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The Ba^{2+} sensitivity of the Na^+-induced Ca^{2+} efflux in heart mitochondria: the site of inhibitory action

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The Na^+-induced Ca^{2+} release from rat heart mitochondria was measured in the presence of Ruthenium red. (1) Ba^{2+} effectively inhibited the Na^+-induced Ca^{2+} release. At 10 mM Na^+ 50% inhibition was reached by 1.51 ± 0.48 (S.D., $n = 8$) μM Ba^{2+} in the presence of 0.1 mg/ml albumin and by 0.87 ± 0.25 (S.D., $n = 3$) μM Ba^{2+} without albumin. (2) In order to inhibit, it was not required that Ba^{2+} ions enter the matrix. $^{140}Ba^{2+}$ was not accumulated in the mitochondrial matrix space; further, in contrast to liver mitochondria, Ba^{2+} inhibition was immediate. (3) The Na^+-induced Ca^{2+} release was inhibited by Ba^{2+} non-competitively, with respect of the extramitochondrial Na^+. (4) The double inhibitor titration of the Na^+-Ca^{2+} exchanger with Ba^{2+} in the presence and absence of extramitochondrial Ca^{2+} revealed that the exchanger possesses a common binding site for extramitochondrial Ca^{2+} and Ba^{2+}, presumably the regulatory binding site of the Na^+-Ca^{2+} exchanger, which was described by Hayat and Crompton (Biochem. J. 202 (1982) 509–518). All these observations indicate that Ba^{2+} acts at the cytoplasmic surface of the inner mitochondrial membrane. The inhibitory properties of Ba^{2+} on the Na^+-dependent Ca^{2+} release in heart mitochondria are basically different from those found on Na^+-independent Ca^{2+} release in liver mitochondria (Lukács, G.L. and Fonyó, A. (1985) Biochim. Biophys. Acta 809, 160–166).

Introduction

Mitochondria from all mammalian tissues possess separate transport pathways for simultaneous Ca^{2+} uptake and release (see for reviews Refs. 1–5). The continuous Ca^{2+} uptake process through the Ruthenium-red-sensitive uniporter is balanced by two types of Ca^{2+} release mechanism. The Na^+-independent pathway, which is postulated to be an H^+-Ca^{2+} antiporter, is highly active in liver and lung mitochondria. The Na^+-dependent Ca^{2+} efflux pathway, which predominates in heart and brain mitochondria, is an Na^+-Ca^{2+} exchanger [36] coupled to a rapid H^+-Na^+ antiporter. However, the Na^+-dependent Ca^{2+} efflux pathway was also found in mitochondria from liver and from other types of 'non excitable tissues' [6,7].

In heart mitochondria there is evidence that Ca^{2+} transport pathways participate in the regulation of the matrix free Ca^{2+} concentration (for review see Ref. [8]). It was proposed that the intramitochondrial free Ca^{2+} concentration regulates the rate of oxidative metabolism through the Ca^{2+}-sensitive dehydrogenases [9–11]. Certain hormonal stimuli elevate the cytoplasmic Ca^{2+} concentration. This change can be relayed to the mitochondrial matrix space either by the activation of the uniporter or by the inhibition of the

Abbreviations: CCCP = carbonyl cyanide *m*-chlorphenylhydrazone; EGTA = ethylene glycol bis(α-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $pCa_o^{2+} = -\log[Ca^{2+}]$ outside the mitochondrial compartment.

Na^+-Ca^{2+} exchange or both [12,13]. In isolated heart mitochondria the Na^+-Ca^{2+} exchange is inhibited by the elevated extramitochondrial Ca^{2+} concentration through a binding site which is distinct from the substrate-binding site of the carrier. On the basis of kinetic analysis of the Na^+-induced Ca^{2+} efflux inhibition by extramitochondrial Ca^{2+}, Hayat and Crompton proposed the existence of a regulatory Ca^{2+}-binding site of the Na^+-Ca^{2+} carrier [14,15].

We found earlier that Ba^{2+} inhibits the Na^+-insensitive Ca^{2+} release from liver mitochondria: in this action they have to enter the mitochondria and they act from the matrix surface of the inner membrane [16]. When we continued the systematic investigation of the effect of Ba^{2+} on Ca^{2+} transport in the heart, we found that Ba^{2+} inhibited Na^+-dependent Ca^{2+} release from these mitochondria acting through the regulatory binding site of the Na^+-Ca^{2+} exchanger.

Besides Ca^{2+}, another inhibitor of the Na^+-Ca^{2+} exchange is the drug diltiazem, which is a 'Ca^{2+} antagonist' acting on the plasma membrane. With double inhibitor titration, the regulatory binding site and the diltiazem binding site of the Na^+-Ca^{2+} exchanger can be differentiated.

Parts of the results have been presented in a preliminary form [18].

Materials and Methods

Materials

Rotenone was purchased from K and K Laboratories (Plainview, NY, U.S.A.), CCCP from Calbiochem, antimycin from Serva and bovine albumin (fraction V.) from Phylaxia (Hungary). Ruthenium red from BDH was used after recrystallization according to Ref. 19. $^{140}BaCl_2$ was obtained from Isocommerz, G.D.R.. Diltiazem, a product of Marion Research Laboratories, Kansas City, MO, U.S.A., was a gift of Dr. P.L. Vághy.

