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MECHANISMS INVOLVED IN THE DISRUPTION
AND RESTORATION OF EXCITATION-
CONTRACTION COUPLING IN THE RAT
MYOCARDIUM BY HYPOCHLOROUS ACID AND
DITHIOTHREITOL

DOUGLAS W. ELEY

A Thesis Submitted to the School of Graduate Studies
of the University of Ottawa in Partial Fulfillment of
the Requirements for the Degree of
Doctorate of Philosophy in Physiology

SUPERVISORS: Dr. B. Korecky
Dr. H. Fliss
Dr. M. Désilets



Douglas W. Eley, Ottawa, Canada, 1991



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II

ABSTRACT

Hypochlorous acid is a powerful oxidant produced by activated polymorphonuclear leukocytes (PMN) during an acute respiratory burst. The influx and activation of PMN has been linked with the development of long-termed cellular damage following reperfusion of previously ischemic myocardium, yet the effects of HOCl on the contractile function of the mammalian myocardium are virtually unknown. In the present studies, the addition of HOCl to the bathing medium for isometrically contracting papillary rat papillary muscles induced the development of contracture. This was characterized by a decline in developed tension (DT) in combination with a rise in resting tension (RT), a prolongation in relaxation kinetics, and a sensitivity of the muscles to stimulation voltage. This response to HOCl was potentiated by preincubation with low extracellular Ca^{2+} or the Ca^{2+} channel antagonist nifedipine. Conversely, the response was attenuated by preincubation with high extracellular Ca^{2+} concentrations or with the Ca^{2+} channel agonist Bay K 8644. The development of contracture was prevented by preincubation with 1 nM ryanodine, an alkaloid compound known to alter Ca^{2+} handling by the sarcoplasmic reticulum.

In isolated whole-cell clamped cardiac myocytes, using the Ca^{2+} fluorescence indicator Fura-2 to monitor $[\text{Ca}^{2+}]_i$, HOCl induced a steady rise in diastolic $[\text{Ca}^{2+}]_i$ even in the absence of extracellular Ca^{2+} . This rise in $[\text{Ca}^{2+}]_i$ was prevented by pre-exposure of the cell to 5 mM caffeine, a compound known to deplete the SR of stored Ca^{2+} . HOCl also induced a loss of Ca^{2+} transient amplitude in response to membrane depolarizations from -40 mV to 0 mV, although the whole cell holding currents and voltage sensitive inward Ca^{2+} currents were relatively unaffected. In oxalate loaded SR vesicles isolated from rat hearts perfused with HOCl, both the activity of the SR Ca^{2+} -

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ATPase (determined by inorganic phosphate production) and the uptake of $^{45}\text{Ca}^{2+}$ were significantly depressed as compared to Control. The 110 kD Ca^{2+} -ATPase protein isolated from these SR microsomes showed evidence of significant levels of thiol depletion. I conclude that the exposure of the rat myocardium to HOCl inactivates the SR Ca^{2+} -ATPase through the oxidation of protein thiols, leading to a cytosolic Ca^{2+} overload and the development of contracture. Cursory evidence also suggests that the Ca^{2+} pump of the sarcolemma and the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger may also be inactivated.

Subsequent exposure of the HOCl-treated myocardium to the disulfide reducing agent dithiothreitol (DTT) resulted in a restoration of contractile function, characterized by a decline in RT concomitant with a recovery of DT in the isolated papillary model, or a recovery of left ventricular end diastolic pressure and developed pressure in the perfused rat heart.. In isolated cardiac myocytes, the treatment with DTT resulted in a restoration of steady-state $[\text{Ca}^{2+}]_i$, as measured by Fura-2 fluorescence, to near-baseline levels. The amplitude of the cellular Ca^{2+} transients was also restored, suggesting that a replenishment of the intracellular releasable Ca^{2+} pool had occurred. In SR vesicles, the activity and function of the Ca^{2+} -ATPase were restored to near control levels following DTT treatment, correlating with a significant restoration of protein thiols in the 110 kD SR protein. We conclude that DTT was able to cross cellular membranes where it restored cellular protein thiol levels by reducing HOCl-induced disulfide formation. In the cell, this is observed as a reactivating the SR Ca^{2+} -ATPase and a partial restoration of cellular Ca^{2+} homeostasis, manifest as a recovery of Ca^{2+} transients, a decline in $[\text{Ca}^{2+}]_i$, and the resultant restoration of contractile function. From these results, I conclude that the effects of HOCl on the contacting myocardium are at least in part through its ability to oxidize protein thiols, leading to an inactivation of normal cellular ionic regulation. The addition of DTT, a disulfide reducing agent, induced a recovery of protein thiols, resulting in a restoration of ionic homeostasis.

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LIST OF ABBREVIATIONS

$\cdot\text{O}_2^-$	superoxide anion
$\cdot\text{OH}$	hydroxyl radical
$[\text{Ca}^{2+}]_e$	extracellular free Ca^{2+} concentration
$[\text{Ca}^{2+}]_i$	intracellular free Ca^{2+} concentration
ADP	adenosine diphosphate
AM	acetoxymethyl ester
AMP	adenosine monophosphate
ATP	adenosine triphosphate
dDT/dt	1st derivative of DT
DT	developed tension
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
EC	excitation-contraction
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol bis(β -aminoethylether)N N N'N' Tetraacetic acid
GSH	glutathione
GSSG	glutathione disulfide
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
IAA	iodoacetic acid
LVEDP	left ventricular end diastolic pressure
min	minute
MPO	myeloperoxidase
ms	millisecond
NP-SH	cellular non-protein sulfhydryls
OFR	oxygen free radical
P-SH	cellular protein sulfhydryls
PMN	polymorphonuclear leukocytes
PP	pulse pressure = end systolic pressure development - LVEDP
RT	resting tension
SDS	Sodium dodecyl sulfate
SOD	superoxide dismutase
V/V	volume/volume

DEDICATION

This thesis is dedicated to my wife, Judy, the reason for it all. And to Dr. G. Mainwood:
knowing you has been an honor and a privilege.

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I would like to thank my supervisors, Dr. B. Korecky for his confidence and continued support over the years, and Dr. H. Fliss for showing me what it takes to do science. I offer my deepest appreciation to Dr. M. Désilets for his relentless patience and unending knowledge. This project was broad, and at times became too much for one set of hands: I owe great deal of thanks to Marika Masika, Isabella Roy, and Michael (Mickey) Menard for their tireless assistance, training, and advice in all stages of the project. Thank you Juliette, all the support staff, and the graduate students within the Department of Physiology, for without them the fun and the science would stop.

And last, but not least, I thank Judy for all you have done.

MECHANISMS INVOLVED IN THE DISRUPTION OF EXCITATION-CONTRACTION COUPLING IN THE RAT MYOCARDIUM BY HYPOCHLOROUS ACID AND DITHIOTHREITOL

GENERAL INTRODUCTION AND LITERATURE REVIEW:

ISCHEMIA-REPERFUSION INJURY IN THE MYOCARDIUM

The occlusion of a major coronary artery will result in a severe reduction of blood flow to the downstream regions of the myocardium. When this occlusion is complete, the loss of oxygen and substrate delivery to the ischemic region results in the depletion of high-energy phosphates (Hearse et al. 1973) leading to a loss of intracellular enzyme activity and ionic regulation. Sustained blockage can result in tissue necrosis (Poole-Wilson 1984) and the formation of a myocardial infarct. To date, reperfusion of an ischemic region of the heart with oxygenated blood is still considered the best treatment available for the prevention and reduction of ischemia-induced myocardial damage (Braunwald and Kloner 1985).

Although the long-term benefits of reperfusion appear obvious, it is not without its own hazards. In recent years, experimental and clinical evidence has determined that the reperfusion of previously ischemic myocardium may lead to accelerated cellular damage (Ferrari et al. 1985, Hearse et al. 1977, Jennings et al. 1960, Jolly et al. 1984, Yano et al. 1987). In 1960, Jennings et al. (1960) described in detail the ultrastructural

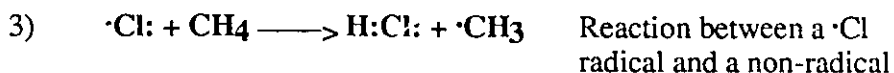
damage associated with coronary reperfusion, including the formation of contracture bands and a separation of the glycocalyx from the basement membrane, and were the first to suggest that reperfusion of previously ischemic myocardium may actually hasten the necrotic process. A major breakthrough occurred in 1973 when Hearse showed that the perfusion of ischemic hearts with oxygenated buffers induced an immediate release of cellular enzymes in combination with ultrastructural changes in the tissue while the perfusion with anoxic buffers did not (Hearse et al. 1973). These results indicated that the damage accrued during the reperfusion phase is not due to the washout of some protective agent, or due to the physical presence of the perfusate itself, but is instead related to the reintroduction of molecular oxygen to the ischemic tissue (Hearse 1977). More specifically, the damage associated with this so-called 'oxygen paradox' (Hearse et al. 1978) has been linked to the endogeneous generation of highly reactive oxygen-free radicals (OFR) (Bolli 1988, Freeman and Crapo 1982, Hess and Manson 1984, Kim and Akera 1987, McCord 1984).

By definition, a 'free radical' is a molecule with a highly reactive unpaired electron (Morrison and Boyd 1974). Should two free radical species encounter and react, their unpaired electrons will share a single molecular orbital, forming a covalent bond, and the two free radicals will be eliminated, as shown in the reaction 2):

UV radiation

- | | | |
|----|--|--|
| 1) | $\text{:Cl:Cl:} \longrightarrow \cdot\text{Cl:} + \cdot\text{Cl:}$ | Formation of two $\cdot\text{Cl}$ radicals |
| 2) | $\cdot\text{Cl:} + \cdot\text{Cl:} \longrightarrow \text{:Cl:Cl:}$ | Reaction between two $\cdot\text{Cl}$ radicals |

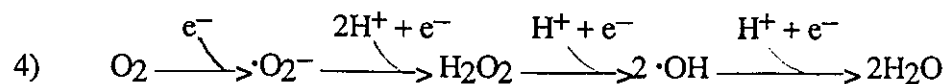
Conversely, if a free radical reacts with a non-radical species, as shown in 3), another free radical will result.



In this manner, free radicals are capable of initiating a potentially endless chain of reactions (Morrison and Boyd 1974) involving perhaps thousands of events, before being eliminated. It is this self-perpetuating property of radicals which make them extremely toxic to living tissue.

IN VIVO SOURCES OF OFR

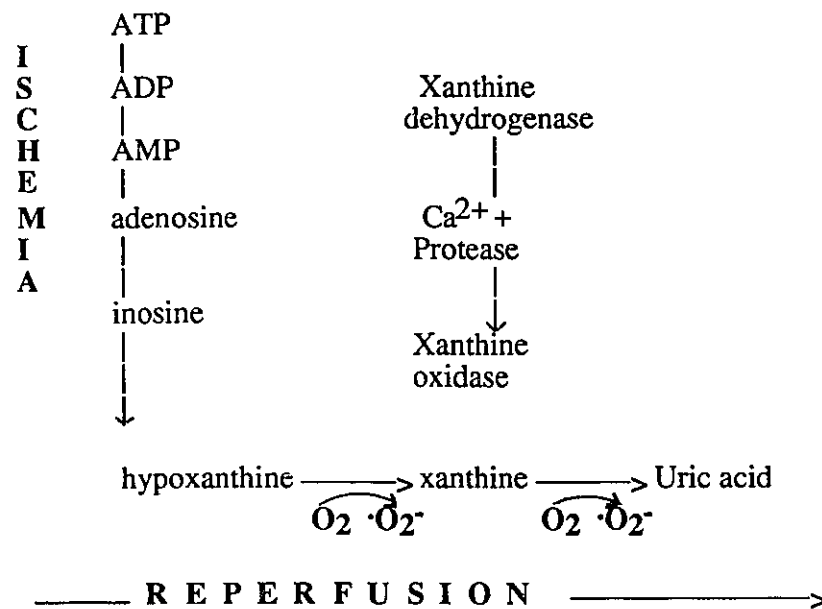
In principle, molecular oxygen (O_2) is a paramagnetic biradical containing two unpaired electrons with parallel electron spins (Hammond et al. 1985), yet because of its unusual electron configuration (Weiss 1986), O_2 is a relatively unreactive compound. However O_2 is capable of being reduced to form highly reactive free radical species. The reduction of O_2 to $2\text{H}_2\text{O}$ requires the addition of four electrons and can proceed by several pathways in living tissues. The mitochondrial enzyme cytochrome oxidase is capable of reducing O_2 to $2\text{H}_2\text{O}$ by tetravalent reduction without the production of reactive intermediate species, and this pathway accounts for greater than 95% of the oxygen consumption by tissues (Fridovich 1978). The remaining 5% proceeds by a univalent pathway first described by Haber and Weiss (1934) in which several highly reactive free-radical intermediates were produced, including superoxide anion radical ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot\text{OH}$) as shown in reaction 4):



The biological significance of these reactions was not fully appreciated until 1969, when McCord and Fridovich (1969) reported that the endogeneous enzyme erythrocuprein was involved in the conversion of $\cdot\text{O}_2^-$ radical to H_2O_2 . The discovery of

this enzyme, now referred to as superoxide dismutase (SOD), has led researchers to the realization that reactive oxygen intermediates are produced in normal, living tissue under physiological and pathophysiological conditions, and opened a whole new field of biomedical research into OFR-induced injury.

At least two major enzyme systems in the body are capable of catalyzing the generation of $\cdot\text{O}_2^-$ from O_2 , including: a) the xanthine oxidase system found in virtually all capillary endothelial cells, and b) the nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase system found primarily in phagocytic cells (Weiss 1986). The proposed chain of events that lead to the formation of $\cdot\text{O}_2^-$ via the xanthine oxidase pathway during ischemia-reperfusion (McCord 1985) is summarized below:



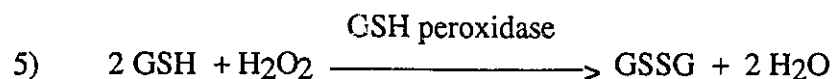
During a prolonged period of ischemia (ie greater than 40 min) (Weiss 1986), the decrease in O_2 available for oxidative phosphorylation leads to the depletion of high energy phosphate production within the cells. The energy available for contraction or maintenance of ionic gradients becomes limited and intracellular $[\text{Ca}^{2+}]_i$ increases. This rise in $[\text{Ca}^{2+}]_i$ activates the proteases required for converting xanthine dehydrogenase to

xanthine oxidase. The degradation of ATP and ADP results in the accumulation of AMP, which is further catabolized to adenosine, inosine and finally hypoxanthine (Van Bilsen et al. 1989). Upon reperfusion, the reintroduction of molecular oxygen initiates the xanthine oxidase catalyzed conversion of hypoxanthine to xanthine and uric acid, with the simultaneous conversion of O_2 to $\cdot O_2^-$. The production of $\cdot O_2^-$ following reperfusion of ischemic myocardium has been recently confirmed by Zweier et al. (1987) using the electron paramagnetic resonance technique. $\cdot O_2^-$ production was shown to peak within 10 seconds of reperfusion, and to decline rapidly thereafter.

CELLULAR DEFENCES AGAINST OFR

Highly reactive oxygen-free radicals such as $\cdot OH$ and $\cdot O_2^-$ are far too toxic to be tolerated within the living system (Fridovich 1978). Therefore, those organisms living in oxygen rich environments have evolved a number of antioxidant defence systems, including both low molecular weight free-radical scavengers and enzymatic defence systems.

The thiol-containing tripeptide glutathione (GSH) is present in concentrations of up to 1.2 μ moles/gram of tissue in the myocardium (Ishikawa and Sies 1984), and can be found in many body fluids as well (Farooqui et al. 1987, Forman et al. 1988, Reed and Farris 1984, Ross 1988). GSH can act as a scavenger for OFR, including $\cdot O_2^-$ and $\cdot OH$, and in the presence of selenium-dependent GSH peroxidase (Forman et al. 1988) can reduce H_2O_2 and other peroxides directly to H_2O with the formation of glutathione disulfide (GSSG) as depicted in reaction 5 below (Ross 1988).



In this manner GSH protects cellular proteins against sulfhydryl oxidation, including the highly sensitive Ca^{2+} -ATPase systems of the SR and sarcolemma (Forman et al. 1988, Grover and Samson 1989). However, as GSSG is considered toxic to the cell (Ishikawa and Sies 1984), the ratio between [GSH] and [GSSG] is kept high through the actions of a NADPH-dependent GSH reductase which converts GSSG to GSH (McCoy et al. 1988). Other endogenous OFR scavengers include α -tocopherol (Pascoe and Reed 1989, Timmenstein and Reed 1989), ascorbate, β -carotene (Burton and Ingold 1984), and uric acid (Becker et al. 1989).

The endogenous enzymatic defence systems include SOD, which dismutates $\cdot\text{O}_2^-$ to H_2O_2 (McCord and Fridovich 1969, Mehta et al. 1989, Naslund et al. 1986), catalase, which converts H_2O_2 to H_2O (Jolly et al. 1984, Lee et al. 1987, Thayer 1986) as well as the GSH peroxidase and reductase described above. Several excellent reviews on cellular defences against OFR are available (Cotgreave et al. 1988, Flaherty and Weisfridt 1988, Freeman and Crapo 1982).

OFR such as $\cdot\text{O}_2^-$ and $\cdot\text{OH}$ have half-lives in the nanosecond to millisecond range (Pryor 1986). While the involvement of OFR in the acute, short-term injury that develops immediately upon reperfusion of the ischemic myocardium is generally accepted, (Aoki et al. 1988, Bajaj et al. 1989, Jolly et al. 1984, Naslund et al. 1986, Werns et al. 1986, Ytrehus et al. 1987), the role of endogenous OFR production in the long termed damage to the myocardium has been seriously questioned (Gallagher et al. 1986, Miura et al. 1988, Richard et al. 1988). The so called "wavefront" of cellular necrosis as described by Reimer and Jennings (Reimer and Jennings 1979) is initiated up to 6 hours after the onset of reperfusion, and may persist for up to 48 hours. This wave of tissue injury coinciding with the influx of neutrophils into the damaged region (Chatelain et al. 1987, Engler et al. 1986b, Smith et al. 1988) and not with endogenous OFR generation. Therefore, the physiological significance of OFR production in the hours

following the onset of reperfusion injury was expanded in 1973 by the discovery by Babior and co-workers that activated neutrophils also produce OFR (Babior et al. 1973).

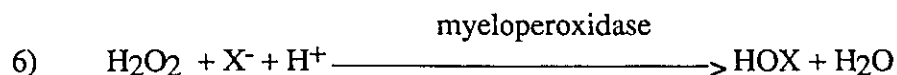
PMN INVOLVEMENT IN MYOCARDIAL DAMAGE

Circulating polymorphonuclear leukocytes (PMN) or neutrophils play an important role in primary host defence response by binding to, ingesting, and destroying pathogenic microorganisms (Weiss et al. 1983) or by removing damaged tissue to allow for the initiation of repair mechanisms. The initial damage caused by the reperfusion of a previously ischemic region of the heart often leads to the accumulation of PMN within the tissue (Chatelain et al. 1987, Loewe et al. 1988), and this accumulation coincides temporally with the initiation of long-term cellular damage (Smith et al. 1988). Mullane and co-workers reported that within 15 minutes of reperfusion, the number of PMN in the injured region can increase by up to 25% (Mullane 1988) often leading to capillary plugging, or the 'no-reflow' phenomena described by Engler et al (1983). Once in the injured area, the PMN will marginate and undergo diapedesis through the capillary wall, entering into the underlying interstitium (Malech 1988). Within 1 hour there is evidence of extravascular PMN accumulation (Mullane et al. 1984, Mullane 1988), and this accumulation may continue for up to 48 hours. Once in the extravascular compartment, the PMN attach onto damaged cells where they may undergo a respiratory burst, consuming large amounts of O₂, and releasing large concentrations of proteases, OFR or related oxidant species onto the surface membrane of these cells (see Figure 1) (Klebanoff 1988, Vissers et al. 1985). Unfortunately, PMN may also attach to and secrete OFR onto surrounding cells which were not previously damaged, thereby leading to the destruction of otherwise healthy tissue.

Several studies (Engler and Covell 1987, Flynn et al. 1984, Korthuis et al. 1988, Kraemer and Mullane 1989, Mullane et al. 1984, Romson et al. 1983, Smith et

al. 1988) have linked PMN oxidant generation with the development of post-reperfusion injury. Engler and co-workers (Barroso-Aranda et al. 1988, Engler et al. 1986a, Engler et al. 1986b) have shown that depleting circulating PMN in dogs results in reduced infarct size and diminished cellular damage in the reperfused myocardium. Specific antibodies which prevent adhesion of circulating PMN to the vascular walls (Romson et al. 1983, Simpson et al. 1988), or drugs which prevent PMN accumulation and activation, including ibuprofen (Flynn et al. 1984, Romson et al. 1982) and nafazatrom (Bednar et al. 1985, Mullane et al. 1984) are also known to reduce infarct size in dogs. Finally, free radical scavengers, including SOD alone (Werns et al. 1985), SOD with catalase (Jolly et al. 1984), or allopurinol, a xanthine oxidase inhibitor, (Godin et al. 1989, Miura et al. 1988) have also provided protection against PMN-induced cellular damage. Taken together, these studies support the hypothesis that a major portion of the damage that is observed following reperfusion of the ischemic myocardium is related to the generation and production of OFR and related oxidant species by activated PMN. Few studies (O'Neill et al. 1989) have failed to show any relationship between PMN and myocardial damage following reperfusion.

As depicted in Figure 1, PMN are also capable of producing a unique class of oxygen metabolites, the hypohalous acids (Klebanoff 1982, Sepe and Clark 1985, Weiss et al. 1983). Granules found within circulating PMN contain high concentrations of the enzyme myeloperoxidase (MPO) which is released upon activation of the PMN during an acute inflammatory response (Halliwell et al. 1987, Mullane et al. 1985, Thomas 1979). This enzyme catalyses the reaction between H_2O_2 and halides (ie chloride or bromide) to form their corresponding hypohalous acid (ie hypochlorous acid or hypobromous acid) as shown in reaction 6) below:

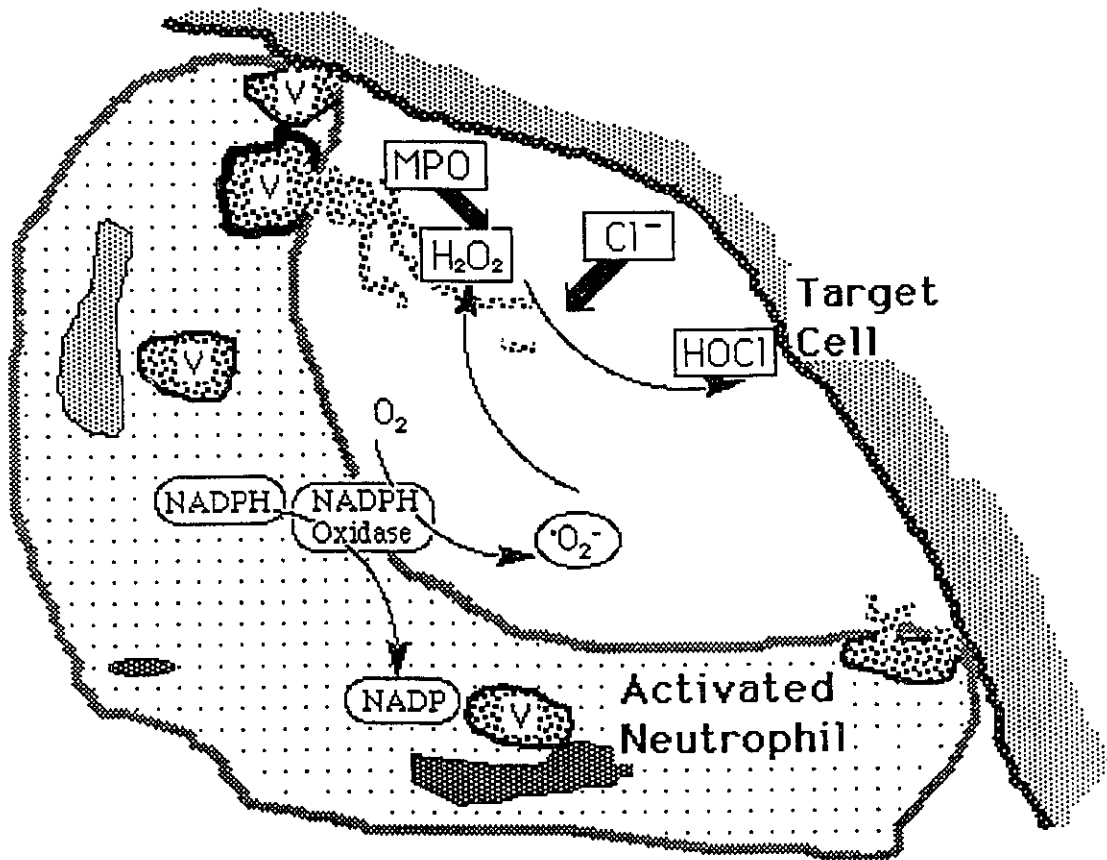


where X^- is the halide species. As chloride is the most abundant halide *in vivo* (Weiss 1989), the most likely hypohalous acid formed via the MPO- H_2O_2 -halide system would be hypochlorous acid (HOCl) (Klebanoff 1988). While HOCl does not have an unpaired electron and hence is technically not an oxygen free radical species, it is often included with the other OFR due to its link with OFR generation in PMN, as well as its high degree of biological reactivity.

HOCl is considered to be the most reactive of the oxidant species generated by PMN (Klebanoff 1988, Winterbourne 1985). Kalyanaraman and Sohnle (1985) showed that 5×10^6 neutrophils per ml of buffer could produce up to 88 μM HOCl when stimulated with phorbol myristate acetate (PMA) *in vitro*. In an important study Vissers et al. (1985) showed that PMN do not release their lysosomal contents randomly into the circulation, but direct their release towards the sites of attachment or into the phagocytotic cleft formed between the PMN and the target cell (see Figure 1). It would follow that the localized concentration of HOCl directly at the site of release could conceivably be much greater than this reported 88 μM . With a pK of 7.53 (Klebanoff 1988), substantial concentrations of HOCl would exist in the nonionized form at normal physiological pH. Hence, under physiological conditions, HOCl would be membrane permeable and able to exert its effects against a variety of cytosolic targets (Weiss 1989) including sulfur containing amino acids, sulfhydryl compounds, thioethers, aromatics, and other unsaturated carbon groups (Albrich et al. 1981, Barrette et al. 1987, Weiss and LoBuglio 1982). Due to its high reactivity, HOCl may not accumulate within the tissue, but may instead react with both primary or secondary amines to form a less

Figure 1 A proposed mechanism for the production of hypochlorous acid (HOCl) by an activated neutrophil. Superoxide radical produced by the NADPH-NADPH Oxidase system is converted into H_2O_2 by SOD. The release of myeloperoxidase (MPO) from cellular vesicles (V) into the cleft between the neutrophil and the target cell will catalyse the conversion of H_2O_2 plus chloride anion (Cl^-) into HOCl. At physiological pH, approximately 50% of the HOCl (pK_a 7.5) will be in the non-dissociated form and hence can cross cellular membranes. The size of the cleft is exaggerated for clarity.

Figure 1



reactive but long-lived oxidant species known as chloramines (Grisham et al. 1984, Test et al. 1984).

OFR AND MYOCARDIAL CONTRACTILE DYSFUNCTION

OFR generation is known to have a negative inotropic effect on the myocardium, leading to the development of contracture. Katz and Tada (1972) described the development of contracture in the ischemic region of a dog heart. This was further explored by Hearse (1977) who showed a similar effect (stone heart) during reperfusion of globally ischemic rat hearts. Hayashi and co-workers (1987) reported that during reintroduction of oxygen to previously hypoxic guinea pig papillary muscles, the muscles developed spontaneous automaticity, aftercontractions, and loss of contractile function. From these studies it is not clear whether this negative inotropic response was due to a loss of high energy phosphates during the ischemic or hypoxic period, or specifically to OFR generation. To examine the effects of OFR upon the myocardial function directly, Blaustein et al. (1986) used an exogenous xanthine-xanthine oxidase system to generate $\cdot\text{O}_2^-$ and $\cdot\text{OH}$, and reported a dramatic decline in active tension development concomitant with a rise of resting tension in isolated rat papillary muscles over a 90 min protocol period. Similarly, Pallandi et al. (1987) showed significant depolarization and decreased action potential amplitude in ventricular strips from the guinea-pig in combination with an increase in arrhythmic activity and contracture during exposure to the xanthine-oxidase free radical generating system. These studies suggested that OFR generation causes damage to the sarcolemmal membrane due to lipid peroxidation, leading to an influx of extracellular Ca^{2+} and the disruption of intracellular Ca^{2+} homeostasis in the isolated myocardium.

In a series of *in vivo* experiments designed to examine the role of PMN-derived OFR generation on the canine myocardium, Rowe and co-workers demonstrated that

PMA-activated human leukocytes induced a significant depression of myocardial contractile function (Rowe et al. 1984, Rowe et al. 1983). Within 30 min following the administration of the PMA they showed that H_2O_2 entered the extracellular space, and that cardiac output, dP/dt , and mean arterial pressure had declined while left ventricular end diastolic pressure had increased by greater than 100%. These effects were inhibited by pretreatment with SOD and catalase, or by neutrophil depletion (Kraemer and Mullane 1989, Rowe et al. 1984). These studies taken in concert further implicate the PMN-derived OFR or related oxidant species in the development of contracture in the myocardium. It was still unclear which of the OFR or oxidants were initiating this contracture

While the data relating the development of contracture to OFR generation or PMN infiltration is extensive, there is a paucity of data concerning the mechanical response of myocardial tissue to specific oxidants. H_2O_2 was once utilized as a source of molecular O_2 during cardiac surgery (Urschel et al. 1966), even though Sellers et al (1962) reported cardiac arrest in a patient in which the myocardium was exposed to 3% H_2O_2 as an antiseptic agent. Olson and Boerth (1978) reported that rabbit hearts perfused with H_2O_2 expressed longer survival time and maintained blood pressure following occlusion of the left main coronary artery *in vivo*, but concluded that the positive inotropic effects of H_2O_2 were due to a direct effect of the oxidant on the excitation-contraction coupling mechanisms, and not to an increase in arterial pO_2 as had been previously proposed by Urschel (1966). Forester et al. (1981) exposed strips of human atrial tissue and rat papillary muscles to H_2O_2 , and reported the development of a concentration dependent 'irreversible' contracture, in contrast to Fliss et al. (1988) who reported a spontaneous recovery from contracture after 40 min of H_2O_2 exposure. More recently, Hayashi et al (1989a) related the arrhythmias and contracture observed in

guinea-pig papillary muscles exposed to 1 mM H₂O₂ to a rise in cytosolic [Ca²⁺]. The involvement of cytosolic [Ca²⁺] in OFR-induced injury will be discussed below.

To date, few direct data are available to elucidate the effects of HOCl on the mechanical function of the myocardium. Sellers and co-workers (1962) injected 0.5% (final concentration 12 mM) Clorpactin WCS 90, an organic derivative of HOCl, into the pericardium of anesthetized dogs and recorded changes in the electrical activity of the heart, as well the development of contractile dysfunction that was considered indicative of oxidative damage to the myocardium. An initial set of studies from our lab showed that rat papillary muscles exposed to 300 µM HOCl developed severe contracture within 30 min that was not reversed upon washout (Eley et al. 1989b). These results, as well as similar results obtained using coronary perfused rat hearts, will be discussed in the following chapters.

OFR AND CYTOSOLIC [Ca²⁺] OVERLOAD

The inability of the myocardium to relax (contracture) is generally associated with a failure of the myocytes to remove cytosolic calcium, leading to the development of a cytosolic Ca²⁺ overload (Daly et al. 1987, Hearse et al. 1977, Lambert et al. 1986). An elevation of intracellular free [Ca²⁺] ([Ca²⁺]_i) has been reported repeatedly as a component of ischemia-reperfusion injury or OFR-induced injury in the myocardium (Buja et al. 1988, Cheung et al. 1986a, Elz and Nayler 1988a, Ito et al. 1987, Kloner 1988, Lambert et al. 1986, Nayler 1987, Otani et al. 1989) and other tissues (Bellomo and Orrenius 1985, Hayashi et al. 1989b, Malis and Bonventre 1986, Nicotera et al. 1988). However the underlying mechanisms responsible for this elevation are still not clear.

Biomembranes are highly sensitive to lipid peroxidation by OFR and related oxidants (Cheung et al. 1986a). Several studies have postulated that this increase in

cytosolic $[Ca^{2+}]_i$ was attributed to increased permeability of the sarcolemmal membrane following lipid peroxidation and subsequent membrane disruption (Chien et al. 1978, Meerson et al. 1982, Sepe and Clark 1985). The removal of Ca^{2+} from the perfusate at the time of reperfusion of previously ischemic hearts can, under certain circumstances, improve the recovery of mechanical function while reducing the development of Ca^{2+} overloading (Bing et al. 1985, Shine and Douglas 1983, Tosaki and Hearse 1987), suggesting that the influx of Ca^{2+} across the sarcolemma is intimately involved. However there is convincing evidence that extracellular Ca^{2+} is essential for the maintenance of membrane integrity (Fariss et al. 1984, Smith et al. 1981), and that the absence of extracellular Ca^{2+} may actually potentiate the onset of membrane damage during reperfusion (Cheung et al. 1986a, Zimmerman et al. 1967). Recent studies (Marban et al. 1989, Steenbergen et al. 1987) have shown that reperfusion with Ca^{2+} -containing perfusates after 20 min of ischemia did not result in a prolonged elevation of $[Ca^{2+}]_i$. Crake and Poole-Wilson also showed (1986) that the rise in cytosolic Ca^{2+} occurred independently of membrane disruption, and Belluk et al. (1988) report no evidence linking the development of contracture with lipid peroxidation in the myocardium. Therefore, while lipid damage may play an important role in the etiology of myocardial damage, the contracture and increase in $[Ca^{2+}]_i$ following OFR exposure may not necessarily be due to a disruption of the sarcolemmal membrane.

Even in the absence of lipid peroxidation, an increase in transsarcolemmal Ca^{2+} influx via the Ca^{2+} channels could still account for a rise in cytosolic Ca^{2+} following OFR mediated injury to the myocardium. Several Ca^{2+} channel antagonists have been shown to reduce the damage to hearts under conditions of experimental ischemia and reperfusion. Henry and co-workers (1978) reported that 1 μ M nifedipine, a dihydropyridine Ca^{2+} channel antagonist was effective in reducing ischemic injury in the canine heart by reducing vascular tone and increasing collateral blood flow to the

infarcting region. Since that time, a multitude of papers (Cheung et al. 1984, Kloner and Braunwald 1987, Pearle 1988, Przyklenk et al. 1989, Watts et al. 1986) have suggested that pharmacological intervention to reduce Ca^{2+} influx may attenuate the injury associated with reperfusion of the ischemic myocardium. Fleckenstein et al (1985) reported that Ca^{2+} channel blockade was effective in reducing myocardial oxygen demand and contractility, thereby preserving the energy demands during ischemia, while also preventing excessive Ca^{2+} influx during reperfusion. As promising as these trials appear, two recent review articles (Kloner and Braunwald 1987, Nayler 1987) indicate that most clinical trials fail to show any reduction in tissue injury in man, unless the Ca^{2+} antagonists are administered prior to the onset of the ischemic period. Therefore, the usefulness of Ca^{2+} channel blockade in reducing myocardial injury in man still requires further investigation

Attention is now being directed towards oxidation damage to the protein components of the cells as possible sites for OFR damage. The generation of OFR, or exposure to H_2O_2 or HOCl , has been shown repeatedly to inactivate a multitude of cellular proteins, including the sarcolemmal Ca^{2+} pump (Kaneko et al. 1989), the Na/Ca exchanger (Antolini et al. 1989), the Na^+/K^+ -ATPase (Dixon et al. 1987, Kako et al. 1988, Kim and Akera 1987), and Ca^{2+} -ATPase of the sarcoplasmic reticulum (SR) (Kukreja et al. 1989, Manson and Hess 1983, Okabe et al. 1988, Scherer and Deamer 1986c) through the oxidation of specific sulfhydryl residues. The SR Ca^{2+} -ATPase, through its ability to transport Ca^{2+} into the SR, is the major ion transport system involved in the relaxation of the myofibril complex, and in maintaining cellular Ca^{2+} homeostasis in the rat myocardium (Carafoli 1985). One could speculate that *in vivo* exposure to OFR or PMN-derived oxidants such as HOCl , could inactivate the Ca^{2+} -ATPase of the sarcolemmal and SR in cardiac myocytes, thereby impairing the removal

of Ca^{2+} from the cytosol, leading to the cytosolic Ca^{2+} overload that is often observed in the damaged myocardium (Elz and Nayler 1988b, Krause and Hess 1984).

OFR, PROTEIN THIOLS AND PROTEIN FUNCTION

The alteration of protein thiol redox status has been postulated as a physiological mechanism for the regulation of various proteins, including the activation of the Ca^{2+} release channel of the SR (Trimm et al. 1986, Zaidi et al. 1989) or the inactivation of specific cellular enzymes (Mannervik 1986, Oliver 1987, Stroncek et al. 1986). In principle, any enzyme containing an accessible cysteine residue that is essential for the activity of that enzyme is susceptible to a decrease (or increase) in activity following the formation of protein-mixed disulfides or intramolecular disulfides (Liu 1977, Matsumura and Matthews 1989). However, the evidence for changes in the redox state of cellular proteins in response to physiological stimuli is very limited (Ziegler 1985). Of perhaps greater interest is the significance of changes in thiol redox state under pathophysiological conditions such as reperfusion injury in the myocardium.

Of the functional groups present in proteins, the sulfhydryl group is usually considered as the one most reactive towards nucleophilic agents (Liu 1977) including oxidants. OFR, especially HOCl, are known to attack a wide range of biologically relevant molecules, including sulfur-containing amino acids and thioethers (Weiss 1989). Fliss and co-workers reported the oxidation of methionine residues in proteins (Fliss et al. 1983, Fliss 1988), while previous reports from our laboratory (Eley et al, 1989) reported extensive loss of protein thiols within the myocardium following HOCl exposure. The high reactivity of HOCl with sulfur-containing amino acids (Albrich et al. 1981, Fliss 1988, Thomas 1979) as well as its capacity to penetrate membranes (Grisham et al. 1984, Test et al. 1984) suggest that this oxidant has the capacity to reach and inactivate many cellular proteins.

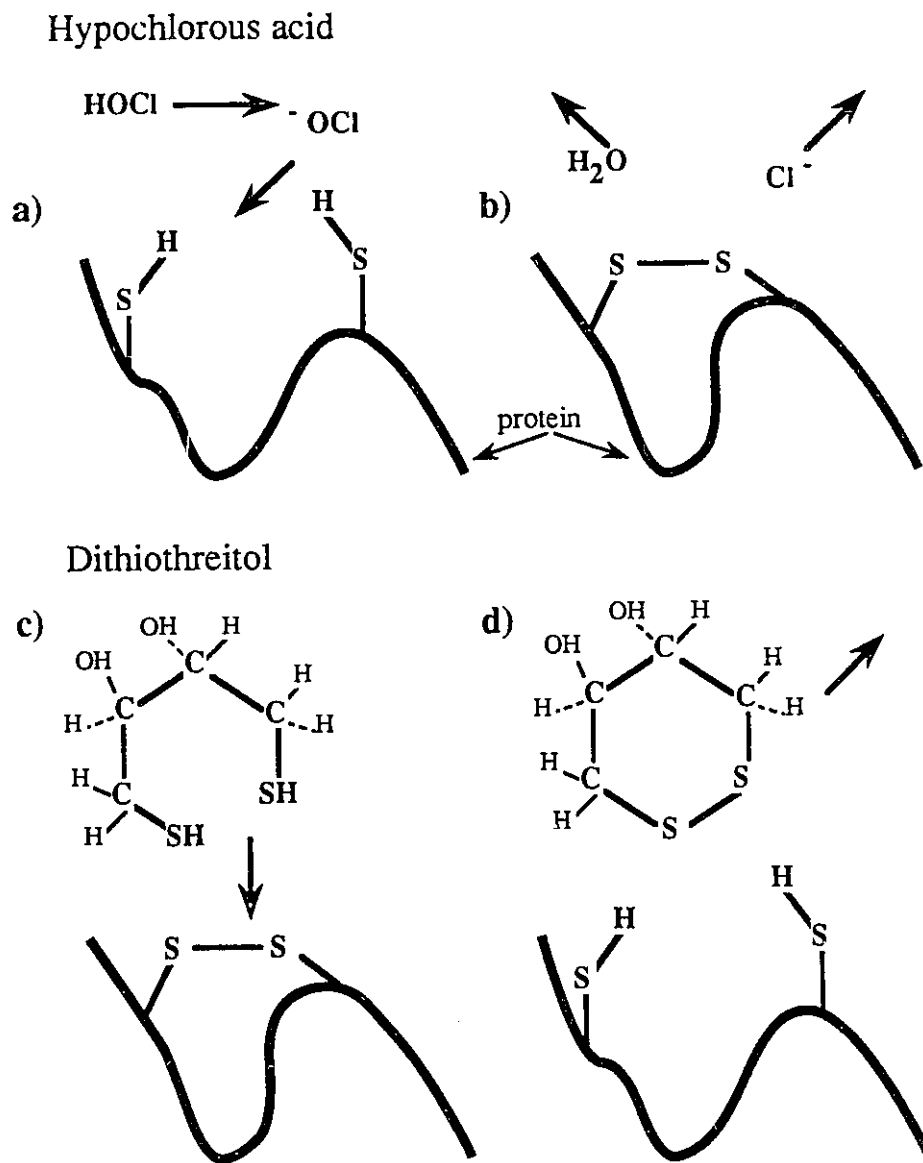
Protein oxidation may increase following the depletion of glutathione (Reed and Farris 1984, Schraufstatter et al. 1985). Previous studies have shown that the depletion of cellular glutathione (GSH) increases susceptibility to OFR injury because of lowered antioxidant capacity (Mirabelli et al. 1988), and oxidants including HOCl and its chloramine derivatives are known to oxidize cellular GSH to glutathione-disulfide (GSSG) (Reed and Farris 1984). The ability of the myocardium to remove oxidized intracellular GSSG is limited (Ishikawa and Sies 1984), and since reduced GSH is important in maintaining the redox state of cellular cysteine residues, the accumulation of GSSG or the diminution of GSH may decrease the ability of the myocytes to resist protein oxidation (Lesnefsky et al. 1989).

DTT, SULFHYDRYL REDUCTION AND PROTEIN FUNCTION

Preliminary work from our lab (Eley et al. 1989b) showed that the mechanical dysfunction and depletion of cellular thiols following HOCl exposure in the rat myocardium was reversed by the addition of dithiothreitol (DTT). DTT is a potent disulfide reducing agent first described by Cleland (Cleland 1964) that is capable of maintaining monothiols, including cysteine, in the reduced state. As shown in Figure 2, DTT consists of a four-carbon carbohydrate skeleton with a single reduced thiol at either end. The reaction between DTT and a disulfide involves a one-step reduction of the disulfide bond, alleviating the necessity for two reducing molecules simultaneously. Previous studies (Lauf 1988, Lee and Hahn 1988) have also shown that DTT can selectively reduce disulfides in cytosolic proteins without first replenishing cellular glutathione levels.

Figure 2 Possible thiol oxidation reaction in the presence of HOCl, showing cross-linking of two cysteine residues leading to a conformational change in the protein structure. The cleavage of the disulfide bond with DTT restores the original protein conformation.

Figure 2



Because of its ability to protect protein thiols from oxidation and its membrane permeability (Depape-Brigger et al. 1977, Klonne and Johnson 1983) DTT has been proposed as a protective agent against oxidative damage in a variety of tissues. Orrenhius and co-workers using isolated hepatocytes have shown repeatedly that the Ca^{2+} overload and resulting cell death associated with menadione or acetaminophen toxicity (Di Monte et al. 1984, Moore et al. 1985), or to the oxidative stress associated with t-butyl hydroperoxide (Jones et al. 1983), are linked directly to protein thiol oxidation. This tissue injury was prevented by the administration of GSH or DTT (Jones et al. 1983, Mirabelli et al. 1988, Nicotera et al. 1988). Blaustein et al (1989) as well as Singh et al (1989) have both reported a strong correlation between myocardial GSH administration and recovery following reperfusion of ischemic tissue. Mandel et al (1990) showed a similar protection of cellular thiols against oxidative damage in the kidney. Of great interest is a study by Nicotera and co-workers (1985) showing that DTT could effectively restore Ca^{2+} ATPase activity in hepatocytes following the oxidation of protein thiols by menadione. Hebbel (1986) also showed a partial recovery of function with DTT in OFR-inactivated Ca^{2+} -ATPase isolated from human erythrocytes. Zaidi and co-workers have shown that disulfide formation will increase the release of Ca^{2+} from the SR via the Ca^{2+} release channels, and that normal activity is restored following the addition of DTT (Zaidi et al. 1989). Therefore, it appears that DTT is not only able to offer protection against thiol oxidation, but is also capable of reversing disulfide formation in cellular enzymes, thereby restoring activity to otherwise inactivated enzyme systems.

In 1978, Mushlin and co-workers performed a significant series of experiments designed to examine the effects of DTT on the cardiac SR Ca^{2+} -ATPase activity *in vivo*. In spontaneously hypertensive rats (SHR), which are known to have impaired vascular relaxation and elevated blood pressures, the injection of DTT induced a significant reduction of relaxation time and contractile activity in aortic strips, in combination with

an increased Ca^{2+} uptake into isolated SR vesicles as compared to untreated SHR rats, leading to a reduction of blood pressure in the intact animal. The addition of DTT did not alter Ca^{2+} uptake in SR preparations from normal skeletal or cardiac muscle (Sreter et al. 1970). Mushlin et al hypothesized (1978) that the the SR Ca^{2+} -ATPase was impaired in these SHR animals, leading to a depressed relaxation response in the vasculature, and that this impairment was related to a loss to reduced thiols that was reversible by DTT. Further studies which have examined the importance of reduced thiols in the uptake and handling of Ca^{2+} by the SR will be discussed in Chapter 3.

SUMMARY

To summarize, the infiltration of PMN into the myocardium following reperfusion of previously ischemic regions has been correlated with the development of membrane disruption, protein inactivation, cytosolic Ca^{2+} overloading, mechanical failure and eventual cell death. HOCl is considered the most powerful oxidant species produced by neutrophils in concentrations approaching 10^{-4}M . To date, the effects of HOCl at these concentrations on the contractile function of the myocardium are virtually unknown.

With a pKa of 7.53, HOCl can cross membranes, allowing it entry to the cell where it can exert its effects against a multitude of targets, including cellular proteins. Sulfur containing amino acids are extremely sensitive to oxidation by HOCl. Many cellular proteins, including those linked to transmembrane ionic movement and regulation, are dependent upon the presence of reduced thiols, and may become inactivated following the oxidation of as few as one cysteine residue. DTT, a disulfide reducing agent, has been shown to restore activity to a variety of enzyme systems *in vitro*, including those involved in maintaining ionic equilibrium within the myocardium. The ability of DTT to protect reduced thiols against oxidation as well as its ability reduce

disulfide bonds in proteins, suggests that this disulfide reducing agent may be an effective agent for restoring the activity of cellular proteins following their inactivation due to thiol oxidation. The possibility of DTT being able to restore contractile function to the myocardium following exposure to HOCl has not yet been fully explored.

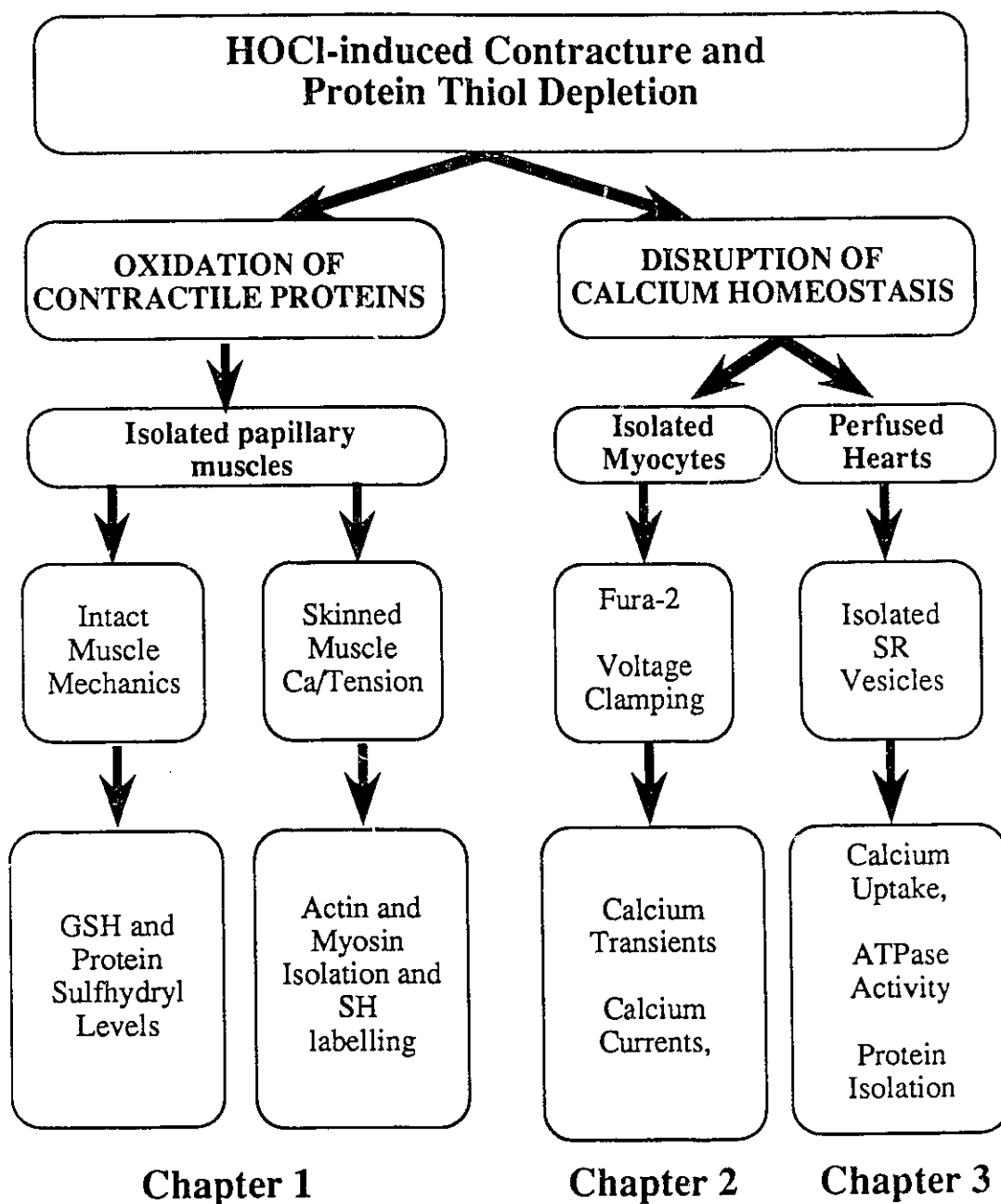
STATEMENT OF THE PROBLEM

The main objective of this project was to examine the effects of HOCl on the contractile performance of the mammalian myocardium. Preliminary results (Eley et al. 1989b) from our laboratory showed that HOCl induced the development of contracture in isolated papillary muscles in a manner symptomatically consistent with a cytosolic Ca^{2+} overload. This mechanical response correlated with a depletion of protein thiols, and subsequent exposure to DTT induced a significant recovery of both protein thiols and contractile function. It is therefore the purpose of this thesis to identify the possible mechanisms associated with this loss and recovery of function.

This thesis is divided into three chapters designed to address these questions (Figure 3). Chapter 1 will examine the effect of HOCl and DTT on the mechanical function of the myocardium. The isometrically contracting left-ventricular papillary muscle from the rat was used as a model for myocardial contractility throughout this chapter, with the inclusion of the chemically skinned papillary muscle preparation to examine more closely the effects of HOCl and DTT on the contractile function of the myofibrils directly. The involvement of thiol oxidation in total cellular proteins, as well as in the individual contractile proteins, will be assessed in this chapter as well.

Figure 3 Flow chart depicting the research questions addressed in this thesis. The development of contracture and oxidation of protein thiols as observed in the isometric papillary muscle following exposure to HOCl may be due to a) a decline in the ability of actin and myosin to form or break cross-bridges; b) an increased sensitivity of the Troponin C to available Ca^{2+} ; c) an increased $[\text{Ca}^{2+}]_i$ due to an increase in membrane permeability to Ca^{2+} ; d) an impairment of intracellular Ca^{2+} regulatory proteins, leading to a breakdown of EC coupling mechanisms. These possibilities will be address using several models, including 1) coronary perfused rat heart for functional measurements; 2) isolated rat papillary muscles for examination of stimulation protocols and the effects of pharmacological agents on isometric contractions; 3) skinned papillary muscles for examining the pCa/Tension relationship; 4) isolated cardiac myocytes for direct assessment of inward Ca^{2+} currents and for fluorometric measurement of $[\text{Ca}^{2+}]_i$; 5) subcellular SR microsomes for the measurement of Ca^{2+} -ATPase activity and function; and 6) isolated proteins for the assessment of protein thiol oxidation. The results will be discussed in three chapters.

Figure 3



Chapter 2 examines the handling of Ca^{2+} in single cardiac myocytes following HOCl exposure. Isolated rat and rabbit myocytes were iontophoretically loaded with the Ca^{2+} indicator Fura-2, then voltage clamped, allowing for simultaneous measurement of diastolic $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} transients, and inward Ca^{2+} current during exposure to HOCl and DTT.

Chapter 3 examines the activity and function of the SR Ca^{2+} -ATPase. Isovolumically contracting rat hearts were perfused with HOCl with or without DTT, then SR microsomes were isolated from the ventricular tissue. The activity and function of the SR Ca^{2+} -ATPase were related to the level of SH oxidation in the Ca^{2+} -ATPase protein, and indirectly to the contractile performance in the whole heart.

The specific objectives and hypotheses tested for each chapter are stated in the appropriate introductory statements. However, throughout the thesis the specific questions addressed include:

- 1) What are the effects of HOCl on the contracting mammalian myocardium?
- 2) Do these effects relate to thiol oxidation? Are these effects reversible with DTT?
- 3) Does the observed mechanical dysfunction relate to an impairment of the myofibrils? Are myofibrillar thiols depleted following HOCl and repleted following DTT?
- 4) Does the observed mechanical dysfunction relate to a disruption of cellular Ca^{2+} homeostasis, and if so, at what level?

CHAPTER 1:

ISOMETRIC CONTRACTILE FUNCTION IN PAPILLARY MUSCLES DURING EXPOSURE TO HOCL AND DTT: THE INVOLVEMENT OF EXTRACELLULAR CALCIUM AND CELLULAR PROTEIN THIOLS

INTRODUCTION

The major aim of *in vitro* cardiac muscle mechanics studies is to explain, in some quantitative terms, the *in vivo* mechanical performance of the functioning, intact heart under normal or stress conditions. Although *in vivo* experimentation most closely represents the physiological norm, the disadvantage of *in vivo* modeling is the limited control that the researcher has over the environment within which the heart exists. A change in the performance of the heart due to an experimental intervention will be counteracted within the body by neural, hormonal, or intrinsic feedback mechanisms in an attempt to maintain cardiac output. As well, the influence from and interactions between adjacent heart chambers, the presence and integrity of the pericardium, the compliance of the aorta, the competence of the valves, resistance offered by the coronary arteries, and the delivery of sufficient oxygen and nutrients to the myocardial tissue will all influence ventricular contraction (Pinto 1987). In order to monitor directly the effects of specific experimental interventions upon the contractile performance of the myocardium, it is often necessary to separate the heart from its normal physiological environment.

The data obtained from *in vitro* whole working-heart preparations are still extremely difficult to interpret. Ideally the heart would act as a three-dimensional expansion of certain unidimensional physical laws (Pinto 1987). However, due to the complex architecture (Sonnenblick 1962) of the mammalian heart, including the multidirectional branching patterns of the cells, such a relationship does not normally exist. A reasonable alternative to whole heart mechanical measurements is to first characterize the unidimensional behavior of a bundle of cardiac muscle, then use this data to make deductions concerning the behavior of the intact heart.

PAPILLARY MUSCLES: A MODEL FOR MYOCARDIAL CONTRACTILITY

While strips of contractile tissue surgically removed from one of the ventricles are potentially available for these types of experiments, the trauma associated with the dissection of these strips as well as the complex geometry of the ventricular myocardium itself, makes them an unattractive model for studying unidimensional muscle mechanics (Pinto 1987). In comparison, the papillary muscle is superior to such strips due to its generally slender, uniform size, its relatively simple geometrical branching pattern (Sonnenblick 1962), and its ease of extraction from the heart. Unfortunately, even the papillary muscle has significant shortcomings as a model for myocardial contractile studies. Damaged regions adjacent to the excision may contribute to the parallel elastic component of the system while the damaged regions of the muscle adjacent to the clamped ends may contribute to the series elastic component (Pinto 1987, Reichel 1976), which may seriously alter contractile parameters. Since these muscles are not coronary perfused, the myocytes are dependent upon oxygen and nutrient diffusion through the tissue for its energy supply. The possibility of developing a hypoxic core in papillary muscles at 37° C (Loiselle 1985) has led researchers to use lower temperatures in an attempt to minimize the metabolic demands.

Despite these technical problems, the papillary muscle preparation has been a favoured model with which to examine the complex mechanical properties of the myocardium in response to various stimulation regimes (Abbott and Mommaerts 1959, Koch-Weser and Blinks 1963, Lee et al. 1970), drug effects (Lues et al. 1988, Saxon and Kobrinski 1988, Singal and Pierce 1986), and atrophy (Korecky et al. 1986) without the influences of changes in coronary perfusion or work load on the heart. The data resulting from studies using the isolated papillary muscle have successfully predicted the behavior of the intact myocardium under complex stress states.

