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Intramitochondrial K^+ as activator of carboxyatractyloside-induced Ca^{2+} release

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The role of intramitochondrial K^+ content on the increase in membrane permeability to Ca^{2+}, as induced by carboxyatractyloside was studied. In mitochondria containing a high K^+ concentration (83 nmol/mg), carboxyatractyloside induced a fast and extensive mitochondrial Ca^{2+} release, membrane de-energization, and swelling. Conversely, in K^+-depleted mitochondria (11 nmol/mg), carboxyatractyloside was ineffective. The addition of 40 mM K^+ to K^+-depleted mitochondria restored the capability of atractyloside to induce an increase in membrane permeability to Ca^{2+} release. The determination of matrix free Ca^{2+} concentration showed that, at an external free-Ca^{2+} concentration of 0.8 μM, control mitochondria contained 3.9 μM of free Ca^{2+} whereas K^+-depleted mitochondria contained 0.9 μM free Ca^{2+}. It is proposed that intramitochondrial K^+ affects the matrix free Ca^{2+} concentration required to induce a state of high membrane permeability.

Introduction

Intramitochondrial Ca^{2+} homeostasis is controlled by a well defined uptake pathway and by release mechanisms that have been only partially characterized. The uptake of Ca^{2+} into the matrix is catalyzed by an electrophoretic uniporter that is driven by the membrane potential [1]. The main efflux mechanisms are through an electroneutral Na^+/Ca^+ exchanger [2], and a H^+/Ca^{2+} exchange reaction [3]. In addition, Ca^{2+} release may involve an unspecific increase in membrane permeability [4]. This latter mechanism requires an inducing agent; the inducing agents are strikingly different, i.e. sulfhydryl reagents [5–9], peroxides [10, 11], heavy metals, such as Pb^{2+} and Hg^{2+} [12–14], metabolites [15–17], benzodiazepines [18], and other pharmacological agents [19,20].

Regardless of the Ca^{2+} releasing agent, the unspecific membrane leakiness can be suppressed by adenine nucleotides, ADP being the most effective [21–23]. The latter observation has led to the conclusion that the adenine nucleotide translocase plays a central role in mitochondrial Ca^{2+} homeostasis [24]. Such a proposal is strengthened by the fact that the external inhibitors of adenine nucleotide translocase (ANT), atractyloside and carboxyatractyloside (CAT) induce mitochondrial Ca^{2+} efflux [25–27]. In contrast, the internal inhibitor, bongkrekic acid, induces mitochondrial Ca^{2+} retention [28]. It has been claimed that the mechanism by which atractyloside and CAT promote calcium release is through the fixing of ANT to the cytosolic side of the inner membrane [26].

The increase in membrane permeability brought about by the toxic agents requires the over-loading of mitochondria with Ca^{2+} [29]. Indeed a critical level of exchangeable Ca^{2+} is required to increase membrane permeability by Hg^{2+} plus dithiothreitol [13,14]. Intramitochondrial Ca^{2+} is also required for the CAT-induced membrane transition [30]. Internal free-Ca^{2+} concentration is probably directly related to the density of intramitochondrial negative charges, which is turn would be controlled by the concentration of internal cations. As K^+ is the main intramitochondrial cation

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

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[31], it was hypothesized that the concentration of matrix K^+ can regulate the amount of free Ca^{2+} available to induce membrane transition. This work describes data that demonstrate that K^+ is an essential factor in the activation of CAT-induced mitochondrial Ca^{2+} release, membrane depolarization, and mitochondrial swelling.

