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RESPIRATORY CONTROL IN HYPERPERMEABLE ADULT HEART MUSCLE CELLS

EFFECTS OF CALCIUM

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SUMMARY

Spontaneously beating myocardial fragments prepared by mechanical disaggregation have hyperpermeable sarcolemmae. Such preparations were used to study mitochondrial function *in situ*. The myocardial fragments suspended in a phosphate-buffered salt solution containing 1–3 mM $MgCl_2$ showed a low rate of oxygen uptake. Addition of succinate, pyruvate plus malate or glutamate was followed by an increase in the rate of O_2 uptake. Addition of ADP to fragments engaged in State 4 respiration was followed by initiation of more rapid State 3 respiration, with respiratory control ratios routinely greater than 3 for succinate and glutamate. If the fragments were suspended in the same medium containing 3 mM ATP (a medium in which contractile activity occurs), State 3 was initiated upon addition of substrate. The suspension medium used in these experiments contained about 8 μM calcium as contamination. Addition of calcium chloride to give a final concentration of 0.14 to 0.57 mM stimulated State 4 respiration of the myocardial fragments. In contrast, similar additions made during State 3 inhibited respiration. The maximum degree of inhibition brought respiration close to the State 4 rate. If calcium was added prior to ADP, respiratory stimulation by the nucleotide was diminished. Respiratory function of myocardial fragments and of mitochondria isolated from them was similar in terms of response to substrate, ADP, and calcium addition in State 4. Response to calcium in State 3 was different in that inhibition was long-lived only at low $[P_i]$ in the case of mitochondria, but at low or high $[P_i]$ in the case of the fragments.

INTRODUCTION

The role of heart mitochondria in regulating the calcium ion concentration and the contraction-relaxation cycle in heart muscle is controversial. Scarpa and Graziotti [1] and Vinogradov and Scarpa [2] have questioned whether mitochondria can segregate calcium rapidly enough to play a physiological role at low calcium concentration. Carafoli et al. [3, 4] and Lehninger [5] maintain, however, that energy-dependent calcium binding by heart mitochondria is sufficiently rapid and the affinity and capacity of the process are compatible with a physiological function in the relaxation of heart muscle.

In the present communication effects of calcium on mitochondrial energy metabolism in mechanically disaggregated mouse myocardium were studied. The muscle cells of these fragments exhibit abnormal sarcolemmal permeability to small charged molecules [6]. In this system organelle and cytoplasmic functions remain integrated, yet substances added to the suspending medium have access to the cell interior.

METHODS

Myocardial fragments were prepared as previously described [6]. The saline-washed ventricular myocardia of three to five random-bred Swiss mice were minced in 20 ml of ice-cold medium in a Virtis-23 homogenizer at 8 000rev./min for 8 s. The standard medium contained 100 mM KCl; 9 mM NaCl; and 20 mM K phosphate buffer, pH 6.8. $MgCl_2$ and ATP · 2 Na were frequently included in the medium and their concentrations were varied, but whenever both were present, their concentrations were equal. All subsequent steps were performed at ice-bath temperatures. After the mince was passed through a plastic "Swinnex" 25 mm filter holder (Millipore Corp.) for removal of large fragments, the suspension was centrifuged in a conical centrifuge tube for 2 min at $50 \times g$. The supernatant was discarded and the fragments were resuspended in 20 ml of fresh iced medium and centrifuged again for 2 min at $50 \times g$. A second resuspension and centrifugation completed the washing of the fragments, which were then suspended in a final volume of 4 ml ice-cold medium per heart.

Mitochondria were isolated from suspensions of myocardial fragments after motor-driven homogenization in a Teflon-glass tissue grinder (6 passes) followed by centrifugation for 10 min at $1000 \times g$. All operations were conducted at ice-bath temperature and in media containing 100 mM KCl and 50 mM Tris · Cl, pH 7.5. EDTA (1 mM) was also present except in the wash and final suspension media. Following the first centrifugation, the pellet was rehomogenized (4 passes) and this homogenate and the first supernatant were centrifuged 10 min at $1000 \times g$. The combined supernatants were centrifuged for 15 min at $12000 \times g$ and the mitochondrial pellet was hand homogenized in EDTA-free media and centrifuged again at $12000 \times g$ for 15 min. The mitochondrial fraction was taken up in a small volume of EDTA-free isolation media, hand homogenized, and samples were taken for protein assay. The mitochondrial suspension usually contained approximately 35 mg protein per ml.

