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Affinity chromatography purification of mitochondrial inner membrane proteins with calcium transport activity

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Abstract

Immobilized calcium affinity chromatography was used to obtain a preparation enriched in calcium transporters from Triton X-100 extracts of rat liver mitochondria inner membranes (PPCT). The PPCT were reconstituted into preformed asolectin liposomes which contained 120 mM KCl as internal high K^+ medium. $^{45}Ca^{2+}$ uptake into proteoliposomes was studied under conditions favoring electrophoretic uptake, and $H_i^+/^{45}Ca_o^{2+}$ or $Na_i^+/^{45}Ca_o^{2+}$ exchange, to test for the presence of the three calcium transport modes present in mitochondria. $^{45}Ca^{2+}$ uptake in liposomes was studied in parallel. $Na_i^+/^{45}Ca_o^{2+}$ exchange activity was not detectable. $H_i^+/^{45}Ca_o^{2+}$ exchange activity measured in the presence of a pH gradient (acid inside) obtained after suspension in low K medium in the presence of nigericin, was 100–200 nmoles $^{45}Ca^{2+}$ per mg protein in 30 s. $^{45}Ca^{2+}$ uptake in voltage-dependent assays (a K^+ diffusion membrane potential induced by valinomycin in the presence of methylamine) was not electrophoretic since it was stimulated by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and probably due to secondary Ca^{2+}/H^+ countertransport. $H_i^+/^{45}Ca_o^{2+}$ uptake showed a saturable component at around 80 μM Ca and was coupled to an increase in internal pH in pyranine-loaded PPCT proteoliposomes. $^{45}Ca^{2+}$ uptake in PPCT proteoliposomes could also be driven by a pH gradient obtained by raising external pH in high K^+ medium. The results are consistent with the presence of a functional nH^+/Ca^{2+} antiporter. Polyclonal antibodies raised against the PPCT were able to immunoprecipitate the $H^+/^{45}Ca^{2+}$ uptake activity and recognized two major bands in the PPCT with molecular masses of about 66 kDa and 55 kDa. This is the first report of a partial purified protein(s) which may represent the H^+/Ca^{2+} exchanger of the inner mitochondrial membrane, and represents an important step towards its identification.

Keywords: Mitochondrion; H^+/Ca^{2+} exchange; Calcium uniporter; Na^+/Ca^{2+} exchange; Affinity purification; Proteoliposome

1. Introduction

The mitochondrial calcium cycle has been known for a long time and it involves four different calcium transport mechanisms (reviewed in [1,2]). Electrogenic ejection of two protons drives electrophoretic uptake of one Ca^{2+} via the inner membrane calcium uniporter, a process inhibited by ruthenium red [3].

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An additional rapid uptake mode (RAM) for calcium influx [4] with a higher calcium affinity and lower ruthenium red sensitivity than the classical calcium uniporter has also been described. Efflux from mitochondria is carried out through two different antiport mechanisms that vary in a tissue specific way. A Na^+/Ca^{2+} antiporter operates in brain, heart and other excitable tissues, and to a minor degree in liver, whereas a H^+/Ca^{2+} exchanger has been described in liver, lung, kidney and smooth muscle. In addition, there is a non-specific pathway for calcium release, the so-called permeability transition pore (PTP), a mechanism whereby the permeability of the inner membrane increases and allows the extrusion of matrix components [5].

The exact function of calcium interaction with mitochondria requires the understanding at the molecular level of the characteristics of these different calcium transporters. With respect to the calcium uniporter, the type of transport mechanism (channel or carrier) or the molecular identity responsible for the transport system has not yet been defined, despite intensive research on this issue. Zazueta et al. [6] reconstituted mitochondrial calcium transport proteins from rat kidney cortex making use of a system where mitochondrial membrane proteins where extracted with 1.6% sodium cholate and incorporated to liposomes reconstituted with cytochrome oxidase. The $^{45}Ca^{2+}$ transporter reconstituted in this system was driven by a membrane potential (negative inside) but was only partially (53%) sensitive to ruthenium red. A 20 kDa protein appeared to be responsible for this activity [7]. Mironova et al. [8,9] isolated calcium transport proteins of 40 kDa and 2 kDa from ethanol extracts of beef heart mitochondria that were able to increase the conductivity of bilayer lipid membranes in a ruthenium red sensitive fashion. The 40 kDa was a glycoprotein and antibodies against it inhibited calcium transport in rat liver mitochondria [10], whereas the 2 kDa protein appeared to be responsible for the increase in conductivity [9]. Thus, it would appear that the calcium uniporter activity is associated with small peptides (2 kDa) and perhaps other larger components that are capable to increase the conductance of lipid membranes in a ruthenium red sensitive fashion [6,8,10].

