Calcium Stimulates ATP-Mg/Pi Carrier Activity in Rat Liver Mitochondria*

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Adenine nucleotide transport over the carboxyatractylodside-insensitive ATP-Mg/Pi carrier was assayed in isolated rat liver mitochondria with the aim of investigating a possible regulatory role for Ca2+ on carrier activity. Net changes in the matrix adenine nucleotide content (ATP + ADP + AMP) occur when ATP-Mg exchanges for P, over this carrier. The rates of net accumulation and net loss of adenine nucleotides were inhibited when free Ca2+ was chelated with EGTA and stimulated when buffered [Ca2+]free, was increased from 1.0 to 4.0 μM. The unidirectional components of net change were similarly dependent on Ca2+; ATP influx and efflux were inhibited by EGTA in a concentration-dependent manner and stimulated by buffered free Ca2+ in the range 0.6-2.0 μM. For ATP influx, increasing the medium [Ca2+]free from 1.0 to 2.0 μM lowered the apparent Km for ATP from 4.44 to 2.44 μM with no effect on the apparent Vmax (3.55 and 3.76 nmol/min/mg with 1.0 and 2.0 μM [Ca2+]free, respectively). Stimulation of influx and efflux by [Ca2+]free was unaffected by either ruthenium red or the Ca2+ ionophore A23187. Calmodulin antagonists inhibited transport activity. In isolated hepatocytes, glucagon or vasopressin promoted an increased mitochondrial adenine nucleotide content. The effect of both hormones was blocked by EGTA, and for vasopressin, the effect was blocked also by neomycin. The results suggest that the increase in mitochondrial adenine nucleotide content that follows hormonal stimulation of hepatocytes is mediated by an increase in cytosolic [Ca2+]free that activates the ATP-Mg/Pi carrier.

In liver, the mitochondrial adenine nucleotide (ATP + ADP + AMP) content changes in a number of specific metabolic states (reviewed in Ref. 1). Dramatic increases occur at parturition in several species (2,8) and upon stimulation by glucagon (9-13). Decreases occur during hypoxic episodes (14) and hibernation (15). It has been suggested that the ATP-Mg/Pi carrier of the mitochondrial inner membrane is responsible for these net changes in adenine nucleotide content because adenine nucleotide transport over this carrier can occur as a counterexchange with P, resulting in a net increase or a net decrease in the matrix adenine nucleotide pool size (1,16).

The ATP-Mg/Pi carrier activity that facilitates net accumulation and net loss of mitochondrial adenine nucleotides in vitro has been characterized in detail (1,16). The direction and magnitude of net transport are determined primarily by the ATP-Mg concentration gradient across the inner membrane and its deviation from equilibrium with the P, concentration gradient (1). A recent report has indicated that in isolated mitochondria, net changes in adenine nucleotide content are stimulated by calcium (20). This calcium effect has important implications for how transport might be regulated in vivo. Direct activation or inhibition of ATP-Mg/Pi transport activity by changes in the cytosolic free Ca2+ concentration ([Ca2+]free) may be part of the mechanism by which physiologic stimuli such as glucagon cause adenine nucleotide recompartmentation between the cytosol and mitochondria in liver.

In this study we examined in greater detail the effects of [Ca2+]free on ATP-Mg/Pi carrier activity in isolated mitochondria. The rate of net change in adenine nucleotide content is equal to the difference between ATP influx and ATP efflux (16). Therefore, in addition to effects of [Ca2+]free on net changes, effects on unidirectional ATP movements over the ATP-Mg/Pi carrier were examined. Ca2+ was found to stimulate carrier activity in a concentration range that is consistent with increases in cytosolic [Ca2+]free, reported for hormonal stimulation of hepatocytes. To our knowledge, this is the first anion carrier in mitochondria that has been shown to be regulated directly by calcium.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rats were obtained from Charles River Laboratories (Wilmington, MA). [3H]Adenine nucleotides and Aquasol were purchased from Du Pont-New England Nuclear, Fura-2 and A23187 from Calbiochem, collagenase, class II, from Worthington, and the remaining materials from Sigma or Fisher. Water was purified by deionization and contained less than 0.5 μM Ca2+.

