Role of Oxidative Stress and Na^+-H^+ Exchanger in Calcium-Handling Abnormalities in Hearts Subjected to Ischemia -Reperfusion

by

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A Thesis submitted to the Faculty of Graduate Studies of
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Institute of Cardiovascular Sciences
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DOCTOR OF PHILOSOPHY

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ABSTRACT

Occurrence of oxidative stress and activation of Na^+-H^+ exchanger (NHE) are considered to be the major mechanisms of intracellular Ca^{2+}-overload and subsequent cardiac dysfunction in hearts subjected to ischemia-reperfusion (I/R). This study was undertaken to test the hypothesis that reduction in the degree of oxidative stress and inhibition of NHE results in the beneficial effects in I/R hearts. For this purpose, I/R was induced in isolated rat hearts by subjecting to global ischemia followed by reperfusion and changes in contractile parameters were monitored. To study Ca^{2+}-mobilization, cardiomyocytes were isolated from hearts and intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$) was measured by employing fura-2 microfluorometry upon exposure to the KCl, catecholamine or ATP. The involvement of oxidative stress was examined by treating hearts with an antioxidant mixture containing superoxide dismutase (SOD) and catalase (CAT) whereas the role of NHE was determined by treating the hearts with 5-(N-Methyl-N-isobutyl) amiloride (MIA), an inhibitor of NHE.

Reperfusion of the 30 min ischemic hearts for different periods (5 to 30 min) revealed marked changes in cardiac function, basal $[Ca^{2+}]_i$ and isoproterenol (ISO)-induced increase in $[Ca^{2+}]_i$ without any alterations in KCl- or S(-)-Bay K8644-induced increase in $[Ca^{2+}]_i$. The I/R-induced alterations in cardiac function, basal $[Ca^{2+}]_i$ and ISO-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes were attenuated by SOD plus CAT as well as by ischemic preconditioning. The observed changes due to I/R were simulated in hearts perfused with hydrogen peroxide for 30 min. These results suggest that abnormalities in basal $[Ca^{2+}]_i$ as well as mobilization of $[Ca^{2+}]_i$ in cardiomyocytes upon stimulation with catecholamines occur due to ischemic injury and are mediated through oxidative stress in I/R hearts.

In another series of experiments reperfusion of 30 min ischemic hearts for 5 to 30 min

manner. The MIA-induced increase in basal $[Ca^{2+}]_i$ was unaffected by extracellular Ca^{2+}, inhibitors of SL L-type Ca^{2+} channels, Na^+-Ca^{2+} exchanger and Ca^{2+}-pump ATPase as well as blockers of mitochondrial Ca^{2+}-uptake. However, the MIA-induced increase in basal $[Ca^{2+}]_i$ was attenuated by inhibitors of SL Na^+-K^+ ATPase and SR Ca^{2+}-transport. MIA-mediated augmentation of the KCl response was dependent on extracellular concentration of Ca^{2+} and attenuated by agents, which inhibit SL L-type Ca^{2+} channels, Na^+-Ca^{2+} exchanger and Na^+-K^+ ATPase as well as SR Ca^{2+}-release channels and Ca^{2+}-pump. Both MIA and a decrease in extracellular pH lowered the intracellular pH and increased the basal $[Ca^{2+}]_i$ whereas the decrease in extracellular pH, unlike MIA, depressed the KCl-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes. These results suggest that oxidative stress and NHE are involved in the regulation of $[Ca^{2+}]_i$ in cardiomyocytes but antioxidants, unlike NHE inhibitor, may exert beneficial effects in attenuating I/R-induced myocardial injury.

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I. REVIEW OF LITERATURE

1. Cardiac dysfunction due to ischemia-reperfusion (I/R)

Myocardial ischemia is known to produce dramatic changes in cardiac function, metabolism and ultrastructure [1,2]; however, the cellular and molecular events leading to contractile dysfunction and derangement of cardiac structure are not clearly understood. Although restitution of coronary flow to the ischemic heart is considered beneficial for the recovery of cardiac pump function, reperfusion after a certain period of ischemia has been shown to aggravate the myocardial abnormalities [3-6]. It is pointed out that cardiac pump failure and changes in cardiac cell ultrastructure due to I/R or hypoxia-reoxygenation involve a wide variety of complex pathophysiological abnormalities and our current information on these aspects is largely based on the beneficial effects of several pharmacological interventions for the treatment of ischemic heart disease (IHD). For example, the beneficial effects of Ca^{2+}-antagonists [7,8] and Na^{+}-H^{+} exchange (NHE) inhibitors [9-12] have supported the concept for the role of intracellular Ca^{2+}-overload [13,14] whereas those of antioxidants [4,5] suggest the involvement of oxidative stress in the pathophysiology of IHD. Both α- and β-adrenergic receptor blockade have also been shown to prevent the I/R-induced abnormalities [15-17], most probably by reducing the development of intracellular Ca^{2+}-overload as a consequence of attenuation of Ca^{2+}-influx in the myocardium. I/R has also been shown to generate different oxyradicals and oxidants such as H_2O_2, peroxynitrite and HOCl and these have been suggested to be responsible for the occurrence of intracellular Ca^{2+}-overload associated with I/R injury [18-22]. Furthermore, some biochemical studies have indicated the involvement of prostaglandins and several metabolic factors to produce cardiac dysfunction at different stages of IHD [23,24] and are considered to induce Ca^{2+}-handling defects in the heart. Thus, it appears that intracellular Ca^{2+}-overload plays a critical role in the

development of I/R-induced injury to the myocardium. A scheme describing various events leading to the occurrence of intracellular Ca^{2+}-overload in I/R hearts is given in **Figure 1**.

In addition to produce dramatic reduction in the high energy phosphate stores (ATP and creatine phosphate), myocardial ischemia results in a large accumulation of free fatty acids (FFA) and their acyl derivatives including palmitoylcarnitine and palmitoyl CoA [25-28]. Such a reason for altered fatty acid metabolism in the cardiac cell due to ischemia has been demonstrated at the level of carnitine palmitoyltransferase [29]. It should be mentioned that accumulation of FFA is considered to have causal significance for cardiac dysfunction in ischemia [30,31] and in fact, FFA have been shown to depress the contractile force development [32]. The long chain acyl esters are known to bind to membranes [33], change their properties [34] and produce intracellular Ca^{2+}-overload. Lysophospholipids are also accumulated in the ischemic heart due to the activation of phospholipases and increased hydrolysis of the membrane phospholipids [35-37] and are known to induce arrhythmias [38,39] upon inducing Ca^{2+}-handling abnormalities in the myocardium. While the accumulation of lipid metabolites in the cardiac cell may play an important role in the genesis of cardiac dysfunction due to ischemia, the reperfusion of the ischemic myocardium is considered to generate oxyradicals, which may account for the reperfusion injury for aggravating cardiac abnormalities [40-44]. In fact, different types of reactive oxygen species have been shown to produce electrical abnormalities [45,46], ultrastructural damage [47,48], intracellular Ca^{2+}-overload [49] and cardiac dysfunction [50,51]. Various oxyradical scavengers have been shown to exert beneficial effects on I/R injury [52-54]. Thus, it is possible that the formation of oxyradicals and accumulation of lipid metabolites due to I/R may result in membrane defects and these can then cause the occurrence of intracellular Ca^{2+}-overload, with subsequent cardiac dysfunction and myocardial cell damage. Intracellular Ca^{2+}-

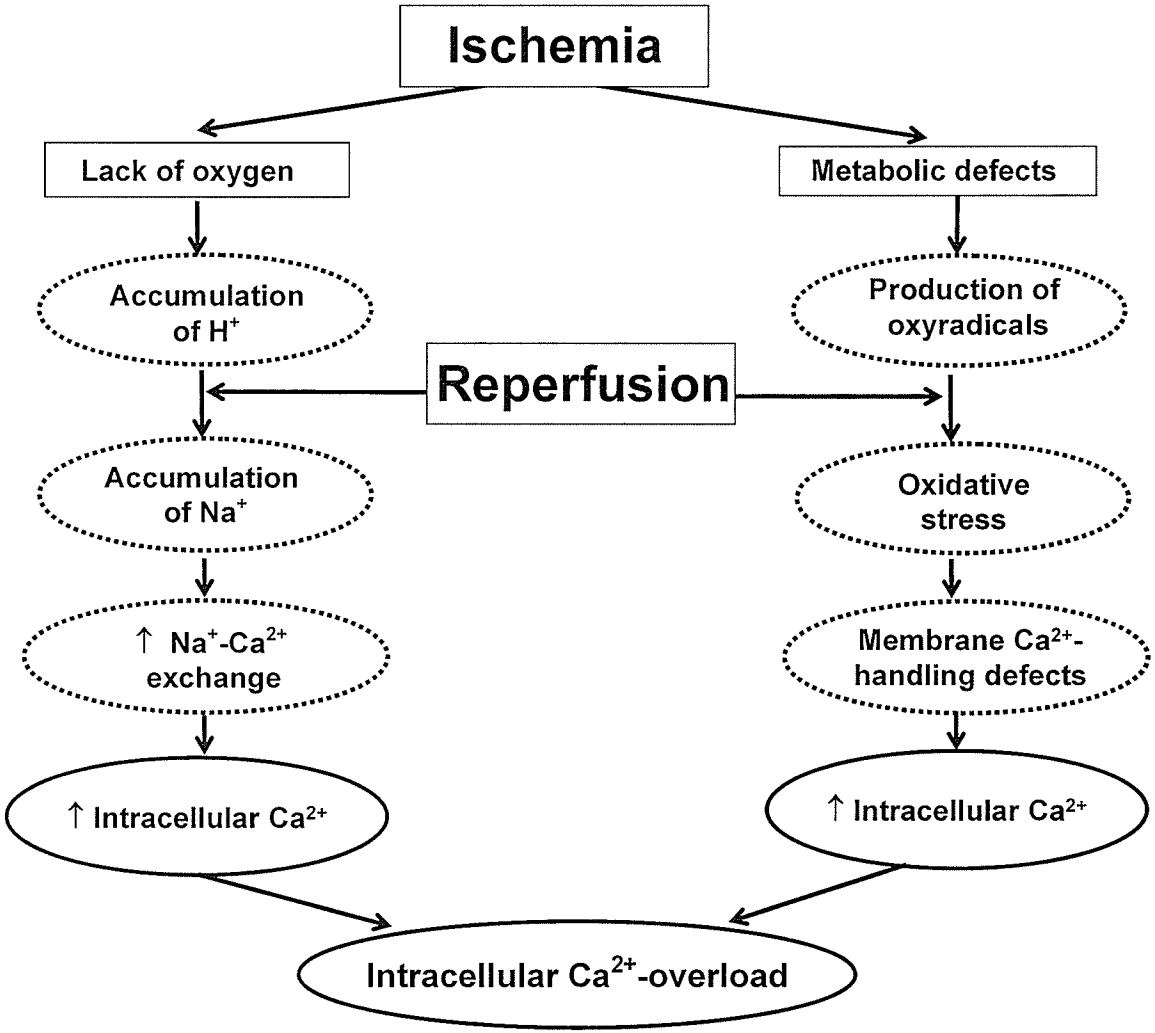


Figure 1. Proposed sequence of events involving the Na^+-Ca^{2+} exchange and oxidative stress for the development of intracellular Ca^{2+}-overload in the ischemic-reperfused hearts

overload has also been shown to cause cardiac dysfunction and cell damage [13,55-58]. Various investigators have shown that alterations in the function of different subcellular organelles such as sarcolemma (SL), sarcoplasmic reticulum (SR), myofibrils (MF) and mitochondria are the major determinants of changes in cardiac function due to I/R. It seems likely that oxidative stress as well as development of intracellular Ca^{2+}-overload play a critical role in inducing subcellular defects in the IHD.

2. Subcellular and molecular abnormalities due to I/R

Over the past 30 years, a wide variety of membrane defects have been observed in the ischemic and I/R hearts [4,5,55-60]. It is now clear that the SR Ca^{2+}-pump (ATP-dependent Ca^{2+}-uptake and Ca^{2+}-stimulated ATPase) and associated regulatory mechanisms are defective due to I/R injury [61-64]; the regulatory mechanisms include both protein kinase A and Ca^{2+}/calmodulin-dependent kinase, which are known to stimulate SR Ca^{2+}-transport activities in the heart [62,63]. Although efficiency of mitochondrial ATP production is impaired at the late stages of I/R injury, depression in both electron transport chain activity and Ca^{2+}-transport in mitochondria occurs at moderate degree of I/R [58,65,66]. Several biochemical activities of the SL membrane including Na^{+}-Ca^{2+} exchange, Ca^{2+}-stimulated ATPase, Na^{+}-K^{+} ATPase and phosphoinositol turnover are markedly altered during myocardial I/R as well as during hypoxia-reoxygenation phases [58,67-76]. Although some investigators [77] have failed to observe any change in the SR Ca^{2+}-release channels during I/R, others have reported a reduction in the density of these channels [61, 78-80]. The oxidants such as HOCl and H_2O_2 as well as hydroxyl radicals were also observed to depress the SR Ca^{2+}-pump activity [81,82]; however, H_2O_2 was found to activate the SR Ca^{2+}-release channels [83]. I/R has also been shown to reduce the sensitivity of myofilaments to Ca^{2+} by causing proteolysis of MF, troponin I and troponin T, in addition to increasing the binding of

cytosolic proteins to MF [84-92]. Reactive oxygen species and oxidants were also observed to alter the MF ATPase and creatine kinase activities and reduce their sensitivity to Ca^{2+} [93-95]. In view of the role of SR and MF in heart function, it appears that cardiac contraction and relaxation abnormalities in the IHD may be due to defects in SR and MF whereas changes in the SL membrane may determine the extent of alterations in cation homeostasis in the myocardium as well as its susceptibility to arrhythmogenic stimuli.

In addition to alterations in the activities of different subcellular organelles, both I/R and oxidative stress have been shown to produce dramatic effects on the cardiac gene expression. It has been demonstrated that mRNA levels for SR Ca^{2+}-pump, Ca^{2+}-channels, phospholamban and calsequestrin proteins were depressed in the I/R hearts; these changes were prevented by superoxide dismutase (SOD) plus catalase (CAT) [61]. Since H_2O_2 was observed to produce similar changes in the SR gene expression, it was suggested that these effects of I/R may be due to oxidative stress [61]. It should be pointed out that I/R has been reported to upregulate the complement gene expression [96] in the heart, whereas hypoxia decreased the ATP synthase subunit gene expression in cell culture [97]. Selective inhibition of muscle cell gene expression by H_2O_2 was seen in cardiomyocytes [98], while oxyradicals were found to stimulate the proto-oncogene expression in vascular smooth muscle cells and proximal tubular epithelium [99,100]. Thus, it appears that oxidative stress may produce marked changes in cellular gene expression, which may be both beneficial and detrimental for cellular function depending upon the time of exposure and concentration of the oxidant in the cell. Recently, it was observed that I/R produced differential changes in gene expression for SL Na^+-K^+ ATPase isoforms and these alterations were simulated by perfusing the hearts with oxyradicals generating system or H_2O_2 [101]; however, the signal transduction mechanisms for such changes were not investigated. The

lack of both substrate and oxygen, which occurs during the ischemic phase [102], was also found to produce marked changes in gene expression for SR proteins [103]. Although proteolysis of SR and MF proteins as a consequence of intracellular Ca^{2+}-overload is known to occur in the I/R hearts [87,91,104,105], it is not known whether alterations in cardiac gene expression are affected by intracellular Ca^{2+}-overload or changes in transcriptional factors due to proteolysis. Likewise, the involvement of TNF-α, which is markedly elevated in I/R hearts [106-108] in inducing changes in cardiac gene expression for subcellular proteins, has not been examined previously. It should be mentioned that the acute effects of I/R injury on cardiac function are considered to be due to changes in the activities of subcellular organelles, whereas the chronic effects of I/R indicating delayed recovery of cardiac function may be the consequence of changes in cardiac gene expression.

3. Role of oxidative stress in hearts subjected to I/R

Reports from various laboratories have indicated that oxyradicals and other oxidants are involved in the genesis of myocardial cell damage and subsequent contractile dysfunction under a wide variety of pathological situations [3-5,59]. Some investigators have demonstrated, by employing electron paramagnetic resonance spectroscopy, the generation of oxygen free radicals after reperfusion of the ischemic heart [109,110]. Since intracellular Ca^{2+}-overload is considered to play a crucial role in I/R injury [111,112], it is possible that several mechanisms, which are involved in the regulation of Ca^{2+}-movements in the myocardial cell, are altered by oxyradicals. It is pointed out that SL Ca^{2+}-channel density was decreased in IHD [113,114] as well as upon treatment of the SL membranes with oxyradicals [115], and this may contribute towards the decreased Ca^{2+}-influx. On the other hand, the activities of both Na^{+}-Ca^{2+} exchange and Ca^{2+}-pump were depressed following hypoxia/I/R [67,68,116] as well as upon the exposure of heart

membranes to oxyradicals [117,118]; these can be seen to decrease Ca^{2+}-efflux. Oxyradicals also affect other SL activities such as Na^{+}-K^{+} ATPase and phospholipid methyltransferase [76,119-121] as well as Ca^{2+}/Mg^{2+} ecto-ATPase and the superficial store of Ca^{2+} [122], and ATP receptors [123], which are known to affect Ca^{2+}-movements in the cell indirectly. SL Ca^{2+}-transporters have also been reported to be altered in hearts subjected to ischemia as well as perfusion with oxyradical generating systems [124-126]. In addition, dramatic changes in β-adrenoceptors, G-proteins and adenylyl cyclase, which regulate Ca^{2+}-movements across cardiac membranes, are altered upon inducing I/R or by exposing heart membranes to oxyradicals and oxidants [127-131]. The SL changes in Na^{+}-K^{+} ATPase and associated currents have been reported oxyradicals and oxidants [132-134]. Several other defects such as changes in SL membrane permeability, loss of dystrophin and alterations in phospholipases have also been observed in the SL membrane due to I/R [135-138]. Thus, it appears that increased formation of oxyradicals and oxidants in ischemic hearts upon reperfusion may induce a complex set of SL and SR defects with respect to mechanisms related to Ca^{2+}-movements and these on balance may result in the development of intracellular Ca^{2+}-overload, myocardial cell injury and contractile abnormalities. It should be emphasized that oxyradical generating systems have been shown to cause alterations in excitation-contraction coupling, heart function and myocardial cell structure [47-50,139-141]; however, the exact mechanisms of these defects due to oxyradicals and oxidants remain to be determined.

H_2O_2 was found to increase Ca^{2+}-uptake in isolated cardiomyocytes [49] whereas the action of oxyradicals on SL Na^{+}-Ca^{2+} exchange seems to depend on the type of radical generating system and the time of exposure to these interventions [111,118,142-144]. The effects of H_2O_2 are shown to be mediated through the activation of protein kinase C, mitogen-activated protein

kinase and/or stress-activated protein kinase [145-147] as well as translocation of protein kinase C has been shown to occur in the ischemic heart [148,149]. Since the activation of protein kinase C by phorbol ester has been observed to produce cardiodepression [150], it is believed that cardiac dysfunction in the I/R heart is elicited through the activation of protein kinase C. Nonetheless, isolated cardiomyocytes exposed to oxyradical generating systems were observed to undergo changes in the electrophysiological behaviour similar to those seen in IHD [45]. It should be mentioned that various oxyradical generating systems are considered to depress the cardiac SR Ca^{2+}-uptake by depressing Ca^{2+}-stimulated ATPase activities [151-153]. A progressive loss of Ca^{2+}-release channels was also seen upon exposing the SR to reactive oxygen species [154]. The rotenone-insensitive NADH cytochrome C reductase activity of the cardiac SR was decreased due to oxyradicals but this effect was seen to be of lesser magnitude when compared to that on the SL Na^{+}-K^{+} ATPase; lipid peroxidation of SL preparations by oxyradicals was also greater than that of the SR [121]. Although ATP-dependent Ca^{2+}-uptake and Ca^{2+}-stimulated ATPase activities of skeletal muscle SR were also depressed by the oxyradicals, this effect was reported to be due to the inhibition of the sulfhydryl groups of the Ca^{2+}-pump ATPase rather than the accumulation of peroxides in the membrane [155,156]. Activated neutrophils, which depress cardiac contractile force development [157], are considered to decrease the SR Ca^{2+}-pump activities due to the generation of oxidants such as HOCl [158]. Some work concerning adverse effects of HOCl derived from neutrophils, which are accumulated in the ischemic heart, on various subcellular organelles has also been carried out [154-159]; however, analysis of the data [160-163] indicate that very little is known about the mechanisms of their alterations due to different oxidants.

Besides the production of oxygen-derived free radicals and formation of oxidants such as H_2O_2 and HOCl, changes in nitric oxide (NO) metabolism have been observed in IHD [109]. NO

is synthesized in endothelial cells and cardiac myocytes and is known to play an important role in the regulation of coronary flow as well as cardiac contractile function. However, excessive formation of NO has been found to be detrimental to heart function. The toxic action of NO is enhanced by its reaction with superoxide anion to form peroxynitrite, which in fact has been demonstrated to impair cardiac function [164,165]. Furthermore, plasma levels of peroxynitrite have been shown to increase upon myocardial infarction [166] and I/R has been reported to promote the formation of peroxynitrite [167], which has been demonstrated to impair the phosphorylation of tyrosine residues of proteins involved in the signal transduction mechanism [168]. On the other hand, peroxynitrite has also been reported to exert cardioprotective effects [169,170]. Such paradoxical effects of peroxynitrite may be due to its concentration dependent actions in the sense that at low concentrations, this agent produces beneficial effects whereas at high concentrations, it causes heart dysfunction in IHD. Although maximal achievable concentrations of peroxynitrite are thought to vary in the range of 2 to 5 μM [169], no information regarding the local concentrations of this agent is available in the literature. In view of the strong oxidant property of peroxynitrite and the possibility of high concentrations of peroxynitrite present locally near the SL, SR and MF, it is possible that subcellular alterations in the I/R heart may be due to peroxynitrite-mediated oxidative stress. The role of oxidative stress in I/R-induced cardiac dysfunction is further supported by observations that some agents, such as vanadate and selenium, produce their beneficial effects on I/R hearts by their antioxidant action [171,172]. Various mechanisms by which oxidative stress is considered to produce subcellular defects and cardiac dysfunction are given in **Figure 2**.

4. Role of intracellular Ca^{2+}-overload and NHE in hearts subjected to I/R

It is now well known that Ca^{2+} plays a critical role in cardiac excitation-contraction coupling,

regulation of myocardial metabolism and maintenance of cardiac cell integrity [56]. The concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in cardiomyocytes is mainly regulated by SL and SR proteins whereas mitochondria and nucleus are also considered to participate in this process to some extent [56,173,174]. It is emphasized that SL L-type Ca^{2+} channels are the major pathways of Ca^{2+} entry whereas SL Ca^{2+}-pump ATPase is involved in Ca^{2+}-efflux from cardiomyocytes. SL Na^+-Ca^{2+} exchanger has been suggested to participate in both Ca^{2+}-entry and Ca^{2+}-removal whereas SL Na^+-pump (Na^+-K^+ ATPase) and NHE are considered to regulate the $[Ca^{2+}]_i$ in cardiomyocytes indirectly through the participation of SL Na^+-Ca^{2+} exchanger [56]. In addition, SR Ca^{2+} ryanodine receptors are associated with the release of Ca^{2+} from the SR stores whereas Ca^{2+} in SR stores is restored by SR Ca^{2+}-pump ATPase [175]. Several studies have been carried out to investigate changes in intracellular Ca^{2+} in hearts subjected to I/R. A marked increase in tissue Ca^{2+} content has been reported in I/R hearts under a wide variety of experimental conditions and this occurrence of intracellular Ca^{2+}-overload has been suggested to explain I/R injury to the myocardium [1,5,176-179]. Different mechanisms by which intracellular Ca^{2+}-overload can be seen to cause different subcellular defects and cardiac dysfunction due to I/R injury are depeicted in **Figure 3**. Although a positive correlation was found to occur between diastolic $[Ca^{2+}]_i$ and left ventricular end diastolic pressure (LVEDP) in I/R rat hearts [180], such a relationship was not evident in post-ischemic ferret myocardium [181,182]. In fact, some investigators [183] have denied a direct role of intracellular Ca^{2+} in ischemic diastolic dysfunction in isolated rat and rabbit hearts. Although various studies have examined the Ca^{2+}-handling ability of cardiomyocytes obtained from I/R hearts or undergoing hypoxia-reoxygenation, the results are conflicting [184-187]. Thus no definitive conclusion regarding the status of Ca^{2+}-handling in I/R hearts can be made on the basis of information available in the literature.

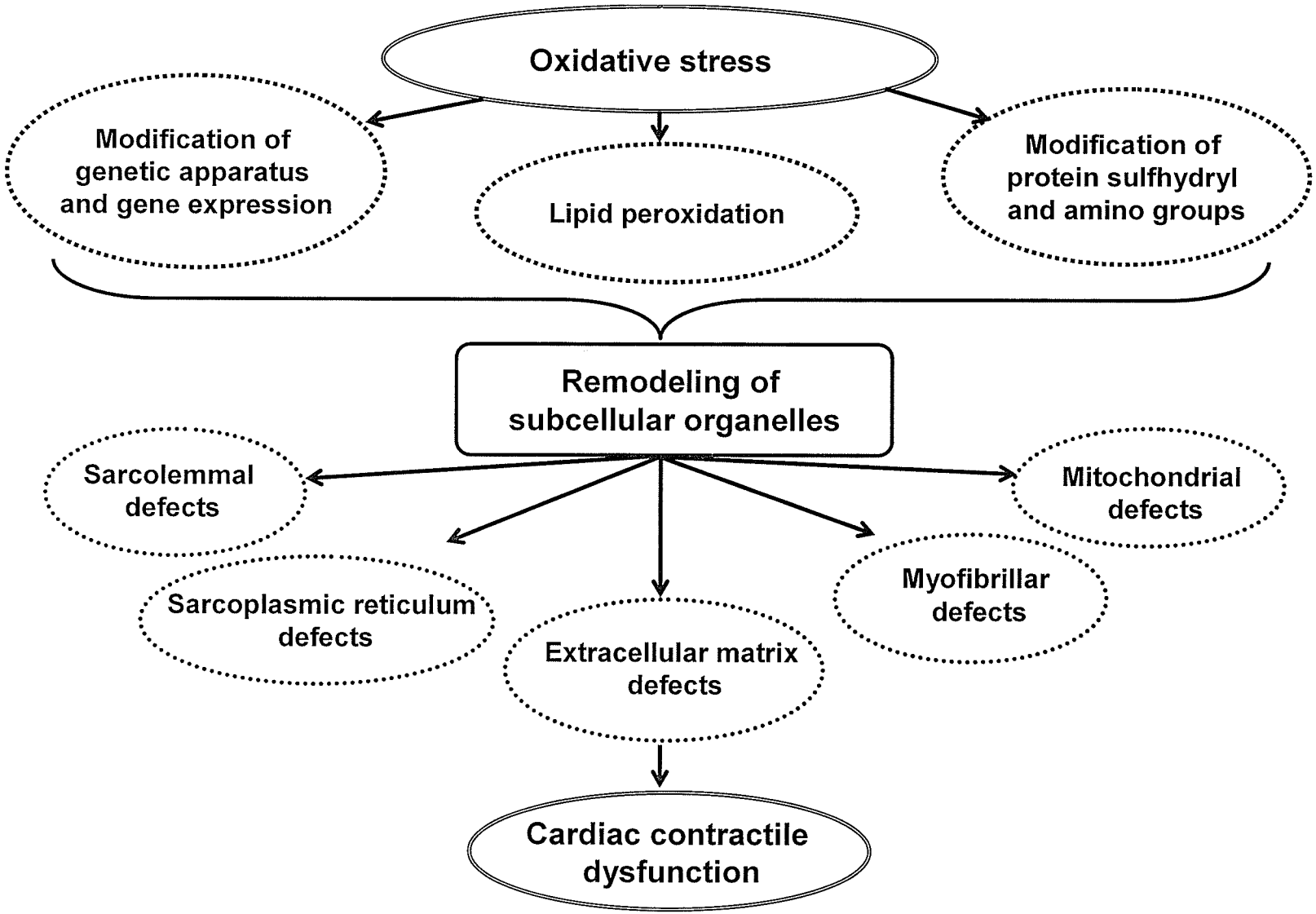


Figure 2. Possible mechanisms for subcellular remodeling and cardiac dysfunction due to oxidative stress in the heart.

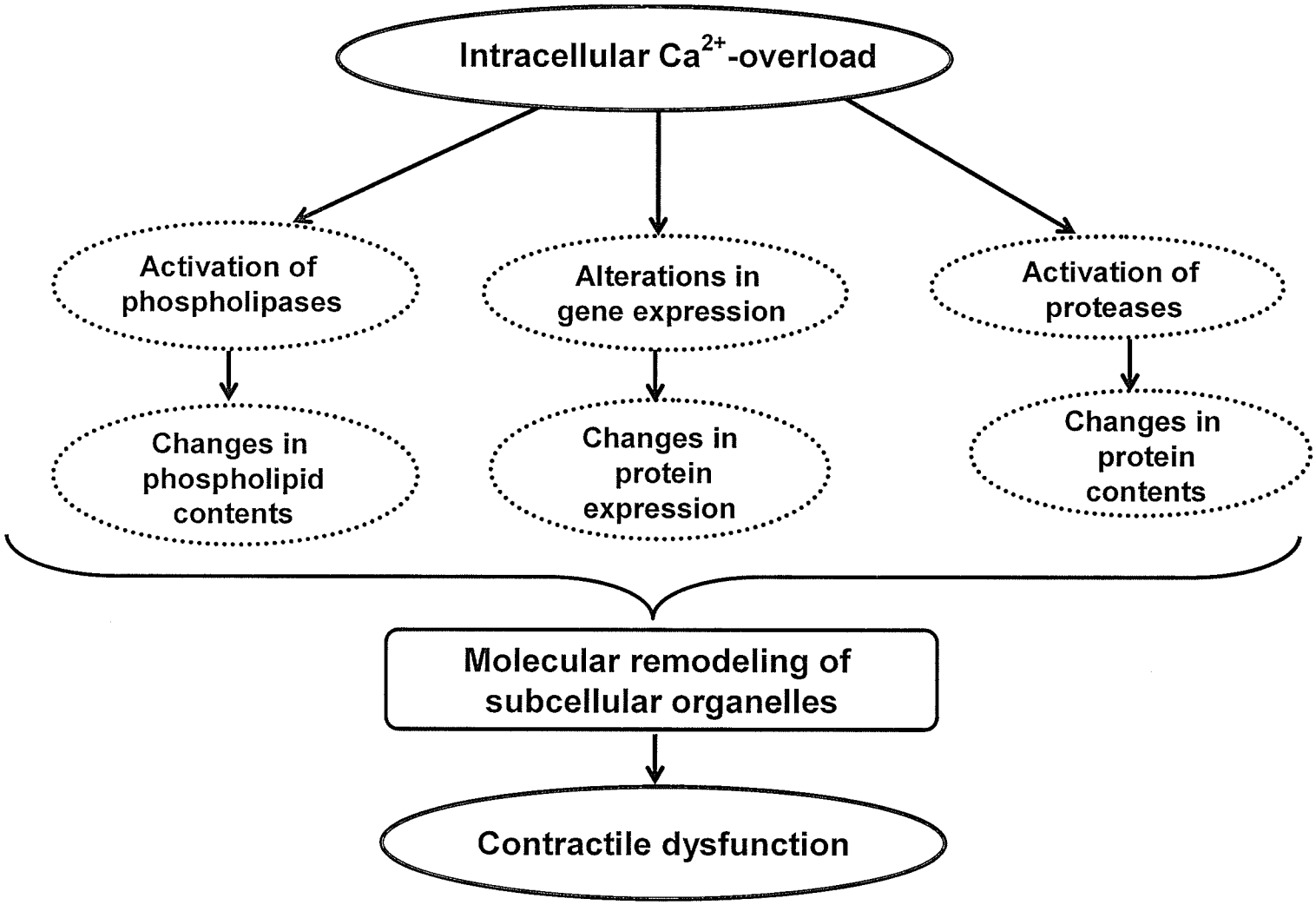


Figure 3. Possible mechanisms associated with changes in the function and molecular structure of different subcellular organelles (subcellular remodeling) and cardiac dysfunction as a consequence of intracellular Ca^{2+}-overload in the myocardium. Different subcellular organelles referred here include sarcolemma, sarcoplasmic reticulum, myofibrils, mitochondria and extracellular matrix, which are directly or indirectly involved in the process of cardiac contractile activity.

Some cation transport studies have revealed that the accumulation of intracellular H^+ by anaerobic metabolism during ischemia activates NHE which causes an increase in intracellular Na^+ with a parallel increase in intracellular Ca^{2+} through the activation of Na^+-Ca^{2+} exchanger. These events result in the development of intracellular Ca^{2+}-overload and depression in cardiac function [188], and are depicted in **Figure 4.** Although experimental studies using various amiloride derivatives as NHE inhibitors have demonstrated the protective effects of these agents in terms of improving I/R-mediated cardiac contractile dysfunction [189,190], the cardioprotective effects of these agents seem to depend upon the animal models, the time of starting the therapy, dose of the agent as well as the end point determined [191]. In fact, amiloride derivatives have been shown to depress the cardiac function under basal conditions [192]. The results of different clinical trials with some of newer NHE inhibitors in patients with IHD were also not promising [193]. It is pointed out that neither the mechanisms of the cardiodepressant effects of NHE inhibitors under basal conditions nor the exact reasons for the lack of beneficial effects under certain pathophysiological situations are completely understood.

5. Subcellular modification due to ischemic preconditioning

Subjecting the heart to brief periods of ischemia has been shown to limit the infarct size, apoptosis as well as cardiac dysfunction due to a sustained period of I/R [194,195]. Although the beneficial effects of ischemic preconditioning are mediated through the participation of adenosine receptors [196] and protein kinase C mediated signal transduction [197,198], the role of both adenosine and protein kinase C seems to be species dependent [199,200]. In view of the fact that inhibition of phospholipase isozymes [201] and attenuation of TNF-α levels [107] have been reported to produce beneficial effects in the I/R hearts, it is possible that such mechanisms may also be involved in modifying the subcellular changes due to ischemic preconditioning. Both

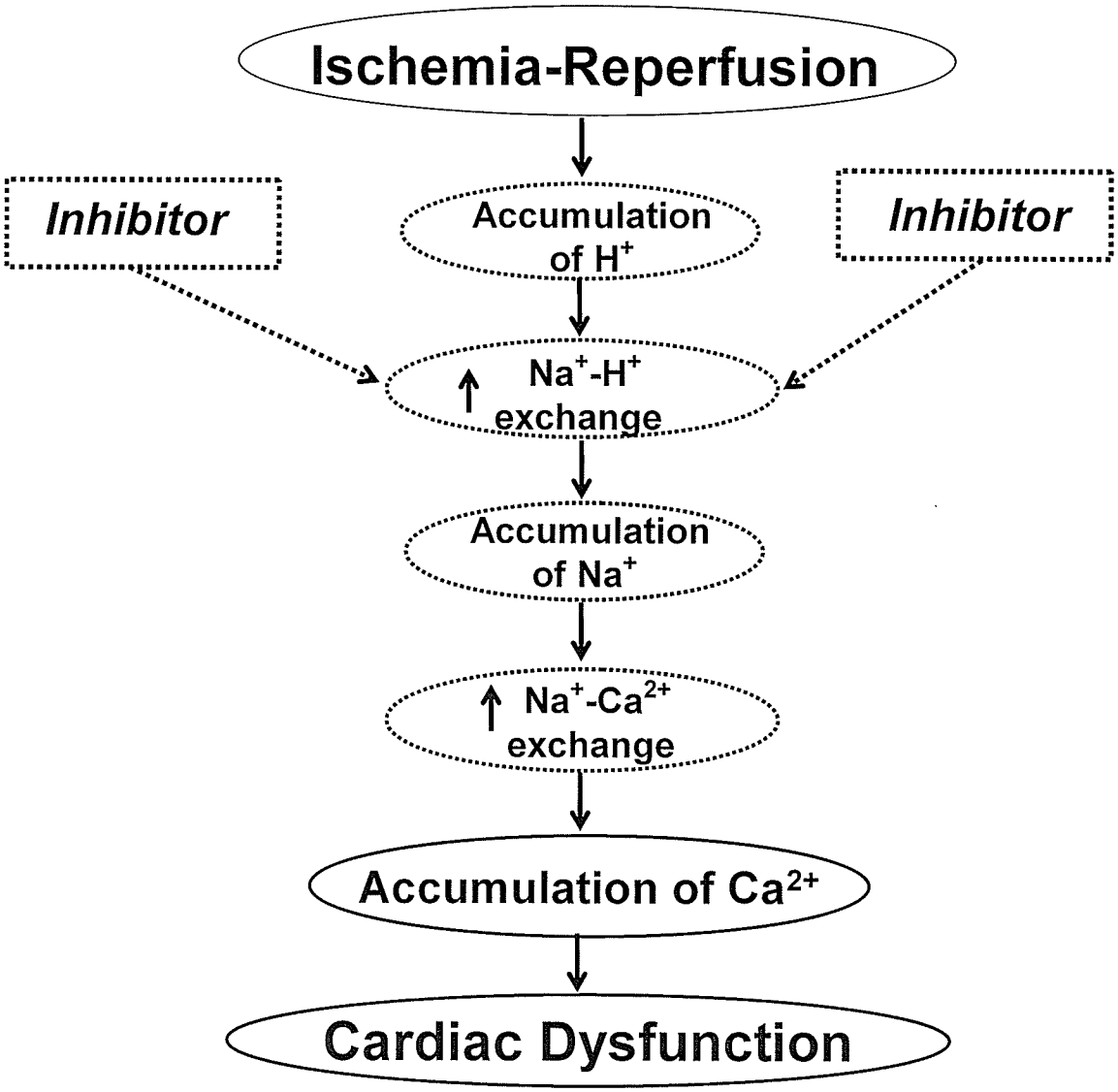


Figure 4. Schematic representation of Na^+-H^+ exchange activation-induced cardiac dysfunction in hearts subjected to ischemia-reperfusion, which is associated with accumulation of intracellular Ca^{2+} through Na^+-Ca^{2+} exchanger. The site of action of Na^+-H^+ exchange inhibitors is shown by dotted arrows. ↑ -increase.