The sodium-dependent calcium efflux has been described as an electroneutral passive transport ($2Na^+/Ca^{2+}$) but recent reports indicate that it works as an electrogenic $3Na^+/Ca^{2+}$ or active $2Na^+/Ca^{2+}$ exchanger [11] or a membrane potential driven electrophoretic antiporter [12]. A protein of about 110 kDa that behaved as an electroneutral $2Na^+/Ca^{2+}$ antiporter has been purified [13]. On the other hand, the sodium-independent mechanism has not been yet identified, and the $H^+:Ca^{2+}$ stoichiometry and/or energy requirements are poorly understood [14].

In the present study we describe the use of immobilized metal affinity chromatography with calcium ions as the immobilized cations, to obtain a preparation of partially purified calcium transporters from the inner mitochondrial membrane. The activity of this preparation has been studied after reconstitution into preformed liposomes. Our results show that the calcium transport activity present in this preparation is largely dependent upon a pH gradient (acid inside), consistent with a H^+/Ca^{2+} exchanger.

2. Methods

2.1. Preparation of submitochondrial particles and solubilization of membrane proteins

Rat liver mitochondria, mitoplasts and submitochondrial particles were isolated from 3-month-old male Wistar rats following published procedures [15]. Livers were homogenized (1:3 w/v) in 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4 at 0°C. Nuclei and cell debris were first removed by centrifugation ($700\times g$, 10 min) and mitochondria then spun down ($14\,500\times g$, 10 min). In order to obtain mitoplasts, the outer mitochondrial membrane was eliminated by hypo-osmotic incubation in 20 mM Tris-HCl pH 7.4 (3 ml/g initial tissue) during 20 min at 4°C and constant stirring and subsequent centrifugation ($12\,000\times g$, 10 min). The pellet, containing mitoplasts, was used as a source of submitochondrial particles (SMPs) which were obtained by incubation in hypo-osmotic medium (1–2 ml/mg protein, 20 min, 4°C). SMPs were collected by centrifugation ($27\,000\times g$, 10 min), washed twice with 20 mM Tris pH 7.4 ($27\,000\times g$, 10 min) and stored at −70°C until used. All the solutions employed were supplemented with the following mixture of protease inhibitors: 1 mM phenylmethylsulfonylfluoride (PMSF,

Fluka), 5 mM iodacetamide (Merck), 0.2 mg/ml benzamidine (Sigma), 0.1 mg/ml trypsin inhibitor (Sigma), 0.1 mg/ml bacitracin (Sigma).

To solubilize inner mitochondrial membrane proteins, SMPs were diluted (10 mg/ml) in 120 mM KCl, 20 mM Tris-HCl, pH 7.4, 3% Triton X-100, and incubated at 4°C and constant stirring during 30 min. Extracted proteins were then separated from unsolubilized material by centrifugation at $100\,000 \times g$ for 30 min at 4°C. Contamination of SMPs by outer mitochondrial membranes was below 6% as determined by the activity of the outer membrane marker NADPH cytochrome *c* reductase [16].