**Mitochondrial Isolation**—Mitochondria were isolated from adult male Sprague-Dawley rats as described previously (18). The isolation medium was 250 mM sucrose, 1 mM Tris-HCl, 0.1 mM EDTA, pH 7.4, with EDTA omitted from the final wash and resuspension. Protein determinations were by the Lowry et al. procedure (21).

1 The abbreviations and trivial names used are: Fura-2, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N',N',N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenediaminetetraacetic acid; GM1, 1-[2,4-bis(carboxymethyl)-aminol]-5-methylphenoxy]-methyl]-6-methoxy-8-bis-(carboxymethyl)-aminominoquinoline; W-7, N-(6-aminoethyl)-5-chloro-1-naphthylisoulefoanilide hydrochloride; calmodizolium, 1-[bis(4-chlorophenyl)-methyl]-3-[2-(2,4-dichlorophenyl)-2-[2,4-dichlorophenyl]-methoxy-ethyl]-1H-imidazolium chloride; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; Tes, N-(2-hydroxyethyl)methyl-2-aminoethanesulfonic acid; Tricine, N-tris(hydroxymethyl)-methylglycine.

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Respiration was measured polarographically with glutamate plus malate as substrates, essentially as described previously (4).

Incubation Conditions—Incubations of mitochondria were carried out at 30 °C in medium containing 225 mM sucrose, 2 mM KH2PO4/KH2PO4, 5 mM MgCl2, 10 mM KCl, 10 mM Tris-HCl, 5 mM glutamate, 5 mM malate; pH was adjusted to 7.4 with NaOH. Mitochondria were added so that the final concentration was approximately 1 mg of protein/ml; ATP was present at concentrations indicated below and controlled using Ca-EGTA buffers. The incubation medium was the same as used for ATP efflux experiments, except that 5 mM EGTA was included along with a variable amount of CaCl2. The total [Ca2+]i needed to produce the desired [Ca2+]i was calculated using the stability constants for Ca-EGTA, Mg-EGTA, Calgrenin, and ATP-EGTA (28). 

Fifteen seconds after the addition of mitochondria, ATP (1.0 mM) on ice for 40 min with 4 nCi of carrier-free (14C) ADP/mg protein of the initial-enzyme assay were performed using a Packard Tricarb liquid scintillation counter. Only those samples that were statistically significant are shown. The specific activity (cpm/nmol) of ATP, ADP, and AMP were measured at the indicated times.

[Ca2+]i was determined in a sample (1 mg of mitochondrial protein) of the initial suspension and dividing this value by the actual ATP + ADP + AMP content determined enzymatically. The initial rates of flux were linear over the time interval measured (usually 90 s). In this study, influx and efflux were both expressed as positive numbers; units are nmol/min/mg mitochondrial protein.

Hepatocyte Isolation, Incubation, and Digitonin Fractionation—Hepatocytes were isolated by a collagenase perfusion method. Livers from anesthetized rats were initially perfused via the portal vein with buffer A (137 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 0.34 mM Na2HPO4, 0.4 mM KH2PO4, 25 mM NaHCO3, 12.5 mM Heps, 60 μM EGTA, 2% bovine serum albumin, pH 7.2). After livers were removed, Cortex was isolated (27). Mitochondria were resuspended in buffer B (125 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 0.34 mM Na2HPO4, 0.4 mM KH2PO4, 25 mM NaHCO3, 12.5 mM Heps, 4 mM CaCl2, 67 mg/ml collagenase, pH 7.2). Buffers A and B were gassed with 95% O2/5% CO2 for 30 min and pH was adjusted just prior to use. Cells were washed twice with buffer C (68 mM NaCl, 5.4 mM KCl, 0.6 mM MgCl2, 2.5 mM CaCl2, 0.86 mM Hepes, 3 mM NaN3, 0.7 mM Na2SO4, 32.7 mM Heps, 30 mM Tris, 36.3 mM Tricine, pH 7.2). Hepatocytes were resuspended in buffer D (142 mM NaCl, 6.7 mM KCl, 25 mM NaHCO3, 2.5 mM CaCl2, 4 mg/ml bovine serum albumin, pH 7.2) and then spun through a Percoll cushion essentially as described elsewhere (27). Cells were then washed and resuspended in buffer D to a final density of 107 cells/ml. Cell viability (as measured by trypan blue exclusion) was always greater than 90%.

