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Biochimica et Biophysica Acta 1659 (2004) 83–91

BIOCHIMICA ET BIOPHYSICA ACTA
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Kinetics and ion specificity of Na^+/Ca^{2+} exchange mediated by the reconstituted beef heart mitochondrial Na^+/Ca^{2+} antiporter

Petr Paucek[a,*], Martin Jabůrek[b]

[a]Department of Biology, Portland State University, Portland, Oregon 97207, USA
[b]Department of Membrane Transport Biophysics, Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 14220 Prague, Czech Republic

Received 12 November 2003; received in revised form 1 March 2004; accepted 5 March 2004
Available online 11 September 2004

Abstract

The Na^+/Ca^{2+} antiporter was purified from beef heart mitochondria and reconstituted into liposomes containing fluorescent probes selective for Na^+ or Ca^{2+}. Na^+/Ca^{2+} exchange was strongly inhibited at alkaline pH, a property that is relevant to rapid Ca^{2+} oscillations in mitochondria. The effect of pH was mediated entirely via an effect on the K_m for Ca^{2+}. When present on the same side as Ca^{2+}, K^+ activated exchange by lowering the K_m for Ca^{2+} from 2 to 0.9 μM. The K_m for Na^+ was 8 mM. In the absence of Ca^{2+}, the exchanger catalyzed high rates of Na^+/Li^+ and Na^+/K^+ exchange. Diltiazem and tetraphenylphosphonium cation inhibited both Na^+/Ca^{2+} and Na^+/K^+ exchange with IC_{50} values of 10 and 0.6 μM, respectively. The V_{max} for Na^+/Ca^{2+} exchange was increased about fourfold by bovine serum albumin, an effect that may reflect unmasking of an autoregulatory domain in the carrier protein.

Keywords: Na^+/Ca^{2+} exchange; Cardiac mitochondria; ATP production

1. Introduction

The Ca^{2+} cycle of cardiac mitochondria is responsible for rapid oscillations in matrix Ca^{2+} [1–6], which result in sustained activation of Ca^{2+}-sensitive matrix dehydrogenases in response to cellular demands for increased ATP production [7–10]. The Na^+/Ca^{2+} antiporter carries out the efflux side of this important cycle, and Cox and Matlib [11] have shown directly that the cardiac mitochondrial Na^+/Ca^{2+} antiporter plays a central role in regulating turnover of the tricarboxylic acid cycle and the rate of oxidative phosphorylation.

Our laboratory has purified the Na^+/Ca^{2+} antiporter from beef heart mitochondria, and the Na^+/Ca^{2+} exchange activity was identified with a 110-kDa inner membrane protein. We also showed that the purified beef heart mitochondrial Na^+/Ca^{2+} antiporter catalyzes Na^+ and Ca^{2+} flux following its reconstitution into liposomes [12]. However, the kinetics, regulation, and pharmacology of the purified reconstituted carrier have not been reported.

In the present study we show that the kinetics of Na^+/Ca^{2+} exchange are consistent with its participation in rapid oscillation in matrix Ca^{2+}. In particular, exchange is strongly inhibited at alkaline pH, an effect mediated by a sharp increase in the K_m for Ca^{2+}. We found that BSA increased the V_{max} of Na^+/Ca^{2+} exchange by three- to fourfold. By exclusion, this effect appears to be due to protein–protein interaction, raising the intriguing possibility that the exchanger is subject to autoregulation by an

Abbreviations: BSA, bovine serum albumin; EGTA, ethylene-bis(oxyethylenenitrilo) tetraacetic acid; HEDTA, *N*-(2-hydroxyethyl) ethylenediamine-*N*,*N*′,*N*′-triacetic acid; SBFI, sodium-binding benzofuran isophthalate; SMPs, submitochondrial particles; TEA^+, tetraethylammonium cation; TES, *N*-tris(hydroxymethyl)methylaminoethenesulfonic acid; TPP^+, tetraphenylphosphonium; XIP, exchange inhibitory peptide

* Corresponding author. Tel.: +1 503 725 8968; fax: +1 503 725 3888.
E-mail addresses: paucek@pdx.edu (P. Paucek), jaburek@biomed.cas.cz (M. Jabůrek).

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doi:10.1016/j.bbabio.2004.03.019

intrinsic peptide, as has been demonstrated for plasma membrane Na^+/Ca^{2+} antiporters [13–15].

2. Experimental procedures

2.1. Materials

SBFI was obtained from Molecular probes and Fura-2 from Calbiochem. Octylpentaoxylethylene was obtained from Bachem. Other chemicals and reagents were from Sigma Chemical Company. Beef heart mitochondria were prepared by differential centrifugation [16].

