns kinase has been described which phosphorylates this compound at the D-3 position of the inositol ring (Whitman *et al.*, 1988; Auger *et al.*, 1989) producing a new series of D-3 polyphosphoinositides. The most investigated enzyme of the phosphoinositide pathway

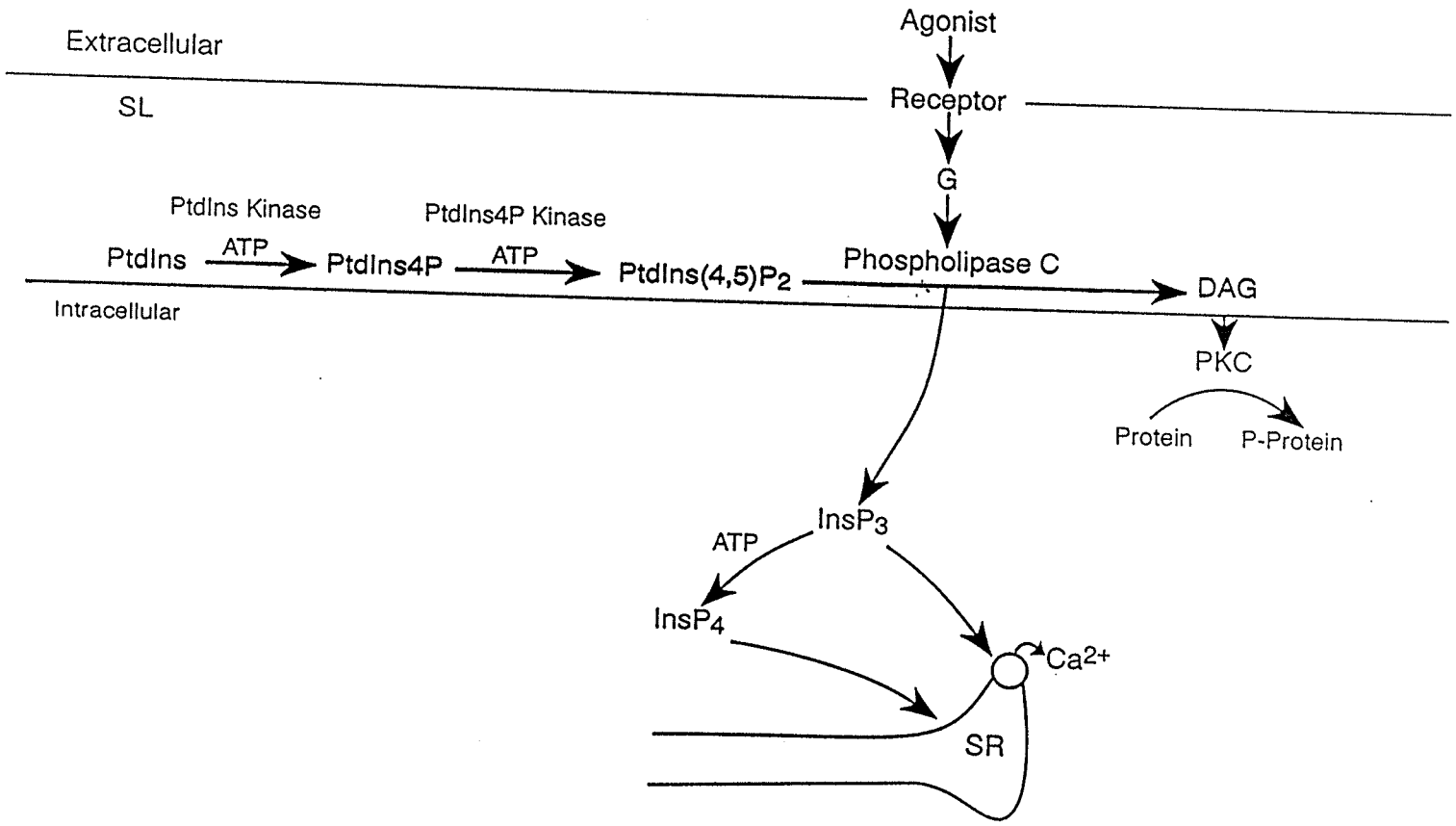

FIGURE 1. Schematic representation of the phosphoinositide pathway.

is PL C, the activity of which results in the formation of two intracellular second messengers, diacylglycerol (DAG) and Ins(1,4,5)P_3. PL C is a cell membrane-associated protein, that is activated upon binding of the agonist to the receptor via G protein. The resulting soluble Ins(1,4,5)P_3 would either be phosphorylated to form multiple forms of highly phosphorylated inositol phosphates or be dephosphorylated to form *myo*-inositol which is recycled back to PtdIns as mentioned above. This turnover of the inositol phospholipids in the cell, known as phosphoinositide cycle, was first described by Hokin and Hokin (1953) in pigeon pancreas slices. They reported an increase in the ^{32}P incorporation into the phospholipids following acetylcholine or carbachol administration (Hokin and Hokin 1954). The phosphoinositide cycle gained more attention after the discovery of protein kinase C (Inoue *et al.*, 1977), and the increasingly important role of Ins(1,4,5)P_3 in mobilizing the intracellular Ca^{2+} stores.

1. Phosphoinositide kinases

Polyphosphoinositides constitute a small percentage (less than 10 %) of the total membrane phosphoinositides. Recently, with the explosion in the number of investigations on the phosphoinositide metabolism field, the simplistic view to this pathway has been changed to a more complex perspective. The occurrence of diverse inositol lipids points to the existence of multiple forms of phosphoinositide kinases as will be discussed below.

a. PtdIns 4-Kinase

PtdIns 4-kinase phosphorylates PtdIns at the fourth position on the inositol ring yielding PtdIns4*P*. Harwood and Hawthorne (1969) were the first to show the existence of two types of PtdIns 4-kinases in the liver plasma membranes and endoplasmic reticulum and called them type 1 and type 2. However, recently Whitman *et al.* (1988) have shown that type 1 PtdIns kinase phosphorylates the D-3 position. Endemann *et al.* (1987) were the first to separate two types of PtdIns 4-kinases from the bovine brain. These two enzymes have apparent sizes of 55 and 230 kDa. The 55 kDa PtdIns 4-kinase is an integral membrane protein, is activated by detergents, has a K_m for ATP of 20-70 μM, and is inhibited by adenosine (Endemann *et al.*, 1987; Whitman *et al.*, 1987; Hou *et al.*, 1988). This type of PtdIns 4-kinase has also been characterized from plasma membranes of human red cell (Endemann *et al.*, 1987) and porcine liver (Hou *et al.*, 1988). The subunit molecular mass of the 55 kDa purified enzyme is similar to that estimated by sucrose density gradient in cholate, which indicates that the protein exist in a monomer form and binds a relatively small amounts of cholate.

The second PtdIns 4-kinase is also a membrane associated protein, and seems to have different molecular weights in the various tissues studied. Generally, the reported molecular weights range from 80 to 230 kDa, being 80 kDa in rat brain (Yamakawa *et al.*, 1988), 200 kDa in bovine uterus (Li *et al.*, 1989), and 230 kDa in bovine brain (Endemann *et al.*, 1987). The activity of this enzyme is stimulated by detergents (Endemann *et al.*, 1987) and is insensitive to adenosine inhibition. The apparent K_m of this enzyme for ATP has been reported to be in the range of 150-750 μM. This enzyme

is different from the 55 kDa enzyme since the monoclonal antibody which causes inhibition of the 55 kDa enzyme has no effect on the 80-230 kDa subtype (Carpenter *et al.*, 1990). Additional reports describe other isoforms of PtdIns 4-kinase, as the 45 kDa membrane-bound enzyme purified from bovine myelin which also can phosphorylate PtdIns4*P* (Saltiel *et al.*, 1987). A PtdIns 4-kinase was also purified from *Saccharomyces cerevisiae* (Belunis *et al.*, 1988) and was activated by non-ionic detergent.

Almost all the PtdIns 4-kinases are membrane-bound enzymes, however, a soluble PtdIns 4-kinase was observed in *S. cerevisiae* (Auger *et al.*, 1989). The majority of this enzyme activity has been reported to be associated to the plasma membrane (Seyfred *et al.*, 1984). In hepatocytes, this activity was also found at the intracellular membrane level (Seyfred *et al.*, 1984). Intracellular PtdIns 4-kinase showed the highest activity in endoplasmic reticulum and Golgi apparatus (Jergil *et al.*, 1983; Lundberg *et al.*, 1988), while some activity was also reported in lysosomes (Seyfred *et al.*, 1984) and nuclear membrane from rat liver (Smith and Wells, 1983).

There is little conclusive evidence for the regulation of the PtdIns 4-kinase activity. Most of the studies with GTP or GTP γ S have concluded that the effect of these compounds on the kinase is via a sparing action on the nonspecific hydrolysis of ATP. Some studies show regulation of this enzyme by cAMP (Holland *et al.*, 1988; Kaibuchi *et al.*, 1986; Kato *et al.*, 1989), phorbol esters, DAG and Ca^{2+} ionophore (Boon *et al.*, 1985; de Chaffoy de Courcelles *et al.*, 1984; Halenda *et al.*, 1984). Walker and Pike (1987) have found an increase in the PtdIns kinase activity of membrane isolated from A431 cells after treatment with EGF. However, a more complete understanding of roles

and cellular regulation of PtdIns 4-kinase will be gained by the use of specific antisera and molecular biology technologies.

b. PtdIns4P 5-Kinase

PtdIns4P 5-kinase activity yields PtdIns(4,5)P_2 which serves as the substrate of PL C. PtdIns4P 5-kinase activity was found in both the cytosolic and membrane fractions from rat brain (Van Dongen *et al.*, 1984; Cochet *et al.*, 1986). This enzyme can be released from the membrane by high salt extraction (Ling *et al.*, 1989). It has been purified from human red cells with an apparent molecular mass of 150 kDa by gel filtration and 53 kDa by SDS-PAGE (Ling *et al.*, 1989). PtdIns4P 5-kinase has a K_m for ATP of 2 μM and is inhibited by its substrate (Downes and MacPhee, 1990). This enzyme showed similarities to the type 2 PtdIns4P 5-kinase isolated by Bazenet *et al.* (1990) from red cell membrane. The other PtdIns4P 5-kinase isolated by Bazenet *et al.* (1990) was called type 1 and using the inhibitory antibodies it has been shown to be responsible for most of PtdIns(4,5)P_2 synthesis in the red cells.

The soluble form of PtdIns4P 5-kinase was purified from rat brain with a molecular mass of 100-110 KDA by gel filtration (Cochet *et al.*, 1986). This preparation showed a molecular mass of 45 kDa on SDS-PAGE. A PtdIns4P kinase, with a molecular mass of 110 kDa (by SDS-PAGE), has also been purified from bovine brain membranes (Moritz *et al.*, 1990); however, the product of this enzyme has not been analyzed. The activity of the membrane-bound PtdIns4P 5-kinase has been located mostly in the plasma membrane (Jergil *et al.*, 1983; Seyfred *et al.*, 1984). PtdIns4P kinase activity has been

also reported in nuclei (Smith and Wells, 1983; Walker *et al.*, 1987; Cocco *et al.*, 1988). The functional importance of the nuclear and cytosolic PtdIns4P kinase needs to be established.

The regulation of PtdIns4P 5-kinase is poorly understood. Some reports support the receptor mediated stimulation of this enzyme (Imai *et al.*, 1986; Renard *et al.*, 1987). In other reports GTP γS has been shown to increase the activity of this kinase in rat brain (Smith and Chang, 1989) and placental membranes (Urumow *et al.*, 1986). However, these results should be considered with caution since other reports have shown the GTP S effect in rat liver plasma membranes could be accounted for by an inhibition of ATP hydrolysis (Benistant *et al.*, 1990). The synthesis of PtdIns(4,5)P_2 has been reported to be increased by phorbol esters, DAG, concanavalin and A23187 (Boon *et al.*, 1985; de Chaffoy de Courcelles *et al.*, 1984; Halenda *et al.*, 1984).

c. PtdIns 3-Kinase

The discovery of the existence of different polyphosphoinositides phosphorylated at the third position of the *myo*-inositol ring (Whitman *et al.*, 1988; Auger *et al.*, 1989b) has drawn the attention to the possible involvement of a D3-kinase in transduction of the signal for different type of receptors. Indeed two products of PtdIns 3-kinase [PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3] were shown to be increased in response to the activation of a variety of cellular tyrosine kinases, e.g. by growth factors and insulin (Ruderman *et al.*, 1990; Endermann *et al.*, 1990). Recently PtdIns 3-kinase activity has also been reported in human platelets (Yamamoto *et al.*, 1990). PtdIns3*P* has been found

in many cell types but it accounts for approximately 2-5% of the total polyphosphoinositide pool except in the yeast *Saccharomyces cerevisiae* which contains equal proportions of PtdIn3*P* and PtdIns4*P*. The other 3-P-containing polyphosphoinositides are present in a very minute amount (0.005% of total inositol lipids) in a typical cell (Stephens *et al.*, 1991; Downes *et al.*, 1991).

The PtdIns 3-kinase was originally isolated as type 1 PtdIns kinase by Whitman *et al.* (1987), and later was identified as a 3-kinase rather than 4-kinase (Whitman *et al.*, 1988). This enzyme was characterized as an adenosine-resistant and detergent inhibitable kinase (Whitmann *et al.*, 1987). Using immunoprecipitation strategies a 85 kDa phosphoprotein was identified which is associated with both the tyrosine kinase and PtdIns 3-kinase activity (Cohen *et al.*, 1990). However, the 85 kDa protein lacks any kinase activity if associated alone with the tyrosine kinase. The 3-kinase activity has been reported to be present in another protein with a molecular mass of 110 kDa (Carpenter *et al.*, 1990; Escobedo *et al.*, 1991; Solnik *et al.*, 1991). Now it is established that in rat liver the PtdIns 3-kinase is a heterodimer comprised of a single 85 kDa and a 110 kDa subunits. Studies on bovine thymus showed the presence of two types of PtdIns 3-kinase, one very similar to the rat liver enzyme (heterodimer), the other a 110 kDa molecular mass monomer with a relatively higher specific activity (Shibasaki *et al.*, 1991).

The PtdIns 3-kinase utilizes PtdIns, PtdIns4*P* and PtdIns(4,5)P_2 as substrates *in vitro* (Auger *et al.*, 1989b; Carpenter *et al.*, 1990; Serunian *et al.*, 1990). The enzyme appears to be Mg^{2+} dependent and its specificity varies with the presence of non-substrate carrier lipids such as phosphatidylserine (Carpenter *et al.*, 1990). The purified

rat liver enzyme has a relatively lower K_m for polyphosphoinositides compared to PtdIns, while V_{max} for PtdIns is higher.

Evidence is accumulating which suggests that PtdIns 3-kinase can be activated through mechanisms involving tyrosine phosphorylation. Stimulation of different growth factor receptors (PDGF and EGF) has been shown to activate intracellular tyrosine kinase. The best studied system is that of PDGF receptor, the activation of which induces receptor autophosphorylation by the tyrosine kinase and activation of a series of proteins including PL C, *ras*GAP, PtdIns 3-kinase, etc. (Kaplan *et al.*, 1987; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990). By using deletion and point mutations of the PDGF β receptor, the binding site for PtdIns 3-kinase has been shown to reside within the kinase insert domain of the receptor around Tyr^{719} (Kazlauskas *et al.*, 1989; Coughlin *et al.*, 1989). The Tyr^{719} phosphorylated peptides efficiently blocked the association of PtdIns 3-kinase with the autophosphorylated receptor.

One current model for activation of PtdIns 3-kinase is that the enzyme is cytosolic in quiescent cells, but upon interaction of the agonist with the receptor and activation of the tyrosine kinase the enzyme relocates to the plasma membrane in close association with its substrate (Downes *et al.*, 1991). The products of the activated PtdIns 3-kinase are the PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Stephens *et al.*, 1991). The rapid increase in the PtdIns(3,4,5)P_3 concentration in response to the stimuli and the direct interaction of the receptor (specially the growth factor receptors) with the PtdIns 3-kinase suggest a possible second messenger role to the PtdIns(3,4,5)P_3 (Downes *et al.*, 1991). However, more detailed studies are required to examine such a role for PtdIns(3,4,5)P_3; in addition, the

target for this messenger also needs to be identified. The role of D-3 phosphoinositides in promoting *in vitro* polymerization of actin by stimulating dissociation of actin/gelsolin complexes (Lassing *et al.*, 1985) and dissociation of profilactin (Janmey *et al.*, 1987) suggests that the 3-phosphorylated inositol phospholipids may be physiologically relevant agents promoting actin polymerization in the stimulated cells. Further studies are required to establish the *in vivo* role of the 3-phosphorylated forms of inositol, but with the current increasing interest in this field, the answers to the various questions will be probably available in the near future.

d. Phosphoinositide kinase in the heart

The so called "phosphoinositide effect" has been shown in myocardium as early as 1966 by Gaut and Huggins. It was not until a decade later that more interest was shown in the phosphoinositide turnover in the heart. Most of the reports on the heart deal with the activity of PL C and very little attention has been directed to the phosphoinositide kinases. Recently PtdIns 4-kinase and PtdIns4P 5-kinase activity have been characterized in the membranes isolated from dog heart (Quist *et al.*, 1989; Kasinathan *et al.*, 1989) and rabbit hearts (Varsanyi *et al.*, 1986; Wolf, 1990). The activity of PtdIns kinase was also shown to be significant in the cardiac sarcoplasmic reticulum (Quist *et al.*, 1989; Wolf, 1990). The studies of cardiac phosphoinositide kinases lack more detailed information about their apparent molecular weight, properties and, more specifically, about the products of their reactions.

2. Phospholipase C

Phosphoinositide-specific PL C plays a crucial role in initiating the surface-mediated signal transduction by generating second messenger molecules, Ins(1,4,5)P_3 and DAG (FIGURE 1). Results accumulated in the last few years clearly indicate that the response of phosphoinositide signalling to a specific stimulus varies depending not only on the tissue and cell type but even when the tissues and cells contain identical receptor subtypes. One of the reasons for such a heterogeneity could be explained by the differential expression of the various post-receptor components, G protein and PL C. Indeed, in the last decade a number of distinct PL Cs have been purified from a variety of mammalian tissues (Rhee *et al.*, 1989) and several isoforms have been cloned and sequenced. PL C can be grouped into one of the five immunologically distinguishable isoforms (PL C- α, ß, γ , δ, and ϵ) (Rhee *et al.*, 1989). The PL C isozymes so far purified, have a molecular size ranging from 70 to 155 kDa (for review see Cockcrof and Thomas, 1992). The cDNAs of three isoforms of PL C (ß, γ, δ) have been cloned and share two regions of homology at 150 and 240 amino acids (60% and 40% homology, respectively) (Rhee *et al.*, 1991). The PL C γ isoform contains the *src* homology domains, SH_2 and SH_3, which governs the protein-protein interaction. The PL C comprises γ_1 and γ_2, which are differentially expressed in cells. The PL C ß family contains three members, PL C $ß_1$, $ß_2$ and $ß_3$, all present in mammalian cells except the myocardium, where they are absent or present in low quantity (Suh *et al.*, 1988; Homma *et al.*, 1989). The PL C δ family contains three members, δ_1, δ_2 and δ_3, that are present

in different tissue including brain (Meldrum *et al.*, 1989), fibroblasts (Kirtz *et al.*, 1990), and myocardium (Wolf, 1992). The majority of these isoforms has been purified from the cytosolic fraction (Ryu *et al.*, 1987; Bennett and Crooke, 1987; Rebecchi and Rosen, 1987; Homma *et al.*, 1988; Meldrum *et al.*, 1989); however, PL C activity was also characterized in the membrane (Lee *et al.*, 1987; Banno *et al.*, 1988; Baldassare *et al.*, 1989; Meij and Panagia, 1992; Wolf 1992). Membrane-associated PL C activity can generally be removed by high salt treatment, indicating that this enzyme is attached to the membranes via ionic interactions. In addition, the hydropathy analysis of the amino acid sequence of PL C β_1 gave no indication of the presence of transmembrane spanning domains (Lee *et al.*, 1987). PL C β_1 showed an equal distribution between the cytosolic and membrane fractions, while PL C γ_1 was predominantly cytosolic (Lee *et al.*, 1987). PL C activity has also been observed in liver nuclei (Kuricki *et al.*, 1992; Martelli *et al.*, 1992). More specifically, in Swiss 3T3 cells PL C β_1 isoform was localized at the nucleus and was regulated by IGF-1 (Divecha *et al.*, 1991). The functional relevance of the nuclear PL C is not yet clear, but it might be postulated to play some role in the regulation of cell growth observed via stimulation of some of the PL C activating receptors.

The distinctive distribution of PL C isozymes (Choi *et al.*, 1989) and the structural differences of the PL C isoform suggests the possibility of multiple regulatory mechanisms for these enzymes (Rhee *et al.*, 1989). The mechanism of regulation of each PL C isoform is not clear yet; nevertheless, many investigators have tried to clarify this point by using permeabilized cells or membrane preparations. Studies on the receptor-mediated

activation of PL C have suggested the involvement of a G protein (called: G_p, G_q and G_h) (as discussed above, and Cockcroft and Lad *et al.*, 1986; Stutchfield, 1988; Dubyak *et al.*, 1988). One of the PL C isoforms that has been linked to the G protein activation is from the ß-family (Bloomquist *et al.*, 1988; Carter *et al.*, 1990; Morris *et al.*, 1990). Cockcroft *et al.* (1991) demonstrated that the PL C Υ isoforms purified from rat brain were not regulated by G proteins while the fraction of the same preparation which contained both PL C ß and PL C δ was activated by G protein. The activation of PL C ß but not PL C Υ by G protein was also reported by Taylor and Exton (1991). The mechanism of regulation of PL C has been shown to involve a tyrosine kinase (for review see Rhee, 1991). The indication that tyrosine phosphorylation plays an important role in the regulation of PL C Υ isoforms came from its structural homology to the tyrosine related oncogenes (Stahl *et al.*, 1988; Suh *et al.*, 1988; Mayer *et al.*, 1988). This led to demonstrate the regulation of PL C Υ by both EGF and PDGF receptors (*in vivo* or *in vitro*) via phosphorylation of tyrosine and serine residues of PL C Υ (Meisenhelder *et al.*, 1989; Margolis *et al.*, 1989; Nishibe *et al.*, 1989).

The activity of PL C is also modulated by the cytosolic concentration of Ca^{2+}. The effect of Ca^{2+} on PL C have been reported in adrenal chromaffin cells (Sasakawa *et al.*, 1987), neuronal cells (Irvine *et al.*, 1984), in the guinea pig ileum longitudinal smooth muscle (Watson *et al.*, 1990) and pancreatic islets (Biden *et al.*, 1987). However, what remains to be investigated is which PL C isoforms activated by Ca^{2+}.

a. PL C in the heart

Several investigators have reported the presence of endogenous PL C activity in cardiac sarcolemmal membranes (Schwertz and Halverson, 1989; Wolf, 1989; Edes and Kranias, 1990; Meij and Panagia, 1992) and cytosolic fractions (Edes and Kranias, 1990). As in other tissues, the presence of multiple isoforms of PL C has been also reported in the myocardium (Low and Weglicki, 1983; Wolf, 1989; 1992; McDonald and Mamrack, 1989). Studies using specific antibodies and cDNA probes indicate that the PL Cγ isoform is the predominant one in bovine, rat and canine heart (Suh *et al.*, 1988; Homma *et al.*, 1989). In addition, the presence of a δ isoform has been reported (Suh *et al.*, 1988; Wolf, 1992), while the ß isoform is absent or very low in abundance in myocardial tissue (Suh *et al.*, 1988; Homma *et al.*, 1989; Rhee *et al.*, 1991). Activation of PL C in the myocardium has been shown to be predominantly regulated by G protein-mediated receptors (as described before); however, whether regulation of PL C by tyrosine phosphorylation plays any role in myocardial tissue has not yet been addressed.

3. Ins(1,4,5)P_3 and its receptors in the heart

Ins(1,4,5)P_3 has been shown to increase intracellular Ca^{2+} in different permeabilized tissues (Sterb *et al.*, 1983; Berridge, 1987; Volpe *et al.*, 1985; Abdel-latif, 1986) including skinned cardiac muscle (Fabiato, 1990). The Ins(1,4,5)P_3-induced Ca^{2+} release from cardiac sarcoplasmic reticulum has been shown in skinned ventricular fibres from rat (Kentish *et al.*, 1990), chick atria (Vites and Pappano, 1990), and cardiac SR

vesicles (Hirata *et al.*, 1984). Ins(1,4,5)P_3 has also been shown to potentiate the effects of caffeine-induced calcium release in skinned guinea pig papillary muscle (Nosek *et al.*, 1986). Using laser photolysis of "caged Ca^{2+}" (Nitr-5) and "caged Ins(1,4,5)P_3" in skinned ventricular trabeculae from rat heart, Kentish *et al.* (1990) showed an increase in intracellular Ca^{2+} concentration. Furthermore, the source of the Ca^{2+} which activated the myofibrils was suggested to be SR, since the Ca^{2+} release was blocked by ryanodine or caffeine. Ins(1,4,5)P_3-induced Ca^{2+} release by α-adrenergic agonists has been reported (Poggioli *et al.*, 1986; Otani *et al.*, 1988). The release of Ca^{2+} induced by Ins(1,4,5)P_3 appears to be through an ion channel (Yamamoto and van Breemen, 1985; Smith *et al.*, 1985). Using a SR fraction incorporated in the lipid bilayer Suarez-Isla *et al.* (1988) recorded Ca^{2+} release by Ins(1,4,5)P_3 as the Ca^{2+} current. A Ca^{2+} current directly activated by Ins(1,4,5)P_3 was also observed in the SR from canine aortic smooth muscle (Ehrlich and Watras, 1988). This channel was voltage independent, activated by ATP and inhibited by heparin. Moreover, Borgatta *et al.* (1991) have recently shown the occurrence of a low-conductance Ca^{2+}-release channel in canine heart SR vesicles which was sensitive to Ins(1,4,5)P_3.

The specific high affinity Ins(1,4,5)P_3-binding sites were first identified in liver, neutrophils and adrenal cortex (Baukal *et al.*, 1985; Spat *et al.*, 1986). Two types (both high and low affinity) of [^{3}H]-Ins(1,4,5)P_3 binding were found in canine cardiac microsomes (Kijima and Fleisher, 1992). In 1988, the Ins(1,4,5)P_3 receptor was purified from brain, independently by two groups and was called Ins(1,4,5)P_3-binding protein (Supattapone *et al.*, 1988) and Purkinje cell-enriched protein P_{400} (Maeda *et al.*, 1988).

These proteins were immunologically identical (Maeda *et al.*, 1990). Then the cDNA of the mouse Ins(1,4,5)P_3 receptor was cloned (Furuichi *et al.*, 1989). Cloning of the Ins(1,4,5)P_3 receptor cDNA from mouse (Furuichi *et al.*, 1989), rat (Mignery *et al.*, 1990; Sudhof *et al.*, 1991) and human (Sudhof *et al.*, 1991) showed that the general structure of the receptor is highly conserved. Further transfection of the cDNA to cell lines showed enhanced Ins(1,4,5)P_3 binding and also Ca^{2+} releasing activity (Miyawaki *et al.*, 1990) suggesting that this protein is an Ins(1,4,5)P_3-gated Ca^{2+} channel. The primary sequence of the Ins(1,4,5)P_3 receptor shares no homology with the Ca^{2+} channels on the plasma membrane (Furuichi *et al.*, 1989; Mori *et al.*, 1991), but shares significant homology with the ryanodine receptor of the sarcoplasmic reticulum in skeletal (Mignery *et al.*, 1989) and cardiac muscle (Takeshima *et al.*, 1989).

The presence of the Ins(1,4,5)P_3 receptors in the myocardial tissue is currently under investigation. The first evidence for the occurrence of these receptors in the heart was provided by detection of low amounts of mRNA encoding the intracellular Ins(1,4,5)P_3 receptor using Northern blot analysis (Furuichi *et al.*, 1990; Marks *et al.*, 1990; Mignery *et al.*, 1990; Nakagawa *et al.*, 1991). Recently, Gorza *et al.* (1993) have demonstrated the presence of Ins(1,4,5)P_3 receptor in rat myocytes. Using Western blot analysis and immunofluorescence (with anti-peptide antibodies specific for the Ins(1,4,5)P_3 receptor), these investigators have shown the highest Ins(1,4,5)P_3 receptor density in the Purkinje myocytes compared to the atrial and ventricular myocytes, and concluded that Ins(1,4,5)P_3-induced Ca^{2+} release evoked by α_1-adrenoceptor stimulation is responsible for the increase in automaticity of Purkinje myocytes shown by del Balzo *et al.* (1990).

Kijima *et al.* (1993) have shown the presence of the Ins(1,4,5)P_3 receptors in ventricular and atrial cardiomyocytes by Western blot analysis. The presence of the Ins(1,4,5)P_3 receptors in atria and ventricular tissue as well as the cardiac vasculature was also reported by Moschella and Marks (1993). By the aid of electron microscopy and immunostaining of the Ins(1,4,5)P_3 receptors Kijima *et al.* (1993) have localized this protein mainly at the intercalated discs of atrial and ventricular cardiomyocytes, and they suggested the possible role of the Ins(1,4,5)P_3 receptor in inducing Ca^{2+} entry through intercalated discs and/or intercellular signalling between cardiomyocytes. However, Gorza *et al.* (1993) using double immunolabelling experiments for Ins(1,4,5)P_3 receptors and calsequesterin showed that the Ins(1,4,5)P_3 receptor was not restricted to the subsarcolemmal SR but possibly present in corbular SR scattered throughout the Purkinje cell. The Ins(1,4,5)P_3 receptor mRNA level in rat myocardium was shown to be about 50 fold lower than the ryanodine receptor (Moschella and Marks, 1993). Although these data provide evidence for the presence of Ins(1,4,5)P_3 receptors in the myocardial tissue, their distribution and functional role in the myocardium needs more detailed investigation.

D. *Effect of Thyroid Hormone on the Heart*

The direct action of thyroid on cardiac function was recognized first in 1785 by C. Parry, who reported hypertrophy of the heart associated with enlargement of the thyroid gland. Following this, more attention was addressed toward the relation between changes

in thyroid hormone level and the heart function. Direct actions of thyroid hormone on the heart, as on other tissues, are mediated via nuclear and extranuclear mechanisms. T_3 receptor proteins in the heart have been characterized and localized. Using radiolabelled hormone of high specific activity T_3 receptors have been localized in isolated nuclei and nuclear extracts in different tissues including the heart (Oppenheimer *et al.*, 1974; Ladenson *et al.*, 1986). The presence of nuclear T_3 receptors was also shown by immunocytochemical localization (Luo *et al.*, 1989) in which receptors bound or unbound to their ligand could be localized using polyclonal or monoclonal antibodies against T_3 receptors. The binding of T_3 to its nuclear receptors has been characterized in ventricular and atrial muscle cell in rabbit heart (Banerjee *et al.*, 1988). The presence of cytosolic T_3 binding protein was also shown in the atrial and ventricular tissues of rat (Osty *et al.*, 1988). The existence of a mitochondrial thyroid hormone binding protein was observed in rat liver (Goglia *et al.*, 1981) and beef heart mitochondria (Sterling, 1987). Furthermore, the mitochondrial T_3 receptor was found to be associated with the inner mitochondrial membrane ADP/ATP carrier and adenine nucleotide translocase purified from beef heart mitochondria (Sterling, 1986). The importance of the mitochondrial T_3 receptor and the change in the level of the electron transport chain and other metabolic enzymes of the mitochondria needs further investigation.

1. Thyroid hormone effect on gene expression and protein synthesis

Thyroid hormone has been shown to affect gene expression and thus protein

synthesis in the heart of different species. This action of thyroid hormone is mediated via its nuclear receptor. Thyroid hormone effects on protein synthesis results in enhanced formation of total cardiac proteins (Sanford *et al.*, 1978), therefore results in cardiac hypertrophy. However, while some new types of proteins are synthesized other specific proteins (e.g., myosin heavy chain ß), are decreased (Gustafson *et al.*, 1987). Changes in the formation of specific proteins can result from the influence of T_3 at the nuclear or extranuclear processes of protein synthesis (Dillmann, 1990). Thus the circulating levels of T_4 and T_3 are very important in expression and synthesis of many cellular proteins such as muscle myosin.

Myosin is the major structural component of the contractile apparatus. It is composed of two heavy chains and four light chains. The myosin heavy chains (class α and ßMHC) possess the ATPase activity (Lompre *et al.*, 1984). The association of the two different classes produces three myosin isozymes of MHC in rat ventricular muscle, which are in order of decreasing ATPase activity, V_1 (two α), V_2 (αß) and V_3 (two ß) (Hoh *et al.*, 1978; Lompre *et al.*, 1981). These MHC isoforms are encoded by multiple genes which appear to be clustered on the same chromosome (Leinwand *et al.*, 1983).

Normally, the MHC isoforms are changed during the developmental stages. During the late embryonic period V_3 (ßß) is the predominant isomyosin in mouse, rat and rabbit ventricles (Lompre *et al.*, 1981; Chizzonite *et al.*, 1982; Everett *et al.*, 1983). After birth the synthesis of the ß class decreases and α is increased, therefore V_3 is gradually replaced by V_1. During the following period of life opposite changes occur, although at a much slower rate (and in older animals V_3 becomes predominant again) (Lompre *et al.*,

1981; Chizzonite *et al.*, 1982; Everett *et al.*, 1983). The changes in the myosin isozymes in different developmental stages were shown to be associated with the change in the serum levels of thyroid hormone (Chizzonite *et al.*, 1984). Chizzonite *et al.* (1984) showed that the rapid increase in V_1 after birth coincides with an intense increase in serum level of thyroid hormone and, when thyroid synthesis is blocked by propylthiouracile (PTU) treatment, V_1 synthesis is prevented. Furthermore, when mature rats were made hypothyroid by PTU treatment or thyroidectomy the synthesis of α-MHC was reduced while the synthesis of ß-MHC was greatly increased (Hoh *et al.*, 1978; Chizzonite *et al.*, 1984). In addition, treatment of both normal and PTU-treated hypothyroid rats with T_3 not only decreased the ß-MHC protein but also the ß-MHC mRNA level, and increased the α-MHC level to an equivalent (Schuyler *et al.*, 1990).

Thyroid hormone regulation of myosin isozymes is mediated via regulation of expression of mRNA, with stimulation of α-MHC mRNA synthesis and inhibition of the expression of ß-MHC mRNA (Lompre *et al.*, 1984; Gustafson *et al.*, 1986). Similar changes were also observed in cultured rat heart cell's MHC isozymes (Nag *et al.*, 1984). The use of cultured cell preparations to determine the effects of thyroid hormone on cardiomyocytes helps to rule out the possible effects of other neurohumoral factors in the expression of cardiac MHC (e.g., sex steroids, glucocorticoids and catecholamines). Recently Gustafson *et al.* (1987) showed a change in expression of MHC isoforms and their respective mRNA in cultured cardiac cells. In addition, these authors showed that various adrenergic agents (isoproterenol, norepinephrine and phenylephrine), steroid hormones, muscarinic agents and glucagon had no effect on expression of MHC genes.

Izumo *et al.* (1986) demonstrated a different mode of regulation of myosin heavy chain gene in different tissues by using a cDNA clone specific for ß-MHC gene (which contain 180 nt of common coding sequence at the carboxyl end of the α- and ß-MHC) and studied their expression in seven different muscles from hypo-, eu- and hyperthyroid animals.

The other contractile protein which can be regulated by thyroid hormone is α-actin. Cardiac α-actin mRNA is expressed in absence of T_3, but its expression can be transiently increased by T_3 treatment (Gustafson *et al.*, 1987). A similar effect is observed in treating hypothyroid hearts with T_3 (Gustafson *et al.*, 1986). However, more research should be carried out on the regulation of α-actin in the myocardium by T_3. The fact that thyroid hormone can regulate the expression of the two important contractile proteins MHC and α-actin demonstrates the importance of this hormone in modulating the heart function. Since myosin ATPase activity is closely related to the speed of contraction in heart and skeletal muscle (Reiser *et al.*, 1985), the effects of thyroid hormone on myosin isozyme composition may explain in part the ability of thyroid hormone to stimulate cardiac performance (Morkin *et al.*, 1983).

2. Effect of thyroid hormone on Ca^{2+} metabolism

In muscle Ca^{2+} plays an important role in contractile activity of the cell. Thus, in conditions associated with increased myocardial performance, which is also accompanied by increase in metabolic activity, Ca^{2+} plays a major role; such conditions

can occur during catecholamine release and/or thyrotoxicosis. In particular, thyroid hormone has an immediate positive inotropic effect, which is characterized by a greater velocity of muscle shortening and abbreviation of the time to reach peak tension (Buccino *et al.*, 1967). The increased contractility of the heart, induced by thyroid hormone suggests that altered Ca^{2+} kinetics contribute to this effect (Klein and Levey, 1984). By using aequorin as an intracellular Ca^{2+} transient monitor in cardiac muscle, MacKinnon and Morgan (1986) showed altered cytoplasmic Ca^{2+} concentration by thyroid hormone. These authors reported a briefer duration of contraction in thyroxine-treated hearts which was accompanied by a faster decay of the Ca^{2+} signal. Fura2 (a Ca^{2+} indicator) used in resting rat cardiomyocytes showed no significant difference in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in euthyroid from the hyperthyroid condition. However, in electrically stimulated myocytes the time to peak fluorescence and 50% decay in peak fluorescence increase in hyperthyroid more than euthyroid and hypothyroid myocytes, which indicates a similar pattern for increase in Ca^{2+} fluxes (Beekman *et al.*, 1988).

The intracellular concentration of Ca^{2+} in cardiac myocytes is regulated via different mechanisms which are mainly associated with sarcolemma (SL) and sarcoplasmic reticulum (SR). Many of these mechanisms are modulated by the thyroid hormone state. In particular, sarcolemmal Ca^{2+} pump was increased in the hyperthyroid chick cardiomyocytes (Kim and Smith, 1985) and SL preparation from rabbit myocardium (Rudinger *et al.*, 1984). However, no reports are available on the SL Ca^{2+} pump activity in hypothyroid conditions. In addition, [^{3}H]nitrendipine binding to the SL Ca^{2+} channel, which is the major pathway for influx of Ca^{2+} from extracellular space, was found to be

significantly depressed in the hyperthyroid rat myocardium (Hawthorn *et al.*, 1988; Kosinski *et al.*, 1990), while it was increased in hypothyroid rat heart SL (Hawthorn *et al.*, 1988). SL Na^+/Ca^{2+} exchange activity, which acts in moving the Ca^{2+} ion in two direction across the membrane, was increased in T_3 treated chick cardiomyocytes (Kim *et al.*, 1985). Na^+-K^+ ATPase is indirectly involved in the maintenance of intracellular Ca^{2+} concentration via altering the cytosolic Na^+, was significantly increased in the hyperthyroid heats (Lin *et al.*, 1978; Gick *et al.*, 1988; Daly *et al.*, 1987).

