ALTERATIONS IN CARDIAC IONIC CURRENTS INDUCED BY OXYGEN-DERIVED FREE RADICAL STRESS

BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

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Dedicated to.....

my parents

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List of Abbreviations

Action potential	AP
Action potential duration after 90% repolarization	APD_{90}
Adenosine 5'-diphosphate	ADP
Adenosine 5'-triphosphate	ATP
4-Aminopyridine	4-AP
Ampere	A
ATP-sensitive potassium current	I_{KATP}
1,2 bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid	BAPTA
Calcium-induced calcium release	CICR
Catalase	CAT
Cumen hydroperoxide	CH
Current	I
Current-voltage	I-V
Cyclic adenosine 3'-5'-monophosphate	c-AMP
Delayed after-depolarization	DAD

Delayed rectifier K^+ current	I_K
Diazenenedicarboxylic acid bis-N-N' dimethylamide	diamide
Dihydroxyfumaric acid	DHF
Dithiothreitol	DTT
Early after-depolarization	EAD
Ethyleneglycol-bis(beta-aminoethyl ether)-N,N'-tetraacetic acid	EGTA
Excitation-contraction	E-C
Extracellular concentration	$[\ ]_o$
Hydrogen peroxide	H_2O_2
Hydroxyl radical	$OH^{\cdot}$
N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid	HEPES
Inward K^+ rectifier current	I_{K1}
Intracellular concentration	$[\ ]_i$
Lipid carbon centered radical	$L^{\cdot}$
Lipid peroxy radical	$LOO^{\cdot}$

Lipid peroxide	LOOH
L-type Ca^{2+} current	$I_{Ca,L}$
Maximal rate of depolarization	$\dot{V}_{max}$
Minute	min
Millivolt	mV
Na^{+}-Ca^{2+} exchange current	I_{NaCa}
Na^{+}/K^{+} pump current	I_{NaK}
NanoAmpere	nA
N-methyl-D-glucamine	NMG
Non-selective cation current	I_{NSC}
Oxidized glutathione	GSSG
Oxygen-derived free radicals	O-R
PicoAmpere	pA
Premature ventricular complex	PVC
Reduced glutathione	GSH
Resting membrane potential	RMP
Reversal potential	E_{rev}

Rose Bengal	RB
Sarcolemma	SL
Sarcoplasmic reticulum	SR
Seconds	sec
Singlet oxygen	$^1O_2$
Superoxide anion	$\cdot O_2^-$
Superoxide dismutase	SOD
tert-butyl hydroperoxide	t-BHP
Tetraethylammonium	TEA^+
Tetrodotoxin	TTX
Transient inward current	I_{ti}
Transient outward current	I_{to}
Ventricular fibrillation	VF
Ventricular tachycardia	VT
Volt	V
Xanthine oxidase	XO

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ABSTRACT

Oxgen-derived free radicals (O-R) are believed to induce arrhythmogenic alterations in cardiac electrical activity. However, the changes in membrane ionic currents which underlie O-R-induced arrhythmogenesis, the mechanisms by which O-R induce their effects and whether there may be differential effects of intracellular versus extracellular O-R are poorly defined. Therefore, in this study we investigated the differential effects of intra- versus extracellular O-R stress on membrane electrical activity, determined the underlying changes in steady-state ionic currents, and identified the cellular mechanisms responsible for modified channel activity. This was achieved by employing whole-cell variant of the patch clamp technique to measure macroscopic currents from guinea pig ventricular myocytes exposed to either intra- or extracellular O-R stress. Oxy-radicals were generated from the combination of dihydroxyfumaric acid (DHF; 3-6 mM) and $FeCl_3$:ADP (0.05:0.5 mM). Intracellular exposure was obtained by adding the O-R generating system to the patch pipette whereas extracellular exposure involved addition of DHF-Fe^{3+}:ADP to the bath solution. Time-dependent changes in resting membrane potential (RMP), action potential (AP) configuration and quasi steady-state currents were measured during exposure to O-R. Intracellular dialysis with O-R generating solution induced three stages of changes; (1) an early depolarization (5-10 mV) and an increase in AP duration accompanied by a

decrease in I_{K1} conductance, (2) delayed after-depolarizations and triggered activity caused by the activation of transient inward currents (I_{ti}) mediated by sodium-calcium (Na^+-Ca^{2+}) exchange as well as failure to repolarize and sustained depolarization (between -35 and -20 mV) due to stimulation of a non-selective cation current (I_{NSC}), and finally, (3) a late stage of marked decline in AP duration, hyperpolarization, and loss of excitability resulting from the activation of outward current through ATP-sensitive K^+ channels. These alterations in electrical activity and membrane currents could be prevented by pretreatment with N-(2-mercaptoproprionyl)-glycine (500 μM), a scavenger of hydroxyl free radicals. The alterations associated with stages 1 and 2 but not stage 3 were completely abolished upon intracellular Ca^{2+} chelation (5 mM EGTA pipette solution) or disruption of SR Ca^{2+} handling with ryanodine (10 μM). These data indicate a dependence of I_{NSC} on abnormal Ca^{2+} handling by the sarcoplasmic reticulum (SR) resulting from O-R stress.

Superfusion of guinea pig ventricular myocytes with bath solution containing O-R generating system (DHF-Fe^{3+}:ADP) showed; (1) sustained depolarization to between -35 and -20 mV and (2) low amplitude oscillations in membrane potential and triggered activity. The oscillations in membrane current were due to transient inward current (I_{ti}) sensitive to Li^+ replacement with external Na^+. Sustained depolarization resulted from the activation of I_{NSC} reversing at ~-20 mV, similar to that observed during intracellular O-R stress, and unaffected by substitutions of Cs^+ and Li^+ for K^+ and Na^+, respectively. However, activation of

I_{NSC} did not appear to require a change in $[Ca^{2+}]_i$. Disruption of SR Ca^{2+} handling by pretreatment with 10μM ryanodine, or intracellular Ca^{2+} chelation with 5 mM EGTA in the pipette solution, blocked I_{ti} evoked by extracellular O-R but did not prevent activation of I_{NSC}. Moreover, I_{NSC} as still observed in the complete absence of Na^+ and Ca^{2+} in the bath solution and 5 mM BAPTA in pipette solution. An alternative mechanism for activation of I_{NSC} was explored. Pretreatment with the sulfhydryl group reducing agent, dithiothreitol (DTT; 1 mM) prevented activation of I_{NSC} and the change in current after O-R could also be reversed with this agent. The sulfhydryl group oxidizing agent, diamide (0.5 mM), was also found to activate I_{NSC}, and the increase in current was also sensitive to DTT. The data indicate that the channels may be directly modified by oxidation of sulfhydryl groups by O-R. In conclusion, this study shows that intracellular and extracellular O-R stress cause specific alterations in membrane ionic currents leading to changes in RMP and AP configuration which are arrhythmogenic. The data provide the first evidence that differential mechanisms can underlie the effects on channel activity of O-R stress in the extracellular compared to the intracellular compartment.

I. REVIEW OF THE LITERATURE

1. Introduction:

Normal heart function depends on periodic waves of electrical activity which trigger contraction in an ordered sequence to pump blood from the ventricular cavities into the pulmonary and peripheral vascular beds (Katz, 1992). Electrical activity of cardiac myocytes is dependent upon the presence of electrochemical gradients for ions across the cell membrane, or sarcolemma (SL), and the selective permeability of this membrane to different ions. Movement of ions across the SL and internal membrane systems is controlled by ionic channels and exchange mechanisms which are integral proteins imbedded in the membrane lipid bilayer. Alterations in the activities of these proteins can result in abnormal electrical activity, such as arrhythmias (Hoffman and Rosen, 1981), leading to contractile failure (Katz, 1992), improper pumping of blood to the periphery, and death.

Arrhythmic electrical activity is frequently observed during reperfusion of the heart after a period of ischemia, open heart surgery, angioplasty or thrombolytic therapy (Goldberg *et al.*, 1983; Tzivoni *et al.*, 1983; Rubin *et al.*, 1985). One of the factors which may play a major role in the genesis of these arrhythmias is a burst of oxygen-derived free radicals (O-R) during early reperfusion (Manning and Hearse, 1984; Garlick *et al.*, 1987). Oxygen-derived free radicals induced

alterations in membrane electrical activity are well described in the literature, however, the underlying changes in ion channel activity are poorly documented. Moreover, the cellular mechanism(s) inducing the changes in ion channel activities due to O-R are unclear. Determining such mechanisms is required in order to be able to provide strategies for the prevention of O-R mediated reperfusion arrhythmias. This review of the literature documents our current understanding of the ionic basis of the cardiac action potential and membrane currents which may contribute to abnormal electrical activity due to O-R stress, and the role of O-R in reperfusion injury.

2. Ionic Basis of the Action Potential:

The electrical activity of the heart *in situ*, as monitored by non-invasive surface extracellular electrodes, reflects the contribution of thousands of electrically coupled individual cells. To measure the electrical activity from single cardiac myocytes in intact multicellular preparations, conventional intracellular microelectrodes are used to impale the cells (Draper and Weidmann, 1951). This technique measures the transmembrane potential difference across the SL between the cytosol of the myocyte and the extracellular space. Excitation produces characteristic changes in transmembrane voltage referred to as an action potential (AP; Fozzard and Arnsdorf, 1992). An AP is a sudden regenerative depolarization followed by repolarization back to a negative resting transmembrane potential (RMP). This event lasts for between 200 and 800 msec depending on the species and region within the heart. The major disadvantage

of the intracellular recording technique is that it does not permit investigations concerning the movements of particular ions across the membrane which underlie the action potential. The recent discovery of the patch clamp technique and methods for isolation of healthy single myocytes provided direct ways to; (1) measure the action potential, (2) determine the underlying macroscopic, whole-cell ionic currents, and (3) record the activity of the single channels contributing to ion movements across the membrane. The following documents our current understanding of the ionic basis of the action potential in ventricular myocytes, which were the subject of my investigation on the effects of O-R on cardiac electrical activity.

The cardiac ventricular myocyte is not a spontaneously active cell type (i.e., it is not a cardiac pacemaker), and therefore, requires an external electrical stimulus to evoke an AP. *In situ*, local circuit currents spreading ahead of a propagating AP serve to depolarize the membrane potential to threshold and evoke an AP in an all-or-none fashion (Fozzard and Arnsdorf, 1992). The changes in transmembrane voltage during an AP are the sum of ionic currents generated by populations of several different ionic channels in the cell membrane. The abundance, activation, conductance and inactivation properties of these channels determine the AP configuration. In general, ion exchange proteins function to maintain ionic gradients across SL, however, some contribution to the AP by electrogenic transporters may be present.

Figure 1 shows diagramatic representation of a ventricular AP. As is

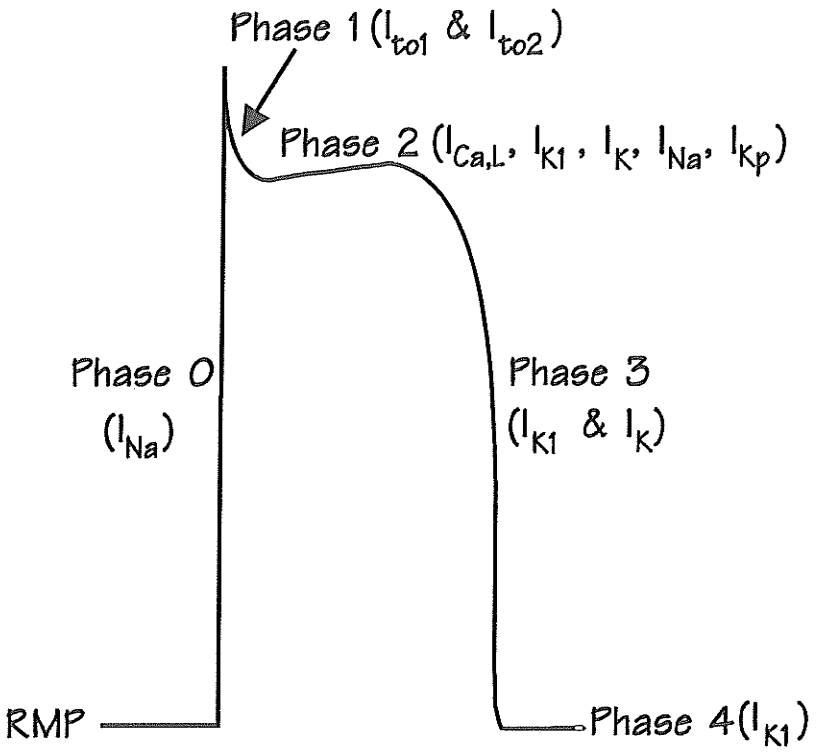

FIGURE 1: ***Diagrammatic Representation of a Cardiac Ventricular Action Potential.***

evident from the figure, the AP has been dissected into four phases (0,1,2,3 and 4), each of which is mediated by specific ionic conductances as described below.

A. Phase 0; Upstroke of the Action Potential:

The depolarization phase of the AP is characterized by a rapid shift of the membrane potential from resting level ~-85 mV to ~35 mV (Doerr *et al.*, 1990; Katz, 1992), with a maximal rate of depolarization ($\dot{V}_{max}$) approaching ~ 200-300 V/sec (Brown *et al.*, 1981). Phase 0 is initiated when membrane potential becomes slightly more positive than the threshold potential (defined as the potential at which inward depolarizing current primarily due to sodium (Na^+) conductance, just surpasses outward current due primarily to the efflux of potassium ions (K^+)). Depolarization to threshold activates Na^+ channels which show an activation range beginning between -70 and -50 mV (Brown *et al.*, 1981; Benndorff *et al.*, 1985). Since the equilibrium potential for Na^+ (E_{Na}) lies between ~ +44 and +60 mV (Lee *et al.*, 1979), the influx of Na^+ through the channels shifts membrane potential towards E_{Na}. Na^+ channels have rapid activation time (<2msec) and a large regenerative inward Na^+ current (I_{Na}) is soon attained, peaking at ~ -50mV with a maximal value of 60-90 nA (Brown *et al.*, 1981; Fozzard *et al.*, 1985). This large magnitude is attributed to the relatively high unitary conductance (10-25 pS) and density (8 per μm^2) of the Na^+ channels (Fozzard *et al.*, 1985). The channels have a short open time and are inactivated within 150-300 msec (Carmeliet, 1987).

Na^+ channels are blocked by inorganic compounds including cadmium (Cd^{2+}) and zinc (Zn^{2+}) in micromolar concentrations. They are also selectively blocked by the naturally occurring organic neurotoxins, tetrodotoxin (TTX) and saxitoxin which bind to an extracellular site of the channel (Fozzard and Hanck, 1992). TTX in concentrations of 10-30 μM is sufficient to decrease the upstroke of the AP and V_{max} (Hume and Uehara, 1985; Fozzard *et al.*, 1985; Kiyosue and Arita, 1989)

B. Phase 1; Early Repolarization of the Action Potential:

The upstroke of the AP is immediately followed by a brief period of membrane repolarization referred to as phase 1 (figure 1). In some species the repolarization can be by as much as 40 mV. Frequently, a second period of depolarization follows phase 1 creating a notch separating the spike (phase 0 and 1) from the subsequent AP plateau. Phase 1 is prominent in ventricular tissues of the following species; rat, mice, rabbit, canine, bovine and feline (Isenberg and Klockner 1982; Gintant *et al.*, 1992). However, in the guinea pig myocyte, phase 1 is generally small or absent. Phase 1 is mediated by transient outward current (I_{to}) (Kukushkin *et al.*,1983; Josephson *et al.*,1984). The ionic basis of I_{to} remains controversial. Two components of I_{to} have been identified (Gintant *et al.*, 1992); the first component, referred to as I_{to1} (Tseng and Hoffman, 1989) (also as long lasting outward current (I_{lo}) (Corabeouf and Carmeliet, 1982), or I_A because it resembles the transient A current of neurons (Connors and Stevens, 1971), and the second component, as I_{to2} (Tseng and Hoffman, 1989) (or I_{bo} (brief outward

current; Corabeouf and Carmeliet, 1982)). The relative contribution of these components to phase 1 appears to vary among species. I_{to1} is Ca^{2+}-insensitive current and is blocked by 4-aminopyridine (4-AP; Kenyon and Gibbons, 1979). In comparison with I_{to1}, I_{to2} appears to be a Ca^{2+}-activated conductance that is resistant to 4-AP (Tseng and Hoffman, 1989; Hiraoka and Kawano, 1989). Previous studies reported that I_{to2} is mainly carried by K^+ in rabbit ventricular myocytes (Giles and Imaizumi, 1988; Hiroaka and Kawano, 1989), but recently, Zygmunt and Gibbons, (1991) identified chloride (Cl^-) as the charge carrier of I_{to2}. These latter authors showed that K^+ replacement was without effect on the magnitude of I_{to2}, yet Cl^- replacement reduced the current. Thus, the present consensus is that I_{to2} is a Ca^{2+}-activated Cl^- current.

Since phase 1 in guinea pig is small or absent, the contribution of I_{to1} and/or I_{to2} to whole-cell membrane conductance is likely very low. For the most part, studies on ventricular myocytes from this species do not consider phase 1 repolarization important to AP configuration.

C. Phase 2; Plateau Phase of the Action Potential:

The sustained depolarization during phase 2 (plateau phase) lasts for a period of 200-400 msec (figure 1). It is this phase which distinguishes cardiac cells from other electrically active cells, such as neurons and skeletal muscle fibers (Katz, 1992). In the first two thirds of this phase membrane potential is generally stable lying between -10 and +30 mV. This relatively stable depolarized potential is maintained by a precise balance between several inward and outward

conductances potentially including; (1) L-type Ca^{2+} current ($I_{Ca,L}$), (2) Na^{+} window current, (3) inward rectifier K^{+} current (I_{K1}), (4) plateau potassium current (I_{Kp}) and (5) delayed K^{+} rectifier current (I_{K}). A role for L-type Ca^{2+}, inward and delayed rectifier currents is established, however, the contribution of the other currents is controversial.

Two types of Ca^{2+} currents were identified in ventricular myocytes (Nilius *et al.*, 1985; Mitra and Morad, 1986) including; (1) a high threshold, transient, rapidly inactivating TTX-insensitive Ca^{2+} current (T-type; $I_{Ca,T}$), and (2) a low-threshold, slowly inactivating, long lasting $I_{Ca,L}$. The former may be important for impulse generation in pacemaker cells. It is activated at potentials negative to -50 mV, a range where I_{Na} is the dominant current in ventricular myocytes. Hence its functional role in non-pacemaker (ventricular) AP generation is controversial. On the other hand, L-type Ca^{2+} channels are considered to be the major route for Ca^{2+} influx during depolarization and the triggering conductance for excitation-contraction (E-C) coupling in the heart (Callewaert, 1992).

L-type Ca^{2+} channels are voltage-dependent and activated at potentials positive to -50 mV (-50 to +10 mV) (Nilius *et al.*, 1985). The channels are highly selective to Ca^{2+} which is evident from the reversal potential of the current between +50 and +60 mV that matches the predicted Nernst value (Lee and Tsien, 1982). Peak current decays or inactivates slowly (0.2-1.0 sec), apparently as a result of the independent influence of voltage- and Ca^{2+}- dependent mechanisms (Hadley and Lederer, 1991). Increased free $[Ca^{2+}]_i$ depress the

current. Physiologically, either rapid Ca^{2+} influx through the channels, an increase in stimulation frequency (increase number of APs/sec), or sarcoplasmic reticulum (SR) Ca^{2+} release triggered by Ca^{2+} influx may bring about a decline in $I_{Ca,L}$ (Cohen and Lederer, 1988).

$I_{Ca,L}$ is enhanced by the dihydropyridine, Bay K8644 (Rosenberg *et al.*, 1986) and blocked by inorganic divalent cations, such as magnesium (Mg^{2+}) (Dichtl and Vierling, 1991), cobalt (Co^{2+}), nickel (Ni^{2+}) and Mn^{2+} (Pelzer *et al.*, 1992) as well as organic compounds including dihydropyridine drugs (e.g., nifedipine), phenylalkamines (e.g., verapamil) and benzothiazepines (e.g., diltiazem). Dihydropyridines bind to the high affinity dihydropyridine receptor (nM-pM) of the alpha subunit of Ca^{2+} channel in a voltage- and temperature-dependent fashion with greater block at depolarized potentials and lower temperatures (Hosey and Lazdunski, 1988). L-type Ca^{2+} channels blockers attenuate phase 2 of the action potential and decrease AP duration leading to a depression of contractility (Fleckenstein, 1983). This reflects the important contribution of $I_{Ca,L}$ in phase 2 and in E-C coupling in the heart.

A steady-state "window" Ca^{2+} current was estimated at plateau voltage range between -30 and 0 mV (Lee *et al.*, 1985; McDonald *et al.*, 1986) which was similar to the range measured directly by Hirano *et al.* (1992). This steady-state, non-inactivating current through L-type Ca^{2+} channels arises as a result of the overlapping of activation and inactivation ranges for this conductance. Window Ca^{2+} current is sensitive to the same agents as the peak $I_{Ca,L}$, i.e., it is enhanced

by Bay K8644 and depressed by the known inorganic and organic blockers of L-type Ca^{2+} channel (Hirano *et al.*, 1992).

It has long been recognized that delayed rectifier K^+ current plays a critical role during phase 2. Indeed, the initiation of repolarization of the AP is mediated mainly by activation of this current. I_K was first characterized in sheep purkinje fibers by Noble and Tsien, (1969) and subsequently studied via patch-clamp technique in a variety of tissues and species (Matsuura *et al.*, 1987; Giles *et al.*, 1988; Kleiman and Houser, 1989; Gintant *et al.*, 1992).

Several discrepancies exist in the early literature regarding I_K in different preparations. However, these were recently resolved by Sanguinetti and Jurkiewicz (1990a) by a detailed examination of tail currents, kinetics of activation and deactivation, reversal potential and pharmacological block of the current. These authors used methanesulfonanilide class III antiarrhythmic drugs (E4031 (Sanguinetti and Jurkiewicz, 1990b) and dofetilide (Jurkiewicz and Sanguinetti, 1993)) which prolong AP duration by lengthening phase 2 as a tool to demonstrate two distinct components of I_K; (1) a high threshold, rapidly activating and rectifying I_{Kr}, and (2) a low threshold, slowly activating component, I_{Ks}. I_{Kr} possessed a reversal potential similar to E_K, activates at potentials positive to -50 mV, peaked at ~ -10 mV, and was evident as a distinct outward hump on the steady-state I-V relationship within the range -30 to +40 mV (Sanguinetti and Jurkiewicz, 1991). I_{Kr} decreases in magnitude upon decreasing extracellular K^+ and it is blocked with low concentrations of lanthanum (La^{3+}) (>10μM) (Sanguinetti

and Jurkiewicz, 1990b), dofetilide (Carmeliet, 1992) and E-4031 (Sanguinetti and Jurkiewicz, 1990a). I_{Kr} has been subsequently identified in guinea pig atria, rat, canine and rabbit ventricular myocytes (Carmeliet, 1992; Gintant *et al.*, 1992). Because of its rapid activation it is thought to contribute significantly to repolarization of AP compared to I_{Ks}, despite the fact that I_{Ks} is much larger in magnitude.

On the other hand, I_{Ks} contributes to the classically evoked I_K measured after long depolarization pulses. It has the properties generally attributed to I_K (Sanguinetti and Jurkiewicz, 1990a and b; Carmeliet, 1992; Jurkiewicz and Sanguinetti, 1993) including for example; (1) a sigmoidal onset of activation positive to +30 mV, (2) a slope constant for activation of +12.7 mV which is less than that for I_{Kr} (7.5 mV), and (3) a reversal potential of -77 mV (when the calculated E_K = -94 mV) due to the partial permeation of Na^+ in the channel (permeability ratio P_K/P_{Na} of 0.016; Matsuura *et al.*, 1987). The magnitude of I_{Ks} when fully activated is about 11.4 times larger than I_{Kr}. At higher frequencies, such as during increased heart rate, the deactivation of I_{Ks} is reduced leading to a build up of current over time. Both I_{Ks} and I_{Kr} are blocked by barium (Ba^{2+}), cesium (Cs^+), tetraethylammonium (TEA^+), 4-AP, quinidine and phencyclidine (Hume, 1988), however, there is no specific blocker for I_{Ks}.

The relative contribution of the two components of I_K to plateau repolarization is still controversial (Gintant *et al.*, 1992). Sanguinetti and Juriewicz (1993) suggested a contribution of similar magnitude since they were

approximately equal in size during 225 msec test pulses to membrane potentials between -20 and +20 mV.

A small outward potassium current carried by inward rectifier K^+ channels (I_{K1}) (described in detail below) was observed during phase 2 by Shimoni *et al.* (1992). These investigators recorded AP from rabbit ventricular myocyte and then employed the AP as the voltage command signal for voltage clamp measurement of I_{K1} in isolation. A slow increase in I_{K1} during phase 2 was evident from the presence of tail currents lasting for 100-300 msec following steps to a range of potentials between -30 and -70 mV after depolarizing pulses.

The possible contribution of I_{Na} to phase 2 and AP duration was postulated based on the reported slow inactivation of residual Na^+ current. The presence of this delayed inactivation is thought to give rise to a window Na^+ current (Attwell *et al.*, 1979; Colatsky 1982). Direct evidence for the contribution of I_{Na} to phase 2 was obtained from chick embryonic ventricular myocytes in simultaneous recordings of single Na^+ channel activity and the AP during phases 1 and 2 (Liu *et al.*, 1992). The Na^+ channels observed during phase 2 conduct Li^+ and are blocked by TTX (Attwell *et al.*, 1979; Liu *et al.*, 1992; Fozzard and Hanck, 1992). Indirect evidence for the contribution of I_{Na} to phase 2 was obtained in studies using TTX to inhibit the current. Concentrations of 0.1-0.33 μM were sufficient to decrease AP duration (Coraboeuf *et al.*, 1979; Kiyosue and Arita, 1989) without substantial effect on AP upstroke or $\dot{V}_{max}$ (Fozzard *et al.*, 1985). The decline in AP duration was attributed to block of a window Na^+ current (Attwell *et al.*, 1979),

carried either by a second population of Na^+ channels with higher sensitivity to TTX or to slowly inactivating component of Na^+ current mediating phase 0. However, the effects of TTX on AP duration are controversial; Hume and Uehara (1985) failed to observe a change in AP duration in isolated guinea pig ventricular myocytes with TTX as high as 30 μM. Possible reasons for the variability in the results include different Na^+ channel density and/or factors influencing the occurrence of long channel openings, such as phosphorylation (Matsuda *et al.*, 1992).

In 1988, Yue and Marban reported that guinea pig ventricular myocytes possess a potassium channel which shows high activity at plateau potentials and a conductance of 14 pS (in physiological K^+ gradient). The channel apparently does not inactivate but remains open and conducting during the AP plateau prompting the suggestion that I_{Kp} is a background K^+ current during phase 2 (Backx and Marban, 1993). This current is the only reported K^+ current which is not blocked by TEA at concentrations up to 135 mM TEA.

D. Phase 3; Rapid Repolarization of the Action Potential:

Figure 1 indicates that phase 3 of the cardiac ventricular AP is a period of rapid repolarization. It is in fact a period of regenerative repolarization in which the rate of change in voltage increases with hyperpolarization. The latter arises as a consequence of the increasing contribution of I_{K1} to membrane conductance as membrane potential repolarizes negative to about -30 mV during continued influence of I_K during phase 2. Since I_{K1} is the conductance exclusively

responsible for maintaining a negative RMP, I will describe its properties in the subsequent section. The functional role for I_{K1} in AP repolarization was obtained using a direct method where an AP from a single myocyte was employed as the voltage command protocol to evoke I_{K1} (Shimoni *et al.*, 1992). A gradual increase in the magnitude of I_{K1} from -30 to -70 mV and a direct correlation between the magnitude of I_{K1} and the rate of change of membrane potential during late repolarization was observed. Further evidence was provided from the observed shortening of AP duration upon elevation in $[K^+]_o$ despite a reduction in driving force for K^+ (Hume and Uehara, 1985). This is believed to be due to a cross-over of the current-voltage relationship of I_{K1} when $[K^+]_o$ is elevated.

E. Phase 4: Resting Membrane Potential:

At resting potential, membrane resistance is low due to the presence of a high resting K^+ conductance (g_K) and lower sodium conductance (P_K/P_{Na} = 10) (Baumgarten and Fozzard, 1992). This high g_K exhibits inward rectification which was found by measuring ^{42}K efflux under voltage clamp conditions in cardiac Purkinje fibers (Haas and Kern, 1966; Vereecke *et al.*, 1980). Membrane conductance is high when membrane potential is negative to E_K and low when it is more positive (Noble, 1985). The reversal potential of I_{K1} matches the predicted Nernst E_K (~-88 mV) which indicates that K^+ is the major permeant ion in the channel. I_{K1} is activated at voltages negative to -30 mV in a time-dependent manner. The activation time constant is 2.8 msec at -90 mV which suggest that the current is not instantaneous as was originally thought (Harvey and TenEick,

1988). The property of inward rectification is caused by the blocking effect of the intracellular Mg^{2+} at physiological concentrations (Matsuda *et al.*, 1987; Ishihara *et al.*, 1989). This is important for stabilizing RMP and preventing K^+ loss during depolarizing potentials (Ishihara *et al.,* 1989) where the magnitude of I_{K1} is small. Also, the absence of rectification would result in significant outward K^+ current at depolarized potentials which would offset and mask the time-dependent inward currents mediating phases 0 and 2 of the action potential. Therefore, this property is essential for achieving a normal electrical activity in the ventricular myocytes.

The current-voltage (I-V) relationship for I_{K1} illustrates positive (negative to -60 mV) and negative slope (-60 to -20 mV) conductances. An estimation for g_{K1} is obtained from calculating the chord conductance which is reported to be about 50-70 nS (measured from the positive slope). This conductance is very much dependent on $[K^+]_o$ (Harvey and Ten Eick, 1988), increasing with elevated $[K^+]_o$. Single channel recordings of I_{K1} indicate a unitary conductance of ~31 pS in symmetrical K^+ and 3.6 pS in physiological K^+ gradient (Sakmann and Trube, 1984). The reported high channel density (~1 channel/μm^2) indicate the importance of g_{K1} for resting membrane conductance. I_{K1} is completely blocked by Cs^+ and Ba^+ and partially by magnesium (Mg^{2+}) (Imoto *et al.*, 1987; Kell and DeFelice, 1988; Shioya *et al.*, 1993).

The contribution of I_{K1} to RMP is evident from the following observations. First, an elevation in $[K^+]_o$ concentration is known to depolarize the RMP. The positive shift in the reversal potential of I_{K1} (E_K) leads to a positive shift in the I-V

relation and hence, depolarization (Hume and Uehara, 1985; Harvey and Ten Eick, 1988). Secondly, inhibition of I_{K1} with Ba^{2+} induced abnormal automaticity in cardiac tissues and myocytes. The automaticity was attributed to slow suppression of I_{K1} leading to slight depolarization in RMP and eventually oscillatory spontaneous activity (Imoto *et al.*, 1987; Valenzuella and Vassalle, 1989). Moreover, suppression of I_{K1} by Cs^{2+} and Ba^{2+} induced a decrease in the positive slope conductance and positive shift in the reversal potential of the steady-state I-V relation. This has two consequences; depolarization of membrane potential, and prolongation in AP duration. The latter may also lead to an increase in intracellular Ca^{2+} because of prolonged activation of I_{Ca} .

Isenberg (1977) reported that an elevation in $[Ca^{2+}]_i$ increased a steady-state potassium conductance which was attributed to I_{K1}. However, single channel recordings of I_{K1} indicate this is unlikely. An increase in $[Ca^{2+}]_i$ in the range of physiological Ca^{2+} transient (0.1 -1 μM) reduced the outward current flow through the channels and contributed to inward rectification. This was apparently mediated by both a decrease in open time probability as well as by favouring transitions into a substate conductance (Mazzanti and DiFrancesco, 1989; Mazzanti and DeFelice, 1990).

F. Contribution of Na^+/K^+ Pump to Cardiac Electrical Activity:

Following an action potential, the intracellular homeostasis of Na^+ and K^+ is disturbed; $[Na^+]_i$ is elevated and $[K^+]_i$ is reduced. The intracellular homeostasis of these ions, and hence cell volume and osmolarity is maintained by the

activation of the Na^+/K^+ pump. Following an AP, a net increase in $[Na^+]_i$ could result from Na^+ entry during phase 0 and from activation of I_{NaCa} during late phase 2 and phase 3 (see below). This will activate the Na^+/K^+ pump and because it is electrogenic (Gadsby, 1984) with a coupling ratio of 3 Na^+ for 2 K^+ ions, its activity will result in a slight hyperpolarization due to pump current (I_{NaK}) (Gadsby *et al.*, 1985). Inhibition of I_{NaK} by cardiac glycosides (such as digitalis and strophanthidine) induced depolarization of membrane potential by 2-13 mV, implying a contribution of I_{NaK} to RMP. However, this remains controversial (Baumgarten and Fozzard, 1992).

G. Contribution of Na^+-Ca^{2+} Exchange to Cardiac Electrical Activity:

Na^+-Ca^{2+} exchange is a countertransport protein in SL membrane which plays a significant role in controlling intracellular Ca^{2+} homeostasis during excitation-contraction coupling. However, it may also contribute to the voltage changes during an AP, and, to RMP (Sheu and Blaustein, 1992). The electrogenicity of the exchanger derives from its stoichiometry of 3 Na^+ to 1 Ca^{2+} as first reported by Blaustein and Hodgkin (1969), and subsequently documented in the heart using a variety of different techniques (Sheu and Blaustein, 1992).

Kimura *et al.* (1986 and 1987) identified Na^+-Ca^{2+} exchange current (I_{NaCa}) in guinea-pig ventricular myocytes by blocking other currents and controlling internal and external $[Na^+]$ and $[Ca^{2+}]$. I_{NaCa} exhibited an exponential I-V relation and its magnitude was Na^+ and Ca^{2+} dependent. Outward I_{NaCa} was induced by increasing $[Ca^{2+}]_o$ or $[Na^+]_i$ and was associated with Ca^{2+} influx, hence, it was

labelled reverse mode of activity (Ehara *et al.*, 1989). On the other hand, forward mode activity mediating Ca^{2+} efflux was recorded as an inward I_{NaCa} after increasing $[Ca^{2+}]_i$ and $[Na^+]_o$ (Kimura *et al.*, 1987). When intracellular and extracellular $[Na^+]$ and $[Ca^{2+}]$ concentrations were set close to physiological levels, the apparent reversal potential fits the following relationship with 3:1 coupling ratio (Mullins, 1979; Difrancesco and Noble, 1985)

(1) $E_{NaCa} = 3E_{Na} - 2E_{Ca}$

Where, E_{Na} and E_{Ca} are the reversal potential of Na^+ and Ca^{2+} respectively. Since I_{NaCa} is voltage-dependent (Lipp and Pott, 1988), the direction of the current and hence net Ca^{2+} flux is determined by the direction of the thermodynamic driving force; $V_m - E_{NaCa}$

(2) $(V_m - E_{NaCa}) = V_m - (3E_{Na} - 2E_{Ca})$

However, under physiological conditions during the AP, V_m, E_{Na}, E_{Ca} and $V_m - E_{NaCa}$ vary dynamically (Blaustein, 1988). Therefore, a fixed value for E_{NaCa} can never be obtained and consequently it has been very difficult to sort out the contribution of I_{NaCa} to the AP.

Changes in V_m, E_{Na}, E_{Ca} and $V_m - E_{NaCa}$ during the AP were predicted by mathematical models using the intracellular Ca^{2+} transient (derived from changes in fluorescence of Ca^{2+} indicator dyes such as Fura-2 and Indo-1) as a measurement of $[Ca^{2+}]_i$ and assuming that $[Ca^{2+}]_o$, $[Na^+]_o$ and $[Na^+]_i$ are constant. These studies showed that E_{NaCa} is positive to RMP during diastole leading to a small net inward current. During the upstroke (phase 0), $V_m - E_{NaCa}$ becomes

positive and tends to drive Ca^{2+} into the cell in exchange for Na^+. This mode may continue during plateau (phase 2) of AP. However, since $[Ca^{2+}]_i$ increases later in phase 2 (mediated by L-type Ca^{2+} channels and Na^+-Ca^{2+} exchange, and Ca^{2+} release from SR), E_{NaCa} shifts to a more positive potential and forward mode is reinitiated. Following repolarization, V_m-E_{NaCa} becomes negative resulting in net Ca^{2+} efflux coupled to Na^+ influx (Blaustein, 1988; Wier and Beuckelmann, 1989).

