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Ba^{2+} ions inhibit the release of Ca^{2+} ions from rat liver mitochondria

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The release of Ca^{2+} from respiring rat liver mitochondria following the addition of either ruthenium red or an uncoupler was measured by a Ca^{2+}-selective electrode or by $^{45}Ca^{2+}$ technique. Ba^{2+} ions are asymmetric inhibitors of both Ca^{2+} release processes. Ba^{2+} ions in a concentration of 75 μM inhibited the ruthenium red and the uncoupler induced Ca^{2+} release by 80% and 50%, respectively. For the inhibition, it was necessary that Ba^{2+} ions entered the matrix space: Ba^{2+} ions did not cause any inhibition of Ca^{2+} release if addition of either ruthenium red or the uncoupler preceded that of Ba^{2+}. The time required for the development of the inhibition of the Ca^{2+} release and the time course of $^{140}Ba^{2+}$ uptake ran in parallel. Ba^{2+} accumulation is mediated through the Ca^{2+} uniporter as $^{140}Ba^{2+}$ uptake was competitively inhibited by extramitochondrial Ca^{2+} and prevented by ruthenium red. Due to the inhibition of the ruthenium red insensitive Ca^{2+} release, Ba^{2+} shifted the steady-state extramitochondrial Ca^{2+} concentration to a lower value. Ba^{2+} is potentially a useful tool to study mitochondrial Ca^{2+} transport.

Introduction

In all mammalian mitochondria there exists a transport system for Ca^{2+} ions which effects the movements of Ca^{2+} along the electrochemical potential gradient (Ca^{2+} uniporter). The function of the Ca^{2+} uniporter results at physiologically high membrane potential in Ca^{2+} uptake (for reviews, see Ref. [1–3]). This system is inhibited by ruthenium red [4] and it is also capable to transport other divalent cations such as Sr^{2+}, Mn^{2+} and Ba^{2+} under experimental conditions [5–7]. The Ca^{2+} uptake process is balanced by one or more ruthenium-red-insensitive Ca^{2+} release systems. One of them, the Na^+/Ca^{2+} exchanger has been well characterized [8,9]. The existence of a Na^+-independent Ca^{2+} release pathway is documented, but its action is much less known [3,10]. Because at the steady-state pCa_0 the rates of the Ca^{2+} uptake and release are equal, the addition of ruthenium red reveals the activity of the release pathway [3,10–12]. In liver mitochondria this release is less dependent on added Na^+ ions than the release in either heart or brain mitochondria (Ref. 3, but see also Ref. 13). Similarly to the in vitro conditions, within the cell the cytoplasmic concentration of free Ca^{2+} is subject to modulation by mitochondrial Ca^{2+} uptake and release processes (see for reviews Refs. 14 and 15). In the present paper, we demonstrate that the addition of Ba^{2+} ions to respiring liver mitochondria inhibits significantly the Na^+-independent ruthenium-red-insensitive and ruthenium-red-sensitive Ca^{2+} release pathways. For the inhibition it is necessary that the Ba^{2+} ions are first taken up by the uniporter.

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Abbreviations: ClCCP, carbonylcyanide-*m*-chlorophenyl-hydrazon; EGTA, ethyleneglycol bis (α-aminoethyl ether)-*N,N,N,N*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulhonic acid; pCa_0, $-\log[Ca^{2+}]$ outside the mitochondrial compartment.

Thus Ba^{2+} performs its inhibitory action from the matrix side of the inner membrane. Parts of the results were presented in a preliminary form [16].

Materials and Methods

Materials

Rotenone was purchased from K and K Laboratories, Inc. (Plainview, NY), ClCCP from Calbiochem. Ruthenium red from BDH was used after recrystallization according to Ref. 17. $^{45}CaCl_2$ and $^{140}BaCl_2$ were obtained from the Institute of Isotopes of the Hungarian Academy of Sciences and from Isocommerz, G.D.R., respectively.

Preparation of mitochondria

Rat liver mitochondria were prepared according to Johnson and Lardy [18], with the modification that the liver was homogenized in 0.25 M sucrose buffered with 2.5 mM Tris-HCl (pH 7.4) in the presence of 1 mM EGTA. Mitochondria were washed in sucrose Tris-HCl in the absence of EGTA. Protein content was determined by the biuret method using bovine serum albumin as standard.

