Calcium ion activation of the anion-conducting channel in the rat liver mitochondrial inner membrane

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Stimulation of the rat liver mitochondrial inner-membrane anion-conducting channel by aeration is dependent on the concentration of Ca^{2+} ions in the assay medium. Ca^{2+} activates anion conduction in both aerated and non-energised mitochondria but acts over a wider concentration range and produces a greater increase in anion-conductivity in aerated mitochondria. EGTA reverses Ca^{2+} stimulation but takes several seconds to act, indicating slow release of Ca^{2+} from the activation site possibly on the matrix side of the inner mitochondrial membrane. It is suggested that this channel may respond to hormone-induced changes in cytosolic Ca^{2+} concentration.

Calcium ion; Anion channel; Mitochondrion; Mitochondrial inner membrane

1. INTRODUCTION

Anion uniport via a pore or channel in the mitochondrial inner membrane has been demonstrated by the techniques of passive osmotic swelling [1,2] and patch clamping [3–5]. However different methods show differing activator and inhibitor characteristics and while there is now little doubt about the existence of anion conducting channels in the IMM both their physiological regulation and function remain unknown.

We have previously reported that the activity of the pH-dependent anion-conducting channel in the rat liver IMM is increased when the mitochondrial stock suspension is aerated by gentle stirring in air at 0°C [6]. The effect of aeration is dependent on energisation of the mitochondria by respiratory chain proton pumping since it is abolished by respiratory chain inhibitors or uncouplers and anion conductivity is correlated with membrane potential [7].

This paper reports investigation of the relation between energisation of the mitochondria by aeration and Ca^{2+} activation of the anion channel assayed in NH_4Cl medium at pH 8.0 and extends previous observations on the effects of divalent cations and chelating agents [1,2,8–10].

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Abbreviations: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; IMM, inner mitochondrial membrane; NEM, *N*-ethylmaleimide

2. EXPERIMENTAL

Hepes, Rotenone and Antimycin A were obtained from Sigma, FCCP from Aldrich and ammonium chloride, analytical grade, from Merck.

Liver mitochondria were prepared from adult Wistar rats, starved overnight, as described previously [11] except that 0.5 mM EGTA was included in the homogenisation and first wash media. The mitochondria were suspended in 0.25 M sucrose containing 5 mM HEPES-KOH pH 7.5 at 40 to 60 mg protein/ml.

Anion uniport was assayed by passive osmotic swelling of mitochondria in 100 mM NH_4Cl adjusted to pH 8.0 with ammonia solution. Rotenone, 0.6 μM and Antimycin A, 0.5 μM, were added to block electron transport and 7.5 μM FCCP was added to allow rapid entry of H^+ ions to balance charge and pH changes produced by anion-uniport and NH_3 entry. Mitochondrial swelling was recorded by changes in % light transmission measured in a cylindrical glass cuvette thermostatted at 30°C [11–14]. A loose-fitting plunger touching the surface of the medium prevented vortex formation at high stirring speeds. The FCCP was added to the medium prior to the mitochondria and the assay initiated by addition of mitochondria. In control experiments mitochondria were added to NH_4Cl medium without FCCP; in all cases, i.e. aerobic or anaerobic storage and ± Ca^{2+}, the control rates (<0.5 %T min^{-1}) were very small compared to the experimental rates. The assay is therefore a reliable measure of chloride uniport.

Calcium concentrations were determined using a Varian SpectrAA-30 atomic absorption spectrophotometer.