Hepatocytes were preincubated in buffer D for 20 min at 37 °C under air in a Buchi rotavapor flask. Five-milliliter aliquots of this hepatocyte suspension were transferred to clean flasks for incubation with hormones. For the experiment involving EGTA, 3.1 mM EGTA was added to the incubation medium. Labeled adenine nucleotides in the mitochondria were determined as for ATP influx, and the specific activity (cpm/nmol) of ATP, ADP, and AMP were measured at the indicated times.

Effects of Extramitochondrial Ca2+ on Net Accumulation and Net Loss of Adenine Nucleotides—Measurements of [Ca2+]i in incubation medium to which no CaCl2 had been added were made using Fura-2. There was 3.28 ± 0.07 μM [Ca2+]i, which was present almost entirely as a contaminant of the MgCl2 reagent. It is important to note that this amount of external Ca2+ was nominally present in all of our previous work.

Lowering external [Ca2+]i, by addition of EGTA affected net accumulation and net loss of adenine nucleotides as shown in Fig. 1. Net changes in the matrix adenine nucleotide content during a 15-min incubation with various external [ATP] occurred as usual in the presence of 1 mM EGTA. Adenine nucleotide accumulation was observed with 4.0 and 2.0 mM ATP, whereas net loss was observed with 0.05 mM ATP. This concentration of EGTA (1 μM) is not sufficient to remove the 3.28 μM [Ca2+]i present in the medium. In contrast, 100 μM EGTA, which chelates most of the [Ca2+]i, almost completely prevented net accumulation and net loss of adenine nucleotides (Fig. 1). In the presence of 1.0 mM ATP, which is close to a steady-state condition (19), very little change in adenine nucleotide content occurred, and this result was unaffected with either 1 or 100 μM EGTA (Fig. 1).

Mitochondrial NAD(H) was determined for the experiment shown in Fig. 1 and did not differ significantly among the eight incubation conditions shown (average = 3.00 ± 0.13 nmol/mg protein).

In further experiments extramitochondrial [Ca2+]i, was controlled using Ca-EGTA buffers. Fig. 2 shows that net accumulation and net loss of adenine nucleotides occurred in the presence of 4.0 μM [Ca2+]i, whereas there was no significant accumulation or loss with 1.0 μM [Ca2+]i.
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Fig. 1. Effect of EGTA on total mitochondrial adenine nucleotide content after incubation with various external ATP concentrations. Incubations were carried out for 15 min as described under “Experimental Procedures” with ATP concentrations as indicated. Incubations included either 1 μM EGTA (open bars) or 100 μM EGTA (hatched bars). Data are expressed as the averages and ranges for two separate experiments. The adenine nucleotide contents of unincubated mitochondria for the two experiments were 14.70 and 10.94 nmol/mg protein.

Fig. 2. The effect of [Ca²⁺]ₘₑₙ on total matrix adenine nucleotide content as a function of time. Incubations were carried out as described under “Experimental Procedures” with [Ca²⁺]ₘₑₙ controlled using Ca-EGTA buffers. Net accumulation (○) and net loss (×) were assayed in the presence of 1.0 μM [Ca²⁺]ₘₑₙ (dashed lines) and 4.0 μM [Ca²⁺]ₘₑₙ (solid lines). For net accumulation, [ATP] was 4.0 mM; for net loss, [ATP] was 0.05 mM. The data shown are from one typical experiment for net accumulation and one for net loss.

