ACTIONS OF OXYGEN-DERIVED FREE RADICALS AND REACTIVE OXYGEN SPECIES ON THE ORGANELLES OF FORCE PRODUCTION AND CALCIUM STORAGE IN CARDIAC MUSCLE.

A Thesis presented for the degree of

Doctor of Philosophy

by

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"Il vaut mieux remuer une question sans décider, que la décider sans la remuer."

Joseph Joubert, 1754-1824, "Pensées"

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Abbreviations

ADP	Adenosine Diphosphate.
ATP	Adenosine Triphosphate.
CABG	Coronary Artery Bypass Grafting.
cAMP	Cyclic Adenosine Monophosphate.
C_{max}	Maximum Calcium-Activated Tension.
CrP	Creatine Phosphate.
DAG	Diacylglycerol.
DIC	Differential Interference Contrast.
EGTA	Ethyleneglycol bis(β-aminoethyl ether) N,N,N',N' Tetraacetic acid.
GSH	Reduced Glutathione.
GSSG	Oxidised Glutathione.
H_2O_2	Hydrogen Peroxide.
HDTA	Hexamethylene N,N,N',N'-diamine Tetraacetic acid.
HOCl	Hypochlorous Acid.
IHD	Ischaemic Heart Disease.
IP_3	Inositol (1,3,5) Triphosphate.
O_2^-	Superoxide Anion.
P_i	Inorganic Phosphate.
PIP_2	Phosphatidylinositol 4,5 bisphosphate.
PTCA	Percutaneous Transluminal Coronary Angioplasty.
SOD	Superoxide Dismutase.
SR	Sarcoplasmic Reticulum.

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Technical assistance was provided by Dr Ian Montgomery in cutting sections for the electron microscopy work (chapter 6). Apart from this the experimental work contained within this thesis was undertaken by myself. None of the material has been previously presented for any other degree. Some of the results have been published during the period of study, details of which are given below.

Publications

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Summary

The work which comprises this thesis concerns the effects of oxygen-derived free radicals and reactive oxygen species on the intracellular organelles of force production and calcium storage in cardiac muscle. In addition, the possibility of endogenous histidine-containing compounds having antioxidant activity has been investigated. Preparations treated with Triton-X100 retain only traces of membrane structure while the contractile proteins remain functional. This form of treatment is ideal for investigating alterations to myofilament calcium sensitivity and maximum calcium-activated force production. Saponin-treated trabeculae retain the intracellular membranes associated with the sarcoplasmic reticulum and the mitochondria, while the sarcolemma is rendered permeable to the experimental solutions. This form of treatment is ideal for investigating alterations to sarcoplasmic reticulum and mitochondrial function.

Chapter 3 investigates alterations to the myofilament responsiveness to calcium due to actions of the superoxide anion in Triton-treated trabeculae. The results presented show that superoxide acts directly on the myofilaments to diminish their force producing ability, with no detectable change in myofilament calcium sensitivity. By studying the preparation in the rigor state it was established that superoxide acts on the active or detached crossbridge and the site of action is most probably on a part of the crossbridge that remains inaccessible in the attached state. The results also suggests that superoxide does not act like a non-specific protease to damage the structural integrity of the myofilament lattice.

Chapter 4 investigates alterations to the myofilament responsiveness to calcium due to actions of hydrogen peroxide and hypochlorous acid in Triton-treated trabeculae. The results presented show that hydrogen peroxide

has little effect of pathophysiological significance on the myofilaments. These findings support the conclusions in the previous chapter that superoxide, and not the downstream production of hydrogen peroxide, was responsible for the loss of force producing ability by the myofilaments. In contrast, however, hypochlorous acid produced significant effects at physiologically relevant concentrations with exposures as short as one minute. Exposing the preparation to hypochlorous acid resulted in an increase in both resting tension and myofilament calcium sensitivity, while maximum calcium-activated force was reduced. By studying the preparation in the rigor state it was established that hypochlorous acid was effective on both cycling and permanently attached crossbridges. The rise in resting tension associated with exposure to hypochlorous acid was unaffected by phosphate, pH or caffeine (after it had been established), but could be diminished by lowering pH prior to exposing the preparation to hypochlorous acid. These results suggest that rigor-like crossbridges are formed and these could account for the increase in calcium sensitivity observed.

Chapter 5 investigates the alterations to sarcoplasmic reticulum calcium handling by the superoxide anion, hydrogen peroxide and hypochlorous acid using saponin-treated trabeculae. The superoxide anion was shown to have no effect on caffeine-induced calcium release from the sarcoplasmic reticulum. Conversely, both hydrogen peroxide and hypochlorous acid effected caffeine-induced calcium release from the sarcoplasmic reticulum. The concentration of hydrogen peroxide required to elicit an effect is greater than that encountered physiologically, and is of little pathophysiological significance. However, the concentration of hypochlorous acid producing an effect was physiologically significant. The time course of the caffeine-induced calcium release suggests that, after exposure to hypochlorous acid, calcium release was slowed but the calcium uptake rate remained unaltered. Exposure to hypochlorous acid also appeared to increase the susceptibility to spontaneous

calcium release from the sarcoplasmic reticulum, but this was not a consistent finding.

Chapter 6 investigates ultrastructural abnormalities associated with exposing the preparations to the superoxide anion and hypochlorous acid. Additionally, the functional and ultrastructural effects of repetitive maximum calcium-activations was studied. Using the calcium jump method of activation, the only ultrastructural abnormality noted in the Triton-treated preparations was rupturing of the intercalated discs. The superoxide anion did not appear to have any ultrastructural effect on the trabecular preparation, whereas exposure to hypochlorous acid resulted in irregularities at the A-band/I-band junction and loss of a distinct M-line.

Chapter 7 investigates the functional significance of mitochondrial generation of the superoxide anion and hydrogen peroxide using saponin-treated preparations. Although the superoxide anion could be produced in functionally significant amounts in the experimental protocols employed. The *in vivo* significance of these findings remains debatable.

Chapter 8 investigates the antioxidant properties of pharmaceutical and endogenous substances. A variety of *in vitro* techniques have been employed to assess the free radical scavenging actions of captopril and endogenous histidyl dipeptides (*e.g.* carnosine and homocarnosine). Such techniques include the skinned-fibre preparations, chemiluminesence of activated neutrophils and spectrophotometric assays. Captopril was able to ameliorate the effects of hypochlorous acid in Triton-skinned fibres at concentrations which would be relevant *in vivo*. The data presented within this chapter could be regarded as evidence in favour of an anti-oxidative function for the histidyl dipeptides. However, further investigation will be required to ascertain the importance of such activities *in vivo* and the possibility of interaction between the compounds resulting in synergistic free radical scavenging.

Chapter 1: General Introduction

Ischaemic Heart Disease

The incidence of ischaemic heart disease (IHD) in industrially developed countries has risen rapidly over the last forty years. Of the 646,181 deaths within the United Kingdom during 1991, 171,179 (approximately 26%) were attributed to IHD (figures supplied by the BHF/British Cardiac Society working party). The condition is a consequence of inadequate blood flow to the myocardium through the coronary circulation. Two important factors that determine the outcome of patients suffering from this condition are *i.* the extent to which myocardial blood flow is decreased, and *ii.* the rate at which blood flow is diminished *i.e.* acutely or progressively.

Slowly progressive coronary insufficiency is usually a consequence of atherosclerosis, with gradual intimal thickening and formation of atheromatous plaques in the major coronary arteries. As this occurs, the gradually increasing hypoxia induces development of a rich collateral circulation which, in many instances, prevents the onset of symptoms despite the presence of severe atherosclerotic lesions. The extensive development of a collateral circulation in older patients with long standing atherosclerosis probably explains why sudden and complete occlusion of a coronary artery is more likely to be fatal in younger than older people. A similar extensive collateral circulation develops in normal individuals who take regular exercise and the incidence of IHD is lower in such individuals than in those who have a more sedentary existence.

Myocardial infarction and reperfusion therapy

Complete and prolonged occlusion of a coronary artery results in myocardial infarction *i.e.* necrosis of the region of myocardium supplied by that artery. Almost half of the people who have a first myocardial infarction die as a result of it. Survival after a myocardial infarction is inversely related to the size of the infarct (Geltman, Ehsani, Campbell, Schechtman, Roberts and Sobel, 1979). Experimental studies have demonstrated that early reperfusion (within 20 minutes of the onset of the ischaemic

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episode) limits infarct size (Reimer, Lowe, Rasmussen and Jennings, 1977). Observations such as these have lead to an exponential increase in interventional recanulization as one of the fundamental strategies in the management of IHD. Coronary Artery Bypass Grafting (CABG) and Percutaneous Transluminal Coronary Angioplasty (PTCA) have been utilised to slow the progression of IHD, while "clot-busting drugs" (thrombolytic therapy) and emergency CABG and PTCA have been used to limit the extent of myocardial infarction. However, although reperfusion halts the progression of ischaemic injury, it predisposes to a form of post-ischaemic myocardial dysfunction which has been termed "myocardial stunning" and may promote chronic heart failure with its attendant morbidity and mortality (Braunwald and Kloner, 1982). Myocardial stunning is the mechanical dysfunction that persists after reperfusion *despite the absence of irreversible damage*. The importance of a clear definition of myocardial stunning is crucial since the terminology is often misapplied to persistent contractile abnormalities in infarcted tissue. The key point to this definition is that myocardial stunning, no matter how severe or attenuated, is *fully reversible*.

In a recent review, Bolli (1990) suggested that myocardial stunning is a multifactorial process involving a complex sequence of cellular perturbations and the interaction of multiple pathogenic mechanisms. Among the numerous mechanisms proposed to account for stunning Bolli suggested three that appear to be the most plausible: *i*. The generation of oxygen-derived free-radicals and reactive oxygen species, *ii*. Calcium overload, and *iii*. Disruption of excitation-contraction coupling.

Such mechanisms are not mutually exclusive and in this review Bolli speculated that oxidants may damage the proteins of the contractile machinery in a manner that reduces their responsiveness to calcium. Therefore, the work which comprises this thesis directly addresses this speculation and investigates the effects of free radicals and reactive oxygen species on the myofilaments directly. Further alterations to excitation-contraction coupling are investigated using *in situ* sarcoplasmic reticulum preparations to simultaneously measure calcium-release and tension generation. Understanding the

mechanisms underlying the pathogenesis of myocardial stunning could allow development of treatment strategies, for this phenomenon, which may significantly delay any benefits that may arise from reperfusion therapy. Pharmaceutical and endogenous compounds have been investigated as possible treatment strategies to ameliorate myocardial stunning. However, before discussing the oxidative processes involved during ischaemia-reperfusion cycles, it is important to consider the processes involved in the development of ischaemia.

Disruptions to excitation-contraction coupling during an ischaemic episode

The purpose of this section of the introduction is not to review extensively the pathogenesis of ischaemia (see Janse and Kleber, 1981; Allen and Orchard, 1987 for reviews of this topic), but rather to consider the functional, metabolic, ionic and hormonal consequences of an ischaemic episode and to assess their contribution to the phenomenon of myocardial stunning.

The effects of ischaemia, anoxia and metabolic blockade on mechanical function of the heart are well recognised (Eisner, Elliot and Smith, 1987; Koretsune, Correti, Kusuoka and Marban, 1991; Elliot, Smith, Eisner and Allen, 1992). Brief (10 minute) episodes of global ischaemia can completely abolish left ventricular developed pressure. Such depressed contractility is associated with a decrease in intracellular creatine phosphate concentration $[CrP]_i$, an increased inorganic phosphate concentration $[P_i]$ and an increased adenosine diphosphate concentrations $[ADP]_i$, but with no change in intracellular adenosine triphosphate $[ATP]_i$ concentration. Intracellular pH falls linearly by *circa* 0.2 pH units every five minutes of ischaemia (Elliot *et al*, 1992). However, if hearts are glycogen depleted $[ATP]_i$ can decrease by 50% during the first ten minutes of that ischaemic episode. In such cases $[ATP]_i$ is undetectable after 70-90 minutes of the ischaemic episode and the concomitant acidosis is less pronounced than in ischaemic hearts that have not been glycogen depleted.

These changes in $[ATP]_i$, $[CrP]_i$, $[ADP]_i$, $[H^+]_i$ and $[P_i]$ will each have pronounced effects on the elements of excitation-contraction coupling. Increasing $[H^+]_i$ and $[P_i]$ during the ischaemic period will depress the responsiveness of the myofilaments to calcium (Fabiato and Fabiato, 1978a; Kentish, 1986, 1987; Mekhfi and Ventura-Clapier, 1988). Low pH reduces calcium-binding by a direct effect on troponin-C, whereas $[P_i]$ has been suggested to decrease the number of actively cycling crossbridges (Kentish and Palmer, 1989). However, repulsive forces between fixed negative charges on the thick and thin filaments are important in the determination of myofilament lattice spacing (Rome, 1967). Consequently, increasing the $[H^+]_i$, and the $[Mg^{2+}]_i$ which is elevated during ischaemia (Murphy, Steenburgen, Levy, Raju and Loudon, 1989; Borchejrevink, Bergan, Bakoy and Jynge, 1989), should reduce these repulsive forces and lattice spacing, giving rise to an increase in calcium sensitivity. X-ray diffraction studies have confirmed such an effect in skeletal muscle (April, Brandt and Elliot, 1972) and further shown that force is inhibited by disruptions to the thin filament arrangement which outweighs the effect on lattice spacing (Matsuda and Podolsky, 1986). However, it is important to remember that an increase in $[ADP]_i$ potentiates maximum calcium-activated force (C_{max}) and calcium sensitivity (Godt and Nosek, 1989).

A decrease in calcium release from the sarcoplasmic reticulum (SR) could also contribute to the fall in contractile force during ischaemia. In the normal myocardium it has been estimated that over 90% of the calcium released into the myoplasm is bound to troponin-C (Bers, 1991). However, lowering the affinity of troponin-C for calcium by increasing $[H^+]_i$ will cause an increase in the free $[Ca^{2+}]_i$; and this has been proposed to account for the elevated $[Ca^{2+}]_i$ that occurs during ischaemia (Lee, Mohabir, Smith, Franz and Clusin, 1988; Allen, Lee and Smith, 1989). This alteration to the intracellular calcium-buffering makes interpretation of $[Ca^{2+}]_i$ transients difficult.

Fabiato and Fabiato (1978) demonstrated that acidosis decreases SR calcium-uptake (so that the subsequent calcium-release was diminished). Additionally,

the process of calcium-induced calcium-release was desensitized at low pH. Also, increased $[P_i]$ and $[ADP]_i$ decreases the calcium-content of the SR in saponin-treated rat ventricular trabeculae, probably by inhibiting calcium-uptake (Smith and Steele, 1992). Such results suggest that ischaemia will decrease SR calcium-release and as such could contribute to decreased contractility.

Various changes are observed in cardiac action potentials during ischaemia (see Janse and Kleber, 1981). However, it is generally accepted that there is a decline in both the duration and the amplitude of the action potential during the ischaemic period (Downer, Janse and Durer, 1977; Russel, Smith and Oliver, 1979). Such changes could clearly reduce sarcolemmal calcium-influx and subsequently SR calcium-release. The extracellular potassium accumulation associated with ischaemia is most probably responsible for the alterations to the cardiac action potential ($[K^+]_i$ tends to leak passively from the cell requiring active pumping to be maintained in the myoplasm). Ion homeostasis will be disrupted when the $[ATP]_i$ falls to a level below that sufficient to maintain the membrane ion pumps. However, cellular calcium overload has been noted early in reperfusion (see Marban, 1991; Kusuoka and Marban, 1992 for review) and long before a significant fall in $[ATP]_i$. It is possible that the calcium-overload results from the $[H^+]_i$ accumulation associated with ATP hydrolysis and lactate production. This would tend to cause sodium overload (via the Na-H exchange system) which will, in turn, lead to calcium overload (via the Na-Ca exchange mechanism). The time course of such an ion pump mechanism to regulate pH_i would be of 10-20 minutes and would be consistent with the time course of the $[Ca^{2+}]_i$ rise. However, the measured increases to $[Ca^{2+}]_i$ have to be interpreted against the alterations to cellular calcium-buffering capacity discussed earlier.

In addition to the metabolic consequences of ischaemia, the reduction in contractility results in an increase to adrenergic drive (Reimmersma, 1982). Two distinct classes of adrenoreceptor exist, termed as α and β. Agonists binding to either type of receptor cause an increase in $[Ca^{2+}]_i$ and the force of contraction. The intracellular events

underlying these responses are, however, quite different. An increase in inositol (1,3,5) trisphosphate concentration [IP$_3$] has been measured in cardiac tissue after α-adrenoreceptor stimulation. An increase in [IP$_3$] may enhance the calcium-sensitivity of the calcium-induced calcium-release mechanism (Nosek, Williams, Zeigler and Godt, 1987). A key feature of this signalling system is that the second messengers are generated by hydrolysing the lipid phosphatidylinositol-4,5,-bisphosphate (PIP$_2$) which is located in the sarcolemma. Agonist binding to the receptor stimulates a phosphoinositidase which cleaves PIP$_2$ into IP$_3$, that is released into the myoplasm, and diacylglycerol (DAG). The DAG can subsequently be hydrolysed by a DAG lipase to form arachidonic acid or phosphorylated to phosphatidic acid by DAG kinase.

β-adrenoreceptor stimulation has been demonstrated to increase cyclic adenosine monophosphate (cAMP) catalysed by adenylate cyclase (Tsien, 1977). This second messenger activates cAMP-dependant protein kinases which phosphorylate various intracellular proteins including troponin-I as well as membrane proteins on the SR and the sarcolemma. cAMP has been suggested to increase calcium-accumulation by the SR (Steele and Miller, 1992) in saponin-treated trabeculae and the calcium-influx via sarcolemmal channels in cardiac myocytes (Osterrieder, Brum, Hescheleller, Trautwein, Flockerzi and Hoffman, 1982). The possibility of a cAMP-induced depression in calcium-sensitivity of the myofilaments directly remains equivocal (Steele and Miller, 1992).

Despite great interest and active investigation the causes of ischaemic contractile dysfunction remain incompletely understood. Much of the evidence discussed above suggests that the depressed contractility during ischaemia is largely due to accumulation of intracellular metabolites (particularly P$_i$ and H$^+$). The metabolic consequences of ischaemia revert relatively rapidly (within an hour or so, GL Smith - personal communication), whereas myocardial stunning is a post-ischaemic condition characterised by diminished myocardial contractility which may last for hours, days or even months. The protracted nature of the stunned myocardium persists longer than the metabolic

consequences of the antecedent ischaemia and would suggest a different or, at least, an additional pathogenic mechanism.

What are free radicals and reactive oxygen species and where do they come from?

A free radical is any species capable of independent existence containing one or more unpaired electron in its outer orbital. Radicals can be formed by loss or gain of a single electron from a non-radical species; and are easily formed when a covalent bond is broken by homolytic fission.

Among the potential mechanisms for generation of free radicals in the myocardium, only two have been extensively explored: Allopurinol, a xanthine oxidase inhibitor, has been shown to promote functional recovery in the stunned myocardium when infused prior to 15 minutes of ischaemia and 4 hours of reperfusion in the dog (Charlat, O'Neill, Egan, Abernethy, Michael, Myers, Roberts and Bolli, 1987). Such data suggest that xanthine oxidase, which catalyses the oxidation of a number of substrates to uric acid producing the superoxide anion (O_2^-) as a by-product, contributes to myocardial stunning in dogs, but reports on the myocardial content of xanthine oxidase in human myocardium are inconsistent (Jarasch, Bruder and Heid, 1986; Eddy, Stewart, Jones, Engerson, McCord and Downey, 1987; Muxfeldt and Schaper, 1987; Huizer, de Jong, Nelson, Czarnecki, Serryus, Bonnier and Troquay, 1989; Grum, Gallagher, Kirsch and Schlafer, 1989).

On activation, which is associated with an increase in oxygen uptake, neutrophils release oxidants which serve as anti-microbial agents, but at the same time these might cause injury of the surrounding native tissue (Fantone and Ward, 1982; Baboir, Kipnes and Curnette, 1973; Rowe, Eaton and Hess, 1984). The superoxide anion is produced as a result of oxygen interacting with electrons released from a leucocyte enzyme system, associated with the plasma membrane, which oxidises NADPH to $NADP^+$. Hydrogen peroxide (H_2O_2) is rapidly formed by the dismutation of O_2^- and, although not a radical

by definition, this reactive oxygen species is a strong oxidising agent that can undergo homolytic fission to produce the hydroxyl radical. The enzyme myeloperoxidase is present within the neutrophil vacuole and is released from the neutrophils during phagocytosis. This enzyme can participate in the killing mechanism by oxidising chlorine into hypochlorous acid (HOCl) which is highly reactive and can oxidise many biological materials, especially thiols. In addition to these two mechanisms free radicals and reactive oxygen species may be produced by the mitochondria, the arachidonic acid cascade, drug toxicity and the auto-oxidation of catecholamines.

Mitochondrial generation of free radicals and reactive oxygen species

Probably the most important source of O_2^- *in vivo* in most aerobic cells is the electron transport chain of mitochondria. An important function of animal mitochondria is the oxidation of NADH and $FADH_2$ produced during the Krebs cycle, β-oxidation of fatty acids and other metabolic pathways. Oxidation is achieved by an electron transport chain located in the inner mitochondrial membrane (see chapter 7 for further details and relevant references). The energy released by oxidation is used to drive ATP synthesis. For every four electrons fed into the cytochrome oxidase complex a molecule of oxygen is reduced to two molecules of water. Cytochrome oxidase releases no detectable oxygen radicals into free solution *i.e.* it has evolved in such a way that all the radical intermediates of oxygen reduction remain firmly bound to the proteins. However, unlike cytochrome oxidase, some other components of the electron transport chain do 'leak' some electrons onto oxygen whilst passing the great bulk of them onto the next component in the chain. As would be expected from the chemistry of oxygen, this leakage produces a univalent reduction to give O_2^-. The main sites of leakage seem to be the NADH-coenzyme Q reductase complex, and the reduced forms of coenzyme Q itself. Mitochondria isolated from several animal tissues have been shown to produce H_2O_2 *in vitro* mostly resulting from the dismutation of the superoxide anion by mitochondrial superoxide dismutase (SOD) activity. Mitochondria possess manganese SOD in the matrix, and may have a little copper-zinc SOD in the space between the inner and outer membranes. The possible

pathogenic significance of mitochondrial generation of H_2O_2 and O_2^- is discussed in chapter 7.

The arachidonic acid cascade

The prostaglandins and related arachidonic acid metabolites are now recognised as potent biologically active substances that play a vital role in myocardial ischaemia and infarction (see Bowman and Rand, 1984). The metabolism of arachidonic acid by prostaglandin synthetase or by cyclooxygenase leads to a host of both inflammatory and anti-inflammatory products by insertion of molecular oxygen which leads to ring formation. Oxygen insertion takes place by a stereo-specific free radical mechanisms at the active sites of the enzymes. Release of arachidonic acid from membrane phospholipids occurs as part of the phenomenon of 'phosphatidylinositol turnover' as previously mentioned and is stimulated by increasing $[Ca^{2+}]_i$. Once arachidonic acid is available, the cyclooxygenase acts upon it to form two hydroperoxides (PGG_2 and PGH_2). If cyclooxygenase is treated with glutathione and glutathione peroxidase (a free radical scavenging system) to remove traces of lipid peroxides then arachidonic acid is not immediately oxidised on addition of the enzyme. Adding PGG_2 or other hydroperoxides (including H_2O_2) can abolish this lag period. Therefore, there appears to be an intimate relationship between non-enzymic lipid peroxidation and arachidonic acid metabolism.

PGG_2 is converted to PGH_2 by a peroxidase that is similar to cyclooxygenase, but is non-specific and co-oxidises a variety of substances while reducing hydroperoxides in a similar way to horseradish peroxidase and myeloperoxidase. *i.e.* oxidation by such 'non-specific' proteases can be represented by the following series of reactions:

$$peroxidase + H_2O_2 \quad \rightarrow \quad compound\ I$$

$$compound\ I + SH_2 \quad \rightarrow \quad SH^\bullet + compound\ II$$

$$compound\ II + SH_2 \quad \rightarrow \quad SH^\bullet + peroxidase$$

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SH_2 is the substrate to be oxidised. The iron in the haem ring of the peroxidase is in the Fe^{3+} state until H_2O_2 removes two electrons to give compound I. The exact structure of this is unknown but is thought to be in the Fe^{4+} state with the extra oxidising capacity located elsewhere on the active site (Kontos, Wei, Ellis, Jenkins, Povlishock, Rowe and Hess, 1985). These two electrons are replaced in one-electron steps in each of which a substrate molecule forms a substrate-derived radical ($SH^{\cdot}$) which usually undergoes a disproportionation reaction in which one molecule is reduced and the other oxidised (*i.e.* 2 x $SH^{\cdot}$ forms $S + SH_2$) but can react with oxygen to form O_2^{-}. Compound II is the intermediate state of the enzyme.

Both PGG_2 and PGH_2 are unstable, having a half-life of minutes in physiological conditions. They can, in theory, exert profound biological effects (including contraction of smooth muscle and stimulation of platelet aggregation) if they are not transformed to other metabolites of arachidonic acid such as PGE_2, PGD_2, PGF_{2a} and Thromboxane A_2. While a detailed review of the tissue-specific complexities of prostaglandin biosynthesis is not appropriate here, it should be mentioned that factors, such as ADP, released by platelets have been claimed to enhance O_2^{-} production by neutrophils.

In addition to the cyclooxygenase pathway for metabolism of arachidonic acid there is the lipoxygenase enzyme. Platelet lipoxygenase acts upon arachidonic acid to form 12-hydroperoxy-5,8,11,14-eicosatatraenoic acid (12-HPETE). This compound is unstable and quickly forms the 12-hydroxy derivative (12-HETE) *in vivo*. This reduction appears to be achieved by platelet glutathione peroxidase. The HPETE compounds are precursors of a range of biologically potent compounds known as the leukotrienes. This pathway is particularly important in neutrophils as LTB_4 is a powerful chemotactic agent which promotes aggregation, degranulation and the respiratory burst. Thus neutrophils generate a mediator to attract more cells to the site of inflammation and promotes their activation. In addition these compounds can increase vascular permeability.

Auto-oxidation of catecholamines

The catabolism of catecholamines proceeds through two major pathways involving the mitochondrial enzyme monoamine oxidase and catechol-*o*-methyltransferase in addition to the minor pathway catalysed by phenolsulfotransferase (Kopin, 1985). However, a non-enzymic pathway should also be taken into account since the catechol moiety can undergo oxidation. The basic oxidation process for catecholamines was proposed many years ago by Raper to explain the mechanism of melanin formation (Raper, 1928).

Catechol (1,2-dihydroxybenzene) is a phenol and is susceptible to oxidation (normally termed auto-oxidation). However, under the conditions likely to be encountered in the physiological environment (*i.e.* air-saturated solutions, *circa* pH 7 and less than 10^{-2}M phenolic material) auto-oxidation is thermodynamically unfeasible (Wradman, 1989) and the equilibrium of the first reaction in auto-oxidation is markedly in favour of the reactants. At physiological pH, most phenols exist in the protonated form and, although deprotonation shifts the equilibrium towards the oxidation products, the reactants are still favoured. Despite the small fraction of phenol converted to oxidation products at equilibrium, auto-oxidation will proceed if these products are removed from equilibrium by their participation in other reactions. An example of this is provided by homogenistic acid (2,5-dihydroxyphenyl acetic acid), whose auto-oxidation in the synovia of patients suffering from alkaptonuria is believed to cause inflammatory arthritis (Martin and Batkoff, 1987) and which is accelerated by the addition of SOD. Superoxide dismutase effectively removes O_2^- from the equilibrium in the first reaction and moves the equilibrium in a second reaction (involving the *para*-semiquinone radical to form the quinone and O_2^-) towards the products (Butler and Hoey, 1986; Mukerjee, Land, Swallow, Guyan and Bruce, 1988).

However, contrary to this effect SOD actually inhibits the auto-oxidation of several reduced quinones, apparently by multiple mechanisms (Bandy, Moon and

Davison, 1990; Cadenas, Mira, Brunmark, Lind, Segura-Aguilar and Ernster, 1988; Ollinger, Buffington, Ernster and Cadenas, 1990; Brunmark and Cadenas, 1988). Adrenaline is a well-known example of the inhibition exerted by SOD (Misra and Fridovich, 1972) and a mechanism for this inhibition has been postulated (Deeble, Parsons, Phillips, Schuchmann and von Sonntag, 1988).

A striking difference in the chemistry of catechols and 1,4-dihydroxybenzenes is the ability of their respective quinones to react with oxygen. The 1,4 semiquinone (*para*-semiquinone), as already mentioned, reacts with oxygen while catechol semiquinones appear to react with much lower rate constants (Kalyanaraman, Korytowski, Pilas, Sarna, Land and Truscott, 1988). It has been proposed that their low reactivity is due to the semiquinone species being stabilised by an intramolecular hydrogen bond (Mukerjee, 1987). On the other hand, catecholamines such as 6-hydroxydopamine and 6-aminodopamine are readily oxidised at physiological pH with a large production of O_2^- and H_2O_2 (Gee and Davison, 1989). Their oxidation appears to be enhanced by the 6-hydroxy group and leads to an *ortho*-hydroxy-*para*-quinone which does not undergo cyclization like simple catecholamines (Adams, Murrell, McCreery, Blank and Karolczak, 1972).

Adriamycin-induced cardiotoxicity

In addition to the endogenous free radical generating mechanisms described above some forms of drug toxicity have been associated with free radical generation (see Singal, Deally and Weinberg, 1987 for review). Adriamycin is a highly effective chemotherapeutic agent utilised in the treatment of solid tumours, leukaemia's and lymphomas. It is an anthracycline antibiotic and is reported to form complexes with DNA and inhibits DNA replication and RNA synthesis. However, patients on long term therapy have developed a dilated cardiomyopathy that often resulted in death after an apparent tumour cure (Bristow MR, 1982; Findlay, 1988).

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The direct effect of the drug on DNA would not appear to be applicable to the induction of cardiomyopathy, since DNA turnover in the adult myocyte is minimal. A second mechanism for cardiotoxicity was suggested when it was demonstrated that adriamycin can be activated to the semiquinone radical by cytochrome P-450 reductase. This enhances NADPH oxidation, oxygen consumption and the production of O_2^- (Bachur, Gordon and Gee, 1977; Minnaugh, Gram and Trush, 1983; Doroshow, 1983). On this evidence several investigators have investigated the effects of antioxidants on the cardiomyopathy induced by adriamycin. The use of acetylcysteine, coenzyme Q and α-tocopherol have ameliorated the negative inotropic effects and electron microscopic evidence of adriamycin-induced cardiomyopathy, while significantly improving mortality and without affecting the anti-tumour activity of the drug (Myers, McGuire, Liss, Ifrim, Grotzinger and Young, 1977; Doroshow, Locker, Ifrim and Myers, 1981; Ohhara, Kanaide and Nakamura, 1981).

The role of free radicals and reactive oxygen species in myocardial stunning

The ability of free radicals and reactive oxygen species to depress myocardial function has been directly demonstrated *in vitro* and *in vivo*. Exposure of isolated rabbit septa (Burton, McCord and Ghai, 1984), isolated rat (Shattock, Manning and Hearse, 1982) or rabbit hearts (Goldhaber, Lamp and Weiss, 1988) to free radical generating systems has uniformly resulted in decreased mechanical function and ATP levels (*i.e.* changes similar to those observed in the stunned myocardium). The severity of post-ischaemic myocardial dysfunction has been suggested to be determined primarily by the severity and duration of the antecedent ischaemia (Bolli, Zhu, Thornby, O'Neill and Roberts, 1988). Whatever the mechanisms responsible for this form of dysfunction they must be initiated and modulated by perturbations associated with ischaemia.

Production of free radicals and reactive oxygen species has been directly demonstrated *in vitro* using isolated rabbit or rat hearts during global ischaemia and

reperfusion (Garlick, Davies, Hearse and Slater, 1987; Krammer, Arroyo, Dickens and Weglicki, 1987; Zweier, Flaherty and Weisfeldt, 1987; Baker, Felix, Olinger and Kalyanaraman, 1988; Zweier, 1988). The use of the spin trap α-phenyl N-*tert*-butyl nitrone (PBN) and electron paramagnetic spectroscopy has detected free radical production in an *in vivo* model of post-ischaemic myocardial dysfunction (regional myocardial occlusion in open-chest dogs). After infusion of PBN, characteristic signals of radical adducts were detected in the venous drainage from the myocardium. Thus, myocardial production of free radicals begins during coronary occlusion and increases dramatically on reperfusion with a peak radical production achieved around three minutes of reperfusion). After the initial burst the production of radicals diminishes but does not cease and persists for as long as three hours after reperfusion.

A linear, positive relationship exists between the magnitude of radical production and the flow reduction during the ischaemic episode. Therefore, the intensity of free radical generation on reperfusion can be determined by the preceding ischaemic episode (Bolli and McCay, 1990; Bolli, Patel, Jeroudi, Lai and McCay, 1988). However, it is not necessary to initiate antioxidant therapy (which will be discussed in chapter 8) prior to the onset of ischaemia. The time course of free radical-induced damage in post-ischaemic myocardial dysfunction appears to be that the substantial portion of cellular damage occurs early on in reperfusion. Such a time course may have therapeutic implications since in the clinical situation antioxidant therapies would require to be established after the onset of ischaemia but could still be effective in attenuating post-ischaemic dysfunction.

Chapter 2: Materials and Methods

This chapter describes the general protocol followed for the majority of experiments described within this thesis. However, specific details of some experimental conditions and techniques used have been described within the appropriate chapters.

Preparation mounting for force measurement

Most experiments were performed on free-running trabeculae isolated from the right ventricle of male Wistar rats (200-240g) which were killed by stunning and cervical dislocation. The heart was rapidly excised via a sternotomy and placed, still beating, in Ringer's solution (composition: 150mM NaCl, 5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 5mM HEPES at pH7.0). The right ventricle was opened by a single cut, close to the interventricular septum, running from the base of the heart to the apex. Preparations ranged from 1-3mm in length and 60-140μm in diameter and were mounted for isometric force measurement.

The preparation was suspended between a fixed point and an Akers AE 875 force transducer secured by nylon monofilament snares. The snares ran within stainless steel tubing (Goodfellows Metals Ltd, Cambridge; o.d. 200μm, i.d. 100μm) and were formed by double threading a 3-4cm length of the stainless steel tube with 25μm diameter monofilament so that a snare was produced at the base of the tube. This could be tightened by pulling the monofilament from the top of the tube. Additional rigidity was provided to the system by further pieces of tubing glued to those forming the snares (as shown in figure 2.1). The compliance of this system has previously been measured and corresponds to a length change of much less than 1% (for a preparation of 2mm in length and producing 60mg.wt.). The assembly was mounted on a Narashige MM3 micomanipulator. This allowed fine movement in three planes, facilitating accurate positioning of the muscle within both the bath change system and the microscope chamber. The force transducer was supported by a second micromanipulator which allowed fine adjustment of the separation between the tubing carrying the snares, thus allowing adjustment of sarcomere length of the preparation.

Transducer

Fixed
Point

Snared Ends
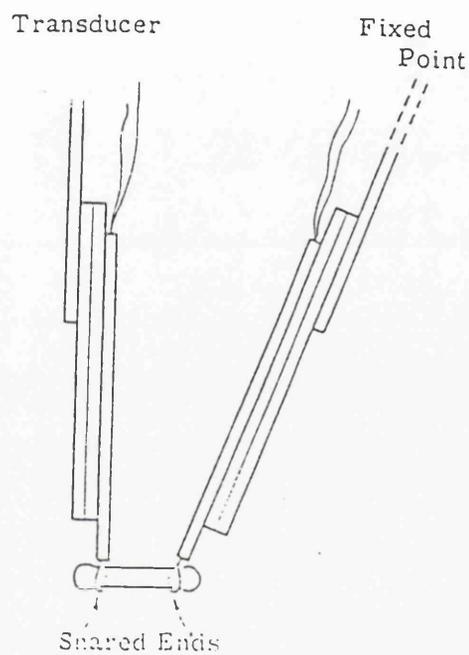
Figure 2.1 Schematic showing the system for mounting the muscle. The trabecula is held by snares running in stainless steel tubes. The tubes are glued to extensions from the force transducer and a fixed point. The seperation between the preparation ends is mounted on a magnetic base to permit transfer from the dissection microscope to the bath-change system and the DIC microscope.

Measurement of sarcomere length

It is crucial to measure accurately and consistently sarcomere length in experiments such as these, since a number of physiological mechanisms are influenced by sarcomere length, particularly calcium-sensitivity. In preference to the laser diffraction system used in most other laboratories a modified Vickers M-16 microscope with Differential Interference Contrast (DIC) optics (Smith, 1969) was used to examine the preparation and set sarcomere length to the desired value (generally 2.1-2.2μm). The microscope lies supine, its stage being replaced by a mounting for the transducer assembly and an observation chamber (so that the preparation can be lowered into the optical path of the microscope, Figure 2.2). The preparation was viewed on a television monitor via a video camera which further enhanced the contrast of the DIC image. A graticule placed in the video camera mounting tube allowed for accurate measurement of the preparation from the monitor.

Automated solution change system

Many of the experiments described in this thesis required accurate control of the duration of exposure to the experimental solutions. For this reason a computer-controlled solution change system was employed (Miller, Sinclair, Smith and Smith, 1982). Solutions were contained within a series of wells cut into a perspex block and were continuously stirred by a small stainless steel paddle driven by an electric motor. Together with mixing the solutions, stirring maintained the solutions saturated with air which was important in the work described later. A solution change was made by lowering this block, moving it horizontally under the preparation and raising it to immerse the preparation in the required new solution. Horizontal and vertical movements of this bath were made by two stepper motors under control of an Apple IIe computer. The speed of these stepper motors was such that a solution change could if necessary be made within 200ms (generally about 1 sec was adopted as a suitable time). The brief exposure to air was without effect on the preparation. The software accompanying this system

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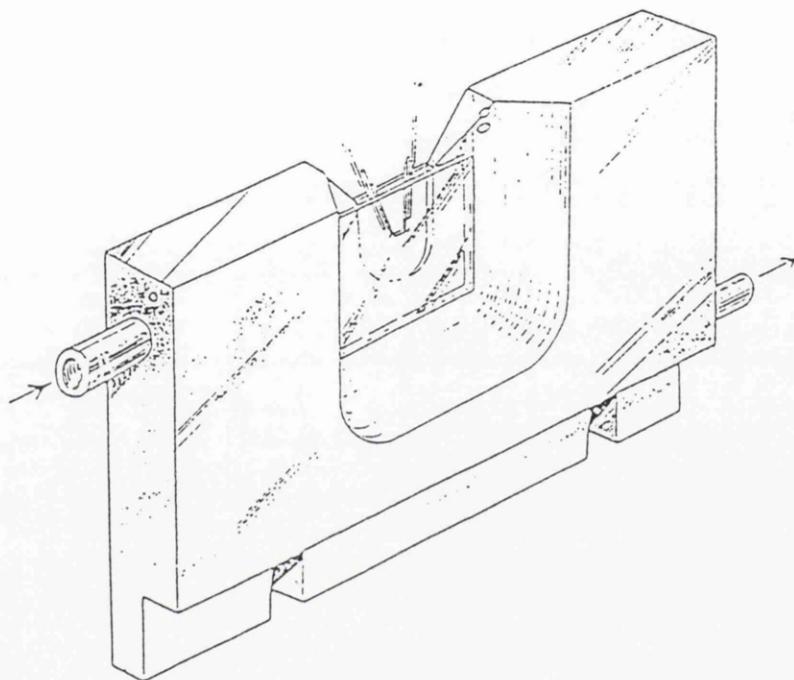
Figure 2.2 Schematic of the chamber used to determine and permit adjustment of sarcomere length. The bath (vol. 0.3ml) is created by two standard coverslips approximately 2mm apart. This permits access from above for the preparation mounting assembly. The temperature of the chamber can be controlled by flushing the brass housing.

allowed the programming of a large number of accurately timed solution changes. Amongst the many advantages of this solution change system is a reduction of 'carry-over' and subsequently contamination of the new experimental solution. Several checks confirm that, in almost every case, solution 'carry-over' adhering to the preparation is trivial. Contamination, for example, of the 'rigor-inducing' solutions by even very small amounts of ATP can have a pronounced effect on force development.

