Calcium Stimulates ATP-Mg/P\textsubscript{i} Carrier Activity in Rat Liver Mitochondria

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Adenine nucleotide transport over the carboxytriacetyl-dihydroxyethylpiperazine-N'-Z-ethanesulfonic acid-insensitive ATP-Mg/P\textsubscript{i} carrier was assayed in isolated rat liver mitochondria with the aim of investigating a possible regulatory role for Ca\textsuperscript{2+} on carrier activity. Net changes in the matrix adenine nucleotide content (ATP + ADP + AMP) occur when ATP-Mg exchanges for P\textsubscript{i} over this carrier. The rates of net accumulation and net loss of adenine nucleotides were inhibited when free Ca\textsuperscript{2+} was chelated with EGTA and stimulated when buffered free Ca\textsuperscript{2+} was increased from 1.0 to 4.0 \textmu M. The unidirectional components of net change were similarly dependent on Ca\textsuperscript{2+}; ATP influx and efflux were inhibited by EGTA in a concentration-dependent manner and stimulated by buffered free Ca\textsuperscript{2+} in the range 0.6-2.0 \textmu M. For ATP influx, increasing the medium [Ca\textsuperscript{2+}]\textsubscript{free} from 1.0 to 2.0 \textmu M lowered the apparent \(K_m\) for ATP from 4.44 to 2.44 nmol/min/mg with no effect on the apparent \(V_m\) (3.55 and 3.76 nmol/min/mg, respectively). Stimulation of influx and efflux by [Ca\textsuperscript{2+}]\textsubscript{free} was unaffected by either ruthenium red or the Ca\textsuperscript{2+} 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 [Ca\textsuperscript{2+}]\textsubscript{free} that activates the ATP-Mg/P\textsubscript{i} 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/P\textsubscript{i} 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\textsubscript{i}, resulting in a net increase or a net decrease in the matrix adenine nucleotide pool size (1,16).

The ATP-Mg/P\textsubscript{i} carrier activity that facilitates net accumulation and net loss of mitochondrial adenine nucleotides in vitro has been characterized in detail (1,16-19). 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\textsubscript{i} 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/P\textsubscript{i} transport activity in changes in the cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{free}) may be part of the mechanism by which physiologic stimuli such as glucagon cause adenine nucleotide recompartmentation between the cytosol and mitochondrion in liver.

In this study we examined in greater detail the effects of [Ca\textsuperscript{2+}]\textsubscript{free} on ATP-Mg/P\textsubscript{i} 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 [Ca\textsuperscript{2+}]\textsubscript{free} on net changes, effects on unidirectional ATP movements over the ATP-Mg/P\textsubscript{i} carrier were examined. Ca\textsuperscript{2+} was found to stimulate carrier activity in a concentration range that is consistent with increases in cytosolic [Ca\textsuperscript{2+}]\textsubscript{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 (Wilmingtom, MA). [\textsuperscript{3}H]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 \textmu M Ca\textsuperscript{2+}.

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).

The abbreviations and trivial names used are: Fura-2, l-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N',N',N'-tetraacetic acid; EGTA, [ethylene-bis(oxyethylenenitriilo)]-tetraacetic acid; Indo-1, 1-[2-amino-5-(6-carboxyindol-2-yl)-phenoxyl]-2-(2'-amino-5'-methylphenoxy)ethane-N,N',N'-tetraacetic acid; Quin2, 2-[2-[bis(carboxymethyl)-amino]-5-methylphenoxy]-methyl]-6-methoxy-8-bis(carboxymethyl)-aminquinoline; W-7, [N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamido]-hydrochloride; calmidazolium, 1-[bis(4-chlorophenyl)-methyl]-3-[2-(2,4-dichlorophenyl)-2-(2,4-dichlorophenyl)-methoxy]-ethyl]-1H-imidazolium chloride; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Tes, N-tris(hydroxyethyl)methyl-2-aminoethanesulfonic acid; Tricine, N-tris(hydroxyethyl)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/KH₃PO₄, 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 in text and in tables. For some experiments the [Ca⁺]ᵢ free was controlled using Ca-EGTA buffers. The incubation medium was the same except that 5 μM EGTA was included along with a variable amount of CaCl₂. The total [Ca⁺]ᵢ needed to produce the desired [Ca⁺]ᵢ free was calculated using the stability constants for Ca-EGTA, Mg-EGTA, Ca-Mg-EGTA from Bartha and Blinks (12). EGTA was present but no CaCl₂ was added. The concentrations of free Ca⁺ were measured with Fura-2 (23, 24) and calculated as [Ca⁺]ᵢ free = Kₐ f [F - Fₐₐ₉]/[Fₐₐ₉ - F].

