CALCIUM TRANSPORT IN MITOCHONDRIA

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1. Introduction

Since Vasington and Murphy [1, 2] and DeLuca and Engstrom [3] found that respiring mitochondria accumulated massive amounts of Ca^{2+} ions a great deal of work has been done on the energy-linked accumulation of divalent cations by mitochondria, (Lehninger, Carafoli and Rossi [4]). It has been shown that when energy is available, either from electron transport or from ATP hydrolysis, mitochondria can accumulate not only Ca^{2+} but also Sr^{2+}, Mn^{2+} and, to a lesser extent Ba^{2+} ions against a very high concentration gradient. This has been interpreted [5, 6] to mean that the mitochondrial membrane contains a Ca^{2+} pump or $Ca^{2+}/2H^{+}$ exchange pump which is driven by the non-phosphorylated high energy intermediate. An alternative explanation proposed by Mitchell [7] is that the membrane contains a Ca^{2+} carrier or Ca^{2+}/H^{+} exchange diffusion carrier, and that Ca^{2+} ions are accumulated in response to the pH and/or electrical potential difference across the membrane.

It has also been reported that mitochondria are able to bind Ca^{2+} in the absence of an energy source but the relation between this binding and the energy-linked massive accumulation is not clear, nor is it clear whether the binding is on the surface of the membrane or inside the mitochondria [8].

The existence of carriers for monovalent cations and for a variety of anions has been investigated by suspending mitochondria in iso-osmotic solutions of salts [9], for when both anion and cation are permeable the solution is osmotically inactive and the mitochondria swell. Furthermore the nature of the translocation of the two ions must be complementary such that no electrical charge or pH imbalance is produced by passage of the ions into the mitochondria. Mitchell and Moyle [10] have established that chloride crosses the mitochondrial membrane slowly by electrogenic uniport and that thiocyanate does so rapidly. Acetate crosses the membrane either as acetic acid or on an acetate/hydroxide anti-porter, the net effect being the same in either case, i.e. an electrically neutral process which produces a pH difference across the membrane. Several non-penetrant anions have been reported but none appears very satisfactory for investigations with divalent metal ions. Investigation showed that rat liver mitochondria have a very low permeability to isethionate an anion which is present in high concentrations in squid axons and therefore likely to be physiologically inert. Furthermore most salts of this ion are very soluble. The set of anions, isethionate, acetate, chloride and thiocyanate, thus provides a means for investigating penetration properties of cations and in particular for testing the validity of Mitchell's suggestion about the divalent cation carriers and, if they are driven by electrochemical gradients, for investigating the nature of the carrier.

2. Materials and methods

Mitochondria were prepared, light scattering measured and reagents obtained as described previously [11].

In addition potassium isethionate was obtained from Kodak Ltd. and divalent metals salts were obtained as reagent grade except for calcium isethionate

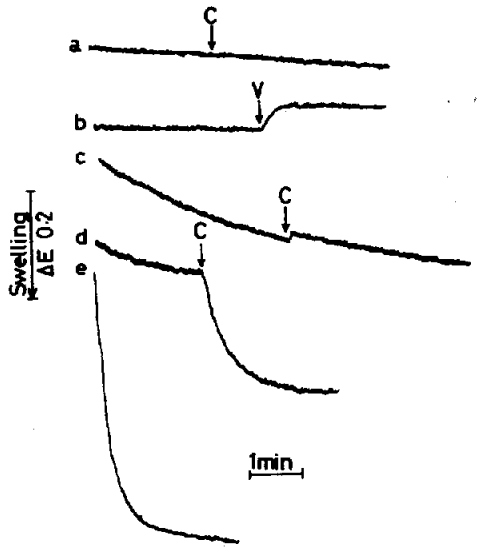

Fig. 1. Swelling of mitochondria in isotonic solutions of calcium salts. The mitochondrial suspension (2.8 mg of protein) was added to an 83 mM solution of the calcium salt containing, in a total volume of 2.5 ml, 2 μg antimycin A, 4 μg rotenone and 5 mM HEPES - tris buffer pH 7.5. (a) and (b) calcium isethionate, (c) $CaCl_2$, (d) calcium acetate, (e) $Ca(SCN)_2$. C indicates the addition of 2.5 nmoles CCCP, V the addition of 50 ng valinomycin. Trace (e) was of identical form when 2.5 nmole CCCP was present initially.

