THE EFFECTS OF HYPOXIA ON INTRACELLULAR ION ACTIVITIES IN THE MAMMALIAN HEART.

BY

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ABSTRACT OF THESIS (Regulation 3.5.10)

This thesis examines the effects of hypoxia on cardiac muscle. Changes in intracellular pH (pH_i) were used as a marker for the cellular changes which occur during hypoxia and anoxia (the latter was produced by chemical removal of oxygen by sodium dithionite). Measurements were made of pH_i in sheep Purkinje fibres and developed tension in ferret papillary muscle during both hypoxia and anoxia. Anoxia caused a larger fall in pH_i and developed tension than hypoxia. The effects of prolonged exposure to hypoxia were examined on pH_i and the ultrastructure in sheep Purkinje fibres and on developed tension and contracture in ferret papillary. Sheep Purkinje fibres appeared to be more resistant to hypoxic damage than ferret papillary muscle. The intracellular acidification and change in developed tension produced by sodium cyanide (NaCN) was compared with anoxia/hypoxia. Anoxia caused the largest intracellular acidification and there was no significant difference between the size of acidification produced by NaCN and hypoxia.

Intracellular potassium activity decreased and intracellular sodium activity increased in hypoxia, both changes were larger in anoxia.

A decrease in temperature from 35°C to 22°C was found to cause an intracellular alkalinisation and resulted in a smaller decrease in pH_i and developed tension during hypoxia.

Replacing glucose pyruvate or acetate had no effect on the decrease in pH_i during hypoxia. Lactate caused a larger intracellular acidification during hypoxia.

Cinnamate was used to inhibit lactate efflux from the cells. It increased the size of the acidification of pH_i during hypoxia and slowed the rate of recovery on reoxygenation. Inhibition of Na/H exchange had no effect on the decrease of pH_i, while inhibition of the Cl^-/HCO_3^- exchanger produced an increased intracellular acidification during hypoxia.

The effect of extracellular pH (pH_o) on pH_i changes seen in hypoxia were investigated. Alkaline pH_o (pH 8.4) resulted in a smaller decrease of pH_i during hypoxia. The fall in developed tension during hypoxia was larger at pH 8.4 than at pH 7.4. Acidifying pH_o (pH 6.4) caused a larger decrease in pH_i during hypoxia and a larger fall in developed tension during hypoxia compared to pH 7.4.

Changing from HEPES-buffered Tyrode to CO_2/HCO_3^- buffered Tyrode resulted in an intracellular acidification but a smaller change in pH_i during hypoxia. Increasing extracellular buffering power (with 40mM HEPES) reduced the size of the acidification of pH_i during hypoxia.

Many of the results obtained during hypoxia can be explained in terms of the production of lactic acid by the tissues and an increase in the intracellular inorganic phosphate concentration.

I, Catherine M. Bright, hereby declare that this thesis, entitled "The Effects of Hypoxia on Intracellular Ion Activities in Mammalian Heart", submitted for the degree of Doctor of Philosophy at the University of Edinburgh, has been composed by myself and is the result of work done entirely by myself.

Catherine Bright
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"THE HEART HAS REASONS WHICH REASON DOES NOT KNOW."

Pascal.

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LIST OF ABBREVIATIONS

All the abbreviations in the text have been specifically defined when initially brought into use. However, a full list of these abbreviations is included below.

a^i_{Ca}	intracellular Ca^{2+} activity
a^i_K	intracellular K^+ activity
a^i_{Na}	intracellular Na^+ activity
$[Ca^{2+}]_i$	intracellular Ca^{2+} ion concentration
$[H^+]_i$	intracellular H^+ ion concentration
$[K^+]_i$	intracellular K^+ ion concentration
$[Na^+]_i$	intracellular Na^+ ion concentration
$[K^+]_o$	extracellular K^+ ion concentration
Em	membrane potential
pH_i	intracellular pH
pH_o	extracellular pH
pH_s	surface pH
PO_2	partial pressure of O_2
DOG	2-deoxyglucose
NaCN	sodium cyanide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
PIPES	pipiperazine-N,N'-bis[2-ethane-sulphonic acid]
TAPS	Tris[hydroxymethyl]methylaminopropanesulphonic acid
SITS	4-acetoamido-4'-isothiocyanatostilbene-2-2'-disulphonic acid disodium salt

The lower case subscripts or superscripts "i" and "o" refer to intracellular and extracellular respectively.

CONTENTS

SUMMARY

(1) The effect of hypoxia was studied on intracellular ion activities in sheep Purkinje fibres and on developed tension of stimulated ferret papillary muscle. The intracellular pH (pH_i), surface pH (pH_s), intracellular potassium activity (a^i_K), and intracellular sodium activity (a^i_{Na}) of sheep Purkinje fibres was recorded using liquid ion exchanger-filled microelectrodes.

(2) Techniques for inhibiting oxidative phosphorylation were compared for their effect on pH_i and developed tension. These techniques were the use of hypoxia, anoxia or NaCN. Hypoxia was produced by degassing solutions under reduced pressure then bubbling with 100% nitrogen gas. Anoxia was produced in a similar manner but with the addition of the reducing agent, sodium dithionite (0.5 mM), to remove all traces of oxygen from the solutions. Anoxia caused the most marked changes in pH_i and developed tension.

(3) The effect of the duration of exposure to hypoxia was examined and was found to cause a larger intracellular acidification after 20 minutes compared with 10 minutes exposure but there was no apparent effect of duration of hypoxic exposure on the rate of recovery after hypoxia. Periods of hypoxia exceeding 1 hour resulted in reversible changes in pH_i in sheep Purkinje fibre but irreversible contracture in stimulated papillary muscle. There was no apparent effect on the ultrastructure or glycogen content of sheep Purkinje fibres.

(4) The effect of hypoxia, saponin and strophanthidin on the a^i_K of sheep Purkinje fibre were compared to try to ellucidate the mechanism for changes in a^i_K during hypoxia and anoxia. All three procedures decreased a^i_K.

(5) The effect of hypoxia and anoxia on a^i_{Na} in sheep

Purkinje fibres were compared. Anoxia caused a larger rise in a^{i}_{Na} than hypoxia.

(6) The effect of temperature on pH_i was examined, a decrease of temperature from 35°C to 22°C caused an alkalinisation of pH_i in sheep Purkinje fibre and a rise in developed tension in ferret papillary muscle. The acidification of pH_i and the fall in tension during hypoxia was smaller at 22°C than at 35°C.

(7) Replacing glucose with the alternative substrates, pyruvate or acetate had no effect on the size of the intracellular acidification during hypoxia in sheep Purkinje fibre. The presence of lactate however caused a larger acidification of pH_i during hypoxia and slowed the rate of recovery of pH_i.

(8) Inhibiting lactate efflux with cinnamate caused an increase in the acidification of pH_i produced during hypoxia and a reduction in the rate of recovery of the tissue from acidosis.

(9) Extracellular acidosis (pH_o 6.4) caused a decrease in developed tension in ferret papillary muscle, the fall in developed tension was greater in pH_o 6.4 hypoxia. Alkalosis of pH_o (pH 8.4) resulted in an alkalinisation of pH_i in sheep Purkinje fibre and a decreased acidification during hypoxia. Alkaline pH_o increased developed tension in ferret papillary muscle during hypoxia and caused a larger fall in developed tension during hypoxia.

(10) Changing the extracellular buffer from HEPES to CO_2/HCO_3^- caused an acidification of pH_i and resulted in a smaller change in pH_i during hypoxia. Extracellular buffering was increased to examine its effects on pH_i changes during hypoxia. Increasing extracellular buffering from 10mM to 40mM HEPES caused a smaller acidification of pH_i during hypoxia.

(11) The role of Na-H exchange in the acidification of

pH_i during hypoxia was examined by inhibiting Na-H exchange with amiloride (1mM). There was no observed affect of amiloride on the acidification of pH_i during hypoxia. However there was a smaller acidification of surface pH (pH_s) during hypoxia in the presence of amiloride than in normal Tyrode in sheep Purkinje fibres.

(12) The anion-exchange inhibitor 4-acetoamido-4'-isothiocyanatostilbene-2-2'-disulphonic acid disodium salt (SITS, 100uM) was used to elucidate the role of Cl^-/HCO_3^- exchange on the acidification of pH_i during hypoxia. SITS resulted in larger changes in pH_i during hypoxia.

(13) The effect of high extracellular potassium concentration ($[K^+]_o$) was examined for its effect on pH_i during hypoxia. There was no effect observed on raising $[K^+]_o$ from 6mM to 12mM in the perfusing solution.

(14) The dependence of the change in pH_i during hypoxia and the effect of the procedures described above are discussed. It is suggested that many of the changes could be due to the production of lactic acid in the cells during hypoxia.

INTRODUCTION

The introduction to this thesis is mainly in the form of a review of the recent literature on the effects of hypoxia, anoxia and ischaemia on the mammalian myocardium. The cellular effects of hypoxia and ischaemia are similar and therefore any study of hypoxia involves a familiarity with the literature concerned with ischaemia.

The introduction is divided into five sections which are:-(1) a general review of the effects of hypoxia, anoxia and ischaemia on the mammalian myocardium, (2) methods of producing hypoxia, anoxia and ischaemia *in vivo*, (3) techniques for studying intracellular ions, (4) a review of the effects of metabolic substrates, H^+ ion buffering and temperature in ischaemia, hypoxia and anoxia and (5) a description of the histology of the myocardium.

(1) EFFECTS OF ISCHAEMIA, HYPOXIA, AND ANOXIA ON THE MAMMALIAN MYOCARDIUM.

The factors limiting myocardial oxygen supply and hence energy production have attracted the interest of physiologists since the beginning of this century. The anatomical basis of this supply boasts a more ancient lineage with Galen's introduction of the term "coronary arteries" in the 1st century A.D. and Harvey's demonstration in the 17th century that there were vessels from which the heart receives its nutrients (Gregg and Fisher, 1963). As might be expected, over the last century, results obtained with the more tractable skeletal muscle have tended to form the framework within which subsequent cardiac data have been interpreted. Although cardiac physiology may have benefited, in part, from this approach it has tended to obscure the unique

nature of myocardial requirements and the devastating outcomes if these requirements are not met.

Myocardial ischaemia is one of the major causes of premature death and serious illness in developed countries. The importance of the effects of ischaemia and hypoxia on the heart and the resulting depression of contractility have therefore been extensively studied.

This thesis describes my investigations of the effects of restricting or removing oxygen supply to isolated cardiac muscle and on its subsequent ability to maintain ionic differences across its membrane.

(A) General Effects

The changes occurring in the mammalian heart during hypoxia and ischaemia are diverse, involving alterations in cellular metabolism and intracellular ion activities (Opie, 1984).

In ischaemia, which is characterised by reduced blood-flow, the heart is deprived of substrates, and metabolites are able to accumulate in the intracellular spaces. These changes lead to decreased tension production (Katz and Hecht, 1969) and possibly to the appearance of arrhythmias. Therefore the effects of ischaemia are of considerable interest, particularly in the understanding of the pathophysiology associated with myocardial infarction (MI). In addition to MI, certain surgical procedures necessitate periods of ischaemia, for instance, bypass and open heart surgery.

Ischaemia can be studied using different experimental models which tend to produce different results. Regional ischaemia is produced by occlusion of a coronary artery which will eventually develop into MI. This evolving process corresponds well with the early stages in MI in man. Another model, is that of global

ischaemia which results when the whole heart is deprived of its blood supply so that the mechanical work of the heart as a whole is stopped. During global ischaemia the systolic pressure (developed pressure) falls rapidly over several minutes resulting in acute ischaemic failure (Shine *et al.* 1976). After 10 to 20 minutes developed pressure is small or absent and a gradual rise in diastolic pressure occurs (ischaemic contracture). After about 1 hour of ischaemia there is considerable evidence of biochemical and histological damage (Ganote et. al. 1983).

Ischaemia is difficult to study experimentally as it is not possible to apply drugs or to alter the extracellular solution composition during ischaemia. In addition, for small isolated muscle preparations it is difficult to simulate ischaemia because of the problem of cannulating the small blood vessels supplying the tissue. For these reasons many studies of this subject have maintained superfusion of the tissue but have removed all or most of the oxygen (anoxia or hypoxia), or have inhibited oxidative phosphorylation in the tissue by chemical means such as cyanide exposure. Hypoxia and anoxia are commonly used in vitro as methods by which some aspects of ischaemic damage can be mimicked. However there are several important differences which will be discussed.

(B) Effects on metabolism

The general changes outlined above are the result of disruptions of the metabolic processes of cardiac tissue. These disruptions produce in turn ionic effects and changes in contractility. One of the most obvious biochemical processes to be affected will be glycolysis.

Glycolysis

When O_2 is excluded from the mammalian myocardium glycolysis is accelerated. The loss of adenosine triphosphate (ATP) and phosphocreatine (PCr), increase in inorganic phosphate (P_i) and adenosine monophosphate (AMP) and decrease in citrate (as a result of decreased flux through the TCA cycle) are all known consequences of blocking oxidative phosphorylation (Kubler and Spieckerman, 1970). Glucose uptake by the cells is also known to increase, as a result of the Pasteur effect (first described in micro-organisms where glucose uptake increases in anaerobic glycolysis facilitating fermentation).

Ischaemia results in a build-up of the products of glycolysis, probably with some residual degree of oxidative metabolism, depending on the extent of ischaemia. Rovetto, Lamberton and Neely (1975) found that in the globally ischaemic rat heart glycolysis was inhibited at the level of glyceraldehyde 3-phosphate dehydrogenase which is the central reaction of glycolysis (Newsholme and Leech, 1983).

The major factors responsible for glycolytic inhibition in the ischaemic compared with the hypoxic/anoxic myocardium appear to be the higher levels of lactate and H^+ ions in the former situation. Rovetto *et al.* (1975) found that increasing extracellular $[H^+]$ concentration inhibited glycolysis in anoxic hearts far more than in aerobically metabolising hearts. Accumulation of tissue lactate and inhibition of glycolysis were directly proportional to the decrease in coronary flow in ischaemic tissue. Rovetto *et al.* (1975) concluded that lactate accumulation represented a major factor in the glycolytic inhibition of ischaemic hearts.

In the ischaemic isolated rat heart O_2 deficiency, as a result of decreased coronary flow, leads initially

to an acceleration of glycolysis due to a more rapid rate of glycogenolysis (Rovetto, Whitmer and Neely, 1973). They also showed that in ischaemia total glycolytic flux decreases below control levels after tissue glycogen stores are depleted (after approximately 8 minutes global ischaemia) in isolated rat heart.

Changes in endogenous energy stores

Interference with glycolysis will inevitably affect the endogenous energy stores in the myocardium, ATP and PCr. During both ischaemia and hypoxia there is a decrease in the O_2 supply to the myocardial cells. This results in a decrease in ATP formation via oxidative phosphorylation. Dhalla *et al.* (1972) found that in isolated rat heart after 7 minutes perfusion with substrate-free (glucose removed) hypoxic medium, the levels of glycogen, PCr and ATP declined whereas the concentration of lactate, ADP, AMP, creatine, and P_i increased during the first minute of hypoxia by which time the contractile force and heart rate decreased to about 20% of control values.

This decrease in high energy phosphates may be an important factor in the contractile failure observed in hypoxic and ischaemic hearts. Hearse (1979) demonstrated that in the isolated rat heart a substantial decrease in ATP and PCr concentration occurs after the onset of anoxia but before the onset of contractile failure. Thus, during the first 5 seconds of anoxia, contractile activity is constant, [ATP] decreases by 25% and [PCr] by 50%. After this contractile failure occurs and therefore utilization of ATP also decreases. However more recent work by Allen *et al.* (1985) has shown much slower changes in high energy phosphate levels. During inhibition of oxidative phosphorylation and glycolysis the [PCr] fell to about 10% of control after 5 minutes and ATP fell to

50% of its control over 10 minutes. This work was performed in isolated ferret heart .

Piper *et al.* (1984) found energy needs in cultured rat ventricular muscle cells decline rapidly during anoxia, yet glycolytic energy production remains inadequate since this also declines and glycogenolysis stops after degradation of only half the glycogen initially present. Once the ATP content falls below 2umol/g wet wt. cells become irreversibly damaged indicating that in cultured cells the anoxic process develops similarly to that of the O_2 deficient heart.

Allen, Morris and Orchard (1985) used ^{33}P NMR to measure the concentration of phosphorous metabolites in Langendorff perfused ferret hearts. Intracellular levels of P_i, PCr, ATP and H^+ were measured either under control conditions or when oxidative phosphorylation and/or glycolysis were prevented (glycolysis was prevented either by removing the extracellular glucose and depleting intracellular glycogen or by removing glucose and blocking glycolysis with 2-deoxyglucose (DOG)). When oxidative phosphorylation was inhibited, developed pressure fell to 35% of control in 5 minutes. PCr fell to 15% of the control level after 5 minutes while $[ATP]_i$ declined very slowly to about 90% of the control value over 10 minutes. It was concluded that when oxidative phosphorylation alone is prevented, changes in pH_i may account for tension changes, while P_i increasing may also contribute to this decline.

A more pronounced decline in pressure was observed in the same set of experiments when both glycolysis and oxidative phosphorylation were inhibited, this decline could not be accounted for solely by changes in pH_i or P_i. It was suggested that a fall in free energy of hydrolysis of ATP may account for the fall in tension. The subsequent hypoxic contracture observed could

adequately be explained as a result of the fall in [ATP] which reaches its lowest levels at about the same time that the contracture develops. It has been suggested that two Ca^{2+} ions are pumped into the sarcoplasmic reticulum (SR) per ATP split (Endo, 1977) and the free energy required from ATP is 38kJ (Allen *et al.* 1983). During hypoxia there is a substantial rise in ADP and P_i, coupled with a small decrease in [ATP]. The free energy available from ATP becomes close to that required to pump Ca^{2+} into the SR. When glycolysis is also inhibited the free energy will presumably decline below 38kJ/mole and there will be decreased Ca^{2+} uptake by the S.R. and myoplasmic Ca^{2+} levels will rise. Alternatively the rise in Pi and ADP (Allen *et al.* 1983) might inhibit the SR [Ca^{2+}] pump. It has been shown that the maintainance of the [ATP] while [PCr] falls and P_i rises results from the resynthesis of ATP from PCr (Garlick, Radda and Seeley, 1979). Although this explains the phosphorous metabolite changes when ATP consumption exceeds production, in hypoxia and ischaemia the procedure is complicated by other factors. These complicating factors include the acceleration of anaerobic glycolysis in the first minute of hypoxia and ischaemia by up to 20 fold. This high rate of glycolysis is continued in hypoxia but as already explained decreases in ischaemia as a result of intracellular acidosis and lactate accumulation (Rovetto et. al. 1975). Also the decrease in tension seen in both hypoxia and ischaemia reduces the major cellular consumption of ATP.

Second messengers

The role of cyclic adenosine monophosphate (cAMP) as a second messenger in cellular functions is well accepted (Tsien, 1977). It is possible that this and cyclic guanosine monophosphate (cGMP), which has antagonistic

properties, may have a role in the changes in metabolism/ contraction observed in hypoxia and ischaemia.

Metsa-Ketala *et al.* (1980) suggested that cAMP and cGMP may play a role in the production of lactate in spontaneously beating rat atria during the early stages of hypoxia (50% O_2 saturation). They found that lactate production decreased after 30 seconds of hypoxia but then accelerated again after 2-4 minutes. It was suggested that at the very early stages of hypoxia, cGMP could inhibit and cAMP could accelerate lactate production.

Protein and amino-acid metabolism

The turn-over of myocardial proteins is influenced by many factors. It may be that removal of O_2 disturbs the equilibrium of the synthesis and degradation of protein leading to damage of the cardiac cells.

The effect of ischaemia on the regulatory sites for proteins in cardiac cells have been investigated (Mudge *et al.* 1976). It has been shown (see Taegtmeyer and Lesch, 1980) that in hypoxia and ischaemia, protein synthesis in the heart is severely depressed, primarily by inhibition of peptide chain elongation. However this is reversible on reoxygenation in the presence of glucose if irreversible damage to the tissue has not occurred. The changes in synthesis are not found to be well correlated with any changes in ATP levels. Myocardial protein degradation can be inhibited by decreased [ATP] under some conditions but in others it can be increased (Taegtmeyer and Lesch, 1980).

Fatty acid metabolism

It has been suggested that an excessive concentration of free fatty acids (FFA) may exert a depressant influence on myocardial metabolism *in vivo* (Evans, 1964). Henderson *et al.* (1970) investigated the

influence of FFA and glucose on the mechanical performance of rat papillary muscles during hypoxia and anoxia. Although FFA did not alter mechanical performance in normoxia, a decrease in contractility and an increased resting tension was observed in the presence of FFA during anoxia. This effect of FFA was modified in part by glucose which counteracted the depression of active tension by FFA. The depressant effect of non-metabolisable FFA was similar, suggesting that the effect was mediated directly by FFA rather than by any product of FFA metabolism.

Katz and Messineo (1982) investigated the effects of saturated (palmitic and stearic acid) and unsaturated (palmitoleic and oleic) fatty acids on Ca^{2+} uptake by isolated rabbit skeletal sarcoplasmic reticulum vesicles. In the presence of phosphate they all inhibited Ca^{2+} uptake. The significance of the findings is equivocal, but alterations of membrane function caused by accumulation of endogenous fatty acids may in part explain some of the functional changes observed in the ischaemic heart.

(C) Ionic Effects

The disruption of metabolism outlined above will of course profoundly affect energy supply to ion regulatory mechanisms and thus intracellular ion activities. The changes in intracellular ion activities can be linked to a number of sarcolemmal exchange mechanisms such as Na/K, Na/H, and Na/Ca exchangers (Page and Storm, 1965, Reuter and Seitz, 1968 and Deitmer and Ellis, 1980). The effects of hypoxia and/or ischaemia will be discussed under the headings of individual ion species.

Intracellular Calcium

Calcium is largely compartmentalized within the cardiac cell. It is therefore difficult to obtain reliable estimates of its concentration in the cellular compartments. Total cell Ca has been shown not to change during the first hour of ischaemia (Shen and Jennings, 1972). Although total $[Ca^{2+}]_i$ appears not to change the distribution in the cell may be altered and this may contribute to the various changes in contractile properties of the heart which occur during ischaemia (the effects of Ca on reperfusion damage will be discussed later).

Jarmakani *et al.* (1979) studied the effects of hypoxia and reoxygenation on Ca flux in neonatal mammalian heart. They found that reoxygenation resulted in an increase in tissue calcium, probably as a result of increased calcium influx since neither hypoxia nor ischaemia affected Ca efflux. An explanation for this may lie in a depression of Na^+/K^+ ATPase activity and therefore an increase in $[Na^+]_i$. This in turn would increase Na^+_i/Ca^+_o exchange and increase Ca influx (Glitsch et.al 1970). It was also found that removing glucose from the perfusate increased Ca uptake on reoxygenation, again suggesting that a depression of [ATP] may be responsible.

Bers and Ellis (1982), measured intracellular Ca activity (a^i_{Ca}) in sheep Purkinje fibres and found that inhibition of the Na^+/K^+ pump using strophanthidin produced a slow increase in a^i_{Ca} and resting tension. They suggested that the rise in a^i_{Ca} occured following loading of the intracellular Ca buffering systems. They found a close relationship between the mechanisms controlling a^i_{Ca}, a^i_{Na} and pH_i, since it appears that changes of $[Na]_o$ or inhibition of the Na^+/K^+ pump can modify the function of sarcolemmal Na^+/Ca^{2+} exchange and

thus cause changes in a^{i}_{Na} and $[Ca^{2+}]_i$ which in turn may affect $[H^+]_i$ via the Na^+/H^+ exchange or intracellular buffering systems (Vaughan-Jones 1986).

Allen and Orchard (1983) examined intracellular Ca in rat, cat and ferret papillary muscles which had been micro-injected with aequorin (which is a photoprotein which emits light as function of Ca concentration). Using this they investigated the effect of cyanide (CN)) and glycolytic inhibition (blocking glycolysis with glucose free solutions and 2-deoxyglucose (DOG)) on early contractile failure and $[Ca^{2+}]_i$. The study revealed that when oxidative phosphorylation is blocked tension development decreases with no change in the calcium transient produced by the cells (a calcium transient is the sudden increase of $[Ca^{2+}]_i$ that initiates contraction and is thought to be caused mainly by release of Ca from the sarcoplasmic reticulum (Chapman, 1979)). Perfusion with glucose-free solution caused a larger decrease of tension in hypoxia accompanied by a decrease in the magnitude of the Ca transients.

When cyanide and DOG were applied together, this caused a rapid decrease in both developed tension and the calcium transients which were not fully reversible. It was concluded that in hypoxia alone the decrease in tension was due to a decrease in the sensitivity of the contractile proteins to Ca , perhaps due to a decrease in pH_i. The fall in tension observed in CN plus DOG or CN in glucose-free solution could be due to a fall in the free energy of hydrolysis of ATP to below the level required to pump Ca into the SR, therefore resulting in contractile failure and a decrease in the size of the Ca transients.

Allen and Orchard (1984), extended their previous work to describe further the decline of cardiac function in hypoxia. Their experiments suggested that two

independent processes are occurring during hypoxia. When only oxidative phosphorylation is inhibited the fall in developed tension is largely attributable to the intracellular acidification which accompanies an increase in the rate of glycolysis. When both oxidative phosphorylation and glycolysis are blocked the contractile failure observed (after 2-4 minutes) occurs when PCr has fallen to zero but ATP levels are still substantial. At the start of contracture (after about 10 minutes), [ATP] has fallen close to zero (see later). Therefore it is suggested that the more extreme contractile failure observed in complete metabolic blockade is a combination of that seen in hypoxia and the free energy of ATP hydrolysis dropping so low that the cell is unable to overcome the energy barrier required to move Ca^{2+} ions into the SR.

Lee *et al.* (1987) used the cell permeant cytosol Ca indicator (Indo-1 AM) to measure Ca transients from the epicardial surface of rabbit ventricle. They showed that myocardial ischaemia produces a rapid and drastic increase in the calcium transients. It was found that ischaemia caused a rapid decrease in resting membrane potential which was initially independent of extracellular K^+ accumulation. Therefore it is possible that the $[Ca^{2+}]_i$ increase which they observed may directly alter the resting potential of ischaemic heart cells by means of a Ca activated inward current (Inesi, 1985). Na^+ may enter the cells either via the Ca^{2+} activated non-specific cation channels (Colquhoun *et al.* 1981) or via the Na^+/Ca^{2+} exchanger.

Intracellular sodium

Current knowledge about the Na pump in cardiac cells is summarized by Eisner (1986). Generally influx of Na^+ into the cell occurs via either voltage dependent channels or exchangers (Na^+/H^+ or Na^+/Ca^{2+} exchange).

In the ischaemic heart the large influx of Ca implicates the sarcolemmal Na^+/Ca^{2+} exchange as a route for Ca^{2+} entry into the cell (Bourdillon and Poole-Wilson 1981).

Kleber (1983) found that a^i_{Na} did not change during a 15 min. exposure to ischaemia. However, subsequently Wilde and Kleber (1986) did show a rise in a^i_{Na} when isolated guinea pig ventricle was exposed to a combination of acidosis, hypoxia and glucose-free conditions. They also found that an increase in $[K^+]_o$ did not change a^i_{Na}.

Guarnieri (1987) showed in experiments on ferret papillary muscle that only during substrate-free hypoxia does a^i_{Na} increase (during this same time period there was no measurable rise in $[Ca^{2+}]_i$) unless the Na^+/K^+ pump was concomitantly inhibited. He suggested that the lack of rise in a^i_{Ca} was consistent with inhibition of the Na^+/Ca^{2+} exchange. Ellis and Noireaud (1987) found a small rise in a^i_{Na} on reoxygenation after hypoxia in the presence of glucose. This small increase in a^i_{Na} was attributed to stimulation of Na^+/H^+ exchange due to intracellular acidification.

MacLeod (1989) also measured a^i_{Na} during metabolic inhibition (CN) and hypoxia and found a rise in a^i_{Na} under these conditions in sheep Purkinje fibres. He also observed a more rapid rise to a greater a^i_{Na} when the tissue was exposed to both CN and glycolytic blockade (DOG). MacLeod also showed that if exposure to CN is followed by strophanthidin (which blocks the Na^+/K^+ pump) the rapid increase in a^i_{Na} does not produce a

contracture. This suggests that whatever mechanism is responsible for the generation of the contracture often seen with hypoxia and metabolic inhibition, a^i_{Na} does not appear to be involved. This finding lends support to the suggestion by Guarnieri (1987) that the Na^+/Ca^{2+} exchanger is inhibited because a rise in a^i_{Na} would be expected to cause a rise in a^i_{Ca} during hypoxia. He found that despite an increase in a^i_{Na} during glucose-free hypoxia there was no change in a^i_{Ca} unless the Na/K pump was inhibited. The lack of rise in a^i_{Na} he attributed to Na/Ca exchange inhibition.

Intracellular and extracellular potassium

The onset of both hypoxia and ischaemia leads to an increased K^+ efflux from myocardial cells (Kleber, 1984). Reduced or absent blood flow results in K^+ accumulation in the extracellular spaces resulting in a depolarization. He showed that $[K^+]_i$ tends to fall while $[K^+]_o$ tends to rise during ischaemia.

Weiss and Shine (1982) showed that during myocardial ischaemia $[K^+]_o$ rose to 10-15mM in the extracellular space during the first 10 minutes of ischaemia. McDonald and McLeod (1973) had shown previously that K^+ loss during anoxia results from a decrease in the rate of influx rather than an increase in the rate of efflux.

Guarnieri and Strauss (1982) also observed a decrease in a^i_K during hypoxia in guinea pig papillary muscle. Poole-Wilson *et al.* (1984) suggested that the increase in $[K^+]_o$ can account for almost all the changes in the action potential observed during ischaemia but not for the changes in contractility.

However in more recent work Gaspardone *et al.* (1986) have attributed the rise in $[K^+]_o$ to inhibition of the

Na^+/K^+ pump, acidosis or a selective increase in cell membrane permeability. Their results indicate that changes in osmolarity and intracellular ion concentrations during ischaemia, can modify K^+ exchange in the myocardium. They suggested that the K^+ loss was due to an increased efflux. There was no evidence of decreased Na^+ pump activity.

Leblanc et. al. (1987) showed that K^+ loss from the hypoxic myocardium can be dissociated from inhibition of the Na^+ pump, at least for a limited period of time. In addition they showed that K^+ loss was modified by extracellular $[K^+]_o$. A decrease in the extracellular concentration enhanced the K^+ loss induced by hypoxia whereas high $[K^+]_o$ prevented or delayed this. This suggested that the K^+ loss was along its electrochemical gradient and therefore passive in nature.

This helps to explain the K^+ loss in ischaemia since an increase of $[K^+]_o$ to levels around 10mM and the subsequent decrease in the electrochemical gradient for K^+ would inhibit further loss by the cells (Noble, 1979), until irreversible membrane damage and massive leakage of K^+ occured in the irreversible phase of ischaemia.

Intracellular and extracellular pH

According to the hypothesis of Katz and Hecht (1969), rapid failure of the mechanical performance of the heart in hypoxia and ischaemia could be due in part to an effect of the associated intracellular acidosis, which results from lactic acid formation by the cell under these conditions. It is known that a decrease in pH decreases the sensitivity of the contractile proteins to Ca^{2+} (Fabiato and Fabiato, 1978).

Cobbe and Poole-Wilson (1980), suggested that an important part of the fall in developed tension in

hypoxia could be attributed to acidosis. In their experiments however tension tended to fall before tissue pH became acidic. It was clear that acidosis was not the sole mechanism for hypoxic contractile failure. More recently it has been suggested that the combined effects of a decrease in pH_i and an increase in inorganic phosphate (Pi) (Allen *et al.* 1985) may be responsible since both will affect the ability of Ca^{2+} to generate force from the myofibrils.

Allen *et al.*(1985) also found an alkalosis of approximately 0.1 pH unit prior to the decline of intracellular pH in hypoxia in the ferret ventricle studied with NMR. This was explained as being due to the breakdown of PCr to P_i and Cr with the net absorption of protons.

Ellis and Noireaud (1987), using ion selective microelectrodes, confirmed that these changes in pH_i occur in ferret papillary muscle and found similar but somewhat smaller changes in sheep Purkinje fibres. They also found a transient decline in developed tension on readdition of O_2. They found that the decline could be explained by a large transient intracellular acidification prior to recovery of pH_i on reoxygenation after hypoxia.

Vanheel *et al.* (1987) measured pH_i in isolated right ventricular muscle from guinea pigs under conditions of simulated ischaemia. The developed tension of the muscles declined and a relatively small decrease in pH_i was observed while the decrease in pH_o was considerably greater. It therefore seems that the contractile failure in early ischaemia cannot be completely explained by intracellular acidification, since this was very small in these experiments. The decrease in pH_o observed in ischaemia may also influence the size of any change in pH_i (Vanheel *et al.* 1986). They found that acidification

at the surface layer by lowering extracellular buffering from 20mM HEPES to 5mM HEPES in sheep Purkinje fibres results in a subsequent acidifying of pH_i which depresses the rate of recovery of pH_i following an imposed acid load. This lowering of pH_i correlated with the depression of twitch tension (de Hemptinne *et al.* 1987) in cat papillary muscle.

The size of pH changes will also be dependent on the buffering capacity of the cells (Ellis and Thomas 1976). This may change during the course of an exposure since intracellular acidification will affect the buffering mechanisms of cells. Several other mechanisms also seem to contribute to pH regulation including Na^+/H^+ exchange, a mechanism mainly used for regulation of an acid load (Deitmer and Ellis, 1980) and a HCO_3^-/Cl^- exchange which tends to be activated under alkaline intracellular conditions (Vaughan-Jones, 1982).

By using NMR Bailey *et al.* (1981) found that during ischaemia intracellular acidosis is of the order of 1.0 pH unit. Obviously a complete understanding of the alterations in pH_i which occur due to hypoxia and ischaemia would enhance our knowledge of the subcellular processes which lead to the various changes (such as contractile changes).

(D) Effects on Contractile Activity

The gross outcome of the metabolic and ionic disruptions outlined above is the failure of the heart as a pump. This failure can be considered in terms of ischaemic and hypoxic failure and contracture.

Ischaemic and hypoxic failure

It has been reported that during what may be considered the reversible phase of hypoxia and ischaemia (i.e. when it is possible to reperfuse or reoxygenate the

preparation without causing further damage) there is an early fall in tension (Carmeliet, 1984). There have been three main suggestions to explain this phenomenon. (1) that there is inhibition of the amount of Ca^{2+} available to the myocardium, (2) that there is a decrease in the myofilament sensitivity to Ca^{2+} as a result of intracellular acidosis and (3) that there is insufficient intracellular ATP for the maintainance of active tension.

The absence of clearly recorded decreases in Ca^{2+} (Allen and Orchard, 1984) suggest that a fall in Ca^{2+} availability is not the likely cause. The effect of acidosis on the myofibrils in early hypoxia/ischaemia may not be the cause of early contractile failure since early and large decreases in pH_i have not been convincingly demonstrated. It seems likely that the earliest effect arises from the inhibitory action of a large rise of Pi as a result of ATP breakdown. This rise is from 1-3mM to 20mM in hypoxia and ischaemia (Kentish 1986). Fabiato (1985) has shown that Pi has an inhibitory effect on developed tension in skinned cardiac preparations.

Activation of the cardiac cell will obviously play an important role in contractility in hypoxia and ischaemia. McDonald and MacLeod (1973) studied the action potential in anoxic guinea pig papillary muscle and found that in general there were large decreases in action potential duration (APD) with only a small depolarization of the resting membrane potential (RMP). In the presence of 5mM glucose the APD fell during anoxia to about 40% of control values over 60 minutes However during the time when contractile failure occured the reduction in APD was less than 5% and therefore unlikely to be responsible for the decrease in contraction observed. Some criticism of the work described above has been levelled in that the action potentials were recorded from only the surface

cells of a large muscle bundle. Carmeliet (1978) suggests that changes in APD and amplitude which occur in hypoxia (with inhibition of glycolysis) and ischaemia may contribute to early contractile failure.

In addition to the biochemical mechanisms already described certain purely mechanical factors may play a role in the early failure of contraction *in vivo*, since this decline occurs during the fall in perfusion pressure and might therefore be attributed to reduced stretch in the heart as a result of the fall in perfusion pressure.

Ischaemic and Hypoxic contracture

The rise in resting tension (ischaemic/hypoxic contracture) which develops after extended exposure to hypoxia or ischaemia has been explained by two mechanisms , one involving a rise in Ca^{2+}, the other a fall in ATP.

Allen and Smith (1985) have shown that at the time the contracture was maximal the resting $[Ca^{2+}]_i$ starts to rise and therefore cannot be responsible for the contracture.

The phenomenon of the "stone heart" was described by Katz (1970) and explained by the attachment of rigor cross-bridges precipitated by low levels of ATP in the cells. Thus any interventions which would increase tension production would lead to more rapid production of rigor.

Miller and Smith (1985) placed a different interpretation on the importance of ATP, by showing that the presence of ADP caused an increase in rigor tension, which was independent of ATP concentration in skinned cardiac muscle. They demonstated that ADP may play a role in the production of rigor.

Generally however, it is accepted that ischaemic and hypoxic contractures are a direct result of ATP

levels dropping to such a low level in the cell, as a result of disruption of the cellular metabolism, that rigor cross-bridges are formed.

(E) Reperfusion Damage

Paradoxically reperfusion does not halt all further damage when a period of total ischaemia extends beyond 30 minutes (see Opie, 1984) as certain ischaemic damage becomes irreversible. This is the result of a critical decrease in the concentration of high energy phosphate, or perhaps on the change in free energy of hydrolysis of ATP. The decrease in energy availabilty inhibits the Na^+ pump which results in calcium overload. In this situation not only does reperfusion fail to produce a recovery but damage may be aggravated by positive feedback mechanisms.

No-reflow phenomena

On reperfusing irreversibly damaged cells, swelling of the cells is observed (Ganote *et al.* 1983), and disruptive contraction of the myofibrils occurs. Ca^{2+} entry into the sarcoplasm is increased which results in contracture and cellular damage leading to wash-out of intracellular enzymes and an influx of extracellular ions.

Humphrey *et al.* (1984) studied the relationship of the release of enzyme (creatine kinase), the loss of vascular competence (the no-reflow phenomenon) and the distribution of morphological changes across the left ventricular wall of the rat myocardium. They found that the amount of creatine kinase released after 60 minutes of global ischaemia was directly proportional to the extent of the no-reflow area. They concluded that the loss of vascular competence has a profound effect on the distribution of myocardial damage and enzyme release which follows reperfusion of ischaemic tissue.

Ca^{2+} Overload

Naylor *et al.* (1979) suggested that mechanical recovery after hypoxia is jeopardised when there is a large net gain of Ca . This re-introduction of oxygen resulting in Ca mediated cell damage is similar to the Ca paradox, which is observed when Ca is re-introduced to a Ca -free solution, resulting in Ca overload of the cells.

Deitmer and Ellis (1978) have shown that during low $[Ca^{2+}]_o$ perfusion, $[Na]_i$ increases. Therefore when $[Ca^{2+}]_o$ is increased, operation of the Ca^{2+}/Na^+ exchanger will result in a rapid increase in $[Ca^{2+}]_i$. It is possible that Ca influx also triggers damage after ischaemia, this being mediated by Na^+/Ca^{2+} exchange and caused by elevation of $[Na^+]_i$.

Higgins *et al.*(1980) found a deleterious effect on membrane integrity of cultured myocytes by Ca during hypoxia. They found that nifedipine and verapamil (both Ca channel blockers) reduced the deleterious effects of hypoxia.

Ganote *et al.* (1983) found that in rat myocardium during Ca -free perfusion, anoxic contraction of the myocardial cells causes separation at the intercalated discs and leads to a release of enzymes. It was proposed that contracture mediates membrane damage and enzyme release from cells exposed to the Ca paradox.

Nakanishi *et al.* (1984) used enzyme release from the heart as a marker for sarcolemmal damage. Their study suggested that although sarcolemmal damage was less in the neonatal heart compared with the adult heart, both produced a measurable release of enzyme without a significant detectable change in permeability to divalent cations. This suggested that (in contrast to previous work) an increased passive permeability to Ca was not responsible for the cellular disruption indicated by

enzyme release.

Hearse *et al.* (1978) suggest that the Ca- and the O_2 paradox (see next section) are facets of the same problem. In both there is an abrupt release of enzymes. They are related also in that they both depend on critical changes in cellular mechanisms which are linked to Ca transport and homeostasis. Hess and Manson (1984) also suggest that the Ca and O_2 paradox have a similar final pathway leading to intracellular Ca^{2+} overload. In both Ca paradox and the O_2 paradox the generation of oxygen free radicals is involved (see later section).

The O_2 Paradox

Hearse *et al.*(1973) described the process whereby reoxygenation of the hypoxic heart resulted in significant damage rather than in the improvement in cardiac function which might be expected. They showed that reoxygenation after 100 minutes of hypoxia resulted in a massive release of creatine phosphokinase (CPK) from the cells. The amount released being over 75% of the total myocardial [CPK].

It was thought that the O_2 paradox may involve a sequence of metabolic events which cause extensive cellular damage. Perhaps the most important mediator of cellular damage on repurfusion being the production of oxygen radicals.

Ferrari *et al.* (1986) suggest that the triggers for cell necrosis on reperfusion include; depletion of high energy phosphates, loss of adenosine nucleotides and catecholamines, accumulation of $[Ca^{2+}]_i$, cell tearing due to contracture, myocardial cell swelling and O_2 generated free radicals.

Oxygen Radicals

If the reintroduction of O_2 causes damage, perhaps some aspect of O_2 metabolism is responsible for the damage. Hess and Manson (1984) describe the reduction of O_2 as following two pathways to the end-product of H_2O. Mitochondrial enzymes convert about 95% of O_2 to H_2O by tetravalent reduction with no production of intermediates. However the remaining 5% proceeds via a univalent pathway in which several intermediates may be produced. The majority of the cell damage problems appear to be caused by the superoxide anion and the hydroxyl radical formed by hydrogen peroxide reduction.

If metabolism of O_2 has an important role in cellular damage on reoxygenation then lipid peroxidation should be measurable and mannitol which is a scavenger of the hydroxyl radical should reduce the extent of lipid peroxidation (Kloner *et al.* 1976) as should application of antioxidants like alpha-tocopherol (Guarnieri *et al.* 1978). The burst of O_2 free radicals on reintroducing O_2 to hypoxic tissue or reperfusing globally ischaemic hearts may result in extensive intracellular and sarcolemmal damage (Guarnieri *et al.* 1980). As a result of this damage extracellular Ca^{2+} would enter the cells resulting in a $[Ca^{2+}]_i$ overload. It therefore seems that O_2 free radical production is a very important factor in the pathophysiology of myocardial ischaemia and hypoxia.

(F) Cardioplegia

To protect the myocardium from the deleterious effects outlined above the process of cardioplegia has been developed. Cardioplegia is defined as an interruption of contraction of the myocardium as may be produced by the use of chemical compounds or of cold, usually applied during surgery on the heart.

It is considered that the major components involved in the cardioplegic protection of the mammalian heart are as follows (for review see Hearse 1980):-

(1) Energy conservation by diastolic arrest.

(2) Slowing the metabolic and degenerative processes (using hypothermia).

(3) Selective prevention or reversal of various unfavourable ischaemic changes.

Diastolic arrest

Melrose *et al.* (1955) produced the first cardioplegic solution which contained high concentrations of K^+, causing reversible depolarization of the membrane potential of the cells and therefore arrest. Other agents will also produce arrest e.g. calcium-free and/or high magnesium solutions. However the deleterious effects of reperfusion with Ca^{2+} containing solution are well established (descibed above) and therefore this technique for inducing arrest is not used.

Hearse *et al.* (1976) used Langendorff perfused rat hearts to show that in high $[K^+]$ (16mM) compared with non-cardioplegic solution (5mM) the [ATP] was greater after 30 minutes ischaemia (induced by aortic clamping). They concluded that rapid diastolic arrest and conserved ATP and PCr levels promote an improved post-ischaemic recovery of cardiac function.

Hypothermia

Barner et. al. (1977) looked at the effects of topical hypothermia on myocardial preservation since it had previously been used as a method for inducing arrest. Myocardial cooling can be produced by either continuous irrigation of the pericardial sac with cold saline or by surrounding the pericardium with ice made from physiological saline. Barner *et al.* (1977) found that

ventricular function was well conserved after 30 minutes ischaemia at 5°C. They concluded that potassium arrest combined with topical hypothermia might provide early blocking of metabolic activity.

Hearse *et al.*(1976) found that, in rat heart at least, the efficacy of hypothermic protection falls off rapidly as the myocardial temperature rises above 28°C. However below 24°C the protection is adequate. It was subsequently suggested (Hearse *et al.*1978) that the sharp transition observed on raising the temperature of the cardioplegic solution may be related to lipoprotein phase transitions in cell membranes. Tyers *et al.*(1977) had previously shown that intracoronary infusion of cardioplegic solution at 10°C and 15°C gave significantly better functional and metabolic protection than perfusate at 4°C and 20°C on recovery from one hour of ischaemia.

Prevention of cellular changes

This is the most variable area in cardioplegic protection. Protective measures can range from the use of additional extracellular buffers to the provision of glucose and insulin to stimulate anaerobic energy production.

Hearse et. al. (1976) investigated the effect of various compositions of cardioplegic solution. They concluded that the most successful formula included high potassium and magnesium concentrations in combination with ATP, PCr and procaine (St Thomas' cardioplegic solution) . However more recent work (Heinmeyer, *et al.* 1987) suggests that inclusion of procaine in cardioplegic solution leads to poor regulation of pH_i. His study compared St Thomas' solution (with procaine) with Bretschneider's solution (without procaine) (Bretschneider, 1980). The inclusion of procaine resulted in incomplete recovery of human left ventricular

papillary muscles after hypoxia which may have been due to the reduction of a^{i}_{Na} and membrane permeabilty which has been observed in the presence of procaine (Deitmer and Ellis, 1980). Heinmeyer et.al. (1987) suggests that poor pH regulation or the direct effects of procaine, are responsible for the poor recovery of the tissue in St Thomas' solution.

(2) EXPERIMENTAL HYPOXIA, ANOXIA AND ISCHAEMIA

Having described the changes produced by ischaemia, hypoxia and anoxia on the mammalian myocardium, the following section describes the techniques for producing these conditions experimentally.

The study of true clinical ischaemia i.e. coronary artery occlusion is not possible in isolated heart tissue. Therefore hypoxia and anoxia coupled with various metabolic blockers have been used to mimic aspects of ischaemia in vitro.

By definition ischaemia means a deficiency of bloodflow to a tissue. In the heart this is an imbalance in the myocardial demand for, and the vascular supply of, coronary blood. Not only does this create a deficit in O_2 and substrates but also results in a reduced capacity for the removal of CO_2, lactate and protons.

Anoxia and hypoxia are different from ischaemia in their origins but not always in their consequences. Hypoxia can be used as a model to ellucidate the relative roles in myocardial ischaemia, of O_2 lack, substrate deficiency and accumulation or redistributon of the products of metabolism.

Pirolo and Allen (1986) suggest that studies of myocardial ischaemia and hypoxia would be greatly assisted if glycolysis could be inhibited completely and reversibly. This might be acheived by three commonly used

methods for inhibiting glycolysis. These are removal of O_2 and depletion of glycogen stores,or the use of DOG and thirdly the use of iodoacetate (this is a non-specific alkylating agent which affects the cysteine residues of enzymes). They conclude that DOG does not completely inhibit glycolysis and iodoacetate blocks glycolysis but irreversibly. The best method appears to be glucose-free solution with depletion of glycogen stores.

During hypoxia, anoxia and exposure to NaCN, oxidative phosphorylation can be inhibited to varying degrees. Various methods can be used to produce hypoxia the most popular being replacing O_2 with N_2 in the superfusate. Metsa-Ketela et.al (1981) has used chemical anoxia produced with sodium dithionite (2mM). This is a powerful reducing agent. A smaller concentration (0.5mM) was used by Dart and Riemersma (1989), the degree of hypoxia being indicated by the dye resazurin changing from blue to colourless on removal of O_2.

(3) METHODS FOR STUDYING INTRACELLULAR IONS

The measurement of intracellular ions can be made using a variety of techniques. The method employed throughout this study to measure intracellular ions was the use of liquid ion exchanger-filled glass microelectrodes for K^+, Na^+ and H^+ measurements. Each type of electrode is calibrated using a variety of concentrations of the ion of interest. The intracellular activity of the ion is recorded by the microelectrode. From this the concentration of the ion can be calculated using the activity coefficient for the ion in question (Robinson and Stokes 1968).

The advantages of using ion-selective microelectrodes are many. The electrodes are very small and therefore cause little damage to cells, they are easy

to fabricate, the calibration procedure is simple and the stability of the electrodes is good. Hinke (1986) suggests that liquid ion exchangers do not function optimally as reliable and stable electrochemical measuring devices. However despite some inadequacies the technique is accessable as a method for elucidating intracellular ion activities and the various ion-carrying systems in the cardiac cell.

(4) THE EFFECTS OF SUBSTRATES, BUFFERING, AND TEMPERATURE ON THE HEART IN ISCHAEMIA, HYPOXIA, AND ANOXIA

(A) Substrates

Cardiac muscle is capable of using a variety of metabolic fuels, the two preferred being fatty acids and glucose (Bing 1965, Opie 1968). In well oxygenated hearts fatty acid is the preferred substrate with energy production resulting from both B oxidation of fatty acids and the tricarboxylic acid cycle. This is prevented in ischaemia with fatty acid metabolism being significantly inhibited by accumulation of long chain acyl CoA groups and other metabolites (Neely *et al.* 1972).

Liedtke *et al.* (1976) investigated whether pyruvate and 2-amino 2-(hydroxymethyl) propane-1,3 diol (tris) buffer (Tris) favourably improved glucose metabolism and energy production in working pigs hearts exposed to ischaemia. They found that pyruvate buffered with Tris improved mechanical function and concluded that metabolic manipulation may provide another mode of therapy for preservation of ischaemic myocardium.

Rau *et al.* (1979) examined the effects of amino acids on the fall of developed tension during anoxic and ischaemic stress. They did not define the exact mechanism of protection but several may be operating, including non-metabolic or transaminase mechanisms. The results

suggest that specific amino-acids (arginine, glutamate, ornithine and aspartate) exert their protective effects via the malate-aspartate shuttle, stimulating mitochondrial metabolism.

Freminet (1981) examined carbohydrate and amino acid metabolism during acute hypoxia in rat hearts. He observed an 85% decrease in the glycogen content of the heart, while glucose, lactate, alanine and succinate all increased. The results appear to demonstrate a utilization of stored glycogen following O_2 deprivation.

Burton *et al.* (1980) showed in feline papillary muscle that glucose availabilty affects the potential for recovery of hypoxia-induced contractile depression, suggesting that glucose alters the progression of damage. This is consistent with the hypothesis that glucose is protective in ischaemia and hypoxia via direct or indirect effects on membrane integrity. Previous work by Apstein *et al.* (1976) also suggested that improved glycolytic substrate availability improved mechanical function in isolated rat trabeculae during severe hypoxia.

It is also suspected that accumulation of free fatty acids elicits a depressant effect on the myocardium, through their direct action on the cell membrane (Harada *et al.* 1984). This accumulation of fatty acids occurs as a result of inhibition of B oxidation.

Lactate appears to be detrimental as an infusion during hypoxia and ischaemia (Hearse *et al.* 1976). In conditions of O_2 deprivation, the resulting metabolic imbalance leads to the production of large quantities of lactate which may be toxic to the tissue in high concentrations.

(B) Buffering of cardiac cells

An understanding of intracellular buffering and of the parameters affecting the degree of buffering is crucial for gaining an insight into pH_i regulation and the transport processes involving H^+ ions.

Buffering is a property of weak acids and bases whereby these compounds minimise shifts in pH by reacting with H^+ according to the equation:

$$B^n + H^+ \rightleftharpoons BH^{n+1}$$

Where B is a weak base of valence n and BH^{n+1} is a weak acid of valence n+1.