EC COUPLING AND Ca^{2+} REGULATION IN THE RAT MYOCARDIUM

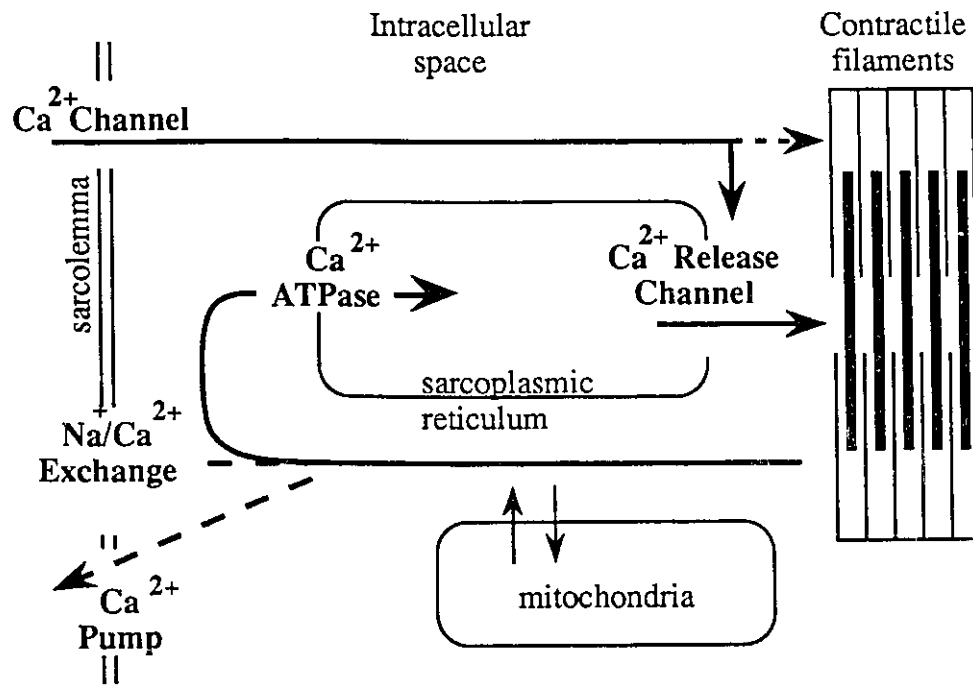
The relationship between excitation, $[\text{Ca}^{2+}]_i$, and contraction (EC coupling) in the myocardium is a complex one, and will be described in greater detail throughout this thesis. For the purpose of this present chapter, a brief discussion describing a current model of EC coupling is required.

As an action potential travels through the syncytium of the heart, the depolarization of the sarcolemmal membrane triggers a transient rise in cytosolic $[\text{Ca}^{2+}]_i$, from a diastolic value of $\approx 10^{-6}$ M to a systolic value of $\approx 10^{-5}$ M (Ruegg 1988, Vaughan-Jones 1986). This elevated $[\text{Ca}^{2+}]_i$ initiates the interaction between actin and myosin leading to contraction (Huxley 1957, Katz 1966, Winegrad 1979). Subsequent to the contraction, diastolic $[\text{Ca}^{2+}]_i$ is rapidly restored as Ca^{2+} is removed from the cell across the sarcolemmal (Carafoli 1989), or resequestered into the SR tubular network (Hasselbach 1979) allowing for relaxation of the muscle.

The regulation of Ca^{2+} during EC coupling in the myocardium can be modeled as two separate parallel pathways to and from the myofibrillar space (see Figure 1.1). The first pathway involves movement of Ca^{2+} through the voltage-sensitive Ca^{2+} channels as measured by the inward Ca^{2+} current upon depolarization (Hilgemann and Noble 1987,

Figure 1.1 A model of Ca^{2+} transport in mammalian myocardium. The arrows represent the pathways for Ca^{2+} movement during the excitation-contraction coupling cycle. Ca^{2+} influx via the Ca^{2+} channels during an action potential may either directly activate the contractile filaments, or trigger Ca^{2+} release from the SR stores. During the repolarizing phase of the action potential, the activator Ca^{2+} is sequestered by the SR or removed from the cell via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the Ca^{2+} pump of the sarcolemma to initiate relaxation. During steady state, Ca^{2+} influx into the cell equals Ca^{2+} efflux from the cell. Any increase in the transsarcolemmal influx may lead to greater Ca^{2+} sequestration by the SR, hence a greater releasable fraction for the next EC coupling cycle.

Figure 1.1



Reuter 1985, Trautwein and Pelzer 1985), and subsequent removal from the cell via the $\text{Na}^+/\text{Ca}^{2+}$ exchange (Blaustein 1989, Philipson and Ward 1986) and sarcolemmal Ca^{2+} pump (Carafoli 1988). The second pathway involves the release of Ca^{2+} from the terminal cisternae of the SR via the Ca^{2+} release channel (Lai et al. 1988a, Meissner 1975) into the myofibrillar space and subsequent resequestration into the SR via the Ca^{2+} -ATPase (Hasselbach 1979, Nayler et al. 1975a). While these two pathways are not physically separate, in the steady state the amount of Ca^{2+} which enters the cell during a single depolarizing event must also be removed during repolarization, while any Ca^{2+} released from the SR must be resequestered. Any change away from steady state will alter the relationship between these two pathways and hence the level of Ca^{2+} released during each contraction cycle, as will be discussed below. A third pathway involving the mitochondria has been proposed, but is too slow to play a significant role during the single contraction cycle (Carafoli 1985, Solaro et al. 1974).

In normal mammalian hearts, the majority of the Ca^{2+} which flows inward across the sarcolemmal membrane during an action potential does not contribute directly to the concomitant contraction (Morad and Goldman 1973), but all or a portion of this Ca^{2+} may be sequestered by the SR and stored until the subsequent release cycle. Similarly, a portion of the Ca^{2+} released from the SR may be lost to the extracellular space. Through this overlap between these two pathways, the movement of Ca^{2+} across the sarcolemma is able to regulate the fraction of activator Ca^{2+} on a beat to beat basis (Schouten 1986). The contribution of each of these pathways to the activator Ca^{2+} pool varies between species, with the rat being more dependent upon the SR than other mammalian species (Bers 1985, Shattock and Bers 1989, Sutko and Willerson 1980).

In the mammalian myocardium, the amount of Ca^{2+} which crosses into the cell from the extracellular space during a single action potential is insufficient to activate the myofilaments directly. The hypothesis of a Ca^{2+} induced Ca^{2+} release (CICR) as

proposed by Fabiato (1972) suggests that the increase of cytoplasmic free Ca^{2+} at the outer surface of the SR resulting from the transsarcolemmal Ca^{2+} influx induces a release of Ca^{2+} from the SR which in turn activates the myofilaments to contract. In mammalian skinned cardiac cells, the CICR can be triggered by a small localized increase in free $[\text{Ca}^{2+}]$ provided that the rate of increase in $[\text{Ca}^{2+}]$ is sufficiently high (Fabiato, 1983). Recently, a number of experiments have been done in intact cardiac cells to test the compatibility of the CICR hypothesis. Nabauer and Morad (1990), using caged Ca^{2+} compounds and flash-photolysis techniques, have shown that a localized release of Ca^{2+} in close proximity to the terminal regions of the SR will induce a much larger release of Ca^{2+} through the SR release channels. Both Cannell et al. (1987) and Callawaert et al. (1988) have shown a strong correlation between the inward Ca^{2+} current and initiation of the SR Ca^{2+} release as detected using fura-2 and indo-1 loaded rat cardiac myocytes, further supporting the CICR hypothesis. While other possible mechanisms have been postulated, the CICR has been generally accepted as the most promising explanation for the coupling link between membrane depolarization and SR Ca^{2+} release (for recent review see Feher and Fabiato, 1990). More specific aspects of CICR will be discussed within the following chapters of this thesis.

STIMULATION PROTOCOLS AND Ca^{2+} HANDLING

Stimulation protocols have been used in the past to examine the inotropic response of the myocardium under physiological and pathophysiological situations (Abbott and Mommaerts 1959, Forester and Mainwood 1974, Korecky et al. 1986). More recently the frequency-force and paired pulsing protocols have been used to examine the regulation of Ca^{2+} in isolated myocardial systems (Lewartowski and Pytkowski 1987a, Schouten and ter Keurs 1986) or in the failing human heart (Phillips et al. 1990). The proposed mechanisms for both frequency-force and paired pulsing is summarized in Figure 1.2. The response of the rat papillary muscle to increasing

stimulation frequency and to paired stimulations was used in the current study to examine the ability of the myocardium to alter Ca^{2+} handling before and after treatment with HOCl.

FREQUENCY-FORCE

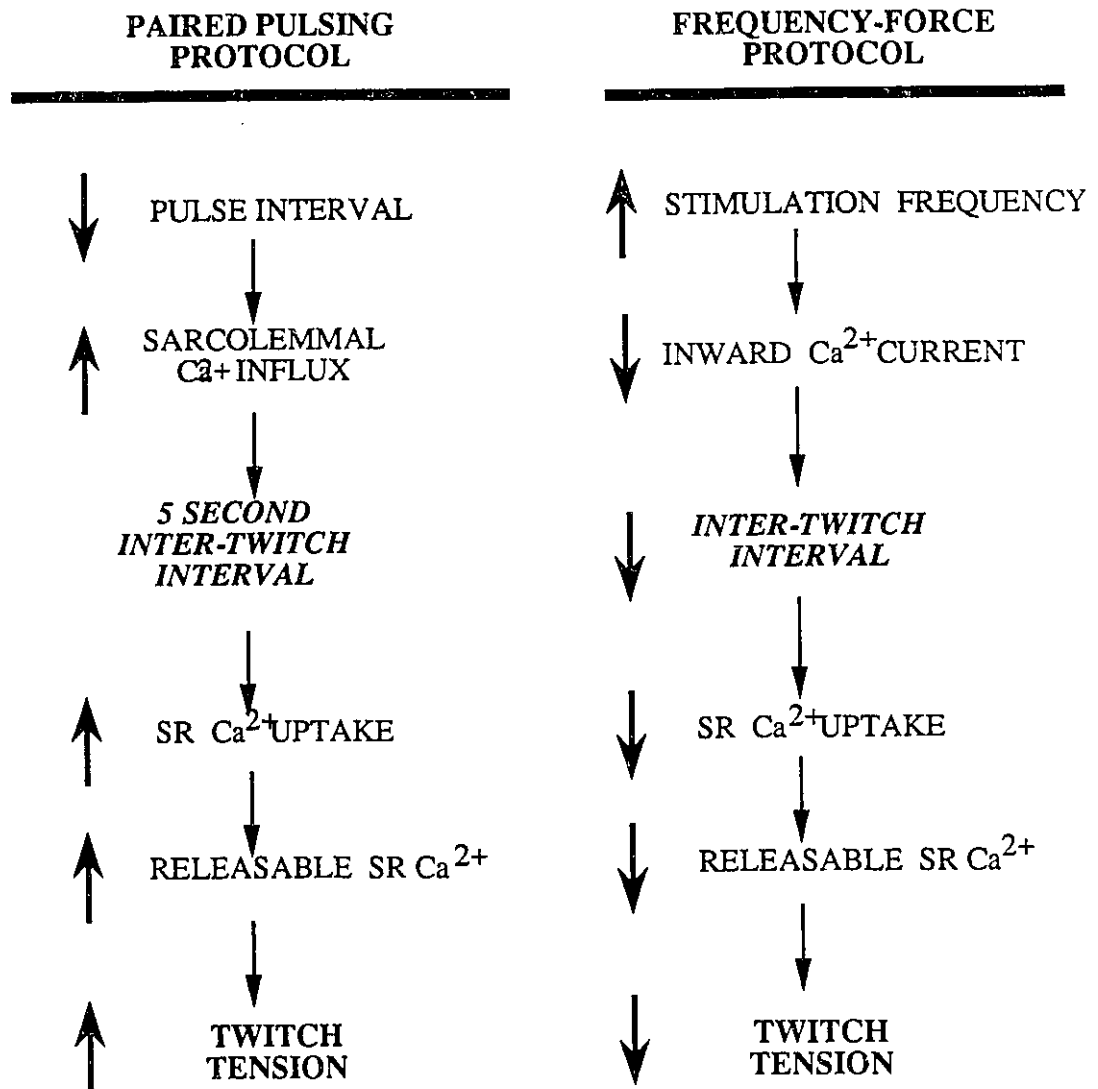
Preparations of mammalian myocardium generally show a positive inotropic response to increasing stimulation frequency (Abbott and Mommaerts 1959) with the exception of the rat (Koch-Weser and Blinks 1963) which exhibits a negative inotropic response. In a recent review, Lewartowski and Pytkowski (1987a) state that this decrease in developed tension (DT) corresponds with a decrease in the inward Ca^{2+} current in isolated rat myocytes but an increase in the inward Ca^{2+} current in rabbit myocytes. A depression of inward movement of Ca^{2+} may lead to a reduction of releasable Ca^{2+} in the SR during the subsequent beats. Orchard and Lakatta (1985) reported a depression of aequorin light transients with increasing stimulation frequency in the rat, and suggested that the negative inotropy is associated with a time-dependent recycling of Ca^{2+} by the SR. Bers and co-workers reported an accumulation of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_e$) during steady state contractions and a net depletion of $[\text{Ca}^{2+}]_e$ during rest in the rat myocardium, presumably reflecting a cellular extrusion of Ca^{2+} during the twitch (Bers and Shattock 1988, Shattock and Bers 1989). The opposite was observed in the rabbit ventricle. Therefore, if the rat myocyte is experiencing an extrusion of Ca^{2+} rather than an influx with increasing stimulation

frequency, this may account in part for the decrease in contractility. Elevation of $[\text{Ca}^{2+}]_e$ increases the tension development with increasing stimulation frequency in all species, including the rat (Forester and Mainwood 1974, Orchard and Lakatta 1985).

Kentish (Kentish 1986) showed that the accumulation of inorganic phosphates may depress the sensitivity of the skinned myofibrils to Ca^{2+} . During high frequency

Figure 1.2 Possible mechanisms behind the paired pulse and frequency-force inotropic response in the rat papillary muscle. During paired pulse stimulation, the second depolarization results in increased Ca^{2+} influx. The five second inter-twitch interval allows for greater SR loading, resulting in increased releasable Ca^{2+} for the subsequent contractions. Conversely, during increasing frequency of stimulation, the decreasing inter-twitch interval results in decreased Ca^{2+} current in combination with a decreased SR loading time. This could lead to a depletion of the releasable pool of Ca^{2+} . (Adapted from Lewartowski and Pytkowski, 1987)

Figure 1.2



stimulations, the accumulation of P_i could account for a depression of contractility, as a greater $[Ca^{2+}]_i$ would be required to attain the same tension development. However, this was also found to occur in skinned muscles from the ferret and rabbit (Allen et al. 1986) both of which exhibit positive inotropy with increasing stimulation frequency. Therefore, the concept of the accumulation of P_i shifting the pCa-tension relationship to the right probably does not play a significant role in the negative frequency-force relationship in the rat myocardium.

PAIRED PULSING

A rate-dependent, slowly exchanging Ca^{2+} fraction has been postulated as a mechanism to explain the negative frequency-force relationship as described above (Lewartowski and Pytkowski 1987a, Orchard and Lakatta 1985). Transport of Ca^{2+} from the sites of uptake to the release compartments of the SR is assumed to occur with a time constant of ~ 1 s (Morad and Goldman 1973). This delayed replenishment of the release compartment has also been postulated as a mechanism to explain the observed phenomena that the addition of a premature stimulation (ie. paired pulse) or post-extrasystolic potentiation have a positive inotropic effect on the subsequent contraction (see Figure 1.2) but not on the concomitant contraction (Lee et al. 1970, Schouten et al. 1987). If we assume that the release site is depleted with every contraction, then the addition of a second stimulation before the release compartment has been replenished (ie. 150 ms interval) will stimulate a second influx of Ca^{2+} from the extracellular pool. If the SR Ca^{2+} sequestering and transport mechanisms are fully functional, then this extra Ca^{2+} will be rapidly taken up into the SR, and the release compartment will become fully saturated within 1-2 seconds. The next stimulation (ie. 5 sec later) will consequently release a larger bolus of Ca^{2+} , leading to an accentuated contraction.

Paired pulsing stimulation and frequency-force protocols have proven useful for comparing the inotropic effects of drugs or pharmacological interventions (MacLeod and Bers 1987), or for examining Ca^{2+} homeostasis before and after specific experimental conditions (Forester and Mainwood 1974, Kennedy et al. 1987, Korecky et al. 1986, Schouten and ter Keurs 1986, Singal et al. 1985). In this chapter, frequency-force and paired pulses stimulation protocols were utilized prior to and subsequent to exposure to HOCl in isolated rat papillary muscles, and compared to the responses observed under various $[\text{Ca}^{2+}]_e$ conditions or following incubation with pharmacological agents known to alter Ca^{2+} homeostasis. In this manner, I hoped to discern the mechanisms involved in the contractile dysfunction which occurs following HOCl exposure.

MYOFIBRIL Ca^{2+} SENSITIVITY AND CONTRACTILE FUNCTION

Contractility in the myocardium can be up or down regulated according to the level of Ca^{2+} occupancy on troponin (Ruegg 1987). In principle, Ca^{2+} occupancy can be increased in two ways. First, interventions which increase the level of free Ca^{2+} within the myofibrillar space will contribute to an increase in the number of Ca^{2+} binding sites being occupied at any given time. Second, any mechanism which increases the sensitivity or affinity of the troponin site for Ca^{2+} will lead to a greater occupancy at any given $[\text{Ca}^{2+}]_i$ (Hofmann and Fuchs 1988, Ruegg 1987). Contractility may also be decreased by negative modulation of Ca^{2+} -ATPase sensitivity. For example, Allen and Orchard showed a reduction of twitch tension that was not accompanied by a reduction in $[\text{Ca}^{2+}]_i$ in the hypoxic rat papillary muscle (Allen and Orchard 1983). They further showed a rightward shift in the pCa-tension relationship in skinned papillary bundles during hypoxia, and concluded that a decline in sensitivity of the troponin Ca^{2+} binding sites may account for the loss of contractile function in the hypoxic cardiac muscle.

The skinned muscle preparation has been used for years as a means of examining the response of the myofibrils to changing $[Ca^{2+}]$ without the influence of cellular EC coupling mechanisms. Fabiato first removed the sarcolemma from isolated rabbit myocytes with fine dissection in the absence of $[Ca^{2+}]_e$, leaving an intact and functional SR (Fabiato and Fabiato 1972, Fabiato 1983). These experiments revealed that a short duration pulse of Ca^{2+} induced a large release of Ca^{2+} from the SR terminal cisternae, sufficient to initiate a contractile interaction between actin and myosin. From this and other skinned muscle experiments Fabiato postulated the Ca^{2+} -induced Ca^{2+} -release as the link between sarcolemmal membrane depolarization and SR Ca^{2+} release in the EC coupling cascade (Fabiato and Fabiato 1979, Fabiato 1983). Specific detergents (eg. saponin) can be used to selectively remove only the sarcolemma and leave the SR intact, in order to monitor the effects of inotropic agents including isoproterenol (Fabiato 1981) or ryanodine (Su 1988) on the function of the SR. Other detergents (eg. Triton X-100, Brij 58) disrupt all cellular membranes including the sarcolemma, SR, and mitochondrial membranes without disturbing the myofibrils themselves (Orentlicher et al. 1974, Veksler 1986). This allows direct control of the $[Ca^{2+}]$ at the myofibrillar level without the uncertain contributions from internal Ca^{2+} stores.

In order to correlate the loss of protein thiols with the loss of contractile function in the muscle independent of cellular Ca^{2+} handling the chemically skinned papillary muscle preparation was ideal. In the present study the skinned papillary muscle from the rat left ventricle was used as a model of myofibrillar function following exposure of the 'intact' papillary muscle to HOCl with or without DTT.

SUMMARY

The isolated papillary muscle preparation has been used extensively over the years to examine the mechanisms involved in cardiac muscle contraction. Several

stimulation protocols have been used to correlate whole muscle contractility with cellular Ca^{2+} homeostasis under both physiological and pathological conditions. The skinned muscle preparation has been developed in order to provide control over the $[\text{Ca}^{2+}]$ at the level of the contractile apparatus without the interference of cellular membranes and intracellular Ca^{2+} stores. These two techniques will be used in Chapter 1 of this thesis in order to examine the contractile function of the myocardium following exposure to HOCl. The two compartment model for cellular Ca^{2+} regulation involves a minimum of Ca^{2+} regulatory five proteins, the activity of which is known to be sensitive to thiol oxidation (Ariki and Shamoo 1983, Debetto et al. 1988, Dixon et al. 1987, Kaneko et al. 1989, Zaidi et al. 1989). Therefore, each of these Ca^{2+} regulatory sites may be extremely susceptible to HOCl-induced inactivation leading to an inability of the cell to regulate cytosolic Ca^{2+} .

OBJECTIVES:

The main objectives of Chapter 1 are as follows:

- 1) To quantitate the changes in myocardial contractility observed in the rat papillary muscle in response to HOCl
- 2) To examine the pCa-tension relationship in skinned muscles prepared from papillary muscles following exposure to HOCl with or without DTT
- 3) To record the changes in thiol redox status in the contractile proteins following exposure to HOCl with or without DTT

MATERIALS AND METHODS

MATERIALS

The model number and source for the equipment used in these studies are identified within the text. All water was deionized using a Millipore Milli-Q filtering system (minimal 18 m Ω resistance). A comprehensive list of the sources for the chemicals, reagents and pharmacological agents used in these studies is located in Appendix I

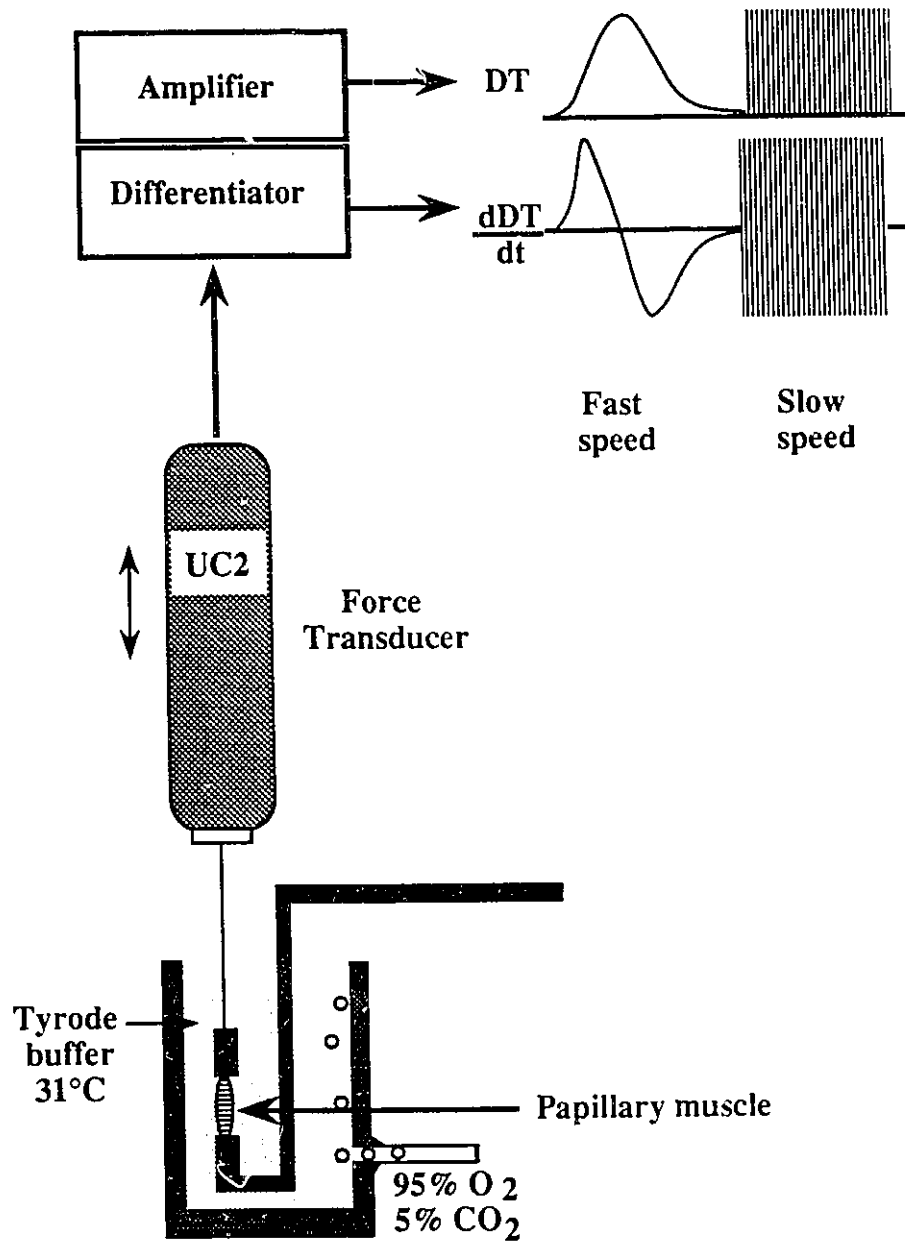
ISOLATED RAT PAPILLARY MUSCLE PREPARATION

Male Sprague-Dawley rats (250–300 g; Charles River) were sacrificed by cervical dislocation, the hearts were removed and immediately immersed in ice cold isotonic saline. The atrial tissue was removed, then the interventricular septum was exposed with one cut through the free wall of the right ventricle from the A/V valve to the apex of the heart. The left ventricle was opened by cutting through the septum from the mitral valve to the apex of the heart, exposing the two papillary muscles attached to the free wall of the left ventricle. Each muscle was excised using fine scissors with a small portion of the free wall attached. The muscles were used only if their free length (excluding the free wall tissue) was undamaged and greater than 3 mm, and their maximum diameter was less than 0.5 mm.

The muscles were immediately mounted onto the myograph (See Figure 1.3). The bottom clip of the myograph was fixed to a plexiglass rod onto which two adjustable platinum field electrodes were attached. The upper clip was attached via a stainless steel wire to a vertically mounted Gould UC2 Force transducer. The position of this transducer could be adjusted vertically, thereby increasing or decreasing the distance between the two clips. This distance between the two clips was measured (to within 0.01 mm) with a

Figure 1.3 Schematic view of the 'isometric' tension myograph used in this study. The papillary muscle was mounted vertically between a fixed clip (bottom) and an adjustable force transducer, and stimulated by field stimulation. Force development was recorded as a polygraph tracing and normalized to muscle cross-sectional area. Muscle length was taken as the distance between the two clips.

Figure 1.3



Starrett dial micrometer attached above the transducer mounting. The muscle preparation was immersed in 31°C Tyrode solution (in mM: NaCl 117, CaCl₂ 2.5, KCl 3.6, MgSO₄ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25.0, D-Glucose 5.5) equilibrated with 95% O₂/5% CO₂ to maintain a pH of 7.40. The total volume of the chamber (40 ml) could be drained and refilled from either of two heated, oxygenated buffer reservoirs within 10 seconds.

The papillary muscles were stretched gently by increasing the distance between the two clips until a resting force (preload) of 500 mg was applied. A continuous 0.2 Hz train of 5 ms square-wave pulses at a minimum of 2x threshold voltage (any exceptions will be noted in figure legends) was used to elicit contraction and the preparations were allowed to equilibrate for a minimum of 45 minutes. The muscles were stretched gradually, increasing the preload in 250 mg increments, until maximal DT was developed upon stimulation (L_{max}). The force developed by each muscle was recorded using a Grass 7P1F Low Level Pre-amplifier and a Grass 7PAG Polygraph Driver Amplifier. The developed force signal was simultaneously differentiated ($\pm dDT/dt$) using a Grass 7P20 differential amplifier and all signals were displayed as a 4 channel polygram. All contractile data were converted to developed tension (DT) or resting tension (RT) by normalizing the force measurements to the cross-sectional area of each muscle, as determined by the formula:

$$\text{Area (mm}^2\text{)} = \frac{\text{Mass (mg)} / \text{Density (mg/mm}^3\text{)}}{\text{length (mm)}}$$

where Density = 1.063 mg/ mm³ (Korecky and Michael 1975)

In order to ensure that only undamaged papillary muscles were used, any muscles which failed to develop less than 1.0 g/mm² DT upon stimulation by the end of the 45 min equilibration period were discarded. Time to maximal tension (TMT), time to 50%

(T50%R) and 90% (T90%R) relaxation were determined directly from the tracings (see Figure 1.11).

EFFECTS OF HOCl AND DTT ON PAPILLARY MUSCLE ISOMETRIC CONTRACTILE FUNCTION

To examine the effects of HOCl on the isometric contractile function of these muscles over time, HOCl was added to the bathing solution directly into the bubble stream as a single bolus of NaOCl (brought to pH 7.4 with 1 N HCl). The concentrations of HOCl initially tested ranged from 50 μ M to 400 μ M as determined using the extinction coefficient ($E_{290} = 350 \text{ M}^{-1}$) given by Morris (Morris 1966). Based on these preliminary studies, the concentration of 300 μ M HOCl was used throughout the papillary muscle studies. For all protocols, the DT, RT, and $\pm dDT/dt$ values immediately prior to addition of HOCl (Time = 0 min) were taken to be 100%.

Where indicated, DTT (final concentration 1 mM) was added at 0, 10, or 40 minutes. In a third groups of muscles the HOCl-containing Tyrode solution was replaced with fresh Tyrode (Washout) at 10 or 40 minutes. Two groups ($n = 8$ in each) of muscles were removed after either 10 minutes or 40 minutes of oxidant exposure specifically for tissue thiol determination. Unless otherwise stated, all protocols ran for a total of 80 minutes after the oxidant addition.

EFFECTS OF $[Ca^{2+}]_e$, TRANSARCOLEMMAL Ca^{2+} INFLUX AND SR Ca^{2+} RELEASE ON THE ACTIONS OF HOCl AND DTT

To examine the effect that extracellular Ca^{2+} has on the contractile dysfunction following HOCl exposure, the above protocols were repeated under elevated (5 mM) or reduced (0.5 mM) $[Ca^{2+}]_e$ conditions. Similar experiments were performed following preincubation with the Ca^{2+} channel antagonist nifedipine (Piacenza et al. 1986), the

Ca²⁺ channel agonist Bay K 8644 (Horackova 1986), or the SR release channel antagonist ryanodine (Bers et al. 1987) as described below.

Rat papillary muscles were initially equilibrated in Tyrode buffer (2.5 mM CaCl₂) for 45 min, then for a further 45 min in:

Normal [Ca²⁺] Tyrode (2.5 mM CaCl₂) ;

Low [Ca²⁺] Tyrode (0.5 mM CaCl₂);

High [Ca²⁺] Tyrode (5.0 mM CaCl₂) ;

Normal Tyrode with nifedipine (1 μM)

Normal Tyrode with Bay K 8644 (1 μM)

Normal Tyrode with ryanodine (1 nM)

Experimental (80 min HOCl ± DTT at 40 min) and control protocols were performed in the above solutions. Nifedipine and Bay K 8644 stock solutions were dissolved in 99% ethanol, producing a final ethanol concentration in the bath of less than 0.01% (V/V).

FREQUENCY-FORCE PROTOCOL

Papillary muscles were stimulated at 3/min to establish the resting state contraction (RSC), then the stimulation frequency was increased stepwise (ie. 3,6,12,24,48,96,192 per min) for a period of 2 min (or until the DT had reached a new steady state), returning to 3/min between steps. Steady state DT was recorded at each stimulation frequency and is expressed as the percentage of the DT observed at RSC. This frequency-force protocol was performed after a 40 min incubation in 0.5, 2.5, or 5.0

mM $[Ca^{2+}]$ Tyrode buffer, or in 2.5 mM $[Ca^{2+}]_e$ Tyrode buffer containing either 1 μ M Bay K 8644, 1 μ M nifedipine, or 1 nM ryanodine.

The frequency-force protocol described above was also performed on a series of papillary muscles before and after exposure to HOCl as follows: 10 min prior to the addition of 200 μ M HOCl (Control); after 40 min of exposure to HOCl (HOCl Only); and after a further 40 min exposure to DTT (HOCl + DTT). A second set of Control muscles were run in conjunction with the experimental muscles and stimulated simultaneously (ie. at 40 and 80 min). These experiments were performed in 2.5 mM $[Ca^{2+}]$ Tyrode buffer, and all muscles were stimulated at 3/min throughout the remainder of the protocol.

PAIRED PULSE PROTOCOL

Papillary muscles were stimulated at 24/min to establish a single pulse (SP) steady state DT, followed by a 2 min train of paired pulses with the interval between pulses varying from 80 to 300 ms. The paired pulse (PP) steady state DT is expressed as a percentage of the single pulse DT (ie. PP/SP). This paired pulse protocol was performed after a 40 min incubation in 0.5, 2.5, or 5.0 mM $[Ca^{2+}]$ Tyrode buffer, or in 2.5 mM $[Ca^{2+}]_e$ Tyrode buffer containing either 1 μ M Bay K 8644, 1 μ M nifedipine, or 1 nM ryanodine.

The paired pulse protocol described above was also performed on a separate series of papillary muscles before and after exposure to HOCl as follows: 10 min prior to the addition of 200 μ M HOCl (Control); after 40 min of exposure to HOCl (HOCl Only); and after a further 20 min exposure to DTT (HOCl + DTT). A second set of Control muscles were run in conjunction with the experimental muscles and stimulated simultaneously. These experiments were performed in 2.5 mM $[Ca^{2+}]$ Tyrode buffer.

TOTAL TISSUE SULFHYDRYL DETERMINATION

Following each protocol, the muscles were removed from the myograph, blotted dry between two pieces of Whatman filter paper, weighed (to the nearest 0.05 mg), and immediately homogenized in 100 μ l of 5 mM ethylenediaminetetraacetic acid (EDTA) in 5% Trichloroacetic acid (TCA), 0°C, using a 0.5 ml glass-glass homogenizer. The homogenate was transferred to a chilled microcentrifuge tube, washing the homogenizer with an additional 100 μ l of the above solution, kept on ice for 30 min, then centrifuged (13,000 x G) for 2 min to pellet the precipitated proteins. The supernatant was collected, brought to pH 8.6 with 1N NaOH and assayed for non-protein (NP-SH) sulfhydryl content using the DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) assay as described by Sedlak and Lindsay (Sedlak and Lindsay 1968). Briefly, the entire supernatant (~150 μ l) was added to 850 μ l of DTNB reagent mixture, containing TRIS-HCl 0.1 M; EDTA 5 mM; DTNB 0.1 mM; pH 8.6 and allowed to stand at room temperature for 30 min. The samples were read at 412 nm (Beckman DU-7), and SH concentration was calculated against a standard curve derived using glutathione (0,20,40,60 nM GSH). (A detailed description of this assay technique is found in Appendix II) Values for NP-SH are expressed in nmoles SH per mg of tissue wet weight.

The protein pellet was washed three times with 100 μ l of 5 mM EDTA in 5% TCA solution (0° C), then resuspended in 150 μ l of 0.5% SDS in 0.1 M TRIS-HCl buffer, pH 7.6. The protein was dissolved with the addition of 1 N NaOH to return the pH to 8.6. Protein (P-SH)sulfhydryl levels were determined using the DTNB assay described above. Briefly, 40 μ l of the protein suspension were added to 960 μ l of DTNB reagent solution, and allowed to stand at room temperature for 30 min. The samples were read at 412 nm against a standard curve derived using GSH (0,20,40,60 nM GSH). All assays were run in duplicate or triplicate. Aliquots of the protein suspension were assayed for total protein content using the Bradford method (Bradford 1976). Briefly, 5 μ l of the protein

suspension were mixed with 995 μ l of 20% BIORAD reagent mix (BIORAD), allowed to stand for 5 min at room temperature, and read at 595 nm. Protein concentration was determined against a standard curve consisting of 0, 2.5, 5, and 7.5 μ g protein (BIORAD standard bovine plasma albumin). All assays were run in triplicate. (A detailed description of this assay technique is found in Appendix II). Values for P-SH are expressed in nmoles SH per mg protein.

SKINNED RAT PAPILLARY MUSCLE PREPARATION

INITIAL CONTRACTILE PROTOCOLS

Papillary muscles were excised from the left ventricles of male rats and mounted on a myograph and exposed to HOCl with or without washout or DTT, following the superfusion protocols described previously (ie. see page 52):

Control (no HOCl) 80 min
 HOCl (300 μ M) 80 min
 HOCl 40 min; Washout 40 min
 HOCl 40 min; DTT 40 min

SKINNING TECHNIQUE

	<u>Skinning</u>	<u>Storage</u>
Caffeine	10 mM	10 mM
EDTA	5 mM	5 mM
Imidazole	20 mM	20 mM
KCl	80 mM	80 mM
MgCl ₂	5 mM	5 mM
Triton X-100	0.1 % (V/V)	-----
Glycerol	-----	50% (V/V)

The muscles were removed from the myograph, gently laid out on a bed of Sylgard submerged under 5 ml of chilled (4°C) skinning solution (see above) in a small Petri dish and pinned down (using the sharp tips of size 26 syringe needles) at resting length to prevent curling of the muscle during skinning. Triton X-100 was the detergent of choice in our experiments because of its ability to solubilize all cellular membranes including the SR and mitochondria, thereby affording complete control over the $[Ca^{2+}]$ at the level of the myofibrils. The muscles remained in this solution for 1 hour at 4° C, then the solution was replaced with 5 ml of storage solution (see above) at 4°C . The muscles were stored for up to 30 days at -20°C, changing the solution once after 2 days. Following this period the storage solution was removed and replaced with oxygenated relaxing solution (pH 7.4, 4°C see below) for a period of 30 minutes prior to each experiment. By this time, all of the skinned muscle preparations appeared translucent and flaccid.

pCa-TENSION RELATIONSHIP

SOLUTIONS

	<u>Relaxing Solution</u>	<u>Test solutions</u>
Imidazole	20 mM	20 mM
KCl	100 mM	100 mM
EGTA	5 mM	5 mM
MgCl ₂	7.5 mM	7.5 mM
ATP	3.3 mM	3.3 mM
Creatine Phosphate	12 mM	12 mM
Creatine Phosphokinase	1500 units	1500 units
CaCl ₂	-----	various

pCa DETERMINATION

Free $[Ca^{2+}]$ in each of the contracting solutions was calculated according to Fabiato (Fabiato 1984). All of the test solutions (see above) were made from the same stock solutions and kept for up to 24 hours in polycarbonate bottles thoroughly rinsed with filtered water to avoid Ca^{2+} contamination from glass or impure water. A stock solution of 1 M $CaCl_2$ was used to bring free Ca^{2+} to the desired concentration. All buffers were mixed thoroughly with the $CaCl_2$ and adjusted to pH 7.4 with NaOH (1N) to correct for the drop in pH associated with Ca^{2+} binding to EGTA (Ethylene glycol bis(β -aminoethyl ether) N,N,N'N' Tetraacetic acid). During the experiments the solutions were maintained at 37°C and were discarded after 8 hours of use.

pCa-TENSION PROTOCOL

The skinned papillary muscles were mounted vertically in the previously described myograph and immersed in relaxing solution (pCa 9.0, pH 7.2) at 37°C at slack length. In an initial series of trial experiments (5 control, 5 HOCl), each muscle was stretched sequentially by applying a preload, equivalent to 100 mg of force per step, until reaching L_{max} (length at which maximal tension was developed upon exposure to pCa = 4.5). For most preparations, averaging between 4-5 mm long, L_{max} was attained with a preload of 500 mg, coinciding with an increase in length of approximately 10 to 12% over slack length. To minimize strain on the experimental preparations, muscle length was set at slack length plus 10%.

The relaxing solution was replaced with a test solution (ie. ranging from pCa = 8 to 4) and the muscle was allowed to contract fully before being returned to the relaxing solution. All contractions were measured from the baseline established in the relaxing solution. Upon completion of the protocol, the muscles were removed and homogenized immediately for the isolation of the contractile proteins.

ACTIN AND MYOSIN ISOLATION AND SH DETERMINATION

SOLUTIONS

Low Salt Buffer (LSB) (pH 6.8, with KOH)

KCl 20 mM

K₂HPO₄ 2 mM

EGTA 1 mM

PMSF (phenylmethylsulfonyl fluoride) (20 µM)

High Salt Buffer (HSB) (pH 9.5, with KOH)

Na₄P₂O₇ 40 mM

MgCl₂ 1 mM

EGTA 1 mM

MYOFIBRIL ISOLATION

Following completion of the pCa-Tension protocol, each muscle was homogenized separately in 100 µl of LSB using a small glass-glass homogenizer for 2 minutes at 0°C. The homogenate was transferred into chilled eppendorf 0.5 ml polyethylene centrifuge tubes and the homogenizer was washed with a further 100 µl of LSB. The tubes were centrifuged (Fisher microfuge, 2500 x G) at 0°C for 45 seconds, and the supernatant was discarded. The pellet was resuspended in 100 µl of HSB on ice for 15 minutes at pH 9.5 (NaOH), then further centrifuged for 10 minutes. The supernatant contained the actinomyosin complex with a protein yield of 50 to 250 µg/mg of tissue homogenized. Protein concentration was determined (BIORAD) and adjusted to 1 mg/ml with HSB.

PROTEIN THIOL LABELLING WITH ^{14}C (IAA)

Iodoacetamide (IAA) is an alkylating agent which binds selectively to reduced protein thiols without significantly altering the proteins charge or molecular mass (Bishop et al. 1988). To 100 μl ($\approx 100 \mu\text{g}$ protein) of the above supernatant, 10 μl (app 0.6 μCi) of ^{14}C IAA (Amersham) was added and the mixture was incubated in the dark at 37° for 60 minutes. The reaction between IAA and protein thiols was stopped by the addition of excess (1 mM) DTT, and the mixture was protected from light at 0°C until subjected to gel electrophoresis.

PROTEIN SEPARATION (SDS-PAGE)

Single dimension SDS-polyacrylamide gels (7.5%) were used for separation of the contractile proteins. A detailed description of the preparation of these gels and the solutions used is located in Appendix II.

Approximately 25 μg of the ^{14}C -IAA labelled proteins were mixed with 25 μl of loading buffer and incubated at 100°C for 3 minutes before being loaded into the wells of the vertical slab gel. Standard samples comprising 1, 5, and 25 μg each of purified rabbit skeletal actin and myosin (Sigma) were run on each gel. The gels were run overnight at 4°C under 100 mA current. The gels were fixed and stained using 0.1% Coomassie Blue in 40% methanol-10% acetic acid for 30 minutes, then destained in 7% acetic acid-20% methanol for up to 4 hours.

DENSITOMETRY

The wet destained gels were sandwiched between two sheets of clear acetate and scanned using a LKB 2222-020 Ultrosan XL laser densitometer. Following background subtraction, the signals were integrated, and protein concentration for actin and myosin were determined from the standard curve.

AUTORADIOGRAPHY

Following densitometric scanning, the gels were immersed in 50 ml of Amplify (Amersham) and shaken gently for 30 minutes prior to drying. Gels were dried onto Whatman filter paper under vacuum at 50°C for up to 3 hours. The dried gels were wrapped in one layer of Saran wrap, taped into a Kodak X-Omatic cassette and exposed for 3-7 days at -80°C against an X-ray film (Kodak). The resulting autoradiograms were developed and photographed.

¹⁴C-IAA BINDING

IAA binding to the reduced protein thiols was estimated by ¹⁴C scintillation counting. The actin and myosin bands were cut from each lane using an oblong punch of known size, and digested in 200 µl of 30% H₂O₂ for 3 hours at 60°C. The unlabeled actin and myosin standards were also cut from the gels, digested and counted for background radioactivity. ¹⁴C-IAA levels were counted using a Beckman LS2800 scintillation counter. Background counts were subtracted from each reading and the level of ¹⁴IAA binding is presented as CPS per µg of protein in each band.

STATISTICAL ANALYSIS

The mechanical data were analyzed at various time points, comparing all group means using one or two-way Analysis of Variance (ANOVA) using time, pretreatment, or treatment as independent variables, as is described in each figure legend respectively. Unless otherwise stated, differences between groups were determined using the Scheffees post-hoc test. Other specific comparisons, were made using paired or unpaired T-tests. Comparisons between NP-SH or P-SH for the various experimental groups were made using one-way ANOVA and Scheffees. For the skinned muscle contractile data, differences between the three treatment groups were examined using a two-way ANOVA,

using pCa and Treatment as the independent variables. The level of significance is as indicated in the figure legends. Values are presented as means $\pm$ SEM. Analysis was done using a MacIntosh SE computer (Apple Computers) and the Statsview 512 (Aldus software) statistical program.

RESULTS

ISOLATED PAPILLARY MUSCLE PREPARATION

DOSE-RESPONSE FOR HOCl

The addition of low concentrations (50-100 μM) of HOCl had no detrimental effects on the mechanical function of rat papillary muscles over the time periods used (Figure 1.4). Concentrations of HOCl in excess of 100 μM induced a concentration dependent decrease in mechanical function. Unless otherwise noted, further experiments were done using 300 μM HOCl.

EFFECTS OF STIMULATION VOLTAGE

Figure 1.5 shows the response of a Control muscle (A) and a HOCl-treated muscle (B) at 40 min after HOCl addition to increasing stimulation voltage. The Control muscle exhibited the classical all-or-none response, such that maximal tension is developed at any voltage above threshold. In contrast, the muscle incubated in HOCl for 40 min exhibited a dependency upon stimulation voltage, developing greater tension at higher stimulation voltages. All further data were collected at a stimulation voltage that elicited maximal DT.

HOCl AND PAPILLARY MUSCLE ISOMETRIC CONTRACTIONS

The effects of 300 μM HOCl addition on the contractile performance of two left ventricular papillary muscles superfused with 2.5 mM Ca^{2+} Tyrode are shown in Figure 1.6. HOCl induced an immediate biphasic decline in the contractile performance of the muscles, consisting of a decline in DT and concomitant rise in RT as the muscles entered into contracture. Within five minutes DT had declined to a mean of $51 \pm 8\%$ of initial values with a concomitant rise in RT (see also Figure 1.8 below). Many muscles

Figure 1.4 Effects of various concentrations (50 to 400 μM) of HOCl on the tension developed by rat papillary muscles. Developed tension (DT) is presented as the mean $\pm$ SEM from at least 4 separate muscles at each concentration, added at Time = 0 min. All papillary muscles were stimulated at 0.2 Hz in buffer containing 2.5 mM Ca^{2+} , $T=31^\circ\text{C}$, pH 7.4.

Figure 1.4

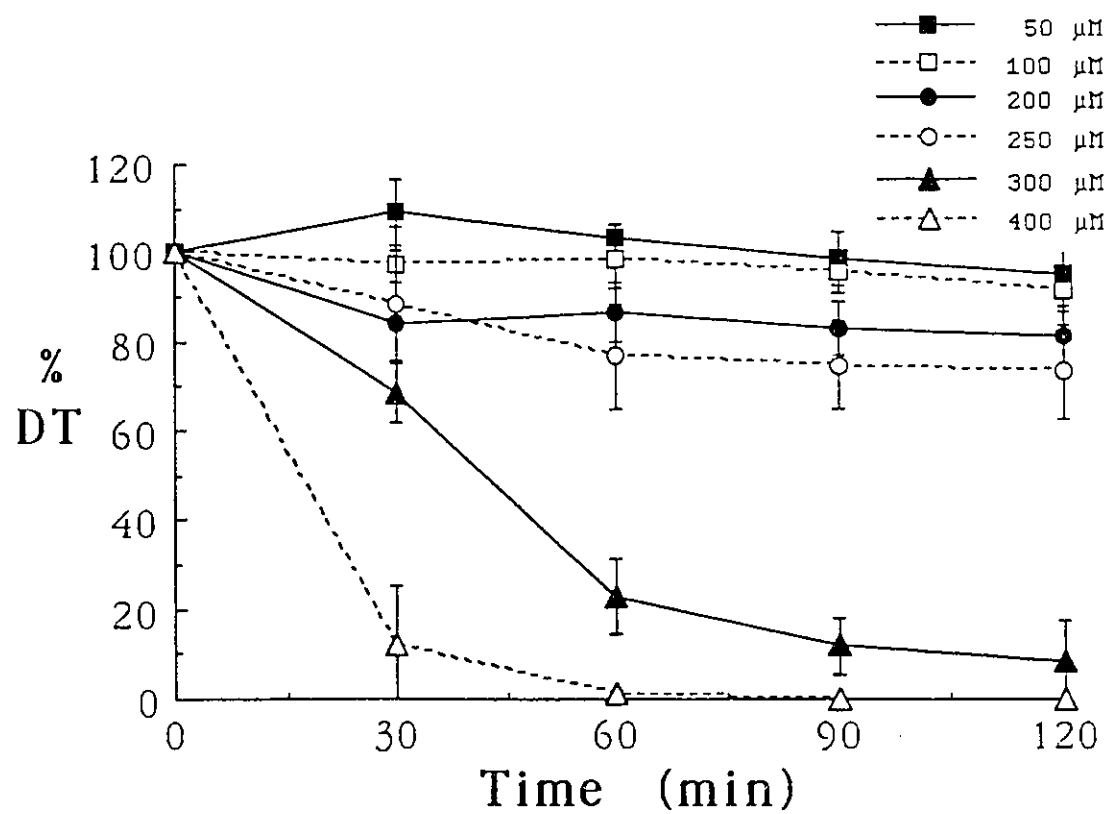


Figure 1.5 Tension development by two papillary muscles, showing the increasing DT with increasing stimulation voltage (from 10 to 40 V) in the HOCl-treated muscle as compared to Control. Both muscles were stimulated with 5 ms square-wave pulses at 0.2 Hz in buffer containing 2.5 mM Ca^{2+} , 31°C, pH 7.4. For the remaining experiments, all HOCl-treated muscles were stimulated at the voltage which elicited maximal DT.

Figure 1.5

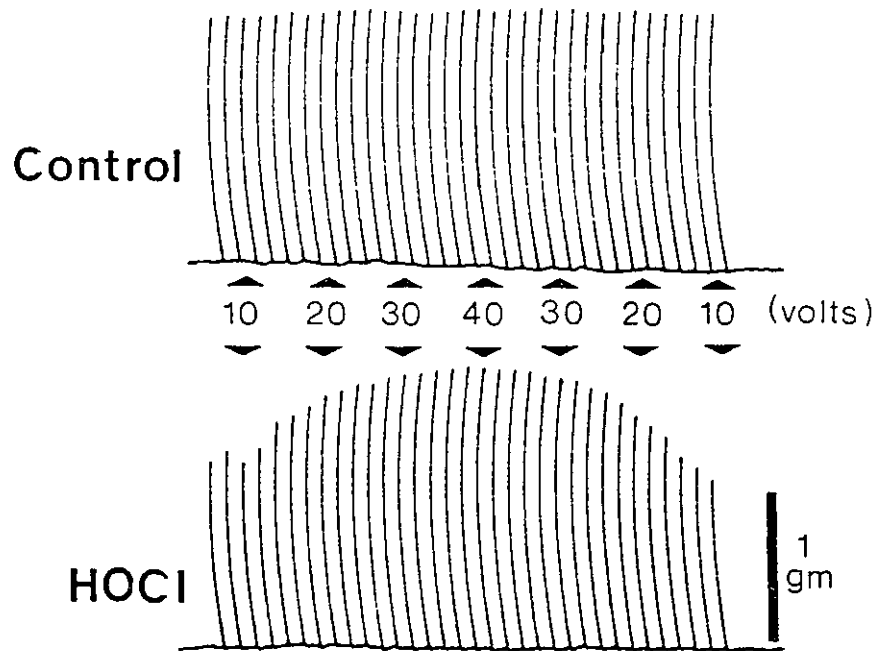
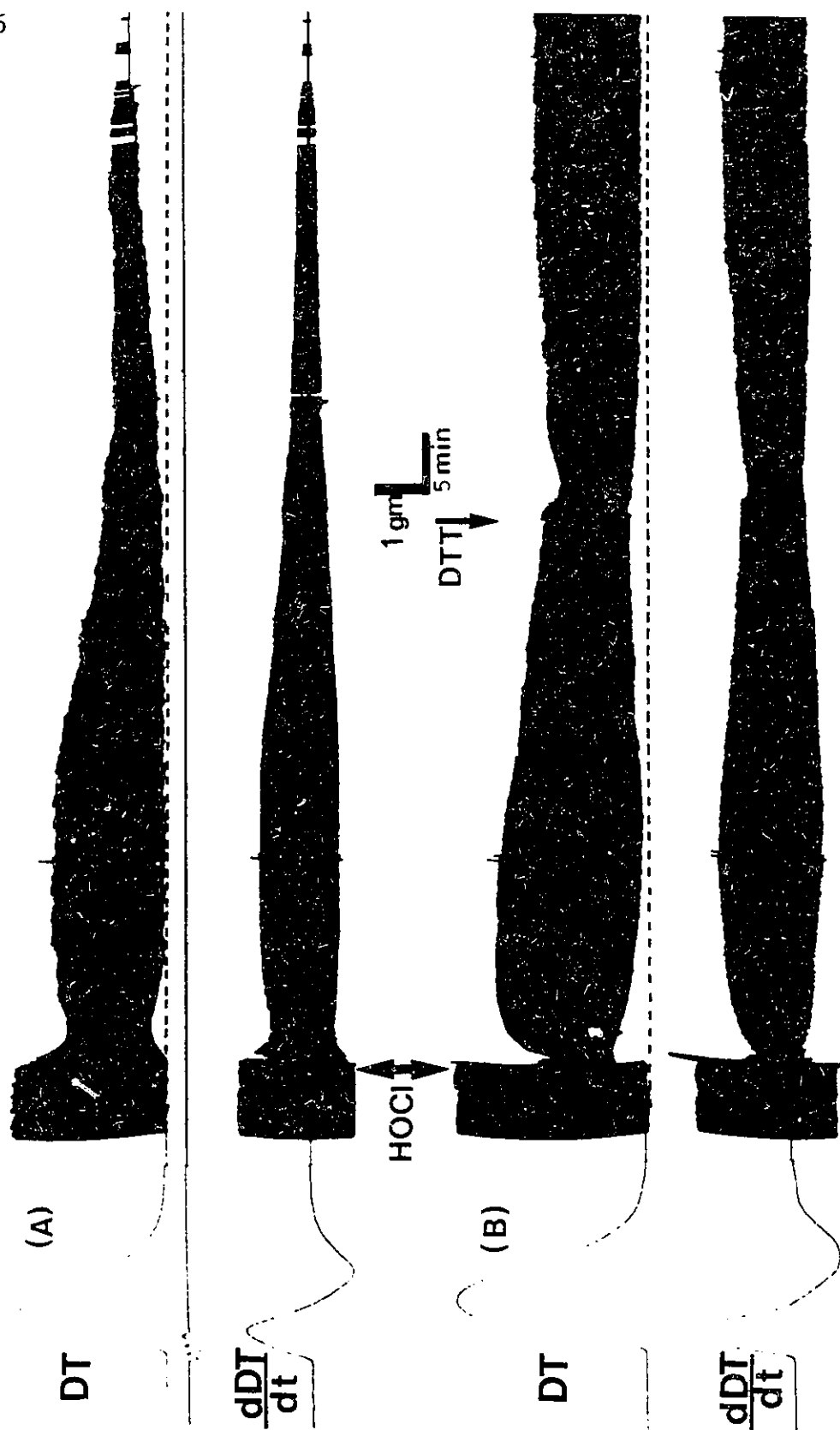


Figure 1.6 A photograph of a typical 80 min polygraph tracing showing the effects of HOCl and DTT on isometric contractile function (DT and dDT/dt) in two rat papillary muscles. HOCl (300 μ M) was added to both muscles as indicated, while DTT was added to the muscle B only at 40 min as indicated. Note that the muscle A was unable to develop any tension in response to electrical stimulation by 70 min. Any contractions beyond this time were spontaneously generated. Preload on these muscles were 1.25 and 1.0 g, respectively. These muscles were contracting in 2.5 mM Ca^{2+} , 31°C, pH 7.4.

Figure 1.6



exhibited a partial spontaneous recovery of DT to $68 \pm 4\%$ of the initial value by ten minutes. In most muscles, after 10 min exposure to HOCl, individual twitches exhibited a significant prolongation of the relaxation phase (see also Figure 1.11 below). In the absence of any further intervention, a gradual decline in DT occurred over the remainder of the protocols, reaching a minimum value of $11 \pm 3\%$ of initial DT by the end of the eighty minute protocol (Figure 1.6 A). Many muscles became unresponsive to electrical stimulation at any voltage by as early as 30 min HOCl (see also Figure 1.7 below). RT increased over the duration of the 80 min protocol. The maximum rate of tension development (+dDT/dt) and relaxation (-dDT/dt) paralleled the decline in DT.

The addition of DTT to HOCl-injured muscles induced a dramatic recovery of function that was dependent upon the time of addition. Figure 1.6 B shows that the addition of DTT at 40 minutes not only prevented further loss of DT, but induced a substantial recovery of mechanical function, shown here as a 35% restoration of initial DT and return of RT to muscles is depicted in Figure 1.7. DTT initiated a restoration of contractility with a decline in RT and a recovery of DT. Higher concentrations of DTT (up to 5 mM), or longer exposure (up to 180 min) did not further enhance the level of recovery.

The results from several experiments are plotted in Figure 1.8, showing the time-dependent effect of DTT on the restoration of contractile function. DTT added at 0 min totally prevented any HOCl-induced functional decline. DTT added at 10 minutes produced a restoration of DT to a value that was only slightly different ($p > 0.1$) from Control while DTT addition at 40 minutes induced a recovery of DT to a mean of 46% of the initial DT values for each muscle. RT returned to baseline levels following DTT addition regardless of the time of addition.

Figure 1.7 A typical polygraph tracing starting 35 min after HOCl addition, showing the isometric contractile function of a single papillary muscle. Note that by 40 min the muscle was unable to respond to electrical stimulation at any voltage and had entered into contracture. The dotted line represents the baseline resting tension prior to HOCl. The addition of DTT at 40 min restored the muscles ability to contract in response to electrical stimulation, and partially restored both RT and DT. This muscle was contracting in 2.5 mM Ca^{2+} , 31°C, pH 7.4.

