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Pathways for Ca^{2+} efflux in heart and liver mitochondria

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1. Two processes of Ruthenium Red-insensitive Ca^{2+} efflux exist in liver and in heart mitochondria: one Na^+-independent, and another Na^+-dependent. The processes attain maximal rates of 1.4 and 3.0 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ for the Na^+-dependent and 1.2 and 2.0 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ for the Na^+-independent, in liver and heart mitochondria, respectively. 2. The Na^+-dependent pathway is inhibited, both in heart and in liver mitochondria, by the Ca^{2+} antagonist diltiazem with a K_i of 4 μM. The Na^+-independent pathway is inhibited by diltiazem with a K_i of 250 μM in liver mitochondria, while it behaves as almost insensitive to diltiazem in heart mitochondria. 3. Stretching of the mitochondrial inner membrane in hypo-osmotic media results in activation of the Na^+-independent pathway both in liver and in heart mitochondria. 4. Both in heart and liver mitochondria the Na^+-independent pathway is insensitive to variations of medium pH around physiological values, while the Na^+-dependent pathway is markedly stimulated parallel with acidification of the medium. The pH-activated, Na^+-dependent pathway maintains the diltiazem sensitivity. 5. In heart mitochondria, the Na^+-dependent pathway is non-competitively inhibited by Mg^{2+} with a K_i of 0.27 mM, while the Na^+-independent pathway is less affected; similarly, in liver mitochondria Mg^{2+} inhibits the Na^+-dependent pathway more than it does the Na^+-independent pathway. In the presence of physiological concentrations of Na^+, Ca^{2+} and Mg^{2+}, the Na^+-independent and the Na^+-dependent pathways operate at rates, respectively, of 0.5 and 1.0 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ in heart mitochondria and 0.9 and 0.2 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ in liver mitochondria. It is concluded that both heart and liver mitochondria possess two independent pathways for Ca^{2+} efflux operating at comparable rates.

INTRODUCTION

Three pathways for Ca^{2+} transport have been described in respiring mitochondria that maintain a high transmembrane electrical potential: (i) the Ca^{2+} uniporter (Selwyn *et al.*, 1970; Scarpa & Azzone, 1970; Rottenberg & Scarpa, 1974; Bernardi *et al.*, 1984); (ii) the putative H^+/Ca^{2+} antiporter (Vasington *et al.*, 1972; Stucki & Ineichem, 1974; Azzone *et al.*, 1975, 1977; Puskin *et al.*, 1976; Nicholls, 1978; Fiskum & Lehninger, 1979; Bernardi & Azzone, 1982, 1983*a*,*b*); (iii) the Na^+/Ca^{2+}antiporter (Crompton *et al.*, 1976, 1978; Nicholls & Scott, 1980; Allshire & Heffron, 1984). In respiring mitochondria, therefore, the uniporter catalyses Ca^{2+} uptake driven by $\Delta\tilde{\mu}_{Ca^{2+}}$, while the H^+/Ca^{2+} and the Na^+/Ca^{2+} antiporters catalyse Ca^{2+} efflux driven by the combination of $\Delta\tilde{\mu}_{H^+}$ and $\Delta\tilde{\mu}_{Ca^{2+}}$ for the former and $\Delta\tilde{\mu}_{Na^+}$ and $\Delta\tilde{\mu}_{Ca^{2+}}$ for the latter (for reviews, see Saris & Åkerman, 1980; Nicholls & Åkerman, 1982; Crompton, 1985). Operation of the antiporters prevents the otherwise harmful Ca^{2+} distribution at electrochemical equilibrium, and allows steady state Ca^{2+} recycling (Stucki & Ineichem, 1974). The energy drain associated with Ca^{2+} cycling is low, due to the low rate of the antiporters.

A highly active uniporter is a common feature of mitochondria from all mammalian tissues, while the antiporters exhibit a tissue specificity (Saris & Åkerman, 1980; Nicholls & Åkerman, 1982). The Na^+/Ca^{2+} antiporter has been originally observed in mitochondria from heart, skeletal muscle, brain, parotid gland and adrenal cortex (Crompton *et al.*, 1976, 1978), while the H^+/Ca^{2+} antiporter has been found in liver, kidney and lung mitochondria (reviewed by Nicholls & Åkerman, 1982). It is commonly thought that in excitable tissues the H^+/Ca^{2+} antiporter operates at a much lower rate than the Na^+/Ca^{2+} antiporter, and hence does not play a role in Ca^{2+} homeostasis. However, the classification of Na^+-sensitive and Na^+-insensitive mitochondria has been challenged by the observation of a Na^+-dependent stimulation of Ruthenium Red-insensitive Ca^{2+} efflux in liver mitochondria (Haworth *et al.*, 1980; Heffron & Harris, 1981; Harris & Heffron, 1982; Nedergaard, 1984).