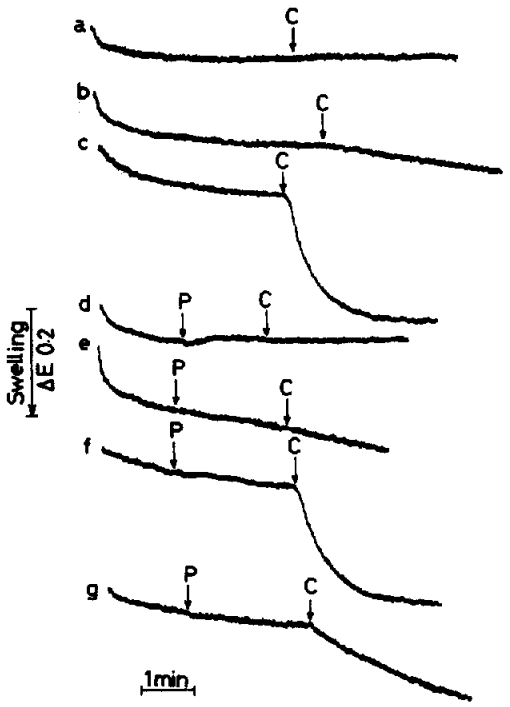

Fig. 2. Swelling of mitochondria in isotonic solutions of divalent metal acetates. (a) Mg^{2+}, (b) Ba^{2+}, (c) Sr^{2+}, (d) Ba^{2+} with 6 ng ions Pr^{3+} added at P, (e) Sr^{2+} with 20 ng ions Pr^{3+} added at P, (f) Ca^{2+} with 10 ng ions Pr^{3+} added at P, (g) Ca^{2+} with 80 ng ions Pr^{3+} added at P. C indicates the addition of 2.5 nmole CCCP. Other conditions were as in fig. 1.

which was prepared from sodium isethionate (Kodak Ltd.) by precipitating the sodium as sodium chloride with HCl gas, removing most of the excess HCl on a rotary evaporator, neutralising with calcium carbonate and recrystallising the calcium isethionate from ethanol:water.

3. Results

When mitochondria are suspended in iso-osmotic solutions of the calcium salts of these anions, fig. 1, rapid swelling is observed only with thiocyanate (e) or with acetate in the presence of an uncoupler which allows protons to cross the membrane (d). This indicates that Ca^{2+} can cross the mitochondrial membrane at a rapid rate as a result of a concentration gradient and in the absence of an energy source. Furthermore the requirement for uncoupler in the acetate system shows that the translocation cannot be a $Ca^{2+}/2H^+$ exchange since this would be balanced for both charge and pH. An exchange of Ca^{2+} for one proton can also be eliminated since in the thiocyanate system this would result in an imbalance of pH with inhibition of the swelling due to lowering availability of protons for exchange with the Ca^{2+} ions. Uncoupler has no effect on swelling in calcium thiocyanate. These results also show that a $Ca^{2+}/2K^+$ exchange does not occur since replacement of the potassium by the divalent Ca^{2+} would lower the osmolarity of the intramitochondrial fluid thus causing shrinkage. This does occur fig. 1(b) in the isethionate medium when valinomycin is added allowing K^+ ions to exchange (on separate carriers) for Ca^{2+} ions. This leaves as the only reasonable possibilities Ca^{2+} entry on an electrogenic uniporter or in exchange for one K^+ ion.