Several studies have indicated that changes in thyroid state are associated with increased Ca^{2+} uptake (Limas, 1978) and Ca^{2+} ATPase activity (Suko, 1973) in cardiac SR. The Ca^{2+} uptake by the myocardial SR depends on both the activity of the SR Ca^{2+} pump and the associated phospholamban. The activities of these proteins could be modified by phosphorylation (Lindeman *et al.*, 1983; Kranias *et al.*, 1985). Beekman *et al.* (1989) showed a decrease in the phosphorylated phospholamban in the direction of hypo-, eu- to hyperthyroid, while the phosphorylated Ca^{2+} pump was increased in the same direction. This result indicate that thyroid hormone induces a decrease in relative amount of phospholamban with respect to Ca^{2+} ATPase. In cultured chick cardiac cells T_3 induced an increase in Ca^{2+} storage capacity indicated by the response to caffeine. Studies on the expression of phospholamban mRNA in rabbits showed a 61% decrease in phospholamban mRNA level after 3 days of treatment with T_3 while hypothyroidism had no effect on phospholamban mRNA levels (Nagai *et al.*, 1989). In contrast the mRNA levels of Ca^{2+} ATPase have shown a rapid increase up to 136% after (2-5 hrs) T_3 treatment of rat (Rohrer *et al.*, 1988) and up to 167% in rabbit myocardium (Nagai

et al., 1989). In hypothyroid animals the Ca^{2+} ATPase mRNA decreased to about 35% of the controls (Rohrer *et al.*, 1988; Nagai *et al.*, 1989). These data support the well established effect of thyroid hormone on improved myocardial contractility and increases speed of diastolic relaxation.

3. Thyroid hormone and the adrenergic system

The relationship between the levels of circulating thyroid hormone and the response of the muscle and glands to adrenergic stimulation has been studied for many years. Thyrotoxicosis presents numerous clinical features suggestive of increased adrenergic activity, such as tachycardia, increased cardiac output, increased glycogen and lipid mobilization, enhanced thermogenesis (Harrison 1964; Waldstein 1966; Irvine, 1976). Conversely, an apparently decreased adrenergic function is often found in the hypothyroid state (Harrison, 1964; Krishna *et al.*, 1968). The serum and urinary level of catecholamines in thyrotoxic patients show a decrease compared to normal subjects (Bayliss *et al.*, 1971; Christensen *et al.*, 1973). In addition, ß blocking drugs only partially blunt the hyperdynamic cardiovascular responses associated with hyperthyroidism (Merillon *et al.*, 1981). This shows that the main changes in the disturbed thyroid state are due to the action of T_3 and thyroxine themselves and not to the blood catecholamines. However, examination of the responsiveness of isolated tissue and organ preparations to catecholamines after T_3 administration or removal of thyroid gland yielded conflicting results. In particular, some investigators have found an increase in

responsiveness of the heart to catecholamine stimulation in hyperthyroid state (Coville *et al.*, 1970; MacLeod, 1981), and decreased responsiveness in hypothyroid state (Kunos *et al.*, 1974). On the other hand, other investigators failed to show any alteration in the catecholamine responsiveness with changed thyroid states (Margolius, 1965; Young and McNeill, 1974).

The other, more direct, approach to the role of catecholamine in disturbed thyroid state is studying the alterations in adrenoceptor density. Several laboratories have utilized the direct measurement of specific adrenergic radioligand binding in ventricular membrane preparations from hypo-, eu- or hyperthyroid animals. The results from such studies have shown a clear and significant increase in cardiac ß receptor number after exposure to excess thyroid hormone *in vitro* (Kempson *et al.*, 1978), *in vivo* administration of T_3 to the hypothyroid rats (Banerjee *et al.*, 1977; Chang and Kunos, 1981), or T_3 addition to cultured rat myocardial cells (Kupfer, 1986). Moreover, the increase in ß receptor number induced by thyroid hormone was associated with a proportional increase in maximal isoproterenol-responsive adenylate cyclase activity (Ciaraldi and Martinetti, 1978; Tse *et al.*, 1980; Krawietz *et al.*, 1982) or in cellular cAMP accumulation (Tse *et al.*, 1980). In contrast, the ß adrenoceptor number has been consistently reported to be decreased in the heart tissue of hypothyroid animals (Banerjee, 1977; Ciaraldi and Martinetti, 1977; Kunos *et al.*, 1980). In addition, the maximal isoproterenol responsiveness was also reduced in hypothyroidism (Nakashima *et al.*, 1971; Ciaraldi and Martinetti, 1978).

The α-adrenoceptor density was also affected by the thyroid state. Using a specific

α radioligand ([^{3}H]prazosin), the density of α receptors was found to be decreased by approximately 40% in hyperthyroid rat hearts (Ciaraldi, 1978; Sharma and Banerjee, 1978; Kunos, 1980; Chang and Kunos, 1981). In addition, some reports have shown a decrease in the antagonist affinity to the α-adrenoceptor (Sharma and Banerjee, 1978; McConnaughey, 1979; Chang and Kunos, 1981) which is in contrast to that seen for the ß receptor. The reports on the state of the α adrenoceptors during hypothyroidism is conflicting. Some reports showed a decrease in the α adrenoceptor density (Ciaraldi and Martinetti, 1978; McConnaughey *et al.*,1979; Noguche *et al.*,1983), while others showed an increase (Sharma and Banerjee, 1978; Kunos *et al.*, 1980; Chang and Kunos, 1981; Fox *et al.*,1985). Kunos *et al.* (1980) have demonstrated an enhanced sensitivity of atrial tissue from hypophysectomized rats to the α-receptor agonists phenylephrine and methoxamine and reduced sensitivity to ß agonists. More recently, Fox *et al.* (1985) showed that the adrenoceptor densities in different tissues respond differently to the disturbed thyroid state. Moreover, these authors showed a clear increase in α receptors in the hypothyroid state, and an increase in the α/ß ratio in this condition. No reports are available on the changes in the α_1-adrenoceptor-associated second messenger system with altered thyroid state. However, functional studies show that the α_1-adrenoceptor mediated changes in contractile force in the hypothyroid hearts are significantly higher than in the euthyroid hearts (Nakashima *et al.*,1971; 1972; Simpson and McNeill, 1980), while the ß-mediated positive inotropy is declined (Nakashima *et al.*, 1971).

The literature available on the changes of α- and ß-adrenoceptors during altered thyroid state postulates the possible regulation of the synthesis of these receptors by the

thyroid hormone. However, no reports are available on the changes of the expression of adrenoceptor genes or their mRNA levels.

In view of the effect of thyroid hormone on the α and ß receptor densities and also the change in the production of cAMP in the heart (Bilezikian and Loeb, 1983), it can be predicted that thyroid hormone may also affect the density of the guanine nucleotide-binding regulatory proteins (G proteins). G protein has several subtypes, two of which are linked to adenylyl cyclase. Recently, the inhibitory G protein subunits (Gi_{α} and ß) have been shown to increase in fat cell membranes of hypothyroid rats (Milligan *et al.*, 1987; Ross, 1988). However, GppNHp activation of adenylyl cyclase activity or the amount of Gs_{α} (determined by cholera toxin-catalyzed [^{32}P]-ADP ribosylation) in rat heart membranes showed no difference with thyroid hormone status (Krawietz *et al.*, 1982). More recently Levine *et al.* (1990) showed a 50%-150% increase in the amounts of the Gi_{α} and $G_{ß}$, respectively, but no increase in Gs_{α} of myocardial membranes isolated from ventricles of hypothyroid rats. There were no significant change with hyperthyroidism. The change in the Gi_{α} and $G_{ß}$ proteins in the hypothyroid rat ventricles were accompanied by concurrent increase in the mRNA transcripts encoding for $Gi_{\alpha\text{-}2}$, $Gi_{\alpha\text{-}3}$ and $G_{ß}$ subunits (Levine *et al.*, 1990). Therefore, in the rat ventricular tissue not only the ß adrenoceptor is decreased in hypothyroidism, but also the ß adrenoceptor effect is further suppressed by an increase in the expression of the inhibitory subunits G_i in the ventricular membranes, which will ensure the hypoactive heart. However, in hyperthyroid state there is only an increase in the ß adrenoceptor density with no change in the associated G protein.

E. *Heart Failure*

The failure of the heart to perform its normal function and the mechanism involved in the development of such abnormality have long intrigued the cardiologist. The diversity of the mechanism of development of heart failure has added to the complexity of this disease. However, the primary defect for development of heart failure, regardless of its origin, is myocardial overload which is accompanied by failure of the contractile apparatus and the compensatory mechanisms to correct this condition. One of the first observed changes in the heart failure is the depletion of myocardial norepinephrine content (Chidsey *et al.*, 1963). This is associated with increased sympathetic nervous system activation and high levels of circulating plasma catecholamines (Thomas and Marks, 1978) in combination with neural re-uptake of norepinephrine in the heart (Liang *et al.*, 1989). Further studies have shown changes in the adrenoceptors and their associated signal transduction as well as in the Ca^{2+} handling processes of the failing myocardium. These studies are discussed below.

1. Adrenoceptors

Heart failure is characterized by reduced effect of sympathomimetic agents on force of contraction. In isolated cardiac preparations a reduced responsiveness to the ß-adrenoceptor agonist, isoprenaline, has been reported (Bristow *et al.*, 1982; 1984;

Ginsburg *et al.*, 1983; Bohm *et al.*, 1988; Brodde, 1991; Dixon and Dhalla, 1991). This decrease in ß-adrenergic mediated positive inotropy has been attributed to the down regulation of the ß-receptors in the failing myocardium (Bristow *et al.*, 1986; Fowler *et al.*, 1986; Brodde *et al.*, 1986; Bristow *et al.*, 1989a; Dhalla *et al.*, 1991). Receptor binding studies have shown a decrease in the $ß_1$-adrenoceptor density in failing myocardial tissue (Bristow *et al.*, 1986; 1989; Bohm *et al.*, 1989; Dixon and Dhalla, 1991; Steinfath *et al.*, 1992a). Fowler *et al.* (1986) have shown a progressive reduction of the endo-myocardial $ß_1$-adrenoceptor density as the heart failure progressed. In spite of normal density of $ß_2$-adrenoceptors in the failing myocardial tissue (Bohm *et al.*, 1989; Steinfath *et al.*, 1992a), $ß_2$-adrenoceptor mediated stimulation of both the adenylate cyclase and muscle contraction were decreased by 30% (Bristow *et al.*, 1986). These changes in the $ß_2$-adrenoceptor density and activity suggest an uncoupling between the receptor and its signal transduction pathway.

The decrease in the inotropic response of the $ß_1$-adrenoceptor stimulation could also be the result of changes in the G protein regulatory proteins. Indeed, pertussis toxin mediated ADP-ribosylation of the Gi_α was increased in failing myocardium from canine (Marzo *et al.*, 1991) and human (Feldman *et al.*, 1988). Furthermore, the mRNA level of the Gi_α was increased in the human end-stage heart failure (Eschenhagen *et al.*, 1992). In studies on canine and human heart failure the Gs protein was not altered (Marzo *et al.*, 1991; Eschenhagen *et al.*, 1992). The adenylate cyclase was also decreased by Gpp(NH)p and forskolin treatment of failing myocardium, an effect which was blocked by pertussis toxin (Felman *et al.*, 1988). The increased Gi_α activity in failing human heart

could also explain the $ß_2$-receptor uncoupling (Bristow *et al.*, 1989b).

The increased circulating level of catecholamines in heart failure implied the regulation of the α_1-adrenoceptor density in a similar manner to that of ß-adrenoceptor. Reports on changes in the α_1-adrenoceptor density in failing heart are conflicting. The density of α_1-adrenoceptors was shown to be increased in pressure overloaded failing guinea pig hearts (Karliner *et al.*, 1980), rat model of congestive heart failure (Dixon and Dhalla, 1991), cardiomyopathic hamsters (Karliner *et al.*, 1981), and human end stage idiopathic dilative cardiomyopathy (Steinfath *et al.*, 1992b). In contrast to these reports, no changes in the failing human hearts were detected compared to non-failing controls (Bohm *et al.*, 1988; Bristow *et al.*, 1989). It is noted that even if no changes could be seen in the absolute density of α_1-adrenoceptors, these receptors reflect a greater proportion of the total adrenergic receptor population in the failing ventricle because of the selective down regulation of the ß-adrenoceptors. Functional studies on the α_1-adrenoceptor stimulation of the failing human hearts showed no change or even a slight decrease in contractile performance (Bohm *et al.*, 1988; Steinfath *et al.*, 1992b), whereas, in the rat model of congestive heart failure the α_1-adrenoceptor mediated inotropism was significantly increased as compared to controls (Dixon and Dhalla, 1991). These results may further suggest changes in the second messenger systems associated with the α_1-adrenoceptor. There is indication for an increased phosphoinositide turnover in ventricular myocytes isolated from infarcted rat hearts 7 days after the left coronary artery ligation (Meggs *et al.*, 1990). Nothing is known about the status of the functional activities of the α_1-adrenoceptor second messenger pathway in CHF due to coronary heart disease.

2. Alteration in Ca^{2+} handling processes in heart failure

Several mechanisms are involved in regulation of the intracellular Ca^{2+} concentration including sarcolemmal (Ca^{2+}-channels, Na^+/Ca^{2+} exchanger, Ca^{2+} pump, and indirectly the Na^+-K^+ ATPase) and sarcoplasmic reticulum (Ca^{2+} pump) activities. Studies on these activities in heart failure demonstrate variations which could be attributed to the type and the stage of heart failure.

Biochemical ligand-binding characteristics of the L-type Ca^{2+} channels in the sarcolemmal membrane have been studied in different experimental models of heart failure. An increased density of Ca^{2+}-channels has been shown in the genetically cardiomyopathic hamsters (Wagner *et al.*, 1987; Kobayashi *et al.*, 1987). However, in older cardiomyopathic hamsters suffering extensive cardiac hypertrophy and congestive heart failure, no changes in Ca^{2+} channel-density were observed (Wagner *et al.*, 1989). Moreover, the number of [^{3}H]-nitrendipine binding sites did not change in hypertrophied right ventricles of rats with congestive heart failure following myocardial infarction (Dixon *et al.*, 1990). However, in the last model the density of Ca^{2+}-channels was decreased in the viable left ventricle, an observation which was persistent in different stages of development of the disease (Dixon *et al.*, 1990).

Alteration in sarcolemmal Na^+/Ca^{2+} exchange and Ca^{2+} pump activities have also been assessed in several experimental models of heart failure. These mechanisms were extensively studied in genetic cardiomyopathic hamster, where a decrease in Na^+/Ca^{2+} exchange and Ca^{2+} pump activity was reported in 120-280 day old as well as depressed

Na^+/Ca^{2+} exchange in 360 day old hamsters (Makino *et al.*, 1985; Wagner *et al.*, 1989). In the rat model of congestive heart failure following myocardial infarction the activity of the sarcolemmal Na^+/Ca^{2+} exchange was depressed in different stages of development of heart failure (Dixon *et al.*, 1992b), whereas, the sarcolemmal Ca^{2+} pump activity was unchanged.

The SL Na^+-K^+ ATPase can indirectly modulate the intracellular concentration of Ca^{2+}. Decreased Na^+-K^+ ATPase has been observed in failing human hearts (Lindenmayer *et al.*, 1971), UM.X7.1 cardiomyopathic hamsters (Panagia *et al.*, 1984), rabbit with left ventricular hypertrophy and failure due to pressure overload (Yazaki and Fujii, 1972) and left ventricles of failing rat heart (Dixon *et al.*, 1992a). However, increased Na^+/K^+ ATPase activity was observed in BIO 14.6 strain cardiomyopathic hamsters (Sulake and Dhalla, 1973) and canine hearts with volume (mitral valve insufficiency) and pressure overload (aortic banding) (Khatter and Prasad, 1976; Prasad *et al.*, 1979).

The sarcoplasmic reticulum plays a very important role in regulating the intracellular Ca^{2+} concentration on a beat to beat basis. This regulation is enforced by the presence of a Ca^{2+} pump in the sarcoplasmic reticulum. Defects in the SR Ca^{2+} pump have been identified in various experimental models of heart failure as well as in failing human hearts (Dhalla *et al.*, 1978). In microsomes derived from failing canine heart, the Ca^{2+} uptake and Ca^{2+} pump were depressed compared to controls (Gertz *et al.*, 1967). Depression in SR Ca^{2+} pump has also been reported in rabbits (Sordahl *et al.*,

1970), hamsters (McCollum *et al.*, 1970), rats (Afzal and Dhalla, 1992) and human (Harigaya and Schwatz, 1971) failing hearts. The SR vesicles isolated from left ventricles of human hearts with idiopathic dilated cardiomyopathy did not show any change in Ca^{2+} uptake properties (Movsesian *et al.*, 1989). Recently, the SR Ca^{2+}ATPase mRNA level was shown to be decreased in hypertrophied rabbit and rat myocardium (following volume overload) (Komuro *et al.*, 1989; Nagai *et al.*, 1989; Da la Bastie *et al.*, 1990), a decrease which was associated with a decrease in protein concentration. The level of mRNA in left ventricular tissue from human end stage heart failure was also decreased as compared to the controls (Mercadier *et al.*, 1990).

F. *Final Considerations*

As discussed in the preceding sections, the α_1-adrenoceptor pathway has been proposed to maintain myocardial responsiveness to catecholamines under pathological conditions in which ß-adrenoceptors are impaired (Bruckner *et al.*, 1985; Osnes *et al.*, 1985; Homcy *et al.*, 1991). Changes in the adrenoceptor densities have been reported in several pathological and clinical conditions such as: congestive heart failure (Bristow *et al.*, 1982; Homcy *et al.*, 1991; Dixon *et al.*, 1991), hypothyroidism (McConnaughey *et al.*, 1979; Kunos *et al.*, 1980; Fox *et al.*, 1985), hypoxia (Heathers *et al.*, 1988; Kagiya *et al.*, 1991) and chronic treatment with ß-adrenergic antagonists (Mugge *et al.*, 1985). The increase in the α_1-adrenoceptor density in these conditions was also associated with an enhanced responsiveness to α_1-adrenergic agonists. An increase in the positive inotropic

effect of α_1-agonists was reported in the myocardium of hypothyroid animals (Nakashima *et al.*, 1971; 1973; Simpson and McNeil, 1980) and in congestive heart failure (Dixon *et al.*, 1991). However, little is known about the status of the functional activities of the α_1-adrenoceptor associated signal transduction pathway (phosphoinositide pathway) under these two conditions.

Given the enhanced relevance of the α-receptors under conditions of impaired ß-adrenoceptor function, we proposed to test the hypothesis that stimulation of the α_1-receptors and subsequent changes in receptor-associated phosphoinositide pathway may serve as a reserve mechanism to maintain the myocardial responsiveness to catecholamines, and may act as a secondary positive inotropic system in pathophysiological conditions of depressed ß-adrenergic activity. The changes in the α_1-adrenoceptor-associated phosphoinositide pathway were studied in the hypothyroid condition and for comparison, some of the pathway's activities were also studied in a rat model of congestive heart failure following myocardial infarction.

III. MATERIALS AND METHODS

A. *Materials*

[Gamma-^{32}P]ATP (specific activity of 10 Ci/mmole), ^{3}H-Prazosin [7-methoxy-^{3}H]-(78.7 Ci/mmol), ^{3}H-Dihydroalprenolol hydrochloride [levo-ring, propyl-^{3}H(N)]-96.8 Ci/mmol), ^{3}H-PtdIns L-α-[*myo*-inositol-2-2^{3}H(N)]-(13 Ci/mmol), ^{3}H-PtdIns(4)*P* [inositol-2-^{3}H(N)]-(9.9 Ci/mmol) and ^{3}H-PtdIns(4,5)P_2 [inositol-2-^{3}H(N)]-(8.8 Ci/mmol) were purchased from DuPont/New England Nuclear (Mississauga, Ont., Canada). D-*myo*-Inositol 1,4,5-trisphosphate [^{3}H]-assay system, [1-^{14}C]20:4*n*-6and *myo*-[2-^{3}H]inositol were from Amersham International (Amersham, U.K.). Alamethicin, neomycin, non-labelled phosphoinositides (PtdIns, PtdIns(4)*P*, PtdIns(4,5)P_2), 6-n-propyl-2-thiouracil, thyroxine, isoproterenol, xanthine, superoxide dismutase (from bovine liver), catalase (from bovine liver), deferoxamine mesylate, dithiothreitol, p-chloromercuriphenylsulfonic acid, N-ethylmaleimide, methylmethanethiosulfonate reduced glutathione and oxidized glutathione (grade III) were from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (30% solution) was obtained from Merck (Toronto, Ont., Canada). Xanthine oxidase (from bovine milk) was from Calbiochem Corp. (LaJolla, CA, USA) and was purified as described by Suzuki *et al.* (1991). Briefly, xanthine oxidase was dialyzed overnight against 0.01M Tris-HCl (pH 7.4) at 4°C to eliminate the contamination by $(NH_4)_2SO_4$ and EDTA which are included in the commercial preparation. Subsequently, it was pretreated with 0.4 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit the associated

trypsin-like activity. PMSF itself did not alter the enzyme activities under study. Hypochlorous acid was prepared from sodium hypochlorite as reported previously (Suzuki *et al., 1991)*. Phenylephrine was from Research Biochemical Incorporation (Natick, MA, USA). Angiotensin-converting enzyme test-combination was from Boehringer Mannheim Canada Ltd. (Laval, Que., Canada). HP-KF silica gel high performance thin layer chromatography plates were obtained from Whatman International Ltd. (Maidstone, England). Dowex 1X8 (formate form, 100-200 mesh) was from BioRad Labs. (Mississauga, Ont., Canada). Kodak X-Omat-R X-ray films and Dupont Cronex intensifying screen were purchased from Picker International (Highland Hts, Ohio, USA). Porous reactive glass beads (Glycophase G/CPG-200, 200-400 mesh) were obtained from Pierce Chemical Co. (Rockford, USA). CytoScint^{TM}ES* is a product of ICN Biomedicals Inc. (Mississauga, Ont., Canada). Growth medium containing Nutrient mixture Ham F10 (Gibco, Scotland), was supplemented with 10% v/v fetal calf serum, 10% v/v horse serum, 200 U/ml penicillin, 0.2 mg/ml streptomycin (all from Boehringer Mannheim, Germany). All other reagents were of analytical grade or of the highest grade available.

B. *Isolation of Sarcolemmal Membranes*

Rats were decapitated, in accordance with the Animal Care Committee, University of Manitoba. After dissecting the hearts, atria and large vessels were removed and the ventricular tissue was used to isolate sarcolemmal-enriched membranes according to the method described by Pitts (1979). All isolation procedures were carried out at 0-4 °C.

The ventricular tissue was washed with 0.6 M sucrose-10 mM imidazole, pH 7.0, to remove excess blood, then the tissue was finely minced in 0.6 M sucrose-10 mM imidazole, pH 7.0 (3.5 ml buffer/g tissue) and homogenized with a polytron PT-20 (6 x 15 s). The resulting homogenate was then centrifuged at 12,000 g for 30 min, and the pellet was discarded. An aliquot of the supernatant was centrifuged at 100,000 g for 60 min to prepare the cytosolic fraction, which was then frozen in liquid nitrogen and stored at -70 °C for later use. The rest of the supernatant was diluted with 140 mM KCl- 20 mM 3(N-morpholino)-propanesulphonic acid (MOPS), pH 7.4 (5 ml buffer/g tissue), and centrifuged at 100,000 g for 60 min. The resulting pellet was then resuspended in 140 mM KCl - 20 mM MOPS, pH 7.4, and layered over a 30 % sucrose solution containing 0.3 M KCl, 50 mM $Na_4PO_4O_7$, and 0.1 M Tris-HCl, pH 8.3. After centrifugation at 100,000 g for 90 min (using a Beckman swinging bucket rotor) the band at the sucrose-buffer interface was taken and diluted with 3 volume of 140 mM KCl, 20 mM MOPS, pH 7.4. The pellet from a final centrifugation at 100,000 g for 30 min was resuspended in 0.25 M sucrose, 10 mM histidine, pH 7.4. This sarcolemma-enriched fraction was divided into aliquots and stored in liquid N_2 till use. The purity of the sarcolemmal membrane prepared by this method was assessed by assaying for the activities of some marker enzymes as described below. The cross contamination of sarcolemmal membranes with endothelial plasma membranes was evaluated by assaying for the angiotensin-converting enzyme (test combination kit, Boehringer, Mannheim). The angiotensin converting enzyme activity in SL (3.4 ± 0.4 μmol/mg/30 min, n=5) was 0.3 fold that of the correspondent value in the heart homogenate.

C. *Marker Enzyme Assays*

Ouabain sensitive K^+-para-nitrophenyl phosphatase (pNPPase) is an accepted SL marker enzyme (Vetter *et al.*, 1991). The K^+-pNPPase was assayed by measuring the formation of para-nitrophenol (pNP) from paranitrophenyl phosphate (pNPP). The assay medium contained: 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 20 mM KCl, 1 mM ouabain (+ or -) and an equal ratio of alamethicin:protein (1μg drug/1μg protein) in a total volume of 1 ml. The mixture was preincubated at 37 °C for 10 min. The reaction was started by the addition of 15 mM pNPP (final concentration) and further incubated for 20 min. The reaction was then stopped by 0.1 ml of ice-cold 50 % trichloroacetic acid followed by the addition of 2 ml Tris (base, 0.5 M). The absorbance was measured at 410 nm by a spectrometer (Spectronic 601, Milton Roy Co., Rochester, N.Y.).

Rotenone-insensitive NADPH cytochrome *c* reductase is considered to be a marker enzyme for endo(sarco)plasmic reticulum (Ragnotti *et al.*, 1969). The enzyme activity was assayed by measuring the rate of change of absorbance at 550 nm at 25 °C, in the initial 3 min. The medium contained 44 mM potassium phosphate buffer (pH 7.6), 66 mM KCl, 0.1 mM NaCN, 1.5 μM rotenone, 0.05 mM oxidized cytochrome *c* and 50 μg membrane protein in a total volume of 2 ml. The reaction was initiated by adding nicotinamide-adenine dinucleotide phosphate (NADP, 0.1 mM final concentration). The blank contained all the components except NADPH. The enzymatic activity was estimated from the difference between the initial rates of cytochrome *c* reduction in the complete reaction mixture and in the blank. The reduction of cytochrome *c* was

calculated from the molar extinction coefficient of 27.7×10^6 cm^2/mole (Ragnotti *et al.*, 1969).

Cytochrome *c* oxidase activity, a mitochondrial marker enzyme, was assayed according to Wharton and Tzagoloff (1967) by measuring the initial rate of change of absorbance at 550 nm. The medium contained 1 mM potassium phosphate buffer (pH 7.0), 20 μg reduced cytochrome *c* and 50 μg membrane protein in a total volume of 1 ml. The blank was oxidized with 10 mM potassium ferricyanide.

D. *PtdIns and PtdIns4P Kinase Assay*

Unless otherwise indicated, the assay was initiated by preincubating 30 μg of SL protein for 30 min at 30 °C in 100 μl (final volume) of 40 mM HEPES-Tris, pH 7.4, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM dithiothreitol and 30 μg alamethicin. The phosphorylation of endogenous PtdIns and PtdIns4*P* was started by the addition of [gamma-^{32}P]-ATP in a final concentration of 1 mM (0.16 Ci/mmol). The reaction was terminated after 1 min by adding 2 ml of ice-cold methanol:13 N HCl (100:1, v/v), and vortexing for 2 min. For polyphosphoinositide extraction, 1 ml of 2.5 N HCl and 2 ml of chloroform were added. The tubes were vortexed for 2 min and centrifuged at 1000 g for 10 min. Then, the aqueous phase was discarded and the chloroform phase was washed with 2 ml of chloroform:methanol: 0.6 N HCl (3: 48: 47, v/v). After a further vortexing and centrifugation step, the final chloroform phase was removed and an aliquot was evaporated almost to dryness under a nitrogen stream. The residue was immediately

redissolved in 100 μl of chloroform: methanol: water (75: 25: 2, v/v) and quantitatively applied under light nitrogen stream to high performance silica gel thin layer plates (0.2 mm thick) that had been previously impregnated with 1 % potassium oxalate in methanol: water (2:3, v/v) and activated at 110 °C for at least 1 h. The test tubes were then washed twice with 30 μl of chloroform: methanol: water (75: 25: 2, v/v) and each washing was again applied to the plate. The chromatogram was developed at room temperature in a solvent system containing chloroform: acetone: methanol: glacial acetic acid: water (40: 15: 13: 12: 8, v/v), as described by Jolles *et al.* (1981). After the solvent front had migrated for approximately 13 cm, the plates were air dried at room temperature. The ^{32}P-labelled phospholipid spots were visualized by overnight autoradiography using X-Omat-R X-ray films and Dupont Cornex intensifying screen. In initial experiments, a 10 μl aliquot of phosphoinositide standards (4 mg/ml) was added to each sample. The spots were also visualized by iodine vapour, and then the autoradiograph was compared to the iodine spots to identify the polyphosphoinositide. The following phospholipids and their relative mobility (R_f) were identified by using authentic lipid standards: PtdIns(4,5)P_2 (R_f= 0.25), PtdIns4P (R_f= 0.31), PtdIns (R_f= 0.46), phosphatidylserine (R_f= 0.57), and phosphatidic acid (R_f= 0.89). PtdIns4P and PtdIns(4,5)P_2 were scraped from the plates, and the radioactivity associated with each spot was determined by scintillation counting. Blanks were carried out under identical conditions except that the membrane proteins were added after terminating the reaction.

In some experiments exogenous PtdIns or PtdIns4P were added to study each kinase activity separately. The exogenous PtdIns or PtdIns4P was prepared by

ultrasonication in a water sonicator (Branson 1200 sonicator) for 30 min and thereafter added to the assay mixture before the preincubation at the concentrations indicated for each experimental protocol.

E. *Phosphoinositide Specific Phospholipase C Assay*

Phospholipase C (PL C) activity was assayed as described elsewhere (Meij *et al.*, 1992). Substrate was prepared by mixing an aliquot of either [^{3}H-]PtdIns(4,5)P_2, [^{3}H-]PtdIns4P or [^{3}H-]PtdIns with an aliquot from the stock solution (in chloroform) of the respective cold substrate. The mixture was evaporated to dryness under a stream of N_2 and redissolved in 10% Na-cholate (232 mM). The substrate solution was kept under N_2 gas overnight at 0-4°C and was diluted to 160 μM substrate/112 mM Na-cholate shortly before use. An aliquot was taken to determine the specific activity.

The PL C assay mixture contained 30 mM HEPES-Tris (pH 7.0), 100 mM NaCl, 2 mM EGTA, 3.13 mM $CaCl_2$ ([free Ca^{2+}]= 1 mM), 15 μg SL protein, 14 mM Na-cholate and 20 μM [^{3}H-]PtdIns(4,5)P_2 , [^{3}H-]PtdIns4P or [^{3}H-]PtdIns (400-500 dpm/μl) in a final volume of 40 μl. The samples were incubated at 37°C for 2.5 min and the reaction was terminated by the addition of 144 μl ice-cold chloroform: methanol: HCl (1:2:0.2,v/v) to each sample. Blanks were carried out under identical conditions except that SL membranes were added after stopping the reaction. Phases were separated by adding 48 μl of 2 M KCl and 48 μl of chloroform (Jackowski *et al.*, 1986). After mixing for 30 sec and 5 min centrifugation in a microcentrifuge (Eppendorf), the upper phase

was aspired and applied to a 300 μl column of Dowex 1X8 (formate form, 100-200 mesh). The columns were rinsed with 0.75 ml of water, then the inositol phosphates were eluted according to Berridge *et al.* (1985) in a gradient steps consisting of 1 ml of each: 5 mM sodium tetraborate in 30 mM sodium formate (to elute Ins), 0.2 M ammonium formate in 0.1 M formic acid (Ins1*P*), 0.4 M ammonium phosphate in 0.1 M formic acid (Ins(1,4)P_2), and finally 1 M ammonium phosphate in 0.1 M formic acid (Ins(1,4,5)P_3). The radioactivity in each eluate was quantitated by liquid scintillation counting in 10 volumes of CytoScint^{TM}ES*.

Free calcium concentrations were determined with the computer program developed by Fabiato (1988).

F. *Pathological Models*

1. Hypo- and hyperthyroidism

Hypothyroidism was induced in male Sprague-Dawley rats weighing 175-200 g. The rats were divided into three groups: euthyroid, hypothyroid and rats with reversed hypothyroidism. The hypothyroid animals received a 0.05% solution of propylthiouracil (PTU) in their drinking water for 7 weeks, while euthyroid groups received only water for the same period of time (Daly *et al.*, 1985). The hypothyroid condition was reversed by injecting, daily for a period of 14 days, the 7-week hypothyroid rats with thyroxine (T_4) (50μg/100g body weight, subcutaneously) dissolved in saline solution. The hypothyroid

animals receiving T_4 (reversal of hypothyroidism) were maintained on 0.05% PTU for the duration of the T_4 treatment (Daly *et al.*, 1987). Age matched control (euthyroid) animals were injected with saline solution.

For comparison, thyroidectomized rats with parathyroid transplant were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA, USA). In these animals (male Spague-Dawley rats, 175-200 g body weight) the thyroid gland has been surgically removed and the concomitantly removed parathyroid were quickly transplanted in the adjacent muscle tissue. The animals were then maintained for 3 weeks on drinking water containing 1 % calcium lactate. Sham-operated (age-matched) rats were used as controls for this group.

The hyperthyroidism studied in male Sprague-Dawley rats (350-400 g) and was induced by daily subcutaneous injection of T_4 (50 μg/ 100g body weight) for 7 days (Williams *et al.*, 1977). Age-matched controls (euthyroid) were injected with saline solution.

All the animals received food and water *ad libitum*. The thyroid status was assessed by measuring the serum levels of both triiodothyronine (T_3) and T_4 at the end of treatment from the above experimental groups. Duplicate aliquots were analyzed for T_3 and T_4 by time resolved Fluoroimmunoassay technique (Wallac, Turku, Finland).

2. Congestive heart failure

Congestive heart failure following myocardial infarction was produced in male Sprague-Dawley rats (200-250 g) by occlusion of the descending left coronary artery as

described by Dixon *et al.* (1990). The animals were anesthetized with ether, the skin incised along the left sternal border, the forth rib was cut proximal to the sternum and retractors were inserted. The pericardial sac was perforated and the heart was exteriorized. The left coronary artery was ligated about 2 mm from its origin by a silk (6.0) suture, and the heart was repositioned in the chest. Throughout the course of the operation, rats were maintained on a positive pressure ventilation delivering a mixture of 95% O_2 and 5% CO_2 mixed with ether. Closure of the wound was accomplished by purse-string suture, and the wound was covered by a mixture of antibiotics. The mortality of all animals operated upon in this fashion was about 35% within 48 h. Sham operated animals were treated similarly except the suture around the coronary artery was not tied. Animals were allowed to recover and were maintained with food and water *ad libitum* for a period of 8 weeks prior to biochemical assessment. To that purpose right and left ventricles from the control and infarcted hearts were separated to isolate the purified sarcolemmal membranes. The scar tissue formed in the left ventricle was removed prior to the isolation procedure. Experimental hearts showing normalized infarcted tissue mass of 30% or more were used in this study. This model have been extensively studied previously (Dixon *et al.*, 1992).

G. *Perfusion of The Hearts*

Experimental animals were killed by decapitation. The hearts were quickly excised and immersed in cold modified Krebs-Henseleit (K-H) buffer saturated by a mixture of

95% O_2-5% CO_2 containing (mM): NaCl 120; $NaHCO_3$ 25; KCl 4.8; KH_2PO_4 1.2; $MgSO_4$ 1.25; $CaCl_2$ 1.25; and glucose 8.6 (pH 7.4) (Gupta *et al.*, 1988). A polyethylene cannula was immediately introduced into the aorta and atrial, fat and connective tissues were removed. The heart was perfused retrogradely with the K-H buffer according to the Langendorff procedure (1895), and the temperature of the perfusion solution was maintained at 37°C. The coronary flow rate was kept constant at 10 ml/min by a Masterflex pump (Cole-Parmer Instrument Co., Chicago, USA), and this flow was monitored during the different interventions by time collection of the coronary effluent. The hearts were electrically stimulated at a constant rate of 330 beats/min using silver electrodes placed at or close to the atrio-ventricular node with 2 msec pulses at 4 Hz and a voltage of 10% above the threshold (Gupta *et al.*, 1987). A latex balloon (Radnoti Glass Technology, INC., CA, USA), connected by a polyethylene catheter to a Statham pressure transducer (model P23XL, Spectramed, CA, USA) was inserted into the left ventricle and secured there. The pressure in the balloon at rest (diastolic pressure) was kept constant at 5 mmHg by filling the balloon with distilled water.

The preparation was left for 15 min to equilibrate in a humidified water (37°C) chamber (non-stabilized preparations were discarded) after which the hemodynamic parameters [maximal left intraventricular pressure (LVP, mmHg) and maximal rates of pressure development (+dP/dt, mmHg/min) and decline (-dP/dt, mmHg/min)] were recorded. The responses were monitored on video, digitized (500 Hz) via an A/D board (TL-125, Axon Instruments, Inc.) and stored in a hard disk in an IBM AT clone computer for analysis. At the end of perfusion the hearts were quickly frozen by a Wollemberger

clamp which was preimmersed in liquid N_2. The frozen ventricles were used to isolate sarcolemmal membranes, and some samples were also used to determine the inositol 1,4,5-trisphosphate levels in the whole tissue. Three perfusion protocols were used.

1. Baseline parameters: hearts from euthyroid and hypothyroid animals were stabilized for 15 min. The contractility parameters were then monitored for 15 min, following which the hearts were freeze clamped for biochemical studies.

2. α_1-Adrenoceptor stimulation: After 15 min stabilization, baseline values of the hemodynamic parameters of euthyroid and hypothyroid hearts were monitored for 2 min. Then, the perfusion medium was switched to a K-H buffer containing 10 μM atenolol, and after 10 min of such perfusion, the contractility parameters were recorded in 2 min. Myocardial perfusion was continued with 10 μM phenylephrine- 10 μM atenolol K-H buffer for 1 min, during which the contractile parameters were recorded. At the peak of positive inotropy the hearts were quickly frozen.

3. ß-Adrenoceptor stimulation: Hearts were stabilized for 15 min with the K-H buffer and contractility parameters were monitored for 14 min. The perfusion was then switched to a K-H buffer containing 0.1 μM isoproterenol for 1 min and the contractile parameters were recorded.