Preparation of heat mitochondria

Rat heart mitochondria were isolated by the method of Ref. 20, with the modification that hearts were homogenized in a sucrose/mannitol (75 : 225 mM) medium containing 0.2 mg/ml albumin, 5 mM Tris-HCl, 0.4 mM EGTA and 0.4 mM EDTA at pH 7.4. Mitochondria were washed once in the same medium without albumin, EGTA and EDTA. Mitochondrial protein content was assayed by biuret method, using bovine serum albumin as standard.

Transport measurements

For Ca^{2+} and Ba^{2+} transport measurements, heart mitochondria (1.7–1.9 mg protein/ml) were incubated at 25°C in a sucrose-based medium (230 mM sucrose, 20 mM Tris-HCl, 2 mM KCl, 0.1 mg albumin/ml at pH 7.4) or in a KCl-based medium (80 mM KCl, 70 mM sucrose, 20 mM Tris-HCl, 0.1 mg albumin/ml at pH 7.4) in an open reaction vessel with magnetic stirring. The respiratory substrate Tris-succinate was added at a final concentration of 5 mM.

Ca^{2+} movements were monitored by a Ca^{2+}-selective electrode (Radelkis, modified as in Ref. 21) connected to Radelkis OP 208/1 precision pH meter and an OP-814 potentiometric recorder. The rate of the initial Ca^{2+} release was calculated on the basis of a multiple point calibration technique combined with an iterative determination of the initial Ca^{2+} concentration of the medium, as described in detail in Ref. 16. To exclude artifactual effects of divalent cations on the Ca^{2+} electrode response, calibrations were carried out at each divalent cation concentration applied in the experiments. The selectivity coefficient of the electrode for Ba^{2+} was 0.01 determined by the fixed interference method [22].

Ba^{2+} transport was measured from the disappearance of $^{140}BaCl_2$ from the supernatant obtained after centrifugation of the mitochondria for 60 s in a table centrifuge (Mechanika Precyzyjna Warsawa, type 320a) at $10000 \times g$. Radioactivity in the supernatant was measured in a Beckman Radioimmuno Analyser. $^{45}Ca^{2+}$ movements were followed in some experiments in the same way as $^{140}Ba^{2+}$ transport.

Na^+-induced ejection of H^+ from heart mitochondria was monitored with a Radiometer pH electrode as described in Ref. 23. The H^+ ejection was initiated with the addition of 10 mM Na^+ in a medium containing 120 mM KCl, 40 nmol/mg mersalyl and 2.4 mg mitochondrial protein/ml.

Membrane potential of the mitochondria was measured with a tetraphenyl phosphonium sensitive electrode as in Ref. 24.

The kinetic analysis of the Na^+ induced Ca^{2+} efflux was performed with the assumption that the carrier was saturated with Ca^{2+} from the matrix space and the stochiometry of the Na^+-Ca^{2+} exchange was 3 : 1 based on the data of Ref. 14, 25. All the Na^+-induced Ca^{2+} release values were corrected for the rate of the basal Ca^{2+} efflux, which latter was measured in the presence of Ruthenium red in an Na^+-free medium. Lines of the plots were drawn after linear regression analysis of the data.

Results

The effect of Ba^{2+} on the rate of Na^+-induced Ca^{2+} efflux and steady-state pCa_o^{2+} in heart mitochondria

Rat heart mitochondria respiring in the presence of succinate accumulate medium Ca^{2+} content until the steady-state extramitochondrial pCa_o^{2+} is reached (Fig. 1A). In our experiments the values of steady-state pCa_o^{2+} varied between 5.6 and 6.1, depending on the mitochondrial preparations. After addition of Ruthenium red, a selective inhibitor of the uniporter [26], the 'basal Ca^{2+} efflux' was observed (Fig. 1A). Subsequent addition of Na^+ (10 mM) induced a rapid release of Ca^{2+} due to the activation of the Na^+-Ca^{2+} carrier (Fig. 1A). However, if Ba^{2+} (4.5 μM) was added after Ruthenium red, the rate of Ca^{2+} release in the presence of Na^+ decreased from 6.7 to 2.2 nmol/mg per min (Fig. 1B).

The balance of the Ca^{2+} uptake and release processes is reflected in the steady-state pCa_o^{2+} value. If the Ca^{2+} efflux was activated by Na^+ in the absence of Ruthenium red, the cycling of Ca^{2+} was enhanced and the steady-state pCa_o^{2+} was shifted to a lower value (Fig. 1C). Conversely, the inhibition of the Na^+-Ca^{2+} exchange by Ba^{2+} ions caused the elevation of the steady-state pCa_o^{2+} (Fig. 1C). Addition of Ba^{2+} in the absence of Ruthenium red and Na^+ did not alter the steady-state pCa_o^{2+}, indicating that Ba^{2+} did not inhibit the activity of the uniporter (Fig. 1C, dashed line). The rapid shift on the record after Ba^{2+} addition, seen also in the absence of mitochondria, is due to the artifactual effect of Ba^{2+} upon the Ca^{2+} electrode and not to a change in the extramitochondrial Ca^{2+} concentration. This was verified by