Figure 1.7

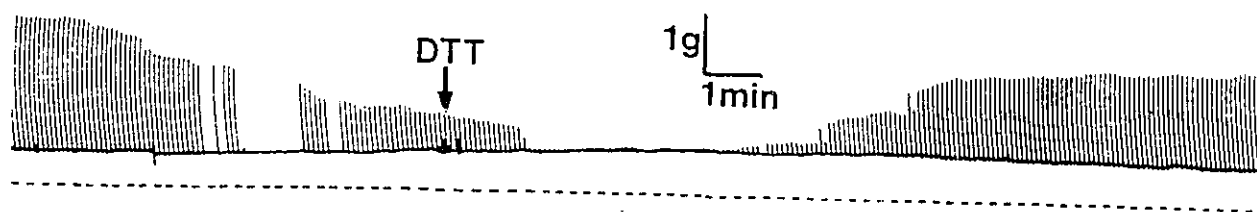
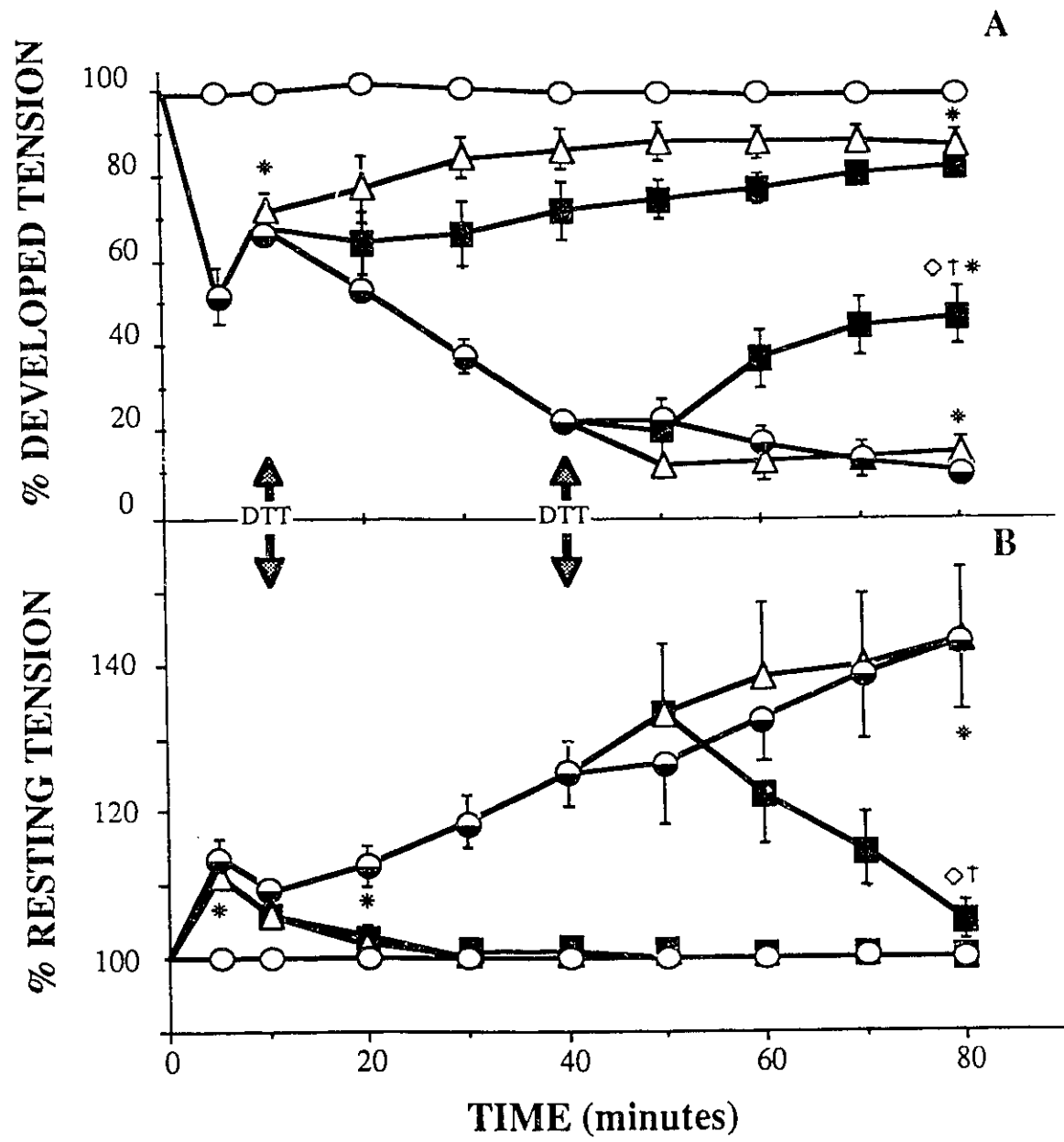


Figure 1.8 Time course of HOCl-induced decline in contractile function and its reversal with DTT. At Time = 0 min, HOCl was added to 68 muscles (●) which were subdivided as follows: 8 were removed at each of 10 min and 40 min for SH determination; 6 each received DTT (■) or Washout (△) at 10 min; 16 received DTT (■) and 11 received Washout (△) at 40 min; 13 remained in HOCl (●) for the full 80 min. 12 Control muscles (○) were not exposed to HOCl. All muscles shown here were contracting in 2.5 mM Ca²⁺, 0.2 Hz, 31°C, pH 7.4. The DT and RT values shown are means ± SEM. Statistical comparisons between all groups at any one time point (eg. at 80 min) were made using one-way analysis of variance (ANOVA) Treatment as the independent variable, and differences were determined using Scheffees post-hoc test. For any one treatment group comparisons were made using one-way ANOVA with Time as the independent variable and differences were determined with Scheffees post-hoc test. Differences are only shown only at specific points for clarity. * = p<0.01 vs Control at the time shown; † = p<0.01 vs HOCl at 40 min; § = p<0.01 vs HOCl at 80 min.

Figure 1.8



Replacement of the Tyrode (Washout) at 10 min also prevented any further decline in DT and allowed for a significant spontaneous restoration of function that was not different from that observed with DTT. In contrast, Washout at 40 min had no ameliorative or restorative effects, and the decline in DT was not different from HOCl Only. DTT alone (Control + DTT) had no positive or negative inotropic effects on muscle function (not shown).

EFFECTS OF $[Ca^{2+}]_e$ ON HOCl-INDUCED CONTRACTILE DYSFUNCTION

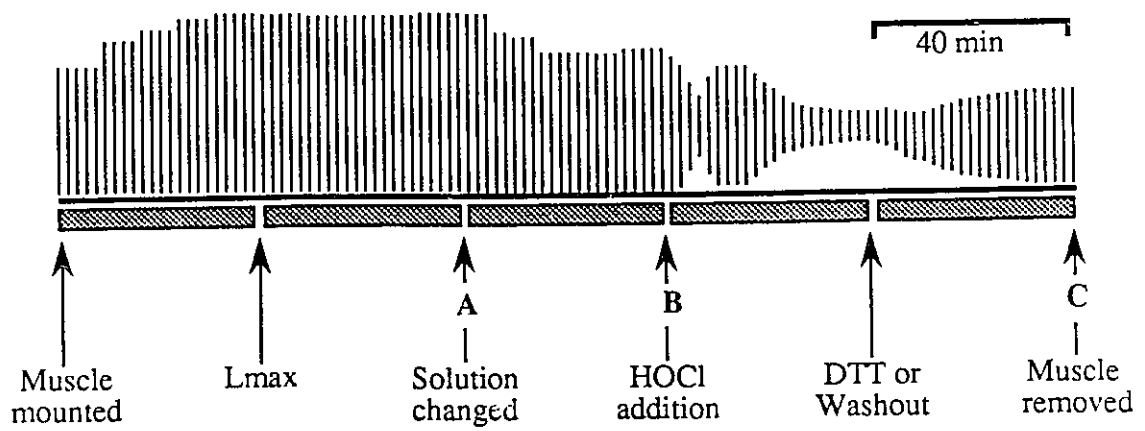
The response of papillary muscles to alterations in $[Ca^{2+}]_e$, or to incubation with agents known to alter Ca^{2+} handling are summarized in Table 1.1. Switching from 2.5 mM to 0.5 mM $[Ca^{2+}]_e$ produced a 56% decrease in DT, from a mean of 2.51 to a mean of 1.15 g/mm². Conversely, switching from 2.5 mM to 5.0 mM $[Ca^{2+}]_e$ produced a 52% increase in DT. Similar changes were observed during incubation with nifedipine (20% decrease) and Bay K 8644 (49% increase). Ethanol alone (up to 0.1 %) had no effect on DT (data not shown). Ryanodine produces a 58% decrease in DT by 45 min preincubation time, however DT continued to decline over the course of the experiment. Control muscles incubated in ryanodine were developing less than 15% of initial L_{max} DT by the end of their 80 min protocols. Changes in $\pm dDT/dt$ paralleled these changes in the DT values. No alterations were observed in RT following these changes.

The contractile response to HOCl was significantly altered in papillary muscles preincubated in 0.5 or 5.0 mM $[Ca^{2+}]_e$, or in the presence of nifedipine, Bay K 8644, or ryanodine. While in all cases, with the exception of the ryanodine treated muscles, the overall effect of HOCl was a decline in DT and concomitant rise in RT, the intensity of the response varied considerably. The drawings shown in Figure 1.9 represent the mean DT values (normalized here for comparison) superimposed on the mean RT values to

Table 1.1 Changes of DT in response to various pretreatments. Panel I illustrates a hypothetical muscle, showing the determination of Lmax in 2.5 mM $[Ca^{2+}]_e$, the effects of switching (A) to one of the pretreatment buffers (as listed in Panel II), the effects of HOCl addition (B) with or without Washout or DTT, and the final DT upon completion of the 80 min protocol (C). Panel II summarizes the response to changing $[Ca^{2+}]_e$ or to preincubation with the Ca^{2+} channel antagonist nifedipine (1 μ M), the Ca^{2+} channel agonist Bay K 8644 (1 μ M), or the SR Ca^{2+} release blocker ryanodine (1 nM) on DT (in g/mm^2). The values at Time B are taken to be 100% of initial DT throughout the following discussions.

Table 1.1

I)



II)

	DT at 'A'	DT at 'B'
0.5 mM Ca	2.51 ± 0.21	1.15 ± 0.13
2.5 mM Ca	2.62 ± 0.31	2.54 ± 0.40
5.0 mM Ca	2.71 ± 0.21	4.06 ± 0.69
Nifedipine	2.55 ± 0.11	2.05 ± 0.27
Bay K 8644	2.49 ± 0.26	3.75 ± 0.45
Ryanodine	2.62 ± 0.23	1.10 ± 0.15

Figure 1.9 Effects of HOCl and DTT on the isometric tension development in various extracellular $[Ca^{2+}]$ or in the presence of pharmacological agents, as described in the Methods. Each panel depicts a 'mean' tracing ($n =$ as given in Table 1.2), with the contractile function from several muscles being presented as the area bound by the RT (bottom border) and DT (upper border) plotted at 5 min intervals. For the sake of clarity, the standard error values are presented only for the initial values. Relative values for the mean $\pm$ SEM at other specific time points are summarized in Table 1.2. The black area represents the contractile function after the addition of HOCl; the stipled area represents the contractile function following subsequent addition of DTT at 40 min. With ryanodine, the stippled area is superimposed onto the black for clarity, and no change in RT occurred in the non-DTT treated muscles.

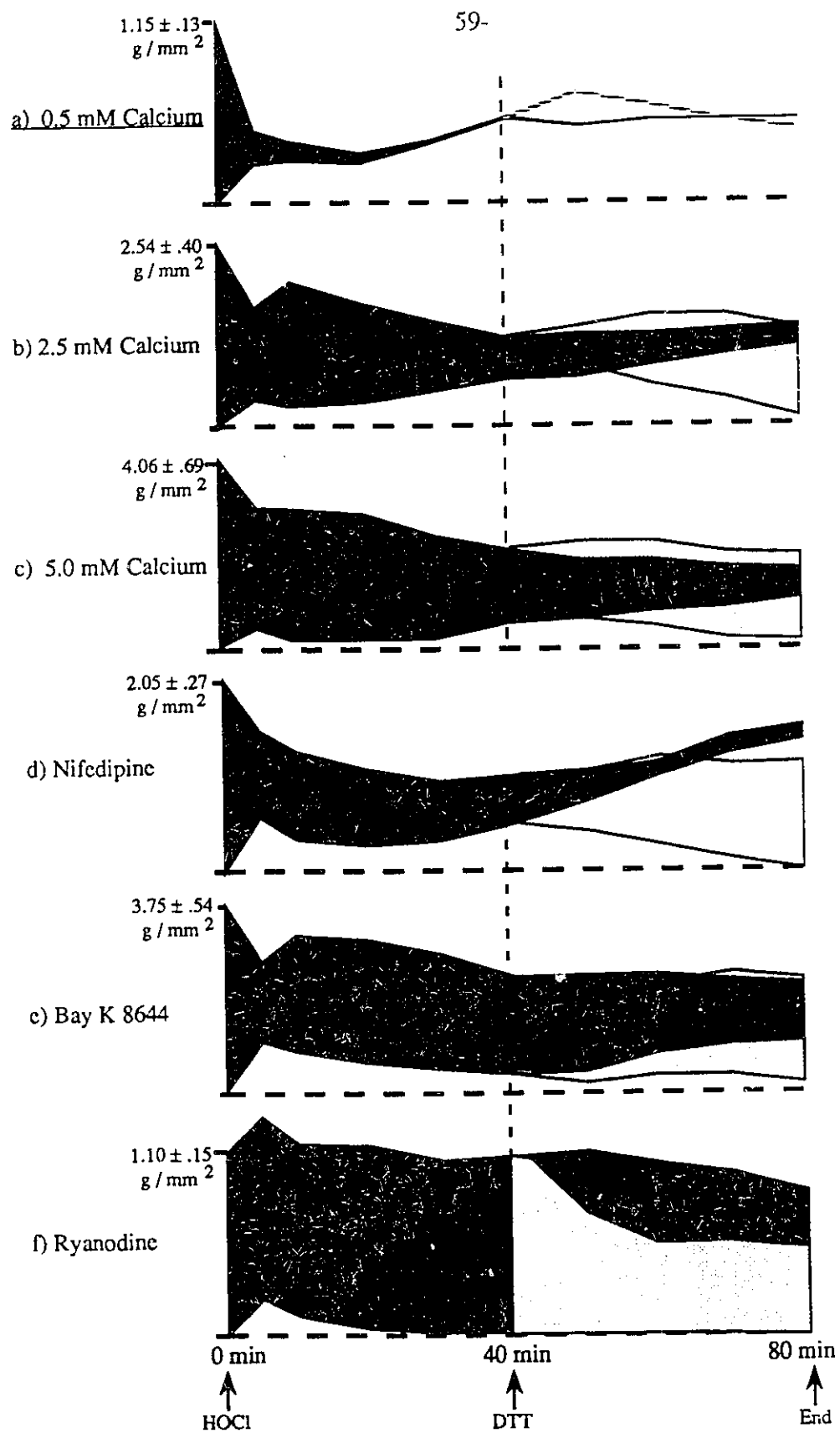


Table 1.2 Relative changes in DT, RT, and $\pm dDT/dt$ in rat papillary muscles during exposure to HOCl with or without the addition of DTT at 40 min. 100% DT represents the values for DT immediately prior to HOCl addition (see Panel II in Table 1.1). Statistical differences between pre-treatment groups were determined at each time point using two way analysis of variance with pre-treatment (ie $[Ca^{2+}]_e$ or pre-incubation with nifedipine, Bay K 8644, or ryanodine) and treatment (ie. Control, HOCl, HOCl + DTT) as independent variables. Differences relating the effects of HOCl over time on any one treatment group were determined using one-way analysis of variance with Time as the independent variable. Scheffees post-hoc analysis was used for determining differences. * = $p < 0.05$ from Control at the same time point; ‡ = $p < 0.05$ from HOCl only at the same time point; § = $p < 0.05$ as compared to 40 min value in the same treatment group. n = as shown in brackets.

Table 1.2

	% DT		% RT		% +dDT/dt		% -dDT/dt	
	40 min	80 min	40 min	80 min	40 min	80 min	40 min	80 min
0.5 mM Ca²⁺								
CONTROL (6)	94 ± 2	87 ± 6	100 ± 0	100 ± 1	96 ± 8	88 ± 10	95 ± 6	86 ± 4
HOCl Only (8)	1 ± 1 *	0 ± 0 *	138 ± 12 *	146 ± 13 *	1 ± 2 *	0 ± 0 *	1 ± 2 *	0 ± 0 *
HOCl+DTT (8)	0 ± 0 *	0 ± 0 *	140 ± 7 *	142 ± 14 *	2 ± 1 *	0 ± 0 *	3 ± 2 *	0 ± 0 *
2.5 mM Ca²⁺								
CONTROL (12)	101 ± 2	101 ± 2	100 ± 0	100 ± 1	100 ± 3	96 ± 5	101 ± 4	95 ± 2
HOCl Only (13)	26 ± 6 *	11 ± 3 *§	129 ± 6 *	143 ± 9 *	25 ± 4 *	8 ± 1 *§	24 ± 5 *	9 ± 2 *§
HOCl+DTT (16)	26 ± 5 *	48 ± 7 *§‡	132 ± 9 *	105 ± 3 §‡	25 ± 6 *	47 ± 5 *§‡	24 ± 6 *	47 ± 5 *§‡
5.0 mM Ca²⁺								
CONTROL (8)	102 ± 1	103 ± 2	100 ± 0	100 ± 1	98 ± 6	97 ± 3	101 ± 2	98 ± 5
HOCl Only (8)	41 ± 7 *	16 ± 7 *§	114 ± 4 *	125 ± 13 *	43 ± 7 *	17 ± 7 *§	45 ± 8 *	19 ± 8 *§
HOCl+DTT (10)	39 ± 10 *	46 ± 6 *‡	120 ± 8 *	101 ± 1 §‡	43 ± 11 *	52 ± 6 *‡	50 ± 3 *	53 ± 5 *‡
NIFEDIPINE								
CONTROL (6)	98 ± 1	95 ± 2	100 ± 0	100 ± 0	96 ± 5	94 ± 4	98 ± 8	97 ± 5
HOCl Only (8)	29 ± 10 *	9 ± 6 *§	127 ± 11 *	167 ± 18 *§	26 ± 11 *	10 ± 7 *	26 ± 9 *	11 ± 7 *
HOCl+DTT (8)	26 ± 8 *	54 ± 4 *§‡	121 ± 10 *	101 ± 1 *	29 ± 9 *	57 ± 4 *§‡	28 ± 9 *	58 ± 4 *‡
BAY K 8644								
CONTROL (6)	101 ± 1	100 ± 3	100 ± 1	100 ± 0	103 ± 2	104 ± 5	100 ± 2	96 ± 5
HOCl Only (8)	57 ± 3 *	31 ± 5 *§	108 ± 6	126 ± 11 *	48 ± 11 *	28 ± 8 *	51 ± 11 *	28 ± 7 *§
HOCl+DTT (8)	49 ± 4 *	50 ± 5 *‡	109 ± 9	103 ± 3 ‡	47 ± 5 *	49 ± 6 *‡	50 ± 3 *	53 ± 5 *‡
RYANODINE								
CONTROL (6)	49 ± 7	28 ± 8 §	100 ± 0	100 ± 1	42 ± 12	22 ± 7 §	40 ± 11	25 ± 6
HOCl Only (8)	100 ± 6 *	78 ± 7 *§	100 ± 1	100 ± 1	94 ± 8 *	68 ± 11 *§	97 ± 6 *	75 ± 4 *§
HOCl+DTT (8)	91 ± 6 *	43 ± 2 §‡	101 ± 1	100 ± 1	88 ± 11 *	40 ± 9 §‡	95 ± 8 *	41 ± 7 §‡

create an average representative 'polygram' tracing similar to those portrayed in Figure 1.6. For the purpose of statistical comparison, the initial RT, DT, and $\pm dDT/dt$ values (ie. immediately prior to HOCl addition) for each muscle were set to be 100%, and subsequent values were normalized to this. Differences in DT, RT, and $\pm dDT/dt$ were examined at 40 and 80 min time points (see Table 1.2) for the three treatment groups (ie. Control, HOCl only, HOCl + DTT) under each of the preincubation protocols (ie. altered $[Ca^{2+}]_e$, Nifedipine, Bay K 8644, Ryanodine). Differences between values are as expressed in the figure legend. The overall mean RT was 1.31 ± 0.03 g/mm² prior to HOCl addition.

The effects of HOCl were potentiated in 0.5 mM $[Ca^{2+}]_e$ (Figure 1.9 a). DT declined rapidly to less than 20% within 5 minutes with no apparent recovery of DT by 10 minutes. By 30 minutes virtually all muscles were non-functional. RT values rose dramatically throughout the protocol and were greater (NS) than those recorded in 2.5 mM $[Ca^{2+}]_e$. The addition of DTT at 40 minutes produced no restoration of either DT or RT. Control muscles in 0.5 mM $[Ca^{2+}]_e$ exhibited a 13% decline in DT over the 80 min protocol, while RT remained stable.

In contrast, the effects of HOCl were attenuated in 5.0 mM $[Ca^{2+}]_e$ (Figure 1.9 c) as compared to 2.5 mM $[Ca^{2+}]_e$. DT values remained significantly higher and RT remained lower (NS) over the first 40 minutes. By 50 minutes there was no significant difference between 5.0 or 2.5 mM $[Ca^{2+}]_e$ values. In 5.0 mM $[Ca^{2+}]_e$, the addition of DTT at 40 minutes produced a restoration of DT (to 46% of initial) and RT returned to baseline values. Control muscles in 5.0 mM $[Ca^{2+}]_e$ remained stable throughout the 80 min protocol.

In muscles preincubated with nifedipine, HOCl induced a rapid decline in DT during the first 5 minutes, with no apparent recovery by 10 minutes (Figure 1.9 d). By 80

min RT was significantly higher and DT significantly lower as compared to those muscles preincubated without nifedipine (ie. 2.5 mM $[Ca^{2+}]_e$ only). The addition of DTT at 40 minutes produced a significant restoration of DT while RT recovered to baseline by 80 minutes. Control muscles with nifedipine remained stable throughout the 80 min protocol.

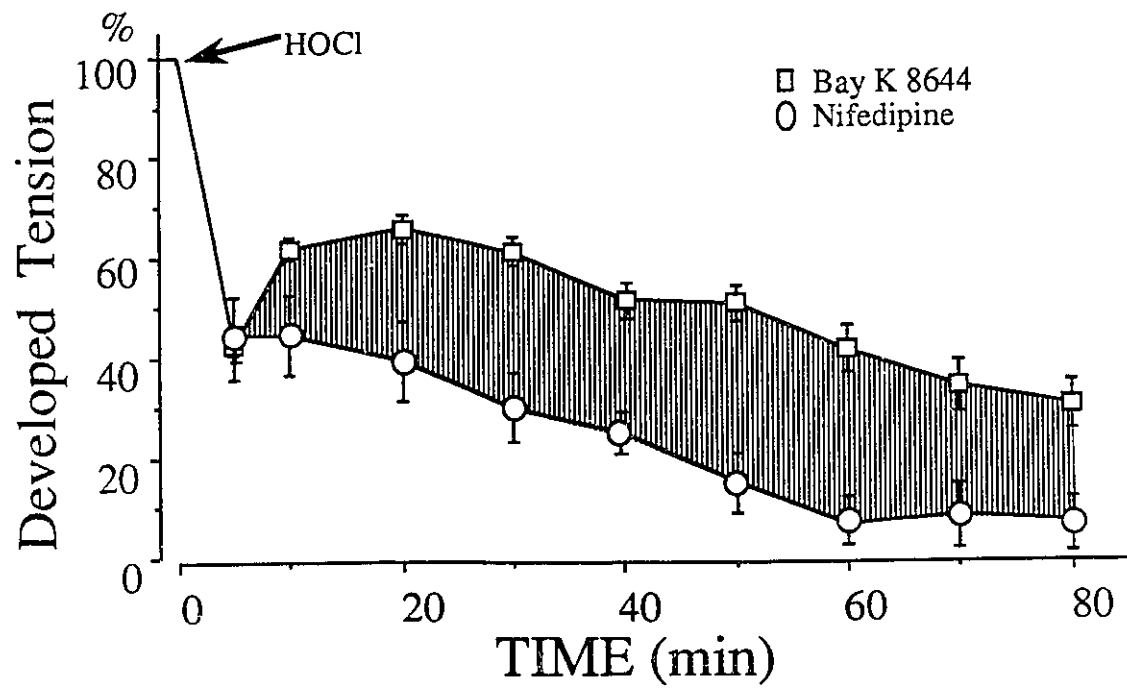
Conversely, those muscles preincubated with Bay K 8644 (Figure 1.9 e) exhibited significantly greater values for DT than those recorded without Bay K 8644, declining to only 31% of initial DT by 80 minutes HOCl exposure (Table 2). RT values became elevated following 10 min of HOCl exposure, but remained lower (NS) than 2.5 mM $[Ca^{2+}]_e$ values. The addition of DTT at 40 minutes produced a significant restoration of DT (to 49% of initial) by 80 minutes, and RT returned to Control values. Control muscles with Bay K 8644 remained stable throughout the 80 min protocol.

Muscles preincubated with ryanodine exhibited a significantly reduced response to HOCl. The addition of HOCl to these muscles produced a transient elevation in RT which recovered by 10 min (Figure 1.9 f). No further increase in RT was observed during the 80 min protocols. By 80 minutes, DT was still 78% of initial and RT remained at baseline. In view of the fact that DT in the ryanodine control muscles continued to decline, HOCl actually improved the contractile function of these muscles (Table 1.2). The addition of DTT at 40 min produced a gradual decline in DT.

Figure 1.10 shows further the effect of HOCl on DT in muscles treated with nifedipine or Bay K 8644. If the shaded region between the two curves is considered to represent the percentage of total DT attributable to Ca^{2+} influx via the slow channels, then the area below the nifedipine line must represent the DT attributable to SR Ca^{2+} release or entry through the Na^+/Ca^{2+} exchange mechanisms. It is noteworthy that as the total DT declines following HOCl addition, the distance between the nifedipine and Bay

Figure 1.10 Replotting of the data from Figure 1.9 comparing DT in muscles exposed to HOCl during a continuous incubation with Bay K 8644 or nifedipine in 2.5 mM Ca²⁺ Tyrodes solution. Note the absence of partial recovery of DT by 10 min in the nifedipine-treated muscles. Values shown are means of n=8 muscles per curve. Statistical comparisons are given in Table 1.2.

Figure 1.10



K 8644 curves remains relatively constant while the distance below the nifedipine curve declines. This suggests that following exposure to HOCl, the DT associated with SR release decreases and the muscle becomes more dependent upon Ca^{2+} influx through the slow channels as its source of activator Ca^{2+} . Note also the absence of partial recovery of DT by 10 min in the nifedipine-treated muscles.

TIME TO MAXIMAL TENSION AND 90% RELAXATION

The time to maximal tension (TMT) and the time to 50% relaxation (T50%R) values were constant (111 ± 4 ms and 89 ± 3 ms respectively) in all the Control groups independent of the preincubation protocol or tension developed with each twitch. The TMT value decreased following HOCl addition in 5.0 mM $[\text{Ca}^{2+}]_e$ or in the presence of Bay K 8644, but this decrease was not significant (Table 1.3). The mean T50%R values (106 ± 18 ms) were not significantly different from Control. However, the final phase of relaxation (T90%R) was significantly prolonged following exposure to HOCl in some of the preincubation groups but not in others. Figure 1.11 shows two contractions from the same muscle in 2.5 mM $[\text{Ca}^{2+}]_e$ 10 minutes prior to and 10 minutes after the addition of HOCl to the bath. This figure clearly shows that the T90%R values were significantly increased in the latter tracing. This prolongation of T90%R following HOCl exposure was also observed under 5.0 mM $[\text{Ca}^{2+}]_e$ conditions or in the presence of Bay K 8644, but never under 0.5 mM $[\text{Ca}^{2+}]_e$ conditions, or in the presence of nifedipine (Table 1.3). This prolongation of T90%R was transient, and by 40 min the T90%R values had returned to normal, however the addition of DTT at 10 min caused a decrease in T90%R to normal values within 10 min after addition. This prolongation of T90%R was never seen in Control tissue from any of the groups with the exception of the ryanodine Controls. While the value for T90%R was increased following incubation in ryanodine, this value did not increase further with the addition of HOCl.

Figure 1.11 Two isometric twitches and their respective dDT/dt tracings from the same rat papillary muscle 10 min prior to (A) and 10 min after (B) the addition of 300 μ M HOCl, showing the effects on the relaxation phase. This muscle were contracting in 2.5 mM Ca^{2+} , 31°C, pH 7.4.

Table 1.3 Effects of HOCl on the time to 50% relaxation (T50%R) and time to 90% relaxation (T90%R) in rat papillary muscles. Although not obvious from the muscle shown in Figure 1.11, no significant differences were observed between Before and After HOCl treatment for T50%R in any of the pretreatment group (see Table 1.3 below). However, T90%R values were significantly prolonged following HOCl addition in muscles bathed in Tyrode buffer containing 2.5 or 5.0 mM $[\text{Ca}^{2+}]_e$ or in 2.5 mM $[\text{Ca}^{2+}]_e$ with Bay K 8644. No changes were observed in the time to maximal tension values in any of the pretreatment groups. The values represent mean (ms) $\pm$ SEM for n=8 muscles in each pretreatment group, measured 10 min prior to and 10 min after the addition of HOCl. * = $p < 0.05$ from Before value.

Figure 1.11

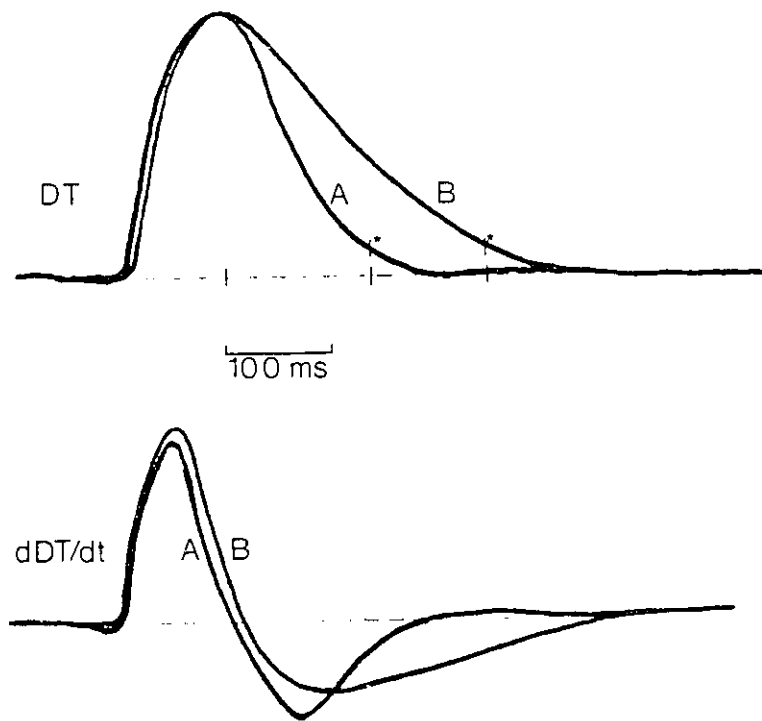


Table 1.3

	BEFORE HOCl		AFTER HOCl	
	T50%R	T90%R	T50%R	T90%R
0.5 mM Ca	86 ± 4	135 ± 3	76 ± 11	117 ± 9
2.5 mM Ca	91 ± 6	138 ± 6	101 ± 7	*184 ± 12
5.0 mM Ca	88 ± 9	140 ± 7	108 ± 14	*208 ± 13
Nifedipine	83 ± 7	145 ± 6	97 ± 8	127 ± 13
Bay K 8644	94 ± 6	145 ± 8	109 ± 11	*198 ± 12
Ryanodine	103 ± 6	186 ± 9	99 ± 10	179 ± 10

FREQUENCY-FORCE RELATIONSHIP:

THE EFFECTS OF $[Ca^{2+}]_e$, NIFEDIPINE, OR BAY K 8644 ON THE FREQUENCY-FORCE RESPONSE

Figure 1.12 shows the negative inotropic response to increasing stimulation frequency as observed in rat papillary muscle under three different $[Ca^{2+}]_e$, as well as in the presence of nifedipine or Bay K 8644. The curves derived from muscles incubated in 5.0 mM $[Ca^{2+}]_e$ or Bay K 8644 were shifted upwards, showing less of a negative inotropic response to the increasing stimulation rate as compared to 2.5 mM $[Ca^{2+}]_e$. Conversely, the curves from muscles incubated in 0.5 mM $[Ca^{2+}]_e$ or with nifedipine were shifted downward, showing a greater negative inotropic response as compared to 2.5 mM $[Ca^{2+}]_e$.

THE EFFECTS OF HOCl ON THE FREQUENCY-FORCE RESPONSE

Figure 1.13 expresses the negative inotropic response to increasing stimulation frequency as observed with the rat papillary muscle after exposure to 200 μ M HOCl. The control and experimental values were taken from the same muscles at: 10 min prior to HOCl addition; 40 min after the addition of 200 μ M HOCl; and after a further 20 min exposure to DTT (total of 60 min). Following exposure to HOCl for 40 min these muscles exhibited a significant upward shift of the curve, as compared to Control, which was indistinguishable from the Bay K 8644 curve shown in Figure 1.12. The addition of DTT for 20 min partially restored (NS) the frequency-force curve although it remained significantly different from Control. Longer exposure to DTT had no greater ameliorative effect. Resting state DT for the muscles treated with 200 μ M HOCl was approximately 45% of Control, while resting state DT for the HOCl + DTT treated muscles was approximately 65% of Control.

Figure 1.12 A) An illustration of the frequency-force protocol, depicting the decline in steady-state DT with increasing stimulation frequency in the rat papillary muscle. The steady-state DT at each frequency is expressed as a percentage of the resting state contraction (RSC) measured at 3 per minute. B) The effects of increasing or decreasing $[Ca^{2+}]_e$, or the presence of 1 μ M Bay K 8644 or 1 μ M nifedipine on the negative inotropic response to increasing stimulation frequency. Values shown are mean $\pm$ SE, n = 8 for each group. Statistical significance was determined using one-way ANOVA at each frequency. * = $p < 0.05$ from the Control (ie 2.5 mM $[Ca^{2+}]_e$) value. T=31°C, pH 7.4.

Figure 1.12

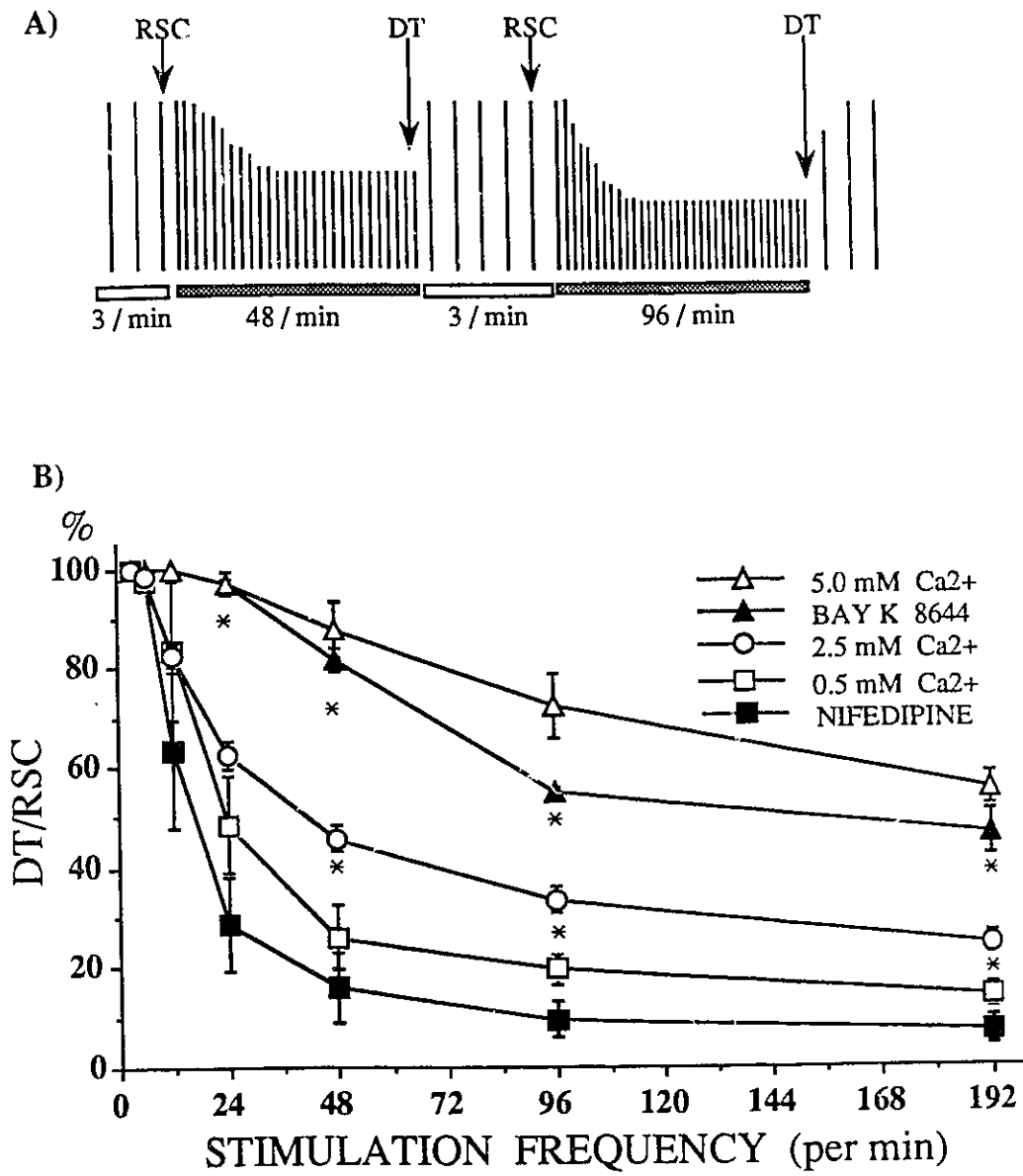
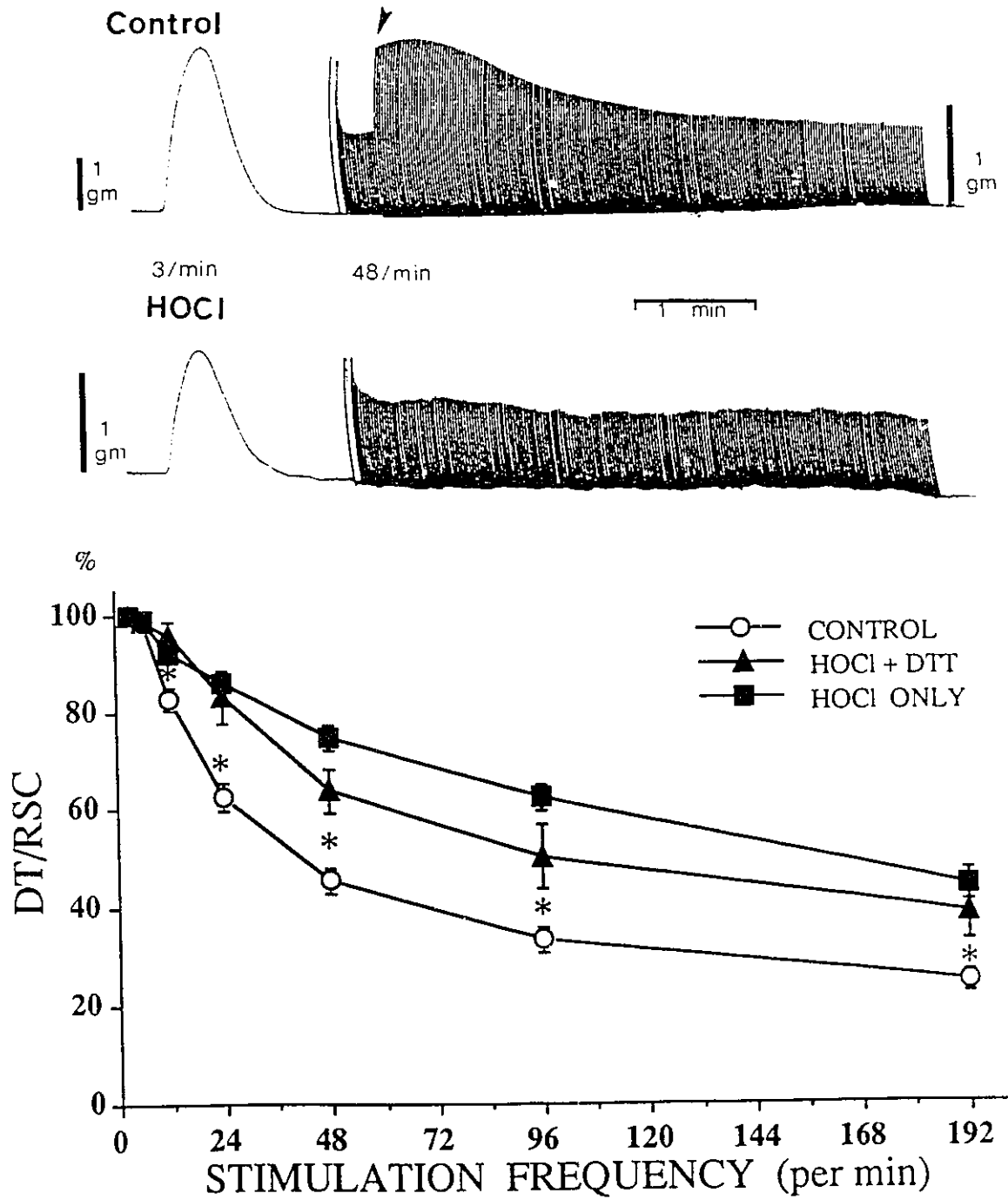


Figure 1.13 A) A photograph showing the tension developed by a Control (top panel) and HOCl-treated (lower panel) rat papillary muscle in response to increasing stimulation frequency. Note the change of scale (arrow) in the Control tracing, such that the DT at 48/min was less than 40% of the RSC. Also note that the decline in DT in the HOCl-treated muscle was rapid, reaching steady state within 15 sec at 48/min, as compared to almost 4 min in the Control muscle. The effects of HOCl with or without DTT are summarized in B). The frequency-force protocol was performed 10 min prior to the addition of HOCl (Control), 40 min after the addition of 200 μ M HOCl (HOCl Only), and again 40 min after the addition of 500 μ M DTT (HOCl + DTT). A second group of Control muscles were stimulated in conjunction with the HOCl protocols and no change in the frequency-force response was observed over time (eg. see A above). Values shown are mean $\pm$ SE, n = 8. Statistical significance was determined using two-way ANOVA, with Treatment and Frequency as the independent variables. * = $p < 0.05$ from Control. No significant differences were found between the HOCl Only and HOCl + DTT values.

Figure 1.13



PAIRED-PULSE RELATIONSHIP

THE EFFECTS OF $[Ca^{2+}]_e$, NIFEDIPINE, OR BAY K 8644 ON THE PAIRED PULSE RESPONSE

Figure 1.14 shows the potentiation of DT as a result of paired pulsing in rat papillary muscles under Control (2.5 mM $[Ca^{2+}]_e$) conditions, or in the presence of nifedipine, Bay K 8644, or ryanodine. Control muscles exhibited a 50% increase in the PP/SP ratio as the interval between the paired pulses decreased from 300 ms to 80 ms. Muscles incubated with nifedipine showed a similar level of potentiation as compared to Control muscles, except that the inotropic response to the paired pulses becomes negative at very long pulse intervals (ie. 300 ms). Muscles incubated with Bay K 8644 exhibited no response to the paired pulsing, suggesting that the SR was fully saturated, and that these muscles were developing the maximum possible tension with each single stimulation. The actual values for the initial DT for each of these pretreatment protocols are similar to those listed previously in Table 1.1

Muscles incubated in ryanodine exhibited a highly significant increase in the PP/SP ratio as compared to Control at all pulse intervals. However, throughout the stimulation protocols, the steady-state DT values for muscles treated with ryanodine decreased over time (not shown) making interpretation of the data difficult.

THE EFFECTS OF HOCl ON THE PAIRED PULSE RESPONSE

Figure 1.15 expresses the response to decreasing paired pulse interval in 2.5 mM $[Ca^{2+}]_e$ 10 min prior to the addition of 200 μ M HOCl, after 40 min exposure to HOCl, and after 40 min exposure to HOCl followed by 20 min of DTT. Prior to HOCl addition, the muscles showed an increase in the PP/SP ratio of about 55% as the interval between the paired pulses decreased from 300 ms to 80 ms. Following exposure to HOCl for 40

Figure 1.14 A) An illustration of the paired pulse protocol, depicting the increase in steady-state DT with paired pulses in the rat papillary muscle. B) The effects of a 45 min preincubation with 1 μ M Bay K 8644, 1 μ M nifedipine, or 1 nM ryanodine on the positive inotropic response to decreasing paired pulse interval. The steady-state DT in response to paired pulses (PP) is expressed as a percentage of the steady-state DT in response to single pulses (SP) at 24/min. Control muscles were incubated in 2.5 mM $[\text{Ca}^{2+}]_e$ only. The values for initial DT for each treatment group are similar to those expressed in Table 1.1. Values shown are mean $\pm$ SE, n = 8 for each group. Statistical significance was determined using one-way ANOVA at each frequency. * = $p < 0.05$ from the Control (ie 2.5 mM $[\text{Ca}^{2+}]_e$) value. T=31°C, pH 7.4.

Figure 1.14

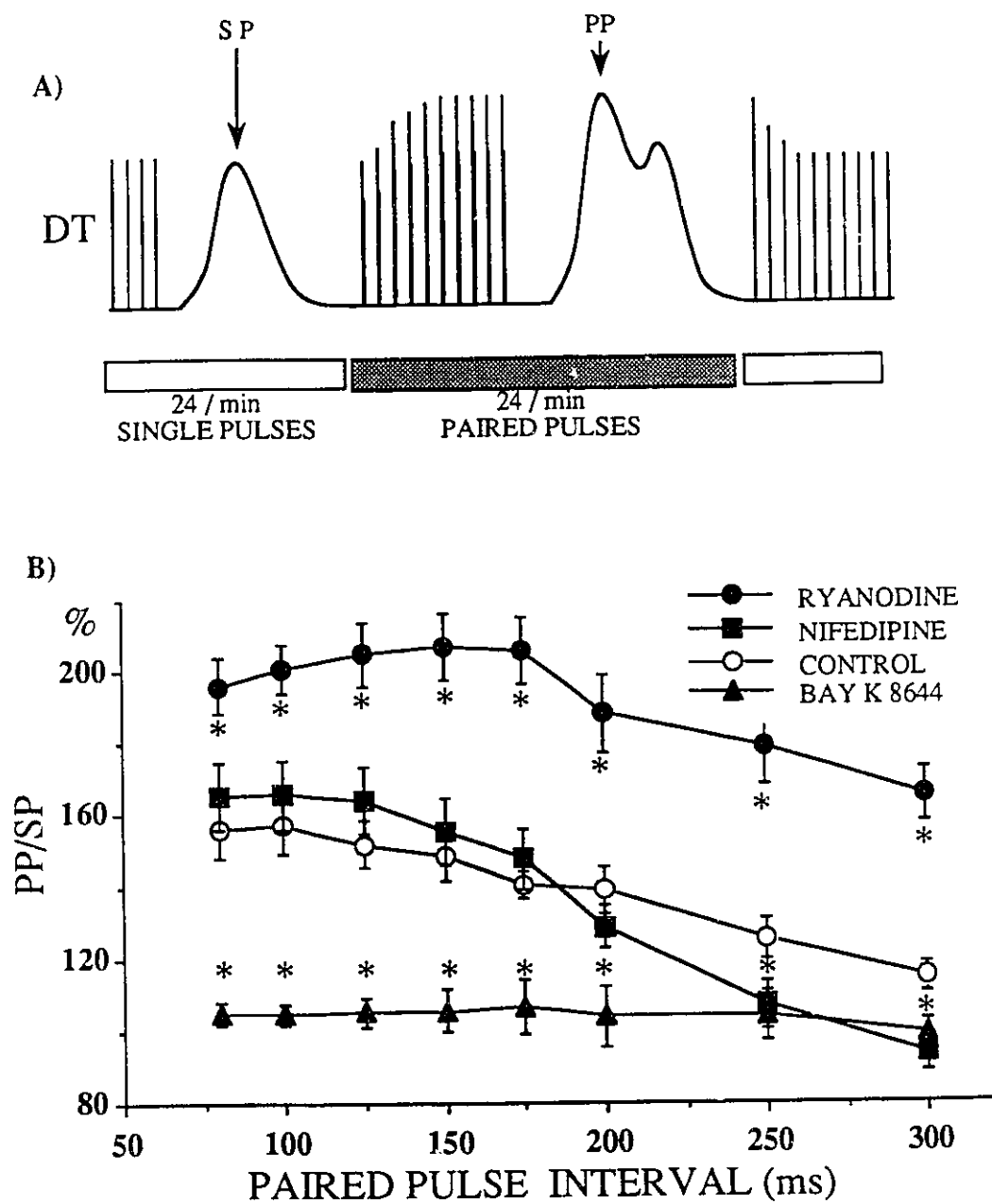
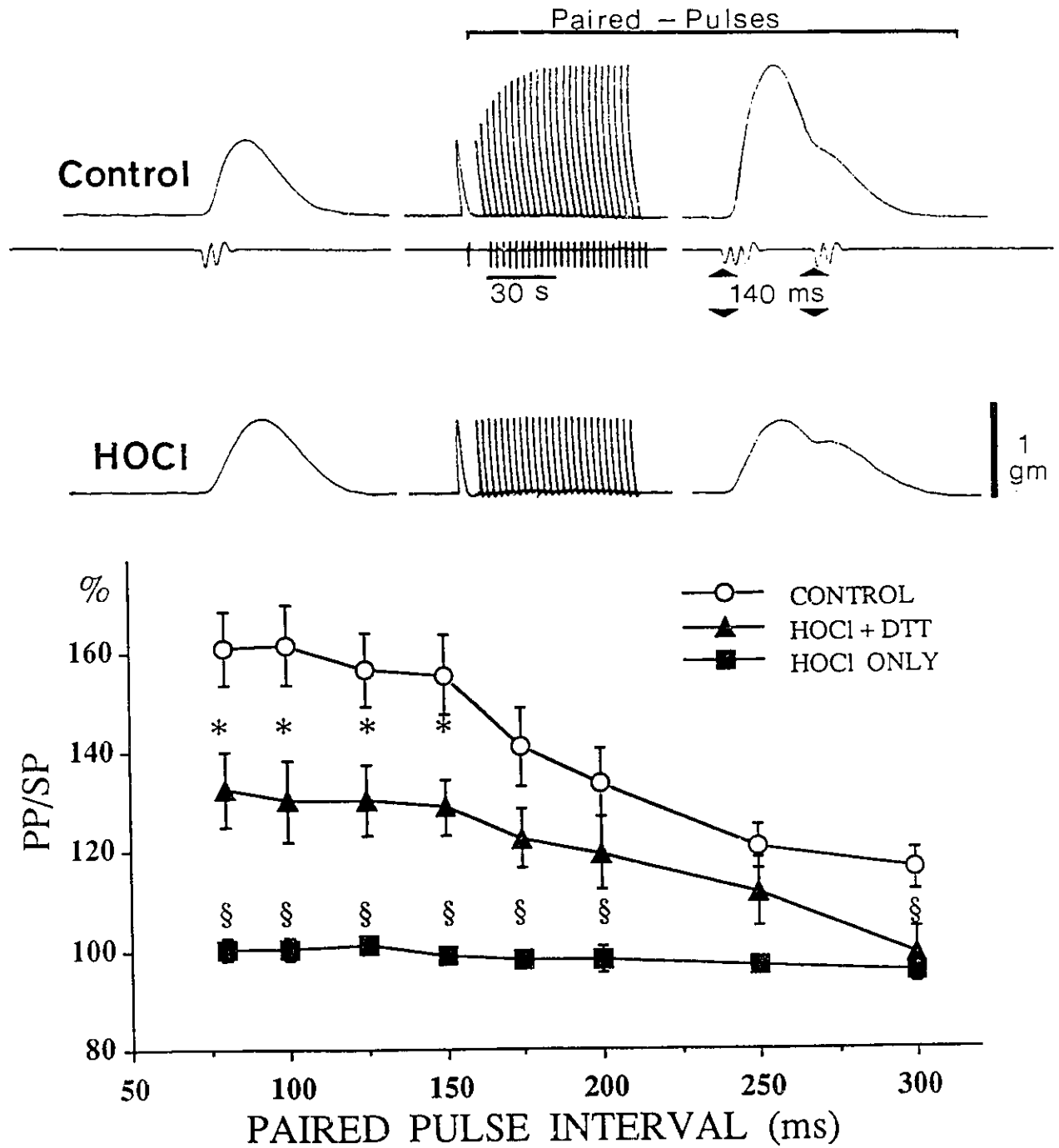


Figure 1.15 A) A photograph showing the tension developed by a Control (top panel) and HOCl-treated (lower panel) rat papillary muscle in response to simultaneous paired pulse stimulation. The effects of HOCl with or without DTT are summarized in B). The paired pulse protocol was performed 10 min prior to the addition of HOCl (Control), 40 min after the addition of 200 μ M HOCl (HOCl Only), and again 40 min after the addition of 500 μ M DTT (HOCl + DTT). Values shown are mean $\pm$ SE, n = 8. Statistical significance was determined using two-way ANOVA, with treatment and paired pulse interval as the independent variables. * = $p < 0.05$ from Control, § = $p < 0.05$ from HOCl + DTT. The differences between Control and HOCl Only were significant at all intervals, and the symbols are omitted for clarity.

Figure 1.15



minutes, the same muscles showed no increase in the PP/SP ratio with decreasing pulse interval, suggesting that the maximal possible contraction was occurring with each single pulse. Subsequent treatment of these muscles with DTT for 20 min partially restored the PP/SP ratio, but the ratio was still significantly different from Control.

PROTEIN AND NON-PROTEIN SULFHYDRYLS

Figures 1.16 to 1.19 summarize the protein(P-SH) and non protein (NP-SH) thiol levels measured following the various perfusion protocols. After 10 min of exposure to 300 μ M HOCl the concentration of NP-SH (Figure 1.16) within the tissue declined to <50% of Control levels, while the concentration of P-SH remained essentially unchanged (Figure 1.17). Washout of HOCl at 10 min attenuated the loss of cellular thiols, such that by 80 min both NP-SH and P-SH levels were not significantly lower than the 10 min values. The addition of DTT at 10 min also prevented any subsequent loss in P-SH, and resulted in a significant increase in NP-SH by 80 min. This significant restoration of NP-SH may not represent a true replenishment of cellular GSH levels, as part or all of the additional NP-SH may be attributed to the sulfhydryls of residual DTT within the tissue.

Longer exposure to HOCl resulted in a further depletion of cellular thiols in a time dependent manner, such that by 40 min both NP-SH and P-SH were significantly lower than the 10 min values. Washout of HOCl at 40 min produced no significant protection against the further loss of either NP-SH or P-SH. Conversely, the addition of DTT at 40 min restored both NP-SH and P-SH back to approximately 30% of Control values by 80 min.

Figures 1.18 and 1.19 express the NP-SH and P-SH values as measured from muscles exposed to HOCl for a total of 80 min (Front row in both figures), or 40 min exposure to HOCl followed by 40 min with DTT (Back row in both figures) under three $[Ca^{2+}]_e$ conditions (0.5, 2.5, and 5.0 mM $[Ca^{2+}]_e$) or in the presence of nifedipine,

Figure 1.16 Non-protein thiol (NP-SH) concentrations (nmoles SH/mg tissue wet weight) in rat papillary muscles following exposure to HOCl with or without DTT or Washout. The clear columns (CON, CON DTT) express NP-SH in Control muscles after 80 min stimulation protocols with or without the addition of DTT at 40 min; the stippled columns (H10, H40, and H80) express NP-SH in muscles after 10, 40, and 80 min exposure to HOCl respectively; the striped columns (W10 and W40) express NP-SH in muscles after Washout at 10 and 40 min respectively; the black columns NP-SH in muscles after the addition of DTT at 10 and 40 min respectively. Values represent mean $\pm$ SEM for n = 8 muscles per column. Statistical comparisons were made using one-way ANOVA with treatment as the independent variable. Differences between the means were determined using Scheffees post-hoc test.

* = $p < 0.05$ vs CON; † = $p < 0.05$ vs H40; § = $p < 0.01$ vs H80.

Figure 1.16

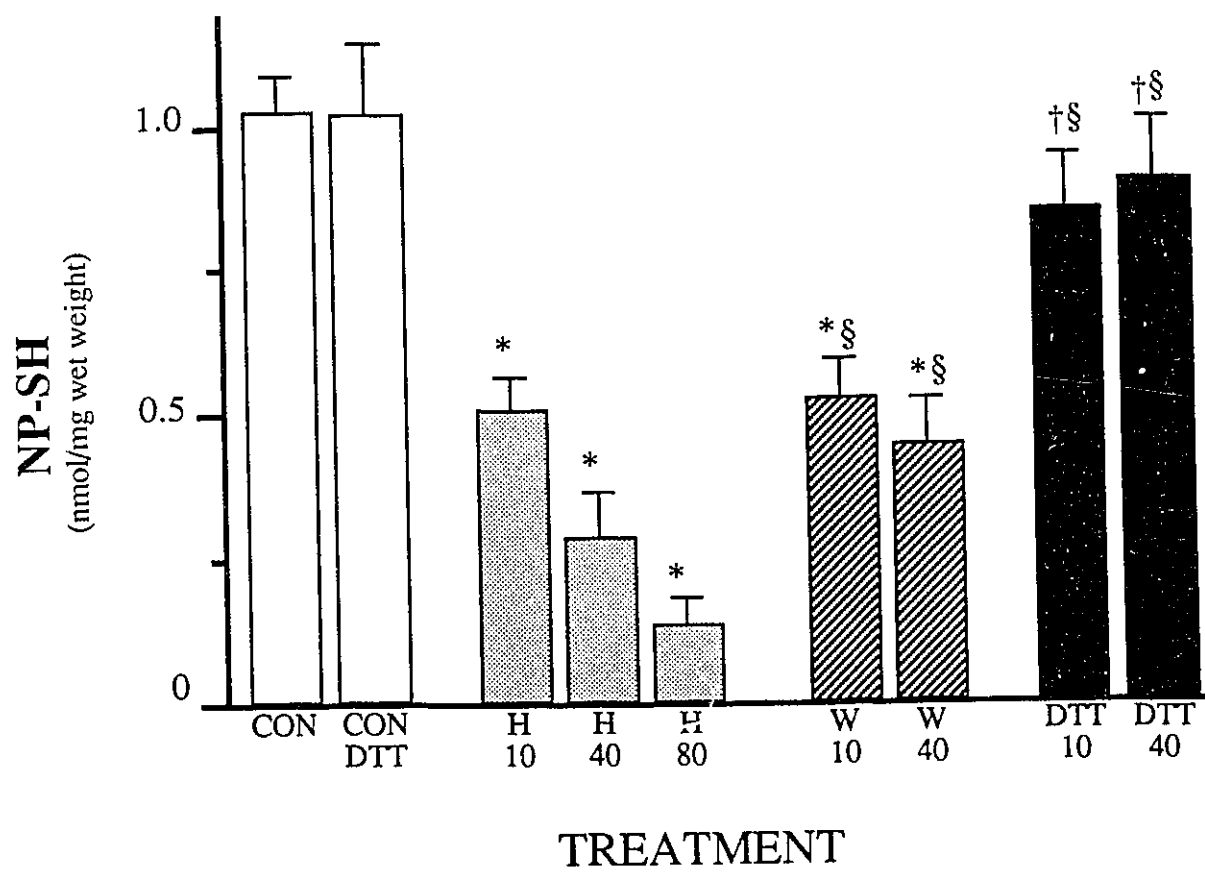


Figure 1.17 Protein thiol (P-SH) concentrations (nmoles SH/mg protein) in rat papillary muscles following exposure to HOCl with or without DTT or Washout. The column labels are as described in Figure 1.16. * = $p < 0.05$ vs Control; † = $p < 0.05$ vs H40; § = $p < 0.01$ vs H80.

