Calcium-dependent opening of a non-specific pore in the mitochondrial inner membrane is inhibited at pH values below 7

Implications for the protective effect of low pH against chemical and hypoxic cell damage

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1. The rate of opening of the Ca^{2+} -induced non-specific, cyclosporin A-inhibited, pore of the mitochondrial inner membrane of rat heart and liver mitochondria at pH 6.0 was less than 10 % of that at pH 7.4. 2. The effect could not be explained by inhibition of Ca^{2+} uptake into the mitochondria, or of the matrix peptidyl-prolyl *cis-trans* isomerase (PPIase), or of the Ca^{2+} -induced conformational change of the adenine nucleotide translocase. 3. It is suggested that the proposed interaction of matrix PPIase with the 'c' conformation of the adenine nucleotide carrier in the presence of Ca^{2+} [Griffiths & Halestrap (1991) *Biochem. J.* 274, 611–614] is inhibited by low pH. 4. The relevance of this to the protective effect of low pH on hypoxic and chemical-induced cell damage is discussed.

INTRODUCTION

Tissues or isolated cells that are made hypoxic become depleted of ATP, but can recover upon re-oxygenation provided that the hypoxic period is short. After longer periods of hypoxia, reperfused tissues are susceptible to substantial damage, which may be irreparable if the hypoxic period is long enough. At this point the plasma membrane of the cell is ruptured and the cell dies (Farber *et al.*, 1981; Naylor, 1981; Steenbergen *et al.*, 1990; Herman *et al.*, 1990). The causes of this irreversible damage are probably several, but two major factors are the observed increase in cellular $[\text{Ca}^{2+}]$ and the production of free radicals (Miccadei *et al.*, 1988; Orrenius *et al.*, 1989; Chien & Engler, 1990; Crompton, 1990; Herman *et al.*, 1990). The latter can cause chemical modification of a variety of cellular components, including the oxidation of phospholipids and protein thiols within membranes and so disrupt mitochondrial membrane function (Olafsdottir *et al.*, 1988; Masaki *et al.*, 1989a; Fagian *et al.*, 1990). Thus manipulation of either cellular Ca^{2+} homeostasis or free-radical metabolism artificially, for example with ionophores or xenobiotics which deplete the cell of GSH, causes similar effects to those of hypoxia (Olafsdottir *et al.*, 1988; Orrenius *et al.*, 1989; Herman *et al.*, 1990). Similar damage occurs to tissues subjected to prolonged treatment with a variety of chemical agents that deplete ATP, such as those which inhibit glycolysis or oxidative phosphorylation. This is sometimes known as chemical hypoxia (Kristensen, 1989; Gores *et al.*, 1989).

Exposure of isolated mitochondria to supra-physiological concentrations of Ca^{2+} or agents that deplete the matrix of NADPH causes the mitochondrial inner membrane to become permeable to small-molecular-mass solutes. This effect is enhanced under conditions of adenine nucleotide depletion (see Crompton, 1990; Gunter & Pfeiffer, 1990). The mechanisms involved in this permeability increase have been studied in several laboratories, and are believed to involve the opening of a non-specific pore within the inner membrane (see Hunter & Haworth, 1979; Haworth & Hunter, 1979; Crompton & Costi, 1988, 1990; Halestrap & Davidson, 1990). The ability of effectors of the adenine nucleotide translocase and cyclosporin A to influence pore opening have implicated both the adenine nucleo-

tide translocase and a cyclosporin-binding protein (cyclophilin) in the process (Le Quoc & Le Quoc, 1988; Crompton *et al.*, 1988; Broekemeier *et al.*, 1989; Halestrap & Davidson, 1990; McGuinness *et al.*, 1990). Data from my laboratory has led to the proposal that the adenine nucleotide translocase itself forms the pore as a result of a substantial conformational change. This may be induced by an interaction of the carrier with peptidyl-prolyl *cis-trans* isomerase (PPIase), whose activity we have demonstrated to be present within the matrix and to be inhibited only by those cyclosporin analogues which inhibit pore formation (Halestrap & Davidson, 1990; Kay *et al.*, 1990; Griffiths & Halestrap, 1991). A different model involving PPIase has been suggested by others (McGuinness *et al.*, 1990) in which a relatively low-abundance membrane protein, distinct from the adenine nucleotide translocase, interacts with a discrete fraction of the mitochondrial PPIase in the presence of Ca^{2+} to form a pore. Whatever the mechanisms involved, a result of pore opening is that mitochondria become uncoupled and so incapable of ATP synthesis and Ca^{2+} accumulation. Should such conditions occur within the cell, protection of the cell from Ca^{2+} overload would no longer be possible and, as phospholipases and proteinases are activated, this would inevitably lead to cell death (Orrenius *et al.*, 1989; Herman *et al.*, 1990).