by Ca²⁺ has been reported previously (20) but over a [Ca²⁺]ₘₑₙ range of 0.4–1.0 μM. We were unable to detect net changes when [Ca²⁺]ₘₑₙ was less than 0.6 μM. However, it is interesting to note that in our initial experiments with Ca-EGTA buffers (1, 29), the pH of the assay medium was adjusted before the addition of Ca²⁺ and thus the pH was accidentally lowered to an extent that was dependent on the total [Ca²⁺]. As a result there was an initial shock to the mitochondria in which the matrix adenine nucleotides were suddenly lost, dropping to about 4 nmol/mg protein within 30 s (a normal initial content is approximately 14–15 nmol/mg protein). This loss was preventable by including ruthenium red. Following the loss there was a recovery of the adenine nucleotide content by net accumulation over several minutes in a manner that was dependent on the concentration of Ca²⁺ which, if pH were 7.4, was supposed to be in the range of 0.1 to 0.8 μM. The actual concentrations of free Ca²⁺ under these conditions, however, were not known because the binding of Ca²⁺ with EGTA is sensitive to pH. These unusual conditions were the only circumstances under which we observed any net uptake that was dependent on [Ca²⁺]ₘₑₙ less than 0.6 μM.

Effects of Extramitochondrial Ca²⁺ on Unidirectional ATP Influx and ATP Efflux—Net transport of adenine nucleotides across the inner membrane via the ATP-Mg/Pₐ carrier has been shown to be equal in magnitude to the difference between ATP influx and ATP efflux (16). In order to understand the role of [Ca²⁺]ₘₑₙ on net changes of adenine nucleotide content it was necessary to examine its effects on the unidirectional fluxes. For these experiments, incubations contained 1.0 mM ATP so that influx and efflux would be approximately equal, producing a steady state in which no net change would occur (16, 19). In the absence of EGTA, addition of extra CaCl₂ up to 10 μM had no effect on the initial rates of ATP influx or ATP efflux (data not shown), but when medium [Ca²⁺]ₘₑₙ (normally 3.28 μM, see above) was chelated with EGTA influx and efflux were inhibited (Fig. 3). Inhibition was equivalent for both processes and was dependent on the concentration of EGTA between 2.5 and 10 μM (Fig. 3).

Fig. 3. The effect of EGTA on the unidirectional rates of ATP influx (○) and ATP efflux (●). Incubations were carried out as described under “Experimental Procedures” with 1.0 mM ATP. Ca²⁺ was unbuffered at 3.28 μM. Data shown are the averages of three separate experiments; bars = S.E.

Fig. 4. The effect of [Ca²⁺]ₘₑₙ on the unidirectional rates of ATP influx (○) and ATP efflux (●). Incubations were carried out as described under “Experimental Procedures” with 1.0 mM ATP. [Ca²⁺]ₘₑₙ was controlled by using Ca-EGTA buffers. Data shown are the averages for three separate experiments; bars = S.E.
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**FIG. 5.** The effect of [Ca$^{2+}$]$_{free}$ on the kinetics of ATP influx. Rates were determined with ATP concentrations of 0.25, 0.5, 1.0, and 2.0 mM. [Ca$^{2+}$]$_{free}$ was buffered at 1.0 μM (○) or 2.0 μM (■). Kinetic constants determined by linear regression analysis of the double-reciprocal plot were as follows: apparent $K_{m}$ (mM) values were 4.44 and 2.44 for 1.0 and 2.0 μM [Ca$^{2+}$]$_{free}$, respectively. $V_{max}$ (nmol/min/mg protein) values were 3.55 and 3.76 for 1.0 and 2.0 μM [Ca$^{2+}$]$_{free}$, respectively. Data shown are averages for two experiments.