Materials and Methods

Rat kidney mitochondria were obtained as reported elsewhere [32]. Potassium-depleted mitochondria were prepared essentially as described by Gómez-Puyou et al. [33]; mitochondria were incubated in a medium containing 100 mM sucrose, 75 mM NaCl, 10 mM phosphate, 10 mM glutamate, and 10 mM EDTA, adjusted to pH 7.3 with Tris base. Protein was determined by the method of Lowry et al. [34]. Calcium movement was followed by dual spectrometry at 685–675 nm with Arsenazo III as indicator [35]. Mitochondrial Ca^{2+} uptake and release was also assayed by using the radionuclide $^{45}Ca^{2+}$ (spec. act. 1000 cpm/nmol). Na^+ and Rb^+ uptake was followed by using $^{22}Na^+$ (spec. act. 23492 cpm/nmol), and $^{86}Rb^+$ (spec. act. 1518 cpm/nmol). Swelling of mitochondria was estimated by measuring the changes in optical density at 540 nm [36]. Mitochondrial transmembrane potential was analyzed spectrophotometrically at 533–511 nm using the dye safranine [37]. Mitochondrial K^+ content was determined flamometrically in acid extracts [33]. Matrix free Ca^{2+} concentration was determined by using the Ca^{2+} fluorescent indicator Fluo 3 [38]; kidney mitochondria were incubated with 10 μM Fluo 3-AM (Molecular Probes) in a medium containing 250 mM sucrose, 10 mM Hepes, 0.5 mM EGTA, 0.5% albumin, 1 mM ADP, 1 mM $MgCl_2$, adjusted to pH 7.4, for 30 min at 30 °C. Fluorescence of Fluo 3-loaded mitochondria was measured in an SPF-Aminco spectrofluorometer equiped for continuous gassing with 100% O_2, magnetic stirring, and thermostated at 25 °C; the excitation wavelength was 500 nm and emission was taken at 530 nm. Calibration of the signal was achieved by reference to a fluorescence minimum generated by addition of 5 mM Tris (pH 10.5), 0.005% deoxycholate, and 5 mM EGTA. The fluorescence maximum was attained by addition of an excess of $CaCl_2$ (10 mM). The dissociation constant of the complex Fluo 3-Ca was assumed to be 400 nM at 25 °C. [38].

Results

Effect of carboxyatractyloside on membrane permeability of K^+-containing mitochondria

Fig. 1A shows that the addition of 5 μM carboxyatractyloside (CAT) to Ca^{2+}-loaded mitochondria induced a fast and extensive release of Ca^{2+}. In contrast, CAT did not stimulate the efflux of accumulated Sr^{2+}. These results support previous evidence that the CAT-induced membrane transition is dependent on Ca^{2+}, and that Sr^{2+} is a poor substitute [30].

