Vasopressin and/or Glucagon Rapidly Increases Mitochondrial Calcium and Oxidative Enzyme Activities in the Perfused Rat Liver

(Received for publication, November 26, 1985)

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Mitochondria were prepared by a method including a Percoll purification step after the rapid homogenization of livers of fed rats which had been perfused either under unstimulated conditions or in the presence of vasopressin and/or glucagon. The two hormones separately or together increased the total calcium content of the mitochondria. This enhancement was accompanied by parallel increases in activities of the Ca$^{2+}$-sensitive intramitochondrial enzymes pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. The effects of the two hormones on total mitochondrial calcium and on the activities of the oxidative enzymes were additive. The persistent enhancements of mitochondrial calcium content and enzyme activities were partially reversed by the addition of Na$^+$ ions to the mitochondrial incubations; these effects of Na$^+$ were blocked by diltiazem, a selective inhibitor of Na$^+$-induced Ca$^{2+}$ release. Mitochondria from control livers were incubated in vitro with CaCl$_2$ to achieve various calcium content, and mitochondrial enzyme activities and calcium content were measured. A good correlation was obtained between the total calcium content and the activities of pyruvate dehydrogenase and oxoglutarate dehydrogenase.

The results obtained are consistent with the hypothesis that vasopressin and glucagon additively cause increases in intramitochondrial [Ca$^{2+}$] and so bring about the activations of these key enzymes of mitochondrial oxidative metabolism.

In the liver of fed rats, the hormones which are known to cause increases in cytosolic [Ca$^{2+}$] (vasopressin, angiotensin II, α-adrenergic agonist, and glucagon (1–3)) also produce an increase in the amount of active, non-phosphorylated pyruvate dehydrogenase (PDHα) (4–6). There is also some indirect evidence of increases in 2-oxoglutarate dehydrogenase activity (7–11), as well as in respiration (5, 12–15), being brought about by these hormones. 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase-phosphate phosphatase (which dephosphorylates and activates pyruvate dehydrogenase) are two of the three Ca$^{2+}$-sensitive intramitochondrial enzymes found in mammalian tissues (16) (the other is NAD$^+$-isocitrate dehydrogenase). Ca$^{2+}$ activates 2-oxoglutarate dehydrogenase by decreasing its $K_m$ value for 2-oxoglutarate. Increases in 2-oxoglutarate dehydrogenase activity and PDHα can be observed within intact mitochondria from several different mammalian tissues (including the liver) as the result of increases in extramitochondrial [Ca$^{2+}$] within the expected physiological range (see Ref. 17). It has been proposed that the higher level of cytoplasmic [Ca$^{2+}$] promoted by these hormones may be relayed into the mitochondrial matrix and thereby lead to the activation of the Ca$^{2+}$-sensitive dehydrogenases (16, 18).

Reports of the measurement of mitochondrial total calcium content in the liver under basal and hormone-stimulated conditions are conflicting and have led to much confusion as to the role of mitochondrial calcium. Basal mitochondrial calcium content has been reported to be at values varying from 2 to 25 nmol/mg of mitochondrial protein (see Ref. 19). Moreover, slight and transient increases (20–22) or, conversely, major decreases (23–27) in mitochondrial calcium have been reported to occur after the addition of vasopressin or α-adrenergic agonists to liver preparations. Recently, the more direct x-ray probe microanalysis technique has been used to estimate total mitochondrial calcium of the liver in situ (28). This gave a value of about 1.1 nmol/mg of protein under unstimulated conditions. From this value and on the basis of estimates of the activity coefficient for mitochondrial Ca$^{2+}$ from in vitro studies (29, 30), a free [Ca$^{2+}$] of about 0.3 μM under basal conditions has been suggested (28). At these concentrations of liver mitochondrial calcium and Ca$^{2+}$, the mitochondrial Ca$^{2+}$-sensitive dehydrogenases would not be expected to be saturated for Ca$^{2+}$ (29, 30). Therefore, any increase in mitochondrial calcium content may thus lead to an increase in the free [Ca$^{2+}$] in the matrix and hence activation of the Ca$^{2+}$-sensitive dehydrogenases.