### TABLE I

<table>
<thead>
<tr>
<th>[Ca$^{2+}$]$_{free}$ μM</th>
<th>Ruthenium red</th>
<th>ATP influx</th>
<th>ATP efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>-</td>
<td>0.56</td>
<td>0.65</td>
</tr>
<tr>
<td>+</td>
<td>0.49</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>-</td>
<td>1.36</td>
<td>1.10</td>
</tr>
<tr>
<td>+</td>
<td>1.25</td>
<td>1.12</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE II

**Effects of calmodulin antagonists on ATP influx and efflux rates**

ATP influx and ATP efflux were assayed as described under “Experimental Procedures” in the presence of 1.0 mM ATP. Calmodulin antagonists at various concentrations were included as indicated. When present oligomycin was 10 pg/ml. Values shown are the rates of flux as a percentage of a control assay that contained no antagonist. The number of experiments for each determination is shown in parentheses. Where $n = 2$, values are the average ± S.E. (ND = not determined.)

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>ATP influx</th>
<th>ATP efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Oligomycin</td>
<td>+Oligomycin</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>58 ± 5 (3)</td>
<td>54 ± 4 (5)</td>
</tr>
<tr>
<td>(50 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>80 ± 6 (2)</td>
<td>83 ± 6 (3)</td>
</tr>
<tr>
<td>(50 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W-7 (200 μM)</td>
<td>50 ± 14 (2)</td>
<td>13 (1)</td>
</tr>
<tr>
<td>Calmidazolium</td>
<td>50 ± 10 (2)</td>
<td>40 (1)</td>
</tr>
<tr>
<td>(25 μM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In further experiments the extramitochondrial [Ca$^{2+}$]$_{free}$ was controlled with Ca-EGTA buffers in the range of 0-4.0 μM. ATP influx and ATP efflux were stimulated in a concentration-dependent manner between 0 and 2.0 μM. Influx, but not efflux, was further stimulated by 4.0 μM [Ca$^{2+}$]$_{free}$ (Fig. 4). The influx of ATP was previously shown to exhibit saturation kinetics with respect to the external concentration of ATP (16). We examined the effect of [Ca$^{2+}$]$_{free}$ (1.0 and 2.0 μM) on the apparent $K_{m}$ and $V_{max}$ of ATP for influx; [ATP] was varied between 0.25 and 2.0 mM. The $V_{max}$ was unaffected by changing the [Ca$^{2+}$]$_{free}$ from 1.0 μM (3.55 nmol/min/mg protein) to 2.0 μM (3.76 nmol/min/mg protein), whereas the apparent $K_{m}$ decreased from 4.44 to 2.44 mM (Fig. 5). The values for apparent $K_{m}$ and $V_{max}$ obtained here in Ca-EGTA buffers are in the range of those reported previously (16).

**Other Characteristics of Ca$^{2+}$-stimulated ATP Fluxes** — Addition of 1 μM ruthenium red had little or no effect on the rates of ATP influx or efflux when [ATP] was 1.0 mM and [Ca$^{2+}$]$_{free}$ was buffered at 1.0 or 4.0 μM (Table I). When [Ca$^{2+}$]$_{free}$ was buffered at 0.6 or 4.0 μM the addition of A23187 (2 μg/ml) to promote Ca$^{2+}$ entry into the matrix did not stimulate ATP influx or efflux (data not shown). Dibutyryl cyclic AMP (250 μM) in the presence of low extramitochondrial [Ca$^{2+}$]$_{free}$...
TABLE III
Mitochondrial adenine nucleotide content in isolated hepatocytes following treatment with glucagon or vasopressin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nucleotide content to controls</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>8.69 ± 0.09 (4)</td>
<td></td>
</tr>
<tr>
<td>Glucagon</td>
<td>11.44</td>
<td>32</td>
</tr>
<tr>
<td>Glucagon + EGTA</td>
<td>8.78</td>
<td>1</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>12.37</td>
<td>55</td>
</tr>
<tr>
<td>Vasopressin + EGTA</td>
<td>8.79</td>
<td>&lt;1</td>
</tr>
<tr>
<td><strong>Experiment II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>10.59 ± 0.16 (4)</td>
<td>29</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>13.70</td>
<td></td>
</tr>
<tr>
<td>Vasopressin + neomycin</td>
<td>10.45</td>
<td>&lt;0</td>
</tr>
</tbody>
</table>

(0.6 μM) had no effect on unidirectional ATP influx (data not shown).