H. *Miscellaneous Assays*

1. Fatty acid composition of phosphoinositides

Freshly obtained ventricular myocardium from adult rats was frozen in liquid N_2 and stored in -80°C. The frozen tissue pieces (total of 10 g) were homogenized for 1.5 min in a microdismembrator (Braun, Melsungen, Germany) at -196°C. The powder was transferred to a glass tube with 9 ml $CHCl_3$:CH_3OH (1:2, v/v) to which 16.8 ml $CHCl_3$:1.2M HCl (1:1, v/v) was then added. After vigorous mixing and centrifugation the interphase and the upper phase were extracted twice with 3 ml $CHCl_3$. The pooled lower phases containing the total tissue lipids were washed once with 6 ml CH_3OH:1 M HCl (1:1, v/v) and then dried under a stream of N_2. The residue was redissolved in CH_3OH:$CHCl_3$ (1:1, v/v) (solvent A) before loading on the neomycin columns, an aliquot was kept for PtdIns separation by direct TLC (see below).

Neomycin sulfate was reductively coupled to reactive porous glass beads as described by Schacht (1981). The neomycin-coated glass beads (2 ml) were packed in small columns, converted to the required salt form, and equilibrated at 0°C with the starting solvent by eluting in order with 3 column volumes each of $CHCl_3$:CH_3OH: 3 M HCl (5:10:2, v/v), $CHCl_3$:CH_3OH:H_2O (5:10:2, v/v) (solvent B), 0.5 M $CHOONH_4$ in solvent B, solvent B, $CHCl_3$:CH_3OH (1:2, v/v) and finally $CHCl_3$:CH_3OH (1:1, v/v). The lipid extracts in solvent A were passed through the columns followed by eluting the phospholipids, except for PtdIns4*P* and PtdIns(4,5)P_2, with 3 column volumes of solvent

A, 3 volumes of CH_3OH:$CHCl_3$ (2:1, v/v), 7 volumes CH_3OH:$CHCl_3$:88% HCOOH (10:5:1, v/v), 2 volumes solvent B and 6 volumes 0.1 M $CHOONH_4$ in solvent B (PtdIns fraction) according to Palmer *et al.* (1981). PtdIns4*P* and PtdIns(4,5)P_2 were eluted with 10 volumes of 1 M $CHOONH_4$ in solvent B. The last two eluates were mixed with 3 ml of 6 M HCl per 10 ml. After centrifugation the resulting lower phase was washed twice with equal volume of CH_3OH: 1 M HCL (1:1, v/v) and dried under N_2. PtdIns4*P* and PtdIns(4,5)P_2 were separated on activated TLC plates (Kieselgel 60 F-254, Merck) developed with $CHCl_3$:CH_3COCH_3:CH_3OH: CH_3COOH:H_2O (40:15:13:12:8, v/v) as described by Jolles *et al.* (1981). The spots were visualized by brief exposure to iodine vapour. The plate was dried under N_2 and the PtdIns4*P* and PtdIns(4,5)P_2 spots were scraped and extracted twice with 1.5 ml CH_3OH for fatty acid analysis. Butylated hydroxytoluene (0.02%, w/v) was included to prevent fatty acid oxydation. The CH_3OH was evaporated under N_2 and the phospholipids were transmethylesterified. Fatty acid methyl esters were analyzed by gas chromatography using a Pye Unicam 102 equipped with a CP-Sil88 (WCOT) capillary column (Chrompack, Delft, The Netherlands) and N_2 gas was used as carrier (Lamers *et al.*, 1992).

The aliquots of the CH_3OH:$CHCl_3$ extracts of rat myocardium kept for PtdIns analysis were directly applied under N_2 to activated TLC plates which were then developed with a solvent $CHCl_3$/CH_3OH/petroleum ether (bp 40-60°C)/acetic acid/boric acid (40:20:30:14:1.8, v/v/v/v/w) containing 0.02% (w/v) butylated hydroxytoluene as described previously (Lamers *et al.*, 1992). The scraped PtdIns spot was extracted twice with 1.5 ml CH_3OH for subsequent fatty acid analysis.

The recoveries of PtdIns and PtdIns4*P* from tissue extracts after elution from the neomycin column and TLC were determined by adding known quantities of unlabelled standards and by employing fatty acid analysis. In-addition the recoveries of [^{3}H]-PtdIns, [^{3}H]PtdIns4*P* and [^{3}H]PtdIns(4,5)P_2 were determined.

2. Primary cultures of neonatal ventricular myocytes

Primary cultures of neonatal ventricular myocytes were prepared from 1-2 day old Wistar rats as described before (Lamers *et al.*, 1992). Cardiomyocytes were seeded in 1.9 cm^2 wells at 150 to 175 x 10^3 cells/cm^2 giving a confluent monolayer of spontaneously contracting cells after 24 hours. The cells were maintained at 37°C and 5% CO_2 in complete growth medium consisting of Ham F10 supplemented with 10% fetal calf serum, 10% horse serum, 200 U penicillin/ml and 0.2 mg streptomycin/ml. Experiments were performed 3-4 days after plating of the cells. Cardiomyocytes were labelled with 0.5 μCi/ml [1-^{14}C]20:4*n*-62 (arachidonic acid) and 2 μCi/ml *myo*-[2-^{3}H]inositol. After the indicated times, incubations were terminated by rapidly washing the cells with ice-cold Ham F10 buffer followed by two successive extractions with ice-cold CH_3OH:HCl (100:1, v/v). Water soluble products were separated from lipids by phase-separation after the addition of 1 volume of $CHCl_3$ and 0.5 volumes of 2.5 M HCl. The resulting organic phase was re-extracted once with 1 volume $CHCl_3$:CH_3OH:0.6 M HCl (3:48:47, v/v) and the upper phases were discarded. The inositol lipids in the organic phase were separated by TLC and were visualized by fluorography after spraying with En^{3}Hance.

Quantification of the separate inositol-containing lipids was carried out by scraping the spots off the plates and counting the scraping in Instagel.

3. α- and ß-Adrenoceptor binding assays

The α and ß adrenoceptor density and dissociation constant (B_{max} and K_D) in the purified sarcolemmal membrane from euthyroid and hypothyroid conditions were quantified according to Bristow *et al.* (1982) using [^{3}H]-prazosin ([^{3}H]-Pz, α-adrenoceptor antagonist) and [^{3}H]-dihydroalprenolol ([^{3}H]-DHA, ß-adrenoceptor antagonist). It is worth noting the [^{3}H]-Pz is a ligand with very high reactivity for α_1-adrenoceptors (Van Zwieten, 1988), and that the α-adrenoceptors of the myocardial cell have been shown to be of the α_1 subtype (Buxton *et al.*, 1986). Sarcolemmal vesicles (50 μg protein/tube) were incubated with six concentrations of either [^{3}H]-Pz or [^{3}H]-DHA. Each tube contained a final concentration of 50 mM Tris, 10 mM $MgCl_2$ (pH 7.4) in a final volume of 0.5 ml. The binding was carried out for 30 min at 37°C. The non-specific binding was measured by adding 5 μM phentolamine hydrochloride (α-adrenoceptor antagonist; Van Zwieten, 1988) or 5 μM l-propranolol (ß-adrenoceptor antagonist; Van Zwieten, 1988) to the incubation medium. Assays were terminated by filtering the tube content by a standard cell-Harvester (Brandel, MD, USA) using Whatman GF/B filters (presoaked in a cold solution of 5 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.4, containing 0.3% polyethylenimine). The filters were then washed thrice with 4.5 ml of ice-cold 5 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.4. The radioactivity of the filters was counted in a Beckman

scintillation counter. The non-specific binding was subtracted from the specific counts. Estimates of the equilibrium binding parameters (K_D, dissociation constant, and B_{max}, maximal density) were obtained from Scatchard plot analysis using the computer program "Ligand" of Munson and Roberts (1980).

4. Inositol 1,4,5-trisphosphate assay

The tissue content of Ins(1,4,5)P_3 was measured using a D-*myo*-inositol 1,4,5-trisphosphate [^{3}H]assay system (Amersham). In brief, a piece of the frozen ventricular tissue was weighed and placed in 0.8 ml of ice-cold 15% (w/v) trichloroacetic acid. The tissue was homogenized with polytron PT-20 for 10 sec at setting 5 as described by Xiang *et al.* (1990). The homogenate was then centrifuged at 2,000 x g for 10 min, and the supernatant was collected and extracted three times with 10 vol of H_2O-saturated diethyl ether. The tubes (containing the water phase) were then placed in a water bath (37°C) for few minutes (Xiang *et al.*, 1990) to evaporate extra diethyl ether. The extracts were titrated to pH 7.5 with 1 mM $NaHCO_3$, and the Ins(1,4,5)P_3 content was then measured in the sample extracts as indicated in the Ins(1,4,5)P_3 assay kit (Amersham). A standard curve was prepared for each experiment by using eight different concentrations of Ins(1,4,5)P_3 (0.19, 0.38, 0.76, 1.5, 3.1, 6.2, 12.5 and 25 pmol). The assay was conducted in duplicate samples containing 100 μl of: assay buffer, deionized water, standard or sample, and [^{3}H]-Ins(1,4,5)P_3. The reaction was then started by addition of 100 μl of the Ins(1,4,5)P_3 binding protein. Total counts were obtained from all the mix without

addition of sample or the binding protein. The non-specific binding was obtained by addition of 100 μl of stock standard, while the blank tubes received all the mixture except the sample or standard. After vortexing and incubation for 15 min on ice, followed by centrifugation at 2000 x g for 10 min at 4°C, the supernatant was discarded, and the pellet was resuspended in 200 μl of water. Then 2 ml of CytoScint^{TM}ES* was added and the radioactivity was measured for 4 min in a scintillation counter.

5. Sulfhydryl group modification

To study the importance of thiol groups in the activities of PtdIns and PtdIns4*P* kinases the SL membranes were pretreated with various thiol-modifying reagents. N-ethylmaleimide (NEM, 0.05 mM) and methylmethanethiosulfonate (MMTS, 0.1 mM) were used to alkylate the sulfhydryl groups. p-Chloromercuriphenylsulfonic acid (pCMPS, 25 μM) was employed to induce mercaptide formation. In some preparations DTT (1 mM) was added along with thiol modifiers to study the protective effect of this synthetic reducing agent against thiol group modification. SL was pretreated with the sulfhydryl modifiers for 10 min at 37°C, and the reaction was stopped by diluting with ice cold sucrose (0.25 M)-histidine (0.01 M), pH 7.4. After centrifugation at 100,000 g for 30 min, the pellet was resuspended in the sucrose-histidine buffer. This step was repeated three times and the SL was used to assay both the kinase activities.

6. Pretreatment of SL with oxidants

The effect of various oxidants on SL PtdIns and PtdIns4*P* kinases was studied by pre-treating the SL membranes with oxyradical generating system or a non-radical oxidants. SL membranes were preincubated (0.3 mg protein/ml) in 10 mM Tris-HCl (pH 7.4) containing the indicated concentrations of reagent, for 10 min at 37 °C. Pretreatment was terminated by cooling on ice, and by two cycles of washing and sedimentation (Beckman type TLA 100.2 rotor; 100,000 x g; 30 min, 4°C) and the resuspended pellet was immediately assayed for the kinases activities as described above.

The action of the superoxide anion radical ($\cdot O_2^-$) was studied by pretreating SL membranes with the xanthine (2 mM) plus xanthine oxidase (0.03 U/ml) system, in the absence or presence of the $\cdot O_2^-$ scavenger SOD (290 U/ml). Furthermore, the effects of HOCl (0.3 mM) were examined. HOCl is a potent non-radical oxidant of sulfhydryl groups (Eley *et al.*, 1989), and is synthesized by myeloperoxidase-catalyzed reactions (Halliwell, 1991) in polymorphonuclear neutrophils. DTT, a synthetic reducing reagent which protects against sulfhydryl group modification (Cleland, 1964), was included in some pretreatment protocols. Also, SL membranes were preincubated with 1 mM H_2O_2 in the absence or presence of the H_2O_2 scavenger catalase (141 U/ml). According to the Fenton reaction (Bast *et al.*, 1991), transformation of H_2O_2 into hydroxyl radicals may occur in the presence of traces of iron. To find out whether the formation of hydroxyl radicals ($\cdot OH$) via the Fenton reaction (Bast *et al.*, 1991) was involved in the H_2O_2 effect, the iron chelator deferoxamine mesylate (1mM) (Gupta *et al.*, 1987) was included in the

pretreatment.

7. Protein assay

The membrane proteins were determined according to Lowry *et al.* (1951) using bovine serum albumin (fraction V) as a standard.

8. Statistics

Results are presented as a mean $\pm$ SEM. All experiments were carried out in duplicate or triplicate determination. The statistical differences between mean values for two groups were evaluated by Student's *t* test. The data from more than two groups were statistically evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. A value of $p < 0.05$ was considered as a significant difference between groups.

IV. RESULTS

A. *Characterization of Rat Heart Sarcolemmal Phosphoinositide Kinases*

The phosphoinositide kinase activities were characterized according to their requirement for detergent, pH, ATP and the divalent cation Mg^{2+}. The presence of detergent has been shown to be important in the activity of both PtdIns 4-kinase and PtdIns4*P* 5-kinases, but inconsistent results were found (Quist *et al.*, 1989, Kasinathan *et al.*, 1989). Furthermore, as discussed above (in Literature Review), PtdIns3-kinase has been recently discovered in non-myocardial cells (Whitman *et al.*, 1987; Yamamoto *et al.*, 1990; Carpenter *et al.*, 1990; Kelly *et al.*, 1992). This enzyme activity, which is profoundly inhibited by nonionic detergent such as Triton X-100 (Whitman *et al.*, 1988; Yamamoto *et al.*, 1990), results in the production of isomers of polyphosphoinositides which are phosphorylated at the D-3 position of the inositol ring. The D-3 kinase, if present in the heart, may potentially interfere with the thin layer chromatographic separation of the D-4 phosphoinositides under study. Therefore, Triton X-100 was used as a tool to discriminate between PtdIns 3- and PtdIns 4-kinases. Increasing the Triton X-100 concentration up to 0.1% significantly increased the activity of PtdIns and PtdIns4*P* kinases (TABLE 1) to a maximum of 12 and 5 fold, respectively. Further increase in Triton X-100 to 0.25% resulted in a decline of the enzyme activities which, however, were still higher than in the absence of detergent. Replacing Triton X-100 by alamethicin resulted in an even higher stimulation of both kinases (TABLE 2). The antibiotic

TABLE 1. Effect of Triton X-100 on PtdIns and PtdIns4*P* kinase activities in rat heart sarcolemma.

Triton X-100	PtdIns kinase	PtdIns4*P* kinase
%	% of Control	
Control	100	100
0.05	1180	396
0.10	1232	500
0.25	659	402

Sarcolemmal membranes were preincubated at 30°C for 30 min with or without Triton X-100 in the presence of 5 mM $MgCl_2$, pH 7.4 as indicated by Quist *et al.* (1989). After adding 0.25 mM [^{32}P]-ATP to the assay mixture, the reaction was terminated after 1 min. The control values (in the absence of Triton X-100) for PtdIns and PtdIns4*P* kinases were 61.05 and 10.28 pmol ^{32}P/mg protein/min, respectively. Results are average of two separate experiments with triplicate determinations (variation was less than 10%).

TABLE 2. Effect of alamethicin on PtdIns and PtdIns4*P* kinase activities in rat heart sarcolemma.

Alamethicin/Protein Ratio	PtdIns Kinase	PtdIns4*P* kinase
	% of Control	
Control	100	100
0.5	643	587
1.0	1801	1159
2.0	1617	757

Sarcolemmal membranes were preincubated at 30°C for 30 min with or without alamethicin in the presence of 5 mM $MgCl_2$, pH 7.4 as indicated by Quist *et al.* (1989). After adding 0.25 mM [^{32}P]-ATP to the assay mixture, the reaction was terminated after 1 min. The control values (in the absence of alamethicin) for PtdIns and PtdIns4*P* kinases were 61.05 and 10.28 pmol ^{32}P/mg protein/min, respectively. Results are average of two separate experiments with triplicate determinations (variation was less than 10%).

alamethicin acts as an ionophore and permeablizes the sarcolemmal vesicles, thus unmasking the latent enzyme activities (Jones *et al.*, 1980). Results in TABLE 2 show that increasing the ratio between alamethicin and membrane protein from 0 to 2 (w/w) increased both PtdIns4*P* and PtdIns(4,5)P_2 formation with an optimal effect at 1:1 ratio (18 and 11 fold increase, respectively). Accordingly, further studies were carried out in the presence of alamethicin (in a 1 μg alamethicin:1 μg protein ratio).

To determine the pH optimum of ^{32}P incorporation in both PtdIns and PtdIns4*P*, SL membranes were incubated at different pH values (from 6 to 9) using established buffer systems. Both enzymes showed a rather broad range (over neutral to a slightly alkaline range) with a maximal activity at pH 7-7.5 (FIGURE 2).

To determine the optimum phosphorylation reaction time, SL proteins were preincubated for 30 min at 30°C with alamethicin. After adding 0.25 mM [^{32}P]-ATP to the assay mixture, the reaction was terminated at different time periods (FIGURE 3). For both kinases the phosphorylation was linear up to 1 min, plateauing at 2 to 10 min. The formation of both PtdIns4*P* and PtdIns(4,5)P_2 was also linear over a concentration range of 15-100 μg SL protein incubated for 1 min (FIGURE 4). The kinetic properties of the kinases were studied by phosphorylating the endogenous phospholipid substrates with 0.1 to 2 mM [^{32}P]-ATP. The Lineweaver-Burk plots of the data obtained in this concentration range were linear (FIGURE 5A and B). The apparent K_m values for ATP were 292 $\pm$ 17 and 398 $\pm$ 25 μM for PtdIns kinase and PtdIns4*P* kinase, respectively, while the correspondent V_{max} values were 1390 $\pm$ 80 and 382 $\pm$ 24 pmol/mg protein/min, respectively. Based on these result, in subsequent experiments 1 mM ATP

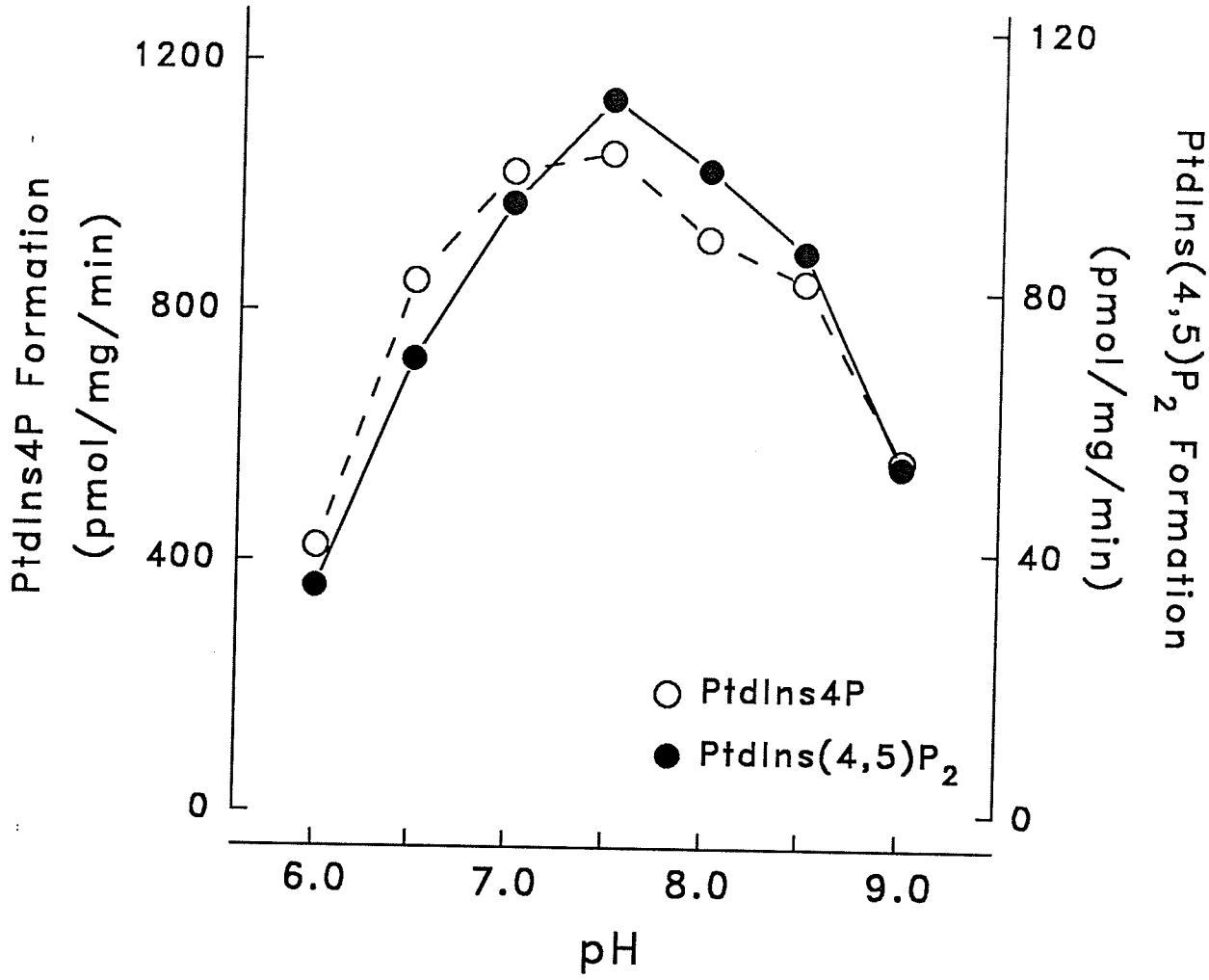

FIGURE 2. Effect of pH on PtdIns and PtdIns4P kinase activities. SL fractions (30 μg) were assayed at 30°C in presence of 5 mM $MgCl_2$, 0.25 mM ATP, 30 μg alamethicin and the following buffers: 40 mM 2-N morpholinoethane sulphonic acid (MES) for pH 6.0-6.5, 40 mM Hepes-Tris for pH 7.0-8.0, and 40 mM Tris for pH 8.5-9.0. Data are from a typical experiment; each point represents the average of triplicate measurements with a variation <10%.

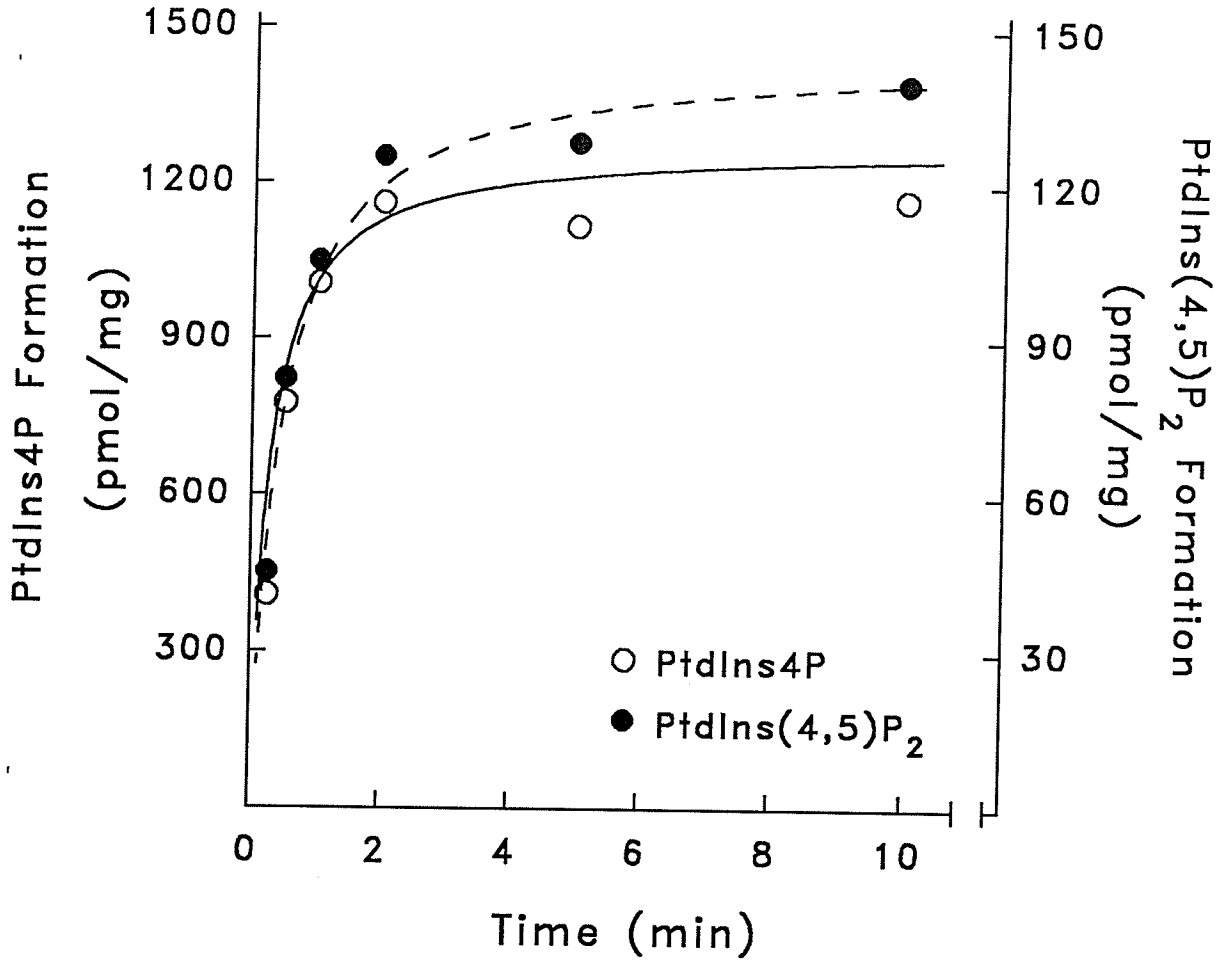

FIGURE 3. Time-course of PtdIns and PtdIns4P kinase activities in rat heart sarcolemma. Membranes were preincubated at 30°C for 30 min with alamethicin (1 mg/mg protein), 2 mM EGTA, 5 mM $MgCl_2$ and 40 mM HEPES buffer, pH 7.4. Phosphorylation was carried out for the indicated times in the presence of 0.25 mM ATP. The phospholipids were then separated as described in "Materials and Methods". Data are from a typical experiment; each point represents the average of triplicate measurements with a variation of less than 10%.

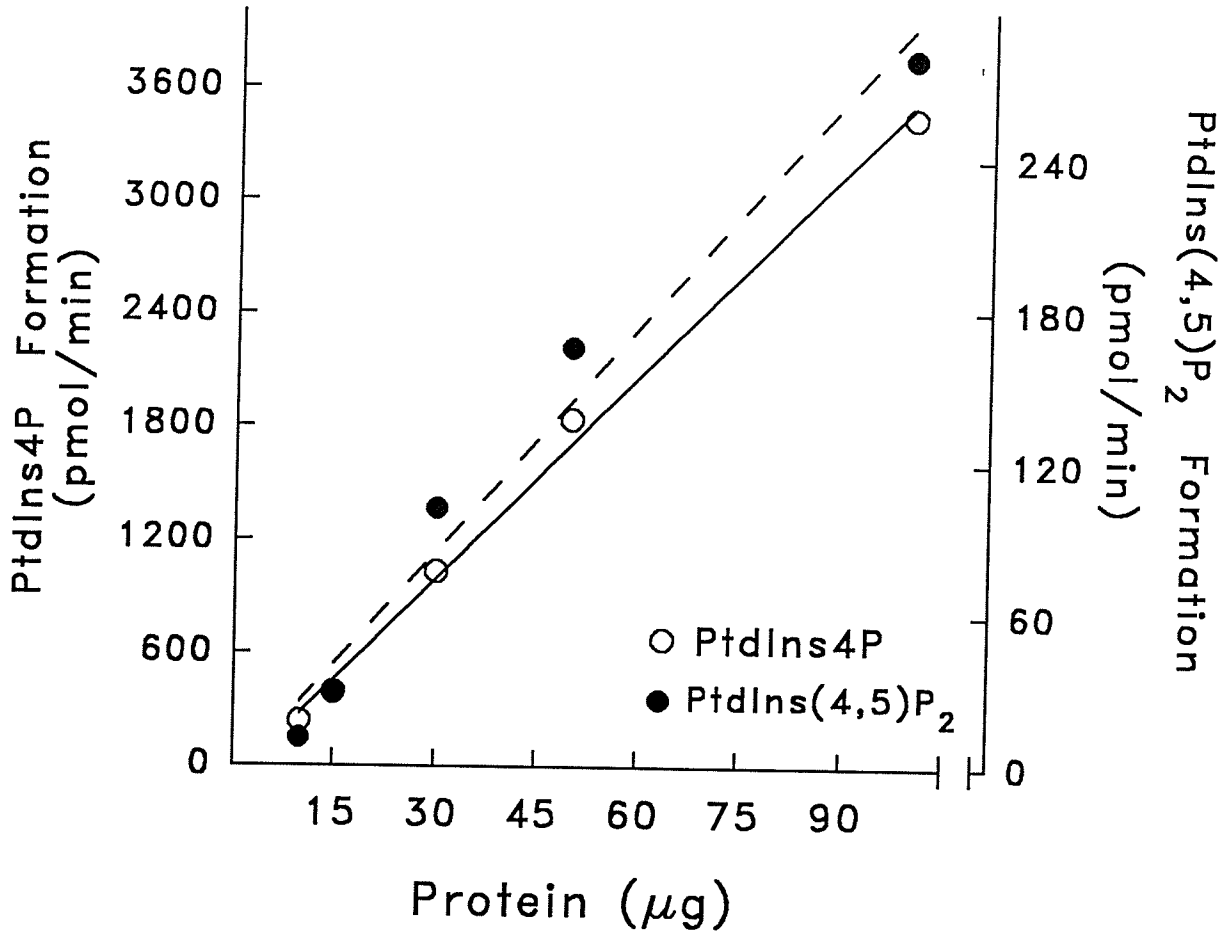

FIGURE 4. Protein dependence of sarcolemmal PtdIns and PtdIns4P kinase activities. The reactions were carried out in the presence of the indicated amounts of SL protein at 30°C for 1 min with alamethicin (1:1 alamethicin to protein ratio), 5 mM $MgCl_2$ and 0.25 mM ATP. Data are from a typical experiment; each point represents the average of triplicate determinations with variation <10%.

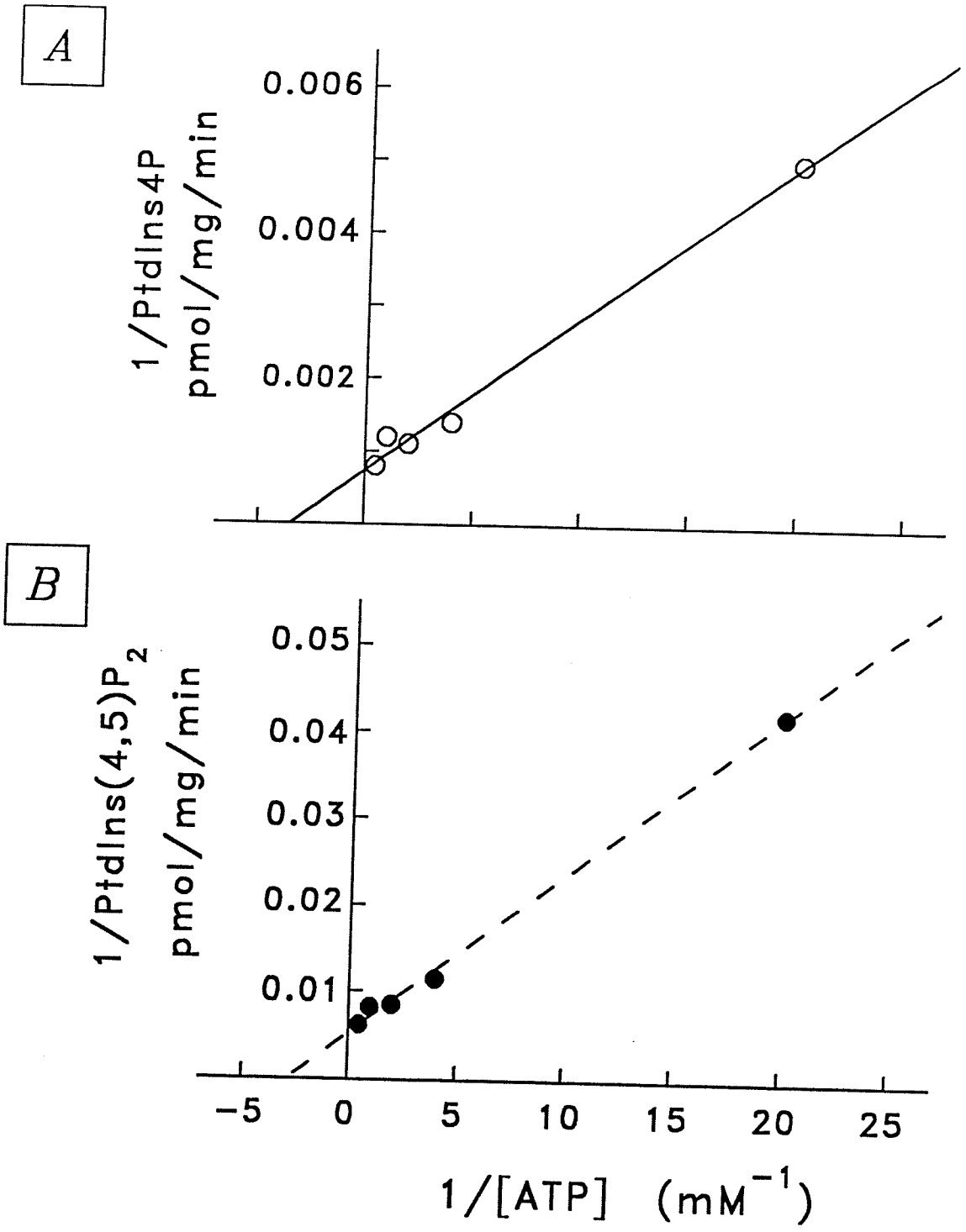

FIGURE 5. Lineweaver-Burk plots of the PtdIns4P (A) and PtdIns(4,5)P_2 (B) formation in rat heart sarcolemma. The SL proteins were incubated in the presence of alamethicin, 5 mM $MgCl_2$, 40 mM HEPES, pH 7.4 and increasing [^{32}P]ATP concentrations (0.1-2 mM). The apparent kinetic parameters (means ± SEM; n=6) were: PtdIns kinase (A), Km= 292 ± 17 μM, Vmax= 1390 ± 80 pmol/mg protein/min; PtdIns4P kinase (B), Km= 398 ± 25μM, Vmax= 382 ± 24 pmol/mg protein/min.

was used in the standard assay for both kinases. The phosphorylation reaction of both PtdIns and PtdIns4*P* were found to be Mg^{2+} dependent in the range of 0.5 to 10 mM $MgCl_2$ (FIGURE 6).

The phosphorylation experiments with endogenous phospholipid substrates showed that the catalytic rate of PtdIns kinase was higher than that of PtdIns4*P* kinase (FIGURES 2-5). This is consistent with previous *in vitro* studies (Quist *et al.*, 1989; Kasinathan *et al.*, 1989) and could be due to either low catalytic rate of PtdIns4*P* kinase or to lack of intramembranal PtdIns4*P* substrate. To clarify this point, exogenous PtdIns4*P* was added to the standard assay medium in a concentration range of 0 to 200 μM. A progressive, concentration dependent increment in the rate of PtdIns(4,5)P_2 synthesis was observed and reached a plateau at 100 μM exogenous PtdIns4*P* (FIGURE 7, inset). The apparent Km (for exogenous PtdIns4*P*) and Vmax values of the PtdIns4*P* kinase were 66 $\pm$ 5 μM and 515 $\pm$ 44 pmol/mg protein/min, respectively (FIGURE 7).

1. The effect of free Ca^{2+} on phosphoinositide kinases

It has been established that the phosphoinositides are located in the inner, cytoplasmic leaflet of the cardiac SL membrane (Post *et al.*, 1988). Therefore, in the beating heart cell, the phosphoinositide kinase activities can be seen to operate in the same region (Quist *et al.*, 1989), and to be exposed to intracellular levels of free Ca^{2+} ranging from 10^{-7} M during rest to 10^{-5} M in response to excitation (Dhalla *et al.*, 1982; Wier, 1990). In view of potential feedback modulation of these kinases by the increased

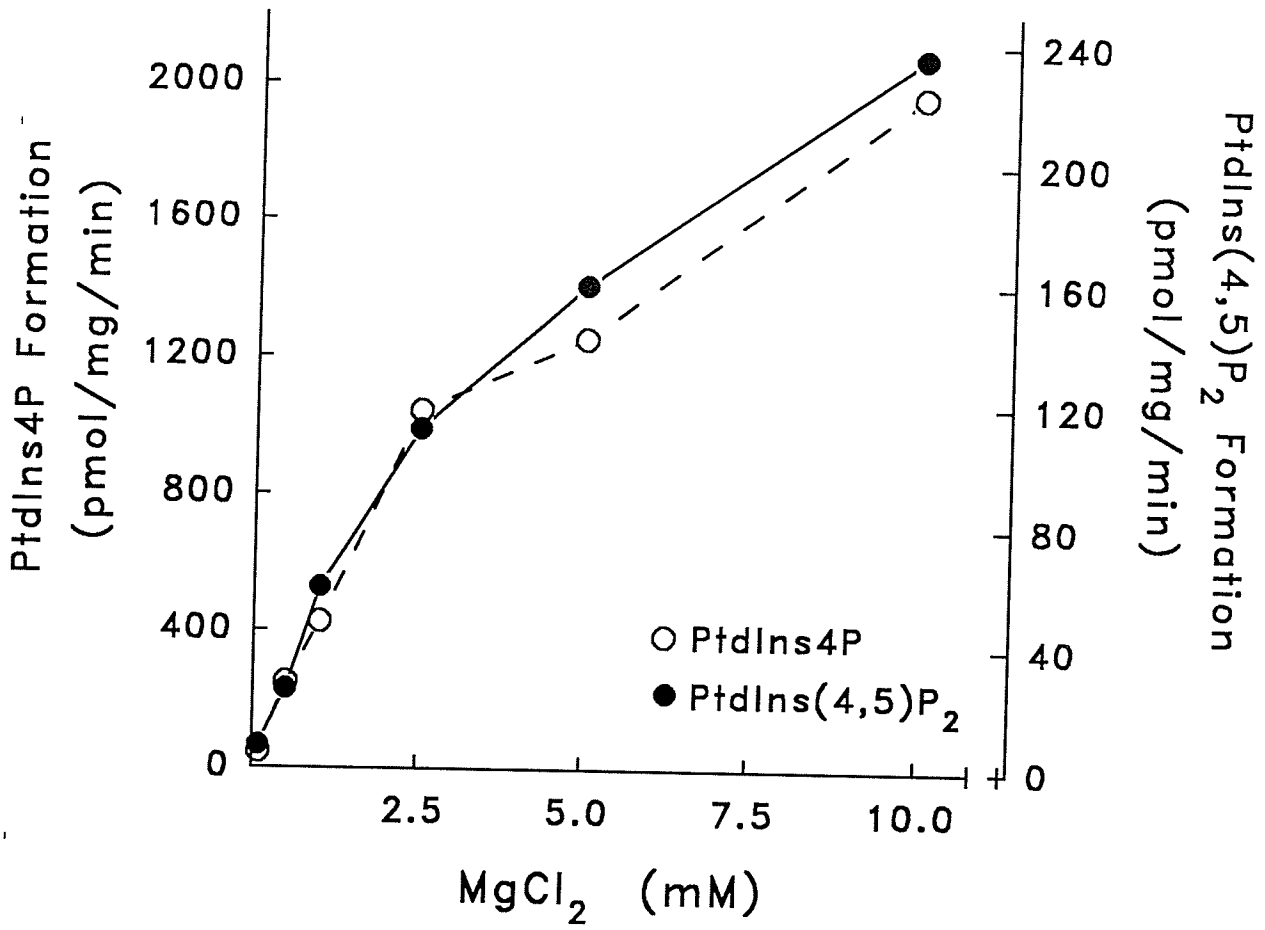

FIGURE 6. Mg^{2+}-dependence of PtdIns4P and PtdIns(4,5)P_2 formation in rat heart sarcolemma. The reaction was carried out for 1 min in presence of 1 mM ATP at 30°C after 30 min preincubation of 30 μg SL protein with 30 μg alamethicin and varying amounts of $MgCl_2$. Data are from a typical experiment; each point represents the average of triplicate measurements with a variation <10%.

