

**MODIFICATION OF CARDIAC REMODELING IN  
FAILING HEART DUE TO MYOCARDIAL  
INFARCTION BY BLOCKADE OF RENIN-  
ANGIOTENSIN SYSTEM**

**BY**

**JINGWEI WANG**

**A Dissertation Submitted to  
The Faculty of Graduate Studies**

**In Partial Fulfillment of Requirements for the Degree of**

**DOCTOR OF PHILOSOPHY**

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together, listening their muttering, and gratifying their growing up. This thesis is dedicated to all of my family members.

## ABSTRACT

Ischemic heart disease, especially myocardial infarction (MI), is the major cause for congestive heart failure (CHF). Cardiac remodeling is considered to contribute to the development of CHF and activation of the renin-angiotensin system (RAS) is known to play an important role in cardiac remodeling. We have therefore employed an angiotensin converting enzyme (ACE) inhibitor (imidapril) and an angiotensin II (Ang II) type 1 receptor (AT<sub>1</sub>R) antagonist (losartan) to explore the therapeutic potential of RAS blockade in preventing CHF due to MI. To understand remodeling of myofibrils, the contractile “motor” of cardiomyocytes, we examined myofibrillar Ca<sup>2+</sup>-stimulated ATPase activities, mRNA level and protein content of  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) and  $\beta$ -myosin heavy chain ( $\beta$ -MHC) in CHF due to MI induced by coronary artery ligation in rats. The control and 3 week infarcted rats were treated with or without different drugs for 4 weeks; hemodynamic assessment was performed at 7 weeks following MI. The myofibrillar Ca<sup>2+</sup>-stimulated ATPase activities, mRNA level and protein level of  $\alpha$ -MHC and  $\beta$ -MHC were examined in the viable left ventricle (LV) tissue and right ventricle (RV). The infarcted hearts exhibited depressed rates of LV pressure development ( $57 \pm 2.4\%$  reduction) and pressure decay ( $55.5 \pm 1.6\%$  reduction). LV myofibrillar Ca<sup>2+</sup>-stimulated ATPase activity, unlike the RV, was decreased in the infarcted animals compared with controls ( $6.8 \pm 0.4$  vs.  $10.3 \pm 0.6$   $\mu\text{mol Pi/mg/hr}$ ). MHC  $\alpha$ -isoform levels were decreased by 47% and 41% whereas those of the MHC  $\beta$ -isoform were increased by 823% and 1200% respectively in the LV and RV due to MI. These results indicate that reduced cardiac myofibrillar Ca<sup>2+</sup>-stimulated ATPase activities are

due to a shift in myosin isozymes from  $\alpha$ -MHC to  $\beta$ -MHC and these changes may explain impaired the LV function in infarcted rats.

To investigate the role of Ang II in myofibrillar remodeling, rats at 3 weeks of MI were treated with imidapril (1 mg/kg/day), an ACE inhibitor for 4 weeks. Imidapril treatment partially prevented changes due to MI in LV function (rate of pressure development,  $24 \pm 2.3\%$  reduction and rate of pressure decay,  $14 \pm 1.8\%$  reduction), myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity ( $81 \pm 4.1\%$  reduction), myosin heavy chain (MHC) protein content ( $\alpha$ -MHC, 24% reduction and  $\beta$ -MHC, 525% increase) and MHC gene expression ( $\alpha$ -MHC, 18% reduction and  $\beta$ -MHC, 15% increase). The results suggest that the beneficial effects of ACE inhibition on the failing heart are associated with improvements in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activities as well as prevention of changes in MHC isozyme protein contents and their gene expression. To explore whether the therapeutic effects of imidapril are due to blockade of the RAS, another series of experimental studies were performed in MI induced CHF model. For the purpose of comparison and studying the effects of a combination of ACE inhibitor and  $\text{AT}_1\text{R}$  antagonist, three groups of rats at 3 weeks of MI were treated for 5 weeks with enalapril (10 mg/kg/day), losartan (20 mg/kg/day) and combined therapy enalapril plus losartan. Alterations in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity as well as MHC protein content and gene expression in the failing heart were similar to that observed in earlier studies. Treatment of animals with enalapril, losartan or a combination of enalapril and losartan partially prevented the MI-induced changes in LV function, myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity, MHC protein expression and MHC gene expression. No additional beneficial effect of the combination therapy was identified. The results suggest that blockade of RAS may produce beneficial effects on cardiac function and myofibrillar

remodeling in heart failure due to MI and that combination therapy with enalapril and losartan does not produce any additional effect.

Due to their multiple functions in regulating myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase in the normal heart, alterations of protein kinase C (PKC) and/or protein kinase A (PKA) may have important roles in MI induced heart failure. The activities of cardiac PKC were examined in hemodynamically assessed rats subsequent to MI. Both  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent PKC activities increased significantly in LV and RV homogenates at 1, 2, 4 and 8 weeks after MI was induced. PKC activities were also increased in cytosolic and particulate fractions of both LV and RV from 8 week infarcted rats. The relative protein levels of PKC- $\alpha$ , - $\beta$ , - $\varepsilon$  and - $\zeta$  isozymes were significantly increased in LV homogenate, cytosolic (except PKC- $\alpha$ ) and particulate fractions from the failing rats. On the other hand, the protein levels of PKC- $\alpha$ , - $\beta$  and - $\varepsilon$  isozymes, unlike - $\zeta$  isozyme, were increased in RV homogenate and cytosolic fractions whereas the RV particulate fraction showed an increase in the PKC- $\alpha$  isozyme only. These changes in the LV and RV PKC activities and protein contents in the infarcted animals were partially corrected by treatment with imidapril (1 mg/kg/day). No changes in PKA activity and its protein content were seen in the 8 week infarcted hearts. The results suggest that the increased PKC activity in cardiac dysfunction due to MI may be associated with an increase in the expression of PKC- $\alpha$ , - $\beta$  and - $\varepsilon$  isozymes, and the improvement of heart function and myofibrillar ATPase activity in the infarcted animals by the RAS blockade may be due to partial prevention of changes in PKC activity and isozyme contents.

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## LIST OF ABBREVIATIONS

$+dP/dt_{\max}$	the maximum rate of pressure development
$-dP/dt_{\max}$	the maximum rate of pressure decay
$\alpha$ -MHC	$\alpha$ -myosin heavy chain
$\beta$ -MHC	$\beta$ -myosin heavy chain
ACE	angiotensin converting enzyme
Ang I	angiotensin I
Ang II	angiotensin II
ANP	atrial natriuretic peptid
AT <sub>1</sub> R	angiotensin II type I receptor
AT <sub>2</sub> R	angiotensin II type II receptor
CHF	congestive heart failure
COM	combined enalapril and losartan treated infarcted (rats)
CPA	cyclopiazonic acid
DAG	diacylglycerol
DTT	dithiothreitol
EDTA	Ethylene Diamine Tetra Acetate
EGTA	EthyleneglucoI-bis( $\beta$ -aminoethylether)N,N,N',N'-tetraacetic acid
ENP	enalapril
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
G <sub>i</sub>	inhibitory guanine nucleotide-binding proteins
G <sub>s</sub>	stimulatory guanine nucleotide-binding proteins
HRP	horse radish peroxidase

IP <sub>3</sub>	phosphatidylinositol 1,4,5-triphosphate
ISO	isoproterenol
LOS	losartan
LV	left ventricle
LVEDP	left ventricle end-diastolic pressure
LVSP	left ventricular systolic pressure
MAP	mean arterial blood pressure
MAPK	mitogen activated protein kinase
MHC	myosin heavy chain
MI	myocardial infarction
MLC	myosin light chain
MLC- <sub>1</sub>	essential myosin light chain
MLC- <sub>2</sub>	regulatory myosin light chain
MOPS	3-[N-morpholino]propanesulfonic acid
PIP <sub>2</sub>	phosphatidylinositolbisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
PVDF	polyvinylidene difluoride
RAS	renin-angiotensin system
RV	right ventricle
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

SHR	spontaneously hypertensive rats
SL	sarcolemma
SR	Sarcomplasmic reticulum
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with 0.1% Tween-2.0
TGF- $\beta$	transforming growth factor- $\beta$
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
wt	weight



## I. LITERATURE REVIEW

Congestive heart failure (CHF) has been recognized for more than a thousand years <sup>1</sup>, and still is a significant public health problem with an unacceptably high morbidity and mortality. It is estimated that more than 5 million people are diagnosed patients with CHF in the United States, while there are over 550,000 new cases <sup>2</sup> and 250,000 deaths <sup>3</sup> annually. From different epidemiology studies, this number is most likely increasing greatly at present, because more patients are surviving acute myocardial infarction (MI). Despite the trend for a decrease in mortality rate due to coronary heart disease, hospitalizations for acute MI and subsequent CHF have remained high <sup>2,4</sup>. Therefore, the combination of increased survival post MI and increased longevity in Western countries has enhanced the increase in overall prevalence of heart failure <sup>5</sup>. Heart failure following the loss of contractile tissue as a result of MI evokes a constellation of responses that act to maintain systemic perfusion. These compensatory mechanisms include an activation of the sympathetic nervous system, alterations in regional vascular resistance, sodium and water retention, and activation of the renin-angiotensin system (RAS) <sup>6</sup>. To study heart failure, different experimental models including tachycardia-induced heart failure model <sup>7</sup> have been utilized. In regarding of MI as the major reason of heart failure in the society, the MI rat model produced by coronary artery ligation has been used extensively to study the remodeling process <sup>8,9</sup>.

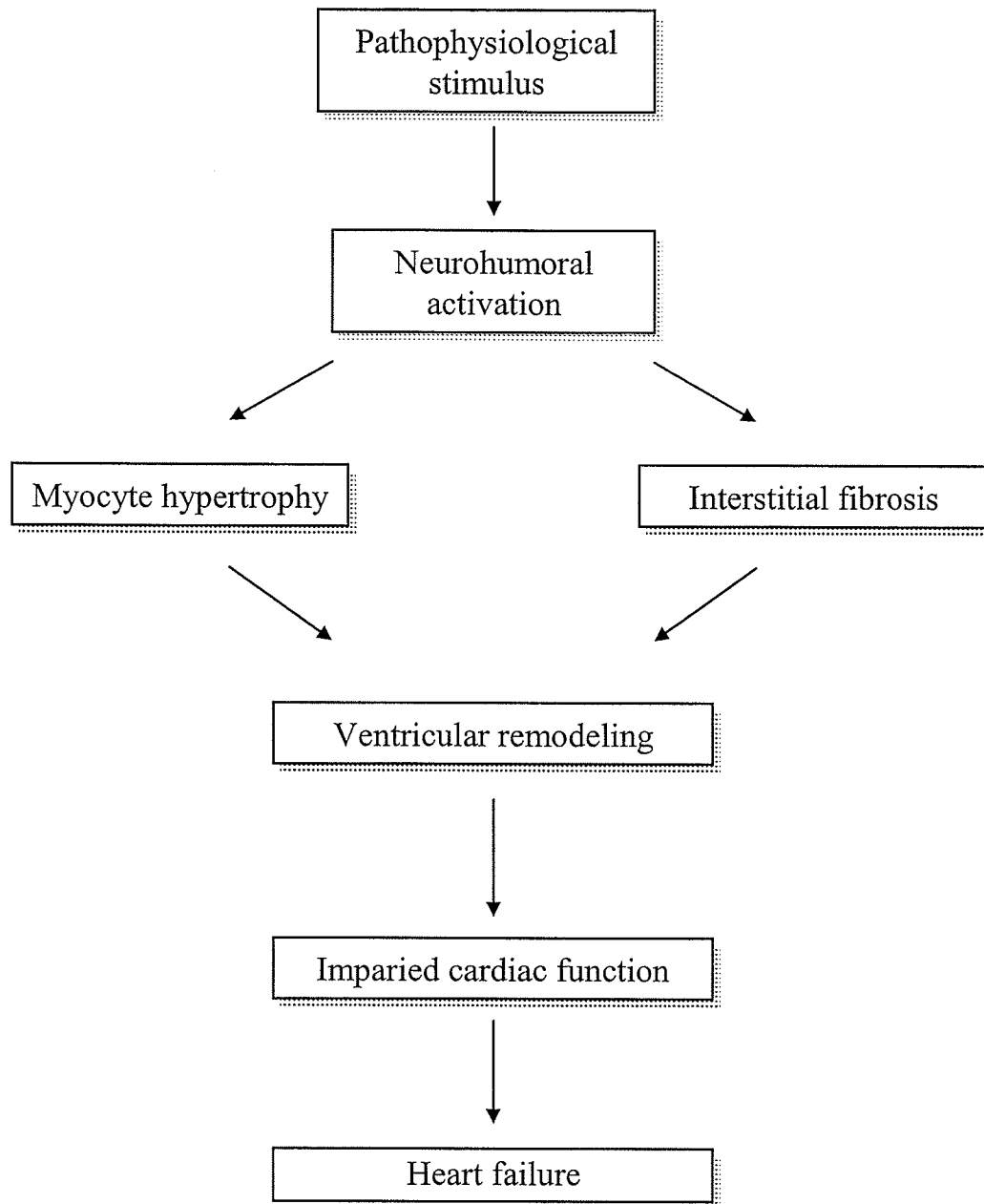
## **A. Pathophysiology of Contractile Dysfunction in Heart Failure**

Heart failure is considered as a clinical syndrome in which the cardiac output is inadequate to meet the metabolic needs of the body <sup>10</sup>. Essentially, it is a pathological state in which impaired cardiac pump activity decreases ejection of the blood and impedes venous return. The pathologic stimuli for the occurrence of heart failure can be categorized as follows: i) conditions which lead to the development of pressure or volume overload, ii) conditions which produce abnormal cardiac muscle contraction and relaxation, and iii) conditions which limit ventricular filling <sup>11</sup>. Various cardiovascular diseases (Table 1) including ischemic heart disease, valvular heart disease, cardiomyopathy, septal defects, hypertension and pericardial disease can result in heart failure <sup>12-15</sup>. The occurrence of heart failure is 1-3% of the population in Western countries and the incidence and prevalence are increasing <sup>2,16-18</sup>. Thus a better understanding of the pathophysiologic mechanisms involved in the genesis of heart failure is necessary in order to have a clear rationale for pharmacologic treatment and development of new agents and procedures to increase survival and improve quality of life.

The sequence of the main pathophysiological processes (Figure 1) which contribute to the development of heart failure includes neurohumoral activation and ventricular chamber remodeling <sup>19</sup>. The heart adapts to the augmented load placed on the myocardium by increasing contractility (systolic reserve) and cavitory volume (diastolic reserve). When the overload on the heart becomes excessive in magnitude and duration, these compensatory mechanisms are exhausted and disparate changes in ventricular mass, cavitory volume, and stiffness become evident. These alterations are considered to result

**Table 1. Types of Heart Failure**

<b>Types of Heart Failure</b>	<b>Causes</b>
Pressure overload	Aortic stenosis Systemic arterial hypertension
Volume overload	Aortic or mitral regurgitation Congenital heart disease Thyrotoxicosis
Primary myocardial disease	Cardiomyopathy Myocarditis
Secondary myocardial abnormalities	Ischemia (coronary heart disease) Inflammation Infiltrative diseases
Impaired ventricle filling	Constrictive pericarditis Restrictive



**Figure 1.** Mechanisms influencing myocardial remodeling in heart failure

in mechanically disadvantageous and compromising ventricular performance<sup>6</sup>. Accordingly, it is proposed to discuss different processes to gain some insight into remodeling of the ventricle and changes in the subcellular organelles such as myofibrils, sarcolemma (SL), and sarcomplasmic reticulum (SR) as well as extracellular matrix in the failing heart.

Activation of the sympathetic nervous system is the first response to left ventricular dysfunction. Sympathetic activation initially compensates for the loss of cardiac output by increasing heart rate and venous return. However, it may also contribute to myocardial cell loss and fibrosis in the chronic phase of heart failure<sup>20,21</sup>. Particularly, high levels of plasma catecholamines for a prolonged period of time have been shown to modulate the function of the  $\beta$ -adrenergic receptor pathway<sup>22</sup>. In fact, the failing heart shows a reduced response to adrenergic stimulation which is associated with alterations in the  $\beta$ -adrenergic signal transduction pathway. Such changes include downregulation of  $\beta_1$ -adrenoceptors, uncoupling of  $\beta$ -adrenoceptor from adenylyl cyclase, and an increase in the functional activity of inhibitory guanine nucleotide-binding proteins ( $G_i$ )<sup>22-24</sup>. The density of  $\beta$ -adrenoceptors has been reported to be decreased in CHF due to idiopathic cardiomyopathy, ischemic cardiomyopathy, as well as MI, and the degree of downregulation is related to the severity of heart failure<sup>25,26</sup>. The decrease in  $\beta_1$ -receptor density and downregulation of the signal transducing mechanism  $\beta$ -adrenoceptors probably accounts for much of the decrease in inotropic response of the failing heart<sup>27</sup>. On the other hand, the density of  $\beta$ -adrenoceptors was reported to be increased in CHF due to aortic constriction in guinea pigs<sup>28</sup>. Furthermore, some investigators have reported both an increase and a decrease in the density of  $\alpha$ -adrenoceptors in a hamster model of

CHF due to genetic cardiomyopathy<sup>29,30</sup>. Other studies have shown either an increase or no change in the density of  $\beta$ -adrenoceptors in CHF in cardiomyopathic hamsters and in patients with heart failure of various etiologies<sup>31-33</sup>. The results from these investigations suggest that the changes in adrenergic receptors in the myocardium may depend on both the etiology of CHF and the stage of the heart failure.

It should be pointed out that the activation of the sympathetic nervous system is accompanied by the activation of the renin-angiotensin-aldosterone system and the release of vasopressin leading to vasoconstriction, retention of sodium, increase of body fluid and formation of edema<sup>34-36</sup>. Angiotensin II (Ang II) has also been shown to increase catecholamine synthesis, produce ventricular hypertrophy and expand the ischemic area due to its vasoconstrictive properties. It has also been reported that chronically elevated endothelin-1 level and subsequent activation of its receptor may play a role in the progression of heart failure<sup>37,38</sup>. On the other hand, atrial natriuretic peptide (ANP) is released in the circulation in CHF and this may exert diuretic and vasodilatory effects which are beneficial to heart failure<sup>24</sup>.

## **B. Cardiac Remodeling in Heart Failure**

The MI-induced rat CHF model is well established for studying pathophysiological mechanisms and pharmacologic effects of different medications<sup>39-43</sup>. It resembles the pathophysiological condition of CHF following MI in human. The most significant change after MI is the formation of scar. There are multiple methods for monitoring the infarct size, however, scar weight is the most convenient one. Scar weight has a direct relationship to the total left ventricle (LV) wall<sup>41</sup> and the total LV mass<sup>44</sup>. It

should be pointed out that this method may underestimate the true percentage of the infarcted LV, due to the edematous condition of viable myocardium, which is not overt in scar fibrous tissue<sup>44</sup>. In the early period of necrosis, edema and vascular congestion after a large transmural MI, the LV may dilate as a result of infarct expansion without any mechanical advantage to the impaired ventricle<sup>45</sup>. The scar size ranges from 1% to 51% following the ligation of coronary artery<sup>46</sup>. Alterations in the infarcted wall are the result of myocyte slippage or a decrease in the number of cells across the wall<sup>47</sup>, and the viable myocyte size is increased by about 31% at 6 weeks after MI<sup>48</sup>. The length of an isolated cardiomyocyte from a CHF group was about 10% longer than that of control at 6 weeks after MI; however, cell width and sarcomere length did not change greatly<sup>49</sup>. It has also been reported that myocardial transverse diameter was significantly greater in the infarcted group than that in the control heart<sup>50</sup>. As necrotic tissue is resorbed and a thin scar is formed, collagen fibers form a strong structural network to limit the infarct expansion<sup>51</sup>. Hypertrophy of the residual viable myocytes takes place, increasing contractile mass to near normal levels in small to moderate infarcts, but not in large infarcts<sup>46,52,53</sup>. In the chronic phase, after the healing process is complete and a discrete scar is formed, changes in ventricular mass and volume may continue. In small infarcts, the ratio of cavity volume-to-mass remains normal, suggesting a normalization of wall stress. On the other hand, in moderate to large infarcts, volume is increased out of proportion to mass as the filling pressure rises, suggesting that the stimulus to volume enlargement is still present<sup>9,54</sup>. Ventricular dilatation may become mechanically disadvantageous in that an augmented shortening load may be sustained throughout ejection because chamber volume is reduced relatively little while ventricular pressure is rising<sup>55</sup>.

Heart failure is characterized by an increase in myocardial mass, an increase in ventricular volume and a change in ventricular shape and interstitial growth<sup>56,57</sup>. Several mechanisms are involved in the structural changes which occur in cardiac remodeling. Cardiac muscle undergoes remodeling by increasing its length (dilatation) or volume (hypertrophy) rather than increasing the cell numbers<sup>58</sup>. Since adult cardiac myocytes cannot divide to increase their numbers, heart chamber enlargement occurs by hypertrophy of cells and is marked by an increase in the number of intracellular sarcomeres. Ventricular dilatation can be due to myocyte slippage between fiber bundles as in cardiomyopathy and in non-infarcted segments after MI produced by activation of collagenase that disrupts the collagen myocyte supports<sup>59,60</sup>. Ultimately, collagen growth including deposition of new collagen and expansion of pre-existing collagen occurs. This collagen overgrowth reduces ventricular distensibility and compliance. Myocardial interstitial fibrosis occurs in heart failure due to both ischemic and dilated cardiomyopathies<sup>61</sup>. Ventricular remodeling is considered to be triggered by mechanical and biochemical factors, including neurohormones, such as norepinephrine, Ang II and vasopressin as well as cardiac growth factors and fibroblast growth factors. In early heart failure, dilatation may increase cardiac performance but chronic enlargement often worsens cardiac function. Although cardiac hypertrophy is a better adaptation than myocardial dilatation for improving ventricular contraction, severe cardiac hypertrophy lasting for a long period results in a loss of contractility. Several surgical treatments have been employed to arrest or reverse the ventricular remodeling. Partial ventriculectomy is performed to remove a substantial portion of the lateral wall to make the dilated heart smaller<sup>62</sup>. Left ventricular assist devices have been shown to unload the failing ventricle, improve systemic blood supply and thereby decrease neurohumoral activation<sup>63</sup>. Lastly,



dynamic cardiomyoplasty (wrapping the heart with skeletal muscle) has been reported to limit cardiac dilation <sup>64</sup>.

Recently, the role of programmed cell death (apoptosis) in ventricular remodeling and the development of heart failure has gained much attention <sup>65,66</sup>. Olivetti *et al.* <sup>21</sup> in a study of human tissue showed that both necrosis and apoptosis cause cell death in patients with ischemic and idiopathic heart failure. Reduced coronary blood flow and increased wall stress are the potential triggers of apoptosis in the failing heart <sup>24</sup>. However, the role of apoptosis is usually questioned on the basis of the fact that the number of myocytes so affected (0.2 to 3.0%) at any given time is too low to account for the impairment of cardiac performance seen in heart failure. Nonetheless, myocardial remodeling is initially compensatory but finally myocardial structure is changed so that the pumping efficiency of the heart is further impaired and the contractility is decreased. Accordingly, cardiac remodeling is critical to the development of progressive heart failure <sup>13</sup>. In advanced cardiac failure secondary to both ischemia and dilated cardiomyopathy, myocyte loss is a feature of the myopathic process and may occur by either necrosis or apoptosis <sup>67</sup>. Apoptosis involves cell shrinkage, condensation of chromatin and fragmentation of chromosomal DNA <sup>68</sup>. It has been demonstrated that apoptosis occurs in constituent myocytes of failed explanted human hearts and in animal hearts with induced heart failure. As well, cardiac myocytes in acute MI, in the hypertrophied heart and in the aging heart, also undergo apoptosis <sup>69</sup>. Furthermore, the p53 gene, which is involved in apoptosis, has been observed in the failing heart and tumour necrosis factor- $\alpha$ , an inducer of apoptosis, is increased in heart failure <sup>56</sup>.

### C. Subcellular Remodeling in Heart Failure

There is a general agreement that  $\text{Ca}^{2+}$  handling by cardiomyocytes is altered both in failing human hearts as well as in animal models of heart failure. Abnormal intracellular  $\text{Ca}^{2+}$  handling is one of the major causes of both systolic and diastolic dysfunction<sup>39,70</sup>. The mechanisms of this abnormal  $\text{Ca}^{2+}$  handling are still unclear, however, possible factors include alterations in SL L-type  $\text{Ca}^{2+}$  channels, SL  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, SL  $\text{Ca}^{2+}$ -pump, SR  $\text{Ca}^{2+}$ -pump and SR  $\text{Ca}^{2+}$  release channels, which participate in the regulation of  $\text{Ca}^{2+}$  movements. Different studies have shown decreased SR  $\text{Ca}^{2+}$  uptake in a variety of animal models of heart failure and in humans; abnormal  $\text{Ca}^{2+}$  release from SR in dilated cardiomyopathy and prolonged duration of intracellular  $\text{Ca}^{2+}$  transients have also been observed in hypertrophied myocytes<sup>71-73</sup>. However, other investigators have found either no change or upregulation in SR  $\text{Ca}^{2+}$  uptake<sup>74,75</sup>. Thus, several other factors such as defects in SL membranes may contribute to the abnormalities of  $\text{Ca}^{2+}$  homeostasis in failing myocardium<sup>76</sup>. Finally it has been suggested that the contractile dysfunction in failing hearts may actually be due to attenuated sensitivity of myofibrils to  $\text{Ca}^{2+}$ . These studies suggest that different subcellular organelles such as SL, SR and myofibril may become remodelled in heart failure.

The status of SL  $\text{Ca}^{2+}$  channels in the failing heart may depend on the type of heart failure<sup>14</sup>. Reports of increased density of  $\text{Ca}^{2+}$  channels from genetic cardiomyopathic hamster hearts imply that the occurrence of intracellular  $\text{Ca}^{2+}$  overload through augmented SL  $\text{Ca}^{2+}$  influx may be the mechanism of pathological alterations in these hearts<sup>77-79</sup>. However, it has been shown that  $\text{Ca}^{2+}$  channel binding densities are reduced in ischemic heart disease induced by global ischemic or hypoxia-reoxygenation

injury<sup>80,81</sup>. Likewise, the density of L-type  $\text{Ca}^{2+}$  channels is decreased in CHF in rats following MI and in dogs with myocardial failure following intracoronary microembolization<sup>82-84</sup>. In addition, a significant decrease in mRNA encoding SL  $\text{Ca}^{2+}$  channels has been reported in the LV of patients with heart failure due to dilated and ischemic cardiomyopathy<sup>85-87</sup>. Finally, in one study the number of  $\text{Ca}^{2+}$  channels in the hypertrophied right ventricle (RV) of rats with CHF secondary to a large left ventricular MI was not changed compared with control value<sup>82</sup>.

Alterations in SL  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and SL  $\text{Ca}^{2+}$ -pump activities have been observed in several experimental animal models of heart failure. Decreased  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and  $\text{Ca}^{2+}$ -pump activities have been seen in 120-180 day old cardiomyopathic hamsters<sup>88,89</sup>. These findings suggest that a depression in  $\text{Na}^+/\text{Ca}^{2+}$  exchanger activity may result in a reduced  $\text{Ca}^{2+}$  efflux from the myocardium, which may contribute to the occurrence of intracellular  $\text{Ca}^{2+}$  overload. Many studies have investigated the status of SL  $\text{Na}^+/\text{K}^+$  ATPase enzyme in both human and experimental heart failure.  $\text{Na}^+/\text{K}^+$  ATPase activity has been observed to be reduced in the failing human heart, in UM-X7.1 cardiomyopathic hamster hearts, in rabbit hearts with left ventricular hypertrophy, in rat hearts with ischemia-reperfusion injury and in the viable LV of rats with CHF due to MI<sup>70,90-93</sup>. These observations have indicated that a reduction in SL  $\text{Na}^+/\text{K}^+$  ATPase in heart failure is important for contractile dysfunction, generation of arrhythmia and for the effectiveness of digoxin treatment<sup>94</sup>. However, increased  $\text{Na}^+/\text{K}^+$  ATPase activity has been observed in the BIO 14.6 strain of cardiomyopathic hamsters and in canine hearts with volume or pressure overload<sup>95-97</sup>. Furthermore, SL  $\text{Ca}^{2+}$ -pump activity was not altered in the failing hearts due to MI<sup>93</sup>. Therefore, the biochemical changes in heart

failure reflecting remodeling of the SL membrane seem to depend on the etiology of the disease.