In some other experiments described in this thesis, specifically those in which simultaneous measurements of calcium and tension were made, the preparation was continuously perfused. In such experiments a solution change was made by exchanging the perfusing solution, but such rapid solution changes could not be obtained. The system utilised for such experiments will be described later in this chapter.

Solution composition

The rationale for solution composition, the method for calculating free ion levels and ionic strength, the choice of ion binding constants for the various ligands, precautions for EGTA purity, calcium contamination determination, the measurement of pH and other details of the experimental solution are described in detail elsewhere (Harrison, Lamont and Miller, 1988; Miller and Smith, 1984). However, a brief description of the main concepts dictating the solution composition are described below.

The components and their respective concentrations of experimental solutions are given in detail in Table 2.1. These solutions were made by mixing the appropriate volume of the following stock solutions, which were prepared on a regular basis: 1M KCl, 1M $MgCl_2$, 100mM EGTA, 100mM CaEGTA, 100mM HDTA and 500mM HEPES. The EGTA and HDTA were dissolved by adding twice the molar amounts of KOH, heating to 50-60°C and stirring before adding distilled H_2O to bring to the desired volume. CaEGTA was prepared in a similar way except that $CaCO_3$ was added to the EGTA and KOH. Prior to and on heating CO_2 is driven off leaving CaEGTA. ATP and

CrP were added in solid form, as sodium salts, and then the solution was titrated to pH_a 7.0 with KOH. ATP, CrP and all other chemicals were obtained from the Sigma Chemical Company, Poole, Dorset, UK., unless otherwise stated. Solutions with different calcium concentrations were obtained by mixing solutions A and B (Table 2.1). Calcium chloride was added to aliquots of solution B (Table 2.1) to achieve pCa 4.00 which, under our standard conditions, ensures maximal activation. To make the activation as rapid and uniform as possible throughout the cross-section of the preparation the 'calcium-jump' method was used. This method produces a rapid increase in $[Ca^{2+}]$, across the whole preparation radius, by increasing the calcium buffer concentration at the same time as the free calcium concentration (Miller, 1975; Moisescu, 1976; Ashley and Moisescu, 1977). *i.e.* the preparation is moved to the test pCa solution after prior equilibration in solution E (Table 2.1), which has its ionic balance (0.2M) maintained by addition of HDTA or KCl.

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Soln	K^{+*}	$Mg^{\dagger}$	ATP	CrP	$Na^{\ddagger}$	EGTA	Hepes	pCa
A	140	7	5	15	40	10	25	9.03
B	140	7	5	15	40	10	25	4.25§
C	140	2.2	5	—	40	10	25	9.03
D	140	2.2	—	—	40	10	25	9.03
E	140	7	5	15	40	0.2¶	25	7.29

Table 2.1 Composition of solutions (in mM except where stated; pH 7.0).

* Potassium ions added as KCl and KOH. †Magnesium added as 1M $MgCl_2$; free Mg^{2+}=2.1-2.5mM in solutions A, B and mixtures of these solutions. ‡Sodium ions from the salts Na_2ATP and Na_2CrP. The Ca_{total} in all solutions was 0.02mM (as contaminating Ca), except solution B where it was 10mM. §An additional 0.1mM $CaCl_2$ was added to aliquots of solution B to yield "full activating" solution (pCa 4.0). Total chloride concentrations varied from about 110 to 120mM. ¶1,6 diaminohexane N,N,N',N'-tetraacetic acid (9.8mM) was added to this solution to maintain equivalent ionic strength.

pH measurement and buffering

Since many of the factors relevant to the work involved in this thesis are influenced by pH it is important that the solution pH is accurately measured and tightly buffered. Within our laboratory, separate reference and pH electrodes are used (Ciba Corning Diagnostics Ltd., Essex, England) to give a reliable measure of pH (Illingworth, 1981; Smith and Miller, 1985). The pH of experimental solutions was adjusted using a null point method. The electrodes were allowed to equilibrate in a standard solution (6.08mM KOH, 197mM KCl and 25mM HEPES) calculated to have the same pH_a and ionic strength as the experimental solutions (Harrison, Lamont and Miller, 1988). HEPES (25mM) was included in all experimental solutions, at pH_a 7.0, to minimise any changes in pH that may occur during contraction.

Calculation of free metal concentration and ionic strength

It is important to note that EGTA binds metal ions other than calcium to various degrees. Therefore, the affinity constants of EGTA and the other ligands (ATP, CrP and HEPES) in these experimental solutions must be known. In order to calculate the free calcium concentration it is important to assess the concentration of other ionic species and the influence of other experimental variables such as pH and temperature.

The REACT computer program, written by Godfrey Smith was used to assess the free ion concentration of the experimental solutions used in the work comprising this thesis (Smith, 1983). The free calcium concentration of the various commonly used mixtures of solutions A and B (Table 2.1) are given in Table 2.2. Ionic strength was defined according to the following equation:

$$I_e = \frac{1}{2} \Sigma c_j \, z_j,$$

where I_e is ionic strength, defined as the total of the ionic equivalents and c_j is the concentration of the jth ionic species and z_j is its valency (Smith and Miller, 1985).

Ratio of solutions B:A	pCa
1:1	6.22
3:1	5.74
5:1	5.52
8:1	5.31
10:1	5.22
15:1	5.04
25:1	4.83
$10_{activating}$	4.25
$10_{relaxing}$	9.03
$0.2_{relaxing}$	7.29

Table 2.2 The free calcium concentration of the basic solution (see Table 2.1) and various commonly used ratios of the solutions.

Chemical skinning

The mounted preparation was exposed to solution A (Table 2.1) containing either 50μg/ml of saponin or 1% v/v Triton X-100 (Pierce Chemicals, Rockford, Illinois, USA.) for 30 minutes. The chemical skinning agent was then removed by washing the preparation in fresh solution A before proceeding with the experiment.

Saponin is a glycoside that was first described as a treatment to skin cardiac muscle preparations chemically by Endo and Kitazawa (1978). At low extracellular calcium concentrations, saponin combines with cholesterol in the sarcolemma and induces small perforations which allow small ions and molecules to permeate (Noireaud, Bright and Ellis, 1989). However, due to the fact that the sarcoplasmic reticulum and the mitochondria have a lower cholesterol content than the sarcolemma, the preparation retains functional sarcoplasmic reticulum and mitochondria, as well as contractile proteins.

Treatment with the non-ionic detergent Triton X-100 results in the complete disruption of surface and intracellular membrane diffusion barriers, while the once again the myofilaments are functionally retained (Miller, Elder and Smith, 1985). Therefore, any intervention that influences the contractile response of a Triton-treated preparation can be attributed to a direct action on the myofilaments. It is well established that disrupting the sarcolemma leads to swelling of the myofilament lattice (Godt and Maughan, 1977) so that results obtained from such preparations must be interpreted with this consideration in mind. A schematic representation of commonly used techniques to disrupt muscle membranes is shown in Figure 2.3.

With both forms of chemical-skinning treatment employed within this thesis the following principle applies. Once skinned, the bathing solution surrounding the preparation becomes an extension of the intracellular environment. The exposure of free-radicals and reactive oxygen species to the intracellular systems under controlled

Cholesterol PPT. agents
Saponin   Digitonin

Non-ionic detergents
Brij-80 Triton-x 100

Collagen/basement membrane

Sarcolemma

Sub-sarcolemmal structures

Contractile proteins & Assoc. enzymes

Sarcoplasmic Retic.

Mitochondria

Diffusible Subst.
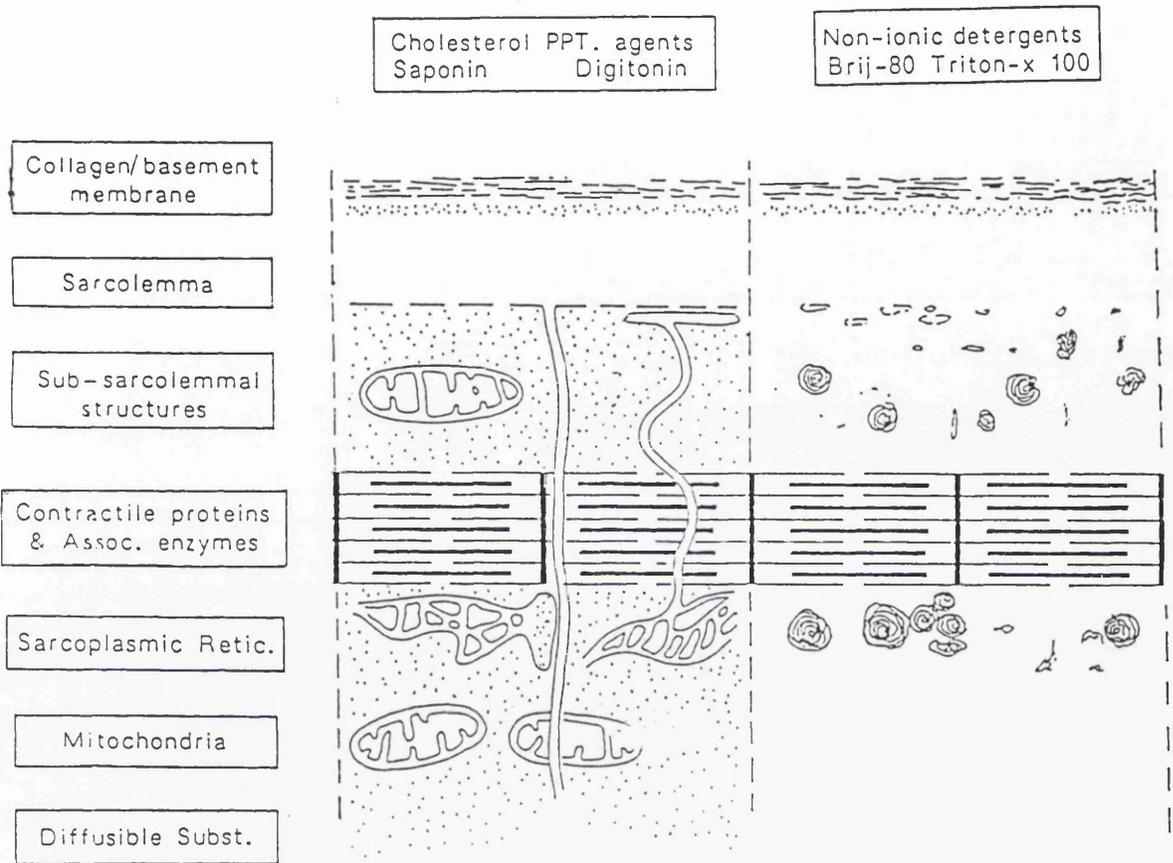
Figure 2.3 Schematic illustrating the muscle in the saponin- and Triton-treated states.

conditions, *i.e.* as dictated by the experimental solutions, allows the effects of such species on these systems to be characterised.

Simultaneous measurement of calcium release from the sarcoplasmic reticulum and tension measurement

The method used to simultaneously measure calcium and tension in chemically skinned muscle was developed by Drs GL Smith, CA Crichton and DS Steele (Smith, Crichton and Steele, 1991). The preparation was mounted between a tension transducer (Akers 46136, SensorNor a.s. Norway) and a fixed point by means of nylon monofilament snares in a similar way to that previously described (Figure 2.4). In contrast to the previous method of supporting the preparation, however, the axis of the steel pins containing the snares lies in the same plane as the preparation (compare Figure 2.4 with Figure 2.1). The preparation was continuously superfused with solutions of low calcium buffering capacity, solution E Table 2.1, at a flow rate of 1ml/min. This perfusing solution was pumped down the centre bore of a 5mm diameter perspex column into a perspex bath whose base was formed from a glass coverslip. The column was lowered to within 5-10μm from the top of the preparation in order to minimise the volume of the perfusing solution above the preparation. The volume of solution surrounding the preparation was estimated at approximately 4μl and excess solution was collected from the edge of the column. Aliquots of the perfusing solution containing 20mM caffeine was injected onto the preparation via the manifold at the base of the column. A solenoid-controlled pneumatic system ensured that a uniform volume of the solution (approximately 100μl) was applied to the preparation during a 50ms injection period. The bath was placed on the stage of a Nikon Diaphot inverted microscope.

Calcium release from the sarcoplasmic reticulum was detected by addition calcium-sensitive fluorescent probes to the perfusing solution. Both indo-1 and fura-2 were employed to detect calcium release in the studies described here. When using indo-1, the preparation was illuminated with light at 360nm via a 20x Fluor objective
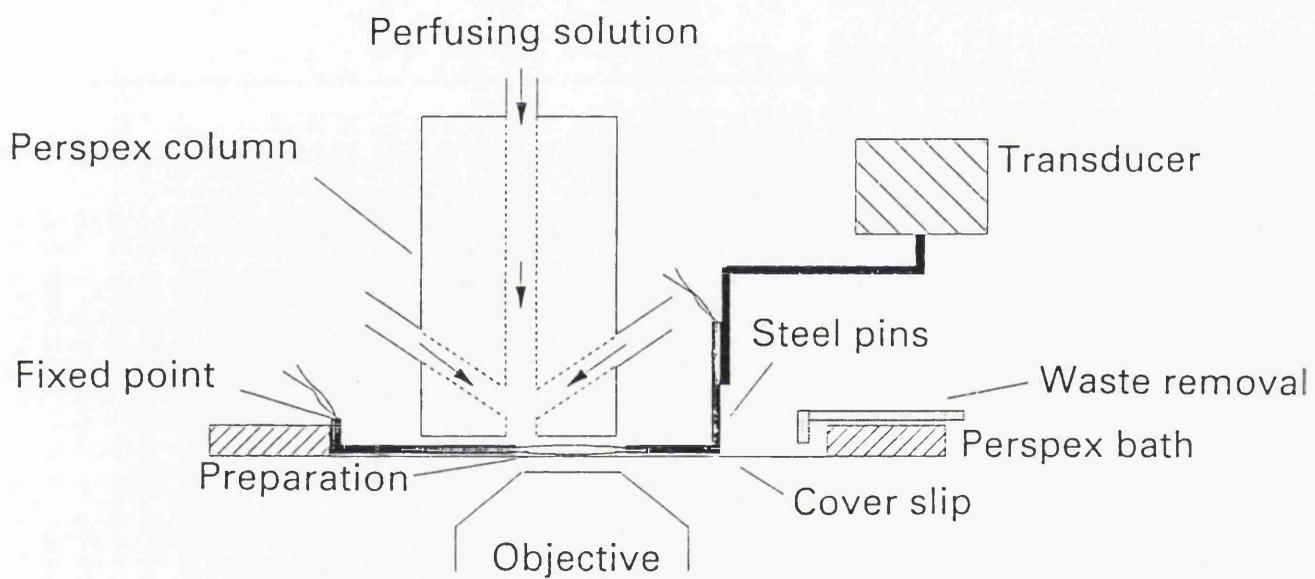
Figure 2.4 Diagram of the apparatus used to make simultaneous measurements of $[Ca^{2+}]$ and tension from saponin-treated preparations.

(Nikon CF Fluor, NA 0.75). The average [Ca^{2+}] within the visual field was indicated by the ratio of light intensities emitted at 405 and 495nm. Whereas with fura-2 the preparation was illuminated with light at 340 and 380nm and the ratio of light intensity at 510nm was measured. In both cases the light emitted from areas of the microscopes field not occupied by the image of the muscle was reduced by shuttering on the side port of the microscope.

Data handling and analysis

In the majority of the experiments described in this thesis, where tension measurement was the only signal being recorded, the output of the tension transducer was displayed on a chart recorder (Astromed Dash 8), after being pre-amplified and filtered at 25Hz. Simultaneously the signal was digitised at appropriate rates using MacLab and the commercially available Chart software. These data can be transferred to Macintosh data handling and graphics programmes such as Igor and MacDraw II, within which they can scaled and annotated prior to obtaining a hard copy via an Apple Laserwriter. Statistical analyses were performed using Excel or Statview software.

In some experiments, where simultaneous measurement of calcium release from the sarcoplasmic reticulum and tension were made, the signal was recorded onto videotape via an A/D VCR adapter (PCM 4/8, Medical Systems Corporation, Greenvale, New York, USA.) and domestic video recorder. The relevant information was then replayed and digitized at a more appropriate rate upon the appropriate PC software.

The tension response of cardiac preparations, skinned with non-ionic detergents such as Triton X-100, is well known to deteriorate with time (Jewell and Kentish, 1981). Since it has been noted that prolonged exposures to high concentrations of EGTA and low concentrations of calcium cause the myofilament lattice to become disorganised in skeletal muscle, which deteriorates very rapidly (Matsubara and Elliot, 1972), the muscle was

bathed in a calcium buffering solution of lower concentration for the majority of the time (solution E, Table 2.1).

The preparation can be activated to produce tension by sequentially stepping up the calcium concentration over the appropriate range. This approach has been widely used in pharmacological studies but has a number of drawbacks. In this laboratory, we occasionally find appreciably different force pCa curves with this approach than with those derived from making a series of single activations to the test pCa. It would seem most likely to be due to local (*i.e.* at the middle of the preparation) increases in phosphate concentration and reductions in pH consequent of the preparation maintaining a substantial force for prolonged periods. Along with the alterations to force-pCa curves noted in this laboratory, prolonged activation also accelerate the deterioration of the preparation. The 'calcium-jump', as previously described, is also useful in diminishing the rate of deterioration in such preparations. The mechanisms underlying the degeneration of preparation performance remain obscure and will be considered later in chapter 6. In saponin-skinned fibres, however, the preparations rarely exhibit any significant functional decay within the time required to carry out the experimental intervention being observed.

The relationship between steady-state force and calcium concentration from all otherwise satisfactory experiments is well described by the Hill equation, allowing objective definition of the position and steepness of the curve relating calcium concentration and tension.

$$\frac{C_x}{C_{max}} = \frac{[Ca_x]^h}{EC_{50}^h + [Ca_x]^h}$$

where C_{max} is the force at a saturating calcium concentration which is $[Ca_x]$; EC_{50} is the calcium concentration which gives 50% of C_{max} and h is the Hill coefficient.

It is generally assumed that the Hill coefficient is an index of the cooperativity of calcium binding to the regulatory protein troponin-C. Skinned cardiac muscle shows high cooperativity (Rüegg, 1986). However, the mechanism underlying cooperativity is poorly understood, so that it is difficult to draw conclusions with regard to small changes in the Hill coefficient that can sometimes be measured.

The curves obtained from the Hill equation were fitted by a least-square procedure after Levenberg and Marquat (Brown and Dennis, 1972). This yields values for EC_{50} and hence $K_{1/2}$, the reciprocal of the calcium concentration producing half-maximal calcium activated force and the Hill coefficient. To avoid the distortions produced by pooling experimental data, curves were fitted on an individual preparation basis so that the $K_{1/2}$s, and any shifts, could be determined for each preparation. The data are presented as the means of such shifts and $K_{1/2}$s and are calculated prior to being expressed as logs.

In chapter 8 of this thesis, analysis of the systemic distribution of histidyl dipeptides was made in human subjects undergoing clinical investigation in the Department of Cardiology at the Western Infirmary, Glasgow. The study protocol was approved by the local hospital ethical committee, and informed consent was obtained prior to the procedure.

Chapter 3: Alterations in the Myofilament Responsiveness to Calcium due to the Action of the Superoxide Anion.

Introduction

During ischaemia, the effects of pH alterations, increases in inorganic phosphate concentration and increased sympathetic drive will all have detrimental effects on the function of myofilaments as described in the general introduction. Calcium sensitivity and the maximum force attainable by the tissue will be reduced during an ischaemic episode. However, myocardial stunning persists long after the metabolic consequences of the antecedent ischaemia, while the alterations of pH and phosphate diminish during the post-ischaemic period. Calcium sensitivity and maximum force attainable by the tissue will return to normal *as far as these factors* are concerned.

One factor implicated in the genesis of experimental post-ischaemic contractile dysfunction is the generation of oxygen-derived free radicals. Myocardial production of radicals begins during coronary occlusion and increases after reperfusion, with a peak at around three minutes, and can persist for hours after the initial burst (Bolli, Patel, Jeroudi, Lai and McCay, 1988). The oxygen-derived free radicals produced during and after the ischaemic episode have been postulated to depress the calcium sensitivity of the contractile proteins (Bolli, 1990). Such speculation arises from the observations of Ito *et al* (Ito, Tate, Koboyashi and Schaper, 1987), who demonstrated that regionally stunned hearts show a contractile reserve when stimulated by positive inotropic interventions and suggested that stunning results from a decrease in contractile protein activation. Such a decrease in activation could occur by either a decrease in the calcium transient or in the calcium sensitivity of the regulatory proteins. It has been demonstrated, however, that in such situations there is a normal or even supra-normal calcium transient and decreased calcium sensitivity has been implicated in the mechanism of stunning (Kusuoka, Koretsune, Chacko, Weisfeldt and Marban, 1990). However, it is important to note that in these experiments, there was a depression of the maximum force attainable by the heart as well as a decrease in calcium sensitivity (E Marban, Personal Communication).

A mechanism implicating oxygen-derived free radicals, resulting from the generation of O_2^-, in the tissue injury seen upon reperfusion of hypoxic tissues was suggested by McCord (1987). Although xanthine oxidase is used as a source of O_2^- *in vitro*, almost all xanthine oxidase in normal tissue exists as a dehydrogenase enzyme. This catalyses the transfer of electrons to NAD^+, and not to oxygen, during the oxidation of xanthine or hypoxanthine to uric acid. McCord suggested that ischaemia/hypoxia might induce the conversion of the dehydrogenase into an oxidase by oxidation of essential thiol groups or by limited proteolysis. One consequence of ischaemia is that the intracellular ATP concentration begins to fall, the AMP concentration rises and is degraded; the hypoxanthine and xanthine that consequently accumulate can be oxidised and participate in the generation of O_2^-. It remains controversial, however, whether xanthine oxidase is present in human myocardium (Eddy, Stewart, Jones, Engerson, McCord and Downey, 1987; Muxfeldt and Schaper, 1987; Huizer, De Jong, Nelson, Czarnecki, Serruys, Bonnier and Torquay, 1989; Grum, Gallagher, Kirsch and Schlafer, 1989). There are, however, other *in vivo* sources of free radicals and non-radical reactive oxygen species, such as mitochondria within the cells (discussed in chapter 7) and activated neutrophils infiltrated between the myocardial cells (discussed in chapter 4).

The aim of the experiments described in this chapter, therefore, was to elucidate the action of O_2^- on the myofilaments in Triton-skinned trabecular preparations from rat heart. The controlled exposure of the myofilaments to the O_2^- was used to determine its effect on C_{max}, resting tension and the calcium sensitivity of the contractile proteins. Some of the results described here and in the following chapter have already been published (MacFarlane and Miller, 1992a,b,d; Miller and MacFarlane, 1992).

Results

The effects of O_2^- on maximum calcium-activated force generated by the myofilaments and resting tension.

The superoxide anion was generated by an established system in which xanthine oxidase (EC 1.1.3.22, Sigma Grade IV (from milk) suspension in 2.3M ammonium sulphate) catalysed the oxidation of xanthine or hypoxanthine to uric acid. The xanthine oxidase concentration was varied by dilution from a stock solution of 2mU/ml (*i.e.* 1.5µl of the commercial stock into 5ml of the appropriate test solution). In all cases the substrate concentration for the reaction (xanthine or hypoxanthine) was 50µM. The addition of 50µM xanthine or hypoxanthine, 70µM ammonium sulphate (*i.e.* the level achieved with the highest concentration of xanthine oxidase employed) or heat denatured xanthine oxidase to the bathing medium had no substantial effect on maximally calcium-activated tension. The effects of the (hypo-) xanthine/ xanthine oxidase system on the peak developed tension is illustrated in Figures 3.1 and 3.2.

Figure 3.1 demonstrates the effect of xanthine oxidase concentrations spanning four orders of magnitude. Initially, three control maximum calcium-induced contractions were evoked by transiently raising the calcium concentration in the bathing medium. The O_2^- generating system was then applied (in a strongly calcium buffered, or "relaxing" solution) for two minutes which had no direct effect upon resting tension. After a brief rinse in the standard relaxing solution, a maximum calcium-induced contraction was again evoked. The effect of each xanthine oxidase concentration was tested for six successive activations, each ten minutes apart. Panels A and B show the results obtained from a typical experiment of this type, with similar results being obtained in eight other preparations. In other experiments, single doses of xanthine oxidase were applied and the tension developed by the preparation stabilised at a reduced level after the exposure.
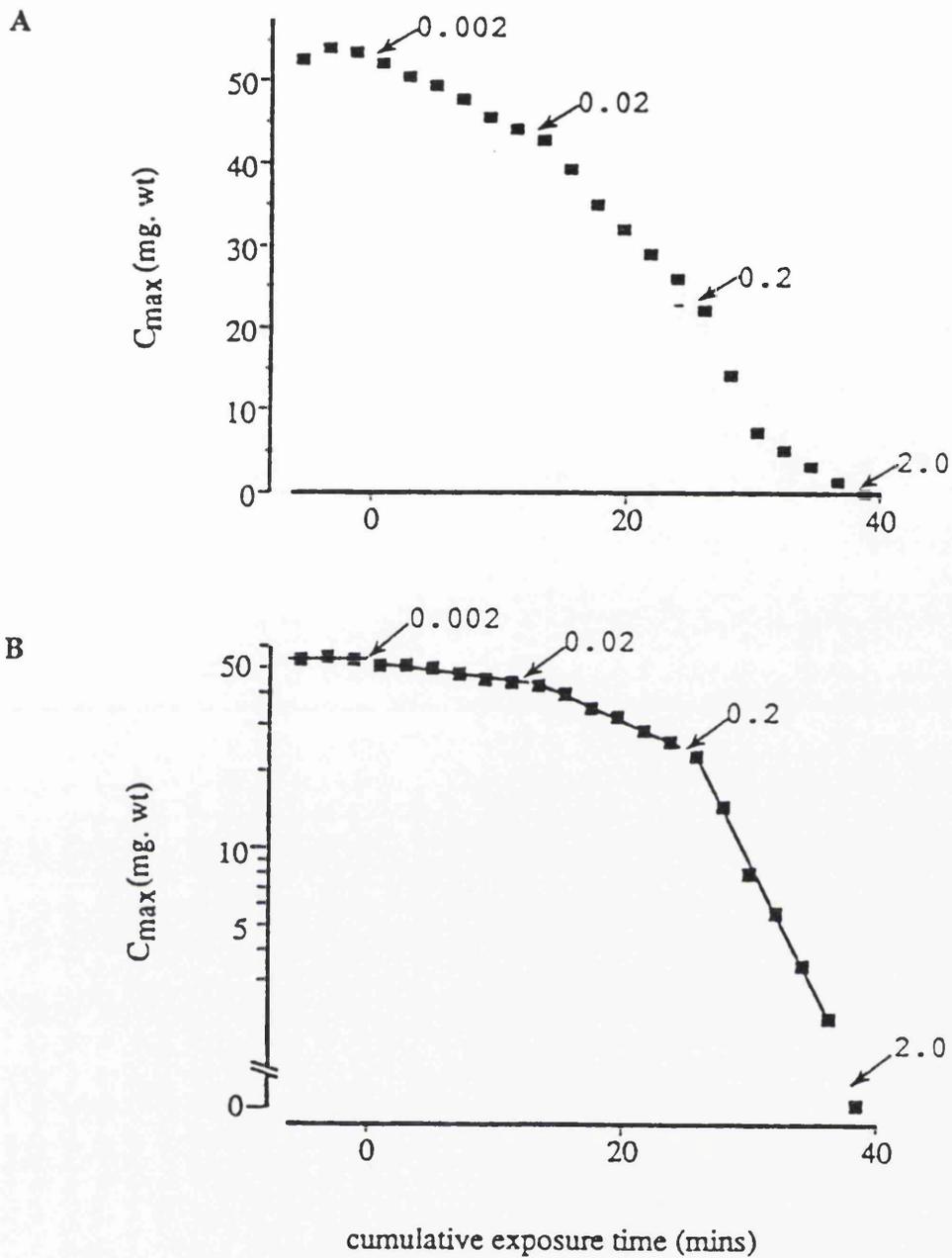
Figure 3.1 The effect of various xanthine oxidase concentrations on maximum calcium-activated force. **Panel A** The ordinate shows the absolute force developed by a typical preparation before and after exposures to the O_2^--generating system. The abscissa shows the cumulative time of exposure to the O_2^--generating system. The experimental protocol is described in the main text (enzyme concentration applied was 0.002, 0.02, 0.2 and 2mU/ml as indicated by the arrows). **Panel B** shows the same data plotted on semi-log axes.

Panel A shows that control C_{max} remains relatively stable. In other experiments, we typically find in this laboratory that C_{max}, when repeatedly evoked, falls by less than 0.2% per minute (Harrison, Lamont and Miller, 1988). As the concentration of xanthine oxidase is increased, C_{max} falls more steeply. In the case shown here, after twenty-two cycles of activation and relaxation (*i.e.* two hundred and twenty-two minutes later), C_{max} had fallen to effectively zero. Panel B shows the same data plotted on semi-logarithmic axes and demonstrates that maximum calcium-activated force falls linearly, *i.e.* exponentially towards zero, at each xanthine oxidase concentration. It was noted that, in conjunction with the depression of C_{max}, exposure to the O_2^- generating system increased the time to half-maximal activation and half-relaxation in every case. Time for half-maximal activation increased from 15.36±7.34seconds to 24.56±14.69seconds, $p < 0.05$, mean±S.D., n=9 preparations and for half-relaxation from 3.11±1.91seconds to 9.08±5.34seconds, $p < 0.005$, in the same preparations.

Figure 3.2 shows the results obtained from a similar protocol except that the xanthine oxidase concentration was kept constant (at 0.02mU/ml) and the time of exposure to the O_2^- generating system was varied. As might be expected, prolonging the exposure time produced a greater relative fall in C_{max}. Panels A and B again show the results of a typical experiment, similar results were obtained in three other preparations.
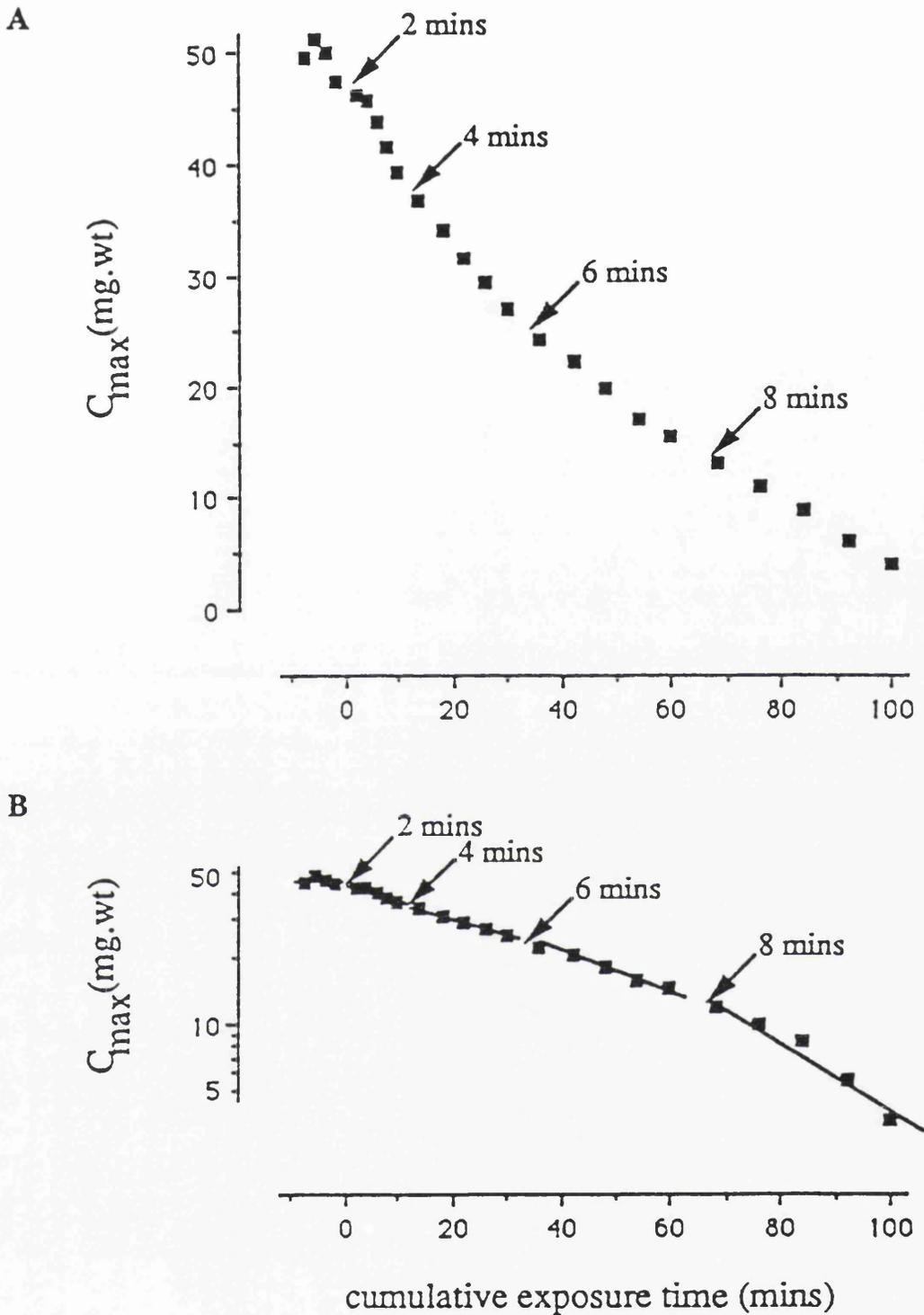
Figure 3.2 The effect of increasing time of exposure to a constant xanthine oxidase concentration (0.02mU/ml). **Panel A** The ordinate shows absolute force developed by a typical preparation before and after exposures to the O_2^--generating system. The abscissa shows the cumulative time of exposure to the O_2^--generating system. The experimental protocol is the same as in figure 1 (except that the arrows indicate increases in exposure times, these being 2, 4, 6 and 8 minutes, respectively). **Panel B** shows the same data plotted on semi-log axes.

The xanthine/xanthine oxidase system employed here generates singlet oxygen in addition to O_2^-. The O_2^- dismutes to hydrogen peroxide, a reaction catalyzed by superoxide dismutase, which in turn can form the hydroxyl radical via the Haber-Weiss reaction (when a suitable metallic cation, such as iron, is present). The Fenton reaction gives rise to the hydroxyl radical from O_2^- directly, in the presence of a substance capable of reducing Fe^{3+} to Fe^{2+}. These conditions might prevail in the experimental solutions used here (but are unlikely due to the strong chelation of relevant cations by EGTA). However, to be sure that the contractile dysfunction associated with exposure to the xanthine oxidase system can be attributed to O_2^- itself, a number of free radical scavenging agents were utilised to identify the culprit species. Figure 3.3 shows that when a preparation was exposed to the O_2^--generating system in the presence of superoxide dismutase, there was little change in the tension generated compared with the control activations. The concentration of xanthine oxidase used in this case would have been sufficient to cause a substantial fall in the force produced by the preparation. In order to demonstrate this, the sequence of activations was continued in the absence of superoxide dismutase and, as expected, the tension generated by the preparation fell (to less than 20% of the control activation within five exposures to the O_2^--generating system). The experimental solutions used here contain HEPES, which is effective as a hydroxyl radical scavenger (Halliwell and Gutteridge, 1989) and so should protect the preparation from the effects of any hydroxyl radical formation occurring downstream from the O_2^--generation. As a further test desferroxamine (another hydroxyl radical scavenger) was added to the solutions and did not protect. Glutathione, which will scavenge the hydroxyl radical, singlet oxygen and organic peroxides, did not confer protection. Addition of catalase (which catalyses the breakdown of hydrogen peroxide) to superoxide dismutase was not necessary to confer protection to the preparation. These findings suggest that the effects of the O_2^--generating system described above can be attributed to O_2^- alone.

Effect of X/XO with & without SOD
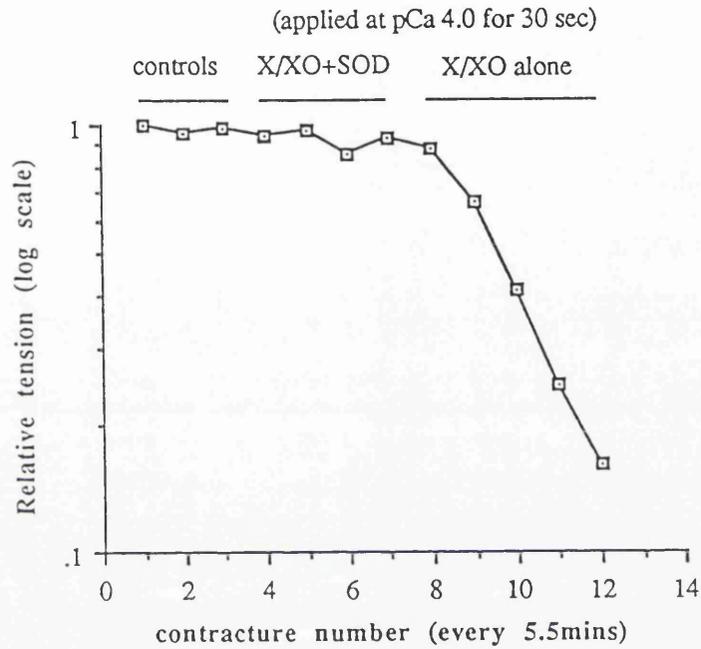
Figure 3.3 The protective effect of superoxide dismutase. The ordinate shows the relative tension developed, on a semi-log scale. The abscissa shows the cumulative time of exposure to the O_2^--generating system. After a series of control activations, the preparation was exposed to the O_2^--generating system (X/XO, 0.2mU/ml xanthine oxidase for thirty seconds at pCa 4.0) in the presence of SOD. The dismutase was then removed and the preparation was exposed again to the O_2^--generating system.

It is important to note that the effects of the xanthine oxidase system can be variable. Since the enzyme preparation is a suspension, great care must be taken to ensure consistent enzyme concentrations are achieved when pipetting the stock solution. For example, an experimental artefact was observed by Dr J J McMurray (Personal Communication) when investigating the effects of the xanthine oxidase system on tension development in a rat aortic ring preparation. Five aortic rings were cut from the same animal and bathed in solutions made from the same batch. The same volume of the commercial stock of xanthine oxidase was pipetted into the five experimental chambers. One aortic ring was able to sustain 100% of the control activation throughout the length of the experiment, whilst two others could only sustain approximately 50%. The final two aortic rings could not be activated to produce tension.

For this reason it is necessary to assess continuously the level of O_2^--production obtained when using the xanthine oxidase suspension. The production of O_2^- can be assessed spectrophotometrically by following the reduction of cytochrome C. The calculated values of O_2^--production can then be compared with the theoretical values for the concentration of enzyme and substrate used. The results of such assays demonstrated that in the experimental solutions used here the expected level of O_2^- production was achieved and not exceeded. These results will be discussed further in chapter 8.

The effects of O_2^- on myofilament calcium sensitivity.