It has been established that lowering the pH of the extracellular medium protects cells from both hypoxic and chemically induced damage and enhances recovery during re-oxygenation (see Herman *et al.*, 1990). This has now been demonstrated in heart (Bielecki, 1969; Poole-Wilson, 1989), liver (Bonventre & Cheung, 1985; Gores *et al.*, 1988, 1989; Masaki *et al.*, 1989a,b; Nieminen *et al.*, 1990; Currin *et al.*, 1991), kidney (Penttilä & Trump, 1974) and tumour cells (Penttilä & Trump, 1974). Low pH tends to accompany hypoxia because of the accumulation of lactic acid within the cell. However, upon reperfusion, this lactic acid will be transported out of the cell on the monocarboxylic acid transporter, with a resultant alkalinization of the cytosol (Halestrap *et al.*, 1990). The protective effect of low pH has usually been ascribed to a general decrease in the activity of ion channels, metabolism and degradative enzymes such as phospholipase A_2 within the cell (see Herman *et al.*, 1990; Currin *et al.*, 1991). However, in the present paper I demonstrate that the opening of

the Ca^{2+} -sensitive pore within the inner membrane of both rat heart and liver mitochondria is inhibited greatly as the pH falls below 7. It is suggested that this may also play an important role in the protective effect of low pH against chemical and hypoxic tissue damage.

EXPERIMENTAL

Materials

The preparation of rat liver and heart mitochondria and their further purification by Percoll-density-gradient centrifugation were performed as described previously (Halestrap, 1987; Whipps *et al.*, 1987). The sources of all chemicals and biochemicals are as given elsewhere (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991). All radiochemicals were from Amersham International, Amersham, Bucks, U.K.

Methods

The pH-sensitivity of Ca^{2+} -induced pore opening was measured by studying the swelling of de-energized mitochondria in KSCN buffer at 30 °C as described previously (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991). The buffer contained 150 mM-KSCN, 10 mM-Mes, 10 mM-Mops, 5 mM-Tris base, 1 μg of rotenone/ml and 1 μg of antimycin/ml adjusted to the pH required with either HCl or KOH. Liver or heart mitochondria were added at approx. 2 mg of protein/ml, and swelling was initiated by addition of 250 μM - CaCl_2 to the reference cuvette of a split-beam spectrophotometer. Swelling was monitored by continuous measurement of A_{520} .

The Ca^{2+} -induced conformational change of energized heart mitochondria was also assayed as a change in A_{520} as described previously (Halestrap, 1987; Halestrap & Davidson, 1990). The buffer contained 125 mM-KCl, 10 mM-Mops, 10 mM-Mes, 5 mM-Tris base, 5 mM-sodium glutamate, 1 mM-L-malate, and was adjusted to the required pH with HCl or KOH. Mitochondria were added at approx. 2 mg of protein/ml and EGTA to a concentration of ($x - 2$ mM), where x is the concentration (mM) of CaEGTA giving the required free $[\text{Ca}^{2+}]$ at the pH used. Free $[\text{Ca}^{2+}]$ was calculated by a ligand-binding program as described previously (Davidson & Halestrap, 1987). The conformational change was initiated by adding to the sample cuvette the required volume of 200 mM- CaEGTA to give x mM final concentration.

Assay of PPIase activity of a liver mitochondrial matrix extract was performed as described previously (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991), but in a buffer containing 10 mM-Mes, 10 mM-Mops and 5 mM-Tris base adjusted to the required pH with KOH or HCl.

Accumulation of $^{45}\text{Ca}^{2+}$ by isolated liver mitochondria was studied in the KSCN buffer described above but supplemented with 2 μM cyclosporin A. Liver mitochondria (approx. 2 mg of protein/ml) were mixed with 3.5 ml of buffer, and, after incubation at 25 °C for 60 s, 250 μM - $^{45}\text{CaCl}_2$ (0.1 $\mu\text{Ci}/\text{ml}$ final) was added, and 90 s later five 0.65 ml samples were centrifuged for 60 s in an Eppendorf 5400 centrifuge. After removal of the supernatant (original supernatant) the mitochondrial pellet was re-suspended in 0.5 ml of water, and in four cases the protein was precipitated with 50 μl of 20% (w/v) HClO_4 . The ^{45}Ca contents of the resulting supernatant and a sample of the original supernatant were assayed by scintillation counting. The remaining sample was used for a biuret protein assay. In parallel experiments the mitochondrial volume was measured by using $^3\text{H}_2\text{O}$ and ^{14}C sucrose as described previously (Halestrap, 1989), and the ^{14}C sucrose space was used to correct total ^{45}Ca in the mitochondrial pellet for that present in the extramitochondrial space.

