ON THE MECHANISM BY WHICH INORGANIC PHOSPHATE STIMULATES MITOCHONDRIAL CALCIUM TRANSPORT

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1. Introduction

Although it is well established that inorganic phosphate (P_i) stimulates the initial rate and extent of Ca^{2+} transport by mitochondria (reviewed in [1]), knowledge about the mechanism of such stimulation is lacking. The view that permeant anions such as P_i stimulate Ca^{2+} transport by donating a proton to the medium in the matrix space and thereby generating an anion gradient, negative inside, has been expressed by Lehninger [2] and Moyle and Mitchell [3] have suggested that the 'calcium' porter might actually be a 'calcium-phosphate' porter catalysing $(Ca_2)^{4+}$–HPO_4^{2-} symport.

The sensitivity of mitochondrial phosphate transport to sulphydryl group reagents was recognised about a decade ago [4] but is only quite recently that this experimental finding has been applied to the study of mitochondrial Ca^{2+} transport. Brand et al. [5] and Moyle and Mitchell [3] used *N*-ethylmaleimide (NEM) to prevent P_i (and OH^-) movement during the quantitative estimation of H^+/site ratios. Still more recently, Harris and Zaba [6] reported that blocking both the entry of exogenous phosphate to the mitochondrial matrix and the generation of endogenous P_i via ATPase activity, completely prevented any Ca^{2+} transport in liver and heart mitochondria. They suggested that under these conditions liver or heart mitochondria are able to transport only about 1 nmol Ca^{2+}/mg mitochondrial protein.

The report of Harris and Zaba [6] together with observations made in this laboratory that reflect a fundamental difference in Ca^{2+} transport between liver and flight-muscle mitochondria in regard to their reactivity towards P_i [7], have led us to consider further the role of P_i in mitochondrial Ca^{2+} transport. Some of these experimental findings and their relevance to this problem are described in this paper.

2. Experimental

Mitochondria were prepared from the liver or heart of adult Wistar strain albino rats [8]. Protease treatment was not used in the preparation of the heart mitochondria. The final pellets were resuspended in 250 mM sucrose, 2.5 mM Hepes (pH 7.4) to give a protein concentration (estimated by the biuret reaction) of about 50 mg. ml^{-1}.

Mitochondria from the flight-muscle of 3–5 h old adult blowflies (*Lucilia cuprina*) were prepared as described earlier [9].

Ca^{2+} transport was measured by either the EGTA–Ruthenium Red quench technique [10] or by means of a Ca^{2+} electrode.

2.1. *Quench-technique*

In the quench, the reaction medium was held in a water-jacketed vessel with constant stirring and contained 150 mM KCl, 3 mM Hepes (*N*-2-hydroxyethylpiperazine–N^1-2-ethane-sulphonic acid) (pH 7.4) and 5 mM succinate (Na^+ salt) or 25 mM α-glycerolphosphate (flight-muscle mitochondria). The solution (2.0 ml total vol.) was allowed to temperature equilibrate after which time 2 mg mitochondrial protein was added and the solution rapidly mixed. Calcium (concentrations as in figure legends and containing 0.2 μCi $^{45}CaCl_2$) was added and the reaction was terminated

at the times indicated in figure legends by transferring 100 μl into Eppendorf tubes containing 100 μM EGTA plus 5 μM Ruthenium Red. The quenched solutions were immediately centrifuged (Eppendorf Microfuge, 2 min at 12 000 × *g*), aliquots of the supernatant transferred to vials containing 10 ml. Scintillation fluid and counted to less than 1% error on a Packard Tri-Carb scintillation counter.

2.2. *Ca-ion specific electrode technique*

In this the Ca^{2+} concentration in the medium was continuously measured using an ion-sensitive Ca electrode (Radiometer, Copenhagen) with a combination pH electrode as reference. The signal from the Ca electrode was converted to give an antilogarithmic output on a Rikidenki recorder. The basic incubation medium in total vol. 4 ml at 25°C, contained 150 mM KCl, 3 mM Hepes (pH 7.4) and 1 μM rotenone. Mitochondria (2 mg protein) were added followed by Ca^{2+} (usually 12.5 μM). Transport was initiated about 1 min later by the rapid addition of 5 mM succinate.

All reagents were of analytical grade. Radioactive $CaCl_2$ was obtained from The Radiochemical Centre, Amersham, Bucks. England.

A stock solution (4.5 mM in absolute ethanol) of tributyltin (British Drug Houses, Poole, England) was prepared by diluting a portion of the concentrated reagent (1.19 g/ml).

Each experiment was carried out at least 3 times. Variation between experiments did not amount to more than about 10%.

3. Results

3.1. *Influence of NEM and oligomycin on Ca^{2+} transport by liver, heart and flight-muscle mitochondria*

Data in fig.1A, obtained with liver mitochondria, show that Ca^{2+} transport as measured by the radioisotope–EGTA–quench technique, can be shown to consist of several basic components.