Since the activity of the mitochondrial Ca^{2+} porters is specifically modulated by divalent cations (Hughes & Exton, 1983; Saris & Bernardi, 1983; Allshire *et al.*, 1985; Favaron & Bernardi, 1985) and depends critically on the free Ca^{2+} concentration (Coll *et al.*, 1982), we have re-examined the relative contribution of the Na^+/Ca^{2+} and H^+/Ca^{2+} antiporters in heart and liver mitochondria: (i) at low Ca^{2+} concentrations and in the presence of physiological concentrations of P_i and Mg^{2+}, and (ii) at moderate Ca^{2+} loads in the presence of Ca^{2+} antagonists

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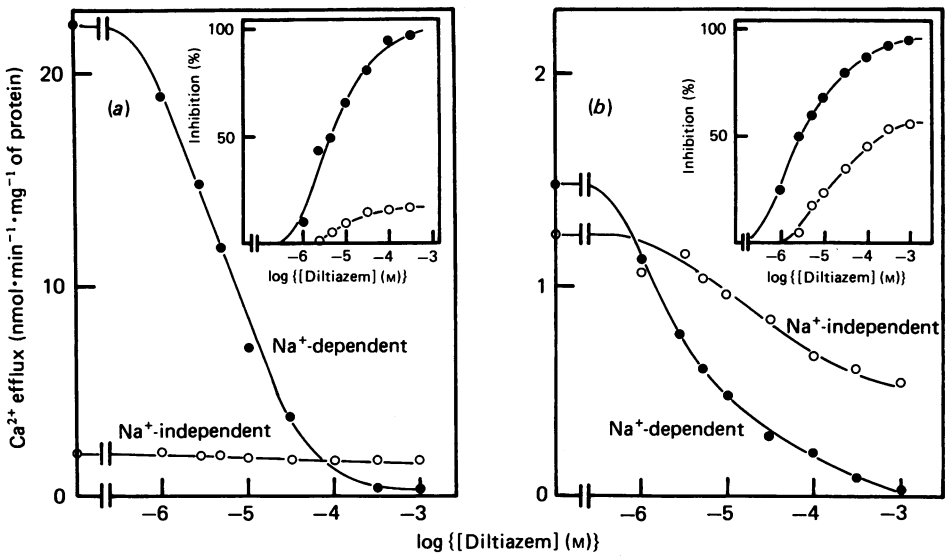

Fig. 1. Effect of diltiazem on Na^+-dependent and Na^+-independent Ca^{2+} efflux in heart and liver mitochondria

The incubation medium contained 100 mM-KCl, 10 mM-Tris/Mops, 5 mM-succinate, 10 mM-acetate, 1 mg of bovine serum albumin/ml, 2 μM-rotenone, 5 μM-cytochrome *c*, 20 μM-Arsenazo III, 40 μM-$CaCl_2$ and diltiazem at the indicated concentrations. Final volume 2 ml, 25 °C, pH 7.0. The experiments were started by the addition of rat heart (*a*) or liver (*b*) mitochondria at 1 mg/ml. After attainment of a steady state Ca^{2+} distribution, Ca^{2+} efflux was initiated by the addition of 80 (*a*) or 250 (*b*) pmol of Ruthenium Red/mg of protein (○) or 80 (*a*) or 250 (*b*) pmol of Ruthenium Red/mg of protein + 20 mM-NaCl (●). The Na^+-dependent rate of Ca^{2+} efflux is calculated as $\Delta \pm Na^+$.

and of various ions acting as inhibitors or activators of the Na^+/Ca^{2+} exchange (Vaghy *et al.*, 1982). Furthermore, we have also tested the effect of membrane stretching and of pH changes on the activity of the H^+/Ca^{2+} and Na^+/Ca^{2+} antiporters. We conclude that excitable and non-excitable tissues possess both a Na^+-dependent and a Na^+-independent pathway for Ca^{2+} efflux. Furthermore, the two pathways seem to correspond to different molecular entities on the basis of their sensitivity to a number of treatments or to inhibitory agents.

MATERIALS AND METHODS

Rat heart and liver mitochondria were prepared as described previously (Favaron & Bernardi, 1985). Ca^{2+} fluxes were monitored either with a Ca^{2+}-selective electrode (W. Moller, Zurich, Switzerland), exactly as described in a previous paper (Bernardi & Azzone, 1983*a*), or using the metallochromic indicator Arsenazo III (wavelength pairs 650 nm minus 690 nm) with an Aminco DW 2A dual wavelength spectrophotometer equipped with magnetic stirring and thermostatic control (final volume 2 ml, 30 °C). Ruthenium Red was purified according to Luft (1971). The dye solutions were prepared daily and the concentration of Ruthenium Red was determined spectrophotometrically on the basis of an ϵ_{533} of 68 $mM^{-1} \cdot cm^{-1}$ (Fletcher *et al.*, 1961). Ruthenium Red was used at 250 pmol·mg^{-1} of protein in liver mitochondria and 80 pmol·mg^{-1} of protein in heart mitochondria, since we found that lower amounts of inhibitor are needed to inhibit Ca^{2+} transport fully in heart mitochondria (results not shown).