2.2. Purification and reconstitution of Na^+/Ca^{2+} antiporter

The protocols for purifying and reconstituting the Na^+/Ca^{2+} antiporter have been described in detail [12,17]. Briefly, beef heart submitochondrial particles were prepared and treated to remove the F_1 subunit of ATPase and other adherent proteins [18]. Proteins (100 mg) are extracted for 1 h in 20 ml of buffer containing 3% Triton X-100, then centrifuged and the supernatant collected for purification [12]. The solubilized protein is loaded onto a 20-ml DEAE-cellulose column and washed with 80 ml of the column-buffer containing 1% Triton X-100. Proteins were then eluted with 2 bed volumes of the column buffer and further purified on a 5-ml DEAE-cellulose column. Na^+/Ca^{2+} exchange activity being found in the second bed volume fraction. This fraction is reconstitutively active and contains the dominant protein band on SDS-PAGE, at 110 kDa. We also noticed a minor (inactive) 70-kDa protein band on SDS-PAGE. [12].

The purified protein fraction was added to a 10:1 mixture of L-α-lecithin (Avanti) and cardiolipin in 10% octylpentaoxyethylene. The buffer composition at this stage defines the internal medium, the composition of which is described in detail in figure legends. This mixture was loaded onto a 2-ml Bio-Beads SM-2 column (Bio-Rad) to remove detergent and form proteoliposomes. After incubation for 90 min at 0–4 °C, the column was centrifuged at 400×*g* for 2 min to collect the proteoliposomes. To remove extravesicular probe, 200-μl aliquots of the proteoliposome suspension were passed twice through 4-ml Sephadex G-25-300 columns. The final stock vesicle suspension (nominally 50 mg lipid/ml) was stored on ice during the experiment. Protein content, measured by the Awmido Black method [19], was normally 50–70 ng protein/mg lipid. Intraliposomal volume of each preparation was estimated from the volume of distribution of entrapped probe and was normally found to be 1 μl per milligram of starting lipid [17].

2.3. Fluorescence measurements

We used Fura-2 (500 μM) to measure internal Ca^{2+} concentration and SBFI (250 μM) to measure internal Na^+ concentration. Proteoliposomes were added to 2 ml of assay medium at a final concentration of 0.5 mg of lipid per milliliter of assay medium, and probe fluorescence was measured on an SLM-Aminco 8000C spectrophotometer [12,16]. Excitation and emission wavelengths corresponded to those described by Molecular Probes.

2.4. Determination of free Ca^{2+}

Internal and external media were buffered with high concentrations of EGTA and/or HEDTA. The pH shift at pH 7.3 caused by additions of $CaCl_2$ to the medium was compensated by immediate addition of an amount of TEA-OH determined by pH electrode to neutralize the effect of the added Ca^{2+}. Free [Ca^{2+}] was estimated using the software "Chelator", which was kindly provided by Schoenmakers et al. [20]. The Chelator software accounts for the effect of medium pH, temperature and ionic strength in the calculation of apparent dissociation constants for CaEGTA or CaHEDTA [21].

2.5. Fluorescent probe calibration

Fluorescence emission, F, by the probe increases with increasing free concentrations of the cationic ligand, $[M]_{free}$. The initial concentration, $[M^o]_{free}$, may be zero or non-zero by experimental design. The following general relationship holds for any value of $[M]_{free}$:

$$F - F_1 = \left\{\Delta[M]_{free}/k'_d\right\}\{F_{max} - F\} \quad (1)$$

where $\Delta[M]_{free} \equiv [M]_{free} - [M^o]_{free}$, F_1 is the fluorescence when $[M]_{free}=0$, and F_{max} is the fluorescence at saturating cation concentration. The apparent probe dissociation constant, K'_d, is related to the true dissociation constant, K_d, as follows:

$$K'_d = K_d + [M^o]_{free} \quad (2)$$

or,

$$K'_d = K^*_d\{F_{max} - F_0\}/\{F_{max} - F_1\} \quad (3)$$

where F_o is the fluorescence in the absence of ligand. Thus, K_d can be obtained from Eq. (2) if $[M^o]_{free}$ is known or from Eq. (3) if it is not.

Calibrations of intraliposomal probe were performed on each preparation. The cation, Na^+ or Ca^{2+}, was added stepwise to the external medium in the presence of ionophore to equilibrate the cation with the intraliposomal space [16]. Monensin was used for Na^+ in calibrating SBFI, and ionomycin was used for Ca^{2+} in calibrating Fura-2. Data were fitted to a linearized form of Eq. (1), yielding the parameters F_{max} and K'_d, and K_d was evaluated according to Eqs. (2) or (3). Calibrations routinely yielded values of K_d in good agreement with the literature. For Na^+ flux measurements, calibration parameters were applied to each

experimental run to convert SBFI fluorescence intensity to $[Na^+]$, from which the initial rates were obtained by linear regression.

For Ca^{2+} flux, the desired quantity is total intraliposomal Ca^{2+} concentration, $[Ca^{2+}]_{total}$. For this purpose, we devised a simple and accurate approximation to Eq. (1) that yields $[Ca^{2+}]_{total}$ directly:

$$F - F_1 = \alpha\Delta[Ca^{2+}]_{total} \quad (4)$$

where α is the proportionality constant, given by

$$\mathrm{Lim}\alpha\{\mathrm{as}\Delta[Ca^{2+}]_{free}\rightarrow 0\} = (K_E/K'_d) \times \{(F_{max}-F_1)/[HEDTA]_{free}\} \quad (5)$$

where $[HEDTA]_{free}$ is free intraliposomal HEDTA concentration, and K_E is the apparent dissociation constant of CaHEDTA, as determined by the "Chelator" program.