Oxygen uptake was measured in a Gilson Oxygraph equipped with a Clark type electrode and a sample chamber of 1.75 ml volume. Temperature was maintained at 25 °C with a refrigerated-heated water pump. The basic high phosphate and ATP-containing medium used in this study was chosen because it had previously been observed to support contractile activity of the cells particularly well. However, for many experiments, ATP was omitted so that the effects of added ADP could be assessed. Other modifications to the media have been noted in the legends of the figures.

Since the myocardial fragments tend to adhere to surfaces, losses occur during pipetting, etc., and cell suspensions with uniform predetermined protein concentrations could not be prepared. Samples of the spent reaction mixtures were therefore set

aside for protein assay according to Lowry et al. [7]. ATP was first removed from the samples by precipitating the protein with 10% (w/v) trichloroacetic acid, and discarding the supernatant containing the nucleotide. The precipitate was digested with 1.5 M NaOH for 15 min at 50 °C and a sample of the hydrolysate was assayed. All samples were subjected to this procedure, including the bovine serum albumin standards. Mitochondrial protein was determined by the biuret reaction [8].

The chemicals used were all of analytical reagent grade. Adenine nucleotides, rotenone, substrates and EGTA (ethylene glycol-bis-aminoethyltetraacetic acid) were obtained from Sigma Chemical Co.

Contaminating calcium levels in the media were estimated by atomic absorption spectroscopy and were in the range of 5 to 10 μM.

RESULTS

Myocardial fragments incubated in phosphate-buffered salt media and supplemented with respiratory substrate exhibited pronounced respiratory stimulation upon introduction of ADP into the suspending medium. Fig. 1 illustrates typical oxygen uptake traces for muscle fragment suspensions supplemented with succinate, pyruvate plus malate, or glutamate, and responses to addition of ADP. Oxygen uptake rates

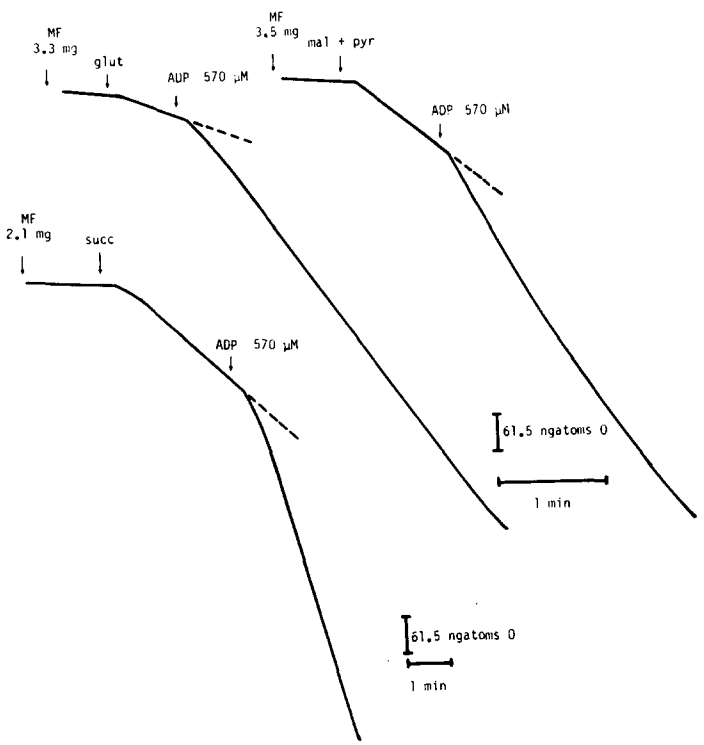

Fig. 1. Succinate, pyruvate+malate, and glutamate-supported respiration of myocardial fragments (MF). Standard incubation medium, containing 3 mM $MgCl_2$; pH 6.8. Substrate added to give a final concentration of 11.4 mM. Temperature maintained at 26 °C. Final volume 1.75 ml.

