Steady State Kinetics of Energy-dependent Ca$^{2+}$ Uptake in Rat Liver Mitochondria*

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The divalent cation ionophore A23187 has been used to examine the steady state kinetics of energy-dependent Ca$^{2+}$ uptake with β-hydroxybutyrate as oxidizable substrate, and these results have been compared to the kinetic parameters obtained with succinate as the available energy source. The kinetics are sigmoidal with the free Ca$^{2+}$ concentration at half-maximal respiratory stimulation ($K_m$) equal to 1.4 μM (β-hydroxybutyrate) versus 3.1 μM (succinate). The respiratory $V_{max}$ is limited by the maximal rate of substrate oxidation which is influenced by the ionic composition of the medium.

With β-hydroxybutyrate, Mg$^{2+}$ is a potent allosteric inhibitor, increasing the $K_m$ for Ca$^{2+}$ to 17.8 μM (25°C) at 2.0 mM added MgCl$_2$. Magnesium inhibition is approximately 2-fold greater at 35°C. When succinate is the oxidizable substrate, the inhibitory effects of Mg$^{2+}$ are similar in magnitude, but the measured values reflect the differences in $K_m$ for Ca$^{2+}$ seen in the absence of added cations.

The respiratory $V_{max}$ is a function of the incubation temperature, but the $K_m$ for Ca$^{2+}$ is affected only when succinate is the energy source. With β-hydroxybutyrate above 20°C, Arrhenius plots of the respiratory $V_{max}$ or uncoupled respiration were linear with an activation energy of 18.7 kcal/mol. The same Arrhenius plots with succinate were not linear, giving activation energies of 8.6 and 13.1 kcal/mol above and below 23°C, respectively. In contrast, ADP-stimulated respiration resulted in linear Arrhenius plots regardless of oxidizable substrate.

The $V_{max}$ (succinate) is enhanced 3-fold over the pH range 6.2 to 7.8 and the $K_m$ for Ca$^{2+}$ progressively increased from 0.5 (pH 6.6) to 3.0 μM (pH 8.2) at 35°C. With β-hydroxybutyrate, the $V_{max}$ was much less affected by pH (40%) and the $K_m$ did not change.

Increasing concentrations of the phosphate transport inhibitor, N-ethylmaleimide, progressively inhibited the succinate-supported respiratory $V_{max}$. Inhibition was partial, specific for N-ethylmaleimide, and the $K_m$ for Ca$^{2+}$ was not affected. The results suggest that N-ethylmaleimide is acting at a site near the Ca$^{2+}$ transport system.

Thus, the steady state kinetic data reflect the properties of the Ca$^{2+}$ transport system plus the available energy source and possibly the number of energy-conserving sites involved in Ca$^{2+}$ transport. The role of Mg$^{2+}$ as a physiological regulator of energy-dependent mitochondrial Ca$^{2+}$ transport is further discussed.

Calcium participates in the basic structural organization of biological systems as well as in the physical and biochemical reactions vital to many cellular processes. The mitochondria, endoplasmic reticulum, and the cell membrane all have an important role in the regulation of cellular free Ca$^{2+}$ concentrations (1, 2). Elucidation of the detailed mechanism(s) of mitochondrial Ca$^{2+}$ transport would be useful in understanding the physiological role played by the mitochondria in the overall regulation of cellular Ca$^{2+}$ fluxes.

The mitochondrial Ca$^{2+}$ pump has been the subject of extensive investigation. Recently, the divalent cation ionophore, A23187, has been used to investigate the kinetics of energy-dependent Ca$^{2+}$ uptake by rat liver mitochondria under steady state conditions (3). At the same time, Heaton and Nicholls (4) used the steady state technique to determine the current of Ca$^{2+}$ flowing across the membrane, the Ca$^{2+}$ electrochemical gradient across the Ca$^{2+}$ carrier, and ultimately the apparent Ca$^{2+}$ conductance. Both steady state kinetics (3, 4) and recent initial velocity data (5) yield $K_m$ values for Ca$^{2+}$ of less than 5 μM for liver mitochondria, near physiological Ca$^{2+}$ concentrations. The higher value of Vinogradov and Scarpa (6) (50 to 60 μM) is probably the result of the presence of inhibitory cations (3).