Stimulation of the ATP-Mg/Pi carrier may be mediated by a Ca" binding site on the carrier, by a mitochondrial calmodulin, or by a calmodulin-like protein associated with the carrier. Calmodulin is too large to cross the outer mitochondrial membrane, and whether or not there is calmodulin associated with mitochondria is still being debated. In any case, Ca" stimulated processes often can be inhibited by calmodulin antagonists whether or not the stimulation is mediated by calmodulin. Several calmodulin antagonists that we tested in ATP influx and efflux assays were found to inhibit calcium-dependent ATP-Mg/Pi, carrier activity (Table II). The percent inhibition was variable for trifluoperazine, chlorpromazine, or calmidazolium, but each antagonist inhibited both ATP influx and efflux to a similar extent. Trifluoperazine was studied in more detail and found to inhibit ATP influx as a linear function of concentration between 10 and 100 μM when [Ca" ]free was unbuffered at 3.29 μM (Fig. 6). The percent inhibition was similar at all inhibitor concentrations whether or not oligomycin was present (Fig. 6) showing that the effect of trifluoperazine was not secondary to a lower matrix ATP/ADP ratio (see below). Chlormpromazine inhibited ATP influx in a similar concentration-dependent manner (data not shown) but less potent than trifluoperazine at all concentrations tested.

The inhibitory effect of trifluoperazine was further tested in both ATP influx and ATP efflux assays over a range of [Ca" ]free (Fig. 7). Trifluoperazine inhibited both fluxes similarly; in general, the percent inhibition was independent of [Ca" ]free.

Mitochondrial respiration was measured under similar assay conditions with the concentrations of trifluoperazine and chlormpromazine reported in Table II. State 3 respiration in the absence of inhibitors was 65.5 nmol \( \text{O}_2/ \text{min/mg protein} \). Partial inhibition was observed with trifluoperazine (42.2 nmol \( \text{O}_2/ \text{min/mg protein} \)) but not with chlormpromazine (65.0 nmol \( \text{O}_2/ \text{min/mg protein} \)). Uncoupled respiration (92.5 nmol \( \text{O}_2/ \text{min/mg protein} \), measured in the presence of 2,4-dinitrophenol) was partially inhibited by both trifluoperazine and chlormpromazine (84.8 and 79.0 nmol \( \text{O}_2/ \text{min/mg protein} \), respectively). The inhibition of state 3 that we observed may have been due in part to inhibition of electron transport (uncoupled respiration also was inhibited), but trifluoperazine is known also to inhibit \( \text{F}_0\text{F}_1 \)-ATPase activity (30). This raised the possibility that inhibition of transport might be secondary to a decrease in the matrix ATP/ADP ratio. To clarify this issue unidirectional ATP influx was measured in the presence of oligomycin. Oligomycin maximally lowers the matrix ATP/ADP ratio so that possible inhibition of ATP flux by calmodulin antagonists could be assessed independently of any effect on the matrix ATP content. In the presence of oligomycin ATP influx was inhibited by trifluoperazine, chlormpromazine, and W-7 to approximately the same extent as with no oligomycin; with calmidazolium, inhibition was greater when oligomycin was included (Fig. 6 and Table II).

**Ca" Dependence of Net Changes in Mitochondrial Adenine Nucleotide Content in Isolated Hepatocytes**—In order to examine if physiological Ca" is important for cellular regulation of the mitochondrial adenine nucleotide content, we studied the effects of glucagon and vasopressin in isolated hepatocytes. Treatment of hepatocytes with glucagon has previously been shown to cause an increase in intramitochondrial adenine nucleotides (9, 11, 13), whereas a similar effect of vasopressin has not as yet been reported. Incubation of isolated hepatocytes with glucagon or vasopressin increased the adenine nucleotide content in the mitochondrial fraction (Table III). The total adenine nucleotide content of the cells did not change (not shown). In the presence of an amount of EGTA (3.1 mM) sufficient to lower extracellular [Ca" ]free to approximately 1 μM, the movement of adenine nucleotides from the cytoplasm into the mitochondrial fraction that was caused by either hormone was prevented (Table III). A 5-min incubation of hepatocytes with 10 mM neomycin (a potent inhibitor of the inositol phosphate pathway) for 5 min prevented the increase in mitochondrial adenine nucleotides observed with vasopressin (Table III).