This approximation is justified on theoretical grounds. Because HEDTA was present in excess, and because initial rates were measured within the first 20 s after initiation of Ca^{2+} flux, $\Delta[Ca^{2+}]_{total}$ was ≤100 μM and $\Delta[Ca^{2+}]_{free}$ was ≤20 nM, satisfying the condition on Eq. (5). Experimental validation of Eqs. (4) and (5) are provided by the calibration curves in Fig. 1. Fluorescence is linear with $[Ca^{2+}]_{total}$, as predicted. Furthermore, the measured values of α yield calculated K_d value, using Eq. (5), of 167 nM for Fura-2, in reasonable agreement with published values [22] and those given by Molecular Probes.

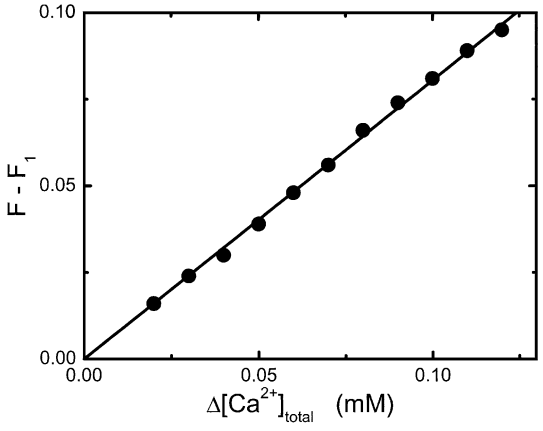

Fig. 1. Dependence of Fura-2 fluorescence on total Ca^{2+}. The Ca^{2+}-dependent increase in fluorescent intensity ($F-F_1$) is plotted versus $\Delta[Ca^{2+}]_{total}$, as defined in Experimental procedures. Calcium was equilibrated in the presence of 250 nM ionomycin, and 2 min was allowed for equilibration after each addition. Linear regression of the data gave values of α=0.805 mM^{-1}. Internal and external media contained 125 mM TEA · Cl, 25 mM NaCl, and 25 mM TEA · TES, pH 7.30. At the start of the experiment, vesicles contained 500 μM Fura-2, 10 mM TEA · HEDTA, and no Ca^{2+}. The following values were used for the calculations in the text: K_E=1.66 μM, $[HEDTA]_{free}$=10 mM, and ($F_{max}-F_1$)=0.81.

Accordingly, it is justifiable to use the following expression for Ca^{2+} flux into the liposomes:

$$d[Ca^{2+}]_{total}/dt = (1/\alpha)dF/dt \quad (6)$$

where α is determined by calibration. The primary advantages of Eq. (6) are that it bypasses ambiguities in estimating K_d and K_E and yields the desired Ca^{2+} flux directly.

3. Results

3.1. Kinetics of the reconstituted Na^+/Ca^{2+} antiporter

Fig. 2 summarizes data relating to the kinetics of the Na^+/Ca^{2+} exchange. Fig. 2A contains the $[Ca^{2+}]$-dependence of Ca^{2+} uptake and Na^+ efflux. The two curves are from parallel reconstitutions that were identical except that one preparation contained SBFI and the other contained Fura-2. In three independent experiments, the K_m for Ca^{2+} was 2.10±0.16 μM in the Ca^{2+} influx experiment and 1.98±0.06 μM in the Na^+ efflux experiment. Fig. 2B demonstrates the $[Na^+]$-dependence of Na^+ influx via the Na^+/Ca^{2+} antiporter. The experiment was carried out in the presence of 10 μM free Ca^{2+} inside the liposomes. The K_m for Na^+ was 8.2±1.4 mM (n=4).

3.2. Regulation of Na^+/Ca^{2+} antiporter by diltiazem and TPP^+

Diltiazem inhibits Na^+/Ca^{2+} exchange in beef heart mitochondria with $K_{1/2}$=7 μM [23], and TPP^+ inhibits Na^+/Ca^{2+} exchange in rat liver mitochondria with $K_{1/2}$=0.2 μM [24]. These agents also inhibited Na^+/Ca^{2+} exchange in the reconstituted system, as shown in Fig. 3. The $K_{1/2}$ for diltiazem was 10±3 μM, and the $K_{1/2}$ for TPP^+ was 0.6±0.2 μM. Complete inhibition of the cardiac mitochondrial Na^+/Ca^{2+} antiporter by TPP^+ has not previously been reported. The data in Fig. 3 are measurements of Ca^{2+}-dependent Na^+ efflux. Nearly identical results were obtained when Na^+-dependent Ca^{2+} efflux was measured.