Ca^{2+}-depletion and repletion as well as tachycardia induced preconditioning, have also been shown to exert protective effects against I/R injury but their mechanisms are different from each other [202-204]. Several trigger mechanisms including the low concentrations of oxyradicals as well as different oxidants such as NO, peroxynitrite and H_2O_2 have been shown to be involved in ischemic preconditioning [167,202,205-212]. Both tyrosine kinase and mitogen-activated protein kinase in addition to different isoforms of protein kinase C have also been demonstrated to participate in ischemic preconditioning [213-219]. A great deal of work involving mitochondrial K_{ATP} channels [220-222] and SL K_{ATP} channels [223-225] in ischemic preconditioning has also appeared in the literature. Although preconditioning has been shown to improve the impaired mitochondrial function [226-228], this phenomenon appears to be independent of changes in mitochondrial ATPase [229]. Preconditioning has been reported to preserve post-ischemic changes in SR Ca^{2+}-release channels and Ca^{2+}-pump protein [63,230,231] and this has been suggested to serve as a mechanism for protecting heart function in I/R. Some work regarding changes in SL Na^+-K^+ ATPase and the mechanisms of these alterations in preconditioning has also been carried out [232]; however the status of changes in other proteins in the SL membrane in preconditioned heart remains to be investigated. In view of the stimulation of NHE and Na^+-Ca^{2+} exchange activities in the I/R hearts [233-235], several investigators have shown beneficial effects of the inhibitors of NHE [236-238] and Na^+-Ca^{2+} exchanger [239-241] on cardiac function, arrhythmias and apoptosis. Nonetheless, very little information regarding the actions of these interventions and ischemic preconditioning on subcellular alterations as well as changes in cardiac gene expression has appeared in the literature [242,243].

II. STATEMENT OF THE PROBLEM AND HYPOTHESES TO BE TESTED

From the foregoing discussion it is evident that conflicting reports regarding the characteristics of $[Ca^{2+}]_i$ in cardiomyocytes from I/R hearts may be due to the reversible and irreversible nature of the ischemic cell injury. This view is based on the observation that excessive accumulation of intracellular Ca^{2+} has been shown to occur during irreversible myocardial injury [1] whereas reversible post-ischemic contractile dysfunction is associated with complete normalization of $[Ca^{2+}]_i$ within a few minutes of reperfusion [244,245]. Accordingly, it was planned to investigate the Ca^{2+}-handling abilities of non-depolarized and KCl-depolarized cardiomyocytes isolated from hearts subjected to different periods of ischemia and reperfusion to test the hypothesis that changes in $[Ca^{2+}]_i$ in cardiomyocytes are related to reversible and irreversible phases of I/R injury. Since catecholamines are known to cause an increase in $[Ca^{2+}]_i$ [246], the effect of isoproterenol (ISO), a β_1-adrenoceptor agonist [127], on intracellular Ca^{2+}-handling was studied in depolarized cardiomyocytes. In view of the important role of oxidative stress in inducing I/R injury, experiments were carried out to examine if I/R-induced Ca^{2+}-handling abnormalities in cardiomyocytes are mediated through the generation of oxyradicals. Since ischemic preconditioning is has been shown to attenuate the I/R-induced cardiac dysfunction [247,248], the status of cardiomyocyte Ca^{2+}-handling was examined in hearts subjected to IP.

Although ATP is an essential source of energy for a wide variety of cellular processes [249], the extracellular ATP has also been recognized as a local regulator of physiologic functions in the cardiovascular system [250]. It has been reported that the ATP-induced increase in $[Ca^{2+}]_i$ is augmented by norepinephrine as a consequence of enhanced inward Ca^{2+} current [251]. It should be pointed out that extracellular ATP is released from the sympathetic nerve terminals as a cotransmitter with norepinephrine [252,253]. The level of extracellular ATP has been shown to

be increased in the interstitial fluid during I/R [254]. Recent studies from our laboratory have shown attenuation of the positive inotropic effect of extracellular ATP in heart failure as well as depression of the ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from the failing hearts [255]. On the other hand, no such defect in ATP responses was observed in cardiomyocytes isolated from the diabetic hearts [256]. Since the effects of exogenous ATP on cardiac performance in I/R hearts as well as intracellular Ca^{2+} mobilization in cardiomyocytes isolated from I/R hearts have not been investigated previously, the present study was undertaken to test the hypothesis that the positive inotropic effect of extracellular ATP and ATP-induced increase in $[Ca^{2+}]_i$ are depressed in I/R hearts. Because oxidative stress has been shown to play an important role in I/R-induced cardiac dysfunction [4,257], this study also examined if the ATP-induced changes in $[Ca^{2+}]_i$ are mediated through oxidative stress. In addition, because ischemic preconditioning is known to prevent I/R-induced changes in cardiac function [248], this study tested if the I/R-mediated alterations in ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes are prevented by ischemic preconditioning.

It is now well known that, unlike quiescent cardiomyocytes, the stimulation of β-ARs by catecholamines causes a marked increase in $[Ca^{2+}]_i$. On the other hand, the stimulation of purinergic receptors by extracellular ATP augments $[Ca^{2+}]_i$ in quiescent cardiomyocytes [258]. It is pointed out that norepinephrine and other catecholamines including ISO have been shown to phosphorylate the L-type Ca^{2+} channels and enhance the inward Ca^{2+} current as a consequence of increased production of adenosine 3′,5′-cyclic monophosphate (cAMP) and the activation of protein kinase A (PKA) [251,259]. Increased Ca^{2+} entry through the SL membrane further triggers the release of Ca^{2+} from the SR stores and thus results in the catecholamine mediated increase in $[Ca^{2+}]_i$ in electrically stimulated cardiomyocytes [260]. In contrast, the norepinephrine

mediated potentiation of the ATP response in quiescent cardiomyocytes has been reported to be independent of Ca^{2+} release from the SR Ca^{2+}-stores [261]. It has been shown that the stimulation of β-ARs by isoproterenol causes an increase in the SL Na^{+}-Ca^{2+} exchange activity in guinea pig ventricular cardiomyocytes [262] and rabbit purkinje fibres [263]. On the other hand, in frog atrial fibres and ventricular cardiomyocytes an increase [264] or decrease [265] in Na^{+}-Ca^{2+} exchange activity was observed upon stimulation by isoproterenol, respectively. Nonetheless, the participation of Na^{+}-Ca^{2+} exchange in the catecholamine mediated increase in ATP response in rat ventricular cardiomyocytes is not known. Accordingly, the present study was undertaken to test the hypothesis that the SL Na^{+}-Ca^{2+} exchanger is involved in the isoproterenol-mediated stimulation of the ATP-induced intracellular Ca^{2+}-mobilization. In addition, the effects of other agents, which are known to modify Ca^{2+}-transport in both SL and SR [266,267], were tested to gain some information regarding the participation of different sites in the ATP and catecholamine-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes.

Although involvement of SL NHE in the development of intracellular Ca^{2+}-overload as a consequence of the activation of Na^{+}-Ca^{2+} exchanger has been suggested in I/R injury [188,190,191], the effect of NHE inhibitors on cardiac function vary under different experimental conditions. Such variable actions of NHE inhibitors may be due to excessive accumulation of H^{+} in the cell. In should be noted that NHE is known to promote the efflux of H^{+} generated by myocardial metabolism [268], the inhibition of NHE is likely to cause accumulation of H^{+} and this may cause the release of Ca^{2+} from SR [269] and thus may lead to intracellular Ca^{2+}-overload. The present study was therefore designed to test the hypothesis that inhibition of NHE may produce intracellular Ca^{2+}-overload and thus may not exhibit beneficial effects on I/R-

induced contractile dysfunction. For this purpose, I/R hearts were perfused with a known NHE inhibitor, 5-(N-Methyl-N-isobutyl) amiloride (MIA) [270] to test its action on I/R-induced injury. In addition, the hearts were treated with MIA before subjecting to mild Ca^{2+}-paradox in order to investigate the effect of NHE inhibition on changes in cardiac function due to intracellular Ca^{2+}-overload. Since neither the participation of Ca^{2+}-regulating sites such as SL Ca^{2+}-channels and SR Ca^{2+}-stores nor the exact mechanisms of NHE-mediated alterations in $[Ca^{2+}]_i$ in cardiomyocytes are completely understood, this study examined the mechanisms of NHE inhibition on Ca^{2+}-mobilization in isolated cardiomyocytes. Accordingly, experiments were undertaken to test the hypothesis that changes in $[Ca^{2+}]_i$ upon inhibiting NHE are mediated through the participation of both SL Na^{+}-Ca^{2+} exchanger and L-type Ca^{2+}-channels as well as SR Ca^{2+}-regulating sites. The participation of NHE in the mobilization of $[Ca^{2+}]_i$ in quiescent as well as KCl-depolarized cardiomyocytes was examined by treatment of cardiomyocytes with different agents, which are known to modify Ca^{2+} transport in SL, SR and mitochondria, to gain some information regarding the interaction of these agents with MIA for Ca^{2+} mobilization.

As the occurrence of oxidative stress and activation of SL NHE are the major mechanisms for the development of intracellular Ca^{2+}-overload during I/R injury to the myocardium, it is proposed to study the role of oxidative stress and NHE inhibition in Ca^{2+}-handling by cardiomyocytes. Accordingly, it is planned to achieve the following objectives in this study:

(i). To investigate alterations in $[Ca^{2+}]_i$ in cardiomyocytes in the absence and presence of catecholamines and ATP during reversible and irreversible stages of I/R injury.

(ii). To understand the role of oxidative stress in causing defects in cardiomyocyte Ca^{2+}-handling in the absence or presence of catecholamines and ATP during the development of cardiac dysfunction due to I/R

(iii). To study the effect of ischemic preconditioning on I/R-mediated changes in Ca^{2+}-handling in the absence or presence of catecholamamines and ATP

(iv). To examine the mechanisms of catecholamine and ATP-induced changes in $[Ca^{2+}]_i$ in cardiomyocytes

(v). To test the effects of NHE inhibition on I/R mediated injury and explore the mechanisms of an NHE inhibitor on Ca^{2+}-handling by cardiomyocytes

III. METHODS

All protocols were approved by the University of Manitoba Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care.

1. Isolated rat heart preparation

Male Sprague-Dawley rats (250-300 g) were anaesthetized with a mixture of ketamine (90 mg/kg) and xylazine (9 mg/kg). The hearts were quickly excised, mounted on the Langendorff apparatus and perfused at 37°C (pH 7.4) with Krebs-Henseleit (K-H) buffer gassed with a mixture of 95 % O_2 and 5% CO_2 at constant flow of 10 ml/min [61,76,102]. The composition of K-H was (mM): 120 NaCl; 4.7 KCl; 1.2 KH_2PO_4; 1.2 $MgSO_4$; 25 $NaHCO_3$; 1.25 $CaCl_2$ and 11 glucose. The hearts were electrically stimulated at 300 beats/min via a square wave current of 1.5 ms duration throughout the experiment using a Phipps and Bird stimulator (Richmond, VA). The left ventricular systolic pressure (LVSP), left ventricular end distolic pressure (LVEDP), the rate of change of pressure development (+dP/dt) and rate of change of pressure decay (-dP/dt) were measured via a transducer (Model 1050 BP-Biopac System Inc., Goleta, CA), which was connected with a water-filled latex balloon inserted into the left ventricle. At the beginning of the experiment, the LVEDP was adjusted to approximately 10 mm Hg by inflating the balloon and the left ventricular developed pressure (LVDP) was taken as the difference between the LVSP and LVEDP. The data were recorded online through an analogue-digital interface (MP-100, Biopac Systems Inc., Goleta, CA) and stored in a computer program (Acqknowledge 3.5.3) by a Biopac Data Acquisition System (Biopac Systems Inc., Goleta, CA). All hearts were stabilized for a period of 20 min and randomly divided into different experimental groups.

In the first set of experiments, control hearts were perfused for 10-60 min after stabilization. As no significant differences were observed in the cardiac performance and intracellular Ca^{2+}-

handling, the control values were grouped together. Some hearts were made globally ischemic for 10 or 20 min by stopping the coronary flow, and then the flow was restored for 30 min whereas other hearts in this group were subjected to 30 min of global ischemia followed by 5, 15 and 30 min of reperfusion. In the second set of experiments, isolated hearts were treated with an antioxidant mixture containing SOD (5×10^4 U/L; Sigma-Aldrich, Oakville, Ont., Canada) and CAT (7.5×10^4 U/L; Fisher Scientific, Nepean, Ont., Canada) for 10 min before inducing ischemia as well as during the reperfusion period as described previously [101,127]. Furthermore, to test if the effects of I/R are simulated by oxidative stress, hearts were perfused with H_2O_2 (50 μM) for 30 min after the stabilization period. In the third set of experiments, ischemic preconditioning in hearts was induced by 3 cycles of 5 min ischemia followed by 5 min reperfusion before subjecting to I/R as described by Temsah et al. [248]. In some experiments, ATP (50 μM) was infused into the perfusion stream to the heart subjected to 30 min ischemia followed by 30 min reperfusion to study the effect of I/R on the positive inotropic effect of ATP. For determining the effect of ISO on Na^+-Ca^{2+} exchanger, a bolus injection of isoproterenol (1 μM) was given to the hearts in the absence or presence of different concentrations of KB-R7943, an inhibitor of Na^+-Ca^{2+} exchanger [271]. For the MIA treatment group, MIA (5 μM) was perfused 5 min before ischemia as well as throughout the reperfusion period. For studying the effects of mild Ca^{2+}-paradox, hearts were perfused with Ca^{2+}-free medium for 3 min followed by 10 min perfusion with normal K-H buffer containing 1.25 mM Ca^{2+}; MIA (5 μM) infusion was started 5 min before inducing Ca^{2+}-paradox and was carried throughout the Ca^{2+}-repletion period. The selection of this concentration of MIA for I/R and Ca^{2+}-paradoxic hearts was based on the previous work carried out by Hoque and Karmazyn [272].

2. Isolation of cadiomyocytes

Ventricular myocytes were isolated using a method described previously [266,267]. In brief,

hearts from different groups were perfused at 37°C for 5 min with Ca^{2+}-free buffer (pH 7.4) containing (in mM): 90 NaCl; 10 KCl; 1.2 KH_2PO_4; 5 $MgSO_4$; 15 $NaHCO_3$; 30 taurine and 20 glucose; gassed with a mixture of 95% O_2 and 5% CO_2. These hearts were then switched to the same perfusion medium containing 0.04% collagenase, 0.1% bovine serum albumin (BSA) and 50 μM $CaCl_2$. At the end of a 25 min recirculation period, except for the hearts treated with H_2O_2, which were perfused for 15 min, the hearts were removed from the cannula. These times of perfusion for I/R hearts or H_2O_2 perfused hearts with medium containing collagenase were found to yield optimal results for cardiomyocyte isolation. The ventricles were cut into small pieces and subjected to another 15 min of digestion in a fresh collagenase solution in the presence of 1% BSA gassed with a mixture of 95% O_2 and 5% CO_2 in a shaking water bath at 37°C. The ventricular fragments were triturated gently (twice per min) with a plastic pipette. The cells from 3 - 4 harvests were combined and filtered through a 200 μm nylon mesh. The myocytes were resuspended for 5 min in buffers containing gradually increasing extracellular Ca^{2+} concentration (250, 500, 750 μM) to a final concentration of 1 mM. The cell viability in all experimental groups was evaluated by using the trypan blue (Sigma-Aldrich, Oakville, Ont., Canada) exclusion method. For this purpose, an aliquot of the cardiomyocyte suspension was added to an equal amount of 0.3% trypan blue in normal saline and incubated for 3 to 5 min. The unstained, stained and total number of cells were counted in the Neubauer chamber. After determining cell viability and yield, the preparation was washed two times by centrifugation at 100 g for 1 min to minimize the contamination from dead cells. All preparations were further purified by subjecting to an isotonic Percoll (Sigma-Aldrich, Oakville, Ont., Canada) gradient (pH 7.3) before Fura-2 acetoxymethyl ester (Fura-2 AM) loading. In some experiments, cardiomyocytes from control and 30 min I/R hearts were examined under light microscope to observe any differences in the

size and shape. The final cell suspension after purification had 90-95% viable cardiomyocytes; 3-5% of cardiomyocytes were observed to beat spontaneously. Since the isolation of cardiomyocytes involved 5 min Ca^{2+}-free perfusion and 25 min collagenase perfusion with oxygenated medium, it is understood that cardiomyocytes obtained from hearts subjected to ischemia or I/R underwent additional reperfusion for 30 min. In some experiments, isolated cardiomyocytes from control hearts were subjected to hypoxia-reoxygenation. For this purpose, cardiomyocytes were suspended in the K-H solution without glucose and gassed with 95% N_2 – 5% CO_2, at room temperature, pH 7.4 for 30 min. Glucose was replaced with Tris-HCl to maintain osmolarity. This cell suspension was then reoxygenated with K-H solution containing 11 mM glucose and gassed with 95% O_2 – 5% CO_2, at room temperature, pH 7.4 for 30 min.

3. Measurement of $[Ca^{2+}]_i$

Isolated cardiomyocytes were incubated with 5 μM Fura-2 AM for 40 min in the buffer (pH 7.4) containing 1 mM Ca^{2+} and washed twice to remove any extracellular dye [256,266]. The final cell number in the cuvette was adjusted to 0.3 million cells/ml for all the experimental groups. Alterations in fluorescence intensity was monitored by a SLM DMX-1100 dual wavelength spectrofluorometer (SLM Instruments, Inc., Urbana, IL) adjusted to an excitation wavelength 340/380 nm; emission wavelength 510 nm; integration time 0.95 sec and resolution time 1.0 sec. The $[Ca^{2+}]_i$ level was calculated according to Grynkiewicz equation [273]:

$$[Ca^{2+}]_i = K_d \times [(R-R_{min}) / (R_{max} - R) \times Sf_2/Sb_2]$$

where K_d is the effective dissociation constant and was taken as 224 for all the $[Ca^{2+}]_i$ measurements. The ratio of the fluorescence signals (R) at 340 and 380 nm was calculated automatically. R_{max} and R_{min} values were determined by addition of 20 μl Triton X-100 (10%) and 40 μl EGTA (400 mM), respectively. Sf_2 and Sb_2 are the fluorescence proportionality

coefficients obtained at 380 nm (excitation wavelength) under R_{min} and R_{max} conditions, respectively. Treatment with different concentrations of ISO was performed by incubating the Fura-2 AM loaded cells in a buffer containing ISO for 5 min at room temperature prior to the measurement of fluorescence. Unless otherwise indicated in the text, 100 μM ISO, which produced a maximal effect, was used in this study. The increase in $[Ca^{2+}]_i$ at peak $[Ca^{2+}]_i$ was calculated as the net increase above the basal value in each experiment. It should be mentioned that ISO was found to have no effect on $[Ca^{2+}]_i$ in unstimulated cardiomyocytes; however, this treatment augmented the KCl, S(-)-Bay K8644- or ATP-induced increase in $[Ca^{2+}]_i$. The difference between the responses in the presence and absence of ISO treatment was taken as the ISO-induced increase in $[Ca^{2+}]_i$. Treatments with different pharmacological agents for the modulation of $[Ca^{2+}]_i$ were performed by incubating the Fura-2 AM loaded cells in the buffer containing the desired concentration of pharmacological agent for 10 min prior to the measurement of fluorescence; the cells were treated with ryanodine and cyclopiazonic acid (CPA) for 20 min prior to the determination of $[Ca^{2+}]_i$. Unless otherwise indicated in the text, 50 μM ATP, which produced a maximal effect, was used in this study. The concentrations of different pharmacological interventions for the present investigation were selected on the basis of our previous studies [246,255,266,267]. For examining the effect of low Na^+ on catecholamine mediated potentiation of $[Ca^{2+}]_i$, cardiomyocytes were treated with K-H buffer (pH 7.4) containing 70 or 35 mM extracellular Na^+ for 10 min at room temperature as described previously and the osmolarity of the solution was maintained by adding choline chloride [266]. It is pointed out that no change in cell viability was observed under different incubation conditions. The increase in $[Ca^{2+}]_i$ at peak $[Ca^{2+}]_i$ was calculated as the net increase above the basal value in each experiment.

4. Measurement of some biochemical parameters of oxidative stress and preparation of crude membranes

In order to gain some information regarding the status of oxidative stress, the lipid peroxidation in control and 30 min I/R heart homogenates was assayed by measuring malondialdehyde (MDA) content [274]. In addition, conjugated diene formation was determined in the heart homogenate according to the method of Esterbauer et al. [275]. For the preparation of crude membranes, hearts subjected to 30 min ischemia as well as 30 min ischemia followed by 30 min reperfusion were removed from the cannula and crude membranes were isolated as described previously [127]. In brief, the ventricular tissue was minced and then homogenized in 50 mM Tris-HCl, pH 7.4 (15 ml/g tissue) with a PT3000 polytron (Brinkman Instruments, Westbury, NY) twice for 20 s each at 15,000 rpm. The resulting homogenate was centrifuged at 1000 × g for 10 min and the pellet was discarded. The supernatant was centrifuged at 48,000 × g for 25 min. The pellet was resuspended and centrifuged twice in the same buffer at the same speed. After determining the protein content by Lowry's method, these membranes were suspended in 0.2 mM sucrose and 10 mM histidine (pH 7.4) at a concentration of 3-5 mg/ml, stored at -80°C, and used within 2-3 weeks without any loss of activity. The purification of isolated membranes was determined by measuring the Na^+-K^+ ATPase (a SL marker enzyme) activity in the presence of 1 mM ouabain and comparing it with that detected in the heart homogenate as described previously [276]. However, the presence of other subcellular fractions including mitochondria in the crude membrane fraction cannot be ruled out.

5. Determination of β_1-adrenoceptors and purinergic receptor binding

Specific binding to β_1-adrenoceptors was calculated as the difference between [^{125}I]cyanopindolol binding values in the presence and absence of CGP-20712A, a selective β_1-adrenoceptor

antagonist [127]. The status of purinergic receptors in the membrane was determined by studying the binding characteristics of a slowly hydrolysable analogue of ATP, [^{35}S]ATPγS (39). 30-50 μg membrane protein was incubated in 0.5 ml medium containing various concentrations (0.1-10 nM for the high affinity site and 1-10 μM for the low affinity site) of [^{35}S]ATPγS (65 Ci/mmol, PerkinElmer Life and Analytical Sciences, Boston, MA) and 50 mM Tris-HCl (pH 7.5) at 37°C for 30 min as described earlier [277]. The reaction was terminated by vacuum filtration over wet Whatman filters (GF/B). The filters were washed three times with 6 ml ice-cold distilled water and radioactivity was counted by using Beckman scintillation counter. The binding was determined in the absence (total) and presence (non-specific) of 4 mM ATP (Tris salt, Sigma-Aldrich, Oakville, Ont., Canada); the specific binding was calculated by subtracting the non-specific binding from the total binding. To avoid possible artifacts, the binding of radioligand GF/B filters was checked in the absence of membrane proteins. The values of dissociation constant (K_d) and maximum receptor density (B_{max}) were calculated by Scatchard plot analysis according to Graph Pad Prism 4 for windows (version 4.02) (GraphPad Software Inc., San Diego, CA).

6. Measurement of intracellular pH in cardiomyocytes

Intracellular H^+ concentration was measured by using 2′, 7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescene acetoxymethyl ester (BCECF AM) loaded cardiomyocytes according to the previous protocol [278]. A calibration curve of BCECF loaded cardiomyocytes was plotted by measuring the fluorescence ratio obtained by exciting the cardiomyocytes at 440 and 500 nm and recording emission at 525 nm with different standard solutions (pH 5.5 – 7.4) containing high K^+ and 10 μM nigericin, a known H^+-K^+ ionophore. No quenching effect of fluorescence of BCECF AM was observed in the pH range of 5.5 – 7.4. In order to measure alterations in the intracellular

pH by different concentrations of MIA, the experiments were performed in HCO_3^- free buffers as described previously [279] to eliminate the activities of other alkalinizing mechanisms such the Na^+-HCO_3^- co-transporter and Cl^--HCO_3^- anion exchanger. In some experiments, pH was measured in the presence of HCO_3^- containing buffer, used for the determination of $[Ca^{2+}]_i$, to mimic the physiological conditions.

7. Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed with Microcal Origin version 6 (Microcal Software, Northampton, MA). The differences between two groups were evaluated by Student's t-test. The data from more than two groups were evaluated by one-way ANOVA followed by the Newman-Keul's test. Values showing $P < 0.05$ were considered statistically significant unless otherwise indicated in the text.

8. Drugs and chemicals

Isoproterenol, ATP, propranolol, EGTA, BSA, MIA, amiloride, nigericin, 5-(N-N)-dimethylamiloride (DMA), verapamil, diltiazem, vanadate, amiloride, nickel chloride, ouabain, sodium azide, ruthenium red, ryanodine, caffeine and CPA were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), whereas KB-R7943 was purchased from Tocris Biosciences (Ellisville, MO). Fura-2 AM and BCECF AM were purchased from Molecular Probes (Eugene, OR). Collagenase (Type II, 265U/mg) was purchased from Worthington Biochemical Co. (Freehold, NJ). All other reagents were of analytical grade and purchased either from Sigma-Aldrich (Oakville, Ontario, Canada) or Fisher Scientific (Fair Lawn, NJ).

IV. RESULTS

1. Catecholamine-induced changes in Ca^{2+}-handling in cardiomyocytes from hearts subjected to I/R

a. Cardiac performance of hearts subjected to different periods of I/R

For studying the effects of I/R on cardiac function, isolated rat hearts were subjected to global ischemia for 10, 20 and 30 min and then reperfused for 5 to 30 min. Alterations in LVDP, LVEDP, +dP/dt and –dP/dt in hearts subjected to 10 and 20 min ischemia followed by 30 min reperfusion are shown in **Figure 5 and Table 1** whereas those in hearts subjected to 30 min ischemia followed by 5, 15 and 30 min of reperfusion are given in **Table 2**. An increase in LVEDP was observed in hearts subjected to 20 and 30 min of ischemia whereas no such alteration was seen in 10 min ischemic hearts. On the other hand, LVDP, +dP/dt and –dP/dt were markedly depressed in all ischemic hearts as compared to control hearts. These changes in cardiac function in hearts undergoing 10 min ischemia were fully reversible after 30 min reperfusion whereas a partial improvement in these parameters was observed in hearts exposed to 20 min ischemia followed by 30 min reperfusion **(Table 1, Figure 5)**. The recovery of cardiac performance was markedly impaired in hearts undergoing 5, 15 and 30 min reperfusion after 30 min ischemia **(Table 2, Figure 5)**. It should be mentioned that the recovery of cardiac function in hearts subjected to 20 min ischemia and 60 min reperfusion was 90 to 95% whereas that in 30 min ischemic and 60 min reperfused hearts was 30 to 40% of the control values (data not shown). Thus it is evident that functional changes in 10 or 20 min ischemic hearts were reversible whereas those in 30 min ischemic hearts were irreversible.

b. Effect of I/R on viability and yield of cardiomyocytes

In order to examine the effect of I/R at the cellular level, cardiomyocytes were isolated from

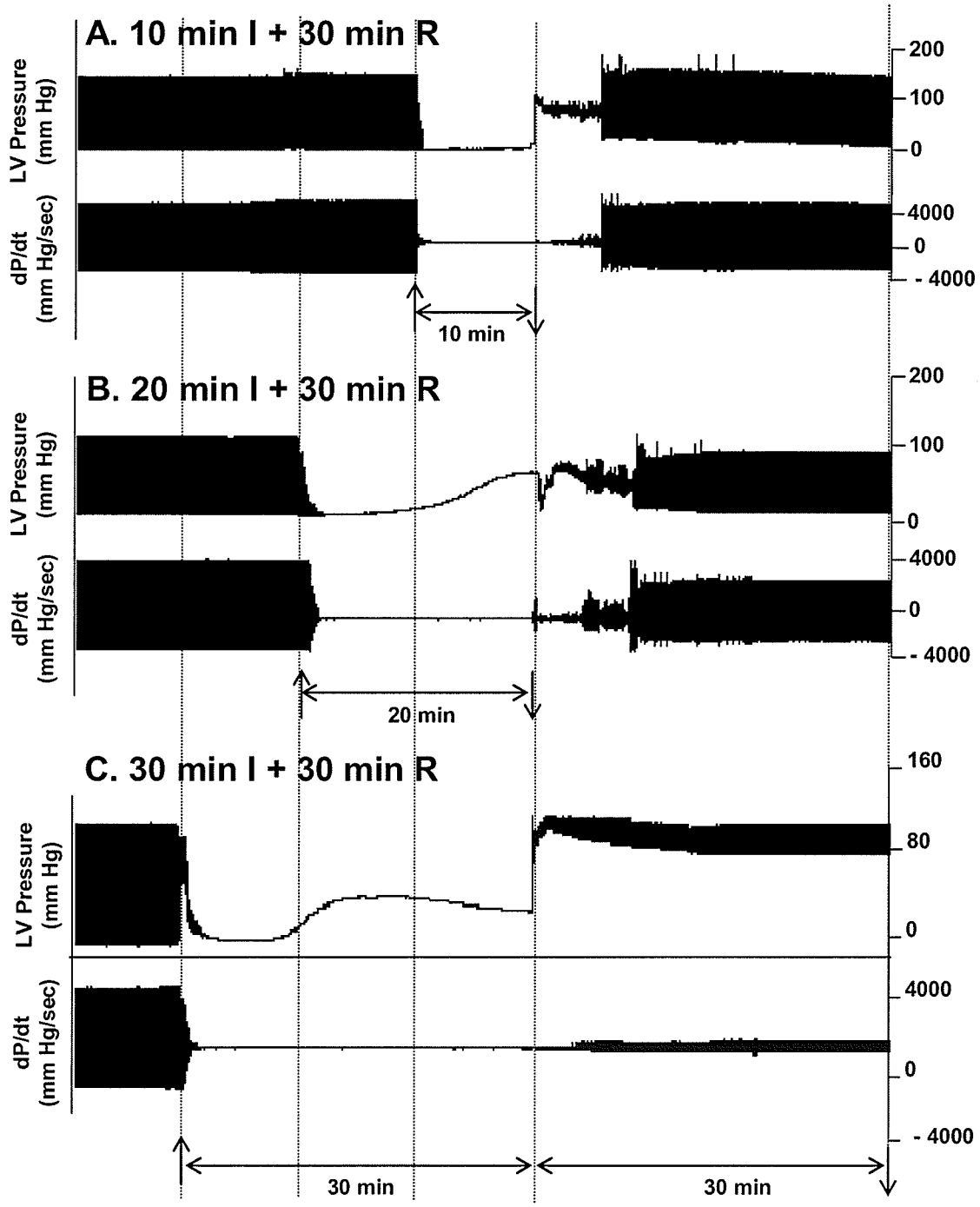


Figure 5. Representative tracings showing the left ventricular (LV) pressure and change of pressure development (dP/dt) in isolated hearts. ***Tracing A:*** Isolated hearts subjected to 10 min of ischemia followed by 30 min of reperfusion. ***Tracing B:*** Isolated hearts subjected to 20 min ischemia followed by 30 min of reperfusion. ***Tracing C:*** Isolated hearts subjected to 30 min of ischemia followed by 30 min of reperfusion.

Table 1: Cardiac performance, cell viability and characteristics of cardiomyocyte Ca^{2+}-handling for isolated hearts subjected to 10 and 20 min of ischemia followed by 30 min of reperfusion.

Parameter	Control	10 min I		20 min I	
		-R	+30 min R	-R	+30 min R
LVDP (mm Hg)	125 ± 12.4	2.2 ± 0.5*	127 ± 5.5†	2.9 ± 0.3*	98 ± 8.0*†
LVEDP (mm Hg)	9.2 ± 2.7	12.8 ± 1.3	7.5 ± 3.0	52.7 ± 3.2*	18.8 ± 2.2*†
+dP/dt (mm Hg/s)	6280 ± 550	35 ± 2.5*	6355 ± 638†	55 ± 10.8*	5072 ± 370*†
-dP/dt (mm Hg/ s)	4120 ± 380	30 ± 2.3*	4089 ± 204†	43 ± 12*	3170 ± 289*†
Viability of cardiomyocytes (%)	84 ± 3.6	82 ± 2.6	78 ± 2.4	77 ± 1.8	76 ± 2.9
Yield of cardiomyocytes (%)	8.6 ± 2.2	8.0 ± 2.4	7.8 ± 1.1	8.1 ± 2.0	8.3 ± 2.5
Basal $[Ca^{2+}]_i$ in cardiomyocytes (nM)	112 ± 3.0	109 ± 2.5	107 ± 3.8	112 ± 4.1	109 ± 3.3
KCl-induced increase in $[Ca^{2+}]_i$ (nM)	111 ± 4.2	113 ± 4.1	115 ± 2.5	107 ± 5.6	112 ± 3.7
ISO-induced increase in $[Ca^{2+}]_i$ (nM)	31 ± 3.5	29 ± 2.8	27 ± 4.5	12 ± 2.2*	30 ± 3.1†

Values are mean ± SEM of 4 hearts in each group except for the control group where 8 hearts were used. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of ventricular pressure development; -dP/dt, rate of ventricular pressure decline; I, ischemia; R, reperfusion; -R, without reperfusion; ISO, isoproterenol. After assessment of cardiac function, hearts were subjected to 5 and 25 min perfusion with Ca^{2+} free and collagenase containing oxygenated solution, respectively, for the isolation of cardiomyocytes. *$p < 0.05$ vs. control. †$p < 0.05$ vs. respective 20 min ischemic group.

Table 2: Cardiac performance and cell viability for isolated hearts subjected to 30 min ischemia followed by different times of reperfusion.

Group	LVDP (mm Hg)	LVEDP (mm Hg)	+dP/dt (mm Hg/s)	-dP/dt (mm Hg/s)	Viability of cardiomyocytes (%)	Yield of cardiomyocytes (millions per heart)
Control	128 ± 8.7	8.8 ± 2.7	6002 ± 480	4074 ± 373	85 ± 3.7	8.5 ± 1.2
30 min I	2.4 ± 0.4*	54 ± 2.5*	97 ± 33*	96 ± 9*	73 ± 1.5*	4.0 ± 1.8*
30 min I+ 5 min R	17.3 ± 3.8*†	105 ± 6.3*†	702 ± 44*†	610 ± 25*†	66 ± 1.3*†	2.9 ± 1.1*†
30 min I+ 15 min R	22.4 ± 1.2*†	83.5 ± 7.6*†	1156 ± 162*†	709 ± 42*†	62 ± 1.6*†	2.5 ± 0.7*†
30 min I+ 30 min R	27.1 ± 3.0*†	81.7 ± 7.4*†	1557 ± 332*†	849 ± 59*†	59 ± 1.1*†	2.1 ± 0.2*†

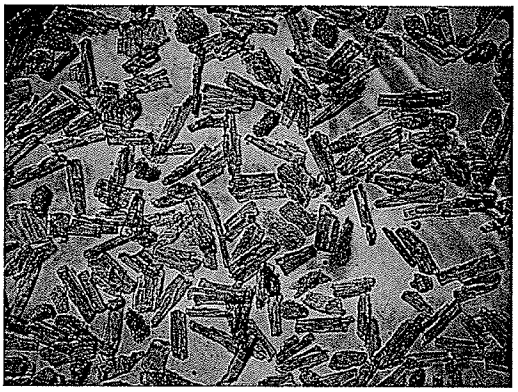
Values are mean ± SEM of 4 hearts in each group except for the control group where 8 hearts were used. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of ventricular pressure development; -dP/dt, rate of ventricular pressure decline; I, ischemia; R, reperfusion. After assessment of cardiac function, hearts were subjected to 5 and 25 min perfusion with Ca^{2+} free and collagenase containing oxygenated solution, respectively, for the isolation of cardiomyocytes. $^{*}p< 0.05$ vs. control; † $p< 0.05$ vs. 30 min ischemia group.

hearts undergoing varying periods of ischemia and reperfusion as shown in Tables 1 and 2. No decrease in cell viability and cardiomyocyte yield was observed in hearts undergoing 10 min ischemia or 10 min ischemia followed by 30 min reperfusion. Likewise, the cell viability and yield of cardiomyocytes did not change significantly in hearts undergoing 20 min ischemia or 20 min ischemia followed by 30 min reperfusion **(Table 1)**. On the other hand, both yield and viability of cardiomyocytes were significantly decreased at different periods (5, 15 and 30 min) of reperfusion in 30 min ischemic hearts **(Table 2)**. However, the size and shape of the viable cardiomyocytes isolated from the 30 min ischemic and 30 min reperfused hearts were not different from the control preparations **(Figure 6)**. This shows that most of dead or damaged cardiomyocytes due to I/R were removed during the process of isolation and purification; this fact is evident from the reduced yield of cardiomyocytes obtained from I/R hearts.

