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PHOSPHATE INDUCED SWELLING, INHIBITION AND PARTIAL UNCOUPLING OF OXIDATIVE PHOSPHORYLATION IN HEART MITOCHONDRIA IN THE ABSENCE OF EXTERNAL CALCIUM AND IN THE PRESENCE OF EGTA

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SUMMARY: Inorganic phosphate (Pi) induced swelling of heart mitochondria and resulted in an inhibition and a partial uncoupling of oxidative phosphorylation in an assay medium that contained 150 mM KCl and 1 mM pyruvate at 37°C. The swelling effect of Pi was partially inhibited and the uncoupling and inhibition of oxidative phosphorylation were completely prevented by ruthenium red (RR) but not by EGTA. The RR-insensitive swelling, on the other hand, was inhibited by rotenone, N-ethylmaleimide and 2,4-dinitrophenol but not by oligomycin. It is suggested that the Pi-induced swelling of mitochondria has two components. One component is insensitive to RR and related to the respiration-dependent uptake of potassium phosphate which by itself does not cause damage to oxidative phosphorylation. The other component of the swelling is RR-sensitive, may involve intramitochondrial calcium and results in inhibition and partial uncoupling of oxidative phosphorylation. Neither component, however, is inhibited by EGTA.

INTRODUCTION: The swelling effect of inorganic phosphate (Pi) on mitochondria was first demonstrated by Raaflaub in 1953 (1). Since then, the mechanism and physiological significance of Pi-induced swelling have been studied in several laboratories (2,3,4). It has been demonstrated that respiration-dependent accumulation of potassium activated by Pi is responsible for the osmotic swelling of mitochondria (5,6). Recent studies carried out in the presence of external calcium indicate that Pi induces an energy-dissipating cycling of

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ABBREVIATION: EGTA, Ethyleneglycol-bis-(β-aminoethyl ether) N, N'-tetraacetic acid.

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calcium across the inner membrane which is related to impairment of oxidative phosphorylation (7,8). It has also been suggested that heart mitochondria containing small amounts of endogenous calcium are insensitive to the effects of Pi (9). In the present study the effects of Pi on isolated heart mitochondria were studied at 37°C in a medium that contained a concentration of potassium similar to that thought to be present in the cytosol and in which a loading of mitochondria by external calcium was avoided. The results indicate the existence of two separable components of the Pi-induced swelling: one, ruthenium red (RR)-insensitive which appears not to cause damage to mitochondria and a second which is RR-sensitive and results in inhibition and partial uncoupling of oxidative phosphorylation. It is of particular significance that neither the swelling nor the damage to oxidative phosphorylation were prevented by EGTA.

METHODS: Isolation of Mitochondria. Male albino rabbits weighing 2-3 kg were used. The chest was opened after cervical dislocation. The heart was quickly removed and immediately placed into ice-cold physiological saline solution to wash the heart free from blood. The heart was then transferred into an ice-cold medium which contained 180 mM KCl, 10 mM EGTA and 0.5% bovine serum albumin (KEA) pH 7.4 at 4°C. All subsequent steps were carried out in a cold room at 4°C. The ventricular tissue was cut free from fat, large vessels and atria, the weight was determined, then it was minced with scissors. Approximately 1.5-2.0g of tissue was homogenized in 10 ml of KEA medium with the aid of an electrically driven teflon pestle at 2,500 rpm in a glass homogenizing vessel immersed in iced-saline solution. Three times three passes were made with a 30 sec rest period after each 3 passes. Then 25 ml of KEA medium was added to the homogenate and 3 x 3 passes were again made as described above. The homogenate was centrifuged at 500 x g for 10 minutes by a Beckman J-21 C type centrifuge fitted with a JA-20 rotor. The supernatant was transferred through two layers of cheesecloth into centrifuge tubes and centrifuged at 8,000 x g for 10 min. The resultant pellet was washed by rinsing, resuspension and centrifugation at 8,000 x g for 10 min. The washing procedure was repeated and the final mitochondrial pellet was resuspended in a small volume of KEA medium to make a mitochondrial protein concentration of 20-30 mg per ml. This isolation method resulted in a yield of 20 mg mitochondrial protein/g tissue. The protein concentration of the final mitochondrial suspension was determined by the method of Lowry et al. (10) with bovine serum albumin as a standard. The oxygen consumption of mitochondria was measured polarographically with the aid of a Clark oxygen electrode fitted to an Oxygraph, model K-1C (Gilson Medical Electronics). State 3 and state 4 respiration, ADP:O and respiratory control index (RCI) were calculated according to Estabrook (11). Swelling of mitochondria was determined by measurement of the light absorbance changes of the sample at 540 nm using an Aminco model DW 2a spectrophotometer. The decrease in the light absorbance was evaluated as swelling (5,6). The total calcium content of mitochondria was determined by atomic absorption spectroscopy after addition of

