PROTECTIVE EFFECT OF 5-(N,N-DIMETHYL)AMILORIDE AND POSSIBLE ROLE OF NA^+-H^+ EXCHANGE IN THE DEVELOPMENT OF ISCHEMIA-REPERFUSION INJURY IN RAT MYOCARDIUM

By

HE-PING MENG

Thesis submitted to the Faculty of Graduate studies of the University of Manitoba in partial fulfilment of the requirement for the Degree of

DOCTOR OF PHILOSOPHY

Department of Physiology
University of Manitoba
Winnipeg, Manitoba

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I dedicate this manuscript to my beloved Chinese people
who are wakening up and fighting for democracy,
social justice and basic human rights.

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SUMMARY

A Na^+-H^+ exchange inhibitor, 5-(N,N-dimethyl)amiloride (DMA) was used to probe the possible role of Na^+-H^+ exchange in ischemia-reperfusion injury in coronary perfused isolated right ventricular wall of rat. Contractile function was evaluated by monitoring resting tension, maximal developed tension and the rates of tension generation and relaxation. The changes of ion content in the right ventricular wall were measured with atomic absorbance spectrophotometry. Coronary effluent was collected for the assay of creatine phosphokinase (CPK) activity which represents the leakage of cellular protein from damaged cells.

It is hypothesized that sarcolemmal Na^+-H^+ exchange may be important for Ca^{2+} overload and cardiac dysfunction during post-ischemic reperfusion. During ischemia, both intra- and extracellular space are very acidic. When reperfusion begins, the pH of extracellular space is restored to normal by a rapid washout of the extracellular H^+, while intracellular space remains acidic. This immediately results in the establishment of a transsarcolemmal H^+ gradient which would strongly stimulate sarcolemmal Na^+-H^+ exchange to extrude the intracellular H^+ in exchange for extracellular Na^+. By this way, Na^+ quickly accumulates inside the myocardium. The intracellular Na^+ loading may in turn stimulate sarcolemmal Na^+-Ca^{2+} exchange to extrude

Na^+ and bring in a large quantity of Ca^{2+}, causing Ca^{2+} overload and subsequent cardiac dysfunction. If this is true, the application of a Na^+-H^+ exchange inhibitor, such as DMA, should be able to protect the heart from reperfusion related damage. Furthermore, if Na^+-H^+ exchange is involved in the Ca^{2+} overload during reperfusion, manipulating cellular Na^+ content or the transsarcolemmal H^+ gradient should be able to induce corresponding ionic and mechanical responses during reperfusion. These responses should also be influenced by the inhibition of Na^+-H^+ exchange.

This study was divided into the following sections:

1) Protective effect of DMA during ischemia-reperfusion insults: In drug-untreated ventricular wall, 60 min global ischemia resulted in a significant rise of resting tension (RT: 174 $\pm$ 8% of the pre-ischemic level). Post-ischemic reperfusion further increased RT (273 $\pm$ 12 % of pre-ischemic level) and induced a poor recovery in developed tension (DT: 28 $\pm$ 4 % of the pre-ischemic level). Both the maximal velocity of tension development (+dT/dt) and the maximal velocity of muscle relaxation (-dT/dt) recovered to a similar degree. When 1, 5 or 20 uM DMA was included in the perfusate (3 min before ischemia and in the first 3 min of reperfusion), the maximal post-ischemic RT of the heart was reduced to 204 $\pm$ 21 %, 166 $\pm$ 15 % and 139 $\pm$ 15 % of the pre-ischemic levels ($p<0.05$), respectively, and DT recovery was 39 $\pm$ 3 %, 63 $\pm$ 10 % and 79 $\pm$ 8 % of the pre-ischemic levels ($p<0.05$), respectively in a dose-dependent manner.

Similar qualitative recovery in +dT/dt and -dT/dt was observed with DMA treatment. Similar post-ischemic recovery of cardiac function was observed if 20 uM DMA was present only during reperfusion. Prolonged adminstration of DMA for up to 30 min during reperfusion did not provide further protection to the ventricular wall. Accordingly, the intracellular Na^+ and Ca^{2+} content in drug-untreated group was significantly increased in the early stage of reperfusion which could be normalized by DMA treatment. DMA treatment also significantly protected against creatine phosphokinase (CPK) leakage from myocardium during reperfusion.

2) The involvement of Na^+ and Na^+-H^+ exchange in ischemia-reperfusion injury: a non-toxic and inotropic dose (35 uM) of ouabain which inhibits sarcolemmal Na^+-K^+ ATPase, was used to alter cellular Na^+ content during ischemia-reperfusion. The mechanical and ionic responses of the ventricular wall were observed in the absence or presence of 20 uM DMA. During post-ischemic reperfusion, DT and RT in the drug-untreated group were 35 $\pm$ 4 % and 221 $\pm$ 12 %, respectively, of the pre-ischemic values. Including 35 uM ouabain in the perfusate 3 min prior to and 6 min after ischemia resulted in a significant rise in cellular Na^+ content which was accompanied by a more severe Ca^{2+} overload, poorer post-ischemic recovery in cardiac function and more leakage of creatine phosphokinase. Introduction of 20 uM DMA 6 min prior to and after ischemia could effectively prevent the increase of cellular Na^+ content and protect the ventricular

wall against Ca^{2+} overload, the post-ischemic dysfunction and CPK leakage induced by ouabain.

3) Modification of the post-ischemic cardiac function by reperfusion buffer pH: Effects of a pH 7.9 and a pH 6.5 reperfusion buffer were evaluated in comparison to that of pH 7.2 buffer in the ventricular walls subjected to 55 min ischemia and 30 min reperfusion in the absence or presence of 20 uM DMA. The purpose using these procedures was to increase or decrease the transsarcolemmal H^+ gradient at the beginning of reperfusion and thus, alter Na^+-H^+ exchange activity. The mechanical and ionic responses of the ventricular wall to the pH changes were observed. During reperfusion at pH 7.2, the recovery of DT and RT of the ventricular wall were 58.8 $\pm$ 6.5 % and 201 $\pm$ 20 %, respectively, of the pre-ischemic values. Reperfusion at pH 7.9 for 6 min resulted in an significantly accelerated accumulation of Na^+ in myocardium which was accompanied by a more severe Ca^{2+} overload, poorer DT recovery and higher RT (40 $\pm$ 5.9 % and 285 $\pm$ 13 %, respectively, of the pre-ischemic values, $p<0.05$ vs. pH 7.2 group). CPK activity in coronary effluent was also elevated by reperfusion at pH 7.9. When DMA was included in the pH 7.9 buffer, the rise in cellular Na^+ following reperfusion at pH 7.9 was normalized. The Ca^{2+} overload, post-ischemic cardiac dysfunction and the CPK leakage were also prevented. Reperfusion with a pH 6.5 media resulted in a gradual rise in cellular Na^+ but did not increase cellular Ca^{2+} in the first 6 min of reperfusion. Furthermore, the DT recovery and the RT rise was

delayed. When the perfusate pH was changed from 6.5 to 7.2, cellular Na^+ and Ca^{2+} were significantly elevated, and cardiac dysfunction developed which was similar to that observed when the ventricular wall was reperfused at pH 7.2 only.

In summary, the results demonstrated that DMA could effectively protect the ventricular wall from ischemia-reperfusion injury in a dose-dependent manner. DMA may exert its protective effect during the early stages of reperfusion. Increasing cellular Na^+ or transsarcolemmal H^+ gradient could cause more severe Ca^{2+} overload and worsen the post-ischemic recovery of cardiac contractile function. These detrimental effects could be prevented by reducing intracellular Na^+ via inhibition of Na^+-H^+ exchange. The data suggest that a reperfusion-related Na^+ loading in the myocytes which precedes the Ca^{2+} overload could be a prerequisite for the subsequent Ca^{2+} overload and cardiac dysfunction. The data strongly support the hypothesis that stimulation of Na^+-H^+ exchange may represent a crucial and primary step for the cascade of ion imbalance during reperfusion. The results are also consistent with the proposed importance of Na^+-H^+ exchange for the development of the post-ischemic damage in myocardium.

I. INTRODUCTION AND STATEMENT OF PROBLEM

The mechanism underlying ischemia-reperfusion injury of the heart is not clear. One of the prominent changes occurring in the early stage of reperfusion is a rapid and excessive accumulation of Ca^{2+} inside the cardiac cell (Shen and Jennings, 1972[b]; Nayler, 1981; Poole-Wilson et al, 1984). This phenomena is termed as "Ca^{2+} overload" which is believed to trigger a series of pathological and biochemical alterations during reperfusion, including cardiac dysfunction, activation of proteases and lipases and inhibition of mitochondrial function (Murphy et al, 1987; Nayler et al, 1988). The mechanism as to how the abnormal, massive Ca^{2+} influx occurs during reperfusion is controversial. Poole-Wilson et al (1984) and Jennings et al (1985) hypothesized that the physical property of the myocardial sarcolemmal membrane was altered during ischemia and becomes fragile. Under this condition, the entry of a relatively small amount of Ca^{2+} during reperfusion may be sufficient to trigger a second chain of events, resulting in additional uncontrolled Ca^{2+} gain and loss of cell integrity. However, this hypothesis is lacking of experimental support. The uncontrolled Ca^{2+} entry seems unlikely to be through voltage dependent Ca^{2+} channel since Ca^{2+} channel blockers did not prevent the Ca^{2+} overload if administered only during reperfusion (Bourdillon and Poole-

Wilson, 1982; Bersohn and Shine, 1983). The stimulation of sarcolemmal Na^+-Ca^{2+} exchange has been proposed as an important pathway for the post-ischemic entry of Ca^{2+}. This hypothesis is supported by many pathological and biochemical evidence (Renlund et al, 1984; Grinwald and Brosnahan, 1987; Nayler et al, 1988; Tani and Neely, 1989; 1990b; Pierce et al, 1990; refer section C.2. of REVIEW OF LITERATURE for details). The stimulus for this accelerated Na^+-Ca^{2+} exchange may come from an elevated intracellular Na^+ ($[Na^+]_i$) loaded during the ischemia-reperfusion period (Lazdunski et al, 1985). Na^+ may enter in the myocardium through Na^+-H^+ exchange, Na^+-Ca^{2+} exchange and voltage-dependent Na^+ channels under physiological conditions. The importance of these Na^+ transport pathways for the contribution of the high $[Na^+]_i$ during reperfusion is not clear. The ionic changes during the early stage of reperfusion favours a Na^+ efflux through Na^+-Ca^{2+} exchange rather than Na^+ uptake (Lazdunski et al, 1985). There is no evidence that Na^+ channel can conduct extraordinarily large amounts of Na^+ entry during reperfusion. The most plausible possibility for the excessive Na^+ entry is via Na^+-H^+ exchange. Cardiac Na^+-H^+ exchange has been indicated to be a major regulatory pathway of $[Na^+]_i$ (Lazdunski et al, 1985; Mahnensmith and Aronson, 1985; Pierce et al, 1990[a]). It is also a very important mechanism for the maintenance of intracellular pH (pH_i), especially at lower pH_i (Lazdunski et al, 1985; Piwnica-Worms et al, 1985). It is well known that ischemia results in tissue acidosis because of the hydrolysis of high

energy phosphates and anaerobic glycolysis (Poole-Wilson, 1978; Couper, 1984). The acidic intracellular environment would favour the exchange of extracellular Na^+ for intracellular H^+ via Na^+-H^+ exchange leading to an abnormal gain of Na_i.

Our working hypothesis is summarized as follows (Figure 1): During prolonged ischemia, both intra- and extracellular pH is very low (Cobbe and Poole-Wilson, 1980; Couper et al, 1984; Vanheel et al, 1989). Upon reperfusion, the extracellular pH can rapidly restore to 7.2-7.4 with the quick washing out of the extracellular H^+. Meanwhile, the intracellular space remains very acidic. This will result in the establishment of an outwardly directed transsarcolemmal H^+ gradient which will strongly stimulate the sarcolemmal membrane Na^+-H^+ exchanger to extrude H^+ out of the cell in exchange for extracellular Na^+ into the cell in the early stage of reperfusion. This entry of Na^+ via Na^+-H^+ exchange leads to a rapid accumulation of intracellular Na^+ (Tosaki et al, 1989). Sarcolemmal Na^+-K^+ ATPase (Na^+ pump) is also inhibited at this time because of the ischemic injury or lack of ATP supply (Bersohn et al, 1982[a]; Jennings et al, 1987; Dhalla et al, 1988). Therefore, intracellular Na^+ can not be efficiently transported out of the cell via Na^+ pump. This may partially contribute to the Na^+ accumulation. The high intracellular $[Na^+]$ can in turn activate sarcolemmal Na^+-Ca^{2+} exchange to transport Na^+ out of the cell for Ca^{2+} entry in a large quantity into the cell (Grinwald and Brosnahan, 1987). This ultimately causes Ca^{2+} overload and subsequent cell damage

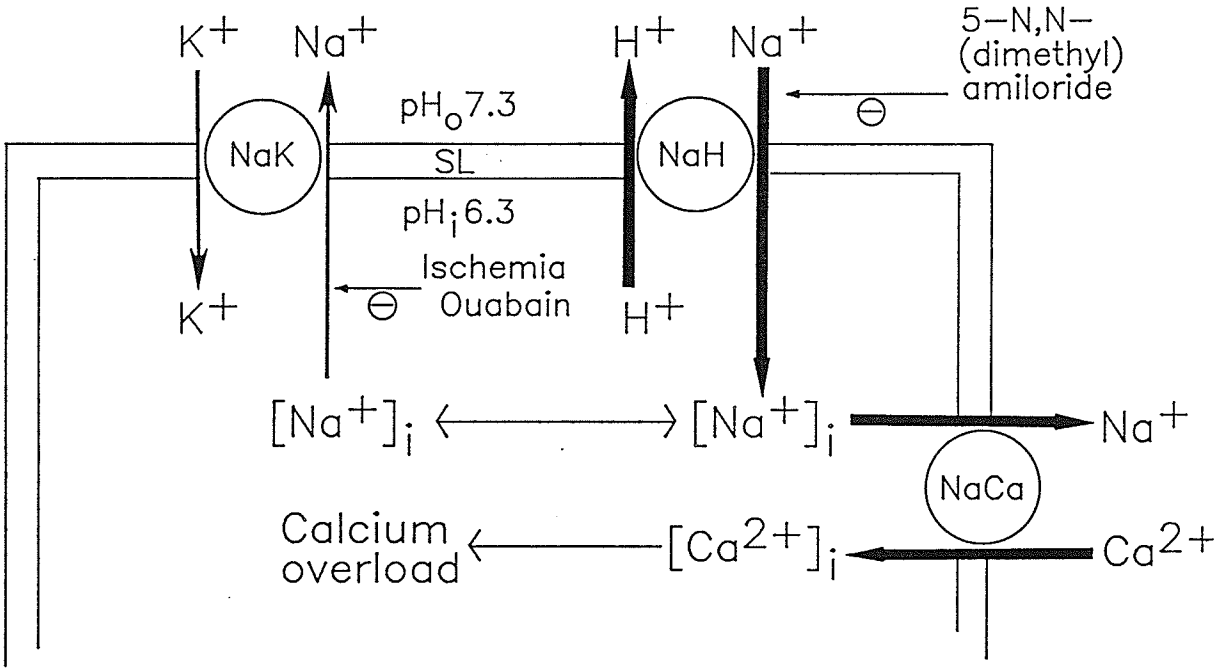


Figure 1. Schematic representation of ionic alterations in the myocardium in the early stages of post-ischemic reperfusion. An outwardly directed H^+ gradient is established during reflow which activates sarcolemmal Na^+-H^+ exchange leading to a dramatic rise in intracellular Na^+ which then stimulates the Na^+-Ca^{2+} exchange pathway resulting in excessive Ca^{2+} entry into the cell and Ca^{2+} overload. The ischemic condition (or the addition of ouabain) will also inhibit the Na^+-K^+ ATPase (Na^+ pump) which will further elevate Na^+ in the cell and worsen the injury. Conversely, DMA may antagonize the Na^+ entry via Na^+-H^+ exchange and ultimately protect the heart.

and cardiac dysfunction (Nayler, 1988). In our working hypothesis, the Na^+ entry via Na^+-H^+ exchange which precedes the Ca^{2+} overload is regarded as a primary and crucial step in the pathophysiological changes during reperfusion since the stimulation of Na^+-H^+ exchange appears to be the prerequisite or a trigger for the subsequent cascade of ionic changes (first a Na^+ loading followed by a Ca^{2+} overload). If this is true, the inhibition of Na^+-H^+ exchange by a Na^+-H^+ exchange inhibitor should reduce Na^+ entry and prevent the following Ca^{2+} overload via activation of Na^+-Ca^{2+} exchange. By this way, the post-ischemic recovery of cardiac function could be improved and the cellular damage attenuated.

Two recent studies using amiloride did demonstrate some protective effects on the heart against ischemic-reperfusion alterations (Karmazyn, 1988; Tani and Neely, 1989). However, the dosages employed were relatively large (0.2-1.0 mM) and in view of the non-specific nature of amiloride's effects (Kleyman and Cragoe, 1988), the mechanism of action may be a combined effect on several ion transport pathways (Na^+-Ca^{2+} exchange, Na^+-H^+ exchange and Ca^{2+} channel). In the present investigation we have employed an analogue of amiloride, 5-(N,N-dimethyl)amiloride (DMA), which is 10-24 times more potent as an inhibitor of Na^+-H^+ exchange than the parent compound, amiloride (IC_{50} for DMA: 7 uM vs. 83.5 uM for amiloride, Kleyman and Cragoe, 1988). The IC_{50} of DMA for the inhibition of Na^+-Ca^{2+} exchange and Na^+-K^+ ATPase activity is from 0.55-3 mM and the IC_{50} for the inhibition of

cardiac Ca^{2+} channel is larger than 0.05 mM (Dennis et al, 1990). The higher selectivity of DMA on Na^+-H^+ exchange, therefore, suggest that it could be a better tool to probe the possible role of Na^+-H^+ exchange in ischemia-reperfusion injury.

If the increased intracellular Na^+ is important for the post-ischemic injury, then changes in Na^+_i loading state in the early stage of reperfusion should be able to modify the cardiac injury. In light of our hypothesis (Figure 1), the partial loss of Na^+-K^+ ATPase activity may contribute to the Na^+ accumulation in cardiac cells. If the Na^+ pump is further inhibited in the early phase of reperfusion by ouabain, a Na^+ pump inhibitor, the Na^+ efflux through this pathway may be further decreased which may lead to a more severe Na^+ loading in the early stage of reperfusion, causing more severe damage on cardiac function. Also if DMA is working through a Na^+-H^+ exchange pathway, then it should be able to reduce Na^+ entry through Na^+-H^+ exchange and reduce the ouabain-induced Na^+ loading as well as the subsequent Ca^{2+} overload.

The activity of Na^+-H^+ exchange is both pH and Na^+ sensitive (Piwnica-Worms and Lieberman, 1983; Lazdunski et al, 1985; Vaughan-Jones, 1988). In vitro studies have demonstrated that the larger the transsarcolemmal H^+ gradient is, the more active the Na^+-H^+ exchange will be (Pierce and Philipson, 1985; Vaughan-Jones, 1988). In isolated heart, when reperfusion at pH 7.2 is initiated after ischemia, a H^+ gradient across the sarcolemma is likely to be formed immediately. The H^+ gradient may serve as a

strong stimulator to Na^+-H^+ exchange. When the heart is reperfused after ischemia at a more alkalinic pH than 7.2 (for example, pH 7.9), the transsarcolemmal H^+ gradient established would be greater which may result in a stronger stimulation of Na^+-H^+ exchange during the early stage of reperfusion. If Na^+ does enter the cells through Na^+-H^+ exchange, the alkalinic reperfusion should induce more Na^+ accumulation and more severe post-ischemic cardiac dysfunction. This pH-related modification of the post-ischemic recovery of cardiac function should also be interfered with by the inhibition of Na^+-H^+ exchange. Nayler and co-workers have demonstrated that tissue ionic changes are exacerbated by a high reperfusion pH and attenuated by a low reperfusion pH (Nayler et al, 1988; Panagiotopoulos et al, 1990). However, no Na^+-H^+ exchange inhibitor was used in their study, hence left the question unanswered as to how the post-ischemic cardiac function is modified by the pH alteration.

The whole study is divided into three parts. The purpose of the first part is to use the more selective Na^+-H^+ exchange inhibitor, DMA, to: 1) probe if it can protect the heart from ischemia-reperfusion injury and contractile dysfunction; 2) determine when it exerts its protective effect during the development of ischemia-reperfusion and 3) examine if the changes of cellular ion content are consistent with the proposed role of Na^+-H^+ exchange in the ischemia-reperfusion injury.

In the second part of the study, ouabain is applied to examine the role of intracellular Na^+ and Na^+-H^+ exchange in the post-

ischemic cardiac dysfunction. The purpose of this part of the study is to: 1) examine if a non-toxic, positive inotropic dose of ouabain could increase Na^+_i and thereby increase cardiac dysfunction during reperfusion period; 2) characterize if ionic changes in the heart, particularly sodium, occurred during reperfusion in the presence or absence of ouabain may influence the recovery of post-ischemic cardiac function and 3) if DMA could protect the right ventricular wall from the detrimental effect of ouabain and attenuate the ionic imbalance during reperfusion.

In the third part of the study, the relationship between the transsarcolemmal H^+ gradient and the post-ischemic cardiac dysfunction is to be examined. The purpose of this part of the study is to: 1) observe if the post-ischemic recovery of cardiac contractility could be modified by manipulating perfusate pH during the early stage of reperfusion; 2) characterize if the changes of cellular ion content were consistent with the proposed role of Na^+-H^+ exchange in the ischemia-reperfusion injury and 3) examine if the Na^+-H^+ exchange inhibitor DMA could antagonize the pH modified post-ischemic recovery of cardiac function.

By collecting the above evidence, we should be able to understand if Na^+-H^+ exchange is an important mechanism for the development of post-ischemic cardiac dysfunction and cell injury.

II. REVIEW OF LITERATURE

A. MYOCARDIAL ISCHEMIA-REPERFUSION: GENERAL DISCUSSION

Myocardial ischemia refers to the imbalance between the demand of the heart for nutrients (such as oxygen and glucose) and the blood supply to the heart (Williamson et al, 1976). The most common cause of the ischemic heart disease is the obstruction of the coronary artery secondary to the formation of vascular atherosclerosis (Meijler, 1983). It also occurs as a complication of cardiac hypertrophy (Marcus et al, 1983), circulatory shock (Hoffman et al, 1983) and diabetes (Meijler, 1983).

In 1912, Dr. Herrick was the first to describe a syndrome which was apparently related to a reduction of blood flow to the heart (Braunwald, 1976). Since then, the importance of ischemic heart disease has been gradually recognized. In humans, a mild, brief decrease of coronary blood flow may induce a transient chest pain (angina pectoris) and incapability. A sudden and continuous coronary occlusion may cause myocardial necrosis (myocardial infarction), leading to the loss of tissue mass or even death. Today, heart disease is the number one killer in Western society. The most current statistical data for the Canadian population show that 40 % of all Canadian male and over 45 % of all Canadian female deaths were a result of circulatory

disease of some kind. Ischemic heart disease constituted the majority of this circulatory failure. It alone is the cause of as many deaths in Canada as all types of cancer combined (Bisch et al, 1989).

The pathophysiological process of ischemia is very complicated. The mechanism for the ischemia-induced lesion in the heart is not clear, although a tremendous amount of work has been done in the past 30 years. In this review, efforts will be focused on the development of ischemia-reperfusion injury, rather than the pathogenesis of ischemia.

In experimental cardiology, cardiac ischemia can be subdivided into four categories: Global ischemia, hypoxia, low flow ischemia and Ca^{2+} paradox.

1. Global ischemia

Whenever the blood supply or perfusate to the heart is completely stopped, the heart is rendered globally ischemic. The severity of the injury in the ischemic myocyte depends on the length of ischemia (Bolli, 1990). If the heart is reperfused after a short period of ischemia, there will be a transient mechanical abnormality following which cardiac function will recover gradually but completely. This post-ischemic cardiac dysfunction is called myocardial stunning (Braunwald and Kloner, 1982). It is fully reversible (reversible ischemia) (Bolli, 1990). If ischemia persists for a prolonged period, reperfusion will not bring the heart back to a normal condition. Instead,

reperfusion leads to further cardiac injury (ischemia-reperfusion injury). This injury is reflected by poor force generation (Meng and Pierce, 1990), elevated muscle resting tension or ventricular end-diastolic pressure (contracture), development of arrhythmias, excessive Ca^{2+} accumulation (Ca^{2+} overload), enzyme release, poor replenishment of the high energy phosphate pool, cellular necrosis and other ultrastructural damage. This is termed irreversible damage (irreversible ischemia). The mechanism of irreversible ischemia will be discussed in detail in the following discussion. The irreversible global ischemia model mimics the pathophysiological process of myocardial infarction and it has been considered appropriate for the investigation of the pathophysiology, biochemistry and pharmacological treatment of ischemia in the heart.

2. Hypoxia

Hypoxia refers to the condition when perfusion is maintained while most or all oxygen in the perfusate is removed (Trump et al, 1976) or mitochondrial oxidative phosphorylation is inhibited by agents like cyanide or dinitrophenol (Dahl and Isenberg, 1980). Under such a condition, the heart is subjected to limited O_2 supply and is forced to convert from aerobic metabolism to anaerobic glycolysis in order to generate new high energy phosphates. This is called metabolic inhibition. In an attempt to mimic ischemia more closely, this model has frequently been combined with procedures that inhibit anaerobic glycolysis

(using 2-deoxy-D-glucose, Barry et al, 1987; using iodoacetate, Koretsune and Marban, 1990). With these procedures, the mechanical responses during hypoxia, such as complete cessation of developed pressure and contracture are similar to those observed in the ischemia model. After restoration of normal O_2 supply (reoxygenation), cardiac cells shift back to aerobic metabolism and accordingly, the energy production gradually returns to normal if hypoxia did not last long. Since the metabolic waste products (such as H^+ and lactate) can be partially washed out during hypoxia, cardiac injury in the hypoxia model is less than that for global ischemia which lasts the same length of time. If hypoxia persists for too long, reoxygenation will induce cell damage and cardiac dysfunction. The mechanism for the post-hypoxia myocardial dysfunction has some similarities but may not be identical to global ischemia (Allen and Orchard, 1987). The advantage of the hypoxia model is that drugs can be applied or extracellular ionic composition can be changed during hypoxia which facilitates the investigation of ischemic metabolism and oxygen toxicity. Hypoxia can also be studied in cell culture. The main limitation of this model is probably that H^+ and lactate are washed out during hypoxia and may mask the effects of these important factors during reperfusion (Poole-wilson, 1989; Marban et al, 1989).

3. Low-flow ischemia

Low-flow ischemia refers to the condition when oxygen and

substrate supply to the heart is significantly reduced by maintaining perfusate rate at a sub-normal level (15-50 % of control; Jennings et al, 1990). It is close to the clinical situation when a compensatory and limited blood supply to different layers of myocardial wall through collateral vessels occurs after the occlusion of a main coronary branch (Jennings et al, 1990). The cardiac dysfunction and cell injury induced in this model is similar to those observed in ischemia model except that it takes longer time to take place. The limitation for the low-flow ischemia is that the accumulation of metabolic wastes in tissue can be partially washed out. Therefore, the mechanism of cardiac dysfunction during reperfusion may not be the same as that in ischemia model (Tani and Neely, 1990[a]; Murry et al, 1990).

4. Calcium paradox (Ca^{2+} paradox)

Calcium paradox (Zimmerman and Hulsmann, 1966; Ruigrok et al, 1975) refers to the condition when the heart is subjected to 2-10 min perfusion with Ca^{2+}-free (< 50 uM) buffer followed by re-admission of Ca^{2+} containing solution (Grinwald and Nayler, 1981). In this model, the heart is not deprived of the supply of oxygen and metabolic substrates. Perfusion with Ca^{2+}-free solution can uncouple excitation-contraction coupling resulting in an immediate decrease of cardiac contraction without significant morphological changes. However, addition of Ca^{2+} in the re-perfusate will induce calcium overload inside the cell

(Zimmerman, 1966; Alto and Dhalla, 1979; Pierce et al, 1990[b]) causing massive enzyme and protein release from the myocardium (Ruigrok and Zimmerman, 1979), irreversible loss of active tension generating activity (Yates and Dhalla, 1975), electrical instability, sustained contracture, depletion of the energy-rich phosphate reserves (Boink et al, 1976) and severe ultrastructural damage (Zimmerman et al, 1967).

The mechanism underlying the Ca^{2+} paradox is controversial. It has been proposed that the severity of the Ca^{2+} paradox is primarily determined by the entry of Na^{+} through the Ca^{2+} channels during Ca^{2+} depletion and the subsequent influx of Ca^{2+} into the cytosol which stimulates Na^{+}-Ca^{2+} exchange during Ca^{2+} repletion (Goshima et al, 1980; Ruano-Arroyo et al, 1984; Chapman et al, 1986; Tunstall et a, 1986; Guarnieri, 1985; Bhojani and Chapman, 1990; Pierce et al, 1990[b]). An alternative hypothesis argues that because Ca^{2+} channel blockers can inhibit Ca^{2+} entry and attenuate cardiac injury (Nayler et al, 1984; Ashraf et al, 1982; Hearse, 1983[a]), the primary event during Ca^{2+} repletion is probably a Ca^{2+} entry via the Ca^{2+} channels which then evokes a massive secondary influx of Ca^{2+}, probably via other routes. A major difference between these two hypotheses is if Na^{+} plays a central role for the uncontrolled Ca^{2+} influx during Ca^{2+} depletion.

Despite the above dispute, Ca^{2+} paradox is used to estimate ion control mechanisms and Ca^{2+}-related morphological alterations during Ca^{2+} repletion (Grinwald and Nayler, 1981; Ruigrok, 1990)

since it exhibits similar Ca^{2+} overload as observed in ischemia model. However, one should take great caution in extrapolating the conclusions from Ca^{2+} paradox to ischemia-reperfusion injury because Ca^{2+} depletion rarely, if ever, occurs except in an experimental setting.

B. PATHOPHYSIOLOGY OF MYOCARDIAL ISCHEMIA

1. Mechanical changes during ischemia

The effects of ischemia on pressure development by the heart are now well recognized (Kubler and Katz, 1977; Nayler et al, 1985). When a beating heart is subjected to global ischemia, the developed tension or pressure starts to decline between 3 (Pirzada et al, 1975) to 10 s (Therous et al, 1974) and it falls rapidly over the first few minutes of ischemia (acute ischemic failure). After 5-20 min, the developed tension is very small or absent. Following this period, a gradual rise in diastolic pressure or resting tension occurs (ischemic contracture, Allen and Orchard, 1987; Meng and Pierce, 1991). After the ischemic contracture is well established, it may stay at the same level or slightly decrease until reperfusion begins.

2. Biochemical changes during ischemia

1) Metabolic changes during ischemia

The most significant feature of the challenge of global ischemia to the heart is the completely cessation in the delivery of oxygen and glucose to myocyte. The oxygen remaining in the

tissue as oxyhemoglobin and oxymyoglobin is used by the ischemic heart. Since this oxygen source is exhausted within 8-10 seconds (Jennings et al, 1963), mitochondria are rapidly deficient in oxygen for its oxidative phosphorylation process and no more ATP can be generated in the mitochondria. The heart must rely on its endogenous high energy phosphate reserves. Adenosine triphosphate (ATP) and creatine phosphate (CP) constitute over 90 % of the high energy phosphate reserve in the myocyte (97 umole/g dry weight tissue, Jennings et al, 1987). This amount is so small that it could only support a few efficient beats (Gordon and Morgan, 1988). However, the high energy phosphate supply is not exhausted immediately after ischemia because new high energy phosphates are produced by anaerobic glycolysis. In addition, the energy demand of the heart is also decreased due to the cessation of contraction. CP levels are reduced more quickly than ATP during ischemia (Steenbergen et al, 1987[b]). Normal [ATP] in myocardium is 6-8 mM (Allen and Orchard, 1987) or 24 umole/g dry weight tissue (Murry et al, 1990). It falls rapidly after ischemia starts (Jennings et al, 1989). In 10 min, cellular [ATP] falls to 36 % of the control level. After 40 min ischemia, [ATP] is near 3% (Murry et al, 1990). Normal adenosine diphosphate (ADP) content is 4 umole/g dry weight tissue and it increases to 150 % of control level during the early phase of ischemia because of ATP hydrolysis and falls to about 50 % of control level at the end of 40 min global ischemia (Murry et al, 1990). Inorganic phosphate (Pi) concentration (normally 1-3 mM)

exhibits a steady and linear rise during ischemia up to 20 mM (Allen et al, 1985; Williamson, 1966; Kubler and Spieckermann, 1970) mainly due to hydrolysis of adenosine phosphates.

Anaerobic metabolism serves as a major compensation mechanism for energy generation during ischemia. After ischemia begins, cytosolic phosphorylase is activated and glycogen is hydrolysed and phosphorylated to form glucose-1-phosphate which enters the glycolytic pathway and generates 3 net molecules of ATP. The initial rate of anaerobic glycolysis in ischemia is 20 times as high as the non-ischemic level (Kubler and Spieckermann, 1970; Williamson, 1966; Braasch et al, 1968). At this rate, 0.1-0.2 mM ATP/second will be produced. Although glycolysis generates 98 % of the ATP during ischemia (Jennings et al, 1987), it can only provide 8 % as much ATP as could be produced from the complete mitochondrial oxidation of the same substrate (Jennings et al, 1987). However, the high anaerobic metabolic rate is not sustained. It decelerates within 60-90 s despite the presence of glycogen stores (Rovetto et al, 1973, Jennings et al, 1990). The slowing is due to the inhibition of glyceraldehyde phosphate dehydrogenase by the high cytosolic reduced nicotinamide adenine dinucleotide-nicotinamide adenine dinucleotide ratio (NADH/NAD ratio), acidosis and high lactate levels (Neely et al, 1976). Glycolysis persists at this slow rate for 40-60 min and eventually ceases (Jennings et al, 1990). The exact cause of the cessation is not known. It seems likely that it is due to low sarcoplasmic ATP and/or allosteric and end-product inhibition of

various glycolytic reaction (Jennings et al, 1987).

The end product of glycolysis is lactate which is formed from pyruvate via catalysis of lactic dehydrogenase. Normal lactate concentration in heart is 2-5 umole/g dry weight tissue (Jennings et al, 1989; Murry et al, 1990). It can be increased to 20 umole/g dry weight tissue in the hypoxia model. Since there is no wash out during global ischemia, lactate concentration can be as high as 200-240 umole/g dry weight tissue (Murry et al, 1990; Tani and Neely, 1990[a]).

2) pH changes during ischemia

Normal intracellular pH (pH_i) in myocardium is maintained at 7.0-7.2 (Poole-Wilson, 1978; Vaughan-Jones, 1988) as protons are generated continuously from mitochondrial respiration and ATP hydrolysis. After the onset of global ischemia, pH_i starts to fall rapidly within 5 s and continuously decreases at a lower rate until the cessation of glycolysis. In severe ischemia, pH_i may fall from 0.6-1.4 pH units (Poole-Wilson, 1978; Cobbe and Poole-Wilson, 1980). Several factors contribute to the intracellular pH changes. They are glycolysis and hydrolysis of ATP and creatine phosphate. During ischemia creatine phosphate breakdown is rapid. Complete breakdown from its normal level of 20 mM would be expected to give a maximum alkalosis of 0.11 pH units (Allen and Orchard, 1987). Allen et al (1985) have observed a transient alkalosis of 0.1 pH unit in the first 1-2 min of hypoxia. The dominating factor for the pH drop is the anaerobic glycolysis which is associated with lactate

production (Wilkie, 1979). If all glycogen stores (20-50 mM glucose units, depending on species (Van der Vusse and Reneman, 1983)) were converted to lactate, it would lead to an acidosis of 0.6-1.4 pH units (Allen and Orchard, 1987). If glycolysis is inhibited, the cellular acidosis can be significantly reduced (Kupriyanov et al, 1988). The other possible H^+ source is carbon dioxide (CO_2) which is generated from mitochondrial respiration (Opie, 1976; Gevers, 1977) and neutralization of HCO_3^- by H^+ (Khuri et al, 1975; Gevers, 1977). Case et al (1979) observed that the myocardial partial pressure of carbon dioxide (PCO_2) was increased immediately after the occlusion of the coronary artery and it reached a peak of 400 mmHg in 20 min which was 6-9 fold as high as the control level. It is not clear how much the accumulation of CO_2 may contribute to cellular acidosis.

The decreased pH results in several important pathophysiological changes in the ischemic myocardium. First, it depresses heart contraction (Katz and Hecht, 1969; Weiss et al, 1984; Steenbergen et al, 1977). Low pH directly reduces the affinity of troponin C for Ca^{2+} (Blanchard and Solaro, 1984; Solaro et al, 1989). When troponin C is in the whole troponin complex or in troponin C-troponin I complex, the inhibitory effect of H^+ for Ca^{2+}-troponin C interaction will become stronger. Second, low pH induces electrophysiological abnormalities (Kagiyama et al, 1980; Weiss and Shine, 1982[a]) and prolong action potential (Orchard, 1989). Third, H^+ accumulation inhibits glyceraldehyde phosphate dehydrogenase so that anaerobic

glycolysis is slowed down (Jennings et al, 1990). Protons also stimulate ATP production by mitochondria although this effect is minimal during ischemia because of the cessation of mitochondrial respiration. Fourth, low pH inhibits ion transport pathways in sarcolemma and sarcoplasmic reticulum and may influence cytosolic ion concentration (Philipson et al, 1982; Vaughan-Jones, 1988). The details of this effect will be discussed in the following section.

Extracellular pH (normal pH 7.4) also decreases dramatically during ischemia because of the passive diffusion of lactate and CO_2 (Couper et al, 1984) or active transport of H^+. Recent study by Vanheel et al (1989) indicated that the inhibition of sarcolemmal Na^+-H^+ exchange by amiloride may result in further pH_i fall during ischemia, suggesting that H^+ extrusion via Na^+-H^+ exchange exists and contributes to extracellular acidosis during ischemia. It is considered that intra- and extracellular pH is not significantly different during ischemia challenge (Couper et al, 1984; Vanheel et al, 1989).

3) Changes of ion transport pathways
and cellular ions during ischemia

Ca^{2+}, Na^+, H^+, Mg^{2+} and K^+ all have an important role in cellular function and the regulation of contraction. Under physiological conditions, the gradient of these ions is maintained by active transport via different pathways. A disturbance of ion homeostasis could result when mechanisms for the entry and removal of ions become defective during

irreversible ischemia.

(1) Ca^{2+}: Total calcium in normal cardiac cells is in the range of 2-4 umole/g dry weight tissue (Alto and Dhalla, 1979; Meng and Pierce, 1991). Intracellular calcium is compartmentalized within sarcoplasmic reticulum (SR) (Van Winkle and Entman, 1979; Fabiato, 1983) and mitochondria (McCormack and Denton, 1986; Fry et al, 1989). It also binds to phospholipid of sarcolemma (Pierce et al, 1985) and proteins in the myoplasm (such as calmodulin and myofilaments; Solaro, 1989). In the cytosolic space, there is only 0.1-0.2 uM free Ca^{2+} during diastole and 10 uM free Ca^{2+} during systole (Solaro et al, 1974; Nayler, 1981).

Total cell Ca^{2+} does not change dramatically during the first hour of ischemia (Shen and Jennings, 1972[a]; Nayler et al, 1976; Ferrari et al, 1988). Although these global measurements of tissue Ca^{2+} show no significant changes, it is suspected that movement and distribution of free Ca^{2+} between compartments is altered and thus, free Ca^{2+} transient may be changed.

A rise in free $[Ca^{2+}]_i$ is suspected to cause lethal cell damage during irreversible ischemia (Steenbergen et al, 1987[b]). If this is true, the rise should precede the cell damage and happen in the early phase of ischemia. Furthermore, the concentration of Ca^{2+} should also be high enough to induce the damage. Jennings et al (1985[a]) considered because only 25 uM of free cytosolic Ca^{2+} was sufficient to induce ischemic contracture and because the ischemic contracture does not accompany

reversible ischemic injury in vivo, the maximal cytosolic free Ca^{+} concentration with brief ischemia could be less than 25 uM. Murphy et al (1987) observed that the $[Ca^{2+}]_i$ in monolayer, cultured myocardium increased only 3 fold after 2 h of severe hypoxia and substrate deprivation. Recently, many investigators demonstrated that an early rise (10-15 min into ischemia) in $[Ca^{2+}]_i$ using NMR (Marban et al, 1989; Steenbergen et al 1987[b]; 1990). Steenbergen et al (1987[b]) observed that the cytosolic free Ca^{2+} did not increase immediately after the onset of ischemia but increased from 0.61 $\pm$ 0.06 uM to 3.0 $\pm$ 0.3 uM in rat heart after 9-12 min global ischemia. This is further confirmed by the observation by Koretsune and Marban (1990). By using NMR technique, they demonstrated that the $[Ca^{2+}]_i$ was increased from 0.25 uM (control level) to 1.7 uM (usual systolic level) within 20-25 min of ischemia and reached a steady level between 2-3 uM by 30-35 min.

Several possibilities have been considered to explain the mechanism of this rise in $[Ca^{2+}]_i$. First, a partial depolarization occurs early during ischemia (Blake et al, 1986) which may keep the voltage-dependent Ca^{2+} channel in a constantly opening state (Steenbergen et al, 1990). Second, sarcolemmal Na^{+}-Ca^{2+} exchange is another potential mechanism to contribute to the rise of cytosolic Ca^{2+}. The Na^{+}-Ca^{2+} exchange in sarcolemmal vesicles is depressed by 30 % during ischemia (Dhalla et al, 1988). Bersohn et al (1982[a]) demonstrated that the velocity of Na^{+}-Ca^{2+} exchange after 60 min ischemia was reduced approximately

50 % due to a reduction of V_{max}. The low cellular pH during ischemia may further inhibit its in vivo activity (Philipson and Nishimoto, 1982). Since Na^+-Ca^{2+} exchange is responsible for the efflux of Ca^{2+} (Bridge et al, 1988), the decreased Na^+-Ca^{2+} exchange would favour an accumulation of intracellular Ca^{2+}. Sarcolemmal Na^+-K^+ ATPase (Na^+ pump) activity is also depressed by an ischemic insult (Bersohn et al, 1982[a]; Dhalla et al, 1988). This may promote an increase of Ca^{2+} influx via Na^+-Ca^{2+} exchange. Third, an inactivated sarcolemmal Ca^{2+} pump (Ca^{2+}-stimulated ATPase) and SR Ca^{2+} pump may be involved (Dhalla et al, 1988; Feher et al, 1980). These Ca^{2+} pumps consume ATP to eliminate Ca^{2+} from the cytosol against a Ca^{2+} gradient (Caroni and Carafoli, 1980; Carafoli, 1984). One would think that the low ATP level during ischemia could result in the inhibition of these pump. However, in vitro studies have indicated that the ATP concentration needed for half maximal activity is 0.03 mM for SL Ca^{2+} pump (Allen and Orchard, 1987) and 0.18 mM for SR Ca^{2+} pump (Shigekawa et al, 1976), respectively which are below the cellular ATP level at 40-60 min ischemia (Murry et al, 1990). Therefore, this possibility needs to be further examined. Dhalla et al (1988) reported that microsomal Ca^{2+}-stimulated pump activity was decreased by about 30 % after 90 min global ischemia. Feher et al (1981) reported a 3-4 fold decrease of SR Ca^{2+} uptake and a 7 fold decrease of SR Ca^{2+} ATPase activity after normothermic ischemia. These in vitro studies may reflect a structural lesion of the pumps by some as yet unknown mechanisms.