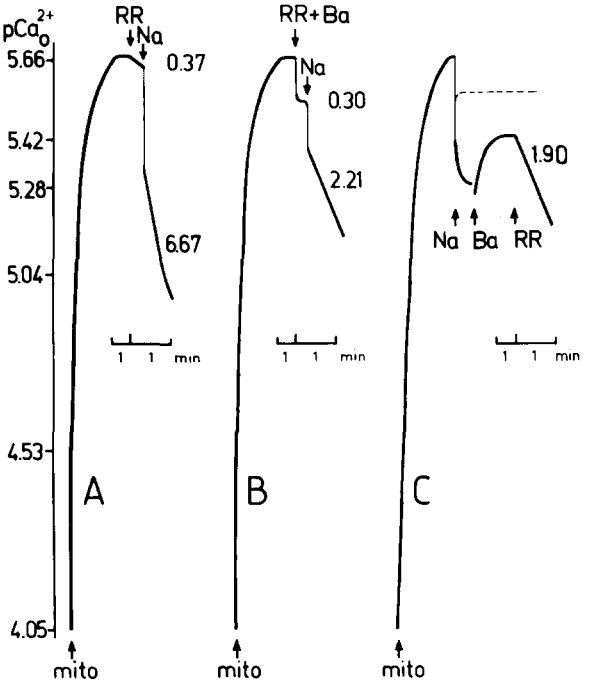

Fig. 1. The effect of Ba^{2+} on the steady-state pCa_o^{2+} and on the rate of the Na^+-induced Ca^{2+} efflux in heart mitochondria. Mitochondria were incubated in the sucrose based medium as described in Materials and Methods. Further additions: 1.2 μM Ruthenium red (RR); 10 mM NaCl (Na); 4.5 μM $BaCl_2$ (Ba); dashed line in panel C: only 4.5 μM $BaCl_2$ was added. The numbers indicate the rate of Ca^{2+} release in nmol/mg per min. The vertical drops are electrode artifacts caused by Na^+ or Ba^{2+} addition. Note that the chart speed was changed in the second part of each experiment.

$^{45}Ca^{2+}$ measurements (not shown).

The effect of various Ba^{2+} concentrations on the rate of Na^+-stimulated and basal Ca^{2+} efflux is reported on Fig. 2A. Ba^{2+} inhibited 50% the rate of the (10 mM) Na^+-induced Ca^{2+} efflux in the sucrose based medium at a concentration of 1.51 ± 0.48 μM (S.D., $n = 8$) in the presence and at 0.87 ± 0.25 μM ($n = 3$) in the absence of albumin. The maximal inhibition (90–95%) of the Na^+ induced Ca^{2+} efflux was reached between 16–24 μM Ba^{2+}. The rate of the basal Ca^{2+} efflux was only slightly inhibited by Ba^{2+} (Fig. 2A).

To elucidate whether Ba^{2+} inhibits the Na^+-Ca^{2+} exchange directly or through the inhibition of the H^+-Na^+ exchange we investigated the effect of Ba^{2+} on the rate of Na^+-induced H^+ ejection. Even the highest Ba^{2+} concentration (30 μM) did

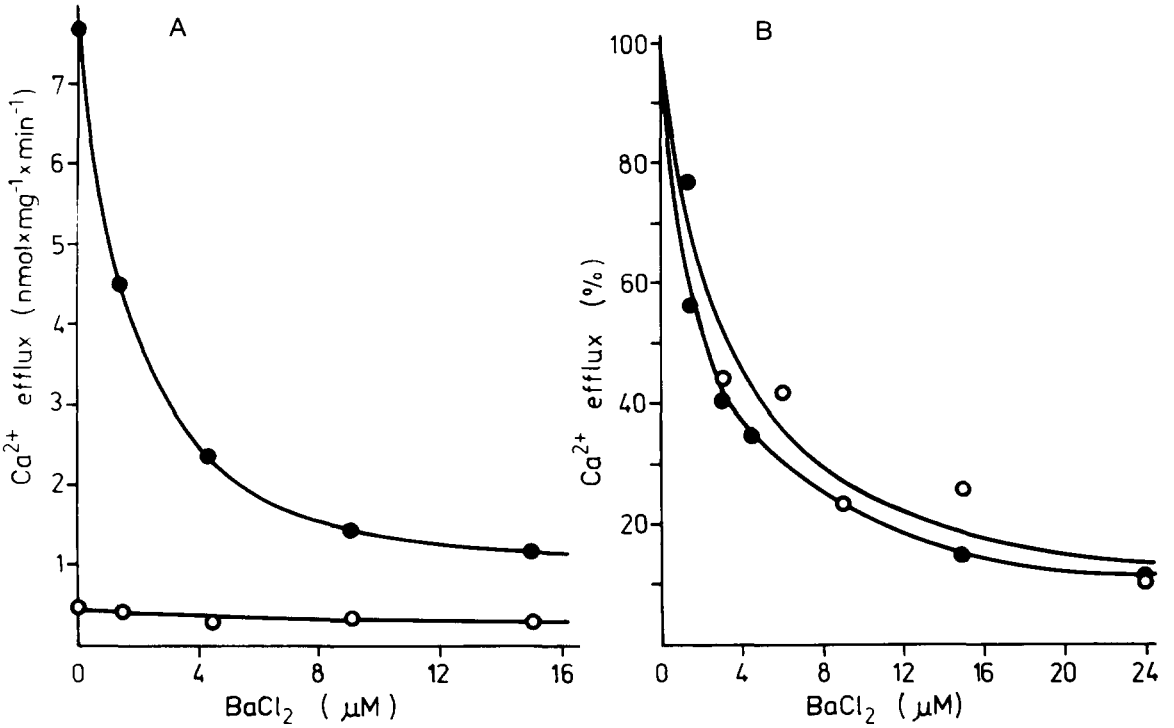

Fig. 2. (A) The effect of Ba^{2+} concentration on the rate of Na^+-induced and basal Ca^{2+} efflux in the sucrose-based medium. The experimental protocol was the same as in Fig. 1B. Symbols: ○, basal Ca^{2+} efflux; ●, in the presence of 10 mM NaCl. (B) The effect of KCl on the Ba^{2+} inhibition of the Na^+-induced Ca^{2+} efflux. The efflux rates are expressed as a percentage of the Na^+-induced Ca^{2+} efflux rates determined in the absence of $BaCl_2$. The measurements were done in the sucrose-based (●) or in the KCl-based (○) medium. The values of the Na^+-induced Ca^{2+} efflux in the absence of Ba^{2+} were 9.6 and 5.9 nmol/min per mg in the KCl- and sucrose-based media, respectively.

not decrease the rate of H^+ efflux induced by Na^+ (not shown).