RESULTS AND DISCUSSION

Effects of pH on Ca^{2+} -induced swelling of de-energized liver and heart mitochondria

The data of Fig. 1 show that the swelling of de-energized liver mitochondria in KSCN medium induced by 250 μM - CaCl_2 is greatly inhibited as the pH falls from 7.4 to 6.0. We have shown previously (Halestrap & Davidson, 1990) that Ca^{2+} -activated swelling measured by this technique accurately reflects the opening of a non-specific pore in the mitochondrial inner membrane. Thus the present data imply that this process is very sensitive to pH in the physiological range. The maximum rates of swelling were measured by differentiation of the traces and expressed as a percentage of the rate observed at pH 7.4. Mean values $\pm$ S.E.M. for four separate experiments are plotted against pH in Fig. 2, where data are also given for rat heart mitochondria. Half-maximal inhibition was seen at about pH 7.0 in liver mitochondria and pH 6.6 in heart mitochondria. No inhibitory effect of lowering pH on the slow rate of Ca^{2+} -independent swelling in KSCN was detected (results not shown).

Fig. 1. Effects of pH on the Ca^{2+} -induced swelling of de-energized liver mitochondria in KSCN medium

The protocol is given in the Experimental section. Swelling was initiated by addition of 250 μM - CaCl_2 to the sample cuvette at the time shown.

Fig. 2. pH-sensitivity of the rate of Ca^{2+} -induced swelling in liver (●) and heart (■) mitochondria

Maximal rates of swelling were measured by differentiating traces from four experiments similar to that shown in Fig. 1. Rates are given as means $\pm$ S.E.M. (error bars), expressed as a percentage of the rates at pH 7.4.

Fig. 3. Effects of Ruthenium Red and A23187 on the Ca^{2+} -induced swelling of liver mitochondria at pH 6.2 and 7.2

Swelling was initiated by addition of $250 \mu\text{M}$ - CaCl_2 to the sample cuvette at the time shown. The pH of the incubation medium is noted alongside the trace, as is the presence of $5 \mu\text{M}$ -A23187 (A23), $5 \mu\text{M}$ -Ruthenium Red (RuR) or $2 \mu\text{M}$ -cyclosporin A (CyA). Further additions of these effectors were made as indicated. In trace v, the pH was raised or lowered by addition of $25 \mu\text{l}$ of 1 M -KOH or -HCl respectively.

One explanation for the inhibitory effect of low pH on Ca^{2+} -mediated swelling would be that Ca^{2+} entry into the mitochondria is inhibited. The data of Fig. 3 suggest that this is not the case. At pH 7.2 the Ca^{2+} ionophore A23187 ($5 \mu\text{M}$) was able to overcome the total inhibition of Ca^{2+} -induced swelling caused by the presence of the inhibitor of the Ca^{2+} uniporter Ruthenium Red ($5 \mu\text{M}$), but not if $2 \mu\text{M}$ -cyclosporin A was present (traces ii and iii). In the absence of Ruthenium Red the ionophore greatly stimulated Ca^{2+} -induced swelling (trace vi), perhaps by allowing Mg^{2+} , an inhibitor of pore formation (Haworth & Hunter, 1979), to leave the mitochondria. However, even in the presence of A23187, no swelling was observed at pH 6.2 (trace i). Furthermore, the swelling initiated at pH 7.2 could be inhibited by rapidly lowering the pH to 6.4 and then re-instated by returning the pH to 7.1 (trace v). In parallel with these experiments, the uptake of $^{45}\text{Ca}^{2+}$ into the mitochondria was measured as described in the Experimental section. Cyclosporin A ($2 \mu\text{M}$) was present in these experiments to prevent loss of accumulated Ca^{2+} occurring owing to pore opening, and uptake was measured after 90 s, since this gave maximal uptake. Slight loss of $^{45}\text{Ca}^{2+}$ was observed if uptake was allowed to continue for 5 min. In one of three such experiments the uptake of $^{45}\text{Ca}^{2+}$ after 90 s at pH 7.2 and pH 6.2 was 14.45 ± 0.03 and 8.22 ± 0.07 nmol of Ca^{2+} /mg of protein respectively (means $\pm$ S.E.M. of four observations). In the presence of $5 \mu\text{M}$ Ruthenium Red the equivalent values were 2.40 ± 0.09 and 1.98 ± 0.05 , and in the presence of $5 \mu\text{M}$ -A23187 19.08 ± 0.16 and 9.12 ± 0.06 . These data imply that lowering the pH does decrease the total uptake of Ca^{2+} by the mitochondria. However, this is unlikely to reflect a decrease in matrix free $[\text{Ca}^{2+}]$, since Ca^{2+} binding to fixed negative charges inside the mitochondria will decrease as these become protonated at the lower pH. In separate experiments (results not shown) it was demonstrated that, even if $[\text{Ca}^{2+}]$ was increased to $500 \mu\text{M}$, little or no swelling was observed at pH 6.2 in either the presence or absence of A23187. However, the uptake of $^{45}\text{Ca}^{2+}$ reached similar values to those measured at pH 7.2 with $250 \mu\text{M}$ - Ca^{2+} , where substantial swelling was observed. Thus the inhibitory effect of low pH on Ca^{2+} -induced swelling must operate on the mechanism of pore opening rather than on Ca^{2+} transport into the mitochondria.