Intracellular buffering can be evaluated by observing the amplitude of the pH_i change following acute alkaline or acid loads of known magnitude. Buffering reflects the contributions of several mechanisms. These include the physicochemical buffers, metabolic processes and sequestration by certain organelles.

Early recordings of pH_i using ion selective microelectrodes were made by Thomas (1976) in snail neurones and the effects of CO_2, HCO_3^-, H^+ and NH_4^+ on pH_i were examined. It was concluded that HCO_3^- made a large contribution to the buffering capacity of the cell. After internal acidification the pH_i was restored to normal by transport of H^+, OH^- or HCO_3^- across the cell membrane. Ellis and Thomas (1976,1977) measured pH_i and subsequently buffering power in mammalian heart cells.

Curtin (1986) examined buffering power in frog sartorius muscle using pH sensitive microelectrodes. Since PCr splitting is a major reaction during contractions of this muscle and this reaction is known to absorb H^+ ions, buffer reactions occur and contribute to the overall change in $[H^+]$. Curtin found that the buffering power of frog skeletal muscle was unexpectably large suggesting that unidentified H^+ ion reactions

exist.

Vanheel *et al.* (1986) observed the effects of external buffering on pH_i regulation and found that lowering external buffering power decreases surface pH (pH_s, the pH measured at the outer surface of the cardiac cell membrane) and causes intracellular acidification. In the less buffered solution recovery of pH_i following an acid loading of the cells was substantially slowed. They concluded that the alteration of the buffering capacity of the superfusate in in vitro experiments causes (via modification of pH_s) changes in pH_i and pH_i recovery from induced acidosis in sheep Purkinje fibre and rabbit papillary muscle.

(C) Temperature and Buffering

Temperature effects on myocardial preservation following hypoxia and ischaemia have already been discussed (see Cardioplegia), but the effects of temperature may also be important to other myocardial processes. The effects of temperature on pH_i have not been studied very extensively.

Work on rat cardiac muscle (Saborowski *et al.* 1973a) has indicated that in the extracellular and intracellular compartments the CO_2 uptake curve (comparing PCO_2 with arterial blood pH) shifted to the left at lower temperature (changing from 38°C to 22°C). They considered that the intracellular buffering may consist of a mixture of phosphates and imidazole. However it is not thought likely that any one buffer is paramount since, for example, a much larger $\Delta pH/\Delta T$ is observed than would be predicted if be predicted if phosphate were the predominant buffer. There is only a small effect of temperature on the pH of phosphate buffer.

More recent work by Dawson and Elliott (1984) used NMR to determine pH_i as a function of temperature in frog

skeletal muscle. It was concluded that maintainance of pH_i close to neutrality is a characteristic of the intracellular buffers and does not depend on blood acid base regulation or the *in vivo* equivalent.

(5) HISTOLOGICAL OBSERVATIONS

The histology of the myocardium is of interest in studies on ischaemia and hypoxia since these interventions may alter tissue structure as well as function.

Glycogen content is generally higher in Purkinje fibres than in ordinary myocardial cells. Thornell (1984) identified glycogen in Purkinje fibres ultrahistochemically and recorded the dimensions and topography using the stain periodic acid-thiosemicarbazide-silver proteinate (PA-TSC-SP). He suggested that glycogen particles are bound to protein *in vivo*, but the extent may vary in different tissues. Enzymes related to the synthesis and degradation of glycogen particles may also be associated. The special character of glycogen in Purkinje fibres may be partly due to the interaction between glycogen particles or between glycogen particles and filaments in the fibre. Polysaccaride deposits were also seen intercellularly but they were thought to be preparatory procedure artifacts or the result of biological degradation.

The conduction system of the heart may be expected to exhibit special morphological features and differs from the contractile myocardium in cell size and content. The Purkinje fibres also contain myofibrillar material with different histochemical, biochemical and morphological properties compared with contractile myocardium (see Thornell and Erikssen 1981, for review). The results of several investigations indicate that the

filamentous systems in Purkinje fibres maintain the structure of the conduction cell bundles, functioning as a cytoskeleton.

Schaper *et al.* (1979) investigated early ultrastructural changes in the myocardial ischaemia and infarction in dog hearts after 45 minutes, 90 minutes, and 48 hours of coronary artery occlusion. Irreversibly damaged cells showed destruction of the mitochondria with mitochondrial debris, progressive destruction of the sarcomeres and swollen or shrunken nuclei. From the study it was evident that 45 minutes of ischaemia caused irreversible damage in some cells. It has been shown that the size of a myocardial infarction depends not only on time but on the rate of O_2 consumption (Schaper 1978).

In this study altering the composition and PO_2 of the perfusing solution were examined for their effects on the extent of acidification of pH_i and the change in active tension produced by the exposure of mammalian myocardial tissue to hypoxia. Measurements of pH_i, a^i_K and a^i_{Na} were made to try to describe the role of ion transport mechanisms in the control of pH_i during hypoxia. The addition of ion exchange blockers, alteration of extracellular pH, raising extracellular buffering and changing the perfusing solution temperature were used to examine the nature of the cellular control of the acidification of pH_i during hypoxia and the recovery of pH_i on reoxygenation.

METHODS

(1)GENERAL

In this study I used sheep Purkinje fibres or ferret papillary muscle. Fresh sheep hearts were obtained from the local slaughter-house, the venticles cut open and the hearts transported to the laboratory immersed in modified St. Thomas' cardioplegic solution (see later) at ambient temperature. Free-running Purkinje fibres were dissected from the left ventricle.

Ferrets were anaesthetised with either intraperitoneal pentobarbitone sodium (Sagatal, May and Baker, U.K.) or ethyl carbamate solution (Urethane, Sigma U.K.), 25% in saline. The heart was removed and washed in modified St. Thomas' cardioplegic solution. Thin trabeculae or papillary muscles were dissected from the right ventricle.

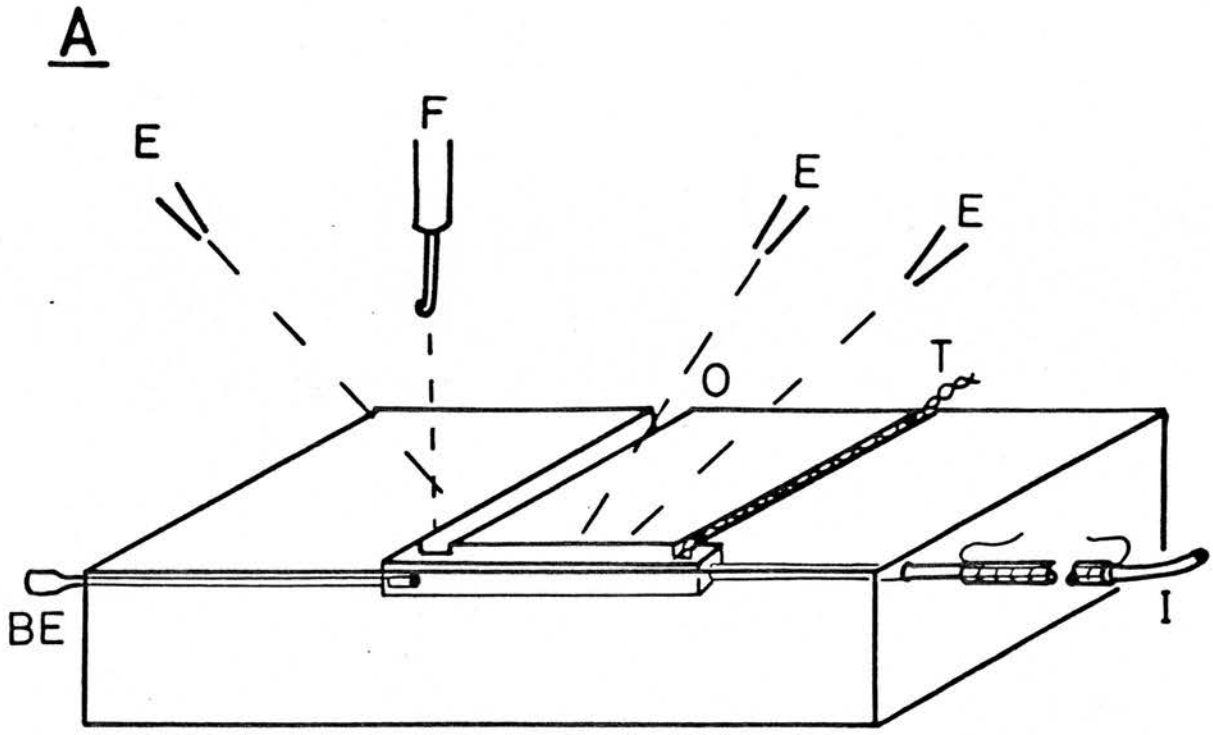
The experiments were carried out in a tissue chamber of perspex with a glass front for viewing the preparation (Fig.2.1). The bath volume was approximately 0.2 ml. At the left end of the bath was a 2mm diameter hole through which the bath electrode could be inserted. In the bottom of the bath a second hole accommodated the oxygen electrode. The bottom of the bath (except at the oxygen electrode position) was covered with a thin layer of silicone rubber (Sylgard, Dow Corning, Belgium). Over this several slightly raised stainless steel wires ran across the chamber. Pinning the preparation across these wires improved electrode penetration. The right end of the preparation was pinned to the floor of the bath using an entomological pin. The other end was attached to the arm of the force transducer (see Fig.2.2 and next section for details) by tying a loop of fine thread round the preparation. The preparation was permanently submerged in

FIGURE 2.1A

A schematic diagram of the experimental bath showing the position of the bath input tube (I), the thermocouple (T), bath output (O), force transducer (F), the bath electrode (BE) and the position of the electrodes (E). Superfusing Tyrodes solution flowed continuosly over the preparation entering the chamber from the right through the input tube (which was heated to maintain a bath temperature of 35^{o}C) and leaving at the back of the bath to the left. The preparation was usually penetrated with two microelectrodes. One was a conventional 3M KCl-filled microelectrode which measured membrane potential and the other was an ion-sensitive microelectrode which measured both membrane potential and the potential due to intracellular ion activity. A third electrode was occasionally used to measure surface pH, this measured the surface ion signal.

FIGURE 2.1B

This shows a more detailed view of the experimental chamber. A silicon rubber base of the bath allowed preparations to be pinned to the bottom of the bath.


A
E
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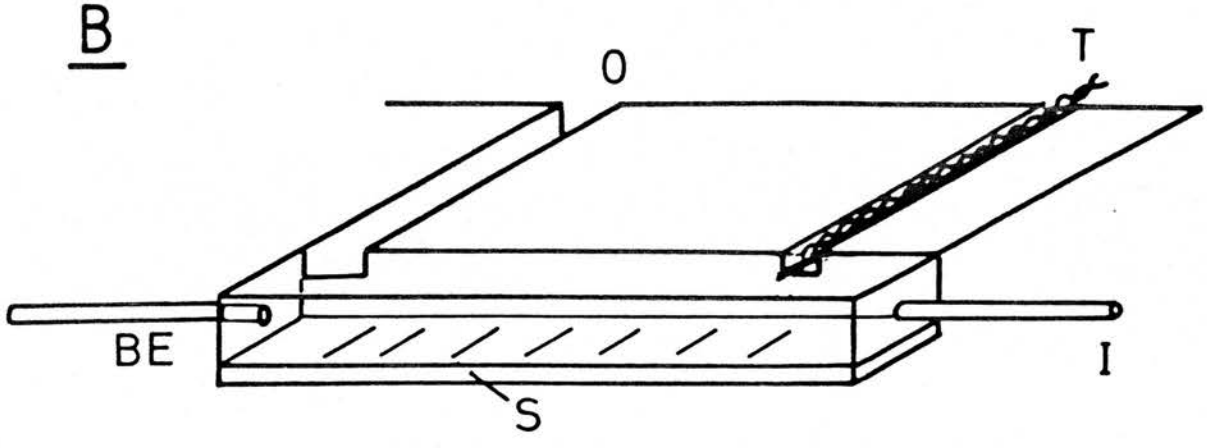
B
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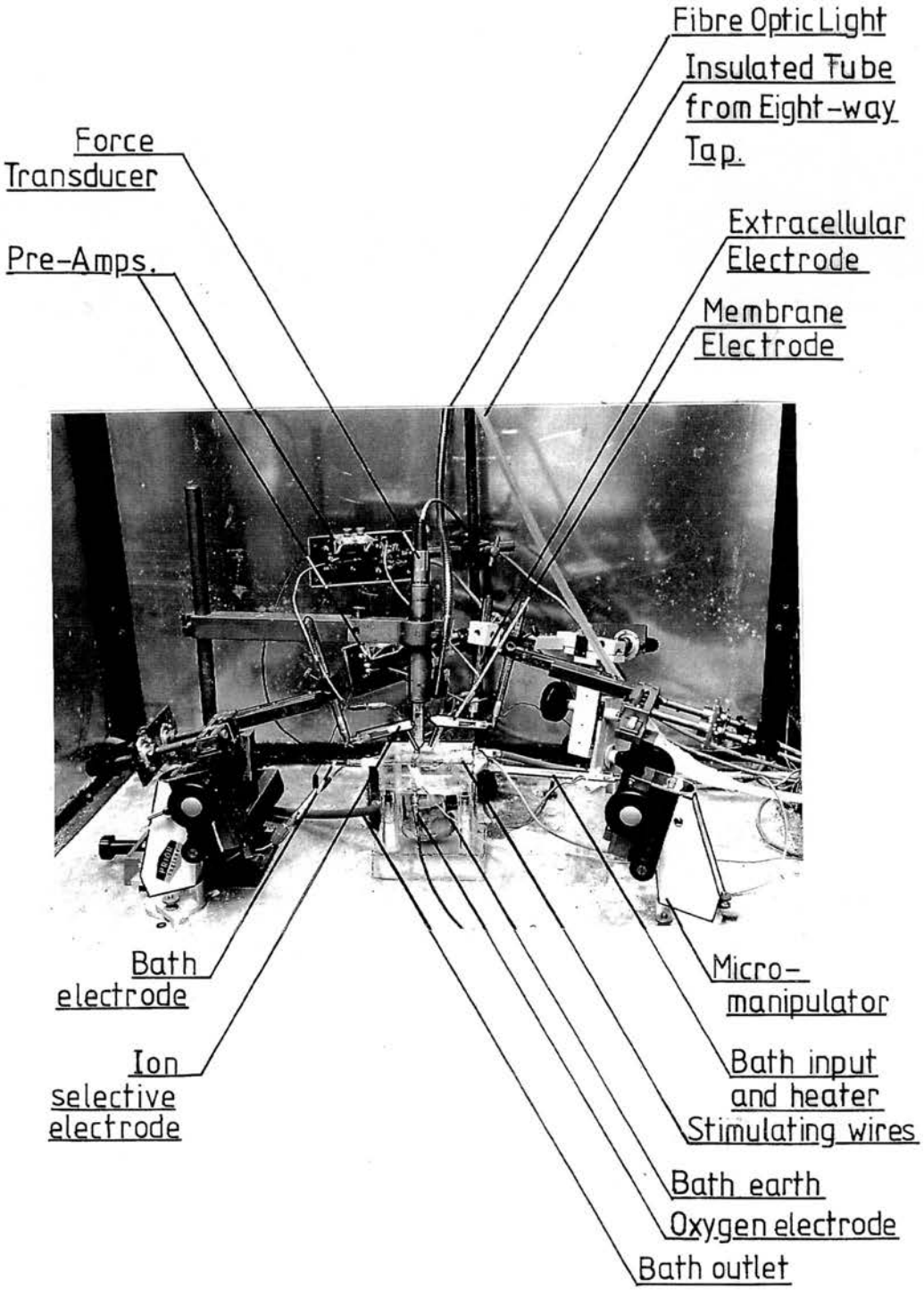
FIGURE 2.2

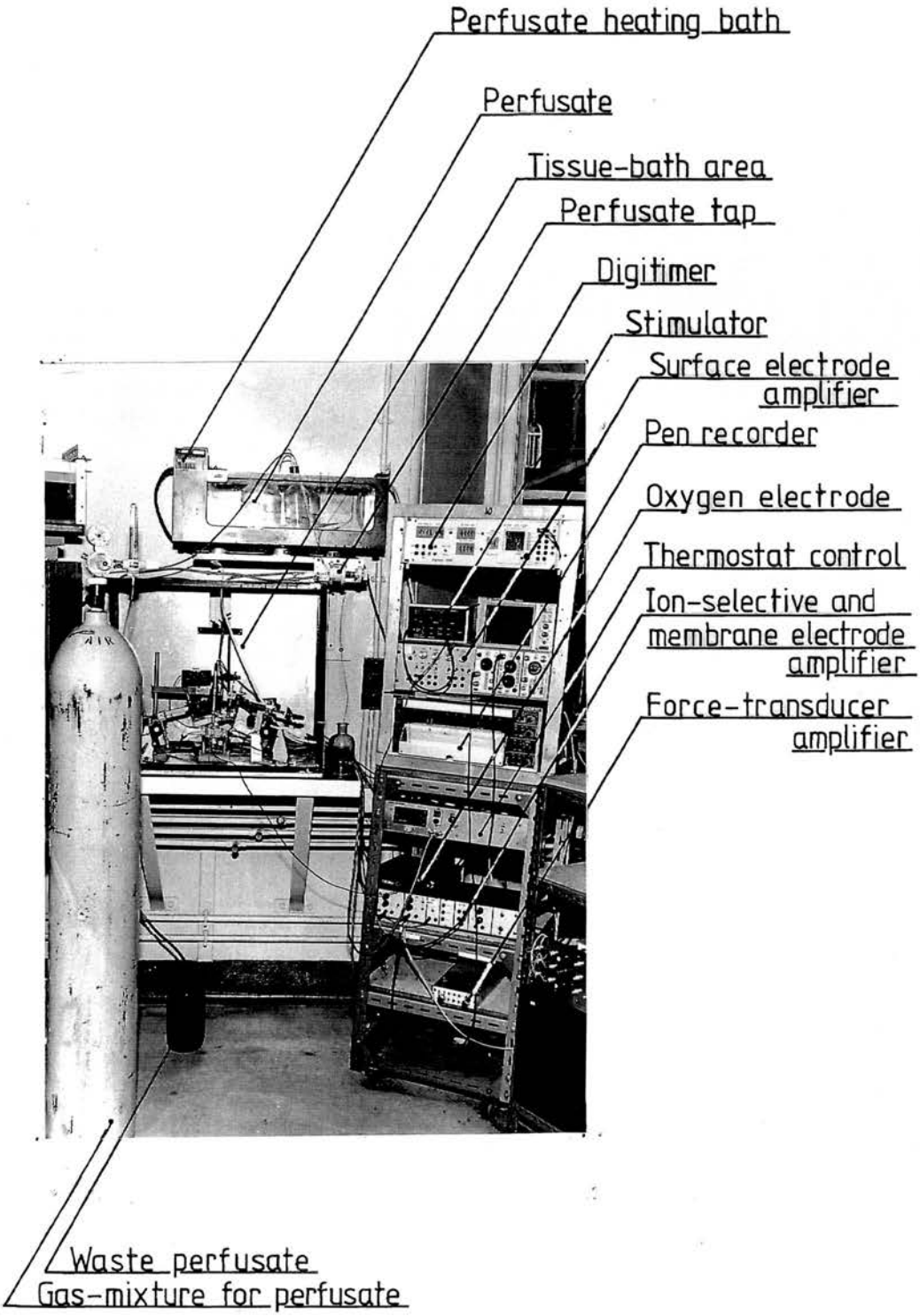
Photograph of bath area and amplifiers for measuring, membrane potential, intracellular ion activities and tension in isolated heart preparations.



Tissue-Bath Area

FIGURE 2.3

Photograph of the general apparatus used for measuring tension, membrane potential and intracellular ion activities in isolated heart preparations. The lid of the Faraday cage has been raised to view the interior.



The Apparatus.

solution. The bath was usually maintained at 35°C ±1.0 by a thermocouple at the bath entrance which controlled a heating element surrounding the glass tube which carried solution into the chamber. An eight way tap was used to change the supply of solutions (similar to a design of Partridge and Thomas, 1975). Solutions were oxygenated with either 100% O_2 or air bubbled directly into the solution with a fish tank pump for normoxic experiments (methods of producing anoxia will be discussed later in this chapter). Solutions were heated to 35°C in a water bath (Fig.2.3) and fed by gravity and gas pressure in the reservoir bottle to the eight way tap by polythene or stainless steel tubing. The metal tubing reduced any diffusion of CO_2 from HCO_3^-/CO_2 solutions or of O_2 into hypoxic solutions. The bath perfusion rate was approximately 10 bath volumes/min and the solution exchange time (time taken for a solution change) as measured using the response of a pH sensitive microelectrode as an indicator was 90% complete in 18-21 sec.

Microelectrodes are prone to electrical interference, that is electrical " noise" can obscure the signal to be measured. In general interference is greatest with high resistance electrodes. Therefore the whole apparatus (see Fig.2.2) except the water bath and eight way tap was enclosed in a Faraday cage which was earthed to the metal conduit carrying the electricity supply. The preparation was illuminated from above and behind using fibre-optic light guides from a light source (Fort EF 150S light unit, France) outside the Faraday cage which minimized electrical noise.

A horizontally mounted microscope (Kyoma 800450, Tokyo) was used to view the preparation through a hole cut in the Faraday cage, the microscope was also earthed.

Stimulating electrodes were placed on either side of

the preparation to check the viability, by stimulating an action potential or eliciting twitch contractions, stimulating electrodes were placed on either side of the preparation. The electrodes were connected to a Digitimer stimulator and pulse generator (Digitimer U.K.).

Oxygen tension was continuously measured in the bath fluid during some experiments using an O_2 electrode (Model 102, Instech Laboratories, USA.). See later for detail of electrode membrane installation and calibration.

(2) FORCE TRANSDUCER

The force transducer which measured tension in the preparation was constructed from piezo-resistive elements (Akers AE801, Aksjeselskapet Microelectronic, Horten, Norway). These elements were composed of a silicon beam (~5mm long, 2mm wide and 0.1mm thick) with planar piezo-resistive surfaces on either side of the beam. The beam was attached to a supporting 'head' to which electrical connections were made. A fine glass capillary tube with the end tapered to form a hook was glued to the beam, the hook was passed through the loop of thread tied to the preparation. On deflecting the beam the diffused resistors on the beam changed resistance, one decreasing, one increasing. This was combined with another beam element providing two passive resistors to form the other two arms of a full Wheatstone Bridge circuit (Fig.2.4). The whole assembly was housed in a brass tube, a perspex tube housed the active beam and its supportive head which was held in place with small screws. The beams were powered by a stabilised 6V DC supply from a Bryans DC bridge amplifier (Model 40550), which also provided preamplification and bridge balance. The voltage output of the force trancsducer was linear over the range of

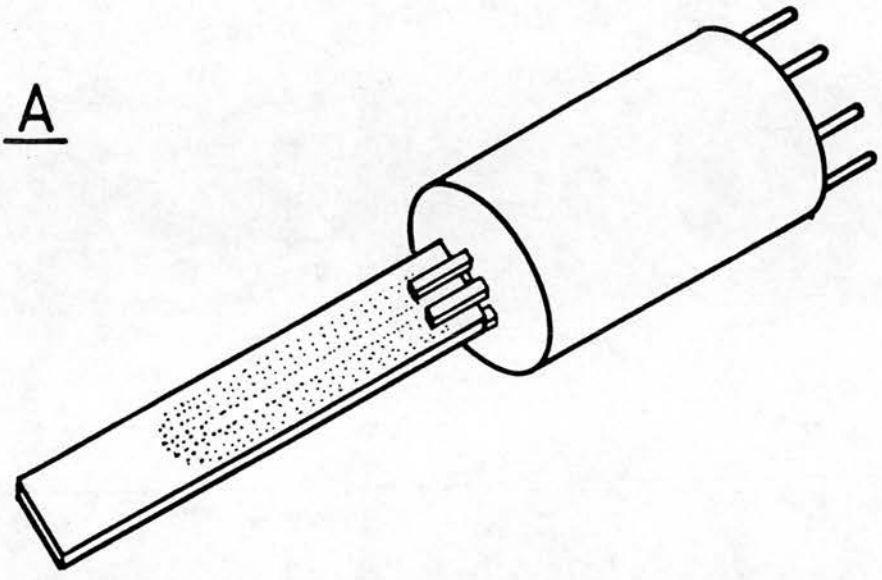
FIGURE 2.4A

Shows a diagram of the force transducer (approx. x30). A glass beam was glued to the silicon beam and it was to this glass rod that preparations were tied. Contraction of the muscle bent the beam and the changes in resistance of the beam were proportional to force.

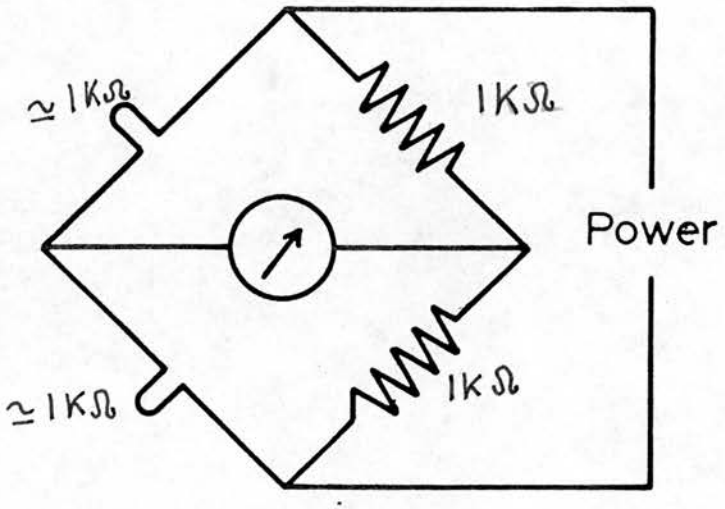
FIGURE 2.4B

A circuit diagram of a full wheatstone bridge circuit, used in the force transducer.

A



B



forces measured (Fig.2.5). Preparations were stretched to a degree that provided maximal twitch tensions. The force transducer output was usually recorded on one channel of a pen recorder (Bryans BS314). However in some experiments an Electromed (Type MX216) chart recorder was used because the Bryans recorder had too slow a response time to record twitch tension accurately but was adequate for observations of contracture development.

(3) MICROELECTRODES

Membrane potentials were measured with conventional 3M KCl-filled glass microelectrodes. These microelectrodes were pulled from lengths of 1.2mm i.d. 2.0mm o.d. borosilicate capillary tubing containing a glass filament (Clark Electromedical Instruments, Reading, U.K.). They were filled with 3M KCl and had resistances between 10 and 25MΩ.

Ion sensitive microelectrodes were fabricated from silanized micropipettes*. These were initially back-filled with aqueous solution by passing a fine plastic tube along the barrel to as near the tip as possible and injecting a suitable solution.The microelectrode tip was then filled with a small column (c.200 um) of liquid ion exchanger by exerting negative pressure (Fig.2.6). Tips were <1um in diameter. Estimates of their response time were limited by the solution exchange time in the bath, which was 90% complete in 18 to 21 s. For any drug used in the experiments, the possibility of interference with the ion selective resin was checked. Extracellular recordings were made using ion sensitive electrodes constructed in the same way except that once filled the tips were broken back (tip diameter 5-20 um) to produce a blunt electrode which was unable to penetrate the tissue.

* Silanised by exposure at 200°C to Tri-n-butyl chlorosilane (Lancaster Synthesis, England).

FIGURE 2.5

A calibration curve for the force transducer at three sensitivities. Note the response of the force transducer is linear over the range of forces measured (lines drawn by eye).

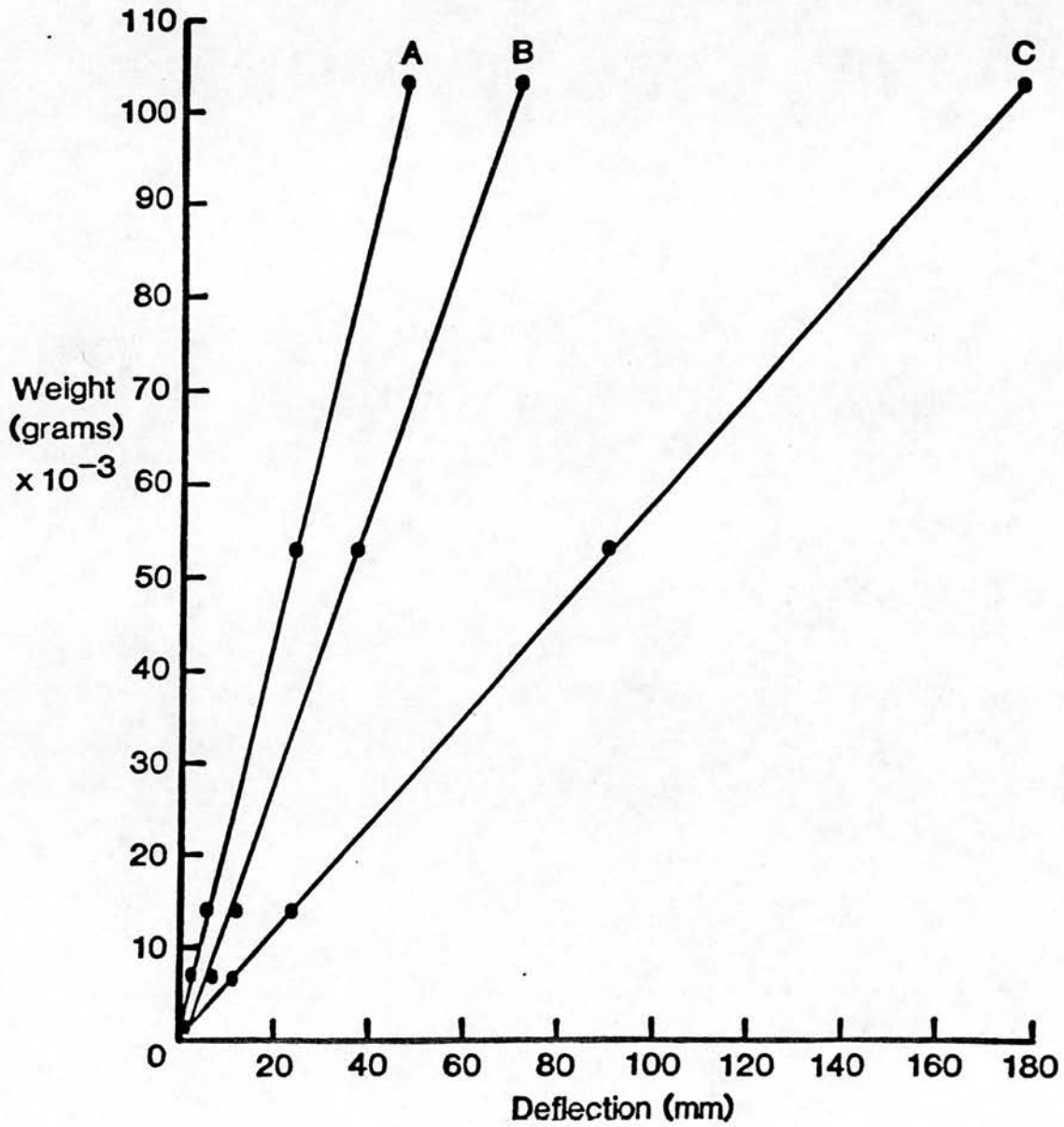
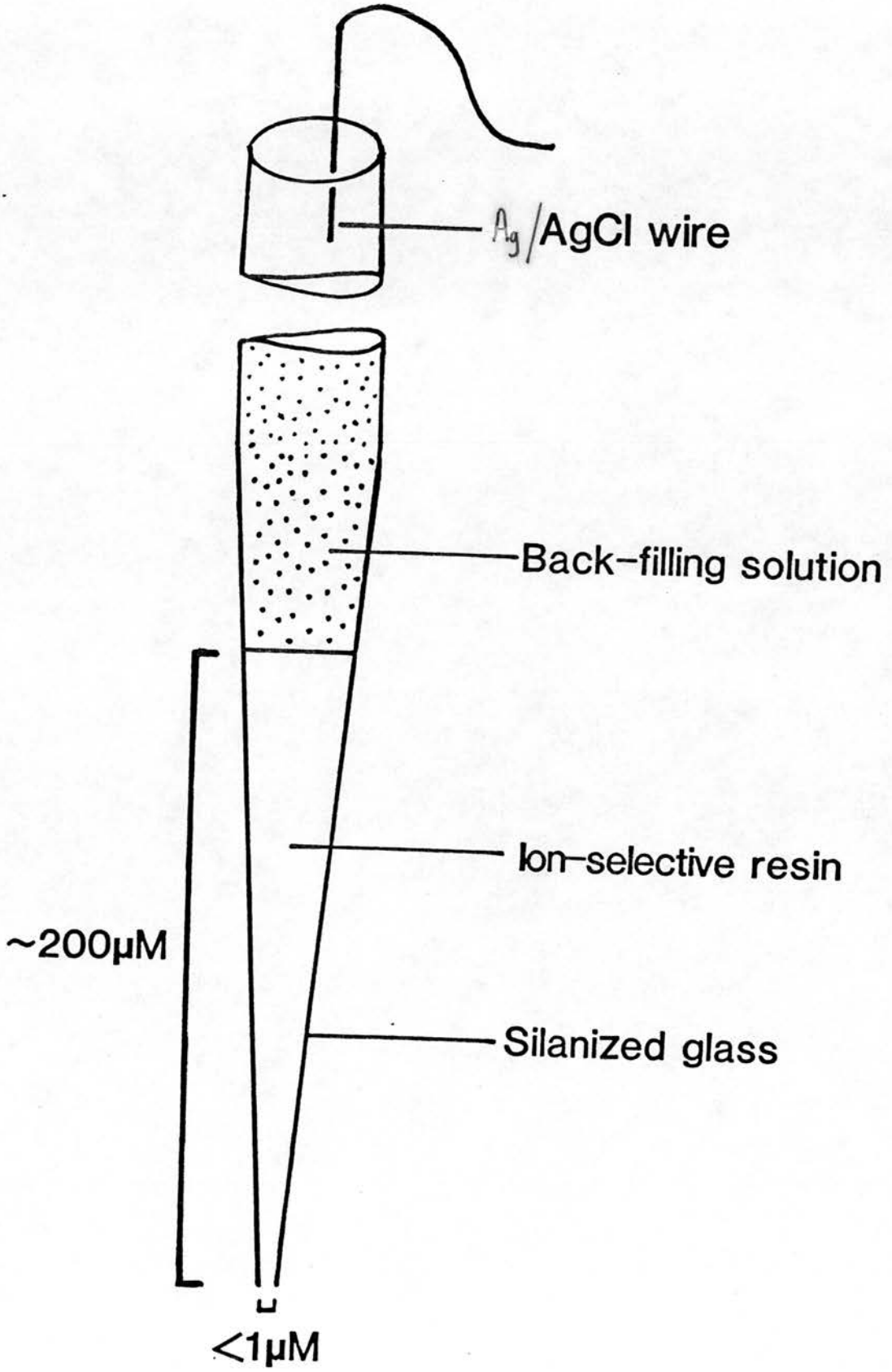

110
100
90
80
70
60
50
40
30
20
10
0
Weight
(grams)
x 10⁻³
A
B
C
20
40
60
80
100
120
140
160
180
Deflection (mm)

FIGURE 2.6

A schematic diagram of a typical ion-selective microelectrode, the values for the size of the column of ion-selective resin and the tip of the electrode are approximate.


Ag/AgCl wire
Back-filling solution
Ion-selective resin
~200μM
Silanized glass
<1μM

Potassium Microelectrodes

The potassium-sensitive microelectrodes were filled with either Corning 44731 ion exchanger (W.P.I, New Haven, U.S.A.) or with Fluka K^+ cocktail (Fluka, Buchs, Switzerland). The silanized micropipettes were filled on the day they were used. They were first back-filled with a solution of 100mM potassium chloride then a column (~200um) of the potassium selective resin was drawn by suction into the tip of the microelectrode.

Sodium Microelectrodes

The sodium sensitive microelectrodes were produced in the same way as the potassium microelectrodes except that they were back-filled with 100mM sodium chloride and 100mM sodium citrate then a column of sodium ionophore (ETH 227, Fluka, Switzerland.) was drawn by suction into the tip.

pH Microelectrodes

The fabrication of the pH electrodes was essentially the same as for Na^+ sensitive electrodes except that pH microelectrodes had neutral H^+ carrier (Fluka, Switzerland) drawn into the tip after back-filling.

(4) SIGNAL RECORDING

Conventional 3M KCl microelectrodes and the ion selective microelectrodes had chlorided silver wires inserted into the barrel of the electrode end. The wires were chlorided by electrolysis in HCl. The chlorided wire forms a stable electrical contact between the filling solution of the microelectrode and the signal

recording system.

The bath electrode (reference electrode) was made by injecting heated Agar (4% dissolved in Tyrode solution) into a glass tube (o.d. 2.0mm) about 30mm in length into which was inserted a chlorided silver wire. The bath was grounded with an earthed chlorided silver wire which dipped into the bath solution. The KCl electrode voltage with respect to the reference electrode was displayed on one channel of a pen recorder (Bryans BS314) or on an oscilloscope (Tektronix 5113 Dual Beam). Signals from the bath electrode and the KCl electrode were pre-amplified using RCA CA 3140 operational amplifiers* wired for unity gain. The signal from the bath electrode was also passed through a CA 3140 pre-amplifier to ensure that any power supply changes or electrical interference would affect each amplifier in the same way and would therefore be cancelled out at the differential input to the recording devices.

The conventional electrode resistance was measured using a triangle wave form generator (Thomas 1978). Triangular voltage signals were fed to a capacitor at the pre-amplifier input. This, together with the resistance of the microelectrode transformed the triangular waves into square waves at the amplifier input. The resistance of the microelectrode was proportional to the height of the square wave and the system was calibrated using known resistors.

Ion sensitive microelectrode potentials were measured using an operational amplifier (Analog Devices 311J) wired for unity gain with a large input impedance ($\sim 10^{14}\Omega$) which makes it suitable for ion sensitive microelectrodes.

The difference between the KCl and the ion-sensitive microelectrode signal, gave the intracellular activity of the ion being measured. The subtraction of signals took

* Bias Current = 5pA, Impedance = $10^{12}\Omega$ c/f impedance of electrode plus cell = $< 10^{8}\Omega$
FET input.

place at the input to the chart recorder.

(5) THE SUBTRACTION PROCESS AND CALIBRATION OF MICROELECTRODES

A diagram of the circuit is shown in Figure 2.7. The KCl microelectrode serves as the differential input to the ion selective electrode. Where an extracellular microelectrode was used, usually to measure extracellular pH, the bath electrode signal was subtracted from the extracellular pH signal at the input to the chart recorder.

The calibration procedure for the three types of ion sensitive electrode used during the experiments was performed at the beginning and end of experiments as a means of checking that there had been no change in electrode characteristics during the course of the experiment.

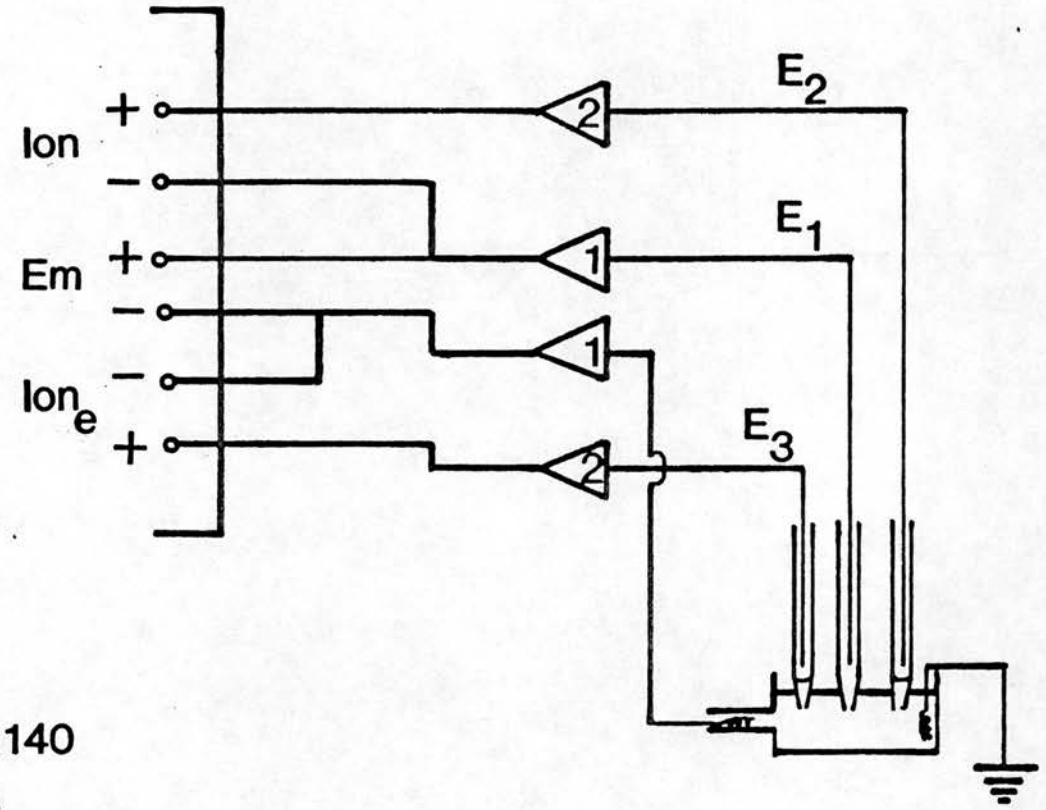
At the beginning of each experiment the preparation and microelectrodes were set up in the bath and left to stabilize for at least 10 minutes.

Potassium Microelectrodes

The calibration of potassium microelectrodes required particular care due to the logarithmic relationship between the activity of an ion and the voltage output of the electrode. Thus at physiological levels of intracellular potassium, relatively large changes in potassium are associated with quite small changes in the voltage output of the microelectrode. For each calibration curve a linear regression was plotted (on log-linear coordinates) to check that the electrode calibration intercepted the x- axis at approximately 6mM which was the $[K^+]$ in normal Tyrode. By using the same

FIGURE 2.7

A schematic diagram of the electrical connections from the microelectrodes to the recording apparatus. The "high" side of the conventional microelectrode which measures membrane potential also serves as the "low" side of the intracellular ion-selective electrode input. The bath electrode signal was subtracted from the extracellular ion-sensitive microelectrode input (E3).



1=CA 3140

2=311J

E_1 = KCl Microelectrode

E_2 = Ion-sensitive Microelectrode

E_3 = Extracellular Microelectrode

Ion = intracellular ion

E_m = membrane potential

Ion_e = extracellular ion

linear regression it was possible to calculate a^i_k directly from the intracellular measurements of the ion sensitive electrode potentials. To check that each impalement was successful the perfusing solution was briefly switched to 12mM [K^+] Tyrode. This produces a slight depolarization in the preparation. The K^+-sensitive microelectrode signal had the membrane potential (Em) signal subtracted from it in order to obtain a value for a^i_k. If both electrodes were intracellular no depolarisation was seen on the a^i_k trace.

K^+ microelectrodes were calibrated using 70, 129, 141 and 151mM K^+ calibration solutions. (Fig.2.8 for calibration and Fig. 2.9 for penetration procedure).

Sodium Microelectrodes

Sodium microelectrodes were calibrated with calibrating solutions containing 14, 8, 5, and 3mM [Na^+]. Potassium was used as a sodium substitute in these solutions (Fig.2.10).

pH Microelectrodes

The calibration procedure for pH microelectrodes was to expose the microelectrode to solutions of pH 7.4 (normal Tyrode solution), 7.0 and 6.4 with a solution of pH 8.0 being added for low temperature experiments. There was an average response of 58mV for a tenfold change in hydrogen ion activity. (Fig. 2.11 for calibration and Fig. 2.12 for penetration procedure).

FIGURE 2.8

The mean (filled circles) $\pm$ s.d. (n=8) and an individual calibration curve (crosses) for valinomycin-filled ion-selective microelectrodes (A) and Corning-filled ion-selective microelectrodes (B) are shown (n=22). The lines are linear regression lines calculated from the four calibration points for each graph.

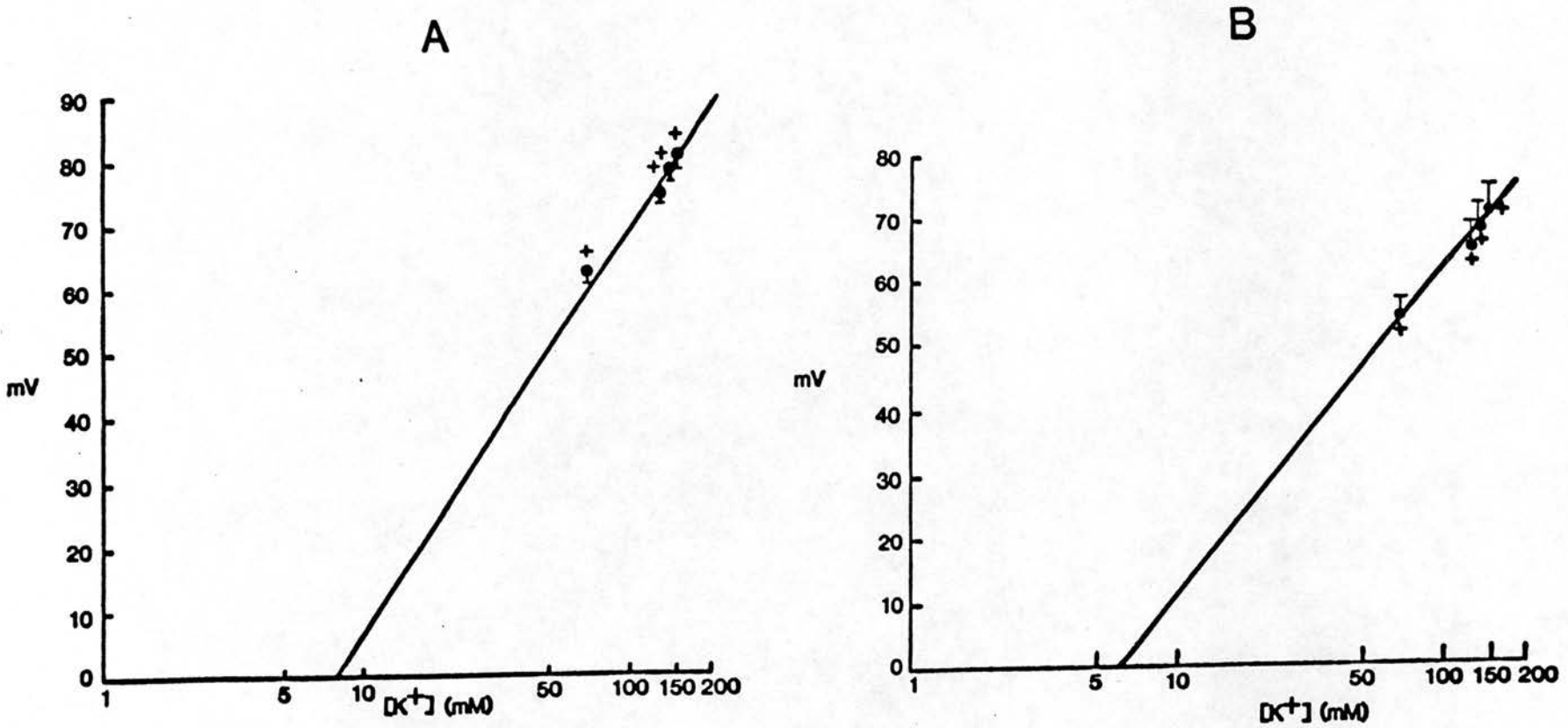

A
B
mV
mV
90
80
70
60
50
40
30
20
10
0
1
5
10
50
100
150
200
[K+] (mM)

FIGURE 2.9

The penetration procedure for a Corning K^+-sensitive microelectrode in ferret papillary muscle. Arrow A shows the K^+-sensitive microelectrode already intracellular. Arrow B shows the membrane electrode intracellular (the ion-selective microelectrode trace moving in an equal and opposite direction). Arrows C, D, E, and F show points where the K^+ microelectrode is intracellular. The spikes on the trace associated with arrows E and F are as a result of stimulating the preparation briefly to induce an action potential to ensure that the K^+ electrode was intracellular.

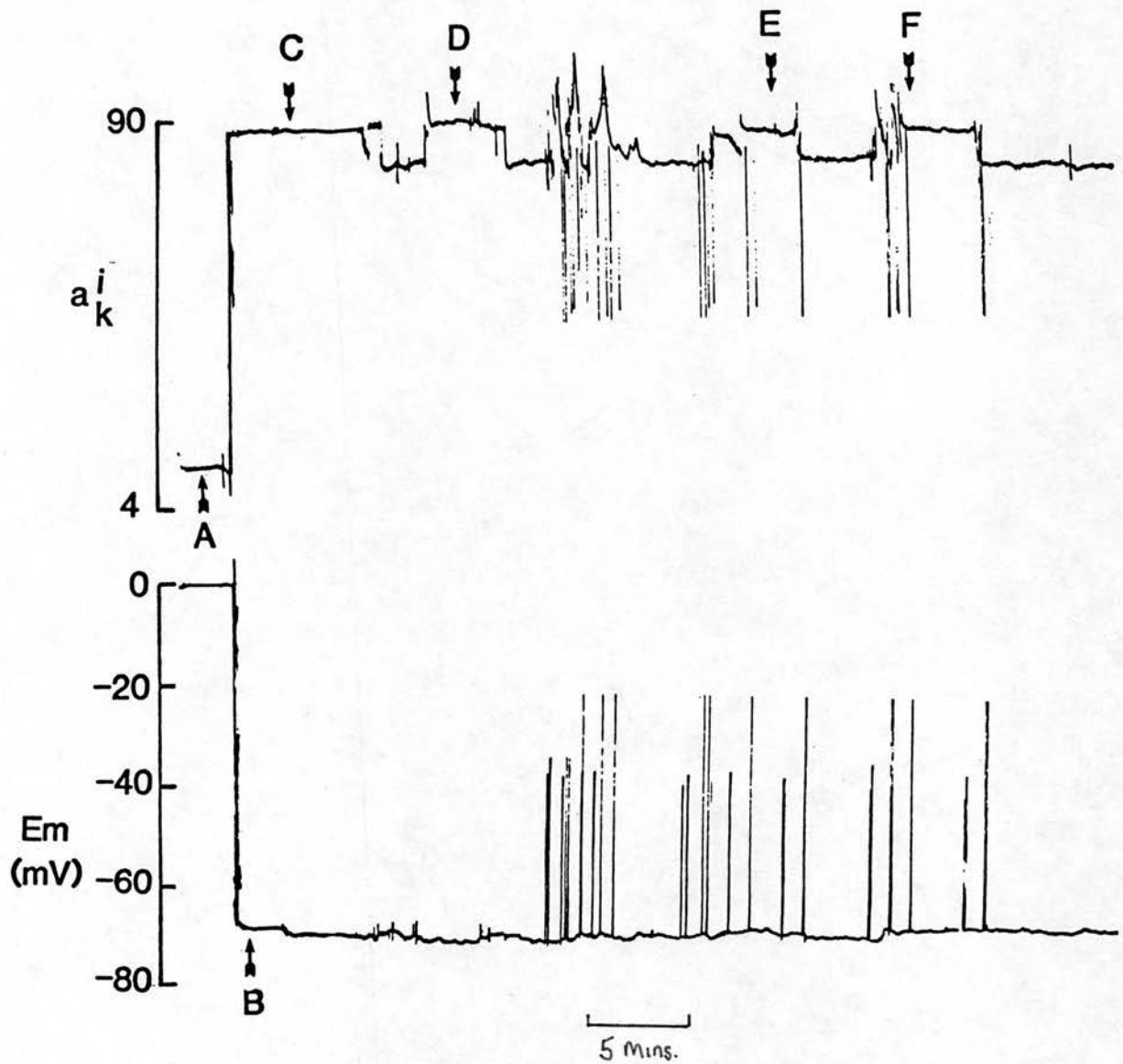

C
D
E
F
90
a_k^i
4
A
0
-20
-40
Em
(mV)
-60
-80
B
5 mins.

FIGURE 2.10

The mean $\pm$ s.d. (n=6) and an individual calibration curve for sodium-selective microelectrodes (line drawn by eye).