The SR plays the most important role in regulating cytoplasmic  $\text{Ca}^{2+}$  during cardiac contraction and relaxation. Calcium is released through the  $\text{Ca}^{2+}$ -release channel (ryanodine receptor) whereas calcium is taken up by the SR via  $\text{Ca}^{2+}$ -pump ATPase which is regulated by phospholamban. The calcium inside the lumen of the SR is stored in a bound form with calsequestrin. The ATP-dependent  $\text{Ca}^{2+}$  sequestration rate is reduced in the animal model of the failing heart from a variety of etiologies including hypertrophy, ischemia, pacing-induced, genetic, diabetic and drug-induced<sup>39,98-105</sup>. The status of SR  $\text{Ca}^{2+}$ -pump ATPase has been studied in different animal models of myocardial failure. A decrease in SR  $\text{Ca}^{2+}$ -pump ATPase protein level was observed in failing guinea pig hearts following banding of the descending aorta as compared to an age-matched sham group and the attenuation in SR  $\text{Ca}^{2+}$ -pump ATPase activity was more than the reduction in protein levels<sup>98,106</sup>. Decreased gene expression of SR  $\text{Ca}^{2+}$ -pump ATPase in Syrian hamsters with hereditary cardiomyopathy has also been observed<sup>107</sup>. In a rat model of MI, SR  $\text{Ca}^{2+}$ -pump ATPase mRNA and protein levels decreased in parallel to the severity of CHF and in the left ventricular myocardium from rats with ascending aortic banding, a decrease in SR  $\text{Ca}^{2+}$ -pump ATPase mRNA level occurred in failing animals<sup>108</sup>. Furthermore, it has been reported that the mRNA levels of SR  $\text{Ca}^{2+}$ -pump ATPase are reduced in the failing heart as compared to the non-failing human heart<sup>109,110</sup>. However, it has been observed that SR  $\text{Ca}^{2+}$ -pump ATPase mRNA levels did not change significantly from the baseline despite development of pacing tachycardia-induced heart failure<sup>111</sup>.

There are indications that ryanodine receptor function may or may not be altered in heart failure. The density of ryanodine receptors was decreased in a rat model of pressure overload cardiac hypertrophy whereas a normal ryanodine receptor protein level was observed in the failing human heart<sup>112,113</sup>. Both reduction and no change in mRNA levels have been observed in dilated cardiomyopathy<sup>114,115</sup>. The results concerning mRNA levels in failing human hearts are somewhat contradictory and appear to be related to the etiology of heart failure. The mRNA and protein levels of phospholamban have been found to be decreased in human heart failure but one study showed a small decrease in phospholamban protein levels relative to total protein in the failing heart due to dilated cardiomyopathy<sup>116-119</sup>. A decrease in phospholamban could be a compensatory change that would relieve inhibition of the SR Ca<sup>2+</sup>-pump ATPase in the failing hearts. A reduced phosphorylation of phospholamban could decrease the rate at which Ca<sup>2+</sup> is sequestered by the SR, and thus result in prolonged Ca<sup>2+</sup> transients and delayed relaxation in the failing heart<sup>120,121</sup>. In left ventricular biopsies from dogs with tachycardia-induced heart failure, no change in phospholamban mRNA levels was observed at the onset of clinical heart failure compared to the baseline<sup>111</sup>. The calsequestrin content of the heart appears to be unchanged in heart failure<sup>122-126</sup>. Studies in the failing human myocardium consistently showed unchanged mRNA and protein level calsequestrin as compared to the nonfailing myocardium<sup>85,113,118,125</sup>. These observations show differential changes in the expression of SR genes and proteins but indicate a great deal of specificity in term of remodeling of the SR membrane during the development of heart failure.

Cardiac myofibrils are composed of repeating contractile units known as sarcomeres<sup>127</sup> and their activity is the ultimate determinant of cellular dynamics and force<sup>128</sup>. Cardiac sarcomeres consist of thick and thin filaments<sup>129</sup> and muscle

contraction involves sliding of the thick filaments (myosin) past the thin filaments (actin) inside the cardiomyocyte<sup>130</sup>. Therefore, myofibrils are the contractile machinery of the cardiac cell and are controlled by the interactions of several myofibrillar proteins<sup>131</sup> through “cross-bridge” cycling which plays an integral role in determining the dynamic properties of the heart<sup>128</sup>. For the biochemical process of contraction, ATP is hydrolyzed and chemical energy is liberated. The myofibrillar proteins undergo physicochemical changes that are manifested by tension development and shortening; all of these reactions are controlled by  $\text{Ca}^{2+}$ <sup>129,131</sup>. Removal of  $\text{Ca}^{2+}$  from myofibrils causes relaxation of the cardiomyocyte<sup>132</sup>.

The thick filament is made of myosin, and the myosin heavy chain (MHC) is regarded as the “molecular motor” of contraction<sup>129</sup>. MHC has two portions: rod and head. The rod portion of myosin constitutes the carboxyl-terminal half of the molecule and serves to integrate MHC into an organized thick filament. The coiled-coil conformation of the filamentous tail confers rigidity to the molecule, which strengthens the aggregated myosin tails in the backbone of the thick filament. The head portion of MHC interacts directly with the thin filament for contraction and relaxation of muscle. The MHC head has the ATPase function units, and two smaller protein subunits, myosin light chain (MLC), which bind to each MHC head<sup>129</sup>. Another “back bone” part of thick filament is titin, which is a 3,000~3,700 kD protein, and due to its connection with the end of sarcomere and thin filament, it is considered as a third filament system<sup>127</sup>.

For the thin filament, the major component is actin<sup>127</sup>, which is directly involved in force generation<sup>133</sup>. There are two actin isoforms,  $\alpha$ -skeletal and  $\alpha$ -cardiac, in human heart;  $\alpha$ -cardiac actin is dominant. In myocardial hypertrophy and cardiomyopathies with

the exception of idiopathic dilated cardiomyopathy<sup>134</sup>, the amount of  $\alpha$ -skeletal actin was significantly increased and expressed in all layers of ventricular myocardium. In the thin filament, both tropomyosin molecules and troponin complex are also present; these interact with each other<sup>127,133</sup>. Tropomyosin lies in the grooves that run longitudinally between the two strands of actin in the thin filament. It adds structural rigidity to the thin filament and, more importantly, interacts cooperatively with the other proteins of the thin filament to allow  $\text{Ca}^{2+}$  to activate excitation-contraction coupling. Tropomyosin regulates the interactions between actin and myosin<sup>135</sup>, and the most important function of tropomyosin is to inhibit the activation of myosin cross bridges by actin in the thin filaments. The inhibition of tropomyosin is reversed when cytosolic  $\text{Ca}^{2+}$  concentration increases and allows  $\text{Ca}^{2+}$  to activate muscle contraction<sup>127,133</sup>.

The troponin complex includes the following three proteins: troponin C (TnC; ~18 kD) contains the regulatory  $\text{Ca}^{2+}$  binding site; troponin I (TnI; ~21 kD) inhibits actin filaments to activate MHC ATPase and thereby produce force and movement; and troponin T (TnT; ~30.5 kD) binds with tropomyosin and may be responsible for the attachment of the troponin complex to the thin filament<sup>136</sup>. TnI, alone is a weak inhibitor of actin-myosin interaction; however, it becomes a powerful regulator when combined with tropomyosin. Cardiac and skeletal muscles contain two isoforms of TnI, one is embryonic TnI, while the other is adult slow skeletal muscle TnI. Switching of TnI has been implicated in developmental changes involving  $\text{Ca}^{2+}$  and pH sensitivity of the contractile system and response to  $\beta$ -adrenergic stimulation<sup>137,138</sup>. Cardiac TnI contains a serine at position 20 which is not found in fast skeletal TnI, but is phosphorylated by cyclic AMP-dependent protein kinase (PKA)<sup>132</sup>. When phosphorylated, cardiac TnI induces

cooperative interactions in the thin filament that reduce the  $\text{Ca}^{2+}$  affinity of TnC, and accelerates relaxation when the heart is exposed to  $\beta$ -adrenergic agonists<sup>139</sup>. Other protein kinases also phosphorylate cardiac TnI, but the functional significance of these phosphorylations remains unclear. In stunned myocardium following ischemia, the most likely damage to contractile regulatory proteins is the proteolysis of TnI, which increases the  $\text{Ca}^{2+}$  concentration requirement<sup>132</sup>.

TnT is the largest of the three troponin components, and has been called the “glue” that holds the regulatory proteins of the thin filaments to one another, especially since it binds the troponin complex to tropomyosin<sup>133,136</sup>. The allosteric effects of TnT within the thin filament influence the  $\text{Ca}^{2+}$  sensitivity for tension development. Furthermore, an abnormal TnT isoform ( $\text{T}_2$ ) is produced in advanced heart failure<sup>140</sup>. On the other hand, TnC with its  $\text{Ca}^{2+}$ -binding potential transmits the signal to the thin filament<sup>136</sup>. Cardiac muscle contraction is under the intracellular  $\text{Ca}^{2+}$  control via the TnC  $\text{Ca}^{2+}$ -specific sites<sup>141</sup>. TnC is a highly conserved protein; only two isoforms, cardiac and skeletal TnC, have been identified in striated muscle. Both contain two  $\text{Ca}^{2+}$ -specific sites and  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$  sites. As the concentration of ionized  $\text{Mg}^{2+}$  in muscle is several orders of magnitude higher than that of  $\text{Ca}^{2+}$ , the  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$  sites are normally occupied by  $\text{Mg}^{2+}$  and so do not play a role in excitation-contraction coupling<sup>142</sup>.

It is now well established that hydrolysis of ATP by actomyosin ATPase provides energy for contraction. Thus any derangements in the organization of the contractile apparatus as well as in myofibrillar ATPase and  $\text{Ca}^{2+}$  binding activities can lead to the development of dysfunction of myocardial contraction and relaxation. Structural changes in actin, myosin, tropomyosin and the troponin complex remain to be carefully evaluated



in different types of heart failure. A marked loss of myofibrillar protein was observed in electron micrographs of the failing human heart and this reduction of contractile units seems to form the basis for the depression of both systolic function and ejection fraction and the prognosis of heart failure <sup>143</sup>.

The fibroblasts synthesize and degrade fibrillar collagen, types I and III, the major structural proteins of the heart. Weber and Brilla <sup>144</sup> showed that the relative proportions of myocardial cells change in pathologic hypertrophy, with collagen concentration rising disproportionately. The extracellular matrix is a flexible, supporting structure that surrounds the cell <sup>11,145</sup>. Although cardiomyocytes occupy 70 to 85% of the volume of the myocardium, 65% of the cells of the heart are fibroblasts, vascular endothelial cells, and smooth muscle cells <sup>146,147</sup>. Changes in the extracellular matrix during the development of heart failure include increases in fibronectin, laminin and vimentin contents, as well as deposition of collagen fibers I, III, IV, and VI in the myocardium <sup>148,149</sup>. There is an increase in collagen concentration in the rat ventricular free wall after MI and fibrosis remote from the infarct site is regarded as “the major cause of ventricular remodeling” in ischemic cardiomyopathy <sup>150,151</sup>. Cardiac remodeling, which occurs after MI <sup>152</sup> and in hypertension <sup>153</sup>, is associated with excessive accumulation of extracellular matrix components, including collagen, and with a reduced activity of the extracellular collagen degradative processes. The resulting increase in extracellular matrix proteins promotes myocardial stiffness and thus impairs contractile activity <sup>152</sup>. Disruption and discontinuity in collagen fibers have also been observed during the development of dilated cardiomyopathy both in animal models and in patients and the equilibrium between proteinase, which is capable of breaking down the extracellular matrix, and antiproteinase is also altered following heart failure <sup>154-156</sup>. In addition, Zellner *et al.* <sup>157</sup> have found a

reduction in myocyte attachment to the basement membrane proteins laminin, fibronectin and collagen IV in tachycardia-induced heart failure. All these extracellular matrix changes can lead to a loss of force transmission via the ventricular free wall and to an alteration in cardiomyocyte alignment which would cause fiber slippage and ventricular free wall thinning<sup>146</sup>.

#### **D. Cardiac Contractile and Regulatory Proteins**

Myosin, the major protein of the thick filament of muscle, is an elongated molecule whose rigid tails are woven in the backbone of this filament. The globular heads, which project as cross bridges, interact with actin in the thin filaments and contain an actin-activated ATPase whose cycling rate is closely correlated with the maximal shortening velocity of the unloaded intact muscle<sup>127,136</sup>. Myosin molecules consist of two MHC and four MLC proteins. Myosin has a molecular weight of ~220,000 and heads of the myosin molecule are paired structures made up of the two MHCs. The ability of heart to generate contractile force is primarily dependent on the myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase which is located at the head of MHC<sup>158</sup>; both the atria and ventricles contain two MHC isoforms that differ in their intrinsic ATPase activities. The so-called  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) has a higher ATPase activity and faster contractile velocity than that of the  $\beta$ -myosin heavy chain ( $\beta$ -MHC)<sup>130,158,159</sup>. While differences in the primary structure of myosin isozymes have been identified with peptide mapping and immunological analysis, complementary DNA and genomic DNA cloning experiments have demonstrated that myosin isozymes are coded by two separate genes that are linked 4 kilo-bases apart in the rat genome<sup>160</sup>. Both  $\alpha$ -MHC and  $\beta$ -MHC proteins<sup>161</sup> form

homodimers and heterodimers with each other resulting in three distinct MHC complexes (MHC isoforms),  $V_1$  (two  $\alpha$ -MHCs,  $\alpha$ -MHC homodimer),  $V_2$  (one  $\alpha$ -MHC and one  $\beta$ -MHC,  $\alpha$ -MHC/ $\beta$ -MHC heterodimer), and  $V_3$  (two  $\beta$ -MHCs,  $\beta$ -MHC homodimer). Therefore,  $V_1$  contracts fastest with increased energy expenditure than  $V_2$ , while  $V_2$  is faster than that of  $V_3$ <sup>130,162</sup>. The human ventricle contains mainly the lower-ATPase  $\beta$ -MHC, the so-called  $V_3$  form, which is similar to the fetal isoform that predominate in the embryonic heart. However, the higher-ATPase  $\alpha$ -MHC, or  $V_1$  is also present in the human ventricle<sup>163</sup>. Adult rodent ventricles, on the other hand, contain mainly the higher-ATPase isoform,  $V_1$ , along with small amounts of a hybrid myosin the so-called  $V_2$ . Selective expression of genes that encode the slow  $\beta$ -MHC isoform plays an important role in the adaptation of cardiac performance to chronic overload. There exists an inverse correlation between ATPase activity of MHC and energetic cost to accomplish a given workload<sup>129</sup>. In heart, myosin contains two pairs of MLC, the regulatory MLC (MLC-2) and the essential MLC (MLC-1). MLC-1 is about 16~27 kD protein, and it is necessary for stability of the myosin head, while MLC-2 is an 18,500 kD protein<sup>164</sup>. However, skeletal muscle myosin is believed to have 3 kinds of MLC<sup>158</sup>.

Myosin has two important biological properties, the first being its ATPase activity for releasing the chemical energy. The second biological property is responsible for contraction of the thick and thin filaments<sup>127</sup>. It is quite clear that muscle contraction speed is directly related to the myosin ATPase activity<sup>158</sup>. Besides the regulatory function of thin filament, MLC is regarded as thick filament regulatory component<sup>158</sup>. In the heart, MLC-2 does not bind  $Ca^{2+}$  and thus does not play a central role in excitation-contraction coupling; however, it is a substrate for MLC kinase, a  $Ca^{2+}$ -dependent protein kinase<sup>165</sup>.

Phosphorylation of the MLC-<sub>2</sub> modifies force development, possibly by bringing the cross-bridges closer to the thin filament<sup>166</sup>. The human atria and ventricles contain at least five different MLC isoforms. Reversal to the fetal phenotype in the overloaded ventricle is accompanied by reappearance of the atrial isoform, which replaces the ventricular isoform<sup>167</sup>. Expression of the atrial MLC-<sub>1</sub> isoform in the hypertrophied human ventricle increases cross-bridge cycling and contractility. It is suggested that MLC-<sub>1</sub> acts as a MHC/actin tether<sup>168,169</sup>.

In heart failure, alterations in the contractile proteins appear to include an initial increase in protein synthesis in response to ventricular overload and a shift to fetal forms of myosin with an ultimate reduction in protein synthesis. A shift in myocardial isozyme content from V<sub>1</sub> ( $\alpha\alpha$ , fast, high ATPase activity) to V<sub>3</sub> ( $\beta\beta$ , slow, low ATPase activity) has been documented in different models of experimental heart disease and is believed to occur at the transcriptional level<sup>170-173</sup>. In response to stimulation of  $\alpha_1$ -adrenoceptors in neonatal rat cardiomyocytes, it has been shown that hypertrophy in these cells is characterized by selective upregulation of early developmental contractile protein isogenes, including those for  $\beta$ -MHC<sup>174</sup>. This shift is not important in humans, since human ventricles contain primarily the  $\beta\beta$  isoform. However, a shift from  $\alpha\alpha$  to  $\beta\beta$  isoforms does occur in the human atrium in heart failure<sup>175,176</sup>. Several functional changes in the failing heart can be explained by an increase in the synthesis of V<sub>3</sub> myosin isozyme with a characteristic derived from ATP used for the depressed rate of myocardial contraction may be beneficial to the failing heart<sup>177</sup>. In addition, changes of contractile proteins are not confined to the MHC because in human heart failure, a marked decreased in the MLC content has also been reported<sup>178,179</sup>. However, the atrial form of MLC-<sub>1</sub> has

been shown to increase in other investigations<sup>180</sup>. Morano *et al.*<sup>168</sup> have demonstrated that in the isolated human myocardium, force development and  $\text{Ca}^{2+}$  responsiveness were profoundly affected by the interaction between MLC and actin. Therefore, alterations in MLC in heart failure may be of functional consequence for contractile activation<sup>181</sup>.

The identification of myosin isoform explains the molecular heterogeneity of sarcomere and distinctive functional properties of myofibers<sup>182</sup>, and shifts involving the contractile proteins have been described in experimental heart failure. The most extensive study of these responses reveals a shift to the fetal isoform of myosin, where chronic overload causes the high-ATPase  $\alpha$ -MHC to be replaced by the slower  $\beta$ -MHC. These alterations have been observed in animal models of both cardiac hypertrophy and heart failure, where there was a shift of  $V_1$  to  $V_3$  MHC isoforms<sup>183,184</sup>. This isoform shift reduces myofibril ATPase activity<sup>162</sup>, and contractility in human atria<sup>185,186</sup> and to a lesser extent, in the failing human ventricle<sup>163</sup>, while  $V_3$  is correlated with a reduction in the maximum velocity of sarcomere shortening<sup>187</sup>. It is clear now that in the nonfailing human heart there is significant amount of  $\alpha$ -MHC mRNA, while in heart failure no  $\alpha$ -MHC mRNA was detected<sup>163</sup>. In different model of failing rodent heart, the shift from  $\alpha$ -MHC to  $\beta$ -MHC was obvious<sup>128</sup>. On the other hand, some researchers did not find myofibrillar changes in failing heart due to MI. Six weeks after MI, there were no significant alteration in the myofilament responsiveness, as measured by fura-2 ratio and mechanical shortening relationship<sup>49</sup>. Isoform shifts have also been found in the MLC<sup>169</sup> and TnI<sup>188</sup>. Whereas no change of tropomyosin has been found in the failing human heart<sup>169</sup>. The status of TnT remains unclear, as reversal to the fetal phenotype has been found by some<sup>140,189</sup> but not all groups<sup>190</sup>, possibly because this isoform shift varies among

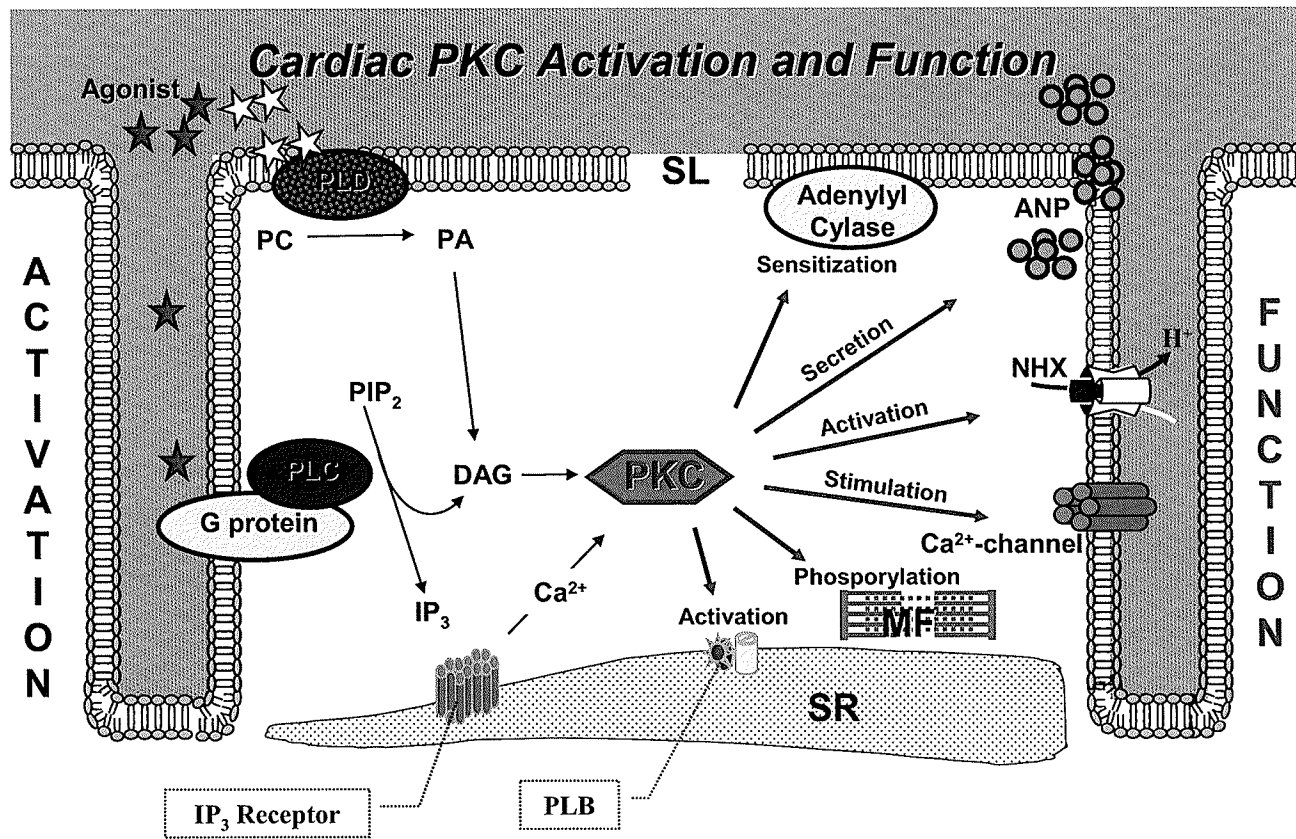
individual patients. Altered phosphorylation of TnI, a post-translational modification that differs fundamentally from the isoform shifts caused by changes in gene expression, has been observed in the failing heart <sup>191</sup>.

## **E. Protein Kinase C in Heart Function**

In view of the fact that protein kinases play a crucial role in intracellular signalling pathways, it is important to explore the biological and pathophysiological functions of protein kinases. In the heart, protein kinases are involved in the regulation of cation transport, cardiac contractility, myocardial metabolism, gene expression, cell growth and apoptosis <sup>192</sup>. Because serine/threonine kinases, particularly protein kinase C (PKC); are known to mediate  $\alpha$ -adrenergic,  $\beta$ -adrenergic, muscarinic, as well as endothelin and Ang II receptor-generated signals in the myocardium, the functional implications of these protein kinases in regulating cardiac performance are readily evident. Alterations in the activities of PKC in cardiac hypertrophy, heart failure, cardiomyopathy and ischemic heart disease seem to depend on the stage, species and type of the disease. The presence of PKC in different isoforms offers a great challenge for the development of specific therapeutic interventions for the manipulation of signal transduction mechanisms in the heart in health and disease.

PKC, a multifunctional protein, is known to play a crucial role in signal transduction; both rat and human tissue homologues appear to exhibit similar isoform expression <sup>193</sup>. PKC isoforms are physiologically activated by neurohormones that bind to G protein-coupled receptors <sup>194,195</sup>. Activation of different receptors, including  $\alpha_1$ -adrenergic receptors, P<sub>2</sub>-purinergic receptors, endothelin receptors, m<sub>1</sub>-muscarinic

receptors and Ang II receptors, most likely activate the G<sub>q</sub>-coupled phospholipase C<sub>β</sub> (PLC-β)<sup>196-200</sup>, which results in the breakdown of phosphatidylinositolbisphosphate (PIP<sub>2</sub>), generating both inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG), whereas DAG is a powerful activator of most PKC isoforms<sup>201</sup>. In addition, phospholipase D (PLD) is considered to be activated via G protein by different stimuli, including growth factors. It should be noted that PLD hydrolyzes phosphatidylcholine with the formation of phosphatidic acid, and DAG is subsequently produced through the action of phosphatidate phosphohydrolase; this complements the formation of DAG through the PLC pathway (Fig. 2)<sup>202</sup>. Activation of PKC may influence the activity of a variety of cation channels with inotropic consequences. Mg<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup> and Ca<sup>2+</sup> channels have been reported to be modulated by PKC (Figure 2)<sup>203-211</sup>; whereas the first well identified cardiac-specific PKC substrate is a membrane-bound 15 kD protein, phospholemman<sup>212</sup>. It has also been suggested that PKC-induced modification of the Na<sup>+</sup>/H<sup>+</sup> exchanger is responsible for cellular alkalization<sup>213-215</sup>. PKC-induced phosphorylation of phospholamban increases SR Ca<sup>2+</sup>-stimulated ATPase activity, which in turn stimulates Ca<sup>2+</sup> uptake from the cytosol and thus promotes the relaxation of cardiac muscle<sup>216</sup>. Contractile proteins are among the best substrates for cardiac PKC because the thick filament proteins (C protein and MLC) and thin filament proteins (TnI and TnT) are targets for this endogenous enzyme<sup>191,217</sup>. Phosphorylation of TnI and TnT by PKC in reconstituted actomyosin results in a decrease of Ca<sup>2+</sup>-stimulated Mg<sup>2+</sup>-ATPase activity, and this is reversed by dephosphorylation<sup>218</sup>. It is reported that PKC plays a role in the myosin isoform shift, which is related to decreased myofibrillar ATPase activity<sup>218,219</sup>. Kariya *et al.*<sup>220</sup> demonstrated that β-MHC is activated preferentially by PKC. On the



**Figure 2.** General scheme of cardiac protein kinase C (PKC) activation and functions. ANP: atrial natriuretic peptide; DAG: diacylglycerol; IP<sub>3</sub>: inositol triphosphate; PA: phosphatidic acid; PC: phosphatidylcholine; PIP<sub>2</sub>: phosphatidylinositol bisphosphate; PLC: phospholipase C; PLD: phospholipase D; SL: sarcolemma; SR: sarcoplasmic reticulum; NHX: Na<sup>+</sup>/H<sup>+</sup>-exchanger.



other hand, PKC is known to phosphorylate a number of cellular proteins mediating  $\beta$ -adrenergic receptor signalling and contractility in the heart. PKC has been reported to phosphorylate contractile proteins <sup>221</sup>, and to alter calcium homeostasis <sup>222</sup> and adenylyl cyclase activity <sup>223</sup>. It was also shown that PKC can phosphorylate and activate  $\beta$ -adrenergic receptor kinase <sup>224</sup> and that the  $\beta_1$ -adrenergic receptor can be desensitized by G protein-coupled receptor kinases <sup>225</sup>. Ang II-induced PKC activation decreased the responsiveness of the rat heart to  $\beta_1$ -adrenergic stimulation, and this PKC activation may differ from that by phorbol esters <sup>226</sup>.

