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Protection against cellular damage in the perfused rat heart by lowered pH

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Abstract

In this comparative study, rat hearts were perfused at 37°C with three clearly defined protocols: the Ca^{2+} paradox, the O_2 paradox and with 20 mM caffeine. Each protocol involved an initial priming (Ca_o^{2+} depletion or anoxia; stage 1) and subsequent full activation (Ca_o^{2+} repletion or reoxygenation; stage 2) of the damage system of the sarcolemma. Creatine kinase release in stage 2 was completely inhibited ($P < 0.001$) in all three protocols when pH was reduced to 6.5 throughout the experiments, or only during stage 1, or only during stage 2. The inhibitor of the Na^+/H^+ antiporter, amiloride (1 mM), completely prevented creatine kinase release in the Ca^{2+} paradox ($P < 0.001$) and markedly reduced damage in the caffeine protocol. Amiloride had no significant effect on creatine kinase release in the O_2 paradox. The possible role of Na_i^+ was studied in the caffeine protocol: ouabain (5×10^{-6} M) had little effect whereas substitution of choline for Na^+ in the perfusion medium reduced creatine kinase release by about 50%. It is suggested that the same damage system is activated in stage 1 in all three protocols and that a key event is the intracellular production of H^+ which are exported via Na_o^+/H_i exchange. Prevention of H^+ efflux by lowered pH_o, even during stage 2, protected against creatine kinase release. The possible role of Na^+ movements in the genesis of sarcolemma damage is discussed. © 1997 Elsevier Science B.V.

Keywords: Ca^{2+} paradox; O_2 paradox; Caffeine; Amiloride; pH; Heart damage

1. Introduction

The Ca^{2+} and O_2 paradoxes are familiar protocols in experimental cardiac physiology and both have been studied in considerable detail (Hearse et al., 1978), although there is wide disagreement concerning the sequence of underlying cellular events that lead to severe damage (Hess and Manson, 1984; Chapman and Tunstall, 1987; Altschuld et al., 1991). The two major signs of overt damage are the release (or escape) of cytosolic proteins and the ultrastructural degradation of the myofilament apparatus; these two damage pathways are separate and can be independently activated (Daniels and Duncan, 1993, 1995). Although there are obvious differences between the two paradoxes, they have characteristic features in common (Hearse et al., 1978), suggesting that the same underlying damage mechanisms may be activated in both (Daniels and Duncan, 1995). Perfusion of the isolated rat heart with caffeine raises $[Ca^{2+}]_i$ and also rapidly causes the release of creatine kinase, but only when initially perfused with Ca^{2+}-free saline (Vander Heide et al., 1986; Daniels and Duncan, 1993) so that this experimental protocol has obvious parallels with the Ca^{2+} paradox. There are two stages in each of these experimental protocols: priming or initial activation is produced in stage 1 (by Ca_o^{2+} depletion in the Ca^{2+} paradox and caffeine protocols or by hypoxic perfusion in the O_2 paradox) and full activation and the leakage of cytosolic proteins in stage 2 (by Ca_o^{2+} repletion or reoxygenation).

Bielecki (1969) has shown that reduced pH_o provides protection against the Ca^{2+} paradox in the perfused rat heart, suggesting the possible involvement of the Na^+/H^+ antiporter which is also inhibited by extracellular acidosis in myocytes from the rat heart (Wallert and Frohlich, 1989). In this comparative study, we explore the protective and inhibitory effects of amiloride (an inhibitor of Na^+/H^+ exchange) and the pH of the perfusion medium against creatine kinase release in each of the three protocols and show that the results are consistent with the suggestion that protons are produced intracellularly during the priming events of stage 1 and that these are transported out of the cell via the Na^+/H^+ antiporter.