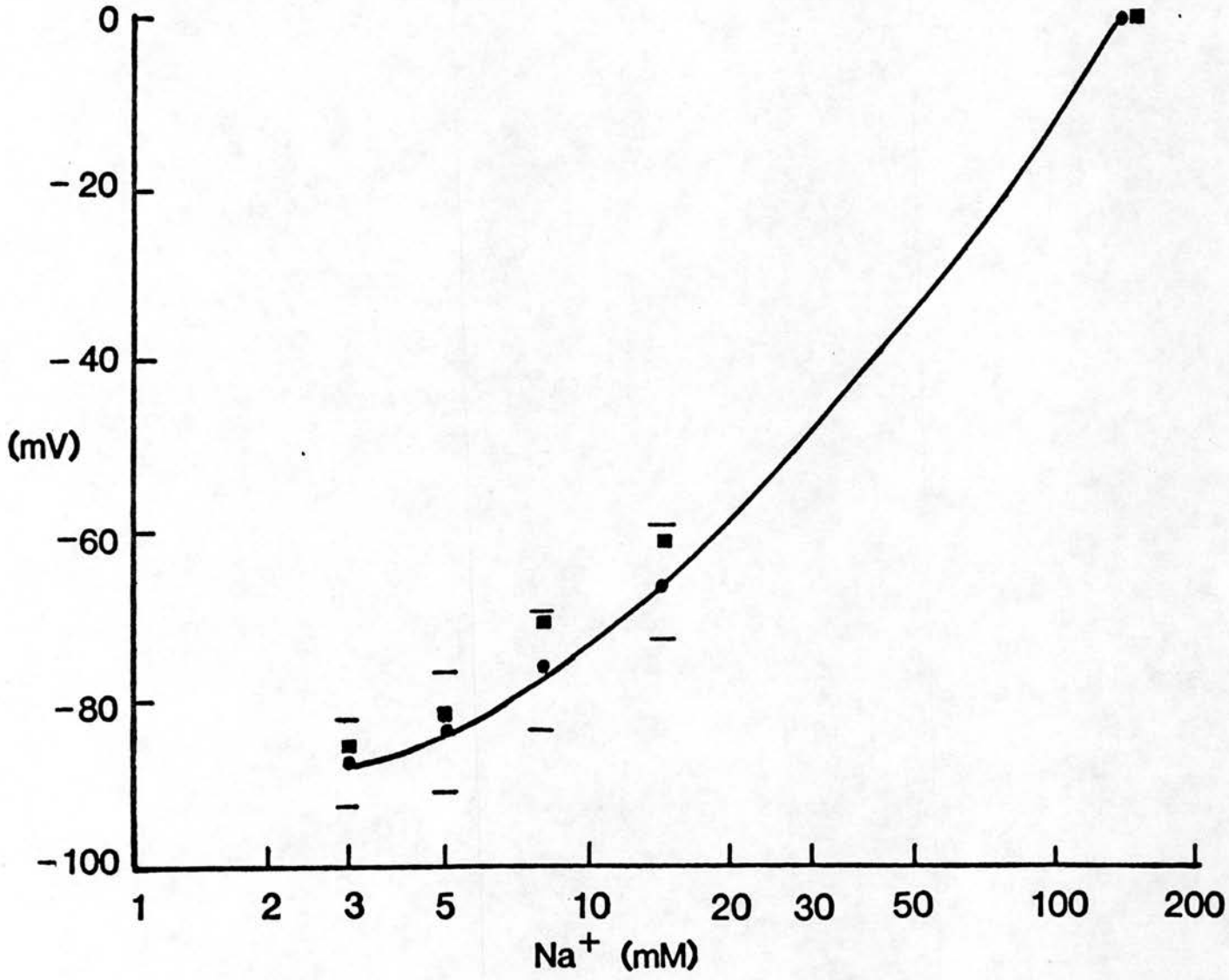

0
-20
-40
(mV)
-60
-80
-100
1
2
3
5
10
20
30
50
100
200
Na+ (mM)

FIGURE 2.11

This shows a mean (n=20)± s.d. (filled squares) and a single calibration curve (filled circles) for pH-sensitive microelectrodes (line drawn by eye).

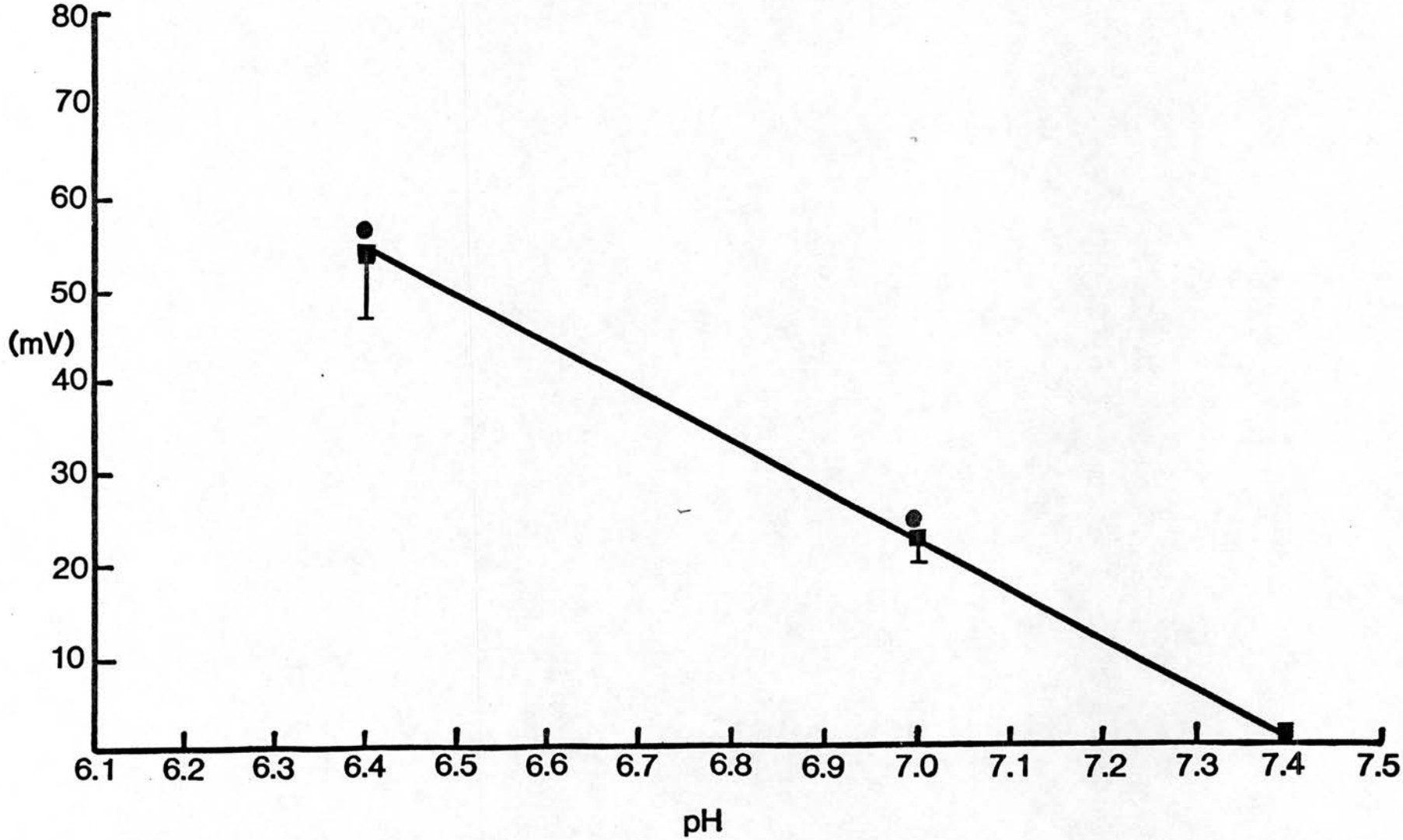
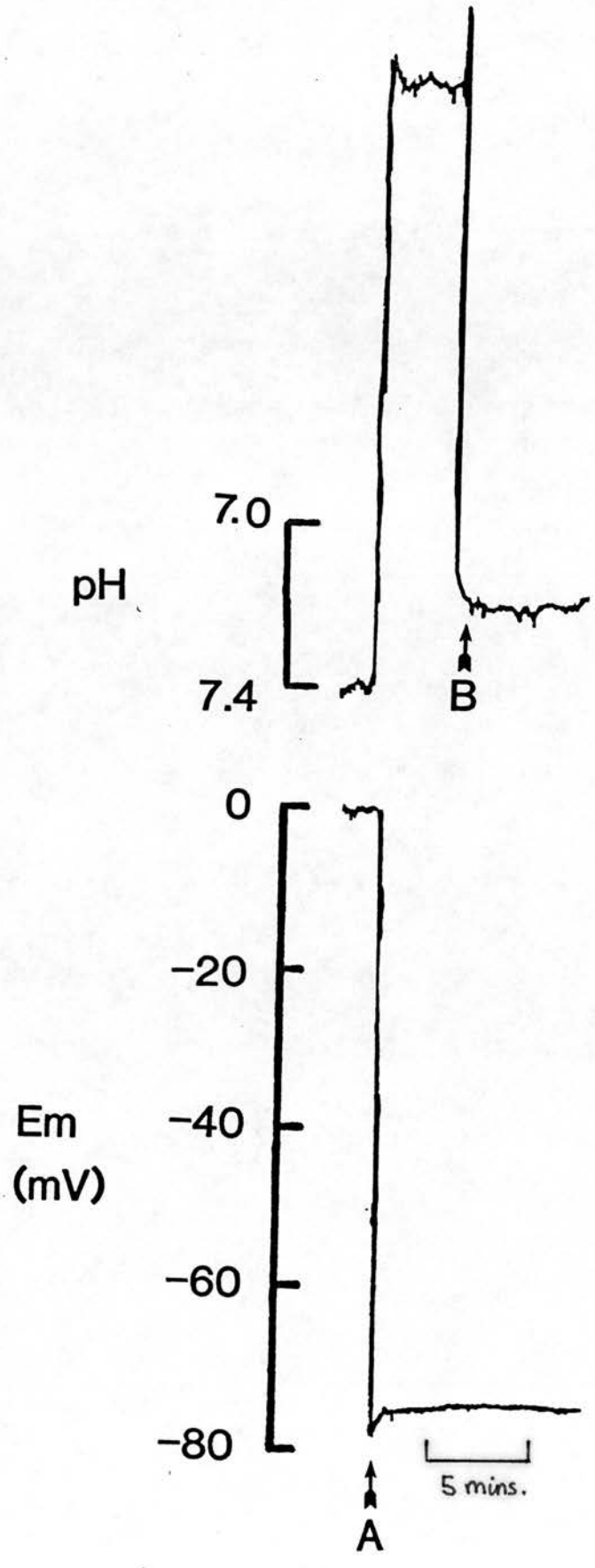

80
70
60
50
(mV)
40
30
20
10
6.1
6.2
6.3
6.4
6.5
6.6
6.7
6.8
6.9
7.0
7.1
7.2
7.3
7.4
7.5
pH

FIGURE 2.12

The penetration procedure for pH-sensitive microelectrodes in a sheep Purkinje fibre. The membrane potential microelectrode penetrates a cell at (A), the pH trace moving in an equal but opposite direction to the Em trace, followed by penetration with the ion selective electrode at (B).


7.0
pH
7.4
B
0
-20
Em
(mV)
-40
-60
-80
5 mins.
A

Testing The Subtraction Procedure

The membrane potential (Em) signal must be subtracted from the ion-sensitive microelectrode's total potential. Therefore, in order that the correct intracellular ion activity is measured it is essential that both microelectrodes measure the same membrane potential, and possibly more importantly that they measure the same change in membrane potential. In this situation the membrane electrode should register a change of Em and, if the subtraction is accurate, the ion-selective microelectrode should not respond. Addition of 12mM $[K^+]$ Tyrode as a method for checking electrode impalement (see page 51) could also be used to test the subtraction procedure. If the subtraction was adequate exposing the intracellular electrodes to 12mM $[K^+]$ would produce no change in the a^i_K signal recorded (a change of less than 1mV was considered acceptable for these experiments). It is difficult to obtain stable recordings when the inter-electrode distance is <100uM. However the cells of the Purkinje fibres and of the papillary muscles are well coupled electrically such that the two electrodes although in different cells measure the same Em under most conditions. It had to be assumed that if the cells became uncoupled, as a result of any of the experimental protocols, that the microelectrodes would measure the same change in Em.

Ion Activities

The activity (a) of an ion (I) is related to its concentration (c) by the equation

$$a_i = f \times c_i$$

where f is the activity coefficient* of i. The activity of a substance can be thought of as the effective free concentration. For electrolytes in very dilute solution,

* f = 0.74 for K^+
= 0.75 for Na^+

activity is nearly equal to the molar concentration. The intracellular level of an ion species which is passively distributed across the cell membrane is determined by the extracellular activity of this ion and the membrane potential. The equilibrium potential of the ion is therefore the same as the membrane potential.

Ion-selective microelectrodes measure intracellular ion activities, these may be converted to the equivalent concentration of ions using the activity coefficient for the ion in question (Robinson and Stokes,1968) if it is assumed that the intracellular activity coefficient of the ion is the same as that in the extracellular solution.

(6) TEMPERATURE CONTROL

Some experiments were performed at low temperature ($22^{o}C$). To achieve a rapid change of temperature, reservoir bottles of solution were pre-cooled in a cooling water bath (Camlab DLK 500, Cooling Unit, Cambridge, U.K.). The bath temperature feedback circuit was altered from $35^{o}C$ to $22^{o}C$. The solutions flowed from the reservoir bottles to the bath through a stainless-steel tube which was encased in plastic tubing through which cold water was pumped using a peristaltic pump (Gilson, Minipuls 2.). Using this cooling system the temperature change from $35^{o}C$ to $22^{o}C$ was 90% complete in 4.5 minutes.

(7) CALIBRATION OF THE OXYGEN ELECTRODE

Oxygen tension in the experimental chamber was constantly measured in some experiments using an oxygen electrode which was connected to one of the channels of the pen recorder. The oxygen electrode was calibrated at

the beginning of each experiment. A zero point was established by exposing the electrode to Tyrode solution containing sodium dithionite which chemically removes oxygen (sodium dithionite is a powerful reducing agent). After a zero point had been established the solution was changed to normal Tyrode solution bubbled with 21% O_2 (approximate PO_2 = 150mmHg) and the amplifier sensitivity was altered to give a suitable sized deflection on the chart recorder trace, the electrode was then tested with Tyrode solution bubbled with 10% O_2. The response of the electrode was linear (Fig.2.13).

(8) SOLUTIONS

The normal Tyrode solutions were composed of Analar grade chemicals. The normal Tyrode contained (mM) Na,140; K,6; Ca,2; Mg,1; Cl,147; Glucose,10; HEPES (N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid),10; and was titrated with NaOH to give a pH of 7.40 $\pm$ 0.05 at 35°C. The solution was equilibrated with 100% O_2 or with 100% N_2 for hypoxic solution. When experiments were performed at room temperature normal Tyrode was titrated to give a pH of 7.4 $\pm$ 0.05 at 22°C. The pH of the solutions was measured with a pH meter (Phillips PW9409 Digital Meter U.K.).

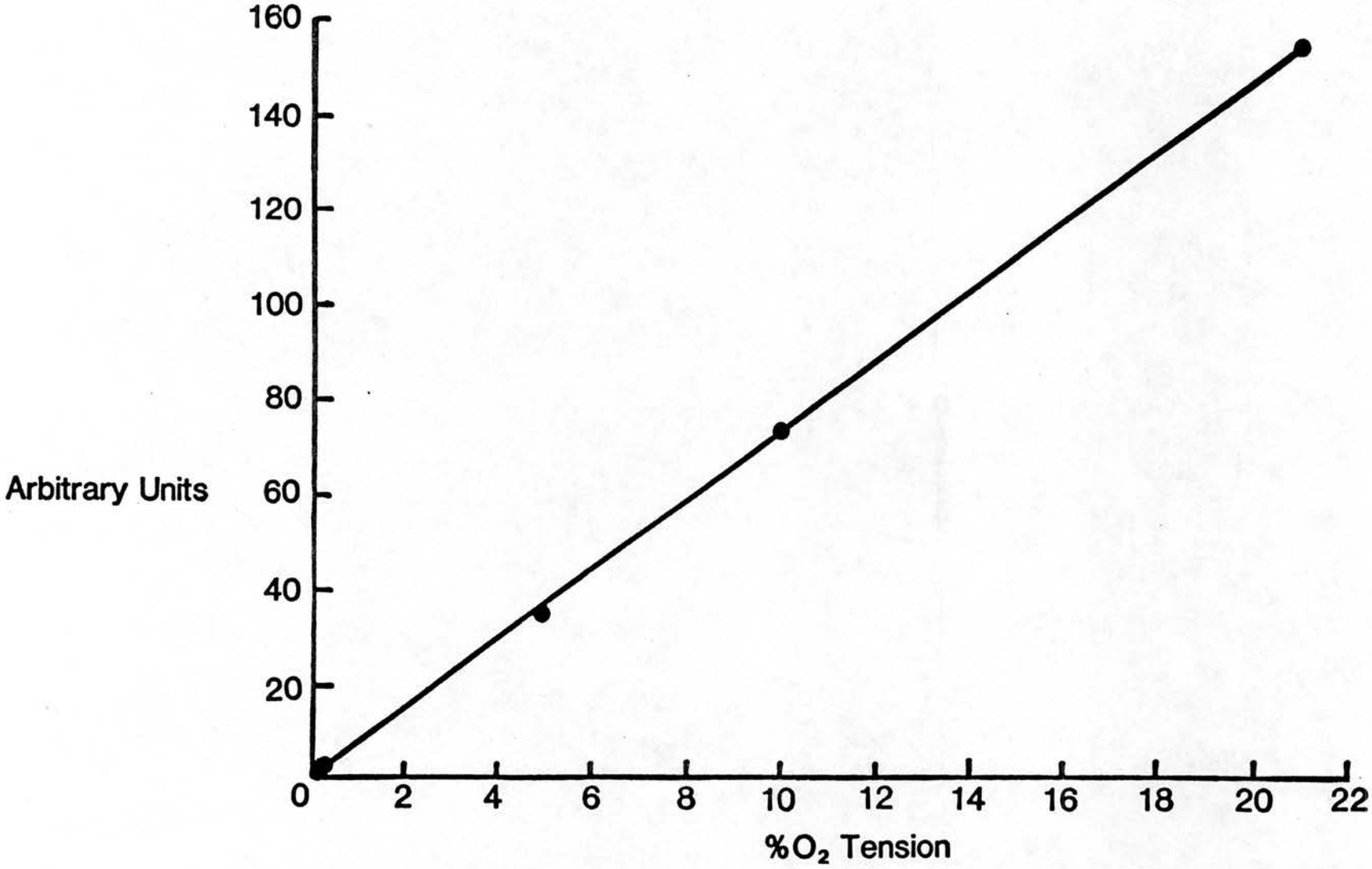
Bicarbonate buffered solutions were similar except that the HEPES was replaced by sucrose (10mM). They contained 24mM $NaHCO_3$, so the NaCl was reduced to 116mM. Bicarbonate solutions were equilibrated with 95% O_2, 5% CO_2 or with 95% N_2, 5% CO_2 for hypoxic solution.

The cardioplegic solution was a modified St. Thomas' solution (mM) NaCl, 100; KCl, 20; $MgCl_2$, 16; Procaine, 0.5; $CaCl_2$, 2; Na_2HPO_4, 1.92; NaH_2PO_4, 6.74.

Saponin (25ugml^{-1}, Sigma, U.K.) was added directly to pre-gassed solution and bubbled very gently with O_2 to

FIGURE 2.13

The calibration procedure for the oxygen electrode. The electrode zero was set using sodium dithionite (approximately 20mM) to remove all O_2 from the solution in the bath. The electrode was calibrated with atmospheric O_2 (150 mmHg), 10% O_2/90% N_2 gas mixture and 5% O_2/95% N_2 gas mixture, these were bubbled into normal Tyrode (line drawn by eye).


160
140
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
22
Arbitrary Units
%O2 Tension

prevent foaming. Strophanthidin (Boehringer Mannheim) was dissolved in 50% ethanol: 50% water (v:v) to produce a stock solution of $2x10^{-2}$M. Thus with a concentration of $2x10^{-5}$M strophanthidin in the Tyrode solution the amount of ethanol was less than 0.1%.

Amiloride (1mM, Sigma, U.K.) was added directly to warmed Tyrode and stirred well before use. SITS (4-acetamido-4-isothiocyanatostilbene-2, 2'-disulphonic acid disodium salt), (100uM, Sigma, U.K.) was added directly to solutions, protected from light and used immediately. Sodium cyanide was added directly to normal Tyrode solution.

To produce a chemically anoxic solution, sodium dithionite (0.5mM) was added directly to Tyrode solution. 50uL/L of resazurin dye (Sigma, U.K.) was added as a 1% aqueous solution which acted as an indicator of the level of O_2 in the solution (the dye changes from blue to colourless on removal of oxygen).

Sodium cinnamate was made up as a 100mM stock solution in 130mM NaOH. 50ml of this stock solution was added to 1 litre of normal Tyrode (with the Na level suitably adjusted) to produce a 5mM cinnamate solution.

Solutions of pH 6.4 and 8.4 were made up using the buffers PIPES (Piperazine-N,N'-bis[2-ethane-sulphonic acid]) and TAPS (Tris[hydroxymethyl]methylaminopropane-sulphonic acid) respectively instead of HEPES.

When experiments were performed with high HEPES levels (high levels of extracellular buffering) the normal [HEPES] Tyrode included 60 mM sucrose to ensure that the osmolarity of the two solutions was the same.

(9) HISTOLOGY

Electron micrographs were produced for experiments on prolonged exposure to hypoxia (1.5 hours) in sheep Purkinje fibres. The fixation and staining protocol was as follows:-

(1) Primary fixation with 2% glutaraldehyde in 0.13M PIPES buffer, pH 7.4 for 2 hours at room temperature.

(2) Brief wash in 0.13M PIPES buffer, pH 7.4.

(3) Post fixed with freshly prepared 1.5% OsO_4 and 2.5% potassium ferrocyanide in 0.13M PIPES buffer, pH 7.4 for 2 hours at room temperature (Russell and Burguet, 1977).

(4) Brief wash in 0.13M PIPES followed by distilled H_2O.

(5) Overnight in 10% ethanol at 4^oC.

(6) Dehydrated in graded ethanol (25%, 40%, 55%, 70%, 85%, 90%, 95% and 100%) 10 minutes each at room temperature.

(7) 3 changes of 100% ethanol, 30 minutes each. 2 changes of 100% ethanol and Spurr resin (1:2), 1 hour each.

(8) Specimens embedded in Spurr resin.

(10) Thin sections were cut with a Porter-Blum MT2B ultramicrotome using glass knives.

(11) Sections were mounted on single-hole type-1000 specimen grids which were covered with Pioloform support film.

(12) Sections were stained with aqueous uranyl acetate followed by lead citrate (Reynolds, 1963), then examined in an AE1EM6B microscope.

(10) THE PRODUCTION OF HYPOXIC SOLUTIONS

During the majority of the experiments it was necessary to produce solutions with oxygen tensions of less than 10mmHg (see Results). It proved to be quite difficult to remove and exclude O_2 from the experimental

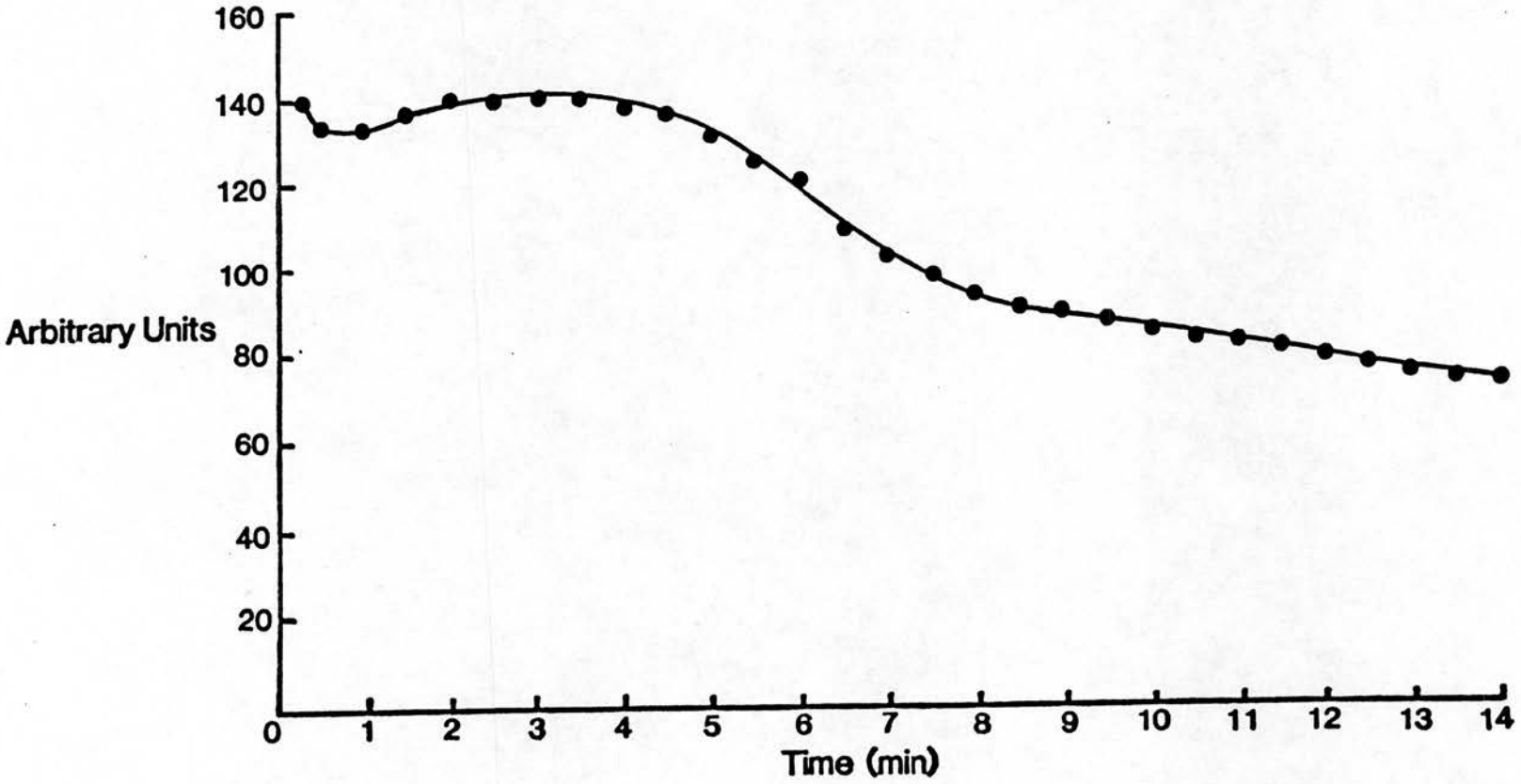
set-up used for these studies. It was also important, when changing from perfusate of one oxygen tension to another, that the time course of the change in partial pressure of oxygen be clearly known, to enable the changes in behaviour of the tissue to be separated from the dynamics of the perfusing apparatus. For these reasons the factors governing the partial pressure of oxygen in the bath and the dynamics of change from normoxic to hypoxic superfusates and vice versa were investigated.

Factors Affecting Partial Pressures Of O_2 Under Equilibrium Conditions

It was initially difficult to produce a stable and reproducible level of hypoxia in the bath. For example, when 250ml of perfusing medium had been bubbled vigorously for 5 minutes with 100% N_2 in an open vessel, a flow through the bath of 3.2ml/min (the flow rate used in these experiments) produced changes in O_2 partial pressure in the bath which were time related (Figure 2.14). This level of stability (during the first 5 minutes) would of course have been useless in the study of the effects of hypoxia. It was initially suspected that diffusion of atmospheric O_2 into the bath was the cause of this stability. Diffusion would have been largely via the uncovered surface of the perfusate in the bath and might perhaps be counteracted by increased rates of flow into the bath, carrying away "oxygen-contaminated" solution. It was therefore decided to investigate the effects of covering the bath and altering superfusate flow rate on O_2 partial pressure in the bath. The bath was perfused with a number of fixed flows of normoxic and hypoxic perfusates a) with the surface uncovered as normal,and b) with the whole surface of the

FIGURE 2.14

Approximately 250ml of normal Tyrode was vigorously bubbled with 100% N_2 in an open vessel. While bubbling was continued the solution was allowed to flow into the bath while continuously measuring the O_2 tension. The figure shows the subsequent changes in partial pressure of O_2 (PO_2) in arbitrary units with time (line drawn by eye)


160
140
120
100
80
60
40
20
Arbitrary Units
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (min)

bath covered with a microscope slide to prevent diffusion. O_2 tension was measured in arbitrary units because the O_2 electrode had not been fully calibrated, however zero on the arbitrary scale represented 0mmHg (PO_2). Oxygen tension in arbitrary units was read continuously after allowing 2 min for equilibration. The effects of varying flow and covering or uncovering the bath surface (Figure 2.15). It can be seen that neither covering the bath nor changing the flow rate materially affected the partial pressure of O_2 in the bath during hypoxia or normoxia. The problems of producing a stable and reproducible hypoxic perfusate were clearly not related to diffusion into the bath due to uncovered surfaces or low flow rates. I therefore turned my attention to the treatment of the perfusate before it entered the bath.

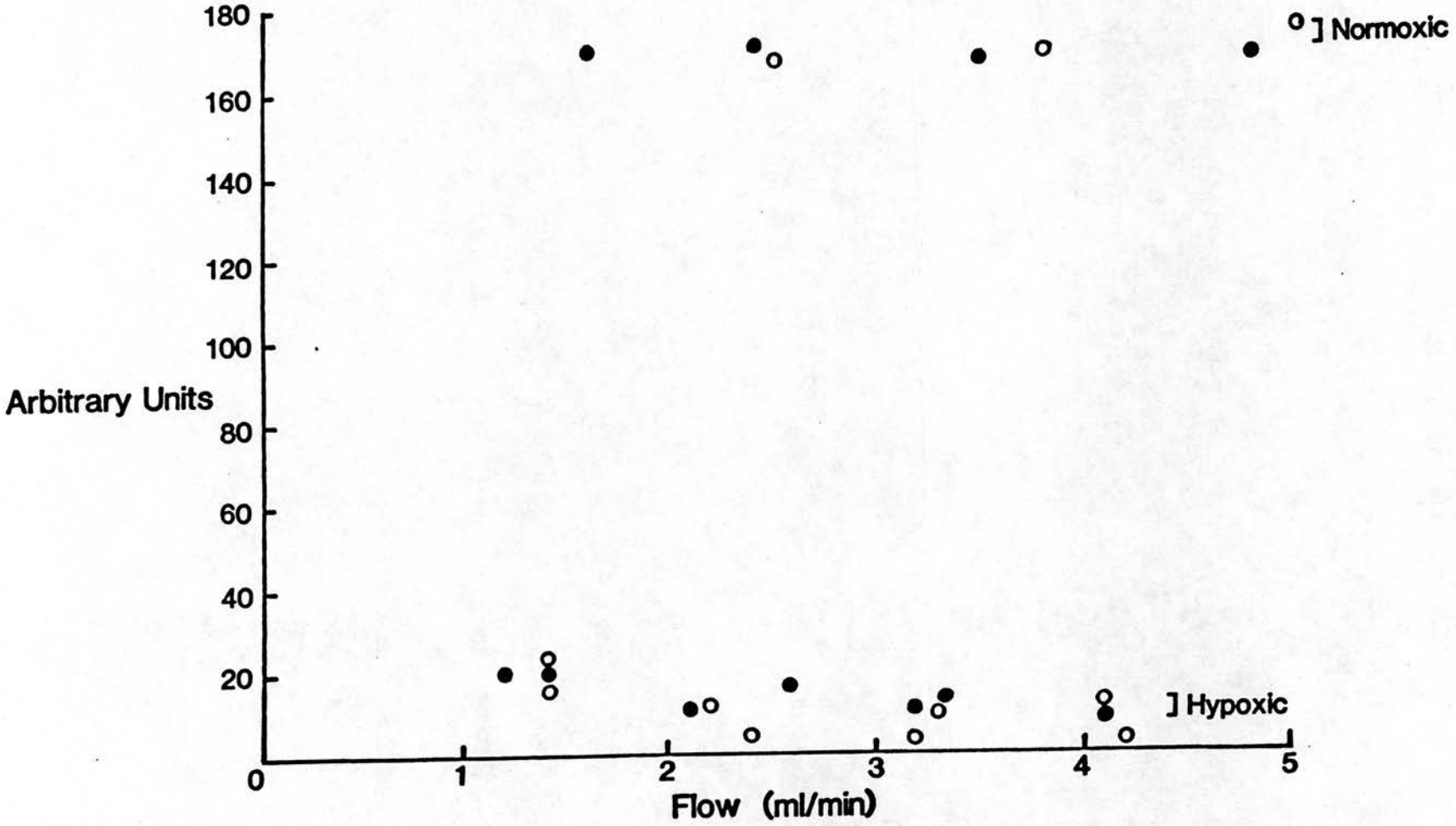
Production Of Hypoxic Perfusate

Movement of gas (O_2 and N_2 in this case) into or out of the superfusate is governed by Henry's Law, which states that "at equilibrium the amount of gas dissolved in a given volume of liquid at a given temperature is proportional to the partial pressure of that gas". The amount of gas dissolved is also proportional to the solubility of that gas.

The solubility of O_2 and N_2 in water at 30^oC is 2.6 and 1.15ml/100gm water respectively (Perry, 1974). Although this relationship is to the disadvantage of producing hypoxia, it cannot be changed and therefore the only way of producing solutions which were hypoxic appeared to be to increase partial pressure of N_2 and to reduce partial pressure of O_2 in contact with the superfusate. This was done by heating the Tyrode solution to 35^oC and then exposing it to low pressure

FIGURE 2.15

The effect of various flow rates on PO_2 measured in hypoxic and normoxic solution. The open circles represent the values obtained when the the bath was uncovered, the closed circles when the bath was covered with a glass coverslip. It can be seen that the partial pressure of O_2 in hypoxia is little affected by uncovering the bath and only slightly lowered in hypoxic solution by increased flow.


180
160
140
120
100
80
60
40
20
0
Arbitrary Units
1
2
3
4
5
Flow (ml/min)
] Normoxic
] Hypoxic

(approximately 100mmHg) for approximately 5 minutes. The solution was then immediately gassed vigorously with 100% N_2, in a reservoir bottle which excluded atmospheric O_2. The superfusate was allowed contact with the air for only the briefest possible time, Figure 2.16 shows the effect of bubbling hypoxic solution with atmospheric O_2. The procedure outlined above proved to be adequate for producing hypoxic solutions (<10mmHg). I then investigated the dynamic properties of the perfusing apparatus.

Dynamic Effects Of Changes In Perfusate

When a reliable and repeatable source of hypoxic perfusate had been established the dynamic effects of changing from normoxic to hypoxic solutions and vice versa was investigated.

The ideal situation would have been an instantaneous change from one solution to the other in the bath. This was not possible but as can be seen the restrictions introduced by the dynamic properties of the system were very small (Figure 2.17). The type of flow existing in the bath was not investigated as it was considered sufficient in practical terms to allow 3 minutes to pass after connection to a particular superfusate, by which time the partial pressure of O_2 in the bath would have reached at least 95% of its equilibrium value.

FIGURE 2.16

The deleterious effects of bubbling a hypoxic solution with air are shown. It can be seen that after exposure to O_2 it only takes 4.5 mins for the solution to be reoxygenated (line drawn by eye).

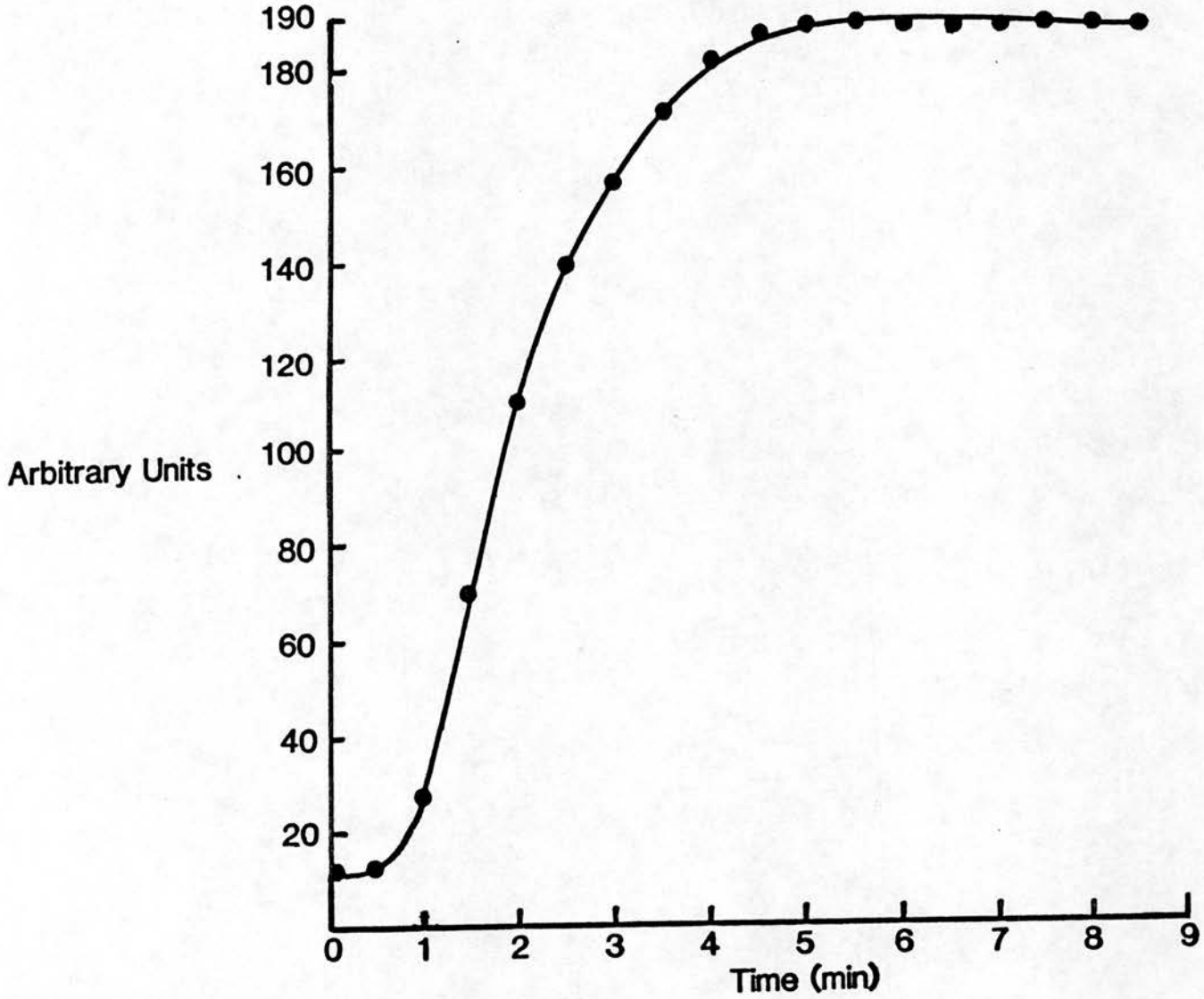
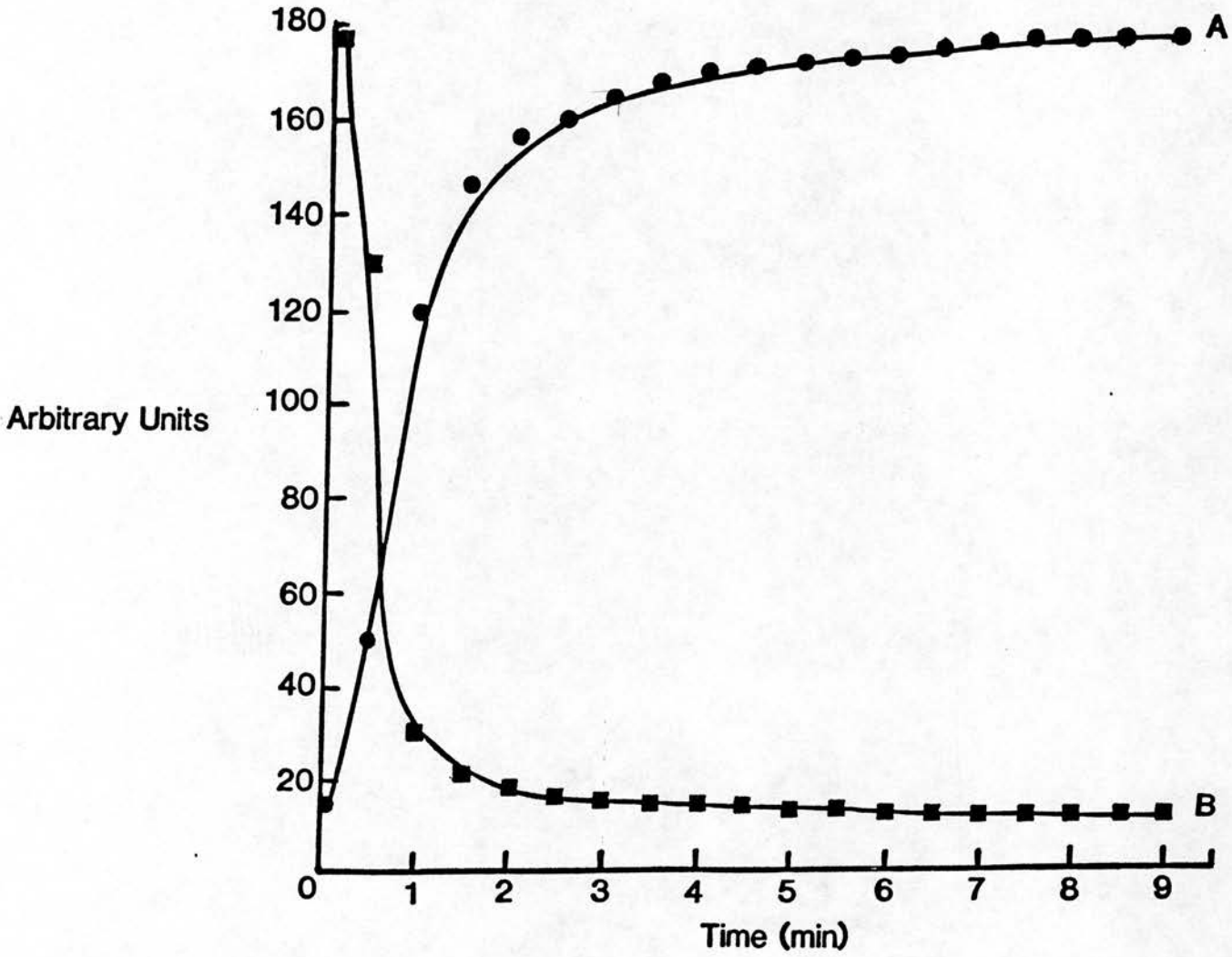

Arbitrary Units
190
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
Time (min)

FIGURE 2.17

Illustration of the dynamic effect of changing from normoxia to hypoxia and vice-versa. This shows that the change to hypoxia from normoxia and vice-versa is not instantaneous but is complete in approximately 2 minutes. This is a sufficiently fast time course for the type of experiments performed in this study. (A) represents the reoxygenation of hypoxic Tyrode solution by bubbling with air, (B) represents the change in PO_2 on switching from Tyrode bubbled with air to hypoxic solution (lines drawn by eye).


Arbitrary Units
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
Time (min)
A
B

RESULTS

The results are presented in 15 sections which are described below. Except where stated the Paired T-test was used to determine significance of difference between means of sample groups. A value of $P<0.05$ was considered to be a significant difference. Except where specifically mentioned means are quoted $\pm$ one standard deviation (S.D.). The mean PO_2 value during hypoxia was 4.8 $\pm$2.6 mmHg (n=19).

(1) GENERAL OBSERVATIONS OF pH DURING HYPOXIA

Ellis and Noireaud (1987) measured cytoplasmic pH_i directly using ion-selective microelectrodes in isolated mammalian myocardial tissue. These experiments revealed large acidifications as a result of hypoxia and showed large and rapid changes in pH_i on re-addition of oxygen. These results are confirmed and expanded by results obtained in this study where exposure to hypoxia for 20 minutes in sheep heart Purkinje fibres resulted in an acidification from pH 7.10 $\pm$0.21 (n=87 experiments) to 6.89 $\pm$0.33 ($P<0.001$) (Figure 3.1). This was accompanied by a small depolarization of the resting membrane potential from -73.9 $\pm$7.5mV to -72.6 $\pm$9.6mV ($P<0.01$). In a few preparations there was no measurable depolarization in some there was even a hyperpolarization of a few mV (e.g. Fig.3.2). The pH_i transiently became significantly more acid on re-oxygenation, decreasing to 6.82 $\pm$0.36 (Fig.3.1). During the transient extra acidification of pH_i on reoxygenation there was normally a small transient hyperpolarization of Em which coincided with the pH_i change perhaps suggesting a weak electrogenic process. In most experiments there was some delay at the beginning of the hypoxic exposure before the development of the intracellular acidification (e.g. Fig.3.4). This is

FIGURE 3.1

The effects on pH_i (top) and membrane potential (Em) (bottom) of 20 minutes exposure to hypoxic Tyrode solution on a sheep heart Purkinje fibre.

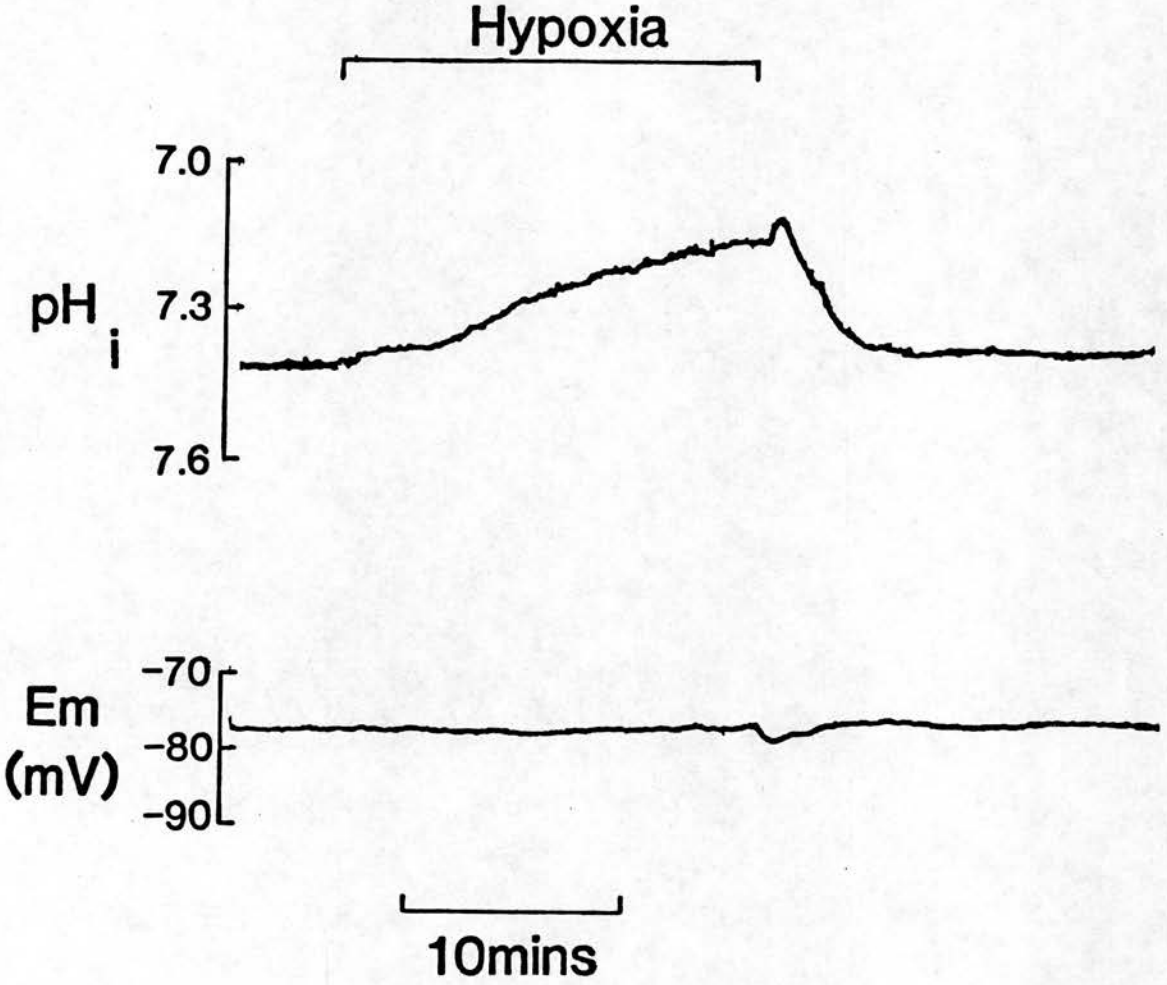
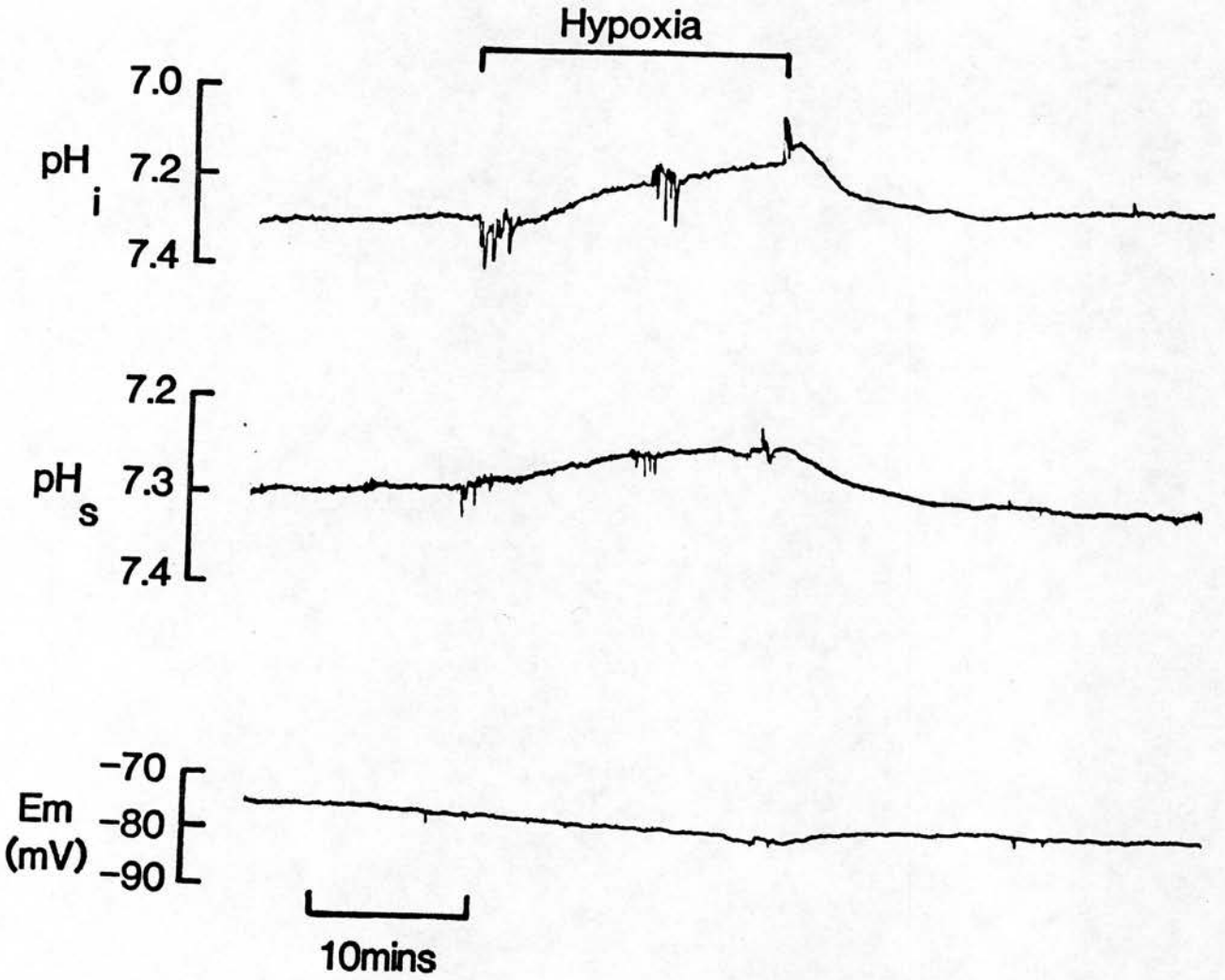

Hypoxia
7.0
pH i
7.3
7.6
-70
Em
(mV)
-80
-90
10mins

FIGURE 3.2

The effects on pH_i (top), surface pH, pH_s (middle) and membrane potential (bottom) of 20 minutes exposure to hypoxic Tyrode, on sheep Purkinje fibre.