The kinetic data are cooperative and Ca$^{2+}$ transport is thought to be mediated by a carrier localized in the mitochondrial inner membrane (2, 7, 8). Specific models have been proposed in which at least two Ca$^{2+}$ ions must be bound to the carrier in order for Ca$^{2+}$ to be transported at significant rates (5, 6).

A23187 catalyzes an electroneutral exchange of a divalent cation for 2 protons. Addition of a high concentration of A23187 results in release of mitochondrial Ca$^{2+}$ (and Mg$^{2+}$) and a stimulated respiratory rate that is dependent on the cyclic flux of Ca$^{2+}$ ions (9, 10) and is a function of the free Ca$^{2+}$ concentration (3, 4). Mitochondrial Ca$^{2+}$ accumulation is a respiration-linked process and is stoichiometric with electron transport (7, 8, 11, 12). Two Ca$^{2+}$ ions are accumulated per pair of electrons per energy site and recently, Brand et al. (12) have shown that 2 protons are ejected for each Ca$^{2+}$ accumulated. It is generally accepted that Ca$^{2+}$ uptake is a consequence of an electrochemical transport of the ion into the matrix in response to a membrane potential (13).
EXPERIMENTAL PROCEDURES

Isolation of Mitochondria—Rat liver mitochondria were prepared from male Sprague-Dawley rats (225 to 250 g) by the method of Johnson and Lardy (14). The homogenizing medium contained 0.22 M mannitol, 0.97 M sucrose, 0.1 M EGTA, 0.5% bovine serum albumin (low fatty acid type), adjusted to pH 7.4, with trisethanolamine at 4°C (3). The livers were perfused with 20 ml of homogenizing medium (minus bovine serum albumin) before homogenization. Protein was determined by the biuret reaction in the presence of 1% sodium deoxycholate, using crystalline bovine serum albumin standards.

Measurement of Respiratory Rates—Respiration was measured with a Clark type oxygen electrode (Yellow Springs Instrument Co.) fitted to the Gilson oxygraph (Gilson Medical Electronics, Middleton, WI). Reported values of Chappell (15) were used to calculate dissolved oxygen at the various temperatures used. The standard incubation medium contained 200 mM mannitol, 63 mM sucrose, 10 mM succinate/triethanolamine, pH 7.4, at 25°C, 1.25 µM rotenone, and 10 mg of mitochondrial protein in a total volume of 4.0 ml. When D-β-hydroxybutyrate (20 mM) was the respiratory substrate, rotenone was omitted from the medium. Commercial preparations of sodium asc-β-hydroxybutyrate were routinely converted to the free acid form using a Dowex 50-H⁺ column. The substrate solution was brought to pH 7.4 with trisethanolamine. When other salts were added to the standard medium, the osmolarity was kept constant by reducing the concentration of mannitol and sucrose.

Determination of Ca²⁺ Concentrations — The free Ca²⁺ concentration in the incubation medium was measured on samples pipetted directly from the oxygraph, using the indicating dye murexide (16) as described by Huisson et al. (3). The sensitivity of murexide to standards Ca²⁺ additions varied with the inorganic component, pH, and temperature of the medium. Calcium standard curves were run on supernatant fractions from untreated control mitochondrial suspensions in each of the various media. The cuvette chamber was maintained at constant temperature. The technique was suitable for determining Ca²⁺ concentrations as low as 0.3 to 1 µM.

Analysis of mitochondrial Ca²⁺, Mg²⁺, and K⁺ was done by atomic absorption spectrophotometry as described by Huisson et al. (3). In all experiments, the total Ca²⁺ concentration was in the range of 84 to 90 µM.

Variation of pH and Temperature—When the pH of the incubation medium was varied, 20 mM Mes (pH 6.2 and 6.6) or 20 mM Heps (pH 7.0) buffer was included in the medium. Above pH 7.4, triethanolamine was used. In all cases, the pH was adjusted with triethanolamine.

Triethanolamine, the buffer used in the standard incubation, was sensitive to variations in temperature. Consequently, for each of the temperatures studied, the incubation medium was adjusted to pH 7.4 at that temperature.

Analysis of Kinetic Data—Analysis of data obtained from measuring respiratory activation as a function of free Ca²⁺ concentration, definition of Vₘₐₓ, and Kₘₐₓ was as described (3).

Materials

CaCl₂ was obtained from Ventron Alfa Products and Murexide from Eastman. N-ethylmaleimide, mersalyl acid, and NBS were obtained from Sigma. A23187 was a gift from Dr. Robert Hamill of Eli Lilly Co. All other reagents were obtained from commercial sources and were at least reagent grade.

