THE EFFECTS OF HYPOXIA, ISCHAEMIA AND INOTROPIC INTERVENTIONS ON MAMMALIAN CARDIAC MUSCLE.

A thesis submitted for the degree of

Doctor of Philosophy

in the Faculty of Science, University of London

by

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October 1989.

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"The heart is a small thing,....it is not sufficient for a kite's dinner.

Francis Quarles (Hugo de Anima).

ABSTRACT.

THE EFFECTS OF HYPOXIA, ISCHAEMIA AND INOTROPIC INTERVENTIONS ON MAMMALIAN CARDIAC MUSCLE.

Variations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) are responsible for much of the modulation of the force of contraction in cardiac muscle. However, in many situations, changes in the sensitivity of the contractile proteins to Ca^{2+} also contribute to alterations of force. If $[Ca^{2+}]_i$ and force production are measured simultaneously, it is possible to identify the contribution of these processes to the changes in the force of contraction produced by various interventions.

Two interventions of clinical importance are hypoxia and ischaemia. Their effects were studied in thin ventricular papillary muscles, which were microinjected with the photoprotein aequorin in order to measure $[Ca^{2+}]_i$. During an initial exposure to hypoxia, the systolic rise of $[Ca^{2+}]_i$ (the Ca^{2+} transient) often showed a moderate increase, but on subsequent exposures this increase disappeared and eventually the Ca^{2+} transient declined on exposure to anoxia. This decline could be converted back to an increase by exposure to an elevated glucose concentration, suggesting that the response was dependent on the metabolic state of the muscle. This was confirmed in a parallel series of experiments in which glycogen and lactate production were measured in Langendorff-perfused hearts exposed to hypoxia.

Ischaemia has previously been difficult to study in isolated cardiac muscle. A new model was developed which allowed ischaemia to be mimicked in an isolated papillary muscle while $[Ca^{2+}]_i$ was measured. Ischaemia caused a dramatic decline in tension, and a large increase in the amplitude of the Ca^{2+} transients developed over several minutes. These changes could be mimicked by the application of 20 mM lactic

acid. After long exposures to ischaemia, resting tension developed and the Ca^{2+} transients declined. Repeated exposures to ischaemia caused an early fall in the Ca^{2+} transients, and the early development of resting tension and raised resting Ca^{2+}. Thus, many of the effects of hypoxia and ischaemia seem to be attributable to an intracellular acidosis due to lactic acid accumulation, which is more severe in ischaemia due to the lack of flow. The severity of the acidosis depends on the initial metabolic state of the tissue.

Although past laboratory studies have concentrated on isometric contraction, *in vivo* the heart has phases of both isotonic as well as isometric contraction, and modern use of cardiac myocytes means that unloaded preparations are now frequently studied. The effects of several positive and negative inotropic interventions on isotonic and isometric contraction were compared. For all interventions, the fractional effect on tension was 1.5 - 2 times larger than that on shortening, and this could be accounted for by the shape of the length-force relation for cardiac muscle in different inotropic states.

Pimobendan is a new inotropic agent, which is thought to have both Ca^{2+} sensitising and phosphodiesterase inhibiting activity. Its effects on tension and Ca^{2+} transients were studied. The results suggest that both these effects are active in living cardiac muscle, and that this combination may be advantageous for an inotropic agent.

These experiments have demonstrated that changes in both the Ca^{2+} transient and Ca^{2+} sensitivity occur when cardiac muscle is exposed to hypoxia or ischaemia. The effects of interventions on isometric force and shortening are similar. Finally, drugs are now available which can alter Ca^{2+} sensitivity as well as the Ca^{2+} transient.

ACKNOWLEDGEMENTS.

Firstly, I would like to thank David Allen for supervising my PhD and for always being available to answer my questions and discuss problems. I have particularly valued his insistence on always questioning assumptions and on uncovering mechanisms behind the phenomena. I am also grateful to him for being willing to argue with me about virtually anything; I have enjoyed and learnt from our exchanges.

I would also like to thank all the people with whom I have worked over the last three years for their help and advice. In particular, I would like to thank Jon Kentish, David Eisner, Max Lab, Godfrey Smith, Caspar Rüegg, Hakan Westerblad, Akira Takai and John Solaro for helping me sort out my thoughts on various matters. In addition, I would like to thank the inhabitants of the Physiology Department at University College for much enjoyable discussion in the Starling room over tea, and members of the "U.K. muscle physiology fraternity" for equally enjoyable discussion at Physiological Society meetings, generally over coffee.

I am grateful to the Medical Research Council for a Research Training Fellowship and for support to attend scientific meetings, and also to the Physiological Society for providing in its meetings such an excellent training ground.

Finally, I would like to thank Lucy for all her encouragement and support.

TABLE OF CONTENTS.

LIST OF FIGURES.

the calcium transients and tension.

Figure 14. The effects of various interventions on the timecourse of the calcium transients and twitch.

Figure 15. The effects of a long exposure to ischaemia on the calcium transients, tension and action potentials.

Figure 16. Comparison of the effects of ischaemia and the application of 20 mM lactic acid.

Figure 17. The effects of repeated exposures to ischaemia.

Figure 18. The effects of ischaemia on resting light.

Figure 19. The effects of 20 mM lactic acid on resting light.

Figure 20. Alternans producecd by ischaemia and the application of 20 mM lactic acid.

Figure 21. Oscillations of intracellular calcium produced by ischaemia and the application of 10 μM strophanthidin.

Figure 22. Calculation of the effects of changing extracellular lactate and pH on intracellular lactate and pH; intracellular buffering assumed to be 70 mM/pH unit.

Figure 23. Calculation of the effects of changing extracellular lactate and pH on intracellular lactate and pH; intracellular buffering assumed to be 20 mM/pH unit.

Figure 24. The effects of increasing extracellular calcium concentration on isometric developed tension and isotonic shortening.

Figure 25. The effects of increasing stimulation frequency on isometric developed tension and unloaded shortening.

Figure 26. The effects of increasing stimulation frequency.

Figure 27. The effects of acidosis on isometric developed tension and isotonic shortening.

Figure 28. The effects of cyanide on isometric developed tension and isotonic shortening.

LIST OF TABLES.

CHAPTER 1. INTRODUCTION.

1.1 The heart.

For centuries authors followed Aristotle in asserting that the heart was the seat of the intellect, pointing out, for example, that the voice issues from the chest. That particular theory was eventually laid to rest by the Roman physician Galen, who traced the course of the recurrent laryngeal nerves from the spinal cord to the chest, and then back up to the larynx, proving that in fact the brain was the organ controlling speech (Jackson, 1988). Although Galen was able to establish the intellect in its correct position, his understanding of the functions of the heart and lungs was not so satisfactory (see Keele, 1965). Fifteen hundred years were to pass before the elegant experiments of William Harvey established the concept of the circulation of the blood (Harvey, 1628), an achievement which marks the beginning of the scientific study of physiology and lead directly on to concepts such as the "milieux interieur" of Bernard (Bernard, 1865) and modern formulations of homeostasis.

An appreciation of the activity and function of the heart leads quite quickly to a feeling of surprise that an organ can be so active for so long, and a desire to know just how "active" the heart is. Indeed, William Harvey himself was the first person in the history of physiology to use a quantitative argument, when he calculated the volume of blood expelled by the heart in half an hour and used the large number so generated as one of the pieces of evidence supporting the existence of a circulation. In fact, a large amount of information is available on the activity of the heart in various animals (Schmidt-Nielsen, 1984). One aspect of this activity is summed up in the allometric equation relating the resting frequency of the heartbeat to body weight in a variety of mammals (Stahl, 1967):

$$\text{Heartbeat frequency} = 241 \times (\text{body weight})^{-0.25} \qquad (1.1)$$

where heartbeat frequency is measured in beats/minute and the body weight in kilograms. For the classical "70 kilogram man", this equation yields a heart rate of 83 beats/minute, quite close to the true value. In other words, when humans are compared with a variety of other mammals, it is seen that our hearts beat at roughly the expected rate for our body weight. (Note: The size of the heart scales with an exponent of unity in mammals, i.e. it is a constant fraction of body weight, about 6%. Since all mammalian hearts are built on the same pattern, it is reasonable to assume that stroke volume is proportional to the size of the heart itself. Hence the frequency of the heartbeat calculated from equation 1.1 would be expected to give a good indication of the resting cardiac output).

Another allometric equation relates the lifespan of mammals in captivity to body weight (Sacher, 1959):

$$\text{Lifespan} = 11.8 \times (\text{body weight})^{0.2} \qquad (1.2)$$

where lifespan is measured in years and body weight in kilograms. This equation indicates an upper limit for lifespan, because animals in captivity have adequate food, are relatively disease free and are not subject to predation. When this equation is applied to the "70 kilogram man", it predicts a lifespan of only 27.6 years. In other words, humans live three or more times longer than is expected on the basis of their body weight (an observation which remains essentially unexplained). When this result is combined with that of equation 1.1, we arrive at the conclusion that although the human heart beats at a rate expected from the body mass, it has to do this for three or more times longer than in other mammals. This amounts to an extra 2076 million beats over a 75 year lifespan or an extra 145 million litres of blood pumped. Perhaps it is only to be expected then, that diseases of

the heart should be prevalent among humans, even without taking into consideration the many additional noxious environmental influences which are known to be damaging to the heart.

In industrially developed nations, diseases of the heart are indeed the commonest cause of death in adults (Peach & Heller, 1984). Of these diseases, the commonest by far is ischaemic heart disease. In England and Wales in 1982 this was responsible for 88,716 deaths in men (31% of total deaths) and 65,889 deaths in women (23% of total deaths). Ischaemic heart disease is usually related to atheroma in the coronary arteries, and manifests as angina pectoris, sudden death, myocardial infarction (fatal or non-fatal) and cardiac failure, which may result from non-fatal infarction(s) or develop insidiously. The high prevalence of ischaemic heart disease, and its high cost in morbidity and mortality, has naturally lead to intense research interest in all of its aspects (see Braunwald, 1988). Some questions which have seemed of particular interest to physiologists are:

1. The mechanism of the fall of force when heart muscle is made ischaemic.
2. The causes of the damage to heart muscle which occurs during ischaemia and on reperfusion.
3. The mechanisms contributing to the generation of the arrhythmias that are frequently found in association with cardiac ischaemia.
4. Ways in which surviving cardiac muscle can be made to contract more strongly in order to ameliorate the effects of death of other parts of the heart.

Aspects of all of these questions are addressed by the experiments described in this thesis. Background relevant to particular experimental series is included in the appropriate places in Chapter 3.

In the rest of this chapter, more general background information is briefly reviewed.

1.2 Cardiac muscle.

In terms of its functioning as a biophysical machine, striated muscle (cardiac and skeletal muscle) is one of the best understood tissues, in that it is now possible to describe fairly completely right down to the molecular level many of the processes that give rise to the generation of force. The discoveries that gave rise to the sliding filament theory of contraction and then lead on to the postulation of "independent force generators" or "cross bridges" as the fundamental units of force generation have been described by Huxley (1980). Much of the work leading to these concepts was performed on skeletal muscle, but the techniques have been extended to cardiac muscle in recent years, and it has been realised that although the two systems have much in common, there are also important differences (for an excellent account of the factors influencing contraction in many different kinds of muscle, see Rüegg, 1988).

1.2.1 Cytoplasmic calcium.

Microelectrode measurements have shown that the intracellular free $[Ca^{2+}]$ in resting cardiac muscle is about 0.1 - 0.2 μM (Marban, Rink, Tsien & Tsien, 1980), below the level at which the myofibrillar ATPase is activated. However, the total Ca^{2+} in cardiac muscle amounts to approximately 1 - 3 mmol/kg tissue (Poole-Wilson, Harding, Bourdillon & Tones, 1984). Most of the Ca^{2+} is thus sequestered in intracellular compartments, of which the most important is the sarcoplasmic reticulum. The sarcoplasmic reticulum contains an active Ca^{2+} pump, which splits adenosine triphosphate (ATP) and moves Ca^{2+} from the cytoplasm into the lumen of the sarcoplasmic reticulum (Inesi, 1985). To a large extent, the level of intracellular free $[Ca^{2+}]$ is self-

regulated under normal conditions, since any increase causes a calmodulin-dependent protein kinase to phosphorylate a regulatory protein in the sarcoplasmic reticulum (phospholamban), which stimulates the Ca^{2+} pump and hence lowers the cytoplasmic [Ca^{2+}] again (Pifl, Plank, Wyskovsky, Bertel, Hellmann & Suko, 1984). (Note that the intracellular [Ca^{2+}] would reach a *stable* level even in the absence of this mechanism, because the sarcoplasmic reticulum uptake will equal the leak only at one [Ca^{2+}]. However, the regulatory mechanism ensures that the intracellular [Ca^{2+}] is kept close to one particular level).

However, the situation is not quite as simple as this, because in cardiac muscle (in contrast to skeletal muscle) there are also important movements of Ca^{2+} across the cell membrane (Reuter, 1984). Ca^{2+} may enter the cell either through voltage-gated Ca^{2+} channels (Beeler & Reuter, 1970 a & b), or by means of an electrogenic Na^{+}/Ca^{2+} exchanger (Reuter, 1974; Eisner & Lederer, 1985), and it may leave by the Na^{+}/Ca^{2+} exchanger working in the reverse direction, or due to an active Ca^{2+} pump located in the plasma membrane (Caroni & Carafoli, 1980). The importance of these sarcolemmal Ca^{2+} movements has been recognised since Ringer demonstrated that cardiac muscle stops contracting if Ca^{2+} is removed from the solution bathing the muscle (Ringer, 1883). (Skeletal muscle is relatively insensitive to this manoeuvre).

The inflow of Ca^{2+} through voltage-gated Ca^{2+} channels occurs during the plateau phase of the cardiac action potential, and is responsible for its distinctive shape (Noble, 1984). As in skeletal muscle, the rapid initial reversal of membrane potential is due to a fast inward current carried by Na^{+} ions. Although the Na^{+} current is inactivated within a few milliseconds, the potential change which occurs during this time is sufficient to open the Ca^{2+} channels, which allows Ca^{2+} to enter the cell, carrying the "slow inward current"

(Beeler & Reuter, 1970a). This current was established as a Ca^{2+} current by the findings that, unlike the fast current, it does not depend on extracellular [Na^+] and is not blocked by tetrodotoxin, but is a function of the extracellular [Ca^{2+}]. Finally the K^+ conductance is activated, giving rise to an outward current carried by K^+ ions. While the Ca^{2+} inward current is still flowing, the net result is that the membrane potential is held at a plateau of about +20 mV. Eventually, however, the Ca^{2+} current becomes inactivated, and at this stage the membrane is rapidly repolarised by the K^+ outward current. The role of the slow inward current is not just to prolong the cardiac action potential, which at 0.15 - 0.3 seconds is 20 - 50 times longer than the action potential of skeletal muscle and helps to prevent the generation of arrhythmias. In addition, the inflow of Ca^{2+} ions has an important part to play in excitation-contraction coupling in cardiac muscle, which is discussed below.

As mentioned, the plasma membrane also contains a Na^+/Ca^{2+} exchanger, which is probably electrogenic, carrying 3 Na^+ ions are transported for every Ca^{2+} ion (Mullins, 1981). This means that the Na^+/Ca^{2+} exchange should be sensitive to the membrane potential. Using the Nernst equation, it is possible to calculate (Blaustein, 1974) that at the known physiological concentrations of Na^+ and Ca^{2+} inside and outside cardiac cells at rest, the Na^+/Ca^{2+} exchange should be in equilibrium at a membrane potential of about -40 mV. At membrane potentials more negative than this, Na^+ enters the cell and Ca^{2+} leaves, while at more positive membrane potentials the reverse movements occur. In resting cardiac muscle therefore, the Na^+/Ca^{2+} exchange acts to remove Ca^{2+} from the cytoplasm, and since it is electrogenic, it also has the effect of generating a net inward current. Because the exchanger is sensitive to membrane potential, it is expected that during the action potential the direction of the

exchanger will reverse, and thus it should help to carry Ca^{2+} into the cell during the action potential. (For a more detailed account of the electrogenicity of the Na^+/Ca^{2+} exchanger, its ability to generate currents and the effects of membrane potential on it, see Eisner & Lederer, 1985).

Thus, the Na^+/Ca^{2+} exchange and the ATP-dependent Ca^{2+} pumps in the cell membrane and sarcoplasmic reticulum are important regulators of the intracellular resting $[Ca^{2+}]$ in cardiac muscle. As is the case with the sarcoplasmic reticulum pump, both of the sarcolemmal systems are sensitive to intracellular free $[Ca^{2+}]$ and are thus able to contribute to its regulation by feedback: if the intracellular free $[Ca^{2+}]$ rises, Ca^{2+} extrusion is stimulated, while if it falls below a certain level, the pump may be switched off and the Na^+/Ca^{2+} exchange slowed or even reversed.

Finally, it should be noted that Ca^{2+} exchange can also take place across mitochondrial membranes via an electrophoretic uptake mechanism, a Na^+/Ca^{2+} exchange and possibly a H^+/Ca^{2+} exchange (Carafoli, 1982). There is now evidence, obtained from experiments with saponin-treated cardiac myocytes, that mitochondrial Ca^{2+} uptake may be important in helping to switch off Ca^{2+}-induced Ca^{2+} release and in accumulating Ca^{2+} during the early part of relaxation (Miller & Fry, 1988). Mitochondrial Ca^{2+} uptake is also important in situations where cytoplasmic $[Ca^{2+}]$ is abnormally high for prolonged periods of time. Under these conditions, mitochondria accumulate Ca^{2+}, and due to the presence in mitochondrial membranes of a phosphate uptake mechanism, are able to precipitate calcium phosphate in their lumen (Carafoli, 1982). In the relatively short term, this is an important Ca^{2+}-buffering mechanism, but if the $[Ca^{2+}]$ remains raised, then the mitochondria can become calcified and damaged, with consequent impairment of ATP production.

1.2.2 Calcium release from the sarcoplasmic reticulum.

The inward flow of Ca^{2+} ions across the cell membrane has important consequences for excitation-contraction coupling in cardiac muscle. In lower vertebrates such as fishes and amphibians, it seems that Ca^{2+} entry during the action potential is able to raise the intracellular free [Ca^{2+}] sufficiently to cause contraction (Morad & Goldman, 1973; Bers, 1985). In mammalian hearts, however, the Ca^{2+} entry seems to perform a trigger function, stimulating Ca^{2+} release from the sarcoplasmic reticulum (in contrast to skeletal muscle, where the electrical events of the action potential directly cause the sarcoplasmic reticulum to release Ca^{2+}). This "Ca^{2+}-induced Ca^{2+} release" from the sarcoplasmic reticulum is now supported by a considerable amount of data (Fabiato, 1983). For example, there is a close relationship between the Ca^{2+} inward current and contraction (e.g. Beeler & Reuter, 1970b; Barcenas-Ruiz & Wier, 1987; Berlin, Cannell & Lederer, 1987), but the intracellular free [Ca^{2+}] expected to result from the influx of Ca^{2+} on the inward current is inadequate to activate the myofilaments directly, although it is sufficient to stimulate Ca^{2+} release from the sarcoplasmic reticulum (Fabiato, 1983).

Evidence for Ca^{2+}-induced Ca^{2+} release in intact cardiac tissue (as opposed to the skinned fibre preparations used by Fabiato) has recently been obtained by raising the intracellular free [Ca^{2+}] directly by using the "caged" Ca^{2+} chelator Nitr-5 (Valdeolmillos, O'Neill, Smith & Eisner, 1989). In these experiments, single rat ventricular myocytes were loaded with Nitr-5. Flashing the cells with ultraviolet light caused the Nitr-5 to release Ca^{2+} and induced a twitch. However, when Ca^{2+} release from the sarcoplasmic reticulum was inhibited by adding ryanodine, twitches could no longer be elicited by flashing Nitr-5 loaded cells, indicating: (i) that the Ca^{2+} release by

Nitr-5 was not in itself sufficient to activate contraction, but (ii) it was sufficient to cause the sarcoplasmic reticulum to release Ca^{2+} and cause a twitch. Furthermore, if the inward Ca^{2+} current was abolished using nickel, stimulated twitches were abolished, but it was still possible to elicit a twitch by flashing the cell, suggesting that the inward flow of Ca^{2+} during the action potential is the usual trigger for release of Ca^{2+} from the sarcoplasmic reticulum. It should be noted that the relative importance of Ca^{2+} induced Ca^{2+} release and trans-sarcolemmal influx of Ca^{2+} for providing Ca^{2+} to activate the myofibrils may vary considerably in different mammals (Bers, 1985). However, ferret myocardium, which was used for all the experiments described in this thesis, seems to be relatively sarcoplasmic reticulum dependent (Wier, Yue & Marban, 1985).

1.2.3 The calcium transient.

The Ca^{2+} which is released into the cytoplasm as a result of the action potential acts as the messenger linking membrane depolarisation to activation of the myofilaments. A major advance of recent years has been the ability to measure this transient intracellular Ca^{2+} signal directly, using photoproteins and fluorescent indicators (Blinks, Prendergast & Allen, 1976; Blinks, Wier, Hess & Prendergast, 1982; Cobbold & Rink, 1987). This was first achieved in cardiac muscle by microinjecting the photoprotein aequorin into frog atrial trabeculae (Allen & Blinks, 1978), and the technique was rapidly extended to mammalian ventricular muscle (Allen & Kurihara, 1980; Wier, 1980; Morgan & Blinks, 1982). The pulse of cytoplasmic Ca^{2+} that accompanies each contraction (the "Ca^{2+} transient") is dependent on all the processes which are contributing Ca^{2+} to and removing it from the cytoplasm. This makes the cytoplasmic Ca^{2+} transient a complicated phenomenon, because it is influenced by:

(i) Entry of Ca^{2+} via the slow inward current.

(ii) Entry or removal of Ca^{2+} by the sarcolemmal Na^{+}/Ca^{2+} exchange.

(iii) Entry of Ca^{2+} due to release of sarcoplasmic reticulum stores.

(iv) Removal of Ca^{2+} by the sarcoplasmic reticulum Ca^{2+} pump.

(v) Removal of Ca^{2+} by the sarcolemmal Ca^{2+} pump.

(vi) Exchange of Ca^{2+} across mitochondrial membranes.

(vii) Binding of Ca^{2+} to intracellular sites. The most important of these are troponin C on the myofibrils (with a concentration of approximately 70 μM - Solaro, Wise, Shiner & Briggs, 1974) and calmodulin (with a concentration of approximately 50 μM - Cheung, 1980). Also, troponin C and calmodulin are the only sites expected to have sufficiently fast kinetics to respond significantly to physiological Ca^{2+} transients (Robertson, Johnson & Potter, 1981).

Quantitatively, it appears that the most important contributor of Ca^{2+} to the cytoplasm in cardiac cells is the sarcoplasmic reticulum (e.g. Fabiato, 1983; Marban & Wier, 1985), while the most important Ca^{2+} sinks are troponin C, calmodulin and the sarcoplasmic reticulum. Because troponin C and calmodulin bind Ca^{2+} almost as fast as it enters the cytoplasm, only a small fraction of the Ca^{2+} involved in producing a contraction is free in the cytoplasm at any given time (Blinks & Endoh, 1986). Nevertheless, the peak Ca^{2+} signal has been found to correlate well with the force produced when inotropic interventions thought to act by changing Ca^{2+} release are applied (see Section 1.3). Although, strictly speaking, comparisons can only be made if the interventions applied have no (or the same) effect on the timecourse of the Ca^{2+} transient (Blinks & Endoh, 1986), in practice, results obtained with intact tissues and skinned fibre preparations show good

agreement, in spite of the small differences in timecourse which are induced by many inotropic interventions.

In rat ventricular muscle at 30°C, the Ca^{2+} transient reaches a peak 25-50 milliseconds after the stimulus, while tension peaks at 75-150 milliseconds (Allen & Kurihara, 1980). Thus, tension lags behind intracellular [Ca^{2+}] and at the time of peak tension, the Ca^{2+} transient had declined to considerably less than 10% of its peak value. (Note that the "Ca^{2+} transient" refers to the aequorin light signal. Because of the non-linear relation between aequorin light and [Ca^{2+}] - see Section 1.4 - the intracellular [Ca^{2+}] actually falls somewhat more slowly than the light signal - see Figure 1 in Allen & Kurihara, 1980). It seems likely that the primary determinant of the decline of the Ca^{2+} transient is uptake of Ca^{2+} by the sarcoplasmic reticulum. Because substantial changes in the timecourse of the decline of the Ca^{2+} transient can occur with little affect on force (Allen & Kurihara, 1980; Kurihara & Allen, 1982), it seems that the decrease in intracellular [Ca^{2+}] is not always the rate-limiting step in relaxation, and that other processes, such as dissociation of Ca^{2+} from troponin C or detachment of cross bridges, may have the dominant effect on relaxation of force.

Although binding of Ca^{2+} to troponin C does not cause the decline of the Ca^{2+} transient, it would be expected to affect the rate of decline (Blinks & Endoh, 1986). During most of the decline of the Ca^{2+} transient, as Ca^{2+} is being pumped back into the sarcoplasmic reticulum, troponin C is releasing Ca^{2+} which has bound to it. This release of Ca^{2+} from troponin C into the cytosol thus tends to slow the decline of the Ca^{2+} transient. Hence, interventions which affect the affinity of troponin C for Ca^{2+}, and thus the rate at which Ca^{2+} is released from troponin C as intracellular [Ca^{2+}] falls, would be expected to influence the decline of the Ca^{2+} transient. An increase

in the Ca^{2+} affinity of troponin C should increase the decline of the Ca^{2+} transient by making it possible for the sarcoplasmic reticulum to pump the intracellular $[Ca^{2+}]$ down more quickly. Also, because the Ca^{2+} now remains attached to troponin C for longer, relaxation of tension would be expected to be slowed. Conversely, a decrease in the Ca^{2+} affinity of troponin C would be expected to have the opposite effects, i.e. a prolonged Ca^{2+} transient and faster relaxation of tension.

1.2.4 Activation of contraction.

The Ca^{2+} which enters the cytoplasm, visualised as the Ca^{2+} transient, activates contraction when it binds to troponin C on the myofibrils (Ebashi, 1980). When troponin C binds Ca^{2+}, a conformational change takes place, resulting in stronger interactions between troponin C and troponin I, while bonds between troponin I and actin become weaker. This has the simultaneous effect of altering the interaction between troponin T and tropomyosin, and is thought to allow tropomyosin to move in the groove between the two actin strands of the thin filament (Squire, 1981; El-Saleh, Warber & Potter, 1986). This movement exposes binding sites on the thin filaments, allowing the thick filament cross bridges to bind to the thin filaments. There follows a complex process (the cross-bridge cycle) in which ATP binds to the actomyosin complex and is split into ADP and inorganic phosphate and force is generated (Huxley, 1980; Woledge, Curtin & Homsher, 1985). ATP is necessary for the dissociation of actin and myosin, which otherwise maintain a very strong interaction. This is why muscle becomes very stiff in energy depleted states ("rigor") and accounts, for example, for the rigor mortis seen after death.

This scheme of muscle activation is similar in cardiac and skeletal muscle: the crossbridge cycling which generates force (often

measured as the myofibrillar ATPase activity - e.g. Holroyde, Robertson, Johnson, Solaro & Potter, 1980) is inhibited by the troponin complex under resting conditions when the intracellular [Ca^{2+}] is low. When the muscle is activated, [Ca^{2+}] rises, the inhibition is relieved, crossbridges cycle and force is generated. There are, however, several differences between the proteins in cardiac and skeletal muscle. One interesting difference is that cardiac troponin C has only one low affinity Ca^{2+}-specific binding site, while skeletal muscle troponin C has two (Holroyde et al, 1980; Pan & Solaro, 1987). Both molecules contain two identical non-specific, high-affinity Ca^{2+} binding sites. It has been suggested that the difference in the Ca^{2+}-specific sites might be responsible for the fact that the relationship between Ca^{2+} and force is less steep in cardiac than skeletal muscle, due to less co-operativity in the Ca^{2+} troponin interaction (Moss, Lauer, Giulian & Greaser, 1986).

1.2.5 Energy for contraction.

As mentioned above, the energy for crossbridge cycling is derived from ATP. ATP is also the energy source for most of the other processes in the cell, including Ca^{2+} uptake by the sarcoplasmic reticulum, Ca^{2+} extrusion by the sarcolemma and Na^+/Ca^{2+} exchange (because it depends on the Na^+ gradient set up by the Na^+/K^+ ATPase). The demand for ATP is not constant, but varies over quite a large range, increasing, for example, when heart rate or the strength of contraction increases (Neely, Liebermeister, Battersby & Morgan, 1967; Neely, Denton, England, & Randle, 1972). Heart muscle therefore has feedback systems which are aimed at maintaining ATP at a high and constant level in spite of variations in demand. The major substrates for ATP production are glucose and fatty acids, which are broken down to two carbon units by glycolysis and beta oxidation respectively. The acetyl units are then oxidised by the citric acid cycle (oxidative

phosphorylation) to carbon dioxide and water. A much larger amount of ATP is produced by oxidative phosphorylation than by either of the other two processes. If oxidative phosphorylation is prevented, glycolysis is able to continue by side-stepping the final reaction in the chain (where pyruvate is converted to acetate) and instead the three carbon unit is removed from the cell as lactate. However, even though glycolysis is stimulated many-fold under circumstances where oxidative phosphorylation is prevented (Rovetto, Whitmer & Neely, 1973), the production of ATP is still much less than could be produced by oxidative phosphorylation.

The feedback mechanism which stimulates ATP resynthesis by oxidative phosphorylation is thought to depend on a rise in one or more of the hydrolysis products of ATP, i.e. ADP, inorganic phosphate or AMP (which is in equilibrium with ATP & ADP due to the myokinase reaction). The ATP level itself is not thought to be involved in the regulation, because it is strongly buffered in the short term by the creatine kinase reaction:

$$ADP + PCr + H^+ \rightleftharpoons ATP + Cr,$$

where PCr is phosphocreatine and Cr is creatine. Because of this reaction, the level of ATP does not change significantly when the muscle activity is altered. The precise way in which the various factors influence the rate of oxidative phosphorylation is at present unclear, although there is evidence supporting a role for both ADP and inorganic phosphate (Hansford, 1980; Brazy & Mandel, 1986). In addition, there is now a growing body of evidence that alterations of cytoplasmic [Ca^{2+}] can regulate oxidative phosphorylation by altering the intra-mitochondrial [Ca^{2+}] and hence the activity of several Ca^{2+}-sensitive dehydrogenases in the citric acid cycle (McCormack & Denton, 1986; Koretsky, Katz & Balaban, 1989). The major control point for glycolysis is the enzyme phosphofructokinase, which is activated by

ADP, inorganic phosphate and also AMP (see Newsholme & Start, 1973).

1.3 Regulation of contraction in cardiac muscle.

Cardiac muscle cells are joined to one another at specialised regions where their cell membranes come into very close contact (the nexuses or gap junctions - e.g. Fawcett & McNutt, 1969). Anatomical evidence suggested that these regions could allow conduction of ions and other small molecules between cardiac cells, and there is now a wealth of physiological evidence that they also provide low resistance pathways for the conduction of electric current (Wiedmann, 1967). Because the cells are electrically coupled in this way, heart muscle contraction always involves all the cells, even if the stimulus is only just above threshold. However, in spite of the fact that all the cells are involved in a contraction, the force developed by cardiac muscle (or the shortening achieved) can be varied over a considerable range. Unlike skeletal muscle, this variation in force development cannot be accomplished by tetanisation under normal conditions, because the refractory period of the action potential lasts almost as long as contraction. Instead, there are three theoretically distinct ways in which the force of cardiac contraction can be controlled at the cellular level. These are:

(i) By changing the myoplasmic calcium concentration achieved as a result of an action potential.

(ii) By changing the calcium sensitivity of troponin C.

(iii) By changing the maximum calcium-activated force which the myofilaments are capable of achieving.

It should be noted that these three mechanisms are not mutually exclusive, and an inotropic intervention may have effects on one, two or all three of them.

1.3.1 Myoplasmic calcium concentration.

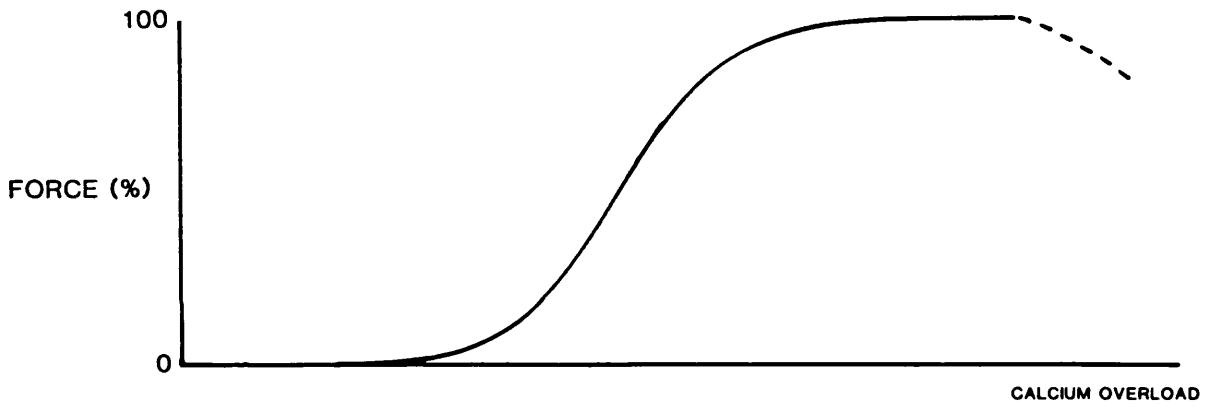
As discussed in section 1.2, a pulse of Ca^{2+} released into the cytoplasm acts as the messenger which triggers contraction. It was also seen that there is a level of cytoplasmic [Ca^{2+}] below which no force is generated by the myofibrils. Clearly, there will also be an upper limit, above which the pulse of Ca^{2+} release becomes so large that the myofibrils are saturated, and no additional force could be generated by increasing the myoplasmic [Ca^{2+}] further. Between these limits, however, it would be expected that the force of contraction could be modified by altering the myoplasmic [Ca^{2+}]. This relationship is shown schematically in Figure 1. The resting [Ca^{2+}] of 1-2 x 10^{-7}M lies just below the tail of the curve. Studies with skinned fibre preparations show that at pH 7.0 (which is thought to be close to the usual pH in cardiac muscle - Ellis & Thomas, 1976; Matthews, Radda & Taylor, 1981; Allen, Morris, Orchard & Pirolo, 1985) activation of contraction begins at approximately 5 x 10^{-7}M Ca^{2+} and saturates between 10^{-5}M and 10^{-4}M Ca^{2+} (e.g. Fabiato & Fabiato, 1978; Kentish, 1984, 1986; Pan & Solaro, 1987).

If this mechanism is to function as an efficient regulator of force in vivo, it is important that the relationship between [Ca^{2+}] and force should not be too steep, otherwise small changes in [Ca^{2+}] could produce large changes in force, making control difficult. As noted in Section 1.2, the Ca^{2+}-force relationship is indeed considerably less steep in cardiac than in skeletal muscle, in keeping with this requirement. The idea that the difference between cardiac and skeletal troponin C is linked to the shallower Ca^{2+}-force relationship of cardiac muscle is strongly supported by experiments in which troponin C from fast skeletal muscle was partially replaced by cardiac troponin C (Moss et al 1986). This had the effect of reducing the steepness of the Ca^{2+}-force relationship.

FIGURE 1.

FIGURE 1.

Graph showing schematically the relation between force and myoplasmic calcium concentration. For explanation, see text.


100
FORCE (%)
0
CALCIUM OVERLOAD
MYOPLASMIC CALCIUM CONCENTRATION

Another important point is that the normal [Ca^{2+}] reached during a twitch should be less than the level needed to saturate the force response of the myofilaments, which would also clearly remove the possibility of control. In fact, most laboratory studies agree that under normal conditions, the myoplasmic [Ca^{2+}] reached during a twitch is well down the force-Ca^{2+} relation, meaning that there is plenty of scope for increases of myoplasmic [Ca^{2+}] to increase force. This is indicated by the large increases in the Ca^{2+} transient and twitch which can be produced by applying inotropic interventions to isolated cardiac muscle (e.g. Allen & Blinks, 1978; Allen & Kurihara, 1980; Kurihara & Konishi, 1987; personal observations).

If sufficient positive inotropic stimulation is applied, a situation is reached where the intracellular Ca^{2+} transient can increase further, although the twitch has saturated (e.g. McIvor, Orchard & Lakatta, 1988; personal observations). This corresponds to the plateau of the curve shown in Figure 1. However, if the intracellular [Ca^{2+}] is increased beyond a certain level, the muscle becomes "Ca^{2+} overloaded" (Allen, Eisner, Pirolo & Smith, 1985). This can cause the sarcoplasmic reticulum to become unstable and spontaneously release Ca^{2+} (Orchard, Eisner & Allen, 1983; Wier Kort, Stern, Lakatta & Marban, 1983), and can be an important factor limiting twitch potentiation (Capogrossi, Stern, Spurgeon & Lakatta, 1988).

The observed fact that many inotropic interventions are able to increase the contractility of cardiac muscle *in vivo*, suggests that cardiac tissue *in situ* functions on a region of the curve similar to that seen in *in vitro* studies, i.e. there is plenty of scope for increasing the force of contraction by inotropic interventions.

1.3.2 Inotropic interventions which act via a change in the myoplasmic calcium concentration.

Most of the well known interventions which alter the force of contraction used in both the laboratory and the clinic have their effect by this mechanism. They are listed in Table 1, and are briefly described below.

If force and Ca^{2+} transients are measured from a papillary muscle while the external [Ca^{2+}] is increased from a low level, it is found that they increase in parallel. If a number of these points are plotted, then a curve similar to Figure 1 is generated, illustrating that the increased force is produced by an increased Ca^{2+} transient. A similar procedure can be carried out for different frequencies of stimulation, and for paired pulse stimulation with varying pairing intervals, and these are found to produce the same result as increasing extracellular [Ca^{2+}]. If all three interventions produce an increase of force by the same mechanism (i.e. by increasing myoplasmic [Ca^{2+}]), then all the points should fall on the same curve. This experiment was performed by Allen & Kurihara (1980), who found that all three interventions did indeed produce points which lay on the same curve, supporting a common mechanism of action.

A similar approach suggested that Ca^{2+} channel blockers also produce their effects by affecting the Ca^{2+} transient (in this case reducing it). These agents have been found to reduce the influx through the slow Ca^{2+} channels, and hence would be expected to reduce the Ca^{2+} transient. Wier & Yue (1985) produced a Ca^{2+}-force curve by varying the extracellular [Ca^{2+}] firstly in the absence of the Ca^{2+} channel blocker nitrendipine and then in the presence of various concentrations of the drug. Although nitrendipine reduced both the force and Ca^{2+} transient, all the points produced by the various manipulations fell on the same curve, supporting the hypothesis that

TABLE 1.

INOTROPIC INTERVENTIONS WHICH ACT BY CHANGING THE MYOPLASMIC $[Ca^{2+}]$.

External calcium concentration

Frequency

Paired pulse stimulation

Cardiac glycosides

Calcium channel blockers

Ryanodine

Beta agonists

Direct alteration of cyclic AMP level by

- adenylate cyclase activation
- phosphodiesterase inhibition

Phosphatase inhibition

the drug was working by reducing the Ca^{2+} transient. It was also noted that the drug concentrations required to half-maximally inhibit the Ca^{2+} transient, Ca^{2+} entry and force were very similar. In contrast to the Ca^{2+} channels blockers, ryanodine does not reduce Ca^{2+} influx, but instead prevents the sarcoplasmic reticulum from releasing Ca^{2+} (e.g. Wier, Yue & Marban, 1985). This appears to be secondary to its effects on the sarcoplasmic reticulum Ca^{2+} release channel, which is held in a partially open state (Fill & Coronado, 1988), causing the sarcoplasmic reticulum to become depleted of Ca^{2+}. It may be noted that there are also agents which increase force by directly opening the Ca^{2+} channels (e.g. BayK 8644 - Schramm, Thomas, Towart Franckowiack, 1983).

Cardiac glycosides have been used extensively for their positive inotropic effects since their careful introduction by Withering over 200 years ago (Withering, 1785). It is thought that their main effect is to inhibit the Na^+/K^+ ATPase, which causes a rise in intracellular $[Na^+]$ sufficient to activate the Na^+/Ca^{2+} exchanger and hence lead to an increase in myoplasmic $[Ca^{2+}]$ (Hansen, 1986). Measurements of the Ca^{2+} transients have shown that these are indeed increased when the force is increased by cardiac glycosides (e.g. Morgan & Blinks, 1982; Wier & Hess 1984). (It is possible that glycosides may have other mechanisms of action which gain importance at low drug concentrations - Noble, 1980; see also Erdmann, Greeff & Skou, 1986).

Another important class of positive inotropic agents, which also have many analogues in clinical use, are the beta agonists. These agents bind to a receptor in the cell membrane and stimulate the intracellular formation of cyclic AMP. This intracellular second messenger affects many cellular systems, but for the effects on contraction, the most important are believed to be: (i) phosphorylation of the Ca^{2+} channel, causing a greater influx of Ca^{2+} with each action potential (see Reuter, 1983); (ii) phosphorylation of phospholamban in

the sarcoplasmic reticulum, causing a greater accumulation of Ca^{2+} in the sarcoplasmic reticulum due to faster Ca^{2+} uptake (Tada, Kirchberger, Repke & Katz, 1974; Tada, Kirchberger & Katz, 1974); (iii) phosphorylation of troponin I, which causes a reduction in the Ca^{2+} sensitivity of troponin C (Solaro, Moir & Perry, 1976). The first two effects cause a very large increase in the size of the Ca^{2+} transient, which underlies the increase in force (e.g. Allen & Kurihara, 1980). However, this increase in force is not as large as it would otherwise be, due to the desensitising effect. Probably the most notable consequence of the desensitisation is that it increases the rate at which relaxation can occur, an important point because beta agonists have other effects causing a large increase in heart rate. At present it is unclear whether this effect is important *in vivo*, or whether other effects such as more rapid sarcoplasmic reticulum uptake predominate in speeding relaxation (McIvor, Orchard & Lakatta).

Other agents have been found which are able to change cyclic AMP levels directly, without acting via a receptor. In the cell, cyclic AMP is synthesised by the enzyme adenylate cyclase and broken down by phosphodiesterase. Some agents (e.g. forskolin) are able to stimulate adenylate cyclase and hence raise cyclic AMP. More interest, however, has focused on phosphodiesterase inhibitors, which increase the cyclic AMP level by reducing the rate of degradation. A number of these agents (e.g. amrinone and milrinone) have been introduced into clinical practice, with at least short term benefit (Colucci, Wright & Braunwald, 1986).

Finally, phosphatase inhibition may be mentioned. If it is true that the cell contains a basal level of cyclic AMP, then it follows that there will be a basal level of phosphorylation due to cyclic AMP-dependent kinases. This is balanced by a basal level of dephosphorylation due to phosphatases. Hence, if the phosphatases are

inhibited, the effects of the basal level of cyclic AMP production will be potentiated due to an increase in the level of cyclic AMP. One such phosphatase inhibitor is the substance okadaic acid, isolated from the Japanese black sponge (Takai, 1988). Application of this substance to cardiac muscle has been found to increase the Ca^{2+} current (Hescheler, Mieskes, Rüegg, Takai & Trautwein, 1988) and also the Ca^{2+} transients (personal observations).

1.3.3 Calcium sensitivity and maximum calcium-activated force.

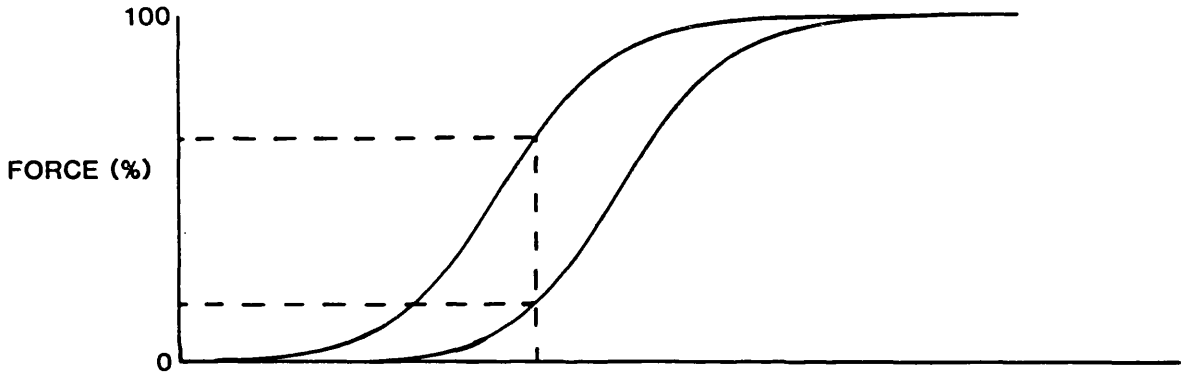
If the curve shown in Figure 1 were unique, then altering myoplasmic $[Ca^{2+}]$ would be the only way in which the force of contraction could be modulated. However, this is not the case. Although the myoplasmic $[Ca^{2+}]$ is the trigger for contraction, the essential step which leads to force generation is the binding of Ca^{2+} to troponin C. Hence the Ca^{2+} occupancy of troponin C at any given myoplasmic $[Ca^{2+}]$ is important in determining how much force is generated. The Ca^{2+} occupancy of troponin C is determined by the ratio of the on-rate and the off-rate for Ca^{2+}. Since it appears that the on-rate is limited by diffusion (Robertson, Johnson & Potter, 1981), changes in the Ca^{2+} occupancy at a given myoplasmic $[Ca^{2+}]$ could only be brought about by changes in the off-rate. Many investigations in recent years have shown that such changes in the Ca^{2+} occupancy of troponin C (i.e. changes in Ca^{2+} sensitivity) can indeed occur. The consequences that this has for force generation are shown in Figure 2. The right-hand curve shows the Ca^{2+}-force relation under control conditions (as in Figure 1), while the left-hand curve shows the effect of increasing the Ca^{2+} sensitivity. This intervention does not directly affect the cross- bridges, and so has no effect on maximum force. However, because the Ca^{2+} occupancy of troponin C is now greater at any given $[Ca^{2+}]$, the effect is a parallel leftward shift of the whole Ca^{2+}-force relation. This has the result that more force is

FIGURE 2.

FIGURE 2.

Graph showing schematically the effects of increasing the calcium sensitivity (i.e. increasing the calcium occupancy of troponin C) on the relation between force and myoplasmic calcium concentration. For explanation, see text.

CALCIUM SENSITIVITY



MYOPLASMIC CALCIUM CONCENTRATION

generated at a given myoplasmic [Ca^{2+}] than was previously the case (illustrated by the dotted lines). Note also that the threshold [Ca^{2+}] for force generation is lowered, as is the [Ca^{2+}] at which the force response saturates. Because this effect is brought about by a reduction in the off-rate of Ca^{2+} from troponin C, it is expected that relaxation of tension will be slower under these conditions.

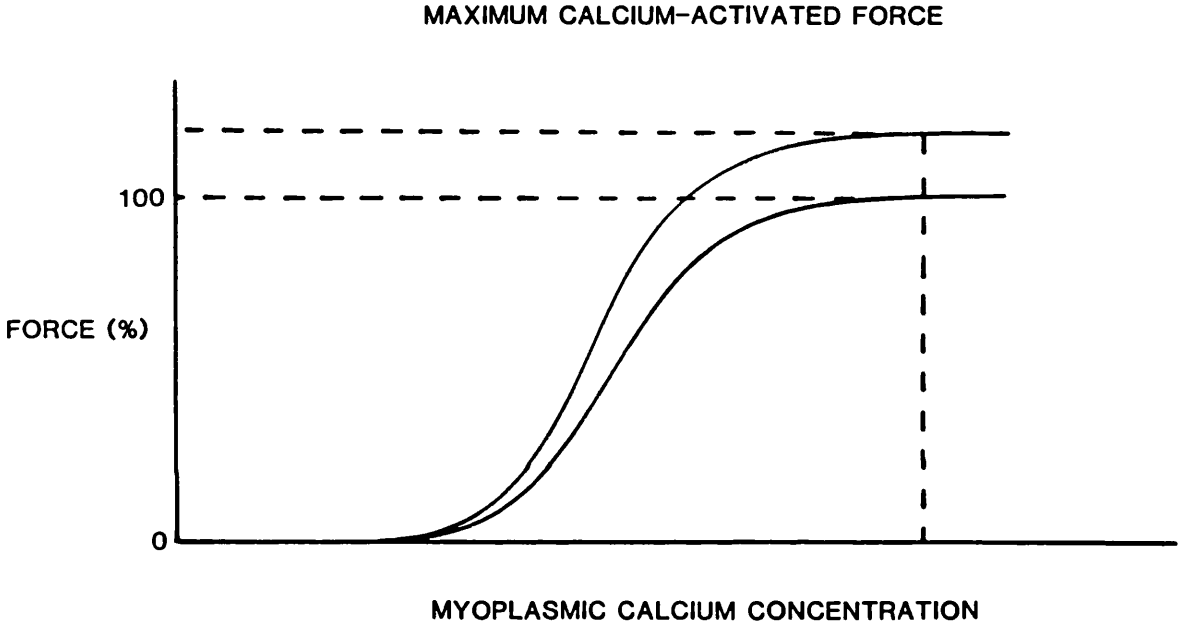
The other way in which the force generation can theoretically be altered is by changing the maximum Ca^{2+}-activated force. Conceptually this could be brought about by altering either the number of cross-bridges, the number of active (i.e. force generating) cross-bridges under any given conditions or the force produced per cross-bridge. The effects which could be produced by purely increasing the maximum Ca^{2+}-activated force are shown in Figure 3. The Ca^{2+}-force relation under control conditions is illustrated by the lower curve, while the upper curve shows the effects of increasing the maximum Ca^{2+}-activated force. In this case the maximum force is of course increased, but the force response saturates at the same myoplasmic [Ca^{2+} as previously. Also, the threshold [Ca^{2+}] for force generation is unchanged. Between these limits, however, the force in increased by the same fractional amount over the whole range of activating [Ca^{2+}]. In practice it is difficult to distinguish between changes in Ca^{2+} sensitivity and maximum Ca^{2+}-activated force unless a wide range of [Ca^{2+}], including saturating [Ca^{2+}], are studied. Changes in maximum Ca^{2+}-activated force are then revealed by changes in maximum force production, while changes in Ca^{2+} sensitivity can be revealed by normalising the curves so that the maximum force generated under each of the various conditions is plotted as 100%.

Although most attention to date has been focused on changes in Ca^{2+} sensitivity, it should be noted that interventions which produce this effect also often have substantial effects on maximum Ca^{2+}-

FIGURE 3.

FIGURE 3.

Graph showing schematically the effects of increasing the maximum calcium-activated force on the relation between force and myoplasmic calcium concentration. For explanation, see text.


MAXIMUM CALCIUM-ACTIVATED FORCE
100
FORCE (%)
0
MYOPLASMIC CALCIUM CONCENTRATION

activated force. Figure 4 illustrates these more complicated effects. The right-hand curve is identical to the control curves shown in the previous Figures. The other two curves illustrate an intervention which produces (i) an increase in both Ca^{2+} sensitivity and maximum Ca^{2+}-activated force (e.g. pimobendan - see Section 3.4) and (ii) an increase in Ca^{2+} sensitivity but a decrease in maximum Ca^{2+}-activated force (e.g. caffeine; Wendt & Stephenson, 1983). The finding that Ca^{2+} sensitivity and maximum Ca^{2+}-activated force can be affected differently by interventions supports the hypothesis that they may be independent mechanisms which could be manipulated separately to affect force if appropriate methods could be found.

1.3.4 Inotropic interventions which affect Ca^{2+} sensitivity and maximum Ca^{2+}-activated force.

Inotropic interventions which are believed to have a substantial effect on calcium sensitivity and maximum Ca^{2+}-activated force are listed in Table 2, and are described briefly below.

It has been known for many years that if cardiac muscle is stretched, it contracts more strongly. The *in vivo* manifestation of this property is known as the Frank-Starling law, whereby if the heart pumps less strongly on a given beat, it is stretched more by the incoming blood and so contracts more strongly on the subsequent beat, tending to restore the initial conditions. The ascending part of the length-force relation for cardiac muscle is much steeper than that for skeletal muscle, and it now appears that this is largely due to length dependence of activation, rather than filament overlap (Jewell, 1977). The most important factor in this length-dependent activation seems to be an increase in the Ca^{2+} sensitivity of the myofilaments as the muscle is stretched (Allen & Kentish, 1985). For example, it has been found that the Ca^{2+}-force relation in skinned cardiac muscle is shifted

FIGURE 4.

FIGURE 4.

Graph showing schematically the effects on the calcium-force relation of interventions which simultaneously affect calcium sensitivity and maximum calcium-activated force. For explanation, see text.

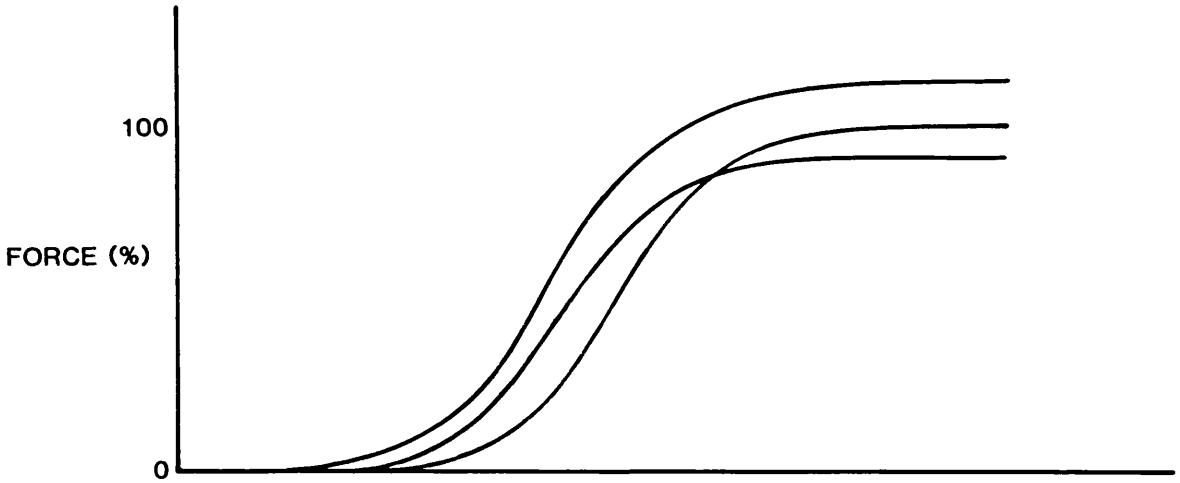

100
FORCE (%)
0
MYOPLASMIC CALCIUM CONCENTRATION

TABLE 2.

INOTROPIC INTERVENTIONS WHICH AFFECT CALCIUM SENSITIVITY AND MAXIMUM CALCIUM-ACTIVATED FORCE.

Length

pH

Phosphate

Hypoxia

Ischaemia

Beta stimulation

Synthetic troponin I peptides

Alpha stimulation

Phosphorylation of the myosin light chain

Natural modulators

Drugs

to the left when the preparation is stretched, indicating an increase in Ca^{2+} sensitivity (Hibberd & Jewell, 1982). Allen & Kentish (1988) investigated myoplasmic [Ca^{2+}] in skinned cardiac muscle fibres using aequorin. They found changes in agreement with a decrease in the Ca^{2+} sensitivity of troponin C when muscle length is reduced (i.e. Ca^{2+} was released, causing an increase in the myoplasmic [Ca^{2+}]). Also in agreement with a change of Ca^{2+} sensitivity underlying length-dependent activation is the finding that the Ca^{2+} transient in intact muscle is little changed by stretching, while the tension increases substantially (Allen & Kurihara, 1982).

Acidosis has been found to cause a decrease in both Ca^{2+} sensitivity and maximum Ca^{2+}-activated force in skinned cardiac muscle fibres (Fabiato & Fabiato, 1978). Results from intact preparations agree with this finding: force decreases on exposure to acidosis, but the Ca^{2+} transients are initially unchanged and then even increase (Allen & Orchard, 1983a). However, it should be noted that the effects of acidosis are more complicated than would be the case if they simply had effects on the myofibrils; for example both membrane currents (Fry & Poole-Wilson, 1981; Sato, Noma, Kurachi & Irisawa, 1985; Irisawa & Sato, 1986) and the sarcoplasmic reticulum (Orchard, 1987; Solaro, Lee, Kentish & Allen, 1988 - see Appendix 1) are affected by acidosis.

Phosphate ions also reduce both Ca^{2+} sensitivity and maximum Ca^{2+}-activated force. Although it has recently been suggested that it is the diprotonated form form of inorganic phosphate ($H_2PO_4^-$) which is responsible for the effects of phosphate on force (Dawson, Smith & Wilkie, 1986; Godt, Fender & Nosek, 1986), it now appears more likely that in fact total phosphate is the most important factor (Eisner, Elliott & Smith, 1987; Cady, Jones, Lynn & Newham, 1989). An acidosis and an increase in the intracellular phosphate concentration are two of the most important changes which occur when cardiac muscle is made

hypoxic or ischaemic. The effects of these changes are considered further in Chapter 3.

As already mentioned, beta stimulation increases cyclic AMP and causes a reduction in the Ca^{2+} sensitivity of the myofibrils due to phosphorylation of troponin I, which in turn affects the Ca^{2+} binding properties of troponin C. Troponin I and troponin C interact through a region which involves amino acid residues 104 - 115 of troponin I and 89 - 100 of troponin C. Rüegg, Zeugner, van Eyk & Hodges (1988) found that a synthetic peptide identical to the 104 - 115 region of troponin I could competitively inhibit the troponin C-troponin I interaction, causing a reduction of both Ca^{2+} sensitivity and maximum Ca^{2+}-activated force in skinned fibres from pig ventricle. This may prove to be a useful approach to unravelling the molecular interactions which occur when myofibrils are activated.

Although beta receptors are the most prevalent adrenergic receptors in heart muscle, alpha receptors are also present in many species (Endoh, 1982). It has recently been found that alpha receptors produce part of their positive inotropic effect by causing an increase in Ca^{2+} sensitivity (Endoh & Blinks 1988), in contrast to beta stimulation, which causes a decrease in Ca^{2+} sensitivity. The mechanism causing the increase in Ca^{2+} sensitivity (revealed as an increase in force disproportionate to the increase in the Ca^{2+} transients on alpha stimulation) is as yet unknown. (It may be noted here that Winegrad and collaborators have proposed that beta adrenergic stimulation may increase the maximum Ca^{2+}-activated force of cardiac muscle by up to 6-fold (Winegrad, 1984). However, this finding remains unconfirmed).

In smooth muscle, contraction is activated when the myosin light chain is phosphorylated by a specific kinase, allowing myosin and actin to interact. Myosin light chain kinase is also present in cardiac

muscle. Interestingly, it has recently been found that phosphorylation of the myosin light chain in cardiac muscle by this kinase can cause a substantial increase in Ca^{2+} sensitivity, although maximum Ca^{2+}-activated force was unaffected (Morano, Hofmann, Zimmer & Rüegg, 1985). The significance of this observation for regulation of cardiac contractility is as yet unclear.

Another interesting observation is the finding that cardiac muscle contains substantial concentrations of what may be "natural modulators" of cardiac contractility. These compounds include N-acetyl anserine, N-acetyl carnosine, N-acetyl histidine and taurine (O'Dowd, Robins & Miller, 1988), and several of them have now been found to have a Ca^{2+} sensitising action on skinned cardiac myofibrils (e.g. Harrison, Lamont & Miller, 1985; Miller, Smith & Steele, 1989). The total concentration of these substances in cardiac muscle is approximately 10 mM, which is sufficient to substantially alter the Ca^{2+} sensitivity of the contractile proteins. It remains to be seen whether their concentrations alter significantly under various physiological and pathological conditions.

Finally, a number of synthetic compounds are now known which have substantial effects on Ca^{2+} sensitivity (Blinks & Endoh, 1986; Rüegg, 1986). The hope is that these compounds will provide a new approach to increasing cardiac contractility in pathological states where this is desirable. Compounds which have a Ca^{2+} sensitising action include caffeine (Wendt & Stephenson, 1983), sulmazole (Solaro & Rüegg, 1982), isomazole (Lues, Siegel & Harting, 1988), pimobendan (see section 3.4), adibendan (Müller-Beckmann, Freund, Honerjäger, Kling & Rüegg, 1988) and DPI 201-106 (Herzig & Quast, 1984). However, all of these compounds seem to combine Ca^{2+} sensitising ability with phosphodiesterase inhibition. This makes their mode of action complex, as discussed in section 3.4. EMD 53998 is the first compound which

appears to have only a Ca^{2+} sensitising activity in intact muscle (Allen & Lee, 1989 - see Appendix 2) and presumably others will be forthcoming.

1.4 Measurement of intracellular free calcium concentration using aequorin.

There are now several well-established methods for measuring the free $[Ca^{2+}]$ in living tissue, including bioluminescent photoproteins such as aequorin, fluorescent indicators, Ca^{2+} sensitive microelectrodes, metallochromic indicators and nuclear magnetic resonance (for reviews see Blinks, Prendergast & Allen, 1976; Blinks, Wier, Hess & Prendergast, 1982; Metcalf, Hesketh & Smith, 1985; Cobbold & Rink, 1987). In a contractile tissue such as cardiac muscle, maintenance of a stable penetration with a microelectrode is clearly very difficult, even in nominally isometric contractions. The use of metallochromic dyes also suffers from movement artefacts, because it is an absorbance method and depends critically on the exact positioning of the tissue with respect to a beam of light. Nuclear magnetic resonance methods have the disadvantage that they require a substantial volume of tissue and have poor time resolution. Fluorescent dyes have mainly been used with single cardiac cells, although it has recently been shown that it is possible to load whole hearts using an ester form of the dye (Lee, Mohabir, Smith & Clusin, 1987) and this method holds promise for the future.

To date, most measurements of intracellular free $[Ca^{2+}]$ in isolated, multicellular cardiac tissue have been obtained using the photoprotein aequorin. This is a 21 kDa protein present in the umbrella of the jellyfish Aequorea forskalea, which emits light in the presence of Ca^{2+} (Shimomura, Johnson & Saiga, 1962). Intracellular free $[Ca^{2+}]$ was first measured with aequorin in barnacle giant muscle fibres in 1967 (Ridgway & Ashley, 1967) and it has since been

extensively used to measure [Ca^{2+}] in a wide variety of tissues. The main disadvantage of aequorin is that due to its large size it has to be microinjected into cells, which can be a difficult and time-consuming process, especially in tissues with a relatively small cell size such as cardiac muscle. Although several "reversible permeabilisation" methods have been published which are able to load aequorin into cells (e.g. liposome fusion, hypo-osmotic shock, permeabilisation by EGTA or ATP, scrape loading), all of them are prone to artefacts, the inter- and intracellular distribution of aequorin has not been determined in any case and they are all more likely than not to introduce stresses to the cell which would probably affect the results of interventions such as hypoxia and ischaemia, which are stressful in themselves. Now that the aequorin gene has been cloned and sequenced (Inouye, Hoguchi, Sakaki, Tagaki, Miyata, Iwanaga, Miyata & Tsuji, 1985; Prasher, McCann & Cormier, 1985; Tsuji, Inouye, Goto & Sakaki, 1986), it may prove possible in the future to use the techniques of molecular biology to introduce aequorin into cells. In spite of the inconvenience of microinjection, it has the advantage that the protein is localised to the cytoplasm (the compartment where it exists in the jellyfish photocytes), where it remains able to give a signal for hours or even days.

The aequorin molecule contains a tightly bound luminophore called coelenterazine (Johnson & Shimomura, 1978). The luminescent reaction involves the oxidation of coelenterazine by oxygen carried by aequorin, and does not require exogenous oxygen or ATP. This is clearly very useful for its use in experiments involving hypoxia and ischaemia. Although it is possible to reconstitute aequorin *in vitro* by incubating the discharged apoprotein with coelenterazine and oxygen in the absence of Ca^{2+} (Shimomura & Johnson, 1975), when it is used as a Ca^{2+} indicator, the reaction can only occur once for each molecule and so

aequorin is gradually "consumed". This has to be taken into account when attempts are made to calibrate aequorin signals in terms of the absolute free [Ca^{2+}] present in cells (Allen & Blinks, 1979; Smith & Allen, 1988).

The essential feature of aequorin which makes it suitable for measuring intracellular [Ca^{2+}] is that the rate of the luminescent reaction is dependent on [Ca^{2+}]. In the absence of Ca^{2+}, a slow reaction rate occurs, which puts a lower limit of approximately 10^{-7}M on the detection of Ca^{2+} (Allen, Blinks & Prendergast, 1977). Above this level the rate of the luminescent reaction depends very steeply on [Ca^{2+}], increasing approximately 10^5-fold as [Ca^{2+}] increases from 10^{-7} to 10^{-5}M, i.e. aequorin light emission is proportional to [Ca^{2+}]$^{2.5}$. At a [Ca^{2+}] of 10^{-5}M the rate of consumption of aequorin is approximately 0.1 per second, while at 10^{-6}M it is approximately 10^{-4} per second. Since the [Ca^{2+}] in cardiac muscle reaches these levels for only a short period of time during each twitch, this means that many thousands of twitches can occur with very little consumption of aequorin. Allen and Kurihara (1980) estimated that approximately 2.5 x 10^{-5} of the injected aequorin was consumed during a twitch in rat cardiac muscle, while only 4 x 10^{-6} was consumed by a twitch in cat cardiac muscle. Clearly, if the resting [Ca^{2+}] rises substantially for any reason, then aequorin consumption can become a limiting factor.

The steep relation between [Ca^{2+}] and light emission is due to the presence of three Ca^{2+} binding sites, predicted from luminescence dose-response curves (Moisescu, Ashley & Campbell, 1975; Allen et al 1977) and confirmed by sequence analysis of the aequorin gene (Inouye et al, 1985; Prasher et al 1985; Tsuji et al 1986). A consequence of the steepness of the relation is that the aequorin signal effectively magnifies changes in intracellular [Ca^{2+}] occurring within the cell, making detection of such changes easier. Although this needs to be

taken into account if it is desired to know the absolute [Ca^{2+}] within the cell, it has been pointed out (Allshire & Cobbold 1987) that many of the biochemical processes within the cell also respond non-linearly to changes in [Ca^{2+}], so the aequorin light signal may in fact mirror the biological signal more faithfully than absolute changes in [Ca^{2+}]. Another important point is that the kinetics of the aequorin reaction in the face of a step change in free [Ca^{2+}] should be sufficiently fast to follow the changes occurring within the cell. This is in fact the case, and the half-time of the change in light emission when [Ca^{2+}] is changed, about 6 milliseconds (Hastings, Mitchell, Mattingly, Blinks & van Leeuwen, 1969), should not be an important influence on the Ca^{2+} transients measured from cardiac muscle. Finally, it may be noted that the intracellular concentration of aequorin necessary to achieve an adequate signal to noise ratio is not expected to buffer intracellular [Ca^{2+}] to any great extent, and so should have little effect on the final free [Ca^{2+}] measured (Allen & Blinks, 1979). Other aspects of the use and interpretation of aequorin signals are considered in Section 2.

CHAPTER 2. METHODS.

2.1 Standard solution.

All solutions were made with distilled water and analytical grade reagents. The standard Tyrode solution contained (mM):

NaCl	93
$NaHCO_3$	20
Na_2HPO_4	1
$MgSO_4$	1
KCl	5
$CaCl_2$	2
CH_3CH_2OONa	20
Glucose	10
Insulin	5U/l (= 40 nM).

This gave a final ionic composition of (mM):

Na^+	135
K^+	5
Ca^{2+}	2
Mg^{2+}	1
Cl^-	102
HCO_3^-	20
HPO_4	1
SO_4	1
Acetate	20
Glucose	10
Insulin	5U/l (= 40 nM).

In order to maintain the pH at 7.4, the solution was bubbled with 5% CO_2/95% O_2. A double strength stock solution was prepared containing no acetate, glucose or insulin, and without calcium chloride. This avoided bacterial contamination of the stock, and also prevented the precipitation of calcium phosphate which occurs in the unbubbled solution due to the alkaline pH. Acetate, glucose and insulin were kept as a separate concentrated stock in the fridge. Fresh solution was prepared at the beginning of each experiment.

2.2. Preparation and use of papillary muscles.

Most of the experiments described in this thesis were performed with ferret right ventricular papillary muscles. Papillary muscles are a useful preparation to work with, because it is possible to control accurately the various factors which influence the strength of

contraction, such as temperature, stimulation frequency, length and composition of the superfusate. In order to obtain papillary muscles, ferrets were deeply anaesthetised with an overdose of pentobarbitone administered intraperitoneally. The chest was opened, and the heart was rapidly removed and washed with cold, bubbled Tyrode solution to remove blood. The heart was then transferred to a dissecting chamber with a strong overhead light. The right ventricle was opened by an incision from the root of the pulmonary artery to the apex. It was found that this approach rarely caused damage to the papillary muscles inside. A thin papillary muscle of < 1 mm diameter (preferably 0.5 - 0.7 mm) was chosen. This diameter was chosen because it allows adequate oxygenation of the tissue during superfusion (see discussions in Blinks & Koch-Weser, 1963; Gibbs, 1978; Allen, 1983).

Once a muscle had been selected, it was secured with 50 /um silver wire at each end. Firstly, a stay knot was tied at the tendon end. Then a small piece of wire was tied first to the tendon and then to itself, forming a loop. The stay knot at the tendon end was necessary because otherwise the loop had a tendency to slip off the muscle. At the ventricular end of the muscle, a stay was not necessary because the cut end of the muscle provided sufficient grip (see Figure 5 C). Although these small loops were used for most experiments, it was found that in experiments concerned with simulating ischaemia, the loops retained by surface tension an unacceptably large amount of solution. For this reason, in these experiments, the securing lengths of wire were not looped, but were instead wrapped around the supporting hooks, i.e. there was now a straight length of wire between the muscle and the hooks. Although inconvenient, this method of attachment gave a much better drainage of solution from the muscle.