Hypoxia
7.0
pH i 7.2
7.4
7.2
pH s 7.3
7.4
-70
Em (mV) -80
-90
10mins

presumably a reflection of not only the time taken to reduce O_2 levels in the bath but also the time taken to inhibit oxidative phosphorylation in the tissue.

In some preparations a small transient alkalosis was observed on changing into hypoxic solution (e.g. Fig.3.9). An alkalosis of pH_i has previously been described in ferret heart by Allen et al. (1983a). There was also a small alkalosis observed on recovery of pH_i after reoxygenation (e.g. Fig.3.9).

In addition to changes in the pH_i, the pH on the surface of the muscle preparation pH_s, (as measured by a blunt pH-sensitive microelectrode pushed against the surface of the tissue) became acidified in hypoxia (see Fig.3.2). The pH_s changing from a mean value of 7.21 $\pm$0.09 (n=14) to 7.07 $\pm$0.25 ($P<0.05$). On reoxygenation the pH_s normally became slightly more acid reaching a pH of 7.06 $\pm$0.25 ($P<0.05$) before recovery.

(2) EFFECT OF HYPOXIA ON FORCE OF CONTRACTION OF FERRET VENTRICULAR MUSCLE

In this study exposing ferret papillary muscle to hypoxia often caused a transient increase in force, possibly as a result of a transient alkalinization of pH_i (Ellis and Noireaud, 1987). This was followed by a falling phase in developed tension during hypoxia. On reoxygenation there is often a transient decrease in force followed by a rapid initial recovery over the first minute of reoxygenation, this is followed by a slower phase of recovery over the following 20-30 minutes.

Hypoxia has been shown to lead to a rapid decline in developed tension due to changes other than a decrease in the $[Ca^{2+}]_i$ available for contraction (Allen and Orchard 1983). In the present study the effect of hypoxia

on developed tension in isolated ferret papillary muscle stimulated at a rate of 0.2 Hertz was investigated. Developed tension fell to approximately 35% ±14% of control tension (mean ±s.d n=15) after 20 minutes of hypoxia (e.g. Fig.3.3).

(3) TECHNIQUES FOR BLOCKING OXIDATIVE PHOSPHORYLATION

Several methods have been used previously to inhibit oxidative phosphorylation. The removal of O_2 inhibits oxidative phosphorylation. Alternatively, NaCN can be used (Allen and Orchard, 1984) since a concentration of 2mM in the perfusing solution reversibly inhibits cytochrome oxidase activity (Pirolo and Allen 1986).

Metsa-Ketela et al. (1981) induced hypoxia in isolated rat atrial cells by using 2mM sodium dithionite in the bathing medium. However, in more recent work Dart and Riemersma (1989) have shown that in bicarbonate buffered solutions bubbled with 95% N_2\5% CO_2 the subsequent addition of sodium dithionite (final concentration 0.5mM) removed all traces of oxygen. The oxygen sensitive dye resazurin ($0.5ugml^{-1}$) was used to indicate anoxia (see methods).

Experiments were performed in the present study to test the effectiveness of hypoxia in blocking oxidative phosphorylation compared with chemical removal of O_2 and metabolic blockade with NaCN.

In 23 experiments where hypoxia and anoxia were directly compared (e.g. Fig.3.4) in sheep Purkinje fibres the pH_i decreased from 7.18 ±0.13 to 7.03 ±0.14 in hypoxia, compared to an acidification to 6.95 ±0.15 during anoxia of 20 minutes. The difference between the acidification of pH_i produced during hypoxia compared with anoxia was significant ($P<0.001$ n=23). On reoxygenation there was a further transient acidification

FIGURE 3.3

The effect of approximately 20 minutes exposure to hypoxic Tyrode solution on developed tension in ferret papillary muscle stimulated at 0.2 Hz.

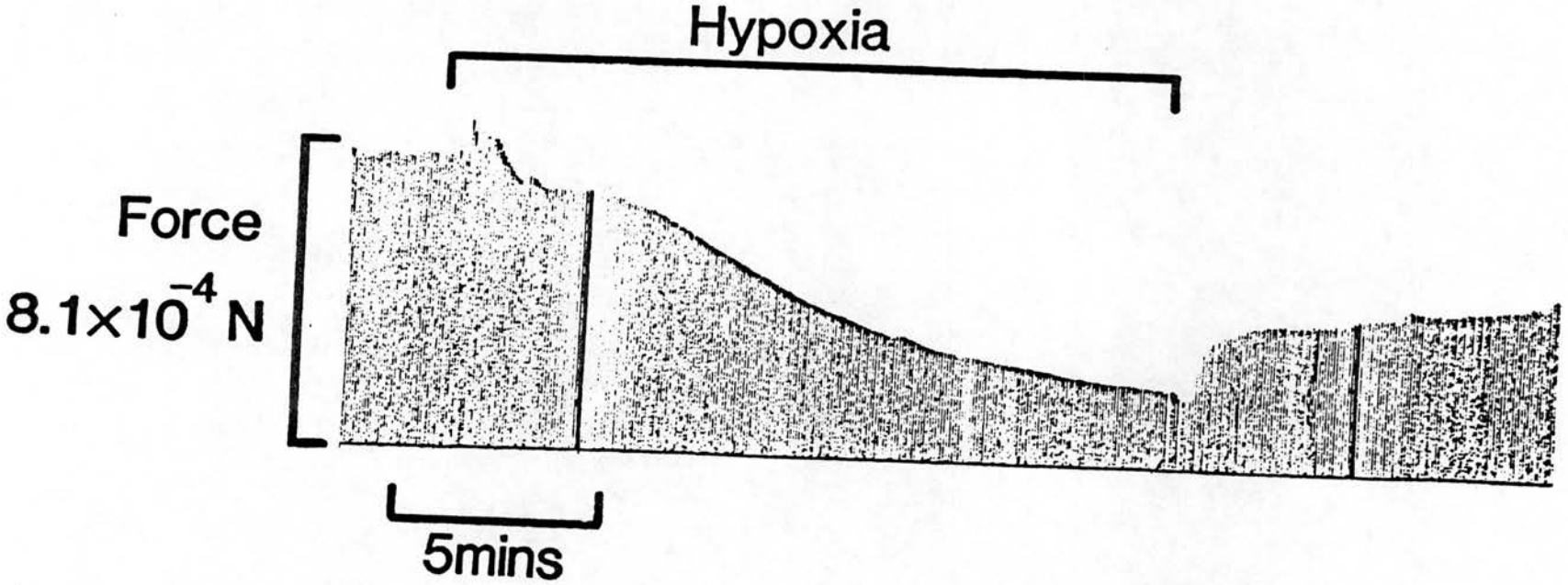
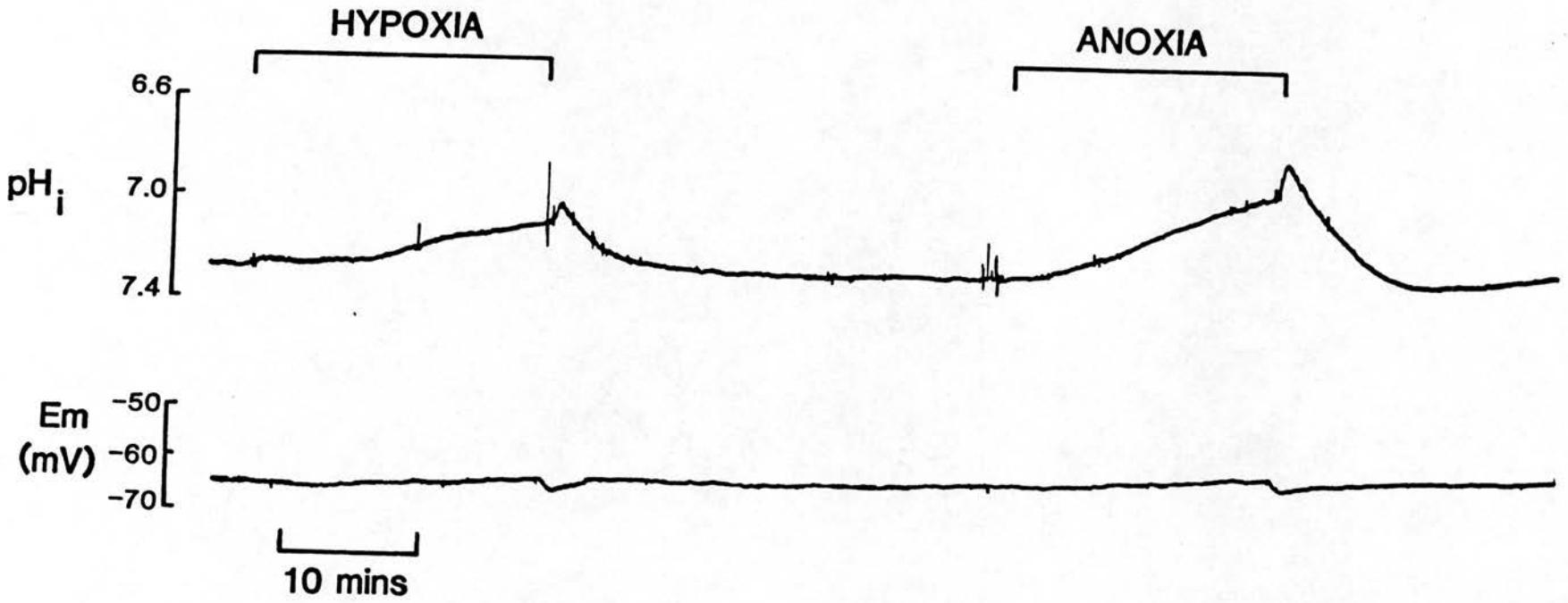

Hypoxia
Force
8.1×10⁻⁴ N
5mins

FIGURE 3.4

The effect of approximately 20 minutes exposure to hypoxia, compared with exposure to anoxia in sheep Purkinje fibre (anoxic solution was produced by adding sodium dithionite to normal Tyrode solution).


HYPOXIA
ANOXIA
6.6
pH$_i$
7.0
7.4
Em
(mV)
-50
-60
-70
10 mins

before recovery, to 6.94 ±0.2 in hypoxia which was significantly smaller ($P<0.001$) than the transient acidification to 6.78 ±0.26 seen on reoxygenation from anoxia.

The onset of acidification of pH_i appeared to be more rapid in anoxic solution compared with hypoxia, perhaps as a result of the more rapid and efficient exclusion of O_2 from the perfusate and thus from the cells, in anoxia.

In 7 experiments where surface pH (pH_s) was compared during hypoxia and anoxia, there was a significant difference in the acidification produced under the two conditions. Hypoxia caused a decrease ($P<0.01$) from 7.19 ±0.11 to 7.10 ±0.17, while anoxia caused an acidification to 7.11 ±0.17 ($P<0.01$). There was no significant difference between the size of acidification of pH_s in hypoxia compared with anoxia.

The effect of hypoxia was compared with blocking oxidative phosphorylation using NaCN (2mM) in 6 experiments (Fig.3.5). Hypoxia resulted in an acidification of pH_i from the control value of 7.26 ±0.08 to 7.11 ±0.08, compared with a change in NaCN to 7.10 ±0.13. The difference between hypoxia and NaCN is not significant. The change in pH_i is not significantly different on reoxygenation in hypoxia (7.03 ±0.11) and NaCN (7.03 ±0.16) although the maximum rate of acidification and recovery on removal of NaCN is slower than in hypoxia. This is presumably as a result of O_2 entry occuring far more rapidly than washout of NaCN from the tissue. In this group of experiments there was no significant difference in membrane potential between the two conditions. In Figure 3.6 the combined results for changes in pH_i under conditions of hypoxia, anoxia and NaCN exposure are compared.

FIGURE 3.5

The effect of approximately 20 minutes exposure to hypoxia compared with exposure to 2mM NaCN.

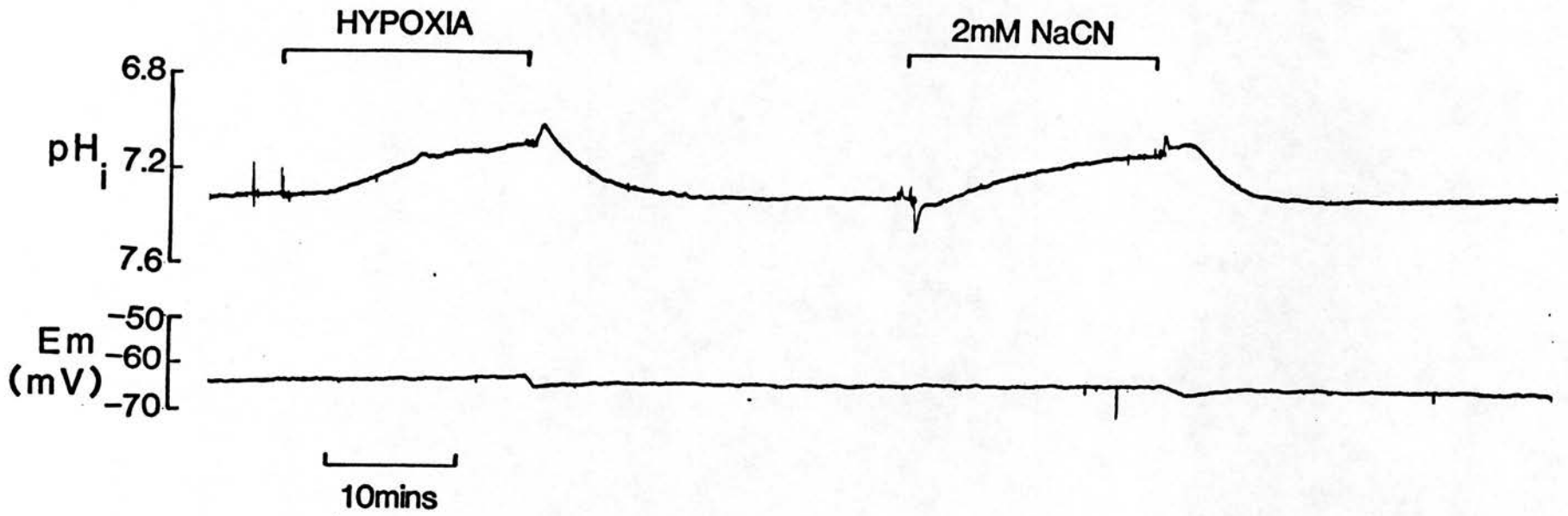
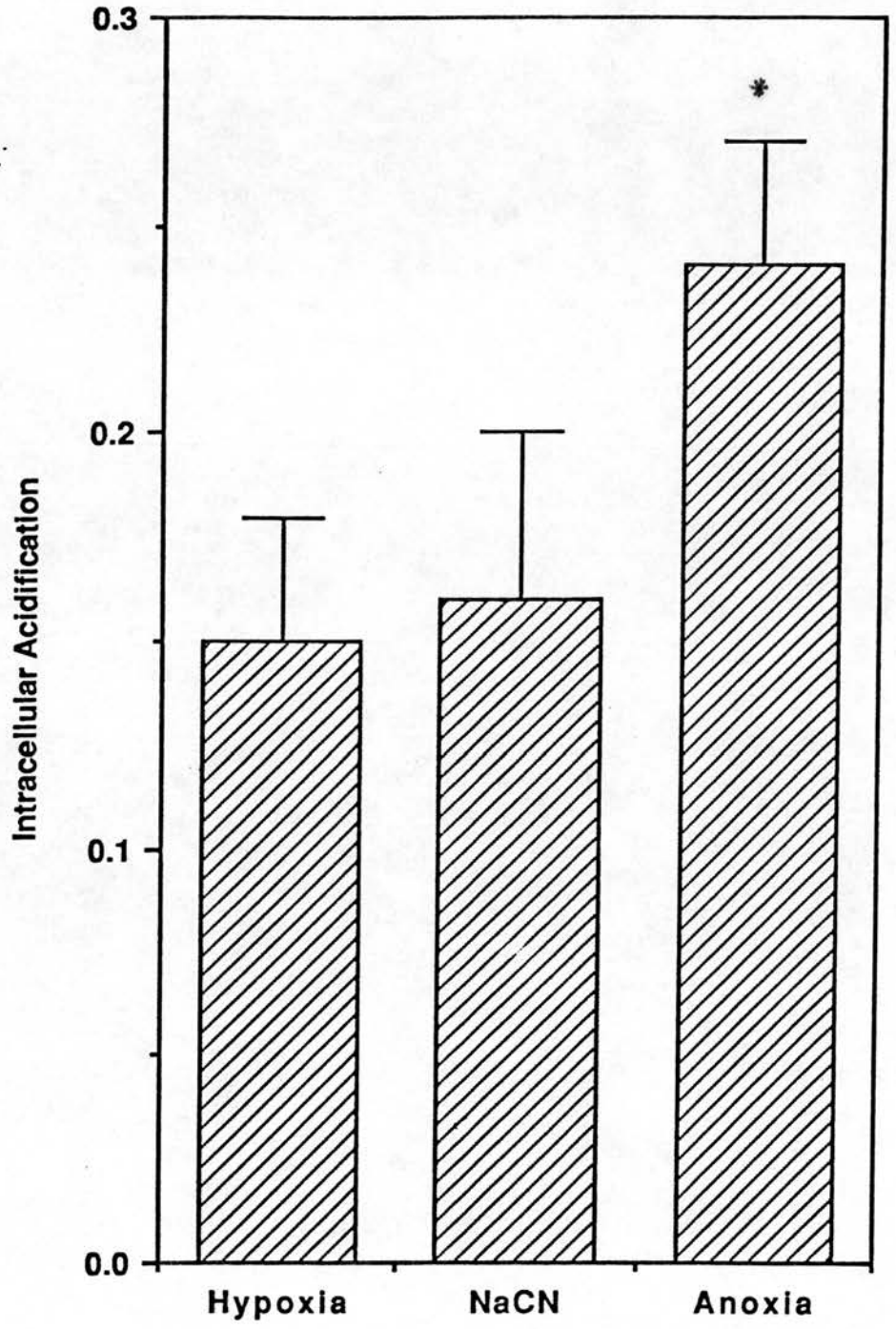

HYPOXIA
2mM NaCN
6.8
pH i 7.2
7.6
-50
Em -60
(mV) -70
10mins

FIGURE 3.6

The change in pH_i during hypoxia compared with NaCN and hypoxia (mean $\pm$ S.E.). The number of separate experiments comparing the three conditions was 23 for hypoxia and anoxia and 6 for NaCN.

$*$ = Significant difference between anoxia and hypoxia ($P<0.01$).


0.3
0.2
0.1
0.0
Intracellular Acidification
*
Hypoxia
NaCN
Anoxia

The effects of these three methods of inhibiting oxidative phosphorylation were compared in ferret papillary muscle for their effect on developed tension (Fig.3.7). Figure 3.7 is unusual in that on reoxygenation there are large transient rises in developed tension after hypoxia and anoxia, this is atypical. During hypoxia developed tension dropped to 34 $\pm$13% (n=14) of the control tension compared with a fall to 24 $\pm$19% (n=5) in anoxia. This decrease in tension in hypoxia compared to anoxia was significant ($P<0.05$). In 3 experiments where the effect of hypoxia on developed tension was compared with NaCN, tension fell to 43 $\pm$16% of the control tension during hypoxia and to 53 $\pm$15% during exposure to NaCN. The latter was significantly less than during hypoxia ($P<0.05$). Figure 3.8 illustrates the mean changes in developed tension caused by the three methods of blocking oxidative phosphorylation.

(4) DURATION OF HYPOXIC EXPOSURE

The severity of the effects of hypoxia on the mammalian myocardium might be expected to be dependent on several factors such as the length of exposure to hypoxia. Figure 3.9 illustrates the effect of varying the length of hypoxic exposure on pH_i in a sheep Purkinje fibre.

The change in pH_i was recorded after 4, 10 and 20 minutes exposure to hypoxia. During 4 minutes of hypoxia pH_i acidified from the control value of 7.09 $\pm$ 0.20 (n=5) to 6.89 $\pm$0.26 compared with the change in pH_i after 20 minutes hypoxia to 6.62 $\pm$0.27. This was significantly larger ($P<0.01$). However the transient acidification on reoxygenation after 4 minutes hypoxia was 0.11 $\pm$0.03 pH units and was significantly greater than that measured after 20 minutes hypoxia, 0.09 $\pm$0.04 pH units ($P<0.01$).

FIGURE 3.7

The effect of 20 minutes exposure to hypoxia, anoxia or 2mM NaCN on developed tension in ferret papillary muscle stimulated at 0.2 Hz.

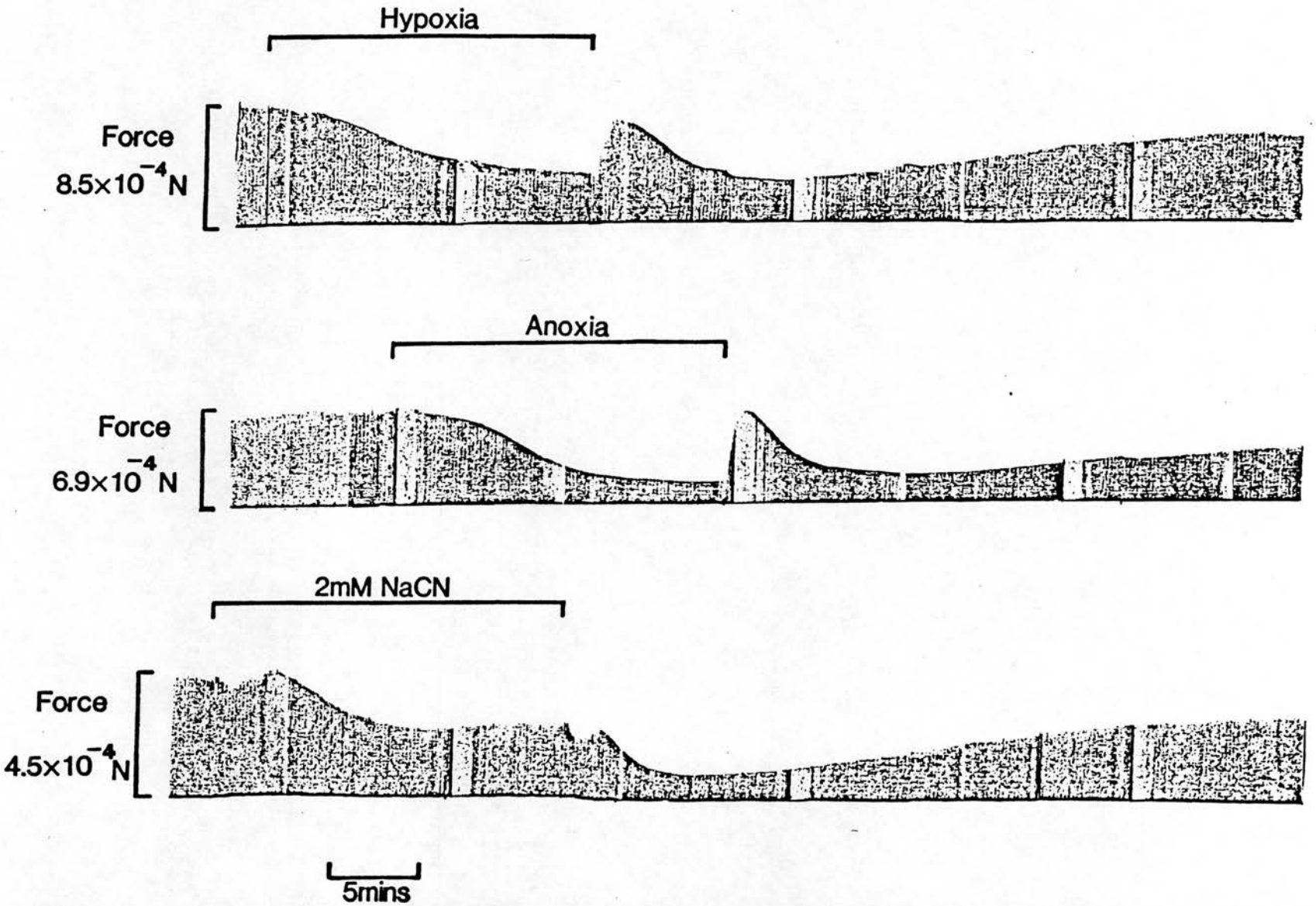

Hypoxia
Force
8.5×10⁻⁴N
Anoxia
Force
6.9×10⁻⁴N
2mM NaCN
Force
4.5×10⁻⁴N
5mins

FIGURE 3.8

The % of control developed tension levels in ferret papillary muscle stimulated at 0.2 Hz. during hypoxia compared with NaCN (n=3) and hypoxia compared with anoxia (n=14). The results are mean values ±S.E.

* = Significant difference between hypoxia and NaCN ($P<0.05$).

** = Significant difference between hypoxia and anoxia ($P<0.01$).

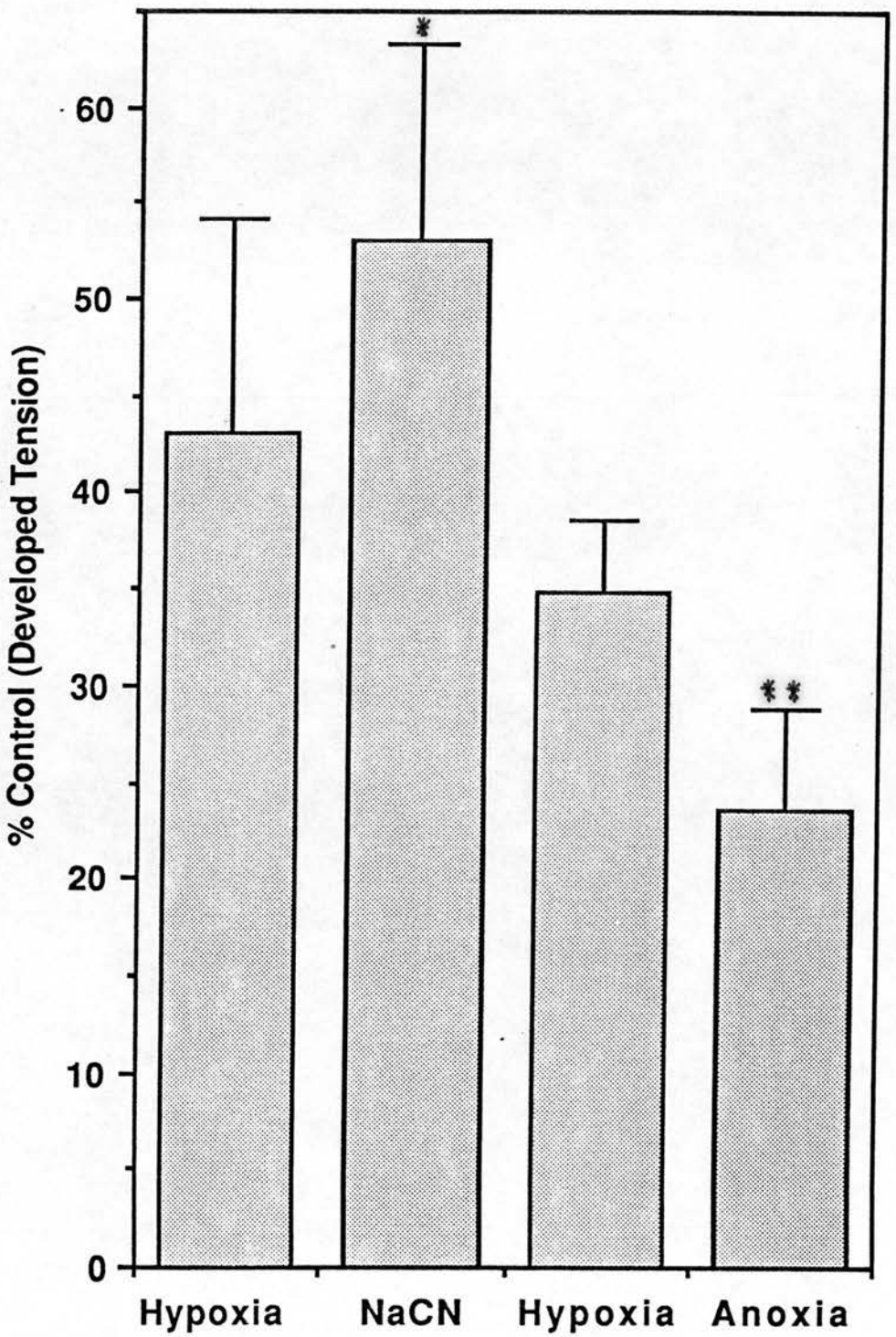
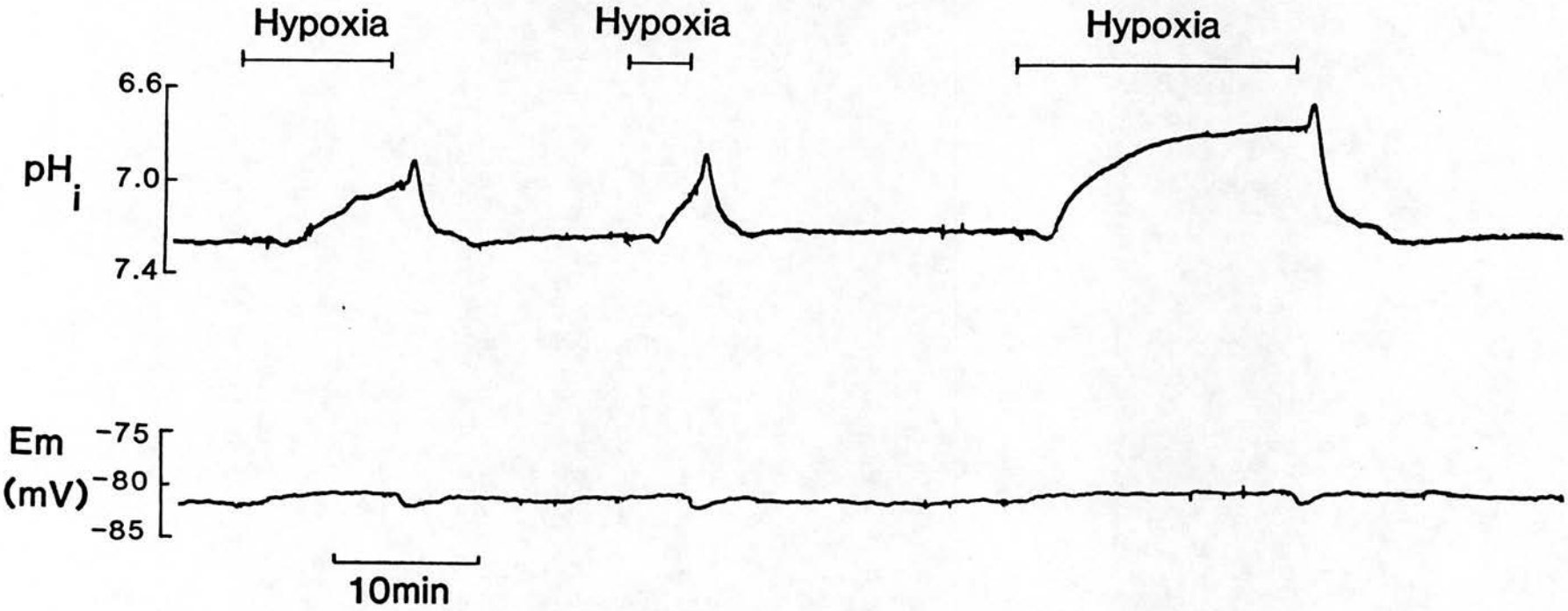

% Control (Developed Tension)
0
10
20
30
40
50
60
*
**
Hypoxia
NaCN
Hypoxia
Anoxia

FIGURE 3.9

The effect of exposure to hypoxia for 4, 10 and 20 minutes on pH_i in a sheep Purkinje fibre.


Hypoxia
Hypoxia
Hypoxia
6.6
pH i
7.0
7.4
Em
(mV)
-75
-80
-85
10min

After 10 minutes of hypoxia pH_i (6.67 $\pm$0.31) was significantly higher than that after 20 minutes ($P<0.05$). The transient change on reintroducing O_2 was significantly larger (0.14 $\pm$0.05, $P<0.05$) compared with the change in pH_i observed following 20 minutes hypoxia.

The effect of longer periods of exposure to hypoxia were investigated. Figure 3.10 shows the effect of one hour of exposure to hypoxia on pH_i and recovery in sheep Purkinje fibre. The recovery from the hypoxic response is very rapid. From Figure 3.10 it can be seen that after only 20 minutes recovery from hypoxia a second exposure results in an acidification of pH_i at only a slightly reduced rate compared to the initial exposure.

In two experiments sheep Purkinje fibres were exposed to 90 minutes hypoxia. These were compared histologically with control fibres (perfused with normal Tyrode for 90 minutes). The fibres were fixed, stained for glycogen and viewed with an electron microscope. The electron-micrographs were examined qualitatively and ranked (blind) for glycogen content (Appendix 1) and a Mann-Whitney U test performed on the mean ranks. The test is rather subjective but since it was not feasible to measure the density of glycogen granules on the electron-micrographs this was considered to be the next best method for assessing glycogen content. There appeared to be no significant difference in the glycogen content between the control and hypoxic preparations. There was also no evidence of any ultrastructural damage in the hypoxic sheep Purkinje fibre samples (e.g. Fig.3.11).

Figure 3.12 illustates the effect of extended hypoxia on developed tension in ferret papillary muscle. A contracture is responsible for the rise in tension after about 45 minutes of hypoxia presumably as a result of depletion of $[ATP]_i$ (Allen et al.1985) as a result of inhibition of oxidative phosphorylation.

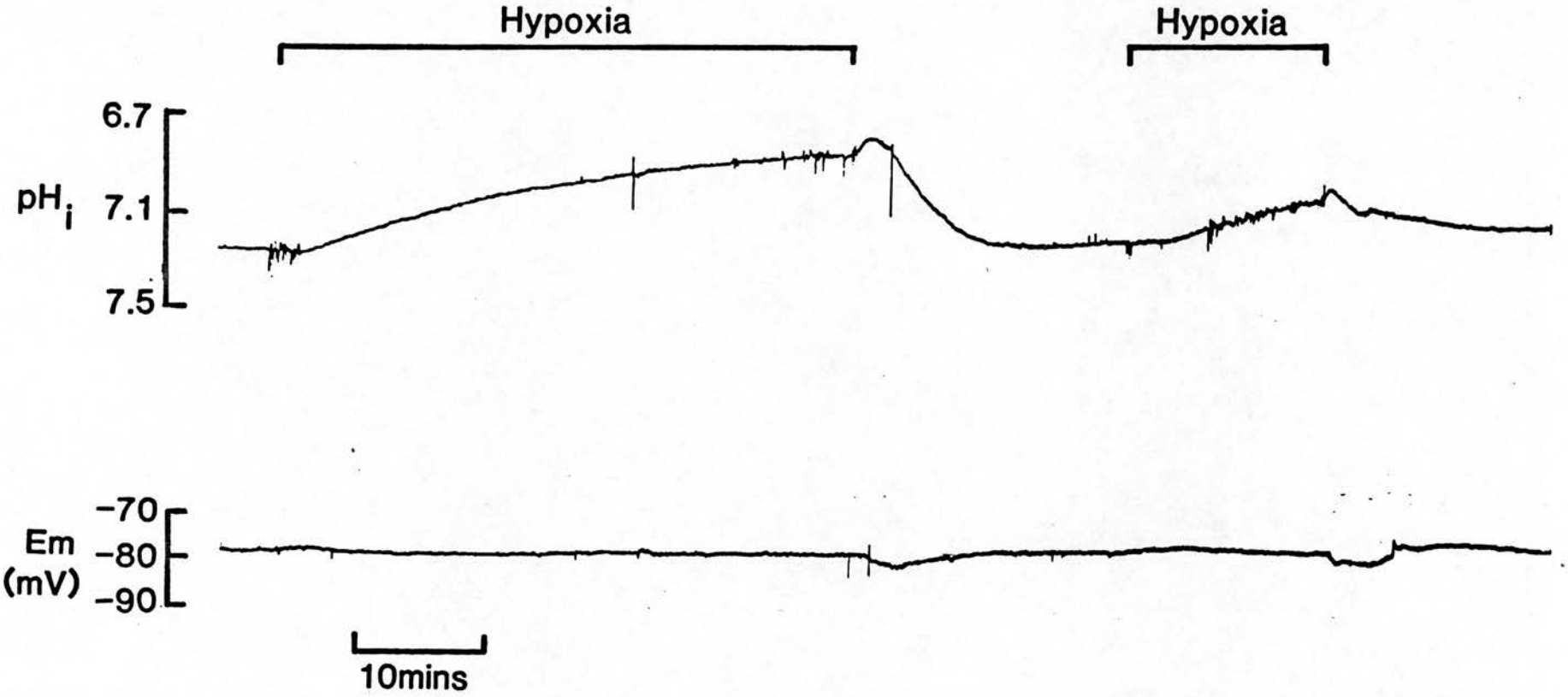

Hypoxia
Hypoxia
6.7
pH$_i$ 7.1
7.5
−70
Em −80
(mV) −90
10mins

FIGURE 3.10

The effect of 60 minutes exposure to hypoxia on pH_i compared with a 20 minute exposure to hypoxia, in a sheep Purkinje fibre.

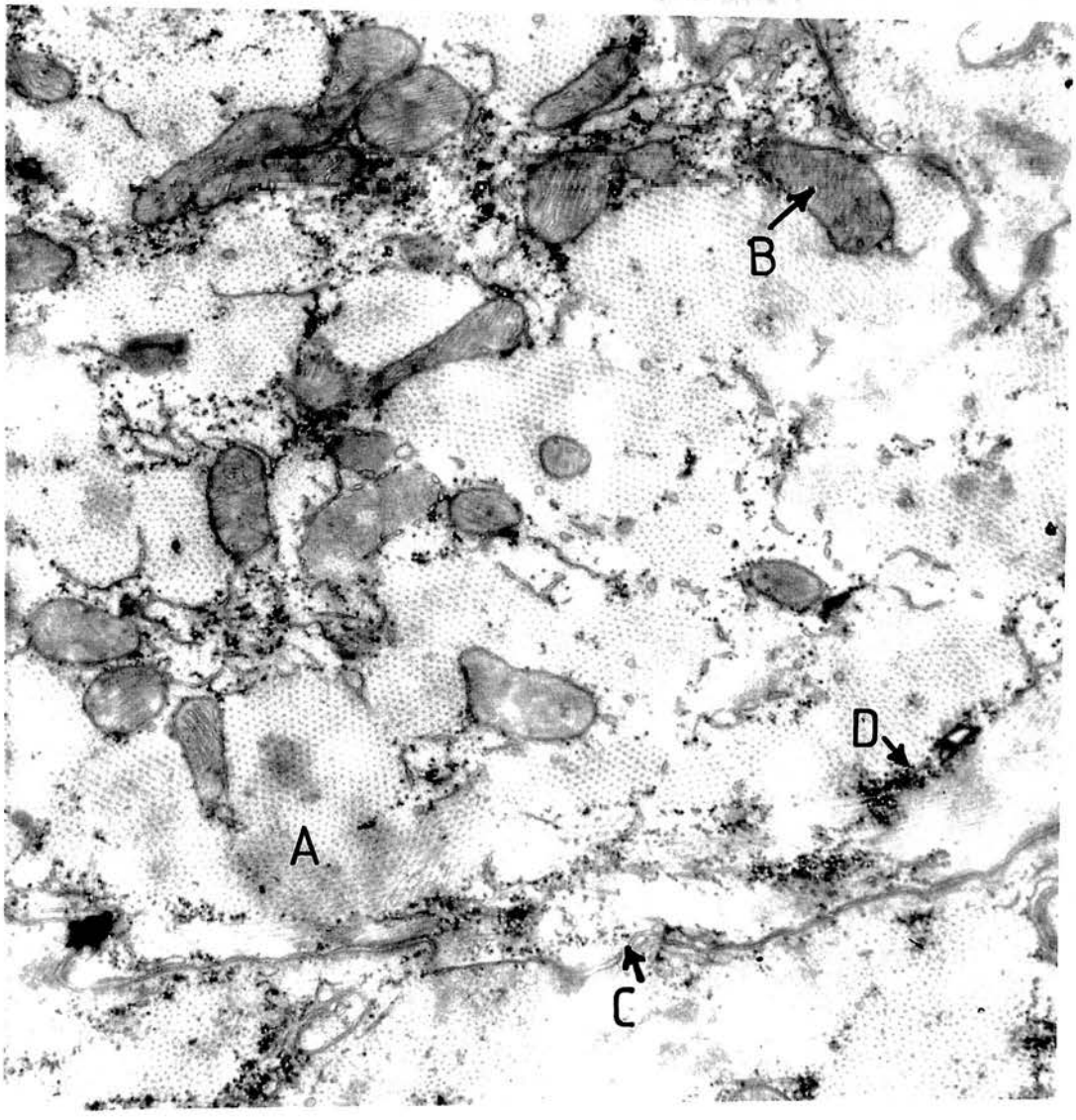
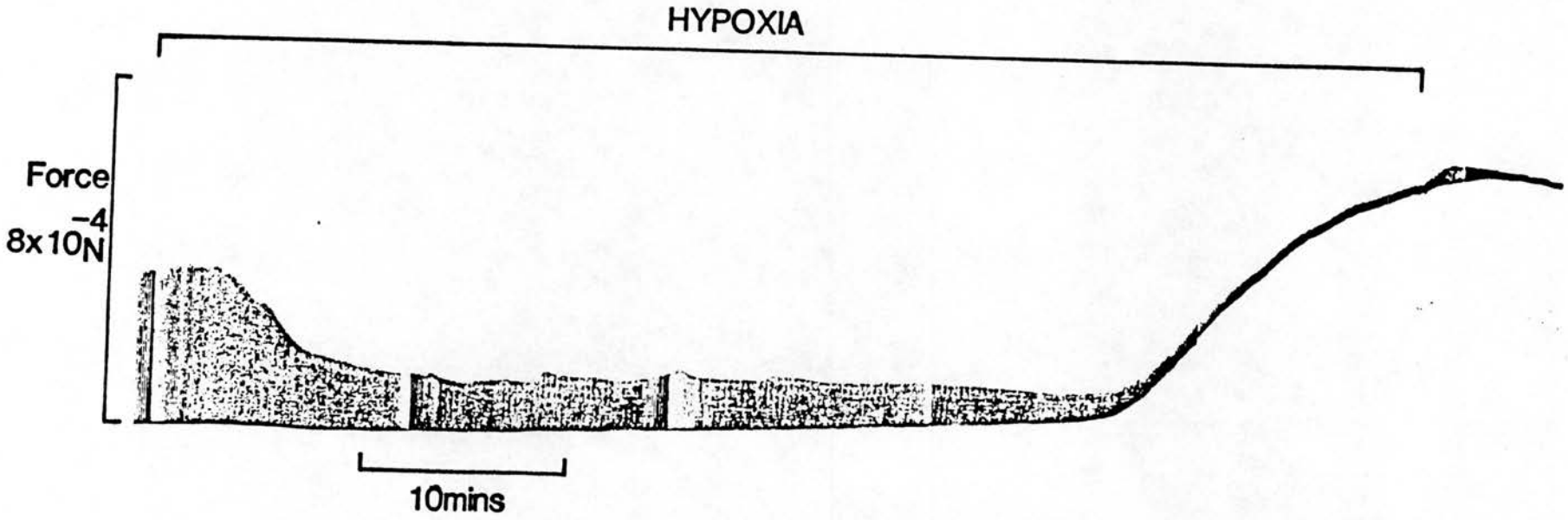

B
D
A
C

FIGURE 3.11

An electron-micrograph of a sheep Purkinje fibre after 90 minutes exposure to hypoxia. (A) indicates the myofibrils, (B) mitochondrion (C) plasmalemma (D) glycogen granules. The magnification is x30,000.


HYPOXIA
Force
8×10^{-4} N
10mins

Ferret papillary muscles exposed to hypoxia for an hour or more did not recover from the contracture, and tended not to recover twitch tension (Figure 3.12).

(5) THE EFFECT OF VARYING OXYGEN TENSION

A group of experiments was carried out to investigate the dependence of the change in pH_i on the concentration of O_2 in the perfusing solution. In 4 experiments the PO_2 in the solution perfusing sheep Purkinje fibres was altered (Figure 3.13) to compare chemically induced anoxia with at least two other O_2 tensions. The results of these experiments are plotted in Figure 3.14 and 3.15 which show the effect of changing PO_2 on the pH_i during hypoxia and on the transient acidification observed during reoxygenation.

Since chemical anoxia (sodium dithionite) was used as a technique to remove all the O_2 from the system it was important to establish that this had an effect identical in mechanism, if greater in severity, on the changes in pH_i. There was no indication that the sodium dithionite produced any extra effect other than might be expected from its efficient removal of O_2 from the solutions.

(6) MEASUREMENTS OF a^i_K AND a^i_{Na}:

Sodium Measurements

Kleber (1983) found that during a 15 minute exposure to global ischaemia in guinea-pig heart the $[Na^+]_i$ did not change. This he suggested was due to a large depolarization. However in a later study Wilde and Kleber (1986) demonstrated small rises in a^i_{Na} when isolated guinea-pig ventricular muscle was subjected to the

Figure 3.12

The effect of 60 minutes exposure to hypoxia on the developed tension in ferret papillary muscle. Note the development of a hypoxic contracture after approximately 45 minutes.

FIGURE 3.13

The effect of varying the PO_2 in the hypoxic solution on pH_i in a sheep Purkinje fibre. (A) is the pH_i change during 20 minutes exposure to solution equilibrated with a PO_2 of 5 mmHg, (B) is the pH_i change during 20 minutes exposure to anoxia (PO_2 0 mmHg), (C) is the pH_i change during 20 minutes exposure to solution equilibrated with a PO_2 of 5 mmHg and (D) is the pH_i change on exposure to solution equilibrated with a PO_2 of 12 mmHg.

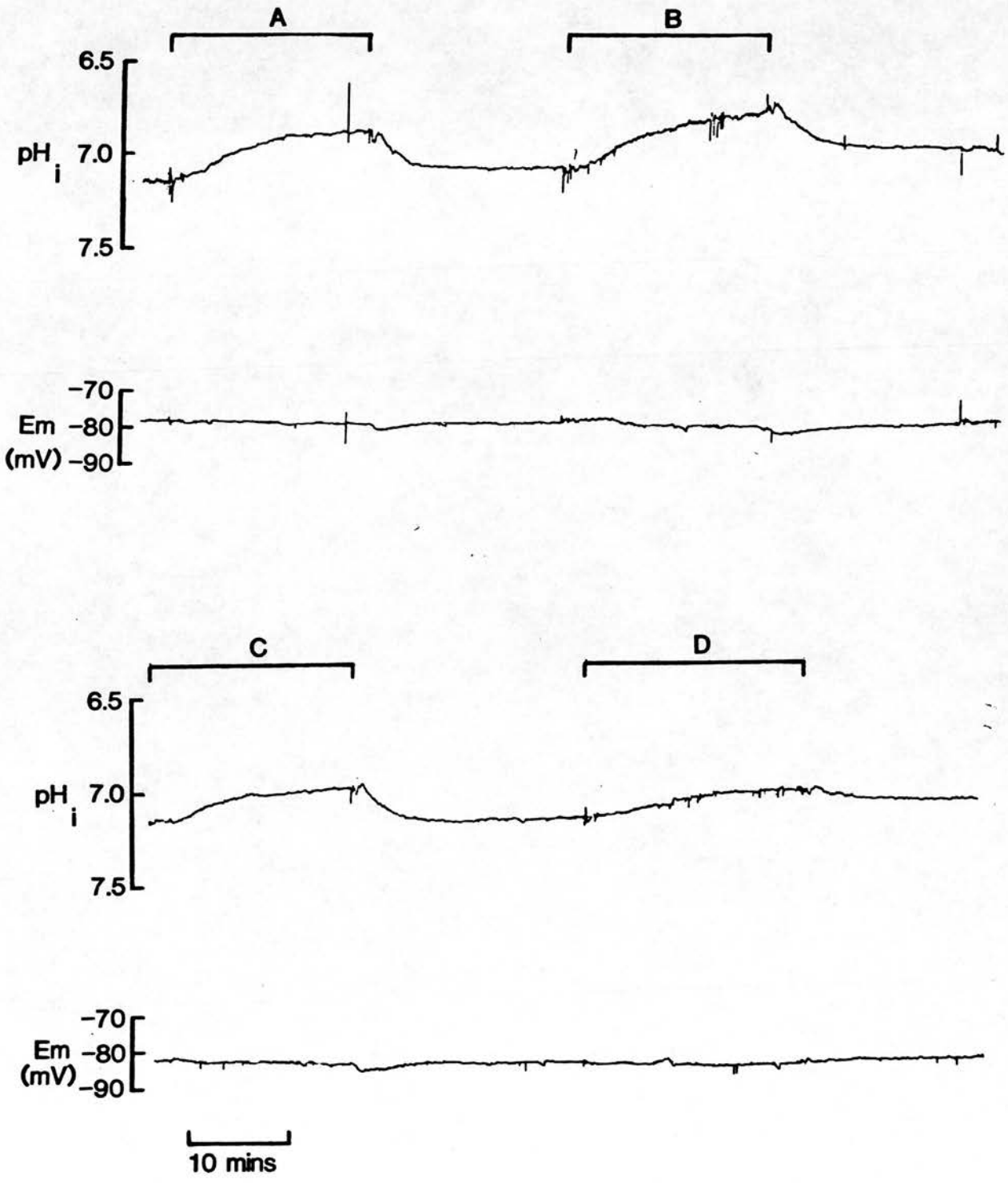

A
B
6.5
pH i 7.0
7.5
-70
Em -80
(mV) -90
C
D
6.5
pH i 7.0
7.5
-70
Em -80
(mV) -90
10 mins

FIGURE 3.14

The change in pH_i in 4 experiments (as indicated by the different symbols) plotted against PO_2 during hypoxia at various O_2 partial pressures in sheep Purkinje fibres.