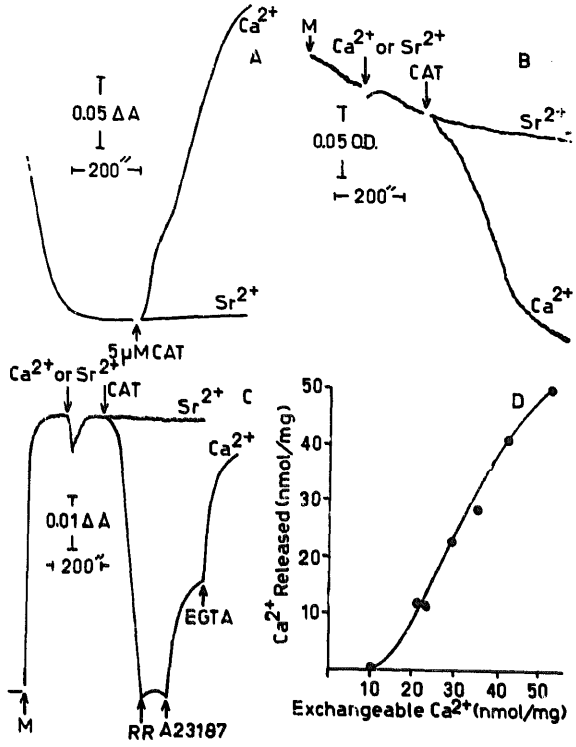

Fig. 1. Effect of carboxyatractyloside on retention of Ca^{2+} swelling, and membrane potential of mitochondria. Mitochondria (M, 2 mg protein) were added to an incubation media containing in A, 250 mM sucrose; 10 mM succinate; 10 mM Hepes; 1 mM Hepes; 1 mM phosphate; 10 mM acetate; 200 μM ADP; 10 μg rotenone; 5 μg oligomycin; 50 μM $CaCl_2$ or 50 μM $SrCl_2$, and 50 μM Arsenazo III. Where indicated carboxyatractyloside was added (CAT). In B, mitochondria were added to incubation mixtures similar to those described for A, except that the media also contained 10 μM EGTA, and that Arsenazo III was omitted. Where indicated, 5 μM CAT was added. In C, mitochondria were incubated similarly to that described for A, except that 10 μM safranine was added. Where indicated, 0.166 μM Ruthenium red (RR), 2 μM A23187, and 100 μM EGTA were added. In D, mitochondria were added to media similar to those described for A, containing in addition $^{45}CaCl_2$, and 0, 1, 2, 2.5, and 3 mM oxalate. After 3 min of incubation, aliquots of 0.2 ml were filtered to estimate the accumulated cation. Immediately, 5 μM CAT was added. After 6 min, aliquots of 0.2 ml were filtered to estimate $^{45}Ca^{2+}$ released. To establish the amount of exchangeable Ca^{2+}, mitochondria were incubated 3 min, and aliquots of 0.2 ml were filtered to know Ca^{2+} accumulation. Immediately, 0.5 μM Ruthenium red and 2 μM A23187 were added to induce Ca^{2+} efflux, then aliquots of 0.2 ml were filtered.

An unspecific increase in membrane permeability can be monitored by mitochondrial swelling. Fig. 1B shows that the addition of 5 μM CAT to mitochondria loaded with Ca^{2+} induced an increase in membrane permeability but failed to do so in mitochondria loaded with Sr^{2+}. Fig. 1C shows that high concentrations of intramitochondrial Ca^{2+} are needed to attain CAT-in-

duced membrane damage, i.e., when 5 μM CAT was added after the addition of 50 μM Ca^{2+}, mitochondria became de-energized. Although the damage caused by Ca^{2+} releasing agents on membrane has been attributed to a Ca^{2+} cycling across the membrane [39], we observed that Ruthenium red did not restore the membrane potential that had been collapsed by CAT in Ca^{2+}loaded mitochondria. However, when Ca^{2+} efflux was induced by the ionophore A23187 and 200 μM EGTA, the membrane potential was restored. Fig. 1C also shows that CAT was unable to induce membrane de-energization in mitochondria loaded with Sr^{2+}.

To ascertain the concentration of internal exchangeable Ca^{2+} required to stimulate CAT-induced Ca^{2+} release mitochondria were incubated with increasing concentrations of oxalate, from 0.5 to 3 mM. It was observed that Ca^{2+} efflux induced by 5 μM CAT was diminished as the concentration of oxalate was increased (not shown). In order to estimate the amount of matrix exchangeable Ca^{2+}, in mitochondria incubated with oxalate, 0.5 μM ruthenium red and 2 μM A23187 were added to the incubation mixture after Ca^{2+} had been accumulated in mitochondria. Under these conditions, it was assumed that Ca^{2+} released corresponds to the fraction of mitochondrial exchangeable Ca^{2+}. With these values and those obtained from CAT-induced Ca^{2+} release, Fig. 1D was configured. It is shown that Ca^{2+} efflux took place when the concentration of exchangeable Ca^{2+} was higher than 20 nmol/mg. Maximal release, i.e. 50 nmol/mg, was attained with a concentration of internal exchangeable Ca^{2+} of 53 nmol/mg. Thus the results presented in Fig. 1 suggest that the generation of an increased permeable state in the inner membrane depends on the internal Ca^{2+} concentration. The site to which Ca^{2+} is bound, most likely is highly specific for this cation, since Sr^{2+}, a cation which can substitute Ca^{2+} in several mitochondrial Ca^{2+} dependent reactions [40–42], did not stimulate the membrane transition.