**DISCUSSION**

This study demonstrates that [Ca" ]free stimulates ATP transport across the inner mitochondrial membrane via the ATP-Mg/Pi carrier. Under steady-state conditions (1.0 mM external ATP), unidirectional ATP fluxes were stimulated over a range of extramitochondrial [Ca" ]free from 0.6 to 2.0 μM; stimulation of ATP influx was very similar in magnitude to stimulation of ATP efflux. The inhibition patterns observed by chelating low level unbuffered Ca" with EGTA were also equivalent for both unidirectional influx and efflux. This is consistent with previous results suggesting that the ATP-Mg/Pi carrier functions as a coupled exchange. With this mechanism in mind, the results of this study suggest that [Ca" ]free stimulates transport activity by a general activation of carrier function, since ATP transport is stimulated equally in both directions. In all of our previous work (reviewed in Ref. 1), maximally stimulating concentrations of [Ca" ]free were present when using the incubation medium described under "Experimental Procedures," as long as MgCl\(_2\) was included as the inadvertent source of Ca".

[Ca" ]free appears to stimulate ATP influx by lowering the apparent \( K_a \) for ATP, with no effect on the apparent \( V_{\text{max}} \). The apparent \( K_a \) was determined only for influx, but the fact that ATP fluxes into and out of the mitochondria are affected similarly suggests that [Ca" ]free lowers the \( K_a \) for efflux as well as influx. Experiments to determine whether or not...
[Ca\(^{2+}\)]\(_{\text{cm}}\) affects the apparent \(K_m\) for \(P_i\), in a similar manner to ATP, are not technically possible at this time. This is because \(P_i\) transport over the ATP-Mg/Pi carrier cannot be measured directly due to much faster rates of \(P_i\) transport over the P/OH and dicarboxylate carriers. No discriminating inhibitor is available (16). This is not critical to understanding how \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) regulates net changes, however, because the current model for the ATP-Mg/Pi carrier suggests that it is the relative difference between ATP influx and ATP efflux that determines net change in the matrix adenine nucleotide content (1). In view of this, increases in \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) permit net changes to occur simply by activating the carrier. Data obtained using calmodulin antagonists suggest that \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) may stimulate ATP-Mg/Pi carrier activity via a Ca\(^{2+}\)-binding site on the transporter itself or perhaps secondarily via an interaction with a separate Ca\(^{2+}\)-binding protein.

The lack of any effects of ruthenium red or A23187 on Ca\(^{2+}\)-stimulation of ATP fluxes (Table I) suggests that changes in extramitochondrial \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) are probably sufficient to regulate the ATP-Mg/Pi, carrier and entry of Ca\(^{2+}\) is not necessary. The possibility that matrix \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) alone can stimulate flux rates has not been studied directly.

Net transport of adenine nucleotides was stimulated by \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) in a manner that is consistent with the effects on unidirectional rates measured under similar conditions. Net accumulation and net loss were both stimulated by Ca\(^{2+}\) and inhibited by EGTA. In general, these results are in agreement with those published by others (20) for net changes except that the effective concentration ranges of Ca\(^{2+}\) stimulation are somewhat different. We were unable to detect net changes when \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) was less than 0.6 \(\mu\)M. This may reflect differences in methods used to set up the incubations (see our comments under “Results”) or in the method used to determine \([\text{Ca}^{2+}]\)\(_{\text{cm}}\); we relied on \(\text{ura}-2\), whereas Haynes et al. (20) used Quin2. Levels of NAD(H) did not change under conditions of net loss and net accumulation. Calcium stimulation of net changes in adenine nucleotide content observed in our experiments is, therefore, not the result of nonspecific Ca\(^{2+}\)-induced damage to the mitochondrial inner membrane. This is particularly important for the case of net loss in which the concentration of external ATP was low.