The Ca^{2+} efflux mode or the inward current of I_{NaCa} has been studied extensively and is recognized to result in a slow transient inward tail current. It was first referred to as creep current based on studies in frog myocytes (Hume and Uehara, 1986) and subsequently identified in mammalian ventricular myocytes as the exchange current (Giles and Shimoni, 1989a). This current was activated upon repolarization of the membrane to potential negative to -35 mV following a depolarizing step pulse, interruptions of the plateau of the AP, or voltage-independent increase in $[Ca^{2+}]_i$ by flash photolysis of caged Ca^{2+} (Fedida *et al.*, 1987; Egan *et al.*, 1989; Giles and Shimoni, 1989; Niggli and Lederer, 1993). Exchange current was also shown to be increased by a rise in $[Ca^{2+}]_i$ due to release from SR and depressed by increasing intracellular Ca^{2+} buffering capacity. The time course of the tail current is thought to be determined by the rate of uptake of Ca^{2+} by SR Ca^{2+}-ATPase. Most studies have focused on this forward mode activity because of its importance in extruding Ca^{2+} under physiological conditions.

The idea that I_{NaCa} could contribute to the plateau during the second half

of AP was suggested by experiments which employed an AP voltage clamp protocol. Applying a low concentration of caffeine (known to release Ca^{2+} from and to impair its reuptake by SR) to enhance Ca^{2+} release from SR in guinea-pig ventricular myocytes (Doerr *et al.*, 1990) was found to lengthen AP duration. This was attributed to the activation of I_{NaCa} because, under AP clamp, inward current was increased during the plateau and reached a peak during repolarization. However, it is difficult to attribute this current solely to I_{NaCa}. Other Ca^{2+}-activated conductances might also mediate such an effect, for example, the Ca^{2+}-activated non-selective cation current (I_{NSC}; Colquhoun *et al.*, 1981; Ehara *et al.*, 1988). It is apparent that assessing and quantitating the contribution of I_{NaCa} to the AP is difficult because of the lack of a selective blocker for the exchanger.

Previous studies have shown that Li^+ (Ponce-Harnos and Langer, 1980; Hale and Keller, 1990) and N-methy-D-glucamine (NMG; Niggli and Lederer, 1993) do not substitute for Na^+ on the exchanger and, therefore, blocked inward I_{NaCa} when applied to the cell exterior. I_{NaCa} is also blocked by heavy metal cations, such as La^{3+}, Cd^{2+}, Mn^{2+} and Ni^{2+} (Kimura *et al*, 1987; Shue and Blaustein, 1992). Nickel is considered as the most selective ionic blocker for I_{NaCa} and was used as a tool for identifying I_{NaCa}. However, Ni^{2+} is known to affect L-type Ca^{2+} channels, depress intracellular Ca^{2+} transient, and alter other $[Ca^{2+}]_i$-dependent conductances directly and indirectly. A number of organic blockers have been reported to block I_{NaCa} such as amiloride, and its analogue 3',4' dichlorobenzamil, quinacrine, verapamil and D600 (Lipp and Pott, 1988; Sheu and

Blaustein, 1992). Recently, the synthesis of the exchange inhibitory peptide (XIP) (Li *et al.*, 1991) made it possible to selectively and specifically block I_{NaCa} in guinea pig ventricular myocytes (Chin *et al.*, 1993).

3. Influence of Steady-State Conductances on RMP:

Under physiological conditions, the steady state I-V relationship in cardiac ventricular myocytes is N-shaped (Baumgarten and Fozzard, 1992). I_{K1} and I_K contribute to the conductance at potentials negative to -40 mV and positive to -50 mV, respectively. The steady-state current intersects the voltage axis at two points (i.e., no net current) representing two stable levels for RMP (Gadsby and Cranefield, 1977; McCullough *et al.*, 1990). However, if the contribution of I_{K1} to resting steady-state conductance is altered, the hyperpolarized value of RMP will change. In the discussion above, the example of Ba^+-induced inhibition of I_{K1} causing depolarization was indicated. However, membrane depolarization may also result from an increase in inward steady-state conductance, independent of changes in I_{K1}.

Steady-state membrane conductance and RMP are known to be modified under pathological conditions, such as conditions of metabolic inhibition (e.g., ischemia, hypoxia and anoxia) and Ca^{2+} overload. The former appears to cause activation of an ATP-sensitive K^+ conductance, and the latter, an increase in Ca^{2+}-activated non-selective cation conductance. Activation of either of these currents will have significant albeit opposite effects on membrane potential.

A. ATP-Sensitive K^+ Conductance:

Under conditions of metabolic inhibition (e.g., anoxia, hypoxia and ischemia) or during prolonged whole-cell recordings, an increase in K^+ efflux and conductance associated with a decline in AP duration has been described in a variety of cardiac preparations (Carmeliet, 1978; Vleugels *et al.*, 1980; Noma and Shibasaki, 1990). Early on, changes in current during whole-cell were found to be reversed by exposure of the cytoplasmic side of the membrane to millimolar concentrations of adenosine 5'-triphosphate (ATP) (Taniguchi *et al.*, 1983). Subsequently, Noma (1983) demonstrated a class of K^+ channels which were normally closed at physiological intracellular ATP concentraion ($[ATP]_i$) and opened when $[ATP]_i$ was depressed, as during metabolic inhibition or prolonged whole-cell recordings. Noma (1983) referred to the channels as ATP-sensitive K^+ channels.

ATP-sensitive K^+ current (I_{KATP}) is voltage and time-independent. The former indicates an ohmic I-V relationship. The reversal potential matches the equilibrium potential of E_K indicating high selectivity for K^+ (Noma, 1983; Kakei *et al.*, 1985). The channel shows inward rectification at potentials positive to +20 mV (Noma,1983), which is due to voltage-dependent block by intracellular divalent cations, mainly Mg^{2+} (Horie *et al.*, 1987; Findlay, 1987). Because of the high density of these channel in ventricular myocytes (0.5-1/μm^2) and their relatively high unitary conductance of ~ 80pS (in symmetrical K^+; Trube and Hescheler, 1984; Noma and Shibasaki, 1985) and 25 pS (in physiological K^+; Findlay, 1987),

only a small number of channels, between 0.5 - 1% of the total cell number of ~ 3000 channels, is required to produce sufficient current to induce considerable decline in the AP. The presence of such an excess of channels is referred to as the "spare channel" hypothesis (Nichols *et al.*, 1991; Findlay and Faivre, 1991).

Intracellular ATP has a dual effect on the ATP-sensitive K^+ channels, i.e, opening and closure of the channels (Edwards and Weston, 1993). ATP bound to Mg^{2+} mediates a phosphorylation of the channel that appears to be a prerequisite for maintaining the channels in an available state and also for reactivation of the channels after rundown during patch voltage-clamp recordings (Findlay and Dunne, 1986; Ashcroft, 1988) .

However, ATP-sensitive K^+ channels are closed when $[ATP]_i$ rises with a concentration of 10-100μM for half maximal block (Edwards and Weston, 1993). Millimolar concentrations of ATP applied to the cytosolic side of the membrane were able to reverse the activation of ATP-sensitive K^+ channels induced by metabolic inhibition (Noma and Shibasaki, 1990). This blockade does not require hydrolysis of ATP, although MgATP was reported to be more effective for the block. ATP is thought to bind to a specific site on the channel, different from the phosphorylation site, leading to the blockade of the channel (Findlay, 1988; Edwards and Weston, 1993). This inhibition is independent of voltage and required only a molecule of ATP to bind (Findlay, 1988; Noma, 1993). Other adenosine nucleotides are less potent in the following order ATP>adenosine diphosphosphate (ADP) >adenosine monophosphate (De Weille and Lazdunski,

1990). Recent evidence suggests that block of ATP-sensitive K^+ channels by ATP is modulated by ADP which competes with ATP for the binding site (in the presence of Mg^{2+}) and decreases ATP's inhibitory effect. This suggest that ATP/ADP ratio may be more important than $[ATP]_i$ itself in regulating channel activity in intact cells (Weiss and Venkatesh, 1993). The effect of ADP can be mimicked by guanosine mono- and diphosphates and their non-hydrolysable analogues (Ashcroft and Ashcroft, 1990). Other stimulatory factors may include depressed cytoplasmic pH, adenosine via G protein activation (Nichols and Lederer, 1991).

There are two sources of ATP production for regulation of ATP-sensitive K^+ channels, aerobic mitochondrial oxidative phosphorylation and anaerobic glycolysis, However, glycolysis appears to be more significant. Evidence from Weiss and coworkers (1987 and 1989) indicates that the key glycolytic enzymes are associated with membrane or cytoskeleton near the channel and thus act as a preferential source for ATP to regulate open probability of ATP-sensitive K^+ channels. Depression in the activity of these enzymes during ischemia could lead to activation of the channel despite only slight changes in cytoplasmic ATP levels (Carmeliet *et al.*, 1990)

ATP-sensitive K^+ channels are blocked by K^+ channel blockers, quinine, 4-AP, TEA, Cs^{2+} and Ba^{2+} (Ashcroft, 1988). However, these channels are selectively blocked by low concentrations of sulfonylurea drugs such as tolbutamide and glibenclamide (De Wielle and Lazdunski, 1990). These agents are lipophilic

hypoglycemic agents used clinically for the treatment of non-insulin dependent diabetes mellitus (Edwards and Weston, 1993).

A number of drugs are known to increase the activity of K_{ATP} channels in cardiac muscle, including; benzopyrans (e.g., cromakalim) and pyridimidines (e.g., nicorandil, pinacidil) (Edwards and Weston, 1993). These drugs appears to compete with ATP binding leading to increase in the open probability of the channels without any effect on the unitary current. All K^+ channels openers decrease AP duration by activating I_{KATP} and mimic changes in electrical activity induced by ischemia, hypoxia and anoxia (Nichols and Lederer, 1992).

B. Ca^{2+}-Activated Non-Selective Cation Conductance:

A second class of steady-state channels were reported to present in ventricular myocytes. These channels were activated when $[Ca^{2+}]_i$ was in μM range (0.3-10μM). Initial reports were limited to neonatal ventricular rat myocytes (Colquhoun *et al.*, 1981) but more recently they were described in adult guinea pig ventricular myocytes (Ehara *et al.*, 1988; Matsuda, 1983). The reversal potential of the channels or macroscopic current does not match the Nernst potential of any major ion (E_{rev}= ~ 0mV). This was attributed to an approximately equal permeability for Na^+ and K^+ in the channels under physiological conditions. Since Li^+ and Cs^+ also permeate the channel but not Cl^-, the lack of selectivity has prompted the name Ca^{2+}-activated non-selective cation channel. These channels in other cell types also conduct Ca^{2+} ions, but whether they pass divalent cations in cardiac myocytes is not known yet. The channels are thought

to be voltage-independent, opening solely as a result of changes in $[Ca^{2+}]_i$. The dose-response relationship has a Hill coefficient of 3.0 which indicates a strong cooperativity among the Ca^{2+}-binding sites on the channel, or an associated regulatory protein. The I-V relationship is linear with a slope conductance for the unitary currents of about 15 pS (Ehara *et al.*, 1988) and 30-40 pS (Colquhoun *et al.*, 1981). Cardiac non-selective cation channels are similar in many aspects to the those found in non-cardiac cells (Yellen, 1982; Maruyama and Peterson, 1982; Maruyama *et al.*, 1985; Von Tscharner *et al.*, 1986; Siemen and Reuhl, 1987).

The physiological role of Ca^{2+}-activated non-selective cation channels in the heart is not known. Their contribution to whole-cell current was estimated from single channel recordings (Ehara *et al.*, 1988) and concluded to be about 72 nS at maximum activation of the channels with calculated density of ~ 0.04- 0.4/μm^2. This conductance is high enough to account for the reported Ca^{2+}-activated background conductance (Ehara *et al.*, 1988). Whole-cell studies on I_{NSC} are limited and have not as yet characterized the current with respect to its ionic selectivity and kinetics if any. Macroscopic I_{NSC} has been identified only on the basis of its Ca^{2+} dependency, ohmic I-V relation, and reversal potential at approximately one half the distance between E_K and E_{Na}. This is critical, because there is always the possibility that simultaneous activation of two different currents which are sensitive to $[Ca^{2+}]_i$ may result in net current similar to I_{NSC}. For example, Matsuda (1983) observed a steady-state Ca^{2+}-activated conductance

in guinea pig ventricular myocytes upon intracellular Ca^{2+} injection through a microelectrode containing 1 mM Ca^{2+}. This current exhibited an almost linear I-V relationship in the voltage range of -60 to +30 mV and reversed at ~ -22 mV. The enhanced current was reduced upon intracellular injection of EGTA. Using an intracellular perfusion technique, Sato *et al.* (1985) and Kimura *et al.* (1987) also reported an increase in Ca^{2+}-activated background current in ventricular myocytes when the level of $[Ca^{2+}]_i$ was increased. Since this current showed a linear I-V relationship with a reversal potential that does not match any major ion, the Ca^{2+}-activated non-selective cation current (I_{NSC}) was considered. The activation of this current was shown to mediate the positive shift in the reversal potential of the whole cell steady-state I-V relation. This shift is expected to induce marked depolarization of the membrane potential. Moreover, from the reversal potential of I_{NSC}, it would be expected that at potentials negative to E_{NSC}, I_{NSC} will be inward. It will tend, therefore, to pull RMP close to its reversal potential of ~ -20 mV leading to marked depolarization. Whether this is true has not been demonstrated as yet in current clamp mode (i.e., while recording the AP). Ca^{2+}-activated current was postulated to mediate the transient inward current (I_{ti}) which developed during Ca^{2+}-overload in Purkinje fibers (Kass *et al.*, 1978; Cannel and Lederer, 1986) (discussed in details below). However, most studies employing single myocytes suggest that I_{ti} is mainly the result of electrogenic Na^{+}-Ca^{2+} exchange activity.

4. $[Ca^{2+}]_i$ Homeostasis and Excitation-Contraction Coupling:

Intracellular Ca^{2+} plays an important second messenger role in the regulation of numerous physiological processes such as E-C coupling and cell-to-cell communication. For this reason appropriate regulation of $[Ca^{2+}]_i$ is critically important for normal cardiac activity (Weir, 1992). Under resting conditions (diastole), $[Ca^{2+}]_i$ is maintained 10^3-10^4 times lower than $[Ca^{2+}]_o$. Free cytoplasmic $[Ca^{2+}]_i$ in isolated cardiac myocytes ranges between 50-200 nM according to measurements made with fluorescence Ca^{2+}-sensitive indicators, such as Indo-1 and Fura 2 (Grynkiewicz *et al.*, 1985). Such a low concentration is maintained by transport proteins in the SL and SR. For example, efflux of Ca^{2+} across the SL is maintained by the high affinity, low capacity ATP-driven SL Ca^{2+}-pump and by I_{NaCa} (Caroni and Carafoli, 1981; Shue and Blaustein, 1992). Excitation or depolarization of membrane potential during an AP triggers Ca^{2+} release from SR into the cytoplasm causing a transient, bell-shaped increase in free $[Ca^{2+}]_i$ which peaks at about 1 μM (Beuckelmann and Wier, 1988; Cannel *et al.*, 1987). This rise in $[Ca^{2+}]_i$ activates the contractile myofilaments and initiates contraction. Following repolarization of the membrane potential, there is a rapid decline in $[Ca^{2+}]_i$ back to resting levels as a result of SL Ca^{2+} extrusion mechanisms and SR Ca^{2+} sequestration, terminating the phasic contraction period and evoking relaxation.

Three different mechanisms have been proposed to explain E-C coupling in cardiac muscle (Callewaert, 1992); (1) charge movement-coupled Ca^{2+} release

in which voltage-dependent movement of fixed charges in the SL mechanically gates Ca^{2+} release from SR, (2) inositol 1,4,5-triphosphate induced Ca^{2+} release, in which inositol 1,4,5-triphosphate acts as a diffusible messenger to trigger Ca^{2+} release from SR, and (3) Ca^{2+}-induced Ca^{2+} release (CICR), in which Ca^{2+} influx across SL triggers the release of Ca^{2+} from SR. Under physiological conditions the third hypothesis seems to be the best candidate to explain E-C coupling in cardiac myocytes.

Ca^{2+}-Induced Ca^{2+} Release:

Experimental evidence obtained using mechanically skinned cardiac myocytes, isolated SR vesicles and intact cardiac myocytes (Fabiato,1983; Chamberlain *et al.*,1984; Fabiato, 1985; Valdeolmillos *et al.*, 1989; Nabauer *et al.*, 1989; Kentish *et al.*, 1990) implies that Ca^{2+} is required to initiate Ca^{2+} release from SR through the activation of ryanodine-sensitive SR Ca^{2+} release channels. Studies on skinned myocytes reported that triggering of CICR depends on the rate Ca^{2+} increase around the SR, a rapid small increase is more effective than a large but slow change (Fabiato, 1985). As indicated above, voltage gated L-type Ca^{2+} channels are considered to be the major pathway for Ca^{2+} entry in ventricular myocytes of different species. Activation and opening of these channels during depolarization of membrane potential (phase 2 of AP), results in a rapid influx of Ca^{2+} which triggers SR Ca^{2+} release. Significantly, the channels are mainly concentrated in sarcolemmal invaginations or transverse tubules (T-tubules) and are physically oriented to oppose the SR Ca^{2+} release channels

concentrated in the junctional sarcoplasmic terminal cisternae (heavy SR). In addition, other studies report that both the intracellular Ca^{2+} transient and force of contraction change in parallel with $I_{Ca,L}$ (Cannell *et al.*, 1987; Beuckelmann and Weir, 1988; Arreola *et al.*, 1991), and are blocked when $I_{Ca,L}$ is blocked (Morad and Cleemann, 1987; Bers *et al.*,1988). Although the role of $I_{Ca,L}$ is significant in CICR, its contribution in initiating contraction independent of SR Ca^{2+} appears to be almost negligible.

Another pathway for Ca^{2+} influx though SL is Na^{+}-Ca^{2+} exchange, as described above. Recent studies suggest a role for Na^{+}-Ca^{2+} exchange in CICR and E-C coupling in guinea pig ventricular myocytes. This was suggested by Leblanc and Hume (1990) who reported that intracellular Ca^{2+} release occurred in the absence of $I_{Ca,L}$, was associated with the activation of TTX-sensitive sodium channels and required extracellular Ca^{2+}. It was concluded that CICR was triggered by Ca^{2+} influx through Na^{+}-Ca^{2+} exchange in response to Na^{+} influx through TTX-sensitive I_{Na}. However, the contribution of this pathway to E-C coupling seems to be species-dependent, and may not be valid for rat myocytes (Sham *et al.*, 1992)

The release of Ca^{2+} from SR triggered by SL Ca^{2+} influx is mediated by opening of 100 pS (in 50 mM Ca^{2+}; Smith *et al.*, 1986) SR Ca^{2+}-activated Ca^{2+} channels (or foot structure proteins), leading to a rapid efflux of Ca^{2+} from SR lumen into the cytosol (Lytton and MacLennan, 1992). These channels are Ca^{2+} gated and maximally activated by free cytosolic [Ca^{2+}] in the range 1-100 μM

(Ashley and Williams, 1990). They are concentrated mainly on the junctional terminal cistern of the SR and along with calsequestrin (the major Ca^{2+} binding protein in SR lumen), lie opposite to L-type Ca^{2+} channels in the SL as indicated above (Lytton and MacLennan, 1992).

Identification and characterization of Ca^{2+} release channels has been greatly facilitated through the use of ryanodine, a plant alkaloid isolated from *ryania speciosa*, a plant native of Trinidad (Janden and Fairhurst, 1969). Because of the specificity of ryanodine binding to these channels, they have been referred to as the ryanodine receptor (Inue *et al.*, 1987; Rardon *et al.*, 1989; Lai and Meissner, 1989; Lidsay and Williams, 1991). Low concentrations of ryanodine (10nM -10μM) are known to lock the channel in a subconductance state, causing a slow leak of Ca^{2+} (Meissner, 1986; Nagasi and Fleischer, 1988). However, concentrations considerably higher than 10 μM are required to close the channels. The former appears due to ryanodine binding to the low affinity binding site and the latter to the high affinity sites (Lytton and MacLennan, 1992).

Measurements of the Ca^{2+} content of the SR in rat ventricles suggests that its storage capacity is sufficient to produce 50-90% of maximal contractile force (Jorgenson *et al.*, 1988). Significantly, ryanodine and caffeine also reduce the initial rise in $[Ca^{2+}]_i$ by 75- 90% induced by depolarization in ventricular myocytes of rats (Callewaert *et al.*, 1989) and guinea pigs (Beuckelmann and Weir, 1988). Direct measurements of Ca^{2+} efflux from SR further confirm the importance of SR Ca^{2+} release in contraction following excitation (Sipido and Weir, 1991).

The increase in $[Ca^{2+}]_i$ following Ca^{2+} release is reported to decrease the activity of Ca^{2+} release channels (negative feedback regulation). The channels also appear to be inhibited by Mg^{2+} (in mM; Meissner *et al.*,1988; Ashley and Williams, 1990), calmodulin (in μM; Smith *et al.*, 1989) and ruthenium red (Rousseau *et al.*, 1986). On the other hand, the channel activity is enhanced by ATP (in mM) and caffeine (10 mM; Rousseau and Meissner, 1989) which is a phosphodiesterase inhibitor that increases open probability of the channel and at the same time, inhibits SR Ca^{2+}-ATPase.

Following repolarization of the AP, the increase in $[Ca^{2+}]_i$ resulting from E-C coupling is restored by extrusion of Ca^{2+} through the SL and its sequestration by SR. This process is important for maintaining resting $[Ca^{2+}]_i$ and prevention of net accumulation.

Two mechanisms account for SL Ca^{2+} efflux in cardiac myocytes; Na^+-Ca^{2+} exchange and ATP-driven SL Ca^{2+}-pump. The latter is a high affinity, low capacity system and saturates when $[Ca^{2+}]_i$ is higher than resting levels (Caroni and Carafoli, 1981; Carafoli, 1991). In the absence of SR Ca^{2+}-ATPase or Na^+-Ca^{2+} exchange, SL Ca^{2+}-ATPase extrudes Ca^{2+} only very slowly, i.e., 8 sec (Weir, 1990). Therefore, its contribution to Ca^{2+} efflux is minimal. On the other hand, Na^+-Ca^{2+} exchange has a low affinity and high capacity for Ca^{2+} and relatively very fast kinetics. Therefore, it likely plays a dominant role in Ca^{2+} extrusion on a beat-to-beat basis and probably contributes to 15 - 20% of Ca^{2+} removal (Bers *et al.*, 1990; Crespo *et al.*, 1990; Bers, 1991). This was suggested by the increase

in the magnitude of the exchange current following peak $[Ca^{2+}]_i$ transient (Sheu and Blaustein, 1992). Moreover, the amount of Ca^{2+} extrusion through I_{NaCa} was suggested to be equal to that entering via $I_{Ca,L}$ (Bridge *et al.*, 1991). However, due to the voltage dependency of I_{NaCa}, it is only effective for removal of Ca^{2+} when membrane potential is negative to E_{NaCa} (Bridge *et al.*, 1988).

The SR Ca^{2+} ATPase (Ca^{2+}-pump) is an enzyme with high affinity for Ca^{2+} and fast kinetics which hydrolyzes one mole ATP in exchange for 2 Ca^{2+} (Katz, 1992). It is mainly distributed over the tubular cistern of the SR which surrounds the contractile myofibrils (Feher and Fabiato, 1990). It is considered to be the major contributor to Ca^{2+} removal and the primary determinant of the decline in the Ca^{2+} transient following E-C coupling (Inesi, 1985; Weir, 1992). This is reflected in the slow decline of the $[Ca^{2+}]_i$ transient and delayed onset of relaxation when the pump was inhibited by caffeine (Bers and Bridge, 1989; Sipido and Weir, 1991) or thapsigargin (Janczewski and Lakata, 1993)

Mitochondria have a large capacity to accumulate Ca^{2+}, however, the kinetics are slower than the rate of Ca^{2+} cycling during an AP (Gunter and Pfeifer, 1990). The half maximal activation for Ca^{2+} sequestration is about 10 μM, which is beyond the normal range of $[Ca^{2+}]_i$ fluctuations during the cardiac cycle (Carafoli, 1987). Therefore, the role of mitochondria in Ca^{2+} homeostasis and beat-to-beat regulation is very likely negligible (Fry *et al.*, 1989).

5. $[Ca^{2+}]_i$ Overload, Delayed After-Depolarizations and Triggered Activity:

As indicated above, $[Ca^{2+}]_i$ is precisely regulated during E-C coupling to

ensure stability of RMP and relaxation of the muscle following an AP. However, when a sustained increase in resting $[Ca^{2+}]_i$ occurs, the SR eventually becomes overwhelmed with Ca^{2+}. In this case, the AP may be followed by a second release of Ca^{2+} by the SR causing secondary electrical and contractile event (Tsien *et al.*, 1979; Orchard *et al.*, 1983; Kort and Lakatta, 1984). Interestingly, spontaneous Ca^{2+} oscillations were observed in stimulated and unstimulated cardiac preparations (Allen *et al.*, 1984; Lakatta, 1992) indicating the lack of any need for prior excitation in triggering the release of Ca^{2+} from overloaded SR.

Ca^{2+} overload in the heart may result during various pathological conditions, such as ischemia-reperfusion (Stern *et al.*, 1989; Weiss *et al.*, 1990), anoxia-reoxygenation (Allshire *et al.*, 1987), free radical stress (Hayashi *et al.*, 1989; Josephson *et al.*, 1991), acidosis, metabolic inhibition (Orchard *et al.*, 1987), and/or use of inotropic drugs or inhibitors of Na^+/K^+ pump (Allen *et al.*, 1984). Each of these conditions may enhance the possibility of spontaneous SR Ca^{2+} release (Lakatta, 1992).

It is now well recognized that spontaneous Ca^{2+} release stimulates an oscillatory, Ca^{2+}-sensitive current referred to as the transient inward current (I_{ti}; Lederer and Tsien, 1976). This current induces low amplitude fluctuations in membrane potential known as oscillatory after-depolarizations, transient depolarizations and/or delayed after-depolarizations (DADs). When DADs are large enough to reach threshold potential for activation of regenerative inward current, an extrasystolic action potential results. This so-called "triggered impulse"

results in after-contractions and can lead to arrhythmias in the intact heart (January and Fozzard, 1988; Wit and Rosen, 1992).

In voltage-clamp experiments on multicellular preparations or single cardiac myocytes in conditions of Ca^{2+} overload due to interventions such as inhibition of Na^+/K^+ pump or Na^+-Ca^{2+} exchange, elevation in $[Ca^{2+}]_o$, cyclic 3',5'-adenosine monophosphate (c-AMP) (Wit and Rosen, 1992), I_{ti} are consistently observed during repolarization of membrane potential following depolarization. The magnitude, time-to-peak and frequency of the I_{ti} are sensitive to the duration of depolarization. The frequency of I_{ti} ranges between 2-7 Hz depending on the level of $[Ca^{2+}]_i$ and membrane potential (Wit and Rosen, 1992).

The idea that I_{ti} underlies DADs caused by a rise in $[Ca^{2+}]_i$ due to abnormal SR Ca^{2+} handling is supported by the following observations; (1) the appearance of I_{ti} coincides temporally with DADs and after-contractions (Kass *et al.*, 1978a and b; Berlin *et al.*, 1989). (2) Power spectrum analysis reveals that the frequency of voltage and current noise were similar and increased during Ca^{2+} overload preparations (Matsuda, 1982; Kass and Tsien, 1982). (3) Intracellular injection of Ca^{2+} elicits I_{ti} and DADs (Matsuda *et al.*, 1982). (4) Increasing $[Ca^{2+}]_i$ buffering capacity with ethyleneglycol-bis(beta-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) into the cell or by diffusing 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) into cardiac tissues abolished Ca^{2+} oscillations, I_{ti} and DADs (Matsuda, 1982; Marban *et al.*, 1986). (5) Depletion of SR Ca^{2+} by ryanodine or caffeine suppressed I_{ti}, DADs and the associated $[Ca^{2+}]_i$ transient

(Matsuda, 1982; Sutko and Kenyon, 1983; Marban *et al.*, 1986; Valdeomillos and Eisner, 1985) .

The exact mechanism underlying I_{ti} is unclear and controversial. Two different intracellular Ca^{2+} activated mechanisms were proposed and distinguished on the basis of the voltage dependency of I_{ti}. The first candidate was the Ca^{2+}-activated non-selective cation conductance as indicated above. Kass *et al.* (1978b) employed digitalis-treated Purkinje fibers and measured a reversal potential for the I_{ti} of ~ -5 mV. At potentials positive to -5 mV, the I_{ti} reversed and there were outwardly directed oscillations in current. The reversal potential was not affected by replacing $[K^+]_o$ with Cs^+ or reducing $[Ca^{2+}]_o$, however, a negative shift was observed when 75% of $[Na^+]_o$ was replaced by nonpermeant cation. All three features are consistent with the non-selective cation conductance. Additional support was provided by Cannell and Lederer (1986) who reported a reversal potential for I_{ti} of -37 mV in Ca^{2+}-loaded Purkinje fibers, superfused with Na^+ free, $CaCl_2$ isotonic solution to block Na^+-Ca^{2+} exchange. They concluded that the activation of I_{ti} was mediated by non-selective cation channels permeable to both Ca^{2+} and K^+. It is important to note these studies were performed on Purkinje fibers only.

The possibility that Na^+-Ca^{2+} exchange could be the main charge carrier for I_{ti} is supported by the following observations; (1) a lack of a reversal potential for I_{ti} in guinea-pig and ferret papillary muscle treated with digitalis (Karagueuzian and Katzung, 1982; Arlock and Katzung, 1985). (2) Replacement of Na^+ with

choline decreased I_{ti} but no outward current was detected (Karagueuzian and Katzung, 1982; Arlock and Katzung, 1985). (3) Substitution of $[Na^+]_o$ with Li^+ in guinea pig ventricular muscles or single myocytes, abolished I_{ti} (Arlock and Katzung, 1985; Fedida *et al.*, 1987). This should not occur if I_{ti} was mediated by Ca^{2+}-activated non-selective cation conductance which is highly permeable to Li^+ (Ehara *et al.*, 1988). (4) No reversal potential was observed in guinea pig atrial and ventricular myocytes in the presence of toxic concentrations of digitalis or low K^+ (Lipp and Pott, 1987; Fedida *et al.*, 1987). (5) The dependency of I_{ti} on membrane potential and electrochemical gradients for Na^+ and Ca^{2+} were as expected for I_{NaCa} (Mechmann and Pott, 1986). Despite the fact that most studies are in favor of I_{NaCa} as the sole contributor to I_{ti}, some reports suggested that both I_{NSC} and I_{NaCa} may be involved (Tseng and Wit, 1987) with a greater contribution from Na^+-Ca^{2+} exchange (Kimura, 1988).

6. Ischemia-Reperfusion Injury:

The heart is an aerobic organ. It depends on oxygen delivered by arterial blood to support high energy phosphate production (ATP and creatine phosphate) by mitochondrial oxidative phosphorylation (Katz, 1992). These high energy phosphates provide the energy to support the continuous metabolic demand of contraction and ion homeostasis imposed during normal activity in cardiac myocytes. During ischemia, an imbalance between supply and demand occurs leading to depletion of high energy stores and accumulation of toxic metabolites (Reimer and Jennings, 1992). Therefore, reinitiation of blood flow to the

myocardium after a period of ischemia is essential for the survival of ischemic tissue (reperfusion). However, reflow is a double edged sword (Braunwald and Kloner, 1985); although it is necessary, it is often associated with serious damage to myocardium referred to as reperfusion injury (Kloner *et al.*, 1983; Hearse, 1977). Reperfusion injury is well-known experimentally and was observed under clinical conditions following periods of ischemia induced by cardiopulmonary by-pass, thrombolytic therapy, percutaneous transmural angioplasty procedures and coronary by- pass following myocardial infarction (Reimer and Jennings, 1992).

Myocardial reperfusion is associated with; (1) disturbances in ionic balance for hydrogen ion (H^+), Na^+ and Ca^{2+} (Hirche *et al.*, 1980; Hill and Gettes, 1980; Kleber, 1984; Dennis *et al.*, 1990; Tani and Neely, 1989), (2) alterations in ultrastuctrure and enzyme activities (Kloner *et al.*, 1983; Reimer and Jennings, 1992), and (3) stimulation of α-adrenoceptors by the catecholamines released during ischemia (Jennings *et al.*, 1960; Sheridan *et al.*, 1980). Such alterations will predispose to myocardial injury manifested in; (1) early alterations in membrane electrical activity including severe arrhythmias (Janse and Kleber, 1981), (2) contractile failure, or stunning (Braunwald and Kloner, 1982; Bolli, 1990) and (3) coronary vasoconstriction leading to no-reflow phenomenon (Kloner *et al.*, 1974; Bernier *et al.*, 1986)

The severity of these manifestations is a function of the duration and severity of the preceding ischemic period as well as rate of reperfusion. The major mechanisms proposed for the genesis of reperfusion injury are impaired energy

metabolism, Ca^{2+} overload and free radical generation (Hess and Manson, 1984; McCord and Fridovich, 1987; Reimer and Jennings, 1992).

7. Reperfusion Arrhythmias:

A. Cardiac Arrhythmias:

Arrhythmias are disturbances in the sequence of activation of atria and ventricles initiated by pacemakers in sinoatrial node. Clinically, ventricular arrhythmias may arise as a result of alterations in impulse generation, conduction, or both (Hoffman and Rosen, 1981; Janse, 1992). These mechanisms contribute to the generation of premature beats in ventricular tissues which are referred to as extrasystoles, ventricular premature beats, or premature ventricular complexes (PVCs) (Janse, 1992).

a. Abnormalities in impulse generation:

Abnormalities in the initiation of action potentials may result from; (1) altered normal automaticity, mainly within the conduction system e.g., sinoatrial node, atrioventricular node or Purkinje fibres, and (2) mechanisms for impulse generation including abnormal automaticity and triggered activity within the atria and ventricles (Hoffman and Rosen, 1981). Normal automaticity is a characteristic of sinoatrial node at depolarized diastolic potentials (~-55 mV). Abnormal automaticity refers to spontaneous diastolic depolarization during phase 4 that occurs at relatively positive membrane potential in atrial, ventricular and Purkinje myocytes that usually possess a stable hyperpolarized resting membrane potential. Triggered activity, on the other hand, is a result of impulse formation

that requires a previous AP to trigger after-depolarizations which lead to extrasystolic excitation (Cranefield, 1977). If the after-depolarizations occur early during repolarization they are referred to as early after-depolarizations (EADs) and when they occur after its completion they are delayed after-depolarizations (DADs; for details see above section on intracellular Ca^{2+}, DADs and triggered activity).

b. Abnormalities in impulse conduction (re-entry):

Re-entrant excitation occurs when a propagating impulse persists to reexcite atria or ventricles after the end of the refractory period. Its occurence depends on the interrelationship between anatomy, initiating source, excitability, conduction velocity and refractoriness (Fozzard and Arnsdorf, 1992). For example, in the presence of a slow conduction velocity or an area of unidirectional block, impulses can propagate around regions of abnormal activity to reexcite tissue proximal to the block. Moreover, under conditions where AP duration is attentuated, a decrease in the effective refractory period (defined as the minimal time required after an AP before a second propagating impulse can be elicited) may predispose to re-entrant excitation.

B. Reperfusion Arrhythmias:

That reperfusion of the ischemic myocardium induces lethal ventricular arrhythmias has been shown experimentally (Harris and Rojas, 1943; Harris, 1950; Manning *et al.*, 1984) and in the clinic following thrombolysis (Goldberg *et al.*, 1983), coronary artery spasm (Tzivoni *et al.*, 1983) or surgery (Rubin *et al.*,

1985). These arrhythmias include PVCs and ventricular tachycardia (VT) which will degenerate into ventricular fibrillation (VF). Three different arrhythmogenic mechanisms are believed to underlie the genesis of abnormal activity during reperfusion including re-entry, enhanced automaticity and triggered automaticity (Kaplinsky *et al.*, 1981; Kabell *et al.*, 1985; Pogwizd and Corr 1987). Pogwizd and Corr (1987) reported that 75% of reperfusion tachycardias were initiated by triggered activity whereas the remaining 25% were compatible with transmural reentry. A bell-shaped time dependency curve was established which showed that the incidence of arrhythmias was mimimal after short ischemic periods (< 20 min), greatest after ischemia of intermediate duration (20-45 min), and declined with prolonged periods of reduced myocardial perfusion (>45 min) (Manning and Hearse, 1984). Ferrier *et al.* (1990) reported that the duration of the ischemic period also influenced which arrhythmogenic mechanism predominated; short ischemic periods were associated with re-entrant VT and longer periods with DADs and triggered activity.