Effect of carboxyatractyloside and Ca^{2+} on the permeability of K^+-depleted mitochondria

Fig. 2 shows that mitochondria with a low amount of internal K^+, i.e. 11 ± 3 nmol/mg protein (n = 4), accumulated Ca^{2+} at a rate and to an extent similar to that of control mitochondria, which had a K^+ content of 83 ± 5 nmol/mg (n = 4) (see Fig. 1). However, in K^+-depleted mitochondria, CAT even at a high concentration of 10 μM failed to stimulate Ca^{2+} release (Fig. 2A). Such a reaction took place after the addition of 40 mM KCl (Fig. 2B). In the absence of CAT, this K^+ concentration did not promote Ca^{2+} efflux.

Fig. 3A shows the effect of CAT on the magnitude of membrane potential in K^+-depleted mitochondria. In these conditions, a high level of membrane energization was maintained despite to the addition of 50 μM Ca^{2+} plus 10 μM CAT. This result contrast with that obtained with mitochondria containing a high level of internal K^+ (see Fig. 1C). Fig. 3B shows the requirement for K^+ of CAT-induced membrane de-energization; as indicated, the addition of 40 mM K^+ promoted a complete collapse of membrane potential, which was partially recovered by removing internal Ca^{2+} with A23187 and EGTA. The requirement for K^+ of CAT-induced mitochondrial swelling in K^+-depleted mitochondria is shown in Fig. 4. Both the rate and extent of mitochondrial membrane permeabilization caused by 10 μM CAT and Ca^{2+} depended on the addition of 40 mM K^+.

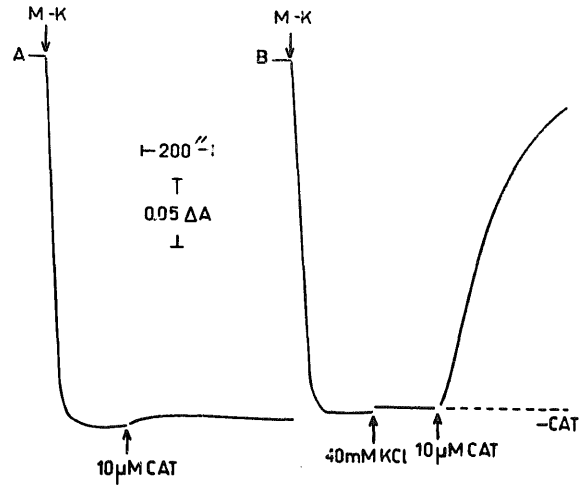

Fig. 2. The effect of K^+ addition of Ca^{2+} release as induced by carboxyatractyloside in K^+-depleted mitochondria. Experimental conditions were as in Fig. 1A, except that K^+ depleted mitochondria (M-K) were added to start the reaction. Other additions were as indicated.

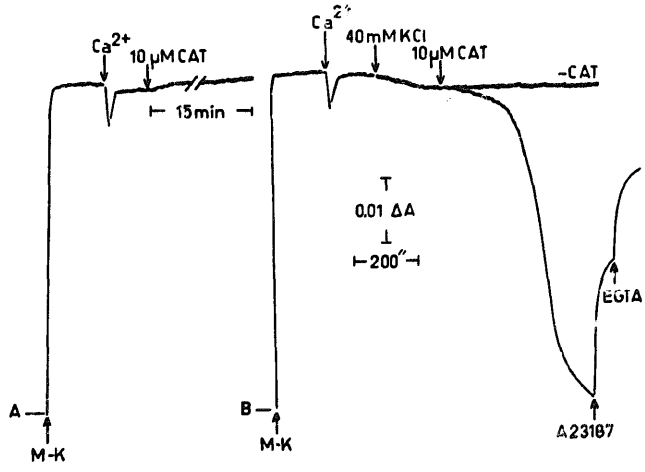

Fig. 3. The effect of K^+ addition on membrane de-energization as induced by CAT in K^+-depleted mitochondria. 2 mg protein from K^+-depleted mitochondria (M-K) were incubated under the conditions described for Fig. 1C. Where indicated, 50 μM $CaCl_2$, 2 μM A23187, and 100 μM EGTA were added. Other additions were as indicated.