Figure 1.17

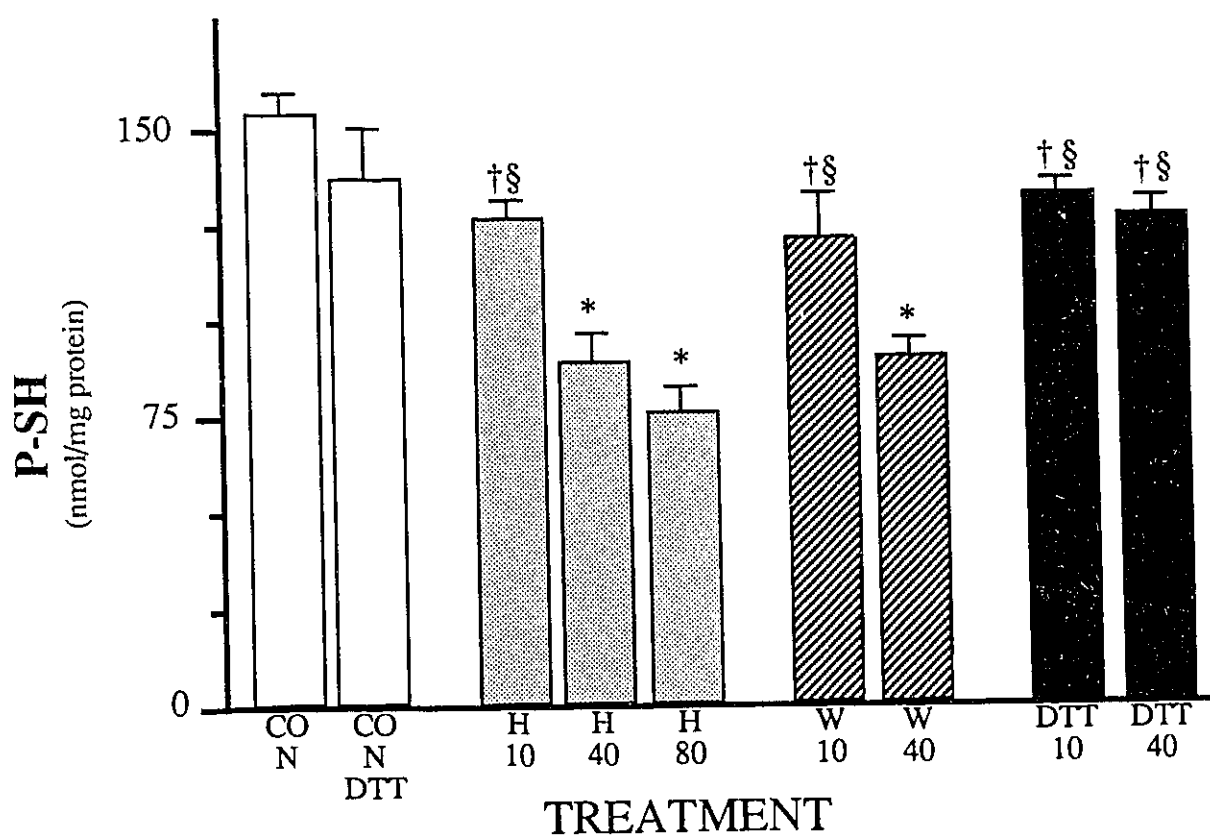


Figure 1.18 Effects of extracellular $[Ca^{2+}]$ or the presence of pharmacological agents on HOCl-induced depletion of non-protein thiols (NP-SH) in rat papillary muscle. The front row (white columns) show NP-SH after 80 min protocols with HOCl only. The back row (stippled columns) show NP-SH after 80 min HOCl protocols in which DTT was added at 40 min. No significant differences were observed between any Control values for any of the preincubation groups so only Control in 2.5 mM $[Ca^{2+}]_e$ is depicted here. No significant differences were observed between any of the HOCl + DTT values with the exception that ryanodine was different from the 2.5 mM Ca^{2+} value. All HOCl + DTT (back row) values were significantly different ($\ddagger = p < 0.05$) from the corresponding HOCl Only (front row) values. Values represent means $\pm$ SEM of a minimum of n=8. * = $p < 0.05$ vs the corresponding Control value; $\S = p < 0.05$ as indicated.

Figure 1.18

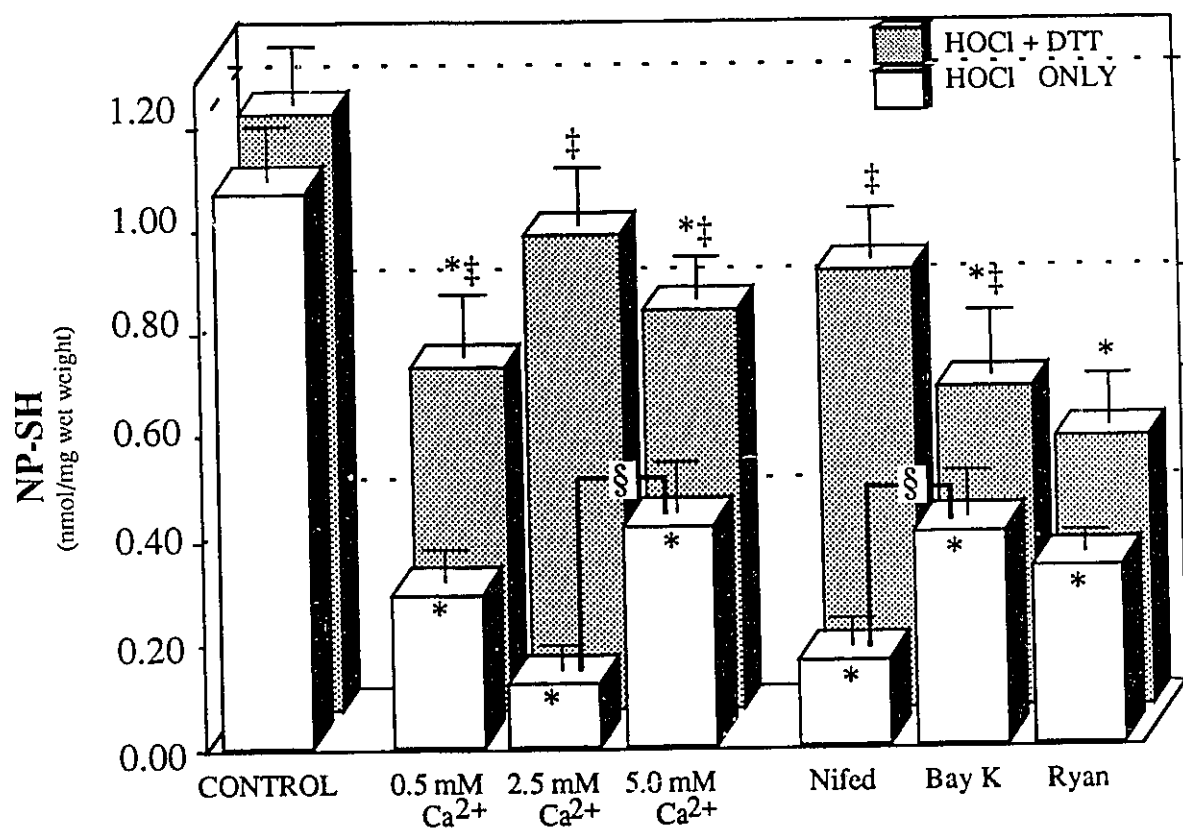
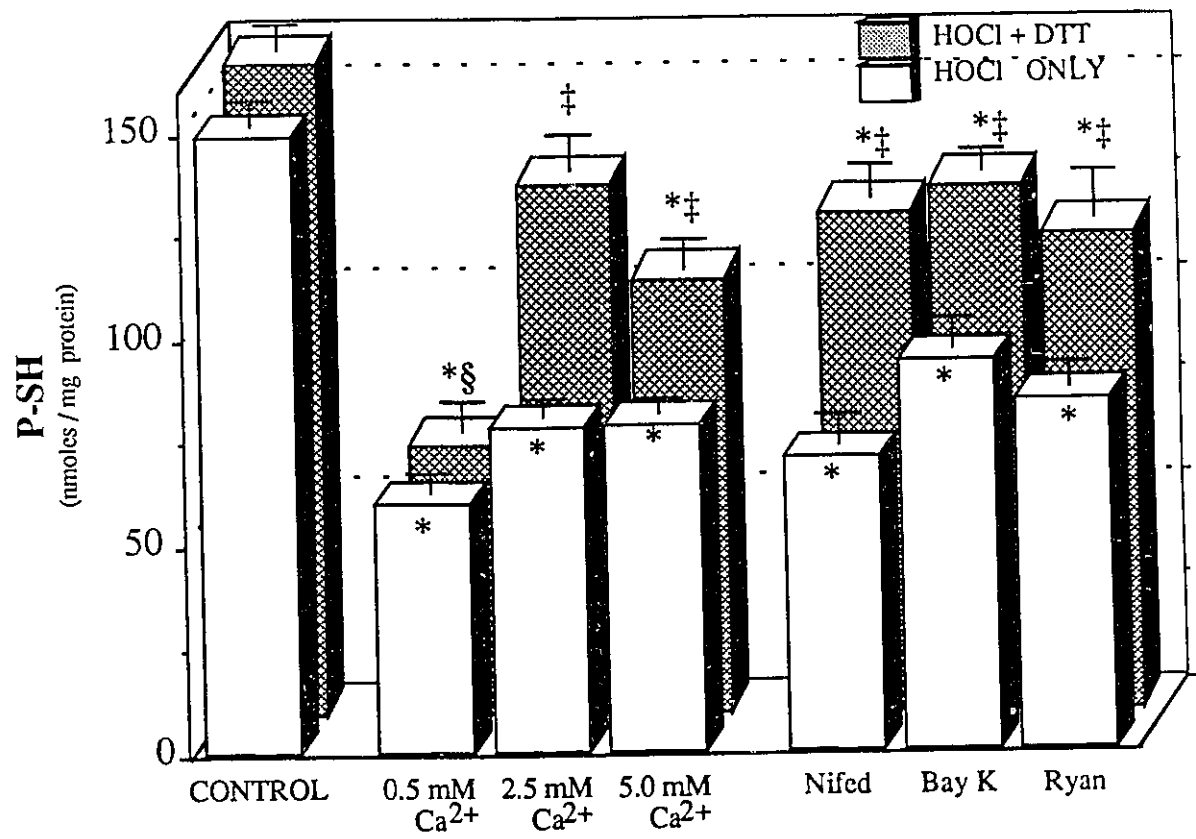


Figure 1.19 Effects of extracellular $[Ca^{2+}]$ or the presence of pharmacological agents on HOCl-induced depletion of protein thiols (P-SH) in rat papillary muscle. The columns are as indicated in Figure 1.18. All HOCl + DTT values (back row) are significantly different ($\ddagger = p < 0.05$) from their corresponding HOCl only values (front row) with the exception of 0.5 mM Ca^{2+} . No differences were found between any HOCl Only values. No significant differences were observed between any of the HOCl + DTT values with the exception that 0.5 mM $[Ca^{2+}]$ was different ($\S = p < 0.05$) from all others. All values represent means $\pm$ SEM of a minimum of n=8. * = $p < 0.05$ vs the corresponding Control value.

Figure 1.19



Bay K 8644, or ryanodine as described in Methods. No differences were found in either the NP-SH or P-SH levels between any of the Control groups, therefore only the values for Control muscles in 2.5 mM $[Ca^{2+}]_e$ are shown here.

NP-SH levels (Figure 1.18) were significantly lower ($p < 0.01$) in those muscles exposed to HOCl for 80 min under all pretreatment conditions (front row) as compared to Control. Preincubation with Bay K 8644, or with 5 mM $[Ca^{2+}]_e$ did appear to offer some protection against NP-SH depletion when compared to 2.5 mM $[Ca^{2+}]_e$ values or nifedipine, but not when compared to all other conditions. No significant differences were noted between 0.5 and 2.5 mM $[Ca^{2+}]_e$ values. The level of NP-SH in muscles treated secondarily with DTT for the final 40 min (back row) was significantly higher in all experimental groups (excluding Control) except Bay K 8644. However, because part or all of this additional NP-SH may be attributed to the sulfhydryls of residual DTT itself, no biological significance could be attributed to this increase.

P-SH levels (Figure 1.19) were significantly lower than Control in muscles exposed to HOCl for 80 min under all the pretreatment conditions. HOCl induced a significantly greater depletion of P-SH in those muscles incubated under 0.5 mM $[Ca^{2+}]_e$ as compared to all the other conditions. Preincubation with 5.0 mM $[Ca^{2+}]_e$ or Bay K 8644 did not offer any significant protection against P-SH oxidation. The level of P-SH in muscles treated secondarily with DTT for the final 40 min (back row) was significantly higher in all experimental groups except 0.5 mM $[Ca^{2+}]_e$. With the latter exception, there were no significant differences between P-SH levels for any of these HOCl + DTT treated muscles. Low $[Ca^{2+}]_e$ may have predisposed cellular proteins to more extensive thiol depletion that was not reversible with DTT reduction.

SKINNED PAPILLARY MUSCLE PREPARATION

INITIAL CONTRACTILE RESPONSE

The 30 isometrically contracting papillary muscles ($n = 10$ for each group) used in this study exhibited a similar decline in contractile function in response to HOCl to that described in the previous sections. Briefly, these papillary muscles showed an elevation of RT with a concomittant depression of DT following exposure to $300 \mu\text{M}$ HOCl. This effect was reversed by subsequent exposure to 1 mM DTT leading to a partial restoration of contractile function within 40 minutes. Following these protocols, the papillary muscles were chemically skinned and glycerinated, then exposed to increasing $[\text{Ca}^{2+}]_e$ as tension development was measured (see Methods).

pCa-RELATIVE TENSION

The relationship between pCa (negative log of $[\text{Ca}^{2+}]$) and the relative contractile response of the skinned muscle is summarized in Figure 1.20. The maximal tension (observed at $\text{pCa} = 4.5$) that each muscle developed was defined as 100% and all other tensions are expressed in relation to this. pCa_{50} , or the concentration of Ca^{2+} at which 50% maximal contraction was attained, was ≈ 5.75 for all three experimental groups. There was no significant shift in the pCa-Tension relationship in the HOCl-treated muscles, suggesting that the sensitivity of the myofibrils to Ca^{2+} was not altered following HOCl exposure.

pCa-ABSOLUTE TENSION

Figure 1.21 expresses the pCa-Tension relationship in terms of absolute developed tension (DT) values (in mg/mm^2). The results show that the ability of the myofilaments to develop tension was impaired following HOCl exposure as compared to

Figure 1.20 Relative tension developed by Triton-X100 treated rat papillary muscles in response to increasing $[Ca^{2+}]_e$. Tension values for each muscle were normalized with respect to the maximum tension developed by that muscle. The pCa-50, or the pCa at which each muscle developed 50% of its maximal tension was 5.72 ± 0.12 for Control vs 5.78 ± 0.13 for HOCl treated muscles. n=10 for each curve.

Figure 1.20

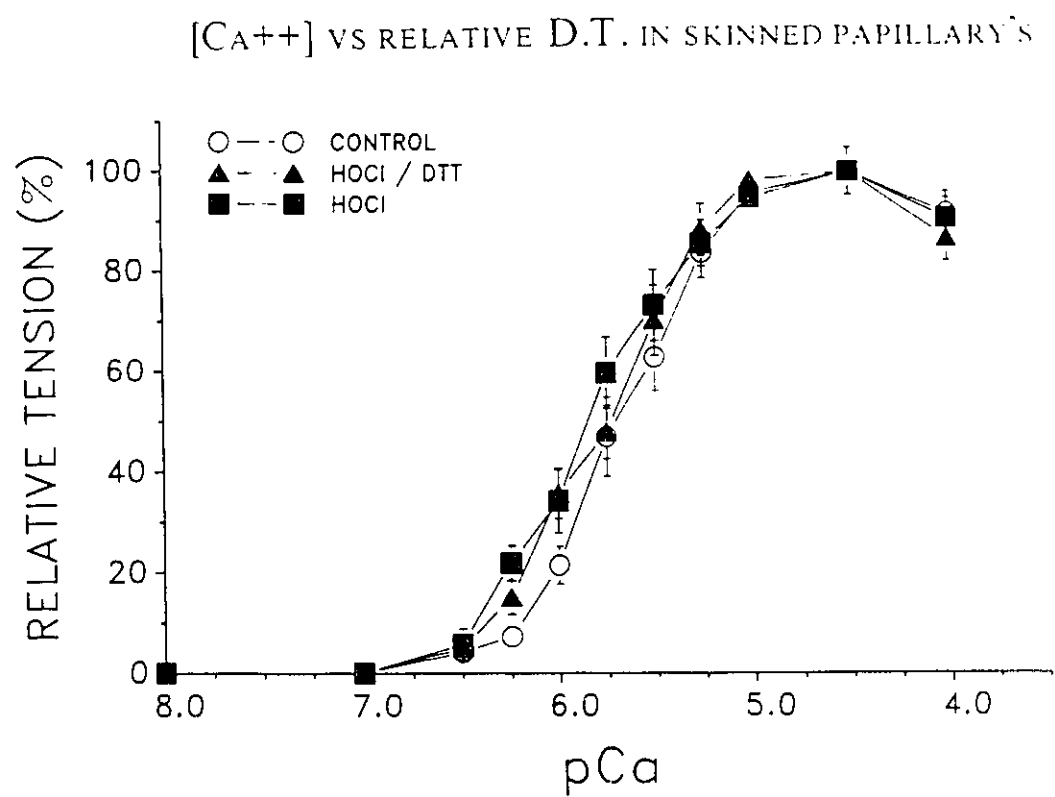
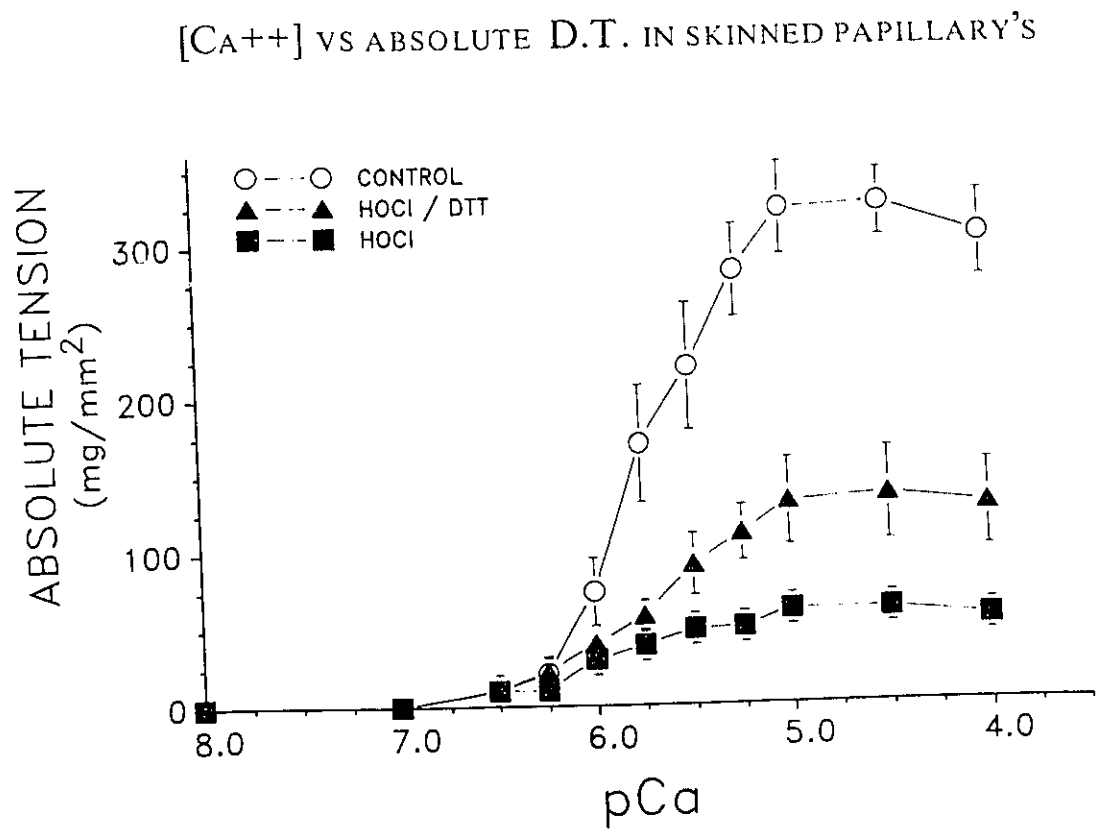


Figure 1.21 Absolute tension values (in mg/mm^2) recorded from the same Triton-X100 treated muscles described in Figure 1.20. $\ast = p < 0.05$ from Control, $\S = 0.05$ from HOCl.

Figure 1.21



control. Those muscles treated with DTT subsequent to HOCl developed significantly greater tension than those exposed to HOCl Only although this was still significantly less than Control.

ACTIN AND MYOSIN SH LEVELS

Figure 1.22 shows 3 lanes from a SDS-PAGE gel used to separate the myofibrillar proteins from the skinned muscle preparations. No change in the migration patterns in the actin and myosin proteins isolated from the HOCl-treated muscles were observed as compared to Control, although some 'smearing' of other minor proteins is observed (see Figure 1.22). The corresponding autoradiogram shows the level of ^{14}C -IAA bound to each protein, taken to represent the level of free thiols. The level of ^{14}C -IAA bound to the contractile proteins from HOCl-Only skinned muscles was significantly reduced as compared to either Control or the HOCl + DTT lanes. The level of ^{14}C -IAA bound to proteins from the HOCl + DTT treated muscles is not different from the Control. The bands were cut from the gel and the level of ^{14}C -IAA bound to each protein was quantified (CPM/ μg protein) using scintillation counting. These results are summarized in Table 1.4. Myosin appears to be more susceptible to thiol oxidation than is actin.

Figure 1.22 Three lanes from a Coomassie Blue stained 7.5% SDS-PAGE gel and its corresponding autoradiogram. Actin and myosin were isolated as described in Methods and labelled with ^{14}C -IAA prior to electrophoresis. Lane 1 = Control muscle, Lane 2 = HOCl only for 80 min, Lane 3 = HOCl for 80 min with DTT added at 40 min.

Figure 1.22

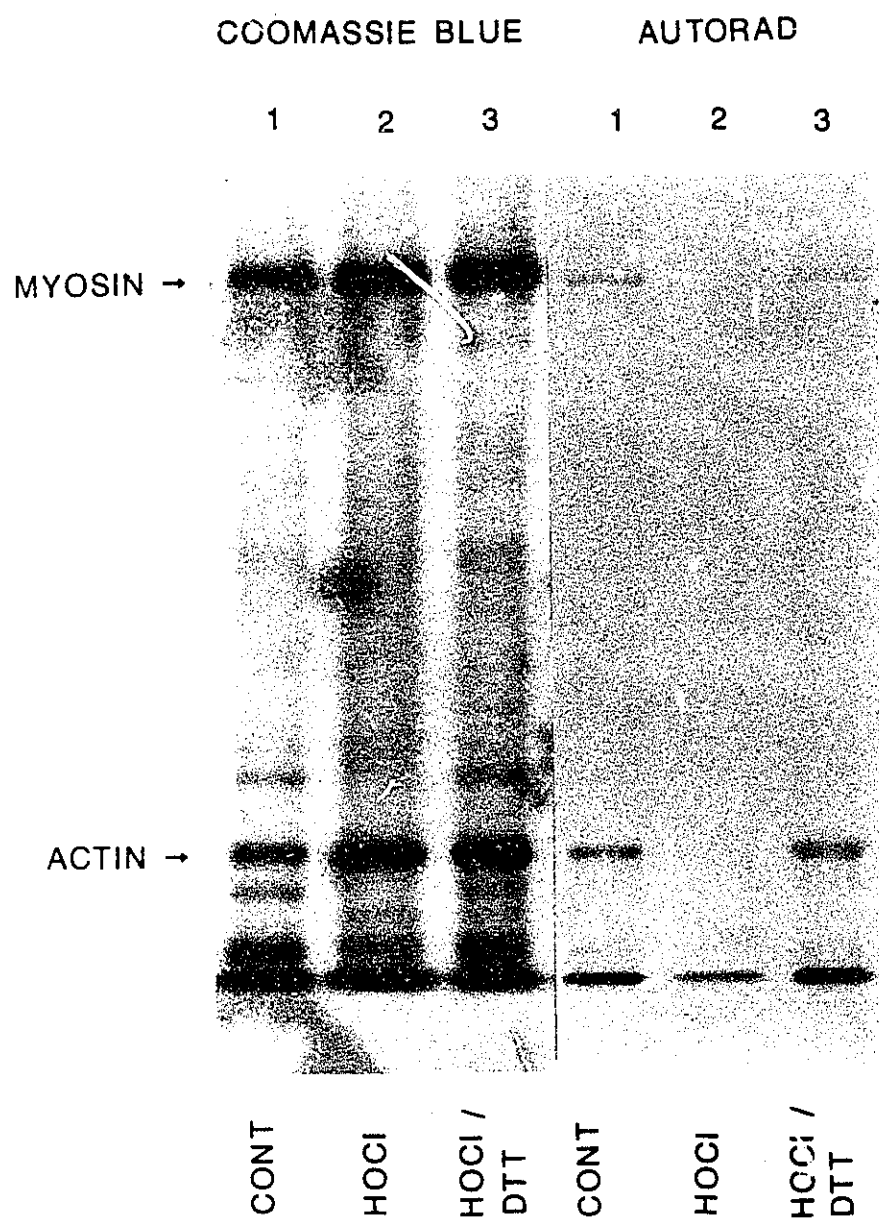


Table 1.4 ^{14}C -IAA binding to actin and myosin. Protein concentration was determined using laser densitometry against a standard curve of rabbit cardiac actin and myosin run on each gel. Actin and myosin bands were cut from each SDS-PAGE gel (eg see Figure 1.22) digested for 3 hours in H_2O_2 at 60°C , then counted in a scintillation counter. Background counts were subtracted, and ^{14}C -IAA binding is expressed as counts per minute (CPM) per μg of protein in each band. * = $p < 0.05$ from Control value, ‡ = $p < 0.05$ from HOCl 80 min value. $n = 10$ for each column.

Table 1.4

	PROTEIN THIOL CONTENT			
	CPM / μ g Protein		Percent of Control	
	Actin	Myosin	Actin	Myosin
CONTROL	200 $\pm$ 18	94 $\pm$ 7	---	---
HOCl 80 min	* 49 $\pm$ 7	‡*10 $\pm$ 2	25%	11%
HOCl + DTT	‡*130 $\pm$ 11	‡*62 $\pm$ 14	65%	66%

DISCUSSION

The results from these studies show that the rat myocardium is susceptible to protein thiol depletion and concomitant mechanical dysfunction following exposure to the neutrophil-derived oxidant, HOCl. Exposure to HOCl for as little as 10 min produced a significant depletion of NP-SH (ie. GSH) with no significant loss of P-SH, suggesting that the protein thiols were protected from oxidation by HOCl at the expense of GSH and other endogenous thiol-containing compounds, as alluded to by Reed (Reed and Farris 1984). Removal of the oxidant (Washout) at 10 min permitted a spontaneous and almost complete recovery in muscle function, but no change of P-SH. The addition of dithiothreitol, a disulfide reducing agent, at 10 min gave no greater recovery of contractile function or P-SH than did Washout. This suggests that the initial decline in function (ie. up to 10 min) was not a direct result of protein thiol depletion.

Prolonged exposure to the oxidant (ie. 40 min) resulted in an apparent 'permanent' injury which was not prevented or reversed by Washout, suggesting that the endogenous scavenging and repair mechanisms may become overwhelmed or inactivated with time (Aruomo and Halliwell 1987). Similar observations showing a time-dependent spontaneous recovery in cells exposed to oxidizing agents have been made previously (Lauf 1988).

Of great interest in the present study was that the addition of DTT after 40 min of HOCl induced a highly significant restoration of P-SH that correlated with a recovery of contractile function in the papillary muscle. These results implicate the oxidation and subsequent reduction of cellular protein thiols in the depression and subsequent recovery of contractile function in the rat myocardium, but do little to indicate which proteins are involved. Given that a large percentage (~50%) of the protein thiols were depleted

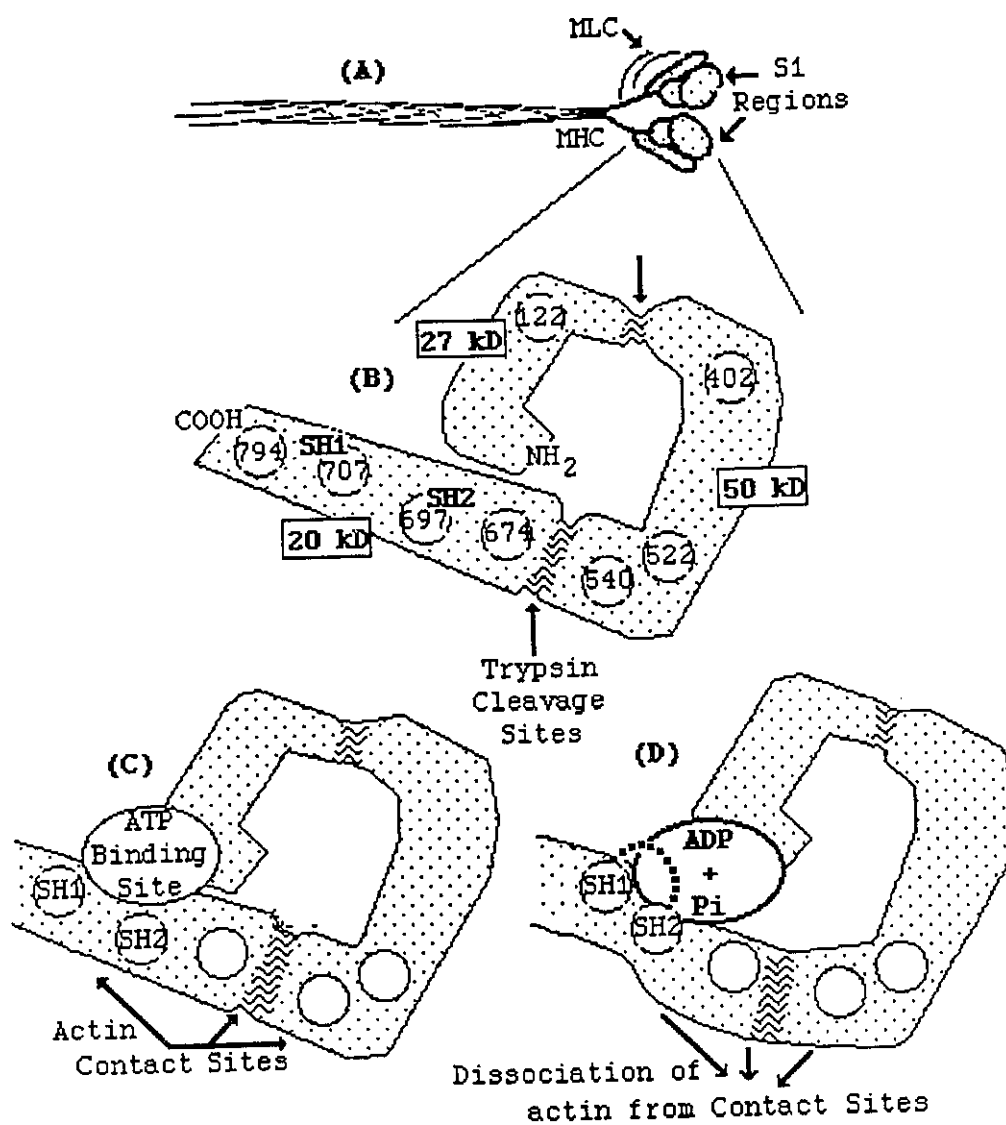
following 80 min of HOCl exposure, it would be reasonable to assume that the major proteins (ie. contractile proteins) of the myocardium must be involved. Both actin (Hinshaw et al. 1986, Mullane 1988) and myosin (Daniel and Hartshorne 1972, Huber et al. 1989, Reisler 1985, Titus et al. 1989) are known to be susceptible to thiol oxidation, and the alteration of the thiol redox status in these proteins may substantially alter their functional ability.

To examine whether the oxidation of sulfhydryl residues within the proteins of the contractile apparatus may have been involved in the HOCl-induced contractile dysfunction, the skinned papillary muscle model was used. Results from these studies showed that the decline in DT as observed in the electrically stimulated membrane intact papillary muscle preparation was also manifest in the free Ca^{2+} stimulated skinned muscle preparations. The finding that the relative tension developed by each muscle was similar at each $[\text{Ca}^{2+}]$ examined suggests that the sensitivity of the muscles to Ca^{2+} was not impaired following exposure to HOCl. However, the ability of the muscles to develop tension at any given Ca^{2+} was significantly impaired following exposure to HOCl. This could be interpreted as the sensitivity of troponin C to the binding of Ca^{2+} was not substantially altered, but that the tension developed as a product of the cross-bridge reaction between actin and myosin was severely depressed.

In order to understand how thiol oxidation within the contractile proteins could alter the contractile performance of the myocardium, we must first consider the structure of the proteins involved. In a recent review paper (Audemard et al. 1988), Audemard describe the structure of the myosin molecule as discerned using various enzymatic treatments. Cleavage of the myosin molecule with chymotrypsin allows for the dissociation into a 200 kD myosin heavy chain (MHC) portion and two different light chains, commonly referred to as the alkali and the regulatory light chains (see Figure 1.23). The MHC can be further cleaved to produce two 95kD S1 segments (the globular

Figure 1.23 A schematic representation (adapted from Audemard et al, 1988) depicting a myosin molecule (A), showing the myosin heavy-chain (MHC), light chains (MLC), and S1 regions of the globular heads (see text). An expansion of the S1 region (B) shows the three major subfragments (27 kD, 50 kD, and 20 kD) as obtained following Trypsin cleavage and the eight cysteine residues (circles). The ATP binding site (C) is dependent upon SH1 and SH2 being in their reduced state (see text). The crosslinking between SH1 and SH2 (D) or with other nearby thiols may induce conformational changes which inhibit the normal cross-bridge cycling interaction between actin and myosin.

Figure 1.23



Dissociation of actin from Contact Sites

heads of the myosin molecule) which have been shown to retain all the ATPase activities of the myosin molecule (Wagner and Giniger 1981). This S1 region has been shown to contain the actin binding domain (Kasprzak et al. 1989) as well as the nucleotide binding site (Walker and Trinick 1988), which are spatially separate but highly interdependent.

Rabbit cardiac myosin contains up to 36 cysteine residues (Tada et al. 1969), of which six are located on the light chains, and a total of eight are distributed throughout each of the S1 heavy chain subfragments (Audemard et al. 1988) as depicted in Figure 1.23. Pfister et al (1975) showed that four reduced SH residues, located adjacent to the nucleotide binding site on the 20 kD S1 subfragment, are essential for maintaining the activity of the K⁺-stimulated myofibril ATPase as well as the Ca²⁺ stimulated myofibril ATPase. These cysteine residues include the two highly reactive thiols depicted as SH1 and SH2, which are known to be critical for maintenance of the ATPase activity (Wells and Yount 1979). The binding of ATP to the nucleotide binding site is thought to induce a conformational change in the 20 kD subfragment, thereby exposing these thiols to oxidation. It has been shown that the crosslinking of SH1 and SH2 following the binding of the nucleotide can effectively 'trap' the nucleotide within the binding site, thereby inactivating the enzyme (Figure 1.23 D). Of great interest was a study by Chaussepied and co-workers (1986) which showed that the modification of SH1 with DTNB leads to a loss of myosin ATPase activity, but subsequent treatment with dithioerythritol (DTE), an isomer of DTT (Cleland 1964), restores this ATPase activity. This suggests that the loss of ATPase activity following thiol oxidation may be a reversible reaction.

In a further study, Kasprzak et al (1989) located the contact site between actin and myosin (actin binding domain) close to the nucleotide binding site (Figure 1.23 C). They showed that the binding of the ATP to the nucleotide binding site caused a significant

decrease in the distance between the SH1 and SH2 thiols, and a concomitant increase in the distance between SH2 and the nearby actin binding domain. This conformational change was shown to disrupt the electrostatic and hydrophobic actin contacts (Chaussepied et al. 1986), producing a dissociation of actin and myosin (Figure 1.23 D). These data suggest that crosslinking between SH1 and SH2, or between SH1 and the other thiols within the myosin molecule will alter the conformation of the actin binding domain and inhibit the binding of actin to the myosin S1 region. In the intact muscle, such a reduction in cross-bridges available for cycling would lead to a decrease in tension development during a contraction. (Eisenberg and Greene 1980).

Actin (rabbit skeletal) has been sequenced and shown to contain 5 free cysteine residues, with no disulfides under normal condition (Collins and Elzinga 1975) . The availability of these thiol residues to bind with iodoacetamide (IAA) changes during the contraction cycle, either due to changes in conformation of the protein during the interaction, or due to the involvement of these thiols in the actual binding of actin to myosin (Collins and Elzinga 1975). This suggests that thiol residues may be actively involved in the contractile process, perhaps by maintaining the integrity of the binding site for myosin. In a study using isolated hepatocytes (Mirabelli et al. 1988), the depletion of free thiols within the actin molecule of the cytoskeleton following exposure to an oxygen free radical generating system resulted in the cross-linking between several actin molecules. This led to the formation of large molecular weight aggregates which severely compromised the viability of those cells. Prior exposure to DTT offered protection against this actin crosslinking, while the subsequent application of DTT reduced the number of aggregates formed and partially reversed the cytoskeletal contractile dysfunctions observed following OFR generation. This suggests that actin is also very sensitive to thiol redox status, and that the presence of reduced thiols in the actin molecule may be essential for its functionality.

In view of the above, it is evident that the modification of thiol redox status within the contractile proteins of the heart may significantly alter their functional characteristics. While the results obtained in this study cannot directly link the decline in contractile performance to a decline in the activity of the myosin ATPase or to a decrease in the availability of actin binding sites on the myosin head, it is interesting to speculate about the possibilities. The results show that protein thiols within the actin and myosin molecules are depleted following exposure to extracellular HOCl, and that this can be reversed by the subsequent exposure to DTT. This repletion of protein thiols with DTT correlates with a significant restoration of contractile function in the isolated myocardium. The fact that DTT does not induce a full recovery of thiols or contractile function suggests that some of the protein thiols may have been irreversibly oxidized, or were in a configuration not affected by DTT. Given that the cysteine residues in actin and myosin become more accessible for binding during the conformational changes involved in each contractile cycle, it may be that these thiols are more susceptible to oxidation than those associated with other cellular proteins.

However, thiol oxidation within the contractile proteins cannot explain fully the development of contracture as observed following exposure to HOCl. The alteration of Ca^{2+} influx via the slow channels by nifedipine or Bay K 8644 either potentiated or attenuated (respectively) the effects of HOCl exposure without significantly changing the level of P-SH oxidation. More interestingly, ryanodine, an alkaloid compound thought to induce a gradual loss of Ca^{2+} from the terminal regions of the SR (MacLeod and Bers 1987, Vierling 1988), totally prevented the contracture observed following HOCl, again without significantly reducing the level of P-SH oxidation. Finally, the functional recovery observed following the addition of DTT was similar in most of the treatment groups (except 0.5 mM Ca^{2+}), but was exactly opposite in the ryanodine treated muscles. Therefore, the ability of these agents to maintain (or depress) DT following HOCl

exposure must be attributed to their ability to alter Ca^{2+} handling at the level of the myocyte. Taken together, these results implicate the disruption in intracellular Ca^{2+} homeostasis following exposure to HOCl.

While our data does not rule out the possibility that an increased influx of extracellular Ca^{2+} is responsible for the observed contracture, they do imply that the presence of functional Ca^{2+} channels is important in the maintenance of DT following oxidative stress. For example, if the shaded region between the two curves in Figure 1.10 is considered as representing the % of DT attributed to the presence of active dihydropyridine sensitive Ca^{2+} channels, then the remaining DT (ie. below the nifedipine line) may be attributed to either Ca^{2+} released from the intracellular stores or to Ca^{2+} entry via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. As shown in Figure 1.10, while total DT declines, this shaded region between the two curves remains constant, suggesting that Ca^{2+} channel activity is maintained. In simplistic terms, this may be interpreted as showing that the decline in DT following exposure to HOCl is related to a depletion of intracellular releasable Ca^{2+} , and not to a loss of Ca^{2+} channel activity. This hypothesis is supported by the results from those experiments where the releasable Ca^{2+} pool is depleted by preincubation with ryanodine. In Figure 1.9 f), the remaining DT, if taken to reflect Ca^{2+} influx from the extracellular pool, remains constant following HOCl exposure. Taken together, this data could suggest that following exposure to HOCl, the rat myocardium becomes more dependent upon transsarcolemmal Ca^{2+} influx for its source of activator Ca^{2+} . While I remain cautious in this interpretation, the results that will be presented in Chapter 2 of this thesis tend to support this hypothesis.

This interpretation is of interest in view of the model being considered. The rat myocardium is thought to be largely dependent upon Ca^{2+} release from the SR stores for contraction (Fabiato 1985b). Papillary muscles isolated from the rat heart typically exhibit a decline in DT with increasing stimulation frequency, referred to as a negative

frequency-force relationship (Orchard and Lakatta 1985, Schouten et al. 1987). In a recent review, (Lewartowski and Pytkowski 1987a) Lewartowski states that this decrease in DT is correlated with a decrease in the inward Ca^{2+} current in the rat myocytes. In the present study, the decline in DT in response to increasing stimulation frequency (see Figure 1.12) was significantly greater in those muscles incubated with nifedipine as compared to control, supporting the hypothesis that a depression of the transsarcolemmal Ca^{2+} movement is instrumental in the development of this phenomenon. Similarly, under conditions of maximal transsarcolemmal Ca^{2+} movement, as for example in the presence of Bay K 8644 or elevated $[\text{Ca}^{2+}]_e$, the contractile response to increasing stimulation frequency is significantly reduced. In those muscles exposed to HOCl (see Figure 1.13), the response to increasing stimulation frequency was similar to that in muscles incubated in Bay K 8644. This suggests that following HOCl exposure, the inward movement of Ca^{2+} through the slow channels is not impaired, but is maximal with every contraction. This hypothesis is supported by recent work which shows that oxidant species such as hydrogen peroxide can increase both action potential duration and inward Ca^{2+} currents in the myocardium of guinea pig (Hayashi et al. 1989a). This question will be address further in Chapter 2.

The delayed replenishment of the release compartment has been postulated (Morad and Goldman 1973) as a mechanism to explain the observed phenomenon that the addition of a premature stimulation (ie. paired-pulse) will have a positive inotropic effect on the subsequent contraction, but not on the concomitant contraction (Schouten et al. 1987). If we assume that the releasable intracellular pool of Ca^{2+} is depleted with every contraction, then the addition of a second stimulation before the release compartment has been fully replenished (see Figure 1.14) will stimulate an extra influx of Ca^{2+} from the extracellular pool. A portion of this extra Ca^{2+} will be available for sequestration by the SR, and hence will be available for release with the subsequent stimulation. However, if

the SR Ca^{2+} -ATPase is impaired, then the amount of Ca^{2+} taken up into the SR will be less, and the amount of Ca^{2+} available for the next release cycle will be reduced.

In the present study, rat papillary muscles exposed to HOCl exhibited no positive inotropic response to paired pulsing (see Figure 1.15). Thiol oxidation within the Ca^{2+} -ATPase protein is known to disrupt Ca^{2+} transport into isolated SR vesicles (Georgoussi and Sotiroudis 1985, Kawakita and Yamashita 1987, Scherer and Deamer 1986c). In an intact cell, the inactivation of the SR Ca^{2+} sequestering system would lead to a depletion of the releasable intracellular pool of Ca^{2+} , and hence the cell would become more reliant upon the extracellular Ca^{2+} pool for contraction. Under these conditions, the addition of a premature stimulation would have little or no positive inotropic effect on the subsequent contraction, as no Ca^{2+} would be stored in the SR from the last beat. At the same time, the failure of the SR to remove Ca^{2+} from the cytosol rapidly would lead to a prolongation of the final phase of relaxation, and the accumulation of Ca^{2+} within the cytosol, leading to the development of contracture. The results from the present studies suggest that the ability of the SR to sequester and store Ca^{2+} has indeed been impaired following exposure to HOCl, and that the myocytes have more reliant upon transsarcolemmal Ca^{2+} for contraction. This question will be addressed in greater detail in Chapters 2 and 3.

SUMMARY AND CONCLUSIONS

The main objectives of this chapter were to quantitate the changes in myocardial contractility observed in the rat papillary muscle in response to HOCl, and relate this to the depletion of protein thiols. The results show clearly that HOCl induced a highly significant depletion of cellular thiols in a time dependent manner which correlated with a dramatic decline in contractile performance in the muscle. The restoration of contractile

function subsequent to treatment with DTT supports the concept of a relationship between thiol oxidation and contractile failure.

A further objective of this chapter was to postulate upon the mechanisms involved in this contractile failure. Results from the skinned muscle experiments show that following exposure to HOCl, the contractile proteins were significantly impaired in their ability to develop tension in response to increasing $[Ca^{2+}]$. This impairment was not related to a change in the sensitivity of the myofibrils to Ca^{2+} , but rather to an inability to develop a normal level of work. Thiol oxidation within the contractile proteins correlated with the decline in contractile function in these skinned muscles.

The results presented in Chapter 1 also suggest that following exposure to HOCl, the handling of Ca^{2+} at the level of the SR is impaired. The loss of normal Ca^{2+} -ATPase activity could lead to a prolongation in the time required for relaxation, and a decrease in the pool of releasable Ca^{2+} available for contraction. As well, the loss of the SR Ca^{2+} sequestration machinery could result in the elevation of cytosolic $[Ca^{2+}]$, especially if other cellular Ca^{2+} extrusion mechanisms (ie. Na^+/Ca^{2+} exchange and the sarcolemmal Ca^{2+} pump) were impaired as well. The presence of an elevated cytosolic $[Ca^{2+}]$ may account at least in part for the contracture observed in these papillary muscle preparations following exposure to HOCl. The restoration of contractile function that was observed following DTT treatment may be due to a restoration of the thiol status in the SR Ca^{2+} -ATPase molecule, and the subsequent recovery of cellular Ca^{2+} homeostasis. These data have shown that altered extracellular Ca^{2+} concentration or the administration of pharmacological agents which alter Ca^{2+} handling can modulate the efficacy of this oxidant.

In conclusion, HOCl induced dysfunction involves the depletion of intracellular glutathione and the oxidation of cellular protein sulfhydryls, possibly within the structure

of the Ca^{2+} regulatory proteins. The addition of DTT produced a significant recovery of contractile function that would be consistent with a removal of a cytosolic Ca^{2+} overload. This recovery of contractile function correlated with a significant recovery of tissue sulfhydryl levels. Taken together, these data imply that if a rise in cytosolic Ca^{2+} is occurring following exposure to HOCl, it occurs prior to the onset of irreversible cellular damage. It will be the main objective of Chapter 2 of this thesis to examine this hypothesis more directly.

CHAPTER 2:

CELLULAR Ca^{2+} HANDLING IN ISOLATED CARDIAC MYOCYTES DURING EXPOSURE TO HOCL AND DTT

INTRODUCTION

THE ISOLATED MYOCYTE AS A MODEL FOR THE MYOCARDIUM

While isolated papillary muscles or trabeculae isolated from the heart of various species have been used as models for cardiac mechanics (Abbott and Mommaerts 1959, Kelly and Hoffman 1960, Koch-Weser and Blinks 1963, Korecky et al. 1986, Pinto 1987, ter Keurs et al. 1987) and for the estimation of cellular Ca^{2+} handling (Allen et al. 1985, Gasser et al. 1989, Gulch and Ebrecht 1987, Marban et al. 1986, Schouten and ter Keurs 1986, Storch et al. 1987) they are not suitable for supplying precise information concerning ionic regulation in the heart. Prior to the late 1960's, attempts to obtain and analyse electrophysiological data from multicellular cardiac preparations were compromised by the complex architecture of the preparations as well as by the presence of low resistance intercellular ionic connections or gap junctions (Lieberman et al. 1987). In order to address specific questions relating to EC coupling in the mammalian myocardium, a preparation equivalent to an isolated skeletal muscle fiber is required. While Kono (1969) first reported the isolation of viable single cardiac myocytes using collagenase digestion in 1969, the significance of using these myocytes as a model for EC coupling was not established until 1972, when Powell and co-workers first described the preparation of cardiac myocytes which remained viable in physiological concentrations of

extracellular Ca^{2+} (Gould and Powell 1972). In the last two decades, this Ca^{2+} -tolerant myocyte preparation has been used extensively for obtaining information concerning transmembrane ionic currents (Clark et al. 1988, Désilets and Baumgarten 1986, Désilets et al. 1989, Giles and Imaizumi 1988, Hearse et al. 1989, Janse and Wit 1989, Patlak and Horn 1982) and Ca^{2+} homeostasis (Barceñas-Ruiz and Wier 1987, Bers et al. 1989, Cannell et al. 1987, Fabiato 1985b, Hume 1987, Reuter and Beeler 1969, Wier et al. 1988) in the myocardium.

Ca^{2+} HOMEOSTASIS IN CARDIAC MYOCYTES

The role of Ca^{2+} in excitation-contraction coupling (EC coupling) is still not completely understood today. From 1883, when Ringer first proposed a role for Ca^{2+} in the contractility of the heart until 1957, when Huxley proposed his theory of muscle contraction, the involvement of Ca^{2+} was virtually unknown. Podolsky and Constantin (1964) showed a direct relationship between iontophoretically-injected Ca^{2+} and sarcomeric contraction, suggesting that this ion was the important link in the EC coupling reaction. However, it was not until the discovery of troponin (Katz 1966) that the mechanisms for Ca^{2+} interaction with actin and myosin were postulated. With the development of techniques including whole-cell voltage clamping and patch clamping (Hamill et al. 1981), fundamental information concerning the properties of transmembrane ionic currents and individual ionic channels has provided much of the evidence necessary for understanding EC coupling. These electrophysiological techniques in combination with recent developments in the field of Ca^{2+} -sensitive fluorescent indicators (Tsien et al. 1984, Tsien 1989) have allowed for direct measurement of the $[\text{Ca}^{2+}]$ required for the initiation of contraction, the source of that Ca^{2+} , the currents generated by movement of Ca^{2+} into the cell, and the rate of force development with relation to $[\text{Ca}^{2+}]_i$ under physiological conditions.

Cardiac myofibrils become activated when the intracellular $[Ca^{2+}]$ increases to greater than 500 nM, and inactivated when the $[Ca^{2+}]$ is lowered to less than 200 nM (Marban et al. 1980, Ruegg 1988, Wier et al. 1988, Yue et al. 1986). Diastolic free $[Ca^{2+}]_i$ in healthy cardiac myocytes has been estimated in the range of 50 to 200 nM using Ca^{2+} sensitive microelectrodes (Marban et al. 1980) and fluorescent indicators such as aequorin (Yue 1987) or Fura-2 (Li et al. 1987, Wier et al. 1988) in the presence of millimolar $[Ca^{2+}]_e$. Since the cytosolic $[Ca^{2+}]$ is so important in the activation of cellular enzymes (Ikemoto 1975, Kunimatsu et al. 1989), mitochondrial function (Cheung et al. 1986b, McCormack et al. 1990) as well as in EC coupling, its regulation must be of paramount importance to the cell. Therefore, myocardial cells have established a very sophisticated and sensitive system for regulating intracellular Ca^{2+} concentrations, as was briefly alluded to in Chapter 1 (see Figure 1.1).

Ca^{2+} CHANNELS

At the onset of contraction, membrane depolarization opens Ca^{2+} channels of the sarcolemma and T-tubule network, leading to a transient influx of Ca^{2+} (Reuter 1969, Trautwein and Pelzer 1985). Most myocytes contain at least two types of Ca^{2+} channels (Hess 1988, Mitra and Morad 1986) which are activated at different voltages and possess different kinetic properties. Ca^{2+} influx through the transsarcolemmal L-type Ca^{2+} channel is responsible for the majority of the inward Ca^{2+} current that is recorded in whole-cell voltage-clamped myocytes (Aaronson et al. 1988, Cannell et al. 1987, Fedida et al. 1988, Hume 1987, Keung 1989, Trautwein and Pelzer 1985). This L-type Ca^{2+} channel is activated at membrane potentials of approximately -40 to -30 mV, and the Ca^{2+} current rises to a peak conductance within 2-4 ms at potentials close to 0 mV (McDonald 1982, Pelzer et al. 1990). Conductance then decays at a 10 to 20 times slower rate, accounting in part for the slow inward current observed during the early plateau component of the cardiac action potential (Trautwein and Pelzer 1985).

Na⁺/Ca²⁺ EXCHANGER

While the contribution made by the Na⁺/Ca²⁺ exchanger to the pool of activator Ca²⁺ during a single depolarization is uncertain, it has been shown repeatedly to play a substantial role in the long-term regulation of myocardial contractility (Barceñas-Ruiz and Wier 1987, Blaustein 1989, Horackova 1989, Philipson and Ward 1986, ter Keurs et al. 1987). This exchanger is electrogenic with a probable stoichiometry of 3 Na⁺ per 1 Ca²⁺ transported (Reeves and Hale 1984). At resting membrane potential or during the repolarization phase of an action potential, Na⁺ ions moving down their electrochemical gradient into the cell are coupled to an outward movement of Ca²⁺ ions, thereby returning [Ca²⁺]_i to the 100 nM level. This exchanger appears capable of operating in either direction across the sarcolemmal membrane, depending upon the relative magnitude of the transmembrane Na⁺ and Ca²⁺ gradients, as well as the magnitude of the membrane potential (Eisner and Lederer 1985, Reeves and Hale 1984). This exchanger is voltage dependent, with the possibility of reversing from a net inward current to a net outward current at between -20 and -30 mV during a depolarizing event (Eisner and Lederer 1985). However, more recent studies (Philipson, 1990, Lederer et al., 1990) have calculated that under normal physiological conditions, during a contractile cycle in the myocardium, the release of Ca²⁺ from the SR will increase [Ca²⁺]_i from approximately 10⁻⁷ to 10⁻⁶, which can effectively reduce the effects of membrane potential on the reversal of the Na⁺/Ca²⁺ exchanger. Lederer (...) shows that in healthy myocytes, the reversal potential remains between 20 and 30 mV higher than membrane potential, even during the plateau phase of the action potential. However, during a depolarizing event, at membrane voltages more positive than -20 mV, the Ca²⁺ efflux through the Na⁺/Ca²⁺ exchange is still reduced inhibited while the possibility for Ca²⁺ influx is enhanced (Philipson and Ward 1986, Shattock and Bers 1989). During repolarization, the opposite changes will occur, facilitating Ca²⁺ efflux via the Na⁺/Ca²⁺

exchange and relaxation of the muscle (Noma and Ehara 1990). While the $\text{Na}^+/\text{Ca}^{2+}$ exchange may add to the influx of Ca^{2+} during the prolonged portion of the normal cardiac action potential, this contribution has been considered insignificant under physiological conditions (Bers et al. 1988, Horackova 1989, Philipson and Ward 1986).

SR Ca^{2+} RELEASE CHANNELS

In both skeletal and cardiac muscle from most mammalian species, the amount of Ca^{2+} which enters the cell, either via the Ca^{2+} channels or the $\text{Na}^+/\text{Ca}^{2+}$ exchange, during a single action potential is considered inadequate to initiate contraction under normal physiological conditions (Morad and Goldman 1973). Although species variations do exist, the major source of activator Ca^{2+} in the rat myocardium is the SR (Fabiato 1983, Fabiato and Fabiato 1972). This SR release of Ca^{2+} occurs as a result of a transient increase in the permeability of the terminal cisternae Ca^{2+} release channel and the subsequent movement of Ca^{2+} down its electrochemical gradient (Bers 1985, Beuckelmann and Wier 1988, Plank et al. 1988). Fabiato (Fabiato 1983, Fabiato 1985a) proposed that the movement of Ca^{2+} into the cell via the sarcolemmal Ca^{2+} channels precipitates this release of Ca^{2+} from the SR (Ca^{2+} -induced Ca^{2+} release or CICR). The structure and function of this SR release channel has been examined extensively (Lai et al. 1988a, Lai et al. 1988b), and is known to consist of a tetrameric arrangement of proteins containing both the release channel and a ryanodine receptor molecule. Inactivation of this protein by ryanodine (Rasmussen et al. 1987, Sutko and Willerson 1980) or by thiol manipulation (Abramson et al. 1988, Trimm et al. 1986, Zaidi et al. 1989) is known to significantly alter myocardial contractility.

SR Ca^{2+} -ATPase

While the $\text{Na}^+/\text{Ca}^{2+}$ exchange has been linked with the extrusion of Ca^{2+} from myocytes following each contraction (Blaustein 1989, Chapman 1983, Hume 1987, Philipson and Ward 1986), on a beat-to-beat basis, the majority of Ca^{2+} is resequestered into the SR via the Mg^{2+} -dependent Ca^{2+} -ATPase (Hasselbach 1964, Hasselbach 1979, Krause and Hess 1984, Nayler et al. 1975a). A component of $[\text{Ca}^{2+}]_i$ may be removed by the high affinity, low capacity, Ca^{2+} pump of the sarcolemma (Carafoli 1988, Carafoli 1989) especially at high $[\text{Ca}^{2+}]_i$. The roles of these transmembrane pumps in the restoration of diastolic $[\text{Ca}^{2+}]_i$ will be addressed further in Chapter 3.

THIOL OXIDATION AND ELECTROPHYSIOLOGICAL CHANGES

Early experiments suggested that the blocking of protein sulfhydryls interfered with the electrical activity of contractile tissue. In 1948 Liu et al. showed that the treatment of skeletal muscle with iodoacetate, an alkylating agent which binds to free sulfhydryls, induced membrane depolarization and a loss of ionic conductance leading to spontaneous contractions and eventual contracture (Liu et al. 1948). In 1968 Kleinfeld and Stein reported a decrease in resting membrane potential, prolongation of the action potential and transient positive inotropy followed by contracture in the rat atrium during treatment with the thiol blocking agent HgCl_2 (Kleinfeld and Stein 1968). Hayashi (Hayashi et al. 1989a) reported recently that treatment of guinea pig papillary muscles with 10 mM H_2O_2 stimulated the generation of triggered arrhythmias and prolonged action potentials, leading to eventual contracture. Exposure of bullfrog atrium to the sulfhydryl blocking reagent N-ethylmaleimide (NEM) produced slightly different results. Aomine and Abe report that 10^{-3} M NEM inhibited the fast inward Na^+ current without altering the resting membrane potential (Aomine and Abe 1978). All authors reported a prolongation of the action potential duration coinciding with an increased Ca^{2+}

conductance through the Ca^{2+} channels and stress that the increase in Ca^{2+} influx is directly related to the blockage of sulfhydryl residues, as it did not occur when NEM was applied in the presence of reactive sulfhydryl groups such as cysteine (Aomine and Abe 1978). A delay in the deactivation kinetics of the Ca^{2+} channel could account for the prolongation of the action potential duration and development of dysrhythmias and subsequent contracture as is often observed in these studies.