Ruthenium Red, diltiazem, Arsenazo III and cytochrome *c* were purchased from Sigma.

The incubation media are specified in the Figure legends, and all chemicals were of the highest purity available.

RESULTS

Effect of diltiazem, stretching and pH

Fig. 1 shows a titration with diltiazem of the Ruthenium Red-insensitive Ca^{2+} efflux from heart and liver mitochondria in the presence and absence of 20 mM-Na^+. The experiment was carried out at moderate Ca^{2+} load and with 10 mM-acetate in order to increase the Ca^{2+} chemical gradient and in KCl media in order to activate the antiporters. Under the conditions of Fig. 1 the rates of the Na^+-dependent and Na^+-independent pathways were, in heart mitochondria, 22 and 2 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$, respectively. The titration of Ca^{2+} efflux in heart mitochondria (Fig. 1*a*) shows a marked sensitivity of the Na^+-dependent efflux and almost no sensitivity of the Na^+-independent efflux to diltiazem. The K_i for the former process was around 5 μM (cf. Vaghy *et al.*, 1982). Furthermore the inhibition by diltiazem was practically complete. In contrast, concentrations of diltiazem up to 1 mM resulted in a depression of the Na^+-independent efflux which was less than 20%. In liver mitochondria (Fig. 1*b*) the rates of the Na^+-dependent and Na^+-independent effluxes were, respectively, about 7% and 60% as compared with those of heart mitochondria. The titrations with diltiazem show that both the Na^+-dependent and Na^+-independent pathways were sensitive to diltiazem although with a different K_i, 5 and 150–200 μM for the Na^+-dependent and the Na^+-independent effluxes, respectively. A further difference between the two pathways is that the Na^+-

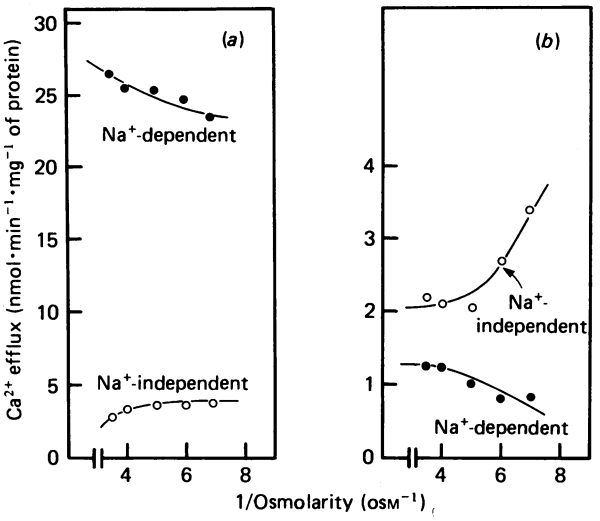

Fig. 2. Effect of hypo-osmotic swelling on Na^+-dependent and Na^+-independent Ca^{2+} efflux in heart and liver mitochondria

The incubation medium contained 20 mM-KCl, 10 mM-Tris/Mops, pH 7.0, 5 mM-succinate, 10 mM-acetate, 1 mg of bovine serum albumin/ml, 2 μM-rotenone, 5 μM-cytochrome *c*, 20 μM-Arsenazo III, 40 μM-$CaCl_2$ and sucrose concentrations varying from 0 to 140 mM. Final volume 2 ml, 25 °C. The experiments were started by the addition of rat heart (*a*) or liver (*b*) mitochondria at 1 mg/ml. After attainment of a steady state Ca^{2+} distribution, Ca^{2+} efflux was initiated by the addition of Ruthenium Red, at the concentrations of Fig. 1, +20 mM-NaCl (●) or KCl (○); the osmolarity reported on the abscissa refers to the final osmolarity. The Na^+-dependent rate of Ca^{2+} efflux is calculated as $\Delta + Na^+$.

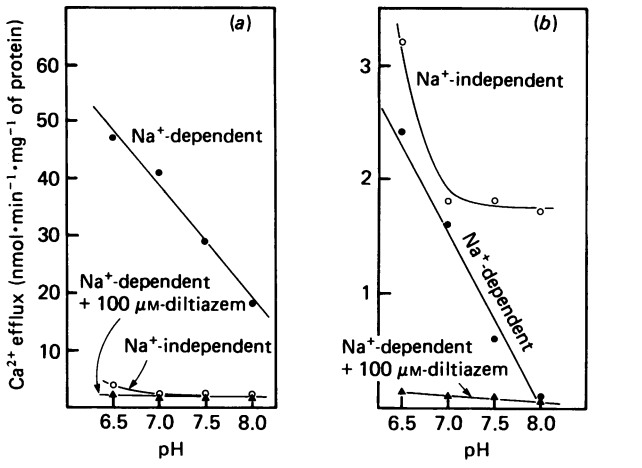

Fig. 3. Effect of pH on Na^+-dependent and Na^+-independent Ca^{2+} efflux in heart and liver mitochondria