In cultured ventricular myocytes, a variety of agonists have been found to stimulate hypertrophy. These are agents such as endothelin-1,  $\alpha_1$ -adrenergic agonists and Ang II, which are coupled to PLC- $\beta$  for increasing the formation of DAG and activation of PKC <sup>227-238</sup>. Activation of PKC, in turn, appears to be one of the events that can initiate a hypertrophic response in cardiomyocytes through the activation of mitogen activated protein kinase (MAPK) <sup>239</sup>. In addition, endothelin-1-induced development of hypertrophy via activation of distinct PKC isozymes may be initiated not only by PLC, but also by the PLD signaling pathway <sup>240</sup>. It should be noted that the hypertrophic process is associated with a genetic program that consists of the expression of immediate early genes (*c-myc*, *c-fos*, *c-jun* and *egr-1*) within 30 to 60 min followed by expression of fetal genes such as skeletal  $\alpha$ -actin,  $\beta$ -MHC and ANP <sup>241-243</sup>. Finally, hypertrophy is accompanied by an overexpression of constitutively expressed MLC-2 and cardiac  $\alpha$ -actin genes, and by an assembly of contractile proteins into sarcomeric units. PKC has been shown to play a role in this genetic program <sup>244</sup>. It is pointed out that PKC does not directly couple the neurohumoral receptor with gene expression, but rather that it belongs

to a cascade of kinases including *Raf*, tyrosine kinase and MAPK, which are also activated during the hypertrophic process<sup>244-247</sup>. Gu and Bishop<sup>248</sup> found a quantitative increase of PKC in the cytosolic, particulate fractions as well as in the nuclear-cytoskeletal fraction of hypertrophied rat left ventricular myocardium. Cardiac PKC- $\beta$  and PKC- $\epsilon$  were increased due to pressure overload, which suggested that during the development of hypertrophy PKC plays an important role in the regulation of cardiac growth and function, and that individual isozymes may perform different functions in response to pathological stimuli.

By increasing myofibrillar protein content and sarcomere assembly in individual myocytes, myocardial hypertrophy provides an adaptive response to hormonal and mechanical stimuli, which increase demand for contractile work. Virtually every phenotypic feature of the hypertrophic response has been shown to be induced by chronic stimulation with phorbol esters. These include an increase in the rate of transcription of rDNA, which leads to an increase RNA content and an increase in the capacity for protein synthesis<sup>241</sup>. These changes are followed by rapid and transient induction of immediate early gene expression, transcriptional activation of several fetal genes, accumulation of contractile proteins, assembly of contractile proteins into organized sarcomeric units and an increase in cell size<sup>249</sup>. It has been suggested that PKC- $\alpha$  and PKC- $\beta$  act simultaneously to induce the central members of the immediate early gene program expression, *c-fos* and *c-jun*, which heterodimerize and bind the consensus activator protein-1 sequence in the promoter region of phorbol ester-inducible genes, thereby exerting regulatory effects on gene transcription<sup>244,248</sup>. The studies implicate PKC in responses leading to myocardial hypertrophy upon transactivation of early immediate genes

and contractile protein genes for an enhanced protein synthesis following hormonal or mechanical stimuli <sup>250</sup>.

Not much information is available in the literature regarding changes in protein kinases in different types of cardiomyopathic hearts. Some investigators have shown that cardiac PKC activities were significantly increased in 180-day-old cardiomyopathic hamsters and that PKC potentiated cAMP-dependent phosphodiesterase activity in hypertrophic cardiomyopathy <sup>251,252</sup>. In diabetic rat cardiomyopathy, it has been reported that cardiac PKC activity is elevated, but changes in different PKC isoforms have not been explored <sup>253,254</sup>. Increased cardiac PKC activity may cause excessive accumulation of intracellular  $Ca^{2+}$  in the myocardium, and this may play an important role in the pathogenesis of cardiac dysfunction in diabetic cardiomyopathy <sup>255</sup>. It has been reported that reduction in  $Na^+/Ca^{2+}$  exchanger activity in the diabetic heart may be caused by impaired translocation of PKC- $\beta$  and/or by activation of PKC- $\alpha$  in the diabetic heart <sup>256</sup>. Recently, Ventura et al <sup>257,258</sup> provided evidence that cardiomyocytes express the prodynorphin gene, a candidate gene for pathological processes involving an impairment of myocardial cell contractility, growth and differentiation, and that this opioid gene is transcriptionally stimulated by PKC activation. The expression of PKC- $\alpha$ , PKC- $\delta$  and PKC- $\epsilon$ , as well as PKC activity, were increased in cardiomyopathic myocytes compared with controls, and it was suggested that PKC activation may be one of the major signalling mechanisms in the induction of the prodynorphin gene in cardiomyopathic cells <sup>259-261</sup>.

Transgenic mice with the specific overexpression of PKC- $\beta_2$  isoform in the myocardium were established to evaluate the effect of PKC- $\beta$  on cardiac functions. These

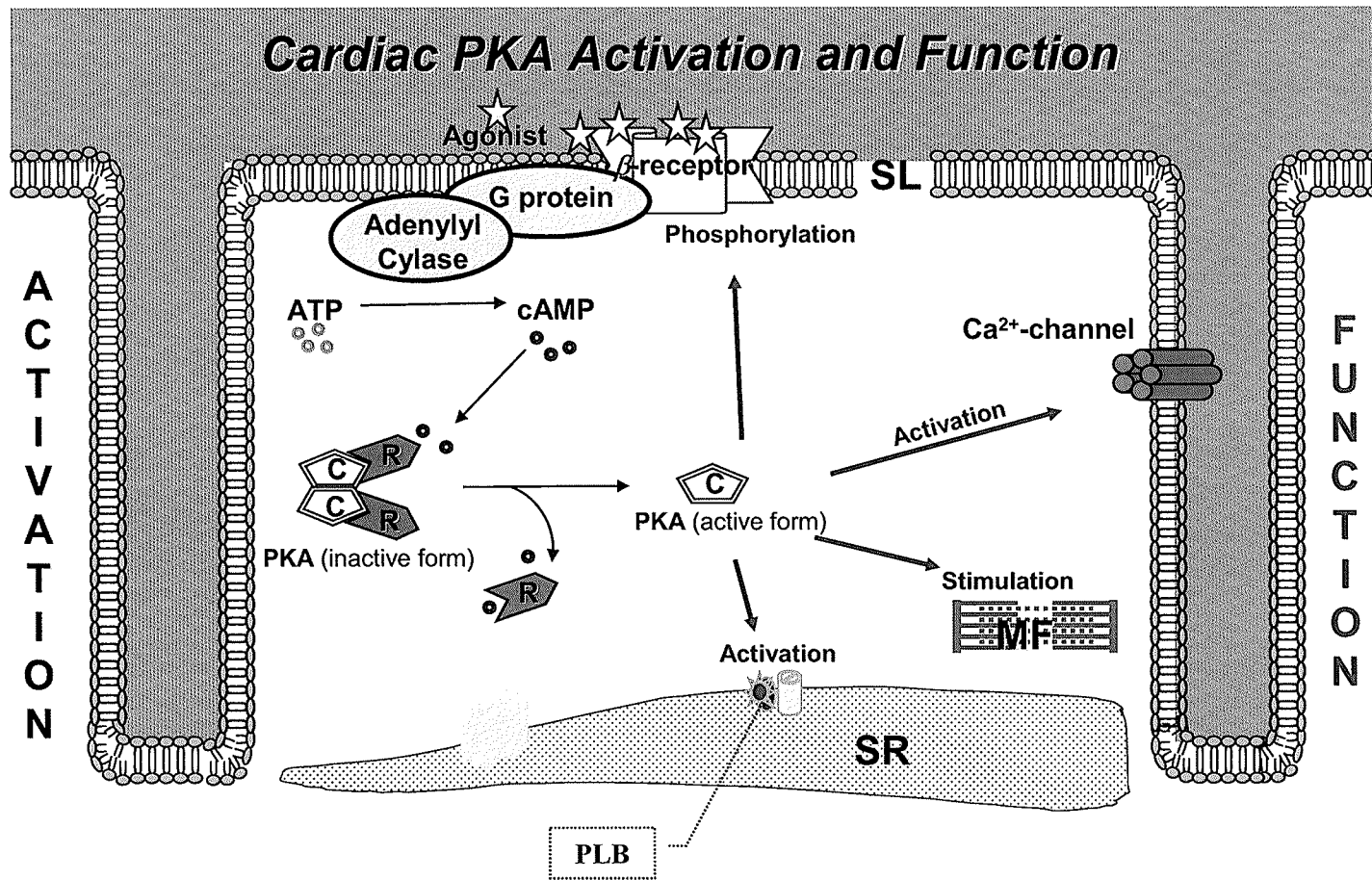
transgenic mice showed left ventricular hypertrophy, cardiac myocyte necrosis, multifocal fibrosis and decreased left ventricular performance, which indicated that these specific cardiac cellular and functional changes due to the activation of PKC- $\beta_2$  may lead to cardiomyopathy<sup>262</sup>. Rouet-Benzineb *et al.*<sup>263</sup> and Mohammadi *et al.*<sup>264</sup> showed that PKC activity and Ca<sup>2+</sup>-dependent PKC isoforms are decreased in adult rabbit heart failure induced by aortic insufficiency followed by aortic stenosis. These results are different from those reported by Gu and Bishop<sup>248</sup>, who demonstrated that PKC activity was increased in the early phase of rat heart failure induced by pressure overload. Such a discrepancy in results may be due to species differences and the fact that the latter study was performed in young developing rats. In view of the availability of very few reports regarding changes in PKC activity or PKC isoforms in heart failure, extensive studies in the failing heart, especially in CHF following MI are required to make any meaningful conclusion.

## F. Protein Kinase A in Heart Function

Over the past 20 years, many laboratories have focused their investigations on the  $\beta$ -adrenergic mediated regulation of cardiac contractility through phosphorylation of cellular proteins by PKA. There is now general agreement that PKA-mediated protein phosphorylation has an important role in heart function. Stimulation of cardiac myocytes by catecholamine and other  $\beta$ -adrenergic agonists produces an elevation of the intracellular concentration of Ca<sup>2+</sup>; this occurs primarily through PKA-mediated phosphorylations at SL Ca<sup>2+</sup> channels and the SR Ca<sup>2+</sup> pump regulatory protein phospholamban<sup>265-268</sup>. The resulting increase in the availability of Ca<sup>2+</sup> to the contractile

apparatus augments the force of cardiac contraction. Direct phosphorylation of the contractile machinery by the activation of PKA itself is also recognized as having an important modulatory role in the cardiac contraction-relaxation cycle. It is also well established that  $\beta$ -adrenergic stimulation of either intact hearts or isolated myocardial cells results in PKA-mediated phosphorylation of C protein and TnI in the thick and thin myofilaments, respectively<sup>269</sup>.

In its holoenzyme state, PKA is an inactive tetramer composed of a regulatory (R) subunit dimer and two catalytic (C) subunits. Each of the PKA subunits consists of a regulatory subunit, which binds two molecules of cAMP and a catalytic subunit. Combination with cAMP causes the R2C2 complex to dissociate and release the active C monomers<sup>270</sup>. When cAMP is elevated in the heart by  $\beta$ -adrenergic stimulation, the holoenzyme is activated. Four molecules of cAMP bind the R subunit dimer causing the release of the C subunits, which phosphorylate a wide variety of substrates such as cation channels, contractile proteins and metabolic enzymes (Fig. 3)<sup>267,271</sup>. The two major classes of holoenzyme, PKA I and PKA II, are present in the heart but their relative abundance is species-dependent; PKA II is by far the most abundant isozyme in bovine heart, whereas PKA I is the predominant isozyme in rat heart<sup>272</sup>. PKA is known to regulate a variety of proteins (Figure 3), such as  $K^+$ ,  $Na^+$  and L-type  $Ca^{2+}$  channels, phospholamban, and the TnI and TnT subunits<sup>273-275</sup>. Regulation of  $\beta$ -adrenergic stimulation of  $Na^+/K^+$  pump current in guinea pig ventricular myocytes is via PKA<sup>276</sup>. Functional and biochemical studies suggest that direct phosphorylation of the  $\alpha_1$  subunit is involved in modulation of cardiac and skeletal muscle L-type  $Ca^{2+}$  channel channel function by PKA<sup>277</sup>. For myofibril proteins, TnI and TnT are the substrates of PKA<sup>278</sup>.



**Figure 3.** General scheme of cardiac protein kinase A (PKA) activation and functions. C: catalytic subunit; R: regulatory subunit; SL: sarcolemma; SR: sarcoplasmic reticulum; MF: myofibrils; PLB: phospholamban.

Two serine residues in the amino terminus of the TnI subunit are believed to be phosphorylated by PKA on stimulation by  $\beta$ -agonists, resulting in decreased sensitivity of myofibrils to  $\text{Ca}^{2+}$  by increasing release of  $\text{Ca}^{2+}$  from the TnC/ $\text{Ca}^{2+}$  complex. Furthermore, it was found that phosphorylation of cardiac TnI by PKA results in decrease in the  $\text{Ca}^{2+}$  sensitivity for muscle contraction <sup>279</sup>. It has been shown that increased dissociation of  $\text{Ca}^{2+}$  from TnC coupled with faster uptake of  $\text{Ca}^{2+}$  by the SR following PKA-dependent phosphorylation of phospholamban can account for the faster relaxation seen in the inotropic response of the heart to catecholamines <sup>278,280</sup>.

The role of PKA in cardiac hypertrophy has been analyzed in different hypertrophic animal models. There may be different biochemical intracellular mechanisms for different models of experimentally induced cardiac hypertrophy. It was reported that cardiac PKA activity from rats with aortocaval shunt increased significantly throughout the study (56 days) with a maximum value at day 7, and decreasing thereafter but remaining higher than the control values <sup>281</sup>. The increase in cardiac PKA activity in this volume overload model may be explained by mechanical stretch, cell deformation or activation of a series of neural and hormonal systems <sup>242</sup>. By using perfused rat hearts, studies on the cAMP transduction pathway provide evidence for stretch-induced activation of adenylate cyclase and PKA in the regulation of protein synthesis and ribosome formation. There are a few reports relating to PKA activity in hypertension. Coquil and Hamet <sup>282</sup> studied PKA activity in spontaneously hypertensive rats (SHR) and found a progressive decrease during five weeks but it was unaltered at 18 weeks compared with normal rat hearts. On the other hand, ventricular PKA activity did not change significantly with respect to the sham group after induction of pressure overload.

For the MI model, ventricular PKA activity increased only seven days after infarction, but it had no relation with the development of cardiac hypertrophy induced by MI <sup>269</sup>. In conclusion, the volume overload model of cardiac hypertrophy exhibits increased intracellular cAMP concentration and a consequent activation of PKA activity, which could be due to increased plasma levels of catecholamines <sup>283</sup>. In contrast, in the pressure overload model of cardiac hypertrophy, it seems that Ang II could play a primary role, activating PKC rather than PKA <sup>242</sup>. The MI model also may share some common intracellular mechanisms with the above two models of hypertrophy because there are both volume and pressure overload stresses during MI <sup>284</sup>.

Although the sympathetic drive is increased in heart failure, the cells of the failing heart become desensitized to the increased level of  $\alpha$ -adrenergic receptor agonists <sup>269,285</sup>.  $\beta$ -adrenergic receptor density is decreased in response to isoproterenol (ISO) exposure, which is accompanied by a decreased stimulation of adenylyl cyclase by the agonist. These effects of high doses of ISO are thought to mimic the *in vivo* situation in heart failure, where the circulating levels of catecholamines are elevated to maintain ventricular function. There is some evidence that the level of stimulatory guanine nucleotide-binding protein ( $G_s$ ) is reduced, whereas that of inhibitory guanine nucleotide-binding protein ( $G_i$ ) is increased, in cardiomyopathic hearts <sup>25,73,286-291</sup>. All these changes may contribute to reducing the contractility and ability of the failing heart to respond to exercise. In heart failure the increase in atrial and ventricular filling pressures results in cardiopulmonary and arterial baroreceptor dysfunction, with a subsequent increase in the activity of the sympathetic nervous system. Therefore, it is quite possible that continued increases in atrial and ventricular filling pressures lead to a rise in plasma catecholamines, with an



increase in cAMP and PKA activation in cardiac tissue <sup>242,286,292</sup>. In addition to phosphorylation of proteins involved in the regulation of cardiac contractility, PKA has been shown to play a role in the regulation of  $\beta$ -adrenoceptor function <sup>293</sup>. Phosphorylation of  $\beta$ -adrenoceptors by  $\beta$ -adrenoceptor kinase occurs at higher agonist concentrations, whereas  $\beta$ -adrenoceptor phosphorylation by PKA occurs at low agonist concentrations; the functional consequence of this phosphorylation is an uncoupling of  $\beta$ -adrenoceptors <sup>294</sup>. In the failing heart,  $\beta$ -adrenoceptor uncoupling has been well defined, and it appears that increased PKA activity may contribute to  $\beta$ -adrenoceptors uncoupling in the failing myocardium. However, it was reported that cAMP concentrations were reduced in the particulate fraction in failing human hearts due to ischemic cardiomyopathy and in the soluble fraction in dilated cardiomyopathy, but there was no change in PKA activity. Phospholamban levels and cAMP-dependent phosphorylation of phospholamban were similar in nonfailing and failing myocardium <sup>267</sup>. However, alterations in PKA activity and content of its isoforms during the development of heart failure remain to be investigated for a full appreciation of defects in signal transduction mechanisms in the failing heart.

## **G. Renin-angiotensin System and the Heart**

RAS is one of the major mechanism for the regulation of cardiovascular system, especially in controlling of blood pressure and cardiac remodeling <sup>295</sup>. RAS is composed by several components, involving the following series endocrine actions: renin secreted by the kidney acts on angiotensinogen released by the liver. The resulting angiotensin I (Ang I) is cleaved by angiotensin converting enzyme (ACE) to generate circulating Ang

II, which binds to its receptors and produces specific biological effects<sup>295</sup>. An increase in the gene transcripts for angiotensinogen and ACE in the ventricular myocardium has been demonstrated in animal models of pressure overload<sup>296</sup>, tachycardia<sup>297</sup> and MI induced heart failure<sup>298,299</sup>. Despite the evidence that expression of angiotensinogen and ACE mRNA in the heart is at least 20-fold lower than in the liver and lung, generation of Ang II occurs in response to a functional overload of the heart, which suggests a potentially important linkage between the cardiac RAS and workload<sup>299,300</sup>. An elevation of ACE activity has been observed in hypertrophied LV of rats with aortic banding<sup>299,300</sup>, in the residual viable myocardium after MI<sup>298</sup>, and in hearts following ISO infusion<sup>301</sup>. ACE activity is increased within the first few days of a MI and remains elevated during the following 80 days<sup>302</sup>. Which cell types within the heart are responsible for the generation of ACE are not well known, but there is increasing evidence that the coronary vascular endothelium may be the major source of cardiac ACE<sup>303</sup>. The activation of RAS plays an important role in the genesis of cardiac remodeling, in terms of stimulation of cardiac myocyte hypertrophy, and modulation of collagen synthesis and degradation. Therefore, great attention has been paid to block RAS, both in research and clinical practice.

ACE inhibitors were first introduced in early 1980 as novel agents for the treatment of hypertension. Twenty years later, ACE inhibitors have become the mainstay for the treatment of CHF, and investigational indications continue to expand as understanding of the pharmacology of these drugs increases<sup>304</sup>. In addition to their vascular effect, ACE inhibitors have multiple functions, especially since they play an important role in the progression of cardiac remodeling<sup>305</sup>. It is worth noting that the effects of ACE inhibitors are dose-dependent as seen in the SHR<sup>153</sup>. The process of ventricular remodeling after MI can be modified by the long-term administration of an

ACE inhibitor<sup>306,307</sup>. Following coronary artery ligation in rat, 3 months of therapy with an ACE inhibitor captopril, reduced ventricular volume. In rats with large infarcts, the reduction in cardiac mass by captopril was proportional to the attenuation in ventricular dilation. In contrast, therapy of animals with moderate infarcts led to a reduction in mass that was less than the attenuation in volume<sup>6</sup>. These alterations in cardiac mass and cavitory volume were associated with a more favorable ventricular performance and a prolongation in survival<sup>306</sup>.

ACE inhibitors are effective as afterload and preload reducing agents. The formation of Ang II, the potent peripheral arterial vasoconstrictor, is greatly reduced by ACE inhibitors, and this relaxes the vascular system<sup>3</sup>. These agents lower LV filling and systemic arterial pressures and decrease systemic vascular resistance but maintain or increase cardiac output without changing the heart rate when administered acutely or chronically in heart failure. ACE inhibitors are arterial vasodilators, but their ability to lower filling pressure post-infarction and to prevent degradation of bradykinin have been attributed to the additive production of venodilatation<sup>305,308</sup>. In patients of hypertension, intracoronary injection of enalapril, an ACE inhibitor, improved the active relaxation, accompanied by a decrease in left ventricle end-diastolic pressure (LVEDP) and LV end-diastolic volume<sup>309</sup>. The rat model of MI has been used to study the short- and long-term hemodynamic effects of ACE inhibitors, and the survival trials with these agents have been successful<sup>306,310</sup>.

The effectiveness of ACE inhibition in reducing cardiac mass and volumes, maintaining cardiac output in hypertension and MI has sparked considerable interest in the role of the RAS in cardiac remodeling<sup>306</sup>. Several studies have indicated that exogenous or locally generated Ang II has a role in altering cardiac gene expression as

Ang II was observed to induce early response genes involved in growth and differentiation in neonatal and adult rat cardiomyocytes<sup>233,311,312</sup>. This induction appears to be mediated via activation by Ang II of the phospholipid-mediated second messenger system, such as PLC and PKC, through the angiotensin II type I receptor (AT<sub>1</sub>R)<sup>233,312</sup>. Within 6 hr in isolated neonatal cardiomyocytes, Ang II induced the late genes expression, including skeletal  $\alpha$ -actin and ANP, and resulted in the upregulation of the genes for angiotensinogen and transforming growth factor- $\beta$  (TGF- $\beta$ ); these observations suggest a positive feedback mechanism for the regulation of cardiac hypertrophy<sup>233</sup>. In hypertrophied hearts induced by banding of the ascending aorta, cardiac ACE activity is increased, as well as ACE mRNA level and protein contents<sup>299</sup>, while ACE inhibitors reduced the intracardiac conversion of Ang I to Ang II in this model<sup>299,300</sup>. An increased expression of *c-myc* and *c-jun* has also been demonstrated in surviving myocytes of RV and LV of rats 2 to 3 days following MI, a time by which an increase in myocytes volume has been documented<sup>284</sup>. Mediation of cardiac hypertrophy by the AT<sub>1</sub>R and upregulation of its gene and that of TGF- $\beta$  have also been shown in rats with coarctation of the abdominal aorta<sup>313</sup>. In SHR and two-kidney renal one-clip renovascular hypertensive rats, ventricular AngII mRNA levels and AT<sub>1</sub>R densities were increased three- and two- fold, respectively<sup>314</sup>. Furthermore, exogenous Ang II has been shown to increase protein synthesis in embryonic and neonatal cells<sup>315</sup> and to reduce atrophy in denervated transplanted rat heart<sup>316</sup>. On the other hand, ACE inhibitors such as enalapril reduced cardiac hypertrophy as indicated by the reduction in ratio of LV to body weight in SHR<sup>153</sup>.

Human clinical trials of ACE inhibitors in the early post-infarction period began when laboratory studies in animal models found that these agents reduced LV dilation after MI. The clinical and hemodynamic benefits of ACE inhibitors in patients with CHF were also established <sup>317</sup>. Therapy with ACE inhibitors improved clinical signs and symptoms, as well as exercise tolerance in patients with moderate to severe CHF <sup>304,318</sup>. ACE inhibitors also slowed the onset of symptomatic CHF and its clinical progression in patients with milder symptoms <sup>319,320</sup>. In addition, ACE inhibitors prolonged survival in patients with CHF, including asymptomatic and symptomatic patients as well as those with symptomatic left ventricular dysfunction in the setting of acute MI <sup>321,322</sup>.

Although the first Ang II receptor antagonist octapeptide, saralasin, was identified early in 1970s, the process of clinical availability of angiotensin II receptor antagonist was very slow <sup>295</sup>. At the beginning of 1990s, nonpeptide AT<sub>1</sub>R antagonists losartan, also named as DuP 753, or MK-954 became available <sup>323,324</sup>. It is a competitive antagonist of the AT<sub>1</sub>R, which has undergone extensive clinical testing in patients with essential hypertension, and was the first nonpeptide Ang II receptor antagonist introduced in 1995 for clinical utilization <sup>295</sup>. Losartan has been developed to overcome potential limitations of ACE inhibitors, *e.g.* insufficient control of tissue Ang II production and bradykinin-related side effects. The clinical study of losartan in heart failure showed that losartan produced dose-dependent vasodilatation and neurohormonal effects <sup>325,326</sup>. In the rat MI model, hemodynamic studies confirmed that reduction in filling pressure by losartan was sustained during chronic therapy. AT<sub>1</sub>R antagonists exert beneficial hemodynamic and neurohormonal effects in human heart failure <sup>325</sup>.

Both ACE inhibitors and AT<sub>1</sub>R antagonists increased the circulating levels of renin and Ang I, and reduced the level of aldosterone and the activity of sympathetic

system. However, these agents produced opposite effects on Ang II levels: ACE inhibitors decreased it and AT<sub>1</sub>R receptor antagonists increased it<sup>295</sup>. When losartan was compared to captopril in the MI rat, the agents were found to be equally effective in attenuating the impairment of LV, reducing LVEDP and LV end-diastolic volume index as well as increasing venous compliance<sup>305</sup>. Losartan reduced LV hypertrophy and completely prevented interstitial collagen accumulation in the myocardium<sup>324</sup>. For the prevention of collagen accumulation in SHR, losartan showed a similar effect as enalapril, and was even much better at lower dose<sup>153</sup>. Losartan was regarded to have comparable efficacy and tolerability with enalapril in the short-term treatment of moderate or severe CHF<sup>327</sup>.

The difference between the two designated sites of action of ACE inhibitors and AT<sub>1</sub>R antagonists makes the drug interaction in RAS an interesting issue for both therapy and research<sup>295</sup>. The pharmacological target of ACE inhibitors is the formation of Ang II, the first step in RAS activation, while that for AT<sub>1</sub>R antagonists is to block the receptor. These two are not co-localized together, but are tissue specific<sup>295</sup>. On the other side, the existence of an alternative pathway for the production of Ang II bypasses the effect of ACE inhibitor, unlike AT<sub>1</sub>R antagonist. Due to these reasons, combination of therapy with ACE inhibitors and AT<sub>1</sub>R antagonists has been investigated in different models and clinical trials. Konstam's group<sup>328</sup> found that combination therapy with enalapril and losartan limited the increase in heart weight/body weight ratio in MI rat. From the clinical trial, echocardiologic studies showed that the combination of enalapril + losartan is more effective than enalapril alone in improving myocardial function both at rest and after stress. The effect is detectable after six weeks of treatment, and it is believed that the combined therapy is perhaps attributable to escape of the "escape phenomenon", which is

common in monotherapy with ACE inhibitors, due to the conversion from Ang I to Ang II by chymase <sup>329</sup>. These observations suggest that the blockade of RAS has beneficial effects in CHF.