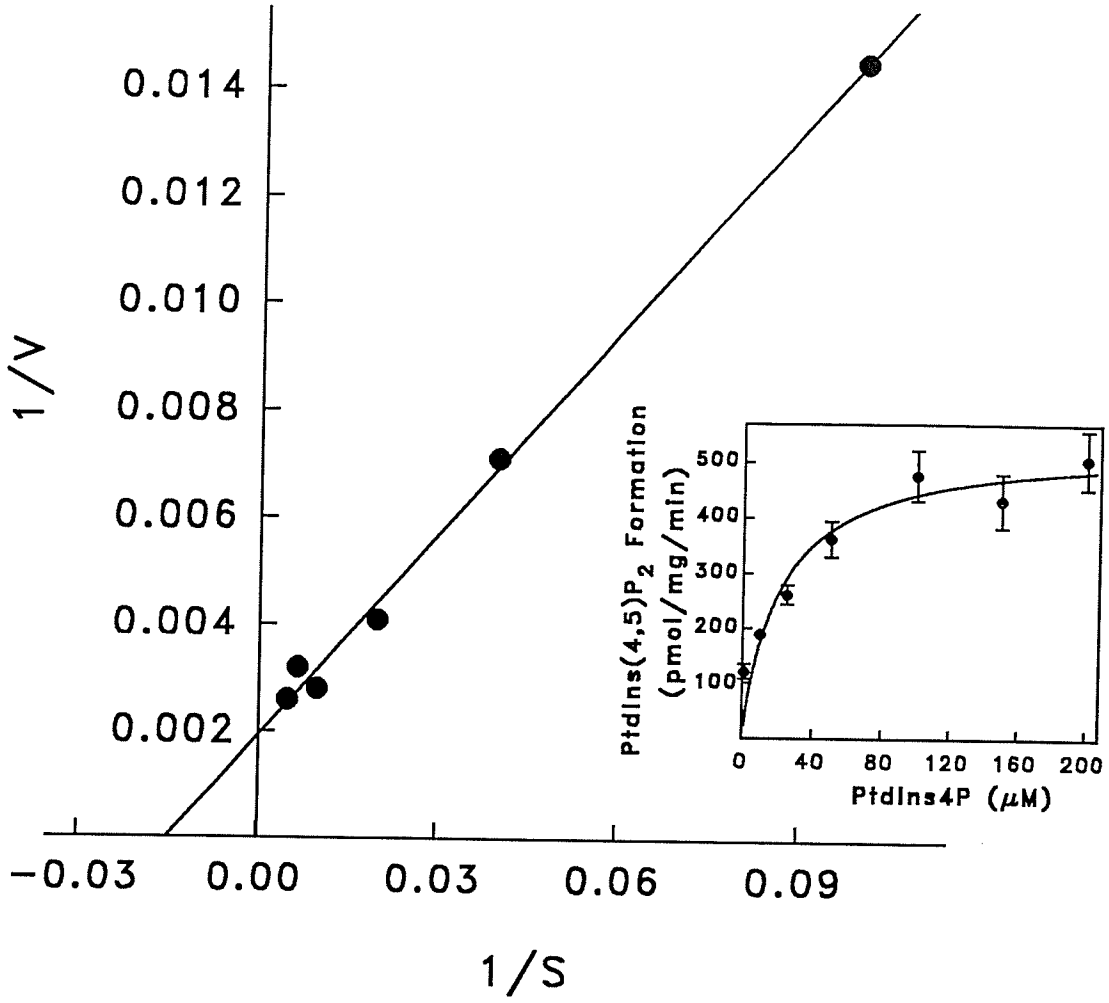

FIGURE 7. Effect of exogenous PtdIns4P on PtdIns4P kinase activity. The exogenous PtdIns4P was prepared by ultrasonication (30 min with Branson 1200 sonicator) and thereafter added to the assay mixture at the indicated concentrations. The reaction was carried out as described in "Materials and Methods". Lineweaver-Burk plots were constructed from the data shown in the inset by subtracting the value at 0 PtdIns4P (due to phosphorylation of the endogenous substrate) from each of the other values. Each point represents the mean ± SEM of five different experiments.

cytosolic Ca^{2+} level which follows the receptor-mediated activation of the phosphoinositide cycle (Rana and Hokins 1990; Meij and Panagia, 1991), we investigated the effect of different free Ca^{2+} concentrations (10^{-9} to 10^{-4}M) on SL PtdIns and PtdIns4*P* phosphorylation (FIGURE 8 A and B). PtdIns and PtdIns4*P* kinase were not affected at 0 to 0.5 μM Ca^{2+}, while a significant inhibition of both kinases occurred, in a concentration dependent manner, at 1 to 100 μM Ca^{2+}. At the latter concentration, the formation of PtdIns4*P* and PtdIns(4,5)P_2 was 40% and 30% of the correspondent control values, respectively.

The aminoglycoside antibiotic, neomycin, is known to bind specifically to the negatively charged PtdIns4*P* and PtdIns(4,5)P_2 (Sastrasinh *et al.*, 1982), thus inhibiting their hydrolysis by phospholipase C (Siess *et al.*, 1986), and probably, their dephosphorylation by specific polyphosphoinositide monophosphoesterases (Rana and Hokins, 1990; Downes and MacPhee, 1990; Meij and Panagia, 1991). Since the decline in phosphorylation products by increasing the free Ca^{2+} concentration (FIGURE 8A and B) could be related to their degradation due to the activation of phospholipase C by Ca^{2+} (Edes and Kranias, 1990; Meij and Panagia, 1992), the above experiments were repeated in the presence of neomycin (FIGURE 8A and 8B). As shown in FIGURE 9, addition of neomycin in a range of 0 to 1 mM in the incubation medium increased both PtdIns4*P* and PtdIns(4,5)P_2 production up to 200 and 180% of the control, respectively. However, neomycin did not modify appreciably the inhibitory effect of Ca^{2+} (FIGURE 8A and 8B); this indicates that the Ca^{2+} -related depression of SL phosphoinositide kinases can not be attributed to any activation of PL C and/or polyphosphoinositide

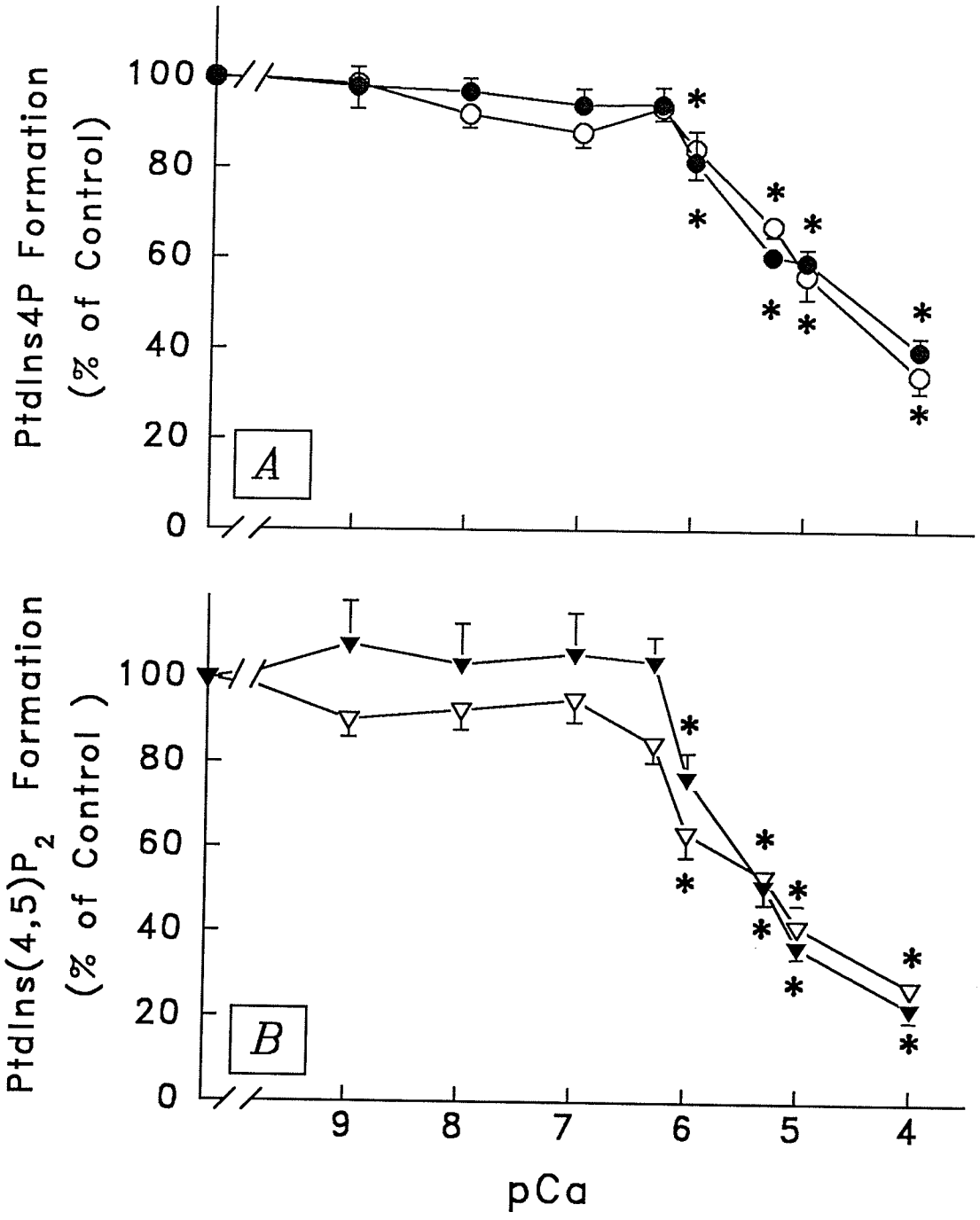

FIGURE 8. Effect of Ca^{2+} on PtdIns (A) and PtdIns4P (B) kinase activities in the presence (●,▼) and absence (○,▽) of 0.25 mM neomycin. The reaction was carried out as described in "Materials and Methods". $CaCl_2$-EGTAbuffer was present to give the indicated free Ca^{2+} concentrations. Control values for PtdIns4P and PtdIns(4,5)P_2 formation in the absence of neomycin were 1101 ± 78 and 115 ± 13 pmol/mg protein/min, respectively, while in the presence of 0.25 mM neomycin were 1409 ± 88 and 148 ± 14 pmol/mg protein/min. Each point represents the mean ± SEM of three to five separate experiments performed in triplicate.

* Significantly different ($p < 0.05$) from control values.

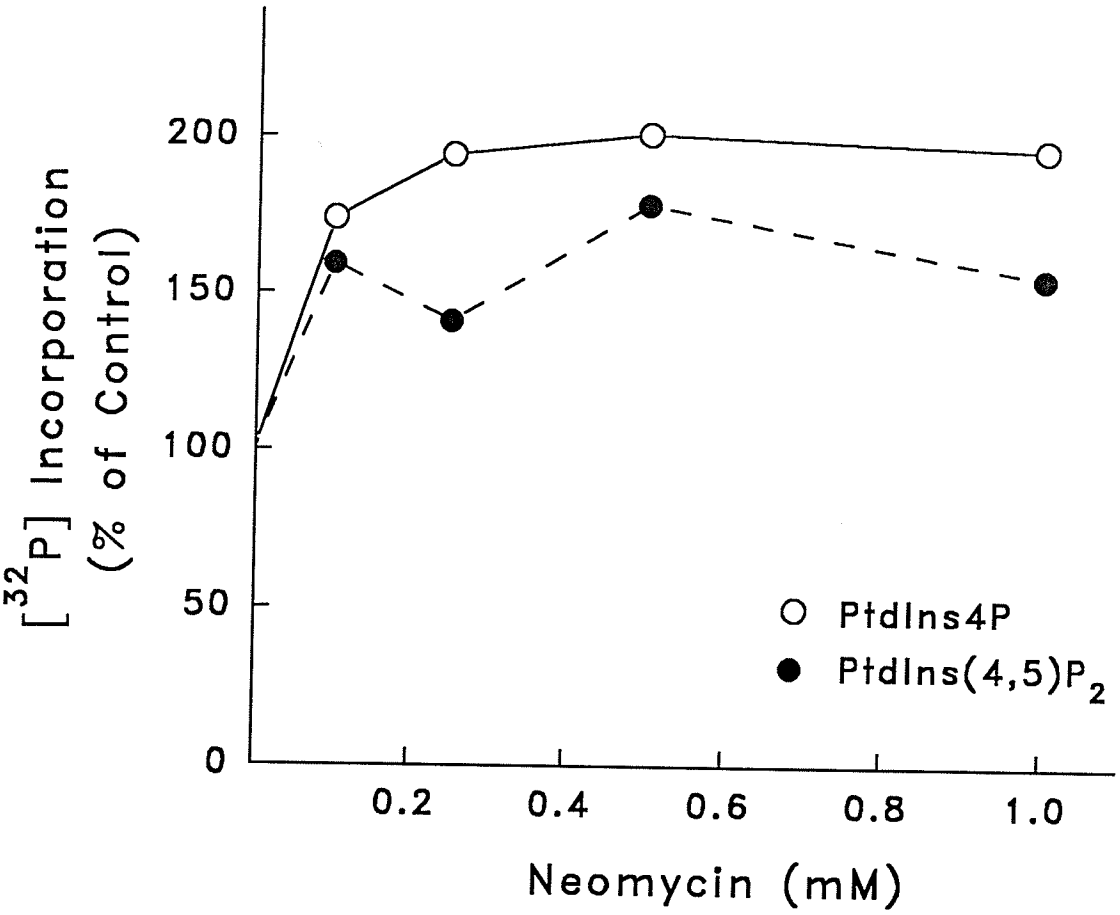

FIGURE 9. Effect of neomycin on PtdIns4P and PtdIns(4,5)P_2 formation in sarcolemma from rat heart. Neomycin was added to the incubation medium at the concentration indicated in the figure. Assay condition were as described in "Materials and Methods". Data are from a typical experiment; control values for PtdIns4P and PtdIns(4,5)P_2 formation were 1063.04 ± 89.0 and 102 ± 7.8 pmol/mg protein/min.

monophosphoesterases by Ca^{2+}. Hypothetically, Ca^{2+} may compete with Mg^{2+} for ATP with resultant inhibition of the kinase activities. This possibility seems unlikely because Ca^{2+} significantly affects both kinases at concentrations (10^{-6}-10^{-5}M, FIGURE 8) markedly lower than the concentration of Mg^{2+} in the assay medium. The PtdIns4*P* kinase appears to be more sensitive to Ca^{2+} than PtdIns kinase (IC_{50} values of the Ca^{2+} effect in the absence of neomycin were 6.3 and 20.0 μM, respectively).

2. Effect of sulfhydryl modification on PtdIns and PtdIns4*P* kinases

Chemical modification of the thiol groups (SH) was used as an approach to study the importance of these groups for the function of myocardial PtdIns and PtdIns4*P* kinase activities. NEM (0.05 mM) and MMTS (0.1 mM), which differ in structure and mode of action (Smith *et al.*, 1975), were used as thiol group alkylating agents, while mercaptide formation was achieved by treatment of SL proteins with pCMPS (25 μM). In a parallel series of experiments, dithiothreitol (DTT, 1 mM) was present during pretreatment of the membranes with thiol modifiers to check the specificity of the changes. As shown in TABLE 3, both types of thiol modification induced a significant inhibition of the phosphoinositide kinases. The inhibition of PtdIns kinase was strongest when SH groups were alkylated, while mercaptide formation had similar action on both PtdIns and PtdIns4*P* kinases. DTT prevented almost completely the inhibitory actions of NEM and MMTS on both kinases; the DTT protection of PtdIns4*P* kinase from pCMPS action was partial but statistically significant ($P < 0.05$).

TABLE 3. Effect of sulfhydryl modifiers on PtdIns kinase and PtdIns4P kinase activities in rat heart sarcolemma.

Experimental conditions	Enzyme Activities (% of control)	
	without DTT	with DTT
a) PtdIns kinase		
Control	100.0 ± 1.7	100.0 ± 9.9
NEM	6.8 ± 0.4*	89.6 ± 3.5
MMTS	9.1 ± 0.5*	75.3 ± 3.8*
pCMPS	26.9 ± 1.1*	95.9 ± 7.8
b) PtdIns4*P* kinase		
Control	100.0 ± 3.7	100.0 ± 2.2
NEM	36.5 ± 2.0*	114.7 ± 10.1
MMTS	38.5 ± 2.1*	96.8 ± 5.2
pCMPS	19.2 ± 1.5*	58.6 ± 2.9*

SL membranes were preincubated with different thiol modifiers in the absence or presence of 1 mM DTT. Thereafter, membranes were washed and assayed for the kinase activities as indicated in "Materials and Methods". Final concentrations of the thiol modifiers were : *N*-ethylmaleimide (NEM, 0.05 mM); methylmethanethiosulfonate (MMTS, 0.1 mM); p-chloromercuriphenylsulfonic acid (pCMPS, 25 μM). Values are expressed as percent of control without or with DTT, and are mean ± SEM of three experiments in triplicate.

* Significantly different ($P < 0.05$) from the control values.

3. Fatty acid composition of phosphoinositides of rat myocardium

We also investigated the products of the kinases' activity for the following reasons. PtdIns is synthesized at the SR and it is transported to the SL by a PtdIns-specific transfer protein (Abdel-Latif, 1986; Wolf, 1990). Once at the SL, PtdIns acquires its characteristic fatty acid composition of predominantly stearic acid (18:0) and arachidonic acid (20:4*n*-6) by deacylation cycle (Holub and Kuksis, 1970). It is generally assumed that the fatty acid composition of PtdIns and its D-4 kinase synthesized derivatives is identical. However, the few studies available show that the phosphoinositides of brain (Holub *et al.*, 1970) and erythrocytes (Chiba *et al.*, 1988) display the same fatty acid profile, unlike those of hepatocytes (Augert *et al.*, 1989). No information is available on the fatty acid composition of myocardial phosphoinositides. Since it has been shown that one of the fatty acid acyl chain of DAG, which may originate from PtdIns(4,5)P_2 hydrolysis (Berridge, 1986), must be unsaturated for optimal activation of protein kinase C (Nishizuka, 1988; Bell and Burns, 1991; Allen and Katz, 1991), we characterized the fatty acid profiles of phosphoinositides in homogenates of rat ventricular myocardium. The amount (quantitated by fatty acid analysis) of PtdIns4*P* and PtdIns(4,5)P_2 extracted from rat myocardium and purified by neomycin columns and TLC were 11.6 $\pm$ 1.1 (n=5) and 14.6 $\pm$ 2.1 (n=5) nmol/g wet weight, respectively. The recovery of PtdIns(4,5)P_2 after column elution and TLC was about 70% as assessed both with [^{3}H]-PtdIns(4,5)P_2 and unlabelled PtdIns(4,5)P_2 standards. The PtdIns fraction isolated by neomycin-coated glass bead column and TLC appeared to be always contaminated by

some endogenous cardiolipin. This resulted mainly in increases of the relative linoleic acid (18:2*n*-6) content of PtdIns. This problem was circumvented by direct separation of PtdIns from the bulk of phospholipids by TLC. The amount of PtdIns extracted from rat was 1485 ± 52 (n=4) nmol/g wet weight. The fatty acid composition of rat myocardial PtdIns4*P* and PtdIns(4,5)P_2 showed significant differences from that of PtdIns (FIGURE 10). The mol percentages (i.e. 100 x mole fraction of the compound in a mixture) of the major fatty acids, stearic (18:0), linoleic (18:2*n*-6) and arachidonic (20:4*n*-6) acid were respectively, 48.8 ± 0.6, 6.9 ± 0.4 and 26.0 ± 1.3 for PtdIns while these values for PtdIns(4,5)P_2 were 63.3 ± 3.2, 4.0 ± 0.7, and 18.4 ± 1.5 (FIGURE 10). The fatty acid composition of PtdIns4*P* was similar to that of PtdIns(4,5)P_2.

In view of the cell heterogeneity of the intact heart we also measured the incorporation of [^{14}C]20:4*n*-6 into the inositol phospholipids of cultured rat ventricular myocytes. Cardiomyocytes were labelled with [^{14}C]20:4*n*-6 and [^{3}H]inositol for 1, 5 and 25 h, then the incorporation of ^{14}C and ^{3}H into the various inositol lipids was determined (TABLE 4). The absolute incorporation of both radioactive tracers increased almost linearly with time in PtdIns, PtdIns4*P*, and PtdIns(4,5)P_2 during the 25 h period. The incorporation of [^{14}C]20:4*n*-6 relative to that of [^{3}H]inositol, however, showed a clear tendency to plateau. The incorporation ratio's (^{14}C/^{3}H) of PtdIns4*P* and PtdIns(4,5)P_2 were always lower than that of PtdIns even after prolonged incubation (25 h).

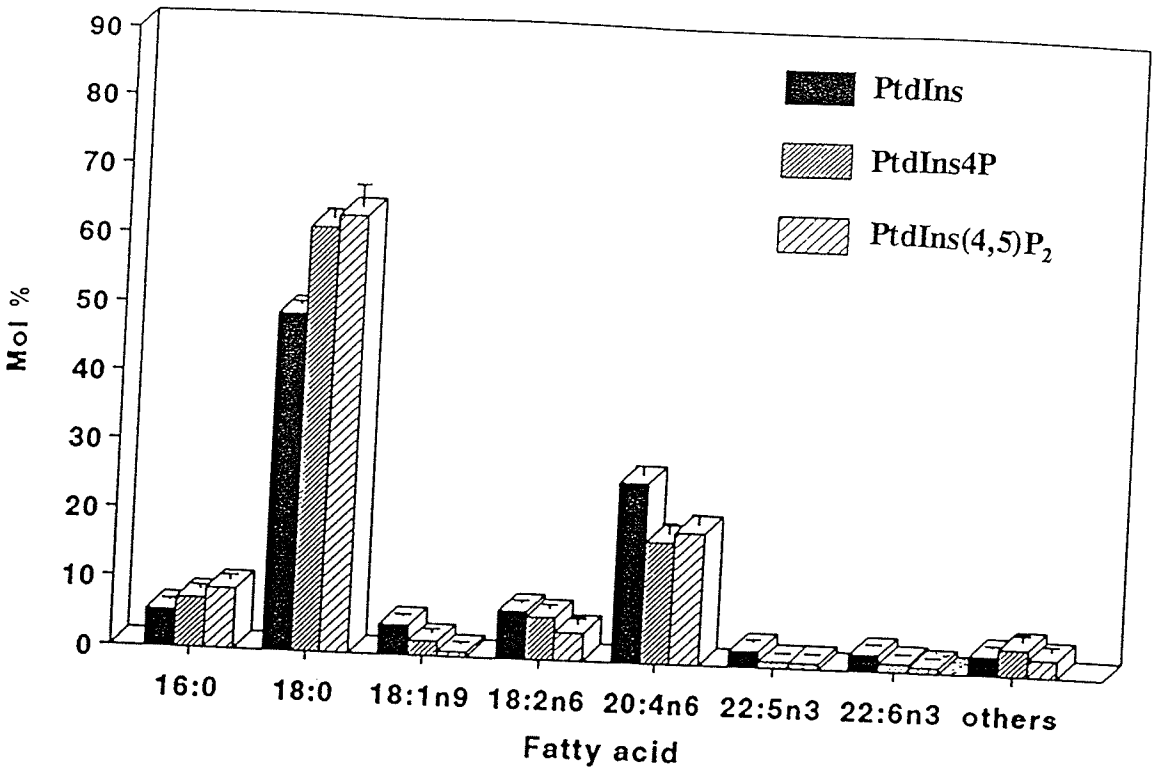

FIGURE 10. Fatty acid composition of the phosphoinositides extracted from rat left ventricular homogenate. Dma is the abbreviation for dimethylacetal. The fatty acids are: palmitic (16:0), stearic (18:0), oleic (18:1n-9), linoleic (18:2n-6) and arachidonic (20:4n-6). Only mol % of major fatty acids are illustrated, and the sum (%) of minor fatty acids (mainly 18:0dma, 18:1n-7, 18:3n-6, and 20:3n-6) is termed "others". The result shown are the mean ± SEM of 4 determinations. All differences of PtdIns versus PtdIns4P and PtdIns(4,5)P_2 are significant ($P < 0.05$) except for "others" and 18:1n-9 of PtdIns(4,5)P_2.

TABLE 4. Time course of incorporation of [1-^{14}C]20:4*n*-6 and *myo*-[2-^{3}H]inositol into the phosphoinositides of cultured rat ventricular myocytes.

	Time (h)	PtdIns	PtdIns4*P* (dpm/well x 10^{-3})	PtdIns(4,5)P_2
[^{14}C]20:4*n*-6				
	1	11.11 ± 0.14	0.40 ± 0.01	0.49 ± 0.02
	5	31.39 ± 1.04	0.99 ± 0.03	1.43 ± 0.07
	25	83.17 ± 2.91	2.24 ± 0.10	3.16 ± 0.07
[^{3}H]inositol				
	1	3.07 ± 0.13	0.14 ± 0.01	0.24 ± 0.01
	5	13.11 ± 0.38	0.49 ± 0.02	0.84 ± 0.04
	25	51.75 ± 0.83	1.66 ± 0.07	2.60 ± 0.05
Incorporation ratio(^{14}C/^{3}H)			([^{14}C]20:4*n*6/[^{3}H]inositol)	
	1	3.65 ± 0.11	3.03 ± 0.23*	2.05 ± 0.09*
	5	2.40 ± 0.07	2.03 ± 0.07*	1.60 ± 0.05*
	25	1.61 ± 0.06	1.35 ± 0.03*	1.22 ± 0.02*

Data are presented as means ± SEM from 7 experiments.

* Significantly different ($P < 0.05$) *vs.* PtdIns.

B. *Hypothyroidism and Hyperthyroidism*

1. General characteristics of hypo-and hyperthyroidism

The serum level of T_3 and T_4 as well as the ventricular to body weight ratio were monitored in normal and experimental animals (TABLE 5). Hypothyroid animals both PTU-fed or thyroidectomized, showed a significant depression of T_3 and T_4 levels compared to euthyroid animals. The ventricular to body weight ratio did not change in the hypothyroid state. This could be attributed to a reduction of both heart and body weight. On the other hand, the hyperthyroid rats showed a marked elevation in the serum levels of T_3 and T_4, as well as a significant increase of the ventricular to body weight ratio (TABLE 5). When the hypothyroid state was reversed by daily injection of T_4 for 14 days upon maintaining the PTU treatment, the increase in heart weight was faster than the increase in body weight (TABLE 5). Unlike T_4, the serum level of T_3 in these animals did not increase because PTU inhibits the conversion of T_4 to T_3 (Chizzonite *et al.*, 1984). To assess the purity of the SL membrane preparations isolated in different thyroid states, homogenate and SL fractions were examined for the marker enzyme activities (TABLE 6). The SL marker enzyme ouabain-sensitive K^+-pNPPase showed a 10 fold increase in SL *versus* homogenate in the euthyroid hearts. This enrichment factor was not changed in hyperthyroidism while it was increased in hypothyroid SL preparations. The activities of both cytochrome *c* oxidase (mitochondrial marker) and rotenone-insensitive NADPH-cytochrome *c* reductase (SR marker) in

TABLE 5. General characteristics of experimental rats in different thyroid states.

Experimental Condition	H. Wt./B. Wt. x 1000	T_4 (nmol/L)	T_3 (nmol/L)
Euthyroid	2.18 ± 0.04	62.0 ± 1.8	1.30 ± 0.10
Hypothyroid:			
A. PTU-treated	2.05 ± 0.03	19.7 ± 0.7*	0.55 ± 0.05*
B. Thyroidectomy	2.15 ± 0.02	23.0 ± 2.1*	0.60 ± 0.09*
Reversal of Hypothyroid (PTU)	2.50 ± 0.05*	>300[a]	0.78 ± 0.09*
Hyperthyroid	3.28 ± 0.06*	>300[a]	>3[a]

Data are expressed as means ± SEM of 10-20 rats. Animals were treated as indicated in "Materials and Methods". T_3 (triiodothyronine) and T_4 (thyroxine) levels were determined by time-resolved fluoroimmunoassay technique (Wallac, Finland). H.Wt. = heart weight; B.Wt. = body weight.

* Significantly different ($P<0.05$) from the euthyroid state.

[a] Values were greater than the upper limit of the assay.

euthyroid SL preparation were 0.61 and 0.58 fold of their respective homogenate values. In addition, these values were not changed with different thyroid state (TABLE 6).

2. [^{3}H]-Dihydroalprenolol and [^{3}H]-prazosin binding in hypothyroid SL

As discussed above (Literature Review), thyroid hormone alters the density of cardiac adrenoceptors. While the changes of α_1-adrenoceptor density in hyperthyroidism have been consistent (Ciaraldi *et al.*, , 1978; Sharma *et al.*, 1978; Kunos, 1980; Chang *et al.*, 1981) the reports on myocardial α_1-adrenoceptors in hypothyroidism have been conflicting (Ciaraldi *et al.*, 1978; Noguche *et al.*, 1983; Kunos, 1980; Fox *et al.*, 1985). Therefore, the binding characteristics of SL ß- and α_1-adrenoceptor were examined in eu- and hypothyroid animals and the results are given in TABLE 7. The K_D value for [^{3}H]-dihydroalprenolol binding was similar in the euthyroid and hypothyroid SL, while the B_{max} value was significantly decreased. The K_D values of [^{3}H]-prazosin binding to euthyroid and hypothyroid SL were not significantly different, the B_{max} value being significantly increased in hypothyroidism (TABLE 7).

3. PtdIns and PtdIns4*P* kinases

As already mentioned, both PtdIns and PtdIns4*P* kinase activities are important to provide the phospholipid substrates for the receptor-mediated phospholipase C action. In view of our aim to study the enzymes involved in the phosphoinositide pathway,

TABLE 6. Marker enzyme activities in heart sarcolemma from rats in different thyroid states.

	Ouabain-sensitive K^+-pNPPase (a)	Cytochrome *c* oxidase (b)	Rotenone-insensitive NADPH-cytochrome *c* reductase (c)
Euthyroid	1.96± 0.29 (10)	55.44 ± 3.41 (0.61)	4.06 ± 0.61 (0.58)
Hypothyroid (PTU)	2.31± 0.24 (12)	57.24 ± 8.94 (0.77)	3.39 ± 0.33 (0.55)
Hypothyroid (Thyroidectomy)	1.95± 0.25 (13)	66.70 ± 9.87 (0.71)	5.63 ± 0.83 (0.65)
Reversal of PTU-Hypothyroid	1.90± 0.15 (9)	70.09 ± 8.72 (1.0)	5.34 ± 0.77 (0.73)
Hyperthyroid	2.33± 0.12 (10)	69.17 ± 5.87 (0.73)	6.54 ± 0.78 (0.78)

Values are means ± SEM of three to four different membrane preparations and are expressed as (a) μmol pNPP/mg/h, (b) nmol cytochrome *c*/mg/min; (c) nmol cytochrome *c* reduced/mg/min. Sarcolemmal membranes were isolated according to Pitts (1979). Data in the parentheses indicate the enrichment factor (specific activity in the SL/specific activity in the homogenate). Assays were performed as indicated in the "Materials and Methods". Ouabain-sensitive K^+-pNPPase activity was determined in the presence of alamethicin (1 mg/mg membrane protein).

TABLE 7. Characteristics of the bindning of ^{3}H-dihydroalprenolol and ^{3}H-prazosin to purified sarcolemmal vesicles prepared from euthyroid and hypothyroid rat hearts.

	Euthyroid	Hypothyroid
A. [^{3}]H-Dihydroalprenolol		
K_D (nM)	0.83 ± 0.11	0.79 ± 0.14
B_{max} (fmol/mg)	259.89 ± 25.20	140.2 ± 22.30*
B. [^{3}H]-Prazosin		
K_D (nM)	0.15 ± 0.01	0.22 ± 0.06
B_{max} (fmol/mg)	209.90 ± 44.9	352.29 ± 35.10*

Data are expressed as mean ± SEM of five to eight experiments performed in duplicate as described in "Materials and Methods". K_D, dissociation constant; B_{max}, maximal density.

* Significantly different ($P < 0.05$) from the respective euthyroid.

both the SL PtdIns and PtdIns4*P* kinases were studied in euthyroid, hypothyroid (PTU-treated, thyroidectomy), and hyperthyroid rats.

FIGURE 11 shows the time-dependence of PtdIns (FIGURE 11A) and PtdIns4*P* (FIGURE 11B) kinases in euthyroid and hypothyroid (PTU-treated) SL. The enzyme activities were not changed in hypothyroidism when the reactions were examined over a range of 0.5 to 5 min. The phosphorylation of PtdIns showed no change in ATP dependency (FIGURE 12) and no significant changes in the apparent K_m and V_{max} values (FIGURE 12, inset) in 0.05 to 2.0 mM ATP range. PtdIns(4,5)P_2 formation in hypothyroid SL was slightly higher than in the euthyroid SL. However, this increase was not significant (FIGURE 13), and the apparent K_m and V_{max} values were similar (FIGURE 13, inset).

PtdIns and PtdIns4*P* kinases of euthyroid and hypothyroid SL were also studied in the presence of their exogenously added phospholipid substrates. In this set of experiments, thyroidectomized and hyperthyroid SL preparations were also examined for comparison. PtdIns phosphorylation was not changed in hypothyroidism (both PTU-treatment and thyroidectomy) under standard conditions (0 PtdIns, FIGURE 14). On the other hand, hyperthyroidism induced a significant increase in the PtdIns phosphorylation. To test if PtdIns kinase was affected by the intramembranal level of its phospholipid substrate, PtdIns (final concentration, 25 μM) was added to the assay medium (FIGURE 14). This resulted in an increase of the PtdIns4*P* formation which was significant in euthyroid and hypothyroid SL but not in the hyperthyroid SL. A similar trend was observed when SL PtdIns4*P* kinase was tested in the absence or presence of 25 μM

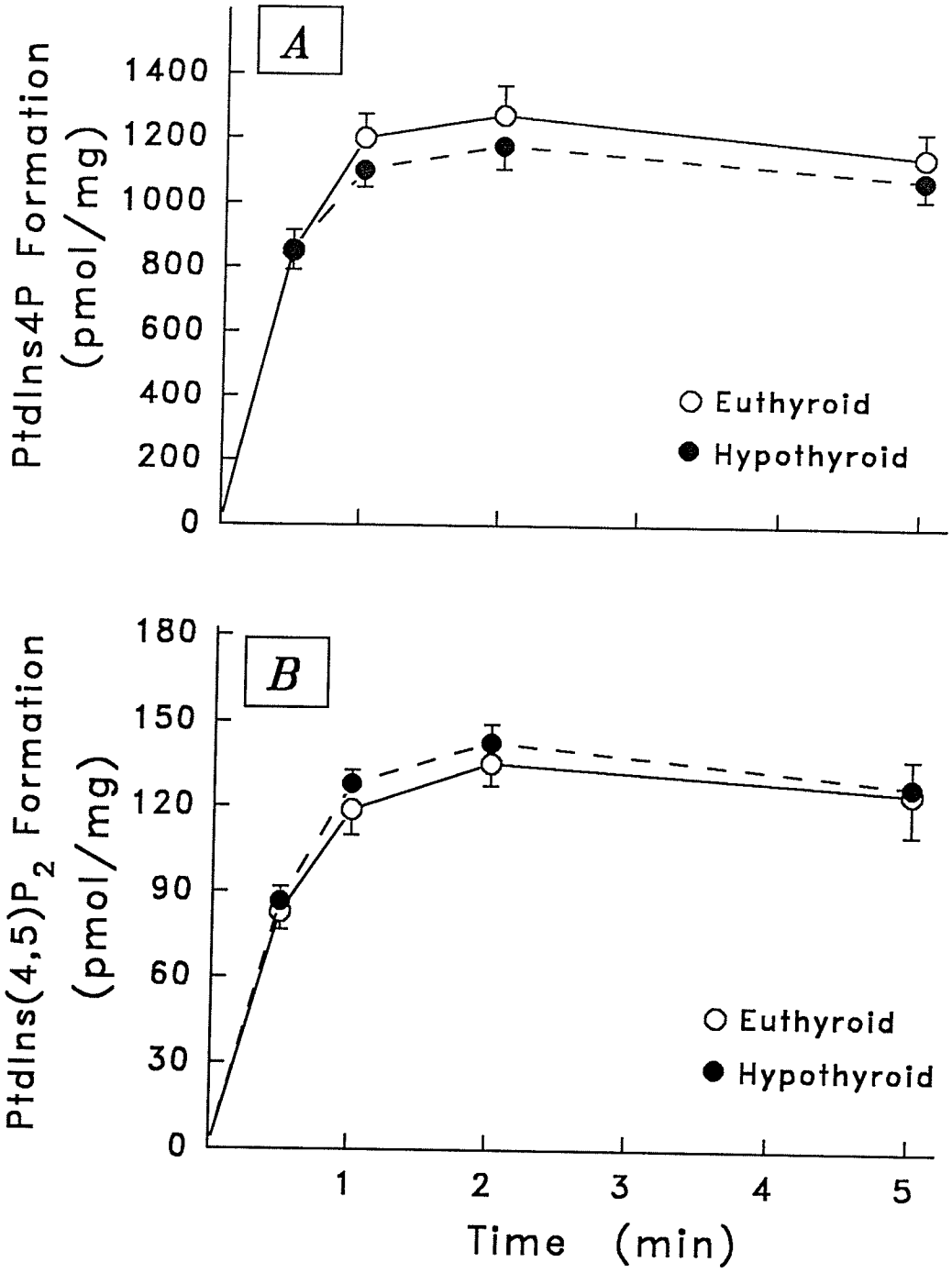

FIGURE 11. Time course of PtdIns4P (A) and PtdIns(4,5)P_2 (B) formation in SL membrane preparations from euthyroid and hypothyroid (PTU-treated) rat hearts. 30 μg of SL protein were incubated for 1 min at 30°C after 30 min preincubation in presence of alamethicin as described in "Materials and Methods". Results represent the mean ± SEM of triplicate determinations in six different preparations.