Fourth, acidosis may also replace Ca^{2+} from its binding sites at myofilaments (Solaro et al, 1989). Protons may have a direct inhibitory effect on SR Ca^{2+} uptake (Fabiato, 1985; Orchard, 1987). However, the relative importance of the above possible sources of cytosolic Ca^{2+} is unclear.

The effects of a high level of cellular Ca^{2+} are as follows: First, more high energy phosphates supply will be hydrolysed during the sequestration of calcium into mitochondria and SR. The excessive gain of calcium into mitochondria will also inhibit oxidative phosphorylation and worsen the imbalance in energy supply and demand (Kusouka et al, 1988). Second, high levels of cellular Ca^{2+} will activate a number of Ca^{2+} sensitive proteases, such as calpain II (Reddy et al, 1975; Toyo-Oka and Masaki, 1979; Mellgren, 1980; Rardon et al, 1990), which are capable of damaging cell membranes and cytoskeleton (Steenbergen et al, 1987[a]). Ca^{2+} can trigger the phosphorylation of a variety of cellular proteins by Ca^{2+} activated calmodulin-dependent protein kinase or by Ca^{2+}-activated phospholipid-dependent protein kinase, thus altering their functions (Katoh et al, 1983). Fourth, an increase in $[Ca^{2+}]_i$ favours triggered arrhythmias mediated by delayed afterdepolarizations (Marban et al, 1986; Ferrier et al, 1985; Coetzee et al, 1987). Finally, the increased cellular Ca^{2+} during ischemia may be related to the development of ischemic contracture (Steenbergen et al, 1990). The controversy concerning this will be discussed in the following section. However, the increase of free Ca^{2+} during

ischemia is relatively small in comparison to that during reperfusion. It is unknown how much damage the small increase in free Ca^{2+} during ischemia can cause.

(2) Na^+: The free myoplasmic concentration of Na^+ ($[Na^+]_i$) is close to 10 mM while the total cellular Na^+ content is in the range of 47-76 mmole/kg dry weight tissue (Alto and Dhalla, 1979; Pierce et al, 1990[b]) or 10 mmole/kg wet weight tissue (Poole-Wilson and Tones, 1988). Total cellular Na^+ has been shown to rise after 60 min of ischemia (Shen and Jennings, 1972[a]). Tani and Neely (1989) reported a 4 fold increase of total $[Na^+]_i$ after 30 min zero-flow ischemia in rat heart. However, Meng and Pierce (1991) demonstrated small (15-20 %) but not significant increase of total $[Na^+]_i$ after 60 min of global ischemia in rat heart. It seems that the increase of total cellular Na^+ occur only after long ischemic period.

If $[Na^+]_i$ does increase during ischemia, it should be a consequence of imbalanced Na^+ influx from extracellular space (via Na^+ channel and Na^+-Ca^{2+} exchange) and Na^+ efflux from the cytosol (via Na^+-H^+ exchange and Na^+-Ca^{2+} exchange), because the compartmentation or binding of $[Na^+]_i$ is not apparent inside the myocardium. The first candidate for an increased $[Na^+]_i$ is Na^+-H^+ exchange since its activity does not depend on ATP and the low pH environment during ischemia stimulates the exchanger. This hypothesis is supported by the observation that if the increase in $[Na^+]_i$ can be blocked by ethylisopropylamiloride, a Na^+-H^+ exchange inhibitor (Anderson et al, 1990). Partially inactivated

Na^+-K^+ ATPase during ischemia also favours Na^+ accumulation (Bersohn et al, 1982[a]; Dhalla et al, 1988). Sodium influx via voltage-dependent Na^+ channel decreases because of a partial depolarization of resting membrane potential (Steenbergen, 1990; Cole and Leblanc, 1990).

The significance of the rise in $[Na^+]_i$ is related to the higher Ca^{2+} level during ischemia. It has been observed that the time courses of the rise in $[Na^+]_i$ and $[Ca^{2+}]_i$ are the same (Pike et al, 1988; Keller et al, 1988). Furthermore, if the increase in $[Na^+]_i$ is blocked by ethylisopropylamiloride, the rise in $[Ca^{2+}]_i$ is also markedly blunted (Anderson et al, 1990). In addition, elevated $[Na^+]_i$ may enhance the release of Ca^{2+} from mitochondria via mitochondria Na^+-Ca^{2+} exchange (Fox et al, 1987).

(3) K^+: Intracellular K^+ (K^+_i) is 130 mM which is much higher than extracellular K^+ (K^+_o; 5 mM). It has been demonstrated that K^+_i is lost from ischemic myocardium (Harris, 1966; Friedrich et al, 1981; Gaspardone et al, 1986; Wilde et al, 1990). The increase in extracellular K^+ during ischemia is characterized by a triphasic time course (Weiss and Shine, 1982[b]; Kleber, 1984). In the first phase, K^+_o begins to rise rapidly within 15 s after interruption of myocardial perfusion and reaches approximately 7.5-15 mM for about 10 min (Hill and Gettes, 1980; Weiss and Shine, 1982[b]; Kleber, 1983). After a further 10 min, during which $[K^+]_o$ is constant (plateau phase; Hirche et al, 1980), a second progressive increase of K^+_o occurs. The initial rise of K^+_o is reversible upon reperfusion,

suggesting that the event is functional in nature whereas the second rise in K^+_o is associated with partial or irreversible loss of cardiac function and with cell death (Rau et al, 1977).

So far, several mechanisms have been proposed for the early loss of K^+_i. Early investigations suggest that the early K^+_i loss is due to an increased K^+ efflux co-transported with the diffusion of intracellular anions from the myocardium (Rau et al, 1977; Kleber, 1984; Gasapardone et al, 1986). During ischemia, weak acids (such as lactate and inorganic phosphate) will be generated intracellularly by the breakdown of high energy phosphates and glycogenolysis. The positively charged part (the protons) of the acids can be partially buffered by the negative charges on intracellular proteins, leaving the anionic part of the acids in the cytosol which then diffuses passively out of the cell. In order to maintain approximate electroneutrality, a cation, potassium, will also be lost from the cell (Gaspardone et al, 1986). Shine (1981) suggested that the K^+_i loss is partially due to a Na^+ pump inhibition. Recently, new evidence indicates that an ATP-regulated K^+ channel may be involved in the K^+ loss (Cole and Leblanc, 1990). The activity of the ATP-regulated K^+ channel is inversely proportional to ATP concentration. Under physiological conditions, this channel is inhibited by levels of ATP above 1 mM (Noma, 1983). When ATP falls below this critical level such as in ischemia, the open probability of the channel markedly increases, leading to K^+ loss. Although the total ATP content in myocardium is not lower than 1 mM at the early phase

of ischemia, the K^+-regulated channel could be opened if ATP is compartmentalized (Noma and Shibasaki, 1985; Weiss and Lamp, 1987). These channels are very densely distributed in the cardiac sarcolemmal membrane (Noma and Shibasaki, 1985). This dense distribution combined with the large conductance of the channel suggest that activation of as little as 2 % of the total number of the ATP-regulated K^+ channels would be necessary to account for a decline in the action potential duration (Fosset et al, 1990).

The immediate consequence of the increased sarcolemmal K^+ conductance is a partial depolarization of resting potential and a decline of action potential duration. As a result, the myocardial cells repolarize more quickly and the plateau phase shortens dramatically. This can strongly offset the net inward current produced by Na^+ and Ca^{2+} channel activation during the action potential. This decrease in the duration and magnitude of action potential results in a parallel decline in the refractory period and the velocity of conduction, creating a situation which is theoretically favourable for ventricular tachyarrhythmias (Harris et al, 1954; Curtis and Hearse,1989). Conversely, the large outward K^+ current may represent a protective mechanism because the shortened action potential can limit Ca^{2+} entry through Ca^{2+} channels and Ca^{2+} release from SR, therefore, diminishing ATP utilization by the contractile mechanism (Stern et al, 1988; Lederer et al, 1989). Cole et al (1991) has demonstrated that the functional recovery of the ischemic heart

during reperfusion could be improved by pretreatment with pinacidil, an ATP-regulated K^+ channel activator.

(4) Mg^{2+}: The total magnesium concentration in normal cardiac cell is in the range between 29-46 umole/g dry weight tissue (Shen and Jennings, 1972[a]; Alto and Dhalla, 1979; Polimeni and Buraczewski, 1988). Mg^{2+} is highly compartmentalized in the myocardium. It is estimated that approximately 12 % of cellular Mg^{2+} is in the mitochondria and 2-3 % associated with the myofibril (Bogucka and Wojtczak, 1971; Polimeni and Page, 1973; Shine, 1979). A large proportion of the intracellular Mg^{2+} is complexed with ATP, ADP and AMP. Additional Mg^{2+} is bound to enzyme-coenzyme complexes for which it plays important roles. The cytosolic free Mg^{2+} measured with NMR is estimated to be less than 1 mmole/L under normal circumstances (Levy et al, 1988; Murphy et al, 1989; Kirkels et al, 1989). Knowledge on the regulation of Mg^{2+} is very limited, the electro-chemical equilibrium for Mg^{2+} suggests that an active extrusion of Mg^{2+} is responsible for the low levels of Mg^{2+} under normal conditions (Murphy et al, 1989).

During ischemia, the total cellular Mg^{2+} does not change (Shen and Jennings, 1972[a]) while the free Mg^{2+} in the heart is rapidly and significantly increased. It has been reported that cytosolic free Mg^{2+} starts to rise within the first 5-10 min of ischemia (Kirkels et al, 1989; Borchgrevink et al, 1989). The free Mg^{2+} concentration can be 6-10 fold as high as control levels at 10-15 min of ischemia. This change in $[Mg^{2+}]$ is

inversely proportional to ATP depletion in the ischemic heart (Murphy et al, 1989). Under physiological conditions, ATP must be bound to Mg^{2+} so that it can be utilized for providing free energy or participating the catalytic processes of enzymes (Murphy et al, 1989; Kirkels et al, 1989). Therefore, the dramatic elevation of free Mg^{2+} in the ischemic heart is the consequence of Mg^{2+} release from its ATP binding sites during the process of ATP depletion (Murphy et al, 1989; Kirkels et al, 1989; Borchgrevink et al, 1989).

Physiologically, Mg^{2+} plays an essential role as a cofactor in many ATP producing and utilizing enzymatic processes (Lawson and Veech, 1979; Altura and Altura, 1985). These include Na^{+}-K^{+} ATPase activity, myofibrillar contraction, oxidative phosphorylation and several steps of glycolysis (Garfinkel et al, 1986; Opie, 1984; Polimeni and Page, 1973). Another important feature of Mg^{2+} is that it can compete with Ca^{2+} both at the sarcolemma (Shine, 1979; Bersohn et al, 1982[b]; Pierce, 1987) and at several intracellular sites, such as the troponin-C binding sites (Shine, 1979) and mitochondrial membranes (Ferrari et al, 1988). Magnesium, therefore, has been called "nature's physiologic calcium blocker" (Iseri and French, 1984). The knowledge about the pathological role of Mg^{2+} during ischemia is very limited. The sulphate and chloride salt of Mg^{2+} have long been used for treatment of atrial, junctional and ventricular tachyarrhythmias occurring in humans during myocardial ischemia (Harris et al, 1953). It was demonstrated that the tolerance of

the heart to ischemia was decreased in Mg^{2+} deficient rat hearts (Borchgrevink and Jynge, 1987; Borgchgrevink et al, 1987). Chang et al (1985) indicated that the infarction size of the heart was larger in Mg^{2+} deficient dogs. The mechanism for this is unknown. Currently, most studies concerning the importance of Mg^{2+} focus on its protective effect during reperfusion that will be dealt with in a following discussion.

4) Receptor changes during ischemia

(1) Catecholamines: The development of acute myocardial ischemia is associated with an increased release of catecholamines from the adrenal medulla and elevated sympathetic neural tone within seconds to minutes of the onset of coronary artery occlusion (Corr and Gillis, 1978[a]; Karlsberg et al, 1979). As a result, plasma catecholamines level can increase 5 times as high as control level or even more (Schomig, 1990). In experimental studies, a good correlation exists between the increased concentration of plasma catecholamines and the anatomic size of myocardial necrosis, as well as the reduction of cardiac output (Karlsberg et al, 1979). Direct effects of the increased plasma catecholamines on cell damage seems not possible since ischemic myocardial areas are not in contact with the systemic circulation and can hardly be reached by circulating catecholamines. Cell damage and malignant arrhythmia observed during ischemia are consequences of increased catecholamines within the ischemic tissue rather than in plasma (see the following discussion). However, the increased plasma

catecholamines can cause an increase in heart rate and blood pressure which may indirectly contribute to progression of cellular damage by increasing myocardial oxygen demands.

More importantly, the activation of the sympathetic nervous system also results in a large local release of catecholamines and hence, an extraordinary accumulation of catecholamines in the extracellular space of the heart. Noradrenaline accounts for 95 % of the total amount of catecholamines whereas adrenaline and dopamine together only account for 5 %. The time course of the catecholamine release can be divided into the following three phases.

Phase 1 (ischemia $\leq$ 10 min): The catecholamine release occurs by exocytosis and depends upon the activity of the efferent cardiac sympathetic nerves which is determined by two mechanisms: a) cardiovascular reflexes, induced by activation of pressor and volume receptors after a decrease in blood pressure and cardiac output and b) reflexes that are activated by afferent nerve from ischemic myocardial areas (Malliani et al, 1969). For activation of these reflexes, local acidosis, accumulation of metabolites and increased wall stretch are important factors. Although the release of catecholamines increases in the first few minutes of ischemia, the heart is prevented from an accumulation of catecholamines because of the mobilization of several mechanisms. The first mechanism is an efficient elimination of noradrenaline from the extracellular space. The excessive accumulation of catecholamines is prevented by a rapid

elimination of the released amine via neuronal uptake ($uptake_1$, Dart et al, 1984). This reuptake mechanism energetically depends upon an intact sodium gradient across the cell membrane of the sympathetic terminal. The second mechanism is a presynaptic inhibition of release by adenosine. Stimulation-induced exocytotic release of noradrenaline in ischemia is modulated via presynaptic receptors (Richardt et al, 1987). While the most effective modulation under normal condition is via $alpha_2$-receptor, in ischemia, inhibition of release by adenosine is more important. After 3 min of ischemia, the extracellular adenosine concentrations that are achieved are sufficient to inhibit noradrenaline release by two thirds (Richardt et al, 1987). This inhibition is transmitted by A_1-adenosine receptors and can be abolished by adequate inhibitors of the receptor such as 8-phenyltheophylline (Richardt et al, 1987). The third mechanism is ATP dependency of exocytosis. With the increase of ischemia duration, the sympathetic neurons become severely depleted of ATP which results in the cessation of exocytotic noradrenaline release since this release process requires high energy phosphates. This happens at the end of Phase 1.

Phase 2 (10-40 min ischemia): The release of catecholamines is determined by local energy exhaustion (metabolic catecholamine release or non-exocytotic catecholamine release) rather than by centrally originating factors. This release has been demonstrated to be a two-step process (Schomig et al, 1987; Schomig et al, 1988). In step 1, noradrenaline

escapes from the storage vesicles, resulting in enhanced axoplasmic amine concentrations. Under normal conditions, a H^+-ATPase in the vesicle membrane can generate a H^+ gradient across the membrane which provides a driving force for the uptake of noradrenaline in exchange for a proton via a reserpine-sensitive carrier (Beers et al, 1982; Phillips, 1982; Winkler et al, 1986). A disturbed neuronal energy metabolism leads to a dissipation of the potential and a loss of noradrenaline from the vesicles into the axoplasm. In step 2, noradrenaline is transported across the plasma membrane into the extracellular space via the $uptake_1$ carrier in reverse of its normal transport direction. This release is independent of calcium and can be specifically inhibited by blockers of neuronal $uptake_1$, such as desipramine (Schomig, 1990). The consequence of the metabolic release of noradrenaline is a massive accumulation of noradrenaline in the extracellular space of the ischemic myocardium. Catecholamine concentration in cardiac tissue can be 100-1000 times as high as the normal plasma concentration (Schomig, 1990) which is capable of producing myocardial necrosis even in the non-ischemic heart (Waldenstrom et al, 1978).

Phase 3 (Ischemia < 40 min): Within the ischemic area, the sympathetic neurons progressively become depleted of noradrenaline. The release occurs in parallel with the development of structural membrane defects and can no longer be blocked by inhibitors of neuronal uptake (Schomig, 1990).

The time course of catecholamine release is derived from

studies in the isolated, globally ischemic heart. It cannot be extrapolated directly to in vivo conditions because in humans, the development of catecholamine release is inhomogeneous and therefore, more complex. For example, in severely ischemic areas, non-exocytotic release may be found and at the same time, in the mildly ischemic area, release preferentially occurs via exocytosis. Thus, the catecholamine accumulation in different ischemic zones can be very different.

In a non-ischemic experimental model, excess catecholamines results in elevation of myocardial cyclic AMP (cAMP) via interaction with the beta-receptor (Thandroyen, 1990), influx of Ca^{2+} into cells, a decrease in energy stores and the development of myocardial necrosis (Fleckenstein, 1971; Opie et al, 1979). Rats given high doses of sympathetic amines, such as isoproterenol, develop myocardial necrosis with histological evidence of hypercontracted fibrils, myofibrillar lysis, swollen mitochondria and derangement of cell structure (Waldenstrom et al, 1978) which are similar to the morphological alterations caused by myocardial infarction (Waldenstrom et al, 1978). In in vivo rat hearts, noradrenaline in high doses decreases the resting membrane potential and action potential amplitude, slows conduction and produces unidirectional block. These alterations predispose the heart to reentrant arrhythmia (Gilmour and Zipes, 1980). Beta-receptor blockers, such as propranolol, prevent both the cAMP accumulation and the myocyte injury induced by epinephrine in the rat heart (Opie et al, 1979). Ca^{2+} channel

antagonists protect the rat heart against myocardial necrosis when administered at the same time that excess isoproterenol is given (Fleckenstein, 1971). These data suggest that the accumulation of catecholamine may play a role in ischemic heart injury. Despite this, these data should be interpreted with caution because they are collected from hearts which have different conditions from the ischemic heart.

(2) Beta-adrenergic receptor (beta-receptor): Stimulation of beta-receptors plays an important role in physiological responses related to cardiac contraction and excitability. During acute myocardial ischemia, beta-receptor together with G-protein and adenylate cyclase mediates catecholamine related cellular responses (Thandroyen et al, 1990). Beta-receptor numbers are increased in myocardium in the early stage of ischemia (within 15-35 min after coronary artery occlusion) (Mukherjee et al, 1982; Maisel et al, 1985; Strasser et al, 1988[a]) which are associated with intact or enhanced coupling with the adenylate cyclase enzyme and elevated levels of cAMP. Beta-receptor numbers are further increased in the late stage of ischemia (60 min or longer ischemia) and persists even after prolonged periods of global ischemia (Strasser et al, 1990[b]). This alteration can be observed both in vivo and in vitro in isolated perfused heart. Strasser et al (1990[b]) observed an approximately 60 % increase of beta-receptor after 50 min of ischemia. Mukherjee et al (1979) demonstrated that the total ^{3}H-dihydroalprinolol binding sites (beta-receptor numbers)

were doubled after 3 h of ischemia in dog's heart, but the beta-receptor was uncoupled from the adenylate cyclase enzyme at the level of the G-protein and/or catalytic subunit of the adenylate cyclase after long period of ischemia (Thandroyen et al, 1990).

Maisel and coworkers (1985) demonstrated an externalization of beta-receptors from an intracellular pool (where they were functionally uncoupled to the adenylate cyclase) to a surface pool where they were coupled to the adenylate cyclase. Muntz et al (1984) found that ischemia was associated with increases in receptor density in the sarcolemmal membrane fraction with concomitant decreases in the intracellular light-vesicle fraction. These findings suggest that retention or redistribution of beta-receptors to the cell surface rather than synthesis of new receptors may be responsible for the increase in functional receptors during acute myocardial ischemia. The mechanism responsible for the redistribution is undefined. Since the process of internalization of membrane receptors is energy dependent (Strittamatter et al, 1979), the high energy phosphate depletion during ischemia may influence the receptor externalization. Buja et al (1985) have shown a temporary relation between depletion of high energy phosphate stores and increases in beta-receptor density under condition of hypoxia. Another consideration is that the increase in receptor density results from an alteration in the membrane phospholipid composition. It has been shown that long-chain fatty acids, which may increase during hypoxia, increase adrenergic receptor

density in isolated ventricular myocytes (Heathers et al, 1987).

Complex dynamic alterations occur in the beta-receptor-G protein-adenylate cyclase system during acute myocardial ischemia. Acute myocardial ischemia sensitizes the beta-adrenergic system by two different mechanisms. One is called receptor-specific sensitization, the other one is called enzyme (adenylate cyclase)-specific sensitization. For the receptor-specific sensitization, the increase in beta-adrenergic receptors on the surface of membrane results in more receptors coupled to stimulatory G protein (G_s) and form a "ternary complex" with adenylate cyclase which consequently activates adenylate cyclase (Mori, 1976; Maisel et al, 1985; Strasser et al, 1988[b]). It was demonstrated that the G_s protein was intact in the early period of ischemia. In contrast, prolonged periods of ischemia (>1 h) result in a 27 % reduction of the G_s protein activity in vivo, indicating a structural defect of the G_s protein (Susanni et al, 1989). The enzyme-specific sensitization is tightly associated with adenylate cyclase and occurs independently of the regulation at the G-protein level or receptor level. In a partially purified preparation, adenylate cyclase retains its ischemia-induced sensitization, indicating that this increased activity may be due to a covalent modification of the enzyme (Strasser et al, 1988[a]; 1989).

In contrast to the dual sensitization of the adenylate cyclase system in early myocardial ischemia, the adenylate cyclase system becomes quite unresponsive with prolonged periods

of ischemia. This unresponsiveness occurs despite the persistent increase in functionally coupled receptors. After 1 h of global ischemia, these receptors capable of forming the ternary complex remain persistently increased, indicating that both the G-protein site of the receptor and the receptor site of the G_s protein remain intact. However, both guanosine triphosphatase activity and G_s-mediated stimulation of adenylate cyclase are greatly diminished after 1 h of myocardial ischemia (Susanni et al, 1989). In addition, the response of the adenylate cyclase to the direct stimulation of forskolin is reduced, indicating that the enzyme itself becomes inactivated after prolonged ischemia (Strasser et al, 1990[a]; 1990[b]). The functional importance of the persistent sensitization at the receptor level for the contribution to inadequate activation of ion channels is unknown.

During the early stage of ischemia, cellular cyclic adenosine monophosphate (cAMP) concentration starts to rise as a result of sensitization of the beta-receptor-G protein-adenylate cyclase cascade (Podzuweit et al, 1978). Cyclic AMP stimulates a protein kinase which phosphorylates the Ca^{2+} channel (Kameyama et al, 1985) and K channel (Walsh and Kass, 1988), thereby increasing the opening probabilities of these channels. Cellular Ca^{2+} overload after a high dose of intravenous catecholamine is probably caused by the activation of the Ca^{2+} channel (Fleckenstein, 1971; Thandroyen, 1990). Cyclic AMP is also considered to be arrhythmogenic. Ionophores of cAMP or 5'-guanylimidotriphosphate can induce slow response action

potentials in single myocardial cell (Sperelakis, 1988). High dose of noradrenaline can increase tissue cAMP levels and enhance the vulnerability to ventricular fibrillation which can be prevented by a beta-blocker, atenolol (Lubbe et al, 1978). Dibutyryl cyclic AMP mimics the arrhythmogenic action of epinephrine which can be antagonized by a Ca^{2+} channel blocker (Thandroyen, 1982). The interaction of intracellular Ca^{2+} overload, depletion of high energy phosphate and "oxygen waste" from cAMP-mediated hydrolysis of triglyceride into free fatty acids and glycerol appears to play a role in the catecholamine-induced, beta-receptor-mediated cell injury (Thandroyen et al, 1990). In spite of the above evidence, the relationship between the beta-receptor activation and ischemic cellular damage is still not clear.

(3) Alpha-adrenergic receptor (alpha-receptor): Much less is known about the alterations and the role of $alpha_1$-adrenergic receptors in acute myocardial ischemia. Using in vivo model of myocardial infarction in the dog (Mukherjee et al, 1980; 1982; 1983) and cat (Corr et al, 1978[a]; 1982; Corr and Crafford, 1981), a significant increase in alpha-adrenergic receptors in the plasma membrane derived from the ischemic zone has been demonstrated. A quite small increase can also be observed in vivo in guinea pigs (Maisel et al, 1985; 1987) whereas no change in alpha-receptor density is observed in the isolated guinea pig heart (Broadley et al, 1985). Similarly, in isolated rat heart, global ischemia failed to increase alpha-adrenergic receptors in

the plasma membrane (Strasser et al, 1989; Dillon et al, 1988). At present, it cannot be ascertained whether these inconsistent data are due to the different species or whether the ischemia-induced increase in $alpha_1$-adrenergic receptors can be observed in vivo only. These varying results on the density of $alpha_1$-receptor in ischemic myocardium are reflected by the controversial results on the role of $alpha_1$-receptor blockade in preventing ischemia-induced arrhythmias (Thandroyen et al, 1983; Dillon et al, 1988; Hamra and Rosen, 1988). The pharmacological characteristics of the $alpha_1$-receptor remain unaltered in acute myocardial ischemia. Their affinities for agonists and antagonists remain constant (Corr et al, 1978^b; 1982). As yet, no biochemical data indicate if the alpha-receptors are active or the regulatory protein (G_p) of the alpha-receptor is altered during ischemia.

3. Electrophysiological changes during ischemia

1) Action potential

Changes of cardiac electrical activity occur within a few minutes of coronary occlusion. Both resting potential and cation potential amplitude decrease, upstroke velocity slows and the action potential duration and refractory period shorten (Janse and Kleber, 1981; Kleber, 1983). When the above alterations are pronounced, a failure of conduction occurs. These rapid changes in electrical activity are accompanied by the first phase of a triphasic accumulation of extracellular

potassium (see K^+ changes in section B.2.3) for details). After 15 min of ischemia, the extracellular potassium concentration is increased from 4 mM to approximately 15 mM while the resting tension is decreased from -82 mV to -49 mV in isolated guinea pig's heart (Kleber, 1983). This suggests that the K^+ efflux in the early phase of ischemia is responsible for the resting tension changes (Carmeliet, 1978). The shortening of action potential duration is related to a metabolic inhibition during ischemia (Carmeliet, 1978). When glycolysis is prevented by glycogen depletion or by 2-deoxyglucose treatment, anoxia leads to a shortening of the action potential duration to 20 % of control within 2-3 min (McDonald et al, 1986). In a study with single myocytes, the decrease of the action potential duration could be reversed by injecting ATP into the cell (Taniguchi et al, 1983). Recently, Cole et al (1991) demonstrated that a selective ATP-regulated K^+ channel inhibitor, glybenclamide, could significantly inhibit the decline of action potential duration in the early stage of ischemia. In their investigation, 20 min of no-flow ischemia resulted in a 63 % decrease in ADP_{90} whereas treatment with 10 uM glybenclamide only produced a 24 % fall in ADP_{90}. In contrast to this, treatment with 10 uM of a selective ATP-regulated K^+ channel opener, pinacidil, caused greater decline of ADP_{90} than control. These data strongly suggest that the activation of the ATP-regulated K^+ channel during early myocardial ischemia is the major factor responsible for the collapse of the action potential after metabolic

inhibition as originally proposed by Noma (1983).

Some early studies demonstrated that a decreased Ca^{2+} current via the Ca^{2+} channel during the plateau phase of the action potential may contribute to the shortening of the action potential duration which takes place after cellular acidosis (Kohlhardt and Kleber, 1975; Kohlhardt et al, 1976). However, the time course of the decrease in pH_i may not be fast enough to account for the shortening (Allen and Orchard, 1987). Experiments by Vleugels et al (1980) suggest that increases in outward K^+ currents are quantitatively more important than reductions in inward current for the shortening of action potential duration. Ca^{2+} channel activity may also be influenced by a phosphorylation process in the early phase of ischemia (Carafoli, 1985). Due to the high concentration of extracellular catecholamines and the subsequent activation of beta-receptor, cellular cAMP level rise significantly which may, in turn, increase the phosphorylation of Ca^{2+} channel gating proteins and therefore, enhance the inward Ca^{2+} current (Lucchesi, 1989). The partial depolarization of sarcolemma may also keep the Ca^{2+} channel open for a longer time (Steenbergen et al, 1990). These effects tend to prolong the plateau phase of the action potential rather than shorten it. Therefore, it seems that the influence of the Ca^{2+} channel on the action potential is not as significant as that of K^+ efflux in the early myocardial ischemia. Furthermore, the decreased Ca^{2+} current seems likely to be a consequence rather than the cause of the action potential

shortening. In prolonged ischemia, with the pH_i decreasing and ATP level dropping, the role of the Ca^{2+} channel for the collapse of action potential will become more and more significant. Na^+ channel activity is directly associated with the membrane resting potential. When partial depolarization occurs, the velocity and magnitude of the zero-phase upstroke will decrease, leading to a slower conduction of the impulse. The role of Na^+-K^+ ATPase activity in the alteration of action potential is not clear because it is still controversial whether it is significantly inhibited in the early phase of ischemia (Shine, 1981; Kleber, 1983; 1984; Gaspardone et al, 1986).

2) Ischemic arrhythmias

Ischemic arrhythmia has been extensively reported (Conrad et al, 1959; Friedman et al, 1973; Lazzara et al, 1978; Hirche et al, 1980; Janse and Van Capelle, 1982). Kaplinsky et al (1979) found that the early ischemic arrhythmias had a biphasic change in canine heart. The first phase occurred between 2-10 min after coronary occlusion and corresponds to the time during which the action potential upstroke velocity and amplitude progressively declined. It ended when the majority of the ischemic cells were inexcitable. This was followed by a period during which sinus rhythm was predominant. The second phase began roughly between 15-20 min after coronary occlusion. It corresponded to the period during which previously inexcitable cells transiently regained the capacity to generate cation potentials. The mechanism for this is not clear. This phasic change is not

reported in other animal models.

Several mechanisms for the ischemic arrhythmias have been proposed. (1) Triggered activity: Triggered activity may be generated by either early or delayed after-depolarization. This may be a mechanism for the ectopic impulse (Cranefield, 1977). Because of technical difficulty, direct proof based on microelectrode recordings is lacking (Janse and Van Capelle, 1982). Early after-depolarization occurs during the repolarization phase of a propagated action potential. It may be present in the in situ heart under a variety of pathological conditions, such as hypoxia, elevated PCO_2 , high concentration of catecholamines, factors which all exist during acute ischemia. Furthermore, stretch, which may occur in subendocardial fibres during acute ischemia due to systolic bulging of the affected myocardium, has also been reported to induce early after-depolarization (Covell et al, 1981). Delayed after-depolarizations, which are transient or an oscillatory depolarization of an action potential, occur under conditions where there is an increase in intracellular Ca^{2+} concentration (Kass et al, 1978; Matsuda et al, 1982). (2) Re-entrant excitation: This mechanism may be the most important cause for ischemia-induced arrhythmias. As mentioned in the previous discussion, the electrophysiological changes during ischemia result in a decrease in the duration and magnitude of the action potential, causing a parallel decline in the refractory period and the velocity of conduction. These alterations constitute a

basis for the development of re-entry arrhythmias. The re-entry hypothesis now has extensive supportive evidence from both in vivo and in vitro studies (Janse et al, 1986). (3) Enhanced automaticity: This is unlikely to occur in ischemic tissue, since the automaticity is suppressed by the elevated extracellular K^+ concentration (Hoffman and Rosen, 1981; Janse et al, 1986).

4. Possible mechanisms underlying ischemic cardiac failure

The mechanism of the acute ischemic cardiac dysfunction is complicated. The consensus of a wide range of studies is that the rapid decline of developed tension or pressure of the heart is a multifactorial process.

ATP concentration is known to provide free energy for the contractile apparatus. After the onset of ischemia, ATP supply could be affected which would, therefore, influence contractile function. However, many studies have demonstrated that the intracellular ATP level falls by only a small amount (Kubler and Spieckermann, 1970; Gibbs, 1978). This has led some groups to speculate that there may be compartmentation of ATP within the myocardium which causes [ATP] fall to much lower levels in some critical region of the cells. This hypothesis is very controversial (Allen and Orchard, 1987). An alternative hypothesis is that the decline of the cardiac contraction is not directly related to [ATP]. At the beginning of ischemia, there are a decrease of creatine phosphate concentration and an increase in inorganic phosphate (Pi) and ADP concentration.

Creatine and ADP have very weak direct effects on tension development in skinned fibres (Kentish, 1986). However, Pi has been shown to have major effect on skinned cardiac fibres. Herzig and Ruegg (1977) showed that Pi exerted a pronounced inhibitory effect on maximum Ca^{2+}-activated tension. Kentish (1986) demonstrated that Pi led to a significant reduction in myofibril Ca^{2+} sensitivity. When intracellular Pi concentration increased from a normal level (1-3 mM) to 20 mM, the activated tension could be reduced to about 50 % of control. The effect of Pi on myofibril Ca^{2+} sensitivity may lead to a further reduction so that the developed tension would be about 20 % of control (Allen and Orchard, 1987). Not only is this effect large, it is also rapid in onset. Many experimental results have shown that intracellular Pi starts to rise as soon as ATP consumption exceeds ATP production or well before there is a significant change in [ATP] (Allen and Orchard, 1987).

A lower intracellular pH may cause the decrease of tension. Steenbergen et al (1977) have demonstrated that intraventricular pressure could be decreased to 20 % of control level if intracellular pH fell by 0.25 pH unit. Protons also compete with Ca^{2+} at the Ca^{2+} binding site of troponin C (Blanchard and Solaro, 1984; Solaro et al, 1986). The occurrence of cellular acidosis during ischemia is a well known phenomena (Poole-Wilson, 1978). It appears possible that a decrease in intracellular pH may be responsible for the decrease of tension. However, Jacobus et al (1982) shown that the decrease in

mechanical performance during ischemia was greater than could be accounted for by the acidosis. Allen et al (1985) demonstrated that in a hypoxia model, the prevention of glycolysis led to a rapid fall in developed tension but without acidosis. The above evidence supports the contention that the decrease of intracellular pH may not be large enough and fast enough in the early phase of ischemia to cause a major inhibition of cardiac contractility.

5. Possible mechanism underlying ischemic cardiac contracture

Ischemic contracture, which is manifested by a gradual elevation of resting tension, ensues after the early decrease of tension development in ischemia or hypoxia. Fully developed ischemic contracture is related to irreversible myocardial damage (Koretsune and Marban, 1990). The mechanisms for the ischemic contracture have been attributed to the rise of intracellular Ca^{2+} or the decrease of ATP content.

1) $[Ca^{2+}]_i$ increase and ischemic contracture

To identify the possible role of Ca^{2+} in the ischemic contracture, several important questions must be answered: (1) Does the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) rise during ischemia ?; (2) Is the increase in $[Ca^{2+}]_i$ high enough ?; and, (3) Is the time course of the $[Ca^{2+}]_i$ changes synchronous with the contracture development. As discussed in the Ca^{2+} section, cytosolic free Ca^{2+} starts to rise after 10-20 min of myocardial ischemia. It may have a 5-10 fold increase during ischemia and

may be beyond its systolic level (Steenbergen et al, 1987[b]; Koretsune and Marban, 1990). Other studies using a metabolic inhibition method (hypoxia model with inhibition of aerobic and/or anaerobic metabolism) also demonstrated that $[Ca^{2+}]_i$ increases to approximately 10 uM (Dahl and Isenberg, 1980; Snowdowne et al, 1985). Allen and Orchard (1987) estimated that in skinned fibre experiments at normal $[ATP]_i$, $[Ca^{2+}]_i$ should reach 5-10 uM to cause the "contracture". The reported Ca^{2+} concentrations in ischemia are close or within this range. However, it does not take the proton effects into consideration. Since H^+ can directly replace Ca^{2+} from its binding sites in troponin C, in ischemia, the real $[Ca^{2+}]_i$ needed for the development of the contracture would be much higher. With respect to the time course work, the results are conflicting. Few studies show that $[Ca^{2+}]_i$ dominates the development of contracture. In hypoxic or metabolic inhibition models, several investigators found that a rise in $[Ca^{2+}]_i$ consistently preceded the contracture in cultured cardiac cells (Hasin et al, 1984; Barry et al, 1987). Another study claimed that the ischemic contracture was a function of both $[Ca^{2+}]_i$ and $[ATP]_i$ (Altschuld et al, 1985). In other studies, the ischemic contracture preceded the $[Ca^{2+}]_i$ increase (Allen and Orchard, 1983; Allshire et al, 1987; Smith and Allen, 1988). Allen and Smith (1985) demonstrated in a hypoxia model after metabolic inhibition that a substantial contracture could develop without significant increase in resting $[Ca^{2+}]_i$ and about the time that the

contracture was close to maximal, the resting $[Ca^{2+}]_i$ started to rise. Cobbold and Bourne (1984) and Guarnieri (1985) reported similar results with aequorin in single cells or using ion-sensitive electrodes. In an ischemia model, Koretsune and Marban (1990) observed that the beginning of the rise in resting pressure was delayed for 15 min after $[Ca^{2+}]_i$ had reached its maximal level when $[ATP]_i$ was near zero. This inconsistency of the time course changes between $[Ca^{2+}]_i$ and the contracture does not support a role of Ca^{2+} for the contracture. In summary, current evidence cannot satisfactorily answer the questions regarding the role of intracellular Ca^{2+} for the development of the ischemic contracture. Ca^{2+} may not play a primary role for the ischemic contracture.

2) Decrease of intracellular ATP concentration ($[ATP]_i$) and ischemic contracture

It is well known that ATP is a crucial factor for muscle relaxation. A decrease in [ATP] keeps the myofibril in a rigor state, thus, relaxation is hampered. A number of experimental observations suggest that ischemic and hypoxic contracture are due to the attachment of rigor crossbridges, which are precipitated by a low $[ATP]_i$ (Haworth et al, 1981). As discussed in a preceding section (metabolic changes), $[ATP]_i$ decreases rapidly. Murry et al (1990) indicated that within 10 min, $[ATP]_i$ was 36 % of control level. Steenbergen et al (1987^b) demonstrated that after 15 min ischemia, there was no detectable ATP in rat heart. Murry et al (1990) and Koretsune and Marban

(1990) demonstrated that ATP level was near zero after 50 min ischemia. Several investigations demonstrated that rigor was observed when $[ATP]_i$ fell below 1 mM (Best et al, 1977; Rupp, 1980). The rigor state in a skinned fibre started when the ATP level fell to 0.1-1 mM (Fabiato and Fabiato, 1975; Miller and Smith, 1985). Miller and Smith (1985) also showed that the ATP level needed for the rigor state was increased when cellular ADP concentration was elevated. It should be noted that the inhomogeneity of tissue [ATP] may exist in intact heart. In NMR studies on whole hearts, contracture can be recorded from left ventricles at a time when the NMR signal from the whole heart suggests a mean $[ATP]_i$ of several mM. This may mean that in the non-working right ventricle, $[ATP]_i$ is close to normal while the working left ventricle has declined to levels capable of producing rigor. In time course studies, the start of contracture was found to be consistent with the progress of ATP depletion (Hearse et al, 1977). Haworth et al (1981) demonstrated that contracture was immediately preceded by a total loss of ATP. This was supported by the observations by Koretsune and Marban (1990) that the start of contracture is consistent with ATP depletion. Steenbergen et al (1990) demonstrated that the occurrence of ischemic contracture could begin earlier if ATP depletion was accelerated by metabolic inhibition. The evidence therefore, favours the hypothesis that a decrease of $[ATP]_i$ is the most plausible candidate for the development of ischemic contracture.

6. Morphological changes

1) Ultrastructure changes in the reversible phase of ischemia

Ultrastructure changes associated with the reversible phase of ischemia (15 min, Jennings and Reimer, 1981) reflect the metabolic changes occurring in the myocardium. There is a progressive edema in ischemic myocytes which is indicated by an increased sarcoplasmic volume. The edema can be observed with transmission electron microscopy as a clearing of the sarcoplasm. The edema appears to be due to the accumulation of osmotically active particles arising from ischemic metabolism within the myocytes. These include glycolytic intermediates, creatine, inorganic phosphate (Pi), ammonia, etc. The edema starts to develop in the myocytes as creatine phosphate (CP) is hydrolysed and can be observed by electronic microscopy after 30 s of ischemia (Jennings et al, 1985[b]). The total osmolarity produced by CP degradation from its normal level of 40 umole/g cell is 80 umole/g cell (Jennings et al, 1990) because of the formation of creatine and Pi. Simultaneously, the lactate level can increase four fold. Together, these particles may create an osmolar load which would exceed 200 milliosmoles per liter of intracellular H_2O if confined to the intracellular space (Steenbergen et al, 1985). The osmolar load can be measured directly in total ischemia by vapour-pressure osmometry (Steenbergen et al, 1985). Water enters the myocyte as a consequence of the load, expanding

sarcoplasmic volume and causing the edema. In addition, fewer glycogen granules are noted as a consequence of its use during anaerobic glycolysis. Widening of the I band of the myofibril becomes apparent, suggesting muscle relaxation. The nuclear chromatin generally is aggregated peripherally. All of these changes become marked with the progression of ischemia.

2) Ultrastructure changes in the irreversible phase of ischemia

The irreversible phase is characterized by all the findings of the reversible phase plus two important additional changes. First, the mitochondria now are markedly swollen and exhibit disorganized cristae and amorphous densities composed of lipid and perhaps denatured protein in the matrix space (Jennings et al, 1978[a]; 1983; Dhalla et al, 1988). The other noteworthy ultrastructural change involves the sarcolemma. The characteristic alteration is focal breaks in the plasma membrane, which are particularly marked over areas of cell edema. In such regions, the sarcolemma is lifted from the underlying myofibril by a subsarcolemmal bleb of edematous fluid. These blebs form as a consequence of rupture of the cytoskeletal attachment complexes, which link the plasma membrane with the underlying myofibril at each Z-line. The cell swelling seems to provide a force for the sarcolemmal rupture. In a study with isolated tissue slices, Steenbergen et al (1985) demonstrated that after 4-5 hours of incubation, there was no cell swelling and sarcolemmal rupture. When the tissue slice was placed in

hypotonic media, myocytes swell and the sarcolemma disrupted. Ashraf and Halverson (1977) found that slight intramembranous particles in sarcolemma were formed during ischemia. Subsequent reperfusion may result in further aggregation of the intramembranous particles and disruption of the sarcolemma, which was attended with the formation and extrusion of multilamellar, lipidic structures. Post et al (1988) hypothesized that these changes were a result of lateral phase separation of the membrane phospholipids and destabilization of the lipid bilayer. This reorganization of phospholipids may be induced by a decrease of the intracellular pH during ischemia and an increase of cellular Ca^{2+} during reperfusion. However, this needs direct evidence to prove. Jennings et al (1990) also proposed that the sarcolemmal disruption during ischemia was due to desegregation or disruption of the attachment complexes between the sarcolemma and the Z-band. These complexes are found to contain several cytoskeletal proteins, such as talin, integrin, alpha-actinin and vinculin. Steenbergen et al (1987^{b}) demonstrated that the normal pattern of vinculin immunofluorescence staining was lost when the sarcolemmal disruption was well developed. The mechanism for the injury of attachment complexes is unclear. Micromolar Ca^{2+}-activated protease, calpain II, was speculated to play a role in degrading the attachment complex proteins (Steenbergen et al, 1987^{b}). In addition, the I-band region of the myofibril becomes superstretched and reveals a band (N-band) which is not usually seen in healthy control tissue (Jennings et al, 1990). After 15

min of ischemia, the disruption of myofibril and sarcoplasmic reticulum became significant (Kloner et al, 1983).