## II. STATEMENT OF THE PROBLEM AND HYPOTHESES TO BE TESTED

Differences in myosin ATPase activities from different sources have been attributed to the presence of different amounts of two MHC isozymes namely  $\alpha$ -MHC and  $\beta$ -MHC<sup>130,159</sup>. While differences in the primary structure of myosin isozymes have been identified with peptide mapping and immunological analysis, complementary DNA and genomic DNA cloning experiments have demonstrated that myosin isozymes are coded by two separate genes that are linked 4 kilo-bases apart in the rat genome<sup>160,330</sup>. Although on the basis of electrophoretic mobility myosin V<sub>1</sub>, V<sub>2</sub> and V<sub>3</sub> isozymes have been identified<sup>331</sup>, these isozymes were found to contain  $\alpha$ -MHC and  $\beta$ -MHC with identical MLC composition. Myosin V<sub>1</sub> is a  $\alpha\alpha$  homodimer and has the highest ATPase activity whereas myosin V<sub>3</sub> is a  $\beta\beta$  homodimer with the lowest ATPase activity; myosin V<sub>2</sub> is a  $\alpha\beta$  heterodimer<sup>162</sup>. Thus a shift in the composition of myosin isozymes with respect to  $\alpha$ -MHC and  $\beta$ -MHC contents can be seen to depress the myosin ATPase as well as myofibrillar Ca<sup>2+</sup>-stimulated ATPase activities, and contractile function of the myocardium<sup>159,332-335</sup>. A wide variety of changes in the distribution of myosin isozymes, myosin ATPase activity and myofibrillar Ca<sup>2+</sup>-stimulated ATPase activity have been reported in cardiac dysfunction and different types of failing hearts<sup>14,131,158,336-339</sup>. Although some investigators have examined changes in myosin isozymes as well as myosin ATPase activities in cardiac hypertrophy and heart failure due to MI, the results are controversial. For example, Geenen *et al.*<sup>340,341</sup> have reported a shift in the myosin isozyme distribution and depression in the actomyosin ATPase activity in the heart at 3



weeks of MI in rats; however, the changes in myosin isozymes were found to be region specific. Although a decrease in  $\alpha$ -MHC and an increase in  $\beta$ -MHC contents were seen in the LV at 4 to 5 weeks following MI in rats,  $\alpha$ -MHC, unlike  $\beta$ -MHC, was not responsive to treatment with thyroid hormones<sup>342</sup>. Alterations in mRNA levels for both  $\alpha$ -MHC and  $\beta$ -MHC in LV as well as RV were seen in rats at 4 weeks following MI<sup>343-345</sup> and these changes were prevented by treatment with  $\text{Ca}^{2+}$ -antagonists<sup>343,344</sup>. On the other hand, an increase in mRNA level for  $\beta$ -MHC in the LV from infarcted rat was detected at 3 days but was not evident at 3 weeks following MI<sup>346</sup>. Some investigators<sup>347</sup> have reported no change in myofilament function as assessed in the RV skinned trabeculae at 4 weeks after inducing MI in rats whereas others<sup>348</sup> have failed to detect any change in MHC isoforms in the LV of rabbit at 3 weeks following MI. These differences in results from various studies may be due to differences in infarct size, duration of coronary occlusion and different species employed for experimentation. In view of such scattered and conflicting information concerning changes in myosin isozymes, mRNA levels and myosin ATPase activity, it is difficult to make any conclusions regarding the significance of changes in cardiac contractile proteins in congestive heart failure due to MI.

Accordingly, the present study is proposed to examine changes in LV myosin isozymes, mRNA levels for  $\alpha$ - and  $\beta$ -myosin isozymes and myofibrillar ATPase activities in a hemodynamically assessed rat model of CHF<sup>39,82</sup>. Since the behaviour of the RV in this experimental model has been reported to be different from that of the LV<sup>39,349</sup>, RV was also used for biochemical analysis for the purpose of comparison. Because treatment of the infarcted animals with ACE inhibitors such as captopril and imidapril, has been shown to exert beneficial effects on CHF<sup>350-352</sup>, we have employed imidapril-treated animals for studying the beneficial effects of ACE inhibitors in CHF. The selection of

imidapril for this study is mainly due to the fact that this long-acting agent has been reported to reduce mortality in a small coronary artery diseased animals to a greater extent than other ACE inhibitors<sup>353</sup>.

The ability of cardiac muscle to generate contractile force is primarily dependent upon myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity<sup>158</sup>. Furthermore, it is known that the myofibrillar ATPase activity is determined by different amounts of MHC isozymes, namely  $\alpha$ -MHC and  $\beta$ -MHC in the myocardium<sup>130,159</sup>. A shift in the composition of myosin isozymes with respect to  $\alpha$ -MHC and  $\beta$ -MHC contents has been shown to depress myosin ATPase as well as myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activities and contractile function in different models of cardiac hypertrophy and heart failure<sup>159,332-335,354</sup>. However, the mechanisms of such a change in the molecular composition of myofibrils (myofibrillar remodeling) in CHF due to MI are not understood. Since the RAS is activated in CHF and its blockade has been shown to prevent cardiac remodeling (changes in cardiomyocyte size and shape) and improve heart function in CHF due to MI in humans<sup>319,355</sup>, and animal models<sup>41,42,350,352,356</sup>, it is likely that myofibrillar remodeling in the failing heart is prevented by the blockade of RAS. Therefore, it is planned to examine the effects of imidapril treatment in infarcted rats on LV function and myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity as well as changes in MHC protein and gene expression<sup>42</sup>. In order to test if the effects of imidapril are mediated through the blockade of RAS, we have used enalapril, a widely used ACE inhibitor, and losartan, an  $\text{AT}_1\text{R}$  antagonist. It should be noted that although these agents have been reported to produce beneficial actions on cardiac remodeling and heart failure<sup>41,357,358</sup>, the effects of

enalapril and losartan on changes in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase and MHC isoforms in CHF have not been examined previously.

The objective of the present study therefore is to investigate if improvement of cardiac function is associated with prevention of changes in myosin isozymes, and gene expression for  $\alpha$ -MHC and  $\beta$ -MHC as well as myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activities in the failing heart upon treatment with enalapril or losartan. In order to test if the effects of losartan are additive, infarcted animals were treated with a combination of both drugs.

Among a wide variety of protein kinases present in mammalian cells, two multifunctional protein kinases, PKC and PKA, are thought to mediate several phosphorylation reactions in the myocardium<sup>359-361</sup>. Both of these protein kinases are known to regulate cation transport, contractile force development, metabolic processes, gene expression, and cellular growth in the heart<sup>360,361</sup>. Molecular cloning studies<sup>360,362</sup> have indicated that PKC exists as a family of at least 12 distinct isoforms. The conventional PKC isoforms ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) contain a  $\text{Ca}^{2+}$ -binding domain which accounts for their activation by  $\text{Ca}^{2+}$ . The novel PKC isoforms ( $\delta$ ,  $\varepsilon$ ,  $\eta$ , and  $\theta$ ) lack the putative  $\text{Ca}^{2+}$ -binding domain and do not require  $\text{Ca}^{2+}$  for maximal enzymatic activation. Atypical PKC isoforms ( $\zeta$ ,  $\lambda$ , and  $\iota$ ) are distinguished from other members of the PKC gene family by the presence of only a single copy of cysteine-rich motif. Activation of Ang II receptors,  $\alpha_1$ -adrenergic receptors, and endothelin-1 receptors has been shown to stimulate PKC via Gq-coupled PLC- $\beta$ <sup>196,360,361</sup>. In contrast, PKA is activated by catecholamines through  $G_s$ -coupled  $\beta$ -adrenergic receptors<sup>265,360</sup>. Previous work from our laboratory<sup>363</sup> has demonstrated increased activities of cardiac PKC and PKA due to CHF

in cardiomyopathic hamsters. Increased cardiac PKC activity has also been shown in pressure-overloaded cardiac hypertrophy in the rat <sup>248</sup> and pressure overloaded heart failure in guinea pigs <sup>364</sup>, as well as in human failing hearts <sup>365</sup>. Varying degrees of changes in PKC activities have been observed in cardiac dysfunction due to diabetes <sup>253,366-369</sup>. Furthermore, transgenic mice with cardiac overexpression of PKC- $\beta$  or PKC- $\epsilon$  were found to exhibit gross cardiac hypertrophy and diminished ventricular function <sup>262,370</sup>. The PKA activity has also been observed to increase in cardiac hypertrophy due to volume overload in rats <sup>281</sup>. Transgenic mice with overexpression of PKA in the heart have been reported to develop dilated cardiomyopathy and reduced cardiac contractility; however no changes in PKA activity were seen in failing human heart or in MI <sup>267,269</sup>. Although cardiac hypertrophy, heart failure and cardiac dysfunction are known to occur as a consequence of MI <sup>39,82,284,351,352</sup>, no information regarding changes in PKC activities in the infarcted heart is available in the literature. Accordingly, this study was undertaken to examine the status of PKC activities during the development of CHF in a rat model of MI. Some experiments were also carried out to examine whether the changes in PKC activities in the failing heart are due to corresponding changes in the contents of PKC isozymes. It should be pointed out that PKC is activated by Ang II through the PLC- $\beta$ -mediated mechanisms in cardiomyocytes <sup>361</sup>. Furthermore, Ang II-induced activation of PKC has been demonstrated to result in the stimulation of cardiac gene expression, cell growth and remodeling of the myocardium <sup>360,361,371</sup>. Accordingly, the stimulation of RAS is considered to play a critical role in the activation of PKC that regulates the hypertrophic process and cardiac performance <sup>364</sup>. Because the RAS is activated in CHF <sup>372</sup> and treatment of infarcted animals with ACE inhibitors has been shown to produce

beneficial effects on heart function and attenuate changes in PLC activities<sup>351,352</sup>, it was planned to test the effect of imidapril on PKC activities and PKC isozyme contents in the failing heart. PKA activity and content of the MI-induced failing hearts with or without imidapril treatment were also maintained to test whether changes in PKC are of a specific nature.

### III. METHODS AND MATERIALS

#### A. Experimental Model

Experiments were conducted in accordance with the "Guide to the Care and Use of Experimental Animals" issued by the Canadian Council of Animal Care, and all experimental protocols were approved by the Animal Care Committee of the University of Manitoba following guidelines established by the Canadian Institutes of Health Research. MI was induced in male Sprague-Dawley rats (175-200g) by occlusion of the left coronary artery as described earlier<sup>39,82,349,352</sup>. Rats were anesthetized with isoflurane and the heart was exposed by opening the chest upon performing left thoracotomy. The left coronary artery was ligated at about 2 mm from origin of the aorta with 6-0 silk suture. The heart was repositioned in the chest and the incision was closed with a purse string suture. A mixed isoflurane with 95% oxygen and 5% carbon dioxide was supplied to the animal under positive pressure during surgery. Mortality of experimental rats was 30~35% within 48 hr. Sham-operated rats were treated in the same way except that the coronary artery was not ligated. Electrocardiography was performed before, and after open-chest to test the success of operation, and also performed at 3 weeks following MI. All animals were fed regular rat chow and were provided water *ad libitum*, and then maintained for 1, 2, 4, 7 and 8 week after the coronary artery ligation before the assessment of cardiac function and biochemical changes.

## **B. Protocol for Drug Treatments**

In one series of experiments (for myosin gene expression), the sham-operated and MI rats were treated at 3 weeks after the surgical operation with or without imidapril hydrochloride (1 mg/kg/day) for 4 weeks; imidapril was dissolved in distilled water (1 mg/ml) and administered once a day by gavage whereas the untreated animals received distilled water. All these animals were assessed hemodynamically and then examined for biochemical changes 7 weeks post-surgery. For PKC isoforms experiment, some sham and myocardial infarcted rats were divided into untreated and treated groups at 4 weeks after the operation. The untreated infarcted animals received distilled water whereas treated animals were given imidapril hydrochloride dissolved in distilled water at a concentration of 1 mg/ml once a day by gavage at a volume of 1 ml/kg/day for 4 weeks. In another series of studies, the experimental animals were randomly divided into 5 groups: sham operated (Sham), infarcted (MI), enalapril treated infarcted (ENP), losartan treated infarcted (LOS), and combined enalapril and losartan treated infarcted (COM). Three weeks after the operation, enalapril (10 mg/kg/day) and/or losartan (20 mg/kg/day), or tap water were given orally via a gastric tube to sham and infarcted groups for 5 weeks. The selection of the doses for these drugs was based on our previous study showing the beneficial effects of these agents on the SR protein and gene expression in this experimental model <sup>41</sup>. Imidapril was kindly supplied by Tanabe Seiyaku (Osaka, Japan). Enalapril and losartan were supplied by Merck Research Laboratories (Rahway, NJ, USA).

### **C. Hemodynamic Studies**

All animals were assessed hemodynamically before sacrifice. The animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (60 mg/kg) and xylazine (10 mg/kg). The right carotid artery was exposed and a cannula with a microtip pressure transducer (model SPR-249, Millar Instruments, Houston, TX) was introduced through proximal arteriotomy<sup>39,82,349</sup>. The catheter was advanced carefully through the lumen of the carotid artery, until it entered the LV, and then was secured with a silk ligature around the artery. The readings were taken using a computer program (AcqKnowledge for Windows 3.0., Harvard Apparatus, Montreal, Canada). The left ventricular systolic pressure (LVSP), LVEDP, heart rate, rate of pressure development (+dP/dt), rate of pressure decay (-dP/dt) and mean arterial blood pressure (MAP) were measured in these anesthetized animals according to the procedure described earlier<sup>39,82,349,352</sup>.

### **D. General Assessment and Tissue Preparation**

At the end of the hemodynamic measurements, the hearts were removed, and the LV (including septum) and RV as well as the scar tissue were quickly dissected, weighed and frozen in liquid nitrogen and stored at -70°C. The lung wet/dry wt ratio, an index of pulmonary congestion, as well as the heart wt/body wt ratio (including both ventricles and infarct scar), an index of cardiac hypertrophy, were measured in these animals. The removal of scar from the LV was necessary to obtain the noninfarcted myocardium (viable LV tissue including septum) for biochemical studies. Since scar wt/total LV wt (including septum and infarcted tissue) ratio was found to exhibit a linear relationship



with infarct size (as measured morphometrically)<sup>350</sup>, the scar wt was used as a marker to determine the extent of scar size<sup>39,41,82,349,352</sup>. It should be pointed out that about 10% of untreated and treated animals showed small infarct (scar wt/total LV ratio < 15% corresponding to scar size < 30% of the free LV wall). Thus the hemodynamic data from the animals showing small infarct were not included and the cardiac tissue from these animals was discarded. Furthermore, in view of the lack of clear demarcation between the adjacent area to the infarcted zone and the remote area, the adjacent segment and remote LV were not separated and thus the noninfarcted LV (including septum) and RV tissues from the infarcted animals were employed in this study. The average scar wt/LV wt ratio in the untreated and treated animals were  $24.4 \pm 0.8$  and  $23.6 \pm 0.6$ , respectively; these values were not statistically different ( $P > 0.05$ ) from each other and corresponded to infarct size of about 40% of the free LV wall area.

## **E. Myofibrillar $Mg^{2+}$ -stimulated ATPase and $Ca^{2+}$ -stimulated**

### **ATPase Activities**

Myofibrils were isolated according to the procedure employed earlier<sup>337</sup>. The myofibrillar fraction was suspended in a final solution containing 100 mM KCl, 20 mM Tris-HCl (pH 7.0).  $Mg^{2+}$ -dependent ATPase activity was determined at 30°C in a medium containing 20 mM imidazole (pH 7.0), 2 mM  $MgCl_2$ , 2 mM  $Na_2ATP$ , 10 mM  $NaN_3$ , 1.6 mM Ethylenegluco-bis( $\beta$ -aminoethylether)N,N,N',N'-tetraacetic acid (EGTA) and 50 mM KCl. Myofibrillar protein concentration varied from 400 to 700  $\mu g/ml$ ; blank tubes did not contain myofibrillar proteins. Total ATPase activity was determined in the same medium except that EGTA was replaced by 1  $\mu M$  of free  $Ca^{2+}$ .  $Ca^{2+}$ -stimulated ATPase

activity was taken as the difference between values obtained for total and  $Mg^{2+}$  ATPase activities. All reactions were terminated after 5 min by the addition of 1 ml of 12% trichloroacetic acid. These samples were centrifuged and the phosphate in the protein-free supernatant was determined<sup>332,338</sup>.

## F. Relative Protein Quantification of Cardiac Myosin

Relative protein content of cardiac MHC and MLC was determined by Western blot. Cardiac muscle homogenate was prepared from 10 mg pieces of ventricles from each experimental group, *i.e.* sham, imidapril-treated sham, infarcted and imidapril-treated infarcted rat heart. Another series of experiments included tissues from Sham, MI, ENP, LOS and COM. The muscle was homogenized using a Brinkmann homogenizer with Kinematica 87/Polytron PTA 7K1 for 4 - 6 s in homogenizing buffer (100  $\mu$ l buffer per 2 mg of muscle), containing 60 mM KCl, 1 mM cysteine, 20 mM imidazole (pH 6.9), 1 mM  $MgCl_2$ , 1 mM ouabain, 10 mM  $NaN_3$ , 1 mM  $CaCl_2$ , 0.01% leupeptin, 250  $\mu$ M phenylmethyl-sulfonylfluoride and 1 mM dithiothreitol (DTT). The homogenization procedure was carried out at 4°C. The concentration of protein in the homogenate was adjusted to 1 mg/ml with the homogenizing buffer. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, which contained 0.25 M Tris-HCl (pH 6.8), 8% (w/v) sodium dodecyl sulfate (SDS), 45% glycerol, 20%  $\beta$ -mercaptoethanol, and 0.006% bromophenol blue, was added into the homogenate buffer (1 part of loading buffer to 3 parts of homogenate)<sup>160</sup>. The samples were boiled for 5 min at 95°C. The proteins in homogenate separated by SDS-PAGE were electroblotted to Immobilon-P transfer membrane (Millipore Company, Bellerica, MA, USA) in a transfer

buffer that contained 25 mM Tris-HCl, 120 mM glycine and 20% methanol (v/v) for the analysis of cardiac MHC with immunoblotting analysis. Then the transferred membranes were shaken for 2 hr in blocking buffer, which contained Tris buffered saline (TBS, 10 mM Tris-HCl, 150 mM NaCl) and 5% fat-free powdered milk, then incubated for 1 or 2 hr at room temperature with monoclonal anti-MHC mouse antibody (1:1000, Sigma Immuno Chemicals, St. Louis, MO, USA), anti-MLC mouse IgG antibody (1:1000, Sigma Immuno Chemicals, St. Louis, MO, USA), or anti-TnI antibody (1:1000, Sigma Immuno Chemicals, St. Louis, MO, USA). The transferred membranes were subsequently incubated with biotinylated anti-mouse IgG (1:1000, Amersham Biosciences Inc, Baie d'Urfe, Quebec, Canada) for 40 min and then finally with streptavidin conjugated horseradish peroxidase (HRP, 1:5000, Amersham Biosciences Inc, Baie d'Urfe, Quebec, Canada) for 40 min. The blots were rinsed in the TBS-T (10 mM Tris-HCl, 150 mM NaCl and 0.2% Tween 20) 3 times (5 min each time) between each of the preceding steps. For chemiluminescent detection, the membrane sheets were developed on Hyperfilm-ECL (Amersham Biosciences Inc, Baie d'Urfe, Quebec, Canada) to visualize proteins. The normal exposure times ranged from 30 sec to 5 min. The relative protein content was determined by a GS-670 Imaging Densitometer (Bio-Rad Laboratories Canada Ltd, Mississauga, Ontario, Canada) with the Image Analysis Software Version 1.0.

### **G. Analysis of Cardiac Myosin Heavy Chain Isoforms**

Cardiac MHC isoforms were determined under denaturing conditions. The  $\alpha$ -MHC and  $\beta$ -MHC isoforms in the SDS-PAGE samples were separated by 4% SDS-PAGE as described<sup>333,339</sup>. The separating gel contained 3.975% (w/v) acrylamide and

0.025% (w/v) bisacrylamide ( $T = 4\%$ ,  $C = 0.625\%$ , where  $T$  = total acrylamide and  $C$  = bisacrylamide concentration as a percentage of total). The height of the stacking gel was reduced to 1 cm. Gels were cast to a thickness of 0.75 mm in glass plates previously silanized on the inner surfaces with dichloromethylsilane to prevent gel adherence. A maximum of 2 - 4  $\mu\text{g}$  of a 1mg/ml protein sample was loaded. Electrophoresis was carried out at a constant 220 V for 3 - 3.5 hr with cooling between 13 and 17°C and continuous stirring of bottom tank buffer. The gels were stained with Coomassie brilliant blue R250 for 2 hr and were destained with 7% acetic acid by diffusion. Relative amounts of isoforms were estimated by Imaging Densitometer (GS-670, Bio-Rad Laboratories Canada Ltd, Mississauga, Ontario, Canada).

#### **H. Preparation of Tissue Extract for Enzyme Determination**

The preparation of tissue extract for PKC analysis was carried out by the method described earlier<sup>363,367</sup>; all procedures were carried out at 4°C. The ventricular tissue (50 mg) was minced in 1 ml of buffer A (50 mM Tris-HCl, 0.25 M sucrose, 10 mM EGTA, 4 mM EDTA, 20  $\mu\text{g}/\text{ml}$  leupeptin, 200 U/ml aprotinin, pH 7.5) and homogenized (Polytron PT3000, Brinkmann Instruments; Mississauga, Ontario, Canada) at a setting of 8 for 2  $\times$  30 sec and sonicated for 2  $\times$  15 sec. In one set of experiments, the homogenate was incubated with 1% Triton X-100 (Sigma Immuno Chemicals, St. Louis, MO, USA) on ice for 60 min to solubilize PKC enzyme which is bound with subcellular structures. This Triton X-100-treated homogenate was then centrifuged at 100,000  $\times$  g for 60 min in an ultracentrifuge (model L70, Beckman Instruments Canada Inc; Mississauga, Ontario, Canada), and the supernatant obtained was labeled as the homogenate fraction. In another

set of experiments, the homogenate without Triton X-100 treatment was centrifuged at  $100,000 \times g$  for 60 min to separate the soluble and particulate-bound enzyme. The resulting supernatant was labeled as the cytosolic fraction, whereas the pellet was resuspended in 1 ml of buffer A with 1% Triton X-100 and incubated on ice for 60 min. The resuspended pellet was centrifuged at  $100,000 \times g$  for 60 min and this supernatant was labeled as the particulate fraction. For preparation of tissue extract for PKA determination, ~50 mg of frozen cardiac tissue were homogenized in 1 ml buffer B at pH 7.4 containing (in mM) 5 histidine-HCl, 0.1 phenylmethylsulphonyl fluoride, 50  $\text{KH}_2\text{PO}_4$ , 25 NaF, 10 EDTA, 750 KCl and 0.2 DTT. After gentle mixing, the homogenate was centrifuged at  $100,000 \times g$  for 60 min at  $4^\circ\text{C}$  and the supernatant was used for analysis of PKA activity and protein level<sup>267,281</sup>.

## I. Assays of PKC and PKA Activities

PKC activities in small samples of nonpurified homogenate, cytosolic and particulate fractions from the ventricular tissue were measured in the presence of okadaic acid, a highly specific inhibitor of type 1 and type 2A phosphatases<sup>373,374</sup>, by following methods described elsewhere<sup>363,367</sup>. The  $\text{Ca}^{2+}$ -dependent PKC activity was determined with a PKC assay kit (Upstate Biotechnology; Lake Placid, NY, USA) in the reaction buffer C containing (in mM) 20 3-[N-morpholino]propanesulfonic acid (MOPS), pH 7.2, 25  $\beta$ -glycerol phosphate, 1 sodium orthovanadate, 1 DTT and 4  $\text{CaCl}_2$ . Substrate cocktail containing 500  $\mu\text{M}$  PKC substrate peptide in buffer C, inhibitor cocktail containing 2  $\mu\text{M}$  PKA inhibitor peptide in buffer C, and lipid activator containing 0.5 mg/ml phosphatidyl serine and 0.05 mg/ml diglyceride in buffer C was used. The  $\text{Ca}^{2+}$ -independent PKC

activity was determined in a reaction buffer D containing (in mM) 20 MOPS, pH 7.2, 25  $\beta$ -glycerol phosphate, 1 sodium orthovanadate, 1 DTT, and 1.25 EGTA. Substrate cocktail (specific for PKC- $\epsilon$  and - $\zeta$  isozymes; Quality Controlled Biochemicals; Hopkinton, MA, USA) containing 500  $\mu$ M PKC substrate peptide in buffer D, inhibitor cocktail containing 2  $\mu$ M PKA inhibitor peptide in buffer D, and lipid activator containing 0.5 mg/ml phosphatidyl serine and 0.05 mg/ml diglyceride in buffer C was used. The sequence of the peptide substrate (Upstate Biotechnology; Lake Placid, NY, USA) used for PKC activity assay was QKRPSQRSKYL. The reactions for both  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent PKC activities were initiated by the addition of [ $\gamma$ - $^{32}\text{P}$ ]ATP (10  $\mu$ l) and allowed to proceed at 30°C for 10 min. The incorporation of  $^{32}\text{P}$  from [ $\gamma$ - $^{32}\text{P}$ ] into a synthesized substrate, which is a more specific substrate for PKC than Histone H-1 protein<sup>253,369</sup>, was measured as described elsewhere<sup>363,367</sup>. On the other hand, PKA activity was determined by using the PKA assay kit (Upstate Biotechnology; Lake Placid, NY, USA). The reaction was initiated by adding [ $\gamma$ - $^{32}\text{P}$ ]ATP (1 part of [ $\gamma$ - $^{32}\text{P}$ ]ATP in 9 parts of kit ATP solution). The PKA activity was assayed as described earlier<sup>363</sup> by measuring the incorporation of  $^{32}\text{P}$  from [ $\gamma$ - $^{32}\text{P}$ ]ATP into the substrate<sup>267,281</sup>.

## J. Analysis of PKC Isozyme and PKA Protein Content

The relative protein content of PKC- $\alpha$ , - $\beta$ , - $\epsilon$ , and - $\zeta$  isozymes was obtained by running SDS-PAGE of samples on 10% minigels followed by Western blot analysis with homogenate, cytosolic and particulate fractions<sup>363,367</sup>. The SDS-PAGE loading buffer contained 0.25 M Tris-HCl (pH 6.8), 8% (wt/vol) SDS, 45% glycerol, 20%  $\beta$ -mercaptoethanol, and 0.006% bromophenol blue. The proteins in both fractions separated

by SDS-PAGE were electroblotted to Immobilon-P transfer membrane (Millipore Company, Bellerica, MA, USA) which were incubated with polyclonal anti-PKC- $\alpha$ , - $\beta$ , - $\epsilon$ , and - $\zeta$  isozyme antibodies (Sigma Immuno Chemicals, St. Louis, MO, USA) for 1 hr at a concentration of 1:1000, respectively, and were subsequently incubated with biotinylated anti-rabbit IgG (1:5000; Amersham Biosciences Inc, Baie d'Urfe, Quebec, Canada) for 40 min and then finally with streptavidin conjugated HRP (1:5000; Amersham Biosciences Inc, Baie d'Urfe, Quebec, Canada) for 30 min. It should be pointed out that a recombinant standard (Bio-Rad Laboratories; Hercules, CA, USA) was run on SDS-PAGE with each sample to confirm molecular weight of PKC isozymes. Ponceau staining of the blots was performed to ensure no difference between control and experimental samples with respect to protein loading and protein transfer. The content of PKC isozyme was determined with an imaging densitometer (model GS-670, Bio-Rad Laboratories Canada Ltd, Mississauga, Ontario, Canada) with the Image Analysis Software Version 1.0. The relative protein content for each experimental sample was expressed as a percentage of the respective control value (band density of the sham control sample was considered as 100%). The information about the cross-reactivity of PKC isoform antibodies (GIBCO-BRL Life Technologies, Burlington, Ontario, Canada) indicated that antibodies directed against PKC- $\zeta$  recognize PKC- $\alpha$  and to a lesser extent PKC- $\beta$ . Nonetheless, the bands for PKC- $\alpha$ , PKC- $\beta$  and PKC- $\zeta$  were distinguished on the basis of molecular weight. Because the antibody against PKC- $\beta$  did not distinguish PKC- $\beta$ I and PKC- $\beta$ II, the band for PKC- $\beta$  was considered to be due to both  $\beta$ I and  $\beta$ II isoforms.