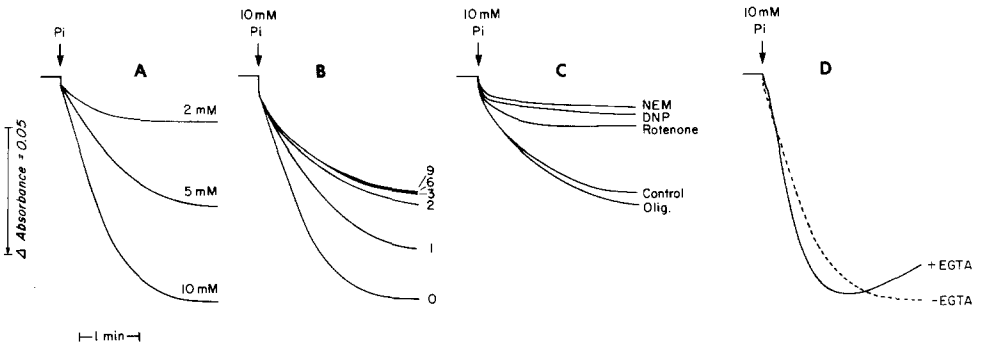

Figure 1. The swelling effect of inorganic phosphate (Pi) on heart mitochondria. Three ml assay medium contained 150 mM KCl, 1 mM sodium pyruvate and 2 mg mitochondrial protein at 37°. A after two minutes of preincubation different concentrations of potassium phosphate buffer PH 7 (Pi) were added. B, effect of ruthenium red (RR) on the swelling produced by 10 mM Pi. The corresponding numbers indicate nmoles(s) RR/mg protein present in the assay medium. C, effects of 100 μM 2,4-dinitrophenol (DNP) 100 μM N-ethylmaleimide (NEM), 4 μM rotenone and 1 μg/mg oligomycin (Olig) on the swelling effect of 10 mM Pi in the presence of 3 nmoles RR/mg protein (control) D, swelling effect of 10 mM Pi in the absence (broken line) and presence of 10 mM EGTA (solid line).

sufficient trichloroacetic acid and $LaCl_3$ to achieve concentrations of 6% and 1% respectively.

All chemicals used in these studies were of analytical grade. Ruthenium red (RR) was purchased from ICN Chemicals, Cleveland, Ohio. RR solutions were prepared daily.

RESULTS: The total endogenous calcium content of our heart mitochondrial preparations were about 2.0 nmoles/mg protein. The assay medium supplemented by 10 mM Pi had a very low calcium contamination (∿2 nmoles/ml). Furthermore, the addition of mitochondria to the assay medium provided the system with about 200 μM EGTA. We took these precautions in order to ensure that the mitochondria used in these experiments were not loaded with calcium and the free calcium concentration in the assay medium was maintained at a very low level. The mitochondria did not swell in the absence of inorganic phosphate (Pi). The addition of Pi in the form of potassium phosphate buffer (pH 7.0), however, induced a decrease in light absorbance which is an index of swelling of mitochondria. Both the rate and extent of swelling were dependent on the amount of Pi added (Figure 1A). Electron transport inhibitors, uncoupling

agents and inhibitors of phosphate uptake prevented the swelling (data not shown). Also, ruthenium red (RR) which is thought to be a specific inhibitor of mitochondrial Ca^{2+} transport (12) inhibited the swelling effect of 10 mM Pi in a dose-dependent manner, although not completely (Figure 1B). Increasing RR up to 9 nmoles/mg protein produced no further inhibition. From these data it is clear that there are RR-sensitive and insensitive components of the Pi-induced swelling. The RR-insensitive component of swelling was completely prevented by N-ethylmaleimide which is an inhibitor of Pi uptake into mitochondria, by the electron transport inhibitor rotenone and by the uncoupler, 2,4-dinitrophenol, but not by the ATPase inhibitor, oligomycin (Figure 1C). These data are in agreement with earlier studies (5,6) and suggest that there is a process of respiration-dependent accumulation of potassium phosphate in the mitochondria which results in osmotic swelling. It is of significance that the swelling effect of Pi was not inhibited but in fact was actually slightly stimulated by 10 mM EGTA (Figure 1D). These data exclude the possible involvement of extramitochondrial Ca^{2+} in the mechanism of Pi-induced swelling, and suggest that an intramitochondrial pool of calcium may be of considerable importance.

Oxidative phosphorylation was substantially inhibited and partially uncoupled in heart mitochondria which were swollen in the presence of 10 mM Pi (Figure 2A). Again, EGTA had no significant effect on oxidative phosphorylation even at concentrations as high as 10 mM (Figure 2B). On the other hand, 3 nmoles of RR/mg protein completely prevented both the inhibition and uncoupling of oxidative phosphorylation (Figure 2C).