Once the wires were in place, the muscle was removed from the heart and transferred to the experimental chamber. The muscle was

mounted horizontally in the chamber and superfused with standard solution (see Figure 5 A & B and Figure 7). The superfusing solution was equilibrated with gas and heated to the chosen temperature in a water bath. Except where specified in the results, experiments were performed at 30°C. Solution was then pumped by a roller pump to a heat exchanger, which lay close to the muscle bath on a temperature controlled table held at the chosen temperature. Solution entered the muscle bath via a stainless steel tube. It was important to preheat the solution in the water bath, because the solubility of gases decreases as temperature rises. Thus if the solution had been equilibrated at room temperature and then heated, bubbles would have appeared in the muscle bath. Solution was removed from the muscle bath by a hypodermic needle, which was attached to the roller pump with a wider bore tubing than the inflow side. The needle thus sucked solution and air and maintained the solution in the bath at a stable level. From the roller pump, solution was returned to the reservoir in the water bath. Solution was recirculated through the chamber at a rate sufficient to achieve a relatively rapid flow of solution past the muscle, e.g. at a rate of approximately one muscle length per second.

The muscle bath itself was approximately 40 mm long, 20 mm wide and 5 mm deep. The body of the chamber was made from black perspex, while the bottom was of glass. The muscle bath was also mounted on the temperature controlled table. Illumination of the muscle was provided by two bulbs set beneath the table. A slot in the table allowed light to fall on the muscle, but the bulbs were positioned to one side, so that light did not directly enter the dissecting microscope. This arrangement thus gave dark field illumination of the muscle, which provided a clear view of the preparation, and facilitated procedures such as microinjection.

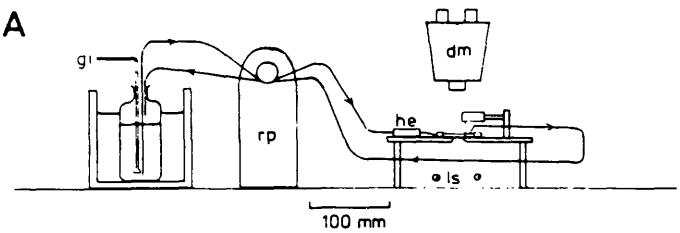
As shown in Figures 5 and 7, the muscle was suspended between a

FIGURE 5.

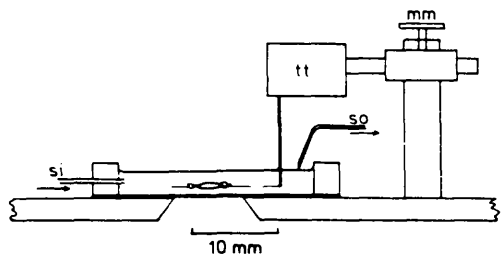
FIGURE 5.

Apparatus for stimulating and recording tension from a horizontally mounted, isolated papillary muscle (from Allen, 1983). A. General view of apparatus. B. Detail of muscle chamber and tension transducer. C. Detail of method of muscle attachment to hooks using silver wire. Abbreviations: gc - gas inlet; rp - roller pump; he - heat exchanger; dm - dissecting microscope; ls - light sources; tt - tension transducer; si - solution inlet; so - solution outlet.

A



B



C

fixed hook attached to the chamber and a hook attached to the tension transducer. The latter hook was movable because the tension transducer was attached to a micrometer, allowing the muscle length to be adjusted accurately. Most experiments were performed with the muscle at the length at which developed tension was a maximum. The muscle bath was attached by an XY micrometer to the tension transducer mounting, so that the transducer and muscle bath could be moved together. This allowed the muscle bath to be positioned in the best place for illumination and then moved, if necessary, to the best place for light collection without disturbing the muscle.

Muscles were stimulated with rectangular voltage pulses delivered through punctate electrodes made of platinum wire. The electrodes were insulated with fine plastic tubing up to the tip to minimise the release of products of electrolysis, and were sufficiently long that they could be manipulated so as to lie close to the muscle. The voltage threshold was determined for each muscle and was periodically checked. An attempt was made to keep the stimulation voltage at approximately 1.2 times threshold in order to minimise catecholamine release from nerve fibres within the muscle (Blinks, 1966). The stimulus pulses were 2 ms in duration and alternated in polarity. In some experiments, particularly those concerned with the phenomenon of alternans, the stimuli were kept at the same polarity throughout the experiment.

Tension, aequorin light, stimuli and, in some experiments, muscle length, were recorded on a pen recorder (Gould Type 2400S) supplied with Gould universal preamplifiers. The output signals from the pen recorder and a trigger signal were also recorded on magnetic tape (Racal Thermionics Store 7D recorder) for later analysis. The tape recorder was run at 15/32 ips. This was the slowest speed available and reduced tape consumption during long experiments. Data could be

signal averaged both during an experiment and later from tape using a programmable signal averager (Datalab Ltd., DL4000 system with DL 450 microprocessor) or a computer based signal averager written for use with IBM compatible computers with a pclab analogue to digital conversion board (Vacuum, by Microbased Computer Systems Ltd.). Averaged traces were plotted using an XY plotter.

The tension transducer used was a semiconductor strain gauge (Akers AE 801, SensoNor Ltd., Norway) with high sensitivity and low thermal drift. The semiconductor element was held in a metal mounting which protected it. A thin carbon fibre extension was glued to the semiconductor element with cyanoacrylate adhesive (Radiospares Ltd.). A smaller piece of carbon fibre was attached to the other side of the element to increase the rigidity of the transducer. A hook was glued to the end of the carbon fibre extension. The compliance of the mounted transducer was typically about 50 μm/g. As mentioned above, the transducer assembly was held firmly on the micrometer stage to which the experimental chamber was attached and the muscle length could be adjusted by moving the transducer mounting parallel to the papillary muscle axis with a micrometer screw. There was also a vertical adjustment which allowed the muscle to be positioned at the correct level within the bath. The elements of the tension transducer formed one half of a Wheatstone bridge whose output passed to a strain gauge amplifier (CIL Electronics Ltd., type SGA 703-14202). This amplifier combined high gain with low drift and also provided a steady current to drive the Wheatstone bridge. The gain of the amplifier was adjusted to 1g/V for convenience. At this gain the output noise was below 5 mV (i.e. below 5 mg), and was therefore essentially negligible.

When it was desired to make measurements of length, the fixed hook was replaced with a hook attached to the rotating arm of a Cambridge Technology series 300 dual mode servo motor. This arrangement allowed

the muscle to be studied in either isometric or isotonic mode by altering the threshold tension at which the rotating arm started to move. The threshold could be altered by applying a voltage signal to the servo motor. It was not possible to reduce the threshold force to arbitrarily low levels in this system because of stability constraints of the servo motor. However, the threshold was always below 10% of the maximum tension, and generally between 2 and 5%. Once the minimum threshold tension had been found, it was kept constant throughout an experiment. The timing of the voltage signal which caused an isometric contraction was automated, and this made it possible to perform experiments in which an isotonic beat could be interpolated at regular intervals when the muscle was in isometric mode, or alternatively an isometric beat could be interpolated when the muscle was in isotonic mode. This arrangement allowed the effects of interventions on both isometric and isotonic contractions to be followed virtually simultaneously.

2.3 Handling and microinjection of aequorin.

As described in section 1.4, aequorin is consumed in the presence of Ca^{2+}. Hence, to prevent consumption during the preparation, storage and use of aequorin, it is essential that all receptacles with which the aequorin must come in contact with should be free of Ca^{2+}. Except where otherwise detailed, pipette tips and containers were washed with 10 mmol/l EGTA and then rinsed with double distilled water before being used with solutions containing aequorin.

Aequorin was obtained from Professor J.R. Blinks (Department of Pharmacology, Mayo Foundation, Rochester, Minnesota, USA). Details of the preparation are described by Blinks, Mattingly, Jewell, van Leeuwen, Harrer & Allen (1978); the current preparation differs from that described in that chelex beads are no longer used. Aequorin was

supplied as a powder in sealed glass ampoules containing the dry lyophilised protein, KCl and HEPES buffer. Each ampoule contained approximately 1 mg of aequorin which was about 99% pure. One ml of double distilled water was added to an ampoule to give a solution containing 150 mmol/l KCl, 5 mmol/l HEPES buffer (pH 7.45) and an active aequorin concentration of about 6×10^{-5} mol/l. This stock aequorin solution was kept frozen at $-20^{o}C$ in a freezer. When required, the stock solution was thawed and 3 - 5 μl was drawn up and temporarily held in a small receptacle, which was made by drawing out a plastic pipette tip over a flame. The receptacle was attached to the tip of a Hamilton syringe. Before and after use it was scrupulously washed with 10 mmol/l EGTA, then 1 mmol/l EGTA and finally double distilled water.

Microelectrodes were pulled using a horizontal microelectrode puller (Industrial Science Associates). Microelectrode glass with an internal diameter of 1.2 mm and an internal thin filament was used (Clark Electromedical Instruments, GC120F - 15). Glass was stored in 10 mmol/l EGTA. On the day of use it was thoroughly washed first with double distilled water and then with alcohol. It was then dried in an oven. It was particularly important for the glass to be free of Ca^{2+}, not only to avoid consumption of aequorin, but also because the presence of Ca^{2+} caused a high background light emission, above which it was difficult to tell if the electrode was ejecting aequorin.

50 - 100 nl of aequorin solution was placed near the tip of a microelectrode using the receptacle mentioned above. The thin filament ensured that the solution rapidly entered the tip of the electrode by capillarity. The microelectrode was then attached to an assembly which allowed the application of high pressure to the solution in the electrode, while holding the electrode firmly enough to prevent movement (Blinks et al 1978). A platinum wire (silver rapidly

inactivates aequorin) passed down the bore of the electrode, stopping just short of the tip and the aequorin solution. (It was found that dipping the wire into the solution often caused increased light emission in spite of prior washing with EGTA and distilled water). Short pulses of pressure were applied using a hand operated valve (Dralim Ltd.). Turning the valve on applied pressure, while turning it off vented to the room. The electrode was held in place by a teflon insert in the holder; when the electrode was screwed in place, the shoulders of the insert pressed on the glass, holding it firmly. The electrode holder was attached to a micromanipulator (Prior), which allowed positioning in the muscle bath. The platinum wire was connected to an amplifier which could either measure the electrode resistance or amplify an input signal. The output of this amplifier passed to a digital voltmeter with an audio output. This allowed membrane potential to be measured during the injection process.

Microinjection of aequorin was then attempted using the following routine:

1. The microelectrode tip was placed in the Tyrode solution in the muscle bath and the light guide of a photomultiplier tube (PMT) was placed near the immersed tip. A light tight box was placed over the apparatus and the output of the PMT was monitored while pressure was applied to the microelectrode.

2. Ejection from the microelectrode was indicated by a signal from the PMT. Microelectrodes which did not eject were discarded.

3. Once an ejecting microelectrode had been obtained, the PMT was replaced with a binocular microscope and the microelectrode was inserted into surface cells of the preparation. Pulses of pressure were applied to eject aequorin into the cells. After 10 - 20 penetrations, the PMT was replaced and the microelectrode was tested again. Microelectrodes often became blocked and had to be discarded.

Microelectrode resistance was also periodically checked during the injection process. Electrodes whose tip resistance fell below about 60 Mohms also usually needed to be discarded (see below).

4. After every 2 - 4 periods of injection, the PMT was placed over the preparation, which was then stimulated. Injection was continued until an adequate signal to noise ratio had been obtained. This procedure usually took 1 - 3 hours, although it sometimes required longer .

The light emitted from the preparation was typically 1 - 2 orders of magnitude less than that emitted by the electrodes. Electrodes which were successfully used for injection typically had a resistance of 60 -80 Mohms as measured with this system (i.e. 150 mmol/l KCl in the electrode rather than 3 mol/l KCl). The electrode resistance used was determined by two opposing factors: higher resistance electrodes had a greater tendency to become blocked, while lower resistance electrodes had a greater tendency to cause damage to the penetrated cells. Damage was indicated either by a sudden and rapid fall in the membrane potential during injection or by a high resting Ca^{2+} and Ca^{2+} oscillations from the preparation during subsequent testing. These findings probably represent either cells with damaged membranes allowing a greater influx of Ca^{2+} than usual, or cells in which the membranes had sealed over, but the previous influx of Ca^{2+} had caused the sarcoplasmic reticulum to become Ca^{2+} overloaded. Nevertheless, these phenomena were fully reversible, and had usually disappeared 30 minutes after injection had been completed.

2.4 Light detection.

The light emitted by aequorin when it combines with Ca^{2+} has a maximum at 470 nm, which is at the blue end of the spectrum. As mentioned in section 1.4, the light emission increases steeply as

$[Ca^{2+}]$ increases, i.e. the light signal changes over a much greater range than the Ca^{2+}. Thus an aequorin light detection system should have sensitivity at the blue end of the spectrum combined with a large dynamic range. It is also desirable that the system should have a small output signal in the absence of any light (i.e. a low "dark current"). These criteria may be met by choosing an appropriate photomultiplier tube (PMT), a device which generates an electric current in response to photons falling on a light sensitive surface.

Because the light emission of aequorin-injected cardiac preparations is generally rather small, it is important to have a system which is as efficient as possible in channelling the photons emitted by the preparation to the light-sensitive surface of the PMT (the photocathode). One simple way to do this is to use a polished methacrylate (Perspex) rod to channel the photons by total internal reflection to the photocathode (Cannell & Allen, 1983). The channelling efficiency of this arrangement is maximal if the end of the perspex rod is close to the photocathode. However, one consideration in using such an arrangement with biological preparations is that it is often interesting to measure the resting light emission of the muscle. In order to do this, it is necessary to have some arrangement for shutting off the collection of light from the preparation by the PMT, so that the dark current can be measured and subtracted from the signal to give the true resting light emission. In order not to break the channelling of photons, it is best therefore if the shutter arrangement does not have an air gap. The way in which this was achieved was to interrupt the perspex column with a glass shutter, while preserving continuity of the light guide with glycerol (see below and Figure 6).

One way in which the dark current of a PMT can be reduced is by cooling it, thus reducing the random thermal emission of apparent photo-electrons. However, in normal working atmospheres this can lead

to problems due to water condensing on the PMT housing and dripping on the preparation. Cooling of the PMT was not used in the experiments described in this thesis. Instead, resting light was measured in preparations which had relatively good light signals (so that the resting light was visible above the room temperature dark current).

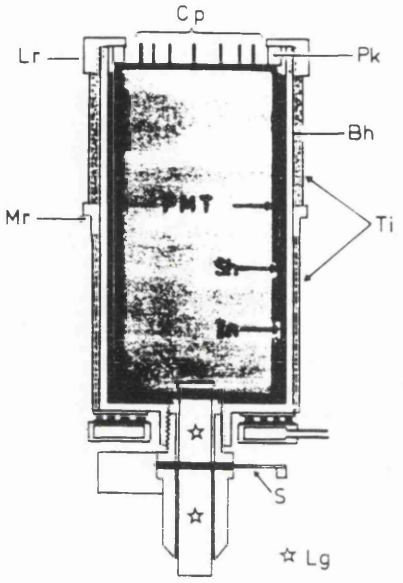
The PMT used combined high efficiency with low dark current (EMI Ltd., type 9789 A). This was mounted in a brass housing which was earthed and insulated from a driven shield which surrounded the glass body of the PMT (see Figure 6A). As mentioned above, a light guide was constructed from two pieces of perspex rod in order to maximise light collection from the preparation. The rods were 10 mm in diameter and 30 mm long and were hand polished using alumina paste. One piece was fixed in a holder which was screwed into the PMT housing. Contact with the photocathode was achieved with a drop of glycerol (which has a similar refractive index to perspex). The second piece of perspex was mounted in a second holder which could be clamped to the first. A small gap was left between the two perspex rods in order to accommodate the shutter, which was made from two glass microscope slide coverslips cut to size. One of the coverslips was silvered over half its length, and the other was then glued over it to protect the silvering from wear. A drop of glycerol provided continuity between the perspex rods and the glass. The shutter was connected to a rod which ran in a cylinder which was connected via an electromechanical miniature solenoid valve (Lee Co.) to a pressure source. Springs attached to the rod were arranged so that the shutter was closed (i.e. the opaque silvered half of the shutter was in line with the perspex light guide) when the gas pressure was shut off by the valve. The valve was connected to a switch outside the light box which could be used to close and open the shutter. This was used during experiments to measure the dark current of the PMT, and hence allowed (by subtraction)

FIGURE 6.

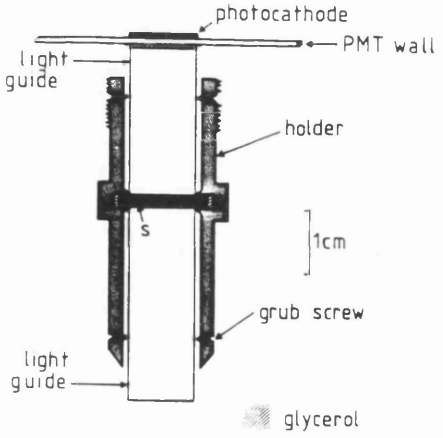
FIGURE 6.

Diagram of apparatus used to collect and measure light from aequorin-injected cardiac papillary muscles (from Cannell & Allen, 1983). A. Cross section of the photomultiplier tube housing. B. Detail of the light guide assembly. In B, the gaps between the two halves of the light guide and between the light guide and the glass of the photomultiplier tube have been exaggerated for clarity. The gaps were filled with glycerol. The shutter moves in and out of the plane of the diagram. Abbreviations: Sh - driven shield; In - electrical insulation; Ti - thermal insulation; Bh - brass housing; Cp - photomultiplier tube connecting pins; Lr - locking ring; Mr - mounting ring; Pc - photomultiplier tube photocathode; S - shutter; Lg - (starred) light guide.

A


Cp
Lr
Pk
Bh
PMT
Mr
Ti
S
Lg

B


photocathode
PMT wall
light guide
holder
S
1cm
grub screw
light guide
glycerol

an estimation of the light emission from a resting muscle. A switch was also arranged so that the valve was automatically closed when the light box was lifted, thus causing the shutter to close and hence protecting the PMT in the event of its inadvertently being left on.

Not all of the light emitted by an aequorin injected muscle is collected by the arrangement described above. Tests with a light standard indicated that approximately 80% of the light entering the end of the light guide reached the photocathode. The cone of light which can enter the end of the light guide is determined by the refractive index of perspex. It can be shown that the solid angle fraction collected by the light guide is (1 - cos T)/2, where T is the angle of incidence of the ray entering the light guide which is contained by total internal reflection (56^{o} for perspex). This means that if there were no losses from the light guide, then 22% of the light emitted by a point source (such as an aequorin injected preparation) would be collected by the light guide. Losses in the light guide probably reduce this figure to approximately 17%. In an attempt to improve this value, silver foil was often inserted below the muscle chamber at the beginning of an experiment to reflect some of the light emitted downwards by the preparation back up to the light guide. On two occasions the usefulness of this manoeuvre was measured and was found to give a small improvement (~10-15%) in the light signal. In spite of the relatively low fraction of total emitted light collected, it did not prove difficult to obtain adequate signal to noise ratios.

During experiments, the level of the bathing solution was approximately 1 mm above the surface of the muscle, and the light guide was lowered until it just contacted the surface of the solution. With this arrangement, the cone of light accepted by the light guide from a point source would have a diameter of approximately 2 mm at the light guide surface. Thus the fraction of light collected by the guide

should not vary if the point source is moved up to 8mm. The papillary muscles used in this study ranged from 2 - 6 mm in length. Thus a safety margin of 8 - 4 mm applied, and movement artefacts should not have contributed to the light signal. Tests with a cylindrical light source 1 mm in diameter and 2 mm long indicated that the fraction of light collected did not vary by more than 2% when the light source was moved 1.5 mm either side of the light guide axis (Allen & Kurihara, 1982).

The PMT was always operated at its optimum voltage, which was 1240 V. At this voltage the dark current of the tube was typically about 1 nA at room temperature. The output from the anode of the PMT was converted to a voltage by an amplifier. This was similar to that described by Blinks et al (1978): it used low drift integrated circuits and was also provided with variable gains and filters. The bandwidth of this amplifier was 10 kHz, but typically this was reduced to 100 - 30 Hz for light recording, and to < 1 Hz when an attempt was made to record resting light levels.

2.5 Inotropic interventions.

Anoxia, which prevents oxidative phosphorylation, was produced by switching from superfusing solution equilibrated with 95%O_2/5% CO_2 to solution equilibrated with 95% N_2/5% CO_2. Ischaemia is more difficult to study in isolated tissue preparations, because in addition to removing oxygen, it is necessary that flow is stopped and the extracellular space restricted. A new model was developed in which ischaemia was simulated by draining away the Tyrode solution in the chamber and superfusing the muscle with water-saturated 95% N_2/5% CO_2 gas (N_2 gas perfusion). Consequently the muscle was surrounded by a thin film of Tyrode solution leaving only a limited extracellular volume in which non-volatile products of metabolism could accumulate.

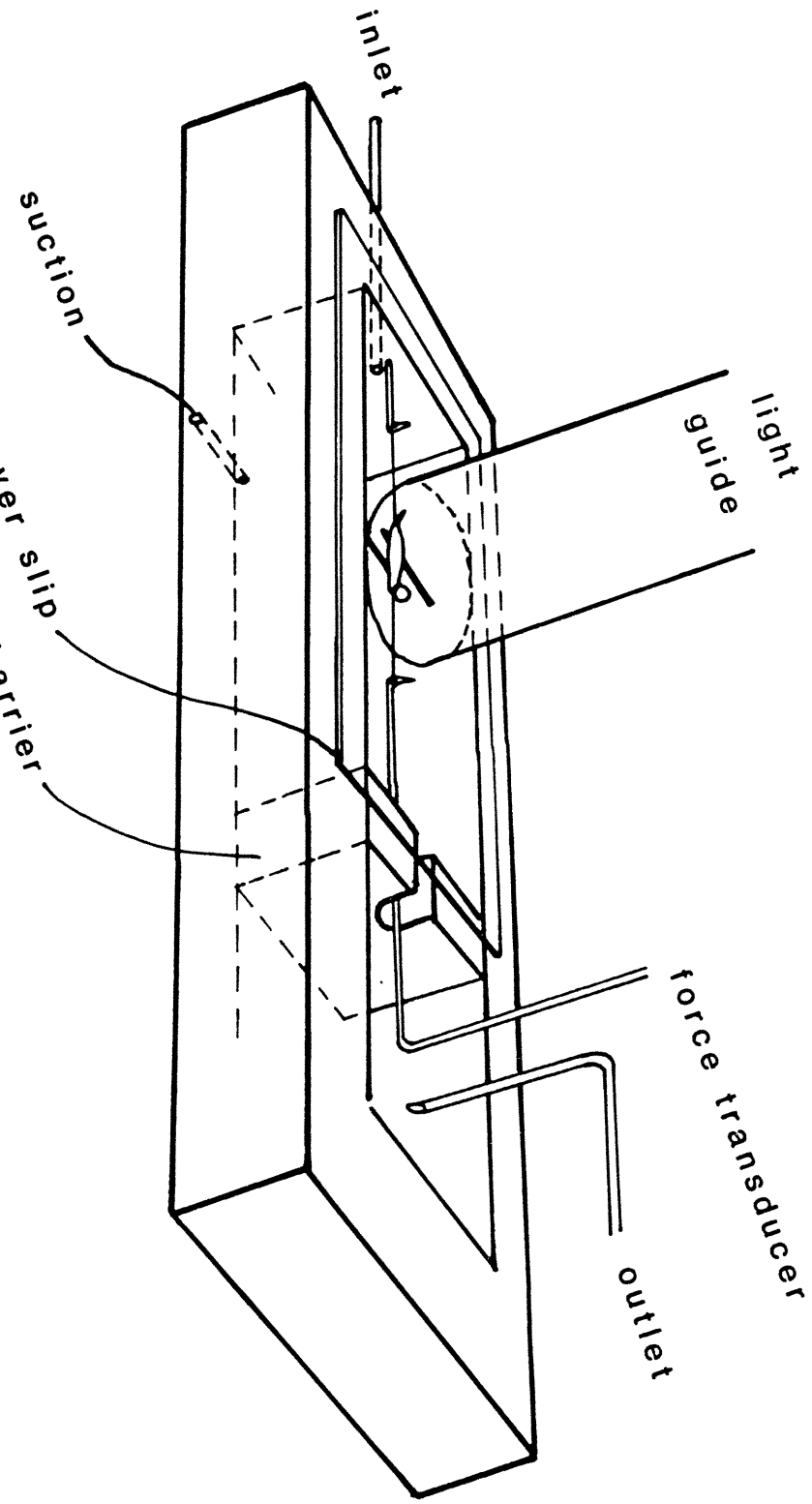
Gas perfusion was carried out in the modified muscle chamber illustrated in Figure 7. To prevent entry of air, the chamber was sealed during use except at 3 points; the fluid inlet, the drainage tube at the base of the chamber (which was sealed except when applying suction), and the fluid outlet, which was a narrow slot in the barrier through which the tension transducer also passed. As described in section 2.2, the muscle was attached to the fixed hook in the chamber and to the tension transducer by single straight lengths of 50 /um silver wire. This arrangement was found by trial and error to minimise the amount of Tyrode solution which remained clinging to the muscle when the chamber was drained. The muscle was stimulated by two platinum wires perpendicular to the muscle and entering the chamber from opposite sides. These lay just beneath the muscle and near to either end. When the chamber was drained, the muscle lay on these two electrodes so that stimulation could continue. During gas perfusion, all the stimulating current was constrained to pass through the muscle and consequently the apparent threshold for stimulation fell. The stimulus voltage was initially set to 10-20% above threshold during superfusion with Tyrode solution and when gas perfusion started the stimulus voltage was reduced to maintain it at approximately this level. This was done in order to minimise catecholamine release from sympathetic fibres within the preparation (Blinks, 1966). In some experiments, blockade of alpha and beta adrenoreceptors was produced before the start of gas perfusion.

The procedure for gas perfusion was as follows. Two bottles of Tyrode solution were maintained in a water bath continuously bubbled with the appropriate gas; 95% O_2/5% CO_2 for the standard Tyrode solution (O_2 Tyrode) and 95% N_2/5% CO_2 for the anoxic Tyrode solution (N_2 Tyrode). Solutions or gases were pumped from one or other bottles to the muscle chamber in tubing which was largely stainless steel to

FIGURE 7.

FIGURE 7.

Muscle bath used for simulating ischaemia in isolated papillary muscles. The muscle was suspended by straight lengths of silver wire between a fixed hook and a hook attached to the arm of a tension transducer. The under surface of the muscle was in contact with two platinum wires which served as stimulating electrodes. The bath was isolated from the atmosphere by a barrier at one end and cover slip on top. During conventional superfusion, solution was pumped in through the inlet, passed through a small gap in the barrier (which also allowed access for the tension transducer hook) and was sucked away at the outlet. During gas perfusion, water-saturated 95% N_2/5% CO_2 or 95% O_2/5% CO_2 was pumped in through the inlet and escaped through the small slot in the barrier. Any residual solution was removed at the beginning of gas perfusion through a small tube near the bottom of the chamber to which suction could be temporarily applied with a syringe. This tube was sealed when not applying suction. Aequorin light signals were collected during these procedures by a light guide in contact with the coverslip.


inlet
suction
cover slip
barrier
light guide
force transducer
outlet

prevent exchange of gas with the atmosphere. For perfusion with Tyrode solution, the outlet in the bottle was below the solution surface: for gas perfusion, the outlet was raised above the solution surface so that the gas pumped to the muscle chamber had bubbled through the Tyrode solution and was thermally equilibrated and saturated with water vapour. At the start of gas perfusion, Tyrode solution in the muscle chamber was sucked away through the drainage tube. Removal of all the solution inevitably entailed sucking a small volume of air into the chamber at the beginning of gas perfusion.

If products of metabolism were to accumulate to the concentrations achieved during ischaemia, it was necessary that only very small quantities of Tyrode solution remained adhering to the external surface of the muscle. This was confirmed visually with a dissecting microscope on a test run at the beginning of each experiment. In two experiments an attempt was made to weigh the solution adhering to the muscle surface during gas perfusion. The chamber was opened and the muscle blotted with small pieces of tissue paper. However, the amount of solution obtained by blotting was at or below the resolution of the balance used (0.1 mg from muscles whose mass was approximately 1-2 mg).

The addition of cyanide mimics hypoxia by blocking oxidative phosphorylation. Because of the volatility of HCN, cyanide solutions were kept as a concentrated stock solution of NaCN (pH 10.5). Immediately before use, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid) solution was added to the cyanide stock to give a solution containing 200 mmol/l NaCN, 500 mmol/l HEPES, pH 7.5. This was then added to the superfusing solution to give 2 mmol/l NaCN buffered with 5 mmol/l HEPES.

Pimobendan was dissolved in dimethyl sulfoxide (DMSO) and added to the superfusing solution at a concentration of 25 μmol/l. DMSO had no

effects on either tension or light emission when added to the superfusing solution alone. When tested *in vitro* neither pimobendan nor DMSO significantly affected aequorin light emission. Although the effects of pimobendan were reversible in skinned fibre experiments, one problem with its use in experiments on intact muscle is that its effects are only poorly reversible, so it was only possible to make one addition of pimobendan in each experiment. This difference between skinned and intact muscle is perhaps due to the highly lipophilic nature of the drug, giving it a very long washout from preparations with intact membranes. In each experiment a series of extracellular [Ca^{2+}] were applied, followed by other interventions (such as addition of isoprenaline), followed by application of pimobendan.

For other inotropic interventions, substances were added to the superfusing solution in the concentrations stated. Results are quoted as mean $\pm$ S.E.M.; statistical significance was tested for using the appropriate paired or unpaired t test.

2.6 Action potential recording.

Action potentials were recorded during gas perfusion using the chamber described in section 2.5, but with the light guide removed. A coverslip with a small hole was used and conventional glass microelectrodes placed through the hole. Once a stable penetration had been obtained and N_2 gas perfusion started, the hole around the microelectrode was sealed with a small drop of glycerol.

2.7 Lactate and glycogen measurements from Langendorff-perfused hearts.

The heart was rapidly removed from the ferret and attached to a Langendorff perfusion apparatus. The methods used were similar to those described by Allen, Morris, Orchard & Pirolo (1985). Hearts were perfused through the aorta at a rate of 5 ml/g/min at 30°C. The right

atrium was removed and a balloon was inflated in the left ventricle in order to measure developed pressure. The heart was stimulated with electrodes placed in the left ventricle at a rate of 1 or 2 Hz. Once the heart was set up, a small receptacle was raised around the heart so that it was fully immersed in Tyrode solution. This served to isolate it as much as possible from atmospheric oxygen during periods of anoxia. In these experiments the glucose under control conditions was varied between 2 and 10 mM (see Chapter 3). Anoxia was produced as described above, by changing from a Tyrode solution equilibrated with 95% O_2/ 5% CO_2, to one equilibrated with 95% N_2/ 5% CO_2.

In order to estimate the glycogen stores of the heart, biopsies were obtained using a hand-held drill with an attached sharpened stainless steel tube (internal diameter 2 mm). Full thickness biopsies were taken from the left ventricle and immediately frozen. Sequential biopsies were taken from the same region of the heart. Although this procedure necessarily produced some local damage, the developed pressure and lactate measurements obtained from such hearts during anoxia were indistinguishable from those obtained in experiments in which no biopsies were taken. Biopsies were analysed for glycogen using an enzymatic assay (Edwards, Jones, Maunder & Batra, 1975). In brief, tissue was homogenised in acetate buffer at pH 4.9, and glycogen was hydrolysed using a mixture of amino glucosidase and alpha-amylase. Free glucose was then estimated using a conventional glucose-6-phosphate dehydrogenase assay for glucose, involving spectrophotometric determination of the conversion of NADP to NADPH. Free glucose measured in the tissue before the hydolysis of glycogen was negligible.

Lactate measurements were made on perfusate collected from the heart during control (aerobic) periods and during anoxia. Lactate was measured using an enzymatic assay (Sigma Diagnostics). In brief, lactate dehydrogenase was used to oxidise lactate to pyruvate, and the

increase in absorption at 340 nm due to conversion of NAD to NADH was measured.

The pH and lactate composition of extracellular fluid during ischaemia was estimated in the following way. Ferret hearts were Langendorff-perfused as described above. The extracellular glucose was 5 mmol/l in these experiments. When developed pressure (measured by a balloon in the left ventricle) was stable, perfusion was stopped (global ischaemia). After 40 minutes of ischaemia, perfusion was restarted for about 4 seconds and the resulting effluent collected in 4 separate tubes (volume ~0.3 ml) which were immediately sealed to prevent loss of CO_2 to the atmosphere. The pH and lactate concentration were measured in each sample. The lowest value of pH and the highest value of lactate were generally found in the 2nd and 3rd samples and these were regarded as representative of extracellular space.

2.8 Interpretation of aequorin signals during ischaemia and hypoxia.

A number of factors may modify the aequorin light emission for a given $[Ca^{2+}]_i$ during N_2 gas perfusion (ischaemia). These are listed below.

(i). Removal of solution from the chamber at the start of gas perfusion may change the efficiency of light collection. Tests with a small cylindrical light source replacing the muscle showed that gas perfusion led to a 10% reduction in the recorded light collection.

(ii). In an ischaemic exposure sufficiently long for the Ca^{2+} transients to fall to zero, there is considerable consumption of aequorin because of the increases in both the amplitude and duration of the Ca^{2+} transients. In 3 experiments the amount of aequorin consumed during such an ischaemic exposure was estimated by integrating all the aequorin light transients. The aequorin remaining in the preparation at the end of the experiment was also estimated by integrating the

aequorin light when the muscle was exposed to Triton-X 100 (for details see Smith & Allen, 1988). The fraction of the total aequorin consumed during an ischaemic exposure varied between 15 and 35% of the total. Light signals towards the end of an ischaemic exposure and on reperfusion will be underestimated to this extent.

(iii). Intracellular pH becomes acid during ischaemia and this decreases the aequorin light signal for a given $[Ca^{2+}]$. Elliott (1987) measured pH_i in Langendorff-perfused ferret hearts (under similar conditions of temperature and stimulation rate to those used in the experiments described in section 3.2), which were then made globally ischaemic. After 25 min, when the Ca^{2+} transients had increased to a maximum (see section 3.2), the intracellular pH had fallen to around 6.2 (change in pH = 0.8 units). *In vitro* aequorin calibration curves (Allen & Orchard, 1983a) show that an acidosis of this magnitude decreases aequorin light by 0.2 log units which represents a 37% decrease in light emission.

(iv). Another possible complicating factor is the changes in $[Mg^{2+}]$ which may occur during ischaemia. This arises because Mg^{2+} binds relatively tightly to ATP and PCr but less strongly to their metabolic products. Thus $[Mg^{2+}]_i$ might be expected to rise during ischaemia, although measurements of $[Mg^{2+}]_i$ during metabolic inhibition failed to show any such rise (Hess, Metzger & Weingart, 1982). A 2-fold increase in $[Mg^{2+}]_i$ would inhibit aequorin light at a given $[Ca^{2+}]_i$, causing about a 30% reduction in light emission. For further discussion see Allen & Orchard (1983b) and Smith & Allen, (1988).

The effects on aequorin light emission noted above all lead to decreases in light emission at a given $[Ca^{2+}]$ during N_2 gas perfusion. The net effect is that the maximum light signals during N_2 gas perfusion are probably underestimated by a factor of X 2-3. Changes in pH and $[Mg^{2+}]$ would also be expected to occur in hypoxia, although to a

lesser extent than in ischaemia. Allen & Orchard (1983b) calculated that this might cause a reduction of light emission by a factor of 1.1 to 1.5 during hypoxia.

2.9 Measurements on skinned cardiac muscle fibres.

Skinned fibres were prepared from ferret papillary muscle as described by Rüegg, Pfitzer, Eubler & Zeugner (1984). Briefly, the fibres were dissected and immersed in an ice-cold extraction solution containing (mmol/l): glycerol 50%, imidazole 20, NaN_3 10, ATP 5, $MgCl_2$ 5, EGTA 4, dithioerythritol 2; pH 7.0 and 1% Triton X-100. After 24 hours of continuous stirring, the solution was replaced with a Triton X-100-free extraction solution and stored at -20°C for three to ten days. Stored fibres were then teased into small bundles of 100-150 /um diameter and mounted horizontally between a glass rod connected to a micromanipulator and the extension of an AME force transducer (type 801, SensoNor, Horten, Norway). Subsequently, the preparation was immersed in relaxing solution containing (mmol/l): HEPES 50, potassium acetate 80, ATP 5, $MgCl_2$ 6, creatine phosphate 10, NaN_3 5, EGTA 5, $CaCl_2$ 5; pH 7.0; 380 units/ml creatine kinase and 1% DMSO. At the beginning of each experiment the fibres were stretched until resting tension just appeared (sarcomere length usually approximately 2.0 /um, as determined by laser diffraction). Contraction was then initiated by replacing EGTA with CaEGTA, and terminated by replacing the fibre in relaxing solution. In a series of contractions, different calcium ion concentrations were obtained by varying the ratio of CaEGTA and EGTA, assuming an apparent dissociation constant of the CaEGTA complex of 0.45 /uM at pH 7.0 (Fabiato & Fabiato, 1979). During the experiments, the 1 ml baths were continuously stirred by rotation at 1 Hz. Test solutions and control solutions also contained DMSO in a final concentration of 1%.

CHAPTER 3. RESULTS AND DISCUSSION.

3.1 THE EFFECTS OF HYPOXIA.

3.1 THE EFFECTS OF HYPOXIA.

3.1.1. Background.

It has been known for over twenty years that if cardiac muscle is made anoxic, the developed tension falls to approximately 30% of the control value within a few minutes, but can then continue at that level for at least 30 minutes (e.g. Weissler, Krüger, Baba, Scharpell, Leighton, & Gallimore, 1968). The reason that tension generation can be sustained for such a long time under anoxic conditions is that although oxidative phosphorylation is prevented during hypoxia, anaerobic glycolysis is stimulated and acts as a source of ATP, using as a substrate either the intramuscular stores of glycogen or external glucose (see Neely & Morgan (1974) for review). The importance of this route of ATP production is emphasised by the very different results obtained if glycolysis is prevented. Glycolysis can be prevented by depleting the endogenous glycogen stores by a period of rapid stimulation in a superfusing solution containing no glucose, or by pharmacological means, using 2-deoxyglucose or iodoacetic acid, both of which interfere with the glycolytic pathway (see Pirolo & Allen 1986 for a discussion of the relative effectiveness of these methods). If cardiac muscle is made anoxic when glycolysis has been prevented ("metabolic blockade"), then developed tension falls rapidly and completely, and a contracture develops within a few minutes (Allen & Orchard 1983b; Smith & Allen 1988). This contracture seemed most likely to be a true rigor, due to very low levels of ATP. NMR measurements have confirmed that the ATP level is well maintained in energy replete muscle exposed to simple hypoxia, while it falls rapidly when muscle is subjected to metabolic blockade (Matthews, Radda & Taylor 1981; Allen, Morris, Orchard & Pirolo 1985; Eisner, Elliott & Smith 1987).

If ATP levels are well maintained during simple hypoxia, the

question arises as to why the developed tension declines at all. One possible cause of the fall of tension is that the Ca^{2+} transient is reduced during hypoxia, a smaller Ca^{2+} release leading to less tension. This possibility was investigated using microinjected aequorin to measure Ca^{2+} in isolated ferret papillary muscles (Allen & Orchard 1983b). These authors found that when glycolysis was able to continue, exposure to hypoxia was not on average associated with any change in the amplitude of the Ca^{2+} transients. This observation indicates therefore, that the decrease in developed tension must be produced by factors which reduce tension even at a constant level of systolic intracellular free $[Ca^{2+}]$. Two metabolic changes which occur during hypoxia are likely candidates for causing such an effect. These are an increase in intracellular phosphate which occurs due to breakdown of phosphocreatine (see model in Allen & Orchard, 1987) and an intracellular acidosis, which is attributable to increased lactic acid production from the stimulated glycolysis. Both phosphate (Herzig & Rüegg 1977; Kentish 1986) and hydrogen ions (Fabiato & Fabiato 1978) have been shown to decrease tension in skinned muscle preparations, and quantitatively these effects appear to be large enough to explain the observed reduction in developed tension. Currently it seems that phosphate has the predominant effect in hypoxia, with only a fairly small component (~20%) being due to acidosis under these conditions (Eisner et al 1987; Kusuoka, Zweier, Jacobus & Marban, 1986). Other metabolites which change substantially under anoxic conditions, such as creatine phosphate, creatine and ADP appear to cause little effect on tension (Cooke & Pate, 1985; Kentish, 1986).

When the Ca^{2+} transients were studied during metabolic blockade, a different result was seen. Under these conditions, the rapid fall of tension was accompanied by a similar rapid decline in the amplitude of the Ca^{2+} transients. This suggests that in metabolic blockade, failure

of the Ca^{2+} transients *is* the cause of the rapid fall of tension.

Although Allen & Orchard (1983b) found that the mean amplitude of the Ca^{2+} transients was unchanged in simple hypoxia, their results were subject to considerable variability. In some experiments the amplitude of the Ca^{2+} transients increased on exposure to hypoxia, while in others it decreased, the range being from 163% to 55% of control values. In that study, no mechanism for this variability was established. More recently, the question of whether the Ca^{2+} transients play a significant role in the fall of tension in hypoxia has been reopened (MacKinnon, Gwathmey & Morgan, 1987). These authors measured Ca^{2+} in ferret papillary muscles which had been "chemically" loaded with aequorin. They found that exposure to hypoxia in the presence of glucose always caused a gradual decline in the amplitude of the Ca^{2+} transients, such that when the tension had fallen to 40% of control, the Ca^{2+} transients had decreased to 71% of control. Thus this observation, unlike the results mentioned above, suggests that a fall in the Ca^{2+} transients may indeed underlie a part of the fall in force in simple hypoxia.

In view of the uncertainty surrounding this question, a series of experiments was performed in order to reinvestigate the problem with a view to establishing a mechanism for the apparent variability of the response of the Ca^{2+} transients to hypoxia both within and between studies. In particular the effects of several exposures to hypoxia in a given preparation were studied and attempts were made to modify the response by various means (see below). In addition to measuring intracellular free $[Ca^{2+}]$ in papillary muscles, measurements of lactate and glycogen were obtained from Langendorff-perfused whole hearts.

3.1.2. The calcium transients during hypoxia.

The first exposure of a papillary muscle to hypoxia following

aequorin injection caused developed tension to fall to 37 ± 4% of control (n = 8) within a few minutes. However, the Ca^{2+} transients demonstrated a variety of responses, as previously noted (Allen & Orchard 1983b). In 4 preparations the Ca^{2+} transients increased as tension fell, in 3 they were unchanged in amplitude, while in 1 preparation the transients fell on the first exposure.

The reasons for this variability became clearer when repeated exposures to hypoxia in the same preparation were studied. In those preparations where the Ca^{2+} transients initially rose, it was observed that on subsequent exposures to hypoxia the rise of the Ca^{2+} transients was reduced, so that a progression was seen from an initial rise, to little or no change and finally to Ca^{2+} transients which declined during hypoxia. Such a progression of events is illustrated in Figure 8A & B, which shows records of the first and seventh exposure to hypoxia in one preparation. A further example is shown in Figure 9A & B. In other preparations, in which the Ca^{2+} transients were little affected by the initial exposure to hypoxia, it was observed that they were more likely to fall on subsequent exposures. Thus, all or part of the sequence of events described above was observed in all the preparations studied.

In the preparations shown in Figures 8 and 9, there appears to be a correlation between the level of tension during hypoxia and the amplitude of the Ca^{2+} transients during hypoxia, i.e. the tension appears to be better maintained in the early exposures to hypoxia, when the Ca^{2+} transients rose, than during later exposures, when they fell. To investigate this point further, the first exposure to hypoxia, in which the Ca^{2+} transients rose, was compared with a later exposure, in which the Ca^{2+} transients declined. On the first exposure the Ca^{2+} transients increased to 167 ± 13% (n = 4) of control, while developed tension decreased to 42 ± 7% of control. On the later exposure, the

FIGURE 8.

FIGURE 8.

The effects of repeated exposures to hypoxia on the Ca^{2+} transients and tension recorded from an isolated ferret papillary muscle stimulated at 1 Hz. Each panel shows original records of aequorin light (above) and tension (below). Hypoxia was produced by switching to Tyrode solution equilibrated with 95% N_2/ 5% CO_2.

A. First exposure to hypoxia.

B. 130 minutes after A, seventh exposure to hypoxia.

C. 70 minutes after B, eighth exposure to hypoxia. This was preceded by a 60 minute period of superfusion with high glucose Tyrode solution (see text), and a reduced stimulation frequency (0.2 Hz).

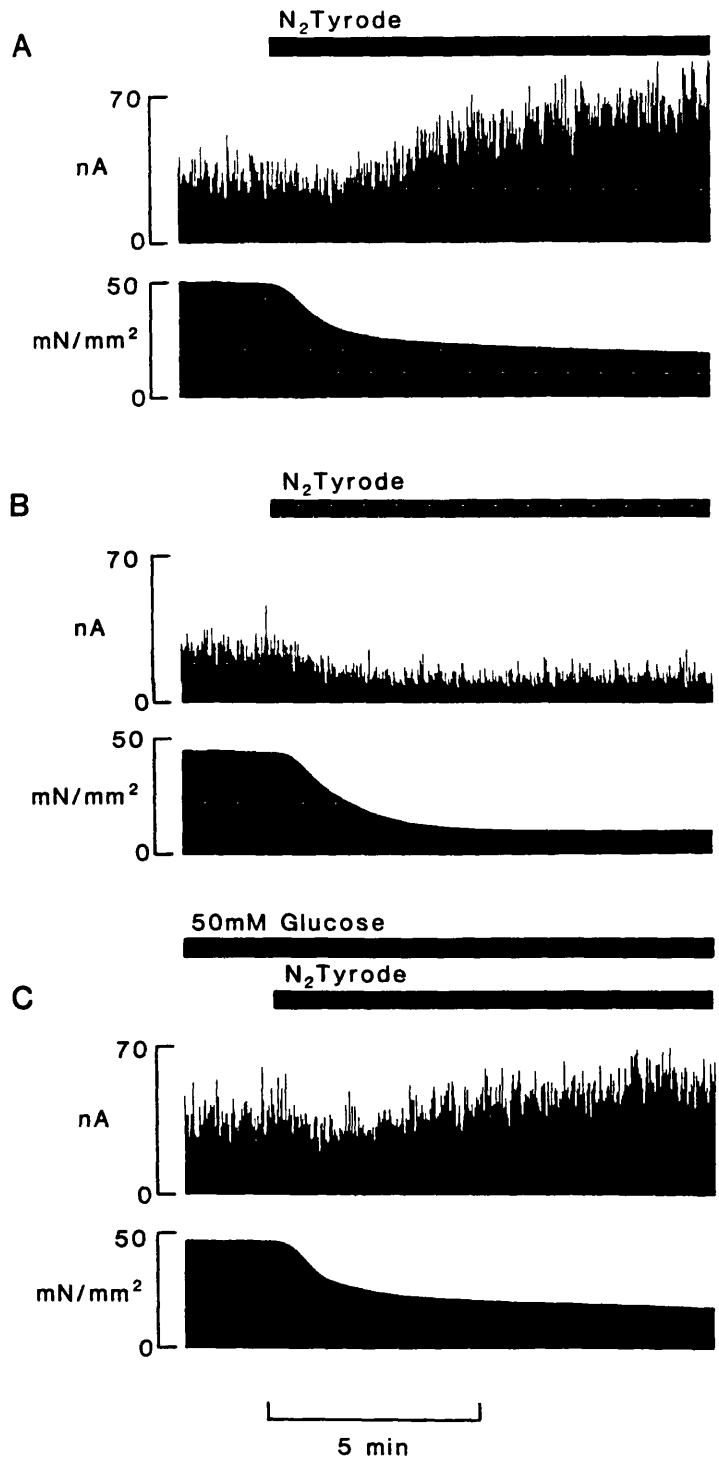

A
N_2Tyrode
70
nA
0
50
mN/mm²
0
B
N_2Tyrode
70
nA
0
50
mN/mm²
0
C
50mM Glucose
N_2Tyrode
70
nA
0
50
mN/mm²
0
5 min

FIGURE 9.

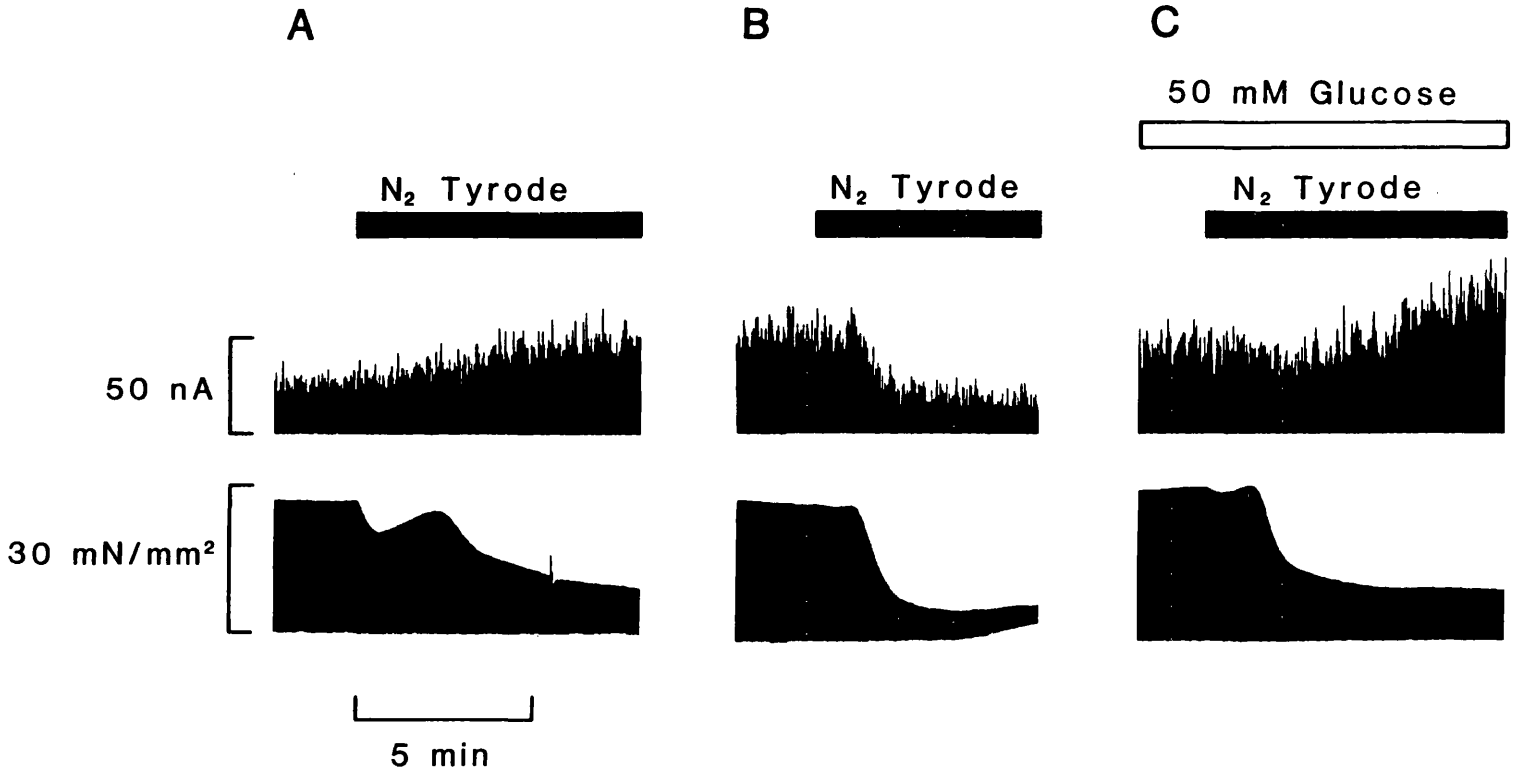
FIGURE 9.

A further example of the effects of repeated exposures to hypoxia on the Ca^{2+} transients and tension recorded from an isolated ferret papillary muscle stimulated at 1 Hz. Each panel shows original records of aequorin light (above) and tension (below). Hypoxia was produced by switching to Tyrode solution equilibrated with 95% N_2/ 5% CO_2.

A. Second exposure to hypoxia.

B. 180 minutes after A, fifth exposure to hypoxia.

C. 20 minutes after B, sixth exposure to hypoxia. Glucose had been increased to 50 mM 5 minutes before this panel.


A
B
C
50 mM Glucose
N_2 Tyrode
N_2 Tyrode
N_2 Tyrode
50 nA
30 mN/mm²
5 min

Ca^{2+} transients decreased to 68 ± 11% and tension decreased to 28 ± 6%. The changes in the Ca^{2+} transients and tension between the two exposures were both significant ($p < 0.05$) on a paired t-test. This result suggests that the decrease in the amplitude of the Ca^{2+} transients during later exposures to hypoxia does make a contribution to the decrease in developed tension.

The response of the Ca^{2+} transients to hypoxia seen in Figures 8B & 9B is similar to that seen in metabolic blockade, as described in section 3.1.1 (Allen & Orchard, 1983b, Smith & Allen, 1988). Metabolic blockade causes a shortening of the action potential (Allen, Harris & Smith, 1987; Hayashi, Watanabe & McDonald, 1987; Lederer, Nicholls & Smith, 1989) and this is thought to be the cause of the decline in the Ca^{2+} transients and tension in this situation.

Thus, the sequence of response patterns observed may be related to a reduced capacity for glycolysis after repeated exposures to hypoxia. In particular, since it is established that an intracellular acidosis causes an increase in the size of the Ca^{2+} transients (Allen & Orchard 1983a; Orchard, 1987), an intracellular acidosis on initial exposure to hypoxia, which is reduced on subsequent exposures, could explain the results. Exposure to hypoxia is known to cause an intracellular acidosis due to lactic acid production by glycolysis (Matthews et al., 1981) which disappears when glycolysis is prevented (Allen et al., 1985). Therefore experiments were carried out to test whether changes in the rate of glycolysis and lactic acid production could account for the results obtained during repeated exposures to hypoxia.

If the pattern of response is dependent on the metabolic status of the tissue, then it should be possible to reverse the progression of events by manoeuvres which increase the rate of glycolysis, either by restoring the tissue glycogen or by increasing glucose influx. This

possibility was tested by raising the external glucose concentration by a factor of 5, from 10 mM to 50 mM. Figures 8C & 9C show that this intervention was capable of reversing both the fall in the Ca^{2+} transients and the greater decline in tension, so that the pattern of response after exposure to high glucose was similar to that seen in the first exposure to hypoxia. This was seen in two out of three preparations tested in which the Ca^{2+} transients initially rose. However, in two other preparations in which the Ca^{2+} transients were initially flat, increasing the external glucose concentration did not lead to an increase in the Ca^{2+} transients during hypoxia. Possible reasons for this are considered in Section 3.1.5.

3.1.3 Comparison of the calcium transients during hypoxia and during the application of lactic acid.

Figure 10 shows in more detail the effects of an initial exposure to hypoxia on the Ca^{2+} transients. Figure 10A shows continuous records of aequorin light and tension from a ferret papillary muscle. Figure 10B shows averaged records of Ca^{2+} transients and tension from the control period (i) and during hypoxia (ii), demonstrating the increase in the size of the Ca^{2+} transients at the time when the tension had fallen. In panels iii and iv, the same averaged records have been scaled to the same size and superimposed. It can be seen that hypoxia causes a prolongation of the Ca^{2+} transients, while simultaneously abbreviating the twitch, as previously noted by others (Allen & Orchard 1983b; MacKinnon et al 1987). In 8 experiments, the duration of the Ca^{2+} transient was increased by 10.4 $\pm$ 3.8% ($p < 0.01$), while the duration of the twitch was decreased by 8.6 $\pm$ 1.3% ($p < 0.001$). (Both the Ca^{2+} transient and twitch duration were measured as time to 50% decline from peak, measured from the stimulus). This is very similar to the effects of acidosis caused by CO_2 on the Ca^{2+} transients (Allen & Orchard, 1983a & b; Orchard, 1987), so it was of interest to see if

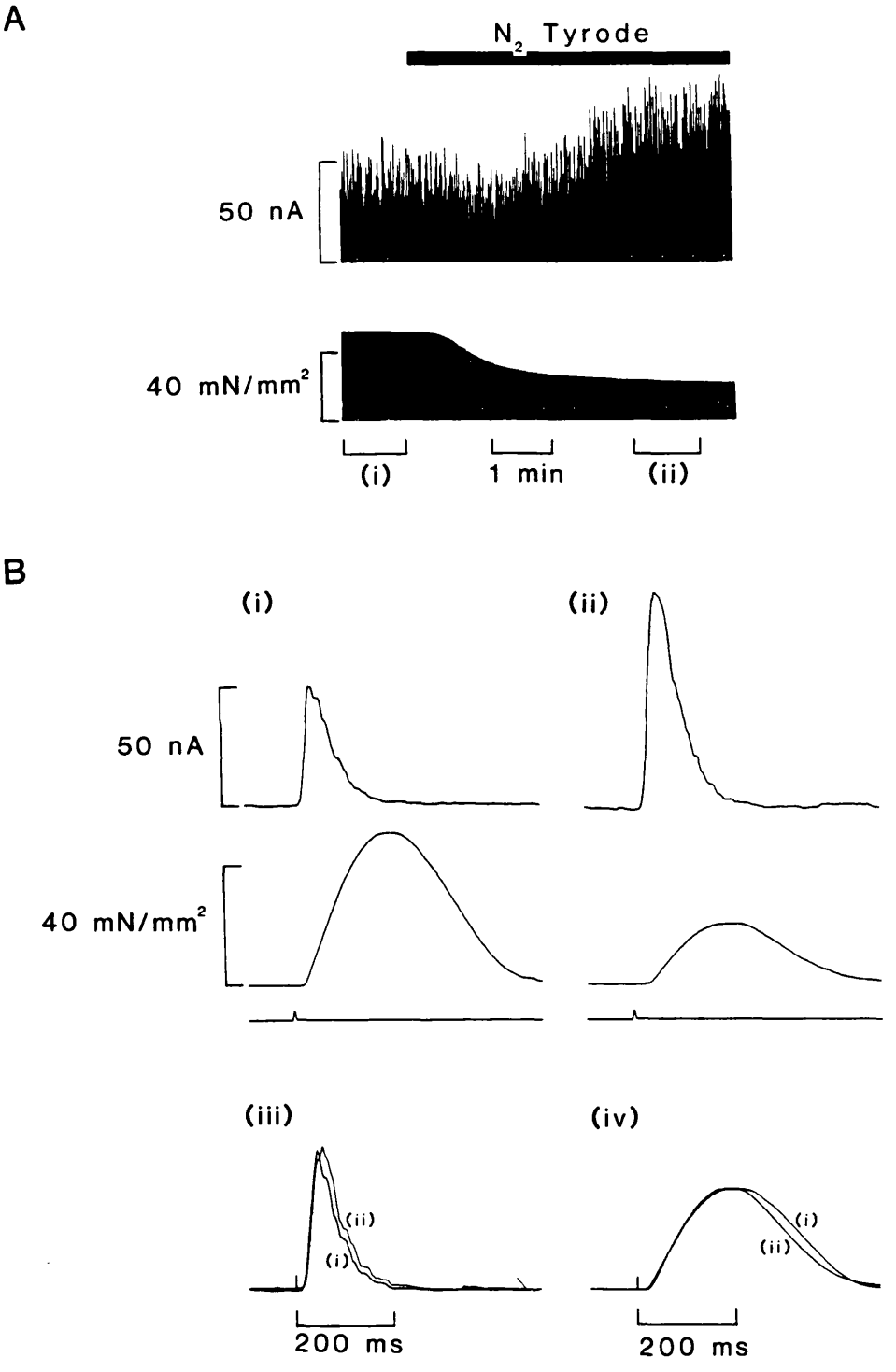
FIGURE 10.

FIGURE 10.

The effects of hypoxia on the Ca^{2+} transients and tension from an isolated ferret papillary muscle.

A. Original records of aequorin light (above) and tension (below) during exposure to hypoxia produced by switching to Tyrode solution equilibrated with 95% N_2/ 5% CO_2.

B. (i) and (ii) show averaged records of aequorin light (above) and tension (below) from the periods indicated in A. In (iii) and (iv) the light and tension records of (i) and (ii) have been scaled to the same size and superimposed. (iii) shows that hypoxia caused a prolongation of the Ca^{2+} transients, while (iv) demonstrates that the twitch was abbreviated by hypoxia.


A
N_2 Tyrode
50 nA
40 mN/mm²
(i)
1 min
(ii)
B
(i)
(ii)
50 nA
40 mN/mm²
(iii)
(iv)
(ii)
(i)
(i)
(ii)
200 ms
200 ms

lactic acid could mimic these effects.

Figure 11 shows an experiment designed to investigate this point. Panels (i) and (ii) show averaged records of Ca^{2+} transients and tension from the control period (i) and 2 min after the application of 10 mM lactic acid (ii). As in the experiments of Orchard (1987), exposure to lactic acid caused an early, transient increase in Ca^{2+} transients and decrease in tension, but this phase was complete before the results shown in (ii), which illustrates steady changes which persisted for several minutes. Panels (iii) & (iv) show that the effects of lactic acid on the timecourse of the Ca^{2+} transients and twitch were very similar to those seen in hypoxia. In 4 experiments, the duration of the Ca^{2+} transients increased by 21.8 $\pm$ 6.3% ($p < 0.05$), while the twitch duration showed an abbreviation of 6.0 $\pm$ 2.9%, which was not, however, statistically significant ($0.2 > p > 0.1$). A possible reason for the changes in the timecourse of the Ca^{2+} transients and tension in both hypoxia and during lactic acid application is that acidosis causes a reduction in the apparent binding constant of troponin for Ca^{2+} (Blanchard & Solaro, 1984). This allows Ca^{2+} to dissociate from troponin faster, leading to an abbreviated twitch, but a prolonged Ca^{2+} transient (see Blinks & Endoh 1986).

As noted above and shown by Orchard (1987), lactic acid produces changes in the Ca^{2+} transients and tension consisting of a transient component which is followed by a more stable response. This is because addition of lactic acid (10 mM) to the perfusate produces changes in extracellular pH with both a transient component (to pH 6.4) and a stable component (to pH 7.0). The transient component causes a temporary elevation of CO_2, which is lost from the solution within a few minutes, while the stable component is due to the replacement of HCO_3^- by lactate. The transiently elevated CO_2 enters cells rapidly and causes a transient intracellular acidosis. This is responsible for the

FIGURE 11.

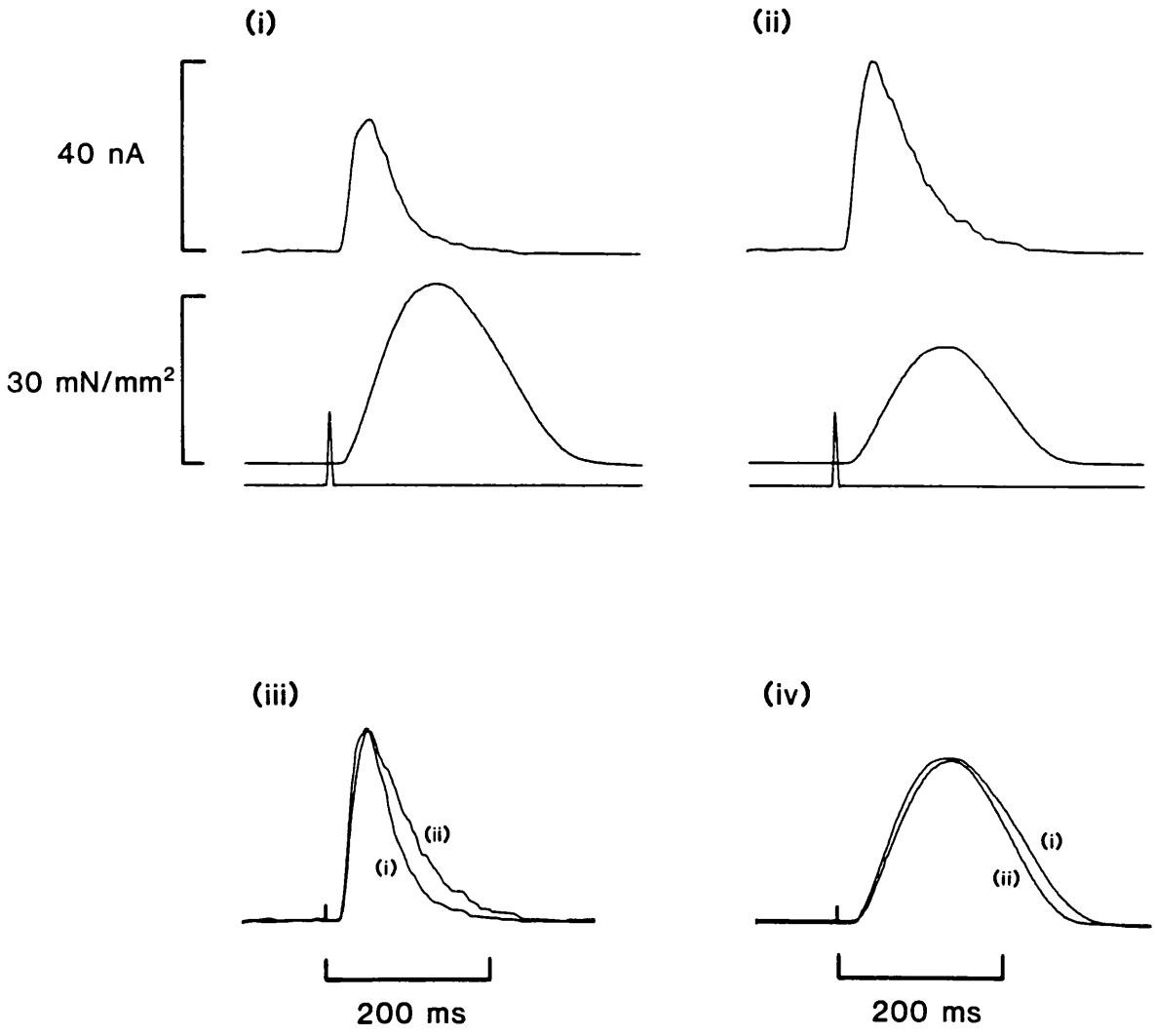
FIGURE 11.

The effects of 10 mM lactic acid on the Ca^{2+} transients and tension from an isolated ferret papillary muscle.

(i) Averaged records of aequorin light (above) and tension (below) during a control period.

(ii) Averaged records from the steady state response to 10 mM lactic acid.

(iii) and (iv) The Ca^{2+} transients and tension from (i) and (ii) have been scaled to the same size and superimposed. 10 mM lactic acid caused the Ca^{2+} transient to be prolonged (iii), while the twitch was abbreviated (iv).


(i)
(ii)
40 nA
30 mN/mm²
(iii)
(iv)
(ii)
(i)
(i)
(ii)
200 ms
200 ms

initial increase in the Ca^{2+} transients and reduction in tension. However, undissociated lactic acid also enters cells and causes a more slowly developing acidosis (de Hemptinne, Marannes & Vanheel, 1983), which is responsible for the more stable increase in the Ca^{2+} transients and decrease in tension. As a control, the effect of 10 mM Na lactate buffered to pH 7.4 before addition was tested. This produced little change in either the Ca^{2+} transients or tension, presumably because the associated intracellular acidosis due to lactate was so small under these conditions (see discussion in Section 3.1.5).

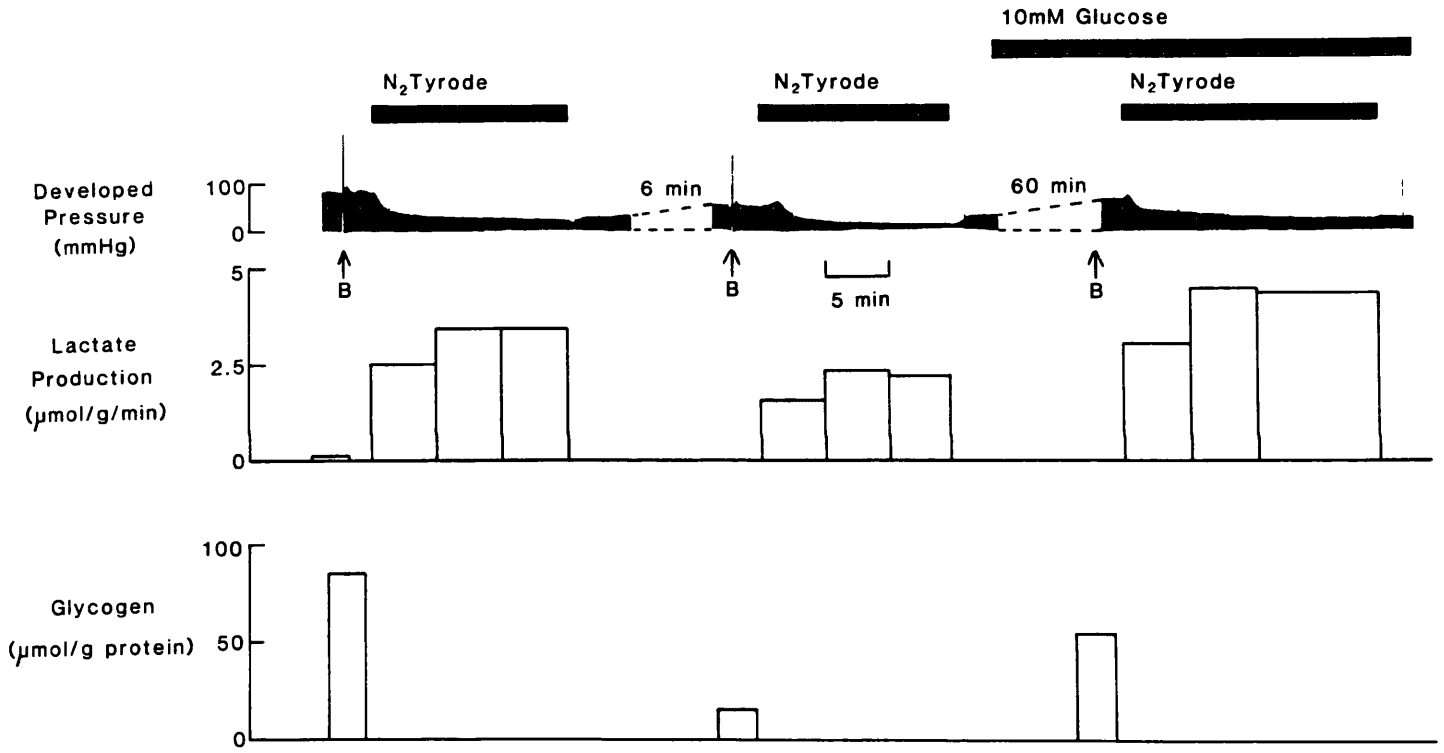
3.1.4 Metabolic measurements on Langendorff-perfused hearts.