2.2. Purification of calcium transporters

The calcium chelating resin used consisted of a chelating ligand, iminodiacetic acid, coupled to a cross-linked agarose matrix (Chelating Sepharose fast flow, Pharmacia). Preparation of the column was performed as described [17]. The resin, 200 ml (5 cm × 10.2 cm), was washed with 10 column vols. of distilled water, loaded with 2.4 column vols. of $CaCl_2$ (3 mg/ml) and washed with 4.8 column vols. of water to remove the remaining calcium. The resin was then equilibrated in 10% glycerol, 500 mM KCl, 1% Triton X-100, 20 mM Tris, pH 8. Solubilized mitochondrial particles (360 mg in 30 ml) were diluted 1/3 with 'Triton-free equilibration medium' (10% glycerol, 500 mM KCl, 20 mM Tris, pH 8) and applied to the column over a period of 12 h at 0°C, by recirculating the sample at a rate of 0.4 ml/min. Unbound proteins were removed by washing with 2 column vols. of 10% glycerol, 500 mM KCl, 1% CHAPS, 20 mM Tris, pH 8. Retained proteins were eluted with 'elution buffer' (10% glycerol, 120 mM KCl, 1% CHAPS, 50 mM EGTA, 20 mM Tris, pH 7.4). The protein peaks eluted from the column were monitored at 280 nm absorbance in an Hewlett Packard 8451A Diode Array Spectrophotometer. The elution fractions (around 100 ml) were pooled and concentrated in a 10 ml ultrafiltration cell (Amicon, PM10 membranes) to a final volume of 0.2 ml.

2.3. Preparation of liposomes

Liposomes were prepared from purified asolectin [18] as described by Lopez-Corcuera and Aragón [19]. In order to eliminate residual Ca^{2+} bound to phospholipids, purified asolectin (35.6 mg/ml in chloroform/methanol, 2:1, v/v) was washed in acid medium as follows: 0.2 vols. of distilled water were layered on top of the asolectin solution and mixed thoroughly. The two phases were separated by centrifugation, the polar (upper) phase was taken to pH 3, the two phases were again mixed, and then separated. The organic (lower) phase was dried under N_2, dissolved in chloroform/methanol (4:1, v/v) to a final concentration of 0.356 g/ml, and stored at −20°C until used.

Aliquots (540 μl) of purified asolectin were dried in a rotatory evaporator and dispersed (192 mg/ml final concentration) in 1 ml HKM containing 120 mM KCl, 20 mM Tris-HCl, pH 7.4. plus 7.43 μl 1 M KOH/ml, and appropriate BCECF, pyranine, or EGTA concentrations. The lipid suspension was then sonicated (3 times for 30 s with 30 s intervals) in an MSE Probe Ultrasonic Disintegrator. Phospholipids were measured by the Bartlett method [20].

2.4. Reconstitution of partially purified calcium transporters

Two different methods were used for reconstitution of partially purified calcium transporters.

2.4.1. Bio-Beads method

In order to eliminate the EGTA present in PPCT before reconstitution, concentrated PPCT fractions were diluted (50-fold) in EGTA-free buffer (120 mM KCl, 20 mM Tris-HCl, pH 7.4 (HKM) plus 1% CHAPS) and again concentrated by ultrafiltration. The procedure was repeated 3 times. 32 μl of the sonicated liposome suspension was then mixed with 0.143 ml of PPCTs or solubilized SMPs (both diluted 1/20 in HKM) or with an equivalent solution lacking proteins to obtain detergent-treated liposomes. To remove CHAPS, 0.175 ml of the liposome/protein mixture were applied to 1.5 ml Bio-Beads SM-2 (Bio-Rad) columns that had been pre-equilibrated with HKM, and proteoliposomes were eluted (flow rate 0.3 ml/min) with the same medium. The elution fractions (500 μl) were applied to 2 ml Sephadex G-50 (Pharmacia) columns pre-equilibrated in 'potassium-free LowKM' (120 mM choline chloride, 20 mM Tris, pH 7.4), in order to remove

external KCl. Detergent-treated liposomes were 'reconstituted' in a similar way to test for non-specific activity.

2.4.2. Dialysis method

The liposomes (loaded with 200 μM pyranine or BCECF and 500 μM EGTA) were first subjected to a purification procedure to obtain a homogeneous preparation. The sonicated lipid suspension was passed through a French Pressure Cell system (Aminco), at 7000 psi, 4 times, then layered (200 μl) on 2.5 ml of 10% glycerol (in HKM), centrifuged (45 min at 200 000 × *g*) and the pellet containing the larger liposomes was resuspended HKM (200 μl). 500 μl of this purified liposomal preparation were mixed with 500 μl of PPCT or solubilized SMP (3 mg/ml), or HKM plus 1% CHAPS, for detergent-treated liposomes. Detergent was eliminated by dialysis (against 1 l HKM, changed once during 15 h). Proteoliposomes and detergent-treated liposomes were centrifuged (200 000 × *g*, 45 min), recovered as pellets, and suspended in a final volume of 200–400 μl of HKM.