Measurements were in good agreement with values calculated from stability constants. A Kₐ of 135 μM was used, which is appropriate for the ionic strength and pH of the incubation medium (24).

The calmodulin antagonists trifluoperazine, chlormazine, calmidazolium, and W-7 were added to mitochondrial incubations from stocks dissolved in dimethyl sulfoxide. The controls for all experiments involving these compounds contained an equivalent volume of dimethyl sulfoxide.

**Net Accumulation and Net Loss of Mitochondrial Nucleotides**—To measure net changes in adenine nucleotide content, mitochondria were added to the incubation medium, followed immediately by the addition of ATP (time = 0). To assay net accumulation the [ATP] was either 4.0 or 2.0 mM; to assay net loss the [ATP] was 0.0 or 0.05 mM. When [ATP] = 1.0 mM a steady state existed and little net change occurred (16, 19). After the desired time intervals, between 2 and 15 min, 1.5 volumes of ice-cold 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, were added to stop the reaction. Samples were then centrifuged (14,000 × g) at 4 °C for 5 min. Tubes were decanted and the pellet was resuspended and washed once with the sucrose-Tris buffer. The final pellet was resuspended to a protein concentration of approximately 10 mg of protein/ml. Neutralized perchloric acid extracts were prepared as described previously (4), and adenine nucleotides (ATP + ADP + AMP) were determined enzymatically (25, 26). NAD(H) was measured in the extracts by reverse-phase high-performance liquid chromatography using a Resolve C₁₈ (8 mm × 10 cm) Radial-Pak column (Waters, Milford, MA). Nucleotides were eluted using a linear gradient of 0.1 M K₂HPO₄/KH₂PO₄, pH 5.8, to 1% methanol, 0.1 M K₂HPO₄/KH₂PO₄, pH 5.8, over 9.5 min, followed by a second linear gradient to 5% methanol, 0.1 M KH₂PO₄/KH₃PO₄, pH 5.8, over 11 min, with a final gradient to 0.1 M KH₂PO₄/KH₃PO₄, pH 5.8, over 14.5 min. Because NADH is converted to NAD during the acid extraction, NADH + NAD can be estimated from a single elution peak at about 25 min corresponding to an NAD standard.

**Unidirectional ATP Fluxes**—ATP influx and ATP efflux were measured essentially as described previously (10) under incubation conditions similar to those used to assay net changes. Mitochondria were added to the incubation medium described above; carboxyatractyslide (5 μM) was included to inhibit the ADP/ATP translocase. Fifteen seconds after the addition of mitochondria, ATP (1.0 mM) was added (time = 0). At specified times thereafter (typically at 30, 60, and 90 s), aliquots (0.15 or 1.0 ml) were applied to Millipore filters (0.45 μM) and further vacuum filtered and immediately placed in 10 ml of ice-cold 150 mM NaCl. For ATP influx the ATP added was traced-labeled with [³²P]ATP (approximately 250 cpm/nmol).

The amount of radioactivity retained on the filters was determined by liquid scintillation counting after the filters were dissolved in Aquasol. For ATP efflux the matrix pool of adenine nucleotides was uniformly labeled during the incubation of mitochondria by counting protein/ml on ice for 40 min with 4 ml of carrier-free (³²P)ADP/mg of protein. The rate of adenine nucleotide efflux was assayed as described for ATP influx except that the extramitochondrial ATP was unlabeled. The radioactivity retained on the filters was determined as for ATP influx, and the specific activity (cpm/nmol) of adenine nucleotides in the mitochondria was determined by counting [³²P] 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 arbitrarily 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 Hapes, 60 μM EGTA, 2% bovine serum albumin, pH 7.2). After livers were removed, 0.5 g liver weight was continued in buffer B (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 Hapes, 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 NaHCO₃, 7.5 mM HEPES, 6.7 mM Na₂SO₄, 32.7 mM Hepes, 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 10⁶ 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 rotovapor 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 or an equivalent volume of buffer D was present in the flask initially, and after 30 s glucagon (1 nM) or vasopressin (10 nM) was added. For experiments involving neomycin, 10 mM neomycin or vehicle was present for 5 min prior to the addition of vasopressin (10 nM). Five minutes after hormone addition 0.5 ml aliquots were subjected to digitonin fractionation, essentially as described by Anderson and Axelrod (1). For net fluxes, controls were assayed first and then EGTA or neomycin was added. Preliminary experiments showed that the mitochondrial adenine nucleotide content was not different among various control samples obtained after preincubation, or from the stock suspension, or after incubation for 5 min with vehicle. Therefore, controls were routinely sampled from the stock suspension for comparison to treated cells.