By studying sub-maximal calcium activations in the Triton-skinned preparations, we are able to investigate the hypothesis that O_2^- depresses calcium sensitivity. Figure 3.4 (upper panel) shows an example of the effect of O_2^- on C_{max}. Prior to being exposed to O_2^- (indicated by the open circles), C_{max} is constant. After two exposures to O_2^- (as indicated by the filled circles), C_{max} fell progressively to less than 20% of the initial level. The lower panel of Figure 3.4 demonstrates the relationship between tension and calcium
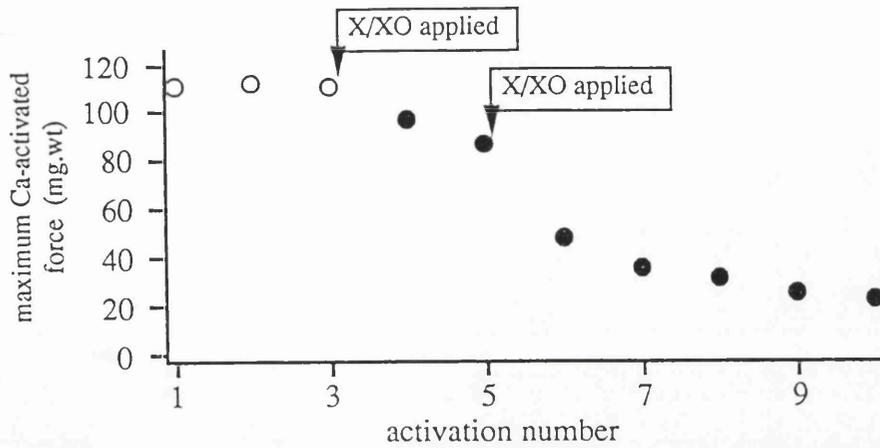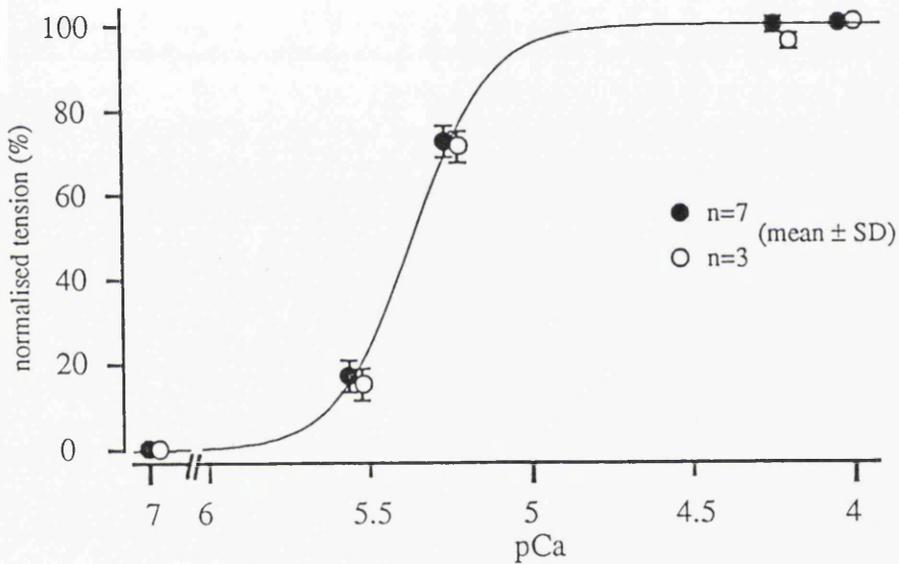
Figure 3.4 The effect of O_2^- on calcium sensitivity. **Panel A** The ordinate shows maximum calcium-activated force developed by a typical preparation.

The abscissa shows contracture number. At the indicated points, the preparation was exposed to the O_2^--generating system (enzyme concentration 0.2mU/ml) for thirty seconds. Open symbols denote control activations and the closed symbols activations obtained after exposure to the O_2^--generating system. **Panel B** The ordinate shows the *relative* force developed by the same preparation. The abscissa shows calcium concentration (expressed as pCa) at which the preparation was activated. Sub-maximal calcium activations are expressed relative to C_{max} obtained for that series of activations. This force pCa curve was constructed using single steps to test pCa's (see main text).

concentration obtained from a range of submaximal calcium activations in the same preparation. Again the open symbols denote data obtained prior to, and closed symbols after, exposure to O_2^- (the symbols have been displaced slightly for clarity). The force-pCa curve was constructed in Figure 3.4 using the 'calcium jump' method of activation. Similar results were found in seven other preparations.

To confirm this lack of effect on calcium sensitivity, and perhaps as a better illustration, Figure 3.5 shows an experimental trace indicative of the calcium sensitivity before and after a single exposure to the O_2^--generating system (10mU/ml Xanthine Oxidase for two minutes). The left panel of this figure shows that C_{max} is approximately 50mg.wt., whereas in the right panel C_{max} has fallen to around 20mg.wt. Rescaling the trace in the right panel, so that the maxima are of similar size, shows that there is very little difference in the relative submaximal levels of activation. This demonstrates again that calcium sensitivity does not alter significantly despite a very large fall in peak force. Similar results obtained with this type of 'cumulative activation' were found in seven other preparations.
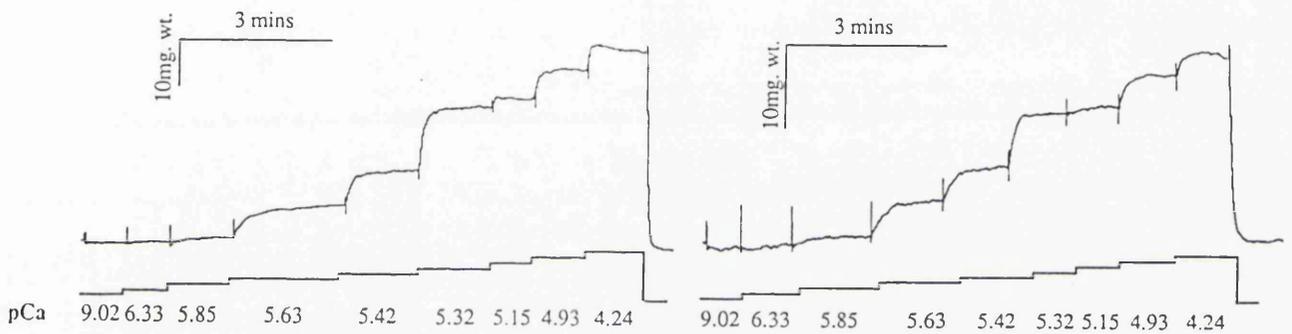
Figure 3.5 Experimental traces demonstrating the lack of effect of O_2^- on calcium sensitivity. The left panel shows a 'cumulative' series of sub-maximal activations prior to exposing the preparation to the O_2^--generating system. The right panel shows a similar series of activations after exposure to the O_2^--generating system (10 mU/ml xanthine oxidase for two minutes). The scale of the right panel has been expanded to facilitate comparison of the relative forces.

The effects of O_2^- on crossbridges in rigor.

The actions of O_2^- were studied on the preparation in the rigor state, *i.e.* during the very strong interaction between actin and myosin filaments that develops in the absence of ATP even at very low calcium concentrations. In these experiments, the preparation was initially relaxed in a solution without creatine phosphate (Solution C, Table 2.1). This manoeuvre was undertaken to prevent creatine phosphate contamination of the 'rigor' solutions (which would otherwise continue to phosphorylate ADP generated by the crossbridges from the residual ATP carried over in the preparation when the solution change is made). The rigor contracture was subsequently induced by exposing the preparation to a similar solution but containing no ATP (Solution D, Table 2.1). Each rigor-inducing solution was only applied once to avoid complications due to cumulative ATP contamination. The $MgCl_2$ was reduced in the rigor-inducing solution to ensure the same free magnesium concentration as in the relaxing solution (Solution A, Table 2.1).

Exposing the preparation to O_2^- in the rigor state can establish whether O_2^- acts on active or non-active crossbridges. Figure 3.6 shows that the amplitude of both C_{max} and rigor contractures can be maintained at a steady level for relatively long periods of time. When O_2^- was applied for a period of two minutes during a rigor contracture, it was without effect on the amplitude of that rigor contracture. The subsequent amplitude of C_{max} was slightly smaller than the control, but not significantly so, given that prolonged activations have a detrimental effect on C_{max} and more than 40 minutes had elapsed since the control activation. When the same concentration of xanthine oxidase was applied for a similar period of time during a maximum calcium-activated contracture, however, its amplitude started to fall steadily within seconds. Similar results were found in seven other preparations. In five other preparations a different protocol was followed to establish whether the fall in tension, if any, noted after exposure to the O_2^--generating system during a rigor contracture was significantly different from the fall noted when

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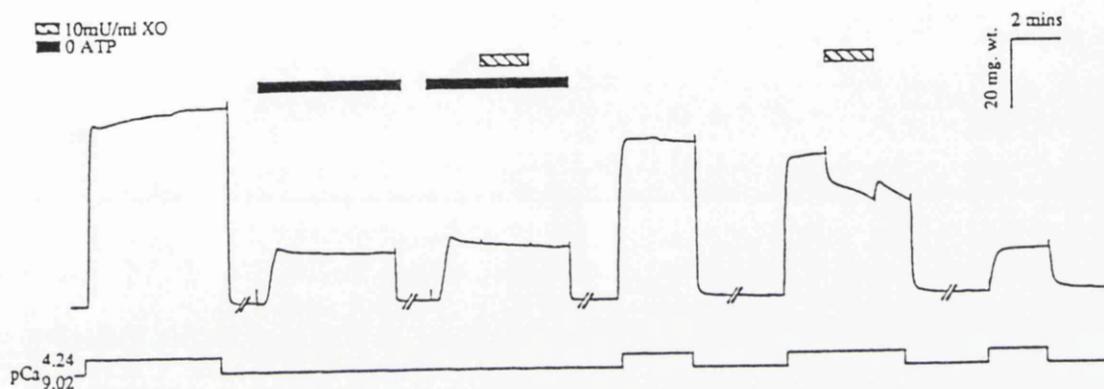
Figure 3.6 Experimental traces demonstrating the lack of effect of O_2^- during a rigor contracture. The preparation was initially activated by raising calcium concentration in the bathing medium, this contracture was maintained for five minutes. A rigor contracture was then induced by removing ATP from the bathing medium. After having allowed the rigor force to develop, the preparation was exposed to the O_2^--generating system (10 mU/ml xanthine oxidase for two minutes). Prior to relaxing the preparation the O_2^--generating system was removed, which did not change the measured force. Subsequent calcium-activations are not diminished beyond the extent which would be expected for the time period elapsed from control (see main text). The preparation was then exposed to the same level of O_2^--generation after calcium-activation. The force developed falls during such exposure and continues to fall after the O_2^--generating system is removed. The force developed during the subsequent calcium-activated contracture is below 50% of that obtained immediately prior to O_2^- exposure during the calcium-activated contracture (and far in excess of that which would be expected). Gaps in the trace indicate periods in 'relaxing' solution of five to ten minutes.

maximum calcium activated force is repeatedly evoked (Harrison, Lamont and Miller, 1988). Five control activations were evoked and the rate of fall in C_{max} calculated. This was compared with the fall, if any, noted after exposure to the O_2^- in the rigor-inducing solution. The rate of fall during the control activations was $0.256\pm0.008\%min^{-1}$ compared with $0.208\pm0.048\%min^{-1}$ after exposure to O_2^- (mean$\pm$S.D.,p>0.2), whereas the rate of fall in tension after exposure to O_2^- in solutions containing ATP was significantly different from the control, $8.04\pm3.86\%min^{-1}$ (mean$\pm$S.D., p<0.02).

Conclusions

Much of the existing work on the phenomenon of myocardial stunning, whether at a cellular, isolated organ or whole animal level, has rested on implicit assumptions about the intrinsic contractile potential of the myocardial cell. Specifically assumptions are made about the contractile 'reserve' available to the heart. An example would be that calcium sensitivity is deduced to have fallen when it is observed that the calcium transient is normal but contraction has reduced (Kusuoka, Koretsune, Chacko, Weisfeldt and Marban, 1990). The force produced during systole is the product of two factors: the relative degree of activation of the contractile proteins and the maximum-achievable force for that tissue. Alterations in the calcium sensitivity of the myofilaments, or the calcium concentration achieved during activation, will change *relative* force, whereas alterations in the maximum force achievable for the tissue will *scale* these effects. It is extremely difficult to detect changes in maximum achievable force in intact preparations for several reasons. In cellular preparations, even maximal positive inotropic interventions may only achieve 85% of C_{max} (Fabiato, 1981). Under the conditions likely to prevail physiologically or even experimentally, the contractile reserve can be several-fold greater than the actual force developed. It follows that a large change in the contractile reserve, either an increase or decrease, could remain undetected. The use of 'skinned-fibres', however, gives sufficient experimental control to allow one to make definitive statements about calcium sensitivity and C_{max} changes. These points are illustrated in Figure 3.7.

The results presented here show that O_2^- acts directly on the myofilaments to diminish their force producing ability. The discussion above, and figure 3.7, emphasise that a reduction in calcium sensitivity will produce a fall in force at submaximal levels of activation. This possibility was explored, and as figures 3.4 and 3.5 reveal, despite a substantial fall in C_{max}, no detectable change in calcium sensitivity occurred. Translated to the intact heart, the fall in C_{max} with no change in calcium sensitivity would account for
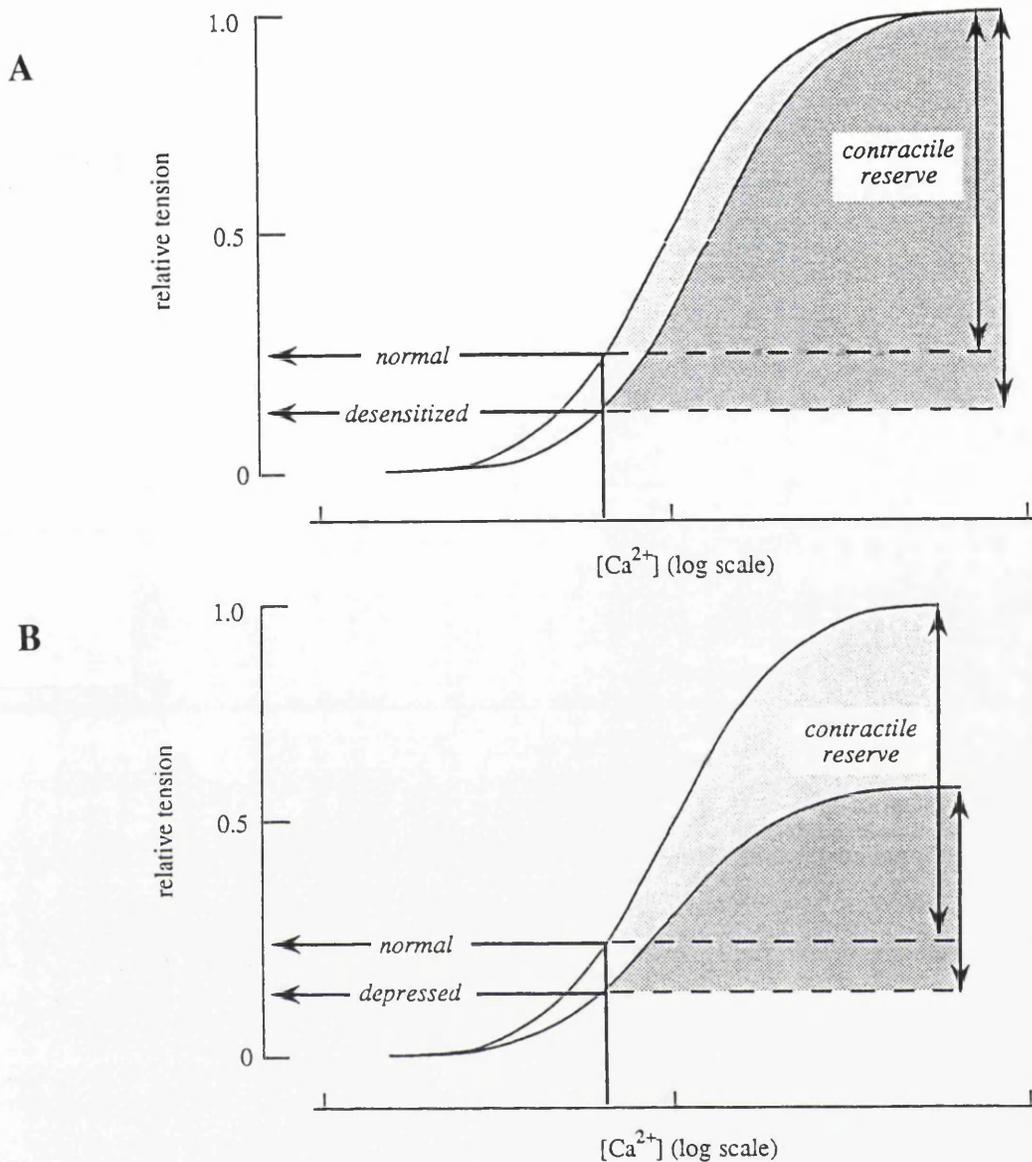
Figure 3.7 Schematic representation of the effect of reduced calcium sensitivity or reduced maximum calcium-activated force. **Panel A** The ordinate shows the relative tension (in arbitrary units). The abscissa shows calcium concentration on a log-scale. When the contractile proteins are desensitized to calcium the force developed at a given calcium concentration is diminished. However, C_{max} does not alter, the pCa-Tension relationship remaining unchanged except that the curve is moved to the right. In this circumstance the contractile reserve is increased. **Panel B** The ordinate and abscissa are as in panel A. When C_{max} is depressed, the pCa-Tension relationship is altered so that the absolute force developed at a given calcium concentration will be diminished. However, force achieved at a sub-maximally activating calcium concentration is simply scaled down, relative to the new C_{max}, so that there is no change in calcium sensitivity (*i.e.* the same calcium concentration would be required for half-maximal calcium activation). In such a situation the contractile reserve will have decreased. It should be noted that the two effects can result in a similar fall in the absolute level of force developed. This is demonstrated by the fact that at the calcium concentration indicated in this figure the same fall in 'absolute' tension was produced.

the observed fall in contractility despite a normal calcium transient. This is the circumstance represented in figure 3.7, panel B.

The results described here indicate that the myofilaments are particularly sensitive to O_2^-. Force is inhibited faster and at a lower enzyme concentration in the O_2^--generating system than has previously been reported for intact experimental preparations (Schrier and Hess, 1988). Falls in force entirely attributable to O_2^- can be produced by as little as $2\mu U/ml$ xanthine oxidase (*e.g.* Figure 3.1) within two minutes; this represents less than 0.01% of the levels frequently employed in experiments with intact cellular systems.

Such results force the conclusion that the myofilaments are very susceptible to damage by O_2^-. This conclusion is particularly important since, in rat heart, physiological concentrations of xanthine oxidase exceed the levels used here (Batteli, Della Corte and Stirpe, 1972). As stated earlier, it remains controversial whether xanthine oxidase is present in human myocardium (Eddy *et al*, 1987; Muxfeldt and Schaper, 1987; Huizer *et al*, 1989; Grum *et al*, 1989).

The effects on C_{max} demonstrated here can be attributed to O_2^- itself rather than the generating system. In the intact cell, there are other sources for O_2^- in addition to xanthine oxidase. Superoxide is produced in the cell as a consequence of normal metabolism. Probably the most important source *in vivo* is from the mitochondrial respiratory chain (Turrens, Beconi, Barilla, Chavez and McCord, 1991). Since the contractile proteins have been revealed by this study to be particularly sensitive to O_2^-, increases in O_2^- production associated with ischaemia and reperfusion might overcome the capacity of endogenous scavengers and threaten the performance of the myofilaments. Aspects of this possibility are investigated in chapter 7.

The exact mode of action of O_2^- on the myofilaments remains to be elucidated, but the results here afford some insight. In the experimental protocols in this study, O_2^- was applied either in the relaxed state or in the calcium-activated state. The relaxed muscle

showed no fall in resting tension (induced by stretching the preparation to set the sarcomere length) when exposed to O_2^-, giving initial evidence to suggest that the effect was more than generalised damage to the structural integrity of the preparation (either by O_2^- directly or by protease contamination of the xanthine oxidase, see chapter 6 for further discussion). By studying the action of O_2^- on the preparation in the rigor state it was sought to establish whether O_2^- affects only active or inactive crossbridges. Figure 3.6 illustrates that even a high level of O_2^- has no immediate effect on force previously developed by the muscle in rigor, unlike the rapid decline of calcium-activated tension that can be induced. The insensitivity of attached rigor crossbridges to alterations in pH has been reported earlier (Smith and Steele, 1989). This result suggests that the O_2^- is not acting in the manner of a non-specific protease to damage the structural integrity of the myofilament lattice, tension would otherwise be lost during the rigor contracture (thus consolidating the initial evidence for such a conclusion when O_2^- is applied in 'relaxed' preparations).

Care was taken in the protocol used for experiments like those described in figure 3.6 to remove the O_2^--generating system fully before relaxing the muscle by replacing ATP. Thus, in these experiments, O_2^- was not present when the crossbridges were actively cycling. The subsequent evidence that no significant damage had occurred implies that O_2^- acts without provoking crossbridge detachment or weakening the crossbridge structure. The site of action of O_2^- is most probably on a part of the crossbridge that remains inaccessible in the attached state (possible sites of action are discussed later in this chapter). The action of O_2^- can be concluded to be on the active or detached crossbridge, for the conditions studied here. This action will compromise one of: subsequent attachment, crossbridge kinetics (such as the transition from the weakly to the strongly attached state and/or ATPase activity).

It is, however, perhaps difficult to reconcile these observations with those of Krause (1990) on myofibrillar ATPase of preparations from stunned rabbit myocardium. He reported no detectable reduction in maximum calcium-activated ATPase, its kinetics or

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calcium sensitivity. To interpret the present results against this background requires that the rate of crossbridge cycling remains essentially unaltered. This would demand a specific alteration to the crossbridge cycle such that attachment time as a fraction of cycle duration would fall substantially, but cycle frequency (and hence the rate of ATP consumption) would remain unaltered. Such a highly specific action seems unlikely. Krause attributed all the post-ischaemic reduction in contractile function to myocardial stunning. The final degree of dysfunction, after only some thirty minutes of reperfusion was only about 27% (as closer inspection of the original figures reveals). At this time, although any reduction in pH would probably have reversed fully, raised phosphate might still decrease contractile function. Additionally, these preparations were unpaced and rate dependent effects on contractility could have contributed to any such decrease. If, for example, only one third of the decrease was due to stunning, and that third was entirely attributable to a reduction in C_{max}, then the myofibrillar ATPase would reduce by as little as 10%, a level difficult to detect reliably with Krause's methods. In contrast, a reduction of C_{max} by 10% is readily detected in the skinned fibres.

It is generally assumed to be the speed of calcium concentration change that determines the speed of tension alteration in skinned fibres. However, changes in the rate of force development and decline, observed when the calcium concentration is raised or lowered, are not readily interpreted. If it is assumed that the diffusion of the CaEGTA buffer system is unaffected by O_2^- treatment then the 60% slowing of activation and 200% slowing of relaxation half-times could indicate altered crossbridge kinetics. This would require that crossbridge attachment and detachment, rather than buffer diffusion, become the rate limiting steps after O_2^- action and would imply a reduced ATPase rate.

In these experimental conditions, a reduction in C_{max} to any degree can readily be produced. The tension responses are slowed accompanying this reduction in C_{max}. There is no change in calcium sensitivity, nor does some non-specific damage appear a major contributor to the altered contractile responses. These findings would be consistent with an alteration to crossbridge kinetics or a reduced overall ATPase activity, as noted

above. Whatever the mechanism of O_2^--induced C_{max} reduction described here, it could contribute to the phenomenon of myocardial stunning. In addition, recent observations using intact buffer-perfused ferret hearts, suggest that maximum force production is diminished in stunned myocardium without alterations in calcium-sensitivity (and only transient calcium overload) (Carrozza, Bentivegna, Williams, Kuntz, Grossman and Morgan, 1992). Such results may suggest a fairly substantial role for O_2^--induced C_{max} reduction in the pathogenesis of post-ischaemic myocardial dysfunction since the effects of pH and phosphate would not discriminate effects on maximal force production and calcium sensitivity.

Possible sites of action for the superoxide anion

Lymn and Taylor (1971) put forward the biochemical context to the concept of the cycling crossbridge. They postulated a cycle such that in the absence of ATP, a permanent actin-myosin link is formed (the rigor-state). Addition of ATP allows the myosin head to detach from actin, and while detached, the hydrolysis products ADP and P_i are formed, but not released. In this state the myosin head can rebind actin and force production occurs with the release of the hydrolysis products. The rigor state is then reformed.

Recent biochemical and mechanical studies support the idea that during activation the crossbridge cycles between two configurations: the weakly-attached state which contributes little to force generation and the strongly-attached state representing the force-generating step. However, in both states crossbridges reversibly attach to actin. The results of studying the actin binding kinetics of both states (Brenner, 1986) suggest that attachment and detachment of crossbridges can occur several times through one cycle from the weakly to the strongly attached state (with hydrolysis of one ATP molecule) although this differs from the working hypothesis, current throughout the 1970's, that a distinct site of interaction exists for actin and myosin. The nature of the relative transfiguration between actin and myosin associated with energy transduction remains

obscure. The most widely accepted model of the crossbridge cycle still views the power stroke as being coupled to the release of ADP and P_i (Cooke, 1986).

In recent years NMR spectroscopy has been used to investigate the interactions of actin and the myosin. Comparing myosin filaments, the myosin molecule, heavy and light meromyosin and the S_1 head has shown that the spectral features of intact myosin are almost completely attributable to the S_1 subfragment (Highsmith, Akasaka, Konrad, Goody, Holmes, Wade-Jardetzky and Jardetzky, 1979). The demonstration of differential flexibility within S_1 could reflect structural elasticity and possibly that the myosin head is involved in mechanical function. NMR spectral studies have demonstrated that addition of F-actin to S_1 gives data consistent with a reduction in S_1 flexibility (Highsmith *et al*, 1979; Prince, Trayer, Henry, Trayer, Dalgarno, Levine, Cary and Turner, 1981).

The regions of actin involved in the interaction with S_1 have been investigated using a nitroxy spin label attached to cysteine residue 705 on the S_1 subfragment (Moir, Levine, Goodearl and Trayer, 1987). Homologous spectral effects are seen on titration of residues 1-82 of actin with spin-labelled S_1. Attachment between these proteins appears to involve two distinct regions within these actin residues which link with the 20kD and 50kD tryptic-cleaved domains of S_1.

The observation that the effects of O_2^- are not revealed when it is applied to preparations in the rigor state implies the conformational change in the strongly attached crossbridge will mask the specific site of O_2^- action. It is assumed that during rigor all the crossbridges are permanently attached, in contrast to the active cycle described above. However, not all the sites of actin's interaction with myosin are occupied during a rigor contracture. For this reason it would appear that the site of O_2^- action must be restricted to myosin.

The NMR spectral studies described earlier suggest that it is the attachment of myosin between residues 522 and 540 on the 50kD domain to actin that restricts its

flexibility. Actin also interfaces with myosin around residues 705-695 on the 20kD domain and appears to act cooperatively with the 50kD domain. Similar structural cooperativity influences the structure of alkali light chain 2.

Modifying the thiol groups on myosin S_1 (see chapter 4) and removing the essential light chains can have a substantial effect on calcium-sensitivity. The fact that the O_2^--induced reduction in C_{max} occurred without alteration in calcium-sensitivity appear hard to reconcile with these postulated sites of action. A further model of crossbridge "cycling" was suggested by Harrington in 1979. In this model the origin of force production is within S_2 subfragment of myosin. ATP is still required for attachment and detachment of the myosin head to actin, but the energy released by ATP hydrolysis is used to trigger the transition of part of the S_2 region from an α-helix to a random coil (which would shorten to give rise to the power stroke). The formation of the hydrolysis products of ATP would be associated with re-crystallisation into a double coiled α-helix. This model does not require the myosin head to rotate on actin, but is still viable even if the head does rotate. If this model is substantiated, a possible site of O_2^- action could be upon subfragment S_2. This possibility has further attractions because the sites of actin-myosin interaction postulated above are most readily associated with thiol residues. However, it requires that the myosin head alone (without the S_2 subfragment) interacts with actin to produce the force level obtained in intact muscle to disprove the Harrington model.

The more classically accepted Huxley and Simmons model of crossbridge cycling would still allow for the S_2 subfragment to contribute to the level of force production. The stiffness or compliance of the myosin rod would obviously influence force transduction during the power stroke of the crossbridge cycle. However, it is generally assumed that such influence would be passive in nature *i.e.* resultant from the power stroke and not contributing to it (being the series elastic element). Therefore, the fact that force can be completely abolished by O_2^- would require that compliance increased enough to allow the myosin head to move in excess of 12nm per half-sarcomere without

generating force, which is unprecedented. The facts that when O_2^- is applied during a rigor contracture tension is maintained and that hypochlorous acid depresses C_{max} could suggest that the O_2^- site of action is on the S_1 subfragment (see chapters 4 and 6 for further discussion).

Chapter 4: Alterations in Myofilament Responsiveness due to Hydrogen Peroxide and Hypochlorous Acid.

Introduction

Neutrophils probably play little role in the contractile dysfunction seen upon reperfusion after brief periods of ischaemia, and it is important to recognise that the phenomenon of stunning is observed in buffer perfused systems where neutrophils can take no part. However, neutrophils may be important source of free radicals and reactive oxygen species after longer periods of ischaemia *in vivo* (Mullane, Salmon and Kraemer, 1987) and after myocardial infarction, when neutrophil accumulation and activation in the myocardium is known to occur (Werns and Lucchesi, 1988; Weiss, 1989; Lucchesi, Werns and Fantone, 1989). Since myocardial stunning has been characterised by prolonged contractile abnormalities it might be appropriate to link the myocardial damage associated with the inflammatory response to that of ischaemia-reperfusion injury (Reimer and Jennings, 1979). Additionally, thrombolytic therapy after acute myocardial infarction can be delayed for up to four hours after the onset of chest pain and reperfusion would therefore be initiated in inflamed tissue.

The demonstration that certain anti-inflammatory agents protect the ischaemic myocardium would give support to the hypothesis that a component of the injury can be mediated by the inflammatory response. *e.g.* Pretreatment with ibuprofen was demonstrated to decrease myocardial damage significantly during coronary occlusion in conscious dogs (Jugdutt, Hutchins, Bulkley and Becker, 1980). Although the mechanism of myocardial protection was not understood, the authors postulated that prostaglandin-induced vasoconstriction was inhibited. However, subsequently it was demonstrated that the protective effects of ibuprofen were mediated without alterations in myocardial blood flow or a decrease in myocardial oxygen uptake (Romson, Bush, Jolly and Lucchesi, 1982). Labelled platelets and neutrophils were used to demonstrate that ibuprofen inhibited neutrophil accumulation but not platelet accumulation in the infarcted myocardium (Romson, Hook, Rigot, Schork and Lucchesi,1982). Importantly, it was

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demonstrated that a 40% reduction in damage to the myocardium after ibuprofen paralleled the reduction in neutrophil accumulation.

It is now appreciated that activated neutrophils release cytotoxic products such as oxygen-derived free radicals, reactive oxygen species and the products of arachidonic acid metabolism. At the onset of phagocytosis, neutrophils show a marked increase in oxygen uptake (which is unrelated to mitochondrial electron transport). Such oxygen uptake is due to activation of a plasma membrane enzyme complex which oxidises NADPH to NADP$^+$, the electrons that are formed reduce oxygen to O_2^- (Baboir, 1978). Neutrophils contain superoxide dismutase so that the O_2^- formed dismutates to hydrogen peroxide in the phagocyte vacuole. Secretion of the enzyme myeloperoxidase into the phagocyte vacuole can oxidise chloride in the phagocytic cytoplasm into hypochlorous acid (HOCl), which is known to react with hydrogen peroxide at high pH to form singlet oxygen, but this reaction does not occur rapidly at pH 7.0.

Human neutrophils stimulated by phorbol myristate acetate (PMA) can significantly alter myocardial function *in vivo* via free radical and reactive oxygen species processes. Consequently the haemodynamic sequelae of PMA infusion could be ameliorated with antioxidants (Rowe, Manson, Caplan and Hess, 1983; Rowe, Eaton and Hess, 1984). The beneficial effects of non-steroidal anti-inflammatory agents could, in fact, be due to their inhibition of free radical and reactive oxygen species production. The purpose of this chapter is to determine the effects of such species on the myofilaments, the fundamental units of force production. The results of this study have been reported in abstract form (Miller and MacFarlane, 1992; MacFarlane and Miller, 1992c) and have been submitted for publication (MacFarlane, dos Remedios and Miller, 1993).

Results

The effects of hypochlorous acid and hydrogen peroxide on maximum calcium-activated force generated by the myofilaments and resting tension

Hypochlorous acid (HOCl) was prepared immediately prior to use by adjusting NaOCl (BDH Ltd, Poole, England) to pH 7.0 with HCl. Hydrogen peroxide was added from a thirty percent stock solution available commercially. The concentrations of hypochlorous acid quoted throughout this thesis refer to the concentration of NaOCl added to the solution. Therefore, at pH 7.0, used for the majority of the experiments, the amount of the dissociated hypochlorite anion (OCl^-)will be approximately 25% (the pK_a of HOCl is 7.5). In the experiments described later in this chapter where pH was lowered to 6.3 the dissociated species will be just in excess of 6%. The concentration of NaOCl added at pH 6.3 was increased by a factor of four to produce a similar concentration of the hypochlorite anion, but a much greater concentration of HOCl. This approach tends to compensate for the slow net loss of HOCl which results from degradation of the anion to gaseous oxygen and HCl and is accelerated at acidic pH. The concentrations are expressed as the total of NaOCl added since the same limitations of dissociation will exist *in vivo* for HOCl generated by activated neutrophils.

Exposure of the preparation to $10\mu M$ hydrogen peroxide for up to 30 minutes had in the relaxed, calcium-activated or rigor states no effect on C_{max} or resting tension. Even at concentrations of 10mM there was no immediate effect of hydrogen peroxide (*i.e.* during a one minute exposure). Increasing the incubation time with 10mM hydrogen peroxide to 60 minutes resulted in a small (<5% of control C_{max}) increase in C_{max} and resting tension (n=4 preparations at each concentration, data not shown).

In contrast, a one minute exposure to $10\mu M$ HOCl had an immediate effect (Figure 4.1). Applying $10\mu M$ HOCl during a maximum calcium-induced contraction
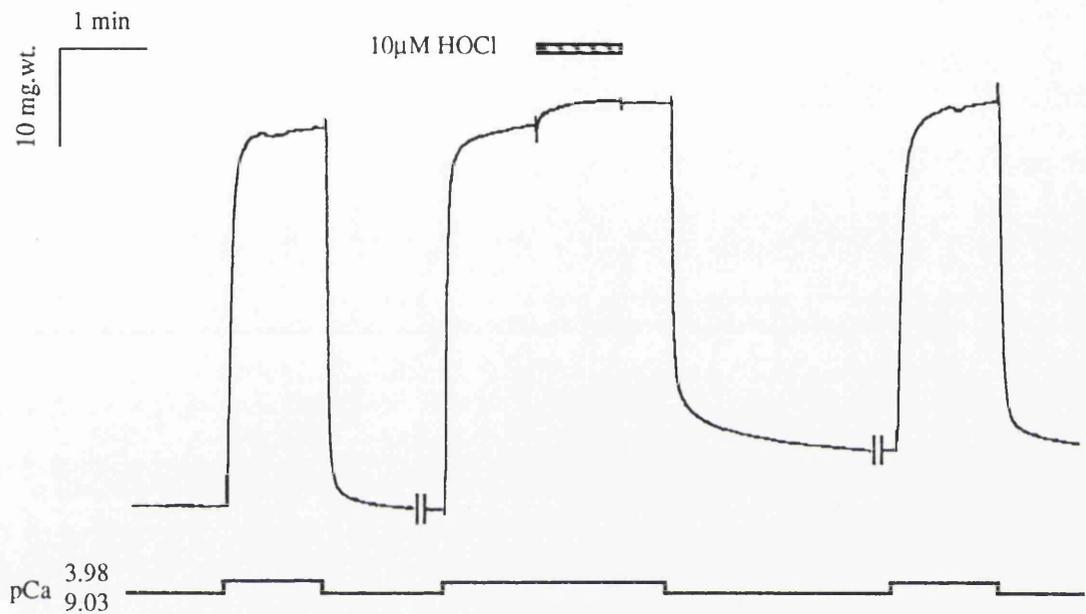
Figure 4.1 Experimental traces illustrating the effect of 10μM HOCl on calcium-activated force and resting tension. The preparation was activated and relaxed by altering the calcium concentration as indicated below the trace. This manoeuvre was repeated except that, once tension had stabilised, 10μM HOCl was applied (hatched bar). Subsequently, resting tension did not return to control level, but once stable, the muscle was fully activated again. Gaps in the trace indicate periods in 'relaxing' solution of five to ten minutes.

increased C_{max}. After relaxing the preparation, with HOCl absent, resting tension failed to return to control levels. The *absolute* tension achieved by preparations in subsequent activations did not change significantly at this dose regime. However, as resting tension increased (from nominally zero to 15.4±8.0% of control C_{max}, n=5) the *relative* level of calcium-activated force diminished (by 18.8±5.8%).

At 50μM HOCl, applied at pCa 3.98 for one minute, C_{max} was reduced by 42.3±23.3% after the exposure and resting tension increased (to 25.0±10.7% of control C_{max}, n=5). Therefore a decrease in the *absolute* level of tension generated was observed (Figure 4.2). In these experiments it was noted that, in addition to the depression of C_{max}, 50μM HOCl increased the time to half-maximal relaxation from a mean value of 1.07 (range 0.4-1.6) to 4.90 (range 0.8-16.2) seconds, ($p < 0.002$, Mann-Whitney U, n=11). Time to half-maximal activation was also increased on exposure to HOCl from a mean value of 1.04 (range 0.5-1.3) to 1.63 (range 0.5-3.7) seconds, but not significantly ($p > 0.06$), in the same preparations. The actions of HOCl were found to be time as well as dose dependant (data not shown).

Applying HOCl to muscle in the relaxed state produced effects similar to that found during a calcium-activation. In one experiment, application of 10μM HOCl for one minute in 'relaxing' solution did not result in an immediate rise in the resting tension. However, the subsequent C_{max} was increased by approximately 10% and, on relaxing the preparation, resting tension did not fall to pre-activation levels (resting tension increased to approximately 7% of the control C_{max}). Similar results were obtained in three other preparations.
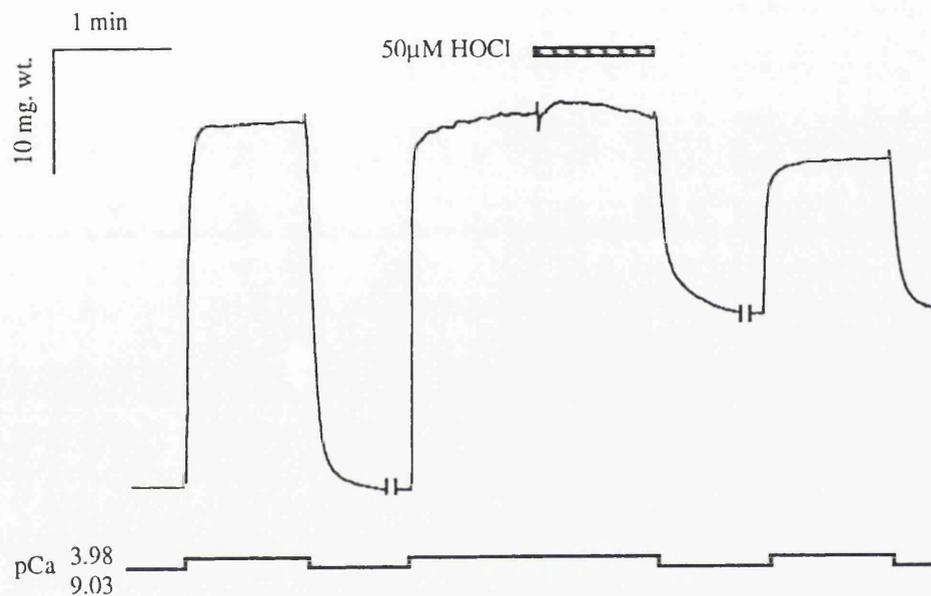
Figure 4.2 Experimental traces demonstrating the effect of 50μM HOCl on calcium-activated force and resting tension. The same protocol was followed as in Figure 1 except that 50μM HOCl was applied during the maximum calcium-activation, as indicated by the hatched bar above the trace. Gaps in the trace indicate periods in 'relaxing' solution of five to ten minutes.

The effects of hypochlorous acid and hydrogen peroxide on myofilament calcium sensitivity

The hypothesis that the reactive oxygen species hypochlorous acid and hydrogen peroxide alter myofilament calcium sensitivity was tested by studying submaximal calcium-activations in Triton-skinned preparations. At concentrations of up to 10mM and incubation periods of up to 60 minutes, hydrogen peroxide had little effect on the myofilament calcium-sensitivity. Log $K_{1/2}$ increased by 0.027 from 5.331 to 5.358 and h from 3.78 to 4.18 in one experiment (Figure 4.3) with similar results being obtained in three other preparations.