C. PATHOPHYSIOLOGY OF REPERFUSION IN HEART

1. Mechanical changes during reperfusion

The post-ischemic recovery of myocardial contractile function is fully reversible if the heart was subjected to a short period of ischemia. Immediately after reperfusion begins, the resting tension may slightly and transiently increase and then returns to normal quickly. The developed tension or pressure gradually rises to a control level. However, if the heart was challenged with ischemia for long time, mechanical functional recovery will be very poor. When reperfusion starts, resting tension or pressure increases quickly and reaches its peak within 10 min (reperfusion contracture). Subsequently, it gradually and slowly decreases but never reaches its pre-ischemic level. Developed tension or pressure may have a transient rise at the beginning of reperfusion which is followed by a subsequent decline. The force or pressure generated by the heart is very poor for the rest of the reperfusion period and cannot return to a control level. Irregular heart beats are also observed.

The mechanisms for the post-ischemic cardiac dysfunction are different from those of ischemia. The reperfusion contracture is mainly attributed to a massive accumulation of cellular [Ca^{2+}] (Murphy et al, 1987; Nayler, et al, 1988) which binds to myofibril and causes shortening of the sarcomere. The

poor recovery of tension development during reperfusion is related to the failure of excitation-contraction, the disturbed regulation of cytosolic Ca^{2+} (Dhalla et al, 1988; Schoutsen et al, 1989) and the ultrastructural damage (Schaper et al, 1979). The continuous low ATP level during reperfusion may also contribute to the impaired cardiac contractile function (Schaper et al, 1979). Krause (1990) demonstrated that Ca^{2+}-sensitive myofibrillar ATPase activity was not affected significantly during reperfusion.

2. Biochemical changes during reperfusion

1) Metabolic changes during reperfusion

Restoration of oxygen and glucose supply to cardiac tissue after ischemia results in a change of the metabolic pattern from anaerobic to aerobic metabolism. Hence, ATP production is primarily through oxidative phosphorylation in mitochondria. However, the repletion of cellular ATP content is incomplete, even after 1 min of ischemia (Jennings et al, 1985[b]). Jennings et al (1987) observed that in in situ dog hearts, 5 min ischemia resulted in a 50 % fall of cellular ATP content and reperfusion increases the ATP level to 57 % of control within 5 min. In subsequent reperfusion, ATP content did not further increase. It may remain at this level for 24 hours. They also demonstrated that the longer the ischemia lasts, the poorer was the partial post-ischemic recovery of ATP. The incomplete repletion of ATP in the first minutes of reperfusion was

attributed to rephosphorylation of ADP and AMP which accumulated while the tissue was ischemic and not to the de novo synthesis of adenine nucleotide (Jennings et al, 1985[b]). Resynthesis of adenine nucleotides eventually occurs, but is incomplete even four days after 15 min ischemia (Reimer et al, 1981). The slow resynthesis rate is due primarily to the slow rate of phosphoribosyl pyrophosphate synthesis in heart because this molecule is rate-limiting for the salvage and de novo pathways of adenine nucleotide synthesis (Zimmer and Berlach, 1978). In the heart which was subjected to long time ischemia, the high energy phosphates remained depleted because mitochondria could not resynthesize ATP due to the loss of adenosine, inosine, hypoxanthine and xanthine (Jennings et al, 1978[b]).

Many studies using instantly frozen biopsies of the zone of injury have shown that creatine phosphate (CP) can reach levels 25-30 umole greater than control (35-40 umole) after a brief period of ischemia and reperfusion (Schaper et al, 1979; Swain et al, 1982; Allison and Holsinger, 1983). This has been termed as the "CP overshoot" (Ichihara and Abiki, 1984). The cause of the CP overshoot is not known at present, but it may be related to an increase in the ratio of ATP/ADP and consequent increased phosphorylation of creatine (Jennings et al, 1987). In reversible reperfusion, ADP and AMP levels decrease to a normal level within 3 min and continue to fall in the first 20 min of reperfusion (Jennings et al, 1985[b]). Lactate levels decrease near normal in the first 3 min of reversible reperfusion

(Jennings et al, 1985[b]).

2) pH changes during reperfusion

Intracellular pH (pH_i) is very low at the end of a relatively long period of ischemia (pH 5.5-6.3, Pooly-Wilson, 1978, Khandoudi et al, 1990). Introduction of reperfusion results in an increase of pH_i. Studies using NMR demonstrated that pH_i could return to normal level within the first 3 min of reperfusion (Tosaki and Braquet, 1990; Khandoudi et al, 1990). The cause of the pH_i recovery was attributed to the stimulation of sarcolemmal Na^+-H^+ exchange which extruded protons from the cytosol to the extracellular space in exchange for extracellular Na^+ (Anderson et al, 1990; Khandoude et al, 1990). In vivo studies have shown that Na^+-H^+ exchange was very sensitive to the transsarcolemmal H^+ gradient. The lower the pH_i, the higher the activity. At pH 6.0, which is a usual value at the end of 60 min global ischemia, Na^+-H^+ exchange is maximally activated (Lazdunski et al, 1985). When pH_i was decreased by loading $NHCl_4$ into myocytes, a quick recovery of pH_i occurred immediately (Arisaka et al, 1988; Vaughan-Jones, 1988; Weissberg et al, 1989; Wallert and Frohlich, 1989). The recovery was accomplished within 3-10 min. Concomitantly, intracellular Na^+ gradually increased (Kaila et al, 1987). This process could be effectively inhibited by Na^+-H^+ exchange inhibitors, such as amiloride (Kaila et al, 1987) and ethylisopropylamiloride (EIPA, Frelin et al, 1985). It was speculated that the activation of Na^+-H^+ exchange was probably one of the earliest events which occurred in

myocardium during reperfusion. It may play a very important role in triggering a cascade of pathological process, such as Ca^{2+}-overload and cardiac dysfunction which will be discussed in the following section.

3) Ion changes during reperfusion

(1) Na^+: Intracellular Na^+ concentration ($[Na^+_i]$) exhibits a fast biphasic change during reperfusion. At the beginning of reperfusion, Na^+_i quickly increases from its already elevated level during ischemia (Tani and Neely, 1989). Na^+_i can be as high as 75-80 umole/g dry weight tissue within the first several minutes of reperfusion (Meng and Pierce, 1991, Tani and Neely, 1989). This is followed by a gradual decline of Na^+_i. After 30 min of reperfusion, it can be near control level (Tani and Neely, 1989). Pike et al (1990) observed a monotonic decrease of Na^+_i during reperfusion with NMR (Pike et al, 1990). The difference for the phasic changes of Na^+ is probably due to the experimental techniques and ischemia models. The source of the Na^+ is from the extracellular space. The major pathway for Na^+ entry in the early stage of reperfusion was attributed to the stimulation of sarcolemmal Na^+-H^+ exchange (Grinwald and Brosnahan, 1987; Nayler et al, 1988; Pike et al, 1990; Crake and Poole-Wilson, 1990). During ischemia, Na^+-H^+ exchange is probably inhibited by a very low transmembrane H^+ gradient (Couper et al, 1984; Vanheel et al, 1989). When perfusion is restored, the rapid wash out of extracellular H^+ immediately results in the establishment of an outwardly directed H^+ gradient

which maximally stimulates the Na^+-H^+ exchange to extrude H^+ in exchange for extracellular Na^+, therefore, causing a temporary rise in Na^+_i (Lazdunski et al, 1985). Studies using cultured myocytes also indicated that the stimulation of Na^+-H^+ exchange by an artificial cellular acidosis accompanied a rise in Na^+_i (Kaila et al, 1987). The voltage-dependent Na^+ channel seems not to play a siginificant role for the rapid accumulation of Na^+_i because at this time, it is inhibited by the low pH_i (Gilliam et al, 1990) and its capacity to convey Na^+ seems to be low (Kim and Smith, 1986). Na^+-Ca^{2+} exchange is involved in the regulation of Na^+_i in normal myocardium (Philipson, 1985, Curtis, 1989). However, in the early stage of reperfusion, its activity is low because of the ischemic damage (Bersohn et al, 1982[a]; Daly et al, 1984; Dhalla et al, 1988) and inhibition by the intracellular acidosis (Philipson et al, 1982). Furthermore, Na^+-Ca^{2+} exchange is bidirectional in transporting either Na^+ or Ca^{2+}. When Na^+_i is high, it is likely that the Na^+-Ca^{2+} exchange functions to remove Na^+ from the cell rather than bring it in (Langer, 1982). Thus, it is unlikely that Na^+-Ca^{2+} exchange plays any major role in the Na^+ accumulation in the early phase of reperfusion. The sarcolemma permeability is not significantly changed at the early phase of reperfusion (Poole-Wilson et al, 1984), therefore, a non-specific Na^+ influx seems unreasonable. Na^+ pump (Na^+-K^+ ATPase) activity is low which may impair Na^+ efflux at this time and thus, favour Na^+_i accumulation (Dhalla et al, 1988). The Na^+_i declines late in reperfusion which reflects a decrease of Na^+

influx and an increase of Na^+ efflux. At this time, pH_i is near normal and the transsarcolemmal H^+ gradient is very small (Tosaki and Braquet, 1990; Khandoudi et al, 1990; Anderson et al, 1990). Under these conditions, Na^+-H^+ exchange and the excessive Na^+ entry will be significantly attenuated. ATP resynthesized during reperfusion can provide free energy to support a submaximal Na^+-K^+ ATPase activity to pump out Na^+ (Jennings et al, 1987). As mentioned above, sarcolemmal Na^+-Ca^{2+} exchange is sensitive to intracellular Na^+ (Philipson, 1985). The high Na^+ environment inside the myocytes during the early stage of reperfusion may activate Na^+-Ca^{2+} exchange to remove Na^+ from the cell and decrease the Na^+_i (Allen and Orchard, 1987; Crake and Poole-Wilson, 1990)。 As yet, there are no direct data about the functional and structural characteristics of Na^+-H^+ exchange during reperfusion.

(2) Ca^{2+}: Intracellular free calcium concentration returns to normal levels within several minutes of reperfusion after a short, reversible ischemia. This has been demonstrated by NMR studies (Steenbergen et al, 1987[b]; Marban et al, 1989). In contrast to this, reperfusion after long term ischemia results in severe Ca^{2+} accumulation (total cellular Ca^{2+}) inside the myocardium. The severity of the Ca^{2+} accumulation is associated with the length of the ischemic period (Shen and Jennings, 1972[b]; Whalen et al, 1974). This phenomena has been extensively observed in the ischemia-reperfusion model and the hypoxia-reoxygenation model (Shen and Jennings, 1972[b]; Poole-Wilson et

al, 1984; Nayler et al, 1988). It has been referred to as "calcium overload". Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) reaches its peak after 10-30 min of reperfusion while Na^+_i is decreasing towards normal level (Shen and Jennings, 1972[b]; Nayler et al, 1988; Tani and Neely, 1989). After the peak, $[Ca^{2+}]_i$ may gradually and slightly decrease (Shen and Jennings, 1972[b]). The redistribution of the cellular Ca^{2+} during reperfusion in the myocardium is not clear. A significant increase of mitochondrial Ca^{2+} has been observed which parallels reperfusion contracture (Wrogeman and Pena, 1976; Henry et al, 1977). Bourdillon and Poole-Wilson (1981) demonstrated that the myocardial Ca^{2+} accumulation upon reperfusion was due to an increased influx of extracellular Ca^{2+} rather than an decreased efflux of intracellular Ca^{2+}. The mechanism for the massive and uncontrolled Ca^{2+} gain is unknown. However, several possibilities exist.

First, Jennings et al (1985[a]) and Poole-Wilson et al (1984) have suggested that the ischemic episode has altered the cell membrane in some way, making it fragile. Under these conditions, the entry of a relatively small amount of Ca^{2+} during reperfusion may be sufficient to trigger a secondary chain of events, resulting in additional uncontrolled Ca^{2+} gain and loss of cellular integrity. The study by Nayler et al (1988) support this hypothesis. They suggested that the early entry of a small amount of Ca^{2+} during ischemia was probably through Na^+-Ca^{2+} exchange. Because of the energy depleted state of the

myocardium, the entry of even a small amount of additional calcium may be sufficient to raise the cytosolic concentration to a level at which the calcium-dependent proteases and phospholipases would be activated. At the same time, myofibril may contract. Under these conditions, the as yet intact, but possibly fragile sarcolemma will lose its integrity, leading to the formation of minute gaps in the sarcolemma through which Ca^{2+} enters the myocardium by inward diffusion. These investigators emphasized that even if the sarcolemma was intact at the moment of reperfusion, it may change dramatically within the first few minutes of reperfusion. However, the failure of lanthanum to penetrate into the myocardium subjected to the similar degree of ischemia seems to cast doubt on the hypothesis (Ashraf et al, 1978).

Second, Ca^{2+} channel blockers have been found to protect the heart from post-ischemic injury. This led many early investigators to speculate that Ca^{2+} might enter the myocardium through voltage-dependent Ca^{2+} channels during early reperfusion (Bush et al, 1981). However, later studies demonstrated that the protective effects of Ca^{2+} antagonists, such as verapamil, diltiazem and nifidipine were partially through the conservation of high energy phosphates prior to and during ischemia due to the inhibition of cardiac contractility (Watts et al, 1990[a]) or through vasodilatation (Watts et al, 1990[b]). If they were administered at the beginning of reperfusion, there would be no protective effect (Watts et al, 1980; Bersohn and Shine, 1983;

Watts et al, 1985). These investigations suggest that the excessive Ca^{2+} entry during post-ischemic reperfusion seems not to be primarily through the Ca^{2+} channel (Watson et al, 1985; Nasa et al, 1990).

Third, Na^+-Ca^{2+} exchange has been proposed as an important pathway for Ca^{2+} entry during reperfusion (Crake and Poole-Wilson, 1990). Several lines of evidence support this hypothesis: a) Na^+_i is high during the early post-ischemic reperfusion (Tani and Neely, 1989); b) the transsarcolemmal Na^+ gradient favours a Na^+ efflux and a Ca^{2+} influx (Nayler et al, 1988); c) Na^+_i accumulation precedes Ca^{2+} overload (Grinwald and Brosnahan, 1987; Tani and Neely, 1989); d) increase Na^+ loading by inhibiting the Na^+ pump induces more severe Ca^{2+} overload (Grinwald and Brosnahan, 1987; Meng and Pierce, 1991); e) a low Na^+ perfusate attenuates Ca^{2+} overload (Grinwald, 1982; Renlund et al, 1984); f) Inhibition of Na^+-Ca^{2+} exchange by an amiloride derivative decreases Ca^{2+} overload in the Ca^{2+} paradox model (Pierce et al, 1990[b]); g) Na^+-Ca^{2+} exchange has a relatively high capacity (Chapman, 1983) and its rate is sensitive to Na^+_i level (Langer, 1980, Philipson, 1985); h) the activity of Na^+-Ca^{2+} exchange is not ATP-dependent (Philipson, 1985, Pierce et al, 1986), thus it may function in the ATP depleted state during reperfusion; i) reperfusion with low Ca^{2+} buffer abolishes the excessive Ca^{2+} gain despite the high $[Na^+]_i$ (Tani and Neely, 1990[b]). To date, the role of Na^+-Ca^{2+} exchange as a mechanism for Ca^{2+} entry in early reperfusion has more supportive evidence than

the other two hypotheses.

In summary, probably no single route is entirely responsible for the uncontrolled entry in calcium that occurs during reperfusion but rather a combined process of separate pathways. The relative importance of these pathways still requires further study.

Ca^{2+} redistribution during reperfusion in mitochondria is suggested to be due to an excessive Ca^{2+} influx secondary to the rise in diastolic Ca^{2+}. The intramitochondrial calcium in normal cells is about 1-3 nmol/mg protein (Buja et al, 1976). The capacity of mitochondria to buffer calcium is very high, about 1,000 nmol/mg mitochondrial protein. The homeostasis of intramitochondrial Ca^{2+} depends on the balance of the influx (by calcium uniporter) and efflux (by sodium-calcium exchange). The physiological concentrations of extramitochondrial Ca^{2+} are well below the Km value for the Ca^{2+} uptake pathway so that the uniporter performs at a rate well below its maximal capacity. In contrast to this, the Km for calcium efflux through Na^{+}-Ca^{2+} exchange is lower than that through the calcium uniporter (McCormack and Denton, 1986) but still can match changes of the influx rate so that no large accumulation of calcium in the mitochondria takes place. During reperfusion or reoxygenation, massive Ca^{2+} influx across the sarcolemma can raise $[Ca^{2+}]_i$ to such a degree that massive mitochondrial calcium accumulation occurs because the efflux pathway becomes saturated while influx of calcium is increasing (McCormack and Denton, 1986). This may

serve as a short-term defense mechanism to prevent cytosolic calcium from rising too high. When the cytosolic Ca^{2+} is far beyond the capacity for mitochondria to buffer, Ca^{2+} precipitation occurs in the matrix of mitochondria (McCormack and Denton, 1986).

Ca^{2+} overload has been proposed as the most important factor to mediate the post-ischemic cell injury (Murphy et al, 1987) which includes: a) development of reperfusion contracture; b) activation of calcium-sensitive ATPases with the subsequent hydrolysis of the already depleted ATP stores (Jennings et al, 1987; Nayler et al, 1988); c) activation of Ca^{2+}-sensitive proteases and lipases which stimulate arachidonic acid metabolism and membrane disruption (Nayler et al, 1988); d) precipitation of Ca^{2+} within mitochondria (Jennings et al, 1976; Nayler et al, 1980) which inhibits substrate respiration (Lehninger, 1974) and enzymes in Kreb's cycle (McCormack and Denton, 1984); e) insufficient ATP to support SR Ca^{2+} sequestration, leading to impaired excitation-contraction coupling and force generation (Feher et al, 1980; Hess et al, 1981); f) reperfusion arrhythmia.

(3) K: Reperfusion after a short period of ischemia results in a fast reuptake of K^+ and the cellular $[K^+]$ returns to a control level (Weiss and Shine, 1982[a]). Reperfusion after a long period of ischemia results in a continuous loss of intracellular K^+ (Tani and Neely, 1989; 1990[b]; Meng and Pierce, 1991). The extent of the K^+_i loss seems proportional to the severity of reperfusion injury (Meng and Pierce, 1991). During

reperfusion, the resynthesis of high energy phosphates may induce reuptake of extracellular K^+ via sarcolemmal Na^+-K^+ ATPase. Since Na^+-K^+ ATPase is structurally damaged during ischemia and further damaged during reperfusion (Dhalla et al, 1988), it may function suboptimally for K^+ uptake. The continuous K^+ loss during reperfusion accompanies cellular enzyme leakage, suggesting that a passive diffusion of K^+_i through the ruptured sarcolemma may exist (Meng and Pierce, 1991). The role of the ATP-regulated K^+ channel for the K^+_i loss is not clear. If the [ATP] level is partially restored, it may not play a primary role for the K^+ loss during reperfusion. The decreased K^+_i is related to reperfusion arrhythmias (Manning and hearse, 1984; Witkowski and Corr, 1984; Curtis and Hearse, 1989).

(4)Mg^{2+}: Intracellular free Mg^{2+} falls rapidly in the early phase of reperfusion which is followed by a slow decline or the establishment of a steady state (Kirkels et al, 1989). After 30 min of reperfusion, free Mg^{2+} may be still 30 % higher than the pre-ischemic level (Kirkels et al, 1989; Borchgrevink et al, 1987). Reperfusion after a long period of ischemia (90 min) results in significant Mg^{2+} release from the myocardium and loss of total cellular Mg^{2+} (Ferrari et al, 1988). The decrease of free Mg^{2+} in early reperfusion is attributed to an increase of Mg^{2+} bound to ATP secondary to the new ATP synthesis (Kirkels et al, 1989). The loss of total cellular Mg^{2+} during reperfusion after long term ischemia is not clear, but may be due to leakage through sarcolemma (Kirkels et al, 1989). The pathophysiological

significance of the post-ischemic Mg^{2+} change is poorly understood. The intracellular Mg^{2+} changes during reperfusion does not limit the metabolic use of the newly synthsized ATP (Kirkels et al, 1989). Nevertheless, the high Mg^{2+} buffer prior to ischemia or during reperfusion may well exert protective effects in ischemia-reperfused heart (Bersohn and Shine, 1982[b]). Ferrari et al (1986) demonstrated that reperfusion with high Mg^{2+} (15 mM) reduced the mitochondrial Ca^{2+} overload and maintained the mitochondria ATP-producing capacity but failed to modify the increase of tissue Ca^{2+} and of diastolic pressure. Borchbrevink et al (1989) demonstrated that during reperfusion after 9 min global ischemia, the recovery of cellular high energy phosphate level was proportional to perfusate $[Mg^{2+}]$. The ATP and CP levels in groups which were reperfused with 15 mM Mg^{2+} were significantly higher than control. The group which was reperfused with 0 Mg^{2+} had the lowest levels of ATP and CP. Reperfusion without Mg^{2+} resulted in more frequent ventricular fibrillation. High $[Mg^{2+}]$ during reperfusion may conserve cellular K^{+} and depress cardiac contractility but did not improve the recovery of developed pressure. The mechanism for the complicated effects of high Mg^{2+} perfusate is very ambiguous and needs further study to clarify.

4) Generation of free radicals during reperfusion

A free radical (oxygen-derived free radical, ODFR) refers to any chemical species that has one or more unpaired electrons in its outermost orbital (Halliwell and Gutteridge,

1984). In ischemia-reperfusion injury, the radicals of primary concern include the hydroxyl radical ($OH^{\cdot}$), the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2). Under normal conditions, a small amount of ODFR can be produced via mitochondrial respiration and miscellaneous oxidative events within the cell (Sevanian and Hochstein, 1985). These ODFR can be quickly eliminated by native antioxidant mechanisms, such as the enzyme superoxide dismutase (SOD; Weiss, 1986), catalase (CAT; Marklund et al, 1982) and glutathione peroxidase (Ursini et al, 1985). No cell injury will result in the normal heart with a functioning antioxidant system (Chance et al, 1979). During ischemia, there is an accumulation of hypoxanthine from the degradation of ATP through ADP, AMP and adenosine. In normal capillary endothelium of the heart, hypoxanthine can be oxidized to xanthine and uric acid by xanthine dehydrogenase with the reduction of $NADP^+$ (Downey et al, 1988). Ischemia results in a dramatic conversion of xanthine dehydrogenase to xanthine oxidase (Parks and Granger, 1986). The metabolism of hypoxanthine by xanthine oxidase uses O_2 as an electron acceptor instead of $NADP^+$. It produces O_2^- (superoxide radical) when oxygen returns upon reperfusion (Parks and Granger, 1986). Another potential source of ODFR during reperfusion is polymorphonuclear neutrophil leukocytes (PMNL). They produce O_2^- through a NADPH oxidase reaction (Weiss, 1986). PMNLs also contain lysosomal myeloperoxidase, which uses the H_2O_2 to produce oxoacids which can in turn oxidize many cellular elements, thus, initiating a

cascade of ODFR injury (McCord, 1985). Ischemia and reperfusion have been reported to decrease the antioxidant content in myocardium. These antioxidants include reduced glutathione (Ferrari et al, 1985; Peterson et al, 1985), SOD (Ferrari et al, 1985), catalase (Peterson et al, 1985) and glutathione peroxidase (Julicher, 1984, Meerson et al, 1982). Under the above pathological conditions, the rapid production of large amounts of ODFR rapidly overcomes the already weakened antioxidant system during reperfusion and can cause cell injury.

The toxic ODFRs directly attack proteins, carbohydrates, nucleic acids and membrane lipids. ODFRs also trigger a chain reaction to form highly toxic lipid hydroperoxides which not only impairs the function of the membrane but also damages other cell components (Freeman and Crapo, 1982; Halliwell and Gutteridge, 1984; Weiss, 1986). Regional myocardial contractile function can be improved following ischemia-reperfusion by the use of ODFR scavengers (exogenous antioxidant enzymes or compounds), suggesting an role of ODFR in the post-ischemic cardiac dysfunction (Myers et al, 1985). In addition to functional impairment, structural damage by ODFR also has been shown in reperfusion injury. The size of necrotic infarct zones can be decreased by pretreatment with ODFR scavengers (Shlafer et al, 1982). ODFR may also promote reperfusion arrhythmias. Studies using the isolated rat heart demonstrates that scavengers are capable of decreasing the incidence of reperfusion arrhythmias (Woodward and Zakaria, 1985). SOD pretreatment can also

significantly improve post-ischemic repletion of cellular ATP in the heart (Das et al, 1986). In vitro studies indicated that activities of many membrane transport proteins like the SR Ca^{2+} pump (Feher et al, 1980; Kim et al, 1988), sarcolemmal Ca^{2+} pump (Kaneko et al, 1989; Dixon et al, 1990), sarcolemmal Na^{+}-Ca^{2+} exchange (Reeves et al, 1986; Shi et al, 1989), Sarcolemmal Na^{+}-K^{+} ATPase (Kukreja et al, 1990) may be inhibited by incubation with ODFR. This suggests a possible role of ODFR in reperfusion-related ion imbalance.

5) Receptor changes during reperfusion

During the first 15 min of reperfusion, the beta-receptor density remains elevated, about 65 % higher than pre-ischemic level (Mukherjee et al, 1982). Reperfusion also reverses the depressed adenylate cyclase activities in the previously ischemic myocardium (Karliner et al, 1989). In addition, intravenous isopreterenol during reperfusion increases cellular cAMP concentration and phosphorylase b to phosphorylase a conversion which is accompanied by a rise in heart rate (Mukherjee et al, 1982). Similarly, epinephrine induced increase of conversion from phosphorylase b to phosphorylase a can also be prevented by propranolol (Thandroyen et al, 1990). These data suggest a recovery of signal transduction during reperfusion through the beta-receptor-G protein-adenylate cyclase pathway in response to beta-agonist stimulation. This may regulate Ca^{2+} influx across membrane of sarcolemma and SR. However, the pathological role of beta-receptor during reperfusion is not

known.

3. Electrophysiological changes and the occurrence of arrhythmias during reperfusion

1) The electrophysiological basis of reperfusion-induced arrhythmias

Reperfusion can result in a rapid recovery in action potential duration, resting membrane potential and the velocity of 0 phase elevation (Corr and Witowski, 1983). The synchronization of depolarization may require several minutes to recover (Kaplinsky et al, 1981). The effective refractory period, which is already shortened during ischemia, can be further shortened during the early seconds of reperfusion (Manning and Hearse, 1984). The electrophysiological changes during reperfusion are not homogeneous in the heart but heterogeneous because of the spatial and temporal heterogeneity of myocardial injury during reperfusion (Boli et al, 1983). This differential return of the excitability within a previously ischemic mass may result in the generation of many small waves of depolarization which travel slowly among multiple islets of conduction block which can change position and magnitude from moment to moment and constitute the basis for reentry and ventricular fibrillation (Janse, 1982). Changes in automaticity may play a role in the genesis of reperfusion arrhythmias. Sheridan et al (1980) demonstrated that a striking increase in idioventricular rate occurred in the cat heart during reperfusion

which may be conductive to the induction of arrhythmia. In summary, different electrophysiological changes may contribute to the development of reperfusion arrhythmia.

2) Factors influencing reperfusion arrhythmias

The duration of the preceding period of ischemia is perhaps the most important determinant of the vulnerability of tissue to reperfusion arrhythmias. Crome et al (1983) described a bell-shaped time dependency curve of reperfusion arrhythmia. They found that the frequency of arrhythmias increased with the length of ischemia up to 15 min. After this time point, the reperfusion incidence of arrhythmia decreased with the length of ischemia. This phenomena was also observed by other investigators (Balke et al, 1981; Corr and Witkowski, 1983; Manning and Hearse, 1984). This leads to a speculation that potentially lethal reperfusion arrhythmias may only occur when the tissue is in a state of reversible injury and is potentially recoverable. Once irreversible injury has occurred, reperfusion may become electrophysiologically benign (Hearse, 1983[b]). Oxygen readmission during reperfusion may induce arrhythmias in the previously ischemic heart. If a globally ischemic heart is reperfused with an anoxic buffer, the reperfusion-related arrhythmias are markedly reduced and the ventricular fibrillation is totally eliminated, suggesting that oxygen is harmful to the restoration of regular rhythm (Carbonin et al, 1981; Corr and Witowski, 1983). Free radical production is closely related to the re-supply of oxygen during reperfusion and free radical

scavengers can reduce the reperfusion-induced ventricular fibrillation (Woodward and Zakaria, 1985). Therefore, free radicals may be involved in the development of the post-ischemic arrhythmias. It is not clear whether free radicals are acting directly on the cardiac rhythm. The role of potassium on reperfusion arrhythmias has been disscused in K^+ section. Calcium overload may cause delayed afterdepolarization and contribute to the increased automaticity (Corr and Witowski, 1983). High concentrations of magnesium may also reduce the vulnerability of the myocardium to reperfusion arrhythmias (Borchgrevink et al, 1989). At present, there is no firm evidence supporting a role for cAMP in the genesis of reperfusion-induced arrhythmias.

4. Morphological changes during reperfusion

During reperfusion after a brief period of reversible ischemia, the ischemic ultrastructural changes will gradually normalizes and no necrosis occurs. After 72 hour reperfusion, the I band has disappeared, nuclear changes and intermyofibrillar edema have mostly dissolved and glycogen is more abundant (Kloner et al, 1983). However, numerous small, clear vacuoles are present throughout the cells which resemble lipid droplets. Some cells still show a limited intracellular edema and mitochondria with an unfolded configuration of the cristae. By 7 days of coronary reperfusion, ultrastructure changes returned to normal

and remained normal at day 14 (Kloner et al, 1983). For irreversible ischemia, reperfusion will exacerbate the already existing ultrastructural changes, including intensified contracture and disruption of myofibril, prominent contraction band, cell swelling (Kloner et al, 1983), rupture and loss of mitochondria, the appearance of intramitochondria crystalline densities which represent a massive precipitation of calcium phosphate (Jennings and Ganote, 1976), development of large subsarcolemmal blebs of fluid, severely damaged sarcolemma in focal areas, formation of circular vesicles of ruptured sarcolemma (Jennings and Reimer, 1983, Nayler et al, 1988). Necrosis of the myocardium thus, ensues. This severe cellular disintegration is accompanied by the leakage of large proteins (such as creatine phosphokinase, lactate dehydrogenase) from intracellular space to the coronary effluent (Jennings and Reimer, 1983).

D. DRUGS THAT IMPROVE THE POST-ISCHEMIC RECOVERY OF CARDIAC FUNCTION

Many drugs have been reported to be effective in protecting ischemia-reperfusion injury in heart. Table 1 lists those which have direct effects on cardiac muscle. They are effective in different pathological models with different mechanisms. Currently, they are still being evaluated in medical laboratories. Ca^{2+} antagonists and beta-adrenergic receptor inhibitors are the most important drugs among them. These two

Table 1.

Drugs reported to improve the post-ischemic recovery of cardiac function

Drugs	Pathological Conditions	Mechanism Involved	Reference
Ca^{2+} channel inhibitors	I-R injury	Preserve energy Inhibit Ca^{2+} influx ?	Watts et al 1985
	High concentration of isopreterenol	Inhibit Ca^{2+} influx	Fleckenstein 1971
	Ischemic arrhythmia	Inhibit Ca^{2+} influx	Thandroyan 1982
	Ca^{2+} paradox	Inhibit Na^{+} influx	Tunstall et al 1986
Beta-receptor inhibitors	High concentration of isopreterenol	Inhibit Ca^{2+} influx ?	Opie et al 1979
	Epinephrine-induced ischemic arrhythmia	Reduce [cAMP]	Lubbe et al 1978
	Myocardial infarction	?	Karlsberg et al, 1979
K_{ATP} channel openers	I-R injury	Promote K^{+} efflux	Cole et al 1991
Calmodulin antagonists	I-R injury	Inhibit Na^{+}-Ca^{2+} exchange ? and protein kinase C ?	Otani et al 1989
Free radical scavengers	I-R injury I-R arrhythmia	Reduce free radicals	Sevanian et al, 1985
Amiloride	I-R injury	Inhibit Na^{+}-H^{+} exchange or Na^{+}-Ca^{2+} exchange or Ca^{2+} Channel ?	Karmazyn 1988
Adenosine A_1 receptor agonists	I-R injury	Raise ATP precursor; Coronary vasodilation	Lasley et al 1990
Transforming growth factor -beta	Tumor necrosis factor(TNF) mimicked ischemic injury	Inhibit TNF release	Lefer et al 1990

I-R: ischemia-reperfusion;

kind of drugs have been extensively used in patients with ischemic heart disease. These agents may also exert their effects through indirect mechanisms, such as vasodilation of coronary and/or systematic vasculature and sympatholysis. In experimental investigations, most drugs listed in Table 1 need pre-ischemic treatment to be effective.

E. Na^+-H^+ EXCHANGE (Na^+-H^+ ANTIPORT, Na^+-H^+ EXCHANGER)

Na^+-H^+ exchange is an ion transport system in biological membrane that catalyses the exchange of Na^+ and H^+ across the membrane in the opposite directions. Na^+-H^+ exchange was first proposed as a mechanism for renal acidification by Pitts et al (1949). The existence of such an exchange system was directly demonstrated by Murer et al (1976) in vesicles prepared from brush border membranes of kidney tubules. In the 1960's, Na^+-H^+ exchange was also proposed to be located in the inner membrane of mitochondria (Mitchell and Moyle, 1969).

The existence of Na^+-H^+ exchange in cultured cardiac cells was first reported by Piwnica-Worms and Lieberman in 1983. The existence of Na^+-H^+ exchange on cardiac sarcolemmal membrane was first demonstrated by Pierce and Philipson in 1985. Later studies indicated that Na^+-H^+ exchange was an important pathway for Na^+ uptake by cardiac cells and may also participate in the regulation of intracellular pH (Lazdunski et al, 1985). More recent data indicated that the Na^+-H^+ exchanger could be actively stimulated when the intracellular pH was lower than normal.

Na^+-H^+ exchange has been demonstrated in most, if not all animal cells and it serves as a H^+ extruding mechanism. The Na^+-H^+ exchanger is a 105-110 kilodalton glycoprotein (Sardet et al, 1990). Molecular cloning work demonstrated that the cDNA for Na^+-H^+ exchanger has a 407 bp noncoding leader, a 2,682 bp open reading frame and a 3' noncoding sequence. The Na^+-H^+ exchange protein contains 894 amino acids which represent a molecular weight of 99,354. The Na^+-H^+ exchange protein has an N-terminal amphipathic domain which contains 10 putative transmembrane segments and 2 potential glycosylation sites, followed by a hydrophilic stretch of 395 amino acid residues, presumably floating in the cytosol (Sardet et al, 1989).

1. Biochemical characteristics of Na^+-H^+ exchange

The Na^+-H^+ exchanger is imbedded in the membrane. Its transport process is independent of ATP supply (Kinsella and Aronson, 1982; Pierce and Philipson, 1985; Grinstein and Rothstein, 1986) and insensitive to transmembrane potential (Kinsella and Aronson, 1980; Pierce and Philipson, 1985). Na^+-H^+ exchange is electroneutral with a 1 H^+ : 1 Na^+ exchange ratio. This has been confirmed in intact cell systems (Cala, 1980; Moolenaar et al, 1981) and membrane vesicles (Kinsella and Aronson, 1980; Cohn et al, 1982). Thermodynamic studies indicated that the interaction of external Na^+ and H^+ with the Na^+-H^+ exchanger conforms to simple saturating Michaelis-Menten kinetics with a Hill coefficient of 1.0, suggesting a 1:1

transporting ratio and a single external binding site for Na^+ or H^+ (Burnham et al, 1982; Aronson et al, 1983). In normal conditions, intracellular H^+ concentration is much larger than extracellular H^+ concentration and the extracellular Na^+ concentration is much larger than intracellular Na^+ concentration ($[Na]_i$). Thus, the Na^+-H^+ exchange will normally operate in the Na^+_o/H^+_i exchanging mode. However, under certain conditions, the direction of exchange can be reversed by inverting the direction of the gradient (Aickin and Thomas, 1977; Vigne et al, 1982; Piwnica-Worms and Lieberman, 1983). The driving force for the operation of Na^+-H^+ exchange is considered to be from the combined chemical Na^+ and H^+ gradients (Kinsella and Aronson, 1982).

One of the most important features for Na^+-H^+ exchange is that its activity is dependent upon external Na^+ concentration. In cultured cardiac cells, the ^{22}Na uptake via Na^+-H^+ exchange is near zero when external $[Na^+]$ is lower than 1 mM. The uptake activity increases with the elevation of extracellular $[Na^+]$ in a sigmoid shaped curve in the logarithmic abscissa and reaches maximum when external $[Na^+]$ is 100 mM (Lazdunski et al, 1985).

The Na^+-H^+ exchange system is also characterized with its dependence on extracellular pH. Increasing the extracellular pH can stimulate Na^+ uptake in cultured cardiac cells. When the external pH is 6.0, the ^{22}Na uptake via Na^+-H^+ exchange is near zero. ^{22}Na uptake increases with increasing extracellular pH in a sigmoid shaped curve and reaches maximum when external pH is

near 8.5 (Lazdunski et al, 1985). The half maximum activity is at pH 7.05.

The dependence of Na^+-H^+ exchange on intracellular pH is another important biochemical feature. Reducing intracellular pH can stimulate Na^+-H^+ exchange in myocytes. When the internal pH is 7.7, no activity can be observed. At an internal pH of 7.0, its activity is maximally stimulated in cardiac cells (Frelin et al, 1985). It is noteworthy that the half maximum activity of Na^+-H^+ exchange is at an internal pH of 7.35 which is close to intracellular pH value that is maintained by cardiac cells under physiological conditions (Poole-Wilson, 1978; Roos and Boron, 1981). These data also show that the internal pH dependence of the system is very steep, its activity being all or none in less that a pH unit (from pH 7.0 to 7.7), indicating that this system can be efficiently mobilized to restore cellular pH to normal after a challenge of acidosis. Furthermore, the dependence of Na^+-H^+ exchange activity on both intra- and extracellular pH indicates that the transsarcolemmal H^+ gradient is as important as the Na^+ gradient for the regulation of Na^+-H^+ exchange activity. A higher H^+ gradient due to decreased pH_i or increased pH_o results in a higher activity for the extrusion of intracellular H^+ and influx of extracellular Na^+ via this system.

Ionic selectivity of Na^+-H^+ exchange has been studied. Li^+ and NH_4^+ are the only monovalent cations that can substitute for Na^+ in the Na^+-H^+ exchange (Moolenaar et al, 1981; Paris and Pouyssegur, 1983; Grinstein et al, 1984). The $K_{1/2}$ for

interaction of external H^+ with the Na^+-H^+ exchange is in the range from 10^{-8} to 10^{-7} M, far lower than the $K_{1/2}$ values for Li^+, NH_4^+ and Na^+ (10^{-3} to 10^{-2}, Aronson, 1985). The apparent selectivity sequence for binding of external cations is $H^+ >> Li^+ > NH_4^+ > Na^+$. There is no appreciable affinity for K^+, Rb^+, Cs^+, tetramethylammonium and choline (Aronson, 1985). Because of the above selectivity and the property of bidirectional transport, the Na^+-H^+ exchanger can mediate Na^+-Na^+ exchange or Li^+-H^+ exchange, depending upon their transmembrane gradient (Mahnensmith and Aronson, 1985). Na^+-Li^+ exchange has been identified in red blood cells and human atrial muscle (Aronson, 1983; Rasmussen et al, 1988). Ions like Mg^{2+} and Ca^{2+} can influence Na^+-H^+ exchange in cardiac sarcolemmal vesicles but it is unclear if these effects are direct or indirect (Pierce, 1987).

Developmental characteristics of Na^+-H^+ exchange have been examined. Na^+-H^+ exchange activity in the sarcolemmal vesicles isolated from newborn rabbit heart is about 40 % higher than that from adult rabbit heart (Meno et al, 1989).

2. Modulation of Na^+-H^+ exchange

1) Protons

As discussed in the above section, the pH_i range (pH 7.0-7.7) for stimulating Na^+-H^+ exchange is much narrower than pH_o (pH 6.0-8.5). This behaviour is not predicted for a simple one-for-one exchanger that obeys Michaelis-Menten kinetics and

suggests that additional effects must exist. Aronson et al (1982) postulated the existence of a second cytoplasmic H^+ binding site that allosterically activates the antiporter. Aronson et al (1982) observed that increasing intracellular H^+ can stimulate Na^+_i efflux, an effect opposite to the expected competition for the internally facing transport site. The allosteric effect of H^+_i is highly asymmetric. The extracellular acidosis fails to significantly increase Na^+ efflux. Therefore, the modifier site appears to be confined to the cytoplasmic side of the antiport, unlike the transport site(s) which must have access to both faces of the membrane. An important consequence of the operation of the modifier site is apparent in the relationship between pH_i and the rate of Na^+-H^+ exchange. The system becomes virtually quiescent at pH 7.7. This threshold is near normal physiological pH_i in cells, consistent with a central role of the Na^+-H^+ exchange in acidic pH_i homeostasis. It may also kinetically protect the cell from approaching an alkalinic cellular pH.

2) Protein Kinase C

In the 1980's, a large number of diverse stimuli were reported to modulate the activity of the Na^+-H^+ exchange in a variety of cells, such as a series of growth factors, thyroid hormone, insulin glucocorticoids, diacylglycerol, phorbol ester, cellular Ca^{2+} and osmotic shrinking. Parathyroid hormone and cyclic AMP were found to inhibit Na^+-H^+ exchange (Grinstein and Rothstein, 1986). Little is known about the specific regulating

mechanisms involved. It was noted that the effectors had a relatively slow time course for the activation or inhibition to start, compared to the virtually immediate effects of protons and Na^+. Some of them needed seconds or even hours or days. This suggested that there may be intervening biochemical reactions which involve intermediate steps. This possibility was further suggested by the reported ATP dependence of the regulation. While acid loading-induced exchange appeared to proceed in the absence of ATP, the stimulation induced by phorbol ester or shrinking were largely eliminated by ATP depletion (Grinstein et al, 1985). Many of the Na^+-H^+ exchange stimulators, like growth factors, insulin, phorbol esters and diacylglycerol, were known to stimulate tyrosine kinase or protein kinase C. Therefore, the Na^+-H^+ exchange protein or proteins capable of regulating the Na^+-H^+ exchanger could be the targets of these kinases (Grinstein and Rothstein, 1986). Stimulation of Na^+-H^+ exchange may be achieved by phosphorylation of the proteins.

3) Other regulating mechanisms

There was direct evidence of Na^+-H^+ exchange stimulation by phospholipase A in osmotically shrunken cells (Dise et al, 1980). Thus, it is possible that one of the products of phospholipid hydrolysis, such as arachidonic acid, could mediate the regulation of Na^+-H^+ exchange activity. Ca^{2+} ionophore A23187 markedly stimulated Na^+-H^+ exchange in certain type of cells, presumably through the phospholipase C and phospholipase A_2 (Villereal, 1981). It was reported that the rate of Na^+-H^+

exchange was reduced by elevating the cytosolic cAMP levels (Kahn et al, 1985). The inhibitory effect of parathyroid hormone on Na^+-H^+ exchange may be through this mechanism.

