The relationship of extramitochondrial \( \text{Ca}^{2+} \) to intramitochondrial \( \text{Ca}^{2+} \) and the influence of intramitochondrial free \( \text{Ca}^{2+} \) concentrations on various steps of the citric acid cycle were evaluated. \( \text{Ca}^{2+} \) was measured using the \( \text{Ca}^{2+} \) sensitive fluorescent dye fura-2 trapped inside the rat heart mitochondria. The rate of utilization of specific substrates and the rate of accumulation of citric acid cycle intermediates were measured at matrix free \( \text{Ca}^{2+} \) ranging from 0 to 1.2 \( \mu \text{M} \). A change in matrix free \( \text{Ca}^{2+} \) from 0 to 0.3 \( \mu \text{M} \) caused a 155% increase in ADP-stimulated oxidation of 0.6 mM \( \alpha \)-ketoglutarate \((K_{0.5} = 0.15 \mu\text{M})\). In the absence of ADP and the presence of 0.6 mM \( \alpha \)-ketoglutarate, \( \text{Ca}^{2+} \) (0.3 \( \mu \text{M} \)) increased NAD(H) reduction from 0 to 40%. On the other hand, when pyruvate (10 \( \mu \text{M} \) to 5 mM) was substrate, pyruvate dehydrogenase flux was insensitive to \( \text{Ca}^{2+} \) and isocitrate dehydrogenase was sensitive to \( \text{Ca}^{2+} \) only in the presence of added ADP. In separate experiments pyruvate dehydrogenase activation (dephosphorylation) was measured. Under the conditions of the present study, pyruvate dehydrogenase was found to be almost 100% activated at all levels of \( \text{Ca}^{2+} \), thus explaining the \( \text{Ca}^{2+} \) insensitivity of the flux measurements. However, if the mitochondria were incubated in the absence of pyruvate, with excess \( \alpha \)-ketoglutarate and excess ATP, the pyruvate dehydrogenase complex was only 20% active in the absence of added \( \text{Ca}^{2+} \) and activity increased to 100% at 2 \( \mu \text{M} \) \( \text{Ca}^{2+} \). Activation by \( \text{Ca}^{2+} \) required more \( \text{Ca}^{2+} \) \((K_{0.5} = 1 \mu\text{M})\) than for \( \alpha \)-ketoglutarate dehydrogenase. The data suggest that in heart mitochondria \( \alpha \)-ketoglutarate dehydrogenase may be a more physiologically relevant target of \( \text{Ca}^{2+} \) action than pyruvate dehydrogenase.

It is generally accepted that movement of \( \text{Ca}^{2+} \) across the inner mitochondrial membrane occurs through separate influx and efflux pathways (1–3) and that the relationship between the concentration of intra- and extramitochondrial \( \text{Ca}^{2+} \) is determined by the relative activity of these two pathways. Moreover, recent observations show that the physiological concentration of matrix free calcium is in the sub-micromolar range and that the activity of three matrix enzymes, pyruvate dehydrogenase, isocitrate dehydrogenase, and \( \alpha \)-ketoglutarate dehydrogenase, are strongly activated by increases in \( \text{Ca}^{2+} \) in that range (4, 5).

Each of these enzymes catalyzes a reaction that is displaced far from equilibrium and hence their modulation by \( \text{Ca}^{2+} \) could contribute significantly to the overall flux of carbon in the citric acid cycle. Recent suggestions that \( \text{Ca}^{2+} \) rather than ADP may control generation of NADH and thus overall flux in the citric acid cycle under certain substrate conditions (6, 7) prompted this investigation of the relative potency of \( \text{Ca}^{2+} \) and ADP and the relative importance of the three \( \text{Ca}^{2+} \)-dependent dehydrogenases in regulating cardiac respiration.

It is important to know which of the three dehydrogenases are physiologically controlled by \( \text{Ca}^{2+} \) because the metabolic consequences of Ca2+ control over each is different. Thus, \( \text{Ca}^{2+} \)-mediated control of pyruvate dehydrogenase would limit the oxidation of glucose, lactate, and pyruvate but not the oxidation of free fatty acids or ketone bodies. On the other hand, if isocitrate dehydrogenase was responsible for rate limitation, in the absence of \( \text{Ca}^{2+} \), feedback control via citrate inhibition of citrate synthase (8) would inhibit oxidation of all substrates which generate acetyl-CoA. Since \( \text{Ca}^{2+} \) effects only the \( K_{c} \) of \( \alpha \)-ketoglutarate dehydrogenase (9, 10), which is in the middle of the citric acid cycle, citric acid cycle flux would not necessarily decrease at low \( \text{Ca}^{2+} \) levels but simply operate at a higher steady-state level of \( \alpha \)-ketoglutarate. Our recent studies indicate that the steady-state level of \( \alpha \)-ketoglutarate can, however, modulate glutamate oxidation to aspartate, because \( \alpha \)-ketoglutarate is a competitive inhibitor of oxaloacetate for aspartate aminotransferase (11). This in turn could modulate the rate of utilization of cytosolic NADH via the malate aspartate cycle. Conclusions concerning the physiological importance of \( \text{Ca}^{2+} \) in control of citric acid enzyme activity was hampered initially by the inability to directly measure mitochondrial free \( \text{Ca}^{2+} \). This difficulty has been overcome by the introduction of the \( \text{Ca}^{2+} \)-sensitive fluorescent probes fura-2 and indo-1 (12–17) and the realization that these indicators may gain access into the mitochondrial compartment under suitable conditions. Although two recent studies have attempted to correlate the activity of either \( \alpha \)-ketoglutarate dehydrogenase (15) or pyruvate dehydrogenase (14) with cytosolic free \( \text{Ca}^{2+} \), flux determinations and comparisons between the effects of ADP and \( \text{Ca}^{2+} \) have not been reported previously.