Re-entry could result from heterogeneity in either injury and/or recovery in ischemic and non-ischemic tissues in regional ischemia, or transmural heterogeneity between the endocardium and the epicardium (Manning and Hearse, 1984). Moreover, early reperfusion in intact preparations was associated with marked shortening in AP duration and effective refractory period which could predispose to re-entrant arrhythmias (Penny and Sheridan, 1983). Differential recovery from ischemia may participate in myocardial arrhythmias. Acute

myocardial ischemia induces the following changes (Reimer and Jennings, 1992); (1) depletion of ATP and creatine phosphate, (2) an increase in carbon dioxide tension, (3) accumulation of myocardial lactate causing cellular acidosis, (4) increases in $[K^+]_o$, $[Ca^{2+}]_i$ and $[Na^+]_i$, (5) increases in lysophophoglycerides and c-AMP, and (6) release of catecholamines from intramyocardial sympathetic fibers. It is possible that reestablishing normal energy levels or ionic balance and/or washout of metabolites may provoke arrhythmic activity. For example, early reperfusion is marked by; (1) washout of K^+ and inhomogeneity of extracellular K^+ gradient, (2) an increase in $[Ca^{2+}]_i$ resulting from depressed forward mode or activation of reverse mode Na^+-Ca^{2+} exchange and Ca^{2+} influx secondary to a rise in $[Na^+]_i$ because of Na^+/H^+ exchange during washout of H^+ from the extracellular space, (3) increased α-adrenoceptors numbers and their stimulation by catecholamines, (4) changes in metabolic status; and (5) accumulation of amphiphiles and c-AMP (Sheridan *et al*, 1980; Witkowski and Corr, 1984). All these changes are potentially arrhythmogenic stimuli. Enhanced automaticity during reperfusion has been attributed to the increase in rate of stimulation of α-adrenoceptors by catecholamines (Sheridan *et al.*, 1980) and/or to injury currents across the border between ischemic and non-ischemic zones (Fozzard and Arnsdorf, 1992). Finally, the late occurence of triggered activity was attributed to Ca^{2+} overload and abnormal Ca^{2+} handling by the SR based on inhibition by ryanodine or caffeine (Thandroyen *et al.*, 1988).

Decreasing the rate of reperfusion was shown to attenuate the incidence

of reperfusion arrhythmias suggesting that the change from ischemia to reflow may be important (Corr and Witkowski, 1983; Manning and Hearse, 1984). One factor known to be involved is the readmission of oxygen during reperfusion. A role for oxygen was suggested by studies indicating reduced incidence of arrhythmias upon reperfusion of globally ischemic hearts with anoxic or hypoxic solutions (Carbonin *et al.*, 1980; Corr and Witowski, 1983). The cytotoxicity of rapidly readmitting oxygen was attributed to a burst of oxygen-derived reactive metabolites, including oxygen-derived free radicals (O-R) (Manning and Hearse, 1984; Garlick *et al.*, 1987). There is now a large body of literature implicating O-R as a factor promoting reperfusion arrhythmias. Evidence for a role of O-R is based on studies using scavengers for these reactive molecules or by applying exogeneous O-R generating systems. Moreover, O-R are known to increase during reperfusion as is evident from their presence in outflow solutions from reperfused myocardium (Bolli *et al.*, 1989), as well as the presence of by-products of their reaction with membrane lipids (Hearse, 1990). It is important however, to emphasize that O-R stress is a contributor but not the only possible cause of reperfusion arrhythmias. Yamada *et al.* (1990) reported that arrhythmias were still evident when reperfusion was carried out with anoxic medium (oxygen tension less than detectable levels), implying that although O-R stress could induce arrhythmias, it was not a prerequisite.

Evidence for the contribution of O-R production to reperfusion arrhythmias was obtained by three strategies; (1) by measuring O-R levels during early

reperfusion, (2) by testing the effect of intervensions that decrease O-R availability, and (3) by exposing tissues to exogenous sources of O-R generation.

A net increase in O-R production during reperfusion was suggested to result from a decrease in antioxidant capacity of the myocardium during ischemia and an increase in O-R production during reperfusion after an ischemic insult (Guarnieri *et al.*, 1980; Julicher *et al.*, 1984; Ferrari *et al.*, 1985; ; Peterson *et al.*, 1985). Elevated levels of O-R during early reperfusion were implicated in reports showing increased levels of lipid peroxidation products including, malondialdeyde and its conjugated dienes during reflow (Meerson *et al.*, 1982). These indirect data are supported by studies which measured O-R levels directly using electroparamagnetic resonance spectroscopy (Zweier *et al.*, 1987), organic spin trapping agents such as 5,5-dimethyl-pirroline-N-oxide (DMPO) and α-phenyl-N-tert-butyl nitrone (PBN) (Arroyo *et al.*, 1987; Bolli *et al.*, 1988; Tosaki and Braquet, 1990) and chemiluminescence techniques (Henry *et al.*, 1990).

That O-R might contribute to reperfusion arrhythmias was indirectly assessed by inhibiting O-R production pathways or by increasing the antioxidant capacity of the heart and compensating for any depletion during ischemia. Electrocardiogram recordings of electrical activity of *in situ* rat hearts demonstrated VT, VF and PVCs during reperfusion after a brief period of ischemia (Manning *et al.*, 1984). The incidence of VT and VF were significantly decreased, but interestingly PVCs were unaffected, when O-R generation was inhibited or O-R were scavenged with antioxidants (Woodward and Zakaria, 1985; Bernier *et al.*,

1986; Bolli *et al.*, 1988).

8. Oxygen-derived Free Radicals:

A. Definition, Chemistry and Mechanisms of Generation:

A free radical is defined as a molecule with an unpaired electron in its last valency shell. They are generated by one electron reduction or oxidation of molecules initially containing a pair of electrons with opposite spin. Molecular oxygen (O_2) contains two unpaired electrons with parallel spins and it generates oxygen-derived free radicals (O-R) when an unpaired electron is added to its last valency shell (Halliwell and Gutteridge, 1984). O-R include superoxide anion radical ($\cdot O_2^-$) and hydroxyl radical ($\cdot OH$). By virtue of their unpaired electron, O-R are unstable, highly reactive and possess very short half lives (nanoseconds to milliseconds). Other oxygen-derived reactive metabolites of importance include hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$).

O-R are normal byproducts of aerobic metabolism in mitochondria during oxidative phosphorylation. They are produced by the univalent reduction of O_2 by four electrons to form water (H_2O) (equation 3). This pathway usually accounts for ~ 5% of normal aerobic metabolism (Thompson and Hess, 1986).

(3) $O_2 \xrightarrow{1e} \cdot O_2^- \xrightarrow{1e} H_2O_2 \xrightarrow{1e} \cdot OH \xrightarrow{1e} H_2O$

$\cdot O_2^-$ is very unstable and spontaneously reacts or dismutates to yield H_2O_2 and O_2 (or $^1O_2$). In an acidic media such as the vacuole of phagocytes or the microenviroment of the cell membrane, $\cdot O_2^-$ is protonated to yield perhydroxy radical ($HO_2\cdot$), this is a stronger oxidant and more cytotoxic than $\cdot O_2^-$ itself

(Kukreja and Hess, 1992).

Both $\cdot O_2^-$ and $\cdot OH$ radicals have the potential to react with biological macromolecules and induce tissue damage. H_2O_2 is a less potent oxidizing molecule, however, in the presence of transition metals such as iron (Fe^{3+}) and copper (Cu^{3+}), $\cdot O_2^-$ can reduce Fe^{3+} in the Haber-Weiss reaction (equation 4), then Fe^+ will reduce H_2O_2 to generate more $\cdot OH$ by Fenton reaction (equation 5) and the net reaction (equation 6) is known as the iron-catalyzed Haber-Weiss reaction (Halliwell and Gutteridge, 1984):

(4) $\cdot O_2^- + Fe^{3+} \longrightarrow O_2 (^1O_2) + Fe^{2+}$

(5) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \cdot OH + OH^-$

(6) Net: $\cdot O_2^- + H_2O_2 \xrightarrow{Fe} \cdot OH + OH^- + O_2 (^1O_2)$

$\cdot OH$ is thought to be the most directly cytotoxic species because of its highly unstable properties and the lack of endogenous antioxidant or defence mechanisms for its metabolism. This radical reacts with macromolecules at very limited diffusion rate within a very close radius to its site of formation. Therefore, it is likely that $\cdot OH$ effects occur at localized cellular sites where transition metals are present such as in mitochondria with their metal containing cytochromes or in metal containing cytosolic proteins and enzymes. Another pathway for $\cdot OH$ production which is iron-independent involves the interaction of $\cdot O_2^-$ with nitric oxide ($\cdot NO$; endothelial relaxing factor) resulting in the production of nitrites and nitrates (Beckman *et al.*, 1990).

$^1O_2$ is formed if one of the unpaired electrons of O_2 is transferred via

energy absorption to a higher energy orbit and its spin is inverted. It could be generated as a product instead of O_2, for example in the Haber-Weiss reaction or dismutation of $\cdot O_2^-$ (Fridovich, 1978; Halliwell and Gutteridge, 1984; Kukreja and Hess, 1992).

B. Mechanisms Responsible for O-R induced Injury:

Two major mechanisms are proposed to account for unjury due to O-R including the oxidation of cellular lipids and proteins. The cell membrane contains large amounts of polyunsaturated fatty acids (PUFA) complexed to membrane phospholipids. The double bonds within PUFA are very sensitive to O-R modification, particularly by ·OH radicals (Kukreja and Hess, 1992). The process of oxygenation of PUFA is known as lipid peroxidation (Yamamato, 1991). In this case a radical, predominantly ·OH, abstracts a hydrogen atom from PUFA to generate a carbon centered radical (L·). Molecular rearrangement results in the formation of conjugated dienes which subsequently react with O_2 to yield an oxygen centered lipid peroxyl radical (L-OO·). This radical is capable of propagating a chain reaction by extracting a hydrogen atom from another fatty acid. These reactions are terminated by annihilation of two radicals to form a nonreactive species (L-L).

(7) Initiation: $LH + \cdot OH \longrightarrow H_2O + L\cdot$

(8) $L\cdot + O_2 \longrightarrow LOO\cdot$

(9) Propagation: $LOO\cdot + LH \longrightarrow LOOH + L\cdot$

(10) Termination: $L\cdot + L\cdot \longrightarrow L\text{-}L$

Lipid peroxidation will alter membrane fluidity and may lead to the loss of membrane integrity. The sensitivity of membranes to peroxidation depends on their PUFA content, with a greater content corresponding to increased potential for O-R modification. In the presence of transition metals such as iron or copper, LOOH will yield aldehydes, such as malondialdehyde and other fragmentation products. Malondialdehyde and conjugated dienes have been extensively used as an indication of lipid peroxidation and oxidative stress (Halliwell and Gutteridge, 1985). Measurements of malondialdehyde are obtained by the thiobarbituric acid test or high performance lipid chromatography. Despite the reported limitations of these methods (Lapenna and Luccurullo, 1993), an increase in the levels of malondialdehyde was demonstrated during exposure to exogenously generated O-R (Janssen *et al.*, 1993). Whether a similar increase occurs during reperfusion is still controversial with some reports showing an increase (Caudray *et al.*, 1992; Cordis *et al.*, 1993) and others no change (Brasch *et al.*, 1989; Janssen *et al.*, 1993).

Alterations in activities of membrane proteins due to O-R may be mediated by changes in the lipid microenviroment around the proteins or by direct oxidation of aromatic (phenylalanine; tryptophan; tyrosine; lysine) and/or sulfhydryl group containing amino acids (cysteine, methionine, or histidine) which play an essential catalytic or structural role (Fliss *et al.*, 1988). Either mechanism may lead to the loss of enzymatic activity or altered function of membrane ionic channels, transporters and/or receptors (Goldhaber and Weiss, 1992). Under severe

oxidative stress, polymerization, protein denaturation, breakage of polypeptide chains and changes in amino acid structure may also occur (Davies, 1987; Davies and Delsignore, 1987; Davies *et al.*, 1987a and b; Fliss *et al.*, 1988). GSH is required to maintain the reduced state of sulfhydryl-group containing amino acids proteins and hence, maintain normal protein activities (Meister and Tate, 1976; Meister and Anderson, 1983). For this reason, O-R induced alterations in proteins or amino acids may also occur when the levels of intracellular reduced glutathione (GSH) are depressed (see below).

C. Endogenous Defence Mechanisms Against O-R (Antioxidants):

Antioxidants are compounds that protect biological systems against the potentially harmful effects of processes or reactions that can cause an increase in the levels of oxidant molecules (Krinsky, 1992). Under physiological conditions, a delicate balance exists between the cellular mechanisms that generate oxygen-derived reactive metabolites and those that maintain antioxidant defense mechanisms (Flaherty and Weisfeldt, 1988). The latter may be classified under two categories; enzymatic and non-enzymatic mechanisms (Krinsky, 1992).

a. Enzymatic Mechanisms:

These mechanisms are considered to be the first line defence against O-R, are targeted to decompose and remove $\cdot O_2^-$ and H_2O_2, and thus prevent the production of $\cdot OH$, the most cytotoxic oxidant.

Superoxide dismutase: This class of intracellular metalloproteins exists as two isoenzymes; a copper zinc containing enzyme located in the cytoplasm and

intramembranal space of mitochondria (CuZnSOD), and as a manganese containing enzyme in mitochondrial matrix (MnSOD) (McCord and Fridovich, 1969; Fridovich, 1978). Both forms of SOD catalyze the dismutation of $\cdot O_2^-$ (as mentioned above) into H_2O_2 and O_2.

Catalase and glutathione peroxidase: The resultant by-product of $\cdot O_2^-$ dismutation, H_2O_2 is in turn reduced by the endogenous peroxidases, catalase (CAT) and glutathione peroxidase, into H_2O minimizing the generation of $\cdot OH$ (Marklund *et al.*, 1982; Flohe, 1982; Halliwell and Gutteridge, 1985; Marlund; 1988). Catalase (CAT) is a cytoplasmic hemoprotein bound to peroxisomes. It reduces H_2O_2 but it does not decompose organic hydroperoxides (LOOH).

(11) $H_2O_2 \xrightarrow{CAT} H_2O + O_2$

Both peroxides can be reduced by glutathione peroxidase. This is a selenium containing protein which is abundant in both the cytosol and mitochondria. Its reducing capacity occurs at the expence of oxidation of GSH to GSSG (oxidized glutathione).

(12) $LOOH + 2GSH \xrightarrow{GSH\text{-}Px} LOH + GSSG + H_2O$

The antioxidant capacity of glutathione peroxidase is tightly coupled to intracellular concentrations of GSH, glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH) (Meister and Tate, 1976; Meister and Anderson, 1983). Once GSH is oxidized, it can be reduced by glutathione reductase with NADPH as the reducing agent. The levels of NADPH are maintained by the hexose monophosphate shunt.

The ability of glutathione peroxidase to reduce organic peroxides including lipid peroxides is an important step terminating the propagation of chain reactions during lipid peroxidation (Krinsky, 1992). Moreover, glutathione peroxidase is effective at low H_2O_2 concentrations and under conditions of low oxidant stress. Therefore, it appears to play a more significant role than catalase in protecting the cells against H_2O_2 mediated injury (Marklund, 1988).

The antioxidants described above are primarily required to decompose $\cdot O_2^-$ and H_2O_2 and, therefore, to prevent the generation of $\cdot OH$ for which there is no specific defence or enzyme system present under physiological conditions. Conditions of elevated levels of $\cdot OH$ are invariably associated with tissue damage. Several exogenous scavengers of $\cdot OH$ have been described, including mannitol, dimethyl sulphoxide (DMSO), dimethylthiourea, and the membrane permeant scavenger N-(2-mercaptopropionyl)glycine (MPG) (Marklund, 1988; Bolli *et al*, 1989).

b. Non-enzymatic mechanisms:

A large number of compounds have been shown to possess some antioxidant activity. In general, this has been determined by their ability to prevent lipid peroxidation. This group of compounds includes lipid soluble and water soluble antioxidants (Krinisky, 1992).

The lipid-soluble antioxidants include; tocopherols (e.g., α-tocopherol; vitamin E), carotenoids (e.g., β-carotene and vitamin A), quinones (e.g., coenzyme Q), and bilirubin. Most of the lipid soluble antioxidants have proven

effective in inhibiting the propagation step of lipid peroxidation. For example, α-tocopherol quenches $\cdot O_2^-$, $\cdot OH$ and $^1O_2$ (Machlin and Bendich, 1987), but it is also known to protect polyunsaturated fatty acids against lipid peroxidation by acting as a chain breaker at the expense of its oxidation into a radical species (Mead, 1980).

Major water-soluble antioxidants include; ascorbic acid (vitamin C), uric acid, GSH, cysteine and creatine (Krinisky, 1992). For example, ascorbic acid can quench $\cdot O_2^-$, $\cdot OH$ and $^1O_2$ (Mechlin and Bendich, 1987) and can behave as an oxidant in the presence of transition metals. However, its main function is to act as a secondary antioxidant which reacts with α-tocopherol radical to regenerate the functional antioxidant α-tocopherol (Packer *et al.*, 1979). GSH is a very important antioxidant in the myocardium. It is an endogenous sulfhydryl containing peptide which prevents O-R formation by acting as a substrate for glutathione peroxidase during reduction of H_2O_2 or organic peroxides (Meister and Anderson, 1983). The resultant GSSG molecule is then either reduced again or transported out of the cell (Ishikawa and Seis, 1984). Therefore, maintaining appropriate levels of GSH, glutathione peroxidase and reductase are essential for protection against oxidative stress. As noted above, normal levels of GSH are required for maintaining sulfhydryl group-containing amino acids of proteins in the reduced state. A decrease in intracellular levels of GSH associated with a large release or accumulation of GSSG results in a decrease in the GSH/GSSG ratio. This ratio is an important measurement of the cellular redox state and its decrease is

indicative of oxidative stress (Brasch *et al.*, 1989; Ferrari *et al.*, 1991). Other important thiol compounds include cysteine, which is very often present in the catalytic subunits of proteins. Other exogenous thiol compounds with similar properties have been used experimentally to prevent oxidative stress, for example, N-acetylcysteine (Ferrari *et al.*, 1991) or the sulfhydryl reducing agent, dithiothreitol (DTT; Fliss *et al.*, 1988). The latter is known to protect the sulfhydryl groups of cysteine from oxidation. Both compounds were capable of attenuating ischemia-reperfusion induced myocardial injury by maintaing the levels of intracellular GSH (Ferrari *et al.*, 1991).

D. Net Increase in O-R During Ischemia-Reperfusion:

Early reperfusion is associated with a net increase in O-R production resulting from; (1) a decrease in the endogenous antioxidants levels, (2) and an increase in O-R production. For example, previous studies have reported decreased levels of endogenous antioxidant myocardial levels during ischemia including, SOD, catalase, GSH and glutathione peroxidase (Guarnieri *et al.*, 1980; Ferrari *et al.*, 1985). The extent of this decrease appears to be dependent on the severity of metabolic alterations in ischemia. Further decreases were shown to occur during reperfusion as a result of O-R induced inactivation of the antioxidants (Julicher *et al.*, 1984; Peterson *et al.*, 1985; Ferrari *et al.*, 1985). Theoretically, depressed antioxidant levels during ischemia or early reperfusion will make the myocardium more susceptible to oxidant stress.

Several sources and mechanisms have been described for O-R production

within or external to cardiac myocytes. These mechanisms appear dependent on, or primed by the preceding ischemic period. Intracellular sources of O-R include mitochondrial respiration and arachidonic acid or prostaglandin metabolism. Mitochondria are considered to be the largest source of intracellular $\cdot O_2^-$ and H_2O_2 produced via electron transport. The primary site of $\cdot O_2^-$ production by mitochondria is the region between quinone and cytochrome B in the internal mitochondrial membrane. $\cdot O_2^-$ is generated by the autooxidation of semiquinone (Freeman and Crapo, 1982; Marklund, 1988). Under normal conditions, the majority of $\cdot O_2^-$ is enzymatically dismutated to H_2O_2 by mitochondrial SOD. During ischemia, however, mitochondrial enzymes such as flavoproteins and ubisemiquinones accumulate in their reduced forms. They will then autooxidize during reperfusion to produce O-R, predominantly $\cdot O_2^-$. In addition, leakage of electron carriers out of the respiratory chain may reduce mitochondrial O_2 and form $\cdot O_2^-$ directly (Kukreja and Hess, 1992). This can occur through two pathways; breakdown of ubisemiquinone and nicotinamide-adenine dinucleotide (NADH) dehydrogenation (Kukreja and Hess, 1992). The increased $\cdot O_2^-$ formation in mitochondria can result in a leak of reactive metabolites into the cytosol of the myocardial cells and initiate the production of other oxygen-derived reactive metabolites, for example $\cdot OH$. Consistent with this mechanism, Guarnieri *et al.* (1985) reported an increased release of oxygen-derived reactive metabolites from mitochondria isolated from reperfused hearts which could be prevented by SOD.

Under normal conditions, $\cdot O_2^-$ is produced during arachidonic acid

metabolism. Cyclooxygenase catalyses the sequential conversion of arachidonic acid into prostaglandin G_2 (Freeman and Crapo, 1982) and then prostaglandin H_2. $\cdot O_2^-$ is produced during this second step catalysed by hydroperoxidase. In addition, the increase in $[Ca^{2+}]_i$ during ischemia may activate phospholipase A_2 and elevate production of arachidonic acid (Karmazyn and Moffat, 1985; Kukreja and Hess, 1992). Reperfusion could potentially stimulate the synthesis of prostaglandins leading to the release of $\cdot O_2^-$ (Kukreja and Hess, 1992). The contribution of this mechanism to reperfusion injury was supported by studies using nafazatrom, an antioxidant and cyclooxygenase blocker, which decreased myocardial infact size (Coker and Parrate, 1984). In addition, several studies have shown that blocking phospholipase A_2 or cyclooxygenase attenuated the incidence of arrhythmias and improved functional recovery in intact heart preparations (Moffat *et al.*, 1988; Moffat and Tsushima, 1989). Whether O-R generation and oxidative stress were involved remain to be elucidated.

Extracellular sources of O-R include endothelial xanthine oxidase activity, polymorphonuclear leukocytes (PMN), and catecholamine autooxidation. It is proposed that the increase in $[Ca^{2+}]_i$ during ischemia (Steenbergen *et al.*, 1987) may activate Ca^{2+}-dependent cytosolic proteases that convert xanthine dehydrogenase into xanthine oxidase (XO) (McCord, 1985). Also during ischemia, accumulation of break down products of ATP hydrolysis occurs (Ip and Levin, 1988). This includes purine, hypoxanthine and/or xanthine. In the presence of oxygen during reperfusion, XO will catalyze the oxidation of purine substrates to

form uric acid, $\cdot O_2^-$, H_2O_2 (McCord, 1985). Ordinarily the activity of XO is very low in the heart and conversion of hypoxanthine to uric acid is catalyzed by xanthine dehydrogenase. This reaction does not produce O-R. However, xanthine dehydrogenase can be converted to XO during ischemia (Chambers *et al.*, 1985) and enhanced XO activity might lead to O-R. This process is species-dependent, for example, XO was found in rat cardiac tissues but was not detectable in rabbit and human (Downey *et al.*, 1988; Grum *et al.*, 1989; Thompson-Gorman and Zweier, 1990). Therefore, it is controversial whether O-R generated by this mechanism is important in the human. On the other hand, this pathway is significant for O-R production in endothelial cells. These cells can also produce O-R through another pathway involving $\cdot NO$ which reacts with $\cdot O_2^-$ to form peroxy nitrite anion ($ONNO^-$), a highly toxic molecular species. Decomposition of these reactive molecules generates $\cdot OH$ (Beckman *et al.*, 1990). The NADPH-dependent oxidase system located on the surface membrane of PMN is also a source of $\cdot O_2^-$. This enzyme is normally dormant (Tauber *et al.*, 1979), however, during ischemia-reperfusion PMN infiltrate the microvasculature and are activated. This results in activity of NADPH-dependent oxidase and O-R production (Engler *et al.*, 1986). This catalyses the production of $\cdot O_2^-$ and H_2O_2 through the so-called respiratory burst which accounts for more than 90% of $\cdot O_2^-$ production (Babior, 1984). Moreover, the secretion of myeloperoxidase from azurophilic granules in PMN can catalyze the formation of another oxidant, hypochlorus acid (HOCl) in the presence of H_2O_2 and chloride ion. The combination of HOCl with nitrogenous

compounds forms the highly toxic lipophilic oxidant, monochloramines. These reactive metabolites may enter cardiac myocytes and induce injury (Kukreja and Hess, 1992). The role of PMN in reperfusion injury was apparent following long periods of ischemia (>90min) (Lucchesi, 1990).

Another extracellular source of O-R is the autooxidation of catecholamines (adrenaline and noradrenaline) (Singal *et al.*, 1980). The latter are released from the sympathetic nerve terminals in the myocardium during ischemia. Upon reperfusion, autooxidation of catecholamines results in the formation of O-quininone, 2 H^+ and 2e. The latter can produce $\cdot O_2^-$ which then forms $\cdot OH$ by the iron catalyzed Haber-Weiss reaction (Kukreja and Hess, 1992).

Thus, several sources and mechanisms may potentially contribute to O-R generated during ischemia-reperfusion. However, the extent to which the site of generation, that is whether the O-R come from intracellular versus extracellular sources, may have differential effects on myocardial viability is still not clear at the present time.

9. Effects of Extracellular O-R Stress on Electrical Activity of The Heart:

A. Exogenous O-R Generating Systems:

In order to understand how O-R stress during reperfusion might affect electrical activity in the heart independent of all other contributing factors, healthy preparations have been exposed to exogenous O-R generating systems. O-R are generated by enzymatic reactions (Kukreja and Hess, 1992), such as the combination of purine metabolites (purine, xanthine or hypoxanthine) with

xanthine oxidase (P:XO;HP:XO:X:XO) to generate $O_2^{\cdot-}$, H_2O_2 and perhaps $^1O_2$. Alternatively, autooxidation of compounds such as dihydroxyfumaric acid (DHF) also produces $O_2^{\cdot-}$, and to a lesser extent H_2O_2 and $\cdot OH$ (Halliwell, 1977). In combination with $FeCl_3$:ADP, DHF autooxidation will result in the production of $\cdot OH$ (Kukreja *et al.*, 1988). Recently, photo-illumination of the flourescein-like compound, Rose Bengal has been employed in several studies. This compound generates $^1O_2$ and to a lesser extend $O_2^{\cdot-}$ when exposed to green light (530-590 nm) (Paczkowski *et al.*, 1985; Kusama *et al.*, 1989). Finally, O-R have also been generated by direct chemical reactions, such as the combination of HOCL and H_2O_2 to yield $\cdot OH$ in the presence of iron (Josephson *et al.*, 1991)

Endogenous antioxidant levels are depleted by cumene hydroperoxides and tert-butylhydroperoxide. Both have the capacity to deplete GSH which will increase the susceptibility of tissue to O-R stress reflected by increased levels of lipid peroxidation (Barrsacchi *et al.*, 1983; Deuticke *et al.*, 1987).

B. Effects oF Exogenous O-R Stress on Electrical Activity and Ionic Currents In the Heart:

Perfusion of isolated hearts with O-R generating systems induced arrhythmias including VT, VF and PVCs similar to those reported during early reperfusion (Bernier *et al.*, 1986; Nakaya *et al.*, 1987; Hearse *et al.*, 1989). These arrhythmias (except for PVCs) were reduced with O-R scavengers, SOD, catalase, mannitol, methionine or desferoxamine (Bernier *et al.*, 1986). This is consistent with the idea that O-R stress contributes to early reperfusion

arrhythmias.

To characterize the changes in action potential configuration and membrane ionic currents which may contribute to O-R arrhythmogenesis, multicellular preparations and/or isolated myocytes have been exposed to exogenous O-R generating systems. The changes in AP configuration induced by exogenous O-R in intact preparations were variable and are summarized in table 1. The most consistent change was a marked decrease in RMP which was associated with DADs, triggered activity and enhanced automaticity (e.g., Nakaya *et al.*, 1987). Interestingly, prolongation, shortening, no change, or an early increase followed by a late decrease in AP duration were all reported (table 1). It is not known why such variable results have been obtained. It is possible that the differences could stem from the O-R generating system used, the experimental preparation, animal species and sensitivity of the preparations to O-R, and/or the levels of O-R produced in the different studies.

The sequence of changes in AP configuration induced by exogenous O-R in isolated myocytes was first described by Barrington *et al.* (1988). Single canine ventricular myocytes were impaled with conventional microelectrodes and exposed to either DHF or X:XO. Three different stages of change were demonstrated; (1) prolongation of AP duration and increase in the plateau height (voltage), (2) developement of early and delayed after-depolarizations (EADs and DADs), which occasionally produced triggered activity, followed by a failure to repolarize beyond -40 mV, and finally (3) a decline in AP duration accompanied

TABLE 1: Summary of Changes in Acion Potential Parameters in Intact Heart Preparations upon Extracellular Exposure to Exogenous O-R Generating Systems (O-R).

PREPARATION	O-R	RMP	$\dot{V}_{max}$	APA	APD	MDP	DADs&TA/ EADs	References
Guinea pig ventricular strip	P:XO	↓	↓	↓	↔		DADs/TA	Pallandi *et al.*, 1986
Guinea pig PM & canine purkinje fibers	CH& t-BHP	↓	↓	↓	↑	↓	DADs/TA	Nakaya *et al.*, 1987
Guinea pig PM	H_2O_2				↑ ↓		DADs/TA	Hayashi *et al.*, 1989
Rabbit intraventricular septum	H_2O_2/ X:XO				↓ ↓		-	Goldhaber *et al.*, 1989
Guinea pig trabeculae	H_2O_2	↓	↓		↑ ↓		DADs	Firek&Berezewicz, 1990
Canine PM/ Purkinje fibers	P:XO	↓/ ↔			↓/↔	-/↔	-	Tsushima&Moffat, 1990
Rabbit+rat PM&trabeculae	RB				↑		EADs	Shattock *et al.*, 1991
Guinea pig PM	CH	↓			↓		-	Nakaya *et al.*, 1992
Guinea pig right ventricular free wall	H_2O_2	↓			↑		DADs/TA	Duan&Moffat, 1992

PM=papillary muscle; V_{max}=maximal rate of depolarization; APA=AP amplitude; APD=AP duration; MDP=maximal diastolic potential; CH= cumene hydroperoxide; t-BHP=tert-butylhydroperoxide; RB=Rose Bengal; P=purine; X=xanthine; XO=xanthine oxidase; ↔=no change; ↓=increase; ↓=decrease; ↑↓= increase then decrease; TA= triggered activity.

by repolarization of membrane potential close to control levels. Stages 1 and 2 were also seen after H_2O_2 superfusion (Barrington, 1990), and the changes in stages 1, 2, and 3 were delayed by SOD or catalase (Barrington *et al.*, 1988). Similar observations were made with myocytes using whole-cell configuration of the patch clamp technique. Prolongation of AP duration accompanied by EADs and DADS were observed in rat and guinea pig ventricular myocytes with minimal intracellular Ca^{2+} chelation following exposure to H_2O_2 (Beresewicz and Horakova, 1991) or DHF (Cerbai *et al.*, 1991). This was followed by either triggered activity, membrane depolarization and loss of excitability (Beresewicz and Horakova, 1991) or by marked shortening of AP duration and inexcitability (Cerbai *et al.*, 1991). Goldhaber *et al.* (1989) reported only marked shortening in AP duration in myocytes with elevated intracellular Ca^{2+} chelation.

Given the highly reactive nature of O-R and their extremely short half life, it is possible that O-R may only affect myocytes within the compartment of their generation. O-R in intracellular versus extracellular compartments may, therefore, have differential effects on AP configuration. This possibility has not been investigated in mammalian ventricular myocytes. However, Tarr and Valenzeno (1989) demonstrated an early increase followed by a decrease in AP duration in frog atrial myocytes during extracellular O-R application. In contrast, intracellular application induced only shortening of AP duration.

The basis for the alterations in AP configuration described above in terms of the underlying changes in ionic currents is poorly defined and controversial.

A summary of effects of extracellular exposure to different generating systems on ionic currents in myocytes obtained from several species is given in table 2. As indicated in table 2, the literature remains insufficient to provide enough information on the effects of a given generating system to formulate any substantial conclusions. In some cases, opposite effects of O-R on some ionic currents is apparent. This could stem from the variability in animal species, the O-R generating sytem used, and/or the recording conditions employed (i.e., with respect to constituents of the internal pipette solution and voltage-clamp protocols used). Unfortunately, no study reported the alterations in ionic currents following given changes in AP in a time-dependent fashion. This would make it easier to identify the underlying alterations in ionic currents.

Table 2 suggests that extracellular O-R has effects on both time-dependent and -independent ionic currents. Most studies to date have focused on the effects of O-R on I_{Na}, $I_{Ca,L}$ and I_K. Unfortunately, these studies showed controversial results (table 2). An increase in steady-state window Na^+ current was reported in frog myocytes (Bhatnagar *et al.*, 1990). This could underlie the increase in AP duration during O-R stress (Bhatnagar *et al.*, 1990; Tarr and Valenzeno, 1991). This idea was supported by the observation that TTX blocked the O-R induced prolongation of AP duration in guinea pig and rat ventricular myocytes (Beresewicz and Horakova, 1991). Alternatively, the prolongation might be mediated by the decrease in I_K reported from guinea pig ventricular myocytes and frog atrial myocytes (Cerbai *et al.*, 1991; Tarr and Valenzeno, 1991). That

TABLE 2: Summary of Effects of Extracellular Application of Exogenous O-R Generating Systems (O-R) on Membrane Ionic Currents.

PREPARATION	O-R	S-S I_{Na}	$I_{Ca,L}$	I_K	I_{K1}	I_{KATP}	Ca^{2+}-Activated currents	References
Guinea pig VM	H_2O_2& X:XO		↓			+		Goldhaber *et al.*, 1989
Guinea pig VM	DHF		↓	↓	⇔			Cerbai *et al.*, 1990
Guinea pig VM	HX:XO		↑		↓			Coetzee & Opie, 1992
Guinea pig & rat VM	H_2O_2		⇔					Beresewicz & Horakova, 1991
Rabbit VM	RB		↓		↓		+ S-S current & + I_{ti}	Matsuura & Shattock, 1991a&b
Frog VM	t-BHP	↑	⇔		⇔			Bhatnagar *et al.*, 1990
Frog AM	RB	↑	↓	↓				Tarr & Valenzeno, 1991

VM=ventricular myocytes; AM=atrial myocytes; ⇔=no change; ↑=increase; ↓=decrease; +=activation; X=xanthine; HX=hypoxanthine; XO=xanthine oxidase; DHF=dihydroxyfumaric acid; H_2O_2=hydrogen peroxide; RB=Rose Bengal; t-BHP=tert-butylhydroperoxide. .

changes in $I_{Ca,L}$ during O-R stress might contribute to changes in AP plateau is still questionable. Table 2 indicates an increase, decrease or no change of $I_{Ca,L}$ was observed in guinea pig myocytes in different studies. Whether O-R affects I_{K1} and the level of RMP is controversial. No change, or a decrease in this current was reported, even within the same species (guinea pig ventricular myocytes). That O-R stress mediates a decrease in I_{K1} was supported by single channel recordings from guinea pig ventricular myocytes (Nakaya *et al.*, 1992). Cell-attached patches exposed to cumene hydroperoxide showed the open probability of the channel to decline. Inhibition of I_{K1} would theoretically lead to depolarization and/or prolongation in AP duration.

Superfusion of rabbit ventricular myocytes with Rose Bengal resulted in the activation of I_{ti} which was mediated by I_{NaCa}. This was demonstrated by the blockade of I_{ti} by equimolar replacement of extracellular Na^+ with Li^+ which, as indicated above, blocks forward mode activity of the Na^+-Ca^{2+} exchanger (Matsuura and Shattock, 1991a). Given that I_{NaCa} is responsible for inducing DADs and triggered activity, it seems likely that DADs and triggered activity account for part of the arrhythmogenesis during O-R stress. Another mechanism may be an O-R induced inhibition of the Na^+/K^+ pump. Superfusion with Rose Bengal was shown to enhance this time-independent conductance (Shattock and Matsuura, 1993). Activation of I_{NaK} could lead to a rise in $[Ca^{2+}]_i$ over time, similar to the effects of ouabain, leading to I_{ti} and DADs.

Alternatively, I_{NSC} could cause depolarization and injury currents across the

border between reperfused and normal myocardium. If I_{NSC} was activated by O-R stress (Matsuura and Shattock, 1991b), then a positive shift in the reversal potential of quasi steady-state I-V would be expected, and this would lead to marked depolarization of RMP.

The final stage of O-R stress marked by shortening of AP duration in guinea pig ventricular myocytes appears to be mediated by activation of I_{KATP} (Goldhaber *et al.*, 1989). This shortening of AP duration could bring about a decline in refractory period, inexcitability and heterogeneity in repolarization, all of which are potentially arrhythmogenic changes.