RESULTS

In a previous publication (3), we reported on the steady state kinetics of energy-dependent Ca²⁺ transport. With succinate as the respiratory substrate in the standard incubation medium, the Vₘₐₓ, ranged from 115 to 135 ng atoms of oxygen/mg of protein/min, and the Kₘₐₓ for free Ca²⁺ was 3.1 ± 0.4 µM (25°C). Magnesium was a potent "competitive-like" inhibitor. Addition of 0.94 and 2.0 mM MgCl₂ raised the Kₘₐₓ for Ca²⁺ to 14.5 and 30.0 µM, respectively (see Figs. 1 and 3 and Ref. 3). To determine whether these kinetic parameters were substrate- and/or site-specific, steady state Ca²⁺ kinetic data have been examined using a DPNH-linked substrate. β-Hydroxybutyrate was chosen because, unlike glutamate/malate or pyruvate, its oxidation is not affected by Mg²⁺ loss (9, 17). In the kinetic experiments, A23187 addition results in release of approximately 10 nmol of Mg²⁺/mg of mitochondrial protein (3).

The effect of varying concentrations of Mg²⁺ on the β-hydroxybutyrate-supported, Ca²⁺-stimulated respiratory activation is shown in Fig. 1. Without added Mg²⁺, the curve is sigmoidal with half-maximal respiratory activation occurring at a free Ca²⁺ concentration of 250 µM. When MgCl₂ was included at 1.0 and 2.0 mM, the curve was shifted to the left, indicating that Mg²⁺ stimulated Ca²⁺-stimulated respiratory activation.

Fig. 1. The effect of free Ca²⁺ concentration on the rate of β-hydroxybutyrate oxidation stimulated by A23187 in the absence and presence of Mg²⁺. Mitochondria incubated in the standard medium (25°C) with 20 mM β-hydroxybutyrate were allowed to accumulate 30 nmol of Ca²⁺/mg of protein. Ca²⁺ accumulation was completed in less than 1 min. After addition of varying amounts of EGTA, A23187 was added to 2.0 nmol/mg of protein and the steady state rate of oxygen consumption was recorded (●). When MgCl₂ was included at 1.0 (□) and 2.0 mM (□), A23187 was 5 and 6 nmol/mg of protein, respectively. Free Ca²⁺ concentrations were measured (as described under "Experimental Procedures") on samples taken directly from the oxygraph. The numbers in parentheses represent the respiratory Vₘₐₓ.
at 1.2 μM free Ca\(^{2+}\) concentration (25). The average \(K_{s}\) for Ca\(^{2+}\) from several experiments was 1.4 μM, and the \(V_{\text{max}}\) ranged from 37 to 51 ng atoms of oxygen/mg/min. Malmström and Carafoli (18) have reported that in rat heart mitochondria oxidizing \(\beta\)-hydroxybutyrate, when endogenous Ca\(^{2+}\) was removed the mitochondrial respiration failed to respond to uncoupler addition. However, using liver mitochondria, neither prior incubation with excess EGTA nor EGTA plus A23187 prevented uncoupling by FCCP.

As shown in Fig 1, Mg\(^{2+}\) dramatically increased the \(K_{m}\) for Ca\(^{2+}\) to 9.5 and 17.8 μM at 1.0 and 2.0 mM added MgCl\(_2\), respectively. While the magnitude of the increase in \(K_{m}\) was similar for both succinate and \(\beta\)-hydroxybutyrate (approximately 10-fold at 2.0 mM MgCl\(_2\)), the measured values reflected the difference in \(K_{m}\) seen in the absence of added cations. The data suggest but do not prove that the apparent affinity of the mitochondrial transport system for Ca\(^{2+}\) may be greater when \(\beta\)-hydroxybutyrate is the energy source.

When succinate was the energy source, Mg\(^{2+}\) had no effect on the Hill coefficient, however, the monovalent cation antagonism of Mg\(^{2+}\) inhibition indicated that Mg\(^{2+}\) was binding to a site distinct from that which binds Ca\(^{2+}\) (3). With \(\beta\)-hydroxybutyrate as oxidizable substrate, Mg\(^{2+}\) increased the Hill coefficient supporting that conclusion. In agreement with previous data with succinate as the substrate (3), phosphate did not influence the steady state kinetics (data not shown).