**EXPERIMENTAL PROCEDURES**

Mitochondrial Isolation—Mitochondria were isolated using a modification of the procedure originally described by Chance and Hagihara (18). The modification was described recently (19) and is specifically designed to obtain mitochondria with very low levels of endogenous \( \text{Ca}^{2+} \). Briefly, after anesthetizing male Sprague-Dawley rats (250–400 g) with 150 mg/kg pentobarbital, hearts were removed with a portion of the aorta intact. After cannulating the aorta, retrograde perfusion of the cardiac tissue was performed using 25 ml of an ice-

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† To whom correspondence should be addressed: Dept. of Surgery, The Milton S. Hershey Medical Ctr., P.O. Box 850, Hershey, PA 17033.
cold solution of 255 mM mannitol, 75 mM sucrose, 5 mM MOPS, and 0.1 mM EGTA (MSE). The perfusate was then changed to a similar one containing 0.3 mg/ml Nagarse (a commercial mixture of proteolytic enzymes). After flushing the heart with 6 ml of the protesase-containing solution, the tissue was minced and rinsed with MSE (no Nagarse) and mitochondria isolated by standard techniques of homogenization and differential centrifugation. Mitochondria isolated by this technique have less than 1 nmol of Ca2+/mg, are unusually stable during incubation in the range 20–37°C and have low levels of Mg2+-stimulated ATPase.

Loading and Incubation of Mitochondria with Fura-2—Mitochondria (50 mg/ml) were loaded at room temperature in a buffer composed of 150 mM KCl, 20 mM HEPES, 5 mM MgCl2, 5 mM ATP, 50 pM EGTA, and 10 pM fura-2-acetoxymethyl ester (fura-2/AM) at pH 7.0. After 20 min loaded mitochondria were centrifuged and resuspended (40–50 mg) in medium B consisting of 130 mM KCl, 20 mM HEPES, 5 mM MgCl2, 5 mM ATP, 5 mM KH2PO4, 5 mM NaCl, and 1 mM EGTA at pH 7.0 and stored in an ice bath. This procedure resulted in mitochondria loaded with approximately 0.54 nmol of fura-2/mg mitochondrial protein (approximately 430 μM inside the matrix).

Measurement of Free Ca2+ in EGTA Buffers—The free Ca2+ concentrations in incubation solutions were determined using calcium electrodes constructed with the calcium ionophore ETH129 (20). This ionophore allows construction of electrodes with a linear response to 10⁻⁹ M free Ca2+ and is thus more sensitive to the more commonly employed ionophore ETH1001 (21). Electrodes were constructed as described previously (21, 22) and were calibrated using the method of Bers (23). Output voltage between the Ca2+ electrode and a reference calomel electrode was fed into an Orion model 811 pH meter and recorded with a Kipp & Zonen model BD41 chart recorder.

Fluorescence Measurements—Free Ca2+ in the mitochondrial matrix was determined by measuring the fluorescence of fura-2-loaded mitochondria with a SPEX dual wavelength fluorometer. The fluorescence emission (510 nm) of fura-2 loaded mitochondria was measured during excitation of the sample with light alternating between 340 and 380 nm at 25 Hz. Since changes in mitochondrial calcium occur at considerably slower rates, the observed ratio between the emission intensity obtained with 340 nm excitation relative to 380 nm excitation can be considered instantaneous. After subtracting the background fluorescence of unloaded mitochondria at each wavelength, this ratio, R, was used to calculate free Ca2+ in a manner that is independent of the degree of mitochondrial dye loading (24). R is related to free Ca2+ by the following equation:

\[
\text{[Ca}^{2+}\text{]} = K_f \frac{(R - R_{\text{min}}) (S_{\text{fura-2}})}{(R_{\text{max}} - R) (S_{\text{fura-2}})}
\]

where \(K_f\) is the apparent dissociation constant of Ca2+ for fura-2; \(R\) is the ratio of emission intensities obtained from excitation at 340 nm relative to excitation at 380 nm; \(R_{\text{max}}\) is R when all of the dye is Ca2+-bound; \(R_{\text{min}}\) is R when all of the dye is in the unbound (free) form; \(S_{\text{fura-2}}\) is the fluorescence proportionality coefficient of the Ca2+-bound dye at 380 nm excitation; and \(S_{\text{fura-2}}\) is the fluorescence proportionality coefficient of the free dye at 380 nm excitation. \(R_{\text{max}}\) was determined by incubation of dye loaded mitochondria with 1 mM EGTA, 3 μM bromo-A23187, 2 ng/mg nigericin, and without added calcium. \(R_{\text{min}}\) was similarly determined using a 1 mM EGTA—calcium buffer containing >30 μM free Ca2+. Since \(R_{\text{max}}\) varied considerably between individual preparations of loaded mitochondria, it was measured in each batch and individual values of \(R_{\text{max}}\) were used in each determination of free matrix Ca2+.