In order to test further the hypothesis that glycogen stores play an important role in determining the response of cardiac muscle to a period of hypoxia, tissue glycogen and lactate production were measured during successive periods of hypoxia. These experiments were performed using a Langendorff-perfused heart preparation. In initial experiments, the solutions used were identical to those used in the papillary muscle experiments. However, it was found that under these conditions (glucose 10 mM, i.e. about 4 times the *in vivo* concentration) glycogen levels, lactate production, and the decline of tension during hypoxia were hardly affected by repeated exposures to hypoxia. In subsequent experiments either 2 or 5 mM glucose was used in the control solutions and, later in each experiment, this was increased 5 fold (to 10 or 25 mM) as in the papillary muscle experiments (see Section 3.1.5 for discussion of this point). Figure 12 shows the result of such an experiment with a Langendorff-perfused heart, in which glucose was initially 2 mM. hypoxia caused developed pressure to fall and lactate production increased greatly, reflecting the increased rate of glycolysis. On a subsequent exposure to hypoxia, however, both glycogen and lactate production had fallen, and the

FIGURE 12.

FIGURE 12.

The effects of repeated exposures to hypoxia on developed pressure, lactate production and tissue glycogen in a Langendorff-perfused ferret heart at 30°C, in Tyrode solution containing 2 mM glucose. Note that during the second exposure to hypoxia, developed pressure fell to a greater extent, lactate production was decreased and tissue glycogen is also decreased when compared to the first hypoxic period. Exposure to 10 mM glucose, coupled with reducing the stimulation frequency from 2 to 1 Hz for one hour, caused the subsequent hypoxic period to closely resemble the initial one. 'B' indicates the time at which a biopsy was taken.


10mM Glucose
N2Tyrode
N2Tyrode
N2Tyrode
Developed Pressure (mmHg)
100
0
6 min
60 min
B
B
5 min
B
5
Lactate Production (μmol/g/min)
2.5
0
100
Glycogen (μmol/g protein)
50
0

tension fell more rapidly to a lower value. The heart was then exposed to 10 mM glucose and a slower stimulation rate for 60 minutes. Following this treatment, it can be seen that the pressure record during hypoxia now closely resembles the initial response. In addition, the glycogen content of the muscle was restored, and lactate production had also increased. Similar results to these were observed in two other experiments with lactate measurements and one other experiment with glycogen measurements. In one experiment at 5 mM glucose, the lactate production averaged 3.4 μmol/g/min on the first exposure, 2.9 on the second, and 2.5 on the third. After one hour in 25 mM glucose the lactate production had increased to 3.5 μmol/g/min.

3.1.5 Interpretation of results.

The main finding of these experiments is that the variability in the response of the Ca^{2+} transients to hypoxia can be observed in a single preparation on repeated exposures to hypoxia. In addition, these changes could be reversed by elevated external glucose. It was also observed that both glycogen stores and lactate production fell on repeated exposures to hypoxia, and that both recovered when extracellular glucose was raised. These results led to the suggestion that the rise in Ca^{2+} transients might be associated with increased levels of intracellular lactic acid, due to the stimulation of glycolysis produced by hypoxia. This idea was supported by exposing aerobic papillary muscles to extracellular lactic acid, which increased the amplitude and duration of the Ca^{2+} transients in a similar fashion to hypoxia. Specifically, the hypothesis suggested by these results is that the intracellular acidosis produced by increased lactic acid production is the cause of the increased Ca^{2+} transients. Repeated or lengthy exposure to hypoxia leads to depletion of glycogen stores (Neely & Grotyohann, 1984); as a result, one contribution to glycolysis is reduced and this causes a smaller lactate production, a smaller

acidosis and a smaller increase in the Ca^{2+} transients during hypoxia. Eventually, the glycogen is exhausted, and in these circumstances exposure to hypoxia causes the action potential to fail rapidly (Allen et al., 1987; Hayashi et al., 1987; Lederer et al. 1989), leading to a decrease in the size of the Ca^{2+} transients. Aspects of this hypothesis are discussed in the following Sections.

3.1.5.1 The effects of hypoxia and acidosis on the calcium transients

The hypothesis outlined above suggests that the increase in the amplitude and duration of the Ca^{2+} transients during hypoxia is caused by intracellular acidosis associated with lactic acid production. Several pieces of evidence support this view:

(i) The similarity between the effects of hypoxia and extracellular lactic acid (Figures 10 & 11).

(ii) There is known to be an accumulation of lactic acid and an acidosis in myocardial cells during hypoxia. Thus intracellular lactate concentration has been shown to increase to 5-10 mM during hypoxia (Rovetto, Whitmer & Neely, 1973) and an intracellular acidosis of 0.15 - 0.3 pH units occurs at the same time (Matthews et al., 1981; Allen et al., 1985; Ellis & Noireaud, 1987), most of which can be accounted for by the lactate accumulation.

(iii). The parallelism between declining Ca^{2+} transients and lactate production and their reversal by increased glucose.

One point which requires further consideration is whether addition of 10 mM lactic acid extracellularly leads to an intracellular acidosis which is comparable in magnitude to that which occurs during hypoxia. Intracellular pH (pH_i) was not measured in these experiments, but it is possible to calculate pH_i and also the intracellular lactate concentration using the equation given by Szatkowski & Thomas (1986). Taking extracellular lactic acid as 10 mM, intracellular buffering as

60 mM/pH unit, pK of lactate as 3.7 and using the measured value of pH_o (7.0), the calculated decrease in pH_i is 0.13 and intracellular lactate is 8 mM. This value for the decrease of pH_i fits well with the direct measurement of a decrease in pH_i of 0.15 on the addition of 10 mM sodium lactate at pH_o of 6.8 by de Hemptinne et al. (1983). Thus, the addition of 10 mM lactic acid, which mimicked the Ca^{2+} transients in hypoxia very closely, does seem to produce an intracellular acidosis and lactate concentration similar to those which have been measured in hypoxia (quoted above). If the calculation is performed for the addition of 10 mM Na lactate buffered to pH 7.4 (i.e. pH_o remains constant at 7.4 during the addition), the decrease in pH_i is found to be only 0.06. Hence it is not surprising that so little effect was observed when buffered lactic acid was added.

3.1.5.2 Mechanism of the increase in the calcium transients.

A likely mechanism for the increase in the Ca^{2+} transients is based on the competition of H^+ for Ca^{2+} binding sites within the cell. This would be expected to raise the resting intracellular $[Ca^{2+}]$, hence increasing loading of the sarcoplasmic reticulum and the size of the Ca^{2+} transients. The similarity of the response of the Ca^{2+} transients to hypoxia and the addition of lactic acid supports this hypothesis.

It has been observed that ischaemia causes an early increase in cAMP (Podzuweit, Dalby, Cherry & Opie, 1978), which might be expected to cause increased Ca^{2+} transients due to phosphorylation of both Ca^{2+} channels and phospholamban in the sarcoplasmic reticulum. However, this seems unlikely to be the cause of the increased Ca^{2+} transients in these experiments, because the transients were prolonged, whereas cAMP-dependent effects lead to shortening of the transients (e.g. Allen & Kurihara, 1980). Also this hypothesis does not explain the similarity of the response to hypoxia and lactic acid, unless acidosis is postulated to cause an increase in cAMP.

Another potential mechanism to explain the increased Ca^{2+} transients would be the operation of the Na^+/H^+ exchange in parallel with the Na^+/Ca^{2+} exchange. In hypoxia, increased intracellular lactic acid production would load to Na^+ influx on the Na^+/H^+, and this in turn would cause Ca^{2+} entry on the Na^+/Ca^{2+} exchange, and hence increase sarcoplasmic reticulum loading and the size of the Ca^{2+} transients. However, this hypothesis fails to explain the response to externally applied lactic acid. In this instance there would be an increased proton gradient into the cells, because for a given change in extracellular pH, intracellular pH changes only about a fifth as much (Ellis & Thomas, 1976). This would cause the Na^+/H^+ exchanger to work in the opposite direction to its action in hypoxia, yet the effects of external lactic acid on the Ca^{2+} transients are very similar to those seen in hypoxia.

3.1.5.3 Accumulation of lactic acid within cells.

One question that arises in relation to the hypothesis described at the beginning of this Section is why do all preparations not show an increase in the Ca^{2+} transients on exposure to hypoxia? A likely reason is that in these preparations glycogen levels are depleted even before the first exposure to hypoxia. Glycogen is presumably depleted to some extent during the period of ischaemia when the heart is removed from the animal. In most preparations, glycogen levels presumably recover during the period of aequorin injection when the muscle is quiescent and the external glucose is relatively high (10 mM). However, some variation in glycogen levels may arise because of differences both in the duration of the initial period of ischaemia and in the duration of the period of quiescence required to inject the preparation. An additional factor which may affect glycogen levels is the diameter of the papillary muscle. Large diameter papillary muscles

tend to have anoxic cores (Blinks & Koch-Weser, 1963) and this leads to increased glycolysis and possible glycogen depletion in the core of the muscle. Presumably the surface cells (which are the aequorin-injected ones) would not be susceptible to this problem. During hypoxia, however, the central, glycogen-depleted cells may become ATP depleted very early, and the consequent action potential shortening could affect all cells in the preparation because of electrical coupling.

One prediction of the hypothesis that lactic acid accumulation inside cells is important in the changes observed in the Ca^{2+} transients during hypoxia, is that in conditions where lactate efflux from the cell is greatly restricted, a consistent and larger increase in the Ca^{2+} transients should be seen. Exactly this situation arises when cardiac muscle is made ischaemic. Experiments describing the effects of ischaemia are described in Section 3.2. However, it may be noted here that, in agreement with the prediction mentioned above, the Ca^{2+} transients in ischaemia always showed a large increase and this was associated with a marked prolongation, as would be expected if a large accumulation of lactate was occurring.

In contrast to the results described above, MacKinnon et al (1987) always observed a decrease in the Ca^{2+} transients during hypoxia. This may be because the rate of glycolysis was reduced in their preparations. They used a "chemical" loading technique to introduce aequorin, which involves exposing the muscle to very low Ca^{2+} concentrations to permeabilise the membrane. It is possible that this damages the glycolytic capability of their muscles, or depletes the glycogen reserves. In the present experiments, it was found that after several exposures to hypoxia, the fall of the Ca^{2+} transients and tension, when compared to an early exposure, do seem to be correlated. Thus, in this situation, when the tissue glycogen is depleted, it seems that the decrease in the amplitude of the Ca^{2+} transients does make a

contribution to the decrease in tension, i.e. a situation more like metabolic blockade begins to develop.

3.1.5.4 Measurements of glycogen and lactate production

The experiments on intact hearts did show reductions in lactate production and glycogen levels on repeated exposures to hypoxia, but only when the glucose level had been reduced from the 10 mM concentration used in the papillary muscle experiments. At these reduced glucose levels, the pressure record from an intact heart was very similar to the tension record of a papillary muscle at the higher glucose. What is the reason for this apparent difference in the behaviour of the intact heart compared to the papillary muscle? A possible explanation is that in the intact heart, perfusion is via the capillary beds, and the diffusion distance to the cells is probably less than 5 /um. However, in the superfused papillary muscle, the diffusion distance to the centre of the muscle is between 250 and 500 /um. Thus, at the same concentration of glucose, the cells in the intact heart are all exposed to close to 10 mM glucose, whereas in a papillary muscle there will be a gradient of glucose across the preparation, with lower levels in the centre due to consumption by the outermost cells. An indication of the magnitude of this problem can be obtained by calculating the critical diameter of a papillary muscle above which the concentration of glucose in the centre is zero. This can be done by using the following equation (Blinks & Koch-Weser, 1963):

$$d_c = 4\sqrt{\frac{C\,D'}{A}}$$

where d_c is the critical diameter (mm), C is the concentration of glucose outside the cell (mmol/l), D' is the apparent diffusion coefficient of glucose through the extracellular space (mm^2/s) and A is

the rate of glucose consumption (mmol/l/s), which in this calculation is assumed independent of the concentration of glucose. D' for glucose is 1.5 x 10^{-4} mm^2s^{-1}, which is the apparent diffusion coefficient for sucrose through the extracellular space of ventricular muscle (Safford, Bassingthwaite & Bassingthwaite, 1978). For A the value of lactate production during a second exposure to hypoxia was used. In this situation, glycogen is depleted so that most lactate comes from glucose influx. This value (2.5 /umol/g/min) was divided by 2 (since 1 mole of glucose produces 2 mole of lactate) and converted to mmol/l/s, giving a value of 0.020 mmol/l/s. With C = 10 mM, a value of d_c = 1.1 mm is obtained, i.e. preparations with a diameter greater than 1.1 mm would have zero concentration of glucose at the core during a period of hypoxia. Although this calculation assumes that glucose consumption continues unchanged until the concentration of glucose reaches zero, it does show, as expected, that in papillary muscles of the diameter used for these experiments (mean 0.65 mm), the glucose concentration at the centre would have been substantially reduced. Thus with an external glucose of 10 mM, the spatially-averaged extracellular glucose across the preparation will be somewhat lower. This presumably explains why in the intact heart the response with a lower level of extracellular glucose matches that observed with a higher level in the papillary muscle experiments.

3.1.5.5 Conclusions.

These experiments show that the response of the intracellular Ca^{2+} transients in cardiac muscle is dependent on the metabolic status of the muscle. In muscles with high levels of glycogen and an adequate rate of glycolysis, an intracellular acidosis occurs on exposure to hypoxia, and the Ca^{2+} transients increase. However, as glycogen levels become depleted and the rate of glycolysis is reduced to that which can

be achieved by glucose influx, the acidosis diminishes, and the increase in Ca^{2+} transients disappears. Finally, when the muscle is completely depleted, exposure to hypoxia causes failure of the action potential, and the Ca^{2+} transients decrease. Since it is known that increases in intracellular Ca^{2+} can activate various currents (see Clusin, 1988), these differences in response might have clinical relevance in relation to arrhythmogenesis in hearts with regional differences in metabolic status and energy supply.

3.2 THE EFFECTS OF ISCHAEMIA.

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3.2.1 Background.

When heart muscle is made ischaemic, there is a rapid decline in contractile performance, a reduction in the action potential duration and amplitude, and arrhythmias often occur (for reviews see Janse & Kleber, 1981; Reimer & Jennings, 1986; Allen & Orchard, 1987). Changes in intracellular free [Ca^{2+}] have long been suspected to occur and to contribute to these alterations in function (e.g. Nayler, Poole-Wilson & Williams, 1979; Clusin, Buchbinder, Bristow & Harrison, 1984).

In the past measurements of intracellular [Ca^{2+}] in ischaemia have been difficult to perform and a variety of related conditions which mimic some aspects of ischaemia have been studied e.g. hypoxia, inhibition of oxidative phosphorylation with cyanide and metabolic blockade (prevention of both oxidative phosphorylation and anaerobic glycolysis) (Dahl & Isenberg, 1980; Allen & Orchard, 1983b; Cobbold & Bourne, 1984). However, in these experimental models perfusate flow continues, so that the consequences of accumulation of ions and products of metabolism, such as K^+, CO_2 and lactate, are not usually observed. Recently, a variety of techniques have been developed which are capable of measuring intracellular [Ca^{2+}] during ischaemia. Reports of the use of F-BAPTA as a Ca^{2+} indicator have appeared (Steenbergen, Murphy, Levy & London, 1987; Marban, Kitakaze, Kusuoka, Porterfield, Yue & Chacko, 1987), but this method suffers from low time resolution (5 minutes per measurement) and studies so far have failed to distinguish between systolic and diastolic [Ca^{2+}]. More recently, the fluorescent indicator indo has been introduced into whole hearts using the AM ester loading technique (Lee, Smith, Mohabir & Clusin, 1987), and has been used to study the effects of ischaemia on intracellular [Ca^{2+}] (Lee, Mohabir, Smith, Franz & Clusin, 1988).

In the experiments described in this section, ischaemia was

simulated in isolated papillary muscles and aequorin was used to monitor the systolic and diastolic intracellular [Ca^{2+}]. Ischaemia was simulated by superfusing a papillary muscle with water-saturated 95% N_2/5% CO_2 (N_2 gas perfusion), as described in Chapter 2. Under these conditions the muscle has only a thin film of Tyrode solution adhering to its surface, so the extracellular space is restricted and non-volatile products of metabolism accumulate.

3.2.2 The effects of ischaemia on developed tension and the amplitude of the calcium transients.

Figure 13A shows the result of perfusing an aequorin-injected ferret papillary muscle with water-saturated 95% N_2/5% CO_2. The irregularities in the tension record indicate when gas reached the chamber, and were due to surface tension effects caused by the temporary formation of bubbles as the fluid surrounding the muscle was removed. Contractions were occasionally missed as the stimulus was readjusted to a level close to the threshold. After this early phase, further adjustments to the stimulus strength were not generally necessary. In the experiment of Figure 13A, developed tension declined to about 15% of control after 10 minutes of ischaemia. In most muscles, the tension decline was even more pronounced than this (see Figures 15A, 16A & 17A). In 10 preparations, developed tension declined to 10 $\pm$ 1 % of control after 10 minutes and to 2 $\pm$ 0.5 % after 20 minutes of ischaemia. For comparison, in Langendorff-perfused ferret hearts which were made globally ischaemic (30^{o}C, 1-2 Hz stimulation rate), developed pressure fell to within a few per cent of zero after 5 minutes and was undetectable after 10 minutes (Elliott, 1987 & personal observations).

The Ca^{2+} transients in Figure 13A showed a small reduction over the first 5 minutes of ischaemia. This effect was not seen in all

preparations, e.g. Figures 15A & 16A, but on average the Ca^{2+} transients had decreased to 79 ± 9 % of control (n = 10) after 5 minutes of ischaemia. However, the most striking finding was a slowly developing, large increase in the amplitude of the Ca^{2+} transients. In the experiment of Figure 13A the Ca^{2+} transients had increased to 400% of control after 15 minutes. On average, the Ca^{2+} transients reached a maximum after 25 minutes of ischaemia (range 20 - 35 minutes) which was 350 ± 60 % of control. Assuming that the aequorin light signal was underestimated by a factor of 2-3 (see section 2.8), the true increase in the peak amplitude of the Ca^{2+} transients may have been by a factor of 7-11. Since aequorin light is proportional to $[Ca^{2+}]^{2.5}$ over this range (Allen, Blinks & Prendergast, 1977), this represents a 2.5-3 fold increase in systolic intracellular $[Ca^{2+}]$.

In the experiment shown in Figure 13A, O_2 Tyrode perfusion was restarted after ~20 minutes of ischaemia and tension recovered to about 80% of control after 5 minutes. The Ca^{2+} transients characteristically showed a short-lived increase when O_2 Tyrode perfusion restarted, but then declined to a level which was below control. In this preparation the Ca^{2+} transients fell to about 25% of the control level, but later recovered somewhat. In 8 experiments, involving exposures to ischaemia of 20 minutes or more, the Ca^{2+} transients during subsequent O_2 Tyrode reperfusion eventually recovered to 56 ± 7 % of the control value. As noted in section 2.8, a large fraction of this decline is simply a consequence of aequorin consumption.

If the accumulation of products of metabolism has functional consequences in ischaemia, then the developed tension and the Ca^{2+} transients should be affected differently in N_2 gas perfused muscles (i.e. ischaemia) and N_2 Tyrode perfused muscles (i.e. hypoxia). To examine this question directly, the responses to both these manoeuvres

FIGURE 13.

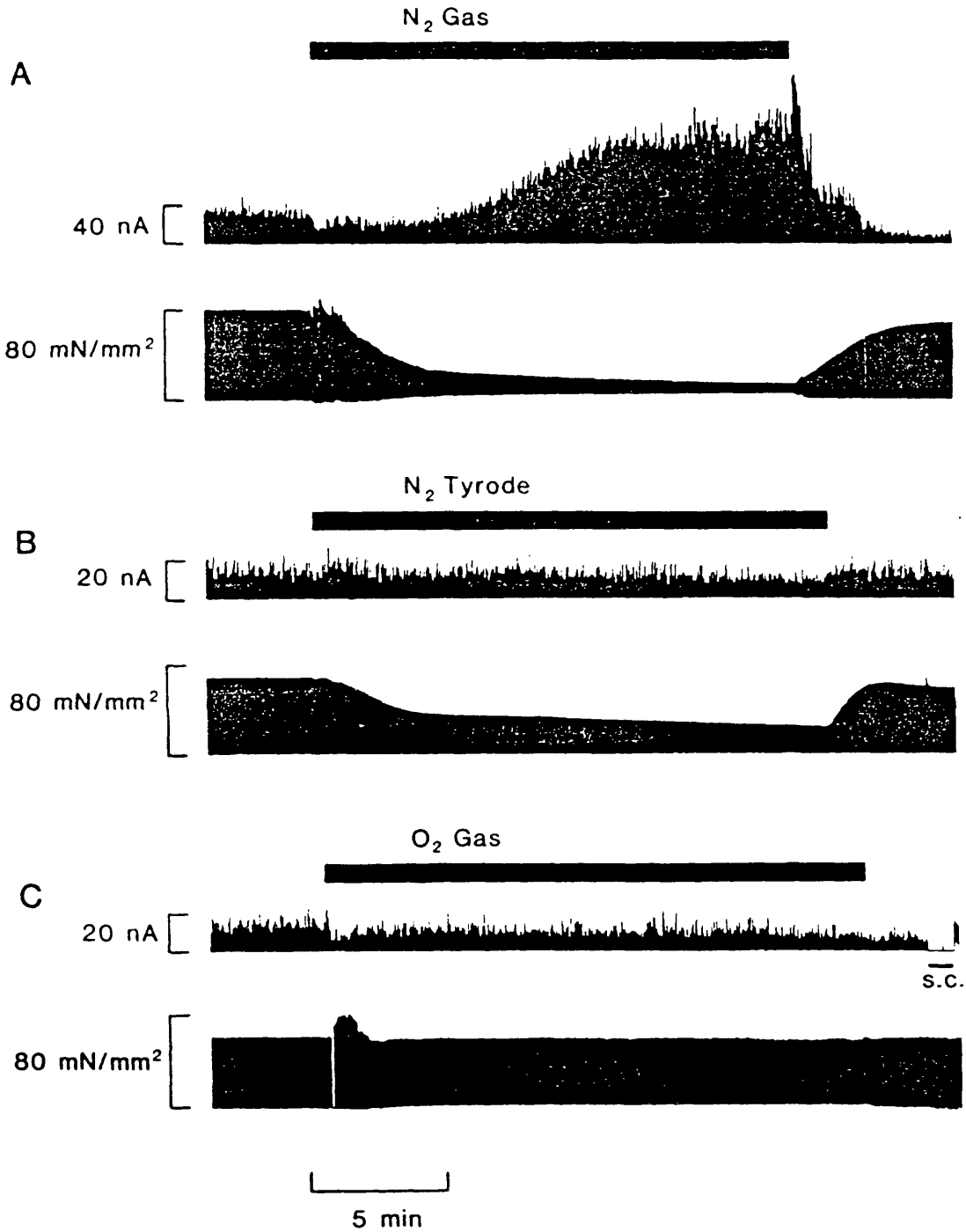
FIGURE 13.

The effects of ischaemia, hypoxia and O_2 gas perfusion on aequorin light and tension from a ferret papillary muscle. All panels are from the same preparation. Each panel shows aequorin light (above) and tension (below).

A. Ischaemia was simulated by switching from superfusion with Tyrode solution to gas perfusion with water-saturated 95% N_2/ 5% CO_2. Recovery was initiated by switching back to Tyrode solution.

B. 40 minutes after A. Hypoxia was produced by superfusing with Tyrode solution equilibrated with 95% N_2/ 5% CO_2.

C. 25 minutes after B. Gas perfusion with 95% O_2/ 5% CO_2 had no deleterious effects on the muscle. sc indicates shutter on photomultiplier closed.


A
N_2 Gas
40 nA
80 mN/mm²
B
N_2 Tyrode
20 nA
80 mN/mm²
C
O_2 Gas
20 nA
S.C.
80 mN/mm²
5 min

were compared in 6 papillary muscles (compare Figures 13A & B). On average, the developed tension fell to a lesser extent during hypoxia than during ischaemia. Developed tension fell to 32 $\pm$ 4 % control after 10 minutes of hypoxia, while it fell to 8.5 $\pm$ 2.5 % when the same muscles were exposed to ischaemia for the same time. The changes in the amplitude of the Ca^{2+} transients were also different in the two groups. As described in Section 3.1, in hypoxic papillary muscles, the Ca^{2+} transients sometimes showed a small increase, especially on the first exposure. On average, the amplitude of the Ca^{2+} transients after 20 minutes of hypoxia in this set of experiments was 105 $\pm$ 25 % of control (n = 4), which was significantly smaller than when the same muscles were exposed to ischaemia (275 $\pm$ 52 %).

In these experiments, where ischaemia was simulated with N_2 gas perfusion, the changes in tension had a slower timecourse, but were otherwise similar to the changes in developed pressure seen in a globally ischaemic heart, suggesting that N_2 gas perfusion provides a reasonable simulation of the effects of ischaemia. On the other hand, such large increases in light transients have not been observed previously in hypoxia and one possibility which needed to be excluded was that they might be an artefact caused by some non-specific consequence of gas perfusion. However, it is known that Langendorff-perfused hearts can be perfused with water saturated 95% O_2/5% CO_2 (O_2 gas) and continue to contract for many hours; in fact, the pressure development of hearts perfused with O_2 gas was better maintained than a conventionally Tyrode-perfused heart (Gabel, Bihler & Dresel, 1966). As a control for the effects of gas perfusion, preparations were perfused with water saturated O_2 gas (Figure 13C). There was an initial transient increase in tension, which is probably due to an alkalosis associated with the fall of CO_2 as air entered the chamber when the Tyrode solution was sucked away. Thereafter the developed

tension was constant for 20 minutes. The Ca^{2+} transients showed an initial short-lived reduction, but were then well maintained for the period of gas perfusion. The amplitude of the Ca^{2+} transients was smaller after recovery in the preparation illustrated, but this was not observed in other experiments of this type.

In order to test the hypothesis that the initial transient increase in tension was due to an alkalosis, three experiments with HEPES buffered solutions were carried out. In these experiments, because buffering was achieved with HEPES, the initial change in CO_2 partial pressure on switching to gas perfusion (now with 100% N_2 or O_2) was negligible. The transient increase in tension did not occur under these conditions, strengthening the interpretation mentioned above, that it is due to a transient alkalosis when the usual CO_2 buffered solutions were used.

3.2.3 The effects of ischaemia on the timecourse of the twitches and calcium transients.

The changes in the timecourse of the twitch and the Ca^{2+} transient are illustrated in Figure 14. In this figure the amplitude of the twitches and Ca^{2+} transients have been normalised, so that changes in the timecourse are clearer. Figure 14A shows the effects of O_2 gas perfusion, which had no significant effect on the timecourse of the Ca^{2+} transient and produced small changes in the timecourse of the twitch, possibly associated with the mechanical effects of gas perfusion. Exposure to hypoxia (N_2 Tyrode; Figure 14B) led to a small prolongation of the Ca^{2+} transient, which was associated with a small abbreviation of the twitch. As noted in section 3.1, these changes are consistent with a reduction in the affinity of troponin C for Ca^{2+} caused by intracellular acidosis. Similar but more pronounced changes in timecourse were seen in ischaemia (N_2 gas perfusion; Figure 14C), which is consistent with the much larger acidosis known to occur in

FIGURE 14.

FIGURE 14.

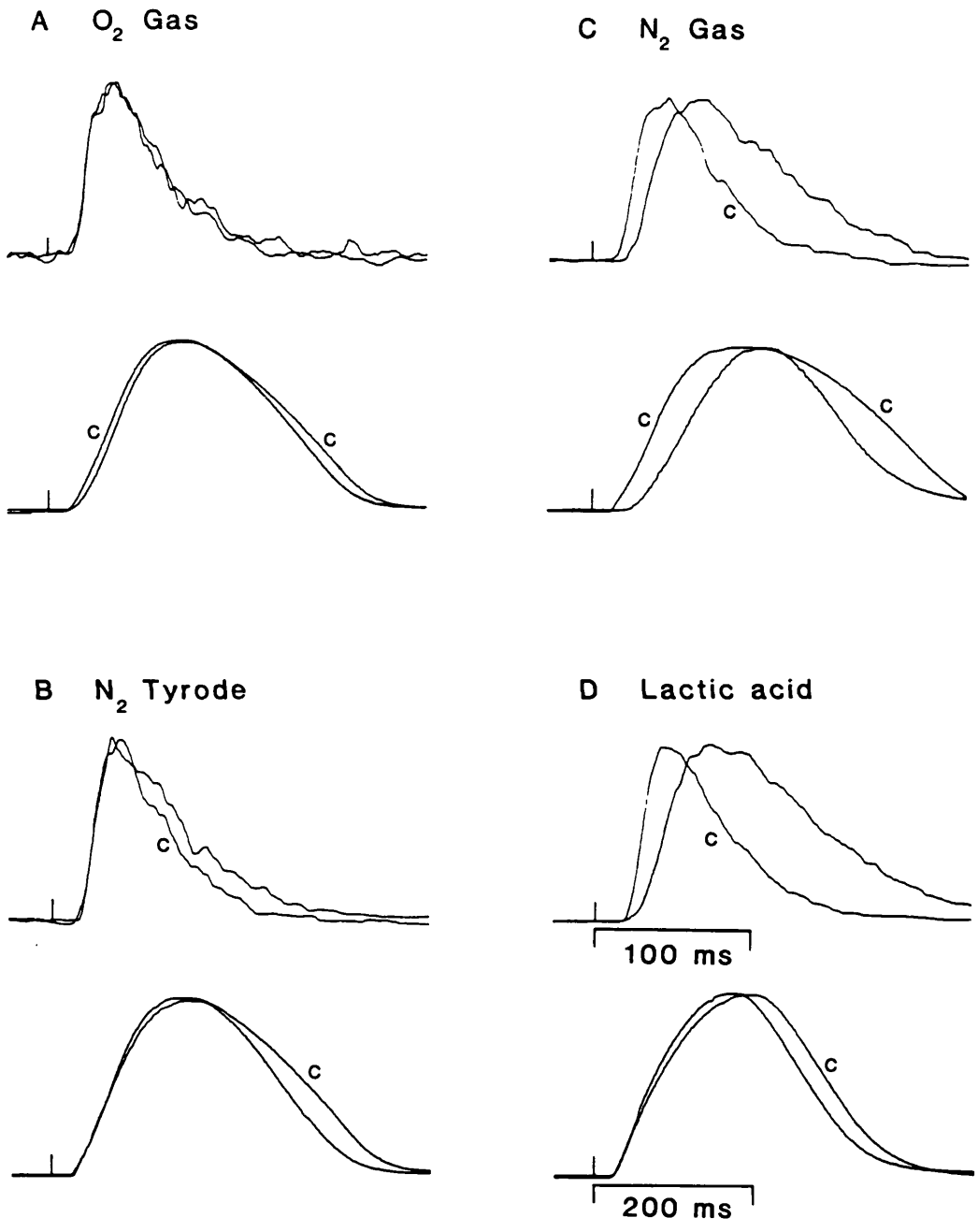
Effects of various interventions on the timecourse of the Ca^{2+} transient and twitch. A, B, and C are from the experiment shown in Figure 13 and were averaged between 10 and 15 minutes after the beginning of the intervention, when the Ca^{2+} transients were stable. D is a similar average taken from another preparation during a period when lactic acid exerted a stable effect. Each panel shows averaged records of aequorin light (above) and tension (below) from a control period and during the intervention. Records have been scaled to the same size and superimposed.

A. Gas perfusion with 95% O_2/ 5% CO_2.

B. Superfusion with Tyrode solution equilibrated with 95% N_2/ 5% CO_2.

C. Gas perfusion with 95% N_2/ 5% CO_2.

D. Superfusion with standard, oxygenated Tyrode solution to which 20 mM lactic acid had been added.


A O_2 Gas
C N_2 Gas
B N_2 Tyrode
D Lactic acid
C
100 ms
200 ms

ischaemia as compared to hypoxia (Bailey, Williams, Radda & Gadian, 1981). In addition, both the Ca^{2+} transient and the twitch showed an increased delay after the stimulus.

Figure 14D shows the effects on the timecourse of 20 mM lactic acid added to O_2 Tyrode (see section 3.2.5). This again prolonged the Ca^{2+} transient but reduced the duration of the twitch.

3.2.4 The effects of longer exposures to ischaemia.

In Figure 15A a 45 minute exposure to ischaemia is illustrated. After about 30 minutes resting tension started to increase slowly. The Ca^{2+} transients reached their maximum at about the same time and after a further 10 minutes started to decline. At about the time when the Ca^{2+} transients had become very small, ischaemia (N_2 gas perfusion) was replaced by O_2 gas perfusion. This has the effect of allowing oxidative metabolism to restart without washing away extracellular metabolites. Resting tension declined rapidly and small twitches reappeared within 20-30 seconds. After 1 hour of O_2 gas perfusion, tension had recovered to 60% of control. Note that the Ca^{2+} transients recovered within 20 - 30 seconds to their previous maximum and then slowly declined (the short break in developed tension and Ca^{2+} transients was caused by a change in stimulus threshold).

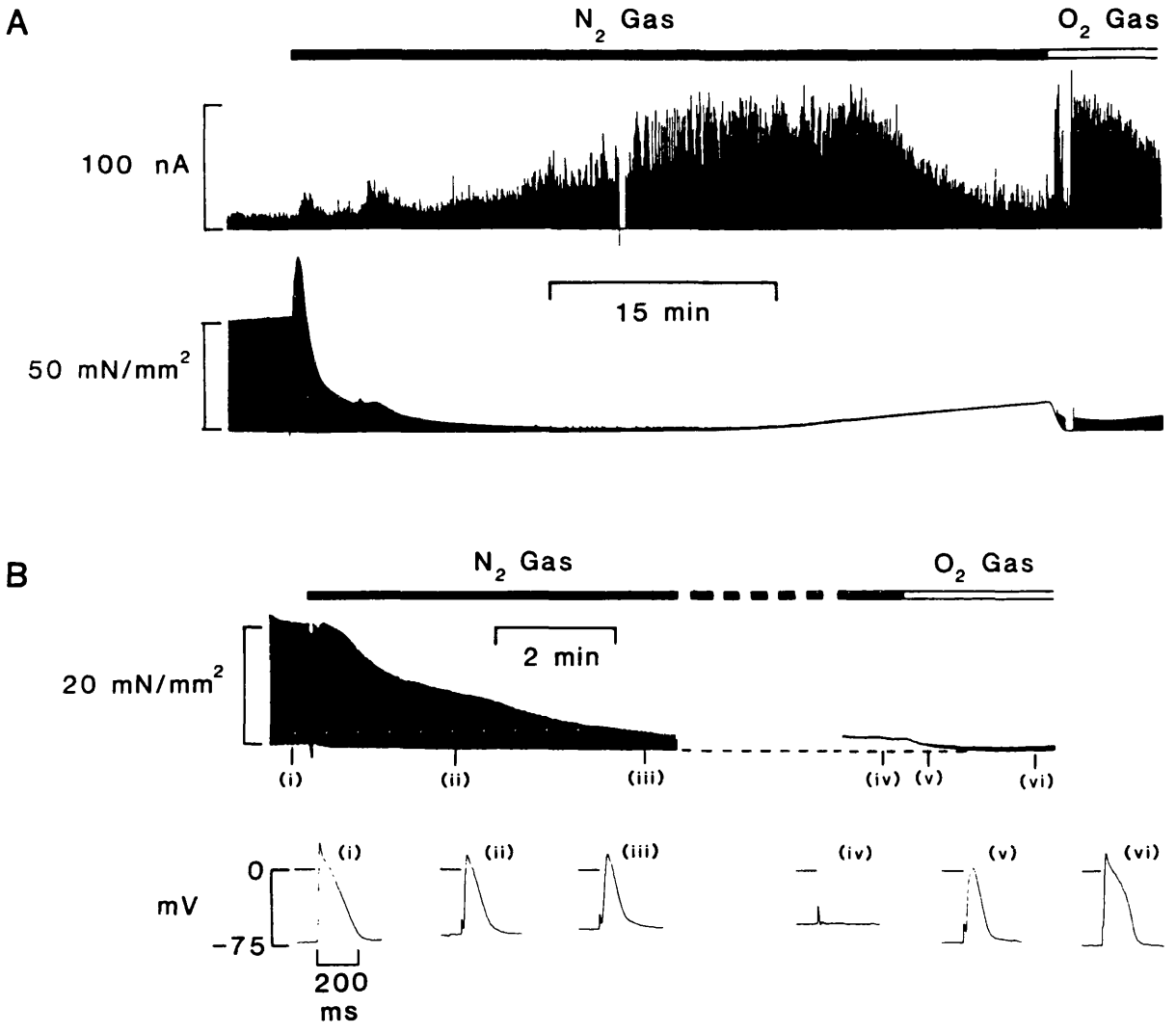
Several factors suggested that the late decline in the Ca^{2+} transients during exposure to ischaemia was because of failure of action potentials, rather than a reversal of the process which had caused the initial increase in the Ca^{2+} transients: (i) In most experiments, after the Ca^{2+} transients had shown a moderate decline, they could be made to recover either by a small increase in the stimulus strength or by introducing a brief rest. It is known that either of these procedures leads to a larger action potential (e.g.

FIGURE 15.

FIGURE 15.

A. Long exposure to N_2 gas perfusion. Record shows aequorin light (above) and tension (below). When the Ca^{2+} transients had become very small, recovery was initiated by switching to O_2 gas perfusion.

B. Effects of N_2 gas perfusion on tension and membrane potential. Different preparation to A. When rigor tension had developed and the action potential had disappeared, recovery was initiated by switching to O_2 gas perfusion.


A
N_2 Gas
O_2 Gas
100 nA
15 min
50 mN/mm^2
B
N_2 Gas
O_2 Gas
20 mN/mm^2
2 min
(i)
(ii)
(iii)
(iv)
(v)
(vi)
mV
0
-75
200 ms

Downar, Janse & Durrer, 1977). (ii) Although the Ca^{2+} transients declined, the resting light (see section 3.2.7) remained elevated.

To examine this further, in some experiments the membrane potential was measured during exposures to ischaemia. The results of such an experiment are shown in Figure 15B. The arrangement of the apparatus did not allow aequorin light and membrane potential to be measured simultaneously, so this result is from a different preparation to that shown in Figure 15A. Panels (i), (ii), and (iii) of Figure 15B show that the resting potential depolarised by 10-20 mV during the first few minutes of ischaemia and this was accompanied by a substantial reduction in action potential duration, by a slowing of the rate of rise of the action potential and by an increase in the delay between the stimulus and the start of the action potential. These findings are similar to many earlier studies of the changes in the action potential during ischaemia (e.g. Downar et al., 1977). After 14-16 minutes, an increase in resting tension became apparent and after 18 minutes of ischaemia, action potentials could no longer be recorded. The record in Figure 15B restarts at the 19th minute when there was clearly detectable resting tension, the membrane potential remained depolarised and there was no action potential following the stimulus artefact (Panel iv). At 20 minutes, O_2 gas perfusion was started and resting tension fell over 60 seconds and the membrane potential repolarised by 20 mV over 30 seconds. Within 30 seconds a substantial action potential was recorded (Panel v) and within 1-2 minutes the action potentials had regained their normal shape and amplitude (Panel vi). This experiment therefore confirms that the action potentials fail at about the time when resting tension appears (and when the Ca^{2+} transients decline) and that they recover very rapidly with O_2 gas perfusion. Possible mechanisms for repolarization and the prolongation of the action potential are considered in the section 3.2.10.

3.2.5 The cause of the increase in amplitude and duration of the calcium transients during ischaemia.

Previous studies have shown that there is no increase in total cell Ca^{2+} during the early part of ischaemia (Shen & Jennings, 1972), suggesting that the increase in systolic intracellular [Ca^{2+}] is not due to changes in total cell Ca^{2+}. NMR studies on Langendorff-perfused ferret hearts (Elliott, 1987) have shown that during global ischaemia, the intracellular ATP concentration fell to 60% of its normal level after 45 minutes. Thus when the Ca^{2+} transients are at their maximal amplitude at around 25 minutes, the various ATP-dependent ionic pumps should still be effective. One likely possibility to explain the large increase in the Ca^{2+} transients is that it is due to the intracellular acidosis which occurs in ischaemia (Bailey et al., 1981; Elliott, 1987). It has been shown that intracellular acidosis in ventricular muscle increases the amplitude and duration of the Ca^{2+} transients (Allen & Orchard, 1983a; Orchard, 1987; see Section 3.1). In these experiments therefore an attempt was made to mimic the changes in pH which occur during ischaemia. This was done by addition of lactic acid to the Tyrode solution, which causes both an extracellular and an intracellular acidosis (de Hemptinne, Marrannes & Vanheel, 1983). In preliminary experiments, it was found that 20 mM lactic acid increased the Ca^{2+} transients to a similar extent to N_2 gas perfusion. An experiment in which the effects of ischaemia were compared to those of 20 mM lactic acid is illustrated in Figure 16. Figure 16A shows an exposure to ischaemia. Once the Ca^{2+} transients had reached their maximum, stimulation was stopped for 2 minutes in order to measure the resting light (see section 3.2.7). Note that when O_2 Tyrode perfusion was restarted, the Ca^{2+} transients showed a short-lived increase.

Figure 16B shows the effects of addition of 20 mM lactic acid directly to O_2 Tyrode perfusing the muscle. pH_o fell to about ~5.2,

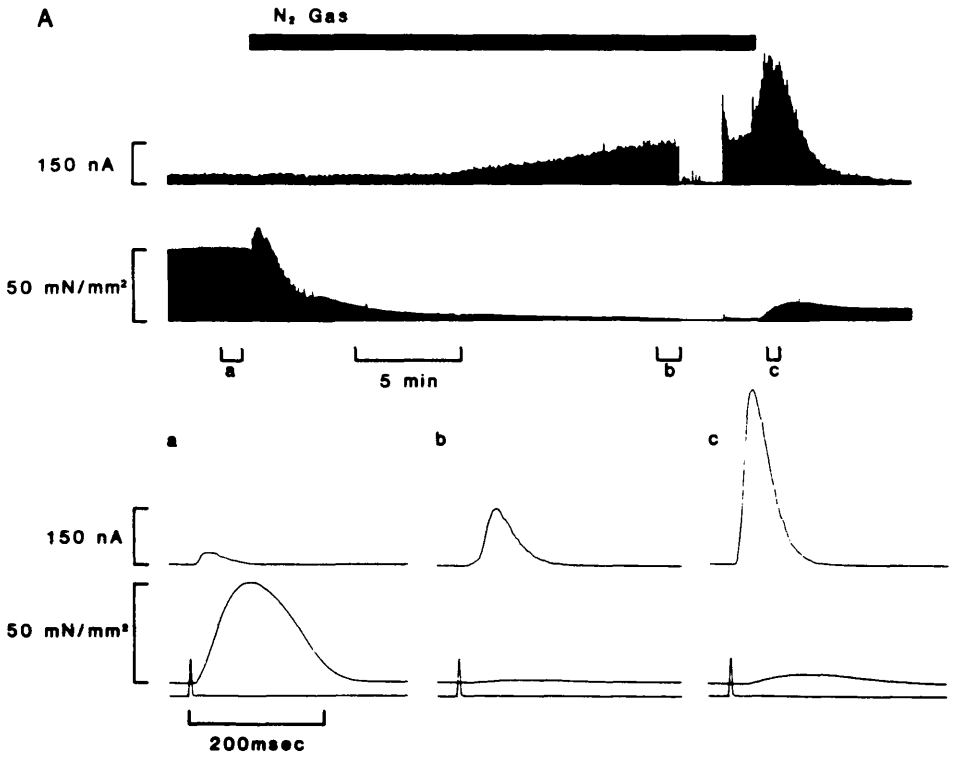
FIGURE 16.

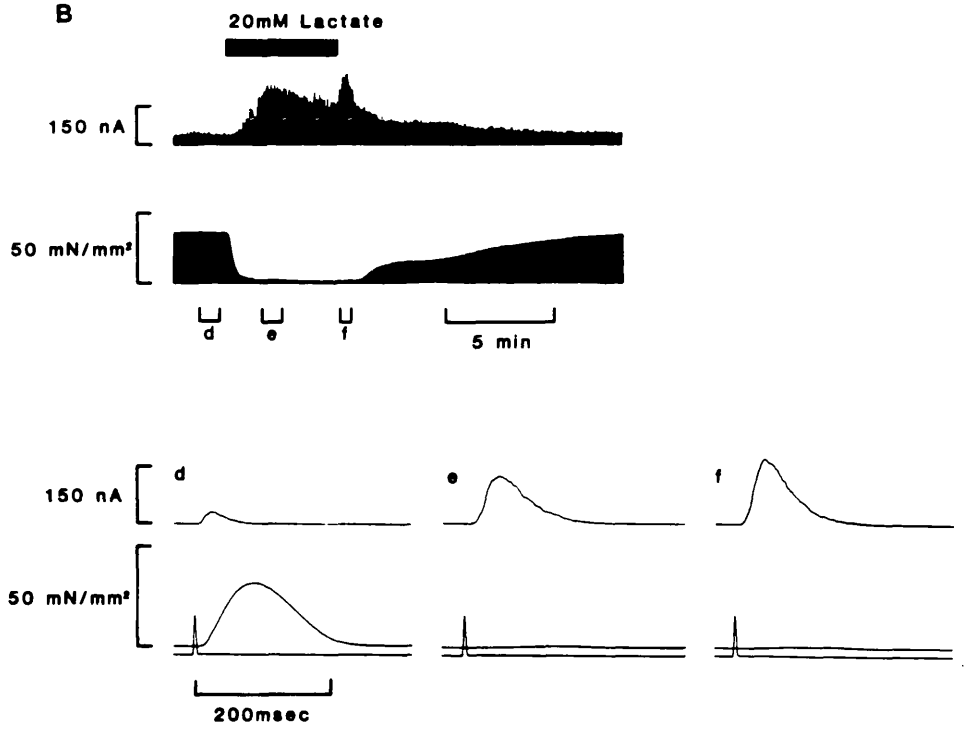
FIGURE 16.

Comparison of the effects of ischaemia and the extracellular addition of 20 mM lactic acid in a single preparation.

A. The effects of N_2 gas perfusion on aequorin light (above) and tension (below). (i), (ii), and (iii) show averaged records of light (above) and tension (below) from the periods indicated in the continuous trace.

B. The effects of 20 mM lactic acid on aequorin light (above) and tension (below). (i), (ii), and (iii) show averaged records from the periods indicated in the continuous record.


A
N_2 Gas
150 nA
50 mN/mm²
a
5 min
b
c
a
b
c
150 nA
50 mN/mm²
200msec


B
20mM Lactate
150 nA
50 mN/mm²
d
e
f
5 min
d
e
f
150 nA
50 mN/mm²
200msec

but then recovered over 2-3 minutes to a steady value of ~6.0 as excess CO_2 was bubbled off. Tension fell rapidly to a low level and the Ca^{2+} transients rose to a peak after about 2 minutes and then declined to a steady value. The initial component of the rise of the Ca^{2+} transients is probably caused by an initial rise in CO_2 as the acidosis caused by adding lactic acid shifts the equilibrium of the carbonic acid reaction. This acidosis causes HCO_3^- to be converted into H_2CO_3 and CO_2. The increased CO_2 causes the early transient fall in pH_o and enters the cell to produce a transient component in the fall in pH_i. If the lactic acid was added to the Tyrode solution 5 minutes before perfusion started, then perfusion of the muscle with pre-equilibrated 20 mM lactic acid caused a slower decline of tension and the rise of the Ca^{2+} transients was also slower and did not show the initial overshoot (e.g. Figure 19A(i)).

There are three points of similarity between the effects of ischaemia and lactic acid on the Ca^{2+} transients: (i) The steady increase in amplitude is similar (to 510% control in A and 420% control in B). (ii). The increase in duration of the Ca^{2+} transients is similar (to 160% of control in A and 190% of control in B, measured from stimulus until the light signal had declined to 50% of maximum amplitude). (iii). There was a short-lived increase in the amplitude of the Ca^{2+} transients at the end of both procedures. Taken together, these data therefore strongly support the idea that the rise in the Ca^{2+} transients is caused by acidosis, due at least in part to lactate accumulation.

If the increase in the amplitude and duration of the Ca^{2+} transients is caused by lactic acid accumulating intracellularly and extracellularly during ischaemia, then it ought to be possible to reduce or abolish the increase by depleting the amount of glycogen present in the preparation. Glycogen is consumed during a period of

ischaemia and then recovers relatively slowly during subsequent perfusion (Neely & Grotyohann, 1984). This property was utilised in the experiment shown in Figure 17. A first period of exposure to ischaemia (Figure 17A) shows all the features described above. The muscle then recovered for 40 minutes in O_2 gas and 30 minutes in O_2 Tyrode, by which time tension was almost fully restored, although the Ca^{2+} transients remained somewhat reduced. Figure 17B shows the effect of a second identical exposure to ischaemia. The response of the muscle differed from the first exposure in three respects: (i) The rise in resting tension was much larger and occurred earlier. (ii) The Ca^{2+} transients showed an initial decline, but did not then recover and they also did not show the characteristic large increase. (iii). There was an increase in resting light (i.e. in resting Ca^{2+}) towards the end of Panel B. (In the slow pen recorder trace in the upper part of Panel B the increase in resting Ca^{2+} appears identical to an increase in the Ca^{2+} transients; the fact that this increase is due to resting Ca^{2+} is clear from the averaged results shown in Panel B(ii).) This experiment therefore supports the hypothesis that the large increase in light transients is associated with lactate accumulation, since it can be abolished by procedures which are known to cause glycogen depletion.

In two experiments it was found that application of large concentrations of alpha and beta adrenoreceptor blockers (5 /uM phentolamine and 5 /uM propranalol) did not prevent the rise of light during ischaemia. In a further experiment it was found that a large concentration of a Na/H exchange inhibitor (1 mM amiloride) also did not prevent the rise of the Ca^{2+} transients during exposure to ischaemia.

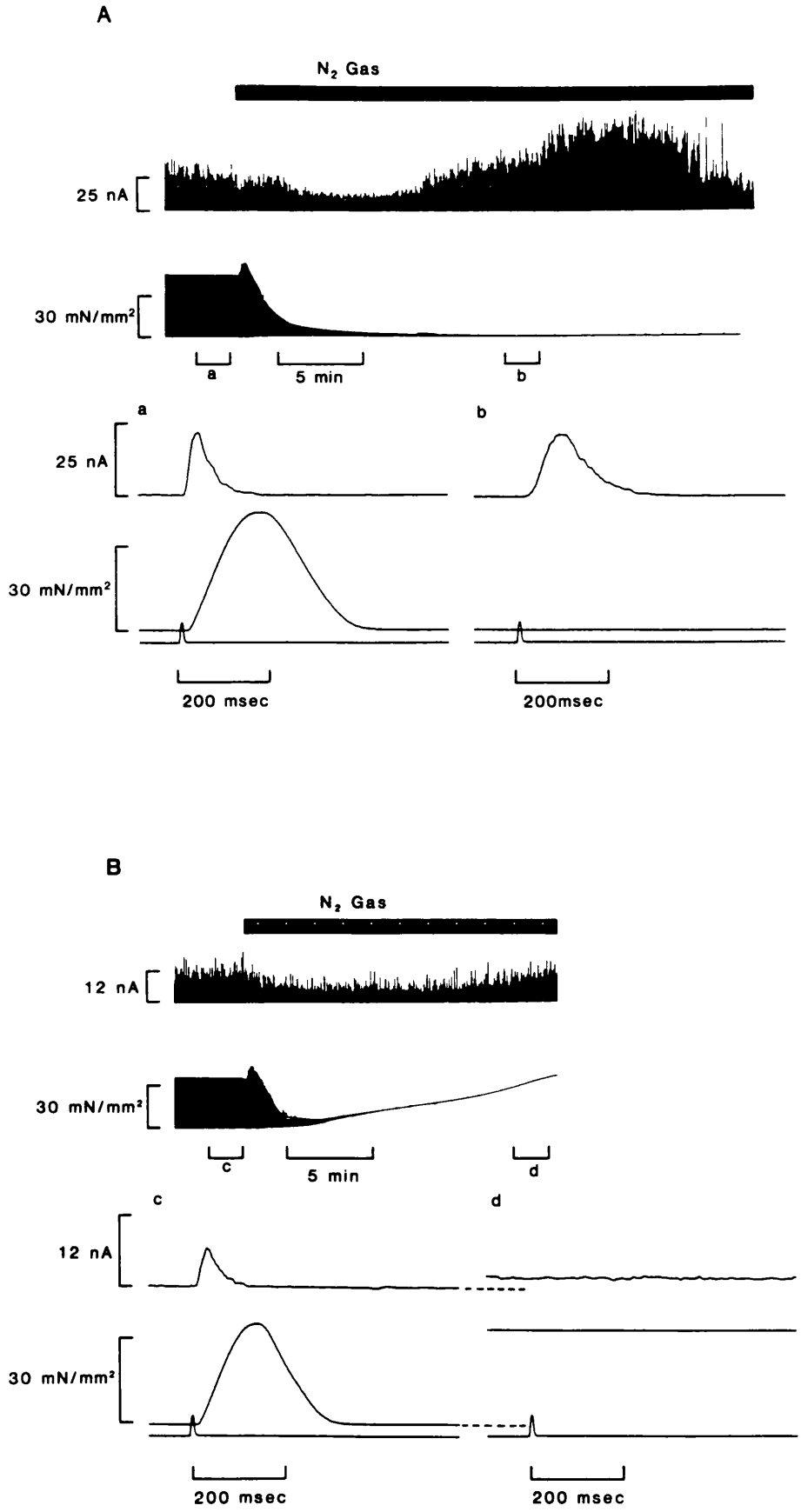
FIGURE 17.

FIGURE 17.

The effects of repeated exposures to ischaemia in a single preparation.

A. The effects of N_2 gas perfusion on aequorin light (above) and tension (below). (i) and (ii) show averaged records of light and tension from the periods indicated in the continuous trace.

B. A further exposure to N_2 gas perfusion. Continuous record shows aequorin light (above) and tension (below). (i) and (ii) show averaged records of light and tension from the periods indicated in the continuous trace.


A
N_2 Gas
25 nA
30 mN/mm²
a
5 min
b
a
b
25 nA
30 mN/mm²
200 msec
200msec
B
N_2 Gas
12 nA
30 mN/mm²
c
5 min
d
c
d
12 nA
30 mN/mm²
200 msec
200 msec

3.2.6 Extracellular pH and lactate in the globally ischaemic heart.

In the experiments described above, 20 mM lactic acid was used because this concentration was found to produce an increase in Ca^{2+} transients which approximately matched that seen in exposure to ischaemia. An important point to establish, therefore, is the actual level of extracellular lactate in ischaemia. Measurements of extracellular lactate and pH were made on small samples of extracellular perfusate flushed from whole hearts after 40 minutes of ischaemia, as described in section 2.7. The lactate and pH_o from 5 hearts averaged 4.1 $\pm$ 1.1 mM and 5.4 $\pm$ 0.1 pH units. Reasons for this apparent discrepancy between the measured lactate and the applied lactic acid will be considered further in Section 3.2.10.

3.2.7 The effects of ischaemia and applied lactic acid on the intracellular resting calcium concentration.

The increased Ca^{2+} transients during ischaemia could be caused by a variety of cellular mechanisms; one possibility is that the diastolic intracellular $[Ca^{2+}]$ is elevated, leading to increased Ca^{2+} uptake by the sarcoplasmic reticulum and hence increased Ca^{2+} release. If this mechanism is responsible for the increased Ca^{2+} transients, then it should be possible to measure an increase in the diastolic intracellular $[Ca^{2+}]$ during ischaemia. However, because of the non-linear relation between $[Ca^{2+}]$ and light for aequorin, the light emission level during diastole is very small and it is necessary to use highly filtered signals to overcome the low signal-to-noise ratio. This is most easily done by stopping stimulation. Figure 18 illustrates an experiment in which this was done. Panel A(i) shows the start of a period of ischaemia and A(ii) shows the Ca^{2+} transients after 20 minutes of exposure when they had substantially increased. At the time indicated by the arrow (25 minutes after the start of ischaemia) stimulation was stopped. Very large, spontaneous increases

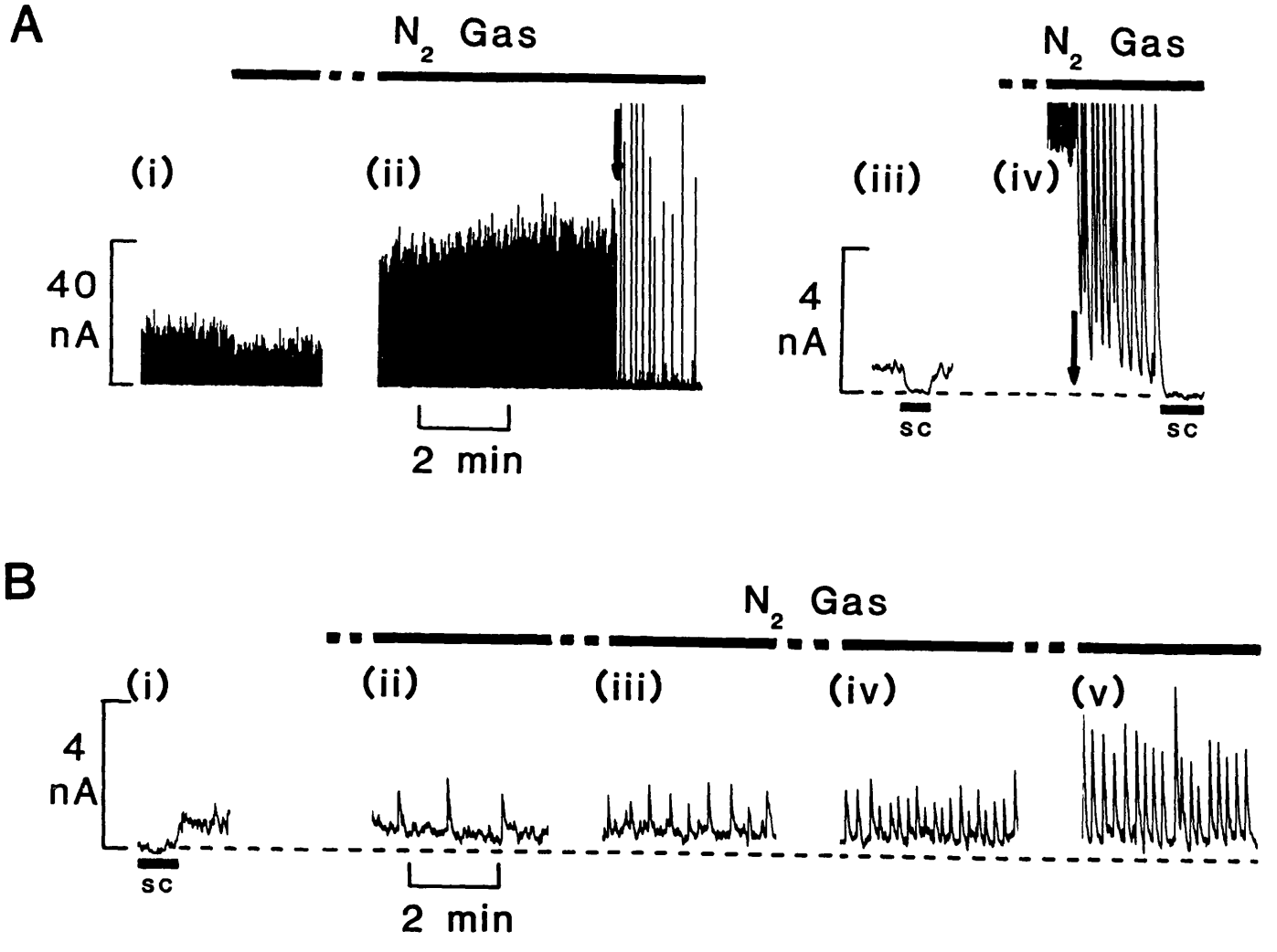
FIGURE 18.

FIGURE 18.

The effects of N_2 gas perfusion on resting light.

A. (i) Continuous record of Ca^{2+} transients in the control period and at the beginning of N_2 gas perfusion. (ii) After 20 minutes of gas perfusion, the Ca^{2+} transients had substantially increased. Stimulation was stopped at arrow, and large spontaneous Ca^{2+} transients can be seen. (iii) High gain, highly filtered (0 - 0.05 Hz) light record from control period showing resting light level. (iv) High gain, highly filtered (0 - 0.05 Hz) record of resting light after stopping stimulation at arrow (as in (ii)). s.c. indicates shutter closed. Dotted line shows baseline.

B. Resting light in an unstimulated exposure to gas perfusion with 95% N_2/ 5% CO_2. Same preparation as A. (i) Control period. Gas perfusion was then started and the effects after 10 (ii), 25 (iii), 40 (iv) and 55 (v) minutes are shown. s.c. indicates shutter closed. Dotted line indicates baseline.


A
N2 Gas
(i)
(ii)
40
nA
2 min
N2 Gas
(iii)
(iv)
4
nA
sc
sc
B
N2 Gas
(i)
(ii)
(iii)
(iv)
(v)
4
nA
sc
2 min

of light (oscillations) occurred at intervals of about 10 seconds and the frequency and amplitude of these spontaneous increases gradually declined. Panels (iii) & (iv) show parts of the same experiment at increased gain and filtering (bandwidth 0-0.05 Hz). Panel (iii) is from a control period without stimulation and indicates the resting light emission. Panel (iv) shows the period of non-stimulation shown in A(ii). In this experiment the light level between the spontaneous increases appeared to return to a level which was not different to the previous control resting level; in other experiments, however, there *was* an apparent increase in the resting level between spontaneous increases.

Because the level of resting light slowly decreased when stimulation was stopped, it was important to establish whether the resting level would rise in the absence of stimulation. On the other hand, the development of ischaemic sequelae will obviously be slower if the metabolic demands of the tissue are reduced by quiescence. To take account of these factors the following protocol was chosen. The muscle was initially rested to establish the resting light level under control conditions (Figure 18, Panel B(i)). The muscle was then stimulated regularly, as in Panel A(i), and N_2 gas perfusion was started. After 9 minutes, when the Ca^{2+} transients were still below the control level, but the tension had declined to virtually zero, stimulation was stopped. Panels B(ii), (iii), (iv) & (v) are after 10, 25, 40 and 55 minutes of ischaemia. There was clearly a gradual increase in the frequency and size of the spontaneous increases in Ca^{2+}. Panel B(iii) is from the same time period as Panel A(iv) and demonstrates that after the same period of N_2 gas perfusion, the increase in resting Ca^{2+} was smaller in a quiescent preparation.

In five experiments resting aequorin light was measured (i) in a control period, (ii) during a short period of quiescence during

ischaemia when the Ca^{2+} transients were maximally elevated and (iii) during a period of quiescence after a longer exposure to ischaemia such that the Ca^{2+} transients had declined to zero. The mean light level was highest in period (ii) because of oscillations, but the light level during period (iii), when oscillations had largely disappeared, was 195 $\pm$ 19 % of the control period (i).

If these increases in resting Ca^{2+} are a consequence of acidosis, then similar effects should occur in perfused preparations subjected to acidosis by means of added lactic acid. Figure 19 shows that very similar phenomena do indeed occur. Figure 19A(i) shows the gradual increase in the Ca^{2+} transients which occurred when Tyrode solution, pre-equilibrated with 20 mM lactic acid, was applied. Stimulation was stopped at the time indicated by the arrow and again substantial spontaneous increases in the light signal occurred but their frequency and amplitude gradually declined. Panels A(ii) & (iii) show at higher gain and filtering the light signals in a period of quiescence preceding lactic acid and the period shown in A(i) when stimulation was stopped during lactic acid exposure. In the latter part of A(iii) there is a period with no obvious spontaneous increases in light and it is apparent that the resting light had increased. Figure 19B(i) & (ii) show resting light before and during lactic acid application in the same muscle during quiescence. There was an increase in the frequency and amplitude of the spontaneous increases in light which eventually fused.

3.2.8 Alternation of the calcium transients and tension.

In all long N_2 gas exposures and virtually all lactic acid applications, alternation of the Ca^{2+} transients was noted i.e. successive Ca^{2+} transients alternated in amplitude. Examples are shown in Figures 20A & B. Usually, there was an associated alternation in

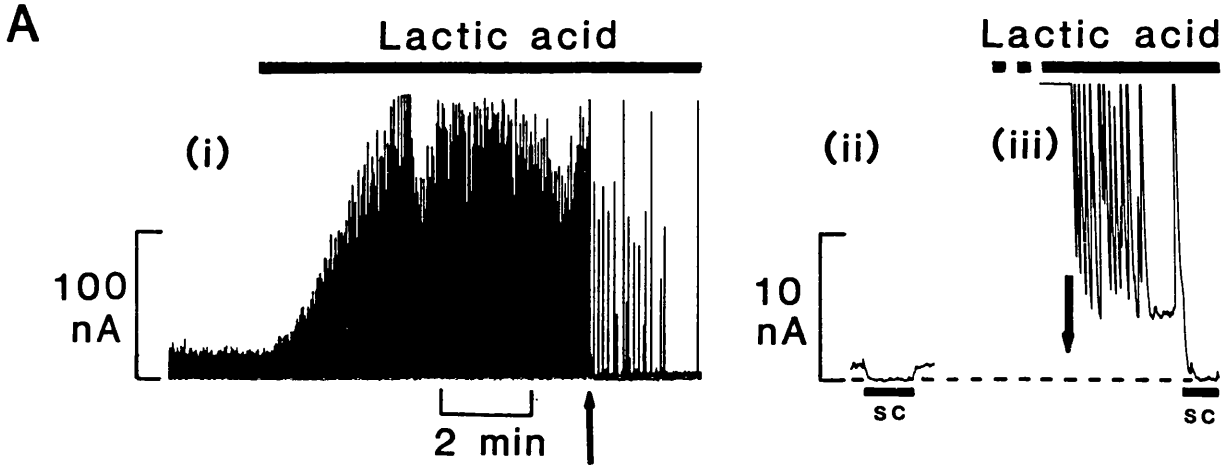
FIGURE 19.

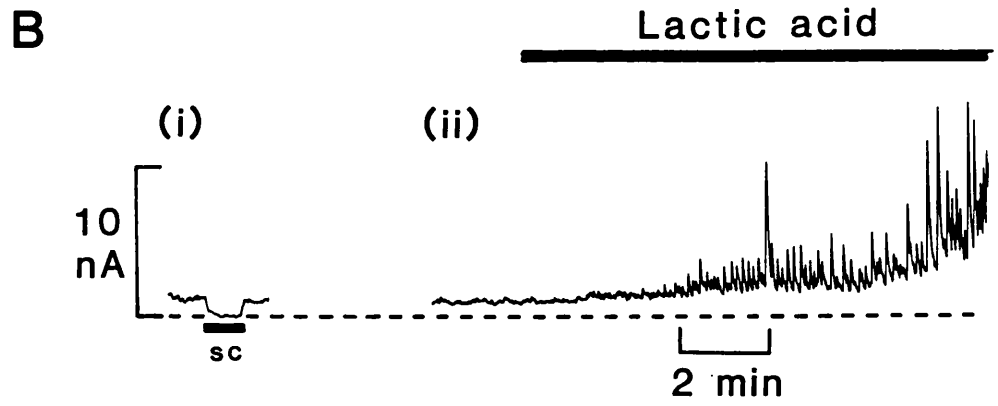
FIGURE 19.

The effects of 20 mM lactic acid on resting light.

A. Control period followed by switching to an identical Tyrode solution to which 20 mM lactic acid had previously been added. At arrow, stimulation was stopped and large spontaneous Ca^{2+} transients can be seen. (ii) High gain, highly filtered (0 - 0.05 Hz) record of resting light level during control period. (iii) High gain, highly filtered (0 - 0.05 Hz) record of resting light after stopping stimulation at arrow (as in (i)). s.c. indicates shutter closed. Dotted line indicates baseline.

B. Resting light in an unstimulated exposure to 20 mM lactic acid. Same preparation as A. (i) Control period. (ii) Application of 20 mM lactic acid. s.c. indicated shutter closed. Dotted line indicates baseline.


A
Lactic acid
(i)
100
nA
2 min
Lactic acid
(ii)
(iii)
10
nA
sc
sc


B
Lactic acid
(i)
(ii)
10
nA
sc
2 min

the tension, but in some exposures (e.g. Figure 20B) there was little detectable alternation in tension despite substantial alternation of the light signal. It should be noted that this phenomenon was only seen when the tension had already declined to very low levels. Once established, alternation of the light signal usually continued until the Ca^{2+} transients and tension became undetectable after 40-50 minutes. Increasing stimulus strength by up to 10 fold did not abolish alternation. The prolongation of the Ca^{2+} transients noted earlier means that detectable light signals continued until the next stimulus and suggests that alternation may be a manifestation of the restitution process between beats, i.e. Ca^{2+} has failed to return to the release sites or the release channel in the sarcoplasmic reticulum remains inactivated. In support of this hypothesis, alternation could often be abolished by small reductions in the stimulus frequency. The effect of increasing the interval between beats was also evident if a single stimulus was omitted. Irrespective of whether the preceding light transient was large or small, the next transient after the increased rest was large and alternation reappeared (not shown).

Alternation of action potentials was also observed which was concordant with the alternation in tension (Figure 20C). The smaller action potentials had reductions in both amplitude and duration but there was no detectable difference in resting membrane potential between the alternating action potentials. It is not clear whether alternation of the light transients causes alternation of the action potential or *vice versa*. This issue is considered in section 3.2.10.

3.2.9 Oscillations of intracellular calcium concentration.

Spontaneous elevations of intracellular $[Ca^{2+}]$ during the diastolic periods between Ca^{2+} transients have frequently been noted in Ca^{2+} overloaded preparations e.g. Orchard, Eisner & Allen, 1983; Wier, Kort, Stern, Lakatta & Marban, 1983). These increases in intracellular

FIGURE 20.

