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Calcium inhibition of the ATP $\rightleftharpoons$ [^{32}P]P_i exchange and of net ATP synthesis catalyzed by bovine submitochondrial particles

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A previous communication (Fagian, M.M., Pereira da Silva, L. and Vercesi, A.E. (1986) Biochim. Biophys. Acta 852, 262–268) indicated that intramitochondrial calcium inhibits oxidative phosphorylation by decreasing the availability of adenine nucleotides to both the ADP/ATP translocase and the F_0F_1-ATP synthase complex. In this work we analyzed the interactions of calcium-nucleotide and magnesium-nucleotide complexes with the ATP synthase during catalysis of ATP $\rightleftharpoons$ [^{32}P]P_i exchange and net synthesis of ATP by submitochondrial particles. Concerning the ATP $\rightleftharpoons$ [^{32}P]P_i exchange reaction, calcium was ineffective as divalent cation when assayed alone. Furthermore, the addition of calcium increased the magnesium concentration required for half-maximal activation of the exchange, without changing V_{max}. With respect to net ATP synthesis, the inhibition by calcium was shown to be due to formation of the $CaADP^-$ complex, which competes with $MgADP^-$ for the active site of the F_0F_1-ATP synthase. Moreover, ATP hydrolysis was competitively inhibited by $CaATP^{2-}$, showing that calcium is able to interact with the enzyme in both forward and backward reactions in the same manner. That high calcium concentrations are required for significant inhibition of ATP synthesis indicates that this inhibition is relevant under conditions in which cytosolic calcium concentrations rise to pathological levels. Therefore, this mechanism may be responsible, in part, for the decrease in cellular ATP content that has been observed to occur when calcium accumulates in the cytosol.

Introduction

In energized mitochondria, calcium distribution between the matrix and extramitochondrial compartments under steady-state conditions is believed to be determined by the simultaneous operation of the electrophoretic calcium influx uniporter and the electroneutral exchange of matrix calcium for external sodium or protons [1–3]. Since the kinetic characteristics of these calcium influx-efflux pathways appear to be incompatible with any role of mitochondria as cytosolic calcium buffers [4], most of the recent studies in this field have been undertaken to investigate the role of this calcium transport system as a regulator of calcium-dependent functions within the mitochondrial matrix [4,5].

Depending on its concentration, extramitochondrial calcium appears to exert two opposite effects on oxidative phosphorylation in intact mitochondria. Calcium concentrations from 0.1 to 1 μM stimulate oxidative phosphorylation, whereas concentrations above 1 μM inhibit it [6,7]. The stimulation of ATP synthesis by low extramitochondrial calcium concentrations is in line with current views of the biological role of the mitochondrial transport system [4,5]. At the free cytosolic calcium concentrations supposed to occur under resting conditions (0.1–0.2 μM), the low-affinity mitochondrial uptake system for calcium is practically inactive and the free calcium concentration in the matrix appears to be lower than 1 μM [8,9]. However, with an increase such as that caused by some hormones [4,10,11] where cytosolic calcium rises from the basal value of 0.1 μM up to about 1 μM, mitochondrial-matrix calcium increases to 5–10 μM. This concentration of calcium activates three calcium-dependent dehydro-

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Abbreviations: SMP, submitochondrial particles; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CAT, carboxyatractyloside; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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genases that catalyze rate-limiting reactions of the Krebs cycle (2-oxoglutarate, NAD^+-linked isocitrate and pyruvate dehydrogenases), and stimulates oxidative metabolism and ATP production [4,5,12]. Because of these calcium effects it has been suggested that the phenomenon of respiratory control, originally proposed by Chance and Williams [13] in terms of the availability of ADP to the mitochondria, may be formulated in terms of the availability of both ADP and calcium [4,5].