3. Physiological significance of Na^+-H^+ exchange

The most important effect of Na^+-H^+ exchange is to regulate intracellular pH. Na^+-H^+ exchange may participate many cellular functions, such as controlling cell volume (Cala, 1980), initiating growth and proliferation (Smith and Rozengurt, 1978; Koch and Leffert, 1979), participating in stimulus-response coupling in neutrophils (Molski et al, 1981), lymphocytes (Gerson and Kiefer, 1983) and platelets (Greenberg-Sepersky and Simons, 1984), mediating the cellular action of hormones (like insulin, glucocorticoids and thyroid hormone; Mahnensmith and Aronson, 1985), fertilization (Busa and Nuccitelli, 1984) and transepithelial ion transport (Murer et al, 1976).

Na^+-H^+ exchange is involved in two important physiological processes in cardiac cells: one is pH_i regulation, the other one is Na^+ uptake.

1) Regulation of pH_i in myocardium

In myocardium, protons are continuously generated by metabolism. However, the pH_i is maintained at 7.3-7.4 (Poole-Wilson, 1978). The extracellular pH (pH_o) is maintained at 7.35-7.45 and the gradient between pH_i and pH_o is relatively small. If there were only passive diffusion of H^+ out of myocardium, pH_i value would be 6.4 and $[H^+]_i$ would be 10 fold as

high as it really is. This means that a transporter is actively pumping H^+ out of the cell at normal pH (Near 7.4).

Two major pH regulating systems have been identified in cardiac cells: Na^+-H^+ exchange and Cl^--HCO_3^- exchange. In cultured myocytes, loading NH_4^+ can result in an immediate intracellular acidosis followed by a rapid restoration of pH_i which is attributed to a proton efflux through Na^+-H^+ exchange (Kaila et al, 1987). Intracellular Na^+ activity exhibits a simultaneous rise with the restoration of pH_i (Deitmer and Ellis, 1980; Kaila et al, 1987). Furthermore, this process can be significantly attenuated by amiloride, a Na^+-H^+ exchange inhibitor (Frelin et al, 1985, Arisaka, 1988). Lowering Na_o to 0.8 mM or application of 1 mM amiloride can cause a rapid cytosolic acidification (Piwnica-Worms and Lieberman, 1983). These data suggest that Na^+-H^+ exchange is the chief, if not the sole pH_i regulator in myocardium.

A Cl^--HCO_3^- system that is independent of Na^+-H^+ exchange also exists in the Purkinje fibres in heart (Vaughan-Jones, 1979; 1981; 1986). It operates to mediate Cl^- influx coupled with a HCO_3^-efflux. This process is equivalent to a net acid influx. The activity of Cl^--HCO_3^- is relatively small in the pH range between 6.7-7.2 whereas it becomes increasingly large in the pH range between 7.2-7.6 and tend to bring the alkalinic cellular pH towards neutral. The Cl^--HCO_3^- can be inhibited by 4',4'-diisothiocyanoto-stilbene-2,2'-disulphonic acid (DIDS, Vaughan-Jones, 1982, Vanheel et al, 1984) which does not affect Na^+-H^+

exchange. In summary, Na^+-H^+ exchange, as an outward acid rectifier, and Cl^--HCO_3^-, as an inward acid rectifier, are symmetrically cooperated in the control of myocardial pH. At lower pH_i, Na^+-H^+ exchange is the major pH_i regulating system on the cardiac sarcolemmal membrane.

2) Regulation of Na^+_i in the myocardium

Among the best identified Na^+ entry pathways in the cardiac cell are Na^+-H^+ exchange (Frelin et al, 1984), Na^+-Ca^{2+} exchange (Langer et al, 1982) and voltage-sensitive Na^+ channel (Renaud et al, 1983). Na^+ efflux is linked to Na^+-K^+ ATPase and Na^+-Ca^{2+} exchange. Blockade of the Na^+-K^+ ATPase increases the $[Na^+]_i$ (Deitmer and Ellis, 1978). For example, in quiescent chick cardiac cells, ouabain blockade of Na^+ efflux through the Na^+-K^+ ATPase can triple $[Na^+]_i$ as compared to control values if pH_o is higher than 7.0 (Frelin et al, 1984). This effect becomes weak when the external pH is close to 6.0. (Lazdunski et al, 1985). The increase of $[Na^+]_i$ by ouabain can be prevented by the treatment of Na^+-H^+ exchange inhibitor, EIPA. These observations strongly suggest that at physiological pH, the Na^+-H^+ exchanger appears to be a major Na^+ entry system in quiescent chick cardiac cells. Therefore, Lazdunski et al (1985) proposed that the order of efficiency of the different Na^+ transporting system in terms of the quantity of Na^+ moved was Na^+-K^+ ATPase > Na^+-H^+ exchange > Na^+-Ca^{2+} exchange. Furthermore, the effect of Na^+-H^+ exchange on $[Na^+]_i$ can in turn influence cellular $[Ca^{2+}]$. Sodium accumulation induced by Na^+-K^+ ATPase inhibitor is known to activate

sarcolemmal Na^+-Ca^{2+} exchange and trigger a Ca^{2+} entry via the Na^+-Ca^{2+} exchange (Eisner et al, 1984; Frelin et al, 1984). This Ca^{2+} uptake can be suppressed when external Na^+ is replaced by Li^+ because Li^+ cannot substitute for Na^+ in the Na^+-Ca^{2+} exchange (Renaud et al, 1983). When the cardiac cells in culture are exposed to ouabain in the presence of Na^+-H^+ exchange inhibitor, EIPA, a reduction of the ^{45}Ca uptake by about 60 % is observed (Frelin et al, 1984). The dose-response curves for the inhibition of the ouabain-induced Ca^{2+} uptake by EIPA are superimposable with the dose-response curves for EIPA inhibition of Na^+ uptake (Lazdunski et al, 1985). The mechanism for the effect of EIPA on $[Ca^{2+}]_i$ is most likely to be due to an inhibition of Na^+-H^+ exchange rather than other mechanisms. In summary, the stimulation of Na^+-H^+ exchange and/or the inhibition of Na^+-K^+ ATPase can raise the $[Na^+]_i$ which in turn increases $[Ca^{2+}]_i$ by stimulating Ca^{2+} entry via Na^+-Ca^{2+} exchange. It is not clear about the quntitative importance of Na^+ entry through voltage-dependent Na^+ channel.

4. Pathological significance of Na^+-H^+ exchange

The involvement and importance of the Na^+-H^+ exchange system in some pathophysiological processes have been examined. It has been demonstrated that the Na^+-H^+ exchange system may be involved in renal acid-base disorders (Struyvenberg et al, 1968; Seifter and Harris, 1984), essential hypertension (Haddy, 1983),

cancer (Burns and Rozengurt, 1983) and organ growth and hypertrophy (Cohn et al, 1982; Harris et al, 1984) and the diabetic cardiomyopathy (Pierce et al, 1990[a]). The role of Na^+-H^+ exchange in ischemic heart disease has been suggested by Lazdunski et al (1985). It has been discussed in the INTRODUCTION part of the thesis.

5. Na^+-H^+ exchange inhibitors

Sensitivity of the plasma membrane Na^+-H^+ exchange to a variety of transport inhibitors has been evaluated. Ouabain, furosemide, disulfonic acid stilbene derivatives and acetazolamide have no direct effect on the pathway. The best inhibitors identified to date are amiloride and its analogues. The synthesis of amiloride and its analogues was first described by Cragoe et al (1967). It was used as a potassium sparing natriuretic (Baer et al, 1967). Amiloride was initially demonstrated to inhibit the Na^+ channel present in urinary epithelia by Beckman et al (1974). In 1977, Aickin and Thomas indicated that amiloride inhibited the activity of the Na^+-H^+ exchanger. Subsequent studies showed that amiloride could also inhibit other ion transport processes (Benos 1982). As more data are obtained, it is increasingly clear that amiloride and its analogues can inhibit several Na^+ transport pathways. Further studies by Kinsella and Aronson (1981), Vigne et al (1982) and Paris and Pouyssegur (1983) revealed that Na^+ acted as a purely competitive inhibitor of amiloride action on Na^+-H^+ exchanger.

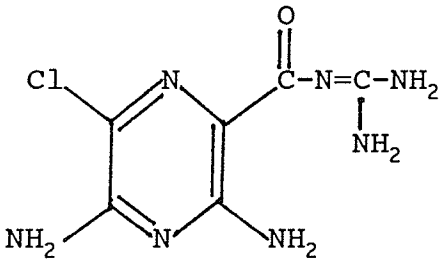
Amiloride and Na^+ compete for access to the external transport site of the Na^+-H^+ exchanger. Amiloride has been extensively used as a useful tool to probe the existence of Na^+-H^+ exchange in other tissues.

1) Chemical characteristics of amiloride and its derivatives

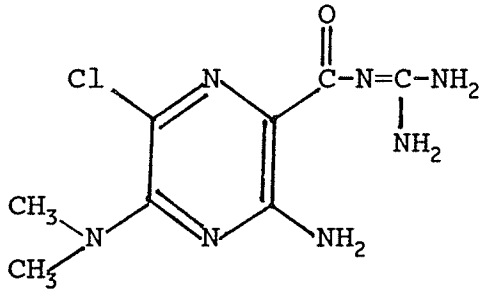
Amiloride is the prototype compound. It is composed of a pyrazine ring and a guanidine group (Figure 2). An amino group is substituted for hydrogen at position 3 and 5 of the pyrazine ring, respectively. A chloride element is substituted for the hydrogen at position 6 of the pyrazine ring. In the present study, a derivative of amiloride, 5-(N,N-dimethyl)amiloride (DMA) is used. The difference between the chemical structure of DMA and amiloride is that the two hydrogens at the amino group of 5 position in the pyrazine ring of amiloride are replaced with two methyl groups in DMA. The pK_a for amiloride and DMA is 8.7 and 8.8, respectively. Protonation of amiloride occurs on the guanidine group, but not on the ring amino groups. Thus, under physiological pH range, amiloride and DMA exist primarily as monovalent cations with positive charge resonating between the terminal amidinium fragment (Benos, 1982). Amiloride is not metabolized in the mammal. It is eliminated from urine in prototype (Benos, 1982).

2) Structure-effect relationship, potency and specificity of amiloride and its derivatives

Structure modification of amiloride can generate



Amiloride



5-(N,N-dimethyl)amiloride

Figure 2. Chemical structure of amiloride and 5-(N,N-dimethyl)-amiloride (DMA).

analogues with increased affinity and specificity for different ion transport systems. Hydrophobic substituents (benzyl or phenyl group) on the terminal nitrogen of the guanidine moiety, such as benzamil and 3',4'-dichlorobenzamil (DCB), can enhance the inhibitory effect on epithelial Na^+ channel and Na^+-Ca^{2+} exchange. Hydrophobic substituents on 5-amino moiety, such as DMA and EIPA, can produce the most potent and specific inhibitors of the Na^+-H^+ exchange. Hydrophobic substituents on either the terminal nitrogen of the guanidine moiety or 5-amino moiety, such as benzamil, DCB and EIPA, can enhance the inhibitory effects on Na^+-Ca^{2+} exchange (Kleyman and Cragoe, 1988). Amiloride is a weak Ca^{2+} channel inhibitor. Structure modification on the guanidino or 5-amino moiety can increase the affinity for binding to the channel (Garcia et al, 1990). Table 2 lists the relative specificity and potency of some amiloride derivatives in the inhibition of different ion transports. It shows that the selectivity of DMA for the inhibition of Na^+-H^+ exchanger is significantly higher than DCB and amiloride.

3) Cardiac pharmacology of amiloride

(i) Inotropism: Amiloride exerts a positive inotropic action in an isolated, blood-perfused canine papillary muscle preparation (Yamashita et al, 1981), and both positive and negative inotropic action in isolated, guinea pig left atrial muscle (Pousti and Khoyi, 1979). Kennedy et al (1986) observed that 0.7 mM amiloride produced a positive inotropic effect in left atrial muscle. The inotropic effect of amiloride started 15

Table 2.

Relative potency of amiloride and its analogues in the inhibition of different ion transports

Drug	Relative Potency Index					
	Epithelial Na^+ Channel	Na^+-H^+ Exchange	Na^+-Ca^{2+} Exchange	Na^+ Pump	Cardiac Ca^{2+} Channel	Cardiac Na^+ Channel
Amiloride	1.0 (0.34 uM)	1.0 (83.8 uM)	1.0 (1100 uM)	1.0 (>3 mM)	1.0 (90 uM)	1.0 (600 uM)
DMA	<0.035	12-24	2.0	1.0	(>50 uM)*	(>50 uM)*
EIPA	<0.035	140-223	8.5	-	30	100
DCB	4.0	-	37.0	-	-	-
Benzamil	9.0	0.08	11.0	3.0	110.0	16.0

Data are from Kleyman and Cragoe, 1988 (* data from Dennis et al, 1990). The relative potency index of amiloride for the inhibition of each ion transport pathway is designated as 1.0. Values in the parentheses represent drug concentrations needed for 50 % inhibition of that transport (IC_{50}).

min after administration and slowly reached its peak (400 % of control level) in 2 h and stayed at that level for another 2 h. At high concentration (1.0-1.5 mM), amiloride exerted a similar positive inotropic effect at the first hour which was followed by a decline in developed tension. The higher the concentration of amiloride was, the more significant the negative inotropism was. Amiloride prolongs the time to peak tensions, increases the rate of tension development and the twitch duration when it exerts its positive inotropic effect (Kennedy et al, 1986). In isolated rat heart, 0.6 and 2.5 mM amiloride exerts a transient positive inotropic effect. The developed force is decreased later in 2.5 mM amiloride group (Kolar et al, 1990). In cultured chick heart cells, 1 mM amiloride produced an immediate increase of cell motion (140 % of control level). But within several minutes, the cell motion declined. After 90 min, the cell motion was about 20 % of control level. At higher (3 mM) or lower (0.1 mM) concentrations of amiloride, cell motion began to decrease right after the administration without any transient positive inotropism (Kim and Smith, 1986). It seems that the above different inotropic responses of the heart are dependent upon the animal species and experimental models used.

Kennedy et al (1986) demonstrated that the inotropic effect of amiloride was not affected by blockers of alpha-, beta-, H_1-, H_2- and muscarinic receptor. In studies with low Na^+ perfusion, an initial positive response could be observed which may be a consequence of Na^+-Ca^{2+} exchange stimulation. This

effect of low Na^+ could be inhibited by 1.5-2.5 mM amiloride (Kennedy et al, 1986; Kolar et al, 1990), suggesting that amiloride may exert its negative inotropic effect by inhibiting Na^+-Ca^{2+} exchange. It is not clear as to the mechanism of the positive inotropic effect of amiloride. Kennedy et al (1986) suggested that it may be due to the inhibitory effect of amiloride on the Na^+ pump. The role of Na^+-H^+ exchange in the inotropism of amiloride is unclear. Toxic ouabain-induced contracture can be prevented by amiloride (Kim and Smith, 1986) and clinically, amiloride can abolish the inotropic effect of intravenous digitalis (Waldorff et al, 1981). These data suggest a decreased availability of intracellular Na^+ and a potential role of Na^+-H^+ exchange in cardiac inotropy (Kim and Smith, 1986).

(ii) Electrophysiology: In spontaneously beating atria of guinea pigs or rat heart, millimolar amiloride treatment results in a concentration-dependent negative chronotropic effect (Floreani and Luciani, 1984; Barrett and Kau, 1986). Early clinical observation showed that digitalis-induced S-T-J segment depression of the electrocardiogram could be reversed by an oral dose of amiloride (Jounela and Pyorala, 1975). Studies using microelectrodes demonstrated that amiloride could significantly prolong action potential duration without an alteration in upstroke velocity of phase 0 in Purkinje fibre (Marchese et al, 1985) and atrial muscle of guinea pig (Kennedy et al, 1986). In contrast to these, Satoh and Hashimoto (1986) demonstrated that

lower concentrations of amiloride (13-870 uM) can decrease the amplitude of the action potential and the maximal velocity of phase 0 in canine ventricular muscle. They also found that higher concentrations of amiloride could suppress the slow inward current (I_{si}) and the outward current (I_k) which may contribute to the negative chronotropic effect. The prolongation of action potential duration may contribute to its antiarrhythmic effect in the ischemia-reperfusion model (Duff et al, 1988).

(iii) Effect of amiloride on ischemia-reperfusion injury has been discussed in the INTRODUCTION part of this thesis.

4) 5-(N,N-dimethyl)amiloride (DMA)

DMA was among the first amiloride derivatives reported by Cragoe et al (1967). Its IC_{50} for the inhibition of Na^+-H^+ exchange is 7 uM (Kleyman and Cragoe, 1988). As yet, there is no systemic investigation of the effects of DMA on the myocardium. Recently, Dennis et al (1990) demonstrated an antiarrhythmic effect of DMA in isolated rat heart after ischemia-reperfusion challenge. The dose range used in the study was 10-50 uM. In a study using human atrial appendage, acid loading into the cardiac cells can induce hyperpolarization of resting membrane potential which can be reversed by acetylstrophanthidin, a Na^+-K^+ ATPase inhibitor. After treatment with 10 uM DMA, acetylstrophanthidin-induced hypopolarization can be abolished which is similar to the effect of reducing extracellular [Na^+] (Rasmussen et al, 1989). This study suggested that DMA may reduce Na^+ availability in human myocardium in an acidic environment, probably through the

inhibition of Na^+-H^+ exchange. In another study using Li^+-loaded human atrial cells, DMA (10 uM) inhibited sarcolemmal Na^+-Li^+ exchange (Rasmussen et al, 1988).

F. EXCITATION-CONTRACTION COUPLING IN MYOCARDIUM

The excitation of the cardiac sarcolemmal membrane followed by the contraction of the myofibril is the basic activity of cardiac cells. Ca^{2+} ion plays a central role in the process. Excitation-contraction coupling includes several consecutive steps which begins with membrane excitation followed by Ca^{2+} influx through the Ca^{2+} channel, Ca^{2+} release from the sarcoplasmic reticulum, the activation of myofibril by Ca^{2+} and the removal of Ca^{2+} from the cytosol.

1. Membrane excitation and the action potential: At the resting state, there is an inwardly directed electrical potential across the sarcolemmal membrane. Inside the membrane is about 90 mV more negative than outside which is caused mainly by a transsarcolemmal K^+ gradient. Once the membrane is stimulated by a propagating impulse, the negative membrane potential will be reversed, causing depolarization and an action potential. The action potential is divided into 5 phases. In phase 0, there is a rapid rise in membrane potential which is accomplished within several milliseconds. The maximal membrane potential can be +30 mV. Na^+ influx through voltage-dependent Na^+ channels is responsible for the fast depolarization. In phase 1, the action potential falls to about 0 mV which is considered to be due to a

Cl^- influx. Phase 2 is characterized by a relatively stable membrane potential near 0 mM It is also called the "plateau phase". It may last for several hundred milliseconds. The plateau phase is caused by an inward Ca^{2+} current via voltage-dependent Ca^{2+} channels and an outward K^+ current. In phase 3, a rapid repolarization of the membrane potential occurs which is mainly due to a K^+ efflux which results in a gradual recovery of the excitability for the membrane. At the end of phase 3, the membrane potential returns to its resting level. The membrane potential is maintained at this level in phase 4 when the sarcolemmal Na^+-K^+ ATPase operates to pump out Na^+ in exchange for extracellular K^+ to re-establish the ionic gradients across the sarcolemma. The cell will then be ready to respond to the next stimulus.

2. Release of Ca^{2+} from SR: During the plateau phase, there is a Ca^{2+} influx through the sarcolemmal Ca^{2+} channel. However, the amount of Ca^{2+} is too small to trigger a maximal twitch in myocardium (Pierce et al, 1985). It will trigger a subsequent release of a larger amount of Ca^{2+} from the intracellular Ca^{2+} pool, the sarcoplasmic reticulum (SR). This process is termed as calcium-induced release of Ca^{2+} (Fabiato, 1983). SR Ca^{2+} release is suggested to be through the Ca^{2+} channels in the SR membrane.

3. Activation of the myofibril: After the substantial increase of cytosolic [Ca^{2+}] secondary to the Ca^{2+} induced release of Ca^{2+}, Ca^{2+} will bind to specific sites in troponin C (Johnson et al, 1980; Putkey et al, 1989) which causes a conformational change of

troponin and releases the inhibitory effect of the troponin-tropomyosin complex on actin. Actin is then able to slide along myosin by using the energy obtained from ATP hydrolysis, leading to the shortening of the myofilament.

4. Removal of Ca^{2+} from the cytosol: Repolarization of membrane potential closes the sarcolemmal Ca^{2+} channels. The high concentration of free cytosolic Ca^{2+} during the plateau phase can stimulate SR Ca^{2+}-ATPase (Ca^{2+} pump) to sequester Ca^{2+} into the SR (Shigakawa et al, 1976). Sarcolemmal Na^{+}-Ca^{2+} exchange (Bridge et al, 1988) and Ca^{2+} pump (Caroni and Carafoli, 1980) will also be operating to extrude cytosolic Ca^{2+} during phase 4 of the action potential. These processes lower the intracellular [Ca^{2+}] down to its resting level which induces the relaxation of the myofibril and prepares the cell for the next cycle of excitation-contraction coupling.

III. MATERIAL AND METHODS

A. ANIMALS

Adult male Sprague-Dawley rats weighing 300-450 grams were used. Animals were housed (2 to 3 in a cage) in the Animal Holding of St. Boniface General Hospital Research Centre and were provided with standard rat chow and water ad libitum.

B. HEART PERFUSION PREPARATION

Male Sprague-Dawley rats, 300 to 450 g, were killed by decapitation. The cardiac muscle preparation used in this study was the isolated right ventricular wall perfused through the right coronary artery as described by Pierce et al (1987). The right ventricular free wall of the rat was dissected from the rat heart together with the ascending aorta. The ventricular wall appeared like a rectangular muscle slice (20 X 10 X 1 mm). A polyethylene cannula (1 mm in diameter) was placed into the right coronary artery and ligated with a silk thread (insert of Figure 3). The ventricular wall was then perfused through the cannula. The perfusion rate was 1.5 ml/min which was controlled by a peristaltic pump. The flow rate was held constant at the same level before and after ischemia and with or without drug perfusion. The ventricular wall was positioned horizontally with three clamps on a plexiglass rack (Figure 3). One of the clamps was connected to an isometric force transducer which measured

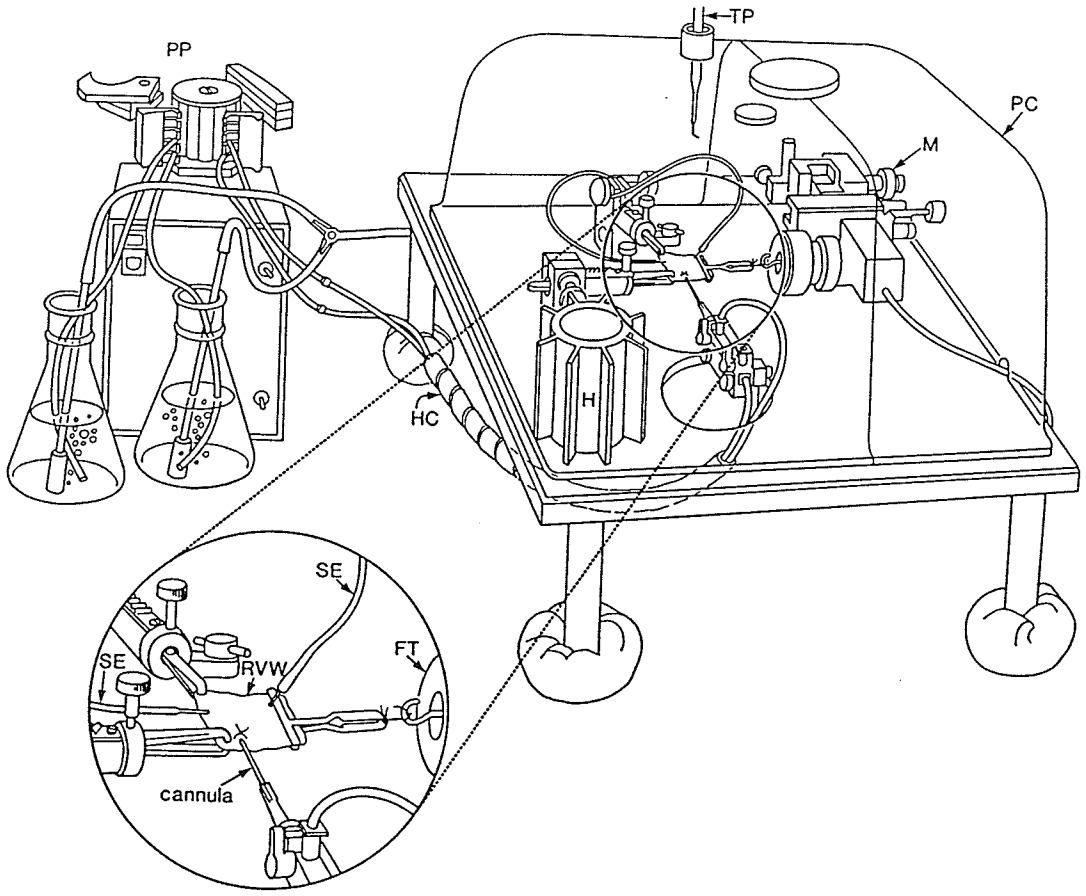


Figure 3. Experimental apparatus for the perfusion of the isolated, perfused right ventricular wall of rat. FT: Force transducer; H: Humidifier in chamber; HC: Heating coil for the perfusate lines; M: Manipulator for force transducer; PC: Plexiglas cover for chamber; PP: Peristaltic pump; SE: Stimulating electrode; TP: Temperature probe to monitor chamber air temperature during reperfusion; RVW: Right ventricular wall perfused via a cannula inserted into the right coronary artery.

resting tension (RT, grams), developed tension (DT, grams), maximal velocity of tension development (+dT/dt, grams/s) and maximal velocity of muscle relaxation (-dT/dt, grams/s) which were recorded with a polygraph (Linearcorder Mark VII WR3101, Graphtec). The right ventricular wall was paced at 200 beats/min at 200 % of threshold voltage with 9 ms of stimulation duration. Electrical stimulation was continued throughout each experiment.

The pre-ischemic level for developed tension of the ventricular wall was between 10 to 26 g and the resting tension was 6-7 g in individual ventricular walls. The ventricular wall was allowed to stabilize for about 60 min prior to ischemia. The contraction of the stabilized ventricular wall may decline by a maximum of 10-15 % during the course of the experiment (about 2 hours). To render the right ventricular wall ischemic, the perfusate flow was completely stopped by turning off the pump. During ischemia, a plexiglas cover was put on top of a plexiglas rack on which the experimental apparatus was installed. This enclosed the right ventricular wall in a chamber (36 X 26 X 18 cm). Inside the chamber was an electrical humidifier which heated water. N_2 was introduced to the humidifier through a bubbler to bubble the warm water to maintain the interior humidity (>70 %). The temperature in the chamber was kept at ($35.5 \pm 0.5^{\circ}C$) throughout the ischemic period (60 min) and was monitored with an electrical thermometer (Model BAT 8, Bailey Instrument). N_2 inside the chamber also served to reduce the possible oxygen attraction by the ischemic ventricular wall

directly from the air. Ischemia lasted for 55 or 60 min. Reperfusion was started by turning on the peristaltic pump. Reperfusion lasted for 30 min. At the end of each experiment, the visible coronary vessels and the clamped portion of the right ventricular wall was dissected out and discarded. The rest of the ventricular wall was considered to have participated in the force generation. All ventricular walls were trimmed identically. The ventricular wall was then briefly blotted and weighed. The average wet weight of the right ventricular wall was 200 $\pm$ 7 mg.

C. MEASUREMENT OF CELLULAR ION CONTENT

Intracellular ion content was measured as described by Alto and Dhalla (1979). In the study, we used exactly the same experimental conditions as stated above except that at defined time points (0 min ischemia, or the end of ischemia, or at the 6th min and /or 12th min of reperfusion), 5 ml of cold sucrose solution (350 mM sucrose, 6 mM HEPES) was injected into the coronary vessel to wash out the extracellular space. Approximately 50-150 mg wet weight tissue was saved for each ventricular wall. The tissue was placed in a plastic vial and dried at 100°C in an oven for 12-24 h. The dried tissue (about 15-35 mg) was digested in 2 ml of acid mixture (nitric acid : perchloric acid = 1:1 (vol:vol)) at 40°C for 24 h. This digestion solution was further diluted 1.25 or 2.5 fold (for

Ca^{2+}), 20 fold (for Na^{+}) and 60 fold (for K^{+}) for the absorption reading in an atomic absorbance spectrophotometer (Model 2380, Perkin Elmer).

A preliminary study was carried out, using ^{3}H-sucrose in the perfusion medium with or without the following washout procedure, to insure that the extracellular space was properly washed out. The radioactive perfusion medium contained the same components of the MOPS solution (pH 7.2) as described above + 5 mM sucrose and 0.5 uCi ^{3}H-sucrose/ml. In the first set of the experiments, the right ventricular wall was equilibrated in normal perfusate then perfused with the radioactive solution for 2 min which should be sufficient to label about 90 % of the extracellular space (Pierce et al, 1987). Then the ventricular wall was taken down, weighed and digested in 1 ml of 1 N NaOH at 80°C for 2 h in a capped plastic tube. After the tissue was completely dissolved, an aliquot of 0.1 ml of 10 N HCl was added and the digestion solution was transferred to a plastic scintillation vials containing 10 ml of scintillation media. The radioactivity was counted. In another set of experiments, the ventricular wall was perfused the same as above and then the extracellular space (the ^{3}H-sucrose portion) was washed out by injecting 5 ml of the cold sucrose solution through the coronary cannula. The muscle was then digested and its radioactivity counted. It is found that the unwashed tissue contained 625 cpm/mg wet weight tissue (n=2) whereas the washed tissue contained only 33 cpm/mg wet weight tissue (n=2). This represented a washout of 94.6 % of the ^{3}H-

labelled sucrose. Thus, our data indicated that the extracellular space could be adequately washed out with the above protocol.

D. ASSAY FOR CREATINE PHOSPHOKINASE (CPK) ACTIVITY IN CORONARY EFFLUENT

In this study, we used creatine phosphokinase (CPK) activity in the coronary effluent as a criterion to estimate the severity of cardiac damage during post-ischemic reperfusion. Normally, CPK exists in the cytosolic space of the myocardium (Jennings et al, 1983). When the sarcolemmal membrane is damaged and loses its integrity during ischemia and reperfusion, the enzyme will leak out of the cardiac cell and its activity becomes detectable in the serum or coronary effluent during reperfusion (Bester et al, 1972). Many investigators have reported that the elevation of CPK activity in serum was correlated to the severity of the damage or infarction size in heart (Staab et al, 1977; Barletta et al, 1978). At present, this method is extensively used to estimate the cardiac damage (Smith et al, 1988; Prasad et al, 1989; Sklar et al, 1991) although some investigators reported disassociation between the elevation of serum CPK and the myocardial lesion in in vivo study (Hjelms et al, 1987). The limitations of the method are 1) the CPK release from skeletal muscle (such as open-chest operation) may provide false information about the cardiac damage and 2) the increase of CPK

exhibits a phasic rather than a constant pattern (Bester et al, 1972; Staab et al, 1977). In the present study, we used isolated right ventricular wall. Therefore, the influence of CPK release from skeletal muscle could be excluded. We also observed in a preliminary experiment the changes of CPK during the reperfusion period. The peak activity of CPK was between 4-10 min of reperfusion (data not shown). Thus, we chose this time period to collect coronary effluent in subsequent experiments. The effluent was saved in plastic vials and stored at 4°C for a maximum of 48 h before the analysis of creatine phosphokinase activity (Okinaka et al, 1961).

E. CHEMICALS AND SOLUTIONS

The bicarbonate perfusate contained the following components (mM): NaCl 120; KCl 6; $MgCl_2$ 1; $CaCl_2$ 1; $NaHCO_3$ 20; dextrose 10 (pH 7.4) and was bubbled with 95% O_2 and 5% CO_2.

The HEPES perfusate contained the following components (mM): NaCl 140; KCl 6; $MgCl_2$ 1; $CaCl_2$ 1; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 6; dextrose 10 (pH 7.4) and was bubbled with 100 % O_2.

The MOPS perfusate contained the following components (mM): NaCl 140; KCl 6; $MgCl_2$ 1; $CaCl_2$ 1; 3-[N-Morpholino]propanesulfonic acid (MOPS) 6; dextrose 10 (pH 7.2) and was bubbled with 100 % O_2. When necessary, the solution pH was adjusted to pH 6.5 with HCl or pH 7.9 with NaOH. The amount of Na^+ added with NaOH is less than 1 mM.

The above solutions were stored at 4°C and the pH of the above solutions was carefully adjusted before and/or during each individual experiment. The perfusion temperature of the above solution was 37 ± 0.5 °C which was calibrated before each experiment.

The sucrose-HEPES solution contained the following components (mM): sucrose 350; HEPES 6. The solution was filtered through a Dowex 50 column to eliminate residual ions and stored at 4°C before use.

All chemicals were of standard reagent grade from Sigma Chemical Company, St. Louis, MO.. 5-(N,N-dimethyl)amiloride hydrochloride was obtained from Research Biochemicals Incorporated, Natick, MA. It was dissolved directly in the perfusate (100 uM). The DMA solution was stored at 4°C and protected from light during storage and during the experiment. It was further diluted to the concentrations needed before each experiment.

F. STATISTICS

Data were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. Values were expressed in mean ± SE.

IV. RESULTS

A. EFFECTS OF DMA ON CARDIAC CONTRACTILE FUNCTION DURING ISCHEMIA-REPERFUSION

1. Effect of DMA in HEPES solution on the recovery of cardiac contractile function during post-ischemic reperfusion

The purpose of this set of experiments was to examine if DMA could protect the ventricular wall from ischemia-reperfusion injury. The ventricular walls were perfused with HEPES solution (pH 7.4) and were subjected to 60 min global ischemia followed by 30 min reperfusion. The ventricular walls were divided into 4 groups: a) drug-untreated; b) treatment with 1 uM DMA for 3 min before and 3 min after ischemia; c) treatment with 5 uM DMA for 3 min before and 3 min after ischemia and d) treatment with 20 uM DMA for 3 min before and 3 min after ischemia. The experimental recordings of the ventricular wall are shown in Figure 4.

1) Resting tension (RT)

Figure 5 shows that before ischemia, the resting tension in the perfused right ventricular wall was 30.3 $\pm$ 1.7 g/g tissue. The RT of the ventricular wall began rising after 30 min ischemia in the drug-untreated group (data not shown) and at the end of 60 min global ischemia, RT was significantly elevated to 174 $\pm$ 8 % of the pre-ischemic level. RT increased to its peak (273 $\pm$ 12 % of the pre-ischemic level) after 6 min of reperfusion. When DMA was included in the perfusate 3 min prior to ischemia and for the

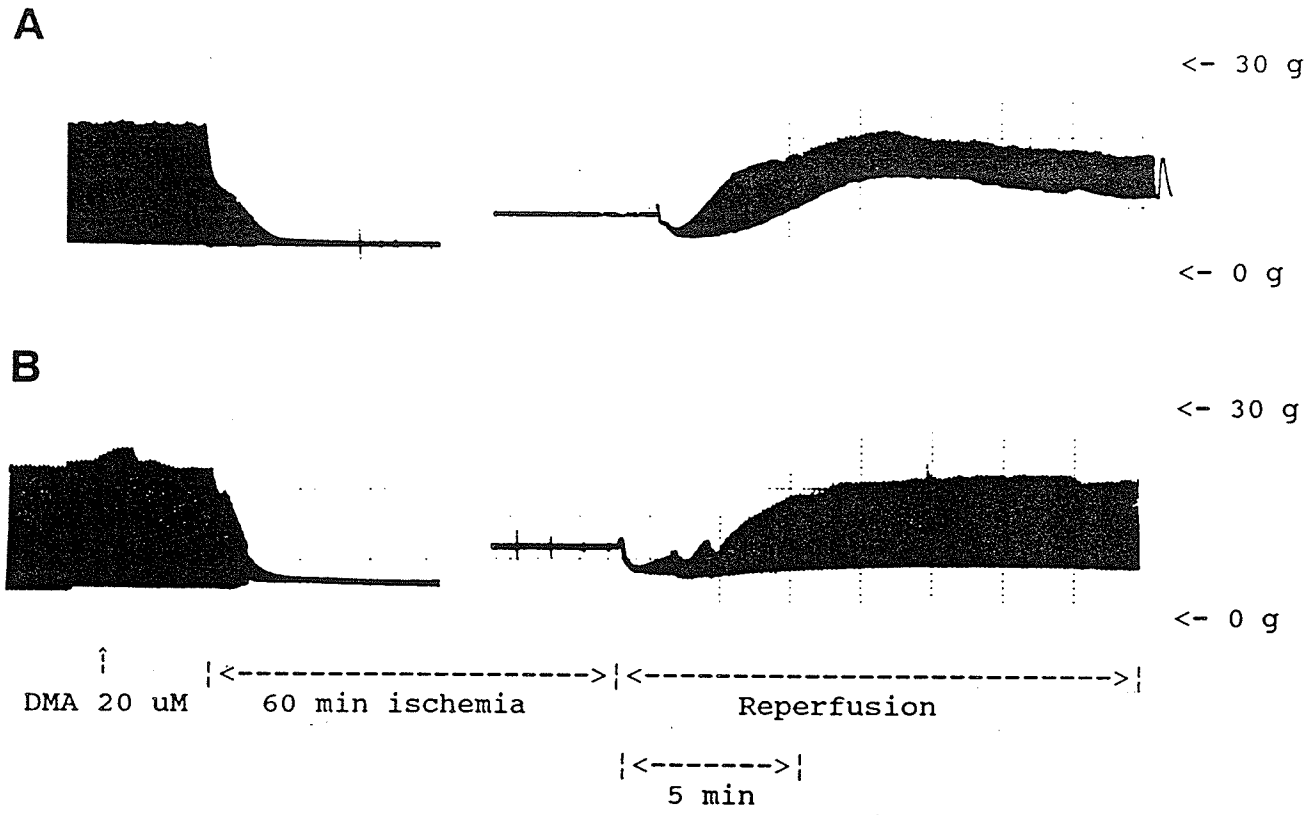


Figure 4. Experimental recordings of the ventricular wall contractile function during ischemia-reperfusion challenge.

Panel A: drug-untreated ventricular wall. Note the resting tension rises and the recovery of developed tension is poor during reperfusion.

Panel B: DMA treatment for 3 min prior to and after ischemia. The rise in resting tension is prevented and the recovery of developed tension is improved by the application of DMA.

first 3 min of reperfusion, the elevation of RT at the end of the 60 min ischemic period was not significantly changed in comparison to drug-untreated group. However, RT was significantly lowered by 1-20 uM DMA during reperfusion in a dose-dependent manner. Twenty uM DMA exhibited the best protective effect, reducing RT to near pre-ischemic levels.

2) Developed tension (DT), +dT/dt and -dT/dt

Developed tension in drug-untreated ventricular wall was severely attenuated by 60 min global ischemia and reperfusion (Figure 6). The DT value in non-ischemic and drug-untreated hearts was 83.7 $\pm$ 6.7 g/g wet weight tissue. The maximal recovery of DT was 28 $\pm$ 4 % of the pre-ischemic levels after 4 min of reperfusion and it was maintained at this level until the end of the 30 min reperfusion (Figure 6). DMA treatment (5 or 20 uM) for 3 min prior to ischemia and 3 min into reperfusion was beneficial in a dose-dependent manner for the recovery of DT. Similar qualitative responses to DMA were demonstrated in the changes of both +dT/dt (Figure 7) and -dT/dt (Figure 8) in the ventricular wall. The pre-ischemic values of +dT/dt and -dT/dt were 2170 $\pm$ 192 and 818 $\pm$ 37 g/s/g wet weight tissue, respectively.

2. Effect of DMA in HCO_3^- solution on the recovery of cardiac contractile function during post-ischemic reperfusion (Table 3)

Since the majority of the present study was done by using

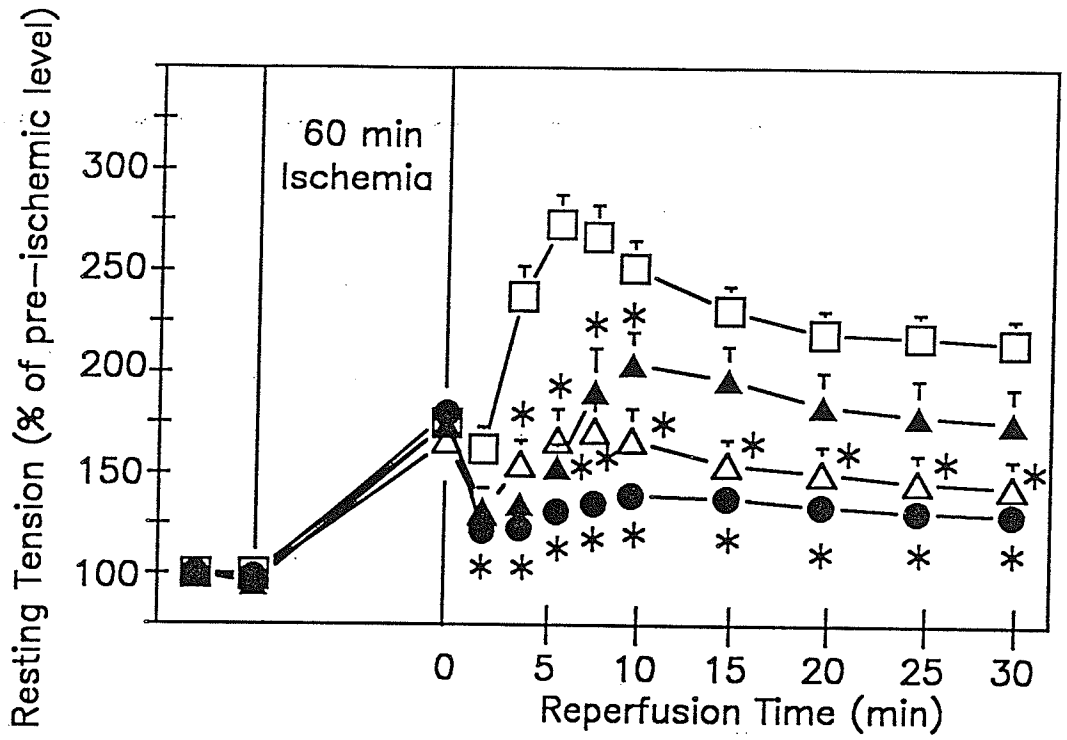


Figure 5. Dose-dependent effect of DMA on the recovery of resting tension in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion. DMA was included in the perfusate for 3 min prior to ischemia and for the first 3 min of reperfusion.