However, after a one minute exposure to 10μM HOCl during a maximum calcium-activation, there was a mean increase in log $K_{1/2}$ of 0.091±0.027 (n=5) from a mean of 5.231 to 5.321 and h from 3.20 to 3.27 (Figure 4.4). This demonstrates that calcium sensitivity increases despite a 20% fall in peak *active* force. Using the same protocol, 50μM HOCl produced a greater increase in calcium sensitivity; the mean increase in log $K_{1/2}$ was 0.238±0.066 (n=3) from a mean value of 5.230 to 5.468 and h from 3.20 to 2.37 (Figure 4.4).
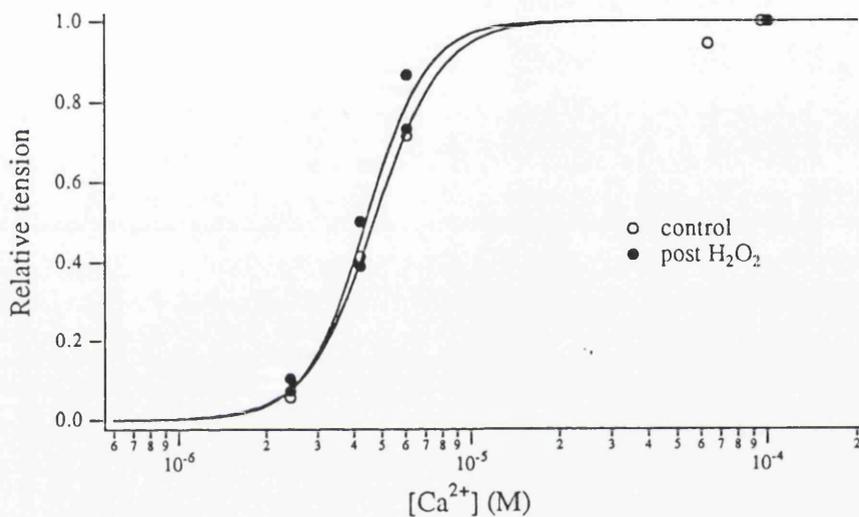
Figure 4.3 Graph showing the effect of 10mM H_2O_2 on calcium sensitivity. The ordinate shows the *relative* tension developed by a typical preparation. The abscissa shows the calcium concentration at which the preparation was activated. Tension is expressed relative to C_{max} obtained for the series of activations. The preparation was exposed to 10mM H_2O_2 for 60 minutes in total, with one series of sub-maximal activations made at 30 and a second at 60 minutes. The best-fit curve for control activations (open circles) and the average of both activations after H_2O_2 (closed circles) are shown.
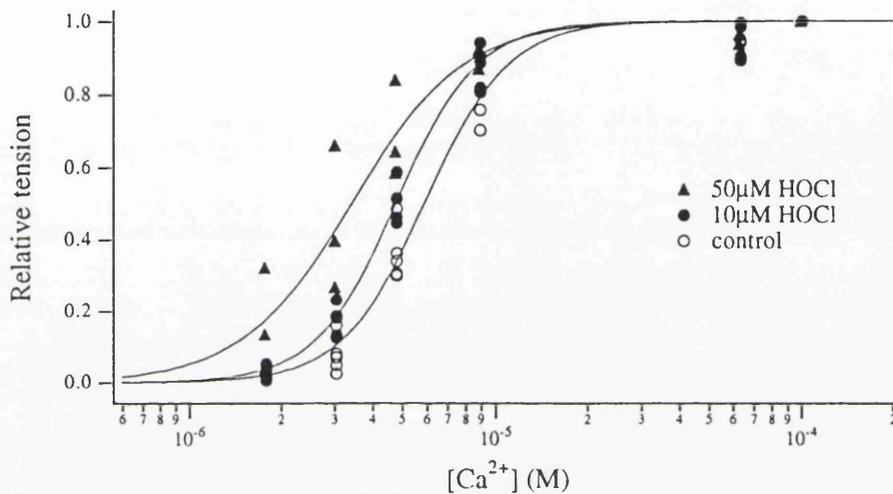
Figure 4.4 Graph showing the effect of 10μM and 50μM HOCl on calcium sensitivity. The ordinate shows the *relative* tension developed by the preparations. The abscissa shows calcium concentration at which the preparations were activated. Tensions are expressed relative to C_{max} obtained for that series of activations: open circles (control), closed circles (after 10μM HOCl), closed triangles (after 50μM HOCl). In each case, the preparation was exposed to HOCl for 1 minute at pCa 3.98. The curves are drawn according to the mean $K_{1/2}$ and Hill coefficient separately determined for each preparation and for each concentration of HOCl applied.

The effects of hypochlorous acid and hydrogen peroxide on the crossbridges in rigor

Hydrogen peroxide had no effect on the level of maintained force when applied for two minutes to a preparation during an established rigor contracture. Subsequent to such an exposure there was no effect on maximum calcium-activated contracture or resting tension (data not shown).

As demonstrated in Figure 4.5, when 50μM HOCl was applied during a rigor contracture its amplitude was not altered but the subsequent C_{max} was diminished (n=11; quantification of this effect is addressed in the conclusions at the end of this chapter). However, unlike the effect of HOCl application during calcium-activation, resting tension generally did not increase after either the rigor contracture or the subsequent maximum calcium-activation. (On some occasions the resting tension increased slightly immediately after the rigor contracture. In some other preparations resting tension increased, but only after a subsequent maximum calcium-activation). With this protocol, exposure during a rigor contracture increased calcium sensitivity in line with the results reported above, but irrespective of what changes to resting tension had occurred. However, the amplitude of subsequent rigor contractions was markedly increased in all preparations. These increases were observed regardless of whether resting tension had changed, or calcium activation was made before or after the rigor contracture subsequent to exposure to HOCl. Repeated exposure to HOCl, independent of the protocol of application, can result in rigor force surpassing calcium-activated force (n=5 preparations, data not shown).
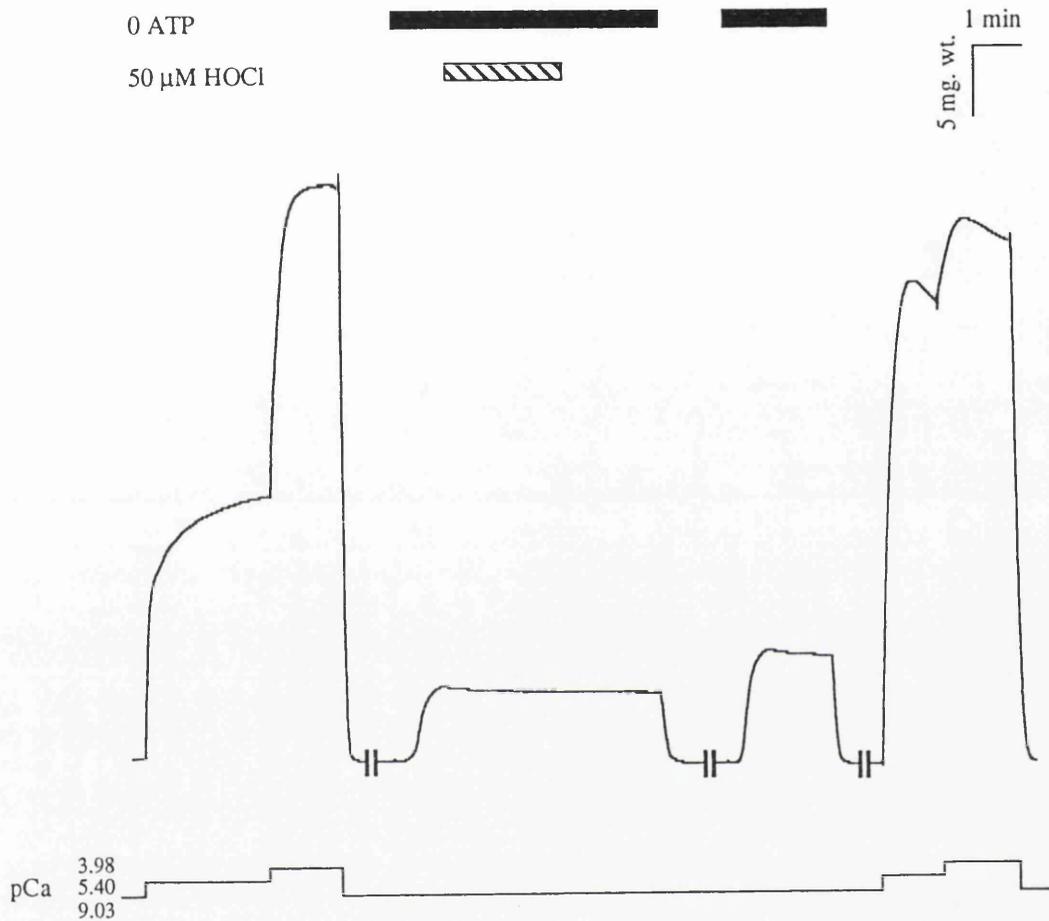
Figure 4.5 Experimental traces demonstrating the effect of 50μM HOCl during a rigor contracture. The preparation was activated and relaxed by altering the calcium concentration as indicated below the trace. A rigor contracture was induced by removing ATP from the bathing medium, as indicated by the solid bar above the trace. The preparation was exposed to 50μM HOCl (for 2 minutes and washed-off for 2 minutes) during an established rigor contracture, as indicated by the hatched bar above the trace. A second rigor contracture was induced after exposing the preparation to HOCl. Gaps in the trace indicate periods in 'relaxing' solution of five to ten minutes.

The effect of caffeine, decreased pH and increased phosphate concentration on the HOCl-induced increase in resting tension

The nature of the resting tension rise seen on exposure to HOCl during a maximum calcium-activated contracture was investigated. The possibility that calcium-independent 'active' force or a rigor-like 'inactive' force was responsible for the resting tension rise was tested by conditions known to influence these two mechanisms. Caffeine (20mM), acidic pH and phosphate (30mM) were found to have no effect on the resting tension in preparations where it had already been increased by a prior exposure to HOCl during a maximum calcium-activation. However, if pH was reduced to 6.3 prior to, and during, the exposure to HOCl, resting tension subsequently rose to only $8.0\pm2.9\%$ of the control C_{max} at pH 7.0. This compares with a $25.0\pm10.7\%$ increase in resting tension ($p<0.015$, unpaired t-test, n=5) at pH 7.0 despite a four-fold increase in the concentration of NaOCl added to the experimental solutions. However, the fall in C_{max} was still apparent (Figure 4.6). There was no further increase in the resting tension or fall in C_{max} on activating the preparation at pH 7.0 after such manoeuvres. Reducing the pH to 6.3 resulted in a 25% drop in the level of maximum calcium-activated force and is consistent with previously reported effects of pH (Fabiato and Fabiato, 1978a). Caffeine and phosphate were without effect when applied prior to, and during, exposure to $50\mu M$ HOCl.

Figure 4.6 Experimental traces demonstrating the effect of acidic pH on the resting tension rise associated with exposing the preparation to HOCl during calcium-activation. The preparation was activated and relaxed by altering the calcium concentration as indicated below the trace. This manoeuvre was repeated except that pH was reduced to pH 6.3 in the bathing medium, as indicated by the solid bar above the trace. 200μM HOCl was applied during the maximum calcium-activation at pH 6.3, as indicated by the hatched bar above the trace. Gaps in the trace indicate periods in 'relaxing' solution of five to ten minutes.

Conclusions

There is wide interest in the possibility that oxygen-derived free-radicals and non-radical reactive oxygen species, produced during ischaemia and reperfusion (pathological or iatrogenic in origin), can prejudice myocardial function. The sites of actions for such species are thought to include the sarcolemma, intracellular membrane systems such as the sarcoplasmic reticulum and the contractile proteins.

There are a number of sites *in vivo* at which hydrogen peroxide, one of the reactive oxygen species studied here, might be produced, *e.g.* within the phagocyte vacuole and within the myocyte mitochondrion (Turrens, Beconi, Barilla, Chavez and McCord, 1991). Physiological concentrations of hydrogen peroxide will not exceed a few micromolar. At these concentrations, even after prolonged exposures, H_2O_2 had no effect on the myofilaments. At much higher concentrations (10mM), and then only after prolonged exposures, hydrogen peroxide marginally increased resting tension, C_{max} and calcium sensitivity. Such concentrations would not be expected even in pathological situations and it can therefore be concluded that hydrogen peroxide has no effects of pathophysiological significance on the myofilaments. These findings support the conclusions of the previous chapter that the superoxide anion, and not the downstream production of hydrogen peroxide, was responsible for depressing maximum calcium-activated force and explains why superoxide dismutase could confer protection from the effects of O_2^- without requiring the addition of catalase.

The local concentration of HOCl produced by activated neutrophils is estimated to be between 60 and 90μM (Wasil, Halliwell, Moorhouse, Hutchison and Baum, 1987; Kalyanaraman and Sohnie, 1985). HOCl will diffuse from the site of its release and might cause more remote damage; concentrations as low as 10-20μM can oxidise membrane proteins (Schraufstätter, Browne, Harris, Hyslop, Jackson, Quehenberger and Cochrane, 1990). At 10μM, which could be attained physiologically some distance from the site of release, a one minute exposure to HOCl led to a significant change in resting

tension, C_{max} and calcium sensitivity of the preparations. At 50μM, which is still lower than *in vivo* extracellular concentrations sustained by activated neutrophils, the effects were much greater.

Since HOCl is generated outside the myocyte, by lymphocytes, it would be unlikely to reach the myofilaments directly. However, as HOCl is a weak acid, at physiological pH but also especially after an ischaemic episode when acidosis prevails, the major portion of the species will be undissociated and permeable. The intracellular concentration of HOCl obtained after neutrophil activation has yet to be determined and might be lower than those used here. However, the results described here relate to short exposures to HOCl whose effects are time, as well as dose, dependant so sustained neutrophil activation could give rise to the effects described here.

The concentration of NaOCl in the experimental solutions was raised in those experiments made at acidic pH (Figure 4.6) to ensure that the hypochlorite anion concentration was initially the same as at pH 7.0 and that the [HOCl] was greater to compensate for any degradation. Despite the substantial increase in the concentration of HOCl, the resting tension increment induced was considerably smaller than at pH 7.0. These findings are consistent with the resting tension increase being due to 'rigor-like' crossbridges forming during exposure to HOCl.

The relative and absolute magnitudes of the change in peak calcium-activated force after HOCl exposure require further consideration. After applying 10μM, C_{max} was reduced as reported above, but superposed on a raised resting tension of similar magnitude to the C_{max} reduction; thus the maximum *absolute* force at maximum calcium-activation was little changed. After applying 50μM HOCl, C_{max} was reduced further, but resting tension increased more so that *absolute* force at maximum calcium-activation did fall, but only by about 17%. It may be significant that the reduction in C_{max} that occurs after exposure to HOCl during rigor is of the same magnitude, but not always associated with any change in resting tension. It is concluded

that the actions of HOCl affecting the *absolute* force that the preparation can sustain are unrelated to the more variable effects on resting tension.

Titus *et al* demonstrated an increase in myosin ATPase activity at a low calcium concentration (Titus, Ashiba and Szent-Gyorgi, 1989). The increase in resting tension observed in this study could be consistent with an increased *acto*myosin ATPase activity as demonstrated by Williams and Swenson (1982) via an apparent increase in calcium sensitivity. However, this increase in resting tension is not likely to be calcium-activated force since caffeine, phosphate or pH, all of which have a marked effect on calcium-activated force, were shown to be without effect on the increased resting tension after it had been established. These results also preclude calcium-independent force being the causative factor in the resting tension rise. Rigor tension can be affected by pH and [P_i] but only during the time when the rigor contracture is developing (Steele and Smith, 1992). Figure 4.6, and the associated text in the results section of this chapter, demonstrates that altering pH during a calcium-activated contracture while exposing the preparation to HOCl diminishes the subsequent increase in resting tension. These results could be consistent with promotion of a 'rigor-like' crossbridge formed by HOCl.

The results of the 'rigor' experiments described here are difficult to interpret since we only occasionally find an increase in resting tension after an exposure to HOCl during a 'rigor' contracture. Any subsequent rigor contracture is potentiated, calcium sensitivity increased and peak force depressed, as with other protocols. This could imply that calcium-activation or crossbridge cycling is necessary for the effects of HOCl to be completed but logically they require some form of activation to be revealed. One would hypothesise that a dormant chemical alteration occurs, acting like a 'memory' of the exposure to HOCl, the effects eventually revealed being dependant upon the changes in spatial orientation of the myofilament lattice associated with the subsequent crossbridge activation either by calcium or zero ATP. The increase in the relative and absolute force of the rigor contracture, after these changes have developed (Figure 4.5), reflects rigor-bond formation against a stiffer preparation. Such increases would be independent

of the nature of the resting tension increase. *i.e.* whether the increase in resting tension was due to 'rigor-bond' formation, calcium-dependent or calcium-independent force (Steele, 1990). However, the previous discussion leads us to conclude that 'rigor-like' crossbridges are involved.

If such crossbridges are always induced during HOCl exposure, independent of the precise protocol involved, it could explain the increased resting tension except that this is only reliably observed after calcium-induced activation. However, the force maintained by a population of 'rigor-like' crossbridges will be markedly different depending on the circumstances prevailing when they were induced. During a rigor contracture, it is generally assumed that *all* possible crossbridges link myosin to actin, whereas during calcium-induced activation, only a fraction of the crossbridges are attached at any one time. However, the forces achieved are quite different. In the case of rigor, under the standard conditions of these experiments, 100% of the crossbridges sustain only about 20-25% of C_{max}. However, full calcium activation might typically involve only 20% of crossbridges attached at any moment. If, for example, one twentieth of crossbridges were permanently converted to the 'rigor-like' state by HOCl (*i.e.* unable to detach), after rigor this fraction would retain around 4-5% of rigor tension *i.e.* only 1-1.25% of C_{max}. After a maximum calcium-activation, however, this same population would retain up to 25% of C_{max} (since at maximal activation just 20% of the crossbridges are responsible for 100% of the force). The precise force sustained by the HOCl-induced crossbridges is variable since it depends upon the other crossbridge interactions prevailing when this type of link forms. This explanation would also account for the increase in calcium sensitivity caused by HOCl, since permanently attached crossbridges will have even greater effect than slowly cycling crossbridges in this regard (Bremel and Weber, 1972).

The possibility of rigor force exceeding calcium-activated force after recurrent exposures to HOCl is also consistent with the discussion given above. At micromolar [$MgATP^{2-}$] the regulation of contraction is more complex than simple troponin and tropomyosin inhibition of the actin and myosin interaction. It is proposed that, when free

of nucleotide, myosin binds actin with a high affinity forming rigor crossbridges (Bremel and Weber, 1972). These crossbridges give rise to a co-operative response disinhibiting the thin filament (after attachment of a critical number of crossbridges) which allows ATP hydrolysis and force production in the effective absence of calcium. In the experiments described here, ATP carried over in the preparations during a solution change to ATP free media will initially diffuse from the preparation and the local [ATP] will fall sufficiently to allow myosin to bind to actin and disinhibit the thin filament. After an exposure to HOCl it is proposed that 'rigor-like' crossbridges will already be attached and may exceed the critical number to enable force production. Rigor bridges may also exist in two states, the ratio of strained (highly stiff) and unstrained (moderately stiff) rigor crossbridges influence the final level of tension produced. These points could perhaps be resolved by measurements of preparation stiffness to yield information about crossbridge attachment and these experiments are planned.

The fact that calcium sensitivity was increased, but C_{max} fell, after HOCl exposure reinforces the idea that attachment of crossbridges *per se* may be more important in influencing calcium sensitivity than the force they generate. An analogous effect was reported for caffeine by Wendt and Stephenson (1983). The apparent disparity between alterations of calcium sensitivity and contractile function could also have important clinical consequences. Homeostatic physiological responses *in vivo* to depressed contractile function in the post-ischaemic period, such as increased adrenergic drive, tend to increase intracellular calcium concentration. In such circumstances any increase in diastolic levels of calcium, in conjunction with an increase in calcium sensitivity, might contribute to diastolic dysfunction which has been noted as an important manifestation of reperfusion injury (Charlat, O'Neill, Hartley, Roberts and Bolli, 1989).

The myosin molecules of the thick filament contain many sulphydryl groups which can be covalently modified by oxidising agents (Reisler, 1982; Wells and Yount, 1982; Chaen, Shimada and Sugi, 1985; Ishiwata, Kinosita, Yoshimura and Ikegami, 1987; Titus *et al*, 1989). Such modifications are widely used to attach fluorescent and

spin labels to components of the contractile proteins in order to report position and conformational changes of these structures during activation. Since several reactive sulphydryls occur at functionally important positions on the myofilaments, modification of these sites can produce a variety of changes in the physiological properties of muscle activation (Moss, Giulian and Greaser, 1982; Williams and Swenson, 1982; Crowder and Cooke, 1984; Wilson, dos Remedios, Stephenson and Williams, 1991). The increase in calcium-sensitivity by the oxidative species HOCl noted in this study (Figure 4.4) could be consistent with modification of the ATPase-related myosin head reactive thiol SH_1, found on the 20kD C terminal sequence of myosin subfragment 1 (Rajasekharan, Sivaramakrishnan and Burke, 1987). Such modification would be analogous to the modification of the Ca^{2+}-ATPase of the SR by oxidative stress (Scherer and Deamer, 1986; Kukreja, Weaver and Hess, 1989). Titus *et al* (1989) reported an increase in myosin ATPase activity, at low $[Ca^{2+}]$, when SH_1 was modified using either $[^{14}C]$-iodoacetamide, 5-(2((iodoacetyl)amino)ethyl) aminoapthalen-1-sulphonic acid (IAEDANS) or 4-(2-iodoacetamido-2,2,6,6-tetramethyl piperidinooxyl (IASL) in glycerinated rabbit skeletal fibres. Modification of rabbit SH_1 has been shown to alter the rate limiting step of the crossbridge cycle (*i.e.* the rate of inorganic phosphate release) (Sleep, Trybus, Johnson and Taylor, 1981) but not the ability of S_1-ADP to bind to actin in a co-operative manner (Green and Eisenberg, 1988). Within the experiments described here alterations to $t_{0.5 \text{ up}}$ and $t_{0.5 \text{ down}}$ might reflect alterations in the rate limiting step which prolongs S_1-ADP binding to actin producing the increase in apparent calcium sensitivity (Güth and Potter, 1987). 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) has been found to affect not only C_{max} but also calcium sensitivity in a complex *fibre-type specific* way that was partially reversible by dithiothreitol (Wilson, dos Remedios, Stephenson and Williams, 1991). A strongly attached SH_1-modified crossbridge would increase calcium sensitivity as proposed by Bremel and Weber (1972) and demonstrated by Güth and Potter (1987) for the rigor crossbridge.

A second possible site on the myosin head for modification by HOCl is another reactive thiol (SH_2) which lies ten residues from SH_1 on the 20kD domain. The region of the S_1 subfragment of myosin containing both these reactive thiols are close to both the other 27 and 50kD domains of S_1 (see Audermand, Bertrand, Bonet, Chaussepied and Mornet, 1988, for review). Furthermore, cross-linking both of these reactive thiols can result in nucleotide entrapment (Dalby, Weiel and Young, 1983). NMR spectral studies have demonstrated that the addition of F-actin to S_1, where SH_1 and SH_2 have been cross-linked, shows the characteristics of rigor complex formation (Highsmith *et al*, 1979; Prince *et al*, 1981).

The previous chapter discussed possible sites of action for O_2^- on the myofilaments. The common feature to both forms of free radical and reactive oxygen species damage is a depression in C_{max}. The discussion above suggests that the 20kD C terminal sequence of myosin subfragment 1 is the most probable site of action for HOCl. As alluded to in the previous chapter, the exact site of O_2^- action would appear unrelated to that of HOCl due to the differential effects on calcium sensitivity. However, the possibility still exists for different residues to be covalently modified by these two species. As suggested previously, the O_2^- site of action would appear very specific and is probably not one of the thiol groups postulated for HOCl (since O_2^- is unreactive with thiol groups, see chapter 8). In contrast the site of HOCl activity need not be so specific. Although tension is unaffected by HOCl exposure during a rigor contracture, the effects on C_{max} and calcium sensitivity are subsequently revealed. It is possible that HOCl exposure may have an allosteric effect *i.e.* SH_1 and SH_2 may be cross-linked, not necessarily to each other, and as a consequence effect the residue responsible for C_{max} reduction in an analogous way to O_2^-, and which could be inaccessible direct modification in the attached state.

The use of dithiothreitol (DTT), an agent which maintains thiol groups in a reduced state or reduces disulphides, did not restore function after exposure to HOCl. Such results would appear to be inconsistent with HOCl acting to modify thiol groups on

the myofilaments. However, the similarities described here (and the protective action of captopril to be described in chapter 8) for thiol modifying agents such as IAEDANS, IASL and DTNB would seem too strong to deny a role for thiol modification as a result of HOCl exposure. It is possible that the modification that occurs is insensitive to DTT, *i.e.* it does not form a disulphide cross-link, since the effects of DTNB were only partially reversed by its application (Wilson *et al*, 1990).

Another limitation to the findings presented in this study is that contractile dysfunction occurs after ischaemia in 'buffer-perfused' isolated heart systems which reveals that neutrophils are not required for the development of 'stunning'. However, the phenomenon of 'stunning' appears to be multifactorial so that neutrophil activation could contribute to the well established clinical manifestations of this condition. Whatever the mechanism of HOCl-induced abnormalities described here, these effects are consistent with the clinical observations noted in post-ischaemic myocardial dysfunction *i.e.* compromised systolic and diastolic function.

Chapter 5: Alterations in Sarcoplasmic Reticulum Calcium Handling due to Free Radicals and Reactive Oxygen Species.

Introduction

The previous chapters have demonstrated that oxygen-derived free radicals and reactive oxygen species can contribute to the depression of contractility noted in the clinical condition of myocardial stunning. Reperfusion of ischaemic myocardium can, additionally, elicit serious ventricular arrhythmias (Manning and Hearse, 1984; Pogwizd and Corr, 1986). Given the observations that such developed arrhythmias are ameliorated by only a few of the drugs which influence ischaemia-induced arrhythmias (Manning and Hearse, 1984), it has been suggested that other or additional pathogenic mechanisms may be involved.

Recent interest has focused on the suggestion that oxygen-derived free radicals and reactive oxygen species may play a role in the initiation of reperfusion-induced arrhythmias. Support for such a hypothesis has been provided by two distinct observations:

1. Free radical scavengers are able to inhibit reperfusion-induced ventricular fibrillation (Woodward and Zakaria, 1985; Bernier, Hearse and Manning, 1986).

2. Free radical generating systems have been demonstrated to have a pro-arrhythmic effect (Bernier, Hearse and Manning, 1986; Pallandi, Perry and Campbell, 1987; Kusama, Bernier and Hearse, 1989).

In addition to these observations, oxygen-derived free radicals and reactive oxygen species can produce peroxidation of the polyunsaturated fatty acids contained in cellular membranes (Thompson and Hess, 1986). Such damage would impair selective membrane permeability and interfere with the function of various cellular organelles.

Evidence for the occurrence of lipid peroxidation in the stunned myocardium has been provided by three recent studies. Initially, an increase in hydroxy conjugated dienes (the products of free fatty acid oxidation) was noted during and after 45 minutes of normothermic ischaemia in open-chest dogs (Romaschin, Rebeyka, Wilson and Mickle,

1987). The maximum concentrations of these products was measured at five minutes of reperfusion. The tissue was dysfunctional, but not necrotic, on examination after reperfusion and thus represented the stunned myocardium. Subsequent to this observation it has been reported that patients undergoing cardioplegic arrest during cardiopulmonary bypass surgery had increased concentrations of conjugated dienes at 3 and 60 minutes of reperfusion (Weisel, Mickle, Finkle, Tumiati, Madonik, Ivanow, Burton and Ingold, 1989). Furthermore, patients with unstable angina (which is characterised by recurring episodes of myocardial ischaemia and reperfusion) have demonstrated increased measures of thiobarbituric acid related substances, another indicator of lipid peroxidation (McMurray, Chopra, Abdullah, Smith and Dargie, 1992).

Sarcoplasmic reticulum (SR) isolated from stunned myocardium has been demonstrated to have a decreased ability to transport calcium concomitant with a reduced Ca^{2+},Mg^{2+}-ATPase activity (Krause, Jacobus and Becker, 1989). These researchers proposed that decreasing the SR calcium store could result in depressed contractile protein activation via an attenuated calcium release during systolic activation. This proposal appears to be an attractive hypothesis. The relative calcium deficiency would be consistent with the published observations that exogenously administered calcium can return contractile function to pre-ischaemic values (Ito, Tate, Kobayashi and Schaper, 1987). It would also be consistent with the notion that inotropic agents (which increase $[Ca^{2+}]_i$) can reverse myocardial stunning (Becker, Irvine, Di Paula, Guarnieri and Aversano, 1986; Ellis, Wynne, Braunwald, Henschke, Sandor and Kloner, 1984). However, since this hypothesis implies that the amplitude of the $[Ca^{2+}]_i$ transient is decreased, it is not easily reconcilable with *in vitro* data (Kuosaka, Koretsune, Chako, Weisfeldt and Marban, 1990).

The reduction in calcium-activated force by oxygen-derived free radicals and reactive oxygen species due to myofilament dysfunction would not be inconsistent with the inotropic responsiveness in the intact preparation and *in vivo*. Figure 3.7 and the

associated text demonstrates that sub-maximal force reduced by a depression in C_{max} could be increased by mechanisms which increase $[Ca^{2+}]_i$.

A similar decrease in calcium uptake and Ca^{2+},Mg^{2+}-ATPase activity has been observed in isolated SR after exposure to free radicals and reactive oxygen species. In addition, the use of free radical scavengers has been demonstrated to preserve SR function (Thompson and Hess, 1986; Rowe, Manson, Caplan and Hess, 1983). Other studies suggest that the sarcolemma may be a critical target of free radical and reactive oxygen species damage. This is especially important since cardiac muscle is generally considered to be dependant upon sarcolemmal calcium influx along with a 'greater' SR calcium release to activate the myofilaments, *i.e.* the process of Ca^{2+}-induced Ca^{2+}-release (Fabiato, 1989). These species have been shown to interfere with calcium transport and calcium-stimulated ATPase activity in the sarcolemma (Kaneko, Elimban and Dhalla, 1989). They have also been demonstrated to interfere with Na^{+}/Ca^{2+} exchange (Reeves, Bailey and Hale, 1986; Okabe, Fujimaki, Murayama and Ito, 1989) and to inhibit Na^{+}/K^{+}-ATPase activity (Kramer, Mak and Weglicki, 1984). Furthermore, Na^{+}/K^{+}-ATPase activity is impaired in the reperfused myocardium (Kim and Akera, 1987) and could result in sodium overload with the consequent activation of the Na^{+}/Ca^{2+} exchange.

All these observations imply that free radicals and reactive oxygen species could result in increased transarcolemmal calcium influx and cellular calcium overload (and would be consistent with the *in vivo* measurements of $[Ca^{2+}]_i$). These postulated mechanisms would alter calcium homeostasis and could reconcile the free radical and calcium-overload hypothesis of post-ischaemic myocardial dysfunction into one pathological mechanism.

Calcium-overload in reperfused myocardium will produce a negative inotropic effect. Fabiato and Fabiato (1972) observed "spontaneous calcium-release" from the SR in skinned rat myocytes when the calcium concentration was raised higher than 100nM.

Also, spontaneous calcium-release has been demonstrated with sodium-pump inhibition (Kort and Lakatta, 1988; Capogrossi, Kort, Spurgeon and Lakatta, 1986). Spontaneous fluctuations in $[Ca^{2+}]_i$ are associated with spontaneous contractile activity. The frequency and amplitude of these oscillations increase with increasing $[Ca^{2+}]_o$ or sodium-pump inhibition.

The functional consequences of these spontaneous SR calcium-releases are important. Experimentally when an activation is made soon after a spontaneous release the $[Ca^{2+}]_i$ transient and the associated contraction are depressed. This weaker calcium-release and activation may be due to a net loss of calcium from the cell or from incomplete mechanical restitution (Ishide, Urayama, Inoue, Komasu and Takashima, 1990). It is important to note that in multi-cellular preparations these spontaneous releases of calcium can occur at random in individual cells. The negative inotropic effect of such releases, however, are not purely additive *i.e.* the negative inotropic effect will be augmented with each increase in the number of overloaded cells. Such augmentation occurs because the smaller calcium release following a spontaneous calcium-release will result in myofilament activation at a 'lower-level' in the overloaded cell. This depressed cell will be more compliant as a result of its weaker activation and could be stretched by adjacent cells which have a greater store of calcium to release and activate their myofilaments. Thus the cell shortening required to stretch this cell will not contribute to the external work of force development.

These spontaneous calcium-releases may also give rise to after-depolarisations by activating a transient inward current (Lederer and Tsein, 1976), The development of oscillatory after-depolarisations can, in turn, lead to triggered arrhythmias in the heart (Wit and Rosen, 1986). While the calcium-overload secondary to sodium-pump inhibition is perhaps the most extensively studied, the same sequelae should occur with other causes of cellular calcium overload.

Previous studies investigating the effects of free radicals and reactive oxygen species on myocardial SR have utilised SR vesicles. The SR is an intracellular membrane-bound compartment which is not continuous with the sarcolemma whose function is to sequester and release calcium into the myoplasm. The autoradiographic studies performed by Winegrad (1965) suggested the terminal cisternae as the site of calcium release from the SR, and were taken to indicate anatomical segregation of transport functions within the SR. Two structural components exist: *i.* junctional or corpuscular SR, including the cisternae which oppose the sarcolemma and the transverse tubules and *ii.* a longitudinal system that is continuous to the junctional SR but surrounds the myofibrils. Obviously SR vesicles formed by homogenisation will lose their structural integrity and form vesicles of both components. It is possible, by differential centrifugation, to form vesicles of junctional SR (which contains calcium channels and pumps) and longitudinal SR (which lack calcium channels but has a high density of calcium pumps). Such a modification to the SR vesicle technique will allow homogeneity in the vesicular membranes. However, as SR Ca^{2+} content is dependant upon the rate of Ca^{2+} uptake (by the pump) and the rate of Ca^{2+} efflux (via the channel), use of such a technique to study relative changes in Ca^{2+} uptake and release appears limited. It is also important to note that the SR interior contains the calcium-binding protein calsequestrin which may be an important factor in increasing the calcium-buffering capacity of the SR and which could be lost in SR vesicles. In addition, the differential centrifugation modification has only rarely been utilised in the previous vesicular studies of free radical and reactive oxygen species damage to the SR and, in general, the solution chemistry of such experiments is highly unphysiological.

The purpose of this chapter, therefore, is to study the effects of free radicals and reactive oxygen species on *in situ* cardiac SR using saponin-treated right ventricular trabeculae isolated from the rat. The benefits of such a study are that the investigation is made in a physiologically relevant environment, the structural integrity of the SR and its matrix are maintained and the mechanical end product of Ca^{2+} release (*i.e.* force

production) can be simultaneously measured. The results of these studies have been published previously in abstract (MacFarlane, Steele, Miller and Smith, 1992; MacFarlane, Steele, Smith and Miller, 1993; MacFarlane, Denvir, Steele and Miller, 1993).

Results

Effect of O_2^- on calcium release from the sarcoplasmic reticulum

The effect of O_2^- (generated by 20mU/ml xanthine oxidase and 50μM hypoxanthine) on caffeine-induced calcium release from the SR is demonstrated in Figure 5.1. Caffeine was injected every 60 seconds and the resultant calcium release from the SR was monitored by indo-1 fluorescence. The amplitude of the caffeine-induced calcium transient can be taken to reflect the calcium content of the SR. Application of O_2^- virtually abolished the tension transient in response to caffeine while the proceeding calcium release was unaffected. Similar results were obtained in three other preparations in which simultaneous measurements of tension and calcium were made.

The O_2^- had no effect upon the caffeine-induced calcium release from the SR. The tension being generated as a result of the calcium release was scaled down due to the depression in calcium-activated force (see chapter 3). Additionally, the time course of the tension transient was slowed, both during activation and relaxation which is consistent with the direct effects of O_2^- on the myofilaments. In other experiments (n>12), where calcium measurements were not made and performed in the standard experimental chamber (see chapter 2 for details), it was observed that it is necessary to increase the xanthine oxidase concentration in the O_2^--generating system by approximately two-fold in these experiments with saponin-treated fibres, in comparison to the 'Triton-skinned' preparations in order to achieve the same effect on calcium-activated force.

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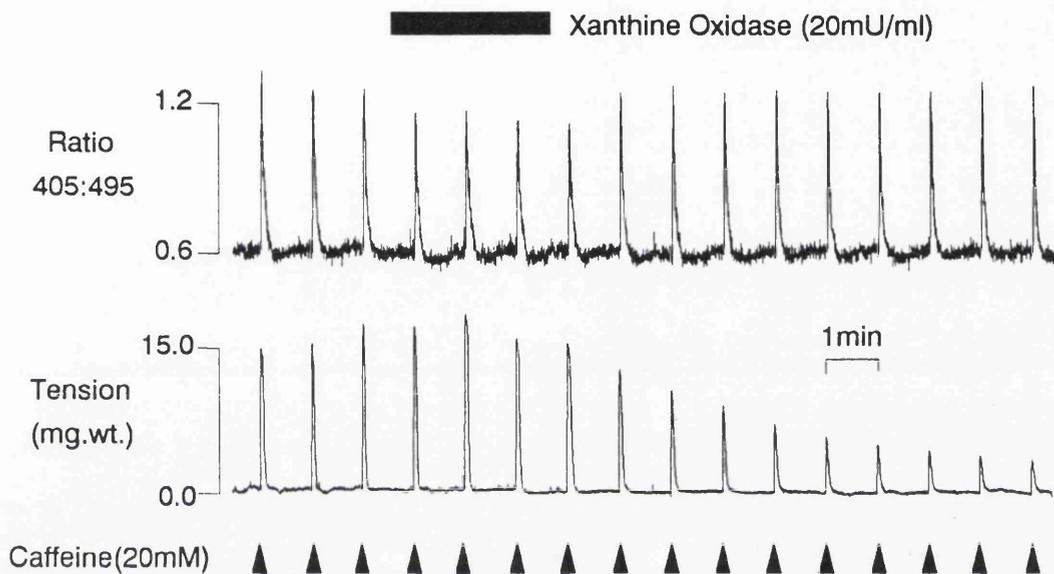
Figure 5.1 Experimental trace showing the effect of O_2^- (generated by 20mU/ml xanthine oxidase and 50µM hypoxanthine) on caffeine-induced calcium release from the SR. Caffeine was injected every 60 seconds (indicated by the arrow head) and the resultant calcium release from the SR was monitored by indo-1 fluoresence. The O_2^--generating system was applied over 3 minutes during cycles of calcium loading of the SR and caffeine-induced calcium release (indicated by the solid bar above the trace). The tension transient in response to caffeine began to fall immediately and was virtually abolished within 10 minutes of the onset of the exposure to O_2^-.

Effect of H$_2$O$_2$ on calcium release from the sarcoplasmic reticulum

The effects of H$_2$O$_2$ on caffeine-induced calcium release are shown in Figure 5.2. The protocol used in these experiments is very similar to that used in Figure 5.1, except that caffeine was injected every 120 seconds and 5mM H$_2$O$_2$ was applied to the preparation. The effects of H$_2$O$_2$ were in marked contrast to that of O$_2^-$. Introduction of H$_2$O$_2$ caused a progressive fall in the caffeine-induced calcium transients (by approximately 50%). In response to the depressed calcium release the peak tension response was diminished (by approximately 25%). At 5mM H$_2$O$_2$ had a slight quenching effect on indo-1 fluorescence and because of this alterations to loading calcium concentration are not readily interpreted. After approximately 3 minutes exposure to the H$_2$O$_2$ a slight increase in resting tension (probably <5% of C$_{max}$) was noted which plateaued after 4 minutes. The time course of the caffeine-induced calcium transient appeared to be slowed. Similar effects were seen in eight other preparations where calcium measurements were not made (Figure 5.2). In such experiments it was noted that the tension response could be diminished within a 10 minute exposure by H$_2$O$_2$ concentrations as low as 1mM (Figure 5.3).