On the other hand, the inhibition of oxidative phosphorylation that occurs with micromolar extramitochondrial calcium concentrations is the consequence of large mitochondrial calcium loads and certainly plays an important role in the development of cell injury associated with disruption of intracellular calcium homeostasis [14–17]. This inhibition has been attributed to different mechanisms, including inhibition of the ADP/ATP carrier [18,19], loss of internal adenine nucleotides [20], inhibition of the release of the ATPase-inhibitor protein from the F_0F_1-ATP synthase [21], and decreased availability of intramitochondrial phosphate [22] or adenine nucleotides [23,24] due to the formation of calcium complexes with these compounds [23,24].

In the present report we have investigated the effects of calcium on the activity of the F_0F_1-ATP synthase of heart submitochondrial particles (SMP). At concentrations supposed to occur normally in the matrix in situ, calcium did not affect the activity of the F_0F_1-ATP synthase. However, at concentrations higher than 50 μM, i.e., a range attained in the matrix when mitochondria accumulate more than 50 nmol Ca/mg protein [25], possibly under severe pathological conditions [14–16], both the hydrolytic and synthetic activities of the enzyme were inhibited by calcium in a purely competitive fashion. The competition found between $MgADP^-$ and $CaADP^-$ complexes during ATP synthesis might be ultimately associated with an overall decrease in the mitochondrial metabolic turnover and subsequent generalized impairment of cell functions.

Materials and Methods

Preparation of submitochondrial particles. Bovine heart mitochondria were prepared according to Löw and Vallin [26], and were stored at −70°C. MgATP submitochondrial particles were prepared as described by Lee and Ernster [27], and stored under liquid nitrogen until use.

Assay of ATPase activity. ATPase activity was assayed colorimetrically by measuring the release of P_i from ATP by the method of Fiske and SubbaRow [28] or by measuring the $[^{32}P]P_i$ released from $[\gamma\text{-}^{32}P]ATP$ (specific activity about 10^4 Bq/nmol ATP). The $[^{32}P]$phosphomolybdate complex formed was extracted using a mixture of acetone and butyl acetate [29]. The reaction mixture contained 65 mM Tris-maleate buffer, 1 μM FCCP, 50 μg/ml SMP protein and different concentrations of ATP, ADP, $CaCl_2$ and $MgCl_2$, in a final volume of 0.5 ml at pH 7.5 and 37°C. An ATP-regenerating system (20 mM KCl, 1 mM phospho*enol*-pyruvate, 10 units pyruvate kinase) and 0.2 mM EGTA were used in the experiments with non-radioactive ATP shown in Fig. 3. The reactions were initiated by the addition of $MgCl_2$ after 5 min preincubation of the particles in the reaction medium, and were stopped 5 min later by the addition of 0.2 M perchloric acid. Under these conditions the reactions follow a linear time-course during the period of assay. The same control preincubation was done in ATP synthesis experiments to ensure linearity of the reaction with time (see below).

Assay of ATP ⇌ $[^{32}P]P_i$ exchange activity. The exchange reaction was assayed by measuring the formation of $[\gamma\text{-}^{32}P]ATP$ from ADP and $[^{32}P]P_i$. The basic medium for the ATP ⇌ $[^{32}P]P_i$ exchange contained 65 mM Tris-maleate buffer, 4 mM potassium $[^{32}P]$phosphate (spec. act. about 10^6 Bq/nmol P_i), 1 mM ATP, 5 μM CAT, 50 μg/ml SMP protein and variable concentrations of EGTA, ADP, $MgCl_2$ and $CaCl_2$, in a final volume of 0.5 ml at pH 7.5 and 37°C. The reactions were initiated by addition of $MgCl_2$ after 5 min preincubation of the particles in the reaction medium, and were stopped 5 min later by the addition of ammonium molybdate in sulfuric acid. The excess $[^{32}P]P_i$ was removed as the phosphomolybdate complex using six consecutive extractions with acetone plus butyl acetate, leaving the $[\gamma\text{-}^{32}P]ATP$ in the aqueous phase [30]. Aliquots of these molybdate extracts were counted in a liquid scintillation counter.