The Ca\(^{2+}\)-stimulated respiratory \(V_{\text{max}}\) is inextricably linked to the maximal rate of electron transport (3); the rate-limiting step is probably the rate of proton translocation by the respiratory chain (4). As with succinate, the \(\beta\)-hydroxybutyrate-supported, A23187-stimulated \(V_{\text{max}}\) was influenced by the ionic composition of the medium. State 3 respiration (ADP present), which is presumably not electron transport rate-limited, was contrasted to the rates in the presence of FCCP or A23187 which presumably are. Both uncoupled and Ca\(^{2+}\)-stimulated respiration are enhanced similarly by KCl and phosphate, while MgCl\(_2\) has no effect on uncoupled respiration. Unlike succinate-supported respiration (3), oxidation of \(\beta\)-hydroxybutyrate was noticeably inhibited by KCl when FCCP or A23187 was present. All of the changes are small, 20% or less. The differences in \(V_{\text{max}}\) (Fig 1) reflect effects of MgCl\(_2\) on the maximal rate of \(\beta\)-hydroxybutyrate oxidation as well as variability associated with different mitochondrial preparations. The ionic composition did not affect State 3 respiration, and this is also true for succinate-supported State 3 (3).

Effects of Temperature and Medium Composition on \(V_{\text{max}}\) — Heaton and Nichols (4) have shown that the apparent calcium conductance of the mitochondrial inner membrane increases 4-fold over the temperature range 23–38°. The effect of temperature on the succinate or \(\beta\)-hydroxybutyrate-supported, Ca\(^{2+}\)-stimulated \(V_{\text{max}}\), uncoupled respiration, and ADP-stimulated respiration are shown in Fig 2. With succinate, it was possible to use a large temperature range (10–40°), but with \(\beta\)-hydroxybutyrate as substrate, respiration stimulated by FCCP and A23187 was nonlinear below 20°. Even at 20°, respiration decreased somewhat with time. This was also true when glutamate/malate was the respiratory substrate. When glutamate/malate was used, the Ca\(^{2+}\)-stimulated \(V_{\text{max}}\) was estimated from the initial rate. With glutamate/malate, the medium was supplemented with 30 mM KCl to ensure maximal linear respiratory rates (19).

FCCP-stimulated or Ca\(^{2+}\)-stimulated respiration resulted in identical Arrhenius plots, but the slope depended on the particular substrate (Fig 2, A and B). With succinate, the Arrhenius plot exhibited a break at approximately 22–23° with activation energies of 8.6 ± 0.8 [3] and 13.1 [2] kcal/mol. With \(\beta\)-hydroxybutyrate there was a 7- to 8-fold increase in the maximal respiratory rate which resulted in a linear Arrhenius plot and an average activation energy of 18.7 ± 1.9 [5] kcal/mol. Since there was only one point below 25°, it was not possible to determine whether there was a break at 22–23° as with succinate. Similar results were obtained with glutamate/malate (18.8 kcal/mol), but there was more scatter in the data and respiration reached a plateau at 40° to this value remains tentative. At 35°, the maximal respiratory rate (\(V_{\text{max}}\)) with glutamate/malate was only slightly less than with succinate. In contrast to FCCP- or Ca\(^{2+}\)-stimulated respiration, ADP-stimulated respiration resulted in a linear Arrhenius plot regardless of substrate, with an average Arrhenius activation energy of 12.9 ± 0.8 [6] kcal/mol (Fig 2). An Arrhenius plot of State 4 respiration was also linear; the activation energies were 13.5 (succinate) and 15 kcal (\(\beta\)-hydroxybutyrate). These data support the hypothesis that the activity of the respiratory chain or the rate of substrate oxidation is rate-limiting for the Ca\(^{2+}\)-stimulated \(V_{\text{max}}\). Furthermore, the \(V_{\text{max}}\) reflects the nature of the respiratory substrate. With State 3 respiration, although the respiratory rates varied among the three substrates, the activation energy was constant.