3.3. Regulation of the Na^+/Ca^{2+} antiporter by K^+

K^+ stimulates both Na^+/Ca^{2+} and Ca^{2+}/Ca^{2+} exchange via the Na^+/Ca^{2+} antiporter in intact mitochondria [25,26]. As summarized in Table 1, K^+ caused an increase in the apparent affinity of the carrier for Ca^{2+} without affecting the V_{max} for Ca^{2+} uptake. The K_m for Ca^{2+} was decreased from 2 μM in the presence of 2.5 mM $[K^+]$ to 0.9 μM in the presence of 100 mM K^+. This effect was only observed when K^+ was present on the same side of the membrane as Ca^{2+}; trans K^+ has no effect on the K_m for Ca^{2+}.

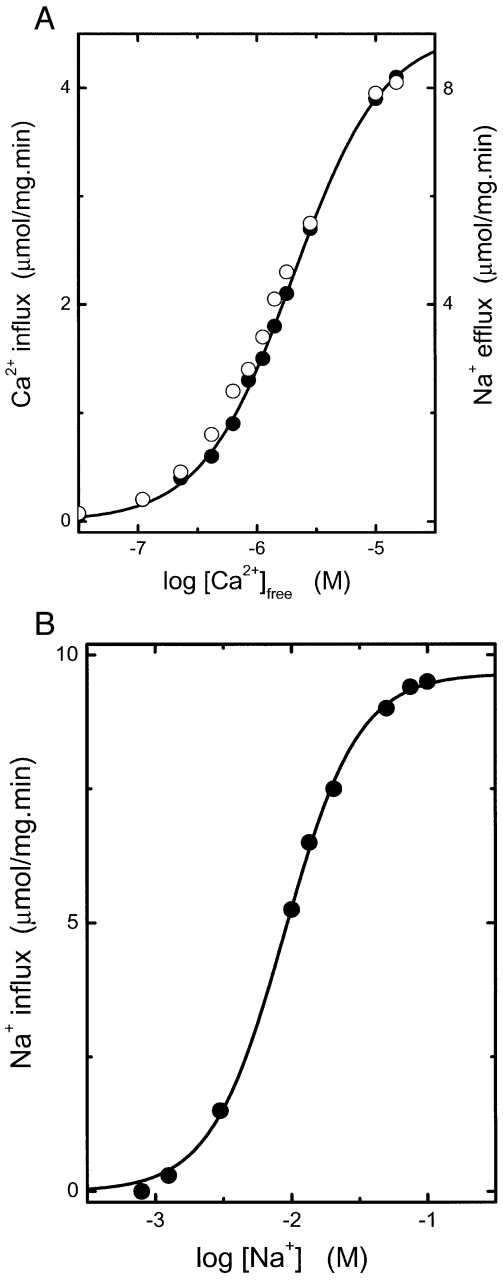

Fig. 2. Kinetics of reconstituted Na+/Ca^{2+} antiporter. Panel (A) Dependence of Na^+/Ca^{2+} Exchange on [Ca^{2+}]. Initial rates of Ca^{2+} influx (●) and Na^+ efflux (○), measured in proteoliposomes reconstituted with the purified Na^+/Ca^{2+} antiporter, are plotted versus external free [Ca^{2+}]. Fura-2 and SBFI were loaded separately into two preparations of proteoliposomes. Internal medium contained 125 mM TEA · Cl, 25 mM NaCl, and 10 mM TEA · HEDTA. External medium contained 5 mM TEA · EGTA, 5 mM TEA · HEDTA and 150 mM TEA · Cl. Both media contained 25 mM TEA · TES, pH 7.30. $CaCl_2$ and TEA · OH were added to external medium in order to vary free [Ca^{2+}] without changing pH, as described in Experimental procedures. Panel (B) Dependence of Na^+/Ca^{2+} exchange on [Na^+]. Initial rates of Na^+ influx were measured in proteoliposomes reconstituted with purified Na^+/Ca^{2+} antiporter as medium [Na^+] was varied. Proteoliposomes were loaded with 250 µM SBFI and 10 µM free Ca^{2+}. External medium contained 150 mM TEA · Cl, and the indicated [Na^+] was obtained by substituting NaCl for TEA · Cl in external medium. Internal medium contained 150 mM TEA · Cl. Both media contained 5 mM TEA · EGTA, 5 mM TEA · HEDTA and 25 mM TEA · TES, pH 7.30.