**RESULTS**

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

Lowering external [Ca⁺]ᵢ free 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 μM 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 [Ca⁺]ᵢ free present in the medium. In contrast, 100 μM EGTA, which chelates most of the [Ca⁺]ᵢ free 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 [Ca⁺]ᵢ free 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 [Ca⁺]ᵢ free; there was no significant accumulation or loss with 10 μM [Ca⁺]ᵢ free. Stimulation of net changes in adenine nucleotide content...
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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 [Ca<sup>2+</sup>]<sub>free</sub> on total matrix adenine nucleotide content as a function of time. Incubations were carried out as described under “Experimental Procedures” with [Ca<sup>2+</sup>]<sub>free</sub> controlled using Ca-EGTA buffers. Net accumulation (○) and net loss (×) were assayed in the presence of 1.0 μM [Ca<sup>2+</sup>]<sub>free</sub> (dashed lines) and 4.0 μM [Ca<sup>2+</sup>]<sub>free</sub> (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 Ca<sup>2+</sup> has been reported previously (20) but over a [Ca<sup>2+</sup>]<sub>free</sub> range of 0.4–1.0 μM. We were unable to detect net changes when [Ca<sup>2+</sup>]<sub>free</sub> 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 Ca<sup>2+</sup> and thus the pH was accidentally lowered to an extent that was dependent on the total [Ca<sup>2+</sup>]. 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 Ca<sup>2+</sup> 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 Ca<sup>2+</sup> under these conditions, however, were not known because the binding of Ca<sup>2+</sup> with EGTA is sensitive to pH. These unusual conditions were the only circumstances under which we observed any net uptake that was dependent on [Ca<sup>2+</sup>]<sub>free</sub> less than 0.6 μM.

Effects of Extramitochondrial Ca<sup>2+</sup> on Unidirectional ATP Influx and ATP Efflux—Net transport of adenine nucleotides across the inner membrane via the ATP-Mg/P<sub>4</sub> 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 [Ca<sup>2+</sup>]<sub>free</sub> 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 [Ca<sup>2+</sup>]<sub>free</sub> (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. Ca<sup>2+</sup> was unbuffered at 3.28 μM. Data shown are the averages of three separate experiments; bars = S.E.

FIG. 4. The effect of [Ca<sup>2+</sup>]<sub>free</sub> on the unidirectional rates of ATP influx (○) and ATP efflux (●). Incubations were carried out as described under “Experimental Procedures” with 1.0 mM ATP. [Ca<sup>2+</sup>]<sub>free</sub> 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 \([Ca^{2+}]_{\text{free}}\) on the kinetics of ATP influx. Rates were determined with ATP concentrations of 0.25, 0.5, 1.0, and 2.0 mM. \([Ca^{2+}]_{\text{free}}\) was buffered at 1.0 \(\mu\)M (○) or 2.0 \(\mu\)M (■). 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 \(\mu\)M \([Ca^{2+}]_{\text{free}}\), respectively. \(V_{max}\) (nmol/min/mg protein) values were 3.55 and 3.76 for 1.0 and 2.0 \(\mu\)M \([Ca^{2+}]_{\text{free}}\), respectively. Data shown are averages for two experiments.

**Table I**

| [Ca^{2+}]_{\text{free}} | Ruthenium red | ATP influx | ATP efflux |
|--------------------------|---------------|------------|------------|
| \(\mu\)M                 | -             | 0.56       | 0.65       |
| +                        | 0.49          | 0.55       |
| 4.0                      | -             | 1.36       | 1.10       |
| +                        | 1.25          | 1.12       |

**Table II**

Effects of calmodulin antagonists on ATP influx and efflux rates

ATP influx and ATP efflux were assayed as described under “Experimental Procedures” with \([ATP] = 1.0 \text{ mM}\) and \([Ca^{2+}]_{\text{free}}\) and 1 \(\mu\)M ruthenium red as indicated. Data are from one typical experiment for ATP influx and one for ATP efflux.