Assay of net ATP synthesis. Net synthesis of ATP from $[^{32}P]P_i$ and ADP was measured by coupling the production of ATP to the phosphorylation of glucose by hexokinase. The glucose 6-$[^{32}P]$phosphate so produced was measured in the aqueous phase after extraction of the excess $[^{32}P]P_i$ as described above. The basic medium for net ATP synthesis contained 65 mM Tris-maleate buffer, 4 mM potassium $[^{32}P]$phosphate (spec. act. about 10^6 Bq/nmol P_i), 0.2 mM EGTA, 10 mM AMP, 5 μM CAT, 26 units hexokinase, 20 mM glucose, 5 mM succinate, 50 μg/ml SMP protein and variable concentrations of ADP, $MgCl_2$ and $CaCl_2$, in a final volume of 1.0 ml at pH 7.5 and 37°C. The reactions were initiated by the addition of $MgCl_2$ after 5 min preincubation of the particles in this reaction medium, and were stopped with a solution of ammonium molybdate in sulfuric acid.

Determination of transmembrane electrical potential in SMP. SMP were incubated in the reaction medium containing 3 μM tetraphenylboron (TPB^-). The concentration of TPB^- in the medium was continuously monitored with a TPB^--sensitive electrode prepared in

our laboratory according to Vuokila and Hassinen [31]. The membrane potential was then calculated assuming that the TPB^- distribution between the medium and the interior of the particles follows the Nernst equation.

Reagents and solutions. $[^{32}P]P_i$ was obtained from the Brazilian Institute of Atomic Energy and purified by the method of Boyer and Bryan [32]. [γ-^{32}P]ATP was prepared according to Glynn and Chappell [33]. Nucleotides, CAT, EGTA, CDTA, FCCP, pyruvate kinase, phospho*enol*pyruvate and hexokinase were from Sigma Chemical Co. Other reagents were of analytical grade.

Calculations. The values shown represent the means of at least four experiments, and standard errors were less than 10%. Concentrations of the ionic species and complexes at equilibrium were calculated employing an iterative computer program [34] that was modified [35] from that described by Fabiato and Fabiato [36] in order to allow different permutations of the magnesium and calcium species. The complexation of calcium and magnesium by maleate was also taken into account [37].

Results

$ATP \rightleftharpoons [^{32}P]P_i$ exchange: comparison of the effectiveness of magnesium and calcium, and effect of calcium in magnesium-containing media

Fig. 1 shows that the rate of exchange increased in a complex way with magnesium in the entire range of concentrations employed, whereas calcium alone had no effect on the formation of [γ-^{32}P]ATP. In magnesium-containing media the addition of calcium (ionized Ca^{2+}, 290–346 μM) promoted inhibition of the exchange, and the $S_{0.5}$ for magnesium increased from 0.62 mM in the absence of calcium to 0.90 mM in the presence of calcium, suggesting that a competition with magnesium may be involved in calcium inhibition of the exchange reaction. The V_{max} of the exchange reaction remained unchanged upon addition of calcium (Fig. 1), thus reinforcing the view that the two cations, or the corresponding nucleotide-divalent cation complexes [38,39], compete for the same site (or sites) of the enzyme. In the absence of contaminating magnesium (0.2 mM CDTA), calcium was unable to sustain $ATP \rightleftharpoons [^{32}P]P_i$ exchange up to 10 mM.

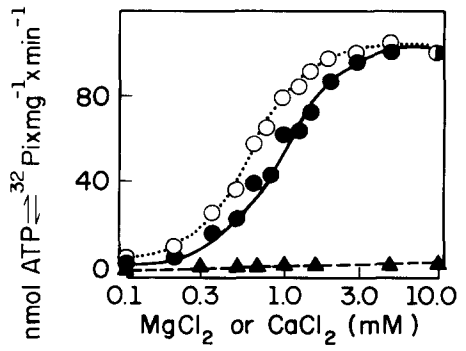

Fig. 1. $MgCl_2$ and $CaCl_2$ concentration dependence of $ATP \rightleftharpoons [^{32}P]P_i$ exchange. The basic medium for $ATP \rightleftharpoons [^{32}P]P_i$ exchange described under Materials and Methods was supplied with 4 mM ADP and the concentrations of $MgCl_2$ shown on the abscissa in the absence of calcium (○) or in the presence of enough $CaCl_2$ to give ionized Ca^{2+} concentrations ranging from 290 to 346 μM and $CaADP^-$ concentrations ranging from 560 to 504 μM (●). ▲, Assays performed in the presence of the $CaCl_2$ concentrations shown on the abscissa and 0.2 mM CDTA. The $S_{0.5}$ values for magnesium (0.62 and 0.90 mM in the absence and in the presence of calcium, respectively) were calculated by non-linear regression.