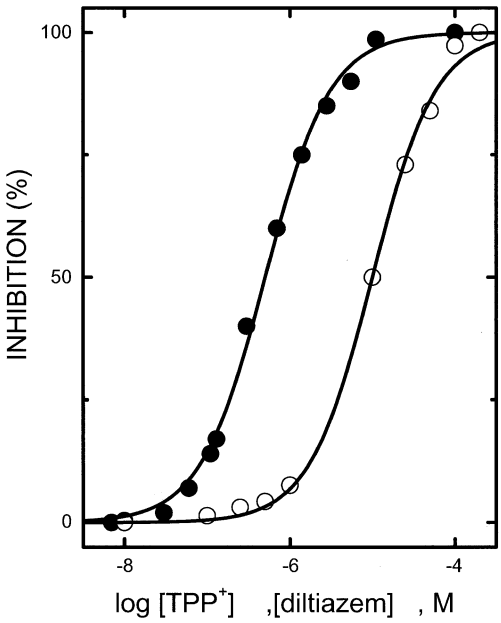

Fig. 3. Inhibition of Na^+/Ca^{2+} exchange by TPP^+ and diltiazem. Percent inhibition of initial rates of Ca^{2+}-induced Na^+ efflux is plotted versus medium [TPP^+] (●) or [diltiazem] (○). Proteoliposomes were reconstituted with purified Na^+/Ca^{2+} antiporter and loaded with 250 µM SBFI. 5 µM free Ca^{2+} was present in external medium. Internal medium contained 125 mM TEA · Cl and 25 mM NaCl. External medium contained 150 mM TEA · Cl. Both media contained 5 mM TEA · EGTA, 5 mM TEA · HEDTA, and 25 mM TEA · TES, pH 7.30.

3.4. Regulation of the Na^+/Ca^{2+} antiporter by pH

Baysal et al. [27] showed, in intact beef heart mitochondria, that the Na^+/Ca^{2+} antiporter is regulated by matrix pH and exhibits maximal activity at pH 7.4 to 7.6. We investigated the effects of pH on Na^+-dependent Ca^{2+} uptake via the reconstituted Na^+/Ca^{2+} antiporter and observed maximum activity at pH 7.0 (Fig. 4). Ca^{2+} flux was strongly inhibited at alkaline pH. This result, which supports the conclusion that pH is a powerful modulator of mitochondrial Na^+/Ca^{2+} exchange [27], does not indicate the target of this effect. This was elucidated in further experiments that examined the effect of pH on the V_{max} and K_m for Na^+/Ca^{2+} exchange. As shown in Fig. 5, the effect of varying pH is mediated entirely via changes in the K_m for Ca^{2+}. In three independent experiments the K_m was shifted

Table 1
Effects of internal and external K^+ on K_m for Ca^{2+} influx

	$[K^+]_i$=2.5 mM	$[K^+]_i$=100 mM
$[K^+]_o$=2.5 mM	1.98±0.12 µM	1.97±0.16 µM
$[K^+]_o$=100 mM	0.94±0.15 µM	0.91±0.11 µM

Shown are K_m values for Ca^{2+} observed when Ca^{2+} influx was measured under conditions varying with respect to intra- and extra-vesicular [K^+]. KCl was substituted for TEA · Cl to obtain the indicated concentrations. Parallel proteoliposome preparations were made for measurements differing with respect to internal [K^+]. Internal and external media were otherwise as described in the legend to Fig. 4, with external [Ca^{2+}] being varied. Data are expressed as the mean obtained in three independent experiments.

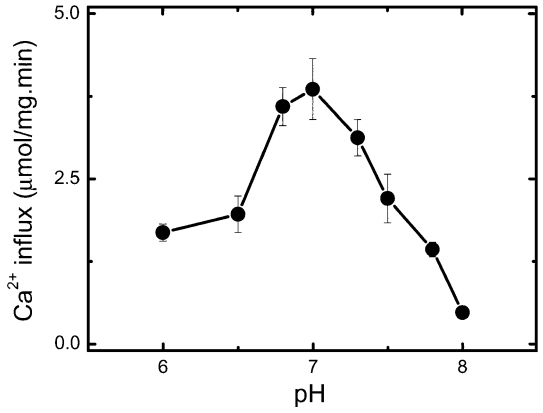

Fig. 4. Regulation of the Na^+/Ca^{2+} antiporter by pH. Initial Ca^{2+} uptake rates were measured in proteoliposomes at different media pH values. Proteoliposomes were loaded with 500 μM Fura-2. Rates at each pH were measured in triplicate, and the error bars reflect the means±standard error of the estimate. 2 μM free Ca^{2+} was present in external medium. Internal medium contained 100 mM TEA · Cl and 50 mM NaCl. External medium contained 150 mM TEA · Cl. Both media contained 5 mM TEA · EGTA, 5 mM TEA · HEDTA, and 25 mM TEA · TES, pH 7.30.

from 0.55±0.16 μM at pH 7.0 to 42±14 μM at pH 6.5, and to approximately 1.0±0.4 mM at pH 7.8.

3.5. Na^+/Li^+ and Na^+/K^+ exchange in the absence of calcium

To our surprise, we observed that the purified, reconstituted Na^+/Ca^{2+} antiporter catalyzed electroneutral Na^+/Li^+ and Na^+/K^+ exchange in the absence of Ca^{2+}, as shown in Fig. 6. The V_{max} was about the same as the V_{max} for Na^+ flux (see Fig. 2). The K_m for K^+ was 20±3 mM (n=3), and the K_m for Li^+ was 27±4 mM (n=3). Na^+/Li^+ and Na^+/K^+ exchange were each inhibited by diltiazem and TPP^+, and the $K_{1/2}$ values for inhibition were approximately the same as those for inhibition of Na^+/Ca^{2+} exchange (not shown).