Effect of HOCl on calcium release from the sarcoplasmic reticulum

The effect of 50μM HOCl on caffeine-induced calcium release from the SR is demonstrated in Figure 5.4. The protocol used in these experiments was again similar to that used in Figure 5.1, except that at a concentration of 50μM hypochlorous acid has a marked quenching effect on indo-1 fluorescence. To overcome this problem the concentration of indo-1 in solutions using this species was increased to 6μM. The effect of HOCl on the SR calcium release was in marked contrast to both H$_2$O$_2$ and O$_2^-$. Introduction of HOCl caused a pronounced fall in the caffeine-induced calcium transients. However, despite the reduction in calcium release from the SR, the amplitude of the associated tension responses were little effected. This is due to the calcium sensitising effect of exposure to hypochlorous acid on the myofilaments (see chapter 4). Similar
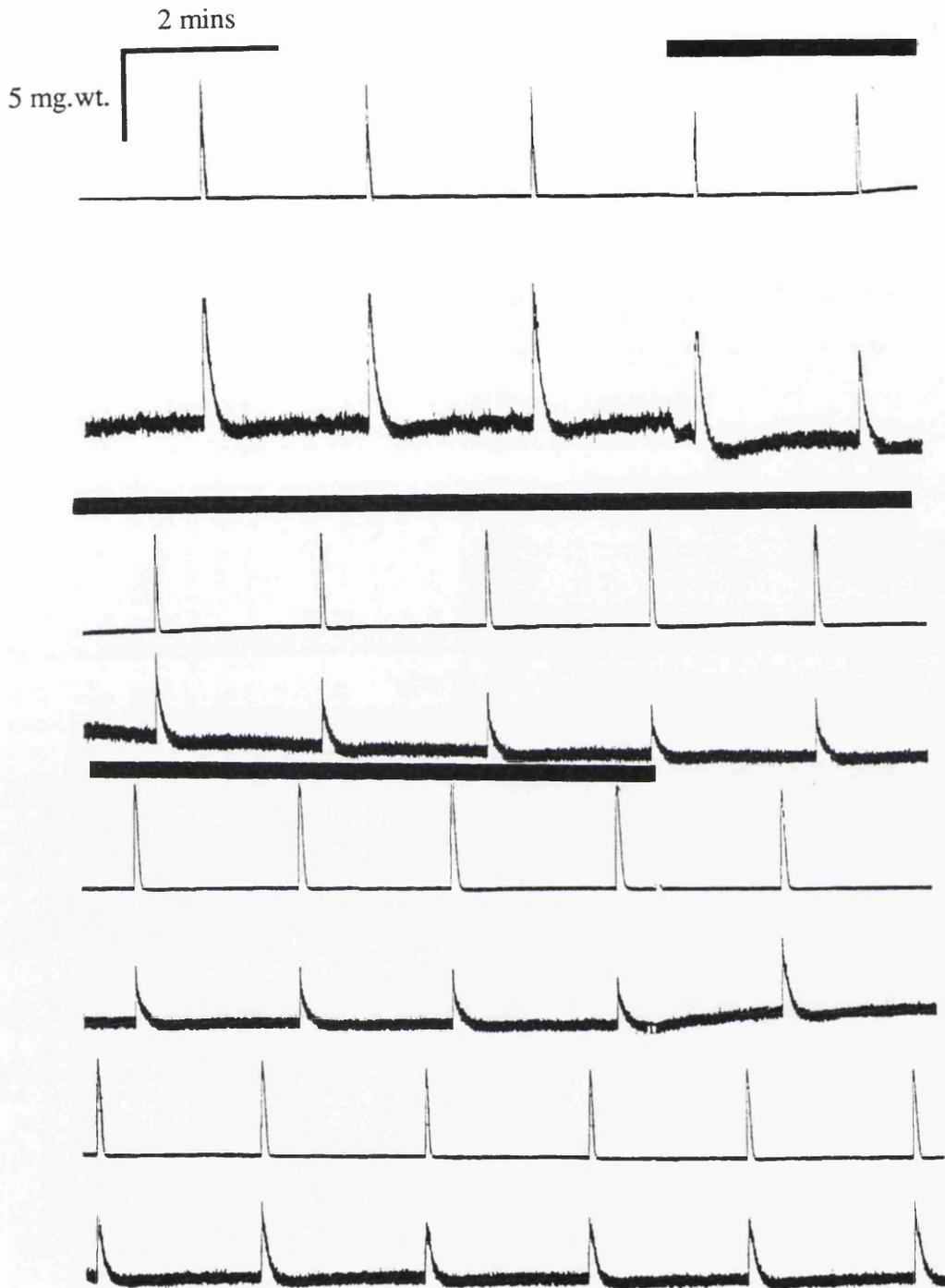
Figure 5.2 Experimental traces showing the effect of H_2O_2 (5mM) on caffeine-induced calcium release from the SR. Caffeine was injected every 120 seconds resulting in calcium release from the SR which was monitored by indo-1 fluoresence. The H_2O_2 was applied for 20 minutes during cycles of calcium loading of the SR and caffeine-induced calcium release (indicated by the solid bar above the trace). There was a 50% fall in caffeine-induced calcium transient and a 25% fall in the associated tension transients.

1mM Hydrogen Peroxide (10 minute exposure)

10 mg. wt.

2 seconds
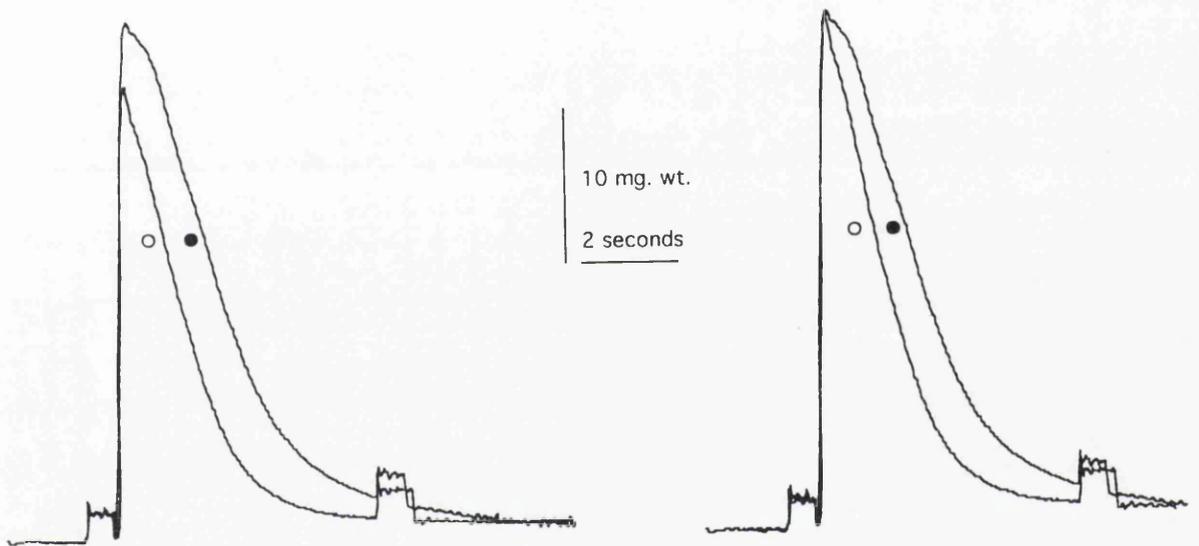
Figure 5.3 Experimental trace demonstrating the effect of a 10 minute exposure to 1mM H_2O_2 on tension generation in a saponin-treated trabecula. The tension response obtained before (solid circle) and after (open circle) H_2O_2 exposure are superimposed. The relative size of the tension responses are shown on the left panel and are scaled to a similar size on the right.
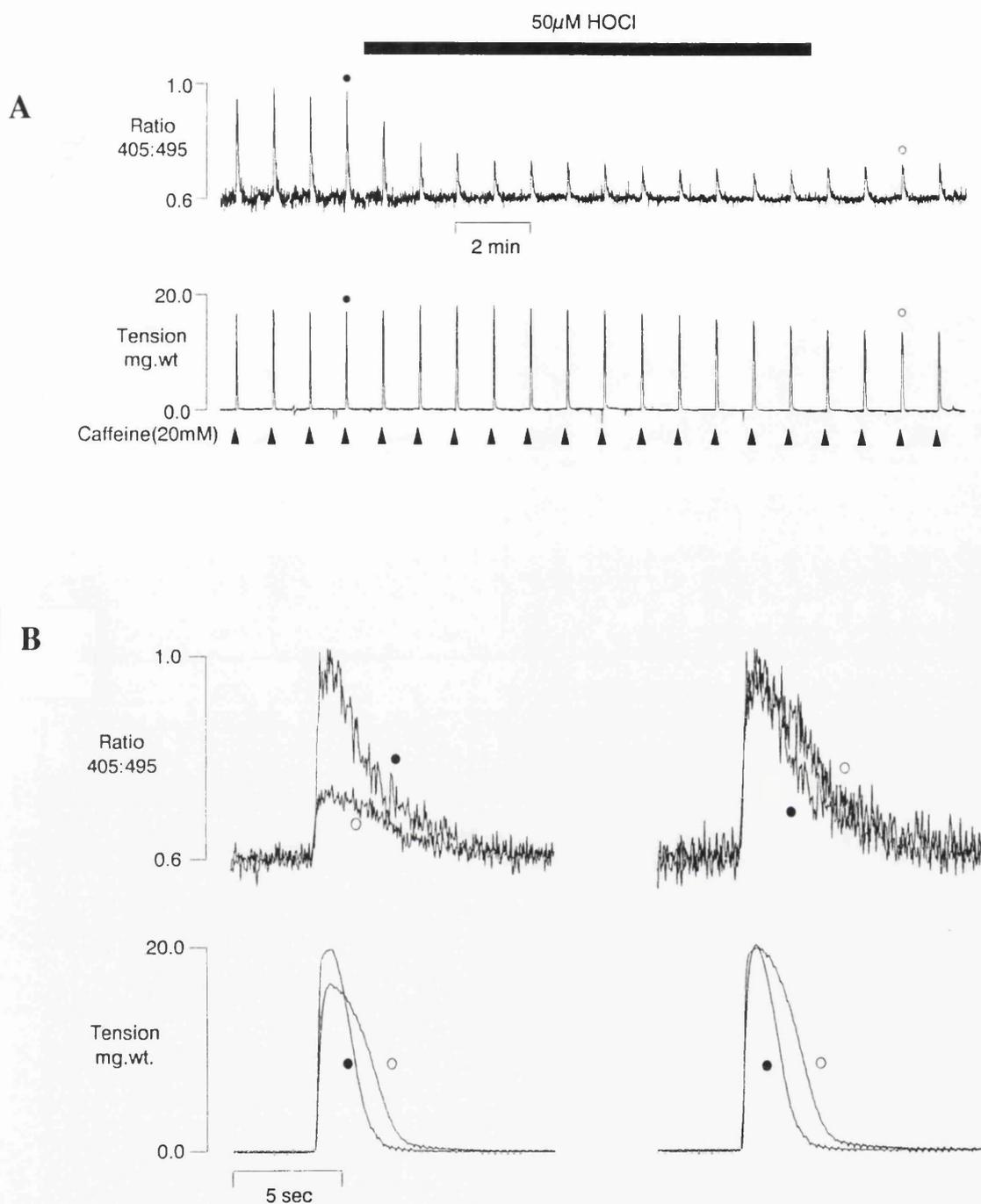
Figure 5.4 Experimental traces showing the effect of HOCl (50μM) on caffeine-induced calcium release from the SR. Caffeine was injected every 60 seconds (indicated by the arrow heads) and the resultant calcium release from the SR was monitored by indo-1 fluoresence. **Panel A**: Demonstrates the protocol in which HOCl was applied for 12 minutes during cycles of calcium loading of the SR and caffeine-induced calcium release (indicated by the solid bar above the trace). There was a pronounced fall in caffeine-induced calcium transient, however, despite this the associated tension transient were little effected. **Panel B**: Shows individual tension and calcium transients before (solid circle) and after (open circle) HOCl exposure.

results were obtained in three other preparations using this protocol of investigation (and at concentrations down to $10\mu M$ HOCl). These effects of HOCl, like those of O_2^-, are not reversible. Function was not restored, nor were the actions of HOCl prevented, by thiol reducing agents including dithiothreitol.

The changes in myofilament calcium sensitivity and the alterations to the level of calcium release make interpretation of the time course of the calcium transient difficult (*i.e.* In Figure 5.4 lower panels the prolonged calcium transient could be due to either an increase in calcium sensitivity or a decrease in SR calcium uptake). For this reason a second experimental protocol was undertaken where the SR loading time was varied. The results of this type of protocol are shown in Figure 5.5. Caffeine was initially injected every 120 seconds and the resultant calcium release from the SR was monitored by fura-2 fluorescence. The period between subsequent caffeine injections was reduced in a stepwise manner (*i.e.* 120 seconds to 60 seconds to 30 seconds to 15 seconds). On this occasion introduction of $50\mu M$ HOCl resulted in a biphasic tension responses. After an initial increase the tension response progressively deteriorated and was subsequently abolished (with a significant increase in resting tension). Once again there was a pronounced fall in the caffeine-induced calcium transient. However, the relationship between the caffeine-induced calcium releases at different SR loading times was little altered. In this case the caffeine-induced calcium release after 15 seconds of loading was only slightly enhanced at 40.0% of the standard 120 second loading period (compared with 34.8% prior to HOCl exposure).
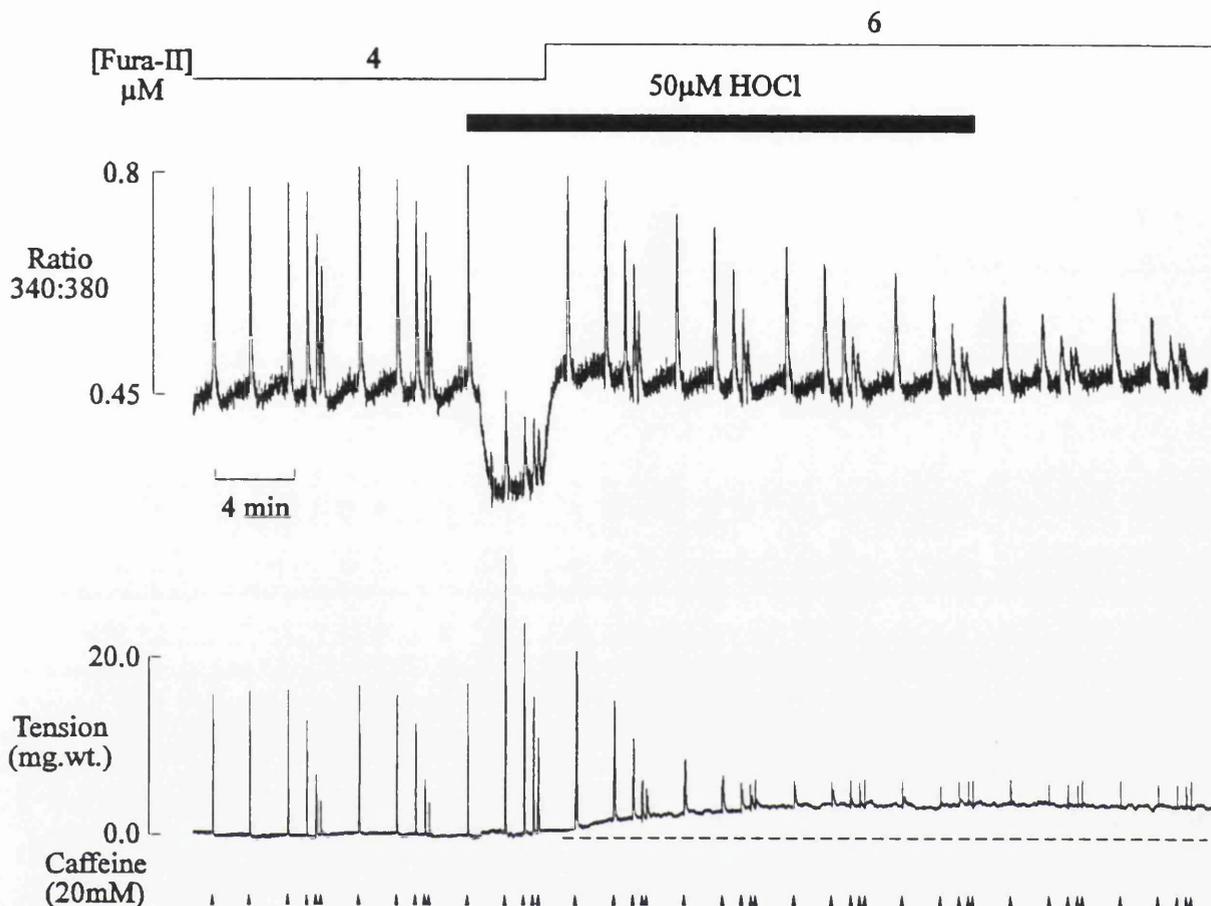
Figure 5.5 Experimental traces showing the effect of HOCl (50μM) on the time course of caffeine-induced calcium release from the SR. Caffeine was initially injected every 120 seconds and then the calcium loading period reduced (indicated by the arrow heads). The resultant calcium release from the SR was monitored by fura-II fluoresence. In this protocol HOCl was applied for 28 minutes during cycles of calcium loading of the SR and caffeine-induced calcium release (indicated by the solid bar above the trace). The HOCl had a marked quenching effect on fura-II fluoresence which was overcome by addition of a further 2μM fura-II to the experimental solutions (indicated by the thin line above the trace). Once again there was a pronounced fall in caffeine-induced calcium transient. However, during this exposure to HOCl the resultant tension responses were abolished and there was a rise in the resting tension.

Susceptibility to spontaneous oscillations associated with free radical mediated mechanisms

Exposing the saponin-permeabilised preparations to HOCl was associated with an apparent increase in the susceptibility to spontaneous releases of calcium. Figure 5.6 demonstrates some of the problems associated with interpreting the appearance of spontaneous oscillations while monitoring only the tension response in cardiac muscle. In this experiment the preparation was 'calcium-loaded' for 2 minutes at approximately 250nM Ca^{2+}. At this $[Ca^{2+}]$ the preparation exhibited very small spontaneous oscillations which became much more apparent after exposing the preparation to $20\mu M$ HOCl. Such an increase in the level of tension generated due to a spontaneous calcium release could be the result of an increase in the amount of calcium released or an increase in the myofilament calcium sensitivity. Therefore, when not directly measuring calcium release from the SR and when applying a substance that increases myofilament calcium sensitivity, the appearance of spontaneous tension oscillations may only reflect calcium releases which were originally present but sub-threshold for tension generation. In six experiments performed where simultaneous measurement of tension generation and calcium release were made only two preparations gave rise to spontaneous calcium oscillations on exposure to HOCl (Figure 5.7) under the standard loading conditions employed.
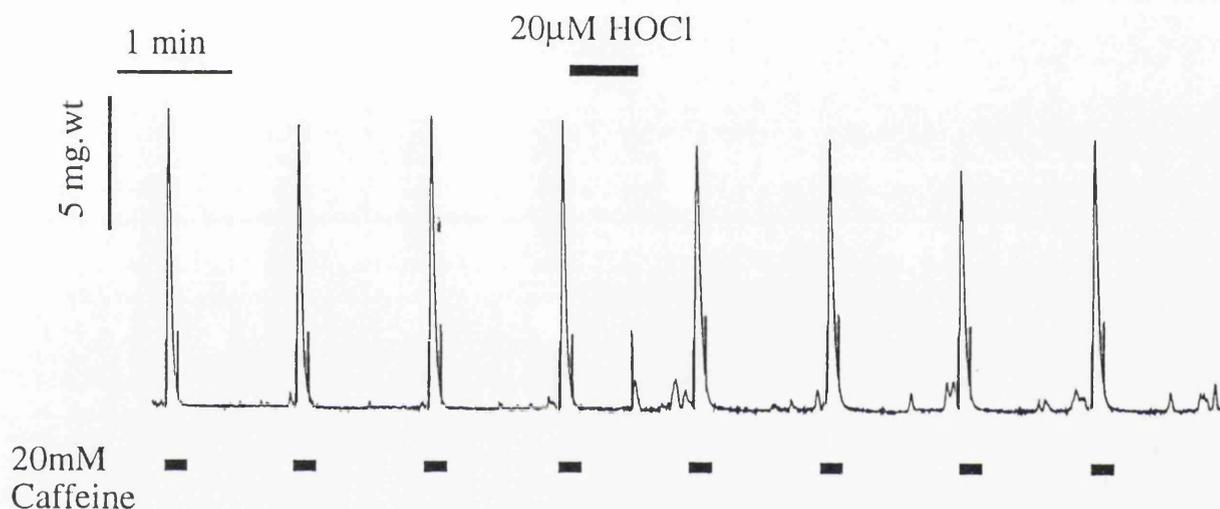
Figure 5.6 Experimental trace demonstrating the problems associated with interpreting the appearance of spontaneous oscillations while monitoring only the tension response in saponin-treated trabeculae. Caffeine was applied every 60 seconds (indicated by the short, solid bars under the trace) and the resultant tension response was monitored. In the protocol shown here, 20μM HOCl was applied for 30 seconds during a period of calcium loading of the SR (indicated by the solid bar above the trace). Prior to the application of HOCl small tension oscillations are apparent during the period of calcium loading, however, after HOCl application these oscillations become more conspicuous.
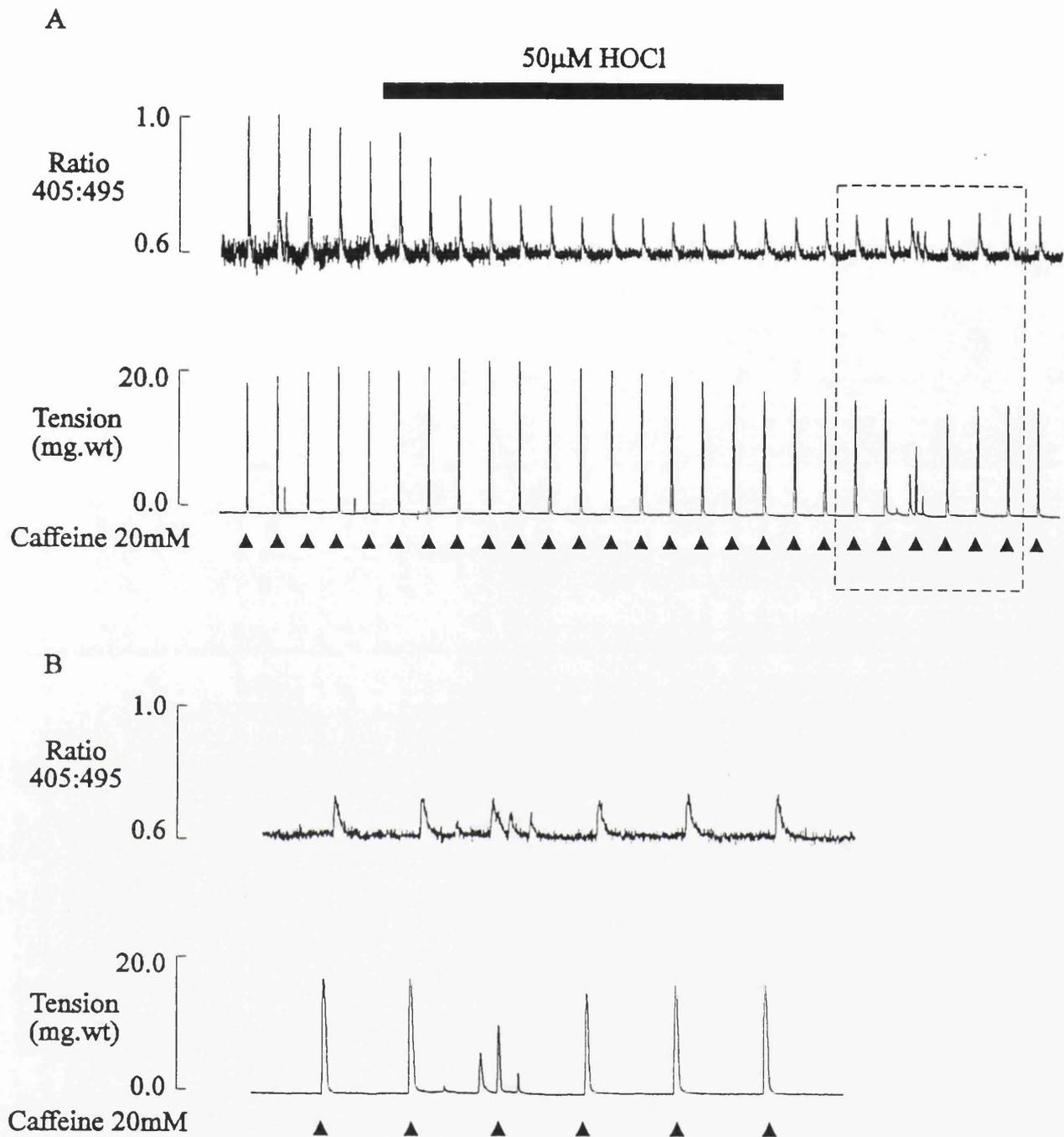
Figure 5.7 Experimental trace demonstrating spontaneous calcium oscillations after exposure to 50μM HOCl (indicated by the solid bar above the trace). **Panel A**: Shows a protocol similar to that applied in Figure 5.4. Caffeine was injected every 60 seconds (indicated by the arrow heads) and the resultant calcium release from the SR was monitored by indo-1 fluorescence. There was a pronounced fall in the caffeine-induced calcium transient during exposure to HOCl, however, despite this the associated tension transients were little effected. **Panel B**: Shows the section contained within the broken-line box in panel A (*i.e.* after removal of HOCl) on an expanded time scale. The caffeine-induced calcium transient has stabilised at a reduced level and there has been a slight fall in the associated tension transient. Prior to and after the third caffeine injection shown in panel B there were spontaneous calcium oscillations which were large enough to produce an associated tension response.

Conclusions

The pathogenic role of calcium overload in post-ischaemic contractile dysfunction has several lines of evidence in its support. Firstly, attenuation of post-ischaemic contractile abnormalities have been demonstrated in ferret hearts when reperfusion is undertaken in solutions with a low calcium concentration (Kusuoka, Porterfield, Weisman, Weisfeldt and Marban, 1987) or if ryanodine is present on reperfusion (Porterfield, Kusuoka, Weisman, Weisfeldt and Marban, 1987). Secondly, calcium overload in the absence of ischaemia can result in similar contractile abnormalities to those seen in post-ischaemic myocardial dysfunction (Kitakaze, Weisman and Marban, 1988). Thirdly, the time course of the rise in $[Ca^{2+}]_i$ is very similar to that of the ischaemic period required to give rise to post-ischaemic myocardial dysfunction (Carrozza, Bentivegna, Williams, Kuntz, Grossman and Morgan, 1992).

The mechanism for this rise in $[Ca^{2+}]_i$ during ischaemia and reperfusion remains unclear. It could be mediated by Na^+-Ca^{2+} exchange as a result of the rise in Na^+_i seen during the ischaemic episode (as a consequence of metabolic inhibition of the Na^+, K^+-ATPase and acidosis increasing Na^+-H^+ exchange). It could also be resultant from decreased calcium uptake by the SR. Evidence has been provided in a variety of studies for a decrease of SR calcium uptake in ischaemic hearts (Feher, Briggs and Hess, 1980; Feher, LeBolt and Manson, 1989). Since the net calcium uptake in the SR results from the activity of both the Ca^{2+}-ATPase and of the Ca^{2+}-release channel, an abnormal calcium uptake may reflect dysfunction of either or both systems. The effect of ischaemia on the Ca^{2+}-release channel remains unclear with one study suggesting that the decreased uptake results from enhanced calcium efflux from the Ca^{2+}-release channel (Limbruno, Zucchi, Ronca-Testoni, Galbani, Ronca and Mariani, 1989) and in another that calcium uptake was reduced (Feher, Lebolt and Manson, 1989). The effect of reperfusion on SR function has been less extensively studied. However, a number of studies suggest that

reperfusion aggravates ischaemic SR dysfunction (Dhalla, Panagia, Singal, Makino, Dixon and Eyolfson, 1988; Yoshida, Shiga and Imai, 1990).

It is probable that multiple mechanisms contribute to the pathogenesis of myocardial stunning. There is considerable evidence to suggest a link between the generation of oxygen-derived free radicals and reactive oxygen species and impaired calcium homeostasis. The results presented here demonstrate for the first time the effects of O_2^-, H_2O_2 and HOCl on SR with normal structural integrity.

The O_2^--generating system has no direct effect upon the calcium handling of the SR in the experiments described in this chapter. These findings are consistent with the previously published effects of O_2^- on $^{45}Ca^{2+}$ uptake (Das, Engelman, Bagchi and Prasad, 1989; Kukreja, Weaver and Hess, 1988) but are inconsistent with the observation that O_2^- generated by xanthine-xanthine oxidase increased the passive calcium efflux from cardiac SR vesicles and reduced the total steady-state intravesicular [Ca^{2+}] (Okabe, Fujimaki, Murayama and Ito, 1989). It is important to note that within the standard experimental conditions of this laboratory the SR is fully loaded within 60 seconds and as such the protocol utilised should reflect the total calcium content of the SR. The observation that it was necessary to increase the xanthine oxidase concentration in the O_2^--generating system in comparison to the 'Triton-skinned' preparation could reflect the more restricted access of xanthine oxidase to the intracellular space in 'saponin-permeabilised' muscle.

Hydrogen peroxide diminished caffeine-induced calcium release from the SR which suggests a decrease in the SR calcium content. However, the lowest concentration at which this effect could be demonstrated was 1mM (at that concentration a 10 minute incubation period was required for a 20% reduction in peak force production). Such results are consistent with previously published experiments using isolated SR vesicles except that concentrations of 2.5-25mM were required over prolonged periods (in excess

of 20 minutes) to obtain any significant effect on calcium-uptake rates and Ca^{2+}-ATPase activity (Kukreja et al, 1988).

The decreased calcium content of the SR could result from an increase in calcium efflux from the SR (via the channel or by an increase in membrane permeability due to lipid peroxidation) or a decrease in the calcium influx (decreased Ca^{2+}-ATPase activity). The previous discussion on the effects of H_2O_2 in SR vesicles would seem to implicate the Ca^{2+}-ATPase. However, subsequent to these observations the same group have demonstrated that H_2O_2, up to 12mM, is without effect on the 97kD Ca^{2+}-ATPase band of cardiac SR (by SDS-PAGE) or the redox status of SR sulphydryl groups (by spectrophotometry with Ellman's reagent) (Kukreja, Kearns, Zweier, Kuppusamy and Hess, 1991). Lipids are of fundamental importance in the maintenance of structure and function of Ca^{2+}-ATPase activity (Warren, Housley, Metcalfe and Birdsall, 1975). Therefore, SR dysfunction could result from increased calcium-efflux and decreased Ca^{2+}-ATPase activity due to lipid peroxidation. The observation that higher concentrations of H_2O_2 are required in isolated vesicle experiments may reflect the use of imidazole as a pH buffer in the experimental solutions since this reagent can ameliorate lipid peroxidation (Aruoma, Laughton and Halliwell, 1989). However, the ability of H_2O_2 to initiate lipid peroxidation within the experimental solutions used in the present experiments is doubtful (see discussion below). Additionally, the concentrations of H_2O_2 required to cause SR dysfunction in the studies reported here and those reported previously are much higher than those obtained physiologically. Activated neutrophils ($1.2 \cdot 10^6$ cells per ml) produce approximately $12.5 \mu moles$ of H_2O_2 within 30 minutes while mitochondria produce approximately 0.8nmol/min/mg (Turrens and Boveris, 1980). Superoxide and hydrogen peroxide can potentially interact to form the highly reactive hydroxyl radical which is toxic to membrane proteins and induces lipid peroxidation (Halliwell and Gutteridge, 1984). However, desferroxamine has been shown to be without significant effect in protecting the SR against damage from activated neutrophils (Kukreja et al, 1989) suggesting little or no role for the hydroxyl radical in

such damage. Additionally, the hydroxyl radical does not cause any structural damage to the 97kD Ca^{2+}-ATPase (Kukreja *et al*, 1991).

Lipid peroxidation can be defined as the oxidative deterioration of polyunsaturated lipids. In a completely peroxide free lipid system the initiation of a peroxidation sequence is caused by any species capable of abstracting a hydrogen atom from a methyl group. Since hydrogen has only one electron, abstraction leaves an unpaired electron forming a carbon radical which forms conjugated dienes. Under aerobic conditions the conjugated dienes combine with oxygen to form peroxy radicals that are able to propagate peroxidation in an adjacent lipid molecule. Hydrogen peroxide is able to initiate lipid peroxidation only in the presence of transition metal ions (probably by generating the hydroxyl radical via the Haber-Weiss reaction). The Haber-Weiss reaction is very slow unless it is metal-catalysed or allowed to proceed at acidic pH; this may be relevant during ischaemia. Since the hydroxyl radical is very unstable (with a half-life of 10^{-9} secs at 37°C) its reactivity is diffusion limited (*i.e.* it reacts with the first molecule it encounters) the ability of deferoxamine to scavenge the radical completely may be inadequate. If initiation of lipid peroxidation is accomplished, the reaction is self propagating. Therefore the possibility of lipid peroxidation occurring *in vivo* in the presence of deferoxamine or within the experimental conditions employed here remains uncertain.

Prior exposure to hypochlorous acid diminished the caffeine-induced calcium release from the SR in addition to its effects on the myofilaments as previously described in chapter 4. The time course of calcium release was altered in such a way as to suggest that both calcium release and uptake are changed. However, interpretation of the time course of the calcium transient is difficult due to the known alteration in myofilament calcium sensitivity. Previous studies have demonstrated that HOCl inhibits ^{45}Ca-uptake and Ca^{2+}-ATPase activity in a dose dependant manner from 2 to 20µM. At concentrations of 10 to 20µM, HOCl has been demonstrated to oxidise plasma membrane thiols and disrupt protein functions in the sarcolemma (Schraufstätter *et al*, 1990) such as ion pumping capacity. Higher concentrations of HOCl (>50µM) are associated with cell

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lysis (Cochrane, 1991) and may, therefore, be associated with lipid peroxidation since cell disruption increases the rate of free radical reactions and activates enzymes (such as cyclooxygenase and lipoxygenase) which produce peroxides (see Halliwell, 1991 for review). The protocol described in Figure 5.4, however, suggests that the calcium uptake rate of the SR is not diminished, in contrast to the depression of Ca^{2+}-ATPase activity observed in SR vesicles. Comparing the time-course of the caffeine-induced calcium release remains difficult (even at similar levels of released calcium *i.e.* comparing calcium signals of a similar size), however, it would appear that the calcium release is slowed.

Spontaneous calcium release due to calcium overload could contribute to reperfusion arrhythmias, as previously described. The results presented here remain too inconclusive to suggest such a pro-arrhythmic role for HOCl, but would suggest that both O_2^- and H_2O_2 do not contribute to reperfusion arrhythmias by this mechanism. These conclusions would be consistent with the observation that reperfusion arrhythmias occur in the effective absence of oxygen (Yamada, Hearse and Curtis, 1990). It still remains possible, however, that increased oxidative stress associated with chronic heart failure (McMurray, McLay, Chopra, Bridges and Belch, 1990) could contribute to the incidence of arrhythmias associated with this condition (see Pye and Cobbe, 1992 for review).

In collaboration with Dr Martin Denvir, Glasgow Royal Infirmary, we have been investigating the calcium handling characteristics of the cardiac SR in two distinct models of chronic heart failure in the rabbit (*i.e.* adriamycin-induced cardiotoxicity and coronary artery ligation). Figure 5.8 shows an example of the increased susceptibility of spontaneous calcium release in the adriamycin-treated animals, with similar observations being made in the coronary artery ligation animals, (Denvir, MacFarlane, Cobbe and Miller, 1993a; Denvir, MacFarlane, Cobbe and Miller, 1993b; Denvir, MacFarlane, Miller and Cobbe, 1993. Further work will be required to determine whether such observations are due to the heart failure *per se* or due to calcium-overload associated with oxidative stress.
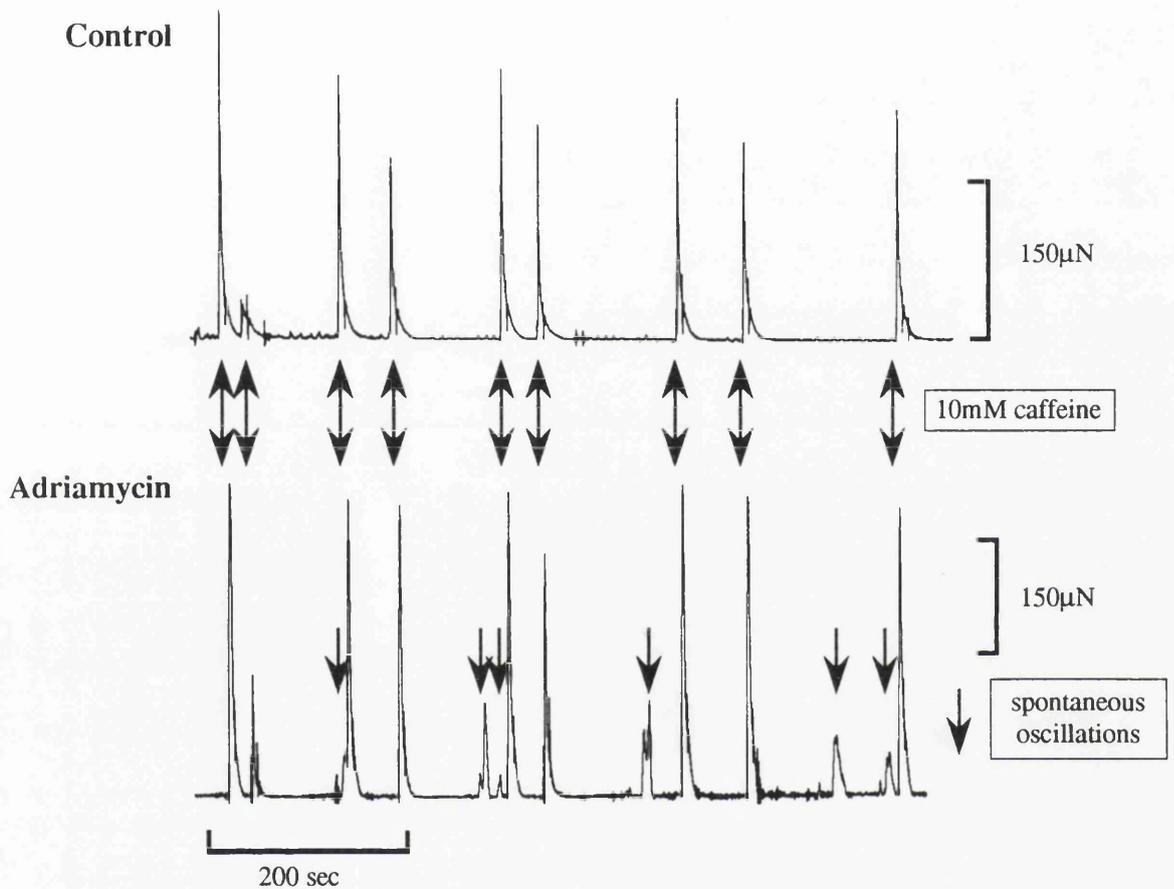
Figure 5.8 Experimental trace demonstrating part of an SR calcium loading protocol (at 0.15μM Ca^{2+}) where the application of caffeine (indicated by the double headed arrow) was monitored by the tension response. The preparation from an adriamycin-treated animal (lower trace) shows spontaneous tension transients (arrowed) indicating spontaneous calcium release from the SR during the loading period.

In summary, therefore, the reactive oxygen species H_2O_2 and HOCl are both capable of damaging SR function while O_2^- is without effect. The effects of HOCl are seen at physiologically relevant concentrations, while unphysiologically high concentrations of H_2O_2 are required. The negatively inotropic effect of SR damage by HOCl may be further enhanced by an increase in the susceptibility to spontaneous releases of calcium, and may also predispose to arrhythmogenesis during the post-ischaemic reperfusion period.