10. Possible Cellular Mechanisms for Effects of O-R on Ionic Channels:

A. Direct Mechanisms:

That O-R induced lipid peroxidation might alter membrane electrical activity and predispose the myocardium to arrhythmias was reported by Nakaya *et al.* (1987). These authors showed that extracellular exposure of isolated perfused guinea pig hearts or papillary muscles to cumene hydroperoxide or tert-butylhydroperoxide led to arrhythmogenic alterations in electrical activities which were associated with an increase in malondialdehyde levels (Hayashi *et al.*, 1989). Although both effects were attenuated by pretreatment with the antioxidant, butylated hydroxytoluene, there is as yet no direct evidence showing alterations in a specific membrane conductances due to lipid peroxidation. On the other hand, direct protein oxidation was implicated in studies in which mechanical dysfunction induced by O-R stress was either reversed or prevented by sulfhydryl

group reducing agents, for example N-acetylcysteine (Ferrari *et al.*, 1991) and DTT (Fliss *et al.*, 1988; Eley *et al.*, 1991). Alterations in the activities of some enzymes and ion transporters were attributed to O-R-induced oxidative modification of sulfhydryl groups in the proteins. For example, inactivation of the SL Ca^{2+}-ATPase (Kaneko *et al.*, 1991), Na^{+}/K^{+}-ATPase (Matsouka *et al.*, 1990) and SR Ca^{2+}-ATPase (Yanagishita *et al.*, 1989; Eley *et al.*, 1991) by O-R stress could all be reversed by DTT (Ziegler, 1985). Significantly, when non-O-R sulfhydryl group oxidizing agents, such as diazenedicarboxylic acid bis-N,N'-dimethylamide (diamide; Kosower *et al.*, 1969; Haest *et al.*, 1979) were employed, similar changes in enzyme activity to those caused by O-R were elicited (Matsouka *et al.*, 1990; Kaneko *et al.*, 1991). Whether changes in electrical activity could also be due to direct sulfhydryl modification of a sarcolemmal ion conductance remains to be determined.

B. Indirect Mechanism:

Two different indirect mechanisms have been implicated to affect membrane ionic currents leading to abnormal membrane electrical activity during O-R stress. These include an increase in $[Ca^{2+}]_i$ and a depletion of $[ATP]_i$.

a. O-R induced rise in $[Ca^{2+}]_i$:

The observation that DADs and triggered activity were induced by O-R in several cardiac intact preparations and isolated myocytes implies the possiblility that Ca^{2+} overload might be involved. Since I_{ti} and DADs could be abolished by ryanodine, abnormal SR Ca^{2+} handling due to O-R seems likely (Hayashi *et al.*,

1988; Beresewicz and Horakova, 1991). Indeed, a rise in free cytosolic $[Ca^{2+}]_i$ was reported by Hayashi *et al.* (1989) to occur in guinea pig ventricular myocytes exposed to extracellular O-R (H_2O_2). Subsequently, studies showing similar results were obtained using myocytes from other species and different O-R generating systems (Burton *et al.*, 1990; Daly *et al.*,1991; Josephson *et al.*, 1991; Peerson-Rothert *et al.*, 1992). The diastolic and systolic increases in $[Ca^{2+}]_i$ observed in these studies are probably not the result of O-R induced non-specific membrane damage, sarcolemmal leakiness or loss of membrane integrity since the myocytes remained impermeable to macromolecules like trypan blue (Josephson *et al.*, 1991).

Several mechanisms could account for the rise in $[Ca^{2+}]_i$ during O-R stress. A possible contribution for I_{Ca} in increasing Ca^{2+} influx is questionable due to the controversy concerning effects of O-R on this conductance. However, Ca^{2+} channels blockers do prevent O-R induced $[Ca^{2+}]_i$ overload in single myocytes (Josephson *et al.*, 1991). Although suggestive, this could be the result of; (1) reducing the overall Ca^{2+} burden on the myocytes and reducing the levels of Ca^{2+} in the SR stores, and/or (2) direct O-R scavenging by L-type Ca^{2+} channels blockers (Mak and Weglicki, 1990).

Studies on isolated SL vesicles reported both stimulation (Reeves *et al.*, 1986; Dixon *et al.*, 1990) and inhibition (Hata *et al.*, 1990; Shi *et al.*, 1989; Okabe *et al.*, 1989) of Na^+-Ca^{2+} exchange by O-R generating systems. On the other hand, an increase in inward current due to forward mode of I_{NaCa} was reported

in whole-cell recordings from rabbit ventricular myocytes exposed to Rose Bengal (Matsuura and Shattock, 1991). Recently, a simultaneous increase in the $[Ca^{2+}]_i$ transient and forward mode I_{NaCa} was reported (Goldhaber and Weiss, 1993). These data suggest that the enhanced I_{NaCa} was secondary to the rise in $[Ca^{2+}]_i$ induced by O-R stress. Whether depressed forward mode activity may contribute to the increase in $[Ca^{2+}]_i$ is still unclear.

Inhibition of two other SL enzyme activities contributing to Ca^{2+} homeostasis are also reported. A decline in SL Ca^{2+}-ATPase by O-R was indicated (Kaneko *et al.*, 1989; Kaneko *et al.*, 1990) which in the long term could lead to an increase in $[Ca^{2+}]_i$. Additionally, several biochemical studies reported that O-R stress decreases the activity of the Na^+/K^+ ATPase which could be prevented by O-R scavengers (Kim and Akera, 1987; Kukreja *et al.*, 1990; Xie *et al.*, 1990). These observations were supported by electrophysiological data obtained by Shattock and Matsuura (1993). These authors noted a decrease in pump current during O-R stress induced by extracellular Rose Bengal. A decrease in the pump current could contribute indirectly to the elevation in $[Ca^{2+}]_i$ by increasing $[Na^+]_i$ and secondarily inhibiting Ca^{2+} efflux via Na^+-Ca^{2+} exchange.

As noted above, ryanodine is well known to reduce the effects of exogeneous O-R on $[Ca^{2+}]_i$ and electrical activity, indirectly supporting a role for abnormal Ca^{2+} handling by the SR during O-R stress. Additionally, a rapid release of Ca^{2+} from SR vesicles (Stuart and Abramson, 1989), a caffeine-sensitive rise in $[Ca^{2+}]_i$ in intact myocytes (Goldhaber *et al.*, 1991) and an increase in the

ryanodine-sensitive, calmodulin-dependent passive Ca^{2+} efflux from SR vesicles (Okabe *et al.*, 1989; Okabe *et al.*, 1991) during O-R stress was also reported. Direct evidence for abnormal Ca^{2+} handling by the SR was recently obtained by Holmberg *et al.* (1991). These authors demonstrated that O-R stress increased the open probability of SR Ca^{2+} release channels incorporated into artificial planar bilayers.

It is also possible that Ca^{2+} uptake by the SR is depressed during O-R stress. Studies on isolated SR vesicles demonstrate depressed Ca^{2+} uptake because O-R reduce the activity of the SR Ca^{2+}-ATPase enzyme (Hess *et al.*, 1981; Kukreja *et al.*, 1988; Okabe *et al.*, 1989; Kukreja 1991). Therefore, direct effects of O-R on the SR would be expected to induce a net Ca^{2+} rise in the cytoplasm and a decrease in SR Ca^{2+} levels. If the SL Ca^{2+} efflux mechanisms were simultaneously depressed a net rise in $[Ca^{2+}]_i$ would occur over time.

As mentioned above, a rise in $[Ca^{2+}]_i$ could theoretically alter the activity of several ionic conductances and transport mechanisms, including stimulation of I_K, I_{NaCa}, I_{NSC} as well as inhibition of $I_{Ca,L}$, I_{K1}. Whether this is true for O-R effects is not well characterized. Matsuura and Shattock (1991b) showed that inclusion of 5 mM EGTA in the pipette solution employed for whole-cell voltage clamp prevented an increase in Ca^{2+}-activated steady-state conductance. Clearly, the rise in I_{NaCa} mediating I_{ti} in rabbit myocytes during O-R stress can be attributed to an increase in $[Ca^{2+}]_i$ (Matsuura and Shattock, 1991a). Finally, the source for the increase in $[Ca^{2+}]_i$, that is extracellular or intracellular, mediating these

alterations in ionic currents is not clear. Beresewicz and Horakova (1991) suggested that abnormal Ca^{2+} release from SR was only responsible for the DADs observed during O-R stress. This conclusion was supported by data from Coetzee and Opie (1992) showing the inability of ryanodine to inhibit contracture induced by O-R stress.

b. O-R induced depletion in $[ATP]_i$:

Several cardiac membrane conductances are known to be controlled by, or dependent on intracellular levels of ATP (Noma and Shibasaki, 1990). A depression in intracellular ATP content of myocardial tissue during O-R stress was reported for intact heart preparations and isolated cultured myocytes (Vlessis *et al.*, 1991; Corretti *et al.*,1991). This decrease was attributed to an inhibition of anaerobic glycolysis as well as mitochondrial oxidative phosphorylation (Hyslop *et al.*, 1988; Corretti *et al.*, 1991; Josephson *et al.*, 1991).

As noted above, depression of $[ATP]_i$ during ischemia or metabolic inhibition activates cardiac ATP-sensitive K^+ channels. A similar mechanism was proposed by Goldhaber *et al.* (1989) to account for the effects of O-R stress on this conductance. These authors showed that O-R activates ATP-sensitive K^+ channels leading to a marked decline in AP duration and an increase in $^{42}K^+$ efflux in intact rabbit ventricle. Depletion of $[ATP]_i$ during O-R stress could also lead to depression of ionic current activities which require phosphorylation, and/or a decrease in the activity of membrane transport mechanisms which are driven by ATP (Noma and Shibasaki, 1990). Such an inhibition might result in a net rise

in $[Ca^{2+}]_i$. Whether $[ATP]_i$ depletion was the consequence or the cause of Ca^{2+} overload remains to be resolved. Inhibition of glycolysis was found to precede the increase in $[Ca^{2+}]_i$ in isolated rabbit hearts exposed to O-R in a nuclear paramagnetic resonance spectroscopy study by Corretti *et al.* (1991). On the other hand, an early increase in $[Ca^{2+}]_i$ measured by Indo-1 was observed to preceed a decline in $[ATP]_i$ measured by the same technique (Josephson *et al.*, 1991).

Some alterations in ionic currents induced by O-R stress can not be attributed to alterations in $[ATP]_i$ or $[Ca^{2+}]_i$. For example, the decrease in I_{K1} induced by O-R in rabbit ventricular myocytes, was not affected by 5 or 10 mM EGTA or 5 mM ATP included in the patch pipette (Matsuura and Shattock, 1991b). Absence of Ca^{2+} sensitivity was also reported by Cerbai *et al.* (1991) who showed that the O-R induced decrease in $I_{Ca,L}$ and I_K was not affected by pipette EGTA. However, their experiments employed lower concentrations of EGTA (0.2 mM) which may have been insufficient to chelate the rise in $[Ca^{2+}]_i$ induced by O-R stress. Whether changes in ionic conductances insensitive to $[Ca^{2+}]_i$ or $[ATP]_i$ are mediated by direct mechanisms, such as lipid peroxidation or oxidative modification of sulfhydryl groups, remains to be defined.

II. AIM OF STUDY

As is apparent from the previous literature review section, oxygen-derived free radicals (O-R) are implicated in myocardial injury during reperfusion after a period of ischemia (Hess and Manson, 1984). A burst of O-R (Garlick *et al.*, 1987; Zweier *et al.*, 1989) is thought to contribute to the generation of early arrhythmias altered mechanical performance and structural damage (Hearse, 1977; Manning and Hearse, 1984; Manning *et al.*, 1984; Woodward and Zakaria, 1985; Jennings *et al.*, 1985; Hearse and Tosaki, 1987). There is abundant evidence indicating that O-R are generated through several different reaction pathways within cardiac myocytes as well as from extracellular sources. Since O-R are highly reactive compounds with extremely short half-lives; (Buettner and Manson, 1990; Hall, 1990), it is possible that they may exert their effects largely within the compartment in which they are generated leading to differential effects on membrane ionic conductances. *To this date, no studies on mammalian myocytes have as yet explored the possibility of differential influences of extracellular versus intracellular O-R stress rising from an identical generating system under identical recording conditions.*

Theoretically, cardiac membrane currents could be affected by O-R stress as a result of; (1) lipid peroxidation and changes in membrane fluidity, (2) direct oxidation of channel proteins or associated regulatory subunits (Hess and Manson, 1984; Halliwell and Gutteridge, 1985; Fliss *et al.*, 1988), or (3) a change

in a cytoplasmic regulatory factor, for example intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Burton *et al.*, 1990, Daly *et al.*, 1991; Persoon-Rothert *et al.*, 1992). Some studies have implicated a rise in $[Ca^{2+}]_i$ as an important factor in causing changes in membrane currents during O-R stress (Beresewicz and Horakova, 1991; Cerbai *et al.*, 1991; Matsuura and Shattock, 1991a and b).

Lipid peroxidation was shown to occur in intact preparations with demonstrable changes in electrical activity (Nakaya *et al.*, 1987). However, there is as yet no direct evidence showing alterations in a specific membrane conductance as a result of lipid peroxidation. On the other hand, direct protein oxidation was shown to account for changes in the activities of several enzymes and ion transporters (Fliss *et al.*, 1988). *However, a role for direct sulfhydryl modification of a cardiac sarcolemmal ion conductance due to O-R leading to abnormal electrical activity has not been described.*

In this study ***we sought to investigate the differential effects of extra- and intracellular O-R sources on alterations in electrical activity and steady-state membrane ionic currents. Second, we attempted to identify and compare the cellular mechanism(s) responsible for the changes in ion channel activity upon intra- and extracellular O-R stress.*** This study provides important insights into the contribution of O-R generated from intracellular sources to the genesis of reperfusion arrhythmias and on the differential ionic and cellular mechanisms which may underlie the arrhythmogenic alterations in electrical activity induced by O-R.

III. MATERIALS AND METHODS

1. Cell Isolation Technique:

Freshly dispersed cardiac ventricular myocytes were isolated from guinea pig hearts using an enzymatic technique modified from Langer *et al.* (1987).

A. Solutions:

Krebs-Henseleit (K-H) solution contained (in mM); NaCl, 120; KCl, 4.8; $NaHCO_3$, 25; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; glucose, 11; taurine, 13; $CaCl_2$, 1.8. After bubbling the solution with 95% O_2- 5% CO_2 for 10-15 min, the pH of solution measured was about 7.4.

IB1 solution contained (in mM): $NaHCO_3$, 50; taurine, 119.9; L-carnitine,4; adenosine, 2.32; $MgCl_2$, 2.26; glucose, 29.9; L-glutamic acid, 21.5; pH adjusted to 7.2 by 1N NaOH. IB2 solution was prepared in the same way as IB1 with the addition of 100μM $CaCl_2$.

MEM Jokliks (MEM; GIBCO BRL, Life technologies Inc., NY) medium was prepared by dissolving 4 packages (11.3 g/pkg) of the powder in 2 L of deionized H_2O. A final concentration of 34.2 mM $NaHCO_3$ was obtained by adding 4g of $NaHCO_3$ to the dissolved medium.

Medium 199 (GIBCO BRL, Life technologies Inc., NY) was prepared by dissolving 2 packages of the powder Medium 199 in 2L of deionized water. A final concentration of 5.9 mM $NaHCO_3$ was obtained by adding 0.7 g of $NaHCO_3$ to the

dissolved medium.

Solutions (IB1; IB2; MEM Jokliks; medium 199) were bubbled with 95% O_2-5% CO_2 for 15 minutes before adjusting their pH to 7.2 with 1N NaOH. Solutions were then filtered under a laminar flow hood using sterile filters (CANLAB, Becton Dickinson Labware, Falcon 7105 Bottle Top Filter; 0.22 micron, NJ). This filtration raised the pH value by 0.1-0.2 resulting in a final pH of 7.3 - 7.4.

IB1-MEM solution was obtained by mixing IB1 solution and MEM-JOKLIKS medium in 1:1 by volume. IB2-MEM solution was obtained by mixing IB2 solution and MEM-JOKLIKS medium in 1:1 by volume. In this solution the final concentration of $CaCl_2$ was 50 μM.

B. Digesting Enzymes:

The following enzymes solution was used to isolate single myocytes from the ventricular myocardium; (1) *collagenase* (74.52 U/ml; type 2, Worthington Biochemical Corp., NJ; Lot. No. M3C601; 295U/mg dry wt.), (2) *hyaluronidase* (0.5 mg/ml; from Bovine testes, Type I-S, lyophilized; Sigma Chemical Co., MO), and (3) *trypsin inhibitor* (0.2 mg/ml; Sigma Chemical Co., MO; Lot No. 82H7016).

C. Cell Isolation Procedure:

Guinea pigs (200-300 g) were anesthetized with CO_2 and then sacrificed by cervical dislocation. Their hearts were quickly excised after opening their chest cavities and placed in well-aerated ice cold K-H solution. The aorta was cannulated and connected to a Langendorff apparatus. Both atria were removed while the heart was perfused in a retrograde fashion with K-H (33-34°C) at a

constant pressure. This closed the aortic valve and directed the perfusate into the coronary circulation (Langendorff, 1895). The temperature of either the left or the right ventricular cavities was monitored during the perfusion period by a temperature probe inserted in the cavity and connected to a thermistor thermometer (model 8402-00, Cole-Parmer Instrument Company, IL). The recorded temperature was about 33 ± 0.2°C during the experimental period. After perfusing the hearts with control K-H solution for a stabilization period of 10-15 min, the solution was switched to nominally Ca^{2+}-free IB1-MEM solution to washout all Ca^{2+} in the extracellular space. After 10 min, the ventricles were perfused in a recirculating manner for 20-30 minutes at a rate of 15 ml/min with a mixture of enzymes added to 50 μM Ca^{2+}-containing IB2-MEM solution. During this period, the ventricles became very soft and were then removed and placed in petri dishes (100 X 15 mm) filled with 20 ml of 50μM Ca^{2+}-containing IB2-MEM solution. Using fine tip forceps, ventricles were torn apart into small pieces which were then washed in several changes of IB2-MEM solution. Ca^{2+} concentration of IB2-MEM solution bathing tissue pieces was then slowly increased by adding 1 ml of Medium 199 containing 1 mM Ca^{2+} at 15 min intervals. This was repeated 3 times after which the supernatant was exchanged with medium 199. The tissue pieces were then washed and stored in medium 199 at room temperature. Single ventricular myocytes were obtained when needed by gentle trituration using a wide tip, heat-polished pipette. This procedure usually yielded 80-90% of Ca^{2+}-tolerant, rod shaped relaxed myocytes which had clear and distinct cross-

striations.

2. Electrophysiological Recording:

A. Patch Clamp Techniques:

Freshly dispersed guinea pig ventricular myocytes were placed in a plexi-glass recording chamber (1ml volume) attached to the stage of an inverted Nikon TMS microscope (MFA 10300; Narishige Scientific Instrument Laboratory, Japan). The microscope was placed within a Faraday cage on a nitrogen-charged Micro-G flotation table (Technical Manufacturing Corp., MA) to prevent interference from external mechanical vibrations. After allowing the myocytes to settle to the glass bottom of the chamber, they were superfused with the bath solution at flow rate of 1.8 ml/min. The time required for complete exchange of the bath contents was 10-30 seconds based on the time required to reach stable positive shift in resting membrane potential upon superfusion with 9.8 mM extracellular potassium concentration ($[K^+]_o$)). Myocytes were monitored during experimentation by a camera (Cohu, CA) and videomonitor connected to the microscope. Most of the experiments were performed at 22°C, however, some experiments were performed at 34°C using a microscope stage heater (Medical Systems Corp., NY) in which the recording chamber was placed.

The membrane electrical activity and ionic currents of single guinea pig myocytes were obtained by employing the standard whole-cell configuration of patch clamp technique described by Hamill *et al.* (1981). This technique is based on the concept that a fine diameter glass patch micropipette can seal to the

membrane of a myocyte when suction is applied.

Patch micropipettes were pulled from capillary glass (inner diameter 1.2 mm, outer diameter 1.67mm; borosilicate glass, Richland Glass Co., NJ) using a two-stage glass puller (PP-83; Narishige Scientific Instrument Lab., Japan) and their tips were then fire-polished using a microforge (MF-83; Narishige Scientific Instrument Lab., Japan), to a final resistance of 0.25-0.5 MΩ when filled with filtered internal pipette solution. Patch micropipettes were then placed in an air-tight pipette holder connected to a low resistance (100 MΩ feedback resistor) head-stage of a Dagan 800 patch-clamp amplifier (Dagan Corp., MN). A silver-chloride wire was immersed in the internal pipette solution within the pipette. Another silver-chloride wire was placed in the bath solution as a grounding electrode. Both wires were connected to the headstage of the amplifier. The movements of the headstage were controlled by coarse and fine manipulators, the latter a three dimension aqua micromanipulator (WR-88; Narishige Scientific Instrument Lab., Japan).

The pipette tip was positioned above the cell and the junction potential was nullified. The pipette tip was pressed against the membrane of the myocyte and a brief pulse of negative pressure (suction) was applied by an air-tight suction syringe (Gilmont Instruments Division, IL) connected to pipette holder by Tygon tubing. This resulted in the formation of tight, and high resistance giga-ohm seal (1-10 GΩ) between the pipette and cell membrane. Giga-seal formation was monitored on an oscilloscope (Hitachi Electronics Co., Japan) as a change in the

pipette current when a test pulse of -20 mV for 20 msec duration was applied.

After giga-seal formation, the pipette, stray and patch capacitance were nullified manually using fast and slow capacitance compensation. In order to obtain the whole-cell configuration, a second pulse of negative pressure was applied to the pipette tip through the syringe until the patch membrane ruptured. This was monitored as a jump in capacitive current during the test pulse. Series resistance and cell capacitance were compensated. The series resistance was on average (1.0- 5.0 MΩ). Whenever cell access deteriorated, additional gentle negative pressure was applied to the pipette and series resistance was readjusted. During whole-cell clamping an exchange of the pipette solution and the intracellular milieu referred to as dialysis occurs, allowing control of the cytoplasmic constituents. However, a decrease in the magnitude of some ionic currents is observed with time during prolonged whole-cell recordings, a phenomenon known as run-down. This could result from the loss (or dilution) of important cytoplasmic constituents required for channel activity such as ATP and second messengers (Belles *et al.*, 1987 and 1988). For this reason, run-down was always monitored in the experiments.

Membrane voltage and whole-cell currents obtained during whole-cell configuration of patch-clamp (filtered at 1 KHz) were recorded directly to hard disk via an analog to digital (A/D) convertor (TL-1-125 LabMaster board, Axon Instruments, CA) interfaced with an IBM clone computer running pClamp software (Axon Instruments, CA). Application of voltage command protocols and analysis

of whole-cell current and voltage data were performed with pClamp software (Axon Instruments, CA).

B. Recording Solutions:

The control bath solution was a HEPES-containing K-H solution with (in mM): NaCl, 120; $NaHCO_3$, 3.6; KCL, 4.8; NaH_2PO_4, 1.2; $MgSO_4$,1.2; glucose, 11.0; $CaCl_2$, 1.8; N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid (HEPES), 5; pH was adjusted to 7.4 using 1N NaOH. *The control pipette solution* contained (in mM): K-gluconate, 130; KCl, 10.0; $MgCl_2$, 1.0; Na_2ATP, 5.0; EGTA, 0.1; HEPES, 5.0. In all experiments 0.1 mM EGTA was used in the pipette unless indicated. The pH of solution was adjusted to 7.2 using 1N KOH. The pipette solution was filtered using a syringe filter (0.45 micron, Nalgene Co. NY).

The exogenous free radical generating system used in this study consisted of dihydroxyfumaric acid (DHF; 3-6 mM) and $FeCl_3$:Na_2ADP (Kukreja *et al*, 1988) (0.05:0.5 mM; Sigma Chemical Co., MO). For intracellular O-R application, DHF and Fe^{3+}:ADP were added to the pipette solution (pH re-adjusted to 7.2 with KOH). For extracellular application, DHF and Fe^{3+}:ADP were added to a bath solution saturated with 100% O_2 (pH re-adjusted to 7.4 with 1N NaOH). This solution was bubbled with 100% O_2 during experimentation.

Dihydroxyfumaric acid is a compound which autooxidizes in the presence of O_2 at physiological pH to produce all oxygen-derived reactive metabolites in the following sequence of reactions (Kukreja *et al.*, 1988):

(13) $DHF + O_2 \longrightarrow DHF^{\cdot} + {}^{\cdot}O_2^-$

(14) $2H^+ + {\cdot}O_2^- + DHF \longrightarrow DHF^{\cdot} + H_2O_2$

(15) $DHF^{\cdot} + O_2 \longrightarrow DKS + {\cdot}O_2^-$

where $DHF^{\cdot}$ is a free radical formed by loss of one electron from DHF; ${\cdot}O_2^-$ is superoxide anion; H_2O_2 is hydrogen peroxide and DKS is diketosuccinate. Moreover, through a non enzymatic dismutation reaction the hydroxyl radical (${\cdot}OH$) and singlet oxygen ($^1O_2$) are also generated:

(16) ${\cdot}O_2^- + {\cdot}O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$

(17) $H_2O_2 + {\cdot}O_2^- \longrightarrow O_2 + {\cdot}OH + OH^-$

(18) $H_2O_2 + {\cdot}O_2^- \longrightarrow {\cdot}OH + OH^- + {}^1O_2$

The presence of Fe^{3+}-ADP in the solution will lead to production of ${\cdot}OH$ through the so-called Fenton and Haber-Weiss reactions:

(19) ${\cdot}O_2^- + Fe^{3+}\text{-ADP} \longrightarrow O_2 + Fe^{2+}\text{-ADP}$

(20) $Fe^{2+}\text{-ADP} + H_2O_2 \longrightarrow Fe^{3+}\text{-ADP} + OH^- + {\cdot}OH$

(21) ${\cdot}O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + {\cdot}OH$

Spin trap measurements showed that this system generates mainly ${\cdot}OH$ radical (Kukreja *et al.*, 1988).

3. Drugs and Chemicals Used:

Water insoluble drugs were made in stock solutions and diluted to appropriate concentrations in HEPES buffered K-H. Stock solutions of glibenclamide (Sigma Chemicals Co., MO) were made in a mixture of dimethyl sulphoxide (DMSO) and PEG (polyethylene glycol). Nicardipine (Research Biochemical Inc., MA) was dissolved in DMSO. The solvents used did not

demonstrate any effect on the electrical activity of cardiac myocytes at the concentrations employed.

Stock solutions of the following water soluble drugs were made in deionized water, and included; ryanodine (Calbiochem. Corp., CA), tetrodotoxin (TTX; 10μM; Sigma Chemical Co., MO), tetraethylammonium chloride (TEA; 10 and 20 mM), barium chloride ($BaCl_2$; 0.2 mM), dithiothreitol (DTT; 0.5 mM) and diazenedicarboxylic acid bis-*N,N'*-dimethylamide (diamide; 1 mM; Sigma Chemical Co., Mo).

N-mercaptopropionyl glycine (MPG; 500 mM) and N-methyl glucamine (NMG; 62.4 mM) (Sigma Chemical Co., MO) were added directly to the bath solution.

The different drug containing bath solutions were kept in four 60 ml reservoirs placed at 15 cm above the level of the recording chamber within the Faraday cage. The solutions were applied to the chamber at a rate of 1.8 ml/min through flexible Tygon tubing from the reserviors and ending in a single outlet in the recording chamber.

4. Statistical Data Analysis:

All data were expressed as means ± S.E.M. Statistical analysis was performed using Student's *t*-test. Figures were prepared using SigmaPlot and/or Corel Draw software and printed using HP Laserjet series IV (Hewlett Packard, CA).

5. Measurement of Junction Potential:

Since the pipette potentials were nulled in external solution, all current clamp traces and voltage clamp protocols required correction for junction potential. Since the junction potential (Neher, 1992) may be expected to be different for each combination of patch and bath solutions employed it was necessary to determine the value for all solutions. Measurements of junction potential were accomplished by recording the difference in potential for 20 pipettes filled with patch pipette solution and nulled in patch solution before being immersed in bath solution. For the standard pipette and bath solutions employed in this study the difference was consistently about -10 mV. This value did not change under different recording conditions employed in this study where various pippete and bath solutions were used. All current clamp data and voltage clamp protocols were corrected for the junction potential.

6. Calculation and Measurement of Equilibrium and Reversal Potentials:

The equilibrium potential is defined as the potential at which the net current passing through the channel pore is zero. It is calculated by the Nerst equation for that ion as follows:

(22) $$E_x = RT/zF \log([X]_o/[X]_i)$$

where R is the gas constant; T is the absolute temperature in Kelvin; z is the valence of the ion X; F is the Faraday constant and $([X]_o/[X]_i)$ represents the ratio of extracellular and intracellular concentration of the ion X. At room temperature of ~22°C, the constant RT/zF is equal to a value of 58.8 mV (Hill, 1992). The

equilibrium potential for the following ions Na^+, K^+ and Cl^- were calculated under various recording conditions in this study. These values were then compared with the measured reversal potential of membrane current during voltage-clamp experiments under the same recording conditions.

IV. RESULTS

1. Effects of Intracellular O-R Stress:

A. Effects of Intracellular O-R Stress on Membrane Electrical Activity :

In the first series of experiments, we monitored the time-dependent changes in electrical activity of isolated guinea-pig ventricular myocytes via whole-cell recording technique in the presence and absence of an O-R generating system in the pipette solution. The generating system consisted of dihydroxyfumaric acid (DHF; 3 mM) and $FeCl_3$:ADP (0.05:0.5 mM). A low concentration of EGTA (0.1 mM) was included in the pipette solution in an attempt to preserve $[Ca^{2+}]_i$ fluctuations and contractions upon electrical stimulation. Initial experiments were conducted at 37°C, resulting in a very rapid progression of changes in electrical activity during exposure to O-R. Representative traces obtained in a single experiment are shown in figure 2. Similar results were obtained from 6 other myocytes. The rapid progression of changes did not provide sufficient time to record the underlying alterations in membrane currents by switching from current- to voltage-clamp and applying command pulses. For this reason, we reduced the temperature to 22°C and compared the changes in activity to those at 37°C. Figure 3 shows results obtained during an experiment at 22°C and illustrates that similar alterations in activity occurred at the two temperatures, although those at 22°C were delayed and slower to develop after

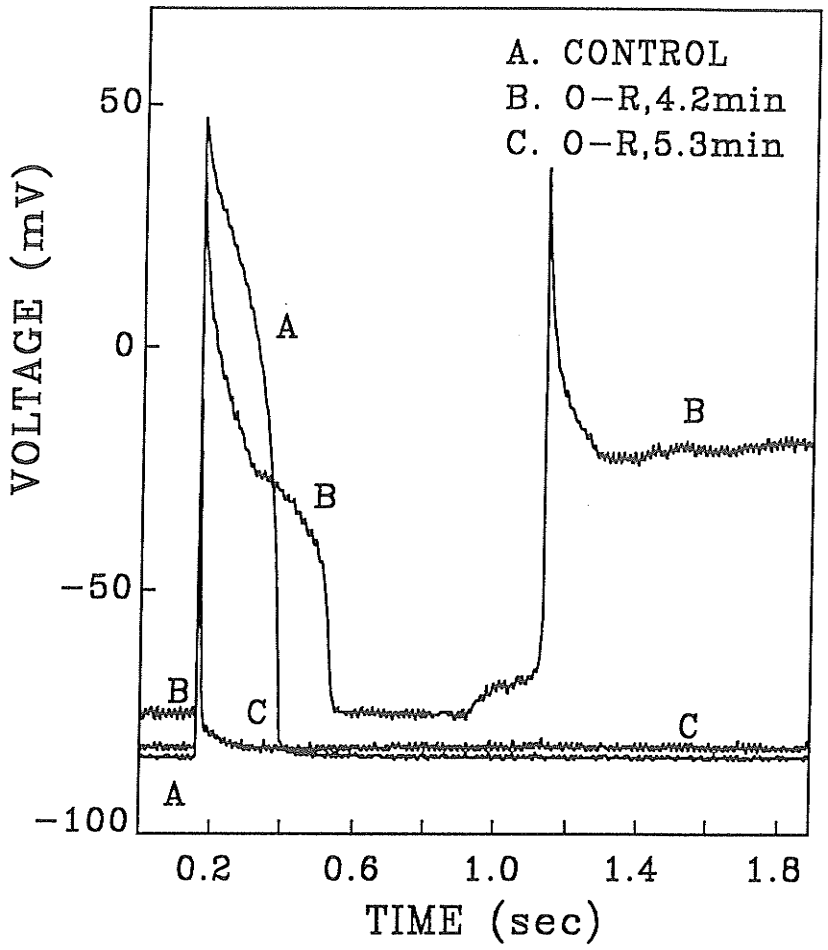

FIGURE 2: ***Time-dependent Changes in Action Potential Configuration Induced by Intracellular O-R Stress at 37°C.***

Three traces (A-C) recorded from a single myocyte upon gaining whole-cell access (trace A) and after 4.2 (trace B) and 5.3 (trace C) min of dialysis with pipette solution containing O-R generating system at 37°C (stimulation frequency 0.25 Hz). Note the rapid progression from normal activity in trace A, through stages of 1) slight depolarization, 2) decline in plateau amplitude and prolongation in action potential duration, and spontaneous activity and failure to repolarize in trace B, and 3) repolarization and decrease in action potential duration.

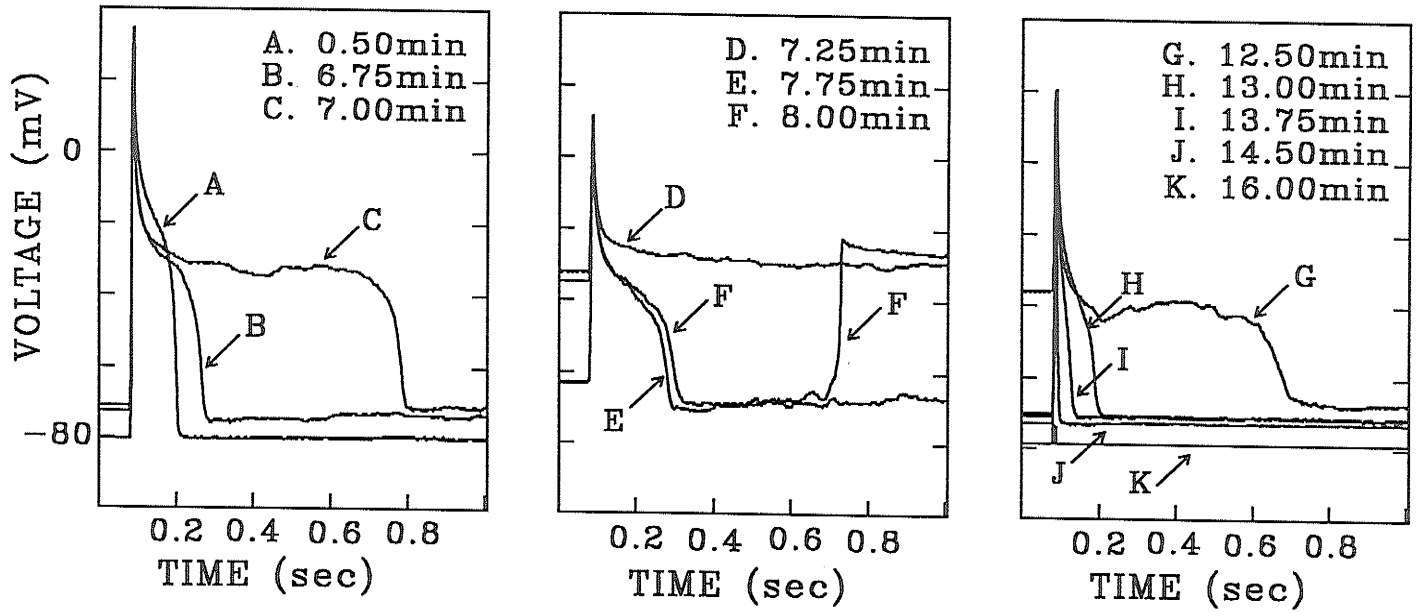

FIGURE 3: ***Time-dependent Changes in Action Potential Configuration Induced by Intracellular O-R Stress at 22°C.***

The three panels show sequential recordings (traces A-K) of the time-dependent changes in electrical activity obtained from a single myocyte during O-R stress at 22°C (stimulation frequency 0.25 Hz). Trace A immediately after obtaining whole-cell access and traces B-K at subsequent times during dialysis as indicated. Note the division of the traces into three stages of change corresponding to (1) early depolarization, decline in AP plateau amplitude and AP prolongation (Traces A-C), (2) failure to repolarize and sustained depolarization as well as spontaneous activity (Traces D-F), (3) decline in AP duration and eventual hyperpolarization and inexcitability (Traces G-K).

achieving whole-cell access. For this reason, the use of the lower temperature to facilitate the switch between recording modes does not compromise the potential relevance of these experiments to pathophysiological events *in vivo* occurring at 37°C.