In this paper, we present evidence that vasopressin and glucagon added to the perfused liver lead to increases in 2-oxoglutarate dehydrogenase activity (at low substrate concentrations) and PDHα content, within subsequently prepared and incubated mitochondria. These persistent activations are accompanied by parallel increases in mitochondrial total calcium content. Incubation of mitochondria with sodium to promote Ca$^{2+}$ efflux (17) produces parallel modifications in the hormone-induced changes in mitochondrial calcium content and stimulation of enzyme activities. The results obtained are consistent with the hypothesis that increases in intramitochondrial [Ca$^{2+}$] mediate the action of these hormones on mitochondrial oxidative metabolism in the liver.

MATERIALS AND METHODS

Livers from fed Sprague-Dawley male rats (250–280 g) were perfused (31) with Krebs bicarbonate buffer (32) containing 3% dialyzed...
bovine serum albumin and 10 mM glucose. The medium was continuously gassed with O₂-CO₂ (95:5) and recirculated (20 ml/min). After 15 min of equilibrium, hormones were added to the reservoir. The time for circulation between the reservoir and the liver was determined, and the time course given presents the real time during which the livers were exposed to the hormones. At various times, one lobe was removed from the liver (approximately 200 mg) being frozen in liquid nitrogen for subsequent determinations of pyruvate dehydrogenase activity (39) and the major part (approximately 1.5 g) being rapidly homogenized for the isolation of mitochondria.

Preparation of Mitochondria — The excised tissue was immediately disrupted in a Polytron (17) homogenizer by 2 × 2-s bursts at setting 5 in ice-cold medium containing 250 mM sucrose, 20 mM Tris (pH 7.4), 2 mM EGTA, and 1% (w/v) bovine serum albumin (approximately 1 g of tissue/4 ml of medium); mitochondria were then prepared as described in Ref. 17. After the initial isolation, a Percoll purification step was performed with the sample being re-centrifuged for this step and then washed once in the same buffer but without albumin (17). The Percoll step allowed the separation from the mitochondrial pellet of a fraction, containing mostly plasma membranes and microsomal marker enzymes (see Table I below).

The mitochondria obtained appeared to be well-coupled and showed good respiratory control (35) (not shown). No changes in respiratory control ratios were noted as the result of the hormone treatments used in the present study. Mitochondrial purity and recovery were assessed by measurements of the specific activity of cytochrome oxidase (34). Possible contamination by other fractions was assessed by measurements of enzyme markers which were galactosidase (cytoplasmic), glucose-6-phosphatase (Golgi apparatus) (36), NADPH-cytochrome-c reductase (endoplasmic reticulum) (37), and 5'-nucleotidase (plasma membranes) (38).

Incubation of Mitochondria, Assay of Enzyme Activities, and Measurement of Total Calcium and Protein and ATP Content —Mitochondria were incubated (1-2 mg of protein/ml) at 30 °C in a basic medium containing 125 mM KCl, 20 mM Tris, 5 mM potassium phosphate, and 0.5 mM EGTA at pH 7.3. When NaCl (10 mM) was present, 115 mM KCl was used. For incubations where pyruvate dehydrogenase was to be measured, the medium contained, in addition, 2 mM 2-oxoglutarate, 0.2 mM malate, and 1 mM pyruvate (K⁺ salts). The addition of pyruvate was required to inhibit partly pyruvate dehydrogenase kinase and to observe an effect on the phosphatase. For the measurement of pyruvate dehydrogenase activity, samples (1 ml) were rapidly sedimented (10,000 × g for 15 s), the supernatant was aspirated off, and the pellet was quickly frozen in liquid N₂. PDHα and 2-oxoglutarate dehydrogenase activities were increased by glucagon and by vasopressin. When both hormones were added together, their effects appeared to be additive on all the parameters measured. Data of Table III were measured 3 min after the addition of the hormones. Fig. 1 shows that 1 min after the addition of glucagon + vasopressin, mitochondrial calcium was then re-isolated using the same full procedure, except homogenization, and calcium content and 2-oxoglutarate dehydrogenase activity were not modified. Possible changes happening during the homogenization cannot be excluded but seem unlikely in the presence of 2 mM EGTA. Hormone treatment did not affect ATP measured in mitochondria incubated for 15 min as described under "Material and Methods": 7.3 ± 0.3, 8.2 ± 0.3, and 9.5 ± 0.8 mmol/mg of protein for control, vasopressin-, and glucagon plus vasopressin-treated livers, respectively.