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2. Materials and methods

Wistar rats (250–400 g) were given an intraperitoneal injection of heparin (1000 U/kg) and were killed by cervical dislocation 20 min later. The heart was dissected out, transferred to ice-cold perfusion medium and then cannulated and mounted in a jacketed chamber maintained at 37°C. The isolated heart was perfused using a Watson and Marlow 503S peristaltic pump to maintain a constant flow rate of 8–10 ml min^{-1}. The three limbs of the perfusion apparatus allowed rapid change from one medium to another and a preheating coil in each limb immediately above the heart had a bubble trap with an overflow that was normally held at 40 cmH_2O pressure head. A stainless-steel heart clip was attached to the base of the left ventricle and linked by a length of cotton to a Biosciences D1 isometric transducer and a Washington MDI oscillograph. The heart was under a tension of 12 g. Mechanical activity was recorded simply to ensure normal contractile activity; any preparations that showed abnormalities or developed an impairment of their mechanical activity during an initial 10 min wash-out and equilibration period were discarded. Coronary flow was measured throughout the perfusion by collection of the coronary effluent at measured intervals and the data were used for the calculation of the rates of creatine kinase efflux.

Hearts were perfused with Krebs-Henseleit medium which contained (mM) NaCl 117, KCl 5.4, $NaHCO_3$ 25, $MgCl_2$ 1, $CaCl_2$ 2.0, NaH_2PO_4 1, glucose 11 made up in distilled water that had been passed through a deionizing column and which was then heavily gassed with 95% O_2 + 5% CO_2 or 95% N_2 + 5% CO_2 ('anoxic medium') and the pH finally adjusted to 7.35 ± 0.05 if necessary. Nominal Ca^{2+}-free solutions had $CaCl_2$ omitted without ionic substitution and were always rigorously prepared with a standardised procedure. They were briefly stored in plastic containers; all glassware was acid-washed. $[Ca^{2+}]$ was $< 10^{-6}$ M and the results were compared with Ca^{2+}-EGTA-buffered solutions; the solutions always primed the hearts satisfactorily in the Ca^{2+} paradox and caffeine protocols. Samples of coronary effluent were stored on ice and measurements of creatine kinase activity were made by linked assay at the end of the experiment, the production of NADPH being followed at 340 nm (Jones et al., 1983).

The standard protocols for the three experimental procedures were:

Ca^{2+} paradox: Initial wash-out and equilibration, 10 min. Ca^{2+} free (stage 1), 3 min. Ca^{2+} repletion (stage 2), 9 min.

O_2 paradox: Initial wash-out and equilibration, 10 min. Anoxic medium with glucose omitted (stage 1), 40 min. Return of O_2 with glucose omitted (stage 2), 18 min.

Caffeine protocol: Initial wash-out and equilibration, 10 min. Ca^{2+} free (stage 1), 3 min. Caffeine (20 mM) in Ca^{2+} free medium (stage 2), 9 min.

The pattern of creatine kinase release over 9 min was sigmoidal and all results were compared over the linear part of the graph by an analysis of covariance program. The program compares linear regression, following Sokal and Rohlf (1981) and the regression statistics are included in the program output. A test of regression slopes precedes a test of elevations. Comparison limits are shown if the regressions differ significantly.

All inorganic salts were AnalaR grade; biochemicals were obtained from Sigma Chemical Co., St. Louis, USA and hexokinase glucose-6-phosphate dehydrogenase preparation was obtained from Boehringer Mannheim UK, Lewes, Sussex, UK.

Experiments carried out under UK Home Office project licence number 40/00928.

3. Results

3.1. Ca^{2+} paradox

Hearts were perfused at 37°C at a constant flow of 8–10 ml min^{-1} at a perfusion pressure of 40 cm water and the cumulative creatine kinase release during Ca^{2+} reperfusion (stage 2) in the standard Ca^{2+} paradox protocol is shown in Fig. 1 line A. Total cumulative release of creatine kinase after 9 min of stage 2 was 678 ± 45.3 (mean ± S.E.M.) IU/g dry weight. No creatine kinase was released when the Ca^{2+} paradox was carried out at 28°C (Fig. 2 line E).