The incubation medium was as in Fig. 1, except for the pH value, which was as indicated. The experiments were initiated by the addition of rat heart (*a*) or liver (*b*) mitochondria (1 mg/ml). After attainment of a steady state Ca^{2+} distribution, Ca^{2+} efflux was initiated by the addition of Ruthenium Red, at the concentrations of Fig. 1 (○), or of Ruthenium Red + 20 mM-NaCl (●). When indicated (▲) 100 μM-diltiazem was also present. The Na^+-dependent rate of Ca^{2+} efflux is calculated as $\Delta + Na^+$.

dependent is almost fully inhibited by diltiazem, while the Na^+-independent is maximally 60% inhibited.

From the titration of Fig. 1 it appears that the Na^+-dependent pathways of heart and liver mitochondria possess similar kinetic properties (equal K_i and complete inhibition at diltiazem concentrations between 0.01 and 1 mM). On the other hand, the Na^+-independent pathways show different kinetic properties (almost complete insensitivity in heart and partial sensitivity in liver mitochondria to diltiazem).

Membrane stretching affects both H^+/K^+ and H^+/Ca^+ antiporters (Bernardi & Azzone, 1983*a*,*b*; Garlid, 1980). Fig. 2 shows that the decrease of medium osmolarity, which causes a swelling-induced stretching of the inner membrane, had an opposite effect to the Na^+-dependent and Na^+-independent Ca^{2+} effluxes. In fact, both in heart and liver mitochondria the decrease of osmolarity was accompanied by an increase of the Na^+-independent and decrease of the Na^+-dependent Ca^{2+} efflux. The effects were proportionally more marked in liver than in heart mitochondria. In fact, the increase of Ca^{2+} efflux was only from about 3 to 4 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$, i.e. 25%, in heart mitochondria, and from 2 to 3.5 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$, i.e. 70%, in liver mitochondria (Bernardi & Azzone, 1983*a*).

Fig. 3 shows that in heart mitochondria, decrease of pH from 8 to 6.5 resulted in a large increase of the Na^+-dependent efflux, say from 18 to 47 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ and in almost no effect on the Na^+-independent efflux. That the pH effect is specific for the Na^+/Ca^{2+} antiporter is supported by the high sensitivity to diltiazem of the pH-stimulated increase of Na^+-dependent Ca^{2+} efflux. In contrast, the acidification of the medium was unable to stimulate the Na^+-independent efflux. In liver mitochondria the effect of pH was similar to that of heart mitochondria, in that the decrease of pH from 8 to 6.5 resulted in an increase of the Na^+-dependent efflux from 0.1 to 2.4 nmol of $Ca^{2+} \cdot min^{-} \cdot mg^{-1}$. Again, the pH-stimulated increase of Na^+-dependent Ca^{2+} efflux was fully sensitive to diltiazem. The rate of the Na^+-independent Ca^{2+} efflux was insensitive to the pH decrease in the range between 8.0 and 7.0, while there was an increase of Ca^{2+} efflux from 1.8 to 3.2 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ at pH 6.5. The pH dependence of the Na^+-independent Ca^{2+} efflux in liver mitochondria of Fig. 3 is different from that reported by Bernardi (1984). However, in those experiments the rate of Ca^{2+} efflux was about 3 times lower than in the present, due to the different experimental conditions, i.e. low Ca^{2+}, no acetate and addition of Mg^{2+}. It seems therefore that the pH-dependence is markedly affected by Ca^{2+}, acetate and Mg^{2+} concentrations.

The Na^+-dependent Ca^{2+} efflux is known to occur at a higher rate in the presence of K^+. In Fig. 4(*a*) the effect of KCl was compared with that of LiCl and of choline chloride. It is seen that addition of increasing salt concentrations in the range between 10 and 100 mM had a completely different effect, namely a marked stimulation of Ca^{2+} efflux in the case of KCl, only a slight stimulation in the case of LiCl and a progressive inhibition in the case of choline chloride (Crompton,