To quantitate the levels of PKC isoforms in the heart, the samples were run on the SDS gels along with recombinant PKC- $\alpha$  and PKC- $\beta$  (Calbiochem; La Jolla, CA, USA).

The membranes were incubated with polyclonal anti-PKC- $\alpha$  and anti-PKC- $\beta$  (Calbiochem; La Jolla, CA, USA) for 1 hr at a concentration of 1:1,000. Different amounts of PKC- $\alpha$  and PKC- $\beta$  (20, 40 and 60 ng) and different exposure times were used for obtaining standard curves for these isoforms; the optimal time period exposure for PKC- $\alpha$  was 30 sec whereas that for PKC- $\beta$  was 3 min. The relative protein content of PKA was obtained by running 12% SDS-PAGE and Western blotting<sup>363</sup>. The anti-PKA polyclonal antibody was from Transduction Laboratories and the concentration of 1:1,000 was utilized for the primary antibody. Cell lysates (5  $\mu$ l) derived from a pituitary tumor of a female Wistar-Furth rat (supplied along with the anti-PKA antibody purchased) was used as a positive control in Western blotting experiments

## **K. RNA Isolation**

Total myocardial RNA preparation was isolated from the LV and RV of sham, imidapril-treated sham, infarcted and imidapril-treated infarcted rats by guanidinium thiocyanate method established by Chomczynski and Sacchi<sup>375</sup> at 7 weeks after the surgery. Briefly, the fresh tissue was washed twice with a solution containing 10 mM MOPS and 10 mM sodium ethylenediaminetetraacetate (EDTA) and frozen in liquid nitrogen. The frozen samples were ground with mortar and pestle while immersed in liquid nitrogen. Powdered samples were suspended in solution D (4 M guanidine isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% N-lauroylsarcosine and 0.1 M 2-mercaptoethanol) and subjected to polytron homogenization. Tissue homogenates were treated with 0.1 volume of 2 M sodium acetate (pH 4.0), equal volume of water-saturated phenol and 0.2 volumes of chloroform-isoamyl alcohol mixture (49:1) and mixed by



inversion. The mixture was cooled on ice for an additional 15 min and was centrifuged at  $6,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ . The RNA-containing aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol and kept at  $-20^{\circ}\text{C}$  for 60 min. RNA was sedimented at  $10,000 \times g$  for 20 min and resuspended in solution D. In another series of experiments, total myocardial RNA preparation was extracted from the viable LV of sham, MI, ENP, LOS, and COM rats by the acid guanidinium thiocyanate-phenol-chloroform method (TRIzol reagent, GIBCO-BRL Life Technologies, Burlington, Ontario, Canada) according to the manufacturer's instructions. Re-precipitation of RNA by isopropanol was carried out at  $-20^{\circ}\text{C}$  for 1 hr and the RNA pellets were suspended in 75% ethanol (molecular biology grade diluted with DEPC-treated water). After sedimentation, RNA pellets were washed second time in ethanol and vacuum dried. Samples were dissolved in DEPC-treated water and the RNA concentration was calculated from the absorbance at 260 and 280 nm.

## **L. Northern Blot Analysis**

Steady-state levels of  $\alpha$ -MHC and  $\beta$ -MHC mRNA were determined by Northern hybridization analysis; 20  $\mu\text{g}$  of total RNA was denatured in 50% formamide, 7% formaldehyde, 20 mM MOPS (pH 7.4), 2 mM EDTA (pH 8.0), 0.1% SDS and was subjected to electrophoresis in a 1.2% agarose/formaldehyde gel to size fractionate the mRNA transcripts. The fractionated RNA was transferred (capillary) to a 0.45 mm positive charge-modified nylon filter (Zeta-Probe membrane, Bio-Rad Laboratories Canada Ltd, Mississauga, Ontario, Canada). After 24 hr, the filter was removed and nucleic acids were covalently crosslinked to the matrix using UV radiation (UV

Stratalinker 2400, Stratagene, Cedar Creek, TX, USA). Each membrane was hybridized with  $^{32}\text{P}$ -labeled cDNA probes at  $42^\circ\text{C}$ . Cardiac MLC,  $\beta$ -actin, as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes (American Type Culture Collection, Rockville, MD, USA) were labeled by random primer DNA labeling system with Klenow fragment<sup>376</sup>. The  $\alpha$ -MHC probe was a 39-mer oligonucleotide derived from the 3'-untranslated region of the rat  $\alpha$ -MHC gene as follows: 5'-GGG ATA GCA ACA GCG AGG CTC TTT CTG CTG GAC AGG TTA-3'. Similarly, the cardiac  $\beta$ -MHC probe was derived from the 3'-untranslated region of the gene (rat genome) and was 5'-CAG GCA TCC TTA GGG TTG GGT AGC ACA AGA-3'. Filters were exposed to x-ray film (Kodak X-OMAT) at  $-80^\circ\text{C}$  with intensifying screens. Results of autoradiographs from Northern blot analysis were quantified by densitometry (GS-670, Bio-Rad Laboratories Canada Ltd, Mississauga, Ontario, Canada). The signals of  $\alpha$ -MHC,  $\beta$ -MHC, MLC, and  $\beta$ -actin mRNA were normalized to that of GAPDH mRNA to account for differences in loading and/or transfer. The signal for GAPDH mRNA in infarcted heart did not change when normalized with respect to that for the 18S or 28S.

## M. Data Analysis

Data are expressed as mean  $\pm$  SE. The differences among various groups were evaluated statistically by one-way ANOVA followed by the Newman-Keuls test. A  $P$  value  $< 0.05$  was taken to represent a significant difference.

## IV. RESULTS

### A. Modification of Myofibrillar ATPase Activity and Myosin

#### Isozymes in Failing Heart by Imidapril

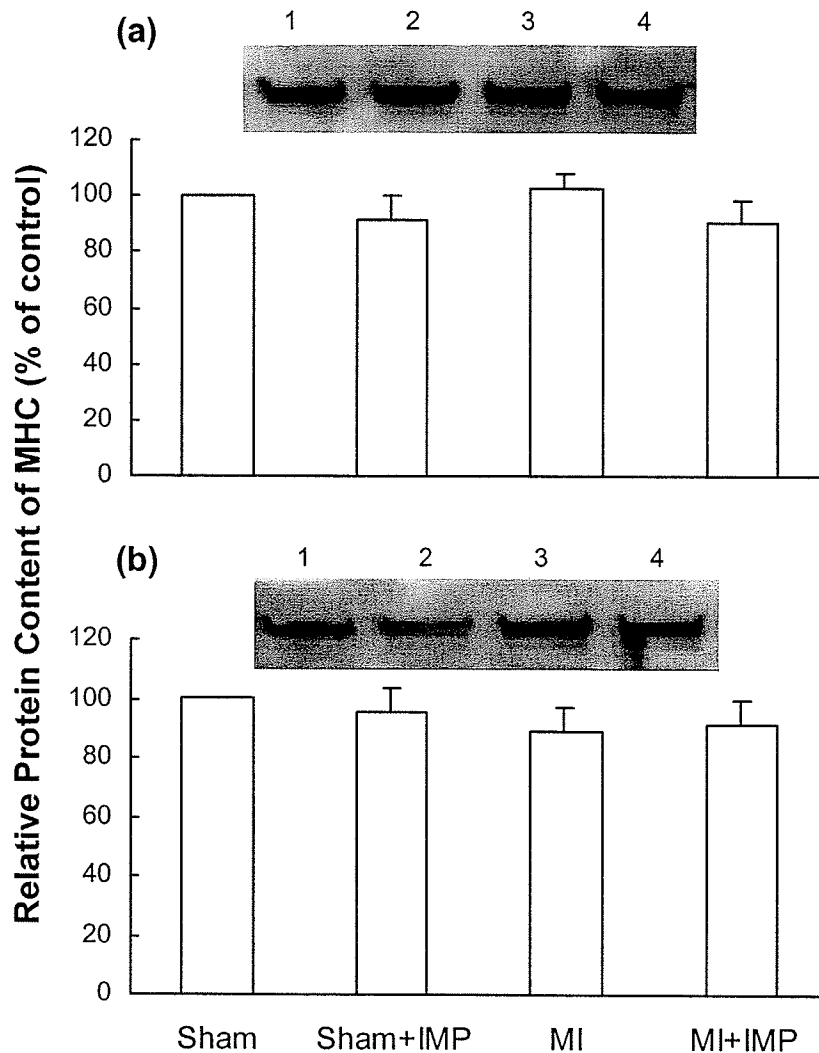
Occlusion of the left coronary artery resulted in scar formation in the LV. The viable cardiac muscle (both LV and RV) in the 7 week infarcted animals underwent hypertrophy; this was indicated by increased ventricular weight (wt) and ratio of the heart wt to body wt compared with sham control values (Table 2). There was a significant increase in wet-to-dry wt ratio of the lungs indicating the presence of pulmonary edema in MI rats. The increase in LVEDP and depression in contractile function with respect to both  $+dP/dt$  and  $-dP/dt$  were observed in the 7 week MI group. All these parameters in general characteristics and hemodynamics were partially prevented by treatment with imidapril (Table 2). No significant changes in the above mentioned parameters were observed in sham control animals treated with imidapril.

The relative protein content of MHC was derived from immunoblots using a monoclonal anti-MHC (a mixture of  $\alpha$ - and  $\beta$ -isoforms) antibody. No significant alteration in the total protein level of MHC was observed in rats with MI irrespective of the treatment (Figure 4). It should be noted that during polyacrylamide gel electrophoresis in the presence of SDS, the MHC  $\beta$ -isoform exhibited a higher electrophoretic mobility than the MHC  $\alpha$ -isoform. The MHC  $\alpha$ -isoform was the dominant (> 90%) isoform in both LV and RV of untreated and imidapril-treated sham rats. In LV and RV of untreated infarcted animals, the MHC  $\alpha$ -isoform was markedly reduced while the MHC  $\beta$ -isoform was increased (Figure 5). The percentage of MHC  $\beta$ -isoform was significantly increased

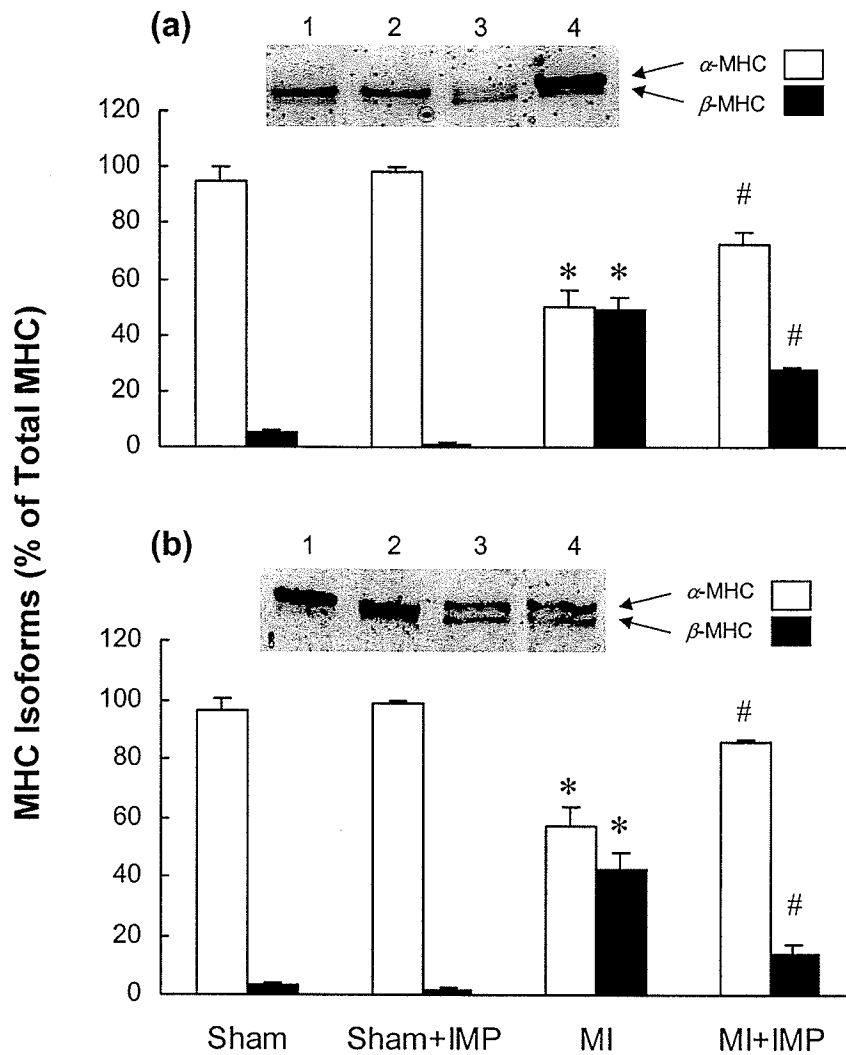
**Table 2. General characteristics of the sham control and infarcted rats with or without imidapril treatment**

	Sham	Sham + IMP	MI	MI + IMP
<b>Body wt (g)</b>	495 ± 8.6	468 ± 8.8*	487 ± 18	463 ± 15.6
<b>Heart wt (mg)</b>	1132 ± 41.6	1141 ± 71.8	1558 ± 59.8*	1267 ± 34.5 <sup>#</sup>
<b>Right ventricular wt (mg)</b>	274 ± 16.6	250 ± 20.1	605 ± 29.3*	420 ± 28.2 <sup>#</sup>
<b>Scar wt (mg)</b>	ND	ND	233 ± 22.5	202 ± 13.4
<b>Heart wt/body wt (mg/g)</b>	2.35 ± 0.11	2.41 ± 0.09	3.08 ± 0.06*	2.65 ± 0.07 <sup>#</sup>
<b>Lung wet/dry wt ratio</b>	4.31 ± 0.15	4.52 ± 0.2	5.41 ± 0.24*	4.89 ± 0.28 <sup>#</sup>
<b>LV +dP/dt (mm Hg/sec)</b>	8856 ± 834	7918 ± 540	3812 ± 252*	6382 ± 552 <sup>#</sup>
<b>LV -dP/dt (mm Hg/sec)</b>	9027 ± 822	9112 ± 322	4021 ± 211*	7813 ± 641 <sup>#</sup>
<b>LVEDP (mm Hg)</b>	4.1 ± 0.3	3.9 ± 0.2	17.6 ± 2.1*	7.4 ± 0.6 <sup>#</sup>

Results are mean ± SE of 12 animals for each group. MI: infarcted; IMP: imidapril; ND: not detected; LV: left ventricle; +dP/dt: rate of pressure development; -dP/dt: rate of pressure decay; LVEDP: left ventricular end diastolic pressure. IMP (1 mg/kg/day) was given orally for 4 weeks starting at 3 weeks after inducing MI. \* P < 0.05 compared to Sham operated control; <sup>#</sup> P < 0.05 compared to infarcted group.



**Figure 4.** Typical immunoblots and relative protein contents of myosin heavy chain in left (a) and right (b) ventricles from sham, imidapril-treated sham, infarcted and imidapril-treated infarcted rats. Relative protein contents of myosin heavy chain were determined by the electrophoresis and immunoblotting assay with monoclonal anti-myosin heavy chain antibody. IMP: imidapril; MI: infarcted; IMP (1 mg/kg/day) was given orally for 4 weeks starting at 3 weeks after inducing MI. The blots from #1 to 4 refer to sham, sham + IMP, MI and MI + IMP, respectively. Values are means  $\pm$  SE of 6 animals for each group.

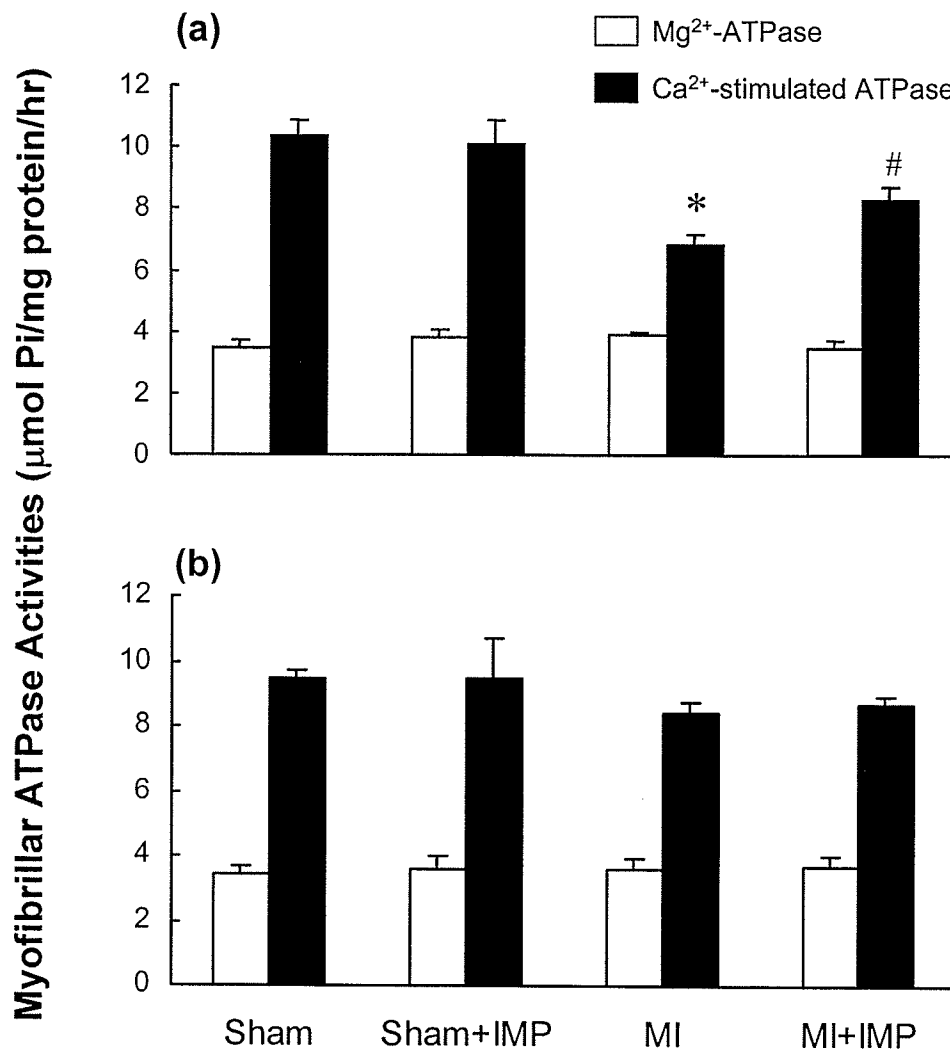


**Figure 5.** Myosin heavy chain  $\alpha$ - and  $\beta$ -isozymes in left (a) and right (b) ventricles from sham, imidapril-treated sham, infarcted and imidapril-treated infarcted rats. The figure shows typical eletrophoresis for myosin heavy chain  $\alpha$ - and  $\beta$ -isozymes and the results for laser densitometric analysis. MHC: myosin heavy chain; IMP: imidapril; MI: infarcted. IMP (1 mg/kg/day) was given orally for 4 weeks starting at 3 weeks after inducing MI. The blots from #1 to 4 refer to sham, sham + IMP, MI and MI + IMP, respectively. Values are means  $\pm$  S.E. of 6 animals for each group. \*  $P < 0.05$  vs Sham; #  $P < 0.05$  vs MI.

from 5% to 50% in the LV and from 3% to 43% in RV of infarcted rats. By contrast, the MHC  $\alpha$ -isoform decreased from 94% to 50% in the LV and from 97% to 57% in the RV of the infarcted rats. The imidapril treatment partially prevented the shift in MHC isoforms in both LV and RV (Fig. 5).

Myofibrils isolated from the LV of infarcted hearts exhibited a lower ( $6.8 \pm 0.4$   $\mu\text{mol Pi/mg/hr}$ )  $\text{Ca}^{2+}$ -stimulated ATPase activity compared with sham-operated rats ( $10.3 \pm 0.6$   $\mu\text{mol Pi/mg/hr}$ ). Imidapril administration to the infarcted rats greatly normalized the activity ( $8.3 \pm 0.42$   $\mu\text{mol Pi/mg/hr}$ ). In sham-operated rats, the imidapril treatment did not significantly alter the myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity (Fig. 6). There was no significant change in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity in the RV. Furthermore, no alterations of myofibrillar  $\text{Mg}^{2+}$  ATPase activity were found among different groups.

In order to examine the significance of changes in the LV myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity with respect to development of heart failure, we determined the myofibrillar ATPase activities in both LV and RV at 1, 2, 4, 8 and 16 weeks of inducing MI. It should be pointed out that studies from our laboratory<sup>39,82,349</sup> with this experimental model have revealed that the infarcted animals at 4, 8 and 16 weeks of coronary occlusion are at early, moderate and severe stages of congestive heart failure, respectively, whereas those at 1 and 2 weeks are at pre-failure stage. The data in Table 3 indicate a progressive decrease in LV myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity at 4, 8 and 16 weeks whereas no change in this parameter was observed at 1 and 2 weeks following MI. On the other hand, myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity in the RV



**Figure 6.** Changes in myofibrillar Mg<sup>2+</sup>- and Ca<sup>2+</sup>-stimulated ATPase activities of the left (a) and right (b) ventricles from sham, imidapril-treated sham, infarcted and imidapril-treated infarcted rats. IMP: imidapril; MI: infarcted. IMP (1 mg/kg/day) was given orally for 4 weeks starting at 3 weeks after inducing MI. Values are means  $\pm$  SE of 6 animals for each group. \* P < 0.05 vs Sham; # P < 0.05 vs MI.



**Table 3. Myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activities in left and right ventricles from sham control and infarcted animals at different intervals following occlusion of the coronary artery**

	Left ventricle		Right ventricle	
	Sham	Infarcted	Sham	Infarcted
<b>1 week</b>	10.4 ± 0.51	10.7 ± 0.42	9.0 ± 0.34	9.1 ± 0.27
<b>2 weeks</b>	10.1 ± 0.44	9.6 ± 0.38	9.3 ± 0.40	9.2 ± 0.36
<b>4 weeks</b>	10.4 ± 0.38	9.0 ± 0.27*	8.7 ± 0.28	8.1 ± 0.33
<b>8 weeks</b>	10.8 ± 0.41	7.2 ± 0.39*	8.9 ± 0.35	8.0 ± 0.38
<b>16 weeks</b>	10.6 ± 0.34	5.8 ± 0.24*	8.4 ± 0.28	7.3 ± 0.23*

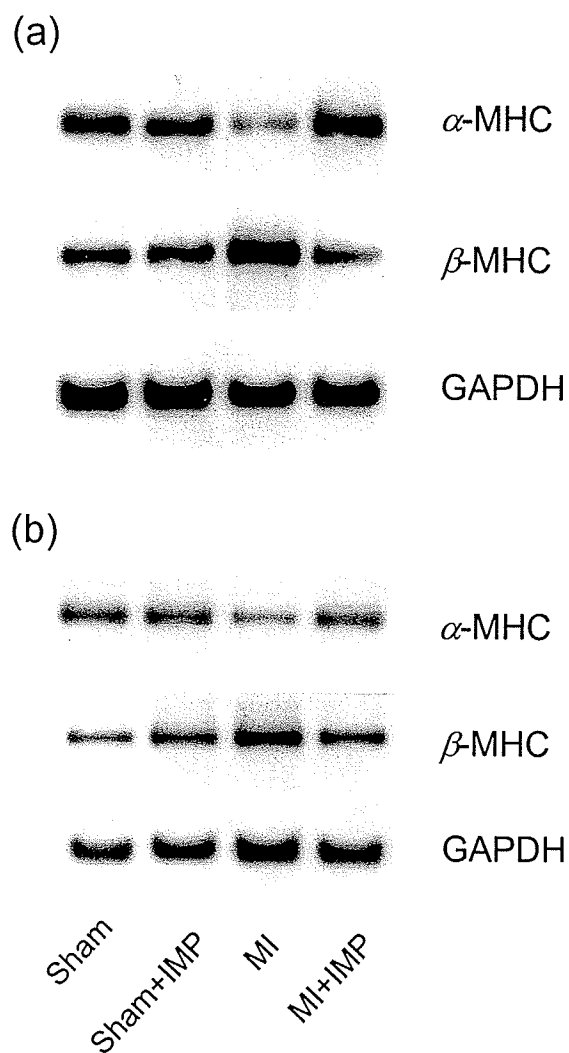
Each value is a mean ± SE of 6 animals for each group. Values for myofibrillar  $\text{Mg}^{2+}$  ATPase in the left ventricles varied between 3.2 to 3.8  $\mu\text{mol Pi/mg protein/hr}$  whereas those in the right ventricle varied between 2.8 to 3.7  $\mu\text{mol Pi/mg protein/hr}$  and there was no statistical difference between sham and infarcted animals. \*  $P < 0.05$  in comparison to respective Sham control.

showed no change at 1, 2, 4 and 8 weeks but was depressed at 16 weeks following coronary occlusion.