Open square: drug-untreated group (n=10);
Filled triangle: DMA 1 uM (n=3);
Open triangle: DMA 5 uM (n=7);
Filled cycle: DMA 20 uM (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

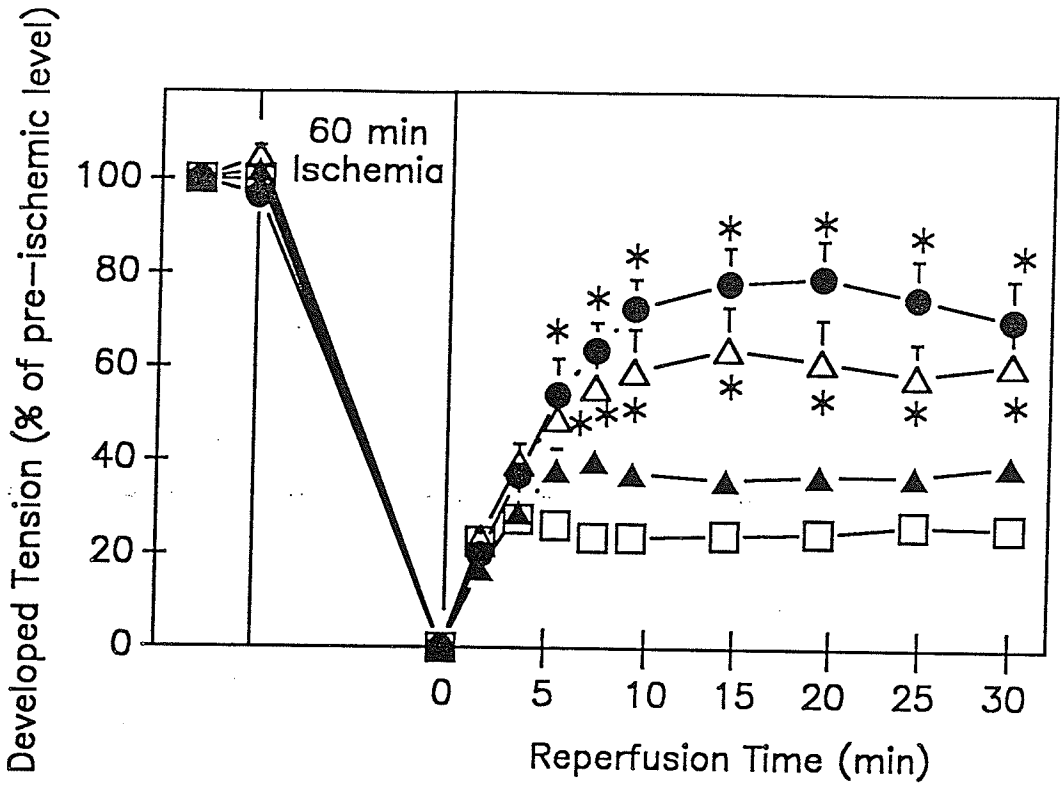


Figure 6. Dose-dependent effect of DMA on the recovery of developed tension in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion. DMA was included in the perfusate for 3 min prior to ischemia and for the first 3 min of reperfusion.

Open square: drug-untreated group (n=10);
Filled triangle: DMA 1 uM (n=3);
Open triangle: DMA 5 uM (n=7);
Filled cycle: DMA 20 uM (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

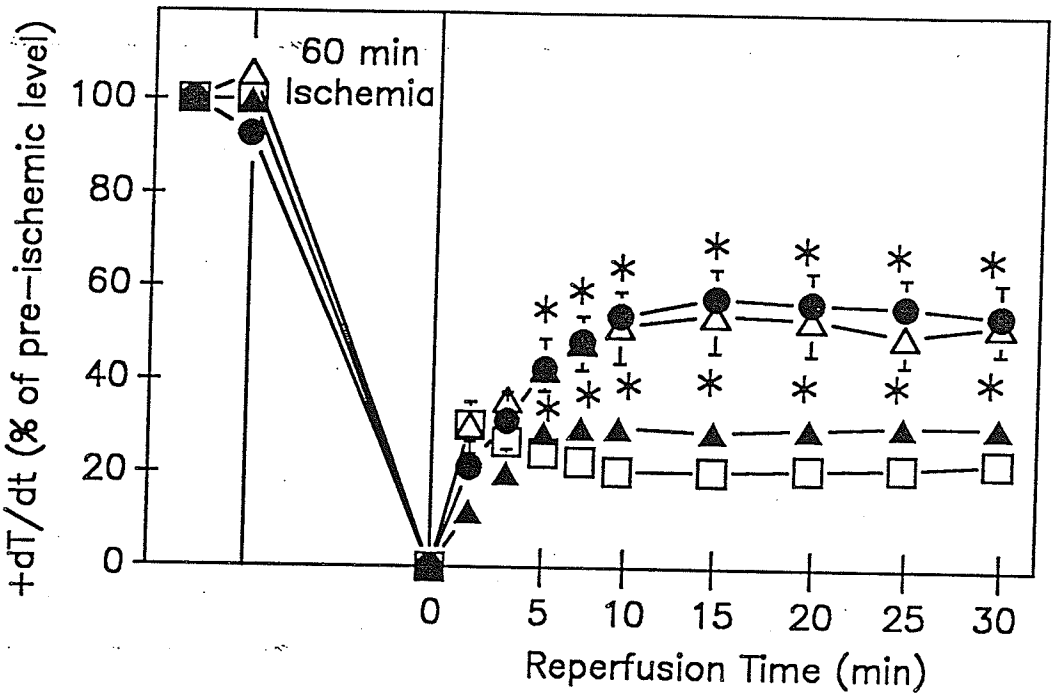


Figure 7. Dose-dependent effect of DMA on the recovery of the maximal velocity of tension development (+dT/dt) in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion. DMA was included in the perfusate for 3 min prior to ischemia and for the first 3 min of reperfusion.

Open square: drug-untreated group (n=10);
Filled triangle: DMA 1 uM (n=3);
Open triangle: DMA 5 uM (n=7);
Filled cycle: DMA 20 uM (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

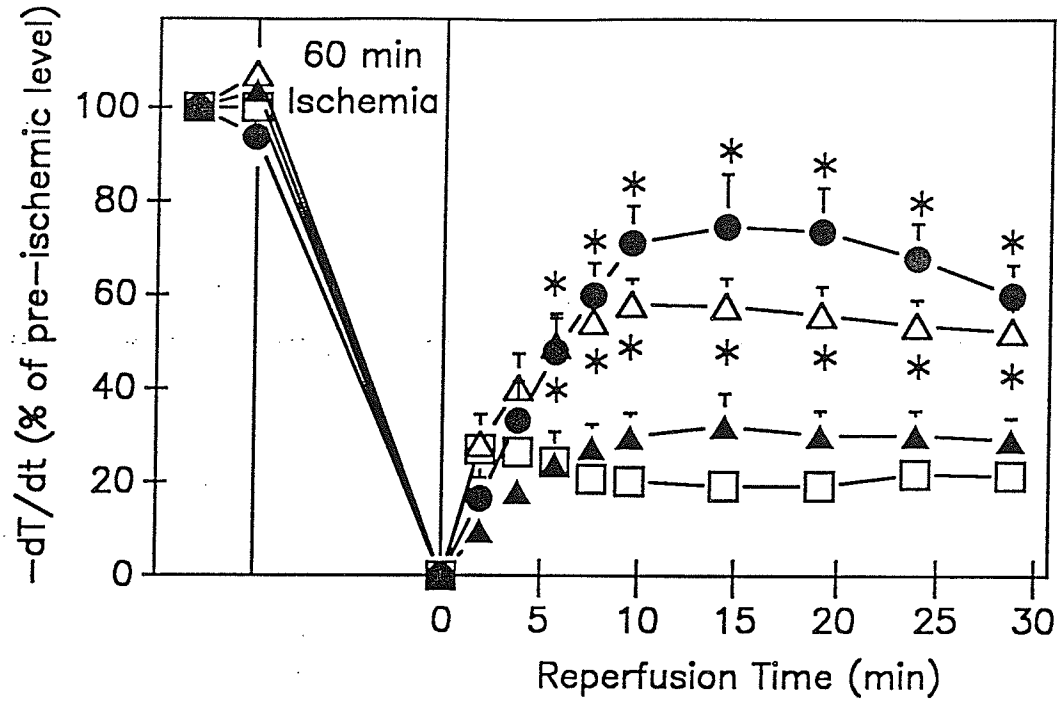


Figure 8. Dose-dependent effect of DMA on the recovery of the maximal velocity of muscle relaxation (-dT/dt) in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion. DMA was included in the perfusate for 3 min prior to ischemia and for the first 3 min of reperfusion.

Open square: drug-untreated group (n=10);
Filled triangle: DMA 1 uM (n=3);
Open triangle: DMA 5 uM (n=7);
Filled cycle: DMA 20 uM (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

HEPES, rather than HCO_3^-, as a buffer in the perfusate to eliminate the possible influence of sarcolemmal Cl^-/HCO_3^- exchange on cellular pH, it was necessary to determine the response of the ventricular wall to the ischemia-reperfusion insult in the presence of bicarbonate. A bicarbonate buffered medium was included and other experimental conditions were identical to the HEPES buffered experiments. After 60 min of ischemia, the RT was 210 ± 11 % of pre-ischemic level. Reperfusion resulted in a further increase of RT (265 ± 34 % of pre-ischemic level) and poor recovery in DT, +dT/dt and -dT/dt. DMA treatment for 3 min prior to ischemia and for the first 3 min of reperfusion led to a lower post-ischemic level of resting tension and a better recovery in DT, +dT/dt and -dT/dt. This pattern is qualitatively similar to that with HEPES buffer.

3. Effect of prolonged administration of DMA on the recovery of cardiac contractile function during post-ischemic reperfusion

To further understand the effects of the drug on ischemia-reperfusion injury, the effects of prolonged administration of DMA was examined. The experimental conditions were the same as the previous experiment except that DMA (5 or 20 uM) was included in the perfusate for 3 min prior to ischemia and throughout the entire 30 min of the reperfusion period. Data in drug-untreated group are taken from Figure 5-8. As shown in Figure 9, treatment with 5 or 20 uM DMA dose-dependently inhibited the post-ischemic

Table 3

Effect of DMA in bicarbonate solution on the recovery of cardiac function in isolated ventricular wall during post-ischemic reperfusion

	Group	Prior to Ischemia	% of Pre-ischemic value				
			0'	3'	6'	10'	20'	30'
RT	Drug-untreated	100 ± 0	210 ± 11	265 ± 34	253 ± 20	230 ± 16	207 ± 21	190 ± 25
	DMA 20uM	100 ± 0	195 ± 11	156* ± 11	185 ± 27	170 ± 26	152 ± 22	152 ± 23
DT	Drug-untreated	100 ± 0	0 ± 0	15.2 ± 4.1	13.2 ± 2.5	13.0 ± 1.9	14.4 ± 1.7	19.2 ± 3.5
	DMA 20uM	100 ± 0	0 ± 0	27.2* ± 3.8	32.4* ± 3.3	35.2* ± 5.2	44.2* ± 8.6	51.4* ± 9.3
+dT/dt	Drug-untreated	100 ± 0	0 ± 0	14.0 ± 2.2	12.2 ± 2.1	13.8 ± 1.8	13.0 ± 2.0	18.0 ± 3.2
	DMA 20uM	100 ± 0	0 ± 0	27.8* ± 4.1	30.4* ± 5.6	35.8* ± 8.3	43.0* ±10.3	58.8* ±13.0
-dT/dt	Drug-untreated	100 ± 0	0 ± 0	16.0 ± 1.8	11.6 ± 2.3	16.6 ± 1.8	17.4 ± 3.1	20.8 ± 4.7
	DMA 20uM	100 ± 0	0 ± 0	31.6* ± 3.4	36.4* ± 5.7	39.4* ± 8.4	55.2* ±12.3	59.6* ±11.1

The ventricular wall was subjected to 60 min global ischemia and 30 min reperfusion. Bicarbonate was substituted for HEPES in the perfusate as described in the Method and Material section. DMA was included in the perfusate for 3 min prior to ischemia and for the first 3 min of reperfusion. RT: resting tension; DT: developed tension; +dT/dt: maximal velocity of tension development; -dT/dt: maximal velocity of muscle relaxation. Values were expressed as percentage of pre-ischemic values (mean ± SE). n=5 in each group. * $p<0.05$. vs. drug-untreated group.

increase in RT which was similar to those observed with shorter DMA exposure (Figure 5). The prolonged treatment with 5 uM DMA during reperfusion did not further improve the recovery of DT (Figure 10). In the 20 uM DMA group, DT had a temporary recovery at the beginning of reperfusion. Longer exposure to 20 uM DMA did not improve DT recovery during reperfusion, instead, DT was gradually inhibited. The recovery of +dT/dt (Figure 11) and -dT/dt (Figure 12) after long exposure to DMA was similar to the DT changes.

4. Effect of different administration protocols of 20 uM DMA on the recovery of cardiac contractile function during post-ischemic reperfusion

This set of experiment is to characterize when DMA may be acting during ischemia-reperfusion challenge. The concentration of DMA used was 20 uM because it had shown the best protective effect to the ventricular wall. The experimental conditions were the same as the previous studies. The ventricular walls were divided into 4 groups: a) drug-untreated group; b) DMA treatment only for 3 min prior to ischemia; c) DMA treatment only for the first 3 min of reperfusion and d) DMA treatment for 3 min prior to and after ischemia. The data in group a and group d were taken from Figure 9-12.

The inclusion of 20 uM DMA in the perfusate for 3 min prior to ischemia did not prevent the development of contracture during ischemia (Figure 13). The post-ischemic recovery of RT (Figure

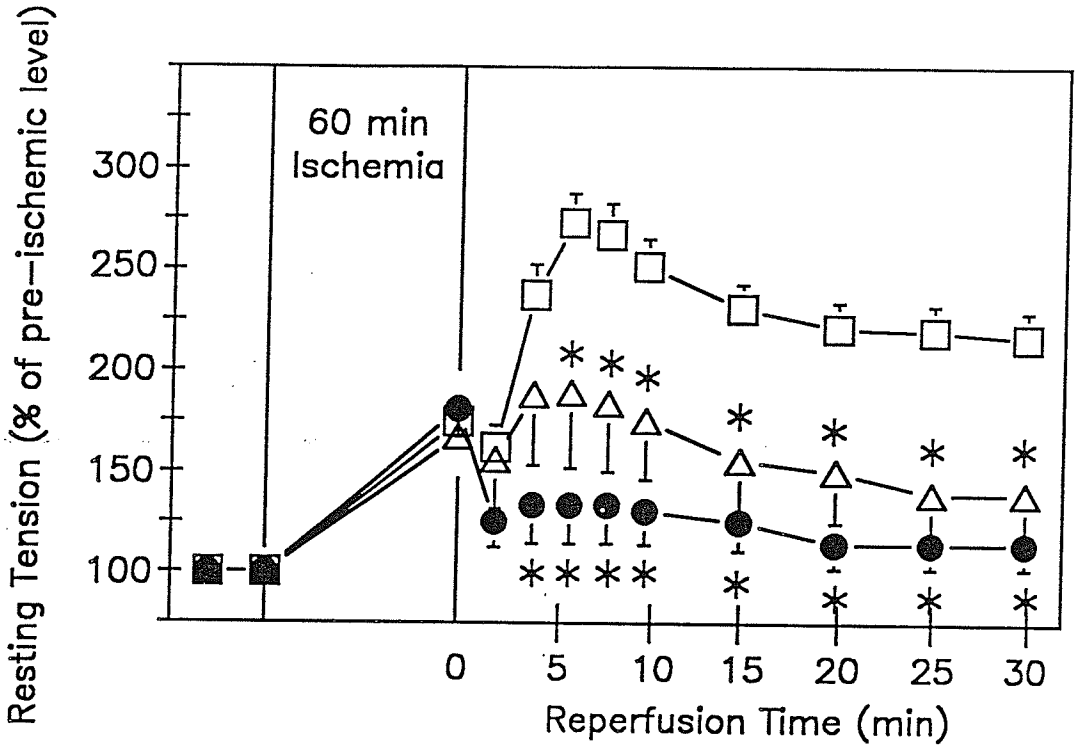


Figure 9. Effect of prolonged administration of DMA on the recovery of resting tension in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion. DMA was included in the perfusate for 3 min prior to ischemia and for the entire period of the 30 min of reperfusion.

Open square: drug-untreated group (n=10);
Open triangle: DMA 5 uM (n=3);
Filled cycle: DMA 20 uM (n=3).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

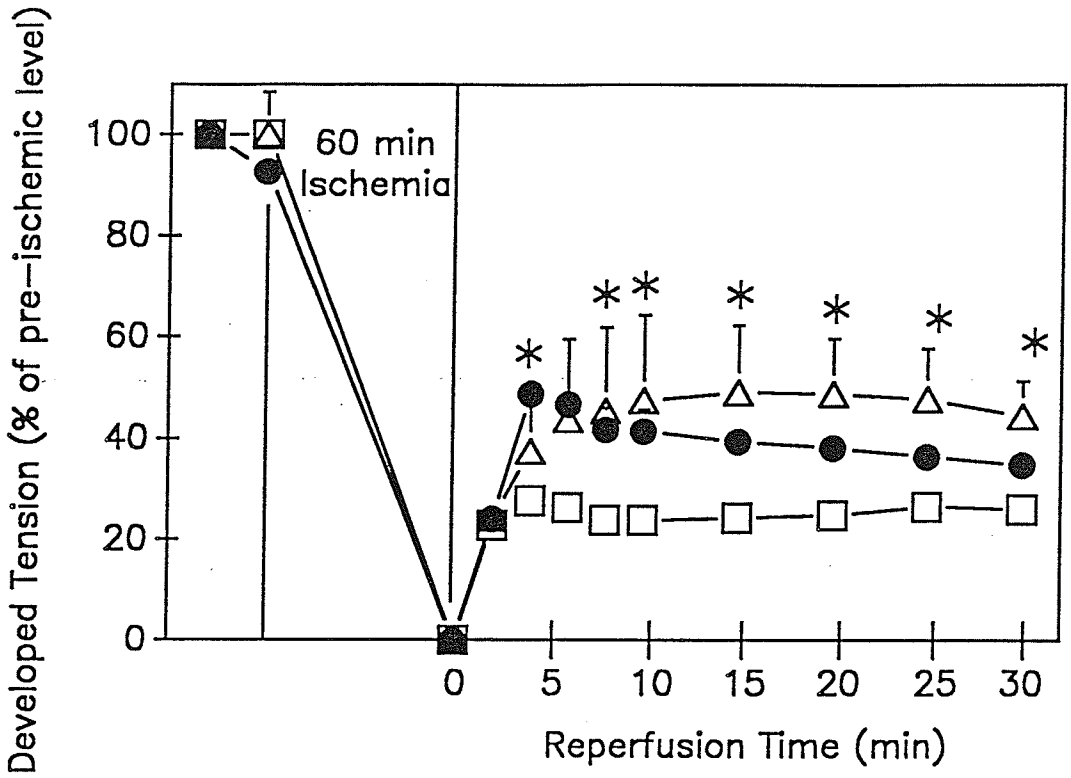


Figure 10. Effect of prolonged administration of DMA on the recovery of developed tension in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion. DMA was included in the perfusate for 3 min prior to ischemia and for the entire period of the 30 min of reperfusion.

Open square: drug-untreated group (n=10);
Open triangle: DMA 5 uM (n=3);
Filled cycle: DMA 20 uM (n=3).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

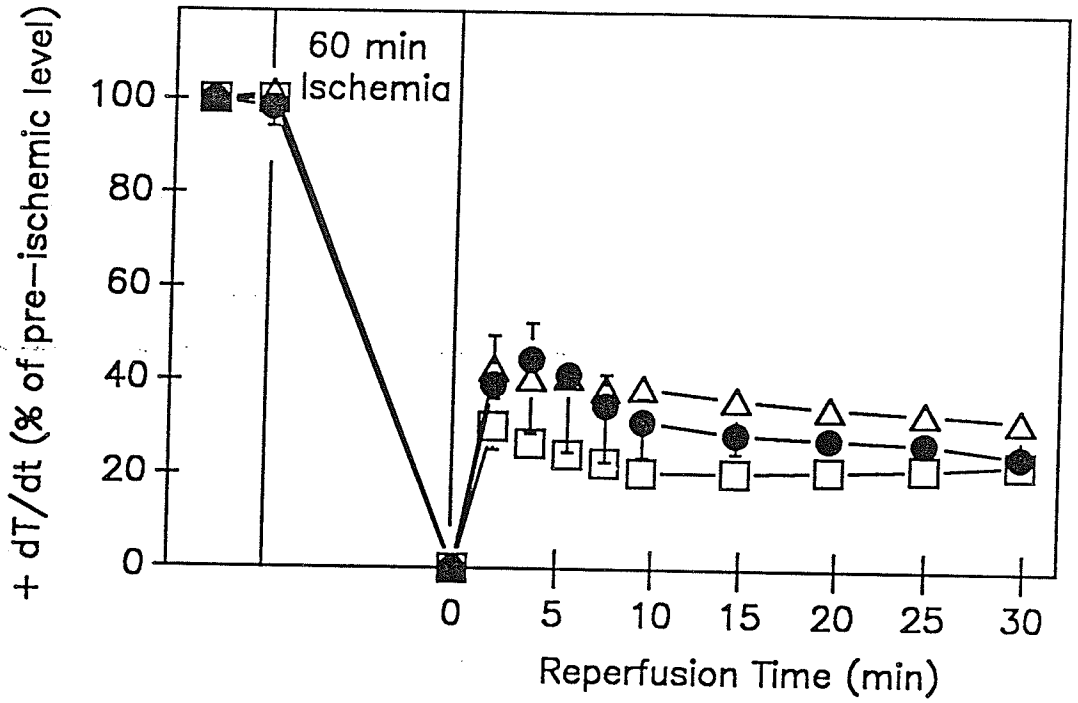


Figure 11. Effect of prolonged administration of DMA on the recovery of the maximal velocity of tension development (+dT/dt) in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion. DMA was included in the perfusate for 3 min prior to ischemia and for the entire period of the 30 min of reperfusion.

Open square: drug-untreated group (n=10);
Open triangle: DMA 5 uM (n=3);
Filled cycle: DMA 20 uM (n=3).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

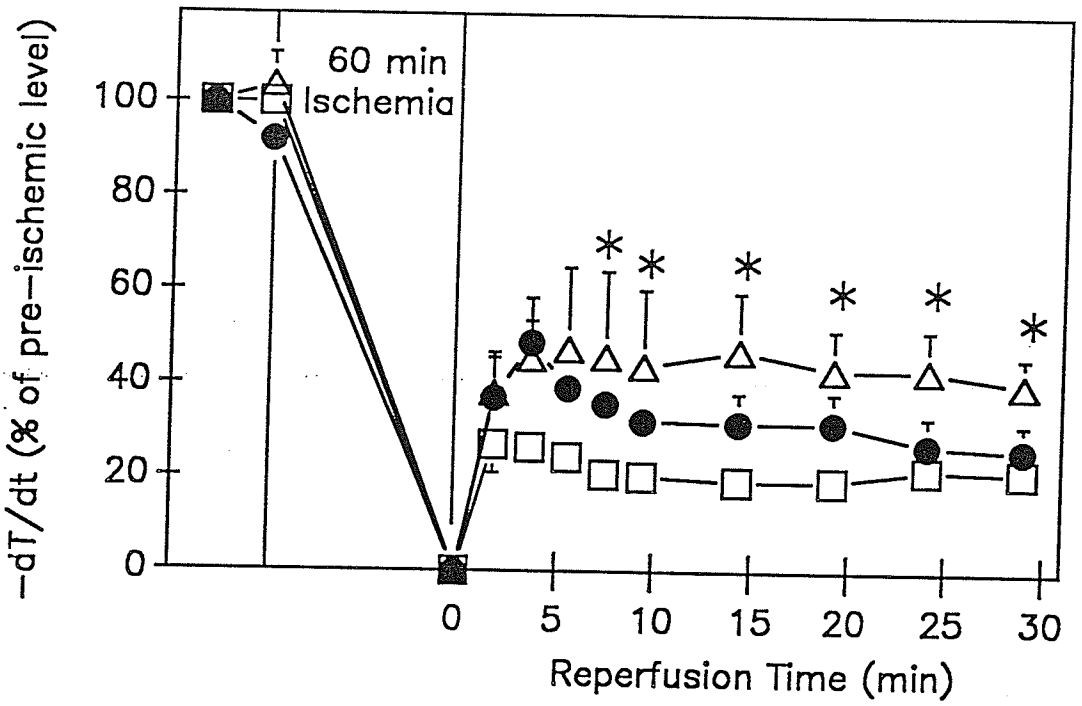


Figure 12. Effect of prolonged administration of DMA on the recovery of the maximal velocity of muscle relaxation (-dT/dt) in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion. DMA was included in the perfusate for 3 min prior to ischemia and for the entire period of the 30 min of reperfusion.

Open square: drug-untreated group (n=10);
Open triangle: DMA 5 uM (n=3);
Filled cycle: DMA 20 uM (n=3).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

13), DT (Figure 14), +dT/dt (Figure 15) and -dT/dt (Figure 16) were as poor as drug-untreated group. When DMA was included in the perfusate only for the first 3 min of reperfusion, the recovery of cardiac contractile function was significantly improved (Figure 13-16) which was similar to that in group d (20 uM DMA for 3 min before and after ischemia).

5. Effect of DMA on cellular Na^+, Ca^{2+} and K^+ content during post-ischemic reperfusion

Since we have proposed that Na^+-H^+ exchange may play a primary role in post-ischemic cardiac ion imbalance, it is important to measure the alteration of cellular ion content, especially for Na^+ and Ca^{2+} during ischemia-reperfusion challenge.

Ion content measurements were carried out in tissues perfused with a HEPES containing solution (pH 7.4). The experimental conditions were the same as those described in section A.1 except that at defined time points, 5 ml of cold sucrose solution was injected into the coronary vessel to wash out the extracellular space of the ventricular wall. In this study, the ventricular walls were divided into 4 groups: a) no-ischemia group: the ventricular wall was not subjected to any ischemia-reperfusion challenge and drug treatment; b) ischemia group: the ventricular wall was sucrosed at the end of 60 min global ischemia without reperfusion; c) drug-untreated group: the ventricular wall was subjected to 60 min global ischemia and 6 min reperfusion without drug intervention; d) DMA group: the

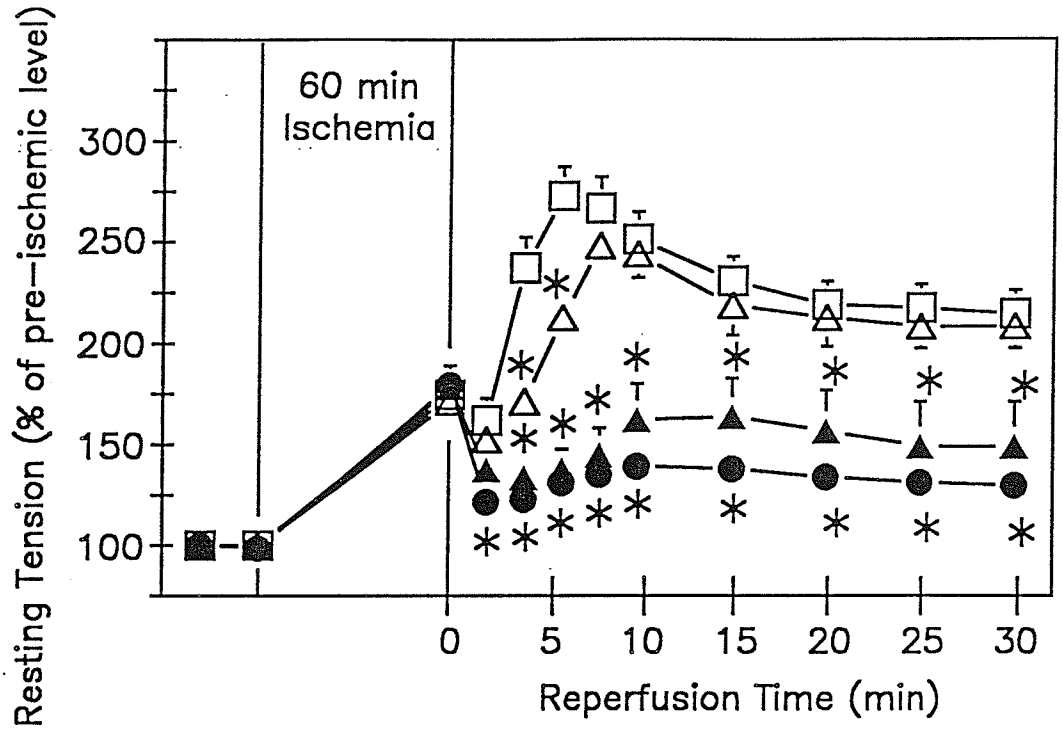


Figure 13. Effect of different administration protocols of 20 uM DMA on the recovery of resting tension in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion.

Open square: drug-untreated group (n=10);
Open triangle: DMA 20 uM was included in the perfusate only for 3 min prior to ischemia (n=4);
Filled triangle: DMA 20 uM was included in the perfusate only for the first 3 min of reperfusion (n=4);
Filled cycle: DMA 20 uM was included in the perfusate for 3 min prior to ischemia and for the first 3 min of reperfusion (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean $\pm$ SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

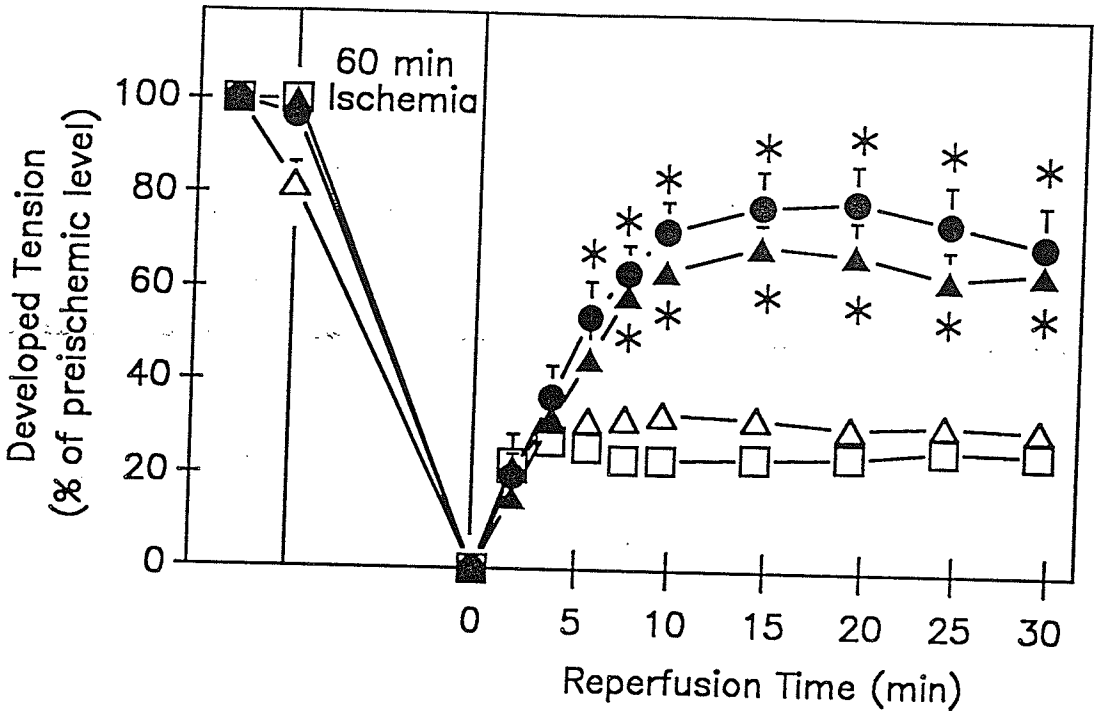


Figure 14. Effect of different administration protocols of 20 uM DMA on the recovery of developed tension in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion.

Open square: drug-untreated group (n=10);
Open triangle: DMA 20 uM was included in the perfusate only for 3 min prior to ischemia (n=4);
Filled triangle: DMA 20 uM was included in the perfusate only for the first 3 min of reperfusion (n=4);
Filled cycle: DMA 20 uM was included in the perfusate for 3 min prior to ischemia and for the first 3 min of reperfusion (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean $\pm$ SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

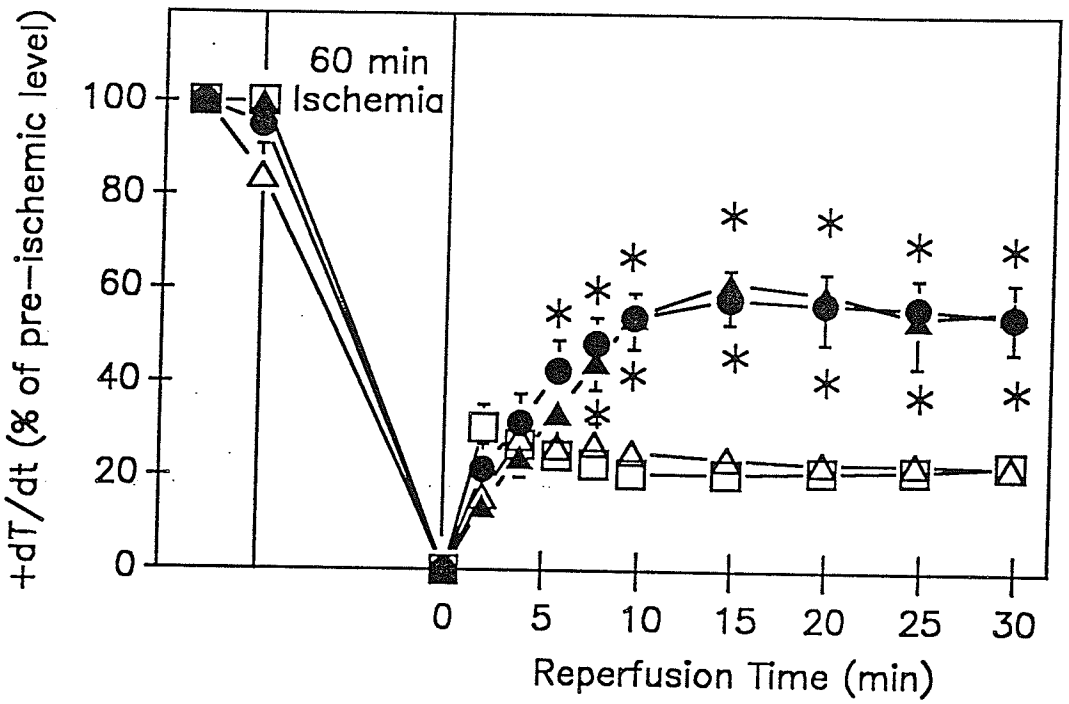


Figure 15. Effect of different administration protocols of 20 uM DMA on the recovery of the maximal velocity of tension development (+dT/dt) in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion.

Open square: drug-untreated group (n=10);
Open triangle: DMA 20 uM was included in the perfusate only for 3 min prior to ischemia (n=4);
Filled triangle: DMA 20 uM was included in the perfusate only for the first 3 min of reperfusion (n=4);
Filled cycle: DMA 20 uM was included in the perfusate for 3 min prior to ischemia and for the first 3 min of reperfusion (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

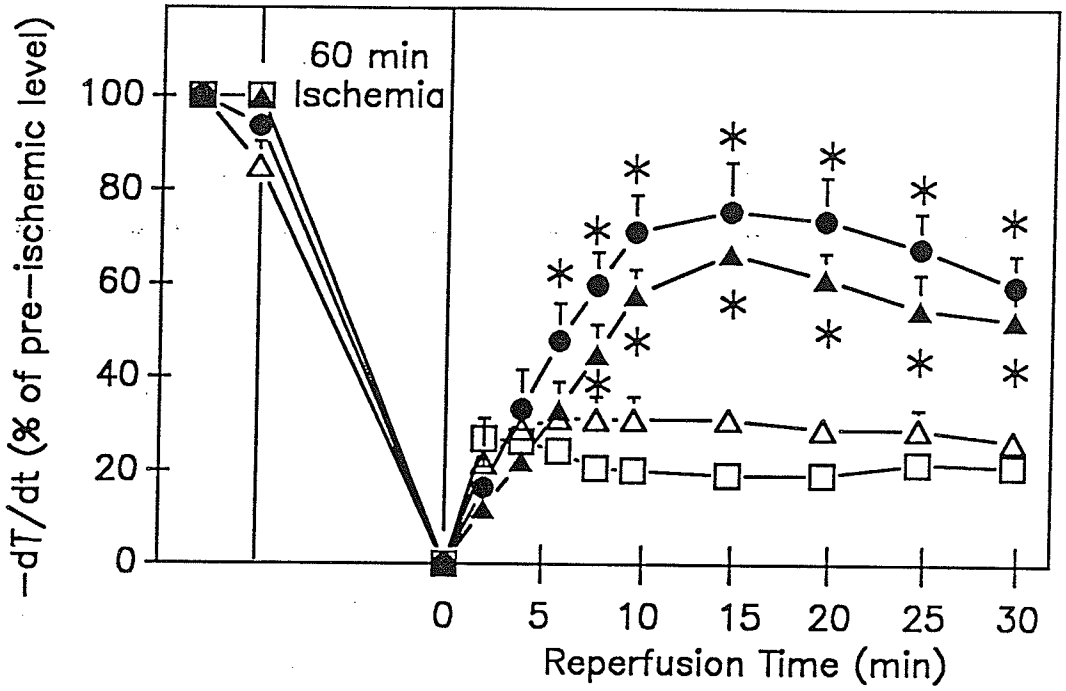


Figure 16. Effect of different administration protocols of 20 uM DMA on the recovery of the maximal velocity of muscle relaxation (-dT/dt) in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion.

Open square: drug-untreated group (n=10);
Open triangle: DMA 20 uM was included in the perfusate only for 3 min prior to ischemia (n=4);
Filled triangle: DMA 20 uM was included in the perfusate only for the first 3 min of reperfusion (n=4);
Filled cycle: DMA 20 uM was included in the perfusate for 3 min prior to ischemia and for the first 3 min of reperfusion (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean $\pm$ SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group.

ventricular wall was exposed to 20 uM DMA 3 min before and after ischemia and subjected to 60 min of ischemia and 6 min of reperfusion, then sucrosed. The mechanical responses of the ventricular wall in the 4 groups during the experiment were similar to those observed in section A.1. Samples were taken at the 6th min of reperfusion because the resting tension was at the maximum at that time point. The values of cellular Na^+, Ca^{2+} and K^+ measured in the no-ischemia group were close to those reported by other investigators (Alto and Dhalla, 1979; Tani and Neely, 1989).

1) Cellular Na^+ content (Figure 17)

Cellular Na^+ content in the no-ischemia group was 53.4 $\pm$ 5.1 umole/g dry weight tissue. After 60 min exposure to ischemia, it was 60.8 $\pm$ 5.1 umole/g dry weight tissue which was not significantly increased in comparison to no-ischemia group. After 6 min of reperfusion, Na^+ was significantly increased to 73.4 $\pm$ 7.2 umole/g dry weight tissue in the drug-untreated group. This elevation of cellular Na^+ could be effectively antagonized by the introduction of 20 uM DMA in the perfusate for 3 min before and 6 min after ischemia.

2) Cellular Ca^{2+} content (Figure 18)

Cellular Ca^{2+} content in no-ischemia group was 2.70 $\pm$ 0.22 umole/g dry weight tissue. After 60 min exposure to ischemia, it was 2.23 $\pm$ 0.27 umole/g dry weight tissue the drug-untreated group. After 6 min of reperfusion, Ca^{2+} was significantly increased to 3.79 $\pm$ 0.31 umole/g dry weight tissue

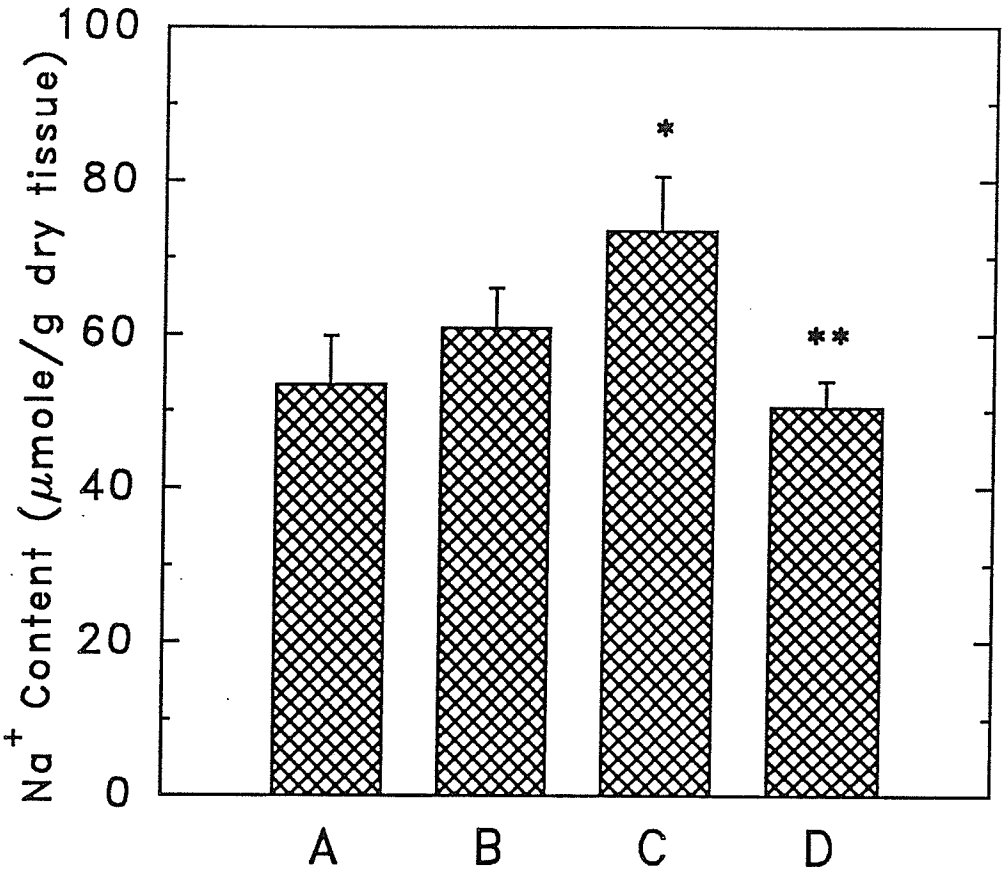


Figure 17. Effect of DMA on cellular Na^+ content in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were equilibrated with HEPES solution (pH 7.4) and subjected to 60 min global ischemia ± 6 min of reperfusion. Sucrose solution was injected into the ventricular wall through coronary artery at the end of each experiment. Other experimental conditions were the same as those described in section A.1.

Column A) no-ischemia group;
Column B) End of 60 min ischemia in drug-untreated group;
Column C) 6 min of reperfusion in drug-untreated group;
Column D) 6 min of reperfusion in DMA group (DMA was applied 3 min before and after ischemia).

Values are expressed in mean ± SE. Each column represents 5 to 13 samples. * $p<0.05$ vs. no-ischemia group (Column A); ** $p<0.05$ vs. 6 min reperfusion in the drug-untreated group (Column C).