2.5. Calcium uptake measurements

Calcium uptake in proteoliposomes was studied with $^{45}Ca^{2+}$. 40 μl for Bio-Beads proteoliposomes, or 4 μl for proteoliposomes reconstituted with the dialysis method, and equivalent volumes for detergent-treated liposomes, were incubated at 37°C in 1 ml of low K^+ medium (LowKM) (120 mM choline chloride, 10 μM KCl, 20 mM Tris, pH 7.4, 0.1 mM $CaCl_2$, 1.2 μCi $^{45}Ca^{2+}$/ml) or high K^+ medium (HKM) (120 mM KCl, 20 mM Tris, pH 7.4, 0.1 mM $CaCl_2$, 1.2 μCi $^{45}Ca^{2+}$/ml) supplemented with 5 nM valinomycin and 10 mM methylamine (voltage-dependent transport) or 1.34 μM nigericin (pH gradient-dependent transport). $^{45}Ca^{2+}$ accumulation was stopped after 10 s (Bio-Beads reconstitution) or 30 s (dialysis reconstitution) by adding 0.2 mM EGTA and dilution with 2 ml of 0.32 M sucrose, 20 mM Tris-HCl, pH 7.4. Then, samples were filtered on nitrocellulose filters (Millipore HAWPO2500), washed twice with 2 ml of the same medium, dried, and counted for radioactivity in 2 ml scintillation cocktail (Optifase 'high safe', Wallac) [21]. Voltage- or pH gradient-dependent $^{45}Ca^{2+}$ uptake was obtained from the difference between uptake in low (10 μM KCl) and high (120 mM KCl) potassium assays. To study $Na_i^+/45Ca_o^{2+}$, assays were essentially as above except that KCl was replaced by NaCl in the composition of the internal and external media.

To study the time course of $^{45}Ca^{2+}$ uptake, 24 μl of proteoliposomes (dialysis reconstitution), were suspended in 6 ml of LowKM. Aliquots (1 ml) were withdrawn at the appropriate times and the reaction was stopped as indicated above.

To study the kinetics of calcium transport in proteoliposomes, the different calcium concentrations were obtained by appropriate dilution of concentrated $CaCl_2$ in calcium-free LowKM (by passage through CHELEX-100, Bio-Rad, or Chelating Resin, Sigma). The final free calcium concentrations obtained were verified with the use of a calcium-selective electrode [22].

2.6. Membrane potential in proteoliposomes

The ability of K^+-loaded proteoliposomes to form a K^+ diffusion potential (negative inside) was studied following the decrease in absorbance of safranine [23,24]. 0.15 ml of proteoliposomes or 0.135 ml of liposomes (Bio-Beads reconstitution method) were added to 1.8 ml of 120 mM choline chloride, 10 M KCl, 20 mM Tris, pH 7.4, 10 mM methylamine and 4.8 μM safranine or medium with the same composition except that the concentrations of KCl were increased and those of choline chloride decreased to maintain isosmosis. When a stable baseline was reached, 5 nM valinomycin was added and the absorbance change of safranine was followed at 530–578 nm in a Hewlett Packard 8451A Diode Array Spectrophotometer.

2.7. Fluorescent dyes

Internal pH was studied in proteoliposomes loaded with 500 μM BCECF (free acid form, Molecular Probes) or 200 μM pyranine (Molecular Probes). Probe-loaded liposomes or proteoliposomes were prepared as described above except that the probe was present in the HKM of liposomes and reconstitution medium. Fluorescence of BCECF-loaded liposomes or proteoliposomes was measured as previ-

ously described [25]. Excitation was at 450 and 508 nm and emission at 531 nm. Calibration of the entrapped dye could not be completed accurately. Thus, calibration of pH_i was carried out at the end of each experiment after addition of 1% Triton X-100 in order to expose the dye to the extracellular medium. Fluorescence of pyranine-loaded samples was measured at 510 nm (emission) and 460 nm (excitation). Calibration of internal pH was carried out after Triton X-100 addition, by exposing the entrapped dye to different external pH. The contribution of external BCECF and pyranine (a maximum of 50%, tested from the changes in fluorescence after rapid changes in external pH) was not corrected for, and, therefore, reported pH_i values are only relative.