Hearts were then perfused with Krebs-Henseleit medium at pH 6.5, against a control pH of 7.35, under standard Ca^{2+} paradox conditions. The hearts were equilibrated

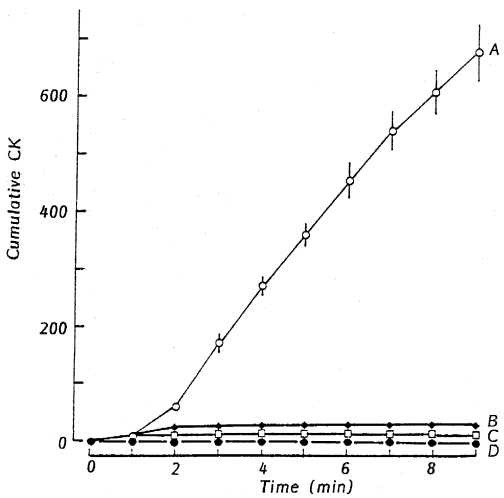

Fig. 1. Effect of pH 6.5 on the Ca^{2+} paradox. In all figures, ordinate shows cumulative mean release of creatine kinase (IU/g dry weight) and abscissa shows duration (min) of stage 2. Vertical lines represent ± S.E.M. where these exceed the diameter of the points. (A) Standard Ca^{2+} paradox, $pH_o = 7.35$ ($n = 7$). (B) $pH_o = 6.5$ throughout stages 1 and 2 ($n = 5$). (C) $pH_o = 6.5$ in stage 1 and 7.35 in stage 2 ($n = 3$). (D) $pH_o = 7.35$ in stage 1 and 6.5 in stage 2 ($n = 3$).

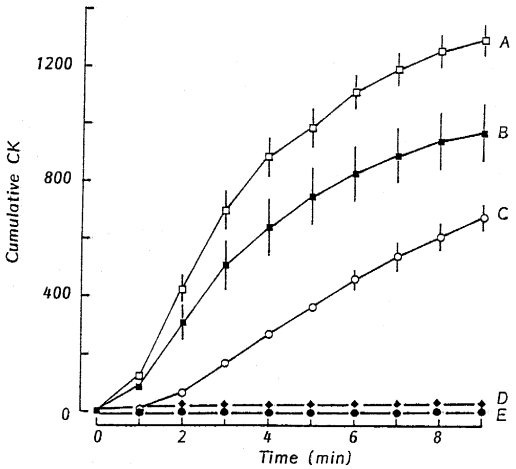

Fig. 2. Protective effect of amiloride on the Ca^{2+} paradox at different perfusion pressures. 1 mM amiloride present during stages 1 and 2. (A) Control Ca^{2+} paradox at 80 cmH_2O ($n = 6$). (B) 80 cmH_2O + amiloride ($n = 5$). (C) Control Ca^{2+} paradox at 40 cmH_2O ($n = 7$). (D) 40 cmH_2O + amiloride ($n = 4$). (E) Control Ca^{2+} paradox at 28°C ($n = 5$).

during wash-out at pH 7.35 and pH_o was then reduced to 6.5 at the start of stage 1; creatine kinase release was completely blocked in this protocol when perfused at pH 6.5 throughout ($P < 0.001$). This total suppression of creatine kinase release ($P < 0.001$) was also seen at pH 6.5 for only stage 1 or only stage 2; see Fig. 1 lines B–D.

Hearts were perfused with 1 mM amiloride (an inhibitor of the Na^+/H^+ antiporter) which was included in the last 5 min of equilibration and was then present for the remainder of the paradox protocol. At a perfusion pressure of 40 cm water, 1 mM amiloride completely blocked creatine kinase release ($P < 0.001$), see Fig. 2 line D.

When perfusion pressure during the standard Ca^{2+} paradox was raised from 40 to 80 cm water, mean total cumulative creatine kinase release rose significantly from 678 ± 45.3 to 1289 ± 60.5 IU/g dry weight ($P < 0.001$; Fig. 2 line A). However, under these conditions of raised perfusion pressure, 1 mM amiloride did not completely suppress creatine kinase release although the mean cumulative total after 9 min of stage 2 fell to 75% (967 ± 100.2 IU/g dry weight; $P < 0.001$; Fig. 2 line B.

In a single experiment, choline chloride was substituted for NaCl throughout stages 1 and 2 of the Ca^{2+} paradox and total cumulative creatine kinase release fell from 687 to 269 IU/g dry weight, a 60% reduction.