and average respiratory control ratios for many such determinations are shown in Table I. The ratios apply only to the initial State 4 to 3 transitions, since in no event was the respiratory rate observed to return to the controlled state, even when ADP additions should have been sufficiently small to so permit. This was not unexpected because of the prevalence of ATPase activities in muscle tissues. Corresponding data for mitochondria derived from the fragments also appear in Table I.

If the fragments were prepared and oxygen uptake was monitored in ATP-containing media, State 3 was initiated immediately upon substrate addition. Cytoplasmic ATPase activity was again thought to be responsible for making available a continuous supply of ADP. Oxygen uptake rates of myocardial fragment suspensions respiring in State 3 in ATP-containing media are shown for the several substrates used in Table I. Also shown are the corresponding respiratory rates of the mitochondrial fraction isolated from the fragments, with succinate or succinate plus rotenone as substrates.

It was perhaps surprising that the succinate-supported respiratory rates of the myocardial fragments and the mitochondria in ATP-free medium were so similar. Since heart muscle has been estimated to contain approximately 45 % mitochondria on a protein basis [1], this finding suggested that the respiratory activity of *in situ* mitochondria exceeded that of the isolated organelles. However, the additional procedures necessary for isolation of mitochondria from the fragments may have been responsible for some loss of respiratory activity.

TABLE I

RESPIRATORY RATES OF MYOCARDIAL FRAGMENTS AND MITOCHONDRIA

All media contain 20 mM potassium phosphate buffer; 100 mM KCl; 9 mM NaCl; pH 6.8 for myocardial fragments (MF), pH 7.4 for mitochondria (MITO). Data expressed as mean ±S.E.M. (N). All reactions were carried out at 25 °C. Substrates were added to a final concentration of 11.4 mM. State 3 was initiated with 0.57 mM ADP. Rotenone, when present, was 1.2 μM.

	Isolation medium	Substrate	Rate of oxygen uptake (ngatoms 0/min/mg protein)		Average respiratory control ratios
			State 4	State 3	
1	MF in ATP-free, 3 mM $MgCl_2$ media	Succinate	32.8±0.88 (51)	118 ± 4.3 (41)	3.6
		Succinate (+rotenone)	43.4±2.1 (9)	127 ± 7.7 (3)	2.9
		Pyruvate+malate	46.1±5.0 (3)	96.5±10 (3)	2.1
		Glutamate	13.6±1.9 (3)	50.8± 7.9 (3)	3.7
2	MF in 3 mM ATP, 3 mM $MgCl_2$ media	Succinate	–	83.3± 6.0 (11)	–
		Succinate (+rotenone)	–	170 ± 5.3 (5)	–
		Pyruvate+malate	–	109 ± 1.9 (2)	–
		Glutamate	–	93.0± 6.8 (2)	–
3	MITO in ATP-free, 3 mM $MgCl_2$ media	Succinate	28.7±1.7 (5)	90.8± 9.0 (4)	3.2
		Succinate (+rotenone)	33.5±3.8 (3)	207 ± 8.5 (2)	6.2
4	MITO in 3 mM ATP, 3 mM $MgCl_2$ media	Succinate	–	40.8± 2.1 (6)	–
		Succinate (+rotenone)	–	106 ± 4.9 (7)	–