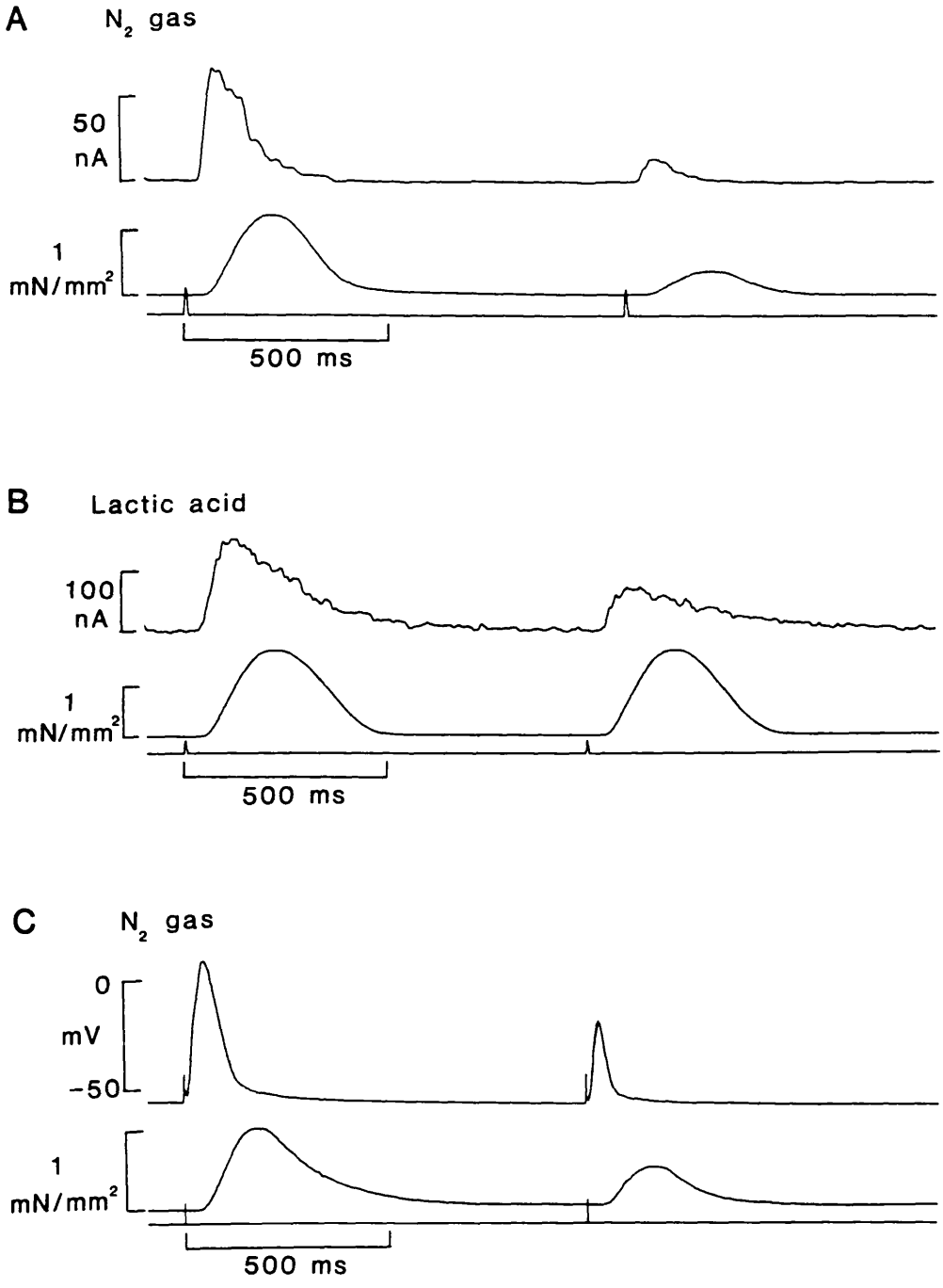
FIGURE 20.

Alternans produced by ischaemia and the application of 20 mM lactic acid.

A. Averaged records of aequorin light (above) and tension (below) during a period of gas perfusion with 95% N_2/ 5% CO_2.

B. Averaged records of aequorin light (above) and tension (below) during a period of superfusion with standard Tyrode with 20 mM lactic acid.

C. Averaged records of membrane potential (above) and tension (below) during a period of gas perfusion with 95% N_2/ 5% CO_2.


A
N_2 gas
50
nA
1
mN/mm²
500 ms
B
Lactic acid
100
nA
1
mN/mm²
500 ms
C
N_2 gas
0
mV
-50
1
mN/mm²
500 ms

$[Ca^{2+}]$ are thought to be caused by spontaneous release from overloaded sarcoplasmic reticulum and underlie the arrhythmogenic transient inward current (Kass, Lederer, Tsien & Weingart, 1978). It was of interest therefore, to see whether such oscillations occurred during N_2 gas perfusion and might contribute to the arrhythmias of early ischaemia. Surprisingly, given the greatly increased Ca^{2+} transients, oscillations were rarely observed during regular stimulation at 1 Hz, in contrast to the situation in Ca^{2+} overload produced by high $[Ca^{2+}]_o$ or cardiac glycoside toxicity. However, as already noted, when stimulation ceased, slow oscillations appeared and increased in amplitude for 10-20 seconds. This situation is shown on a faster timebase in Figure 21. Note that there were no detectable oscillations during stimulation, but oscillations developed over 10-20 seconds when stimulation was stopped. After 20 seconds rest the first contraction and accompanying light transient were greatly increased and alternation of both light and tension was increased in subsequent contractions.

Figure 21B shows spontaneous diastolic oscillations of intracellular $[Ca^{2+}]$ from another preparation exposed to 10 /uM strophanthidin and 10 mM external $[Ca^{2+}]$. The time base is identical to Figure 21A and the amplitude has been set so that the Ca^{2+} transients in both panels have similar magnitude. Note that the dominant frequency of oscillations is 3-4 Hz in Figure 21B but only 0.5 Hz in Figure 21A.

3.2.10 Interpretation of results.

3.2.10.1 N_2 gas perfusion as a model of ischaemia.

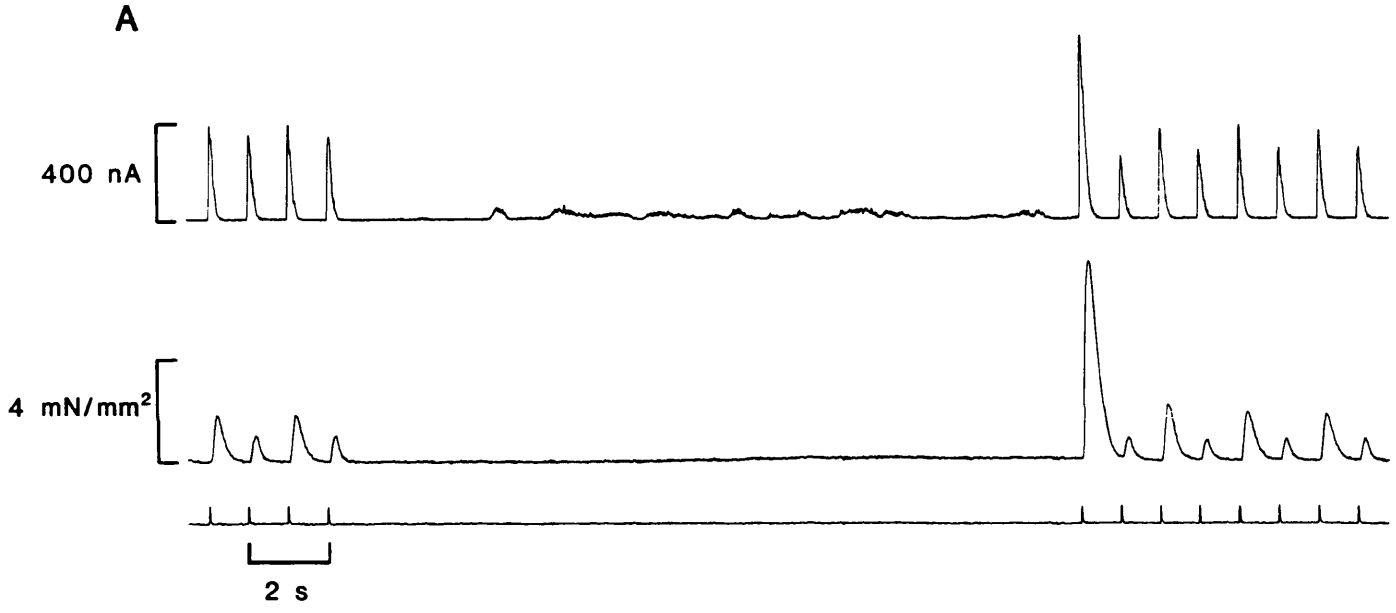
These experiments have examined the effects of superfusing isolated ventricular muscle with N_2 gas, and the changes in tension, membrane potential and intracellular $[Ca^{2+}]$ which occur under these conditions have been described. It is necessary to consider first the

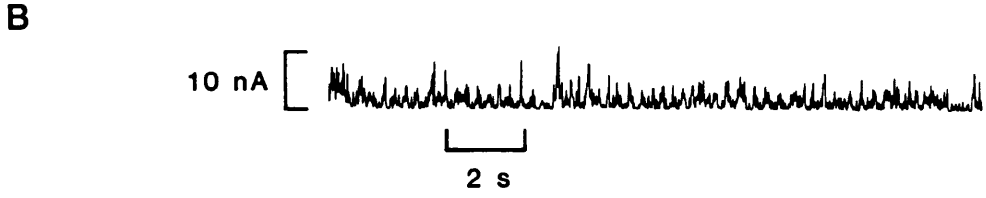
FIGURE 21.

FIGURE 21.

A. Fast timebase recording of aequorin light (above), tension (middle) and stimulus (below) during a period of N_2 gas perfusion. When stimulation was stopped, spontaneous light oscillations appeared after a few seconds. When stimulation was restarted, the first Ca^{2+} transient and twitch were large and alternans can be clearly seen.

B. Resting light from another preparation in 10 /uM strophanthidin and 10 mM extracellular Ca^{2+}. The light gain has been set so that Ca^{2+} transients in Panels A and B had similar amplitudes.


A
400 nA
4 mN/mm²
2 s


B
10 nA
2 s

extent to which N_2 gas perfusion can be regarded as an adequate model of ischaemia. As noted in the results, the decline of tension in a papillary muscle during N_2 gas perfusion was slower than the decline of pressure in the whole heart during ischaemia, but in other respects the mechanical failure was similar. The changes in the resting potential and action potential are similar in N_2 perfused muscle and the ischaemic heart (e.g. Downar et al., 1977). In addition, measurements of intracellular $[Ca^{2+}]$ by two quite different methods have also shown increases during ischaemia. Studies with F-BAPTA (Steenbergen et al., 1987; Marban et al., 1987) indicated that time-averaged intracellular $[Ca^{2+}]$ rose 5 - 10 minutes after the start of ischaemia. Studies with indo (Lee et al., 1987, 1988) show increases in both diastolic and systolic $[Ca^{2+}]$ starting within 1 minutes of exposure to ischaemia. The indo method also showed a prolongation of the Ca^{2+} transient similar to that observed in the present experiments. These similarities suggest that N_2 gas perfusion is an adequate model of ischaemia.

There are two respects in which N_2 gas perfusion does not perfectly mimic ischaemia:

(i). Volatile products of metabolism will not accumulate for any significant time in this model. Specifically CO_2, which can reach a tissue partial pressure of 400 mm Hg after 20 minutes of ischaemia (Case, Felix & Castellana, 1979), will not accumulate in this model. This large rise in pCO_2 in the intact heart is probably caused mainly by lactic acid displacing HCO_3^- to CO_2 (Ichihara, Haga & Abiko, 1984), which can then only leave the ischaemic tissue by diffusion. Since the papillary muscles used in this study were less than 1 mm in diameter, metabolically generated CO_2 would have diffused away rapidly and hence the early acidosis in this preparation would have been smaller than in the intact heart. This is probably part of the explanation for the slower fall of tension in this model. Although not attempted in this

study, it would be possible to achieve the increase in CO_2 partial pressure in this model by superfusing with an appropriately changing mixture of gases.

(ii). The restriction of the extracellular space in this model depends on how much Tyrode solution remains attached to the surface of the muscle. As noted in section 2.6, this was usually too small to measure, i.e. it was less than 5 - 10 % of the muscle weight. Nevertheless even such a small amount of fluid as 5% of the muscle weight represents a considerable increase in the extracellular fluid volume and would cause a larger dilution of products of metabolism such as lactic acid than would occur in the intact heart. This would lead to a smaller extracellular and intracellular acidosis in the papillary muscle preparation and represents a further reason for the slower fall in tension in this model when compared to ischaemia in the intact heart.

3.2.10.2 Mechanism of the changes in intracellular calcium concentration.

During the first 5-10 minutes of exposure to ischaemia, the Ca^{2+} transients were slightly reduced in amplitude and this may be a consequence of the reduction of amplitude and duration of the action potential observed at this time. Subsequently, the resting $[Ca^{2+}]$ and the Ca^{2+} transients increased and oscillations of intracellular $[Ca^{2+}]$ appeared. Evidence has been presented that the increases in intracellular $[Ca^{2+}]$ can be reproduced by application of lactic acid and can be prevented by prior exposure to N_2 gas, which is likely to decrease lactic acid production in the muscle due to glycogen depletion (Neely & Grotyohann 1984; see also section 3.1). Since the rise in resting $[Ca^{2+}]$ and oscillations appeared in unstimulated preparations, it seems that activity and action potentials are not required for this

rise, although the rise is much greater after a period of activity. It has been known for some years that intracellular acidosis increases intracellular [Ca^{2+}] (Bers & Ellis, 1982; Allen & Orchard, 1983a) and it has also been shown that increases in intracellular [Ca^{2+}] can lead to an intracellular acidosis (Meech & Thomas, 1977; Vaughan-Jones, Lederer & Eisner 1983). However, the mechanism by which this occurs is not known and is not revealed by the present experiments. It seems likely that the rise in resting [Ca^{2+}] leads to greater sarcoplasmic reticulum loading and that this is the cause of the increased Ca^{2+} transients. A second factor may be that the apparent binding constant of troponin C for Ca^{2+} is reduced, so that an unchanged release of Ca^{2+} leads to a larger Ca^{2+} transient. However, the fact that the time course of the rise of Ca^{2+} lags behind the timecourse of the decline of tension during acidosis (e.g. Figure 16B) argues against this mechanism. The prolongation of the Ca^{2+} transient and the shortening of the time course of tension are at least qualitatively in accord with the reduced apparent troponin binding constant in acidosis (Blanchard & Solaro, 1984; Blinks & Endoh, 1986). In contrast, if the slowing of the Ca^{2+} transient were due to H^+ or phosphate slowing the sarcoplasmic reticulum Ca^{2+} pump, then one would expect to see the timecourse of tension prolonged rather than abbreviated.

The elevation of resting Ca^{2+} seen during exposure to a second period of ischaemia is probably due to a different mechanism. In this case lactate accumulation would not occur to the same degree because of glycogen depletion. However, under these conditions ATP will fall much more rapidly, because little energy is available from glycolysis. Hence ATP dependent pumps fail sooner after the onset of ischaemia, allowing the resting Ca^{2+} to rise. This hypothesis is supported by the fact that a contracture develops much more rapidly in this situation.

Reperfusion of a preparation after a period of N_2 gas perfusion

sufficiently long to elevate the Ca^{2+} transients, usually led to a further transient increase in Ca^{2+} transients before they returned towards control values (Figures 13A & 16A). It is possible that this increase in the Ca^{2+} transients could be produced by reactivation of the Na^+/H^+ exchanger which had been inhibited by the extracellular acidosis produced by ischaemia, a mechanism suggested by Lazdunski, Frelin & Vigne (1985). Rapid extrusion of protons by the Na^+/H^+ exchanger would lead to Na^+ influx, which would trigger Ca^{2+} influx on the Na^+/Ca^{2+} exchanger.

3.2.10.3 Changes in intracellular and extracellular pH in ischaemia.

These experiments have suggested that accumulation of intracellular lactic acid is the cause of the rise in both resting and systolic $[Ca^{2+}]$. However, it is clear from the results that the extracellular lactate measured in whole hearts in ischaemia (4 mM) was substantially less than the level which was applied to produce similar effects on the Ca^{2+} transients (20 mM). Much of this discrepancy disappears if the intracellular lactate in each situation is calculated.

If lactic acid is produced in the cell, it diffuses across the membrane and appears in the extracellular space. Conversely the addition of lactic acid to the extracellular space causes entry of lactic acid into the cell. It is possible to calculate the equilibrium concentrations of lactic acid in the extracellular and intracellular compartments on the basis of the following assumptions:

(i) The cell membrane is permeable to undissociated lactic acid (HL), but not to H^+ or L^-.

(ii) At equilibrium, extracellular [HL] ($[HL]_o$) and intracellular [HL] ($[HL]_i$) are equal.

(iii) Metabolism of lactic acid and pH regulating mechanisms have negligible effects over the timescale of interest.

Because pH affects the equilibrium between HL and L^-, the extracellular and intracellular pH influence the distribution of lactic acid across the cell membrane. The following approach is similar in principle to that of Szatowski & Thomas (1986).

The various species of lactic acid in the extracellular space are related as follows:

$$pH_o = pK_L + \log [L^-]_o/[HL]_o \quad (1)$$

where pH_o is the extracellular pH, pK_L is the pK of lactic acid, and $[L^-]_o$, $[HL]_o$ are the dissociated and undissociated forms respectively of lactic acid in the external solution. Likewise, the intracellular pH in the presence of lactic acid (pH_{iL}) is given by

$$pH_{iL} = pK_L + \log [L^-]_i/[HL]_i \quad (2)$$

where pH_{iL} is the intracellular pH after the addition of lactic acid.

The change in pH on the entry of lactic acid is determined by the buffering power of the cell, B, defined as

$$B = [L^-]_i/(pH_i - pH_{iL}) \quad (3)$$

where pH_i is the initial intracellular pH before the addition of lactic acid. Because of the assumption that $[HL]_o = [HL]_i$ at equilibrium, and expression can be derived for pH_{iL} which depends only on B, pK_L, pH_i and $[L^-]_o$, all of which are known.

$$pH_{iL} = pK_L + \log(B\,(pH_i - pH_{iL})(10^{pH - pK})/[L^-]_o \quad (4)$$

This may be further simplified to eliminate pK_L, giving

$$pH_{iL} = pH_o + \log\,(B\,(pH_i - pH_{iL})/[L^-]_o \quad (5)$$

Equation (5) cannot be solved analytically, but it can be solved iteratively for a range of $[L^-]_o$ and pH_o. The initial intracellular pH (pH_i) was assumed to be 7.0 (e.g. Ellis & Thomas, 1976). There is some discussion in the literature as to the appropriate value of buffering power to use. Ellis & Thomas (1976) measured buffering in ferret ventricular muscle to be 69 mM/pH unit. More recently, however, Bountra, Powell & Vaughan-Jones (1987) found buffering power to be 72

mM/pH unit in multicellular guinea pig preparations, but only 18 mM/pH unit in isolated myocytes from the same species. In order to span this range of apparent intracellular buffering, Figure 22 shows the results of the calculation with buffering set at 70 mM/pH unit, while Figure 23 shows the results when buffering is set at 20 mM/pH unit. The Figures also show the expected levels of intracellular lactate. It can be seen that the main effects of decreasing buffering power are to decrease the intracellular lactate necessary for a given intracellular pH change, and to increase the effect of a given change in extracellular pH.

When lactic acid was applied externally (external lactate = 20 mM, pH_o = 6.0), the calculated intracellular lactate (using a buffering power of 70 mM/pH unit) is 45 mM (pH_i = 6.35). In ischaemia, the measured extracellular lactate was 4 mM and the measured pH_o was 5.4. This gives a calculated intracellular lactate of 38 mM (pH_i = 6.45). If 20 mM/pH unit is used for the buffering power, the values of pH_i and intracellular lactate are different (see Figure 23), but the similarity between applied lactic acid and ischaemia is still present. Thus if intracellular pH_i is the most important consequence of lactic acid accumulation, then the effects of applied lactate and ischaemia are quite comparable.

3.2.10.4 Mechanical consequences of the changes in intracellular calcium concentration in ischaemia.

An important goal of studies of ischaemia is to identify the mechanism of the rapid decline in tension. The contribution of metabolic factors is now relatively well understood. After 10 minutes of ischaemia, tension is reduced to about 10% in this model and the Ca^{2+} transients are virtually unchanged from control. Intracellular inorganic phosphate, measured by nuclear magnetic resonance (NMR) in whole hearts, has increased from about 3 to 15 mM (Elliott, 1987) and

FIGURE 22.

FIGURE 22.

The effects of changing extracellular lactate (L_o) and pH (pH_o) on intracellular lactate (L_i) and pH (pH_i). The lines were calculated using the model described in the text. Intracellular buffering power was assumed to be 70 mM/pH unit and the initial intracellular pH was assumed to be 7.0.

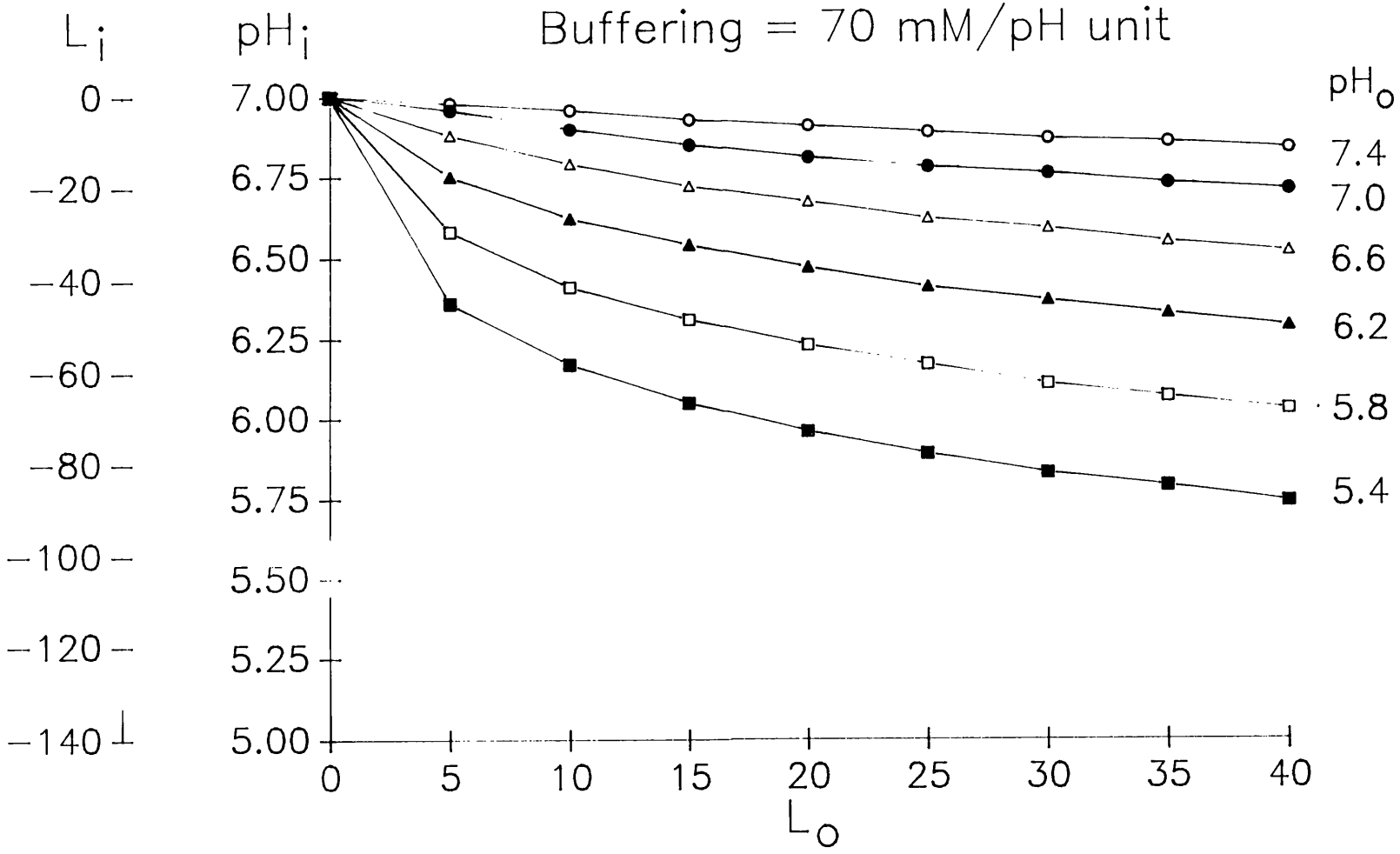
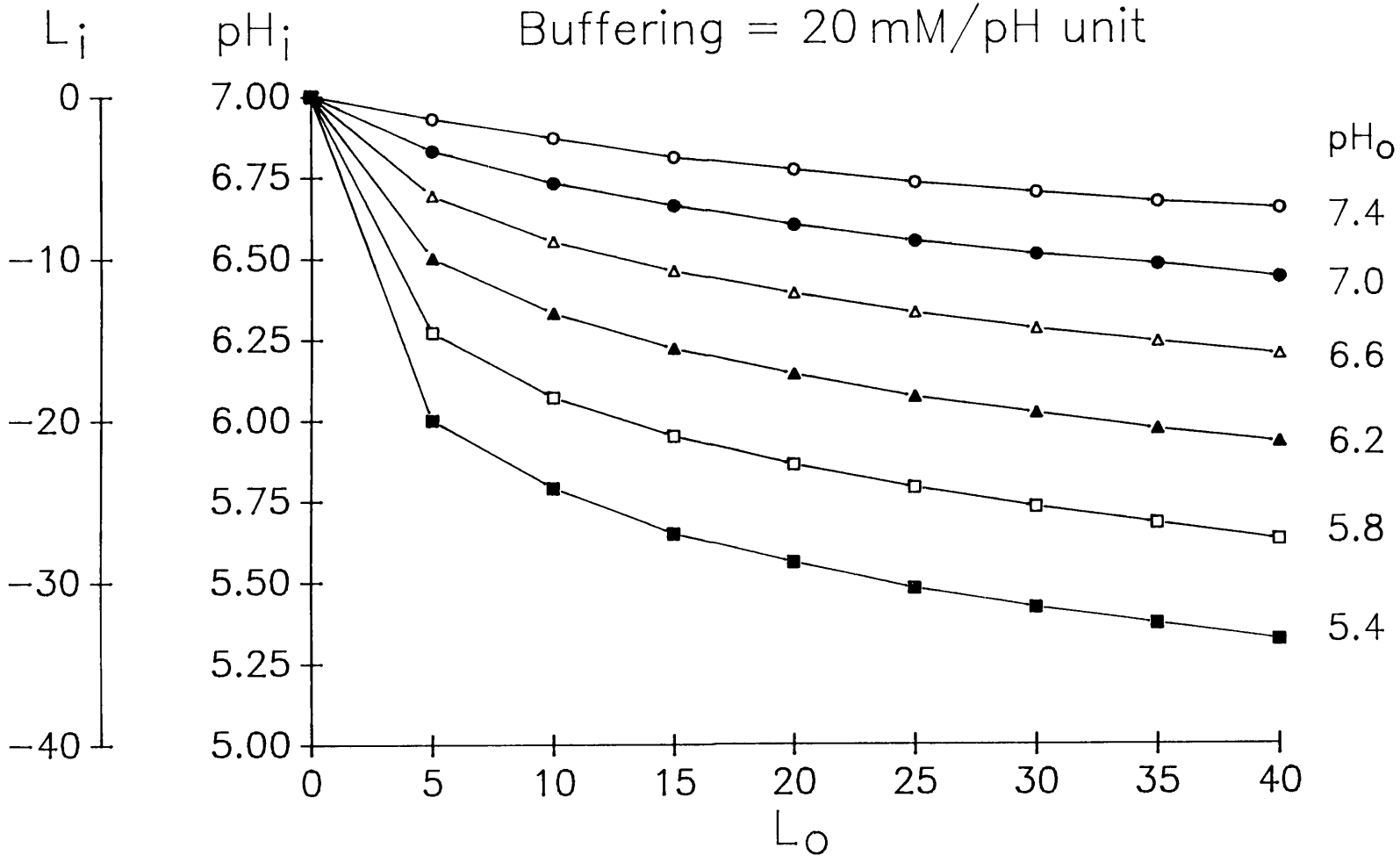

Buffering = 70 mM/pH unit
L_i
pH_i
pH_o
0
−20
−40
−60
−80
−100
−120
−140
7.00
6.75
6.50
6.25
6.00
5.75
5.50
5.25
5.00
7.4
7.0
6.6
6.2
5.8
5.4
0
5
10
15
20
25
30
35
40
L_o

FIGURE 23.

FIGURE 23.

The effects of changing extracellular lactate (L_o) and pH (pH_o) on intracellular lactate (L_i) and pH (pH_i). The lines were calculated using the model described in the text. Intracellular buffering power was assumed to be 20 mM/pH unit and the initial intracellular pH was assumed to be 7.0.


Buffering = 20 mM/pH unit
L_i
pH_i
0
−10
−20
−30
−40
7.00
6.75
6.50
6.25
6.00
5.75
5.50
5.25
5.00
0
5
10
15
20
25
30
35
40
L_o
pH_o
7.4
7.0
6.6
6.2
5.8
5.4

data from skinned ventricular muscle suggests that this would decrease developed tension to about 12% of control (Kentish, 1986). Intracellular pH, also measured by NMR, has decreased from about 7.0 to 6.6 (Elliott, 1987) and skinned fibre data suggest that this would also reduce tension to around 12% (Fabiato & Fabiato, 1978). Thus the combined effect would be expected to reduce tension to about 1% (12% X 12%). This is substantially smaller than the tension measured in the present experiments (10%), but similar to the measured tension in the intact ischaemic heart (<1%). As noted above the larger fall of tension in the intact heart at this stage may represent the greater initial acidosis which probably occurs due to the accumulation of CO_2.

After 20 minutes of ischaemia, developed tension was about 2% of control in these experiments and it was found that the peak $[Ca^{2+}]$ has increased about 2 fold. At this stage, intracellular phosphate has increased only slightly further to 16 mM (Elliott, 1987) which would reduce developed tension to around 10 % control, assuming intracellular $[Ca^{2+}]$ remained unchanged, or to 75 % control, taking account of the 2 fold increase in intracellular $[Ca^{2+}]$ (Kentish, 1986). pH_i has decreased from 7.0 to 6.3 (Elliott, 1987) which would reduce tension to 5 % of control (unchanged intracellular $[Ca^{2+}]$) or 30 % of control (with observed intracellular $[Ca^{2+}]$ changes). On this basis, the expected tension for an unchanged intracellular $[Ca^{2+}]$ is 0.5 % and for the observed increase in intracellular $[Ca^{2+}]$ is 20 % (measured value 2 % in these experiments, undetectable in the ischaemic heart). These discrepancies between measured and calculated tension should probably not be taken too seriously because of the different preparations from which the metabolic measurements and intracellular $[Ca^{2+}]$ were obtained. In addition the use of skinned fibre data involves additional uncertainties. Nevertheless it is clear that a complete understanding of the changes in tension in ischaemia will require

reliable measurements of mechanical and metabolic values and intracellular [Ca^{2+}] from the same preparation.

The appearance of alternation of tension, Ca^{2+} transients and action potentials was often noted in these experiments, and in the intact heart similar alternation of the action potential often precedes arrhythmias (Dilly & Lab, 1988). One possible mechanism for this alternation is that it is caused by the slow recovery of the Ca^{2+} current and action potential duration which has been observed under conditions of depolarisation (Gettes & Reuter, 1974). In this situation, a long action potential is followed by a shorter one because of failure of recovery of i_{Ca}. Since the stimulus interval is constant, the short action potential will be followed by a longer period for recovery of i_{Ca} and so the subsequent action potential will be longer again. This variation in Ca^{2+} current or action potential duration could lead to changes in Ca^{2+} release from the sarcoplasmic reticulum and explain the alternation in Ca^{2+} transients and tension. A quite different mechanism is that the return of Ca^{2+} to release sites in the sarcoplasmic reticulum is slowed in ischaemia, particularly after a large release. This could then lead to alternate amounts of calcium release from the sarcoplasmic reticulum (Lab & Lee, 1988 - see Appendix 3). Because Ca^{2+} activated currents are increased when the Ca^{2+} transient is larger, the changes in intracellular [Ca^{2+}] could also lead to changes in the action potential. These possibilities cannot be distinguished by the present experiments, but they could be distinguished in a voltage clamp experiment.

An interesting feature of the results is the occasional appearance during the last phase of the decline in tension of alternation in Ca^{2+} transients without any detectable alternation of tension (e.g. Figure 20B). This could occur if intracellular [Ca^{2+}] was so high that maximal tension activation was occurring, but this explanation is made

unlikely by results (e.g. Figure 21A) in which a short rest in a preparation showing alternation caused a large increase in tension immediately after the rest. Another possibility is that there were conduction blocks to some parts of the preparation which appeared only on alternate contractions. This kind of conduction block is known to be a feature of ischaemia (Janse & Kleber, 1981). If a small fraction of the preparation, which included half of the aequorin injected cells, was not invaded by alternate action potentials, this could produce a record similar to Figure 20B, in which the light signal was approximately halved on alternate contractions while the tension was hardly changed. This kind of mechanism could also explain alternation of tension and light where the two responses are more nearly concordant (e.g. Figure 20A).

3.2.10.5 Electrophysiological consequences of the changes in intracellular calcium concentration in ischaemia.

Clusin et al. (1984) have accumulated considerable evidence that the depolarization during ischaemia, which is one cause of the arrhythmias, is a consequence of elevated intracellular [Ca^{2+}]. The main strands to this argument are as follows. (i) Ca^{2+} channel blockers or lowered extracellular [Ca^{2+}] reduce the injury current between normal and ischaemic myocardium and also reduce the incidence of arrhythmias. (ii). Application of metabolic inhibitors, which have been shown to elevate intracellular [Ca^{2+}] under some circumstances (Dahl & Isenberg, 1980; Barry, Peeters, Rasmussen & Cunningham, 1987) induce a current which leads to diastolic depolarisation (Clusin, 1983). (iii). Ca^{2+}-activated currents which could underlie these effects have been identified (Colquhoun, Neher, Reuter & Stevens, 1981; Kimura, Miyamae & Noma, 1987).

The present results support this hypothesis to the extent that a rise in diastolic intracellular [Ca^{2+}] was seen and a depolarisation

occurred during exposure to N_2 gas. The magnitude of the increase in resting $[Ca^{2+}]$ is not clear from these experiments. Although the increases in resting aequorin light were very small, the relation between aequorin light and $[Ca^{2+}]$ is relatively flat for $[Ca^{2+}]$ in the range of the resting intracellular $[Ca^{2+}]$, so that the increase in the resting $[Ca^{2+}]$ may in fact be substantial. The studies by Lee et al. (1987, 1988) using indo appeared to show very large increases in diastolic $[Ca^{2+}]$ (above the control systolic level). However, because the Ca^{2+} transients had not returned to a steady level before the next stimulus and because Ca^{2+} transients get both larger and more prolonged during ischaemia, part of the apparent increase in diastolic $[Ca^{2+}]$ seen in those experiments presumably represents the summation of successive Ca^{2+} transients, rather than a true increase in resting $[Ca^{2+}]$. Further assessment of the role of intracellular $[Ca^{2+}]$ in ischaemic depolarisation will require measurements of K_o as well as $[Ca^{2+}]$ and membrane potential in the same preparation throughout ischaemia.

One interesting situation is the rapid repolarisation of the membrane potential and prolongation of the action potential (largely complete within one minute) which occurs when N_2 gas perfusion is terminated with O_2 gas perfusion. The recovery cannot be due to washout of extracellular metabolites, such as K^+ or lactic acid, since only O_2 gas entered the chamber. The prolongation of the action potentials could be related to the recovery of $[ATP]_i$ and its effects on the ATP-dependent K channel (Noma, 1983; Elliott, Smith & Allen, 1989), but this seems unlikely as the sole explanation because reduction in K^+ permeability would be expected to lead to depolarization rather than repolarization. One mechanism which could explain the repolarization is if the elevated resting $[Ca^{2+}]$ observed during N_2 gas perfusion fell rapidly during O_2 gas perfusion. Elevated

intracellular [Ca^2] opens Ca^{2+} -activated channels leading to depolarization and this would be reversed when intracellular [Ca^{2+}] fell. An attempt was made to measure resting [Ca^{2+}] under these circumstances but, although it was raised, the increase was very small and it was not possible to detect any change when O_2 was substituted for N_2. However, aequorin signals are very small under these circumstances so this result is subject to considerable uncertainty. Nevertheless, there are additional reasons to doubt whether resting [Ca^{2+}] changes sufficiently fast. (i) The Ca^{2+} transients decline quite slowly over this period (e.g. Figure 15A), requiring 5-10 minutes to return to control. (ii) The elevated resting [Ca^{2+}] was probably caused by increased intracellular lactic acid. However, it is unlikely that the lactic acid would decline this fast, because when O_2 gas was readmitted, the extracellular lactic acid would not be washed away, and would have to be removed metabolically.

Another possibility is that the rapid repolarisation is caused by either reuptake of extracellular K^+ due to reactivation of the Na^+ pump as ATP recovers or by the effect of the pump current which would also lead to hyperpolarization.

The oscillations of intracellular [Ca^{2+}] seen during ischaemia will lead to transient depolarizations and can cause focal arrhythmias. The possible importance of this mechanism in ischaemia is suggested by recent work of Thandroyen, McCarthy, Burton & Opie (1988). They showed that agents, such as ryanodine and caffeine, which are known to suppress oscillatory Ca^{2+} release, reduced the incidence of ischaemic arrhythmias. In the present experiments, oscillations were only observed when stimulation was stopped: perhaps in intact, ischaemic hearts conduction block occurs first and oscillations may then arise in the regions which are not invaded by the action potential.

3.2.10.6 Gap junctions.

There is good evidence that cells uncouple during ischaemia and this contributes both to the slowing of conduction and to the arrhythmias (Janse & Kleber, 1981). Both acidosis (Reber & Weingart, 1981) and increases in intracellular [Ca^{2+}] (de Mello, 1975) cause closure of gap junctions and result in uncoupling. In a study of the effects of ischaemia on intra- and extracellular resistance in arterially perfused rabbit papillary muscles, Kleber, Riegger & Janse (1987) found that a sharp increase in intracellular resistance occurred after 15-20 minutes of ischaemia. A substantial acidosis would be expected to occur well before this, but the time of rapid uncoupling coincides reasonably well with the marked increase in the Ca^{2+} transients observed in the present experiments.

3.2.10.7 Conclusions.

It is clear that intracellular [Ca^{2+}] rises substantially in the early phase of ischaemia and this is probably a consequence of intracellular acidosis. The increased intracellular [Ca^{2+}] will modify the effect of metabolic factors on the contractile machinery and is probably involved, by a variety of mechanisms in the arrhythmias of early ischaemia.

3.3 COMPARISON OF THE EFFECTS OF INOTROPIC INTERVENTIONS ON CONTRACTION IN ISOMETRIC AND ISOTONIC CARDIAC MUSCLE.

3.3 COMPARISON OF THE EFFECTS OF INOTROPIC INTERVENTIONS ON CONTRACTION IN ISOMETRIC AND ISOTONIC CARDIAC MUSCLE.

3.3.1 Background.

During the normal cardiac cycle there are phases of isovolumetric pressure development and of ejection at approximately constant pressure. In other words, there are times when the muscle develops tension at a constant length (isometric contraction) and when it maintains a constant tension while shortening (isotonic contraction). In spite of the fact that the heart constantly utilises these two modes of contraction, the majority of studies on the effects of inotropic and other interventions on isolated cardiac tissue have, for reasons of simplicity, involved measurements of isometric tension only. In fact, the cardiac cycle *in vivo* involves two other phases as well - isovolumetric relaxation and diastolic filling. In a study where the physiological "four phase" contraction cycle of cardiac muscle was closely mimicked in a papillary muscle preparation, it was found that the length of time a muscle spent at short diastolic length could influence the inotropic state of the muscle (Hanck & Jewell, 1985). The negative inotropic effect of short diastolic muscle length has been confirmed (Nicholls, 1985), and appears to be due to an effect of short diastolic length which reduced the resting intracellular [Ca^{2+}], and hence the Ca^{2+} transients (Allen, Nicholls & Smith, 1988). "Physiological" contraction of cardiac muscle differs from simple isotonic contractions in that: (i) relaxation takes place at short length (to mimic isovolumetric relaxation) and (ii) restretching of the muscle occupies most of the diastolic period (to mimic diastolic filling). Although physiological contraction does show differences from isotonic contraction (Hanck & Jewell, 1985), these differences are quantitatively small, being less than 5% of the control contraction strength. Thus isotonically contracting cardiac muscle provides a

reasonably good model for the last three phases of the physiological contraction cycle, the main differences being accounted for by the shorter time spent at short diastolic length in isotonic contractions.

There are, however, a number of indications that the response of shortening cardiac muscle may differ from that of the isometric preparation. For example, the time course of the lightly loaded isotonic contraction is much shorter than that of isometric tension development (Brady, 1966), and it has also been observed that the oxygen consumption of a papillary muscle falls to approximately one third in unloaded shortening when compared to isometric contractions (McDonald 1966; Coleman 1968). In addition, some recent work with skinned fibre preparations of skeletal muscle (Cooke & Pate, 1985) has indicated that the maximum velocity of shortening is much less sensitive to intracellular pH and intracellular inorganic phosphate (Pi) than is isometric tension. It is thus possible that factors which influence the inotropic state of the muscle and hence also alter intracellular pH and Pi, might influence shortening and isometric tension development differently.

Another reason for interest in differences which may exist between unloaded shortening and isometric tension is that modern studies of the mechanical responses of single cardiac myocytes are largely restricted to unloaded shortening (e.g. Mitchell, Powell, Terrar & Twist, 1985; Stern, Silverman, Houser, Josephson, Capogrossi, Nicholls, Lederer & Lakatta 1988). This is due to the cell isolation procedure, which intentionally removes all the connective tissue in order to separate cells and expose the cell membrane. The inotropic state of these preparations is then measured optically (typically using a photodiode array or an automatic edge following device), using shortening and/or velocity of shortening as the "index of contractility". Although attempts have been made to re-attach cells after isolation in order to

measure tension (e.g. Fabiato, 1981; Copelas, Briggs, Grossman & Morgan 1987; Argibay, Garnier, Le Guennec, Nigretto & Peineau, 1989), such procedures are technically demanding and not always successful - e.g. Copelas et al (1987) managed to record only tonic tension, the cells tearing away when stimulated. Furthermore, in the absence of a direct comparison of isometric and isotonic contraction, it is unclear whether there are likely to be significant advantages to be obtained by measuring tension.

A further reason for interest in a comparison between isometric and isotonic contraction is that when differences occur between studies on shortening measurements in single cardiac cells, and tension measurements in isolated multicellular preparations (e.g. papillary muscles), it is not clear whether these differences are due to the different mechanical conditions, or because of other differences between isolated cells and multicellular preparations (Stern et al, 1988; Krueger, 1988). For example, studies on isolated papillary muscles (Allen & Orchard, 1983b) showed that anoxia or cyanide caused a small transient increase of tension, followed by a sustained decline in tension, and measurements on Langendorff perfused isolated hearts indicated that the reduced tension was associated with an intracellular acidosis (Allen, Morris, Orchard & Pirolo, 1985). However, others (Stern et al, 1988; Eisner, Lederer, Nicholls, O'Neill, Smith & Valdeolmillos, 1989) working with single cells have found that exposure of isolated cardiac cells to cyanide caused an increase in shortening which could be sustained for many minutes. This difference was attributed to a prolonged alkalosis measured in the isolated myocytes (Eisner et al, 1989), but it is also possible that these differences might in part be due to the different mechanical conditions of the preparations (Stern et al, 1988).

In this series of experiments, some of these issues have been

examined by comparing the response of isolated ferret papillary muscles to a range of positive and negative inotropic interventions while they were contracting (i) isometrically and (ii) isotonically at low load, i.e. shortening. The experimental arrangement was such that either isometric or isotonic beats could be interpolated into a protocol consisting mainly of the other mode (see section 2.2). This allowed the effects of interventions on both isometric and isotonic contractions to be followed virtually simultaneously.

3.3.2 Results.

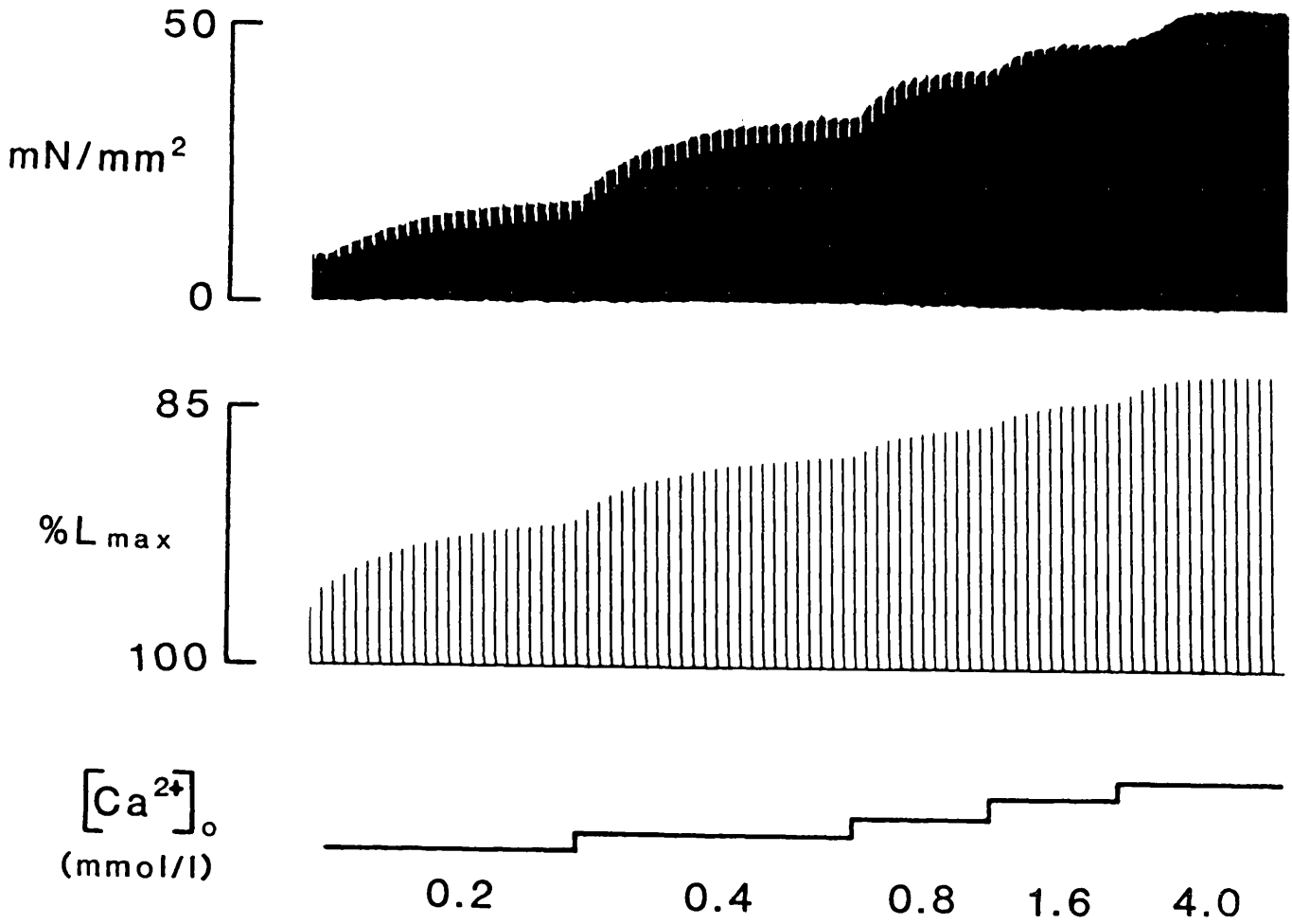
Figure 24 shows the effects of increasing the extracellular Ca^{2+} concentration in the superfusing Tyrode solution from 0.2 mmol/l to 4 mmol/l. The muscle was in mainly isometric mode, with isotonic shortening at low load interpolated every tenth beat. It can be seen that the responses of tension and shortening were broadly similar over this range of extracellular [Ca^{2+}], both showing, as expected, a stepwise increase with increasing [Ca^{2+}]. In other experiments with a similar protocol, it was found that the addition of either isoprenaline (10^{-7} mol/l) or strophanthidin (10^{-5} mol/l) also caused tension and shortening to increase in a parallel fashion. These inotropic interventions thus seem to have very similar effects on tension and shortening.

When the effects of these interventions on tension and shortening were compared quantitatively, it was noted that there was generally a larger fractional change in isometric tension than in shortening. This feature was also seen with all the other interventions studied, and in order to describe this difference and to compare the effects of different interventions the following numerical index was chosen. The fractional change in isometric tension was compared with the fractional change in isotonic shortening when an intervention was applied by using

FIGURE 24.

FIGURE 24.

The effects of increasing extracellular Ca^{2+} concentration on isometric developed tension (above) and isotonic shortening (below) in an isolated ferret papillary muscle stimulated at 0.5 Hz. The muscle was performing mainly isometric contractions, but every tenth beat an isotonic contraction at low load was interpolated. As extracellular $[Ca^{2+}]$ was increased, developed tension and isotonic shortening increased in parallel.


50
mN/mm²
0
85
%L max
100
[Ca²⁺]o
(mmol/l)
0.2
0.4
0.8
1.6
4.0

Note: R was calculated from values of tension and shortening before and after each intervention, and not related to one baseline value taken before any interventions had been applied.

the equation:

$$R = \frac{(x_2 - x_1)/x_1}{(y_2 - y_1)/y_1,}$$

where x_1 = isometric tension before the intervention,

x_2 = isometric tension after the intervention,

y_1 = isotonic shortening before the intervention,

y_2 = isotonic shortening after the intervention.

R is thus a dimensionless quantity which indicates, for any given intervention, whether the fractional change in tension was greater or smaller than the fractional change in shortening. When R was computed for changes in extracellular $[Ca^{2+}]$, the value was 1.49 $\pm$ 0.14 (n = 8); in other words, for a given increase in extracellular $[Ca^{2+}]$, tension increased 1.49 times more than shortening.

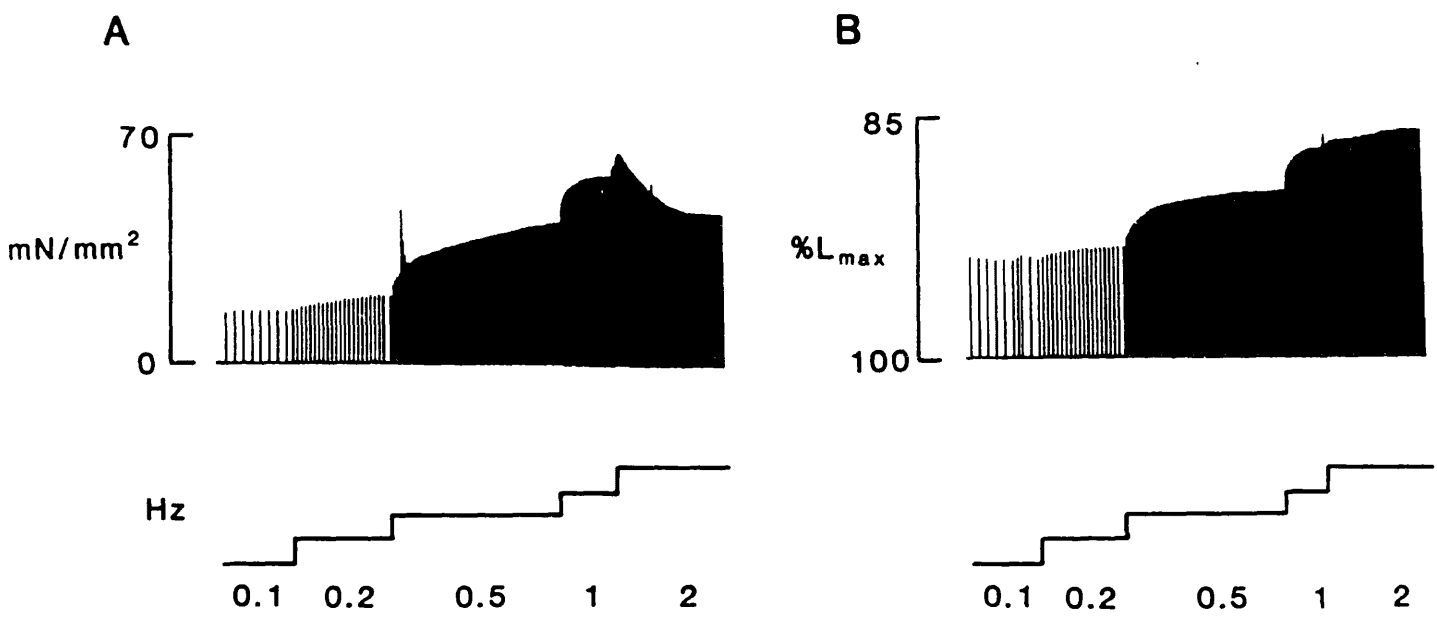
Figure 25 shows the effects of increasing the frequency of stimulation on tension and shortening. Increasing the frequency from 0.1 Hz to 1 Hz caused both tension and shortening to increase. When R was calculated for changes in this frequency range, the value was 2.04 $\pm$ 0.31 (n = 6). Studies with the photoprotein aequorin (e.g. Allen & Kurihara, 1980) have demonstrated that both increasing extracellular $[Ca^{2+}]$ and increasing frequency cause tension to increase by increasing the Ca^{2+} transient. Hence, since the increase in tension is apparently brought about by the same mechanism, it would be expected that the values of R for these two interventions would not be significantly different, and this is indeed the case ($p > 0.1$).

Although increasing frequency caused tension and shortening to increase in parallel at lower frequencies, Figure 25 illustrates that something different happened at the highest frequency. At this frequency, shortening was well maintained, but tension, after showing an initial increase, then declined substantially. When R was calculated for the initial change in tension and shortening after

FIGURE 25.

FIGURE 25.

The effects of increasing stimulation frequency on isometric developed tension (A) and unloaded shortening (B) in an isolated ferret papillary muscle. As the frequency was increased from 0.1 to 1 Hz, tension and shortening increased in parallel. At the highest frequency, however, tension showed a marked decline, while shortening was well maintained.


A
70
mN/mm²
0
Hz
0.1
0.2
0.5
1
2
B
85
%L max
100
0.1
0.2
0.5
1
2

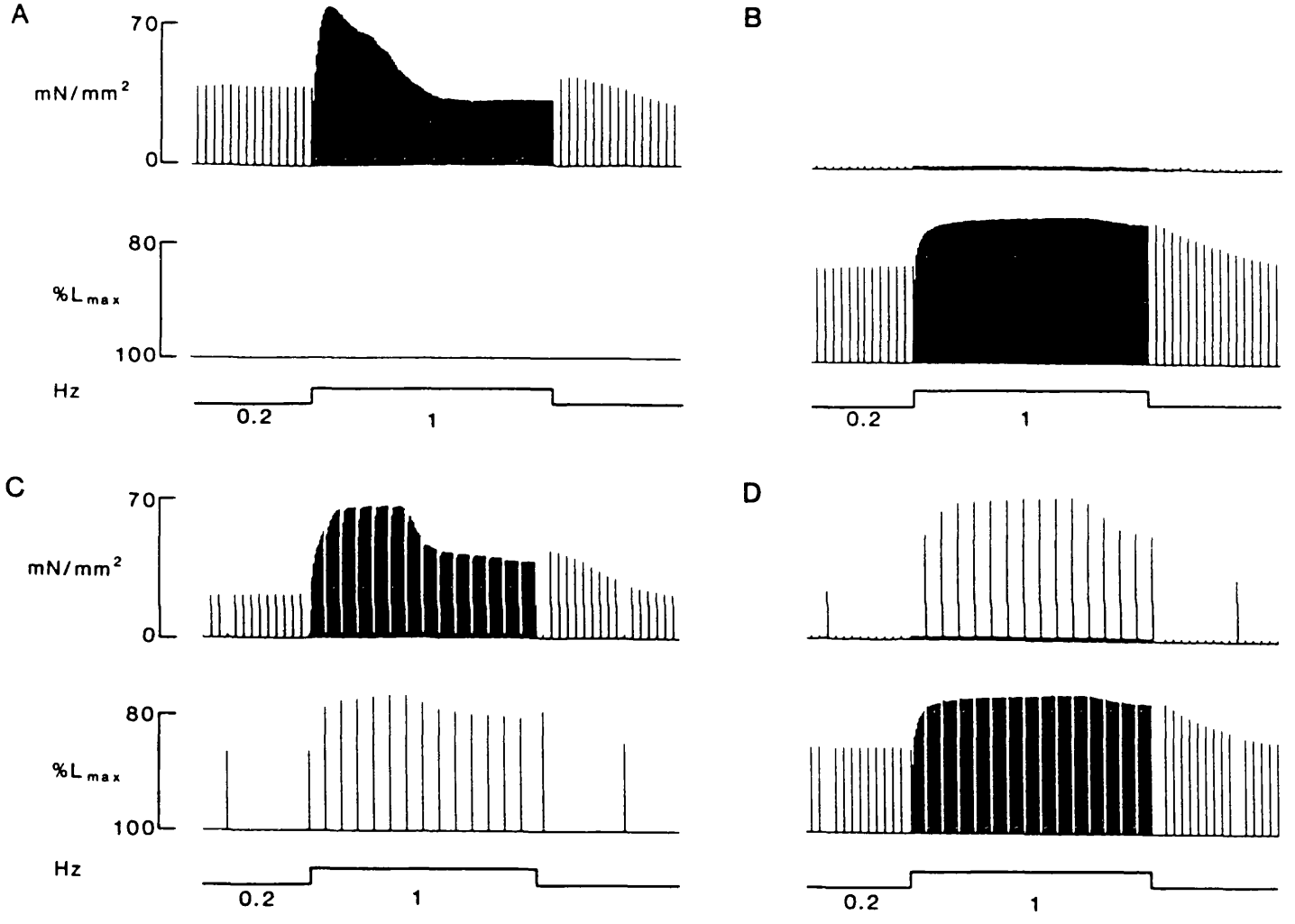
changing to high frequency, the value was 1.72 ± 0.20, not significantly different from the values quoted above. However, when R was determined for the subsequent change compared with the initial increase, the value was 7.49 ± 1.73 (n = 12), clearly different from the other values. This indicates that different processes may be affecting tension and shortening in this situation.

Figure 26 shows an experiment designed to investigate this phenomenon further. In this experiment, the frequency was increased from 0.2 Hz to 1 Hz, at which the preparation showed a marked decline in isometric tension (Figure 26A), while unloaded shortening (Figure 26B) was well maintained. In Figure 26C, unloaded shortening was allowed to occur every tenth beat, thus monitoring tension and shortening in predominantly (90%) isometric mode. In Figure 26D, unloaded shortening occurred 90% of the time, while every tenth beat was an isometric contraction, thus monitoring tension and shortening in predominantly isotonic mode. Since it is known that an isometric contraction consumes more energy than an isotonic contraction (McDonald, 1966; Coleman, 1968), Figure 26 illustrates a gradation in energy consumption from isometric (A), 90% isometric (C), 10% isometric (D), to fully isotonic contraction (B). It can be seen that as energy consumption decreased (A,C,D), so the decline in isometric tension at 1 Hz stimulation frequency also decreased. Conversely, as energy consumption increased (B,D,C), so the small decline in isotonic shortening at 1 Hz stimulation frequency increased. Figure 26C & D show that when tension and shortening were monitored simultaneously, shortening showed less of a decline than tension. When R was calculated in this situation, the value was 2.46 ± 0.53 (n = 5). Although the mean was larger, this value was not significantly different from the values for extracellular [Ca^{2+}] and frequency mentioned above. The significance of these results is considered

FIGURE 26.

FIGURE 26.

The effects of increasing stimulation frequency. A. Isometric contraction. B. Isotonic shortening. C. Isometric contraction with shortening interpolated every tenth beat. D. Isotonic shortening with every tenth beat isometric. Tension exhibited a decline which became less pronounced as more shortening was interpolated (A, C, D). Shortening showed a small decline which became larger as more isometric beats were interpolated (B, D, C).


A
70
mN/mm²
0
80
%L max
100
Hz
0.2
1
B
0.2
1
C
70
mN/mm²
0
80
%L max
100
Hz
0.2
1
D
0.2
1

further in Section 3.3.3.

Much recent work on both multicellular cardiac preparations and single cardiac myocytes has been concerned with the effects of acidosis, hypoxia and metabolic blockade, and the way in which these relate to events occurring during ischaemia. The effects of these negative inotropic interventions on tension and shortening were also compared in the papillary muscle preparation.

Figure 27 illustrates the effects of acidosis produced by increasing the CO_2 in the superfusing solution from 5% to 15%. Acidosis caused a rapid depression of tension, which was then followed by a slower recovery to less than the control level, as has been previously noted (e.g. Allen & Orchard 1983a; Orchard 1987; Solaro, Lee, Kentish & Allen, 1988 - see Appendix 1). It can be seen that the effects on shortening were similar, but as was the case with the positive inotropic interventions mentioned above, the fractional effect was smaller. Figure 27B shows that essentially the same results were obtained when the muscle was exposed to acidosis under predominantly isotonic conditions. The computed R value was 1.61 $\pm$ 0.09 (n = 6), and this was not significantly different from the values for increasing extracellular $[Ca^{2+}]$ or frequency.

Figure 28 shows the effects of 2 mmol/l cyanide on tension and shortening. In Figure 28A the muscle was in isometric mode with shortening interpolated every tenth beat. Cyanide caused a rapid decline in tension to 30 - 40% of control, which was then relatively well maintained (see Section 3.1). Again the effect on shortening was similar, but the fractional effect was less. When cyanide was applied with the muscle in predominantly isotonic mode (Figure 28B), broadly similar results were seen. However, it should be noted that cyanide had a smaller effect on both tension and shortening than when it was applied with the muscle in predominantly isometric mode (Figure 28A).

FIGURE 27.

FIGURE 27.

The effects of acidosis on isometric developed tension and shortening in an isolated ferret papillary muscle. A. Isometric contraction with every tenth beat unloaded. B. Unloaded shortening with every tenth beat isometric. There was an initial decline and a subsequent small recovery of both tension and shortening.

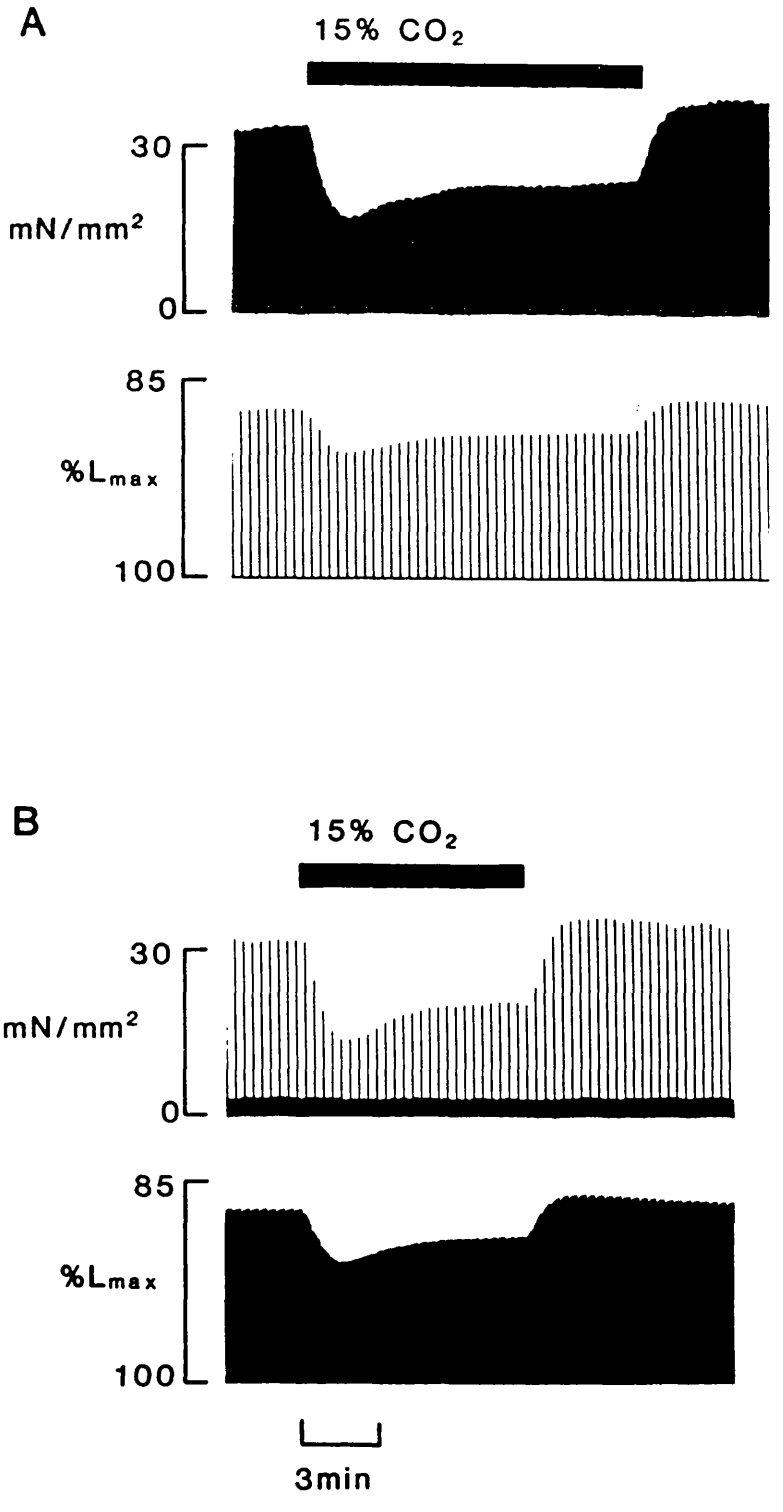
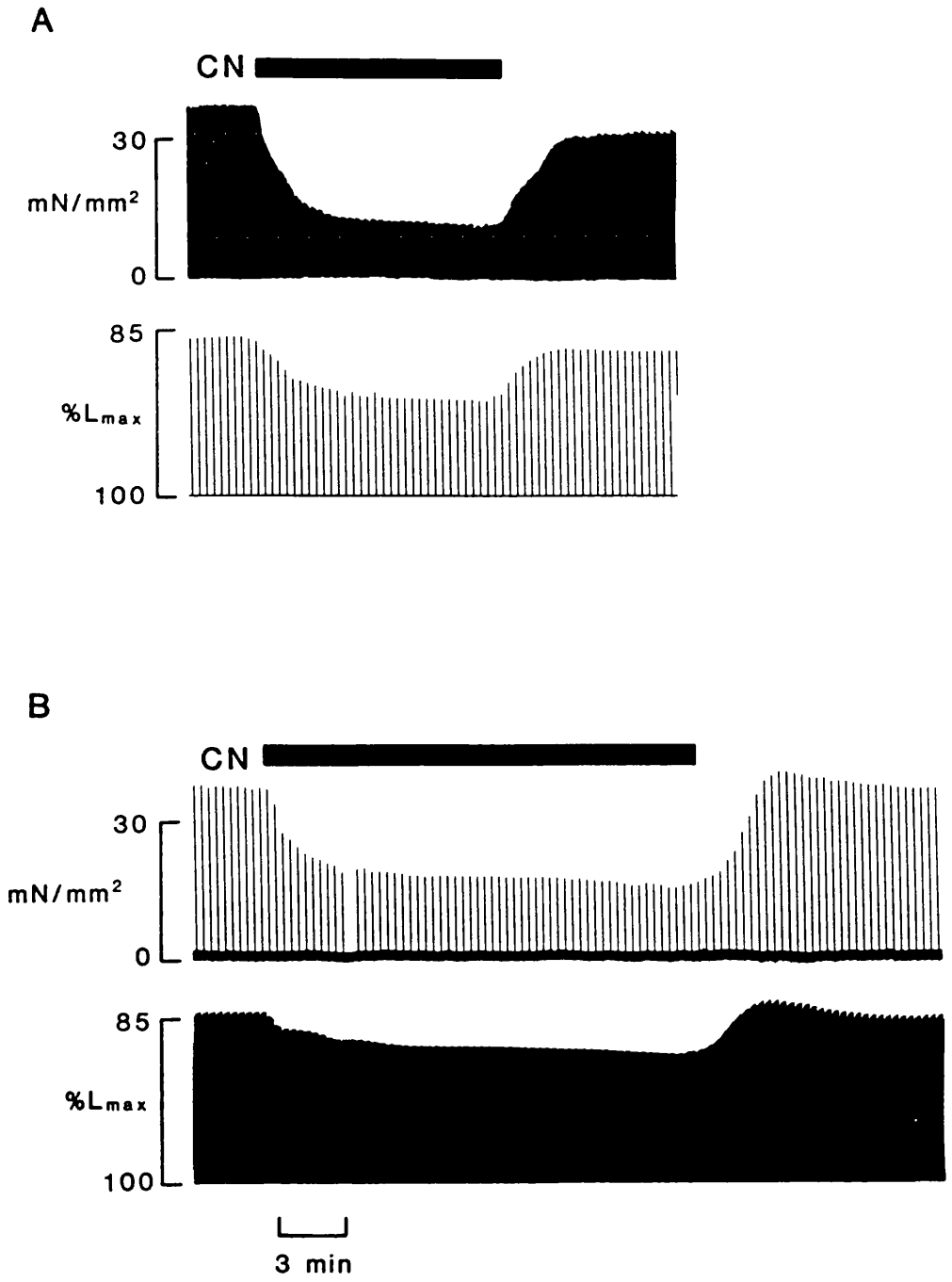

A
15% CO_2
30
mN/mm²
0
85
%L_{max}
100
B
15% CO_2
30
mN/mm²
0
85
%L_{max}
100
3min

FIGURE 28.

FIGURE 28.

The effects of 2 mmol/l NaCN on isometric developed tension and unloaded shortening in an isolated ferret papillary muscle. A. Isometric contraction with every tenth beat unloaded. B. Unloaded shortening with every tenth beat isometric. Both tension and shortening declined when cyanide was applied. Note that the effect of cyanide was less pronounced with the muscle in predominantly isotonic mode.


A
CN
30
mN/mm²
0
85
%Lmax
100
B
CN
30
mN/mm²
0
85
%Lmax
100
3 min

As noted above, energy consumption is smaller during isotonic contractions; the contribution this may make to the effects of cyanide is considered further in Section 3.3.3. The computed R value for cyanide was, 1.95 $\pm$ 0.26 (n = 6), which was not significantly different from the values for increasing extracellular [Ca^{2+}] or frequency.

