CONTROL OF CATION MOVEMENTS IN LIVER MITOCHONDRIA BY A CYTOPLASMIC FACTOR.

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SUMMARY: Externally added Ca^{2+} above 20 n moles Ca^{2+} / 1 mg mitochondrial protein causes ejection of Mg^{2+} and K^+ from liver mitochondria suspended in sucrose-Tris medium. Energy dependent Ca^{2+} uptake or retention of K^+ in mitochondria depends on bound Mg^{2+}. A trace amount of a cytoplasmic factor counteracts the Mg^{2+} and K^+ labilizing effect of Ca^{2+} and causes rapid uptake of Ca^{2+}. Uncoupling agents inhibit the effect of the cytoplasmic factor while oligomycin but not aurovertin mimic its effect on Mg^{2+} and Ca^{2+} but not on K^+. The cellular role and mode of action of the cytoplasmic factor is interpreted in terms of regulation of energy transfer.

The ability of mitochondria to respond with increased metabolism to uncouplers (1,2) or to retain intramitochondrially formed α-ketoglutarate (3), depends on the presence of inner membrane-bound Mg^{2+}. The inducibility of latent mitochondrial ATP-ase by uncouplers and oxidative phosphorylation itself were also shown to depend on bound Mg^{2+} (4). A trace amount of an as yet chemically unidentified cytoplasmic metabolic factor (CMF)** counteracted Mg^{2+} labilization (by uncouplers + ADP) and simultaneously preserved various energy coupled mitochondrial processes (1-5). It was concluded that the bound Mg^{2+} containing system on which CMF acts is an essential component of the mitochondrial energy transducing apparatus. In view of the artificial experimental conditions necessary to demonstrate a mitochondrial CMF requirement, no CMF-dependent physiological process has so far been identified.

Because of well-known similarities between certain effects of uncouplers and of Ca^{2+} (cf. 6), we assumed that a CMF sensitive in vitro system may be found if artificial uncouplers were replaced by low levels of Ca^{2+}. In agreement with this expectation, we found that CMF not only counteracts Ca^{2+} induced labilization of bound Mg^{2+}, but greatly augments Ca^{2+} uptake and inhibits the outward movement of intramitochondrial K^+. Since no artificial reagents were necessary to show a large in vitro effect of CMF, this experimental design tends to identify CMF as a regulat of energy coupled mitochondrial ion movements.

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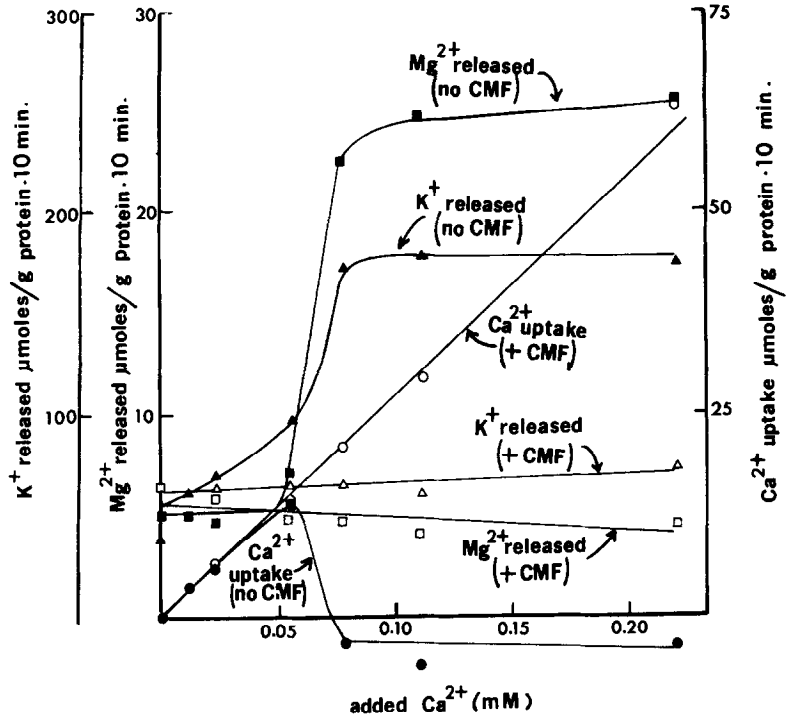
**Abbreviations: CMF - cytoplasmic metabolic factor; DNP - 2,4-dinitrophenol; BSA bovine serum albumin.