Chapter 6: Ultrastructural Abnormalities Associated with the Superoxide Anion and Hypochlorous Acid.

Introduction

Major physiological changes occur during the first hour of myocardial ischaemia produced by sudden occlusion of a major coronary artery. Within 10 seconds the demand of the affected tissue for oxygen exceeds the supply available from the collateral arterial flow and (in most mammalian species) the intramyocardial oxygen tension decreases; coincident with the onset of hypoxia, energy metabolism converts from aerobic to anaerobic pathways. However, although acontractile, the cells are not dead; if arterial flow is restored within the first 20 minutes, the injured cells resume electrical and mechanical activity. This time period is termed the *reversible* phase of ischaemic injury. On the other hand, if ischaemia persists, some of the affected cells die. Restoration of arterial flow to these cells is associated with explosive cell swelling and the appearance of overt necrosis *i.e.* the injury is *irreversible*.

Although many of the molecular events that occur in acutely ischaemic myocardium at the time ischaemic injury becomes irreversible have been identified (Jennings and Wartman, 1957), the events or series of events which cause the transition to irreversibility have yet to be established. The ultrastructural changes that appear during the late reversible and early irreversible phase of ischaemic injury are as follows: Tissues exposed either to 15 or 40 minutes of ischaemia show marked glycogen depletion, margination of nuclear chromatin and mitochondrial swelling. However, small breaks in the sarcolemma are the striking structural change characteristic of irreversible injury. The mechanism of damage is unknown but could be due to increased $[Ca^{2+}]_i$ and/or free radicals and reactive oxygen species. The ultrastructural effects of direct exposure to the myofilaments of free radicals and reactive oxygen species have not been previously investigated. The only directly relevant investigation has involved infusion of purine and xanthine oxidase isolated rat ventricular septa via a cannulated septal artery (Burton, McCord and Ghai, 1984). In this study the O_2^--generating system had no effect on the contractile performance of the septa or on myofilament ultrastructure.

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The previous results and discussion from chapters 3 and 4, relating specifically to the experiments where free radicals and reactive oxygen species are applied during a rigor contraction, lead to the conclusion that the observed depression in C_{max} is more specific than 'protease-like' damage of the myofilament lattice or to the S_2 subfragment. Such conclusions can be tested by the use of electron microscopy to visualise the myofilament ultrastructure after tissue fixation subsequent to obtaining functional data in protocols similar to those described in chapters 3 and 4. Additionally, the determination of any structural abnormalities after a series of maximum calcium-activations in cardiac muscle is important since prolonged exposures to high [EGTA] and low [Ca^{2+}] have been associated with accelerated deterioration of skeletal muscle fibres (April et al, 1972). Although no similar investigations have been performed in cardiac muscle, the fall in C_{max} often observed in cardiac skinned muscle preparations ($e.g.$ Kentish, 1986) has been proposed as a limitation of this technique in following changes in calcium sensitivity associated with an experimental intervention (Kusuoka and Marban, 1992). Within this laboratory we have been able to diminish the rate of deterioration in chemically skinned cardiac muscle by employing the 'calcium jump' method of activation (Miller, 1975; Moisescu, 1976; Ashley and Moisescu, 1977). However, the mechanisms underlying this degeneration remain obscure and merit investigation.

The procedure followed for these experiments was as follows: Trabeculae were isolated from the right ventricle of male Wistar rats and mounted for isometric force measurement and chemically skinned with Triton X-100 as previously described. After a series of maximum calcium-activations (6 activations each 10 minutes apart, with or without exposure to O_2^- or HOCl during the series of activations) the tissue was fixed for 1 hour using 2% glutaraldehyde in 0.1M sodium cacodylate buffer (NaCac) at pH 7.2 (Sabatini, Bensch and Barnett, 1963). Tension was monitored during the fixation process and the muscles remained relaxed throughout. The muscle was then removed from the snares (without distortion) and the specimens were the given three 20 minute washes in 0.1M NaCac and post-fixation was carried out using 1% osmium tetroxide in

0.1M NaCac. Following fixation the specimens were dehydrated with graded alcohol and embedded in araldite (Glauert, 1991). Ultrathin sections were cut with a diatome diamond knife on an LKB 3 ultrotome and mounted on formvar coated 1000μm aperture grids. The ultrathin sections were double stained with uranyl acetate (Stempak and Ward, 1964) and lead citrate (Reynolds, 1963). The stained sections were examined using a Zeiss EM 109 electron microscope at 50kV and electronmicrographs taken on Ilford FP4 film. This results from this study will be presented at the American Heart Association in November, 1993 (MacFarlane, Montgomery and Miller, 1993).

Results

Figure 6.1 demonstrates the normal myofilament structure obtained in the Triton-skinned ventricular muscle. Panel A shows a longitudinal section at a relatively low magnification (x8500). Triton X-100 treatment has completely dissolved all the membranes as would be expected, however, the structure of the myofilaments is maintained. Prior to fixation this specimen had been subjected to a series of 6 maximum calcium-activations each 10 minutes apart. In four preparations the rate of fall in these control activations was calculated as $0.184 \pm 0.009\% min^{-1}$, which is close to the rate of fall seen normally in this laboratory when calcium activated force is repeatedly evoked (Harrison *et al*, 1988). The only structural abnormality noted in the specimens treated in this manner was rupturing of the intercalated discs, an example of which is illustrated in panel B (magnification x18000).

Figure 6.2 demonstrates once again the normal myofilament ultrastructure obtained after a series of maximum calcium-activations as described in Figure 6.1. However, in this figure longitudinal sections are shown a three magnifications (for comparison with the O_2^- and HOCl treated trabecula shown later). The inset within panel C shows a transverse section from a trabeculae treated in the same way. The points of note in both Figure 6.1 and 6.2 are that the sarcomeres are very regular, the Z-lines are parallel to one another, even at the ruptured intercalated disc (Figure 6.1, panel B), the
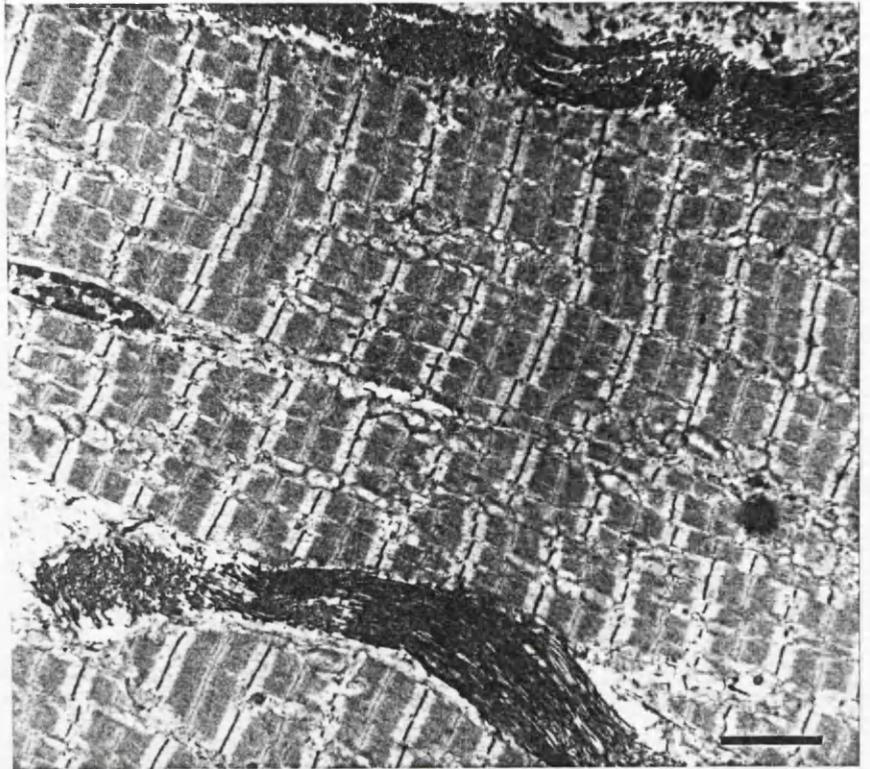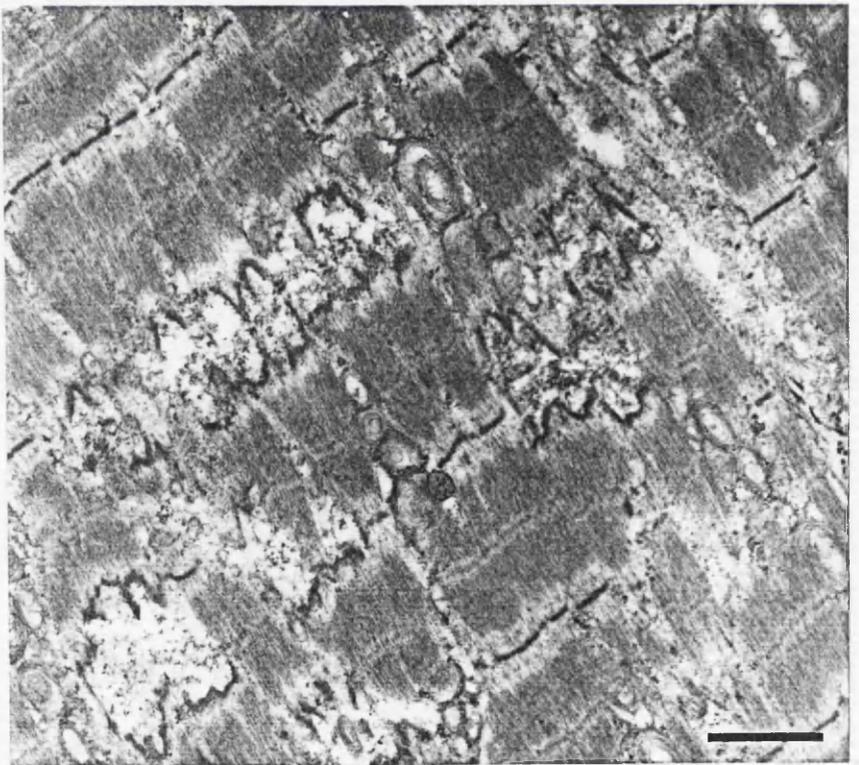
Figure 6.1 Electron micrograph of a Triton-skinned trabecula after a series of maximum calcium-activations. **Panel A:** Longitudinal section of Triton-treated rat ventricular muscle (x8500, scale bar 2.5μm). **Panel B:** Longitudinal section of Triton-treated rat ventricular muscle (x18000, scale bar 1.2μm).
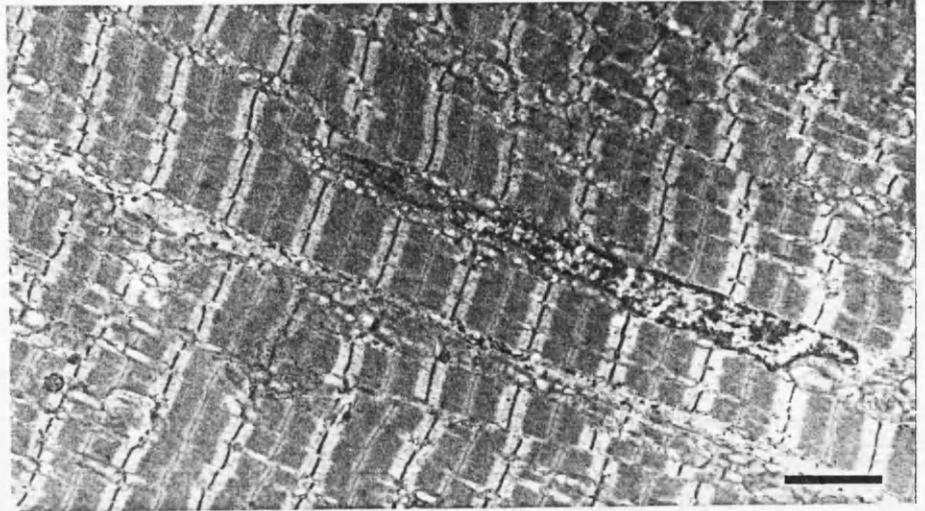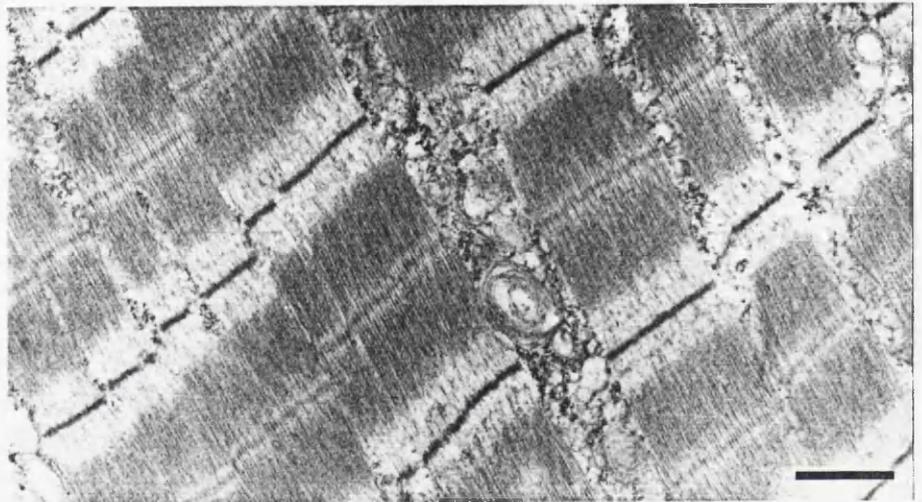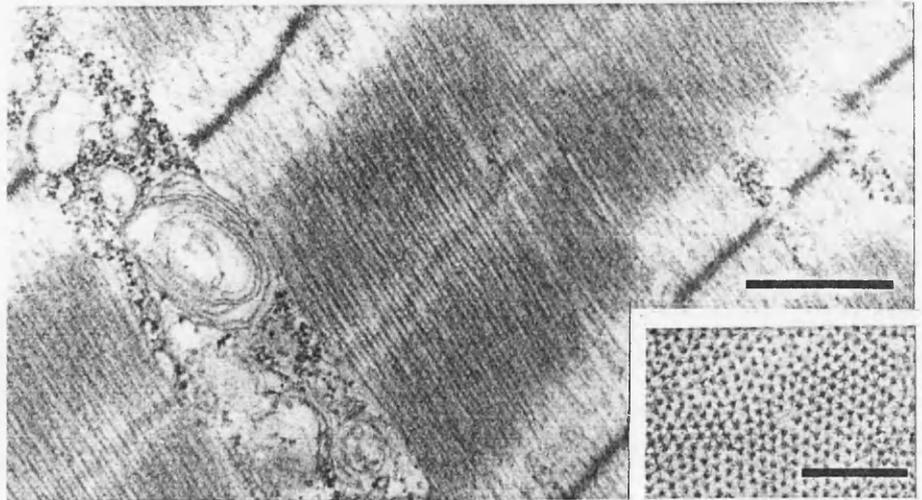
Figure 6.2 Electron micrograph of a Triton-skinned ventricular trabecula after a series of maximum calcium-activations. **Panel A**: Longitudinal section of Triton-treated rat ventricular muscle (x8000, scale bar 2.5μm). **Panel B**: Longitudinal section of Triton-treated rat ventricular muscle (x16000, scale bar 1.0μm). **Panel C**: Longitudinal section of Triton-treated rat ventricular muscle (x50000, scale bar 1.0μm). Inset: Transverse section of Triton-treated rat ventricular muscle (x86000, scale bar 0.2μm).

M-lines are apparent and the A-band/I-band junctions are regular and symmetrical. The filament lattice in the overlap region of the A-band is shown in the inset to panel C, Figure 6.2 shows a normal symmetrically spaced hexagonal lattice arrangement.

Figure 6.3 demonstrates the myofilament ultrastructure after exposure to the xanthine-xanthine oxidase O_2^--generating system sufficient to produce a large fall in C_{max} during a series of activations similar to those described for Figure 6.1. Exposure to O_2^- resulted in an increased rate of fall in tension to $9.87\pm2.43\%min^{-1}$, n=4, p<0.01 compared with control activations. Despite the low contrast in this example, the appearance of the sarcomere pattern is very similar to that of the controls even although C_{max} has fallen by some 50-60%.

Figure 6.4 demonstrates the effect of 50μM HOCl on the myofilament ultrastructure, but in contrast to the other figures the trabecula had been saponin-skinned. The rate of fall in C_{max} increased after exposure to HOCl and was calculated as $14.6\pm5.2\%min^{-1}$, n=4, p<0.005 compared with controls, after one exposure to HOCl in Triton-skinned preparations. The appearance of the sarcomere pattern in the HOCl treated saponin-skinned trabeculae is the same as that observed in the Triton-skinned preparations i.e. in contrast to the normal and O_2^--treated trabeculae, the Z-lines no longer remain parallel, the M-line has become indistinguishable and the A-band/I-band junctions are no longer symmetrical. A saponin-skinned fibre was used to show the myofilament ultrastructure so that it could be demonstrated simultaneously that no gross alterations to SR structure was observed despite the SR dysfunction noted in chapter 5. The inset in panel C, Figure 6.4 shows the lattice arrangement in a transverse section of a HOCl treated Triton-skinned preparation. The lattice was consistently very difficult to visualise in transverse sections of the HOCl-treated tissues.

The qualitative analysis obtained by using standard electron microscopy is very difficult to describe quantitatively. Obviously no commercially available image analysis systems is as sensitive as the human eye and, as such, attempts to quantify the disruptions
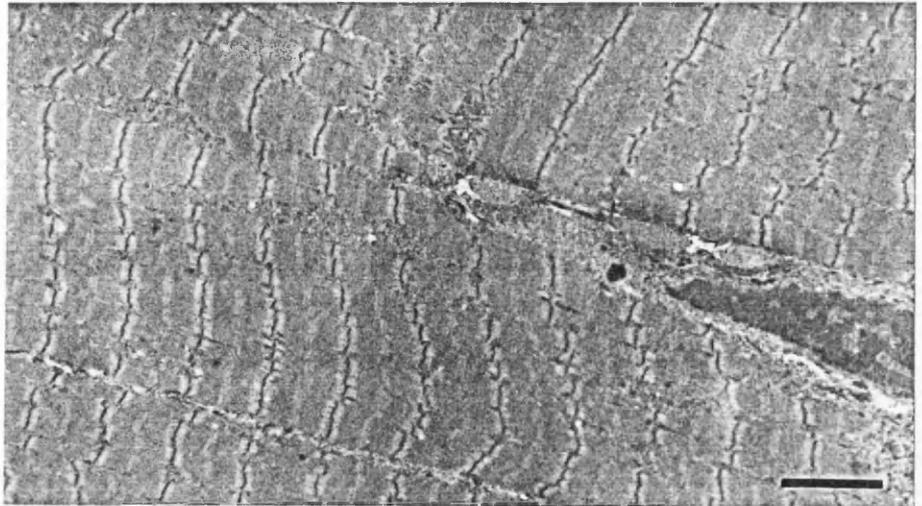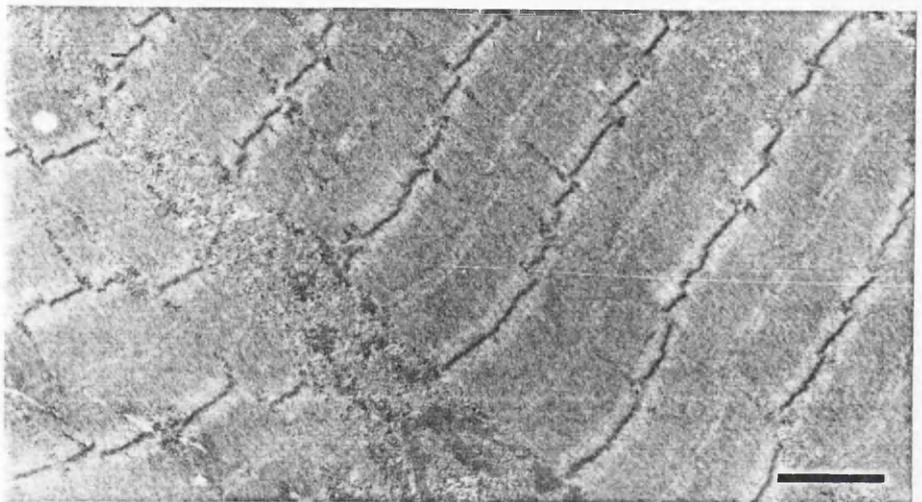
Figure 6.3 Electron micrograph of a Triton-skinned ventricular trabecula after exposure to O_2^- during a series of maximum calcium-activations. **Panel A**: Longitudinal section of Triton-treated rat ventricular muscle (x8000, scale bar 3.0μm). **Panel B**: Longitudinal section of Triton-treated rat ventricular muscle (x17000, scale bar 1.5μm). **Panel C**: Longitudinal section of Triton-treated rat ventricular muscle (x48000, scale bar 1.0μm).

D
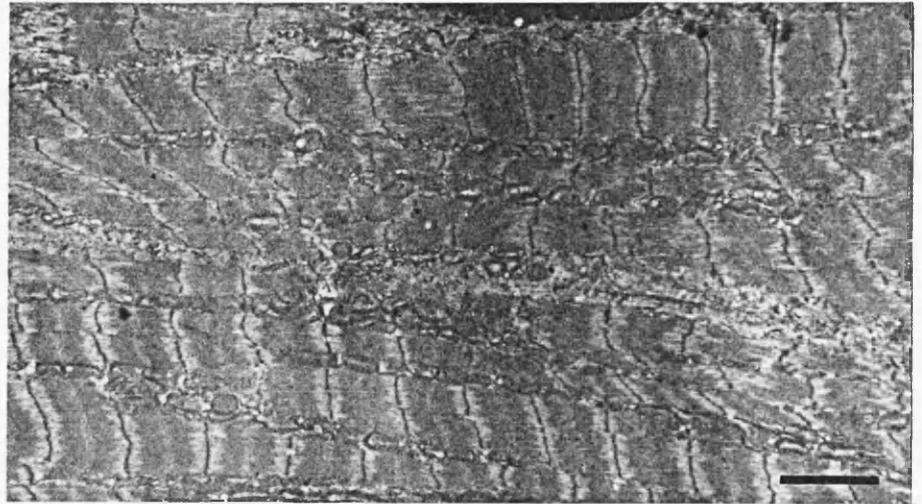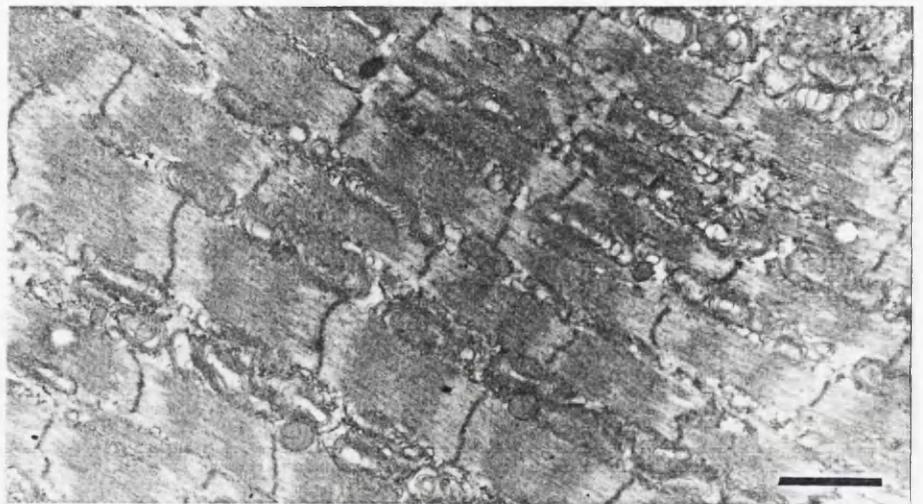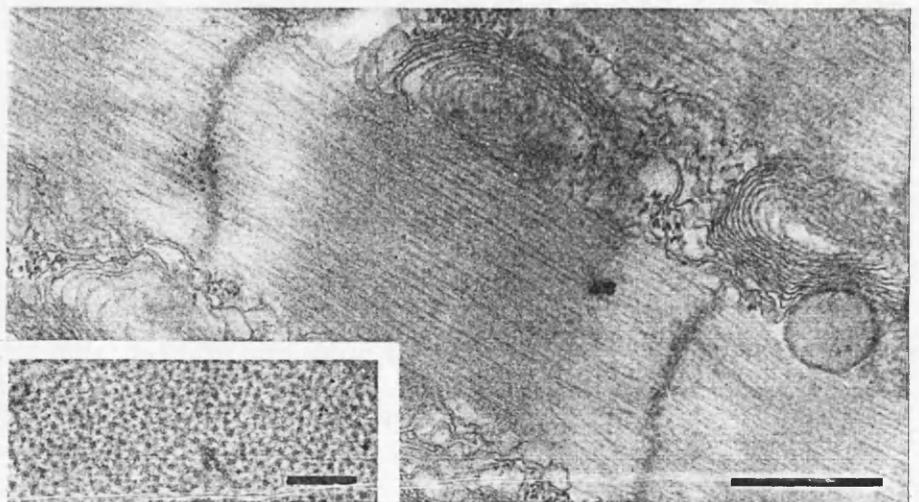
Figure 6.4 Electron micrograph of a saponin-skinned ventricular trabecula exposed to 50μM HOCl during a series of caffeine-induced calcium-activations. **Panel A**: Longitudinal section of saponin-treated rat ventricular muscle (x8000, scale bar 2.5μm). **Panel B**: Longitudinal section of saponin-treated rat ventricular muscle (x17000, scale bar 1.5μm). **Panel C**: Longitudinal section of saponin-treated rat ventricular muscle (x50000, scale bar 1.0μm). Inset: Transverse section of *Triton*-treated rat ventricular muscle (x108000, scale bar 0.1μm).

Panel D: (Shown on page opposite). Longitudinal section of saponin-treated rat ventricular muscle without exposure to HOCl.

to myofilament ultrastructure observed on exposure to HOCl have met with only limited success. The results from one of the image analysis packages employed to assess the ultrastructural changes observed are shown as a histogram in Figure 6.5 and numerically in Table 6.1. The method employed to obtain these results is as follows:

An acetate sheet was placed over the electron micrographs and the contours of the Z-lines, A-band and M-line traced from a single sarcomere. The tracings obtained in this manner were then imported into a Macintosh IIci computer (by the Applescan software and a scanner), along with an individual calibration mark for each electron micrograph. The traced contours were then transformed into x and y co-ordinates along their length via commercially available 'Data-thief' software (the y co-ordinates reflecting the distance (in μm) from the first Z-line traced) and generated approximately 80 values per micron of Z-line or A/I boundary. The mean and standard deviation of the position for each of the traced lines was calculated. This allows sarcomere length, A-band length *etc.* and their variance to be estimated for the two groups (control and HOCl-treated) from the combined data and statistical analysis to be performed (see legend to Table 6.1 for further details). The individual y co-ordinates obtained for these two groups via the data-thief software were used to produce a histogram (shown in Figure 6.5), which gives a graphical representation of the increased variance of the sarcomere pattern in the HOCl treated trabeculae.

	Z-line	A-band	M-line	A'-band	Z'-line
Control mean±SD	0.0±0.042	0.311±0.052	1.043±0.048	1.779±0.044	2.105±0.04
HOCl mean±SD	0.0±0.093	0.341±0.065	1.055±0.095	1.781±0.076	2.075±0.044
Significance[*]	0.48	0.01	0.36	0.34	0.16
Control var[†]	0.0953	0.122	0.1122	0.137	0.105
HOCl var[†]	0.2001	0.6162	0.048	0.383	0.297
F-test[‡]	4.452	25.76	0.187	7.94	8.018

Table 6.1 Statistical analysis of grouped electron micrographs (see Figure 6.5 for schematic of structural regions refered to here). The table shows the mean and SD of the y co-ordinate data points obtained by the Data-thief image analysis system from trabeculae that had been calcium-activated only (Control, n=29 sarcomeres from 6 preparations) and exposed to 50μM HOCl during a calcium-activation (HOCl, n=27 sarcomeres from 5 preparations). The data represent the distance between each of the ultrastructural features listed and the first Z-line traced (*e.g.* mean sarcomere length in the HOCl group was 2.075μm and the A-band length was 1.44μm *i.e.* A'-band minus A-band). [*] = p value obtained by an unpaired t-test of the Control and HOCl data points. [†] = mean SD from variance of the Control and HOCl data points. [‡] = Snedecor's F-distribution (*i.e.* F = largest [mean SD from variance]^2 divided by the smallest [mean SD from variance]^2. F≥ 1.0 for 1% significance with these sample sizes.).

Effect of HOCl treatment on sarcomere structure (from analysis of EMs)

Control
HOCl

80x10⁻³ →

60

40

20

0

Fraction of observations

0.0   0.5   1.0   1.5   2.0

A-band

A'-band

Z-line

M-line

Z'-line
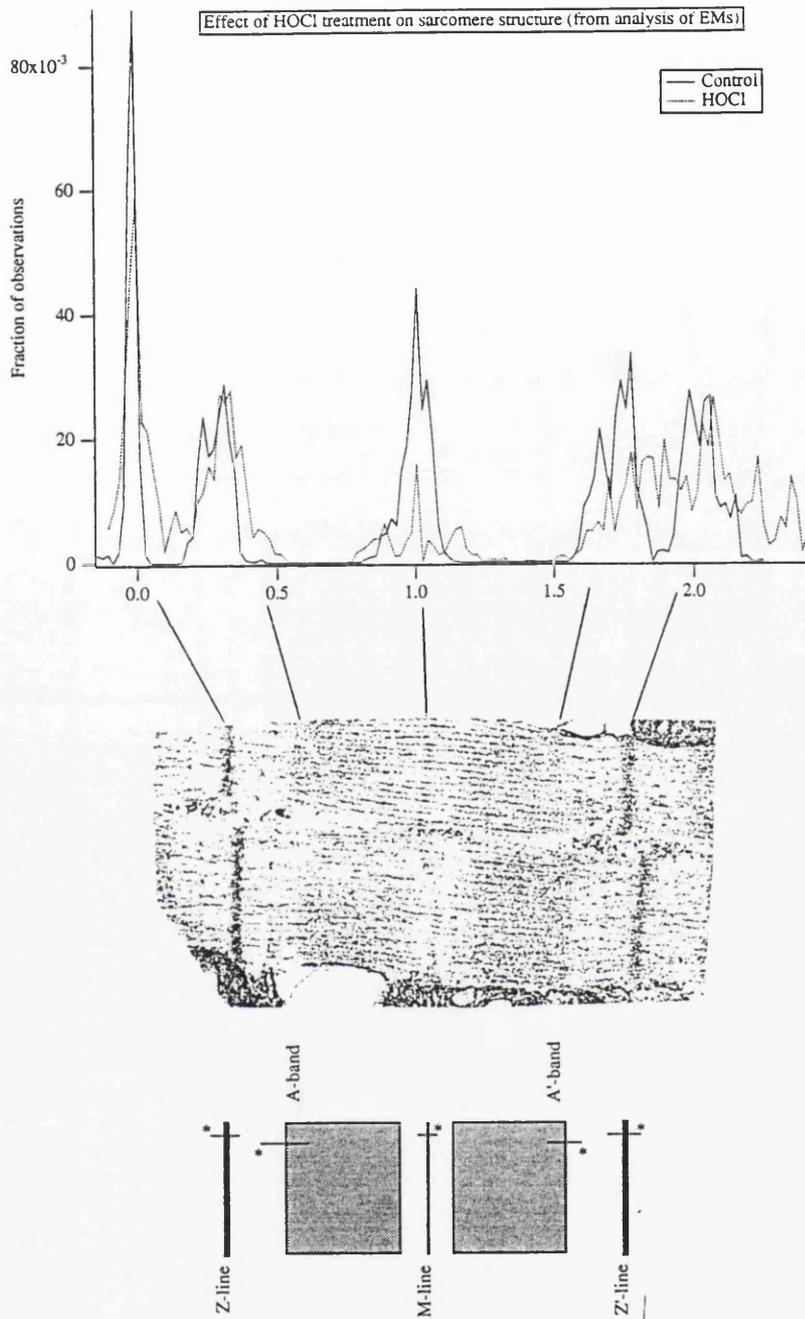
Figure 6.5 Histogram demonstrating the effect of HOCl on myofilament ultrastructure. The top panel shows the histogram constructed using the y-coordinates (*i.e.* the sarcomere being traced and scanned would be perpendicular to that shown in the middle panel) obtained using the data-thief software. The middle panel shows the corresponding structural regions on an electron micrograph to the graphical representation of the sarcomere pattern shown in the histogram (corresponding regions are connected by a solid line). The lower panel shows a schematic of the sarcomere structure and shows the structural regions and the mean SD from variance for HOCl data points (indicated by the asterix) refered to in table 6.1.

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Conclusions

The purpose of the experiments described within this chapter is two-fold. First, it is important to demonstrate that the conclusions reached from the functional data obtained in chapters 3 and 4 are valid *i.e.* that the free radical and reactive oxygen species do not have a non-specific 'proteolytic' effect upon the myofilament structure. Secondly, the ultrastructural abnormalities associated with the tension deterioration during sequential maximum calcium-activations have not been studied in chemically-skinned cardiac muscle, although previous studies have demonstrated a disruption to the myofilament lattice in skeletal muscle (Matsubara and Elite, 1972).

With reference to the first point of the experiments, Figure 6.3 demonstrates that the contractile dysfunction associated with exposure to O_2^- would appear to be specific damage which does not disrupt the myofilament ultrastrucure. Such observations are consistent with the only other studies to date assessing myofilament structural integrity after exposure to O_2^- (Burton *et al*, 1984; Miki *et al*, 1988). However, the major damage observed in those studies were at the vascular level and limited access of the O_2^--generating system in those preparations may not be sufficient to allow assessment of the effects O_2^- on the myofilaments even although systems within the myocyte are capable of generating O_2^- directly (see chapter 7).

In contrast, Figure 6.4 and 6.5 do suggest some ultrastructural alterations result from exposure to HOCl. The irregularities within the A-band/I-band junction and the loss of a distinct M-line in the longitudinal sections along with aberrant appearance of the transverse sections are difficult to interpret. Nevertheless it would seem that the damage is not merely a 'proteolytic-like' cleavage of the thick or thin filaments. The consistent finding that poor quality transverse images were obtained from HOCl-treated preparations (n=4) may be of significance when considered along with the loss of a distinct M-line (and hence the M-line is the only image where the F-test does not reach a high level of significance). The M-line is the point where cross-links occur between adjacent myosin

filaments (known as M-bridges) and a connecting point for the muscle cytoskeletal protein titin (Squire, Luther and Trinick, 1987) both of which may contribute to sarcomere stability. Protein structures such as these will be susceptible to oxidative damage and although there is no evidence as yet concerning the fate of such structures during reperfusion injury, the stunned myocardium has been associated with collagen loss (Charney, Takahashi, Zhao, Sonnenblick, Factor and Eng, 1989; Whittaker, Przyklenk, Boughner and Kloner, 1989) which might disrupt the cytoskeletal protein structures around the myocyte and could contribute to the sarcomere instability.

The transverse sections of the HOCl-treated trabeculae observed in this study might be interpreted as a shrinkage of the myofilament lattice since the thick filaments appear to be more densely packed (86 per $0.2\mu m^2$ *vs* 62 per $0.2\mu m^2$) in addition to an apparent disruption to the lattice. The tension development by skeletal muscle fibres that have been subjected to osmotic shrinkage of the lattice have been extensively studied in skeletal muscle and suggest that peak force is depressed at a critical level of lattice shrinkage (Kawai and Schulman, 1985; April and Maughan, 1986). The change in myofilament calcium sensitivity associated with such fibre treatment are more controversial with studies showing an increase (Godt and Maughan, 1981), no change (Gulati and Babu, 1985) or a decrease (Maughan and Godt, 1981) in sub-maximal force production. Similar studies in cardiac muscle are sparse and similarly controversial (Fabiato and Fabiato, 1978b; Lamont and Miller, 1987). However, the disparities most probably arise due to the different extents of lattice shrinkage employed within these studies. Therefore, the HOCl-induced disruptions to lattice structure could contribute to the contractile abnormalities previously noted, however, the functional evidence from chapter 4 would suggest some form of permanent crossbridge interaction to account for the dysfunction noted.

With reference to the second point of the experiments, the ultrastructural abnormalities associated with the tension deterioration during sequential maximum calcium-activations in chemically skinned cardiac muscle have not been studied

previously. Figures 6.1 and 6.2 demonstrate that the only structural abnormality observed in these cardiac preparations, after a series of 6 brief maximum calcium-activations with the protocol lasting one hour in total, was rupturing of the intercalated discs. However, the deterioration in the tension response observed within this laboratory is not as marked as has been published for other laboratories (Kentish, 1986). One possibility to account for the more sustained contractile performance within this laboratory is the use of the 'calcium-jump' method of activation (see methods, chapter 2). In addition making the preparation activation as rapid (and therefore brief) and uniform as possible throughout the cross-section of the whole preparation, the preparation spends the majority of the time between activations in solution E, Table 2.1. This solution is different from standard relaxing solutions (solution A, Table 2.1) in that the calcium buffer concentration is lower and the free [Ca^{2+}] is higher (*i.e.* 0.2mM EGTA and pCa 7.29). Exposure to low [Ca^{2+}] and high [EGTA] has also associated with a deterioration in skinned skeletal muscle fibres (Matsubara and Elliot, 1972).

In addition, the experiments performed in this laboratory usually employ very small (< 120μm major diameter) trabecular preparations to ensure complete intracellular membrane disruption. The use of larger diameter preparations might result in incomplete chemical skinning and leave a hypoxic, ATP depleted, unskinned core. In such preparations the activations might have to be prolonged to achieve C_{max} (due to the restricted diffusion in the central core) and the activation not uniform (since the core would probably be in rigor) so that more rapid deterioration of the tension response might be expected. A further consideration could be that within the central core of an incompletely skinned preparation there might be functional hypoxic mitochondria. If this were the case then the possibility exists for mitochondrial free radical generation which could also contribute to the deterioration in tension (see chapter 7 for further details). The observation that dithiothreitol ameliorates the tension deterioration in skinned cardiac muscle (Kentish and Jewell, 1981) is suggestive that some form of oxidative damage

occurs but would be inconsistent with the actions of O_2^-, H_2O_2 and HOCl that the previous chapters demonstrate.

Whatever the mechanism of the deterioration in the tension response in skinned cardiac muscle, the use of the 'calcium-jump' method of activation and preparations of a suitable size allows sufficient experimental control to make definitive statements about calcium sensitivity and C_{max} changes.

Chapter 7: Functional Significance of Mitochondrial Generation of Oxidants.

Introduction

The previous discussion within this thesis demonstrates that the myofilaments are sensitive to oxidative damage by O_2^- since enzyme concentrations as low as 0.002mU/ml have significant effects on contractile function in triton-skinned cardiac muscle fibres (within the standard conditions employed in skinned-fibre experiments in this laboratory such enzyme concentrations would produce *circa* O_2^- to pmol $min^{-1}ml^{-1}$) and that there are many sites and systems *in vivo* capable of producing such amounts of O_2^-. One such system previously described is the electron transport chain of the mitochondria.

The main function of mitochondria is the energy-yielding oxidation of pyruvic acid, fatty acids and certain amino acids. These substances are first metabolised to acetyl or other groups which are oxidised, coenzymes being reduced and carbon dioxide liberated. The coenzymes are re-oxidised with the consumption of oxygen and the formation of water. The energy made available from these oxidations (known collectively as the Krebs cycle) is utilised in the formation of ATP from ADP and inorganic phosphate. Comparatively little is known of the permeability of the mitochondrial membranes, but presumably they are freely permeable to a wide range of substrates and products of the mitochondrial enzymes. The possibility of these membranes being permeable to O_2^- has yet to be investigated.

The mammalian mitochondrion was first identified as a source of H_2O_2 in the early 1970's (Boveris, Oshino and Chance, 1972; Loschen, Azzi and Flohe, 1973). However, the release of H_2O_2 was later found to be consequent on the dismutation of O_2^- (by the mitochondrial form of SOD) occurring in the mitochondrial matrix (Boveris and Cadenas, 1975). The unstable nature of free radical species within biological systems makes accurate quantification or detection of small amounts of such species difficult. Therefore, the possibility exists for release of O_2^- from the mitochondrial matrix and the purpose of the experiments described in the following chapter are to assess the functional

significance of O_2^- and H_2O_2 production by *in situ* cardiac mitochondria on myofilament contractile performance.