Fig. 4. Effects of pH on the Ca^{2+} -induced conformational change of energized heart mitochondria

The protocol is given in the Experimental section. The conformational change was initiated at the time shown by addition to the sample cuvette of the volume of CaEGTA required to give the free $[\text{Ca}^{2+}]$ indicated.

Haworth & Hunter (1979) used shrinkage of pre-swollen bovine heart mitochondria induced by polyethylene glycol in the presence of different concentrations of Ca^{2+} to study the effect of pH on the opening of the non-specific pore. They also found the pore opening to be inhibited between pH 7.2 and 6.7, and suggested that the effect was one of competition between H^+ and Ca^{2+} at the site which triggers pore opening. In the model that we have proposed for pore opening (Davidson & Halestrap, 1990; Griffiths & Halestrap, 1991), this could be reflected in an effect of pH on the activity of PPIase or on the conformation of the adenine nucleotide translocase. I have assayed the activity of PPIase towards its artificial substrate *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide at pH values between 6.2 and 7.8 and found no major differences (results not shown).

Effects of pH on the Ca^{2+} -induced conformational change of heart mitochondria

The data of Fig. 4 investigate the effect of pH on the light-scattering change of energized heart mitochondria induced by the addition of sub-micromolar Ca^{2+} . Previous data from this laboratory have shown this light-scattering change to reflect a conformational change of mitochondria, rather than an increase in their matrix volume (Halestrap, 1987). The effect is reversed by ADP and bongkreikic acid, the action of ADP being overcome by carboxyatractylide, which implies that a conformational change of the adenine nucleotide translocase is involved (Halestrap & Davidson, 1990). It is clear from Fig. 4 that the sensitivity of this conformational change to $0.3 \mu\text{M}$ - Ca^{2+} decreases as the pH decreases from 7.2 to 6.2. However, a maximal response can still be observed if the extramitochondrial $[\text{Ca}^{2+}]$ is raised to $3 \mu\text{M}$. This is in contrast with the effects of low pH on Ca^{2+} -induced swelling, which is very much decreased at pH 6.2 (Figs. 1 and 2). The conformational changes induced by ADP, carboxyatractylide and bongkreikic acid were similar at both pH 6.2 and 7.2, although bongkreikic acid acted more rapidly at the lower pH (results not shown). This probably reflects an increase in the concentration of the free acid form of the inhibitor, which allows it to diffuse across the membrane and reach its site of action more rapidly. The data of Fig. 4 could be explained by an effect of pH on the equilibration of $[\text{Ca}^{2+}]$ across the mitochondrial membrane rather than on the proposed binding of Ca^{2+} to the adenine nucleotide translocase, just as was argued for the effects of pH on Ca^{2+} -induced swelling. It is well documented

Fig. 5. Effects of pH on the PCMBs-induced swelling of de-energized liver mitochondria in KSCN medium

The protocol is given in the Experimental section. Swelling was initiated by addition of 60 μ M-PCMBs to the sample cuvette at the time shown.