The ionic composition of the medium has an influence on the Ca\(^{2+}\)-stimulated \(V_{\text{max}}\) (3). Since the various cations and anions affected the maximal rate of electron transport at 25°, their effects were also examined at 35°. The results for FCCP-stimulated, Ca\(^{2+}\)-stimulated, and ADP-stimulated respiration are compared in Table I. There was an increased sensitivity to changes in medium composition at 35°. State 3 respiration, which is presumably not electron transport limited, was unaffected by Mg\(^{2+}\) and K\(^{+}\) at 25° (3), but these ions enhanced respiration at 35°. With one or two exceptions, FCCP-stimulated respiration and Ca\(^{2+}\)-stimulated respiration showed parallel responses. This suggests that the ionic composition of the medium is largely influencing the rate of substrate oxidation and/or uptake. However, each substrate responded differently to the various ions. Mg\(^{2+}\) was generally stimulatory, but the effects of K\(^{+}\) and P\(^{3-}\) were variable. Potassium usually enhanced respiration at 35°, but had no effect or was inhibitory at 25° (3).


TABLE I

Effects of cations and anions on maximal respiratory rates at 35°C

Mitochondria were incubated in the standard incubation medium with 20 mM dl-β-hydroxybutyrate or 10 mM succinate. Salts were added at the following concentrations: 2.0 mM MgCl₂, 100 mM KCl, and 1.0 mM phosphate (pH 7.4, triethanolamine). FCCP was added at 0.42 μM and A23187 was 2.0 nmol/mg of protein; however, when Mg²⁺ was present, it was increased to 8 nmol/mg of protein. In experiments with A23187, mitochondria were allowed to accumulate 30 nmol of Ca²⁺/mg of protein before ionophore addition as in Fig. 1. State 3 respiration was measured after addition of 0.05 mM ADP (pH 7.4, triethanolamine).

<table>
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<th>Additions</th>
<th>Succinate</th>
<th>β-Hydroxybutyrate</th>
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<tbody>
<tr>
<td>FCCP</td>
<td>A23187</td>
<td>FCCP</td>
</tr>
<tr>
<td>None</td>
<td>266</td>
<td>224</td>
</tr>
<tr>
<td>[MgCl₂]</td>
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<tr>
<td>[KCl]</td>
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<tr>
<td>[P]</td>
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<td>216</td>
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<tr>
<td>[KCl, P]</td>
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<td>266</td>
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<tr>
<td>[MgCl₂, KCl]</td>
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<td>214</td>
</tr>
<tr>
<td>[MgCl₂, P]</td>
<td>293</td>
<td>261</td>
</tr>
<tr>
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<td>293</td>
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<tr>
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<th>FCCP</th>
<th>ADP</th>
</tr>
</thead>
<tbody>
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<td>116</td>
</tr>
<tr>
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<td>138</td>
</tr>
<tr>
<td>KCl</td>
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<td>149</td>
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<tr>
<td>MgCl₂, KCl, P</td>
<td>172</td>
<td>134</td>
</tr>
</tbody>
</table>

Influence of Temperature on K₉₅ and Mg²⁺ Inhibition—When β-hydroxybutyrate was the oxidizable substrate, there was apparently no effect of temperature on the K₉₅ for Ca²⁺; K₉₅ = 1.4 and 1.7 μM free [Ca²⁺] at 25°C and 35°C, respectively. With succinate there was a decrease in K₉₅ for Ca²⁺ (3.1 to 1.9 μM) when the temperature was increased from 25 to 35°C. However, at 15°C the results were quite variable (2.1 ± 1.4 μM), so it is not clear whether there was any significant pattern. However, the identity of the available energy source does not appear to result in any significant difference in K₉₅ for Ca²⁺ at 35°C.

The fact that Mg²⁺ is a very potent inhibitor of steady state Ca²⁺ cycling at 35°C (near the physiological temperature range) is illustrated in Fig. 3, A and B. The inhibitory effects of Mg²⁺ were approximately 2-fold greater than at 25°C (see Fig. 1 and Ref. 3). Addition of 1.0 and 2.0 mM MgCl₂ to the standard medium with succinate raised the K₉₅ for Ca²⁺ from 1.9 to 22.5 and 38 μM, respectively (Fig. 3A). With β-hydroxybutyrate the K₉₅ was increased from 1.7 to 16.5 and 34 μM (Fig. 3B). This corresponds to about a 10- and 20-fold change in K₉₅. In the absence of added MgCl₂, the K₉₅ for Ca²⁺ is the same with succinate or β-hydroxybutyrate as substrate, but with added MgCl₂ there is a small but consistent difference. MgCl₂ increased the Hill coefficient, supporting the hypothesis that Mg²⁺ is probably binding to an allosteric site.