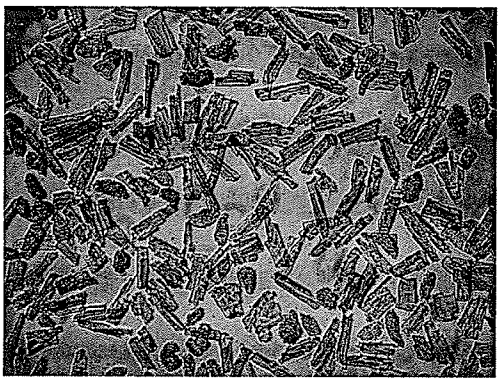
c. Basal $[Ca^{2+}]_i$ and KCl-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from I/R hearts

To investigate alterations in Ca^{2+}-handling by I/R, $[Ca^{2+}]_i$ was measured in isolated cardiomyocytes by using Fura-2 microfluorometry. Representative tracings for KCl (30 mM), a known depolarizing agent [266], induced increases in $[Ca^{2+}]_i$ in cardiomyocytes isolated from control and I/R hearts as shown in **Figure 7A**. The basal $[Ca^{2+}]_i$ was not altered in cardiomyocytes isolated from hearts undergoing 10 and 20 min ischemia **(Table 1)**, whereas a gradual increase in basal $[Ca^{2+}]_i$ was observed at different times of reperfusion in cardiomyocytes isolated from 30 min ischemic hearts **(Figure 7A)**. The data in **Table 1** indicate that the KCl - induced increase in $[Ca^{2+}]_i$ was not altered in cardiomyocytes isolated from hearts subjected to 10 and 20 min ischemia followed by 30 min reperfusion. Similarly, no alterations in KCl-induced increase in $[Ca^{2+}]_i$ were observed in hearts subjected to varying periods of reperfusion (5, 15 , 30 min) after 30 min ischemia **(Figures 7A and 8B)**. It is pointed out that after measuring the

A. Cardiomyocytes from hearts subjected to control perfusion (x10)



B. Cardiomyocytes from hearts subjected to 30 min ischemia and 30 min reperfusion (x10)



C. Cardiomyocytes from hearts subjected to 30 min ischemia and 30 min reperfusion (x25)

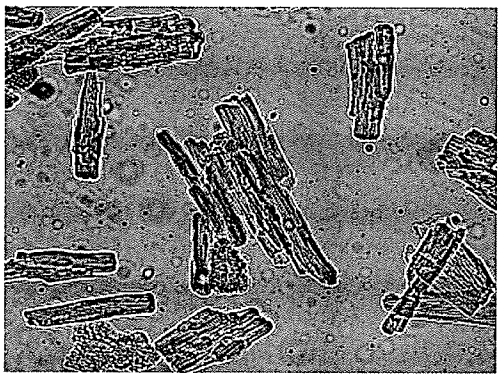


Figure 6: Size and shape of cardiomyocytes isolated from control and ischemic-reperfused (I/R) hearts. ***A:*** Cardiomyocytes isolated from control-perfused hearts (X10) ***B:*** Cardiomyocytes isolated from hearts subjected to 30 min of ischemia followed by 30 min of reperfusion (X10). ***C:*** Cardiomyocytes isolated from hearts subjected to 30 min of ischemia followed by 30 min of reperfusion (X25). Each micrograph is the representative of cardiomyocytes isolated from 4 different preparations from control or I/R groups after application of Percoll gradient and cell pellet was used for taking the micrograph. The number of cardiomyocytes in preparation from the I/R hearts was adjusted equal to that from the sham control hearts.

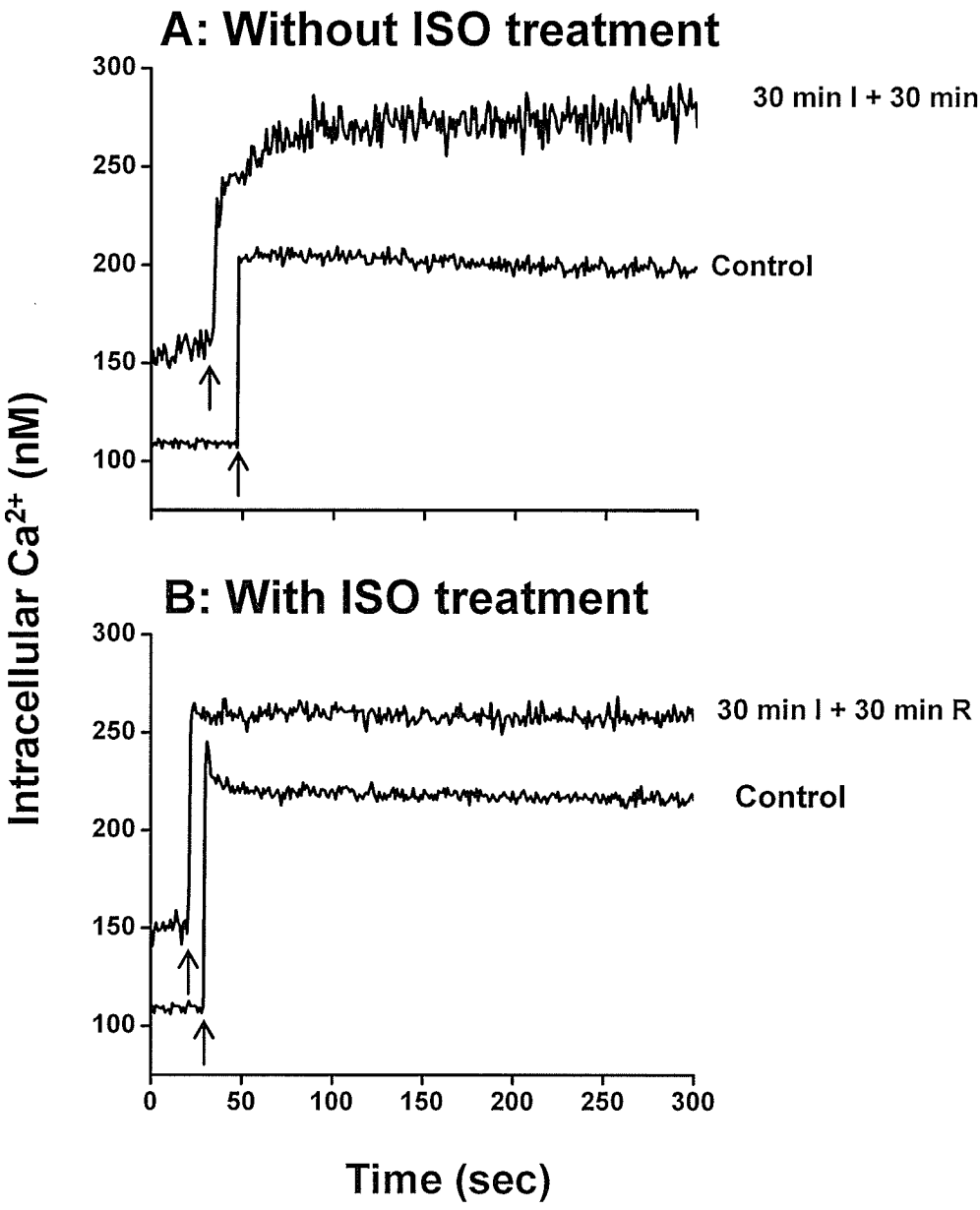


Figure 7: KCl -induced increase in $[Ca^{2+}]_i$ in cardiomyocytes, with or without isoproterenol (ISO) treatment, isolated from hearts subjected to 30 min ischemia followed by 30 min of reperfusion. ***Tracing A:*** KCl-induced increase in $[Ca^{2+}]_i$ in untreated cardiomyocytes isolated from control and I/R hearts. ***Tracing B:*** KCl-induced increase in $[Ca^{2+}]_i$ in ISO-treated cardiomyocytes isolated from control and I/R hearts. ↑— indicates the time when the preparation was exposed to 30 mM KCl. Treatment with 100 μM was carried out for 5 min prior to intracellular Ca^{2+} measurements.

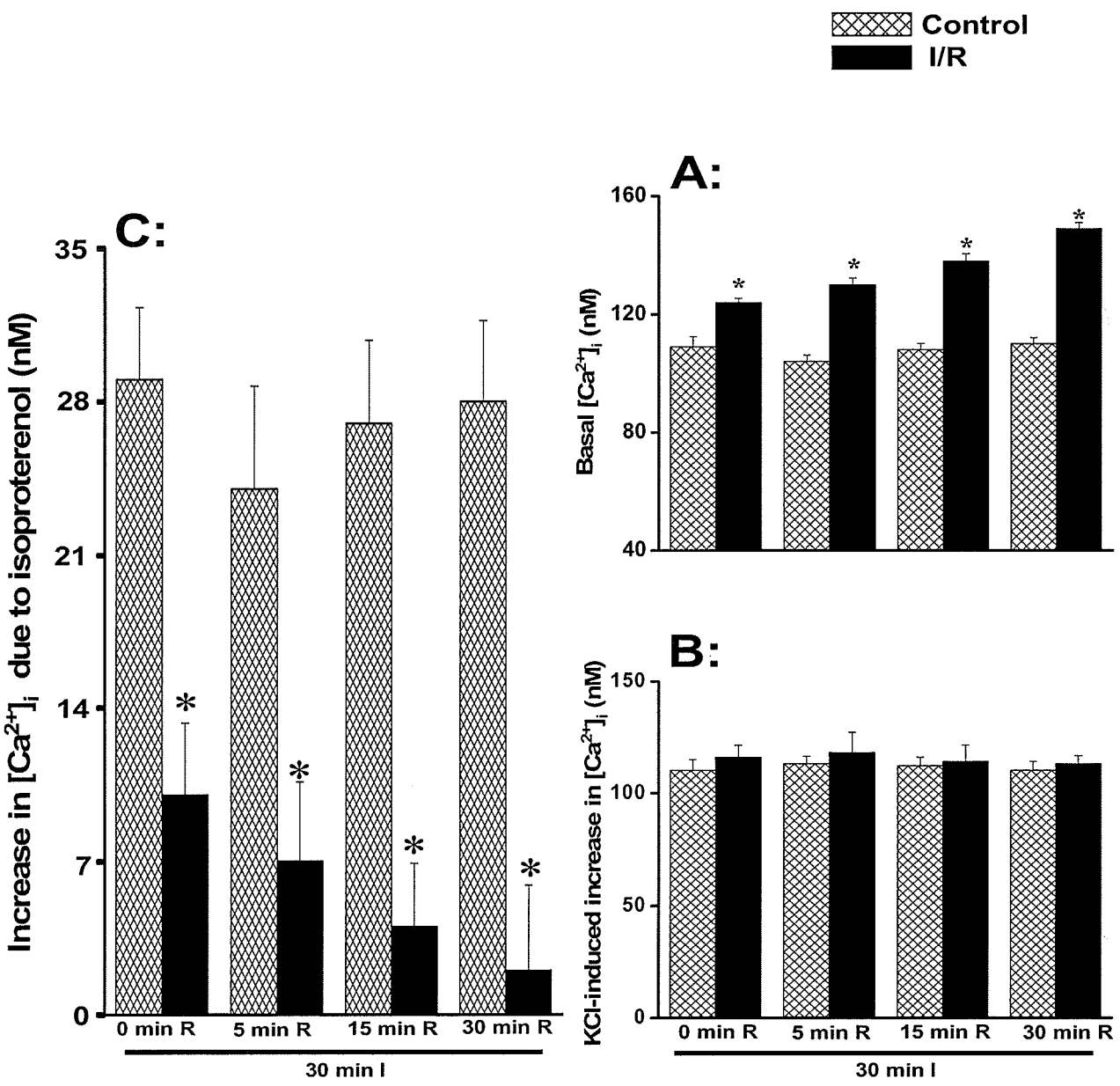


Figure 8: Effect of 30 min of ischemia and different times of reperfusion on $[Ca^{2+}]_i$ in isolated cardiomyocytes. ***Panel A:*** Effect of ischemia-reperfusion (I/R) on basal $[Ca^{2+}]_i$ in isolated cardiomyocytes. ***Panel B:*** Effect of I/R on KCl-induced increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes. ***Panel C:*** Effect of I/R on isoproterenol (ISO)-induced increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes. The basal $[Ca^{2+}]_i$ represents $[Ca^{2+}]_i$ before the addition of KCl. The increase in $[Ca^{2+}]_i$ was calculated as the difference between the peak value and the basal value in each experiment. ISO-induced increase was calculated by subtracting the values for KCl-induced increase in $[Ca^{2+}]_i$ in untreated cardiomyocytes from those treated with 100 μM ISO. The concentration of KCl was 30 mM. Each point represents mean ± SEM of 4 experiments. *$p<0.05$ vs. control.

cardiac performance, both ischemic and I/R hearts were further perfused for 5 min with Ca^{2+}-free medium and 25 min perfusion with medium containing collagenase; this 30 min of additional reperfusion period should be taken into consideration while interpreting the data in cardiomyocytes isolated from ischemic or I/R hearts.

d. Effect of ISO on $[Ca^{2+}]_i$ in KCl-depolarized cardiomyocytes isolated from I/R hearts

Since treatment of mainly quiescent cardiomyocytes with ISO, a known β_1-adrenoceptor agonist [127], did not affect the $[Ca^{2+}]_i$, KCl-depolarized cells were used to examine the effect of 100 μM ISO at the receptor level. ISO was observed to increase $[Ca^{2+}]_i$ in KCl-depolarized cardiomyocytes obtained from control hearts **(Figures 7B and Table 1)**. No alterations in ISO-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes was observed in hearts exposed to 10 min ischemia with or without 30 min reperfusion **(Table 1)**. In contrast, a significant depression in ISO-induced increase in $[Ca^{2+}]_i$ was seen in hearts subjected to 20 min ischemia. However, this alteration in 20 min ischemic hearts was fully reversible after 30 min of reperfusion **(Table 1)**. On the other hand, in hearts subjected to 30 min of ischemia followed by 5, 15 or 30 min of reperfusion, a significant depression in ISO-induced increase in $[Ca^{2+}]_i$ was detected at each of these time points **(Figure 8C)**. It is pointed out that ISO-induced increase in $[Ca^{2+}]_i$ in KCl-depolarized cells was depressed at different concentrations of ISO (10 - 150 μM) in hearts subjected to 30 min of ischemia followed by 30 min of reperfusion **(Figure 9)**. Furthermore, the increase in $[Ca^{2+}]_i$ by 100 μM ISO was mediated by β-adrenoceptors because propranolol (50 μM) was found to inhibit this effect completely in cardiomyocytes isolated from both control and I/R hearts.

e. Effect of S(-)-Bay K8644 on $[Ca^{2+}]_i$ in cardiomyocytes isolated from I/R hearts

Although no difference in KCl responses was seen in control and I/R cardiomyocytes, KCl-induced increase in $[Ca^{2+}]_i$ in both control and I/R hearts was inhibited up to 70 to 80% by

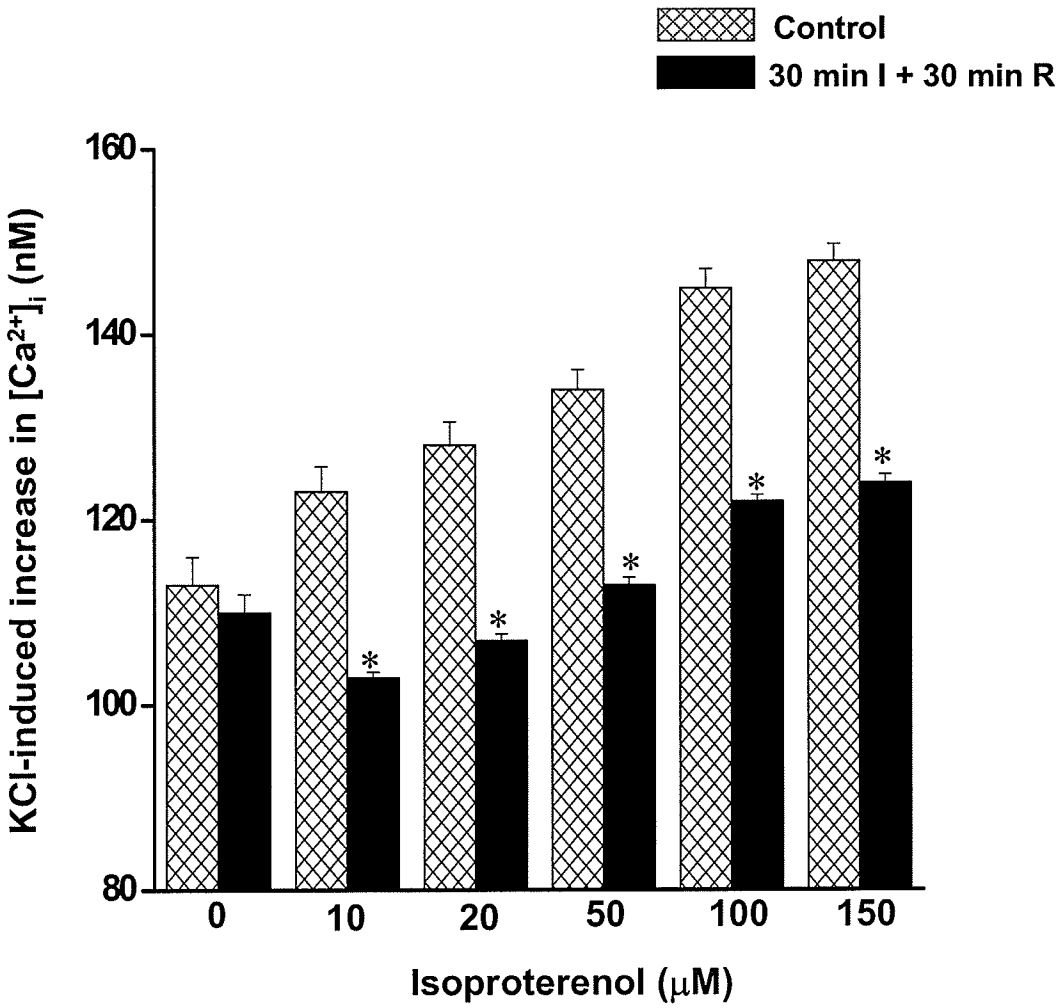


Figure 9: Effect of treatments with different concentrations of isoproterenol (10 - 150 μM) on KCl-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from hearts subjected to 30 min of ischemia followed by 30 min of reperfusion. Each point represents mean ± SEM of 4 experiments in each group. *$p<0.05$ vs. respective control group.

verapamil (10 μM), a well known antagonist of L-type Ca^{2+}-channels, but was unaffected by propranolol (50 μM). The involvement of L-type Ca^{2+}-channels in I/R-induced changes in Ca^{2+}-handling was further verified by using S(-)-Bay K8644 (2 μM), a specific dihyropyridine (DHP) receptor agonist, which promote the opening of L-type Ca^{2+}-channels [280]. No change in Bay K8644-induced increase in $[Ca^{2+}]_i$ was observed after 30 min ischemia or after 30 min of ischemia followed by 30 min of reperfusion. On the other hand, ISO-induced increase in $[Ca^{2+}]_i$ in the presence of Bay K8644 was significantly depressed in all these groups **(Figure 10)**. It should be mentioned that the Bay K8644-induced increase in $[Ca^{2+}]_i$ in both control and I/R hearts was 90 to 95% inhibited by 10 μM verapamil but was not altered by 50 μM propranolol.

f. Status of oxidative stress and β_1-adrenoceptors in I/R hearts

In order to gain some information regarding the status of oxidative stress in I/R hearts under the experimental conditions used in the present study, we measured the level of MDA and the formation of conjugated dienes. The data shown in **Table 3** reveal an increase in the level of MDA and conjugated dienes formation in I/R hearts in comparison to control. Since the responsiveness of I/R cardiomyocytes to ISO was attenuated, the status of β-adrenoceptors in control and I/R hearts was also determined. The binding characteristics of β_1-adrenoceptors indicated that both the density (B_{max}) and affinity ($1/K_d$) of β_1-adrenoceptors were depressed in I/R hearts **(Table 3).**

g. Effect of SOD plus CAT on intracellular Ca^{2+}-handling in isolated cardiomyocytes

To investigate the involvement of oxyradicals in I/R-induced alteration in intracellular Ca^{2+}-handling, isolated hearts were treated with an antioxidant mixture containing SOD and catalase. Treatment of hearts with SOD plus CAT did not exert any effect on LVDP, LVEDP, +dP/dt, and –dP/dt or cell viability and cardiomyocyte yield in control and 30 min ischemic hearts **(Table 4).**

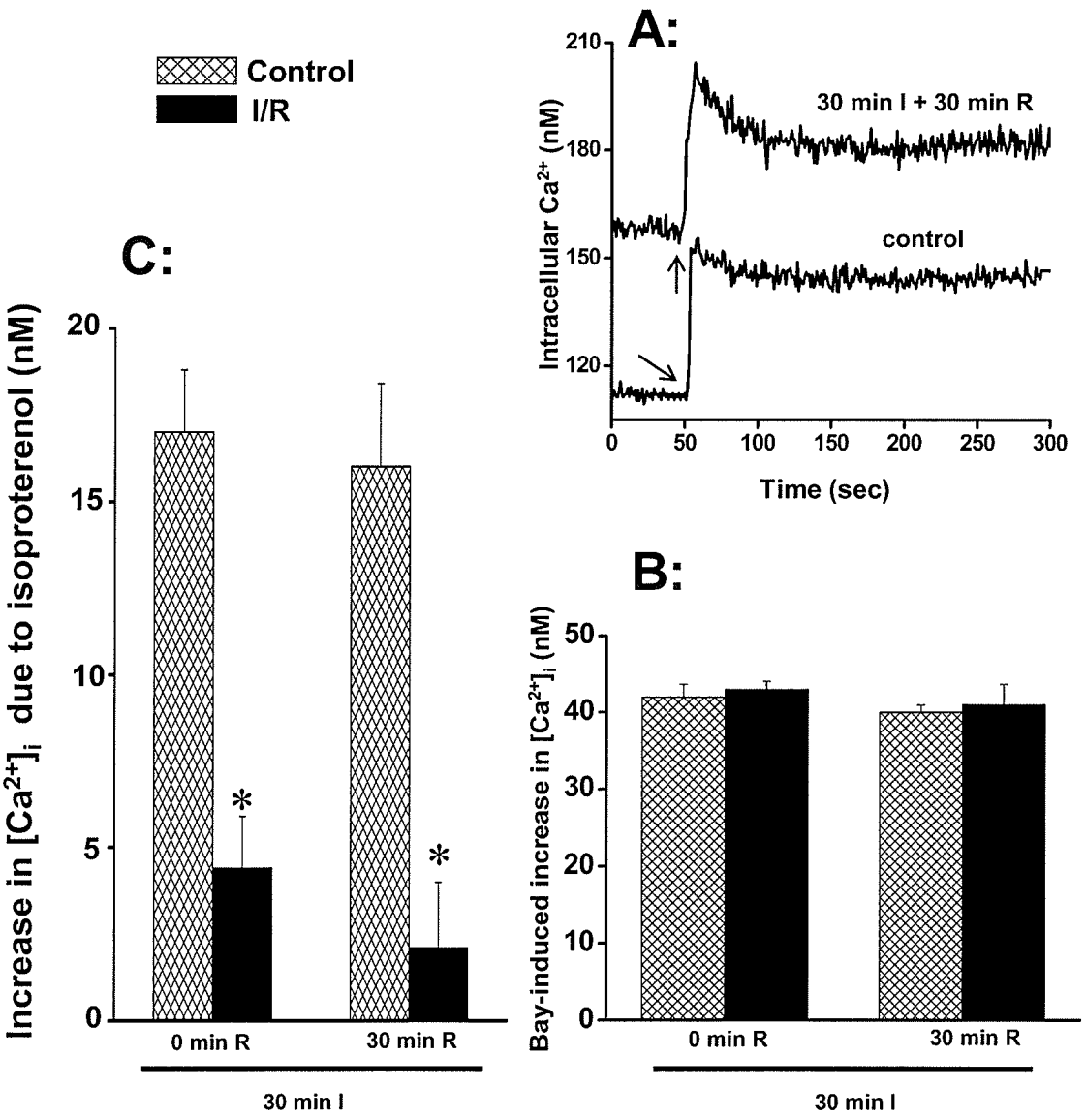


Figure 10: Effect of 30 min of ischemia and 30 min of reperfusion (I/R) on $[Ca^{2+}]_i$ in isolated cardiomyocytes. ***Panel A:*** Representative tracing showing the S(-)-Bay K8644 (Bay)-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from control and I/R hearts. ***Panel B:*** Effect of I/R on Bay-induced increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes. ***Panel C:*** Effect of I/R on isoproterenol (ISO)-induced increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes. The basal $[Ca^{2+}]_i$ represents $[Ca^{2+}]_i$ before the addition of S(-)-Bay K8644 (2 μM). ISO-induced increase was calculated by subtracting the values for Bay-induced increase in $[Ca^{2+}]_i$ in the untreated cardiomyocytes from those for the 100 μM ISO treated cardiomyocytes. Each point represents mean ± SEM of 4 experiments in each group. ↑— indicates the time when the cardiomyocytes were exposed to 2 μM Bay. *$p<0.05$ vs. control.

Table 3: Alterations in some parameters of oxidative stress and binding characteristics of β_1-adrenoceptors in hearts subjected to 30 min ischemia and 30 min reperfusion.

	Control hearts	I/R hearts
Malondialdehyde levels (nmol/mg tissue lipids)	3.6 ± 0.7	14.8 ± 1.5*
Conjugated dienes (nmol/mg tissue lipids)	27.4 ± 2.1	60.5 ± 3.9*
Maximal β_1-adrenoceptor binding (B_{max}) (fmol/mg protein)	64.5 ± 4.1	27.5 ± 2.7*
Dissociation constant (K_d) for β_1-adrenoceptors (pmol)	32.3 ± 2.6	48.9 ± 2.3*

Values are mean ± SEM of 5 hearts in each group. *$p < 0.05$ vs. respective control values.

On the other hand, the increase in basal $[Ca^{2+}]_i$ and decrease in ISO-induced increase in $[Ca^{2+}]_i$ in 30 min ischemic cardiomyocytes, unlike control preparations were attenuated by SOD plus CAT. From the data given in **Table 4**, it can be seen that treatment of hearts with SOD plus CAT attenuated the I/R-induced alterations in cardiac function, cell viability, cardiomyocyte yield as well as basal $[Ca^{2+}]_i$ and ISO-induced increase in $[Ca^{2+}]_i$. Although SOD plus CAT treatment was observed to increase the KCl-induced increase in $[Ca^{2+}]_i$ in both control and 30 min ischemic as well as I/R cardiomyocytes **(Table 4)**, no attempt was made to understand the mechanisms of such an effect.

h. Effect of H_2O_2 on intracellular Ca^{2+}-handling in isolated cardiomyocytes

In order to examine if the effects of I/R are mimicked by oxidative stress, isolated hearts were treated with 50 μM H_2O_2 for 30 min. H_2O_2 caused a depression in LVDP, +dP/dt and –dP/dt with an elevation in LVEDP as shown in **Table 5**. The selection of this concentration and time period of perfusion with H_2O_2 was based on comparable depression of cardiac performance in 30 min I/R hearts in the present study. A marked decrease in cell viability and number of cardiomyocytes and an increase in basal $[Ca^{2+}]_i$ were observed after H_2O_2 treatment, whereas ISO-induced increase in $[Ca^{2+}]_i$ was significantly depressed in H_2O_2 treated hearts **(Table 5)**. Although I/R and H_2O_2 are considered to produce oxidative stress, it is pointed out that unlike I/R, H_2O_2 was able to augment the KCl-induced increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes **(Table 5)**. This effect is in accordance with a previous study [281] and may be due to the activation of SR Ca^{2+} release channels and inhibition of SR Ca^{2+}-pump activity in cardiomyocytes [281]. It is also possible that I/R-induced injury may not produce sufficient amount of H_2O_2 as employed in our study. Furthermore, the concentration of H_2O_2 as employed in the present study may be affecting some

Table 4: Cardiac performance, cell viability and characteristics of cardiomyocyte Ca^{2+}-handling for isolated rat hearts treated with superoxide dismutase (SOD) plus catalase (CAT) 10 min before ischemia and during reperfusion period.

Group	Control	SOD+CAT	30 min I		30 min I + 30 min R	
			(-) SOD+CAT	(+) SOD+CAT	(-) SOD+CAT	(+) SOD+CAT
LVDP (mm Hg)	119 ± 10.2	125 ± 9.3	2.9 ± 1.7*	1.9 ± 0.2	30 ± 4.0*	71 ± 4.3†
LVEDP (mm Hg)	8.9 ± 2.9	10.2 ± 3.6	57 ± 4.4*	63 ± 3.1	85 ± 9.9*	43 ± 3.2†
+dP/dt (mm Hg/s)	6112 ± 450	6230 ± 530	99 ± 12*	101 ± 24	1598 ± 340*	4241 ± 284†
-dP/dt (mm Hg/s)	4065 ± 390	4225 ± 410	101 ± 11*	93 ± 20	890 ± 60*	2790 ± 126†
Viability of cardiomyocytes (%)	84 ± 3.3	82 ± 5.1	73 ± 2.3*	66 ± 3.7	58 ± 2.2*	67 ± 2.9†
Yield of cardiomyocytes (millions per heart)	8.0 ± 2.1	8.3 ± 0.7	4.3 ± 2.0*	5.8 ± 1.2	2.5 ± 0.5*	5.5 ± 0.9†
Basal $[Ca^{2+}]_i$ in cardiomyocytes (nM)	112 ± 3.0	111 ± 2.4	127 ± 2.8*	113 ± 3.2†	150 ± 3.1*	115 ± 2.1†
KCl-induced increase in $[Ca^{2+}]_i$ (nM)	120 ± 6.3	140 ± 3.9*	109 ± 4.3	144 ± 5.0†	115 ± 3.9	147 ± 4.0†
ISO-induced increase in $[Ca^{2+}]_i$ (nM)	26 ± 4.2	25 ± 3.7	6.2 ± 3.0*	16.7 ± 2.0†	2.7 ± 3.5*	15.5 ± 2.5†

Values are mean ± SEM of 4 hearts in each group. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of ventricular pressure development; -dP/dt, rate of ventricular pressure decline; I, ischemia; R, reperfusion; ISO, isoproterenol; (-) SOD+CAT, without SOD plus CAT treatment; (+) SOD+CAT, with SOD plus CAT treatment. After assessment of cardiac function, hearts were subjected to 5 and 25 min perfusion with Ca^{2+} free and collagenase containing oxygenated solution, respectively, for the isolation of cardiomyocytes. *$p < 0.05$ vs. control; † $p < 0.05$ vs. 30 min ischemia and 30 min reperfusion group.

Table 5: Cardiac performance, cell viability and characteristics of cardiomyocyte Ca^{2+}-handling for isolated rat hearts treated with 50 μM H_2O_2 for 30 min

Parameter	Control	H_2O_2
LVDP (mm Hg)	124 ± 9.8	45 ± 2.6*
LVEDP (mm Hg)	8.3 ± 3.7	36 ± 4.1*
+dP/dt (mm Hg/s)	6529 ± 539	2565 ± 85*
-dP/dt (mm Hg/s)	4129 ± 214	1152 ± 100*
Viability of cardiomyocytes (%)	84 ± 2.7	57 ± 3.3*
Yield of cardiomyocytes (millions per heart)	8.3 ± 1.7	2.4 ± 0.5*
Basal $[Ca^{2+}]_i$ in cardiomyocytes (nM)	108 ± 4.5	146 ± 3.8*
KCl-induced increase in $[Ca^{2+}]_i$ (nM)	117 ± 6.5	148 ± 5.6*
ISO-induced increase in $[Ca^{2+}]_i$ (nM)	28 ± 2.7	9 ± 1.2*

Values are mean ± SEM of 6 hearts in each group. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of ventricular pressure development; -dP/dt, rate of ventricular pressure decline; ISO, isoproterenol. *$p < 0.05$ vs. control. After assessment of cardiac function, hearts were subjected to 5 and 25 min perfusion with Ca^{2+} free and collagenase containing oxygenated solution, respectively, for the isolation of cardiomyocytes. *$p < 0.05$ vs. control.

other sites in addition to those affected by I/R. However, no experiments were carried out to establish the mechanisms of the observed differences in cardiomyocytes isolated from H_2O_2 and I/R hearts with respect to the KCl-induced increase in $[Ca^{2+}]_i$.

i. Effect of ischemic preconditioning on intracellular Ca^{2+}-handling in isolated cardiomyocytes

To determine the significance of I/R mediated changes in intracellular Ca^{2+}-handling at the level of isolated cardiomyocytes, we examined the effect of ischemic preconditioning, which is known to prevent I/R-induced changes in cardiac function [247,248]. Although ischemic preconditioning depressed the cardiac performance (LVDP, LVEDP, +dP/dt and –dP/dt) in control hearts, alterations in cardiac function in I/R hearts, unlike the ischemic hearts, were attenuated **(Table 6)**. Both cell viability and yield of cardiomyocytes in I/R hearts, unlike control hearts were improved by ischemic preconditioning. On the other hand, cell viability in ischemic heart was improved by ischemic preconditioning without any changes in cardiomyocyte yield **(Table 6)**. Attenuation of basal $[Ca^{2+}]_i$ and a marked improvement of ISO-induced increase in $[Ca^{2+}]_i$ were observed in cardiomyocytes isolated from both ischemic and I/R hearts subjected to ischemic preconditioning (Table 6). The KCl-induced increase in $[Ca^{2+}]_i$ in control, ischemic and I/R groups was not affected by ischemic preconditioning **(Table 6)**. It is also pointed out that a strong correlation (r^2= 0.9814, $p<0.001$) was observed between IR-induced changes in LVEDP and LVDP in ischemic preconditioned hearts. Similar correlation (r^2= 0.9179, $p<0.001$) was also found between IR-induced changes in LVDP and cell viability in cardiomyocytes isolated from ischemic preconditioned hearts. Such relationships suggest that cardiomyocyte preparations obtained from I/R hearts under different experimental conditions including ischemic preconditioning may show corresponding magnitude of I/R injury.

Table 6: Cardiac performance, cell viability and characteristics of cardiomyocyte Ca^{2+}-handling for isolated hearts subjected to ischemic preconditioning (IP) before the induction of 30 min ischemia and 30 min reperfusion.

Group	Control	IP	30 min I		30 min I + 30 min R	
			(-) IP	(+) IP	(-) IP	(+) IP
LVDP (mm Hg)	117 ± 12.5	92 ± 3.5*	3.5 ± 2.2*	3.8 ± 0.5	33 ± 4.9*	89 ± 8.7†
LVEDP (mm Hg)	8.0 ± 2.3	16.1 ± 2.2*	60 ± 3.8*	64 ± 5.4	80 ± 10.0*	27.4 ± 12.3†
+dP/dt (mm Hg/s)	6540 ± 520	4399 ± 280*	100 ± 13*	105 ± 14	1615 ± 385*	5263 ± 507†
-dP/dt (mm Hg/s)	4489 ± 568	2353 ± 292*	98 ± 10*	99 ± 11	925 ± 70*	3105 ± 426†
Viability of cardiomyocytes (%)	85 ± 3.8	81 ± 3.5	72 ± 2.8*	83 ± 3.5†	57 ± 2.8*	80 ± 3.9†
Yield of cardiomyocytes (millions per heart)	7.7 ± 1.9	8.2 ± 2.0	4.5 ± 1.7*	5.4 ± 0.2	2.6 ± 1.3*	5.5 ± 0.3†
Basal $[Ca^{2+}]_i$ in cardiomyocytes (nM)	110 ± 2.3	109 ± 3.5	128 ± 2.7*	108 ± 2.7†	154 ± 4.8*	110 ± 4.1†
KCl-induced increase in $[Ca^{2+}]_i$ (nM)	115 ± 9.1	110 ± 6.9	120 ± 6.5	132 ± 5.0	119 ± 7.9	137 ± 11.1
ISO-induced increase in $[Ca^{2+}]_i$ (nM)	28 ± 4.7	25 ± 3.7	7.1 ± 3.3*	27 ± 3.9†	2.0 ± 0.5*	30 ± 4.3†

Values are mean ± SEM of 4 hearts in each group. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of ventricular pressure development; -dP/dt, rate of ventricular pressure decline; I, ischemia; R, reperfusion; IP, ischemic preconditioning; ISO, isoproterenol; (-) IP, without ischemic preconditioining; (+) IP, with ischemic preconditioning. After assessment of cardiac function, hearts were subjected to 5 and 25 min perfusion with Ca^{2+} free and collagenase containing oxygenated solution, respectively, for the isolation of cardiomyocytes. * $p < 0.05$ vs. control; † $p < 0.05$ vs. 30 min ischemia and 30 min reperfusion group.