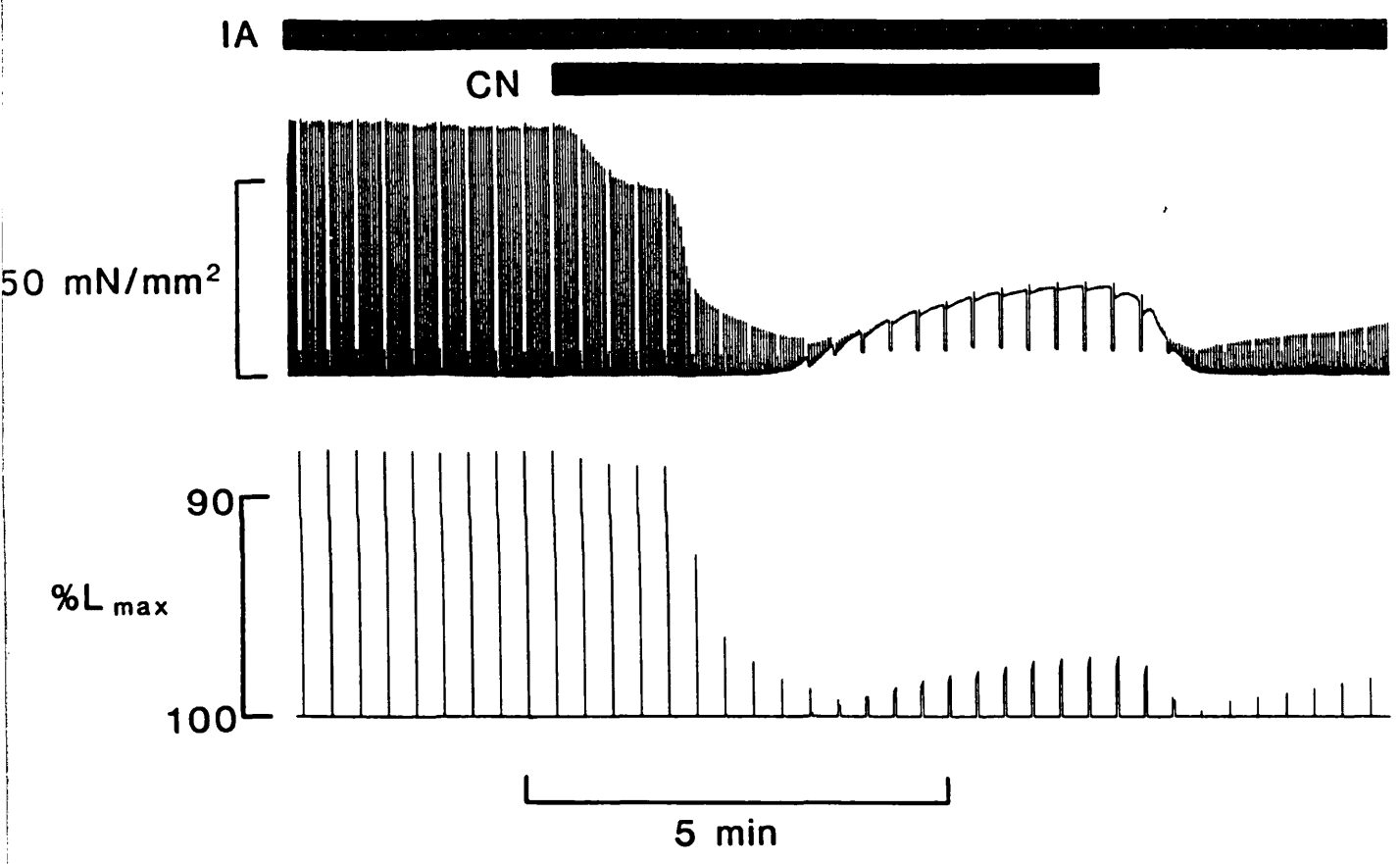
Figure 29 illustrates the effect of metabolic blockade on tension and shortening. Metabolic blockade was produced by first exposing the muscle to iodoacetic acid to inhibit glycolysis and then adding cyanide to inhibit oxidative phosphorylation. Iodoacetate on its own had little effect on either tension or shortening, but when metabolic blockade was produced by adding cyanide, tension and shortening both rapidly declined to zero, and shortly afterwards a rigor contracture began to develop. It can be seen that the "shortening contracture" began to develop at the same time as the tension contracture. Furthermore, when metabolic blockade was relieved by the removal of cyanide, the isometric and isotonic contractures relaxed at the same time, and recovery of twitch tension and shortening also occurred together. A similar result was seen with the muscle in predominantly isotonic mode.

Because shortening eventually reaches a limit set by the steady-state length-tension relation, it is possible that the peak shortening velocity during a contraction could be a more sensitive indicator of the inotropic state than shortening. For this reason, peak shortening velocity was also examined over the range of inotropic intervention studied. It was found that the ratio comparing peak shortening velocity with shortening (calculated in the manner shown at the beginning of this Section) was 1.64 $\pm$ 0.19 (n = 32). In other words, peak shortening velocity was a more sensitive index of contractility than shortening, being roughly comparable to isometric tension.

FIGURE 29.

FIGURE 29.

The effects of metabolic blockade with 100 μmol/l iodoacetate and 2 mmol/l NaCN in an isolated ferret papillary muscle in predominantly isometric mode. Iodoacetate on its own had little effect, but when cyanide was added, developed tension and shortening rapidly declined to zero, and a contracture in both modes began to deveop at the same time. When cyanide was removed, the contractures relaxed together and tension and shortening recovered with a similar time course.


IA
CN
50 mN/mm²
90
%L max
100
5 min

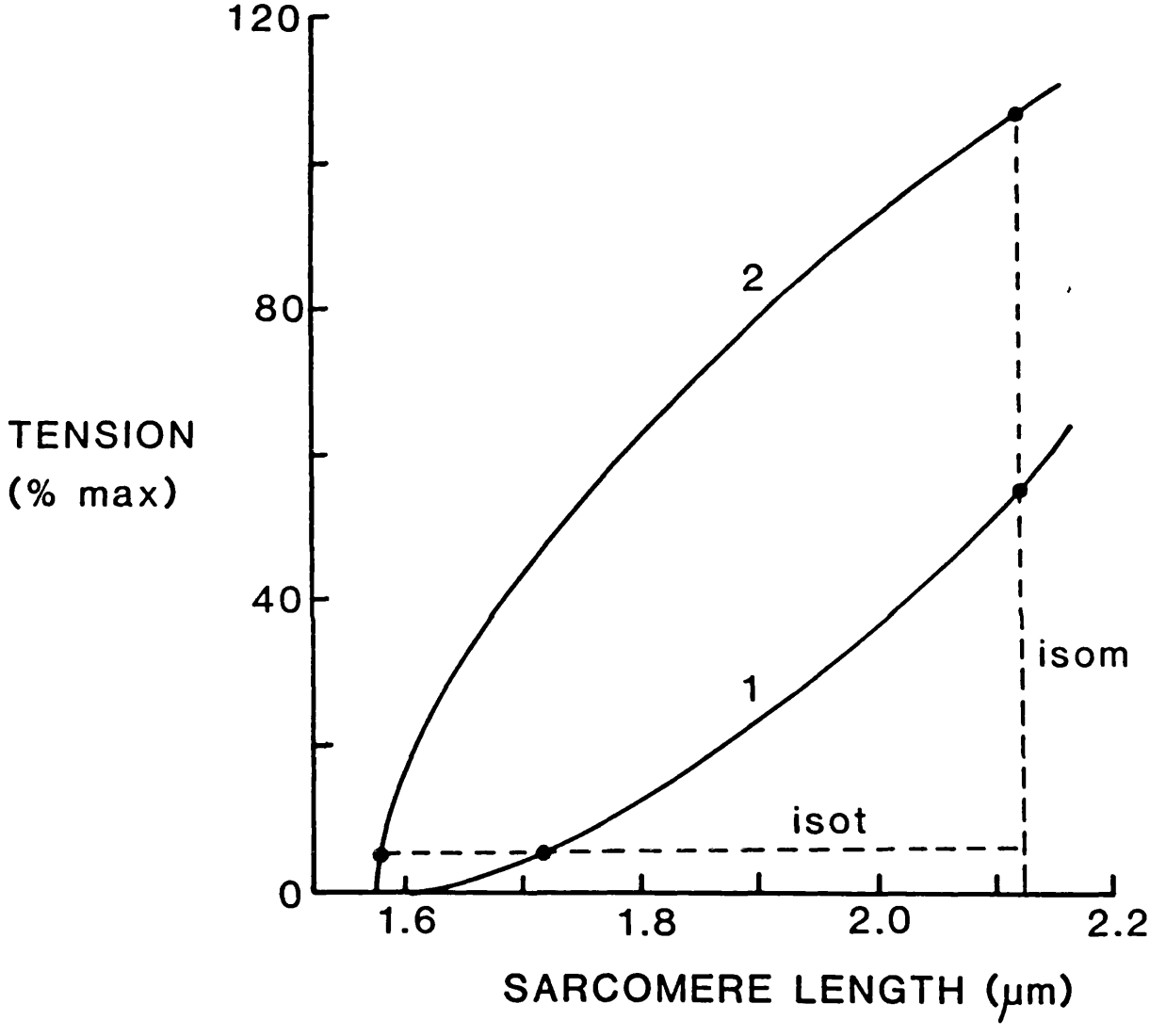
3.3.3 Discussion.

In these experiments, the effects of inotropic interventions on papillary muscles which were allowed to contract under isometric and isotonic conditions were examined. The main finding was that most interventions affect tension and shortening in a similar manner. This is encouraging for comparisons between studies using multicellular preparations, in which isometric tension is generally measured, and studies using isolated cardiac myocytes, in which unloaded shortening is generally used as a measure of contractility (see Section 3.3.1). One point which emerged from the results was that both positive and negative inotropic interventions had a larger fractional effect on tension than shortening. The magnitude of this effect was 1.5 - 2 for the interventions studied. On the other hand, peak shortening velocity responded in a way which was quite similar to isometric tension. The differences between tension and shortening may arise from the shape of the cardiac tension-length relation, as illustrated in Figure 30. The curves 1 and 2 in Figure 30 are taken from the data of Kentish, ter Keurs, Ricciardi, Bucx & Noble (1986), and represent tension-length relations for intact cardiac muscle in two different inotropic states. The course of an isometric contraction is illustrated by "isom". The muscle contracts until it reaches the tension indicated by the tension-length relation for that inotropic state, and moves back along the same line during relaxation. The course of an isotonic contraction is indicated by "isot". When the muscle performs an isotonic contraction, it develops tension to a preset value and then shortens down until it reaches the intersection with the tension-length relation. During relaxation, the muscle lengthens again. If a positive inotropic intervention is applied, the muscle moves to a new tension-length relation, for example from curve 1 to curve 2. It can be seen that for curves of this general shape, the relative change in tension produced

FIGURE 30.

FIGURE 30.

The effects of changing the inotropic state of a muscle on tension and shortening (after the data of Kentish et al, 1986). Curves 1 and 2 represent different inotropic states of the muscle. "Isom" indicates the course taken by an isometric contraction and "isot" that taken by an isotonic contraction.


120
80
40
0
TENSION
(% max)
2
1
isom
isot
1.6
1.8
2.0
2.2
SARCOMERE LENGTH (μm)

is greater than the relative change in shortening.

When preparations were stimulated at high frequency, isotonic shortening increased to a level which was well maintained, but tension showed a considerable decline after the initial rise. This was reflected in the calculated R value for the sag, which was significantly different from other interventions. What is the cause of the difference of behaviour under these conditions? It has been known for some years that the oxygen consumption of isotonic contractions is approximately one third that of isometric contractions (McDonald, 1966; Coleman 1968). Because of the greater energy consumption of isometric contractions, more ATP is consumed and hence, due to a greater conversion of creatine phosphate to creatine, a greater build-up of intracellular inorganic phosphate would be expected. Inorganic phosphate is well known to reduce the force of contraction in cardiac muscle (Kentish, 1986). Also, in a perfused muscle preparation of the type used for these experiments, a greater oxygen consumption would lead to a greater depletion of oxygen in the core of the muscle. This causes cells to rely more on anaerobic energy production, and hence lactic acid production from glycolysis is increased in these cells, leading to an intracellular acidosis. Intracellular acidosis also reduces the force of contraction in cardiac muscle (Fabiato & Fabiato, 1978). Hence these two factors associated with increased energy consumption might account for the greater sag of isometric tension as compared with isotonic shortening.

However, an additional factor which might be implicated has recently been described. Cooke and Pate (1985), working with skinned rabbit psoas muscles, found that the maximum velocity of shortening was unaffected by increases in inorganic phosphate or by decreases in pH, in marked contrast to isometric tension production. Although it has subsequently been found that these factors may in fact have some effect

on shortening velocity (Metzger & Moss, 1987; Cooke, Franks, Luciani & Pate, 1988), this nevertheless raised the possibility that part of the observed difference in behaviour was due to a differential sensitivity of tension and shortening to the effects of phosphate and pH, rather than solely due to the differences in oxygen consumption. This possibility was investigated in experiments where either isometric beats were interpolated during predominantly isotonic contraction, or isotonic beats were interpolated during predominantly isometric contraction. With this protocol it was found that the R value, although greater than those found for other interventions was not significantly greater than them. In other words, the factors causing the negative inotropic effect occurring during the secondary sag produced a fractional effect on tension and shortening which was statistically indistinguishable from other positive and negative inotropic effects studied. Furthermore, peak shortening velocity was even more similar to isometric tension than shortening. This suggests that differential sensitivity of tension and shortening to inorganic phosphate and pH was not a major factor in this situation, and implies that the difference in behaviour observed can be largely ascribed to differences in oxygen consumption under these conditions.

Single cardiac myocytes are now widely used for the study of cardiac muscle. This study demonstrates that when inotropic interventions are applied, unloaded shortening does not produce unduly different results from isometric contraction, although the fractional effect on shortening is smaller. Inotropic interventions had a greater fractional effect on peak shortening velocity than on shortening per se, so peak shortening velocity may be a useful additional index of contractility in isotonic preparations, because it is more sensitive to changes in the inotropic state.

Under conditions when the energy supply to a muscle fails

completely, as occurs in metabolic blockade, contractures of tension and shortening developed at about the same time when measured simultaneously in the same muscle. Thus there appears to be no fundamental difference in the sensitivity of crossbridges to lack of ATP when operating isometrically or isotonically, and hence the rounded forms seen in single cardiac myocytes under conditions of de-energisation (Cobbold & Bourne, 1984; Stern et al, 1988; Li, Altschuld & Stokes, 1988) are likely to correspond to rigor contractures in the isometric preparation. On the other hand, because the work output, and hence energy consumption, of unloaded preparations is smaller, shortening may be able to continue for longer in situations where the energy supply is limited, and development of a contracture may be delayed in these circumstances. For example, cyanide produced a slower effect with a muscle in predominantly isotonic mode as compared to predominantly isometric mode (compare Figure 28A & B).

In conclusion, the results of these experiments demonstrate that most inotropic interventions produce qualitatively very similar effects on tension and shortening, but have a fractionally larger effect on tension. Other interventions may produce effects which appear qualitatively different, and where this occurs, the differences can be largely explained by the different energy consumption of the preparation under the two conditions.

3.4 THE EFFECTS OF PIMOBENDAN, A NEW INOTROPIC AGENT.

3.4 THE EFFECTS OF PIMOBENDAN, A NEW INOTROPIC AGENT.

3.4.1 Background.

As discussed in Chapter 1, cardiac muscle generates tension when an action potential causes entry of Ca^{2+} through the surface membrane and release of Ca^{2+} from the sarcoplasmic reticulum. The main groups of inotropic agents in clinical use until recently all achieve their inotropic effects by means of an increase in the level of intracellular free [Ca^{2+}] during systole. Cardiac glycosides such as digitalis inhibit the Na^+/K^+ ATPase, and the most widely accepted theory for their mechanism of action is that inhibition of the Na^+/K^+ ATPase leads to a rise in the intracellular [Na^+]. Na^+ is then exchanged for Ca^{2+} on the Na^+/Ca^{2+} exchanger, leading to a greater intracellular accumulation of Ca^{2+}, greater Ca^{2+} release with each action potential, and hence more tension (Hansen, 1986). The beta adrenergic agonists elevate intracellular cyclic AMP, the level of which is controlled by a balance between synthesis by adenylate cyclase and breakdown by phosphodiesterase. This then activates cyclic AMP-dependent protein kinases, which are responsible for phosphorylating many proteins in the cell, including a Ca^{2+} channel in the surface membrane, leading to greater Ca^{2+} influx, and phospholamban in the sarcoplasmic reticulum, leading to more rapid Ca^{2+} uptake (Tsien, 1977; England, Pask & Mills, 1984). These changes also cause greater Ca^{2+} release with each beat (e.g. Allen & Kurihara, 1980), and hence greater tension.

However, in spite of the use of cardiac glycosides, beta adrenergic agonists and other supportive agents, the prognosis of congestive heart failure has remained very poor (Franciosa, Wiler, Ziesche & Cohn, 1983). Therefore, during the last few years, there has been considerable interest in the development of new inotropic agents with novel mechanisms of action. Two mechanisms have generated particular interest. In the first of these, a rise in cyclic AMP

levels is achieved by inhibition of phosphodiesterase activity (Scholz & Meyer, 1986). A new group of agents which have this effect have been synthesised and include amrinone and milrinone, which have both begun to be clinically evaluated (Colucci, Wright & Braunwald, 1986). The other mechanism involves agents which can increase the Ca^{2+}-sensitivity of the cardiac myofibrils (Rüegg, 1986; Blinks & Endoh, 1986). That is, they alter the coupling between Ca^{2+} and tension production, so that more tension is generated for the same myoplasmic $[Ca^{2+}]$ (see section 1.3).

Pimobendan (UDCG 115 - BS) is a new inotropic agent which is currently undergoing limited clinical trials. Early studies (Honerjager, Heiss, Schafer-Korting, Schonsteiner & Reiter, 1984; Berger, Meyer, Scholz & Starbatty, 1985) suggested that this agent worked as a phosphodiesterase inhibitor, causing a rise in the levels of cyclic AMP. More recently, however, experiments on skinned cardiac myofibrils have suggested that pimobendan may also have a direct Ca^{2+}-sensitising effect (Rüegg, 1986; van Meel, 1987; Fujino, Sperelakis & Solaro, 1988). This agent thus apparently possesses both of the newer mechanisms of inotropic action. However, it has not yet been ascertained whether both of these mechanisms are active in intact myocardium. In this series of experiments, the effects of pimobendan on skinned ferret cardiac myofibrils was examined, to establish its direct effect on the Ca^{2+} sensitivity of the myofibrils in this preparation. Its effects were then studied on isolated, intact ferret ventricular muscle which was microinjected with the photoprotein aequorin in order to measure the Ca^{2+} transients.

3.4.2 The effects of pimobendan on skinned ventricular muscle.

In order to confirm previous work and establish that pimobendan is able to produce a direct sensitisation of the skinned fibres in this

preparation, its effects on force production in skinned ferret ventricular muscle were tested. The preparation was first exposed to a series of increasing [Ca^{2+}] (Figure 31), and were then returned to the relaxing solution. 20 /umol/l pimobendan was added to the solutions, and the skinned fibres were again exposed to the same series of [Ca^{2+}]. Finally, the skinned fibres were once more exposed to the series of [Ca^{2+}] in the absence of pimobendan. Control exposures before and after exposure to pimobendan were averaged in order to correct for any deterioration of the preparations. Note that pimobendan increased tension at submaximal Ca^{2+}-activation, while at maximal Ca^{2+}-activation (13 /umol/l) the effect of pimobendan on tension was very slight (to 112% of control). Figure 32 shows the results of the experiment shown in Figure 31 plotted with tension at maximal [Ca^{2+}] normalised to 100% in the presence and absence of pimobendan. It can be seen that pimobendan produces a shift of the curve to the left. This indicates that more tension is being produced for a given [Ca^{2+}], i.e. a Ca^{2+}-sensitisation effect has occurred. Similar results were seen in a total of 11 experiments. At the Ca^{2+} concentration of 1.7 /umol/l, 20 /umol/l pimobendan increased tension production by 53 $\pm$ 3% (mean $\pm$ S.E.M., n = 4), while 100 /umol/l pimobendan increased tension production by 124 $\pm$ 31% at 1.7 /umol/l Ca^{2+} and by 12.9 $\pm$ 2.8% at 13 /umol/l Ca^{2+} (n = 7).

3.4.3 The effects of pimobendan on intact ventricular muscle.

Figure 33 shows the effects of adding 25 /umol/l pimobendan to an isolated ferret papillary muscle injected with aequorin. Preliminary experiments showed that increasing pimobendan to 50 /umol/l had no further inotropic effect. It can be seen that this caused a slowly developing positive inotropic effect, which was mirrored by a similar slow increase in the light signal. The averaged records below show that the increase in the amplitude of the twitch was accompanied by an

FIGURE 31.

FIGURE 31.

The effects of pimobendan on the relation between Ca^{2+} concentration and tension in skinned cardiac muscle fibres. Records show tension (above) and step changes in Ca^{2+} concentration (below). Skinned muscle from a ferret right ventricle was exposed to a series of increasing Ca^{2+} concentrations in the presence of 20 /umol/l pimobendan (middle panel). Controls were performed before and after exposure to pimobendan (first and last panels).

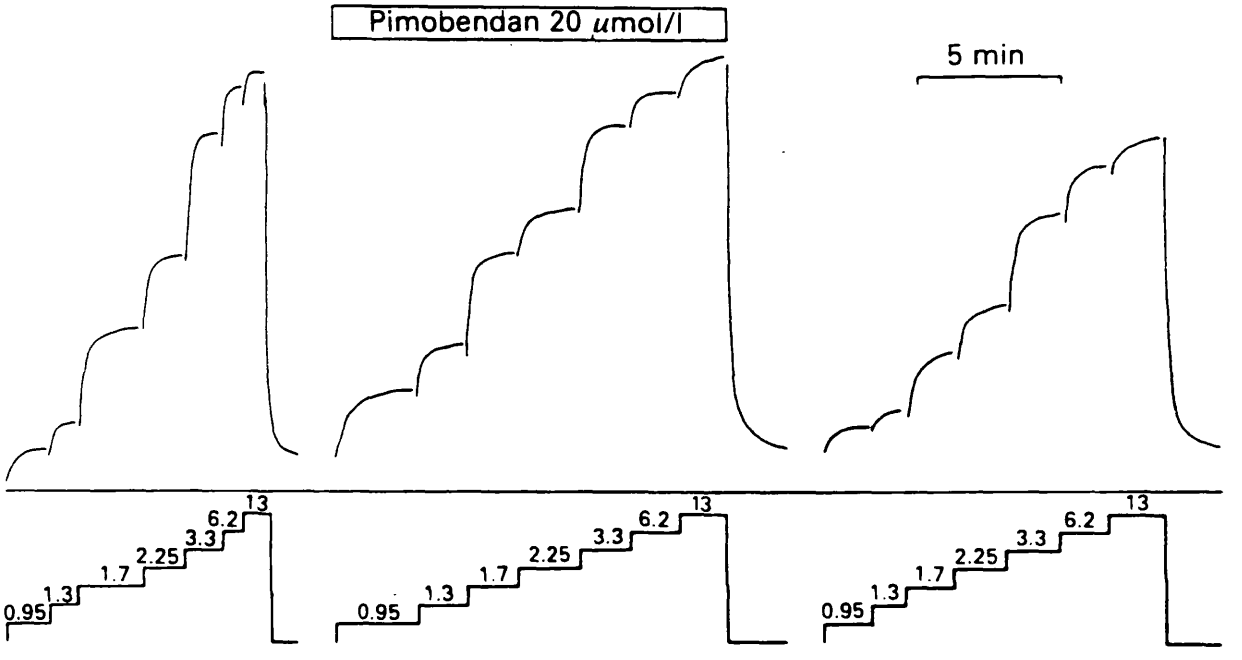
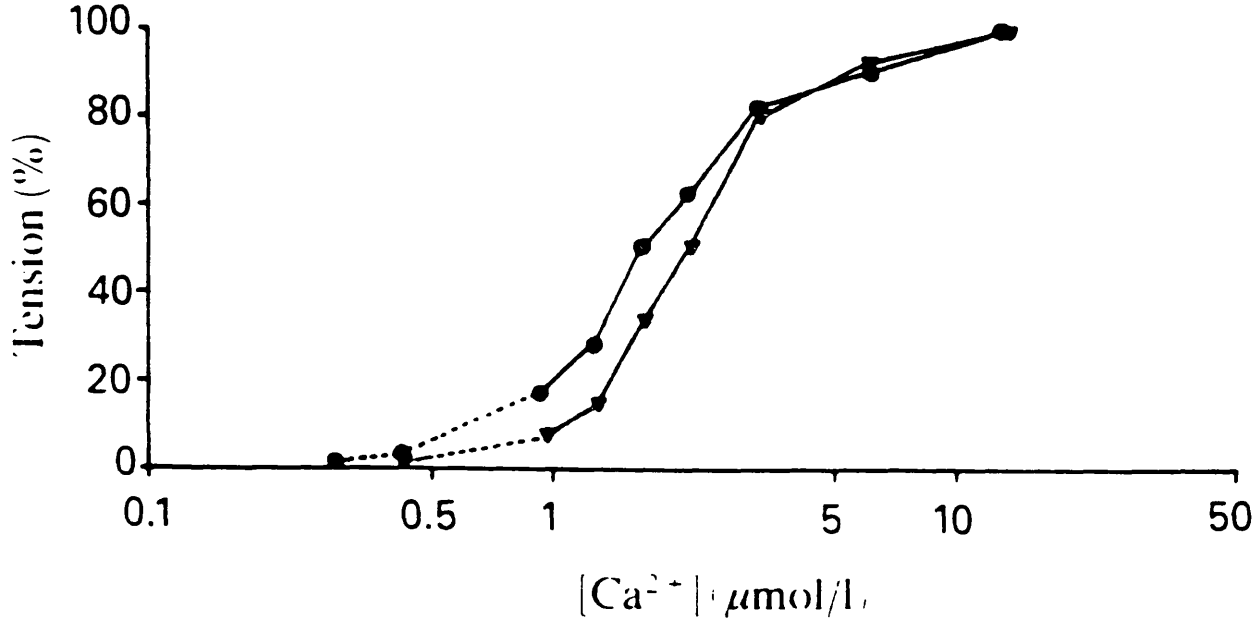

Pimobendan 20 μmol/l
5 min
0.95
1.3
1.7
2.25
3.3
6.2
13

FIGURE 32.

FIGURE 32.

The effects of pimobendan on the relation between Ca^{2+} concentration and tension in skinned ferret ventricular muscle. The results of the experiment illustrated in Figure 31 have been normalised with respect to activation at 13 μmol/l Ca^{2+} and plotted to show the relationship between log $[Ca^{2+}]$ and tension under control conditions (average of before and after) and in the presence of pimobendan. Pimobendan produced a Ca^{2+}-sensitisation; i.e. the $[Ca^{2+}]$ required for half maximal activation was lowered.


100
80
60
40
20
0
Tension (%)
0.1
0.5
1
5
10
50
[Ca2+] µmol/l

increase in the size of the systolic rise in $[Ca^{2+}]_i$ (Ca^{2+} transients). In Figure 33B (iii) & (iv), the same averaged records have been normalised to the same amplitude and superimposed. It can be seen that pimobendan (dotted record) caused an abbreviation of the timecourse of the Ca^{2+} transients, while simultaneously causing the twitch to be slightly prolonged. This is a surprising combination because one would predict that if the timecourse of the Ca^{2+} transients was abbreviated, the timecourse of the tension would also be shorter. This observation is considered further in section 3.4.5.

In order to determine whether pimobendan produces an in vivo sensitisation effect, it is necessary to determine whether the tension produced in the presence of pimobendan is greater than 'expected' for the size of the Ca^{2+} transients. Figure 34 illustrates the experimental protocol we used in order to do this. The muscle was first exposed to a series of increasing extracellular $[Ca^{2+}]$. This caused the Ca^{2+} transients and the twitch tension to increase, as shown in the upper part of the Figure. The muscle was then returned to a $[Ca^{2+}]$ at which the twitch tension was 20 -30% of maximum, and 25 μmol/l pimobendan was added. This produced an increase in the amplitude of both the Ca^{2+} transients and the twitch as mentioned above, and shown in the lower part of Figure 34. The light signal and tension in the presence of pimobendan could then be compared with those of the extracellular $[Ca^{2+}]$ series to see if a sensitisation effect had occurred.

The results of 5 such experiments are shown in Figure 35, in which the peak of the aequorin light signal is plotted against the peak developed tension. All the points have been normalised to the control period immediately before adding pimobendan (open triangle). It can be seen that the extracellular $[Ca^{2+}]$ series forms an approximately linear relationship, analogous to the pCa/tension curve shown in Figure 33.

FIGURE 33.

FIGURE 33.

The effects of pimobendan on aequorin light and tension in an isolated ferret papillary muscle.

A. A continuous record showing aequorin light (above) and tension (below). The addition of 25 /umol/l pimobendan caused a slowly developing positive inotropic effect which was accompanied by an increase in the size of the Ca^{2+} transients.

B. Panels (i) and (ii) show averaged records of aequorin light (above) and tension (middle) from the control period (i) and in the presence of pimobendan (ii). The stimulus marker is also shown (below). The same averaged records were then normalised to the same amplitude and superimposed to show the effects of pimobendan on the timecourse of the Ca^{2+} transient (iii) and twitch (iv). Note that the timescale on (i) applies to (i), (ii) and (iv), while panel (iii) is displayed on double this timescale for clarity.

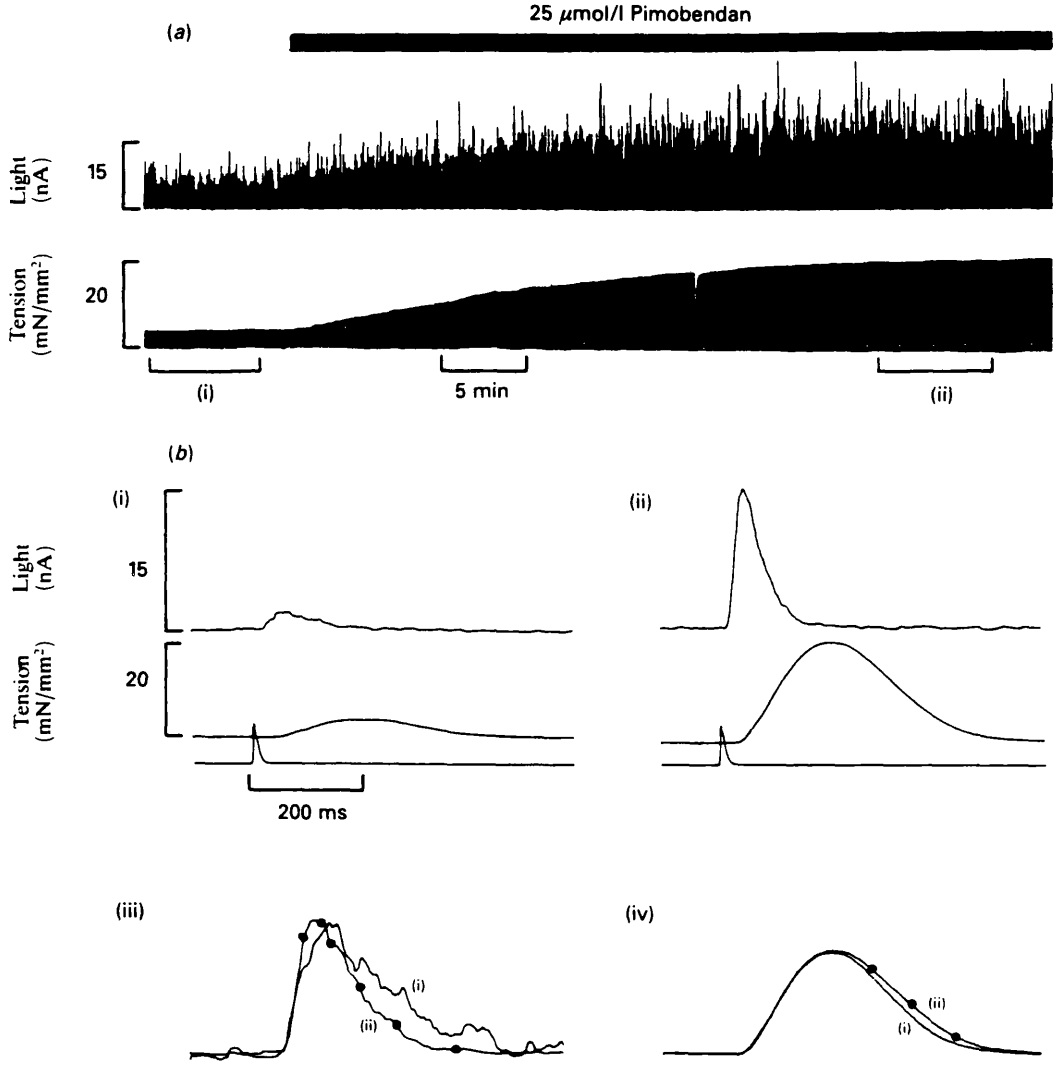

(a)
25 μmol/l Pimobendan
Light (nA)
15
Tension (mN/mm²)
20
(i)
5 min
(ii)
(b)
(i)
(ii)
Light (nA)
15
Tension (mN/mm²)
20
200 ms
(iii)
(iv)
(i)
(ii)
(ii)
(i)

FIGURE 34.

FIGURE 34.

The protocol used to determine whether pimbendan caused a change in Ca^{2+}-sensitivity. Each panel shows averaged records of aequorin light (above), tension (middle) and stimulus marker (below). A muscle was first exposed to a series of extracellular Ca^{2+} concentrations (top three panels). The extracellular Ca^{2+} concentration was then adjusted so that the muscle produced 20 - 30% of maximum tension, and 25 μmol/l pimobendan was added (bottom panel). See text for further explanation.

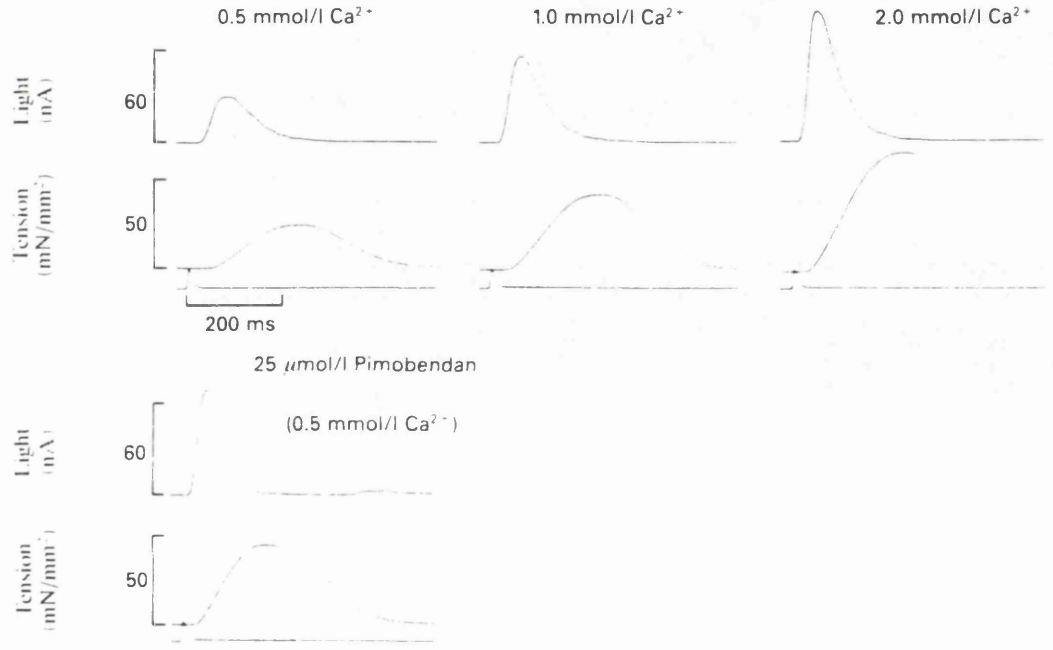
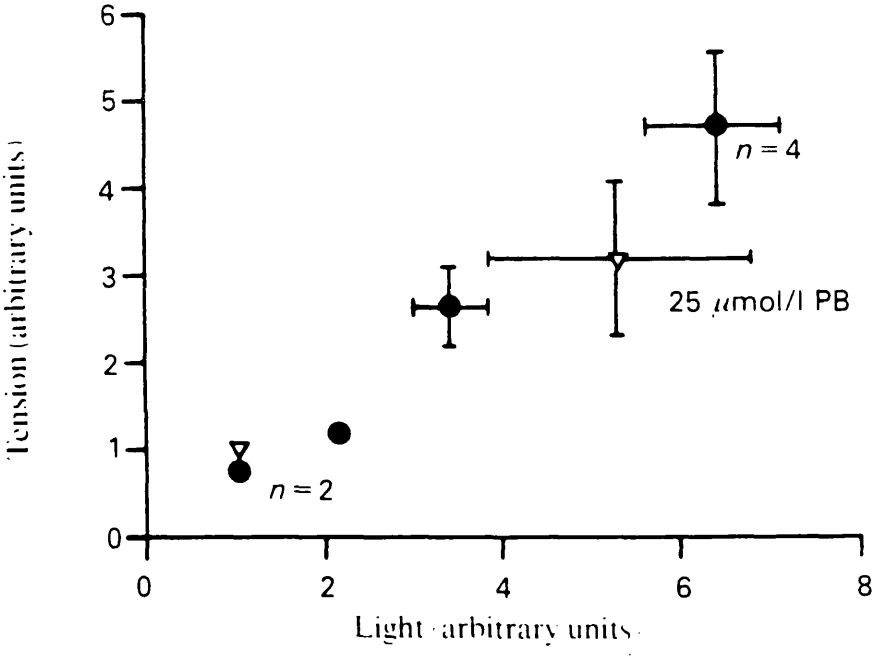

0.5 mmol/l Ca2+
1.0 mmol/l Ca2+
2.0 mmol/l Ca2+
Light (nA)
60
Tension (mN/mm2)
50
200 ms
25 µmol/l Pimobendan
(0.5 mmol/l Ca2+)
Light (nA)
60
Tension (mN/mm2)
50

FIGURE 35.

FIGURE 35.

Graph showing the absence of a sensitivity change when pimobendan was applied to an intact papillary muscle. Tension is plotted against aequorin light to produce a plot analogous to that shown in Figure 32 for skinned fibres. All points have been normalised to the pre-pimobendan control (open triangle). The point produced by the addition of pimobendan (25 μmol/l) is not significantly different from those of the control series (filled circles).


Tension (arbitrary units)
6
5
4
3
2
1
0
n = 4
25 μmol/l PB
n = 2
0
2
4
6
8
Light (arbitrary units)

Relative to this series, sensitisation is indicated by more tension for a given light, i.e. points lying to the left of the extracellular [Ca^{2+}] series, while desensitisation is indicated by points lying to the right of the series. It can be seen that addition of 25 /umol/l pimobendan caused a variable response in which both light and tension increased (to 341 $\pm$ 91% and 545 $\pm$ 137% of control respectively; mean $\pm$ S.E.M., n = 5). It is also clear that the point produced by the addition of pimobendan was <u>not</u> significantly different from the control [Ca^{2+}] series, and in fact lies slightly to the right of the curve, indicating a tendency towards desensitisation.

3.4.4 Comparison of the effects of pimobendan and isoprenaline.

Thus although skinned fibre experiments indicate a direct sensitising effect of pimobendan, it appeared that it was not possible to demonstrate it in intact muscle. However, it will be recalled that there is also evidence indicating that pimobendan may cause a rise in the level of cyclic AMP by inhibiting phosphodiesterase activity. One of the effects of increased cyclic AMP is that troponin I becomes phosphorylated, causing a <u>decrease</u> in Ca^{2+}-sensitivity (Ray & England, 1976; Solaro, 1986). Thus, it is possible that this effect could mask the direct Ca^{2+}-sensitising effect of pimobendan in intact muscle. To test this hypothesis, the effects of pimobendan were compared with those of isoprenaline, a beta adrenergic agonist which is believed to have its effects mainly by elevating cyclic AMP. This comparison is illustrated in Figure 36. Isoprenaline caused an increase in both the twitch tension and the Ca^{2+} transients. The normalised records demonstrate that it also caused an abbreviation of the timecourse of the Ca^{2+} transients, <u>and</u> an abbreviation of the timecourse of the twitch. These are thus the actions of an agent which functions predominantly by causing an increase in the levels of cyclic AMP. The

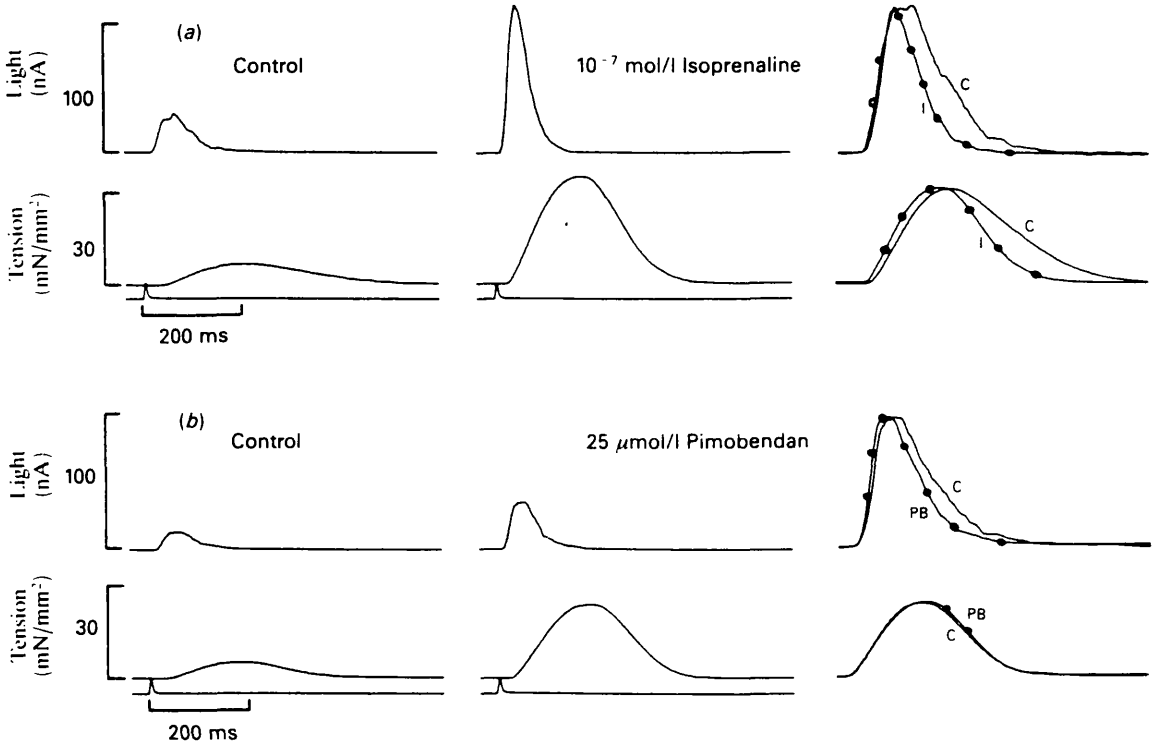
FIGURE 36.

FIGURE 36.

Comparison of the inotropic effects of isoprenaline and pimobendan in an isolated ferret papillary muscle.

A. Averaged records of aequorin light (top) and tension (middle) show that isoprenaline produced an increase in tension and in the Ca^{2+} transients. The stimulus marker is also shown (below). The same averaged records, when normalised (right), show that isoprenaline also caused an abbreviation of both the Ca^{2+} transient and the twitch.

B. Averaged records of aequorin light (top) and tension (middle) showing that pimobendan also caused an increase in both the tension and the Ca^{2+} transients. The normalised, superimposed traces (right) show that pimobendan abbreviated the Ca^{2+} transient, but caused a slight prolongation of the twitch. Note that in both A and B the normalised light records have been displayed on double the timescale for clarity.


(a)
Control
10⁻⁷ mol/l Isoprenaline
Light (nA)
100
Tension (mN/mm²)
30
C
I
200 ms
(b)
Control
25 μmol/l Pimobendan
PB
200 ms

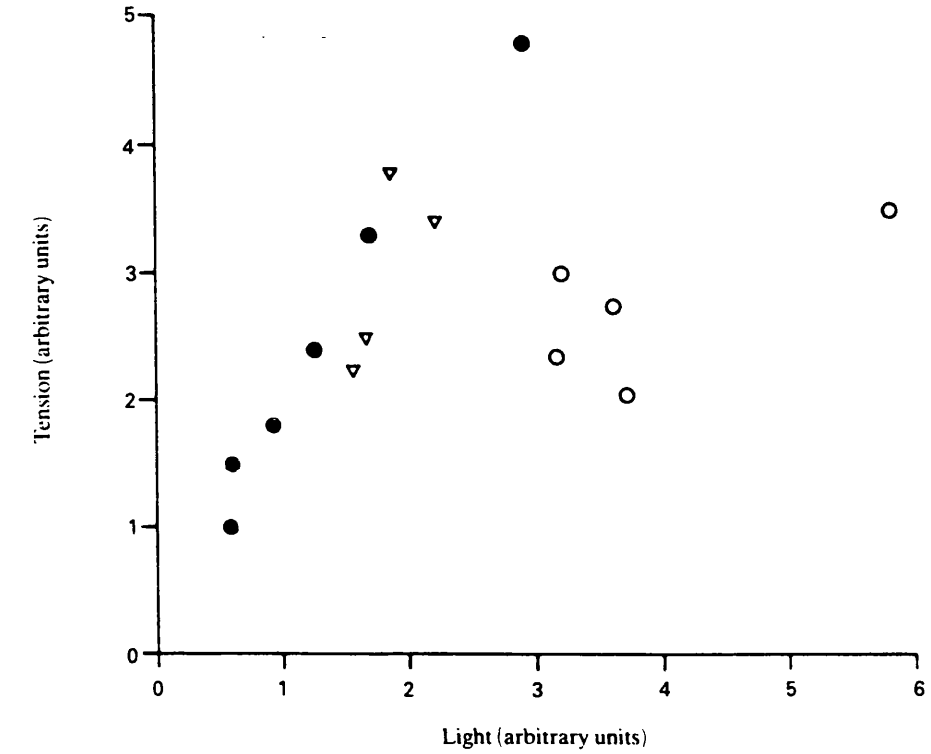
effects of pimobendan (see above) showed two notable differences from those of isoprenaline. Firstly, the increase in the size of the Ca^{2+} transients for a given inotropic effect associated with pimobendan was less than the increase produced by isoprenaline. Secondly, although pimobendan produces an abbreviation of the Ca^{2+} transients, as expected for an agent which increases cyclic AMP, it does not produce an abbreviation of the twitch, but rather a slight prolongation. Since prolongation of the twitch is an expected property of a Ca^{2+}-sensitising agent, this suggests that pimobendan may indeed be having this additional action in vivo.

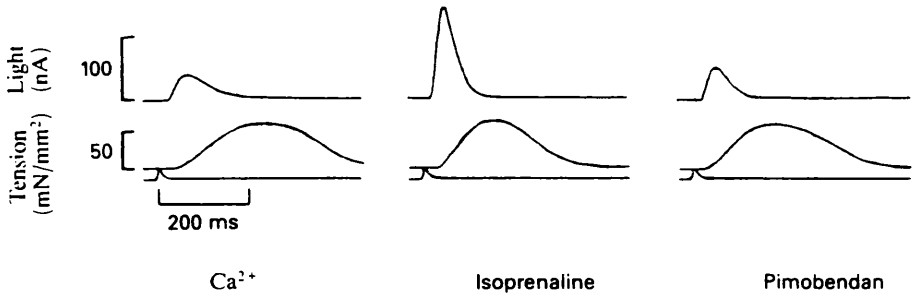
Figure 37 illustrates an experimental protocol designed to test this hypothesis further. First an extracellular $[Ca^{2+}]$ series was performed as described above (filled circles). Then the muscle was exposed to isoprenaline (open circles), and the $[Ca^{2+}]$ was again varied to produce a further series of points. Although there is some variability, it is clear that all the points lie well to the right of the control curve, illustrating the desensitisation that occurs in the presence of isoprenaline, as shown previously (Allen & Kurihara, 1980; Endoh & Blinks, 1988; Kurihara & Konishi, 1987). Isoprenaline was then washed off, and the muscle was exposed to a series of extracellular $[Ca^{2+}]$ in the presence of pimobendan (open triangles). If pimobendan had been acting purely by increasing cyclic AMP, it is clear that these points should lie close to those produced by isoprenaline. Instead, however, they lie close to the control curve. Thus although pimobendan does not produce a sensitisation relative to control, it produces a clear sensitisation relative to another agent which acts mainly by increasing cyclic AMP. This point is emphasised in the inserts below the graph, which show that for a given inotropic effect, the Ca^{2+} transients in the presence of pimobendan are much smaller than those in the presence of isoprenaline.

FIGURE 37.

FIGURE 37.

A comparison of the relation between tension and light during increases in extracellular Ca^{2+} and during the application of isoprenaline and of pimobendan. Isoprenaline (open circles) produced a marked desensitisation relative to control (filled circles). Pimobendan (open triangles) did not sensitise relative to control, but did cause a sensitisation relative to isoprenaline. Inserts below the graph show the size of the Ca^{2+} transients for similar inotropic effects obtained by varying the extracellular $[Ca^{2+}]$ under control conditions, in the presence of isoprenaline and in the presence of pimobendan. Each panel shows aequorin light (above), tension (middle) and stimulus marker (below).


Tension (arbitrary units)
Light (arbitrary units)
0
1
2
3
4
5
6


Light (nA)
100
Tension (mN/mm²)
50
200 ms
Ca²⁺
Isoprenaline
Pimobendan

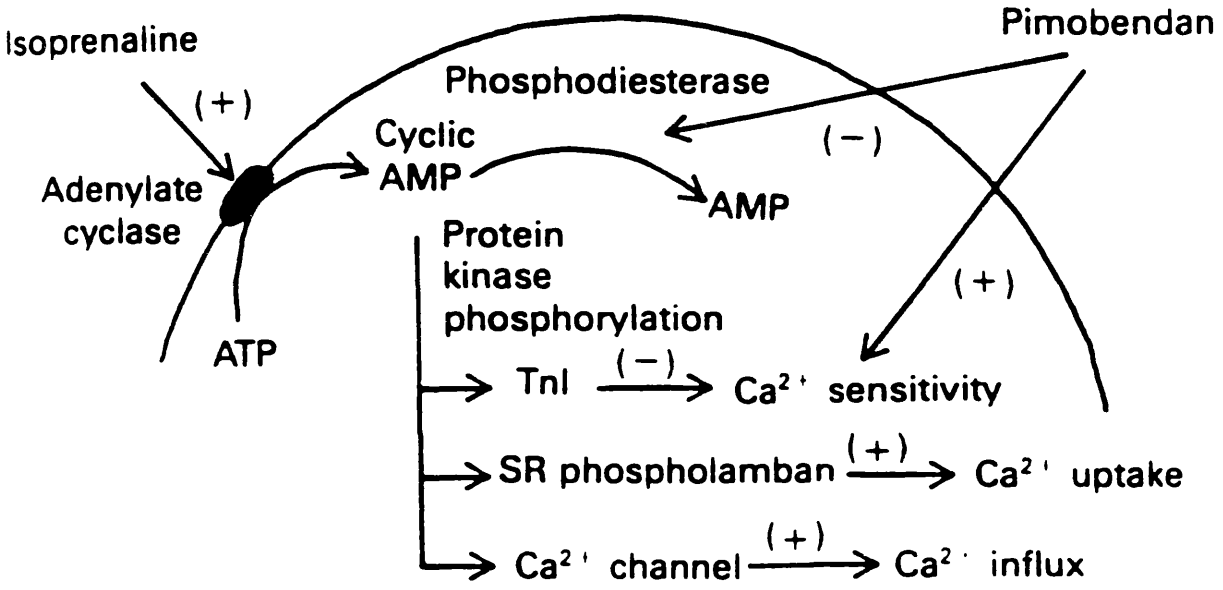
3.4.5 Interpretation of results.

These experiments have investigated the actions of pimobendan, a novel inotropic agent, on isolated ferret ventricular muscle. Since previous studies have suggested that this agent can cause both a rise in cyclic AMP and an increase in the Ca^{2+}-sensitivity of cardiac myofibrils, it was of particular interest to determine whether either or both of these actions could be demonstrated in intact working muscle. First the effects of pimobendan on skinned cardiac myofibrils were tested and these experiments confirmed that it has a direct Ca^{2+} sensitising effect on the myofibrils. When pimobendan was tested on intact muscle, it caused an increase in both the tension and the Ca^{2+} transients, but surprisingly an increase in Ca^{2+} sensitivity above control could not be demonstrated. However, when pimobendan was compared with isoprenaline, although it was found to abbreviate the Ca^{2+} transients as expected for an agent which increases cyclic AMP, the twitch was prolonged as expected for a Ca^{2+} sensitising agent. The reason why a Ca^{2+} sensitising agent might be expected to prolong the twitch is that if an agent increases the Ca^{2+} sensitivity by increasing the Ca^{2+} affinity of troponin, this is likely to occur by a decrease in the off-rate constant, since the on-rate constant is diffusion limited (Robertson, Johnson & Potter, 1981). Since Ca^{2+} then leaves troponin more slowly, this would be expected to slow the timecourse of relaxation and hence to prolong the twitch. Further comparison showed that pimobendan produced a sensitisation relative to isoprenaline. Pimobendan thus produced effects which are consistent with it causing both a rise in cyclic AMP and a Ca^{2+}-sensitisation in vivo. However, because an increase in cyclic AMP itself causes a desensitisation, this masks the sensitising effect of pimobendan when compared with control. Figure 38 presents a schematic diagram illustrating the various actions of pimobendan and isoprenaline.

FIGURE 38.

FIGURE 38.

Schematic diagram showing the effects of isoprenaline and pimobendan on a cardiac muscle cell.


Isoprenaline
(+)
Adenylate cyclase
ATP
Cyclic AMP
Phosphodiesterase
AMP
Pimobendan
(−)
(+)
Protein kinase phosphorylation
TnI
(−)
Ca2+ sensitivity
SR phospholamban
(+)
Ca2+ uptake
Ca2+ channel
(+)
Ca2+ influx

Specific points of interest and interpretation are considered below.

3.4.5.1 Calcium sensitising agents.

A considerable amount of interest has been generated recently by the idea that increasing the Ca^{2+}-sensitivity of the myofibrils may be a means of increasing the tension generated in cardiac contractions (Colucci et al, 1986; Rüegg, 1986; Blinks & Endoh, 1986). Theoretically, such an effect could be brought about either by increasing the affinity of troponin C for Ca^{2+}, or by increasing the amount of tension generated for a given amount of Ca^{2+} bound, although distinction between these possibilities requires techniques which monitor the amount of Ca^{2+} bound to troponin (e.g. Zot, Guth & Potter, 1986). A number of agents which have a sensitising action on cardiac myofibrils are now known, for example sulmazole (Herzig, Feile, & Rüegg, 1981; Solaro & Rüegg, 1982), pimobendan (Rüegg, 1986; van Meel, 1987; Fujino et al 1988), isomazole (Lues, Siegel & Harting, 1988) and BM 14.478 (Muller-Beckmann, Freund, Honerjager, Kling & Rüegg, 1988). However, there is a problem associated with agents whose sensitising action is caused by an increase in the affinity of troponin for Ca^{2+}. As explained above, these agents tend to slow relaxation. This effect is clearly seen with isomazole (Lues et al, 1988). This is obviously a major disadvantage for an agent aimed at the therapy of heart failure, where slowing of relaxation has been implicated as an important factor limiting cardiac output in diseased states (Bourdillon, Lorell, Mirsky, Paulus, Wynne & Grossman, 1983; Aroesty, McKay, Heller, Royal, Abo & Grossman, 1985). However, as discussed below, this is probably not an insurmountable problem.

3.4.5.2 The use of aequorin for the determination of calcium sensitivity *in vivo*.

This topic has been discussed in detail by Blinks & Endoh (1986)

and Endoh & Blinks (1988). The main theoretical problem with estimating *in vivo* Ca^{2+} sensitivity as has been done in this study, is that agents frequently alter the timecourse of the Ca^{2+} transients, as well as the amplitude. If other factors remain the same, then abbreviation of the Ca^{2+} transients might be expected to generate less tension, thus causing an *apparent* desensitisation (i.e. less tension for a given amplitude of the Ca^{2+} transients). For example, isoprenaline is thought to cause a direct desensitisation by the mechanism illustrated in Figure 38, and results obtained with aequorin appear to support this hypothesis. However, isoprenaline also abbreviates the Ca^{2+} transients, which could cause an apparent desensitisation, and it is not possible from results of this type to decide on the relative importance of these two effects, thus leaving some uncertainty about the importance of an *in vivo* desensitising action. Nevertheless, two pieces of evidence suggest that the contribution of the timecourse effect may be quite small. In a recent study, Kurihara & Konishi (1987) examined the effects of isoprenaline on tension and aequorin light in contractures induced by reducing extracellular sodium. Under these conditions, tension and light change very slowly, so timecourse effects of the changes in intracellular $[Ca^{2+}]$ are eliminated. In these experiments, isoprenaline still caused a marked desensitisation relative to control. In addition, there is good agreement between studies on skinned myofibrils and those which have studied sensitivity *in vivo* using aequorin. Thus it seems that although the use of aequorin is theoretically subject to certain disadvantages, it is nevertheless a useful way to qualitatively evaluate whether a substance has actions affecting Ca^{2+}-sensitivity *in vivo*.

3.4.5.3 The actions of pimobendan.

It was initially surprising when the results showed an absence of a sensitising action of pimobendan *in vivo*, even though it had a sensitising effect on skinned myofibrils of the same preparation. However, this can be explained by the fact that it simultaneously causes a rise in cyclic AMP by inhibiting phosphodiesterase. As illustrated in Figure 38, this would be expected to cause an abbreviation of the timecourse of the Ca^{2+} transients by the same mechanisms as isoprenaline, but would also be expected to cause a simultaneous desensitisation of the myofibrils due to phosphorylation of troponin I. This is in agreement with the findings that the Ca^{2+} transients are abbreviated by pimobendan, but the Ca^{2+}-sensitivity is virtually unaffected. It is also in agreement with the observation that pimobendan has little effect on the timecourse of the twitch, compared with the considerable abbreviation seen with isoprenaline. As discussed above, agents with a purely Ca^{2+}-sensitising activity cause a prolongation of the twitch. However, when combined with an action which causes abbreviation of the twitch, it seems that these effects can largely counteract each other. Combination of these actions in new inotropic agents could thus be a particular advantage. The main problem with Ca^{2+}-sensitising agents, namely slowing of relaxation, is avoided, and furthermore a given inotropic effect can be achieved with smaller Ca^{2+} transients than with conventional beta agonists (see inserts at bottom of Figure 37). This is important because large increases in intracellular [Ca^{2+}] lead to the phenomenon of Ca^{2+} overload (Allen, Eisner, Pirolo & Smith, 1985) and intracellular Ca^{2+} oscillations (e.g. Capogrossi, Stern, Spurgeon & Lakatta, 1988), which can cause a *decrease* in tension due to desynchronisation of different cells in the muscle, and can have potential arrhythmogenic effects. Thus agents with an appropriate combination of Ca^{2+} sensitising and

cyclic AMP raising effects might have considerable advantages over agents with either of these mechanisms of action present in isolation.

In the above discussion, it has been assumed that pimobendan has a direct sensitising action on troponin on the thin filaments. An alternative mechanism is that pimobendan affects the myosin cross-bridge kinetics, and this could explain both the small increase in maximum Ca^{2+}-activated tension and the apparent increase in Ca^{2+} sensitivity (see discussion in Brenner, 1986). Direct measurements of Ca^{2+} binding to troponin or of cross-bridge kinetics will be needed to distinguish between these possibilities.

CONCLUSION

The experiments described in this thesis have addressed the mechansims by which certain interventions affect the force production of cardiac muscle. The approach taken was to monitor intracellular calcium concentration using the photoprotein aequorin, while simultaneously measuring force. This allows an assessment to be made of the contribution of changes in the calcium transient or the calcium sensitivity (and/or maximum calcium-activated force) to the change in force brought about by an intervention (see Chapter 1). Distinguishing between these mechanisms is important, because they represent distinct ways in which modulation of cardiac contraction may be achieved. Previous investigations (see pages 33 - 37) have found that many well-known inotropic interventions, such as increasing the extracellular calcium concentration or increasing the frequency of stimulation, affect force primarily by altering the calcium transient. It has also been shown that digitalis, and indeed all the other positive inotropic agents used clinically, produce their increase in force by this mechanism. More recently, however, it has been appreciated that many interventions may affect force through actions on calcium sensitivity or maximum calcium-activated force (see pages 43 - 50). This has raised the possibility that it may prove possible to find a new class of positive inotropic agents which act via this mechanism. This is an attractive idea because there are strong reasons to suspect that calcium sensitivity and maximum calcium-activated force may be depressed in the diseased heart (e.g. due to accumulation of hydrogen and phosphate ions in the myoplasm; see Chapters 3.1 and 3.2). Since the commonest heart diseases in our society cause an

inadequate supply of oxygen to the heart (see p.16), particular attention was paid to the effects of hypoxia and ischaemia.

Experimentally, hypoxia (Chapter 3.1) is characterised by a great reduction of oxygen availability, while flow of superfusate past the preparation continues. When cardiac muscle is made hypoxic, the force declines, but there is variability in the behaviour of the calcium transients, which are unaltered in some experiments, but increase or decrease in others. The experiments described in Chapter 3.1 investigated the cause of this variability. It was found that in sequential exposures to hypoxia, all three patterns could be seen in the same preparation and appeared in the order - increase, unaltered, decrease. The latter two patterns could be converted back to an increase by exposure to a raised extracellular glucose concentration. These observations, coupled with measurements of glycogen and lactate in anoxia and similiarities betweeen the effects of anoxia and applied lactic acid, supported the hypothesis that the variability in the response of the calcium transients to hypoxia was due to differences in the metabolic state between preparations. Glycolysis is stimulated by hypoxia, resulting in the production of lactic acid, which may then diffuse from cells into the superfusate. In preparations with substantial glycogen stores, increased lactate production on exposure to anoxia seems to produce a sufficient intracellular acidosis to cause an increase in the calcium transients. In other preparations an acidosis sufficient to affect the calcium transients is not achieved, while in the third group the preparations appear to be

glycogen depleted, so that anoxia effectively results in metabolic blockade, which causes reduced calcium transients due to failure of the action potential. Most preparations exposed to anoxia fell into the first two groups (i.e. the calcium transients were increased or unaltered). Since this occurred when the force had fallen, it is clear that hypoxia causes a fall in the calcium sensitivity of force production in intact cardiac muscle.

In some respects, ischaemia (Chapter 3.2) might be expected to produce effects which are similar to those of anoxia, but more severe. This is because in addition to lack of oxygen, ischaemia is characterised by absence of flow past cells, and hence a greatly restricted extracellular space. Thus, hydrogen ions produced from stimulation of glycolysis would be expected to accumulate within cells to a much greater degree than in hypoxia. The experiments described in Chapter 3.2 investigated the effects of ischaemia on the calcium transients using a new model which allowed ischaemia to be studied in the isolated papillary muscle preparation. It was found that ischaemia did indeed cause a large increase in the amplitude of the calcium transients, while force declined rapidly. This indicates a very large decrease in the calcium sensitivity of force production in ischaemic cardiac muscle. These changes were closely paralleled by extracellular application of lactic acid to the preparation in a concentration which was calculated to cause a similar intracellular acidosis to that occurring in ischaemia. Also, if the preparation was initially glycogen depleted, the increase of the calcium transients on exposure to ischaemia was abolished. These

observations thus support the hypothesis that the metabolic state of the muscle is important in the response of the calcium transients to ischaemia and that, as with hypoxia, an important determinant of this response is the degree of intracellular acidosis produced by stimulated glycolysis. The experiments described in Chpaters 3.1 and 3.2 have also confirmed that hypoxia or ischaemia cause a reduced calcium sensitivity of force production in living cardiac muscle. There is thus good reason to suppose that agents which increase calcium sensitivty may be useful in clinical situations.

The experiments of Chapters 3.1, 3.2 and 3.4, in common wtih most other experiments performed on isolated cardiac tissue, examined the muscle in the isometric state. However, the working heart has phases of isotonic contraction, in which the muscle shortens, and indeed it is these isotonic contractions which are responsible for expelling the blood from the cardiac chambers. It is thus of some importance to know whether inotropic interventions produce similar effects on isometric and isotonic contractions. The experiments described in CHapter 3.3 examined this question for a variety of positive and negative inotropic interventions (including hypoxia). It was found that isometric and isotonic contractions were affected similarly by the interventions studied, with the fractional effect on tension being 1.5 - 2 times greater than that on shortening. Thus comparisons between the effects of interventions on isomteric and isotonic contractions may readily be made. The only complication is that isometric contractions use more energy that isotonic

ones, and this can lead to differences in behaviour where the oxygen supply is limited in part of the preparation (e.g. in the centre of isolated papillary muscles - see discussion in Chapter 3.3). However, in the intact heart, where all cells are within a few microns of a capillary, this should not be a problem.

Finally, the experiments described in Chapter 3.4 investigated the effects of a new positive inotropic agent called pimobendan. It was initially thought that pimobendan produced its inotropic effects purely by phosphodiesterase inhibition. However, subsequent evidence from experiments with skinned fibres suggested that it might also have a calcium sensitising action. The data presented in Chapter 3.4 support the idea that both of these mechanisms combine to produce an inotropic effect in intact muscle. As discussed on pages 212 - 213, the combination of these two mechanisms of action may be of particular interest as new inotropic agents are developed. Pimobendan is currently undergoing trials, and seems to be useful in the clinical setting. In this respect it is interesting to note that pimobendan is able to produce a substantial positive inotropic effect <u>in vitro</u> in papillary muscles where the force of contraction has been reduced by hypoxia (personal observations). A number of other compounds with putative calcium sensitising actions are currently under investigation; EMD 53998 (see Appendix 2, pages 253 - 254) is the first compound which has been demonstrated to have a predominantly calcium sensitising action in living cardiac muscle. It seems likely that agents which modulate calcium sensitivity will become increasingly important therapeutic agents in the future. Experiments in which

intracellular calcium is measured during contraction of living muscle will continue to play a useful role in establishing the mechanisms by which these new agents and indeed all other inotropic interventions affect the contraction of the heart.

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APPENDICES.

APPENDIX 1.

Effects of acidosis on ventricular muscle from adult and neonatal rats, by R.J. Solaro, J.A. Lee, J.C. Kentish & D.G. Allen.

A paper published in Circulation Research 63, 779 - 789 (1988).

Effects of Acidosis on Ventricular Muscle From Adult and Neonatal Rats

R. John Solaro, John A. Lee, Jon C. Kentish, and David G. Allen

We compared the response of ventricular muscle from adult and neonatal rats to hypercapnic acidosis. In adult muscle, acidosis caused an initial rapid fall of developed tension to 30±5% of control (mean±SEM, $n=6$). However, tension recovered slowly to a steady state that was 56±6% of control. In neonatal muscle, acidosis caused a significantly smaller initial fall in tension to 43±3% ($n=8$, $p<0.05$), but the tension then showed a subsequent slower fall to a steady state that was 29±4% of control, significantly less than in the adult ($p<0.01$). We have attempted to identify the mechanisms underlying these differences in response. In detergent-skinned myofibrils, reducing the pH from 7.0 to 6.5 caused a reduction in the pCa_{50} of 0.61 units in the adult muscle, but only 0.27 units in the neonatal ventricular muscle. Myofibrillar Ca^{2+} sensitivity in neonatal ventricular muscle is thus less susceptible to the effects of acidic pH than that of adult muscle. Since intracellular pH decreases rapidly on application of increased external CO_2, these results are consistent with the finding that, initially, developed tension in neonatal muscles is less sensitive to the effects of acidosis. Sodium dodecylsulfate gel electrophoresis of myofibrillar preparations from adult and neonatal rats demonstrated differences in thin filament proteins, including troponin I, which may underlie the observed differences in Ca^{2+} sensitivity. In adult rat ventricular muscles, the slow recovery of tension during acidosis is associated with an increase in the amplitude of the Ca^{2+} transients to 263±34% of control ($n=4$). In neonatal ventricular muscle, however, the Ca^{2+} transients decreased to 91±3% of control during acidosis ($n=3$). This difference in Ca^{2+} handling probably contributes to the difference in the slow mechanical response and could be related to the smaller amount of sarcoplasmic reticulum in neonatal muscle. Our results thus indicate that the difference in the initial rapid effect of acidosis on neonatal and adult rat heart can be explained by the effects of acidosis on the myofibrils, whereas the difference in the later, slower changes may be caused by differences in Ca^{2+} transport. (*Circulation Research* 1988;63:779–787)

It has long been known that acidosis is associated with a decrease in the ability of the heart to generate tension.[1] In the case of hypercapnic acidosis, produced by increasing the CO_2 concentration from 5% to 15% in the solution perfusing a papillary muscle, there is initially a rapid fall in developed tension.[2] This is followed by a second phase in which tension slowly recovers to a steady-state level that is less than the control level. In experiments in which the systolic rise in Ca^{2+} (Ca^{2+} transient) was measured with the photoprotein aequorin,[3] it was found that the initial fall occurs without change in the Ca^{2+} transient, while the slow recovery of tension is associated with a concurrent increase in size of the systolic rise of the transients.

Although the mechanisms responsible for these phenomena are not completely understood, there is reason to believe that the effects of acidosis on the myofilaments and sarcoplasmic reticulum (SR) play major roles. The initial rapid fall in tension, which occurs without change in the intracellular Ca^{2+} transient, is thought to be due to a reduction in the sensitivity of the myofilaments to Ca^{2+}, thus leading to less tension. Studies with skinned-fiber preparations have shown that both maximum tension and Ca^{2+} sensitivity are reduced when pH is reduced.[4]

The slower recovery of tension in the second phase may be related to increased Ca^{2+} accumulated in, and released from, the SR. This interpretation has recently been reinforced by the demon-

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Supported by a grant from the British Heart Foundation and National Institutes of Health Grant HL-22231. R.J.S. was supported as a Senior International Fellow of the Fogarty Foundation. J.A.L. is a Medical Research Council Training Fellow.