2.8. Antibodies

Polyclonal antibodies were raised against PPCT in rabbits. For immunization, 50 μg of PPCT were emulsified with complete Freund's adjuvant and injected subcutaneously into New Zealand white rabbits. For boostings (four), similar amounts of PPCT were emulsified with incomplete Freund's adjuvant and injected with a periodicity of 10 days or more. Antibodies were purified by affinity chromatography with protein A (Pharmacia) and stored at −70°C in 0.02% mercury[(*o*-carboxyphenyl)thio] ethyl sodium salt (thymerosal, Sigma).

2.9. Western blots

SDS-PAGE was carried out according to Laemmli [26]. Proteins were transferred from gels to PVDF (InmobilonP, Millipore), using a Bio-Rad transblot apparatus at 40 mV overnight in 25 mM Tris, 192 mM glycine, 0.5% SDS and 10% methanol. PVDF membranes were activated with methanol during 30 min before use, and then washed with water.

For immunoblot analysis, PVDF membranes were blocked with PBS containing 5% casein (Molico, Nestle), for 1 h at room temperature, washed 3 times with PBS (1 min each) and incubated with primary antibodies (1/1000 dilution) in PBS 5% casein (3 h). Membranes were washed with PBS, 0.05% Tween 20, 2% casein (6 times, 7 min each), and with PBS alone (3 times, 5 min each), then incubated with the secondary antibody (goat anti-rabbit, linked to peroxidase, Bio-Rad) diluted 1/4000 in PBS, 2% casein during 20 min, and processed with the luminescence technique (ECL, Amersham), following the indications of the suppliers.

2.10. Immunoprecipitation

The PPCT was diluted 5-fold in 20 mM Tris HCl pH 7.4, 0.5% Triton X-100 and incubated with PPCT antibodies, total antiserum or with non-immune antibodies (at 1/2–1/50 dilution) in the presence of protease inhibitors (PMSF, 0.18 μg/180 μl) overnight at 4°C and constant gentle mixing. Immunoprecipitates were removed by adding protein A (0.3 mg/ml) and after an incubation of 2–3 h, the mixture was centrifuged for 1 min at top speed in a table top centrifuge. The supernatants were collected, made 120 mM KCl, reconstituted with the Bio-Beads method, and assayed for activity.

3. Results

3.1. Membrane potential and pH gradient in proteoliposomes

Fig. 1 shows that a K^+ diffusion membrane potential (negative inside), manifested as a decrease in safranine absorbance, is formed upon suspension of liposomes or proteoliposomes in LowKM and after valinomycin addition. No decrease in absorbance occurred in high K^+ medium but a slow decrease also occurred in LowKM in the absence of valinomycin (results not shown). Nigericin addition collapsed the diffusion potential (see Fig. 1). On the other hand, Fig. 2B shows that the suspension of liposomes or proteoliposomes in LowKM results in a drop of the internal pH. This acidification was not observed in the absence of a KCl gradient (Fig. 2A), indicating that H^+ entry might be driven by the membrane potential. Fig. 2C shows that this acidification was not avoided even in the presence of methylamine.

It has been shown that artificial membranes formed with different phospholipids have a relatively high passive K^+ and proton permeability [27,28]. With appropriate K^+ or H^+ concentration gradients, most of the K^+ flux appears to be electrogenic and that of H^+ electrophoretic, but in KCl medium, both