Results

A variety of metabolic inhibitors (*e.g.* cyanide, azide, antimycin A) have been applied to saponin-treated trabeculae while providing 10mM pyruvate as a mitochondrial respiratory substrate and 0.5mM NADH. Figure 7.1 shows the basic protocol applied in the experiments described within this chapter (in this example 1.5μM antimycin A was the respiratory blocker being used). Cycles of calcium-loading and caffeine-induced calcium release were initiated and a reproducible train of tension responses obtained. Antimycin A was applied in the presence of SOD for thirty minutes after which no significant depression in the caffeine-induced contracture was noted. Antimycin A was removed in the presence of SOD, which was also then removed. The preparation was then exposed to antimycin A alone for a further thirty minutes, during which the tension response fell by approximately 30%. On 'washing off' the antimycin A the tension response to the caffeine-induced calcium release fell steeply to less than 10%. Similar results were seen in three other preparations.

Figure 7.2 demonstrates the results of a similar protocol in which 1mM azide was used to block the mitochondrial electron transport chain in the presence of 10mM pyruvic acid and 0.5mM NADH. In this example, after a thirty minute exposure to the respiratory blocker alone, there was a 20% reduction in the tension response. However, in this example there was no a further fall in the caffeine-induced tension response upon removing azide (not shown).
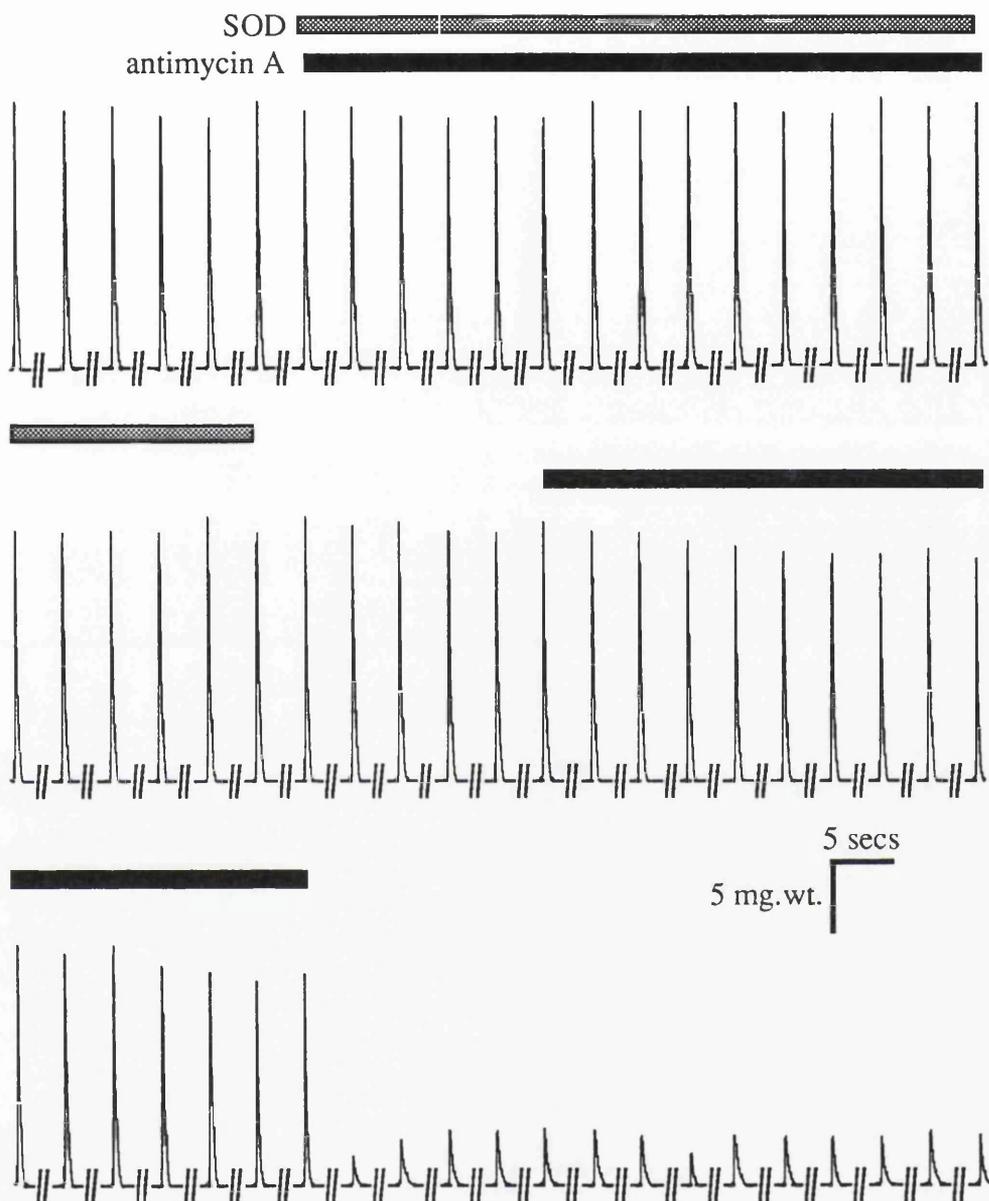
Figure 7.1 Experimental trace demonstrating the protocol used to investigate the possibility of functionally significant generation of O_2^- by the mitochondria. Caffeine was applied every two minutes and resulted in a transient tension response which was monitored. A series of six stable tension responses was obtained in the presence of 10mM pyruvate and 0.5mM NADH. The preparation was then exposed to 1.5μM antimycin A (indicated by the black bar above the trace) in the presence of 10μg/ml SOD (indicated by the grey bar above the trace) for 30 minutes. The antimycin A was removed in the continuing presence of SOD and there was little effect on the caffeine-induced tension response. When applied alone, however, antimycin A resulted in a small fall in the tension response (over the 30 minutes). On removing the antimycin A there was a pronounced reduction in the caffeine-induced tension response. The breaks in the trace represent periods in a loading solution of two minutes.
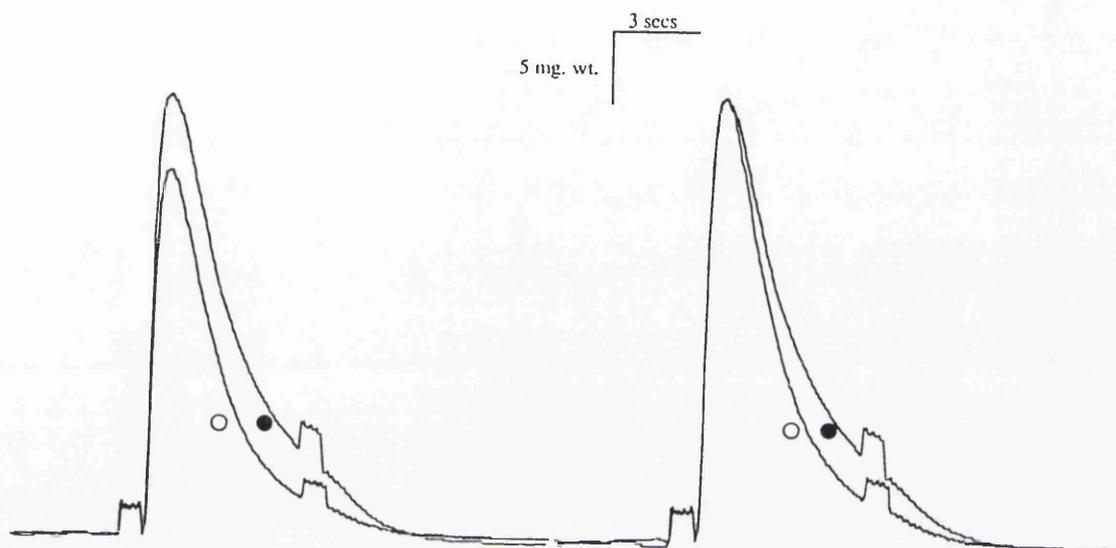
Figure 7.2 Experimental trace showing the effect of a 30 minute exposure to 1mM azide. The conditions of the protocol applied was the same as in Figure 7.1 except that 1mM azide was employed as the respiratory blocker rather than antimycin A. The tension responses obtained before (solid circle) and after (open circle) are superposed on the left panel and then scaled to the same size on the right panel. Metabolic blockade was associated with a 20% reduction in the caffeine-induced tension response.

The anthracycline quinone adriamycin has been widely used as an anti-neoplastic agent with substantial therapeutic activity against a broad variety of human cancers. However, the use of this agent is limited by a unique cardiac toxicity which has been suggested to be due the result of anthracycline-induced free radical formation (Davies and Doroshow, 1986; Doroshow and Davies, 1986). In the presence of molecular oxygen adriamycin has been suggested to undergo one-electron reduction to a free radical semiquinone species which is rapidly re-oxidised in a process that generates O_2^- and which is catalysed by the mitochondria (Doroshow and Davies, 1986). The adriamycin-quinone is then available to participate in further redox reactions (Lown, Chen, Plambeck and Acton, 1982).

The possibility of enhanced O_2^- generation within the mitochondria in the presence of adriamycin was investigated in saponin-skinned fibres. Figure 7.3 demonstrates the effect of metabollic blockade (with 1mM azide) in the presence of $0.2\mu M$ adriamycin. A train of stable caffeine-induced tension responses was obtained prior to the preparation being exposed to adriamycin. Exposure to adriamycin resulted in an increase in the caffeine-induced tension response (not shown) while addition of 1mM azide to the adriamycin caused an irreversible decrease in the caffeine-induced tension response by approximately 30% despite a recurrence of the increase in the caffeine-induced tension response seen in the presence of adriamycin. The observed reduction in caffeine-induced tension responses during azide-induced metabolic blockade in the presence $0.2\mu M$ adriamycin was similar to that obtained with 1mM azide alone except that the tension response was markedly slowed. Increasing the adriamycin concentration to 0.2mM completely, and irreversibly, abolished the caffeine-induced tension response (data not shown).
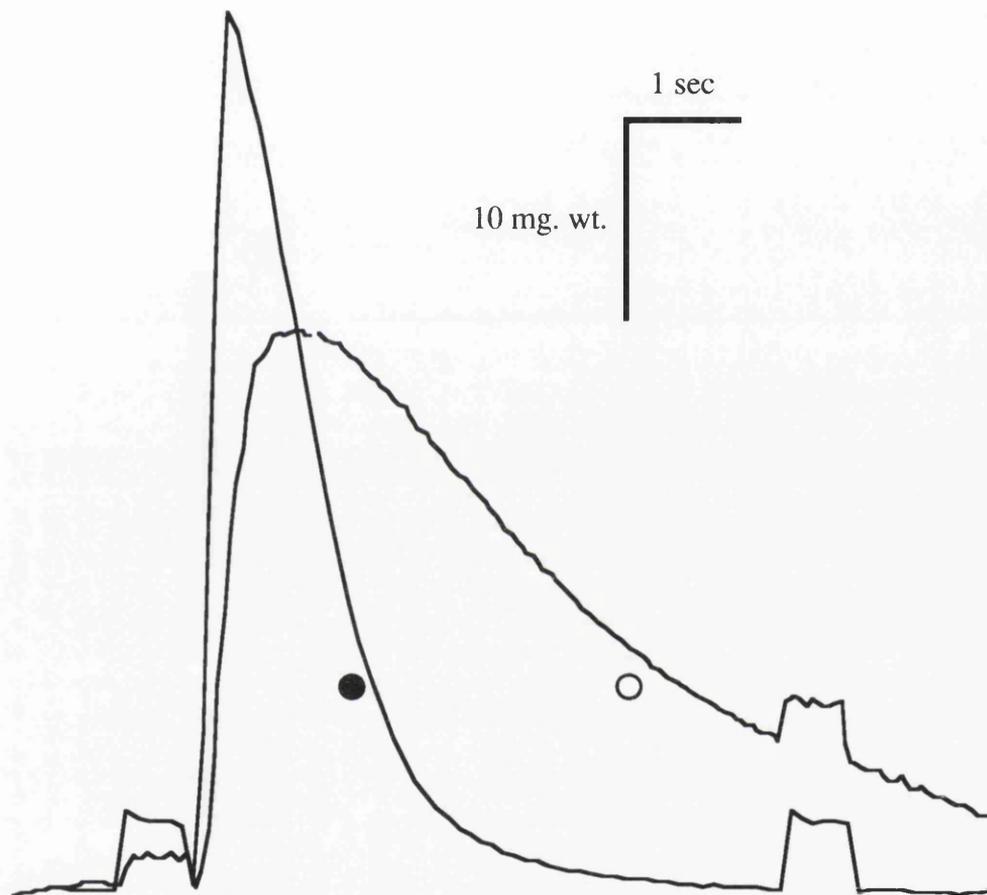
Figure 7.3 Experimental trace showing the effect of a 30 minute exposure to 1mM azide. The conditions of the protocol applied was the same as in Figure 7.1 except that 1mM azide was employed as the respiratory blocker rather than antimycin A and 0.2μM adriamycin was added to the solutions throughout. Metabolic blockade was again associated with a reduction in the caffeine-induced tension response (30% in this example) but additionally the tension response was markedly slowed.

Conclusions

A number of previous studies have demonstrated that sub-mitochondrial particles are capable of generating both O_2^- and H_2O_2 (*e.g*. Turrens and Boveris, 1980; Turrens, Beconi, Barilla, Chavez and McCord, 1991), however, it remains debatable whether O_2^- would be able to cross mitochondrial membranes and what the functional significance of the low level of O_2^- generation would be.

Reduced coenzymes and hydrogen ions generated by the oxidation of metabolites in the Krebs cycle are re-oxidised and the hydrogen is converted to water by a series of interlocking enzymes and cofactors known as the respiratory chain. These enzymes and enzyme-bound cofactors are embedded in and form part of the inner mitochondrial membrane. The links in the respiratory chain are as follows:

Reduced nicotinamide-adenine-dinucleotide (NADH) is oxidised by a flavoprotein enzyme consisting of a dehydrogenase with a prosthetic group of flavin mononucleotide (FMN) or flavin-adenine-dinucleotide (FAD). The reaction liberates energy that is utilised to generate ATP from ADP and phosphate. The oxidation of reduced nicotinamide-adenine-dinucleotide-phosphate (NADPH) is coupled to the reduction of NAD. These reduced flavins are oxidised by a dehydrogenase containing the cofactor ubiquinone *i.e*. the coenzyme Q reductase complex (the reduced form of which is in turn oxidised by cytochrome *b*).

Cytochromes, or tissue respiratory pigments, are conjugated proteins containing *haem* prosthetic groups. The change from the reduced to the oxidised form of a cytochrome is essentially the change of the iron atom from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state and amounts to a transfer of an electron from the substance. These are recombined with hydrogen ions which are released in the oxidation of ubiquinone and react with oxygen to form water at the end of the chain. The cytochrome portion of the respiratory chain has been termed the electron transport chain.

Reduced cytochrome b is oxidised by the reduction of cytochrome c_1 and the energy output from this reaction is coupled to the phosphorylation of ADP. Cytochrome c_1, c, and a are linked in the electron transport chain in the same way. Finally, reduced cytochrome a is cytochrome oxidase which is the third point at which the redox reaction is coupled with the formation of ATP.

The mammalian mitochondrion was first identified as a source of H_2O_2 in the early 1970's (Boveris, Oshino and Chance, 1972; Loschen, Azzi and Flohe, 1973). Later, the release of H_2O_2 was found to be consequent on the dismutation of O_2^- occurring in the mitochondrial matrix (Boveris and Cadenas, 1975). Reactions in two segments of the respiratory chain have been found to be responsible for the partial reduction of oxygen to O_2^-. The first is located in complex I of the respiratory chain and most probably produces O_2^- via auto-oxidation of the reduced flavin mononucleotide of NADH dehydrogenase. The second segment is located in the ubiquinone-cytochrome c reductase system of complex III, and both ubisemiquinone and reduced cytochrome b have been suggested to auto-oxidise and transfer electrons directly to oxygen generating O_2^- (Turrens and Boveris, 1980).

Recently, mitochondria isolated from rat hearts after ischaemia and reperfusion have demonstrated a specific inhibition in the electron transport chain between NADH dehydrogenase and ubiquinone. Such inhibition was associated with an increase in H_2O_2 formation in the presence of NADH-dependent substrates (Turrens et al, 1991). A similar inhibition was produced in control heart mitochondria by increasing the bathing $[Ca^{2+}]$, but was partially prevented by albumin. Such results suggest that the Ca^{2+}-dependent effect could be due to phospholipase A_2 activation and the subsequent release of fatty acids. Addition of unsaturated fatty acids mimic the effects of ischaemia-reperfusion and calcium-overload (Turrens et al, 1991).

The substrate supply to the heart is complex and during ischaemic episodes would not depend on NADH-dependent substrates so that the electron transport inhibition noted

above might not occur. However, as previously discussed, during the ischaemic episode α-adrenoreceptor activation stimulates a phosphoinositidase which cleaves PIP_2 into IP_3 and DAG. DAG can subsequently be hydrolysed, by a DAG-lipase to form arachidonic acid, or phosphorylated, to phosphatidic acid by DAG-kinase. The possibility exists, therefore, for such a mechanism to inhibit the electron transport chain which would predispose to O_2^--generation. In addition, the calcium-overload noted during ischaemia and transiently on reperfusion could have a similar effect.

The energy for the production of ATP ultimately comes from the energy in reduced metabolic substrates oxidised via intermediary metabolism. This energy, in the form of reducing equivalents, is delivered to the mitochondrial respiratory chain through NADH and FADH. To increase ATP synthesis in the steady-state the delivery of reducing equivalents to the cytochrome chain must match its utilisation in oxidative phosphorylation. Therefore, the flux of reducing equivalents into the cytochrome chain must be directly or indirectly regulated by the rate of ATP hydrolysis or work.

Early experiments on isolated mitochondria demonstrated a direct relation of the concentration of ATP hydrolysis products to the rates of oxygen consumption and ATP production (Heineman and Balaban, 1990). Such observations resulted in an elegantly simple model of respiratory control proposing a feedback mechanism between the cellular energy demands and the rate of ATP production. However certain controversies arose over these initial *in vitro* observations about the respiratory control, *i.e.* whether a kinetic or thermodynamic mechanism is responsible, and data acquired under more physiological conditions argue against the fundamental requirement of both kinetic and thermodynamic models - that changes in the $[P_i]$ are necessary for the changes in the rate of oxidative phosphorylation.

It has been demonstrated that the maximum rate of respiration can be controlled by the delivery of reducing equivalents or the levels of mitochondrial NADH in liver mitochondria (Heineman and Balaban, 1990). Studies on perfused hearts also indicate

that increases in mitochondrial NADH can stimulate the rate of oxidative phosphorylation (Doroshow and Davies, 1986).

The results shown here, that tension is irreversibly depressed during and after metabolic blockade and that SOD protects such depression, demonstrate that O_2^- is produced in pathologically significant amounts by respiring mitochondria and can interact with the myofilaments directly in saponin-skinned cardiac muscle fibres. However, interpretation of the results described within these protocols to the *in vivo* phenomenon of ischaemia reperfusion is limited. Firstly, in the saponin-skinned fibres the external mitochondrial membrane is disrupted. Cytochrome oxidase is a typical integral membrane protein 'which is plugged through the membrane'. Re-oxidation of NADH and the reduction of O_2 takes place at the inner surface of the inner mitochondrial membrane and the cytochrome c portion of the electron transport chain is found at the outer surface of this membrane (Bennett, 1977) and might not be totally shielded by the phospholipid bilayer. Secondly, in these protocols it was necessary to add reducing equivalents in the form of NADH to ensure that electron transport continues in the presence of the metabolic blocker and excess ATP *i.e.* the electron transport chain may be blocked prior to the electron transfer to O_2 by cytochrome a but continue to lose electrons, in the presence of a reducing agent, at complex I and III of the respiratory chain. However, during ischaemia within the intact heart the NADH is quickly oxidised to NAD^+ (GL Smith, personal communication) and the tissues are hypoxic so that oxidative phosphorylation will be disrupted. Thirdly, redox energy is conserved in the electron transport chain by generation of an electrochemical proton gradient by the translocation of H^+ from the mitochondrial matrix to the cytoplasm. The profound intracellular acidosis observed during an ischaemic episode would tend to diminish this proton gradient and prevent continued electron transport. However, during reoxygenation after 20-30 minutes of ischaemia the rapid increase in mitochondrial oxygen consumption, mitochondrial swelling, intracellular calcium-overload and the decrease in intramitochondrial antioxidants might allow O_2^- production and release.

Comparison of Figures 7.2 and 7.3 suggest that addition of adriamycin to azide treated saponin-skinned trabeculae does little to aggrevate the contractile dysfunction observed after azide treatment alone. The slowin of the tension response is difficult to interperate due to the effect of adriamycin on the caffeine-induced tension response. Nevertheless, such results do not preclude a role for free radical production participating in the mechanisms inducing heart failure on chronic exposure to adriamycin.

Interestingly exposure to 0.2μM adriamycin increased the calcium load of the SR (*i.e.* an increase in the caffeine-induced tension response was observed). This increase could result from either an increase in calcium influx (via the Ca^{2+}-ATPase) or a decreased efflux (via the calcium channel). Doxorubicinol (the major metabolite of adriamycin) has previously been identified as a potent inhibitor of membrane-associated ion pumps using SR vesicles (Boucek, Olson, Brenner, Ogunbunmi, Inui and Fleischer, 1987), whereas adriamycin (or doxorubicin) is with little effect at therapeutically relevant concentrations. However, adriamycin is an inhibitor of the calcium-release channel (S Fleischer - personal communication). This effect is could underly the mechanism by which we observed the increased susceptibility to spontaneous calcium release in adriamycin-treated rabbits (see Figure 5.8) and might contribute to the development of chronic heart failure in these animals.

Chapter 8: Endogenous and Pharmaceutical Free Radical Scavengers.

Introduction

The aerobic myocardium maintains a delicate balance between cellular systems that generate various oxidants and those maintaining the antioxidant defence mechanisms. In the heart, these defence mechanisms include the enzymes SOD, catalase and glutathione peroxidase (GPD) plus other endogenous antioxidants such as vitamin E, ascorbic acid and cysteine (Roy and McCord, 1983; Diplock and Lucy, 1974). The primary mechanism for O_2^- clearance is SOD, which catalyses the dismutation of O_2^- to H_2O_2 and O_2. The reaction proceeds spontaneously, but SOD increases the rate of intracellular dismutation by a factor of 10^9 (Halliwell and Gutteridge, 1989). At least three forms of SOD have been characterised. One contains copper and zinc and is present in the cytosol. Another contains manganese and it is present in the cytosol. The third contains iron and is found in the cytoplasm of *Escherichia coli.*

Two enzyme systems are important in the metabolism of H_2O_2 produced by the univalent reduction of O_2^- (Roy and McCord, 1983; Chance, Sies and Boveris, 1979). The first is catalase present in the cytosol that catalyzes the reduction of H_2O_2 to water and the second is GPD (a selenium-dependent enzyme). Catalase is present at only very low concentrations in the myocardium while GPD is found at a significant concentration in the cytosol of cardiac cells (Lawrence and Burk, 1978). The hexose monophosphate shunt produces, via glucose-6-phosphate oxidation, the reducing equivalents as nicotinamide adenine dinucleotide phosphate (NADPH) to activate glutathione reductase. Reduced glutathione (GSH) is utilised by GPD to form oxidised glutathione (GSSG). Changes in glutathione status provide important information on the cellular oxidative events, and tissue accumulation and/or release of GSSG in the coronary effluent is a sensitive and accurate index of oxidative stress (Currello, Ceconi, Medici and Ferrari, 1986).

Glutathione is in dynamic equilibrium with all cellular sulphydryl groups. Glutathione and mixed disulphides with proteins constitute an important part of the total

cellular GSH pool and the equilibrium is regulated by thiol transferases (Mannervick and Axelsson, 1980). Alterations to the sulphydryl:disulphide ratio modulates the activity of a number of enzymes and is involved in the transport of amino acids across the cell membrane (Mannervick and Axelsson, 1980; Meister and Tate, 1976). During periods of reperfusion after an ischaemic episode myocardial tissue GSSG is significantly increased (Ferrari, Ceconi, Currello, Guarneri, Caldarera, Albertini and Visioli, 1985). The resultant alteration to the sulphydryl:disulphide ratio could increase the level of disulphide interactions with cellular proteins. The actions of GSSG on the myofilaments appear analogous to that of HOCl (Prof JC Rüegg, personal communication).

Vitamin E, an antioxidant that has long been recognised in biological systems, has been identified at significant concentrations in the myocardial, cytosolic and mitochondrial membranes (Guarnieri, Ferrari, Visioli, Caldarera and Nayler, 1978). *In vitro* studies have shown that vitamin E functions as a free radical scavenger and protects the heart membranes from lipid peroxidation. It functions synergistically with ascorbic acid (which reacts with vitamin E radicals to regenerate vitamin E). Vitamin C radicals are reduced by NADH reductase. Due to its lipophilic nature, vitamin E serves as an antioxidant within membranes and vitamin C serves as a water-soluble electron transport system in the cytosol or extra-cellular fluid. Although there is sufficient *in vitro* and *in vivo* evidence to support vitamin E as an important antioxidant, the protective role for this compound, at physiologically relevant concentrations, has yet to be demonstrated.

The timely restoration of blood flow to severely ischaemic myocardium limits infarct size. However, experimental studies demonstrate that the myocardial salvage achieved is limited because of the additional injury that occurs during reperfusion. This is due in part to the generation of free radicals and reactive oxygen species which overcome the delicate balance between the antioxidant systems and the oxidant-generating cellular systems (Lucchesi, 1990; Downey, 1990; Kloner, Przyklenk and Whittaker, 1989; Werns, Shea and Lucchessi, 1986; Forman, Virmani and Puett, 1990). Despite numerous studies (Engler and Gilpin, 1989; Shirato, Miura, Ooiwa, Toyofuku, Willborn

and Downey, 1989; Richards, Murry, Jennings and Reimer, 1987; Jolly, Kane, Bailie, Abrams and Lucchessi, 1984; Nejima, Knight, Fallon, Uemura, Manders, Canfield, Cohen and Vatner, 1988) the ability of exogenously applied SOD with or without catalase to reduce infarct size remains controversial. Both SOD and catalase are large enzymes, with short intravascular half-lives and poor entry into cells (Engler and Gilpin, 1989). If O_2^- and H_2O_2 are generated in the extracellular space, then they may be accessible to these enzymatic scavengers. However, O_2^--generation at neutrophil-target cell interfaces or intracellular O_2^--generation would be inaccessible to these enzymatic scavengers. Superoxide might also enter cells via anion channels (Kontos et al., 1985). Additionally, it now appears that SOD has a "bell-shaped" therapeutic dose response curve with protection being obtained at only the mid-range of doses (Omar and McCord, 1990).

The short intravascular half-life and impermeability of unconjugated SOD means that it is rapidly cleared from the circulation and is probably does not have access to some of the environments where O_2^- is generated. While early-reperfusion bolus regimens of SOD might protect against the early 'burst' of free radical generation the protective effect might not be sustained and could account for the variation in protection by antioxidants during reperfusion. Experimental infarct size is more consistently reduced by therapy initiated immediately prior to reperfusion with cell permeable, long half-life free radical scavengers e.g. by polyethylene glycol modified SOD, PEG-SOD, which is a SOD conjugate with a prolonged intravascular half-life of greater than 30 hours, and mercaptopropionyl glycine, MPG, which readily enters the intracellular space and is active when taken orally (Tamura, Chi, Driscoll, Hoff, Freeman, Gallagher and Lucchesi, 1988; Tanaka, Stoler, Fitzharris, Jennings and Reimer, 1990; Bolli, Jeroudi, Patel et al, 1989). This is consistent with the previously discussed experimental data suggesting that free radical and reactive oxygen species damage occurs at intracellular sites and continues over a long period of time. Thus, it is not surprising that short-acting, extracellular scavengers have not convincingly reduced infarct size and, despite the controversy,

unconjugated SOD the PEG-SOD and MPG studies suggest a possible therapeutic benefit from cell-permeable scavengers with prolonged half-lives.

The purpose of this chapter is to assess the ability of captopril, a clinically-relevant drug in the treatment of post-ischaemic contractile dysfunction and which is postulated to have free radical scavenging activity, by testing it in 'skinned-fibre' experiments with HOCl. In addition, the possible role of endogenous histidine-containing compounds as free radical scavengers during post-ischaemic contractile dysfunction, and alterations to the range and concentration profile of these compounds in ischaemia and heart failure, will be assessed. A variety of assay methods have been employed to investigate the antioxidant activities of the histidyl dipeptides. For this reason, unlike the other experimental chapters within this thesis, this chapter will not be broken-down into Introduction, Results and Discussion sections. The results of some of these studies have been published previously (MacFarlane, McMurray, O'Dowd, Dargie and Miller, 1991; MacFarlane and Miller, 1992c; O'Dowd, O'Dowd, O'Dowd, MacFarlane, Abe and Miller, 1992) or have been submitted for publication (MacFarlane, dos Remedios and Miller, 1993).

Effects of captopril on hypochlorous acid-induced contractile dysfunction

The use of thiol-containing agents to 'scavenge' HOCl and protect against local coronary vasoconstriction has been examined as a therapeutic defence against reperfusion injury. Studies have identified endothelium-derived relaxing factor (EDRF) as either the free nitric oxide (NO) radical (Opie, 1989) or a chemically bound form of NO (S-nitrosothiol). Nitroglycerin is frequently used in the management of acute myocardial infarction and undergoes bioconversion, via a sulphydryl-dependent process, to nitric oxide which could potentially act to correct a localised deficiency of EDRF levels (Horowitz, 1991). However, nitrate tolerance represents a failure of the bioconversion step which can be ameliorated by addition of a thiol-containing compound to the nitrate. Thiol containing drugs such as N-acetylcysteine (NAC) potentiates the peripheral and

coronary vasodilator effects of nitrates (Winniford, Kennedy, Wells and Hillis, 1986). Additionally, NAC provides a degree of protection against ischaemia and reperfusion (oxidative stress does not occur, mitochondrial function is maintained, enzyme release is reduced and contractile recovery is increased). The protective effect of NAC *in vivo* probably occurs as a result of its ability to maintain the GSH/GSSG ratio (Ferrari, Ceconi, Currello, Cargnoni, Condorelli and Raddino, 1985). Captopril is a thiol-containing angiotensin converting enzyme (ACE) inhibitor that has attracted much attention due to its ability to scavenge HOCl directly *in vitro*. Peak plasma concentrations of approximately 17µM have been measured after a single 100mg oral dose in man (Creasey, Funke, McKinstry and Sugerman, 1986). In preliminary clinical studies higher plasma concentrations have been measured after intravenous administration of captopril (Rademaker, Shaw, Williams, Duncan, Corrie, Eglen and Edwards, 1986). Such therapeutic plasma concentrations have been suggested to offer protection against HOCl up to 50µM by *in vitro* experiments (Chopra *et al*, 1992; Chopra *et al*, 1993). Therefore, on the basis of these previous experiments it would be expected that 20µM captopril would be sufficient to ameliorate the functional consequences of HOCl exposure in the 'skinned-fibre' experiments previously described in chapter 4.

Captopril (20µM) has no direct effect on C_{max} or resting tension when applied to 'triton-skinned' cardiac muscle. However, the effects of 50µM HOCl were ameliorated when captopril was present prior to and throughout the exposure. The changes in C_{max} and resting tension produced by HOCl were significantly different in the presence of captopril. The former fell by 10.2±3.7% (compared with 42.3±23.2%, $p<0.02$, unpaired t-test, n=5) and the latter increased by 2.8±1.3% of control C_{max} (compared with 25.0±10.7%, $p<0.005$, n=5). Captopril had no direct effect on calcium sensitivity but, as with peak force and resting tension, diminished the effects of 50µM HOCl on calcium sensitivity when present throughout the exposure. The mean increase in log $K_{1/2}$ was only 0.024±0.009 (compared with 0.238±0.066, $p<0.01$, unpaired t-test, n=3) from a mean of 5.308 to 5.332 and *h* from 3.37 to 3.61 (Figure 8.1).
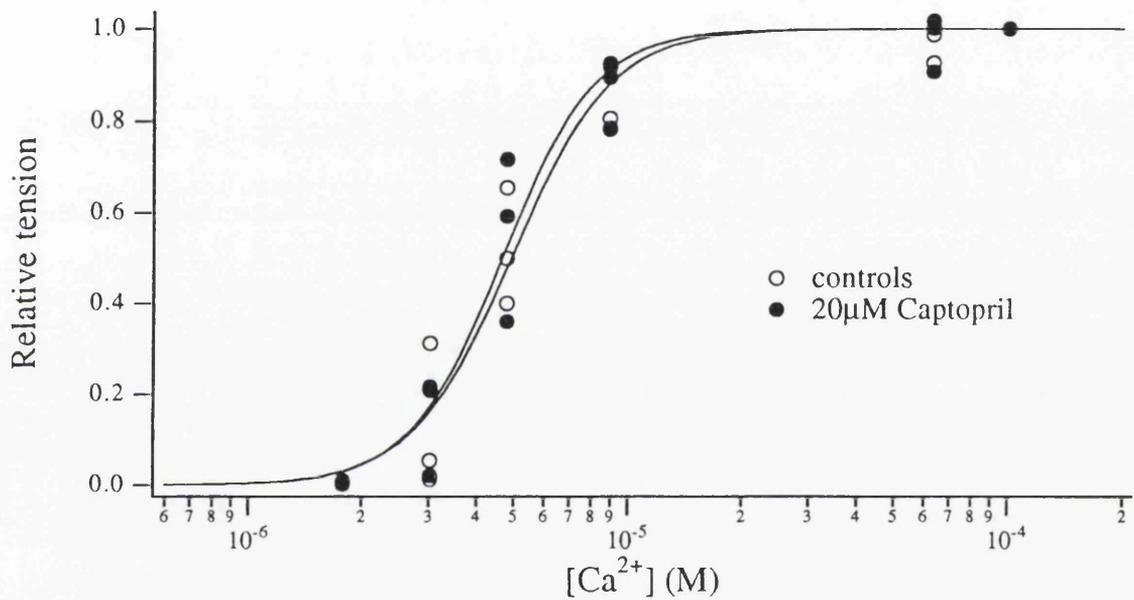
Figure 8.1 Graph showing the ameliorative effect of 20μM Captopril on the calcium sensitivity increase caused by application of 50μM HOCl. The ordinate shows the *relative* tension developed by the preparations. The abscissa shows calcium concentration at which the preparation were activated. Tension is expressed relative to C_{max} obtained for that series of activations: open circles (control), closed circles (50μM HOCl with 20μM Captopril present throughout the exposure). The preparation was exposed to HOCl for 1 minute at pCa 4.0. The curves are drawn according to the mean $K_{1/2}$ and Hill coefficient separately determined for each preparation.

Systemic distribution of endogenous histidyl dipeptides

Histidyl dipeptides such as carnosine (β-alanyl-L-histidine) and homocarnosine (γ-amino-butyryl-L-histidine) are reported to be present at millimolar concentrations in several mammalian tissues (O'Dowd, Robins and Miller, 1988; House, Miller and O'Dowd JJ, 1988), but their precise physiological function, or functions, is uncertain. These compounds are known to be potent buffers at physiological pH (Davey, 1960). They are also able to restore functional capacity to fatigued muscle preparations, stimulate some glycolytic enzymes, maintain coupling between mitochondrial oxidation and phosphorylation (Severin, 1964), cause vasoconstriction and modulate the calcium sensitivity of the contractile proteins (Lamont and Miller, 1992; Miller, Lamont and O'Dowd, 1993; Melville, Trainor, McGrath, Daly, O'Dowd and Miller, 1990).

Histidyl dipeptides may also have antioxidant activity, though this finding is controversial and will be investigated later in this chapter. For example, Aruoma *et al* (1989) have argued that these compounds, individually, are unable to scavenge O_2^-, H_2O_2 or HOCl at rates which could offer antioxidant protection *in vivo*.

Forms of these histidyl compounds exist that are acetylated and could contribute to the circulating and cellular levels of these imidazoles which could have a significant effect on cardiovascular performance and oxidative status. To investigate the distribution of these compounds in the heart and circulation two analytical methods have been developed in our laboratory by Drs Anne and John O'Dowd. The use of a detection system in the low-UV wavelength has permitted post-chromatographic detection without prior derivatisation (see O'Dowd, Robins and Miller, 1988; O'Dowd, Cairns, Trainor, Robins and Miller, 1990; O'Dowd, O'Dowd, O'Dowd, MacFarlane, Abe and Miller, 1992 for further details). Extractions of imidazoles from tissue were performed as follows. Portions of tissue were collected, blotted and frozen in liquid N_2 and then homogenised in an 80% solution (v/v) of ethanol prior to incubation and extraction (60°C, 90 minutes). Extracts were centrifuged (2000g, 15 minutes) and the supernatant removed and

evaporated under reduced pressure before re-suspension in phosphate buffer (Na_2HPO_4, 0.1M. pH 7.0, 2ml/100 mg tissue wet weight) for examination by analytical high performance liquid chromatography (HPLC). Extraction of imidazoles from blood was effectively the same as tissue except that the blood was collected in pre-cooled tubes containing the anti-coagulant K_2-EDTA. The blood was then centrifuged (2000g, 20 minutes) and the plasma removed and treated as the tissue samples.

In the first method applied (method A) reverse-phase analytical HPLC was performed using a Gilson/Apple liquid chromatography system (Gilson, Villiers le Bel, France/Apple Computers, Hemel Hempstead, UK.). This will separate histidyl ring-substituted and *N*-acetylated amino acids and dipeptides chemically unchanged, allowing their direct identification and quantification. There relative hydrophobicities allow the prediction of elution order in HPLC systems with aqueous eluent (Molnar and Horvath, 1977). For such systems with a non-polar stationary phase (such as octadecylsilane, ODS) eluted with a polar mobile phase, the retention time increases with hydrophobicity.

Samples dissolved in distilled water and were injected (20μl) onto a column (250x50mm) packed with Hypersil ODS (5μm; Anachem Ltd, London, UK.). The column was eluted isocratically at room temperature with phosphate buffer (Na_2HPO_4, 0.1M. pH2.0) over 30 minutes at a flow rate of 1.0ml/minute. Eluates were monitored for UV absorbance at 210nm. The output was relayed to a Chromatopac C-RIB data processor (Shimadzu Corporation, Kyoto, Japan.) and the areas under the peaks were used to provide data for calibrating the system. Subsequent analyses utilised stored chromatographic calibration data for comparison.

In the second method applied (method B) reversed-phase-ion-pair analytical HPLC was performed using the same system as above. Columns were eluted isocratically at 50°C with phosphate buffer (70mM KH_2PO_4, pH 3.3) containing 10mM 1-heptanesulphonic acid over 60 minutes at a flow rate of 1.0ml/minute. Eluates were

monitored for UV absorbance at 210nm and the output was recorded as described above. In contrast to method A, this method relies upon the interaction of a hydrophobic surfactant anion (1-heptanesulphonic acid) with a solute cation (the pH-dependent protonated terminal amino group) to form a 'solvophobic' uncharged group which then absorbs onto the non-polar stationary phase (Melander, Kalhatghi and Horvath, 1980). This 'ion-pair' may be eluted from the column in a temperature-dependent manner using an eluant of appropriate ionic strength (Riley, Tomlinson and Hafkenscheid, 1981).

In patients with chronic heart failure (compared with age-matched controls) a recent study has demonstrated, amongst the many compounds analysed, raised concentrations of histidine (64.0±14.1 vs 46.4±4.0µM, mean±SEM, p=0.12), carnosine (55.9±3.6 vs 42.4±4.4µM, p=0.013) and homocarnosine (22.2±11.7 vs not detectable, p=0.03) were found. However, in a coronary artery ligation model of heart failure no significant differences in carnosine were found (O'Dowd, O'Dowd, McMurray, Denvir and Miller, 1993).