Ca\(^{2+}\) stimulation of the ATP-Mg/Pi carrier may be part of the mechanism(s) by which physiological changes in mitochondrial adenine nucleotide content occur. There is general agreement that treatment of isolated hepatocytes with glucagon and other hormones causes an increase in cytosolic \([\text{Ca}^{2+}]\)\(_{\text{cm}}\). Cytosolic \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) has been reported to increase from 0.2 to 0.6 \(\mu\)M after glucagon treatment using the indicator dye Quin2 (31). Using Indo-1, hormone-stimulated increases in \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) were reported from a resting value of 0.5 to 1.6 \(\mu\)M for glucagon and to 2.9 \(\mu\)M for vasopressin (32). Our data demonstrate that in isolated mitochondria unidirectional ATP fluxes are stimulated by \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) free in the physiological range, between 0.6 and 2.0 \(\mu\)M. In addition, net changes in adenine nucleotide content are stimulated over this same \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) range in a manner consistent with the proposed mechanism of transport (1, 16).

Glucagon and vasopressin both increase intracellular \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) by the release of Ca\(^{2+}\) from intracellular stores and by stimulating Ca\(^{2+}\) influx from the extracellular media. EGTA pretreatment of isolated hepatocytes decreases the glucagon- and vasopressin-induced increases in intracellular \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) (33). In our experiments EGTA prevented the increase in the mitochondrial adenine nucleotide content caused by these hormones. Dibutyryl cyclic AMP, which mimics the glucagon-induced increase in intracellular \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) (32), caused an increase in the mitochondrial adenine nucleotide content. For vasopressin, an increase in intracellular \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) is mediated by inositol polyphosphates (34) but not by cyclic AMP (35). Neomycin, shown to decrease the intracellular \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) caused by vasopressin (32), also completely prevented vasopressin-induced adenine nucleotide accumulation by mitochondria in hepatocytes. These results are consistent with the hypothesis that the net increase in mitochondrial adenine nucleotide content that follows glucagon or vasopressin administration may occur because of a change in cytosolic \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) which regulates the ATP-Mg/Pi, carrier. An increase in cytosolic \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) may translate into an increase in matrix \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) as well (36–38), but for ATP-Mg/Pi carrier stimulation an increase in cytosolic \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) is probably sufficient.

Adenine nucleotide recompartmentation between the cytosol and mitochondria occurs as an adaptive response to changing physiological conditions, with important effects on metabolic activity (9-8, 12, 13). The shift in adenine nucleotides from the cytosol to the mitochondria observed at parturition in the rat and rabbit has already been shown to be affected by the changing hormonal status (increasing glucagon/insulin ratio) of the newborn animal (7, 39, 40). This is probably related to an increase in cytosolic \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) which may now be presumed to stimulate the ATP-Mg/Pi carrier. In the normal newborn and in normoxic adults, activation of the carrier normally results in net uptake of adenine nucleotides into the mitochondria, because the normal cytoplasmic and matrix ATP concentrations favor net movement in that direction (1). If the cytoplasmic ATP concentration falls to very low levels, as it does in hypoxia, less uptake or even net loss of adenine nucleotides from mitochondria is predicted to occur whenever Ca\(^{2+}\) is present to activate the carrier. This prediction is consistent with published observations (7, 14, 40). Further investigations of cellular mechanisms of adenine nucleotide recompartmentation that occur via the ATP-Mg/Pi carrier will require careful evaluation of both cytosolic \([\text{Ca}^{2+}]\)\(_{\text{cm}}\) and ATP concentration gradients across the inner mitochondrial membrane.

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REFERENCES

Calcium Stimulation of ATP-Mg/Pi Carrier Activity

Calcium stimulates ATP-Mg/Pi carrier activity in rat liver mitochondria.
M T Nosek, D T Dransfield and J R Aprille


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