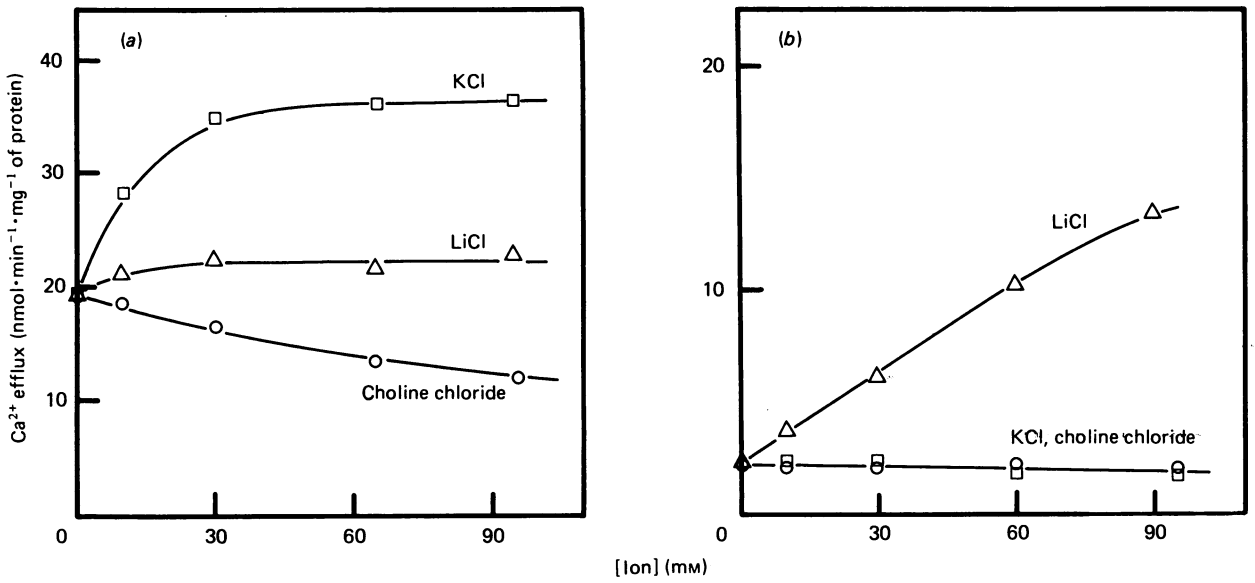

Fig. 4. Effect of K^+, Li^+ and choline$^+$ on Na^+-dependent and Na^+-independent Ca^{2+} efflux in heart mitochondria

The incubation medium contained 10 mM-Tris/Mops, pH 7.0, 5 mM-succinate, 10 mM-acetate, 1 mg of bovine serum albumin/ml, 2 μM-rotenone, 5 μM-cytochrome *c*, 20 μM-Arsenazo III, 40 μM-$CaCl_2$, the indicated concentrations of KCl (□), LiCl (△) or choline chloride (○) and the concentration of sucrose necessary to reach a final osmolarity of 295 mM. The experiments were initiated by the addition of rat heart mitochondria (1 mg/ml). After attainment of a steady state Ca^{2+} distribution, Ca^{2+} efflux was initiated by the addition of 80 pmol of Ruthenium Red/mg of protein + 20 mM-NaCl (*a*) or 80 pmol of Ruthenium Red/mg of protein (*b*).

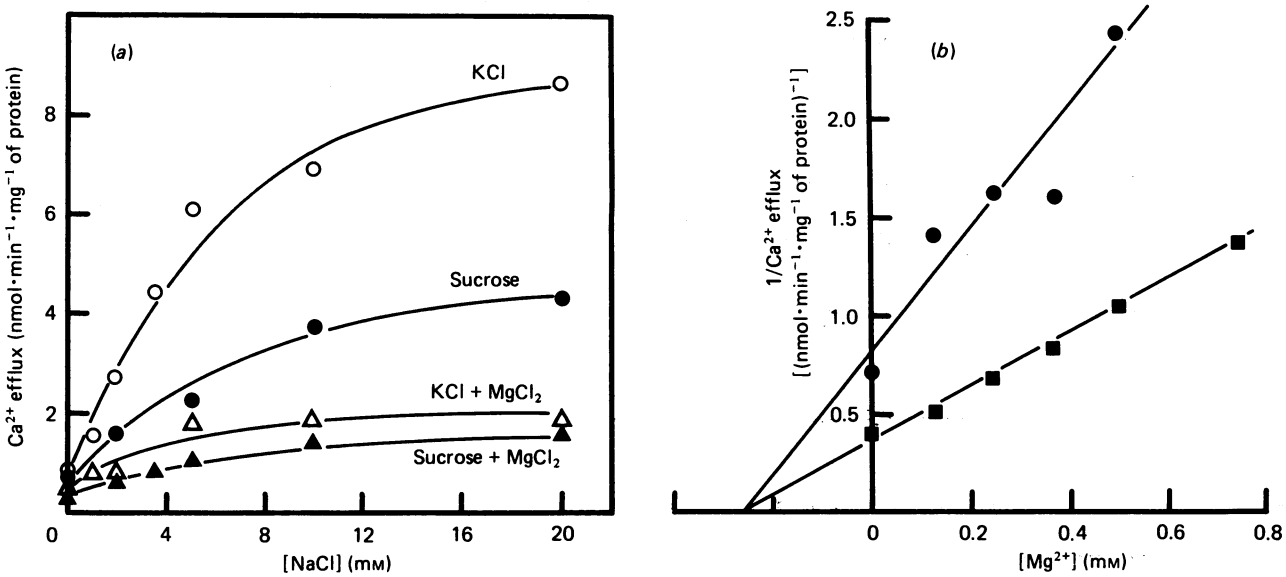

Fig. 5. Na^+-induced changes of Ca^{2+} efflux rates in heart mitochondria: effect of Mg^{2+}