| Antagonist     | ATP influx | ATP efflux |
|----------------|------------|------------|
| +Oligomycin    |            |            |
| -Oligomycin    |            |            |
| Trifluoperazine| 58 ± 5 (3) | 54 ± 4 (5) |
| (50 \(\mu\)M) | 59 ± 6 (2) |            |
| Chlorpromazine | 80 ± 6 (2) | 83 ± 6 (3) |
| (50 \(\mu\)M) | 88 ± 1 (2) |            |
| W-7 (200 \(\mu\)M)| 50 ± 14 (2) | 13 (1) | ND |
| Calmidazolium  | 50 ± 10 (2) | 40 (1)     | 60 (1)    |

In further experiments the extramitochondrial \([Ca^{2+}]_{\text{free}}\) was controlled with Ca-EGTA buffers in the range of 0-4.0 \(\mu\)M. ATP influx and ATP efflux were stimulated in a concentration-dependent manner between 0 and 2.0 \(\mu\)M. Influx, but not efflux, was further stimulated by 4.0 \(\mu\)M \([Ca^{2+}]_{\text{free}}\) (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 \([Ca^{2+}]_{\text{free}}\) (1.0 and 2.0 \(\mu\)M) on the apparent \(K_m\) and \(V_{max}\) of ATP for influx; \([ATP]\) was varied between 0.25 and 2.0 \(\mu\)M. The \(V_{max}\) was unaffected by changing the \([Ca^{2+}]_{\text{free}}\) from 1.0 \(\mu\)M (3.55 nmol/min/mg protein) to 2.0 \(\mu\)M (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 \(\mu\)M ruthenium red had little or no effect on the rates of ATP influx or efflux when \([ATP] = 1.0 \text{ mM}\). ATP influx varied between 0.25 and 2.0 \(\mu\)M. The \(V_{max}\) was unaffected by changing the \([Ca^{2+}]_{\text{free}}\) from 1.0 \(\mu\)M (3.55 nmol/min/mg protein) to 2.0 \(\mu\)M (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 in the presence of low extramitochondrial \([Ca^{2+}]_{\text{free}}\)
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 glucagon (pretreatment was for 5 min prior to addition of hormone (either glucagon (1 mM) or vasopressin (10 mM)). After 5 min aliquots were subjected to digitonin fractionation, and the adenine nucleotides were measured in the mitochondrial fraction. Controls were samples from the untreated cell suspension and represent the average (±S.E.) of replicates for each experiment.

| Experiment | Mitochondrial adenine nucleotide content | Increase relative to controls |
|------------|-----------------------------------------|-----------------------------|
|            | nmol/10^6 cells                         | %                           |
| Controls   |                                        |                             |
| Glucagon   | 11.44 ± 0.09 (4)                        | 32                          |
| Glucagon + EGTA | 8.78                      | 1                           |
| Vasopressin | 12.27 ± 0.53 (5)                      | 53                          |
| Vasopressin + EGTA | 8.79                     | <1                          |
| Controls   | 10.59 ± 0.16 (4)                        | 29                          |
| Vasopressin | 13.70                                    |                             |
| Vasopressin + neomycin | 10.45                  | <0                          |

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

Stimulation of the ATP-Mg/Pi carrier may be mediated by a Ca^{2+}-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^{2+}-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^{2+}]_{free} was unbuffered at 3.28 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). Chlorpromazine inhibited ATP influx in a similar concentration-dependent manner (data not shown) but was 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^{2+}]_{free} (Fig. 7). Trifluoperazine inhibited both fluxes similarly; in general, the percent inhibition was independent of [Ca^{2+}]_{free}.

Mitochondrial respiration was measured under similar assay conditions with the concentrations of trifluoperazine and chlorpromazine reported in Table II. State 3 respiration in the absence of inhibitors was 63.5 nmol %O_{2}/min/mg protein. Partial inhibition was observed with trifluoperazine (42.2 nmol %O_{2}/min/mg protein) but not with chlorpromazine (65.0 nmol %O_{2}/min/mg protein). Uncoupled respiration (92.5 nmol %O_{2}/min/mg protein, measured in the presence of 2,4-dinitrophenol) was partially inhibited by both trifluoperazine and chlorpromazine (84.8 and 79.0 nmol %O_{2}/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 F_{0}-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 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, chlorpromazine, 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).

**DISCUSSION**

This study demonstrates that [Ca^{2+}]_{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^{2+}]_{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^{2+} 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^{2+}]_{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^{2+}]_{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^{2+}.