Figure 3 was divided into three panels to indicate that the changes in electrical activity induced by O-R stress occurred in three stages. However, it should be noted that the alterations in activity in our three stages of intracellular O-R stress do not correspond exactly to those observed by Barrington *et al.*, (1988) during extracellular O-R stress (see dicussion section 1). Trace A in figure 3 shows a control AP obtained immediately after membrane rupture and before dialysis of the cell interior with the O-R containing pipette solution. The subsequent traces (i.e. B-K) were obtained at various time intervals until the myocytes became inexcitable (i.e. no AP regardless of stimulus strength).

The alterations in activity associated with stage 1 consisted of a slow, progressive 5-10 mV depolarization of RMP and increase in AP duration, especially at 90% of repolarization (APD_{90}). During the initial phase of depolarization little change or a slight increase in the AP plateau was apparent. However, a subsequent decline in the plateau amplitude was noted especially when the AP was markedly prolonged (figure 3, traces B and C).

The second stage began with the appearance of DADs and low amplitude oscillations of membrane potential during the plateau. In 10 of 26 myocytes spontaneous APs were noted as result of DADs or triggered automaticity.

Following the appearance of DADs, the myocytes then failed to repolarize after an AP and showed sustained depolarization between -35 and -20 mV (figure 3, trace D). This stage was observed in all but 2 myocytes exposed to O-R. In 15 myocytes this stage was a transient event, lasting for 0.5-3 min before spontaneous recovery of resting potential (figure 3, trace E). In 9 of 26 myocytes, however, membrane potential did not repolarize and the myocytes were observed to shorten significantly (hypercontracture) and depolarize to 0 mV. In those myocytes showing repolarization after stage 2, a second period of large oscillations in membrane potential, DADs and spontaneous activity followed (figure 3, traces E and F).

The third stage of change was marked by hyperpolarization, decline in AP duration and loss of excitability. After spontaneous repolarization from ~-35 mV, DADs and oscillations in potential during the plateau disappeared, the myocytes gradually hyperpolarized, and AP duration decreased markedly (figure 3, traces H, I and J). Eventually, the myocytes hyperpolarized to or beyond the value of RMP measured initially upon membrane rupture. At this time, all myocytes were inexcitable and would not fire APs even in response to current pulses more than 10 - 15 times greater strength than immediately after whole-cell access (figure 3, trace K).

Some variability in the behaviour of the myocytes during intracellular O-R stress was evident. However, all changes in activity induced by DHF-$FeCl_3$:ADP were evident within 20 min. The average time required for O-R stress to induce

stage 1 was calculated based on changes in RMP and APD_{90} of greater than 2 and 10%, respectively. Table 3 summarizes the number of myocytes demonstrating each stage of change in electrical activity and the latency for the onset of these stages. A total of 26 myocytes were exposed to O-R generating system (in the absence of any other treatments); 10 myocytes demonstrated all 3 stages, 9 demonstrated stages 1 and 2 but failed to recover from stage 2. Only 2 myocytes failed to exhibit stage 2, but these myocytes did show stages 1 and 3 at similar times as in the previous groups. Thus, all myocytes which demonstrated stage 3 (17 of 26) first demonstrated stage 1 and/or 2. Interestingly, in the 5 myocytes which failed to exhibit stage 1, the latency to the onset of stages 2 and 3 was reduced. Note that in these myocytes stage 3 also occurred with a reduced latency.

The whole-cell recording technique is well known to cause time-dependent changes in electrical activity. For example a decline in AP duration in the absence of any treatments occurs presumably as a result of the dilution of intracellular constituents important to membrane channel activity by the pipette solution (Hamill *et al.*, 1981; Belles *et al.*, 1988). For this reason, we compared the time course and nature of the changes in myocytes dialyzed with internal solution lacking the O-R generating system. That the O-R generating system caused marked changes in electrical activity compared to untreated myocytes or those dialyzed with internal solution containing $FeCl_3$:ADP alone (and no DHF) is evident from a comparison of figures 3 and 4. Panel A of figure 4 shows APs

TABLE 3: Summary of the Latency for the Onset of Membrane Electrical Alterations Induced during Intracellular O-R Stress.

Cells	Stage 1 (min)	Stage 2 (min)	Stage 3 (min)
n=10	3.3 ± 1.1	5.5 ± 1.1	8.4 ± 1.8
n=9	3.3 ± 0.6	5.4 ± 0.9	---
n=5	---	2.9 ± 1.0	4.9 ± 0.7
n=2	3.7	---	9.7
N=26	21	24	17

n, Indicates number of cells demonstrating a given sequence of stages;
N, total of n cells. Values are means ± S.E.M.

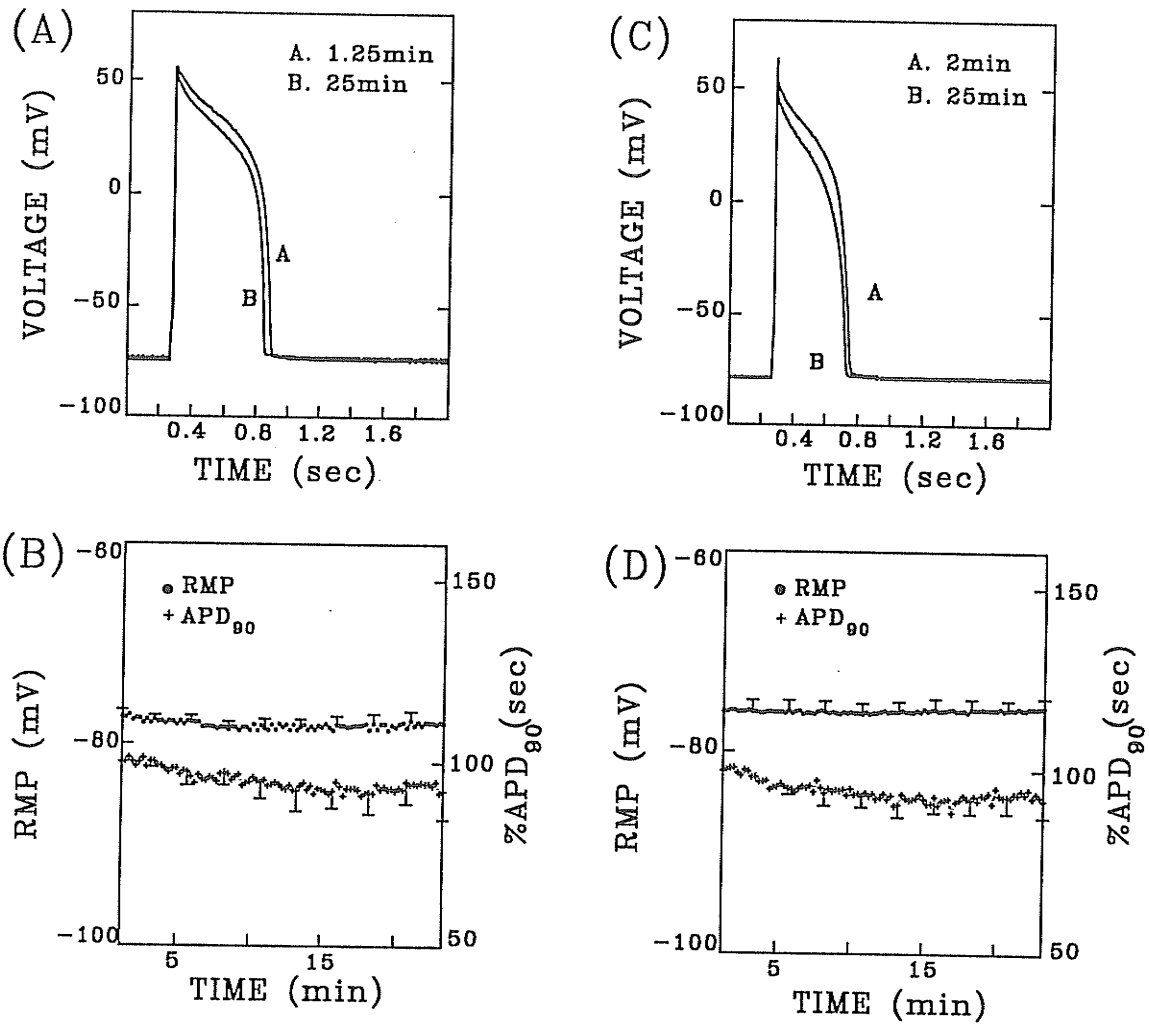

FIGURE 4: ***Time-Dependent Changes in Action Potential Configuration in the Absence of O-R Stress.***

Panels A and C show representative action potentials recorded from myocytes dialyzed with 0.1 mM EGTA in the absence (A) and presence (C) of $FeCl_3$:ADP (stimulation frequency 0.25 Hz; 22°C). Traces A and B in each panel were obtained after gaining cell access at the times indicated. ***Panels B and D*** show the mean (± S.E.M.) changes in RMP (●) and $\%APD_{90}$ (+) for myocytes dialyzed with 0.1 mM EGTA in the absence (n = 8; Panel B) and presence (n = 7; Panel D) of $FeCl_3$:ADP. ● RMP- resting membrane potential; + $\%APD_{90}$- action potential duration at 90% of repolarization as a percentage of control.

recorded immediately upon rupture of the membrane (trace A) and after 25 min (trace B) in a myocyte dialyzed with control internal solution lacking both DHF and $FeCl_3$:ADP. Neither APD_{90} (measured as percent of control) nor RMP were significantly altered over the first 25 min (figure 4, panels A and B). Similar results were obtained with 7 other myocytes. Myocytes (n = 7) dialyzed with internal solution containing $FeCl_3$:ADP alone (i.e. without DHF) also showed no significant change in APD_{90} and RMP over 25 min (figure 4, panels C and D). These data imply that rundown and/or $FeCl_3$:ADP can not account for alterations in activity observed in myocytes exposed to the complete O-R generating system.

B. Effects of Intracellular O-R Stress on Quasi Steady-State Currents:

In the second series of experiments, the changes in membrane ionic currents induced by the O-R generating system were investigated. The conventional step-type voltage clamp protocols could not be employed because of the time required to record families of membrane currents over the physiological range of voltages; the changes in electrical activity induced by O-R stress were often very rapid (even at 22°C) so alterations in the magnitude of the whole-cell currents would be expected during the protocols. As an alternative, a ramp protocol which required 8 sec to obtain a quasi steady-state I-V relation for the range of potentials between -130 and +30 or +60 (depolarization at a rate of 20 or 25 mV/sec, respectively) was employed. This protocol reflects net steady-state current produced by the myocyte over the selected range of voltages, which under normal conditions largely reflects inward rectifying K^+ current (I_{K1}) at

potentials negative to -40 mV. The nature of the current at more positive potentials under control conditions remains to be defined. Some contribution from I_{K1} (Sakmann and Trube, 1984; Shimoni *et al.*, 1992), the fast and slow components of delayed rectifier K^+ current (I_K) (Sanguinetti and Jurkiewicz, 1990) and/or plateau background current may be involved (Yue and Marban, 1988).

Stage 1: The changes in quasi steady-state currents during O-R stress occurred in three stages at times corresponding to the periods described above for current clamp data. The first phase of change was marked by a decline in the magnitude of current at potentials near RMP which accompanied the slight initial depolarization in current clamp (figure 5). Panels A and B of figure 5 show representative examples of the depolarization and change in membrane current. A fall in the amplitude of the positive and negative slope regions of the I-V relation negative to -40 mV, as well as, marked decline in the current at potentials near RMP (inset of panel B) was observed when myocytes exhibited the slight early depolarization. That the decline in current was due to a decrease in I_{K1} and not the result of an increase in an inward current was suggested by the minimal shift in reversal potential and the decrease in current at potentials negative to the expected equilibrium potential for K^+ under our recording conditions (E_K = -86 mV). Activation of an inward current would be expected to cause a marked positive shift in reversal potential and increase in current negative to E_K (as was observed during the phase of marked depolarization described below). That I_{K1} was depressed at all potentials negative to -40 mV is apparent in figure 6 which

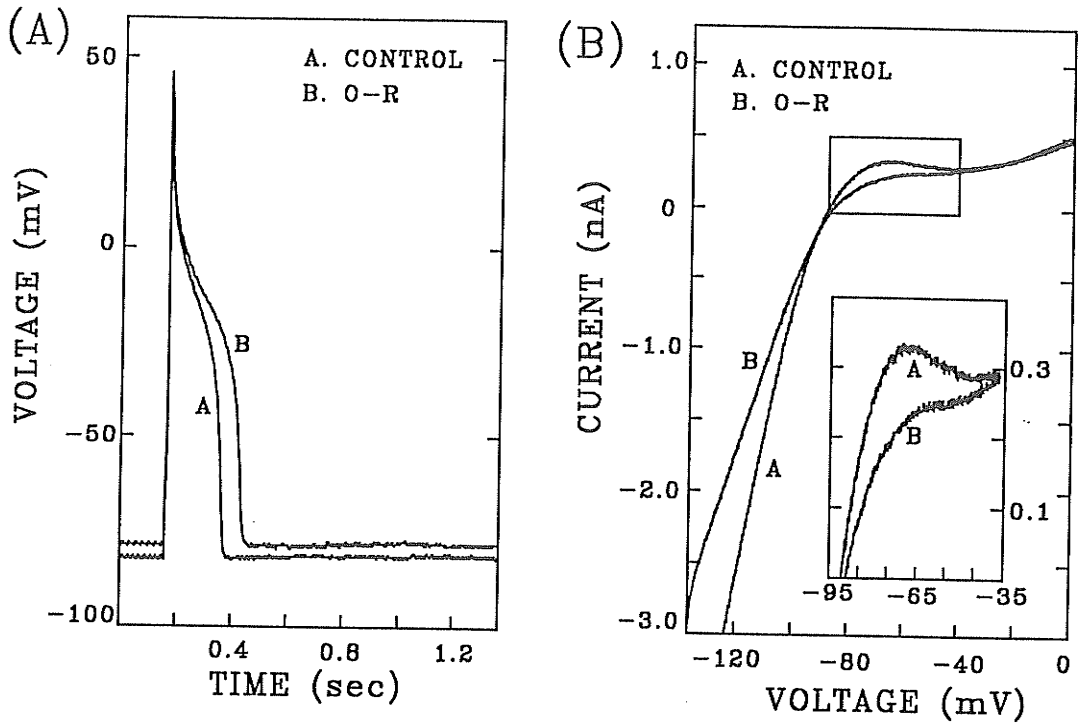

FIGURE 5: ***Alteration in Quasi Steady-State Current during Stage 1 of Intracellular O-R Stress.***

Panel A shows representative current clamp records obtained from a single myocyte upon gaining cell access (trace A, control) and after 6.00 min (trace B, O-R) of dialysis with O-R containing pipette solution when the initial stage of depolarization was observed (stimulation frequency 0.25 Hz; 22°C). ***Panel B*** shows a representative example of the initial change in steady-state current from that observed immediately after gaining cell access (trace A, control) and at 3.1 min (trace B, O-R) when the slight depolarization was observed. Quasi steady-state current in this myocyte was evoked by ramping membrane potential between -130 and 0 mV over 6 sec from a holding potential of -85 mV. The inset figure shows an expanded version of the region of traces A and B within the box on the I-V relation and depicts the decline in steady-state current at voltages negative to -35 mV.

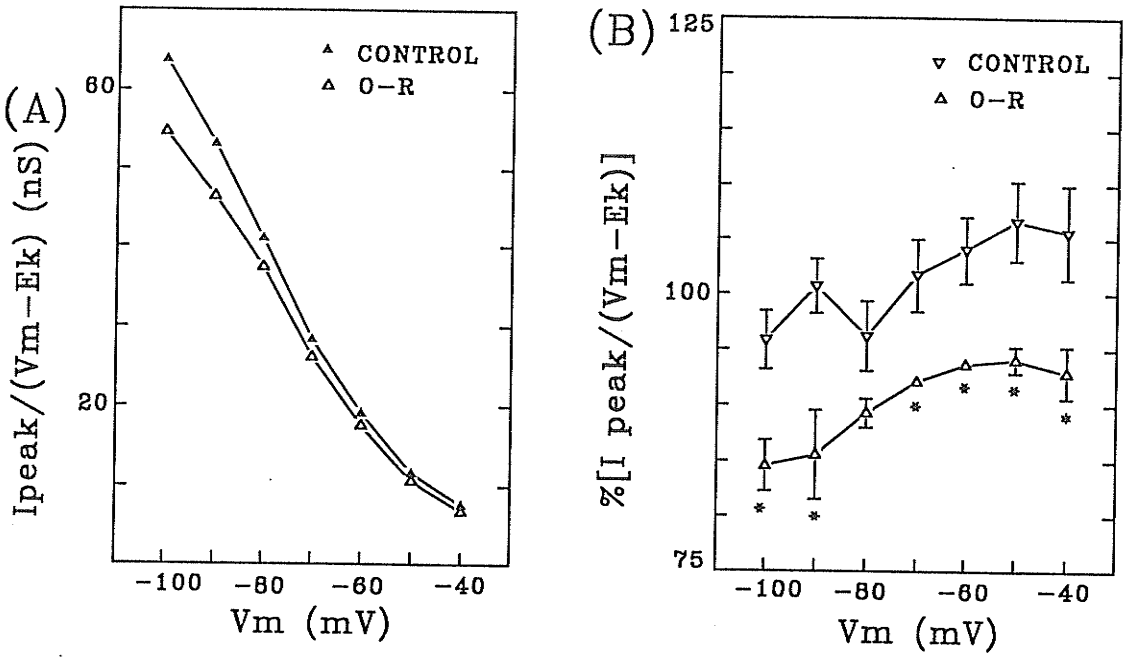

FIGURE 6: ***Alteration in Chord Conductance Negative to -40 mV during Stage 1 of Intracellular O-R Stress.***

Panel A: Representative decline in chord conductance (I_{peak}/V_m-E_K) at potentials negative to -40 mV in a single myocyte during O-R stress (O-R - △) compared to that measured upon gaining cell access (Control - ▲). ***Panel B:*** Average change in chord conductance with time (i.e. 8 min) at potentials negative to -40 mV as a % of that obtained upon gaining cell access in several myocytes in the absence (Control -▽ ; n = 5) and presence of O-R stress (O-R -△; n = 4). * indicates a significant difference at $P < 0.05$. (Note absence of significance at -80 mV reflects similarity of current magnitudes in the two groups of myocytes at potentials near to the reversal potential for the steady-state I-V relation.)

shows a representative example of the decrease in chord conductance for potentials between -100 and -40 mV (Ipeak / V_m - E_K) in a single myocyte (panel A) and the average change in chord conductance (as a % of the conductance measured upon cell access) in 4 myocytes exposed to O-R versus 5 dialyzed with control pipette solution during the first 8 min of recording. The average decline in chord conductance by ~10 % over potentials negative to -40 mV (figure 6, panel B) was similar to the decline in slope conductance measured at the reversal potential in myocytes exposed to O-R (i.e. 11.2 ± 1.8 % from 76 ± 8 to 67 ± 7 nS (n = 4)). The latter change was significantly different ($P < 0.05$) from that observed in untreated myocytes (no DHF in pipette) (2 ± 3 % from 61 ± 8 to 60 ± 7 (n = 5)). This early depression in I_{K1} persisted until masked by changes in other currents.

Stage 2: Figure 7 illustrates the changes in membrane currents observed during the phase of DADs, failure to repolarize and sustained depolarization; panels A and C show current clamp records obtained upon gaining cell access and during this second phase, panels B and D show the corresponding steady-state currents recorded immediately after switching to voltage clamp. Several changes in the currents are apparent; (1) a marked positive shift in reversal potential of the I-V relation, (2) inwardly directed oscillations in current, i.e., transient inward currents (I_{ti}), recorded upon repolarization to the holding potential of -85 mV, (3) a large negative increase in holding current, i.e. the I_{ti} were frequently superimposed on a sustained inward current, and (4) inward deflections

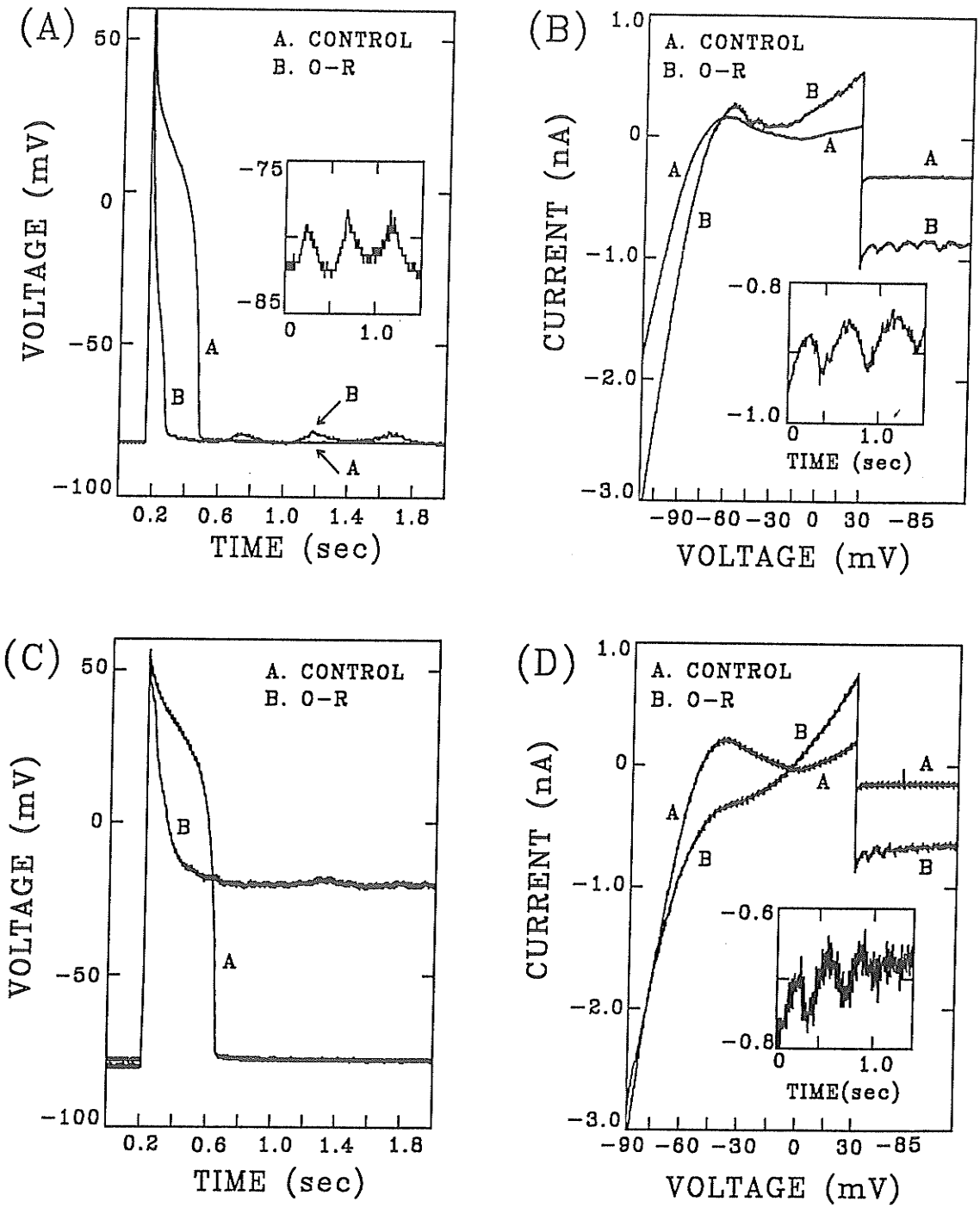
(A)
A. CONTROL
B. O-R
VOLTAGE (mV)
TIME (sec)
(B)
A. CONTROL
B. O-R
CURRENT (nA)
VOLTAGE (mV)
TIME (sec)
(C)
A. CONTROL
B. O-R
VOLTAGE (mV)
TIME (sec)
(D)
A. CONTROL
B. O-R
CURRENT (nA)
VOLTAGE (mV)
TIME(sec)

FIGURE 7: ***Alterations in Quasi Steady-State Current Associated with Delayed-After Depolarizations and Action Potential Plateau Decline and Failure to Repolarize Induced by Intracellular O-R Stress.***

Panel A: Traces A and B are current clamp recordings obtained upon gaining cell access and at 15.08 min when DADs were evident during dialysis with pipette solution containing O-R generating solution, respectively (stimulation frequency 0.25 Hz; 22 °C). The inset shows a magnified version of the delayed after-depolarizations (DADs) in trace B. ***Panel B:*** Quasi steady-state currents recorded from the same myocyte after the traces in Panel A were obtained (trace A - 0.63 min and trace B - 15.2 min after cell access). Membrane potential was ramped between -130 and +30 mV over 8 sec from a holding potential of -85 mV. The inset shows a magnified version of the inward oscillations in current (I_{ti}) in trace B that were recorded upon returning to the holding potential (-85 mV). Note that the frequency of the DADs and I_{ti} in the inset figures of panel A and B are identical (~2 Hz). ***Panel C:*** Traces A and B were obtained upon gaining cell access and at 3.7 min during dialysis with pipette solution containing O-R generating system, respectively (stimulation frequency 0.25 Hz; 22 °C). Note the decline in plateau amplitude and failure to repolarize in trace B. ***Panel D:*** Traces A and B are quasi steady-state current records evoked by ramp protocols (as in Panel B) applied immediately after traces A and B in Panel C, respectively. Note the positive shift in reversal potential of steady-state current, increase in outward current positive to -20 mV, negative shift in holding current, and I_{ti} evoked upon stepping back to the holding potential of -85 mV in trace B. The inset shows a magnified version of the I_{ti} in trace B.

of membrane currents in the negative slope region of the I-V relation. A magnified version of the I_{ti} is shown in the inset boxes of panels B and D. These oscillations occurred with a similar frequency to the DADs shown in the inset of panel A (i.e. ~2.0 Hz). Both the frequency and magnitude of these I_{ti} are similar to those occurring during Ca^{2+} overload as reported by other investigators (Matsuda *et al.*, 1982; Orchard *et al.*, 1983; Giles and Shimoni, 1989b; Berlin *et al.*, 1989).

In order to identify the ionic mechanism(s) mediating I_{ti}, two sets of experiments were performed. Firstly, we determined the reversal potential of this current by stepping to a range of potentials between -80 and +25 mVs at the end of the ramp (figure 8, panel A). The oscillatory currents were observed at all potentials and no distinct reversal potential was apparent between these voltages. Secondly, the bath solution was exchanged for one in which Na^+ ($[Na^+]_o$) was replaced with an equimolar concentration of Li^+ immediately after the onset of stage 2. In this case, the I_{ti} were completely blocked as shown in figure 8 (panel B, trace C). Similar results were obtained in 7 myocytes. Interestingly, replacement of $[Na^+]_o$ with Li^+ did not, however, alter the reversal potential of the current responsible for the shift in steady-state I-V relation during stage 2 (figure 8, panel B).

The positive shift in reversal potential of the steady-state current and inwardly directed increase in holding current were transient phenomena which showed reversibility. Figure 9 (panel A) illustrates this complete reversibility; the reversal potential and holding current recorded after (trace C), were the same as

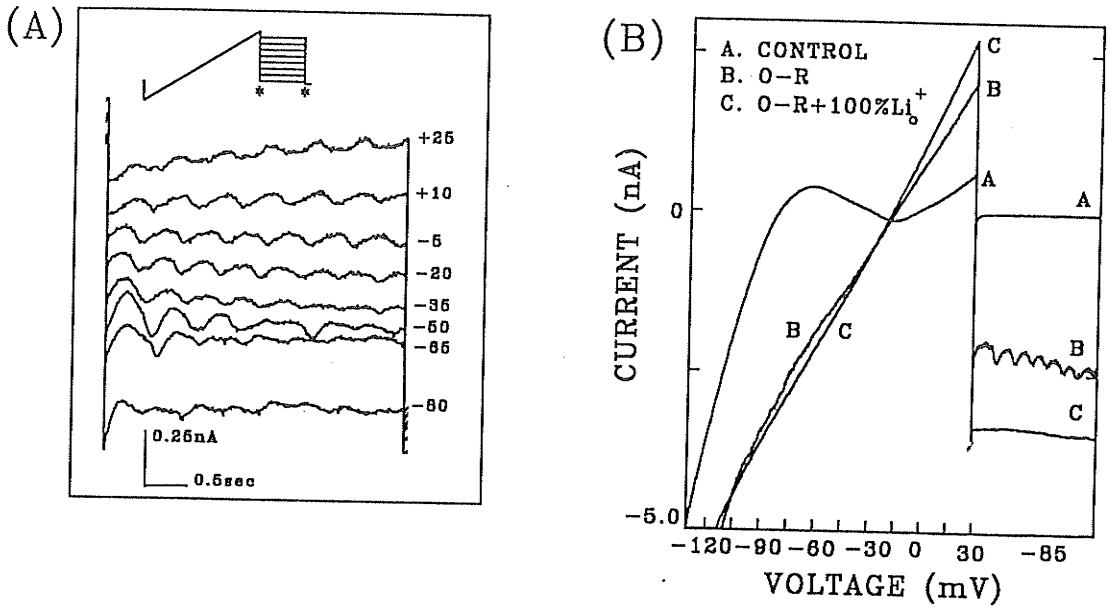

FIGURE 8: ***Blockade of Intracellular O-R Stress-Induced Transient Inward Current (I_{ti}) upon 100% $[Na^+]_o$ Replacement with Li^+.***

Panel A shows representative current recordings obtained during stage 2, by stepping to different potentials ranging between -80 to +25 mV at the end of the ramp protocol. Note the direction of I_{ti} was inward at all potentials and did not demonstrate a reversal potential. ***Panel B*** shows representative recordings of quasi steady-state currents obtained from myocyte dialyzed with O-R generating system immediately after gaining access (trace A, control) and during stage 2 (trace B, O-R). Note the positive shift in steady-state current associated with I_{ti} evoked upon stepping back to the holding potential -85 mV. Trace C was obtained 1 min after complete $[Na^+]_o$ replacement with Li^+.

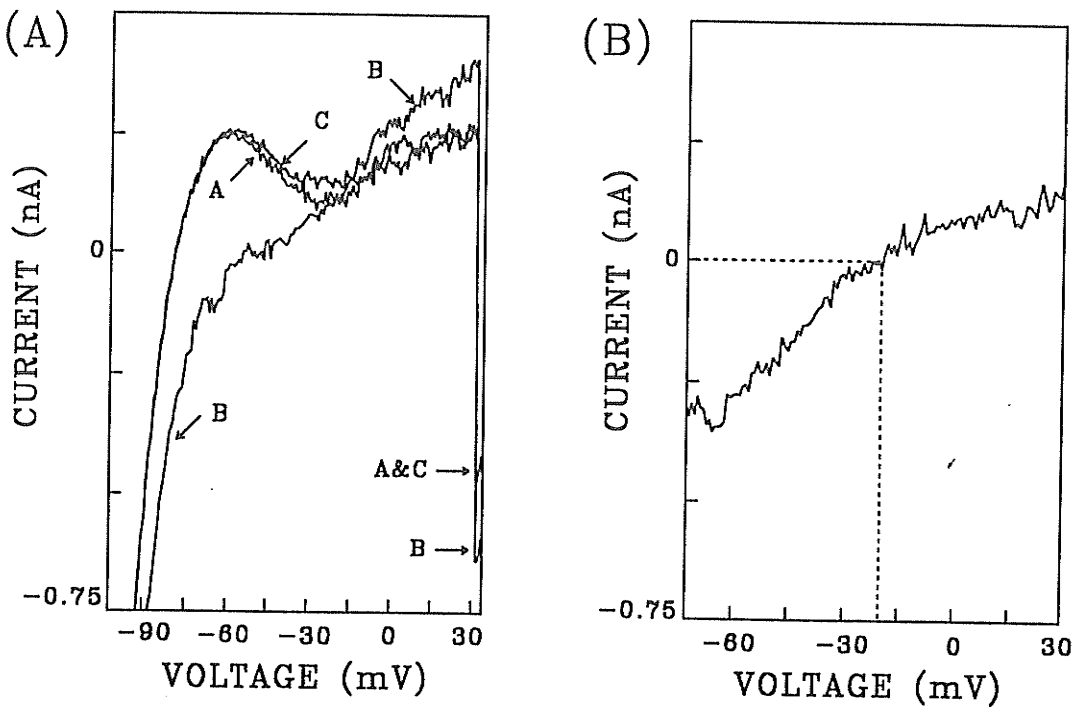

FIGURE 9: ***Intracellular O-R Stress Activates a Quasi Steady-State Current During Stage 2.***

Panel A: Quasi steady-state current records evoked before (trace A), during (trace B), and after (trace C) the phase of failure to repolarize and sustained depolarization in a single myocyte via a ramp protocol (-130 to +30 mV over 8 sec from a holding potential of -85 mV). Note that the changes in (1) reversal potential for the steady-state current, (2) current at voltages positive and negative to -30 mV, and (3) holding current in trace B compared to trace A were completely reversed in trace C. ***Panel B:*** Difference current obtained by digital subtraction of trace A from trace B. The reversal potential for the difference current in this myocyte was -19.5 mV.

those recorded immediately before (trace A) the shift in reversal potential which occurred during the period of sustained depolarization (trace B). To determine the O-R stress-activated current responsible for the shift we calculated the difference current by digital subtraction of the I-V relation recorded before, from that obtained during the reversal potential shift. The current was largely voltage insensitive and possessed a reversal potential of -19.5 mV in this myocyte (figure 9, panel B). The average reversal potential for the difference current recorded from 16 myocytes was -19.9 ± 0.7 mV. This is not the equilibrium potential corresponding to any single ion under the recording conditions used. However, it is close to the reversal potential of the non-selective cation current of cardiac myocytes which Matsuda (1983) identified to be Ca^{2+}- activated. With this in mind, we first altered K^+ and Na^+ concentrations in the bath when stage 2 was observed. Representative results are shown in figure 10. Panels A and B show that changing external K^+ concentration from 4.8 to 9.6 mM during stage 2 caused a positive shift in reversal potential from -23.5 to -14.8 mV. The average shift in reversal potential for the difference current calculated from 6 myocytes was from -24.4 ± 2.5 to -15.6 ± 1.7 mV ($P< 0.05$) or 8.8 ± 1.7 mV. In contrast, panels C and D of figure 10 shown that changing $[Na^+]_o$ from 124.8 to 62.5 mM by 50% replacement of Na^+ with NMG, induced a negative shift in the steady-state current and reversal potential of the difference current, respectively. The average shift in reversal potential was from -19.3 ± 2.1 to -28.5 ± 2.1 mV ($n = 4$; $P<0.01$) or 8.9 ± 1.1 mV. Given the effects of Na^+ replacement with NMG, we tested for a role

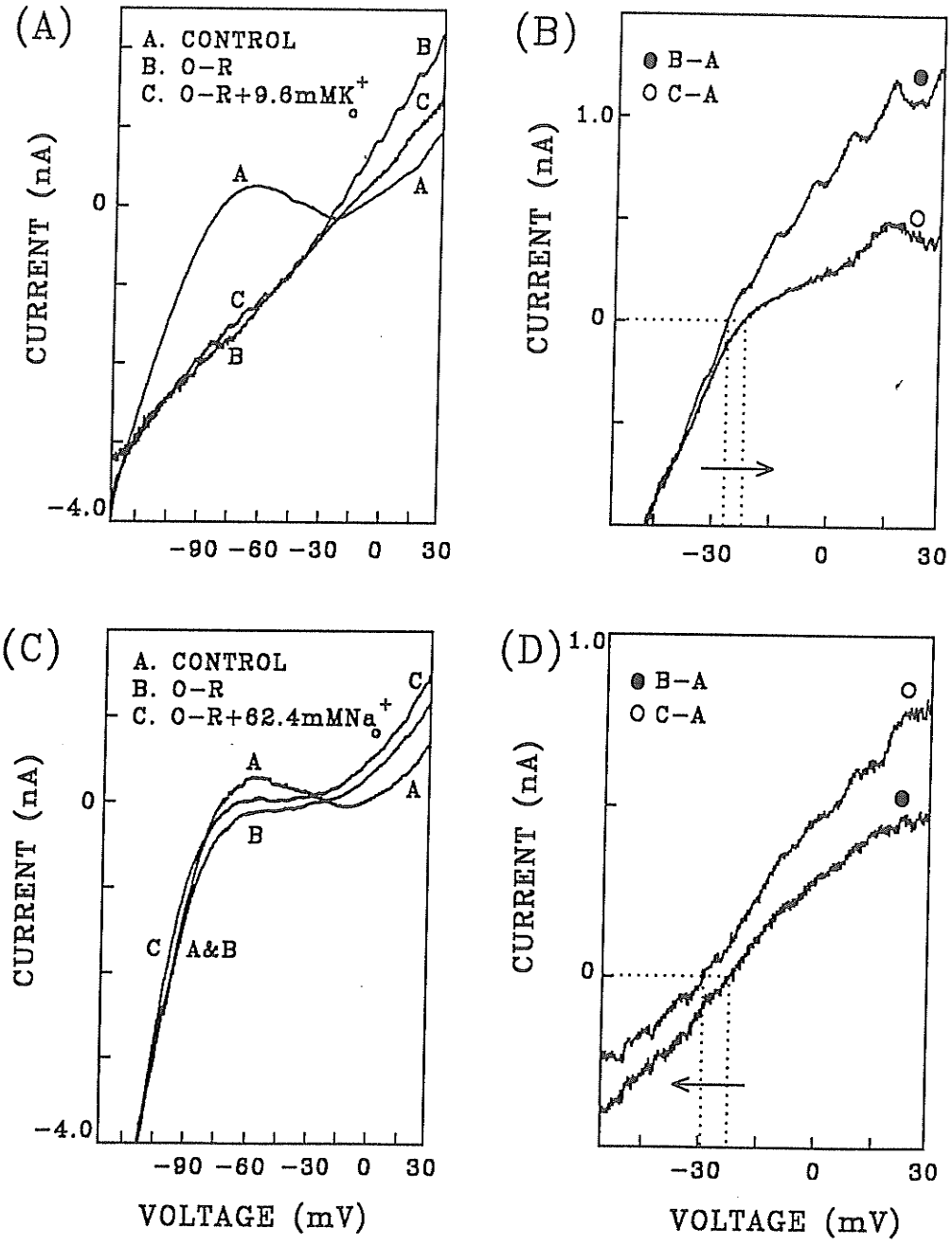
(A)
A. CONTROL
B. O−R
C. O−R+9.6mMK$_o^+$
CURRENT (nA)
0
−4.0
−90 −60 −30 0 30
(B)
B−A
C−A
1.0
CURRENT (nA)
0
−30 0 30
(C)
A. CONTROL
B. O−R
C. O−R+62.4mMNa$_o^+$
CURRENT (nA)
0
−4.0
A&B
−90 −60 −30 0 30
VOLTAGE (mV)
(D)
1.0
B−A
C−A
CURRENT (nA)
0
−30 0 30
VOLTAGE (mV)

FIGURE 10: ***Alterations in Steady-State Current During Stage 2 by Changing $[K^+]_o$ and $[Na^+]_o$.***

Panel A: Traces A and B are quasi steady-state current recordings obtained immediately after gaining cell access and during stage 2, respectively. Trace C was recorded following exchanging $[K^+]_o$ from 4.8 to 9.6 mM. ***Panel B:*** Difference currents obtained by digital subtraction of trace A from trace B (●) and trace C (○) in Panel A, respectively. Note the positive shift in the reversal potential of the difference current from -23.5 to -14.8 mV. ***Panel C:*** Traces A and B are quasi steady-state currents obtained via a ramp protocol immediately after gaining cell access and during stage 2, respectively. Trace C was recorded upon replacement of 50% $[Na^+]_o$ with NMG. Note the negative shift in the steady state current. ***Panel D:*** Difference current obtained by digital subtraction of trace A from trace B (●) and trace C (○) in Panel C, respectively. Note the negative shift in the reversal potential of the difference current from -21.2 to -28.3 mV.

of TTX-sensitive Na^+ channels in the failure to repolarize by applying 10 μM TTX (which was sufficient to block APs and Na^+ current under control conditions) to 4 myocytes after stage 2 occurred. This manipulation failed to change membrane potential suggesting TTX-sensitive Na^+ channels are likely not involved in stage 2 (data not shown).