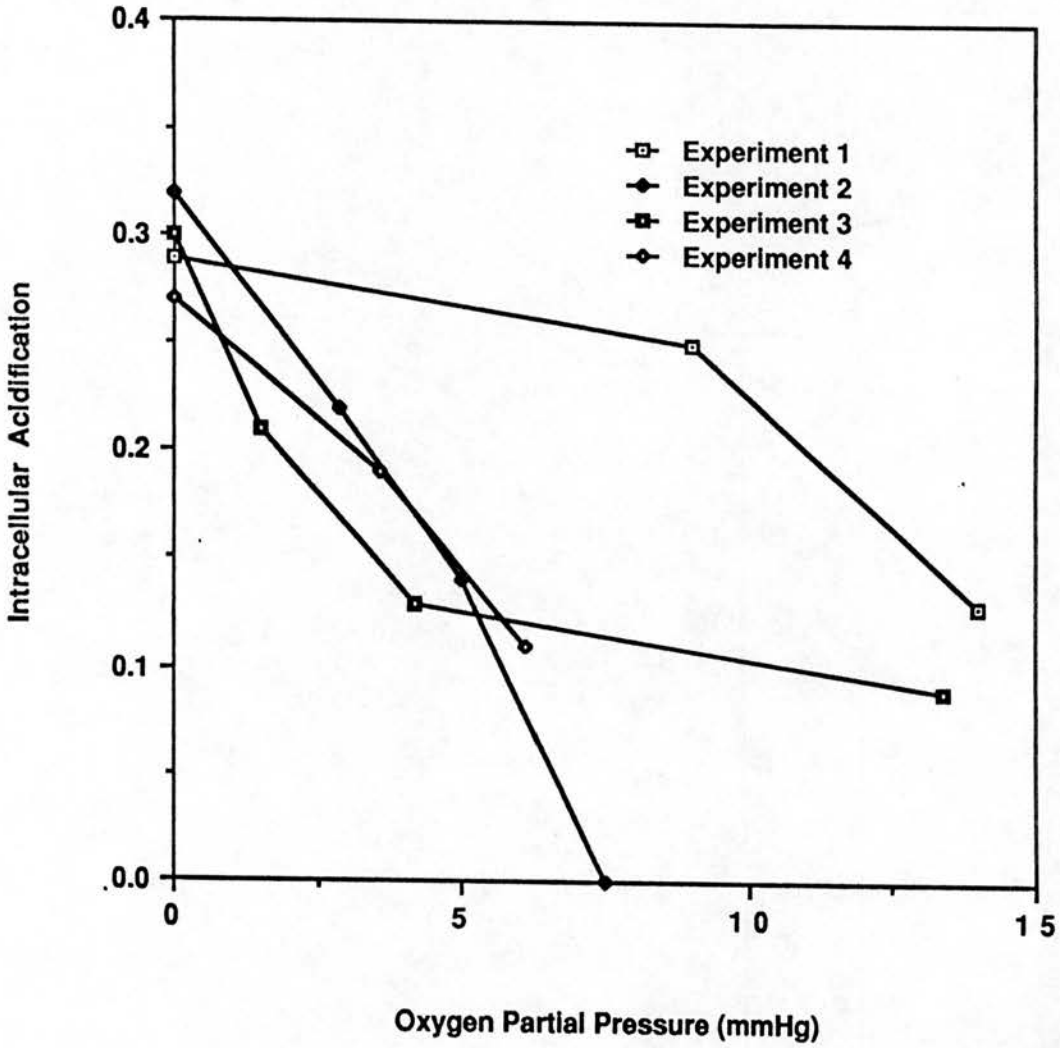
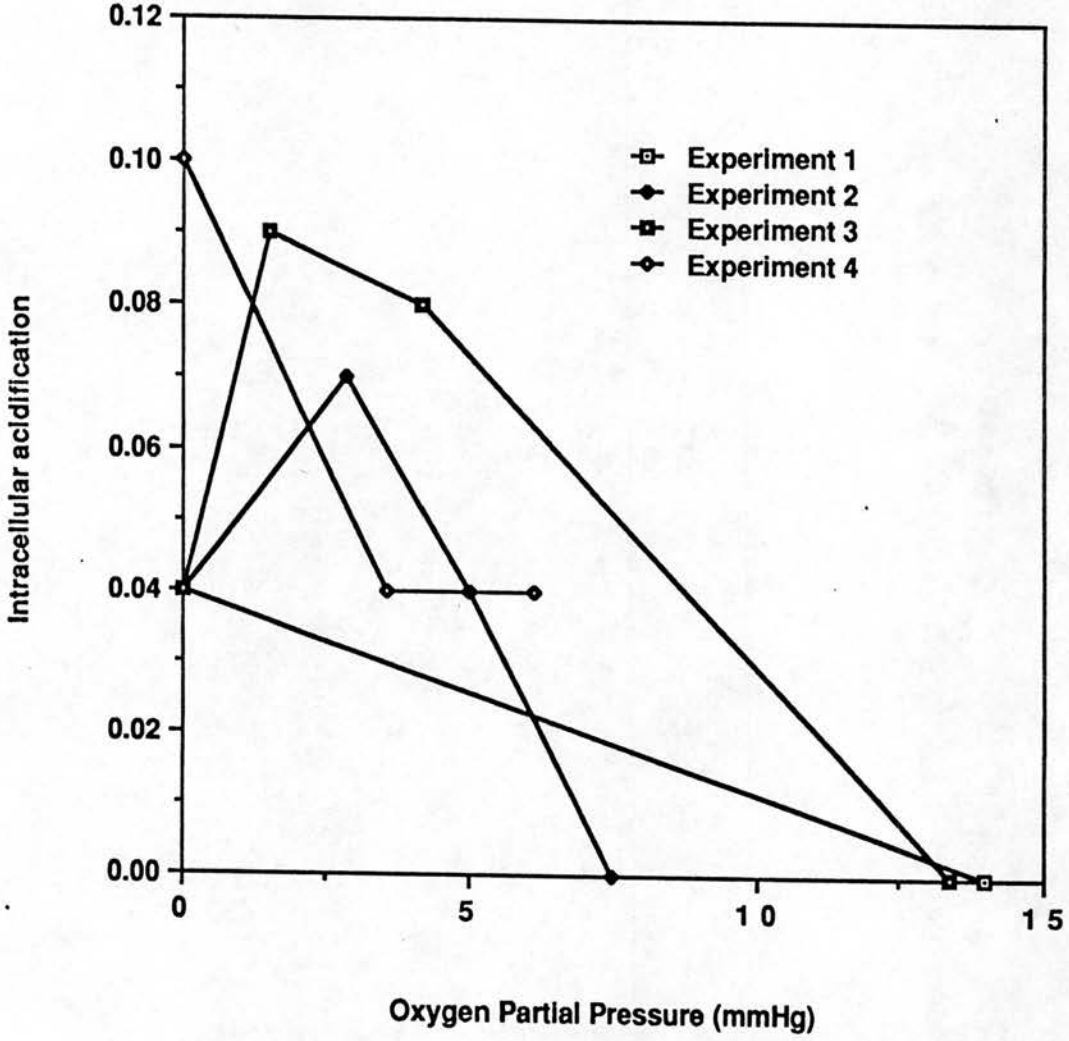

0.4
0.3
0.2
0.1
.0.0
0
5
10
15
Experiment 1
Experiment 2
Experiment 3
Experiment 4
Intracellular Acidification
Oxygen Partial Pressure (mmHg)

FIGURE 3.15

The change in pH_i occuring as a result of the transient acidification on reoxygenation of a sheep Purkinje fibre plotted against PO_2 during hypoxia at various O_2 partial pressures in 4 experiments.


0.12
0.10
0.08
0.06
0.04
0.02
0.00
Intracellular acidification
0
5
10
15
Oxygen Partial Pressure (mmHg)
Experiment 1
Experiment 2
Experiment 3
Experiment 4

combined effects of hypoxia, acidosis, and removal of glucose as a substrate in an attempt to mimic certain aspects of ischaemia. Ellis and Noireaud (1987) found that during hypoxia there was a small rise in a^i_{Na}, and on reoxygenation a transient rise after hypoxia (with glucose present in the solution). This agrees with the results of the present study where it was found that after 20 minutes hypoxia a^i_{Na} increased from 5.5 $\pm$2.9mM (n=6) to 5.8 $\pm$3.1mM$_\Lambda$ ($P<0.05$). This is equivalent to a mean increase in $[Na^+]_i$ of 0.43 $\pm$0.29mM. There was no significant change in membrane potential ($P>0.1$).

Anoxic exposure (Fig.3.16), induced by adding 0.5mM sodium dithionite, caused a larger rise in a^i_{Na} from 5.5 $\pm$3.1mM (n=6) to 6.8 $\pm$2.8mM$_\Lambda$ ($P<0.01$). This is equivalent to a rise in $[Na^+]_i$ from 7.4mM to 9.0mM, assuming an intracellular activity coefficient of 0.75 (Ellis, 1977). These a^i_{Na} changes were accompanied by a significant depolarization ($P<0.05$) of Em from 70.5 $\pm$6.0mV to 61.2 $\pm$11.7mV (Figure 3.16 is slightly atypical in that it shows no evidence of a depolarization during anoxia)

The effects of hypoxia and anoxia on $[Na^+]_i$ are summarized in Figure 3.17.

Potassium Measurements

It was of interest to measure intracellular potassium because this is another indicator of the activity of the Na^+/K^+ pump activity. The results above describe a rise in a^i_{Na} which might be accounted for by some inhibition of the Na^+/K^+ pump. Gaspardone et al. (1986) examined potassium loss from rabbit myocardium during hypoxia. They postulated that this loss could be caused by Na/K pump inhibition, acidosis and/or a selective increase in permeability of cell membranes to K^+ ions. They could find no evidence of a decrease in

FIGURE 3.16

The effect of 20 minutes anoxia on intracellular sodium activity (a^{i}_{Na}) in a sheep Purkinje fibre.

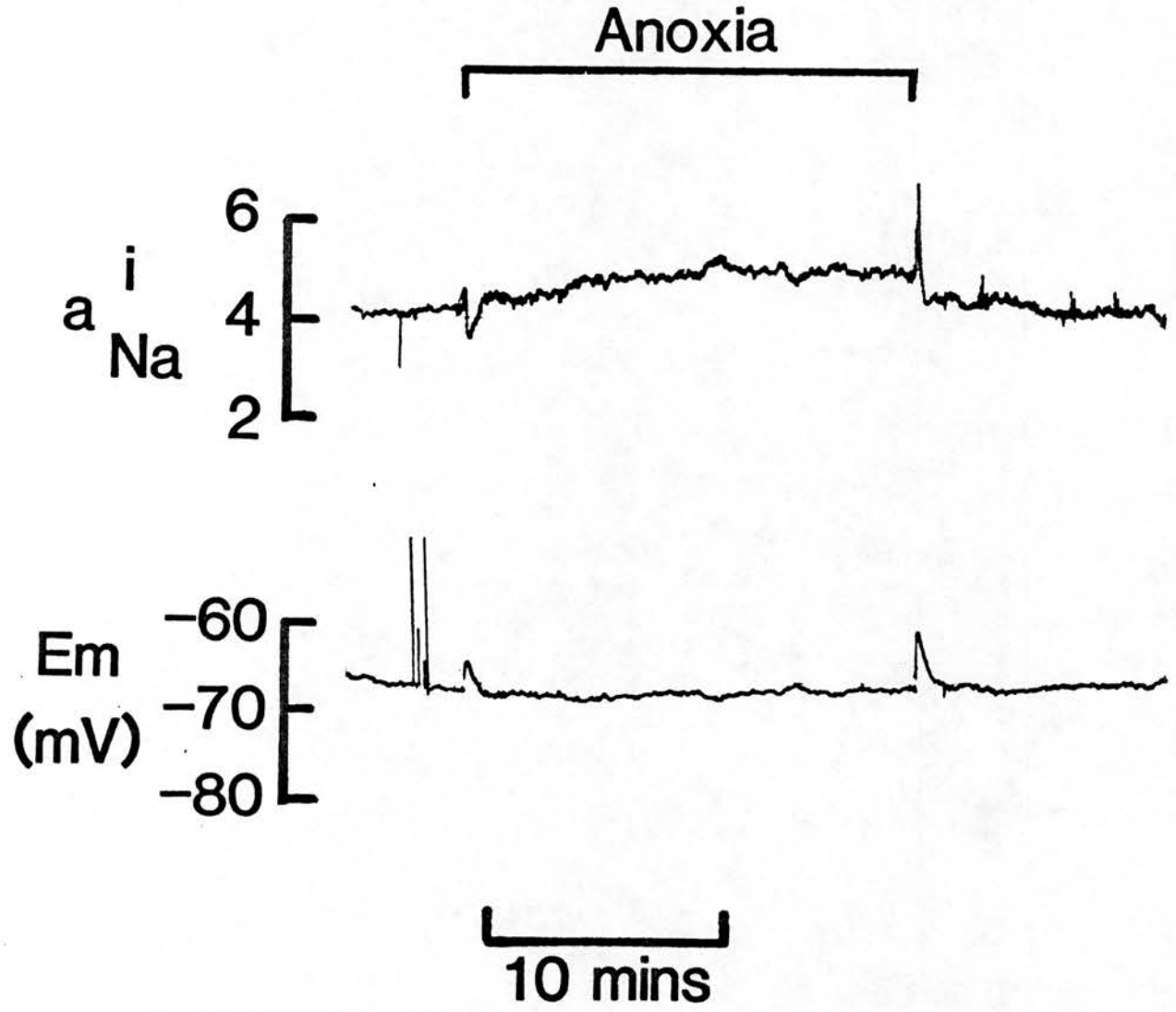
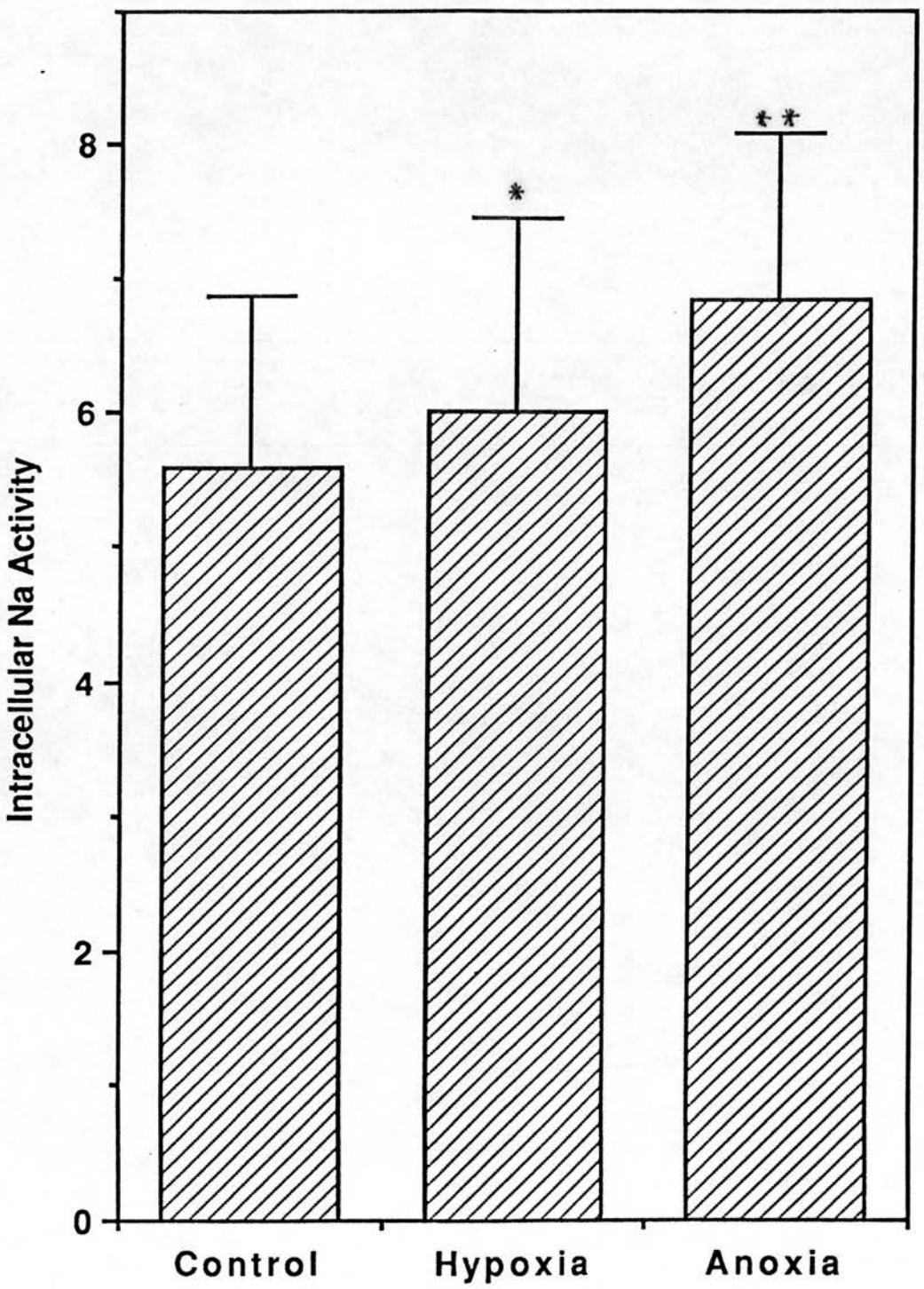

Anoxia
6
4
2
a_{Na}^{i}
−60
−70
−80
Em
(mV)
10 mins

FIGURE 3.17

The effect of hypoxia and anoxia on a^{i}_{Na} in sheep Purkinje fibres. The results are mean changes in a^{i}_{Na} ($\pm$S.E.) after 20 minutes for 6 experiments.

* = Significant ($P<0.05$).

** = Significant ($P<0.01$).


Intracellular Na Activity
0
2
4
6
8
*
**
Control
Hypoxia
Anoxia

sodium pump activity (they detected no change in $[Na^+]_i$ after 15 minutes exposure to hypoxia).

There are two popular types of potassium ion-selective ligands available. These are the Corning K^+-selective liquid ion exchanger and the WPI valinomycin-based liquid ion exchanger. Most previous recordings have been made with the Corning ligand. Recent work by Reverdin et al. (1986) has suggested that the Corning K-selective liquid ion exchanger (Corning Medical No 477317) can give spuriously high values for a^i_K in ferret papillary muscle. A series of experiments were therefore performed to test whether there was a significant difference between values for a^i_K measured in the same ferret papillary muscle using the two types of ion-sensitive resin (Table 1). The measurements were made by alternate penetrations of Corning and Valinomycin-filled electrodes starting with a different ligand at the start of each experiment. Although the Corning ligand gave higher values for a^i_K in ferret papillary muscle the difference was not significant between the two sets of recordings (2-sample T-Test $P>0.1$). Corning-filled microelectrodes measured an a^i_K of 85.1 $\pm$16.3mM (n=19) while valinomycin-filled microelectrodes measured an a^i_K 77.0 $\pm$20.7mM (n=17). These are equivalent to values of $[K^+]_i$ of 115mM and 104mM respectively. The values of the membrane potential for these experiments where a^i_K was measured were 65.0 $\pm$5.3mV for the Corning impalements (n=19) and 67.0 $\pm$5.0mV (n=17) for the valinomycin recordings.

Having established that the type of electrode used to measure $[K^+]_i$ was not of paramount importance, recordings of $[K^+]_i$ were made in sheep Purkinje fibres during 20 minute periods of hypoxia or anoxia (Fig 3.18). The Corning resin was used since the electrodes are of lower resistance and therefore pick up less electrical

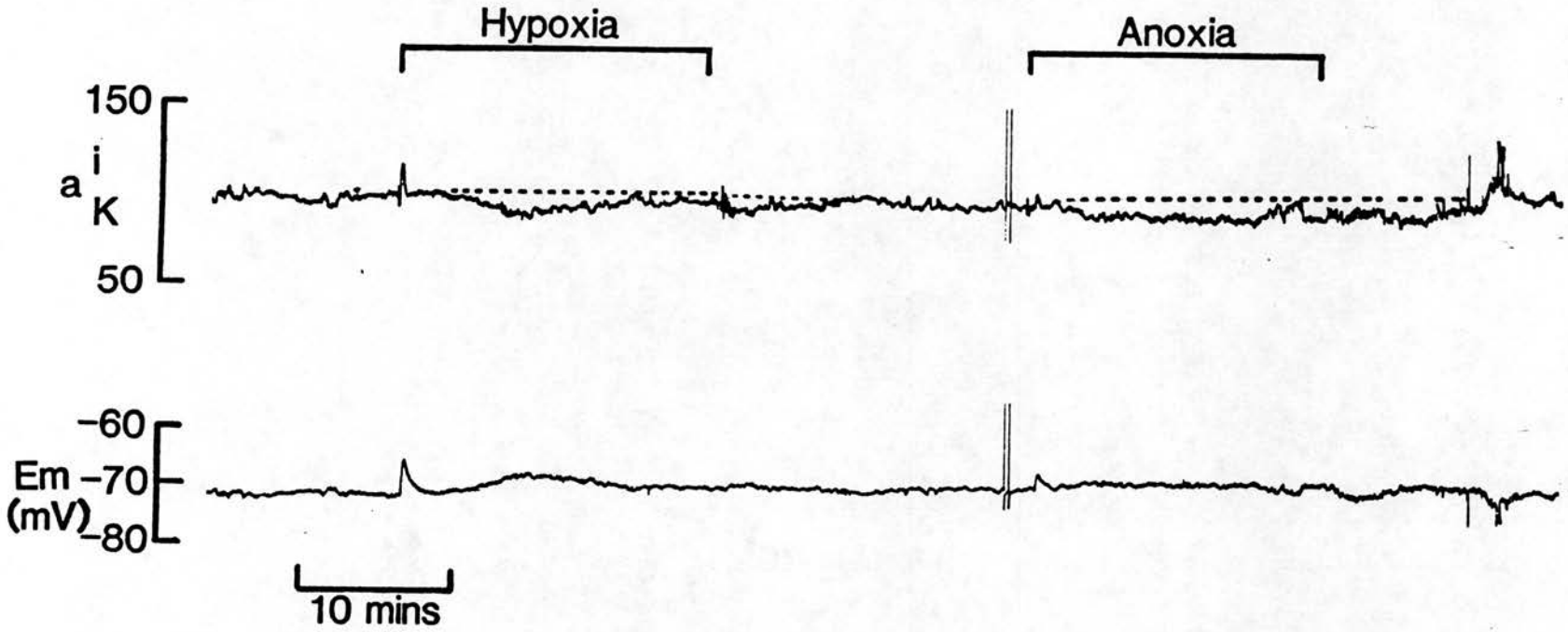
TABLE 1

Compares the measurement of a^i_K using Corning sensor-filled and valinomycin sensor-filled microelectrodes in 5 experiments on ferret papillary muscle. A-E label the individual experiments, 1-4 indicate the number of penetrations with the ion-selective electrodes.

EXPERIMENT No.	CORNING SENSOR PENETRATION				VALINOMYCIN SENSOR PENETRATION			
	1	2	3	4	1	2	3	4
A	79.2	97.7	94.0	-	68.1	100.0	92.5	-
B	78.4	60.7	98.4	122.5	57.7	55.5	-	-
C	61.4	84.4	93.2	80.0	108.0	97.7	105.8	83.6
D	64.4	71.8	71.8	65.1	51.1	62.9	40.0	52.5
E	100.0	100.0	100.0	107.3	87.3	87.3	83.6	74.0

FIGURE 3.18

The change in a^{i}_{K} during 20 minutes exposure to hypoxia and anoxia in a sheep Purkinje fibre measured with a Corning sensor-filled microelectrode.


Hypoxia
Anoxia
150
$a_i K$
50
−60
Em −70
(mV)
−80
10 mins

noise and thus permit more accurate measurements. No previous problems have been reported (apart from Reverdin *et al.* (1986)) when this type of ligand has been used in mammalian heart muscle preparations. Browning et. al. (1981) measured a^i_K in cat papillary muscle and Guarnieri and Strauss (1982) in guinea-pig papillary muscle.

The a^i_K decreased ($P<0.05, n=5$) from 97.6 $\pm$8.1mM to 91.8 $\pm$4.9mM during hypoxia. Assuming an activity coefficient for K^+ of 0.74 (Davies, 1938), this is equivalent to a change in $[K^+]_i$ from 131.9mM to 124.1mM.

During anoxia, produced by the addition of 0.5mM sodium dithionite ($n=2$), there was a mean decrease in $[K^+]_i$ of 9.7mM.

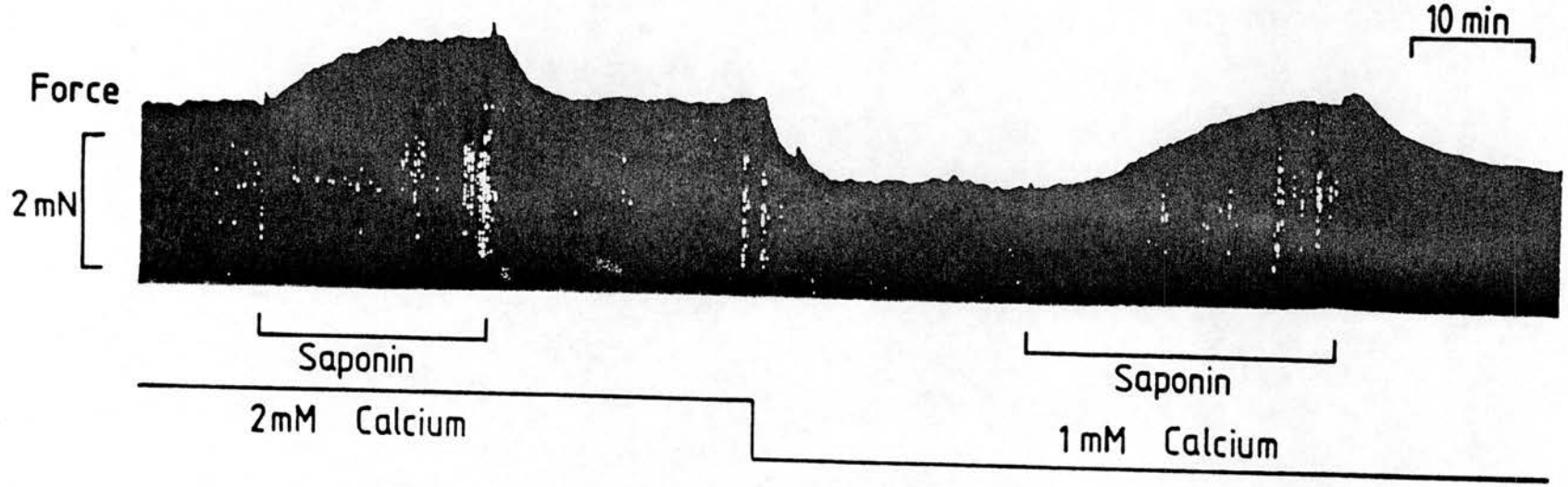
Effects of Saponin and Strophanthidin on a^i_K

Other procedures which are known to alter $[K^+]_i$ were compared with the effects of hypoxia and anoxia. These included the cholesterol attacking substance, saponin and inhibition of Na^+/K^+ pump with the synthetic cardioactive steroid strophanthidin. Saponin can be used to chemically "skin" muscle cells e.g. to permit investigation of the calcium dependence of force production. It has been postulated that at sub-skinning concentrations it can increase the passive permeability of the cell membrane (Yamasaki et. al 1987) and it is known to increase developed tension in myocardial tissue (Enomoto et. al. 1986). Thus the effect of hypoxia and anoxia on a^i_K could be compared with a substance known to inhibit the Na^+/K^+ pump (strophanthidin) and with a factor that probably increases the K^+ permeability of the cell membrane (saponin).

Figure 3.19 shows an experiment where the effect of saponin at a sub-skinning concentration 25ugml^{-1} was applied to ferret papillary muscle at two calcium

FIGURE 3.19

The effect of saponin on developed tension in ferret papillary muscle. The muscle was stimulated at 1Hz in Tyrode solution containing either 2mM Ca^{2+} (normal $[Ca]_o$) or 1mM Ca^{2+}. During the period indicated by the bars 0.025 mg/ml saponin was added to the superfusing solution.


10 min
Force
2 mN
Saponin
2 mM Calcium
Saponin
1 mM Calcium

concentrations. It shows that saponin produced rapid and reversible increases in developed tension, the effect being more marked, though slower to develop, at lower Ca^{2+} concentrations.

The effect of a sub-skinning concentration of saponin ($25ugml^{-1}$) was compared with the effect of strophanthidin ($2x10^{-5}M$) on the a^i_K. At the concentration of strophanthidin used it is known to almost completely inhibit the Na^+/K^+ pump (Deitmer and Ellis , 1978). Treatment with $2x10^{-5}M$ strophanthidin (Fig.3.20) resulted in a decrease of a^i_K ($P<0.01$) from 93.2 $\pm$11.3mM to 80.1 $\pm$9.8mM with an accompanying depolarization from 66.4 $\pm$4.9mV to 59.2 $\pm$6.1mV ($P<0.001$).

Saponin (Fig.3.21) resulted in a decrease ($P<0.05$, $n=5$) in a^i_K from 93 $\pm$7.2 to 78 $\pm$15.5mM). This was accompanied by a large and significant ($P<0.02$) depolarization of Em from 69.0 $\pm$5.0mV to 51.2 $\pm$14.3mV.

(7) TEMPERATURE EFFECTS

Hypothermia has been shown to be effective in combating the deleterious effects of ischaemia (Barner,1977). It is therefore of interest to investigate the effects of hypothermia on the changes of pH_i observed during hypoxia.

The effect of reducing temperature from 35°C to 22°C was tested in 14 experiments (Fig.3.22) on the acidification observed during 20 minutes hypoxia. On changing the bath temperature from 35°C to 22°C resting pH_i rose approximately 0.3 pH units from 7.11 $\pm$0.17 to 7.42 $\pm$0.18. This change was significant ($P<0.001$ Two sample T-Test). There was no significant difference between the values for Em which were 73.6 $\pm$7.5mV and 73.9 $\pm$8.3 at 35°C and 22°C respectively.

The change in pH_i observed in hypoxia was 0.26 $\pm$0.18

FIGURE 3.20

The effect of strophanthidin on a^i_K (top trace), the membrane potential (Em, middle trace) and the resting tension bottom trace in a sheep Purkinje fibre. Strophanthidin (2×10^{-5} M) was applied during the period indicated by the bar.

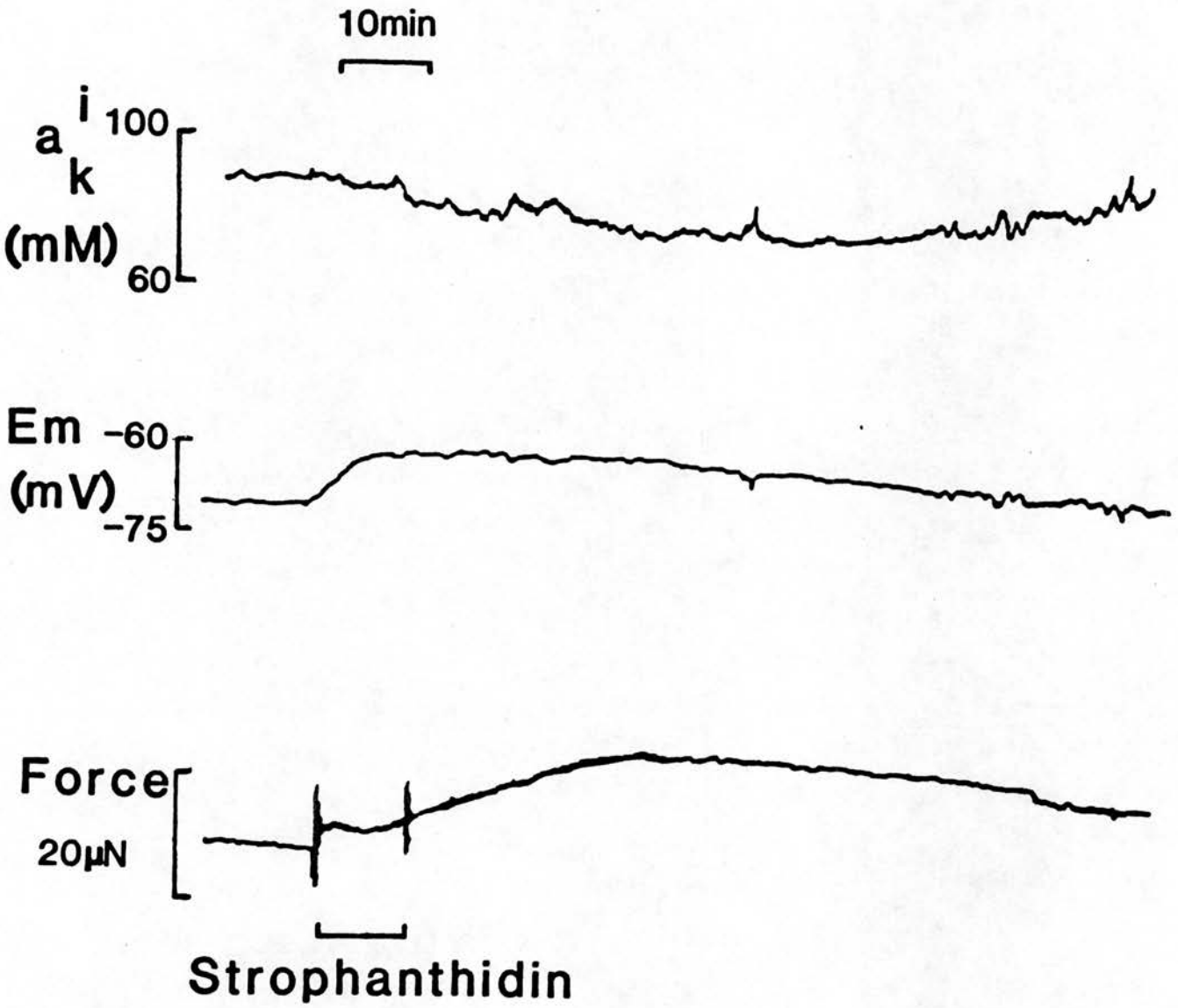

10min
a_k^i
100
(mM)
60
Em
-60
(mV)
-75
Force
20μN
Strophanthidin

FIGURE 3.21

The effect of saponin on a^i_K (top trace), the membrane potential (Em, middle trace) and resting tension (bottom trace) in a sheep heart Purkinje fibre. The bar indicates the priod of exposure to 0.025 mg/ml saponin.

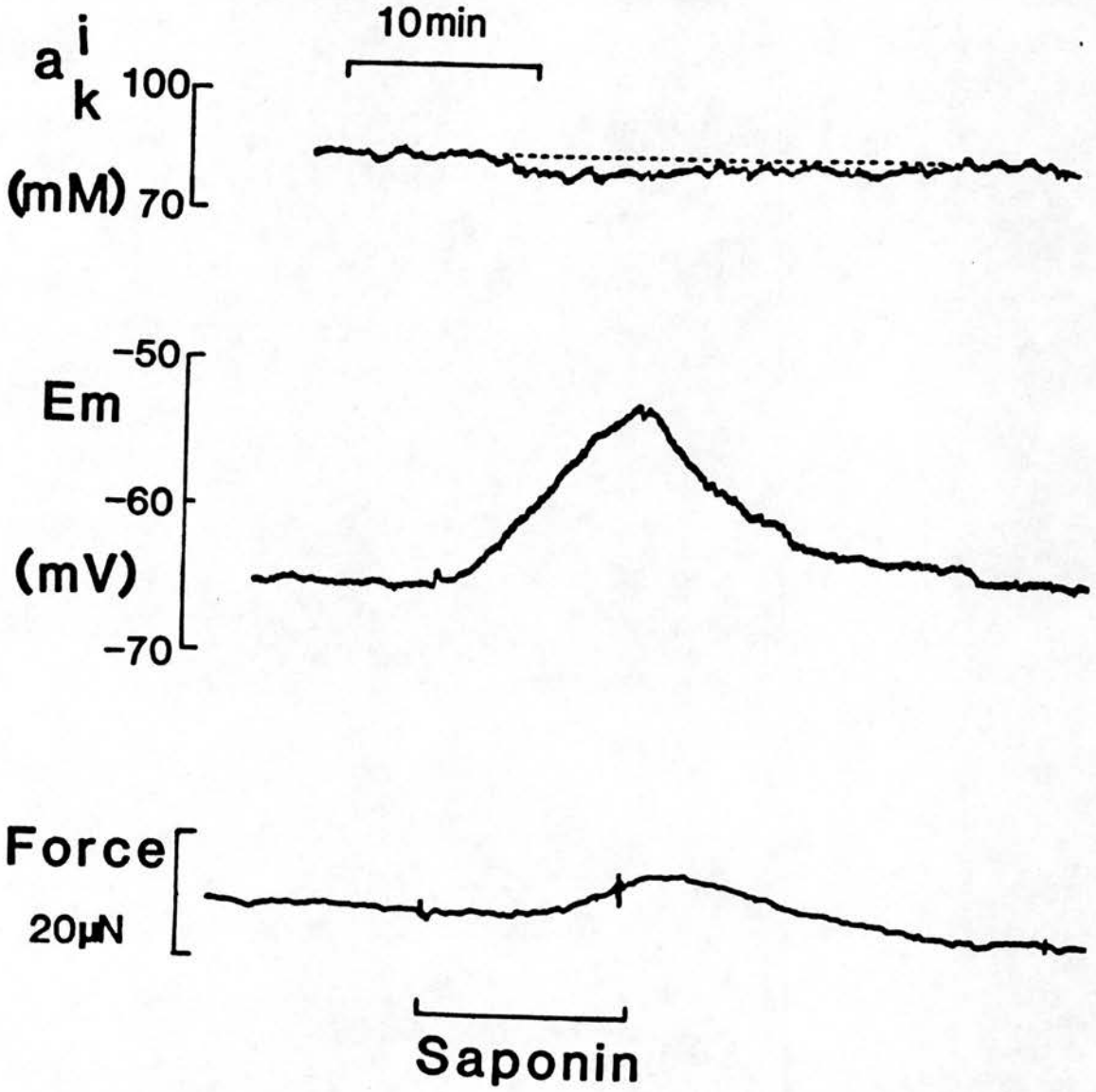
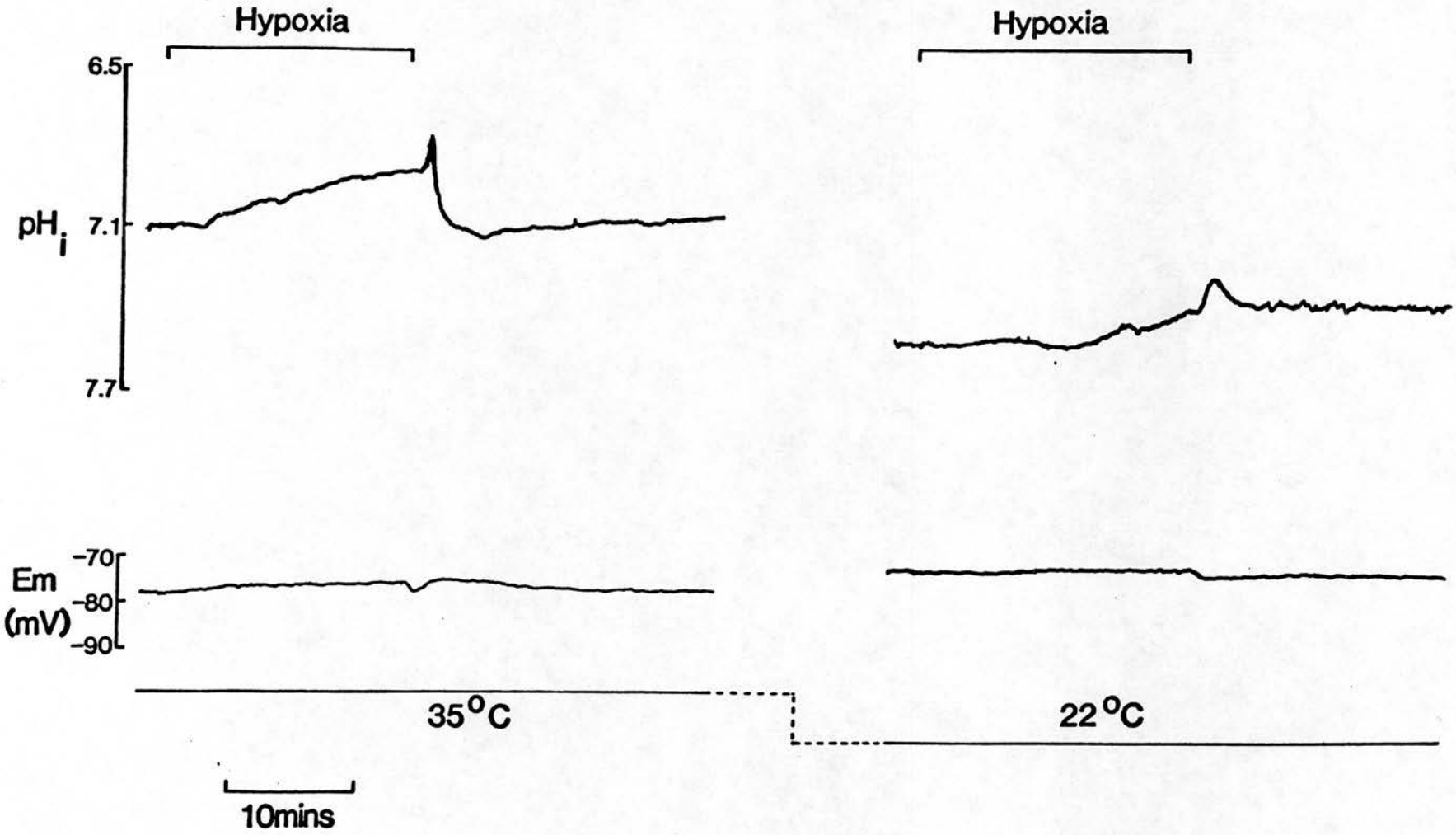

10min
a_k^i
100
(mM)
70
-50
Em
-60
(mV)
-70
Force
20μN
Saponin

FIGURE 3.22

The effect of hypoxia on pH_i in sheep Purkinje fibres at 35°C and 22°C. The dotted line indicates the period during which temperature was lowered. This was complete in less than 4.5 minutes. The pH electrodes were calibrated only at 35°C and at 22°C. Recordings of pH_i during temperature changes could not therefore be calibrated.


Hypoxia
Hypoxia
6.5
pH i
7.1
7.7
Em
(mV)
-70
-80
-90
35°C
22°C
10mins

at 35°C, (equivalent to a change in H^+ ion concentration of $6.3x10^{-8}M$). This was significantly greater ($P<0.01$ Two sample T-test) than the acidification of 0.09 $\pm$0.12 produced by hypoxia at 22°C, (equivalent to a $[H^+]_i$ change of $9.0x10^{-9}M$). The transient acidification on the readdition of O_2 was 0.09 $\pm$0.06 at 35°C compared with 0.06 $\pm$0.08 at 22°C (no significant difference $P<0.1$). During hypoxia at 35°C there was a significant depolarization of Em ($P<0.001$ Two sample T-test) of 1.5mV and a depolarization of 2mV during hypoxia at 22°C ($P<0.01$ Two sample T-test). Comparing hypoxia with anoxia at 22°C (n=4) revealed a larger acidification of 0.17 $\pm$0.07 in anoxia compared with an acidification of 0.05 $\pm$0.05 in hypoxia, this difference was significant. There was no significant difference in the transient change in pH_i observed on reoxygenation ($P>0.1$). Figure 3.23 shows absolute changes in pH_i produced by hypoxia at 35°C and 22°C at three different durations of hypoxia. This type of presentation was used because there is a different time course of pH changes at the two different temperatures. Figure 3.24 shows the maximum rate of change of pH_i during hypoxia against temperature in eight experiments showing that for seven of the experiments the rate of acidification was greater at 35°C than at 22°C.

In other experiments the effect of temperature on developed tension in ferret papillary muscle was tested. Hypothermia increased developed tension (Fig.3.25) to 129 $\pm$13% (n=3) of control (35°C) developed tension. The decrease in tension during hypoxia at 22°C was to 54 $\pm$19% of the control (22°C before hypoxia) compared with a fall to 33 $\pm$13% in hypoxia at 35°C.

In two experiments the effect of raising temperature to 38°C was tested on the response of developed tension in ferret papillary muscle to hypoxia

FIGURE 3.23

The effect of hypoxia on pH_i at durations of 4, 10 and 20 minutes at temperatures of 22°C and 35°C. The change in pH_i is shown as the mean ±S.E. for 6 experiments in sheep Purkinje fibres.

* = Significant difference between 22°C and 35°C ($P<0.001$).

** = Significant difference between 22°C and 35°C ($P<0.01$).

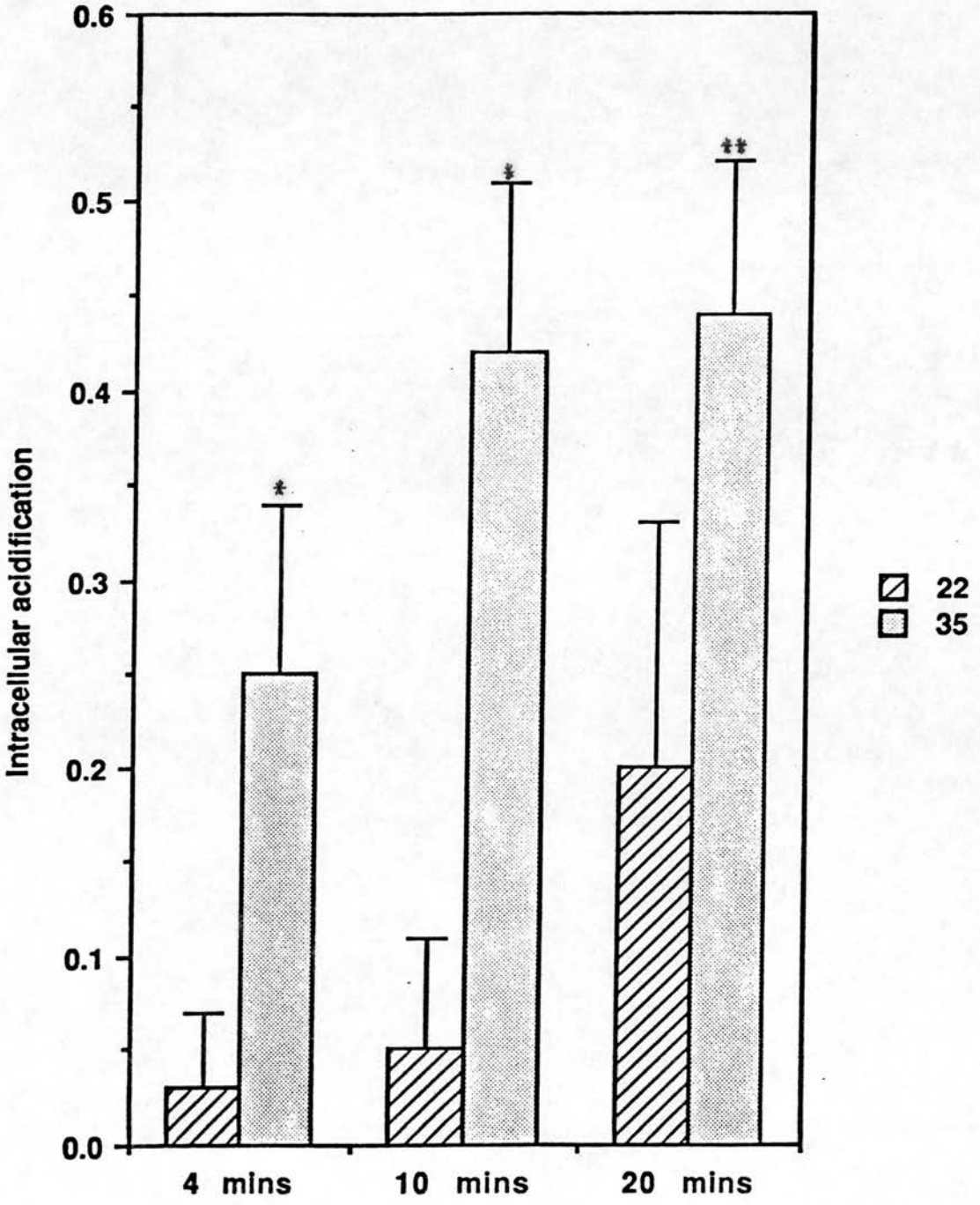

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Intracellular acidification
*
*
**
22
35
4 mins
10 mins
20 mins

FIGURE 3.24

The effect of temperature on the maximum rate of fall of pH_i in hypoxia. The results are from 8 experiments on sheep Purkinje fibres.

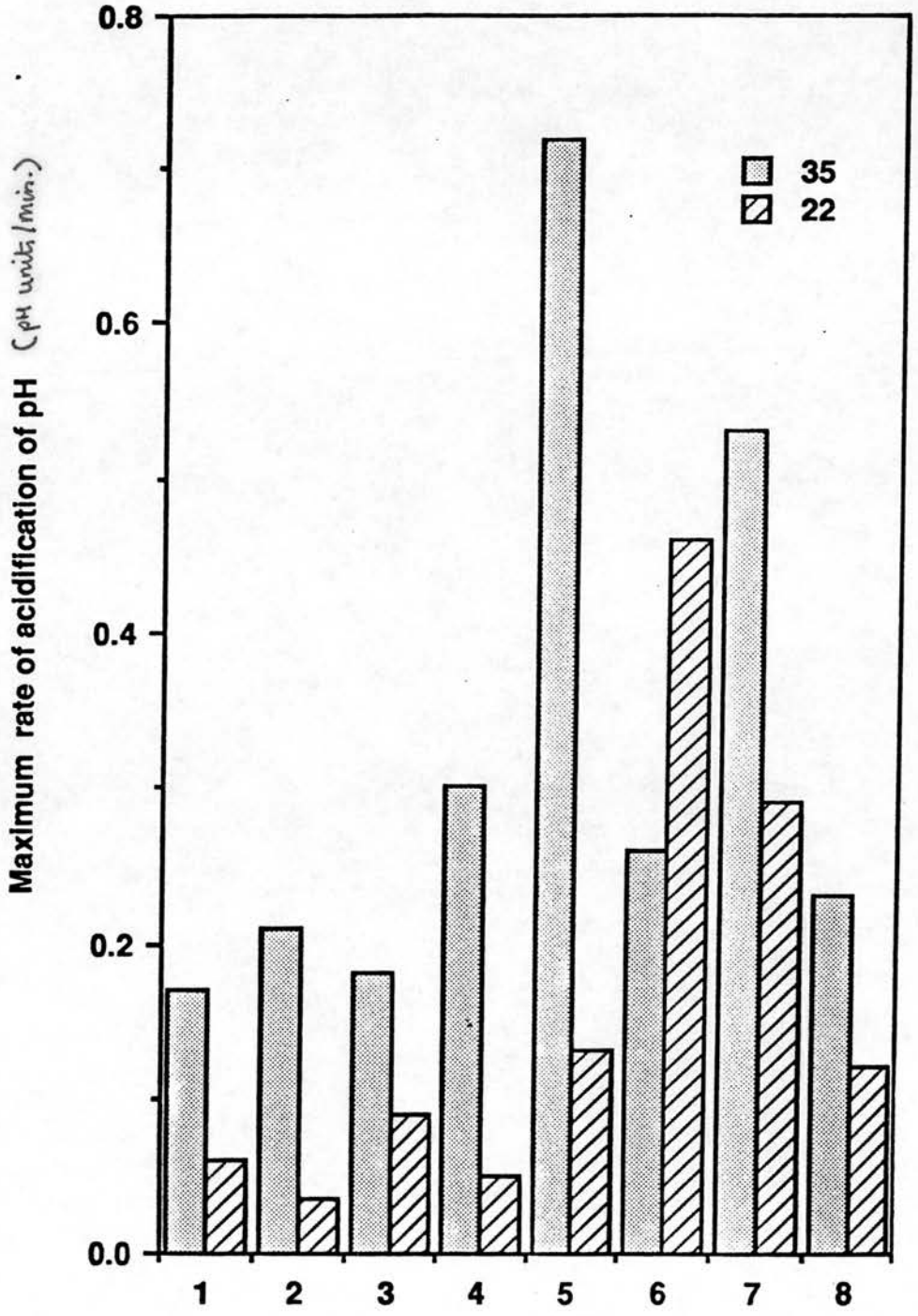

0.8
0.6
0.4
0.2
0.0
35
22
Maximum rate of acidification of pH (pH units/min.)
1
2
3
4
5
6
7
8
Experiment Number

FIGURE 3.25

The effect of hypoxia on developed tension in ferret papillary muscle at 35°C and 22°C, stimulated at 0.2 Hz.