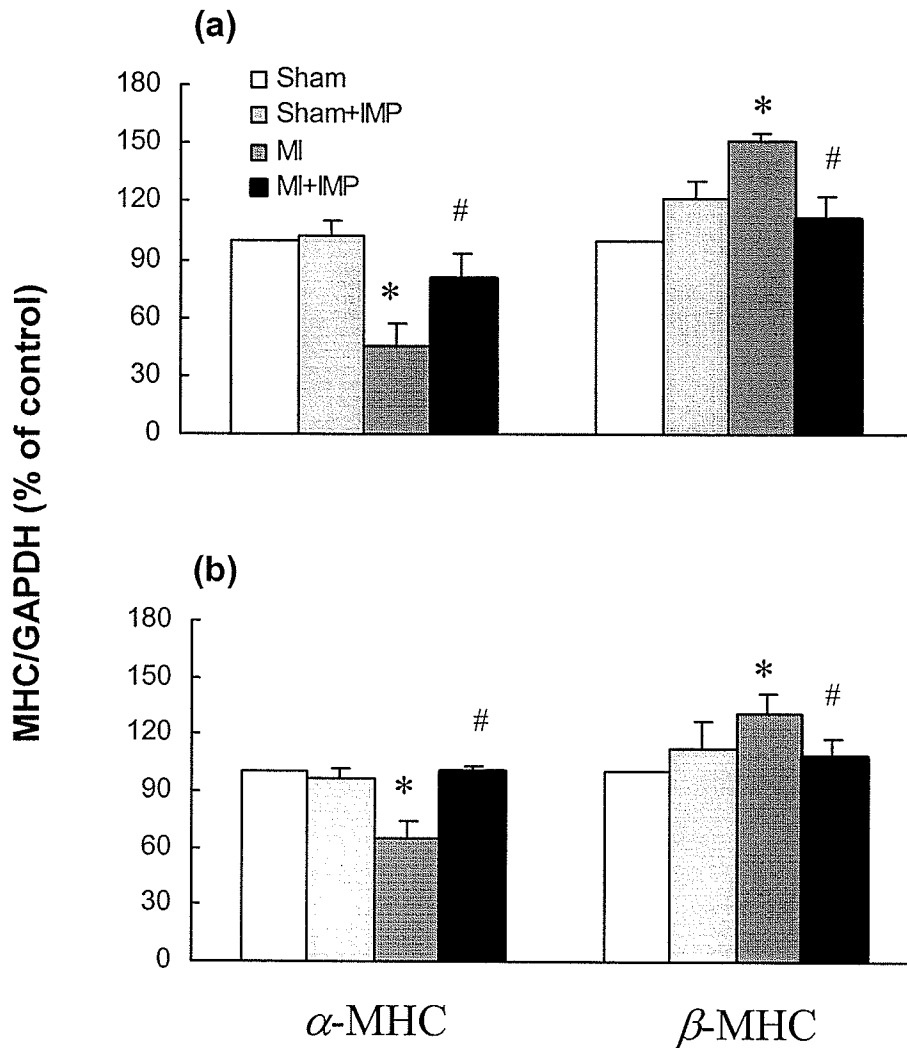
The mRNA abundance of MHC  $\alpha$ -isoform and  $\beta$ -isoform was determined by Northern blot analysis (Fig. 7 and 8). The mRNA abundance of the MHC  $\alpha$ -isoform and  $\beta$ -isoform was expressed as MHC  $\alpha$ -isoform/GAPDH and MHC  $\beta$ -isoform/GAPDH ratio, respectively. The MHC  $\alpha$ -isoform mRNA was decreased by about 35% to 55% and MHC  $\beta$ -isoform mRNA was increased by about 30% to 50% in LV and RV from the infarcted rats, respectively, and these changes were partially reversed by treatment with imidapril (Fig. 8).

## **B. Effects of Enalapril or Losartan on Myofibrillar ATPase and Myosin Isozymes in Failing Heart**

In this series of experiments, occlusion of the left coronary artery also resulted in scar formation in the LV, while the viable cardiac muscle in the 8 weeks infarcted animals underwent hypertrophy as indicated by increased ventricular wt compared to sham control values (Table 4). A significant increase in wet wt/dry wt ratio of the lungs indicated the presence of pulmonary congestion in MI rats. The liver wet wt/dry wt ratio was not altered. An increase in LVEDP and depression in contractile function with respect to both  $+dP/dt$  and  $-dP/dt$  were observed in the 8 weeks untreated MI group (Table 5). All these parameters in general characteristics and hemodynamics were partially prevented by treatment with enalapril, losartan or combination of enalapril and losartan. It was interesting to observe that the combination therapy did not produce additive effects as these values were not different from those obtained by treatments with



**Figure 7.** Typical Northern blots showing mRNA abundance for myosin heavy chain  $\alpha$ - and  $\beta$ -isozyme from the left (a) and right (b) ventricles from sham, imidapril-treated sham, infarcted and imidapril-treated infarcted rats. IMP: imidapril; MI: infarcted. IMP (1 mg/kg/day) was given orally for 4 weeks starting at 3 weeks after inducing MI.  $\alpha$ -MHC: mRNA blot for myosin heavy chain  $\alpha$ -isozyme;  $\beta$ -MHC: mRNA blot for myosin heavy chain  $\beta$ -isozyme; GAPDH: mRNA blot for glyceraldehyde-3-phosphate dehydrogenase. Values are means  $\pm$  SE of 6 animals for each group.



**Figure 8.** Densitometric analysis for mRNA abundance for myosin heavy chain  $\alpha$ -isozyme and  $\beta$ -isozyme in the left (a) and right (b) ventricles from sham, imidapril-treated sham, infarcted and imidapril-treated infarcted rats. The mRNA abundance of myosin heavy chain  $\alpha$ - and  $\beta$ -isozymes were expressed as ratio of these isozymes mRNA and GAPDH mRNA levels. All other details are same as in Fig. 7. Values are means  $\pm$  SE of 6 animals for each group. \* P < 0.05 vs Sham; # P < 0.05 vs MI.

**Table 4. General characteristics of myocardial infarcted rats with or without enalapril, and/or losartan treatment for 5 weeks starting at 3 weeks after coronary occlusion.**

	Sham	MI	ENP	LOS	COM
<b>Body weight (g)</b>	505 ± 11	503 ± 13	499 ± 19	505 ± 22	500 ± 23
<b>Heart weight (mg)</b>	1210 ± 90	1610 ± 110 *	1421 ± 68 #	1387 ± 28 #	1341 ± 27 #
<b>Scar wt (mg)</b>	ND	230 ± 15	231 ± 18	228 ± 11	229 ± 16
<b>Heart wt/BW (mg/g)</b>	2.40 ± 0.12	3.20 ± 0.25 *	2.85 ± 0.22 #	2.74 ± 0.13 #	2.68 ± 0.20 #
<b>Lung wet/dry wt</b>	4.35 ± 0.1	5.51 ± 0.33 *	4.85 ± 0.18 #	4.90 ± 0.30 #	4.95 ± 0.31 #
<b>Liver wet/dry wt</b>	3.16 ± 0.3	3.26 ± 0.22	3.21 ± 0.16	3.19 ± 0.20	3.34 ± 0.16

Values are mean ± SE of 7 animals in each group. ENP: myocardial infarcted treated with enalapril; LOS: myocardial infarcted treated with losartan; MI: myocardial infarcted; COM: myocardial infarcted treated with both enalapril and losartan; ND: not detected; \* P < 0.05 compared with Sham group; # P < 0.05 compared with MI group.

**Table 5. Hemodynamic parameters in myocardial infarcted rats with or without enalapril, and/or losartan treatment for 5 weeks starting at 3 weeks after coronary occlusion.**

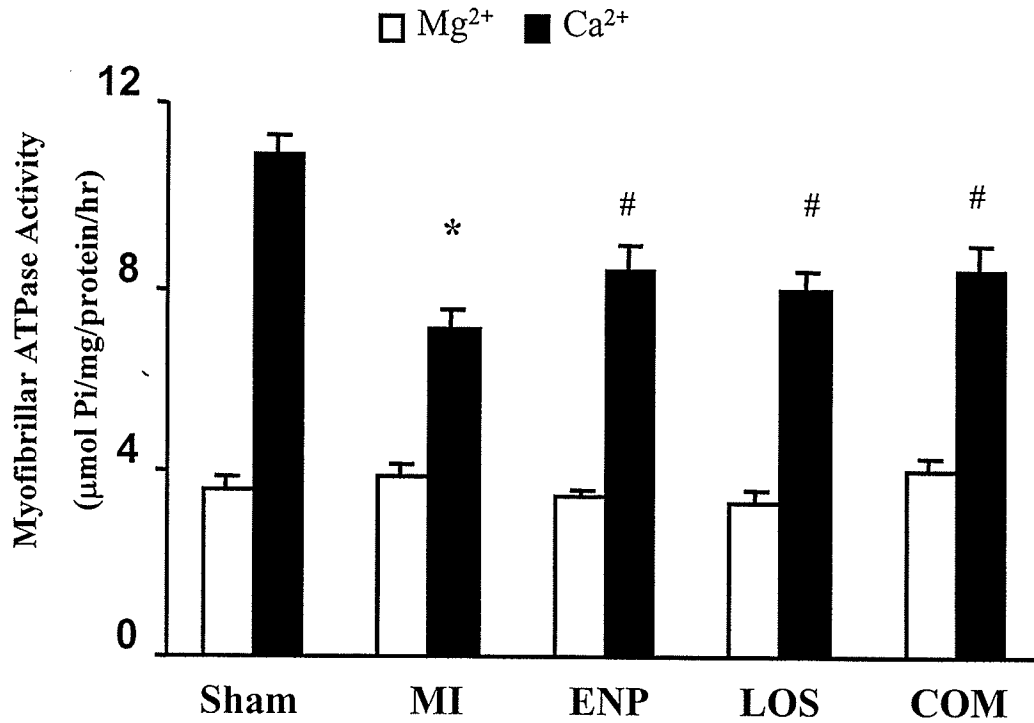
	Sham	MI	ENP	LOS	COM
Heart rate (bpm)	265 ± 9	270 ± 13	272 ± 19	258 ± 15	267 ± 15
LVSP (mm Hg)	122 ± 6.5	118 ± 4.6	115 ± 8.5	116 ± 9.5	114 ± 8.6
LVEDP (mm Hg)	4.2 ± 0.3	18.9 ± 4.6 *	8.9 ± 1.2 #	9.0 ± 0.7 #	8.5 ± 0.6 #
+dP/dt (mm Hg/sec)	11228 ± 850	8409 ± 687 *	9521 ± 459 #	9362 ± 562 #	9533 ± 625 #
-dP/dt (mm Hg/sec)	12518 ± 1050	8524 ± 950 *	10057 ± 1012 #	9895 ± 762 #	9959 ± 721 #
MAP (mm Hg)	102 ± 9	100 ± 10	95 ± 7	101 ± 7	97 ± 5

Values are mean ± SE of 7 animals in each group. ENP: myocardial infarcted treated with enalapril; LOS: myocardial infarcted treated with losartan; COM: myocardial infarcted treated with both enalapril and losartan; LVEDP: left ventricular end diastolic pressure; LVSP: left ventricular systolic pressure; MAP: mean arterial pressure; MI: myocardial infarcted; \* P < 0.05 compared with sham group; # P < 0.05 compared with MI group.

either enalapril or losartan alone (Table 4 and 5). The scar wt in the untreated MI group was not different from the treated groups.

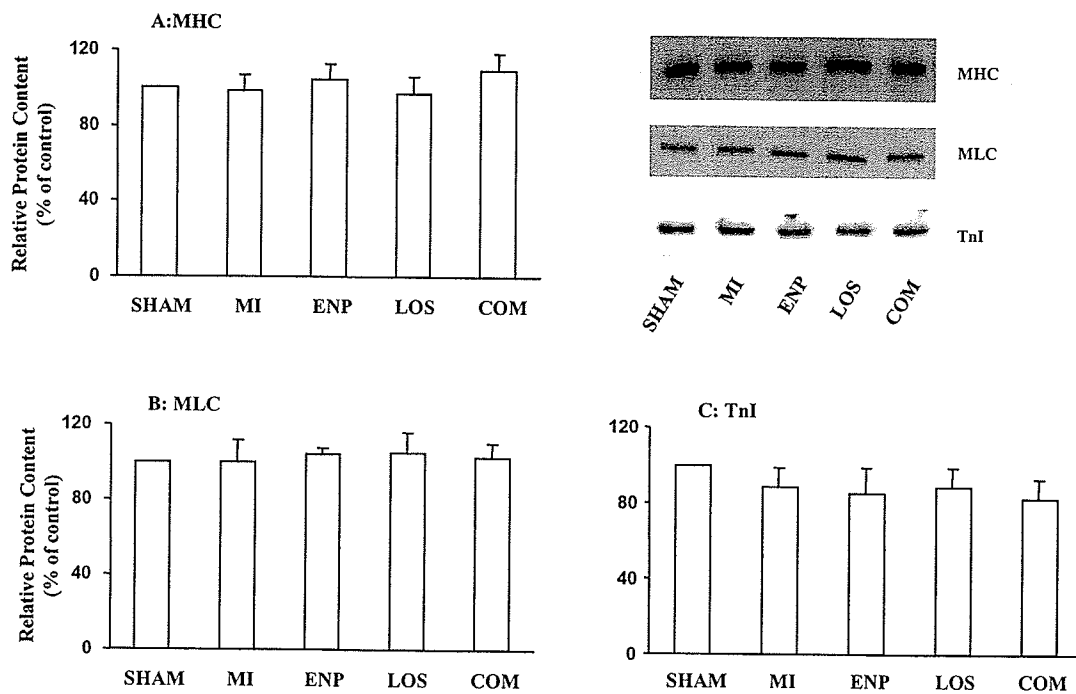
Myofibrils isolated from the viable LV of infarcted hearts exhibited a lower ( $7.1 \pm 0.4$   $\mu\text{mol Pi/mg/hr}$ )  $\text{Ca}^{2+}$ -stimulated ATPase activity ( $P < 0.05$ ) compared with sham-operated rats ( $10.8 \pm 0.4$   $\mu\text{mol Pi/mg/hr}$ ). Blockade of RAS with enalapril and/or losartan partially normalized the activity ( $8.3 \pm 0.65$ ,  $7.9 \pm 0.52$ , and  $8.4 \pm 0.58$   $\mu\text{mol Pi/mg/hr}$ ) in ENP, LOS and COM groups, respectively (Fig. 9). The values for  $\text{Ca}^{2+}$ -stimulated ATPase activity in COM group were not different from that in ENP or LOS groups. Furthermore, no alterations of myofibrillar  $\text{Mg}^{2+}$ -stimulated ATPase activity were found among different groups (Fig. 9).

The relative protein content of MHC was determined from immunoblots using a monoclonal anti-MHC (a mixture of  $\alpha$ -MHC and  $\beta$ -MHC) antibody. No significant alterations in protein contents for MHC, MLC and TnI were observed in rats with MI with or without any drug treatments (Fig. 10). It should be noted that during polyacrylamide gel electrophoresis,  $\beta$ -MHC exhibited a higher electrophoretic mobility than  $\alpha$ -MHC which was the dominant (>90%) isoform in sham group (Fig. 11). In MI group, protein content for  $\alpha$ -MHC was reduced significantly while that for  $\beta$ -MHC was increased markedly (Fig. 11);  $\beta$ -MHC content was increased from 6.5 to 29% of total MHC in the viable tissue of LV whereas  $\alpha$ -MHC content was decreased from 93.5 to 71.0% of total MHC. Blockade of RAS with ENP, or LOS partially prevented the increase in  $\beta$ -MHC as well as the decrease in  $\alpha$ -MHC due to MI. Furthermore, the combination therapy did not show any additive effects (Fig. 11).

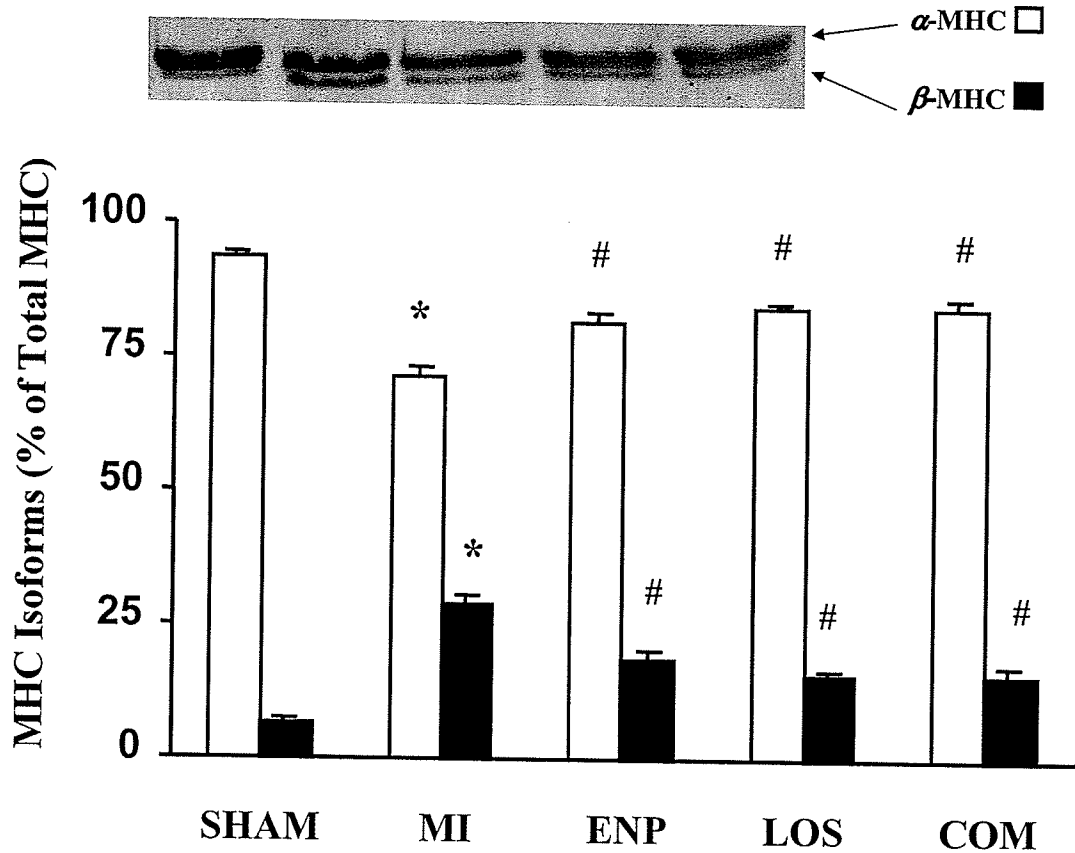


**Figure 9.** Myofibrillar Mg<sup>2+</sup>- and Ca<sup>2+</sup>-stimulated ATPase activities of the left ventricles from Sham, infarcted (MI), enalapril treated infarcted (ENP), losartan treated infarcted (LOS), and combined enalapril and losartan treated infarcted (COM) rats. ENP and/or LOS were given orally for 5 weeks starting at 3 weeks after inducing MI. Values are means  $\pm$  SE of 7 animals for each group. \* P < 0.05 vs Sham; # P < 0.05 vs MI.





**Figure 10.** Typical immunoblots and relative protein contents of myosin heavy chain (MHC) (A), myosin light chain (MLC) (B), and troponin I (TnI) (C) in the left ventricles from Sham, infarcted (MI), enalapril treated infarcted (ENP), losartan treated infarcted (LOS), and combined enalapril and losartan treated infarcted (COM) rats. ENP and/or LOS were given orally for 5 weeks starting at 3 weeks after inducing MI. Values are means  $\pm$  SE of 7 animals for each group. \*  $P < 0.05$  vs Sham; #  $P < 0.05$  vs MI.

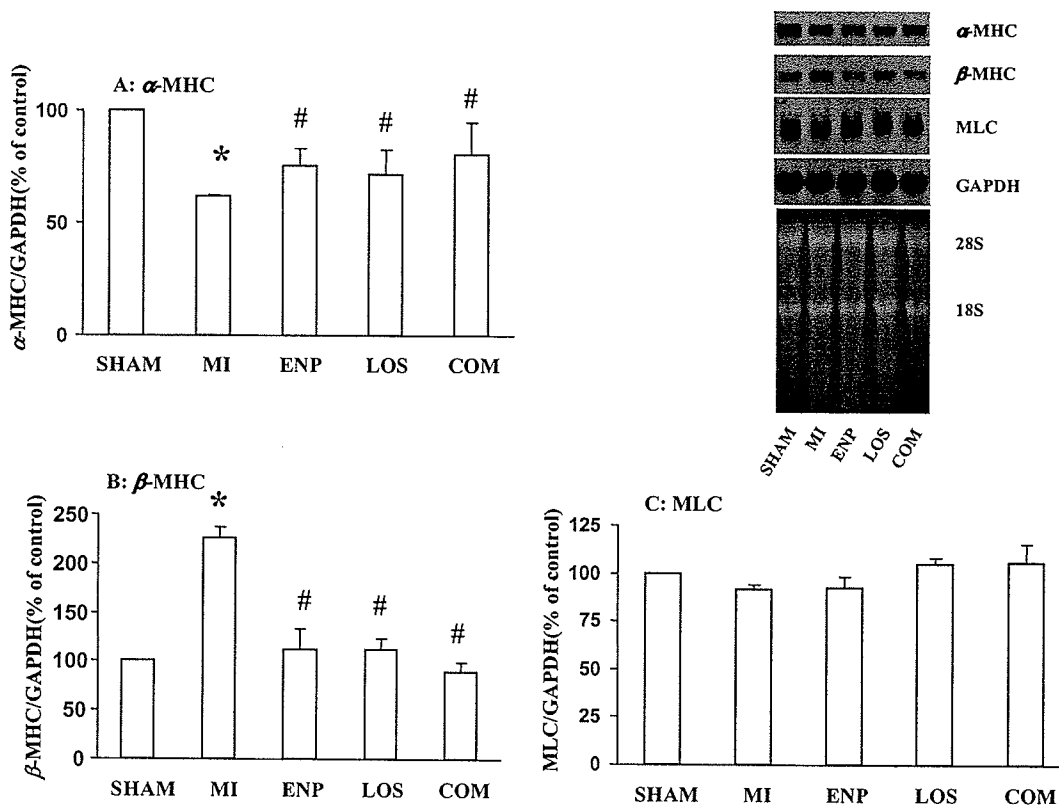


**Figure 11.** Myosin heavy chain  $\alpha$ - and  $\beta$ - isozymes ( $\alpha$ -MHC and  $\beta$ -MHC) in left ventricles from Sham, infarcted (MI), enalapril treated infarcted (ENP), losartan treated infarcted (LOS), and combined enalapril and losartan treated infarcted (COM) rats. The figure shows typical electrophoresis for myosin heavy chain  $\alpha$ - and  $\beta$ - isozymes and results for laser densitometric analysis. % of total MHC is calculated as density of  $\alpha$ - or  $\beta$ -MHC divided by sum of  $\alpha$ - and  $\beta$ -MHC. ENP and/or LOS were given orally for 5 weeks starting at 3 weeks after inducing MI. Values are means  $\pm$  SE of 7 animals for each group. \*  $P < 0.05$  vs Sham; #  $P < 0.05$  vs MI.

The mRNA levels for  $\alpha$ -MHC and  $\beta$ -MHC were determined by Northern blot analysis (Fig. 12). The  $\alpha$ -MHC mRNA level was decreased by 39% and that for  $\beta$ -MHC mRNA was increased by 125% in LV from the infarcted rats. These changes were partially reversed by treatment with ENP, LOS and COM. The mRNA levels for MLC in the sham group were not different from those in the untreated and drug treated MI groups (Fig. 12).

### C. Protein Kinase Isozymes in Heart Failure

As discussed earlier, coronary occlusion in rats resulted in extensive LV infarction and the noninfarcted cardiac muscle underwent significant hypertrophy at 1, 2, 4 and 8 weeks after the operation. These changes are reflected by the presence of large scar (average infarct size varied from 38 to 42% of the LV free wall corresponding to the average values in the range of 19 ~ 21% for scar wt-to-total LV wt ratio in different groups) and increased ratio of heart wt-to-body wt ratio at all time points (Table 6). These values for infarct size in animals used in this study are comparable to those reported by others<sup>377</sup>. The RV wt and viable LV wt were increased at 2, 4 and 8 weeks after MI was induced compared with the respective sham control animals. There was a significant increase in the wet wt-to-dry wt ratio of the lungs in 4- and 8-weeks infarcted animals, indicating the presence of pulmonary edema (Table 6). An increase in LVEDP and a decrease in both  $+dP/dt_{\max}$  and  $-dP/dt_{\max}$  were observed in 1, 2, 4 and 8 weeks myocardial infarcted animals (Table 6). These results are consistent with earlier observations in this experimental model<sup>39,82,349</sup>, which have indicated that the experimental animals at 4 and 8 weeks after the coronary occlusion are at early and moderate stages of CHF,



**Figure 12.** Typical Northern blots showing mRNA abundance for myosin heavy chain  $\alpha$ -isozyme (A), myosin heavy chain  $\beta$ -isozyme (B), and myosin light chain (C) in left ventricles from Sham, infarcted (MI), enalapril treated infarcted (ENP), losartan treated infarcted (LOS), and combined enalapril and losartan treated infarcted (COM) rats.  $\alpha$ -MHC: mRNA blot for myosin heavy chain  $\alpha$ -isozyme;  $\beta$ -MHC: mRNA blot for myosin heavy chain  $\beta$ -isozyme; MLC: mRNA blot for myosin light chain; GAPDH: mRNA blot for glyceraldehydes-3-phosphate dehydrogenase; 28S/18S: the quality of mRNA preparation is evident from the ethidium bromide staining of the 28S and 18S ribosomal RNA. Values are means  $\pm$  SE of 7 animals for each group. \*  $P < 0.05$  vs Sham; #  $P < 0.05$  vs MI.

**Table 6. General and hemodynamic characteristics and cardiac homogenate PKC activity of infarcted rats 1, 2, 4 and 8 wk after left coronary artery ligation**

	1 week		2 weeks		4 weeks		8 weeks	
	Sham	MI	Sham	MI	Sham	MI	Sham	MI
<b>Body wt (g)</b>	279 ± 8.5	273 ± 15.6	297 ± 8.5	298 ± 13.7	378 ± 13.9	393 ± 12.2	491 ± 7.5	482 ± 12.7
<b>RV (mg)</b>	157 ± 13.7	181 ± 10.1	163 ± 5.2	216 ± 17.2*	172 ± 12.6	306 ± 33.1*	278 ± 17.1	600 ± 19.8*
<b>Viable LV (mg)</b>	672 ± 21.1	669 ± 25	660 ± 25.3	705 ± 11.6*	771 ± 37.8	867 ± 24.7*	861 ± 27.3	942 ± 39*
<b>Scar wt (mg)</b>	ND	156 ± 13.4	ND	184 ± 14.2	ND	201 ± 11.2	ND	237 ± 21.4
<b>Heart wt/Body wt ratio (mg/g)</b>	2.92 ± 0.06	3.67 ± 0.13*	2.79 ± 0.12	3.71 ± 0.1*	2.49 ± 0.07	3.5 ± 0.17*	2.33 ± 0.12	3.7 ± 0.1*
<b>Lung wet/dry wt ratio</b>	5.23 ± 0.07	5.36 ± 0.15	5.28 ± 0.19	5.17 ± 0.05	5.01 ± 0.08	5.47 ± 0.06*	4.58 ± 0.25	5.32 ± 0.15*
<b>+dP/dt (mm Hg/sec)</b>	9438 ± 341	6227 ± 436*	9922 ± 576	6882 ± 447*	9102 ± 579	6910 ± 336*	8763 ± 412	5468 ± 389*
<b>-dP/dt (mm Hg/sec)</b>	10259 ± 527	6639 ± 303*	9908 ± 575	7361 ± 638*	10065 ± 562	7182 ± 344*	8906 ± 501	6574 ± 409*
<b>LVEDP (mm Hg)</b>	8.4 ± 0.9	18 ± 1.2*	9.8 ± 2.1	19.8 ± 2.6*	8.6 ± 0.8	17 ± 2.3*	8.1 ± 0.9	19 ± 2*
<b>Ca<sup>2+</sup>-dependent PKC activity (pmol/min/mg)</b>								
<b>LV</b>	117 ± 15.4	179 ± 6*	104 ± 9	187 ± 5.6*	87.4 ± 4	128 ± 12.4*	110 ± 7.5	285 ± 18.3*
<b>RV</b>	99 ± 6.3	152 ± 9.3*	126 ± 4.7	156 ± 15.3*	98.7 ± 6.8	132 ± 13.4*	93.5 ± 6.4	184 ± 14*
<b>Ca<sup>2+</sup>-independent PKC activity (pmol/min/mg)</b>								
<b>LV</b>	82.9 ± 10.7	122 ± 8.5*	84.4 ± 9.5	159 ± 15.1*	66.7 ± 4.9	86.7 ± 6.7*	145 ± 10.2	437 ± 30.2*
<b>RV</b>	93.1 ± 4.1	137 ± 4.4*	118 ± 4.9	146 ± 8.9*	106 ± 2.6	137 ± 4.7*	70.5 ± 5.2	238 ± 18.5*

These observations are means ± SE of 6 animals for each group. MI: myocardial infarction; RV: right ventricle; LV: left ventricle; LVEDP: left ventricular end-diastolic pressure; +dP/dt: rate of pressure development; -dP/dt: rate of pressure decay; ND: not detectable; PKC: protein kinase C; \*: Significantly different from Sham-control group (P < 0.05). Left ventricular pressure was not altered in the failing heart.

respectively. The  $\text{Ca}^{2+}$ -dependent PKC activity was increased by 53, 79, 47 and 159% in the LV homogenate, whereas the  $\text{Ca}^{2+}$ -independent PKC activity was elevated by 47, 88, 30 and 201% in the LV homogenate at 1, 2, 4 and 8 weeks of MI compared with control values, respectively (Table 6). On the other hand, the  $\text{Ca}^{2+}$ -dependent PKC activity in the RV homogenate was increased by 54, 24, 34 and 97% and the  $\text{Ca}^{2+}$ -independent PKC activity was augmented by 47, 24, 29 and 238% of control values at 1, 2, 4 and 8 weeks of MI, respectively.