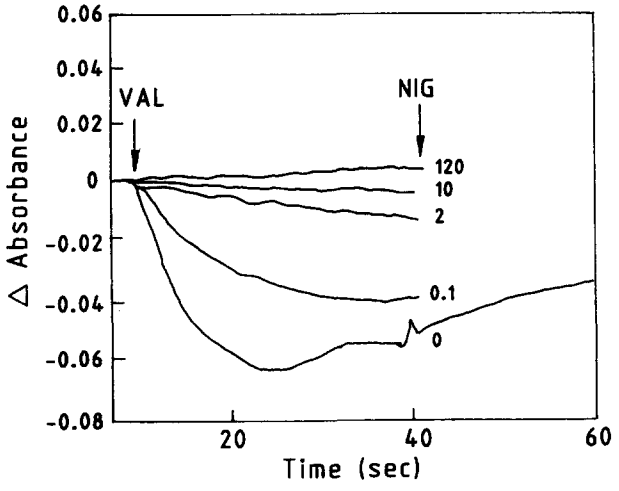

Fig. 1. Diffusion potential formation in K^+-loaded PPCT proteoliposomes. PPCT proteoliposomes (0.150 ml) prepared with the Bio-Beads reconstitution method were suspended in 1.8 ml of 120 mM choline chloride, 20 mM Tris, pH 7.4, 10 mM methylamine and 4.8 μM safranine or medium with the same composition except that it included the KCl concentrations (mM) indicated, and the concentrations of choline chloride were adjusted to maintain isosmosis. Where indicated 5 nM valinomycin (VAL) or 1.34 μM nigericin (NIG) were added.

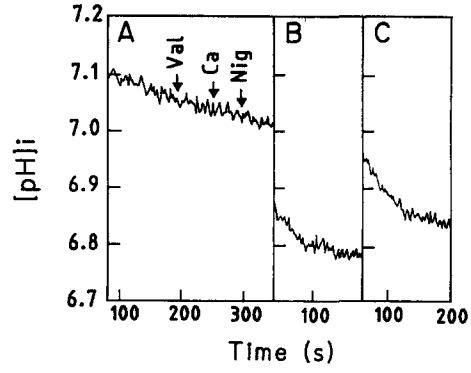

Fig. 2. Internal pH in K^+-loaded PPCT proteoliposomes. BCECF-loaded PPCT proteoliposomes (0.03 ml) prepared with the Bio-Beads reconstitution method were suspended in 3 ml of 120 mM KCl, 20 mM Tris pH 7.4 (A), or 120 mM choline chloride, 10 μM KCl, 20 mM Tris pH 7.4 (B,C). 10 mM methylamine was present in A and C. Where indicated, 5 nM valinomycin (Val), 0.1 mM $CaCl_2$ (Ca), or 1.34 μM nigericin (Nig) were added. The pH_i values shown are relative rather than absolute values, since it was not possible to obtain a correct calibration of the probe while trapped in liposomes or proteoliposomes.

H^+-Cl^- codiffusion and H^+-K^+ exchange may operate [28]. Our results are consistent with the existence of a large passive proton permeability in our proteoliposomal preparation. Attempts to reduce this permeability, using different buffers (zwitterionic, such as PIPES) or sulfate salts instead of chloride salts as reported by Levy et al. [28] were unsuccessful, as judged by the fact that the internal pH in the proteoliposomes suspended in LowKM was more acidic than in HKM (results not shown).

3.2. *Calcium transport activity in PPCT proteoliposomes*

The proteoliposomes prepared by the dialysis reconstitution method were homogeneous with an average diameter of 73 nm (see Fig. 3). The internal volume of these proteoliposomes was calculated to be around 3.6 μl/mg phospholipid, within the range of that used by Levy et al. [28]. PPCT proteoliposomes and preformed liposomes were assayed for $^{45}Ca^{2+}$ uptake activity under conditions where a membrane potential (negative inside) was present or absent. These conditions were (i) a K^+ diffusion potential which was induced by valinomycin in the presence of methylamine; and (ii) a pH gradient obtained in the presence of nigericin. These same assays were conducted with the unpurified Triton X-100 extracts of inner mitochondrial membrane proteins (SMPs).

