Mitochondrial dehydrogenase activity affects adaptation of cardiac oxygen consumption to demand

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Mitochondrial dehydrogenase activity affects adaptation of cardiac oxygen consumption to demand. Am. J. Physiol. 264 (Heart Circ. Physiol. 33): H448–H453, 1993.—The effect of regulation of mitochondrial dehydrogenase activities on the mean response time of mitochondrial oxygen consumption, which characterizes the delay between changes in ATP hydrolisis and changes in oxygen consumption, was investigated in isolated rabbit hearts and perfused with Tyrode solution at 28°C. Perfusion with ruthenium red (RR) blocks mitochondrial calcium uptake and thus decreases mitochondrial dehydrogenase activities. Perfusion with pyruvate increases pyruvate dehydrogenase (PDH) activity. The mean response time was 11.8 ± 0.7 s (means ± SE) during control, 12.2 ± 1.2 s during perfusion with 0.9 μg/ml RR, and 20.7 ± 3.4 s during perfusion with 2.1 μg/ml RR. Blockade with 0.9 μg/ml RR, which is presumably partial, did not slow the response, suggesting that mitochondrial calcium uptake may not be rate limiting. Strong blockade of mitochondrial calcium uptake increases the mean response time, presumably due to decreased calcium activation of the mitochondrial dehydrogenases. Perfusion with pyruvate significantly decreased the mean response time to 10.0 ± 1.4 s compared with 11.9 ± 0.7 s during perfusion with glucose. This decrease with pyruvate is not compatible with a shift to regulation by high-energy phosphates but may reflect increased mitochondrial oxidative capacity caused by increased NADH levels.

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MATERIALS AND METHODS

Fourteen New Zealand White rabbits of both sexes, weight 2.9 ± 0.1 kg (means ± SE), were anesthetized with 9.3 ± 0.2 mg/kg fluanisone and 0.29 ± 0.01 mg/kg fentanyl citrate (Hypnorm, Janssen Pharmaceutica, Beerse, Belgium) injected intramuscularly. They were then artificially ventilated. Before the thorax was opened, 10 mg/kg of pentobarbital sodium was administered in the ear vein. After administration of heparin (at least 2.500 IU, Leo Pharmaceutical Products, Weesp, The Netherlands), the aorta was cannulated in situ whereafter the heart was excised and placed in the experimental setup. In this way coronary perfusion stopped for a few seconds only. Tyrode solution was used as perfusion medium (in mM: NaCl 128.3, KCl 4.7, CaCl, 1.36, MgCl, 1.05, NaHCO3, 20.2, NaH2PO4, 0.42, and glucose 11.1) and gassed with 95% O2–5% CO2. Perfusate pH was 7.3 throughout the experiments. Adenosine (10–5 M) was added to the perfusion medium to obtain maximal vasodilation. A funnel-shaped cannula was pierced through the apex of the papillary muscle and connected to a water-jacketed infusion device (2°C) to enable continuous perfusion with drugs at a flow of 150 ml/min at 37°C, which is consistent with the coronary blood flow during aortic occlusion in rabbits. Isolated hearts were stimulated at a basic rate of 2 Hz. The response of myocardial performance to changes in stimulation frequency was determined with a stimulation protocol, as previously described (27). At each level of stimulation (5, 10, 15, and 20 Hz), the rate of change in cardiac output was determined as a function of time from the beginning of stimulation. At least 20 min were allowed between measurements to ensure equilibration of the heart to the experimental conditions. Changes in cardiac output were expressed as a percentage of control values. Ratios of diastolic to systolic wall stress were calculated from the ratio of diastolic to systolic wall thickness and a mean wall stress of 150 dyne/cm2, as determined by myocardial biopsy. These data are expressed as the percentage of change from baseline. The cardiac output was calculated as the product of stroke volume and heart rate. The speed at which mitochondrial ATP production adapts to cardiac energy demand can be obtained from steps in heart rate or left ventricular balloon volume and characterized by the mean response time of mitochondrial oxygen consumption (27). This mean response time, defined as the first statistical moment of the impulse response function, was previously determined in saline-perfused isolated rabbit heart and is ~15 s at 28°C (11).