TABLE I

Calcium inhibition of the $ATP \rightleftharpoons [^{32}P]P_i$ exchange reaction without added ADP in the presence of different $MgCl_2$ concentrations

Experimental conditions were those of Fig. 1, except that ADP was omitted (ADP was only that derived from ATP hydrolysis) and $MgCl_2$ concentrations were those shown in the Table. The ionized Ca^{2+} concentrations were 334, 365 and 446 μM in the presence of 0.1, 2 and 10 mM $MgCl_2$, respectively.

$MgCl_2$ (mM)	$ATP \rightleftharpoons [^{32}P]P_i$ (nmol·mg^{-1}·min^{-1})		Inhibition by Ca^{2+} (%)
	without Ca^{2+}	with Ca^{2+}	
0.1	19	5	74
2	125	73	58
10	142	134	6

Since changes in ADP concentrations modify the interactions of magnesium with the enzyme [40], the effect of calcium on the $ATP \rightleftharpoons [^{32}P]P_i$ exchange reaction was also explored in media in which the sole ADP present was that derived from ATP hydrolysis, at saturating (10 mM), subsaturating (2 mM) and very low (0.1 mM) $MgCl_2$ concentrations (Table I). In the presence of 10 mM $MgCl_2$ the rate of exchange was not modified by calcium as in a medium with high ADP (4 mM; Fig. 1). Calcium inhibition attained 58% and 74% with 2 mM and 0.1 mM $MgCl_2$, respectively.

The inhibition promoted by calcium was not due to partial collapse of the transmembrane electrical potential ($\Delta\Psi$). Regardless of whether mitochondrial particles were energized by ATP hydrolysis ($ATP \rightleftharpoons [^{32}P]P_i$ exchange conditions; Fig. 1) or by succinate oxidation (net ATP synthesis conditions; see below Fig. 2), there was no significant difference in $\Delta\Psi$ when it was measured in the absence or in the presence of calcium (data not shown).

Effect of increasing $CaADP^-$ and Ca^{2+} on net ATP synthesis at fixed $MgADP^-$ concentrations

$ATP \rightleftharpoons [^{32}P]P_i$ exchange and net ATP synthesis have different responses towards the same ligands, and this has been ascribed to differences in the mechanism by which the particles are energized: by ATP hydrolysis or by electron transport [41]. For this reason, the influence of calcium were also studied in the presence of an ATP

trap, oxidative phosphorylation being sustained by the addition of succinate.

The experiments depicted in Fig. 2 were done to establish whether or not the $MgADP^-$ and $CaADP^-$ complexes compete for the same site of the synthase, and to determine the kinetic parameter of inhibition, K_i. The Dixon plot of inverse velocity against [$CaADP^-$] at two different fixed $MgADP^-$ concentrations (0.12 and 0.52 mM) gave straight lines that intersected above the [$CaADP^-$] axis. In addition, when [$CaADP^-$] was equal to the K_i (0.19 mM), $1/v$ was equal to $1/V_{max}$ measured with 20 mM $MgCl_2$ ($MgADP^- = 1.67$ mM), confirming that the $CaADP^-$ complex acts as a pure competitive inhibitor with respect to the true substrate $MgADP^-$.