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Received January 25, 1988; accepted May 9, 1988.

stration that the recovery phase of tension in adult rat ventricular muscle can be abolished by various interventions known to interfere with SR activity.[5]

In the present study, we explored these mechanisms by comparing the effects of acidosis on preparations from neonatal (1-day-old) and adult rat hearts. There is evidence that newborn rabbit hearts are less sensitive than adult hearts to the effects of acidosis.[6] It has also been found that Ca^{2+} activation of myofibrils from perinatal dog hearts is relatively insensitive to acidosis.[7] This effect may be related to differences in the isotype population of the thin filament proteins existing in the neonatal heart.[7] In addition, it has been demonstrated that the SR is poorly developed in the ventricular muscle of neonatal mammals.[8] Neonatal heart muscle thus provides a natural variation in SR and myofilament activity that appears useful in the investigation of the response of the heart to acidosis.

Our results show that, compared with the adult, tension development by neonatal rat ventricular muscle is less sensitive to acidosis in the initial rapid phase of the response to increased CO_2, but more sensitive in the subsequent slower response.[9-11] Studies with detergent-skinned preparations suggest that the decrease in sensitivity to acidosis in the initial phase of the response may be related to differences in the effect of acidosis on tension development of the neonatal versus adult myofilaments. During the slow response to acidosis, tension fell significantly more in the neonatal muscle than in the adult muscle. This difference may be related to a difference in the Ca^{2+} transients, which increased in the adult, but decreased slightly in the neonatal heart preparations.

Materials and Methods

Intact Muscle

The adult rats used were 8–10 weeks old and weighed approximately 200 g. Neonatal rats were obtained within 24 hours of birth. The animals were killed by cervical dislocation and the heart was rapidly removed and immersed in Tyrode's solution equilibrated with 5% CO_2-95% O_2. The right ventricle was opened and, in the case of adult hearts, a papillary muscle or trabecula of uniform thickness (<1 mm) was chosen. In the case of neonatal hearts, a papillary muscle or ventricular strip was used. The ends of the preparation were tied with 50 μm silver wire or silk monofilaments. Loops extending from the ties were used to mount the muscle in the experimental chamber. The muscles were superfused with Tyrode's solution at 30° C. The composition of the standard Tyrode's was (in mM): Na^+ 135, K^+ 5, Ca^{2+} 2, Mg^{2+} 1, Cl^- 102, HCO_3^- 20, HPO_4^{2-} 1, SO_4^{2-} 1, acetate 20, glucose 10; and insulin 5 units/l (40 nM). When equilibrated with 5% CO_2-95% O_2, the pH of this solution was 7.36; when equilibrated with 15% CO_2-85% O_2, the pH was 6.91.

In four experiments, preparations from neonatal and adult hearts were mounted side by side in the same chamber, which was similar to that described by Blinks.[12] This ensured that during changes of superfusate CO_2, the muscles were exposed to identical conditions. In experiments in which aequorin was used, individual preparations were mounted horizontally.[3] In both kinds of experiment, the muscles were stimulated by two platinum wire electrodes whose ends approached within 0.5 mm of one end of the muscle. The stimuli were 2 msec pulses at about 1.3 times threshold, given at 1–2 second intervals. The muscle was stretched to the length where developed force was at a maximum. The stimulus and tension signal were amplified and displayed on a pen recorder, and simultaneously recorded on tape for later analysis. Accurate measurements of the cross-sectional area of neonatal preparations are difficult to make because of their small size and irregular contour. For this reason the figures show absolute tension rather than tension per cross sectional area.

Detergent-Skinned Muscle

In some experiments, trabeculae or papillary muscles prepared as described above were detergent-skinned after measurements had been made on the intact preparation. In this case, the muscles were mounted in the chamber described in detail by Kentish,[13] and the methods followed were essentially similar. In brief, once measurements on the intact preparation were complete, the Tyrode's solution was cooled to room temperature (20–22° C), and was then replaced with a detergent "skinning solution" consisting of; K propionate 100 mM; BES [*N,N'*-bis-(-2-hydroxyethyl)-2-aminoethanesulfonic acid] 60, K_2Na_2ATP 5.2, $MgCl_2$ 6.8 (giving a calculated [Mg^{2+}] of 2 mM), Na creatine phosphate 10, dithiothreitol 1, K_2H_2EGTA 10, and Triton X-100 1% (vol/vol); pH 7.00; pCa>8. The rest of the experiment was performed at room temperature.

The muscles were skinned in the detergent solution for 30 minutes. After skinning was complete, contractures were produced by transferring the skinned preparation to a series of chambers containing solutions in which the free Ca^{2+} concentration was varied with CaEGTA buffers between pCa ($-\log_{10}[Ca^{2+}]$) of 4 and 7. Between measurements the preparation was placed in a "high-EGTA relaxing solution", which had the same composition as the skinning solution except that Triton X-100 was omitted, and then in a "low-EGTA relaxing solution" containing 9.85 mM HDTA (1,6-diaminohexane-*N,N,N',N'*-tetraacetic acid) and 0.15 mM EGTA (instead of 10 mM EGTA), in order to wash most of the EGTA out of the skinned muscle; this reduced the time needed to reach a steady tension in the subsequent contraction. Sarcomere lengths were monitored with a helium-neon laser. In adult rat preparations, the sarcomere lengths were between 2.2 and 2.4 μm. However, in neonatal

preparations, diffraction patterns were unsatisfactory, so it was not possible to measure the sarcomere length. This is possibly due to the lower density of myofibrils present in the newborn rat myocardium.[8] So that the sarcomere lengths were as similar as possible, all preparations were stretched to the length at which the Ca^{2+}-activated force at pCa 4.0 was maximal.

In six experiments with detergent-skinned trabeculae, their uniform shape allowed measurement of tension per cross-sectional area. In the adult, the maximal Ca^{2+}-activated tension per cross-sectional area was 39.9 ± 8.6 mN/mm^2 (mean $\pm$ SEM, $n=3$), whereas in the neonate it was 8.7 ± 2.2 mN/mm^2 ($n=3$). This is also in agreement with the lower density of myofibrils in the neonatal myocardium.[8]

Tension-pCa relations were first measured at either pH 7.0 or pH 6.5. They were then measured at the other pH, and again at the original pH. A solution producing maximal activation (pCa 4.0 or 4.5) was applied repeatedly during the experiment to correct for any deterioration of the preparation. All forces were expressed relative to the maximum Ca^{2+}-activated tension. Curves were fitted to the results with nonlinear least-squares analysis to produce a Hill plot of form:

$$\text{relative tension} = [Ca]^n/(K + [Ca]^n)$$

where n is Hill coefficient and K is a compound association constant.

Aequorin Light Measurements

The use of aequorin luminescence as a measure of intracellular Ca^{2+} ($[Ca^{2+}]_i$) has been reviewed by Blinks et al.[14] The techniques used with adult heart preparations have been described by Allen and Orchard[3] and by Orchard.[5] In brief, aequorin was pressure-injected into 50–100 cells on the surface of the preparation, and the light emission (a measure of $[Ca^{2+}]_i$) was monitored with a photomultiplier. It was considerably more difficult to obtain an adequate light signal in the neonatal preparations, presumably due to the smaller size of the cells compared with the adult. Consequently the neonatal preparations required about twice the number of penetrations. Signal averaging of the light signals was performed to improve the signal-to-noise ratio. Because of the pH sensitivity of aequorin light emission, the decrease in intracellular pH in these experiments would be expected to cause a small reduction of about 10% in aequorin light emission (see discussion by Allen and Orchard[3]). The size of the Ca^{2+} transients would thus be slightly underestimated under acidic conditions. Because the aequorin experiments reported here were concerned mainly with qualitative differences, we have not corrected for this effect.

Gel Electrophoresis

Preparations from the right ventricle were skinned with Triton X-100 as described above, and were then examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), essentially as described by Laemmli.[15]

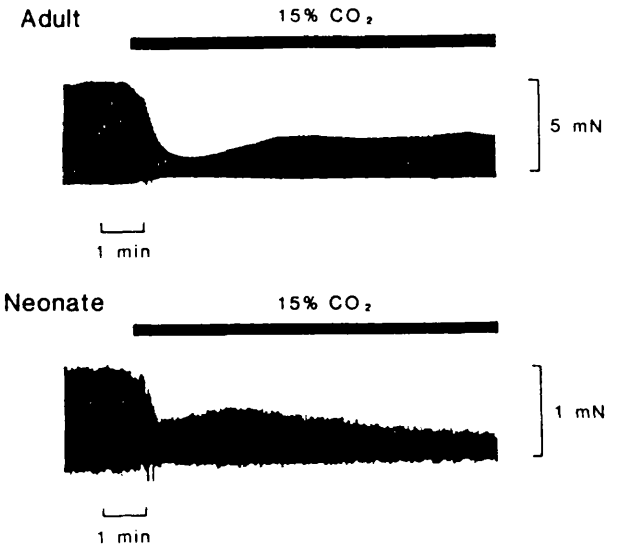

FIGURE 1. *Effect of changing from normal Tyrode's solution (5% CO_2, pH 7.36) to Tyrode's solution equilibrated with 15% CO_2 (pH 6.91).* Top panel: *Tension record from papillary muscle from adult (9-week-old) rat.* Lower panel: *Tension record from neonatal (1-day-old) papillary muscle. Muscles were mounted side by side in the same superfusion chamber. Small increases in resting tension seen in these preparations were not consistently observed in other preparations.*

Statistics

Statistical significance was determined by the two-tailed Student's *t* test.

Results

Studies with Intact Muscle

Figure 1 shows the responses of adult and neonatal ventricular muscle to an increase in CO_2 concentration from 5% (pH 7.4) to 15% (pH 6.9). In this experiment, the muscles were mounted side by side in the same chamber. There was an initial fall in developed tension, which was more pronounced in the adult muscle than in the neonatal muscle. The adult muscle subsequently exhibited a slow, partial recovery of tension, whereas the neonatal preparation showed only a transient recovery, followed by a further decrease in tension in the steady state. Because of the slow changes, the steady-state tension was higher (relative to control at pH 7.4) in the adult than in the neonatal muscle. The mean results, shown in Figure 2, demonstrated that the higher tension in the initial response of the neonate ($43 \pm 3\%$ of control, $n=8$) was significantly different from the initial response of the adult ($30 \pm 5\%$ of control, $n=6$, $p<0.05$). Also, the steady-state tension developed by neonatal preparation ($29 \pm 4\%$, $n=8$) was significantly lower than the steady-state tension in the adult muscle ($59 \pm 6\%$ of control, $n=6$, $p<0.01$).

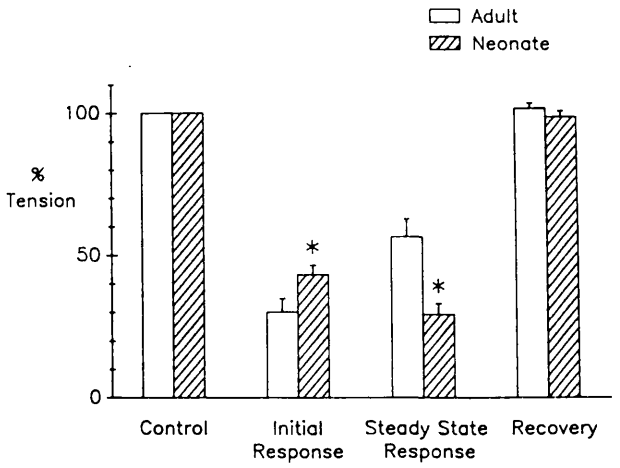

FIGURE 2. *Histogram of the effects of hypercapnic acidosis on initial and steady-state tension in adult and neonatal ventricular muscle from rat. Recovery shows that steady-state tension on removal of acidosis was same as control. Error bars show SEM. *Significantly different ($p<0.05$) responses of adult and neonatal muscle.*

Once the developed tension had reached an apparent steady state, which typically took 5–10 minutes, the muscles were returned to 5% CO_2 superfusate. The subsequent changes in tension were normally the inverse of those seen on application of 15% CO_2 (results not shown), and the final steady-state tension recovered completely to control levels (Figure 2). It was possible to make several (3–5) such exposures to acidosis in each muscle.

We reasoned that the differences between adult and neonatal muscle in the immediate response might be an indicator of differences in the myofibrillar proteins of the two preparations, while the differences in steady-state tension might be related to variations in Ca^{2+} handling. To investigate these possibilities, we measured the effect of pH on Ca^{2+} activation of myofibrillar tension and the effect of acidosis on Ca^{2+} transients.

Myofibrillar Tension Production

Figure 3 presents averaged data on detergent-skinned myofibrillar preparations from adult and neonatal rats. The reduction of pH from 7.0 to 6.5 caused a similar decrease in the maximum Ca^{2+}-activated tension at pCa <4.5 (Table 1). In the adult, tension declined to 65.7±5.8% of control ($n=3$), while in the neonate tension fell to 68.4±1.1% ($n=3$). In addition, acidic pH also caused a decrease in the Ca^{2+} sensitivity of the myofibrils, which is manifested in the shift of the tension-pCa curve to the right. At 50% maximal activation, there was a shift of 0.61±0.03 pCa units in the adult ($n=3$), but only 0.26±0.02 pCa units in the neonate ($n=3$, $p<0.001$) (Table 1). Thus the effect of a reduction in pH on Ca^{2+} sensitivity in the neonate was less than half that in the adult.

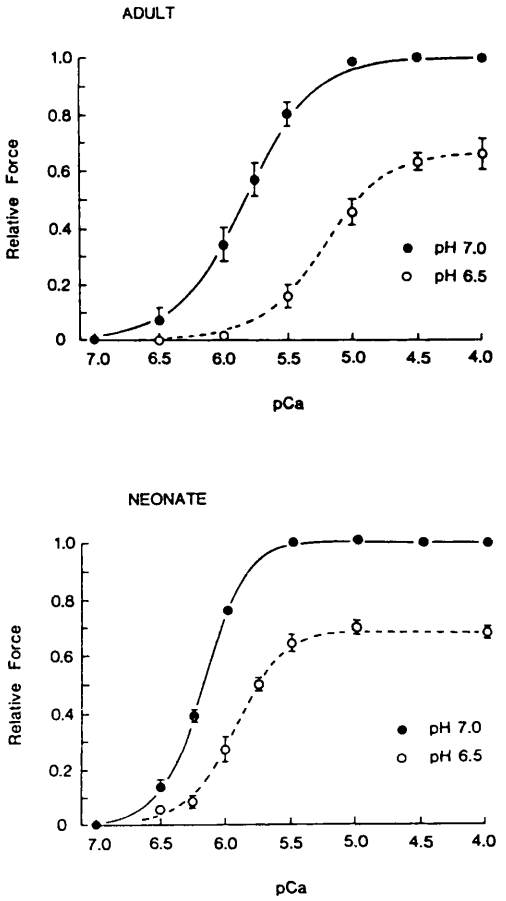

FIGURE 3. *Effect of decreasing pH from 7.0 to 6.5 on tension production in skinned myofibrils from adult and neonatal rat. Tension is expressed as percentage of maximum tension measured at pH 7.0. Error bars show SEM, n=3 for both graphs. For some points, error bars are smaller than the symbol. Sigmoid curves are drawn according to the Hill equation (see text).*

This difference is brought out more clearly in Figure 4, which shows the same data after normalization of maximum tensions. Figure 4 also demonstrates that myofibrils from the neonate are more sensitive to Ca^{2+} than those from the adult: at pH 7.0 the pCa_{50} (pCa at half maximal activation) for adult myofibrils was 5.83±0.06 (Table 1) whereas for the neonate it was 6.18±0.01 ($p<0.01$). At acidic pH this effect was even more marked; the adult pCa_{50} was 5.22±0.09, while the neonate value

TABLE 1. Ca^{2+} Activation Parameters for Tension Developed by Adult and Neonatal Myofibrils

	Adult		Neonate	
	pH 7.0	pH 6.5	pH 7.0	pH 6.5
Max force*	100	65.7±5.8	100	68.4±1.1
pCa_{50}	5.83±0.06	5.22±0.09	6.18±0.01	5.91±0.03
Hill n	1.81±0.11	2.10±0.24	2.81±0.28	2.59±0.35

Values are mean±SEM from three adult and three neonatal preparations.
*Relative to force at pH 7.0.

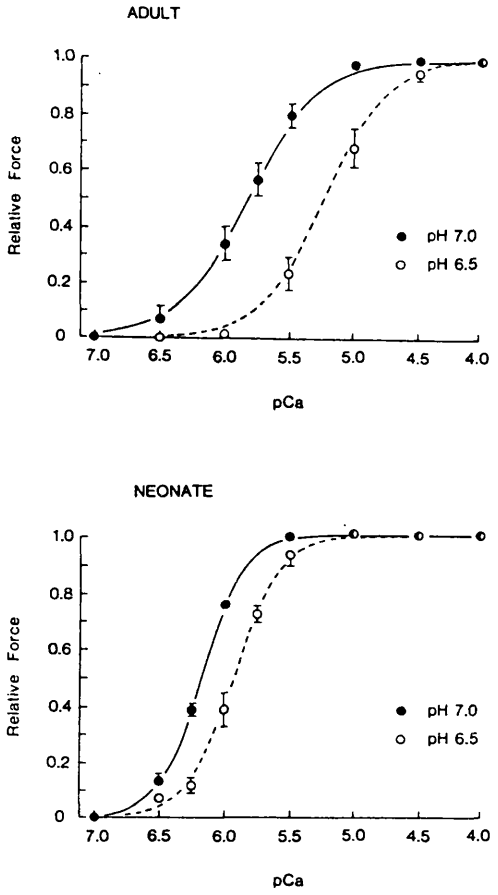

FIGURE 4. *Normalized curves showing effect of decreasing pH from 7.0 to 6.5 in skinned myofibrils from adult and neonatal rat. Tension on each curve is expressed as percentage of maximum tension measured at that pH. Error bars show SEM,* n=*3.*

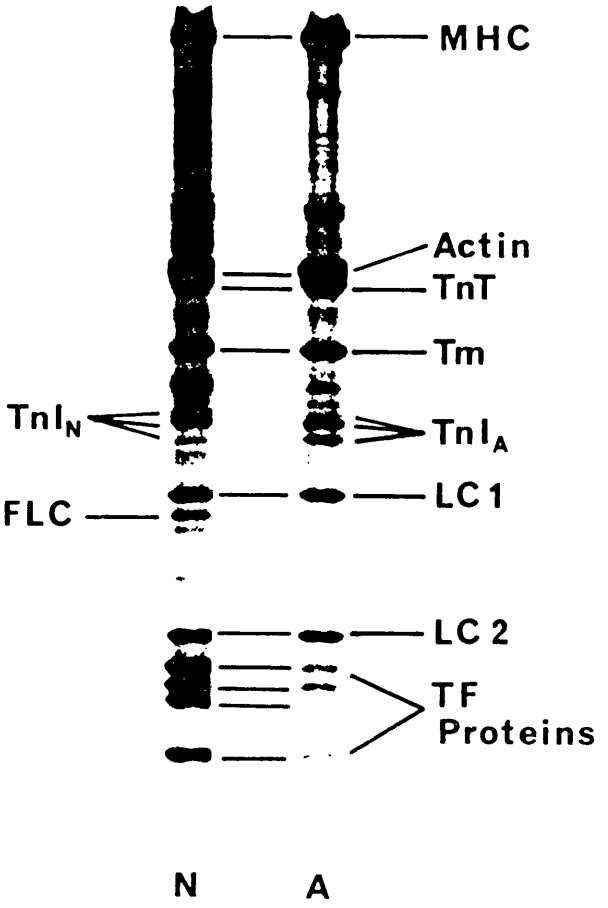

FIGURE 5. *Separation of myofibrillar proteins from neonatal (N) and adult (A) rat ventricular muscle on 15% polyacrylamide gels in presence of 1% sodium dodecylsulfate. Both gels were loaded to approximately the same extent. MHC, myosin heavy chain; TnT, troponin T; TnI$_N$ or $_A$, neonatal or adult pattern of troponin I; FLC, fetal light chain; LC, light chain; TF proteins, thin filament proteins. (See text for details).*

was 5.91 ± 0.03 ($p<0.01$). A further difference between the adult and neonatal muscles was evident from the curve-fitting procedure. The Hill coefficient, which is a measure of the steepness of the curves, was significantly greater for the neonate than for the adult at pH 7.0 ($p<0.05$). At pH 6.5 this difference was still present, although no longer significant ($p>0.1$).

The results of the skinned-fiber experiments suggest that the proteins responsible for Ca^{2+} regulation in neonatal myofibrils differ in some way from those of the adult. We investigated this possibility by analysis of the myofilament proteins with SDS polyacrylamide gel electrophoresis. Figure 5 shows SDS gels prepared from adult and neonatal rat right ventricles. These were loaded to approximately the same extent with respect to the density of the myosin light chains. There are obvious differences between the electrophoretic profiles of the two preparations, especially in the case of proteins migrating with a mobility similar to that of troponin I and those migrating to the bottom of the gel, which have previously been identified as thin filament–associated proteins.[7] Troponin I in the adult preparation ran as two distinct bands with a faint band above them. In the neonatal preparation, however, the troponin I ran as two distinct bands with a faint band below them. These bands have been shown to cross react with a polyclonal antibody to skeletal muscle troponin I and to bind to troponin C attached to an affinity column (P. Kumar, A.F. Martin, and R.J. Solaro, unpublished observations). It thus seems likely that during the development of rat heart, there is a change in isotype population from a troponin I neonatal pattern to an adult pattern, as has been demonstrated in dog heart.[7]

Ca^{2+} Transients

As described above, after the initial effect on tension of exposure to acidic pH, the adult muscle showed a slow recovery of tension, while the neonate, after a brief recovery, showed a slow decline to the steady state. To investigate whether alterations in the Ca^{2+} transient could account for the changes underlying these longer term changes in

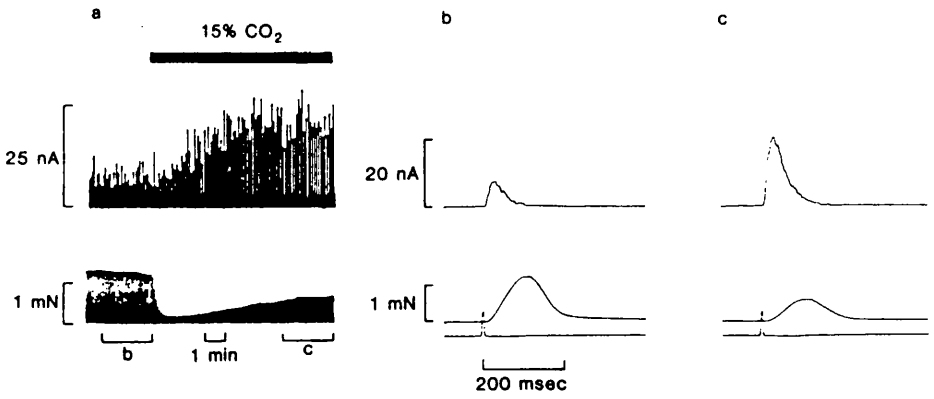

FIGURE 6. *Effect of changing from normal Tyrode's solution (5% CO_2) to acid Tyrode's solution (15% CO_2) on light and tension in an adult rat papillary muscle.* Panel A: *Continuous chart record of light (retouched), measured as current from photomultiplier (above) and developed tension (below).* Panels B *and* C: *Averaged records* (n=*32*) *of light (above) and tension (middle) with a stimulus marker (below) from the periods indicated in Panel A.*

tension, we injected adult and neonatal papillary muscles with aequorin. Figure 6 shows a representative experiment on an adult heart preparation. The results are similar to those previously reported.[3,5] During the initial rapid fall of tension, there was no significant change in the size of the Ca^{2+} transients, whereas during the slower recovery of tension, the Ca^{2+} transients showed a monophasic rise. In four preparations, the amplitude of the Ca^{2+} transients in 15% CO_2 rose to 264±34% of control ($n=4$). There was no indication of this increase subsequently declining, even when experiments involving longer exposures to acidosis (up to 20 minutes) were performed.

In the neonatal preparations the response was different. A representative example is shown in Figure 7. Again, no significant change in the Ca^{2+} transient is apparent during the initial fall of tension. Figure 7 shows a small increase in the size of the systolic rise of the Ca^{2+} transient that roughly correlates with the transient recovery of tension. However, this was not consistently seen. The most dramatic difference of the neonate from the adult muscle response is that during the period when the adult tension recovers and the systolic rise of the Ca^{2+} transient more than doubles in size, the neonate tension decreases and the Ca^{2+} transient also decreases. In three preparations, the Ca^{2+} transients decreased to 91±3% of control ($p<0.1$). Although not statistically significant, this decrease suggests that part of the slow fall of tension in the neonate may be caused by a reduction in the supply of Ca^{2+} to the myofilaments.

Figure 8 demonstrates that the twitch is slower in the neonate than the adult, as has been previously noted.[16] It can be seen that the time course of the Ca^{2+} transient is also considerably slower in the neonate. The time from stimulus to 75% decline of light was 117±3 msec in the neonate ($n=3$) and 73±5 msec in the adult ($n=4$, $p<0.001$). The slower

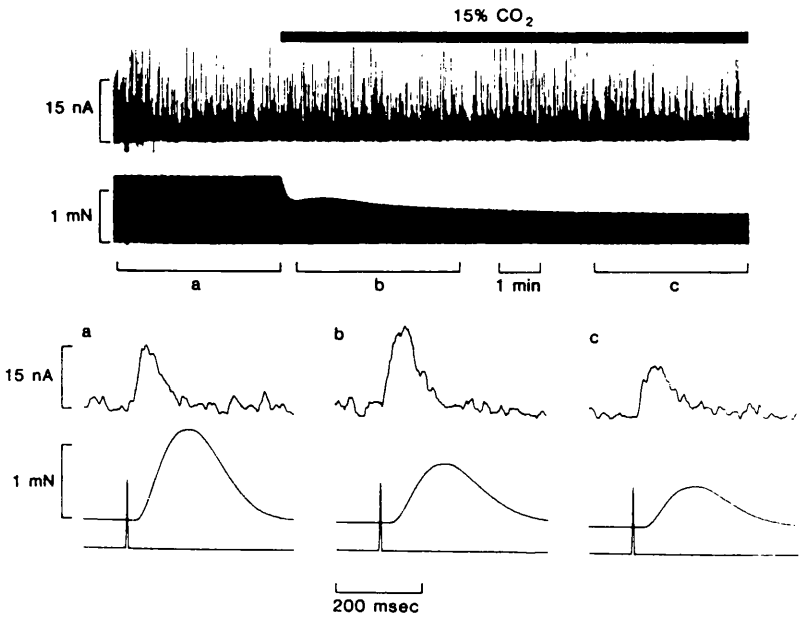

FIGURE 7. *Effect of changing to Tyrode's solution equilibrated with 15% CO_2 on light and tension in neonatal ventricular strip.* Upper panel: *Continuous record of light (above) and tension (below);* Bottom panel: *Averaged records* (n=*512*) *of light (above), tension (middle), and a stimulus marker (below) from periods indicated in upper panel. Note that aequorin traces show an increased noise when compared with Figure 6, reflecting the greater difficulty of injecting aequorin into neonatal preparations.*

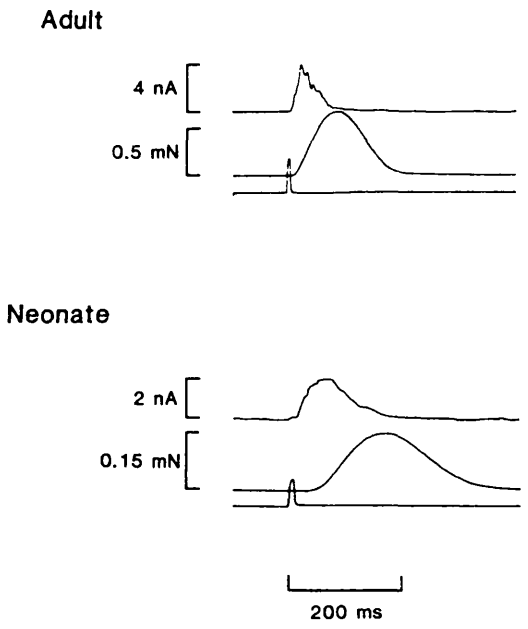

FIGURE 8. *Comparison of time-course of Ca^{2+} transient and contraction in ventricular muscle from adult and neonatal rat.* Upper panel: *Averaged records (n=32) of light (above), tension (middle), and a stimulus marker (below) recorded from an adult rat papillary muscle;* Lower panel: *Averaged records (n=512) of light (above), tension (middle), and a stimulus marker (below) recorded from neonatal rat ventricular strip.*

time course of the Ca^{2+} transient in the neonate probably contributes to the slower time course of tension, and may be related to the poorer development of the SR in the neonate.

Our results are different from those obtained by Nakanishi et al,[6] who compared adult and neonatal rabbits. They found that the neonate was less sensitive to the effects of acidosis in the steady state. In three experiments we mounted muscles from neonatal (0–5-day-old) rabbits and adult rabbits in the same chamber and observed their response to an increase in CO_2 from 5% to 15%. These experiments confirmed the results of Nakanishi et al,[6] in that the neonatal muscle showed a smaller reduction in steady state tension than the adult. There thus appears to be a significant species difference between the rat and rabbit in this respect.

Discussion

We used ventricular preparations from neonatal and adult rats in an attempt to define the mechanisms involved in the response of cardiac muscle to hypercapnic acidosis. It is known from other studies with microelectrodes[17] and with NMR[18] that in the adult heart, application of CO_2 leads to a rapid intracellular acidosis, which is then more or less stable for the relatively short exposures we used. The results show that adult and neonatal hearts differ in both their initial, rapid response and in the slower phase of their response to acidosis. These will be discussed separately.

Initial Decrease of Developed Tension

In adult muscle, the developed tension fell initially to 30% of control levels on exposure to 15% CO_2. However, in neonatal muscle the tension fell less, to 43% of control. These results are qualitatively consistent with the skinned-fiber studies reported here, which demonstrate that the tension production of neonatal myofibrils, at Ca^{2+} concentrations below that needed to saturate the myofilaments, is less sensitive to pH than is the tension production of adult myofibrils. A more quantitative correlation of these two sets of results is complicated by two factors: intracellular pH buffering and the degree of Ca^{2+} activation of the preparations.

It is generally agreed that CO_2 rapidly crosses cell membranes and causes a decrease in pH_i.[3,16] This is likely to be true for neonates as well as adults, though the extent to which pH_i changes on the application of a given external CO_2 concentration will depend on the degree of intracellular buffering. Because neonatal rat muscle has a much lower concentration of myofibrillar proteins than adult rat muscle it is possible that the intracellular buffering will be lower in the neonate than the adult. If this is true, then pH_i will decrease more in the neonate than the adult on exposure to a given level of external CO_2. The measurements on the intact preparations would then underestimate the difference in sensitivity between the adult and neonate. Nakanishi et al,[6] from titration measurements made on preparations of homogenized adult and neonatal rabbit heart, have suggested that the buffering capacity may be greater in the neonate. However, there is a clear species difference between the rat and rabbit in the response of neonatal muscle to acidosis (see "Results"), and perhaps a difference in buffering is part of the explanation.

At maximal Ca^{2+} activation of the skinned muscles, a reduction of the pH from 7.0 to 6.5 causes a reduction of developed tension to approximately 65% of control in both the adult and the neonate. At 50% maximal activation, however, the same pH change causes the adult tension to decrease to about 5% of control, but the neonate decreases to only 15% of control (Figure 3). It is thus clear that the degree of activation of the muscle can have a considerable quantitative effect on the result observed. Added to this is the fact that at pH 7.0 the higher Ca^{2+} sensitivity of the neonatal myofilaments, as compared with the adult myofilaments, would cause them to be more fully activated (and so less sensitive to pH) for a given systolic rise in Ca^{2+}. Thus, although it is not possible to correlate quantitatively the intact preparation results with the skinned-fiber studies, the results are consistent with the hypothesis that the initial fall of tension is smaller in the neonate than in the adult because of a difference in the myofibrillar proteins.

On the basis of studies with adult and perinatal dog hearts, Solaro et al[7] have suggested that the difference in pH sensitivity of adult and neonatal myofibrils resides in the thin filaments. Using SDS gel electrophoresis, they detected differences in the troponin I and thin filament proteins between neonatal and adult dog heart muscle, although troponin C showed no changes. The gels of rat heart muscle (Figure 5) confirm this finding. There are differences in the troponin I pattern, and in the bands tentatively identified by Solaro et al[7] as thin filament proteins. Which of these is most important in determining the myofibrillar pH sensitivity remains to be established. It is of interest that during development the pH sensitivity of pCa_{50} decreases, while that of maximum tension development remains the same (Figure 3). This suggests that the effects of pH on pCa_{50} and maximum tension may involve different mechanisms.

We did not detect any changes in the Ca^{2+} transients during the early period of exposure of neonatal muscle to 15% CO_2 when tension fell rapidly. This result agrees with previous findings on adult preparations.[3] Although it is expected that an acidosis will be associated with a decrease in Ca^{2+} release from the SR,[4,19] the unaltered Ca^{2+} transient could arise from the net effect of a reduction in Ca^{2+} release from the SR and a decrease in Ca^{2+} bound to the myofilaments.[5] However, the fact that developed tension is reduced in the presence of an unchanged $[Ca^{2+}]_i$ is consistent with the hypothesis that the initial effects of acidosis are mediated directly via the myofibrillar proteins.

Slower Changes of Developed Tension

After the initial decrease of developed tension, adult rat muscle showed a recovery that was correlated with an increase in the size of the systolic rise of the Ca^{2+} transients, as described previously.[3] The mechanism that underlies this increase is not clear, but may be related to an increased resting $[Ca^{2+}]_i$ caused by acidosis.[20] This could be due to displacement of Ca^{2+} from intracellular stores or buffers by hydrogen ions, or to an increased Ca^{2+} influx, or to decreased efflux across the surface membrane. An increased resting $[Ca^{2+}]_i$ would lead to greater uptake by the SR and hence greater Ca^{2+} transients.

In contrast, neonatal muscle showed a quite different response. After a transient recovery, the tension showed a decline to a steady-state level, and this was associated with a small (although not statistically significant) decrease in the amplitude of the Ca^{2+} transients. A most striking difference between adult and neonatal rat ventricular muscle is the greatly reduced myofibrillar protein content and SR content in the neonate.[8] It seems possible that these differences underlie the observed difference in response. Our finding that the time course of the Ca^{2+} transient in the neonate is longer than in the adult (Figure 8) is consistent with the hypothesis that the neonate has less functioning SR.

There are a number of mechanisms that could account for the differences in steady-state responses between adult and neonatal muscle. It is known that the slow inward current is reduced by acidosis.[21,22] Also, it has been shown that Ca^{2+} uptake and release from the SR in skinned cardiac cells at constant Ca^{2+} is maximum at pH 7.4.[4] Normal pH_i is about 7.0, and a further decrease would be expected to reduce the Ca^{2+} transients. However, an opposing factor may be that acidosis increases the $[Ca^{2+}]_i$,[5,20] so that SR loading and release in acidosis might increase because of a raised $[Ca^{2+}]_i$. It is possible that because of the small amounts of SR in neonatal rat heart muscle the reduced Ca^{2+} current dominates, but in the adult the greater loading of SR dominates the changes in Ca^{2+} transients. These speculative ideas could potentially be tested in single cardiac cells in which both Ca^{2+} currents and the contribution of SR Ca^{2+} release can be examined more directly.

Comparison of the effects of acidosis on adult and neonatal rat ventricular muscle suggests that the smaller initial response in the neonate can be accounted for by the reduced effect of acidosis on the Ca^{2+} activation of the myofilaments. However, the steady-state tension, which is lower in the neonate than in the adult, cannot be explained by the differences in myofilament properties, but may be due to the slow changes in the magnitude of the Ca^{2+} transient.

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KEY WORDS • acidosis • detergent-skinned myofibrils • aequorin • troponin

APPENDIX 2.

EMD 53998 increases tension with little effect on the amplitude of the calcium transients in isolated ferret ventricular muscle,

by D.G. Allen & J.A. Lee.

An abstract published in the Journal of Physiology **416**, 43P (1989).

[*From the Proceedings of the Physiological Society*, 19–20 *May* 1989
Journal of Physiology, **416**, 43*P*, 1989]

EMD 53998 increases tension with little effect on the amplitude of calcium transients in isolated ferret ventricular muscle

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EMD 53998 (a thiadiazinone) is a new inotropic agent which has been shown to increase the sensitivity of skinned ventricular muscle to Ca^{2+} (Merck Pharmaceuticals, Darmstadt, FRG). We have investigated its effects on tension and intracellular calcium ($[Ca^{2+}]_i$) measured with aequorin in intact ventricular muscle. Fig. 1 shows that EMD caused a substantial, reversible increase in developed tension, while the aequorin light transients showed a small decrease (to 88% of control). When a comparable increase in tension was achieved by elevating extracellular Ca^{2+} concentration, the light transients increased to 150% of control. Fig. 1*B* (i) and (ii) show responses before and in the presence of EMD; (iii) shows that EMD abbreviated the light transient, but prolonged the tension. The increase in tension without increase in $[Ca^{2+}]_i$ and the changes in time course are all consistent with EMD causing an increase in the calcium sensitivity of the contractile proteins in intact muscle.

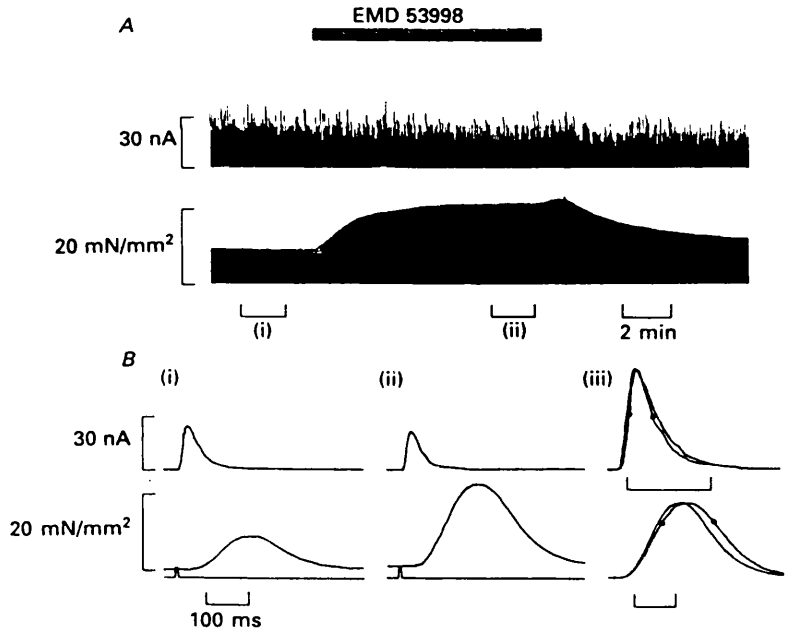

Fig. 1. Effect of 10 μM EMD (solvent acetone) on aequorin light (a measure of $[Ca^{2+}]_i$) and tension from an isolated ferret papillary muscle; 37 °C, 0·5 Hz, 0·5 mM extracellular Ca^{2+}, preparation pre-treated with guanethidine to minimize noradrenaline release. *A*, Continuous record. *B*, (i) and (ii), averaged records from the periods indicated in *A*. (iii), normalized, superimposed records from (i) and (ii). Time bars in (iii) both represent 100 ms, dots indicate EMD record.

J.A.L. is an MRC Training Fellow. We thank Merck for providing the drug and for defraying in part the cost of experiments.

APPENDIX 3.

Changes in intracellular calcium during mechanical alternans in isolated ferret ventricular muscle,

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A paper published in Circulation Research (in press).

Changes in intracellular calcium during mechanical alternans in isolated ferret ventricular muscle.

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Keywords: Aequorin, alternans, intracellular calcium, calcium cycling, sarcoplasmic reticulum, ischemia.

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Abstract

Mechanical alternans in heart muscle is a beat to beat alternating amplitude of force or pressure at a steady heart rate. Despite its potentially important role in clinical conditions such as pulsus alternans in cardiac failure and electrophysiological alternans in myocardial ischemia, the explanation of this phenomenon is still unclear. Several mechanisms have been proposed, including alternation of the action potential duration, incomplete relaxation and calcium cycling involving the sarcoplasmic reticulum. In this study, we attempted to investigate the cellular mechanisms of alternans by measuring intracellular free calcium concentration ($[Ca^{2+}]_i$) with the photoprotein aequorin in isolated ferret papillary muscles. Tension and length were also recorded simultaneously. Transient mechanical alternans lasting 5 - 20 contractions could be reliably induced in this preparation by following a 30 s rest period with stimulation at a fast rate (2-4 Hz). In order to produce sustained mechanical alternans, which lasted longer than twenty and could persist for several hundred contractions, it was necessary to use additional interventions. These were a lower temperature (25^{o}C), a lower external calcium concentration (1 mM) and a lower pH (6.91) than control conditions (0.33 - 0.5Hz, 30^{o}C, 2 mM Ca^{2+}, pH 7.36). Transient mechanical alternans was associated with transient in-phase alternation of aequorin light and hence $[Ca^{2+}]_i$. Sustained mechanical alternans was associated with sustained in-phase alternation of aequorin light, and was also associated with incomplete relaxation of tension. However, when muscles were switched from isometric to virtually unloaded isotonic contraction, relaxation between stimuli was complete, but contraction and the aequorin light signal continued to alternate. The addition of 10 mM caffeine or 10 μm ryanodine abolished transient and sustained mechanical alternans and also abolished the associated alternation of aequorin light. Commensurate with the action of ryanodine, which allows the sarcoplasmic reticulum to

reaccumulate calcium to a limited extent after a period of rapid stimulation, sustained mechanical alternans sometimes reappeared in an attenuated form 30 - 50 contractions after the addition of ryanodine. These results demonstrate that incomplete muscle relaxation between beats need not be present for alternans to occur, and support the hypothesis that alternans is caused by intracellular calcium cycling involving the sarcoplasmic reticulum.

Introduction

Mechanical alternans in cardiac muscle is defined as alternating large and small amplitude force (or pressure) while the heart rate is steady. It has long been of interest because of the clinical phenomenon of pulsus alternans, which was first described by Traube in 1872[1] in association with the failing heart, and may also be observed with cardiac anoxia or ischemia[2-4], and with increased ventricular loading[5-7]. Electrical alternans (alternation of action potential amplitude or duration), is also frequently seen during anoxia or ischemia[8,9] and has recently been observed to consistently precede severe arrhythmia in regional ischemia[10].

Despite its clinical relevance, however, the mechanism of mechanical alternans remains unclear. There have been several mechanisms proposed to explain this phenomenon. Early work concentrated on haemodynamic changes, and suggested, for example, a role for changes in end diastolic volume or initial muscle length[11-14]. However, several authors have demonstrated that it is possible to observe mechanical alternans in intact hearts in situ without significant differences in these parameters[15-18], and it has therefore been suggested that an 'alternating contractile state' is an independent mechanism for pulsus alternans in the intact animal[18]. This suggestion is supported by the well-known observation that alternans can occur in isolated papillary muscle preparations[19-22], in which haemodynamic factors cannot be operating.

Current interest is thus focussed on providing a cellular explanation to account for mechanical alternans. Most suggestions have been phenomenological in nature, and include what has been called alternating 'deletion and potentiation of contractility'[23], and 'beat to beat variation of the inotropic state'[16,24,25]. Other authors have suggested more specific mechanisms for mechanical alternans, such as alternation of the action potential duration[20-22,26], incomplete relaxation[19] and intracellular Ca^{2+}-cycling involving the sarcoplasmic reticulum (SR)[27].

Studies of the relation between action potential duration and mechanical alternans, have shown that where both mechanical and electrical alternans are present, the large beat of mechanical alternans may be associated with either prolonged[19,20,26] or shortened[19,21,22] action potentials, and may also be found in the absence of alternation in electrical activity[15,19,28,29]. On the basis of their studies of mechanical and electrical alternans in myocardium from a variety of mammals, Spear & Moore (1971)[19] suggested that action potential alternation was secondary to an alternation of the contractile state (see Lab (1982)[30] for review of possible mechanisms). They also suggested that incomplete relaxation, which is frequently seen in association with mechanical alternans, might be casually involved in producing it[19]. In contrast, Adler, Wong & Mahler (1985)[27] produced a mathematical model involving SR Ca^{2+}-cycling, which could closely reproduce many of the features of mechanical alternans. However, there has been little evidence for either of these mechanisms at a cellular level. In particular, the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) has not previously been studied under conditions in which mechanical alternans is provoked.

In this study, we have attempted to further define the cellular mechanisms of alternans by measuring $[Ca^{2+}]_i$ with aequorin in isolated ferret papillary muscles, while simultaneously measuring tension and length. The results demonstrate that mechanical alternans is associated with an in-phase alternation of $[Ca^{2+}]_i$, which was not abolished by allowing the muscle to relax completely, but was abolished (along with mechanical alternans) by caffeine or ryanodine. The results thus support the hypothesis that mechanical alternans is caused by intracellular Ca^{2+}-cycling involving the sarcoplasmic reticulum. A preliminary account of some of this work has been presented[31].

Methods

Ferrets were deeply anaesthetised by an overdose of pentobarbital administered intraperitoneally. The heart was removed, rapidly washed in cold physiological salt solution and transferred to a dissecting bath. Thin papillary muscles (mean diameter 0.75 $\pm$ 0.02 mm, n = 10) were dissected from the right ventricle and secured with a wire loop at each end. The muscle was then mounted horizontally in a tissue bath and superfused with physiological salt solution. One end of the muscle was secured to a force transducer and the other to a hook attached to the rotating arm of a Cambridge Technology series 300 dual mode servomotor. This arrangement allowed the muscle to be studied in either isometric or isotonic mode by altering the threshold force at which the rotating arm started to move. At the beginning of the experiment, muscles were stretched to the length at which developed tension was a maximum.

$[Ca^{2+}]_i$ was monitored using the photoprotein aequorin[32]. The methods were essentially similar to those described by Allen & Orchard (1983)[33]. Briefly, aequorin was microinjected into cells on the surface of the preparation, and the resulting light emission (a function of $[Ca^{2+}]_i$) was measured with a photomultiplier tube. The light collecting system consisted of a light guide which completely covered the muscle, and was designed so that movements of the muscle did not significantly affect light collection[34]. Force, light and stimulus signals were recorded on tape and also displayed on a four channel pen recorder. During analysis signal averaging was used to improve the signal to noise ratio of the light records.

The solution used to superfuse the muscles contained (in mM): Na^+, 135; K^+, 5; Ca^{2+}, 2; Mg^{2+}, 1; Cl^-, 102; HCO_3^-, 20; HPO_4^{2-}, 1; SO_4^{2-}, 1; acetate, 20; glucose, 10; and insulin 5U/L (40 nM). Initially the solution was equilibrated with 95% O2/5% CO2 to give a pH of 7.36, and the temperature was 30^{o}C. The muscle was stimulated with punctate electrodes using 2 ms

pulses at approximately 30% above the threshold. The initial stimulation frequency was 0.33 to 0.5 Hz.

We found that alternans could be reliably induced in this preparation by switching to a higher stimulation rate (2-4 Hz). Transient mechanical alternans lasting 5 - 20 contractions was always produced by this procedure. In order to produced sustained mechanical alternans, which lasted longer than twenty and could remain stable for several hundred contractions, it was necessary to vary other conditions as well as increase the stimulation rate. The interventions used were a lower temperature ($25^{o}C$), a lower external calcium concentration (1mM), and a lower pH (6.91; produced by changing the CO_2 in the superfusing solution from 5% to 15%) than control. Changing all of these paramenters produced the most reproducible sustained alternans. In some preparations sustained alternans could be produced by lowering only temperature and extracellular calcium concentration. Because of the pH sensitivity of aequorin light emission, the changes in intracellular pH produced by increasing the CO_2 would be expected to produce a small reduction of aequorin light emission by about 10%[33]. The size of the calcium transients would thus be slightly underestimated under acidic conditions. However, because the experiments reported here were concerned mainly with qualitative differences, this effect has not been corrected for.

Results

Two forms of mechanical alternans were observed[19,21,27,35]. When stimulation was started after a short rest period, a transient, self-correcting mechanical alternans was seen, which disappeared after 5 - 20 contractions. However, by adjusting the conditions, it was also possible to induce sustained mechanical alternans, which lasted for more than twenty and could remain stable for hundreds of contractions. In individual preparations under a given set of conditions, transient mechanical alternans was observed at lower stimulation frequencies than sustained mechanical alternans.

Figure 1 shows examples of transient mechanical alternans from one preparation at different stimulation frequencies. In this preparation, stimulation at a cycle length of 250 ms after a 30s rest (Fig. 1A), produced a transient alternation in tension which disappeared after 10 - 12 contractions. The mechanical alternans was matched by the light record, which indicates that there was an accommpanying alternation in $[Ca^{2+}]_i$. Note that the small Ca^{2+} transients became larger, until the alternation had virtually disappeared. Figure 1B shows the effect of stimulation at a cycle length of 300 ms. The alternans now lasted for a shorter time, and had disappeared after 6 - 8 contractions. Figures 1C & D show the effect of further increasing the cycle length, and demonstrate that the alternans had almost disappeared in this preparation at a cycle length of 400 ms and had gone at a cycle length of 500 ms. These results suggest that transient mechanical alternans is caused by transient alternation of $[Ca^{2+}]_i$. The fact that alternation of $[Ca^{2+}]_i$ is closely dependent on the frequency of stimulation is behaviour that would be expected of a phenomenon that involved Ca^{2+} cycling between Ca^{2+} stores and the myofilaments.

Figure 2 shows an example of sustained mechanical alternans. The original record (Figure 2A) illustrates that there was an initial large contraction, followed by a complex series of changes out of which sustained alternans (see fast playout section) of both tension and light developed.

This is clearer in Figure 2B, which shows an averaged record taken from the period when the alternans had become stable. The aequorin light record demonstrates that $[Ca^{2+}]_i$ was alternating in phase with the mechanical alternans.

Averaged records of tension and light during sustained mechanical alternans were made, and measurements were taken where the alternation of tension exceeded 5%. The tension and light measurements were then expressed as ratios of the large signal to the small signal, and these have been plotted against each other in Figure 3. There was a significant correlation ($R = 0.78$; $p < 0.01$) between the tension ratio and the light ratio during sustained mechancial alternans, supporting the suggestion that alternation of $[Ca^{2+}]_i$ causes the mechanical alternans.

Figure 2B shows that during sustained mechanical alternans, the large contraction did not relax completely before the small contraction began, leaving open the possibility that incomplete relaxation was involved in the production of mechanical alternans and that alternation of the Ca^{2+} transients was somehow a consequence of this. However, we consistently saw examples of mechanical alternans in which relaxation <u>was</u> complete or near complete, which were nevertheless accompanied by simultaneous alternation in $[Ca^{2+}]_i$. However, in order to investigate more carefully the role of incomplete relaxation in the production of alternans, we examined the effects of allowing preparations to perform lightly loaded isotonic contractions. This maneuver shortened the duration of contraction[36], and allowed the muscle to relax completely between stimuli. Figure 4 shows an example of such an experiment. During isotonic contractions relaxation was essentially complete, yet both contraction and light exhibited alternans. When the muscle was switched to isometric contractions, alternation of tension and light continued, although relaxation was now grossly incomplete. Finally, when the muscle was switched back to isotonic mode relaxation became complete again and alternation of tension and light continued. This result thus demonstrates that incomplete

relaxation is not necessary for the production of alternans. Figure 4B also illustrates that during isotonic contractions, the decline of the calcium transient was prolonged when compared with isometric contractions, as has been previously demonstrated[37].

As mentioned above, an alternative hypothesis to explain mechanical alternans is that it is due to a Ca^{2+} cycling phenomenon, probably involving the SR. If this were so, it should be possible to modify or abolish alternation by using substances which affect the ability of the SR to function as a Ca^{2+} store. Two such substances are caffeine, which affects cardiac cell function at several levels, but has a predominant effect on the SR handling of calcium[38,39], and ryanodine, which has a more specific effect on the SR[40,41]. Figure 5 shows the effect of 10 mM caffeine on alternation of tension and light. Addition of 10 mM caffeine to the superfusing solution reduced the size of the twitch, and also virtually abolished mechanical alternans (Figure 5A). The averaged records of Figure 5B show that caffeine simultaneously reduced the amplitude of the aequorin light signals and also abolished alternation of light. Caffeine also abolished the transient mechanical alternans observed after a rest period.

Figure 6 illustrates the effects of ryanodine on mechanical alternans. Figure 6A shows that 10 μM ryanodine completely abolished transient mechanical alternans. This was associated with a large reduction in the size of the calcium transients, which also showed no discernable alternation in the presence of ryanodine. Sustained mechanical alternans was also largely abolished by ryanodine, as shown in Figure 6B. Ryanodine caused a reduction in tension and light and also abolished the alternation in both. However, greatly reduced mechanical alternans reappeared in the presence of ryanodine after 30-50 contractions (not shown). This is in agreement with the known actions of ryanodine, which causes depletion of Ca^{2+} in the SR during a rest period, but allows a small amount of Ca^{2+} reaccumulation during stimulation at a rapid rate[41]. Under control conditions, the first contraction after a rest period was always considerably larger than the second contraction (e.g. Fig.

6A (i)). In the presence of both caffeine and ryanodine, however, the first contraction was always smaller (e.g. Figure 6A(ii)).

Another way in which the force of contraction can be modulated in cardiac muscle is by spontaneous Ca^{2+} release from the SR under conditions of high Ca^{2+} loading. This could conceivably give rise to mechanical alternans if the oscillation frequency became synchronised with the stimulation frequency. If this happened, it would be expected that during mechanical alternans, the small contraction would be preceded by a Ca^{2+} oscillation, while the large one would not. We looked for evidence that oscillations of $[Ca^{2+}]_i$ might be involved in the production of alternans. However, oscillations were only rarely seen under our conditions, and did not apparently contribute to the development or maintenance of alternans in these experiments.

Discussion

In this study we have used the photoprotein aequorin to measure $[Ca^{2+}]_i$ under conditions in which mechanical alternans was produced in isolated ferret papillary muscles. The experiments were performed in a specially designed chamber, in which light collection was independent of muscle position[34], and it is therfore unlikely that movement artefacts contribute to the results. When either transient or sustained mechanical alternans was induced, it was found that the Ca^{2+} transients alternated in phase with the tension. In the following discussion, we will concern ourselves mainly with sustained mechanical alternans, because this is the type most often described in association with pathological situations. However, it is likely that transient mechanical alternans is produced by similar mechanisms, because it is observed under similar conditions to sustained mechanical alternans and also responds similarly to pharmacological interventions such as caffeine and ryanodine. Also, mathematical modelling of alternans is able to produce both transient and sustained mechanical alternans by assuming the same mechanism.

There have been three specific hypotheses proposed to explain mechanical alternans in isolated superfused muscle: (i) incomplete relaxation; (ii) alternation of the action potential duration; and (iii) a Ca^{2+} cycling phenomenon, resulting in alternating release of Ca^{2+} from the SR. We will discuss these mechanisms in turn.

Incomplete relaxation

Incomplete relaxation of cardiac muscle is often seen under conditions in which mechanical alternans is present, and Spear & Moore[19] proposed that this could be responsible for the production of mechanical alternans. They suggested that re-exciting the muscle before it had completely relaxed prevented the myofibrils from being fully re-activated in the next contraction. Hence less tension was generated during this contraction, and relaxation proceeded to a greater degree before the next stimulus. Our experiments show that although changing from an isometric to an isotonic

arrangement caused a marked change in contraction (from complete to incomplete relaxation), mechanical alternans and alternation of the Ca^{2+} transients was little altered. Thus, incomplete relaxation is unlikely to be fundamental to the mechanism of mechanical alternans, i.e. mechanical alternans is not simply a consequence of high stimulation rates not allowing enough time for relaxation between contractions when muscles are contracting isometrically. It is possible that the prolongation of the time course of decay of the calcium transient on changing from isometric to lightly loaded isotonic contractions[37] could influence calcium cycling within the muscle. In fact, load changes have been reported to trigger alternans under experimental conditions[21] and in clinical studies[5-7]. Thus, the loading conditions of the muscle (and hence indirectly the extent of relaxation) should be considered as one of the factors modulating alternans. The mechanism of this modulation is probably due to the changes in $[Ca^{2+}]_i$, rather than to the mechanical response of the muscle (i.e. the extent of relaxation) per se.

Action potential alternans

Evidence that alternation of the action potential duration is the cause of sustained mechanical alternans is inconsistent. The large beat of mechanical alternans has been associated with prolongation[19,20,26], abbreviation[19,21,22], and no change[15,19,28,29] in the action potential. In their careful studies on heart muscle from a variety of mammals, Spear & Moore (1971)[19] found all three of these patterns. For example, guinea pig myocardium showed both prolongation and abbreviation of the action potential, while in rat myocardium, even large degrees of mechanical alternans were not accompanied by alternation of the action potential. Spear & Moore also observed that when the stimulation rate was increased, mechanical alternans appeared before alternation in the action potential, and they suggested that alternation of the action potential duration was secondary to the mechanical alternans. This hypothesis, that mechanical activity may feed back and influence electrical activity, now has considerable

evidence to support it[30]. The most likely mediator of this interaction is $[Ca^{2+}]_i$[30,37] acting via Ca^{2+}-activated currents[42]. Alternation of $[Ca^{2+}]_i$ during mechanical alternans provides a plausible mechanism for observations that mechanical alternans may be associated with electrical alternans. Although alternation of the action potential may be a secondary phenomenon, it could contribute to the maintenance of alternans, and it is also of considerable interest in relation to the generation of arrhythmias in the intact heart (see below).

Ca^{2+} cycling

Many studies of mechanical restitution (i.e. the recovery of contraction as the interval preceding the test beat is lengthened) confirm the classical studies of Koch-Weser & Blinks[43], and suggest that some form of intracellular Ca^{2+} cycling occurs in cardiac cells[44-48]. Most authors favor the simplest model of excitation-contraction coupling which can account for many of these findings. This model suggests that at normal physiological steady states the intracellular Ca^{2+} store consists functionally of two separate compartments[44-45]. An 'uptake' compartment recovers Ca^{2+} from the myoplasm, and then passes the Ca^{2+} to a 'release' compartment, which, in turn, empties it into the myoplasm when the cell is stimulated. The mechanical restitution curve is thought to represent the time course of the transfer process between the uptake and the release sites, with the plateau of the curve reflecting the maximum amount of Ca^{2+} which can be accumulated in the release sites. A recent study[49] has supported this hypothesis by demonstrating that restitution of $[Ca^{2+}]_i$ is very similar to mechanical restitution.

We tested the idea that Ca^{2+}-cycling could be involved in the production of mechanical alternans by applying either caffeine or ryanodine. Although caffeine has several actions on cardiac muscle[38,39], one of its main effects is to impair the ability of the SR to function as a Ca^{2+} store. In these experiments, caffeine abolished both transient and sustained

mechanical alternans and the associated alternation of $[Ca^{2+}]_i$, strongly indicating that the SR is closely involved in the production of mechanical alternans. The experiments with ryanodine also support the Ca^{2+}-cycling hypothesis. Ryanodine depletes the SR during rest periods but allows it to load with Ca^{2+} during stimulation[41]. Mechanical restitution in the presence of ryanodine shows an initial very steep slope after which it is flat; i.e. it reaches maximum restitution at very short test intervals and shows little change at the longer intervals[49]. In our studies, during control conditions, the first beat on beginning stimulation was always larger than the second and transient mechanical alternans was always seen. Ryanodine abolished both the initial large beat and transient mechanical alternans; it also initially abolished sustained mechanical alternans, but then allowed attenuated mechanical alternans to reappear after 30 - 50 contractions during continued rapid stimulation. These observations are commesurate with the known actions of ryanodine and strongly implicate the SR in the production of mechanical alternans with a dominant role being played by recirculating Ca^{2+}.

This hypothesis can account for many of the features of mechanical alternans and has been effectively mathematically modelled[27]. The suggestion is, that under conditions in which mechanical alternans is present, there is not enough time between alternate contractions for Ca^{2+} to reaccumulate in the release sites to a level which will allow activation of the contractile proteins to the same extent as the in preceding contraction. This reduced Ca^{2+} release produces small, say even numbered, beats. The cycling process is quickly over and a large proportion of the interval between this and the following beat is available for fuller restitution of odd numbered beats. The resultant large contraction has a longer cycling process leaving a shorter interval for restitution which is thus incomplete again. The intracellular microenvironment does not allow a dampening of this alternation and the system shows sustained oscillation between large and small beats. In the preparations used in these experiments, maximum

stimulation frequency alone produced a transient mechanical alternans, suggesting that it was not possible to stimulate faster than the restitution of the Ca^{2+} cycling process under control conditions. However, if high stimulation frequency was combined with other interventions such as reduced temperature, reduced $[Ca^{2+}]_e$ and acidosis, sustained mechanical alternans was produced. Although the precise details of how these interventions produce sustained mechanical alternans are unknown, it is clear that they all affect intracellular Ca^{2+} homeostasis. Reduced temperature is known to increase the permeability of the SR to Ca^{2+} [39], acidosis affects SR Ca^{2+} handling[33,50], and altering $[Ca^{2+}]_e$ changes both diastolic and systolic $[Ca^{2+}]_i$ and would be expected to lead to a different steady state in the SR compartments. Whatever the exact mechanism, the alterations in intracellular Ca^{2+} handling induced by these interventions are clearly sufficient to put the muscle into a condition in which a 'two state' release of Ca^{2+} is stable and preferred[27].

Calcium oscillations

One possibility that seemed an attractive candidate to explain mechanical alternans was that oscillations of $[Ca^{2+}]_i$ could become synchronised with the frequency of stimulation. Oscillations of $[Ca^{2+}]_i$ have been directly observed in other circumstances and they may be spontaneous, especially if diastolic calcium is high[51,52]. Thet have also been shown to influence the Ca^{2+} transient and the tension generated by a succeeding beat[51,52]. If this mechanism was operating, the small beat of alternans would be preceded by an oscillation, while the large beat would be preceded by a smaller oscillation or no oscillation. We found no evidence to support this hypothesis in these experiments. However, in order to produce mechanical alternans in this preparation we needed to use high frequencies of stimulation. If the stimulation frequency was faster than the natural frequency of any oscillations which would have been present, then oscillations could not develop and contribute to mechanical alternans. It is thus possible that oscillations might be important in the generation and maintenance of

mechanical alternans in circumstances where it occurs at lower contraction frequencies.

Clinical implications

Mechanical alternans, and alternation of the action potential, have been linked with a variety of clincial conditions. Two notable examples are pulsus alternans found in association with cardiac failure and ischemia[1-4] and electrical alternans found in association with ischemic heart disease[8-10]. The former represents contractile failure at the cellular level, and the latter has been observed to precede ventricular arrhythmias. It has recently been shown that alternation of monophasic action potentials recorded from the epicardial surface of open chested pigs is almost invariably associated with the development of early arrhythmias following ligation of a coronary artery[10]. In this study, adjacent regions of myocardium were frequently found to exhibit alternation of action potential which was out phase, thus increasing the possbility of current flow in abnormal pathways. Interestingly, many of the conditions we used to elicit alternans may be expected to exist in ischaemic myocardium. Tissue CO_2 is known to rise rapidly following cessation of blood supply[53], pH falls[54] and tachycardia is often present. It is thus likely that mechamisms similar to those observed in this study produce alternation in ischemic hearts. A recent study in which Ca^{2+} transients in ischemic heart muscle have been investigated[55], has demonstrated that alternation of both tension and Ca^{2+} transients does indeed occur under ischemic conditions.

In summary the results presented in this study demonstrate that an alternation of $[Ca^{2+}]_i$ underlies mechanical alternans, and support the general hypothesis that the alternation of $[Ca^{2+}]_i$ is due to a form of intracellular Ca^{2+} cycling.

Acknowledgements: This work was supported by the British Heart Foundation and the Medical Research Council. M.J.L. is a Wellcome Senior

Research Fellow. J.A.L. is an MRC Research Training Fellow. We would like to thank Dr David Allen for the facilities of his laboratory and for much helpful discussion. We also thank Dr. J.R. Blinks (Department of Pharmacology, Mayo Medical School, Rochester, Minnesota) for aequorin, which was purified in his laboratory with support from NIH grant HL 12186.

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Figure Legends

Figure 1. Transient mechanical alternans during stimulation at different cycle lengths following a 30 s rest. Each panel is the average of 4-6 stimulation periods and shows aequorin light (above), tension (middle) and stimulus (below). At each cycle length the mechanical alternans was closely mirrored by alternation of the aequorin light signal. Alternation of both tension and light lasted for a shorter period and became less marked as the cycle length was increased A. Cycle length 250 ms. The first stimulus caused a large Ca^{2+} transient and also a large contraction; however, the second produced only a very small transient and no discernable effect on tension. Alternation of tension and light then diminished in parallel over the next 6-8 contractions. B. Cycle length 300 ms. Again, the first stimulus produced a large Ca^{2+} transient and tension, while the second had little effect. The alternans dissapeared over the next 4-6 contrqactions. C. Cycle length 400 ms. The first stimulus produced a large Ca^{2+} transient and contraction, while the second stimulus now produced a small Ca^{2+} transient and twitch. Alternation of light was not apparent after the third stimulus and mechanical alternans had disappeared by the fifth stimulus. D. Cycle length 500 ms. The large first Ca^{2+} transient and twitch were succeeded by a larger second transient and twitch than any seen at shorter cycle lengths. Alternans was not apparent after the first two stimuli. In B, C & D the initial twitch has been allowed to saturate in order to show the succeeding twitches more clearly. (Temperature 30°C, $[Ca^{2+}]_e$ 2mmol/l, pH_e 7.36; similar results were seen in 10 preparations).

Figure 2. Sustained mechanical alternans in an isolated ferret papillary muscle. A. Original record on a slow timebase showing the development of sustained mechanical alternans following rapid stimulation (cycle length 400 ms) after a rest period. Figure shows aequorin light (above) and tension (below). The envelope of tension indicates the magnitude of the larger contraction, while the central darker part indicates the magnitude of the

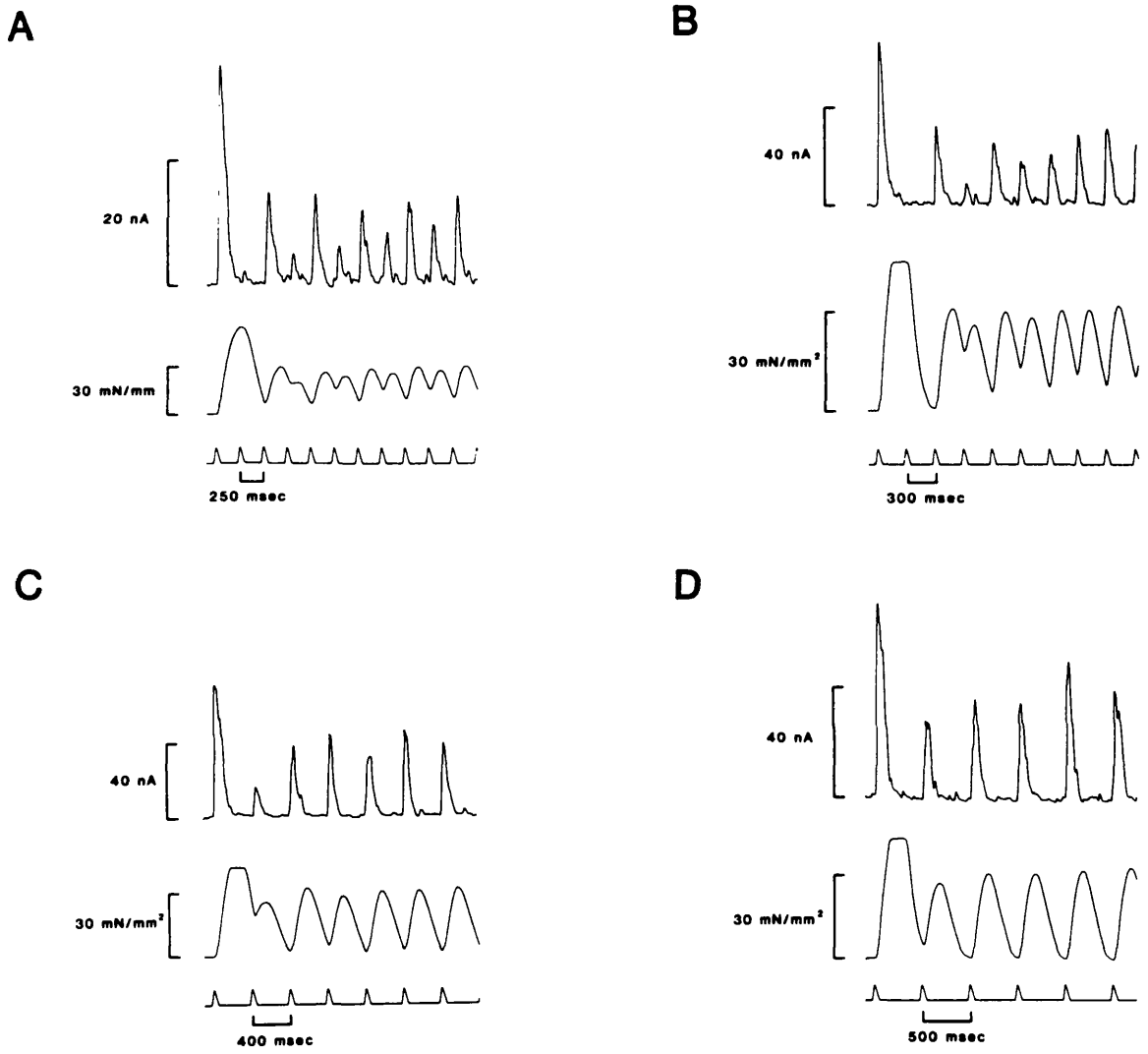
smaller contraction. The same is true of the light record. A short period of faster playout shows the individual twitches and Ca^{2+} transients. B. Averaged records showing aequorin light (above), tension (middle) and stimulus (below) from the period indicated in A. Mechanical alternans is accommpanied by an in phase alternation of aequorin light. Note the incomplete relaxation of tension, and also the absence of oscillations or a raised baseline in the light record. (Temperature 25°C, $[Ca^{2+}]_e$ 1 mmol/l, pH_e 6.91; similar results were seen in 10 preparations).

Figure 3. Graph of light alternans against tension alternans. Averaged records were obtained from periods of sustained mechanical alternans. Measurements of tension and light were taken when the mechanical alternans exceeded 5%, and light and tension expressed as ratios of the large to the small signals. There was a significant correlation between the light ratio and the tension ratio ($r=0.78$, $p<0.01$), supporting the hypothesis that alternation of $[Ca^{2+}]_i$ causes mechanical alternans. Pooled data from 10 preparations.