3.2. O_2 paradox

In the standard O_2 paradox at 37°C (40 min anoxia in stage 1), with glucose omitted from the perfusion medium, a cumulative total of 444 ± 54.6 IU creatine kinase/g dry weight was released during the 18 min reoxygenation in stage 2 (Fig. 3 line B). In single experiments, 20 min anoxia in stage 1 produced a cumulative total of 117 IU (25%; Fig. 3 line C) whereas 60 min anoxia produced

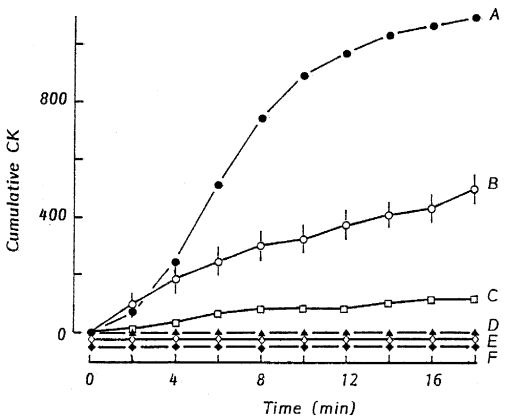

Fig. 3. O_2 paradox. Effect of time of anoxic perfusion and pH_o. (A) Stage 1 = 60 min anoxia. (B) Control O_2 paradox; stage 1 = 40 min anoxia ($n = 5$). (C) Stage 1 = 20 min anoxia. (D–F) Effect of pH_o on anoxia control O_2 paradox. (D) $pH_o = 6.5$ throughout stages 1 and 2 ($n = 3$). (E) $pH_o = 6.5$ in stage 1 and 7.35 in stage 2 ($n = 3$). (F) $pH_o = 7.3$ in stage 1 and 6.5 in stage 2 ($n = 3$).

1097 IU creatine kinase/g dry weight (250%; Fig. 3 line A). Thus, prolongation of stage 1 progressively increased creatine kinase release in stage 2. No creatine kinase was released when the standard O_2 paradox was carried out at 28°C (Fig. 4 line C).

Hearts were perfused with Krebs-Henseleit medium at pH 6.5 at 37°C, the reduction in pH_o being introduced at the start of stage 1. Reduced pH_o of 6.5 completely suppressed creatine kinase release when present in both stages ($P < 0.001$) or only in stage 1 ($P < 0.001$) or only in stage 2 ($P < 0.001$); see Fig. 3 lines D–F.

Amiloride (1 mM) included for the last 5 min of the equilibration period and then included for the duration of the O_2 paradox protocol produced only a very small reduction in the mean cumulative release of creatine kinase (Fig. 4 line B) which was not statistically significant.

3.3. Caffeine protocol

Hearts were perfused with Ca^{2+}-free saline for 3 min (stage 1) before perfusion with Ca^{2+}-free saline containing

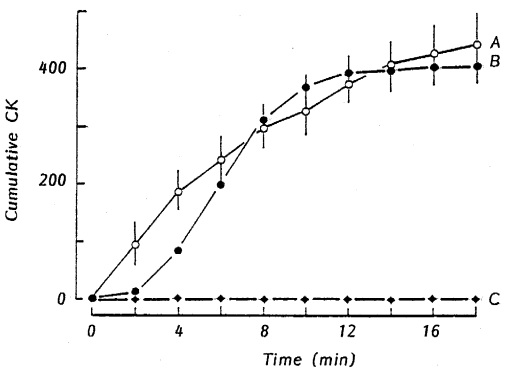

Fig. 4. Effect of amiloride on O_2 paradox. (A) Control O_2 paradox ($n = 5$). (B) Plus 1 mM amiloride ($n = 3$). (C) O_2 paradox at 28°C ($n = 5$).