Transport measurements

For all the transport measurements (uptake and release) reported below mitochondria (1.5 mg protein/ml) were incubated in a basic medium containing 240 mM sucrose/15 mM Hepes/2 mM KCl/1.5 mM $MgCl_2$/3.2 mM Tris-acetate/1 μM rotenone/3.3 mM Tris-ascorbate and the indicated concentration of $CaCl_2$ at pH 7.1 in open reaction vessels with magnetic stirring. Oligomycin (6.6 ng/mg protein), sodium ATP (0.2 mM) and *N,N,N,N*-tetramethylparaphenylenediamine (53 μM) were added successively. In those experiments in which Ca^{2+} release was measured, sufficient time was allowed to obtain the steady-state distribution of Ca^{2+} ions and then 1.3–13 nmol ruthenium red/mg protein was added to induce net Ca^{2+} release. In those experiments in which $^{45}Ca^{2+}$ was employed to measure Ca^{2+} release, duplicate samples were removed immediately before and 1 min after ruthenium red addition. The samples were centrifuged for 60 s in a table centrifuge at $10000 \times g$ (Mechanika Precyzyjna Warsawa, type 320a). Radioactivity of the supernatant was measured in a Beckman LS 250 liquid scintillation spectrometer. In separate experiments the initial Ca^{2+} content of the mitochondria was measured with Ca^{2+} electrode, in the absence of respiratory substrate. In calculation of the release rates the specific activity of labelled Ca^{2+} was corrected by this unlabelled intramitochondrial calcium pool. Ca^{2+} release was monitored by Ca^{2+}-selective electrode [19] connected to a Radelkis OP-205 pH meter and OH-814 potentiometric recorder. Since the presence of Ba^{2+} ions the conventional calibration method based on standard calcium-EGTA buffer mixtures could not be used [20], a multiple point calibration procedure was employed with an iterative determination of the initial Ca^{2+} concentration. To perform this, known amounts of $CaCl_2$ pulses were added to the suspension after each experimental run until the Ca^{2+} concentration of the medium reached about 0.1 mM. The initial Ca^{2+} concentration was iterated as described in Ref. 20. The prerequisite for this calculation was that the electrode responses to the log $[Ca^{2+}]$ should be linear between 0.1 μM and 0.1 mM Ca^{2+} concentration. The linearity of the electrode response was verified by Ca^{2+}-EGTA buffer mixtures according to Ref. 21. The Ca^{2+} concentration of the medium for each calibration point were then calculated from the amount of Ca^{2+} added and the initial Ca^{2+} concentration. Regression line was calculated for the log values of the corrected Ca^{2+} concentrations thus obtained and the recorder deflections. A possible error of the calibration could be the continuous Ca^{2+} release from the mitochondria through the ruthenium-red-insensitive efflux pathway during the calibration procedure. Under conditions when the Ca^{2+} release was blocked by 80% this error was found to be less than 10%. The selectivity coefficient of the electrode was 0.01 for Ba^{2+} ions determined by the fixed interference method [20]. Ca^{2+} binding by the ATP added could be neglected because of the concentration ratio of Mg^{2+} ions and ATP added (see above).

Ba^{2+} uptake was measured from the disappearance of $BaCl_2$ from the supernatant obtained after centrifugation of the mitochondria as described above. In preliminary experiments using quenching techniques with ruthenium red and

various chelators for Ba^{2+}, we found that adsorption of $^{140}Ba^{2+}$ to mitochondria was negligible as compared to $^{140}Ba^{2+}$ uptake. $^{140}Ba^{2+}$ was counted in a Beckman gamma-counter.

Membrane potential of the mitochondria was measured with a tetraphenyl phosphonium sensitive electrode as described in detail previously [22].