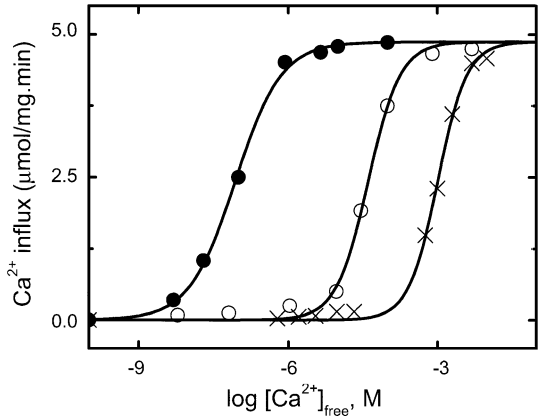

Fig. 5. The effect of pH on the kinetics of Na^+/Ca^{2+} exchange. Initial rates of Ca^{2+} uptake into proteoliposomes were measured as medium $[Ca^{2+}]$ was varied at three different values of medium pH and the K_m was shifted from 0.55±0.16 μM at pH 7.0 (●) to 42±14 μM at pH 6.5 (○), and to approximately 1.0±0.4 mM at pH 7.8 (×). Internal and external media were as described in the legend to Fig. 4. The estimated K_m values are given in the text. The curves are plotted with Hill coefficients of 1.

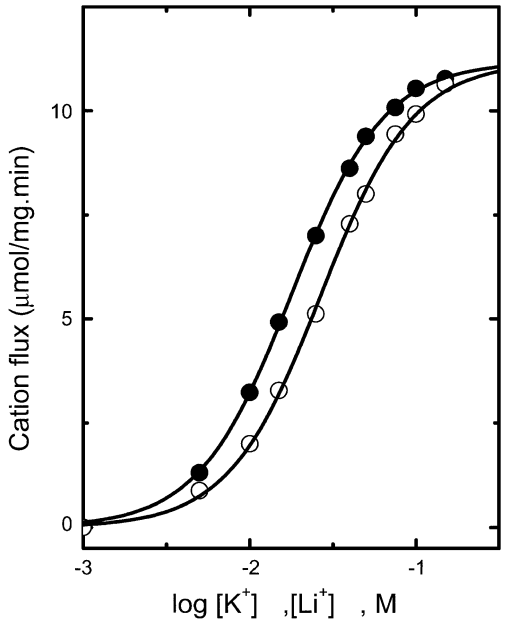

Fig. 6. Na^+/Li^+ and Na^+/K^+ exchange via the Na^+/Ca^{2+} antiporter in Ca^{2+}-free media. Initial rates of Na^+ efflux are plotted versus medium $[K^+]$ (●) and medium $[Li^+]$ (○). Proteoliposomes contained 125 mM TEA · Cl, 25 mM NaCl and 250 μM SBFI. External medium contained 150 mM TEA · Cl, substituted with LiCl or KCl to achieve the indicated cation concentrations. Both media contained 1 mM TEA · EGTA, and 25 mM TEA · TES, pH 7.30.

It is important to emphasize that these experiments were carried out in the absence of Ca^{2+}. In the presence of Ca^{2+}, Na^+ did not induce K^+ flux, K^+ was not competent to induce Ca^{2+} counter-flux, and Ca^{2+} did not induce K^+ counter-flux (data not shown).

3.6. Regulation of Na^+/Ca^{2+} exchange by bovine serum albumin

During development of a protocol to help preserve the activity of the purified Na^+/Ca^{2+} antiporter for longer periods of time, we found that the addition of BSA stimulated Na^+/Ca^{2+} exchange. The following experiments show the effect of BSA when added to the assay medium. Fig. 7A contains representative traces from proteoliposomes incubated with BSA for 2 min, followed by the addition of 5 μM free Ca^{2+} to initiate Na^+/Ca^{2+} exchange. Ca^{2+} uptake on the exchanger was about twofold higher in the presence of BSA (trace c of Fig. 7A), as compared with control (trace b of Fig. 7A). Note that the fluorescent traces is the absence (trace c of Fig. 7A) and presence (trace c, Fig. 7A) of BSA read the same value, showing that the fluorescence is not affected by presence of BSA prior to the addition of Ca^{2+}.