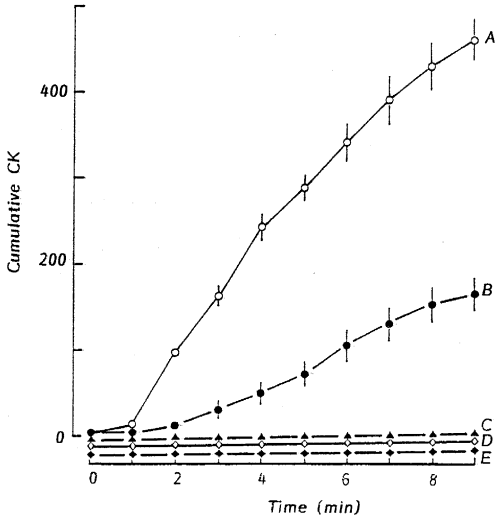

Fig. 5. Effect of pH 6.5 on the caffeine protocol. (A) Standard caffeine protocol with Ca^{2+}-free priming ($n = 5$). (B) No priming by Ca_o^{2+} depletion ($n = 3$). (C) pH_o 6.5 throughout stages 1 and 2 ($n = 3$). (D) $pH_o = 6.5$ in stage 1 and 7.35 in stage 2 ($n = 3$). (E) $pH_o = 7.35$ in stage 1 and 6.5 in stage 2 ($n = 3$).

20 mM caffeine for 9 min (stage 2). Mean total cumulative creatine kinase release was 458 ± 27.7 IU/g dry weight (Fig. 5 line A). Omission of stage 1 (i.e. perfusion of caffeine in Ca^{2+}-free saline with no Ca^{2+}-free priming) significantly reduced cumulative creatine kinase release to only 160 ± 21.8 IU/g dry weight ($P < 0.001$; Fig. 5 line B) showing the importance of initial Ca_o^{2+}-free priming, as in the Ca^{2+} paradox. Creatine kinase release was completely inhibited when the perfusion temperature was reduced to 28°C (Fig. 6 line E).

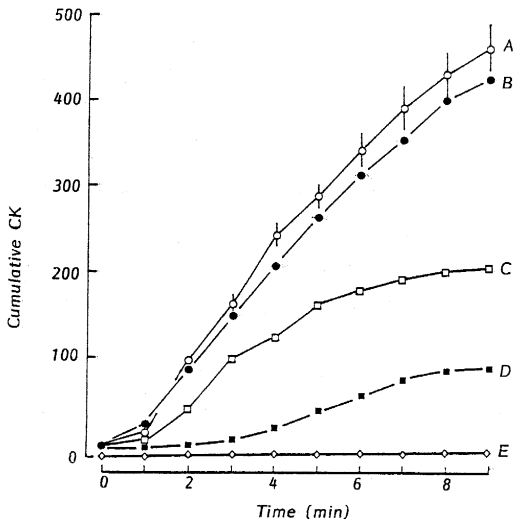

Fig. 6. Caffeine protocol. (A) Standard caffeine protocol ($n = 5$). (B) Plus 5×10^{-6} M ouabain throughout stages 1 and 2. (C) Replacement of Na^+ in Krebs-Henseleit medium by an equiosmotic concentration of choline. (D) Plus 1 mM amiloride. (E) Caffeine protocol at 28°C ($n = 5$).

The reduction of pH_o to 6.5 was introduced at the start of stage 1. Creatine kinase release was completely suppressed when pH_o was reduced in both stages of the caffeine protocol ($P < 0.001$) or only during stage 1 ($P < 0.001$) or only during stage 2 ($P < 0.001$); see Fig. 5 lines C–E.

The inclusion of 1 mM amiloride in the perfusion medium during the last 5 min of equilibration until the end of stage 2 of the caffeine protocol resulted in the release of only 88 IU creatine kinase/g dry weight, an 80% reduction from control values (Fig. 6 line D).

The addition of 5×10^{-6} M ouabain to the perfusion medium during the last 5 min of equilibration and throughout the caffeine protocol resulted in a cumulative total creatine kinase release of 424 IU/g dry weight, little different from control values (Fig. 6 line B). The equiosmotic substitution of choline for Na^+ in the Krebs-Henseleit perfusion medium from the start of stage 1 in the caffeine protocol resulted in a cumulative creatine kinase release of 201 IU/g dry weight, 44% of the release from control hearts (Fig. 6 line C).