In (*a*) the incubation medium contained either 120 mM-KCl (○, △) or 140 mM-sucrose and 40 mM-choline chloride (●, ▲), 10 mM-Tris/Mops, 5 mM-succinate, 0.5 mM-phosphate, 1 mg of bovine serum albumin/ml, 2 μM-rotenone, 1 μg of oligomycin/ml and 7 μM-free Ca^{2+}; NaCl was as indicated. Final volume 2 ml, 30 °C, pH 7.0. ●, ○, No Mg^{2+}; ▲, △, +1.5 mM-Mg^{2+}. The experiments were started by the addition of 1 mg of rat heart mitochondria/ml. After attainment of a steady state Ca^{2+} distribution, Ca^{2+} efflux was initiated by the addition of 80 pmol of Ruthenium Red/mg of protein. In (*b*) the experiment was carried out in the sucrose medium of (*a*): ●, +6 mM-NaCl; ■, +20 mM-NaCl. Mg^{2+} was as indicated.

1985). Fig. 4(*b*) shows that neither KCl nor choline chloride were able to stimulate to any degree the Na^+-independent Ca^{2+} efflux. This supports the view that KCl cannot replace Na^+ as the transported ion in exchange with Ca^{2+}, but rather acts as an allosteric activator of the Na^+/Ca^{2+} exchange. Conversely, choline acts as an allosteric inhibitor of the exchange. On the other hand and in accord with previous reports (Crompton *et al.*, 1976) Li^+ ions are able to replace Na^+ ions in view of their capacity to stimulate Ca^{2+} efflux in

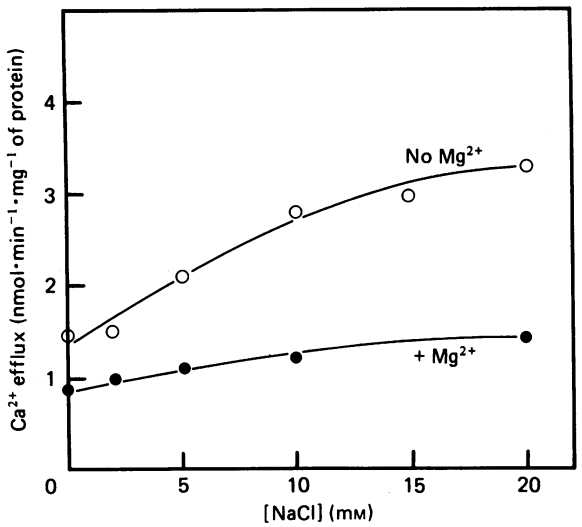

Fig. 6. Na^+-induced changes of Ca^{2+} efflux rates in liver mitochondria: effect of Mg^{2+}

The incubation medium was as in Fig. 5(*a*) with 120 mM-KCl. The experiments were started by the addition of liver instead of heart mitochondria (1 mg/ml). Ca^{2+} efflux was initiated with 250 pmol of Ruthenium Red/mg of protein. Data from two independent experiments are collected: ○, no Mg^{2+}; ●, +1.5 mM-Mg^{2+}.

the absence of Na^+. However 50% stimulation of Ca^{2+} efflux requires Li^+ concentrations higher than those of Na^+.

Effect of Mg^{2+}

Fig. 5(*a*) analyses the effect, in the absence and in the presence of 1.5 mM-Mg^{2+}, of increasing Na^+ concentrations on Ca^{2+} efflux in heart mitochondria either in sucrose or in KCl media in the presence of 0.5 mM-P_i. In the absence of Mg^{2+} and in sucrose media an increase of Na^+ from 0 to 20 mM resulted in an increase of the rate of Ca^{2+} efflux from 0.8 to 4.2 nmol·min^{-1}·mg^{-1}, i.e. a 5-fold stimulation. Addition of 1.5 mM-Mg^{2+} resulted in an almost 3-fold decrease of both the Na^+-independent and Na^+-dependent (Bernardi & Azzone, 1984; Lukacs & Fonyo, 1986) rates of Ca^{2+} efflux (0.3 and 1.5 nmol·min^{-1}·mg^{-1} in the absence and presence of 20 mM-NaCl). Replacement of the sucrose with the KCl media resulted in a marked enhancement of the Na^+-dependent Ca^{2+} efflux, from 0.9 nmol·min^{-1}·mg^{-1} in the absence to 8.6 nmol·min^{-1}·mg^{-1} in the presence of 20 mM-NaCl. However, parallel with the more marked stimulation of the Na^+-dependent Ca^{2+} efflux, in KCl media also the Mg^{2+} inhibition became more marked (0.5 in the absence and 1.5–1.9 nmol·min^{-1}·mg^{-1} in the presence of 5–20 mM-NaCl). In the presence of physiological concentrations of Na^+, Mg^{2+} and Ca^{2+}, therefore, the Na^+-dependent and the Na^+-independent pathways contribute to Ca^{2+} efflux from heart mitochondria in the ratio of 2:1. Fig. 5(*b*) analyses the Mg^{2+} inhibitory effect in sucrose media, shown in Fig. 5(*a*), according to a Dixon plot, where the rate of Ca^{2+} efflux has been tested at 6 and 20 mM-NaCl. The inhibition was non-competitive, with a K_i of 0.27 mM.