PROTEIN THIOLS AND Ca^{2+} REGULATION

The effects of thiol oxidation on the kinetics of the Ca^{2+} release channel of the SR has been explored in more detail. Thiol oxidation within the 400 kDalton Ca^{2+} release channel following exposure to HOCl (Trimm et al. 1986) chloramine T (Aoki et al. 1986) or other sulfhydryl blocking agents (Zaidi et al. 1989) resulted in a significant increase in the channel activity and subsequent release of Ca^{2+} from heavy SR vesicles isolated from the rabbit myocardium. This effect of thiol oxidation was reversed by DTT (Scherer and Deamer 1986a, Trimm et al. 1986). Aoki et al. also reported that the effects of chloramine T were prevented by DTT (Aoki et al. 1986). Abramson et al. reported that preincubation of heavy SR vesicles with DTT or cysteine caused a complete loss of Ca^{2+} release by the SR vesicles, while thiol oxidation with phthalocyanine dyes induced a rapid release. (Abramson et al. 1988). While this may not be evidence that a manipulation of the thiol redox status within the Ca^{2+} release protein is involved in normal EC coupling, it does show that thiol oxidation will substantially increase SR Ca^{2+} release.

The effects of thiol oxidation have been reported for other Ca^{2+} regulatory proteins as well. Debetto et al. report that thiol oxidation resulted in a loss of $\text{Na}^+/\text{Ca}^{2+}$ exchange activity (Antolini et al. 1989, Debetto et al. 1988) while Reeves et al. reported the opposite effect (Reeves et al. 1986) in sarcolemmal preparations prepared from

myocardial tissue. The Ca^{2+} -ATPase of the SR can be totally inhibited by the oxidation of as few as one sulfhydryl residue (Ariki and Shamoo 1983, Hasselbach and Seraydarian 1966, Kawakita and Yamashita 1987). Similar results have been reported for the sarcolemmal Ca^{2+} pump (Kaneko et al. 1989, Nicotera et al. 1985). Taken together, thiol oxidation within the various proteins involved in the regulation of cellular Ca^{2+} could result in an increased influx of Ca^{2+} into the cytosol (from the SR and via the Ca^{2+} channels) combined with an impairment of the Ca^{2+} extrusion mechanisms, leading to a cytosolic Ca^{2+} overload and a depletion of SR stores. In the contracting myocardium, this would manifest itself as a rise in diastolic tension and an eventual loss of transient contractile function. The muscle would enter into a state of contracture.

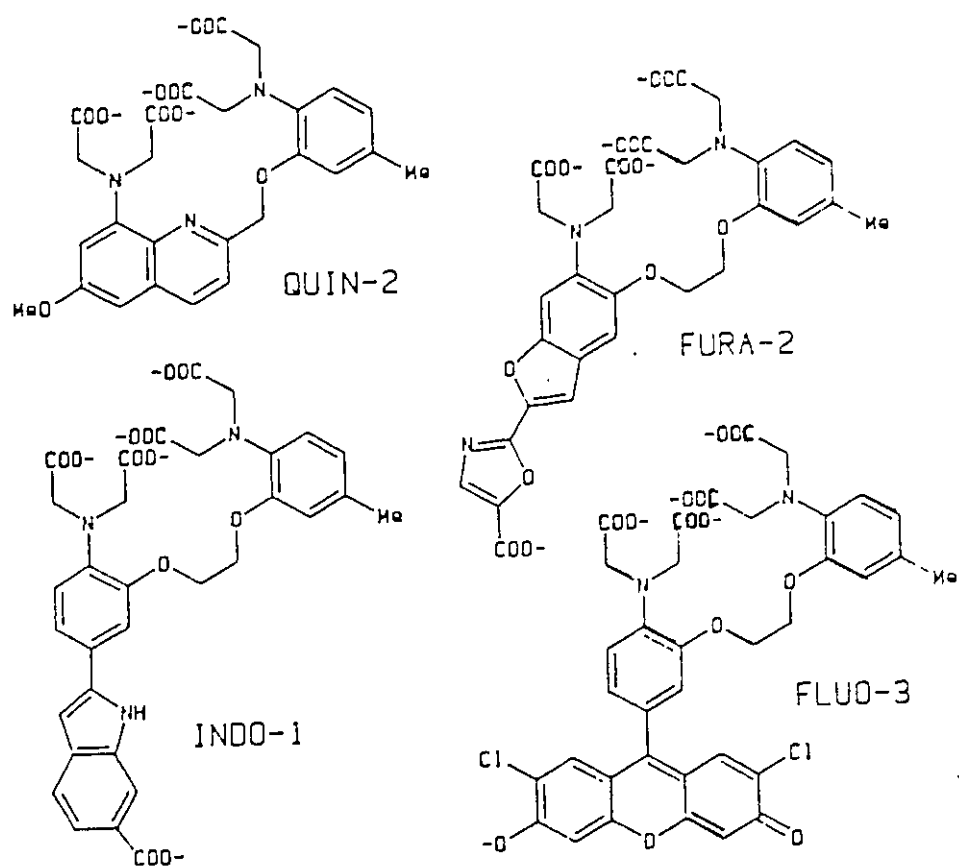
MEASUREMENT OF $[\text{Ca}^{2+}]_i$ AND Ca^{2+} TRANSIENTS WITH FURA-2

The results presented in Chapter 1 of this thesis showed that papillary muscles exposed to HOCl developed contracture, and that contractile function was restored by the subsequent exposure to DTT. The effects of HOCl were prevented by preincubation with ryanodine but not nifedipine, suggesting that Ca^{2+} handling by the SR was impaired, leading to the development of a cytosolic Ca^{2+} overload. To test this hypothesis $[\text{Ca}^{2+}]_i$ must be measured. With the development of Ca^{2+} sensitive indicators such as Fura-2 it became possible to measure $[\text{Ca}^{2+}]_i$ in living cells (Grynkiewicz et al. 1985).

Fura-2 is an EGTA derivative possessing a fluorescent sidechain that fluoresces at 510 nm in response to excitation by UV radiation at specific frequencies (see Figure 2.1 below). The frequency to which the fluorophore responds is dependent upon the presence or absence of Ca^{2+} , such that the fluorescence excitation spectrum shifts to shorter wavelengths as $[\text{Ca}^{2+}]$ increases. The binding of Ca^{2+} to Fura-2 shifts the emission maxima much less than it shifts the excitation maxima (Grynkiewicz et al. 1985) thereby allowing for dual-beam excitation and single beam emission to be used for quantification

Figure 2.1 Structure of four hydrophilic Ca^{2+} indicators including Fura-2 {Grynkiewicz, 1985 #258}, showing their common EGTA-derived Ca^{2+} binding domain. The octacoordinate binding site is selective for divalent cations as most small monovalent ions fail to contact sufficient ligand groups simultaneously. The larger fluorophore of Fura-2 gives it slightly longer wavelengths of excitation as compared to Quin-2, allowing better compatibility with glass optics. The green emission spectrum (505-520 nm) does not shift with Ca^{2+} binding. Fura-2 is also more resistant to photodestruction than Quin-2. In the acetoxymethyl (AM) form, each $-\text{COO}^-$ group is replaced by $-\text{COOCH}_2\text{OCOCH}_3$. (Adapted from Tsien, 1989)

Figure 2.1



of $[Ca^{2+}]$. For example, in 0 mM Ca^{2+} , Fura-2 in the unbound form will be maximally excited by UV light at 362 nm and will emit at 510 nm. In contrast, in high Ca^{2+} , Fura-2 in the bound form will be maximally excited at 340 nm and will emit at 505 nm (Grynkiewicz et al. 1985). By comparing against a set of excitation spectra over a range of known $[Ca^{2+}]$ as shown in Figure 2.3 (below), the $[Ca^{2+}]$ in an unknown sample or within a cell loaded with Fura-2 can be approximated.

One problem arising from the use of fluorescent dyes to measure ionic concentrations is that a change in the concentration of the dye will also produce a change in the fluorescence signal. By exciting Fura-2 at 340 nm only, one could theoretically record changes in $[Ca^{2+}]$, as the fluorescence signal would increase with increasing $[Ca^{2+}]$. However, unless one has absolute control over the dye concentration, it would be difficult to quantitate those changes in $[Ca^{2+}]$. The loss of Fura-2 from the cell, or the loss of its fluorescence intensity due to photobleaching (Becker and Fay 1987) would be interpreted as a decline in $[Ca^{2+}]$. This problem can be overcome by using the ratio technique. By stimulating the dye at 340 and 380 nm 'simultaneously' and monitoring the 340/380 emission, one can remove the influence of dye concentration on the final $[Ca^{2+}]$ calculations. (for example, see Figure 2.6 below).

Similarly, by knowing the ratio of emitted fluorescence induced by 340 to 380 nm excitation in 0 mM Ca^{2+} and saturated Ca^{2+} solutions, the $[Ca^{2+}]$ can be calculated using the following equation:

$$[Ca^{2+}] = K_d \frac{R - R_{min}}{R_{max} - R} \frac{Sf2}{Sb2}$$

where R = the 340/380 fluorescence ratio from the sample, $R_{\min}$ = the 340/380 fluorescence ratio at 0 mM $[Ca^{2+}]$, $R_{\max}$ = the 340/380 fluorescence ratio at saturating $[Ca^{2+}]$, K_d = the dissociation constant for Fura-2 at 37°C, S_{f2} = the free dye fluorescence signal at 380 nm, and S_{b2} = the bound dye fluorescence signal at 380 nm as described by Tsien and co-workers (Grynkiewicz et al. 1985, Tsien 1988, Tsien 1989).

Two forms of Fura-2 are currently available: the membrane permeable acetoxymethyl ester form (Fura-2 AM), and the membrane impermeable pentapotassium salt form (Fura-2) (Figure 2.1).

Fura-2 AM contains four acetoxymethyl ester groups which afford membrane permeability to this otherwise impermeable compound. Once inside the cell, intracellular esterases cleave these AM group, leaving the membrane-impermeable form of Fura-2 trapped within the cytosol (for a more detailed description see (Gunter et al. 1988)). The AM form is an extremely valuable tool for loading many cells simultaneously without altering the integrity of the cellular membrane. However, recent evidence has shown that Fura-2 AM may not remain within the cytosol, and may move into the SR or mitochondria as well (Highsmith et al. 1986, Scanlon et al. 1987). Any distortion of the fluorescence signal caused by the high $[Ca^{2+}]$ found in the SR or mitochondria will substantially increase background interference. As well, as the Fura-2 within the SR would be saturated with Ca^{2+} , any release of Ca^{2+} from the SR in response to a perturbation may result in a reduction of the Fura-2 signal from this SR source, cancelling out the rise in the signal from cytosolic Fura-2. Therefore, the use of Fura-2 AM for fine monitoring of $[Ca^{2+}]_i$ has to consider the influence from other intracellular stores of Ca^{2+} .

The membrane impermeable pentapotassium form of Fura-2 can be injected directly into a single cell, where it will remain specifically within the cytosol and will not

move out of the cell, nor into intracellular compartments such as the SR or mitochondria. The main advantage of this form is that the researcher has greater control over the location and concentration of the Fura-2 used. The difficulty is that the Fura-2 must be injected into each cell separately. Beyond the technical difficulty of such an injection, the necessary damage to the sarcolemma at the site of injection could lead to increased noise or artifactual data if the cell becomes leaky to extracellular Ca^{2+} . However this is outweighed by the advantage of being able to stimulate the cell while simultaneously monitoring the membrane potential via the injection electrode, or to inject current to clamp the cell for the measurement of inward Ca^{2+} currents. Several laboratories have successfully combined the whole-cell voltage clamp technique with high speed dual-excitation beam spectrofluorometer and fluorescence microscopy, and have quantitated the rapid fluctuations in $[\text{Ca}^{2+}]_i$ which occur during single depolarizing events in Fura-2 loaded isolated cardiac myocytes (Barcenas-Ruiz and Wier 1987, Beuckelmann and Wier 1988, Cannell et al. 1987, Wier et al. 1988, Wier et al. 1987).

SUMMARY

In Chapter 1 it was shown that exposure of rat ventricular muscle to the oxidant HOCl induced a decline in tension development and concomitant rise in resting tension. While this decline in force development may be due to a direct effect of the oxidant on the protein thiols of the myofibrils, there is evidence that a disruption of cellular Ca^{2+} handling is also involved. Thiol oxidation in any of the major Ca^{2+} regulatory proteins has been shown to significantly alter their function, and could account for the development of a cytosolic Ca^{2+} overload. It is important to ascertain that exposure to HOCl does induce a rise in $[\text{Ca}^{2+}]_i$, and that this effect is reversed by the addition of DTT.

With the advent of Ca^{2+} indicators such as Fura-2, it is possible to measure intracellular $[\text{Ca}^{2+}]_i$ in isolated cells. Because the AM form of Fura-2 may diffuse throughout the cell and into subcellular compartments, the use of this form to determine cytosolic $[\text{Ca}^{2+}]_i$ has been questioned. Therefore, by injecting the pentapotassium salt form of Fura-2 directly into isolated cardiac myocytes, it is possible to monitor not only changes in diastolic $[\text{Ca}^{2+}]_i$, but the dynamic changes in $[\text{Ca}^{2+}]_i$ during a single excitation/contraction event. It will be the aim of Chapter 2 to apply these techniques to examine the effects of HOCl on cellular Ca^{2+} homeostasis in the mammalian myocardium.

OBJECTIVES:

The main objectives of Chapter 2 are as follows:

- 1) To determine whether exposure of isolated myocytes to HOCl leads to an increase in diastolic $[\text{Ca}^{2+}]_i$.
- 2) To determine whether exposure to DTT subsequent to HOCl induces a recovery of diastolic $[\text{Ca}^{2+}]_i$.
- 3) To determine the source of this Ca^{2+} .
- 4) To compare the effects of HOCl and DTT on both rat and rabbit myocytes.

METHODS

ISOLATION OF CARDIAC MYOCYTES

SOLUTIONS:

	A	B	C	D
CaCl ₂	2.0 mM	-----	25 µM	2.0 mM
Collagenase	-----	-----	1 mg/ml	-----
Creatine	20.0 mM	20.0 mM	20.0 mM	-----
Glucose	10.0 mM	10.0 mM	10.0 mM	10.0 mM
Hepes	-----	-----	-----	5.0 mM
KCl	5.0 mM	5.0 mM	5.0 mM	5.0 mM
MgSO ₄	1.2 mM	1.2 mM	1.2 mM	1.2 mM
Na ₂ HPO ₄	1.37 mM	1.37 mM	1.37 mM	1.37 mM
NaCl	130.0 mM	130.0 mM	130.0 mM	145.0 mM
NaH ₂ PO ₄	0.43 mM	0.43 mM	0.43 mM	0.43 mM
NaHCO ₃	23.0 mM	23.0 mM	23.0 mM	-----
Amino Acids	yes	yes	yes	no
(MEM-Gibco)				
NaOH (5 N) to pH 7.4				

MYOCYTE ISOLATION

Cardiac myocytes were isolated from adult rabbit ventricular septum by a procedure similar to that described previously (Désilets and Baumgarten 1986). Briefly, hearts were retrogradely perfused at 37°C with a modified Tyrode (Solution A) equilibrated with 95% O₂-5% CO₂ to maintain a pH of 7.4) in the following sequence: 4 min with nominally Ca²⁺-free (Solution B), 15 min (minimum) with 25 µM CaCl₂

(Solution C) and collagenase (Worthington CLS II), and a final 2 min with nominally Ca^{2+} -free (Solution B). The myocytes were harvested from the ventricular septum by gentle mincing of the tissue in 10 ml of Solution B and incubated with shaking at 37°C for 5 to 10 min as required to separate the individual myocytes. The isolated cells were passed through a 200 μm nylon mesh and washed three times with fresh Buffer B. The $[\text{Ca}^{2+}]$ was gradually brought to 2 mM, then the viable cells were stored in 2 mM HEPES buffer (solution D) at 37°C for up to 8 hours. The percentage of rod-shaped cells ranged from 40 to 50%.

Ventricular cells were isolated from male Sprague-Dawley rats (275-300 grams) using a similar protocol to that described above with the following exception: during the final 5 min of collagenase perfusion the $[\text{Ca}^{2+}]$ was brought to 1 mM as described by Haworth (Haworth et al. 1989). The whole ventricle was used for this isolation. The percentage of rod-shaped cells ranged from 60 to 80%.

NOTE:

The majority of the results presented here relate to rabbit cardiac myocytes, simply due to the relative ease of acquiring data from these cells in comparison to rat. Quiescent rat myocytes often contracted spontaneously upon impalement leading to irreversible contracture. However, representative examples are presented here to show that the response to HOCl and DTT was similar in rat and rabbit myocytes. Where possible, mean values will be presented for both species.

ELECTRICAL RECORDINGS:

Electrical recordings were obtained with small suction electrodes (Désilets et al. 1989) containing (in mM): K aspartate, 95; KH_2PO_4 , 1; K_2ATP , 5; K_2 -phosphocreatine, 2.5; MgCl_2 , 2.5; KCl, 15; taurine, 10; HEPES, 5; pH 7.3 with 1 N KOH, (DC resistance

in HEPES buffer $\approx 50 \text{ m}\Omega$). These electrodes were chosen to minimize intracellular dialysis and prevent rundown of the slow inward currents. Upon contact with the plasma membrane, a gentle suction was applied via the electrode to seal the 'patch' and rupture the membrane. Single electrode voltage clamp experiments were performed by use of the chop-clamp technique (Axoclamp 2A, Axon Instruments) with a chopping frequency of $\approx 2\text{-}3 \text{ KHz}$. A 3M KCl bridge was in contact with the solution in the superfusion chamber as a ground. Membrane potential values were corrected for a 10 mV underestimation caused by changes of junction potential (Désilets and Baumgarten 1986). Current and voltage values were monitored using a Tektronix four-beam oscilloscope and were stored on video tape using a modified VCR (Panasonic).

Data acquisition, analysis, and digital plotting were performed using the ASYST (Keithley Asyst) integrated software package. Current signals were filtered using a 8-pole Bessel filter at half the sampling rate (ie. 1 kHz for Ca^{2+} currents and 100 Hz for steady state signals) prior to analysis.

MEASUREMENT OF INWARD CALCIUM CURRENT:

At the onset of each experiment, each myocyte was clamped at membrane potential ($E_m = 0 \text{ nA}$ of holding current) and any changes in the membrane potential during the experiments were recorded as changes in the holding current required to maintain E_m . In order to estimate the whole cell Ca^{2+} current, the cell would be clamped at a membrane potential at -40 mV then exposed to a 1 Hz train of 300 ms depolarizing pulses (-40 to 0 mV) while monitoring the inward current. While no attempt was made to block all other transarcolemmal conductances, for the purpose of this study, the resulting net whole-cell inward current during these pulses will be considered to represent the inward Ca^{2+} current via the L-type Ca^{2+} channels.

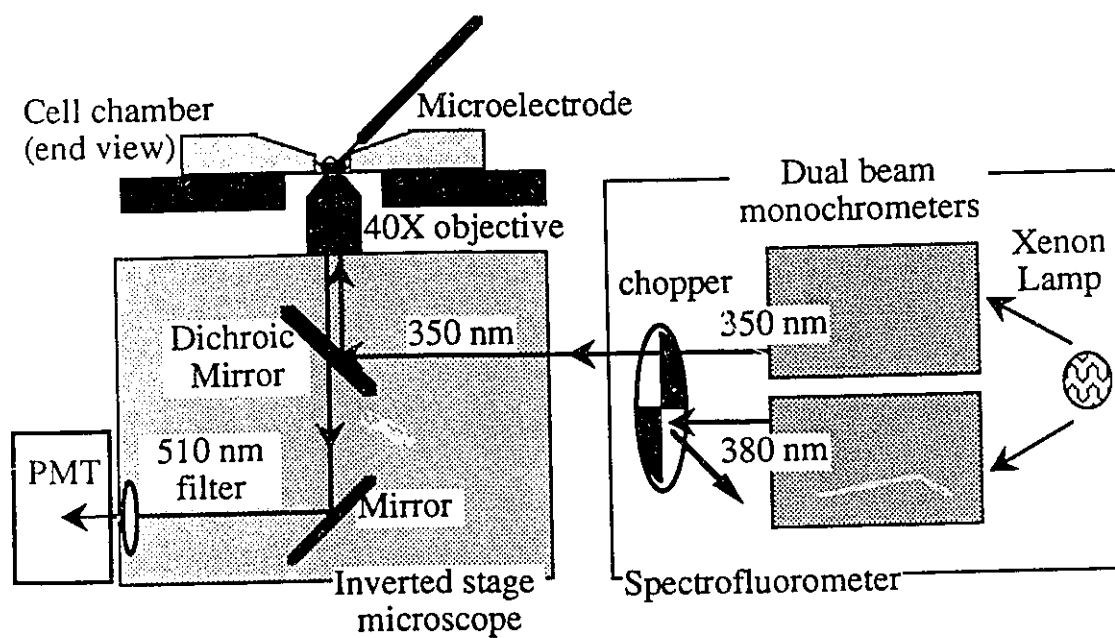
SINGLE CELL SPECTROFLUOROMETRY

Freshly isolated cardiac myocytes, or myocytes preloaded with Fura-2 AM were plated in a monolayer on the glass bottom of a 0.3 ml superfusion chamber mounted on the stage of an inverted-stage Nikon diaphot fluorescence microscope as shown in Figure 2.2. The superfusate (Buffer D) was equilibrated with 95% O₂-5% O₂ at pH 7.4 and heated immediately prior to being passed over the cells at a flow rate of ≈ 5 ml/min, maintaining a bathing temperature of $37 \pm 0.5^\circ\text{C}$. Solution changes occurred with a dead-space of less than 0.5 ml. Cells attached to the coverslip in this chamber remained viable for up to 2 hours.

The two beams of monochromatic light required for exciting the Fura-2 were derived using a SPEX Fluorolog dual-wavelength spectrofluorometer (Rayonics Scientific Inc.). Briefly, white light from a Xenon light source was split into two beams of equal intensity, each of which passed through a separate monochromator system to produce monochromatic light (± 5 nm) at 350 and 380 nm. The 350 beam was used instead of 340 nm to account for a 10 nm spectral shift associated with our optical system. These twin excitation beams were directed in parallel through an eight-blade mirror chopper to produce alternating pulses at 200 Hz. These pulses were focused together and channeled using a Nikon 400 nm dichroic mirror through a 40x fluorescence objective onto a single cell attached to the described superfusion chamber (See Figure 2.2). The resultant epifluorescence from the cell was directed downward through the same objective and dichroic mirror, through a camera port of the microscope, and along a side-arm assembly where it passed through a 505 nm band-pass filter prior to reaching the photomultiplier tube (PMT). The resulting signal was digitized and correlated with its corresponding excitation wavelength signal using the DM3000CM (SPEX) program on a IBM-clone AT computer. Background fluorescence was subtracted from each signal prior to ratioing.

Figure 2.2 Diagrammatic representation of the inverted stage microscope and Ca^{2+} fluorescence apparatus. White light from the Xenon lamp source is split into two equal beams, which pass through separate monochrometers to produce twin beams of monochromatic light at different frequencies (ie. 350 and 380 nm). The beams pass through an eight-blade mirror chopper to allow alternating pulses of both beams. The light is focused into the base of an inverted stage microscope and onto a dichroic mirror which reflects light at < 400 nm and passes light at > 400 nm. The beam is focused onto a Fura-2 loaded cell, emitting peak fluorescence at about 510 nm which in turn will pass through the same dichroic mirror. The final fluorescence signal is reflected through a 500 nm band pass filter and onto a photomultiplier tube. An AT type computer links the resulting fluorescence signal with the appropriate excitation pulse. The cell can be accessed from above by a microelectrode for Fura-2 loading and voltage clamping.

Figure 2.2



IONTOPHORETIC INJECTION OF FURA-2:

A single cell within the perfusion chamber was oriented such that both excitation beams illuminated the entire cell. Cells were iontophoretically loaded by including 0.9 mM Fura-2 (pentapotassium salt, Molecular Probes) in the solution at the tip of the fine suction electrode described below and applying a -1nA current following attachment to the cell (see Figure 2.6 below). Injection was complete within 3-5 min when the fluorescence signals had increased to 2-3x background. Fluorescence signals were recorded from a portion of the cell distant from the site of impalement, thereby minimizing fluorescent interference from the electrode. Background fluorescence and autofluorescence were determined prior to loading and subtracted from the Fura-2 fluorescence signals. The final ratio signals were digitally filtered at half the sampling rate (100 Hz) prior to analysis.

FURA-2 AM LOADING

Fura-2 AM was used in earlier protocols prior to the availability of iontophoretic injection. For these experiments, an aliquot of myocytes was incubated with intermittent mixing for 1 hour at 37°C in Buffer D containing 2 μ M Fura-2 AM in DMSO (final concentration of DMSO was <0.01% V/V). The cells were washed twice with oxygenated Buffer D and maintained in fresh Buffer D at 37°C for 60 min prior to use. For AM cells, background autofluorescence was determined for a minimum of five unloaded cells and the average value was subtracted from the loaded cell signals.

MEASUREMENT OF $[Ca^{2+}]_i$

Intracellular $[Ca^{2+}]_i$ was calculated by the ratio method (Grynkiewicz et al. 1985) with a K_d of 230 nM (see Figure 2.3 below) determined from our *in vitro* calibration solutions, containing (in mM): KCl, 145; HEPES, 5; EGTA, 5; pH 7.2

adjusted with 1N KOH, with pCa values ranging between 3 and 9 (Fabiato 1984) (see Figure 2.3). Minimum and maximum ratios (0.9 and 13.9 respectively) were similar to those measured in Digitonin-treated cells (0.5 ± 0.1 and 12.2 ± 0.5 , $n=6$). Although subject to uncertainty related to the properties of Fura-2 inside the cells (Becker et al. 1989, Cannell et al. 1987), the accurate determination of $[Ca^{2+}]_i$ does not affect the main conclusions of these results.

The effects of 100 μ M HOCl and 500 μ M DTT on the fluorescent characteristics of Fura-2 itself were examined in 5 μ M Fura-2 solutions at various $[Ca^{2+}]$. The effect of superfusion with 100 μ M HOCl or 500 μ M DTT on cell autofluorescence in unloaded myocytes were also examined.

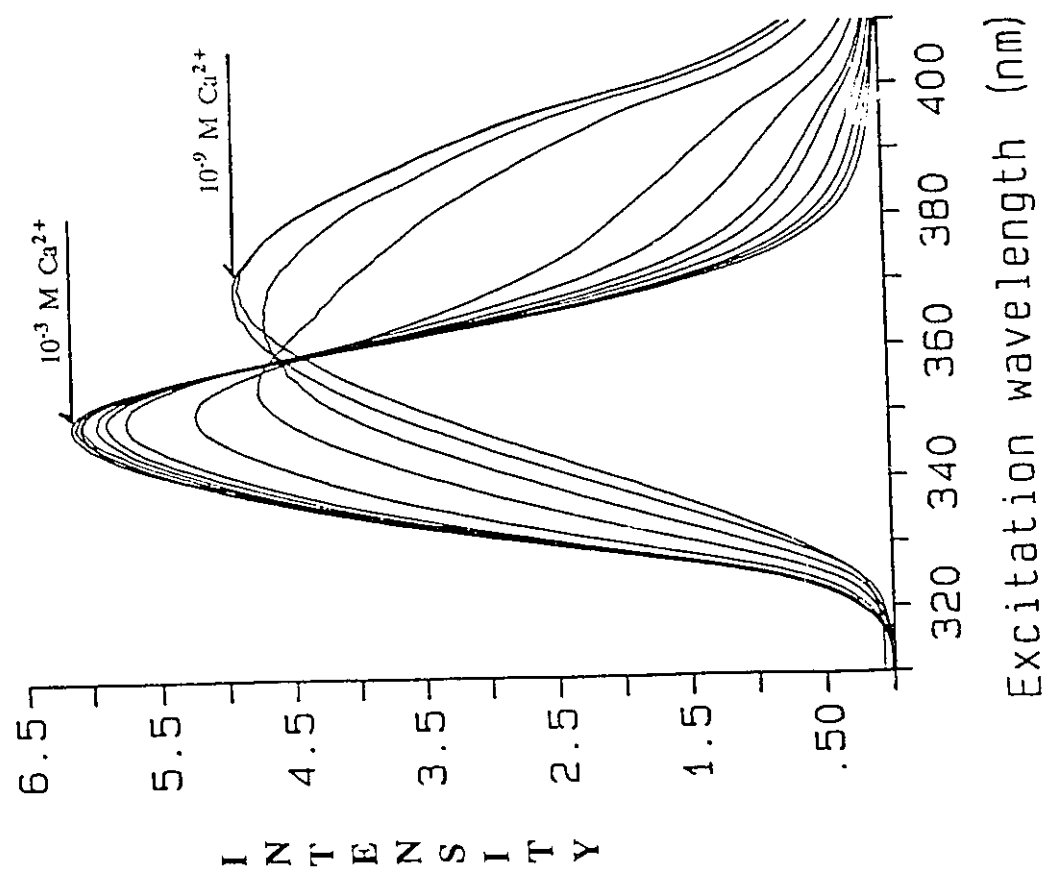
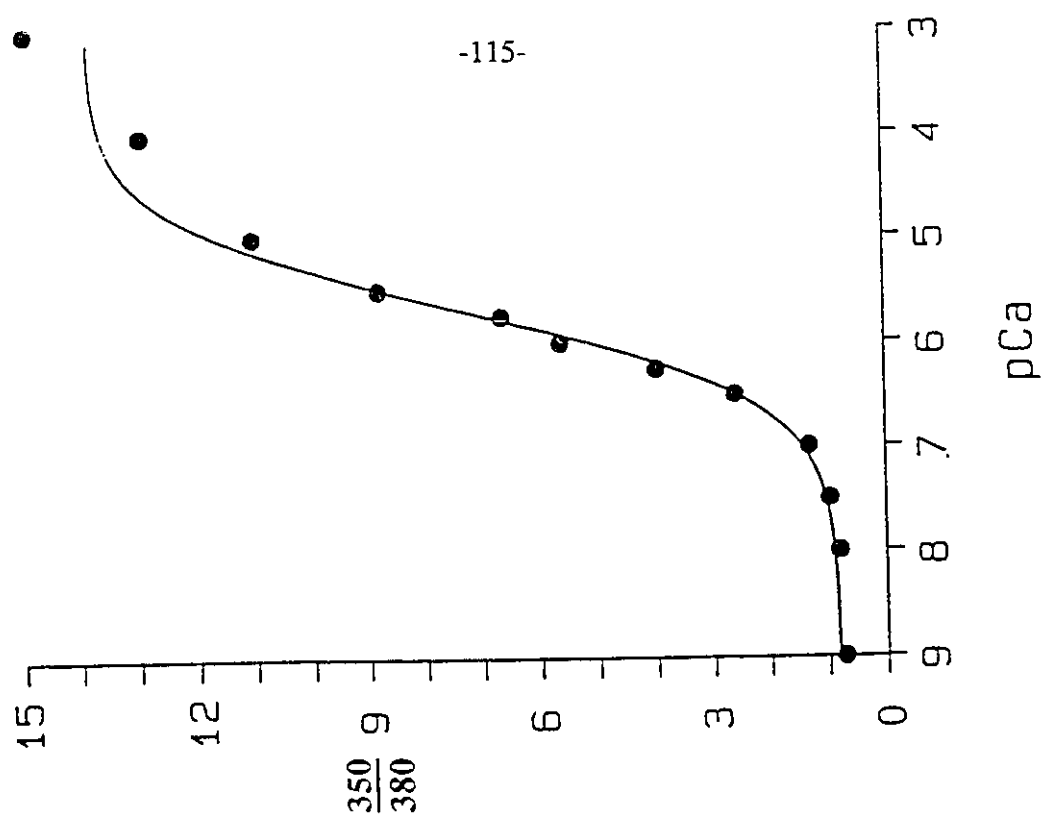
EXPERIMENTAL PROTOCOLS:

FURA-2 AM LOADED MYOCYTES

During a typical experimental protocol, Fura-2 AM loaded rabbit myocytes were superfused for up to 40 min while their fluorescence signals were recorded. Experimental protocols involved superfusion with HOCl (Buffer D with 100 μ M HOCl, prepared immediately prior to the start of each experiment) with or without subsequent superfusion with DTT (500 μ M) or Washout. Other cells were superfused with Buffer D containing 0 mM Ca^{2+} (Mg^{2+} replacement + 0.5 mM EGTA) for 10 min with or without a 20 sec pulse of caffeine (10 mM) (Koshita and Oba 1989, Wyskovsky et al. 1988) prior to perfusion with 0 mM Ca^{2+} with HOCl (100 μ M). Control experiments were performed in 2 mM and 0 mM Ca^{2+} with or without DTT. Further details are given in the legend to each figure.

Figure 2.3 Excitation spectrum for 0.5 mM Fura-2 at 37°C in buffers containing free $[Ca^{2+}]$ ranging from 10^{-9} M to 10^{-3} M. The emission energy was monitored at 505 nm and corrected for excitation lamp intensity fluctuations by continuously normalizing the signal against a rhodamine B signal. With increasing pCa, the 380 nm stimulated emission declined as the 350 nm stimulated emission increased, with the isobestic point (362 nm) remaining relatively stable. The ratio of the 350 nm to 380 nm for each pCa is also plotted, showing a sigmoid shaped calibration curve with an apparent dissociation constant (K_D) for Ca^{2+} of 230 nM.

CALIBRATION CURVES FOR FURA-2



FURA-2 INJECTED MYOCYTES

Following the iontophoretic injection of Fura-2, cells were whole-cell voltage clamped as described previously. Following an initial stabilization period of 5 min, the myocytes were superfused with 100 μ M HOCl for up to 10 min with or without DTT at 5 min while holding current and diastolic $[Ca^{2+}]_i$ were recorded. Trains of depolarizing pulses were used to elicit Ca^{2+} currents and Ca^{2+} transients in these cells immediately prior to switching to HOCl superfusion, as well as at various times after HOCl, or subsequent to the secondary superfusion with DTT. Cells were also exposed to caffeine prior to HOCl as described above. Further details are given in the legend to each figure.

RESULTS:

CHARACTERIZATION AND CALIBRATION OF FURA-2:

Figure 2.3 shows the excitation scans for Fura-2 as recorded *in vitro* in various concentrations of Ca^{2+} . With increasing $[Ca^{2+}]$, the maximal excitation frequency decreases (left panel), peaking in saturated Ca^{2+} at 350 nm. By taking the ratio of the emitted fluorescence intensity at 350 and 380 nm from each scan, a relationship between $[Ca^{2+}]$ and the 350:380 ratio was established (right panel). The solid line represents the $[Ca^{2+}]$ values as calculated using the equation described on page 124 of this thesis. The K_d calculated from this curve is 230 nM. As shown in Figure 2.3 (right panel) the relationship is virtually linear over the working range of pCa 7.5 to pCa 5. All values of $[Ca^{2+}]_i$ found in this chapter were calculated using the equation as discussed above (Grynkiewicz et al. 1985)

EFFECTS OF HOCl AND DTT ON FURA-2 FLUORESCENCE

Before determining the effects of HOCl on the intracellular $[Ca^{2+}]_i$ in superfused isolated cardiac myocytes, the effects of HOCl upon the fluorescent characteristics of Fura-2 itself were examined. Figure 2.4 shows the 350nm and 380nm fluorescence signals and their ratio for Fura-2 in 0 mM Ca^{2+} buffer in response to the addition of equal volumes of H_2O , HOCl (final concentration 100 μM) or DTT (final concentration 500 μM) (top panel). No significant change in either the 350 or 380 signals or in the 350:380 ratio was observed. No change was observed in the excitation scans for 5 μM Fura in either 0 mM or 1 mM Ca^{2+} (Figure 2.4; bottom panel), confirming that neither HOCl or DTT had any direct effect on the fluorescent properties of Fura-2. For the purpose of this thesis, any changes in the 350/380 ratio are taken to represent changes in $[Ca^{2+}]_i$.

EFFECTS OF HOCl AND DTT ON CELL AUTOFLUORESCENCE

Figure 2.5 shows the effect of superfusion with HOCl or DTT on background fluorescence in a single isolated myocyte, showing no significant change in autofluorescence following exposure to either of these agents. Autofluorescence during HOCl treatment was recorded and averaged for at least three unloaded (no Fura-2 AM) cells prior to each experimental protocol. The mean background autofluorescence values were subtracted from the Fura-2 AM fluorescence signals prior to ratioing. Autofluorescence was generally approximately 10-15% of the Fura-2 AM fluorescence values.

IONTOPHORETIC LOADING OF FURA-2

Figure 2.6 shows the effects of iontophoretic injection of Fura-2 into an isolated

Figure 2.4 The effects of HOCl and DTT on Fura-2 fluorescence. The cell chamber was filled with 700 μl of 0 mM Ca^{2+} solution containing 1 μM pentapotassium Fura-2 and the solution was excited using 350 and 380 nm UV pulses at 1 Hz. In the upper panel, 10 μl of either distilled H_2O , HOCl (final $[\text{HOCl}] = 100 \mu\text{M}$), or DTT (final $[\text{DTT}] = 500 \mu\text{M}$) was added to the bath as shown (at the appropriate arrow) and mixed thoroughly. The Fura-2 containing solution was replaced between tests (ie. at 'new'). Downward spikes are flashes of low-intensity light used to indicate solution changes. No change other than dilution artifacts were observed in either emission signal (a) or in the 350/380 ratio (b) upon exposure to HOCl or DTT. Similar results were observed using 2 mM Ca^{2+} solution with Fura-2 (data not shown). The lower panel shows the spectral excitation scan (from 250 to 450 at 2 nm intervals) of Fura-2 containing buffer with either 0 mM or 1 mM Ca^{2+} before and after the addition of HOCl (100 μM) or DTT (500 μM). The slight upward shift in the 0 mM spectrum with HOCl could be explained by small variations in light path due to a slight pH or temperature effect, but had no significant effect on the 350/380 ratio. The units for the emission values are arbitrary and are omitted here for clarity.

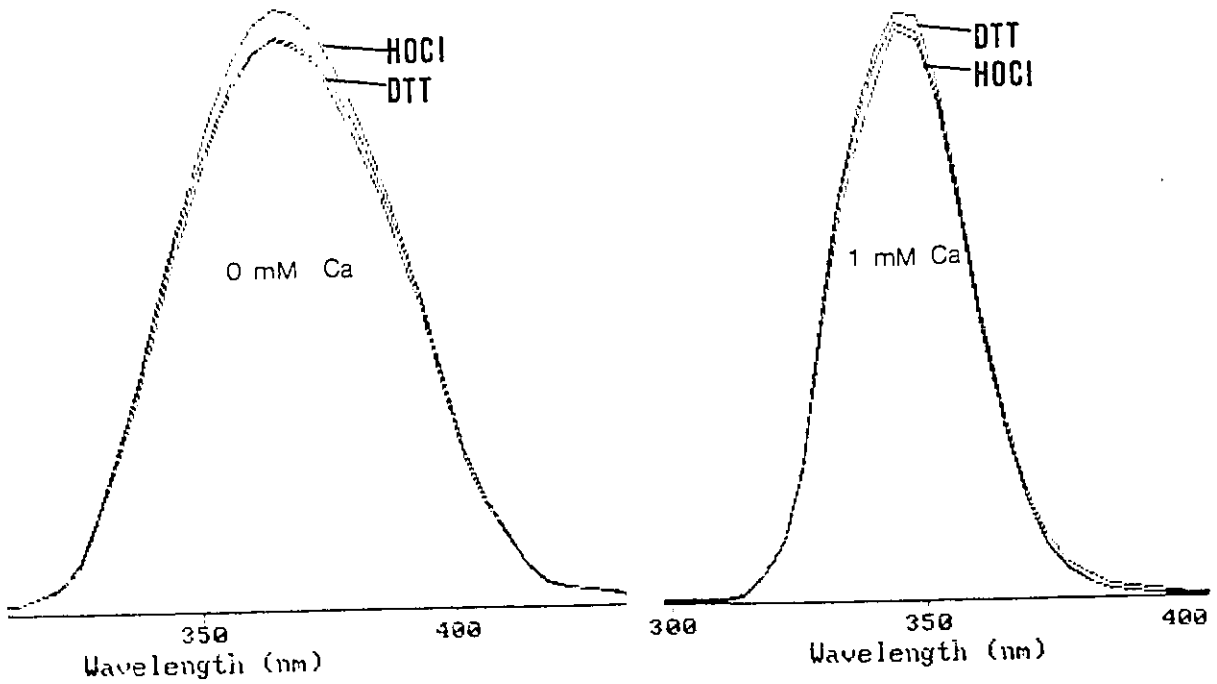
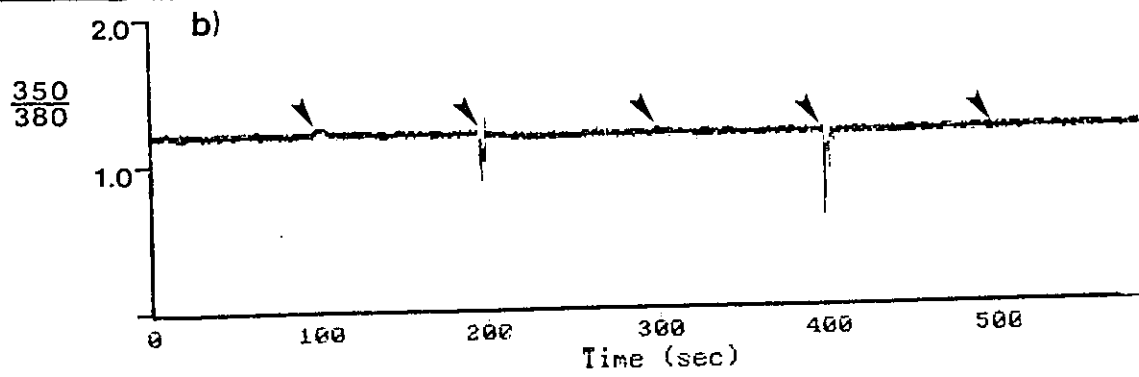
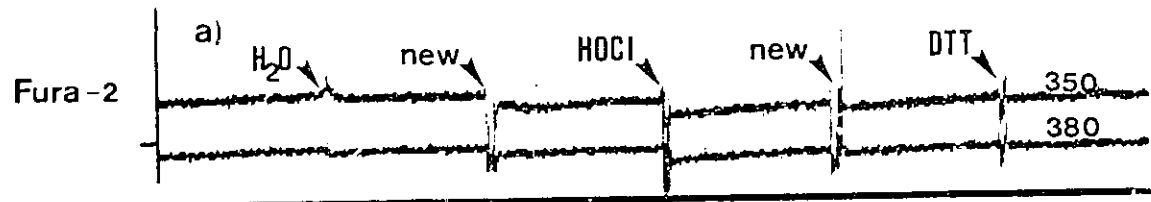
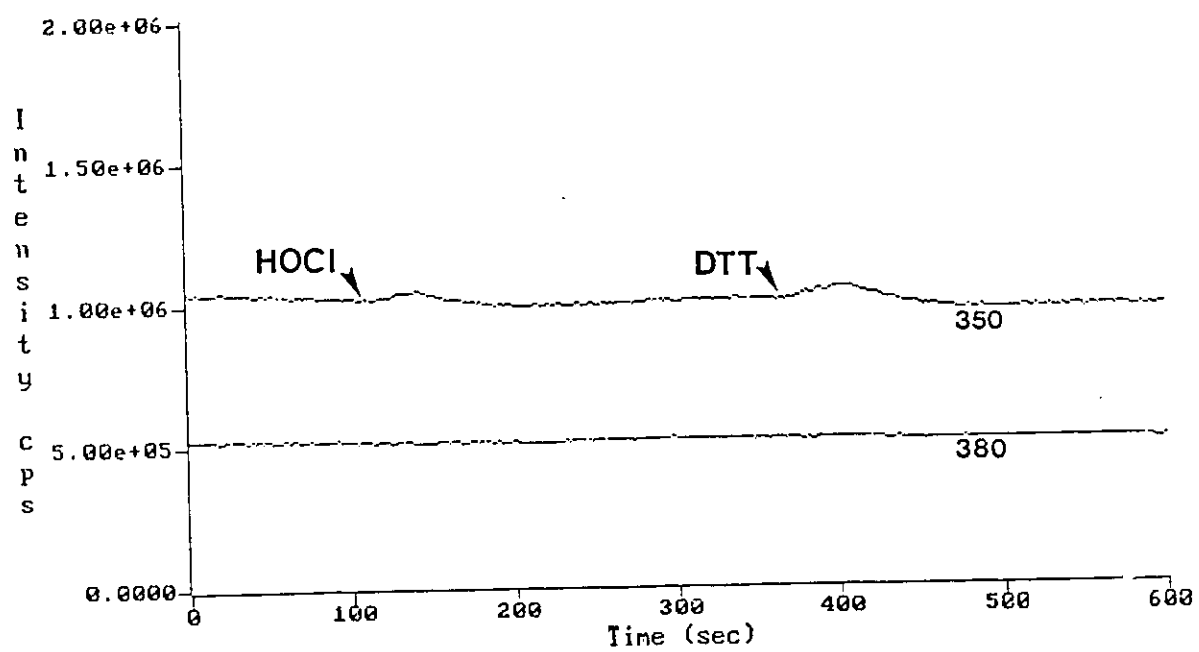


Figure 2.5 Cellular autofluorescence signals collected from a single rabbit cardiac myocyte during superfusion at ≈ 5 ml/min with superfusate containing a) 100 μ M HOCl and b) 500 μ M DTT. $[\text{Ca}^{2+}]_i = 2$ mM, $T=37^\circ\text{C}$, pH 7.4. The cell was sequentially excited with 350 and 380 nm light at 1 Hz, and fluorescence was monitored at 505 nm. Autofluorescence was measured in at least 3 unloaded cells by following the same perfusion protocol as the Fura-2 AM loaded cells. The mean fluorescence signals were subtracted from Fura-2 AM signals prior to ratioing. The units for emission intensity are arbitrary.

Figure 2.5



rabbit myocyte. Note that Fura-2 did not diffuse from the fine suction pipette into the cytosol prior to the application of the hyperpolarizing current. This loading technique allowed control over the amount of indicator being injected. To minimize Ca^{2+} buffering by the Fura-2, current injection was stopped when the fluorescence signals were 2-3x background. The lower panel shows the 350/380 ratio after background subtraction, and clearly shows that $[\text{Ca}^{2+}]_i$ was not perturbed during the injection procedure.

Figure 2.7 shows a typical Ca^{2+} transient (upper panel) and simultaneous transmembrane current (lower panel) recorded from an isolated rabbit cardiac myocyte during a 500 ms voltage clamp pulse from -40 mV to 0 mV in 2 mM $[\text{Ca}^{2+}]_e$ buffer. The Ca^{2+} transient can be divided into 2 distinct phases: a rapid but transient rise in $[\text{Ca}^{2+}]_i$ followed by a sustained elevation of steady-state $[\text{Ca}^{2+}]_i$. The fast component has been attributed to the entry of Ca^{2+} via the Ca^{2+} channels and the release of Ca^{2+} from the internal stores (Callewaert et al. 1988, Cannell et al. 1987). The potential-dependent, sustained component can be accounted for by a reduction of Ca^{2+} efflux through the electrogenic $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Cannell et al. 1987) and/or by an increase of background Ca^{2+} influx (Callewaert et al. 1988). The relaxation of the Ca^{2+} transient upon repolarization follows the small inward current tail, as expected from a relaxing $\text{Na}^+/\text{Ca}^{2+}$ exchange current (Giles and Shimon 1989).

It should be mentioned that the Ca^{2+} transient amplitude tended to decrease progressively throughout the duration of the experiments, such that their maximal amplitude (ie. peak minus steady-state $[\text{Ca}^{2+}]_i$ at -40 mV) was generally less than 500 nM in myocytes studied 3 to 4 hours after isolation. This decline was related to a diminution of the fast component, and may be explained by a gradual depletion of Ca^{2+} from the SR in the unstimulated myocytes.

Figure 2.6 Iontophoretic injection of Fura-2 into a single rabbit myocyte. After accessing the interior of the cell with the Fura-2 filled tip of a fine suction pipette, a continuous hyperpolarizing current (ie. -1 nA) was applied. The gradual increase in fluorescence intensity of both the 350 and 380 nm signals (sampling rate = 20 Hz, intensity units are arbitrary) is indicative of increasing [Fura]_i (upper panel). The upward spikes signal the start and end of current injection, and injection was stopped when fluorescence reached 2-3X background. Background fluorescence for each frequency was taken to be the values immediately prior to injection, and was subtracted prior to ratioing (lower panel): the period of large noise was caused by ratioing these small fluorescence values prior to the increase in intracellular Fura. During injection the 350/380 ratio remained constant, indicating that no change in [Ca²⁺]_i occurred during loading. Cell was superfused during injection with 2 mM [Ca²⁺]_e buffer at 37°C, pH 7.4.

Figure 2.6

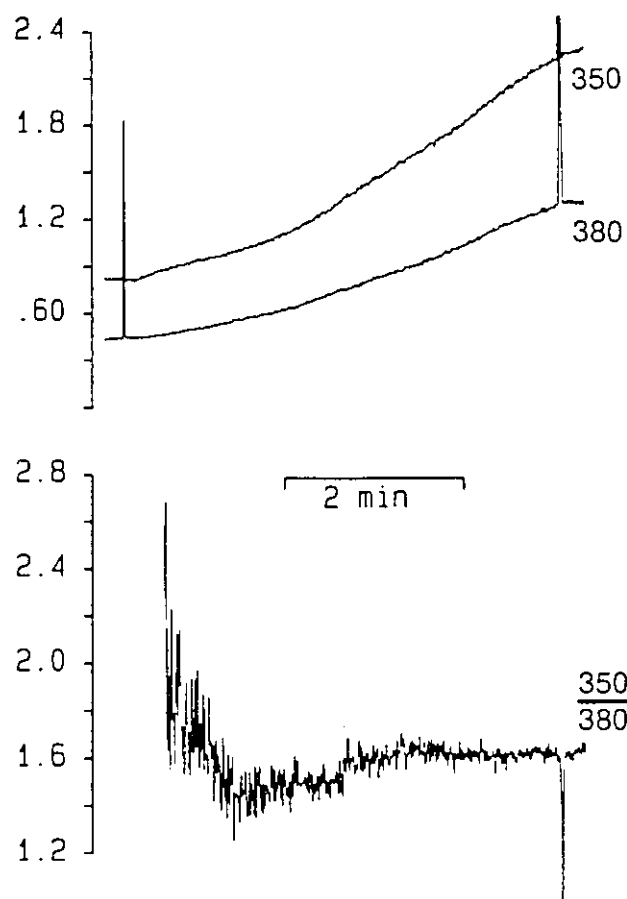
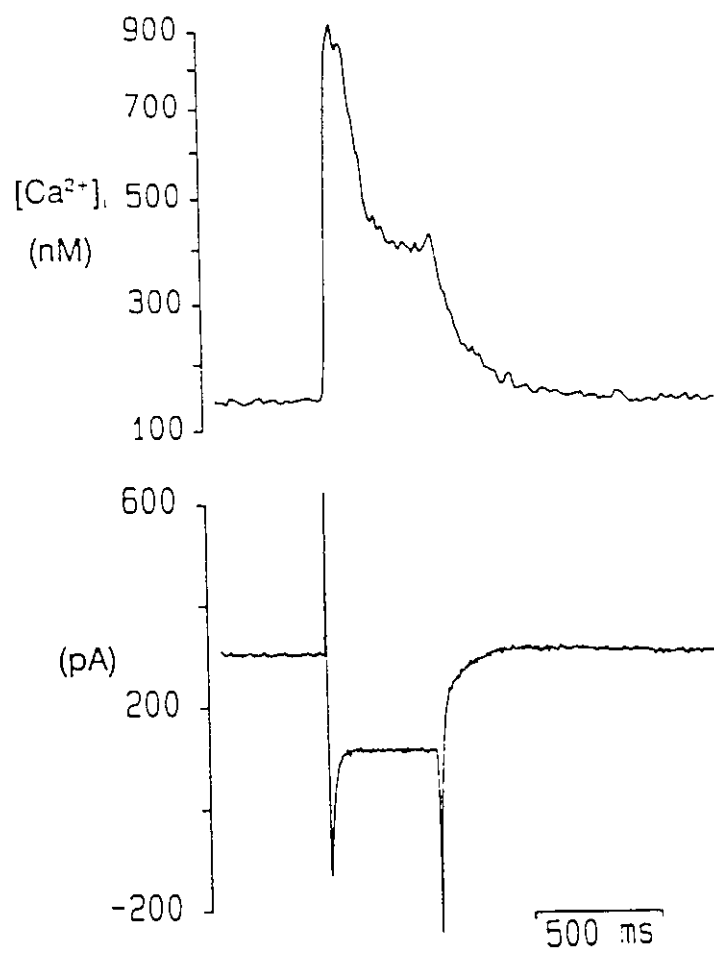


Figure 2.7 A representative Ca^{2+} transient (upper panel), recorded here as the 350/380 Fura-2 ratio, and inward whole cell Ca^{2+} current tracings (lower panel) in response to a 500 ms depolarizing pulse from -40 mV to 0 mV. Fluorescence and current recordings were digitized at 0.1 and 1 kHz respectively, and each signal represents the average of eight consecutive pulses (pulse frequency = 0.5 Hz). Depolarization of the cell caused a rapid, transient (≈ 200 ms) rise in $[\text{Ca}^{2+}]_i$ followed by a sustained component which lasted the duration of the pulse. $[\text{Ca}^{2+}]_i$ was calculated as described in Methods. $[\text{Ca}^{2+}]_e = 2$ mM, $T=37^\circ\text{C}$.

Figure 2.7



EFFECTS OF HOCl ON DIASTOLIC $[Ca^{2+}]_i$, Ca^{2+} TRANSIENTS, AND Ca^{2+} CURRENT MEASUREMENTS

Figure 2.8 shows the effects of HOCl on steady-state $[Ca^{2+}]_i$ in unstimulated Fura-2 loaded rat myocytes. The relative rise in the Fura-2 fluorescence signal in 2 mM $[Ca^{2+}]_e$ (increase by a factor of 3.1 ± 0.2 ; $n=6$) was not significantly different from that in nominally Ca^{2+} -free $[Ca^{2+}]_e$ (2.7 ± 0.3 ; $n=5$). The finding that $[Ca^{2+}]_i$ increased in response to HOCl even in the absence of $[Ca^{2+}]_e$ strongly suggests that the source of this Ca^{2+} is the intracellular stores. This is supported by the studies using caffeine, as shown in the lower panel in Figure 2.8. A single 20 s pulse of 5 mM caffeine induced a transient rise in $[Ca^{2+}]_i$, and virtually abolished any further response to HOCl (see also Figure 2.11 below). It should be noted that the cells were washed with superfusate containing no caffeine for 30 s prior to superfusion with HOCl, hence the presence of residual caffeine should be minimized. Further, the constant delivery of 100 μ M HOCl over the subsequent minutes should overcome any suspected scavenging effects of this residual caffeine. The insert in the lower panel of Figure 2.8 shows that a second 20 s pulse of caffeine after a 45 s washout period failed to produce a second rise in $[Ca^{2+}]_i$, suggesting that the SR was fully depleted by the initial caffeine pulse. Therefore, the finding that caffeine prevented any rise in $[Ca^{2+}]_i$ in response to treatment with HOCl is most probably related to its effects on the SR Ca^{2+} stores.

As shown in Figure 2.9, washout of HOCl after a 3 min superfusion period did little alleviate the elevated $[Ca^{2+}]_i$ condition (upper panel). In contrast, the exposure of HOCl-treated myocytes to 500 μ M DTT induced a rapid decrease in the fluorescence signals indicative of a reduction in steady-state $[Ca^{2+}]_i$ (lower panel). The rise in steady-

Figure 2.8 Effects of HOCl on steady-state Fura-2 AM signals in unstimulated rat cardiac myocytes under 2 mM (top panel) or 0 mM $[Ca^{2+}]_e$ (middle and bottom panels). Continuous superfusion with 100 μ M HOCl (started at the arrow) initiated a rise in $[Ca^{2+}]_i$ within 30 s which was independent of $[Ca^{2+}]_e$. In the lower panel, a 20 s prepulse of 10 mM caffeine caused a rapid, transient increase in the fluorescence ratio. The insert depicts a second cell under 0 mM $[Ca^{2+}]_e$, and shows that a second pulse of caffeine (insert) fails to elicit any further response, suggesting that the SR was fully depleted of Ca^{2+} . The units represent the value for the 350/380 ratio and not $[Ca^{2+}]_i$. $T=37^\circ C$.