EXPERIMENTAL PROCEDURES

Rat liver mitochondria from 24 hour starved male rats (200 g body wt.) were prepared in mannitol-sucrose-BSA (7), washed twice in BSA free medium and resuspended in 0.25 M sucrose + 30 mM Tris-HCl (pH 7.4). Mitochondria prepared in this manner contained 29-35 μ moles Mg^{2+}, 12-16 μ moles Ca^{2+} and 210-310 μ moles K^+ per 1 g mitochondrial protein. K^+, Mg^{2+} and Ca^{2+} movements were assayed by direct analyses for cations by atomic absorption (Perkin Elmer 403 atomic absorption instrument) as described earlier (1, 2), following aerobic incubation for 10 minutes at 30^0 (in a Dubnoff shaker). The assay system consisted of 15 mg mitochondria (in terms of protein), suspended in 6 ml sucrose-Tris medium and additions as specified in Results. Ion movements were calculated from ion distribution in mitochondria and in the suspending medium separated by centrifugation at 0 time and after 10 minutes incubation (cf. 1, 2). CMF was the same preparation from pig liver as employed previously (1, 2). Highly purified CMF (up to 100,000 fold) gave the same results as the relatively crude (5 to 800 fold purified), but more stable preparations. Scarcity of CMF of highest purity prohibited its routine use.

RESULTS

The effect of externally added Ca^{2+} on ion movements of mitochondria in sucrose-Tris is shown in the Figure. The range of Ca^{2+} concentration (0.0 to 0.2 mM) corresponded with the amount of Ca^{2+} found by atomic absorption analysis in the cytosol of rat liver (0.18 μ mole Ca^{2+} per cytosol, corresponding to 1 g of rat liver). In terms of Ca^{2+}/mitochondrial protein ratio, our range varied between 3 n moles Ca^{2+}/1 mg to 120 n moles Ca^{2+}/1 mg mitochondrial protein. Up to a critical level of Ca^{2+} it was taken up into mitochondria and only a relatively slow rate of K^+ ejection was noticeable. Above a critical Ca^{2+} concentration (0.05 mM, or 20 n moles Ca^{2+}/1 mg protein), rapid ejection of bound Mg^{2+} and K^+ took place. Simultaneously Ca^{2+} uptake was not only inhibited, but some originally mitochondrial-bound Ca^{2+} was also extruded. This experiment shows that Ca^{2+} uptake is dependent on mitochondrial bound Mg^{2+} and so is the ability of mitochondria to retain large concentrations of K^+. All effects of Ca^{2+} are reversed by CMF. Not only Ca^{2+} induced Mg^{2+} and K^+ ejection are prevented by CMF, but all externally added Ca^{2+} is quantitatively absorbed into mitochondria. When Ca^{2+} levels are raised to 0.5 to 1 mM, the effect of CMF fails (not shown). CMF alone, without added Ca^{2+}, inhibits spontaneous loss of mitochondrial K^+, but this process is much slower than the large effects observed in the presence of added Ca^{2+}. In collaboration with Dr. H.A. Lardy, the kinetics of Ca^{2+}-induced K^+ ejection was studied with the aid of ion specific electrodes, (cf. 8). In agreement with the results obtained by atomic absorption, addition of 0.1 mM Ca^{2+} caused a significant increase in spontaneous K^+ ejection which was inhibited by CMF. Apart from some increase in endogenous metabolism, no changes in light scattering or H^+ ejection were observed during a 10 minute period (at 30^0) of incubation.



Experimental details for results shown in the Table and Figure are given in Methods. Abbreviations for the Table: Oligo = oligomycin (2.5 µg/1 mg protein); aurov = aurovertin (2.0 µg/1 mg protein). The amount of CMF was 2.7 µ moles reducing equivalent/1 ml incubation mixture (cf. 1, 2). (Both antibiotics were generous gifts of Dr. H.A. Lardy).

TABLE

The effects of Ca^{2+}, CMF and reagents affecting energy transfer on ion movements

(Results are expressed as µmoles ions/1 g mitochondrial protein, translocated in 10 minutes at 30°)

Batches	No.	Variable Conditions	Mg^{2+} Ejection	Ca^{2+} Uptake	Ca^{2+} Ejection	K^{+} Ejection
A	1.	Ca^{2+} (0.1 mM)	19.4	7.5	-	210
	2.	Ca^{2+} " + CMF	1.6	56.3	-	80
	3.	Ca^{2+} " + DNP (50µM)	18.0	0.8	-	180
	4.	Ca^{2+} " + DNP " + CMF	24.5	1.6	-	190
B	5.	Ca^{2+} (0.15 mM)	31.2	-	4.2	230
	6.	Ca^{2+} " + CMF	3.6	61.5	-	70
	7.	Ca^{2+} " + Oligo.	10.4	49.0	-	163
	8.	Ca^{2+} " + Oligo. + CMF	3.9	59.9	-	71
	9.	Ca^{2+} " + Aurov.	29.1	1.4	-	218
	10.	Ca^{2+} " + Aurov. + CMF	3.8	59.8	-	70.
C	11.	No Ca^{2+}	3.0	-	1.2	70
	12.	No Ca^{2+} + CMF	4.6	-	1.2	70
	13.	No Ca^{2+} + DNP (50 µM)	3.0	-	10.6	100
	14.	No Ca^{2+} " DNP " + CMF	5.3	-	9.0	110