[Ca^{2+}]_{free} appears to stimulate ATP influx by lowering the apparent K_{m} for ATP, with no effect on the apparent V_{max}. The apparent K_{m} was determined only for influx, but the fact that ATP fluxes into and out of the mitochondria are affected similarly suggests that [Ca^{2+}]_{free} lowers the K_{m} for efflux as well as influx. Experiments to determine whether or not

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**TABLE III**

**Mitochondrial adenine nucleotide content in isolated hepatocytes following treatment with glucagon or vasopressin**

Inclusions of hepatocytes were carried out as described under "Experimental Procedures." In Experiment I, pretreatment with EGTA (5.1 mM) was for 30 s, and in Experiment II, 10 mM neomycin pretreatment was for 5 min prior to addition of hormone (either glucagon (1 mM) or vasopressin (10 mM)). After 5 min aliquots were subjected to digitonin fractionation, and the adenine nucleotides were measured in the mitochondrial fraction. Controls were samples from the untreated cell suspension and represent the average (±S.E.) of replicates for each experiment.
[Ca\textsuperscript{2+}]\textsubscript{free} affects the apparent $K_m$ for P, in a similar manner to ATP; are not technically possible at this time. This is because P, transport over the ATP-Mg/P, carrier cannot be measured directly due to much faster rates of P, transport over the P, OH and dicarboxylate carriers. No discriminating inhibitor is available (16). This is not critical to understanding how [Ca\textsuperscript{2+}]\textsubscript{free} regulates net changes, however, because the current model for the ATP-Mg/P, 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 [Ca\textsuperscript{2+}]\textsubscript{free} permit net changes to occur simply by activating the carrier. Data obtained using calmodulin antagonists suggest that [Ca\textsuperscript{2+}]\textsubscript{free} may stimulate ATP-Mg/P, carrier activity via a Ca\textsuperscript{2+}-binding site on the transporter itself or perhaps secondarily via an interaction with a separate Ca\textsuperscript{2+}-binding protein.

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

Net transport of adenine nucleotides was stimulated by [Ca\textsuperscript{2+}]\textsubscript{free} 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\textsuperscript{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\textsuperscript{2+} stimulation are somewhat different. We were unable to detect net changes when [Ca\textsuperscript{2+}]\textsubscript{free} 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 [Ca\textsuperscript{2+}]\textsubscript{free}; we relied on Fura-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\textsuperscript{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\textsuperscript{2+} stimulation of the ATP-Mg/P, 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 [Ca\textsuperscript{2+}]\textsubscript{free}. Cytosolic [Ca\textsuperscript{2+}]\textsubscript{free} 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 [Ca\textsuperscript{2+}]\textsubscript{free} 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 [Ca\textsuperscript{2+}]\textsubscript{free} in the physiological range, between 0.6 and 2.9 $\mu$M. In addition, net changes in adenine nucleotide content are stimulated over this same [Ca\textsuperscript{2+}]\textsubscript{free} range in a manner consistent with the proposed mechanism of transport (1, 16).

Glucagon and vasopressin both increase intracellular [Ca\textsuperscript{2+}]\textsubscript{free}, by the release of Ca\textsuperscript{2+} from intracellular stores and by stimulating Ca\textsuperscript{2+} influx from the extracellular media. EGTA pretreatment of isolated hepatocytes decreases the glucagon- and vasopressin-induced increases in intracellular [Ca\textsuperscript{2+}]\textsubscript{free} (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 [Ca\textsuperscript{2+}]\textsubscript{free} (32), caused an increase in the mitochondrial adenine nucleotide content. For vasopressin, an increase in intracellular [Ca\textsuperscript{2+}]\textsubscript{free} is mediated by inositol polyphosphates (34) but not by cyclic AMP (35). Neomycin, shown to decrease the intracellular [Ca\textsuperscript{2+}]\textsubscript{free} 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 [Ca\textsuperscript{2+}]\textsubscript{free} which regulates the ATP-Mg/P, carrier. An increase in cytosolic [Ca\textsuperscript{2+}]\textsubscript{free} may translate into an increase in matrix [Ca\textsuperscript{2+}]\textsubscript{free} as well (36-38), but for ATP-Mg/P, carrier stimulation an increase in cytosolic [Ca\textsuperscript{2+}]\textsubscript{free} 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 (9-12, 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 [Ca\textsuperscript{2+}]\textsubscript{free} which may now be presumed to stimulate the ATP-Mg/P, 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\textsuperscript{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/P, carrier will require careful evaluation of both cytosolic [Ca\textsuperscript{2+}]\textsubscript{free} and ATP concentration gradients across the inner mitochondrial membrane.

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