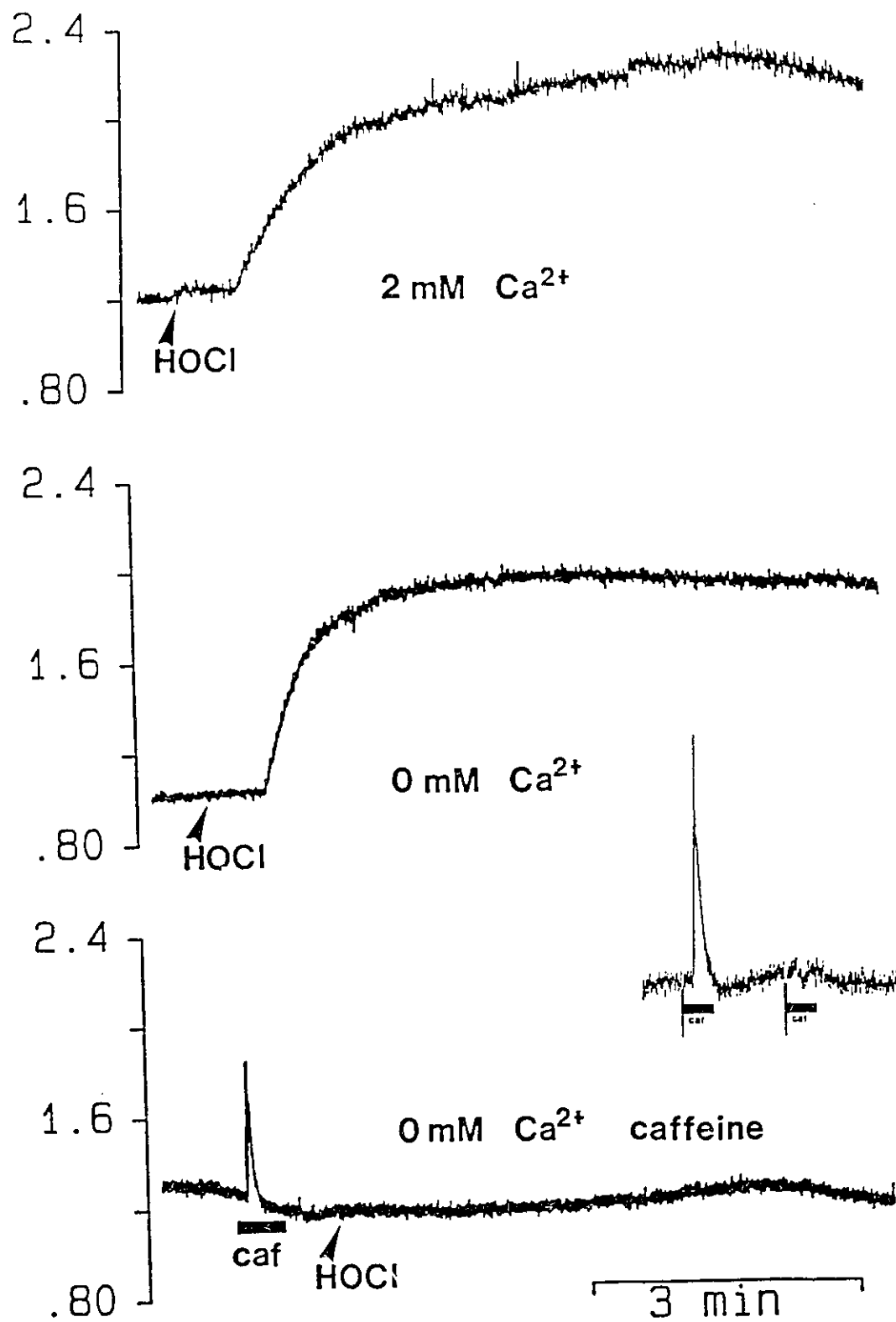
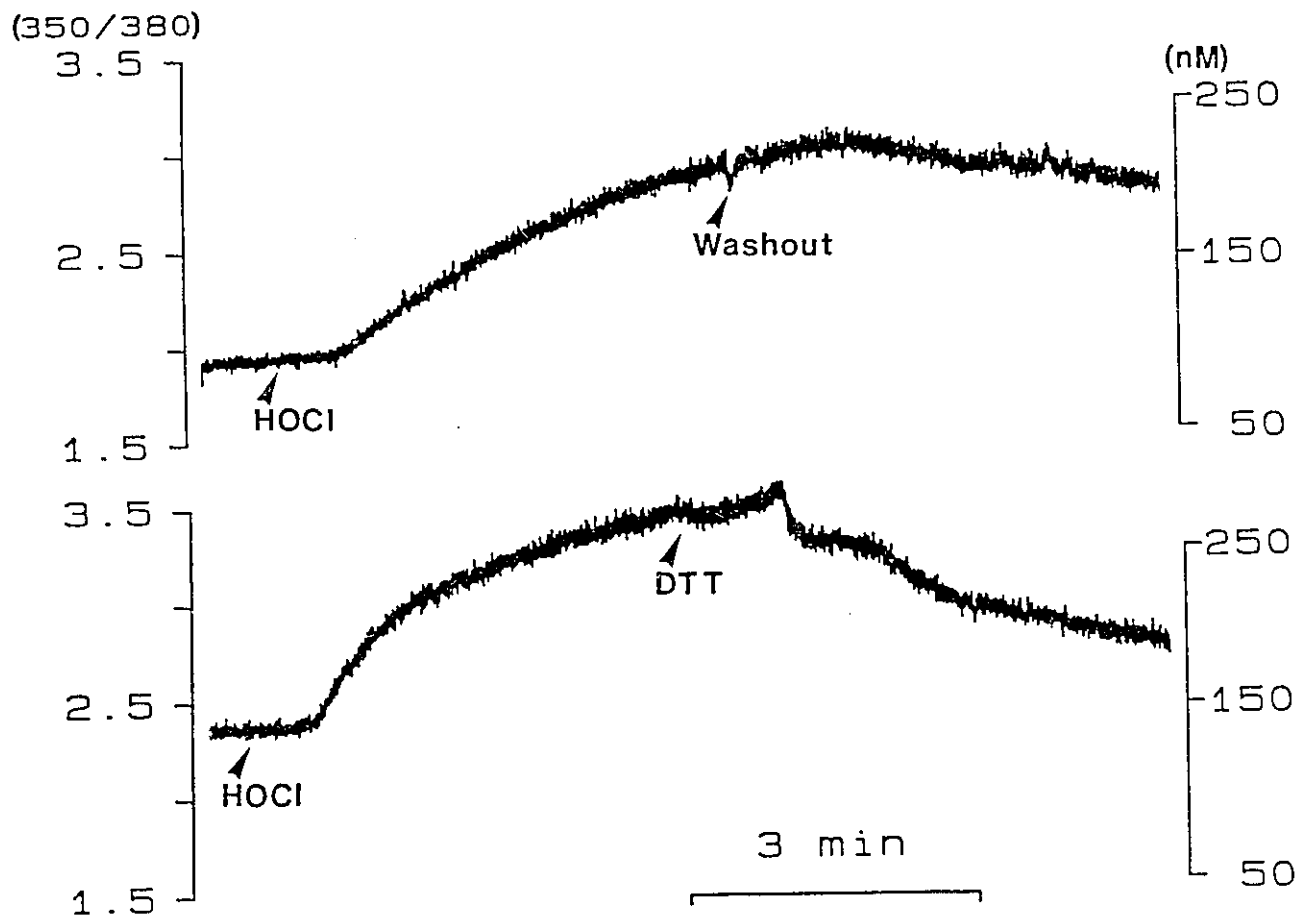


Figure 2.9 Steady-state Fura-2 AM ratio and the calculated $[Ca^{2+}]_i$ in two unstimulated rabbit myocytes during continuous superfusion with 100 μ M HOCl. In the cell shown here, subsequent Washout (upper panel) induced a small decline in $[Ca^{2+}]_i$ that remained elevated (185 nM) as compared to initial $[Ca^{2+}]_i$ (75 nM). Continuous superfusion with 500 μ M DTT (lower panel) caused an 'immediate' decline in steady-state $[Ca^{2+}]_i$ to 180 nM. Note that the initial steady-state $[Ca^{2+}]_i$ in the bottom panel was higher (ie 145 nM) than the upper panel (75 nM). T=37°C, $[Ca^{2+}]_e = 2$ mM.

Figure 2.9



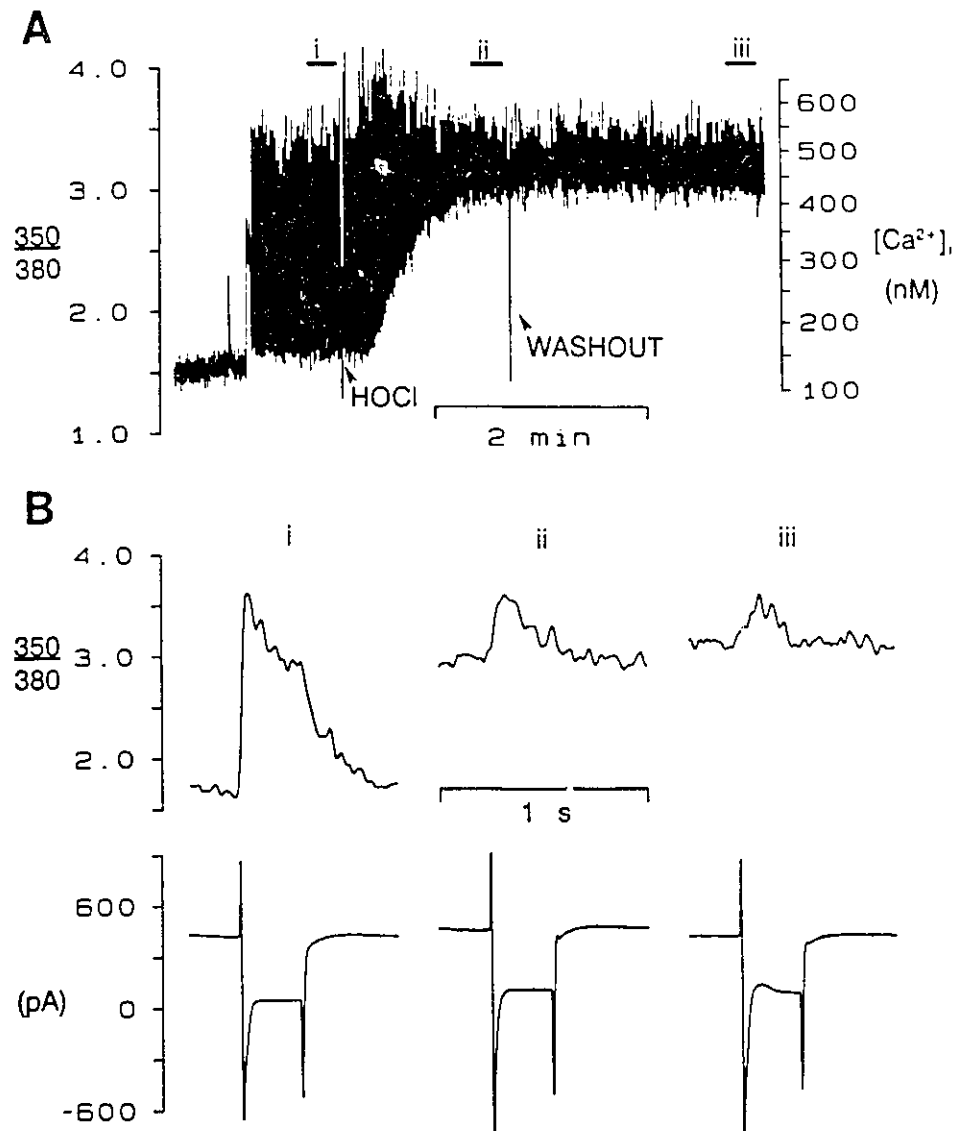
state $[Ca^{2+}]_i$ in response to HOCl, and the subsequent decline with DTT was similar in both rat and rabbit myocytes.

Figure 2.10 shows the Fura-2 fluorescence signal from a whole-cell clamped, Fura-2 loaded, rabbit myocyte stimulated at 1 Hz with 300 ms depolarizing pulses from -40 to 0 mV. As shown in the upper panel, superfusion with 100 μ M HOCl induced a marked rise in diastolic $[Ca^{2+}]_i$ over time. This effect was highly reproducible, and steady-state $[Ca^{2+}]_i$ increased on average from 78 ± 16 nM to 265 ± 48 nM ($n = 11$) within the first 3 min of exposure to HOCl. Similar changes were observed in rat cardiac myocytes with an average increase in steady-state $[Ca^{2+}]_i$ from 103 ± 16 to 388 ± 65 nM ($n = 5$). These effects of HOCl were not attenuated with Washout, and intracellular free Ca^{2+} remained elevated. All rat myocytes and several of the rabbit myocytes exhibited weak, non-synchronized, spontaneous contractions located mainly near the extremities during this period of elevated $[Ca^{2+}]_i$. After exposure to HOCl for more than 10 min, most cells spontaneously contracted, either very slowly to form square-shaped cells, or more rapidly to form rounded cells.

The lower panel in Figure 2.10 shows Ca^{2+} transients and simultaneous Ca^{2+} current tracings on an expanded time scale, depicting the values shown at the corresponding time on the upper panel. In all of the myocytes studied, HOCl caused a pronounced depression of the Ca^{2+} transients, with the maximum amplitude decreasing on average from 402 ± 89 nM to 82 ± 19 nM ($n=11$) after 3 min of exposure. Similar effects were observed in stimulated rat myocytes, with maximal amplitude decreasing from 660 ± 96 nM to 63 ± 33 nM ($n=5$). It is noteworthy that both the fast and the sustained components of the transients were depressed, suggesting that the intracellular release of Ca^{2+} was reduced following HOCl. Conversely, the amplitude of the inward Ca^{2+} current was, if anything, slightly increased by HOCl treatment ($9.5 \pm 5\%$ greater

Figure 2.10 Effects of HOCl superfusion on $[Ca^{2+}]_i$ and transmembrane currents. Panel A: Continuous recording of Fura-2 fluorescence ratio. After approximately 45 s, holding potential was switched from resting potential value of -80 mV to -40 mV, and 300-ms pulses to 0 mV were elicited at a frequency of 1 Hz. The initiation of superfusion with 100 μ M HOCl (first marker) caused an abrupt rise in steady-state $[Ca^{2+}]_i$ with a reduction of Ca^{2+} -transient amplitude. Washout of HOCl (second marker) failed to induce recovery of $[Ca^{2+}]_i$ levels. Panel B: Ca^{2+} transients and corresponding membrane currents are represented on an expanded time scale. Data were digitized at 0.1 Hz and 1 Hz respectively. These traces represent averages of 8 consecutive signals taken immediately prior to the addition of HOCl (at i), following 200 s exposure to HOCl (at ii) and following 200 s of washout (at iii) as indicated by the horizontal bars in Panel A. HOCl induced a depression of both components of the Ca^{2+} transients while increasing the average peak amplitude of the slow inward current from 710 to 860 pA by 200 s. Following washout, Ca^{2+} transient amplitude continued to decline while the inward Ca^{2+} current remained stable at 830 pA after 200 s of washout. Note also that with the exception of an initial outward shift, steady-state currents showed little variation throughout the protocol. Depolarization-induced outward currents, or conversely, slowly activating inward currents sometimes developed after prolonged exposure to HOCl, as observed in Panel B iii).

Figure 2.10



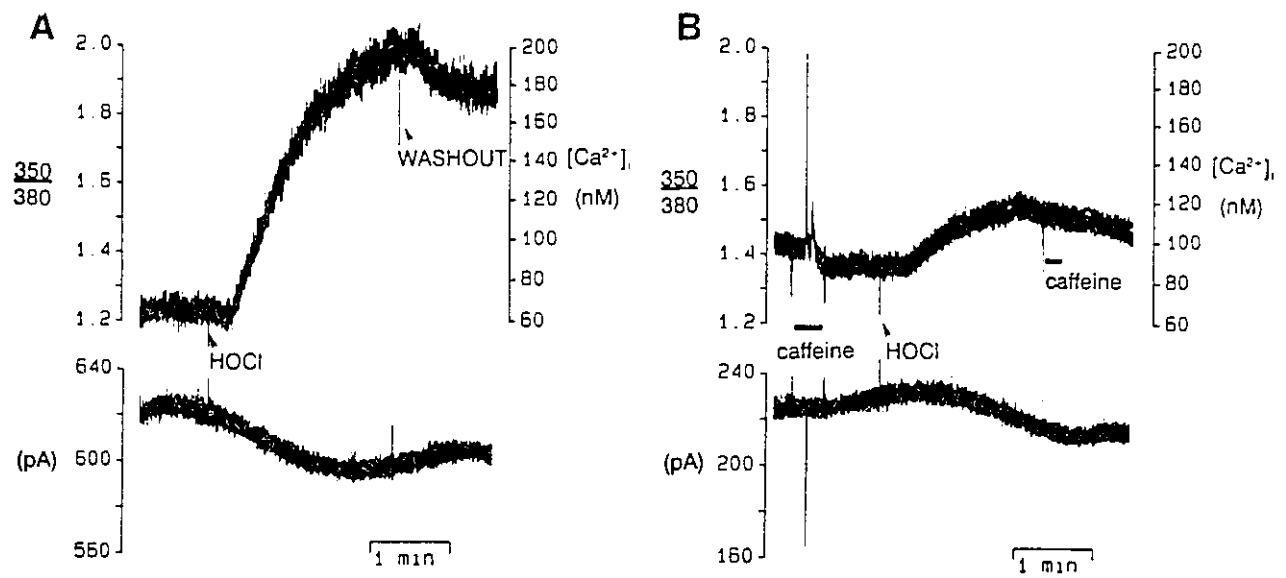
than initial amplitude, $n=11$). The whole cell holding current remained stable throughout the protocols, although small outward shifts were often observed (eg see Figure 2.10). The fact that HOCl induced only small variations of transmembrane ion currents indicates that the rise of steady-state $[Ca^{2+}]_i$ was not due to an augmentation of Ca^{2+} influx, and could therefore be of intracellular origin. Further, the loss of both the fast and prolonged components of the transients points towards an impairment of SR function.

In order to verify this conclusion, similar protocols to those described above were performed on voltage-clamped rabbit and rat myocytes in nominally Ca^{2+} free superfusate. As shown in Figure 2.11 even in the absence of extracellular Ca^{2+} , $[Ca^{2+}]_i$ rose from an average value of 87 ± 22 nM to 210 ± 16 nM ($n = 6$) following exposure to HOCl. These values were not statistically different from those recorded in 2 mM Ca^{2+} . In the experiment illustrated in Figure 2.11, membrane potential was held at 0 mV. This observed HOCl-induced rise in $[Ca^{2+}]_i$ (ie. 130 nM) was similar to that observed at resting membrane potential, therefore it appears that HOCl-effects are independent of membrane potential. A similar rise was observed in Fura-2 loaded rat cardiac myocytes (106 ± 34 to 303 ± 45 nM; $n = 4$) under 0 mM $[Ca^{2+}]_e$ conditions. Washout did not alleviate this rise in $[Ca^{2+}]_i$. These experiments show clearly that the main source of this $[Ca^{2+}]_i$ must be the internal stores.

The involvement of the SR was further examined by exposing myocytes to 10 mM caffeine for a 20 s pulse prior to HOCl treatment, as shown in Figure 2.11 B. Caffeine induced a large, transient increase of intracellular Ca^{2+} (mean amplitude of 199 ± 31 nM; $n = 6$) that was consistent with a release of Ca^{2+} from the SR. A second pulse of caffeine prior to HOCl (see Figure 2.8 insert) or following HOCl failed to release any further Ca^{2+} , verifying that the caffeine pulse had effectively depleted the releasable pool of SR Ca^{2+} as expected (Salviati and Volpe 1988). Subsequent exposure to HOCl failed

Figure 2.11 Effects of HOCl on steady-state $[Ca^{2+}]_i$ and holding current in nominally Ca^{2+} -free solution (Ca^{2+} replaced with 2 mM Mg^{2+} and 0.5 mM EGTA). Panel A: Steady-state recordings from an unstimulated myocyte exposed to 100 μ M HOCl after 3 min superfusion with nominally Ca^{2+} -free solution. HOCl induced a rapid rise of the fluorescence ratio corresponding to an increase of $[Ca^{2+}]_i$ from 60 to 200 nM. Holding current decreased by 25 pA. Washout of HOCl caused only a small recovery of $[Ca^{2+}]_i$ and holding current. Holding potential was set at zero mV throughout. Panel B: Prior to the addition of HOCl the cell was exposed to nominally Ca^{2+} -free solution for 1 min, then Ca^{2+} -free solution with 10 mM caffeine for 20 s. Caffeine induced a large but brief ($\approx$ 2 s) Ca^{2+} spike which corresponded to an amplitude of 100 nM Ca^{2+} and coincided with a 65 pA inward spike of the holding current. Under these conditions, HOCl induced only a small rise of $[Ca^{2+}]_i$ from 90 to 120 nM. Re-exposure to caffeine, together with a washout of HOCl, failed to elicit any further release of Ca^{2+} or any current spike. Holding potential was set at -60 mV throughout. Recordings in Panels A and B are from two different cells. Sampling rate was 100 Hz for all tracings.

Figure 2.11



to induce any substantial increase in the fluorescence signal, with a mean rise from 59 ± 19 to 81 ± 17 nM ($n = 6$). These data suggest that the main source of the HOCl-induced rise in Ca^{2+} is the SR stores.

Even in the absence of extracellular Ca^{2+} , HOCl had little effect on the holding currents, which remained stable within 20 pA during the first 5 min of exposure. This contrasts with the relatively large inward current induced by the first exposure to caffeine (see Figure 2.11). This transient current can be attributed to the Ca^{2+} -activated electrogenic $\text{Na}^+/\text{Ca}^{2+}$ exchange (Callewaert et al. 1988), or possibly to some Ca^{2+} -dependent nonspecific ionic conductances (Colquhoun et al. 1981). As expected, the second exposure to caffeine during HOCl treatment failed to induce any further transient current.

Figure 2.11 also confirms that the *in vivo* effects of HOCl on Fura-2 fluorescence properties are similar to those observed *in vitro* (ie. no effect), as exposure of the cell to HOCl does not significantly alter the fluorescence signals.

EFFECTS OF DTT ON THE HOCl-INDUCED Ca^{2+} IMBALANCE

As shown in Figure 2.10, Washout after 2 min exposure to HOCl failed to attenuate or reverse the oxidant's effects. However, a substantial reversal of these effects was obtained repeatedly by subsequent superfusion of the cells with 500 μM DTT. Figure 2.12 clearly illustrates this reversal. The replacement of HOCl with DTT rapidly induced a decline in steady-state $[\text{Ca}^{2+}]_i$ towards control levels, declining from a mean of 288 ± 70 nM to 132 ± 27 nM ($n = 6$) in rabbit cells. A similar reversal was observed in rat myocytes (Figure 2.13), where steady-state $[\text{Ca}^{2+}]_i$ declined from a mean of 376 ± 83 nM to 190 ± 78 nM ($n=4$) after 5 min exposure to DTT. This decline in $[\text{Ca}^{2+}]_i$ paralleled a significant restoration of Ca^{2+} transient amplitude (Figure 2.12, lower

Figure 2.12 Effects of DTT on $[Ca^{2+}]_i$ and transmembrane currents in a cell previously exposed to 100 μ M HOCl. Panel A: Membrane potential was held at -40 mV and Ca^{2+} transients were elicited by 300-ms depolarizing pulses (1 Hz) as described in Figure 2.10. HOCl was replaced by 500 μ M DTT after approximately 200 s (second marker). This induced a rapid decline in $[Ca^{2+}]_i$ in combination with a recovery of Ca^{2+} transient amplitude. Panel B: Ca^{2+} transients and corresponding Ca^{2+} currents on an expanded time scale, representing the average of eight consecutive pulses during the periods indicated by the horizontal bars in Panel A: immediately prior to HOCl superfusion (at i), after 2 min of HOCl superfusion (at ii), and after 2 min (iii) and 4 min (iv) of DTT superfusion. DTT induced a rapid and substantial restoration of both components of the Ca^{2+} transients as well as steady-state $[Ca^{2+}]_i$. On the other hand, prolonged exposure to DTT resulted in a decrease in the peak amplitude of the slow inward Ca^{2+} current. DTT also caused an inward shift of steady-state holding current at -40mV (iii).

Figure 2.12

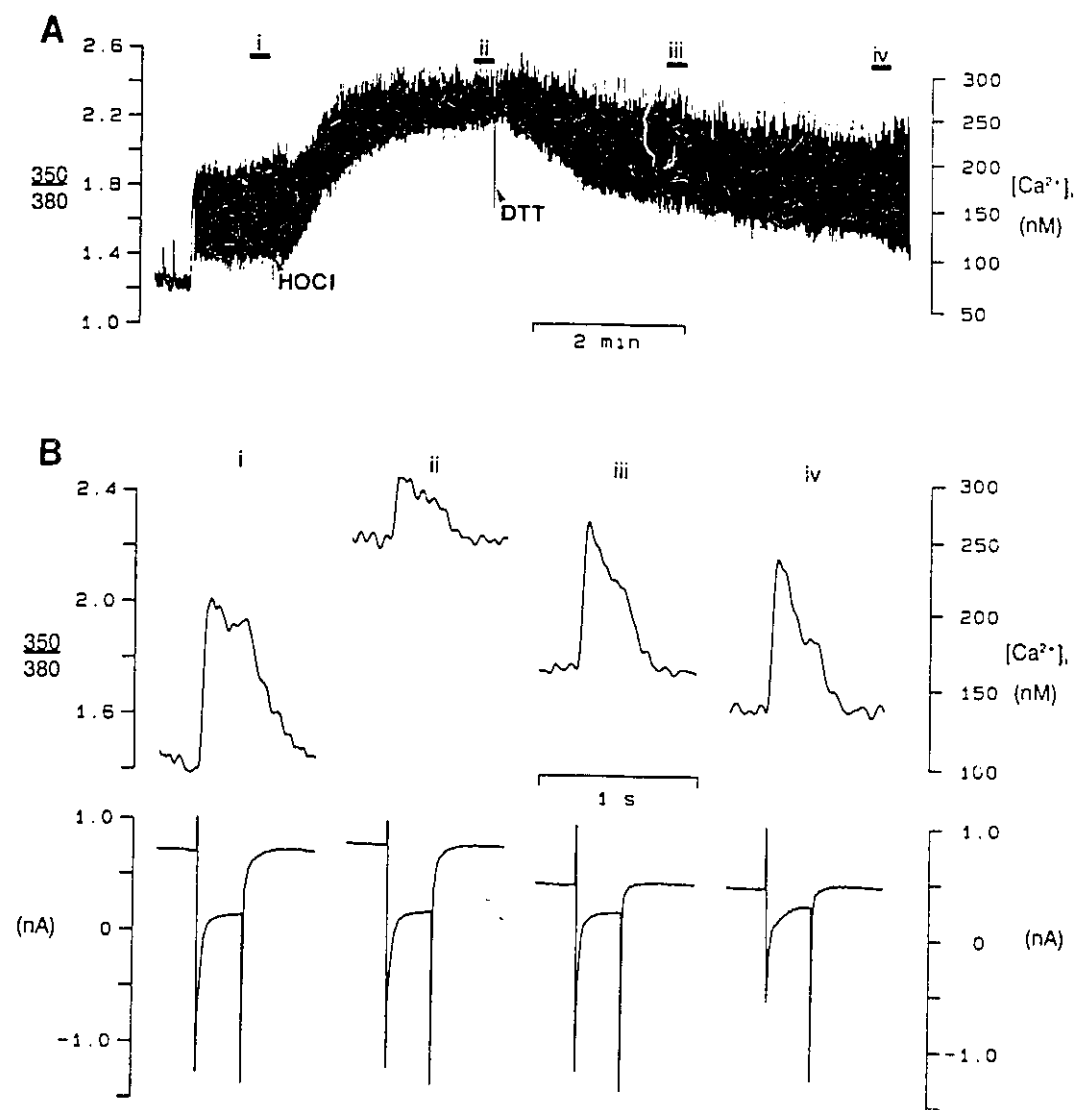
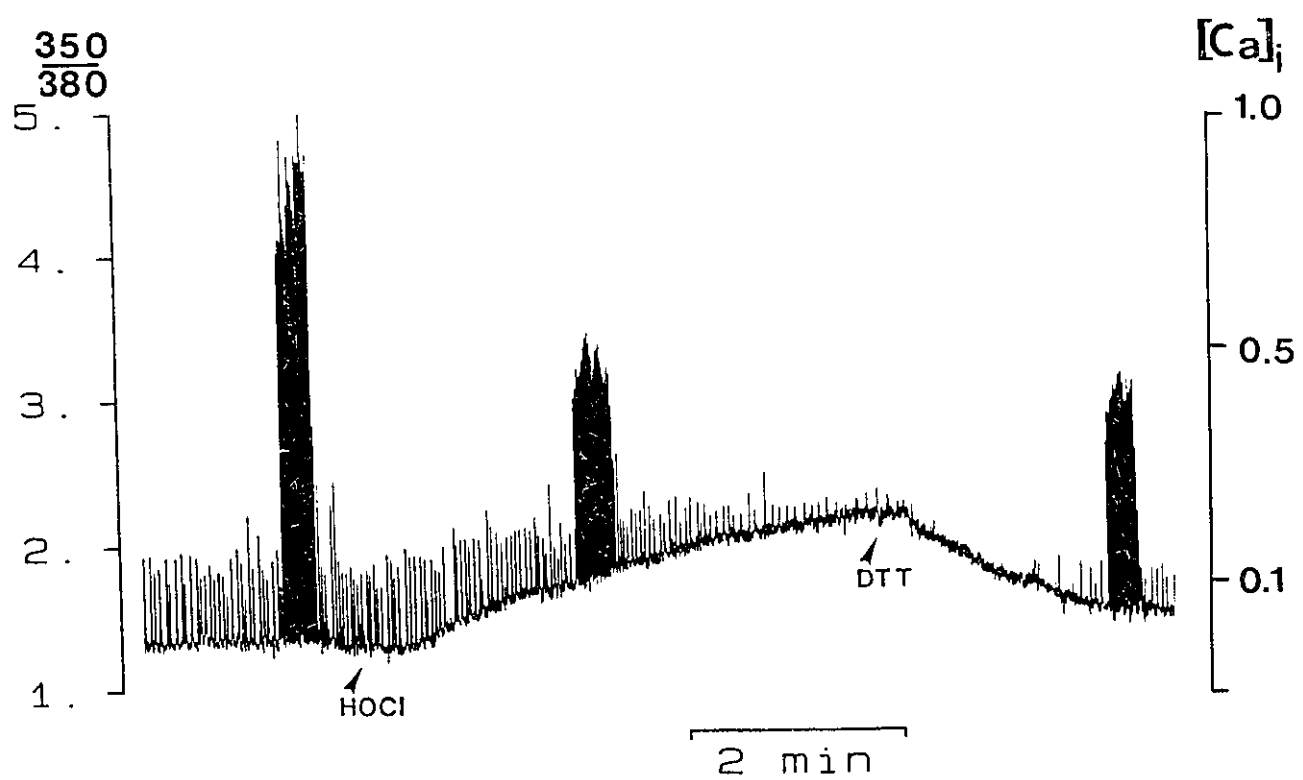


Figure 2.13 Effects of DTT on $[Ca^{2+}]_i$ in a rat cardiac myocyte exposed to 100 μM HOCl. This cell was held at resting membrane potential (-65 mV) and stimulated by 300 ms pulses to 0 mV during the periods indicated by the vertical bars. Note the presence of spontaneous activity, as was common in the rat cells, that diminished upon exposure to HOCl and reappeared following treatment with DTT. Ca^{2+} transient amplitude decline to less than 40% of original as steady-state $[Ca^{2+}]_i$ increased following HOCl. The initiation of superfusion with DTT (second marker) produced a rapid recovery of steady state $[Ca^{2+}]_i$ concomitant with a return of the spontaneous activity. This cell was superfused in 2 mM $[Ca^{2+}]_e$, pH 7.4, 37°C.

Figure 2.13



panel). Maximal transient amplitude increasing from a mean of 92 ± 52 nM to 233 ± 67 nM ($n = 6$) in rabbit cells and 34 ± 21 nM to 188 ± 76 nM in the rat ($n=4$). These data suggest that a replenishment of the releasable SR pool of Ca^{2+} has occurred, possibly due to a recovery of Ca^{2+} uptake from the cytosol. Any cells which exhibited spontaneous contractile activity during exposure to HOCl were returned to a quiescent state with DTT.

Interestingly, the DTT-induced restoration of Ca^{2+} transients occurred in spite of a depression of the inward Ca^{2+} currents. Initially, DTT had no effect on the holding current or inward Ca^{2+} currents, but by 3 min a significant depression of the inward Ca^{2+} was observed, suggesting that thiol reduction with DTT may have adverse effects on the transarcolemmal influx of Ca^{2+} via the Ca^{2+} channel. A similar decrease in Ca^{2+} current amplitude was also observed in control cells exposed to DTT.

DISCUSSION

The results from this study show clearly that exposure of ventricular myocytes to the oxidant HOCl causes rapid changes of both steady-state $[Ca^{2+}]_i$ and Ca^{2+} transient dynamics. While these effects were irreversible by washout, they were substantially reversed by DTT. These results are analogous to the effects of HOCl and DTT on the diastolic and systolic contractile function of rat papillary muscles (see Chapter 1 of this thesis) and in the retrograde perfused rat heart (Eley et al. 1990b). It is therefore likely that one of the major factors contributing to the HOCl-induced mechanical dysfunction in the myocardium is the disruption of cellular Ca^{2+} homeostasis.

HOCl CAUSES THE RELEASE OF Ca^{2+} FROM INTRACELLULAR STORES

The observed rise in steady-state $[Ca^{2+}]_i$ in response to HOCl could theoretically be attributed to either an increased influx of Ca^{2+} from the extracellular compartment and/or to an increased release of Ca^{2+} from intracellular stores. Evidence in the literature has shown that oxidative stress may damage the integrity of the sarcolemmal membrane through lipid peroxidation, leading to a non-specific influx of Ca^{2+} into the cell (Albrich et al. 1986, Huber and Weiss 1989, Sepe and Clark 1985). However, the present data clearly demonstrate that, at least over the short time scale examined here, the integrity of the sarcolemmal membrane was not altered upon exposure to HOCl. Any increase in membrane permeability should result in a decrease in input resistance and hence an inward shift in holding current at negative potentials. Yet transmembrane ion currents remained stable or slightly outward at -40 mV. Furthermore, $[Ca^{2+}]_i$ did not rise to match $[Ca^{2+}]_e$, but rather reached a new steady-state level approximating the amplitude of the caffeine-induced Ca^{2+} transient (Figure 2.11). Most importantly, the rise in $[Ca^{2+}]_i$ in response to HOCl occurred even in the absence of external Ca^{2+} . The

magnitude of this rise in $[Ca^{2+}]_i$ (141 ± 21 nM) was similar to that seen in 2 mM $[Ca^{2+}]_e$ (187 ± 40 nM) indicating that a major portion of the Ca^{2+} originated from the intracellular stores.

This latter observation also rules out several other plausible HOCl-dependent mechanisms which could lead to Ca^{2+} overload due to an influx of Ca^{2+} from the extracellular milieu. For instance, thiol oxidation has been shown to inhibit the function of the Na^+/K^+ -ATPase of the myocardium (Kako et al. 1988, Kim and Akera 1987, Temma et al. 1978). While the consequent rise in intracellular Na^+ and hence of $[Ca^{2+}]_i$, could occur during exposure to HOCl, these experiments in 0 mM $[Ca^{2+}]_e$ argue against such a mechanism as the principal cause of the intracellular rise in Ca^{2+} . As well, previous reports have shown that oxidative stress can increase Ca^{2+} influx via the Ca^{2+} channels, leading to cellular depolarization, arrhythmias, and subsequent Ca^{2+} overloading (Aomine and Abe 1978, Hayashi et al. 1989a, Hayashi et al. 1989b). While the present experiments do show a slight increase in the inward Ca^{2+} currents (app 10%) this is unlikely to be sufficient to induce the observed rise in $[Ca^{2+}]_i$. No significant change in background current was recorded in either stimulated or unstimulated cells during the initial phases of HOCl exposure, showing that the cells were not depolarizing. Finally, $[Ca^{2+}]_i$ increased in unstimulated cells, as well as in 0 mM $[Ca^{2+}]_e$ during exposure to HOCl, showing conclusively that the observed increase in the inward Ca^{2+} current was not a major cause of the observed rise in $[Ca^{2+}]_i$.

Finally, in *E. Coli*, HOCl has been shown to cause ATP depletion (Barrette et al. 1987) leading to a loss of cellular ionic regulation and intracellular Na^+ loading. In a contracting muscle it would be expected that ATP utilization would be use dependent; ie. contracting cells would use more ATP than non-contracting cells under steady-state conditions. Therefore, if HOCl is acting to deplete ATP in our cells, $[Ca^{2+}]_i$ would have risen faster in stimulated cells than in non-stimulated cells. In the present study, the rate

and magnitude of rise in intracellular $[Ca^{2+}]_i$ were shown to be independent of stimulation frequency, suggesting that at least within the time period examined in this study, ATP depletion is not the main factor precipitating the rise in $[Ca^{2+}]_i$.

That this rise in the Fura-2 fluorescence signal was prevented by a 20-second pre-exposure to caffeine also confirms that HOCl does not increase the fluorescence ratio directly by altering the properties of the fluorophore. Further, this absence of response following caffeine precludes other possible factors which could alter Fura-2 fluorescence intensity, such as changes in intracellular pH (Ganz et al. 1990) or an HOCl-induced release of heavy metals which may bind to Fura-2 (Grynkiewicz et al. 1985). Although caffeine is known to have effects other than to stimulate the release of Ca^{2+} from the SR (Blanchard and Alpert 1987, Wyskovsky et al. 1988), the inhibition of this HOCl-induced rise in $[Ca^{2+}]_i$ by caffeine strongly suggests that the SR is the source of this $[Ca^{2+}]_i$.

An impairment of SR function is also well supported by the HOCl-induced inhibition of the Ca^{2+} transients. If we assume that the majority of the Ca^{2+} associated with the rapid rise in $[Ca^{2+}]_i$ during a depolarization-induced Ca^{2+} transient is due to the release from the SR, the loss of this component suggests that this 'releasable' pool of intracellular Ca^{2+} has been depleted. This would be in agreement with data showing that HOCl both increases SR Ca^{2+} release (Trimm et al. 1986, Zaidi et al. 1989) and impairs SR Ca^{2+} uptake (Eley et al. 1990a, Kukreja et al. 1989) in isolated cardiac microsomes.

HOCl IMPAIRS CELLULAR Ca^{2+} -EXTRUSION MECHANISMS

If the sole action of HOCl was to deplete the SR of Ca^{2+} , the corresponding rise in $[Ca^{2+}]_i$ should be transient, in a manner comparable to that observed during exposure to caffeine. However, following exposure to HOCl, the rise in $[Ca^{2+}]_i$ was not transient

but remained sustained for several minutes. This suggests that the Ca^{2+} was effectively “trapped” within the cytosol, leading to the conclusion that HOCl exposure induces an impairment of the cellular Ca^{2+} extrusion mechanisms as well. This would be in accordance with the reported impairment of the $\text{Na}^+/\text{Ca}^{2+}$ exchange (Dixon et al. 1987, Pierce et al. 1986) and the sarcolemmal Ca^{2+} pump (Kaneko et al. 1989) as well as the SR Ca^{2+} -ATPase (Aoki et al. 1986 Kukreja, 1989 #468) by thiol oxidation following exposure to oxidants.

An interesting observation arising from these results concerns the fact that the magnitude of the HOCl-induced rise of $[\text{Ca}^{2+}]_i$ in the absence of external $[\text{Ca}^{2+}]$ was not significantly different from that in the presence of 2 mM external $[\text{Ca}^{2+}]$. If we assume that the cellular Ca^{2+} extrusion mechanisms were inhibited, and consider that the inward Ca^{2+} influx through the voltage-dependent Ca^{2+} channels were if anything enhanced, we should have observed a greater HOCl-induced rise in $[\text{Ca}^{2+}]_i$ in the presence of 2 mM $[\text{Ca}^{2+}]_e$. Several mechanisms could be proposed to account for this apparent discrepancy. First, it is possible that intracellular Ca^{2+} binding sites or stores other than the SR, such as the mitochondria, may become effective in buffering intracellular Ca^{2+} only at elevated $[\text{Ca}^{2+}]_i$. However, under conditions of constant influx these systems would eventually saturate, leading to a secondary rise of $[\text{Ca}^{2+}]_i$. This would be in agreement with our observation that the prolonged (ie.10 min) exposure of myocytes to HOCl in 2 mM $[\text{Ca}^{2+}]_e$ resulted in spontaneous synchronized contractions and eventual contracture, however no secondary elevation of $[\text{Ca}^{2+}]_i$ was observed prior to cell contracture. Second, the affinity of the transsarcolemmal Ca^{2+} extrusion mechanisms towards Ca^{2+} may be reduced following HOCl exposure. This would result in the elevation of the steady-state $[\text{Ca}^{2+}]_i$ to a level at which the rate of Ca^{2+} efflux would again equal Ca^{2+} influx. In other words, an HOCl-induced shift of the dissociation curve of the Ca^{2+} extrusion mechanisms would cause a rise of $[\text{Ca}^{2+}]_i$ that could appear

to be relatively independent of Ca^{2+} influx, and hence of extracellular $[\text{Ca}^{2+}]$. Finally, the possibility exists that the absence of external Ca^{2+} could enhance the deleterious effects of HOCl (Smith et al. 1981). Under 0 mM $[\text{Ca}^{2+}]_e$ conditions, the inhibition of the Ca^{2+} extrusion mechanisms would be more pronounced than under 2 mM $[\text{Ca}^{2+}]_e$ conditions.

DTT-INDUCED RESTORATION OF Ca^{2+} HOMEOSTASIS

The recovery of steady-state $[\text{Ca}^{2+}]_i$ following superfusion with DTT but not with Washout implicates a reversible thiol oxidation in the proteins responsible for Ca^{2+} extrusion. Similarly, the recovery of the Ca^{2+} transients shows that the releasable intracellular pool of Ca^{2+} has been replenished, and implicates a restoration of the SR Ca^{2+} -ATPase function in this DTT-induced recovery. Thiol oxidation is known to impair the function and activity of the SR Ca^{2+} -ATPase *in vitro* (Ariki and Shamoo 1983, Georgoussi and Sotiroudis 1985, Henao and Gutierrez-Merino 1989, Kawakita and Yamashita 1987, Kukreja et al. 1988, Scherer and Deamer 1986b). There is also evidence that HOCl perfusion of the rat heart reduces the activity and function of the SR Ca^{2+} -ATPase *in situ* to less than 20% of control (see Chapter 3 below). There is also evidence to suggest that this impairment is reversible with thiol reduction (Eley et al. 1989a). Taken together, the present data support the concept that DTT induced an intracellular restoration of the SR Ca^{2+} -ATPase function, leading to a repletion of the releasable SR Ca^{2+} pool. This would account in part for the decrease in $[\text{Ca}^{2+}]_i$, and the restoration of the Ca^{2+} transients observed following exposure to DTT.

While this replenishment of the releasable Ca^{2+} pool could be due to either an increased loading of the SR following reactivation of the Ca^{2+} -ATPase, or an increase in the activity of the Ca^{2+} release channels of the SR, there is strong evidence to suggest that thiol reduction with DTT decreases rather than increase Ca^{2+} release from heavy SR

vesicles (Trimm et al. 1986, Zaidi et al. 1989). This makes the latter possibility less probable. The fact that cytosolic $[Ca^{2+}]$ decreases while the intracellular releasable pool increases further implicates a restoration of the SR Ca^{2+} -ATPase function in this DTT-induced recovery. This possibility will be examined further using isolated SR vesicles in Chapter 3 of this thesis.

SUMMARY

The data presented in these studies suggest that under the present experimental conditions, the early events associated with HOCl exposure consist of: 1) release of Ca^{2+} from the SR which results in an inhibition of the depolarization-induced Ca^{2+} transients concomitant with a rise in diastolic free Ca^{2+} level, and 2) an impairment of the sarcolemmal Ca^{2+} extrusion mechanisms, leading to a maintenance of an elevated $[Ca^{2+}]_i$. HOCl had little effect on the inward Ca^{2+} currents or time-independent background conductances, further supporting the notion that the rise in $[Ca^{2+}]_i$ was not dependent upon an influx of Ca^{2+} from the extracellular milieu. The reduction of both steady-state $[Ca^{2+}]_i$ and Ca^{2+} transient amplitude by subsequent exposure to DTT suggests that an alteration of protein thiol redox status is involved in the HOCl-induced disruption of cellular Ca^{2+} homeostasis.

CHAPTER 3:

MYOCARDIAL CONTRACTILITY AND SARCOPLASMIC RETICULUM FUNCTION FOLLOWING HOCl PERFUSION OF ISOLATED RAT HEARTS: RECOVERY WITH DTT

INTRODUCTION:

THE ROLE OF THE SARCOPLASMIC RETICULUM IN EC COUPLING

The classic studies by Ringer showed that the presence of extracellular Ca^{2+} was necessary for the maintenance of contraction in cardiac muscle (Ringer 1883), and he proposed that the influx of Ca^{2+} was the activator signal. However, in 1948, Hill calculated that, at least in skeletal muscle, the minimal diffusion time for any ionic substance to reach the myofibrils from the extracellular compartment was far too great to allow for such diffusion to be directly responsible for contraction during a single action potential (Hill 1948). Similarly, in 1958, Huxley determined that the amount of Ca^{2+} crossing the sarcolemmal membrane during a single action potential was insufficient to elicit contraction in skeletal muscle (Huxley and Taylor 1958). From this came the suggestion that the source of activator Ca^{2+} must be intracellular, and that the depolarization of the cell must in some way stimulate a synchronous release of this internal Ca^{2+} . By the early 1960's it was established that the internal membrane system known as the sarcoplasmic reticulum (SR) was a major Ca^{2+} source during EC coupling in skeletal muscle (Ebashi and Lipmann 1962, Huxley 1957, Podolsky and Constantin 1964, Winegrad 1965). However, this has been shown to be highly species and tissue

dependent (Aoki et al. 1986, Fabiato 1982, Horackova 1986, Kort and Lakatta 1988, Nayler et al. 1975a, Nayler et al. 1975b, Sutko and Willerson 1980). For example, the frog myocardium exhibits a prolonged action potential duration in combination with a poorly developed SR network, and is almost completely dependent upon transsarcolemmal Ca^{2+} influx for contraction. Conversely, the rat myocardium exhibits a shortened action potential duration in combination with an extensive SR network, and is more dependent upon SR function. It has also been shown that the contribution to the activator Ca^{2+} pool by the SR in the rat myocardium is highly dependent upon the level of SR loading following the previous depolarizing event, as previously discussed in Chapter 1.

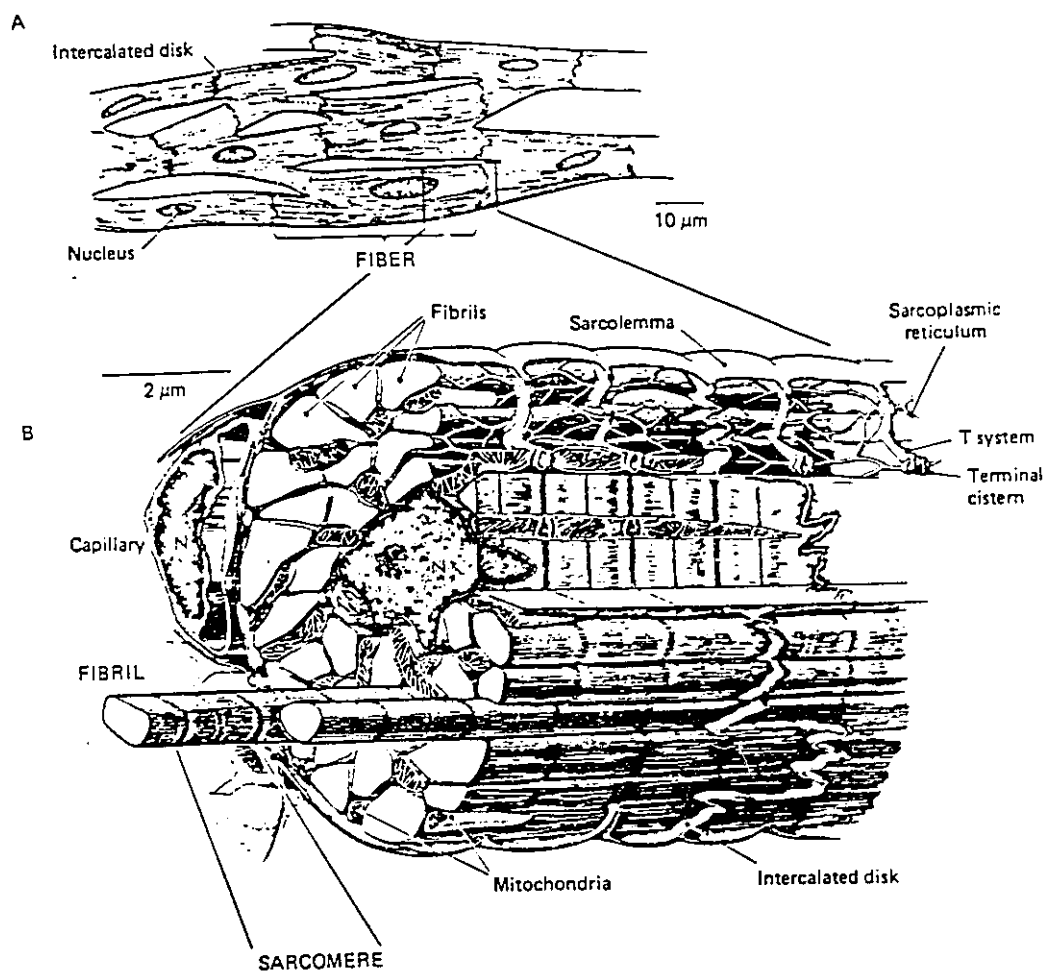
The involvement of the SR in EC coupling was further enhanced by the discovery and characterization of an ATP dependent Ca^{2+} pump in a membrane fraction isolated from the SR by Hasselbach and co-workers (Hasselbach 1964, Hasselbach and Makinose 1961) and Ebashi and Lipmann (1962). The ability of this intracellular pump to lower intracellular $[\text{Ca}^{2+}]$ to a level at which contraction would not occur established the SR as the major relaxing factor in skeletal muscle (Hasselbach 1964). Since this time, the role of the SR as a relaxing factor in skeletal muscle, as well as in cardiac muscle, has been examined extensively under both physiological and pathological conditions.

STRUCTURE AND FUNCTION OF THE SARCOPLASMIC RETICULUM

The sarcoplasmic reticulum is a highly specialized form of endoplasmic reticulum consisting of an intracellular network of interconnecting tubules ranging from 20-60 nm in diameter (Severs 1990, Van Winkle 1988) which surrounds the myofibrillar apparatus as shown in Figure 3.1. In cardiac muscle, the SR is segmented with repeating bands of outpocketings or junctions overlying the M-band of each sarcomere. The entire network of the SR can be crudely divided into the junctional SR or terminal cisternae, which are

Figure 3.1 Drawing of a cardiac myofibril showing the relationship between the sarcoplasmic reticulum (SR) and the contractile apparatus. Adapted from Braunwald E, Ross, J, Sonnenblick, EH: Mechanisms of contraction of the normal and failing heart. N. Engl. J. Med. 1967;277:794.

Figure 3.1



closely apposed or attached to the T-tubular network of the sarcolemma, and the non-junctional or longitudinal SR (Van Winkle 1988) which are distributed in close proximity to the myofibrils and are not physically connected with the outer membrane system. Unlike the skeletal muscle SR, where the junctional complex is composed of two separate sacs of the terminal cisternae on opposite sides of the T tubule, the cardiac junctional SR commonly encircles the entire T tubule (Forbes and Sperelakis 1982). These enlarged junctional cisternae are thought to be the main storage sites for Ca^{2+} . Bridging the gap between the T tubule and the junctional SR are densely staining structures referred to as the feet structures (Forbes and Sperelakis 1982, Franzini-Armstrong and Nunzi 1983) which have recently been determined to contain the ryanodine receptor and Ca^{2+} release channel (Lai et al. 1988a, Lai et al. 1988b, Meissner 1975). The remaining longitudinal regions of the SR spread across the myofibrils and are responsible for the re-uptake of Ca^{2+} from the myoplasm (Carafoli 1985, Entman et al. 1986, Hasselbach 1979, Meissner 1975).

The membrane of the SR can be isolated in vesicular form from muscle homogenates by a series of differential centrifugations, as first described by Ebashi (1958). Further purification of this initial vesicular preparation by the use of sucrose gradient centrifugation reveals separate Ca^{2+} uptake (light SR) and Ca^{2+} release (heavy SR) vesicles, corresponding to regions of the longitudinal and terminal SR respectively (Meissner 1975). The light SR vesicles retain an efficient Ca^{2+} transport system mediated by a membrane-bound Mg-dependent ATPase enzyme (Ebashi and Lipmann 1962, Hasselbach 1964). Electrophoretic analysis of isolated SR proteins has revealed the presence of several prominent bands, including the 110 kilodalton Ca^{2+} -ATPase (MacLennan et al. 1985), and the 55 kD high-affinity Ca^{2+} binding protein (Fliegel et al. 1989), the 44 kD calsequestrin (Ostwald et al. 1974, Scott et al. 1988), and the 22 kD regulatory protein phospholamban (James et al. 1989, Kirchberger et al. 1974). The

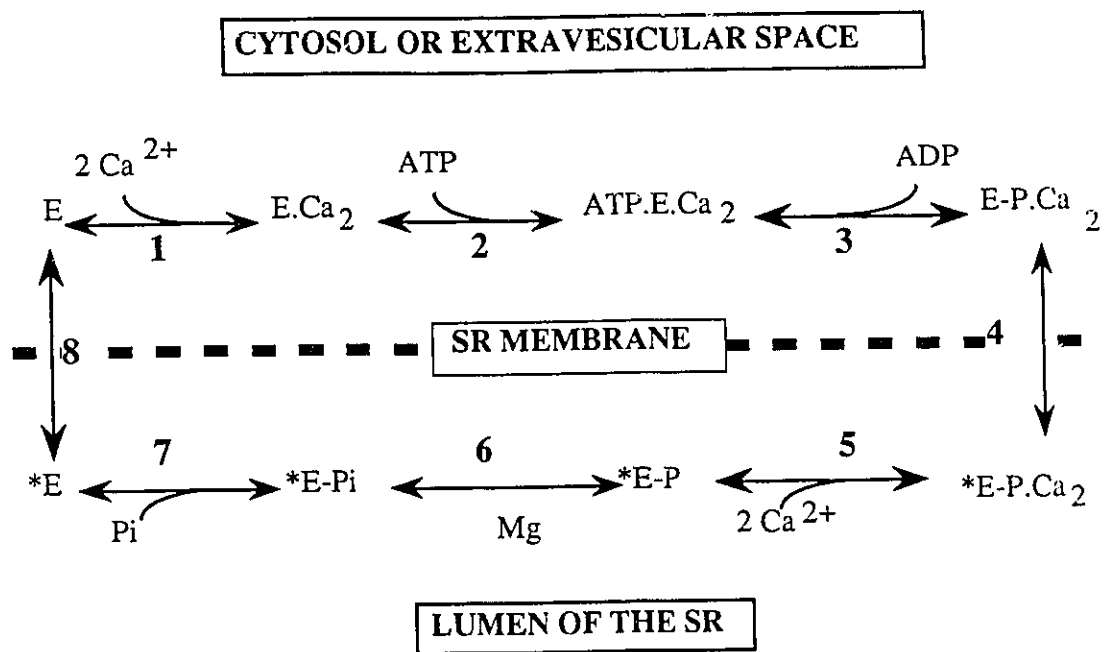
SR Ca^{2+} -ATPase represents between 60 and 80% of the total protein content of the light SR membrane (de Meis and Vianna 1979, MacLennan 1975). MacLennan showed that tryptic digestion of the Ca^{2+} -ATPase released two major fragments; a 45 kD helical portion which acts as a Ca^{2+} ionophore, and a 55 kD component which contained the functional ATPase activity (MacLennan 1975). Recent work has also resulted in the isolation of a 400 kD tetrameric Ca^{2+} release channel protein which is involved in linking sarcolemmal depolarization and SR Ca^{2+} release during excitation-contraction coupling in both skeletal and cardiac muscle (Hymel et al. 1988, Lai et al. 1988a, Lai et al. 1988b). A 106 kD protein has also been recently isolated which may represent a second Ca^{2+} release channel (Zaidi et al., 1989).

Ca^{2+} -ATPase OF THE SR: ACTIVITY AND FUNCTION

When ATP is added to SR vesicles in a neutral pH buffer containing Mg^{2+} and Ca^{2+} , the vesicles take up and store Ca^{2+} from the medium. The energy required for Ca^{2+} transport and the establishment of the Ca^{2+} gradient across the SR membrane is derived from the hydrolytic cleavage of the terminal phosphate group from the ATP molecule (de Meis and Vianna 1979). The reaction sequence (Figure 3.2) as described by de Meis and co-workers (1979) is similar to that given for many ATP-driven ion pumps within the cell, including the Na^{+} - K^{+} ATPase and sarcolemmal Ca^{2+} -ATPase (Stein 1986). This sequence includes the interconversion between two distinct functional states of the enzyme, shown here as E and $*E$. This scheme suggests that for the active transport of Ca^{2+} to occur, the Ca^{2+} affinity of the ATPase must be different at the external and internal surfaces of the SR membrane. The Ca^{2+} binding site in the E form faces the outer surface of the vesicle and has an apparent K_m for Ca^{2+} in the 0.2 - 2.0 μM range (high affinity), while the Ca^{2+} binding site faces the inner surface in the $*E$ form and has a much lower affinity for Ca^{2+} (ie. 1-3 mM) (de Meis and Vianna 1979). During the ATPase catalytic cycle the high affinity sites are converted to a low affinity

Figure 3.2 Reaction sequelae for the SR Ca^{2+} -ATPase, including the two functional states E and *E. The high affinity binding site in the E form faces the outer surface of the SR while the lower affinity *E form faces the inner surface. As cytosolic $[\text{Ca}^{2+}]$ rises, Ca^{2+} binding to E (1) in the presence of ATP (2) will induce a phosphorylation-dependent conformational change (4) coupled with a translocation of the binding site and Ca^{2+} ion to the inner region of the SR. This conformational change results in a decrease in the binding site affinity for Ca^{2+} , which is released into the lumen (5). The loss of the Ca^{2+} and phosphate group restores the original configuration to the enzyme (7). The binding of Ca^{2+} to the high affinity site(1) induces a modification of specific SH groups, and the oxidation of these thiols prevents the further conversion into the lower affinity form, hence the loss of pump function. (Adapted from de Meis and Vianna, 1979)

Figure 3.2



state following enzyme phosphorylation by ATP, resulting in the release of the bound Ca^{2+} molecule (Ikemoto 1975). The binding of Ca^{2+} to the high affinity binding site is accompanied by a protein conformational change that has been postulated to correlate with a translocation of Ca^{2+} through the membrane (Andersen and Møller 1977, Murphy 1978).

Under ideal steady-state conditions, a maximal value of two Ca^{2+} ions are translocated for each molecule of ATP consumed (de Meis and Inesi 1982) in SR isolated from mammalian skeletal muscle. The accumulation of Ca^{2+} within the vesicles will occur with a time constant of 60-90 s as the ATP-dependent Ca^{2+} uptake is impaired when intravesicular $[\text{Ca}^{2+}]$ reaches the millimolar range (Scarpa et al. 1972). The addition of Ca^{2+} -precipitating anions such as oxalate or phosphate will reduce the high intravesicular $[\text{Ca}^{2+}]$ (Hasselbach 1964, Hasselbach 1979), thereby allowing both the transport of Ca^{2+} and the hydrolytic activity of the Ca^{2+} -ATPase to be measured *in vitro* by using radio tracer and colorimetric techniques respectively. The activity and function of the SR Ca^{2+} -ATPase has been studied extensively using SR vesicles isolated from skeletal or cardiac muscle from various species (Hasselbach and Seraydarian 1966, Jones and Besch 1979, Kargacin et al. 1988, Nayler et al. 1975b, Rowe et al. 1983, Selinsky et al. 1988). With rat myocardium, in the presence of oxalate, the maximal rate of $^{45}\text{Ca}^{2+}$ Uptake into highly purified preparations of isolated SR vesicles has been reported to be as high as 130 nmol/mg protein/min (Barker et al. 1988) coinciding with a reported Ca^{2+} -ATPase activity, measured as the P_i liberated following activation with ATP, of 510 nmol/mg protein/min. These values are significantly depressed in SR vesicles isolated from diabetic hearts (Lopaschuk et al. 1983), atrophic hearts (Korecky et al. 1986), hypertrophied hearts (Gwathmey and Morgan 1985, Kimura et al. 1989, Morgan et al. 1990) or from hearts following ischemic injury (Buja et al. 1988, Krause and Hess 1984, Lee et al. 1987).