3. RESULTS

Typical light scattering changes recorded after adding mitochondria to NH_4Cl medium containing FCCP are shown in Fig. 1. Without added Ca^{2+}, the rate of swelling after aerated storage (a) was lower than after storage under nitrogen (b). When the medium contained an additional 5 μM Ca^{2+} ions the rate was higher after aerobic storage (c) than after storage under

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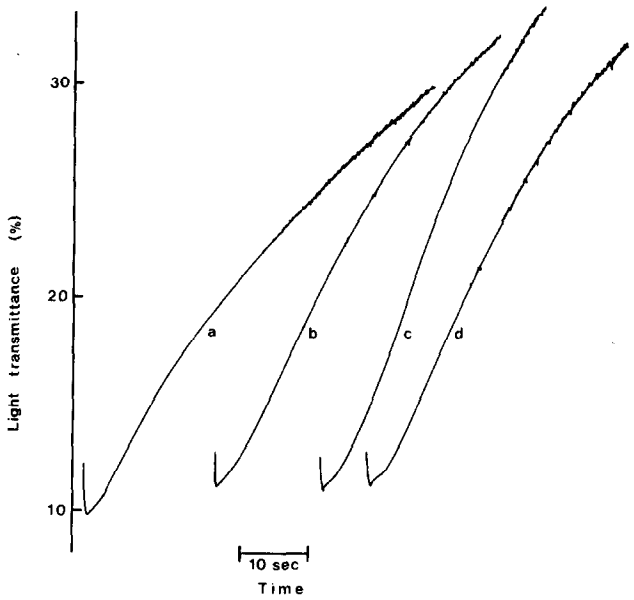

Fig. 1. Effect of added Ca^{2+} on Cl^- entry after aerobic and anaerobic storage of mitochondria. Mitochondrial suspension, 1.5 mg protein, was injected into NH_4Cl medium containing 7.5 μM FCCP as described under section 2. (a,c) Mitochondria aerated during storage; (b,d) mitochondria kept under nitrogen; (c,d) 10 μM $CaCl_2$ added to the assay medium.

nitrogen (d). The effect of varying the concentration of added Ca^{2+} ions is shown in Fig. 2. Added Ca^{2+} had little effect on the rate with mitochondria kept under nitrogen. When the mitochondrial stock suspension had been kept aerobic the rate increased steeply with increasing Ca^{2+} concentration, approximately 2 μM added Ca^{2+} produced half-maximal stimulation at which

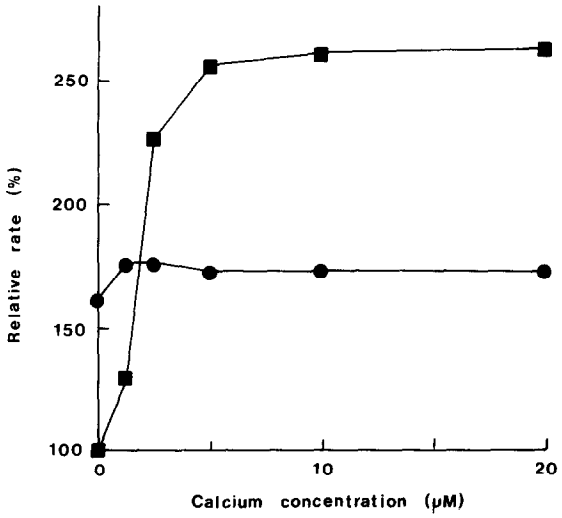

Fig. 2. Effect of concentration of added Ca^{2+} on the rate of mitochondrial swelling in NH_4Cl medium after aerobic and anaerobic storage. Assay as described in Fig. 1, using 1.6 mg mitochondrial protein per assay. Rates are relative to that after aerobic storage in the absence of added Ca^{2+}. Mitochondrial stock suspension stirred in air (■) or nitrogen (●).

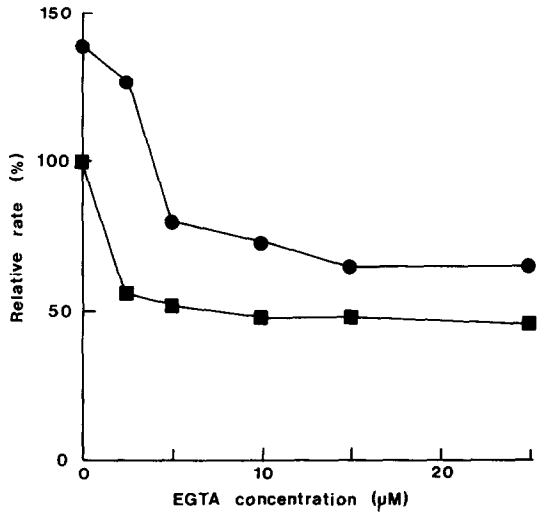

Fig. 3. Effect of EGTA on the rate of mitochondrial swelling in NH_4Cl medium after aerobic and anaerobic storage. Assay as described in Fig. 1, using 1.6 mg mitochondrial protein per assay. Rates are expressed relative to that after aerobic storage in the absence of added EGTA. Mitochondrial stock suspension stirred in air (■) or under nitrogen (●).