Autofluorescence at 340 and 380 nm excitation of unloaded mitochondria was determined in each batch and subtracted from fluorescence values at 340 and 380 nm of loaded mitochondria. When 10 mg/ml mitochondria were loaded with 10 pM fura-2/AM (1 nmol/mg protein), autofluorescence was 8–10% of the fluorescence of fura-2-loaded mitochondria. The \(K_f\) of the intramitochondrial dye for Ca2+ was determined using bromo-A23187 (3 μM) and nigericin (2 ng/mg) to remove permeability restrictions to K+, H+, and Ca2+. R of ion permeable mitochondria was measured in buffers known Ca2+ concentration, as measured by Ca2+ selective electrode.

\(^1\)The abbreviations used are: MOPS, 3-(N-morpholino)propane-sulfonic acid; EGTA, (ethylendiaminetetraacetic acid); FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; AM, acetoxymethyl ester.
FIG. 1. Fura-2 is localized in mitochondrial matrix. Washed, fura-2-loaded mitochondria were incubated in medium B (with the exception that EGTA was 20 μM and NaCl was omitted) at 28 °C in the Spex fluorometer. The fluorescence intensity ratio, R, was recorded as a function of time. A, additions were as follows: 40 μM Ca²⁺ (a), 5 mM EGTA (b), 10 mM NaCl (c), and 3 μM bromo-A23187 (d). B, additions were: 40 μM Ca²⁺ (a), 3 μM bromo-A23187 (b), 10 mM NaCl (c), and 5 mM EGTA (d).

not decrease in response to Na⁺ addition but showed the expected rapid decline when EGTA was subsequently added. Taken together, these data demonstrate that fura-2 was localized within the mitochondrial matrix.

An accurate $K_D$ value is necessary for converting $R$ values into matrix free [Ca²⁺] values. Since the $K_D$ value for matrix-localized dye may differ from that for fura-2 in solution, experiments were performed to measure this $K_D$. Fura-2-loaded mitochondria were treated with bromo-A23187 and nigericin to equilibrate intra- and extramitochondrial free Ca²⁺ and $R$ values measured. From Equation 1, it can be seen that a plot of free Ca²⁺ versus $(R - R_{min})/(R_{max} - R) \times (S_{500}/S_{360})$ yields a straight line with a slope equal to $1/K_D$. Fig. 2 illustrates the data obtained at pH 7.7 using mitochondria containing either 0.6 or 5 nmol/mg fura-2. The alkaline pH was used because the ionophores also collapse the mitochondrial pH gradient, and this is the likely intramitochondrial pH under these conditions. The data were linear with respect to free Ca²⁺ and the $K_D$ was independent of the degree of loading and equal to 0.379 ± 0.019 μM. This value was used in subsequent experiments and agrees well with that reported by Gunter et al. (17) for fura-2 in liver mitochondria. Determination of Ca²⁺ Gradient across the Mitochondrial Membrane as a Function of Na⁺, Mg²⁺, and ADP—Recent studies (13, 14) of matrix free Ca²⁺ in heart mitochondria using the trapped dye technique have indicated large (10-fold) inverse gradients (out:in) of Ca²⁺ across the mitochondrial inner membrane especially when the external Ca²⁺ is in the

![Image of graph](https://example.com/graph.png)

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physiological submicromolar range. The gradient diminishes sharply with increasing external Ca\(^{2+}\). Although previous studies have documented the inhibitory effect of Mg\(^{2+}\) on Ca\(^{2+}\) entry \((K_i = 30 \text{ mM})\) and the stimulatory effect of Na\(^{+}\) on Ca\(^{2+}\) efflux \((K_i = 4-5 \text{ mM})\), no previous studies have investigated in a systemic way the effect of Na\(^{+}\) and Mg\(^{2+}\) on the Ca\(^{2+}\) gradient across intact mitochondrial membranes. Moreover, recent studies of Azzone et al. (30) suggest that ADP may inhibit efflux of Ca\(^{2+}\) from mitochondria more than it inhibits Ca\(^{2+}\) entry, thus potentially altering the gradient as a function of external ATP/ADP ratio. Therefore, the effect of ADP on the Ca\(^{2+}\) gradient was also monitored under approximately physiological conditions. The minimal effect of ADP is shown in Fig. 3. The effect of varying Na\(^{+}\) is shown in Fig. 4A. As in Fig. 2, the relationship of internal to external Ca\(^{2+}\) is sigmoidal and Na\(^{+}\) decreases matrix Ca\(^{2+}\) and its effect saturates at 5 mM Na\(^{+}\). The sigmoidicity of these curves might have been anticipated, because the Ca\(^{2+}\) uniporter kinetics are sigmoidal (second order) with respect to Ca\(^{2+}\). At 5 mM Na\(^{+}\) the inverse gradient diminishes to nearly one when external free Ca\(^{2+}\) is 1 \(\mu\text{M}\). Fig. 4B shows the effect of Mg\(^{2+}\). Mg\(^{2+}\) as opposed to Na\(^{+}\), changes the sigmoidal nature of the internal/external relationship. Indeed, in the absence of added external Mg\(^{2+}\) the relationship appeared hyperbolic and an inverse gradient was not observed. It is important to note that the concentration of added Mg\(^{2+}\) is not equivalent to free Mg\(^{2+}\) due to the presence of 5 mM ATP which binds Mg\(^{2+}\). Thus, the data are in approximate agreement with previous kinetic determinations (28) of the \(K_i\) of Mg\(^{2+}\) on Ca\(^{2+}\) influx. In further studies of the effect of matrix Ca\(^{2+}\) on citric acid cycle flux, data were gathered using medium B which contains 5 mM Na\(^{+}\), 5 mM Mg\(^{2+}\), and 5 mM ATP. In some cases (State 3) ATP was replaced with ADP.