2. ATP-induced changes in Ca^{2+}-handling in cardiomyocytes from hearts subjected to I/R

a. Effect of I/R on ATP-induced increase in $[Ca^{2+}]_i$

In order to examine the effect of I/R on ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes, isolated hearts were subjected to 10, 20 or 30 min of global ischemia followed by 30 min of reperfusion. It can be seen from **Table 7** that chages in both LVDP and LVEDP due to different periods of ischemia followed by different periods of reperfusion are similar to those observed earlier under similar experimental conditions **(Tables 1 and 2).** Measurement of $[Ca^{2+}]_i$ in purified preparations revealed an increase in basal $[Ca^{2+}]_i$ in cardiomyocytes isolated from 30 min I/R hearts **(Table 7 and Figure 11)**. Extracellular ATP (50 μM), a purinergic receptor agonist (37), caused a significant increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from control hearts **(Figure 11)**; this is in agreement with the previous studies (31, 39). However, the increase in $[Ca^{2+}]_i$ by ATP was significantly depressed in purified cardiomyocytes isolated from 30 min I/R hearts **(Table 7 and Figure 11)**. The depression in ATP-induced increase in $[Ca^{2+}]_i$ in I/R hearts may not be due to elevated level of basal $[Ca^{2+}]_i$ because the increase in $[Ca^{2+}]_i$ by KCl (30 mM) was not altered in 30 min I/R hearts (110 ± 5.5 vs 112 ± 4.0 nM in control) **(Figure 11)**. To gain further information regarding the effects of I/R on Ca^{2+}-handling by cardiomyocytes, $[Ca^{2+}]_i$ measurements were carried out in preparations isolated from hearts subjected to 10 or 20 min ischemia followed by 30 min of reperfusion as well as 30 min ischemic hearts followed by 5 or 15 min reperfusion. The data in **Table 7** indicate that basal $[Ca^{2+}]_i$ was not altered in cardiomyocytes isolated from hearts undergoing 10 or 20 min of ischemia as well as 10 or 20 min ischemia followed by 30 min reperfusion. On the other hand, in cardiomyocytes isolated from hearts undergoing 30 min ischemia or 30 min ischemia followed by 5 and 15 min of reperfusion, a significant increase in basal $[Ca^{2+}]_i$ was observed **(Table 7)**. ATP-induced increase

Table 7: Various parameters of isolated hearts subjected to 10, 20 and 30 min of ischemia followed by different times of reperfusion.

Group	LVDP (mm Hg)	LVEDP (mm Hg)	Basal $[Ca^{2+}]_i$ (nM)	ATP-induced increase in $[Ca^{2+}]_i$ (nM)
A. 10 and 20 min ischemia followed by 30 min of reperfusion				
Control	130.7 ± 8.4	9.5 ± 2.3	112 ± 4.5	47 ± 3.3
10 min	2.1 ± 0.7*	15.5 ± 3.1	109 ± 5.0	45 ± 3.5
10 min I+ 30 min R	125.3 ± 7.1	10.9 ± 3.7	108 ± 5.3	47 ± 3.1
20 min I	3.0 ± 0.5*	55.0 ± 4.8*	114 ± 2.1	27 ± 2.6*
20 min I+ 30 min R	95 ± 10.0*	25.4 ± 3.6*	111 ± 4.2	37 ± 1.9*
B. 30 min of ischemia followed by 5, 15 and 30 min of reperfusion				
Control	129.3 ± 11.5	11.4 ± 2.8	113 ± 4.1	45 ± 3.9
30 min I	2.0 ± 1.1*	56.7 ± 4.3*	127 ± 2.3*	24 ± 4.1*
30 min I+ 5 min R	20.2 ± 3.8*	98 ± 7.7*	132 ± 1.8*	28 ± 1.7*
30 min I+ 15 min R	25.0 ± 1.7*	80.5 ± 6.5*	141 ± 2.9*	30 ± 2.4*
30 min I+ 30 min R	30.2 ± 3.9*	76.4 ± 9.6*	153 ± 2.9*	28 ± 2.7*

Values are mean ± SEM of 4 hearts in each group except for the control group where 8 hearts were used. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; I, ischemia; R, reperfusion. After assessment of cardiac function hearts were subjected to 30 min perfusion with collagenase containing oxygenated solution for the isolation of cardiomyocytes. The cell viability before purification in control or 10 to 20 min ischemic and I/R groups was 82 ± 3.5 and 79 ± 4.3%, respectively, whereas that in 30 min ischemic, 5 min reperfusion, 15 min reperfusion and 30 min reperfusion groups was 72 ± 1.9, 68 ± 2.7, 63 ± 2.4 and 60.2 ± 1.4%, respectively. *$p < 0.05$ vs. control.

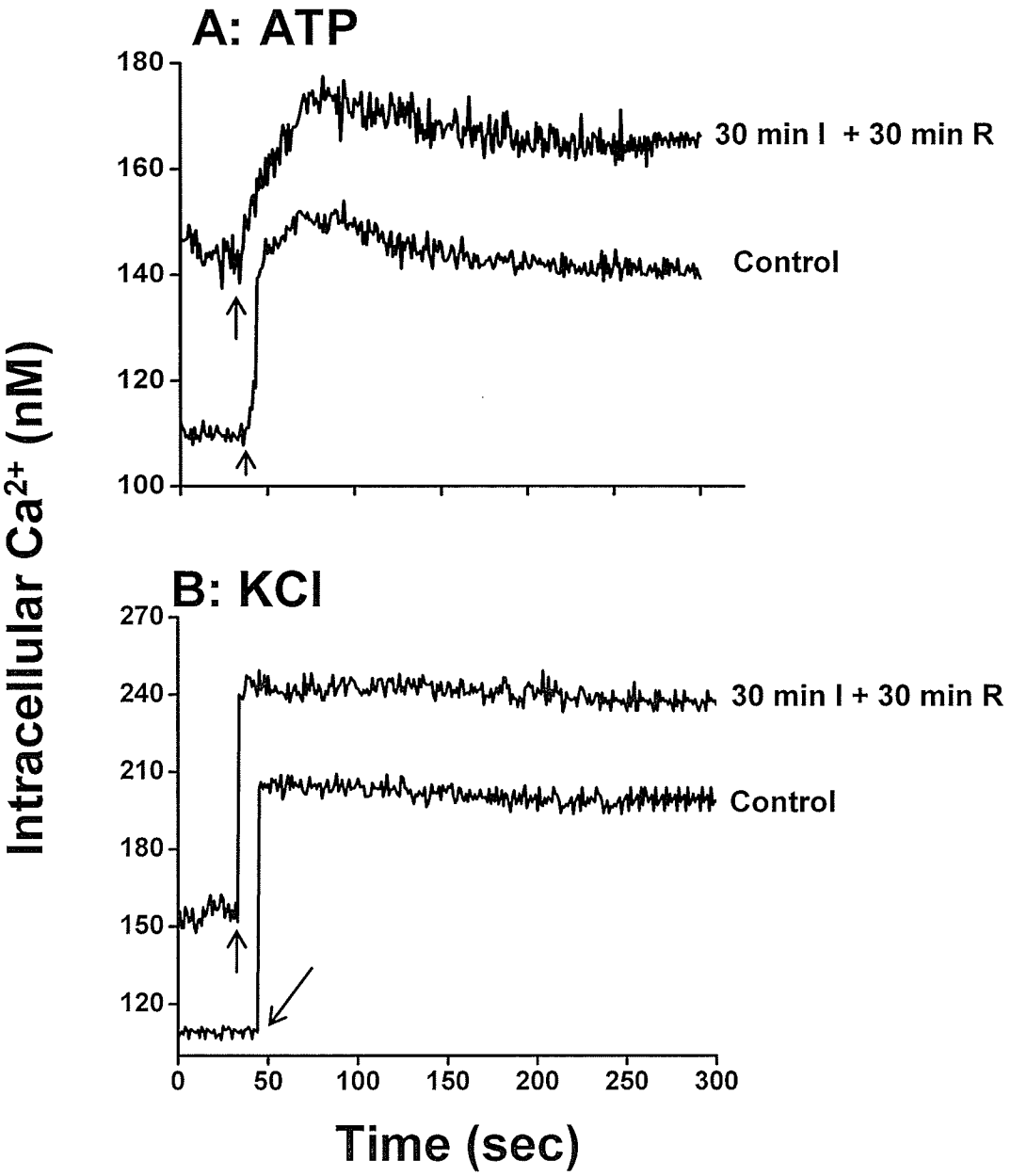


Figure 11. Representative tracings showing the increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from hearts subjected to 30 min ischemia and 30 min of reperfusion. ***Tracing A:*** ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from control and I/R hearts. ***Tracing B:*** KCl-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from control and I/R hearts. The increase in $[Ca^{2+}]_i$ was calculated as the difference between the peak value and the basal value in each experiment. ↑— indicates the time when the preparation was exposed to 50 μM ATP or 30 mM KCl.

in $[Ca^{2+}]_i$ was depressed in cardiomyocytes isolated from hearts undergoing 20 or 30 min of ischemia, unlike the 10 min ischemic hearts. This attenuation in ATP-induced increase in $[Ca^{2+}]_i$ in 20 min ischemic hearts was partially reversed by 30 min of reperfusion, whereas ATP responsiveness remained markedly depressed in 30 min ischemic hearts undergoing 5 or 15 min of reperfusion **(Table 7)**. Analysis of data from 10, 20 and 30 min ischemic-reperfused hearts **(Table 7)** revealed that changes in LVEDP were linearly related to alterations in ATP-induced changes in $[Ca^{2+}]_i$ (r^2=0.8737; p<0.0001) as well as basal $[Ca^{2+}]_i$ (r^2=0.8033; p<0.0001). It is also observed that ATP-induced increase in $[Ca^{2+}]_i$ was depressed at different concentrations of ATP (10 – 100 μM) in cardiomyocytes isolated from hearts undergoing 30 min of ischemia followed by 30 min of reperfusion **(Figure 12)**.

b. Effect of ISO on ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes

To examine the effect of I/R on catecholamine-mediated potentiation of ATP response, cardiomyocytes isolated from I/R hearts were treated with ISO (100 μM). As shown in **Figure 13**, ISO treatment caused an augmentation in ATP-induced increase in $[Ca^{2+}]_i$; this is in agreement with previous findings [251]. On the other hand, a significant depression in ISO-induced increase in $[Ca^{2+}]_i$ was observed in cardiomyocytes isolated from hearts undergoing 30 min of ischemia followed by 30 min of reperfusion **(Figure 13)**. It should be mentioned that ISO treatment did not have any effect on basal $[Ca^{2+}]_i$ in cardiomyocytes isolated from control and I/R hearts.

c. Effect of SOD plus CAT on ATP responsiveness in cardiomyocytes

To investigate the involvement of oxidative stress in I/R-mediated alterations in ATP-induced increase in $[Ca^{2+}]_i$, isolated hearts were treated with an antioxidant mixture containing SOD and catalase. The SOD plus CAT treatment caused a significant improvement in heart function in terms of increase in LVDP and decrease in LVEDP in hearts undergoing 30 min of ischemia

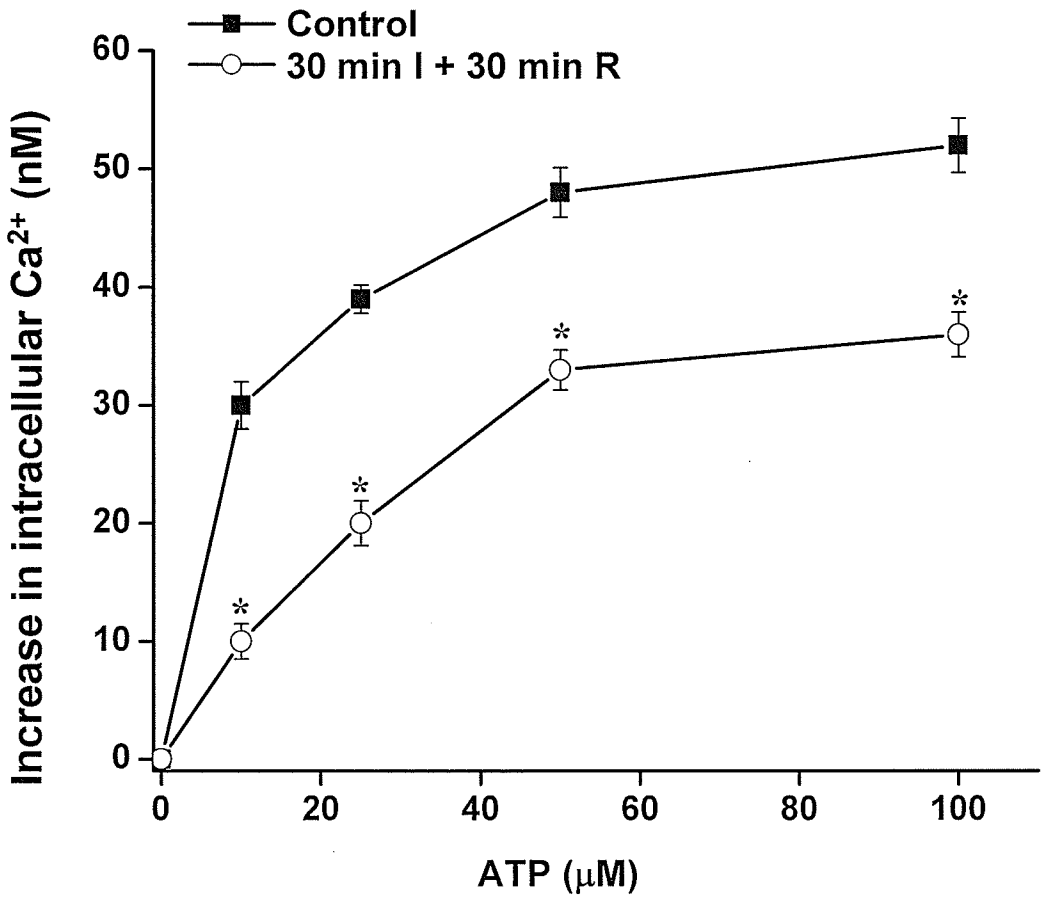


Figure 12. Effect of different concentrations of ATP (10 – 100 μM) on the increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from control and 30 min ischemic 30 min reperfused hearts. Each point represents mean ± SEM of 4 experiments in each group. *$p<0.05$ vs. control group.

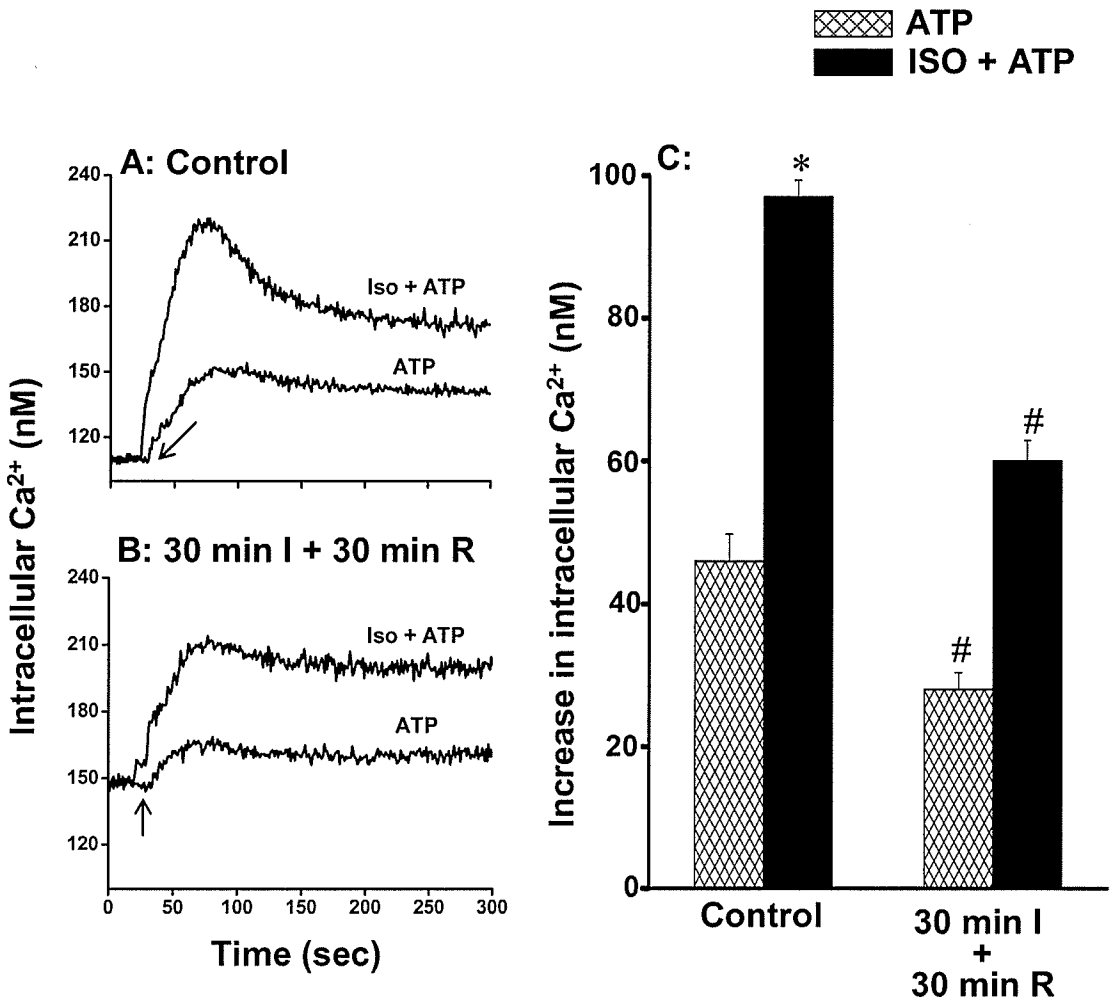


Figure 13. ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes, with or without isoproterenol (ISO) treatment, isolated from hearts subjected to 30 min ischemia followed by 30 min of reperfusion. ***Panel A:*** Representative tracings showing the ATP- and ISO-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from control hearts. ***Panel B:*** Representative tracings showing the ATP- and ISO-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from I/R hearts. ***Panel C:*** Effect of I/R on ISO-induced increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ was calculated as the difference between the peak value and the basal value in each experiment. ISO-induced increase was calculated by subtracting the values for ATP-induced increase in $[Ca^{2+}]_i$ in untreated cardiomyocytes from those treated with 100 μM ISO. ↑— indicates the time when the preparation was exposed to 50 μM ATP. Treatment with 100 μM ISO was carried out for 5 min prior to intracellular Ca^{2+} measurements. Each point represents mean ± SEM of 4 experiments in each group. *$p<0.05$ vs. ATP; #$p<0.05$ vs. control group.

followed by 30 min of reperfusion **(Table 8)**. In addition, I/R-induced increase in basal $[Ca^{2+}]_i$ was significantly reduced by SOD plus CAT treatment. Furthermore, the depression of ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from I/R hearts was markedly attenuated by the antioxidant mixture **(Table 8)**. From the data given in Table 8, it can be seen that treatment of SOD and catalase itself had no effect on LVDP, LVEDP and basal $[Ca^{2+}]_i$. Although SOD plus CAT treatment was observed to augment the ATP-induced increase in $[Ca^{2+}]_i$ in control and I/R cardiomyocytes **(Table 8)**, no attempt was made to understand the mechanisms of such an effect.

d. Effect of H_2O_2 on ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes

In order to test if the effects of I/R are mimicked by oxidative stress, isolated hearts were perfused with 50 μM H_2O_2 for 30 min. H_2O_2 caused a depression in LVDP, +dP/dt and –dP/dt as well as an elevation in LVEDP **(Table 9)**. A marked increase in basal $[Ca^{2+}]_i$ was observed, whereas ATP-induced increase in $[Ca^{2+}]_i$ was significantly depressed in cardiomyocytes isolated from H_2O_2 treated hearts **(Table 9)**. Similarly, ISO-induced increase in $[Ca^{2+}]_i$ was also depressed in H_2O_2 treated hearts.

e. Effect of ischemic preconditioning on ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes

To determine the significance of I/R-mediated changes in ATP-induced increase in $[Ca^{2+}]_i$ at the level of isolated cardiomyocytes, we examined the effect of ischemic preconditioning, which is known to prevent the I/R-induced changes in cardiac performance (36). Although ischemic preconditioning caused a partial depression in cardiac performance in control hearts, the I/R-induced alterations in cardiac function were improved by ischemic preconditioning **(Table 10)**. Attenuation of basal $[Ca^{2+}]_i$ and a marked improvement in ATP- induced increase in $[Ca^{2+}]_i$ were observed in cardiomyocytes isolated from I/R hearts subjected to ischemic preconditioning **(Table 10).** The basal $[Ca^{2+}]_i$ and ATP-induced increase in $[Ca^{2+}]_i$ did not change in

Table 8: Various parameters of isolated rat hearts treated with superoxide dismutase and catalase 10 min before ischemia and during reperfusion period

Group	LVDP (mm Hg)	LVEDP (mm Hg)	Basal $[Ca^{2+}]_i$	ATP-induced increase in $[Ca^{2+}]_i$
Control	129.3 ± 11.5	9.7 ± 3.1	110 ± 3.5	45 ± 3.9*
SOD + CAT	132.0 ± 9.7	10.8 ± 2.5	115 ± 4.4	95 ± 8.2*
30 min I + 30 min R	32.1 ± 3.3*	72.3 ± 6.9*	151 ± 3.6*	27 ± 5.3*
SOD + CAT + 30 min I + 30 min R	72.3 ± 8.5†	40.0 ± 4.7†	132 ± 1.8†	92 ± 7.4†

Values are mean ± SEM of 4 hearts in each group. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; I, ischemia; R, reperfusion. After assessment of cardiac function hearts were subjected to 30 min perfusion with collagenase containing oxygenated solution for the isolation of cardiomyocytes. The cell viability before purification in control, SOD + CAT, untreated I/R and treated I/R was 85 ± 4.2, 83 ± 5.7, 59 ± 2.5 and 68 ± 2.1%, respectively. * $p < 0.05$ vs. control; † $p < 0.05$ vs. 30 min ischemia and 30 min reperfusion group.

Table 9: Various parameters of isolated rat hearts treated with H_2O_2 for 30 min.

Parameter	Control	H_2O_2
LVDP (mm Hg)	129.8 ± 12.3	43.3 ± 4.7*
LVEDP (mm Hg)	10.1 ± 2.2	37.9 ± 5.4*
+dP/dt (mm Hg/s)	6200 ± 495	2490 ± 110*
-dP/dt (mm Hg/s)	4312 ± 317	1250 ± 130*
Basal $[Ca^{2+}]_i$	115 ± 4.9	150 ± 4.7*
ATP-induced increase in $[Ca^{2+}]_i$	47 ± 5.3	30 ± 1.5*
ISO-induced increase in $[Ca^{2+}]_i$	55 ± 3.7	29 ± 3.3*

Values are mean ± SEM of 4 hearts in each group. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of ventricular pressure development; -dP/dt, rate of ventricular pressure decline; ISO, isoproterenol. Cardiomyocytes were isolated from after 30 min perfusion with collagenase containing oxygenated solution. The cell viability before purification in control and H_2O_2 groups was 81 ± 3.6 and 54 ± 3.3%, respectively. *$p < 0.05$ vs. control.

Table 10: Various parameters of isolated hearts subjected to ischemic preconditioning before the induction of ischemia and reperfusion.

Group	LVDP (mm Hg)	LVEDP (mm Hg)	Basal $[Ca^{2+}]_i$	ATP-induced increase in $[Ca^{2+}]_i$
Control	127.0 ± 12.7	8.6 ± 4.0	108 ± 5.5	48 ± 6.9
IP	99.3 ± 7.8	9.9 ± 3.1	107 ± 4.2	50 ± 5.2*
30 min I + 30 min R	33.4 ± 3.6*	80.7 ± 4.9*	155 ± 3.6*	25 ± 5.1*
IP + 30 min I+ 30 min R	102.6 ± 5.3†	26.4 ± 10.6†	114 ± 3.2†	52 ± 3.8†

Values are mean ± SEM of 4 hearts in each group. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; I, ischemia; R, reperfusion; IP, ischemic preconditioning. After assessment of cardiac function hearts were subjected to 30 min perfusion with collagenase containing oxygenated solution for the isolation of cardiomyocytes. The cell viability before purification in control, IP, untreated I/R and treated I/R was 83 ± 2.9, 80 ± 3.9, 57 ± 3.6 and 79 ± 3.3%, respectively. * $p < 0.05$ vs. control; † $p < 0.05$ vs. 30 min ischemia and 30 min reperfusion group.

cardiomyocytes isolated from ischemic preconditioned hearts in comparison to control hearts **(Table 10).**

f. Inotropic effect of ATP in I/R hearts

In order to investigate the relationship between reduced responsiveness of ATP in cardiomyocytes isolated from I/R hearts and the response of ATP in I/R hearts, ATP (50 μM) was infused in the hearts subjected to 30 min of ischemia followed by 30 min of reperfusion. Representative tracings showing the positive inotropic effect of ATP in control and I/R hearts are given in **Figure 14.** The positive inotropic effect of ATP in control hearts in terms of increase in LVDP, +dP/dt, and –dP/dt was 33.4 ± 1.2, 36.7 ± 1.9 and 38.1 ± 2.1% of the respective basal values, respectively. On the other hand, in hearts undergoing 30 min ischemia followed by 30 min reperfusion, ATP-induced increase in these parameters (LVDP, 11.5 ± 0.9%, +dP/dt, 8.7 ± 1.4%, and –dP/dt 9.2 ± 1.7% of the respective basal values) was significantly depressed as compared to control hearts. No significant difference was observed in LVEDP after ATP infusion in both the groups (3.1 ± 1.7 and 2.4 ± 1.3% for control and I/R hearts, respectively).

g. Mechanisms of I/R-induced alterations in cardiomyocytes

In order to assess if the attenuation of ATP-induced increase in $[Ca^{2+}]_i$ in I/R cardiomyocytes was due to a defect at the purinergic receptor level, the specific binding of [^{35}S]ATPγS, a slowly hydrolysable analogue of ATP [277], to cardiac membranes isolated from ischemic and I/R hearts, was determined. As reported earlier [277], ATP was found to bind cardiac membranes at high and low binding sites. Although the affinity ($1/K_d$) of purinergic receptors to ATP at both high affinity and low affinity binding sites was increased in 30 min ischemic as well as 30 min ischemia followed by 30 min reperfused hearts, a significant depression in maximal number of purinergic receptors (B_{max}) at both sites was observed in these hearts **(Table 11).**

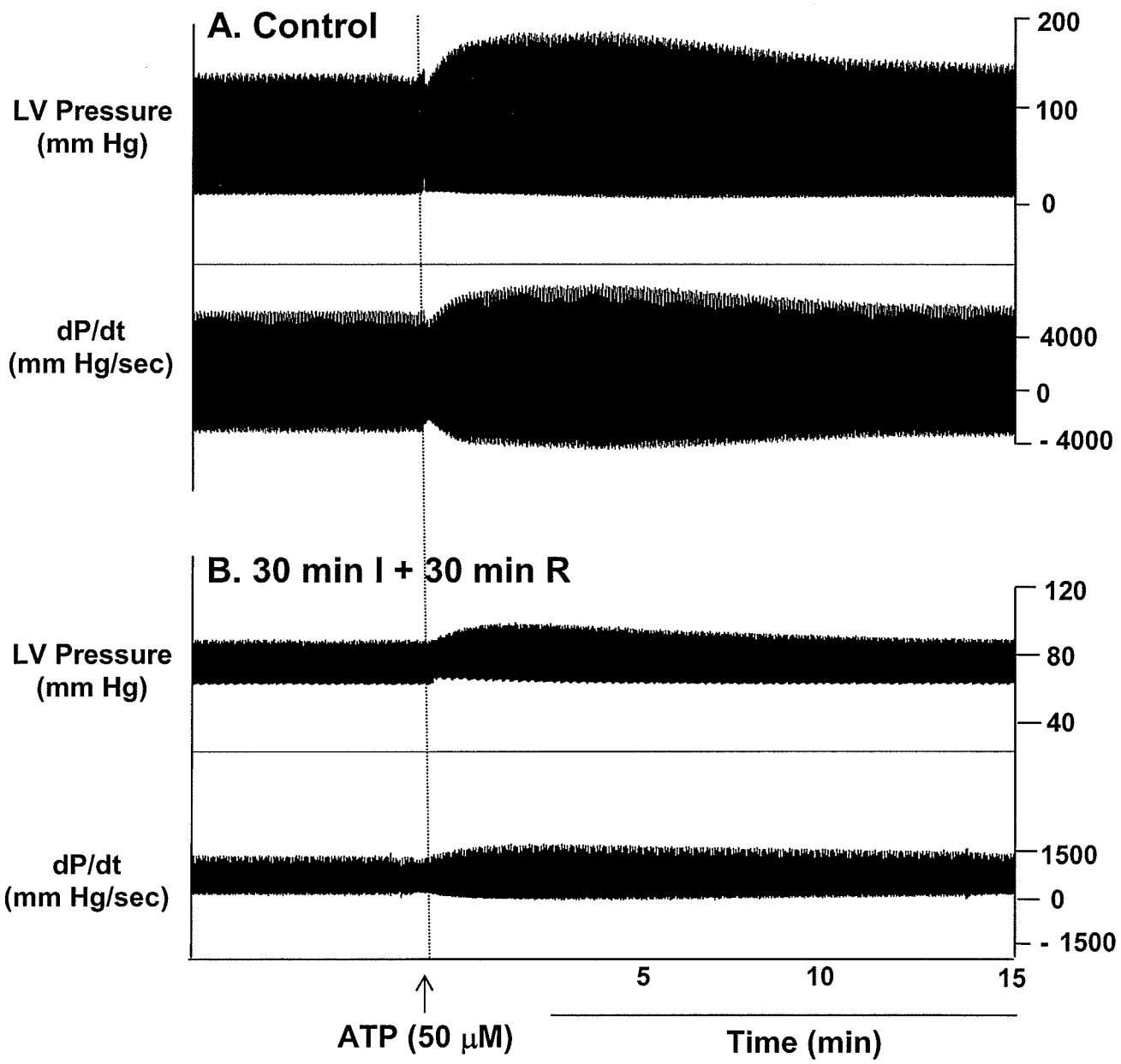


Figure 14. Representative tracings showing the effect of ATP on left ventricular (LV) pressure and change of pressure development (dP/dt) in isolated hearts. ***Tracings A.*** Effect of ATP on cardiac function in control perfused hearts. ***Tracings B.*** Effect of ATP on cardiac function in hearts subjected to 30 min ischemia and 30 min reperfusion. ↑— indicates the time when the hearts were perfused with 50 μM ATP.

To determine the mechanism of depression of ATP responsiveness at cellular level, cardiomyocytes isolated from control and 30 min ischemic and 30 min reperfused hearts were incubated with verapamil, an L-type Ca^{2+} channel blocker in SL membrane [266], cibacrone blue, an ATP receptor blocker [255], or ryanodine, an agent which prevents the release of Ca^{2+} from SR by opening the ryanodine receptor channel to subconductance level or by locking the channel [282,283]. Treatments with different concentrations of verapamil (1 and 10 μM) and cibacrone blue (50 and 100 μM) resulted in a significant reduction of ATP-induced increase in $[Ca^{2+}]_i$ in both control and I/R hearts **(Table 12)**. On the other hand, ryanodine (2 and 10 μM) treatment caused a significant decrease in ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from control perfused hearts but did not affect the ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from I/R hearts **(Table 12)**. None of these drugs altered the basal $[Ca^{2+}]_i$ in cardiomyocytes isolated from both control and I/R hearts.

h. Effect of hypoxia-reoxygenation on ATP-induced increase in $[Ca^{2+}]_i$

To eliminate the possibility of any artifact caused by cell damage during the isolation process, cardiomyocytes isolated from control hearts were subjected to 30 min hypoxia (in the absence of glucose) followed by 30 min reoxygenation. The results in **Table 13** show that the yield and viability of cardiomyocytes were decreased after hypoxia-reoxygenation; however, an increase in basal $[Ca^{2+}]_i$ was observed in cardiomyocytes after hypoxia-reoxygenation. Extracellular ATP (50 μM) caused a significant increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from control hearts; this increase in $[Ca^{2+}]_i$ by ATP was significantly depressed in purified hypoxic-reoxygenated cardiomyocytes **(Table 13)**. The depression in ATP-induced increase in $[Ca^{2+}]_i$ in hypoxic- reoxygenated cardiomyocytes may not be due to elevated level of basal

Table 11: Changes in ATP binding characteristics in crude membranes isolated from hearts undergoing 30 min ischemia and 30 min ischemia followed by 30 min of reperfusion.

	High affinity		Low affinity	
	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)
Control	33.1 ± 1.8	15.0 ± 1.9	4518 ± 278	686 ± 8.6
Ischemia	17.9 ± 1.3*	9.2 ± 1.8*	3400 ± 148*	591 ± 27.1*
Ischemia/Reperfusion	18.4 ± 3.6*	7.1 ± 0.4*	2363 ± 91*	498 ± 39.6*

Values are mean ± SEM of 3 experiments in each group. *$p < 0.05$ vs. respective control. Maximal binding (B_{max}) and dissociation constant (K_d) were determined from the Scatchard plot for the specific ATP binding.

Table 12: Effect of various pharmacological agents on ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from control and I/R hearts.

Group	Control $[Ca^{2+}]_i$ (nM)		I/R-induced increase in $[Ca^{2+}]_i$ (nM)	
	Basal	**ATP-Induced**	**Basal**	**ATP-Induced**
Without drug	110 ± 3.8	48 ± 3.4	153 ± 4.7	30 ± 3.3
Cibacron blue (50 μM)	109 ± 3.6	20 ± 2.3*	154 ± 2.3	18 ± 1.7*
Cibacron blue (100 μM)	110 ± 2.5	8 ± 3.5*	153 ± 3.6	9 ± 0.8*
Verapamil (1 μM)	114 ± 5.1	30 ± 1.9*	150 ± 2.9	19 ± 1.7*
Verapamil (10 μM)	107 ± 6.8	22 ± 2.3*	152 ± 3.3	14 ± 2.8*
Ryanodine (2 μM)	110 ± 4.4	39 ± 1.8*	152 ± 4.8	28 ± 2.3
Ryanodine (10 μM)	115 ± 5.2	32 ± 2.7*	150 ± 3.7	25 ± 3.1

Values are mean ± SEM of 4 hearts in each group. I, ischemia; R, reperfusion. After 30 min of ischemia and 30 min of reperfusion, hearts were subjected to 30 min perfusion with collagenase containing oxygenated solution for the isolation of cardiomyocytes. *$p < 0.05$ vs. without drug. The concentration of ATP was 50 μM.

Table 13: Various parameters of control cardiomyocytes subjected to 30 min hypoxia followed by 30 min of reoxygenation at room temperature.

Parameter	Control	Hypoxia-Reoxygenation
Basal $[Ca^{2+}]_i$ (nM)	112 ± 5.8	142 ± 6.3*
ATP-induced increase in $[Ca^{2+}]_i$ (nM)	48 ± 2.3	35 ± 1.1*
ISO-induced increase in $[Ca^{2+}]_i$ (nM)	59 ± 6.6	15 ± 3.8*
KCl-induced increase in $[Ca^{2+}]_i$ (nM)	120 ± 7.9	125 ± 8.6

Values are mean ± SEM of 4 hearts in each group. Cardiomyocytes were isolated from control hearts and then subjected to hypoxia reoxygenation. The yield and viability of cells were determined before purification. The isoproterenol (ISO) induced increase was calculated as the difference between values for ISO + ATP and ATP. The cell viability of control and experimental preparations was 76 ± 3.1 and 5.5 ± 4.4%, whereas the yield of cardiomyoctyes (millions per heart) was 6.8 ± 1.9 and 2.9 ± 0.2, respectively. *$p < 0.05$ vs. control.