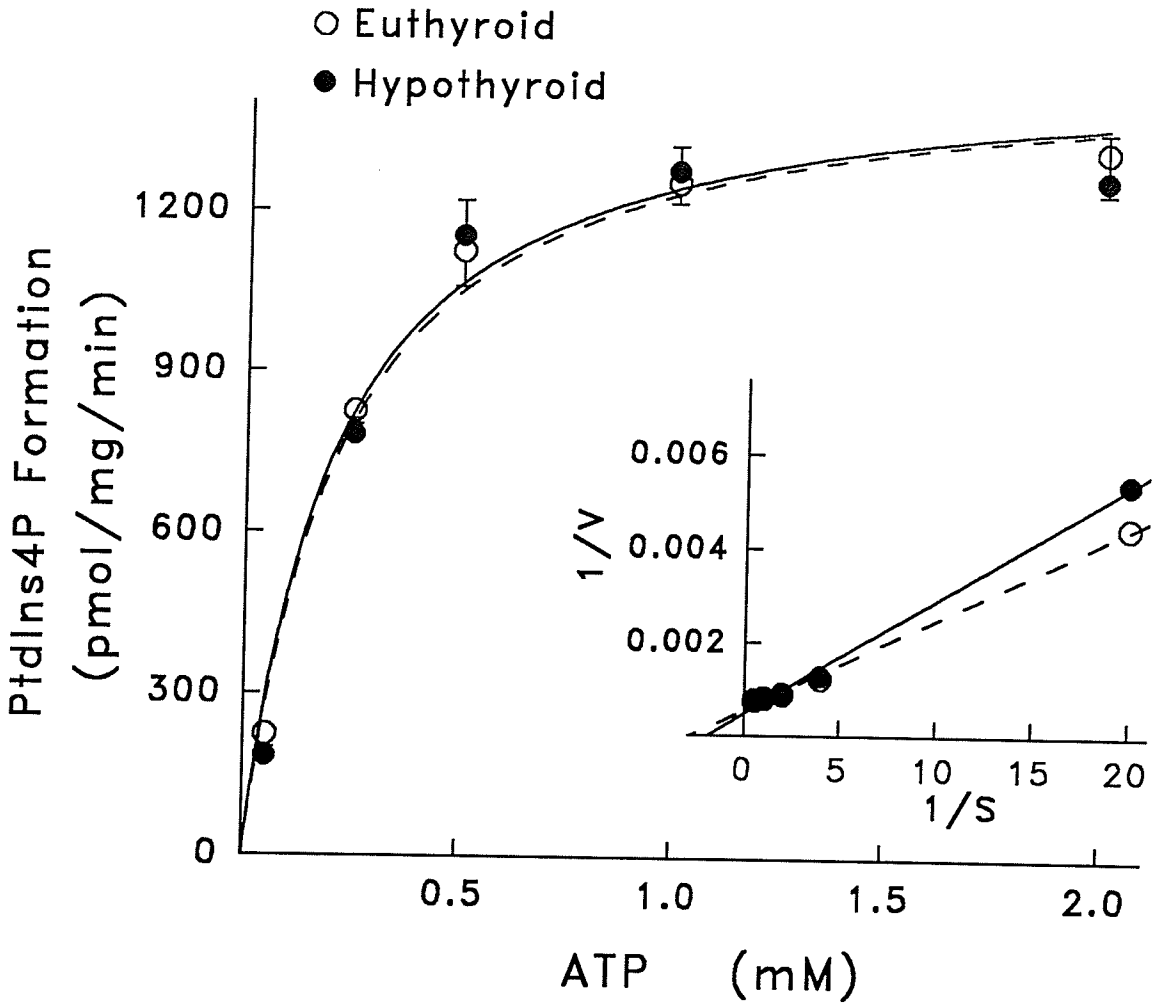

FIGURE 12. Effect of ATP on PtdIns kinase activity in euthyroid and hypothyroid SL membranes. 30 μg of SL protein were incubated in the presence of increasing ATP concentrations (0.05-2.0mM) under the assay conditions described in "Materials and Methods". Values are means $\pm$ SEM of six experiments performed in triplicate. The Lineweaver-Burk plot (inset) was constructed from the values of the enzyme specific activity at different concentrations of ATP.

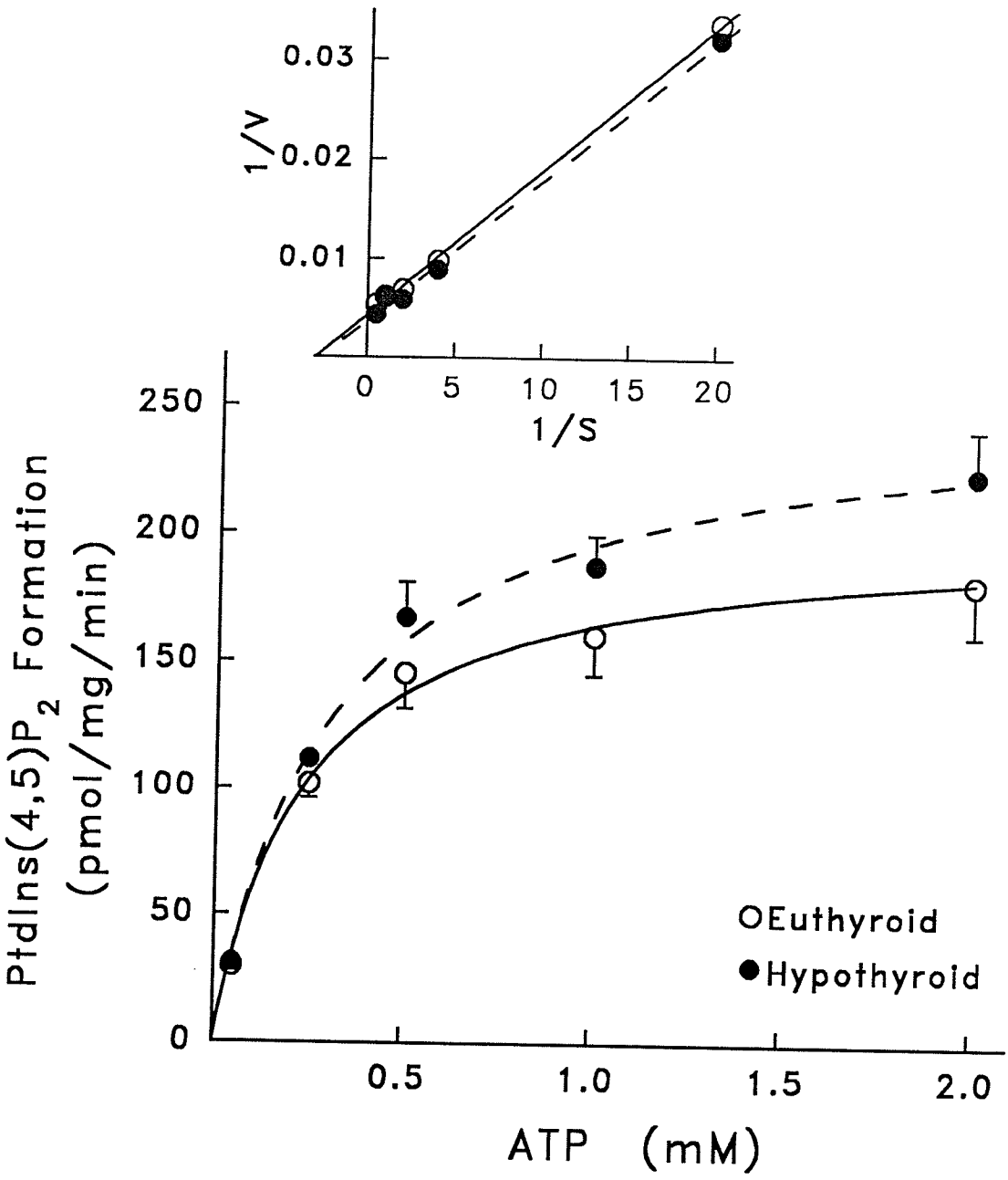

FIGURE 13. Effect of ATP on PtdIns4P kinase activity in euthyroid and hypothyroid SL membranes. SL proteins (30 μg) were incubated in the presence of increasing ATP concentrations (0.05-2.0 mM) as indicated in the legend of FIGURE 12. Results are means $\pm$ SEM of six experiments in triplicate. The Lineweaver-Burk plot (inset) was constructed from the values of the enzyme specific activity at different concentrations of ATP.

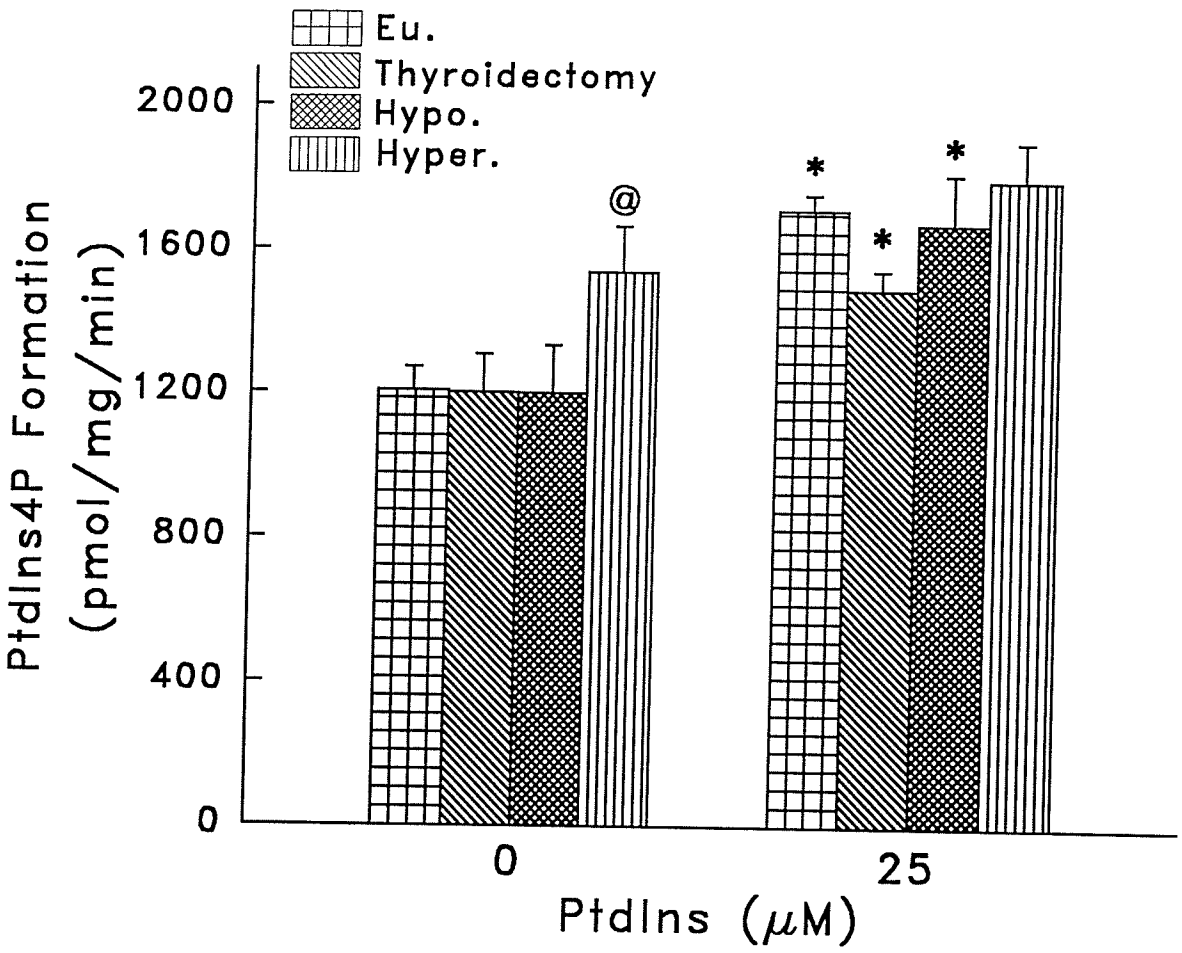

FIGURE 14. PtdIns kinase activity in heart SL from euthyroid, thyroidectomized, hypothyroid (PTU-treated), and hyperthyroid rat heart SL in the absence and presence of 25 μM exogenously added PtdIns. Values are means $\pm$ SEM of at least four to five experiments carried out in triplicate. The data from euthyroid animals (age-matched controls for hypothyroidism, thyroidectomy and hyperthyroidism) were pooled since there was no difference (variability < 10%) in the enzyme activity among different groups. Exogenous PtdIns was prepared by ultrasonication (30 min in Branson 1200 sonicator) and thereafter added to the incubation medium.

* Significantly different ($P < 0.05$) from the respective 0 PtdIns.

@ Significantly different ($P < 0.05$) from euthyroid.

exogenous PtdIns4*P*, the only difference being that the activity of the hyperthyroid SL at 25 μM PtdIns4*P* was significantly higher than the absence of the phospholipid substrate (FIGURE 15).

4. Basal phospholipase C activity in hypo- and hyperthyroidism

Sarcolemmal phospholipase C (PL C), which hydrolyzes PtdIns(4,5)P_2 to yield Ins(1,4,5)P_3 and DAG is the only enzyme of the phosphoinositide pathway that has been shown to be directly regulated by receptor stimulation via a G protein. This enzyme activity was studied in SL isolated from euthyroid, hypothyroid and hyperthyroid SL from rat myocardium. For comparison purposes, some studies were also carried out on rat heart cytosolic PL C under the different thyroid conditions.

FIGURE 16D, shows the time dependent formation of total inositolphosphates (InsP_s), which is the sum of Ins(1)*P*, Ins(1,4)P_2 and Ins(1,4,5)P_3 formed in euthyroid and hypothyroid (PTU) SL. The InsP_s formation was significantly higher in hypothyroid SL over all the time periods studied (0.5 to 5 min) (FIGURE 16D). This significant increase was also observed when each inositol phosphate was measured individually (FIGURE 16 A, B, and C). It is important to notice that when PtdIns(4,5)P_2 is used as a substrate for PL C the major product of the reaction is Ins(1,4,5)P_3 followed by Ins(1,4)P_2 (FIGURE 16B, 16C). Ins*P* which is the product of dephosphorylation (Downes and MacPhee, 1990) is minimal (FIGURE 16A) while under the current assay conditions, no inositol formation was detectable. The time dependent changes in SL PL C from reversed hypothyroid and

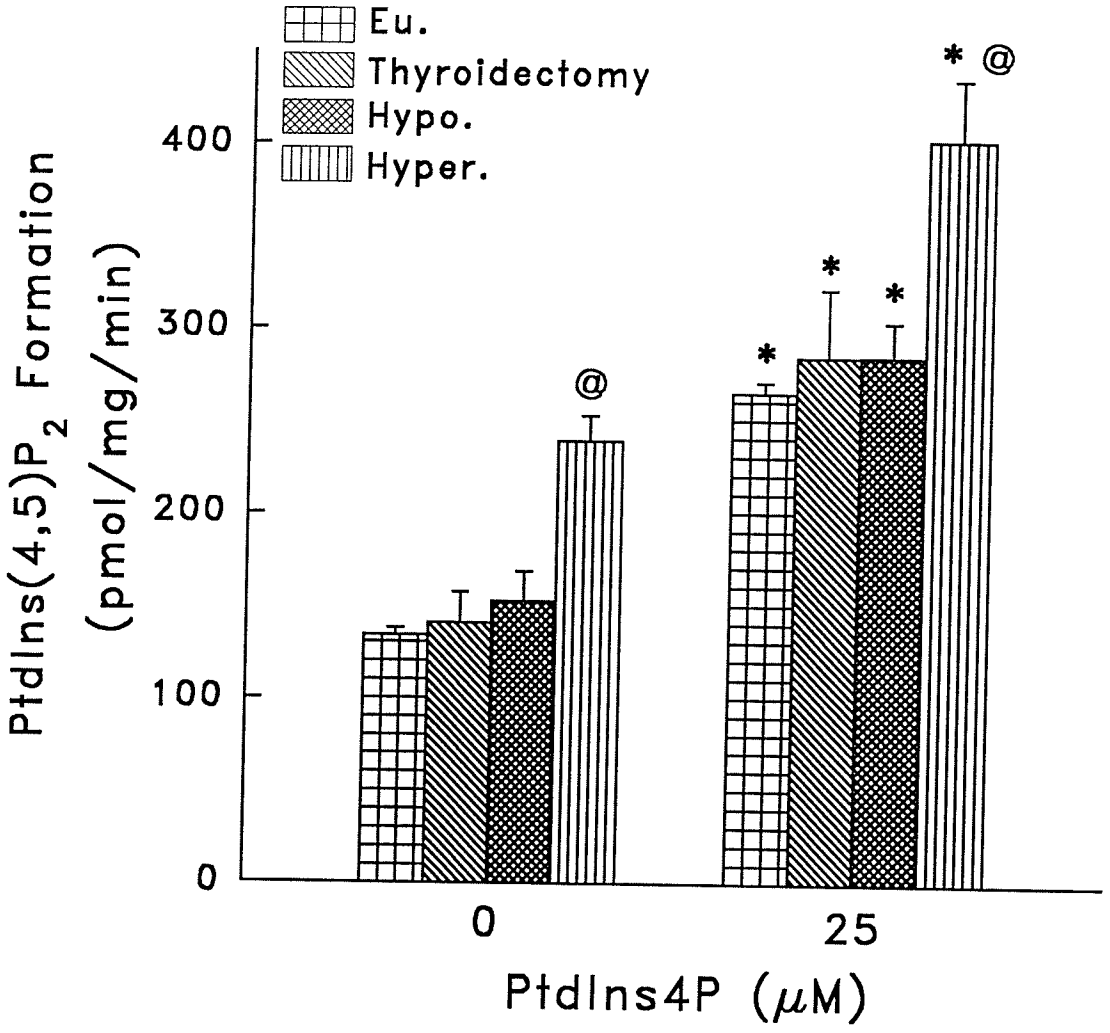

FIGURE 15. PtdIns4P kinase activity in heart SL from euthyroid, thyroidectomized, hypothyroid (PTU-treated), and hyperthyroid rat in the absence and presence of 25 μM exogenously added PtdIns4P. Values are means ± SEM of at least four to five experiments carried out in triplicate. Euthyroids were pooled from different groups as indicated in the legend of FIGURE 14. Exogenous PtdIns4P was prepared by ultrasonication (30 min in Branson 1200 sonicator) and thereafter added to the incubation medium.

* Significantly different ($P < 0.05$) from the respective 0 PtdIns4P.

@ Significantly different ($P < 0.05$) from euthyroid.

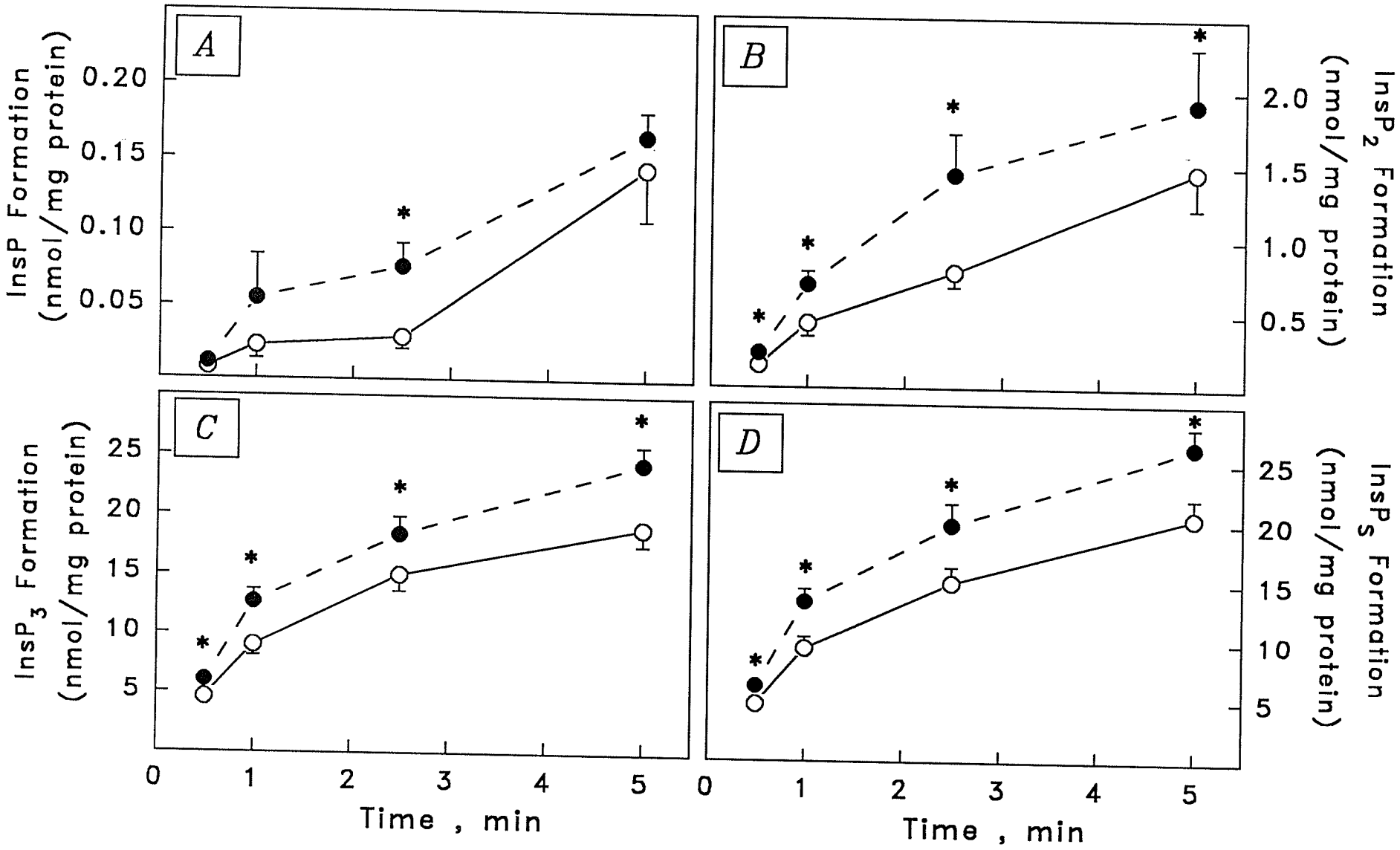

FIGURE 16. Time-dependent hydrolysis of PtdIns(4,5)P_2 by SL PL C in euthyroid (○) and hypothyroid (PTU, ●) rat heart SL. Sarcolemmal membranes were incubated for the indicated times at 37°C with 20 μM [^{3}H]-PtdIns(4,5)P_2. Ins(1)P (A) Ins(1,4)P_2 (B) and Ins(1,4,5)P_3 (C) were separated as in "Materials and Methods", total inositol phosphates (InsPs, D) were the sum of the three inositol phosphates. Values are means ± SEM of four different preparations in triplicate.
* Significantly different ($P < 0.05$) from the euthyroid values.

hyperthyroid rat hearts were also studied. When the PTU-induced hypothyroid condition was reversed (14 days injection with T_4, while keeping the rats on 0.05% PTU containing water), the increase in the SL PL C was abolished (FIGURE 17). Furthermore, hyperthyroidism induced a significant decline in the activity of SL PL C, an effect which was observed during 1-5 min reaction time (FIGURE 18).

To establish if there was any change in substrate specificity of the SL PLC as a consequence of the hypothyroid state, three putative substrates [PtdIns, PtdIns4*P* and PtdIns(4,5)P_2, (Meij and Panagia, 1992)] of the phosphoinositide specific PL C were used. As shown in FIGURE 19 the PL C activity in both euthyroid and hypothyroid SL increased by increasing the PtdIns(4,5)P_2 concentration from 10 to 200 μM, with the increase in the hypothyroid SL PL C being significantly higher than that of euthyroid SL. However, when PtdIns4*P* (FIGURE 20) or PtdIns (FIGURE 21) was used as a substrate for PL C, similar increases of the enzyme activity with increasing the substrate concentration were observed in eu- and hypothyroid (PTU-treated) rat heart SL. In agreement with a previous report (Meij and Panagia, 1992), PtdIns was hydrolyzed at a very low rate (FIGURE 21), and the main products from PtdIns4*P* and PtdIns hydrolysis were Ins(1,4)P_2 and Ins*P*, respectively (data not shown). TABLES 8 and 9 summarizes the hydrolytic activity of SL and cytosolic PL C on different phosphoinositide substrates in the various thyroid states under study. As already observed (FIGURE 16, 19), hypothyroidism (both PTU treatment and thyroidectomy) significantly increased the rate of PtdIns(4,5)P_2 hydrolysis by SL PL C, while this effect was not observed when either PtdIns4*P* or PtdIns were used as a substrate (TABLE 8). In the reversal of PTU-induced

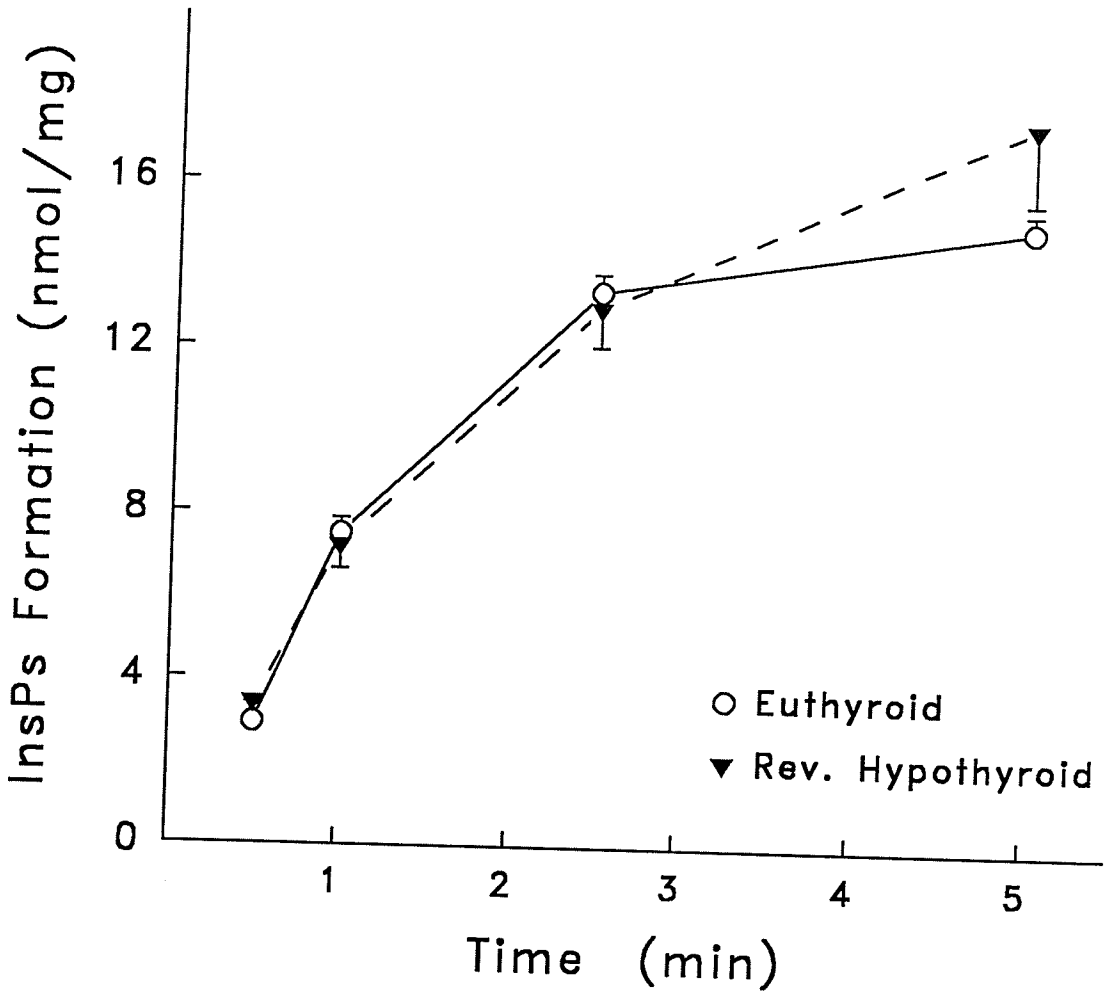

FIGURE 17. Time-dependent hydrolysis of PtdIns(4,5)P_2 by SL PL C in euthyroid (age-matched) and reversed hypothyroid rat heart. Animals were treated as indicated in "Materials and Methods". Sarcolemmal membranes were incubated for the indicated times at 37°C with 20 μM [^{3}H]-PtdIns(4,5)P_2; thereafter, inositol phosphates (InsPs) were separated as in "Materials and Methods". Values are means ± SEM of four different preparations in triplicate.

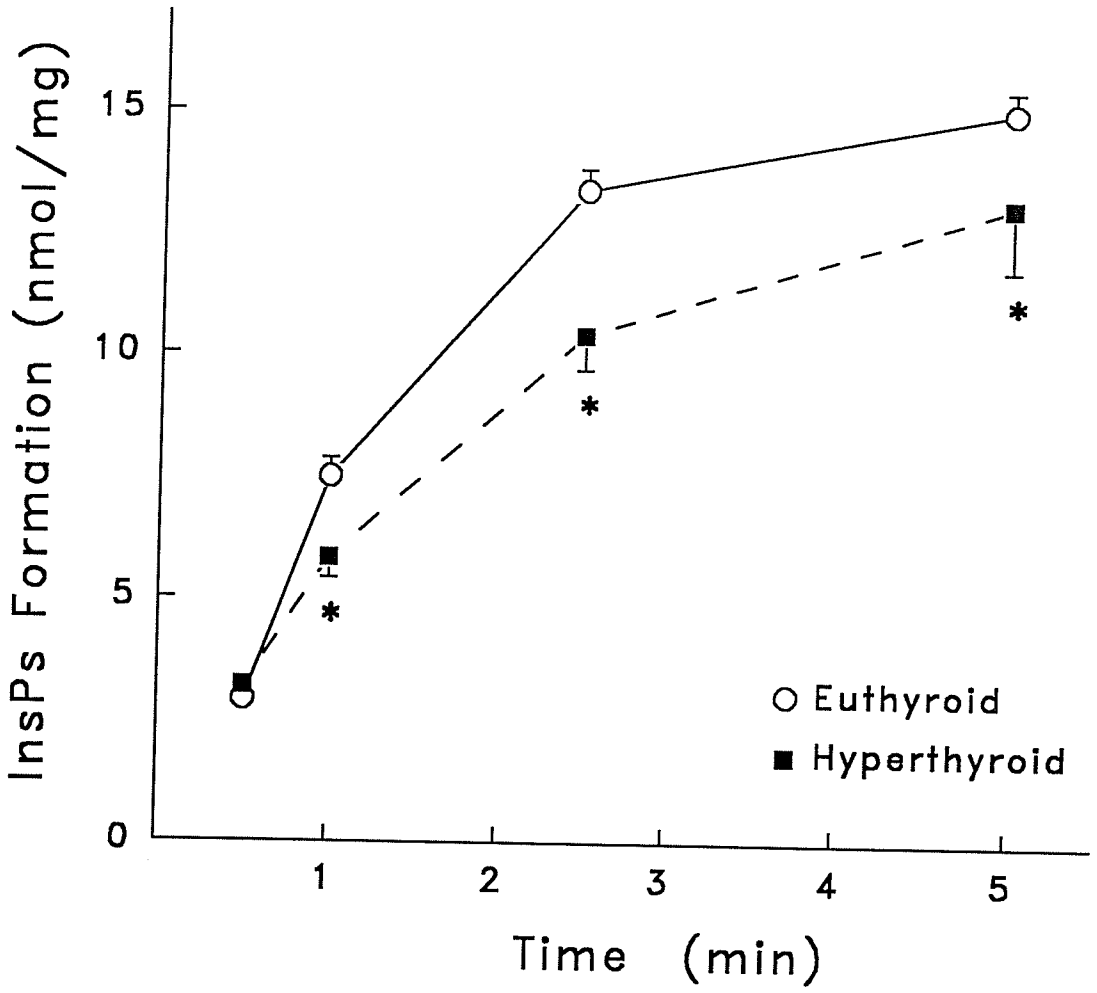

FIGURE 18. Time-dependent hydrolysis of PtdIns(4,5)P_2 by SL PL C in euthyroid (age-matched) and hyperthyroid rat heart. Hyperthyroidism was induced as described in "Materials and Methods". Sarcolemmal membranes were incubated for the indicated times at 37°C with 20 μM [^{3}H]-PtdIns(4,5)P_2; inositol phosphates (InsPs) were separated as in "Materials and Methods". Values are means ± SEM of four different preparations in triplicate.

* Significantly different ($P < 0.05$) from the euthyroid values.

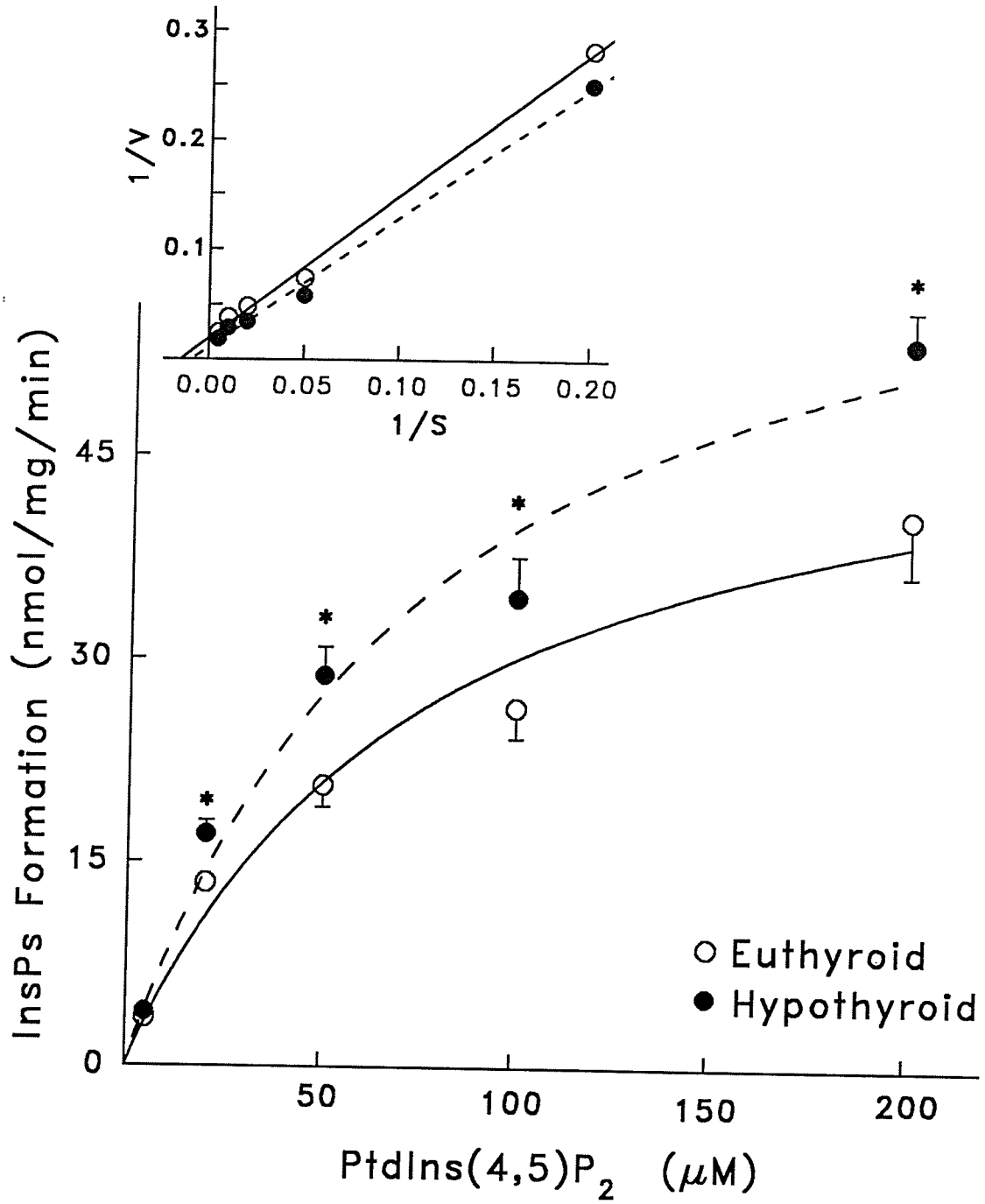

FIGURE 19. PtdIns(4,5)P_2 concentration-dependent changes in sarcolemmal PL C from euthyroid and hypothyroid (PTU) rats. SL membranes were incubated in the presence of increasing concentration of PtdIns(4,5)P_2 under standard conditions as described in "Materials and Methods". Values are means ± SEM of four experiments done in triplicate. The Lineweaver-Burk plot (inset) constructed from the values of the enzyme specific activities corresponding to different PtdIns(4,5)P_2 concentrations indicates that the apparent K_m (μM) and V_{max} (pmol/mg/min) values are, euthyroid: K_m= 69.4 ± 8.0, V_{max}= 53 ± 7.1; hypothyroid: K_m= 113.7 ± 15.6, V_{max}=95.15 ± 11.5.

* Significantly different ($P < 0.05$) from the euthyroid values.