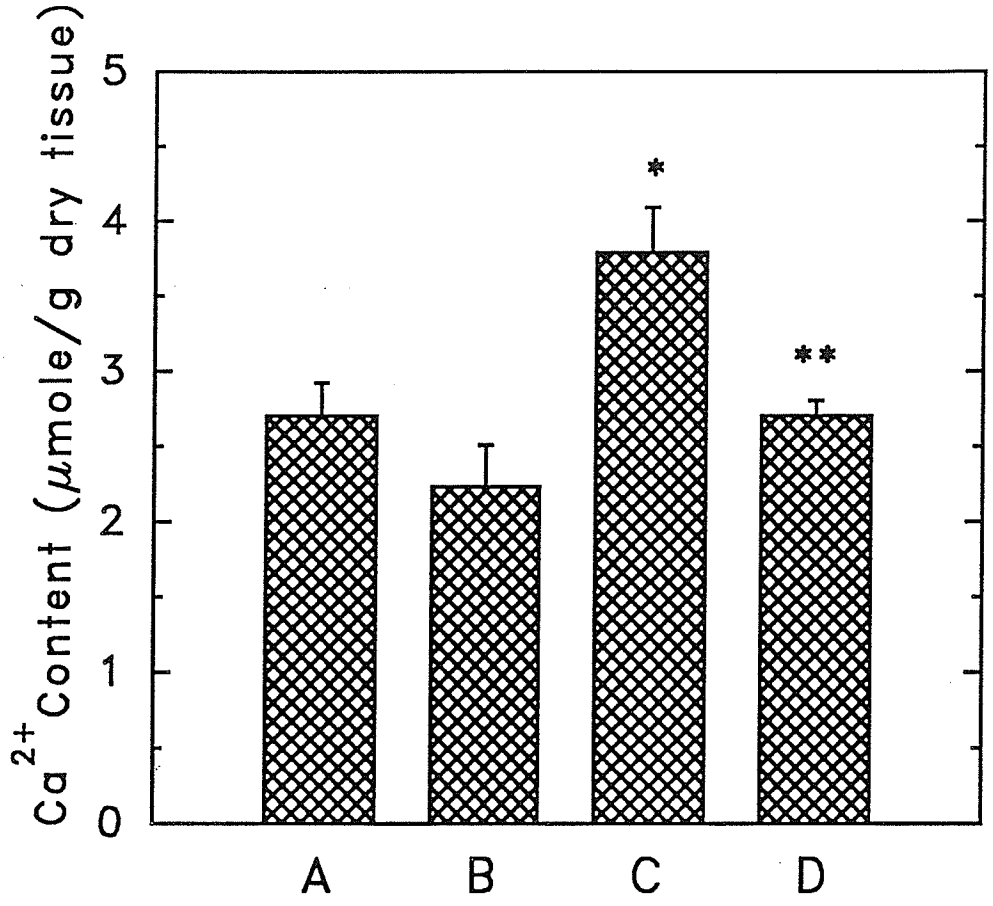


Figure 18. Effect of DMA on cellular Ca^{2+} content in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were equilibrated with HEPES solution (pH 7.4) and subjected to 60 min global ischemia ± 6 min of reperfusion. Sucrose solution was injected into the ventricular wall through coronary artery at the end of each experiment. Other experimental conditions were the same as those described in section A.1.

Column A) no-ischemia group;
Column B) End of 60 min ischemia in drug-untreated group;
Column C) 6 min of reperfusion in drug-untreated group;
Column D) 6 min of reperfusion in DMA group (DMA was applied 3 min before and after ischemia).

Values are expressed in mean ± SE. Each column represents 5 to 13 samples. * $p<0.05$ vs. no-ischemia group (Column A); ** $p<0.05$ vs. 6 min reperfusion in the drug-untreated group (Column C).

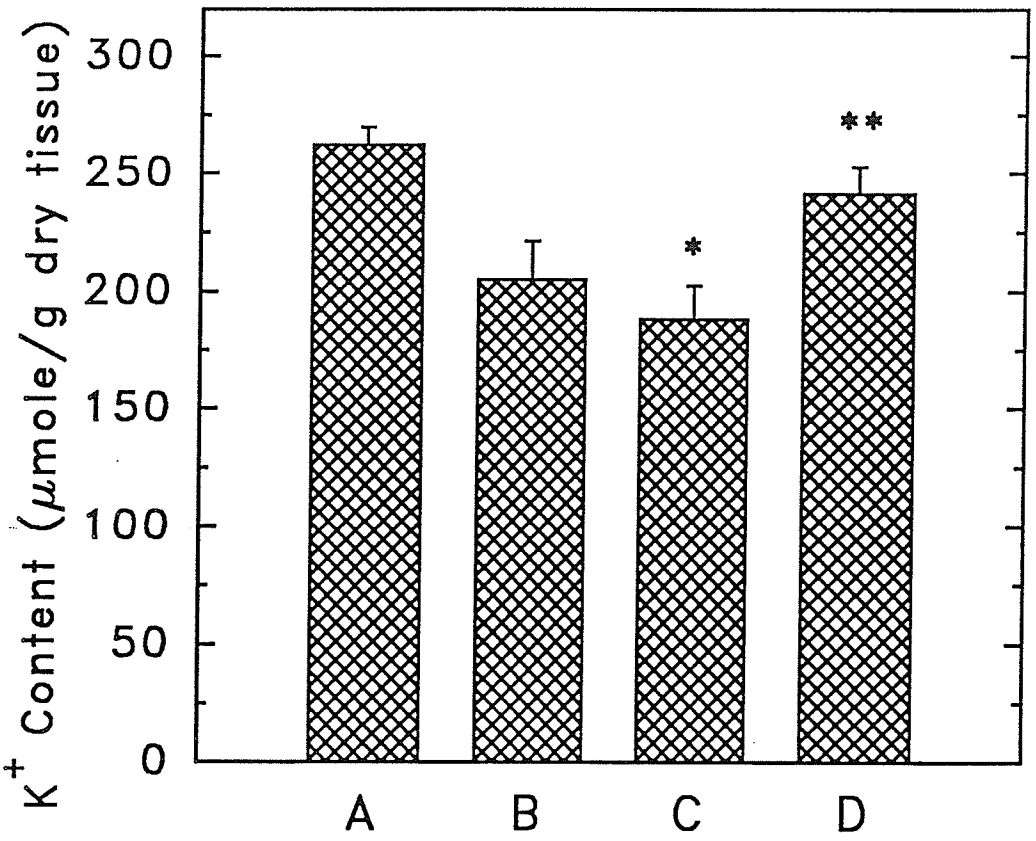


Figure 19. Effect of DMA on cellular K^+ content in isolated ventricular wall during post-ischemic reperfusion. Ventricular walls were equilibrated with HEPES solution (pH 7.4) and subjected to 60 min global ischemia ± 6 min of reperfusion. Sucrose solution was injected into the ventricular wall through coronary artery at the end of each experiment. Other experimental conditions were the same as those described in section A.1.

Column A) no-ischemia group;
Column B) End of 60 min ischemia in drug-untreated group;
Column C) 6 min of reperfusion in drug-untreated group;
Column D) 6 min of reperfusion in DMA group (DMA was applied 3 min before and after ischemia).

Values are expressed in mean ± SE. Each column represents 5 to 13 samples. * $p<0.05$ vs. no-ischemia group (Column A); ** $p<0.05$ vs. 6 min reperfusion in the drug-untreated group (Column C).

in drug-untreated group. This could be effectively antagonized by the introduction of 20 uM DMA in the perfusate.

3) Cellular K^+ content (Figure 19)

Cellular K^+ content in no-ischemia group was 262 $\pm$ 7.7 umole/g dry weight tissue. After 60 min exposure to ischemia, it was 205 $\pm$ 16.5 umole/g dry weight tissue in drug-untreated group. After 6 min of reperfusion, K^+ was significantly decreased to 181 $\pm$ 15 umole/g dry weight tissue. This could be effectively normalized by the inclusion of 20 uM DMA in the perfusate.

6. Effect of DMA on creatine phosphokinase (CPK) activity in coronary effluent during post-ischemic reperfusion (Table 4)

Creatine phosphokinase (CPK) activity in the coronary effluent was determined as a criteria of cardiac damage in response to the ischemia-reperfusion challenge (Jennings et al, 1983). The CPK sample were taken from the ventricular walls described in section A.1 and A.3. CPK activity in the effluent in the drug-untreated group increased about 5 fold during post-ischemic reperfusion. DMA treatment in no-ischemia group did not influence CPK activity. When 5 or 20 uM DMA was applied for 3 min before and after ischemia, the CPK activity was significantly attenuated in a dose-dependent manner. Longer exposure to 5 or 20 uM DMA also protected against CPK release, a result consistent with the inhibition of reperfusion contracture (Figure 9).

Table 4

Effect of DMA on creatine phosphokinase (CPK) activity in coronary effluent of isolated ventricular wall during post-ischemic reperfusion

Groups	n	CPK Activity (unit/ml/g wet weight)
No-ischemia group	20	5.5 ± 1.1
No-ischemia + 20 uM DMA	8	4.9 ± 1.4
Drug-untreated (60 min ischemia + reperfusion)	9	31.5 ± 6.1 #
DMA 5 uM (3 min pre + 3 min post ischemia)	5	20.9 ± 5.8 # *
DMA 20 uM (3 min pre + 3 min post ischemia)	5	12.8 ± 3.3 *
DMA 5 uM (3 min pre + 30 min post ischemia)	3	6.2 ± 1.1 *
DMA 20 uM (3 min pre + 30 min post ischemia)	5	6.0 ± 1.2 *

The ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion. Samples were collected between 5-10 min of reperfusion. n is sample number in each group. Values are expressed as mean ± SE. * $p<0.05$ vs. drug-untreated group. # $p<0.05$ vs. no-ischemia group.

B. INVOLVEMENT OF SODIUM IN THE PROTECTIVE EFFECT OF DMA ON ISCHEMIA-REPERFUSION INJURY

1. Effect of DMA on the recovery of cardiac contractile function during post-ischemic reperfusion in the presence of ouabain

In order to understand the mechanism of action of DMA, the effects of DMA was observed in the presence of ouabain. Ouabain is a Na^+-K^+ ATPase inhibitor which can increase intracellular Na^+. The response of the ventricular wall to ouabain and the effects of DMA on the increased cellular Na^+ were examined. In this study, the experimental conditions were the same as the previous studies. The right ventricular walls were divided into 4 groups: a) drug-untreated group: the ventricular wall was subjected to 60 min of ischemia and 30 min of reperfusion without any drug interventions; b) Ouabain group: 35 uM ouabain was included in the perfusate for 3 min prior to ischemia and for the first 6 min of reperfusion and c) Ouabain + DMA group: ventricular walls were perfused with 20 uM DMA for 3 min, then switched to a solution which contained 35 uM ouabain and 20 uM DMA for another 3 min prior to ischemia and for the first 6 min of reperfusion; d) 20 uM DMA was included in the perfusate 3 min before and after ischemia. In groups c and d, the drugs were present in the ventricular wall during ischemia. In a preliminary experiment, 35 uM ouabain induced a 20-25 % increase of the cardiac contraction without any irregular beating rhythm. The dose of ouabain was well within the mild inotropic and non-toxic range

reported by other investigators using rat heart (Kolar et al, 1990, Tani and Neely, 1990[c]). Therefore, the ouabain concentration was considered to be non-toxic and chosen for our study. The experimental recordings of the ventricular wall in response to ouabain and DMA are shown in Figure 20.

1) Resting tension (RT) (Figure 21)

In drug-untreated right ventricular wall, the resting tension before ischemia was 32.6 ± 1.7 g/g wet weight tissue. It began to rise after 30 min ischemia (data not shown) and at the end of 60 min global ischemia, RT was significantly elevated to 165 ± 5.6 % of the pre-ischemic level. RT increased to its peak (221 ± 10 % of the pre-ischemic level) after 8 min of reperfusion. When 35 uM ouabain was included in the perfusate (3 min before ischemia and for the first 6 min of reperfusion), the RT at the end of the ischemic period was not significantly changed in comparison to that in the drug-untreated group. However, during reperfusion, RT was significantly elevated. It reached its peak (342 ± 11 % of pre-ischemic value, $p<0.05$ vs. the drug-untreated group) at the 6th min of reperfusion and remained at a significant higher level for the rest of the 30 min reperfusion. When 20 uM DMA was added to the perfusate for 6 min before ischemia and for 6 min during reperfusion (DMA + ouabain group), the ouabain-induced rise in RT observed during reperfusion was significantly decreased. The highest RT in this group was 145 ± 11 % of the pre-ischemic level ($p<0.05$ vs. the drug-untreated group or ouabain-treated group) at the 15th min of

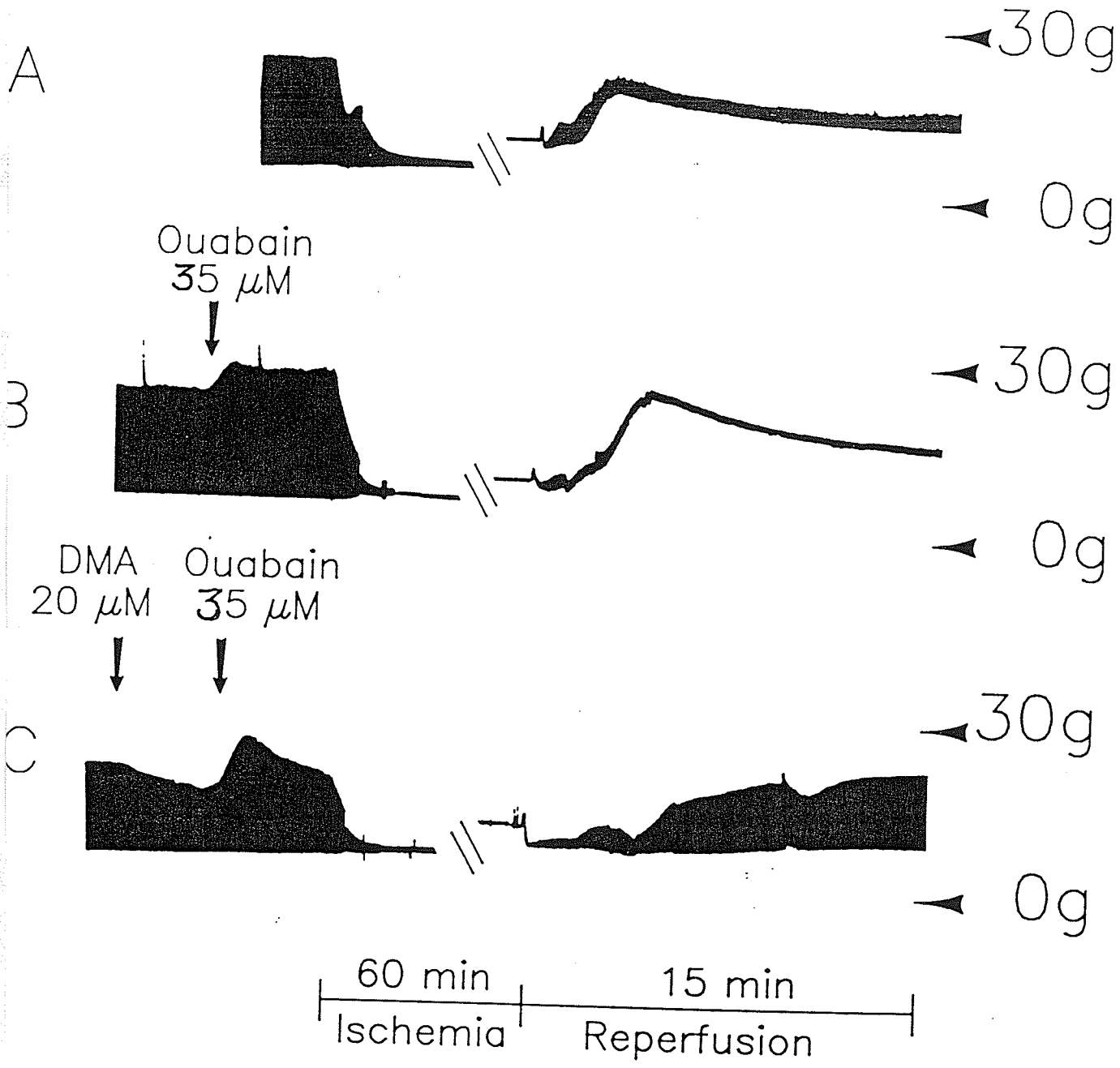


Figure 20. Experimental recordings of the ventricular wall contraction during ischemia-reperfusion challenge in the presence or absence of 35 uM ouabain.

Panel A: Drug-untreated ventricular wall;
Panel B: Ouabain-treated ventricular wall:
Panel C: Ouabain- and DMA-treated ventricular wall.

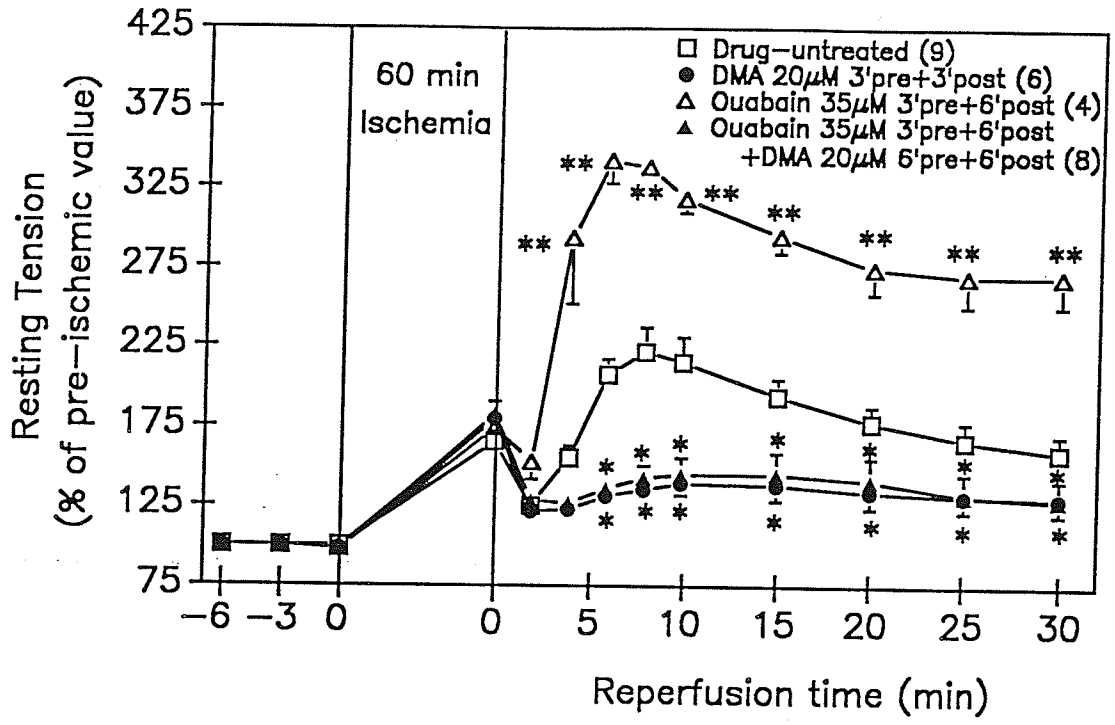


Figure 21. Effect of DMA on the recovery of resting tension in isolated ventricular wall during post-ischemic reperfusion in the presence of 35 uM ouabain. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion.

Open square: drug-untreated group (n=9);
Open triangle: ouabain 35 uM was included in the perfusate for 3 min prior to ischemia and for 6 min during reperfusion (n=4);
Filled triangle: DMA 20 uM was included in the perfusate for 6 min before ischemia and for 6 min during reperfusion (n=8);
Filled cycle: DMA 20 uM was included in the perfusate for 3 min prior to and after ischemia (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group. ** $p<0.05$ vs. all other groups.

reperfusion which is similar to that in group d.

2) Developed tension (DT) (Figure 22)

The developed tension of the muscle in non-ischemic ventricular wall was 87 ± 9.4 g/g wet weight tissue. It was severely attenuated following 60 min global ischemia and reperfusion in the drug-untreated group. The maximal recovery of DT in drug-untreated group was 35 ± 4.4 % of the pre-ischemic levels after 6 min of reperfusion and it was maintained at this level until the end of the 30 min reperfusion. Ouabain treatment (35 uM) elicited a mild positive inotropic effect in non-ischemic hearts (123 ± 1.7 % of pre-ouabain treatment level, $p<0.05$). The presence of 35 uM ouabain in reperfusion media for 6 min did not induce a significant positive inotropic effect. Instead, the recovery of DT in the ouabain-treated group was further worsened during reperfusion. The maximal DT recovery in this group was 14 ± 5.8 % of pre-ischemic level ($p<0.05$ vs. the drug-untreated group). The ouabain-induced positive inotropic effect in the ventricular wall was transient and could be attenuated by DMA pretreatment. After 20 uM DMA had been introduced into the perfusate for 3 min, introduction of ouabain could only induce a temporary positive inotropic effect which rapidly declined below control levels before the start of ischemia (Figure 20 and 22). DMA treatment for 6 min before ischemia and for 6 min into reperfusion dramatically improved the post-ischemic recovery of DT in spite of the presence of ouabain. In the DMA + ouabain group, DT gradually improved during reperfusion and the peak

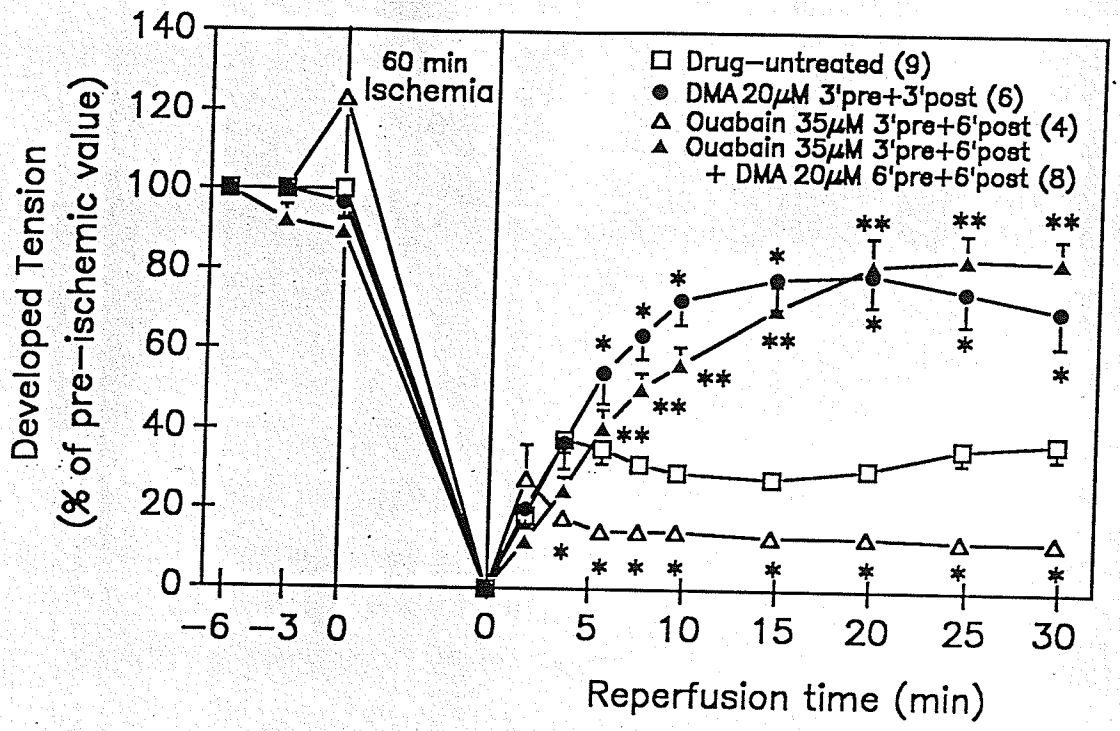


Figure 22. Effect of DMA on the recovery of developed tension in isolated ventricular wall during post-ischemic reperfusion in the presence of 35 uM ouabain. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion.

Open square: drug-untreated group (n=9);
Open triangle: ouabain 35 uM was included in the perfusate for 3 min prior to ischemia and for 6 min during reperfusion (n=4);
Filled triangle: DMA 20 uM was included in the perfusate for 6 min before ischemia and for 6 min during reperfusion (n=8);
Filled cycle: DMA 20 uM was included in the perfusate for 3 min prior to and after ischemia (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group. ** $p<0.05$ vs. ouabain-treated group.

3) +dT/dt (Figure 23) and -dT/dt (Figure 24)

The pre-ischemic values of +dT/dt and -dT/dt were 2329 ± 295 and 1172 ± 110 g/s/g wet weight tissue, respectively. They had similar qualitative responses to the administration of ouabain ± DMA as observed in DT. The ischemia-reperfusion insults resulted in a poor recovery in both +dT/dt and -dT/dt during reperfusion (36 ± 3.8 and 34 ± 4.7 % of the pre-ischemic value, respectively). Ouabain treatment further attenuated the post-ischemic recovery of +dT/dt and -dT/dt to 11 ± 6.8 and 13 ± 7.0 % of the pre-ischemic level ($p<0.05$ vs. the drug-untreated group), respectively. Treatment with 20 uM DMA for 6 min prior to ischemia and for 6 min of reperfusion significantly improved the recovery of +dT/dt and -dT/dt (67 ± 4.8 and 70 ± 5 %, respectively, of the pre-ischemic value, $p<0.05$ vs. ouabain group) despite the presence of 35 uM ouabain.

2. Effect of DMA on cellular Na^+, Ca^{2+} and K^+ content during post-ischemic reperfusion in the presence of 35 uM ouabain

Ion content measurements were carried out in tissues perfused with a HEPES containing solution (pH 7.4). The experimental conditions were the same as those described in section A.1 and B.1 except that at defined time points, 5 ml of cold sucrose solution was injected into the coronary vessel to

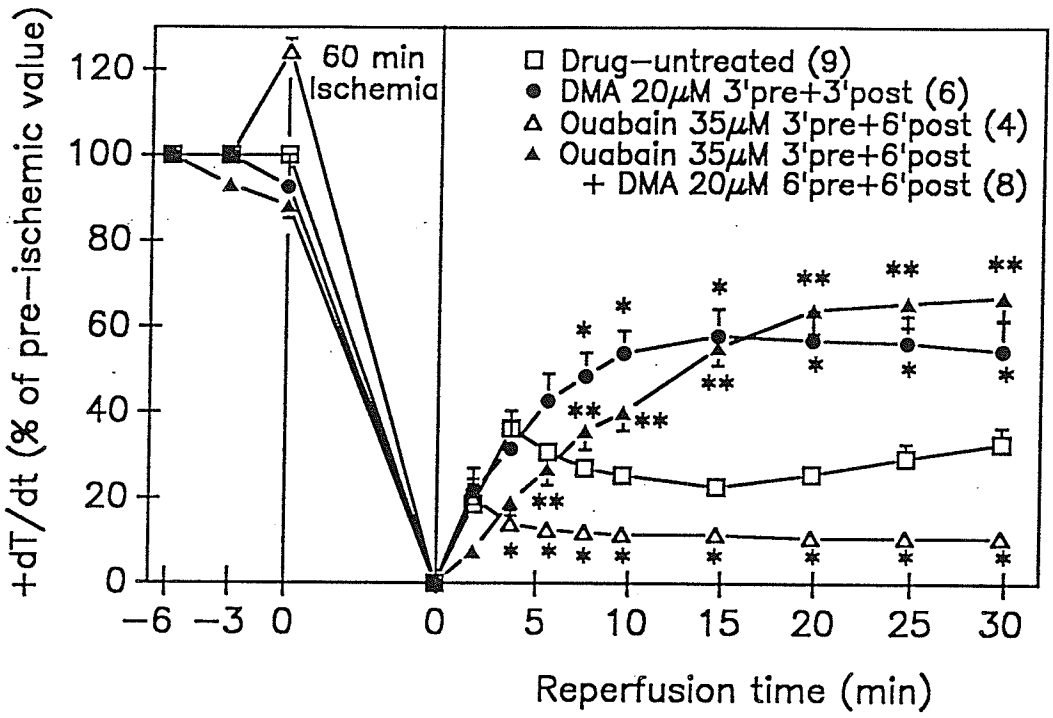


Figure 23. Effect of DMA on the recovery of the maximal velocity of tension development (+dT/dt) in isolated ventricular wall during post-ischemic reperfusion in the presence of 35 uM ouabain. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion.

Open square: drug-untreated group (n=9);
Open triangle: ouabain 35 uM was included in the perfusate for 3 min prior to ischemia and for 6 min during reperfusion (n=4);
Filled triangle: DMA 20 uM was included in the perfusate for 6 min before ischemia and for 6 min during reperfusion (n=8);
Filled cycle: DMA 20 uM was included in the perfusate for 3 min prior to and after ischemia (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group. ** $p<0.05$ vs. ouabain-treated group.

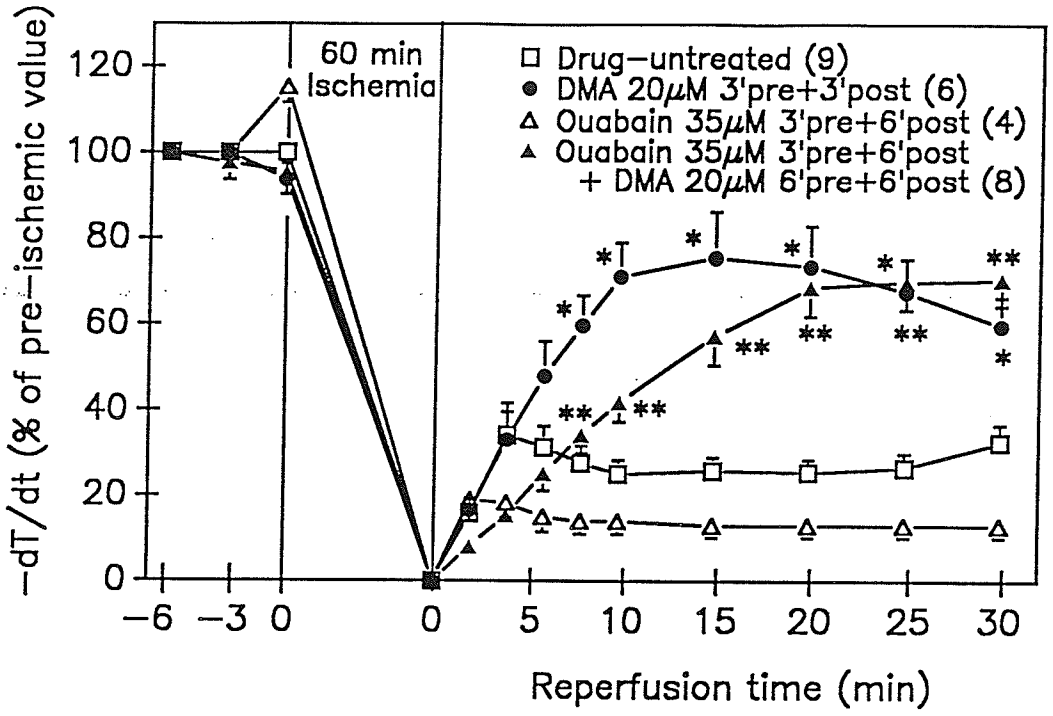


Figure 24. Effect of DMA on the recovery of the maximal velocity of muscle relaxation (-dT/dt) in isolated ventricular wall during post-ischemic reperfusion in the presence of 35 uM ouabain. Ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion.

Open square: drug-untreated group (n=9);
Open triangle: ouabain 35 uM was included in the perfusate for 3 min prior to ischemia and for 6 min during reperfusion (n=4);
Filled triangle: DMA 20 uM was included in the perfusate for 6 min before ischemia and for 6 min during reperfusion (n=8);
Filled cycle: DMA 20 uM was included in the perfusate for 3 min prior to and after ischemia (n=6).

All values are expressed as the percentage of pre-ischemic levels (Mean ± SE). If no error bar is presented in the figure, the error of the mean was smaller than the symbol size. *$p<0.05$ vs. drug-untreated group. ** $p<0.05$ vs. ouabain-treated group.

wash out the extracellular space of the ventricular wall. In this study, the ventricular walls were divided into 6 groups: a) no-ischemia group: the ventricular wall was not subjected to any ischemia-reperfusion challenge and drug treatment; b) ischemia group: the ventricular wall was sucrosed at the end of 60 min global ischemia without reperfusion and drug intervention; c) ischemia + ouabain group: the ventricular wall was exposed to 35 uM ouabain for 3 min before ischemia and sucrosed at the end of 60 min ischemia; d) ischemia + DMA + ouabain group: the ventricular wall was exposed to 20 uM DMA for 6 min and 35 uM ouabain for 3 min before ischemia and sucrosed at the end of 60 min ischemia; e) drug-untreated group: the ventricular wall was subjected to 60 min of ischemia and 6 min reperfusion without drug intervention; f) ouabain group: the ventricular wall was exposed to 35 uM ouabain for 3 min before ischemia and for 6 min during reperfusion; g) ouabain + DMA group: the ventricular wall was exposed to 20 uM DMA for 6 min and 35 uM ouabain for 3 min prior to ischemia, and both drugs were present in the perfusate for 6 min during reperfusion. During ischemia, drugs were present in the cardiac tissue. Data in group a, b and e were taken from the study described in section A.5. The mechanical responses of the ventricular wall in these groups during the experiment were similar to those observed in section A.1 and A.5. Samples were taken at the 6th min of reperfusion because the resting tension was at the maximum at that time point.

1) Cellular Na^+ content (Figure 25)

Cellular Na^+ content in no-ischemia group was 53.4 ± 5.1 umole/g dry weight tissue. After 60 min exposure to ischemia, it was 60.8 ± 5.1, 53.3 ± 8.2 and 52.4 ± 7.3 umole/g dry tissue, respectively in the ischemia group, ischemia + ouabain group and ischemia + ouabain + DMA group which were not significantly increased in comparison with no-ischemia group. After 6 min of reperfusion, Na^+ was significantly increased to 73.4 ± 7.2 umole/g dry weight tissue in the drug-untreated group. Na^+ was further increased to 95.6 ± 4.3 umole/g dry weight tissue in ouabain group and this elevation of Na^+ could be normalized by the exposure to 20 uM DMA.

2) Cellular Ca^{2+} content (Figure 26)

Ca^{2+} content in non-ischemic right ventricular wall was 2.70 ± 0.22 umole/g dry weight tissue. After 60 min exposure to ischemia, it was 2.23 ± 0.27, 2.03 ± 0.60 and 1.23 ± 0.11 umole/g dry weight tissue, respectively in ischemia group, ischemia + ouabain group and ischemia + ouabain + DMA group. After 6 min of reperfusion, Ca^{2+} was significantly increased to 3.79 ± 0.31 umole/g dry weight tissue. Ca^{2+} was further increased to 4.14 ± 0.40 umole/g dry weight tissue in ouabain group. This elevation of Ca^{2+} could be normalized by 20 uM DMA.

3) Cellular K^+ content (Figure 27)

Cellular K^+ content in non-ischemic right ventricular wall was 262 ± 7.7 umole/g dry weight tissue. After 60 min exposure to ischemia, it was 205 ± 16.5, 166 ± 15.6 and 155 ± 15.6 umole/g dry tissue, respectively in ischemia group, ischemia

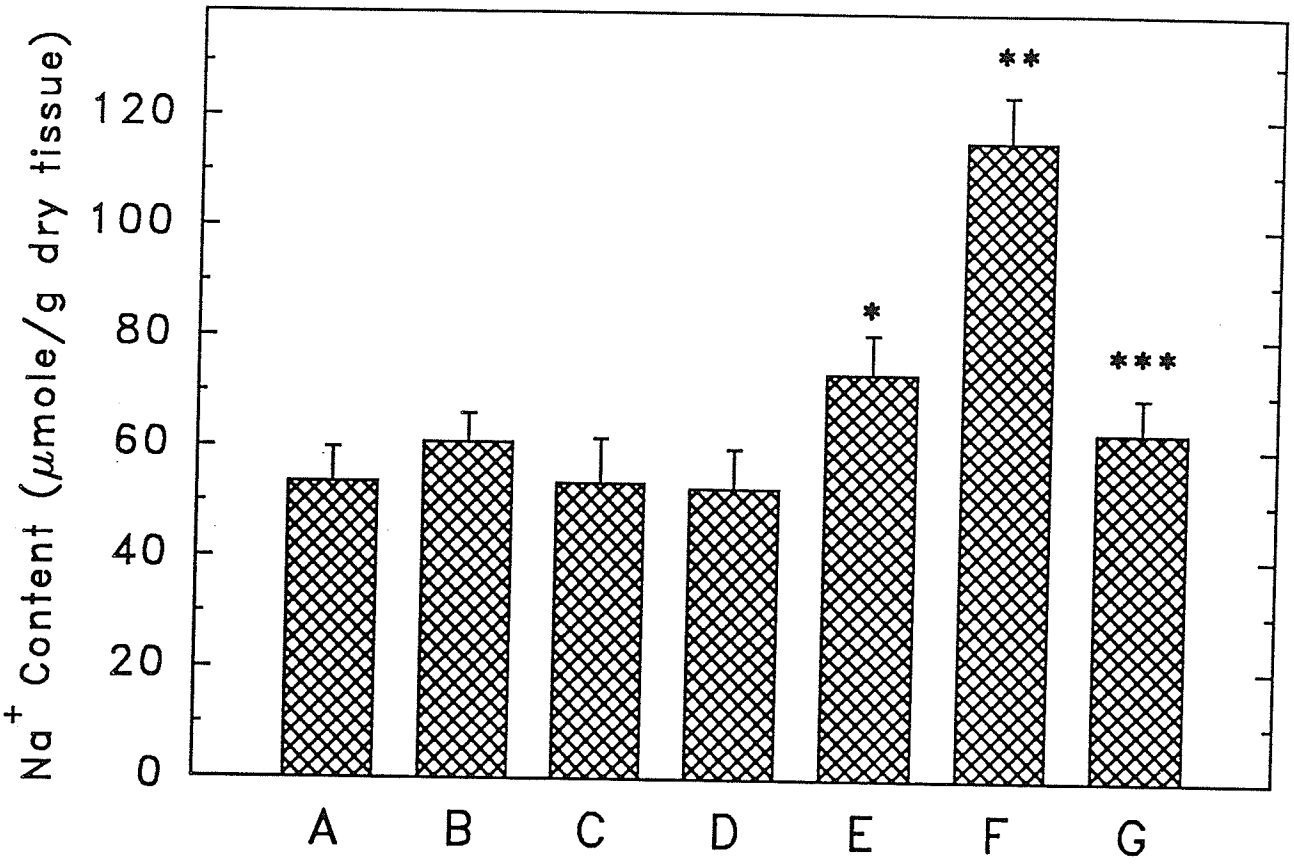


Figure 25. Effect of DMA on cellular Na^+ content in isolated ventricular wall during post-ischemic reperfusion in the presence of 35 uM ouabain. Ventricular walls were equilibrated with HEPES solution (pH 7.4) and subjected to 60 min global ischemia ± 6 min of reperfusion. Sucrose solution was injected into the ventricular wall through coronary artery at the end of each experiment. Other experimental conditions were the same as those described in section A.5.

Column A) no-ischemia group;
Column B) Ischemia group;
Column C) Ischemia + ouabain group;
Column D) Ischemia + DMA + ouabain group;
Column E) 6 min of reperfusion in drug-untreated group;
Column F) 6 min of reperfusion in ouabain group;
Column G) 6 min of reperfusion in ouabain + DMA group.

Values are expressed in mean ± SE. Each column represents 5 to 13 samples. * $p<0.05$ vs. no-ischemia group (Column A); ** $p<0.05$ vs. 6 min reperfusion in the drug-untreated group (Column E); *** $p<0.05$ vs. 6 min reperfusion in ouabain group (Column F).

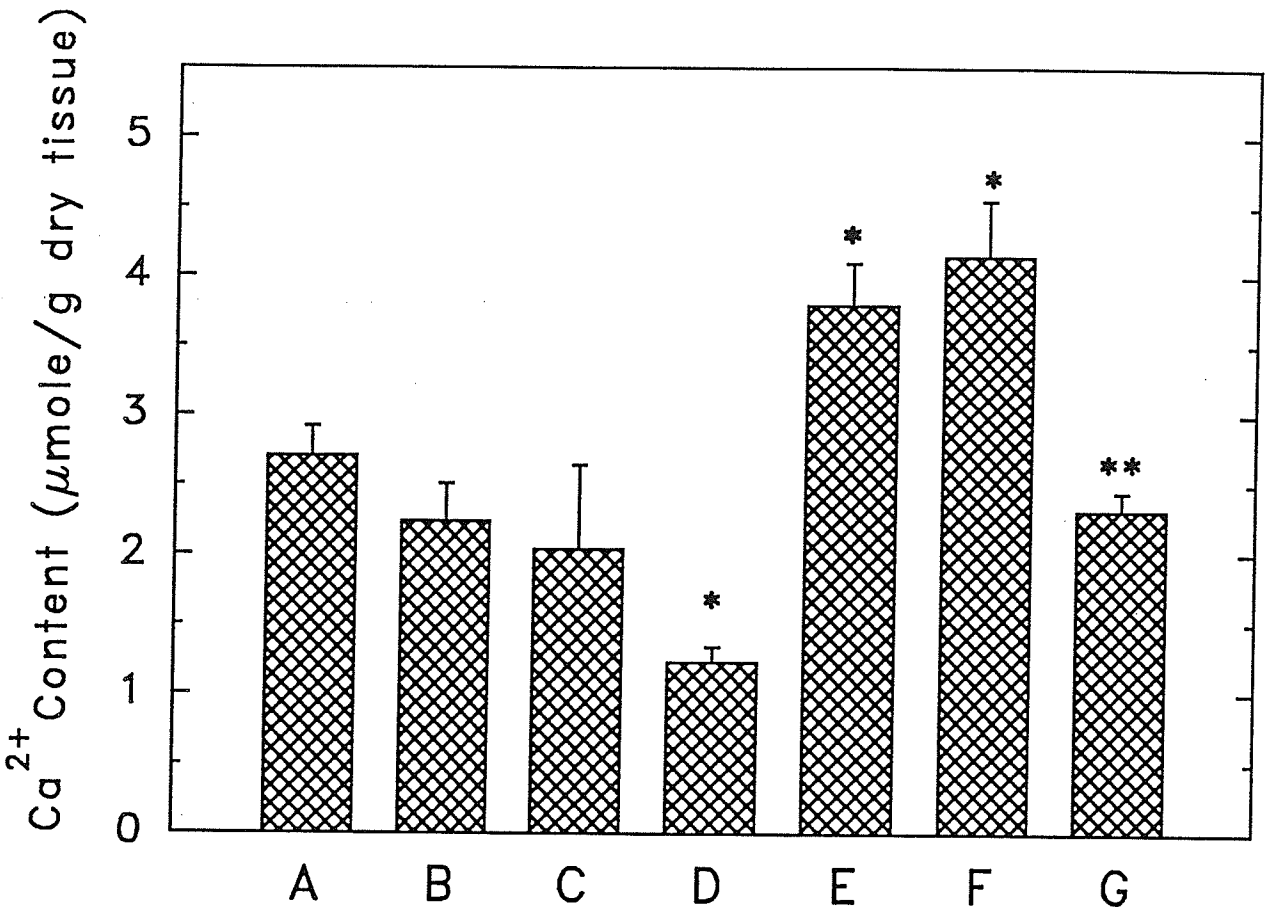


Figure 26. Effect of DMA on cellular Ca^{2+} content in isolated ventricular wall during post-ischemic reperfusion in the presence of 35 uM ouabain. Ventricular walls were equilibrated with HEPES solution (pH 7.4) and subjected to 60 min global ischemia ± 6 min of reperfusion. Sucrose solution was injected into the ventricular wall through coronary artery at the end of each experiment. Other experimental conditions were the same as those described in section A.5.

Column A) no-ischemia group;
Column B) Ischemia group;
Column C) Ischemia + ouabain group;
Column D) Ischemia + DMA + ouabain group;
Column E) 6 min of reperfusion in drug-untreated group;
Column F) 6 min of reperfusion in ouabain group;
Column G) 6 min of reperfusion in ouabain + DMA group.

Values are expressed in mean ± SE. Each column represents 5 to 13 samples. * $p<0.05$ vs. no-ischemia group (Column A); ** $p<0.05$ vs. 6 min reperfusion in ouabain group (Column F).