$[Ca^{2+}]_i$ because the increase in $[Ca^{2+}]_i$ by KCl (30 mM) was not altered under similar conditions. On the other hand, ISO-induced increase in $[Ca^{2+}]_i$ was significantly depressed in hypoxic-reoxygenated cardiomyocytes **(Table 13).**

3. Mechanisms of ATP- and catecholamine-induced changes in Ca^{2+}-handling by cardiomyocytes

a. Effect of β-AR stimulation on ATP-mediated intracellular Ca^{2+}-mobilization

To examine the effect of β-AR activation on ATP-induced Ca^{2+}-mobilization, isolated cardiomyocytes were treated with ISO prior to the addition of ATP (50 μM). Representative tracings showing the effects of ATP and ISO on $[Ca^{2+}]_i$ are shown in **Figure 15**. ATP caused a significant (43 ± 4.2%) increase in $[Ca^{2+}]_i$, this increase in $[Ca^{2+}]_i$ by ATP was further augmented by 55 ± 5.7% upon ISO treatment. It is pointed out that ISO-induced potentiation of ATP response, as shown in **Figure 15**, was completely blocked by a β-AR antagonist, propranolol (50 μM). It can also be seen from **Figure 15** that ISO and propranolol treatments had no effect on basal $[Ca^{2+}]_i$. Preliminary experiments in the cardiomyocytes treated with different concentrations of ISO (10, 25, 50 and 100 μM) revealed a concentration dependent effect on the potentiation of ATP response; however, the maximal effect of ISO treatment was seen at 100 μM under in vitro experimental conditions employed in this study.

b. Effects of L-type Ca^{2+} channels and SL Ca^{2+} -pump ATPase inhibitors on catecholamine-or ATP-induced increase in $[Ca^{2+}]_i$

In order to investigate the involvement of L-type Ca^{2+} channels in catecholamine- or ATP-mediated increase in $[Ca^{2+}]_i$, isolated cardiomyocytes were treated with verapamil and diltiazem, well known L-type Ca^{2+} channel blockers [266]. Both verapamil (1 and 10 μM) and diltiazem (1

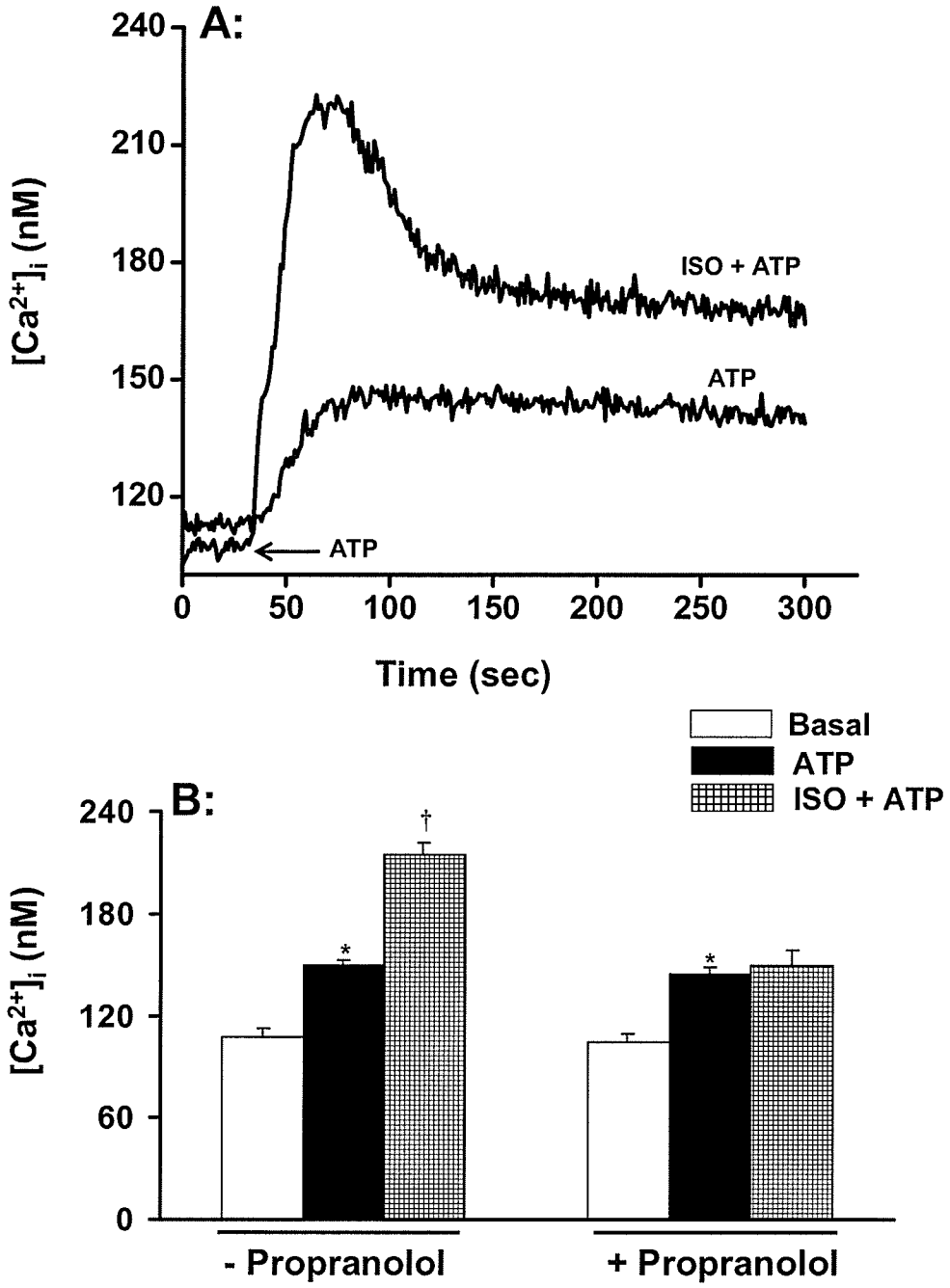


Figure 15. Isoproterenol (ISO)- or ATP-induced alteration in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated cardiomyocytes. **Panel A:** Representative tracings showing the effect of ATP and ISO on $[Ca^{2+}]_i$ in isolated cardiomyocytes. **Panel B:** Effect of propranolol (50 μM) on ISO- and ATP-induced increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes. ↑— indicates the time when the preparation was exposed to 50 μM ATP. Treatment with 100 μM ISO was carried out for 5 min prior to intracellular Ca^{2+} measurements. Each point represents mean ± SEM of 4 experiments in each group. *$p<0.05$ vs. control group; †$p<0.05$ vs. ATP.

10 μM) caused a significant attenuation of the ATP- or the ISO-induced increase in $[Ca^{2+}]_i$ **(Table 14)**. On the other hand, basal $[Ca^{2+}]_i$ remained unaltered in the presence of these agents **(Table 14)**. The participation of SL Ca^{2+}-pump ATPase in catecholamine-or ATP-mediated increase in $[Ca^{2+}]_i$ was determined by treating the isolated cardiomyocytes with low concentrations of vanadate (1 and 2 μM), which is known to inhibit SL Ca^{2+}-pump ATPase [284]. Vanadate caused a significant depression in ISO-induced increase in $[Ca^{2+}]_i$ whereas the ATP-mediated increase in $[Ca^{2+}]_i$ was augmented **(Figure 16)**. The results in **Figure 16** indicate that basal $[Ca^{2+}]_i$ was not affected by vanadate treatment .

c. Role of intracellular Ca^{2+}-stores in catecholamine- or ATP-induced increase in $[Ca^{2+}]_i$.

To test the contribution of intracellular Ca^{2+}-stores in ISO- or ATP-induced increase in $[Ca^{2+}]_i$, isolated cardiomyocytes were treated with ryanodine, an agent that prevents the release of Ca^{2+} from SR by opening the Ca^{2+} release channel to subconductance level or by locking the channel [282,283] and CPA, a well known inhibitor of SR Ca^{2+}-pump ATPase [285]. Both ryanodine (2 and 4 μM) and CPA (20 and 40 μM) were observed to decrease the ATP-induced increase in $[Ca^{2+}]_i$, whereas the ISO-mediated increase in $[Ca^{2+}]_i$ remained unaltered by these agents **(Table 15).** Basal $[Ca^{2+}]_i$ was not changed by treatment with these agents **(Table 15)**. It is pointed out that the ATP-induced increase in control cardiomyocytes in this group was 21 ± 3.4% higher than that of other experimental groups **(Table 15)**. Incubation of cells for 20 min under control conditions required for experiments involving 20 min treatment with ryanodine or CPA instead of 10 min appears to be the reason for an increase in the sensitivity of control cardiomyocytes for ATP.

d. Effects of Na^+-Ca^{2+} exchange inhibitors on catecholamine- or ATP-mediated increase in $[Ca^{2+}]_i$

In order to examine the role of Na^+-Ca^{2+} exchanger in ISO-or ATP-mediated increase in $[Ca^{2+}]_i$,

Table 14: Effect of L-type Ca^{2+} channel blockers on isoproterenol-mediated potentiation of ATP-induced increase in $[Ca^{2+}]_i$

Group	Basal $[Ca^{2+}]_i$ (nM)	ATP-induced increase in $[Ca^{2+}]_i$ (nM)		ISO-induced increase in $[Ca^{2+}]_i$ (nM)
		(-) ISO	(+) ISO	
Without drug	112 ± 5.2	45 ± 2.3	92 ± 9.1*	52 ± 4.9
Verapamil (1 μM)	105 ± 7.0	30 ± 2.5†	50 ± 5.2*	22 ± 3.7$^{\#}$
Verapamil (10 μM)	113 ± 3.5	21 ± 2.9†	35 ± 3.7*	12 ± 2.3$^{\#}$
Diltiazem (1 μM)	115 ± 4.3	32 ± 2.0†	69 ± 3.8*	37 ± 1.8$^{\#}$
Diltiazem (10 μM)	110 ± 3.8	25 ± 3.3†	51 ± 2.1*	26 ± 2.7$^{\#}$

Values are mean ± SEM of 4 hearts in each group. Cardiomyocytes were incubated with different concentrations of verapamil and diltiazem for 10 min including 5 min incubation with 100 μM isoproterenol (ISO) before 50 μM ATP. $^{\dagger}p< 0.05$ vs. without drug in the presence of ATP; $^{*}p< 0.05$ vs. without ISO in the presence of ATP and $^{\#}p< 0.05$ vs. without drug in the presence of ISO.

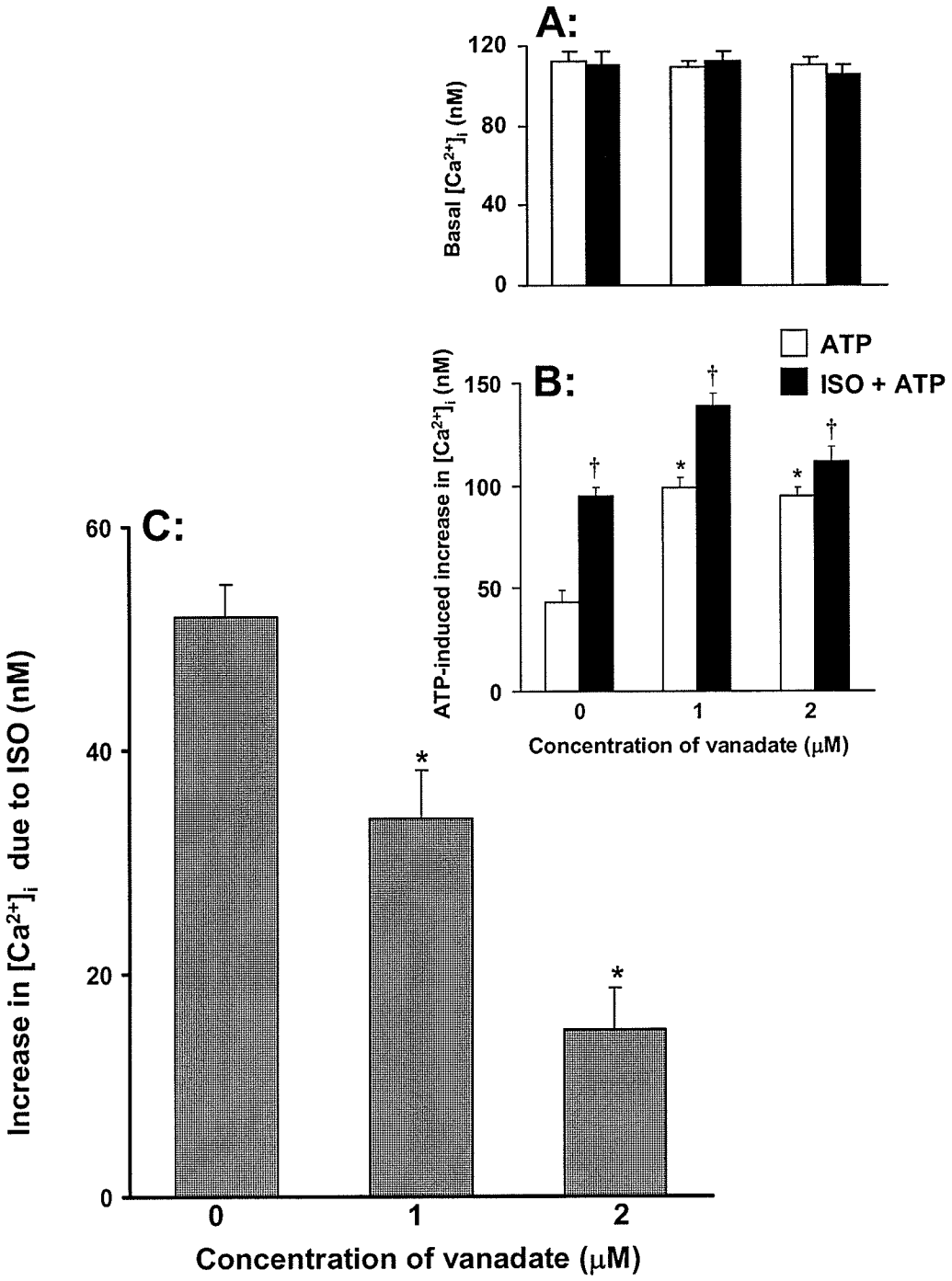


Figure 16. Effect of different concentrations of vanadate on isoproterenol (ISO)-or ATP-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated cardiomyocytes. **Panel A:** Effect of different concentrations of vanadate on basal $[Ca^{2+}]_i$. **Panel B:** Effect of different concentrations of vanadate on ATP-induced increase in $[Ca^{2+}]_i$. **Panel C:** Effect of different concentrations of vanadate on ISO-induced increase in $[Ca^{2+}]_i$. ISO-induced increase in $[Ca^{2+}]_i$ is a difference between ATP-induced increase in the presence and absence of ISO. Each point represents mean ± SEM of 4 experiments in each group. $^{\dagger}p<0.05$ vs. ATP; $^{*}p<0.05$ vs. in the absence of vanadate

Table 15: Effect of sarcoplasmic reticulum Ca^{2+} store depletion on isoproterenol-mediated potentiation of ATP-induced increase in $[Ca^{2+}]_i$

Group	Basal $[Ca^{2+}]_i$ (nM)	ATP-induced increase in $[Ca^{2+}]_i$ (nM)		ISO-induced increase in $[Ca^{2+}]_i$ (nM)
		(-) ISO	(+) ISO	
Without drug	107 ± 4.3	74 ± 3.2	120 ± 2.5*	48 ± 5.7
Ryanodine (2 μM)	108 ± 7.7	62 ± 1.7†	107 ± 3.8*	45 ± 3.8
Ryanodine (4 μM)	113 ± 6.4	60 ± 3.0†	100 ± 3.5*	40 ± 8.8
CPA (20 μM)	110 ± 4.7	55 ± 2.0†	96 ± 2.2*	43 ± 4.7
CPA (40 μM)	108 ± 6.9	46 ± 3.0†	83 ± 4.7*	38 ± 6.2

Values are mean ± SEM of 4 hearts in each group. Cardiomyocytes were incubated with different concentrations of ryanodine and cyclopiazonic acid (CPA) for 20 min including 5 min incubation with 100 μM isoproterenol (ISO) before 50 μM ATP. †$p < 0.05$ vs. without drug in the presence of ATP and *$p < 0.05$ vs. without ISO in the presence of ATP.

cardiomyocytes were treated with amiloride and Ni^{2+}, inhibitors of Na^{+}-Ca^{2+} exchanger [286,287]. Results in **Figures 17 and 18** indicate that both ATP- and ISO-mediated increase in $[Ca^{2+}]_i$ were significantly attenuated by these treatments. Basal $[Ca^{2+}]_i$ remained unaltered in cardiomyocytes treated with amiloride, whereas a significant decrease in basal $[Ca^{2+}]_i$ was observed in Ni^{2+} treated cells **(Figures 17 and 18)**. Such a decrease in basal $[Ca^{2+}]_i$ by Ni^{2+} seems to be due to some non-specific effect of this agent because no such depression was seen by other inhibitors of Na^{+}-Ca^{2+} exchanger. No attempt was made to understand the reason for such an effect of Ni^{2+} on basal $[Ca^{2+}]_i$. Since both amiloride and Ni^{2+} are non-specific inhibitors of Na^{+}-Ca^{2+} exchanger [288,289], the cells were also treated with KB-R7943, a moderately specific inhibitor of Ca^{2+} entry mode of Na^{+}-Ca^{2+} exchanger [271] to confirm the involvement of Na^{+}-Ca^{2+} exchanger in the ISO-mediated increase in $[Ca^{2+}]_i$. KB-R7943 (25 and 50 μM) caused a significant depression in both ATP- and ISO-mediated increase in $[Ca^{2+}]_i$ without having any effect on basal $[Ca^{2+}]_i$ **(Figure 19)**. To further reveal the participation of Na^{+}-Ca^{2+} exchanger in ISO- or ATP-mediated increase in $[Ca^{2+}]_i$, cardiomyocytes were treated with KB-R7943 (50 μM) in the presence of different concentrations of verapamil (0.25, 0.5, 1 μM). Unlike ATP-induced increase in $[Ca^{2+}]_i$, an additive inhibitory effect on ISO-mediated increase in $[Ca^{2+}]_i$ was observed after treatment with a combination of KB-R7943 and verapamil **(Table 16)**.

e. Involvement of Na^{+}-Ca^{2+} exchanger in catecholamine- or ATP-induced increase in $[Ca^{2+}]_i$.

In view of the fact that low Na^{+} has been shown to cause an increase in $[Ca^{2+}]_i$ by activation of Na^{+}-Ca^{2+} exchanger [290,291], the cells were exposed to 30 mM KCl in the presence of low Na^{+} (35 mM). It can be seen from **Figure 20** that the KCl-mediated increase was further augmented in the presence of low Na^{+}; this finding is in agreement with our previous observations [266]. In addition, the ISO-induced increase in $[Ca^{2+}]_i$ in KCl-depolarized cardiomyocytes was potentiated

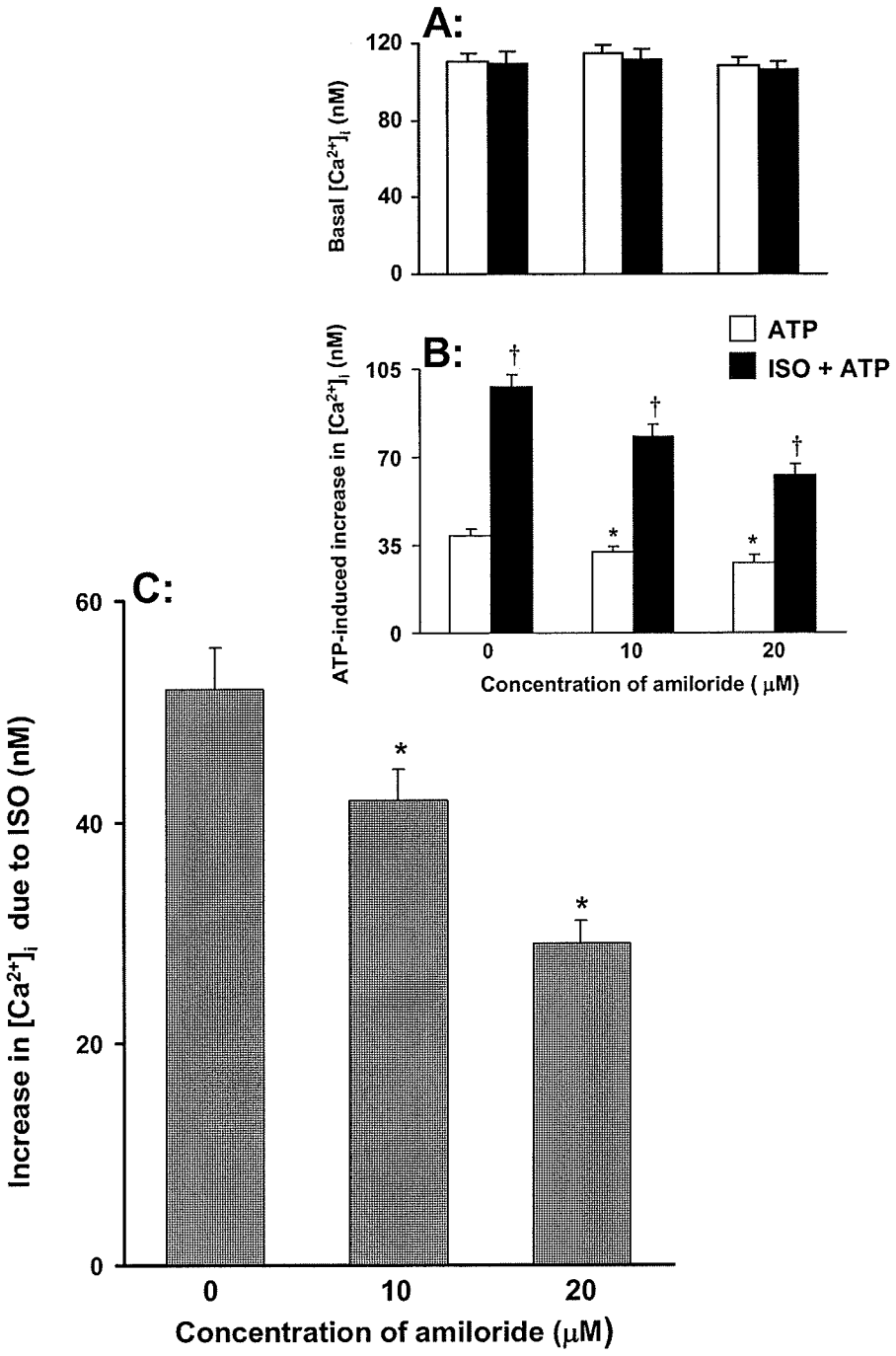


Figure 17. Effect of different concentrations of amiloride on isoproterenol (ISO)-or ATP- induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated cardiomyocytes. **Panel A:** Effect of different concentrations of amiloride on basal $[Ca^{2+}]_i$. **Panel B:** Effect of different concentrations of amiloride on ATP-induced increase in $[Ca^{2+}]_i$. **Panel C:** Effect of different concentrations of amiloride on ISO-induced increase in $[Ca^{2+}]_i$. ISO-induced increase in $[Ca^{2+}]_i$ is a difference between ATP-induced increase in the presence and absence of ISO. Each point represents mean ± SEM of 4 experiments in each group. $^{\dagger}p<0.05$ vs. ATP; $^{*}p<0.05$ vs. in the absence of amiloride.

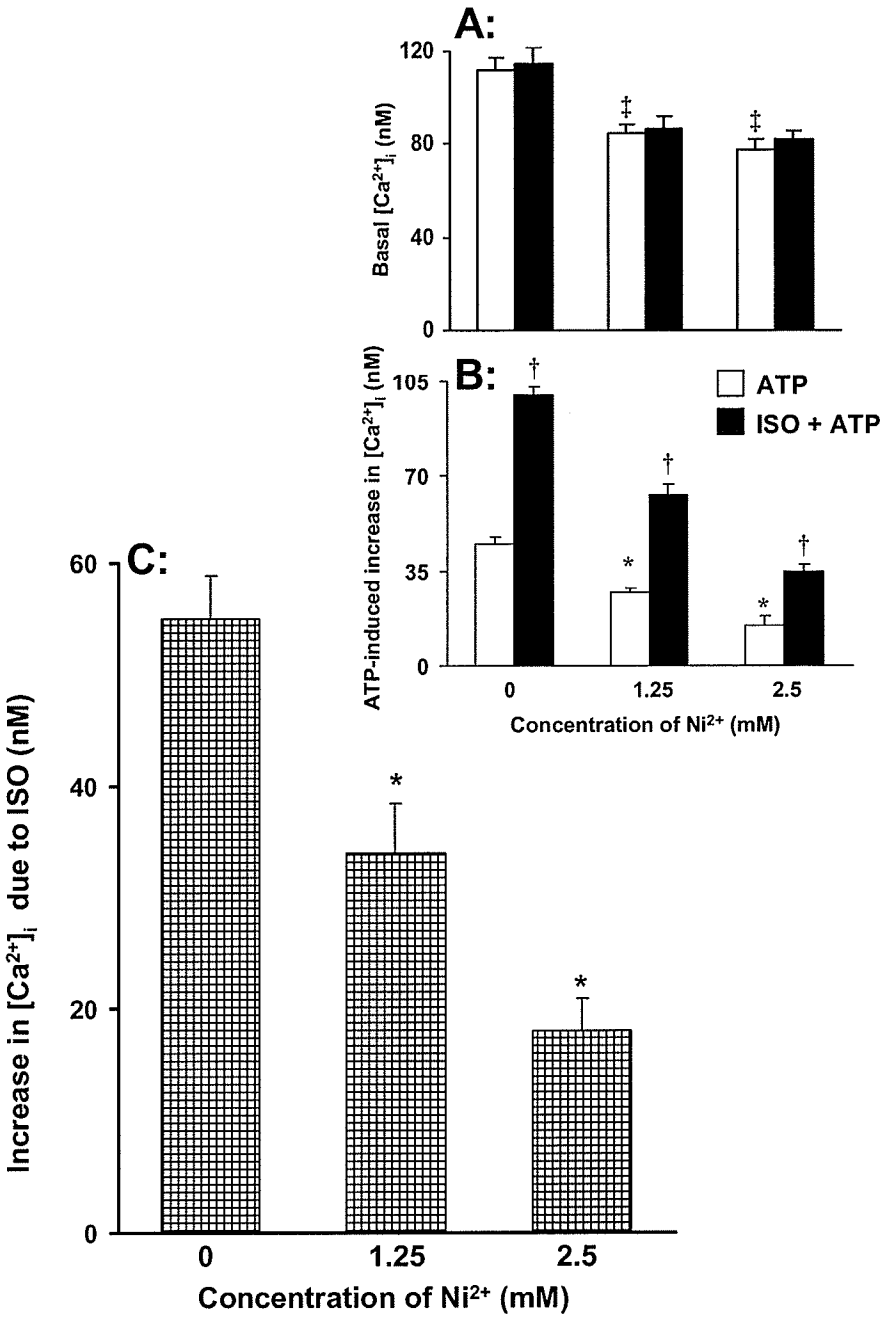


Figure 18. Effect of different concentrations of Ni^{2+} on isoproterenol (ISO)-or ATP-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated cardiomyocytes. **Panel A:** Effect of different concentrations of Ni^{2+} on basal $[Ca^{2+}]_i$. **Panel B:** Effect of different concentrations of Ni^{2+} on ATP-induced increase in $[Ca^{2+}]_i$. **Panel C:** Effect of different concentrations of Ni^{2+} on ISO-induced increase in $[Ca^{2+}]_i$. ISO-induced increase in $[Ca^{2+}]_i$ is a difference between ATP-induced increase in the presence and absence of ISO. Each point represents mean ± SEM of 4 experiments in each group. $^{\dagger}p<0.05$ vs. ATP; $^{*}p<0.05$ vs. in the absence of Ni^{2+}.

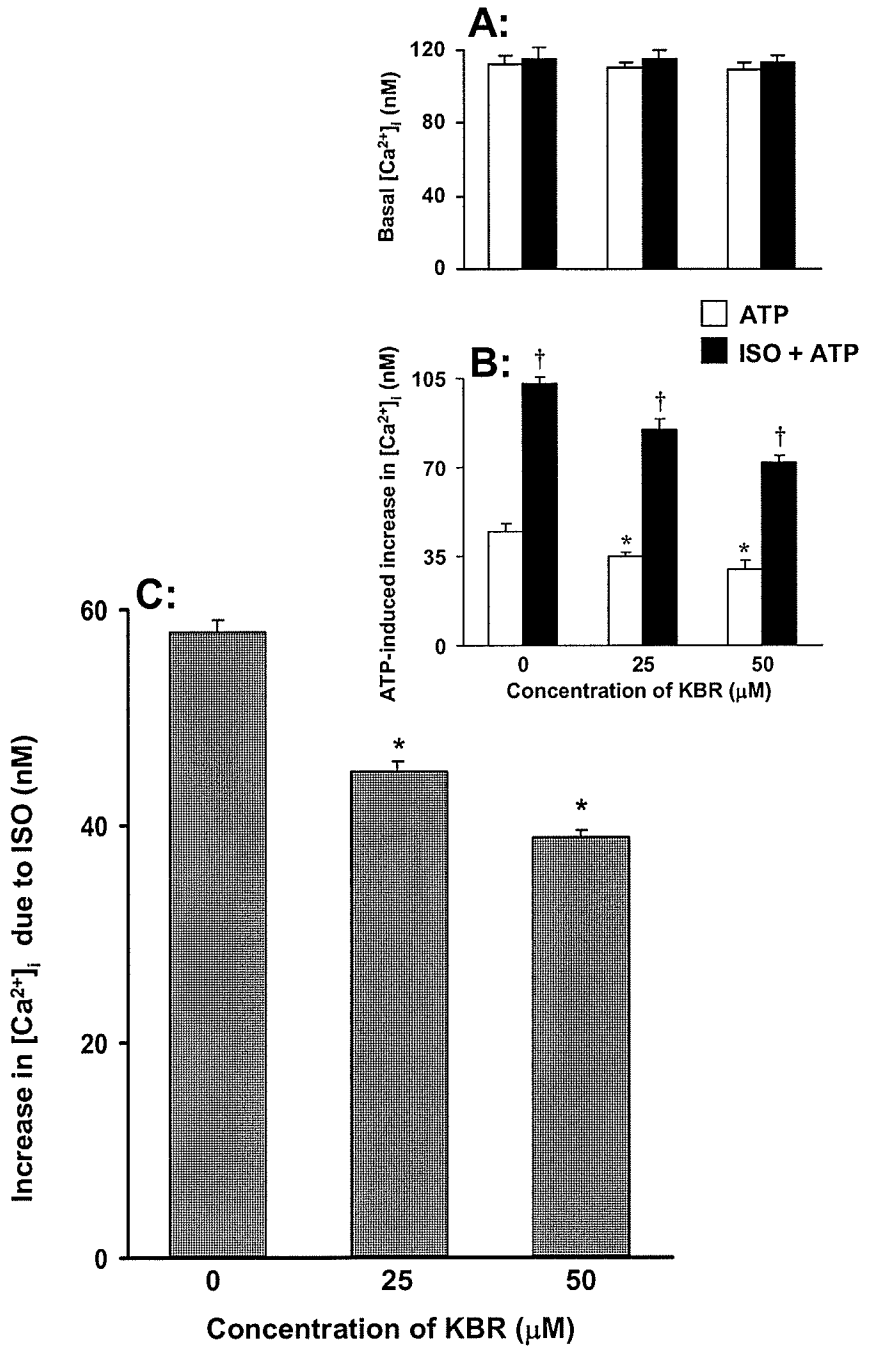


Figure 19. Effect of different concentrations of KB-R7943 (KBR) on isoproterenol (ISO)-or ATP-induced increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated cardiomyocytes. **Panel A:** Effect of different concentrations of KBR on basal $[Ca^{2+}]_i$. **Panel B:** Effect of different concentrations of KBR on ATP-induced increase in $[Ca^{2+}]_i$. **Panel C:** Effect of different concentrations of KBR on ISO-induced increase in $[Ca^{2+}]_i$. ISO-induced increase in $[Ca^{2+}]_i$ is a difference between ATP-induced increase in the presence and absence of ISO. Each point represents mean ± SEM of 4 experiments in each group. $^{\dagger}p<0.05$ vs. ATP; $^{*}p<0.05$ vs. in the absence of KBR.

Table 16: Effect of combination of verapamil and KB-R7943 on isoproterenol- or ATP-induced potentiation of ATP-induced increase in $[Ca^{2+}]_i$

Group	Basal $[Ca^{2+}]_i$ (nM)	ATP-induced increase in $[Ca^{2+}]_i$ (nM)		ISO-induced increase in $[Ca^{2+}]_i$ (nM)
		(-) ISO	(+) ISO	
Without drug	110 ± 7.1	47 ± 3.2	95 ± 7.8	53 ± 2.9
KB-R7943 (50 μM)	108 ± 4.9	37 ± 2.4*	73 ± 4.6*	37 ± 0.8*
Verapamil (0.25 μM)	114 ± 5.1	39 ± 1.2*	79 ± 2.3*	40 ± 1.4*
Verapamil (0.25 μM) + KB-R7943 (50 μM)	111 ± 3.6	38 ± 1.9	58 ± 2.1†	20 ± 1.5†
Verapamil (0.5 μM)	107 ± 5.8	35 ± 1.3*	71 ± 1.0*	34 ± 2.1*
Verapamil (0.5 μM) + KB-R7943 (50 μM)	110 ± 6.3	37 ± 1.4	56 ± 4.1†	16 ± 2.5†
Verapamil (1 μM)	109 ± 4.6	29 ± 2.3*	55 ± 2.9*	25 ± 3.1*
Verapamil (1 μM) + KB-R7943 (50 μM)	113 ± 5.3	33 ± 1.8	47 ± 4.5#	12 ± 1.6†

Values are mean ± SEM of 4 hearts in each group except from 12 hearts each, from without drug and KB-R7943 groups. Cardiomyocytes were incubated with KB-R7943 and different concentrations of verapamil for 10 min including 5 min incubation with 100 μM isoproterenol (ISO) before 50 μM ATP. *$p < 0.05$ vs. respective values of without drug group; †$p<0.05$ vs. respective values of KB-R7943 or verapamil groups; # $p<0.05$ vs. KB-R7943 group in the presence of ISO.

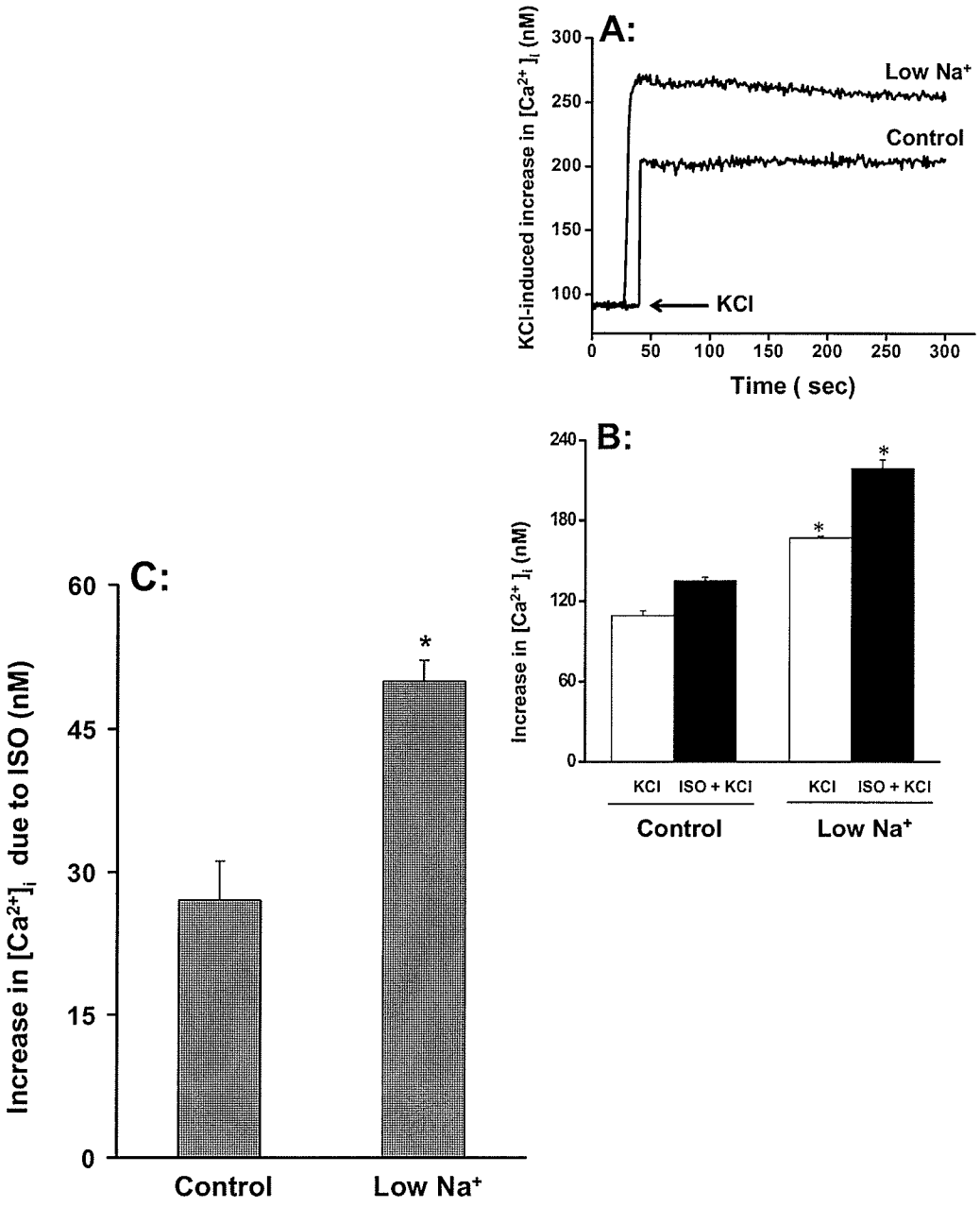


Figure 20. Effect of low Na^+ on isoproterenol (ISO)-induced alteration in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated cardiomyocytes. **Panel A:** Representative tracings showing the effect of low Na^+ on KCl-induced increase in $[Ca^{2+}]_i$. **Panel B:** Effect of different concentrations of Na^+ [90 mM (control) and 35 mM (low Na^+)] on KCl-induced increase in $[Ca^{2+}]_i$. **Panel C:** Effect of low Na^+ on ISO-induced increase in $[Ca^{2+}]_i$ in presence of KCl. ISO-induced increase in $[Ca^{2+}]_i$ is a difference between KCl-induced increase in the presence and absence of ISO. Each point represents mean ± SEM of 4 experiments in each group. $^*p<0.05$ vs. respective control value.

by low Na^+ (35 mM). It can be seen from **Figure 20** that the KCl-mediated increase was further augmented in the presence of low Na^+; this finding is in agreement with our previous observations [266]. In addition, the ISO-induced increase in $[Ca^{2+}]_i$ in KCl-depolarized cardiomyocytes was potentiated by low Na^+ **(Figure 20)**; this increase in $[Ca^{2+}]_i$ by ISO in KCl-depolarized cardiomyocytes was significantly depressed by different concentrations of KB-R7943 without any alteration in basal $[Ca^{2+}]_i$ **(Table 17)**.

f. Modification of positive inotropic effect of ISO by inhibition of Na^+-Ca^{2+} exchanger

Treatment of the isolated heart with different concentrations of KB-R7943 was found to depress the LVDP **(Table 18)**. The data in **Table 18** show that the positive inotropic effect of ISO measured in terms of an increase in LVDP was significantly depressed by treatment with different concentrations of KB-R7943. Likewise, the basal as well as the increase in ±dP/dt by ISO were also depressed by KB-R7943 (data not shown). It is also pointed out that the positive inotropic effect of ISO was completely blocked by 10 μM propranolol (data not shown).