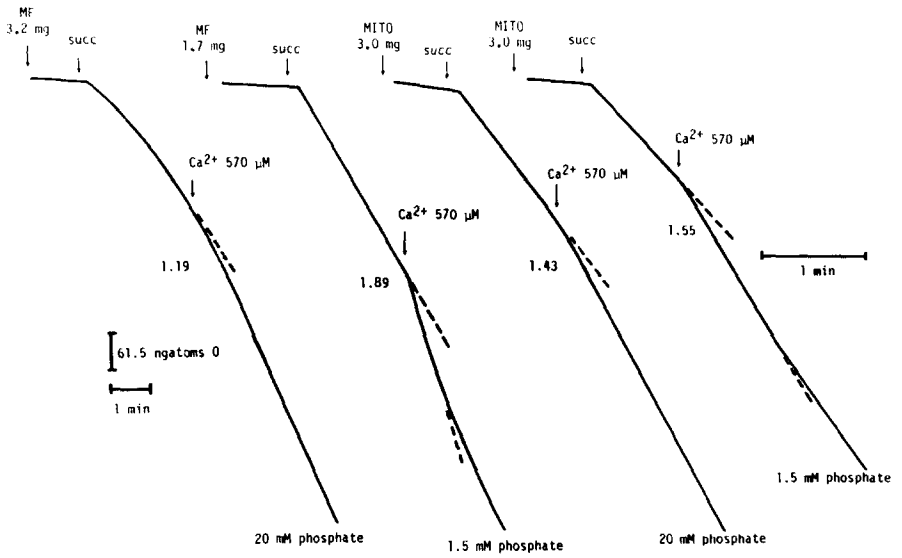

Fig. 2. The effect of calcium on State 4 respiration of myocardial fragments (MF) and mitochondria (Mito), at two phosphate concentrations. Standard incubation medium with 20 mM phosphate; 3 mM $MgCl_2$ or medium containing 20 mM Tris · Cl; 1.5 mM phosphate; 3 mM $MgCl_2$ and concentrations of KCl and NaCl identical to standard incubation medium. Temperature maintained at 26 °C. Final volume 1.75 ml, pH 6.8 for myocardial fragments, pH 7.4 for mitochondrial incubations.

Effects of calcium on state 4

Calcium like ADP stimulates State 4 respiration of the myocardial fragments, as well as that of the derived mitochondrial fraction (Fig. 2). The ratios of calcium stimulated to unstimulated State 4 rates shown in the figure indicate that stimulation was much more pronounced at lower phosphate concentrations, particularly in the case of the myocardial fragments. In addition, the stimulated rates in low phosphate medium undergo a spontaneous decrease, implying completion of energy-linked mitochondrial calcium uptake, both in the fragments and the mitochondrial fraction, allowing calculation of the $Ca^{2+}/0$ ratio for the process. The resulting value was 3.68 ± 0.25 ($N = 4$), or 1.84 Ca^{2+} per site, which approached the expected value when succinate was the respiratory substrate [9]. The absence of a spontaneous rate reduction in the presence of high phosphate was consistent with results of Rossi and Lehninger [9], who fould that at 2 mM or higher phosphate, no respiratory cutoff could be observed following calcium uptake by isolated rat liver mitochondria. These results suggest that the mitochondria within the fragments accumulate calcium in classic energy-linked fashion.

Effects of calcium on state 3

In contrast to the stimulation of State 4 respiration by calcium, introduction of calcium during respiration in State 3 evoked an abrupt inhibition of oxygen uptake by the myocardial fragments. Fig. 3 illustrates the calcium inhibition of succinate supported respiration in the presence of ATP, and its release by EGTA. In the corresponding experiment in ATP-free media, where ADP addition initiated State 3, addition of calcium likewise produced a distinct inhibition of oxygen uptake. EGTA

relieves the inhibition in this case as well (Fig. 3). Similar observations of the inhibitory effect of added calcium on State 3 respiration of the myocardial fragments have been made with the other respiratory substrates we have employed, including succinate in the presence of rotenone.