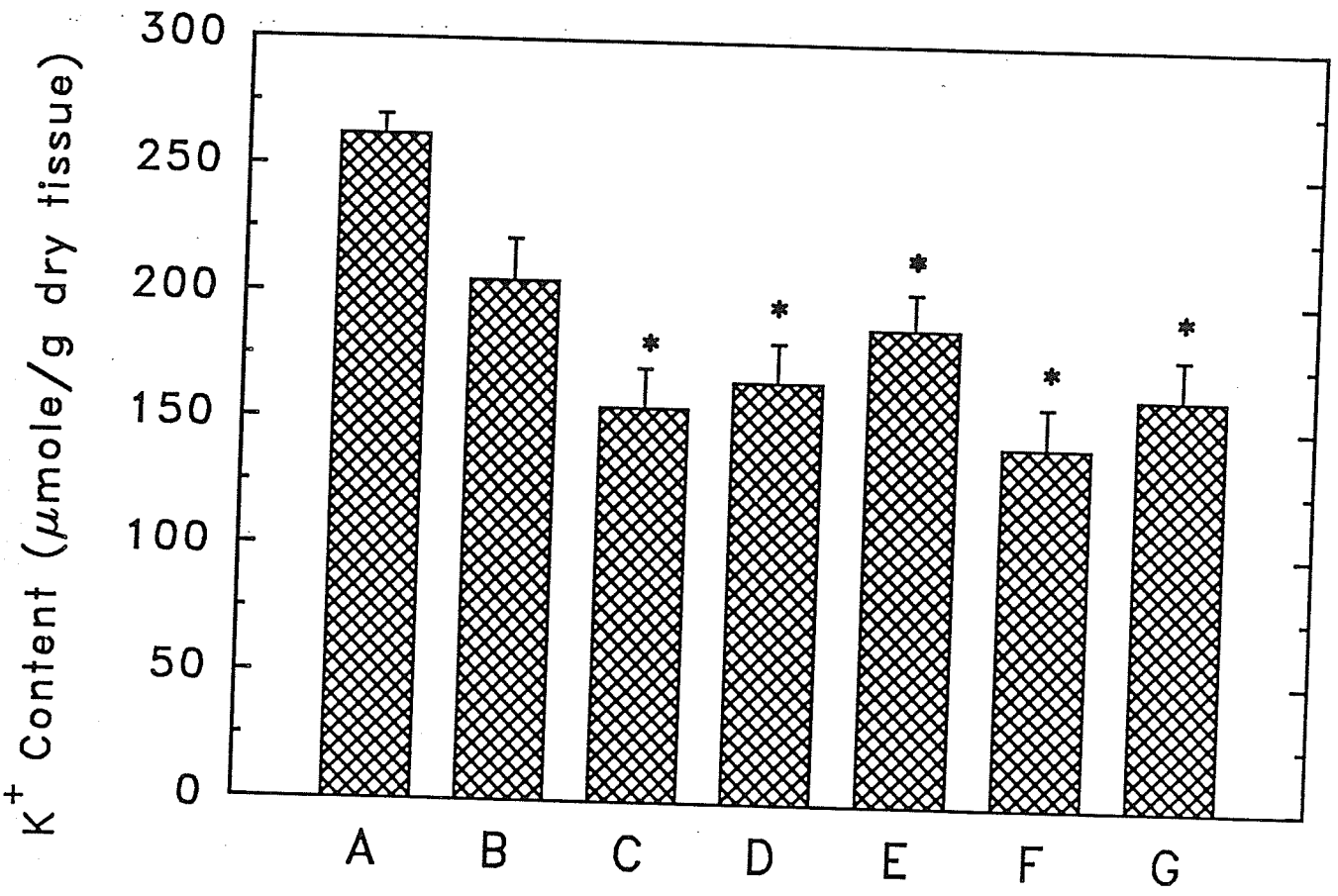


Figure 27. Effect of DMA on cellular K^+ content in isolated ventricular wall during post-ischemic reperfusion in the presence of 35 uM ouabain. Ventricular walls were equilibrated with HEPES solution (pH 7.4) and subjected to 60 min global ischemia ± 6 min of reperfusion. Sucrose solution was injected into the ventricular wall through coronary artery at the end of each experiment. Other experimental conditions were the same as those described in section A.5.

Column A) no-ischemia group;
Column B) Ischemia group;
Column C) Ischemia + ouabain group;
Column D) Ischemia + DMA + ouabain group;
Column E) 6 min of reperfusion in drug-untreated group;
Column F) 6 min of reperfusion in ouabain group;
Column G) 6 min of reperfusion in ouabain + DMA group.

Values are expressed in mean ± SE. Each column represents 5 to 13 samples. * $p<0.05$ vs. no-ischemia group (Column A).

+ ouabain and ischemia + ouabain + DMA group. After 6 min of reperfusion, K^+ was significantly decreased to 181 $\pm$ 15 umole/g dry weight tissue. K^+ was further reduced to 143 $\pm$ 16.2 umole/g dry weight by reperfusion with 35 uM ouabain. DMA treatment failed to normalize K^+ content in the presence of ouabain. Cellular Na^+ and Ca^{2+} contents presented in Figures 17, 18, 25 and 26 were also plotted against resting tension to determine if there was a relationship between Na^+, Ca^{2+} and resting tension (Figure 28). The cellular ion content and the value for resting tension were obtained at the 6th min of reperfusion (except for the non-ischemic ventricular wall) from the same experimental groups. The intracellular Ca^{2+} content was elevated in proportion to the increase of intracellular Na^+ in the early stage of reperfusion. Correspondingly, the rise in resting tension demonstrated a good correlation with the increase of cellular Na^+ and Ca^{2+}.

3. Effect of DMA on creatine phosphokinase (CPK) activity in coronary effluent during post-ischemic reperfusion in the presence of 35 uM ouabain (Table 5)

In this study, the CPK sample were taken from the ventricular walls described in section B.1. CPK activities in the effluent in no-ischemia and drug-untreated group were close to those in our study described in section A.6 and Table 4. Reperfusion after 60 min global ischemia significantly increased CPK activity in the coronary effluent. Administration of 35 uM

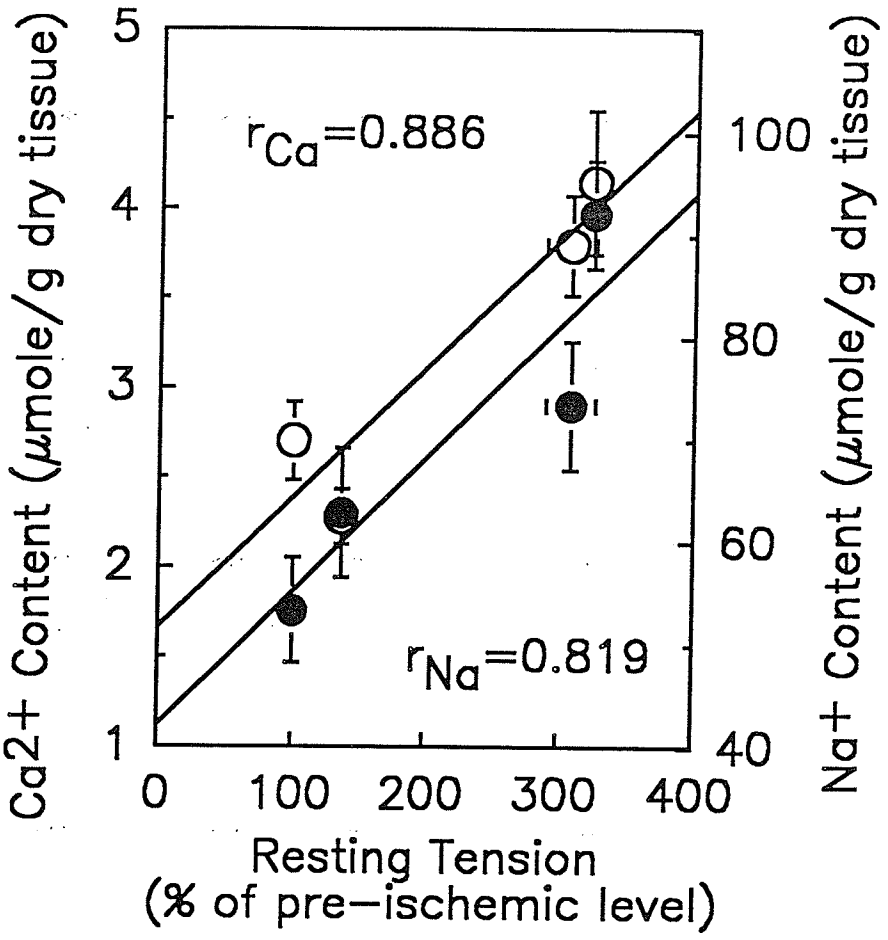


Figure 28. The relationship between cellular Na^+, Ca^{2+} and resting tension in isolated rat right ventricular wall during post-ischemic reperfusion.

Filled circle: resting tension plotted vs. Na^+ content. The filled circles from left to right represent no-ischemia group, ouabain + DMA group, 6 min reperfusion in drug-untreated group and ouabain group, respectively.

Open circle: resting tension plotted vs. Ca^{2+} content. The open circles from left to right represent no-ischemia group, ouabain +DMA group, 6 min reperfusion in drug-untreated group and ouabain group, respectively.

Values are expressed as mean ± SE. Each point represents 5 to 13 samples. If no horizontal error bar is presented at data points, error of the mean was smaller than symbol size. Correlation coefficient value for each ion with resting tension is indicated by r.

Table 5

Effect of 20 uM DMA on creatine phosphokinase (CPK) activity in coronary effluent of isolated ventricular wall during post-ischemic reperfusion in the presence of 35 uM ouabain

Groups	n	CPK Activity (unit/ml/g wet weight)
No-ischemia group	32	6.1 ± 2.4
Drug-untreated group	7	23.6 ± 4.2 * #
Ouabain group	9	40.6 ± 3.7 *
Ouabain + DMA group	6	16.9 ± 4.5 #

The ventricular walls were subjected to 60 min global ischemia and 30 min reperfusion. Samples were collected between 5-10 min of reperfusion. Ouabain (35 uM) was applied for 3 min before and 6 min after ischemia. DMA (20 uM) was applied for 6 min before and after ischemia. n is sample number in each group. Values are expressed as mean ± SE. * $p<0.05$ vs. no-ischemia group. # $p<0.05$ vs. ouabain group.

ouabain resulted in a further elevation of CPK activity which could be effectively inhibited by the simultaneous treatment of 20 uM DMA.

C. POST-ISCHEMIC RECOVERY OF CARDIAC CONTRACTILE FUNCTION MODIFIED BY REPERFUSION pH AND THE PROTECTIVE EFFECT OF DMA DURING ISCHEMIA-REPERFUSION INJURY

1. Effect of DMA on the recovery of cardiac contractile function during post-ischemic reperfusion at different pH

To further understand the mechanism of action of DMA, different reperfusion pH was used to observe the response of the ventricular wall and the protective effect of DMA. All ventricular walls were perfused with MOPS solution at pH 7.2 for pre-ischemic equilibration. Then the ventricular walls were subjected to 55 min global ischemia and 30 min reperfusion. In this investigation, the right ventricular walls were divided into 4 groups according to reperfusion pH: a) pH 7.2 group: the ventricular wall was subjected to 30 min continuous reperfusion with pH 7.2 media following 55 min global ischemia; b) pH 7.9 group: reperfusion with pH 7.9 media for the first 6 min then changed to a pH 7.2 media for the rest of reperfusion; c) pH 7.9 + DMA group: reperfusion with pH 7.9 media + 20 uM DMA for the first 6 min then changed to a pH 7.2 media (without DMA) for the rest of reperfusion; d) pH 6.5 group: reperfusion with pH 6.5 media for the first 6 min then changed to a pH 7.2 media for the rest of reperfusion. MOPS was chosen as a buffer because its

effective buffer range is between pH 6.5-pH 7.9. Since MOPS is membrane impermeable, it does not directly influence intracellular pH. DMA was present in the perfusate only for the first 6 min of reperfusion. No DMA treatment was applied before or during ischemia.

1) Resting tension (RT) (Figure 29)

Figure 29 presents the changes of the resting tension (RT) in the ventricular wall. Before ischemia, RT was 29.6 $\pm$ 1.0 g/g wet weight tissue. It began to rise after 30 min ischemia (data not shown) and at the end of 55 min global ischemia, RT was significantly elevated to 154 $\pm$ 5.0 % of the pre-ischemic level (average of four groups). RT increased to its peak after 6 min of reperfusion at pH 7.2 which is followed by a gradual decline of RT. RT was further elevated when pH 7.9 media was applied in the first 6 min of reperfusion. After changing pH from 7.9 to 7.2, there was a rapid decline of RT in this group. However, RT remained high at the end of 30 min reperfusion. Inclusion of 20 uM DMA into the pH 7.9 media could effectively antagonize the high pH induced rise in RT ($p<0.05$ vs. pH 7.9 group). After changing pH from 7.9 to 7.2 (without DMA), RT remained at a low level (near normal) until the end of reperfusion. In the ventricular wall reperfused at pH 6.5, RT was low at the beginning of reperfusion. It gradually increased at the 6th min of reperfusion. After changing to a pH 7.2 perfusate, RT increased and the pattern was similar to that in pH 7.2 only group.

2) Developed tension (DT) (Figure 30)

Before ischemia was 57 ± 3.7 g/g wet weight tissue. It was reduced to zero approximately 10 to 15 min after the introduction of ischemia. When reperfusion started, DT in pH 7.2 group gradually increased. In pH 7.9 group, there was a transient, rapid increase of DT in the first 2 min of reperfusion followed by a gradual decline. Changing perfusate pH from 7.9 to 7.2 did not significantly improve DT recovery. The presence of 20 uM DMA in the first 6 min of reperfusion significantly improved DT recovery. After changing perfusate pH from 7.9 to 7.2 (DMA was absent in pH 7.2 media), DT had a transient, small decrease followed by a gradual and steady increase ($p<0.05$ vs. pH 7.9 group). When the ventricular wall was reperfused at pH 6.5, DT recovery was slow and poor. Changing perfusate pH from 6.5 to 7.2 at the 6th min of reperfusion induced a further small increase in DT which remained at this level throughout the rest of reperfusion.

3) +dT/dt (Figure 31) and -dT/dt (Figure 32)

The pre-ischemic values of +dT/dt and -dT/dt were 1574 ± 123 and 835 ± 75 g/s/g wet weight tissue, respectively. The post-ischemic recovery in +dT/dt and -dT/dt was similar to that of developed tension with or without DMA treatment.

2. Effect of DMA on cellular Na^+, Ca^{2+} and K^+ content during post-ischemic reperfusion at different pH

Ion content measurements were carried out in tissues were

subjected to 55 min global ischemia and reperfused with MOPS containing solutions with different pH (pH 6.5, pH 7.2 or pH 7.9). DMA, where applicable, was only present in the perfusate for the first 6 min of reperfusion. The experimental conditions were the same as those described in section C.1 except that at defined time points of, 5 ml of cold sucrose solution was injected into the coronary vessel to wash out the extracellular space of the ventricular wall. In this study, the ventricular walls were divided into 8 groups: a) no-ischemia group: the ventricular wall was not subjected to any ischemia-reperfusion challenge and drug treatment; b) ischemia group: the ventricular wall was sucrosed at the end of 55 min global ischemia without reperfusion and drug intervention; c) pH 7.2, 6 min group: the ventricular wall was reperfused at pH 7.2 for 6 min and sucrosed; d) pH 7.9 group: the ventricular wall was reperfused at pH 7.9 for 6 min and sucrosed; e) pH 7.9 + DMA group: the ventricular wall was reperfused for 6 min with a pH 7.9 solution containing 20 uM DMA and sucrosed; f) pH 6.5 group: the ventricular wall was reperfused for 6 min at pH 6.5 and sucrosed; g) pH 6.5 + pH 7.2 group: the ventricular wall was reperfused at pH 6.5 for 6 min and shifted to pH 7.2 solution for another 6 min and sucrosed; H) pH 7.2, 12 min group: the ventricular wall was reperfused at pH 7.2 for 12 min and sucrosed. The mechanical responses of the ventricular wall in these groups during the experiment were similar to those observed in section C.1. Samples were taken at the 6th min of reperfusion because the resting tension was at the

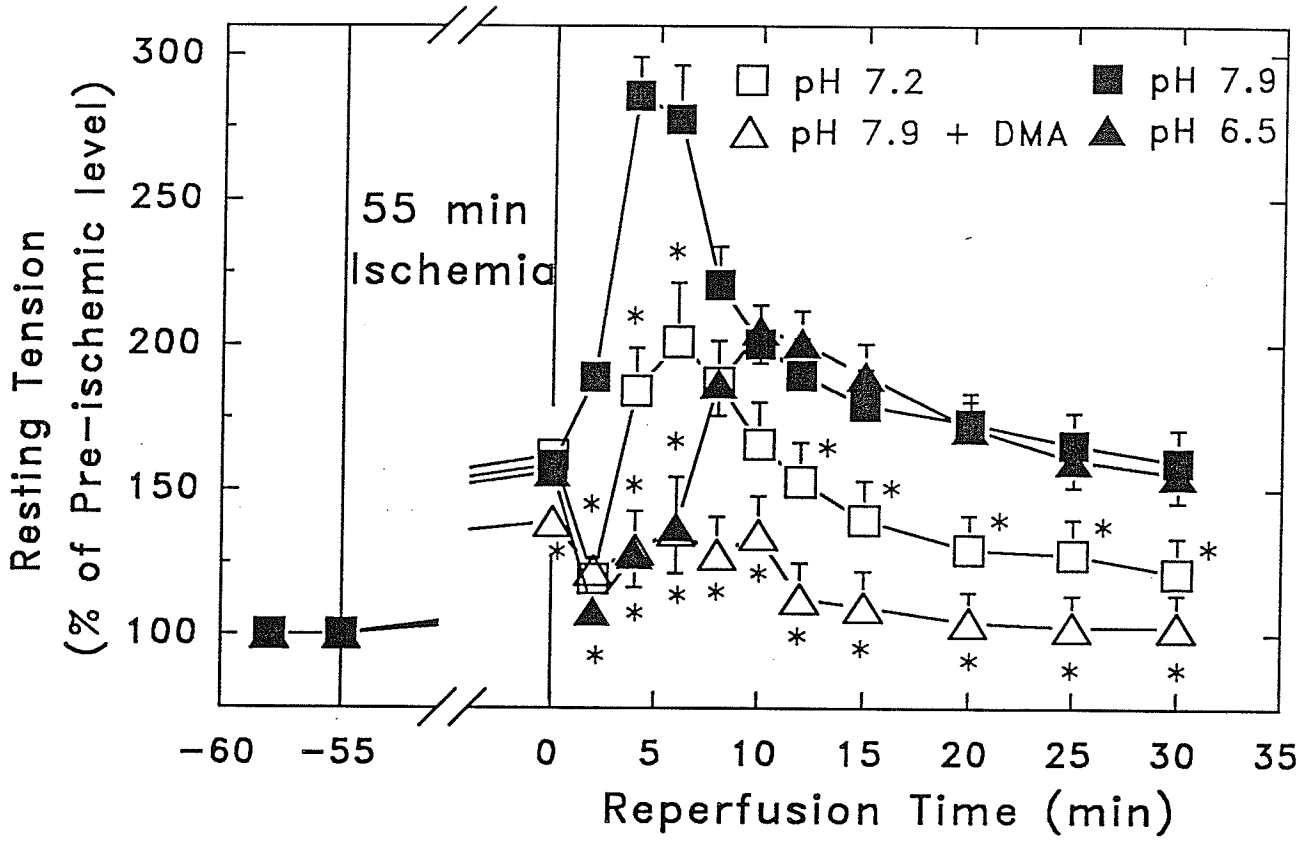


Figure 29. Effect of DMA on the recovery of resting tension in isolated ventricular wall during post-ischemic reperfusion at different pH. All the ventricular walls were equilibrated with pH 7.2 MOPS solution and subjected to 55 min global ischemia and 30 min reperfusion.

Open square: the ventricular wall was subjected to 30 min reperfusion at pH 7.2;
Filled square: reperfusion at pH 7.9 for the first 6 min of reperfusion then changed to a pH 7.2 media for the rest of reperfusion;
Open triangle: reperfusion at pH 7.9 + 20 uM DMA for the first 6 min then changed to a pH 7.2 media (without DMA) for the rest of reperfusion;
Filled triangle: reperfusion with pH 6.5 media for the first 6 min then changed to a pH 7.2 media for the rest of reperfusion.

Values are expressed as mean ± SE of the pre-ischemic level. If no error bar is presented at some data points, the error of the mean was smaller than the symbol size. Each data point represent 5-8 observations. * $p<0.05$ vs. pH 7.9 group (filled square).

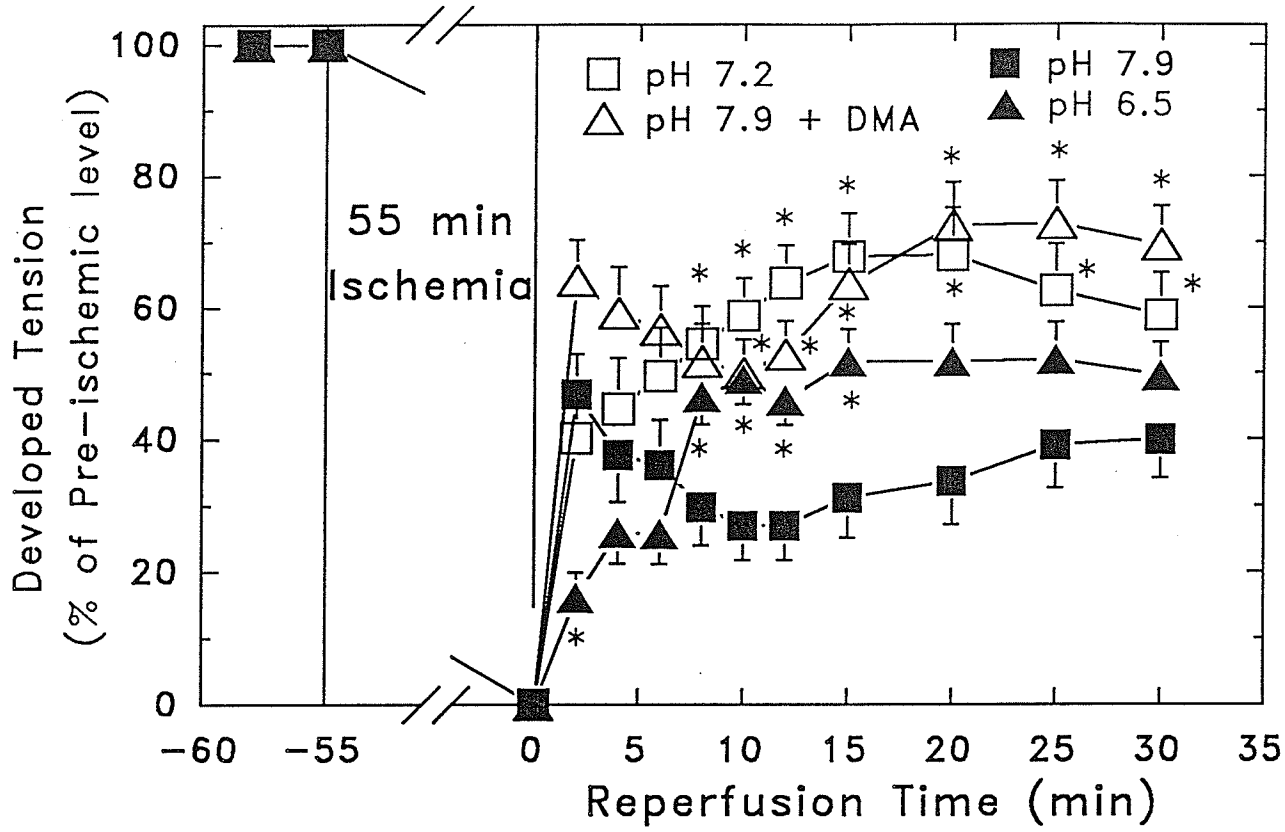


Figure 30. Effect of DMA on the recovery of developed tension in isolated ventricular wall during post-ischemic reperfusion at different pH. All the ventricular walls were equilibrated with pH 7.2 MOPS solution and subjected to 55 min global ischemia and 30 min reperfusion.

Open square: the ventricular wall was subjected to 30 min reperfusion at pH 7.2;
Filled square: reperfusion at pH 7.9 for the first 6 min of reperfusion then changed to a pH 7.2 media for the rest of reperfusion;
Open triangle: reperfusion at pH 7.9 + 20 uM DMA for the first 6 min then changed to a pH 7.2 media (without DMA) for the rest of reperfusion;
Filled triangle: reperfusion with pH 6.5 media for the first 6 min then changed to a pH 7.2 media for the rest of reperfusion.

Values are expressed as mean ± SE of the pre-ischemic level. If no error bar is presented at some data points, the error of the mean was smaller than the symbol size. Each data point represent 5-8 observations. * $p<0.05$ vs. pH 7.9 group (filled square).

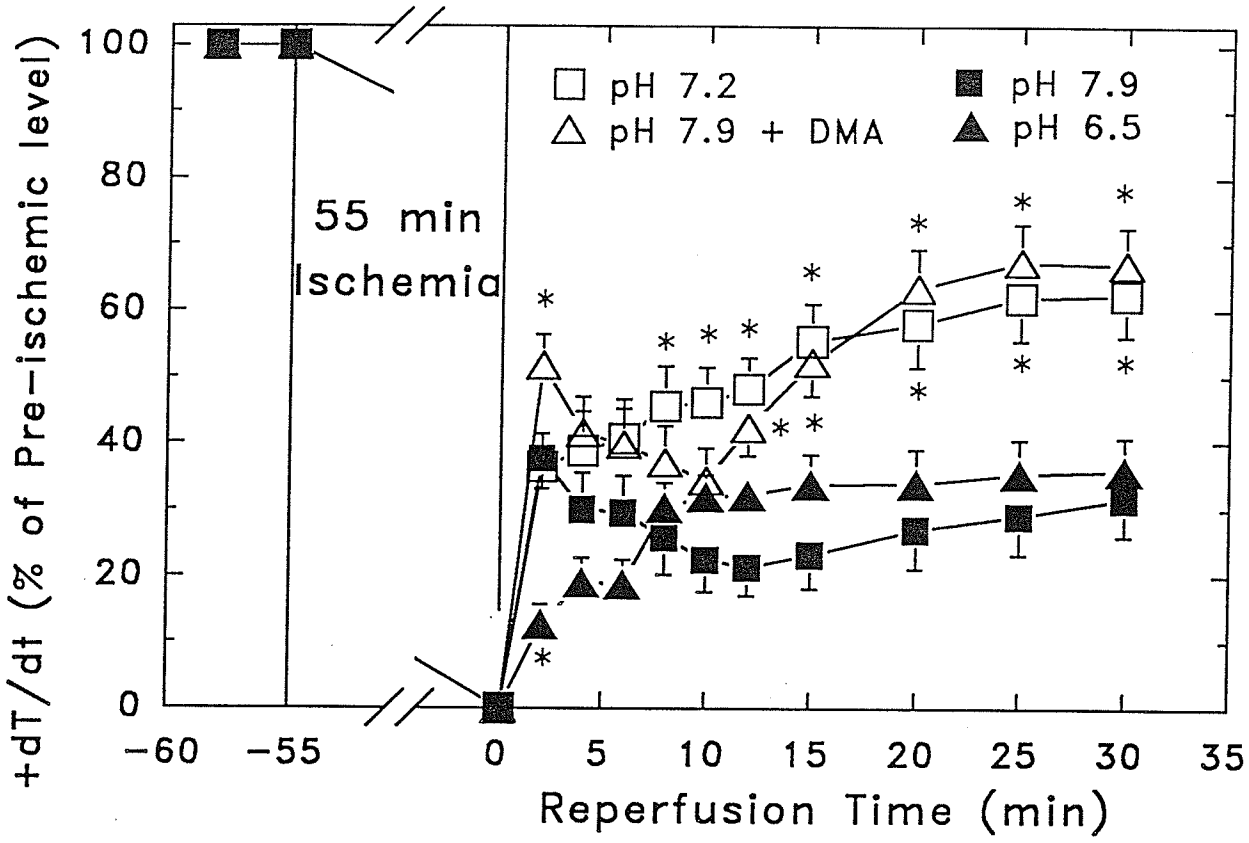


Figure 31. Effect of DMA on the recovery of the maximal velocity of tension development (+dT/dt) in isolated ventricular wall during post-ischemic reperfusion at different pH. All the ventricular walls were equilibrated with pH 7.2 MOPS solution and subjected to 55 min global ischemia and 30 min reperfusion.

Open square: the ventricular wall was subjected to 30 min reperfusion at pH 7.2;
Filled square: reperfusion at pH 7.9 for the first 6 min of reperfusion then changed to a pH 7.2 media for the rest of reperfusion;
Open triangle: reperfusion at pH 7.9 + 20 uM DMA for the first 6 min then changed to a pH 7.2 media (without DMA) for the rest of reperfusion;
Filled triangle: reperfusion with pH 6.5 media for the first 6 min then changed to a pH 7.2 media for the rest of reperfusion.

Values are expressed as mean ± SE of the pre-ischemic level. If no error bar is presented at some data points, the error of the mean was smaller than the symbol size. Each data point represent 5-8 observations. * $p<0.05$ vs. pH 7.9 group (filled square).

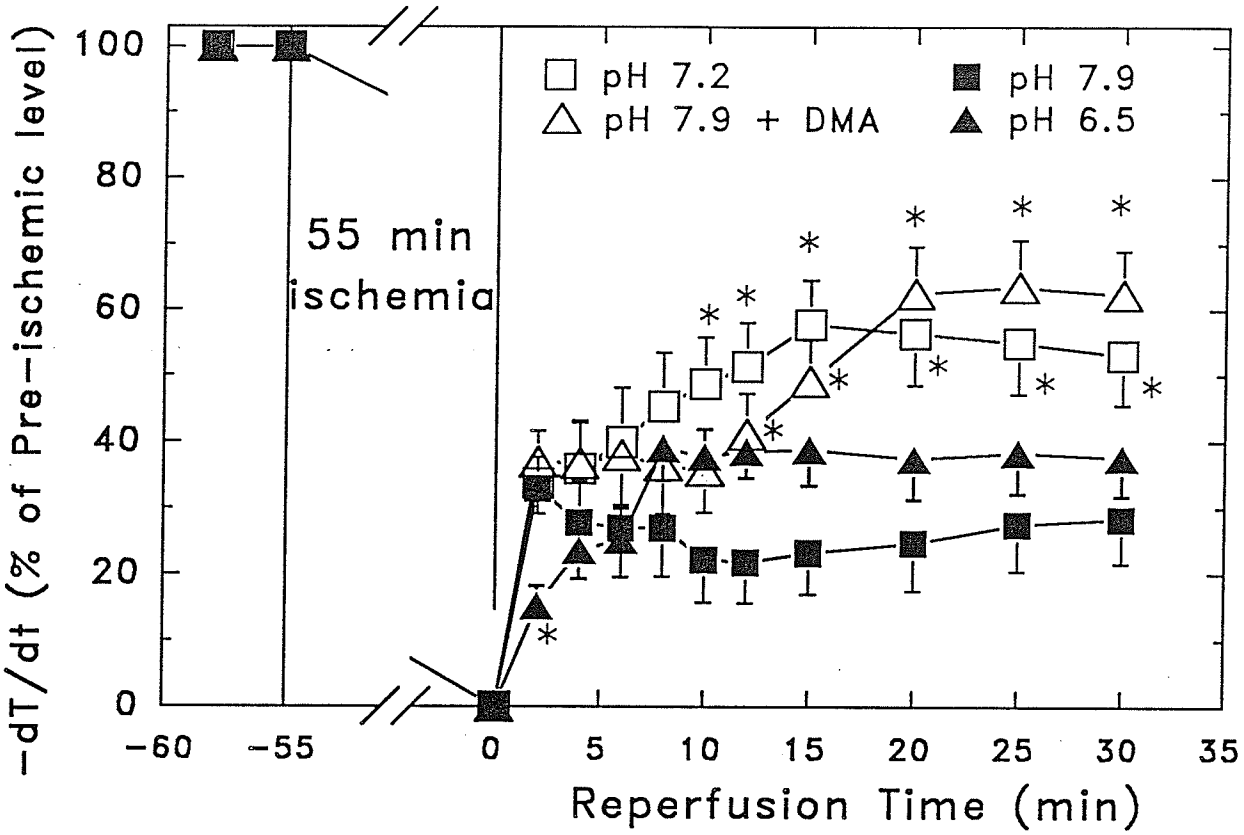


Figure 32. Effect of DMA on the recovery of the maximal velocity of muscle relaxation (-dT/dt) in isolated ventricular wall during post-ischemic reperfusion at different pH. All the ventricular walls were equilibrated with pH 7.2 MOPS solution and subjected to 55 min global ischemia and 30 min reperfusion.

Open square: the ventricular wall was subjected to 30 min reperfusion at pH 7.2;
Filled square: reperfusion at pH 7.9 for the first 6 min of reperfusion then changed to a pH 7.2 media for the rest of reperfusion;
Open triangle: reperfusion at pH 7.9 + 20 uM DMA for the first 6 min then changed to a pH 7.2 media (without DMA) for the rest of reperfusion;
Filled triangle: reperfusion with pH 6.5 media for the first 6 min then changed to a pH 7.2 media for the rest of reperfusion.

Values are expressed as mean $\pm$ SE of the pre-ischemic level. If no error bar is presented at some data points, the error of the mean was smaller than the symbol size. Each data point represent 5-8 observations. * $p<0.05$ vs. pH 7.9 group (filled square).

maximum at that time point. The values of cellular Na^+, Ca^{2+} and K^+ measured in no-ischemia group were close to those reported by other investigators (Alto and Dhalla, 1979; Tani and Neely, 1989) and our study with HEPES solution (see section B.1).

1) Cellular Na^+ content (Figure 33)

Cellular Na^+ content in no-ischemia group was 53.6 $\pm$ 7.0 umole/g dry weight tissue. After 55 min exposure to ischemia, it was not significantly increased. In the ventricular wall reperfused at pH 7.2, Na^+ content was 76.4 $\pm$ 6.3 umole/g dry tissue. Reperfusion at pH 7.9 doubled the Na^+ content. DMA treatment during reperfusion significantly inhibited the pH 7.9 media-induced rise in Na^+ content ($p<0.05$ vs. pH 7.9 group). Na^+ content was elevated in pH 6.5 group after 6 min of reperfusion. Changing perfusate pH from 6.5 to 7.2 for 6 min resulted in a further small increase in Na^+ content.

2) Cellular Ca^{2+} content (Figure 34)

Cellular Ca^{2+} content in no-ischemia group was 2.10 $\pm$ 0.24 umole/g dry weight tissue. After 55 min exposure to ischemia, it was not significantly increased. In pH 7.2 group, Ca^{2+} content was significantly increased ($p<0.05$ vs. no-ischemia group). Reperfusion at pH 7.9 resulted in a further increase in cellular Ca^{2+} ($p<0.05$ vs. pH 7.2 group). DMA treatment during reperfusion significantly inhibited the rise in cellular Ca^{2+} ($p<0.05$ vs. pH 7.9 group). Reperfusion at pH 6.5 for 6 min did not significantly increase cellular Ca^{2+}. Changing perfusate pH from 6.5 to 7.2 resulted in a significant increase in Ca^{2+}

content which was similar to that observed in pH 7.2 group.

3) Cellular K^+ content (Figure 35)

Cellular K^+ content in no-ischemia group was 251 $\pm$ 3.4 umole/g dry weight tissue. It was not significantly changed after 55 min global ischemia. Reperfusion at pH 7.2 decreased cellular K^+ content. Reperfusion at pH 7.9 resulted in a further drop of cellular K^+. This could be partially antagonized by inclusion of 20 uM DMA in the pH 7.9 media. Reperfusion at pH 6.5 resulted in a decrease of cellular K^+ which had a further decrease after changing perfusate pH from 6.5 to 7.2.

3. Effect of DMA on creatine phosphokinase (CPK) activity in coronary effluent during post-ischemic reperfusion at different pH (Table 6)

In this study, the CPK sample were taken from the ventricular walls described in section C.1. CPK activity in the effluent in no-ischemia group was close to that in our study described in section A.6 and B.3. In the effluent of pH 7.2 group, CPK activity was tripled during reperfusion in comparison to no-ischemia group. Reperfusion at pH 7.9 further increased the CPK activity. DMA treatment during reperfusion effectively inhibited this elevation. Reperfusion at pH 6.5 for 6 min resulted in a mild increase of CPK activity which was further elevated by subsequent reperfusion at pH 7.2.

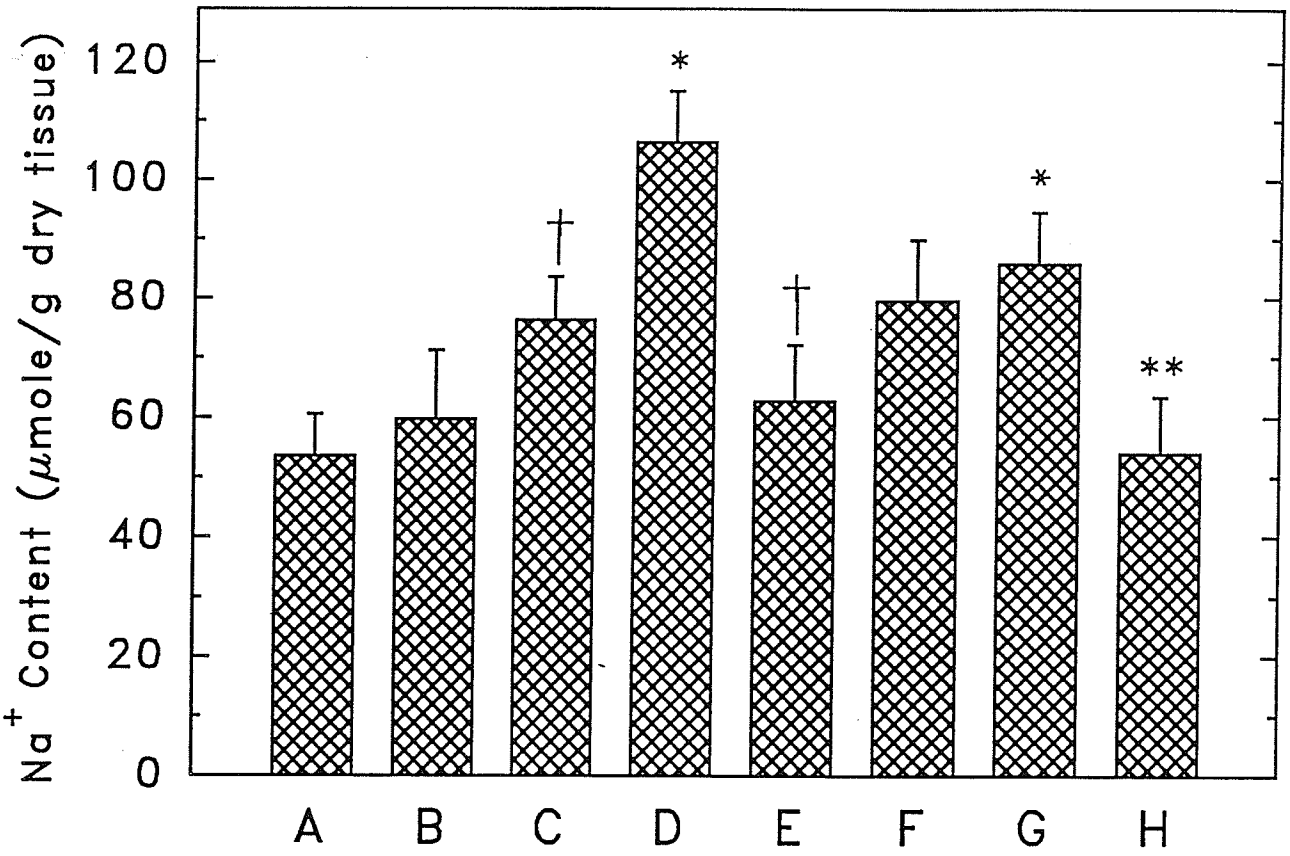


Figure 33. Effect of DMA on cellular Na^+ content in isolated ventricular wall during post-ischemic reperfusion at different pH. Ventricular walls were equilibrated with MOPS solution (pH 7.2) and subjected to 55 min global ischemia with 0-12 min of reperfusion. Sucrose solution was injected into the ventricular wall through coronary artery at the end of each experiment. Other experimental conditions were the same as those described in section C.1.

Column A: No-ischemia group;
Column B: Ischemia group;
Column C: pH 7.2, 6 min group;
Column D: pH 7.9 group;
Column E: pH 7.9 + DMA group;
Column F: pH 6.5 group;
Column G: pH 6.5 + pH 7.2 group;
Column H: pH 7.2, 12 min group.

Values are expressed in mean $\pm$ SE. Each column represents 4-10 samples. * $p<0.05$ vs. no-ischemia group (Column A); ** $p<0.05$ vs. pH 6.5 + 7.2 group (Column G).

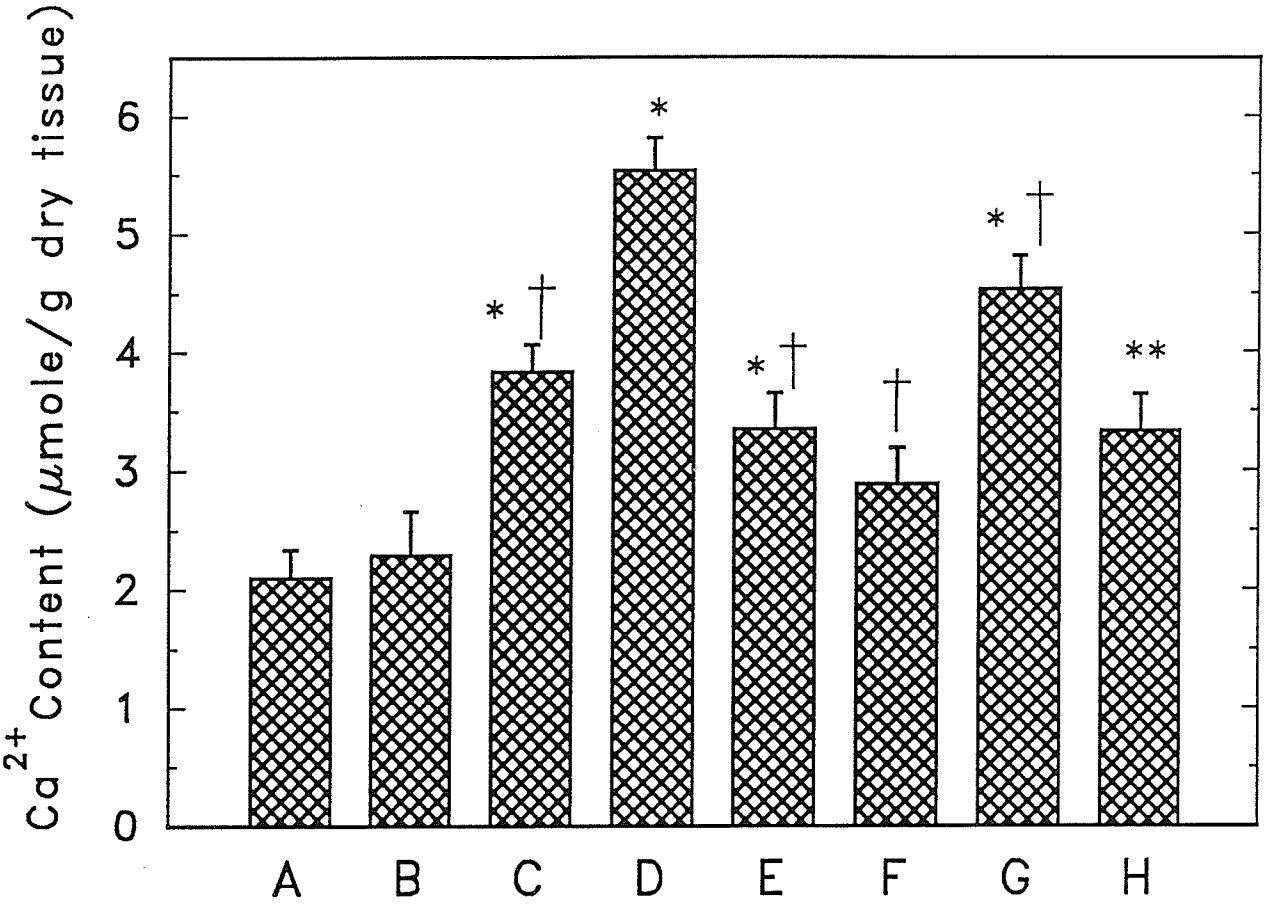


Figure 34. Effect of DMA on cellular Ca^{2+} content in isolated ventricular wall during post-ischemic reperfusion at different pH. Ventricular walls were equilibrated with MOPS solution (pH 7.2) and subjected to 55 min global ischemia with 0-12 min of reperfusion. Sucrose solution was injected into the ventricular wall through coronary artery at the end of each experiment. Other experimental conditions were the same as those described in section C.1.