4. Involvement of NHE in I/R-induced cardiac dysfunction and mechanisms of Ca^{2+}-handling in cardiomyocytes due to NHE inhibition

a. Modulation of I/R-mediated alterations in cardiac function in presence of MIA

Since the activation of NHE in I/R hearts is considered to produce intracellular Ca^{2+}-overload and cardiac dysfunction, the effect of a NHE inhibitor, MIA, were examined in the isolated heart preparations. To investigate the status of NHE inhibition in I/R injury, hearts were pretreated with MIA (5 μM) before the induction of global ischemia and during the reperfusion period. Representative tracings showing the effect of I/R and MIA treatment on I/R-mediated alterations in cardiac function are given in **Figure 21.** I/R caused a significant increase in LVEDP with a marked decrease in LVDP, +dP/dt and –dP/dt **(Table 19 and Figure 21).** Treatment with MIA

Table 17: Effect of different concentrations of KB-R7943 on isoproterenol-induced increase in $[Ca^{2+}]_i$ by low Na^+ in KCl-depolarized cardiomyocytes

Group	Basal $[Ca^{2+}]_i$ (nM)	KCl-induced increase in $[Ca^{2+}]_i$ (nM)		ISO-induced increase in $[Ca^{2+}]_i$ (nM)
		(-) ISO	(+) ISO	
Without drug	108 ± 4.9	175 ± 4.1	235 ± 5.9	58 ± 4.3
KB-R7943 (10 μM)	107 ± 4.9	168 ± 3.2	218 ± 3.8*	49 ± 3.7
KB-R7943 (25 μM)	115 ± 6.1	159 ± 2.8*	190 ± 5.7*	32 ± 3.5*
KB-R7943 (50 μM)	113 ± 4.2	145 ± 2.5*	130 ± 1.5*	17 ± 3.8*

Values are mean ± SEM of 4 hearts in each group. Cardiomyocytes were incubated with different concentrations of KB-R7943 for 10 min including 5 min incubation with 100 μM isoproterenol (ISO) in the low Na^+ containing medium before 50 μM ATP. *$p<0.05$ vs. without drug in the presence of low Na^+.

Table 18: Effect of different concentrations of KB-R7943 on left ventricular developed pressure in isolated rat heart with or without stimulation by 1 μM isoproterenol

Concentration KB-R7943 (μM)	Basal LVDP (mm Hg)	Decrease in LVDP due to KB-R7943 (% of basal)	Increase in LVDP due to ISO (% of basal)
Control	70 ± 8.7	—	123 ± 5.3
1	75 ± 9.1	50 ± 2.5*	72 ± 3.5*
3	73 ± 9.8	66 ± 3.1*	64 ± 2.8*
5	77 ± 9.3	78 ± 3.8*	58 ± 2.7*

Values are mean ± SEM of 4 hearts in each group. After stabilization, isolated hearts were perfused with or without (control) different concentrations of KB-R7943 for 10 min before bolus injection of isoproterernol (ISO) into the perfusion stream. LVDP: left ventricular developed pressure. *$p < 0.05$ vs. control.

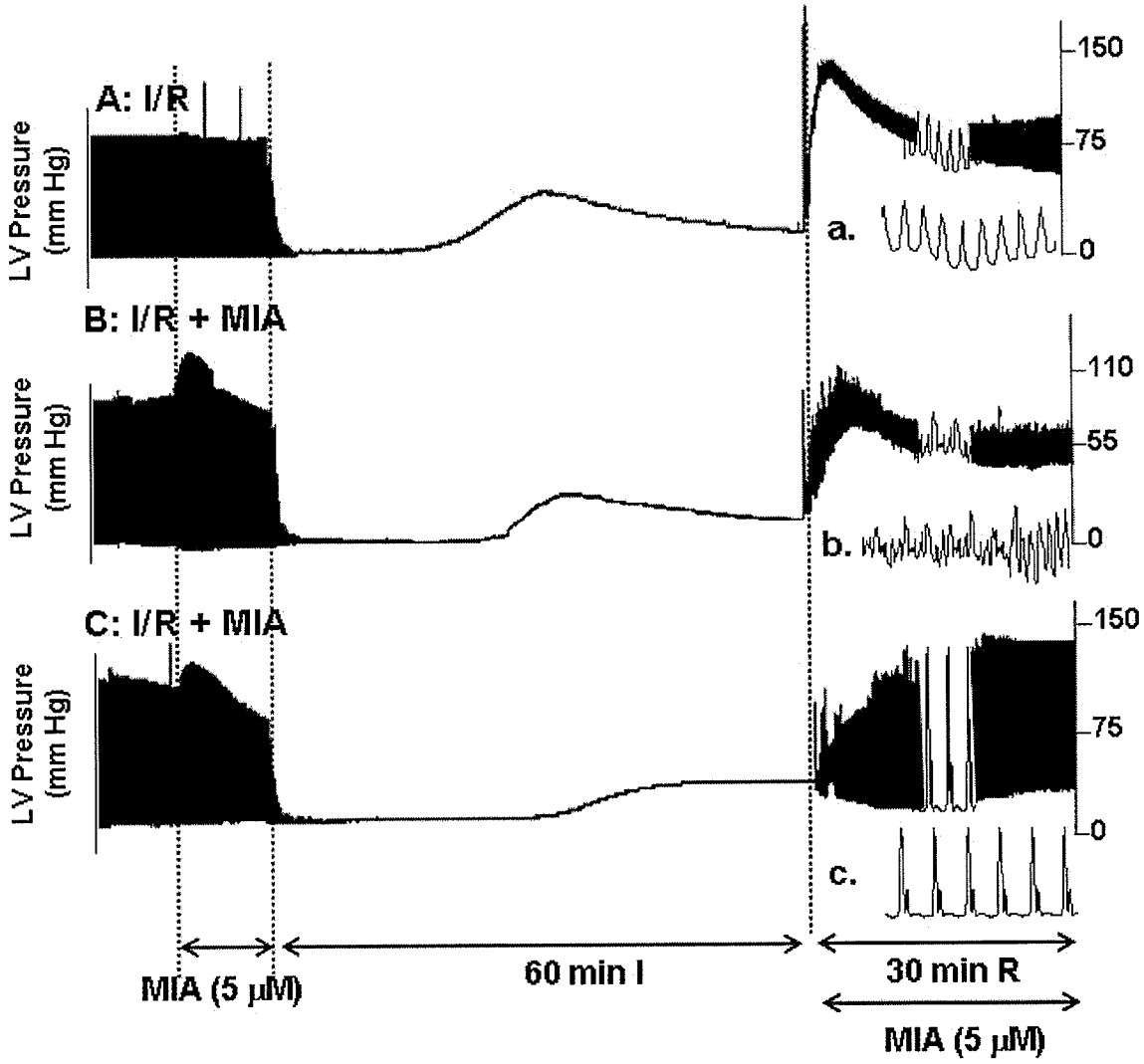


Figure 21. Representative tracings showing the effect of 5-(N-Methyl-N-isobutyl) amiloride (MIA) treatment on ischemia-reperfusion (I/R)-mediated alterations in cardiac function. **Panel A:** Effect of I/R on left ventricle (LV) pressure. **Panel B:** Effect of MIA (5 μM) treatment on left ventricle (LV) pressure (6/10 hearts) in hearts subjected to I/R. **Panel C:** Effect of MIA (5 μM) treatment on left ventricle (LV) pressure (4/10 hearts) in hearts subjected to I/R. The pattern shown in panels a, b and c represents the LV pressure at an expanded scale in the end of 30 min reperfusion. MIA (5 μM) infusion was started 5 min before ischemia and continued throughout the reperfusion period.

Table 19: Effect of 5-(N-N)-dimethylamiloride (MIA) on cardiac performance in isolated hearts subjected to 60 min ischemia followed by 30 min of reperfusion.

Group	LVDP (mm Hg)	LVEDP (mm Hg)	+dP/dt (mm Hg/s)	-dP/dt (mm Hg/s)
Control	94 ± 10.2	9.0 ± 3.5	4998 ± 380	3281 ± 415
I/R	31.7 ± 5.3*	72.5 ± 8.0*	1796 ± 250*	1255 ± 370*
I/R + MIA (6 out of 10)	25.7 ± 7.1*	65.7 ± 10.3*	1572 ± 318*	823 ± 280*
I/R + MIA (4 out of 10)	80 ± 5.0#	25.0 ± 5.6#	4246 ± 410#	3070 ± 295#

Values are mean ± SEM of 4 hearts in each group. Hearts were treated with MIA (5 μM) 5 min before ischemia followed by throughout the period of reperfusion. LVDP, left ventricular developed pressure; LVEDP: left ventricular end diastolic pressure; +dP/dt: rate of change of pressure development; -dP/dt: rate of change of pressure decay. * $P < 0.05$ vs. control; # $P < 0.05$ vs. ischemia-reperfusion (I/R).

had an inconsistent effect; 6/10 hearts did not show any improvement in cardiac function after treatment with MIA, whereas 4/10 hearts showed a significant recovery in I/R-mediated depression in cardiac function **(Table 19)**. It is pointed out that MIA treatment had an arrhythmogenic effect on the heart in the post-ischemic period both in hearts showing recovery in cardiac function as well as the hearts which did not exhibit any functional recovery **(Figure 21)**.

b. Cardiac dysfunction due to mild Ca^{2+}-paradox mediated injury

Three minutes of Ca^{2+}-free perfusion (depletion phase) followed by 10 min of perfusion with Ca^{2+}-containing solution (repletion phase) caused a dramatic impairment in cardiac performance **(Figure 22).** These changes in LV function due to mild Ca^{2+}-paradox were reflected by an increase in LVEDP and a marked decrease in LVDP **(Figure 22)**. Treatment of hearts with MIA (5 μM) prior to the Ca^{2+}-depletion period and during the Ca^{2+}-repletion period produced no improvement in LVDP, whereas LVEDP was further augmented in the presence of MIA **(Figure 22).**

c. Effect of MIA on $[Ca^{2+}]_i$ in isolated cardiomyocytes

In first set of experiments, isolated cardiomyocytes were pretreated with different concentrations of MIA (1-10 μM) for monitoring the basal $[Ca^{2+}]_i$ as well as the changes in $[Ca^{2+}]_i$ upon the addition of KCl (30 mM). Typical tracings reflecting changes in $[Ca^{2+}]_i$ are shown in **Figure 23A**; an increase in basal $[Ca^{2+}]_i$ and augmentation of KCl-induced increases in $[Ca^{2+}]_i$ were observed upon pretreatment of cells with MIA (5 μM). As shown in **Figures 23B and C**, the increase in basal and $[Ca^{2+}]_i$ augmentation of KCl response were dependent upon the concentration of MIA (1-10 μM). To test if the augmentation of KCl-induced increase in $[Ca^{2+}]_i$ is of specific nature, the effect of MIA on $[Ca^{2+}]_i$ was observed in the presence of ATP. The representative tracing showing the effect of MIA (5 μM) on basal and ATP response are shown in

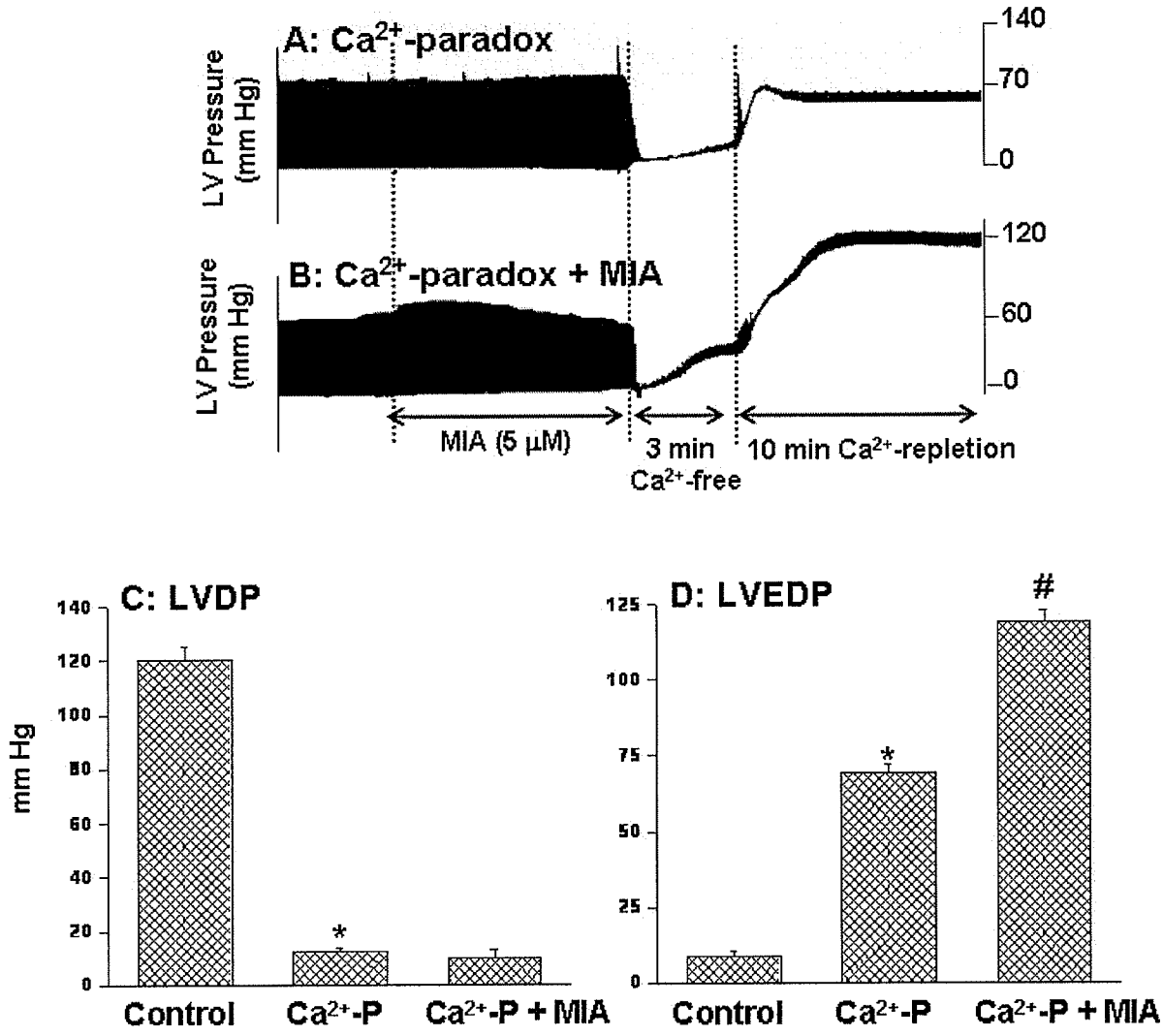


Figure 22. Effect of 5-(N-Methyl-N-isobutyl) amiloride (MIA) treatment on mild Ca^{2+}-paradox (Ca^{2+}-P) (3 min Ca^{2+}-depletion phase followed by 10 min Ca^{2+}-repletion phase)-mediated alterations in cardiac function. **Panel A:** Representative tracings showing the effect of Ca^{2+}-P on left ventricle (LV) pressure. **Panel B:** Representative tracings showing the effect MIA (5 µM) on Ca^{2+}-P-induced alterations in LV pressure. **Panel C:** Effect of MIA (5 µM) on Ca^{2+}-P mediated alterations in left ventricular developed pressure (LVDP). **Panel D:** Effect of MIA (5 µM) on Ca^{2+}-P mediated alterations in left ventricular end diastolic pressure (LVEDP). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. Ca^{2+}-P.

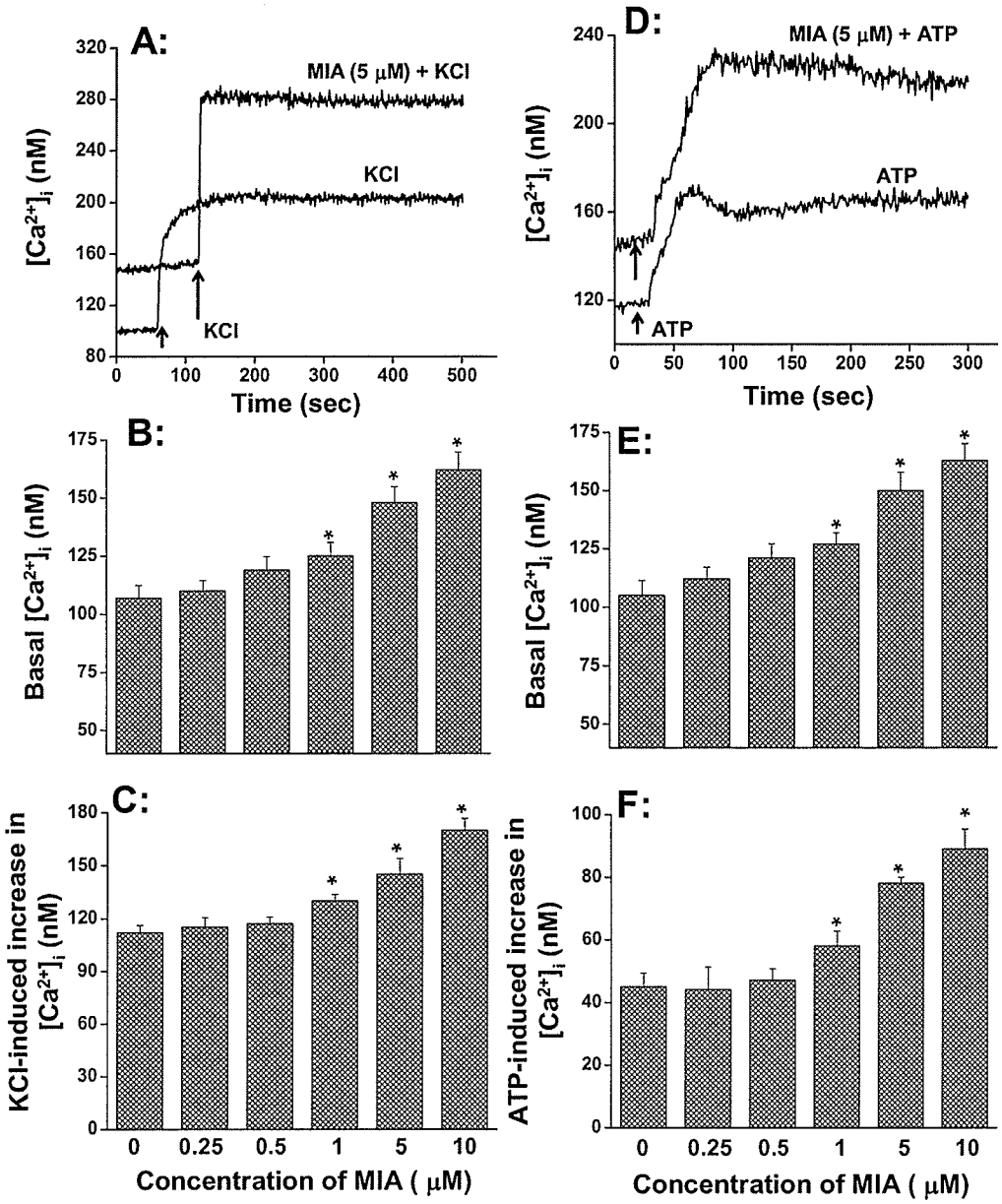


Figure 23. Effect of different concentrations (1-10 μM) of 5-(N-Methyl-N-isobutyl) amiloride (MIA) pretreatment on basal $[Ca^{2+}]_i$ as well as KCl (30 mM) and ATP (50 μM)-mediated increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes. ***Panel A:*** Representative tracings showing the effect of 5 μM MIA on basal and KCl-mediated increase in $[Ca^{2+}]_i$. ***Panel B:*** Effect of different concentrations of MIA on basal $[Ca^{2+}]_i$ before the addition of KCl. ***Panel C:*** Effect of different concentrations of MIA on KCl-mediated increase in $[Ca^{2+}]_i$. ***Panel D:*** Representative tracing showing the effect of 5 μM MIA on basal and ATP-mediated increase in $[Ca^{2+}]_i$. ***Panel E:*** Effect of different concentrations of MIA on basal $[Ca^{2+}]_i$ before the addition of ATP. ***Panel F:*** Effect of different concentrations of MIA on ATP-mediated increase in $[Ca^{2+}]_i$. Each value is a mean ± SEM of 4 preparations in each group. $^*P<0.05$ vs. control in the absence of MIA.

Figure 23D. An increase in basal $[Ca^{2+}]_i$ and an augmentation of ATP-induced increase in $[Ca^{2+}]_i$ were also observed after pretreatment of cells with 1-10 μM MIA **(Figures 23E and F)**.

The other set of experiments were carried out to examine the possibility of any artifact due to pretreatment of cardiomyocytes with MIA. For this the effects of MIA on $[Ca^{2+}]_i$ were studied in the cuvette and real-time tracings were recorded. It can be seen from the representative tracings in **Figures 24A** that MIA increased the basal $[Ca^{2+}]_i$ in a concentration dependent manner similar to that observed with pretreatment of cardiomyocytes. A typical tracing after addition of 5 μM MIA inside the cuvette followed by the addition of KCl is shown in **Figure 24B**; an augmentation of KCl-mediated increase in $[Ca^{2+}]_i$ was also observed at different concentrations of MIA **(Figure 24B, D and F)**. The maximal increase in basal $[Ca^{2+}]_i$ and MIA-induced augmentation of the KCl response was observed at 10 μM MIA **(Figure 24C, D, E and F)**; further increase in MIA concentration caused no additional potentiation in these parameters. Since 5 μM MIA produced a substantial increase in basal $[Ca^{2+}]_i$ as well as augmentation of KCl response, all further experiments were performed by using 5 μM MIA in the presence of different pharmacological interventions.

To establish if the effects of MIA on $[Ca^{2+}]_i$ are simulated by amiloride or its other derivatives, changes in the $[Ca^{2+}]_i$ in cardiomyocytes were measured in the presence of amiloride, a non-specific NHE inhibitor [266], as well as DMA, a specific NHE inhibitor [236]. Amiloride (5-20 μM) caused no alteration in basal $[Ca^{2+}]_i$, whereas KCl-induced increase in $[Ca^{2+}]_i$ was significantly attenuated by amiloride in a concentration dependent manner **(Table 20)**. It is pointed out that alterations in $[Ca^{2+}]_i$ due to amiloride do not represent the effects of NHE inhibition as amiloride is also known to inhibit SL Ca^{2+} regulating sites such as Na^+-Ca^{2+}

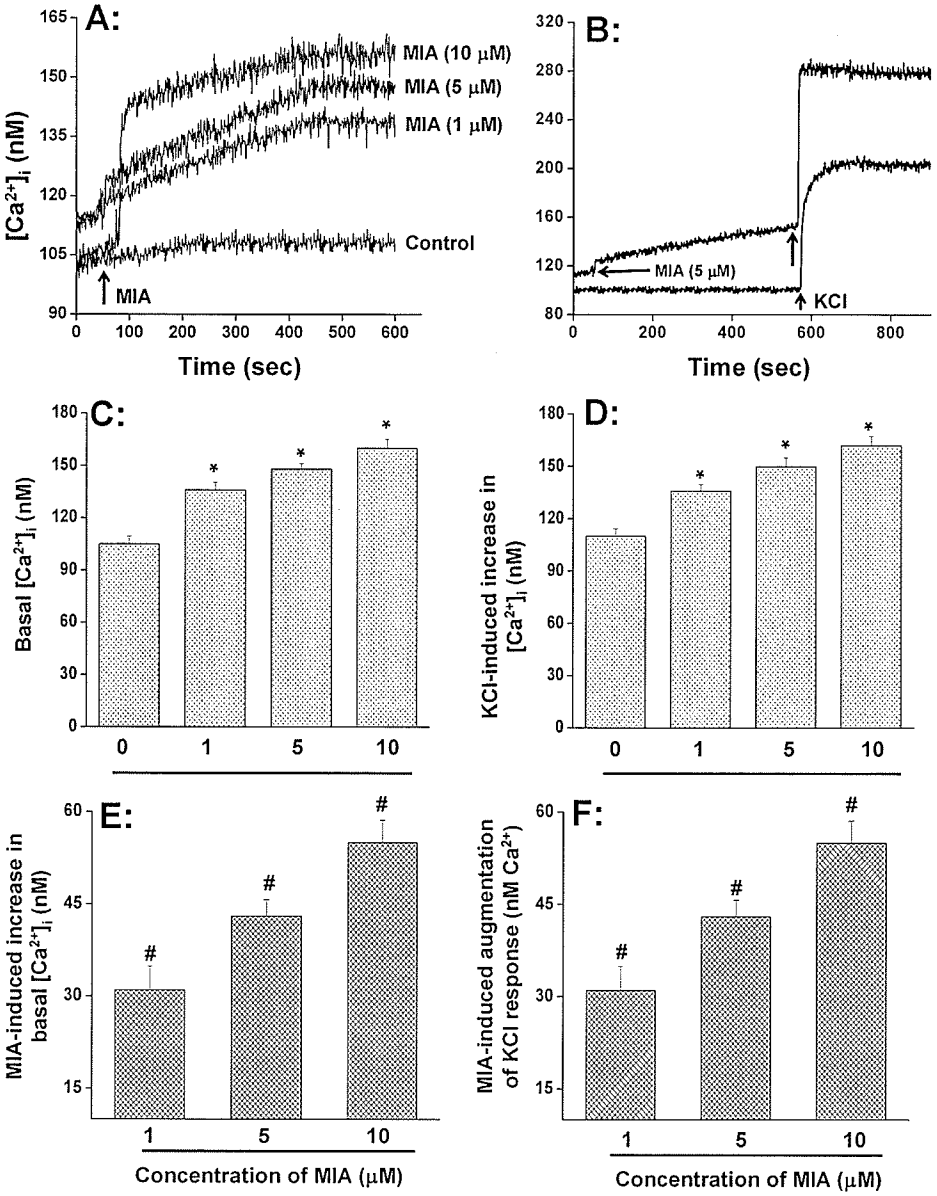


Figure 24. Effect of different concentrations (1-10 μM) of 5-(N-Methyl-N-isobutyl) amiloride (MIA), when added in the cuvette, on basal $[Ca^{2+}]_i$ and KCl-mediated increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes. ***Panel A:*** Representative tracings showing the effect of different concentrations (1-10 μM) of MIA on basal $[Ca^{2+}]_i$. ***Panel B:*** Representative tracings showing the effect of 5 μM MIA on basal and KCl-mediated increase in $[Ca^{2+}]_i$. ***Panel C:*** Effect of different concentrations of MIA on basal $[Ca^{2+}]_i$. ***Panel D:*** Effect of different concentrations of MIA on KCl-mediated increase in $[Ca^{2+}]_i$. ***Panel E:*** Dose response for MIA-induced increase in basal $[Ca^{2+}]_i$. ***Panel F:*** Dose response for MIA-induced augmentation of KCl response. MIA-induced increase in $[Ca^{2+}]_i$ is a difference between basal or KCl-induced increase in $[Ca^{2+}]_i$ in the absence and presence of MIA. Each point represents the mean ± SEM of 4 experiments in each group. $^{*}P<0.05$ vs. control in the absence of MIA; $^{\#}P<0.05$ vs. blank which did not shown any increase

Table 20: Effect of different concentrations of amiloride and 5-(N-N)-dimethylamiloride (DMA) on basal $[Ca^{2+}]_i$ and KCl-induced increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes

Group	Basal $[Ca^{2+}]_i$ (nM)	Drug-induced increase in basal $[Ca^{2+}]_i$ (nM)	KCl-induced increase in $[Ca^{2+}]_i$ (nM)	Drug-induced alterations of KCl response (nM Ca^{2+})
Without drug	111 ± 4.2	—	118 ± 5.4	—
Amiloride (5 μM)	112 ± 5.1	2 ± 1.2	91 ± 3.3*	-27 ± 2.6
Amiloride (10 μM)	114 ± 6.4	3 ± 1.0	80 ± 2.9*	-38 ± 3.4
Amiloride (20 μM)	117 ± 4.0	5 ± 1.3	65 ± 7.1*	-53 ± 4.3
DMA (5 μM)	154 ± 3.0*	41 ± 3.2	140 ± 2.1*	+22 ± 3.2
DMA (10 μM)	168 ± 4.5*	55 ± 4.1	150 ± 4.3*	+32 ± 2.9
DMA (20 μM)	182 ± 3.7*	69 ± 3.6	175 ± 3.6*	+57 ± 3.2

Values are mean ± SEM of 4 hearts in each group. Cardiomyocytes were incubated with different concentrations of DMA or amiloride for 10 min before the addition of KCl. The concentration of KCl for intracellular Ca^{2+} measurement was 30 mM. Since DMA showed varying degrees of autofluorescence at all concentrations employed here, the basal $[Ca^{2+}]_i$ for DMA was adjusted after subtracting the autofluorescence at each concentration of the drug. *$p < 0.05$ vs. respective value without drug.

exchanger, Na^+-K^+ ATPase, Na^+-channels and T-type Ca^{2+}-channels [192]. On the other hand, DMA (5-20 μM) caused a significant increase in both basal and KCl-mediated increase in $[Ca^{2+}]_i$ **(Table 20)**. Since DMA had some effects on the autofluorescence of unloaded cardiomyocytes, no further experiments were performed in the presence of DMA. On the other hand, it is pointed out that MIA in the concentration used in the present study dose not show any autofluorescence and thus does not interfere with the measurement of $[Ca^{2+}]_i$ by fura-2 microfluorometric technique.

d. Effect of extracellular Ca^{2+} on MIA-induced increase in $[Ca^{2+}]_i$

To examine the direct involvement of extracellular Ca^{2+} in MIA-mediated increase in basal $[Ca^{2+}]_i$ and augmentation of KCl response, isolated cardiomyocytes were treated with low (0.5 mM) and high (2.5 mM) concentration of extracellular Ca^{2+}. It was observed that basal $[Ca^{2+}]i$ and MIA-induced increase in basal $[Ca^{2+}]_i$ remained unaltered at different concentrations of extracellular Ca^{2+} (0.5-2.5 mM). On the other hand, KCl-induced increase in $[Ca^{2+}]_i$ and MIA-mediated augmentation of KCl response in cardiomyocytes (1.25 mM extracellular Ca^{2+}) was significantly depressed by decreasing extracellular Ca^{2+} to 0.5 mM **(Table 21)**. On the other hand, increasing the extracellular concentration of Ca^{2+} to 2.5 mM produced a significant potentiation of KCl-mediated increase in $[Ca^{2+}]_i$ as well as augmented the MIA-induced increase in KCl response **(Table 21)**.

e. Modulation of MIA-induced increase in $[Ca^{2+}]_i$ by L-type Ca^{2+}-channels

To investigate the involvement of extracellular Ca^{2+} in MIA-induced increase in basal $[Ca^{2+}]_i$ and augmentation of KCl response, isolated cardiomyocytes were treated with two well known L-type Ca^{2+}-channel antagonists, verapamil and diltiazem [266]. Preincubation of cells with verapamil (1 and 5 μM) or diltiazem (1 and 5 μM) did not produce any significant change in basal $[Ca^{2+}]_i$ as

Table 21: Effect of different concentrations of extracellular Ca^{2+} on basal $[Ca^{2+}]_i$ and KCl-induced increase in $[Ca^{2+}]_i$ in absence or presence of MIA in isolated cardiomyocytes

Group	Basal $[Ca^{2+}]_i$ (nM)	MIA-induced increase in basal $[Ca^{2+}]_i$ (nM)	KCl-induced increase in $[Ca^{2+}]_i$ (nM)	MIA-induced augmentation of KCl response (nM Ca^{2+})
A: Control Ca^{2+}				
1.25 mM Ca^{2+}	108 ± 4.9	—	112 ± 5.9	—
1.25 mM Ca^{2+} + MIA (5 μM)	155 ± 4.5*	47 ± 4.3	155 ± 3.9*	43 ± 3.5
B: Low Ca^{2+}				
0.50 mM Ca^{2+}	112 ± 4.6	—	84 ± 5.2	—
0.50 mM Ca^{2+} + MIA (5 μM)	154 ± 3.6*	42 ± 4.1	78 ± 5.1	6 ± 1.9†
C: High Ca^{2+}				
2.50 mM Ca^{2+}	115 ± 4.2	—	150 ± 4.3	—
2.50 mM Ca^{2+} + MIA (5 μM)	155 ± 5.8*	40 ± 5.7	220 ± 6.5*	70 ± 2.1†

Values are mean ± SEM of 4 hearts in each group. Cardiomyocytes were incubated for 10 min with different concentrations of Ca^{2+} before the addition of MIA (5 μM). The concentration of KCl for intracellular Ca^{2+} measurement was 30 mM. *$p < 0.05$ vs. respective value without MIA in the presence of each concentration of Ca^{2+} and †$p < 0.05$ vs. MIA response in the presence of KCl at 1.25 mM Ca^{2+}.

well as MIA-mediated increase in basal $[Ca^{2+}]_i$ **(Table 22)**. On the other hand, KCl-induced increase in $[Ca^{2+}]_i$ and MIA-mediated augmentation of KCl response were significantly attenuated by verapamil or diltiazem treatment **(Table 22)**.

f. Role of SL Ca^{2+}-pump ATPase and Na^+-K^+ ATPase in MIA-induced increase in $[Ca^{2+}]_i$

For studying the involvement of SL Ca^{2+}-pump ATPase and SL Na^+-K^+ ATPase in MIA-induced increase in $[Ca^{2+}]_i$, cells were treated with specific inhibitors of these enzymes. Preincubation with vanadate (0.5, 1 and 2 μM), a known inhibitor of SL Ca^{2+} pump ATPase [284] caused no alteration in basal $[Ca^{2+}]_i$ and MIA-mediated increase in basal $[Ca^{2+}]_i$. Similarly, KCl-mediated increase in $[Ca^{2+}]_i$ and MIA-mediated augmentation of KCl response remained unaltered by treatment with vanadate **(Table 23)**. On the other hand, treatment with ouabain (0.05, 0.1, and 0.3 mM), a known inhibitor of Na^+-K^+ ATPase [292], significantly attenuated the MIA-mediated increase in both basal $[Ca^{2+}]_i$ and KCl response **(Table 23)**. It can also been seen from the data given in Table 4 that ouabain (0.05-0.3 mM) caused a significant increase in basal $[Ca^{2+}]_i$ and augmented the KCl-mediated increase in $[Ca^{2+}]_i$. On the other hand, low concentration of ouabain (0.01 mM) did not cause any alteration in basal $[Ca^{2+}]_i$ or KCl-mediated increase in $[Ca^{2+}]_i$ in absence or presence of MIA **(Table 23)**.

g. Involvement of Na^+-Ca^{2+} exchanger in MIA-induced increase in $[Ca^{2+}]_i$

To test the role of Na^+-Ca^{2+} exchanger in MIA-induced increase in basal $[Ca^{2+}]_i$ and KCl response, cells were treated with KB-R7943, a known inhibitor of Na^+-Ca^{2+} exchanger [271]. KB-R7943 (10 and 25 μM) caused no alterations in basal $[Ca^{2+}]_i$ and MIA-induced increase in basal $[Ca^{2+}]_i$ **(Table 24)**. On the other hand, KCl-induced increase in $[Ca^{2+}]_i$ and MIA-mediated augmentation of KCl response was significantly attenuated by KB-R7943 treatment **(Table 24)**. In view of the fact that low Na^+ has been shown to cause an increase in $[Ca^{2+}]_i$ by the activation

Table 22: Effect of different concentrations of verapamil and diltiazem on basal $[Ca^{2+}]_i$ and KCl-induced increase in $[Ca^{2+}]_i$ in absence or presence of MIA in isolated cardiomyocytes

Group	Basal $[Ca^{2+}]_i$ (nM)	MIA-induced increase in basal $[Ca^{2+}]_i$ (nM)	KCl-induced increase in $[Ca^{2+}]_i$ (nM)	MIA-induced augmentation of KCl response (nM Ca^{2+})
Without drug	109 ± 5.3	—	108 ± 7.5	—
MIA (5 μM)	152 ± 5.1*	43 ± 4.6	150 ± 5.5*	42 ± 4.5
Verapamil (1 μM)	107 ± 6.3	—	62 ± 5.9	—
Verapamil (1 μM) + MIA (5 μM)	157 ± 7.7*	50 ± 3.9	67 ± 6.1	5 ± 1.7†
Verapamil (5 μM)	108 ± 5.2	—	35 ± 5.8	—
Verapamil (5 μM) + MIA (5 μM)	148 ± 5.4*	40 ± 5.8	42 ± 7.3	7 ± 2.4†
Diltiazem (1 μM)	109 ± 7.3	—	92 ± 3.6	—
Diltiazem (1 μM) + MIA (5 μM)	150 ± 4.9*	41 ± 3.7	100 ± 8.3	8 ± 3.3†
Diltiazem (5 μM)	110 ± 5.0	—	70 ± 5.8	—
Diltiazem (5 μM) + MIA (5 μM)	153 ± 5.3*	43 ± 4.0	78 ± 5.6	8 ± 2.1†

Values are mean ± SEM of 4 hearts in each group. Cardiomyocytes were incubated with different concentrations of verapamil or diltiazem for 10 min before the addition of MIA (5 μM). The concentration of KCl for intracellular Ca^{2+} measurement was 30 mM. *$p< 0.05$ vs. respective values without MIA in the absence or presence of verapamil or diltiazem and †$p< 0.05$ vs. MIA response in the absence of verapamil or diltiazem.