In this study we investigate to what extent the mean response time of mitochondrial oxygen consumption is determined by intramitochondrial calcium-mediated changes. To this end we block mitochondrial calcium uptake with ruthenium red, both partially and almost completely. A supraphysiological concentration of pyruvate as exogenous substrate leads to maximal activation of PDH by dephosphorylation of the enzyme and a severalfold increase of the mitochondrial NADH level (6). We therefore studied the mean response time during perfusion with glucose or during perfusion with pyruvate. As a result of blocking the mitochondrial calcium uptake or of pyruvate perfusion we expect that fast changes of intramitochondrial NADH do not occur and that changes of the high-energy phosphates take over regulation (9, 16, 30). Changes in the cytosolic ADP concentration are buffered by the creatine kinase reaction, and larger changes in ADP lead to larger changes in phosphocreatine (PCr). It has been shown that larger decreases in PCr for a given increase in ATP hydrolisis indicate a slower response of mitochondrial ATP synthesis (27) so that we may conclude that a shift to regulation by high-energy phosphates means a slower response of oxidative phosphorylation to demand. Steady-state high-energy phosphate concentrations at different work loads have been studied with 31P nuclear magnetic resonance (NMR) spectroscopy using similar interventions (16, 19, 26). However, the concentrations of high energy phosphates are the result of breakdown and resynthesis. Here we monitor the transient change of the mitochondrial oxygen consumption after changes in metabolic demand, which gives direct information on the mitochondrial ATP synthetic flux.
the left ventricle via the left atrium to drain the thebesian flow. A saline filled latex balloon was inserted in the left ventricle to measure the left ventricular pressure. Hearts were electrically paced. Experiments were performed at 28°C to obtain a high oxygen solubility and a low metabolic demand resulting in a sufficient oxygen supply (11). Arterial and venous oxygen tensions were continuously monitored by two oxygen electrodes (17). Oxygen concentrations were calculated with an oxygen solubility of 1.55 μmol O₂·1−1 Tyrode·1−1·mmHg−1 in saline solution at 28°C (11). Details of the preparation and measurement procedures have been described previously (11, 27).

Mitochondrial ATP production, which is related directly to oxygen consumption, adapts to a change in metabolic demand with a certain speed that can be described by a mean response time. The mean response time of mitochondrial oxygen consumption is defined as the first statistical moment of the impulse response function (27) and is a generalized version of the time constant that is also valid when the response is not monoexponential. This mean response time can be viewed as the average delay between changes in ATP hydrolysis and changes in oxygen consumption. The method to calculate the mean response time of mitochondrial oxygen consumption (tmito) used in this paper has been developed in our laboratory and has been described in detail (27). The essence of this method is given by a simple equation: tmito = t - ttransport. The measured response time (t) is obtained from venous oxygen tension transients after changes in heart rate or changes in left ventricular balloon volume. To obtain tmito, the transport time (ttransport), which corrects for diffusion of oxygen between capillaries and mitochondria, and convective transport through the coronary vessels has to be subtracted from t. The ttransport can be obtained from venous oxygen tension transients after stepwise changes in perfusion flow or arterial oxygen tension in combination with data on intravascular and right heart chamber volumes (27).

Ruthenium red (Sigma R-2751, St. Louis, MO) was added in the perfusion medium in six hearts using an infusion pump. Ruthenium red was not further purified, as was the case in other studies (10, 16, 26). Before ruthenium red entered the perfusion medium it passed a 0.22-μm filter (Millipore, Bedford, MA). First, the protocol to determine the tmito (11, 27) was performed without ruthenium red (Fig. 1). Then infusion of ruthenium red was started 30 min before the second measurement of the mean response time at a concentration of 0.9 μg/ml to obtain partial blockade of the mitochondrial calcium-uptake channels. Thereafter the higher concentration of ruthenium red (2.1 μg/ml) was infused to obtain a strong blockade, and after 30 min the third response time measurement was performed. We did not change the sequence in which the two concentrations and control were applied because washout of ruthenium red is slow.