Effects of Free Ca\(^{2+}\) on Mitochondrial Metabolism—Using fura-2 as an indicator for matrix free Ca\(^{2+}\), it is possible to assess the effects of intramitochondrial free Ca\(^{2+}\) on mitochondrial metabolism. Control (unloaded) mitochondria and mitochondria loaded with 0.6 nmol of fura-2/mg were incubated in medium B with various amounts of added Ca\(^{2+}\) plus 5 mM ADP. Free Ca\(^{2+}\) ranged from 0 to 1.2 \(\mu\text{M}\) and the matrix free Ca\(^{2+}\) was measured in each buffer. In order to identify Ca\(^{2+}\)-sensitive enzymes in functioning mitochondria, O\(_2\) consumption by fura-2 loaded mitochondria was measured when incubated with different substrate combinations at each level of free Ca\(^{2+}\). Unloaded and fura-2 loaded mitochondria displayed the same respiration rates under similar incubation conditions (but see Ref. 16). This indicated that fura-2 does not inhibit citric acid cycle enzymes, does not affect Ca\(^{2+}\) gradient across the membranes or coupling of oxidation to phosphorylation.

When mitochondria were incubated with various concentrations of pyruvate, altering matrix free Ca\(^{2+}\) appeared to have little effect on pyruvate oxidation (Fig. 5A). Double-reciprocal plots indicated that increasing matrix free Ca\(^{2+}\) primarily elevated \(V_{\text{max}}\) with little to no effect on the \(K_m\) of pyruvate dehydrogenase for pyruvate (Fig. 5B). Since Ca\(^{2+}\) promotes the conversion of pyruvate dehydrogenase from the inactive (phosphorylated) to active (dephosphorylated) form (31, 32), the increase in \(V_{\text{max}}\) by Ca\(^{2+}\) is an expected finding. What is unexpected, however, is the small magnitude of the observed effect. On the other hand, when the same experiment was performed using \(\alpha\)-ketoglutarate as substrate, a slight increase in intramitochondrial free Ca\(^{2+}\) from 0 to 0.3 \(\mu\text{M}\) had a dramatic effect on substrate oxidation (Fig. 6A). The effect of Ca\(^{2+}\) is more clearly shown in Lineweaver-Burk plots (Fig. 6B).
Thus, the steady-state level of NADH provides an index of

...ments.

Fig. 3 legend.

genases produce NADH when they oxidize substrates and on a-ketoglutarate dehydrogenase. Mitochondrial dehydrogenases (31), the effects of Ca2+ on enzyme activity may affect the ability of Ca2+ to interact with Ca2+-sensitive dehydrogenase activity. To achieve a steady state with respect to NADH, mitochondria were incubated for 4 min in the presence of the specific substrate prior to measuring NADH fluorescence. If Ca2+ activates a particular dehydrogenase in the presence of its specific substrate, the effect should be observed as an increase in NADH fluorescence. These experiments were performed in mitochondria not loaded with fura-2, since fura-2 interferes with measurement of endogenous NADH. The results, illustrated in Fig. 7, again demonstrate that pyruvate dehydrogenase was relatively insensitive to increases in matrix free Ca2+, whereas a-ketoglutarate dehydrogenase flux (at low substrate concentration of 0.6 mM) was highly sensitive. With 0.2 mM pyruvate plus 1 mM malate as substrates, increasing intramitochondrial free Ca2+ from 0 to 0.8 gM increased the percent reduction of NADH from 42 to 65 (Fig. 7B). By contrast, in the presence of 0.6 mM a-ketoglutarate, similar increases in matrix free Ca2+ increased the percent reduction of NADH from 0 to 40 (Fig. 7A). With pyruvate and malate as substrates, the half-maximal effective concentration of matrix free Ca2+ (Kc5) was estimated to be 0.21 gM in the presence of ADP and phosphate (Fig. 5A) and 0.2 gM in the presence of ATP (Fig. 7B). With a-ketoglutarate as substrate the Kc5 for Ca2+ was approximately 0.12 gM, both

6B) which indicate that the Kmax rather than the Vmax of a-ketoglutarate dehydrogenase was affected. Previous workers using other techniques and broken mitochondria have also shown that the effect of Ca2+ on a-ketoglutarate is on the substrate Kmax (9, 10). Increasing matrix free Ca2+ from 0 to 0.64 gM decreased the apparent Kmax for a-ketoglutarate from 2.5 to 0.6 mM (Fig. 6B). Stated in another way, at 0.6 mM a-ketoglutarate, an increase of matrix free Ca2+ from 0 to 0.3 gM caused a 135% increase in O2 consumption (Fig. 5A).