Ca²⁺ ATPase FUNCTION AND THIOL OXIDATION

The amino acid sequence of the skeletal SR Ca²⁺-ATPase as described by MacLennan and co-workers (1985) contains 26 sulfhydryl residues, with three disulfide bonds (Ariki and Shamoo 1983, Thorley-Lawson and Green 1977). Murphy (1976) reported that this ATPase contains almost 95% of the sulfhydryl groups found in the sarcoplasmic reticulum. Early work by Hasselbach and Seraydarian (1966) revealed that the presence of reduced sulfhydryls was essential for the maintenance of normal ATPase activity in SR isolated from skeletal muscles. The reactivity of specific sulfhydryl residues has been examined extensively. Andersen and Møller (1977) showed that all free sulfhydryl groups in purified Ca²⁺-ATPase from rabbit skeletal muscle were reactive with the thiol blocking agent, DTNB, in the absence of Ca²⁺ or ATP, but their availability to react decreased upon the addition of 0.1 mM ATP. They also showed that prior modification of an increasing number of free sulfhydryls with DTNB produced a near linear decrease in ATPase activity, with complete inactivation occurring upon reaction with twelve SH groups. Measuring the rate of the ATP hydrolysis reaction and the ability of Ca²⁺ to bind with the enzyme following treatment of SR vesicles with DTNB, Murphy (1976) showed a similar modification of the reactivity of sulfhydryl groups upon binding of Ca²⁺ to the high affinity site. He concluded that this decreased accessibility of sulfhydryls was related to structural changes which occurred during the formation of the E-P complex. Recent studies examining the involvement of sulfhydryl oxidation in the Ca²⁺-ATPase protein have revealed that the oxidation of as few as one thiol residue will compromise the Ca²⁺ pumping action of this enzyme (Aoki et al. 1986, Ariki and Shamoo 1983, Kawakita and Yamashita 1987, Saito-Nakatsuka et al. 1987). This is of particular interest to researchers studying the function of the SR in hearts following reperfusion injury.

The effects of oxygen free radical damage on the SR has been examined by several groups and their results exhibit great consistency. For example, Hess and co-workers (Krause and Hess 1984, Manson and Hess 1983, Schrier and Hess 1988) using SR vesicles isolated from canine hearts showed a significant decline in both $^{45}\text{Ca}^{2+}$ uptake and Ca^{2+} -ATPase activity following exposure to the OFR-generating system xanthine-xanthine oxidase. More recent work showed that neutrophil-derived oxidants including H_2O_2 and HOCl induced similar loss of Ca^{2+} -ATPase function (Kaminishi et al. 1989, Kukreja et al. 1988, Kukreja et al. 1989). The SR Ca^{2+} -ATPase shares greater than 84% homology with other ATPase molecules (Bastide et al. 1973, Taniguchi and Post 1975) and the reaction pathway is similar to that given for many ATP-driven ion pumps within the cell, including the Na^+ - K^+ ATPase and sarcolemmal Ca^{2+} -ATPase (Stein 1986). Both of these enzyme systems are also inhibited by thiol oxidation (Kako et al. 1988, Kaneko et al. 1989), suggesting that the necessity for free sulfhydryl residues is important for the catalytic process of many ATP-dependent pumps.

These aforementioned studies have generally involved exposing isolated SR microsome preparations to OFR or oxidative stress, without the benefit of normal cellular protective mechanisms such as GSH and tocopherol as described previously in the Review of Literature. To date, no studies have examined the effects of exposing the intact heart to oxidative stress, and then examining the activity of the SR Ca^{2+} -ATPase. As suggested earlier, *in vivo* exposure of the myocardium to PMN-derived oxidants may inactivate the Ca^{2+} -ATPase of the sarcolemma and SR in the cardiac myocytes, thereby impairing the removal of Ca^{2+} from the cytosol and leading to the elevation of cytosolic Ca^{2+} that is often observed in the damaged myocardium (Elz and Nayler 1988b, Krause and Hess 1984). However, to date it has not been adequately shown that exposure of the whole heart to oxidative stress will lead directly to an impairment of the SR Ca^{2+} -ATPase through thiol oxidation, or whether this impairment is reversible.

SUMMARY

The role of the SR as a major storage and release site for cellular Ca^{2+} and its involvement in EC coupling has been examined extensively over the past two decades. The SR Ca^{2+} -ATPase system is known to be extremely sensitive to thiol oxidation *in vitro* when isolated SR vesicles are exposed to various OFR or thiol blocking agents. However little evidence is available to show that the exposure of the intact myocardium to oxidative stress will lead to thiol oxidation and subsequent inactivation of this protein. As well, there is little evidence to date to suggest that this enzyme can be reactivated by thiol reduction.

The contracture as observed in isolated papillary muscles following exposure to HOCl has been shown to be prevented by ryanodine but not by nifedipine. This impaired contractile function correlated with a decrease in P-SH, suggesting that a relationship exists between the oxidative state of protein sulfhydryl groups and the maintenance of cellular Ca^{2+} homeostasis at the level of the SR (Chapter 1). The data from Fura-2 loaded isolated myocytes supported this hypothesis, showing that the rise in cytosolic $[\text{Ca}^{2+}]_i$ following exposure to HOCl was prevented by caffeine but not by the absence of $[\text{Ca}^{2+}]_e$. The fact that the rise in cytosolic $[\text{Ca}^{2+}]_i$ in response to HOCl was reversed by DTT suggested that a link existed between the thiol redox status of the Ca^{2+} -ATPase of the SR and this HOCl-induced contractile failure (Chapter 2).

In view of these previous findings, the overall objective of Chapter 3 is to ascertain whether the uptake of Ca^{2+} via the Ca^{2+} -ATPase into the SR is impaired in the intact coronary perfused rat heart exposed to HOCl, and whether DTT is able to restore the same.

OBJECTIVES:

The main objectives of this chapter are as follows:

- 1) To ascertain that the previously described recovery from HOCl-induced mechanical failure afforded by DTT addition in the isolated papillary muscle preparation is also observed in the coronary perfused rat heart;
- 2) To examine the activity (phosphate production) and function ($^{45}\text{Ca}^{2+}$ Uptake) of the Ca^{2+} -ATPase in isolated SR microsomes prepared from HOCl-perfused hearts as compared to both perfused and nonperfused control hearts;
- 3) To correlate this decrease in SR function with a depletion of free sulfhydryls in the Ca^{2+} -ATPase enzyme, as well as with the decline in contractile function observed in these perfused hearts.
- 4) To correlate a recovery in contractile function subsequent to perfusion with DTT with a restoration of free sulfhydryls in the SR Ca^{2+} -ATPase enzyme.

MATERIALS AND METHODS

ISOLATED HEART PREPARATIONS:

Hearts were excised from male Sprague-Dawley rats (275 - 300 g) following cervical dislocation, and mounted on a modified Langendorff perfusion apparatus. The hearts were perfused at constant flow with oxygenated (95% O₂ / 5% CO₂) Krebs Ringer (KR) buffer (in mM: NaCl 120; D-glucose, 10; KCl 4.8; CaCl₂ 2.5; MgSO₄ 1.2; KH₂PO₄ 1.2; NaHCO₂ 25.3, EDTA 0.5) pH 7.40, 37°C via the coronary arteries at an initial mean aortic root perfusion pressure (ARP) of 50 mm Hg. The atria were removed and the hearts were paced at 270/min with an electrode inserted through the free wall of the right ventricle. A latex balloon attached to a Statham P23 pressure transducer was inserted through the mitral valve into the left ventricle and gradually distended with water until the hearts attained maximal developed pressure upon stimulation. The preparations were considered acceptable if the left ventricular end diastolic pressure (LVEDP) was between 0 and 10 mm Hg, and end systolic pressures (LVESP) between 90 and 120 mm Hg prior to the initiation of the experimental procedures. Left ventricular pressure measurements as well as ARP, which represents the perfusion pressure for the coronary vasculature, were recorded using a Grass 4 channel polygraph.

Two identical systems were set up in parallel, allowing for the perfusion of a control and experimental, or two experimental, preparations simultaneously from the same reservoirs contained the following: I) normal KR buffer; II) KR with HOCl (100 mM); or III) KR with DTT (500 µM). Following a 15 min stabilizing period, the hearts were perfused as follows:

- 1) **Control** (normal KR for 60 minutes);
- 2) **Control + DTT** (KR for 15 minutes, followed by KR with DTT for a further 45 minutes);
- 3) **HOCl 15 min** (KR with HOCl for 15 minutes);
- 4) **HOCl 30 min** (KR with HOCl for 30 minutes);
- 5) **HOCl 60 min** (KR with HOCl for 60 minutes);
- 6) **HOCl + Washout** (KR with HOCl for 15 minutes, then KR with no HOCl for a further 45 minutes);
- 7) **HOCl + DTT 30 min** (KR with HOCl for 15 minutes, then KR with DTT for a further 15 minutes);
- 8) **HOCl + DTT 60 min** (KR with HOCl for 15 minutes, then KR with DTT for a further 45 minutes).

Following each experimental period, the hearts were perfused with normal KR buffer for 2 minutes to remove any residual solutions, then rapidly chilled by dropping into chilled (0°C) normal saline. A final group of hearts which were chilled immediately upon removal from the rat and not perfused (NP) were used as an absolute control for the SR Ca²⁺ ATPase assays.

DRY / WET RATIO

Approximately 50 mg (wet weight) of myocardium was excised from the left ventricular free wall, blotted dry between two pieces of Whatman filter paper for 15 seconds, then weighed (nearest 0.05 mg) before and after drying at 90°C for 24 hours. The dry weight to wet weight ratio is expressed as a percentage.

SR Ca^{2+} ATPase ACTIVITY AND FUNCTION

SR MICROSOME PREPARATION

The remaining ventricular tissue was minced in 2 ml of 20 mM TRIS maleate (pH 7.2) buffer, and SR microsomes were isolated following a variation of the technique described by Harigaya and Schwartz (Harigaya and Schwartz 1969). All isolation steps were done at 0°C. Briefly, the minced tissue was transferred to a glass homogenizer tube (40 ml) containing 5 ml of the above TRIS buffer and homogenized with a motor-driven Teflon pestle (Fisher) at 2100 R.P.M. for a total of 30 seconds. The same mortar and pestle combination was used for all preparations. The homogenate was transferred to a 40 ml centrifuge tube and centrifuged (IEC-B20A centrifuge) at 9000 x G for 10 min. The supernatant was collected and centrifuged again at 9000 x G for 20 min. The latter supernatant was then centrifuged at 120,000 x G for 60 min (Beckman Ultracentrifuge, SW28 rotor). The resulting pellet was resuspended with 6 strokes of a teflon pestle in a 1 ml Potter-Elvehjem glass mortar with 0.5 ml of 20 mM TRIS, 0.6 M KCl (pH 6.8), and centrifuged again in 40 ml of this buffer at 120,000 x G for 60 min. The final pellet was resuspended in 0.3 ml of 50 mM KCl, 250 mM sucrose, 20 mM TRIS maleate pH 6.8, and adjusted to 1 mg protein / ml. An estimated yield of approximately 1 mg SR protein per gram of ventricular tissue was attained by this method.

SR Ca^{2+} -ATPase ACTIVITY

Ca^{2+} dependent and independent ATPase activities were determined (in triplicate) at 37°C using 50 μl of SR (50 μg protein) in a total volume of 1 ml containing (in mM): KCl 100; EGTA 0.1; NaN_3 5; MgCl_2 5; Histidine 25; in the presence or absence of 0.1 mM CaCl_2 . Na^+/K^+ ATPase activity was estimated in the presence or absence of 1 mM ouabain and subtracted from the Ca^{2+} -ATPase activity. The reactions were initiated by the addition of 50 μl of 100 mM ATP and terminated after 10 min by

the addition of 1 ml of cold (0°C) 12% TCA. The samples were centrifuged at 3000 x G for 10 min at 0°C, then 1 ml (50% of total) of the supernatant was added to 1 ml of 6% TCA. One ml of reagent mixture, containing 88.5 mM NH₃Mb; 330 mM FeSO₄ in 10% H₂SO₄ was added and allowed to stand on ice for 10 min. The liberated inorganic phosphate (Pi) was determined spectrophotometrically at 700 nM against a standard curve derived using 0, 100, 200, and 300 pmoles KH₂PO₄ as described by Nakamura et al. (1983). The [Pi] produced /mg protein was calculated using the formula:

$$1) \quad [\text{Pi}](\text{nmoles/mg protein/min}) = \text{Pi (pmoles / 10 min)} \times P$$

$$\begin{aligned} \text{where } P &= \frac{2 \text{ (half of sample was used)}}{10 \text{ (convert to 1 min value)}} \times \frac{1000}{50 \text{ } \mu\text{g protein}} \\ &= 200 / \mu\text{g protein} \end{aligned}$$

The values for Pi production in the absence of Ca²⁺ were subtracted to determine the Ca²⁺-activated ATPase activity.

A more detailed description of the SR microsome isolation procedures, ⁴⁵Ca²⁺ Uptake, and Ca²⁺-ATPase assays can be found in Appendix II.

SR ⁴⁵Ca²⁺ UPTAKE

ATP dependent ⁴⁵Ca²⁺ uptake into the isolated SR microsomes was measured in triplicate at 37° C using 25 µl of SR (25 µg protein) in a total volume of 675 µl containing (in mM): KCl 100; ATP 5; NaN₃ 5; MgCl₂ 5; K-oxalate 10; and TRIS maleate 20; pH 6.8. A similar set of experiments were performed simultaneously in the absence of K-oxalate to assess the degree of Ca²⁺ binding, and subtracted from the final Ca²⁺ uptake values. The reaction was initiated by the addition of 0.1 µCi ⁴⁵CaCl₂ (Amersham) in 0.1 mM CaCl₂, 0.1 mM EGTA, 20 mM TRIS, and the ⁴⁵Ca²⁺ uptake

was determined over 1, 10, 20, and 30 minutes in the presence or absence of ATP to correct for ATP independent binding of Ca^{2+} to the SR membrane. The reaction was terminated by filtering 50 μl aliquots of the mixture through 0.45 μm pore nitrocellulose filters (Millipore) at the four time points and washing with 5 ml of ice cold 20 mM TRIS maleate pH 6.8, 0.1 mM EGTA and 0.1 mM CaCl_2 . Vesicular $^{45}\text{Ca}^{2+}$ retained on the filters was measured in a liquid scintillation counter (Beckman LS2800) along with a standard aliquot (50 μl) of the $^{45}\text{Ca}^{2+}$ solution. Assuming that the uptake and binding of $^{45}\text{Ca}^{2+}$ is identical to Ca^{2+} , the theoretical Ca^{2+} Uptake into the microsomes was calculated using the formula:

$$2) \quad \text{Ca}^{2+} \text{ Uptake} = \frac{\text{CPM (Sample-background)}}{\text{CPM (Standard-background)}} \times N$$

$$\begin{aligned} \text{where } N &= \frac{67.5 \text{ nmoles } \text{Ca}^{2+} / \text{min}}{0.025 \text{ mg protein}} \times \frac{675 \mu\text{l total volume}}{50 \mu\text{l sampled}} \\ &= 36450 \text{ nmoles / mg protein / min} \end{aligned}$$

SR PROTEIN ISOLATION AND SH DETERMINATION

Iodoacetamide (IAA) is an alkylating agent which binds covalently to reduced thiols but not to oxidized thiols (Bishop et al. 1988), without significantly altering the structure or charge of the protein involved. Aliquots of microsomes (50 μg protein) were incubated in the dark at 37°C for 60 min with 0.6 μCi of ^{14}C -IAA as previously described (see Chapter 1, Page 62). The binding was terminated by the addition of excess DTT.

Approximately 30 μg of the protein were dissolved in Loading buffer, containing: 25% glycerol; 2.5% SDS, 0.05% Bromophenol Blue; 2.5% β -mercaptoethanol in 125

mM TRIS pH 6.5 and incubated at 100°C for 5 min, before loading onto 8% polyacrylamide slab gels. The proteins were separated by electrophoresis and stained with Coomassie Blue. Protein concentration in the 110 kD band was determined using laser densitometry (LKB 2222-020) against a standard curve (0, 1, 5, and 25 µg) of purified rabbit skeletal Ca^{2+} ATPase, then the gels were photographed, dried and exposed against an X-Ray film for 3 days at -80°C.

Several major protein bands, including the 110 kD protein which co-migrated with the purified Ca^{2+} ATPase standards, were excised from the gels using an oblong stainless steel punch, digested at 60°C for 3 hours in 30% H_2O_2 , and counted using liquid scintillation. Background counts were subtracted by digesting and counting non-labelled regions of each gel, and the level of ^{14}C -IAA bound to the proteins was calculated as ^{14}C -CPM / µg protein.

PROTEIN DETERMINATION

Protein determinations were performed in triplicate using the Biorad method as previously described (See Chapter 1, Page 56), using Bovine plasma albumin as the standard.

STATISTICAL ANALYSIS

All values are expressed as means $\pm$ SE with n=8 for each group. For the contractile data, differences were tested between the treatment groups using a repeated measures 2 way ANOVA with time and treatment as independent variables and Scheffes post-hoc test. For comparing the effects of treatment on SR function a one-way ANOVA was used with Scheffes. A difference of $p < 0.05$ was considered significant. Correlation coefficients were determined using the specified data for each heart individually.

RESULTS

MECHANICAL DATA

The Control hearts performed at steady state for up to three hours under these described perfusion conditions. Control hearts did not exhibit any significant degree of edema following the 60 minute perfusion protocol (Table 3.1) and showed no appreciable increase in aortic root perfusion pressure (ARP) or loss of pulse pressure ($PP = LVESP - LVEDP$) (Figure 3.3). Control hearts perfused with DTT for the final 45 min also performed at steady state for the duration of the perfusion protocol, however 4 of the eight hearts exhibited a slight (NS) rise in ARP by 60 min coinciding with substantial edema formation (Table 3.1). Two Control + DTT hearts had episodes of transient arrhythmic contractions during the final five minutes of perfusion, however this was rapidly reversed during the brief washout period following each protocol. No differences were observed for LVEDP or PP between Control and Control + DTT hearts.

Hearts perfused with 100 μ M HOCl exhibited substantial edema formation and distinct alterations in mechanical performance. By 15 minutes ARP and LVEDP had increased significantly without any accompanying degree of edema, and continued to rise throughout the duration of the protocol. Pulse pressure declined slowly over the first 15 minutes, but by 30 minutes the mechanical function of most hearts was severely compromised. Substantial edema was observed in these hearts by 60 minutes.

If the HOCl was removed from the perfusate (Washout) as early as 15 minutes, no ameliorative effects were observed, with the exception of a slight (10 mm Hg) reduction in ARP by 60 min. This suggests that the damage induced by HOCl perfusion had been initiated within the first 15 min.

Figure 3.3 Mechanical properties of rat hearts during 60 min retrograde perfusion with 100 μ M HOCl, with or without the addition of DTT or Washout at 15 min. ARP = aortic root pressure as an index of coronary resistance; PP = pulse pressure (systolic - diastolic pressures); LVEDP = left ventricular end diastolic pressure. Values represent mean $\pm$ SEM for n = 8 experiments each. Significance was determined by two-way analysis of variance with time and treatment as independent variables * = p<0.05 from Control; § = p<0.05 from HOCl Only; ‡ = p<0.05 from the 15 min time point within the same treatment group.

Figure 3.3

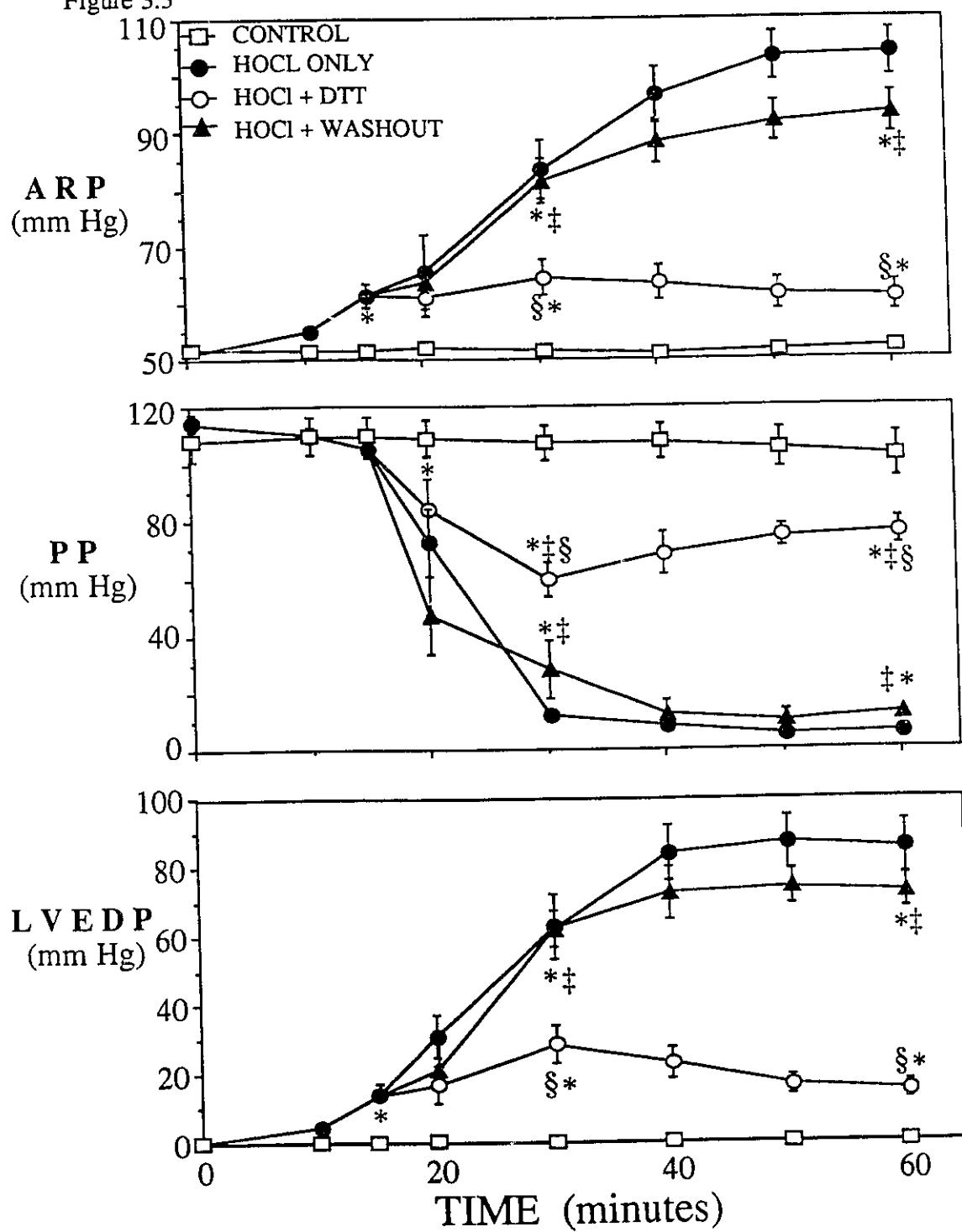


Table 3.1 Dry / wet weight ratio as an index of tissue edema in rat hearts following the described perfusion protocols. The values given are mean $\pm$ SEM for n = 8 hearts per group. * = p<0.05 from Control Perfused.

Table 3.1

	DRY/WET ratio (%)
NOT PERFUSED	20.7 ± 0.4
CONTROL PERFUSED	20.2 ± 0.7
CONTROL + DTT	16.5 ± 1.0*
HOCl FOR 15 MIN	21.0 ± 0.3
HOCl FOR 30 MIN	18.3 ± 1.1
HOCl FOR 60 MIN	16.8 ± 0.6*
HOCl + DTT 30 MIN	19.0 ± 0.9
HOCl + DTT 60 MIN	16.3 ± 0.5*
HOCl + WASHOUT	16.5 ± 0.4*

In contrast to Washout, the addition of DTT at 15 minutes attenuated any further decline in mechanical performance and, with continued perfusion, induced a partial functional recovery. For example, following DTT administration, LVEDP continued to rise slowly for the first 10-15 min, peaking (29 ± 5 mm Hg) by about 30 minutes, then recovered gradually back to near-baseline (15 ± 3 mm Hg) values by 60 min. Similarly, PP declined (55 ± 6 mm Hg) until 30 minutes, then recovered back to about 75 ± 4 mm Hg. ARP rose slightly (from 58 ± 3 mm Hg at 15 min to 65 ± 3 mm Hg at 30 minutes), then stabilized at 61 ± 3 mm Hg. DTT did not prevent edema formation in these hearts.

SR Ca^{2+} ATPASE ACTIVITY AND $^{45}\text{Ca}^{2+}$ UPTAKE:

Figures 3.4 and 3.5 depict the maximal Ca^{2+} dependent ATPase activity (in nmoles Pi/mg SR protein/min) and the ATP-dependent oxalate supported uptake of $^{45}\text{Ca}^{2+}$ (in nmoles Ca^{2+} /mg SR protein/min) into the SR microsomes isolated from the hearts described above. In each graph, the first three columns represent results from the three control groups (Normal, Control, and Control + DTT), and show that perfusion of the hearts with KR buffer for 60 min (Control) with or without DTT did not significantly alter either the ability of the enzyme to hydrolyze ATP (Figure 3.4) or the ability of the microsomes to sequester Ca^{2+} (Figure 3.5) as compared with the non-perfused hearts.

In contrast, both the activity of the Ca^{2+} -ATPase and $^{45}\text{Ca}^{2+}$ uptake were highly sensitive to HOCl perfusion. Microsomes isolated from hearts perfused with HOCl for only 15 minutes show a significant depression of ATP activity (350 ± 66 vs 595 ± 74 nmoles Pi/mg/min), combined with a depression in $^{45}\text{Ca}^{2+}$ uptake (34 ± 5 vs 46 ± 4 nmoles /mg/min) as compared to Control. A further depression in both Ca^{2+} -ATPase activity and $^{45}\text{Ca}^{2+}$ Uptake was observed following 30 min (207 ± 57 nmoles Pi/mg/min and 22 ± 5 nmoles/mg/min) and 60 min (123 ± 40 nmoles Pi/mg/min and 12 ± 5 nmoles/mg/min) of perfusion with HOCl in a time-dependent manner.

Figure 3.4 Maximal rate of Ca^{2+} -ATPase activity (nmoles Pi/mg SR protein/min) in SR microsomes isolated from perfused rat hearts. The first three columns represent the three Control preparations (N = Control non-perfused hearts, CP = Control perfused hearts, CD = Control hearts perfused with DTT for the final 45 min). The second group of three columns represents the effects of HOCl perfusion over time (H15, H30, and H60 = 15, 30, and 60 min of HOCl perfusion respectively). HW represents the effects of HOCl for 15 min, followed by 45 min of Washout. The last two columns represent the effects of HOCl for 15 min followed by DTT perfusion until 30 min (HD 30) or 60 min (HD 60). Values shown are mean $\pm$ SEM for n = 8 experiments. Significant differences ($p < 0.05$) were determined using one-way analysis of variance and Scheffes post-hoc test: * = from CP; ** = from CD; § = from H60; ‡ = from H15.

Figure 3.4

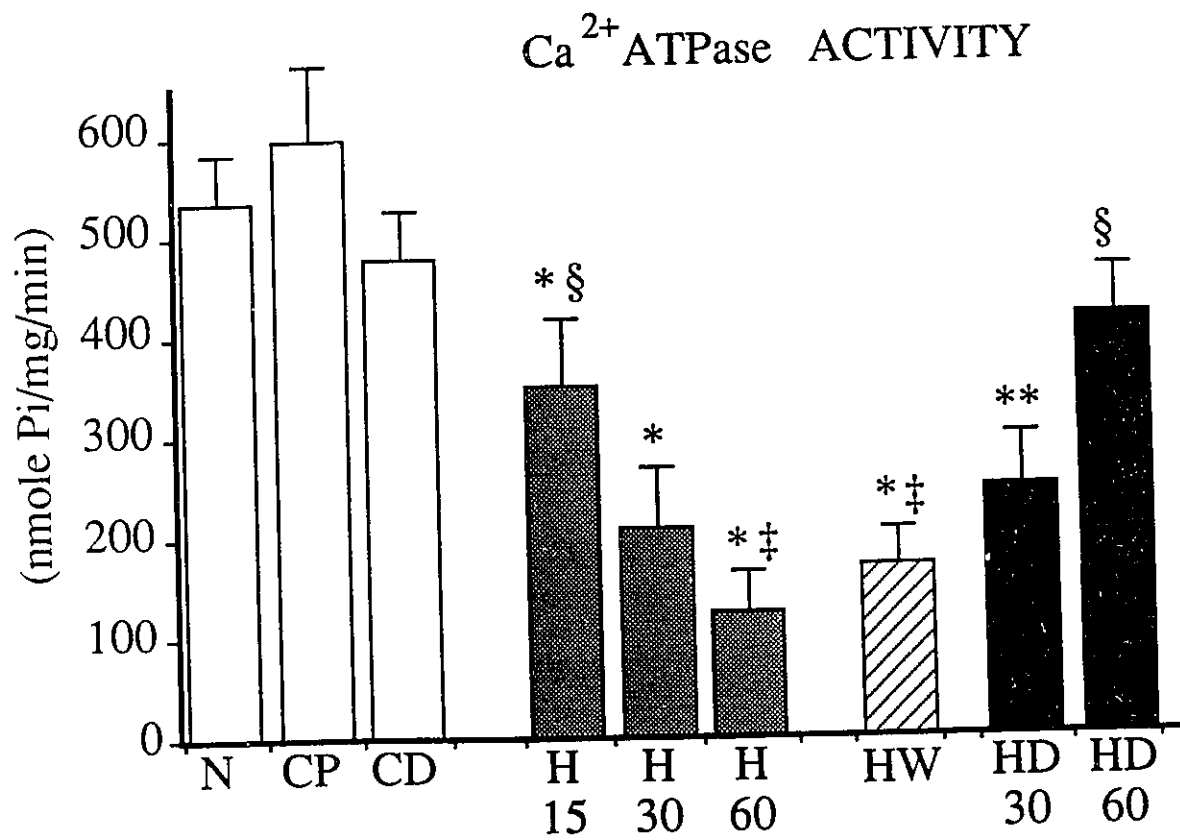
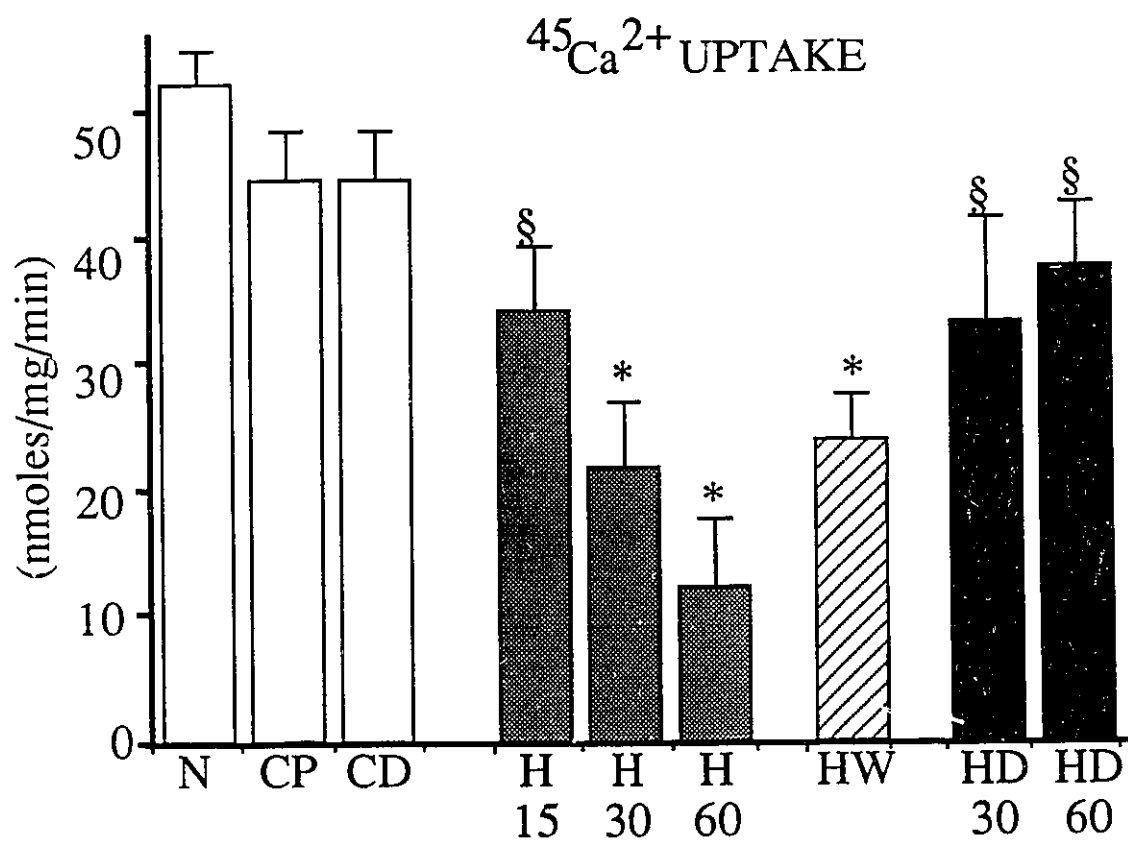


Figure 3.5 Maximum rate of $^{45}\text{Ca}^{2+}$ Uptake (nmoles $^{45}\text{Ca}^{2+}$ /mg SR protein/min) into oxalate-loaded SR microsomes isolated from perfused rat hearts. The identification for each column and the significance symbols are as described in Figure 3.4. Values shown are mean $\pm$ SEM for n = 8 experiments.

Figure 3.5



Removal of the oxidant (Washout) at 15 minutes had no protective or restorative effect, as both Ca^{2+} ATPase activity and $^{45}\text{Ca}^{2+}$ Uptake continued to decline, and by 60 min were significantly lower (169 ± 34 nmoles Pi/mg/min and 24 ± 3 nmoles/mg/min respectively) than the 15 min time point values.

In contrast, DTT added at 15 minutes produced a recovery of Ca^{2+} ATPase activity (415 ± 47 nmoles Pi/mg/min) and $^{45}\text{Ca}^{2+}$ uptake (38 ± 5 nmoles/mg/min) back to values that were not significantly different from the Control + DTT values (473 ± 46 nmoles Pi/mg/min and 46 ± 4 nmoles/mg/min respectively), although ATPase activity was still significantly lower than the Control (no DTT) perfused group.

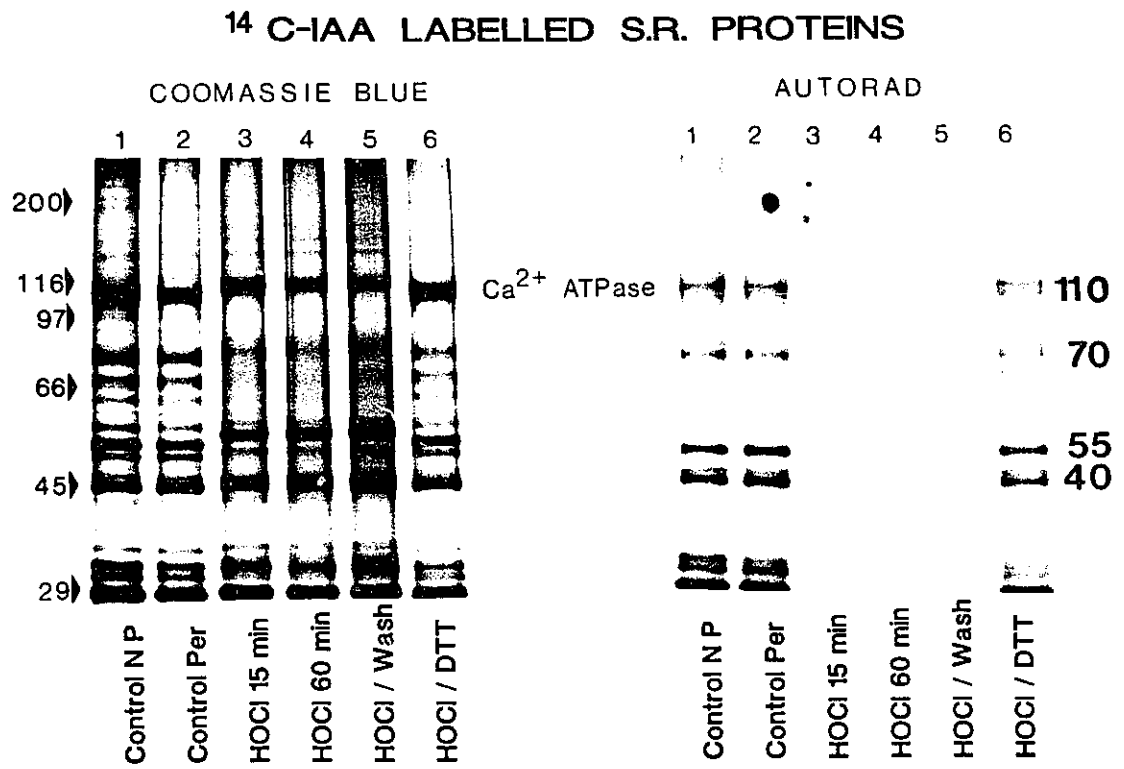
SR PROTEIN ISOLATION AND SH OXIDATION

Figure 3.6 depicts 6 lanes from a representative 8% polyacrylamide gel showing the proteins separated from the above described microsomes. The protein profile was identical in all three control groups. Several major bands were observed, including a 110 kD protein which co-migrated with purified Ca^{2+} ATPase (rabbit skeletal), and several other unidentified protein bands (eg. 70, 55, and 40 kD). In those microsomes from HOCl-perfused hearts, the 110 kD and 55 kD proteins appeared to migrate slower as compared to Control, perhaps due to charge alteration or the presence of cross-linked proteins. Other bands appeared more diffuse in those HOCl-treated tissues, even after as little as 15 min exposure. Washout had no restorative effects following 15 min of exposure to the oxidant. However, microsomes isolated from hearts secondarily treated with DTT at 15 min exhibited a near-normal protein banding pattern by 60 min.

The corresponding autoradiogram shows a high level of ^{14}C -IAA binding in the proteins isolated from Control hearts (Figure 3.6), while very low levels of ^{14}C -labelling are observed in all the IAA-reactive proteins following HOCl exposure. Some ^{14}C

Figure 3.6 A Coomassie-blue stained gel and its corresponding autoradiogram showing the proteins extracted from the SR-enriched microsomes prepared from individual isolated rat hearts as described in Methods. The numerical values to the left of the gel indicate molecular weights. The numbers on the right indicate the approximate molecular weight of the proteins isolated for quantification of the ^{14}C -IAA binding (see Table 3.2 below). Diminished ^{14}C -IAA binding to all proteins including the 110 kD Ca^{2+} -ATPase are seen in lanes 3-5. Lane 1 shows SR proteins from a non-perfused control heart. The mean values (n=8 gels) of ^{14}C -IAA per μg of protein are given in Table 3.2.

Figure 3.6



binding is still visible in the HOCl 15 min lane, but has virtually disappeared by 60 min, with or without Washout. In contrast, perfusion of the HOCl-treated hearts with DTT at 15 min restored protein thiols to near Control levels by the end of the 60 min protocols

Table 3.2 expresses the level of reduced thiols in terms of ^{14}C -IAA CPM / μg of protein in four of the protein bands cut from the gels. HOCl perfusion for 15 min reduced the level of IAA binding in the 110 kD Ca^{2+} -ATPase band to approximately 50% of Control, while longer exposure further reduced this to less than 15% of control by 60 min. Washout at 15 min did not prevent further loss of protein thiols. Conversely, DTT addition at 15 min restored levels of IAA binding to Control values by 60 min. Similar results were observed in the 70, 55 and 40 kD protein bands.

Figures 3.7 to 3.9 illustrate the relationships between protein thiol levels, Ca^{2+} -ATPase activity, SR $^{45}\text{Ca}^{2+}$ Uptake, and the myocardial contractile function. In each of the graphs, the correlation coefficients were determined using the data from each preparation individually (eg. Figure 3.7). However, in Figures 3.8 and 3.9, the mean values ($\pm$ SE) are plotted for clarity and in order to show how the data were grouped according to their respective experimental protocols.

Figure 3.7 shows the correlation between the level of reduced thiols (in ^{14}C -IAA CPM/min) and the activity of the SR Ca^{2+} -ATPase enzyme. This figure verifies that the Ca^{2+} -ATPase protein is dependent upon reduced thiols for activity and function. The restoration of Ca^{2+} -ATPase activity as observed in the proteins isolated from HOCl + DTT perfused hearts is closely linked to the restoration of protein thiols.

Figure 3.8 shows the correlation between Ca^{2+} -ATPase activity and $^{45}\text{Ca}^{2+}$ Uptake into the microsomes. This figure shows that the function of the Ca^{2+} pump was not uncoupled from the hydrolysis of ATP, but rather that the activity and function of this

Table 3.2 Quantification of ^{14}C -IAA binding in the 110,70,55, and 40 kD proteins from rat SR as shown in Figure 3.6 (above) Protein concentration was determined using densitometric scanning of Coomassie-blue stained gels against a standard curve of known concentrations of purified Ca^{2+} -ATPase protein run concurrently on each gel. IAA binding is expressed in CPM per μg of protein in each band. Values given are mean $\pm$ SEM for n=8 gels. Significant differences ($p<0.05$) are noted as follows: * = from Control Perfused; ‡ = from HOCl 15 min; § = from HOCl 60 min.

Table 3.2

	14C-IAA CONTENT (CPM/ μ g protein)			
	110 kD	70 kD	55 kD	40 kD
NOT PERFUSED	199 $\pm$ 26	304 $\pm$ 36	63 $\pm$ 9	272 $\pm$ 37
CONTROL PERFUSED	205 $\pm$ 25	332 $\pm$ 38	58 $\pm$ 12	297 $\pm$ 36
CONTROL + DTT	210 $\pm$ 21	340 $\pm$ 25	54 $\pm$ 12	328 $\pm$ 33
HOCI FOR 15 MIN	*104 $\pm$ 18	*133 $\pm$ 17	* 15 $\pm$ 3	* 79 $\pm$ 11
HOCI FOR 30 MIN	‡* 32 $\pm$ 14	‡* 26 $\pm$ 21	‡* 5 $\pm$ 2	‡* 22 $\pm$ 9
HOCI FOR 60 MIN	‡* 26 $\pm$ 2	‡* 9 $\pm$ 5	‡* 5 $\pm$ 1	‡* 5 $\pm$ 1
HOCI + WASHOUT	‡* 14 $\pm$ 8	‡* 12 $\pm$ 3	‡* 9 $\pm$ 3	‡* 8 $\pm$ 2
HOCI+DTT 30 MIN	* 85 $\pm$ 21	* 77 $\pm$ 18	* 38 $\pm$ 8	‡*176 $\pm$ 22
HOCI+DTT 60 MIN	§‡203 $\pm$ 16	§‡284 $\pm$ 41	§‡52 $\pm$ 14	§‡245 $\pm$ 38

Figure 3.7 The relationship between thiol oxidation and enzymatic activity in the 110 kD Ca^{2+} -ATPase isolated from rat myocardial SR. The correlation coefficient was determined using the data from each heart separately.

Figure 3.7

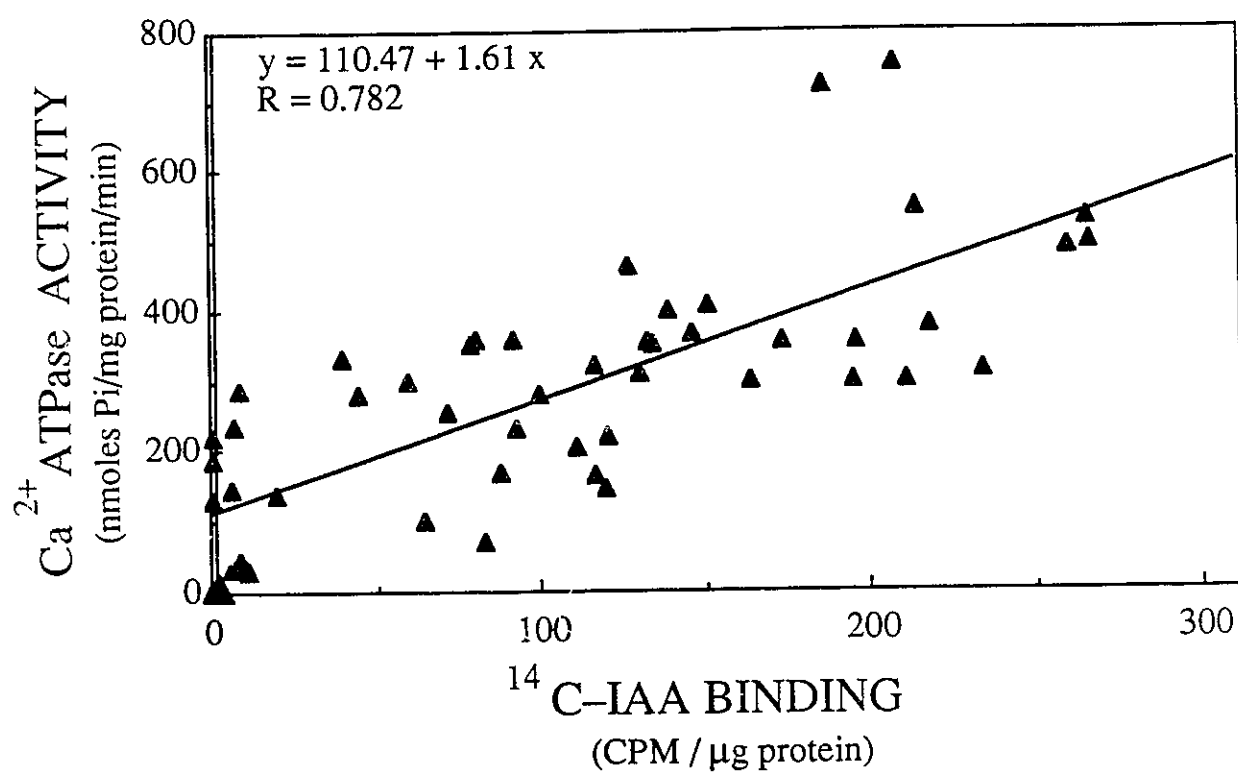


Figure 3.8 The relationship between Ca^{2+} -ATPase activity (nmoles Pi/mg SR protein/min) and $^{45}\text{Ca}^{2+}$ Uptake (nmoles/mg protein/min) into oxalate-loaded SR microsomes. The correlation coefficient (R) for this relationship was derived using the data from each individual preparation. The mean values $\pm$ SEM are plotted here to show the arrangement of the various experimental groups. CNP and CP = Control not-perfused and perfused, respectively; H15, H30, and H60 = HOCl perfusion for 15,30, and 60 min respectively; HD30 and HD60 = HOCl for 15 min, followed by DTT until 30 or 60 min respectively; HW = HOCl for 15 min followed by Washout until 60 min.

Figure 3.8

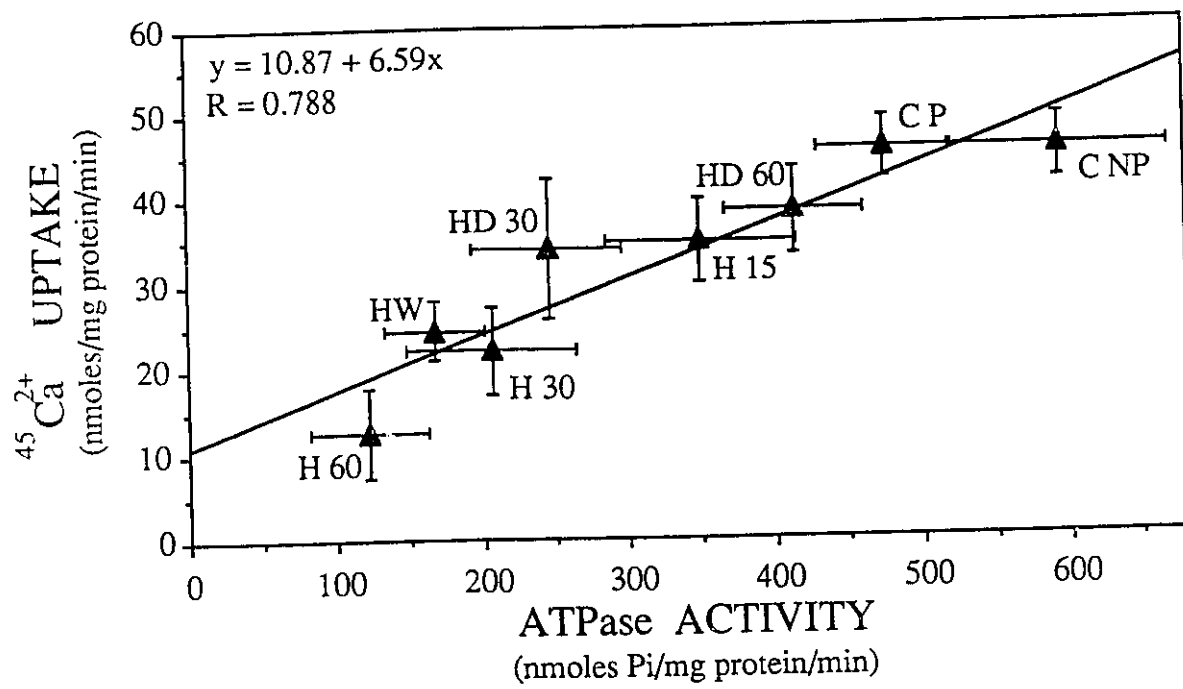
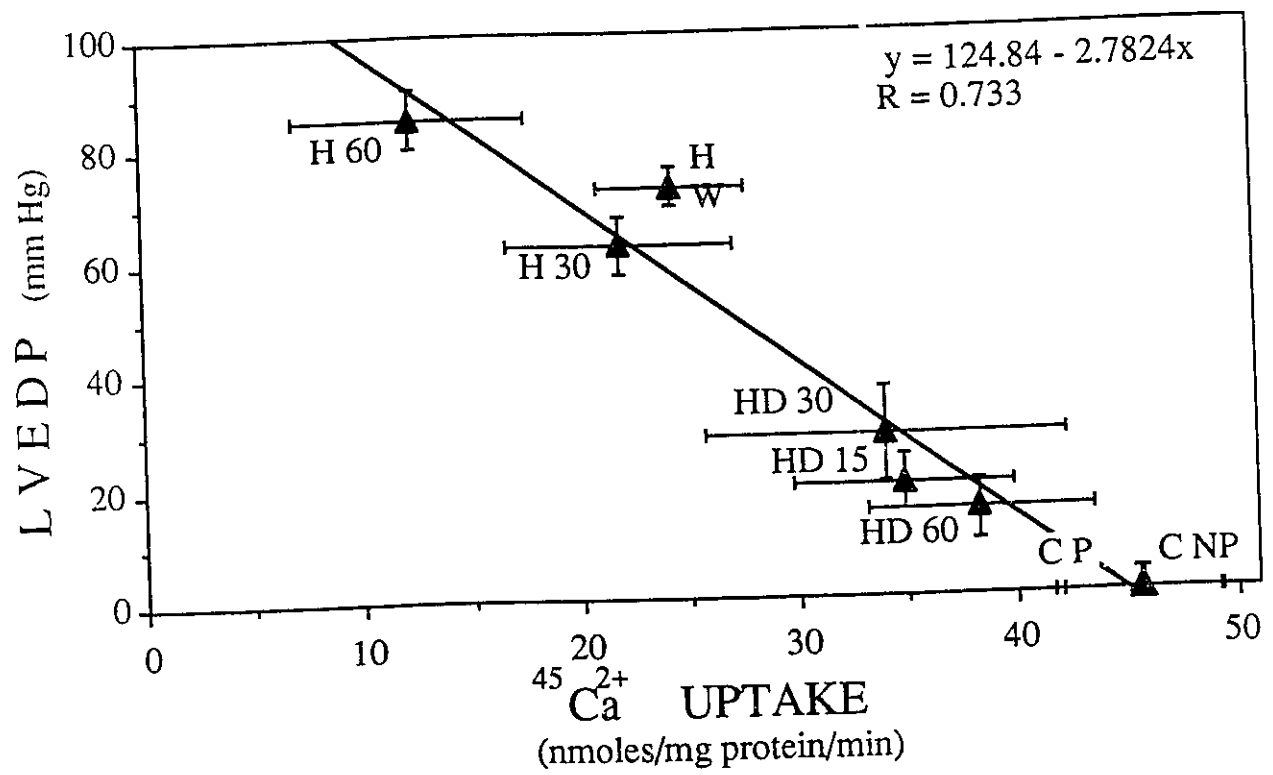


Figure 3.9 The relationship between SR $^{45}\text{Ca}^{2+}$ Uptake (nmoles/mg protein/min) and LVEDP (mm Hg) in perfused rat hearts. The correlation coefficient (R) for this relationship was derived using the data from each individual preparation. The mean values $\pm$ SEM are plotted to show the arrangement of the various experimental groups. CNP and CP = Control not-perfused and perfused respectively; H15, H30, and H60 = HOCl perfusion for 15,30, and 60 min respectively; HD30 and HD60 = HOCl for 15 min, followed by DTT until 30 or 60 min respectively; HW = HOCl for 15 min followed by Washout until 60 min.

Figure 3.9



enzyme declined in parallel with increasing time of exposure to HOCl. The restoration of $^{45}\text{Ca}^{2+}$ Uptake into microsomes isolated from hearts exposed to HOCl + DTT (30 min and 60 min) correlated with a recovery of enzymatic activity.

Figure 3.9 shows the relationship between $^{45}\text{Ca}^{2+}$ Uptake into SR microsomes and the intact heart LVEDP. This figure shows that as the ability of the SR microsomes to sequester $^{45}\text{Ca}^{2+}$ declined, the ability of the heart to relax became severely compromised. Hearts exposed to HOCl + DTT were able to relax fully, coinciding with a restoration of the ability of the SR microsomes to resequester Ca^{2+} .

DISCUSSION

The overall purpose of this study was to determine whether a depression in SR function could be correlated with the contractile dysfunction observed following HOCl exposure. Following perfusion with HOCl, these rat hearts entered into a state of contracture which was consistent with the mechanical failure observed in the papillary muscle preparations described in Chapter 1. Subsequent perfusion with DTT induced a substantial recovery of contractile function, while washout had no restorative effects. The response of the myocardium to 100 μ M HOCl was more pronounced in the perfused hearts as compared to the superfused papillary muscle preparations.

The SR microsomal preparations isolated from the 60 min Control perfused rat hearts exhibited values for Ca^{2+} -ATPase activity and $^{45}\text{Ca}^{2+}$ Uptake that were not significantly different from the non-perfused (Normal) preparations. The microsomal protein composition as determined by gel electrophoresis, as well as the relative affinity of the various proteins for ^{14}C -IAA were also similar in the perfused and non-perfused Control preparations. Therefore, any differences observed in the microsomes isolated from HOCl-perfused hearts are probably due to the experimental protocol and not to the perfusion or isolation procedures per se.

The final objective of this study was to correlate changes in the function and activity of the SR Ca^{2+} -ATPase to changes in the contractile function of the perfused heart, as well as to the level of thiol oxidation within the protein itself. The data show that the oxidation of thiol residues within the 110 kD Ca^{2+} -ATPase protein correlated with a decline in the ability of this enzyme to hydrolyze ATP, as well as its ability to sequester $^{45}\text{Ca}^{2+}$ into the SR microsomes. Thiol oxidation further correlated with the decline in contractile function, as characterized by a decrease in PP and a concomitant increase in both LVEDP and ARP. The addition of DTT to the perfusate restored the

level of reduced thiols within the 110 kD Ca^{2+} -ATPase protein, correlating with a restoration of the Ca^{2+} -ATPase activity and $^{45}\text{Ca}^{2+}$ sequestration function of the SR, and a recovery of contractile function in the perfused heart, depicted by a decrease in LVEDP and ARP, and a concomitant recovery of PP.

Several previous studies have already shown that HOCl is capable of inactivating the SR Ca^{2+} -ATPase when added directly to a suspension of isolated microsomes (Kaminishi et al. 1989, Kukreja et al. 1989), but none have examined the more physiologically relevant perfused organ model. When dealing with an intact organ rather than isolated membrane preparations, one needs to consider the specific cellular defence systems (ie. glutathione, superoxide dismutase, vitamin E) (Pascoe and Reed 1989, Reed and Farris 1984, Thayer 1986) which are effective at diminishing the effects of exogenously applied oxidants. An extracellular HOCl concentration of 10^{-5} M range would, in all likelihood, be dissipated by cytosolic scavengers and hence would be considerably lower at the level of the SR. Therefore, the application of high μM concentrations of oxidants directly to isolated SR vesicle preparations must be considered as non-physiological, and caution must be exercised when extrapolating these data to the whole organ level. However, the data presented here strongly support the results obtained using the isolated SR preparation, and clearly show that perfusion of the intact organ with a continuous flow of HOCl at 'physiological' concentrations may indeed override the cellular defence systems and compromise the ability of the SR to sequester and store Ca^{2+} *in situ*.

The correlation between LVEDP and the function of the Ca^{2+} -ATPase implicated the inactivation of this SR protein in the development of the observed contractile dysfunction. The recovery of function and activity of the SR Ca^{2+} -ATPase with DTT correlated well with both contractile recovery and thiol redox status in this protein, suggesting that the relationship between these events is probably not due to chance.

However, full functional recovery was not attained with DTT, suggesting that either other amino acid residues besides sulfhydryls may have been oxidized, or that the damage is not restricted to the SR proteins in question. Other proteins, including the 55 kD band thought to be calsequestrin, also show evidence of thiol depletion. As addressed in Chapter 2 of this thesis, it would also appear that other systems involved in Ca^{2+} homeostasis, ie. the sarcolemmal Ca^{2+} pump and the Na/Ca^{2+} exchanger, were also compromised following HOCl exposure. Similarly, oxidation of the contractile proteins may play a significant role in the observed contractile dysfunction (Eley et al. 1990b). Because no attempt was made in this study to determine the identity or activity of all affected proteins, their contribution to the contractile dysfunction can not be ascertained at this time.

The coupling ratio expressed in this study is comparable to that reported by several laboratories using a similar preparation methodology (ie Nayler et al. 1975, Lopaschuk et al 1983, Korecky et al 1986, Kaminishi et al 1989, Limbruno et al 1989). Because the major objective of this study was to correlate the whole heart contractile function with the ability of the SR to sequester Ca^{2+} , we attempted to minimize the time required for isolation. Similarly we were unable to isolate microsomes in the presence of DTT, as is often done in laboratories requiring highly purified SR preparations, as we wished to measure thiol oxidation. Therefore, our assay used an SR enriched preparation only. The possibility of contamination with sarcolemmal vesicles, or the presence of a significant amount of Ca^{2+} release channels must be considered. Recent finding by Zaidi and coworkers have shown the presence of a 106 kD Ca^{2+} release protein in isolated SR that was sensitive to thiol modification (1989a, 1989b). Given that this protein may make up a portion of the 110 kD band observed in the gels presented here, it must be considered that HOCl may have increased Ca^{2+} release from these vesicles. Therefore, the values for Ca^{2+} uptake represent the net accumulation of Ca^{2+} , and may not truly

represent the actual uptake per se. A more thorough examination of this protein band would be required to specifically address this question.