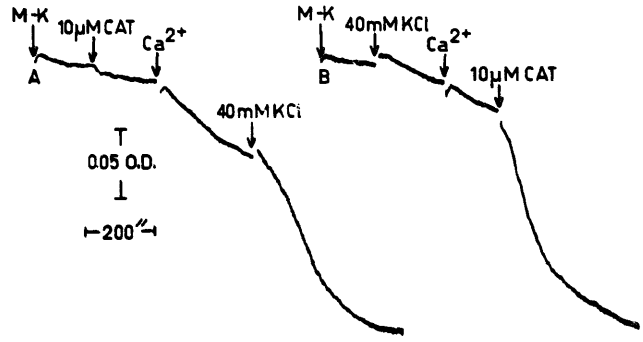

Fig. 4. The effect of K^+ on mitochondrial swelling as induced by CAT in K^+-depleted mitochondria. Experimental conditions were as in Fig. 1B, except that K^+-depleted mitochondria (M-K) were added to start the reaction. Where indicated, 50 μM $CaCl_2$ was added.

The amount of K^+ necessary to induce CAT-dependent Ca^{2+} release was determined (Fig. 5). As shown, the Ca^{2+} release pathway functioned at maximal extent, i.e. 42 nmol Ca^{2+} released/mg, when the concentration of added K^+ was 40 mM, with a $K_{0.5}$ of approx. 20 mM. Interestingly the kinetics of Ca^{2+} efflux followed a sigmoidal pattern, similar to that observed in control mitochondria incubated with increasing concentrations of oxalate (see Fig. 1D). It appears that K^+ displaces Ca^{2+} from unspecific internal binding sites, and thereby increases the matrix free-Ca^{2+} concentration. In fact, determinations of matrix free-Ca^{2+} by using Fluo 3 indicated that at an external Ca^{2+} concentration of 813 nM, stabilized by a Ca^{2+}/EGTA buffer, the free matrix Ca^{2+} concentration was 3.9 ± 0.8 μM ($n = 4$) in control mitochondria (K^+-containing), and 0.9 μM ± 0.3 μM ($n = 3$) in K^+-depleted mitochondria.

The cationic selectivety of CAT-induced Ca^{2+} release is shown in Table I. The following order of effectiveness was found: $K^+ > Cs^+ > Rb^+ \gg Li^+ = Na^+$

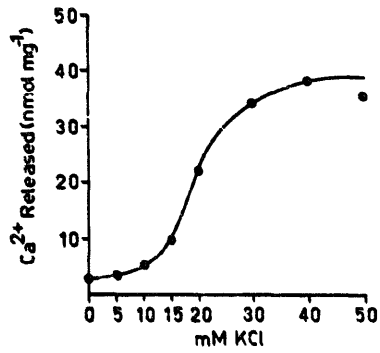

Fig. 5. The effect of increasing concentrations of K^+ on Ca^{2+} release induced by CAT in K^+-depleted mitochondria. 2 mg protein from K^+-depleted mitochondria were incubated in a basic medium as described for Fig. 1A, except that $^{45}Ca^{2+}$ was used, and the indicated concentrations of KCl were added. After 3 min of incubation, aliquots of 0.2 ml were filtered to estimate the accumulate cation. Immediately 10 μM CAT was added. After 6 min, aliquots of 0.2 ml were filtered to estimate the $^{45}Ca^{2+}$ released.

TABLE I

Cationic selectivity for CAT-induced Ca^{2+} release in K^+ depleted mitochondria

Two mg protein were incubated as described for Fig. 5, except for the addition of 40 mM of the indicated chloride salts. Ca^{2+} release was estimated as described for Fig. 5.

Cation added	CAT-induced Ca^{2+} release (nmol/mg)
Li^+	10
Na^+	10
K^+	32
Rb^+	21
Cs^+	28
Choline $^+$	5

> choline$^+$. The fact that choline did not stimulate CAT-induced Ca^{2+} release ruled out the possibility that the effect of K^+ may be due to the presence of external possitive charges. On the other hand, it is likely that the pattern of ionic selectivity depends on the extent to which the various cations are accumulated in the matrix space. To test this point, Na^+ and Rb^+ accumulation in K^+-depleted mitochondria was analyzed. The results indicate that Na^+ was accumulated at a concentration of 37 nmol/mg, whereas Rb^+ was accumulated to a value of 109 nmol/mg.