Results

The effect of Ba^{2+} ions on the ruthenium-red-insensitive Ca^{2+} release

Ba^{2+} ions added to mitochndria inhibit the Ca^{2+} release process revealed by the addition of ruthenium red. $BaCl_2$ in a concentration as low as 15 μM inhibited measurably the Ca^{2+} release and 75 μM resulted in 80% inhibition (Fig. 1). The inhibition can equally be demonstrated by Ca^{2+} selective electrode and by $^{45}Ca^{2+}$ technique. Comparison of the right and left panels of Fig. 1 reveals that the absolute values of the Ca^{2+} release rates differ by a factor of two, possibly due to the time required for separation of the mitochondria, causing an apparently higher rate of $^{45}Ca^{2+}$ release than that measured by the continous monitoring by Ca^{2+} electrode. Using both methods however, the Ba^{2+} concentration yielding 50% inhibition of Ca^{2+} release was 40 μM. The inhibitory effect of Ba^{2+} ions was equally observed if the incubation

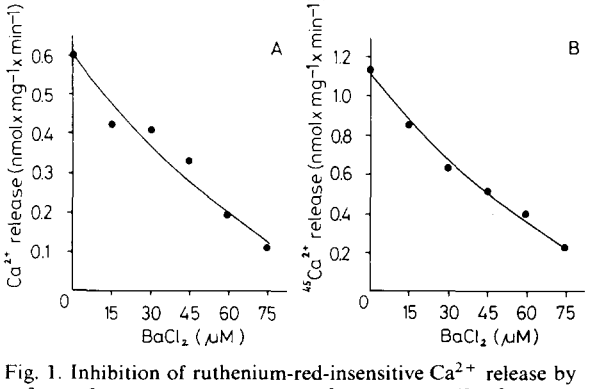

Fig. 1. Inhibition of ruthenium-red-insensitive Ca^{2+} release by Ba^{2+}. Ca^{2+} measured with (A) Ca^{2+} electrode; $^{45}Ca^{2+}$ technique (B). Mitochondria were incubated inthe standard medium in the presence of Ca^{2+} and the indicated concentration of Ba^{2+} for 12 min before the addition of ruthenium red. In (A) 26 μM Ca^{2+} and in (B) 30 μM Ca^{2+} (0.5 μCi/ml $^{45}CaCl_2$) was present. Further details are given in Methods.

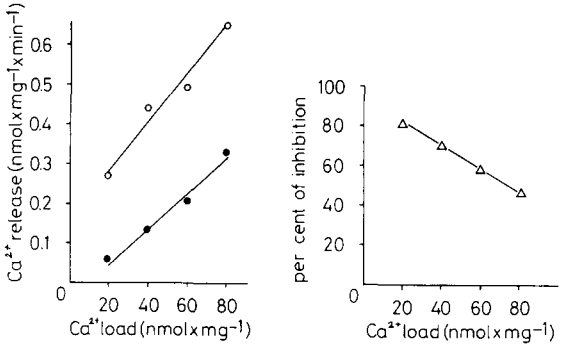

Fig. 2. The effect of Ca^{2+} load on Ba^{2+} inhibition of Ca^{2+} release. Ca^{2+} electrode measurements. After the indicated amount of Ca^{2+} was accumulated and the steady state pCa_0 was attained, Ba^{2+} was added. 4 min later Ca^{2+} release was started by 1.3 nmol/mg protein ruthenium red. ○, without Ba^{2+}; ●, 60 μM Ba^{2+}.

medium contained Na^+ up to a concentration of 40 mM, or at pH values between 6.5 and 7.8.

The rate of Ca^{2+} release from mitochondria is a function of the Ca^{2+} load added to mitochondria prior to ruthenium red addition [23–25]. By increasing the Ca^{2+} load from 20 to 80 nmol/mg protein, the rate of release doubled (Fig. 2). Ba^{2+} (60 μM) inhibited the rate of Ca^{2+} release by 80% at low Ca^{2+} load, whereas only 50% inhibition was observed at the highest Ca^{2+} load applied (Fig. 2). These experiments were carried out in the presence of oligomycin, ATP, acetate and only endogenous phosphate was present. Thus a specific efflux due to the excessive formation of calcium-phosphate precipitate was avoided [3,23]. The presence of acetate ions might have caused a moderate and reversible osmotic swelling [25] and an elevation of the matrix-free Ca^{2+} concentration [23,26].