These observations can be criticised on the basis that the observed swelling is not due to osmotic effects

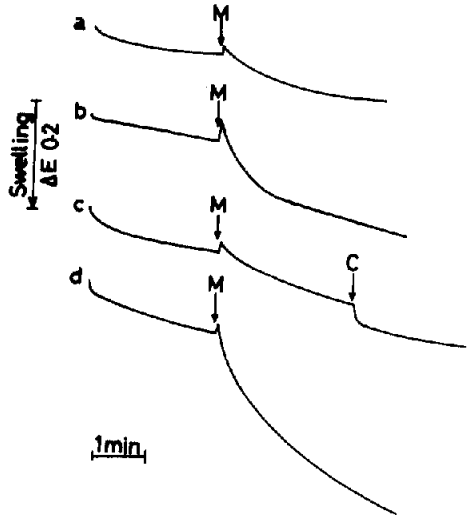

Fig. 3. Mitochondrial swelling induced by the addition of calcium salts to mitochondria suspended in isotonic solutions of potassium salts of the same anions. The mitochondrial suspension (4.2 mg protein) was added to 2.5 ml of 100 mM potassium salt containing 2 μg antimycin A, 4 μg rotenone and 5 mM HEPES-tris buffer pH 7.5. Swelling was initiated by addition, at the arrows M, of 20 μl of a 1.0 M solution of the calcium salt. The anions were: (a) isethionate, (b) chloride, (c) acetate, C indicates addition of 2.5 nmole CCCP, (d) thiocyanate. Conditions were as in fig. 1.

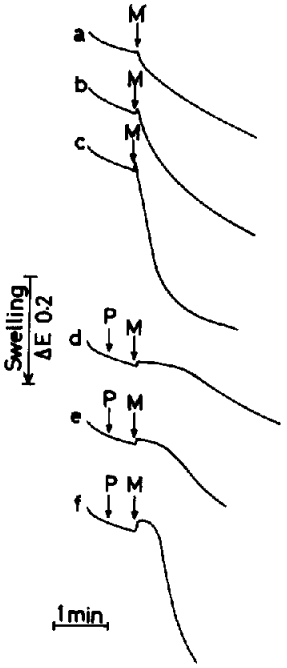

Fig. 4. Inhibition by praseodymium of calcium induced swelling. Various volumes of 1.0 M $Ca(SCN)_2$ were added at arrow M to mitochondria (2 mg protein) suspended in 2.5 ml of 0.1 M KSCN containing 2 μg antimycin A, 4 μg rotenone, 5 mM HEPES-tris buffer pH 7.5. The volumes of $Ca(SCN)_2$ added were: (a) and (d) 10 μl, (b) and (e) 20 μl, (c) and (f) 100 μl. In (d), (e) and (f), P shows the addition of 4 ng ions Pr^{3+}. Other conditions as fig. 1.

of accumulated calcium but is some lytic or structural effect and on the basis that the effects are unrelated to the energy-linked accumulation of calcium.

The following observations have been made to test these possibilities but it is clear that even if the first criticism were justified the method is still testing for the penetration of calcium since an equal external concentration is present in the isethionate medium when no swelling is observed.

Fig. 2 shows that swelling of the mitochondria is also observed with Sr^{2+} or Ba^{2+} ions but not with Mg^{2+} ions. These effects show a similar specificity to the energy-linked accumulation, the order of effectiveness being $Ca^{2+} > Sr^{2+} > Ba^{2+}$ in both cases, with Mg^{2+} ineffective. Furthermore under conditions when Ca^{2+} causes anomalous swelling Sr^{2+} does not or even causes shrinkage of mitochondria [12] so that in this case the swelling is almost certainly due to osmotic effects. Fig. 2 also shows that the uncoupler-stimulated swelling in solutions of the acetate salts of the penetrant metal ions is inhibited by Pr^{3+} (praseodymium) ions, which have been shown by Mela [13] to inhibit the energy-linked accumulation of divalent metal ions.