In control experiments, Ba^{2+} in the concentration range of 1–30 μM did not alter the rate of oxygen consumption of respiring mitochondria either in the presence or absence of ADP or an uncoupler (not shown).

The regulatory binding site of the Na^+-Ca^{2+} exchanger confers Ba^{2+} sensitivity to the Na^+-dependent Ca^{2+} release pathway

Ba^{2+} might interact with the Na^+-dependent Ca^{2+} release pathway at several different cation-binding sites: possible candidates are the substrate- (Na^+, Ca^{2+}) binding sites facing either the cytosol or the matrix space and the regulatory binding site facing the cytosol. In the following paragraph we report evidence that Ba^{2+} inhibits the Na^+-dependent Ca^{2+} release after binding to the regulatory binding site of the Na^+-Ca^{2+} carrier.

First, we wanted to localize that side of the mitochondrial inner membrane on which Ba^{2+} inhibits Ca^{2+} release. If Ba^{2+} interacts with the substrate-binding site of the carrier facing the matrix space, Ba^{2+} ions have to enter the mitochondria and the Ba^{2+} inhibition has to develop parallel with the Ba^{2+} uptake process, as was shown by us in rat liver mitochondria [16].

However, there was negligible amount of $^{140}Ba^{2+}$ uptake in the case of either respiring or deenergized mitochondria (Fig. 3A) as compared to liver mitochondria. That small amount (0.4–0.6 nmol/mg protein) of $^{140}Ba^{2+}$ found in the mitochondrial pellet was further diminished if EGTA (1 mM) was added to the mitochondria just before the centrifugation procedure (Fig. 3A). A similar effect was obtained if the transport was

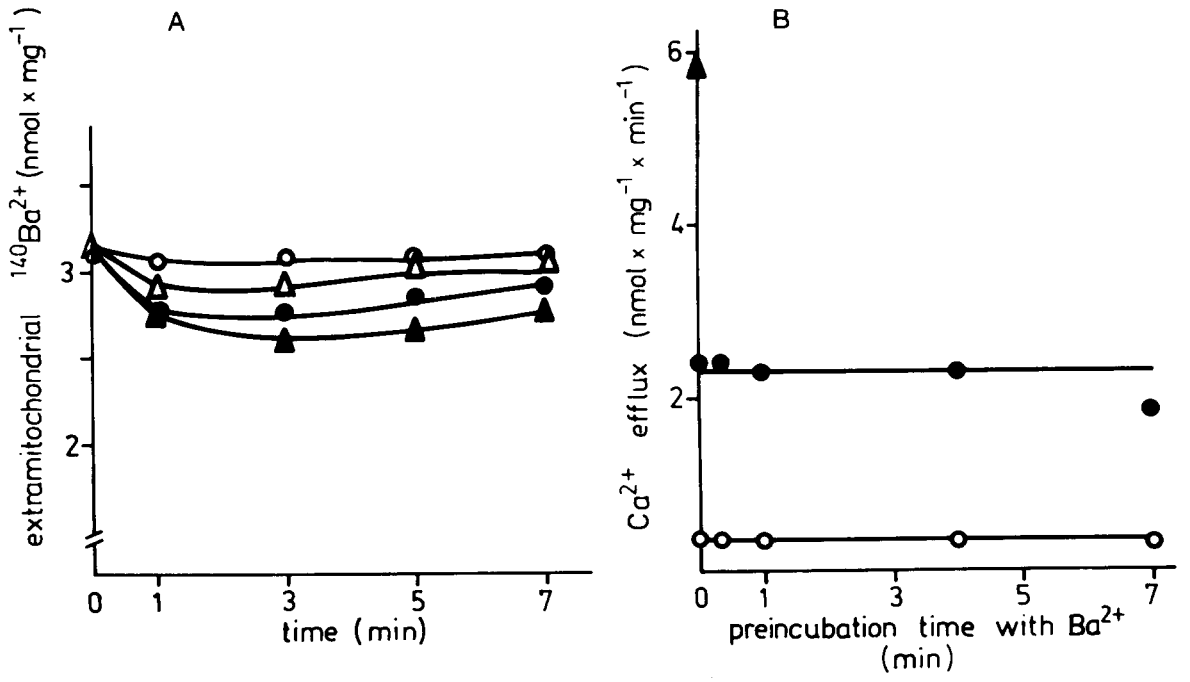

Fig. 3. (A) The association of $^{140}Ba^{2+}$ with heart mitochondria. Mitochondria were preincubated for 3 min in the sucrose-based medium before 6 μM $^{140}BaCl_2$ (10 μCi/ml) was added. Samples were taken for centrifugation after the time indicated on the abscissa. In the presence of 5 mM succinate (▲, △), or with 4 μM CCCP, 5 μg/ml antimycin and 2 μM rotenone (●, ○). Open symbols indicate that 1 mM EGTA was added to the samples just before centrifugation. (B) The effect of preincubation time with Ba^{2+} on the rate of Na^+-induced Ca^{2+} efflux. $BaCl_2$ (3 μM) was added to mitochondria after the steady-state pCa_o^{2+} was reached. At the time indicated, the basal (○) and the (10 mM) Na^+-induced Ca^{2+} effluxes (●) were measured in the presence of 1.2 μM Ruthenium red. In the absence of $BaCl_2$ the activity of the Na^+-induced Ca^{2+} efflux was 5.9 nmol/mg per min (▲).

measured in a KCl-based medium in which the surface potential was decreased (not shown).