4. Discussion

The results from the three experimental protocols described here have features in common, suggesting that the same damage mechanism underlies the leakage (or release) of creatine kinase (see Duncan, 1990, 1997). In each protocol, the damage system (i) is initially activated (or primed) in stage 1 and fully activated in stage 2, (ii) is completely protected by reducing the perfusion temperature in stage 1 to 28°C (Hearse et al., 1978; Harding and Duncan, 1996), and (iii) is critically dependent on a marked rise in $[Ca^{2+}]_i$ (Grinwald and Brosnahan, 1987; Opie et al., 1988; Matsuda et al., 1992). However, there are also clear differences between the protocols. Caffeine and the Ca^{2+} paradox both require priming via Ca^{2+} depletion in stage 1 and $[Ca^{2+}]_i$ rises in stage 2 (although the intracellular Ca^{2+} has different origins in the two protocols, with Ca^{2+} influx during Ca^{2+} repletion in stage 2 of the paradox whereas Ca^{2+} is released from intracellular storage sites with caffeine). On the other hand, the system is primed by a rise in $[Ca^{2+}]_i$ in stage 1 of the O_2 paradox because the reduced supply of high-energy phosphates results in a progressive loss of Ca^{2+} homeostasis (Duncan, 1990; Daniels and Duncan, 1994); the longer the period of anoxia in stage 1, the greater is the rise in $[Ca^{2+}]_i$ with a corresponding exacerbation of creatine kinase release (see Fig. 3). Extracellular Ca^{2+} is not removed in either stage of the O_2 paradox and full activation is achieved by the return of molecular O_2 in stage 2.

Reducing pH_o to 6.5 either only in stage 1 or only in stage 2 completely suppressed creatine kinase release in all three protocols, an additional feature that confirms their

underlying similarity. Other studies have shown a protective effect with reduced pH_o in the Ca^{2+} paradox and O_2 paradox: it has been suggested that the alteration of pH_o might interfere with the transmembrane fluxes of Ca^{2+} during the Ca^{2+} paradox (Bielecki, 1969) or that reduced pH_o might induce a membrane phase change (Trauble and Eibl, 1974). However, this is believed to be the first study which shows that both stages of each of the three protocols are independently and critically sensitive to pH_o. The caffeine protocol in the present experiments was conducted with Ca^{2+}-free solutions throughout so that reduced pH_o cannot be affecting Ca^{2+} influx, as previously suggested (Bielecki, 1969).

The protection produced by lowering the incubation temperature to 28°C suggests that mobility of a molecular complex within the bilayer is critical for activation of the damage system (Duncan, 1990). It is possible that lowered pH_o results in a comparable phase transition of the phospholipid domain (Houslay and Stanley, 1982) and so provides protection against creatine kinase release, as suggested above (Trauble and Eibl, 1974), similar to the protective effect of lowered temperature. However, no protection is found if the incubation temperature is reduced to 28°C only during stage 2 (Hearse et al., 1978), so that it is only the priming during stage 1 that is dependent on molecular mobility. In contrast, a reduction of pH_o only during stage 2 provided complete protection in all three protocols and it is therefore concluded that pH_o of 6.5 does not act via a membrane phase change.

A reduction in intracellular pH has been demonstrated during cardiac ischemia (Meng and Pierce, 1990; Moffat and Karmazyn, 1993) and during the Ca^{2+} paradox (Pierce et al., 1990), suggesting that activation of the damage system in both paradoxes is associated with the intracellular production of H^+, the efflux of which via the antiporter would be blocked at lowered extracellular pH because the cardiac Na^+/H^+ exchanger in rat is inhibited by extracellular acidosis (Wallert and Frohlich, 1989). Such a conclusion is supported by the protective effect of 1 mM amiloride (the Na^+/H^+ antiporter inhibitor) in the Ca^{2+} paradox and caffeine protocols (Fig. 2 line D and Fig. 6 line D). This concentration of amiloride was chosen because earlier studies had shown that lower concentrations were without effect (Daniels and Duncan, 1995).