The alkali metal cations are weak inhibitors of steady state Ca²⁺ accumulation and antagonists of Mg²⁺ inhibition (9). The effect of K⁺ (100 mM) on Mg²⁺ (2.0 mM) inhibition at 25°C is shown in Fig. 4, A and B. Potassium reduced the K₉₅ for Ca²⁺ to 22 (succinate, Fig. 4A) and 17 μM (β-hydroxybutyrate, Fig. 4B), essentially the same K₉₅ seen in the presence of 1.0 mM MgCl₂. Although K⁺ is a slightly better antagonist at 35°C than 25°C, the potency of magnesium increases much more dramatically. Potassium did not change the Hill coefficient value for Ca²⁺ seen in the presence of Mg²⁺.

Dependence of Kinetic Parameters on pH of Medium—The pH of the incubation medium has a marked influence on the Ca²⁺ conductance of the mitochondrial membrane and on the Ca²⁺ electrochemical gradient required to drive the current (4). There have also been reports on the effects of pH on substrate oxidation (20) and the kinetics of substrate anion uptake (21). Table II illustrates the effect of medium pH on the K₉₅ for Ca²⁺ at 35°C. With β-hydroxybutyrate as respiratory substrate, there was essentially no change in the K₉₅ for Ca²⁺ over the pH range 6.6 to 8.2. In contrast, with succinate, the K₉₅ steadily increased over this pH range. This was also true at 25°C where the K₉₅ for Ca²⁺ was determined at pH 6.6 and 7.4; the corresponding values were 1.4 ± 2.1 and 3.1 ± 0.4 μM, respectively.

In Fig. 5, the influence of medium pH on Ca²⁺-stimulated respiratory activation at 35°C. Conditions were the same as in Fig. 1, except the medium was adjusted to pH 7.4 at 35°C. The MgCl₂ concentrations were 0 (●), 1.0 mM (○), and 2.0 mM (□), and A23187 was added at 2.0, 0.2, and 0 nmol/mg of protein, respectively. The oxidizable substrate was succinate (A) or β-hydroxybutyrate (B). The numbers in parentheses represent the respiratory V₉₅.

Fig. 3. The effects of varying concentrations of Mg²⁺ on the Ca²⁺-stimulated respiratory activation at 35°C. Conditions were the same as in Fig. 1, except the medium was adjusted to pH 7.4 at 35°C. The MgCl₂ concentrations were 0 (●), 1.0 mM (○), and 2.0 mM (□), and A23187 was added at 2.0, 0.2, and 0 nmol/mg of protein, respectively. The oxidizable substrate was succinate (A) or β-hydroxybutyrate (B). The numbers in parentheses represent the respiratory V₉₅.

Fig. 4. The effect of K⁺ on Mg²⁺ inhibition of Ca²⁺-stimulated respiratory activation at 35°C. Conditions were the same as in Fig. 1, except the medium was adjusted to pH 7.4 at 35°C. MgCl₂ was added at 2.0 mM (●) and at 2.0 mM together with 100 mM KCl (○). A23187 was 8.0 nmol/mg of protein. The oxidizable substrate was succinate (A) and β-hydroxybutyrate (B).
Table II

Effect of pH on \( K_{\text{m}} \) for Ca\(^{2+} \) at 35°

Experimental procedure was essentially the same as in Fig. 1, and the media are described under "Methods." The \( K_{\text{m}} \) for Ca\(^{2+} \) was determined as described (3).

<table>
<thead>
<tr>
<th>pH</th>
<th>Respiratory substrate</th>
<th>( K_{\text{m}} ) μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.6</td>
<td>Succinate</td>
<td>0.5</td>
</tr>
<tr>
<td>7.0</td>
<td>Succinate</td>
<td>0.95</td>
</tr>
<tr>
<td>7.4</td>
<td>Succinate</td>
<td>1.9</td>
</tr>
<tr>
<td>7.8</td>
<td>Succinate</td>
<td>2.6</td>
</tr>
<tr>
<td>8.2</td>
<td>Succinate</td>
<td>3.0</td>
</tr>
<tr>
<td>7.0</td>
<td>( \beta )-Hydroxybutyrate</td>
<td>0.95</td>
</tr>
<tr>
<td>7.4</td>
<td>( \beta )-Hydroxybutyrate</td>
<td>1.7</td>
</tr>
<tr>
<td>7.8</td>
<td>( \beta )-Hydroxybutyrate</td>
<td>1.1</td>
</tr>
<tr>
<td>8.2</td>
<td>( \beta )-Hydroxybutyrate</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\( V_{\text{max}}/K_{\text{m}} \) versus pH fell off slightly at higher pH, but the changes were small and inconclusive. Interpretation of the significance of these plots is limited because of the complexity of the system.