Continuous monitoring of the mitochondrial membrane potential by the tetraphenylphosphonium electrode revealed that addition of Ba^{2+} did not alter the membrane potential, whereas addition of the same amount of permeating Ca^{2+} or Sr^{2+} decreased the membrane potential by 15 mV (not shown). This was again in contrast to the membrane potential decreasing effect of Ba^{2+} in liver mitochondria [16].

These observations led us to the conclusion that Ba^{2+} did not penetrate the mitochondrial matrix space but only adsorbed to the phospholipid membrane under our experimental conditions.

There was no time requirement for the development of Ba^{2+} inhibition of the Na^+-induced Ca^{2+} efflux (Fig. 3B). In contrast, liver mitochondria had to be preincubated with Ba^{2+} to obtain the inhibition of the Na^+-insensitive Ca^{2+} efflux [16]. The conclusion that Ba^{2+} interacts with Ca^{2+} release at the cytosolic side of the membrane seems inevitable.

The interaction between the binding of extramitochondrial Na^+ (as substrate) and Ba^{2+} (3 μM) as an inhibitor was investigated as reported in Fig. 4A. Plots of $1/v_i$ versus $1/[Na^+]$ are linear and show Ba^{2+} to be a non-competitive inhibitor of the Na^+-induced Ca^{2+} release with respect to the extramitochondrial Na^+. We obtained the same type of Ba^{2+} inhibition if the Na^+-induced Ca^{2+} release was plotted according to Dixon (Fig. 4B). The inhibitor constant obtained by this way was 1.69 μM for Ba^{2+}, in agreement with the 50% inhibitory concentration of Ba^{2+} (1.51 μM). Sr^{2+}, in contrast to Ba^{2+}, inhibited the Na^+-induced Ca^{2+} release competitively on the substrate-binding site (Fig. 4A), as was shown earlier [14].

These results indicate that Ba^{2+} did not inhibit

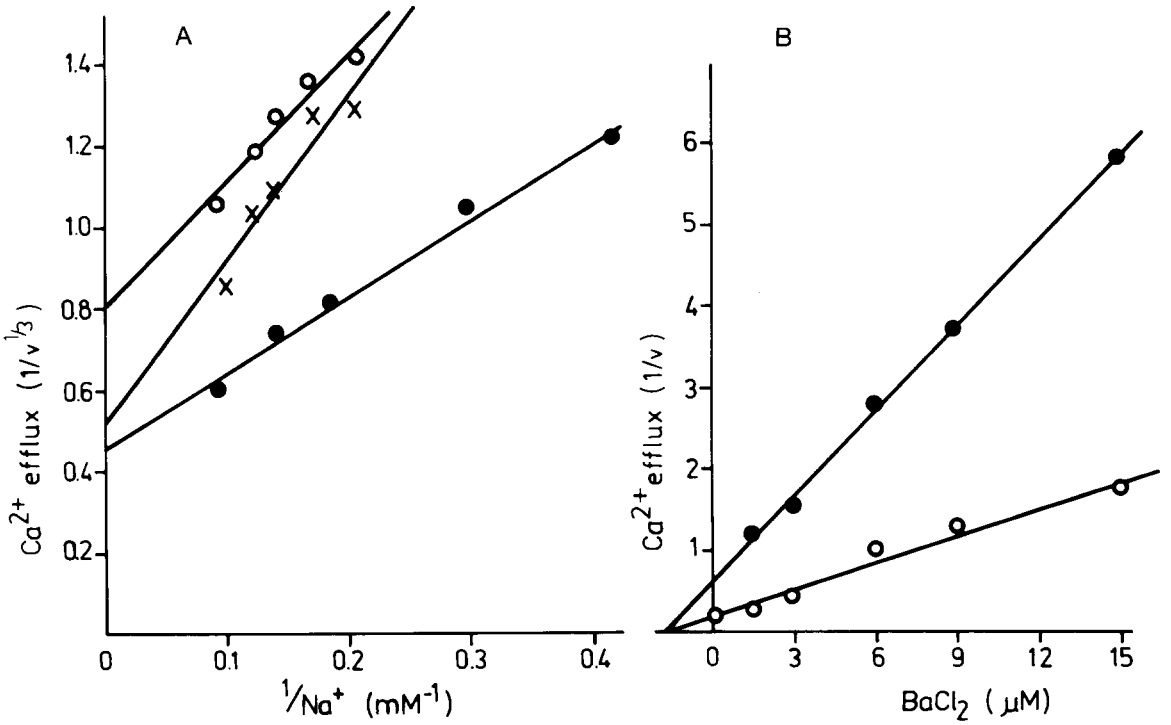

Fig. 4. The kinetic analysis of the inhibition by Ba^{2+} of the Na^+-induced Ca^{2+} efflux: (A) plotted as a double-reciprocal plot, (B) as a Dixon plot. The Ca^{2+} efflux (v, nmol/mg per min) was measured in the sucrose-based medium as in Fig. 1B, (A) in the absence (●) or in the presence of either 3 μM $BaCl_2$ (○) or 10 μM $SrNO_3$ (×); (B) after the addition of 7.1 mM (●) or 14.3 mM (○) NaCl.

the Na^+-dependent Ca^{2+} release through the externally oriented substrate-binding site, but rather by interacting with the regulatory site of the carrier.