The beneficial effects of imidapril treatment on heart function and cardiac PKC activities were tested by treating the 4-week experimental animals with imidapril for 4 weeks. The results in Table 7 indicate cardiac hypertrophy, lung congestion, elevated LVEDP, depressed  $+dP/dt$  and  $-dP/dt$  as well as increased  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$ -independent PKC activities in both LV and RV homogenates in the 8-weeks infarcted animals. All of these changes were partially normalized by treatment with imidapril. Treatment of sham control animals with imidapril did not show any significant effect on any of these parameters (Table 7). The values for LVSP and MAP in the untreated and treated groups were not different from each other. Furthermore, the average scar wt-to-total LV wt ratio in the untreated and treated animals were  $20.3 \pm 0.08$  and  $22.8 \pm 1.1$ ; these values correspond to infarct size of ~41 and 46% of the free LV wall area and were not different ( $P > 0.05$ ) from each other.

To determine the contribution of the cytosolic and particulate fractions to the increased homogenate PKC activity in infarcted rat hearts, PKC activities from cytosolic and particulate fractions were determined in the failing LV and RV from sham, imidapril-treated sham, untreated-infarcted and imidapril-treated infarcted rat hearts 8

**Table 7. General and hemodynamic characteristics and homogenate PKC activity of MI rats with or without imidapril treatment for 4 weeks starting at 4 weeks after coronary occlusion.**

	Sham	Sham + IMP	MI	MI + IMP
Body wt (g)	495 ± 8.6	486 ± 8.6	487 ± 18	480 ± 15
RV (mg)	274 ± 16.6	270 ± 20	605 ± 29.3*	402 ± 28.2 <sup>#</sup>
Viable LV (mg)	858 ± 32.1	871 ± 28.5	946 ± 41.2*	834 ± 38.4 <sup>#</sup>
Scar wt (mg)	ND	ND	241 ± 25	247 ± 18
Heart wt/Body wt (mg/g)	2.31 ± 0.15	2.43 ± 0.09	3.68 ± 0.07*	3.15 ± 0.11 <sup>#</sup>
Lung wet/dry	4.69 ± 0.24	4.47 ± 0.19	5.37 ± 0.21*	4.86 ± 0.27 <sup>#</sup>
+dP/dt (mm Hg/sec)	8019 ± 554	7125 ± 461	5321 ± 470*	6435 ± 594 <sup>#</sup>
-dP/dt (mm Hg/sec)	8319 ± 622	8054 ± 467	6296 ± 302*	7810 ± 569 <sup>#</sup>
LVEDP (mm Hg)	7.9 ± 0.6	8.2 ± 0.7	18.2 ± 1.1*	10.3 ± 0.9 <sup>#</sup>
LVSP (mm Hg)	121 ± 5.4	114 ± 8.1	113 ± 9.7	107 ± 3.6
MAP (mm Hg)	104 ± 7	95 ± 8	101 ± 11	97 ± 8
<b>Ca<sup>2+</sup>-dependent PKC activity (pmol/min/mg)</b>				
LV	102 ± 8.1	101 ± 4.4	295 ± 20.3*	220 ± 18.4 <sup>#</sup>
RV	91.6 ± 5.6	89.8 ± 6.2	177 ± 10.6*	129 ± 9.8 <sup>#</sup>
<b>Ca<sup>2+</sup>-independent PKC activity (pmol/min/mg)</b>				
LV	139 ± 9.4	148 ± 12.3	442 ± 29.6*	305 ± 28.9 <sup>#</sup>
RV	65.3 ± 5.7	65.8 ± 6.5	246 ± 16.9*	117 ± 5.1 <sup>#</sup>

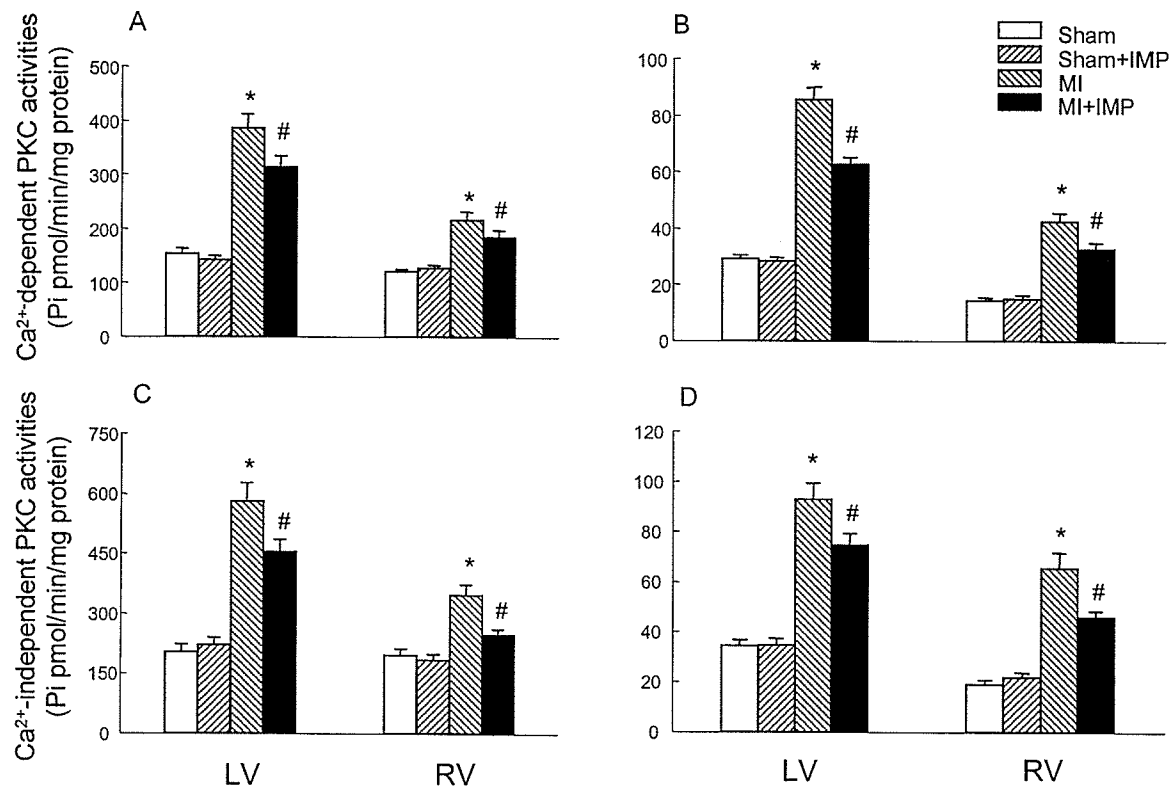
Values are means ± SE of 6 animals for each group. Sham + IMP: imidapril treated Sham; MI: myocardial infarcted; MI + IMP: imidapril treated myocardial infarcted; RV: right ventricle; LV: left ventricle; +dP/dt: rate of pressure development; -dP/dt: rate of pressure decay; LVEDP: left ventricular end diastolic pressure; LVSP: left ventricular systolic pressure; MAP: mean arterial pressure; ND: not detectable; \* Significantly different from sham-control group (P < 0.05); <sup>#</sup> P < 0.05 compared with MI group.

weeks after the operation. As shown in Fig. 13, Panels *A* and *B*, the  $\text{Ca}^{2+}$ -dependent PKC activities were increased by 150% and 80% in cytosolic fraction and 192% and 190% in particulate fraction from LV and RV of the infarcted animals compared with sham controls, respectively. Likewise, the  $\text{Ca}^{2+}$ -independent PKC activities were increased by 186% and 80% in cytosolic fraction and 170% and 241% in particulate fraction of LV and RV from the infarcted animals 8 weeks after coronary ligation compared with the values of sham controls (Fig. 13, Panels *C* and *D*). The increase in both LV and RV cytosolic and particulate fractions due to MI were significantly attenuated by imidapril treatment (Fig. 13). There was no significant difference between sham and imidapril-treated sham animals with respect to  $\text{Ca}^{2+}$ -dependent PKC and  $\text{Ca}^{2+}$ -independent PKC activities in LV and RV cytosolic and particulate fractions.

To examine if the observed changes in PKC activities in the homogenate, particulate, and cytosolic fractions from the untreated and treated infarcted animals are due to alterations in the protein concentrations of these fractions, the protein yields in different fractions were determined. The results in Table 8 indicate no difference in the LV homogenate, particulate, or cytosolic fractions obtained from the untreated and imidapril-treated animals.

The relative protein contents of PKC- $\alpha$ , - $\beta$ , - $\epsilon$  and - $\zeta$  isozymes in homogenate, cytosolic, and particulate fractions of the LV and RV from 8-week sham, imidapril-treated sham, infarcted and imidapril-treated infarcted animals were determined by Western blot analysis. The typical bands representing PKC- $\alpha$ , - $\beta$ , - $\epsilon$  and - $\zeta$  isozymes in these fractions of rat hearts are shown in Figs. 14-16. Polyclonal antibodies to PKC isozymes detected proteins at 76 kDa for  $\alpha$ -isozyme, 77 kDa for  $\beta$ -isozyme, 83 kDa for



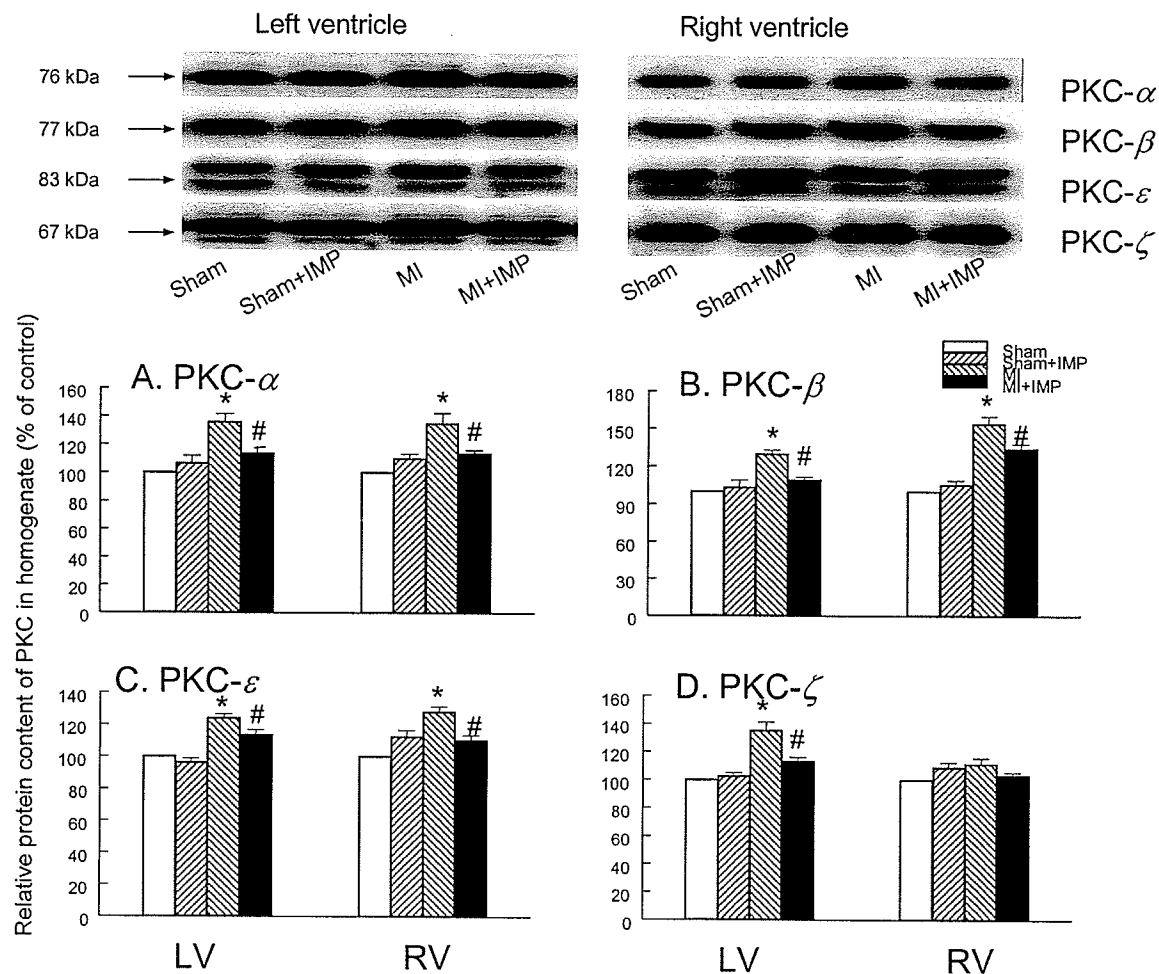


**Figure 13.** Protein Kinase C (PKC) activities in cytosolic and particulate fractions in left (LV) and right (RV) ventricles from sham-control (Sham), imidapril treated sham (Sham + IMP), myocardial infarcted (MI) and imidapril treated myocardial infarcted (MI + IMP) rats 8 weeks after operation. **A:**  $\text{Ca}^{2+}$ -dependent PKC activity in cytosolic fraction; **B:**  $\text{Ca}^{2+}$ -dependent PKC activity in particulate fraction; **C:**  $\text{Ca}^{2+}$ -independent PKC activity in cytosolic fraction; **D:**  $\text{Ca}^{2+}$ -independent PKC activity in particulate fraction. Values are means + SE of 6 experiments in each group. \*  $P < 0.05$ , significantly different from sham-control; #  $P < 0.05$ , significantly different from MI.

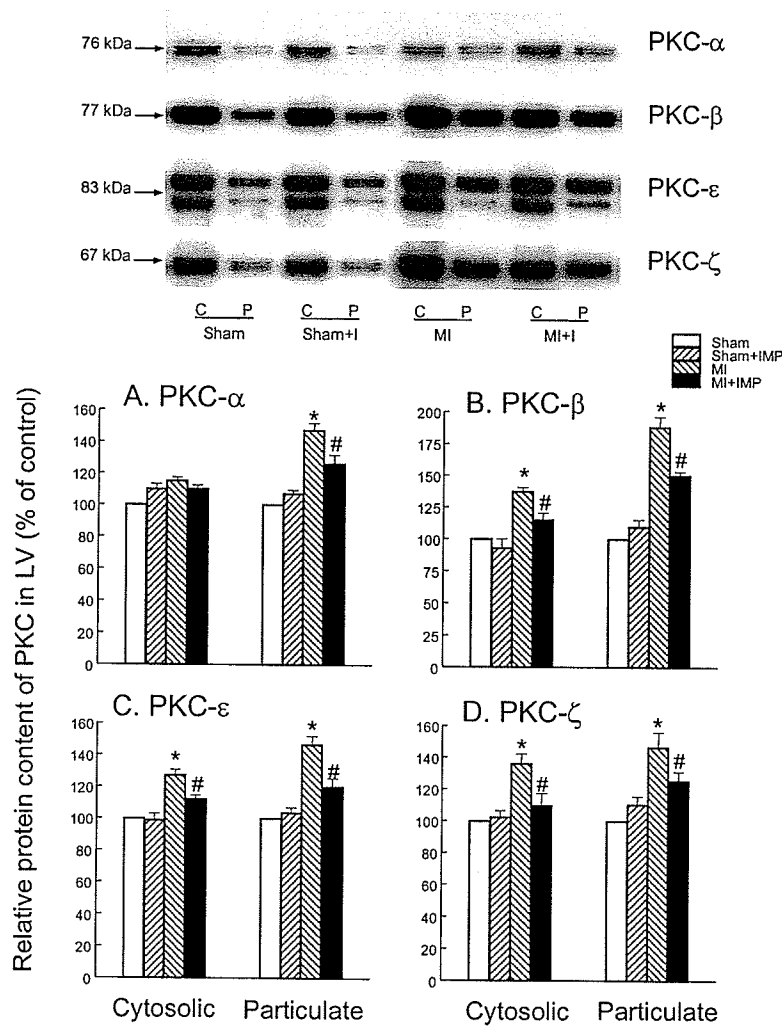
**Table 8. Protein concentration per unit of heart tissue in homogenate, cytosolic and particulate fractions isolated from myocardial infarcted rats with or without imidapril treatment for 4 weeks starting at 4 weeks after coronary occlusion**

	<b>Sham</b>	<b>Sham + IMP</b>	<b>MI</b>	<b>MI + IMP</b>
<b>Homogenate</b>	52.21 ± 4.36	53.08 ± 2.18	52.72 ± 3.82	51.89 ± 4.17
<b>Cytosolic</b>	26.13 ± 1.32	25.56 ± 2.03	25.04 ± 2.71	24.87 ± 1.85
<b>Particulate</b>	19.76 ± 1.65	19.48 ± 1.40	19.07 ± 0.98	19.61 ± 2.07

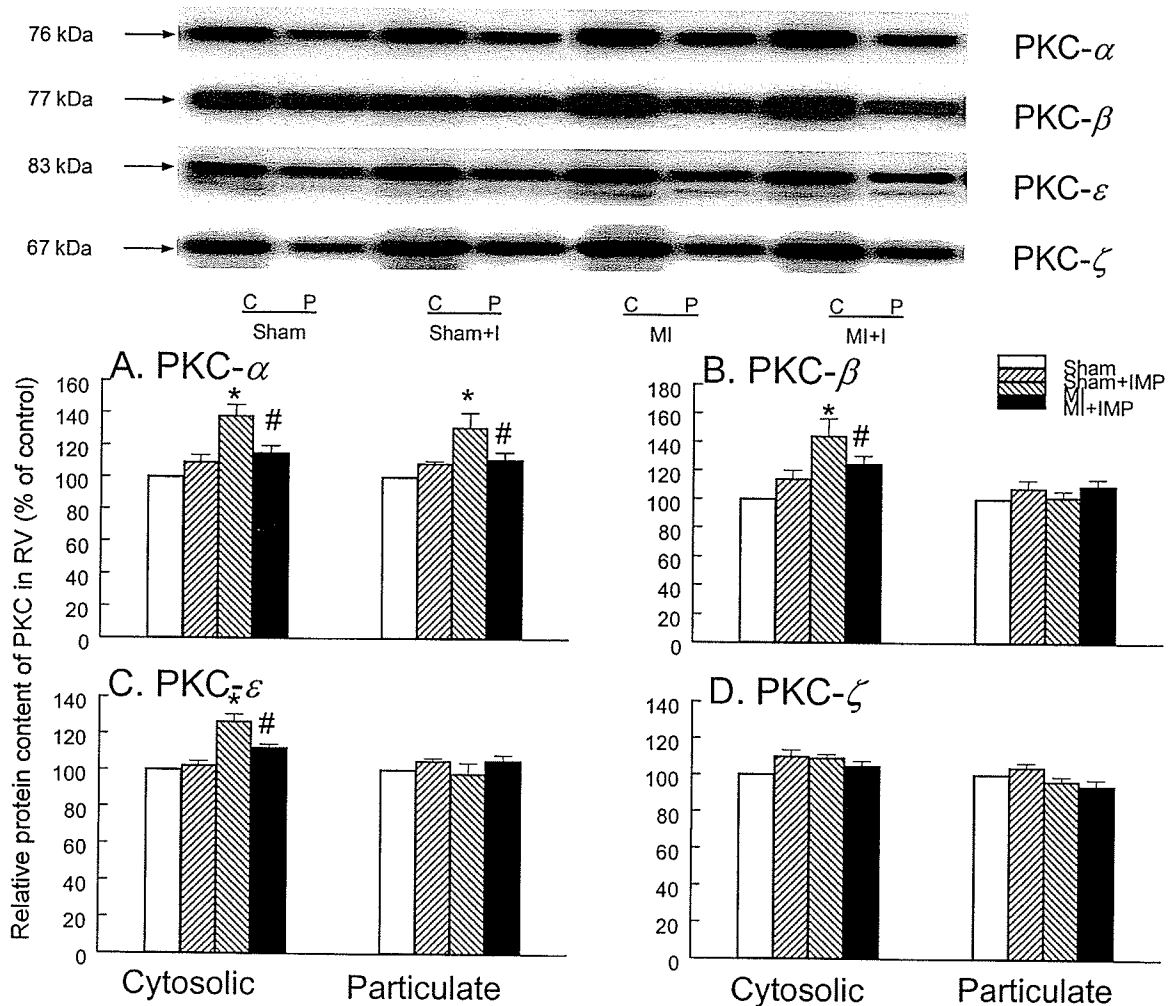
Values are means ± SE; n= 6 animals for each group. Sham + IMP: imidapril treated Sham; MI: myocardial infarcted; MI + IMP: imidapril treated myocardial infarcted.



**Figure 14.** Typical immunoblots (top) and analysis of results (A-D) for the relative protein contents of cardiac PKC isoforms ( $\alpha$ ,  $\beta$ ,  $\varepsilon$  and  $\zeta$ ) in homogenate fraction in left ventricle (LV) and right ventricle (RV) from sham-control (Sham), imidapril-treated sham (Sham + IMP), myocardial infarcted (MI) and imidapril treated myocardial infarcted (MI + IMP) rats at 8 weeks after surgery. Values are means  $\pm$  SE of 6 experiments in each group. \*  $P < 0.05$ , significantly different from sham-control; #  $P < 0.05$ , significantly different from myocardial infarcted.



**Figure 15.** Typical immunoblots (top) and analysis of results (A-D) for the relative protein contents of cardiac PKC isoforms ( $\alpha$ ,  $\beta$ ,  $\epsilon$  and  $\zeta$ ) in cytosolic and particulate fractions in left ventricle from sham-control (Sham), imidapril treated sham (Sham + IMP), myocardial infarcted (MI) and imidapril-treated myocardial infarcted (MI + IMP) rats 8 weeks after surgery. Values are means  $\pm$  SE of 6 experiments in each group. \*  $P < 0.05$ , significantly different from sham-control; #  $P < 0.05$ , significantly different from myocardial infarcted.



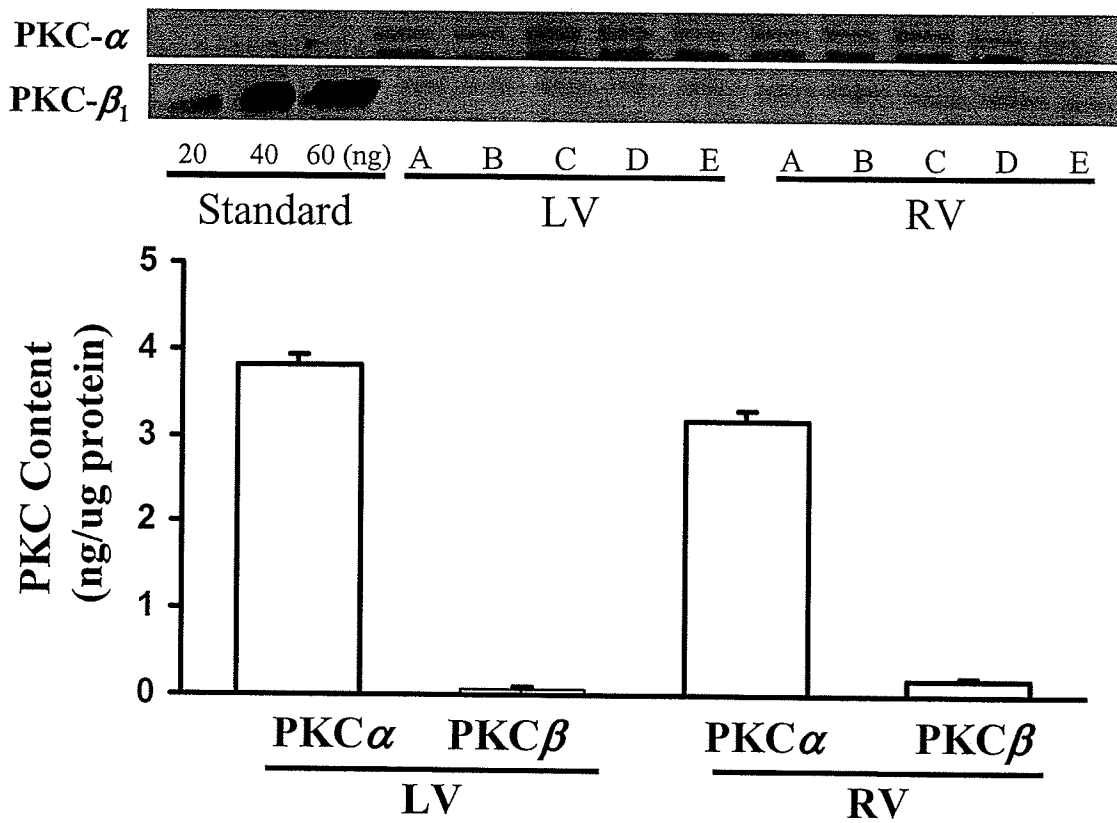
**Figure 16.** Typical immunoblots (top) and analysis of results (A-D) for the relative protein contents of cardiac PKC isoforms ( $\alpha$ ,  $\beta$ ,  $\epsilon$  and  $\zeta$ ) in cytosolic and particulate fractions in right ventricle from sham-control (Sham), imidapril treated sham (Sham + IMP), myocardial infarcted (MI) and imidapril-treated myocardial infarcted (MI + IMP) rats 8 weeks after operation. Values are means  $\pm$  SE of 6 experiments in each group. \*  $P < 0.05$ , significantly different from sham-control; #  $P < 0.05$ , significantly different from myocardial infarcted.

$\varepsilon$ -isozyme and 67 kDa for  $\zeta$ -isozymes. There was a nonspecific band at ~80 kDa below the bands for  $\varepsilon$  isozyme in Figs. 14-16; however, because its identity is unknown, this band was not included in the densitometric analysis. In LV homogenate, the relative protein contents of PKC- $\alpha$ , - $\beta$ , - $\varepsilon$  and - $\zeta$  isozymes were significantly elevated, whereas in RV homogenate, only PKC- $\alpha$ , - $\beta$ , and - $\varepsilon$  isozymes were increased in the infarcted animals (Fig. 14). The relative protein contents for PKC- $\alpha$ , - $\beta$ , - $\varepsilon$  and - $\zeta$  isozymes were also increased in LV cytosolic and particulate fractions in the infarcted animals except PKC- $\alpha$  isozyme content in the cytosolic fraction was unaltered (Fig. 15). On the other hand, PKC- $\alpha$ , - $\beta$  and - $\varepsilon$  isozymes, unlike PKC- $\zeta$  isozyme, the contents were increased in the RV cytosolic and particulate fractions from infarcted animals, except that no changes were seen in the RV particulate fraction (Fig. 16). The MI-induced increases in PKC isozymes in both LV and RV cytosolic and particulate fractions were attenuated by imidapril treatment, which showed no effect on the sham-control animals (Figs. 14-16).