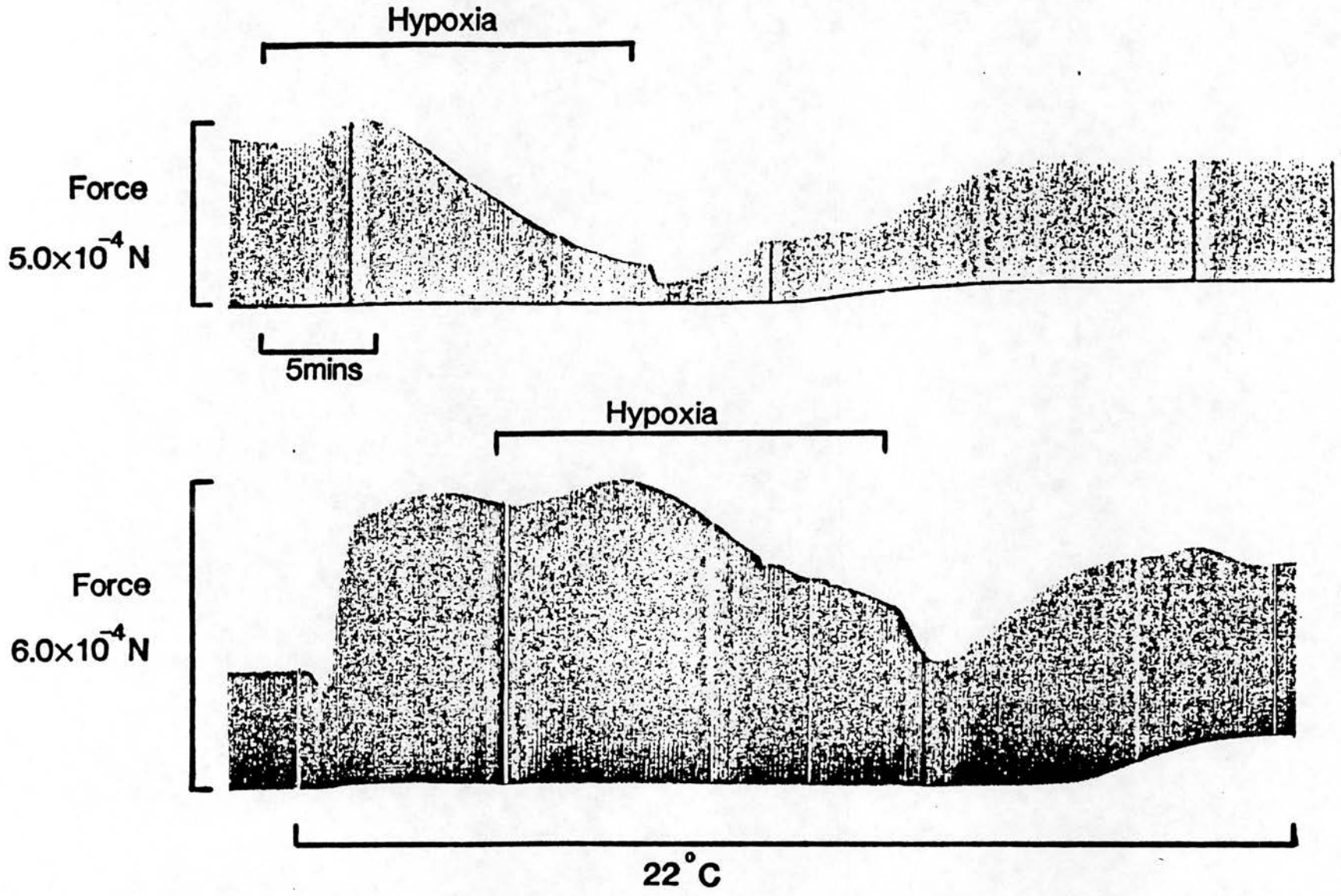
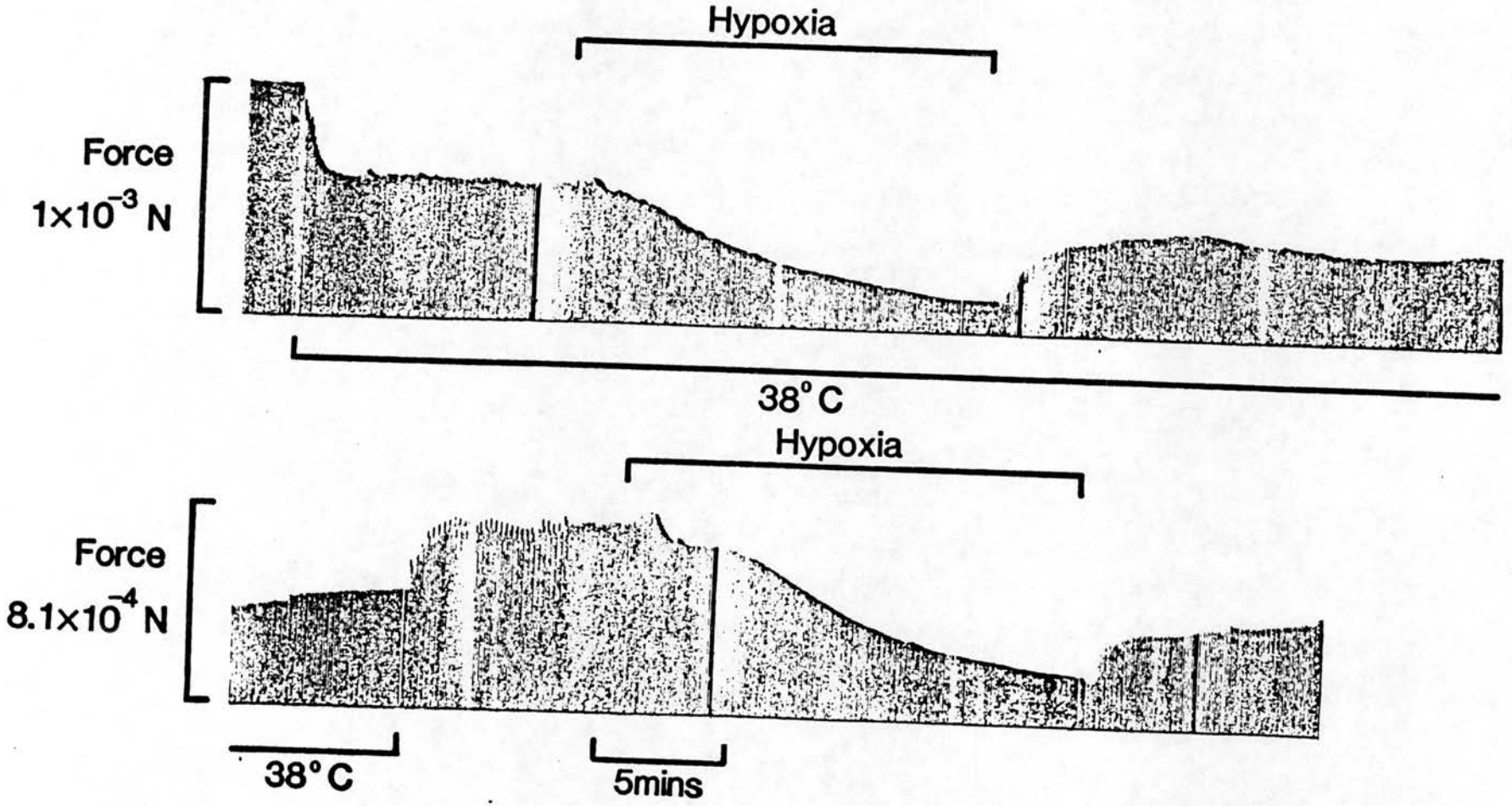

Hypoxia
Force
5.0×10⁻⁴N
5mins
Hypoxia
Force
6.0×10⁻⁴N
22°C

FIGURE 3.26

The effect of increased temperature (38°C) on developed tension in ferret papillary muscle compared with the effect of hypoxia at 35°C. The preparation was stimulated at 0.2 Hz.


Hypoxia
Force
1×10⁻³ N
38° C
Hypoxia
Force
8.1×10⁻⁴ N
38° C
5mins

(Fig.3.26). The developed tension fell to approximately 44% of the control on raising the temperature by 3°C. During hypoxia at 35°C developed tension fell to 51% of control tension compared with a fall to approximately 36% of the control tension at 38°C.

(8) SUBSTRATE EFFECTS

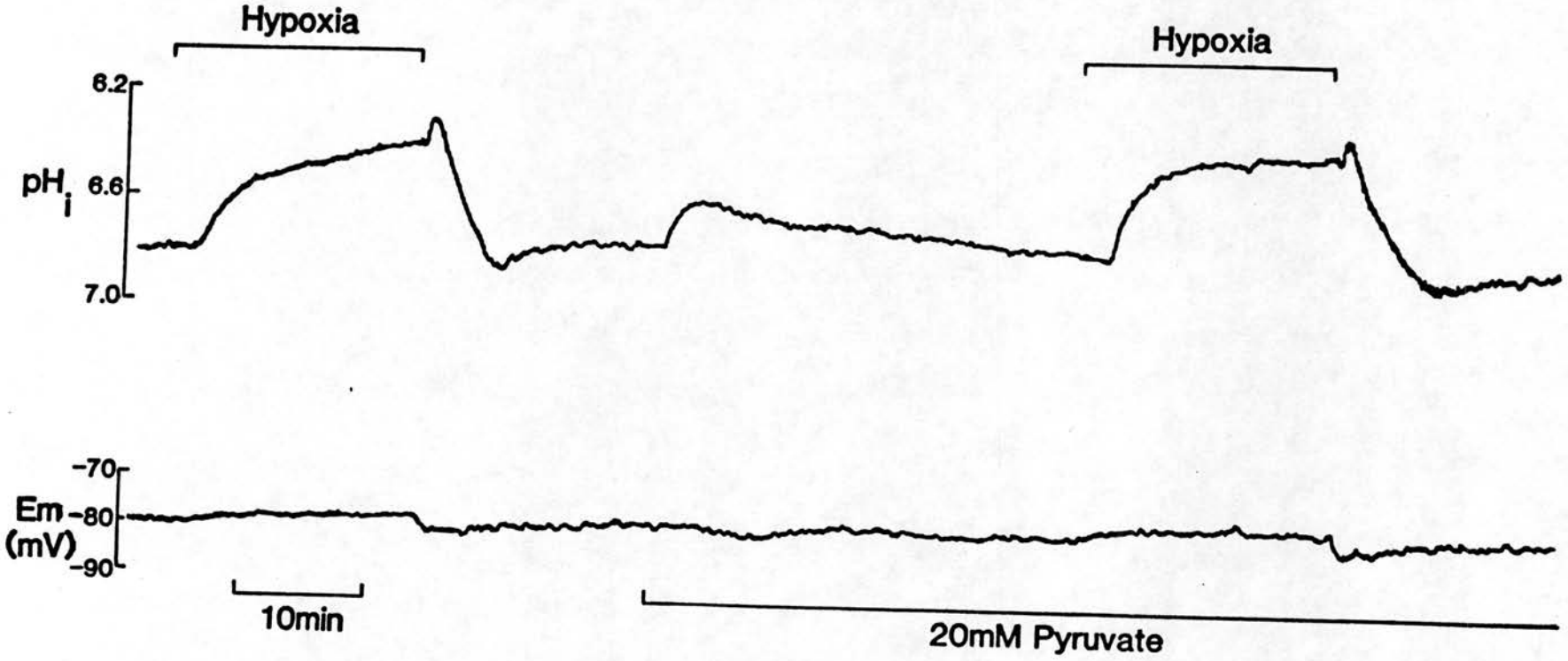
The mammalian heart is able to utilize a number of substrates in the absence of glucose. It has been suggested (Liedtke et al.1976) that in globally ischemic perfused heart preparations, pyruvate in a suitably buffered medium reduces the severity of ischemic injury. The effect of a number of substrates were compared in the absence of glucose to see if they could protect sheep Purkinje fibre preparations against hypoxia-induced changes in pH_i. The substrates used were 20mM pyruvate, acetate, lactate or 20mM glucose. The preparation was exposed to the substrates in glucose-free Tyrode solution for 30 minutes before a 20 minute exposure to hypoxia and a 20 minute recovery period in the substrate. Pyruvate like other weak acids produces a transient acidification of pH_i (de Hemptinne et al.1983). This recovers in about 20 minutes (Fig.3.27).

There was no significant decrease in pH_i on adding pyruvate to normal Tyrode solution. There was no significant change in membrane potential.

There was no significant difference ($P>0.1$) between the acidification of pH_i during hypoxia compared with that during hypoxia in pyruvate. There was no significant difference between the membrane potential changes (Fig.3.27). Although Figure 3.27 appears to show a more rapid rate of intracellular acidification during hypoxia in the pyruvate solution, this was not the usual finding in these experiments.

FIGURE 3.27

The effect of 20 mM pyruvate on the change in pH_i in hypoxia compared with normal Tyrode (10mM glucose, 10mM sucrose) during hypoxia in a sheep Purkinje fibre.


Hypoxia
Hypoxia
6.2
pH$_i$ 6.6
7.0
-70
Em -80
(mV) -90
10min
20mM Pyruvate

Acetate did not alter pH_i except transiently in the first 20 minutes of the equilibration period. The Em was also unaffected by acetate. Hypoxia caused a decrease in pH_i from 7.0 $\pm$0.21 to 6.72 $\pm$0.35(n=8) which was not significantly different from the acidification during hypoxia in acetate solution which was 6.65 $\pm$0.34.

High glucose concentration (20mM) caused no increase in pH_i from 6.9 $\pm$0.3 (n=5) to 6.94 $\pm$0.35 in 20mM glucose the difference was not significant (P>0.1). There was no significant change of Em. During hypoxia there was a decrease in pH_i to 6.55 $\pm$0.37 in normal Tyrode which was not significantly different from the fall in 20mM glucose to 6.6 $\pm$0.36. The cells depolarised in normal hypoxia from 80.0 $\pm$5.6mV to 79.4 $\pm$8.1mV, which was not significantly different (P>0.1) from the 80.0 $\pm$5.6mV to 74.6 $\pm$7.6mV change in hypoxia in 20mM glucose (P<0.02).

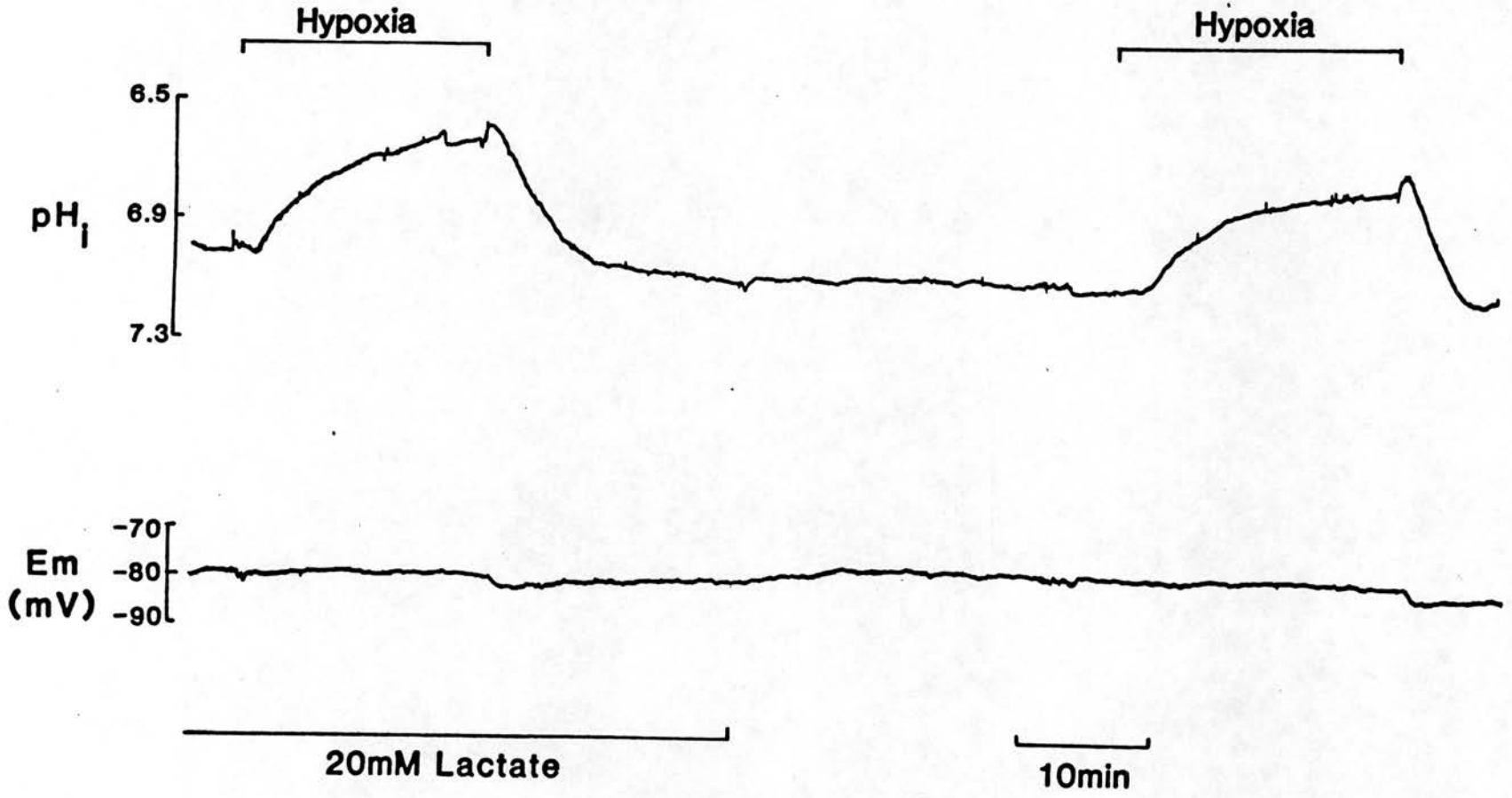
In 2 experiments involving 20mM lactate as a substrate, the pH_i transiently decreased from a mean control value of pH 7.32 to pH 7.20 before returning to the control level. The Em hyperpolarised slightly in lactate from the control value of 70mV to 75mV during the 30 min equilibration period. During hypoxia pH_i decreased to a mean value of pH 7.10 in normal Tyrode, compared with an acidification to pH 6.90 in lactate solution (e.g. Fig. 3.28). The transient acidosis on reoxygenation was approximately 0.1 pH units in both normal Tyrode and lactate Tyrode.

(9) EFFECTS OF CINNAMATE

Previous work (Halestrap and Denton 1974), has shown that cinnamate depresses pyruvate transport across the cell membrane of human erythrocytes and the mitochondrial membrane of rat liver cells. De Hemptinne et al. (1983)

FIGURE 3.28

The effect of 20 mM lactate on the pH_i change during hypoxia compared with that seen in normal Tyrode (10mM glucose, 10mM sucrose) during hypoxia in a sheep Purkinje fibre.


Hypoxia
Hypoxia
6.5
pH$_i$
6.9
7.3
-70
Em
(mV)
-80
-90
20mM Lactate
10min

showed that cinnamate also had an effect on the transport of a number of organic acids including lactic acid. Mason and Thomas (1988) used cinnamate to block the carrier mediated component of L-lactate transport across the membrane of frog sartorius muscle.

The effect of blocking lactate transport during hypoxia was investigated in sheep Purkinje fibres by equilibrating the preparation for 20 min with Tyrode to which was added 5mM sodium cinnamate. The preparation was then subjected to 20 min hypoxia followed by 20 min recovery still in the presence of cinnamate.

In 4 experiments on sheep Purkinje fibre (Fig.3.29) pH_i appeared to decrease in the presence of 5mM cinnamate from a 7.21 $\pm$0.10 pH units in normal Tyrode to 7.11 $\pm$0.11 pH units however this change was found to be not significant ($P>0.1$). In hypoxia pH_i acidified by 0.15 $\pm$0.05 which was significantly less than the change in pH_i in hypoxia in the presence of cinnamate ($P<0.05$) of 0.21 $\pm$0.16 pH units. On reoxygenation of the preparation the pH_i transiently decreased further by 0.34 $\pm$0.25 pH units in normal Tyrode and by 0.40 $\pm$0.42 pH units in cinnamate. This transient change in pH_i was not significantly greater in cinnamate ($P<0.1$).

Figure 3.30 illustrates the effect of cinnamate in substantially slowing the maximum rate of recovery of pH_i on reoxygenation in 4 experiments. The recovery of pH_i was also shown to be slower in the presence of extracellular lactate as the cell substrate (see above). Thus the decrease in lactate efflux caused by cinnamate appears to have a similar effect on the recovery of pH_i on reoxygenation.

In 3 experiments the effects of hypoxia on pH_s of sheep Purkinje fibres in normal Tyrode and in cinnamate were compared (not illustrated). On changing from normal Tyrode to cinnamate the pH_s became slightly more

FIGURE 3.29

The effect of 5mM sodium cinnamate on the size of the change in pH_i during hypoxia compared with that in normal Tyrode in a sheep Purkinje fibre.

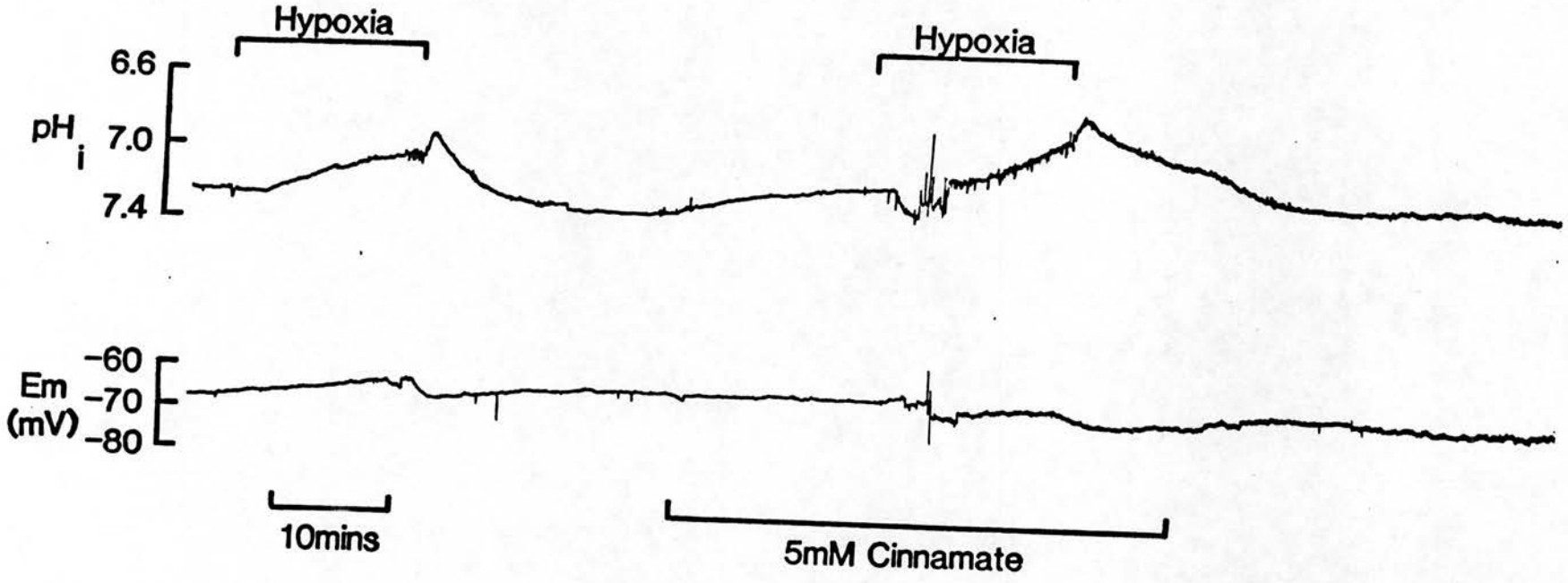
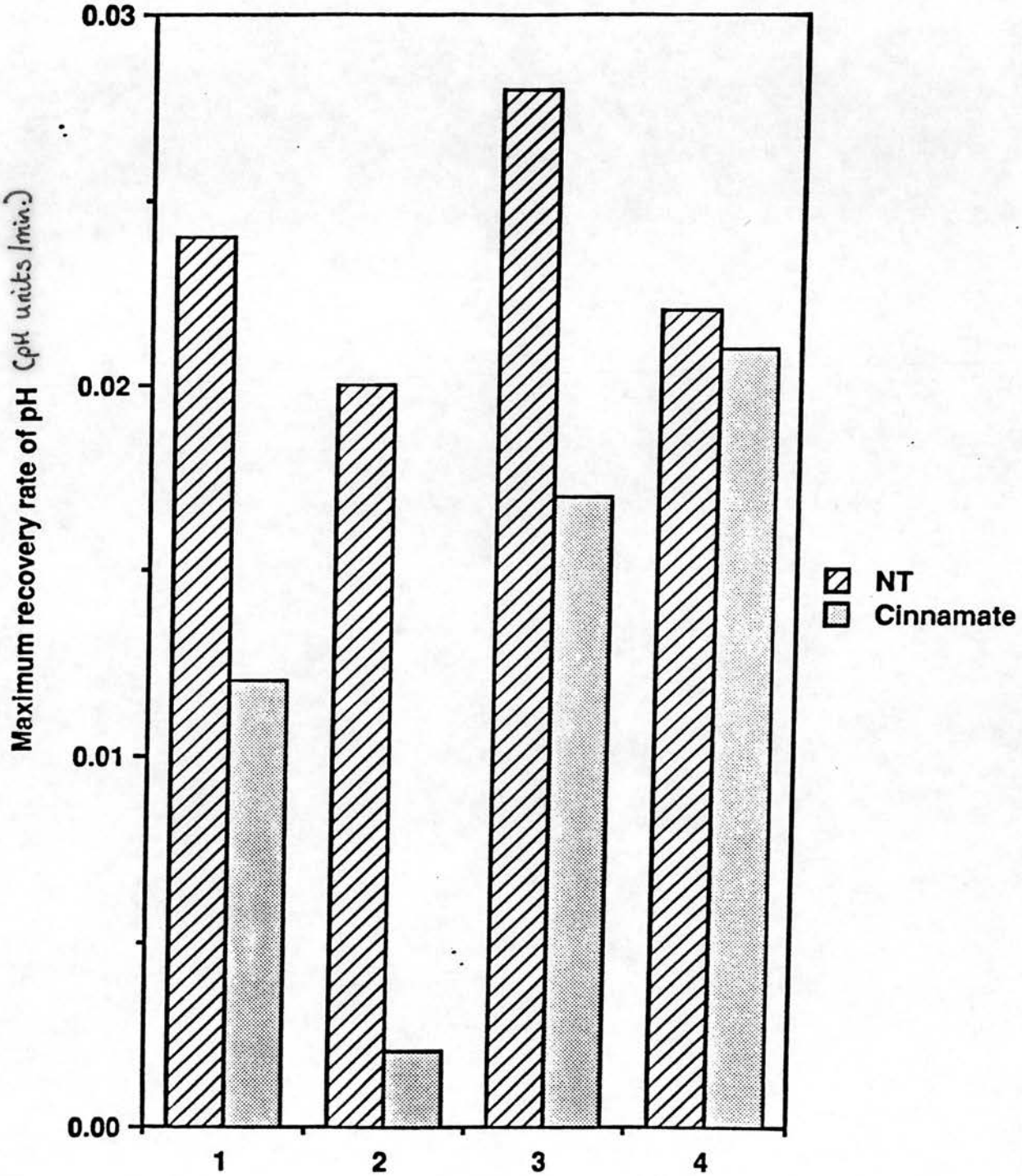

Hypoxia
Hypoxia
6.6
pH i 7.0
7.4
-60
Em -70
(mV) -80
10mins
5mM Cinnamate

FIGURE 3.30

The effect of 5mM sodium cinnamate on the maximum recovery rate of pH_i on reoxygenation after hypoxia compared with the recovery rate on reoxygenation in normal Tyrode in sheep Purkinje fibres (n=4).


0.03
0.02
0.01
0.00
Maximum recovery rate of pH (pH units /min.)
1
2
3
4
Experiment Number
NT
Cinnamate

alkaline, changing from pH 7.2 ±0.1 to 7.25 ±0.1. During hypoxia there was very little difference in the acidification (7.14 ±0.11 in normal Tyrode and 7.13 ±0.11 in cinnamate).

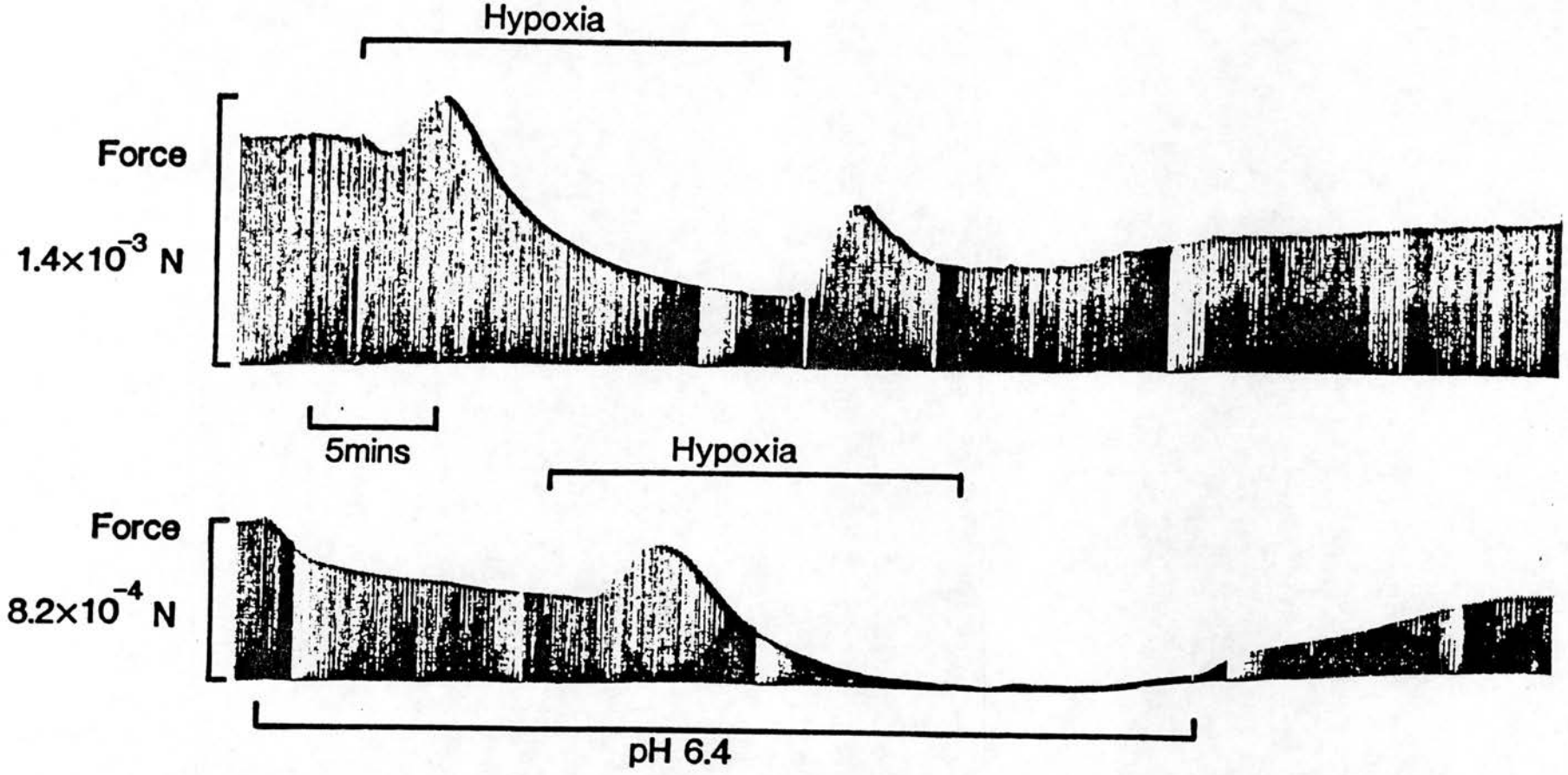
(10) EXTRACELLULAR ACIDOSIS

During ischemic changes in the heart extracellular pH becomes more acidic as H^+ ions accumulate in the extracellular space as a result of reduced or absent blood flow (Katz and Hecht, 1969). Acidosis has also been shown to depress the contractility of cardiac myofibrils (Fabiato and Fabiato, 1978).

In one experiment the effect of low pH_o (6.4) on the size of the acidification of pH_i during hypoxia was investigated (not illustrated). Exposure to pH 6.4 Tyrode for 20 minutes resulted in an intracellular acidification of about 0.1 pH unit. Exposure to hypoxia (pH 6.4) caused a further acidification of pH_i by 0.3 pH units (compared to an acidification of 0.11 pH units in hypoxia at pH_o 7.4). On reoxygenation there was a transient acidification of pH_i of 0.16 pH units at pH_o 6.4 (compared to 0.17 units at pH_o 7.4). The experiment was repeated on developed tension of ferret papillary muscle and is illustrated by Figure 3.31. In 2 experiments on ferret papillary muscle developed tension dropped to a mean value of 27% of the control tension on switching to pH 6.4 Tyrode (PIPES buffered). During hypoxia in normal Tyrode tension dropped to 30% of the control. In pH 6.4 Tyrode hypoxia produced a decrease of developed tension to 9% of the control.

FIGURE 3.31

The effect of acid extracellular pH (pH 6.4) on developed tension in a ferret papillary muscle during hypoxia compared with hypoxia at pH 7.4.


Hypoxia
Force
1.4×10⁻³ N
5mins
Hypoxia
Force
8.2×10⁻⁴ N
pH 6.4

(11) EXTRACELLULAR ALKALOSIS

Figure 3.32 illustrates the effect of increasing the pH of the superfusion solution to pH 8.4 (TAPS buffered). The pH_i changes to a new alkaline steady-state level within about 30-40 minutes. The hypoxic acidification is reduced under these conditions as is the acidification on reoxgenation.

The experiment was repeated to test the effect of extracellular pH on developed tension in 2 ferret papillary muscle preparations. Changing to pH 8.4 perfusate caused a large rise in developed tension (Fig.3.33) to a mean value of 154% of the control tension. During hypoxia in normal Tyrode tension dropped to 30% of control, compared with a drop to 14% of the control tension in pH 8.4 solution. In these experiments 20 minutes exposure to hypoxia resulted in the development of a contracture soon after the readdition of O_2 to the pH 8.4 solution.

(12) EXTRACELLULAR BUFFERING

Extracellular buffering may have an important influence on the acidification of pH_i during hypoxia since the level of extracellular buffering will affect the H^+ ions at the outer surface of the cell and therefore the pH inside the cell. De Hemptinne et al. (1987) showed that high extracellular buffer capacity gave a more alkaline pH_i perhaps due to an acceleration of proton extrusion. HEPES and bicarbonate buffered solutions were compared for their effects on pH_i during hypoxia.

In 15 experiments where a direct comparison was made between the two types of buffer (e.g. Fig.3.34), the pH_i became acidified on changing to bicarbonate buffer

FIGURE 3.32

The effect of alkaline pH_o (pH 8.4) on the change in pH_i during hypoxia compared with hypoxia in pH 7.4 solution in a sheep Purkinje fibre. The dotted lines indicate breaks in the trace of the same length during which time both electrodes remained intracellular.

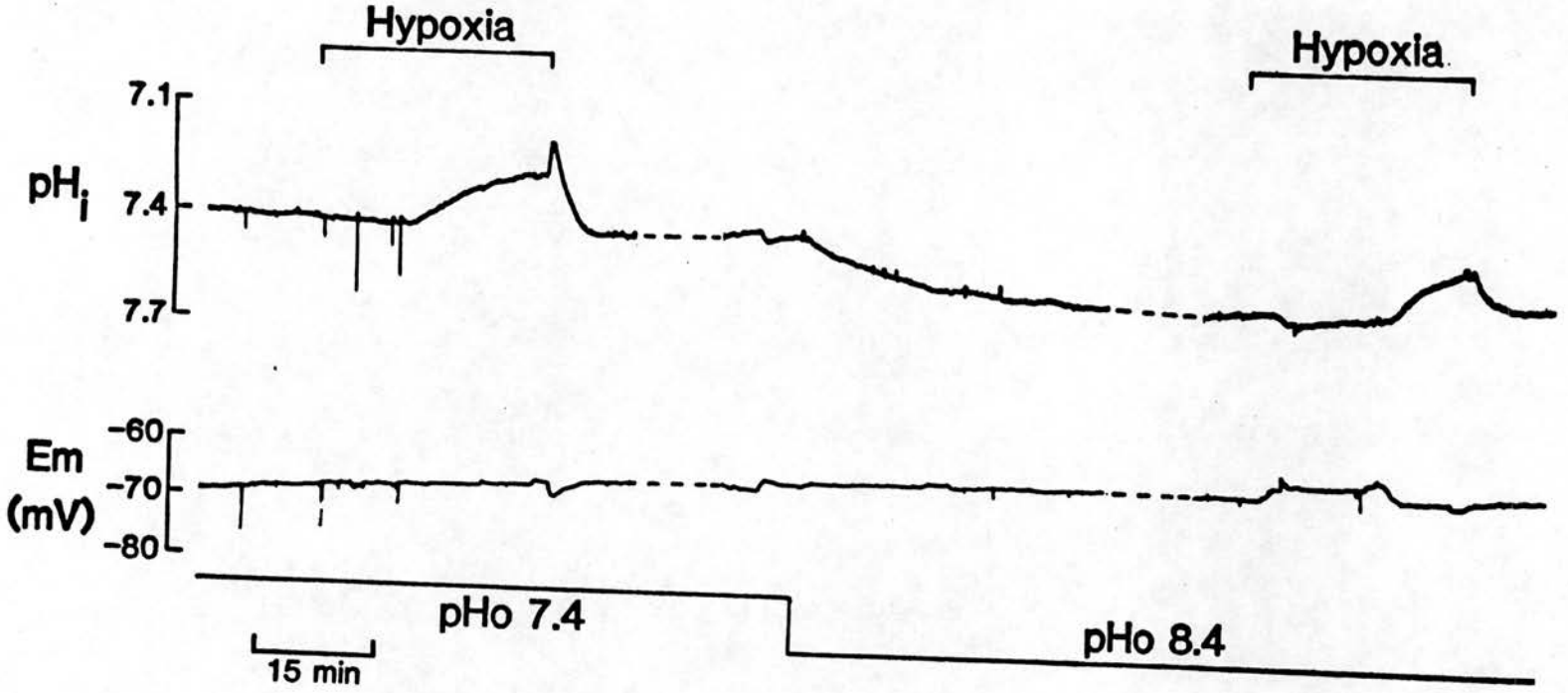

Hypoxia
Hypoxia
7.1
pH$_i$
7.4
7.7
-60
Em
(mV)
-70
-80
pHo 7.4
pHo 8.4
15 min

FIGURE 3.33

The effect of alkaline extracellular pH (pH 8.4) on the developed tension during hypoxia compared with the effect of hypoxia at pH 7.4 in a ferret papillary muscle.

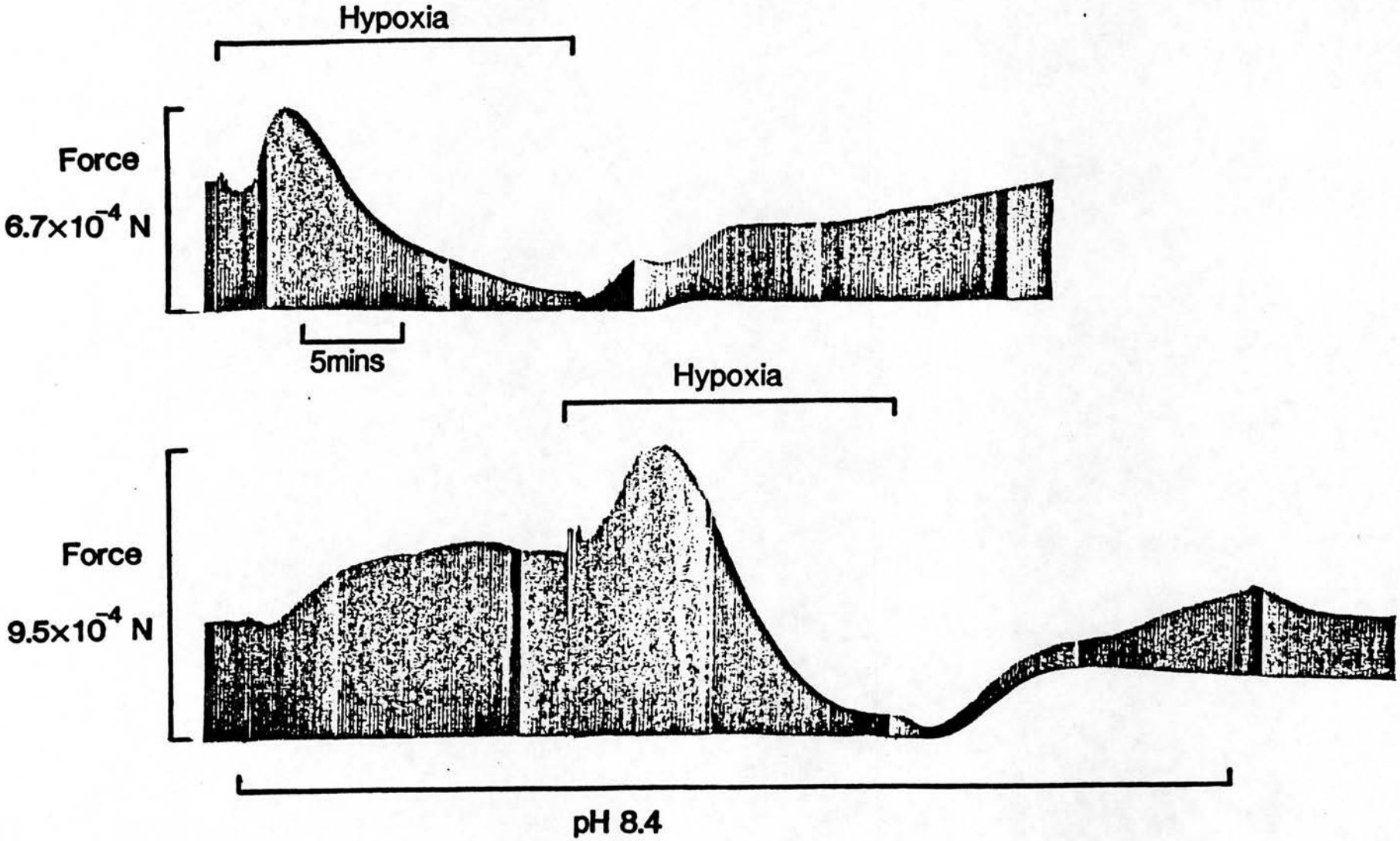
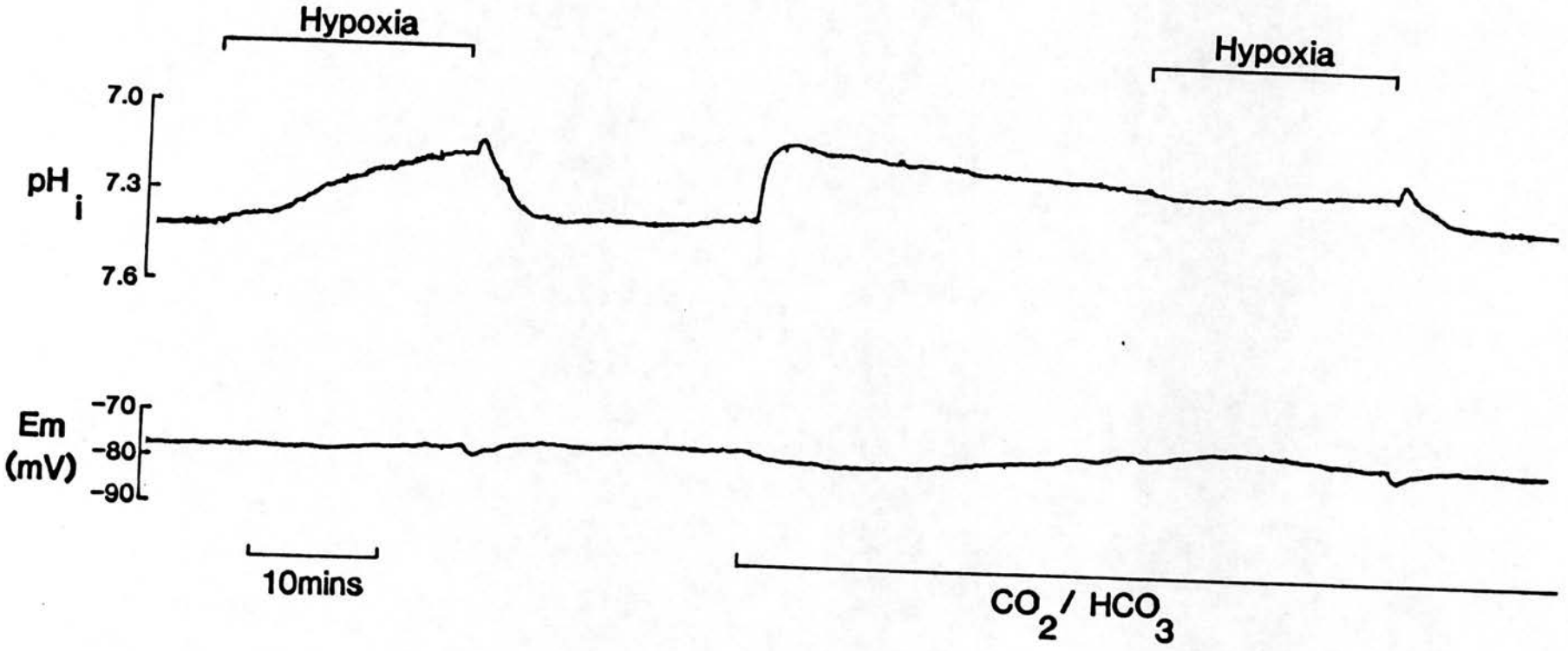

Hypoxia
Force
6.7×10⁻⁴ N
5mins
Hypoxia
Force
9.5×10⁻⁴ N
pH 8.4

FIGURE 3.34

The effect of extracellular buffering on the change in pH_i seen during hypoxia in sheep Purkinje fibre. The effect of hypoxia in normal Tyrode (10mM HEPES) was compared with the effect hypoxia in CO_2/HCO_3^- buffered solution (24mM $NaHCO_3$, 5% CO_2).


Hypoxia
Hypoxia
7.0
pH i
7.3
7.6
-70
Em
(mV)
-80
-90
10mins
CO2 / HCO3

(24mM HCO_3, 5% CO_2). The pH_i decreased from 7.06 $\pm$0.2 in HEPES to 6.83 $\pm$0.25 in bicarbonate buffer. This difference was significant ($P<0.001$). There was no significant change in membrane potential from its control value of 73.7 $\pm$5.6mV. The change in pH_i produced during 20 minutes of hypoxia was significantly greater in HEPES buffered solution (0.38 $\pm$0.29 pH unit) compared with bicarbonate solution (0.20 $\pm$0.17 pH unit) ($P<0.002$). The transient acidification on reoxygenation was not significantly different in HEPES (0.11 $\pm$0.04 pH unit) compared with bicarbonate (0.14 $\pm$0.10 pH unit) ($P<0.1$). In HEPES buffered hypoxia there was a significant depolarization of Em by 3.3 $\pm$1.9mV ($P<0.001$). There was also a significant depolarization by 3.0 $\pm$1.4mV in bicarbonate buffered solution.

It could be suggested that the smaller change in pH_i observed in bicarbonate buffered Tyrode is a direct result of the more acid pH_i before hypoxia. If this were the case one might expect to see a correlation between the pH_i before exposure and the amplitude of the acidification produced by hypoxia. To investigate this possibilty the Pearson correlation coefficient was calculated for data from 64 experiments and a scatter diagram was plotted (Figure 3.35). There was found to be no correlation (positive or negative association) between the initial pH_i and the extent of acidification of pH_i during hypoxia.

In 5 experiments the effect of increasing the extracellular buffering power was investigated. The effect of hypoxia on pH_i of sheep Purkinje fibres in 40mM HEPES Tyrode was compared with normal Tyrode (10mM HEPES), to which was added 60mM sucrose to produce a solution of the same osmolarity (Fig.3.36). On changing from normal Tyrode (high osmolarity) to high HEPES solution, pH_i appeared to increase slightly from 7.31

FIGURE 3.35

Scatter plot showing the change in pH_i during hypoxia compared with the pH_i before hypoxia for 64 experiments. There was no correlation between the size of the acidification in hypoxia and the initial pH_i in sheep Purkinje fibres.

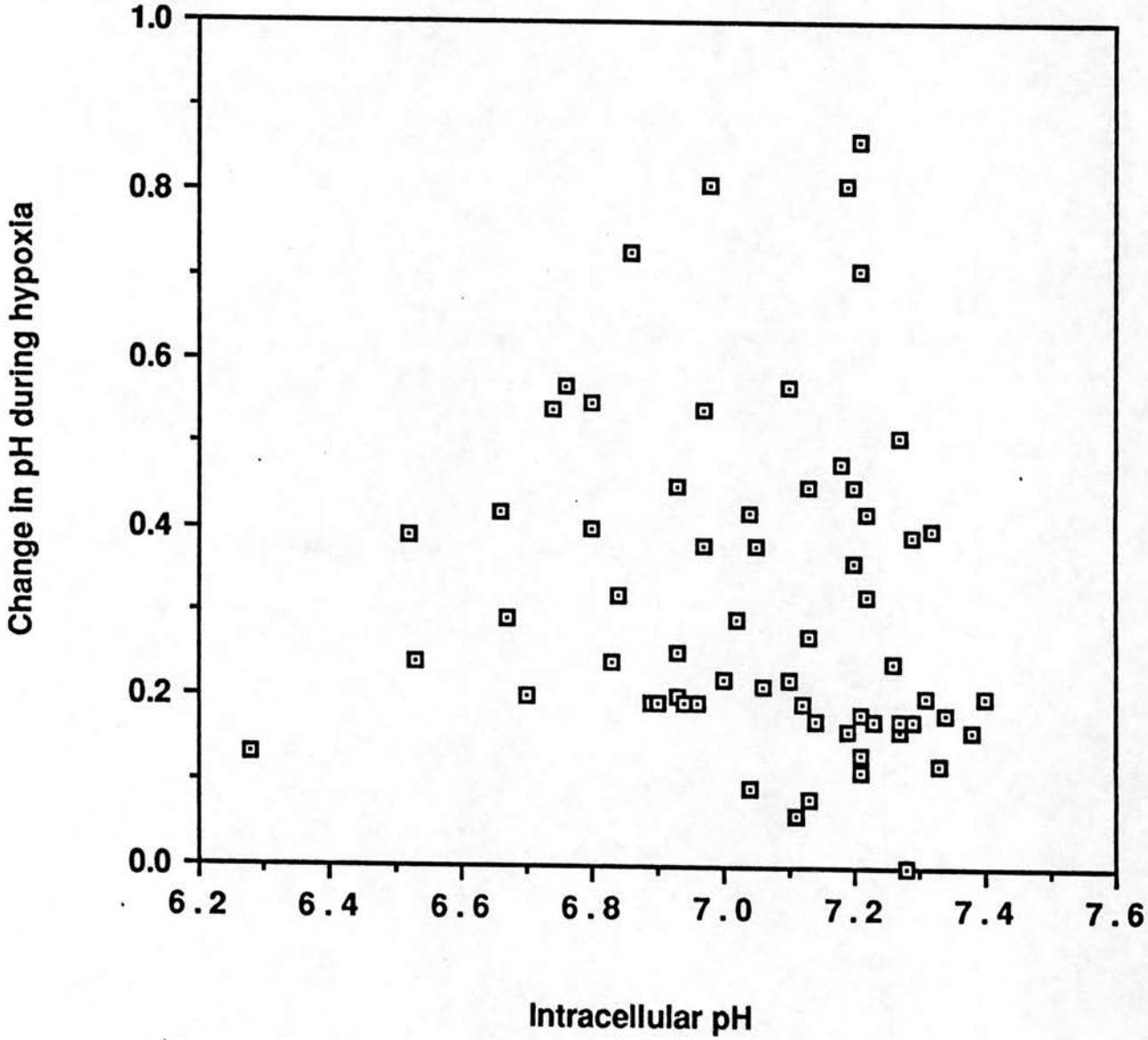

Change in pH during hypoxia
1.0
0.8
0.6
0.4
0.2
0.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
Intracellular pH

FIGURE 3.36

The effect of high extracellular buffering (40mM HEPES) on pH_i in a sheep Purkinje fibre during hypoxia compared with the change in pH_i on exposure to normal 10mM HEPES Tyrode (with 60 mM sucrose added).