The inhibition of Ca^{2+} release by Ba^{2+} in the absence of ruthenium red is reflected by an increase of the steady-state pCa_0 value (Fig. 3). When the Ca^{2+} load was 20 nmol/mg protein the steady-state extramitochondrial Ca^{2+} concentration was 0.14 μM lower in the presence of 60 μM Ba^{2+} than in the absence of Ba^{2+}. The same result was observed with $^{45}Ca^{2+}$ technique (data not shown). At higher mitochondrial Ca^{2+} load, the difference between the steady pCa_0 in the presence and absence of Ba^{2+} was gradually diminished (Fig. 3), probably due to the decreasing

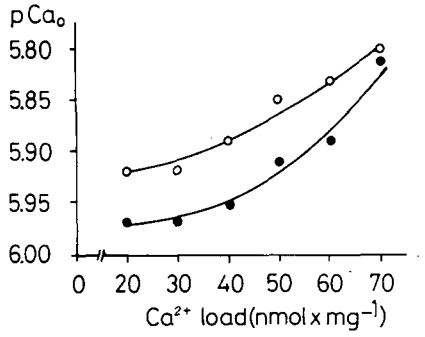

Fig. 3. The effect of Ba^{2+} on the steady state pCa_0. The experimental condition of the ca^{2+} electrode measurements was as in Fig. 1A. After the steady state pCa_0 was reached the mitochondria were incubated for 10 min in the presence of 60 μM $BaCl_2$ (●) or in the absence of $BaCl_2$ (○). Then Ca^{2+} pulses of 10 nmol/mg protein were added consecutively.

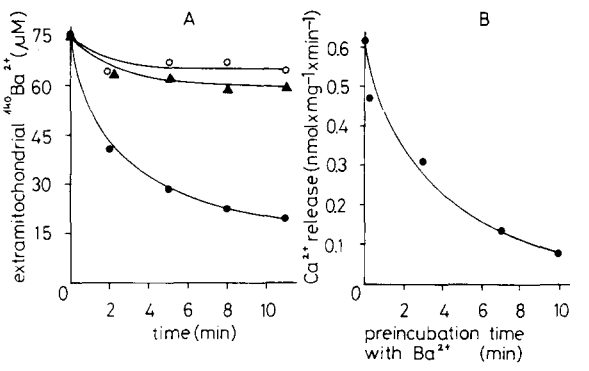

Fig. 4. (A) Ruthenium red and uncoupler sensitivity of $^{140}Ba^{2+}$ uptake by mitochondria. Mitochondria were incubated in the standard medium with 75 μM $^{140}BaCl_2$ (10 μCi/ml). ●, no further addition; ▲, with 6.0 nmol/mg protein ruthenium red; ○, with 2 μM ClCCP. (B) The effect of the preincubation time with Ba^{2+} on the inhibition of ruthenium-red-insensitive Ca^{2+} release. Ca^{2+} electrode measurements. At the steady state pCa_0 75 μM $BaCl_2$ was added. At times indicated Ca^{2+} release was started by addition of 13 nmol/mg protein ruthenium red. The initial Ca^{2+} concentration was 30 μM.

inhibitory effect of Ba^{2+} on the Ca^{2+} efflux pathway at higher Ca^{2+} load (Fig. 2).

The functional integrity of the mitochondria was not affected by Ba^{2+} ions up to a concentration of 75 μM. Neither the ADP nor the uncoupler-stimulated respiration was inhibited by 75 μM Ba^{2+}. The rate of Ca^{2+} uptake was inhibited by less than 8% in the presence of 25 μM Ca^{2+} and 75 μM Ba^{2+} measured with Ca^{2+} electrode or $^{45}Ca^{2+}$ (data not shown).

The site of Ba^{2+} ion inhibition is on the matrix side of the membrane

Ba^{2+} ions enter the mitochondrial matrix compartment and they inhibit Ca^{2+} release only from the matrix surface.

TABLE I

THE EFFECT OF Ba^{2+} ACCUMULATION WITHIN THE MITOCHONDRIA ON THE RATE OF $^{45}Ca^{2+}$ RELEASE

Experimental conditions were as those in Fig. 1B. The final concentration of $BaCl_2$, added either before or after ruthenium red, was 75 μM. Ba^{2+} was preincubated with the mitochondria for 10 min before ruthenium red addition. Identical preincubation was performed in the other samples (first and third line) without Ba^{2+} ions.