Since free Ca^{2+} at concentrations supposed to occur in mitochondria in situ (below 10 μM [8,9]) stimulate the overall oxidative phosphorylation process by activating the rate-limiting dehydrogenase-catalyzed reactions [4,5], we also studied the effect of Ca^{2+} in this concentration range on the net synthesis of ATP to verify whether the activation of dehydrogenases could be accompanied by a simultaneous stimulation of the F_0F_1 system by the cation. Contrary to what happens with the dehydrogenases, the ATP synthase itself was not activated by Ca^{2+} concentrations between 0.9 and 30 μM (experiments not shown).

Hydrolysis of ATP: dependence on $CaATP^{2-}$ concentration

In a previous report [24] it was shown that the addition of calcium inhibited ATP hydrolysis catalyzed by submitochondrial particles. To verify whether or not nucleotide-divalent metal complexes compete during

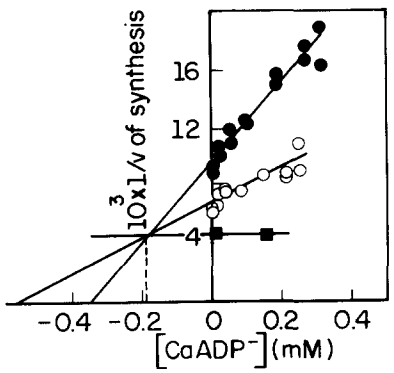

Fig. 2. Dixon plot of net ATP synthesis. Dependence on $CaADP^-$ concentration. The reaction was performed using the basic medium for net ATP synthesis described under Materials and Methods, the concentrations of $CaADP^-$ shown on the abscissa, and three fixed $MgADP^-$ concentrations (0.12 mM, ●; 0.52 mM, ○; or 1.67 mM, ■). The total amounts of ADP, $MgCl_2$ and $CaCl_2$ necessary to form the desired cation-complex concentrations were calculated as described under Materials and Methods. The v values whose reciprocal are shown on the ordinate are in nmol·mg^{-1}·min^{-1}. The K_i value for $CaADP^-$ was calculated by the least squares method.

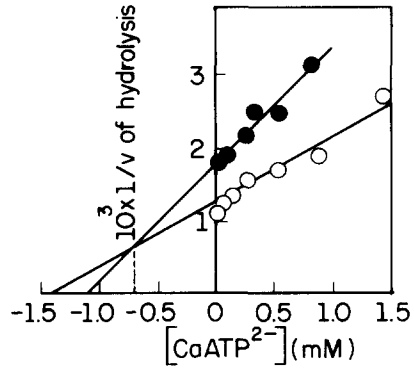

Fig. 3. Dixon plot of ATP hydrolysis: Dependence on $CaATP^{2-}$ concentration. The reaction was carried out as indicated under Materials and Methods at various $CaATP^{2-}$ concentrations with fixed $MgATP^{2-}$ concentrations (0.15 mM, ●; or 0.57 mM, ○). The total ATP, $MgCl_2$ and $CaCl_2$ concentrations needed to give the desired concentrations of each complex were calculated as indicated under Materials and Methods. The v values on the ordinate are in nmol $P_i \cdot mg^{-1} \cdot min^{-1}$. The K_i value for $CaATP^{2-}$ was calculated by the least-squares method.

cycles of hydrolysis as well as during the cycles of synthesis, the following experiments were done. When ATP hydrolysis was measured at two different $MgATP^{2-}$ concentrations, there was a progressive inhibition by $CaATP^{2-}$, with a competitive pattern (Fig. 3). The K_i value for $CaATP^{2-}$ calculated at the intersection of both lines was 0.71 mM.

Discussion

Previous data indicated that the inhibition of oxidative phosphorylation by intramitochondrial calcium was due to a decrease in the availability of adenine nucleotides to both the ADP/ATP carrier and the ATP synthase caused by the complexation of these nucleotides with calcium [24]. Those conclusions were based on measurements of the hydrolytic activity of submitochondrial particles in the presence or absence of calcium.

In the present study, the data obtained with the ATP $\rightleftharpoons$ [^{32}P]P_i exchange reaction indicate that calcium competes with magnesium when ATP synthesis occurs at the same time as hydrolysis (Fig. 1). Fig. 2 suggests that this phenomenon is accounted for through the formation of $CaADP^-$, which competes with the kinetically competent $MgADP^-$ complex.