point the rate was similar to that of the anaerobically stored mitochondria and near maximal rate was produced by 5 μM added Ca^{2+}.

Chloride entry was inhibited in both aerobically and anaerobically stored mitochondria when EGTA was added to chelate Ca^{2+} present in the NH_4Cl medium, Fig. 3. Anion-conductivity decreased sharply between 2.5 and 5 μM EGTA in accord with measurements of Ca^{2+} concentration, 2–4 μM, by atomic absorption spectrophotometry. Maximal inhibition (54%) was

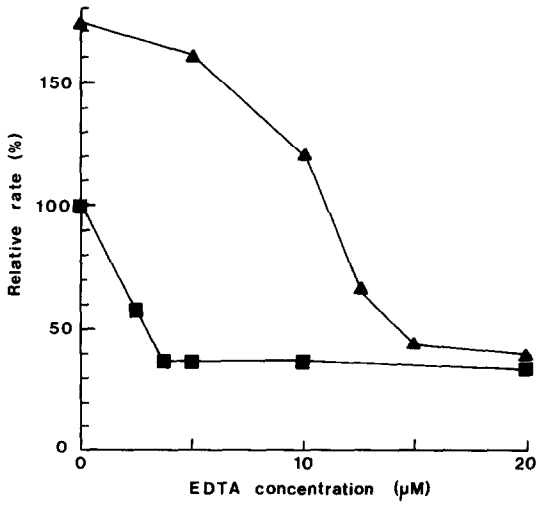

Fig. 4. Effect of EDTA in the presence and absence of added Ca^{2+} on the rate of mitochondrial swelling in NH_4Cl medium after aerobic storage. Assay as described in Fig. 1, using 1.75 mg mitochondrial protein per assay. (■) No added Ca^{2+}; (▲) 10 μM $CaCl_2$ added to assay medium.

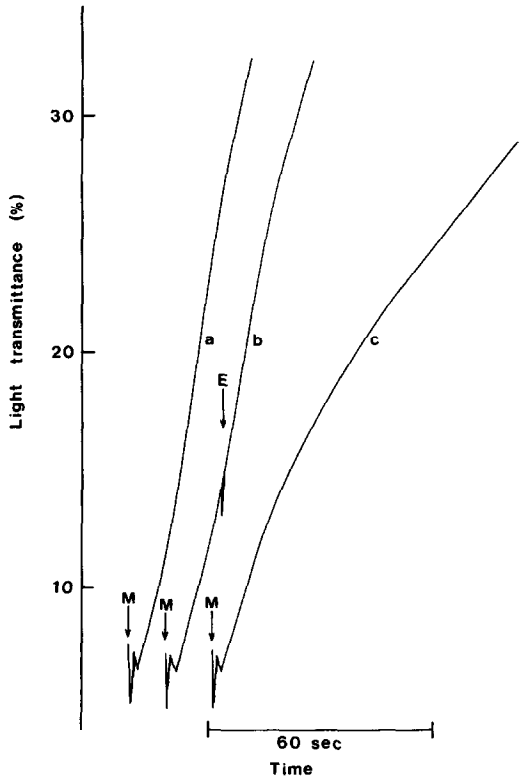

Fig. 5. Effect of addition of EGTA subsequent to addition of Ca^{2+} ions. Recordings obtained as in Fig. 1; in each experiment 2.5 μM $CaCl_2$ was added to the NH_4Cl assay medium prior to the injection, at arrows M, of 1.5 mg mitochondrial protein. (a) No further additions; (b) EGTA, 20 μM final concentration, added to assay 15 s (arrow E) after mitochondria; (c) 20 μM EGTA added to assay medium before injection of mitochondria.