DISCUSSION: The experiments reported here were carried out on heart mitochondria which were isolated in the presence of EGTA and therefore contained only about two nmoles of endogenous calcium/mg protein. The results indicate that 5-10 mM Pi can induce swelling of mitochondria, inhibition and partial uncoupling of oxidative phosphorylation even in the absence of free extra-

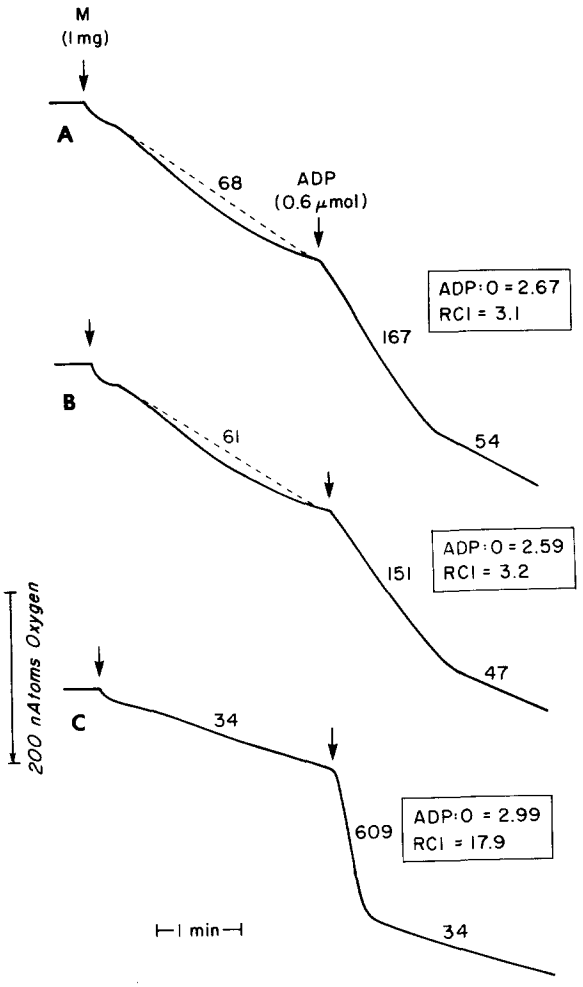

Figure 2. Effects of EGTA and ruthenium red (RR) on the oxidative phosphorylation of heart mitochondria. The assay medium contained 150 mM KCl, 1 mM sodium pyruvate and 10 mM potassium phosphate buffer pH 7 at 37°. The reaction was started by the addition of 1 mg mitochondrial protein to 1.5 ml medium. Three minutes later state 3 respiration was induced by the addition of ADP. The rate of oxygen consumption was expressed as nanoatoms oxygen$\cdot$ min^{-1}.mg $protein^{-1}$ A, is a control (no other addition). B, effects of 10 mM EGTA. In experiments A and B the resting state 4 respiration was not linear. Therefore, the corresponding numbers indicate the average rate of oxygen consumption within 3 minutes indicated by the broken lines. C, effects of 3 nmol RR.

mitochondrial calcium provided that the experiments are carried out in a KCl medium at 37°C. Our results are different from those reported previously which suggested that mitochondria prepared in the presence of EGTA are insensitive to Pi (9). The difference in results can be explained by differences in the

experimental conditions used. It has been previously shown that heart mitochondria are more resistant to the swelling effect of Pi in a nonphysiological sucrose containing medium than in a KCl medium (13, 23). Recent studies carried out in the presence of exogenous calcium show that the cycling of calcium across the mitochondrial membrane is the factor which causes swelling and loss of membrane potential (7,8). The inhibition of the Pi-induced swelling by RR in the present study strongly suggests the involvement of calcium in the Pi-induced damage to oxidative phosphorylation. It is of considerable interest that the calcium chelator EGTA was unable to inhibit swelling and the related uncoupling and inhibition of oxidative phosphorylation. Therefore, if the very small amount of endogenous (i.e., intramitochondrial) calcium present is cycling because of the high Pi concentration used in the present study, then it is occurring in an EGTA-inaccessible compartment. The sensitivity of the Pi-induced mitochondrial damage to RR suggests that a very small endogenous pool of calcium may have some heretofore unknown role in the regulation of membrane permeability and oxidative phosphorylation. The results also show that the RR-insensitive swelling of mitochondria does not cause any damage to oxidative phosphorylation. Our data support the recent view that both calcium and Pi are involved in the damage to oxidative phosphorylation (7,8,9) and further suggest that exogenous calcium is not essential. It is also possible that the loss of Mg^{2+}, which has been demonstrated in the presence of Pi (8,14), is a critical factor in the mechanism of Pi-induced damage to heart mitochondria, although this latter point remains to be proven.

The data presented here may also be of significance in myocardial ischemia. Intracellular Pi elevation is one of the early metabolic changes in the ischemic myocardium (15). This may induce swelling of mitochondria, inhibition and partial uncoupling of oxidative phosphorylation, i.e., changes that are known to occur very early after the onset of myocardial ischemia (16-18). In addition, drugs such as calcium channel blockers that

are effective in preventing ischemic damage to mitochondria in vivo (19-22) may exert a beneficial effect by a heretofore unrecognized mechanism, viz., by preventing the damaging effects of increased intracellular Pi concentration on heart mitochondria (23-25).

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