It should be emphasized that at saturating $MgCl_2$ concentrations, the V_{max} of exchange is not affected by the presence of calcium regardless of whether a high or a low ADP concentration is used (Fig. 1; Table I). This observation indicates that ATP synthesis is not affected by calcium when the synthase is saturated with magnesium, and we infer that the effects of calcium at any ADP/ATP ratio depend, at least in part, on intramitochondrial magnesium levels.

Fig. 1 also shows that although calcium is able to compete with magnesium, it is not able to promote the formation of [γ-^{32}P]ATP during simultaneous cycles of ATP hydrolysis and ATP synthesis, when it is assayed as the unique divalent cation. This observation agrees with data of Yohda et al. [42], obtained with the use of F_1-ATPase from a thermophilic bacterium. They showed that calcium was not able to sustain ATP synthesis, ATPase activity or proton translocation, which are partial reactions of the ATP $\rightleftharpoons$ [^{32}P]P_i exchange reaction described in this manuscript. The magnesium specificity of the F_0F_1-ATP synthase appears to be related with the amino acid structure of the enzyme, since it was shown to be modified by mutations around the catalytic site [42,43].

The observation that ATP $\rightleftharpoons$ [^{32}P]P_i exchange is more strongly inhibited by calcium in the presence of a lower magnesium concentration (Table I), reinforces the hypothesis that a competition between $CaADP^-$ and $MgADP^-$ is responsible for the inhibition by calcium. Indeed, the data of Fig. 2 show a purely competitive pattern of inhibition by $CaADP^-$, similar to the effect of $CaATP^{2-}$ on hydrolysis (Fig. 3). Thus, the results presented in this report show, to our knowledge for the first time, that calcium inhibits both synthetic and hydrolytic cycles catalyzed by the mitochondrial F_0F_1-ATPase, by forming complexes with ADP and ATP that compete with the corresponding magnesium complex substrates. According to recent findings [38,39], the true hydrolytic substrate of F_0F_1-ATPase is the Δ,β,γ-ATPMg bidentate complex. It may be that displacement of the magnesium-AT[D]P chelate with this stereospecific configuration by calcium-AT[D]P involves the amino acids which have been shown to be essential for catalysis by the F_1 portion of F_0F_1-ATP synthase. This sequence, which is present in the β-subunits and is conserved in other nucleotide binding proteins [44,45], appears to be located close to the catalytic site, and in the presence of magnesium may undergoe a conformational change essential for the catalysis step. In this view, the movement could not occur in the presence of the calcium chelates.

In the present experiments, a parallel effect of free Ca^{2+} can not be completely ruled out, since there is evidence for a site whose occupancy by free Mg^{2+} accelerates the turnover of the enzyme [40]. Moreover, whether or not the effects of calcium are modulated by its binding to the recently isolated Ca^{2+}-binding ATPase inhibitor protein [46] remains to be elucidated.

Conclusion

Although the calcium concentrations that caused appreciable inhibition of both ATP synthesis (this manuscript) and ATP hydrolysis (Fig. 3, this manuscript; and Ref. 24) are in the range that causes inhibition of oxidative phosphorylation in isolated mitochondria [6,18–23,47], they are much higher than those supposed to occur in the matrix of mitochondria in situ under normal resting conditions [8,9]. However, these results are certainly relevant to conditions in which intracellular calcium homeostasis is disrupted and the cytosolic calcium increases to pathological levels [14–16]. Such changes occur with several toxic agents [16,17,48], ischemia [16,48–50], and other injuries [51–54] that appear to be mediated by an increase in cytosolic calcium. In this regard, it is noteworthy that the consequent accumulation of the cation in the mitochondrial matrix will tend to overwhelm recovery of the normal cellular functions, if it inhibits ATP synthesis and allows ATP in the cytosol to fall below the level necessary to sustain the energy-utilizing processes.

Acknowledgments

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