Finally, to determine whether L-type Ca^{2+}, Na^+ and/or K^+ channels were contributing to the steady-state difference current we blocked these conductances with nicardipine (10μM), TTX (10μM) and complete K^+ replacement in the pipette and the bath with Cs^+. Figure 11 shows that these manipulations failed to prevent activation of a steady-state difference current with a reversal potential of -18 mV in this myocyte. The average reversal potential of the difference current calculated from 7 myocytes was -18.3 $\pm$ 3.5 mV which was not different from that recorded in the absence of the blockers and Cs^+ replacement. Moreover, it is also significant that changing E_{Cl} in these experiments to -2.4 mV from -60 mV as in all other experiments had no effect on the reversal potential of the difference current.

Stage 3: The final changes in electrical activity induced by O-R stress included a marked shortening of AP duration, a gradual hyperpolarization and loss of excitability as indicated in panel A of figure 12. Recordings of the quasi steady-state I-V relation during this period are shown in panel B which illustrates the gradual departure from the control recording (trace A) in successive traces obtained at 30 sec intervals after the first sign of a change in outward current (i.e.

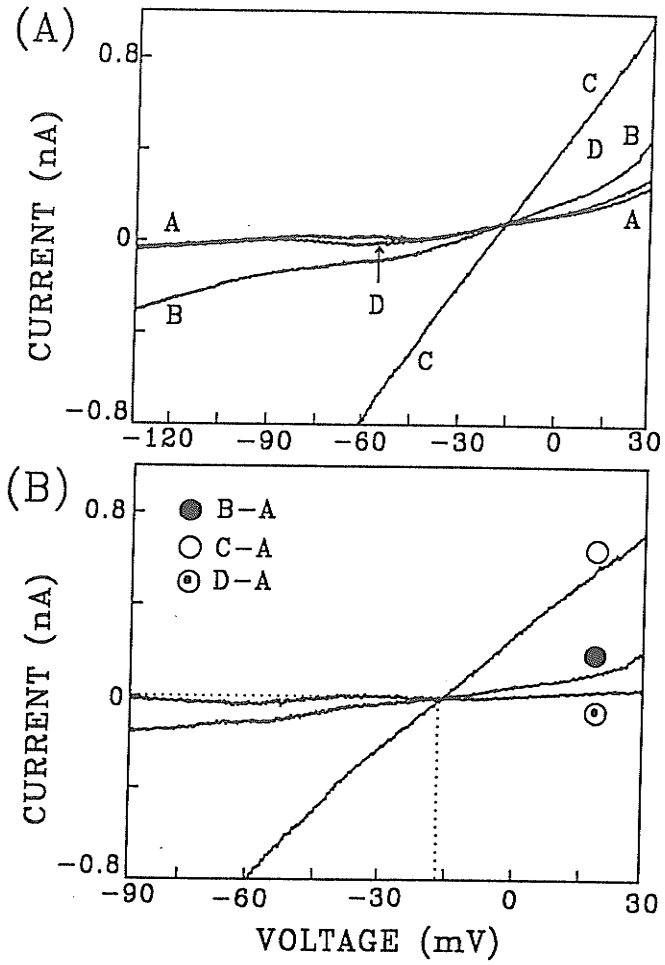

FIGURE 11: ***Intracellular O-R Activates the Steady-State Difference Current after the Blockade of L-type Ca^{2+}, TTX-Sensitive Na^{+} and All K^{+} Currents.***

Panel A shows quasi steady-state current records evoked by a ramp protocol (-130 to +30 over 8 sec from a holding potential of -85 mV). In this experiment 10 μM TTX, 10 μM nicardipine were added to the bath solution and $[K^{+}]_o$ and $[K^{+}]_i$ were completely replaced with Cs^{+}. Trace A was obtained immediately after gaining access from a myocyte dialyzed with 0.1 Mm EGTA and O-R generating system. Traces B, C and D were consecutive recordings obtained when the shift in steady-state was evident. Note the complete reversibility of the changes in steady-state current during O-R stress. ***Panel B*** shows the steady-state difference current obtained by digital subtraction of trace A from trace B (○), C (●) and D (⊙) respectively. The difference currents had a common reversal potential of -18 mV in this myocyte.

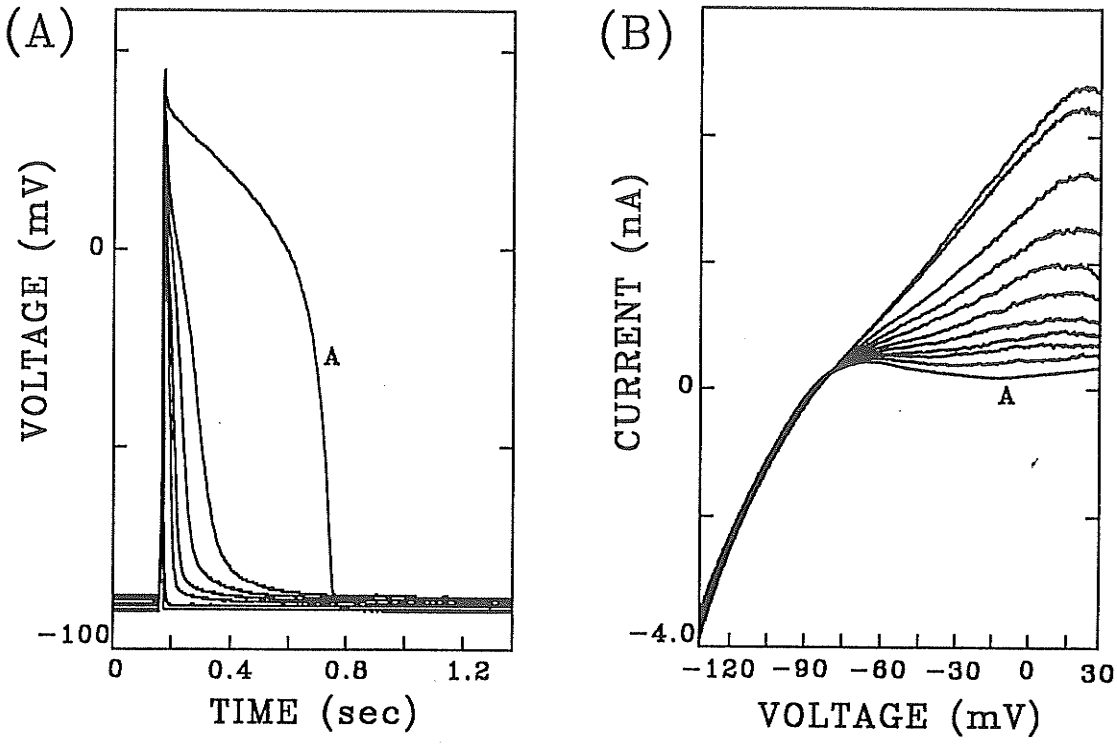

Figure 12: ***Late Changes in Action Potentials and Quasi Steady-State Current during Stage 3 of Intracellular O-R Stress.***

Panel A: Action potentials recorded under current clamp from a single myocyte upon gaining cell access (Trace A) and subsequently during the phase of decline in AP duration and hyperpolarization (stimulation frequency 0.25 Hz; 22°C). ***Panel B:*** Quasi steady-state current records obtained sequentially (at 30 s intervals) during stage 3 of O-R stress via a ramp protocol (-130 to +30 mV over 8 sec from a holding potential of -85 mV). Trace A was recorded immediately after obtaining cell access (control) and the remaining traces were recorded successively after the first sign of a change in outward current.

at 8.5 min in this myocyte). Outward current was enhanced at all potentials positive to E_K, and when markedly activated was largely linear between -80 and +20 mV but showed strong inward rectification at potentials positive to the latter voltage. This current resembled that reported to result from ATP-sensitive K^+ channels (I_{KATP}) (Noma, 1983). For this reason myocytes were exposed to the I_{KATP} inhibitor, glibenclamide (De Weille and Lazdunski, 1990) after the outward current was increased to test for the involvement of I_{KATP} in stage 3. Figure 13 demonstrates that 3 min exposure to glibenclamide (10 μM) blocked the outward current and restored membrane current to a level similar to that recorded immediately upon gaining whole-cell access. A similar result was obtained in three other myocytes. Thus, activation of I_{KATP} appears to account for the decline in AP duration, hyperpolarization, and loss of excitability observed in current clamp during O-R stress.

Quasi steady-state currents were also recorded from myocytes which were dialyzed with control pipette solution or solution containing only $FeCl_3$:ADP (no DHF) to determine the changes in current due to rundown. Figure 14 shows representative recordings obtained on achieving whole-cell access and after extended recording times. Only very slight changes in steady-state currents were evident in both groups over considerably longer recording times than that required for the alterations occurring in the presence of DHF. Similar results were obtained for 4 additional myocytes in each group.

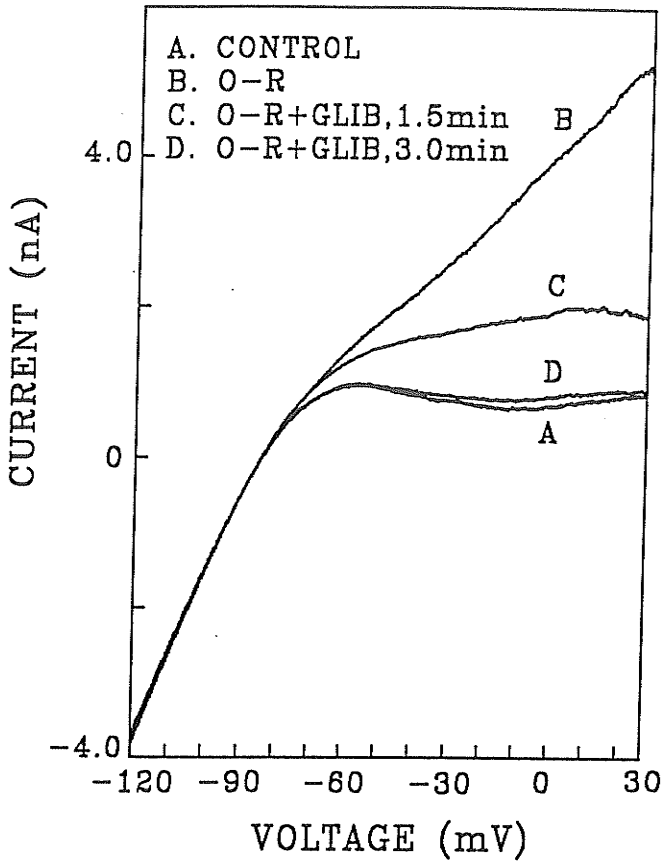

Figure 13: ***A Glibenclamide-Sensitive Outward Current is Activated during Stage 3 of Intracellular O-R Stress.***

Steady-state outward currents in a single myocyte evoked by a ramp protocol (-130 to +30 mV from a holding potential of -85 mV; 22°C) upon gaining cell access (trace A), during stage 3 of O-R stress (O-R) when substantial outward current was evident (trace B), and after 1.5 (trace C) and 3 (trace D) minutes exposure to 10 μM glibenclamide (GLIB).

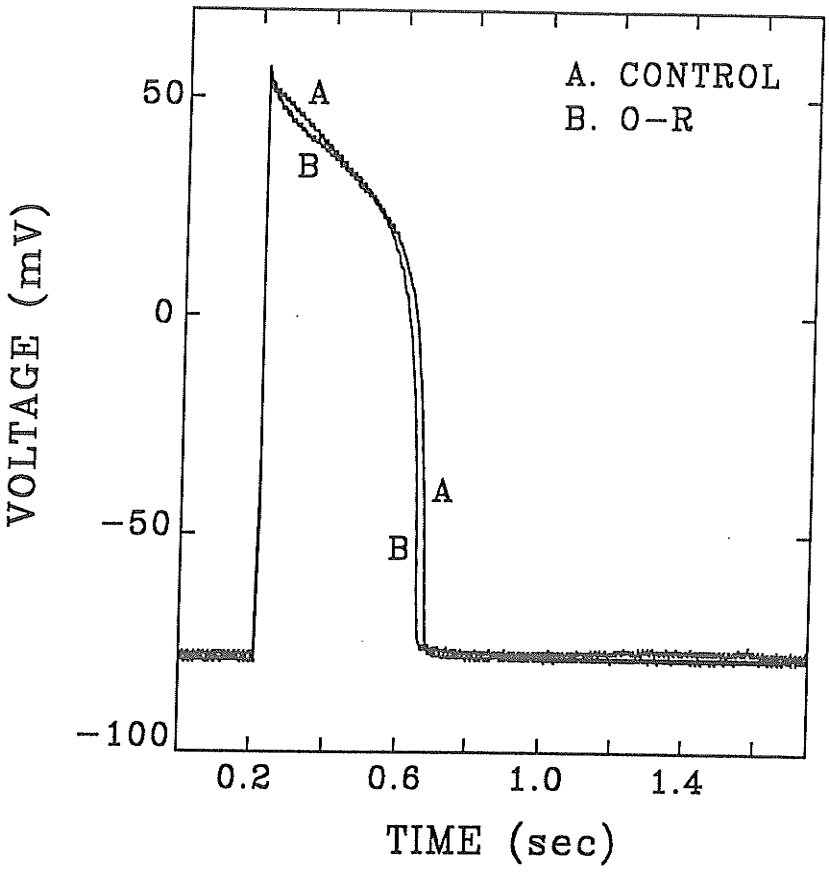

FIGURE 14: ***Changes in Quasi Steady-State Current in the Absence of O-R Stress.***

Panel A: Traces A and B show steady-state currents from a myocyte dialyzed with 0.1 mM EGTA (no O-R or $FeCl_3$:ADP) obtained via a ramp protocol (-130 to +30 mV over 8 sec from a holding potential of -85 mV) at the times following membrane rupture as indicated in the panel (22°C). ***Panel B:*** Traces A and B show steady-state currents from a myocyte dialyzed with 0.1 mM EGTA and $FeCl_3$:ADP at the times indicated in the panel (ramp protocol as in panel A; 22°C).

C. Effects of O-R Scavengers on O-R Induced Changes in Electrical Activity and Quasi Steady-State:

To provide evidence that alterations in electrical activity and membrane currents were due to O-R stress, we employed the membrane permeant O-R scavenger, MPG (500 μM) (Bolli *et al.*, 1989). Pretreatment of myocytes with MPG for at least 30 min prior to exposure to generating system did not affect AP configuration or steady-state membrane currents recorded immediately upon gaining cell access, however, all three stages of change in electrical activity during O-R stress were prevented. Figure 15 shows representative data from a single experiment. Similar results were obtained from an additional 5 myocytes. This suggests that the alterations in electrical activity and membrane currents described above were due to O-R stress rather than non-specific effects of treatment with DHF or $FeCl_3$:ADP.

D. Role of Elevated $[Ca^{2+}]_i$ and SR Ca^{2+} Release on Changes in Electrical Activity and Quasi-steady State Currents due to O-R Stress:

We consistently noted that intracellular O-R stress caused marked cell shortening and since O-R are reported to elevate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Burton *et al.*, 1990; Daly *et al.*, 1991; Peerson-Rothert *et al.*, 1992), we considered the possibility that alterations in this ion may have played a role in inducing the changes in steady-state membrane currents. To determine whether $[Ca^{2+}]_i$ was involved we increased the concentration of EGTA in the pipette solution to 5 mM. All myocytes (n = 5) which were dialyzed with 5 mM EGTA and

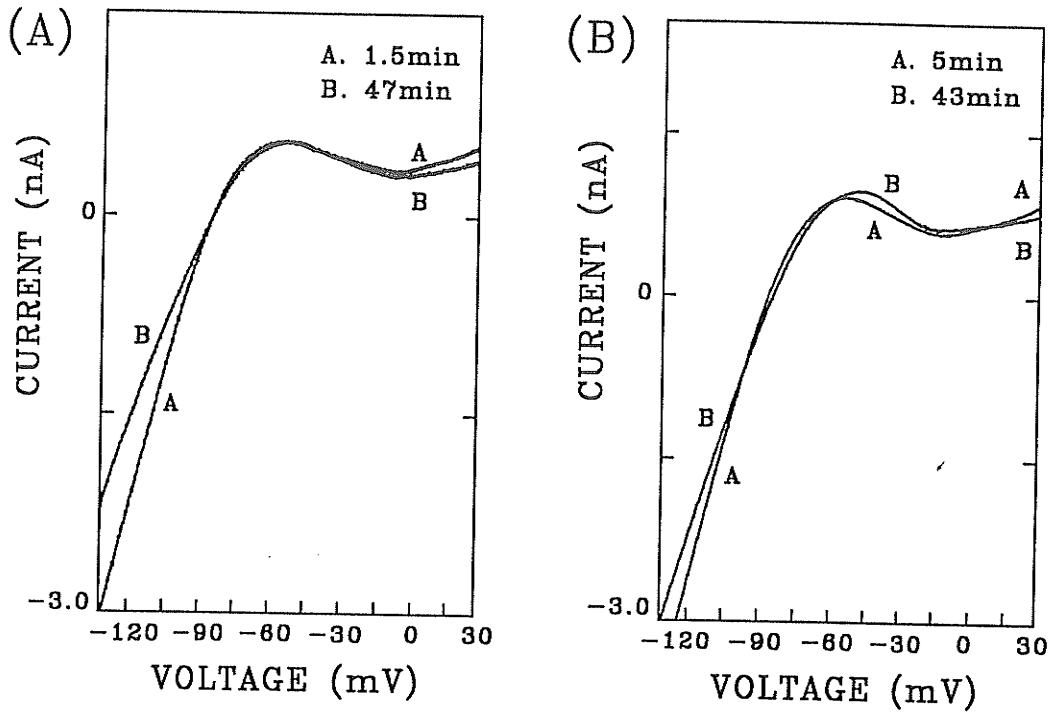

FIGURE 15: ***Prevention of Intracellular O-R Stress-induced Alterations in Electrical Activity following Pretreatment with O-R Scavenger.***

Representative current clamp records obtained from a single myocyte pretreated with 500 μM MPG for 30 min. Traces A (Control) and B (O-R) were recorded upon gaining cell access and after 22.6 min of dialysis with pipette solution containing O-R generating system (stimulation frequency - 0.25 Hz; 22°C).

O-R generating system failed to exhibit the changes in resting membrane potential or AP prolongation associated with stages 1 and 2 but APD_{90} still shortened due to activation of outward current (figure 16, panel B) with a time delay to onset of shortening of 10.7 ± 2.0 min. This absence of depolarization and increase in APD_{90} during O-R stress is shown in figure 16 (panel A) which plots the average RMP and APD_{90} as a percent of control over a period of 16 min after achieving access. Since the delay to onset of shortening was not different from that required for stage 3 in myocytes dialyzed with 0.1 EGTA it seems unlikely that elevated Ca^{2+} is necessary for the initiation of this stage. In light of the inhibition of stages 1 and 2 by Ca^{2+} chelation, we sought to define the source of Ca^{2+}. To test for a role of intracellular Ca^{2+} stores we pretreated myocytes for 30 min with ryanodine at a concentration (10 μM) which is thought to lead to a depletion of SR Ca^{2+} stores (Beuckelmann and Weir, 1988) prior to intracellular exposure to O-R. Figure 16 (panels C and D) summarizes the effects on RMP and APD_{90} in 5 myocytes and shows a representative example of the increase in outward current associated with the decline in AP duration. All myocytes pretreated with ryanodine failed to demonstrate stages 1 and 2, but again, action potential shortening associated with stage 3 still occurred with a delay of 7.6 ± 2.2 min which was not different from that required for this stage in the absence of ryanodine pretreatment.

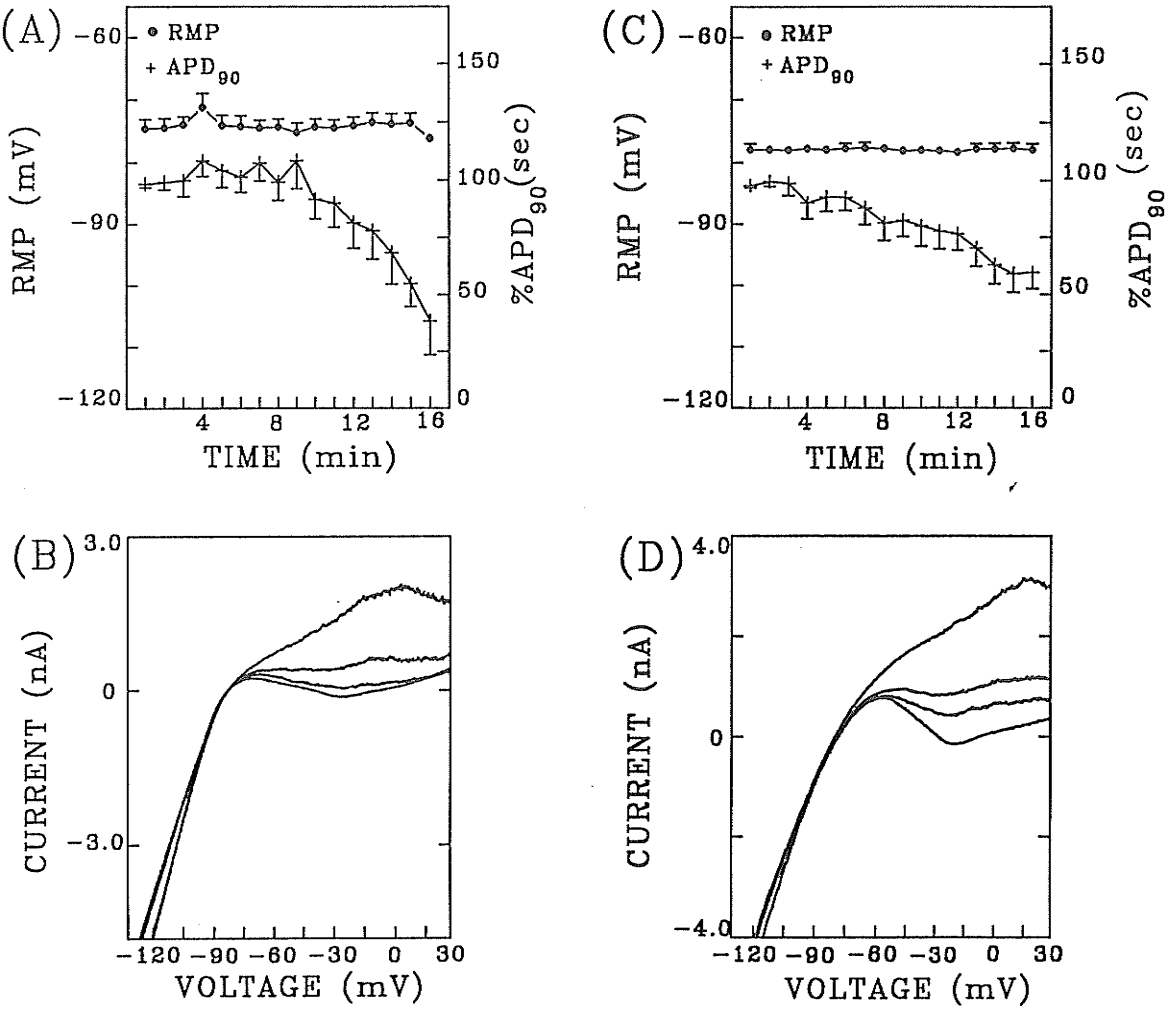

FIGURE 16: ***Blockade of Intracellular O-R Induced Alterations in Electrical Activity and Steady-State Currents during Stages 1 and 2 Upon Intracellular Ca^{2+} Chelation or SR Ca^{2+} Depletion.***

Panels A and C show the mean (± S.E.M.) changes in RMP (●) and $\%APD_{90}$ (+) for myocytes dialyzed with O-R and either 5 mM EGTA (n = 5; Panel A) or 0.1 mM EGTA after pretreatment with 10 μM ryanodine (n = 5; Panel C). ● RMP - resting membrane potential; + $\%APD_{90}$ - action potential duration at 90% repolarization as a percentage of control. ***Panels B and D*** show representative quasi steady-state current recordings obtained from myocytes dialyzed with 5 mM EGTA (panel B) or 0.1 mM EGTA after pretreatment with 10 μM ryanodine (panel D) when the shortening in APD_{90} was evident.

2. Effects of Extracellular O-R Stress:

A. Effects of Extracellular O-R Stress on Electrical Activity and Quasi Steady-State Current:

In the first series of experiments, we employed whole-cell recording technique to monitor the time-dependent changes in electrical activity and net quasi steady-state membrane currents of isolated guinea-pig ventricular myocytes superfused with O-R generating system consisted of DHF (3-6 mM) and $FeCl_3$:ADP (0.05:0.5 mM). A low concentration of EGTA (0.1 mM) was included in the pipette solution in an attempt to preserve intracellular calcium ($[Ca^{2+}]_i$) fluctuations and contractions upon electrical stimulation. Experiments were conducted at 22°C to slow the progression of changes in electrical activity during exposure to O-R and provide sufficient time to record the underlying alterations in membrane currents by switching from current- to voltage-clamp mode. Panel A of figure 17 shows that extracellular O-R stress caused depolarization of RMP which was associated with a depression of the plateau voltage and prolongation in AP duration (trace B) and/or low amplitude membrane oscillations in membrane potential (trace C). Following the initial depolarization, myocytes failed to repolarize after an action potential and showed a sustained depolarization to between -35 and -20 mV (trace D). These changes were observed in additional 5 myocytes. In 3 out of 6 myocytes we observed DADs and triggered activity prior to the period of sustained depolarization. This sequence of changes was similar to those we observed during intracellular O-R stress and to those previously

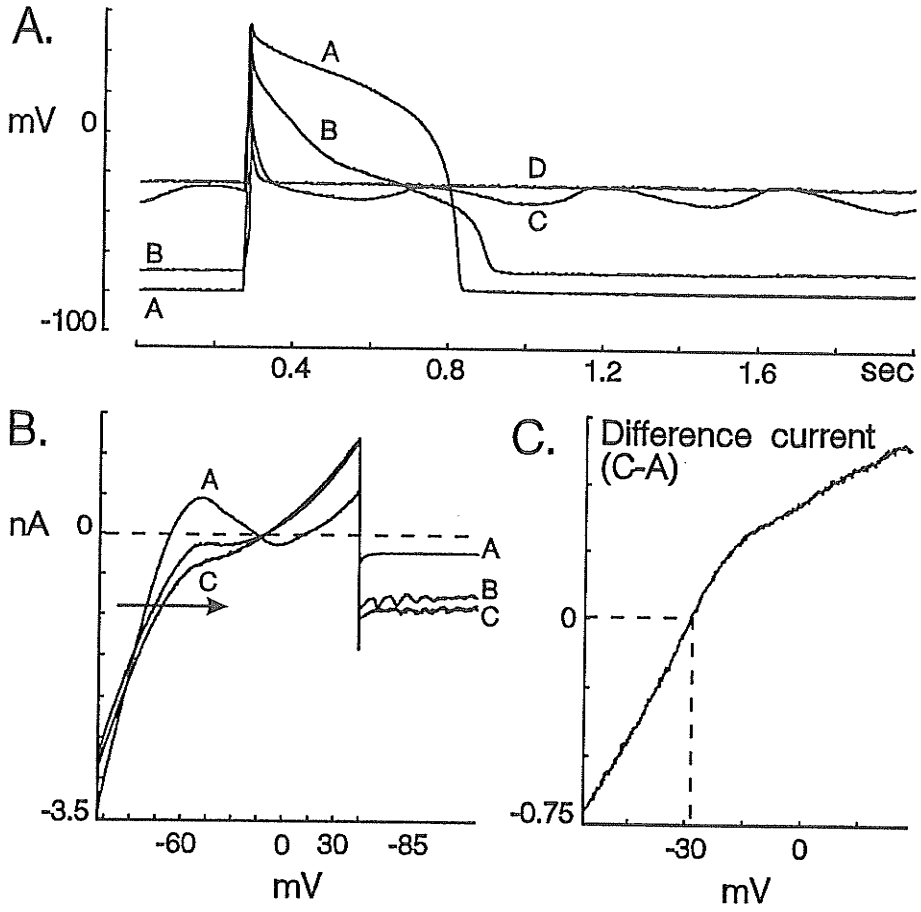

FIGURE 17: ***Extracellular O-R Stress Induced Alterations in Membrane Electrical Activity and Quasi Steady-State Current.***

Panel A shows representative current-clamp recordings obtained from a single myocyte dialyzed with 0.1 mM EGTA (stimulation frequency = 0.25 Hz). Trace A was obtained upon gaining whole-cell access and traces B, C and D were obtained following 9.2, 11, and 12.8 min of superfusion with O-R, respectively. ***Panel B:*** Traces a, b and c were quasi steady-state currents obtained from the same myocyte after current-clamp recordings A, C, and D of panel A, respectively. Membrane potential was ramped between -130 and +30 mV over 8 sec from a holding potential of -85 mV. Note the positive shift in reversal potential of steady-state current, the increase in outward current positive to -20 mV, the negative shift in the holding current and the transient inward currents (I_{ti}) evoked after stepping back to the holding potential in tracings B and C. ***Panel C:*** Difference current obtained by digital subtraction of trace a from C in panel B. The reversal potential of the difference current in this myocyte was -24.2 mV.

reported for extracellular O-R stress in isolated myocytes from different species (Barrington *et al.*, 1988; Beresewicz and Horakova, 1991; Matsuura and Shattock, 1991a). To investigate the underlying changes in the net whole-cell steady-state current mediating the period of sustained depolarization, we switched from current- to voltage-clamp mode and applied a ramp protocol to determine the I-V relation between -130 to +30 mV (8 sec ramp from a holding potential of -85 mV; ramp rate of 25 mV/sec). Panel B of figure 17 shows the changes in net quasi steady-state current obtained during extracellular O-R stress. Compared to steady-state current recorded under control conditions (trace A), the period of sustained depolarization (traces B and C) was associated with the following changes; (1) a positive shift in the reversal potential of the quasi steady-state I-V relation (average values in table 4); (2) positive shift in the holding current, and (3) inward oscillations in membrane current or transient inward currents (I_{ti}), upon stepping back to -85 mV at the end of the ramp. In an attempt to determine the change in steady-state current responsible for the shift in the reversal potential, we calculated the O-R sensitive difference current by digital subtraction of steady-state current recorded before (trace A) from that obtained during O-R stress (trace C, panel C, figure 17). The current was largely voltage independent and possessed a reversal potential of -24.2 mV in this myocyte. Similar results were obtained from additional 5 myocytes and average reversal values are given in table 4. That extracellular O-R stress led to the activation of a steady-state current which reversed at ~-20 mV is similar to our observation during intracellular O-R

TABLE 4: Reversal Potential for Quasi Steady-State I-V Relation and O-R Sensitive Difference Current during Extracellular O-R Stress ±SR Ca^{2+} Depletion or $[Ca^{2+}]_i$ Chelation.

Reversal potential (E_{rev})	0.1mM EGTA (pipette) (n=6)	10μM Ryanodine + 0.1mM EGTA (pipette) (n=3)	5mM EGTA (pipette) (n=4)
Control (C)	-78.3 ± 1.4	-80.9 ± 1.8	-75.8 ± 2.3
O-R	-27.6 ± 4.8***	-40.8 ± 13.2*	-30 ± 11.7*
Difference Current [(O-R) - C]	-18.3 ± 2.1	-20.8 ± 0.8	-23.2 ± 3.8

E_{rev} - reversal potential for the quasi steady-state I-V relation and for the difference current. Values are mean ± S.E.M. * and *** indicate significant difference ($P< 0.05$ and $P< 0.001$ respectively) from values recorded prior to O-R .

stress or intracellular Ca^{2+} injection (Matsuda, 1983) and attributed to a non-selective cation conductance (I_{NSC}).