Table 23: Effect of different concentrations of ouabain and vanadate on basal $[Ca^{2+}]_i$ and KCl-induced increase in $[Ca^{2+}]_i$ in absence or presence of MIA in isolated cardiomyocytes

Group	Basal $[Ca^{2+}]_i$ (nM)	MIA-induced increase in basal $[Ca^{2+}]_i$ (nM)	KCl-induced increase in $[Ca^{2+}]_i$ (nM)	MIA-induced augmentation of KCl response (nM Ca^{2+})
Without drug	112 ± 4.3	—	110 ± 4.6	—
MIA (5 μM)	152 ± 5.9*	40 ± 3.9	160 ± 7.8*	50 ± 3.7
Vanadate (0.5 μM)	105 ± 6.9	—	112 ± 6.7	—
Vanadate (0.5 μM) + MIA (5 μM)	148 ± 5.3*	43 ± 4.2	154 ± 5.0*	42 ± 4.3
Vanadate (1 μM)	112 ± 5.3	—	111 ± 6.0	—
Vanadate (1 μM) + MIA (5 μM)	162 ± 7.8*	50 ± 5.1	160 ± 2.6*	49 ± 3.8
Vanadate (2 μM)	110 ± 5.7	—	109 ± 7.5	—
Vanadate (2 μM) + MIA (5 μM)	161 ± 5.0*	51 ± 5.7	157 ± 3.3*	48 ± 4.9
Ouabain (0.01 mM)	120 ± 4.2	—	130 ± 2.1	—
Ouabain (0.01 mM) + MIA (5 μM)	165 ± 3.9*	45 ± 2.9	170 ± 4.4	40 ± 3.8
Ouabain (0.05 mM)	137 ± 3.3		163 ± 3.9	—
Ouabain (0.05 mM) + MIA (5 μM)	167 ± 2.4*	30 ± 2.7*	192 ± 2.8	29 ± 3.1†
Ouabain (0.1 mM)	149 ± 3.9	—	180 ± 5.6*	—
Ouabain (0.1 mM) + MIA (5 μM)	159 ± 5.2	10 ± 2.5*	196 ± 9.2*	16 ± 2.4†
Ouabain (0.3 mM)	161 ± 3.8	—	260 ± 5.8*	—
Ouabain (0.3 mM) + MIA (5 μM)	177 ± 8.4	16 ± 3.3*	272 ± 4.0*	12 ± 2.1†

Values are mean ± SEM of 4 hearts in each group. Cardiomyocytes were incubated with different concentrations of vanadate or ouabain for 10 min before the addition of MIA (5 μM). The concentration of KCl for intracellular Ca^{2+} measurement was 30 mM. *$p < 0.05$ vs. respective value without MIA at different concentrations of vanadate or ouabain and †$p < 0.05$ vs. MIA response in the presence of KCl without vanadate or ouabain.

Table 24: Effect of different concentrations of KB-R7943 and low-Na^+ on basal $[Ca^{2+}]_i$ and KCl-induced increase in $[Ca^{2+}]_i$ in absence or presence of MIA in isolated cardiomyocytes

Group	Basal $[Ca^{2+}]_i$ (nM)	MIA-induced increase in basal $[Ca^{2+}]_i$ (nM)	KCl-induced increase in $[Ca^{2+}]_i$ (nM)	MIA-induced augmentation of KCl response (nM Ca^{2+})
Without drug	110 ± 6.2	—	113 ± 4.9	—
MIA (5 μM)	155 ± 4.9*	45 ± 3.9	153 ± 4.8*	41 ± 3.8
KBR (10 μM)	111 ± 6.3	—	103 ± 4.8	—
KBR (10 μM) + MIA (5 μM)	155 ± 4.5*	44 ± 3.7	112 ± 5.0	9 ± 2.5†
KBR (25 μM)	106 ± 5.2	—	79 ± 4.3	—
KBR (25 μM) + MIA (5 μM)	148 ± 4.8*	42 ± 4.2	83 ± 2.6	4 ± 1.1†
Low Na^+ (70 mM)	114 ± 4.1	—	140 ± 3.7	—
Low Na^+ (70 mM) + MIA (5 μM)	156 ± 3.5*	42 ± 2.4	149 ± 3.0	9 ± 1.4†
Low Na^+ (35 mM)	118 ± 5.7	—	169 ± 4.9	—
Low Na^+ (35 mM) + MIA (5 μM)	158 ± 4.1*	40 ± 5.6	163 ± 9.7	-6 ± 3.2†

Values are mean ± SEM of 4 hearts in each group. Cardiomyocytes were incubated with different concentrations of KB-R7943 and 35 mM Na^+ for 10 min before the addition of MIA (5 μM). The concentration of KCl for intracellular Ca^{2+} measurement was 30 mM. *$p < 0.05$ vs. respective value without MIA and †$p < 0.05$ vs. MIA response in the presence of KCl without KBR or low Na^+.

of SL Na^+-Ca^{2+} exchanger, the cells were exposed to K-H solution containing 70 mM or 35 mM Na^+ before measurement of fluorescence. No depression in MIA-mediated increase in basal $[Ca^{2+}]_i$ was observed in the presence of low Na^+ **(Table 24)**. On the other hand, MIA-induced augmentation of KCl response was markedly depressed in low Na^+ solution **(Table 24)**. Low Na^+ treatment itself had no effect on basal $[Ca^{2+}]_i$, whereas KCl-induced increase in $[Ca^{2+}]_i$ was significantly potentiated by low Na^+.

h. Involvement of mitochondrial Ca^{2+}-regulatoy mechanisms in MIA-induced increase in $[Ca^{2+}]_i$

The role of mitochondria in MIA-induced increase in $[Ca^{2+}]_i$ was examined by treating cardiomyocytes with different concentrations of sodium azide (0.5-10 mM) and ruthenium red (0.5-10 μM), inhibitors of mitochondrial Ca^{2+}-uptake [293,294]. Both these agents did not show any effect on basal $[Ca^{2+}]_i$ and KCl-induced increase in $[Ca^{2+}]_i$. Similarly, MIA-mediated increase in basal $[Ca^{2+}]_i$ (in nM; control 42 ± 3.4 vs. 0.5 mM sodium azide 40 ± 5.1; 1 mM sodium azide 41 ± 3.3; 0.5 μM ruthenium red 42 ± 3.7; 1 μM ruthenium red 41 ± 4.0) as well as augmentation of KCl response (in nM Ca^{2+} control 42 ± 3.9 vs. 0.5 mM sodium azide 46 ± 4.7; 1 mM sodium azide 45 ± 4.3; 0.5 μM ruthenium red 41 ± 3.8; 1 μM ruthenium red 42 ± 4.7) remained unaltered in the presence of both of these agents

i. Role of SR Ca^{2+}-stores in MIA-induced increase in $[Ca^{2+}]_i$

In order to assess the involvement of SR Ca^{2+}-stores in MIA-mediated alterations in basal Ca^{2+} and KCl response, cardiomyocytes were treated with different agents, which are known to modulate SR Ca^{2+}-stores. Preincubation of cells with ryanodine (2 and 5 μM), a blocker of SR Ca^{2+} release channel [285], attenuated the MIA-mediated increase in basal $[Ca^{2+}]_i$ and KCl-induced increase in $[Ca^{2+}]_i$. Similarly, pretreatment of cells with cyclopiazonic acid (CPA) (20

and 50 μM), a known SR Ca^{2+}-pump ATPase pump inhibitor [285], caused a significant decrease in MIA-induced increase in basal $[Ca^{2+}]_i$ **(Table 25)**. Representative tracings showing the effect of caffeine pretreatment on MIA-induced increase in basal $[Ca^{2+}]_i$ and augmentation of KCl response are shown in **Figure 25A**. Caffeine (10 and 20 mM), which keeps Ca^{2+}-release channels in an open state [295], attenuated the MIA-induced increase in basal $[Ca^{2+}]_i$ **(Figure 25B and D)**. In addition, MIA-mediated augmentation in KCl response was significantly depressed by treatment with these agents **(Table 25, Figures 25C and 25D)**. It can be noted from the data given in **Table 25 and Figure 25** that preincubation with ryanodine, CPA and caffeine caused no effect on basal $[Ca^{2+}]_i$, whereas KCl-mediated increase in $[Ca^{2+}]_i$ was significantly depressed. In contrast to a typical Ca^{2+}-transient obtained upon electrical stimulation of a single cardiomyocyte, the increase in $[Ca^{2+}]_i$ after KCl addition, as measured in the preparation used in this study, did not decrease over a period of time. The presence of KCl in the cuvette at all the time points during the measurement of florescence may be the reason for such a response, which is in accordance with our previous studies [255,266,296].

j. Effect of lowering intracellular pH on $[Ca^{2+}]_i$

To understand the mechanisms of MIA-induced increase in basal $[Ca^{2+}]_i$ and augmentation of KCl-mediated increase, the effects of extracellular pH and MIA on intracellular pH were studied. Reducing the extracellular pH from 7.4 to 5.5 caused a decrease in intracellular pH **(Figure 26A)**. A significant increase in basal $[Ca^{2+}]_i$ was observed upon reducing the pH to 5.5 **(Figure 26B)**. On the other hand, no significant change in KCl-induced increase in $[Ca^{2+}]_i$ was observed upon reducing the extracellular pH from 7.4 to 6.5; this parameter was significantly decreased at pH 6.0 and 5.5 **(Figure 26C)**. MIA (1-10 μM) caused a significant reduction in pH in

Table 25: Effect of different concentrations of ryanodine and cyclopiazonic acid (CPA) on basal $[Ca^{2+}]_i$ and KCl-induced increase in $[Ca^{2+}]_i$ in absence or presence of MIA in isolated cardiomyocytes

Group	Basal $[Ca^{2+}]_i$ (nM)	MIA-induced increase in basal $[Ca^{2+}]_i$ (nM)	KCl-induced increase in $[Ca^{2+}]_i$ (nM)	MIA-induced augmentation of KCl response (nM Ca^{2+})
Without drug	112 ± 5.9	—	105 ± 4.0	—
MIA (5 μM)	160 ± 6.7*	48 ± 5.2	149 ± 6.3*	44 ± 3.7
Ryanodine (2 μM)	109 ± 5.3	—	93 ± 5.3	—
Ryanodine (2 μM) + MIA (5 μM)	141 ± 3.2*	32 ± 2.3*	87 ± 5.0	-6 ± 1.3†
Ryanodine (5 μM)	112 ± 5.9	—	72 ± 2.3	—
Ryanodine (5 μM) + MIA (5 μM)	135 ± 5.4*	23 ± 3.2*	75 ± 4.9	-3 ± 1.2†
CPA (20 μM)	108 ± 6.3	—	90 ± 3.2	—
CPA (20 μM) + MIA (5 μM)	142 ± 3.1*	34 ± 2.1*	93 ± 5.0	-3 ± 1.1†
CPA (50 μM)	109 ± 6.7	—	60 ± 3.6	—
CPA (50 μM) + MIA (5 μM)	133 ± 2.7*	24 ± 1.7*	65 ± 8.9	-5 ± 2.3†

Values are mean ± SEM of 4 hearts in each group. Cardiomyocytes were incubated with different concentrations of ryanodine or CPA for 20 min before the addition of MIA (5 μM). The concentration of KCl for intracellular Ca^{2+} measurement was 30 mM. *$p < 0.05$ vs. respective value without MIA in the absence or presence of ryanodine or CPA and †$p < 0.05$ vs. MIA response in the presence of KCl without ryanodine and CPA.

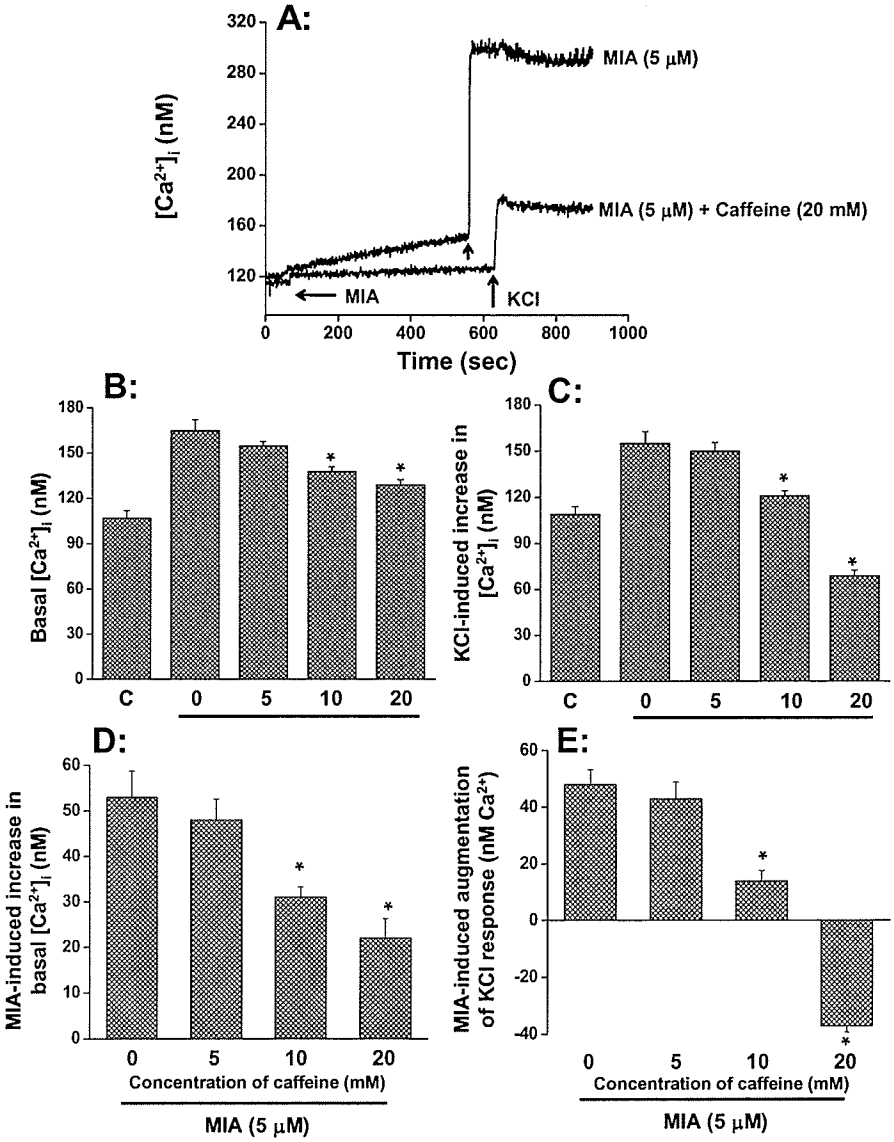


Figure 25. Effect of different concentrations of caffeine (5-20 mM) on 5-(N-Methyl-N-isobutyl) amiloride (MIA) (5 μM)-mediated increase in basal and KCl-induced augmentation of $[Ca^{2+}]_i$. ***Panel A:*** Representative tracings showing the effect of caffeine (20 mM) on MIA-mediated increase in basal and KCl-induced augmentation of $[Ca^{2+}]_i$. ***Panel B:*** Effect of different concentrations of caffeine on basal $[Ca^{2+}]_i$ in the presence of MIA. ***Panel C:*** Effect of different concentrations of caffeine on KCl-mediated increase in $[Ca^{2+}]_i$ in the presence of MIA. ***Panel D:*** Dose response for caffeine showing the effect on MIA-induced increase in basal $[Ca^{2+}]_i$. ***Panel E:*** Dose response for caffeine showing the effect on MIA-induced augmentation of KCl-mediated increase in $[Ca^{2+}]_i$. MIA-induced increase in $[Ca^{2+}]_i$ is a difference between basal or KCl-induced increase in $[Ca^{2+}]_i$ in the absence and presence of MIA. Each point represents the mean ± SEM of 4 preparations in each group. C represents the control cardiomyocytes in the absence of MIA or caffeine. $^*P < 0.05$ vs. control in the presence of MIA without pretreatment with caffeine

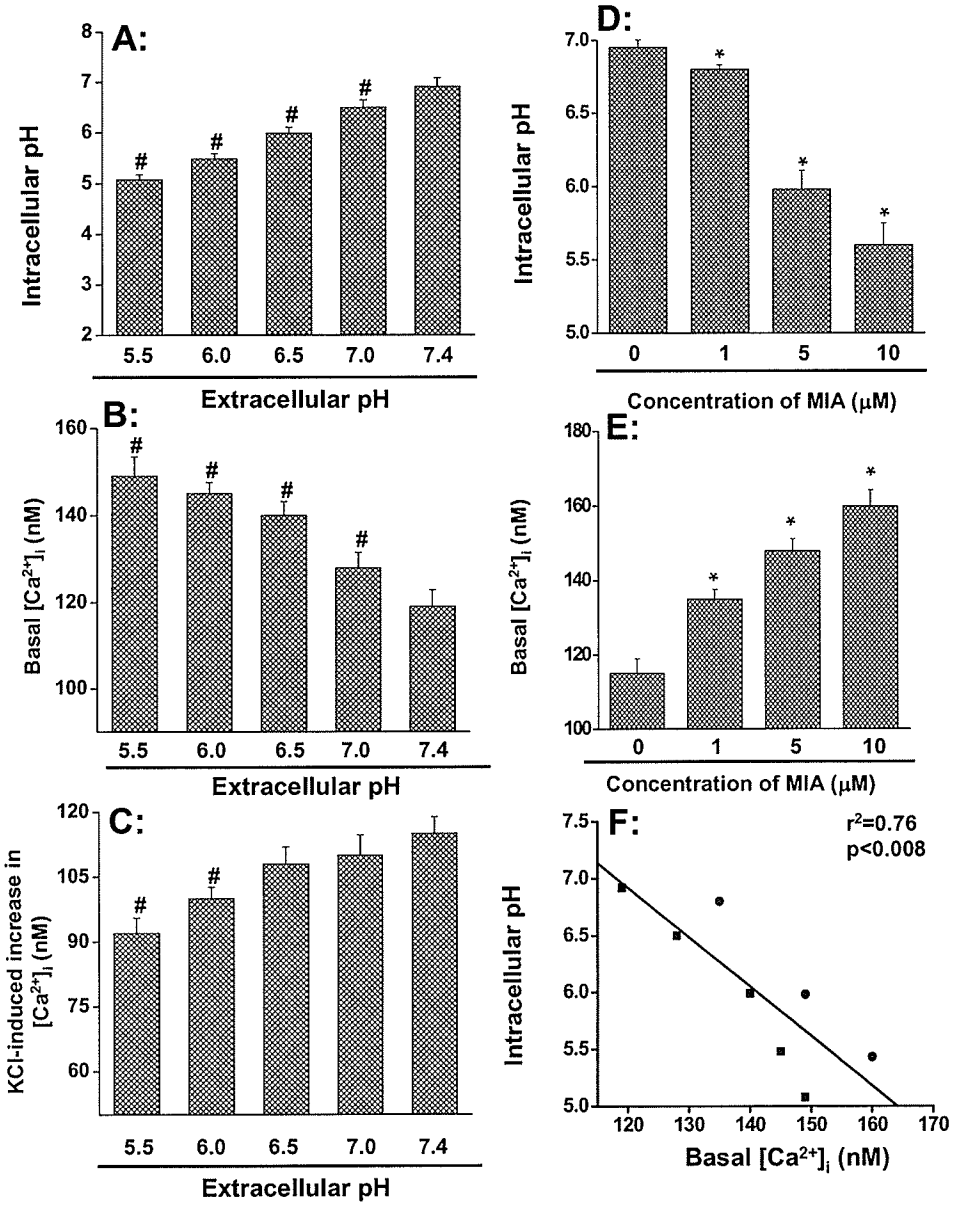


Figure 26. Effect of extracellular pH and 5-(N-Methyl-N-isobutyl) amiloride (MIA) on intracellular pH and increase in basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated cardiomyocytes. ***Panel A:*** Effect of extracellular pH (5.5-7.4) on intracellular pH. ***Panel B:*** Effect of extracellular pH (5.5-7.4) on basal $[Ca^{2+}]_i$. ***Panel C:*** Effect of extracellular pH (5.5-7.4) on KCl-induced increase in $[Ca^{2+}]_i$. ***Panel D:*** Effect of different concentrations of MIA (1-10 μM) on intracellular pH. ***Panel E:*** Effect of different concentrations of MIA (1-10 μM) on basal $[Ca^{2+}]_i$ under the same conditions those employed for the determination of pH. ***Panel F:*** Correlation of intracellular pH and basal $[Ca^{2+}]_i$ in isolated cardiomyocytes. These data are taken from the mean values of intracellular pH at different extracellular pH (■) and different concentrations of MIA (●). Each point represents the mean ± SEM of 4 experiments in each group. $^{*}P < 0.05$ in the absence of MIA and $^{\#}P < 0.05$ vs. extracellular pH of 7.4.

cardiomyocytes in HCO_3^- free buffer **(Figure 26D)**; this agent also produced an increase in basal $[Ca^{2+}]_i$ under the experimental conditions employed for pH measurements **(Figure 26E)**. A highly significant linear relationship was observed for changes in basal $[Ca^{2+}]_i$ and decrease in pH, unlike for KCl-induced increase in $[Ca^{2+}]_i$ and decrease in pH, both in acidic environment as well as in the presence of MIA **(Figure 26F).** In another set of experiments using 3 separate preparations and HCO_3^- containing buffer, the values for intracellular pH were: control 7.12 ± 0.03; 1 μM MIA 6.90 ± 0.10; 5 μM MIA 6.32 ± 0.04; 10 μM MIA 5.95 ± 0.05. These values at various concentrations of MIA in the presence of HCO_3^- containing buffer were not different qualitatively from those in the absence of HCO_3^- buffer.

V. DISCUSSION

As oxidative stress and activation of NHE are considered to produce intracellular Ca^{2+}-overload and cardiac dysfunction following I/R, this work examined the effects of an antioxidant mixture and a NHE inhibitor on cardiac function and Ca^{2+}-handling by cardiomyocytes obtained from I/R hearts. Alterations in Ca^{2+}-mobilization by a depolarizing agent, a β-adrenoceptor agonist and a purinergic receptor agonist in cardiomyocytes from I/R hearts were determined. The effects of an oxidant on these parameters were studied to establish if these chages simulate those seen in I/R. In addition, the mechanisms of action of catecholamines, ATP and an NHE inhibitor on Ca^{2+}-handling by cardiomyocytes were investigated. The results obtained from this study are discussed below:

1. Defects in catecholamine-induced Ca^{2+}-mobilization in cardiomyocytes isolated from hearts subjected to I/R

In the present study, basal $[Ca^{2+}]_i$ was increased in purified cardiomyocytes isolated from hearts subjected to 30 min of global ischemia followed by different times of reperfusion whereas no change in basal $[Ca^{2+}]_i$ was observed in hearts undergoing 10 or 20 min ischemia and subsequent reperfusion. By employing the whole heart preparations, Seki et al. [297] have also shown an increase in diastolic Ca^{2+} in hearts undergoing 30 min of low-flow ischemia followed by reperfusion whereas no difference was observed in hearts subjected to reperfusion after 10 min ischemia. Although Meissner and Morgan [180] have shown a significant increase in diastolic $[Ca^{2+}]_i$ in isolated hearts exposed to 30 min of global ischemia followed by 60 min of reperfusion, these investigators were also able to detect an increase in basal $[Ca^{2+}]_i$ in 10 or 20 min ischemic hearts upon reperfusion. Similarly in isolated rat cardiomyocytes, hypoxia-reoxygenation has been shown to cause persistent elevation in cytosolic Ca^{2+} concentration, which is associated with

cell injury [298]. An increase in $[Ca^{2+}]_i$ has been observed in rat cardiomocytes after 45 min of anoxia followed by 10 min of reperfusion [187]. On the other hand, Chandrashekhar et al. [184] have reported that the $[Ca^{2+}]_i$ does not change in cardiomyocytes isolated from rat hearts subjected to 20 min global ischemia and 20 min reperfusion and have suggested that impairment of cardiac function in I/R is not linked with abnormal Ca^{2+}-handling in cardiomyocytes. Furthermore, Kim et al. [185] have shown that impaired Ca^{2+}-handling in I/R hearts is species dependent because no defect was evident in cardiomyocytes isolated from rat I/R hearts while a significant impairment in Ca^{2+}-handling was seen in cardiomyocytes isolated from swine I/R hearts. It is to point out that Dhalla et al. [5] have reported an increase in total tissue Ca^{2+} in isolated hearts after 30 min I/R. Likewise, Tani and Neely [179] observed an increase in $^{45}Ca^{2+}$-uptake in hearts undergoing 30 min zero-flow global ischemia with 30 min of aerobic perfusion. Although accumulation of $[Ca^{2+}]_i$ during the ischemic period appear to be a consistent finding, it is pointed out that the gradual increase in $[Ca^{2+}]_i$ during reperfusion period depends on the duration of ischemic insult. Different studies subjecting the isolated hearts or trabeculae muscles to ischemia for less than 20 min have shown that the increased $[Ca^{2+}]_i$ during ischemia normalizes to preischemic values in the first few min of reperfusion [182,244,299]. Thus it appears that the ability of cardiomyocytes isolated from the I/R heart to exhibit increased level of basal $[Ca^{2+}]_i$ may be related to the irreversible nature of the ischemic injury. It should be noted that cardiac dysfunction in hearts subjected to 10 min of ischemia was fully reversible upon reperfusion for 30 min and that in hearts subjected to 20 min ischemia, unlike 30 min ischemic hearts, was reversible upon reperfusion for 60 min. No changes in cell viability and cardiomyocyte yield in hearts subjected to 10 or 20 min ischemia followed by reperfusion, unlike in the 30 min I/R hearts, provide further evidence that ischemic insult for a period of 10 to 20 min may produce

reversible alterations whereas irreversible changes are induced by 30 min of global ischemia. This view is consistent with the observations by other investigators [300,301] that 20 to 40 min of ischemia in rat and mouse heart is responsible for transition from reversible to irreversible injury associated with cardiac dysfunction.

It is now well known that $[Ca^{2+}]_i$ is regulated by the coordinated functions of SL, SR and mitochondria [178,179,186,248]. Accordingly, the observed increase in basal $[Ca^{2+}]_i$ in cardiomyocytes isolated from I/R hearts may be due to defects in one or more of these subcellular mechanisms. It should be noted that both I/R and hypoxia/reoxygenation have been shown to produce marked changes in SL Na^+-K^+ ATPase, Na^+-Ca^{2+} exchange, Ca^{2+}-pump and Ca^{2+}-channels [71,179,298,302,303]. Furthermore, alterations in SR Ca^{2+}-uptake and Ca^{2+}-release activities have been observed in I/R hearts [248]. Excessive amount of Ca^{2+} has also been shown to accumulate in mitochondria during reperfusion of the ischemic myocardium [178]. Thus abnormalities in different subcellular organelles and particularly in the SL Na^+-Ca^{2+} exchange system due to I/R can be seen to produce defects in Ca^{2+}-handling by cardiomyocytes and explain the observed increase in basal $[Ca^{2+}]_i$. Nonetheless, an increase in basal $[Ca^{2+}]_i$ reflects the occurrence of intracellular Ca^{2+}-overload and is likely to explain the increased LVEDP and depressed ability of I/R hearts to generate contractile force [176,304]. In fact, increases in LVEDP in I/R hearts in all untreated and treated groups were found to be linearly related to increases in basal $[Ca^{2+}]_i$ as well as decreases in LVDP, cardiomyocyte yield and cell viability. By using a different experimental design, other investigators [180] have also reported a correlation between diastolic Ca^{2+} and LVEDP in I/R hearts. In view of the role of intracellular Ca^{2+}-overload in inducing cell injury [304], the increased level of basal $[Ca^{2+}]_i$ in cardiomyocytes from I/R hearts may also explain the observed decrease in cell viability and cardiomyocyte yield

from I/R hearts. In addition, rapid normalization of tissue pH and osmolarity at the time of reperfusion also causes severe mechanical stress on cardiomyocytes leading to their loss in I/R hearts [298].

The results in this study indicate an augmentation of the KCl-induced increase in $[Ca^{2+}]_i$ upon treatment of cardiomyocytes with ISO. Since such an augmentation of the KCl-induced increase in $[Ca^{2+}]_i$ by ISO was prevented by propranolol, a β-adrenoceptor blocker, it is evident that the ISO-induced increase in $[Ca^{2+}]_i$ is mediated through the activation of β_1-adrenoceptors in cardiomyocytes. These results are consistent with earlier observations showing an increase in $[Ca^{2+}]_i$ by ISO in KCl-depolarized as well as electrically stimulated cardiomyocytes [246,305]. The data described here show that the ISO-induced increase in $[Ca^{2+}]_i$ in KCl-depolarized cells is attenuated in I/R hearts. A highly significant linear relationship between LVEDP and decrease in ISO-induced increase in $[Ca^{2+}]_i$ was also evident in I/R cardiomyocytes from all untreated and treated groups used in this study. Because attenuation of the ISO-induced increase in $[Ca^{2+}]_i$ was seen in cardiomyocytes simulated by BAY K8644, an activator of L-type Ca^{2+}-channel in the SL membrane, it is likely that such an effect of I/R on Ca^{2+}-influx may be mediated through changes in the L-type Ca^{2+}-channels. The ISO-induced increase in $[Ca^{2+}]_i$ in control and I/R preparations was markedly prevented by verapamil (10 μM), a Ca^{2+} channel antagonist (data not shown) without having any effect on basal $[Ca^{2+}]_i$. Although a depression in the density of L-type Ca^{2+}-channels has been reported to occur in I/R hearts [280], Ca^{2+}-channel antagonists have been shown to prevent the I/R-induced increase in $[Ca^{2+}]_i$ [177]. Since the KCl-induced or BAY K8644-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes was not altered by I/R, it is unlikely that the observed decrease in ISO-induced increase $[Ca^{2+}]_i$ is due to any direct defect in L-type Ca^{2+}-channels. As verapamil in high concentrations (10 μM) as used in the present study has been

shown to inhibit Na^+-Ca^{2+} exchanger as well as the increase in $[Ca^{2+}]_i$ in KCl-depolarized cardiomyocytes [266], the role of Na^+-Ca^{2+} exchanger in causing Ca^{2+}-handling abnormalities in I/R hearts can not be ruled out. On the other hand, the attenuation of ISO-induced increase in $[Ca^{2+}]_i$ may be related to changes in β-adrenoceptors. In fact, both the affinity and density of β_1-adrenoceptors were reduced in I/R hearts. Similar alterations in binding characteristics of β_1-adrenoceptors in I/R hearts have been shown previously [127]. Thus the observed changes in Ca^{2+}-mobilization by ISO, reflecting Ca^{2+}-handling abnormalities in I/R hearts, may be due to defects at the level of β-adrenoceptors. However, in view of the dramatic changes in Ca^{2+}-release and Ca^{2+}-uptake activities in SR from I/R hearts [248], the contribution of attenuated Ca^{2+}-induced Ca^{2+}-release from SR cannot be ruled out from the observed depression of ISO-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes obtained from I/R hearts.

There is a good possibility that changes in cardiac function as well as basal and ISO-induced increase in $[Ca^{2+}]_i$ in I/R hearts may be due to oxidative stress. This view is supported by the observations that the concentration of MDA and conjugated diene formation were increased in I/R hearts. In fact oxidative stress has been known to produce cardiac dysfunction, alterations in β_1-adrenoceptor mediated signal transduction mechanisms and changes in subcellular functions in I/R hearts [4,5,129]. The participation of oxidative stress in inducing changes in Ca^{2+}-handling by cardiomyocytes as well as cardiac function is further evident from the finding that these alterations in I/R hearts were attenuated by treatment with mixture of SOD and CAT, which is known to scavenge the oxyradicals [101,127]. By employing a similar experimental model, SOD plus CAT treatment has also been shown to cause reduction in MDA and conjugated diene content in I/R hearts [5]. In addition, the changes observed in I/R hearts and cardiomyocytes were simulated by treatment with H_2O_2, a well-known oxidant [129,281]. It may be noted that

treatment of I/R hearts with SOD plus CAT improved the cell viability whereas H_2O_2 produced a reduction in cell viability. The results described in this study also indicate that ischemic preconditioning, which is known to upregulate the antioxidant enzymes in I/R hearts [4], was found to prevent changes in cardiomyocyte Ca^{2+}-handling. Ischemic preconditioning-mediated reduction in cytosolic Ca^{2+} has been previously observed in isolated hearts [306]. It is pointed out that the changes in Ca^{2+}-handling by I/R are fully reversible by ischemic preconditioning whereas these parameters remained attenuated in SOD plus CAT treated I/R hearts. Although Persad et al. [127] have demonstrated that the binding characteristics of β_1-adrenoceptors (K_d and B_{max}) were completely normalized to control values in I/R hearts after treatment with SOD and catalase under the same experimental conditions used in this study, a partial improvement was observed for I/R-induced changes in adenylyl cyclase activity in presence of ISO. The poor recovery of cell viability by antioxidant mixture may also be responsible for incomplete restoration of ISO response in cardiomyocytes isolated from SOD plus CAT treated I/R hearts. In addition, the present study suggests that mechanisms other than increasing the antioxidant reserve may be involved in the improvement of defective Ca^{2+}-handling in I/R hearts by ischemic preconditioning. Synchronization of SR Ca^{2+}-pump and release channels [248], translocation and activation of specific PKC isoforms [247] followed by phosphorylation of myofilament regulatory proteins [307] and reduction of H^+ accumulation during ischemia [308] may be associated with improved Ca^{2+}-handling by ischemic preconditioning.