BSA is a potent activator of Na^+/Ca^{2+} exchange, as illustrated by the dose–response curves in Fig. 7B. The apparent K_m for this effect was about 250 nM (16 μg/ml), and maximal stimulation was threefold in this experiment and up to fourfold in other experiments. Removal of

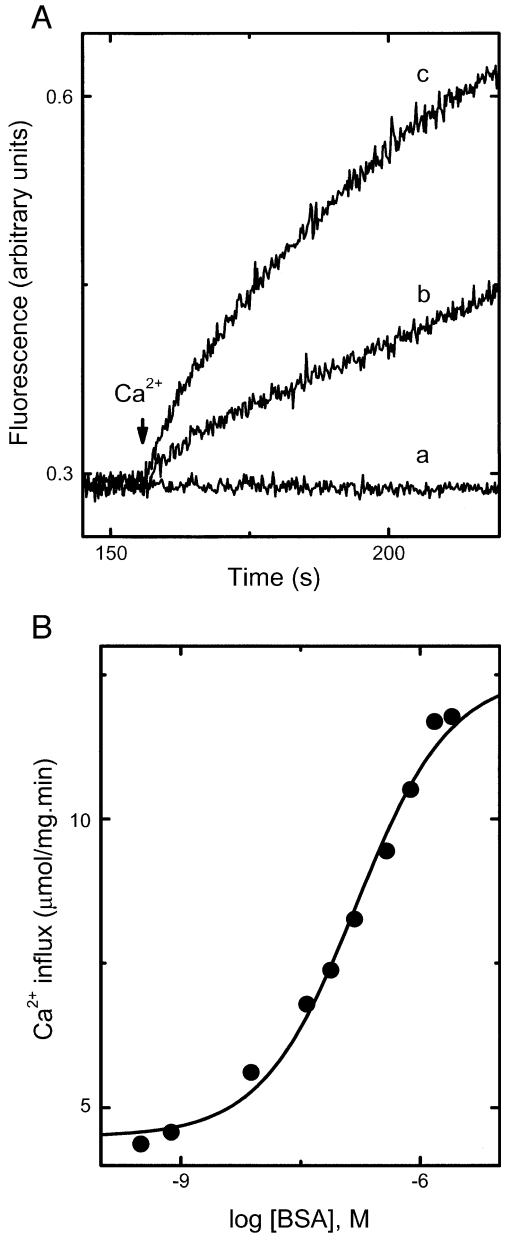

Fig. 7. Stimulation of Na^+/Ca^{2+} exchange by BSA. Ca^{2+} uptake was initiated by adding 5 μM free Ca^{2+} after proteoliposomes were preincubated for 2 min in assay medium in the absence or presence of BSA, as indicated. Fluorescence traces prior to the addition of Ca^{2+} is a control showing that the system is not affected by the presence of BSA. Internal and external media were otherwise as described in the legend to Fig. 4. Panel (A) Fura-2 fluorescence intensity is plotted versus time. Trace a, no Ca^{2+}; trace b, 5 μM free Ca^{2+}; trace c, 5 μM free Ca^{2+} and 1 μM (64 μg/ml) BSA. Panel (B) Ca^{2+} influx is plotted versus [BSA].

endogenous fatty acids, which are very low in this preparation, did not appear to be responsible for this effect, because fatty acids had no effect on Na^+/Ca^{2+} exchange. Moreover, BSA removal of a heavy metal inhibitor can be excluded, because a high concentration of HEDTA was present on both sides of the membrane. It is worth noting that there was no decay of Na^+/Ca^{2+} exchange activity during these incubations, and the BSA stimulation was unchanged when BSA and control runs were compared at the beginning and end of a 4-h experiment.

4. Discussion

Understanding the kinetics and regulation of the mitochondrial Na^+/Ca^{2+} antiporter is essential for assessing its role in regulating mitochondrial metabolism and for comparing its structure–function with other members of this expanding gene family. Reconstitution is a particularly useful technique for these purposes, because it provides reliable estimates of kinetic parameters that are difficult to obtain in isolated mitochondria. Reconstitution is also capable of revealing new properties that could not readily be obtained in the complex setting of intact mitochondria.

4.1. Kinetics and regulation of the mitochondrial Na^+/Ca^{2+} antiporter

Studies on mitochondria have indicated a K_m for Ca^{2+} between 2 and 13 μM Ca^{2+} [28–30]. In the reconstituted system, we estimate the physiologically relevant K_m to be 0.9 μM in the presence of K^+ (Table 1). The effect of K^+, which has also been observed in intact mitochondria [25], is mediated by an increase in the apparent affinity for Ca^{2+}. The K_m for Ca^{2+} is profoundly affected by pH with a profile in general agreement with the one observed in isolated mitochondria [27]. As with all such measurements, the K_m values for Ca^{2+} depend strongly on the estimate of free [Ca^{2+}]. We note that the Chelator program [20] yields values of free [Ca^{2+}] that are two and three times higher than those estimated by Grynkiewicz et al. [22] and McCormack et al. [31], respectively.

The K_m for Na^+ was 8 mM when proteoliposomes contained 10 μM free Ca^{2+}, in agreement with values obtained in isolated mitochondria, which vary between 2.6 and 12 mM [26,28,32,33]. Cytosolic [Na^+] has been estimated between 5.7 and 10 mM in heart [34,35].

The Na^+/Ca^{2+} antiporter is sensitive to a variety of pharmacological agents, including trifluoperazine [36], diltiazem [23] and amiloride [37]. Our studies show that diltiazem and tetraphenylphosphonium cation inhibit the antiporter with potencies similar to those observed in isolated mitochondria [23,24].