B. Effects of Ryanodine Pretreatment or Internal Dialysis with EGTA (5mM) on O-R Activated I_{NSC}:

The sustained depolarization and activation of I_{NSC} with a reversal potential of ~-20 mV during intracellular O-R stress was due to an increase in $[Ca^{2+}]_i$ resulting from mishandling of Ca^{2+} by the SR. Because of the importance of marked depolarization in genesis of arrhythmias we sought to determine whether a similar cellular mechanism was involved in the activation due to extracellular O-R stress. First, we pretreated the myocytes with 10 μM ryanodine (Beuckelmann and Weir, 1988) for 30 min prior to O-R stress and second, we increased the concentration of EGTA in the pipette solution to chelate intracellular Ca^{2+}. Comparison of panel A of figure 18 with figure 17 shows that similar changes in electrical activity were obtained despite pretreatment with ryanodine. Superfusion with O-R still caused depolarization, prolongation of AP duration and depression in plateau amplitude followed by a period of sustained depolarization. Under voltage-clamp conditions we also found that the positive shift in the reversal potential of quasi steady-state I-V and the negative shift in the holding current were unaffected by ryanodine pretreatment (figure 18). However, it is significant that we never observed low amplitude oscillations in membrane potential and I_{ti} during the period of sustained depolarization after depletion of SR Ca^{2+} stores with ryanodine. The difference current (panel C, figure 18) obtained by digital

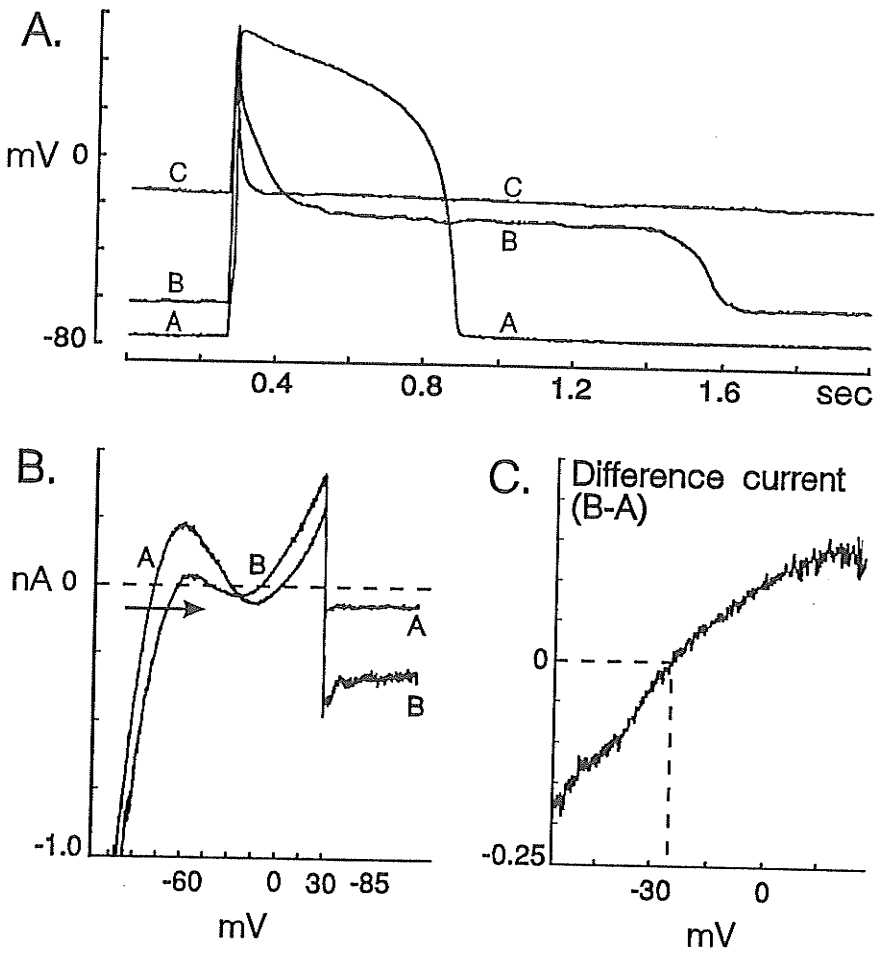

FIGURE 18: Sarcoplasmic Reticulum Ca^{2+} Depletion Blocked O-R-Induced I_{ti}, DADs and Membrane Oscillations but not the Activation of I_{NSC} and Sustained Depolarization.

Panel A: Action potentials recorded from a single myocyte pretreated with 10 μM ryanodine and dialyzed with 0.1 mM EGTA (stimulation frequency = 0.25 Hz). Trace A was obtained immediately after gaining cell access whereas B and C were after 4.7 and 4.8 min of O-R superfusion, respectively. ***Panel B:*** Quasi steady-state currents evoked by 8 sec ramp protocol between -130 and +30 mV in the same myocyte as in panel A. Traces A and B were obtained after traces A and C in panel A, respectively. Note the similarity of these recordings to those in figure 17, except for the absence of transient inward currents (I_{ti}) evoked upon stepping back to holding potential. ***Panel C:*** Difference current obtained by digital subtraction of trace A from B in panel B. The reversal potential of the difference current in this myocyte was -22.3 mV.

subtraction of control from O-R traces consistently revealed a current with an I-V relation that reversed at -22.3 mV and similar to that obtained in the absence of ryanodine. Average values for the reversal potential before and after O-R obtained from 3 myocytes are shown in table 4. These data exclude abnormal Ca^{2+} release from SR as a cause of sustained depolarization and the change in steady-state difference current during O-R stress.

Whether the change induced by extracellular O-R stress was due to an increase in $[Ca^{2+}]_i$ derived from a Ca^{2+} source other than the SR was explored by dialyzing myocytes with a high concentration of EGTA (5 mM). Figure 19 shows that 5 mM EGTA failed to suppress the changes in AP configuration, RMP and net whole-cell steady-state current induced by O-R which were similar to those observed in ryanodine pretreated and/or control myocytes (figures 17 and 18). The average value for E_{rev} of the steady-state current from 4 myocytes is shown in table 4 and was not different from that obtained with low EGTA in the pipette solution. However, as with the ryanodine experiments, myocytes dialyzed with 5 mM EGTA failed to exhibit DADs or I_{ti} upon superfusion with O-R. These data suggest that the sustained depolarization and the change in steady-state current due to I_{NSC} during extracellular O-R stress might not be caused by an increase in $[Ca^{2+}]_i$. This result was surprising in that it was not consistent with our findings for intracellular O-R stress. It implied the possibility that different cellular mechanisms might be involved in the effects of O-R stress depending upon the source of O-R, i.e., intracellular versus extracellular.

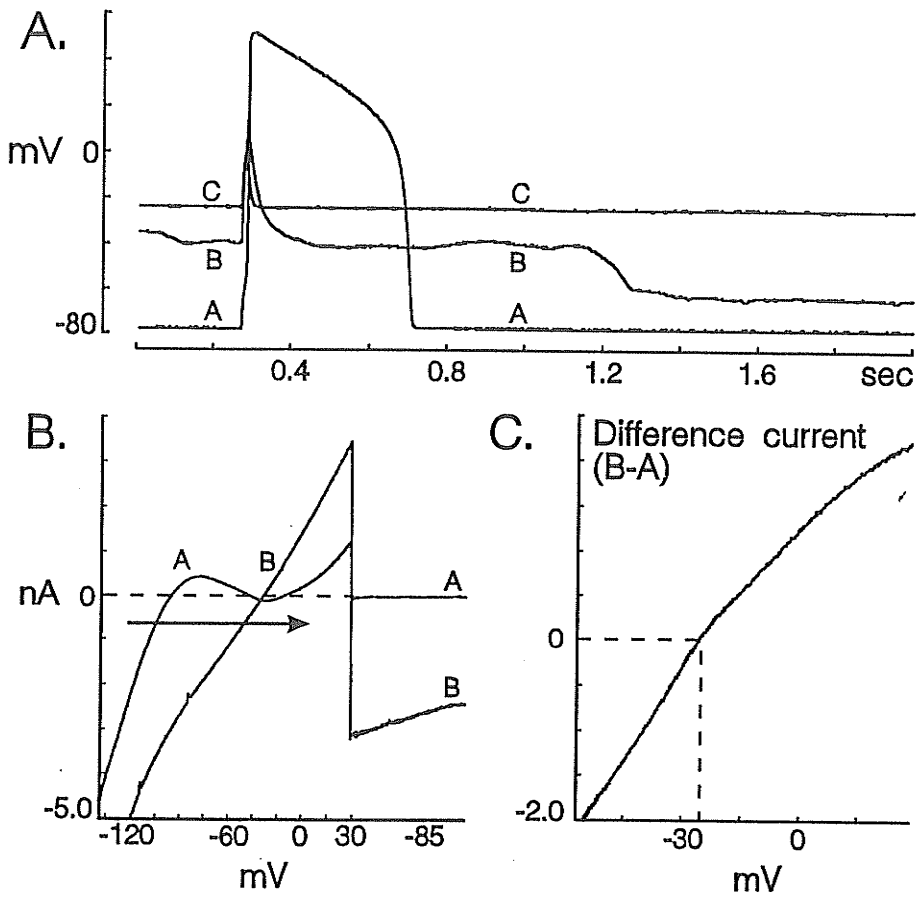

FIGURE 19: Intracellular Ca^{2+} Chelation Abolished O-R-Induced I_{ti}, DADs and Membrane Oscillations but not Activation of I_{NSC} and Sustained Depolarization.

Panel A: Action potentials recorded from a single myocyte dialyzed with 5 mM EGTA (stimulation frequency = 0.25 Hz). Trace A was recorded immediately after gaining cell access and traces B and C were obtained after 0.5 and 2.33 min of superfusion with O-R, respectively. *Panel B:* Quasi steady-state currents evoked by 8 sec ramp protocol between -130 and +30 mV in the same myocyte. Traces A and B were obtained after traces A and C in panel A, respectively. Note the similarity to data in figure 17 except for the absence of transient inward currents (I_{ti}) evoked upon stepping back to the holding potential. ***Panel C:*** Difference current obtained by digital subtraction of trace A from B of panel B. The reversal potential of the difference current in this myocyte was -21.9 mV.

C. Effects of Ryanodine Pretreatment or Internal Dialysis with EGTA (5mM) on O-R Activated I_{NSC} recorded in isolation:

To provide further evidence that activation of I_{NSC} was unaffected by pretreatment with ryanodine or high EGTA in the pipette solution we repeated the experiments under recording conditions to monitor non-selective cation current while depleting SR Ca^{2+} stores or chelating $[Ca^{2+}]_i$. In these experiments, the pipette solution was changed to a solution containing (in mM): CsCl, 140; $MgCl_2$, 1; Na_2ATP, 5; TEA^+ 20; EGTA (0.1 or 5). The bath solution contained (in mM): NaCl, 120; $NaHCO_3$, 3.6; NaH_2PO_4, 1.2; CsCl, 4.8; $MgSO_4$, 1.2; HEPES, 5; TEA^+, 10; $BaCl_2$, 0.2; nicardipine, 10μM; TTX, 10μM. Under these conditions, the reversal potential of Cl^- (E_{Cl}) was shifted from -60.4 to -2.9 mV and all K^+, Na^+ and L-type Ca^{2+} currents were blocked. Figure 20 shows that in a myocyte pretreated with ryanodine, extracellular O-R stress led to the activation of a steady-state current which was largely voltage independent and reversed at -13.4 mV in this myocyte. Average values for the reversal potential of the difference current from 5 myocytes are given in table 5. The average value for O-R activated current with ryanodine was not different from the value for I_{NSC} we previously recorded during intracellular O-R stress. Whether an increase in $[Ca^{2+}]_i$ could mediate the activation of I_{NSC} by extracellular O-R was explored by using 5 mM EGTA in the pipette solution. Figure 21 shows similar changes in steady-state I-V relation were observed from a myocyte dialyzed with pipette solution containing 5 mM EGTA compared to that shown for 0.1 mM EGTA and ryanodine above.

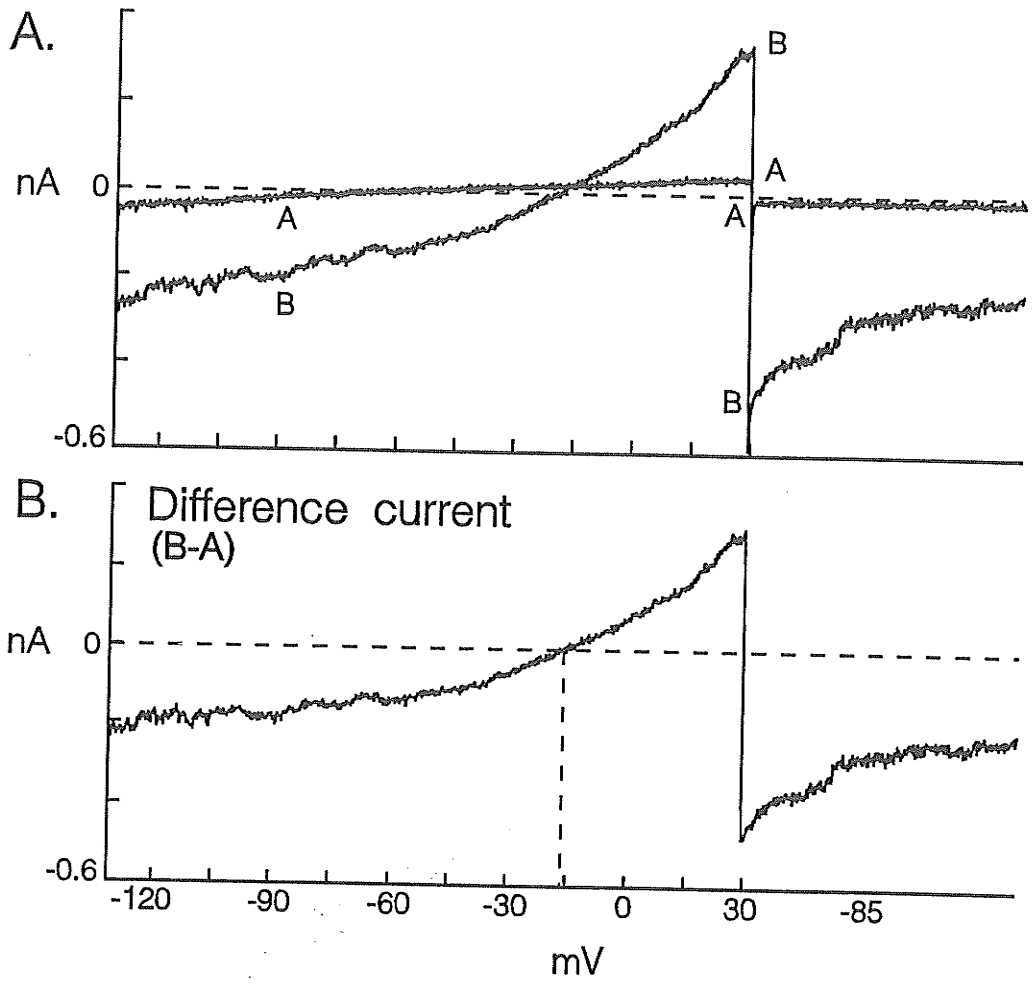

FIGURE 20: ***Lack of Effect of Sarcoplasmic Reticulum Ca^{2+} Depletion on Extracellular O-R Induced Activation of I_{NSC}.***

Panel A: Quasi steady-state current recordings evoked by a ramp protocol (from -130 to +30 mV over 8 sec from a holding potential of -85 mV) before and after activation of I_{NSC} by extracellular O-R. In this experiment, L-type Ca^{2+}, Na^+ and all K^+ currents were blocked and E_{Cl} was changed from -60.4 to -2.9 mV. Trace A was obtained immediately after gaining cell-access from A myocyte pretreated with 10 μM ryanodine and dialyzed with 0.1 mM EGTA. Trace B was obtained 5 min after superfusion with O-R. Note positive shift in reversal potential of steady-state current reflecting activation of I_{NSC}. ***Panel B:*** Difference current obtained by digital subtraction of trace A from B in panel B. The reversal potential of the difference current in this myocyte was -13.4 mV.

TABLE 5: Reversal Potential Shift due to Non-Selective Cation Current during Extracellular O-R Stress with SR Ca^{2+} Depletion, $[Ca^{2+}]_i$ Chelation and Replacement of External Ca^{2+}.

Reversal potential (E_{rev})	10μM Ryanodine+ 0.1mM EGTA (pipette) (n=5)	5mM EGTA (pipette) (n=4)	5mM BAPTA (pipette)+ $0Ca^{2+}_o$ + $0Na^{+}_o$ (n=7)
Control (C)	-44.4 ± 5.8	-49.6 ± 4	-55.3 ± 11.3
O-R	-23.7 ± 3.7*	-26.5 ± 6.3*	-21.1 ± 1.7*
Difference Current [(O-R) - C]	-17.9 ± 1.7	-14.8 ± 1.8	-14.9 ± 1.5

E_{rev} - reversal potential for the quasi steady-state I-V relation and for the difference current.
Values are mean ± S.E.M. * indicates significant difference ($P < 0.05$) from values recorded prior to O-R .

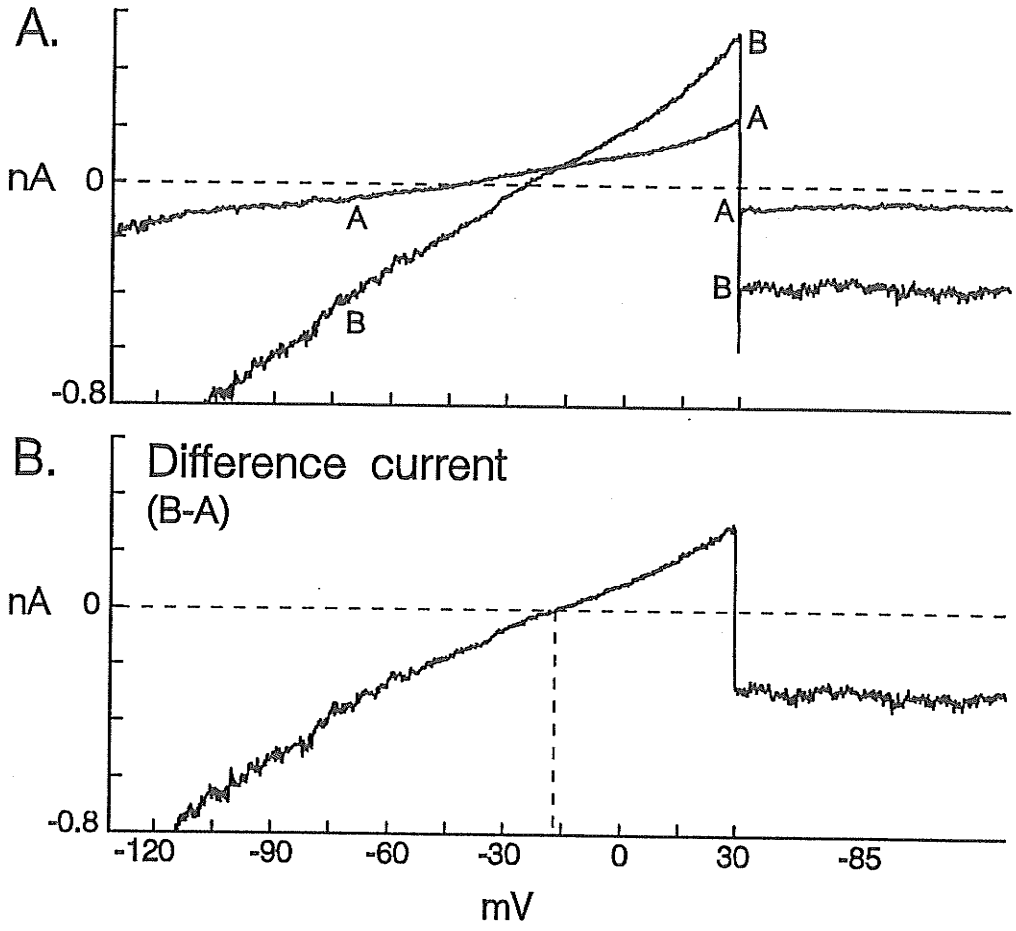

FIGURE 21: ***Lack of Effect of Intracellular Chelation of Ca^{2+} on Extracellular O-R Induced Activation of I_{NSC}.***

Panel A: Quasi steady-state current recordings evoked by a ramp protocol (from -130 to +30 mV over 8 sec from a holding potential of -85 mV). In this experiment, L-type Ca^{2+}, Na^+ and all K^+ currents were blocked and E_{Cl} was changed from -60.4 to -2.9 mV. Traces A and B were obtained before and 8.7 min after superfusion with O-R, respectively. Note the positive shift in the reversal potential of steady-state current reflecting activation of I_{NSC}. ***Panel B:*** Difference current obtained by digital subtraction of trace a from B of panel B. The reversal potential of the difference current in this myocyte was -15.3 mV.

The average value for the reversal potential of steady-state current in 4 myocytes was not different from that obtained from myocytes dialyzed with 0.1 mM EGTA solution (table 5). The effects of extracellular O-R on I_{NSC} following ryanodine and high EGTA are consistent with the net whole-cell current data and imply that an increase in $[Ca^{2+}]_i$ due to abnormal Ca^{2+} mishandling by the SR does not mediate the effects of extracellular O-R on I_{NSC}.

D. Effects of Internal Dialysis with BAPTA and Nominal $[Ca^{2+}]_o$ on O-R Activated I_{NSC} recorded in isolation:

Despite the results obtained with 5 mM EGTA in the presence of nicardipine, we felt that it was possible that a localized increase in $[Ca^{2+}]_i$ in the subsarcolemmal space resulting from Ca^{2+} entry through Na^+-Ca^{2+} exchange (Lederer *et al.*, 1990) might occur during O-R stress. This increase in $[Ca^{2+}]_i$ might activate I_{NSC} due to an inadequate intracellular Ca^{2+} chelation by EGTA. We sought to rule out this possibility by (1) dialyzing myocytes with 5 mM BAPTA, a Ca^{2+} chelator known to have a higher Ca^{2+} binding affinity than EGTA (Tsein, 1980), and (2) superfusing myocytes with a bath solution in which Na^+ and Ca^{2+} were substituted by equimolar replacement with LiCl and $MgCl_2$, respectively. Figure 22 shows that superfusion with O-R under these conditions still induced a positive shift in reversal potential of steady-state I-V and a negative shift in the holding current (panel A). The reversal potential of I_{NSC} was -18.2 mV in this myocyte. Similar results were obtained in another 6 myocytes and the average values of the reversal potential are given in table 5. We did not find any difference

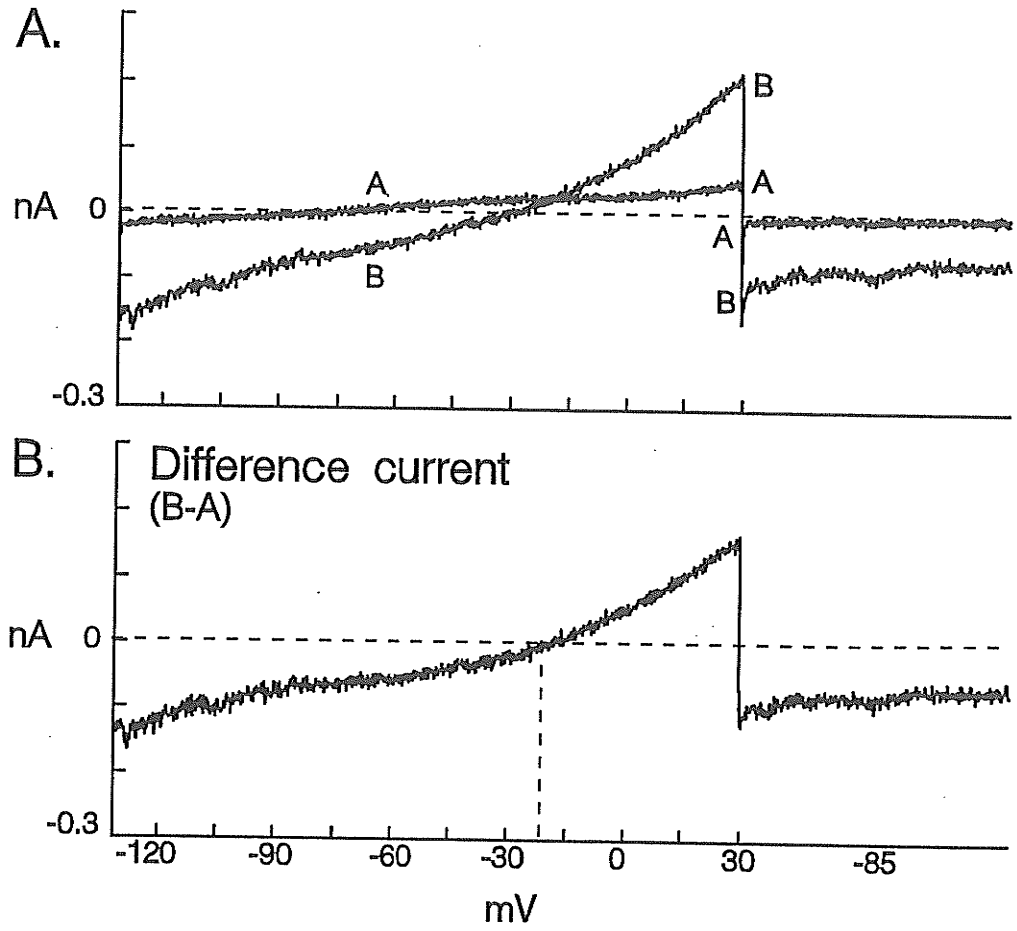

FIGURE 22: ***Lack of Effect of Intracellular Chelation of Ca^{2+} with BAPTA on Extracellular O-R Induced Activation of I_{NSC}.***

Panel A: Quasi steady-state current recordings evoked by a ramp protocol (from -130 to +30 mV over 8 sec from a holding potential of -85 mV). In this experiment, L-type Ca^{2+}, Na^+, Na^+-Ca^{2+} exchange and all K^+ currents were blocked and E_{Cl} was changed from -60.4 to -2.9 mV. Traces A and B were obtained from a myocyte dialyzed with 5 mM BAPTA before and after 10.2 min of O-R superfusion, respectively. Note positive shift in reversal potential of steady-state current reflecting activation of I_{NSC}. ***Panel B:*** Difference current obtained by digital subtraction of traces A from B of panel A. The reversal potential of the difference current in this myocyte was -18.2 mV.

in the reversal potential compared to that with high EGTA and with both Ca^{2+} and Na^{+} in the bath solution. These data provide further evidence that activation of I_{NSC} by extracellular O-R may occur independently of changes in $[Ca^{2+}]_i$. We concluded, therefore, that the activation of I_{NSC} by extracellular O-R might be the result of some other change in the myocytes, such as direct oxidative modification of the channel protein or lipid peroxidation.

E. Effects of DTT on Extracellular O-R Activated I_{NSC}:

It was previously reported that O-R stress alters the activation of several enzymes and ion transport proteins in the heart by oxidation of sulfhydryl containing amino acids. These changes in activity could be prevented or reversed by the sulfhydryl group reducing agent, DTT (Fliss *et al*, 1988; Kaneko *et al.*, 1991; Matsouka *et al.*, 1990; Yanagishita *et al.*, 1989; Eley *et al.*, 1991). We felt that if activation of I_{NSC} in the present study was due to oxidative modification of the non-selective cation channel proteins, then the change in I_{NSC} should be sensitive to DTT (Ziegler, 1985). We conducted DTT experiments in two different ways; myocytes were either; (1) pretreated with DTT (1 mM) for 30 minutes to prevent the change in current and then exposed to O-R, or (2) they were exposed to O-R and then to DTT in an attempt to reverse the changes in current. Figure 23 shows that O-R stress failed to activate I_{NSC} in a myocyte pretreated with DTT. A similar result was obtained in an additional 3 myocytes. In all cases, the time over which we monitored for a change in current was significantly longer (25 $\pm$ 1.1 min; n=4) than that required for O-R to activate I_{NSC} in the absence of DTT (10.1

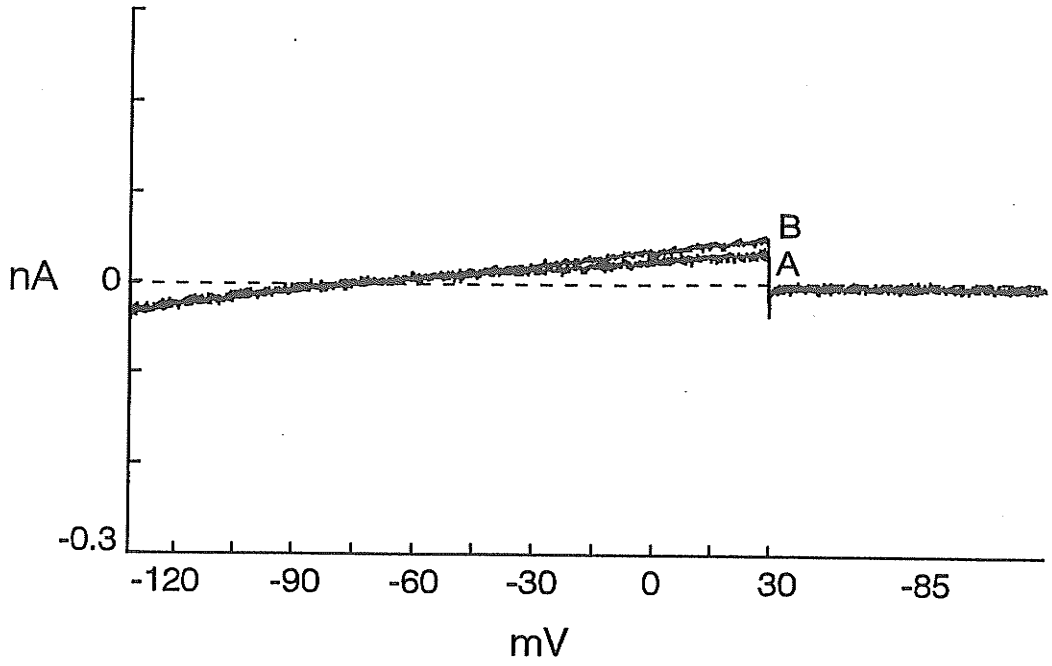

FIGURE 23: ***Pretreatment with DTT Prevented the Activation of I_{NSC} by Extracellular O-R.***

Quasi steady-state current recordings evoked by a ramp protocol (from -130 to +30 mV over 8 sec from a holding potential of -85 mV). In this experiment, L-type Ca^{2+}, Na^{+}, Na^{+}-Ca^{2+} exchange and all K^{+} currents were blocked and E_{Cl} was changed from -60.4 to -2.9 mV. Traces A and B were obtained from a myocyte pretreated with 1 mM DTT and dialyzed with 5 mM BAPTA before and after 26 min of O-R superfusion, respectively.

± 1.6 min; n=8; p< 0.001). These data suggest that pretreatment with DTT was able to protect sulfhydryl groups in the I_{NSC} channels from oxidative modification by extracellular O-R. Figure 24 shows that application of DTT in the continued presence of O-R, reversed the positive shift in reversal potential of steady-state current and the negative shift in the holding current caused by O-R stress. Average values for reversal potential of the difference current from 4 myocytes are shown in table 6. The reversal potential of steady-state I-V with DTT was significantly different from that during O-R stress, but was not different from that prior to O-R (table 6).

F. Effects of Diamide on I_{NSC}:

If oxidation of sulfhydryl groups is involved, then similar changes in steady-state current should be obtained with a sulfhydryl oxidizing agent, such as diamide (Kosower *et al.*, 1969; Haest *et al.*, 1979). Significantly, we found that diamide (0.5 mM) induced identical changes in the steady-state I-V relation to those observed during O-R stress (traces A and B, panel A, figure 25). In addition, the positive shift in Er was reversed (trace C, panel A, figure 25) following superfusion with DTT (1 mM) in the continued presence of diamide. Similar results were obtained from additional 4 myocytes and the average values for the reversal potential during diamide and after DTT in the presence of diamide are given in table 6. The difference current activated by diamide shows a largely ohmic I-V relation and possessed a reversal potential of -12.7 mV in this myocyte (panel B, figure 25). The average value for the diamide-activated current was not

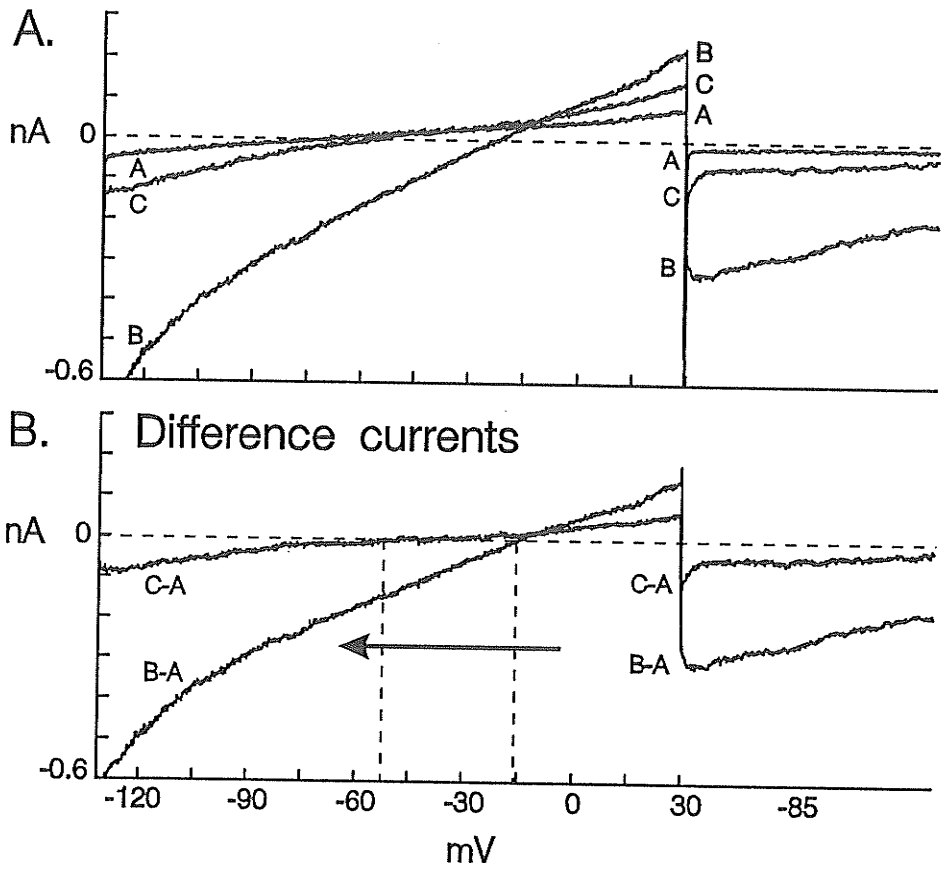

FIGURE 24: ***DTT Reversed the Activation of I_{NSC} by Extracellular O-R.***

Panel A: Quasi steady-state membrane current recordings evoked by a ramp protocol (-130 and +30 over 8 sec from a holding potential of -80 mV) in a myocyte dialyzed with 5 mM BAPTA. In this experiment, L-type Ca^{2+}, Na^+, Na^+-Ca^{2+} exchange and all K^+ currents were blocked and E_{Cl} was changed from -60.4 to -2.9 mV. Traces were obtained before (A) and after 3.6 min of O-R superfusion (B) and then after 3.6 min of 1 mM DTT in presence of O-R (C). Note reversal of positive shift in steady-state current with DTT. ***Panel B:*** Difference current obtained by digital subtraction of trace A from B of panel A. The reversal potential of the difference current in this myocyte was -15.7 mV.

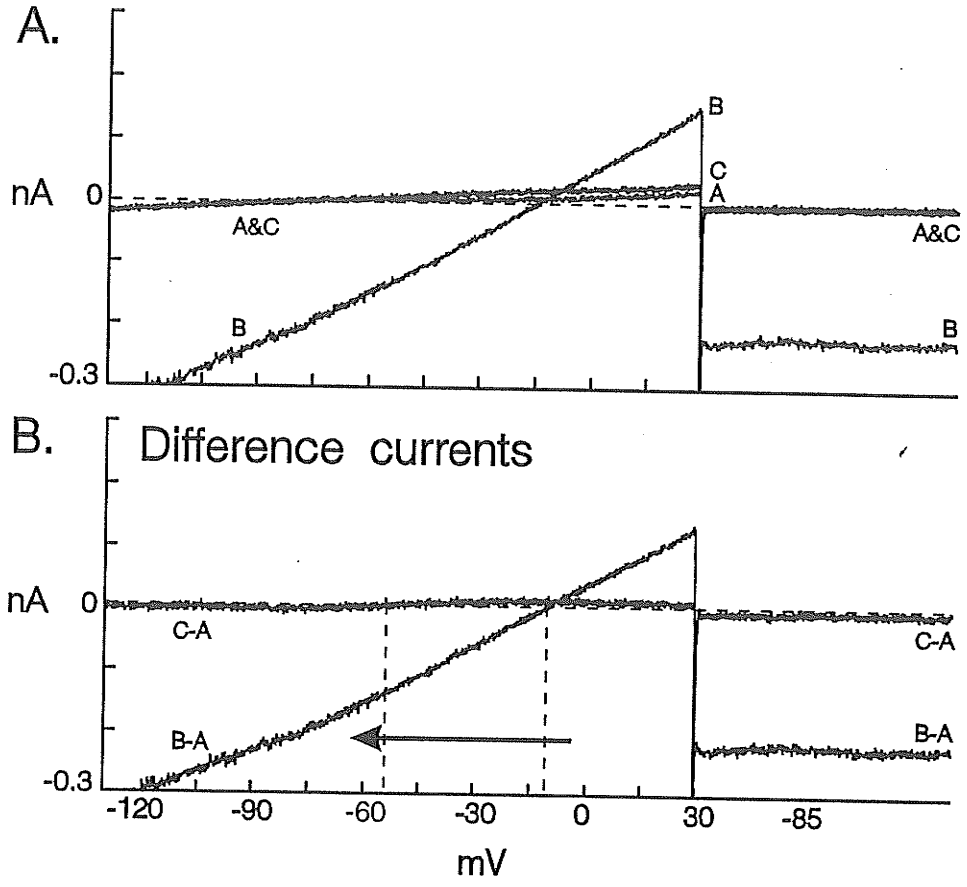

FIGURE 25: ***Activation of I_{NSC} by Diamide was Reversed by DTT.***

Panel A: Quasi steady-state membrane current recordings evoked by a ramp protocol (-130 and +30 over 8 sec from a holding potential of -80 mV) in a myocyte dialyzed with 5 mM BAPTA before (A) and after 4.5 min diamide (B). Trace C was after 4.8 min of superfusion with 1 mM DTT in presence of 0.5 mM diamide (C). Note the positive shift in the reversal potential of steady-state current with diamide and its block with DTT (compare with figure 24). ***Panel B:*** Difference current obtained by digital subtraction of trace A from B in panel A. The reversal potential of the difference current in this myocyte was -12.7 mV.