Additions	$^{45}Ca^{2+}$ efflux (nmol·mg^{+1}·min^{-1})
Ruthenium red only	1.25
Ba^{2+} before ruthenium red	0.30
Ruthenium red before Ba^{2+}	1.29

Ba^{2+} transport via the uniporter was proposed earlier on the basis of indirect experimental evidences: measuriang the respiration burst, H^+ ejection or absorbtion changes of cytochrome *b* and murexide after the addition of Ba^{2+} [5,6]. In our experiments Ba^{2+} transport was followed directly, applying $^{140}Ba^{2+}$ isotope. Fig. 4A shows that $^{140}Ba^{2+}$ is slowly taken up into mitochondria by a process that is sensitive to ruthenium red and also to uncoupler. The uptake pathway is thus probably identical with the Ca^{2+} uniporter. This is substantiated by the fact that Ca^{2+} ions were found to be competitive inhibitors of $^{140}Ba^{2+}$ uptake (Fig. 5). In contrast to Ca^{2+}, Ba^{2+} is firmly retained within the matrix: after addition of either an uncoupler or ruthenium red only negligible $^{140}Ba^{2+}$ release was observed (data not shown).

An indirect evidence of Ba^{2+} uptake was also found by monitoring the membrane potential. After the addition of 75 μM Ba^{2+} the membrane potential was slightly and transiently depressed, the decrease being 6 mV. This depolarization was prevented by ruthenium red. As the ruthenium-red-insensitive Ca^{2+} efflux was measured under conditions of constantly high membrane potential, the membrane-potential-mediated inhibition of

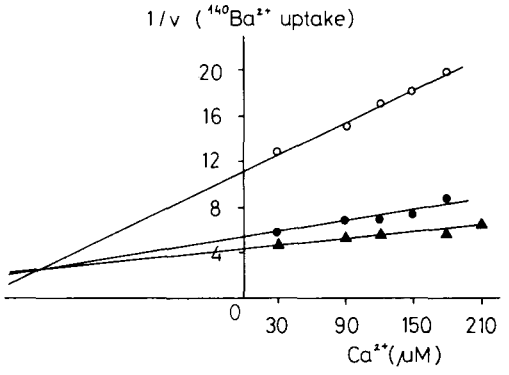

Fig. 5. Inhibition of $^{140}Ba^{2+}$ uptake by extramitochondrial Ca^{2+} plotted as a Dixon plot. Experimental conditions were as in Fig. 4A. Samples were removed 1.5 min after the transport was started. $BaCl_2$ concentrations were: ○, 60 μM; ●, 100 μM; ▲, 150 μM. Transport rate ($1/v$) was expressed as mg·min·nmol^{-1}. The lines were derived by linear regression analysis.

Ca^{2+} release reported by Ref. 27 could be excluded.

Ba^{2+} ions inhibited Ca^{2+} release only if they were added before ruthenium red (Table I). Addition of 75 μM Ba^{2+} after ruthenium red did not influence the rate of Ca^{2+} release at all. This fact strongly indicates that Ba^{2+} has first to enter before it is able to inhibit Ca^{2+} release. In accordance with this, it was found that the development of inhibition by Ba^{2+} requires a certain time after the addition of $BaCl_2$: the uptake of Ba^{2+} and the development of inhibition of Ca^{2+} release run parallel (Fig. 4).

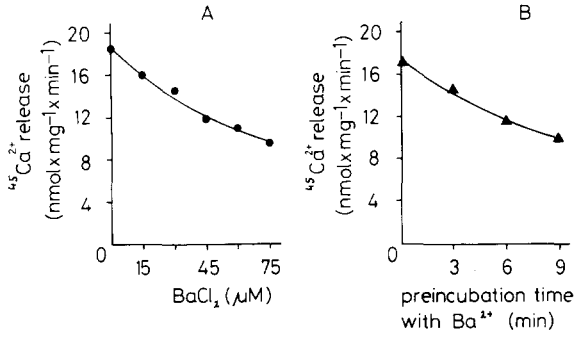

Fig. 6. Inhibition of the uncoupler induced $^{45}Ca^{2+}$ release by $BaCl_2$. The effect of the preincubation time with Ba^{2+} on the uncoupler induced $^{45}Ca^{2+}$ release. Experimental conditions were as in Fig. 1B. Concentration of the ClCCP was 2.9 μM. (A) Mitochondria were incubated in the standard medium with $BaCl_2$ as indicated. (B) At steady state pCa_0 75 μM $BaCl_2$ was added. At times indicated Ca^{2+} release was started by the addition of ClCCP.