In a second group of eight hearts the mean response time during perfusion with 11 mM pyruvate (16) was compared with the mean response time measured during perfusion with 11 mM glucose. In four hearts the first response time measurement was done during perfusion with glucose followed by a response time measurement during pyruvate perfusion. In four other hearts the sequence was reversed. Between the series of measurements there was an equilibration period of 30 min (Fig. 1).

Data are means ± SE unless indicated otherwise. Oxygen consumption during the various interventions and the mean response times of mitochondrial oxygen consumption were compared by means of analysis of variance for repeated measurements or paired t test (25). The null hypothesis was rejected when P < 0.05.

RESULTS

Wet weight of the hearts measured just after the experiments was 11.0 ± 0.4 g, and dry weight was 1.5 ± 0.1 g. Paced heart rate, perfusion pressure, and flow are given in Table 1. During perfusion with ruthenium red, perfusion pressure increased slightly and to compensate this, perfusion flow was decreased somewhat. The oxygen consumptions during the different perfusion conditions are presented in Table 1. Oxygen consumption increased significantly during perfusion with 0.9 μg/ml ruthenium red but did not increase further with the higher concentration of ruthenium red. Also a statistically significant increase of oxygen consumption during perfusion with pyruvate compared with perfusion with glucose was found. There were significant increases in oxygen consumption by 16 ± 2% after increases in heart rate and by 6 ± 1% after changes in left ventricular balloon volume. After flow or arterial oxygen tension reduction, there was no statistically significant change in oxygen consumption for all perfusion conditions, indicating that oxygen supply did not limit oxygen consumption. Left ventricular diastolic and systolic pressure changed under all perfusion conditions after increases in heart rate or left ventricular balloon volume (Fig. 2) but did not change when arterial oxygen tension or perfusion flow was decreased, again indicating a surplus of oxygen supply. At increased heart rate, end-diastolic pressure increased due to incomplete relaxation at this relatively low temperature.

Calculations of tmito. Steps in heart rate and left ventricular balloon volume were made during all perfusion conditions. Within all these perfusion conditions, the measured t obtained from upward and downward steps of the metabolic interventions were not significantly different. Also there was no statistically significant change between t obtained from heart rate and left ventricular balloon volume steps. The average t0 of the upward and downward changes in heart rate and left ventricular balloon volume for each perfusion condition is given in Table 2. An example of the venous oxygen transients after an increase in heart rate during perfusion with glucose and pyruvate is presented in Fig. 3.

The ttransport can be obtained from the venous oxygen tension transient after changes in flow or after changes in

<table>
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<tr>
<th>Ruthenium red experiments</th>
<th>preparation first series</th>
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<td>[RR] = 0.9 μg/ml</td>
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<td>[RR] = 2.1 μg/ml</td>
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<td>pyruvate</td>
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Fig. 1. Time table of experiments. Excision of heart occurs at time 0. During each series of measurements 5 interventions (stepwise decrease in arterial oxygen tension, stepwise increase in heart rate, stepwise decrease in perfusion flow, stepwise increase in left ventricular balloon volume and intravascular indicator step, respectively) were applied to determine mean response time. Eq, Equilibration period; [RR], concentration of ruthenium red. During ruthenium red experiments, hearts were perfused with 11 mM glucose.
Table 1. Experimental parameters

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<th>[RR], µg/ml</th>
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<th>Pyruvate (11 mM)</th>
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<td>10.4±0.4</td>
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<td>8.2±0.4</td>
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<tr>
<td>2.1</td>
<td>5.8±0.5</td>
<td>7.8±0.5</td>
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</table>

Values are means ± SE. Oxygen consumption is given at basal heart rate. [RR], concentration of ruthenium red.

arterial oxygen tension in combination with the intravascular volume (27). Perfusion flow was lowered stepwise by 8.9 ± 0.2% during ruthenium red experiments and by 10.4 ± 0.4% during pyruvate-glucose experiments. The transport derived from upward and downward changes in perfusion flow (27) were not significantly different, and their average is presented in Table 2 for the ruthenium red and substrate experiments. The increase in ttransplant during the ruthenium red experiments is a result of the decrease in flow brought about to prevent a large increase in perfusion pressure. The ttransplant derived from upward and downward changes in arterial oxygen tension in combination with the indicator dilution method (27) were not significantly different, and their averaged value is presented in Table 2 for the ruthenium red and substrate experiments. Because there was no statistically significant difference found between the ttransplant calculated from the stepwise change in perfusion flow and the stepwise change in arterial oxygen tension, their mean (Table 2) is used for further calculation of the tmito.