The Hill coefficient for the Ba^{2+} inhibition of the Na^+-induced Ca^{2+} release was 0.86 ± 0.11 ($n = 7$). This value may be slightly underestimated, as the free Ba^{2+} ion concentration is probably decreased by the binding of Ba^{2+} ions to albumin present in the incubation medium.

In all the above experiments, the steady-state extramitochondrial Ca^{2+} concentrations were between 0.95 and 2.60 μM. This level of Ca^{2+} concentration partially inhibits in itself the Na^+-Ca^{2+} exchange [14,15]. To verify that two different inhibitors (i.e., Ba^{2+} and Ca^{2+}) act on the same binding site one can use double inhibitor titration [27]. Accordingly we measured the effect of Ba^{2+} on the rate of Na^+-induced Ca^{2+} efflux at low (1.6 μM) and at elevated (16.6 μM) extramitochondrial Ca^{2+} concentration. In the latter case, to keep the mitochondrial Ca^{2+} content at the same level, the extra Ca^{2+} was added after Ruthenium red. Fig. 5 shows that 16.5 μM extramitochondrial Ca^{2+} reduced the Na^+-induced Ca^{2+} release from 6.75 to 4.40 nmol/mg per min. On increasing the medium Ba^{2+} concentration the extent of the inhibition by added Ca^{2+} on Na^+-induced Ca^{2+} release decreased progressively (Fig. 5). The lines on the Dixon plot of the Ba^{2+} inhibition in the presence and absence of high extramitochondrial Ca^{2+} concentrations are almost parallel (slopes are 0.110 and 0.117), which means that the Na^+-Ca^{2+} exchanger possesses a common binding site (i.e., the regulatory binding site) for extramitochondrial Ca^{2+} and Ba^{2+} (Fig. 5 inset). The inhibitory effect of the elevated extramitochondrial Ca^{2+} concentration on Na^+ association to the substrate binding site was negligible at 10 mM Na^+ [14].

The association of external Na^+ (and Sr^{2+}) to the substrate-binding site was promoted by external K^+ (3-fold decrease in the $K_s^{Na^+}$ value and

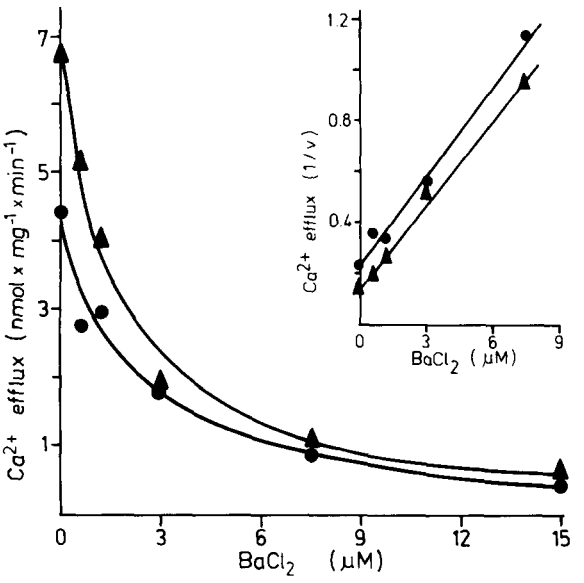

Fig. 5. Effect of extramitochondrial Ca^{2+} on the Na^+-induced Ca^{2+} efflux. The (10 mM) Na^+-induced Ca^{2+} efflux was measured as in Fig. 1B. When the steady-state pCa_o^{2+} was obtained, $BaCl_2$ (▲) or $BaCl_2$ together with 15 μM $CaCl_2$ (●) were added. Inset: Dixon plot from the data of the same experiment.

1.2-fold increase in the V_{max} value, if the medium K^+ was changed from 0 mM to 120 mM, see Ref. 14). In contrast, the inhibition of Na^+-Ca^{2+} exchange by Ca^{2+} [14] and in our case by Ba^{2+} was affected little by extramitochondrial K^+ (Fig. 2B). The Na^+-Ca^{2+} exchange was inhibited 50% by 2.3 ± 0.87 ($n = 4$) μM Ba^{2+} in the KCl- and 1.51 ± 0.48 ($n = 8$) μM Ba^{2+} in the sucrose-based medium.

Interaction between diltiazem and Ba^{2+} binding site of the Na^+-Ca^{2+} antiporter

The 'Ca^{2+} antagonist' compound diltiazem proved to be a selective inhibitor of the Na^+-Ca^{2+} antiporter in heart mitochondria [17]. The interaction between the two inhibitor sites, i.e., diltiazem- and Ba^{2+}-binding sites, was investigated with double inhibitor titration, as shown in the Dixon plot of Fig. 6A. The value of the interaction constant of the two inhibitors calculated as in Ref. 27 was 0.3, suggesting that the binding sites of the two inhibitors are largely different but some groups that take part in the binding might be common [27]. A similar conclusion was drawn from experi-