Addition of NEM (to 200 nmol/mg protein) reduces by about 25% the initial rate of Ca^{2+} transport, and increases by about 1 min the time at which the steady-state concentration of Ca^{2+} in the mitochondria is reached. Following the attainment of the steady-state, a gradual loss of Ca^{2+} from the mitochondria occurs so that by about 8 min the NEM-sensitive component comprises some 80% of the Ca^{2+} transported overall (the control value). The NEM-insensitive component in turn consists of oligomycin-sensitive and oligomycin-insensitive components.

The initial rate of transport of the oligomycin-insensitive component is about 50% of that of the control and the steady-state concentration is reached in less

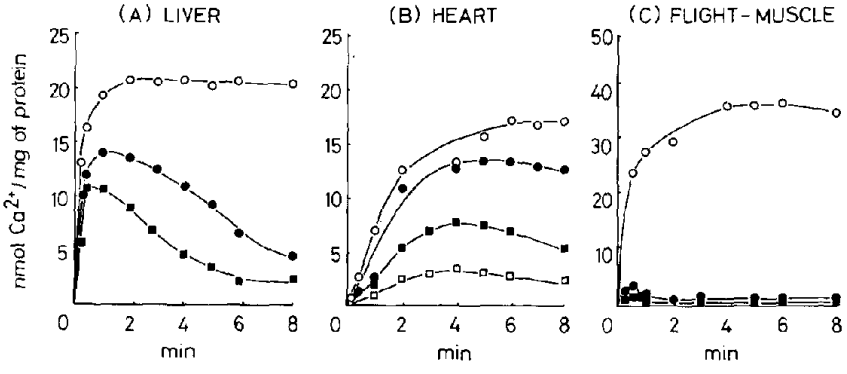

Fig.1. Effect of NEM and oligomycin on Ca^{2+} transport by mitochondria from liver and heart tissue of the rat and flight-muscle of the blowfly *Lucilia cuprina*. Reaction media were as described in the Experimental section. In experiments A and B, liver and heart mitochondria, respectively, were used. $^{45}Ca^{2+}$ was present at 25 μM. The temperature was 15°C. Experiment A: (○) control; (●) plus NEM (200 nmol/mg protein; (■) plus NEM plus oligomycin (5 μg/mg protein). Experiment B: (○) control; (●) plus NEM (200 nmol/mg protein); (■) plus NEM plus oligomycin (5 μg/mg protein); (▫) plus NEM (800 nmol/mg protein) with or without oligomycin (5 μg/mg protein). Experiment C: (○) Plus 2 mM P_i, (●) no added P_i; (■) plus 2 mM P_i plus NEM (100 nmol/mg protein). $^{45}Ca^{2+}$ was present at 50 μM. The temperature was 25°C.

than 30 s after the addition of Ca^{2+} to the mitochondria. This steady-state concentration is approx. 50% of that observed for the control. A loss of Ca^{2+} immediately ensues so that by 6 min after Ca^{2+} addition, the oligomycin-insensitive component is reduced to only about 10% of the total in the mitochondria. Data similar to these were obtained when NEM was present at 800 nmol/mg protein (results not shown).

It seems particularly noteworthy that in the absence of the inhibitors, Ca^{2+} remains within the mitochondria following its accumulation but that when access or internal generation of P_i is prevented, Ca^{2+} is spontaneously released from the mitochondria.

A similar trend is seen also in heart mitochondria (fig.1B) in that once the steady-state concentration has been reached, the accumulated Ca^{2+} is released from the mitochondria. It should be noted that the NEM concentrations used (200 nmol/mg protein) are considerably in excess of those required to inhibit the P_i/OH^- exchange system [11]. As the NEM concentration is increased further to 800 nmol/mg of protein, i.e., approaching that employed by Harris and Zaba [6], the oligomycin- and NEM-sensitive components merge, i.e., the 3-component becomes a 2-component system. These effects are under further study.

Data in fig.1C highlight the inability of flight-muscle mitochondria to transport Ca^{2+} in the absence of exogenous P_i (see also ref. [7,12]) despite the existence in 3–5 h old flight-muscle mitochondria of an endogenous P_i concentration of about 17 nmol/mg protein [7]. Consequently, these mitochondria almost completely lack the components of the mitochondrial Ca transport system that exist in liver and heart mitochondria as described in figs 1A and 1B.

3.2. *Inability of tributyltin or acetate to promote Ca^{2+} influx in mitochondria treated with NEM and oligomycin*

If the role of the penetrant anion is confined to donating a proton to the matrix space and thereby establishing an anion gradient, negative inside, which then acts as the pulling force for mitochondrial Ca^{2+} transport [2], it would be anticipated that any other compound able to carry out this task might also promote Ca^{2+} transport despite the presence of NEM and oligomycin in the reaction medium. Two such compounds are tributyltin (TBT), which catalyses a Cl^-/OH^- exchange across the inner mitochondrial membrane [13] and acetate, which readily diffuses across this membrane into the matrix space.