For each heart during each perfusion condition the tmito was calculated, whereafter the average of the mean response times is presented in Fig. 4. The low concentration, 0.9 µg/ml ruthenium red, did not prolong the mean response time. However, perfusion with 2.1 µg/ml ruthenium red resulted in a significant increase of the mean response time. Substitution of glucose by pyruvate resulted in a small but significant decrease of the tmito. The sequence of glucose or pyruvate perfusion (Fig. 1) did not significantly influence the results.

In our calculations we assumed that changes in metabolic demand are stepwise. If rate-pressure product, i.e., heart rate times peak systolic pressure (RPP) (16) is taken as a measure for ATP hydrolysis, the assumption can be investigated. The RPP reached a steady state after an initial overshoot so that the mean response time for RPP is negative (11). RPP reached a new steady state with a mean response time of -1.7 ± 0.9 s for perfusion without ruthenium red, -1.3 ± 0.8 s for perfusion with 0.9 µg/ml ruthenium red, and -1.5 ± 0.8 s for perfusion with 2.1 µg/ml ruthenium red. During perfusion with glucose...
RPP reached a steady state with a mean response time of 
-0.9 ± 0.4 s and during perfusion with pyruvate with a 
mean response time of -1.8 ± 1.0 s. From the negative 
value of the RPP response time, it follows that we have 
slightly underestimated the \( t_{\text{mito}} \). Our conclusions will not 
be affected, since there were no significant differences 
between the RPP response times.

**DISCUSSION**

In this study the role of calcium signaling in the regu-
lation of mitochondrial metabolism was assessed by 
blocking the mitochondrial calcium uptake with ruthen-
ium red so that the calcium-sensitive intramitochon-
drial dehydrogenases should be stimulated slower and to 
a lesser extent during increased cytosolic calcium concen-
tration. Ruthenium red was found in the cytosol of the 
cardiac myocytes after 15 min of perfusion of isolated rat 
hearts at a dye concentration of 2.1 \( \mu \)g/ml (10). Concentra-
tions of ruthenium red as small as 21 ng/ml are already 
slowing calcium uptake in isolated cardiac mitochondria 
(10). A ruthenium red concentration of 2.5 \( \mu \)g/ml blocks 
the interconversion of the PDH complex to its active 
dephosphorylated form after epinephrine stimulation of 
isolated rat heart (21, 26). Higher concentrations of ru-
thenium red (10–21 \( \mu \)g/ml) lead to contracture within half 
an hour in rat heart (10). We found that concentrations 
higher than 2.5 \( \mu \)g/ml ruthenium red lead to contracture 
in the isolated rabbit heart, i.e., at lower concentrations 
than in the rat heart. The data from the literature suggest 
that the concentration of 2.1 \( \mu \)g/ml that we used blocks 
the mitochondrial calcium-uptake channels almost com-
pletely.

Considering the strong blockade with 2.1 \( \mu \)g/ml ru-
thenium red, perfusion with 0.9 \( \mu \)g/ml ruthenium red might 
lead to partial inhibition of the mitochondrial calcium 
uptake channels in our preparation. Although 0.9 \( \mu \)g/ml 
ruthenium red might partially block the calcium chan-
nels, we found that the \( t_{\text{mito}} \) after steps in metabolic de-
mand remains unchanged. Metabolic Control Theory 
states that if a partial blockade of an enzyme or transport 
protein has negligible effect on a process, then the control 
strength of that enzyme or transport protein with regard 
to that process is low (14). Thus we suggest that the flux 
of calcium ions into the mitochondria after an increase in 
cytosolic calcium concentration may not be a major de-
terminant of the mean response time. The statement that 
oxidative phosphorylation in rabbit myocardium is not 
stimulated via calcium-mediated increases in NADH is in 
accordance with the finding that NADH decreases with 
contraction in isolated rabbit papillary muscle as meas-
ured by fluorometry (2) and by biochemical measure-
ments on frozen samples from isolated rabbit heart (15). 
However, during perfusion with 2.1 \( \mu \)g/ml ruthenium red, 
the mean response time increased from 12 to 21 s. We 
postulate that this may be due to a major decrease in 
calcium content of the mitochondria. Indeed, this is 
found in isolated mitochondria incubated with high con-
centrations of ruthenium red, since calcium uptake is 
inhibited while the calcium egress process is not influ-
enced (24). This low intramitochondrial calcium concen-
tration may lead to decreased activity of mitochondrial 
matrix dehydrogenases and a fall in mitochondrial 
NADH level. A lower NADH level in the mitochondria 
previously causes a decrease in oxidative capacity, as 
evidenced by the correlation between NADH level and 
state 3 oxygen consumption found in isolated rat liver 
mitochondria by Koretsky and Balaban (18). Decreased 
mitochondrial oxidative capacity in turn leads to a slower 
response of mitochondrial oxygen consumption according 
to Meyer’s (22) model of the regulation of oxidative phos-
phorylation in muscle.