that the relationship between intramitochondrial $[Ca^{2+}]$ and extramitochondrial $[Ca^{2+}]$ is dependent on the relative activity of the influx and efflux pathways for Ca^{2+} transport across the inner membrane (see McCormack *et al.*, 1990). In the presence of 5 mM- Na^+ and no Mg^{2+} , as in the present experiments, the intramitochondrial $[Ca^{2+}]$ rises extremely sharply as the extramitochondrial $[Ca^{2+}]$ rises above about 0.3 μ M (Moreno-Sanchez & Hansford, 1988; McCormack *et al.*, 1989; Wan *et al.*, 1989). Thus effects of pH on the Ca^{2+} -transport processes could have profound effects on the sensitivity of the conformational change to extramitochondrial $[Ca^{2+}]$. Indeed, the available data on heart mitochondria suggest that, although both transport pathways are inhibited by a decrease in pH from 7.8 to 6.6, the net accumulation of Ca^{2+} does decrease as the pH falls (Fry & McGuigan, 1990). Nevertheless it must be recognized that the Ca^{2+} -sensitivity of the conformational change has been studied in energized mitochondria, whereas Ca^{2+} -mediated pore opening was studied in de-energized mitochondria. Since the adenine nucleotide translocase catalyses an electrogenic transport process (Klingenberg, 1976), this difference in energization may influence the conformation of the carrier and thus the binding of Ca^{2+} to it. Indeed, under de-energized conditions I was unable to detect a conformational change induced by Ca^{2+} concentrations up to 3 μ M at either pH 7.2 or 6.2. Thus the present data cannot rule out an inhibitory effect of low pH on the Ca^{2+} -induced conformation of the adenine nucleotide carrier that interacts with PPIase. However, an alternative explanation would be that the interaction between the carrier- Ca^{2+} complex and the PPIase is decreased as the pH is lowered.

Effect of pH on swelling mediated by thiol reagents

It has been demonstrated that treatment of the purified and reconstituted aspartate/glutamate and ATP/ADP carriers of the mitochondrial inner membrane with low concentrations of organomercurials such as *p*-chloromercuribenzenesulphonate (PCMBs) does induce non-specific pore-like behaviour (Dierks *et al.*, 1990a,b). In Fig. 5 I demonstrate that 60 μ M-PCMBs causes an increase in the permeability of the inner membrane of de-energized liver mitochondria incubated in KSCN medium, which probably reflects this phenomenon. Swelling accelerates after a brief lag, the length of which is substantially decreased as the pH is decreased from 7.2 to 6.0. Unlike the Ca^{2+} -induced swelling, the maximal rate of swelling increases as the pH decreases (Fig. 5). This is in contrast with the overall reactivity of mitochondrial-membrane thiol groups that has been reported

to decrease markedly in this pH range (Zimmer *et al.*, 1990). The PCMBs-mediated swelling was insensitive to both cyclosporin A and bongkreic acid (results not shown). Thus the two processes are quite distinct in mechanism, although both may involve the transition of a specific carrier into a non-specific pore. There are data to suggest that some of the swelling induced by glutathione oxidation of isolated mitochondria may be insensitive to cyclosporin A (Broekemeier & Pfeiffer, 1989). Since GSH is essential to maintain mitochondrial integrity and protects protein thiol groups from oxidation or mixed-disulphide formation (Beatrice *et al.*, 1984; Olafsdottir *et al.*, 1988; Le Quoc & Le Quoc, 1988; Fagian *et al.*, 1990), it is possible that a mechanism like that for PCMBs-induced swelling may operate under such conditions. However, it is unlikely that this is a major cause of mitochondrial damage *in situ*, since cell death caused by oxidative stress is also decreased by low pH (Herman *et al.*, 1990). Rather, it is probable that oxidative stress leads to Ca^{2+} overload of cells and consequent mitochondrial damage (Orrenius *et al.*, 1989; Herman *et al.*, 1990).

General conclusions

The data in the present paper show clearly that low pH protects mitochondria from the non-specific pore opening induced by Ca^{2+} overload. This provides a plausible explanation for at least some of the protective effects of low pH against cellular damage caused by both toxic chemicals and hypoxia/reperfusion, since irreparable mitochondrial damage is considered to lead inevitably to cell death (see the Introduction). It is known that cyclosporin A can protect the liver from reperfusion injury (Hayashi *et al.*, 1988; Kawano *et al.*, 1989; Goto *et al.*, 1990), and it would be expected that cyclosporin A should protect other cells from chemical or hypoxic damage. This has yet to be tested.

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