The above experiments were performed in the presence of excess ADP and phosphate, conditions in which mitochondrial NADH and ATP levels are low. Since these metabolites may affect the ability of Ca2+ to interact with Ca2+-sensitive dehydrogenases (31), the effects of Ca2+ on enzyme activity was also measured in the presence of ATP and absence of added ADP. This was achieved by measuring endogenous NADH fluorescence of intact mitochondria exposed to different concentrations of extramitochondrial free Ca2+, a technique previously employed (15, 34) to assess effects of Ca2+ on a-ketoglutarate dehydrogenase. Mitochondrial dehydrogenases produce NADH when they oxidize substrates and thus the steady-state level of NADH provides an index of  

Fig. 6. Effect of Ca2+ on kinetic parameters of a-ketoglutarate (aKG) oxidation by intact mitochondria. Experiments were performed as in Fig. 5 except that pyruvate was substituted by a-ketoglutarate and the concentration of fura-2 loaded mitochondria was 0.5 mg/ml. A, effect of matrix free Ca2+ on respiration in mitochondria incubated with various concentrations of a-ketoglutarate. ▲, 0.03; ○, 0.06; ●, 0.2; and ▼, 5 mM pyruvate. B, Lineweaver-Burk plot of O2 consumption versus a-ketoglutarate concentration. ▲, 0.04 mM matrix free Ca2+ and ○, 0.64 mM matrix free Ca2+. Values shown are the mean ± S.E. of three experiments.

Fig. 5. Effect of Ca2+ on kinetic parameters of pyruvate oxidation by intact mitochondria. Fura-2 loaded mitochondria (0.25 mg/ml) were incubated with Ca2+-EGTA buffers containing 5 mM ADP, 1 nM malate, and various concentrations of pyruvate and free Ca2+. Intramitochondrial free Ca2+ was measured as described in Fig. 3 legend. A, effect of matrix free Ca2+ on respiration in mitochondria incubated with various concentrations of pyruvate. ▲, 0.03; ○, 0.06; ●, 0.2; and ▼, 5 mM pyruvate. B, Lineweaver-Burk plot of O2 consumption versus pyruvate concentration. ▲, 0.04 mM and ○, 0.64 µM matrix free Ca2+. Values shown are mean ± S.E. of three experiments.

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Regulation of Citric Acid Cycle by Ca²⁺

Pyruvate oxidation when matrix free Ca²⁺ levels were extremely low and constant in the presence of Ca²⁺ and 1 mM malate, increases in matrix free Ca²⁺ from pyruvate oxidation in the citric acid cycle.

It was not possible to use either NADH fluorescence or O₂ consumption measurements to evaluate isocitrate dehydrogenase activity since citrate and isocitrate are not readily transported across the inner membrane of cardiac mitochondria (35). A different approach was used to evaluate the effect of Ca²⁺ on isocitrate dehydrogenase flux. Mitochondria were incubated with [1-¹⁴C]pyruvate and the rate of ¹⁴CO₂ production was measured as described under “Experimental Procedures” and plotted as a function of time. At 0 and 0.8 µM extramitochondrial Ca²⁺, 5 mM ADP; 0, 0 µM Ca²⁺, 5 mM ATP. At 6 and 0.8 µM extramitochondrial Ca²⁺, matrix free Ca²⁺ were 0.04 and 0.62 µM, respectively. Values shown are the mean ± S.E. of three separate experiments.

Fig. 7. Effect of Ca²⁺ on NADH fluorescence of mitochondria in State 4 respiration. Mitochondria (0.25 mg/ml) not loaded with fura-2 were incubated for 4 min at 28 °C in Ca²⁺-EGTA buffers containing 5 mM ATP, 1 mM malate, and various amount of pyruvate or α-ketoglutarate. NADH fluorescence was measured as described under "Experimental Procedures." Percent of total mitochondrial NAD⁺ reduced was calculated by the equation:

\[
\frac{\% \text{ NAD}^+ \text{ reduced} = \frac{F_i - F_0}{F_o - F_0} \times 100}
\]

where \(F_i\) = mitochondrial fluorescence intensity under experimental condition i, \(F_0\) = mitochondrial fluorescence intensity in the absence of substrate. Mitochondria were incubated with [1-¹⁴C]pyruvate and the rate of ¹⁴CO₂ production was measured as described under "Experimental Procedures" and plotted as a function of time. At 0 and 0.8 µM extramitochondrial Ca²⁺, 5 mM ADP; 0, 0 µM Ca²⁺, 5 mM ATP.