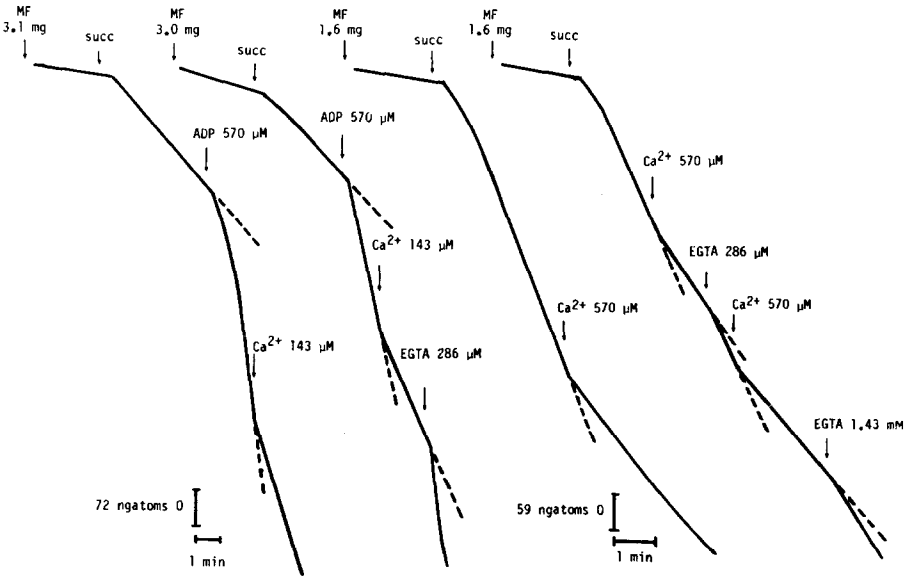

Fig. 3. The influence of calcium on State 3 respiration of myocardial fragments in the presence and absence of ATP. Standard incubation medium containing 3 mM $MgCl_2$, pH 6.8 for the two traces at the left; standard incubation medium containing 10 mM ATP, pH 6.8 for the two traces at the right. Temperature 26 °C. Final volume 1.75 ml.

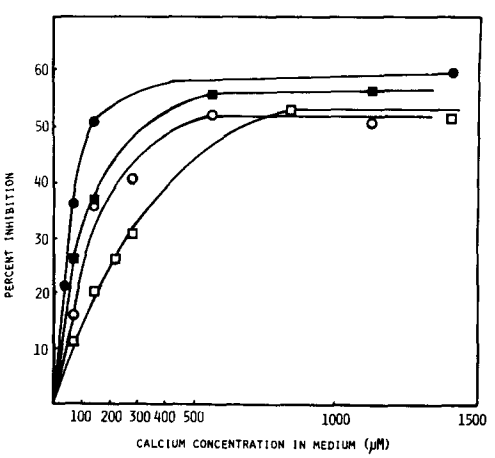

Fig. 4. Inhibition of State 3 respiration of myocardial fragments as a function of $[Ca^{2+}]$. All standard incubation media with the following additions: 1 mM Mg ATP (●—●); 3 mM Mg ATP (■—■); 10 mM Mg ATP (○—○); 0.57 mM ADP; 3 mM $MgCl_2$ (□—□). All at pH 6.8, temperature 26 °C; succinate, 11.4 mM; $CaCl_2$ as indicated. Final volume 1.75 ml.

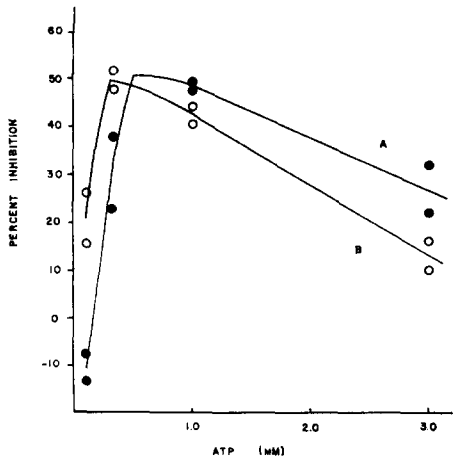

Fig. 5. Ca^{2+} inhibition of State 3 respiration of myocardial fragments as a function of [ATP]. Standard incubation medium with 20 mM phosphate; 3 mM $MgCl_2$; pH 6.8 and varied ATP concentrations. Following addition of succinate, $CaCl_2$ was added to give a final concentration of 280 μM. Temperature 26 °C, final volume 1.75 ml. Curve A, Reaction mixture contained indicated concentration of ATP; Curve B, 50 μm ADP present in addition to indicated [ATP].