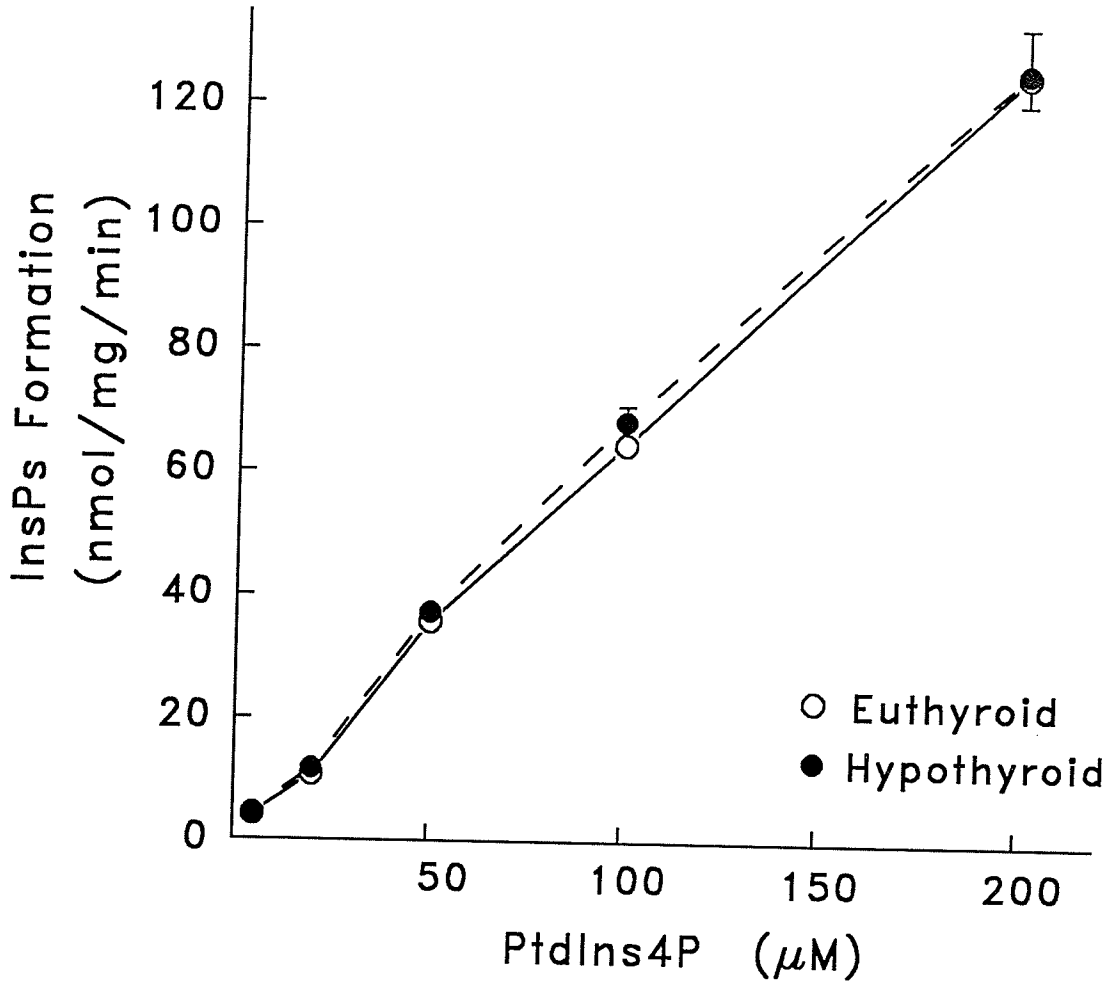

FIGURE 20. PtdIns4P concentration-dependence of PL C activity in SL from euthyroid and hypothyroid (PTU) rats. SL protein (15 μg) was incubated with increasing concentration of PtdIns4P as described in "Materials and Methods". Values are means ± SEM of four different preparations carried out in triplicate.

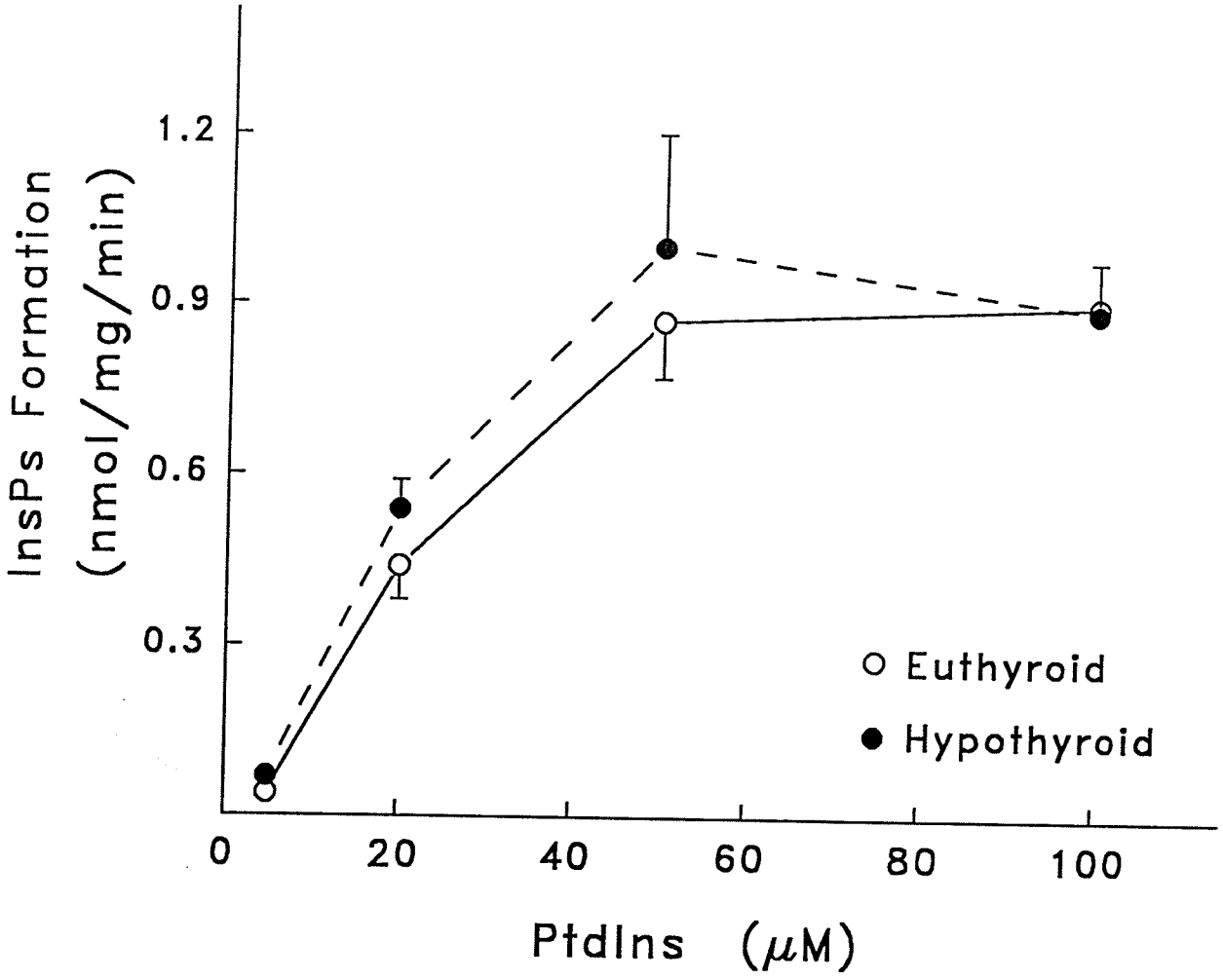

FIGURE 21. PtdIns concentration-dependence of PL C activity in SL from euthyroid and hypothyroid (PTU) rats. SL membranes were incubated with increasing concentration of PtdIns as described in "Materials and Methods". Values are means ± SEM of three experiments carried out in triplicate.

TABLE 8. Hydrolytic activity of sarcolemmal phospholipase C on phosphoinositide substrates in different thyroid states.

	Phosphoinositide Substrate		
	PtdIns	PtdIns4*P*	PtdIns(4,5)P_2
		(nmol/mg protein/min)	
Euthyroid	0.18 ± 0.01	9.67 ± 0.19	13.46 ± 0.13
Hypothyroid (PTU)	0.18 ± 0.02	9.97 ± 0.67	17.05 ± 0.69*
Hypothyroid (Thyroidectomy)	0.17 ± 0.03	10.80 ± 0.38	17.34 ± 1.61*
Reversal of PTU-hypothyroid	0.16 ± 0.06	9.92 ± 0.45	14.22 ± 0.98
Hyperthyroid	0.15 ± 0.02	9.65 ± 1.10	10.84 ± 0.64*

PL C activity in sarcolemmal membranes from each group was assayed under standard condition as described in "Materials and Methods" in the presence of 20 μM [^{3}H]-PtdIns, [^{3}H]PtdIns4*P* or [^{3}H]-PtdIns(4,5)P_2, for 2.5 min at 37°C. Values are means ± SEM of total Ins*P* formation in at least four to six experiments in triplicate. The data from euthyroid animals (age-matched controls for each hypothyroid conditions, as well as for reversed hypothyroidism and hyperthyroidism) were pooled since there was no difference (variability < 10%) in the activity among the different groups.

* Significantly different ($P < 0.05$) from euthyroid values.

TABLE 9. Cytosolic phospholipase C activity in different thyroid states and with different phosphoinositide substrates.

	Phosphoinositide Substrate		
	PtdIns	PtdIns4*P*	PtdIns(4,5)P_2
	(nmol/mg protein/min)		
Euthyroid	0.17 ± 0.007	10.71 ± 0.25	10.10 ± 0.197
Hypothyroid (PTU)	0.27 ± 0.027*	13.38 ± 1.03*	13.13 ± 0.54*
Hypothyroid (Thyroidectomy)	0.21 ± 0.009*	15.53 ± 1.00*	13.43 ± 0.50*
Reversal of PTU-hypothyroid	0.18 ± 0.021	10.21 ± 0.62	10.49 ± 0.48
Hyperthyroid	0.13 ± 0.012*	8.46 ± 0.95*	7.98 ± 0.35*

Cytosolic fraction from each group was assayed under standard condition as described in "Materials and Methods" in the presence of 20 μM [^{3}H]-PtdIns, [^{3}H]PtdIns4*P* or [^{3}H]PtdIns(4,5)P_2 for 2.5 min at 37°C. Values are means ± SEM of total Ins*P* formation in at least four to six experiments in triplicate. Other details are as in the legend to TABLE 8.

* Significantly different ($P < 0.05$) from euthyroid values.

hypothyroidism, the SL PL C-dependent hydrolysis of PtdIns(4,5)P_2 was normalized (TABLE 8). SL PL C was significantly depressed in hyperthyroidism when PtdIns(4,5)P_2 was used as a substrate, while no change from euthyroid was observed with either PtdIns4P or PtdIns (TABLE 8). The cytosolic PL C activity in different thyroid states with the three phospholipid substrates is shown in TABLE 9. The changes in cytosolic PL C with PtdIns(4,5)P_2 as a substrate were similar to those of SL PLC, i.e., the enzyme activity was significantly increased in hypothyroidism, normalized by reversal of hypothyroidism and significantly depressed in the hyperthyroid condition (TABLE 9). Unlike SL, however, these changes were also observed when the other two substrates, PtdIns and PtdIns4P, were used (TABLE 9).

5. Myocardial responsiveness to isoproterenol and phenylephrine

TABLE 10 shows the contractile response of isolated eu- and hypothyroid hearts which were perfused according to the Langendorff procedure and stimulated by either isoproterenol or phenylephrine. As compared to respective baseline values, stimulation by 0.1 μM isoproterenol induced a significant increase in the maximal developed left intraventricular pressure (LVP_{max}), and in the maximal rates of pressure development and decline of both euthyroid and hypothyroid hearts. However, in the hypothyroid hearts this increase was significantly lower than the euthyroid hearts. On the other hand, when the hearts were stimulated by phenylephrine, the increment of the contractile parameters over the baseline values was significantly higher in the hypothyroid than in the euthyroid

TABLE 10. Effect of different adrenergic agonists on the contractile parameters of euthyroid and hypothyroid hearts.

	% of Basal		
	LVP_{max}	+dP/dt	-dP/dt
A. Euthyroid:			
Isoproterenol	214.5 ± 16.9	208.1 ± 12.9	234.9 ± 27.1
Phenylephrine	184.7 ± 10.2	177.3 ± 14.2	212.1 ± 20.9
B. Hypothyroid:			
Isoproterenol	149.9 ± 15.3*	144.1 ± 10.04*	165.2 ± 14.5*
Phenylephrine	219.9 ± 12.8*	205.0 ± 9.8*	246.7 ± 13.4

Values are means ± SEM of 6-8 perfused hearts and expressed as % change from the basal values. The hearts were stimulated with 0.1 μM isoproterenol for 1 min. For the α_1-adrenoceptor stimulation, following 10 min perfusion with K-H containing 10 μM atenolol the hearts were perfused for 1 min with 10 μM phenylephrine in presence of 10 μM atenolol. LVP_{max}= maximum developed pressure in the left ventricle; ±dP/dt= maximal rates of pressure development and decline.

* Significantly different ($P < 0.05$) from their respective euthyroid values.
hearts.

6. Phospholipase C activity under α_1-adrenoceptor stimulation

As mentioned above, SL PL C is a key enzyme in transducing the extracellular adrenergic message, via α_1 receptor and G protein, to intracellular effector(s). Given the increased α_1-adrenoceptor density and improved contractile function in the hypothyroid hearts under α_1-receptor stimulation, it was important to study the effect of α_1-adrenoceptor stimulation on the activity of SL PL C. In preliminary studies the *in vitro* addition of 10 μM phenylephrine or 10 μM atenolol to the PL C assay medium did not have any effect on the PL C activity (data not shown). FIGURE 22 shows the hydrolysis of [^{3}H]-PtdIns(4,5)P_2 by PL C in SL isolated from hearts perfused with 10 μM phenylephrine (PE) in the presence of 10 μM atenolol. The basal SL PL C was significantly higher in hypothyroid than in euthyroid hearts (FIGURE 22). The enzyme was significantly stimulated by PE in both conditions. However, the PE-stimulated PL C activity in hypothyroid SL was significantly greater than in euthyroid SL (FIGURE 22). To test if there were any change in substrate specificity of the SL PL C under α_1-adrenoceptor stimulation, SL membranes isolated from euthyroid and hypothyroid hearts perfused with or without 10 μM phenylephrine were assayed in the presence of [^{3}H]-PtdIns4*P*. No difference in the basal activity was observed in the two conditions (FIGURE 23). However, under phenylephrine stimulation the hydrolysis of PtdIns4*P* was significantly increased to a similar extent in both eu- and hypothyroid SL.

The basal PtdIns(4,5)P_2 hydrolysis by cytosolic PL C was significantly higher in hypothyroid compared to euthyroid hearts (FIGURE 24). Significant stimulation over basal

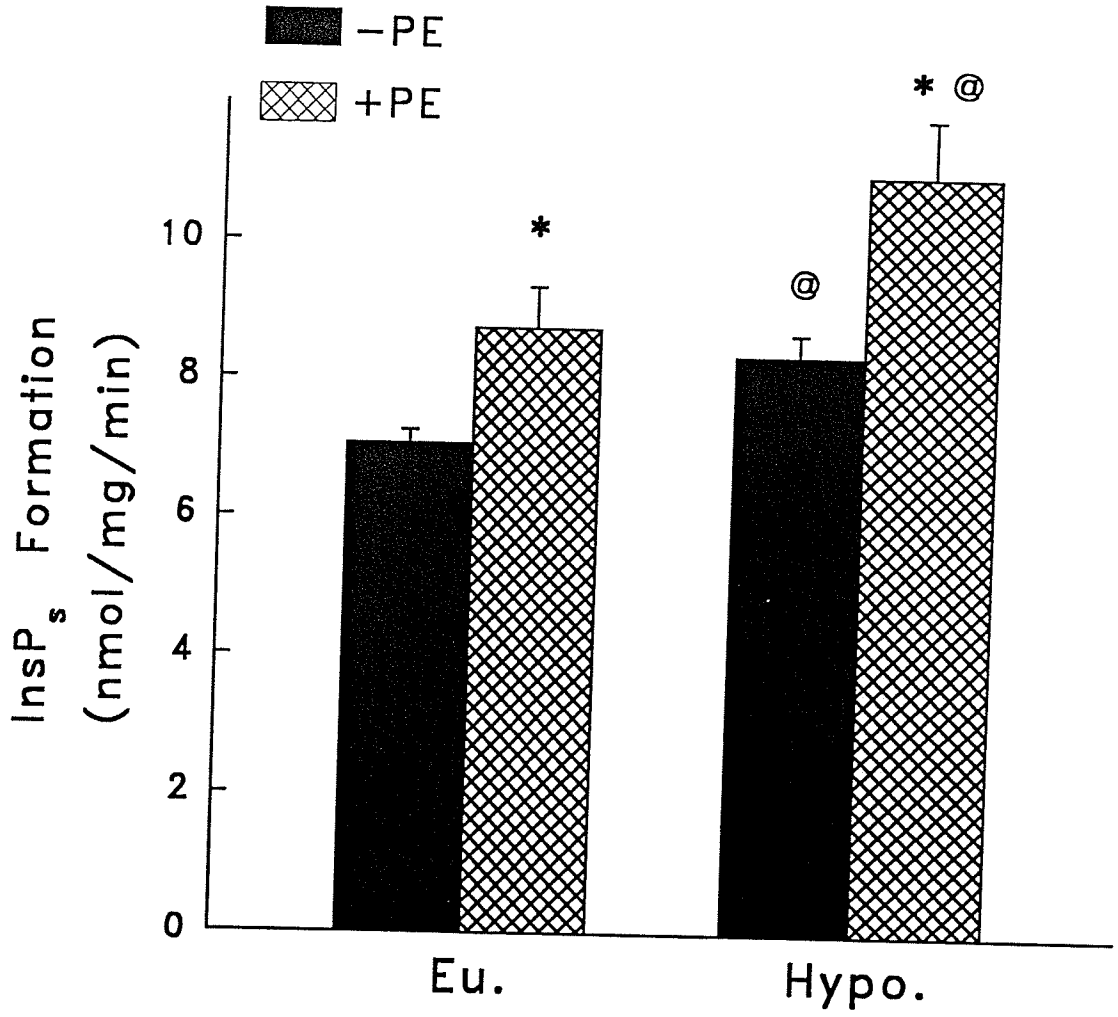

FIGURE 22. Effect of stimulation of euthyroid and hypothyroid Langendorff hearts with phenylephrine (PE) on PtdIns(4,5)P_2 hydrolysis by sarcolemmal PL C activity. Values are means ± SEM from five separate preparations assayed in triplicate. SL membranes were isolated from rat hearts perfused for 12 min with K-H medium containing 10 μM atenolol followed by 1 min perfusion with 10 μM PE and 10 μM atenolol, and frozen at the peak of positive inotropy as described in "Materials and Methods". 15 μg of SL protein was incubated in the presence of 20 μM [^{3}H]-PtdIns(4,5)P_2 for 2.5 min at 37°C, and inositolphosphates (InsP_s) were separated on Dowex 1X8 columns.

* Significantly different ($P < 0.05$) vs. - PE.

@ Significantly different ($P < 0.05$) from euthyroid.

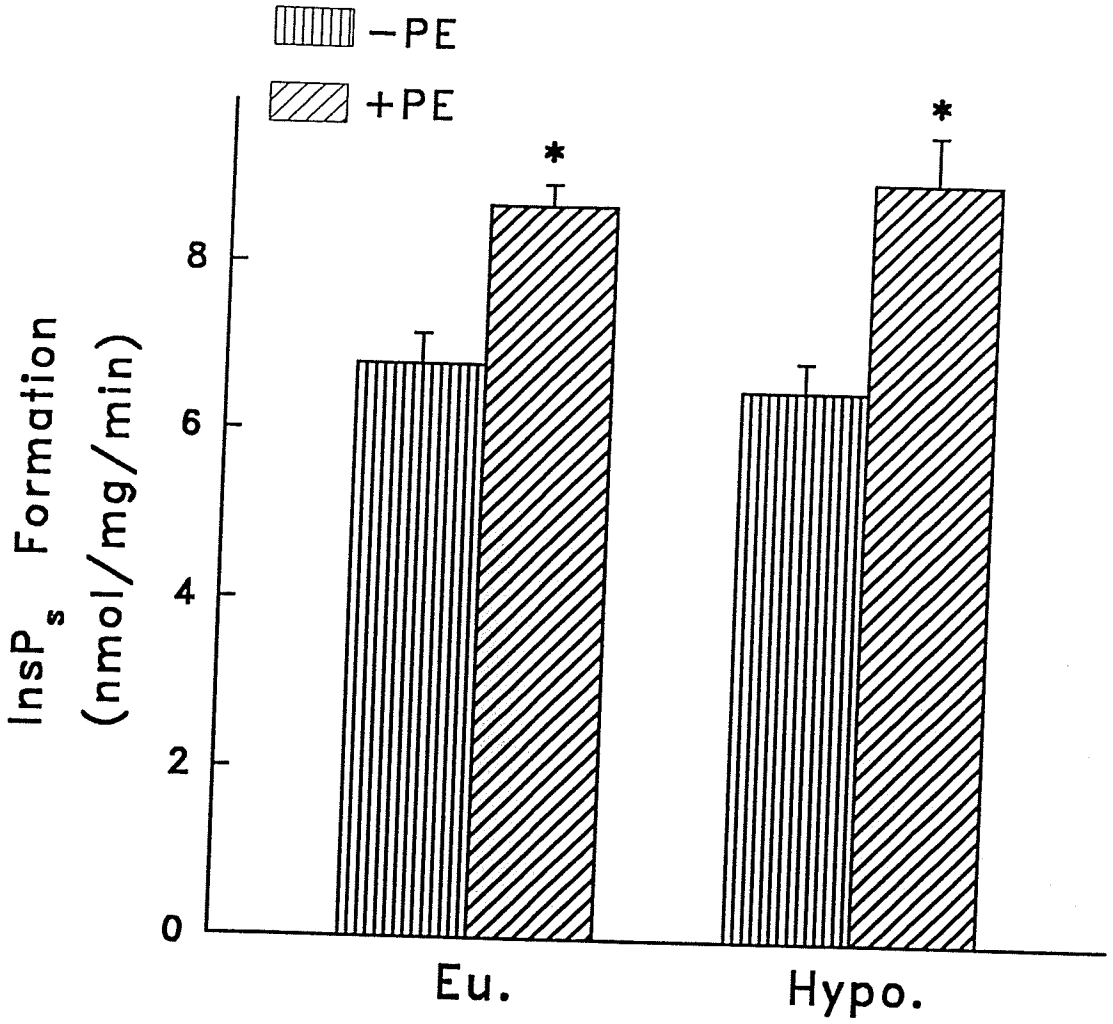

FIGURE 23. Hydrolysis of PtdIns4P by SL PL C after perfusion of euthyroid and hypothyroid rat hearts with phenylephrine (PE). Values are means ± SEM from five separate preparations assayed in triplicate. SL proteins (15 μg) were incubated in the presence of 20 μM [^{3}H]-PtdIns4P for 2.5 min at 37°C. Other detailes are as in the legend of FIGURE 22.

* Significantly different ($P < 0.05$) vs. - PE.

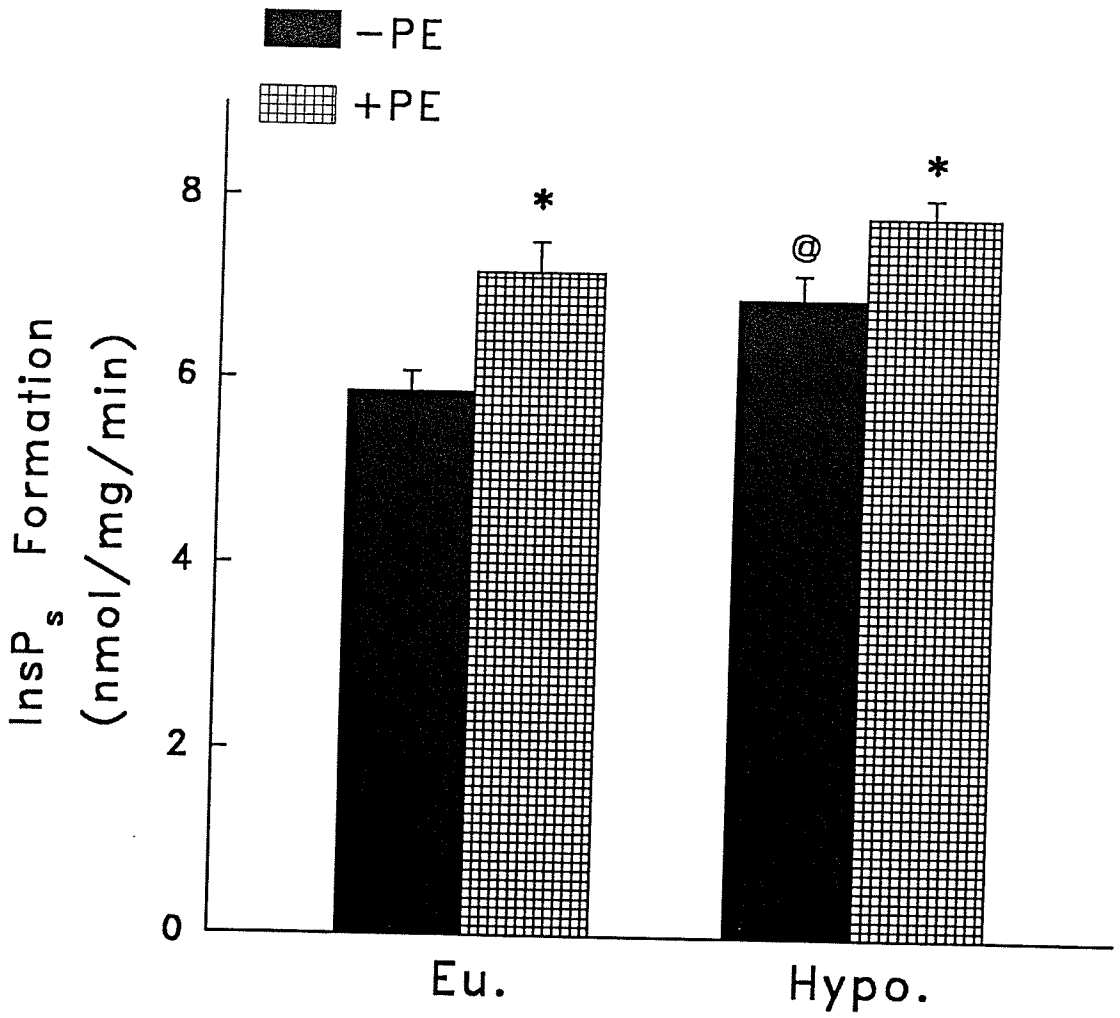

FIGURE 24. Hydrolysis of PtdIns(4,5)P_2 by cytosolic PL C following perfusion of euthyroid and hypothyroid rat hearts with phenylephrine (PE). Values are means ± SEM of five different preparations in triplicate. Cytosolic fraction was obtained from rat hearts perfused and frozen as described in the legend of FIGURE 22. 15 μg of cytosolic protein was incubated in the presence of 20 μM [^{3}H]-PtdIns(4,5)P_2 for 2.5 min at 37°C, and inositol phosphates were separated as described in "Materials and Methods".

* Significantly different ($P < 0.05$) vs. -PE.

@ Significantly different ($P < 0.05$) vs. euthyroid.

values was induced by PE in both states. Unlike SL, however, the degree of stimulation of euthyroid and hypothyroid cytosolic PL C was similar (FIGURE 24). The hydrolysis of PtdIns4*P* by cytoplasmic PL C was not affected by α_1-adrenoceptor stimulation (FIGURE 25), while this activity was significantly higher in hypothyroid compared to euthyroid hearts. The hydrolysis of [^{3}H]-PtdIns by either SL or cytosolic PLC from the perfused hearts was undetectable (data not shown).

7. Ins(1,4,5)P_3 content of euthyroid and hypothyroid hearts

The Ins(1,4,5)P_3 content of freeze-clamped ventricles from both euthyroid and hypothyroid rats perfused with or without phenylephrine according to the protocol described in "Materials and Methods" is shown in TABLE 11. No significant difference was observed in the basal Ins(1,4,5)P_3 content of eu- and hypothyroid hearts. Under phenylephrine stimulation, the Ins(1,4,5)P_3 content increased significantly in the hypothyroid hearts.

C. *Congestive heart failure*

After the above in depth study of the status of the phosphoinositide pathway at the SL level in hypothyroidism, a series of experiments was conducted to see whether the changes in polyphosphoinositide synthesis and degradation seen in hypothyroidism could be also seen in postinfarcted congestive heart failure (CHF). As mentioned above, this

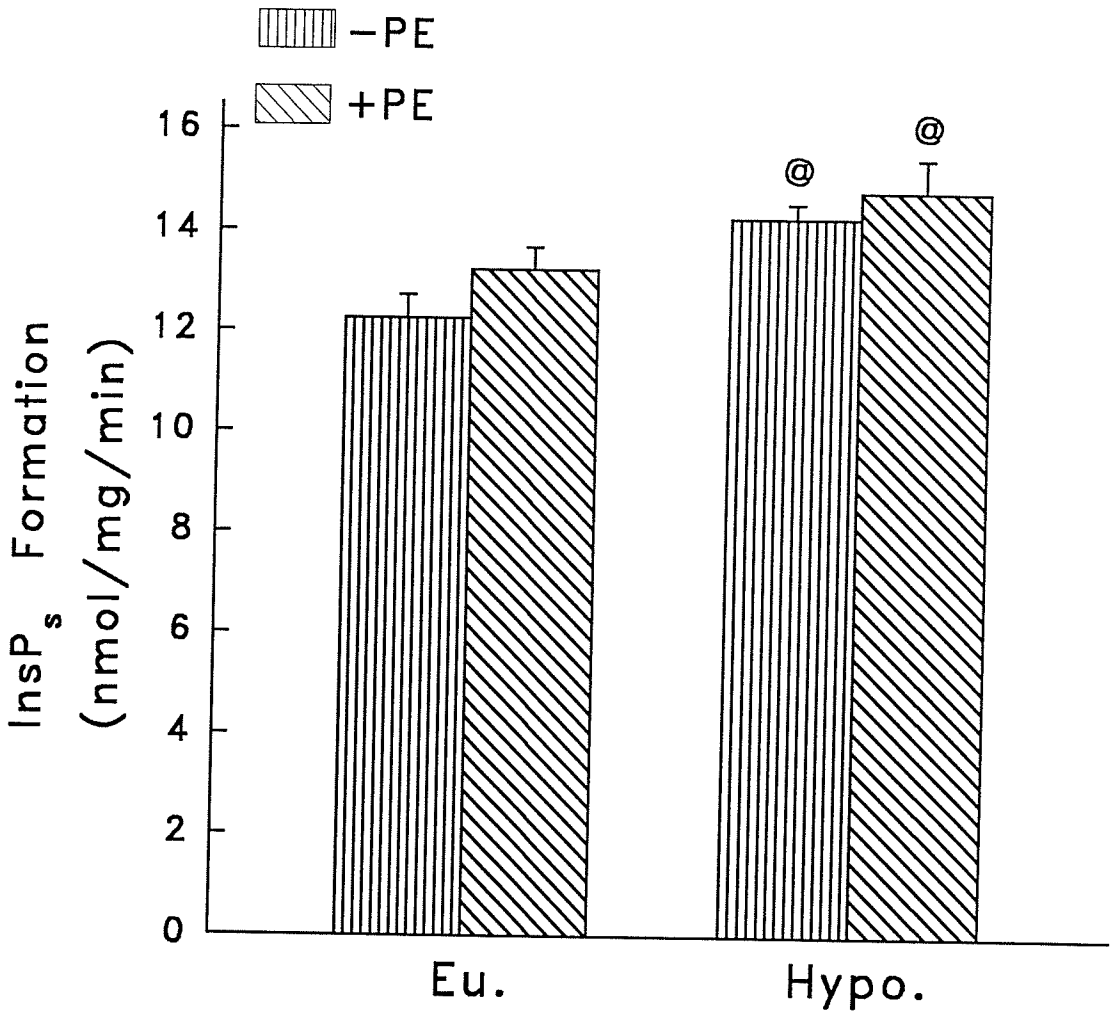

FIGURE 25. Hydrolysis of PtdIns4P by cytosolic PL C after phenylephrine perfusion of euthyroid and hypothyroid rat hearts. Data are means ± SEM of five separate preparations in triplicate. 15 μg of cytosolic protein was incubated in the presence of 20 μM [^{3}H]-PtdIns4P for 2.5 min at 37°C. Other details are as in the legend of FIGURE 24.

@ Significantly different ($P < 0.05$) vs. euthyroid.

TABLE 11. Effect of phenylephrine on the Ins(1,4,5)P_3 content of euthyroid and hypothyroid Langendorff perfused hearts.

	Basal	10 μM PE
Euthyroid	0.35 ± 0.07	0.41 ± 0.06
	(n=4)	(n=4)
Hypothyroid	0.37 ± 0.03	0.47 ± 0.02*
	(n=5)	(n=5)

Values are mean ± SEM and expressed as pmol Ins(1,4,5)P_3/mg tissue. Hearts from euthyroid and hypothyroid rats were perfused with K-H buffer in the absence or presence of 10 μM phenylephrine plus 10 μM atenolol, and were freeze clamped at the peak of the positive inotropic response, as described in "Materials and Methods". Ins(1,4,5)P_3 levels were determined using a protein binding assay kit (Amersham, UK). PE = phenylephrine, n = number of experiments.

* Significantly different ($p < 0.05$) from Basal.

cardiac pathology is characterized by a decrease in ß-adrenoceptor and a concomitant increase in α_1-adrenoceptor density. Thus, for comparison the phosphoinositide pathway was studied in animals at a moderate stage of CHF occurring at 8 weeks post-coronary ligation.

1. General characteristics in CHF animals subsequent to myocardial infarction

TABLE 12 shows the general characteristics of the controls and 8 weeks CHF. Evidence of cardiac hypertrophy in 8 weeks CHF was noted by the significant increase in the viable left ventricle to body weight ratio. This was accompanied by a significant increase in the mass of CHF right ventricles.

2. Phosphoinositide pathway in CHF

The phosphorylative activity of PtdIns kinase was significantly depressed in SL from viable left ventricles of CHF rats as compared to the control left ventricle (FIGURE 26), and this depression was observed at the different reaction times (0.5 to 5 min). A similar degree of depression was also observed in the SL PtdIns4*P* kinase activity from failing viable left ventricles studied at different (0.5-5 min) reaction times (FIGURE 27). The possibility of any change in the affinity of both enzymes for ATP was studied by employing different concentrations of ATP in the reaction medium. FIGURE 28 shows that increasing the ATP concentration (0.01-2 mM final concentrations) did not normalize

TABLE 12. General characteristics of sham operated control and failing rat hearts (8 weeks after coronary ligation).

	Sham	8 week CHF
Body weight (g)	495.0 ± 8.0	477.0 ± 6.0
RV weight (g)	0.22± 0.01	0.32 ± 0.01*
LV weight (g)	0.87± 0.01	0.90 ± 0.01
Scar weight (g)	N.D.	0.21 ± 0.01
LV wt./body wt. (mg/g)	1.76± 0.03	2.03 ± 0.03*

Data are expressed as means ± SEM of eight experiments. CHF= congestive heart failure at 8 weeks after coronary ligation; RV= right ventricle; LV= left ventricle; wt.= weight; N.D.= not detectable.

* Significantly different ($P < 0.05$) from the respective sham operated.

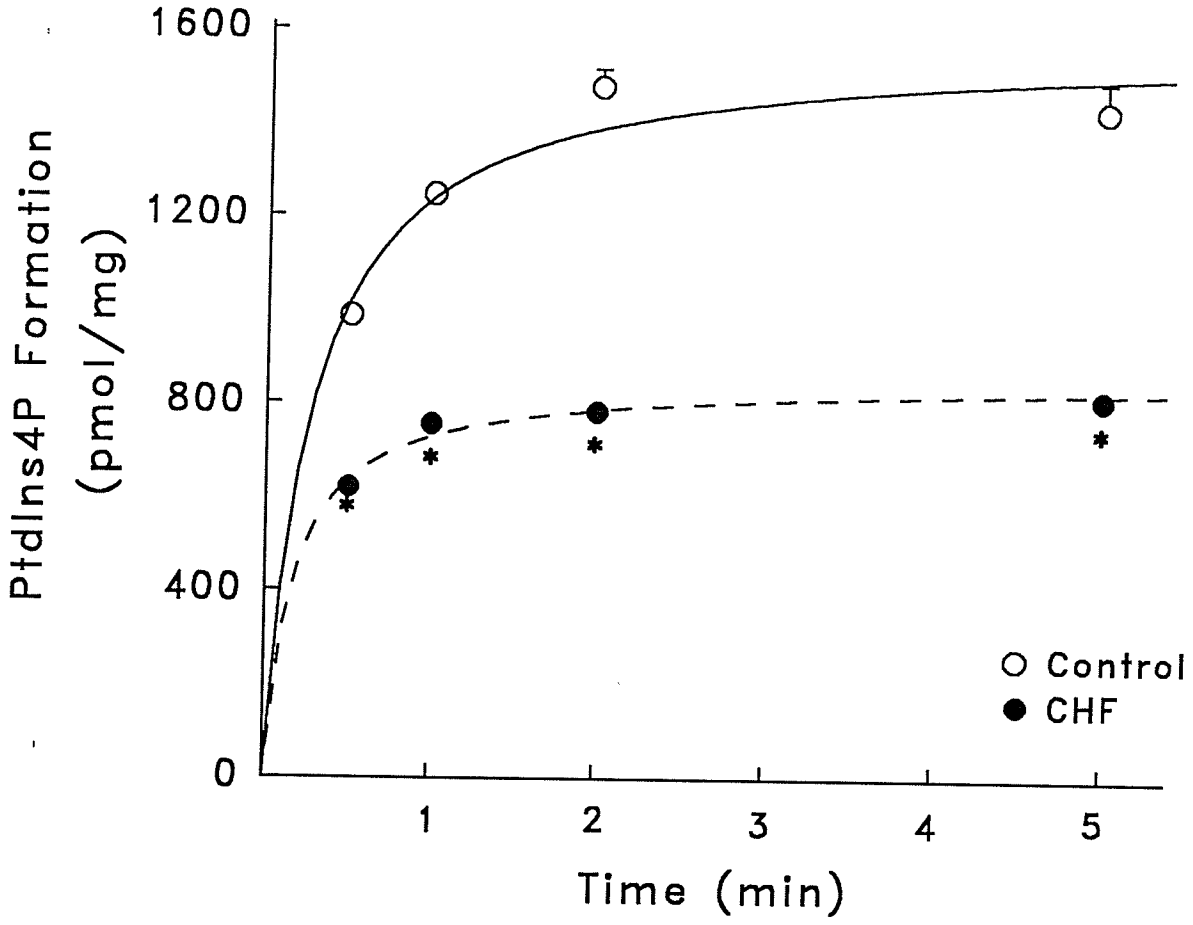

FIGURE 26. Time dependent change in PtdIns4P formation in SL membrane from left ventricles of sham-operated control and congestive heart failure (CHF, 8 weeks post-coronary ligation) rats. Values are means ± SEM of four to six experiments carried out in triplicate. SL membranes were incubated in the presence of alamethicin, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT and 1 mM [^{32}P]-ATP for the indicated times.