It is clear that the respiratory \( V_{\text{max}} \) is much less affected by medium pH when \( \beta \)-hydroxybutyrate is the available substrate. Plots of log \( V_{\text{max}} \) or log \( V_{\text{max}}/K_{\text{m}} \) versus pH (\( \beta \)-hydroxybutyrate) exhibited essentially no change over the entire pH range. With either substrate, ADP-stimulated respiration was maximal around pH 7.0, and the P:O ratio was constant between pH 6.2 and 8.2.

Phosphate Transport Inhibitors—The permeant anions, phosphate and acetate, which can enter the matrix compartment of respiring mitochondria as counterions with Ca\(^{2+} \), do not have any significant effect on the steady state Ca\(^{2+} \) kinetics (3). Both anions have been reported to increase the initial velocity and extent of energy-dependent Ca\(^{2+} \) accumulation (5, 11). The transport of phosphate is inhibited by sulphydryl reagents (23-25). It seemed logical to assume that under the limited loading conditions used in the steady state experiments, phosphate transport inhibitors would have no effect on the kinetics. However, as shown in Fig. 6A, increasing concentrations of N-ethylmaleimide progressively inhibited the respiratory \( V_{\text{max}} \) (succinate). Inhibition saturated at 50 to 80 nmol of N-ethylmaleimide/mg of protein, and maximal inhibition ranged from 35 to 60% in various mitochondrial preparations.

As Fig. 6A suggests, N-ethylmaleimide did not change the \( K_{\text{m}} \) for Ca\(^{2+} \), which was 2.8 μM (25°) in the presence of 75 nmol of N-ethylmaleimide/mg of protein. This concentration was sufficient to inhibit the respiratory jump initiated by ADP. Curiously, inhibition was eliminated by low concentrations of phosphate added immediately before N-ethylmaleimide (see Fig. 6B). Prior addition of 1.0 mM phosphate only partially reversed N-ethylmaleimide inhibition of State 3, and relief was only temporary.

In order to determine whether the effects of N-ethylmaleimide were a general property of sulphydryl reagents, experiments were conducted using the phosphate transport inhibitors NBS, and mersalyl. NBS, (45 nmol/mg of protein) had no effect on Ca\(^{2+} \)-stimulated respiration, but completely inhibited State 3. Similar results were obtained with \( \beta \)-hydroxybutyrate as oxidizable substrate over a concentration range of NBS, from 18 to 45 nmol/mg of protein. Mersalyl (18 nmol/mg) inhibited succinate oxidation, had no effect on the oxidation of \( \beta \)-hydroxybutyrate stimulated by A23187 or FCCP, and inhibited State 3. To eliminate the possibility that N-ethylmaleimide was merely inhibiting succinate oxidation, the effects of the various inhibitors on uncoupled respiration were examined. Oxidation of \( \beta \)-hydroxybutyrate in the presence of the uncoupler, FCCP, was unaffected by NBS, or mersalyl. Similarly, the uncoupled oxidation of succinate was not inhibited except at high N-ethylmaleimide concentrations. For example, in one experiment, A23187-stimulated respiration (succinate) was inhibited 52% by 90 nmol of N-ethylmaleimide/mg of protein, whereas FCCP- and DNP-stimulated respiration were inhibited 9% and 7%, respectively. At 80 nmol of N-ethylmaleimide/mg of protein, inhibition of Ca\(^{2+} \)-stimulated respiration was the same, while FCCP and DNP were affected 15% and 10%.