Although from the Western blots (Figs. 14-16) it appears that PKC- $\beta$  expression is equal to or slightly greater than the expression of PKC- $\alpha$  in the LV and RV fractions, these data should be interpreted with a great deal of caution. In this regard, it is pointed out that the relative protein content for each of the PKC isozymes was determined in the untreated sham, sham + imidapril-treated, untreated infarcted, and infarcted + imidapril-treated animals under identical conditions where the densitometric intensity value for each isozyme band in the untreated sham control was taken as 100%. Thus the relative protein content for one isozyme should not be compared with that of another in any of the fractions from different groups. To better evaluate the significance of changes in PKC isoforms in the experimental group, we have measured the absolute levels of

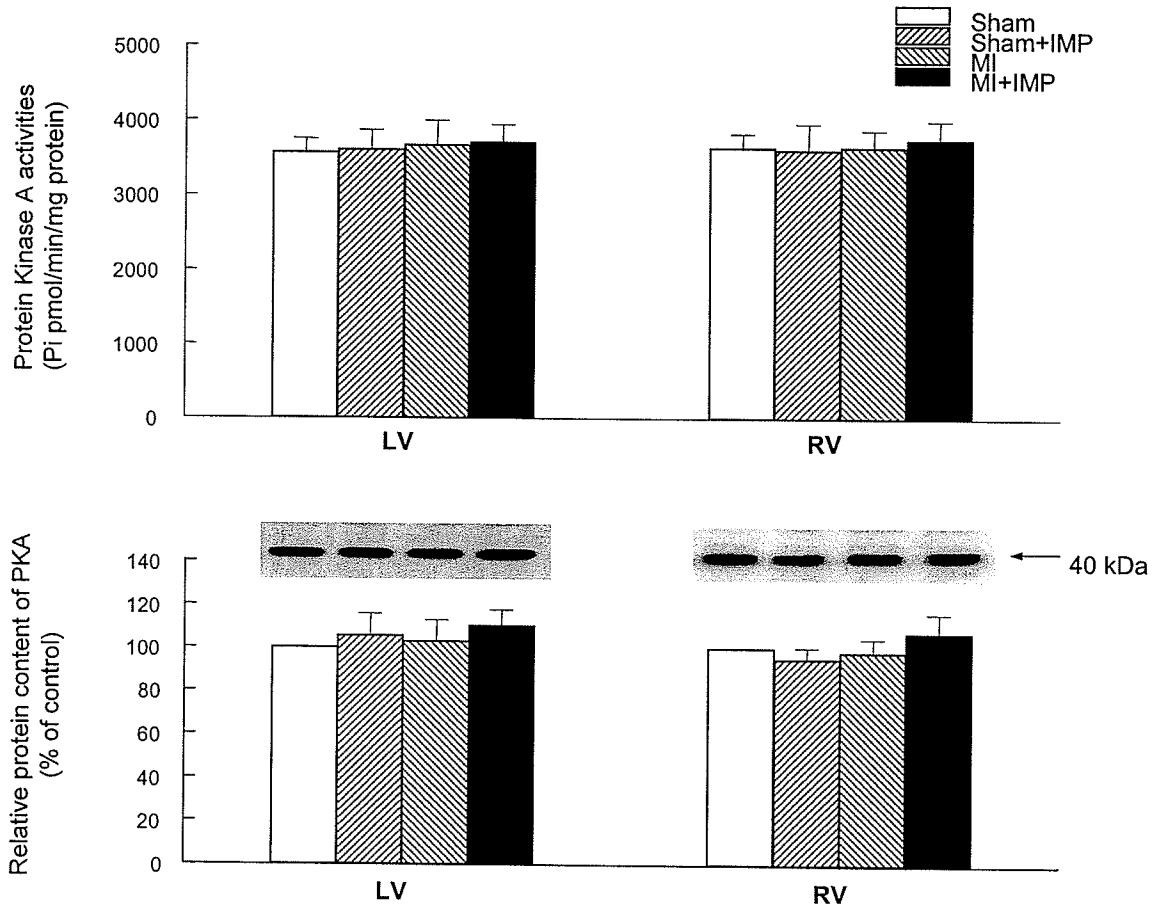
PKC- $\alpha$  and PKC- $\beta$  isoforms in the control myocardium. The results shown in Fig. 17 indicate that PKC- $\alpha$  content is ~50 times of PKC- $\beta$  in the LV and ~17 times in the RV. In view of the relatively low value for PKC- $\beta$  content in the normal heart, the observed increase in the relative protein content for PKC- $\beta$  isoform in the failing heart as well as its reduction by imidapril treatment are significant findings.

The activity and relative protein content of PKA were examined in homogenate from the LV and RV of sham, imidapril-treated sham, untreated infarcted, and imidapril-treated infarcted animals. The results showed that there were no significant changes in LV and RV PKA activity and protein content in the infarcted animals (Fig. 18). Imidapril treatment did not affect PKA activity and protein content in the sham or infarcted animals compared with values from untreated animals.



**Figure 17.** Immunoblots of standard and different samples (*A-E*) and analysis of results for the absolute levels of PKC isoforms ( $\alpha$  and  $\beta$ ) in the left ventricle (LV) and right ventricle (RV) of control rats.





**Figure 18.** Cardiac PKA activities and relative protein content in left ventricle (LV) and right ventricle (RV) from sham-control (Sham), imidapril-treated sham (Sham + IMP), myocardial infarcted (MI) and imidapril treated myocardial infarcted (MI + IMP) rats 8 weeks after operation. Values are means  $\pm$  SE of 6 experiments. Bottom, typical immunoblots for PKA protein are also shown. No change was found in these groups.

## V. DISCUSSION

### A. Modification of Myofibrillar ATPase Activity and Myosin

#### Isozymes in Failing Heart by Imidapril

In this study we have shown that levels of  $\alpha$ -MHC protein were decreased whereas those of  $\beta$ -MHC were increased in both LV and RV from the 7 week infarcted animals. Such changes in the  $\alpha$ -MHC and  $\beta$ -MHC contents may not be due to proteolysis or some other degradative process because the total MHC content in both LV and RV were unaltered in the 7 week infarcted animals. Since mRNA levels for  $\alpha$ -MHC were decreased and those for  $\beta$ -MHC were increased in both LV and RV from the 7 week infarcted animals, it appears that the observed changes in both  $\alpha$ -MHC and  $\beta$ -MHC contents are due to alterations of their genes expression in the myocardium. Similar changes in mRNA levels for  $\alpha$ -MHC and  $\beta$ -MHC proteins in the 4 weeks infarcted rats have been reported by other investigators<sup>343-345</sup>. Furthermore, we have observed that myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity in the LV, unlike that in the RV, was decreased in the 7 week infarcted animals. Regional variations in rat cardiac myosin isozymes and ATPase activities due to MI have also been reported by Geenen *et al*<sup>340,341</sup>. In view of the functional significance of both  $\alpha$ -MHC and  $\beta$ -MHC in terms of ATPase activities<sup>159,332-335</sup>, a shift from  $\alpha$ -MHC to  $\beta$ -MHC protein content may explain the depressed myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity in LV of the 7 week infarcted animals. However, such an explanation for alterations in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activities on the basis of a shift in the  $\alpha$ -MHC and  $\beta$ -MHC proteins may not hold good for the RV because no changes in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity

were observed in the RV from 7 week infarcted animals. In view of the finding that MLC kinase-mediated phosphorylation, which has been shown to increase the actomyosin ATPase activity<sup>378</sup>, was decreased and increased in the LV and RV from 7 weeks infarcted rats, respectively<sup>379</sup>, the observed differences between the LV and RV myofibrillar Ca<sup>2+</sup>-stimulated ATPase in the 7 week infarcted animals may partly be due to differences in the MLC phosphorylation. Other mechanisms<sup>130,158</sup> such as the interaction of actin with myosin as well as the interaction of Ca<sup>2+</sup> with troponin underlying the difference between LV and RV myofibrillar Ca<sup>2+</sup> ATPase activities in the infarcted animals remain to be explored.

The results described in this study indicate that myofibrillar Ca<sup>2+</sup>-stimulated ATPase activity in the LV, unlike that in the RV except at 16 weeks, from the infarcted animals was lower than that in the LV from the sham control animals. Such changes in the LV may be of some specific nature because neither the myofibrillar Mg<sup>2+</sup> ATPase activity nor the total MHC content in the experimental LV and RV were different from their respective sham control values. These differences between the LV and RV from infarcted animals with respect to contractile proteins and Ca<sup>2+</sup>-stimulated ATPase activity may be due to differences in the behaviour of these ventricles during the development of congestive heart failure following occlusion of the coronary artery. This view is supported by previous studies on adenylyl cyclase<sup>349</sup>, G-protein content<sup>380</sup>, SR Ca<sup>2+</sup>-transport<sup>39</sup>, MLC phosphorylation<sup>379</sup> as well as antioxidant responses and content<sup>381,382</sup>, have revealed differences between LV and RV following MI in rats. The biochemical differences between LV and RV in the MI model may also be due to the fact that the LV is subjected to changes in wall stress with an alteration in geometry whereas the RV is experiencing pressure overload. Depressed myofibrillar Ca<sup>2+</sup>-stimulated ATPase activity

in the RV from the 16 weeks infarcted animals appears to suggest that contractile dysfunction in the RV may become evident at late stages of congestive heart failure in this MI rat model. Nonetheless, differences between the LV and RV cannot be explained on the basis of changes in the circulating levels of hormones and other factors but instead may be due to differences in the basal regulatory mechanisms present in these ventricles. This suggestion is supported by the fact that the characteristics of the adrenergic nerves present in the LV and RV from the infarcted animals were found to be dramatically different from each other<sup>383</sup>.

Since the magnitude of cardiac contractile force is linearly related to myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity<sup>158</sup>, the observed decrease in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity in the LV from the infarcted animals would result in depression of cardiac function. A progressive decrease in LV myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity at 4, 8 and 16 weeks of inducing MI was seen in this experimental model exhibiting early, moderate and severe stages of heart failure<sup>39,82,349</sup>. Furthermore, treatment of infarcted animals with an ACE inhibitor, imidapril, was found to improve heart function partially and attenuate the depression in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity in the LV. Alterations in  $\alpha$ -MHC and  $\beta$ -MHC protein content and mRNA levels in both LV and RV from the infarcted animals were also corrected partially upon treatment with imidapril. Attenuation of MI-induced changes in heart function, cardiac hypertrophy, different clinical signs of heart failure and phospholipase C activity by imidapril has also been observed previously<sup>351</sup>. Treatment of infarcted rats with an ACE inhibitor, perindopril, was reported to partially prevent changes in the MHC isozyme composition in the non-infarcted hypertrophied LV<sup>384</sup>. Since other ACE inhibitors such as captopril, enalapril and trandolapril were shown to produce beneficial effects on

cardiac performance in this experimental model of heart failure<sup>350,352,356</sup>, it is likely that the observed effects of imidapril are due to ACE inhibition *per se*. However, the role of bradykinin, which is known to be accumulated in the cell upon treatment with ACE inhibitors and produce beneficial effects in the heart<sup>385,386</sup>, cannot be ruled out with respect to the action of imidapril. Nonetheless, treatment of infarcted animals with angiotensin II receptor antagonists, candesartan, losartan, has also been shown to partially prevent changes in heart function due to MI<sup>345,347,358,387</sup>. Thus it is likely that the beneficial effects of imidapril in attenuating MI-induced changes in heart function as well as contractile protein content and gene expression are partly due to the blockade of renin-angiotensin system in congestive heart failure due to MI. The partial attenuation of MI-induced changes by imidapril is in agreement with the view that other mechanisms such as the activation of sympathetic nervous system and elevated levels of several hormones in addition to angiotensin II play an important role in the development of cardiac dysfunction in this experimental model of congestive heart failure<sup>14,388,389</sup>.

In this study, we have shown changes in myofibrillar  $\text{Ca}^{2+}$  ATPase, MHC isoforms and cardiac function upon inducing MI in rats and have observed attenuation of these biochemical alterations and improvement of cardiac function upon treatment with imidapril. We have attempted to explain the impairment as well as improvement of cardiac function on the basis of corresponding changes in myofibrillar  $\text{Ca}^{2+}$  ATPase activities; however, it is possible that the biochemical alterations as seen in this study are the consequence of changes in heart function. In this regard, improved function of LV by decreasing the LV afterload as a consequence of altered remodeling and that of RV by decreasing passive pulmonary hypertension as a consequence of decreased LVEDP by ACE inhibition can be seen to produce beneficial effects on both LV and RV myosin

isozyme expression upon treating the MI animals with imidapril. Nonetheless, the observed alterations in MHC isozyme proteins and mRNA levels as well as myofibrillar  $\text{Ca}^{2+}$  ATPase activity in both untreated and imidapril-treated hearts suggest that there occurs a dramatic defect in the contractile machinery of cardiomyocytes in heart failure due to MI. However, measurement of mechanical activity of cardiomyocytes from rats with MI has revealed controversial data as some investigators<sup>390,391</sup> have observed contractile abnormalities whereas others<sup>392,393</sup> have failed to do so. Such conflicting results from different laboratories may be due to differences in the techniques employed for the isolation of cardiomyocytes, infarct size, duration of MI or the stage of congestive heart failure in the experimental animals. It may be noted that no changes in LV myofibrillar  $\text{Ca}^{2+}$  ATPase activity were apparent in 1 or 2 week infarcted animals whereas significant depression in RV myofibrillar  $\text{Ca}^{2+}$  ATPase became apparent only at 16 weeks of inducing MI. Although our observations, particularly with respect to changes in myosin isozyme shift, in a rat model of heart failure should be extrapolated with some caution, similar changes in MHC gene expression have been shown to occur in patients with heart failure<sup>394,395</sup>.

## **B. Effects of Enalapril or Losartan on Myofibrillar ATPase and Myosin Isozymes in Failing Heart**

As described earlier, we have observed depressions in both  $+dP/dt$  and  $-dP/dt$  whereas LVEDP was markedly increased in the infarcted animals in this series of experiments. In addition, a decrease in myofibrillar ATPase activity, an increase in MHC- $\beta$  protein and gene expression, as well as a decrease in MHC- $\alpha$  protein and gene

expression were also evident in hearts following MI. These hearts were hypertrophied because the heart wt/body wt ratio was increased and the animals showed signs of CHF because the lung wet wt/dry wt ratio was increased. These results, showing depressed cardiac function and myofibrillar ATPase activity as well as changes in MHC protein and gene expression, are in agreement with our results reported previously in this experimental model<sup>42,43,340,341</sup>. In view of the critical role of  $\alpha$ -MHC and  $\beta$ -MHC in determining the velocity of cardiac contraction<sup>130,158,159,332</sup>, it is likely that the depressed myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity in the failing heart may be due to the observed increase in  $\beta$ -MHC protein content and a decrease in  $\alpha$ -MHC protein content. Since the protein content of total MHC was not altered, it appears that there occurs a shift in MHC isozymes in the failing myocardium. Such a change in the protein contents of MHC isozymes seems specific because the protein content of both MLC and TnI in the failing hearts was unchanged. Nonetheless, the observed alterations in the protein contents of MHC isozymes can be seen to result in changes in the composition as well as molecular structure of myofibrils and thereby represent the process of myofibrillar remodeling during the development of heart failure. This process of myofibrillar remodeling in the failing heart may be occurring at the level of gene expression because the mRNA levels for  $\alpha$ -MHC were decreased and those for  $\beta$ -MHC were increased.

Treatment of infarcted animals with enalapril, an ACE inhibitor, was observed to partially prevent alterations in cardiac hypertrophy, lung congestion, heart function, myofibrillar ATPase activity and a shift in MHC isozyme protein and gene expression. Since neither enalapril nor losartan affected the protein contents and mRNA levels for MLC, it is evident that the observed effect of these drugs in MHC protein and gene

expression is specific in nature. These results are consistent with the beneficial effects of imidapril, an ACE inhibitor, described earlier in this experimental model. Since the effect of both enalapril and imidapril were simulated by losartan, an AT<sub>1</sub>R antagonist, it appears that the beneficial actions of both enalapril and imidapril on heart function and cardiac as well as myofibrillar remodeling are due to the blockade of RAS in animals with heart failure. Both enalapril and losartan as well as other ACE inhibitors such as captopril and trandolapril have also been shown to partially prevent cardiac remodeling and changes in sarcoplasmic reticular function, protein content and gene expression in the failing hearts<sup>352,396,397</sup>. Furthermore, treatment of infarcted animals was found to partially prevent changes in sarcolemmal phospholipase C isozyme expression<sup>351</sup>. Since ACE inhibitors are also known to prevent the breakdown of bradykinin<sup>385,386</sup>, it can be argued that the beneficial effects of both enalapril and imidapril are mediated through the actions of bradykinin. Although we have not carried out any experiment to rule out this possibility, this mechanism in the experimental model used may not be of any major significance. Based on our observation, the combination therapy with both enalapril and losartan did not produce an additive effect on heart dysfunction or myofibrillar remodeling.

### **C. Protein Kinase Isozymes in Heart Failure**

In this study, we have observed an increase in both Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent PKC activities in the viable LV as well as LV dysfunction in 1-, 2-, 4-, and 8-weeks infarcted animals. Because the activation of PKC by phorbol esters has been shown to exert a negative inotropic effect on the heart due to phosphorylation of troponin I and T and subsequent inhibition of myofibrillar ATPase activity<sup>398</sup>, it is possible that



the sustained increase in PKC activity may be involved in depressing the LV function on inducing MI. Stimulation of PKC by phorbol esters has also been shown to produce changes in cytosolic  $\text{Ca}^{2+}$  and negative inotropic effect in cardiomyocytes<sup>222</sup>. Although the activation of PKC by phorbol esters has been reported to decrease cardiac SR  $\text{Ca}^{2+}$ -transport<sup>399</sup> and can explain the depression in LV  $+dP/dt$  and  $-dP/dt$  in the infarcted hearts, the mechanism of decrease in SR  $\text{Ca}^{2+}$  uptake by the activation of PKC are not clear. Nonetheless, increased PKC activities and cardiac dysfunction have also been observed in diabetic animals<sup>366-368</sup>. Furthermore, attenuation of both increased PKC activities and LV dysfunction in the infarcted animals was found to occur on treatment with imidapril, which has been reported to produce beneficial effects as a consequence of ACE inhibition in this model of heart failure<sup>351</sup>. Increased PKC activities have also been observed in failing human hearts<sup>365</sup> as well as in different experimental models of heart failure<sup>263,363,364</sup>. Thus in view of such observations, it appears that a sustained increase in PKC activities may be involved in the genesis of contractile dysfunction in heart failure. This suggestion is further supported by the fact that overexpression of PKC isozymes resulted in diminished heart function in transgenic mice<sup>262</sup>. Whereas the attenuation of increased PKC activity and depressed cardiac function by treatment of MI animals with imidapril can be explained on the basis of suppression of the RAS, imidapril treatment was found to exert no effect in the control animals. Such results may indicate that both cardiac function and PKC isozymes in normal physiological conditions may not be under the influence of RAS.

The increased PKC activities in the LV homogenate in infarcted animals does not appear to be due to translocation of cytosolic enzyme to the particulate compartment of the cell because the PKC activities in both LV cytosolic and particulate fractions were

increased due to MI. Such an increase in PKC activities in the LV is likely to be due to increase in the expression of PKC- $\alpha$ , - $\beta$ , - $\epsilon$  and - $\zeta$  isozymes in the myocardium because the relative contents of these isozymes (except cytosolic PKC- $\alpha$  content) were increased in LV homogenate, cytosolic and particulate fractions on inducing MI. Furthermore, treatment of infarcted animals with imidapril not only partially prevented the increase in LV PKC activities, it also had similar effect on the isozyme contents in the LV homogenate, cytosolic and particulate fractions. Whether the observed increase in LV PKC isozymes is due to translational or transcriptional changes in the myocardium remains to be investigated. However, it should be pointed out that PKC- $\epsilon$  isozyme, a predominant isoform in cardiomyocytes<sup>400</sup>, has been shown to be associated with sarcomeres on activation<sup>401</sup> and be responsible for the phosphorylation of troponin I<sup>368,398</sup>. On the other hand, PKC- $\beta$  isoforms have been reported to stimulate the promoter of  $\beta$ -myosin heavy chain in the myocardium<sup>220</sup>. Accordingly, it seems possible that the depressed LV function in the infarcted heart may be due to increases in both PKC- $\epsilon$  and PKC- $\beta$  isozyme contents. Because the role of PKC- $\alpha$  and - $\zeta$  isoforms in altering the function of any subcellular organelle or metabolic site in the myocardium has not been well established at present, it is difficult to speculate on the exact functional significance of increased PKC- $\alpha$  and - $\zeta$  isozymes in failing LV from the infarcted animals. However, the translocation of PKC- $\alpha$  and PKC- $\epsilon$  has been reported to occur in failing hearts due to aortic banding in guinea pigs and this change was attenuated by treatment with ramipril, an ACE inhibitor<sup>364</sup>.

PKC isozymes are known to serve in the signal transduction mechanism and thus play a crucial role in the development of cardiac hypertrophy<sup>201,370,400,402</sup>. Transfection of cardiomyocytes with constitutively active PKC was demonstrated to activate genes for

atrial natriuretic factor and  $\beta$ -myosin heavy chain, which are associated with cardiac hypertrophy<sup>220,403,404</sup>. Previous studies<sup>248</sup> have shown that the expression of both PKC- $\beta$  and - $\epsilon$  isozymes is increased in cardiac hypertrophy induced by aortic banding in rats. Furthermore, mechanical stretch has been reported to increase PKC- $\epsilon$ , but not PKC- $\alpha$ , and induce cardiac hypertrophy<sup>405</sup>. It is thus possible that cardiac hypertrophy observed in the LV due to MI may be caused by an increase in the content of PKC isozymes including content of PKC- $\epsilon$ . This view is consistent with the observations that treatment of infarcted animals with imidapril was found to not only reduce the extent of LV hypertrophy but also the level of LV PKC isozymes.

Whereas the Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent PKC activities were increased in both LV and RV homogenates on inducing MI, some differences between LV and RV were apparent with respect to changes in PKC isozyme contents. For example, the infarcted LV showed an increase in PKC- $\zeta$  content in homogenate, cytosolic, and particulate fractions, but no such changes were seen in the RV. Furthermore, unlike RV, no increase in cytosolic PKC- $\alpha$  content was detected in the LV. On the other hand, no changes in particulate PKC- $\beta$  and - $\epsilon$  isozyme contents were seen in the RV, whereas the contents of these isoforms were increased in the LV after MI. Such differential changes in the LV and RV in the infarcted heart indicate that PKC isozymes in different regions of the heart may be regulated differentially. Differences in the behaviour of LV and RV with respect to changes in SR Ca<sup>2+</sup>-pump as well as adenylyl cyclase activities have also been reported during the development of CHF due to MI<sup>39,349</sup>. Nonetheless, the increase in PKC activities as well as PKC isozymes in both LV and RV in the infarcted animals may be of some specific nature because neither the PKA activities nor PKA protein contents were altered in both LV and RV upon inducing MI. Although activation of PKA has been

shown to represent a growth promoting signal<sup>281,406</sup>, our data are in agreement with other reports that PKA activity had no relation to the development of cardiac hypertrophy due to MI or pressure overload<sup>269</sup>. Furthermore, unlike PKC, no change in PKA activity was observed in the failing human heart<sup>267</sup>, as observed in our study. Therefore, the relationship of Ang II and PKA is not clear in MI-induced CHF model.

Taken all together, the data in this study are consistent with the view that the increased PKC activities in the hypertrophied and failing heart subsequent to MI are due to increased expression of PKC isozymes and that the sustained increase in PKC activity may be involved in cardiac dysfunction on occluding the coronary artery. However, it should be recognized that the observed changes in cardiac function in the MI-induced heart failure may not be entirely due to cardiomyocyte-specific adaptation in PKC signalling because the contribution of alterations in PKC activity from other cell types such as fibroblasts<sup>407</sup> cannot be excluded. Furthermore, in spite of the association of increased PKC activity and cardiac dysfunction in the failing heart, the exact significance of the observed changes in PKC isozymes in heart failure due to MI remains to be established by the use of PKC inhibitors in this experimental model. The partial prevention of changes in cardiac PKC isozymes and cardiac dysfunction in heart failure due to MI by an ACE inhibitor, imidapril, indicates that mechanisms other than those mediated by increased formation of Ang II in heart failure may also be implicated in the genesis of cardiac dysfunction. Although ACE inhibition is generally considered to confer beneficial effects on the failing heart by reducing afterload, we did not observe any changes in the MAP in the MI animals on treatment with imidapril for a period of 4 wk. Also, this study does not provide any information regarding the cause-and-effect relationship between changes in heart function and PKC isozyme expression.

Accordingly, the exact mechanisms responsible for the observed increase in PKC isozymes during the development of heart failure due to MI as well as for the partial prevention of PKC activities in the failing hearts on treatment with imidapril require further studies.

#### **D. Inter-relationships Among Studies**

From the foregoing discussion, it is clear that cardiac remodeling due to MI in rats is associated with remodeling of myofibrils and increase of PKC activity. In view of the role of PKC in signal transduction mechanisms, it is likely that both cardiac hypertrophy and depressed myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity in the infarcted heart due to increased PKC activity. Such a role of PKC may be of some specific nature because the activity of PKA, which is also known to be involved in signal transduction, was unaltered in the failing hearts. Furthermore, it appears that activation of PKC may also result in cardiac dysfunction due to a depression in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity in the failing heart. Since cardiac hypertrophy, increased PKC activity, depressed myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase and cardiac dysfunction in the infarcted hearts were partially prevented by treatment with imidapril, an ACE inhibitor, it is suggested that activation of RAS may play a critical role in the activation of PKC, depression in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase, heart dysfunction and cardiac remodeling in heart failure due to MI.

This study has revealed that the augmented PKC activity may be due to increased expression of different PKC isozyme contents in the failing hearts. On the other hand, the increased PKC activity does not seem to be due to translocation of the PKC isoforms

because the increase in the PKC isozyme contents was evident in the homogenate, particulate and cytosolic fractions from the infarcted hearts. This study has also shown that depressed myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity may be due to a shift in the expression of MHC proteins in the failing hearts. Since changes in mRNA for the MHC proteins were associated with corresponding changes in MHC proteins, it is evident that remodeling of myofibrils may occur at the molecular level during the development of CHF due to MI.

It was observed that treatment of infarcted animals with imidapril prevented change in PKC activity, protein isoform content, myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity, shift in MHC protein content and gene expression for MHC proteins in the failing hearts. Since cardiac remodeling, heart dysfunction and remodeling of myofibrils were partially prevented by treatment of infarcted animals with enalapril, an ACE inhibitor, it appears that the beneficial effects of imidapril on heart function, cardiac remodeling and myofibrillar remodeling may be due to blockade of the RAS in CHF. This view is further substantiated by our observations that the beneficial effects of both imidapril and enalapril in infarcted hearts were simulated by losartan, an  $\text{AT}_1\text{R}$  blocker. Partial prevention of MI induced changes in cardiac remodeling, heart function, myofibrillar remodeling and PKC protein contents in the failing heart by the blockade of RAS seems to suggest that other mechanisms in addition to RAS may be involved in CHF due to MI.

## VI. CONCLUSIONS

This study was undertaken to test the beneficial effects of imidapril on cardiac remodeling, heart function, myofibrillar remodeling and PKC activation in CHF due to MI in rats. From the results obtained in this study, the following conclusions can be made.

1. Cardiac remodeling, heart dysfunction, and changes in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity may be due to an increase in PKC activity in CHF.
2. Alteration in PKC activity may be due to increased expression of PKC isozyme contents rather than changes in the translocation of PKC isozymes.
3. Heart dysfunction in the infarcted animals may partially be due to a depression in myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity.
4. Depressed myofibrillar  $\text{Ca}^{2+}$ -stimulated ATPase activity in the failing heart appears to be due to a shift in MHC isozymes. The alterations in MHC isozymes may be due to corresponding changes in gene expression of MHC isozymes in CHF.
5. Treatment of infarcted animals with imidapril prevented the MI-induced changes in cardiac hypertrophy, heart function, myofibrillar remodeling and PKC activation partially.
6. The beneficial effects of imidapril were simulated by enalapril and/or losartan indicating the involvement of RAS in the MI-induced CHF.

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