Although the Na^+/H^+ antiporters from different tissues differ markedly in their sensitivity to amiloride, the concentration of this inhibitor required is high leading to the suggestion that it may be having a non-specific action. Amiloride is known to inhibit a number of ion transporters, ion channels and enzymes (Kleyman and Cragoe, 1988) of which the Na^+/Ca^{2+} exchanger may be the most relevant because, it is suggested (Chapman and Tunstall, 1987), Na^+/Ca^{2+} exchange is responsible for the rise in $[Ca^{2+}]_i$ in stage 2 of the Ca^{2+} paradox. However, as shown in this comparative study, amiloride had significant inhibitory effects in the caffeine protocol in which extracellular Ca^{2+} is omitted throughout (see above) and it is concluded that the Na^+/Ca^{2+} exchanger is probably not the main site of action. By the same argument, amiloride is not inhibiting the voltage-gated Ca^{2+} channel, another known site of its action (Kleyman and Cragoe, 1988). The Na^+/K^+-ATPase is inhibited by amiloride at high concentrations ($IC_{50} > 3$ mM; Kleyman and Cragoe, 1988) but since ouabain had little protective effect it is concluded that amiloride is not inhibiting active Na^+/K^+ transport here. Amiloride has other known inhibitory targets: the K^+ channel, acetylcholinesterase, the muscarinic acetylcholine receptor and the α- and β-adrenoceptors (Kleyman and Cragoe, 1988), although it is difficult to suggest how these might be implicated in the pathways that culminate in sarcolemma damage. Perhaps more relevant is the inhibition of protein kinases by amiloride because it has been suggested (Duncan, 1997) that protein phosphorylation may be involved in the sequence of events of myocardial preconditioning. These non-specific effects of amiloride cannot be ignored, but the correspondence between the protective effects of lowered pH_o and amiloride suggest that their common action is probably via an inhibition of the Na^+/H^+ antiporter.

It is interesting that in the present experiments the protective effect of amiloride was markedly reduced at a higher perfusion pressure (Fig. 2, lines A and B) and we conclude that raised perfusion pressure can interact synergistically with priming via Ca_o^{2+} depletion and so augment creatine kinase release and that protection is less effective under these conditions of exacerbated damage.

In contrast, creatine kinase release in the O_2 paradox was almost completely unaffected by amiloride, whereas lowered pH_o provided the same protection as in the two other protocols. Improvement in functional recovery after reperfusion injury in the anaesthetised animal has been reported with the inclusion of amiloride or other Na^+/H^+ exchange inhibitors in the perfusate (Matsuda et al., 1992; Moffat and Karmazyn, 1993; Pike et al., 1993; Slack et al., 1994), although there was no measurement of creatine kinase release in these studies, and it is possible that amiloride is having its main protective effect on reperfusion injury in the intact animal via an inhibition of superoxide production by neutrophils (Besterman and Cuatrecasas, 1984; Grinstein and Furuya, 1986) which accumulate at the site of injury in the damaged heart (Duncan, 1994).

Do these apparent differences in the different inhibitory effects of amiloride in the three protocols reveal details of the sequence of damage events that culminate in creatine kinase release? Stage 1 in both the Ca^{2+} paradox and caffeine protocols is initiated by a temperature-sensitive, brief Ca_o^{2+} depletion which might (i) activate the damage system via membrane perturbation – creatine kinase release in the Ca^{2+} paradox is augmented by increased perfusion pressure which reduces the inhibitory action of amiloride (Fig. 2); (ii) activate via membrane depolariza-

tion (Chapman and Tunstall, 1987); or (iii) produce a modification of the L-type Ca^{2+} channels which now permit Na^+ entry (Chapman and Tunstall, 1987).