There is an interesting relation between the mean re-
response time in this study and the steady-state changes in 
PCr levels observed after a heart rate step in isolated rat 
heart. Katz et al. (16) found a decrease in PCr when heart 
rate was increased during perfusion with 2.5 \( \mu \)g/ml ru-
thenium red, while PCr did not change in the absence of 
ruthenium red. Given this decrease in PCr, one expects 
an increase in the \( t_{\text{mito}} \), to a step in heart rate. This was 
found for 2.1 \( \mu \)g/ml ruthenium red but not for 0.9 \( \mu \)g/ml 
ruthenium red. Thus it is apparent from both Katz et al. 
(16) and our data that blockade of mitochondrial calcium 
uptake has major effects on the regulation of oxidative 
phosphorylation. Unitt et al. (26) studied the effect of 
ruthenium red on the response of isolated rat heart to
stimulation with isoprenaline. Their findings are in general in agreement with those of Katz et al. (16) and our results. They found a slower transient response of the high-energy phosphates to isoprenaline stimulation when 2.5 µg/ml ruthenium red was present in the perfusate. The transient response of oxygen uptake during isoprenaline stimulation was also measured, although with more than an order of magnitude lower time resolution (1 min) than in our study and therefore a change due to ruthenium red could not be detected. It should also be noted that they did not correct for oxygen transport delay as we did in the present study and that the isoprenaline did not reach the heart cells instantaneously, while a heart rate step leads to very fast stimulation. As in Katz et al. (16) study, the phosphorylation potential remained low after 10 min of isoprenaline stimulation (26). It remains to be seen what the effect might be of partial blockade of the mitochondrial calcium channels in a study as done by Unitt et al. (26). From our study we predict that moderate blockade will have a negligible effect. Although in some studies ruthenium red modified the steady state relation between oxygen consumption and high-energy phosphates (16, 26), Lew et al. (19) found that 2 µg/ml ruthenium red did not modify the relation between ADP and myocardial oxygen consumption over a wide range of values in perfused rat heart with glucose as exogenous substrate. Despite the presence of ruthenium red, the isolated rat hearts attained high work levels and oxygen consumption rates. This suggests that the calcium regulatory mechanism is not obligatory with regard to coupling ATP consumption to production. However, in isolated rat cardiac myocytes it has been shown that the mitochondrial calcium concentration increased with stimulation frequency (23). This keeps alive the possibility that calcium still plays a role, although probably not obligatory, in the physiological regulatory response of oxidative phosphorylation to demand.

Besides the explanation of the NADH level affecting the ATP synthetic capacity, another explanation can be found in the influence of calcium on the ATP synthase complex. It has been shown in several studies that calcium can affect the activity of this enzyme (3, 29). Because of a fall in intramitochondrial calcium concentration, the activity of ATP synthase may decrease, which might also prolong the mean response time according to Meyer's model (22). We cannot, based on the results obtained from ruthenium red perfusion, discriminate between these two explanations.