Figure 4. Alternation of tension and light under isotonic and isometric conditions. A. Original records showing Ca^{2+} transients (above) isometric tension (middle) and muscle length (below). The muscle was initially contracting isotonically with a low load. It was then switched to isometric and finally back to isotonic contractions. It is clear that contraction continued to alternate in spite of marked changes in the degree of relaxation. B. Averaged records from the periods indicated in A. (i) is from the period of isometric contraction and (ii) is from the period of isotonic contraction. It can be seen that the Ca^{2+} transients (above) showed alternation in both situations, and were little affected by switching from isometric to isotonic contraction. Note that the calcium transients became prolonged under isotonic conditions (Temperature 25°C, $[Ca^{2+}]_e$ 1 mmol/l, pH_e 7.36; similar results were seen in 4 preparations).

Figure 5. The effects of 10 mM caffeine on alternans. A. Original record with a slow timebase showing envelopes of light (above) and tension (below). Two short periods of faster timebase illustrate the form of the mechanical alternans and the effect of caffeine. B. Averaged data from the periods indicated in A, showing light (above) and tension (below). Alternation of tension and light (i) was abolished by caffeine (ii). Caffeine also reduced the amplitude of both tension and light. (Temperature 25 ^{o}C, $[Ca^{2+}]_e$ 1 mmol/l, pH_e 7.36; similar results were seen in 3 preparations).

Figure 6 The effects of 10 μM ryanodine on alternans. A. Each panel shows aequorin light (above) and tension (below). (i) Transient alternans produced by stimulating at a cycle length of 400 ms. (ii) In the presence of ryanodine, the amplitude of tension and light were reduced, and the transient alternation of both was abolished. Note that ryanodine also abolished the initial large contraction when stimulation was restarted. B. Each panel shows aequorin light (above), tension (middle) and stimulus marker (below). (i) Averaged record obtained during sustained alternans. (ii) Alternation of light and tension was abolished in the presence of ryanodine. (Temperature 25^{o}C, $[Ca^{2+}]_e$ 1 mmol/l, pH_e 6.91; similar results were seen in 3 preparations).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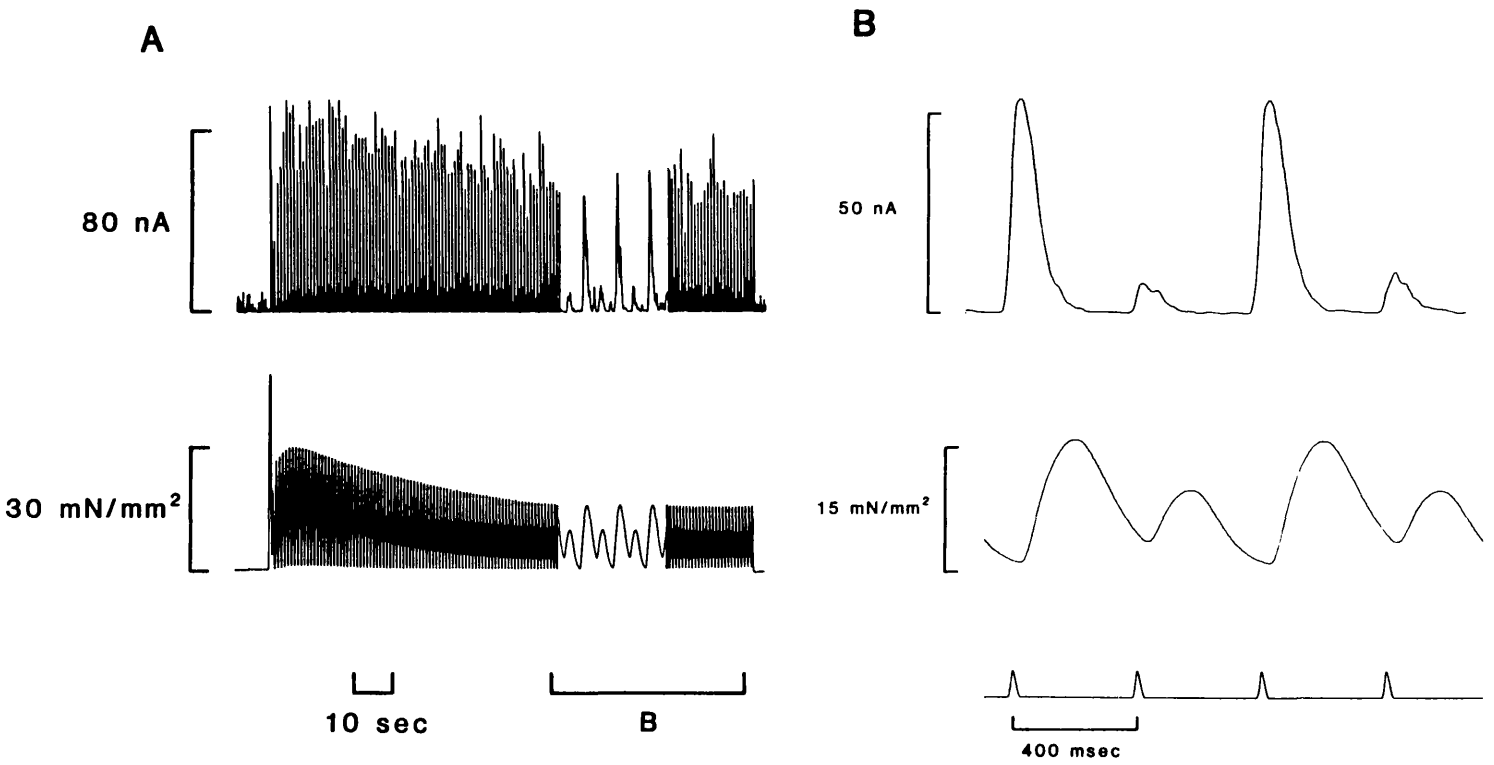
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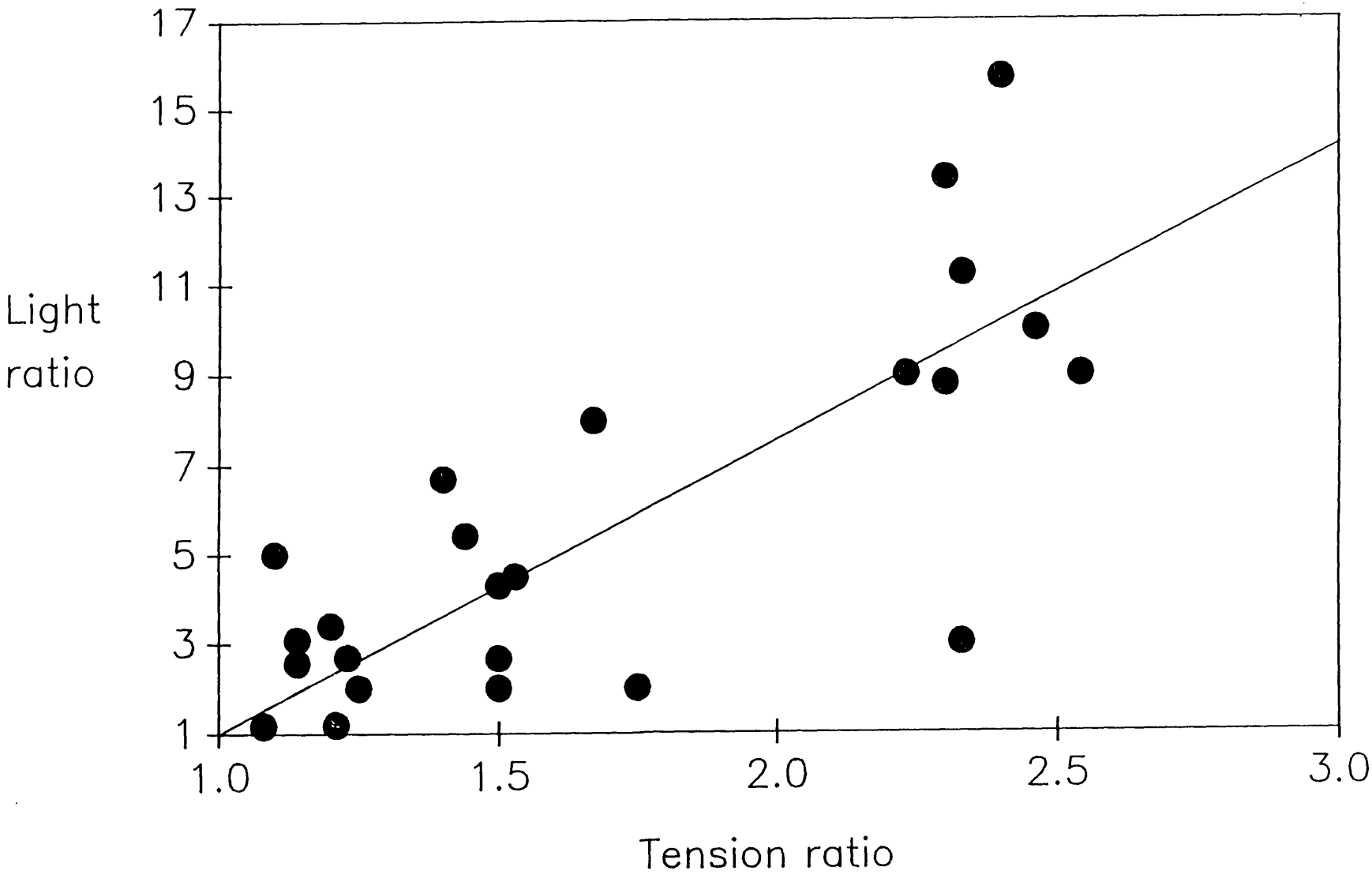
17
15
13
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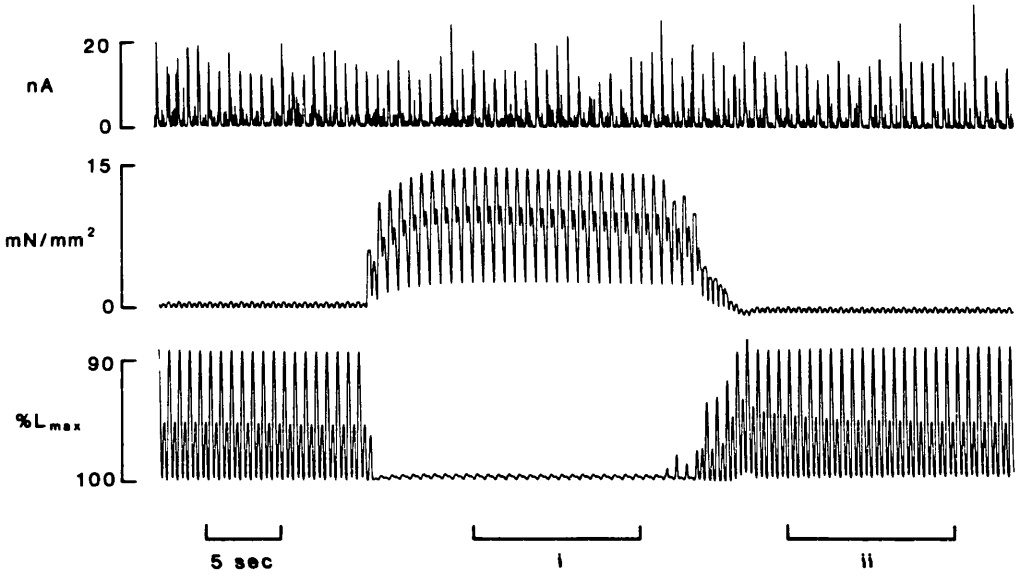
Tension ratio

A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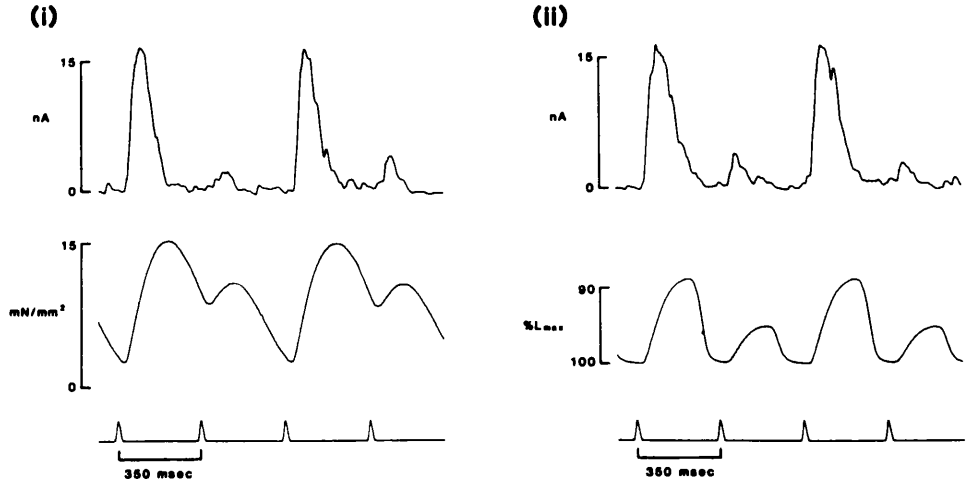
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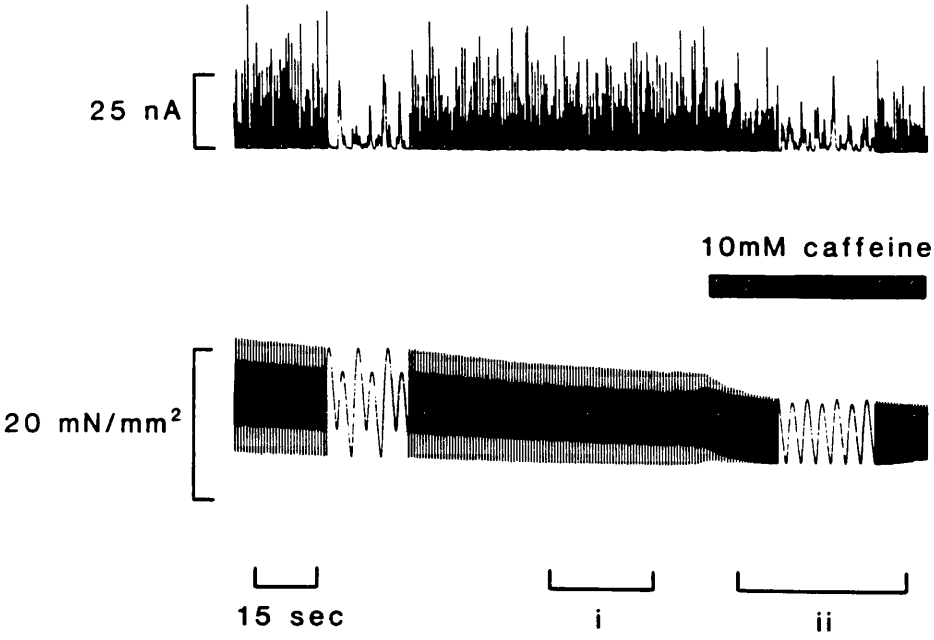
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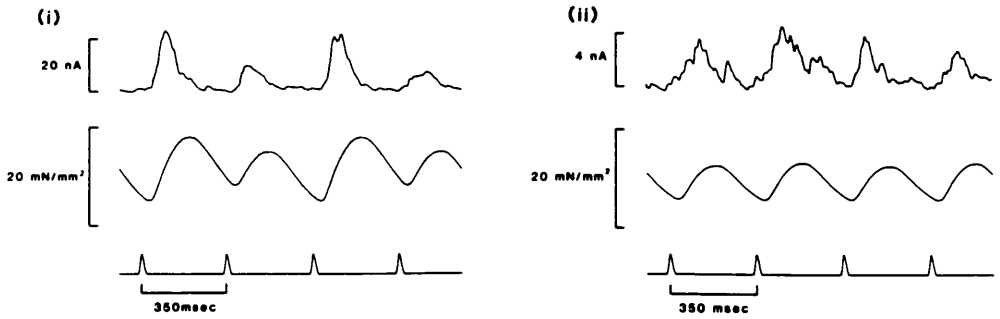
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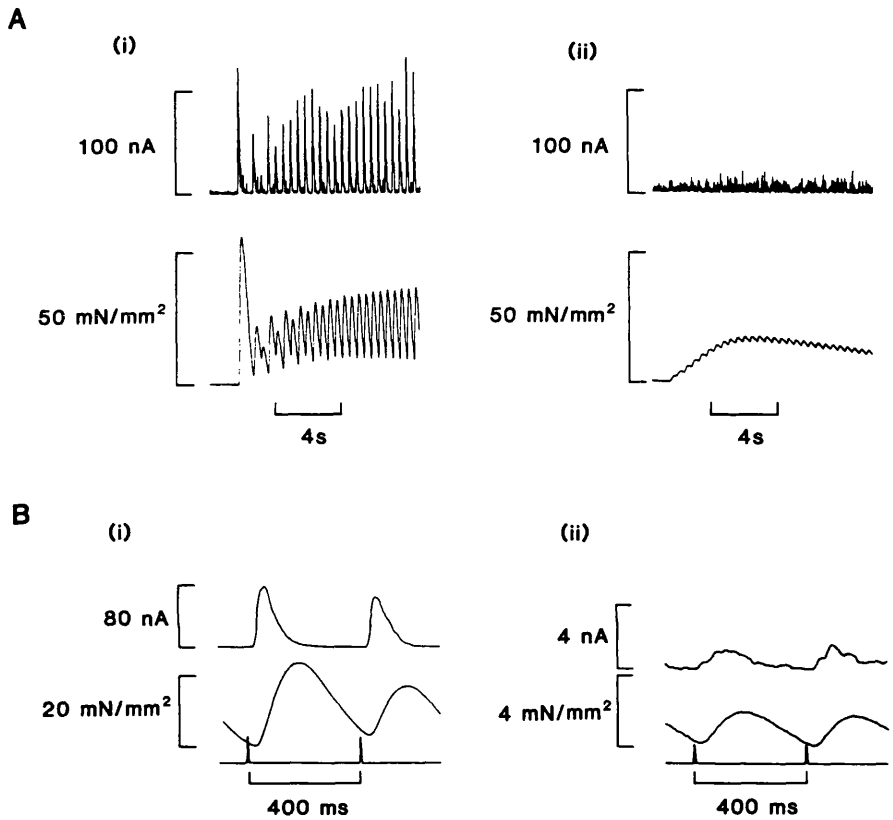
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20 mN/mm²
400 ms
(ii)
4 nA
4 mN/mm²
400 ms

APPENDIX 4.

Intracellular calcium and tension during fatigue in isolated single muscle fibres from *Xenopus laevis*,

by D.G. Allen, J.A. Lee & H. Westerblad.

A paper published in the Journal of Physiology **415**, 433 - 458 (1989).

Journal of Physiology (1989), **415**, *pp.* 433–458
With 12 text-figures
Printed in Great Britain

INTRACELLULAR CALCIUM AND TENSION DURING FATIGUE IN ISOLATED SINGLE MUSCLE FIBRES FROM *XENOPUS LAEVIS*

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(*Received 8 February 1989*)

SUMMARY

1. Single muscle fibres were dissected from *Xenopus* lumbrical muscles and microinjected with the photoprotein aequorin in order to measure the myoplasmic free calcium concentration ($[Ca^{2+}]_i$). Fatigue was produced by repeated intermittent tetanic stimulation continued until tension had declined to approximately 50% of the initial level. Fibres were then allowed to recover by giving tetani at less frequent intervals. Aequorin light (a measure of $[Ca^{2+}]_i$) and tension were measured during fatiguing stimulation and recovery.

2. During fatiguing stimulation, tetanic tension decined steadily, but peak aequorin light first increased before declining substantially. The largest light signal was about 155% of initial control while at the end of fatiguing stimulation the tetanic light fell to about 14% of control.

3. Fibres showed a characteristic slowing of relaxation in the fatigued state. This was associated with a slowing of the rate of decline of the aequorin light signal.

4. Intracellular acidosis produced by equilibrating the Ringer solution with either 5 or 15% CO_2 caused an increase in the light signal associated with a tetanus. Carbon dioxide also caused a reduction of tension and a slowing of relaxation.

5. *In vivo* pCa–tension curves were constructed by exposing the fibres to a series of K^+ concentrations which produced contractures of different sizes. Light and tension were measured during periods when both were relatively stable and the light signal was subsequently converted to pCa.

6. Exposure of fibres to 5 or 15% CO_2 caused the pCa–tension curve to be shifted to the right of the control curve. This indicates a reduced Ca^{2+} sensitivity of the contractile proteins, which is in agreement with results from skinned fibre studies.

7. The pCa–tension points obtained from tetani during the early part of fatiguing stimulation also deviated to the right of the control pCa–tension curve, suggesting a reduced Ca^{2+} sensitivity of the contractile proteins. At the end of fatiguing stimulation, however, pCa–tension points did not differ greatly from the control pCa–tension curve, suggesting that Ca^{2+} sensitivity was approximately normal. Thus the reduced $[Ca^{2+}]_i$ during tetani at the end of fatiguing stimulation (when tension was reduced to approximately 50%) could explain all of the reduction in tension.

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8. After fatiguing stimulation, tension and light recovered monotonically in some fibres; however, in the majority of fibres, tension and light showed a secondary decline followed by a slower recovery (post-contractile depression).

9. During post-contractile depression, caffeine contractures or tetani in the presence of caffeine gave increased aequorin light signals and the tension developed was close to that produced in an unfatigued tetanus.

10. The results of this study show that changes of $[Ca^{2+}]_i$ during fatigue can account for much of (i) the tension reduction during fatigue, (ii) the slowing of relaxation, and (iii) the tension reduction during post-contractile depression.

INTRODUCTION

Repeated tetanic contractions of skeletal muscle eventually lead to a reduction in the developed tension, i.e. fatigue develops. In intact animals, this familiar phenomenon is known to have contributions from the central nervous system, the neuromuscular junction and the muscle itself (Bigland-Ritchie & Woods, 1984). However, many features of fatigue may be seen in isolated muscle preparations, suggesting that processes within the muscle make a large contribution.

In isolated muscles, the intracellular mechanisms which cause the various manifestations of fatigue are not well understood. One hypothesis to explain reduced tension in fatigue is that it is due to the changes in intracellular metabolites which accompany prolonged activity. These include reduced concentrations of phosphocreatine (PCr) and ATP, and increased concentrations of creatine (Cr), inorganic phosphate (P_i), ADP, lactic acid and hydrogen ions (i.e. a reduced pH) (Dawson, Gadian & Wilkie, 1978; Nassar-Gentina, Passonneau, Vergara & Rapoport, 1978). Further, it is established from skinned fibre studies that increased P_i and H^+ have marked tension-reducing properties when applied to isolated myofibrils (Fabiato & Fabiato, 1978; Cooke & Pate, 1985; Kentish, 1986; Godt & Nosek, 1989).

An alternative hypothesis suggests that changes in excitation–contraction coupling may underlie the fall in tension. This was first suggested by Eberstein & Sandow (1963) on the basis that the tension developed by fatigued muscles could be substantially increased by exposure to high potassium or caffeine, procedures which bypass certain steps in the excitation–contraction sequence. While these observations have been confirmed, and it is widely accepted that changes in excitation–contraction coupling have some role in fatigue (Grabowski, Lobsiger & Lüttgau, 1972; Lännergren & Westerblad, 1989), there is little information on the precise step in excitation–contraction coupling which might be affected.

Several other features of fatigued muscle may also be related to changes in intracellular Ca^{2+} movements resulting from abnormal excitation–contraction coupling. Slowing of relaxation is a characteristic feature of fatigue and has been postulated to be due to a reduced rate of uptake of Ca^{2+} by the sarcoplasmic reticulum (Dawson, Gadian & Wilkie, 1980). Also, recent studies have shown that following stimulation there is often a reversible secondary decline of tension (post-contractile depression), which has been attributed to failure of excitation–contraction coupling (e.g. Lännergren & Westerblad, 1989). However, direct evidence on both these points is lacking.

In this study, we have investigated the role of excitation–contraction coupling in

fatigue by using the Ca^{2+}-sensitive photoprotein aequorin to measure the myoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Single muscle fibres were fatigued with intermittent tetanic stimulation which continued until tension was reduced to about 50% of control. The results show that there are changes in $[Ca^{2+}]_i$ during fatigue and recovery which can explain much of (i) the tension reduction during fatigue, (ii) the slowing of relaxation, and (iii) the tension reduction during post-contractile depression. Preliminary accounts of some of these results have been presented (Allen, Lee & Westerblad, 1988, 1989*a*).

METHODS

Single fibre dissection and mounting

Experiments were performed on single muscle fibres from adult female *Xenopus laevis*. The frogs were killed by stunning followed by decapitation. Single fibres were dissected from any of the lumbrical muscles II–IV under dark field illumination. The trimmed tendons of the fibres were held with platinum foil micro-clips. After dissection, the largest and smallest diameters of the fibres were measured using an ocular scale and the cross-sectional area of the fibre was calculated. Fibres were then transferred to the perfusion channel of the experimental chamber. One end was attached to a horizontally mounted tension transducer (Akers AE 801, SensoNor, Norway) via a glass extension with a fine hook at the end. The other end was attached to a movable hook which allowed the length of the fibre to be adjusted so that maximum tetanic tension was obtained. Fibres were superfused with a standard Ringer solution containing (mM): Na^+, 120; K^+, 2·5; Ca^{2+}, 1·8; Cl^-, 121; HPO_4^{2-}, 2·15; $H_2PO_4^-$, 0·85; pH, 7·0. All experiments were performed at 21 °C.

Microinjection of aequorin

Fibres were injected with aequorin essentially as described by Blinks, Rüdel & Taylor (1978). Briefly, the procedure was as follows. Aequorin solution (approximately 50 μM in 140 mM-KCl, 5 mM-HEPES, pH 7) was placed at the end of a conventional microelectrode (resistance 30–50 MΩ). The microelectrode was inserted into the fibre under visual control and while monitoring the electrode potential. When the microelectrode was in the fibre, pulses of pressure from a gas cylinder were applied. After several pressure pulses, the electrode was removed and a photomultiplier tube with an attached acrylic light guide was placed over the fibre. The light emitted by the fibre was then monitored during a test stimulus. Often several impalements with different microelectrodes were necessary to achieve an acceptable aequorin signal. In some experiments the external $[Ca^{2+}]$ was raised to 5 mM during the injection period because this was found to decrease the risk of damage to the fibre during injection.

Experimental protocol

Fibres were stimulated with platinum electrodes using alternating current pulses with a duration of 0·5–1·0 ms and an intensity of approximately 1·2 × threshold. The stimulation frequency was 40 or 50 Hz. Fatigue was produced using a protocol similar to that of Westerblad & Lännergren (1986). Initially, 500 ms tetani were given every 4 or 5 s and the inter-tetanus interval was then successively decreased every 2 min to 4·0, 3·0, 2·5, 2·0, 1·7 and 1·5 s. Stimulation was continued until the tension was reduced to about 50% of control. To study recovery from fatigue, test tetani were given at various times after fatiguing stimulation. These times were usually 1, 5, 10, 20, 30 and 40 min into recovery and then every 20 min until tension was stable.

The perfusion channel of the experimental chamber was provided with a side arm which allowed injection of solutions. In most experiments K^+ contractures were produced before fatiguing stimulation by rapid injection, via this side arm, of solutions containing varying concentrations of K_2SO_4. These solutions were constructed by mixing 75 mM-K_2SO_4 (150 mM-K^+) with Ringer solution in various proportions. In some experiments 150 mM-K^+ was applied at various times during the recovery period. Applications of Ringer solution containing high doses of caffeine (10 mM) were performed in a few experiments. These caffeine contractures generally resulted in irreversible fibre damage. In other experiments low doses of caffeine (3 mM) were instead combined with tetanic stimulation; this procedure decreased the risk of fibre damage.

In some experiments the effects of acidosis were examined before fatiguing stimulation. Fibres were exposed to Ringer solution which had been equilibrated with either 5 or 15% CO_2, which reduced the external pH to 6·3 and 5·9, respectively.

Tension, aequorin light and stimuli were displayed on a pen recorder and also recorded on tape for later analysis. Values in the results are presented as mean ± S.E.M.

Interpretation and calibration of aequorin light signals

During fatiguing stimulation there are a number of metabolic changes inside muscle fibres which might affect aequorin light emission. Decreased pH is known to occur in muscle fibres exposed to repeated tetani, due to lactic acid production (Dawson *et al.* 1978). In the fibre types used in these experiments, intracellular pH fell during fatiguing stimulation by about 0·6 pH units in easily fatiguable fibres and by 0·3 pH units in fatigue-resistant fibres (Westerblad & Lännergren, 1988). Acidosis reduces aequorin light emission, but this effect is small at intracellular levels of $[Ca^{2+}]$: an acidosis of 0·5 pH units at a $[Ca^{2+}]$ of 10 μM caused a 30% (0·15 log unit) reduction in light emission, but had no effect at $[Ca^{2+}] = 0{\cdot}1$ μM (Allen & Orchard, 1983). Others have found no change in aequorin light emission for a 0·5 pH unit change at $[Ca^{2+}]$ of 2–6 μM (e.g. Fabiato, 1985).

Magnesium (Mg^{2+}) is another ion which can compete with Ca^{2+} for aequorin and hence cause a reduction in light emission. Its concentration might be expected to rise during fatigue because of release from ATP and possibly also because of displacement from parvalbumin if the average $[Ca^{2+}]_i$ during fatiguing stimulation is increased. However, to our knowledge, $[Mg^{2+}]_i$ has not been measured during fatigue. Thus the changes in the intracellular environment of fatigued fibres may cause a small reduction in aequorin light for a given $[Ca^{2+}]$. In view of these uncertainties, it is important to note that the Ca^{2+} signals during fatigue have now been measured with Fura-2 (Allen, Lee & Westerblad, 1989*b*), a fluorescent Ca^{2+} indicator which is not sensitive to $[Mg^{2+}]$ and has quite different properties to aequorin. The Fura-2 signals confirmed all the major features observed in the present study, making the presence of major artifacts due to changes in intracellular metabolites most unlikely.

Consumption of aequorin will reduce the aequorin light signals during fatigue. To quantify this effect we have compared the integrated light in one tetanus with the light produced when all the remaining aequorin was discharged at the end of an experiment with Triton X-100. On average, a normal tetanus (50 Hz, 0·5 s, unfatigued) consumed 0·07% (range 0·04–0·13%, $n = 6$) of the aequorin, while a maximal K^+ contracture (150 mM-K^+) consumed 3·3% (range 1–8%). In the six experiments examined, the train of tetani during fatigue consumed 5·9% (range 1–13%) of the aequorin, the variability arising both from the size in aequorin transients and the number of tetani required for fatigue. Correction for this small effect is made in the Discussion. Potassium contractures were never produced during a fatigue run so that, although they produce significant consumption, they do not affect our estimate of the fall of light during fatigue.

In some experiments (see Figs 5, 6 and 10) the light signals have been converted to $[Ca^{2+}]$ using a calibration procedure, details of which are given in Smith & Allen (1988). In principle this involves normalizing the light signal for the amount of aequorin in the preparation and then converting the normalized light signal to $[Ca^{2+}]$ by means of an appropriate *in vitro* calibration curve. It should be noted that this calibration has a number of well-recognized weaknesses (e.g. Blinks, Wier, Hess & Prendergast, 1982; Smith & Allen, 1988) and provides an indication of $[Ca^{2+}]$ rather than a precise calibration.

RESULTS

It is well recognized that single muscle fibres vary substantially in their susceptibility to fatigue. Following previous studies with *Xenopus* single fibres (Westerblad & Lännergren, 1986) we have defined easily fatiguable fibres as those in which tetanic tension declined to less than 50% at stimulation intervals of 2·5 s or longer, and fatigue-resistant fibres as those in which shorter interstimulus intervals than 2·5 s were needed for tension to fall to 50%. In this study we report results from seven easily fatiguable and from three fatigue-resistant fibres.

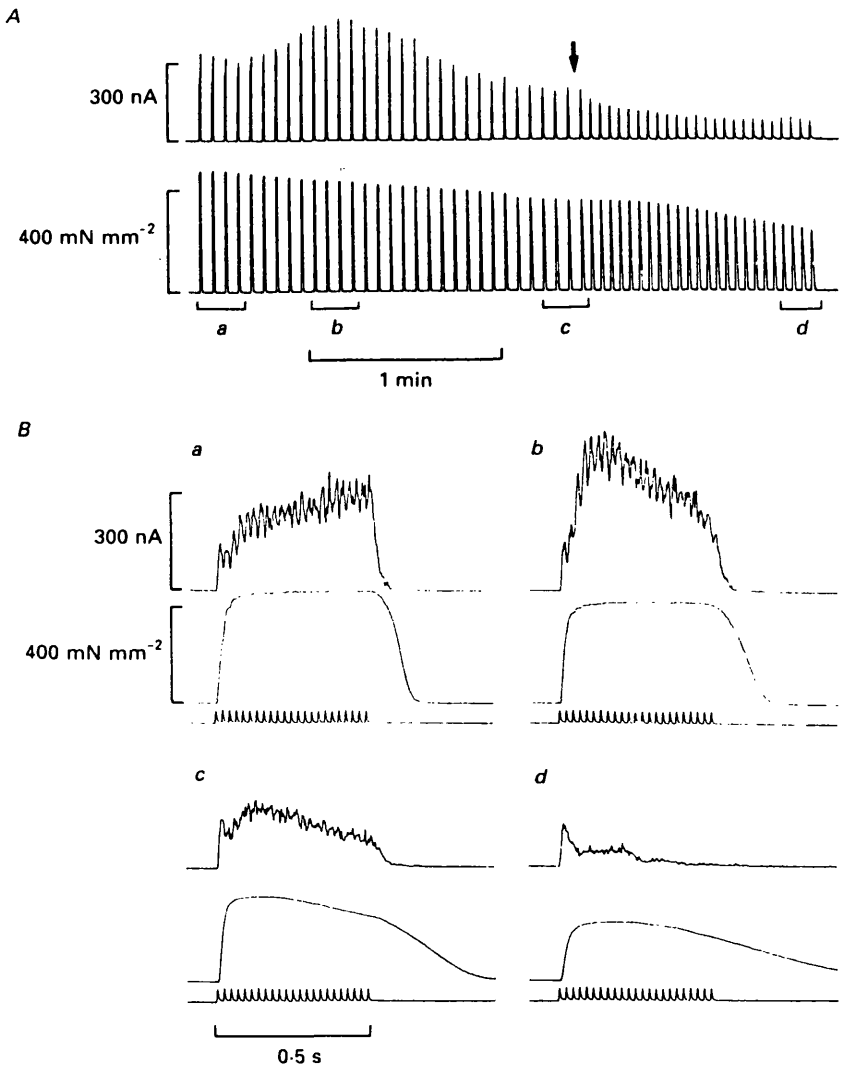

Fig. 1. Records of aequorin light and tension from an easily fatiguable fibre during fatiguing stimulation (fifty-five tetani). *A*, continuous record of aequorin light (above) and tension (below). Arrow marks the point at which stimulation was changed from 4 to 3 s interval. *B*, averaged records ($n = 4$) of aequorin light (above) and tension (below) from the periods shown in *A*. The stimulus marker is also shown. Note the changes in the form of the aequorin light transient and the slowing of tension relaxation in fatigue.

The amplitude of aequorin signals during fatigue

Figure 1 shows a representative example of aequorin light and tetanic tension from an easily fatiguable fibre. Figure 1*A* shows a continuous record, while Fig. 1*B* shows averaged records from the periods indicated in *A*. It can be seen that while tetanic tension declined more or less steadily during fatiguing stimulation, the amplitude of the aequorin signal showed a more complex pattern, increasing over the first twelve

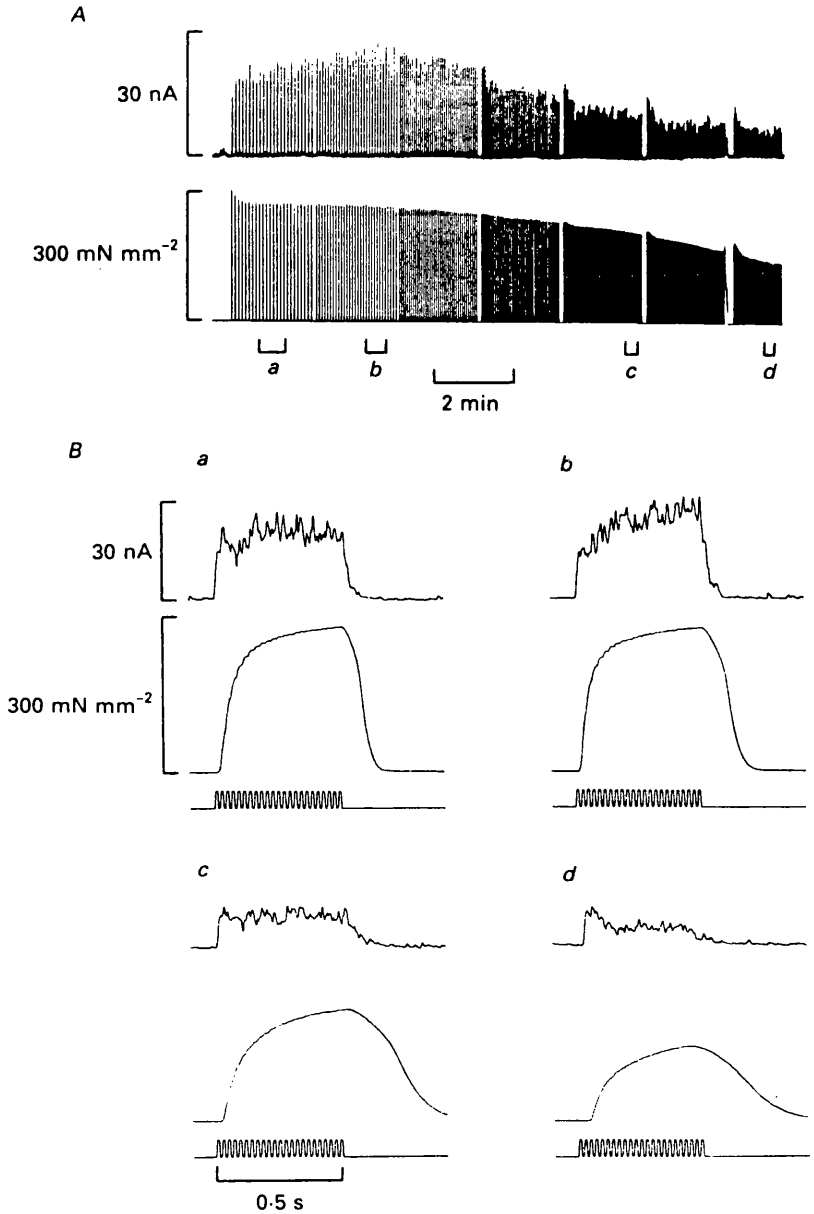

Fig. 2. Records of aequorin light and tension from a fatigue-resistant fibre during fatiguing stimulation (311 tetani). *A*, continuous record of aequorin light (above) and tension (below). *B*, averaged records ($n = 8$) of aequorin light (above) and tension (below) from the periods shown in *A*. The stimulus marker is also shown. Note the changes in the form of the aequorin light transient and the slowing of tension relaxation in fatigue.

tetani and thereafter showing a pronounced decline. For the easily fatigued fibres, if the amplitude of the first aequorin transient is defined as 100%, the largest transient was 162 ± 28% and occurred, on average, on the eighth tetanus (range three to twelve). By the time tension had declined to 50%, which required, on average, fifty-

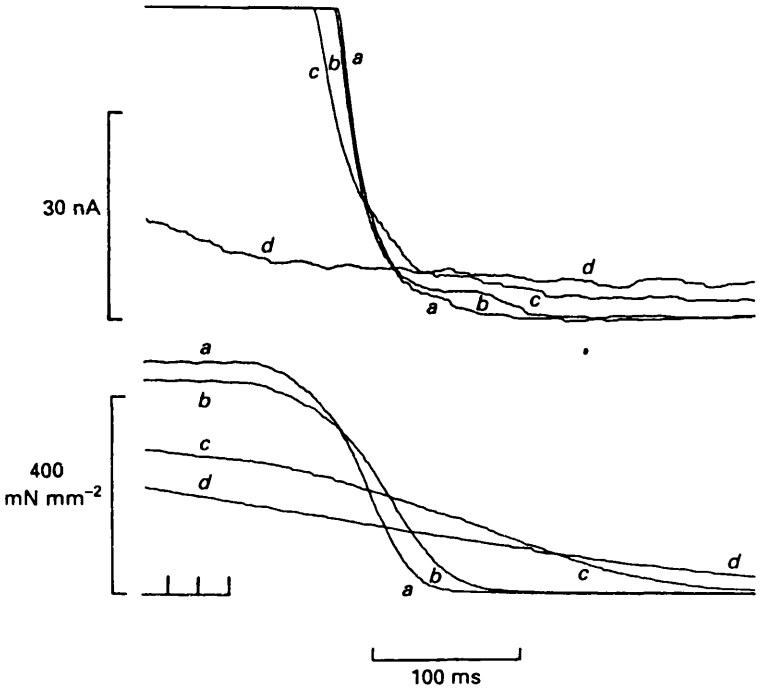

Fig. 3. Slowing of tension relaxation during fatigue is associated with slowing of the rate of fall of aequorin light. The averaged records of Fig. 1 are displayed here with higher gain and time resolution. It can be seen that as tension relaxation slows, the rate of fall of aequorin light is also slowed. Also, in the later tetani, the minimum level of the light signal is higher than in the earlier tetani, i.e. there is a raised resting light level.

nine tetani (range twenty-five to ninety-four tetani), the light signal had declined to $13 \pm 2\%$.

Figure 2 shows an example of aequorin light and tetanic tension from a fatigue-resistant fibre. Figure 2*A* shows a continuous record, while Fig. 2*B* shows averaged records from the periods indicated in *A*. In these fibres the pattern was similar to that seen in the easily fatiguable fibres, with tension declining steadily throughout fatiguing stimulation, while the light signal first showed an increase in amplitude and then a substantial decrease. In fatigue-resistant fibres the maximum aequorin light was $140 \pm 15\%$. In comparison with easily fatiguable fibres, the increase in aequorin light occurred somewhat later in fatigue-resistant fibres (maximum, on average, at thirty-third tetanus, range fourteen to forty-three). The reduction of tension to 50% required 340 tetani (range 216–495) and at that time the peak aequorin signal was $18 \pm 6\%$.

Panels *B* of Figs 1 and 2 show that there were also changes in the form of the aequorin signals as fatigue developed. The light signals often changed from a form which was increasing throughout stimulation (as in Fig. 1*Ba*) or flat, to one which was largely decreasing (as in the other panels in Fig. 1*B*). Also, the early peak in the transient at the later stage of fatigue was characteristic (e.g. Figs 1*Bd* and 2*Bd*).

The slowing of relaxation and the decline of light

Slowing of relaxation is a characteristic feature of fatigue and is clear in the later tetani shown in Figs 1*B* and 2*B*. This could arise either because the rate at which Ca^{2+} is removed from the myoplasm is slowed during fatigue or because of changes in cross-bridge cycling. If slowed relaxation is due to $[Ca^{2+}]_i$ falling more slowly in a fatigued fibre, then it should be possible to observe a slower fall of the aequorin light signal at the end of a tetanus.

The fall in aequorin light at the end of a tetanus in an unfatigued fibre has been described as falling in three phases (Cannell, 1986). There is a rapid exponential phase, which includes more than 95% of the signal, followed by a period in which the fall of light slows or even reverses slightly and finally a long slow tail of light. We did not regularly observe the second phase described by Cannell (1986), probably because this phase is most obvious in tetani of 1 s or greater in duration.

The simplest way to determine whether the rapid phase of decline of the aequorin signal was altered in the fatigued state and contributed to the slowing of relaxation, would be to measure the time for the light signal to decline to, say, 25% of its level after the last stimulus (t_{25}(light)) and to compare this with the time taken for tension to decline to some fraction of its level at the last stimulus. There are, however, several problems with this approach. Towards the end of fatiguing stimulation both tension and light start to decline in individual tetani well before the last stimulus. This can lead to an artificial reduction in the apparent rate of decline of tension when measured by this criterion. Furthermore, in some preparations (e.g. Fig. 3, trace *d*) the fast phase of decline of light is completely absent by the end of fatigue, so that measurement of t_{25}(light) as defined above probably measures the characteristics of the slow phase of light decline. To minimize these problems, we measured t_{25}(light) and t_{50}(tension) (the time taken for the tension to decline to 50% of its level after the last stimulus) for the first tetanus of fatiguing stimulation and the last tetanus which still had a fast component of decline of light and in which t_{50}(tension) was still increasing. In eight experiments t_{50}(tension) for the first tetanus was 106 ± 6 ms and t_{25}(light) was 43 ± 1 ms. Towards the end of fatiguing stimulation t_{50}(tension) had increased to 246 ± 30 ms while t_{25}(light) had increased to 103 ± 13 ms. Both increases were highly significant ($P < 0.01$ on a paired t test).

Within individual experiments there was a clear correlation between the gradual slowing of relaxation and the decline of light. This is obvious when records at various stages of fatiguing stimulation are superimposed as in Fig. 3. In this record the aequorin signals are displayed at high gain so that only the lowest 10% of the control signal is visible. It is clear that the aequorin signals slowed progressively throughout fatigue and that, while there is some slowing of the rapid phase, there is in addition a large increase in the amplitude of the slow phase. The similarity of the slow mechanical relaxation in the last tetanus (*d* in Fig. 3) and the slowed decline of the accompanying aequorin signal suggests that a reduced rate of fall of $[Ca^{2+}]_i$ was making a substantial contribution to the reduced rate of relaxation. A similar tendency for slowing of the fall of light to accompany slowing of the fall of tension was seen in all experiments. There was no obvious difference between easily fatiguable and fatigue-resistant fibres in this respect, except that more tetani were required to produce these changes in fatigue-resistant fibres.

Towards the end of fatiguing stimulation, the aequorin light was still detectably elevated in some fibres when the next tetanus started (see figure in Allen, Lee & Westerblad, 1988). This could occur either because the steady state resting $[Ca^{2+}]_i$ was elevated, or because the tail of decline of aequorin light was now so long that it

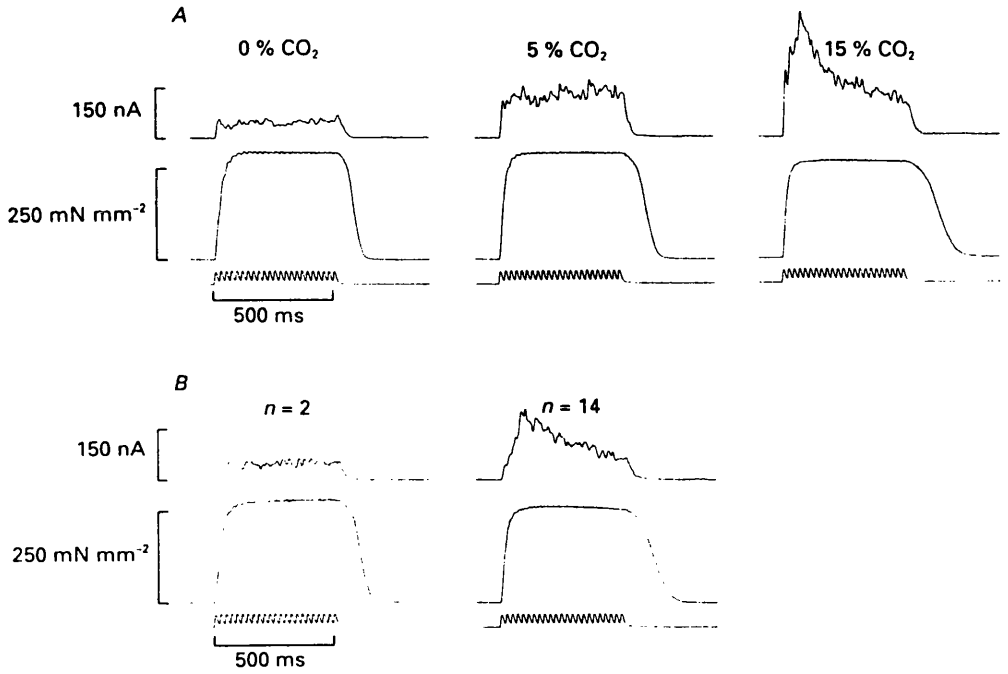

Fig. 4. Comparison of the effects of acidosis and fatigue on aequorin light and tension in the same muscle fibre. Each panel shows aequorin light (above), tension (middle) and stimulus marker (below) from a single contraction. *A*, the three panels show from left to right the effects of 0, 5 and 15% CO_2. *B*, the two panels show the second and fourteenth tetani of fatiguing stimulation in the same muscle fibre and illustrate the early rise in tetanic $[Ca^{2+}]$.

merged with the next tetanus (i.e. had the inter-tetanus interval been long enough, the light would have declined to the original resting level). Unfortunately, the relation between $[Ca^{2+}]$ and light for aequorin is such that low calcium levels are very difficult to detect (Blinks *et al.* 1982), and so it is not an ideal tool with which to distinguish between these possibilities. Nevertheless, in three fibres which had a large light signal, it was established by photon counting that the resting light 30 s after the last tetanus was elevated. Thus, although it was not visible in all fibres, this suggests that a raised resting $[Ca^{2+}]_i$ is present towards the end of fatiguing stimulation and in the early part of recovery.

The effect of intracellular acidosis on aequorin light and tension

One possible explanation for the early increase in the light transients during fatigue is that it is due to an intracellular acidosis caused by lactic acid production. An increased hydrogen ion concentration could reduce the effectiveness of intracellular calcium buffers, leading to a greater free $[Ca^{2+}]$ inside the cell for a given release of calcium. It has been shown in cardiac muscle that acidosis, due to either

increased CO_2 or lactic acid accumulation, may increase the calcium transients (Allen & Orchard, 1983). Evidence supporting the role of hydrogen ions in the early increase in aequorin light during fatiguing stimulation is shown in Fig. 4. Panel *B* shows that a substantial increase in aequorin light had occurred by the fourteenth tetanus in this experiment. Panel *A* shows the effects of increasing the CO_2 concentration in the same fibre. This series of contractions preceded the fatigue series; the muscle fibre was perfused with Ringer solution equilibrated with 5 or 15% CO_2. Several tetani at 2 min intervals were given until the light and tension response at the new CO_2 level was stable. Carbon dioxide causes an extracellular acidosis (see Methods) and, more important, also causes an intracellular acidosis (Bolton & Vaughan-Jones, 1977; Curtin, 1986, 1987). It can be seen that changing the CO_2 in this way had marked effects on the light signals. The light signal was increased by 5% CO_2, while 15% CO_2, in addition to causing a further increase, also changed the shape of the signal so that it exhibited an early peak and subsequent decline. The form of this transient is similar to that seen in the fourteenth tetanus shown in panel *B*. In four experiments 5% CO_2 caused light to increase to a peak of 218 ± 31% of control, whereas the maximum light signal during fatiguing stimulation was 188 ± 38% of control in these fibres.

At both CO_2 levels there was obvious slowing of relaxation and also a small reduction in tension, in agreement with previous results (Edman & Mattiazzi, 1981; Curtin, 1986). In 5% CO_2 t_{50}(tension) (as defined above) was increased by 22 ± 3 ms ($n = 5$) while in 15% CO_2 t_{50}(tension) increased by 52 ± 6 ms. However, the rate of decline of light was not significantly affected by either level of CO_2; t_{25}(light) decreased by $1{\cdot}8 \pm 2{\cdot}9$ ms in 5% CO_2 and increased by $3{\cdot}7 \pm 2{\cdot}2$ ms in 15% CO_2.

The contribution of reduced tetanic $[Ca^{2+}]_i$ *to reduced force during fatigue*

In a previous study of aequorin signals in frog skeletal muscle (Blinks *et al.* 1978), it was pointed out that the aequorin signal during a tetanus could change over a considerable range with little change in tension. It was suggested that this arose because myoplasmic $[Ca^{2+}]$ under their conditions was considerably above the level required to saturate troponin binding sites. Thus, in order to show that the reduced tetanic $[Ca^{2+}]_i$ in fatigued muscle is the cause of reduced tension, it was desirable to (i) work under conditions in which the $[Ca^{2+}]_i$ in a control tetanus was only just maximal and (ii) to determine the relation between $[Ca^{2+}]_i$ and tension for submaximal levels of tension. To achieve (i), the stimulus frequency was set close to the fusion frequency. To achieve (ii) we attempted to construct an *in vivo* pCa–tension curve using submaximal K^+ contractures. The basis of this method was to produce a series of K^+ contractures which gave different levels of light and tension. Suitable values of light and tension were then plotted so as to obtain a pCa–tension curve under control conditions.

We identified two potential problems with using K^+ contractures in this way. First, because $[Ca^{2+}]_i$ was changing continuously throughout a K^+ contracture, it is difficult to be sure that $[Ca^{2+}]_i$ and tension had reached a steady-state relationship. Second, if there was inhomogeneity of activation, the non-linearity of the relation between aequorin light and $[Ca^{2+}]_i$ would lead to aequorin signals which would exaggerate the spatially averaged $[Ca^{2+}]_i$ (e.g. Blinks *et al.* 1982). Our approach to the

first problem was to select periods of K^+ contractures when both tension and light were relatively constant. After some experimentation we chose periods of 200 ms where tension changed by less than 10% of its maximum value and where light was not rising or reached a peak. (Examples of the method are shown in Fig. 5*B* and *C*

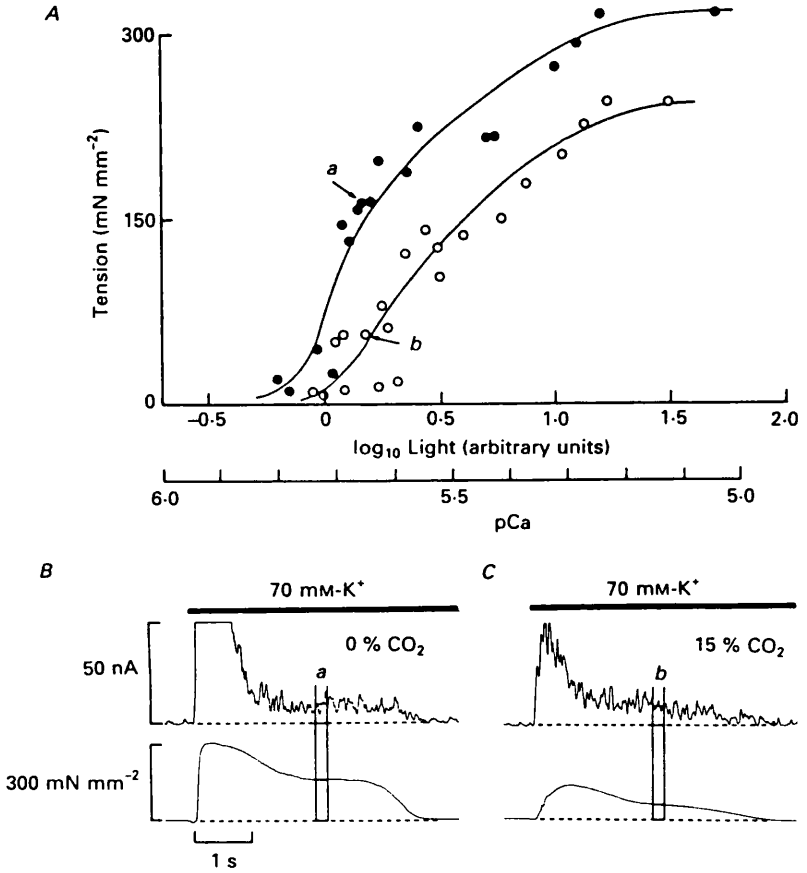

Fig. 5. *In vivo* pCa–tension curves were constructed with the use of K^+ contractures. *A*, *in vivo* pCa–tension curves under control conditions, pH_o 7·1 (●) and in the presence of 15% CO_2, pH_o 6·3 (○). *B* and *C*, examples of records from which the pCa–tension curves were constructed. Both of these records were obtained with a K^+ contracture using 70 mM-K^+. Note that for a similar light level, the contracture in 15% CO_2 produces considerably less tension. Measurements at *a* and *b* are shown on the curves in *A*.

and Fig. 6*B*, *C* and *D*.) Tension and light were averaged over periods fulfilling these criteria, and in this way a sufficient number of points to define a pCa–tension curve were obtained from four or five K^+ contractures. Our evidence that the problem of inhomogeneity cannot be large is that the scatter on these graphs is reasonably small. In addition, in several experiments, points with similar tension levels were measured from contractures at different levels of K^+, but the light signals proved reasonably comparable.

Figure 5 illustrates an attempt to validate this method by examining the effects of increasing the concentration of CO_2 in the Ringer solution. In panel *A*, the filled

circles represent contractures produced by 50, 60, 70, 80 and 150 mM-K^+. Panel *B* shows one such record, from a K^+ contracture at 70 mM-K^+, and illustrates how the measurements were made. This record is particularly satisfactory, since tension and light were virtually constant over about 1 s, giving a high degree of confidence that

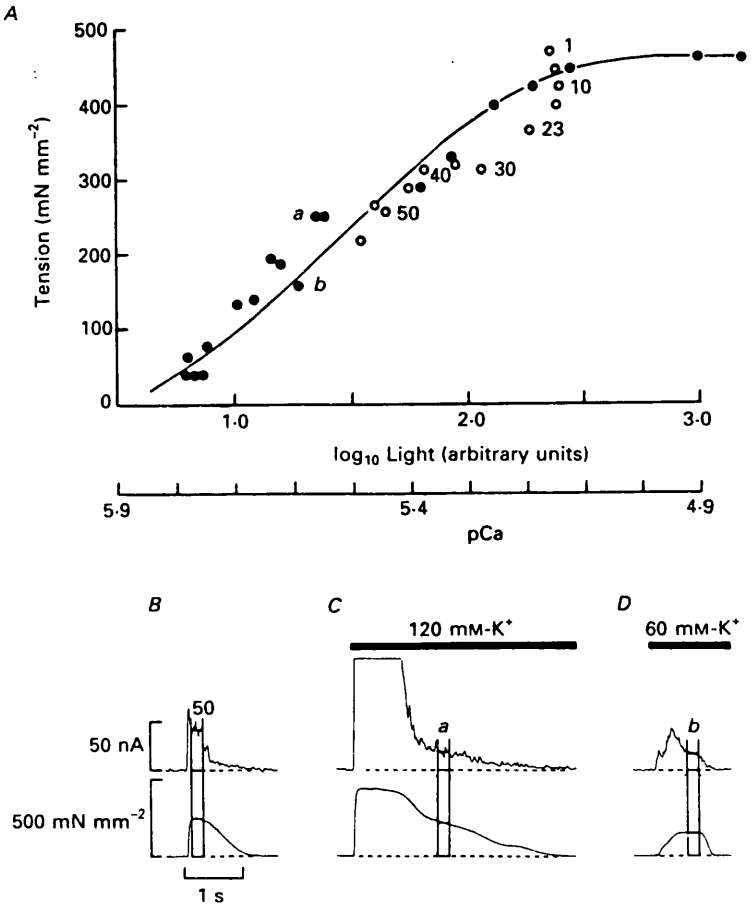

Fig. 6. Comparison of pCa–tension curve under control conditions with points obtained from tetani during fatigue. *A*, filled circles represent control pCa–tension points obtained with K^+ contractures. Open circles are those measured from records of light and tension obtained during fatiguing stimulation. Numbers indicate tetani during the stimulation period. *B*, *C* and *D*, examples of records from which the curves in *A* were constructed. *B* shows the calcium transient and tension from the fiftieth tetanus. *C* and *D* show the K^+ contractures from which the points (*a*) and (*b*) on the curve in *A* were measured.

tension and $[Ca^{2+}]_i$ were close to steady state. Potassium contractures were then examined in the same muscle fibre after the Ringer solution had been equilibrated with 15% CO_2. Panel *C* illustrates another K^+ contracture at 70 mM-K^+ during exposure to CO_2. By chance this gave a stable light level which was very similar to that before exposure to CO_2 (panel *B*), but it can be seen that the steady tension is less than half of that in the absence of CO_2. The curve constructed from such measurements in the presence of CO_2 is shown by the open circles in panel *A*. It can

be seen that it is shifted to the right, in agreement with studies on skinned muscle (Fabiato & Fabiato, 1978). The acidosis produced by CO_2 reduced the maximum developed tension by 25% in this experiment, and shifted the pCa giving 50% maximum tension by 0·11 pCa units. A curve constructed in the same muscle for an exposure to 5% CO_2 (not shown) lay in between the other two.

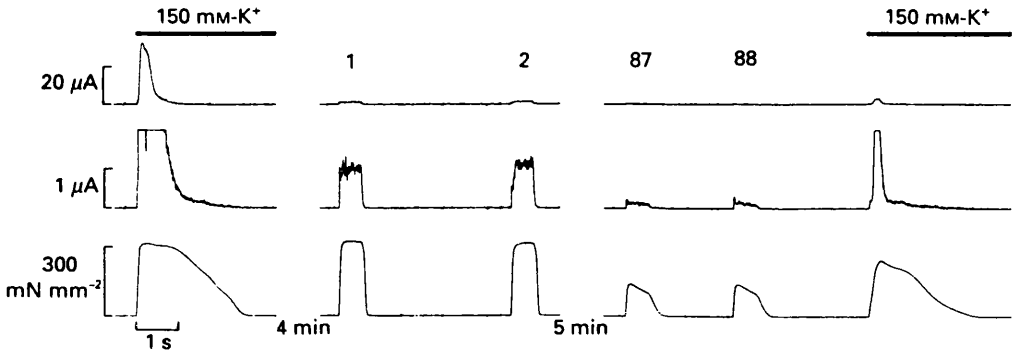

Fig. 7. The effects of maximal depolarization with 150 mM-K^+ before and after fatiguing stimulation. Records show low gain light (above), standard gain light (middle) and tension (below). The control K^+ contracture gave a very large peak light and maximum tension. Fatigue was then produced by repeated tetanic stimulation (numbers refer to tetani). The K^+ contracture given in place of the eighty-ninth tetanus produced a much reduced peak light and 79% of maximum tension.

Figure 6*A* shows a further example of an *in vivo* pCa–tension curve constructed from K^+ contractures (filled circles). The open circles represent points measured in a similar manner from tetani during fatiguing stimulation (panel *B* shows an example). In this experiment the first tetanus appears to lie above the constructed line, but this was not generally the case (e.g. Fig. 10). As described above, during the first ten to twenty tetani tension declined, but light increased, so the points move down and to the right of the constructed curve. In subsequent tetani, both tension and light declined, so the points fall more or less parallel to the constructed curve. However, towards the end of fatiguing stimulation, the points return towards the curve, so that the final tetani do not differ from it. Similar plots were performed for five other preparations and it was generally the case that the first tetani lay close to the constructed line, that tetani during the early part of fatiguing stimulation had a tendency to lie to the right of the curve, and that tetani towards the end of fatiguing stimulation lay close to the curve again. Thus it appears that towards the end of fatiguing stimulation the tension produced is what would be expected from the $[Ca^{2+}]_i$ observed, suggesting that the fall of $[Ca^{2+}]_i$ is making the major contribution to the reduced tension in fatigue.

Figure 7 shows the effects of exposure to 150 mM-K^+ before and after fatiguing stimulation. Before fatigue, this produced a tension similar to tetanic tension, while peak light was approximately twenty times greater than the light obtained from a tetanus at 50 Hz. By the end of fatiguing stimulation the light signal in a tetanus had declined to about 10% of its pre-fatigue level in this experiment. The potassium contracture at the end of fatiguing stimulation produced 79% of maximum tension, and the light signal was reduced to ~ 8% of its pre-fatigue level and its duration was

considerably reduced. Note that although the light in the K^+ contracture is considerably reduced by fatigue, its peak remains substantially larger than the light in a normal tetanus before fatiguing stimulation. On average a maximal K^+ contracture immediately after fatiguing stimulation produced $77 \pm 3\%$ of the tension

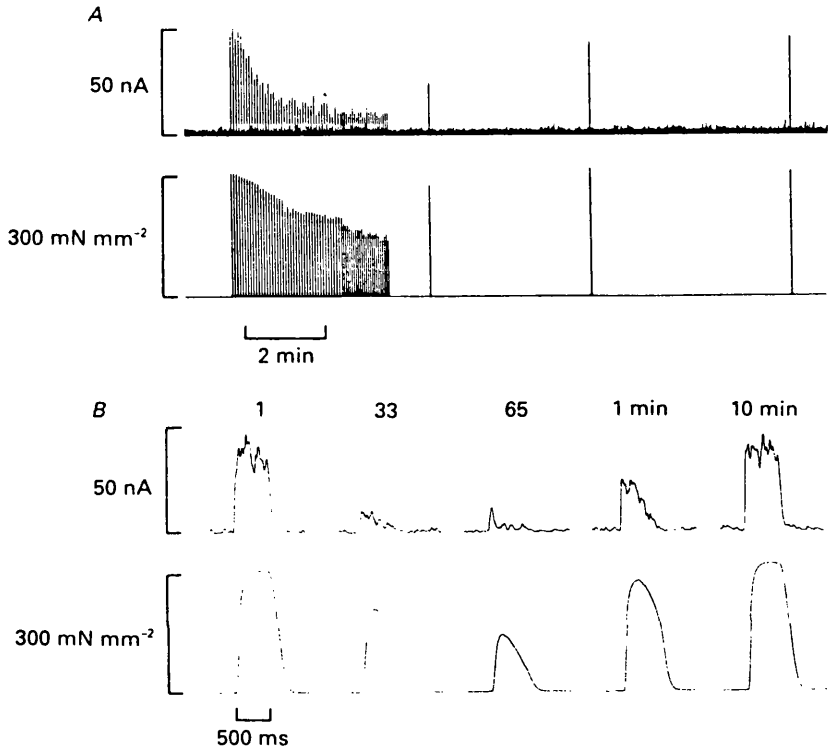

Fig. 8. Recovery from fatiguing stimulation in a fibre which recovered monotonically. *A*, continuous record of aequorin light (above) and tension (below) during and after fatiguing stimulation. By the end of the stimulation period light had declined substantially. Test tetani at 1, 5 and 10 min after the end of fatiguing stimulation show a smooth recovery of both light and tension to levels which are similar to control. *B*, individual tetani from the same experiment displayed on a faster time base. The first, thirty-third and last (sixty-fifth) tetani are shown, together with the tetani after 1 and 10 min of recovery.

given by one before stimulation, while the peak light was reduced to $16 \pm 7\%$. Despite the reduction of peak light in K^+ contractures in the fatigued state, the light signal was markedly greater than in the preceding tetani. This result shows that a substantial recovery of tension can be produced in a fatigued muscle by a procedure which increases the $[Ca^{2+}]_i$. Possible reasons for the decline of light in a K^+ contracture during fatigue will be considered in the Discussion.

Recovery from fatiguing stimulation

Of the ten fibres studied during fatigue development, eight were followed throughout recovery; the other two fibres showed considerable recovery before the experiment was terminated prematurely by a caffeine contracture or technical

problems. Two patterns of tension recovery were observed. In some fibres tension recovered monotonically, while in others a further reduction of tension after the end of fatiguing stimulation (post-contractile depression) was followed by a gradual recovery to pre-fatigue tensions (Westerblad & Lännergren, 1986).

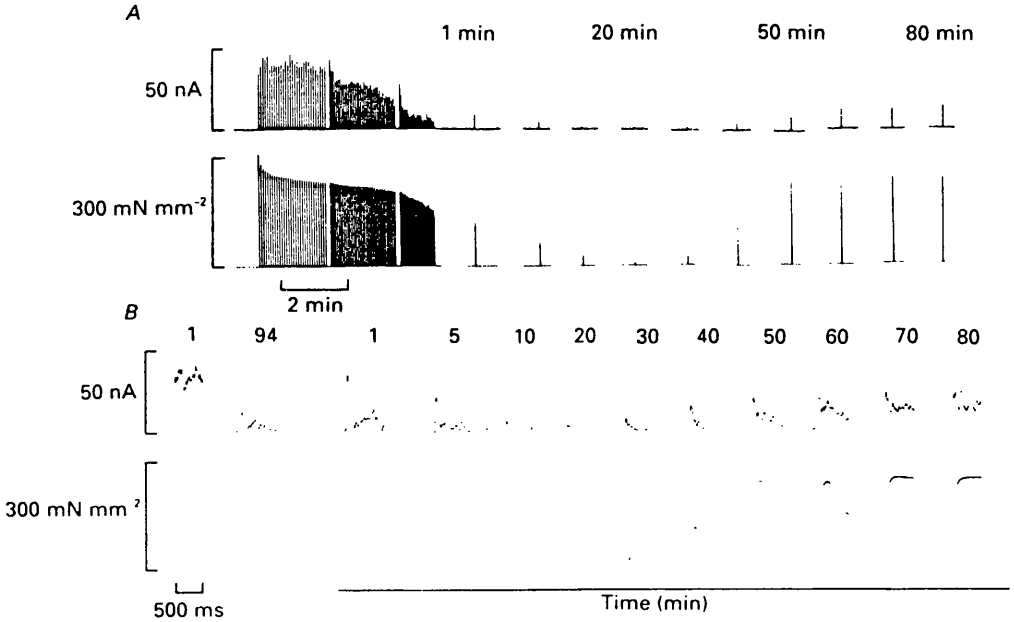

Fig. 9. Recovery from fatiguing stimulation in a fibre which showed post-contractile depression. *A*, continuous record of aequorin light (above) and tension (below) during and after fatiguing stimulation. Following the end of fatiguing stimulation, tension showed a further decline to a minimum at 20 min before finally recovering to a value which was similar to control. Light showed a small recovery at the 1 min test, but then also declined to a minimum at 20 min before recovering to a value which was substantially smaller than the control even after 80 min of recovery. *B*, individual tetani from the same experiment displayed on a faster time base. The first and last tetani of fatiguing stimulation are shown, together with test tetani at 1, 5, 10, 20, 30, 40, 50, 60, 70 and 80 min. The slight recovery of the light signal at 1 min and the sagging form of the tetanus during recovery are typical.

Figure 8 illustrates a fibre which recovered monotonically. In this fibre the tension recovered within 10 min and the light signal had almost fully recovered in the same time. In fibres which showed this pattern of recovery ($n = 3$), tension was $99 \pm 1\%$ of control and light was $89 \pm 14\%$ of control within 30 min after the end of fatiguing stimulation.

Figure 9 shows records from a fibre in which post-contractile depression developed. Tetani identical to those used during the control period and fatiguing stimultion were given during the recovery period. It can be seen that the tension fell to a minimum and then gradually recovered. In this fibre tension reached a minimum of 4% of control 20 min after the end of fatiguing stimulation. The fall of tension during post-contractile depression was accompanied by a fall of the light signal, which in Fig. 9 reached 2% of the control value before it started to recover. In the fibres which showed post-contractile depression, a minimum tension of $7{\cdot}4 \pm 3{\cdot}3\%$ ($n = 5$) of

control was produced at 10–20 min of recovery. The light signal at this time was 2·8±0·7% of control.