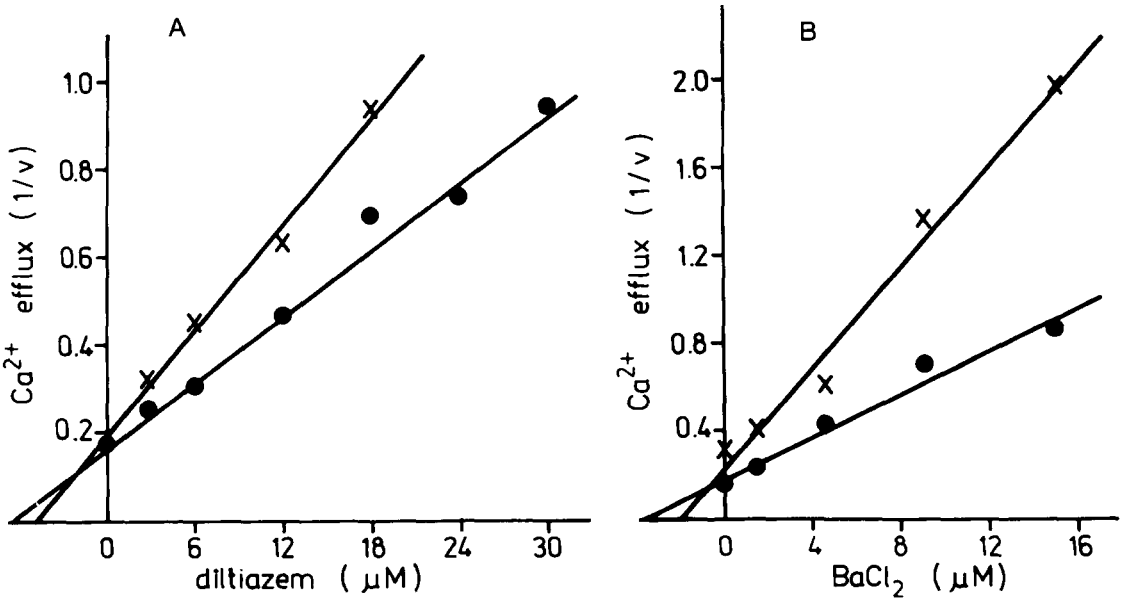

Fig. 6. Double inhibitor titration of the Na^+-induced Ca^{2+} efflux with $BaCl_2$ and diltiazem plotted as a Dixon plot. The (10 mM) Na^+-induced Ca^{2+} efflux (v, nmol/mg per min) was measured as in Fig. 1B. (A) Diltiazem without (●) or with 1.5 μM $BaCl_2$ (×); (B) $BaCl_2$ without (●) or with 3 μM diltiazem (×).

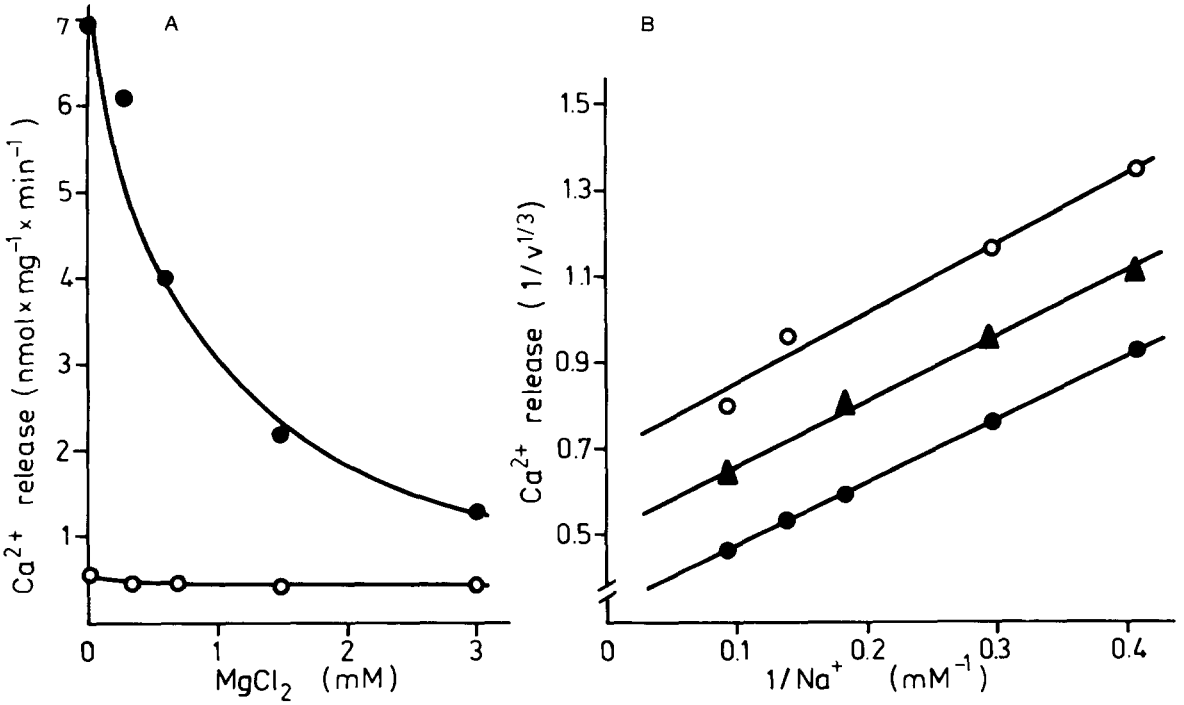

Fig. 7. The effect of Mg^{2+} on the activity of the Na^+-Ca^{2+} exchanger. Experimental conditions were as in Fig. 1B. (A) Basal (○) and the (10 mM) Na^+-induced (●) Ca^{2+} efflux were measured in the sucrose based medium. $MgCl_2$ was added after Ruthenium red. (B) The Ca^{2+} efflux (v, nmol/mg per min) was induced in the absence (●) or in the presence of 0.6 mM (▲) or 3 mM (○) $MgCl_2$ and the results are presented as a double-reciprocal plot.

ments in which the Na^+-Ca^{2+} exchange activity was measured at different Ba^{2+} concentrations in the presence and absence of diltiazem (Fig. 6B).