Fig. 8. Effect of Ca²⁺ on [1-¹⁴C]pyruvate oxidation by mitochondria in State 3 or State 4 respiration. Mitochondria (1 mg/ml) were incubated at 28 °C in Ca²⁺-EGTA buffers (buffer B) containing 5 mM [1-¹⁴C]pyruvate (4.0 µCi/ml) and 1 mM malate. ¹⁴CO₂ production was measured as described under "Experimental Procedures" and plotted as a function of time. At 0 and 0.8 µM extramitochondrial Ca²⁺, matrix free Ca²⁺ were 0.04 and 0.62 µM, respectively. Values shown are the mean ± S.E. of three separate experiments.

exerted via stimulation of α-ketoglutarate dehydrogenase flux. Similar results were obtained when extramitochondrial pyruvate concentrations were kept low (10 µM rather than 5 mM) by inclusion of [1-¹⁴C]lactate (5 mM), lactate dehydrogenase (10 units) and NAD⁺ (100 µM) in the incubation medium. Rates of ¹⁴CO₂ production were much lower (25 nmol/min·mg) in the presence and absence of ADP, but increasing matrix free Ca²⁺ had no affect on the rates. Acetyl-CoA levels could not be detected and much more α-ketoglutarate accumulated in the absence than in the presence of Ca²⁺ (data not shown).

When mitochondria were incubated under State 3 condi-
Regulation of Citric Acid Cycle by Ca\(^{2+}\)

**Fig. 9.** Effect of Ca\(^{2+}\) on citrate accumulation in rat heart mitochondria during State 3 and State 4 respiration. Mitochondria (1 mg/ml) were incubated as described in Fig. 8 except that the incubations were conducted in open vessels gassed continuously with 100% O\(_2\). Samples were withdrawn at times indicated, deproteinized with 3% perchloric acid, neutralized, and assayed for citrate. A, citrate levels in the mitochondria incubated with 5 mM ADP and either 0 (△) or 0.8 μM (▲) extramitochondrial free Ca\(^{2+}\). B, citrate levels in the mitochondria incubated with 5 mM ATP and either 0 (○) or 0.8 μM (●) extramitochondrial free Ca\(^{2+}\). Values shown are the means of two separate experiments.

Since citrate is a competitive inhibitor of citrate synthase (8), Ca\(^{2+}\) regulation of isocitrate dehydrogenase could influence flux in the citric acid cycle. Flux in the first committed step of the citric acid cycle could be decreased if citrate levels became high enough. If this were an important consideration in the present experiment, acetyl-CoA would have accumulated when citrate levels were high. However, this was not observed.

Since previous studies (31, 32) indicate that conversion of pyruvate dehydrogenase from inactive to active form is modulated by increases in matrix free Ca\(^{2+}\), and since our data did not demonstrate a significant effect of Ca\(^{2+}\) on pyruvate dehydrogenase flux, we performed further experiments to reconcile these apparently contradictory findings. With pyruvate and malate as substrate (in the absence of added α-ketoglutarate), mitochondrial pyruvate dehydrogenase was nearly completely activated regardless of the external Ca\(^{2+}\) concentration or the external ATP/ADP ratio (Fig. 11). On the other hand, in the presence of α-ketoglutarate (in the absence of pyruvate), activation of pyruvate dehydrogenase in mitochondria incubated under State 4 conditions was very low when external Ca\(^{2+}\) was low, but progressively increased with increasing Ca\(^{2+}\) (Fig. 12), confirming previous findings that pyruvate dehydrogenase activation can be in part mediated by Ca\(^{2+}\). The lack of effect of Ca\(^{2+}\) on pyruvate dehydrogenase activation in mitochondria incubated with α-ketoglutarate in the presence of ADP may be due to low ATP levels in the matrix. This would result in depressed pyruvate dehydrogenase kinase activity and thus most of the pyruvate dehydrogenase would remain in the active form.

**DISCUSSION**

Most studies of the control of respiration have focused on the role of ADP, the ratio of ATP/ADP or the phosphorylation potential ATP/ADP-P, as potential modulators of respiration. The classical studies of Chance and Williams (36) demonstrated large increases in respiration of isolated mito-
on whether ADP controls respiration but on how (19,37,38). Creases hepatic ADP, a potent such ATP consumption, it seemed reasonable to assume that in the concentrations of ADP and phosphate do not appear in intact tissue. In the past, debate centered not isolated mitochondria, might be responsible for control of respiration in mitochondria on addition of ADP. With few exceptions, respiration and thus mitochondrial ATP synthesis adjusts to the needs of intact tissue for ATP utilization. Thus, ATP utilization for muscle contraction is accompanied by increased ATP consumption. Since ADP is the product of metabolism of mitochondria incubated with 0.1 mM pyruvate and either 5 mM ATP (O) or 5 mM ADP (D). Values shown are the mean ± S.E. of four separate experiments.

**Fig. 11.** Effect of matrix free Ca²⁺ and energy state on pyruvate dehydrogenase (PDH) activity in mitochondria using pyruvate as substrate. A, effect of matrix free Ca²⁺ on pyruvate dehydrogenase activity of mitochondria incubated with 0.1 mM pyruvate and either 5 mM ATP (O) or 5 mM ADP (D). Values shown are the mean ± S.E. of four separate experiments.