After reaching a minumum, tension gradually increased until a relatively stable level was attained after 60–140 min of recovery. The tension produced was then 91±3% of control. The light signal also increased, but it did not recover as completely, being 36±3% of control in the same fibres. (Correction for aequorin

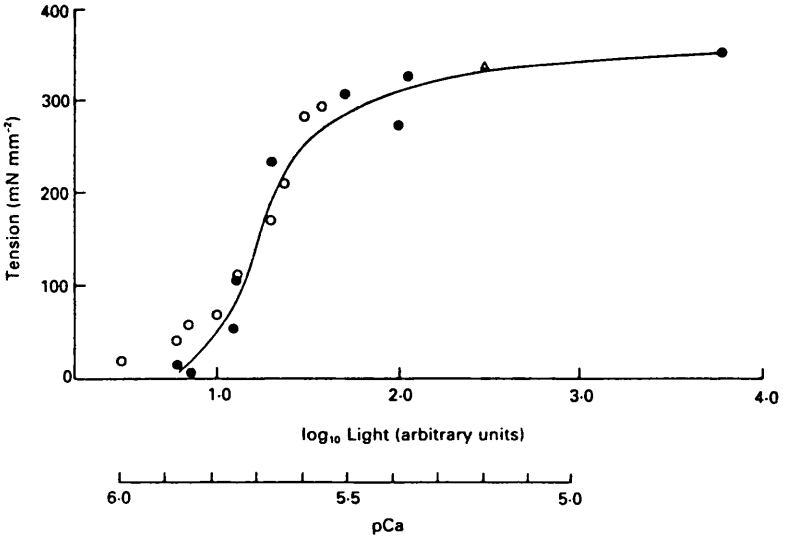

Fig. 10. The relationship between light and tension during recovery. Filled circles represent a control pCa–tension curve measured from K^+ contractures. Open circles represent measurements from records of light and tension obtained during recovery. The points lie very close to the control curve. Open triangle represents results obtained from control tetani before fatiguing stimulation.

consumption increases this figure only to about 40% of control). This lower level of $[Ca^{2+}]_i$ caused the tetani to be less fused after recovery from fatigue, as can be seen in the 60–80 min tests shown in Fig. 9*B*. In some experiments test tetani at higher stimulation frequencies were given. These caused a substantial increase of the light signal and also the contraction became more fused (not shown).

In order to determine whether the $[Ca^{2+}]_i$ during the recovery period could account for the reduced tension during post-contractile depression, we plotted tetani during recovery on pCa–tension curves. One such plot is shown in Fig. 10. The line plotted through the filled circles is a control pCa–tension curve, which was measured from K^+ contractures before fatiguing stimulation. The open circles represent measurements from tetani during recovery. It can be seen that these lie very close to the curve; in other words the tension generated for the level of $[Ca^{2+}]_i$ present during the tetanus is what would be expected from the control pCa–tension curve. Also, it should be noted that when a stable level had been reached in this fibre, the light level was close to the shoulder of the curve. This contrasts with the control, unfatigued tetanus (triangle) which has a light level which is above saturation.

While it is known that the action potentials during post-contractile depression appear normal (Westerblad & Lännergren, 1986), it remains possible that action potentials are no longer able to release an adequate amount of Ca^{2+} from the

sarcoplasmic reticulum and that this causes the decline in $[Ca^{2+}]_i$ and tension. We therefore examined the relation between light and tension produced by K^+ contractures and caffeine during post-contractile depression. Figure 11 shows the peak light and tension in tetani (panel *A*) and K^+ contractures (panel *B*) before,

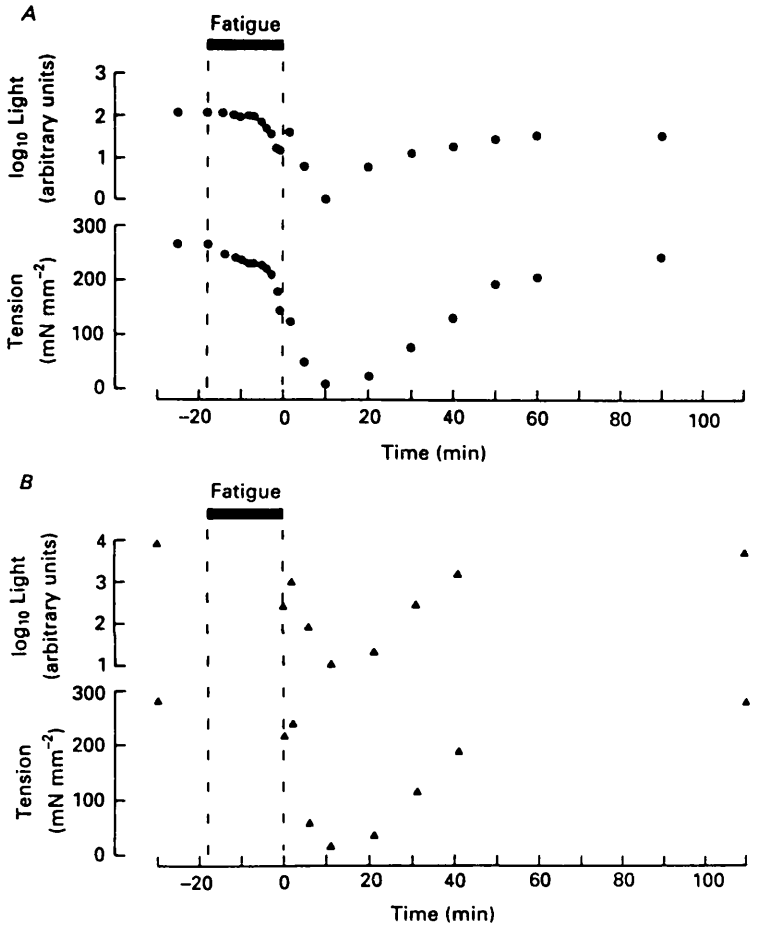

Fig. 11. Aequorin light and tension measured from tetani and maximal K^+ contractures during and after fatiguing stimulation in a fibre which showed post-contractile depression. *A*, light and tension measured from tetani. Note the similarity in the pattern shown by tension and light. *B*, peak light and tension measured from maximal K^+ contractures with 150 mM-K^+ during the same experiment. The light and tension show a similar pattern both to each other and to the pattern observed with tetani.

during and after a period of fatigue. As described above, tetanic light and tension fell during fatigue, and this was then followed by a secondary decline of light and tension, and finally by recovery. Panel *B* shows that peak light and tension elicited by maximal K^+ contractures in the same fibre also showed a decline during post-contractile depression, followed by a recovery. Thus tension produced by K^+ contractures, which cause a continuous depolarization of the membrane, is affected in a similar way to tetanic tension, and so also is the peak light caused by this manoeuvre. (Note that the K^+ contractures caused a much larger peak light signal

than tetani, presumably because they cause continuous depolarization, and hence the ordinate is at least an order of magnitude greater in panel *B*.) Thus the process causing post-contractile depression also affects Ca^{2+} release by continuous depolarization of the membrane, suggesting either that the sarcoplasmic reticulum is

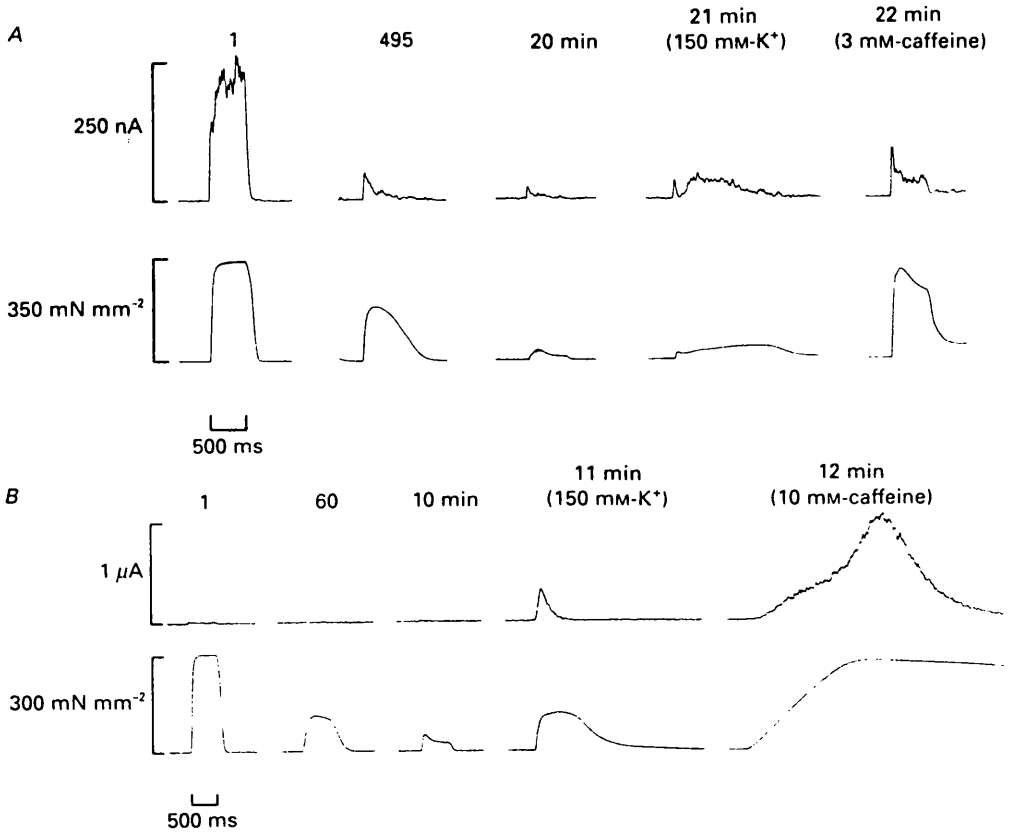

Fig. 12. Effect of high K^+ and caffeine on aequorin light and tension during post-contractile depression. *A*, leftmost panels show the first and last tetani of fatiguing stimulation. Panel marked 20 min shows a tetanus given 20 min after the end of fatiguing stimulation. Panel marked 21 min (150 mM-K^+) shows a K^+ contracture caused by 150 mM-K^+ given 1 min after the tetanus. One minute later still (panel marked 22 min (3 mM-caffeine)) a tetanus after 20 s exposure to 3 mM-caffeine was given. Note that the fibre did not relax completely after the tetanus in 3 mM-caffeine and subsequently went into a contracture. *B*, different experiment. The first four panels are similar to those in *A*, except that in this muscle tension was greatly reduced after 10 min. Final panel, marked 12 min (10 mM-caffeine), shows a contracture provoked by 10 mM-caffeine applied at the start of the twelfth minute.

depleted of Ca^{2+} or that the release mechanism initiated by T-tubular depolarization has failed.

The hypothesis that reduced tetanic $[Ca^{2+}]_i$ (myoplasmic free calcium concentration during a tetanus) is the cause of post-contractile depression is further supported by the finding that caffeine can produce similar tension in unfatigued fibres and during post-contractile depression (Lännergren & Westerblad, 1989).

Figure 12 shows two experiments illustrating this result and, in addition, shows that the $[Ca^{2+}]_i$ can be very large under these conditions. Figure 12*A* shows a control tetanus and the last tetanus of fatiguing stimulation. Post-contractile depression then occurred, as shown by a tetanus at 20 min, when tension was much reduced. One minute later a K^+ contracture produced a slightly larger tension and a moderate increase in light. After another minute a tetanus was given in 3 mM-caffeine and produced a large tension (90% of control), with a light signal which was similar to the preceding K^+ contracture, but only one-fifth of the initial control tetanus. Figure 12*B* shows a similar protocol. When the fibre was in post-contractile depression, as indicated by the reduced tetanic tension, a K^+ contracture was followed by a contracture produced with 10 mM-caffeine. Although the K^+ contracture increased light and tension somewhat, the most dramatic increase was produced by caffeine, which gave a very large light signal (40 × control) and a tension which was 91% of control. Thus, in this experiment there was clearly a large releasable store of Ca^{2+}. Both these experiments show that caffeine was able to increase $[Ca^{2+}]_i$ and tension in post-contractile depression, and was more effective than K^+ contractures. More puzzling is the large variability of the peak light produced by these procedures during post-contractile depression. Thus in the K^+ contracture of Fig. 12*A*, peak light was about one-sixth of that during a control tetanus, while in that of Fig. 12*B* the peak light was about 10 × bigger than the control tetanus. It is possible that spatial gradients of $[Ca^{2+}]_i$ may be produced by K^+ contractures during post-contractile depression (hence exaggerating the light signal with respect to the tension), while caffeine contractures, acting directly on the sarcoplasmic reticulum, give a more accurate picture of the releasable pool of Ca^{2+}.

DISCUSSION

Isolated single fibres show at least two distinct kinds of fatigue. When continuously stimulated at high frequencies (e.g. 100 Hz) there is a rapid and often irregular decline of tension (high frequency fatigue) which is characterized by failure of action potentials (Lüttgau, 1965; Lännergren & Westerblad, 1986) and rapid recovery. Our conditions of intermittent tetani stimulated at low frequency largely avoids this kind of fatigue and leads to a slowly developing fatigue in which action potentials show only minor changes in form (Grabowski *et al.* 1972; Westerblad & Lännergren, 1986).

In principle the tension reduction during fatigue could be due to (i) reduced maximum Ca^{2+}-activated tension i.e. reduced tension production per cross-bridge or a reduced number of cross-bridges participating in contraction, (ii) reduced myoplasmic $[Ca^{2+}]$ during a tetanus, (iii) reduced Ca^{2+}-sensitivity at the contractile proteins or a combination of these effects. We will discuss each of these possibilities in turn.

Reduced maximum Ca^{2+}-activated tension

A variety of metabolic changes occur during fatiguing stimulation, but the tension produced by the contractile proteins is known to be particularly sensitive to the increased inorganic phosphate (P_i) and the decreased pH_i (Cooke & Pate, 1985; Godt & Nosek, 1989). In fact, if the concentrations of P_i and pH_i which are thought to occur in fatigue are applied to skinned fibres, then reductions in tension equal to or

greater than those observed in our fatigued fibres can be obtained. For instance, Godt & Nosek (1989) used solutions with 1 mM-P_i and pH 7·0 to mimic normal conditions and 17 mM-P_i and pH 6·65 to mimic fatigue and observed a 33% reduction in maximum Ca^{2+}-activated tension and a much greater reduction at submaximal $[Ca^{2+}]$.

In opposition to this view is the observation that caffeine can largely or completely reverse the decline of tension in fatigue (Eberstein & Sandow, 1963; Grabowski *et al.* 1972; Lännergren & Westerblad, 1989; present study). Since caffeine directly releases Ca^{2+} from the sarcoplasmic reticulum (SR), raises the myoplasmic $[Ca^{2+}]$ (Fig. 12), and also increases the Ca^{2+} sensitivity of the contractile proteins (Wendt & Stephenson, 1983), there is no difficulty in explaining its action, but this observation does seem to exclude a substantial role for reduction in maximum Ca^{2+}-activated tension in the reduced tension seen in fatigued fibres.

The discrepancy between skinned fibre experiments, which suggest a major role for reduction in maximum Ca^{2+}-activated tension, and application of caffeine in intact fibres, which suggests little or none, could have several explanations. (i) Metabolic changes and tension have not yet been simultaneously measured in individual fibres with known fatigue characteristics. (ii) The P_i sensitivity and the pH sensitivity of different muscles are known to vary (Fabiato & Fabiato, 1978; Godt & Nosek, 1989). Preliminary experiments by R. E. Godt & J. C. Kentish (personal communication) suggest that the P_i sensitivity of the fibres used in this study may be lower than that of some other skeletal muscles. (iii) The observed effects of P_i and pH on skinned fibres may somehow be masked in intact fibres. (iv) The possibility that caffeine may overcome the inhibitory effects of P_i and pH on maximum Ca^{2+}-activated tension has been eliminated by direct experiment. R. E. Godt & J. C. Kentish have shown in preliminary experiments (personal communication) that caffeine applied to skinned fibres does not reverse the inhibition of tension by P_i or pH.

Changes in $[Ca^{2+}]_i$ during fatigue and their contribution to the decline of tension

At the stage of fatigue when tension is reduced to about 50%, the evidence that reduced $[Ca^{2+}]_i$ contributes to the decline of tension is now very strong. We find that the tetanic light signal is reduced to ~ 15%. Correction for aequorin consumption increases this figure to ~ 16% (i.e. by 6% of 15; see Methods). Because of the non-linearity of the relation between aequorin light and $[Ca^{2+}]$, this represents a fall in $[Ca^{2+}]_i$ to 40–50% of control levels (0·3–0·4 pCa units). Our pCa–tension curves show that this fall in $[Ca^{2+}]_i$ was capable of explaining all the fall in tension at the end of fatiguing stimulation. This conclusion is further supported by the fact that application of caffeine or high K^+ during fatigue was capable of substantially increasing $[Ca^{2+}]_i$ and produced increased tension, which was close to control levels in the case of caffeine.

Tetanic $[Ca^{2+}]_i$ changed in a complex way during fatigue and we will discuss separately possible mechanisms for the early rise in tetanic $[Ca^{2+}]_i$ and the late fall.

Possible mechanisms for the early rise in $[Ca^{2+}]_i$

The first obvious change during fatiguing stimulation is an increase in tetanic $[Ca^{2+}]_i$. Two possible mechanisms for this increase are (i) an effect of acidosis on $[Ca^{2+}]_i$ and (ii) an effect of repeated tetani on the distribution of Ca^{2+} between various

compartments within the cell. In support of hypothesis (i), we found that application of extracellular CO_2, which causes an intracellular acidosis, increased tetanic $[Ca^{2+}]_i$. Five percent CO_2, which increased $[Ca^{2+}]_i$ to a similar extent to fatiguing stimulation, caused an intracellular acidosis of between 0·15 and 0·25 pH units (Bolton & Vaughan-Jones, 1977; Curtin, 1987). Rough calculations from the acidosis measured at the end of fatiguing stimulation suggest that a pH_i reduction of 0·05–0·1 units should have been present when the maximum light signals occurred (Westerblad & Lännergren, 1988). Thus quantitative considerations of this sort suggest that acidosis may not be the only mechanism involved.

The second hypothesis above suggests that repeated activity may lead to a rise in tetanic $[Ca^{2+}]_i$ due to redistribution of Ca^{2+} within the fibre. Such redistributions were invoked in a model (Cannell & Allen, 1984) which reproduced the rise in $[Ca^{2+}]_i$ during a tetanus (Blinks *et al.* 1978; present study). In the model, as a tetanus progressed, Ca^{2+} was gradually transferred from calsequestrin sites in the SR to troponin and parvalbumin binding sites in the myoplasm. Thus, although Ca^{2+} release from the SR gradually declined throughout a tetanus, the consequence of increased saturation of Ca^{2+} binding sites in the myoplasm was that the $[Ca^{2+}]_i$ gradually rose.

Possible mechanisms for the late fall in tetanic $[Ca^{2+}]_i$

When tetanic $[Ca^{2+}]_i$ falls, this could arise either because of reduced Ca^{2+} release from the SR or because of increased Ca^{2+} buffering in the myoplasm. Since acidosis *reduces* Ca^{2+} buffering by troponin (Blanchard, Pan & Solaro, 1984) but has no effect on Ca^{2+} binding to parvalbumin (Pechere, Derancourt & Haiech, 1977), we assume that the reduced tetanic $[Ca^{2+}]_i$ represents a reduction in Ca^{2+} release from the SR. Three possible causes of the late fall in tetanic $[Ca^{2+}]_i$ are as follows.

Gradients of Ca^{2+} *release.* The action potential changes in form during fatigue (Westerblad & Lännergren, 1986) and this might be associated with impaired action potential conduction in the T-tubule network leading to reduced Ca^{2+} release in the centre of the fibre. There is some evidence for this theory from observations of wavy myofibrils in the centre of single fibres during fatigue (Gonzalez-Serratos, Garcia, Somlyo, Somlyo & McClellan, 1981). However, we have recently measured the spatial distribution of Ca^{2+} in *Xenopus* fibres using identical protocols to those used in this study and found no inhomogeneities across fibres as tension was reduced to 50% (Allen, Bolsover, Lamb, Lee, Silver & Westerblad, 1989).

Ca^{2+} is present in the SR but is not being released by action potentials. This hypothesis can explain the reduced tetanic $[Ca^{2+}]_i$ and the fact that high K^+ or caffeine applied immediately after fatiguing stimulation increases $[Ca^{2+}]_i$ with recovery of all or most of the tension. The fact that caffeine, which directly opens Ca^{2+} channels in the SR (Suarez-Isla, Orozco, Heller & Froehlich, 1986), is more effective than high K^+ (Lännergren & Westerblad, 1989), which causes continuous depolarization of the T-tubules, suggests that the defect in Ca^{2+} release during fatigue lies in the coupling between T-tubule depolarization and opening of SR Ca^{2+} channels. In support of this theory, it has been found, using electron microprobe techniques, that the SR contained Ca^{2+} even during severe fatigue (Gonzalez-Serratos, Somlyo, McClellan, Shuman, Borrero & Somlyo, 1978).

Ca^{2+} in the SR is greatly reduced during fatigue. This is an attractive hypothesis

because if SR Ca^{2+} uptake is impaired, this can explain all the main changes in $[Ca^{2+}]$ which we observe. Thus a slowed SR Ca^{2+} uptake would reduce the rate of fall of $[Ca^{2+}]_i$ after a tetanus and would increase resting $[Ca^{2+}]_i$. If the mean $[Ca^{2+}]_i$ is elevated, more Ca^{2+} will bind to parvalbumin and, in addition, there will be increased mitochondrial calcium uptake. As a consequence, SR Ca^{2+} will be reduced, leading to reduced tetanic $[Ca^{2+}]_i$. However, this hypothesis does not naturally explain why caffeine can still produce a maximal contracture during fatigue. Perhaps the amount of Ca^{2+} in the SR *is* reduced, but caffeine can still release sufficient to produce a full contracture when coupled with the increase in Ca^{2+} sensitivity which it also produces (Wendt & Stephenson, 1983).

Changes in Ca^{2+} sensitivity during fatigue

It is known that pH_i falls and P_i accumulates during fatigue and that both of these lead to reduction in the Ca^{2+} sensitivity (Fabiato & Fabiato, 1978; Kentish, 1986; Godt & Nosek, 1989) and would therefore be expected to contribute to reduced tension during fatigue. If this reduced Ca^{2+} sensitivity was present, then the pCa–tension points from tetani during fatigue ought to lie to the right of the control curves, as was seen when acidity was induced with CO_2 (Fig. 5). However, our data during fatigue show a shift to the right for tetani early in fatigue, but by the end of fatigue this shift was no longer apparent. Yet the combination of acidosis and P_i accumulation (and changes in ATP and ADP, see below) in skinned fibres led to a desensitization of between 0·25–0·3 pCa units (Godt & Nosek, 1989). Here again there exists an unexplained discrepancy between our results and those that we might expect on the basis of skinned fibre data. Some possible explanations have been discussed in the section on maximum Ca^{2+}-activated tension. Other possible explanations are as follows. (i) It could arise artificially as a consequence of reduced Ca^{2+} sensitivity of aequorin. As discussed in the Methods it is possible that acidosis and changes in $[Mg^{2+}]$ reduce the aequorin sensitivity. It is impossible to quantify this effect with data available, but we do not believe that the true $[Ca^{2+}]_i$ is underestimated by more than 0·1 pCa units and if such an error were present there would remain a substantial discrepancy to explain. (ii) There may be a genuine resensitization to Ca^{2+} superimposed on the desensitization caused by acidosis and P_i. For instance, large increases in ADP (e.g. to 5 mM) have been shown to increase Ca^{2+} sensitivity by about 0·6 pCa units (Hoar, Mahoney & Kerrick, 1987) but it seems unlikely that sufficient change in ADP occurs in fatigue to have much effect. Alternatively, a fall in ATP is known to sensitize the myofibrils to Ca^{2+} (Godt, 1974), but again measured falls in ATP are small (Dawson *et al.* 1978; Nassar-Gentina *et al.* 1978) and are likely to have only a small effect. Furthermore in the study of Godt & Nosek (1989), a realistic increase in ADP (to 0·7 mM) and decrease in ATP (from 6 to 5 mM) was incorporated in the 'fatigue' solution (in addition to the changes in pH and P_i) but a net decrease in Ca^{2+} sensitivity was still observed.

Slowing of relaxation

It seems clear that the slowing of relaxation observed in fatigue has more than one cause. Edman & Matiazzi (1981) and Curtin (1986) have shown that intracellular acidosis slows the rate of relaxation. We confirmed this finding but did not observe

any effect of pH on the rate of decline of $[Ca^{2+}]_i$ after a tetanus. Thus our conclusion is that pH causes a moderate slowing of relaxation, which is caused by a direct effect of pH on cross-bridge cycling and detachment rates. This conclusion is supported by the reduction of V_{max} which Edman & Matiazzi (1981) observed in acid solutions. Our conclusion differs from that of Curtin (1986) who suggested that, at least for long tetani, the effects of pH might be mediated by a slowing of SR Ca^{2+} uptake.

A further Ca^{2+}-independent slowing of relaxation may be caused by the rise in ADP. Cooke & Pate (1985), using maximally Ca^{2+}-activated skinned fibres, found that 1–4 mM-ADP slows V_{max} and Lännergren & Westerblad (1989) showed that V_{max} was reduced during fatigue. Although the ADP levels used by Cooke & Pate (1985) were above those likely to occur in fatigue it seems probable that some of the reduced shortening velocity and the slowing of relaxation arises from this source.

Our experiments suggest that there may also be a Ca^{2+}-dependent component to the slowing of relaxation. We found that the rate of fall of $[Ca^{2+}]_i$ was reduced substantially during fatiguing stimulation and this is likely to cause a further slowing of relaxation. Dawson *et al.* (1980) showed a close correlation between the free energy of ATP hydrolysis and the slowing of relaxation throughout a period of fatigue and suggested that this arose because of a slowing of the SR Ca^{2+} pump. The free energy of ATP sets an upper limit on the concentration gradient of $[Ca^{2+}]$ which the SR Ca^{2+} pump can achieve. Dawson *et al.* (1980) argued that, as the free energy of ATP fell, the rate of pumping of the SR Ca^{2+}pump would also decline. Our finding of a slower rate of fall of $[Ca^{2+}]_i$ supports their hypothesis but it seems likely that direct effects of pH_i and ADP on crossbridge cycling also contribute to slowing of relaxation.

Events during recovery

In some fibres, tension and light recovered quickly and fully, while in others, which exhibited post-contractile depression, full recovery of tension could take more than 2 h and light only recovered partially. This delayed recovery is not due to either a prolonged acidosis (Westerblad & Lännergren, 1988), or to membrane inexitability (Westerblad & Lännergren, 1986). Phosphocreatine has been found to recover five times faster than pH after fatigue (Kushmerick & Meyer, 1985), so it seems unlikely that this or other phosphorus metabolites contribute to the phenomenon. Furthermore, application of caffeine at the time of maximum tension depression (about 20 min after the end of fatiguing stimulation), was able to produce close to full tension (Lännergren & Westerblad, 1989; present study, Fig. 12*B*) and the reduced V_{max} had also almost fully recovered at this time (Lännergren & Westerblad, 1989). These results suggest that post-contractile depression is due to a failure of excitation–contraction coupling, rather than to an inability of the cross-bridges to generate tension and to cycle. This hypothesis is supported by the present finding that tetanic $[Ca^{2+}]_i$ is greatly reduced in post-contractile depression. Comparison of the $[Ca^{2+}]$ with a pCa–tension curve (Fig. 10), suggests that the reduced tension may be entirely explained by the reduced $[Ca^{2+}]_i$.

The reason for the reduced tetanic $[Ca^{2+}]_i$ during post-contractile depression is not clear, but it appears that recovery processes may under certain circumstances lead to transient block of the coupling process. This block may be related to the appearance of vacuoles during fatigue (Gonzales-Serratos *et al.* 1978), which could

conceivably contribute to mechanical disruption of connections between the T-tubules and the SR. The observation that light only recovered partially after more than 2 h indicates that fatiguing stimulation can cause a long-lasting depression of excitation–contraction coupling which persists even when tension has largely recovered.

Conclusions

In this model of fatigue, complex changes in $[Ca^{2+}]_i$ occur which make a major contribution to the changes in mechanical performance observed in fatigue. A substantial part of (i) the slowing of relaxation, (ii) the tension reduction during fatiguing stimulation, and (iii) the secondary tension depression during the recovery period can be ascribed to altered handling of Ca^{2+}. The most probable cause of this alteration is either abnormal coupling between the T-tubules and the SR or Ca^{2+} depletion in the SR.

This work was supported by the Nuffield Foundation. J.A.L. is an MRC Research Training Fellow, H.W. was supported by the Swedish Medical Research Council. We would like to thank Drs D. A. Jones and J. C. Kentish for their comments on the manuscript and Dr J. R. Blinks (Department of Pharmacology, Mayo Medical School, Rochester, Minnesota 55901, USA) for supplying us with aequorin, which was purified in his laboratory with support from NIH Grant HL 12186.

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APPENDIX 5.

Spatial gradients of intracellular calcium in skeletal muscle during fatigue,

by H. Westerblad, J.A. Lee, A.G. Lamb, S.R. Bolsover & D.G. Allen.

A paper published in Pflügers Archiv (in press).

Spatial gradients of intracellular calcium in skeletal muscle during fatigue.

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Abstract

We have measured the distribution of intracellular calcium concentration in isolated single muscle fibres from Xenopus laevis using the fluorescent calcium indicator fura-2 with digital imaging fluorescence microscopy. Under control conditions, resting and tetanic calcium were uniform throughout a fibre. When fatigue was produced using a prolonged, high frequency tetanus, the distribution of calcium within muscle fibres became non-uniform, with greater levels near the outer parts of a fibre than near the centre. This non-uniform distribution of calcium was rapidly abolished by lowering the stimulation frequency. When fatigue was produced using a series of repeated intermittent tetani, tetanic calcium showed an initial small increase, followed by a decrease as stimulation was continued. The distribution of calcium remained uniform under these conditions. Calcium distribution was also uniform during recovery from intermittent tetanic stimulation. Although fibres varied considerably in their fatigue resistance, the time for tension to fall to 50% was correlated with the reduction in tetanic calcium seen at this time. These results indicate that there are at least two patterns of reduced calcium relase that can contribute to the development of fatigue. The appearance of a calcium gradient is consistent with impaired t-tubular conduction, while a uniform reduction of calcium is likely to be due to the action of metabolic factors on systems controlling calcium homeostasis within the cell.

Keywords: Skeletal muscle, fatigue, calcium, fura-2.

Introduction

The tension produced by skeletal muscle declines after prolonged or repeated activity. This phenomenon of fatigue represents one fundamental limit on physical activities. Although fatigue in intact animals is known to involve the central nervous system, the neuromuscular junction and the muscle itself (Bigland-Ritchie & Woods, 1984), many features of fatigue may be seen in isolated muscle preparations, suggesting that processes within the muscle make an important contribution. Two main mechanisms have been proposed to explain fatigue in isolated muscle: (i) the effects of metabolic products on the contractile proteins, causing a reduction of the maximum tension-generating capacity (Fabiato & Fabiato, 1978; Cooke & Pate, 1985; Kentish 1986; Godt & Nosek, 1989) and (ii) failure of calcium release, causing reduced activation of the contractile proteins (Eberstein & Sandow, 1963; Grabowski et al, 1972; Lännergren & Westerblad, 1989). Current evidence favours a major contribution from the latter mechanism, because caffeine, which releases calcium from the sarcoplasmic reticulum, can overcome the reduced tension in fatigued muscle (Eberstein & Sandow, 1963; Grabowski et al, 1972, Lännergren & Westerblad, 1989). In addition, it has recently been shown using the calcium indicator aequorin, that intracellular calcium release during a tetanus is reduced in fatigued muscle (Allen, Lee & Westerblad, 1989).

Reduced tetanic calcium release in fatigued muscle could occur in two distinct ways: (i) by failure of inward conduction of the action potential, causing reduced calcium release in the central part of a muscle fibre (Gonzalez-Serratos et al, 1978); (ii) by a spatially uniform failure of calcium release. In order to understand mechanisms of fatigue, it is important to know which of these patterns actually occurs, but no data is available on this point. In this study, we have used the calcium indicator fura-2 (Grynkiewicz et al, 1985) with digital imaging fluorescence microscopy (Williams et al, 1985) to distinguish between these two possibilities. The results show that the decline of tension in a prolonged tetanus produced by high frequency stimulation is associated with spatial gradients of calcium

across a muscle fibre. Repeated short tetani, on the other hand, lead to a form of fatigue in which calcium is uniformly reduced across muscle fibres. A preliminary account of these results has been presented (Allen, Lee & Westerblad, 1989).

Methods

Single fibre dissection and mounting. Experiments were performed on single muscle fibres dissected from adult female Xenopus laevis. Frogs were killed by stunning followed by decapitation and the second, third and fourth lumbrical muscles were removed. Single fibres were dissected from any of these muscles using dark field illumination. Tendons were trimmed close to the muscle fibre and were held with platinum foil micro-clips. The largest and smallest diameters of fibres were measured using an ocular scale and the cross-sectional area was calculated. Fibres were then transferred to an experimental chamber which allowed stimulation and recording of tension. Fibres were stimulated with platinum electrodes using pulses of 0.5 - 1 ms duration and an intensity of approximately 1.2 x threshold. Tension was measured with a horizontally mounted tension transducer (Akers AE 801, SensoNor, Norway), attached to the fibre via a glass extension with a fine hook at the end. The other end of the fibre was attached to a moveable hook which allowed the length of the fibre to be adjusted so that maximum tension was obtained. Fibres were superfused at 21^{o}C with a standard frog Ringer solution containing (mM): Na^{+} 120, K^{+} 2.5, Ca^{2+} 1.8, Cl^{-} 121, HPO_4^{2-} 2.15, $H_2PO_4^{-}$ 0.85 (pH 7.0).

Measurement of the spatial distribution of intracellular calcium. Once in the experimental chamber, fibres were microinjected with the calcium indicator fura-2. A small quantity of a solution containing 10 mM fura-2 and 150 mM KCl was placed in the tip of a conventional glass microelectrode of resistance 20 - 60 Mohms . The microelectrode was inserted into the fibre under visual control, while simultaneously monitoring the membrane potential. When the electrode was inside the fibre (as indicated by the potential), controlled pulses of pressure from a gas cylinder were applied using an electronically operated valve (Picospritzer II, General Valve Corporation, New Jersey, USA). This method of microinjection avoided mechanical vibration and allowed precise control of the duration and intensity of the pulse. Injection of fura-2 was monitored visually and by measuring the change in fluorescence

elicited by a test stimulus when the fibre was illuminated with ultraviolet light. In order to achieve an acceptable light signal, several impalements with different electrodes were sometimes necessary. The fura-2 injections used in this study had little effect on the amplitude or timecourse of a twitch, indicating that fura-2 was not significantly buffering $[Ca^{2+}]_i$.

Once an acceptable fura-2 signal had been achieved, the experimental chamber was transferred to the stage of an imaging microscope. This apparatus has been described in detail by Silver et al (1989). For imaging the spatial distribution of calcium in single muscle fibres, the procedure was as follows. Fibres were illuminated with ultraviolet light of 350 or 380 nm wavelength, which was focussed on the long axis of the fibre. An image of emitted light at 500 nm was obtained by averaging over a 267 ms period. A ratio image was then produced by dividing the 500 nm image obtained with illumination at 350 nm by that obtained with illumination at 380 nm. The ratio image has the advantage that it largely corrects for variations of intensity associated with uneven fibre thickness and for changes in dye concentration due to, for example, diffusion or bleaching. The two collection periods required for each ratio image were either at identical times in the latter part of successive 500 ms tetani or closely spaced (1 s apart) in a long tetanus. The ratio values (R) shown in the figures are monotonically related to $[Ca^{2+}]_i$, but the relationship is non-linear because at the $[Ca^{2+}]_i$ which occurs in an unfatigued tetanus the binding of Ca^{2+} to fura-2 is close to saturation. The relationship between R and $[Ca^{2+}]$ in vitro can be deduced from the two colour scales in Fig. 3, where the upper shows values for R and the lower indicates $[Ca^{2+}]$. However, it is not correct to assume that the intracellular $[Ca^{2+}]$ is that given by the in vitro calibration, because Konishi et al (1988) have shown that soluble muscle proteins substantially change the Ca^{2+} binding properties of fura-2 in vivo.

Stimulation protocol. Fatigue was produced by two different stimulation protocols. One method was to use a prolonged, high frequency tetanus given

until tension had declined to approximately 50% of control. The other method was to use repeated, intermittent tetani, according to a protocol decribed by Westerblad & Lännergren (1986). Tetani were 500 ms in duration and at a stimulation frequency of 50 Hz. The intertetanus interval was 4 s for the first 2 minutes, and then decreased to 3, 2.5, 2, 1.7, 1.5 and 1.2 s in subsequent 2 minute intervals until tension had decreased to 50% of control. Stimulation was then stopped, and recovery of the fibre was followed by giving pairs of test tetani at 1, 5 and 10 minutes and every 10 minutes thereafter until a stable level was reached.

Interpretation of ratio images. The ratio images illustrated in this paper are distorted because the signal from the centre of the fibre contains contributions from peripheral parts of the fibre above and below the plane of focus. The net effect of this is to reduce the apparent size of any gradient of $[Ca^{2+}]_i$ from the periphery to the centre of a fibre. It is possible to make a correction for this effect based on the following assumptions: (i) the fluorescent intensity recorded at any region represents the sum of all light emitted by dye in a vertical column passing through this region, (ii) the distribution of $[Ca^{2+}]_i$ is radially symetrical. Images were divided into 14 segments and the outermost segments assumed to contain no errors due to thickness. The estimated signal for each segment was calculated by subtracting the contribution of outer elements which lay above and below the plane of focus. This correction did not produce a gradient of $[Ca^{2+}]_i$ where none existed in the uncorrected signal, but increased the size of the gradient where one was present. For instance in Fig. 2C(3) the minimum ratio at the centre is 77% of that at the edges; after applying the correction described, the minimum was 57% of that at the edges.

Results

Many different protocols have been used to produce fatigue in muscle preparations. In this study we have compared the effects of prolonged high frequency tetani and repeated short tetani on the spatial distribution of intracellular calcium during a tetanus.

Fig. 1 illustrates the effects of prolonged high frequency stimulation on tension and on the distribution of the myoplasmic free calcium concentration ($[Ca^{2+}]_i$) in an isolated muscle fibre. Fig. 1A shows the high and uniform level of $[Ca^{2+}]_i$ associated with a high frequency tetanus in the unfatigued state. Fig. 1B shows that after 8s of high frequency stimulation, a spatial gradient of calcium developed, with a greater $[Ca^{2+}]_i$ near the edges of the fibre than at the centre. It can be seen that $[Ca^{2+}]_i$ near the edges of the fibre in Fig. 1B was somewhat greater than control, while $[Ca^{2+}]_i$ at the centre of the fibre was less than control. Note that $[Ca^{2+}]_i$ at the centre of the fibre is overestimated in this figure due to the contribution of the edges of the fibre above and below the plane of focus, as described in Methods. The increase of $[Ca^{2+}]_i$ near the edges of the fibre, which can also be seen in Fig. 2, may be related to the early increase of $[Ca^{2+}]_i$ seen with intermittent tetanic stimulation (Allen, Lee & Westerblad, 1989, and see below).

The pattern of $[Ca^{2+}]_i$ seen in Fig. 1 is what would be expected if high frequency stimulation was causing failure of calcium release in the centre of the fibre, for example because of failure of conduction down the t-tubular network. Fig. 2 shows an experiment in which this possibility was investigated further. Two control tetani at 100 Hz were given, followed by a prolonged tetanus. During the control tetani, $[Ca^{2+}]_i$ was again uniform across most of the fibre. In this example it can be seen that a somewhat lower concentration was found near (i.e. < 10 μm from) the edges of the fibre (Figs. 2A (Control) & 2C (1)). This appearance was seen in several fibres. Although we are not certain of the cause of the lower $[Ca^{2+}]_i$ at the edges, it should be noted that the edges are probably most susceptible to artefactual ratio signals due to slight differences in the focussing of the different

wavelengths of illuminating light. At the start of the long tetanus, which had an initial stimulation frequency of 100 Hz, $[Ca^{2+}]_i$ was uniform across most of the fibre (not shown), as in the control tetani. However, after 8s, when tension had declined to 82% of control (Fig. 2B (2)), a gradient had developed, and $[Ca^{2+}]_i$ was clearly lower in the centre of the fibre than close to the edges, giving the fibre a banded appearance (Figs. 2A (100 Hz, 8s) & 2C (2)). After 27s of stimulation at 100 Hz, tension was reduced to 49% of control (Fig. 2B (3)). At this time, the gradient of $[Ca^{2+}]_i$ into the centre of the fibre remained and in addition, the $[Ca^{2+}]_i$ close to the edges of the fibre had fallen (Figs. 2A (100Hz, 27s) & 2C (3)). This may be related to the fall in tetanic $[Ca^{2+}]_i$ seen in fatigue due to intermittent tetanic stimulation (Allen, Lee & Westerblad, 1989, and see below). Shortly after this point, the stimulation rate was reduced to 20 Hz. This caused a rapid increase in tension (Fig. 2B), which was accompanied by a marked alteration in the distribution of $[Ca^{2+}]_i$ within the fibre. Although $[Ca^{2+}]_i$ was almost unaltered close to the edges of the muscle fibre, the gradient into the centre was abolished (Figs. 2A (20 Hz, 37s) & 2C (4)). The timecourse of the increased tension and the redistribution of $[Ca^{2+}]_i$ were rapid, and were complete within 2-3s.

Figure 3 shows results from a muscle fibre stimulated with repeated, short tetani. The protocol (see Methods) involved gradual shortening of the inter-tetanus interval until tension was reduced to approximately 50% of control. The distribution of $[Ca^{2+}]_i$ in resting fibres was uniform (Fig. 3A and 3C). The ratio image produced from the first and second tetani of fatiguing stimulation (F1) illustrates the increase of $[Ca^{2+}]_i$ produced by tetanic stimulation and is also uniform (except right at the edges, see comment above). After fifty tetani (F50), tension had fallen to 84% and tetanic $[Ca^{2+}]_i$ had <u>increased</u> slightly, but there was still a largely uniform distribution of calcium. After 210 tetani, when tension had fallen to 44% (F210), the tetanic $[Ca^{2+}]_i$ was substantially reduced but remained spatially

uniform. Thus, a spatial gradient of $[Ca^{2+}]_i$ was not seen at any time during the development of fatigue due to intermittent tetanic stimulation.

After repeated tetani, Xenopus muscle fibres often show a further decline in tension, before slowly recovering to control levels (Westerblad & Lännergren, 1986). This 'post-contractile depression' has been found to coincide with reduced tetanic $[Ca^{2+}]_i$ (Allen, Lee & Westerblad, 1989). Pairs of tetani after 1, 30 and 100 minutes of recovery (Figs. 3A and 3D (R1min, R30min & R100min)) illustrate this pattern, and in addition show that the spatial distribution of calcium is also uniform throughout the recovery period. To illustrate that it was possible to detect gradients of calcium in this fibre, the final panel shows an image produced from tetani performed with the external sodium concentration reduced to 50% by substitution with N-methyl glucamine. This procedure is known to impair t-tubular action potential conduction and hence to cause failure of contraction of the central myofibrils (Bezanilla et al, 1972). It can be seen that tetanic $[Ca^{2+}]_i$ in the centre of the fibre was dramatically inhibited by these conditions, being scarcely greater than resting levels, while calcium release could still occur at the edges of the fibre (Figs. 3A and 3D).

In five muscle fibres fatigued with intermittent tetani, the number of tetani required to reduce tension to 50% varied from 40 to 500, in accordance with the known variability of fatigue resistance of these fibres (Westerblad & Lännergren, 1986). The fura-2 ratio at the time when tension was reduced to 50% was reduced to 73 $\pm$ 3% (mean $\pm$ S.E.) of the unfatigued control. It should be noted that the change in $[Ca^{2+}]_i$ is probably larger than this, since the fura-2 signal is close to saturation during a control tetanus. We found that the reduction of the fura-2 ratio at the time when tension was reduced to 50% was independent of the fatigue resistance of the muscle fibres. For example, in the fibre which required 500 tetani to be fatigued, the fura-2 ratio fell to 77% of control, while in the fibre which required only 40 tetani, the fura-2 ratio fell to 83%. Thus the fall in tension in the fatigued state correlates with the decline of $[Ca^{2+}]_i$.

Discussion

Recent direct measurements of $[Ca^{2+}]_i$ in single muscle fibres (Allen, Lee & Westerblad 1989) have confirmed suggestions (Eberstein & Sandow, 1963; Grabowski et al, 1972; Lännergren & Westerblad, 1986) that failure of release of calcium from the sarcoplasmic reticulum is an important factor contributing to the decline of tension seen during fatigue. The previous measurements of $[Ca^{2+}]_i$, however, represent average values for the whole of a single fibre, and provide no information on the distribution of calcium within the fibre. The results of this study now demonstrate that there are at least two patterns of reduced calcium release which may be seen in fatigued muscle.

In fatigue due to prolonged high frequency stimulation, the decline of tension is associated with a gradient of $[Ca^{2+}]_i$, which is high close to the outer parts of the fibre, but falls towards the centre. This pattern is what would be expected if high frequency stimulation was causing failure of conduction of the action potential to central parts of the fibre. In agreement with this is the rapid recovery of tension seen when the stimulus frequency was lowered. This recovery, which was complete in 2-3 s, is too fast to be caused by metabolic recovery, which takes several minutes (Kushmerick & Meyer, 1985), but has a time course similar to the diffusion of ions in or out of the t-tubules (Nakajima et al, 1973). The presence of $[Ca^{2+}]_i$ gradients which rapidly disappear when stimulation frequency is reduced, thus support the hypothesis that failure of t-tubule conduction contributes to fatigue during prolonged high frequency tetani. Possible mechanisms are that K^+ accumulation or Na^+ depletion in the t-tubules causes failure of inward conduction of action potentials (Jones et al, 1979) or a reduction in their amplitude, hence causing less effective calcium release from the sarcoplasmic reticulum. Also in agreement with this interpretation is the observation that substitution of half the external sodium by N-methyl glucamine, a procedure known to cause impaired t-tubular action potential conduction, also caused a gradient of $[Ca^{2+}]_i$.

As well as the changes of $[Ca^{2+}]_i$ seen in the central parts of fibres, high frequency stimulation also caused an initial increase in the calcium release near the edges, which was then followed by a decline of calcium release as fatigue developed further. These changes were superimposed on the gradient of $[Ca^{2+}]_i$, and are similar to those seen in fatigue due to intermittent tetani (Allen, Lee & Westerblad, 1989; present study). With intermittent tetani, a uniform increase in tetanic calcium across the fibre was seen during the initial part of the stimulation period, which later developed into a uniform decrease in tetanic calcium as stimulation was continued.

The initial increase in tetanic calcium may result from a reduction of intracellular Ca^{2+} buffering capacity produced by an intracellular acidosis due to lactic acid production, as glycolysis is stimulated by the increased activity of the muscle. An intracellular acidosis produced by increasing the CO_2 in the perfusing solution was found to increase tetanic calcium in fibres studied with aequorin (Allen, Lee & Westerblad, 1989). Since this small increase in tetanic $[Ca^{2+}]_i$ was associated with a moderate decline in tension, it seems likely that the early tension loss is caused by metabolic effects on the contractile proteins.

The mechanism of the fall in tetanic $[Ca^{2+}]_i$ which occurs later during a series of fatiguing tetani is as yet unclear, although the present results demonstrate that the mechanism which produces it must lead to a spatially uniform decrease. In other words, in fatigue due to intermittent tetanic stimulation, t-tubule conduction was adequate throughout the stimulation period. We have also shown that there is a correlation between reduction in tension and reduction in $[Ca^{2+}]_i$ which is independent of the time required for fatigue. Since fatigue resistance is probably related to metabolic capacity (Lännergren & Smith, 1966), it seems likely that there exists a link between these factors on the one hand, and the ability to maintain tetanic $[Ca^{2+}]_i$ on the other. Two possible sites for this link between metabolism and calcium release are: (i) impaired function of the ATP driven calcium pump of the

sarcoplasmic reticulum, which might cause a reduced calcium content in the SR and therefore reduced release (Dawson et al, 1980), and (ii) inhibition of the calcium release channels of the sarcoplasmic reticulum due to a low ATP concentration (Smith et al, 1985). In the first situation, the Ca^{2+} would presumably be redistributed to parvalbumin or mitochondria, while in the second it would remain in the sarcoplasmic reticulum. Ion probe measurements of sarcoplasmic reticulum Ca^{2+} content during fatigue (Gonzalez-Serratos et al, 1978), which indicate that the sarcoplasmic reticulum was not depleted of calcium, appear to support the latter hypothesis.

During recovery from fatigue due intermittent tetanic stimulation, $[Ca^{2+}]_i$ remained uniform. As previously noted (Allen, Lee & Westerblad, 1989), $[Ca^{2+}]_i$ showed the same behaviour as tension, initially declining during 'post-contractile depression' and then recovering to a level which, while it was able to produce full tension, was nevertheless considerably reduced when compared with control. Thus, even when tension had fully recovered, tetanic calcium release was still depressed. The cause of this long-term reduction of calcium release is unclear, although it may be related to phenomenon of 'low frequency fatigue' (Edwards et al, 1977), in which low frequency tetanic force in human muscle is reduced for many hours after fatiguing exercise.

These experiments extend earlier studies (Eberstein & Sandow, 1963; Grabowski et al, 1972; Gonzalez-Serratos et al, 1978; Jones et al, 1979; Lännergren & Westerblad, 1989; Allen, Lee & Westerblad, 1989) which suggested that an important element in the decline of tension during fatigue is reduced Ca^{2+} release. We conclude that this reduced Ca^{2+} release may be due to two different mechanisms: (i) impaired conduction of action potentials into the t-tubular system, resulting in lower $[Ca^{2+}]_i$ in the central part of fibres, and (ii) a uniformly reduced Ca^{2+}-release due to metabolic factors.

Acknowledgements: This work was supported by the Wellcome Trust and the Nuffield Foundation. H.W. was supported by funds from the Karolinska

Institute. J.A.L. is an MRC Training Fellow. We thank R.A. Silver for his contribution to setting up the microscope and imaging camera.

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Figure Legends

Figure 1.

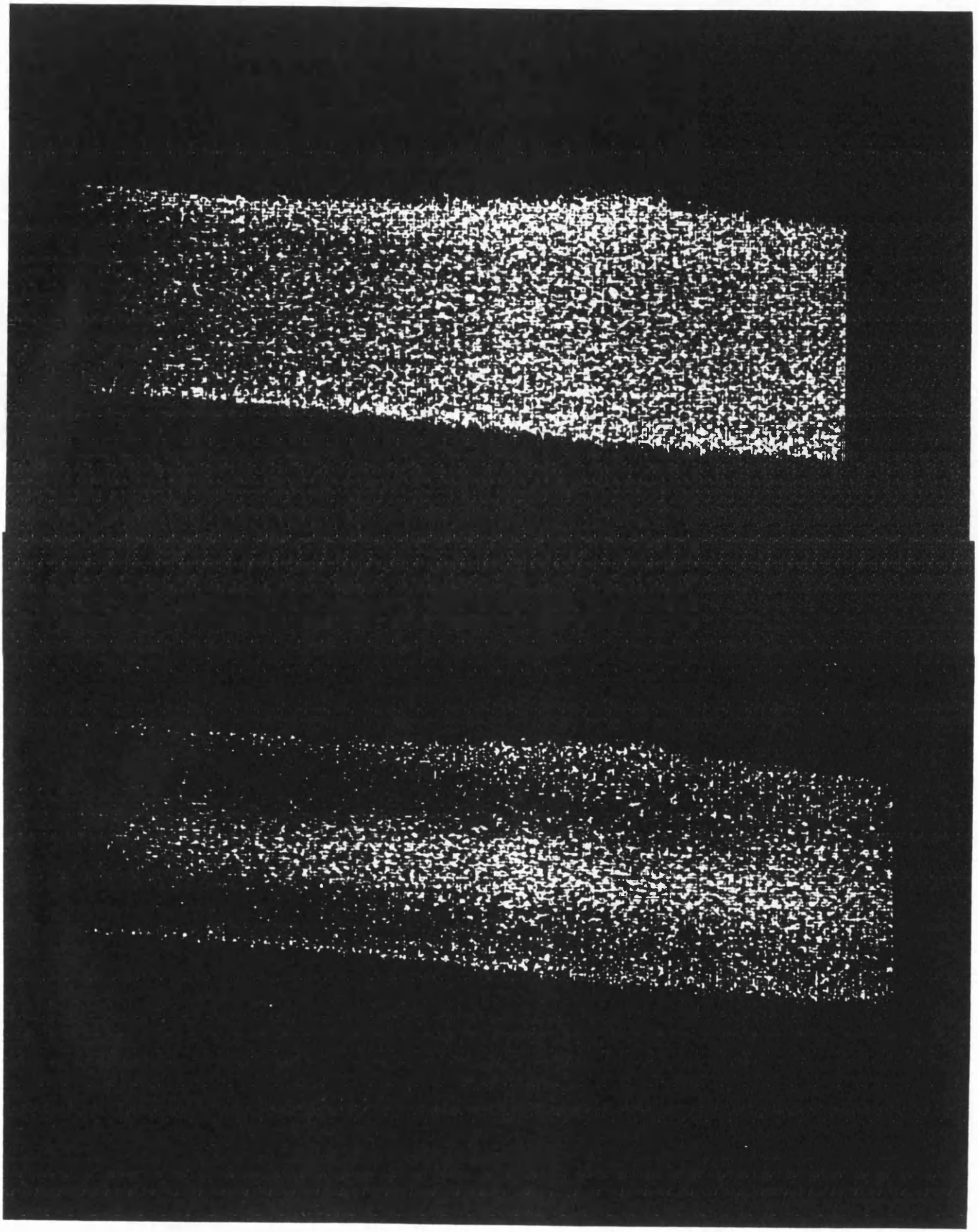
Ratio images illustrating the distribution of $[Ca^{2+}]_i$ across a 500 /um length of a single muscle fibre. A. Image from a pair of control tetani; 500 ms, 100 Hz. B. Image after 8s of a prolonged tetanus at 100 Hz stimulation frequency. Similar results were seen in 3 experiments.

Figure 2.

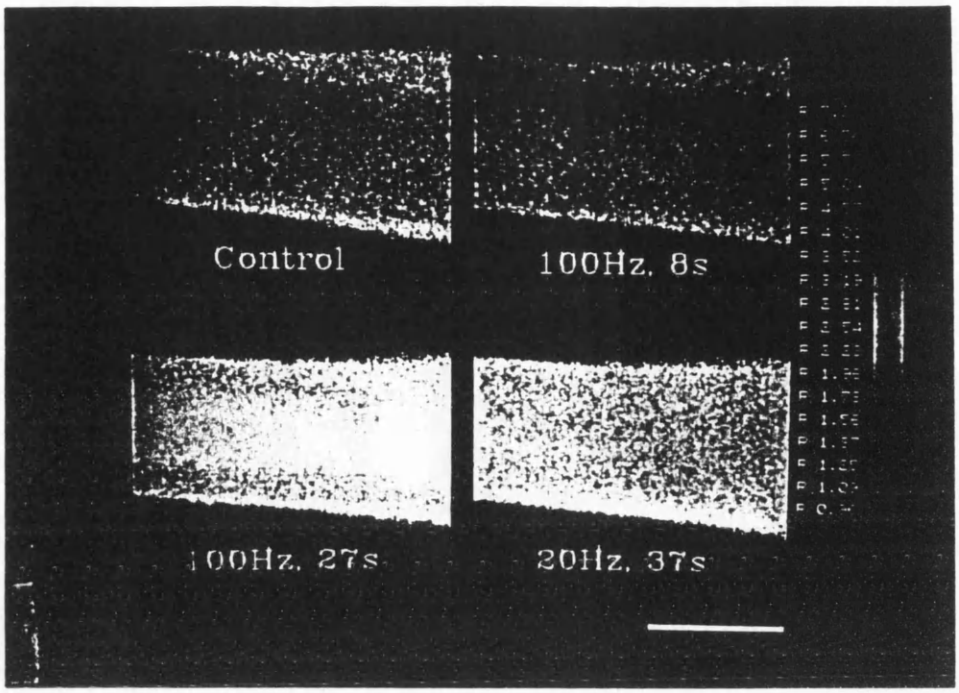
A. Ratio images representing the distribution of $[Ca^{2+}]_i$ across a 250 /um length of the fibre (total fibre length ~1500 /um). (1) Control image from two 500 ms, 100 Hz control tetani separated by 2 min. (2) 8s into a continuous tetanus at 100 Hz. (3) 27s into the continuous tetanus. (4) 37s; stimulus frequency now 20 Hz. Bar represents 100 /um. B. Tension record from control tetani and long high frequency tetanus. Times at which images were collected are indicated. C. Variations of R with distance across the fibre. Each point represents the average of 300 pixels from a rectangle 37 X 7 /um. Standard errors of each point were less than the size of the symbol.

Figure 3.

A. Ratio images as in Fig. 1. (i) Resting fibre. (ii) Beginning of fatiguing stimulation (first two tetani). (iii) After 50 tetani. (iv) End of fatiguing stimulation. (v) Tetanus 1 minute after end of fatiguing stimulation. (vi) After 30 minutes of recovery. (vii) After 100 minutes of recovery. (viii) Gradients produced by a tetanus in low sodium solution (half sodium was replaced by N-methyl glucamine). B. Tension records from periods at which the images in A were taken. Panels C & D. Plots of the variation in R with distance across the muscle fibre. Each point represents the average of ~800 pixels from a rectangle 94 X 6.5 /um. Standard errors of each point were less than the size of the symbol. Similar results were seen in 5 experiments.



A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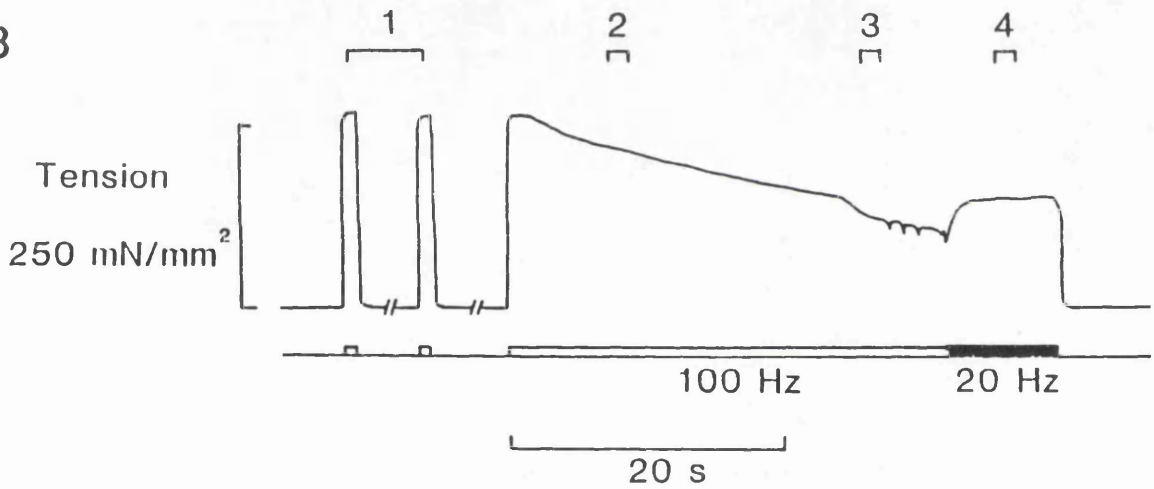
100Hz, 27s
20Hz, 37s

B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2
3
4
Tension
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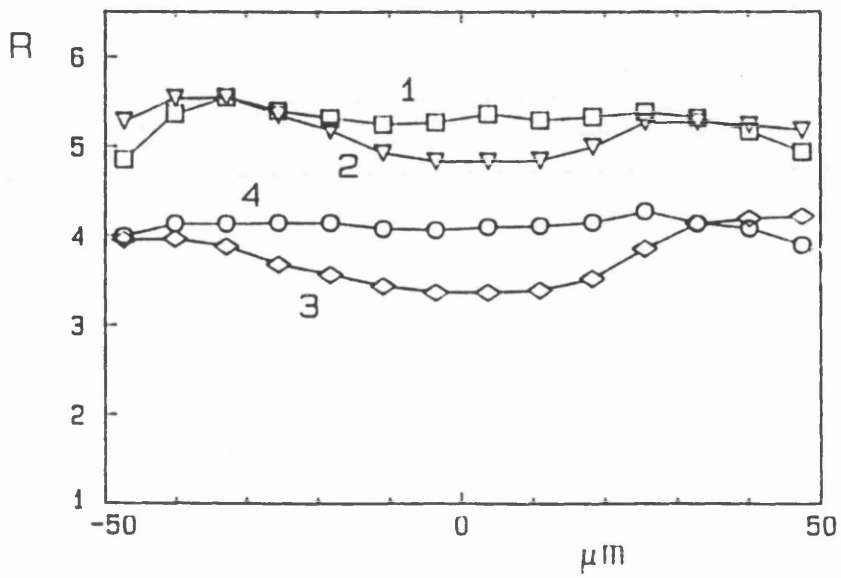
100 Hz
20 Hz
20 s

C


R
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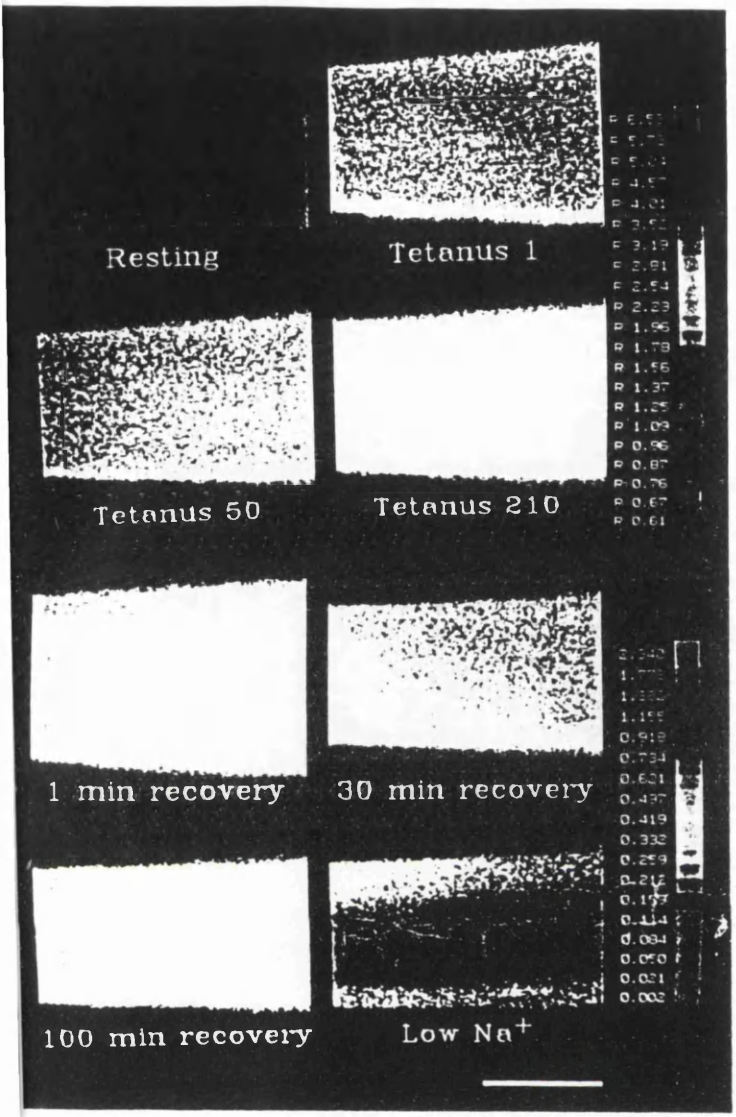
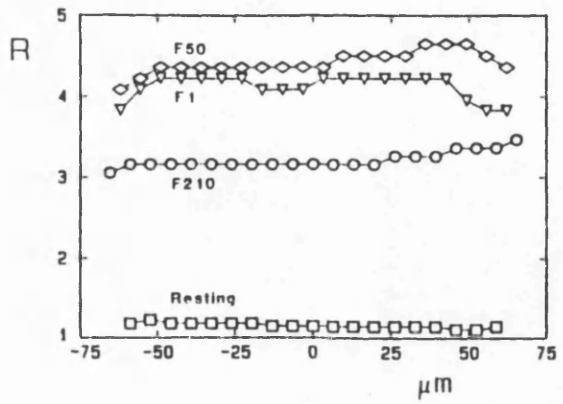
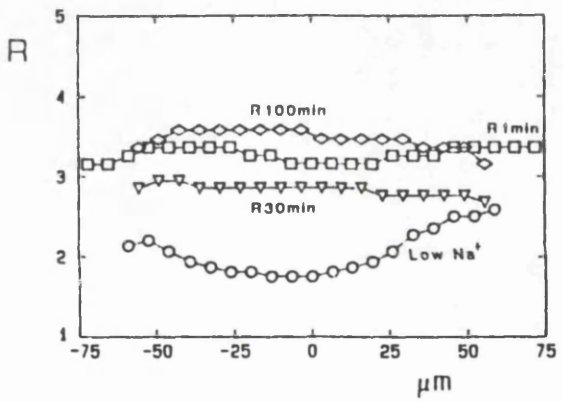
5
4
3
2
1
1
2
3
4
-50
0
50
μm

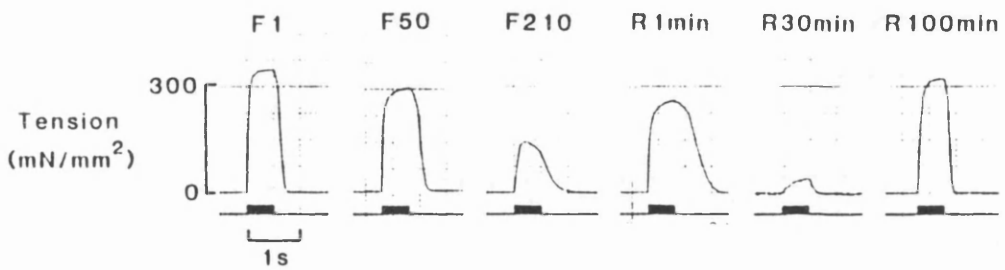

Resting
Tetanus 1
Tetanus 50
Tetanus 210
1 min recovery
30 min recovery
100 min recovery
Low Na+

C


R
F50
F1
F210
Resting
μm

D


R
R100min
R1min
R30min
Low Na+
μm


F1
F50
F210
R1min
R30min
R100min
Tension
(mN/mm2)
300
0
1s

APPENDIX 6.

The effects of fatigue on intracellular calcium measured with fura-2 in isolated single muscle fibres from *Xenopus*,

by D.G. Allen, J.A. Lee & H. Westerblad.

An abstract published in the Journal of Physiology **414**, 49P (1989).

[*From the Proceedings of the Physiological Society*, 10–11 *February* 1989
Journal of Physiology, **414**, 49*P*, 1989]

The effects of fatigue on intracellular calcium measured with fura-2 in isolated single muscle fibres from *Xenopus*

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We have recently reported measurements of intracellular free calcium ($[Ca^{2+}]_i$) using the photoprotein aequorin in *Xenopus* single-muscle fibres during fatigue (Allen *et al.* 1988). Intermittent tetani were continued until developed tension had fallen to 50%; by this stage tetanic $[Ca^{2+}]_i$ had fallen substantially and this could explain much of the reduction in tension.

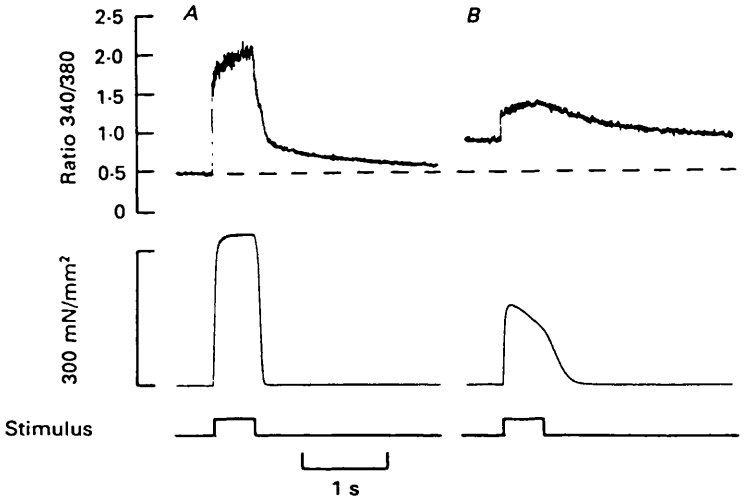

Fig. 1. Repeated tetani (duration 0·5 s, stimulus frequency 50 Hz, cycle length 4 s for 2 min, then 3 s) were used to produce fatigue. Each panel shows the ratio of fura-2 fluorescence at 340 nm and 380 nm (above) and tetanic tension (below). (*A*) Control. (*B*) Tetanic tension reduced to 50% (after 39 tetani in this experiment). Temperature 21 °C.

We have now repeated these experiments using the fluorescent Ca^{2+} indicator fura-2. Compared with aequorin, fura-2 has the advantage that it is more sensitive to low levels of Ca^{2+} and is not affected by consumption or by changes in Mg^{2+} or pH. The changes in tetanic $[Ca^{2+}]_i$ measured with fura-2 during fatigue were qualitatively similar to those seen with aequorin, but in addition it was possible to observe a pronounced slowing of the fall in $[Ca^{2+}]_i$ at the end of a tetanus and a substantial rise in resting $[Ca^{2+}]_i$ during fatigue (Fig. 1). The raised resting $[Ca^{2+}]_i$ took about 20 min to recover after fatiguing stimulation had stopped. This slow fall of $[Ca^{2+}]_i$ after a tetanus and increase in resting $[Ca^{2+}]_i$ could arise because sarcoplasmic reticulum function is impaired during fatigue.

Supported by the Nuffield Foundation.

REFERENCE

Allen, D. G., Lee, J. A. & Westerblad, H. (1988). *J. Physiol.* **407**, 75*P*.

* J.A.L. is an MRC Training Fellow.