Column A: No-ischemia group;
Column B: Ischemia group;
Column C: pH 7.2, 6 min group;
Column D: pH 7.9 group;
Column E: pH 7.9 + DMA group;
Column F: pH 6.5 group;
Column G: pH 6.5 + pH 7.2 group;
Column H: pH 7.2, 12 min group.

Values are expressed in mean ± SE. Each column represents 4-10 samples. * $p<0.05$ vs. no-ischemia group (Column A); ** $p<0.05$ vs. pH 6.5 + 7.2 group (Column G); + $p<0.05$ vs. reperfusion at pH 7.9 (Column D).

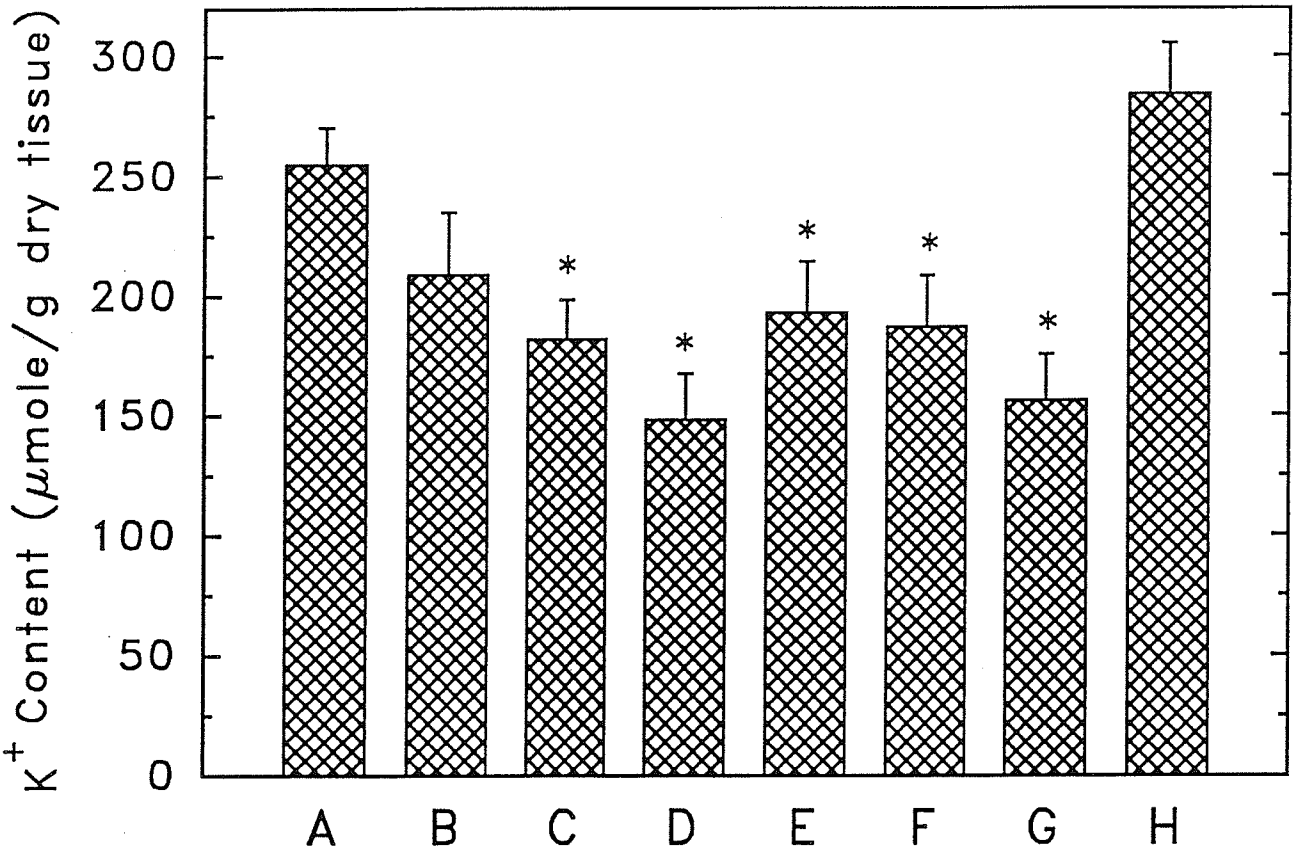


Figure 35. Effect of DMA on cellular K^+ content in isolated ventricular wall during post-ischemic reperfusion at different pH. Ventricular walls were equilibrated with MOPS solution (pH 7.2) and subjected to 55 min global ischemia with 0-12 min of reperfusion. Sucrose solution was injected into the ventricular wall through coronary artery at the end of each experiment. Other experimental conditions were the same as those described in section C.1.

Column A: No-ischemia group;
Column B: Ischemia group;
Column C: pH 7.2, 6 min group;
Column D: pH 7.9 group;
Column E: pH 7.9 + DMA group;
Column F: pH 6.5 group;
Column G: pH 6.5 + pH 7.2 group;
Column H: pH 7.2, 12 min group.

Values are expressed in mean ± SE. Each column represents 4-10 samples. * $p<0.05$ vs. no-ischemia group (Column A).

Table 6

Effect of DMA on creatine phosphokinase (CPK) activity in coronary effluent of isolated right ventricular wall during post-ischemic reperfusion at different pH

Groups	n	CPK Activity (unit/ml/g wet tissue)
a. No-ischemia group	34	9.0 ± 1.2
b. Reperfusion at pH 7.2 for 6 min	8	27.2 ± 2.3 * #
c. Reperfusion at pH 7.9 for 6 min	7	39.4 ± 7.9 *
d. Reperfusion at pH 7.9 + 20 uM DMA for 6 min	8	18.8 ± 3.4 * #
e. Reperfusion at pH 6.5 for 6 min	12	17.3 ± 3.4 * #
f. Reperfusion at pH 6.5 for 6 min then at pH 7.2 for another 6 min	12	24.7 ± 5.0 * #

The ventricular walls were subjected to 55 min global ischemia and 30 min reperfusion. Samples were collected between 5-10 min of reperfusion. n represents sample number in each group. Values are expressed as mean ± SE. * $p<0.05$ vs. no-ischemia group. # $p<0.05$ vs. pH 7.9 group (group C).

V. DISCUSSION

A. PROTECTIVE EFFECTS OF 5-(N,N-DIMETHYL)AMILORIDE (DMA) IN ISOLATED RIGHT VENTRICULAR WALL OF RAT DURING ISCHEMIA-REPERFUSION INJURY

In the present study, the poor post-ischemic recovery of contractile function (RT, DT, +dT/dt and -dT/dt) of the ventricular wall was significantly improved by DMA in a dose-dependent manner in the reperfusion period. Treatment with 1 uM DMA only exhibited a marginal and paradoxical improvement of cardiac function during reperfusion. When the concentration of DMA was increased to 5 uM, a significant improvement of the post-ischemic recovery was observed. At the concentration of 20 uM, DMA exerted the best effect on the recovery of the mechanical function. It is important to note that the ischemic protocol used in this study was severe which further emphasizes the efficacy of the drug. It is also noteworthy that creatine phosphokinase (CPK) release from the intracellular space, which has been frequently employed as a marker of myocyte damage (Bersohn and Shine, 1983; Karmazyn, 1988), was also inhibited by DMA treatment of the heart. These would strongly suggest, when taken in conjunction with the contractile data, that DMA protected against ischemia-reperfusion damage in the heart.

The different administration protocols of DMA elicited

different functional recovery. Prolonged administration of 20 uM DMA during 30 min reperfusion resulted in a similar decrease in RT as observed in the ventricular walls with shorter DMA treatment. The low CPK activity in coronary effluent was coincident with the depressed RT rise. However, the recovery of cardiac contractile function was not further enhanced with long term treatment of DMA. Instead, DT, +dT/dt and -dT/dt, after a transient increase in the early phase of reperfusion, were gradually decreased during the later phase of reperfusion. In consideration of the normalized RT and CPK activity in this group, the depressed contractile function seems not due to the ischemia-reperfusion injury itself. It is most likely due to the negative inotropic effect of the drug. When used in high concentrations or perfused for longer periods of time, DMA will depress force generation in control, non-ischemic ventricular walls. For example, 30 min of perfusion with 20 uM DMA reduced tension development in non-ischemic hearts to 16.0 $\pm$ 4.6 % (n=5, data not shown in RESULTS section) of drug-untreated level. However, under the shorter DMA pre-ischemic perfusion time (3 min) used in the majority of experiments here, DMA did not manifest a significant inotropic effect. When 5 uM DMA was used for the prolonged administration during reperfusion, the improved recovery of cardiac function during reperfusion was not significantly influenced in comparison to the shorter DMA (5 uM) treatment. It was also observed in a preliminary experiment that the depressed cardiac contractile function by the prolonged

treatment of 20 uM DMA could gradually increase after subsequent reperfusion with DMA free buffer.

Significant information on ischemia-induced damage was obtained by introducing DMA prior to or after ischemia. The inclusion of 20 uM DMA in the perfusate ONLY for the first 3 min of reperfusion exhibited a protective effect similar to the protocol with DMA treatment for 3 min prior to and after ischemia. The exposure of the hearts to 20 uM DMA only for 3 min prior to ischemia failed to result in any significant protection and DMA did not to inhibit the formation of ischemic contracture.

It should be noted that the protective effects of cardioactive agents, such as those listed in Table 1, are dependent on pre-ischemic treatment. If they are administered only during reperfusion, their protective effect will be very weak or even abolished (Watts et al, 1980; Bersohn and Shine et al, 1983; Nayler et al, 1988). The effectiveness of DMA during reperfusion is one of our major findings in the present study which has very important pathophysiological significance. First, this clearly indicated that DMA exerted its effect primarily during the early phase of reperfusion period rather than during ischemia. Second, since DMA could almost normalize DT and RT despite the occurrence of ischemic contracture, our data suggest that irreversible damage may not occur during ischemia but during the first minutes of reflow in these experimental conditions. DMA, if present, can prevent the events during reperfusion which cause that damage. These data, therefore, support the argument that reperfusion

causes the death of cells which were potentially viable at the time of reperfusion and irreversible injury may not occur to any discernible degree during ischemia. This matter is currently controversial (Hearse, 1988). Third, the effectiveness of DMA during reperfusion can be used to distinguish the effect of DMA from other cardioactive agents (see section E of DISCUSSION).

In our experimental condition, the ion imbalance was not significant at the end of ischemia although intracellular Na^+ and Ca^{2+} tended to increase. The reason for this can be attributed to the method used to measure the ion content. Our method has the advantage in measuring the total accumulation of ion inside the cell, but it cannot detect the increase of the free form of ion (such as free Na^+ and free Ca^{2+}) in the cytosol. K^+ content was decreased by about 20 % at the end of ischemia which was consistent with previous report (Weiss and Shine, 1984; Gaspardone et al, 1986; Tani and Neely, 1989; Wilde et al, 1990) although there is no statistical difference in comparison to the pre-ischemic value. Significant ion imbalance occurred during reperfusion.

When HEPES in the perfusate was replaced with bicarbonate, DMA treatment could also result in a better recovery of the contractile function in the ventricular wall (Table 3). This suggests that the Cl^-/HCO_3^- system may not play a major role in the post-ischemic recovery of the contractile function of the ventricular wall.

B. EFFECTS OF CELLULAR Na^+ ALTERATION BY OUABAIN ON POST-ISCHEMIC CARDIAC DYSFUNCTION AND CELL INJURY IN THE ABSENCE OR PRESENCE OF DMA

The present data demonstrated that a non-toxic, mildly inotropic concentration of ouabain could aggravate the ischemia-reperfusion-induced cardiac dysfunction observed in the isolated rat right ventricular wall. Ouabain-treated ventricular wall had higher RT, and poorer recovery of DT, +dT/dt and -dT/dt during reperfusion than the drug-untreated ventricular wall. The mechanism of action for ouabain was likely to be through an inhibition of the membrane Na^+-K^+ ATPase (Na^+ pump). In support of this, the intracellular Na^+ was significantly increased and intracellular K^+ decreased. It is important to re-emphasize that these effects were caused by a relatively low dose of ouabain which had no toxic action alone. Interestingly, similar qualitative findings have been shown by subtoxic doses of ouabain in a model of hypoxia-reoxygenation challenge to heart (Cunningham et al, 1989). The relationship among Na^+, Ca^{2+} and resting tension (Figure 28) indicated that the rise in resting tension was correlated with the increase of intracellular Na^+ and Ca^{2+}. It was also demonstrated (Figure 5-8, 17, 18, 20-26 and 28) that the lower the intracellular Na^+, the lower the intracellular Ca^{2+} and the better was the post-ischemic recovery of contractile function. These data, therefore, are consistent with a central role for the disturbance of intracellular Na^+ homeostasis in ischemic-reperfusion-induced cardiac dysfunction.

As outlined earlier in the text, the increased intracellular Na^+ concentration may in turn stimulate Ca^{2+} entry via Na^+-Ca^{2+} exchange which is crucial for the subsequent Ca^{2+} overload.

It is likely that Na^+-K^+ ATPase activity is partially inactivated during ischemia due to lack of ATP supply (Bersohn et al, 1982[a]) and ischemic damage (Jennings et al, 1987; Dhalla et al, 1988). Its activity remains relatively low during the early stage of reperfusion (Daly et al, 1984; Dhalla et al, 1988). The lowered Na^+-K^+ ATPase activity reduces Na^+ efflux during reperfusion and favours Na^+ accumulation inside the cell. Under this condition, if ouabain is applied to further inhibit the Na^+ pump, it will further increase intracellular Na^+ and lead to more severe damage of the cell. The present study has clearly demonstrated the deleterious effect of low concentrations of ouabain in ischemia-reperfusion.

Consistent with our data reported in section IV.A.1-4, DMA demonstrated potent protective action against reperfusion-induced cardiac dysfunction in the presence of ouabain. The concentration (20 uM) of DMA in the present study was chosen from previous experiments which demonstrated that this concentration was optimal. It is proposed that the mechanism by which DMA antagonized the detrimental effect of ouabain on the reperfused ventricular wall is through an inhibition of transsarcolemmal Na^+-H^+ exchange. This inhibition of Na^+-H^+ exchange results in a reduction of Na^+ entry from the extracellular space and a lower concentration of Na^+ inside the myocytes. Therefore, even if the

Na^+ pump is inhibited by ouabain during reperfusion, Na^+ cannot accumulate in the cells. This hypothesis is based upon several lines of evidence.

First, there is no evidence that DMA can directly antagonize ouabain's action by stimulating Na^+-K^+ ATPase activity. On the contrary, Zhuang et al (1984) reported an IC_{50} of 3 mM for DMA to inhibit the Na^+ pump. Thus, DMA must be acting via an ion transport pathway other than the Na^+ pump.

Second, since the Na^+-H^+ exchange pathway can bring large amount of Na^+ into the myocardial cell (Frelin et al, 1984), the inhibition of the Na^+-H^+ exchanger could antagonize the increase in $[Na^+]_i$ caused by the depressed activity of the Na^+ pump. Frelin et al (1984) reported that the application of ouabain to quiescent chick cardiac cells tripled intracellular Na^+ and this increase could be normalized by ethylisopropylamiloride (EIPA), another Na^+-H^+ exchange inhibitor. Similar data were obtained by Kim and Smith (1986) with beating cultured heart cells. These studies demonstrated that the ouabain-induced elevation in $[Na^+]_i$ could be antagonized by blocking Na^+-H^+ exchange (Lazdunski, 1985). The data from the present study in cardiac tissue suggest that the decreased cellular $[Na^+]$ by DMA is due to an decreased Na^+ entry rather than an increased Na^+ efflux.

Third, the dose of DMA used in the present study (20 uM) is close to the IC_{50} for DMA to block Na^+-H^+ exchange in sarcolemmal vesicles (7 uM, Kleyman and Cragoe, 1988).

Fourth, the antagonistic effect of DMA on the Na^+ loading-

related cardiac dysfunction does not appear to be due to the inhibition of other sarcolemmal Na^+ entry pathways, such as the Na^+ channel or Na^+-Ca^{2+} exchange. The IC_{50} for DMA to inhibit the voltage-gated Na^+ channel was > 50 uM (Dennis et al, 1990), therefore, the influence of Na^+ channel blockade in this study would be minimal. The DMA concentration (20 uM) used was far below its IC_{50} (550 uM) for the inhibition of Na^+-Ca^{2+} exchange (Kleyman and Cragoe, 1988). DMA is also unlikely to be acting directly via K^+ transport pathways because, in spite of its protective effect on cardiac function in the presence of ouabain, it could not normalize intracellular K^+ content (Figure 27). Therefore, the most plausible Na^+ transport pathway through which DMA is acting is sarcolemmal Na^+-H^+ exchange.

C. MODIFICATION OF THE POST-ISCHEMIC CARDIAC FUNCTION BY REPERFUSION BUFFER pH

The present study indicates that perfusate pH can modify the post-ischemic recovery in rat ventricular wall. Reperfusion with an alkalinic media can significantly increase cellular Na^+ and Ca^{2+} content and accordingly, worsen cardiac contractile dysfunction and cell damage as indicated by a higher resting tension, poorer recovery of contractility and a significantly elevated CPK activity in the coronary effluent. When DMA is included in the pH 7.9 media for 6 min reperfusion, the ionic imbalance in the cardiac tissue can be normalized and accordingly, the loss of cardiac function and cell damage can be

effectively antagonized. Reperfusion at a lower pH than 7.2 can delay the recovery of developed tension and the rise in resting tension, CPK, cellular Na^+ and Ca^{2+}. Subsequent perfusion at pH 7.2 resulted in cellular ion imbalance and cardiac dysfunction similar to those observed in pH 7.2 only group. This suggests that the acidic reperfusion can only delay the occurrence of cell damage rather than provide protection. These results are consistent with those reported by Williamson (1976) and Panagiotopoulos et al (1990).

Although the effects of reperfusion pH and the action of DMA within this intervention may provide evidence regarding the role of Na^+-H^+ exchange in the development of ischemia-reperfusion injury, caution should be exercised during interpretation of the results. Since protons have extensive effects on biochemical processes, several different ion transport pathways could be involved in the pH-related post-ischemic recovery of contractile function.

1. Na^+-Ca^{2+} exchange

Low pH inhibits sarcolemmal Na^+-Ca^{2+} exchange and high pH stimulates it (Philipson et al, 1982). Under our experimental conditions, Na^+-Ca^{2+} exchange was likely to be activated by pH 7.9 media to stimulate Ca^{2+} entry or inactivated by pH 6.5 media to inhibit Ca^{2+} entry. However, this modification of Na^+-Ca^{2+} exchange activity by pH cannot convincingly explain the mechanism underlying the improved recovery of cardiac function with DMA.

First, a stimulation of Na^+-Ca^{2+} exchange by pH 7.9 media cannot explain a high cellular Na^+ content during reperfusion because the stimulated Na^+-Ca^{2+} exchange would favour Na^+ transport out of the cell. Second, the concentration of DMA used was 20 uM, which is far lower than its IC_{50} (550 uM) for the inhibition of Na^+-Ca^{2+} exchange (Kleyman and Cragoe, 1988). Third, if DMA did inhibit Na^+-Ca^{2+} exchange, both Na^+ efflux and Ca^{2+} influx would be blocked which would be followed by an increase of cellular Na^+ and a decrease of cellular Ca^{2+}. However, this was not the case in our experiment. Both cellular Na^+ and Ca^{2+} were reduced by DMA treatment.

2. Na^+-K^+ ATPase (Na^+ pump)

Another Na^+ transport pathway, the sarcolemmal Na^+-pump, is also influenced by cellular pH (Sulakhe et al, 1985). However, the antagonism of the pH effects by DMA cannot be explained on the basis of an effect on the Na^+-K^+ ATPase. DMA is a very weak Na^+-K^+ ATPase inhibitor (IC_{50}: 3 mM, Zhuang et al, 1984). It does not seem possible that DMA had any effect on Na^+-K^+ ATPase activity in our experimental conditions. Furthermore, previous work (Figure 20-24) demonstrated that the inhibition of Na^+-K^+ ATPase did not improve post-ischemic recovery but instead, aggravated it.

3. Na^+ and Ca^{2+} currents

It is possible that the high reperfusion pH may enhance Na^+

and Ca^{2+} current through potential-dependent Na^+ and Ca^{2+} channels which would result in increases in cellular Na^+ and Ca^{2+} content. DMA may be protecting the heart by blocking these channels more effectively during the alkalotic reperfusion period. Although the possibility exists, there is no supportive evidence that the primary action of DMA is via an inhibition of these alkalosis-enhanced current. Amiloride and DMA are weak bases (pKa=8.8) which interact with the channels only in their protonated form (Kleyman and Cragoe, 1988). As the pH increases, protonation of DMA will decrease (Kleyman and Cragoe, 1988). Thus, on the basis of the chemical characteristics of DMA, one would predict the drug to interact even less with the Na^+ and Ca^{2+} channel under our alkalotic reperfusion condition. Despite this, DMA potently protected the heart. This is entirely inconsistent with DMA exerting its effects through ion channels yet perfectly consistent with an action on the Na^+-H^+ exchanger.

4. Effects of low pH

At pH 6.5, one would expect a smaller transsarcolemmal H^+ gradient, depressed Na^+-H^+ exchange, no Na^+ accumulation and protection against reperfusion damage. However, the increased cellular Na^+ during reperfusion at pH 6.5 appears to argue against this. Several factors may contribute to the Na^+ accumulation in the low pH reperfusion. First, the imposed electrical stimulation may open the voltage-dependent Na^+ channel to allow Na^+ entry even at an acidic pH (Gilliam et al, 1990).

Second, if the intracellular pH is lower than 6.5 (for severe ischemia, this is possible (Jacobus et al, 1977; Cobbe and Poole-Wilson, 1980)), a small outwardly directed H^+ gradient may still exist for Na^+ influx via Na^+-H^+ exchange in the early stage of reperfusion. Third, the inhibition of Na^+-Ca^{2+} exchange and Na^+ pump by the acidic media will retard Na^+ efflux. Thus, the combination of a small, continuous entry of Na^+ coupled with an inhibition of Na^+ efflux may explain the elevated tissue [Na^+] during the reperfusion period with pH 6.5 media.

The main limitation of the present study was that a direct measurement of intracellular pH was not taken during ischemia and reperfusion. Because of this, it is not known how large the transsarcolemmal H^+ gradient is nor how long it remains during reperfusion. However, as indicated by many other investigators (as reviewed by Poole-Wilson, 1978), intracellular pH is certainly very acidic at the end of ischemia (pH 6.0-6.8). The extracellular pH during reperfusion will certainly be governed by the pH of the reperfusion solution which is usually near neutral. Thus, it is highly unlikely to be anything other than an outwardly directed H^+ gradient across the sarcolemmal membrane at the start of the reperfusion. Although the central assumption of our study is not directly supported by pH measurement, it is certainly not unreasonable and it is consistent with the existing literature. Our work has demonstrated that the first 3 min of reperfusion was important (Figure 13-16) which would suggest that the H^+ gradient was maintained within this critical time period.

This is also consistent with other observations using nuclear magnetic resonance which indicated a recovery of intracellular pH during the first 3 min of reperfusion (Tosaki and Braquet, 1990).

The observation that post-ischemic recovery of cardiac function was pH sensitive did not provide any direct support for the conclusion that it depends on Na^+-H^+ exchange. However, the data from the present study did provide evidence that the sarcolemmal Na^+-H^+ exchange was involved. First, tissue Na^+ increased significantly when the reperfusion solution was alkalotic. This demonstrates an important association between extracellular pH and tissue Na^+. Secondly, DMA could block this association by reducing the tissue Na^+ loading. DMA could also protect the myocardial function. Finally, the DMA concentration was within the IC_{50} for inhibition of Na^+-H^+ exchange but no other ion transport pathway as discussed above. Thus, the pH sensitivity of cardiac recovery during reperfusion on its own allows us to draw no firm conclusions but the tissue Na^+ data coupled with the DMA effects in altered reperfusion pH do permit us to make some rather clear and significant conclusions about the role of Na^+-H^+ exchange for the development of ischemia-reperfusion injury. This will be discussed in detail in the next section.

D. ROLE OF Na^+-H^+ EXCHANGE IN THE ISCHEMIA-REPERFUSION INJURY IN MYOCARDIUM

1. Biochemical evidence supporting the involvement of Na^+-H^+ exchange for the development of post-ischemic reperfusion injury in myocardium

If the Na^+-H^+ exchange pathway is involved in the ionic disturbances associated with contractile dysfunction during ischemia-reperfusion challenge, the Na^+-H^+ exchanger must be proven to: 1) be present in the myocardial sarcolemmal membrane, and 2) have the biological characteristics compatible with the transport of Na^+ into the myocardial cell and H^+ out of the cell during ischemia-reperfusion conditions. These conditions must be satisfied before the Na^+-H^+ exchange hypothesis may be considered tenable.

The independent labs of Lieberman (Piwnica-Worms et al, 1985) and Lazdunski (Frelin et al, 1984; Lazdunski et al, 1985) were the first to identify and characterize the Na^+-H^+ exchange pathway in isolated myocardial cells. Pierce and Philipson (1985) and Seiler et al (1985) independently identified that the primary site for the exchanger in the myocardial cell was located in the sarcolemmal membrane of the heart. These works together demonstrated the biochemical characteristics of the Na^+-H^+ exchange transport system in the heart. The Na^+-H^+ exchanger in the heart appears to be active primarily when the intracellular pH is acidic (Lazdunski et al, 1985; Piwnica-Worms et al, 1985). When stimulated, this transport pathway acts to transport H^+ out

of the cell in exchange for extracellular Na^+ (Frelin et al, 1984; Lazdunski et al, 1985; Piwnica-Worms et al, 1985). It is found to be the primary mechanism for normalizing intracellular pH under acidic conditions inside the cell (Kaila et al, 1987; Vaughan-Jones, 1988; Weissberg et al, 1989). The quantity of Na^+ which enters the myocardial is very large (Frelin et al, 1984; Vaughan-Jones, 1988). This transport pathway may allow more Na^+ influx into the myocardial cell than any other pathway (Lazdunski et al, 1985). For example, if the cell $[Na^+]$ increases after Na^+ pump inhibition, application of a blocker of Na^+-H^+ exchange (to reduce Na^+ entry) will antagonize the effects of ouabain and effectively normalize cell Na^+ levels (Frelin et al, 1984, Kim and Smith 1986). In studies of cardiac Purkinje fibres, a significant increase of cellular $[Na^+]$ rises occurs within 1 min after acid loading (Kaila et al, 1987). The activity of Na^+-H^+ exchange is ATP-independent. Considering the ATP depleted state at the beginning of reperfusion, this feature has special importance. Therefore, the biochemical characteristics of Na^+-H^+ exchange described above are compatible with its involvement in the schema of ischemia-reperfusion injury events outlined in Figure 1.

2. Pharmacological evidence supporting the involvement of Na^+-H^+ exchange in post-ischemic reperfusion injury in myocardium

   If Na^+-H^+ exchange is an important mechanism to mediate

ischemia-reperfusion damage to the heart, then application of a pharmacological agent which blocks this transport pathway should be able to protect the heart. According to the schema depicted in Figure 1, an inhibition of Na^+-H^+ exchange would block Na^+ entry into the cell during the early phase of reperfusion and therefore, Ca^{2+} overload and its detrimental consequences would be avoided. In the present study, DMA was proved to be a potent protective agent against ischemia-reperfusion. It significantly improved the post-ischemic recovery of contractile function, normalized cellular Na^+ and Ca^{2+} content and prevented creatine phosphokinase leakage from myocardium. These results were consistent with DMA's pharmacological effect, inhibition of Na^+-H^+ exchange.

Several important characteristics of DMA during the ischemia-reperfusion challenge should be emphasized for understanding its mechanism of action. First, the effective concentrations of DMA used in the present study were 5-20 uM which were well within the reported IC_{50} (~ 7 uM) of DMA for inhibiting Na^+-H^+ exchange in isolated vesicles (Kleyman and Cragoe, 1988). The DMA concentrations we used were much lower than those reported by other investigators using amiloride (200-1500 uM, Karmazyn, 1988; Tani and Neely, 1989). This effective dose range was also in parallel with the increased selectivity of DMA on Na^+-H^+ exchange in comparison to amiloride.

Second, DMA treatment prior to ischemia was not essential for its protective effect. It remained effective if introduced

only upon reperfusion. Furthermore, this effect of DMA occurred in the early stage of reperfusion (3-6 min). Prolonged exposure to DMA during reperfusion could not induce further improvement of cardiac function. This was again entirely consistent with the sequence of events proposed in Figure 1 that the Na^+-H^+ exchange pathway would be stimulated primarily during the initial stage of reperfusion.

Third, our study suggests that Na^+ plays an important role in the development of ischemia-reperfusion injury. A significant rise in cellular Na^+ has been observed in the present study which was accompanied by a corresponding Ca^{2+} overload and cardiac dysfunction. Administration of ouabain to the ventricular wall to block the Na^+ pump exacerbated the sodium increase and accordingly, worsened the recovery from ischemia during reperfusion. Perfusion with DMA antagonized the detrimental effects of ouabain by reducing cellular Na^+. This effect can only be explained with the inhibition of Na^+-H^+ exchange. Thus, it is apparent that Na^+ entry through Na^+-H^+ exchange is the cause of cellular Na^+ loading. Further, the reperfusion-induced Na^+ loading in myocardium in the early phase precedes the Ca^{2+} overload (Tani and Neely, 1989) and is closely related to the reperfusion injury. This association between the ion imbalance and reperfusion contracture was presented in Figure 28 which was also in full agreement with the proposed cascade of ion changes during reperfusion. In the present study, Na^+ content was almost doubled after reperfusion for 6 min with ouabain. This value did

not include the amount of Na^+ which had been extruded via Na^+-Ca^{2+} exchange in the first minutes of reperfusion. Tani and Neely (1989) demonstrated that the peak value of cellular Na^+ was at the first 1 or 2 min of reperfusion which was followed by a gradual decline. In the present study, the time point (6 min reperfusion) for sampling may not be at the peak of cellular Na^+. Therefore, actual value for cellular Na^+ in the present study could be higher than we presented. In spite of this, the measured Na^+ level was already high enough to activate Na^+-Ca^{2+} exchange and cause Ca^{2+} overload. Sonn and Lee (1988) demonstrated that when cellular Na^+ activity increased from 9.5 mM to 11 mM, the developed tension of cardiac muscle (as a function of cellular Ca^{2+}) increased 2 fold.

Fourth, reperfusion injury is sensitive to the pH of the reperfusion solution (Nayler et al, 1988; Meng et al, 1991). One would hypothesize according to the schema in Figure 2 that the greater the transsarcolemmal H^+ gradient at the beginning of reperfusion, the greater the stimulation of the Na^+-H^+ exchange pathway and the greater was the Na^+ entry and subsequent damage. Consistent with this line of reasoning, our study demonstrated that reperfusion with an alkalinic solution significantly increased cellular Na^+ content. This was associated with a greater Ca^{2+} overload and worsened cardiac damage and contractile recovery. The relationship among the higher transsarcolemmal H^+ gradient, cellular Na^+ and Ca^{2+} content, and contractile dysfunction strongly indicated that the stimulated Na^+-H^+

exchange played a crucial role in the process. When the ventricular wall was reperfused at a lower pH, cellular Na^+ was increased without Ca^{2+} overload and the formation of reperfusion contracture. As explained in the previous section, the mechanism for the rise in intracellular [Na^+] may be different from that in alkalinic reperfusion. The Na^+ accumulation is most likely to be due to the reduction of Na^+ efflux via the inhibited Na^+-Ca^{2+} exchange by acidosis. Under the acidic experimental condition, the influx of Na^+ may be slow because of the lowered H^+ gradient across the sarcolemma. After a certain time period, cellular Na^+ may increase to a high level. However, the total amount of Na^+ entering the cell through Na^+-H^+ exchanger could be much less since the efflux was very slow. The Na^+ accumulation during acidic reperfusion preconditioned the ventricular wall for the subsequent Ca^{2+} overload and cardiac dysfunction when it was subsequently reperfused at pH 7.2.

3. Pathological evidence supporting the involvement of Na^+-H^+ exchange in post-ischemic reperfusion injury in myocardium

To date, the functional integrity of the Na^+-H^+ exchange in heart has been examined only in one disease condition, diabetes mellitus. Na^+-H^+ exchange was significantly depressed in cardiac sarcolemmal vesicles isolated from chronically diabetic rats as compared to non-diabetic control preparations (Pierce et al, 1990[a]). This depression in Na^+-H^+ exchange, which is not drug-

induced, may provide a biochemical mechanism for the altered contractile response of the diabetic heart to acidotic challenge and ischemic-reperfusion conditions.

One may hypothesize that if the Na^+-H^+ exchange pathway is the primary mechanism for the regulation of intracellular pH in the heart, the diabetic heart may be more sensitive to an acidotic challenge when Na^+-H^+ exchange is defective. Papillary muscles from diabetic rats subjected to intracellular acidification recovered from the acidosis much more slowly than control muscles (Feuvray et al, 1990). In view of the known role for the Na^+-H^+ exchanger in regulating intracellular pH, a depressed sarcolemmal Na^+-proton exchange was suggested to be involved. In further work elsewhere, hearts from diabetic animals were reported to be more resistant to ischemia-reperfusion challenge than control hearts (Tani and Neely, 1988). Khandoudi et al (1990) demonstrated that in the diabetic rat heart, the post-ischemic recovery of intracellular pH was 25-30 min which was significantly slower than non-diabetic heart (2.5 min). This was accompanied by a higher stroke volume and aortic flow, indicating a better post-ischemic recovery of cardiac function. Administration of amiloride induced similar changes in cellular pH and mechanical responses. Therefore, the evidence obtained from an unrelated disease model, diabetes mellitus, also strongly suggests the involvement of Na^+-H^+ exchange in ischemia-reperfusion injury which is consistent with the proposed role of Na^+-H^+ exchange in the present manuscript.

E. OTHER ION TRANSPORT PATHWAYS WHICH MIGHT BE INVOLVED IN THE PROTECTIVE EFFECT OF DMA ON THE RECOVERY OF CARDIAC FUNCTION DURING POST-ISCHEMIC REPERFUSION

DMA, like the vast majority of drugs, is selective but not specific. It does act on other ion transport pathways and cellular processes besides the Na^+-H^+ exchanger. The possibility exists that its protective effects during reperfusion might be partially due to an action on one of these pathways. Thus, the role of these transport pathways should be carefully excluded before making a conclusion on the mechanism of action of DMA.

1. Voltage-dependent Ca^{2+} channel

Ca^{2+} entry via Ca^{2+} channels can be inhibited in an acidic environment (Kohlhardt et al, 1976). Application of Ca^{2+} channel blockers is known to promote post-ischemic recovery (Bush et al, 1981; Bersohn and Shine, 1983; Watts et al, 1985). Recent in vitro study by Garcia et al (1990) indicated that amiloride derivatives have Ca^{2+} channel binding affinity or block the Ca^{2+} current. It is possible, therefore, that DMA could be acting in our study by blocking the Ca^{2+} channel. However, evidence obtained from the present investigation did not support that the inhibition of Ca^{2+} channel by DMA played any important role for the improvement of post-ischemic cardiac function. First, the protective effect of Ca^{2+} channel blockers is dependent on the application prior to ischemia. Ca^{2+} channel blocking drugs (verapamil, nifedipine and diltiazem) with far more potency and

affinity for the Ca^{2+} channel than DMA did not demonstrate protective effect during ischemia-reperfusion challenge if they were introduced only in the reperfusion period (Bersohn and Shine, 1983; Watts et al, 1990[b]). Clearly, if the primary ion transport pathway which DMA were acting upon were the Ca^{2+} channel, then it would act like the other Ca^{2+} channel antagonists and have very weak or no effect if introduced solely during reperfusion. However, it has been clearly demonstrated that the protective effect of DMA did not require pre-ischemic perfusion and instead, appeared to act solely during the several minutes of reperfusion (Figure 13-16 and Figure 29-32). This is very distinctive from the pattern of action of classic Ca^{2+} channel blockers. Second, the half maximal effective concentration for DMA to affect rat myocardial Ca^{2+} channels is > 50 uM (Dennis et al, 1990). This is at least 2.5 fold higher than the concentration that we employed. Third, the reduction of cellular Na^{+} by DMA treatment in the absence or presence of ouabain is very hard to explain with the inhibition of Ca^{2+} channels. Therefore, the protective effect of DMA would be minimal at best through the reduction of Ca^{2+} entry via Ca^{2+} channel in our experiment.

2. Na^{+}-Ca^{2+} exchange

DMA is unlikely to be protecting via inhibition of Na^{+}-Ca^{2+} exchange. The 1-20 uM dose of DMA employed is much lower than the IC_{50} of DMA (550 uM) required for Na^{+}-Ca^{2+} exchange inhibition

(Table 2). Furthermore, DMA is twice as effective as amiloride as an inhibitor of Na^+-Ca^{2+} exchange (Kleyman and Cragoe, 1988) yet the protective effect of 20 uM DMA was as good as (Tani and Neely, 1989) or better than (Karmazyn, 1988) 0.2-1.5 mM amiloride. If DMA was protecting the heart through Na^+-Ca^{2+} exchange inhibition, we would expect it to be less effective than amiloride at this low concentration, not more effective as it was. If DMA did attenuate Ca^{2+} overload by inhibiting Na^+-Ca^{2+} exchange, one would expect a decreased Na^+ efflux which would result in a lower Ca^{2+} and higher Na^+ content during reperfusion. This was not the case in our study.

3. Other mechanisms

The concentration of DMA used does not influence 0 phase of the action potential (Dennis et al, 1990), thus, the decrease of Na^+ entry is unlikely through the inhibition of cardiac Na^+ channel. The [DMA] employed in the present study was also 50 fold below its IC_{50} for the inhibition of protein kinase activity (Kleyman and Cragoe, 1988). Thus, this effect is also unlikely to be involved. DMA may be interacting with various cell receptors (Kleyman and Cragoe, 1988) but the relevance of this, if true, is speculative at present. DMA was also unlikely to be acting directly via K^+ transport pathways because, in spite of its protective effect on cardiac function in the presence of ouabain, it could not normalize intracellular K^+ content (Figure 27). To date, no data indicates that DMA can reduce free radical

production.

F. THE SIGNIFICANCE OF THE PRESENT STUDY

Two predominant hypotheses have evolved over the last three decades to explain the ischemia-reperfusion injury to the heart. One involves metabolic changes, the second one involves ionic alteration. In the former, muscle metabolism occurs through anaerobic pathways during ischemia. Coupled with the decreased removal of metabolic end-products, this leaves the cell acidotic (Poole-Wilson, 1978). The limited O_2 and substrate supply results in a loss of high energy phosphate stores (Murry et al, 1990; Koretsune and Marban, 1990; Steenbergen et al, 1990). This depressed energy status of the cell during ischemia has been thought to be a major contributory factor to ischemia-reperfusion injury in the heart (Murry et al, 1990; Koretsune and Marban, 1990; Steenbergen et al, 1990). Alternatively, reperfusion is believed to initiate an excessive accumulation of cellular calcium (through mechanisms which has not been clearly defined) (Nayler, 1981; Allen and Orchard, 1987; Nayler et al, 1988; Marban et al, 1989). This Ca^{2+} overload may start several pathological processes in the cell which ultimately end in contractile dysfunction, damage and death (Murphy et al, 1987). Thus, generally, ischemia-reperfusion injury to the heart may be due to metabolic aberrations or ionic imbalance. Unfortunately, neither hypothesis on its own has conclusively explained the mechanism responsible for the pathology of ischemia-reperfusion

injury to the heart and considerable controversy still exists (DeBoer et al, 1980; Koretsune and Marban, 1990; Steenbergen et al, 1990). The present study has suggested that the Na^+-H^+ exchange pathway may represent an important new mechanism involved in ischemia-reperfusion damage to the heart. Our work links the two hypotheses described above and connects the ischemic event with the reperfusion events. The present work also demonstrated the possibility that the Ca^{2+} overload during reperfusion may be preceded by a Na^+ loading state in myocardium which is considered to be the cause or prerequisite of the Ca^{2+} overload. Na^+-H^+ exchange may play a crucial role in connecting the ischemic event (cellular acidosis) to the reperfusion event (the cascade of ion imbalance). The effects of DMA during ischemia-reperfusion were first characterized in this investigation. Its effectiveness during reperfusion is indeed an important finding which suggests that the Na^+-H^+ exchange inhibitors could be protective in reducing post-infarction necrosis in myocardium. The investigation with different reperfusion pH indirectly examined an important feature of Na^+-H^+ exchange in the pathological process. It will be of interest in the future to further examine if the relationship observed here in diabetes model can be extended to other pathological models and disease states.

For several reasons, caution should be exercised when extrapolating the significance of this study to other pathological situations. Firstly, since the experimental model

used here was the isolated right ventricular wall of the rat, the responses of the whole heart or different species to ischemia-reperfusion challenge and/or DMA may vary. Secondly, the pathological model used was global ischemia and the metabolic and ionic changes in other pathological models (such as low-flow ischemia and hypoxia-reoxygenation) may be different. Thirdly, ischemia-reperfusion injury is a multifactorial event which involves many pathological, pathophysiological and biochemical changes. Emphasizing the importance of Na^+-H^+ exchange in our experimental conditions does not exclude the possibility that other mechanisms may also contribute to the ischemia-reperfusion injury. Lastly, the features of Na^+-H^+ exchange have not been fully characterized in the present study. Therefore, the role of Na^+-H^+ exchange in ischemia-reperfusion injury has not been completely examined in the present study.

VI. CONCLUSION

1. The post-ischemic recovery of the contractile function in isolated right ventricular wall of rat can be effectively improved by DMA, a selective Na^+-H^+ exchange inhibitor. This is accompanied by a decreased cellular Na^+ and Ca^{2+} content and creatine phosphokinase leakage from myocardial cells.

2. The protective effect of DMA seems to occur mainly during the first few minutes of reperfusion.

3. Ouabain treatment increased the cellular Na^+ content and further worsened the Ca^{2+} overload and post-ischemic cardiac dysfunction. This detrimental effect can be effectively antagonized by DMA treatment through normalization of cellular Na^+ and Ca^{2+} content.

4. Increasing reperfusion pH may result in an increase of intracellular Na^+ and Ca^{2+} followed by a poorer recovery of cardiac function. DMA, by reducing cellular Na^+, can prevent this process.

5. Na^+ entry in the early phase of reperfusion is pH sensitive.

6. The data are consistent with the hypothesis that stimulation of sarcolemmal Na^+-H^+ exchange and the entry of Na^+ through the Na^+-H^+ exchanger in the early phase of reperfusion may play a primary role for the development of the post-ischemic cardiac injury.

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