Fig. 6 analyses the effect, in the absence and in the presence of 1.5 mM-Mg^{2+}, of increasing Na^+ concentrations on Ca^{2+} efflux in liver mitochondria, under the same conditions as Fig. 5(*a*) without Mg^{2+}. Ca^{2+} efflux increased from 1.4 in the absence to 2.1–3.3 nmol·min^{-1}·mg^{-1} in the presence of 5–20 mM-NaCl, whereas with 1.5 mM-Mg^{2+} Ca^{2+} efflux increased only from 0.9 to 1.1–1.4 nmol·min^{-1}·mg^{-1} in the presence of 5–20 mM-NaCl. In summary, in the presence of physiological concentrations of Mg^{2+}, Na^+ and Ca^{2+}, the rates of the Na^+-dependent and the Na^+-independent pathways for Ca^{2+} efflux were, respectively, 0.2 and 0.9 nmol·min^{-1}·mg^{-1}.

DISCUSSION

Properties of the Na^+-dependent and Na^+-independent pathway

Whether Na^+ is able to promote Ca^{2+} efflux in liver mitochondria has been a matter of dispute, and a number of conflicting reports have appeared (cf. Crompton, 1985). Haworth *et al.* (1980) reported that Na^+ stimulates Ca^{2+} efflux in liver, kidney and lung mitochondria. In this study, however, Ca^{2+} distribution never attained the stationary state that Na^+ would modify. Heffron & Harris (1981) and Harris & Heffron (1982) also observed a Na^+ stimulation of Ca^{2+} efflux which however was abolished by oligomycin. The view that liver mitochondria possess a Na^+/Ca^{2+} antiporter was then reproposed by Nedergaard (1984). The topic has been recently reviewed by Crompton (1985). Gunther *et al.* (1983) have provided some experimental evidence against the view that the Na^+-independent Ca^{2+} efflux in liver mitochondria involves a H^+/Ca^{2+} exchange and favoured the concept of an active Ca^{2+} efflux. In our view a H^+/Ca^{2+} antiporter remains the most attractive hypothesis for a H^+–Ca^{2+} cycling in steady state involving independent pathways for Ca^{2+} influx and efflux. However, the conclusions of the present study hold also in the case the Na^+-independent pathway involves an active Ca^{2+} efflux mechanism.

At this point it is necessary to emphasize a crucial feature of the Na^+-independent Ca^{2+} efflux observable at high $\Delta\tilde{\mu}_{H^+}$ after addition of Ruthenium Red. Such a Na^+-independent Ca^{2+} efflux should not be confused with the Ca^{2+} release following changes of membrane permeability as induced by hydroperoxides, SH reagents, menadione and inorganic phosphate. In fact the former process occurs against, while the latter occurs down, the Ca^{2+} electrochemical gradient. The distinction implies that the former process must occur via a specific carrier mechanism, distinct from the uniport pathway, while the latter may occur either through the uniport or any unspecific pathway opened during the permeability change.

Two factors have limited the establishment of the Na^+-dependent pathway in liver mitochondria. First, the low rate of the process of Ca^{2+} efflux following addition of Ruthenium Red. Second, the fact that the extent of Na^+ stimulation may vary in a range between 30 and 70% among the various mitochondrial preparations. The reason for this variability is not understood at present.

In our study the Na^+-independent and the Na^+-dependent have been characterized on the basis of the following kinetic properties: (i) sensitivity to diltiazem, high for the Na^+-dependent and very low or almost absent for the Na^+-independent pathway; (ii) sensitivity

to changes of medium pH, activation of the Na^+-dependent pathway at acidic pH and practical insensitivity of the Na^+-independent pathway at pH changes; (iii) effect of membrane stretching, depression of the Na^+-dependent and activation of the Na^+-independent pathway; (iv) sensitivity to ions, activation by K^+ ions and inhibition by choline of the Na^+-dependent and insensitivity to ions of the Na^+-independent pathway. A further characterization of the kinetic properties of the antiporters is provided by the fact that the pH-activated Na^+-dependent pathway maintains both in liver and heart mitochondria full sensitivity to diltiazem.

The sensitivity to diltiazem and the pH-dependence are presumably the reflection of the molecular properties of the Na^+/Ca^{2+} antiporters. This is in accord with the observation that both in heart and liver mitochondria the acid stimulation of the Na^+-dependent efflux maintains full sensitivity to diltiazem. It would therefore seem that the acid stimulation is due to the pH-dependence of the carrier. Note that in the case of the H^+/Na^+ and H^+/K^+ antiporters there is an alkaline rather than an acid stimulation and this may reflect a competition of H^+ ions and cations for the cation-binding site of the antiporters.