Em
(mV)
-60
-70
-80
10 mins
pH
i
7.6
7.2
6.8
Hypoxia
Hypoxia
40mM HEPES

$\pm$0.05 to 7.34 $\pm$0.04 but this was not significant ($P>0.1$). During hypoxia there was a significantly larger decrease in pH_i in normal Tyrode than in high HEPES solution ($P<0.01$). The pH_i decreased to 7.11 $\pm$0.12 in normal Tyrode compared with 7.21 $\pm$0.09 in high HEPES (e.g. Fig.3.36). On reoxygenation the transient acidification was greater in normal Tyrode ($P<0.05$ for the change in pH_i), 7.07 $\pm$0.17 compared with 7.18 $\pm$0.10 in high HEPES. In Figure 3.36 a large alkaline undershoot occurs. However this was not common to the other experiments. Figure 3.37 summarizes the results of experiments comparing the change in pH_i during hypoxia in normal Tyrode (high osmolarity) with that in high HEPES hypoxia. 10mM HEPES (plus 60mM sucrose) produced the same intracellular acidification as normal Tyrode (10mM HEPES, no sucrose), there appeared to be no effect of sucrose on pH_i.

An experiment showing the effects of these procedures on pH_s during hypoxia is shown in Figure 3.38. It can be seen that on changing from normal Tyrode to 40mM HEPES there is an alkalinization of pH_s as might be expected (Vanheel et al.,1986). However the reason for the acidification of pH_s in 10mM HEPES + 60 mM sucrose Tyrode before hypoxia is unclear but may be the result of the solution affecting the bath electrode.

(13) EFFECT OF AMILORIDE

Amiloride has been shown to inhibit the Na/H exchanger in cardiac tissues (Deitmer and Ellis, 1980). The effect of exposing sheep Purkinje fibres to 1mM amiloride for 20 minutes before, during and after hypoxia, was examined to try to elucidate what role (if any) that Na/H exchange has in pH changes associated with hypoxia and recovery from hypoxia.

FIGURE 3.37

The effect of high concentration (40mM) HEPES buffered solution compared with 10mM HEPES Tyrode (60 mM sucrose) on the acidification during hypoxia and the transient extra acidification on reoxygenation after hypoxia. The results are from 6 experiments on sheep Purkinje fibres and are represented as mean $\pm$S.E.

*= Significant difference between high sucrose and high HEPES ($P<0.01$).

**= Significant difference between high sucrose and high HEPES ($P<0.05$).

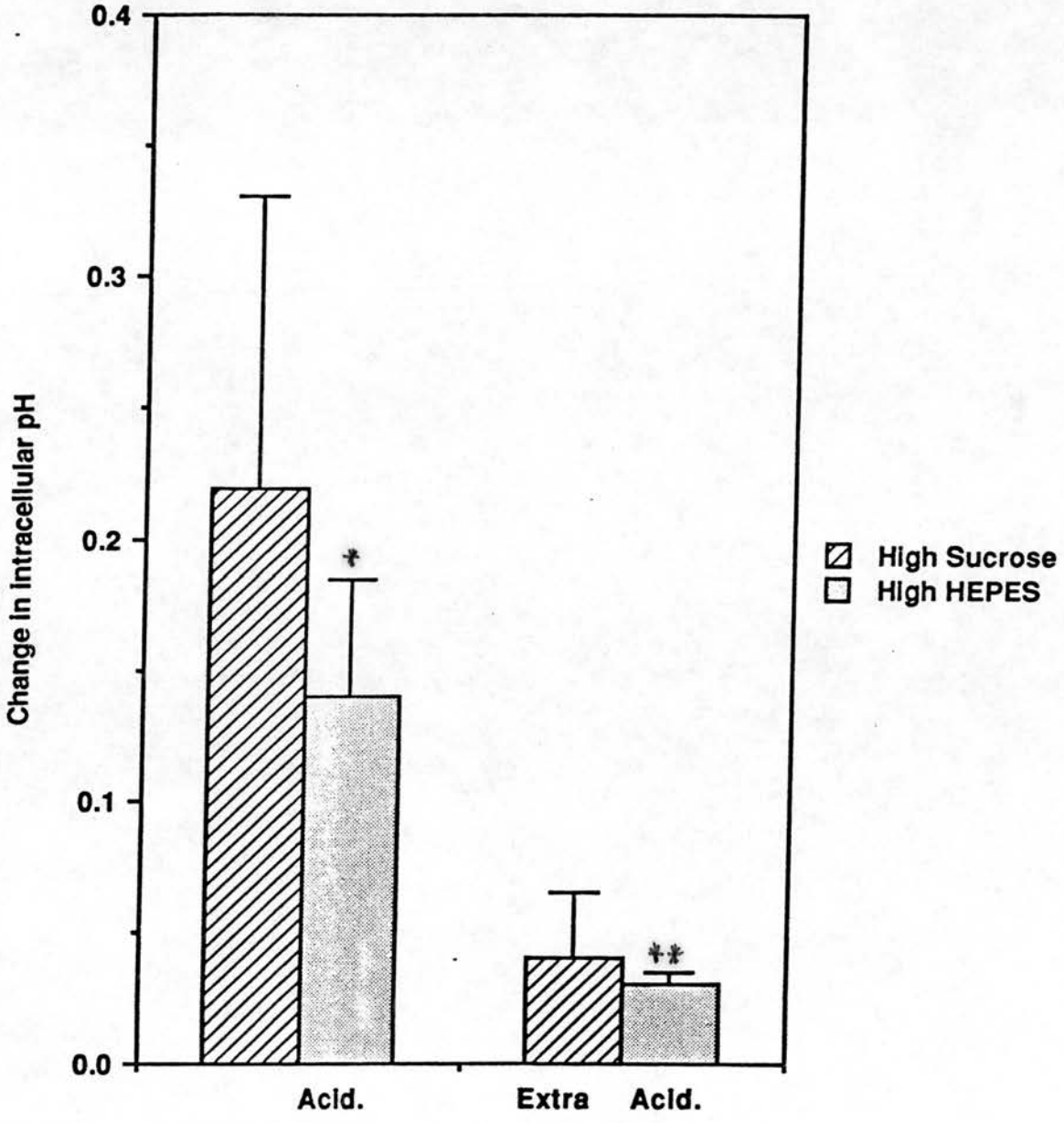
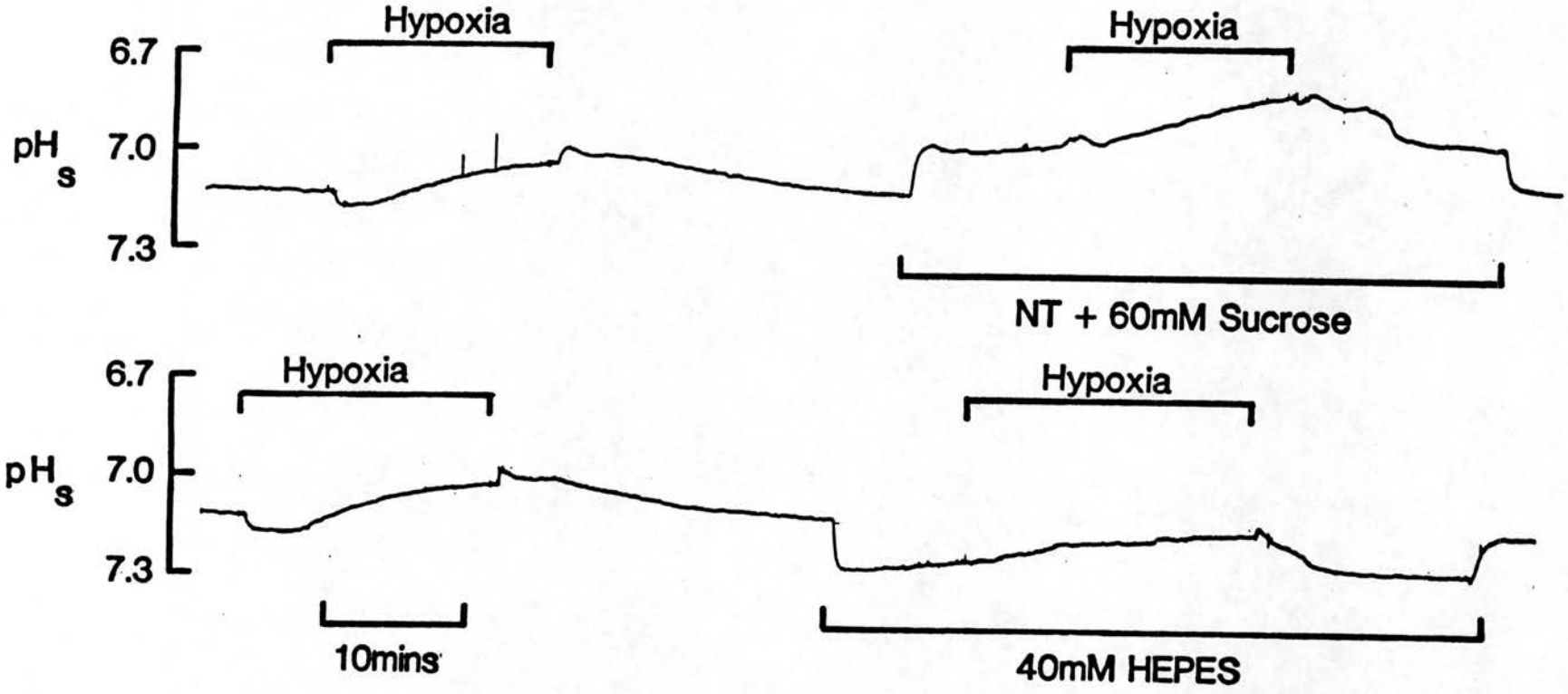

0.4
0.3
0.2
0.1
0.0
Change in Intracellular pH
*
**
Acid.
Extra
Acid.
High Sucrose
High HEPES

FIGURE 3.38

The effect of high extracellular HEPES on the surface pH (pH_S) changes during hypoxia compared with the effect of 10 mM HEPES Tyrode (60 mM sucrose) in a sheep Purkinje fibre.


Hypoxia
Hypoxia
6.7
pH s
7.0
7.3
NT + 60mM Sucrose
Hypoxia
Hypoxia
6.7
pH s
7.0
7.3
10mins
40mM HEPES

Changing to amiloride appeared to cause a slight acidification of pH_i from 7.14 $\pm$0.13 in normal Tyrode to 7.11 $\pm$0.15. This was not significant ($P>0.1$, $n=12$) although previous work has shown a slow progressive intracellular acidification (Ellis and MacLeod, 1985). The mean Em was 73.5 $\pm$5.9mV under both conditions. In hypoxia (e.g. Fig.3.39) the presence of amiloride did not result in a significantly different acidification (0.26 $\pm$0.22) compared with that in normal Tyrode (0.29 $\pm$0.17) ($P<0.01$). On reoxygenation there was no significant difference in the transient acidification in normal Tyrode (0.10 $\pm$0.13) compared to that in amiloride (0.12 $\pm$0.14) ($P>0.1$). Figure 3.40 compares the mean results for changes in pH_i during hypoxia in normal Tyrode and amiloride.

In 5 experiments (not illustrated) the effect of amiloride in hypoxia on pH_s was compared with that in normal Tyrode. The pH_s appeared to become slightly more acid in amiloride changing from 7.22 $\pm$0.07 in normal Tyrode to 7.15 $\pm$0.13 but this difference was not significant ($P>0.1$). In hypoxia there was a significantly greater change in pH_s in normal Tyrode (0.13 $\pm$0.13) than in amiloride (0.10 $\pm$0.15 $P<0.05$). There was no transient acidification on reoxygenation.

(14) EFFECT OF SITS

SITS has been shown to inhibit HCO_3^-/Cl^- exchange in mammalian cardiac muscle (Vaughan-Jones, 1979). The role of HCO_3^-/Cl^- exchange in pH regulation may be of importance in cardiac tissue under some conditions. Therefore it was of interest to observe the effect of blocking this anion exchange on the process of hypoxic acidification.

In 4 experiments where hypoxia in normal Tyrode and

FIGURE 3.39

The effect of 1mM amiloride on the change in pH_i during hypoxia compared with the change in pH_i in normal Tyrode during hypoxia in a sheep Purkinje fibre.

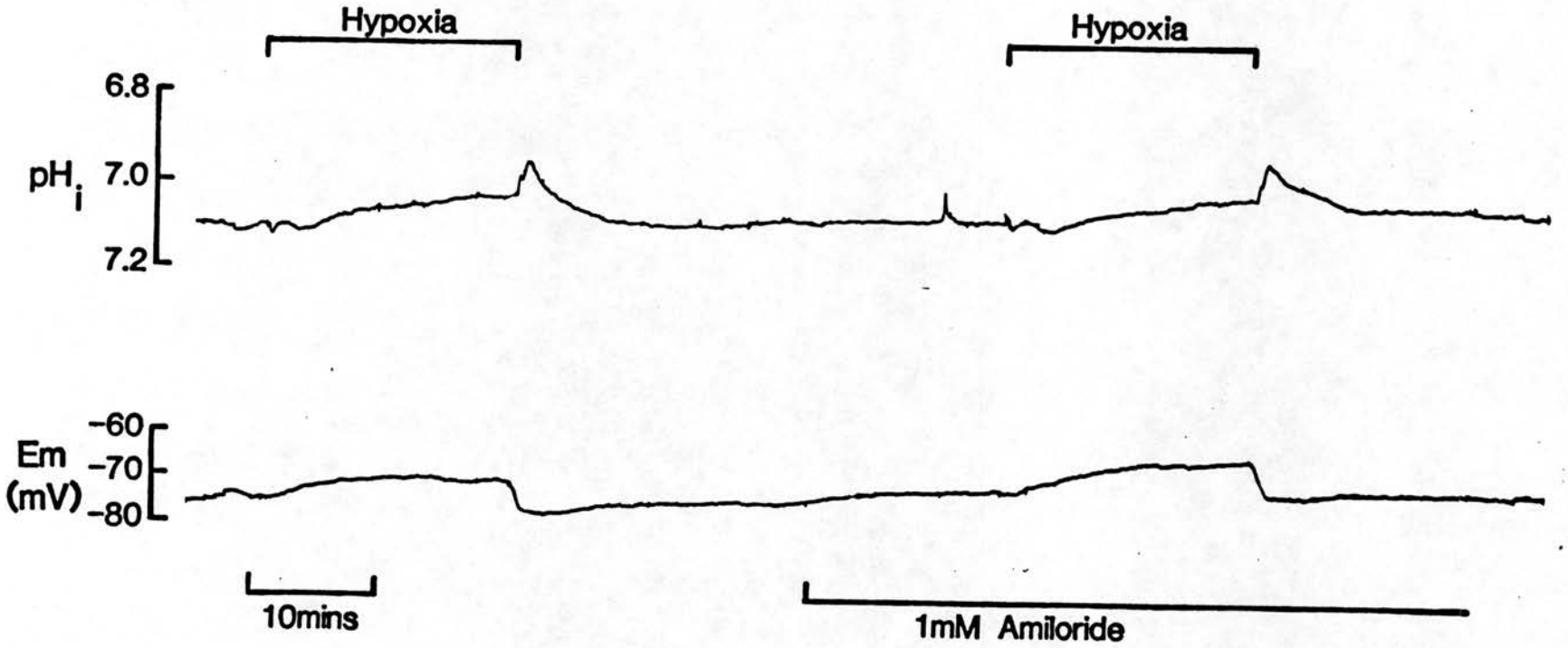
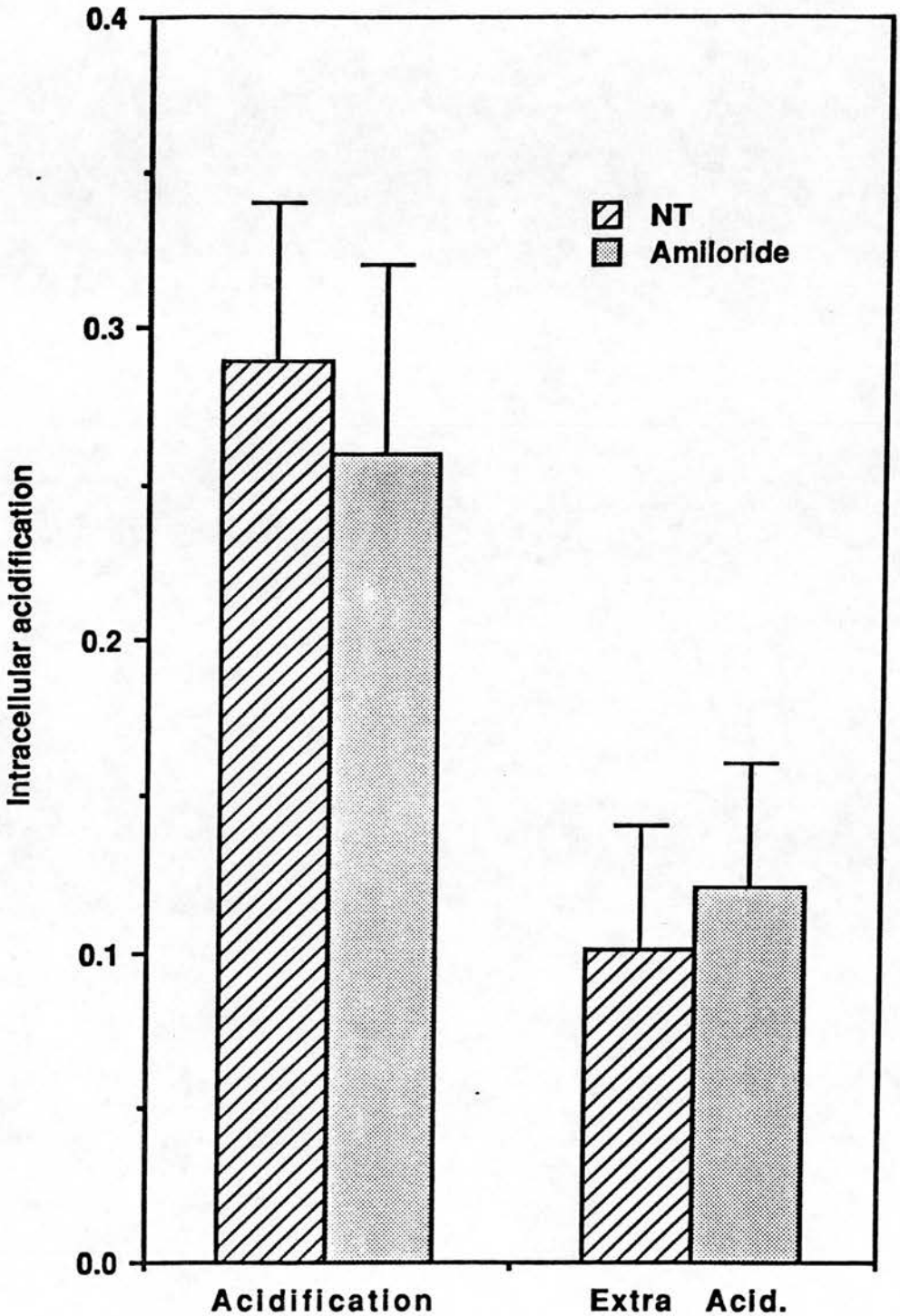

Hypoxia
Hypoxia
6.8
pH$_i$ 7.0
7.2
-60
Em -70
(mV) -80
10mins
1mM Amiloride

FIGURE 3.40

The effect of 1mM amiloride on the change in pH_i during hypoxia and on the transient extra acidification on reoxygenation after hypoxia. The change in pH_i is the mean $\pm$S.E. for 12 experiments on sheep Purkinje fibres.


0.4
0.3
0.2
0.1
0.0
Intracellular acidification
NT
Amiloride
Acidification
Extra Acid.

SITS were compared the preparation was exposed to 100uM SITS for 30 minutes before exposure to hypoxia. The control pH_i was found to be 7.16 $\pm$0.05 in both solutions.
Intracellular acidification during hypoxia was greater in the presence of SITS (not illustrated). The intracellular acidification was 0.27 $\pm$0.22 in normal Tyrode hypoxia compared with 0.33 $\pm$0.26 in SITS hypoxia ($P<0.05$). There was no significant difference in the amplitude of the transient acidification on reoxygenation ($P>0.1$). Figure 3.41 compares the mean change in pH_i during hypoxia and the transient acidification of pH_i on reoxygenation in normal Tyrode and SITS.

(15) EFFECT OF HIGH EXTRACELLULAR POTASSIUM

During ischemia $[K^+]_o$ is known to accumulate in the extracellular space causing depolarization of the Em (Kleber, 1984). It has been estimated (Weiss and Shine, 1982) that in ischaemia the $[K^+]_o$ rises to 10-15mM. It was therefore of interest to investigate how this facet of ischaemia may affect pH changes in hypoxia. Changing to 12mM $[K^+]_o$ caused a significant depolarization of Em from 72.5 $\pm$9.9mV (n=3) to 58 $\pm$8.2mV (Fig.3.42) but no change in pH_i at 7.2 $\pm$0.2. After 20 min hypoxia there was found to no significant difference in the decrease in pH_i, which changed to 7.07 $\pm$0.24 in normal Tyrode and to 7.08 $\pm$0.24 in 12mM $[K^+]_o$. On reoxygenation there was no significant difference in the further acidification of pH_i (7.03 $\pm$0.27 in normal Tyrode, 6.99 $\pm$0.26 in high $[K^+]_o$). Figure 3.42 shows an experiment where the preparation was returned to 6mM $[K^+]_o$ before reoxygenation. However in the other two experiments 12mM $[K^+]_o$ was maintained during the reoxygenation, producing similar results.

FIGURE 3.41

The effect of 100uM SITS on the change in pH_i during hypoxia and the transient extra acidification on reoxygenation compared with hypoxia in normal Tyrode. The change in pH_i is the mean $\pm$S.E. for 4 experiments on sheep Purkinje fibres.

$\ast$ = Significant difference between normal Tyrode and SITS ($P<0.05$).

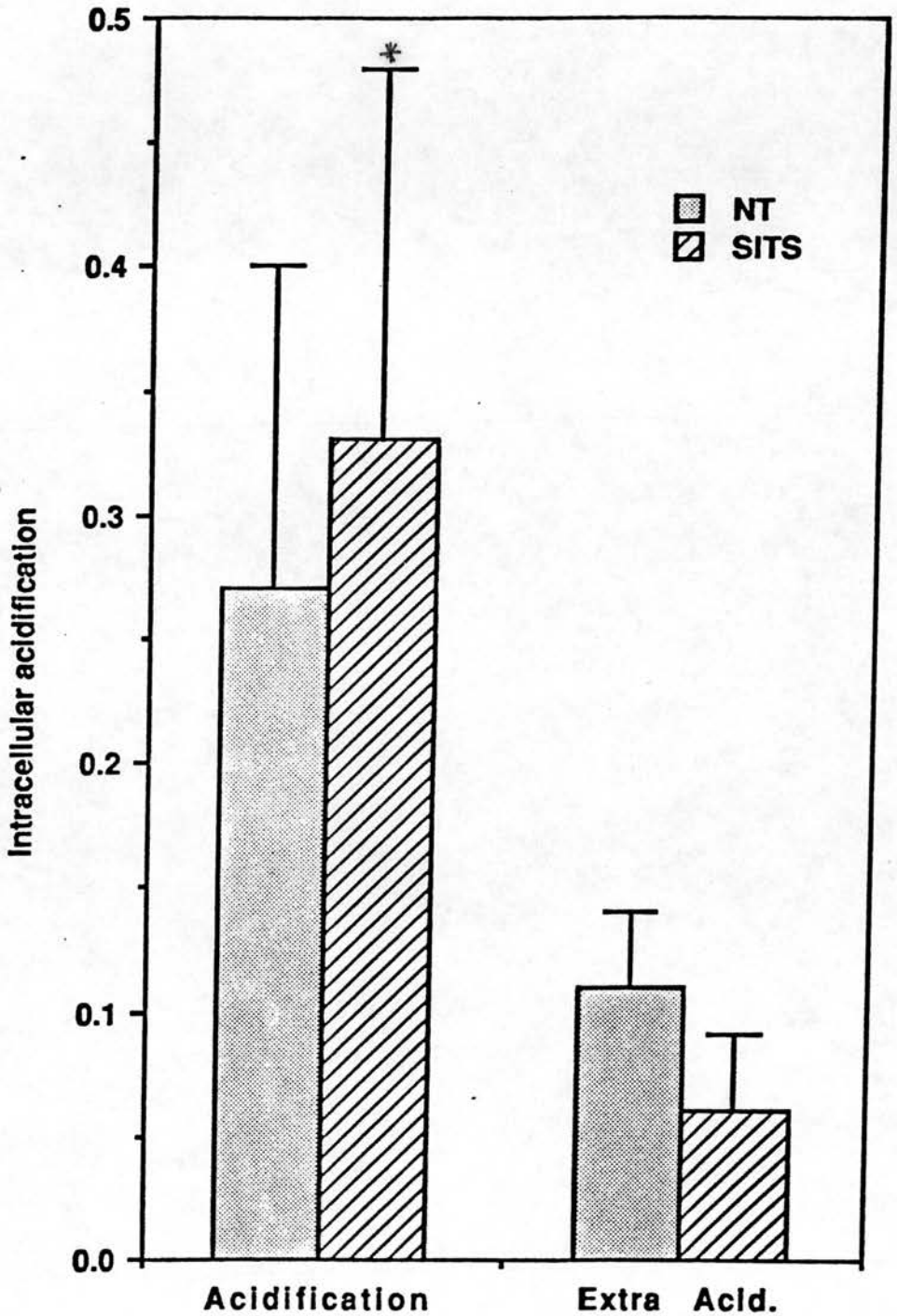
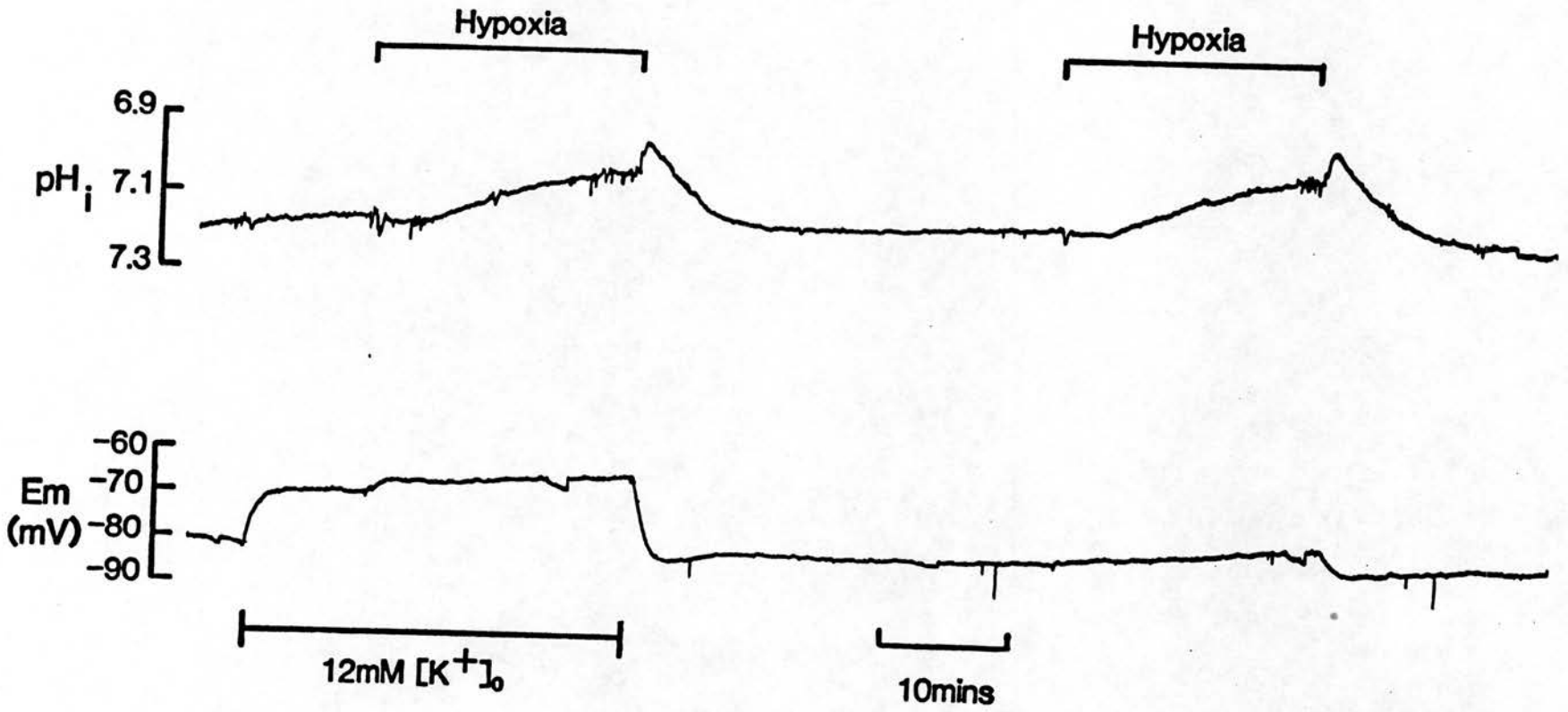

0.5
0.4
0.3
0.2
0.1
0.0
Intracellular acidification
*
NT
SITS
Acidification
Extra Acid.

FIGURE 3.42

The effect of increasing $[K^+]_o$ from 6mM (normal Tyrode to 12mM on the change in pH_i during hypoxia in asheep Purkinje fibre.


Hypoxia
Hypoxia
6.9
pH$_i$ 7.1
7.3
-60
-70
Em
(mV) -80
-90
12mM [K$^+$]$_o$
10mins

DISCUSSION

(A) GENERAL DISCUSSION

(1) GENERAL EFFECTS OF HYPOXIA ON pH_i

This study shows that hypoxia results in an acidification of approximately 0.2 pH unit after 20 minutes exposure (PO_2 mean value was 4.8 $\pm$2.6 mmHg n=19). This is a similar change to that seen in previous studies of hypoxia (see Ellis and Noireaud 1987). In the ischaemic heart, when lactate cannot leave the cells, an intracellular acidosis of 1.0 pH unit has been observed (Bailey *et al.* 1981). Allen *et al.* (1985) found a decrease of pH_i during hypoxia of 0.13 pH units during 15 minutes exposure to hypoxia in ferret papillary muscle using NMR techniques. The acidification of pH_i during hypoxia was usually accompanied by a small depolarization of the membrane potential (approximately 1.3 mV). In some preparations however there was no measurable depolarization or even a slight hyperpolarization (e.g. Fig.3.2). These experiments indicate that when oxidative phosphorylation is inhibited by hypoxia there is a decrease of pH_i which may be due to the production of lactic acid by anaerobic glycolysis (Williamson, 1966). Several mechanisms seem to contribute to the regulation of intracellular pH including Na^+/H^+ exchange which is mainly active under acid load (Deitmer and Ellis, 1980) and a Cl^-/HCO_3^- exchange which is mainly active under alkaline conditions (Vaughan-Jones, 1982).

Allen *et al.* (1985) described a transient intracellular alkalinisation prior to acidification on addition of NaCN in stimulated ferret heart. The alkalinization was attributed to PCr breakdown absorbing protons.

Fry *et al.* (1987) describe a transient alkalosis in ferret muscle exposed to NaCN. It was found to be transient but did not develop into an acidosis in their quiescent preparations.

In the present study, on reoxygenation after hypoxia, the pH_i became transiently more acid presumably due to production of protons on resynthesis of PCr (the Pi being derived from ATP). The net reaction is:-

$$Cr + P \rightleftharpoons PCr + H$$

However extrusion of protons by mitochondria as ATP synthesis is restarted or the hydration of CO_2 on reactivation of aerobic respiration may also be involved. An extra acidification on reoxygenation after hypoxia has also been reported in rat slow-twitch skeletal muscle after hypoxia (de Hemptinne and Hugeunin, 1984).

(2) GENERAL EFFECTS OF HYPOXIA ON CONTRACTION

In this study hypoxia has been found to cause a decrease in developed tension to approximately 35% of control values in ferret papillary muscle after 20 minutes exposure (e.g.Fig.3.3). Allen and Orchard (1984) have shown that [ATP] does fall during CN exposure but that the fall was not large, reaching 90% of the control value after 15 minutes exposure to hypoxia. Unless the free energy of hydrolysis of ATP is decreased below a critical level, this would not greatly affect developed tension. It seems likely that the free energy of hydrolsis of ATP is reduced in hypoxia (Fiolet *et al.*, 1984) and that this may well decrease the force of contraction of cardiac muscle.

Another explanation for the fall in tension during hypoxia may be a reduction in the amount of Ca^{2+} available for contraction. However, Allen and Orchard (1984) have also shown that the action potential induced

calcium transient does not seem to change during hypoxia and therefore a limitation of the amount of Ca^{2+} available for contraction is probably not the cause of hypoxic contractile failure.

Acidosis is known to decrease the sensitivity of myofibrils to Ca^{2+} (Fabiato and Fabiato 1978), and hypoxia has been shown to produce intracellular acidosis. However it has been shown that the size of the decrease in pH_i is very small when contractile force is reduced (Ellis and Noireaud 1987, Vanheel *et al.*1987). Despite the fact that intracellular pH may not be solely responsible for the early contractile failure, it is inevitable that as pH_i decreases in hypoxia this will contribute to the further reduction of developed tension.

Another important factor which may affect twitch tension in hypoxia is the level of inorganic phosphate (Pi) (Allen *et al.* 1985). The large rise in [Pi] may play a role in inhibition of tension production during hypoxia (Allen and Orchard, 1987). Kentish (1986) has shown that PCr and ADP have little direct effect on developed tension in skinned fibres. However increases in the concentration of Pi from 1-3mM to 20mM occur in hypoxia and ischaemia. This produces a 50% fall in developed tension in skinned fibres compared with controls. It seems likely that increases in $[Pi]_i$ during hypoxia exert a major depressant effect on tension. Kentish (1986) has shown that Pi inhibits maximum Ca^{2+}-activated tension by reducing the Ca^{2+}-sensitivity of the myofilaments. It is possible that an explanation of the decrease in contractile force observed during hypoxia in this study is as a result of a combination of the explanations offered above.

The phenomenon of hypoxic contracture did not occur after only 20 minutes of hypoxia and is therefore discussed in section 5 in the context of prolonged

exposure to hypoxia.

(3) HYPOXIA VERSUS ANOXIA

The experiments comparing the effects of hypoxia and anoxia indicate as might be predicted that anoxia is more effective for inhibiting the metabolic processes of the myocardium, since 20 minutes of anoxia produced a larger decrease in pH than did hypoxia (e.g.Fig.3.4). In this study, anoxia (produced by sodium dithionite, 0.5mM) reduced PO_2 to zero whereas hypoxia reduced PO_2 to approximately 5 mmHg. The PO_2 in anoxia in this study is in keeping with that found in recent work (Dart and Riemersma 1989). However Metsa-Ketela (1981) suggested that sodium dithionite (2mM) reduced PO_2 to only 50%. However their work was performed in isolated rat atria, and it may be that they found it difficult to exclude O_2 from their experimental apparatus. The O_2-reducing capability of sodium dithionite is well documented, indeed it is the standard method used to calibrate the zero position for oxygen electrodes.

Anoxia caused a marked fall in developed tension (e.g. Fig.3.7) to approximately 25% of the control value, in ferret papillary muscle. The severe exposure to oxygen starvation probably causes radical intracellular changes. For example pH_i becomes more acidic in anoxia and may therefore have a greater effect on contractility (Fabiato and Fabiato,1978). There may also be a greater effect on the free energy of hydrolysis of ATP during anoxia (Fiolet *et al.*,1984).

It was of interest to see how varying the PO_2 of the hypoxic solution affected the pH_i changes in hypoxia. The extent of hypoxic changes in the heart might be predicted to depend on the severity of the hypoxia to which the heart is exposed i.e. the PO_2 of the perfusing

solution. Anoxia was shown to produce a larger acidification of pH_i than hypoxia. The results are variable but suggest that a PO_2 below 10 mmHg is required to produce an acidification of pH_i after 20 minutes exposure to hypoxia (Fig.3.14 and Fig.3.15). There was a general increase in the size of the acidification, dependent upon the decrease in PO_2. The effect of decreasing PO_2 on the size of the transient reoxygenation acidification seems more variable, but the amplitude of this change is much smaller and therefore more difficult to measure accurately.

The results of these experiments suggest that the effects of sodium dithionite on pH_i in sheep Purkinje fibres are mediated via a decrease in PO_2 rather than by any other action of the chemical on the heart (Figure 3.14). Figure 3.14 shows continuous curves to a PO_2 of 0mmHg. The effect of decreasing PO_2 is different in the case of the transient reoxygenation acidification (Figure 3.15) since the size of the acidification falls on exposure to anoxia in two experiments but this may just reflect the time taken to remove sodium dithionite from the bath and perfusing solution.

While the bathing solution has a known PO_2 this does not necessarily mean that cellular $[O_2]$ is the same. It will be less due to the O_2 consumption by the cells. Mitochondria are able to function at very low levels of O_2 (Newsholme and Leech, 1983).

(4) INHIBITING OXIDATIVE PHOSPHORYLATION

NaCN is commonly used for inhibiting oxidative phosphorylation and therefore mimicking hypoxia *in vivo*

(Pirolo and Allen, 1986). Therefore it was of interest to compare this method with that of true hypoxia.

Comparing the effects of NaCN and hypoxia on pH_i showed no significant difference in the size of the acidification produced. On removal of NaCN the recovery was relatively prolonged (Fig.3.5) presumably reflecting the gradual washout of NaCN from the tissue whereas the O_2 level only has to be raised by a few percent to overcome the effects on pH_i. The fall in tension observed during NaCN exposure in ferret papillary muscle was approximately 10% less than in hypoxia.

Usually the physical methods of removal of O_2 and NaCN exposure are considered interchangeable techniques for inhibiting oxidative phosphorylation (Allen *et al.*,1985). The results of the present study indicate that NaCN and hypoxia may not be identical in their effects on isolated heart preparations. It seems that although a convenient method for mimicking true hypoxia, NaCN has limitations in that the decrease in tension in ferret papillary muscle is not the same as in hypoxia, and that the transient acidification of pH_i in sheep Purkinje fibre, on removal of NaCN, is slower than that observed on reoxygenation after hypoxia.

Fry *et al.* (1987) have shown that addition of NaCN can not only alter the response of ferret and rat cardiac muscle to decreased $[Na]_o$ but also has transient effects itself on $[H^+]_i$, $[Ca^{2+}]_i$ and $[Na^+]_i$.

(5) DURATION OF EXPOSURE TO HYPOXIA

In the ischaemic heart the duration of exposure to ischaemia dictates the extent and reversibility of cell damage. It seemed likely that the length of exposure to

hypoxia would dictate the extent of intracellular acidification. As can be seen from Figure 3.9, exposures to hypoxia over the range of exposure periods in these experiments appear to have little effect on the rate of recovery of pH_i on reoxygenation. However the size of the pH_i obviously increases with the length of hypoxic exposure. The transient acidification seen on reoxygenation was larger after 4 minutes (0.11 $\pm$0.04 pH units) and 10 minutes (0.14 $\pm$0.05 pH units) than after 20 minutes (0.09 $\pm$0.03) hypoxia. This might indicate that either more PCr is resynthesised (or is synthesised more rapidly) after 10 minutes hypoxia than after 20 minutes. This is perhaps the result of a higher $[H^+]_i$ after 20 minutes inhibiting the resynthesis reactions or a greater decrease in [ATP] after 20 minutes hypoxia making resynthesis of PCr slower.

The progressive acidification during hypoxia would be in agreement with the theory that the intracellular acidification is as a result of an increase in lactic acid production produced by anaerobic respiration. This might be accompanied by some inhibition of Na^+/H^+ exchange at the cell membrane if a^i_{Na} rises during hypoxia. However the increased intracellular acidification is more likely to have a significant stimulatory action on Na^+/H^+ exchange than is the relatively small increase in a^i_{Na} (see section 7).

It could be postulated that extended periods of hypoxia may result in an adaptive recovery of the Na^+/H^+ exchanger rate, by stimulation of the Na^+/H^+ exchanger by intracellular acidification. This might result in some recovery of pH_i during extended hypoxia. During extended hypoxia (1 hour) no recovery of pH_i is observed (Fig.3.10) although the acidification reached a plateau level when presumably acid production in the cell and extrusion from the cell are balanced, perhaps with a

degree of stimulation of the Na^+/H^+ exchanger by the acid pH_i. The effect of amiloride, which was used to block Na/H exchange (see section 14), suggests that amiloride has little effect on the intracellular acidification produced in hypoxia. Therefore the proposed saturation of Na/H exchange does not account solely for the sustained intracellular acidification during extended periods of hypoxia.

In the experiment illustrated in Figure 3.10 recovery of pH_i on reoxygenation was complete suggesting that the exposure to hypoxia had not resulted in any permanent cell damage. This was normally the case in sheep Purkinje fibres.

Extended exposure to hypoxia has been previously shown to cause the formation of a hypoxic contracture in ventricular muscle and this has been suggested to be caused by depletion in [ATP] levels resulting in rigor cross-bridge formation (Katz, 1970). Allen *et al.*(1985) have verified that $[ATP]_i$ is very low when ferret heart is in hypoxic contracture. Figure 3.11 shows the effect of hypoxia after 1 hour on the developed tension in ferret papillary muscle. The preparation did not recover on reoxygenation and had presumably gone into contracture as a result of depletion of ATP levels in the cell i.e. the tissue was irreversibly damaged.

The effect of 90 minutes hypoxia on glycogen levels in sheep Purkinje fibres was examined using electron-microscopy (Fig.3.10a). There was no obvious depletion in the glycogen content of the fibres and no evidence of cellular or sub-cellular damage. Thus it appears that sheep Purkinje fibres are more resistant to hypoxic damage than ferret ventricular preparations. However it should be remembered that the Purkinje fibres were quiescent and ferret heart was stimulated. This would result in faster ATP depletion in the ventricular muscle.

(B) EFFECTS OF HYPOXIA AND ANOXIA ON OTHER INTRACELLULAR IONS

I aimed to observe how hypoxia and anoxia affect the concentrations of other intracellular ions as alteration of these ions may also affect pH_i and pH_i regulation via a variety of effects including cellular ion exchange mechanisms. The ions studied were Na^+ and K^+.

(6) INTRACELLULAR SODIUM

This study showed that 20 minutes exposure to hypoxia and anoxia caused a small rise in a^i_{Na} in sheep Purkinje fibres, anoxia causing a larger rise than hypoxia (e.g. Fig.3.16).

Hypoxia has previously been reported to produce a large rise in a^i_{Na} in guinea pig papillary muscle (McDonald and Macleod, 1973a). In a later study, Kleber (1983) found no change in a^i_{Na} during 15 minutes of ischaemia in guinea-pig heart. Nakaya *et al.*(1985) were also unable to demonstrate a rise in a^i_{Na} under the combined conditions of hypoxia, acidosis and no glucose in dog ventricular muscle. Results from my experiments are in agreement with a recent study by MacLeod (1989) who found a rise in a^i_{Na} which became greater when glycolysis was blocked by DOG.

The rise in a^i_{Na} in hypoxia which has been observed may be accounted for by inhibition, to some extent, of Na^+/K^+ exchange, although this seems unlikely since the total cell $[ATP]_i$ does not fall very much in the first 15 minutes of hypoxia (Allen and Orchard, 1984). Guarnieri (1981) suggests that the rise in a^i_{Na} which he observed was in fact due to inhibition of the Na^+/K^+ pump by a fall in [ATP] in the cell. The lack of any large effect of hypoxia and anoxia on a^i_{Na} favours the explanation

that stimulation of Na^+/H^+ exchange is responsible for increased a^i_{Na} (see also Ellis and Noireaud 1987) rather than any substantial inhibition of the Na^+/K^+ pump. Kaila and Vaughan-Jones (1987), have shown that in sheep Purkinje fibres a rise in a^i_{Na} produced by inhibition of the Na^+/K^+ exchange (they used strophanthidin) leads to a stimulation of Na^+/H^+ exchange. However, this apparently paradoxical result occurs because elevated a^i_{Na} produces inhibition of Na^+/Ca^{2+} exchange leading an intracellular acidosis via interactions between Ca^{2+} and H^+ at various intracellular sites. This leads to stimulation of Na^+/H^+ exchange. Thus if the Na^+/K^+ pump is inhibited in hypoxia it may be responsible for the acidification of pH_i but not directly due to the rise in a^i_{Na}. This question is addressed in section 14 on the results of amiloride exposure.

(7) INTRACELLULAR POTASSIUM

Before studying a^i_K levels in sheep Purkinje fibres and ferret papillary muscle during hypoxia and anoxia it was important to establish that the K-selective microelectrodes gave valid and accurate measurements of a^i_K. Reverdin *et al.* (1986) have suggested that the most commonly used K-sensitive ligand (Corning) gave spuriously high recordings of $[K^+]_i$ in ferret papillary muscle (they report a mean value of 195mM but they also recorded values of several thousand mM). They suggested that the ligand reacted not only to K^+ ions but possibly to some other biological substance or substances within the cells. I performed a series of experiments comparing the recordings obtained using a valinomycin-based sensor with a Corning ion-selective sensor in ferret papillary muscle (see Table 1). Corning-filled microelectrodes

measured a slightly higher a^i_K but this was not significantly different from the valinomycin measurements and no extremely high values for a^i_K were recorded like those reported by Reverdin *et al.*(1986). It is difficult to account for these differences although no attempt was made to use identical electrodes to those produced by Reverdin *et al.* (1986). The silanization procedure used in the production of the microelectrode might have influenced results. They used both single and double-barrelled electrodes and a different batch of Corning ion-selective resin. Corning resin has been used with success in a variety of mammalian cardiac preparations in previous studies (Browning *et al.* 1981, Baumgarten *et al.* 1981), producing measurements for a^i_K of similar magnitude to those of a^i_K in ferret papillary muscle in this study. Therefore in most subsequent experiments Corning-filled K-sensitive microelectrodes were used because these electrodes were less noisy, of lower resistance and proved to be more stable. Having established that the technique for measuring a^i_K was satisfactory in normoxia the measurements could be made in hypoxia.

(i) Effects of Hypoxia and Anoxia on a^i_K

The experiments on the effects of 20 minutes exposure to hypoxia and anoxia on a^i_K showed that both conditions resulted in a decrease of a^i_K (a^i_K decreased by 5.8mM in hypoxia and 9.7mM in anoxia) which recovered on reoxygenation (e.g. Fig. 3.17). Guarnieri and Strauss (1982) reported that $[K^+]_i$ fell by 70% (measured with ion-selective microelectrodes) during a three hour exposure to hypoxia in guinea pig papillary muscle.

Gaspardone *et al.* (1986) observed a rise in $[K^+]_o$ during hypoxia which they attributed to Na^+/K^+ pump

inhibition. They suggested that the cellular potassium loss which occurs in hypoxia may also be attributed to an increased efflux rather than a reduced influx of K^+ ions.

Nakaya *et al.*(1985) found that after 30 minutes exposure to hypoxia, acidosis and no glucose the a^i_K in dog ventricular muscle decreased but they found no change in a^i_{Na}. They therefore prefer the hypothesis that Na^+/K^+ exchange continues to function in hypoxia. An increase in potassium outward current may play a role in the loss of potassium from the cell or potassium may efflux concomitantly with lactate on a carrier as was suggested by Kleber (1983). Leblanc (1986) suggested that loss of K^+ can be dissociated from the inhibition of the sodium pump at least for a limited period.

It is possible that hypoxia may cause a small rise in $[K^+]_o$ in the intercellular spaces at the tissue surface (perhaps an area of "dead space" where washout of K^+ by the perfusate is slow) due to K^+ leaving the cell along its concentration gradient. Therefore with an increased efflux of K^+, a^i_K will fall slightly. If the Na^+/K^+ pump is inhibited by hypoxia then this would also contribute to the decrease in a^i_K observed in hypoxia. Thus it appears that more than one factor may be responsible for the decrease in a^i_K during hypoxia and anoxia.

(ii) The Effects of Strophanthidin and Saponin.

Other procedures known to alter a^i_K were used to compare with the effects of hypoxia and anoxia. Strophanthidin was used to reversibly inhibit the Na^+/K^+ pump (Ellis and Deitmer 1978). This produced larger decreases in a^i_K (e.g. Fig. 3.19) which were approximately twice as great as the change in a^i_K (approximately 13mM) observed in hypoxia (e.g. Fig.3.17).

In addition a depolarization was observed in strophanthidin of about 7mV. These results suggest that the Na^+/K^+ pump is probably only slightly inhibited in hypoxia.

Saponin (0.25 $ugml^{-1}$, 10 minute exposure) caused a decrease in a^i_K of about the same magnitude as exposure to hypoxia (approximately 7mM), but the size of the decrease will be dependent on the concentration of saponin and the duration of exposure. The reversible decrease in a^i_K on exposure to saponin (e.g. Fig.3.20) could be accounted for by an increased permeability of the cell membrane to K^+ (saponin is known to increase the passive permeability of cell membranes (Yamasaki *et al.*1987)). Some inhibition of the Na^+/K^+ pump by saponin cannot be ruled out. Since saponin produced a large depolarization (approximately 18mV) of the cell this may in part be responsible for some of the decrease in a^i_K.

It therefore seems likely that the change in a^i_K during anoxia and hypoxia cannot be accounted for in terms of complete inhibition of Na^+/K^+ exchange since the decrease in a^i_K during anoxia and hypoxia is less than that observed during pump inhibition in strophanthidin. However partial inhibition of the Na^+/K^+ or an increased efflux of K^+ may explain the small decrease in a^i_K observed under these conditions. Experiments performed on cardiac tissue with saponin show that there is almost no change in pH_i in saponin (Noireaud *et al.* 1989). Since the, changes in a^i_K are of the same magnitude as the changes seen in hypoxia, it seems unlikely that the change in a^i_K seen in hypoxia can be responsible for the acidification of pH_i, unless this is mediated via a different mechanism than in the saponin experiments.

(C) FACTORS AFFECTING THE CHANGE IN INTRACELLULAR pH DURING HYPOXIA

(8) TEMPERATURE EFFECTS

Lowering ambient temperature is a frequently used technique for protecting the myocardium during heart surgery (Barner *et al.* 1977). It was therefore of interest to investigate the effect of low temperature on pH_i changes during hypoxia.

Samson *et al.* (1977) studied the effects of lowering temperature (from 33°C to 20°C) on intracellular ion concentrations in isolated rabbit heart. They found that low temperature caused a decrease of a^i_{Na} and an increase of a^i_K. They also found an increase in action potential amplitude at low temperature. If a decrease in a^i_{Na} occurs this may affect the pH_i of the preparation, since if the a^i_{Na} falls it may stimulate the Na^+/H^+ exchanger causing an intracellular alkalinization. Glitsch and Pusch (1984) described the effect of temperature on active Na^+ transport (measured with ion-selective microelectrodes) and compared this to the Na^+ K^+-ATPase activity in Purkinje fibres. They found an increase in a^i_{Na} at 22°C compared with 35°C. Isenberg and Trautwein (1975), have shown that an outward current caused by electrogenic Na-pumping is strongly reduced by cooling from 37°C to 21°C in Purkinje fibre.

In this study, decreasing the temperature in the bath from 35°C to 22°C resulted in a smaller acidification of pH_i during hypoxia (the intracellular acidification in hypoxia was approximately 0.17 pH unit smaller than that seen at 35°C).

It has been shown that a 9°C decrease in temperature slows the formation and breakdown of high energy phosphates to about 50% the rate at 35°C (Jones *et*

al. 1982). It is possible that at 22°C the slowing of the high energy phosphate metabolism may affect the the acidification of pH_i in hypoxia.

In the present study lowering temperature from 35°C to 22°C caused a rise in pH_i of approximately 0.3 pH units. The effects of cooling may not be solely on the rate of production of H^+ ions due to slowing of anaerobic glycolysis, there could also be a reduction in the rate of H^+ entry into the cells or an increase in the rate of H^+ ion efflux. A stimulation of the sodium hydrogen exchange would lead to an intracellular alkalinization, but much more slowly than the pH_i change produced by lowering the temperature. The temperature dependence of buffers must also be an important factor influencing the increase in pH_i on lowering temperature.

Several studies on cold-blooded vertebrates have suggested that the regulation of pH_i is dependent on temperature (Reeves, 1972). In measurements on whole animals Howell *et al.*(1970) showed that between 5°C and 37°C as temperature rises PCO_2 increases and HCO_3^- decreases in frogs, toads and turtles. They suggest that the temperature behaviour of certain protein systems led to their selection as the most important buffers in vertebrates. The bicarbonate-carbonic acid systems could be adapted to temperature changes by controlling PCO_2 by ventilation and HCO_3^- by renal regulation. Care is required when interpreting these types of experiment since the animals were usually acclimatised first before measurements were taken. Aicken and Thomas (1977) found that under conditions of constant pH and CO_2 the measurement of pH_i changes may be influenced by the temperature dependence of the intracellular buffers.