greater in the case of aerobically stored mitochondria than with those stored under nitrogen (35%). Confirmation that the effect of EGTA is due to chelation of Ca^{2+} was provided by the use of the alternative chelator EDTA and by the effects of EDTA or EGTA on the rate of swelling when Ca^{2+} was added to the medium. As shown for EDTA in Fig. 4, inhibition of swelling occurred when the chelator concentration was sufficient to complex both added and endogenous Ca^{2+}.

Ca^{2+} activation was reversed by adding EGTA after Ca^{2+}, Fig. 5, but there was a delay of several seconds before reversal. This indicates slow release of Ca^{2+} from the activating site and eliminates the possibility of Ca^{2+}-cycling across the IMM as a mechanism of Ca^{2+}-activation since this would be inhibited immediately by chelation of external Ca^{2+}.

Assuming that EGTA acts solely as a Ca^{2+} chelator and combining the information in Figs. 2 and 3 and similar experiments we calculate that the rate of chloride uniport is increased 2.2 ± 0.5 (6) times by external Ca^{2+} with non-energised mitochondria with half-maximal effect at 1 μM Ca^{2+} while with energised mitochondria the stimulation is 5.0 ± 0.7 (6) times with half-maximal effect at 4 μM Ca^{2+}.

4. DISCUSSION

Thus, stimulation by energisation is dependent on the Ca^{2+} concentration and below a critical Ca^{2+} concentration aeration of the stock mitochondrial suspension produces lower rather than higher rates of anion conduction. We conclude that in previously reported experiments [6,7], conducted in a hard water area, the level of Ca^{2+} contamination, probably about 10 μM [15], was above this critical concentration.

The present observations together with those of Halle-Smith and Selwyn [7] can be explained by the hypothesis that the Ca^{2+} activation site is on the matrix site of the IMM. Thus aeration, which increases the membrane potential, would favour retention of Ca^{2+} in the mitochondrial matrix. Although under the assay conditions, i.e. in the presence of respiratory chain inhibitors and uncoupler, Ca^{2+} leaks out of the mitochondria, the matrix Ca^{2+} concentration at equilibrium will depend on the external Ca^{2+} concentration. The slow release of Ca^{2+} into the external medium from the activation site, shown by the delay in reversal of Ca^{2+}-activation by EGTA is also in accord with this hypothesis.

Beavis and coworkers [2,9,10] have reported inhibition of the IMM anion-conducting channel at higher Ca^{2+} concentrations, 50% inhibition of 17 μM Ca^{2+}, but used mitochondria which had been depleted of both endogenous Mg^{2+} and Ca^{2+} by treatment with A23187 and EDTA. Since Mg^{2+} was inhibitory it may be that Ca^{2+} binds at the inhibitory Mg^{2+} site in the absence of Mg^{2+}. We have found that, under our assay conditions, Mg^{2+} in the range 0.2–1.0 mM inhibits anion conductivity but increases the % stimulation by Ca^{2+}.

The action of Ca^{2+} ions on the pH-dependent anion-conducting channel can be distinguished from other effects of Ca^{2+} on mitochondrial permeability. The non-specific pore [16,17] and phospholipase A_2-dependent permeability changes [18,19] require higher concentrations of Ca^{2+}, agents such as phosphate, NEM or peroxide and take 2 or more minutes to develop. The K^+ uniport produced by low Ca^{2+} concentrations [20] depends on a similarly slow increase in matrix inorganic pyrophosphate concentration.

Denton and McCormack [21] have proposed that changes in cytosolic free Ca^{2+} concentration, in the range 10^{-7} M to 2 μM Ca^{2+}, produced by hormones such as vasopressin, adrenalin and glucagon are relayed to the mitochondrial matrix and regulate the activity of certain matrix enzymes. The results described here suggest that Ca^{2+}-activation of the IMM anion-conducting channel may be part of the mitochondrial response to these hormones.

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