The Na^+-independent pathway shows different kinetic properties both in liver and in heart mitochondria: (i) it is almost completely insensitive to diltiazem in heart and 50 times less sensitive (as compared with the Na^+-dependent pathway) in liver mitochondria; (ii) it is almost pH-insensitive; (iii) it is activated by membrane stretching, and (iv) it is insensitive to the presence of K^+ or choline$^+$ ions. The activation due to membrane stretching in liver mitochondria resembles the similar activation recorded in the case of the H^+/Na^+ and H^+/K^+ antiporters, suggesting that the antiporter activity is partly masked in the native mitochondrial membrane and becomes unmasked under stretching. The concept of the Na^+-dependent pathway as a molecular entity distinct from the Na^+-independent pathway is therefore strengthened by the present observations. Diltiazem sensitivity, acid activation and presence of the carrier both in heart and liver mitochondria may be taken as specific molecular properties of this class of ion carriers.

Pathways and regulation of Ca^{2+} efflux in heart mitochondria

Although the presence of a Na^+/Ca^{2+} exchange in heart mitochondria is well documented (Crompton *et al.*, 1976, 1978), its role in the modulation of mitochondrial transmembrane Ca^{2+} distribution *in vivo* remains unclear. In the living cell, the activity of the Na^+/Ca^{2+} antiporters depends on: (i) the cytosolic free Na^+ concentrations; (ii) the concentration of positive or negative effectors; (iii) the matrix free Ca^{2+} concentration.

The intracellular free Na^+ concentration is close to 6 mM (Lee & Fozzard, 1975) and is unlikely to undergo wide variations during the cardiac action potential. The calculated changes of intracellular free Na^+ in the 0.1–0.5 mM range are presumably large overestimates (Crompton *et al.*, 1976). Since the apparent K_m for Na^+ of the Na^+/Ca^{2+} antiporter *in vitro* is 8 mM (Crompton *et al.*, 1976), it is unlikely that the rate of Ca^{2+} efflux is modulated by changes of intracellular free Na^+ during the action potential.

The intracellular free Mg^{2+} maintained in the intact, perfused guinea pig heart is 2.5 mM (Wu *et al.*, 1981). This value can be compared with the data of Figs. 5 and 6 of the present study, indicating that 50% inhibition of the Na^+/Ca^{2+} antiporter is obtained at Mg^{2+} concentrations 10-fold lower than those occurring *in vivo*. It seems therefore likely that the activity of the Na^+/Ca^{2+} antiporter *in vivo* is very low, its rate being comparable with that of the H^+/Ca^{2+} antiporter. The mitochondrial matrix Ca^{2+} *in vivo* is lower than 2 nmol/mg of protein (Somlyo *et al.*, 1985). Assuming a matrix volume of 1 μl/mg of protein, and a matrix activity coefficient of 4×10^{-4} (Coll *et al.*, 1982), the intramitochondrial free Ca^{2+} is in the micromolar range. Since the apparent K_m for Ca^{2+} for the Na^+/Ca^{2+} antiporter in heart mitochondria is 5.7 μM (Coll *et al.*, 1982), it appears that the rate of operation of this antiporter is essentially regulated by changes of matrix free Ca^{2+}. The present data indicate that, at physiological Ca^{2+}, Na^+ and Mg^{2+} concentrations and in KCl media the Na^+-sensitive and Na^+-insensitive pathways operate at rates of approx. 1.0 and 0.5 nmol of $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$ of protein, respectively. These rates suggest that Na^+/Ca^{2+} and H^+/Ca^{2+} exchanges are about equally active in mitochondria from excitable tissues.

Physiological contribution of the H^+/Ca^{2+} and Na^+/Ca^{2+} carriers

The present study indicates a fundamental similarity between mitochondria from excitable and non-excitable tissues. In KCl media and in the presence of Mg^{2+}, the rates of Ruthenium Red-insensitive Ca^{2+} efflux are in heart mitochondria 0.5, 1.5 and 2.0 and in liver mitochondria 0.9, 1.1 and 1.4 $nmol \cdot min^{-1} \cdot mg^{-1}$ of protein at 0, 5 and 20 mM-NaCl respectively. Thus, while in heart mitochondria the Na^+-insensitive pathway accounts for about 33% with respect to the total rate of Ca^{2+} efflux in 5 mM-Na^+, in liver mitochondria in the presence of 5 mM-NaCl the Na^+-insensitive pathway accounts for about 80% of the total rate of Ca^{2+} efflux. Conversely, the Na^+-sensitive pathway accounts for 67% and 20% of the total Ruthenium Red-insensitive Ca^{2+} efflux in heart and liver mitochondria, respectively. These numbers indicate that presumably both the H^+/Ca^{2+} and Na^+/Ca^{2+} exchanges fulfill the role of physiological regulators of the Ca^{2+} distribution in heart or in liver mitochondria.

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