TABLE 6: Shift in Reversal Potential due to Non-Selective Cation Current Induced by Extracellular O-R or Diamide and Inhibition by DTT.

	Reversal Potential (E_{rev})	
	O-R (n=4)	Diamide (DIAM) (n=5)
Control (C)	-57.6 ± 3.6	-60.3 ± 4.1
O-R or DIAM	-16 ± 4.2***	-15.4 ± 1.9***
Difference Current [(O-R) or DIAM-C]	-13.2 ± 3	-12.4 ± 1.6
+ DTT	-60.1 ± 3.4	-63.1 ± 5.2

E_{rev} - reversal potential for the quasi steady-state I-V relation and for the difference current. Values are mean ± S.E.M. *** indicates significant difference ($P < 0.001$) from values recorded prior to O-R .

different from that due to extracellular O-R (table 6).

V. DISCUSSION

The major goals of this study were; (1) to investigate the possible differential effects of O-R generated from intracellular versus extracellular compartments on membrane electrical activity, and (2) to determine and compare the underlying ionic and cellular mechanisms responsible for these alterations. This was achieved by employing whole-cell variant of patch clamp technique to record the electrical activity and quasi steady-state ionic current from guinea pig ventricular myocytes exposed intracellularly or extracellularly to an O-R generating system (DHF-Fe^{3+}:ADP). Intracellular O-R induced three stages of changes in activity including; (1) early depolarization and prolongation in AP duration mediated by a decrease in I_{K1}, (2) DADs and low amplitude membrane oscillations were caused by stimulation of I_{ti} mediated by the forward mode of I_{NaCa} as well as sustained depolarization due to the activation of I_{NSC}, and (3) marked shortening in AP duration and hyperpolarization mediated by the activation of I_{KATP}. Stages 1 and 2 appeared to result from an O-R induced abnormal Ca^{2+} handling leading to an increase in $[Ca^{2+}]_i$. Extracellular O-R application induced some similar changes in AP configuration including DADs, low amplitude membrane oscillations and marked sustained depolarization but changes in I_{K1} or I_{KATP} were not observed in net current recordings. The DADS were clearly dependent on changes in $[Ca^{2+}]_i$. However, the activation of I_{NSC},

which mediated the sustained depolarization, appeared to result from a $[Ca^{2+}]_i$-independent mechanism apparently involving direct oxidative modification of sulfhydryl groups on the channels or associated regulatory proteins.

This study is the first to; (1) identify a role for intracellular O-R in inducing arrhythmogenic alterations in membrane electrical activity and ionic currents in cardiac ventricular myocyte, (2) identify both direct and indirect cellular mechanisms affected by O-R which are responsible for the arrhythmogenic changes in ionic channel activity, and (3) provide the very first evidence for altered cardiac sarcolemmal ion channel activity due to direct oxidative modification.

1. Intracellular O-R Stress:

This study is the first to describe the effects of intracellular O-R stress on electrical activity and quasi steady-state membrane ionic currents of mammalian ventricular myocytes. Intracellular application of an O-R generating system caused three stages of change in electrical activity, including; (1) an early slight depolarization and AP prolongation, (2) delayed after-depolarizations (DADs), failure to repolarize and a sustained marked depolarization, and (3) a marked decline in AP duration, hyperpolarization and loss of excitability. On the basis of whole-cell voltage clamp experiments, these alterations in electrical activity appear to involve specific changes in membrane ionic currents carried by I_{K1}, forward mode Na^+-Ca^{2+} exchange activity, non-selective cation and I_{KATP} channels. Moreover, the data indicate an important role for elevated $[Ca^{2+}]_i$ due

to abnormal Ca^{2+} handling by the SR in inducing the changes in electrical activity during intracellular O-R stress.

Autooxidation of DHF results in the formation of DHF radical (DHF·) and superoxide radical ($\cdot O_2^-$) (Kukreja *et al.*, 1988). These species will subsequently lead to the generation of other reactive metabolites of oxygen, including hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH). $FeCl_3$:ADP was included in the generating system to enhance the formation of ·OH from H_2O_2 and $\cdot O_2^-$ or the latter directly (Kukreja *et al.*, 1988). Thus, this generating system can be expected to produce the species of O-R specifically relevant to ischemia-reperfusion (Bolli *et al.*, 1988; Zweier *et al.*, 1989). However, we found that the changes in electrical activity produced by dialysis with DHF-$FeCl_3$:ADP were inhibited by pretreatment with MPG. Bolli *et al.* (1989) indicate that this is primarily a scavenger of hydroxyl radical, suggesting that this species of oxygen metabolite may be primarily responsible for the alterations in steady-state membrane currents described in this study.

That O-R stress may produce alterations in electrical activity is apparent from a variety of studies on intact cardiac preparations and isolated myocytes (Barrington, 1990), however, the specific ionic conductances affected by O-R are poorly characterized. We found that guinea-pig ventricular myocytes dialyzed with pipette solution containing 0.1 mM EGTA and O-R generating system demonstrated three stages of alterations in electrical activity. We concentrated on identifying the underlying changes in steady-state membrane currents in an effort

to understand the basis for; (1) the slight depolarization during stage 1, (2) the failure to repolarize and sustained depolarization in stage 2, and (3) the hyperpolarization and collapse of AP duration noted during stage 3. Thus, the three stages in this study reflect three periods during which there were distinct changes in steady-state membrane conductances induced by O-R stress. We do not rule out the possibility that intracellular O-R stress may also have affected time-dependent currents as well (particularly I_{Ca} and I_K), and that such changes may also contribute to the observed alterations in electrical activity.

The electrophysiological features of the three stages of intracellular O-R stress are similar but not identical to those occurring during extracellular O-R stress as described by Barrington and co-workers (1988). The following differences should be noted; (1) Stage 1 in both studies was associated with AP prolongation but Barrington *et al.* did not observe the slight depolarization described in this study. (2) DADs were observed during stage 2 in both studies, but we did not record EADs which were frequently present in stage 2 of Barrington *et al.* (3) Finally, stage 3 of Barrington *et al.* was marked by a loss of excitablilty due to either a failure to repolarize or marked depolarization. In the present study, we have included failure to repolarize in the second stage and limited stage 3 to the period of hyperpolarization and decline in AP duration. This was based on (1) the appearance of DADs before and after the myocytes failed to repolarize, (2) the different ionic mechanisms underlying the inexcitability during the failure to repolarize and hyperpolarization (see below), and (3) the similar

sensitivity of the DADs and failure to repolarize to intracellular Ca^{2+} chelation and ryanodine pretreatment as well as the apparent lack of effect of these manipulations on the hyperpolarization and decline in AP duration.

A. Stage 1 of Intracellular O-R Stress:

The first alterations in electrical activity noted during O-R stress were a decline in RMP of 5-10 mV and lengthening in AP duration. We attribute the slight depolarization to reduction in resting K^+ current through inward rectifier channels (I_{K1}). A marked decline in outward current in the negative slope region of the steady-state I-V relation and a decline in chord conductance at all potentials negative to -40 mV were observed in the absence of a significant change in the reversal potential for the quasi steady-state currents. This implies that the initial change in steady-state current was due to a decline in I_{K1} in the absence of a change in any other steady-state current(s), for example, activation of an inward current as was observed during the second stage of O-R stress. Inhibition of I_{K1} was also reported to occur in rabbit (Matsuura and Shattock, 1991b) and guinea-pig (Coetzee and Opie, 1991; Nakaya *et al.*, 1992) myocytes in response to extracellular O-R stress. The change in the current apparently arises because of a decrease in the opening probability of the channels rather than a decline in unitary conductance based in cell-attached recordings (Nakaya *et al.*, 1992). However, no studies have reported early depolarization in RMP induced by O-R stress.

Although alterations in I_{K1} may contribute to changes in the plateau and

repolarization phases of the action potential (Shimoni *et al.*, 1992), it is also possible that the changes in AP duration in stage 1 may have resulted from alterations in other currents as well. Firstly, the increase in AP plateau amplitude and prolongation of AP duration during early stage 1 may also have involved changes in time-dependent currents, such as I_{Ca} or I_K, which were reported to be influenced by extracellular O-R stress (Matsuura and Shatock,1990; Cerbai *et al.*, 1991; Tarr and Valenzeno, 1992; Coetzee and Opie, 1992). Secondly, during late stage 1 and immediately before the onset of stage 2, the AP plateau phase declined in amplitude when AP duration was very prolonged due to delayed repolarization negative to -30 mV. It is possible that activation of the non-selective cation current mediating the failure to repolarize during stage 2 (see stage 2 of O-R stress below) contributed to this change in activity. Activation of non-selective current would tend to enhance repolarization positive to, and inhibit repolarization negative to, its reversal potential of ~-20 mV. Hence the activation of this current would be expected to cause a concomitant decline in AP plateau potential and delayed repolarization such as were observed during late stage 1. Further experiments are required to determine whether the changes in AP duration during stage 1 reflect a contribution of these other conductances or are due solely to I_{K1}.

B. Stage 2 of Intracellular O-R Stress:

Following the initial depolarization, intracellular O-R stress induced DADs and triggered activity, as well as, failure to repolarize and sustained depolarization at -35 to -20 mV. The presence of DADs in this study is similar to that reported

previously in guinea-pig papillary muscles (Hayashi *et al.*, 1989), ventricular strips (Pallandi *et al.*, 1987) and canine (Barrington *et al.*, 1988), rat and guinea-pig (Beresewicz and Horakova, 1991) myocytes during extracellular exposure to O-R. We noted that the frequency of DADs and I_{ti} were similar, as previously reported, implying a causal relationship between the inward currents and depolarizations (Matsuda *et al.*, 1982; Orchard *et al.*, 1983). The ionic basis of I_{ti} due to Ca^{2+} overload is controversial; the inward current has been attributed to (1) Ca^{2+}-activated non-selective cation channels and/or (2) forward mode activity of the Na^+-Ca^{2+} exchanger (Matsuda, 1983; Giles and Shimoni, 1989). A role of Na^+-Ca^{2+} exchange current was indicated by sensitivity of the inward currents to Li^+ replacement for $[Na^+]_o$ and the absence of a unique reversal potential for the I_{ti} under recording conditions in which both $[Ca^{2+}]_i$ and $[Na^+]_i$ are dynamic (Fedida *et al.*, 1987). In contrast, the non-selective cation conductance is not sensitive to Li^+ replacement (Ehara *et al.*, 1988) and reverses at a voltage approximately halfway between E_K and E_{Na}, or ~-20 mV (Matsuda, 1983). In this study, we could not identify a reversal potential for the I_{ti} of stage 2 over a voltage range of -80 to +25 mV and the currents were inhibited when Na^+_o was completely replaced with Li^+. For these reasons, it would appear that the I_{ti} induced by intracellular O-R stress reflects forward mode Na^+-Ca^{2+} exchange activity rather than non-selective cation current. It should also be noted that Matsuura and Shattock (1990) previously reported a similar involvement of exchange current in the I_{ti} occurring during extracellular O-R stress.

The predominant feature of stage 2 was a failure to repolarize after an AP and sustained depolarization to between -35 and -20 mV. The results of our experiments favour the interpretation that activation of an inward current through non-selective cation channels accounts for the marked change in membrane potential during stage 2. Neither a decline in seal resistance nor O-R mediated breakdown of the sarcolemmal integrity appear to be involved because the changes in holding current and reversal potential of the difference current activated during this stage were completely reversible in 15 myocytes (Table 3) which would not be expected if seal leak or membrane disruption were involved. Moreover, the reversal potential for the difference current evoked during this stage was -19.9 ± 0.7 mV rather than 0 mV as would be expected for a non-specific leak conductance. It is also unlikely that the current was due to Cl^-, L-type Ca^{2+}, TTX-sensitive Na^+, typical K^+ channels or Na^+-Ca^{2+} exchange current because; (1) the reversal potential for the difference current was not affected by changes in E_{Cl}, (2) membrane potential was unaffected by exposure to TTX after failure to repolarize occurred during stage 2, (3) the reversal potential of the difference current induced by O-R stress was unaffected by exposure to nicardipine, TTX and Cs^+, (4) the difference current had a demonstrable reversal potential under conditions in which $[Na^+]_i$ and $[Ca^{2+}]_i$ were dynamic and (5) the current was not affected by Li^+ replacement of Na^+.

On the other hand, the linear I-V relationship and reversal potential of -19.9 ± 0.7 mV for the difference current activated during stage 2 are similar to that

reported by Matsuda (1983) (-22 ± 11 mV) for the voltage-independent non-selective cation current evoked by elevating intracellular Ca^{2+} concentration. That intracellular O-R stress leads to the activation of a non-selective cation conductance is supported by the following observations; (1) a 50% increase in $[K^+]_o$ (4.8 to 9.8 mM) or a 50% decrease in $[Na^+]_o$ by NMG substitution shifted the reversal potential for the difference current by +8.8 ± 1.7 mV and -8.9 ± 1.1 mV, respectively, values which are compatible with that expected (i.e. +8.83 mV and -8.83 mV) assuming an equal permeance of K^+ and Na^+ in the channel (Colquhoun *et al.*, 1981; Ehara *et al.*, 1988). (2) The reversal potential of the difference current activated by O-R stress was unaffected by 100% replacement of $[K^+]_o$ and $[Na^+]_o$ with Cs^+ and Li^+, respectively, as would be expected for a conductance which was not selective for different cations (Ehara *et al.*, 1988).

C. Stage 3 of Intracellular O-R Stress:

The decline in AP duration and hyperpolarization during the final stage of intracellular O-R stress were due to the activation of an outward K^+ current which was; (1) voltage-insensitive negative to +20 mV yet showed strong inward rectification positive to this potential, (2) a "noisy" current suggestive of a large single channel conductance, and (3) blocked with glibenclamide (10 μM). These properties are all consistent with those reported for ATP-sensitive K^+ current (Noma, 1983). Goldhaber *et al.* (1989) also observed a decline in AP duration during extracellular O-R stress due to activation of I_{KATP}.

D. Mechanism(s) for Alterations in Membrane Ionic Currents during Intracellular O-R Stress:

O-R stress causes oxidation of membrane phospholipids and/or sulfhydryl-containing proteins which could lead to alterations in channel activity and changes in the membrane currents described above. Alternatively, it is possible that other more indirect mechanisms are also involved since ion channels are sensitive to a variety of intracellular factors which might be influenced by O-R stress. For example, intracellular Ca^{2+} and ATP affect the non-selective cation conductance (Colquhoun *et al.*, 1981; Matsuda, 1983; Ehara *et al.*, 1988) and I_{KATP} (Noma, 1983; De Weille and Lazdunski, 1990), respectively and the levels of both factors are known to be altered by O-R stress (Josephson *et al.*, 1991). The results of the present study suggest that intracellular O-R stress indirectly leads to stages 1 and 2 because of elevated $[Ca^{2+}]_i$ due to abnormal SR Ca^{2+} release. Increasing EGTA from 0.1 to 5 mM in the pipette solution to improve intracellular Ca^{2+} chelation prevented stages 1 and 2 but not stage 3. Moreover, pretreatment with ryanodine to deplete SR Ca^{2+} stores prior to application of O-R also prevented stage 1 and 2 but was without effect on stage 3. These data suggest that the effects of intracellular O-R stress on I_{K1}, I_{ti} and non-selective cation current are mediated by an elevation in $[Ca^{2+}]_i$ due to release of SR Ca^{2+} stores. The data are consistent with the reported effects of elevated $[Ca^{2+}]_i$ on these currents and influence of O-R stress on $[Ca^{2+}]_i$ and SR Ca^{2+} handling. Elevated $[Ca^{2+}]_i$ activates I_{ti} (Orchard *et al.*, 1983; Fedida *et al.*, 1987; Giles and Shimoni, 1989;

Berlin *et al.*, 1989) and non-selective cation current (Matsuda, 1983) but inhibits I_{K1} (Mazzanti and Difrancesco, 1989). A ryanodine-sensitive rise in $[Ca^{2+}]_i$ was observed in myocytes during extracellular O-R stress (Hayashi *et al.*, 1989) consistent with several studies indicating that SR Ca^{2+}-handling is affected by O-R stress; e.g. passive Ca^{2+} leak from the SR is increased (Stuart and Abramson, 1989; Okabe *et al.*, 1989 and 1991), SR Ca^{2+} uptake is depressed (Kukreja *et al.*, 1988), and the open probability of SR Ca^{2+}-release channels is increased (Holmberg *et al.*, 1991) by O-R leading to net elevation in $[Ca^{2+}]_i$. Moreover, intracellular Ca^{2+} chelation was previously reported to block some changes in electrical activity induced by extracellular O-R stress (Cerbai *et al.*, 1991). Interestingly, Beresewisz and Horakova (1991) reported that ryanodine did not block similar changes in electrical activity to those in stage 2 during exposure to extracellular H_2O_2.

The mechanism by which ATP-sensitive K^+ channels are activated during stage 3 of intracellular O-R stress to cause hyperpolarization and decline in AP duration is unknown. It is clear from the absence of an effect of Ca^{2+} chelation or ryanodine pretreatment on stage 3 that I_{KATP} activation is not dependent upon elevated $[Ca^{2+}]_i$ and is not dependent on the presence of stages 1 and 2 due to O-R. The earlier onset of stage 3 in 5 myocytes following premature stage 2, however, suggests that changes in $[Ca^{2+}]_i$ homeostasis associated with stage 2 was sufficient to evoke early activation of I_{KATP}. Additional experiments are required to determine whether the channels are affected by O-R directly or

activation results from a decline in intracellular ATP levels. It is possible that the onset of stage 2 provoked a more rapid decline in ATP levels due to increase consumption or decreased synthesis. With regards to the latter, Goldhaber *et al.* (1989) concluded that I_{KATP} was activated during extracellular O-R stress because of a depression of glycolytic enzyme activity and reduced ATP production rather than a direct effect of O-R on the channels.

Some variability in the effects of intracellular O-R were noted in this study. As indicated in table 3 all three stages occurred in 38% of the myocytes. The absence of stage 3 in 35% of the myocytes may be attributed to loss of membrane seal or cell death during the hypercontracture associated with stage 2. Stage 1 was not observed and stage 2 had an early onset in 19% of the myocytes. In this case, it seems likely that the premature onset of stage 2 masked the more subtle changes associated with stage 1. Finally, in approximately 8% of the myocytes stage 2 did not occur but stages 1 and 3 were both evident. The reason for the premature onset of stage 2 in some myocytes but its absence in others is unknown. It is possible that the variability in stage 2 may relate to (1) differences in intracellular O-R levels due to variations in the extent of dialysis and/or (2) variations in the amount and/or rate of Ca^{2+} release from the SR stores during O-R stress. On the basis of our data none of the stages appears to be linked to the others in an obligatory fashion.

2. Extracellular O-R Stress:

This study reports the novel finding that oxidative stress due to reactive O-R species in the extracellular space activates the non-selective cation current in isolated guinea pig ventricular myocytes by directly modifying sulfhydryl groups. Moreover, the data provide the first support for the hypothesis that mammalian cardiomyocytes may be differentially influenced by O-R in the extracellular versus intracellular space. In this study extracellular oxidative stress was shown to activate I_{NSC} under conditions which prevent changes in $[Ca^{2+}]_i$. In the presence of intracellular BAPTA (5 mM) and no external Na^+ or Ca^{2+} (replaced with Li^+ and Mg^{2+}, respectively), O-R generated from the combination of DHF and $FeCl_3$:ADP, as well as, the sulfhydryl group oxidizing agent, diamide, led to the activation of a largely voltage-independent, steady-state current reversing at approximately the halfway point between the equilibrium potentials for K^+ and Na^+ (or Cs^+ or Li^+) under the recording conditions employed. We previously failed to observe I_{NSC} during intracellular O-R stress in the absence of changes in $[Ca^{2+}]_i$ under identical recording conditions. These observations imply that the sulfhydryl groups affected by extracellular O-R stress may be localized on the extracellular surface of the channel or an associated regulatory protein.

A. Effects of Extracellular O-R Stress on Electrical Activity:

In this study extracellular O-R stress induced alterations in electrical activity similar to those reported previously including prolongation in AP duration, DADs and sustained depolarization (Barrington *et al.*, 1988; Beresewicz and Horakova,

1991). However, we did not observe EADs which were reported to result from extracellular O-R stress in three other studies (Barrington *et al.*, 1988; Beresewicz and Horakova, 1991; Cerbai *et al.*, 1991). This could be attributed to the differences in animal species, O-R generating system and/or recording conditions.

The changes in AP configuration induced by extracellular O-R were similar to those of stage 2 observed during intracellular exposure. For example, prolongation in AP duration, low amplitude membrane oscillations, DADs and sustained depolarization. However, we failed to observe the early slight depolarization or marked shortening in AP duration and hyperpolarization associated with stages 1 and 3, respectively. This point is significant in that it reveals the importance of the differential effects of O-R from the intra- versus extracellular compartment. Activation of I_{NSC} during intracellular O-R stress required time and was reversible, presumably because of depletion of the SR Ca^{2+} stores. This permitted the changes in I_{K1} during stage 1 and I_{KATP} during stage 3 to dominate membrane conductance and cause depolarization and hyperpolarization, respectively. On the other hand, I_{NSC} activation occurred early during extracellular O-R stress, and the current did not decrease with time. It is possible therefore, that the changes in I_{NSC} may have masked alterations in I_{K1} and/or I_{KATP}.

The differences in electrical activity observed during intra- and extracellular O-R stress in this study were not the same as those reported to occur in frog

atrial myocytes by Tarr and Valenzeno (1989). These authors showed that intracellular O-R caused a decline, but extracellular O-R led to a biphasic increase followed by a decrease in AP duration. Perhaps the absence of an SR Ca^{2+} pool in the frog myocytes accounts for the divergent of observations of shortening of AP duration but prolongation during intracellular O-R stress in this study.

B. Ionic and Cellular Mechanisms for Abnormal Electrical Activity Induced by Extracellular O-R stress:

We found that the difference current underlying sustained depolarization during extracellular O-R stress was largely voltage-independent and possessed a reversal potential of -18.3 ± 2.1 mV consistent with the idea that it is due to a non-selective cation conductance. This value is identical to that which we reported during intracellular O-R stress (E_{rev}= -19.9 ± 0.7 mV) and is similar to that reported by Matsuda (1983) for whole-cell current induced by intracellular Ca^{2+} injection (-22 ± 11 mV). The apparent insensitivity of the reversal potential of the current to TTX, nicardipine, Cs^+ and Li^+ and changes in E_{Cl} was also similar to that reported for I_{NSC} during intracellular O-R stress. However, we do not attribute the activation of I_{NSC} during extracellular O-R stress to changes in $[Ca^{2+}]_i$ as was concluded for the change in this current during intracellular O-R stress. Activation of I_{NSC} during extracellular O-R stress was not affected by; (1) depletion of SR Ca^{2+} stores by ryanodine pretreatment, (2) elevated intracellular Ca^{2+} chelation with 5 mM EGTA or BAPTA in the pipette solution, or (3) complete replacement of external Na^+ and Ca^{2+} to prevent Ca^{2+} influx on the Na^+-Ca^{2+} exchange and

a localized elevation of $[Ca^{2+}]_i$ near the sarcolemma. The apparent insensitivity to intracellular Ca^{2+} chelation is different from that reported of Matsuura and Shattock (1991b). The authors found that 5 mM EGTA in the pipette solution blocked I_{NSC} activation following O-R stress due to photo-illumination of extracellular Rose Bengal. The reason(s) for this difference is unknown but they may derive from the different generating systems employed. Rose Bengal produces singlet oxygen ($^1O_2$) (Kusama *et al.*, 1989) whereas the dominant species produced by the combination of DHF and $FeCl_3$:ADP is the hydroxyl radical (Kukreja *et al.*, 1988). Further studies are required to assess the divergent effects of different species of O-R.

a. Role of $[Ca^{2+}]_i$ on activation of I_{NSC} by extracellular O-R stress:

We are confident that the changes in I_{NSC} in this study may not be attributed to changes in $[Ca^{2+}]_i$ for two reasons. First we went to considerable lengths to eliminate $[Ca^{2+}]_i$ as a factor by chelating this ion with 5 mM BAPTA in the pipette solution and bathing the myocytes in bath solution in which Ca^{2+} and Na^+ were replaced with Mg^{2+} and Li^+ to eliminate Na^+-Ca^{2+} exchange (Kimura *et al.*, 1987; Fedida *et al.*, 1987; Miura *et al.*, 1989). In addition, in four myocytes exposed to O-R or diamide after pretreatment with ryanodine and under these recording conditions still demonstrated activation of I_{NSC} (data not shown). Secondly, we failed to observe low amplitude membrane oscillations, triggered activity and delayed after-depolarizations mediated by the activation of the transient inward currents (I_{ti}) in myocytes pretreated with ryanodine or dialyzed

with 5 mM EGTA. Despite these treatments, I_{NSC}, sustained depolarization and a positive shift in the reversal potential for the whole-cell steady-state I-V relation were still observed. As noted above, I_{ti} are thought to reflect stimulation of forward mode of Na^+-Ca^{2+} exchange due to increased $[Ca^{2+}]_i$ caused by abnormal release of Ca^{2+} by SR (Fedida *et al.*, 1987). The absence of I_{ti} after ryanodine or dialysis with EGTA suggests adequate block of changes in $[Ca^{2+}]_i$ due to O-R stress. Additionally, the data indicate that O-R may cause I_{ti} and DADs because of changes in $[Ca^{2+}]_i$ regardless of whether the reactive species are applied from the intracellular or extracellular compartment. However, we do not believe that this is the case for the activation of I_{NSC}. Our data imply that a change in $[Ca^{2+}]_i$ is not necessary for an increase in I_{NSC} during extracellular O-R stress and indicate the presence of another mechanism for activating the current.

B. Role of oxidative modification of non-selective cation channel by extracellular O-R stress:

A non-$[Ca^{2+}]_i$ mediated mechanism by which extracellular O-R activates I_{NSC} was identified in this study. The data indicate that oxidative modification of sulfhydryl groups on non-selective cation channels or an associated regulatory protein may lead to the activation of this conductance in cardiac ventricular myocytes. The activation of I_{NSC} was reversed or prevented by sulfhydryl-group reducing agent, DTT. This result eliminates the possibility that membrane lipid peroxidation and a localized change in the phospholipid milieu surrounding the channel proteins mediates the change in activity. The ability of DTT to reverse the

effects of extracellular O-R on I_{NSC} was similar to that reported for several ion transporters such as SL Ca^{2+}-pump (Kaneko *et al.*, 1991), Na^{+}-K^{+} ATPase (Matsouka *et al.*, 1990) and SR Ca^{2+}- ATPase (Yanagishita *et al.*, 1989; Eley *et al.*, 1991). Moreover, that DTT is capable of protecting cysteine amino acids (Ziegler, 1985; Fliss *et al.*, 1988) implies that a cysteine residue on the channel or an associated regulatory protein was oxidized by O-R.

Secondly, we found that an increase in I_{NSC} could be induced by a non-O-R oxidizing agent, diamide. We do not attribute the effects of diamide to alterations in phospholipid asymmetry. The time required for diamide to induce I_{NSC} was too short (3.9± 0.8 min) compared to that necessary for phospholipid translocation (over 5 hr incubation period) (Middelkoop *et al.*, 1989). We also found that the effects of diamide on I_{NSC} were reversed with DTT. This would not be expected if the change in current were due to a diamide induced alteration in membrane phospholipids. However, this would suggest that diamide oxidized cysteine amino acids on the channels that were sensitive to DTT. Interestingly, only I_{NSC} and SR Ca^{2+} release appear to increase as a result of oxidative stress (Holmberg *et al.*, 1991) which could be mediated by sulfhydryl oxidation (Prabhu and Salama, 1990). The sarcolemmal Ca^{2+}-pump (Kaneko *et al.*, 1991), Na^{+}-Ca^{2+} exchange (Antolini *et al.*, 1991), and Na^{+}-K^{+} ATPase (Matsouka *et al.*, 1990) enzymes are all reported to be inhibited. The similarity of the effects of O-R and diamide on I_{NSC} and the sensitivity of both treatments to DTT provides strong evidence for the idea that extracellular O-R stress activates I_{NSC} by inducing

oxidative modification of sulfhydryl group containing amino acids. We do not know whether the sulfhydryl groups sensitive to O-R and diamide are on the non-selective channel per se or on an associated regulatory protein. However, it seems likely that the groups affected by O-R are on the portion of the protein within the extracellular compartment. We previously showed that intracellular O-R failed to increase I_{NSC} when $[Ca^{2+}]_i$ was prevented from rising. If an intracellular oxidative reaction was involved, then an increase in I_{NSC} should still have been observed during Ca^{2+} chelation in our previous study. The continued presence of an increase in I_{NSC} during extracellular O-R stress despite intracellular Ca^{2+} chelation provides evidence that the sites for oxidative modification are very likely extracellular.

In summary, this study is the first to report the sensitivity of a sarcolemmal ion channel to direct oxidative stress and to indicate a differential mechanism of modulation of a membrane current due to O-R stress depending on the compartment in which the reactive species are generated. Our data implicating direct oxidative modification of a membrane conductance leading to altered electrical activity agrees with prior studies in which sulfhydryl group modifying agents were found to block abnormal electrical activity believed to arise as a result of altered redox state due to O-R in reperfusion (Sochman *et al.*, 1990; Qiu *et al.*, 1990). N-acetylcysteine reduced the incidence of arrhythmias caused by reperfusion in Langendorff perfused rat hearts (Qiu *et al.*, 1990) and canine hearts *in vivo* following coronary artery ligation (Sochman *et al.*, 1990).

3. Potential Mechanisms for Arrhythmogenesis of O-R:

The alterations in quasi steady-state conductances induced by O-R stress as described in this study are potentially arrhythmogenic and may, in theory, induce two different types of arrhythmias in the whole heart as summarized in figure 26. Triggered automaticity could occur as a result of extrasystolic APs due to I_{ti} mediated by stimulation of forward mode of I_{NaCa}. The depression of I_{K1} and/or the activation of I_{NSC} by O-R would be expected to enhance the opportunity for triggered activity by reducing the magnitude of net outward current at membrane potentials close to RMP indicated by the dashed arrows in figure 26. In addition, the decline in I_{K1} and increased I_{NSC} can directly lead to enhanced automaticity like those evoked by barium treatment (Imoto *et al.*, 1987; Valenzuela and Vassale, 1989) Re-entrant arrhythmias may result from the activation of I_{KATP} and consequential decline in AP duration and refractory period. Alternatively, injury current and depolarization may be expected to cause focal regions of slow conduction which could predispose intact myocardium to re-entrant excitation.

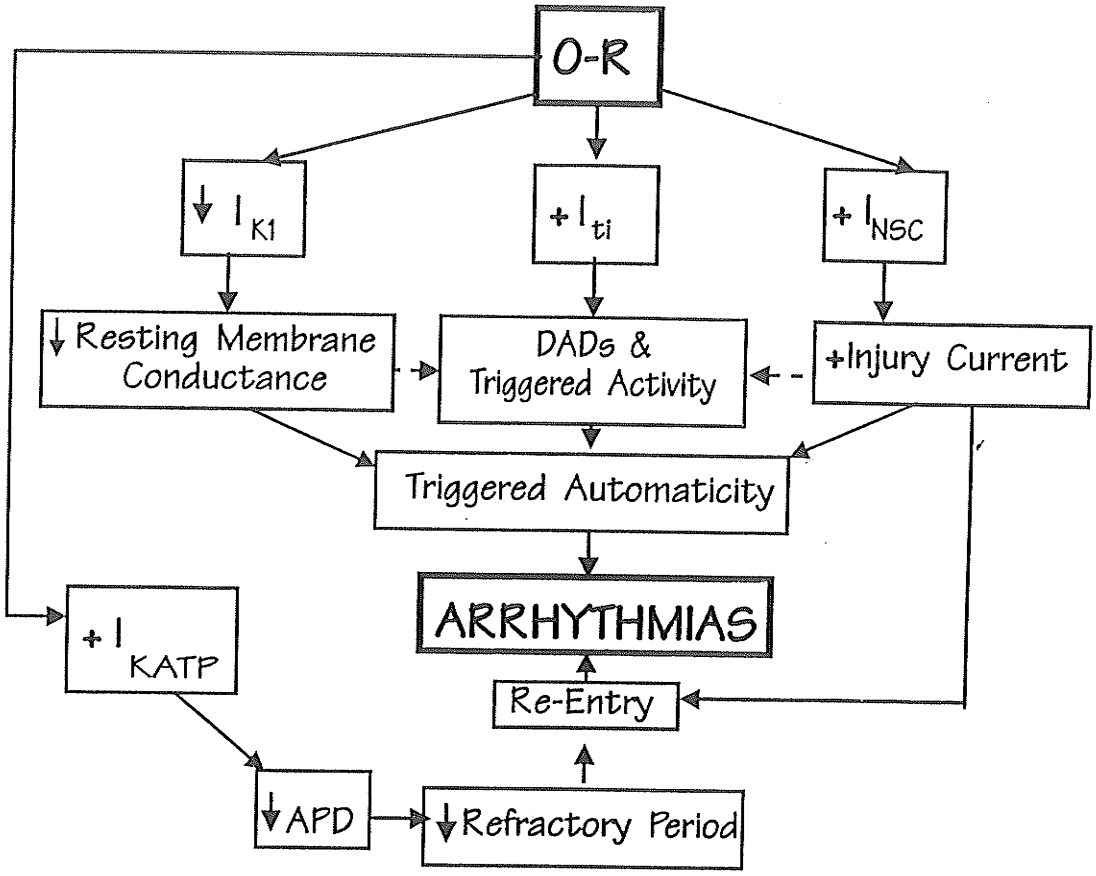

FIGURE 26: ***Possible Mechanisms for Proarrhythmogenic Role of O-R-Induced Alterations in Membrane Ionic Currents.***

This flow chart representation shows the consequences of, and possible mechanisms by which a decrease in I_{K1} or increase in either I_{ti}, I_{NSC} or I_{KATP} could lead to reperfusion arrhythmias.

VI. SUMMARY AND CONCLUSIONS

1. Intracellular O-R stress (DHF-Fe^{3+}:ADP) induced three stages of changes in membrane electrical activity and quasi steady-state currents in guinea pig ventricular myocytes including; (1) an early depolarization in RMP and prolongation in AP duration due to a decrease in I_{K1}, (2) DADs and triggered activity caused by activation of I_{ti} (i.e., forward mode activity of the Na^+-Ca^{2+} exchange), with failure to depolarize and sustained depolarization between -35 to -20 mV, reflecting the stimulation of I_{NSC}, and (3) a late stage of marked decline in AP duration, hyperpolarization and loss of excitability due to the activation of I_{KATP}. The changes during stage 1 and 2 were mediated by O-R induced abnormal SR Ca^{2+} handling. Stage 3 may arise because of a depletion of high energy phosphate.

2. Extracellular exposure of guinea pig ventricular myocytes to O-R generating system induced some similar alterations in membrane electrical activity but differences were apparent as well compared to intracellular stress. Activation of I_{ti} was sensitive to elevation in $[Ca^{2+}]_i$ mediated by abnormal SR Ca^{2+} release similar to that during intracellular O-R stress. However, the sustained depolarization caused by the activation of I_{NSC} did not require a rise in $[Ca^{2+}]_i$ or abnormal SR Ca^{2+} release. The dominant feature was activation of I_{NSC}

associated with stage 2 of intracellular stress. Stages 1 and 3 were not readily apparent during extracellular O-R stress. It is concluded that the change in this conductance was due to oxidative modification of sulfhydryl groups on the non-selective cation channel or associated regulatory protein. Stages 1 and 3 of intracellular O-R stress were not observed, possibly because they were marked by the irreversible increase in I_{NSC}. The data indicate that differential cellular mechanisms for modulation of ionic conductances due to O-R stress are involved depending upon the compartment within which they are generated.

3. The alterations in membrane currents due to O-R are all potentially arrhythmogenic, and therefore, these changes may contribute to genesis of early reperfusion arrhythmias .

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