The effect of Mg^{2+} and other divalent cation on the activity of the Na^+-Ca^{2+} antiporter

The evidence described above indicates that the regulatory site of the Na^+-Ca^{2+} antiporter is not absolutely selective for Ca^{2+}. As the Mg^{2+} concentration in heart cytoplasm is relatively high (1.1–2.5 mM [28,29]), we measured the effect of Mg^{2+} concentrations on the rate of Na^+-induced Ca^{2+} release (Fig. 7A). The inhibitory effect of Mg^{2+} on the uniporter was excluded, as Mg^{2+} was added in the presence of Ruthenium red. The (10 mM) Na^+-induced Ca^{2+} release in sucrose- and KCl-based media was inhibited 50% at 0.51 ± 0.21 ($n = 5$) mM and at 1.32 ± 0.23 ($n = 4$) mM $MgCl_2$, respectively. However, at 1.5 mM $MgCl_2$, which is in the physiological concentration range of free Mg^{2+} in the sarcoplasm, the Na^+-Ca^{2+} exchange activity was attenuated by 54% in KCl and 80% in sucrose-based medium. Mg^{2+} is an uncompetitive inhibitor of the Na^+-Ca^{2+} exchange with respect to extramitochondrial Na^+ (Fig. 7B). This excludes the possibility that Mg^{2+} competes with Na^+ on the substrate-binding site of the antiporter.

Other divalent cations inhibited the Na^+-Ca^{2+} carrier less effectively than did Ba^{2+}. In the presence of 40 μM Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Fe^{2+}, the (10 mM) Na^+-induced Ca^{2+} efflux was inhibited by 32%, 35%, 2%, 25% and 13%, respectively (not shown).

Discussion

It has been proposed recently that the Na^+-Ca^{2+} exchanger possesses a regulatory binding site that renders the carrier sensitive to extramitochondrial Ca^{2+} [14,15]. Data reported in this paper are best explained on the basis of the interaction of Ba^{2+} ions with this regulatory site.

The following evidence supports the idea that

Ba^{2+} ions, similarly to Ca^{2+} ions, interact with the regulatory site of the Na^+-Ca^{2+} antiporter facing the cytosol:

(1) Under the experimental conditions, Ba^{2+} did not accumulate in the matrix space and there was no time-dependence of the Ba^{2+} inhibition (see data in Ref. 16 for comparison). Consequently, the site of Ba^{2+} action is located on the cytosolic surface of the inner membrane.

(2) The non-competitive type of Ba^{2+} inhibition on Ca^{2+} release with respect of extramitochondrial Na^+.

(3) With double inhibition titration, it was revealed that the binding sites for Ca^{2+} ions and Ba^{2+} ions are identical.

(4) The inhibition of the Na^+-Ca^{2+} exchanger by Ca^{2+} [14] or Ba^{2+} was only slightly affected by the extramitochondrial K^+ concentration. In contrast, the affinity of Na^+ (or Sr^{2+}) for the substrate-binding site was strongly dependent on the K^+ concentration of the medium [14].

However, the characteristics of the Ba^{2+} inhibition of the Na^+-induced Ca^{2+} release differ in some respects from the Ca^{2+} inhibition of the same process. The Hill coefficient was 2 for Ca^{2+} [14] and approx. 1 for Ba^{2+} inhibition. The maximal inhibition of the Na^+-Ca^{2+} carrier was 70% by Ca^{2+} [14] and 90% by Ba^{2+}. One possible explanation is that Ba^{2+} is able to induce a more inactive conformation of the carrier than is Ca^{2+}.

The basically different characteristics of Ba^{2+} inhibition on Na^+-dependent Ca^{2+} efflux in heart and Na^+-independent Ca^{2+} efflux in liver mitochondria are in agreement with earlier suggestions that these Ca^{2+} efflux pathways are not only functionally but also structurally distinct (e.g., basically different sensitivity to lanthanides [30]). While the Na^+-dependent pathway binds Ba^{2+} to its regulatory site, facing the cytosol, the Na^+-independent pathway is inhibited exclusively on the substrate-binding site, facing the matrix space [16].

Release of Ca^{2+} from heart mitochondria is usually measured in the absence of Mg^{2+}, as Mg^{2+} strongly inhibits the Ca^{2+} uptake process [31,32]. Our results show that Mg^{2+} concentrations occurring in vivo inhibit the Na^+-dependent Ca^{2+} efflux at least 50%. Consequently, the rate of Ca^{2+} cycling is decreased and the steady-state pCa_o^{2+} is increased. Mg^{2+} inhibited the Na^+-induced Ca^{2+} release in an uncompetitive manner, indicating that Mg^{2+} is presumably bound to the regulatory site of the Na^+-Ca^{2+} antiporter, although with a substantially higher K_i than either Ba^{2+} or Ca^{2+}. Mg^{2+} might decrease the mitochondrial membrane fluidity, as was shown in liver mitochondria [33], though this is very unlikely at 25°C and in the range of sarcoplasmic Mg^{2+} concentration [33].

As Ba^{2+} ions could permeate the plasma membrane of certain cells [34,35] and are able to inhibit the Na^+-Ca^{2+} exchange at micromolar concentrations, Ba^{2+} might prove to be a useful tool to elucidate the role of the mitochondrial Na^+-Ca^{2+} carrier also in the intact cell.

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