It appears that some of the protective effects of hypothermia in cardioplegia (Barner *et al.* 1977) are as a result of the alkalinization of pH_i and the smaller

acidification which occurs during hypoxia.

Tension Experiments

When the bath temperature was decreased from 35°C to 22°C in stimulated papillary muscle the developed tension appeared to increase by about 30% (Fig.3.24) If the Na^+/K^+ pump was inhibited and a^i_{Na} increased in the cell this in turn would inhibit Na^+_o/Ca^{2+}_i exchange, cause an increase in a^i_{Ca} and subsequently allow larger release of Ca^{2+} from the S.R. and therefore the development of a larger contractile force. In most mammalian heart muscle preparations, cooling leads to a large increase in developed tension and an increase in active tension duration due to a reduced rate of Ca^{2+} uptake by the SR delaying relaxation. If cooling leads to an increase in the Ca^{2+} sensitivity of the myofilaments (Fabiato, 1985) this could contribute to the hypothermia-induced positive inotropy. Shattock and Bers (1987) have shown the increase in developed tension to be fivefold in rabbit and rat ventricle.

Rapid cooling to below 4°C induces contracture in skinned cardiac muscle, thought to be due to release of Ca^{2+} from the S.R. (Bridge, 1986). It is likely that a lesser degree of cooling may also affect Ca^{2+} uptake and release from the S.R., Harrison and Bers (1989) suggest that between 36°C and 29°C, when there is maximal hypothermic positive inotropy, there is little or no change in myofibril Ca^{2+} sensitivity in skinned rabbit ventricular muscle.

If cooling leads to an alkalinization (as shown in the present study), the increase in pH_i would also lead to increased Ca^{2+} sensitivity of the myofilaments (Fabiato, 1985). In experiments where ryanodine was used to inhibit S.R.

function, Shattock and Bers (1987) have shown that a functional S.R. is not essential for hypothermic inotropy.

The fall in tension during hypoxia is less in 22°C solution than in 35°C solution which may be predicted if the [ATP] is conserved for longer at low temperature as a result of the reduction in the rate of breakdown of high energy phosphate described by Jones *et al.* (1982).

The effect of raising the perfusing fluid temperature to 38°C causes a fall in developed tension to almost half the developed tension at 35°C (Fig.3.25). This may be the opposite of the effect observed at 22°C in that Na^+/K^+ exchange may be accelerated at high temperature and therefore so would Na^+/Ca^{2+} exchange resulting in less Ca^{2+} being available for contraction. The fall in developed tension during hypoxia at 38°C was greater than that observed at 35°C, perhaps as a result of an acceleration of high energy phosphate metabolism by the 3°C rise in perfusate temperature.

It therefore seems that low temperature is beneficial to the preservation of cardiac function during hypoxia both in terms of pH_i and developed tension.

(9) THE EFFECT OF ALTERNATIVE SUBSTRATES

It is known that the mammalian heart will utilize a number of substrates in the absence of glucose (Opie, 1968). The rates of utilization and selection of any one substrate are dependent on a number of factors including the concentration of that substrate in plasma, the availabilty of alternative competitive substrates, O_2 delivery to the myocardium and the mechanical activity of the heart. In well-oxygenated heart, fatty acid is utilized in preference to other substrates. It has also been suggested that increased levels of pyruvate and

buffer may help to protect the mechanical function of the heart in ischaemia (Liedtke *et al.*,1976).

The effects of removing glucose from the perfusate and providing an alternative substrate have been investigated. The utilization of 20mM glucose, acetate or pyruvate appeared to have no effect on the size of the acidification produced in hypoxia compared with that produced in 10mM glucose (normal Tyrode).The lack of difference between glucose, acetate and pyruvate (Fig.3.26) might be predicted if the acidification in hypoxia is produced by lactic acid formation by the anaerobic glycolysis pathway, although a difference might have been expected between 10 and 20mM glucose. The use of 20mM lactate as substrate (which is one of the main products of anaerobic glycolysis) resulted in a larger acidification of pH_i during hypoxia. This could be due to the reduced ability of lactate to leave the cells in the presence of a high extracellular concentration. The larger transient acidification on reoxygenation in lactate might be explained by the fact that lactate is a good substrate and may cause faster or greater resynthesis of PCr after hypoxia and hence a larger transient acidification.

It seems that during inhibition of oxidative phosphorylation the deleterious effects of intracellular acidification are not reduced when glucose is replaced with pyruvate or acetate. An aggravation of the deleterious effects of hypoxia is observed only in the presence of extracellular lactate. This mimics the rise in extracellular lactate predicted in the ischaemic heart (Katz and Hecht, 1969). This finding is in accord with the work of Hearse *et al.* (1976) who suggested that lactate should not be present in cardioplegic solutions since, lactate is produced in large quantities in ischaemia and can have a toxic effect.

Apstein *et al.* (1976) found that increasing substrate glucose concentration from 5.5 to 22mM improved active tension development during the hypoxic period and after reoxygenation and prevented the occurence of rigor or contracture, in rat papillary muscle. They also found that an increase in glucose after 30 minutes of hypoxia was still effective in improving cardiac performance and recovery. The effect of hypoxia on intracellular pH_i does not appear to be affected by glucose, since doubling the concentration of glucose had no effect on the size of the acidification produced by hypoxia. This may just reflect the fact that the preparation is different, for instance, Purkinje fibres are known to have large intracellular stores of glycogen. The comparision of glycogen levels in electron- micrographs from sheep Purkinje fibres exposed to 90 minutes hypoxia with controls does not reveal a fall in glycogen levels (e.g. Fig.3.10a). It might be suggested that sheep Purkinje fibres are resistant to hypoxic damage, perhaps as a result of their large intracellular glycogen stores prolonging the supply of ATP and therefore retarding cell damage.

On changing from 10mM glucose to pyruvate or acetate there was a transient acidification of pH_i lasting about 15 minutes (see Fig 3.26). This occurs because the two substances are weak organic acids and produce a transient acidification as a result of their entry into the cell in their undissociated acid form followed by dissociation in the cell (de Hemptinne *et al.*, 1983). In the present study the tissue was left to equilibrate in the new substrate for about 30 minutes. Therefore the transient acidification would have had little or no effect on the acidification produced in hypoxia.

It might therefore be concluded that under conditions where oxidative phosphorylation was severely restricted, the addition of replacement substrates

(pyruvate and acetate) appeared to have little effect on the extent of anaerobic glycolysis as measured by the degree of acidification of pH_i produced by exposure to hypoxia.

(10) EFFECT OF CINNAMATE

Cinnamate has been shown to inhibit pyruvate (Halestrap and Denton, 1974) and lactate transport in human erythrocytes.

In this study treatment with cinnamate did not result in a significant acidification of pH_i in sheep Purkinje fibres (the mean change in pH was 0.11 $\pm$0.08, n=4) although Figure 3.28 seems to suggest that the pH change is significant. de Hemptinne *et al.* (1983) observed an acidification of pH_i of 0.1 pH unit on average in 4mM cinnamate. In the present study 5mM cinnamate was used. The lack of a significant pH change may have been because a small sample group was used. The mean acidification is of the same magnitude as that measured by de Hemptinne *et al.* (1983) but the individual measurements were very variable.

During hypoxia there was a larger acidification in cinnamate treated fibres than was observed in normal Tyrode, again presumably due to inhibition of lactate efflux from the cells. On reoxygenation cinnamate caused a marked slowing in the rate of recovery of pH_i (Fig.3.29 and 3.30), indicating that the recovery of pH_i from hypoxia is dependent on an efficient lactate efflux from the tissue.

It has been shown that cinnamate slows the rate of acidification of pH_i caused by addition of lactate, in sheep Purkinje fibres (de Hemptinne *et al.*,1983) although it does not affect the rate of acidification by acetic or proprionic acid. This suggests that lactate crosses the

cell membrane via a carrier-mediated process and that inhibition of lactic acid efflux results in increased acidosis during hypoxia and slowing of the recovery of pH_i on reoxygenation.

(11) ALTERATION OF EXTRACELLULAR pH

Acidification of extracellular pH is known to have an effect on contractility (Katz and Hecht,1969). It was of interest to observe the effect of changing pH_o on developed tension in ferret papillary muscle exposed to hypoxia.

Acidification of pH_o to pH 6.4 caused a fall of developed tension in normoxia, with a greater fall in hypoxia than that seen at pH 7.4. This was presumably as a result of an acidification of pH_i in pH_o 6.4 solution which became further acidified in hypoxia. Acidification is known to decrease the sensitivity of the myofibrils to Ca^{2+} (Fabiato and Fabiato, 1978) and could therefore account for the difference in developed tension during hypoxia at pH 7.4 compared with pH 6.4.

There are differences in the estimates of the effect of pH_o on intracellular buffering capacity. It has recently been shown that buffering capacity in sheep Purkinje fibres is not constant over the physiological range but increases approximately linearly as pH_i falls, a doubling of buffering capacity occurring as pH_o falls from 7.4 to 6.4 (Vaughan-Jones and Wu, personal communication). It therefore seems unlikely that effects of pH_o on intracellular buffering power are responsible for the changes in tension which occur at pH 6.4 since a high intracellular buffering would help to absorb excess protons and thus decrease the effect of H^+ ions in reducing developed tension in myofibrils.

Altering the pH_o to a more alkaline pH (pH 8.4)

caused a 50% rise in developed tension. The subsequent decrease in developed tension during hypoxia was approximately half the size of the fall recorded at pH 7.4. Curtin (1978) has shown that in amphibian skeletal muscle an alkaline pH increases twitch tension.

At pH_o 8.4 the decrease in the developed tension which occurred during hypoxia was accompanied by a contracture (see Fig.3.33). The reason for this contracture is unclear but it may be that the larger twitch tensions in pH 8.4 solution put a greater metabolic stress on the muscle. Therefore in hypoxia there is a faster exhaustion of high energy phosphate compounds and a faster development of contracture.

The effect of an alkalinization of pH_o was an alkalinization of pH_i in sheep Purkinje fibres. During hypoxia a slower and smaller acidification of pH_i was observed than at normal extracellular pH. This was perhaps due to the alkaline pH_o making acid efflux easier.

(12) EXTRACELLULAR AND INTRACELLULAR BUFFERING

Changing the extracellular buffer from HEPES to CO_2/bicarbonate resulted in a transient acidification followed by a smaller sustained acidification of pH_i. This has been explained to be as a result of the cell's high permeability to CO_2 which enters causing the transient acidification (Thomas, 1976). The sustained acidification has been suggested be due to bicarbonate permeability (Vanheel *et al.* 1985). The change in pH_i during hypoxia was less than that observed in HEPES buffered solution. This can be explained by an increased intracellular buffering capacity in CO_2/bicarbonate buffer (Thomas, 1976).

In one series of experiments high HEPES solution

(40mM HEPES) was compared with 10mM HEPES (plus 60mM sucrose). The acidification in hypoxia and transient reoxygenation acidification were both found to be smaller in 40mM HEPES compared with 10mM HEPES (Fig.3.36). This suggests that raising the external buffering power reduces the intracellular acidification observed during hypoxia. It has been shown that superfusion of the sheep Purkinje with low buffered solution (5mM HEPES compared with 20mM in normal Tyrode) produced an acidification of pH at the surface layer (pH_s) and therefore a depression of proton extrusion following an acid load (de Hemptinne *et al.* 1987).

This study has illustrated that both raised external buffering (HEPES) and intracellular buffering (CO_2/HCO_3 reduce the acidification during hypoxia. In the case of increased HEPES buffering the transient acidification on reoxygenation was also reduced compared to normal Tyrode solution.

Surface pH (pH_s) became more acid during hypoxia (by approximately 0.14 pH units). This might be explained by pH_i becoming more acidic during hypoxia, the H^+ ions being removed from the cell via the Na^+/H^+ exchanger and the outward movement of lactic acid. Lactic acid then accumulating in the intercellular spaces and at the surface of the tissue. This mechanism is probably similar to the processes occuring in ischaemia when reduced flow of blood to the tissue results in accumulation of large amounts of H^+ ions in the intercellular space (Katz and Hecht 1969). Hypoxia is different in that there is continued perfusion and therefore less H^+ ions and lactate will be able to accumulate.

The work of de Hemptinne and Hugeunin (1984), with mammalian skeletal muscle, suggests that the difference between pH_s and pH_o (the pH in the bathing or bulk solution) is due to an H^+ gradient in the unstirred

layers of fluid surrounding the cells which forms as a result of metabolically produced CO_2 and lactic acid. As a result the H^+ gradient between the extracellular medium close to the surface and the intracellular fluid is smaller than the gradient of H^+ activity between intracellular fluid and the bulk solution.

Vanheel *et al.* (1986), conclude that in superfused multicellular preparations buffer concentration of the superfusate and therefore pH_s can considerably influence steady-state pH_i and pH_i recovery from an acid load. They found that decreasing extracellular buffering from 20mM to 5mM produces acidosis at the preparation surface and slows the rate of acid extrusion after an imposed acid load.

In mammalian preparations it has been demonstated that the intracellular buffering capacity of cardiac muscle is better than skeletal muscle (Clancy and Brown, 1966). Saborowski *et al.* (1973) suggest that the buffering capacity is linked to the mechanical work load which is to be performed by the muscle. This was found to be the case when pH_i was compared in normal and hypertrophic rat hearts. Buffering capacity was found to greater in the hypertrophic heart which performed more mechanical work.

Several factors may contribute to intrinsic buffering power. Metabolic changes in PCr and ATP can either liberate or consume H^+ (Allen *et al.* 1985). Passive buffering may occur by ATP, PCr, and Pi. Buffering by cytoplasmic proteins also occurs. Intracellular acidosis has been shown not to significantly alter intracellular phosphate compounds (Allen *et al.* 1987a) so that changes in energy metabolism (for instance in hypoxia) are unlikely to influence intracellular buffering capacity. Passive buffering from Pi will be less than 1mM under resting conditions $[Pi]_i$

is less than 1mM,(Allen *et al.* 1985) but rises to 20 mM in hypoxia when it may affect buffering (Kentish, 1986). Recently, House *et al.* (1989) have shown that in rat ventricular muscle there is up to 10mM imidazole buffering and therefore this may make up a large proportion of the intracellular buffering capacity. Vaughan-Jones and Wu (personal communication) have estimated the total intracellular intrinsic (non-CO_2) buffering capacity as 20mM in sheep Purkinje fibres. It therefore seems that several factors may influence the intracellular buffering capacity of cardiac tissue and therefore pH regulation during hypoxia.

(13) EFFECT OF AMILORIDE

Amiloride is a potassium-sparing diuretic and has been reported to inhibit Na^+/H^+ exchange in sheep cardiac Purkinje fibres (Deitmer and Ellis, 1980) as well as Na,K-ATPase in human cardiac sarcolemma (Erdman and Bolte, 1977).

In addition it would seem that inhibition of Na^+/H^+ exchange can affect myocardial activity by altering intracellular H^+ and Na^+ concentrations. Changes in $[H^+]_i$ may influence contractility by effects related to pH sensitivity of the contractile proteins (Fabiato and Fabiato, 1978), whereas, changes in $[Na^+]_i$ may alter contractile force by action on the Na^+/Ca^{2+} exchange. Changes in the Na^+ and Ca^{2+} levels within the cells may have further effects on intracellular pH via various cellular mechanisms (Deitmer and Ellis, 1980).

Kennedy *et al.* (1986), suggest that the positive inotropic action of amiloride (at low concentration (0.3mM)) is reduced and the negative inotropic action (at high concentration (1.5mM)) is enhanced in low $[Na^+]$ solutions i.e. under conditions likely to favour Ca^{2+}

influx via Na^+/Ca^{2+} exchange. This suggests that there is a complex interaction of amiloride with cardiac muscle. Amiloride may produce its inotropic effects in cardiac muscle by several mechanisms including, sodium pump inhibition, Na^+/Ca^{2+} exchange inhibition, or by effects of Na^+/H^+ exchange inhibition.

In this study the effect of amiloride was tested (at a concentration known to inhibit Na^+/H^+ exchange and not produce ionotropic changes) on the acidification of pH_i produced during exposure to hypoxia. Hypoxia did not cause a significantly different acidification of pH_i in the presence of 1mM amiloride compared with hypoxia in normal Tyrode solution.

An explanation of these results is rather difficult since it might be expected that amiloride inhibition of Na^+/H^+ exchange might result in a larger acidification of pH_i during hypoxia. However, in view of the results obtained using cinnamate, it seems likely that a proportion of the acidification seen in hypoxia is the result of lactate production in the cell during anaerobic glycolysis. Perhaps the major factor controlling the amplitude of the acidification of pH_i during hypoxia and the rate of recovery of pH_i on recovery is the cinnamate inhibited lactic acid carrier.

Ellis and Macleod (1985) have measured the rate of pH recovery from various intracellular acid loads produced by acid pH_o or CO_2. These recoveries were shown to be primarily due to Na/H exchange. Their measurements of the time course of recovery from acid loads appear to be much slower than the time course of recovery of pH_i measured in this study after hypoxia. This suggests that Na/H exchange is not the main controller of intracellular acidification in, and recovery from, hypoxia and therefore amiloride might be expected to have little effect.

(14) EFFECT OF SITS

The amine-reactive drug, SITS has been shown to inhibit transmembrane anion-exchange carriers (Russell and Boron, 1976).

Vaughan-Jones (1979), measured a^i_{Cl} in sheep Purkinje fibre and found that inward pumping of Cl^- was substantially inhibited by 100uM SITS. Baumgarten and Duncan (1987), suggest that an alternative method for Cl^- uptake is Na^+-dependent Cl^- co-transport. Their experiments indicate that while Cl^-/HCO_3^- exchange occurs, it does not physiologically control a^i_{Cl} in the heart.

Ellis and Thomas (1976) looked at the rapid recovery of pH_i from acidosis in sheep Purkinje fibres and found that unlike the situation in snail neurones where active outward transport of H^+ ions is selectively inhibitable by SITS, recovery of Purkinje fibre pH_i from CO_2-induced acidosis was unaffected by SITS (Ellis and Thomas, 1976a). It was concluded that Cl^-/HCO_3^- exchange, Na-dependent or otherwise, is not involved in pH_i recovery from acidosis.

The role (or lack of one) for Cl^-/HCO_3^- exchange in pH_i regulation during hypoxia was therefore of some interest. In this study it was found that in the preparations exposed to SITS (100uM), hypoxia produced a larger acidification of pH_i. If SITS were inhibiting a Cl^-/HCO_3^- exchange mechanism intracellular HCO_3^- levels would rise, there would be a more efficient removal of H^+ ions by HCO_3^- to produce H_2O and CO_2. These would leave the cell therefore reducing $[H^+]_i$ and causing a more alkaline pH_i. However in this study exposure to SITS caused a larger acidification of pH_i than was observed in normal Tyrode. The weight of evidence is generally

against Cl^-/HCO_3^- exchange having an important role in pH_i regulation. It is also possible that SITS may have effects on the tissue other than that of inhibiting Cl^-/HCO_3^- exchange. The only HCO_3^- available for exchange in HEPES buffered solutions is that produced by the cellular processes of the tissue. The results of these experiments remain ambiguous and further experiments would be required to elucidate the role of Cl^-/HCO_3^- exchange in pH_i regulation under conditions of hypoxia.

(15) EXTRACELLULAR POTASSIUM

It has been shown that in ischaemia $[K^+]_i$ tends to decrease and there is an accumulation of K^+ outside the cell (Kleber,1984). K^+ may also play a role in the development of arrhythmias. Harris (1954) found increasing levels of K^+ in coronary venous blood during the onset of arrhythmias after coronary ligation. The accumulation of K^+ during ischaemia may have other deleterious effects on the heart and it was of interest to mimic this aspect of ischaemia in the hypoxic heart.

The application of 12mM $[K^+]$ caused a depolarization of the membrane potential of about 15mV, with no measurable change in pH_i. The depolarization therefore appears not to affect Na^+/H^+ exchange or also affects another process in an equal and opposite fashion. The acidification of pH_i in hypoxia was not significantly different in high (12mM) and low (6mM) $[K^+]_o$ (e.g. Fig.3.41), suggesting that the mechanisms of regulation of pH_i are not dependent on $[K^+]_o$ or the membrane potential in this range. This would fit in with an electroneutral regulatory mechanism e.g. a lactic acid carrier. The lack of large Em changes during hypoxia also suggest an electroneutral process. However a noticable small hyperpolarisation of Em on the readdition of O_2

suggests a slight contribution of an electrogenic mechanism.

In summary, this study indicates that the effects of hypoxia and anoxia on the pH_i of sheep Purkinje fibres may be largely due to the production of lactic acid by the hypoxic tissue. This hypothesis is substantiated by the experiments investigating the effect of cinnamate on the extent of the acidification of pH_i during, and the recovery of pH_i, after hypoxia. The experiments performed using amiloride to inhibit Na/H exchange indicate that Na/H exchange is not solely responsible for the regulation of pH_i during hypoxia.

Hypoxia, anoxia and NaCN were compared for their effects on pH_i and contraction, it should be noted that although all three may be assumed to inhibit aerobic glycolysis to some extent there was some variation between the three conditions.

It was shown that there was no adaptive recovery of pH_i during prolonged hypoxia and that there appears to be no decrease in stored glycogen levels in sheep Purkinje fibres after extended periods of hypoxia.

Temperature, extracellular buffering and extracellular pH were all found to alter the extent of the acidification of pH_i during hypoxia while changing the extracellular substrate or raising extracellular $[K^+]$ had little effect on pH_i during hypoxia.

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APPENDIX 1

	S1	S2	S3	S4	S5	MEAN
5H	1	2	5	2	2	2·4
6H	4	6	6	6	5	5.4
8H	7	8	8	8	7	7.6
4H	8	7	4	5	7	6.0
1H	10	9	9	9	9	9.2
2C	2	1	1	1	1	1.2
3C	3	4	3	4	3	3.4
10C	5	3	2	3	4	3.4
9C	6	5	7	7	8	6.6
7C	9	10	10	10	10	9.8

The table shows the subjects (S1-S5) and the rank they assigned to electron-micrographs (labelled 1-10) from either hypoxic (H) or control (C) Purkinje fibres. This data was used to perform a Mann-Whitney U test on the mean ranks.

The effect of temperature on intracellular pH (pH_i) and the response to hypoxia in quiescent sheep heart Purkinje fibres *in vitro*

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When oxidative phosphorylation is blocked by hypoxia there is an intracellular acidification which is followed on reoxygenation by a further transient intracellular acidification before recovery (Ellis & Noireaud, 1987). Low temperature has been shown to have an effect on the ischaemic myocardium (Jones *et al.* 1982) by delaying the depletion of high energy phosphate. This study was designed to investigate whether hypothermia could delay the onset and the extent of the intracellular acidification caused by hypoxia. Experiments were performed to measure changes in pH_i during hypoxia at 35 and 22 °C.

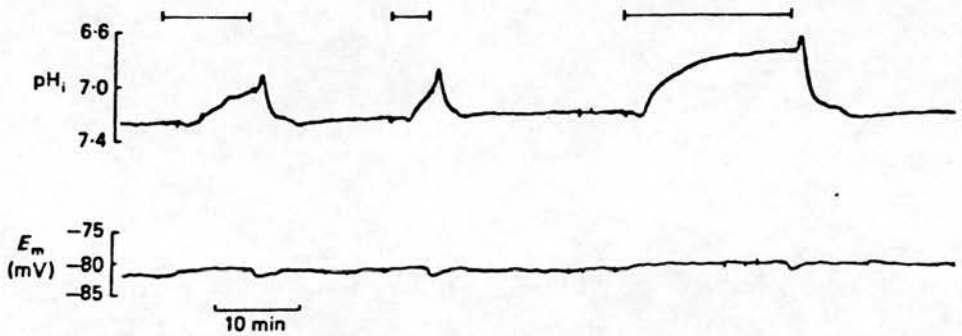

Fig. 1. The changes of intracellular pH and membrane potential (E_m) during exposures to 10, 4 and 20 min of hypoxia (as indicated by the bars) at 35 °C. Solutions were buffered with 10 mM-HEPES. Hypoxic solutions were bubbled with 100% N_2.

The resting level of pH_i at 35 °C was 7·11 ± 0·17 (mean ± S.D., $n = 14$). Following 20 min exposure to hypoxia the pH_i became more acidic by 0·26 ± 0·18 pH units ($n = 14$). On reoxygenation a further transient acidification of 0·09 ± 0·06 pH units occurred before recovery. At 22 °C the resting pH_i was 7·42 ± 0·18 ($n = 14$). During 20 min of hypoxia it became more acid by 0·09 ± 0·12 pH units ($n = 14$). The transient extra acidification on reoxygenation was 0·06 ± 0·08 pH units. The acidification during hypoxia was significantly greater at 35 °C than at 22 °C ($P < 0·01$ on a two sample t test).

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J Mol Cell Cardiol **20** (Supplement IV) (1988)

P-55 THE EFFECT OF METABOLIC SUBSTRATES ON INTRACELLULAR pH (pH_i) DURING HYPOXIA IN QUIESENT SHEEP HEART PURKINJE FIBRES. C.M. Bright and D. Ellis. Department of Physiology, University Medical School, Teviot Place, Edinburgh, EH8 9AG.

When oxidative phosphorylation is blocked by hypoxia there is an intracellular acidification whioh is followed on reoxygenation by a further transient acidification before recovery (Ellis & Noireaud, 1987. J. Physiol. 383, 125-141). Cardiac tissue can utilize different metabolic substrates so the effects of glucose, pyruvate or acetate (20mM) during hypoxia were investigated. 30 minutes exposure to the substrate was followed by 20 minutes of hypoxia. In 6 experiments there appeared to be no significant difference ($P>0.1$ in a 2 sample t-test) between hypoxia induced acidification in 20mM glucose, acetate or pyruvate compared to that in normal Tyrode (10mM glucose). This lack of significance could have been due to the wide variation in the effects of the various substrates. It is possible that the large glycogen stores of Purkinje fibres prevent the different effects of the substrates from being apparent in these relatively short term exposures. (Supported by the British Heart Foundation).

EFFETS DE LA SAPONINE SUR LES ACTIVITES IONIQUES INTRACELLULAIRES (Na^+, K^+, H^+) DE LA FIBRE DE PURKINJE DE MOUTON.

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Utilisée à des doses n'induisant pas un pelage de la membrane cellulaire, la saponine possède une action inotrope positive sur le muscle papillaire de mammifère (Enomoto et al, Brit J Pharmacol 1986, 88: 259). Le but de ce travail fut d'examiner si des variations concommittante du sodium et/ou du pH intracellulaire pourraient permettre d'interpréter les effets de ce glycoside sur la force contractile du muscle cardiaque.

Les activités sodique (Nai), potassique (Ki) et protonnique (Hi) de la fibre de Purkinje de mouton ont été mesurés à l'aide de la technique des micro-électrodes sensibles.

En présence de 0.015-0.04 mg/ml de saponine, on observe une dépolarization d'environ 20 mV de la membrane, une augmentation de 40% de Nai, une diminution de 9% de Ki et aucun effet sur le pHi.

Les effets de la saponine semblent donc liés à une perméabilisation non spécifique de la membrane et l'augmentation résultante de Nai permet d'expliquer via l'échange Na/Ca l'effet inotrope positif de cette substance.

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Effects of Saponin on Contractile Force and Intracellular Ion Activities of Cardiac Tissues

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J. Noireaud, C. M. Bright and D. Ellis. Effects of Saponin on Contractile Force and Intracellular Ion Activities of Cardiac Tissues. *Journal of Molecular and Cellular Cardiology* (1989) **21**, 291–298. The effects of the glycoside saponin on ferret ventricular muscle have been investigated. Saponin produced a positive inotropic effect, the extent of which was determined by the bathing calcium concentration. If the bathing calcium concentration is reduced to levels equivalent to those found intracellularly then similar saponin concentrations are able to "skin" the cardiac cells. The effects were further investigated in sheep heart Purkinje fibres. In the presence of normal extracellular calcium concentrations (to prevent skinning), saponin produced increases in sodium activity, decreases in potassium activity but little change in intracellular pH. The decreases of potassium activity were compared to the effects of strophanthidin. The changes in intracellular ion levels were accompanied by the development of a contracture. The effects of saponin could be explained by its interaction with cholesterol in the cell membrane resulting in an increase in membrane permeability to sodium which may be part of a nonspecific increase in membrane permeability.

Key Words: Intracellular pH; Intracellular K; Intracellular Na activity; Cardiac Purkinje fibre; Cardiac ventricular muscle.

Introduction

Saponins form a complex with cholesterol, resulting in the production of holes in a cholesterol-containing membrane (Bangham and Horne, 1962; Glauert *et al.*, 1962). This makes these molecules useful tools to skin muscle preparations chemically (Endo *et al.*, 1977). At 25°C, 30 min treatment with 0.05 mg/ml saponin is a standard procedure for chemical skinning of cardiac muscles (Endo and Kitazawa, 1978). Using lower concentrations, which did not skin the muscle membranes, Enomoto *et al.* (1986) have shown that several saponins of animal and plant origin, have a positive inotropic action on atrial and papillary muscles of the guinea-pig. They suggested that modification of the calcium channel could be involved in this effect. Recently Yamasaki *et al.* (1987) have suggested that the passive permeability of sarcolemmal vesicles could be increased and that there was an indication of the stimulation of sodium–calcium exchange. "Sub-skinning" concentrations of saponins also induced depolarization in cardiac muscles (Enomoto *et al.*, 1986), smooth muscles (Osa and Ogasawara, 1984) and squid axons (De Groof and Narahashi, 1976). The latter authors concluded that holothurin A (a saponin obtained from a sea cucumber) increased sodium permeability. In heart, the positive inotropic effect of increasing the intracellular sodium activity (a^{i}_{Na}) is well documented and probably occurs via an effect on the sodium–calcium exchange system (Reuter and Seitz, 1968). On the other hand, an increase of intracellular pH (pH_i) is also known to potentiate the developed tension of muscles

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(Fabiato and Fabiato, 1978). The interaction of saponins with cholesterol could have perturbated such membrane functions as the regulation of pH_i and thus have affected force production.

The aim of the present experiments has been to study whether or not the saponin induced modification of the mechanical performance of cardiac muscles could be related to changes in a^i_{Na} and/or pH_i.

Methods

General

Fresh sheep hearts were obtained from the local slaughterhouse. They were cut open and transported to the laboratory immersed in a Tyrode solution at ambient temperature that was either bicarbonate-buffered and bubbled with 95% O_2, 5% CO_2 gas mixture or HEPES (n-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) buffered and bubbled with O_2. No differences were noticed between the experiments using hearts collected in the two types of solution. Free-running Purkinje fibres were removed from the left ventricle. Ferrets were anaesthetized with intraperitoneal pentobarbitone sodium (Sagatal, May and Baker). The heart was removed rapidly and washed in Tyrode solution. Thin papillary muscles were dissected from the right ventricle. Methods of mounting both types of preparation in the experimental chamber to record tension were essentially the same as described previously (Bers and Ellis, 1982; Ellis and MacLeod, 1985).

Solutions

The normal Tyrode solution contained (mM): Na^+ 140, K^+ 6, Ca^{2+} 2, Mg^{2+} 1, Cl^- 152, glucose 10, HEPES 5, and was titrated with NaOH (*c.* 6 mM) to give a pH of 7.4 ± 0.05 at 35°C. The solution was equilibrated with 100% O_2.

Solutions for calibrating sodium-sensitive microelectrodes were made by substituting potassium for sodium in Tyrode solution. The Tyrode solutions used to calibrate the sodium- and potassium-sensitive microelectrodes had no calcium added. The calibrating solutions for potassium had the following potassium concentrations (mM) 70, 110, 129, 141 and 151. The latter was slightly hypertonic (by 20 mOsmol). This hypertonicity had such a small effect on the response of the electrode that the effect was neglected. The 12 mM $[K^+]$ Tyrode used to check impalement with the potassium-sensitive electrode was identical to normal Tyrode except that it contained (mM) Na^+ 128, and K^+ 12.

Saponin was obtained from Sigma. It contains about 20% of sapogenin isolated from the white roots of *Dianthus gypsophila*. Strophanthidin (Boehringer Mannheim) was used at a concentration of 2×10^{-5} M. This concentration was obtained by diluting 10^{-2} M strophanthidin (dissolved in a 50/50 mixture of ethanol and water) in normal Tyrode solution so that the final solution did not contain more than 0.5% ethanol.

Micro-electrodes

Membrane potentials (E_m) were measured with conventional 3 M-KCl-filled microelectrodes with resistances 10 to 25 MΩ. pH-sensitive and sodium-sensitive microelectrodes were filled with neutral H^+ carrier (Fluka) and sodium-ionophore I (ETH 227, Fluka) respectively. The potassium-sensitive electrodes were filled with Corning liquid ion exchanger (W.P.I.). The silanized micropipettes were filled on the day they were used. The micropipettes were first back-filled with a solution of 100 mM sodium chloride and 100 mM sodium citrate for both hydrogen ion and sodium-sensitive electrodes or 100 mM potassium chloride for potassium electrodes. Then a column (*c.* 200 μm) of the hydrogen, sodium or potassium selective resin was drawn by suction into the tip of the microelectrode. Their tips were < 1 μm in diameter. Estimates of their response time were limited by the solution exchange time in the bath. This was 90% complete in 18 to 21 s. For each drug used, the possibility of an interference with the ion-sensitive electrode was checked.

Calibration of electrodes

All electrodes were calibrated at 35°C in the experimental chamber before and after each experiment as described previously for sodium and hydrogen-sensitive electrodes (Ellis, 1977; Deitmer and Ellis, 1980). Particular care is necessary in calibrating potassium-

sensitive electrodes due to the logarithmic relationship between the activity of an ion and the voltage output of the electrode. Thus at physiological levels of intracellular potassium, relatively large potassium changes are associated with quite small changes in the voltage output of the microelectrode. For each calibration curve a linear regression was plotted to check that the electrode calibration intercepted the x-axis at approximately 6 mM which was the $[K^+]$ in normal Tyrode. By using the same linear regression it was possible to calculate a_K^i directly from the intracellular measurements of the ion-sensitive electrode potentials. To check that each impalement was successful the perfusing solution was briefly switched to 12 mM $[K^+]$ Tyrode. This produces a slight depolarization in the preparation, the K^+-sensitive micro-electrode signal has the E_m signal subtracted from it in order to obtain a value for a_K^i. If both electrodes are intracellular no depolarization is seen on the a_K^i trace.

Results

Effects of saponin on the developed tension of ferret papillary muscle

The effects of saponin, at various $[Ca]_0$, were measured in ferret papillary muscles. The papillary muscles were stretched to a length giving optimal force production and stimulated at a frequency of 1 Hz for about 30 min in order to allow the developed tension to equilibrate to a steady amplitude. Thereafter saponin was applied to the muscle. Typical records of the effect of adding 0.025 mg/ml saponin on the developed tension are shown in Figure 1. Saponin at this concentration added to normal Tyrode ($[Ca]_0 = 2$ mM) increased the amplitude of the developed tension by 35% compared to the control. Then $[Ca]_0$ was reduced to 1 mM and the same procedure repeated. Now the amplitude of the developed tension was increased by 63% on adding an identical amount of saponin. A subsequent reduction of $[Ca]_0$ to 0.5 mM (not illustrated) further potentiated the effect of 0.025 mg/ml saponin. The amplitude of the developed tension was increased by 74% compared to the control in the absence of saponin.

Saponin concentrations of 0.025 mg/ml resulted in the development of a contracture in some preparations. A contracture was always apparent if a saponin concentration of 0.05 mg/ml or higher was used.

Effects of saponin on membrane potential, intracellular pH, and intracellular sodium activity of sheep Purkinje fibres

Figure 2 shows the effect of adding 0.04 mg/ml saponin to the Tyrode solution while pH_i, a_{Na}^i and membrane potential were being recorded with ion-sensitive micro-electrodes in a Purkinje fibre of sheep heart. Saponin treatment was accompanied by a depolarization (of 14 mV in this case). During the change of E_m, there was an increase of a_{Na}^i (46% compared to the control) but little or no change of pH_i. The effects were completely reversible on return to normal Tyrode solution. Different concentrations of saponin have been tested up to 0.1 mg/ml and under these experimental conditions, the half-maximum effect on E_m and a_{Na}^i seems to occur with approximately 0.03 mg/ml saponin. The effects were however

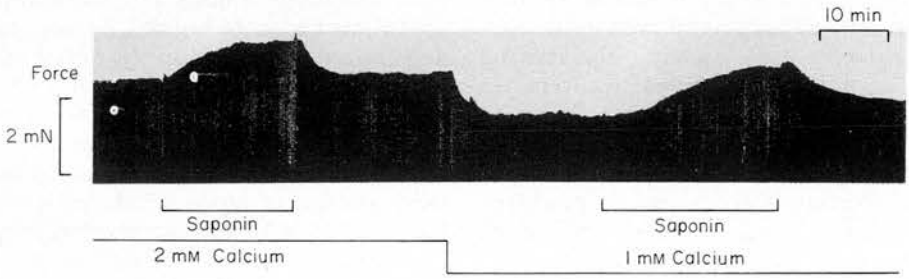

FIGURE 1. The effect of saponin on developed tension from ferret papillary muscle. The muscle was stimulated at 1 Hz in Tyrode solution containing either 2 mM Ca (normal $[Ca]_0$) or 1 mM Ca. During the period indicated by the bars 0.025 mg/ml saponin was added to the superfusing solution.

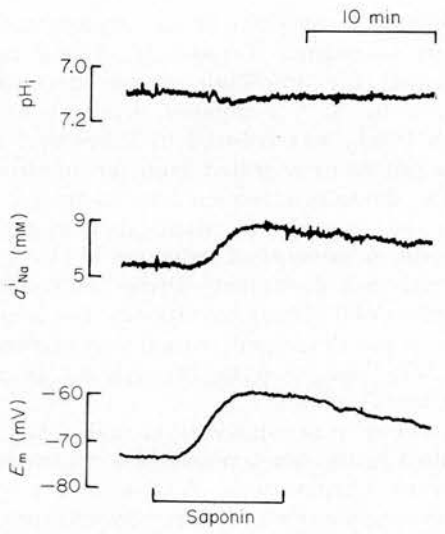

FIGURE 2. Effect of saponin on the pH_i (top trace), the intracellular Na^+ activity (a^i_{Na}, middle trace) and the membrane potential (E_m, bottom trace) of a Purkinje fibre from sheep heart. During the period indicated by the bar 0.04 mg/ml saponin was added to the superfusing solution. After 10 min the muscle was returned to the control Tyrode solution.

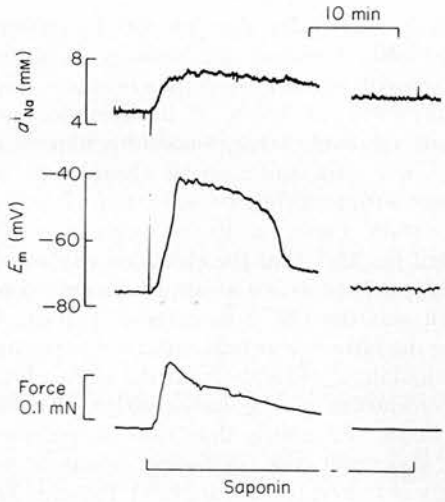

FIGURE 3. The effect of a long exposure to saponin on the intracellular Na^+ activity (a^i_{Na}, top trace) the membrane potential (E_m, middle trace) and on the resting tension (bottom trace) of a Purkinje fibre from sheep heart. During the period indicated by the bar, 0.065 mg/ml saponin was added to the superfusing solution. After 70 min the muscle was returned to the control Tyrode solution. The break in the trace was for approximately 45 min.

rather variable. When higher concentrations were used (> 0.1 mg/ml), reversibility was often difficult to obtain.

Figure 3 shows that in some experiments the effects of saponin appeared to be reversible even in the continued presence of the compound. Figure 3 shows the effects of a long exposure (70 min) to a Tyrode solution containing 0.065 mg/ml saponin, while resting tension, a^i_{Na} and membrane potential were recorded in a Purkinje fibre of sheep heart. The addition of saponin induced a depolarization of about 35 mV of the membrane. The intracellular sodium activity increased in parallel by 54% compared to the control. However, after about 25 min even in the continued presence of saponin, the resting tension, a^i_{Na} and E_m had almost recovered to their control values.

The sodium-potassium pump appeared to be still functional in the presence of saponin as removal of extracellular potassium produced an extra increase of a^i_{Na} (not illustrated) which recovered on adding back potassium.

In another series of experiments undertaken in Purkinje fibres of sheep heart, the ammonium rebound technique (Boron and de Weer, 1976) was used to test the integrity of pH_i regulation after a long exposure to saponin. After 60 min in the presence of a high concentration of saponin (0.15 mg/ml), pH_i was still able to recover both on addition and removal of NH_4Cl in some preparations while in others there was inhibition of the pH_i recovery following acid loading even at a concentration of 0.05 mg/ml.

Calcium and magnesium dependency of the effects of saponin

Figure 4 shows an example of the effect of adding 0.025 mg/ml saponin to Tyrode solutions containing 2 mM Ca^{2+} (normal $[Ca]_0$) or 0.5 mM Ca^{2+}. In normal Tyrode, saponin depolarized the membrane by only 5 mV and no change of a^i_{Na} was recorded. The membrane potential had recovered to its control value after less than 3 min on removal of saponin. On reducing calcium to 0.5 mM, the same amount of saponin induced a depolarization of 15 mV, and increased a^i_{Na} by 20%. Recovery was much more difficult to obtain. Similar effects were obtained by varying the external magnesium concentration in the presence of low concentrations of calcium.

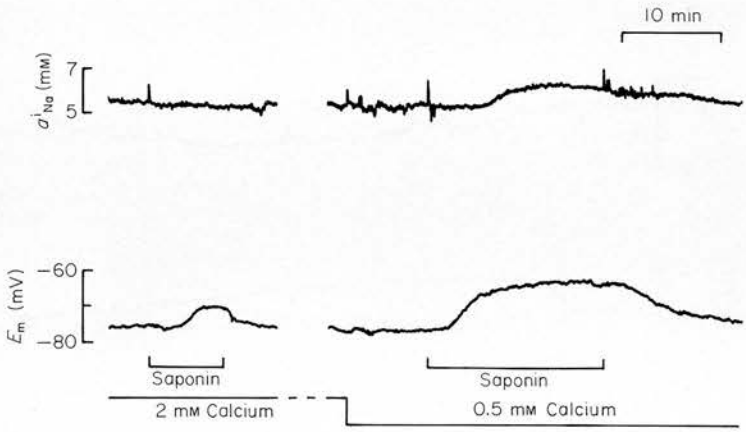

FIGURE 4. The effect of saponin on the intracellular Na^+ activity (a^i_{Na}, top trace) and the membrane potential (E_m, bottom trace) of a Purkinje fibre from sheep heart. During the periods indicated by the bars 0.025 mg/ml saponin was added to the superfusing solution containing either 2 mM Ca (normal $[Ca]_o$) or 0.5 mM Ca.

Measurement of intracellular potassium activity and the effects of saponin

Measurements were made of a^i_K in order to try to assess the possible effect of saponin on K^+ permeability. For comparison, changes of a^i_K were also induced by application of high concentrations of cardiac glycosides to inhibit the sodium–potassium pump.

The effects of saponin on a^i_K were investigated in both sheep Purkinje fibres and ferret papillary muscle with Corning sensor filled electrodes. In Purkinje fibres on exposure to saponin (0.025 mg/ml) a significant ($P < 0.01$) decrease in mean a^i_K was recorded from 82.4 ± 15.2 mM (control value mean ± S.D.) to 75.4 ± 15.5 mM ($n = 5$). Saponin also had a considerable effect on E_m, decreasing it by a mean 20.4 ± 13.2 mV ($n = 5$). In addition to these effects saponin also caused the development of a contracture (see Fig. 5). We have compared the effects of saponin on a^i_K with strong inhibition of the sodium-potassium pump using strophanthidin (2×10^{-5} M). Strophanthidin (10 min exposure) caused a significant ($P < 0.002$) decrease in mean a^i_K from 90.1 ± 16.4 mM to 77.7 ± 14.0 mM ($n = 8$). This was accompanied by a depolarization of 9.6 mV ± 3.0 mV ($n = 8$). In addition, strophanthidin also caused an increase in resting tension (Fig. 6).

In two experiments performed on isolated ferret papillary muscle, exposure to saponin caused a decrease in a^i_K from a mean value of 101.5 mM to 90.3 mM ($n = 2$). The membrane potential decreased from 67 mV to 53 mV after saponin treatment. For comparison, strophanthidin (10 min exposure of 2×10^{-5} M) caused a decrease in the mean a^i_K from 101.7 to 90.7 mM. This was accompanied by a decrease of E_m from a mean value of 67 to 55 mV ($n = 2$).

Discussion

The results reported here have confirmed that saponin induces a depolarization of cardiac

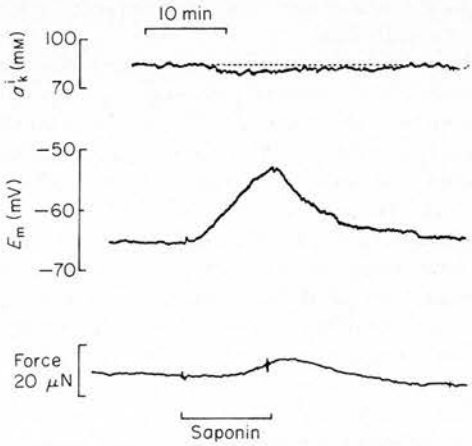

FIGURE 5. The effect of saponin on the intracellular potassium activity (a^i_K, top trace), the membrane potential (E_m, middle trace) and resting tension (bottom trace) in a sheep heart Purkinje fibre. The bar indicates the period of exposure to 0.025 mg/ml saponin.

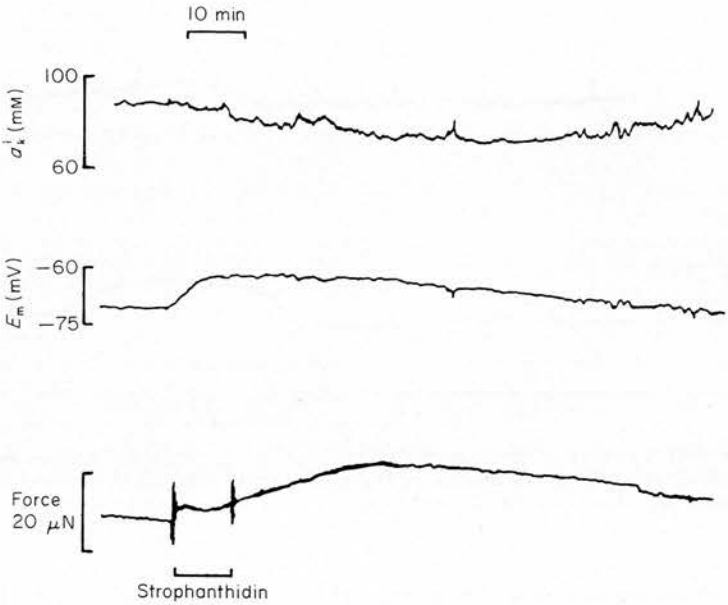

FIGURE 6. The effect of strophanthidin on the intracellular potassium activity (a_K^i, top trace), the membrane potential (E_m, middle trace) and the resting tension (bottom trace). Strophanthidin (2×10^{-5} M) was applied before the period indicated by the bar.

cell membranes (Enomoto *et al.*, 1986) and have shown that the depolarization is concomitant with an increase of the intracellular sodium ion activity and a decrease of intracellular potassium. The cardiac tissue was depolarized to about −40 mV in Tyrode solution following treatment with 0.15 mg/ml saponin for about 1 h. Therefore the skinning of the surface membrane was incomplete under these conditions.

In "sub-skinning" concentrations of saponin the sodium–potassium pump still appears to be operative. Under these conditions the removal of extracellular potassium caused an extra increase of intracellular sodium. Subsequent readdition of potassium was followed by a reduction of sodium. Also, in some experiments, there was an apparent partial reversal of the effects of saponin on a_{Na}^i and on membrane potential even in the continued presence of the glycoside. This effect is difficult to explain. Following the rise of sodium there might have been a slow recruitment of extra sodium pumps from a reserve pool as was suggested by Lamb and Ogden (1982) in HeLa cells.

The literature concerning the relationship of cholesterol to the sodium pump enzyme system is often contradictory (see e.g. Peters *et al.*, 1981). Some results appear to indicate that cholesterol is not essential for Na-K-ATPase activity (Roelofsen *et al.*, 1966; Wheeler and De Caldentey, 1980). The present results suggest that the interaction of saponin with cholesterol in these experiments did not impair greatly the function of the sodium–potassium pump. An alternative explanation for the partial recoveries of a_{Na}^i and membrane potential in the continued presence of saponin may be that either the sodium permeability decreased, or the sodium–potassium pump activity increased, due to changes in other intracellular factors.

One possible explanation for the apparent recovery of a_{Na}^i in saponin was that cells penetrated by the ion-sensitive and by the conventional microelectrodes became electrically uncoupled (the voltage recorded by the conventional electrode is electronically subtracted from that recorded by the ion-sensitive electrode in order to obtain the a_{Na}^i). This seems unlikely as quite large and rapid changes of membrane potential occurred without apparent artifacts on the recording of a_{Na}^i.

Saponin caused reversible decreases of a_K^i

(Fig. 5). These effects could be accounted for by an increased permeability of the cell membrane to potassium but some inhibition of the sodium–potassium pump cannot be ruled out. However, complete inhibition of the sodium–potassium pump with high concentrations of cardiac glycosides normally produces membrane depolarization of only 10 mV or less (Fig. 6; Deitmer and Ellis, 1978) whereas larger depolarizations were produced by saponin. It is conceivable that the large depolarizations were at least partly responsible for the observed decreases of intracellular potassium activity.

Saponin had relatively little effect on steady-state pH_i. Even if the resting H^+ influx was increased in saponin little change in pH_i would be expected due to the high buffering capacity of these cells and efficient proton pumping (Ellis and Thomas, 1976). In some experiments pH_i recovery from an acidosis was inhibited but this might have occurred as a result of an increase of a^i_{Na}. The latter would be expected to inhibit sodium–hydrogen exchange, the major factor involved in pH_i recovery from acidosis (e.g. Ellis and MacLeod, 1985).

Divalent cations and saponin

Interaction between the effect of saponin and the stabilizing action of divalent cations has already been reported to exist in smooth muscles (Osa *et al.*, 1985). The present results indicate that in cardiac tissue, changing $[Ca]_o$ or $[Mg]_o$ modulates the effect of saponin, it being more effective at low divalent cation concentrations. This could explain why incomplete skinning was obtained in the present experiments when concentrations as high as 0.15 mg/ml were added to the normal Tyrode solution (which contains 2 mM calcium and 1 mM magnesium). When such concentrations were added in the presence of only trace amounts of $[Ca]_o$ (plus 130 mM $[K]_o$, 10 mM $[Na]_o$ to mimic the intracellular medium) then the cells became skinned. Saponin has surfactant properties. However the divalent cation dependence does not appear to be like the interaction of calcium and soap (where insoluble calcium stearate is produced thereby lowering the soap concentration) as calcium does not cause precipitation in saponin solutions (100 mM calcium added to 50 mg/ml saponin).

To conclude, therefore, saponin is able to produce a positive inotropic effect on cardiac muscle and to produce cell skinning. Saponin is more effective the lower the divalent cation concentration. At subskinning concentrations there is an increase of a^i_{Na} and a decrease of a^i_K that appears not to be accounted for solely by an inhibition of the sodium–potassium pump. Even small increases of a^i_{Na} can produce large increases in force (Eisner *et al.*, 1984) via the sodium–calcium exchange mechanism. Thus the positive inotropic effects, the changes in intracellular ion levels and the depolarization could be explained by a saponin induced increase in membrane permeability to sodium which may be part of a nonspecific increase in ion permeability. The positive inotropy is probably not due to a change in intracellular pH.

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