* Significantly different ($P < 0.05$) from control.

the PtdIns kinase activity in the viable left ventricles of CHF rats. The apparent V_{max} (FIGURE 28, inset) was significantly ($P < 0.05$) decreased in CHF (1658 ± 100.9 and 994.7 ± 88.5 pmol/mg protein/min, for control and CHF respectively), while the K_m was significantly ($P < 0.05$) increased (340 ± 15 and 632 ± 69 μM, for control and CHF respectively). A similar alteration was observed for the PtdIns4*P* kinase in left ventricles of CHF (FIGURE 29) with a significant depression in the apparent V_{max} (215.8 ± 20.8 and 147.5 ± 15.1 pmol/mg protein/min, in control and CHF respectively) (FIGURE 29, inset) and a significant increase in the apparent K_m (382 ± 28 and 536 ± 60 μM, in control and CHF, respectively). Since the depression observed in the PtdIns4*P* phosphorylation might have been due to the diminished synthesis of PtdIns4*P* by reduced PtdIns kinase activity, the experiments in FIGURE 29 were repeated by including 25 μM of exogenous PtdIns4*P* in the phosphorylation reaction. As shown in FIGURE 30, the activity of the SL PtdIns4*P* kinase in CHF was not significantly different from that of the controls when 25 μM exogenous PtdIns4*P* was added to the reaction medium. To test if the observed alteration in PtdIns kinase was also due to the limitation of its phospholipid substrate, 25 μM PtdIns was added to the reaction medium. However, the depression in the PtdIns kinase activity in CHF was persistent (FIGURE 31).

Since in this model of CHF the right ventricle undergoes different changes than the left ventricle (Anversa *et al.*, 1984; Anversa *et al.*, 1986; Green *et al.*, 1989), the phosphoinositide kinases were also studied in SL isolated from the right ventricles of control and failing hearts (8 weeks post-coronary ligation). FIGURE 32 shows the PtdIns phosphorylation in SL from right ventricles of CHF rats in presence and absence of

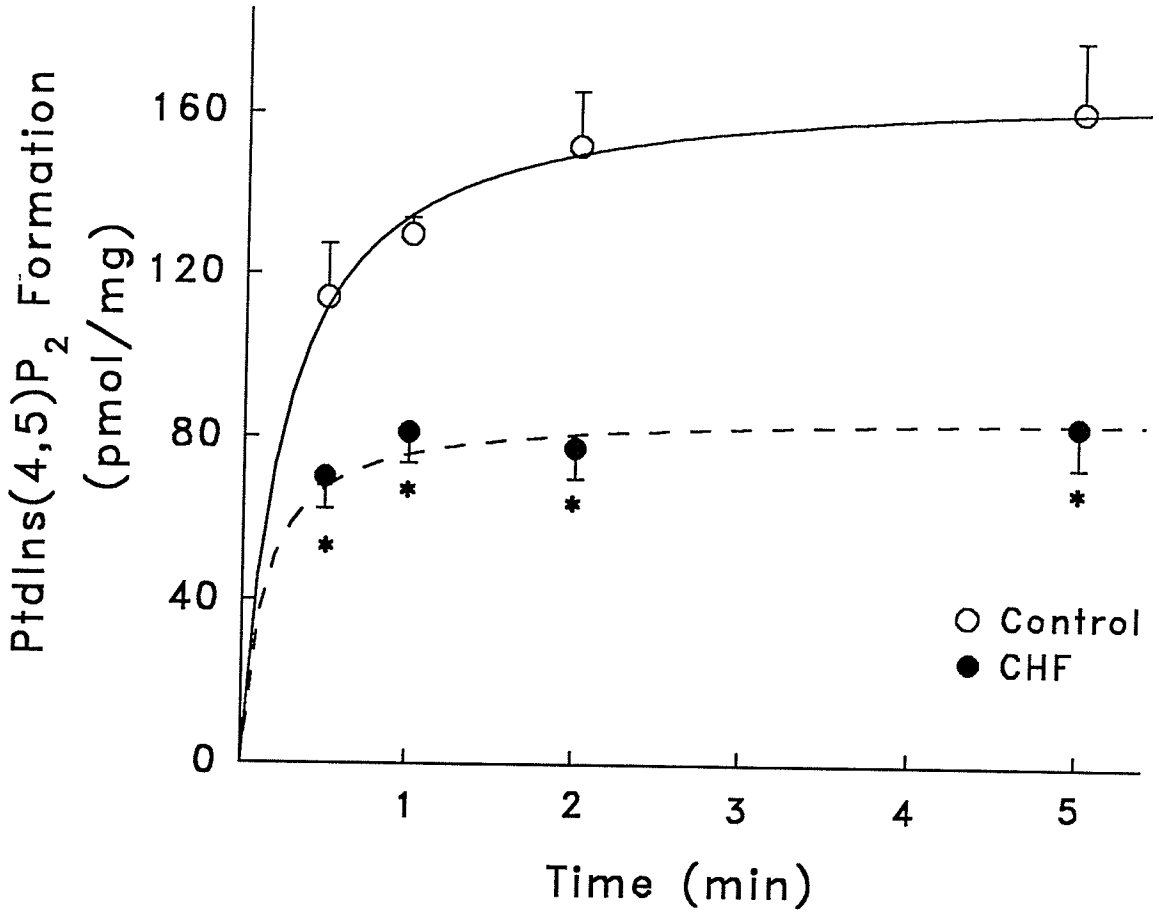

FIGURE 27. Time dependent change in PtdIns(4,5)P_2 formation in SL membranes from left ventricles of sham-operated control and congestive heart failure (CHF, 8 weeks post-coronary ligation) rats. Values are means ± SEM of four to six experiments carried out in triplicate. SL membranes were incubated in the presence of alamethicin, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, HEPES buffer (pH 7.4) and 1 mM [^{32}P]-ATP for the indicated times.

* Significantly different ($P < 0.05$) from control.

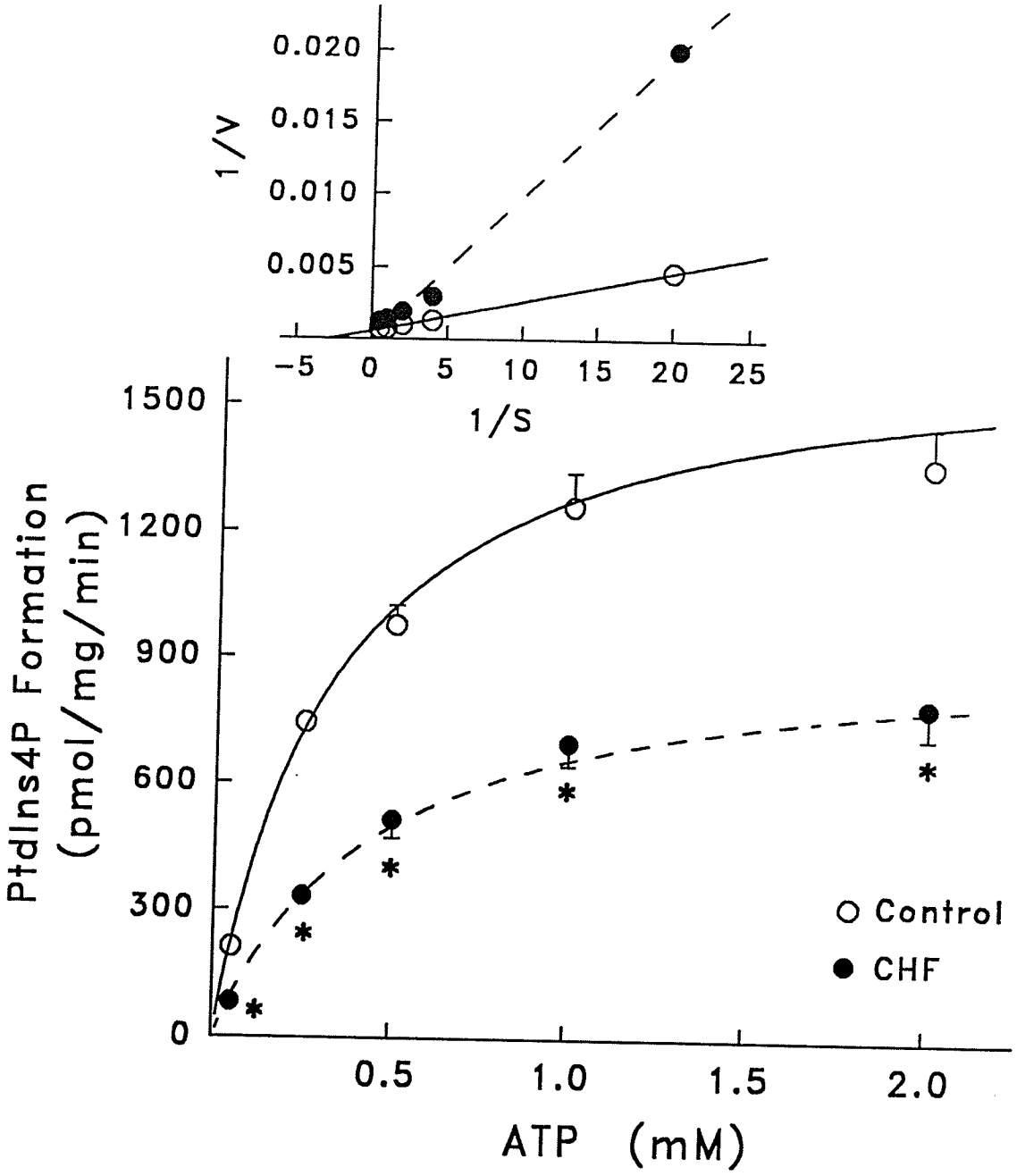

FIGURE 28. ATP dependent changes in PtdIns4P formation in SL membranes from viable left ventricles of control and CHF (8 weeks post-coronary ligation) rats. Data are means ± SEM from four to six experiments in triplicate determinations. 30 μg of SL protein was incubated in the presence of increasing ATP (0.05-2.0 mM) concentration, and 30 μg alamethicin, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, HEPES buffer (pH 7.4) for 1 min at 30°C. The Lineweaver-Burk plot (inset) was constructed from the values of the enzyme specific activity at different concentrations of ATP.

* Significantly different ($P < 0.05$) from the controls.

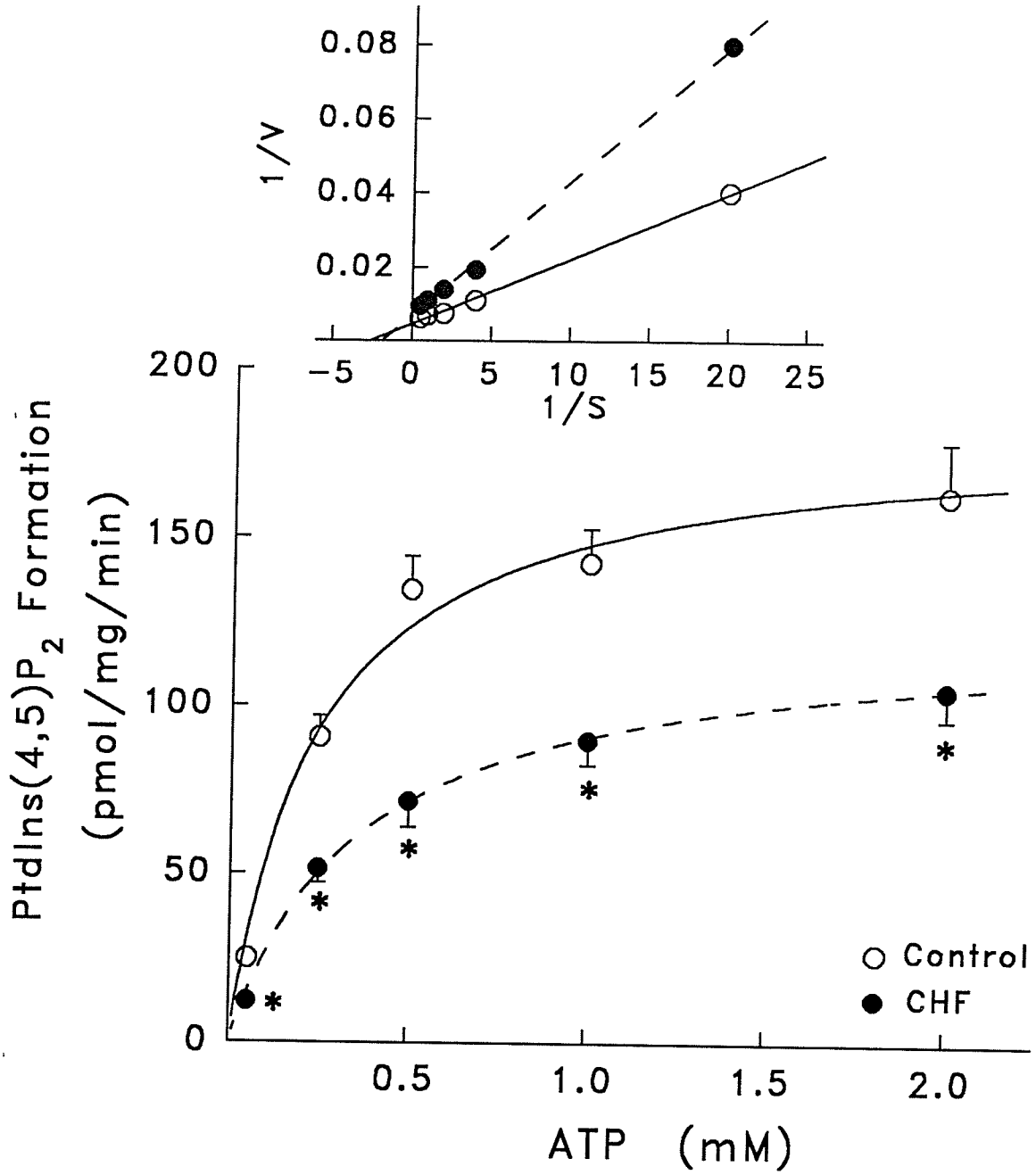

FIGURE 29. ATP dependent changes of PtdIns(4,5)P_2 formation in SL membranes from viable left ventricles of control and CHF (8 week post-coronary ligation) rats. Data are means ± SEM from four to six experiments in triplicate determinations. 30 μg of SL protein was incubated in the presence of increasing ATP (0.05-2.0 mM) concentration, and 30 μg alamethicin, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, HEPES buffer (pH 7.4) for 1 min at 30°C. The Lineweaver-Burk plot (inset) was constructed from the values of the enzyme specific activity at different concentrations of ATP.

* Significantly different ($P < 0.05$) from the controls.

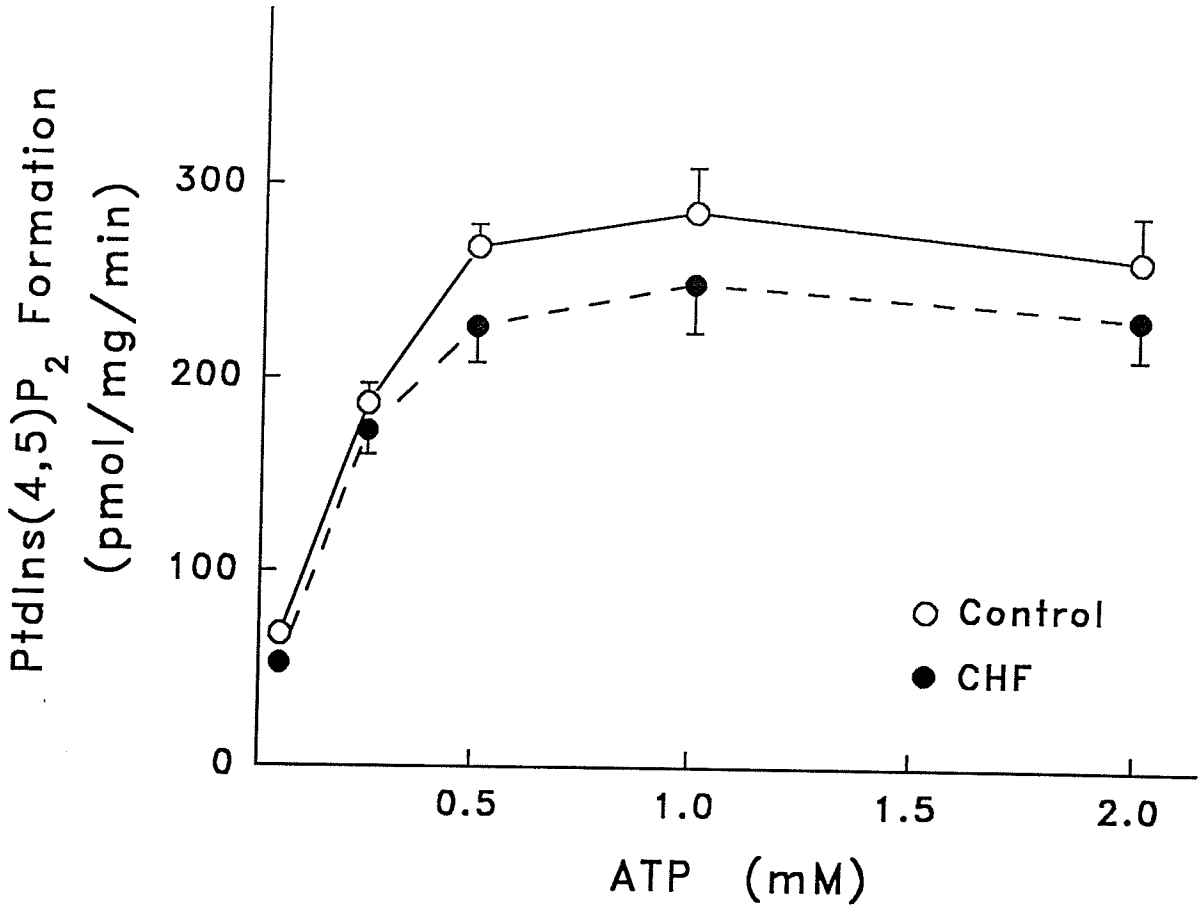

FIGURE 30. **PtdIns(4,5)P_2 formation in SL membranes from left ventricles of control and CHF (8 weeks post-coronary ligation) in presence of exogenously added PtdIns4P (25 μM) and increasing ATP concentrations. Data are means $\pm$ SEM from four to six experiments in triplicate determinations. 30 μg of SL protein was incubated in the presence of increasing ATP (0.05-2.0mM) concentration and 30 μg alamethicin, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, HEPES buffer (pH 7.4) for 1 min at 30°C.**

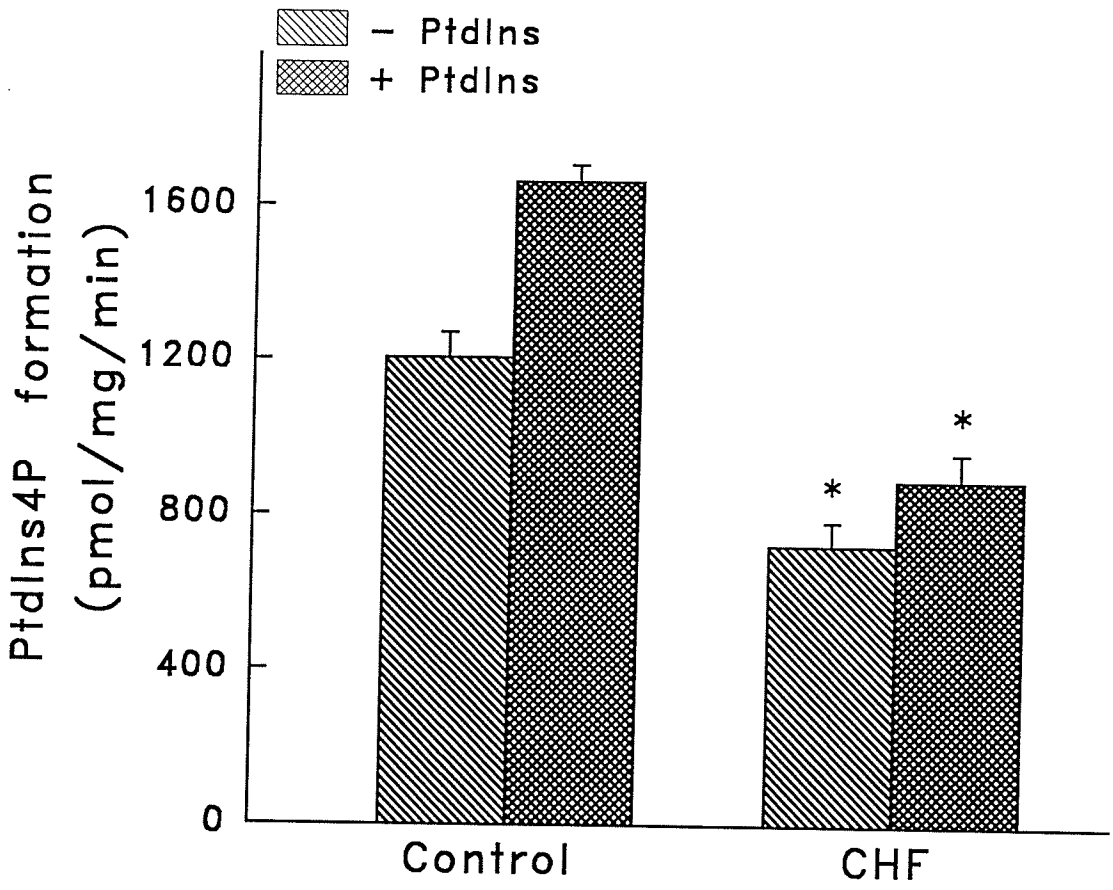

FIGURE 31. PtdIns kinase activity in SL from left ventricles of control and CHF rats in absence and presence of 25 μM exogenously added PtdIns. Values are means ± SEM of five experiments carried out in triplicate. Exogenous PtdIns was prepared by ultrasonication (30 min in Branson 1200 sonicator) and thereafter added to the incubation medium with the rest of conditions as indicated in "Materials and Methods".

* Significantly different ($P < 0.05$) from control values.

25 μM PtdIns. The PtdIns kinase activity was significantly higher in SL from right ventricles of CHF rats (FIGURE 32). Addition of 25 μM PtdIns significantly enhanced the PtdIns kinase activities in both control and CHF right ventricles; however the increase in CHF with 25 μM exogenous PtdIns was still significantly higher than the correspondent control values. The PtdIns4*P* kinase activity in right ventricles from controls and CHF was not different (FIGURE 33), and the exogenous addition of 25 μM PtdIns4*P* increased the phosphorylation of PtdIns4*P* in both control and CHF SL to a similar degree.

The phospholipase C activity in right and left ventricles from 8 week CHF was also studied in our laboratory. In brief, the PtdIns(4,5)P_2-PL C activity was significantly depressed in SL isolated from the viable tissue of left ventricles (Meij *et al.*, 1991). In some preliminary experiments on SL at an advanced stage of heart failure (16 weeks after coronary ligation) changes in PL C and phosphoinositide kinase activities were similar to those observed at 8 week CHF.

3. Effect of oxidants on SL phosphoinositide kinases

Inspite of the fact that the two pathological states under study, i.e. hypothyroidism and CHF, share the same characteristics of increased α/ß adrenoceptor ratio, the SL enzymes of the phosphoinositide pathway showed opposite alterations. In an attempt to unravel the molecular mechanism(s) responsible for this diversity, we hypothesized that oxidants, which are produced during CHF (Prasad *et al.*, 1988) may affect the enzyme activities by modifying essential thiol residues on their molecules.

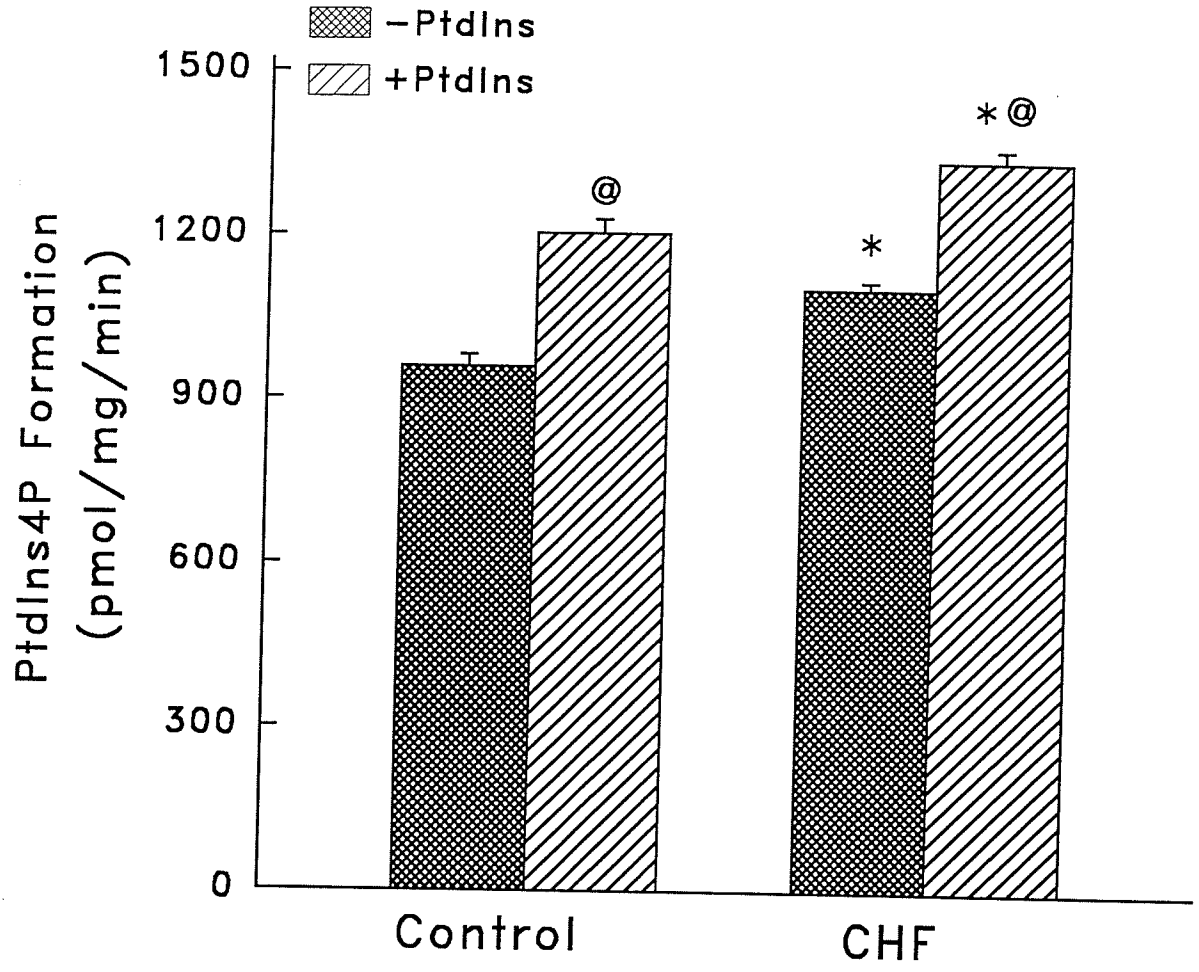

FIGURE 32. PtdIns kinase activity in SL from right ventricles of control and CHF rats in absence and presence of 25 μM exogenously added PtdIns. Values are means ± SEM of five experiments carried out in triplicate. Exogenous PtdIns was prepared by ultrasonication (30 min in Branson 1200 sonicator) and thereafter added to the incubation medium with the rest of conditions as indicated in "Materials and Methods".

* Significantly different ($P < 0.05$) from respective control values.

@ Significantly different ($P < 0.05$) vs. - PtdIns.

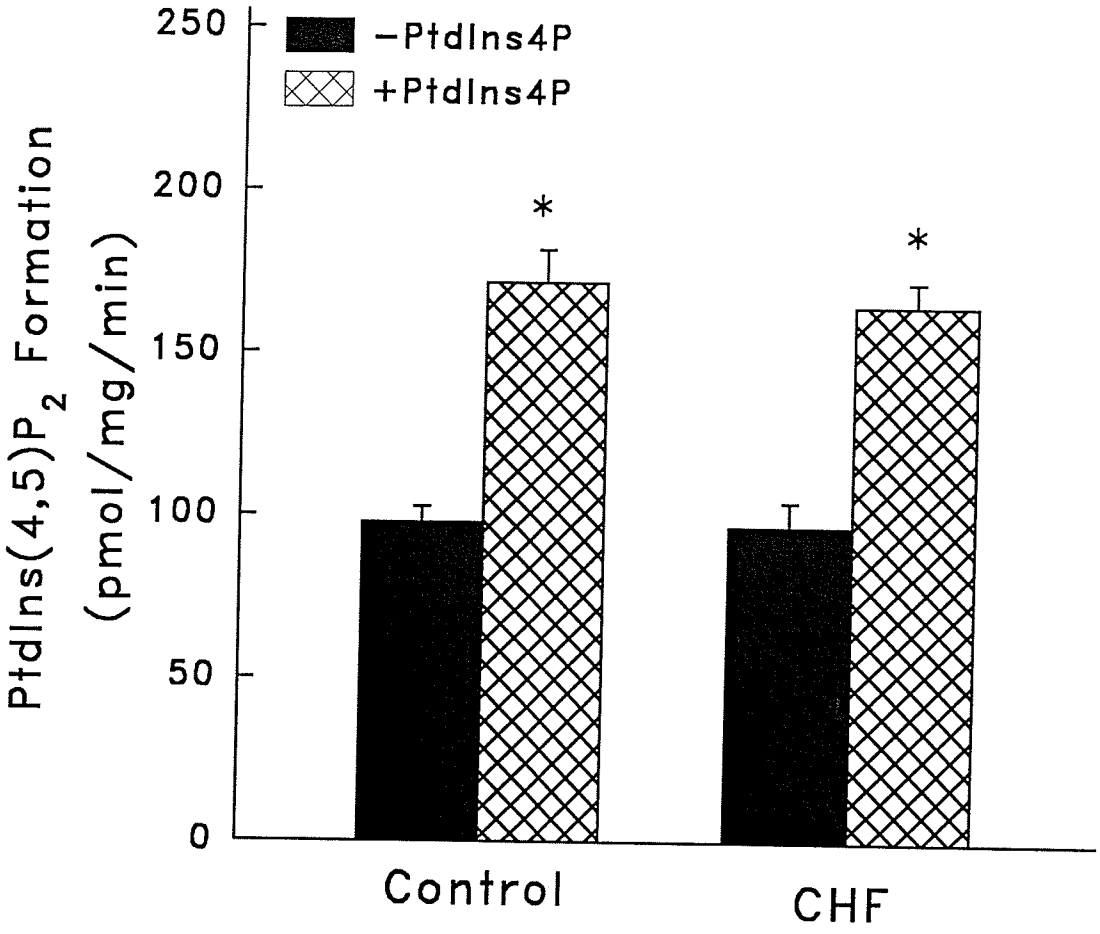

FIGURE 33. PtdIns4P kinase activity in SL from right ventricles of control and CHF rats in absence and presence of 25 μM exogenously added PtdIns4P. Values are means ± SEM of five experiments carried out in triplicate. Exogenous PtdIns4P was prepared by ultrasonication (30 min in Branson 1200 sonicator) and thereafter added to the incubation medium.

* Significantly different ($P < 0.05$) from vs. PtdIns4P.

TABLE 13 shows the effects of different oxidants on the phosphoinositide kinases of SL membranes isolated from normal rat hearts. Incubation of SL membranes in the presence of 2 mM xanthine plus 0.03 U/ml xanthine oxidase, a superoxide anion generating system in which some H_2O_2 is also produced (Fridovich, 1970; Weiss, 1986), significantly depressed both PtdIns kinase and PtdIns4*P* kinase activity by 80 and 60%, respectively (TABLE 13). SOD, a superoxide anion scavenger, could not prevent the deleterious effect of X+XO. However, simultaneous addition of CAT to the X , XO and SOD medium was able to protect both PtdIns and PtdIns4*P* kinase activities, implicating H_2O_2 as the effective species. In fact, the full protection effect of catalase in the X+XO system suggests that after generation of the superoxide anion, its subsequent conversion to H_2O_2 by spontaneous dismutation or by SOD (Halliwell, 1991) may be responsible for the observed PL C depression. Previous evidence has shown that myocardial reperfusion injury in the isolated rat heart is mediated by H_2O_2 derived from xanthine oxidase (Brown *et al.*, 1988). Indeed, 1 mM H_2O_2 (a non-radical oxidant) inhibited both the kinase activities to the same extent as that of X+XO (TABLE 13), an effect which was completely abolished by CAT but not by iron chelation with deferoxamine. This suggests that the hydroxyl radical formation via the Fenton reaction was not involved in the inhibitory effect of H_2O_2. Treating the SL membranes with X, XO, SOD or CAT separately had no significant effect on these enzymes. However, deferoxamine alone resulted in 28% and 16% depression of PtdIns and PtdIns4*P* kinases, respectively.

The effects of another non-radical oxidant, hypocholorus acid (HOCL) which is produced in neutrophils by the myeloperoxidase enzyme, were also studied. As shown in

TABLE 13. Effect of reactive oxidants on PtdIns kinase and PtdIns4P kinase activities in rat heart sarcolemma.

Experimental conditions	PtdIns Kinase	% of Control	PtdIns4*P* Kinase	% of Control
Control	585.3 ± 14.2	100.0	69.9 ± 2.7	100.0
(a)				
X+XO	122.3 ± 4.8*	20.9	27.9 ± 1.4*	40.0
X+XO+SOD	123.5 ± 2.7*	21.1	30.1 ± 1.8*	43.0
X+XO+SOD+CAT	424.3 ± 7.0*@	72.5	65.8 ± 1.4@	94.1
SOD	649.5 ± 17.5	110.9	80.3 ± 3.9	115.0
(b)				
H_2O_2	149.8 ± 12.2*	25.6	32.1 ± 1.1*	45.9
H_2O_2+CAT	527.4 ± 39.5	90.1	72.1 ± 4.8	103.2
H_2O_2+ Deferoxamine	179.7 ± 13.1*	30.7	32.1 ± 2.6*	46.0
H_2O_2 + DTT	656.4 ± 57.2	112.1	76.4 ± 6.1	109.2
CAT	577.1 ± 9.7	98.6	78.8 ± 3.2	112.8
Deferoxamine	422.0 ± 26.7*	72.1	58.5 ± 3.5*	83.7
(c)				
HOCl	91.0 ± 10.8*	15.5	23.1 ± 1.6*	33.0
HOCl + DTT	618.1 ± 40.5	105.6	75.1 ± 9.6	107.4

X, xanthine; XO, xanthine oxidase; SOD, superoxide dismutase; CAT, catalase. Values are expressed as pmol/mg protein/min and are mean ± SEM of five experiments in triplicate. Sarcolemmal membranes were preincubated for 10 min at 37°C. The final concentration of the chemicals were X, 2mM; XO, 0.03 U/ml; SOD, 80 μg/ml; H_2O_2, 1 mM; CAT, 10 μg/ml and deferoxamine, 1 mM. The pellet was sedimented by ultracentrifugation, washed and used to determine the PtdIns and PtdIns4*P* kinase activities as described in the "Material and Methods".

* Significantly different ($P < 0.05$) from controls.

@ Significantly different ($P < 0.05$) *vs.* X+XO+SOD.

TABLE 13, HOCL (0.3 mM) inhibited both the kinases to a similar extent as H_2O_2. Treatment of the SL membrane with 1mM DTT, a potent reducing agent, in combination with H_2O_2 or HOCL prevented their oxidative effects (TABLE 13). It is worth noting that PtdIns kinase resulted to be more sensitive to the oxidative stress than PtdIns4*P* kinase (TABLE 3, 13).

Since H_2O_2 was found to be the major oxygen derived inhibitor of PtdIns and PtdIns4*P* kinases, its inhibitory action was further detailed. Both PtdIns kinase and PtdIns4*P* kinases were inhibited by H_2O_2 in a dose dependent manner (FIGURE 34A and 34B). PtdIns and PtdIns4*P* kinases were significantly depressed at 1 μM and 10 μM H_2O_2, respectively. The depression was progressively aggravated with increasing the H_2O_2 concentrations reaching a minimum at 10 mM H_2O_2. The IC_{50} of the H_2O_2 action on PtdIns kinase was 27 μM while that on PtdIns4*P* kinase was 81 μM. Parallel experiments conducted in our laboratory showed a thiol-dependent deactivation of the SL PL C the activity under oxidative stress (Meij *et al.*, 1993).

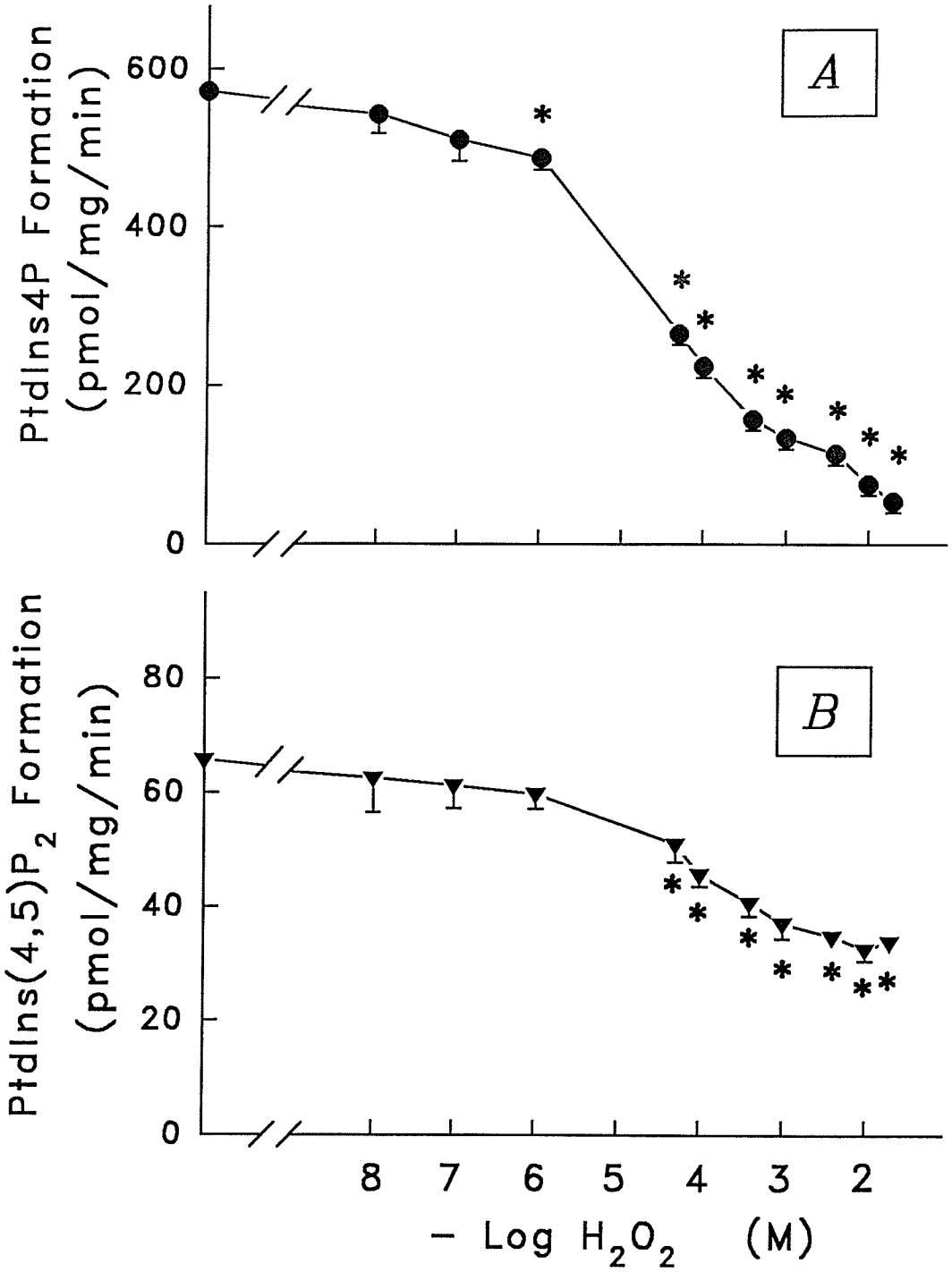

FIGURE 34. Concentration dependence of H_2O_2 effects on SL PtdIns (A) and PtdIns4P (B) kinase activities. Results are means ± SEM of four experiments carried out in triplicate determinations. SL membranes were pretreated with increasing concentrations of H_2O_2 for 10 min at 37°C, the samples were then placed on ice and processed as described in " Materials and Methods".