Another variation of the osmotic swelling technique is to suspend the mitochondria in an iso-osmotic solution of a potassium salt and then add a small volume of a strong solution of the calcium salt of the same anion. Since the calcium concentration is smaller the conditions are more physiological than in the previous type of experiment. As shown in fig. 3 a similar dependence on the anion is observed. Pr^{3+} ions inhibit the swelling produced by addition of Ca^{2+} ions to mitochondria suspended in potassium thiocyanate solution, fig. 4, and the concentration of Pr^{3+} required is less than that required in the type of experiment shown in fig. 2 but is higher than that used by Mela. Scarpa and Azzone [14] have reported that the inhibition of calcium accumulation by Pr^{3+} is competitive. Our results also indicate the competitive nature of this inhibition. First, when swelling is ini-

tiated by addition of Ca^{2+} after prior addition of Pr^{3+}, fig. 4, there is a lag period during which swelling is very slow, this suggests that in the absence of Ca^{2+} most of the carrier is blocked by Pr^{3+} which is slowly displaced by Ca^{2+}. Second, the length of the lag is shorter with higher Ca^{2+} concentrations. Third, the maximal rates of swelling in the presence of Pr^{3+} are 97%, 78% and 66% of the rates in the absence of Pr^{3+} for Ca^{2+} concentrations of 40, 8 and 4 mM, respectively. Mela [13] reported that the inhibition by Pr^{3+} was non-competitive but Ca^{2+} uptake was initiated by addition of Ca^{2+}. Our results indicate that this measurement is during the lag phase and thus reflects the binding of Pr^{3+} in the absence of Ca^{2+}, that is, before Pr^{3+} is displaced by the added Ca^{2+}; in these circumstances the inhibition would be expected to be non-competitive. Fig. 2 shows that the inhibition of swelling in iso-osmotic solutions of Sr^{2+} and particularly Ba^{2+} by Pr^{3+} is more effective than in the Ca^{2+} medium which is in accord with the findings of Vainio, Mela and Chance [15]. This inhibition by Pr^{3+} is a further indication that the divalent cation transport observed in the experiments is mediated by the same carrier as in the energy-linked accumulation.

Since Vainio, Mela and Chance have shown that potassium ions inhibit the transport of Ba^{2+} ions it would be of great interest to discriminate between the possibilities of a Ca^{2+} uniporter or the Ca^{2+}/K^+ exchange. Preliminary observations with a K^+ selective glass electrode suggests that although K^+ leaks out of the mitochondria it does so only as the electrical potential drops and not in a compulsory exchange process.

4. Discussion

Both calcium uniport and exchange of one calcium ion for one potassium ion are electrogenic processes and the calcium ion gradient across the membrane will be affected by the electrical potential difference across the membrane. Respiration and ATP driven calcium accumulation are thus directly accounted for on the chemi-osmotic hypothesis of Mitchell [7] in which energy from respiration or ATP hydrolysis is conserved by translocation of protons outward across the mitochondrial membrane thus rendering the inside alkaline and negatively charged. Our observations appear to be incompatible with the non-phosphorylated high energy intermediate of the chemical hypothesis being a direct participant in a calcium pump. However, Chappell and Crofts [16] have proposed that this intermediate could drive a proton pump – the overall effect being similar to that proposed in the chemi-osmotic hypothesis. While our observations do not discriminate between the two hypotheses of energy conservation it may be significant that one energy-linked function, divalent ion transport, which was previously supposed to be directly mediated by the non-phosphorylated high energy intermediate now appears to require proton transport (or possibly K^+ transport) as a compulsory intermediate stage.

This electrophoretic transport of calcium ions is in accord with the observed features of respiration driven calcium accumulation. It accounts for the requirement, for massive accumulation, of a counter ion such as acetate or phosphate which is translocated in an electrically neutral process but which neutralises the pH difference generated by respiration. The variable stoichiometry of calcium uptake in relation to proton ejection is also accounted for since the net changes observed will depend on the initial and final charge and pH differences across the mitochondrial membrane and will be affected by the movement not only of potassium and substrate ions out of the mitochondrion but also of chloride into the mitochondrion in response to the changed electrochemical gradients across the membrane.

Acknowledgement

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