During the brief experimental period (10 minutes), mitochondria exhibited significant endogenous metabolism (0.3 to 0.4 μ moles O_2 absorbed in 10 minutes per 10 mg mitochondrial protein). It was therefore assumed that energy required for Ca^{2+} uptake as well as K^+ retention was derived from endogenous metabolism. The nature of ion movements and energy dependence of the effect of CMF was further studied with the aid of reagents known to influence mitochondrial energy transfer. Results are shown in the Table. Since different batches of mitochondria show some variation in the magnitude of Ca^{2+} induced ion movements, comparable series of measurements performed with the same batch are also indicated in the Table. In agreement with results shown in the Figure, Ca^{2+} releases bound Mg^{2+} and mitochondria lose their K^+ (Exp. 1, 5). These effects of Ca^{2+} are prevented by CMF and there is large Ca^{2+} uptake (Exp. 2, 6). A typical uncoupler, DNP, inhibits the Ca^{2+} accumulation induced by CMF and simultaneously prevents the protective effect of CMF on bound Mg^{2+} and K^+ (Exps. 3, 4). Oligomycin alone has a similar effect to CMF, inasmuch as it diminishes the Ca^{2+} induced Mg^{2+} ejection, supports Ca^{2+} accumulation but not K^+ retention (Exp. 7). On the other hand, oligomycin at the level tested does not interfere with the action of CMF (Exp. 8). In contrast to oligomycin, aurovertin alone has no effect and does not influence the action of CMF either (Exps. 9, 10). In the absence of Ca^{2+}, neither oligomycin nor aurovertin has any effect on ion movements (not shown); however, DNP causes a significant loss of mitochondrial Ca^{2+}, and this effect is uninfluenced by CMF (Exps. 13 & 14). CMF alone has no effect (Exp. 12; compare with 11).

DISCUSSION

A biochemical regulatory function of CMF is indicated by the following experimental results. Added Ca^{2+} in amounts present in liver cytoplasm, in the absence of any other agent can labilize bound mitochondrial Mg^{2+}, an effect which coincides with the extrusion of mitochondrial K^+. CMF prevents these events initiated by Ca^{2+} and greatly augments Ca^{2+} uptake. Since CMF alone has no effect, Ca^{2+} in this system plays the role of an apparent cofactor of CMF. Cation (i.e. K^+) retention and Ca^{2+} uptake depend on bound Mg^{2+}. Once bound Mg^{2+} is labilized by Ca^{2+} (or by DNP + ADP), the ability of mitochondria to retain K^+ or to accumulate Ca^{2+}, even in the presence of externally applied energy sources (ATP or substrates), is irreversibly lost. The same is true for valinomycin induced energy coupled K^+ uptake, a process which critically depends on mitochondria bound Mg^{2+}*. In all systems where bound Mg^{2+}-dependent energy transfer can be demonstrate CMF serves as a cofactor and experimental demonstration of CMF requirement depends on the chosen model where a certain type of energy transfer has to be made rate limiting.

Interpretation of the mode of action of CMF on Ca^{2+} accumulation and K^+ retention has to take into account the fact that the prerequisite of CMF dependent phenomena is an appreciable rate of endogenous metabolism as observed with freshly prepared intact

*Unpublished results.

mitochondria. It is significant that in our system, oligomycin (cf. 9), a well known stabilizer of some non-phosphorylated energy rich mitochondrial component (or system), acts in a similar manner to CMF (see Table) with respect to Ca^{2+} uptake and Mg^{2+} retention, but not K^{+} translocation. Previous work already disclosed a certain similarity of action between CMF and oligomycin. It is well known that oligomycin is in certain respects an antagonist of DNP-type uncouplers. Since CMF also antagonizes DNP (+ ADP) induced Mg^{2+} ejection, it is apparent that a definite similarity exists between mitochondrial effects of oligomycin and CMF. This experimental evidence supports the view that CMF acts on energy transduction. That aurovertin fails to mimic CMF, but oligomycin does, suggests that CMF may interact with a site which transfers energy also to the ATP synthesizing system.

A critical aspect of the mode of action of CMF depends on compartmentalization: i.e. intramitochondrial localization of energy source and extramitochondrially imposed energy requiring process: in our case Ca^{2+} uptake. It is highly probable that Ca^{2+} translocation is a carrier mediated process (10, 11) and CMF in some way provides coupling of Ca^{2+} transfer to intramitochondrial energy sources, presumably via a bound Mg^{2+}-containing system. An entirely different situation is created when Ca^{2+} uptake is supported by externally supplied energy sources (ATP, substrates) and this system can obscure the regulatory role of CMF which requires anisotropic orientation of intramitochondrial energy generation.

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