SUMMARY

The evidence from this study further implicates the proteins involved in Ca^{2+} handling, specifically the Mg^{2+} -dependent Ca^{2+} -ATPase of the SR, in the observed mechanical dysfunction following exposure to HOCl. These results show that the degree of SR Ca^{2+} -ATPase impairment is related to the duration of HOCl perfusion. The activity of the Ca^{2+} -ATPase is correlated with the level of oxidized thiols within this protein, and that exposure to HOCl for as little as 15 min leads to an impairment of enzyme activity which can only be restored by thiol reduction with DTT. These data clearly demonstrate a functional restoration in an intact organ model that may be attributed at least in part to the reduction of oxidized protein thiols in a specific cellular ion transport system of the SR.

OVERALL SUMMATION AND CONCLUSIONS

The series of studies presented within the preceding chapters of this thesis examined the effects of a putative neutrophil-derived oxidant on several of the mechanisms involved in EC coupling in the mammalian heart. In order to address these objectives, several models representing specific aspects of the myocardium were employed, including:

- 1) the isovolumically contracting coronary-perfused rat heart for monitoring the mechanical function at the 'whole' tissue level
- 2) the isometrically contracting left ventricular papillary muscle to monitor 'unidimensional' myocardial contractility
- 3) the skinned rat papillary muscle preparation to examine the sensitivity of the myofibrils to $[Ca^{2+}]_e$, as well as to measure force development in response to $[Ca^{2+}]_e$
- 4) Fura-2 loaded whole-cell voltage-clamped isolated rat and rabbit myocytes in order to record transsarcolemmal Ca^{2+} currents, diastolic $[Ca^{2+}]_i$, and Ca^{2+} transients
- 5) the subcellular SR membrane vesicle preparation for determining the function and activity of the Ca^{2+} -ATPase
- 6) isolated proteins, including actin, myosin, and the Ca^{2+} -ATPase for the estimation of thiol oxidation.

The purpose of this final section of the thesis is to bring together the results from these different models with respect to these original objectives.

WHAT ARE THE EFFECTS OF HOCl ON THE CONTRACTING MAMMALIAN MYOCARDIUM?

In both the isometrically contracting rat papillary muscle and the isovolumically contracting rat heart, HOCl induced a time and dose-dependent decline in muscle contractility. This was measured as a rise in resting tension or diastolic pressure, combined with a decline in developed tension or systolic pressure, respectively. In papillary muscles, the time required to reach maximal tension during a single twitch was unaffected, while the time required for relaxation was significantly prolonged. This was coupled with the development of a sensitivity to stimulation voltage. Given that the normal mammalian myocardium responds as a syncytium, contracting in an all-or-none fashion, due to the presence of low resistance intercellular connections (ie gap junctions), this response to HOCl suggests that these connections were compromised.

The response of these muscles to stimulation regimes designed to address specific aspects of cellular Ca^{2+} handling suggested that Ca^{2+} homeostasis in the heart was impaired following HOCl exposure. The effects of HOCl were potentiated under low $[\text{Ca}^{2+}]_e$ or in the presence of the Ca^{2+} channel antagonist nifedipine, and were partially attenuated in elevated $[\text{Ca}^{2+}]_e$ or in the presence of the Ca^{2+} channel agonist Bay K 8644. The development of contracture was prevented by preincubation with ryanodine, an alkaloid compound known to inhibit SR Ca^{2+} release. Taken together, the results from the isolated papillary muscle studies suggest that HOCl altered the ability of the cell to regulate cytosolic Ca^{2+} handling, leading to a breakdown of normal EC coupling mechanisms.

DO THESE EFFECTS RELATE TO THIOL OXIDATION? ARE THESE EFFECTS REVERSIBLE WITH DTT?

The HOCl-induced decline in contractile function correlated with a substantial and highly significant depletion of cellular GSH, followed temporally by a loss of protein thiols. Subsequent exposure of this HOCl-treated myocardial tissue to the disulfide reducing agent DTT provided a significant restoration of both protein thiols and contractile performance in both of the models described. These data linked the observed loss in contractile function following HOCl exposure to the depletion of protein sulfhydryl residues. The restoration of protein thiol levels following exposure to DTT suggests that this depletion is at least partially reversible.

DOES THE OBSERVED MECHANICAL DYSFUNCTION RELATE TO THE IMPAIRMENT OF THE MYOFIBRILS? ARE MYOFIBRILLAR THIOLS DEPLETED FOLLOWING HOCl AND REPLETED FOLLOWING DTT?

The development of contracture as observed following exposure to HOCl may be related to 1) an increased sensitivity of the contractile proteins to the available $[Ca^{2+}]_i$, resulting in the myofibrils becoming activated at a lower Ca^{2+} concentration; 2) a decrease in the ability of actin and myosin crossbridges to cycle, leading to an impairment of the release following the power stroke and a subsequent decrease in DT. In skinned muscles prepared from papillary muscles previously exposed to HOCl, no change in the pCa / relative tension relationship was observed, suggesting that the development of contracture in the isolated myocardium was not related to an increased sensitivity of the myofibrils to $[Ca^{2+}]_i$. However, the loss of myofibrillar thiols correlated with a decline in the absolute tension developed by the skinned muscles at any given pCa greater than 7. In myofibrils isolated from those muscles treated with DTT subsequent to HOCl, the total force

developed was significantly greater than the HOCl-only treated muscles, suggesting that a thiol-dependent mechanism was involved, possibly at the level of the contractile proteins.

The relationship between thiol depletion and loss of myosin ATPase function or the inability of actin and myosin to interact have already been well established, as discussed in Chapter 1. In the present study, the fact that the depletion of protein thiols within the actin and myosin molecules correlated with a loss of contractile function of the muscle following exposure to HOCl suggested that a possible correlation may be found. The fact that DTT induced a partial recovery of contractile function, correlating with a restoration of protein thiols within the actin and myosin molecules supports this hypothesis. Therefore, I conclude that HOCl-induced oxidation of thiol residues within the contractile proteins may account, at least in part, for the loss of tension development in the intact muscle following exposure to HOCl.

However, the data from Chapter 1 also implicate a disruption of cellular Ca^{2+} homeostasis in the development of the contractile dysfunction. The HOCl-induced development of contracture in the isolated papillary muscle preparation was prevented by preincubation with ryanodine even though the degree of thiol oxidation was similar to the non-ryanodine treated muscles. Similar results were observed in muscles preincubated in agents known to alter Ca^{2+} handling at the level of the sarcolemmal Ca^{2+} channels. Therefore, the contribution of intracellular Ca^{2+} homeostasis was also examined.

**DOES THE OBSERVED MECHANICAL DYSFUNCTION RELATE TO A
DISRUPTION OF CELLULAR Ca^{2+} HOMEOSTASIS, AND IF SO, AT
WHAT LEVEL?**

The data discussed in the preceding chapters suggested that Ca^{2+} handling within the myocardium may have been disrupted following exposure to HOCl. The use of these 'specific' pharmacological agents such as nifedipine, Bay K 8644, ryanodine, and caffeine suggested that an increased influx of extracellular Ca^{2+} was not directly involved in the onset of contracture following HOCl, but that the Ca^{2+} release from intracellular stores was.

This involvement of the SR was confirmed by estimating $[\text{Ca}^{2+}]_i$ directly using the Ca^{2+} sensitive indicator Fura-2 under whole-cell voltage clamp conditions. In isolated rat or rabbit myocytes, exposure to HOCl induced a substantial rise in resting steady-state or diastolic $[\text{Ca}^{2+}]_i$ independent of whether the muscle was being stimulated to contract or not. This rise in $[\text{Ca}^{2+}]_i$ occurred in the absence of $[\text{Ca}^{2+}]_e$ and was prevented, or at least significantly attenuated, by a 20 second pre-exposure to caffeine, a compound known to induce a depletion of SR Ca^{2+} stores. The fact that diastolic $[\text{Ca}^{2+}]_i$ did not rise to the same level as $[\text{Ca}^{2+}]_e$ suggests that the sarcolemma was still intact following superfusion with HOCl. Taken together, these data suggest that HOCl induced a rise in diastolic $[\text{Ca}^{2+}]_i$ by inducing a loss of Ca^{2+} from the SR, coupled with a decrease in Ca^{2+} extrusion from the cytosol: in short, the Ca^{2+} was effectively "trapped" within the cytosol.

This hypothesis is supported by the data which show that HOCl caused a dramatic decline in individual Ca^{2+} transients in voltage-clamped Fura-2 loaded myocytes, yet had little effect on the inward Ca^{2+} currents. The loss of the transients must therefore represent the loss of SR Ca^{2+} rather than a decline in the inward Ca^{2+} movement via the

Ca^{2+} channels. While the inward Ca^{2+} currents were not altered by HOCl, the Ca^{2+} transients were significantly diminished or lost, again confirming that the depletion of the SR-releasable pool of Ca^{2+} and not the loss of transarcolemmal Ca^{2+} influx was the most important aspect in the loss of force development. The depletion of the SR may be due to an inability of the Ca^{2+} -ATPase to take up cytosolic Ca^{2+} , thereby leading to cytosolic Ca^{2+} overloading. The work using the SR microsomal preparations showed a significant loss of Ca^{2+} -ATPase activity and Ca^{2+} uptake into the SR fraction, correlating with a loss of reduced thiols in the Ca^{2+} -ATPase protein. In the whole tissue, the loss of SR uptake can lead to a cytosolic Ca^{2+} overloading and subsequent increase in resting force.

Superfusion of the HOCl-treated Fura-2 loaded myocytes with DTT induced a decline in the diastolic $[\text{Ca}^{2+}]_i$ concomitant with a restoration of Ca^{2+} transient activity (Chapter 2). These data suggest that a restoration of intracellular Ca^{2+} homeostasis and a repletion of the releasable pool of Ca^{2+} had occurred, perhaps due to a restoration of the Ca^{2+} sequestration capacity of the SR Ca^{2+} -ATPase. The restoration of SR Ca^{2+} handling could in turn account for the recovery of resting force and developed force in the papillary muscle and perfused heart models.

The major protein involved in the sequestration of Ca^{2+} by the SR is the Ca^{2+} -ATPase. In isolated SR vesicles obtained from rat hearts perfused with HOCl, both the ATPase activity and Ca^{2+} transport function of the Ca^{2+} -ATPase were impaired. This impairment correlated with a loss of protein thiols, as has been confirmed in other laboratories. However, in hearts perfused with DTT subsequent to the HOCl, both the activity and function of the SR Ca^{2+} -ATPase were significantly restored, correlating with a restoration of protein thiol levels. This restoration of SR Ca^{2+} sequestration correlated with a partial restoration of contractile function in the perfused heart. These data serve to

confirm the involvement of the SR in the HOCl-induced loss of contractile function in the rat myocardium.

CONCLUSIONS

In conclusion, exposure of the myocardium to HOCl appears to induce an initial increase in Ca^{2+} influx into the cytosol, mainly from the SR, and possibly from the extracellular compartment. This may be coupled with an impairment of the ability of the Ca^{2+} -ATPase to resequester Ca^{2+} into the SR, and possibly with an impairment of the sarcolemmal Ca^{2+} pump and $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger as well. The final effect is that the cellular Ca^{2+} is effectively trapped within the cytosol, and can not cycle freely during EC coupling. This may account at least in part for the development of contracture in the intact muscle, with increasing RT due to the elevated diastolic $[\text{Ca}^{2+}]_i$, the prolongation of the relaxation phase, and concomitant loss of DT due to a depletion of the releasable pool of SR Ca^{2+} . The recovery of contractile function as observed following the addition of DTT appears linked to a restoration of Ca^{2+} -ATPase function, and hence a restoration of cellular Ca^{2+} homeostasis. The fact that DT does not recover fully may be the result of an irreversible loss of contractile function at the level of the myofibrils.

In light of the two-compartment model for the cellular Ca^{2+} handling (Schouten et al. 1987), it appears that the connections between the sarcolemma and the SR may have become uncoupled. With the addition of DTT, the amplitude of the Ca^{2+} transients were restored, suggesting that the function of the SR was restored, at least in part, while the inward Ca^{2+} currents were impaired. The loss of this inward current did not affect the generation of these transients, suggesting that alternate mechanisms were in play

Thiol oxidation is known to inactivate both the Ca^{2+} -ATPase of the SR (Aoki et al. 1986, Kukreja et al. 1989) and the sarcolemma (Kaneko et al. 1989), while impairing the function of the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (Dixon et al. 1987, Pierce et al.

1986). Thiol oxidation has also been linked to an increase in Ca^{2+} release from the SR via the release channels (Trimm et al. 1986, Zaidi et al. 1989), and to a prolongation of action potential duration due to increased inward Ca^{2+} current (Aomine and Abe 1978, Hayashi et al. 1989a). Many of these reported effects were reversed by the subsequent addition of thiol reducing agents, including DTT (Aoki et al. 1986, Trimm et al. 1986, Zaidi et al. 1989). Taken together, indiscriminant thiol modification by HOCl would lead to an increase in Ca^{2+} movement into the cytosol, combined with a loss of extrusion mechanisms. The recovery of steady-state $[\text{Ca}^{2+}]_i$ following DTT implicates a reversible thiol oxidation in the proteins responsible for Ca^{2+} extrusion. The recovery of the fast component of the Ca^{2+} transients shows that the releasable pool of SR Ca^{2+} has been replenished, and implicates a restoration of the SR Ca^{2+} -ATPase function in this DTT-induced recovery.

The use of specific reducing agents such as DTT as an *in vivo* therapeutic agent is not without precedent, and may not be unreasonable. In 1978, Mushlin (Mushlin et al. 1978) reported that the injection of DTT into spontaneously hypertensive rats (SHR) led to a decline in systemic blood pressure and a reduction of vascular smooth muscle tone. This effect of DTT was linked to an increase in aortic smooth muscle SR Ca^{2+} -ATPase activity and function. DTT had no effect on the blood pressure, vascular tone, or Ca^{2+} -ATPase function in the Control rats (Sreter et al. 1970). While it has yet to be shown, DTT may also prove effective *in vivo* for reducing the contracture that often leads to cardiac failure following an oxidative insult.

Irreversible myocardial damage may turn out to be not so irreversible after all.

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APPENDIX I

Chemicals and Reagents:

<u>Abbreviation</u>	<u>Chemical name</u>	<u>Source</u>
	β -Mercaptoethanol	BDH
	Acetic Acid	Fisher
	Acrylamide	Biorad
	Ammonium Persulfate	Biorad
	Aspartic Acid	BDH
	Bis-Acrylamide	Biorad
CaCl ₂	Calcium Chloride	BDH
	Collagenase CLS II	Worthington
	Coomassie Brilliant Blue	Biorad
CPK	Creatine Phosphokinase	Sigma
	D-Glucose	BDH
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)	Sigma
DTT	Dithiothreitol	Sigma
EDTA	Ethylenediaminetetraacetic acid	BDH
EGTA	Ethylene glycol bis(β -aminoethyl ether)N N N'N' Tetraacetic acid	BDH
FeSO ₄	Ferrous Sulfate	BDH
	Glycerol	BDH
	Glycine	Biorad
GSH	Glutathione	Sigma
H ₂ SO ₄	Sulfuric acid	BDH
HEPES		Sigma
	Histidine	Sigma
HOCl	Hypochlorous Acid	Fisher
IAA	Iodoacetamide (¹⁴ C labelled)	Amersham
	Imidazole	BDH
K-oxalate	Potassium oxalate	Baker
KCl	Potassium Chloride	BDH
KH ₂ PO ₄	Potassium Phosphate dibasic	BDH
MeOH	Methanol	Fisher

MgSO ₄	Magnesium Sulfate	Fisher
Na ₂ -ATP	Adenosine Triphosphate (disodium)	Sigma
Na ₄ P ₂ O ₇	Sodium Pyrophosphate	BDH
NaH ₃ PO ₄	Sodium Phosphate	Fisher
NaCl	Sodium Chloride	BDH
NaHCO ₂	Sodium Bicarbonate	BDH
NaN ₃	Sodium Azide	BDH
NaOH	Sodium Hydroxide	Fisher
NH ₃ Mb	Ammonium Molybdate	BDH
PC	Phosphocreatine	Sigma
PMSF	Phenylmethylsulfonyl fluoride	Sigma
SDS	Sodium dodecyl sulfate	Biorad
TCA	Trichloroacetic acid	BDH
TEMED		Biorad
TRIS	Trizma base	Sigma
	Triton X-100	BDH

Pharmacological agents:

<u>Name</u>	<u>Source</u>
Bay K 8644	Miles Laboratory
Caffeine	BDH
Fura-2	Molecular Probes
Nifedipine	Sigma
Ouabain	Sigma
Ryanodine	Calbiochem
Somnatol	MTC Pharmaceuticals

Equipment List:

Slab gel apparatus	LKB 2297 Macrodrive Power supply
Spectrophotometer	LKB Ultrospec II
	Beckman DU-7
Densitometer	LKB 2222-020 Ultrosan XL
Centrifuges:	Fisher Microfuge
	Beckman Ultracentrifuge
	IEC B20A

Scintillation Counter	Beckman LS2800
Polygraph	Grass 7P1F Preamplifier
	Grass 7PAG Driver amplifier
Transducers	Statham P-23 Pressure Transducers
	Gould UC-2 Force Transducers
Homogenizer	Fisher D220
Spectrofluorometer	SPEX Fluorolog
Microscope	Nikon Diaphot
Voltage Clamp Amplifier	Axoclamp 2A
Computer	***** <u>MACINTOSH</u> *****

APPENDIX II

DETAILED DESCRIPTION OF METHODOLOGY,

for the benefit of those who may wish to repeat these techniques

PART I

ACTIN AND MYOSIN ISOLATION

LOW SALT BUFFER (pH 6.8, using KOH if necessary)

KCl	20 mM	1.49 g / L
K ₂ HPO ₄	2 mM	0.348 g / L
EGTA	1 mM	0.380 g / L

HIGH SALT BUFFER (pH 9.5, using KOH or HCl if necessary)

Na ₄ Pyrophosphate	40 mM	17.80 g / L
MgCl ₂	1 mM	0.20 g / L
EGTA	1 mM	0.38 g / L

freeze in 1 ml aliquots until used

PMSF (phenylmethylsulfonyl fluoride) (1 mM final solution)

Dilute 50 µl of 100 µM PMSF with 200 µl of 2-propanol to make a 20 µM solution. This agent inhibits protease activity in the muscles to prevent degradation during the homogenization

[¹⁴C] IODOACETAMIDE SOLUTION (IAA)

-¹⁴C IAA as delivered from Amersham, 50 µCi, with activity of 58.3 m Ci per millimole : 0.84 µMoles of IAA are in the tube.

-dissolve this in 1 ml of warm H₂O giving a final concentration of 0.86 millimolar.

-freeze in 100 µl aliquots until used.

ISOLATION TECHNIQUE:

-THAW SOLUTIONS AND ENSURE THAT THE CENTRIFUGE IS IN THE COLD ROOM.

-WARM THE IAA SOLUTION SLIGHTLY BEFORE USE, BUT KEEP AWAY FROM LIGHT.

Homogenize papillary muscle (5 mg) in 100 µl of LSB with 5 µl of 20 mM PMSF solution using small glass/glass homogenizer for 2 minutes, and transfer into chilled Eppendorff tube. Wash the homogenizer with additional 100 µl of LSB and transfer into the tube as well (final volume 200 µl)

Centrifuge for 45 seconds or less, to ensure pellet is not tightly packed. Discard the supernatant, or keep for DTNB assay if desired. (Remember... pH must be near 8 for that.)

Add 100 µl of HSB, resuspend the pellet with a glass rod and extract for 15 minutes at 0 ° C, ensuring pH is 9.5. Centrifuge for 10 minutes, perform the Biorad assay on the

supernatant to determine protein concentration. This supernatant should contain the actin, myosin, tropomyosin and troponin complex proteins for separation. Protein yield should be in the range of 50 - 250 μg / mg tissue. Take an aliquote of this (enough to make 100 μl of 1 mg/ml solution) and adjust to 1 mg / ml (if possible) with HSB in a cooled tube.

Add 10 μl of the cold (0° C) ^{14}C IAA solution (approximately 0.6 μCi , see below for calculations), and extract in a darkened waterbath at 37° C for 60 minutes. Stop the reaction with the addition of excess DTT (eg 2 μl of 0.5 M DTT stock = 10 mM).

Assuming 1 mg protein / ml, we want to load 10-15 μg of total protein onto the gel. We would therefore add 10-15 μl of the sample to each well.

Actin and Myosin subaliquots prelabelled with [^{14}C] IAA are in the freezer in a concentration of 2 mg / ml total (therefore 1.0 mg / ml of each protein). For the Densitometer readings we need three concentrations of these standards. Therefore, aim for 0.0, 5.0, and 20.0 μg of each protein per lane.

Lane 1; add 10 μl of High Molecular Weight standard.

Lane 2; add 20 μl of straight loading buffer,

Lane 3; add 5 μl of actin / myosin mix, 10 μl water, and 15 μl of loading buffer

Lane 4; add 20 μl of actin / myosin mix and 15 μl of loading buffer

Lane 5 to 10, add 15 μl of the samples (1 μg / μl) and 15 μl of loading buffer

GELS:

SOLUTIONS:

1) 30% Acrylamide **CAUTION: NEUROTOXIN**

-30 g Acrylamide / 100 ml H_2O

-0.8 g BIS acrylamide / 100 ml H_2O

2) Lower Buffer:

-1.5 M TRIS - HCl pH 8.8 18.1 g / 100 ml H_2O

-0.4 % SDS (w / v) 0.4 mg / 100 ml H_2O

3) 10 % Ammonium Persulfate **CAUTION: TOXIC**

-0.1 g / ml H_2O

-make fresh daily

4) Upper Buffer (Stacking Buffer)

-0.5 M TRIS - HCl pH 6.8 6.01 g / 100 ml H_2O

-0.4 % SDS (w / v) 0.4 mg / 100 ml H_2O

5) Running Buffer

-25 mM TRIS 3.03 g / litre

-0.1 % SDS 1.00 g / litre

-0.192 M Glycine 14.4 g / litre

Make 10 X stock (10 X the above values) and dilute before use

6) Loading Buffer (Sample Buffer)

-2 ml 50 % Glycerol

-1 ml 10 % SDS

-1 ml Upper Buffer (4)

-0.1 ml β Mercaptoethanol

-0.1 ml Bromo Phenol Blue (2% in 40% Sucrose)

(add 0.1 g of BPB to 5 ml 40% sucrose)

- 7) Staining Solution
 -10% Acetic Acid 100 ml / litre
 -40% Methanol 400 ml / litre
 -0.1 % Coomasi Blue 1 g / litre
 or
 -50% TCA
 -0.1 % Coomasi Blue
 8) Destaining Solution
 - 7% Acetic Acid 70 ml / litre
 -20% Methanol 200 ml / litre
 9) ENHANCE
 10) To prevent cracking of the gel upon drying
 -10% Glycerol
 -5% Acetic Acid

TO MAKE GELS:

Running gel recipe (for one gel only)

		6%	8%	10%	12%	15%
30% Acrylamide (1) <u>(TOXIC)</u>	ml	6.6	8.8	11.0	13.2	16.5
50% Glycerol	ml	5.3	5.3	5.3	5.3	5.3
Lower Buffer (2)	ml	8.3	8.3	8.3	8.3	8.3
Water	ml	12.9	10.6	8.5	6.3	3.0

10% Am. persulfate(3) (TOXIC) µl 120 120 120 120 120

TEMED (TOXIC) ADD LAST µl 15 15 15 15 15

For stacking gel (one gel only)

30% Acrylamide (1) <u>(TOXIC)</u>	ml	1.5
Upper Buffer (4)	ml	2.5
Water	ml	6.0

10% Am. persulfate(3) (TOXIC) µl 60.0

TEMED (TOXIC) ADD LAST µl 20.0

PROCEDURE:

-----Clean the glass plates thoroughly with methanol, ensuring there are no cracks or chips in the plate. Place a spacer (black plastic) between the two plates (as shown) and mount in the plate clamps. The plates should extend slightly above and below the clamps to ensure a tight seal when mounted later in the running apparatus. Tighten the clamps and insert the plates into the pouring base. Using the turn cocks, tighten the plates against the rubber seal of the base. Make sure no leaks can occur!!!!

-----Pour the running gel between the plates by pouring from a beaker along a long Pasteur Pipette. Add enough (about 12 cm) to leave about 3-5cm at the top for the stacking gel. Remove any bubbles from the surface, then GENTLY add water to the surface area, using a glass syringe and size 26 long needle. DO NOT DISTURB SURFACE, if possible. This will prevent a meniscus from forming which will alter the running of the proteins. Wait about 30-60 minutes for the gel to polymerize. More A.P. and Temed will speed up this process.

-----Remove water from the surface using the same syringe. Insert a paper towel bringing the edge into contact with the gel to remove any residual water.

-----Pour the stacking gel (same way as the running gel) up to about 1 cm from the top of the plates. Insert the appropriate comb into the stacking gel immediately, but be careful to not get any bubbles beneath the comb itself. Insert about 2 cm if possible, but leave at least 2 cm of stacking gel between the comb and the running gel for the protein to run through. Leave for about 30-60 minutes to polymerize.

-----Gently remove the comb and flush the wells with fresh Running Buffer to remove any debris. Leave the wells filled with buffer.

-----Fit the top chamber onto the top of the plates, again ensuring that a tight seal is made between the glass and the top. If necessary, loosen the plate clamps and adjust the plates to allow a good seal. The plates can be removed from the turn cocks at the base at this point, and these same turn cocks can be used to tighten down the top. Check for leaks by adding running buffer to the top reservoir. MAKE SURE BOTH SIDES HAVE A GEL OR A DUMMY GEL (a piece of 3/8" plexiglass can be used) IN PLACE!! Clean sides of the glass plates.

-----Put about 1 litre of running buffer in the gel box and remove as many bubbles as possible. Remove the base assembly from the glass plates and place the assembly (top is still attached I hope) into the gel box. Shake the assembly to ensure no bubbles are along the bottom of the gel itself. Fill up the top reservoir (about 600 ml) with running buffer.

-----Samples are added right through the top reservoir into each well of the gel. Use a Hamilton Syringe to insert the samples, ensuring that all the sample is applied. When complete, attach the lid, connect the lines to the power supply and start the run. 60-80 V overnight should do the trick, if the amperage is turned to full clockwise position. If higher voltage is used, run cooling water through the apparatus to prevent smiling. Approximately 800 V hours are required

STAINING / DESTAINING

Once the gel has run to within 1 cm of the bottom, disconnect power and remove gels from the buffer. Undo the clips and gently push the black spacers out from between the

glass plates. Use one of the spacers to gently pry the glass plates apart. **IMPORTANT!!!!** Do not damage the gels at this step. Mark the gel with an identifying notch in one corner and note this for future reference. Submerge the glass plate with the gel attached into a tupperware container containing staining solution (7), seal, and swirl gently for 1-2 hours. When completed, pour off the staining solution (keep for a few more gels), rinse with H₂O, and add Destaining solution (8). Put in sponge pieces to absorb the stain and shake gently for several hours as required (ie. overnight). Gel should become transparent. If bands are not adequately stained, repeat process, possibly with fresh staining solution.

DENSITOMETRY

using the LKB 2222-020 ultrosan XL laser densitometer in McBurneys lab.

Put wet gel onto a clean acetate sheet directly onto the densitometer plate. Make sure no bubbles or water are beneath the acetate to ensure even readings. Wet the gel lightly with H₂O. Set lane parameters and read. From the standard curve, and from the known [protein] added, calculate concentrations of the proteins desired.

AUTORADIOGRAPHY

IN THE FUMEHOOD:.....

Place destained gel into tupperware container with enough ENHANCE solution (DUPONT) to cover the gel. Seal container and shake gently for 1 hour at room temperature. IN FUMEHOOD, discard the ENHANCE into a waste bottle and wash gel with H₂O for several minutes to remove and precipitate remaining solution. Submerge the gel into 10% glycerol solution for 1 hour to saturate the gel with glycerol. This prevents excess cracking of the gel during drying. Carefully pick up the gel on a piece of Whatman filter paper and put onto the drying apparatus. **NOTE: TURN ON THE FREEZE DRYER 15 MINUTES PRIOR TO ENSURE IT IS COLD. TURN ON VACCUUM PUMP TO THE FREEZER UNIT.** Make sure there are two layers of filter paper cut to fit the dryer itself, leaving room at edges for sealing. Cover the gel with clear saran or gel paper and trim to fit. Put the nylon shield over the saran and close the rubber lid. Make sure there is room for vacuum to form a tight seal. Turn on the vacuum to the dryer and set timing (heater) to 2 hours (or longer if required). Wait.

Turn off the vacuum line, open dryer and remove the gels. Leaving the saran on the gel transfer it directly to a KODAK X-OMATIC cassette with intensifying screen. Tape into place. In the dark room, insert a sheet of X-RAY film into the cassette directly in contact with the saran covered gel. Seal, put into black plastic bag and into - 80 freezer for 7 days. Develop the autorad at the General Hospital radiology department.

PHOTOGRAPHY

Dried gel and its accompanying autorad should be photographed for future reference using PANATOMIC X film. Gel should be taken at grey card $\pm$ 2 stops (4 clicks) bracketing under tungsten lights. The autorads should be taken from a light box at

SCINTILLATION COUNTING

Bands can be cut from the gels using a oblong shaped hole punch and digested in 200 μ l of H₂O₂ (30%) at 60 °C for 3 hours directly in the small scintillation tubes. Add scintillant (ACS), shake gently for 15 minutes, then read on the β counter, program # 2 for 10 minutes.

[¹⁴C] IAA CALCULATIONS

We found from the skinned muscles isolated using the above extraction method that about 35 - 50 nmoles SH per mg protein were present. We want to add excess [¹⁴C] IAA (eg 100 nmoles per mg protein) to the HSB. Assuming 1 mg protein per ml, we should have about 0.1 mg protein in the sample. Therefore we want to add 10 nmoles of [¹⁴C] IAA. Assuming the stock IAA concentration is 1 mM, we would want to add about 10 µl to each sample

$$\frac{0.001 \text{ moles IAA}}{1000 \text{ ml}} = \frac{10 \text{ nano moles IAA}}{x \text{ ml}} \quad x = 10 \mu\text{l}$$

If we assume that the [¹⁴C] IAA was at 1 mM, and we have added 10 µl of the IAA solution to 100 µl of sample, we have a final [¹⁴C] IAA concentration of 100 µM. This should also contain 1% of the total radioactivity (as the IAA was dissolved in 1 ml), or .5 µCi per sample. If we add 10 µl of the mixture to each well, we can expect about 0.05 µCi per well, or greater than 1,000,000 degradations per minute. We can expect that not all will be bound, but a sufficient amount should be present for counting and autoradiography.

PROTEIN DETERMINATION

Set spectrophotometer at 595 nm, visible lamp (Tungsten, not deuterium).

Standards for BIORAD protein are in the freezer (0.5 mg / ml). In labelled Epindorff tubes put 0, 5, 10, and 15 µl of standard (corresponding to 2.5, 5, and 7.5 µg protein), or 2 and 5 µl of sample. Add 800 µl (minus the amount already added as sample or standard) of H₂O and vortex lightly. Add 200 µl of BIORAD reagent (at room temperature) and vortex again (lightly).

Transfer the solutions into 1 ml cuvettes and read in spectrophotometer within 10 minutes. The concentration of the protein can be determined as follows:

Plot the standard curve with the amount of protein added (per ml.) on the ordinate and absorbance on the abscissa and determine the slope (units will be mg protein / Absorbance Unit) (eg 17.5 µg / AU). From this curve, the unknown protein can be determined per µl of sample added (eg if absorbance is .282 AU for 5 µl of unknown, then the amount of protein per µl will be:

$$17.5 \mu\text{g} / \text{AU} \times .282 \text{ AU} / 5 \mu\text{l of sample} = 0.98 \mu\text{g} / \mu\text{l} \\ = 0.98 \text{ mg} / \text{ml}$$

If muscle weight was 5 mg, then 1 µg protein / µl X 100 µl
 = 100 µg protein / 5 mg tissue
 = 200 mg protein / gram tissue yield

PART III

MICROSOMAL ISOLATION PROCEDURE PREPARATIONS

SOLUTIONS:

solutions are pH'd with saturated Maleate (5-10 grams in enough water to dissolve).
pH each buffer before bringing to final volume, and check final pH just before using each solution

1) **BUFFER 1** (Homogenizing buffer) **pH 7.2** with Maleate

20 mM TRIS (mw 121.14)

-2.423 g / litre

dissolve in 800 ml water, pH with Maleate, then bring to volume

2) **BUFFER 2** (Low speed spin buffer) **pH 6.8** with Maleate

20 mM TRIS (mw 121.14)

-2.423 g / litre

600 mM KCl (mw 74.56)

-44.70 g / litre

3) **BUFFER 3** (Final suspension buffer) (200 ml)

- 20 mM TRIS (mw 121.14)

-0.484 g

- 50 mM KCl (mw 74.56)

-0.745 g

- 250 mM Sucrose (mw 342.3)

-17.16 g

bring to 100 ml with Distilled water, **pH 6.8** with Maleate then

bring to 200 ml with distilled water

Slow speed centrifuge: IECB20A

-MAIN power on

-set control to REFRIGERATE

-set green needle to 0°C

-adust rotor so that lines match, put top on, close lid.

Ultra centrifuge: BECKMAN

-POWER on ,

-press DISPLAY, hold down while setting conditions

-set TEMP to 1.0° C

-set VACCUUM to ON

- check settings by DISPLAY
- use rotor SW28, (kept in cold room). Leave this in centrifuge during cooling to ensure even temperature

Other centrifuge

- power on
- set temperature at 4°C and close lid
- bring buckets into cold room or fridge
- set speed at 3000 R.P.M.

Homogenizing equipment:

- ICE--large bucket, 2 smaller buckets, 1 large and 1 small plastic beaker
- for each heart, put one 25 ml beaker containing 2 ml of the pH 7.2 **BUFFER 1** on ice in the first small ice bucket, and bury the small scissors (blades) into the ice. (To be used for mincing the heart)
- put a second 25 ml beaker in fridge (4°C) half filled with isotonic saline (to be used for cooling the heart).
- in 200 ml beaker, pH **BUFFER 1** to 7.2 with Maleate You need about 150 ml for two hearts. Put on ice in large bucket
- for each heart, put two 40 ml slow-speed centrifuge tubes and the large homogenizer / pestle on ice in the large bucket.
- put the ultracentrifuge rotor and the plastic ultracentrifuge tubes which fit it in the cold room. If you are doing only one heart, you only need two of the plastic ultracentrifuge tubes, but all six of the buckets must be used at all times. Put the plastic tubes on ice in the large ice bucket.
- label plastic (3 ml) test tubes (1 per heart) and put on ice in second small bucket in cold room
- put the small glass/teflon homogenizer (for resuspending the pellet between spins) on ice in the second small ice bucket and put in cold room. Also take 100 µl, 1 ml and 5 ml pipetters along with plenty of tips, plus disposable pasteur pipettes (plastic) to the cold room

Filters:

- you need one 0.45 µm filter for each time point, for each duplication, for each sample. ie for 1 heart and 4 time points (1,10,20,30 min) you need 8 filters X (multiplied by) the number of replications desired. Pre-soak these filters in a shallow weigh boat with WASH MEDIUM (Solution 8 on Page 7). Always soak a few extras in

case of emergency (they tear easily). **Do not soak for more than two hours or the filters will clog.**

a) Just prior to completion of the perfusion protocol:

- Remove saline, **BUFFER 1**, scissors, from fridge but leave on ice.
- insert pestle into the motor driven homogenizer and set speed at 2100 R.P.M.: do not turn on motor yet. Use a stand to support the large plastic beaker filled with ice such that the pestle is suspended in the ice. Remember to dry the pestle before homogenizing the tissue.

b) Upon completion of the perfusion protocol,

- immerse the heart in the iced isotonic saline, then remove, blot dry and immerse in the chilled 2 ml of **BUFFER 1**-mince the ventricular tissue using the cooled scissors into 1 mm³ pieces, taking about 2 minutes.
- add 5 ml of **BUFFER 1** using 5 ml pipetter, swirl and transfer into chilled glass homogenizer tube.

c) Homogenization

This is the most important step. We don't want to homogenize too long or oxidation of the protein occurs. Too short and the yield is low. We used the following protocol with good results.

- set speed at **2100 RPM**, but do not turn on yet. Turn on stopwatch
- place pestle into the mortar (still on ice I hope) and push mortar up until pestle contacts the surface of the buffer.
- Hold the mortar steadily, still immersed in ice, and turn on motor.
- push mortar up until homogenizer starts to slow down. Release slightly to maintain speed for about 4-5 seconds, then withdraw until the top of the pestle just contacts the surface of the buffer. Do not go further, or this will bring air into the mixture and increase oxidation.
- Repeat this twice more (total three strokes) Each stroke takes 10 seconds (4 seconds at bottom at slightly less than maximal speed, 6 seconds drawing pestle up and down at maximal speed), so total homogenization takes 30 seconds.
- withdraw the pestle carefully (keeping mortar still on ice). The slurry appears light red colour, with a few pieces still present. Do not attempt to further homogenize unless necessary.

- pour homogenate into a chilled slow speed centrifuge tube
- wash mortar and pestle with 2 ml **BUFFER 1**, and pour it into the centrifuge tube.

NOTE: this mortar and pestle will be reused, so during the first spins, wash them several times with distilled water, rinse with buffer 1, dry, and return to ice.

- if only homogenizing one heart, remember to use a second centrifuge tube for balance

d) Slow speed centrifugation

- balance tubes to within 100 mg, but do not fill tubes.
- spin in the IECB centrifuge for **10 MINUTES @ 7000 RPM.**
(9000 g)
- take tubes from centrifuge, put on ice and take to the cold room.
- With 5 ml pipetter, transfer the **SUPERNATANT** to the second clean 40 ml tube, balance with **BUFFER 1** and spin again, **20 MINUTES @ 7000 RPM** Discard the pellet

e) Ultracentrifugation

- take tube(s) from centrifuge into the cold room again and transfer **SUPERNATANT** to 40 ml **ULTRACENTRIFUGE** tube(s). Fill tubes (**BUFFER 1**) to within 5 mm of top, then place small beaker, on balance, and fill to top (**BUFFER 1**) caps (or during spin) will Balance the other tube(s) against this.

VERY IMPORTANT!!!!

- Place tubes into centrifuge buckets and screw on caps tightly and hook all six buckets onto the rotor, ensuring the numbers on the buckets match the numbers on the rotor.

Check the position of the buckets carefully, and ensure they are hooked correctly and move freely!!!!

- Release the vacuum on the ultracentrifuge, open the lid and place the rotor into position. Test the rotor by spinning in freehand, checking that the buckets swing freely Close the centrifuge, restore vacuum, press **DISPLAY** and set speed **@ 28,000 RPM for 65 minutes** (5 minutes to reach speed, 60 minute run). Push **TIME & AUTORUN**. Ensure that the speed does reach 10,000 before leaving.
- During this spin, pH **BUFFER 2** to 6.8.

- Wipe
- Upon completion of this spin, release vacuum, carry buckets to cold room, remove the ultracentrifuge tubes and discard the supernatant. tube walls dry
 - dislodge the **PELLET** in 0.5 ml of **BUFFER 2** using the small teflon pestle. Transfer this into the small glass homogenizer using the plastic pasteur pipettes, and resuspend pellet by 2-3 spins of the pestle (on ice). Transfer back to the ultracentrifuge tubes with the plastic pasteur pipette and rinse homogenizer with 1 ml of **BUFFER 2**.
 - Balance tubes with **BUFFER 2**, filling tubes to the top as before, and repeat the ultracentrifugation step (28000 RPM for 65 minutes).
 - During this spin, pH **BUFFER 3** to 6.8, wash the small homogenizer and pestle, rinse with distilled water, dry in oven (or under a stream of air) and return to ice for resuspension of the final pellet.
- f) Final suspension of vesicles:
- remove samples from ultracentrifuge and discard supernatant
 - resuspend as above in 400-500 μ l of **BUFFER 3**
 - transfer from homogenizer into chilled test tubes using the plastic pasteur pipettes, washing the homogenizer once with 50 μ l of **BUFFER 3**.

VOILA -- VESICLES

- g) **DILUTE** vesicles preparation to 1 mg / ml (using BIORAD ASSAY as described below) with **BUFFER 3**.

BIORAD PROTEIN ASSAY

Protein Standards:

We used Biorad protein assay because of its simplicity and high sensitivity, allowing us to use very little protein.

- Standard used here is the BIORAD bovine plasma albumin, diluted to 0.5 mg / ml in distilled H₂O
- Prepare BIORAD reagent mix. This mix consists of 1 part BIORAD reagent, 4 parts distilled H₂O, mixed then vacuum-filtered (Whatman #1) to remove particulate matter.
- standards should be run in triplicate with 2.5, 5, and 7.5 µg protein. To get these concentrations, add 5,10,15 µl of the standard (above) to 1 ml of BIORAD reagent mix. These should not be prepared until needed, and should be read (595 nm) within 10 minutes of being prepared.

BIORAD Protein assay:

-Assuming a protein concentration of about 1-2 mg / ml in the vesicle preparations, we want to assay approximately 5 µg to be on the standard curve. Therefore, we add 3-4 µl of vesicle suspension to 1 ml of BIORAD reagent mix, mix gently and read immediately at 595 against the standard curve described above. Samples should be run in triplicate

PART IV

CA⁺⁺ UPTAKE ASSAY:

4) **BUFFER 4** (Ca⁺⁺ Uptake Medium) (makes 1 litre)

we will eventually add solutions 4 to 7 together to equal 675 ml. Therefore, 500 µl of this solutions will be diluted by factor of 675 / 500 or 1.35. Solutions 5,6 & 7 will also be diluted accordingly

135 mM KCl (mw 74.56) (100 mM final conc during assay)

-10.066 g / litre

6.75 mM MgCl₂ (mw 203.33) (5 mM final conc during assay)

-1.372 g / litre

6.75 mM NaN₃ (mw 65.01) (5 mM final conc during assay)

-0.439 g / litre

- 27 mM TRIS (mw 121.14) (20 mM final conc during assay)
-3.271 g / litre **pH 6.8 with Maleate**
- 5) 135 mM K oxalate (10 ml) (10 mM final conc during assay)
- for dipotassium salt (mw 182.4)
-0.246 g / 10 ml H₂O
- 6) 67.5 mM ATP (10 ml) (5 mM final conc during assay)
(mw 551.2)
- 0.372 g / 10 ml of BUFFER 4 (TRIS pH 6.8)

MAKE FRESH EACH DAY

- 7) ⁴⁵Ca⁺⁺ mixture (100 ml) pH 6.8 with Maleate
Ca / EGTA ratio = 1.0, want final values to be 0.1 mM Ca / 0.1 mM EGTA,
giving a final [Ca] of about 100-200 nM
50 µl of this solution will be added to the reaction mixture, so this solution
will be diluted by factor 675 / 50, or 13.5
- a) CaCl₂ (mw 147.02) (0.1 mM final conc during assay)
-0.020 g / 100 ml
- b) EGTA (mw 380.4) (0.1 mM final conc during assay)
-0.051 g / 100 ml
- c) TRIS (mw 121.14) (20 mM final conc during assay)
-3.272 g / 100 ml

To 10 ml of this mixture add 10 µl of ⁴⁵Ca⁺⁺ solution described below (final [⁴⁵Ca⁺⁺] about 1 µCi / ml). Aliquot (1 ml) and store in freezer
(Whatever ⁴⁵Ca⁺⁺ comes in the vial (~ 1.0 mCi) is diluted in 1 ml of distilled water.
T_{1/2} = 165 days).

8) Wash Medium

20 mM TRIS maleate pH 6.8
-2.423 g / litre
0.1 mM CaCl₂ (mw 147.02)
-0.0147 g / litre
0.1 mM EGTA (mw 380.4)
-0.038 g / litre

PROCEDURE:

Set up a water bath at 37° C containing a test tube rack.

Have available 100 µl and 1 ml pipetter and tips, plus at least one test tube rack
outside of the waterbath.

Set up vacuum filtration device, with Millipore scinttered glass base set into a vacuum flask, 5 ml Millipore glass funnel attachment and clamp.
Have presoaked filters on hand with set of blunt forceps. Put one filter on the scinttered glass (see Page 2)
Have two beakers (50 ml and 300 ml) of chilled Wash solution (**Solution 8**) by the filtration apparatus with a 100 μ l and a 5 ml pipetter plus tips.
Have squeeze bottle of distilled water available by the filtration apparatus.
Label 4 - 3 ml **plastic** tubes per sample, with duplicates (2) of each of the following, with the sample #, and:

- + ATP
- ATP (blank)

Label the same number of scintillation vials (10 ml) as above plus one labelled
STANDARD

a) Into each test tube put

- 500 μ l Uptake medium (**BUFFER 4**)
- 50 μ l oxalate solution (**Solution 5**)
- 25 μ l vesicles (25 μ g of protein)

-To those tubes labelled "+ ATP", add

-50 μ l ATP (**Solution 6**)

-To those tubes labelled "- ATP", add

-50 μ l (**BUFFER 4**)

Preincubate 5 minutes in 37°C water bath.

b) To initiate the uptake reaction, add

- 50 μ l of $^{45}\text{Ca}^{++}$ mix (**Solution 7**) (total of 675 μ l in each tube)
- vortex thoroughly and put back into waterbath
- start stopwatch

c) At timepoint (eg 1 minute)

- vortex tube, remove 50 μ l of mixture, and apply to the centre of the filter paper on the vacuum.
- Wash the pipette tip 3 X (from 50 ml beaker of **Solution 8 - Wash Medium**) to remove all vesicles, applying the washes onto the filter paper as well
- mount the glass funnel with the clamp, then wash the filter by applying 5 ml of chilled Wash solution (**Solution 8**)

- remove the filter paper into the appropriate scintillation vial
- clean the filter apparatus with distilled water, blot dry, and put a new filter in place

Repeat for each sample and for each timepoint.

- d) To the scintillation vial marked STANDARD, add 50 μ l of the $^{45}\text{Ca}^{++}$ solution (Solution 7).
- e) To each scintillation vial, add ACS and count. On our Beckman counter, use USER #6 (put the USER 6 card on the front rack, press AUTOCOUNT and count for 10 minutes.

CALCULATIONS: (from CPM ^{45}Ca to Ca^{++} Uptake)

Assuming that $^{45}\text{Ca}^{++}$ and Ca^{++} uptake and binding are identical, we can use $^{45}\text{Ca}^{++}$ to monitor Ca^{++} uptake.

- 1) Each tube contains 675 μl , and $[\text{Ca}^{++}]$ is 0.1 mM, therefore the total Ca^{++} added to each tube = 67.5 nmoles per sample .
- 2) Assume that the 50 μl of (containing 67.5 nmoles of Ca) counted as 150,000 CPM
Assume that 50 μl of the sample counted at 1 minute was 300 CPM, and that background was 50 CPM, then in total sample (675 μl) we should have counts of $(300 - 50 \times 675 / 50) = 3375$ CPM per 25 μg protein
- 3) If 150,000 CPM = 67.5 nmoles Ca^{++} , then 3375 sCPM = 1.52 nmoles Ca^{++}
If 25 μg protein takes up 1.52 nmoles Ca^{++} , then 1 mg will take up 60.8 nmoles Ca^{++} per minute.

FORMULA:

$$\text{Ca}^{++} \text{ Uptake} = 67.5 \times \frac{1}{0.025} \times \frac{675}{50} \times \frac{\text{sample} - \text{background}}{\text{standard} - \text{background}}$$

$$\frac{\text{nmoles}}{\text{mg protein}} \quad \text{total Ca}^{++} \quad \text{25 } \mu\text{g} \quad \text{to convert} \quad \frac{\text{C.P.M}}{\text{C.P.M}}$$

$$\text{in tube} \quad \text{of protein} \quad \text{to the total in the} \quad \text{tube being sampled}$$

$$= 67.5 / 0.025 \times 13.5 \times 300 - 50 / 150000 - 50$$

$$= 60.8 \text{ nmoles Ca}^{++} \text{ uptake per mg protein}$$

Ca++ ATPase assay

adapted from Wang, Nakamura, and Schwartz, Ch. 4. Cardiac S.R.

SOLUTIONS:

- 1) Working buffer: pH 7.0 with Maleate, 37° C (important)
This solutions will be mixed with others, so the following will be diluted by a factor of 1.82. Similar dilutions will occur for solutions 2 & 4 below
45.5 mM Histidine (25 mM final conc during assay)
-3.53 g / 500 ml
9.1 mM MgCl₂(·6H₂O) (5 mM final conc during assay)
-0.925 g / 500 ml
182.0 mM KCl (100 mM final conc during assay)
-6.785 g / 500 ml
182.0 µM EGTA (100 µM final conc during assay)
-0.035 g / 500 ml
9.1 mM NaN₃ (5 mM final conc during assay)
-0.296 g / 500 ml
- 2) Ouabain: (1 mM final conc during assay)
-33.13 mg dissolve in 25 ml Working Buffer each day,
MAKE FRESH EACH DAY takes about 15 minutes
-ensure pH is still 7.0 to 7.05 with KOH
-(0.5-1 mM will inhibit Na/K ATPase)
-enough for about 4 samples with repeats
- 3) 1 mM CaCl₂
-.074 g / 500 ml distilled H₂O
(store for 1 week in fridge, 4 weeks in freezer)
- 4) 100 mM ATP (5 mM final conc during assay)
-0.551 g / 10 ml H₂O, (Na⁺ salt) (frozen)
- 5) 0.5 M DTT
(frozen IN 500 µl aliquots)
- 6) 6% TCA (60 g / litre)
- 7) 12% TCA (120 g / litre)
- 8) Molybdate / H₂SO₄ solution
-20 g NH₃Mb dissolved in 54 ml H₂SO₄ (concentrated) and
100 ml H₂O. Make to 200 ml with H₂O when cooled
(Final conc = 885 mM)

9) FeSO_4 (2.5 g)

add last to a mixture of 5 ml Mb/ H_2SO_4 solution and 45 ml H_2O
(final conc Mb = 88.5 mM, FeSO_2 = 330 mM)

10) Standard 1 mM KH_2PO_4

-0.136 g / litre

standard curve 0.00 mM Pi 1 ml H_2O

0.10 mM Pi 900 μl H_2O + 100 μl standard

0.20 mM Pi 800 μl H_2O + 200 μl standard

0.30 mM Pi 700 μl H_2O + 300 μl standard

Label 4 - 3 ml **plastic** tubes with the above values for standards. Put standard solutions in (as above) with 1 ml 12% TCA (total 2 ml in each tube) and store in fridge

Label 16 - 3 ml **plastic** tubes per sample, with quadruples (4) of each of the following with the sample #;

Mg -No calcium added

Mg + -No calcium added, plus 10 mM DTT

Ca -Calcium added

Ca + -Calcium added, plus 10 mM DTT

The first two tubes will be for determining the Mg^{++} ATPase activity (with or without DTT) (background), and the second two tubes will be for determining the Ca^{++} ATPase activity (with or without DTT)

-Two tubes from each set are put in fridge, each containing 1 ml of 6% TCA, for a total of 8 tubes per sample, to be used later in the assay.

PROCEDURE:

set up

a) -prepare a heated water bath (37°C) and an ice bath with tube holders

-turn on centrifuge to 4° C, bring small buckets to lab and put in ice

-have spare ice bucket handy

-labeled tubes on counter near heated water bath

-working buffer with ouabain prepared (takes about 15 minutes to stir)

-ATP at 37°, CaCl_2 , DTT at room temperature near water bath

-12% TCA (1 ml per tube will be required) on ice near water bath

-measure out (for one sample)

-45 ml of H_2O

- 5 ml of Mb/ H_2SO_4 mixture

into small beaker and weigh out 2.5 gr of FeSO_4 . Do not mix yet.

-label cuvettes for each sample, including standard and have ready.

-turn on spec at 700 nm, 25° C

-S.R. samples thawed but on ice

-SR should be at about 1 $\mu\text{g} / \mu\text{l}$ in pH 6.8 BUFFER 3 (final suspension buffer TRIS 6.8). We will use about 50 μg of S.R. per tube (50 μl), therefore need 400 μg (or 400 μl). Save a bit for protein determination after the assay.

-pipettors near bath

50 μl for SR and ATP

1 ml for TCA

tips for each

-vortex near water bath

-stopwatch for timing reactions

- b) Add 550 μl working Buffer with Ouabain (solution 2) to each of the eight labelled tubes.
- c) Add 250 μl H_2O into two tubes marked Ca (RED)
230 μl H_2O into two tubes marked Ca + plus 20 μl DTT (RED and BLUE)
350 μl H_2O into two tubes marked Mg (NO MARK)
330 μl H_2O into two tubes marked Mg + plus 20 μl DTT (BLUE)
- d) Add 100 μl of 1 mM CaCl_2 solution into tubes marked Ca and Ca +

.....**VORTEX ALL TUBES**.....

START ASSAY:

- e) Vortex SR sample well initially, then every second sample or so to ensure adequate mixing. Pipet 50 μl SR into each tube at 15 second intervals, vortex, then put into water bath (Add buffer without SR into **Blank** tubes). Use fresh pipet tip for each new sample. Incubate each for 10 minutes
- f) Add 50 μl of ATP to each tube at 15 second intervals. Vortex and return to water bath. Incubate each for 10 minutes
- g) Add 1 ml 12% TCA to each tube at 15 second intervals. Vortex and put tubes into ice bath immediately. Transfer into chilled centrifuge buckets and centrifuge for 10 minutes at 3000 rpm.

- h) During spin, add the FeSO_4 to the Mb/ H_2SO_4 / H_2O mixture and stir. Remove tubes containing the 6% TCA from fridge and put on standards Krepice as well.
- i) Immediately after spin, remove 1 ml of supernatant and add to the TCA containing tubes. (rest can be discarded) Add 1 ml of Mb solution to each tube (on ice) including the standards and wait 15 minutes should be 2n minute water bath to bring temperature up for reading.
- j) Read at 700 nm

CALCULATIONS

- 1) Determine [Pi] produced from standard curve (in nmoles / 10 min)
- 2) Determine [protein] per μl using Biorad or Lowry
Determine protein (μg) used per tube ($50 \mu\text{l} \times [\text{protein}]$)
- 3) Calculate:

$$\frac{[\text{Pi}] \times 2}{10 \text{ min}} \times \frac{1000}{\mu\text{g protein per tube}}$$

nmoles only half convert to
10 min was used 1 min

(Short cut----- $[\text{Pi}] \times 200 / [\text{protein}]$)

UNITS: nmoles Pi / mg protein / minute
- 4) subtract Mg value from Ca value to get the $\text{Ca}^{2+}\text{ATPase}$ activity only

PART II

MYOCYTE ISOLATION PROCEDURE

BUFFERS:

	<u>Solute</u>	<u>Final</u> <u>Concentration</u>	<u>Stock/litre</u>	<u>ml of</u> <u>(M)</u>	<u>Stock Solution</u> <u>[gr/litre]</u>
<u>Buffer A:</u>	NaCl	130.0 mM	50	2.6	151.90
	KCl	5.0 mM		0.1	7.46
	MgSO ₄	1.2 mM		0.024	5.92
	Glucose	10.0 mM	10	1.0	45.04
	Na ₂ HPO ₄	1.37 mM	20	0.068	9.71
	NaH ₂ PO ₄	0.43 mM		0.021	3.34
	Hepes	5.0 mM	20	0.25	59.58
	Amino Acid mix (MEM-Gibco)		20		
	Taurine	60.0 mM	7.51 grams/litre		
	Creatine	20.0 mM	2.62 grams/litre		
	NaOH (5 N) to pH 7.4		≈ 2.1 ml		

Buffer B:

To 200 ml of Buffer A, add 1 ml of 0.4 M CaCl₂ stock to make 2 mM Ca⁺⁺ solution

Buffer C:

To 50 ml of Buffer A, add 50 mg Collagenase (Worthington CLS II) or about 700 units. Add 5 µl of 0.4 M CaCl₂ stock to bring [Ca] to 40 µM (to activate the collagenase)

Buffer D:

To 200 ml of Buffer A, add 3.0 g of BSA (Fraction V) to make 1.5 % W/V

PROCEDURE:

- 1) Use a constant volume Langendorff apparatus with four heated reservoirs arranged as shown. Put Buffers A, B, and C into the reservoirs while bubbling gently with 95% O₂ / 5% CO₂ and heat to 37° C. Buffer D is put into a 37° waterbath until later.
- 2) Remove heart from anesthetized rat (300 grams, 0.12 ml somnotol / 100 grams body weight) and mount onto a size 16 steel cannula (as described in Langendorff technique) and onto the perfusion apparatus. Initiate perfusion with **Buffer B** at 60 mm Hg for two minutes, or until the heart is thoroughly washed free of blood and is beating strongly. Ensure that temperature is 37° above the heart.
- 3) Switch to **Buffer A** (0 Ca⁺⁺) for two minutes to remove calcium solution from the heart. Then switch to **Buffer C** (collagenase) solution. Collect the Buffer C below the heart for recirculation. Perfuse with collagenase until digested; perfusion pressure (pp) should be about 50-60 mm Hg, with flow between 15-20 ml/min. This pp should rise, perhaps as high as 100 mm Hg, by about 5-7 minutes. If pressure attains higher than 100, reduce flow slightly. PP should drop by 12-15 minutes. If PP drops to less than 50mm, increase flow up to a maximum of 30 ml/min. PP will eventually drop low (eg 40 mm Hg at 30 ml/min) and heart may look brownish and flaccid (although my best isolations have come from very pink hearts). Switch to **Buffer A** again for 2 minutes.

OPTION: At 15 minutes, I add Ca⁺⁺ to the collagenase solutions, bringing concentration up to 1 mM. This appears to give a BETTER yield of calcium tolerant cells. See Haworth et al, 1989 Cell Calcium paper

- 4) Into a 50 ml beaker (siliconized) put 5-10 ml of warmed **Buffer D**. Remove heart (cutting atria off as well) into this beaker and mince for 1 minutes with small (4") scissors into app. 1 mm³ cubes. Cover and put into a 37 ° shaking (optional) water bath for 5 minutes.
- 5) Meanwhile, put 5 ml **Buffer D** with 5 ml **Buffer C** into a second beaker. After incubation period, disturb the partly digested tissue by drawing it back and forth into a 5 ml pipetter (with the tip cut off to allow a 5 mm orifice) for about 10 times, then strain the mixture through a 200 µm mesh into a 50ml centrifuge tube (polypropylene)

containing 20 ml of **Buffer D**, and return the residual tissue to the second beaker for an additional 5 minutes. Strain into a second 50 ml centrifuge tube with 20 ml of **Buffer D** and allow both tubes to settle (10 minutes) in the water bath.

6) Remove the supernatant and replace with 20 ml **Buffer D**. Allow to settle for 10 minutes, or centrifuge for 2 minutes at 40 G, then remove supernatant and relace with **Buffer A** (no BSA, as this will prevent cells from plating later on) Repeat this washing twice, replacing with **50/50 Buffer A / Buffer B** in the final wash.

7) Check both tubes and compare cell viability. If they are comparable, combine the contents of the tubes and replace the supernatant with 20 ml of **Buffer B** (Ca^{++}). These cells should now be calcium tolerant and ready to use. Replace the buffer every 30-60 minutes to reduce the level of cellular debris or toxic compounds.