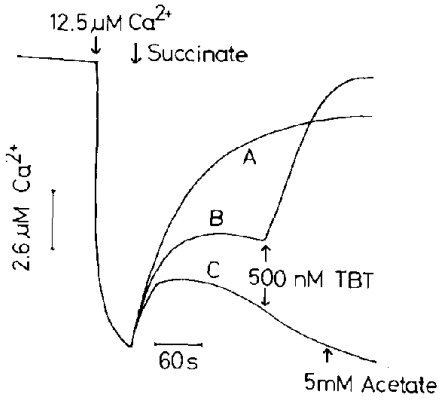

Fig.2. Ca^{2+} electrode traces showing the effect of tributyltin on Ca^{2+} transport by liver mitochondria in the presence and absence of NEM and oligomycin. Incubation conditions were as described in the Experimental section. TBT and acetate were added where indicated at 500 nM and 5mM, respectively. The temperature was 25°C. Curve A: control (no other additions). Curve B: NEM (200 nmol/mg protein) added before Ca^{2+}. Curve C: NEM plus oligomycin (5 μg/mg protein) added before Ca^{2+}.

Data in fig.2 were obtained from experiments in which Ca^{2+} movements into mitochondria were followed by means of a Ca^{2+} ion-specific electrode coupled to a pH electrode. As shown in fig.2A, addition of TBT to NEM-inhibited mitochondria, promotes a rapid and immediate stimulation of Ca^{2+} influx (curve B) in confirmation of experiments previously made in this laboratory [14]. However, TBT fails to promote Ca^{2+} influx when oligomycin also is present in the reaction mixture (curve C). Subsequent addition of acetate or its addition before that of TBT (data not shown) also fails to promote Ca^{2+} transport in the presence of oligomycin. In other experiments we have shown that TBT promotes Ca^{2+} transport in the presence of oligomycin alone indicating that the data seen in curve C are not the result of inhibition of TBT action by oligomycin. Moreover (a) addition of TBT in both experiments of curves B and C, promotes an instantaneous Cl^-/OH^- exchange across the inner membrane that is altered little by the presence of NEM and/or oligomycin and (b) the $\Delta\psi$ and $-Z\Delta pH$ components of the proton electrochemical gradient in the steady state in curves B and C were

practically identical (C. Ramachandran and F. L. Bygrave, unpublished findings).

4. Discussion

Data in this study confirm the finding of Harris and Zaba [6] that the initial rate of Ca^{2+} transport in liver and heart mitochondria is significantly depressed when steps are taken to prevent cycling of P_i via P_i carrier systems or generation of internal P_i via ATPase. However, and due in part to the limited reaction time in their experiments (ca. 60 s), Harris and Zaba [6] were not in a position to detect a number of other features of Ca^{2+} movements in mitochondria of liver and heart tissue revealed in the present study.

The first point to be gathered from this work is that a finite concentration of internal P_i is necessary in order for Ca^{2+} to be retained by these mitochondria for prolonged periods in a KCl-containing medium. Lowering the internal P_i concentration with the use of appropriate inhibitors clearly leads to a release of the accumulated Ca^{2+}. This observation is fully consistent with the proposal of Reed and Bygrave [15] that with little permeant anion, the Ca^{2+} cycles in and out of mitochondria continuously and that the anion reduces the rate of efflux.

A second point is that the amount of Ca^{2+} able to be transported into liver mitochondria, in which all precautions have been taken to minimise internal P_i, is about 7–10 nmol/mg protein as determined by two types of measurement. This amount is significantly greater than the 1 nmol/mg protein reported by Harris and Zaba [6] but still quite less than the 37 nmol/mg protein reported by Brand et al. [5]. Recalculation of data in the report of Moyle and Mitchell [3] also indicates that rat liver mitochondria will transport at least 10 nmol Ca^{2+}/mg protein in the presence of NEM and oligomycin.

A third point and one that relates directly to the hypothesis of Lehninger [2], is that agents other than P_i which also donate a proton to the matrix space (TBT and acetate), have no effect on Ca^{2+} transport in the presence of both NEM and oligomycin. This finding raises the possibility that some other unique feature(s) exists about internal P_i that is fundamental to mitochondrial Ca^{2+} transport. It is pertinent to note in this regard that, although flight-muscle mitochondria of *Lucilia* possess as much if not more endogenous P_i than liver on a mg protein basis, they are still incapable of transporting Ca^{2+} in the absence of exogenous P_i [7].

Alternatively, and because the presence of oligomycin does not alter the proton electrochemical gradient across the inner membrane of NEM-treated liver mitochondria (fig.2, curves B and C), it is possible that the mitochondrial Ca^{2+} carrier is inhibited directly by the unique combination of NEM and oligomycin.

Finally it seems possible that we may have detected in mammalian mitochondria, a Ca^{2+}-translocation cycle with properties quite different to that which functions in the presence of exogenous P_i. The possible existence of this cycle has implications for several fundamental aspects of mitochondrial Ca^{2+} transport including its molecular mechanism and its role in the overall control of cell Ca^{2+}. These problems are under further study in this laboratory.

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