The interpretation of the experiments with ruthenium red should be done carefully because ruthenium red has many effects besides the blockade of the mitochondrial calcium-uptake channels. Ruthenium red is known to block calcium binding to the sarclemma, change the affinity of sarcolemmal calcium adenosinetriphatase to calcium, and to block calcium release from the sarcoplasmic reticulum (10). By acting on these and possibly other sites, ruthenium red affects contractility and leads to contracture at high concentrations. However, at the concentrations of ruthenium red that we used there were only minor changes in systolic pressure, and indeed the concentrations of ruthenium red necessary to block other systems are considerably higher than for the mitochondrial calcium uptake channels (10). It is very likely that the somewhat increased oxygen consumption during perfusion with ruthenium red is caused by changes in cytosolic calcium concentration, which cause alterations in cytosolic ATP turnover. Because this study addresses the speed of adaptation of mitochondrial ATP synthesis to cytosolic ATP hydrolysis, it is of little consequence that cytosolic ATP hydrolysis is somewhat affected by ruthenium red perfusion. This holds true as long as ruthenium red does not interfere with signaling pathways between ATP hydrolysis and mitochondria other than mitochondrial calcium uptake, as is the case to the best of our knowledge.

It should be realized that it is still impossible to measure mitochondrial calcium uptake or the capacity of mitochondrial calcium-uptake channels in the intact heart so that the extent of calcium channel blockade cannot be quantitatively assessed. Thus the present study has the advantage of investigating the t_mito, in the intact heart, which at the same time makes quantitative evaluation of the extent of calcium channel blockade very difficult.

The role of activation of intramitochondrial dehydrogenases was also investigated by infusion of supraphysiological concentrations of pyruvate that maximally activate PDH. The result is that intramitochondrial NADH levels are high (2, 6). The regulation of oxidative phosphorylation is expected to shift from mitochondrial NADH to the high-energy phosphates (9, 30). As a consequence it is predicted that, with increased work load, there is a more extensive change of the cytosolic ADP concentration and consequently of the PCr concentration. It is the buffering of the ADP concentration by the creatine kinase reaction that makes this response slow. A larger decrease in the PCr content for a given increase in ATP hydrolysis has been shown to indicate an increase in the t_mito (27, Eq. 14). The prediction of a larger mean response time is not fulfilled, since the mean response time decreased from 12 to 10 s when replacing glucose by pyruvate. This decrease of the mean response time suggests that increases of oxidative phosphorylation in the glucose-perfused rabbit heart are not to a major extent brought about by increases in NADH that were thought to take place soon after an increase in heart rate or left ventricular balloon volume (1). Again this is in accordance with the findings of Chapman (2) and Katz et al. (15), who found that NADH decreases on stimulation of rabbit myocardium, and with recent findings of Heineman and Balaban (13), who found that NADH did not increase with changes in work load in the isolated rabbit heart.

The large increase in mitochondrial NADH with pyruvate compared with glucose may lead to increased mitochondrial capacity for oxidative phosphorylation (18). Meyer's (22) model for the regulation of muscle metabolism predicts that when mitochondrial oxidative capacity is increased, the mean response time of mitochondrial ATP synthesis should be reduced, which would explain our experimental findings. The relatively small change in
mean response time with pyruvate perfusion may mean that with glucose perfusion the mitochondrial NADH content was already fairly close to saturating concentrations for oxidative phosphorylation. During pyruvate perfusion the mitochondrial oxidative capacity may be slightly increased. With glucose as exogenous substrate, substrate supply may thus slightly limit mitochondrial oxidative phosphorylation. The increased mitochondrial NADH content may also explain why cytosolic Pi concentration is found to decrease with pyruvate (9). Because of increased stimulation of oxidative phosphorylation via NADH, the high-energy phosphate concentrations will increase and ADP and P_i concentrations will be lowered. As a result, P_i may have less of an inhibiting effect on force development as suggested by the increase in developed pressure with pyruvate (4).

In summary, our results suggest that mitochondrial calcium-uptake channels have little control strength with respect to the fast response of oxidative phosphorylation. However, when mitochondrial calcium-uptake channels are blocked more fully, the speed of response of oxidative phosphorylation to metabolic demand is slowed, presumably due to the decreased calcium activation of mitochondrial dehydrogenases. Activation of PDH by pyruvate increases the speed of the response of oxidative phosphorylation to metabolic demand.

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