The concentration dependence of the calcium effect on State 3 respiration of the myocardial fragments is shown in Fig. 4, in which percent inhibition is plotted as a function of added calcium concentration for representative experiments carried out in media at several ATP concentrations, and an analogous experiment conducted in ATP-free media, where State 3 was initiated with ADP. The maximum degree of respiratory inhibition was similar with ATP and ADP-containing reaction mixtures. At calcium concentrations producing less than maximum inhibition there was a dependence of inhibition on concentration of ATP, and a clear difference between reaction mixtures containing ATP and ADP. When calcium was added to a concentration of 286 μM the extent of inhibition varied with the ATP content of the reaction mixture (Fig. 5). Under these conditions, maximum inhibition was observed at about 1 mM ATP. Addition of 50 μM ADP to each reaction mixture produced an effect greater than would be expected from a corresponding amount of ATP. These results are consistent with the fact that only State 3 respiration is inhibited, and that this may come about through ADP · Ca^{2+} competition.

Similar or slightly greater levels of inhibition of State 3 were observed when exposure of the myocardial fragments to calcium occurred in a five minute preincubation before substrate and ADP addition. In such experiments, recording of oxygen uptake did not begin until after mitochondrial calcium binding or uptake should have been largely completed. The State 4 rate recorded under these conditions was not measurably increased (Fig. 6). However, the response to the subsequent addition of ADP decreased as the calcium concentration to which the fragments were exposed was increased. Fig. 6 shows respiratory control ratios and rates of State 3 and 4 oxygen uptake plotted as functions of calcium concentration. These data imply that calcium, even at the highest concentration used, did not uncouple respiration in these cells, since in this case a sustained increase in State 4 respiration toward the State 3 would be expected. Rather, calcium seems to prevent the stimulation of respiration by ADP.

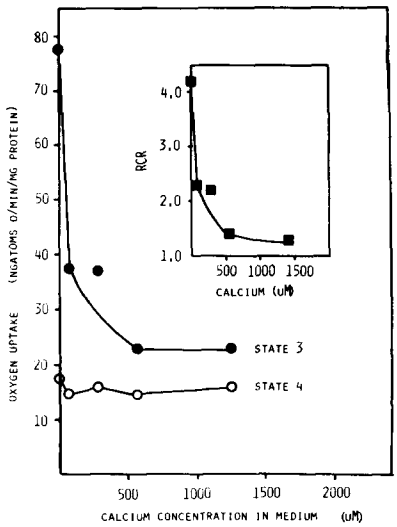

Fig. 6. The effect of prior exposure to calcium on State 4 and 3 oxygen uptake and respiratory control ratio of myocardial fragments. Standard incubation medium with 3 mM $MgCl_2$; pH 6.8; 26 °C. Additions: succinate, 11.4 mM; ADP, 0.57 mM; $CaCl_2$ as indicated. Final volume 1.75 ml. Protein content approximately 1 mg/ml in each incubation.