Little is known of both carnosine and homocarnosine, and nothing of their relatives, in plasma. Concentrations are thought to be very low due to a circulating carnosinase, EC 3.4.13.3, (Perry, 1974; Gardner, Illingworth, Kelleher and Wood, 1991). Figure 8.2 shows an example of the HPLC traces (method B) obtained from a heart failure patient during cardiac catheterization for a routine clinical investigation (prior to triple valve replacement). Samples were taken from the aorta and from the venous drainage of different organs and anatomical regions (hepatic, renal, femoral, internal jugular and the great cardiac vein). The aortic blood sample (Ao) shows many of the compounds noted in peripheral blood by O'Dowd *et al* with the carnosine peak equivalent to a concentration of 57.8µM (Figure 8.2, Panel A). The great cardiac vein was cannulated via the coronary sinus and a blood sample from this region is referred to as a coronary sinus sample (CS) (Figure 8.2, Panel B). The early peaks, which characterise the acetylated histidyl dipeptides in this method, greatly exceed those observed in the Ao and represent at least a four-fold increase in their concentration. However, the CS sample

shows additional peaks representing homocarnosine (trace amounts) and an unidentified peak (Q). This peak is speculated to be homoanserine from the retention time and the hydrophobicity of homoanserine, however, as this compound is not commercially available proper identification is difficult. Figure 8.2, Panel C shows the HPLC trace obtained from a superior vena cava (SVC) blood sample. This sample was taken because in some patients it is very difficult to cannulate the internal jugular vein. The catheter was positioned in the SVC as close as possible to the internal jugular vein (under X-ray screening). The SVC sample taken was markedly different from the femoral vein sample in that there was an increase in the homocarnosine content (to $25.6\mu M$), but which may be diluted. This might be expected from the relatively high homocarnosine levels in brain, and especially hypophysial portal blood (Mitchell, Grieve, Dow and Fink, 1983). The blood samples obtained from the renal, hepatic and femoral veins were indistinct from peripheral venous samples obtained prior to cardiac catheterization.

In a further study the effects of ischaemia-reperfusion on the distribution of these compounds in the myocardium of Langendorff-perfused rabbit hearts. New Zealand White rabbits (2-2.25 kg) were sacrificed by a lethal injection (sodium pentobarbitone, 400mg/Kg). The hearts were rapidly removed, cannulated and perfused with Kreb's buffer (mM: NaCl, 119; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 24.8, glucose, 5) bubbled with $95\%O_2$-$5\%CO_2$. A pressure transducer on the perfusion catheter recorded perfusion pressure, the heart was not paced and tension recorded via a transducer attached to the apex of the heart. After the heart had stabilised tissue samples (10mg) were removed by means of a pre-cooled rotating hollow steel tube (*i.e.* the hollow tube replaced a drill in the bit of a hand-held power drill). A 5 minute re-stabilisation period was allowed, even though mechanical function was not affected by this manoeuvre, before global ischaemia was induced by stopping the perfusion pump and clamping the perfusion catheter. A further tissue sample was removed after 10 minutes of ischaemia and the heart was reperfused. After 10 minutes of reperfusion the last tissue sample was removed. All samples were flash frozen in liquid N_2 prior to extraction. No
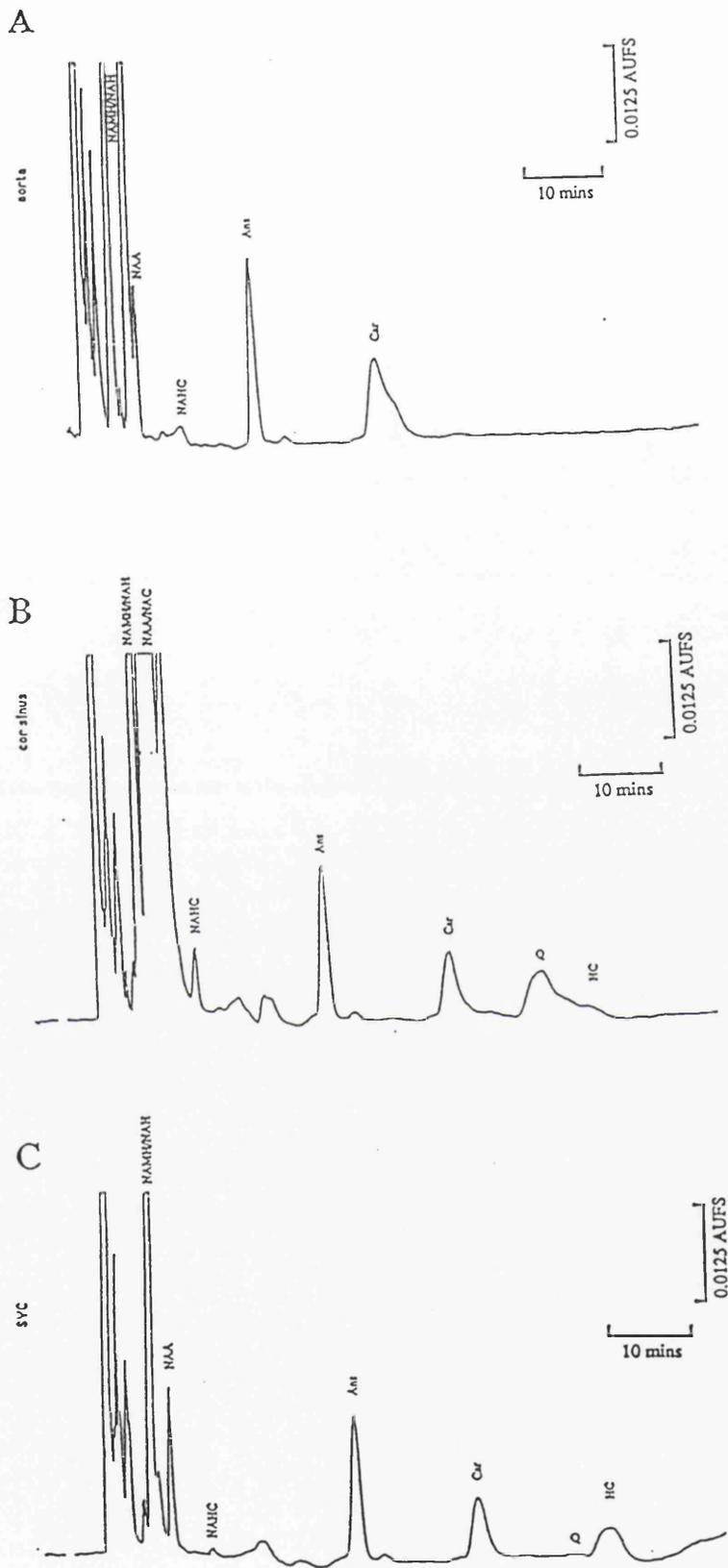
Figure 8.2 HPLC chromatographs of human serum extracts (see main text for details). Ordinate: Absorbance at 210nm (arbitrary units), Abscissa: Elution time (minutes). **Panel A**: Aortic serum. **Panel B**: Coronary sinus serum. **Panel C**: Superior vena cava serum. The concentrations detected by the peak areas represent *e.g.* NAMH/NAH A: 6.9µM **B**: 0.6mM **C**: 80µM-5mM.

alteration in the tissue concentrations of these compounds were noted during ischaemia or reperfusion. However, the presence of significant amounts of the compounds were obtained in the buffer solution which had reperfused the heart.

The diversity in the variety and concentration of these compounds, particularly homocarnosine, in the venous drainage from the heart and brain may be of importance in their postulated activity as endogenous free radical scavengers. The increase in plasma concentrations of these compounds appears to be related to ischaemia (since the CS sample from the heart failure patients bear a strong similarity to the histidyl dipeptide profile obtained from the perfusate of isolated perfused rabbit hearts).

Histidyl dipeptides as antioxidants: i. Photo-oxidation assay

The method used to assess the possible free radical scavenging ability of histidyl dipeptides was that described by Misra and Fridovich (1977). In this assay free radicals are generated by photo-oxidation of dianisidine sensitised by riboflavin. This involves a series of free radical reactions, involving O_2^- as the propagating species (Figure 8.3). A non-superoxide radical scavenger will inhibit this reaction, leading to a decrease in oxidised dianisidine measurable by a u.v./visible spectrophotometer. By contrast, a scavenger specific for O_2^- will remove this species from steps (3) and (4) in the reaction, increasing the amount of oxidised dianisidine and augmenting the assay. Compounds with no free radical scavenging activity will not affect this assay, as has been validated in other studies (Chopra, Scott, McMurray, McLay, Bridges, Smith and Belch, 1989; McNeil, Banford, Brown and Smith, 1981). Standard scavengers behave in a predictable way in this assay. For example, the non-superoxide scavengers ascorbate, uric acid, glutathione and ergothioneine, at $4 \times 10^{-5}M$, inhibit by 23, 31, 74 and 52% respectively (means of three observations). By contrast, superoxide dismutase (1.44U/ml) produced an augmentation by 179%.

Riboflavin solution (1.3×10^{-5}M) was prepared in 0.01M potassium phosphate buffer pH 7.5 and *o*-dianisidine solution (10^{-2}M) was prepared in ethanol (Sigma Chemicals Ltd, Poole, Dorset, UK.). Illumination for the photochemical reaction was provided by a pair of parallel 10 watt white fluorescent tubes mounted 15cm apart in an enclosed box. These tubes were used to provide a constant source of wide band radiation. Statistical analysis was performed by one-way analysis of variance and any significant variances subjected to an unpaired t-test with the Bonferroni correction applied.

The procedure for the assay was as follows: 60µl of *o*-dianisidine solution was added to 0.94ml of the riboflavin solution. Absorbance of light was measured at 460nm using a Pye Unicam PU8600 u.v./visible spectrophotometer. The cuvette was then transferred to the illumination box, illuminated for 4 minutes and the absorbance re-measured at 460nm. The change in absorbance after 4 minutes illumination served as the control reading. 60µl of the test solution was then added to 0.88ml of riboflavin followed by 60µl of *o*-dianisidine and measurements carried out as above. Different concentrations (1-50mM) of each test compound were tested individually. Mixtures of the compounds (total concentration 10mM) in concentration ratios near to those reported in cardiac and skeletal muscle (8:1:1:2 and 1:8:1:2, respectively) were also tested. Each assay was repeated five times with intra-assay variation of less than 8%. The histidyl dipeptides, alone or in combination, had no effect on absorbance at 460nm by the reactants without illumination (*i.e.* without free radical generation).

Rb	+	hv	--------->	Rb^*					(1)
Rb^*	+	DH_2	--------->	$RbH^\cdot$	+	$DH^\cdot$			(2)
$RbH^\cdot$	+	O_2	--------->	Rb	+	O_2^-	+	H^+	(3)
$DH^\cdot$	+	O_2^- + H^+ ----->		DH_2	+	O_2			(4)
$DH^\cdot$	+	$DH^\cdot$	--------->	D	+	DH_2			(5)
O_2^-	+	O_2^-	--------->	H_2O_2					(6)

Rb: Riboflavin

hv: energy of photon of light

Rb^*:   excited riboflavin

DH_2: o-dianisidine

O_2^-:   superoxide anion

D: product formed by photo-oxidation measured at 460nm

Figure 8.3 Photo-oxidation of o-dianisidine.

Panel A in Figure 8.4 shows the effect of the final concentration of each individual test compound in the assay. All the compounds, with the exception of homocarnosine, inhibited this assay over the range of concentrations tested, suggesting that they are general rather than superoxide-specific free radical scavengers. It is uncertain, however, whether this degree of scavenging (approx. 40%) would be significant *in vivo*. Homocarnosine, however, inhibits the assay at low concentrations and augments it at higher concentrations.

Figure 8.4, panel B, shows the effect of a combination of the histidyl-containing compounds at a total concentration of 10mM, compared with the effect of 10mM of the individual compounds. In combination there is an apparent potentiation of O_2^- preferential scavenging by homocarnosine. This can be described as a synergism between homocarnosine and related histidyl dipeptides, being most clearly seen in the concentration ratio thought to be associated with cardiac muscle (*i.e.*. one predominantly containing homocarnosine).

These results show that, in this assay, individual histidyl dipeptides have antioxidant activity consistent with that previously reported (Aruoma, Laughton and Halliwell, 1989; Boldyrev, Dupin, Batrukova, Bauykina, Korshunova and Shuachkin, 1989). It is uncertain how much *in vivo* protection may be provided by these levels of activity (Aruoma *et al*, 1989), but it has been suggested that these compounds may be a useful tool in the therapy of various diseases associated with oxidative stress (Boldyrev *et al*, 1989). However, these data suggest that, in combination, histidyl dipeptides exhibit synergistic antioxidant activity.
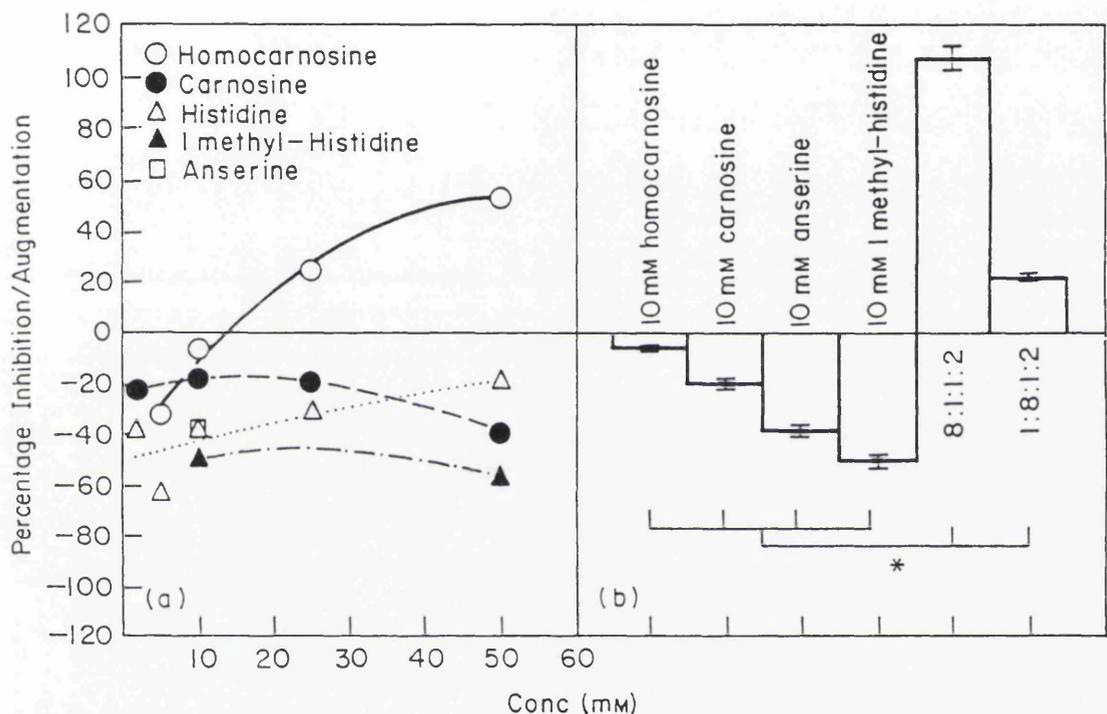
Figure 8.4 Photo-oxidation of *o*-dianisidine sensitised by riboflavin. **Panel** (a): The effect of concentrations of individual compounds on the assay (mean of 5 results for which the S.D. were smaller than the symbols). **Panel** (b): Comparison of 10mM homocarnosine, carnosine, anserine and 1-methyl histidine individually and in concentration ratios of 8:1:1:2 and 1:8:1:2 (mean of 5 results ± S.D., *=p<0.001).

Histidyl dipeptides as antioxidants: ii. Cytochrome-*c* assay

Superoxide dismutases are unique among enzymes in that their substrate is an unstable free radical. This complicates the measurement of their catalytic activity. Convenient assays of SOD have necessarily been of an indirect type. Such assays consist of two components: a O_2^- generator and a superoxide detector. The generator serves to produce the radical at a controlled rate and in the absence of SOD the radical reacts with the detector for O_2^-. Of the many assays which have been proposed, one of the most reliable is the spectrophotometric assay initially described at the time the SOD function was identified (McCord and Fridovich, 1968), in which xanthine/xanthine oxidase is used as a source of O_2^- and cytochrome *c* is used as the indicating scavenger for the radical. The reduction of cytochrome *c* by the xanthine oxidase enzyme is dependent upon oxygen (Fridovich and Handler, 1962) and can be attributed generation of O_2^-.

A unit of SOD (as for any enzyme) is defined functionally. It is the quantity of SOD required to produce 50% inhibition of the rate of reduction of cytochrome *c* under specified conditions. This unit represents a concentration of SOD; thus, the absolute amount of enzyme required to produce this inhibition varies with the volume of the assay mixture. When working with purified preparations of SOD the rate of change in absorbance is zero before the addition of xanthine oxidase. Crude tissue homogenates may cause small rates of change in the baseline that can be subtracted from the reaction rate. The control reaction rate can be completely inhibited by large amounts of SOD if a xanthine oxidase of high quality is being used. Partially degraded xanthine oxidase can, to a small extent, reduce cytochrome *c* by a non-O_2^- mediated mechanism.

The possibility exists, therefore, to utilise this assay in the investigation of the SOD-like activity of the histidyl dipeptides and also to determine whether the xanthine oxidase concentrations used in chapter 3 are producing the predicted concentrations of O_2^- within the conditions specified by these experiments. These possibilities are discussed below.

The procedure for the assay was as follows: Cytochrome c (0.05mM) and 50μM xanthine were dissolved in solution A, table 2.1 and then placed in a 1ml cuvette in the light path of a Cecil CE 4400 UV-double beam scanning spectrophotometer. After recording the baseline absorbance at 550nm had been recorded, the reaction was initiated by the addition of 20mU/ml of xanthine oxidase, both in the presence and absence of SOD, and the rate of increase in absorbance was measured. At this concentration of xanthine oxidase the rate of increase should be of the order of 0.020 absorbance units per minute (Crapo, McCord and Fridovich, 1978). The amount of O_2^- required to reduce cytochrome c at this rate can be calculated from the molar extinction coefficients (ε) given by Massey (1959) using the Beer-Lambert law (*i.e.* Absorbance $= \log_{10}(I_0/I) = \varepsilon cl$, where l is the length of the light path, c is the concentration of the substance causing the absorbance change and I_0/I is the transmittance) as *circa* 10nmol O_2^-min^{-1}. Within the experimental solutions described in chapter 3 the concentration of O_2^- produced was calculated as 6.6$\pm$0.3nmol O_2^-min^{-1} (from a rate of increase in absorbance of 0.014$\pm$0.002, mean$\pm$S.D.). Therefore, the conclusion that the myofilaments are particularly susceptible to O_2^- damage cannot be explained by higher than expected levels of O_2^- were being produced within the experimental solutions.

By adding a variety of the histidyl dipeptides to the reaction mixture in this assay it is possible to investigate further the SOD-like activity of homocarnosine and the possibility of a synergistic action between the compounds. Additionally, this assay will allow determination of the free radical scavenging activity of these compounds in relation to a 'SOD-unit of activity'. Figure 8.5 demonstrates the effect of 10mM homocarnosine and the mixture of histidyl dipeptides representing cardiac muscle (which gave the greatest synergistic effect within the photo-oxidation assay described previously). 10mM homocarnosine itself depresses the level of cytochrome c reduction by around 40% over the 5 minutes in which the reaction was followed spectrophotometrically. Under the previously discussed definition of a unit of SOD activity, the 10mM homocarnosine would be approximately equivalent to 1 unit of SOD per ml under these conditions. The

cardiac mixture of histidyl dipeptides did not augment the SOD-like activity of homocarnosine to the same extent as in the photo-oxidation assay and after 5 minutes the reduction in cytochrome c had been reduced by only 30%.

The results obtained in this assay may help to explain the apparent synergistic antioxidant activity obtained in the photo-oxidation assay previously described. In this assay, the cytochrome c reduction is solely dependent on the generation of O_2^-, therefore, the effect of the histidyl dipeptides on other radical species are not taken into account. Within the photo-oxidation assay the augmentation or inhibition of the assay is dependent upon the combined effect of the test substance on O_2^- and non-O_2^- radical species. Decreasing the concentration of homocarnosine in the mixture of histidyl dipeptides to just over 7mM had little effect on it's O_2^--scavenging activity (equivalent to 1 SOD unit), as demonstrated by the cytochrome c assay. In the photo-oxidation assay the mixture of histidyl dipeptides augmented the assay by approximately two-thirds of the level obtained by 1.44U/ml of SOD (*i.e.* equivalent to 1 unit of SOD). These points will be discussed in further detail later within this chapter.
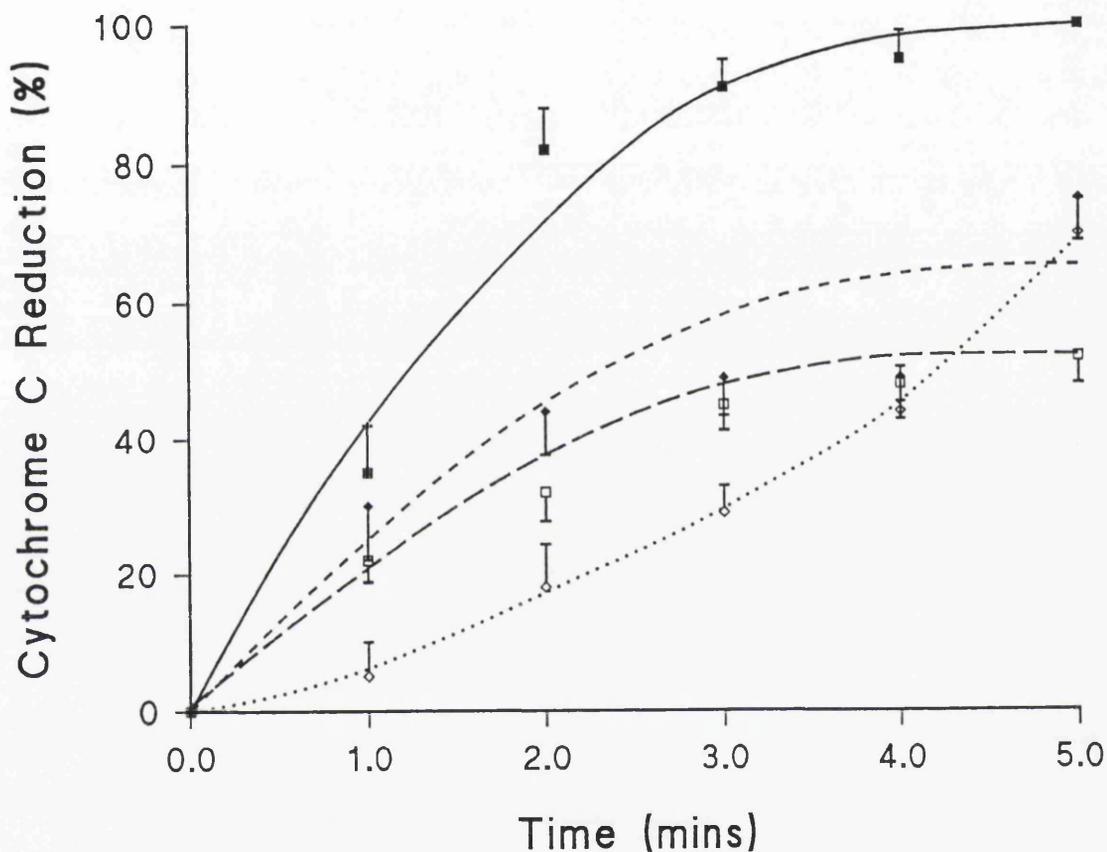
Figure 8.5 Graphs showing the effect of histidyl dipeptides on cytochrome *c* reduction. The ordinate shows the relative level of cytochrome *c* reduction as a percentage of the peak reduction in control experiments. The abscissa shows the time-course of the assay in minutes. Control experiments (inicated by solid squares and a solid line), homocarnosine alone (indicated by the solid diamonds and the short broken line), the mixture of histidyl dipeptides representing skeletal muscle (indicated by the open squares and long broken line) and the mixture of histidyl dipeptides representing cardiac muscle (indicated by the open diamonds and the dotted line) are plotted as means±SEM.

Histidyl dipeptides as antioxidants: iii. Neutrophil activation

The physical and biochemical processes whereby phagocytic cells such as neutrophils engulf and subsequently destroy bacteria have been the target of active research in recent years. Hypochlorous acid formed during the course of the myeloperoxidase reaction contributes to the bactericidal effects of neutrophils and it has long been known that the interaction of hypochlorite (OCl^-) and H_2O_2 results in light emission (Seliger and McElroy, 1960). Such chemiluminescence occurs due to the release of singlet oxygen, which emits a photon, during the reaction between OCl^- and H_2O_2.

In determining the instrumentation capable of detecting the chemiluminescence response of cells undergoing phagocytosis, it is necessary to realise, in relative terms, the amount of light being generated and the wavelength. Basically, there are two systems which may be employed: *i.* measurement of the weak inherent cellular light which has a broad wavelength spectrum (with the highest intensities in the red region) using photomultiplier tubes, and *ii.* an amplified-light system dependent on the reactive oxygen species generated during phagocytosis.

The methodology described in this section of the chapter is based on the method used to measure polymorphonuclear leucocyte chemotaxis described by Wilkinson (1974) which has been developed by Dr JJ McMurray, Western Infirmary, Glasgow. It is an amplified-light system utilising the chemiluminescent compound luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), which is converted to an excited aminophthalate ion in the presence of an oxidising species and emits blue light at 425nm (Vilim and Wilhelm, 1989). Agents which interfere with the activity of reactive oxygen species formed by neutrophils (*e.g.* SOD and catalase) or inhibit certain metabolic events (*e.g.* sodium azide) lead to a decreased chemiluminescence response in otherwise normally functioning neutrophils.

The procedure for the assay was as follows: a 5ml peripheral venous blood sample is mixed with 5IU/ml of sodium heparin (preservative-free) and layered onto 4ml 'lymphoprep' (9.6%(w/v) sodium metrizoate, 5.6% Ficoll from Nycomed, Norway) in a sterile conical tube. The blood/lymphoprep mixture is spun at 1800rpm for 15 minutes at 4^oC. The plasma and monocyte layers are removed with a Pasteur pipette after centrifugation. 15ml of erythrocyte lysis buffer (0.1155M NH_4Cl, 0.01M $KHCO_3$, 0.1mM Na_2EDTA) is added to the remaining erythrocyte/PMN leucocyte layer. The mixture is gently mixed, left for 15-20 minutes at room temperature with occasional mixing until the liquid becomes clear and claret in colour. The mixture is then spun at 1000rpm for 10 minutes at 4^oC. The supernatant is discarded and the PMN pellet is re-suspended in 15ml erythrocyte-lysis buffer and re-spun at 1000rpm for 10 minutes at 4^oC.

The PMN is suspended in 1ml Gey's Bovine Serum Albumin (BSA, 2% w/v) solution. $10\mu l$ of the PMN suspension is added to $90\mu l$ of white cell diluting fluid. The diluted PMN suspension is placed into a Neubauer chamber and the number of PMN leucocytes is counted per 16 squares using a low power magnification. The number of PMN leucocytes counted is then divided by a factor of ten and the figure so obtained represents the amount in mls (minus 1ml) of Gey's BSA solution to be added to the PMN suspension and mixed to give an approximate count of 0.3×10^6 cells/ml.

Gey's BSA (2% w/v) is prepared from three stock solutions as follows: Stock solution A contains NaCl 28.313g, KCl 1.875g, $Na_2HPO_4.2H_2O$ 0.75g, KH_2PO_4 0.125g, $NaHCO_3$ 1.25g and D-glucose 10.0g in 500ml of distilled water. Stock solution B contains NaCl 11.687g in 250ml of distilled water. Solution C contains $CaCl_2.6H_2O$ 0.687g and $MgCl_2.6H_2O$ 0.525g in 250ml of distilled water. 10ml of solution A and 5ml of solution B are added to 80ml of distilled water. Solution C is added last to make up a final volume of 100ml in order that calcium precipitation is prevented. Bovine serum albumin (2g, fraction V) is dissolved in 100ml Gey's solution.

A 10mM stock solution of luminol was dissolved in dimethyl sulphoxide and added to the PMN Gey's solution reaction mixture ($50\mu M$) with a final volume of 1ml. The reaction was initiated by addition of $1\mu g$ phorbol myristate acetate (PMA) to the reaction mixture and the effect of the test compounds upon luminol chemiluminescence was measured by a LKB 1251 Wallac Luminometer at 37^oC.

Figure 8.6 is a graph showing the effect of 10mM homocarnosine and the cardiac and skeletal profile of histidyl dipeptides on PMA-induced neutrophil activation as measured by chemiluminescence. The graph shows that the cardiac mixture of histidyl dipeptides have a similar effect to that of 10mM homocarnosine alone, both tending to maintain a 40-60% inhibition on chemiluminescence compared with the control cells. The time course of the chemiluminescence response to neutrophil activation is markedly different in the presence of the histidyl dipeptides which could contribute to their antioxidant activity. It is important to note that in both the previous assays the change in absorbance was monitored over 4-5 minutes, at the point where the greatest difference in chemiluminescence was observed. These results are further discussed later in this chapter.
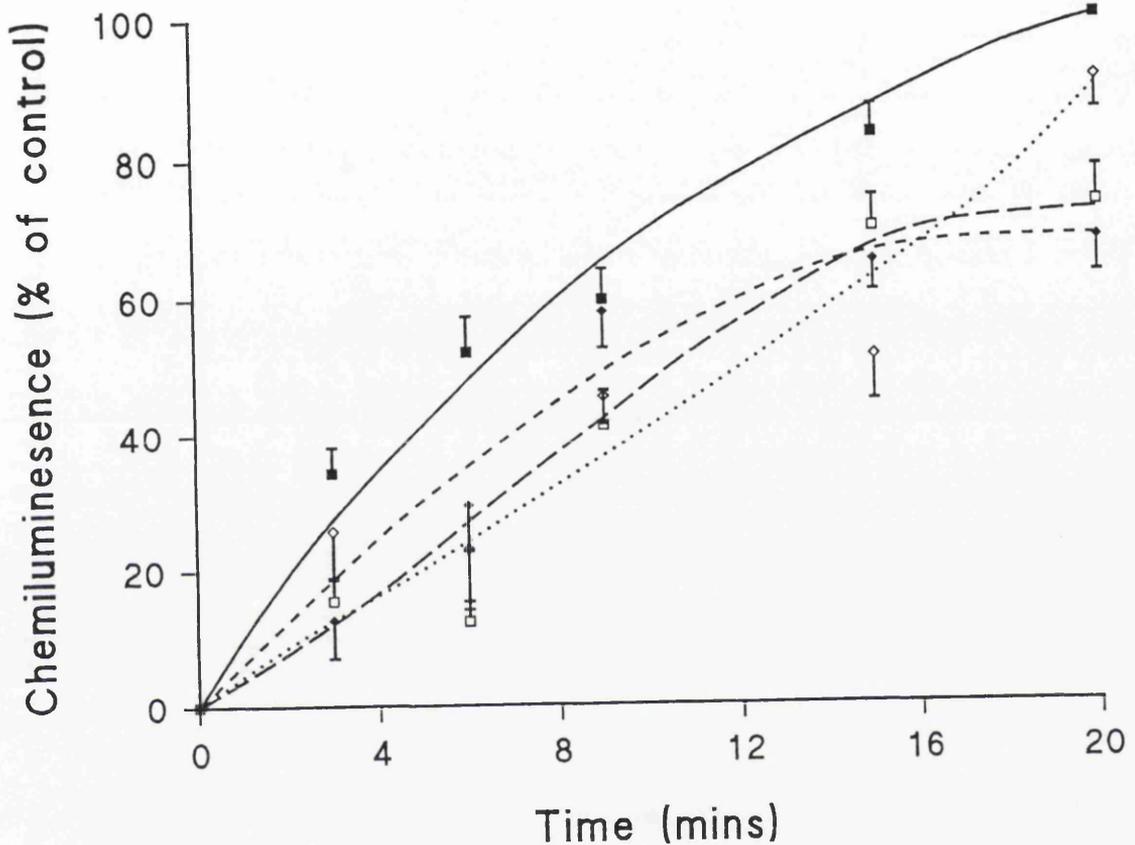
Figure 8.6 Graph showing the effect of histidyl dipeptides upon chemiluminesence due to neutrophil activation. The ordinate shows the chemiluminesence relative to that obtained in control experiments. The abscissa shows the time-course of the assay in minutes. Control experiments (inicated by solid squares and a solid line), homocarnosine alone (indicated by the solid diamonds and the short broken line), the mixture of histidyl dipeptides representing skeletal muscle (indicated by the open squares and long broken line) and the mixture of histidyl dipeptides representing cardiac muscle (indicated by the open diamonds and the dotted line) are plotted as means±SD.

Conclusions

There is much interest in the role of free radicals and/or reactive oxygen species during ischaemia and reperfusion under a variety of experimental and clinical circumstances. Considerable emphasis has been given to regional ischaemia and evolving myocardial infarction, where early reperfusion by thrombolysis has now become a routine clinical procedure. It has become clear that it would be of considerable importance if interventions could be developed that, if given just prior to (or at the time of) reperfusion would reduce tissue injury and limit the size of the evolving infarct. In the surgical arena, there is interest in improving the rate and extent of contractile recovery following the period of global ischaemia that occurs during cardiopulmonary bypass surgery or cardiac transplantation.

An array of experimental and clinical studies with agents as diverse as SOD, catalase, mercaptopropionylglycine and N-acetylcysteine have been undertaken. While a number of impressive results have been presented it must be conceded that controversy remains and there are some negative reports with antioxidant therapy. One difficulty which may account for some studies that have not produced positive results has been the failure of many investigators to establish adequate dose-response curves. Many cardioactive drugs, including antioxidants, show a bell-shaped dose-response curve, with narrow therapeutic ranges. Not only does this make it difficult to select the correct dose for study, but failure to observe a protective effect might incorrectly result in a drug being dismissed as ineffective.

The results described in this chapter confirm that captopril is effective in protecting against physiologically-relevant levels of HOCl at therapeutically-relevant doses. In addition the histidyl dipeptide homocarnosine appears to have SOD-like activity (10mM being equivalent to 1U/ml) as determined by three distinct assays, which could be important physiologically due to the apparent mobilisation of this compound during ischaemia and reperfusion (as established by HPLC techniques). The increase in the

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circulating levels of the homocarnosine in heart-failure patients could have dual effects, both as an antioxidant and as a calcium-sensitiser (Lamont and Miller, 1993; Miller, Lamont and O'Dowd, 1993).

The broad definition of a biological antioxidant includes compounds which can prevent oxidative damage to lipids, nucleic acids and proteins. The majority of antioxidants are specific, providing only one form of protection (*e.g.* blocking initiation of free radical production, removing mediators which catalyse free radical damage or reacting with the reactive species to spare the biological target). The mechanisms which defend against oxidative stress are the most important in several categories of antioxidant mechanisms. The enzymes SOD, catalase and glutathione peroxidase serve in the first line of defence against free radicals and reactive oxygen species. Other small antioxidants, such as ascorbic acid and glutathione can protect against reactive oxygen species in areas where the defence enzymes have limited access. Beside the enzyme SOD, there are other small molecules which posses SOD-like activity *in vitro*, usually small complexes of amino acids and copper ions, so that investigation of the antioxidant activity of the histidyl dipeptides seemed appropriate.

One mechanism by which antioxidants can protect their biological targets is the chelation of transition metals such as copper and iron thus preventing them from participating in the deleterious Haber-Weiss and Fenton reactions. Carnosine, homocarnosine and anserine have been shown to be efficient copper chelating agents *in vivo* (Brown, 1981) which could further contribute to their possible antioxidant activity. Additionally, a recent study has demonstrated SOD-like activity in copper:carnosine and copper:homocarnosine complexes (Kohen, Misgav and Ginsburg, 1991). Interestingly, carnosine (3mM) and homocarnosine (3mM) were demonstrated to decrease chemiluminescence in phorbol miresitate acetate or histone-opsonized streptococci A stimulated neutrophils by approximately 15% when added alone. These results are not inconsistent with the chemiluminescence experiments described in this chapter.

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The additional SOD-like activity of the histidyl dipeptides when chelated with copper is intriguing. The active site of the SOD enzyme is formed by four imidazole groups of histidine with a Cu^{2+}, further linked to a Zn^{2+} which stabilises the catalytic site (Getzoff, Cabelli, Fisher, Parge, Viezzoli, Banci and Hallewell, 1992). Bis[cyclo(histidyhistidine)]copper(II) complexes, which also have SOD-like catalytic activity, has recently been reported to have crystalline structure with the Cu^{2+} tightly co-ordinated with four nitrogen atoms of the imidazole groups in a plane square (Hori, Kojima, Matsumoto, Ooi and Kuroya, 1979). Although the solid-state structure of such a complex might not be preserved in solution, the bis[cyclo(histidyhistidine)]copper(II) complex has been found to be a rigid structure in aqueous solution and not easily broken up by other chelating agents (Kubota and Yang, 1984), so that chelated histidyl dipeptides may resemble the active site of the SOD enzyme more closely. SOD has evolved to be one of the fastest known enzymes ($V_{max} \sim 2 \times 10^9 \ M^{-1}s^{-1}$; Klug, Rabani and Fridovich, 1972), however, even faster SOD mutants have been designed by site-specific mutations that increase local positive charge in the amino acid side chains (Getzoff *et al*, 1992) and enhance electrostatic guidance which has been demonstrated to increase the dismutation reaction normally considered to be limited by diffusion (Getzoff, Tainer, Weiner, Kollman, Richardson and Richardson, 1983).

The results of the photo-oxidation assay described in this chapter suggest an apparent synergism between the histidyl dipeptides which produce a potentiation of the SOD-like scavenging activity of homocarnosine. However, this effect is not sustained within the other assays utilised and probably results from as an experimental 'artefact' of that assay. Perhaps the major difference in the methodology of three assays described previously is in the buffers used in the experimental solutions. The cytochrome *c* assay and the chemiluminescence assay contain either EGTA or EDTA in their solutions whereas the photo-oxidation assay does not. The remote possibility exists for the solutions in the photo-oxidation assay to be sufficiently contaminated with transition metals which could alter the antioxidant activity of the histidyl dipeptides. This alteration

might be further enhanced by a mixture of dipeptides which facilitate diffusion of O_2^- to a SOD-like imidazole-transition metal dismutation site. However, such a possibility appears unlikely since the mixture of histidyl dipeptides in the concentration ratio similar to that found in cardiac muscle appears to have equivalent scavenging activity (relative to SOD activity) to the 10mM homocarnosine alone in the other assays. An alternative explanation for this apparent 'artefact' could be that the cytochrome c and the chemiluminescence assays contain biological material, and thus have greater transition metal contamination. The resultant imidazole-transition metal dismutation site might not be easily disrupted by other chelating agents (Kubota and Yang, 1984) and could enhance the effect of homocarnosine alone in these assays. It could be postulated that a mixture of histidyl dipeptides in the photo-oxidation assay form a similar rigid structure in aqueous solution, with similar enhanced catalytic activity to copper:homocarnosine in the other assays described. However, such an interaction would be unprecedented.

It is important to remember that it is not necessary to chelate the histidine-containing compounds in order that they have antioxidant activity. Carnosine, anserine and homocarnosine inhibit lipid peroxidation when added alone (Boldyrev, Dupin, Pindel and Severin, 1988). This scavenging activity will result in accumulation of the products of their oxidative reduction. In some situations the products of free radical scavenging can be more reactive than the initial radical, but in the case of the histidyl dipeptides imidazolones should be formed (Prof DJ Robins, personal communication) which have antioxidant and cardiotonic activities of their own (Smith, Reeves, Dage and Schnettler, 1987). The time course of the chemiluminescence assay suggest that the histidyl dipeptides act by reacting with the reactive species to spare the biological target, and that there is still significant amounts of the histidyl dipeptides or their oxidative products after 15 minutes of exposure to oxidative species. However, the increase in chemiluminescence appears to be exponential, suggesting that no antioxidant activity would be seen after 20 minutes (which is a similar time course to the length of ischaemia required to produce myocardial stunning).

The data presented within this chapter could be regarded as evidence in favour for an anti-oxidative function of the histidyl dipeptides during ischaemia and reperfusion. However, further investigations are required to ascertain whether they have important *in vivo* activity as antioxidants and possible interaction between these compounds in their antioxidant activity.

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