In conclusion, the findings presented in this study demonstrate that Ca^{2+}-handling abnormalities occur in cardiomyocytes isolated from I/R hearts and this alteration is dependent on the period of ischemic insult. The method employed for assessing changes in $[Ca^{2+}]_i$ in cardiomyocytes can be seen to have advantage over that used for the measurement of $[Ca^{2+}]_i$ in

intact heart [180-183]. This view is based on the observations that diastolic Ca^{2+} measurements in the intact heart are associated with methodological problems including the motion artifacts of the beating hearts, absorbance of light by chromatic molecules, contribution of changes in signal due to the presence of other types of cells and the effect of temperature/pH on the intracellular Ca^{2+}-induced signal emission [309]. Since a collective response has been taken from a large number of cells, the possibility of biasing associated with selection of single cells is eliminated. Although the preparation employed in this study has been used previously to examine the effect of ATP [255,261], low Na^{+} [266] and phosphatidic acid [267] on the intracellular levels of Ca^{2+}, some caution should be exercised while interpreting the data presented here. Since the hearts in all experimental groups were subjected to 5 and 25 min perfusion with Ca^{2+}-free and collagenase containing solution, the effect of ischemia alone cannot be assessed on the basis of experimental design used in this study. Furthermore, cardiac performance was measured in the isolated hearts and Ca^{2+}-handling studies were performed in isolated cardiomyocytes. Therefore, the correlation between these parameters is of an indirect nature. Although we have removed the dead cells from the preparation by applying Percoll gradient, the contamination by some trypan blue permeant cells cannot be ruled out. The low yield of cardiomyocytes isolated from I/R hearts may be a consequence of decreased cell viability due to ischemic insult as well as increased susceptibility to stress during the isolation procedures used in this study. In addition, the data are representative of viable cardiomyocytes and exclude a large population of cells, which are lost during the I/R insult and the isolation procedure. Thus it can be argued that remaining viable cells from I/R hearts used in this study are I/R resistant. However, it is important to point out that the remaining viable cells exhibit rod shaped structure and show no difference in KCl or BAY K8644-induced increase in $[Ca^{2+}]_i$ whereas these cells had an increased basal levels of $[Ca^{2+}]_i$ and depressed ISO-

induced increase in $[Ca^{2+}]_i$. These observations clearly indicate that there occurs a Ca^{2+}-handling defect at the level of cardiomyocytes in I/R hearts. Such an abnormality can be seen to produce intracellular Ca^{2+}-overload and decrease the cell viability leading to cell damage. Furthermore, the experiments described here indicate that the oxidative stress may be an important mechanism for causing changes in $[Ca^{2+}]_i$ handling in the I/R hearts. It seems that there is a functional relationship between cardiac function of isolated hearts and intracellular Ca^{2+}-handling by cardiomyocytes, because ischemic preconditioning, which prevents changes in cardiac function, also prevents the alterations in intracellular Ca^{2+}- handling.

2. Defects in ATP-induced Ca^{2+}-mobilization in cardiomyocytes isolated from hearts subjected to I/R

The results of this investigation indicate that infusion of exogenous ATP caused a significant increase in LV function in control hearts and produced an increase in $[Ca^{2+}]_i$ in control cardiomyocytes; these observations are in agreement with our previous studies [255,277]. On the other hand, the positive inotropic effect and increase in $[Ca^{2+}]_i$ in cardiomyocytes by extracellular ATP were attenuated in hearts subjected to 30 min of ischemia followed by 30 min of reperfusion. This depression in ATP-induced increase in $[Ca^{2+}]_i$ after 30 min of ischemia was evident even after 5 and 15 min of reperfusion, whereas no depression in ATP responsiveness was observed in hearts undergoing 10 min of ischemia and 30 min of reperfusion. Although a depression in ATP-mediated increase in $[Ca^{2+}]_i$ was observed after 20 min ischemia, a partial recovery was observed after 30 min of reperfusion. A highly significant relation between depression in ATP-induced increase in $[Ca^{2+}]_i$ and LVEDP as well as basal $[Ca^{2+}]_i$ was observed in I/R hearts. In view of the fact that the previous studies have demonstrated that the positive inotropic effect of ATP in control hearts is mediated by its action on purinergic receptors in

cardiomyocytes and subsequent increase in $[Ca^{2+}]_i$ [259,277,310], it is possible that the attenuated response to ATP in 30 min I/R hearts may be due to alterations in purinergic receptors or defects in associated signal transduction mechanisms. Since the affinity ($1/K_d$) of ATP, as indicated by the specific binding of [^{35}S]ATPγS, to purinergic receptors was increased, and the density (B_{max}) was decreased after 30 min of ischemia as well as 30 min ischemia followed by 30 min of reperfusion, it appears that I/R-induced alterations at the purinergic receptor level are of complex nature. On the other hand, depression in ATP-induced increase in $[Ca^{2+}]_i$ in cardiomyocytes isolated from 30 min I/R hearts by cibacrone blue, a well known ATP receptor antagonist, rule out the possibility of any major defect at the receptor level. It is possible that the decreased density of purinergic receptors may explain the attenuated responses of I/R hearts to ATP whereas the increased affinity to [^{35}S]ATPγS may serve as a compensatory mechanism to the decreased receptor density for maintaining the purinergic receptor function in I/R hearts. Therefore, post-receptor defects may be responsible for the altered ATP response in I/R hearts and cardiomyocytes isolated from these hearts. In view of the involvement of phospholipase C (PLC) in the signal transduction of ATP-induced increase in $[Ca^{2+}]_i$ [311] and marked alterations in PLC isoforms during I/R [137], the participation of altered PLC mediated signal transduction mechanisms in depressed ATP responsiveness during I/R seems likely. On the other hand, the depression in ISO, a known β_1-adrenoceptor agonist [246], mediated increase in $[Ca^{2+}]_i$ during I/R hearts as explained earlier may be related to defects at the level of β_1-adrenoceptors in both affinity and density of β_1-adrenoceptors in I/R hearts.

Previous studies have shown the involvement of SL L-type Ca^{2+} channels in ATP-induced increase in $[Ca^{2+}]_i$ [258], which further trigger the mobilization of Ca^{2+} from SR [259]. Since verapamil, a known Ca^{2+} channel antagonist, caused a depression in ATP-induced increase in

$[Ca^{2+}]_i$ in both control and I/R hearts, it is likely that influx through L-type Ca^{2+} channels remain preserved during I/R. It should be noted that Ca^{2+} channel antagonists are known to prevent the I/R-induced increase in $[Ca^{2+}]_i$ [312]; however some investigators have shown the downregulation of SL L-type Ca^{2+} channels [280], while others have revealed no alterations in these receptors after I/R [313]. Furthermore, no difference in KCl, a known depolarizing agent as well as Bay K 8644, a dihydropyridine receptor agonist, mediated increase in $[Ca^{2+}]_i$ in control and I/R hearts as observed earlier rules out the possibility of any defect in L-type Ca^{2+} channels. On the other hand, ATP-induced increase in $[Ca^{2+}]_i$ in I/R cardiomyocytes, unlike control preparations, was not altered by ryanodine, an agent that depresses Ca^{2+} release by depleting SR Ca^{2+} stores as well as by blocking Ca^{2+} channels [266]. Accordingly, it is suggested that abnormal Ca^{2+} handling at the level of SR may be responsible for the depressed ATP-induced increase in $[Ca^{2+}]_i$ in I/R cardiomyocytes. In this regard, decreased protein content and gene expression of ryanodine receptors have been demonstrated in I/R hearts [248]. Furthermore, Zucchi et al. [78] have shown a significant reduction in the density of high affinity and low affinity ^{3}H-ryanodine binding sites in I/R hearts. In addition, protein kinase C, which is known to be activated during I/R [314] and causes decrease in ATP-induced as well as catecholamine-induced increase in $[Ca^{2+}]_i$ [259], may be involved in the depressed ATP and ISO responsiveness in I/R hearts.

There is a possibility that the depressed responsiveness to ATP in I/R hearts may be due to the oxidative stress. This view is supported by the observation that the attenuated ATP-induced increase in $[Ca^{2+}]_i$ in I/R hearts was prevented by an antioxidant mixture containing SOD plus CAT. In fact, oxidative stress has been shown to cause cardiac dysfunction during I/R, which was prevented by SOD plus CAT [101]. In addition, I/R-induced increase in basal $[Ca^{2+}]_i$ was also attenuated by the antioxidant mixture. Furthermore, changes observed in I/R hearts and

cardiomyocytes isolated from these hearts were simulated by H_2O_2 because like I/R hearts, an increase in basal $[Ca^{2+}]_i$ and a depression in ATP-mediated increase in $[Ca^{2+}]_i$ were observed in cardiomyocytes isolated from hearts treated with H_2O_2. On the other hand, Wang et al. [281] have shown that H_2O_2 at high concentrations caused an augmentation of ATP-induced increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes. Although H_2O_2 at the concentration used in the present study has been shown to cause an increase in [^{35}S]ATPγS binding in purified SL membranes [123], defects in SR Ca^{2+} handling may be the major reason for the depression in ATP-induced increase in $[Ca^{2+}]_i$. In addition, H_2O_2 has been known to cause a depression in PLC activity in SL membranes [315], which is associated with ATP-induced increase in $[Ca^{2+}]_i$. The results described in this study also indicate that ischemic preconditioning, which is known to upregulate the antioxidant enzymes in I/R hearts [4] was found to prevent the attenuation of ATP-induced increase in $[Ca^{2+}]_i$ in I/R hearts. It may be noted that ischemic preconditioning caused a depression in basal $[Ca^{2+}]_i$ in I/R hearts and this is in agreement with the previous studies [306]. Furthermore, the participation of IP mediated synchronization of SR Ca^{2+}-pump and release channels [80,248] may be involved in the restoration of ATP-induced increase in $[Ca^{2+}]_i$ in I/R hearts.

In summary, this series of experiments have shown that 30 min of ischemia followed by 30 min of reperfusion caused a marked increase in basal $[Ca^{2+}]_i$ as well as a significant depression in ATP-induced increase in $[Ca^{2+}]_i$; these changes may be associated with defective Ca^{2+} handling at the level of SR. Furthermore, cardiomyocytes isolated from control hearts and undergoing hypoxia-reoxygenation have shown a similar depression in ATP- and ISO-induced increase in $[Ca^{2+}]_i$ as well as a significant increase in basal $[Ca^{2+}]_i$ without any change in KCl response. These alterations in $[Ca^{2+}]_i$ in hypoxic-reoxygenated cardiomyocytes are similar to that observed

in cardiomyocytes isolated from I/R hearts. In addition, the results of the present study indicate that the depression in ATP-induced increase in $[Ca^{2+}]_i$ may explain the attenuated positive inotropic effect of ATP in I/R hearts. Development of oxidative stress due to I/R appears to be an important mediator in causing this decrease in ATP response in I/R cardiomyocytes.

3. Mechanisms of Ca^{2+}-handling by cardiomyocytes upon exposure to catecholamine and ATP

This study has shown that ATP causes an increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes, which effect was potentiated by treatment with ISO. This finding is in agreement with the previous observations [259]. The ISO mediated increase in $[Ca^{2+}]_i$, unlike that due to ATP, was markedly inhibited by propranolol indicating the involvement of β-ARs in the ISO-mediated increase in $[Ca^{2+}]_i$. Similarly, Zheng et al. [251] have shown that the ISO-induced increase in the ratio of changes in florescence level ($\Delta F_{ATP}/\Delta F_{KCl}$) was significantly attenuated by propranolol after the addition of ATP and KCl. The effect of β-AR blockade on ISO-induced Ca^{2+}-mobilization may be of some specific nature because basal $[Ca^{2+}]_i$ remained unaltered in the presence of propranolol. The involvement of extracellular Ca^{2+} entry by L-type Ca^{2+} channels in the ISO-mediated increase in $[Ca^{2+}]_i$ was evident from the observation that low concentrations (1 μM) of verapamil and diltiazem, which are sufficient to block the SL L-type Ca^{2+} channels [266], produced a marked reduction in the ISO-induced increase in $[Ca^{2+}]_i$. The ATP-induced increase in $[Ca^{2+}]_i$ was also attenuated by treatment with these antagonists. Thus an increase in the amplitude of inward Ca^{2+} current through the SL L-type Ca^{2+} channels [251] and β-AR—cAMP—PKA pathway mediated phosphorylation of L-type Ca^{2+} channels [316] may be the mechanism of ISO and ATP mediated increase in Ca^{2+}-influx. Although β-AR stimulation mediated Ca^{2+} entry by L-type Ca^{2+} channels has been linked with Ca^{2+} release from the SR Ca^{2+}-stores [317], the ISO-

mediated potentiation of the ATP-induced increase in $[Ca^{2+}]_i$ seems to be independent of Ca^{2+} release from the intracellular stores because ryanodine, an inhibitor of the SR Ca^{2+} release channel [282,283], did not affect the ISO-mediated increase in $[Ca^{2+}]_i$. Similarly, DeYoung and Scarpa [261] have shown that the potentiation of ATP response by norepinephrine, a non-specific β-AR agonist, remained unaltered by depletion of the intracellular Ca^{2+}-stores by ryanodine. The involvement of SR Ca^{2+}-stores in the ISO-mediated increase in $[Ca^{2+}]_i$ was further ruled out by the observation that CPA, a well known inhibitor of the SR Ca^{2+}-pump ATPase [285], did not alter the ISO responsiveness in isolated cardiomyocytes. On the other hand, the ATP-mediated increase in $[Ca^{2+}]_i$ was depressed in the presence of both ryanodine and CPA demonstrating that SR Ca^{2+}-stores play an important role in the ATP-mediated increase in $[Ca^{2+}]_i$.

The contribution of SL Ca^{2+}-pump ATPase, which is known to cause efflux of intracellular Ca^{2+} [318], in the ATP-mediated increase in $[Ca^{2+}]_i$ was apparent from the observation that low concentrations of vanadate (1 and 2 μM), a SL Ca^{2+}-pump ATPase blocker [284], caused a significant elevation of the $[Ca^{2+}]_i$. In contrast, the ISO-mediated potentiation of the ATP-induced increase in $[Ca^{2+}]_i$ was attenuated by vanadate treatment. Since the level of $[Ca^{2+}]_i$ is determined by a balance between Ca^{2+}-influx and Ca^{2+}-efflux, and catecholamines are known to augment both these processes [316,318], the observed decrease in the potentiation of ATP-induced increase in $[Ca^{2+}]_i$ by ISO in the presence of vanadate may be due to greater stimulation of Ca^{2+}-efflux than Ca^{2+}-influx. Such an effect of ISO in the vanadate treated preparations may occur as a consequence of release of the inhibitory action of vanadate on the SL Ca^{2+}-pump ATPase by ISO. This and other possibilities such as the status of Ca^{2+}-pump mechanisms in the SR membrane in the presence of vanadate remain to be examined for a meaningful conclusion.

Since high concentrations of verapamil and diltiazem (10 μM) have been shown to depress the SL Na^+-Ca^{2+} exchange activity [319], it is possible that inhibition of the ISO or ATP mediated increase in $[Ca^{2+}]_i$ by these agents may be due to a non-specific inhibition of Na^+-Ca^{2+} exchanger in addition to the SL L-type Ca^{2+} channels. The involvement of Na^+-Ca^{2+} exchanger in catecholamine mediated increase in $[Ca^{2+}]_i$ was evident upon the use of amiloride and Ni^{2+}, inhibitors of Na^+-Ca^{2+} exchanger, which caused a marked attenuation of the ISO-mediated increase in $[Ca^{2+}]_i$; similar depression in ATP response was also observed by these blockers. It is pointed out that amiloride has also been reported to cause inhibition of NHE [288]; therefore, the involvement of NHE in the ISO- or ATP- mediated increase in $[Ca^{2+}]_i$ cannot be ruled out. The participation of the Na^+-Ca^{2+} exchanger in the catecholamine-mediated increase in $[Ca^{2+}]_i$ was further demonstrated by treatment of cardiomyocytes with KB-R7943, a specific inhibitor of Ca^{2+}-influx mode of Na^+-Ca^{2+} exchanger [271]. Both ISO- and ATP-mediated increases in $[Ca^{2+}]_i$ were depressed by KB-R7943 treatment. From these observations it seems likely that extracellular Ca^{2+} entry via SL Na^+-Ca^{2+} exchanger plays a crucial role in the ATP- or ISO-mediated increase in $[Ca^{2+}]_i$. This observation was further confirmed by the finding that the combination of KB-R7943 and verapamil showed an additive effect on the ISO-mediated increase in $[Ca^{2+}]_i$. This experiment indicates that both SL L-type Ca^{2+} channels and SL Na^+-Ca^{2+} exchanger may be involved in eliciting the increase in $[Ca^{2+}]_i$ due to ISO. Although under physiological conditions Na^+-Ca^{2+} exchanger is an important mechanism of Ca^{2+}-efflux [320], an increase in intracellular Na^+ or a change in trans-sarcolemmal potential causes the reversal of the exchanger to mediate Ca^{2+} entry [291]. In this context, ATP has been shown to cause depolarization of the membrane [251,258] and the induction of $^{22}Na^+$ influx in the isolated cardiomyocytes [321]. In addition, PKA-mediated phosphorylation of Na^+-Ca^{2+} exchanger

subsequent to increase in cAMP concentration by ISO treatment [262] may be the mechanism of catecholamine-induced increase in $[Ca^{2+}]_i$.

While low Na^+ has been shown to cause an increase in Ca^{2+} entry in isolated cardiomyocytes by activation of SL Na^+-Ca^{2+} exchanger [266], the contribution of Na^+-Ca^{2+} exchanger in catecholamine-induced increase in $[Ca^{2+}]_i$ was observed in the presence of low Na^+. KCl, a known depolarizing agent, was found to cause a significant increase in $[Ca^{2+}]_i$ in presence of low Na^+; this is in agreement with our previous observations [266]. In addition, ISO- mediated potentiation of KCl response was further augmented by low Na^+. It is pointed out that this increase in $[Ca^{2+}]_i$ by ISO treatment in KCl depolarized cardiomyocytes was markedly depressed by KB-R7943 indicating that Ca^{2+}-influx through Na^+-Ca^{2+} exchanger may be involved in catecholamine-mediated increase in $[Ca^{2+}]_i$. Furthermore, the positive inotropic effect of ISO in terms of increase in LVDP, which is known to be associated with an increase in $[Ca^{2+}]_i$ [322], was significantly depressed in the presence of KB-R7943; this observation confirms the contribution of reverse mode of Na^+-Ca^{2+} exchanger in the ISO-mediated increase in $[Ca^{2+}]_i$. The depressant effect of KB-R7943 on the basal cardiac function in the isolated rat heart is in agreement with previous report [323] and is likely to be due to the inhibition of SL Na^+-Ca^{2+} exchanger.

In conclusion, our observations suggest that SL Na^+-Ca^{2+} exchanger plays an important role in catecholamine-mediated increase in $[Ca^{2+}]_i$ besides the Ca^{2+} entry through L-type Ca^{2+} channels. However, it cannot be determined on the basis of data presented here, that the increase in $[Ca^{2+}]_i$ by catecholamines is induced by a decrease in Ca^{2+}-efflux or an increase in Ca^{2+}-influx by Na^+-Ca^{2+} exchanger, as non-specific inhibitors of Na^+-Ca^{2+} exchanger, amiloride and Ni^{2+}, are known to depress both the transport modes of the exchanger [289,324]. Additionally, ISO has

been shown to activate both the inward and outward current generated by Na^+-Ca^{2+} exchanger [262]. Nonetheless, the results of the present study in terms of inhibition of ISO response by a selective inhibitor of Ca^{2+} entry mode of Na^+-Ca^{2+} exchanger, KB-R7943, particularly when stimulated by low Na^+, indicate an important role of reverse mode of Na^+-Ca^{2+} exchanger in catecholamine-mediated increase in $[Ca^{2+}]_i$. Although the results in this study indicate that both SL L-type Ca^{2+} channels and SL Na^+-Ca^{2+} exchanger may participate in eliciting the ISO-induced increase in $[Ca^{2+}]_i$ in isolated cardiomyocytes, the exact contribution of each site cannot be determined on the basis of pharmacological approach employed here.

4. Role of NHE in cardiac dysfunction due to I/R and mechanisms of its inhibition in cardiomyocyte Ca^{2+}-handling

The results described in this study show an inconsistent beneficial effect of MIA on the I/R-induced alterations in cardiac function. Although 40% of the hearts recovered from I/R-mediated depression in cardiac function, no recovery in heart function was observed in the remaining 60% of I/R hearts treated with MIA. Similarly, Shimida et al. [325] have shown no improvement in cardiac function in isolated hearts treated with 5-(N,N-dimethyl) amiloride (DMA), a known NHE inhibitor, under similar experimental conditions. Although Moffat and Karamazyn [270] have shown the protective effect of MIA in terms of reduction in I/R-induced alterations in rates of force development and decay, no effect of MIA on high energy phosphate stores or on the levels of creatine kinase release was observed. No improvement in heart function was observed by treating the I/R hearts with NHE inhibitors, DMA or HOE-642, in HCO_3^- containing buffer during the reperfusion period [325]. By using other amiloride derivatives such as DMA, 5-(N,N-hexamethyl) amiloride (HMA) and ethylisopropyl amiloride (EIPA), Meng et al. [189] have shown the improvement in I/R-induced alterations in developed and resting tension in right

ventricular wall by perfusing the drug only for 10 min during the reperfusion period after 60 min of global ischemia in HCO_3^- free buffer. One of the reasons for the lack of beneficial effect in our study may be due to the presence of Na^+-HCO_3^- symporter in the HCO_3^- containing K-H buffer as the Na^+-HCO_3^- symporter plays an important role in the recovery of acidic pH during the reperfusion period [326]. It is pointed out that activation of Na^+-HCO_3^- symporter leads to the accumulation of Na^+ with subsequent accumulation of Ca^{2+}. Furthermore, various studies have shown that inhibition of Na^+-HCO_3^- symporter, in addition to NHE, may be a better option to protect the hearts due to I/R-induced cardiac dysfunction [327-329]. Since the effect of NHE inhibition in I/R hearts has been reported to be overestimated in HCO_3^- free buffer [325], no attempt was made to determine the effect of MIA on I/R-mediated injury in HCO_3^- free buffer.

The increase in mitochondrial Ca^{2+}-overload may also be responsible for the lack of beneficial effect of MIA in the present study. In this context, various investigators have shown that an increase in mitochondrial Ca^{2+} attenuates the reperfusion-induced recovery in ischemic myocardium [178,226]. Another NHE inhibitor, cariporide, has also been shown to enhance the mitochondrial Ca^{2+}-overload during simulated ischemia [330]. On the other hand, Yamamoto et al. [331] have shown the protective effect of SM-20550, a known NHE inhibitor, on I/R-induced changes in mitochondrial respiration, oxidative phosphorylation rate and Ca^{2+}-overload in mitochondria. However, this effect of SM-20550 may not be solely related to NHE inhibition property of this agent as other agents of this category have been shown to have free radical scavenging property [332]. In addition, EMD87580, another NHE inhibitor, which has an anti-hypertrophic effect and improves the mitochondrial function, has been shown to reduce the phenylephrine-induced superoxide generation [333]. It was noted that all the I/R hearts treated with MIA, irrespective of the recovery, showed higher rate of ventricular arrthymias as compared

to I/R hearts. Similarly, 50-100% of I/R hearts treated with other NHE inhibitors such as DMA and HOE-642 exhibited tachyarrhythmias during the reperfusion period [325]. Amiloride derivatives which are commonly used as NHE inhibitors have also the tendency to produce extra systoles, the irregular beats which do not follow the electrical stimulus [192]. Although Tani et al. [334] have shown the anti-arrthymic effect of MIA in I/R hearts, the time period of ischemia was 25 min as compared to 60 min in the present study. It is important to note that the extent of reperfusion-induced arrthythmias are linearly related with the occluded ischemic-zone size [335] and 60 min of ischemia may cause a greater increase in reperfusion-arrhythmias that cannot be attenuated by treatment with MIA.

The treatment with MIA did not exert any effect on Ca^{2+}-paradox induced depression in LVDP. Previous studies have also shown no improvement in contractile function after treatment with HMA on hearts undergoing 5 min Ca^{2+}-free perfusion followed by 30 min of Ca^{2+}-repletion period. It was concluded that the damage caused by this Ca^{2+}-paradoxic protocol was not reversible by pharmacological inhibition of NHE [336]. Even in the mild Ca^{2+}-paradoxic conditions (3 min Ca^{2+}-depletion followed by 10 min of Ca^{2+}-repletion phase), as employed in the present study, LVEDP was significantly higher in MIA treated Ca^{2+}-paradoxic hearts than the untreated hearts. The increase in basal $[Ca^{2+}]_i$ by MIA as seen in cardiomyocytes may be the reason for such an effect. In this context, we have shown in this study that there is a positive correlation between the LVEDP and the increase in basal $[Ca^{2+}]_i$ in I/R hearts. Nonetheless, the lack of beneficial effects of MIA in the I/R myocardium may be due the development of intracellular Ca^{2+}-overload and the tendency to enhance reperfusion-induced arrhythmias. The failure of NHE inhibitors in clinical trials [193] in terms of reducing the mortality seems to be primarily inconclusive because preclinical studies were based upon the protective effects of NHE

inhibitors on I/R-mediated depression in cardiac function. In particular, these studies were concerned with respect to the specificity of different compounds as NHE inhibitors rather than their protective effects. It is pointed out that the effects of MIA in the concentration range (1 to 10 μM) employed in this study are due to selective inhibition of NHE because the IC_{50} value for MIA with respect to NHE inhibition was 14 μM whereas those for Na^+- Ca^{2+} exchanger, Ca^{2+}-pump and Na^+-pump were 84, 70 and >300 μM, respectively [337]. However, the results presented in this thesis do not support the beneficial effect of NHE inhibitors on the ischemic myocardium.

By employing cardiomyocyte preparations, it was observed that inhibition of NHE by MIA caused a significant increase in basal $[Ca^{2+}]_i$. This increase in basal $[Ca^{2+}]_i$ seems to be specific to the derivatives of amiloride as amiloride itself did not show any increase in basal $[Ca^{2+}]_i$ under similar experimental conditions, whereas DMA, a selective NHE inhibitor [236], caused a significant increase in basal $[Ca^{2+}]_i$. The increase in basal $[Ca^{2+}]_i$ by MIA appears to be independent of extracellular Ca^{2+} concentration because inhibition of SL L-type Ca^{2+} channels or Na^+-Ca^{2+} exchanger did not affect the MIA-mediated increase in $[Ca^{2+}]_i$. In addition, the MIA-induced increase in basal $[Ca^{2+}]_i$ remained unaltered by changing the extracellular Ca^{2+} concentration. It should also be noted that MIA-mediated increase in basal $[Ca^{2+}]_i$ was not altered by treatment with SL Ca^{2+}-pump ATPase and mitochondrial Ca^{2+}-uptake inhibitors. On the other hand, the involvement of SR Ca^{2+}-stores in modifying MIA-mediated increase in basal $[Ca^{2+}]_i$ is apparent from the observation that depletion of SR Ca^{2+} stores by ryanodine, caffeine and CPA attenuated the MIA-induced increase in basal $[Ca^{2+}]_i$ in quiescent cardiomyocytes. Since basal Ca^{2+} was increased upon reducing the intracellular pH as observed in the present study as well as previously [338,339], it is likely that MIA-mediated reduction in intracellular pH is responsible

for such an increase in basal $[Ca^{2+}]_i$. Orchard et al. [269] have shown that acidosis facilitates the spontaneous SR Ca^{2+} release in rat myocardium which can be abolished by treatment with ryanodine and caffeine. Furthermore, acidosis has been shown to reduce SR Ca^{2+}-uptake [340] as well as affect the L-type Ca^{2+}-channels and Na^{+}-Ca^{2+} exchange activities [341,342]. In view of the linear relationship between decrease in intracellular pH and basal $[Ca^{2+}]_i$ observed in this study, it appears that the increase in basal $[Ca^{2+}]_i$ by NHE inhibition may be as a consequence of decrease in pH with a subsequent release of Ca^{2+} from SR as well as changes in SL Ca^{2+}-transport. It is also pointed out that MIA was found to exert no direct effect on SR Ca^{2+}-uptake or SR Ca^{2+}-release under in vitro conditions (unpublished observations). Although the increase in basal $[Ca^{2+}]_i$ by NHE inhibition may not represent a marked intracellular Ca^{2+} overload, it can affect the steady state $[Ca^{2+}]_i$ over a certain period of time.

It is now well documented that KCl-mediated increase in $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+} concentration and is mediated by both SL and SR Ca^{2+} regulating sites [266,296]. The results of the present study show that KCl-mediated increase in $[Ca^{2+}]_i$ was augmented by treatment with MIA. Such an augmentation seems to be a consequence of changes in both SL and SR Ca^{2+} regulating sites. This view is based on the observations that SL L-type Ca^{2+} channel blockers, Na^{+}-Ca^{2+} exchange inhibitor and SR Ca^{2+} store modulating agents attenuate the MIA-mediated augmentation of KCl response. On the other hand, mitochondrial Ca^{2+}-uptake and SL Ca^{2+}-pump ATPase inhibitors did not prevent the KCl-induced increase in $[Ca^{2+}]_i$ or MIA-mediated potentiation of KCl response. Cui et al. [343] have shown that DMA, another NHE inhibitor, increased the Ca^{2+}-transient and cell shortening in isolated cardiomyocytes, which are mediated by stimulating the Na^{+}-Ca^{2+} exchanger without the involvement of Ca^{2+} entry through L-type Ca^{2+} channels. The differences in the regulation of

$[Ca^{2+}]_i$ in electrically stimulated Ca^{2+} transients and KCl-depolarized cardiomyocytes may be the reason for such discrepancy. It is important to note that extracellular K^+ has been reported to inhibit NHE-1, the most abundant NHE isoform in cardiomyocytes [344] and thus the participation of such an inhibition by KCl under the experimental conditions used in the present study cannot be ruled out. It should also be noted that the KCl-induced increase in $[Ca^{2+}]_i$ in contrast to the basal $[Ca^{2+}]_i$, was depressed by decreasing the extracellular pH from 6.0 to 5.5. This is consistent with the observations for the decrease in Ca^{2+}-transient due to acidosis [338].

It was interesting to observe that ouabain, a Na^+-K^+ ATPase inhibitor [292], caused a marked increase in basal as well as KCl-mediated augmentation of $[Ca^{2+}]_i$. These results are in agreement with our previous observations [266]. Since ouabain is known to increase $[Ca^{2+}]_i$ by promoting the entry of Ca^{2+} through Na^+-Ca^{2+} exchanger [292], it was expected that MIA-induced increase in $[Ca^{2+}]_i$ will be depressed after treatment with ouabain. This was found to be the case in the present study indicating the interaction of ouabain with MIA. Nakanishi et al. [345] have also shown the attenuation of ouabain-induced increase in $[Ca^{2+}]_i$ after NHE inhibition. It should be mentioned that Bolck et al. [346] have demonstrated that Na^+-Ca^{2+} exchanger overexpression results in reduction of cell shortening at higher stimulation frequencies and after inhibition of Na^+-K^+ ATPase by ouabain. Furthermore, low Na^+, which is known to enhance the Na^+-Ca^{2+} exchanger activity [266], did not show any effect on the MIA-induced increase in basal $[Ca^{2+}]_i$ whereas it depressed the MIA-mediated augmentation of KCl response. Thus it appears that the results regarding the interaction of MIA and ouabain on the KCl-induced increase in $[Ca^{2+}]_i$ may be a consequence of both the direct action of ouabain on SL Na^+-K^+ ATPase and indirect effect of MIA on SL Na^+-Ca^{2+} exchanger. In this regard, the contribution of

alterations in intracellular pH cannot be ruled out as ouabain has been shown to produce a significant decrease in pH both in the presence and absence of a NHE inhibitor [292].

The results in this study suggest that MIA-mediated alterations in $[Ca^{2+}]_i$ mobilization are regulated differently in quiescent and depolarized cardiomyocytes. Although NHE is an important SL site for promoting the efflux of H^+ and thus leading to the decrease of metabolic acidosis due to its inhibition by agents such as MIA [347,348], its contribution under normal physiological conditions is controversial [349-351]. Kusuoka et al. [352] have demonstrated that NHE works actively in physiological range of pH in isovolumically- contracting hearts and blockade of this exchanger caused a decrease in pH under steady state conditions. Prolonged inhibition of NHE by MIA with a significant decrease in pH as observed previously [270] and in the present study indicates that this exchanger is active under the conditions employed here. Since we have used a pharmacological approach to obtain some information regarding the role of NHE in regulating the $[Ca^{2+}]_i$ in cardiomyocytes, it is understood that further experiments by employing molecular approaches are needed to investigate the involvement of a particular site in NHE mediated alterations in $[Ca^{2+}]_i$. Studies employing site-specific mutagenesis and transgenic animals with NHE knock out may further unravel the mechanisms for such an effect of NHE inhibition on Ca^{2+}-handling by cardiomyocytes. Although it can be argued that the Ca^{2+}-handling experiments in the present study were conducted in the presence of buffer containing HCO_3^- and thus the changes observed here may not be the true reflection of NHE inhibition in cardiomyocytes, it is important to note that changes in basal $[Ca^{2+}]_i$ and MIA-mediated augmentation of KCl response as well as MIA-induced alterations in pH were qualitatively similar in the absence and presence of buffer containing HCO_3^-. In this context, Ruiz-Meana et al. [353] have also shown that metabolic-inhibition mediated increase in intracellular Ca^{2+} and Na^+ in the presence of HOE 642

(cariporide), a selective Na^+-H^+ exchange inhibitor, was independent of HCO_3^- in the perfusion medium.

Although cardiotoxicity of amiloride derivatives have limited their use as an experimental tool [337], no such effect of MIA on cardiomyocyte viability was observed in the present study. Nonetheless, the occurrence of intracellular Ca^{2+}-overload by MIA in cardiomyocytes as observed in this study can be considered to explain the cardiotoxic effects of high concentrations of amiloride derivatives [337]. Since we did not compare the effects of MIA with some of the newer NHE inhibitors such as cariporide, it is difficult to comment on the clinical significance of the results presented in this study. However, it should be mentioned that cariporide has been reported not to depress the anoxia-induced intracellular Ca^{2+} overload [279]. Similarly, the metabolic inhibition-mediated increase in intracellular Na^+ and Ca^{2+} was not prevented by cariporide treatment [353]. On the other hand, chemical hypoxia-mediated Ca^{2+}-accumulation was attenuated by cariporide treatment [354]. Although cariporide was observed to produce a beneficial effect on heart function in a clinical trial (23), this agent was found to produce an increase in the incidence of stroke. In fact, thrombotic stroke and platelet aggregation in cariporide treated patients [355] were apparently not a class effect of NHE inhibitors. Thus a great deal of caution should be exercised while interpreting the observations with MIA in this study in terms of explaining the potential toxicity of the newer NHE inhibitors.

5. Significance of the study in relation to IHD

While IHD is a major and multifacet problem, the present study has attempted to focuses only on one issue which is related to establishing reflow to the ischemic myocardium. It is now becoming evident that cardiac dysfunction is invariably apparent while instituting reflow by procedures such as coronary bypass, angioplasty and thrombolytic therapy used for removing the blockade of

coronary flow in the ischemic heart. This phenomenon has been attributed to reperfusion injury but inspite of extensive efforts by several investigators no satisfactory solution has been found for the salvage of the ischemic heart. This study has provided evidence that cardiac dysfunction of the ischemic heart becomes irreversible depending upon the duration of ischemic insult and this reperfusion injury is associated with Ca^{2+}-handling abnormalities in cardiomyocytes. It has also been shown that the signal transduction mechanisms for both catecholamines and ATP, which provide support for appropriate cardiac performance, become defective in I/R hearts. Furthermore, this study has demonstrated that the I/R-induced abnormalities in cardiac function and Ca^{2+}-handling by cardiomyocytes are not only simulated by an oxidant H_2O_2, but are also attenuated by an antioxidant mixture containing SOD and CAT. Thus it appears that an appropriate antioxidant therapy before reinstituting reflow may prove beneficial in preventing adverse effects of I/R injury. Although activation of NHE, like oxidative stress, is considered to play a critical role in the development of intracellular Ca^{2+}-overload and cardiac dysfunction in the I/R hearts, inhibition of NHE by MIA was not observed to be beneficial. The ineffectiveness of NHE inhibition in I/R hearts was primarily due to the accumulation of H^+ and subsequent release of Ca^{2+} from SR Ca^{2+}-stores. The results described in this study therefore support the view that antioxidants, unlike NHE inhibitors, may offer cardioprotection to the ischemic myocardium upon reperfusion.

VI. CONCLUSIONS

1. By employing isolated perfused rat heart, the irreversible stage of I/R injury with respect to cardiac dysfunction and Ca^{2+}-handling abnormalities in cardiomyocytes was observed to depend upon the duration of ischemic insult.
2. There occurs a loss of adrenergic and purinergic support in the I/R hearts because both catecholamine- and ATP-induced increase in intracellular Ca^{2+} in cardiomyocytes from I/R hearts were impaired.
3. I/R-induced cardic dysfunction as well as increase in basal $[Ca^{2+}]_i$ and defects in both catecholamine- and ATP-induced changes in Ca^{2+}-handling in cardiomyocytes were attenuated by an antioxidant mixture containing SOD and CAT as well as by ischemic preconditioning.
4. Alterations in cardiac function and Ca^{2+}-handling abnormalities in I/R hearts were simulated by perfusing the hearts with an oxidant, H_2O_2.
5. Although both catecholamine and ATP-induced an increase in intracellular Ca^{2+} in cardiomyocytes, these agents were observed to affect different subcellular sites involved in the regulation of $[Ca^{2+}]_i$.
6. An inhibitor of NHE, MIA, did not produce any beneficial effect on I/R-induced or Ca^{2+}-paradox induced defects in cardiac function. The ineffectiveness of MIA in producing beneficial effects in I/R hearts was observed to be due to accumulation of H^+ and subsequent release of Ca^{2+} from SR.
7. This study suggest that both the occurrence of oxidative stress and activation of NHE are involved in the regulation of $[Ca^{2+}]_i$ but antioxidant, unlike NHE inhibitors, may exert beneficial effects in attenuating I/R injury.

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