**Fig. 12.** Effect of matrix free Ca²⁺ and energy state on pyruvate dehydrogenase (PDH) activity in mitochondria using a-ketoglutarate as substrate. Conditions are the same as those described in Fig. 11 except that 5 mM a-ketoglutarate rather than pyruvate was used as substrate. 5 mM ATP (O) or 5 mM ADP (D) was present in the incubation. Values shown are the mean ± S.E. of four separate experiments.

to adequately account for changes of O₂ consumption. Moreover, changes in NADH during increased cardiac work are opposite to those expected in the presence of increased ADP (E, 7). Therefore, other modes of stimulation of cardiac respiration in response to increases in contraction have been sought. Ca²⁺ is a potential regulator of cardiac metabolism since it modulates muscle contraction, and simultaneous stimulation of contraction and respiration would result in the observed increases in respiration without large changes in tissue free ADP.

Certain mitochondrial dehydrogenases are known to be sensitive to Ca²⁺ (40), but the physiological significance of these observations were not immediately appreciated since the Ca²⁺ sensitivity of the dehydrogenases was in the low micromolar range, whereas early estimates were on the basis of equilibrium thermodynamic considerations (41) suggested values of 0.7 to 1.9 mM for free intramitochondrial Ca²⁺. Total matrix Ca²⁺ contents of mitochondria as usually isolated averaged 25–39 nmol/mg protein (42). Later estimates based on mitochondrial Ca²⁺ activity coefficient and liver mitochondrial Ca²⁺ content of at least 16 nmol/mg protein gave values of about 16 μM (43). This derived value was still more than 1 order of magnitude above the range of free Ca²⁺ concentrations required to activate mitochondrial Ca²⁺-sensitive enzymes. These high matrix free Ca²⁺ estimates may relate to the fact that the mitochondrial total Ca²⁺ contents (42, 43) are much higher than the 1 nmol of Ca²⁺/mg mitochondrial protein measured by electron probe x-ray microanalysis of tissues frozen rapidly in vivo (44). It is known that matrix free Ca²⁺ increases directly with total mitochondrial Ca²⁺ content (45) and that total Ca²⁺ content in mitochondria can vary manyfold depending on isolation conditions (46).

The first direct measurement of matrix free Ca²⁺ using the so-called null-point titration method gave values of 0.5 and 1.5 μM, corresponding to mitochondrial total Ca²⁺ contents of 1 and 2 nmol/mg protein, respectively (45). Despite the critical importance of these observations on the physiological relevance of Ca²⁺ in regulating mitochondrial metabolism, the null-point titration technique is cumbersome, measures only steady-state values, and is inaccurate at low matrix free Ca²⁺ levels. The recent introduction of the Ca²⁺-sensitive fluorescent probe fura-2 (12, 13, 15–17) and indo-1 (14) and the observation that fura-2 may distribute in mitochondria in vivo (44, 46) have made it possible to monitor matrix free Ca²⁺ changes in response to experimental manipulations.

Since matrix free Ca²⁺ levels are critically dependent on total Ca²⁺ content of isolated mitochondria (45), our method for mitochondrial isolation (19) was specifically designed to yield mitochondria containing <1 nmol of Ca²⁺/mg protein. These Ca²⁺ content values have been previously shown to reflect those present in vivo (44, 46). Particular attention was paid to the fura-2 loading conditions since overexposure of cardiac mitochondria to fura-2/AM may result in a decrease in the dynamic range (ratio of R_{max} to R_{min} of the trapped dye). The optimal loading conditions established in our study (10 μM fura-2/AM with 10 mg/ml mitochondrial protein for 20 min or 1 nmol/mg protein) resulted in R_{max}/R_{min} ratio of 11.1 which is still somewhat lower than that determined for fura-2 free acid in solution (19.60 ± 3.20, n = 5). This is most likely due to incomplete ester hydrolysis in the matrix (49). For this reason, individual R_{max} and R_{min} values of each batch of mitochondria were determined and used in the calculation of free matrix Ca²⁺ levels.

Previous studies of matrix free Ca²⁺ using trapped dye techniques are in general agreement with the present studies though none of them have simultaneously compared and
examined all three Ca²⁺-sensitive matrix dehydrogenases nor compared the effect of ADP with the effect of Ca²⁺ on fluxes. The recently published studies of Reers et al. (13) are in excellent agreement with those reported here, especially with respect to the Kₕ of intramitochondrial fura-2 (0.37 μM). These authors find a somewhat steeper gradient of Ca²⁺ (out/in) than our current results, especially in the higher range (>1.0 μM Ca²⁺). This is not surprising since they used a higher ratio of Mg²⁺/ATP. Their conditions are different in other respects as well, since they employ perfused mitochondria attached to glass coverslips. On the other hand, Lukacs and Kapos (17) and Lukacs et al. (15) have reported the relationship of matrix Ca²⁺ levels to α-ketoglutarate dehydrogenase activity measured at low concentrations of α-ketoglutarate in State 3 (using the NADH fluorescence method). They report a Kₕ value of Ca²⁺ for α-ketoglutarate dehydrogenase of 0.8 μM but use a different Kₕ value (0.135 μM, assumed) for fura-2 in calculating matrix free Ca²⁺. Their results would be in good agreement with ours using a fura-2 Kₕ of 0.37 μM. However, previous studies of partially purified α-ketoglutarate dehydrogenase and of α-ketoglutarate dehydrogenase in intact mitochondria made permeable to Ca²⁺ reported significantly higher values (1 μM) of Kₕ than those found in the present study.