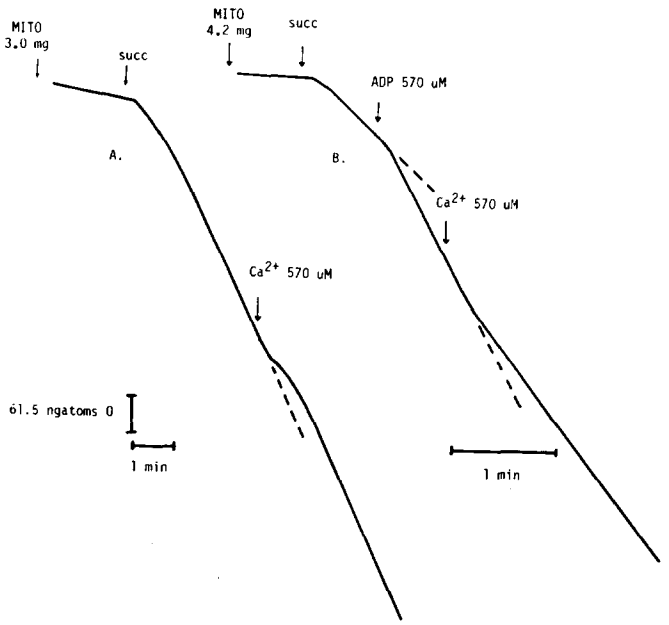

Fig. 7. The influence of calcium on State 3 respiration of mitochondria. A. Standard incubation medium containing 3 mM Mg ATP, pH 7.4. B. Medium containing 20 mM Tris · Cl, 3 mM $MgCl_2$, 1.5 mM phosphate. Concentrations of KCl and NaCl identical to standard incubation medium, pH 7.4. Final volume 1.75 ml, temperature 26 °C.

This finding was similar to that of Thorne and Bygrave [10] who demonstrated the failure of calcium to uncouple oxidative phosphorylation in mitochondria isolated from Ehrlich ascites tumor cells, as well as a similar inhibition of State 3 respiration by calcium. Recently, Wohlrab [11] also obtained data on calcium inhibition of State 3 respiration in blowfly flight muscle mitochondria.

The mitochondria isolated from the myocardial fragments also experience inhibition of State 3 respiration by calcium, but the effect was somewhat different than that observed with the fragments. In Mg · ATP containing medium with 20 mM phosphate, mitochondria undergoing succinate-supported State 3 respiration exhibited a brief inhibition of oxygen uptake upon introduction of 570 μM $CaCl_2$, as shown in Part A of Fig. 7. The inhibition was short-lived, however, and the oxygen uptake rate then increased so that it often actually exceeded the State 3 rate. If this increased respiratory rate was due to uncoupling secondary to calcium sequestration, then it should not occur in low phosphate medium [9]. This proved to be the case: respiration remained depressed after calcium addition to mitochondria suspended in low phosphate medium (Fig. 7B).

DISCUSSION

The experiments reported here reveal two findings which are important to the study of myocardial biology. First, mechanically disaggregated myocardial fragments provide a suitable preparation for the study of mitochondrial function. Methods similar to those used for isolated mitochondria are applicable. This allows evaluation of function closer to the *in situ* state than is possible with purified mitochondrial preparations. Secondly, it has been shown that Ca^{2+}, at concentrations presumably attained during pathological states in which sarcolemmal permeability is increased *in situ*, causes a reduction of the State 3 respiratory rate.

The observed inhibition of State 3 respiration by calcium does not correspond to the State 6 of Chance [11], since an excess of the permeant anion P_i was present. Calcium does inhibit respiration by induction of State 6 when a permeant anion is not available, but this inhibition is relieved by P_i (data not shown). The fact that the maximum degree of inhibition of State 3 results in a respiratory rate which corresponds to State 4 suggests the possibility that in the presence of high cytosolic [Ca^{2+}] ADP availability or interaction with ATP synthetase is interfered with. This possibility is strengthened by preliminary experiments in which the maximum inhibition of State 3 respiration by atractyloside and Ca^{2+} was found to be similar, and Ca^{2+} did not produce any further decrease in respiration when added to atracyloside-inhibited preparations.

Calcium inhibition of coupled respiration has been described in mitochondrial preparations derived from blowfly muscle [11] and cultured tumor cells [10]. This inhibition may, therefore, represent a general biochemical mechanism. The possible physiologic role of this process is not obvious. However, since calcium influx into damaged tissue is known to occur [13–15], this respiratory inhibition may play a role in the response to injury. The calcium levels observed to inhibit respiration in this study are probably similar to those attained in damaged cells equilibrating with plasma. In the case of anoxic injury, inhibition of respiration by calcium could serve to decrease oxygen consumption by irreversibly injured cells, thus sparing available oxygen for adjacent cells which may be able to survive.

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