Since almost complete protection is provided by lowered pH_o or amiloride in stage 1 in both protocols, it is concluded that the result of this initial activation involves the intracellular production of H^+ which efflux via a Na^+/H^+ antiporter; if this efflux is inhibited no creatine kinase release occurs in stage 2. It is clear that stage 2 in the Ca^{2+} paradox and caffeine protocols is characterised by a marked and persistent rise in $[Ca^{2+}]_i$ (Chapman and Tunstall, 1987). How then, does the intracellular production of H^+ in stage 1 of the Ca^{2+} paradox result in a rise in $[Ca^{2+}]_i$ in stage 2? Chapman and Tunstall (1987) suggest that a modification of L-type Ca^{2+} channels during Ca_o depletion permits Na^+ entry and consequently a rise in $[Na^+]_i$ in stage 1 of the Ca^{2+} paradox; during Ca_o^{2+} repletion in stage 2 extracellular Ca^{2+} enters via Na_i^+/Ca_o^{2+} exchange. Thus, Na^+ would act as the link between the two stages but it is difficult to explain the inhibitory actions of lowered pH_o and amiloride on this hypothesis. Alternatively, with the results presented here, entry of Na^+ in stage 1 might be via the antiporter, accompanying the efflux of intracellularly generated H^+.

However, stage 1 of the caffeine protocol is inhibited by the same treatments as the Ca^{2+} paradox but extracellular Ca^{2+} is not returned in stage 2, so that any rise in $[Na^+]_i$ cannot promote Ca^{2+} influx. The caffeine experiments, therefore, provide a good protocol for elucidating the possible role of Na^+ movements in the sequence of events that culminate in sarcolemma damage. Inhibition of the Na^+-K^+-ATPase with ouabain had little effect on cumulative creatine kinase release, but substitution of choline for NaCl reduced creatine kinase release by approximately 50% in both the Ca^{2+} paradox and caffeine protocols. We conclude that a rise in $[Na^+]_i$ may not be as important as Na^+ entry via the antiporter in the sequence of damage events, i.e. lowered pH_o, amiloride and reduced $[Na^+]_o$ have their major protective effects via an inhibition of Na^+/H^+ antiporter activity which is integral to the genesis of sarcolemma damage.

Even if the activation events of stage 1 proceed normally at pH 7.35, lowering pH_o only during stage 2 still provides complete protection in all three protocols, i.e. no creatine kinase release occurs in the Ca^{2+} paradox even though H^+ efflux and Na^+ entry have been initiated during normal stage 1 activation. We conclude that (i) lowered pH_o is acting in the same way in stages 1 and 2, namely preventing H^+ efflux, (ii) the damage system and the molecular events initiated in stage 1 are fully activated in stage 2 by raised $[Ca^{2+}]_i$ (Chapman and Tunstall, 1987), (iii) creatine kinase release in stage 2 is critically dependent on this synergistic activation of the damage mechanism that is probably located at the sarcolemma, (iv) activity of the Na^+/H^+ antiporter is intimately linked to the damage system, and (v) these findings are consistent with an earlier suggestion that the priming events in stage 1 activate a transmembrane oxido-reductase which is central to sarcolemma damage and which generates H^+ intracellularly (Duncan, 1990; Harding and Duncan, 1996).

The sequence of events in the O_2 paradox is almost the reverse of the Ca^{2+} paradox: the damage complex is initially activated in stage 1 by the rise in $[Ca^{2+}]_i$ produced by prolonged anoxia. This activation is completely inhibited by lowered pH_o, suggesting that, again, H^+ efflux is critically implicated in these initial events and that the intracellular production of H^+ can be triggered in stage 1 of the O_2 paradox by a prolonged rise in $[Ca^{2+}]_i$. Full activation in stage 2 is produced by reoxygenation suggesting that activation of an oxidase complex may have a central role in the release of creatine kinase. Lowered pH_o also completely protects in stage 2 of the O_2 paradox, suggesting that creatine kinase release is dependent on H^+ efflux as well as raised $[Ca^{2+}]_i$.

We conclude that the damage mechanism that is central to the O_2 paradox is the same as that operating during the Ca^{2+} paradox and caffeine protocols and hence it can be initially activated in stage 1 either externally by Ca_o^{2+} depletion or internally by a marked and persistent rise in $[Ca^{2+}]_i$, suggesting that it is a transmembrane system. In the O_2 paradox, as in the other protocols, H^+ are generated intracellularly during initial activation and this activation is inhibited if H^+ efflux is blocked by lowered pH_o, although amiloride was ineffective under the experimental conditions of this study. This difference from the other two protocols may reflect the relative importance of H^+ and Na^+ movements in the link between stages 1 and 2 of the O_2 paradox.

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