The relationship of free mitochondrial Ca²⁺ to pyruvate dehydrogenase was recently explored by Moreno-Sanchez and Hansford (14). These authors use indo-1 as the trapped Ca²⁺ indicator dye and measure the relationship of free mitochondrial Ca²⁺ to the activation state of pyruvate dehydrogenase. They find that activated pyruvate dehydrogenase in State 4 increases with matrix Ca²⁺ level and that the Kₕ of Ca²⁺ is 0.3 μM. This value is lower than the value of approximately 1 μM which we found. However, again the disagreement may lie in the measurement of the Kₕ of indo-1, since the value used by these workers is almost one-third lower than that reported for the free dye in solution and was moreover determined at neutral pH rather than the more alkaline values likely present in the intact mitochondrial matrix.

Moreno-Sanchez and Hansford (14) have also measured Ca²⁺ gradients across the mitochondrial membranes and, in agreement with the present study and that of Reers et al. (13), find lower values in the mitochondrial matrix than outside in the presence of 1 mM Mg²⁺ (but not in its absence).

Our data demonstrated that α-ketoglutarate dehydrogenase in the mitochondrial matrix is more sensitive to lower levels of Ca²⁺ than pyruvate dehydrogenase. The physiological significance of this is of course uncertain until measurements of mitochondrial Ca²⁺ can be made in situ heart mitochondria. Since the cytosolic Ca²⁺ of heart cells oscillates below 0.1 to perhaps over 1 μM from beat to beat, matrix free Ca²⁺ is difficult to predict even from the present steady-state measurements of Ca²⁺ gradients. Moreover, numerous previous studies have demonstrated the fact that pyruvate dehydrogenase is subject to Ca²⁺ activation due to the Ca²⁺-sensitive pyruvate dehydrogenase phosphatase. Nevertheless it is also clear that other modes of control of pyruvate dehydrogenase may override that of Ca²⁺ (50, 51). For example, although conversion of pyruvate dehydrogenase to its active dephosphorylated form is readily observed on addition of epinephrine to perfused hearts (50, 52), Hiraoka et al. (50) have shown that this occurs only when low concentrations of pyruvate are available. In the absence of additional exogenous substrates (β-hydroxybutyrate), oxidation of pyruvate may actually decrease, despite the hormone-dependent enzyme activation. However, since the normal physiological condition in vivo is one in which pyruvate concentrations are relatively low and other substrates are present, Ca²⁺-dependent activation/deactivation of pyruvate dehydrogenase cannot be dismissed as physiologically insignificant. It is apparent from the present study, as well as those of others, that Ca²⁺ does not provide overriding control of pyruvate dehydrogenase (50, 51) and moreover Ca²⁺ control of pyruvate dehydrogenase is unlikely to control overall rates of cardiac respiration. Likewise, it seems unlikely from the present study that isocitrate dehydrogenase would provide a severe limitation for citric acid cycle flux in the absence of Ca²⁺.

On the other hand, under some physiological circumstances Ca²⁺ control of α-ketoglutarate may contribute significantly to the rate of generation of ATP, which cannot be overcome even by increases in ADP. Numerous studies of liver metabolism demonstrate that Ca²⁺ levels may control the flux through the malate aspartate cycle (53–55) and therefore transport of reducing equivalents from the cytosol to the mitochondria. Our recent studies (11) indicate that this Ca²⁺ effect is mediated via the Ca²⁺ sensitivity of α-ketoglutarate dehydrogenase. Ca²⁺-mobilizing hormones cause large decreases in hepatic α-ketoglutarate levels. The decrease in α-ketoglutarate in turn causes significant stimulation of aspartate aminotransferase (11). Since the mitochondrial enzymes (aspartate aminotransferase and α-ketoglutarate dehydrogenase) involved in Ca²⁺ control of the malate/aspartate shuttle in the liver are very similar to those of the heart, a Ca²⁺-linked control of the malate/aspartate shuttle may occur in cardiac tissue. This would explain anomalous increases in mitochondrial NADH fluorences following an increase in work when hearts are perfused with glucose as substrate (7). When hearts are perfused with pyruvate, whose oxidation is not dependent on oxidation of cytosolic NADH, the expected decrease in NADH fluorescence occurs (56, 57). Clearly Ca²⁺ stimulation of the malate/aspartate shuttle would lower lactate/pyruvrate ratios in the heart and thus promote pyruvate and glucose oxidation. Ca²⁺ activation of pyruvate dehydrogenase at higher levels of Ca²⁺ would augment the effect of Ca²⁺ on reducing equivalent transport. Thus, these two Ca²⁺-sensitive dehydrogenases may work synergistically providing a two-tiered system of activation of glucose and lactate oxidation as cardiac contractility increases.

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