* Significantly different ($P < 0.05$) vs. 0 H_2O_2.

V. DISCUSSION

A. *Characteristics of Sarcolemmal PtdIns and PtdIns4P Kinases*

PtdIns kinase and PtdIns4*P* kinase activities of the rat heart SL exhibited an absolute requirement for Mg^{2+} as reported for the heart of other species (Quist *et al.*, 1989). Their pH profiles, which had not yet been examined in cardiac membranes, were similar and showed the highest activities at pH 7.5, in agreement with findings in other cell types (Harwood and Hawthorne, 1969). The apparent Km values for ATP were in a μM range higher than that reported for the dog heart (Quist *et al.*, 1989; Kasinathan *et al.*, 1989) but comparable to other published values (Wolf, 1990; Kanoh *et al.*, 1990). In this study we have shown that Triton X-100 (0.05-0.25%, v/v), a non-ionic detergent, and alamethicin (0.5-2.0,drug/SL protein ratio), an ionophore polypeptide antibiotic that permeabilizes SL membrane vesicles (Lamers *et al.*, 1983), enhanced the synthesis of both endogenous PtdIns4*P* and PtdIns(4,5)P_2. Other studies in dog (Quist *et al.*, 1989; Kasinathan *et al.*, 1989) and rabbit (Wolf 1990) cardiac membranes using our range of Triton X-100 concentrations showed decreased (Quist *et al.*, 1989) or increased (Kasinathan *et al.*, 1989) endogenous PtdIns4*P* formation, while PtdIns(4,5)P_2 synthesis was negligible (Quist *et al.*, 1989) or undetectable (Kasinathan *et al.*, 1989; Wolf, 1990). In particular, PtdIns4*P* kinase was completely inactivated by 0.25% Triton X-100 (Kasinathan *et al.*, 1989; Wolf, 1990) so that exogenous PtdIns4*P* could not be used as a substrate to establish the maximal catalytic activity of the enzyme (Wolf, 1990). These

conflicting observations may be attributed either to species specificity of the phosphoinositide kinases with respect to their organization in the heart SL membrane, as reported for other SL enzymes (Panagia *et al.*, 1986), or to the inter-species occurrence of distinct enzyme forms (Downes and Macphee, 1990; Carpenter and Cantley, 1990). At any rate, our finding that Triton X-100 strongly (12 fold) stimulates the rat heart SL PtdIns kinase indicates that this enzyme phosphorylates the D-4 position of the inositol ring to form PtdIns4*P*, as opposed to the D-3-phosphorylating PtdIns kinase which is profoundly inhibited by Triton X-100 (Downes and Macphee, 1990; Carpenter and Cantley, 1990; Downes and Carter, 1991). The latter kinase has been recently found in several nonmyocardial cells, is responsible for the synthesis of 3-phosphate containing polyphosphoinositides, and responds to the activation of a variety of cellular tyrosine kinases (Carpenter and Cantley, 1990; Majerus *et al.*, 1990; Downes and Carter, 1991). These D-3 phosphorylated phosphoinositides represent a maximum of 2-5% of the polyphosphoinositide pool, are not intermediates of the phosphoinositide cycle, and are not hydrolysed by phospholipase C (Carpenter and Cantley, 1990; Majerus *et al.*, 1990; Downes and Carter, 1991).

In our *in vitro* experiments with endogenous phospholipid substrate, the catalytic rate of PtdIns kinase far exceeded that of PtdIns4*P* kinase, and this was observed also by others in a similar conditions (Quist *et al.*, 1989; Kasinathan *et al.*, 1989). However, we were able to maximize PtdIns4*P* kinase activity by the exogenous addition of its phospholipid substrate, which suggests that: (1) the PtdIns4*P* level in isolated SL membrane is subnormal, possibly due to PtdIns4*P* dephosphorylation and/or hydrolysis

by the action of specific membrane monophosphoesterase and/or PL C (Rana and Hokin, 1990; Meij and Panagia, 1991; Meij and Panagia 1992), respectively, during the isolation or the pre-incubation steps; (2) this seems to be a rate-limiting factor for PtdIns4*P* kinase and, therefore, (3) exogenous PtdIns4*P* is needed for assessing the optimal PtdIns4*P* kinase activity in studies with isolated SL membranes.

Although other membranous and cytosolic factors might influence the kinase activities in the intact cardiomyocytes, as already shown for other cell types (Downes and Macphee, 1990; Carpenter and Cantley, 1990), it was interesting to find that the phosphoinositide phosphorylation system of the heart SL was inhibited by micromolar (1-10μM) concentrations of Ca^{2+}, independently of either Ca^{2+}-induced activation of PL C and monophosphoesterases or low levels of ATP. Another study on dog cardiac membranes showed no effect by 0.1 to 30 μM Ca^{2+} (Kasinathan *et al.*, 1989). However, an increase in free Ca^{2+} concentration inhibited both kinases associated with the T-tubule membranes from mammalian skeletal muscle (Carrasco *et al.*, 1988; Heilmeyer *et al.*, 1990). At present, the biological significance of the above finding is only theoretical but some possibilities could be considered. Firstly, both kinase activities are regulated by changing Ca^{2+} from 10^{-7} to 10^{-5} M which are, respectively, the diastolic and systolic intracellular Ca^{2+} concentration (subsarcolemmal levels) during the cardiac cycle. That may be simply coincidental or it may imply a participation of these enzymes in the biochemistry of the normal cycle. On the other hand, the phosphoinositide cycle has a rapid turnover only when the receptor interacts with the activating hormone. In such a situation, the phosphorylation rate of the coupled PtdIns and PtdIns4*P* kinases become

critical in maintaining the intrasarcolemmal level of PtdIns(4,5)P_2, the precursor to the PL C-dependent formation of Ins(1,4,5)P_3 and DAG. The Ins(1,4,5)P_3-induced mobilization of Ca^{2+} from cardiac SR (Nosek *et al.*, 1986; Kentish *et al.*, 1990), and the subsequent activation of SL Na^+/Ca^{2+} exchanger (Gilbert *et al.*, 1991) result in higher cytosolic Ca^{2+} concentrations. Therefore, the observed Ca^{2+}-induced depression of the kinases could be potentially important in the feedback inhibition of Ins(1,4,5)P_3 formation. As well, in pathological situations of Ca^{2+} overload the combined inhibition of phosphoinositide kinases and the activation of PL C (Edes and Kranias *et al.*, 1990; Meij and Panagia, 1992) by Ca^{2+} can be seen to ultimately decrease the SL polyphosphoinositide level, the Ins(1,4,5)P_3 formation and the subsequent Ca^{2+} release from SR, thus limiting further rise of cytosolic Ca^{2+} through this pathway.

The regulation of the catalytic activity of various enzymes by modulation of the thiol-disulfide exchange has been shown in several instances (Vetter *et al.*, 1991; Dai *et al.*, 1992; Meij *et al.*, 1993; for review see Ziegler 1985). The depression of SL PtdIns and PtdIns4*P* kinases after treatment with sulfhydryl group modifiers (TABLE 3) and oxidants (TABLE 13), and the protective role of dithiothreitol demonstrate the functional importance of thiol groups in these kinases, and suggest that changes in the redox state of the thiol groups by oxidants could impair these enzyme activities. The differential protection of PtdIns and PtdIns4*P* kinases by DTT in the presence of NEM or MMTS (TABLE 3) could be attributed to the different action of these modifiers on the PtdIns and PtdIns4*P* kinases. NEM an alkyl-introducing compound also reacts with other protein residues (Van Iwaarden *et al.*, 1992), while MMTS action on proteins is mediated via

introduction of methanethio groups to the protein thiol groups and leads to the formation of mixed disulfides (Smith *et al.*, 1975; Van Iwaarden *et al.*, 1992). Inactivation of these enzymes also occurred when pCMPS was used (TABLE 3). pCMPS is an organomercurial compound which has poor lipid solubility and the ability to reach partially masked sulfhydryl groups (Van Iwaarden *et al.*, 1992). This compound caused a similar depression in both kinases, inducing a stronger inhibition on PtdIns4*P* kinase compared to NEM and MMTS (TABLE 3). The inhibition of PtdIns4*P* kinase by pCMPS was only partially protected by DTT. Thus, our findings could be explained, at least in the case of PtdIns4*P* kinase, by the existence of two classes of reactive sulfhydryl groups located in hydrophilic regions; one reactive class buried within the protein and a more accessible class which is readily affected by different factors. Furthermore, the naturally occurring oxidant, H_2O_2 and HOCL, which have been previously shown to oxidize protein sulfhydryl groups (Kaneko *et al.*, 1989; Suzuki *et al.*, 1991; Eley *et al.*, 1989; 1991), induced a significant inhibition of the PtdIns and PtdIns4*P* kinases (TABLE 13). DTT prevention of this depression confirms the essential role of the thiol groups in these kinases. In particular, the deactivation of the phosphoinositide kinases occurred at low levels of H_2O_2 (1-10 μM, FIGURE 34). Previous reports have shown the regulatory action of H_2O_2 (in small quantities) by modifying the cellular thiol-disulfide redox balance (for review see Ziegler, 1985). Hydrogen peroxide (1μM) was also able to trigger the onset of stress response via induction of heat shock proteins (Becker and Mezger, 1991). Therefore, the sulfhydryl-mediated action of H_2O_2 on the phosphoinositide kinases may play a role in pathological conditions and, in the case of PtdIns kinase, also under

physiological state.

The physiological importance of PtdIns and PtdIns4*P* kinases has been associated with the production of the substrate for the catalytic activity of PL C. In addition, polyphosphoinositide and the DAG formed by their PL C-dependent hydrolysis have long been suggested to be an important source of arachidonic acid, and thus of prostaglandins and eicosanoids. This suggestion was mainly derived from the fatty acid composition of PtdIns (Holub, 1987), being generally assumed that the fatty acid composition of PtdIns and its D-4 derivatives were identical. The few studies available show that the phosphoinositides of brain (Holub *et al.*, 1970) and erythrocytes (Chiba *et al.*, 1988), but not of hepatocytes (Augert *et al.*, 1989), share the same fatty acid profile. In this study we demonstrated that the fatty acid profile of rat heart PtdIns4*P* and PtdIns(4,5)P_2, containing lower arachidonic acid component, significantly differ from that of the PtdIns. This observation is further supported by previous reports of low 18:2*n*6 and 20:4*n*6 content of cellular DAG in α_1-adrenergically stimulated perfused hearts (Okumura *et al.*, 1990) and cultured rat ventricular myocytes (Bordoni *et al.*, 1991). Thus our results imply the specificity of the PtdIns kinase for a pool of PtdIns molecules with low arachidonic acid content. Furthermore, our finding that PtdIns(4,5)P_2 represents a relatively modest source of unsaturated fatty acids, in particular of arachidonic acid (20:4*n*-6), indicates that the myocardial PtdIns(4,5)P_2 and its hydrolysis product DAG may be less important source for the eicosanoid synthesis. Previous reports have shown that one of the fatty acid acyl chain of DAG must be unsaturated for the optimal activation of PK C (Nishizuka, 1988; Bell and Burns, 1991; Allen and Katz, 1991), thus in the myocardium

the hydrolysis of polyphosphoinositides may be less important source for the activation of protein kinase C.

B. *Role of α_1-Adrenoceptor Associated Phosphoinositide Pathway in Pathological Conditions*

1. Hypothyroidism

Cardiac α_1-adrenoceptor mediate positive inotropism in the mammalian hearts (Benfey, 1980; Brodde *et al.*, 1978; Bruckner *et al.*, 1984) including the human ventricular myocardium (Bruckner *et al.*, 1984; Aass *et al.*, 1986), a response which is masked by the activity of ß-adrenoceptors under physiological conditions. However, in certain pathological conditions characterized by compromised ß-adrenoceptor system, the increased α_1/ß adrenoceptor density and the α_1-adrenoceptor mediated positive inotropy have been suggested to compensate for the reduced ß-adrenoceptor mediated response. In this study we utilized two pathological models (hypothyroidism and post-infarcted congestive heart failure in rats) in which an increase in α_1/ß ratio has been reported to examine the above hypothesis and to test its universality.

Thyroid regulation of the adrenoceptor density has been pursued by many investigators. Reports on hyperthyroidism have clearly showed an increase in the ß-adrenoceptor density (Banerjee *et al.*, 1977; Kempson *et al.*, 1978; Chang *et al.*, 1981; Kupfer *et al.*, 1986) which was accompanied by a proportional increase in the maximal

isoproterenol-responsiveness (Coville *et al.*, 1970; MacLeod, 1981) and adenylate cyclase activity (Ciaraldi *et al.*, 1978; Tse *et al.*, 1980; Krawietz *et al.*, 1982). A significant depression in the α_1-adrenoceptor density has also been reported in the hyperthyroid hearts (Ciaraldi *et al.*, 1978; Sharma *et al.*, 1978; Kunos *et al.*, 1980; Chang and Kunos, 1981). Opposite changes in the ß-receptor density were reported in the hypothyroid hearts (Banerjee *et al.*, 1977; Ciaraldi *et al.*, 1977; Kunos *et al.*, 1980) and that were associated with a decline in the response to ß-agonists (Nakashima *et al.*, 1971; Ciaraldi *et al.*, 1978). Despite the consistent observation of augmented α_1-adrenoceptor mediated positive inotropy (Nakashima *et al.*, 1971; Simpson and McNeil, 1980), reports on the α_1-adrenoceptors have been conflicting, although mainly pointing to an increased density (Ciaraldi *et al.*, 1978; McConnaugehey *et al.*, 1979; Sharma *et al.*, 1978; Kunos, 1980; Fox *et al.*, 1985). In our hands, SL purified from PTU-treated rat hearts showed a decrease in the density of the ß-adrenoceptors with a concomitant increase in the α_1-adrenoceptor density (TABLE 7). In addition, there were no changes in the affinity of these receptors for their respective antagonists. Therefore, in the PTU-treated hypothyroid hearts, the depressed ß and enhanced α_1-adrenoceptor density tend to support the importance of the latter receptors in the responsiveness to circulating catecholamines. Moreover, we have shown that in the perfused PTU-treated hypothyroid hearts the α_1-adrenoceptor agonist induces a higher increment in LVP_{max} and $\pm dP/dt$ compared to the ß-adrenoceptor agonist (TABLE 10), which indicates an increase in the responsiveness of the α_1-associated signalling pathway in hypothyroidism. The enhancement of $\pm dP/dt$ by α_1-adrenoceptor agonist demonstrates an increase in the

rate of contraction and relaxation which would result in a decrease in the duration of contraction. A chronotropic effect via α_1-adrenoceptor stimulation has been previously reported by exposure of the spontaneously beating rat atria to methoxamine or phenylephrine (α_1-adrenoceptor agonists) (Nakashima *et al.*, 1973; Wagner and Brodde, 1978; Simpson and McNeill, 1980; Flavahan and McGarth, 1981). The chronotropic effect was higher in the hypothyroid compared to the euthyroid rats and was blocked completely by the α_1-adrenoceptor antagonists phentolamine (Nakahshima *et al.*, 1973) and phenoxybenzamine (Simpson and McNeil, 1980). In contrast to these observations in rat, studies on rabbit myocardium stimulation by phenylephrine or methoxamine have resulted in an inotropic response which was not accompanied by any change in the myocardial chronotropy (Talosi and Kranias, 1992; Jahnel *et al.*, 1992). These differences could be attributed to species difference. In dog heart, the positive inotropic response of sympathomimetic amines (phenylephrine, norepinephrine, dopamine and epinephrine) is not mediated by the α_1-adrenoceptors (Yamashita and Endoh, 1981; Schumann *et al.*, 1978; Endoh *et al.*, 1991). The differences in the α_1-adrenoceptor mediated response in various species could be readily explained by the presence of multiple α_1-receptor subtypes as described above (see Literature Review).

Our observation of a decreased positive inotropic response (both LVP_{max} and $\pm dP/dt$) to isoproterenol in the hypothyroid rat hearts could be justified by the depression in the ß-adrenoceptor density. In addition, recent reports have illustrated an increase in the inhibitory G protein (Gi) and the ß-subunit of G protein in hypothyroid rat ventricular tissue (Levine *et al.*, 1990). In contrast, in the hypothyroid rat heart

membranes the Gpp(NH)p activation of the adenylate cyclase activity and the [^{32}P]-ADP ribosylation of the α-subunit of the stimulatory G protein (Gs_α) were not altered (Krawietz *et al.*, 1982). Therefore, the combined effects of declined ß-receptor density, increased G protein ß subunit and increased level of Gi_α protein could result in the depression of the ß-adrenoceptor mediated responses in the hypothyroid myocardium. The observed increase in the density of α_1-adrenoceptor could in part explain the augmented α_1-induced contractile response of the hypothyroid hearts. However, the not yet investigated possibility of hyperfunctioning of the mechanisms involved in mediating the α_1-receptor response should not be ignored. One way to enhance the α_1-mediated contractility is the increased sensitivity of the contractile proteins. Nevertheless, previous reports have clearly shown a switch of the myosin isozymes from the V_1 (with higher ATPase activity) to V_3 (with lower ATPase activity) in the PTU-treated and thyroidectomized rats (Hoh *et al.*, 1978; Chizzonite *et al.*, 1984). The altered cellular Ca^{2+} handling mechanisms also was suggested to play a role in the α_1-mediated responses and are discussed in the "Literature Review". To the above mechanism the phosphoinositide pathway should be added, which plays a significant role in the transduction of the α_1-adrenoceptor mediated response in the normal heart and has not been yet explored in hypothyroidism.

The present study, for the first time, examined the changes of PtdIns kinase, PtdIns4*P* kinase and PL C activities in the SL preparations from hypothyroid and hyperthyroid rat hearts. We demonstrated that the basal activities of PtdIns and PtdIns4*P* kinases were not altered in the hypothyroid rat heart SL and no changes in kinetic

parameters for ATP were observed (FIGURE 11, 12, 14, 15). However, in hyperthyroidism both these kinases were significantly increased (FIGURE 14, 15). No other reports are available on the basal activities of these kinases in the hypothyroid state in another tissue. Few studies have investigated the impact of hypothyroidism on the α_1-adrenoceptor-induced phosphoinositide turnover in adipose tissue (Garcia-Sainz and Fain, 1980; Fain 1981; Andersen *et al.*, 1991). These reports have shown an increase in the labelling of the phosphoinositides in both eu- and hypothyroid adipocytes after α_1-adrenoceptor stimulation, but no differences were observed between these two thyroid states. Our data on the phosphoinositide kinases suggest that these enzymes are not directly involved in the amplification of the α_1-adrenoceptor signal in the hypothyroid state. However, the increased activity rate of phosphoinositide kinases under stimulation (Carpenter and Cantley, 1990) would assure the presence of sufficient substrate for the PL C activity, thus positively regulating the signal amplification in subsequent steps of the pathway. In this study we have also shown that the activities of the phosphoinositide kinases were augmented when their phospholipid substrates (exogenously added) were increased in the hypo- and hyperthyroid hearts in the same manner as in the euthyroid hearts, an observation which supports the increased turnover of the phosphoinositide pathway under α_1-stimulation. Whether the observed increases in phosphoinositide kinase activities in hyperthyroid SL are simply due to an increased catalytic rate of the phosphoinositide kinases or are mediated by an increased number of the enzyme molecules requires further investigation. Nevertheless, the observed increase in PtdIns and PtdIns4*P* kinase activities in the hyperthyroid hearts might be considered as an

attempt to compensate for the decreased α_1-receptor density in this case.

In this study we have also demonstrated a significant increase in the basal activity of both the SL and cytosolic PL C in the hypothyroid hearts (FIGURE 16 and 19). These activities were further enhanced after α_1-adrenoceptor stimulation (FIGURE 22, 24). Recently, Andersen *et al.* (1991) have shown a slight, but not significant, increase of the PL C activity in the unstimulated hypothyroid adipocytes compared to the euthyroid ones. In addition, these authors have also observed an increase in the PL C activity after α_1-adrenoceptor stimulation. However, this increase was not significantly different in the hypothyroid compared to the euthyroid rat adipocytes. The quantitative differences between these authors' observation and our current study could be due to several factors; (1) subtle diversity may exist among tissues with respect to the intramembranal organization of PL C activity; (2) the occurrence of different PL C isozymes; (3) their cellular localization, since the variability between the preparations used (cell cultures versus purified cell membranes and cytosol) should not be overlooked. As described above (Literature Review), five isoforms of PL C (α, ß, γ, δ and ϵ) have been identified in different tissues (Cockroft and Thomas, 1992). The presence of only three PL C subtypes, namely PL C γ, δ and ß in lower quantities, was reported in the myocardium (see Literature Review). These isoforms differ in their localization (Rhee *et al.*, 1991), affinity for the different phosphoinositide substrates (PtdIns, PtdIns4*P* or PtdIns(4,5)P_2) (Rhee *et al.*, 1989) and are stimulated via different mechanisms (Cockcroft and Thomas, 1992; Rhee and Choi, 1992). Since most of the PL C isoforms have been localized in the cytoplasmic fraction and only two have been shown to be associated with the membrane

fraction (PL C ß and δ), it was important to test the effects of the circulating thyroid hormone on PL C activity separately in each fraction. Indeed, we have shown that changes in the SL PL C activity at various circulating thyroid hormone levels were limited to PtdIns(4,5)P_2 hydrolysis (TABLE 8), with a significant increase in both K_m and V_{max} values in the PL C activity in the hypothyroid rat heart SL. The cytosolic PL C showed similar hydrolytic changes with changes in thyroid hormone, which were common to all the three substrates used (TABLE 9). Previous reports have shown the association of the PL C δ isoform to the cardiac SL and cytosol (Suh *et al.*, 1988; Wolf, 1992), the PLC γ isoform being only cytosolic fraction (Suh *et al.*, 1988; Homma *et al.*, 1989). No reports are available on the presence of the PL C α in the myocardial tissue. Our observations could not indicate which of the PL C isoform(s) is regulated by thyroid hormone. In addition, whether the modulatory effect of the thyroid hormone is mediated via regulation of the enzyme synthesis (at transcription or translation level) or changes in the catalytic activity of this enzyme awaits further investigation. Interestingly, we have observed that the increase in the PL C (both SL and cytosolic) activity in the hypothyroid heart was completely reversible by T_4 administration to the experimental animals (TABLE 8, 9), which might suggest an extranuclear (post-transcriptional) regulation of this enzyme by the thyroid hormone. Recently, Kato *et al.* (1992) have reported an increase of PL C activity in the aorta from spontaneously hypertensive rats. This increase was associated with the PL C δ isoform, and shown to be the consequence of a three point mutation in the PL C δ cDNA which resulted in two amino acid substitution (Yagisawa *et al.*, 1991). Changes of myocardial PL C activity have also been reported in different pathological

conditions. In cardiomyocytes isolated from the spontaneously hypertensive rat hearts PL C activity was significantly higher than that of control animals (Kawaguchi *et al.*, 1992). However, whether this response is due to a change similar to that of the aortic PLC δ has not been investigated. The cardiac SL PL C has been shown to slightly increase at 5 min ischemia then decreasing significantly at 10 min ischemia, while the changes in the cytosolic PL C were opposite to those of the SL PL C (Schwertz and Halverson, 1989).

The enhanced basal PL C activity combined with an increased α_1-adrenoceptor density will induce a greater augmentation of the α_1-adrenoceptor mediated response in the hypothyroid hearts. Indeed, in streptozotocin diabetic rat heart despite the reported depression of the α_1-adrenoceptors' density (Heyliger *et al.*, 1982; Williams *et al.*, 1983), Xiang and McNeil (1990) have reported an increase in the myocardial content of Ins(1,4,5)P_3 following α_1-adrenoceptor stimulation which was accompanied by a higher positive inotropic response as compared to the control hearts. In addition, in the spontaneously hypertensive and hypertrophic hearts, the increase of the α_1-adrenoceptor density (Hanna and Khairallah, 1986) was accompanied by an augmented PL C activity under α_1-adrenoceptor stimulation. Our results from the hypothyroid hearts are in agreement with the latter case. The increased hydrolysis of PtdIns(4,5)P_2 by SL PL C demonstrated in this study after α_1-adrenoceptor stimulation would further ensure the amplification of the α_1 agonist signal as it is transduced down the signalling pathway. We have also observed an increase in the cytosolic PL C activity after α_1-stimulation. However, the mechanism of this increase is not clear and might be partly due to an increase in the intracellular Ca^{2+} concentration under α_1-stimulation or to other yet

unidentified factor(s). All three types of inositol phospholipids (PtdIns, PtdIns4*P* and PtdIns(4,5)P_2) are hydrolysed by the PL C isoforms (Rhee and Choi, 1992), with PtdIns4*P* and PtdIns(4,5)P_2 being the preferred substrates. In an attempt to examine possible changes of PtdIns4*P* hydrolysis after α_1-adrenoceptor agonist administration, we have shown a similar increase of the PL C-dependent PtdIns4*P* hydrolysis in SL fractions isolated from euthyroid and hypothyroid Langendroff hearts perfused with phenylephrine (FIGURE 23). The α_1-stimulation did not significantly change PtdIns4*P* hydrolysis by cytosolic PL C (FIGURE 25), whereas the hydrolysis of [^{3}H]PtdIns by either cytosolic or SL PL C after α_1-stimulation was not detectable.

In agreement with the above observation of increased PL C activity under α_1-adrenoceptor stimulation, we have observed an increase in the myocardial content of Ins(1,4,5)P_3 (TABLE 11). Nevertheless, given the cell heterogeneity of the heart, this result is only indicative because the Ins(1,4,5)P_3 was measured in the total myocardial tissue homogenate, and one could not eliminate the contribution of other cell types apart from the cardiomyocytes (fibroblasts and smooth muscle cells of the cardiac vessels). Recently Kawaguchi *et al.* (1992), using the same procedure as ours, have demonstrated an increase of the Ins(1,4,5)P_3 content in cardiomyocytes from the hypertensive rat hearts. This may suggest that our results are representing the Ins(1,4,5)P_3 changes in the cardiomyocytes of the hypothyroid hearts.

Finally, our detailed study of the α_1-adrenoceptor-associated phosphoinositide pathway in hypothyroid rat hearts is clearly in agreement with the proposed compensatory role of α_1-adrenoceptors under pathological conditions of depressed ß-adrenoceptor

density and response. Whether or not this hypothesis could be generalized was also our concern.

2. Congestive heart failure

Congestive heart failure following myocardial infarction was the second animal model with an increased α_1/ß ratio which we utilized as a comparative model to test whether or not the proposed hypothesis could be generalized. The rat model of congestive heart failure that we used has been studied extensively in the last few years. The viable LV of CHF rats showed a decrease in the ß-adrenoceptor density (at 4, 8 and 16 weeks post-left coronary ligation) associated with an increase in the α_1-adrenoceptor density (at 8 and 16 weeks). No alteration in these receptors' affinity for the respective antagonists were observed (Dixon and Dhalla, 1991). The changes in adrenoceptors were accompanied by depressed ß- and enhanced α_1-adrenergic response (Dixon and Dhalla, 1991). This model was also characterized by depression of sarcolemmal [^{3}H]-nitrendipine binding (Dixon *et al.*, 1990), Na^+-K^+ ATPase (Dixon *et al.*, 1992) and Na^+/Ca^{2+} exchange (Dixon *et al.*, 1992), whereas the SL Ca^{2+} pump was not altered (Dixon *et al.*, 1992) in the viable LV of CHF rats. The onset of these changes was not simultaneous but developed with the progression of the disease and all were present at the moderate stage of congestive heart failure (8 weeks post-coronary occlusion). The SR Ca^{2+} uptake was decreased in the viable LV of this model. However, opposite changes were observed in the RV at 4 and 8 weeks post-ligation with no change at 16 weeks (Afzal and Dhalla,

1992). These differential changes in RV and LV were attributed to the differences in the compensatory hypertrophy of the surviving LV, and of the RV (Anversa *et al.*, 1984; 1986; Greenen *et al.*, 1989). Whether the SL functional activities are differentially regulated in the RV and LV at different stages of CHF has not yet been reported.

Our results demonstrated, for the first time, that the polyphosphoinositide synthesis was significantly depressed in the left ventricular SL from CHF animals (8 weeks post-coronary ligation) (FIGURE 25-29). The analysis of the kinetic parameters of PtdIns and PtdIns4*P* kinase showed a significant decrease in the V_{max} accompanied by increased affinity of these enzymes for ATP. We also have demonstrated that the observed depression of the PtdIns4*P* kinase in the LV of CHF was not due to alteration of the enzyme molecule but to insufficient amount of the PtdIns4*P* substrate as a result of the diminished PtdIns kinase activity (FIGURE 30). Lack of phospholipid substrate was not a factor in the depression of the LV SL PtdIns kinase since the addition of exogenous PtdIns could not improve this activity (FIGURE 31). In contrast to the viable LV, the RV PtdIns kinase was significantly higher in CHF (FIGURE 32) and was further increased by the addition of exogenous PtdIns to the assay medium. On the other hand, the PtdIns4*P* kinase was not different in the RV of CHF from sham-operated controls because the addition of the exogenous phospholipid substrate seemed to increase this kinase activity to the same extent in control and CHF (FIGURE 33). These results further suggest that although PtdIns4*P* kinase is the rate limiting step of the D-4 phosphoinositide phosphorylation the regulatory mechanisms and the for microdomains of the two kinases are different.

In addition to the depressed polyphosphoinositide synthesis in the LV of CHF, previous report from our laboratory has shown a similar degree of depression in the LV SL PL C activity at 8 weeks post-coronary ligation (Meij *et al.*, 1991a). In preliminary experiments on an advanced stage of heart failure (16 weeks after coronary ligation) changes in the PL C and phosphoinositide kinase activities were similar to those seen at 8 weeks CHF. These data suggest that the above proposed hypothesis of compensatory role of α_1-adrenoceptors could not be generalized to include the CHF model. However, Dixon and Dhalla (1991) have reported a higher increase in the contractile force of the isolated and perfused heart from CHF rats compared to the controls following α_1-adrenoceptor stimulation. Although we could not provide any data on the activity of the phosphoinositide pathway under α_1-adrenoceptor stimulation, our finding on the differential changes in the phosphoinositide kinases in the RV and LV could suggest that the observed increase in force of contraction of the whole heart would be partly due to the RV change in force of contraction. A recent study by Rowley *et al.* (1991) has shown a decreased ability of the α_1-adrenoceptor agonist to induce a chronotropic action in the spontaneously beating atria from the rats with chronic LV infarction when compared to non-infarcted rats. In the same model, the α_1-mediated positive inotropy was also impaired, while no changes in α_1-adrenoceptors or phosphoinositide turnover were observed (Rowley *et al.*, 1991). On the other hand, Meggs *et al.* (1990) have reported an α_1-adrenoceptor mediated increase (3.1 fold) of the phosphoinositide turnover in the viable myocytes isolated from the infarcted rat hearts. However, the latter study fail to differentiate between RV and LV and has been done at an early (7 days) post-infarction

stage.

Our observation on the differential alterations of the phosphoinositide kinase activities in the RV and LV from the CHF rats led us to speculate a role for the oxidant species. Ligation of the descending left coronary artery will result in ischemic insult to the surrounding tissue. Rao *et al.* (1983) have observed an increase in free radical concentration in the coronary venous blood within 5 min of coronary ligation using an electron spin resonance spectrometer. These authors also observed the depletion of ascorbic acid in left ventricular tissue after 15 min of coronary occlusion while the decrease in glutathione peroxidase was delayed until 45 min following occlusion. Prolonged occlusion of the coronary artery will lead to the death of the tissue and formation of a scar and inflammation (Fishbein *et al.*, 1978). Increased potential for the production of free radicals by polymorphs in the plasma (Parsad *et al.*, 1989) and increased lipid peroxidation in the myocardium (Singal *et al.*, Kobayashi *et al.*, 1987) have been reported in different models of heart failure. Recently, in a study of patients with congestive heart failure, malondialdehyde levels were significantly higher, and plasma thiols were seen to be low (Belch *et al.*, 1991). The augmented plasma levels of catecholamines in failure may also contribute to the free radical damage (products of oxidation of catecholamines). Indeed, autooxidation of catecholamines and subsequent formation and myocardial accumulation of adrenochrome, free radicals or other highly cytotoxic species has been shown to impair the contractile function of the heart (Singal *et al.*, 1982). Thus this led to the hypothesis that oxygen-free radicals derived from persistent sympathetic drive play an important role in the genesis of various adverse

effects of catecholamines on the failing myocardium.

In this context, our study demonstrated a strong inhibition of both PtdIns and PtdIns4*P* kinase activities by oxidant stress (TABLE 13). The full protective effect of catalase (not SOD) in the xanthine-xanthine oxidase system suggests the involvement of H_2O_2, but not the superoxide radicals or the hydroxyl radical. As already discussed, the effects of H_2O_2 and HOCl are mediated by the modification of the kinases' sulfhydryl groups. Data from our laboratory have demonstrated the functional importance of the thiol groups present in some enzymes involved in signal transduction such as phosphatidylethanolamine N-methylation (Vetter *et al.*, 1991); PL D (Dai *et al.*, 1992) and PL C (Meij *et al.*, 1993). Thus the observed depression in the phosphoinositide kinases and PL C activity in moderate stages of CHF could be in part explained by oxidant-induced alteration of the thiol groups of these enzymes.

In conclusion, the role of the α_1-adrenoceptor and its associated phosphoinositide pathway in CHF remains unclear. Further studies on the changes in the α_1-adrenoceptor-associated signalling pathway and the resulted response under α_1-stimulation in each ventricle from the post-infarcted failing hearts would give more insight on the role of myocardial α_1-adrenoceptor in the development of the heart failure.

VI. CONCLUSIONS

1. The PtdIns and PtdIns4*P* kinases are associated with myocardial sarcolemmal membranes. These enzymes are Mg^{2+} dependent and inhibited by micromolar Ca^{2+} concentrations. Furthermore, these enzymatic activities were enhanced by non-ionic detergent Triton X-100 and ionophore alamethicin. Both PtdIns and PtdIns4*P* kinases contain functionally important thiol groups which are readily affected by sulfhydryl modifiers and the naturally occurring oxidants. DTT could prevent the oxidation of these thiol groups. The PtdIns kinase activity seemed to be the rate limiting step in the synthesis of PtdIns(4,5)P_2. In addition, the SL PtdIns(4,5)P_2 represents a relatively modest source of unsaturated fatty acids, and thus may be less important source for the DAG mediated activation of protein kinase C and for eicosanoid synthesis.

2. Thyroid hormone upregulated the activity of PtdIns and PtdIns4*P* kinases, whereas its low level did not affect these enzymes in the hypothyroid rat hearts. In contrast, thyroid hormone decreased the basal activity of both SL and cytosolic PL C while hypothyroidism resulted in a significant increase of the SL and cytosolic PL C (in both PTU-induced and thyroidectomized animals) of the rat myocardium. PL C activities were normalized upon reversal of hypothyroidism. The changes observed in SL PL C activity with different thyroid levels were specific to the PtdIns(4,5)P_2 as a substrate, while the changes in cytosolic PL C were also seen when PtdIns and

PtdIns4*P* were used as substrates.

3. Thyroid hormone regulation of the synthesis and degradation of the myocardial phosphoinositides could result in the alteration of membrane lipid microenvironment, thus affecting the functional activities such as Na^+/Ca^{2+} and Ca^{2+}-pump.

4. In hypothyroid cardiac SL the density of the ß-adrenoceptors was significantly decreased, while that of the α_1-adrenoceptor was elevated as compared to the euthyroid hearts. These changes were also apparent by the responses of the hypothyroid perfused hearts to the α- and ß-adrenoceptor agonists. The isoproterenol-induced increase in LVP_{max} was significantly depressed in hypothyroid perfused rat hearts, while the phenylephrine-induced positive inotropy in these hearts was significantly augmented.

5. Under phenylephrine stimulation of the Langendorff perfused hearts (in the presence of atenolol, a $ß_1$ blocker), the phenylephrine-induced increase of PL C activity in hypothyroid SL was significantly greater than in euthyroid SL. Unlike SL, cytosolic PL C was significantly stimulated to a similar extent in both eu- and hypothyroid hearts. The elevation of the PL C activity in the hypothyroid myocardium upon stimulation with phenylephrine was further confirmed by the increased levels of Ins(1,4,5)P_3 in these hearts.

6. The results of this study support the compensatory inotropic role for the the α_1-adrenoceptor and its associated phosphoinositide pathway in the hypothyroid rat hearts where the ß-adrenoceptor density and response are compromised. Furthermore in hyperthyroid rat hearts where the ß-adrenoceptors are up-regulated, the α_1-adrenoceptor and its associated phosphoinositide pathway are depressed.

7. In moderate stage of congestive heart failure (8 weeks post-coronary ligation), the activities of PtdIns kinase and PL C from the viable LV were significantly diminished, whereas PtdIns4*P* kinase was depressed due to substrate limitations. In contrast, the PtdIns activity in RV was increased, with no change in RV PtdIns4*P* kinase or PL C. These differences were attributed to the variation in the development of the disease.

8. Overall, the results of the present study which do not support the compensatory role of the α_1-adrenoceptor in CHF, emphasizes the necessity of defining the compensatory role of the α_1-adrenoceptor and post-receptor mechanisms in each cardiac pathology associated with enhanced α_1/ß ratio.

VII. References

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