Effects of Glucagon, Vasopressin, and Glucagon plus Vasopressin on Mitochondrial Enzyme Activities and Calcium Content — Glucagon or vasopressin does increase the amount of pyruvate dehydrogenase in its active form (PDHα) in the perfused rat liver (Table III, legend). This effect is additive. When mitochondria are isolated from these livers and incubated in the absence of Na⁺, increases in the activities of PDHα and 2-oxoglutarate dehydrogenase at low substrate concentration were observed (Table III). Total calcium measured in mitochondria incubated under the same conditions as for the oxidative enzyme activities was increased by glucagon and by vasopressin. When both hormones were added together, their effects appeared to be additive on all the parameters measured. Data of Table III were measured 3 min after the addition of the hormones. Fig. 1 shows that 1 min after the addition of glucagon + vasopressin, mitochondrial calcium as well as oxidative enzyme activities were increased. The effects increase with time, at least up to 10 min (Fig. 1). 1 min after the addition of vasopressin (10⁻⁸ M), the activity of 2-oxoglutarate dehydrogenase is 217 ± 32%, and total mitochondrial calcium is 193 ± 21% of the values observed in mitochondria from control livers.

Effects of Na⁺ on Oxidative Activities and Calcium Content in Mitochondria from Control or Hormone-perfused Livers — Recent data have demonstrated that liver mitochondria do exhibit a Na⁺-dependent calcium egress (43, 44). Mitochondria from control and hormone-treated livers were therefore incubated with 10 mM Na⁺ for various times, and calcium content as well as oxidative enzyme activities were measured. In mitochondria from vasopressin and glucagon plus vasopressin-treated livers, a time-dependent effect of Na⁺ on calcium content and oxidative enzyme activities was observed (Fig. 2). The effects of Na⁺ on calcium content and on oxidative enzyme activities are quite parallel, and all these parameters return toward basal control values upon incubation.
Hormonal Control of Liver Mitochondrial Metabolism by \( \text{Ca}^{2+} \)

**Table I**

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>Cytochrome oxidase</th>
<th>5'-Nucleotidase</th>
<th>Glucose-6-phosphatase</th>
<th>Galactosyltransferase</th>
<th>NADPH-cytochrome-c reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>123 ± 6</td>
<td>10.0 ± 0.4</td>
<td>3.5 ± 0.3</td>
<td>1.8 ± 0.1</td>
<td>12.2 ± 0.4</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>643 ± 69°</td>
<td>19.6 ± 0.5</td>
<td>1.7 ± 0.1</td>
<td>0.1 ± 0.05°</td>
<td>2.2 ± 0.1°</td>
</tr>
</tbody>
</table>

* \( p < 0.001 \) compared to homogenate.

**Table II**

Effect of "re-isolation" of low (control) or high (glucagon + vasopressin) \( \text{Ca}^{2+} \)-containing mitochondria on their total calcium content and 2-oxoglutarate dehydrogenase activity

Mitochondria from control livers or livers perfused for 3 min with glucagon \( (10^{-9} \text{ M}) \) plus vasopressin \( (10^{-8} \text{ M}) \) were prepared as described under "Materials and Methods." An aliquot (MI) was incubated with 300 \( \mu \text{M} \) diltiazem for 5 min for the determination of total calcium or 2-oxoglutarate dehydrogenase activity at 50 \( \mu \text{M} \) substrate concentration. The remaining mitochondria were re-isolated (through all of the steps of the preparation procedure), and calcium and 2-oxoglutarate dehydrogenase were remeasured (MII).