It is worth noting that the exchange discussed in this study was obligatorily electroneutral, because no counterion was present to support an electrophoretic movement. Although we reported earlier that valinomycin stimulated Na^+/Ca^{2+} exchange in the reconstituted system in the presence of a K^+ gradient, to answer the question of stoichiometry of the Na^+/Ca^{2+} exchange requires detailed study which is beyond the scope of this paper. Electro-

phoretic Na^+/Ca^{2+} exchange in intact heart mitochondria was reported by Jung et al. [38].

4.2. Ion selectivity of the mitochondrial Na^+/Ca^{2+} antiporter

The observation that this carrier mediates Na^+/K^+ and Na^+/Li^+ exchange in the absence of Ca^{2+} would be difficult to discern in mitochondria due to the multiplicity of transport pathways for alkali cations. Li^+ can induce Ca^{2+} efflux from mitochondria [33], but there was no previous indication of Na^+/Li^+ exchange in the absence of Ca^{2+}.

K^+ could not substitute for Na^+ in inducing Ca^{2+} transport, nor did Ca^{2+} induce K^+ transport. Nevertheless, the Na^+/Ca^{2+} antiporter catalyzed Na^+/K^+ exchange. This is reminiscent of the K^+/H^+ antiporter, in which cation/cation exchange proceeds normally when K^+/H^+ exchange is fully inhibited by DCCD [39,40]. The cation exchange pathway in both carriers is nonselective for alkali cations. If the Na^+/Ca^{2+} antiporter contains separate pathways for alkali cations and Ca^{2+}, it follows that Na^+ and Li^+, but not K^+, induce a conformational change that permits Ca^{2+} movement across the protein. K^+ is also transported by the electrophoretic Na^+/Ca^{2+} exchanger of bovine rod outer segment, which exchanges 1 K^+ and 1 Ca^{2+} for 4 Na^+ [41]. Na^+ and Li^+ ions also appear to be mediated by the mitochondrial calcium uniporter in the absence of Ca^{2+}, suggesting similar loss of specificity in the absence of Ca^{2+} for both mitochondrial transporters [42].

4.3. Regulation of Na^+/Ca^{2+} exchange by BSA

BSA stimulated Na^+/Ca^{2+} exchange, primarily by increasing V_{max}. We consider it reasonable that the effect is due to protein–protein interaction. Plasma membrane Na^+/Ca^{2+} antiporters from a variety of tissues contain an intrinsic exchange inhibitory peptide (XIP) [13–15] which has been suggested to react with another region of the exchanger to produce an inactive conformation or with neighboring lipid to produce an active conformation [43,44]. In view of these results, we are investigating the possibility that BSA is unmasking an intrinsic autoregulatory peptide of the mitochondrial carrier.

4.4. The role of the Na^+/Ca^{2+} antiporter in regulating matrix Ca^{2+}

It was proposed some years ago that mitochondrial Ca^{2+} cycling is responsible for the energetic response to signals calling for increased work and, hence, ATP production [45–48]. These concepts have now been extended by studies, beginning with Rizzuto et al. [6], showing that matrix $[Ca^{2+}]$ increases rapidly and transiently upon stimulation with agonists coupled to IP3 generation. The phenomenon of rapid Ca^{2+} oscillations has been observed by several laboratories in a variety of tissues [49]. It was also demonstrated that Ca^{2+} oscillations, and not steady state increases in matrix Ca^{2+}, are responsible for sustained activation of Ca^{2+}-sensitive dehydrogenases [7,9].

These new findings impose new requirements on regulation of the Na^+/Ca^{2+} antiporter, because it must exchange slowly during the Ca^{2+} uptake phase and rapidly during the ejection phase. Inhibition of Na^+/Ca^{2+} exchange during Ca^{2+} uptake can be understood in the following way: Rapid Ca^{2+} uptake will transiently alkalinize the matrix and lower $\Delta\psi$. As shown here, the effect of pH will severely inhibit Ca^{2+} efflux on the exchanger, causing a reshaped profile of the Ca^{2+} spike. pH and $\Delta\psi$ will soon be restored toward normal values by uptake of phosphoric acid on its carrier, thereby reactivating the Na^+/Ca^{2+} antiporter as the cytosolic Ca^{2+} spike begins to decay. We note that inhibitors of the exchanger (diltiazem, CGP37157) have been shown to cause only a small if any increase in the magnitude of the internal $[Ca^{2+}]$ spikes but to prolong the decay phase of the matrix $[Ca^{2+}]$ rise during both IP3 receptor and ryanodine receptor induced oscillating calcium spikes [50,51]. It is not clear that these effects are sufficient to account for the observed decay phase of matrix Ca^{2+}, which is extremely rapid. Thus, further regulation, such as exists for the plasma membrane Na^+/Ca^{2+} exchanger in the form of XIP, may be required to support rapid Ca^{2+} oscillations in mitochondria.

Acknowledgments

We would like to thank Dr. Keith D. Garlid for his excellent suggestion about the research strategies, and Dr. Ian C. West for reading of the manuscript.

This research was supported by the Grant 0140138N (to P.P.) from the American Heart Association.

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