Discussion

In agreement with previous studies [26,30], this work shows that carboxyatractyloside induces release of matrix Ca^{2+}, complete collapse of membrane potential, and mitochondrial swelling. It is also shown that, similarly to other Ca^{2+} releasing agents [5–20], the increased membrane permeability induced by CAT requires the addition of Ca^{2+}, and that Sr^{2+} cannot substitute for Ca^{2+}.

The damaging effect of Ca^{2+} on membrane integrity has been ascribed to: (a) Ca^{2+} cycling across the membrane [39,43,44]; (b) Ca^{2+} binding to external sites [45]; or (c) Ca^{2+} binding to internal specific sites [46,47]. The fact that Ruthenium red did not restore the membrane potential, which had been collapsed after the addition of Ca^{2+} plus CAT (see Fig. 1C), indicates that Ca^{2+} cycling is not involved in the mechanism of CAT-induced unspecific membrane transition. However, the fact that mitochondrial Ca^{2+} depletion, induced by A23187 and EGTA, restored the permeability barrier (see Fig. 1C) seems to indicate that Ca^{2+} loading is a necessary condition for the development of increased membrane permeability.

Haworth and Hunter [48] have proposed that mitochondria contain hydrophilic channels that become operative upon Ca^{2+} binding. The experiment with oxalate would be in agreement with this claim. This

experiment, indicated that Ca^{2+} release was attained only after the concentration of exchangeable Ca^{2+} was higher than 20 nmol/mg. The cooperative kinetics followed by Ca^{2+} release, appears to indicate that a critical level of matrix free-Ca^{2+} is required to activate this process.

It is conceivable that the free Ca^{2+} concentration in the matrix space can depends on two factors, in addition to the rates of uptake and release: (a) the degree of precipitation of the cation as Ca-phosphates [49], or (b) its degree of binding to internal negative charges of membrane phospholipids [50]. The density of membrane negative charges, available for Ca^{2+} binding, would be governed by the concentration of internal cations, such as K^+, which is the predominant cation in the mitochondrial matrix space [31]. Accordingly, the experiment with the fluorescent Ca^{2+} indicator, Fluo 3, showed that the concentration of free matrix Ca^{2+} in control mitochondria was 4.3 times higher than in K^+-depleted mitochondria.

We cannot to rule out the regulating role of adenine nucleotide translocase in mitochondria Ca^{2+} content [22,24,51]. We must also consider the experiments by Meisner [52], which indicate that K^+ increases the inhibitory effect of atractyloside on the exchange of adenine nucleotides; however, we observed that CAT inhibited ADP and ATP exchange reactions to a similar extent either in K^+-containing or K^+-depleted mitochondria (not shown).

In conclusion, it is proposed that the concentration of matrix free-Ca^{2+} is regulated by intramitochondrial K^+. The mechanism proposed would involve the masking or unmasking of anionic binding sites for Ca^{2+}. Accordingly, K^+ could be a modulator of the metabolic effects of Ca^{2+} in mitochondria. This is of significance since it is well established that Ca^{2+} is involved in the activation of pyruvate dehydrogenase phosphatase [53,54], NAD-isocitrate dehydrogenase [55,56], and 2-oxoglutarate dehydrogenase [56–58]. Thus, the effect of Ca^{2+} on these dehydrogenase systems would be directly related to the intramitochondrial K^+ content. To determine if this interpretation is correct, experiments in such direction are being carried out; the results will be presented elsewhere.

In addition, it is conceivable that a combined effect of K^+ and Ca^{2+} would be reflected in an improvement in the phosphorilating capacity of mitochondria, particularly if it is considered that the increased NADH levels, brought about by Ca^{2+} activation of mitochondrial NAD-linked dehydrogenases, become rapidly oxidized due to the activating effect of K^+ on NADH dehydrogenase of respiratory chain Site I, as was demonstrated by Gómez-Puyou et al. [33]. However, it remains to be established if indeed, there are variations on intramitochondrial K^+ concentration under physiological conditions.

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