<table>
<thead>
<tr>
<th>Mitochondria from</th>
<th>Calcium content</th>
<th>2-Oxoglutarate dehydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MI</td>
<td>MII</td>
</tr>
<tr>
<td></td>
<td>nmo/\text{mg protein}</td>
<td>nmo/min/\text{mg protein}</td>
</tr>
<tr>
<td>Control</td>
<td>0.83 ± 0.11</td>
<td>0.89 ± 0.08</td>
</tr>
<tr>
<td>Glucagon ( (10^{-9} \text{ M}) ) + vasopressin ( (10^{-8} \text{ M}) )</td>
<td>2.23 ± 0.19°</td>
<td>2.95 ± 0.53°</td>
</tr>
</tbody>
</table>

* \( p < 0.02 \) compared to control values.

**Table III**

Calcium content and oxoglutarate dehydrogenase and PDHa activities in mitochondria isolated from control, vasopressin-, glucagon-, and glucagon + vasopressin-treated livers

Mitochondria were incubated at 30 °C for 5 min as described under "Materials and Methods." PDHa activity measured in livers was 5 ± 0.6, 27 ± 5.2, 12 ± 1.4, and 44.6 ± 4.1% of total activity for control, vasopressin-, glucagon-, and glucagon + vasopressin-treated livers, respectively. Data are mean ± S.E. of four to nine preparations.

<table>
<thead>
<tr>
<th>Calcium 2-Oxoglutarate dehydrogenase</th>
<th>PDHAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmo/\text{mg protein}</td>
<td>nmo/min/\text{mg protein}</td>
</tr>
<tr>
<td>Control</td>
<td>1.15 ± 0.12</td>
</tr>
<tr>
<td>Vasopressin ( (10^{-8} \text{ M}) )</td>
<td>2.14 ± 0.10°</td>
</tr>
<tr>
<td>Glucagon ( (10^{-7} \text{ M}) )</td>
<td>2.29 ± 0.52°</td>
</tr>
<tr>
<td>Glucagon ( (10^{-8} \text{ M}) ) + vasopressin ( (10^{-8} \text{ M}) )</td>
<td>4.89 ± 0.44°</td>
</tr>
</tbody>
</table>

* \( p < 0.005 \) compared to control.

**DISCUSSION**

The present study provides strong evidence that vasopressin and glucagon, each and additively, activate pyruvate and 2-oxoglutarate dehydrogenases in rat liver by causing increase in mitochondrial calcium. The recent publication reporting total calcium contents obtained using x-ray probe microanalysis shows that the endoplasmic reticulum is the main calcium-containing organelle (28). The purity of the mitochondrial preparation is therefore critical. The use of the Percoll purification step in the isolation of the mitochondria is a mandatory step to almost fully remove the microsomal fraction (Table I). There are also suggestions that the time needed for the preparation of the mitochondria may be critical (19). Table II shows that this is not the case for calcium content and oxidative enzyme activity. These are very stable throughout the separation procedure, provided that no Na" is present and that the mitochondria are kept in ice-cold medium, which contains an adequate amount of EGTA. Indeed, we would suggest perhaps that the latter feature is the reason behind the high values reported in previous studies where lower concentrations of EGTA were used in the homogenizing medium. This low [EGTA] would thus allow sufficient calcium to be available for uptake (even at 0 °C and in the presence of ruthenium red, especially if the perfusate [calcium] is at a physiological level (>1 mm)). Indeed, the basal values of total mitochondrial calcium measured in the present study and the values reported using x-ray probe microanalysis techniques are quite comparable and lower than any of the previously reported estimates.

It has been proposed (30) that mitochondria can either regulate dehydrogenase (at low calcium content) or buffer the cytosol (at high calcium content), but not do both. The data obtained in the present study support the proposal that the...
Hormonal Control of Liver Mitochondrial Metabolism by Ca^{2+}

Intramitochondrial [Ca^{2+}] in liver (and other mammalian tissues) (16, 18) is within the regulatory range for the Ca^{2+}-sensitive dehydrogenases. This regulatory range appears to correspond to total mitochondrial contents of between ~0.5 and 6 nmol/mg of mitochondrial protein. The latter range would appear to be below that (approximately 10 nmol/mg of protein) at which liver (and other mammalian) mitochondria can exhibit buffering behavior toward the extramitochondrial environment (46-47). Significantly, the range of total mitochondrial calcium content over which enzyme regulation could be observed in the present study with liver mitochondria is very similar to those which have previously been reported for heart mitochondria (29, 30).