Ba^{2+} also inhibits Ca^{2+} release caused by the collapse of the membrane potential

Besides the ruthenium-red-insensitive Ca^{2+} release system, Ca^{2+} can leave the matrix space also via the Ca^{2+} uniporter. This release, due to the substantial decrease of the membrane potential is the consequence of the reversal of the uniporter, and could be inhibited by ruthenium red in a widely different extent by various authors [10,23,26]. If the experimental conditions were carefully standardized in our experiments, the reversed uniporter was inhibited by ruthenium red by 90% in accordance with the results of [26].

If mitochondria were preincubated in the presence of 75 μM Ba^{2+} the uncoupler induced Ca^{2+} release was inhibited by 50% (Fig. 6A). Similarly to their effect on the ruthenium red insensitive Ca^{2+} release, Ba^{2+} ions had to enter the matrix space in order to inhibit the ruthenium-red-sensitive Ca^{2+} release (Fig. 6B). If Ba^{2+} ions (75 μM) were added just after ruthenium red, the rate of ClCCP induced Ca^{2+} release was unchanged (data not shown).

Discussion

The data reported in this paper suggest that the various transport pathways involved in mitochondrial Ca^{2+} transport are able to interact with other divalent catiaons, such as Ba^{2+}. Remarkable is that in liver mitochondria for Ba^{2+} ion there is a serious difference in affinity at the two sides of the membrane, or more precisely, the Ca^{2+}/Ba^{2+} affinity ratios are different at the two sides. The slow uptake kinetics of Ba^{2+} relative to that of Ca^{2+} and the slight inhibition of Ca^{2+} uptake by extramitochondrial Ba^{2+} [28] indicate that at the cytoplasmic side the uniporter has much higher affinity for Ca^{2+} than for Ba^{2+} uptake. This is probably not the case for the Ca^{2+} release process. Under conditions when the inhibition of Ca^{2+} release by Ba^{2+} was found to be maximal, the intramitochondrial Ba^{2+} and Ca^{2+} contents were approximately equal. As acetate ions were present

in excess and phosphate was limited, it can be safely assumed that the intramitochondrial concentration ratio of Ba^{2+}/Ca^{2+} was approaching unity. At this intramitochondrial concentration ratio the uncoupler-induced Ca^{2+} release was inhibited about 50%. Under basically similar conditions the ruthenium-red-insensitive Ca^{2+} release was about 80% inhibited by Ba^{2+} at an intramitochondrial Ba^{2+}/Ca^{2+} ratio of 1, while an even higher extramitochondrial Ba^{2+}/Ca^{2+} ratio (the value being 75 in the experiments shown) did not affect the release process at all. These facts indicate very strongly that both the ruthenium-red-sensitive and insensitive Ca^{2+} channels have an asymmetric affinity for Ba^{2+}.

Because of the similarities in the action of Ba^{2+} and Sr^{2+} [29] on the inhibition of the ruthenium-red-insensitive Ca^{2+} efflux, it is reasonable to assume that the binding sites for Ba^{2+} and for Sr^{2+} at the matrix surface of the ruthenium red insensitive Ca^{2+} release pathways are identical. Probably, once Ba^{2+} or Sr^{2+} is bound at the inner surface of the membrane, the complex transports neither Ca^{2+} nor Ba^{2+} or Sr^{2+} [29,30].

The asymmetric inhibition of the reversed uniporter was observed with Sr^{2+} as well (Lukács, G.L., unpublished results). Whether the asymmetry between the two openings of the uniporter channel is a performed characteristics or induced by the collapse of the membrane potential remains to be established.

The results reported here may have a methodological significance too. Although Ba^{2+} was applied in a wide variety of cells (e.g., pancreatic β-cell [31], adrenal medulla cell [32], neuronal cell [33], renal tubules cell [34]) to investigate different Ca^{2+}-dependent physiological functions, the interactions of Ba^{2+} with the mitochondrial Ca^{2+} transport systems have not been studied yet. Thus Ba^{2+} might be a useful tool in studying mitochondrial Ca transport both in vitro and in vivo.

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