N-ethylmaleimide binds to many mitochondrial proteins (26) besides inhibiting the phosphate carrier. N-ethylmaleimide is also able to perturb ADP translocation, the reaction is time-dependent, and traces of ADP strongly potentiate the inhibitory effect of N-ethylmaleimide. Mersalyl is not inhibitory and FCCP prevents N-ethylmaleimide inhibition (for review, see Ref. 27). The conformational state of the mitochondria...
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N-ethylmaleimide is fairly permeable to the inner mitochondrial membrane (28), and it is very possible that N-ethylmaleimide is reacting with a protein(s) on the matrix side of the membrane. The partial nature of the inhibition and the lack of effect on \(K_m\) for Ca\(^{2+}\) suggest that N-ethylmaleimide is acting at a site near the Ca\(^{2+}\) transport system. The protection by phosphate might suggest that the Ca\(^{2+}\) and phosphate transport carriers and/or ADP translocator are closely associated but this remains speculative. Alternatively, N-ethylmaleimide could be interfering with energy transfer from the respiratory chain to the Ca\(^{2+}\) transport system.

**DISCUSSION**

The steady state kinetic approach to studying energy-linked mitochondrial Ca\(^{2+}\) transport has focused attention on the driving force for Ca\(^{2+}\) uptake and the limitations of treating the Ca\(^{2+}\) transport system like an enzyme system. Our work (3) and that of Heaton and Nicholls (4) have emphasized that the rate-limiting step is probably at the level of the respiratory chain rather than at the calcium carrier. Heaton and Nicholls (4) have suggested that the rate of proton translocation by the respiratory chain is actually rate-limiting. The data presented here illustrate that the nature of the respiratory substrate is important, and that the substrate dehydrogenases or the associated enzymes of the respiratory chain could actually be limiting the \(V_{max}\).

Originally, we had thought that the Arrhenius plots would provide information on the mechanism of Ca\(^{2+}\) transport. Two groups have proposed models for Ca\(^{2+}\) transport based on transport of two Ca\(^{2+}\) ions by a diffusible carrier (5, 6); however, a "gated pore" mechanism that does not involve shuffling protein through a membrane can be kinetically indistinguishable from a mobile carrier (29). Presumably, this model might require a lower activation energy than the mobile carrier model, depending on the rate-limiting step (30), or might be expected to be less affected by phase changes in the membrane lipids. It appears, however, that the temperature profiles probably reflect kinetic properties of the enzyme(s) associated with oxidation of the energy sources – β-hydroxybutyrate or succinate. This would suggest that the activation energy for Ca\(^{2+}\) transport is actually less than the observed values, and a pore mechanism cannot be ruled out based on the available data.

The temperature profiles of the respiratory \(V_{max}\) were very substrate-specific, and uncoupled respiration generally paralleled the results with A23187. The temperature profile for the β-hydroxybutyrate-supported \(V_{max}\) indicated a significantly greater activation energy (18.7 kca/mol) than with succinate (8.6 kca/mol above 22-23°C). The break in the Arrhenius plot in the vicinity of 22-23°C seen with succinate agrees with earlier observations (31, 32), although one could argue the data may fit a single curved line. The break in the Arrhenius plot at 22-23°C has been attributed to a phase change in the mitochondrial lipids (32, 33).

In contrast to the results of Lyons and Raison (31) and Raison (32), there was no break in the Arrhenius plot of ADP-stimulated respiration, and the activation energy was constant regardless of substrate (12.9 kca/mol). The exact reason for this discrepancy is not known and it is difficult to compare the data because our protein concentrations, media composition, and substrate concentrations were different from those employed by Raison (31, 32). During State 3, substrate oxida-

tion is probably not rate-limiting (Table II; Ref. 3). The transi-
tion point for adenine nucleotide translocation is at 8°C; above 8°C, the Arrhenius activation energy has been reported as 19 and 21 kca/mol (34, 35). Phosphate transport at 0°C is 20 times faster than ADP translocation (26), suggesting that ADP translocation may be rate-limiting here. Our ADP concentrations greatly exceeded the \(K_m\) for the translocase (27). However, the \(K_m\) for phosphate transport during oxidative phosphorylation is not known.

The kinetic data of the dicarboxylic acid carrier in non-syn-
tion of intracellular Ca\(^{2+}\) and the presence of Mg\(^{2+}\) probably minimize competition between calcium transport and ATP synthesis. State 4 respiration can be used to support Ca\(^{2+}\) uptake (10, 46, 47) and is not insignificant at 37\(^\circ\); so raising the \(K_m\) for Ca\(^{2+}\) merely modifies the rate of respiratory activation or rate of Ca\(^{2+}\) uptake. What still remains largely unknown is the mechanism(s) that control Ca\(^{2+}\) uptake and release in situ.

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