Hormones which are known to increase liver cytosolic Ca^{2+}, like vasopressin or glucagon, do increase PDHa in perfused livers (5) (Table III) and in isolated hepatocytes (4, 6). This increase is still measured in mitochondria in which an increase in activity is the activity of 2-oxoglutarate dehydrogenase at low substrate concentration is also observed (Table III and Fig. 1). The increase in oxidative enzyme activities is always paralleled by an increase in mitochondrial total calcium (Fig. 1 and Table III). Glucagon is known to increase mitochondrial ATP/ADP, acetyl-CoA/CoA and NADH/NAD ratios (48). Pyruvate dehydrogenase kinase is activated by these changes. An action of the hormone at the level of the kinase may explain why PDHa is less increased by glucagon in samples from liver than in isolated mitochondria. These samples are incubated in the presence of 1 mM pyruvate in order to inhibit pyruvate dehydrogenase kinase and to study pyruvate dehydrogenase phosphatase (see "Material and Methods").

When mitochondria with a high calcium content are incubated with Na^+, a correlation is observed between the decrease in calcium content and the decrease in oxidative enzyme activities (Fig. 2). The effect of Na^+ in liver mitochondria is, however, smaller and slower than in heart mitochondria (49). Strong correlations were also observed between values obtained with mitochondria prepared from livers exposed to different treatments and those obtained with mitochondria which were loaded in vitro with different amounts of calcium (Fig. 3). Therefore, the most plausible interpretation of the results is that mitochondrial matrix free [Ca^{2+}] (at least when it is within the regulatory range for the enzymes) varies in a parallel manner with total mitochondrial calcium content. Hence, the most likely mechanism for the parallel actions of these hormones on matrix [Ca^{2+}] and total mitochondrial calcium content is that they are the consequences of the increases in cytoplasmic [Ca^{2+}] being relayed into the matrix through the action of the Ca^{2+}-transport system of the mitochondrial inner membrane. The results obtained in the present paper fully support and substantiate the similar conclu-
are performed at protein/ml and 30°C in the absence or presence of 10 mM NaCl and vasopressin; hence, it is unknown if an increase in cytosolic hormones when they are acting together (50), and content. Therefore, the intriguing possibilities remain that between the action of calcium-mobilizing hormones and those discussed (50). In this work, however, vasopressin added by vasopressin and/or glucagon in the present study. However, the present work, in addition to showing for the first time that vasopressin works in this manner and that total mitochondrial calcium varies in parallel with the oxidative enzyme activities, has the major additional advantage over the in vivo work that the effects observed can be truly assigned to the hormones, rather than to indirect effects of the hormones acting elsewhere in the intact animals. Interaction between the action of calcium-mobilizing hormones and those increasing CAMP on the level of mitochondrial calcium has been described (50). In this work, however, vasopressin added alone decreased mitochondrial calcium (50).

The source of the calcium mobilized may be different for the two hormones: mobilization of calcium from pools in the endoplasmic reticulum by vasopressin (51, 52) and/or opening of Ca2+ channels and thereby increasing calcium influx by vasopressin and/or glucagon (53). The present experiments are performed at 2.4 mM Ca2+ in the perfusate, a concentration above the K_m of Ca2+ influx as stimulated by glucagon and/or vasopressin (53).

It is not accurately known, however, if cytoplasmic [Ca2+] is increased to the same extent, or additively, by glucagon and vasopressin; hence, it is unknown if an increase in cytosolic [Ca2+] can fully explain the increase in mitochondrial calcium content. Therefore, the intriguing possibilities remain that (a) the mitochondria may represent the major target for these hormones when they are acting together (50), and (b) these hormones may bring about changes in the activities of the Ca2+-transport systems of the mitochondrial inner membrane.

Although these questions remain unanswered, the present data clearly show that hormones which increase cytosolic Ca2+ also increase total mitochondrial calcium and, by doing so, activate key oxidative enzymes.

Acknowledgements—We thank Dr. J. Cox (Department of Biochemistry, University of Geneva) for his advice and for making the Perkin-Elmer atomic absorption spectrophotometer available. We are grateful to Francine Monsch for her excellent technical assistance.

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