Table 1 summarizes the results obtained. The membrane potential- or pH gradient-dependent cal-

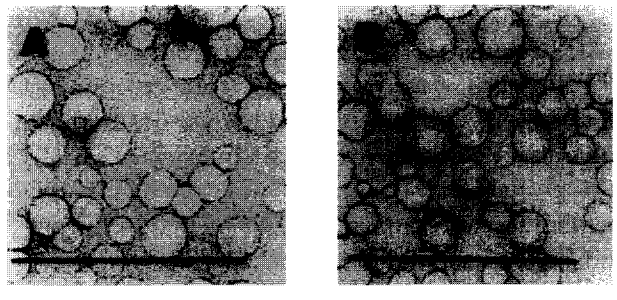

Fig. 3. Negative stain electron microscopy of proteoliposomes and liposomes. Samples obtained by the dialysis reconstitution method (A, liposomes; B, proteoliposomes; bar, 500 nm) were stained with uranyl acetate. Both liposomes and proteoliposomes had an average diameter of 73.3 nm, ranging from 30 to 100 nm. The internal volume calculated from the relation $E = 1/3\ Ar$ [41] (where A is the area of the membrane occupied by 1 mg of phospholipid, r is the radius of the vesicle, and E is the entrapped volume expressed as μl/mg phospholipid) was 3.6 μl/mg phospholipid.

Table 1
Characterization of PPCT proteoliposomes

	Lipid/assay (mg)	Protein/assay (μg)	Nig activity	Val activity
PPCT proteoliposomes	0.2	3–6	1.54	1.06
SMPs proteoliposomes	0.2	18	0.83	0.62

PPCT proteoliposomes were prepared by the dialysis reconstitution method. Nigericin activity and valinomycin activity correspond to $^{45}Ca^{2+}$ uptake in pH gradient- or membrane potential-dependent assays, carried out as indicated in Section 2, and expressed as nmoles $^{45}Ca^{2+}$ (mg phospholipid)$^{-1}$ 30 s^{-1}. The lipid:protein ratio (w/w) was 36–70:1 in PPCT proteoliposomes and 10:1 in SMP proteoliposomes. $^{45}Ca^{2+}$ uptake in liposomes (0.2 mg phospholipid per assay) was 0.86 or 0.48 nmoles $^{45}Ca^{2+}$ (mg phospholipid)$^{-1}$ 30 s^{-1} for nigericin- or valinomycin-dependent activity. For PPCT proteoliposomes reconstituted with the Bio-Beads method, the corresponding $^{45}Ca^{2+}$ uptake activities were 0.18 and 0.13 nmoles $^{45}Ca^{2+}$ (mg phospholipid)$^{-1}$ 10 s^{-1} for nigericin and valinomycin assays, respectively.

cium uptake activity observed in SMP proteoliposomes did not exceed that of liposomes. On the other hand, the results indicate that PPTC is enriched in proteins that are able to increase calcium uptake in proteoliposomes (0.68 or 0.48 nmoles $^{45}Ca^{2+}$ (mg phospholipid)$^{-1}$ 30 s^{-1}, after subtracting $^{45}Ca^{2+}$ in nigericin- or valinomycin-dependent assays).

The results obtained when the PPCT were reconstituted by the Bio-Beads method were similar. These proteoliposomes were heterogeneous in size, with a smaller average internal volume (0.12 μl/mg lipid, estimated from the relative distribution of $^{3}H_2O$ and ^{14}C-sucrose as markers of total and external milieu), and $^{45}Ca^{2+}$ uptake (corrected for $^{45}Ca^{2+}$ uptake in liposomes) in nigericin and valinomycin assays was also smaller, 0.18 or 0.13 nmoles $^{45}Ca^{2+}$ (mg phospholipid)$^{-1}$ 10 s^{-1}, respectively. $Na_i^+/^{45}Ca_o^{2+}$ uptake was studied in PPCT proteoliposomes and preformed liposomes, and found to be not detectable (results not shown).

$^{45}Ca^{2+}$ accumulation in PPCT proteoliposomes was due to the transport of calcium across the phospholipid membrane. Thus, provided that the calcium gradient was adequate (i.e., in the presence of external EGTA), the calcium ionophore A23187 was able to promote the complete efflux of the accumulated $^{45}Ca^{2+}$ (results not shown; see also Fig. 5C).

3.3. Kinetics of calcium transport

To investigate the existence of a facilitated diffusion pathway for calcium uptake in our PPCT prep-

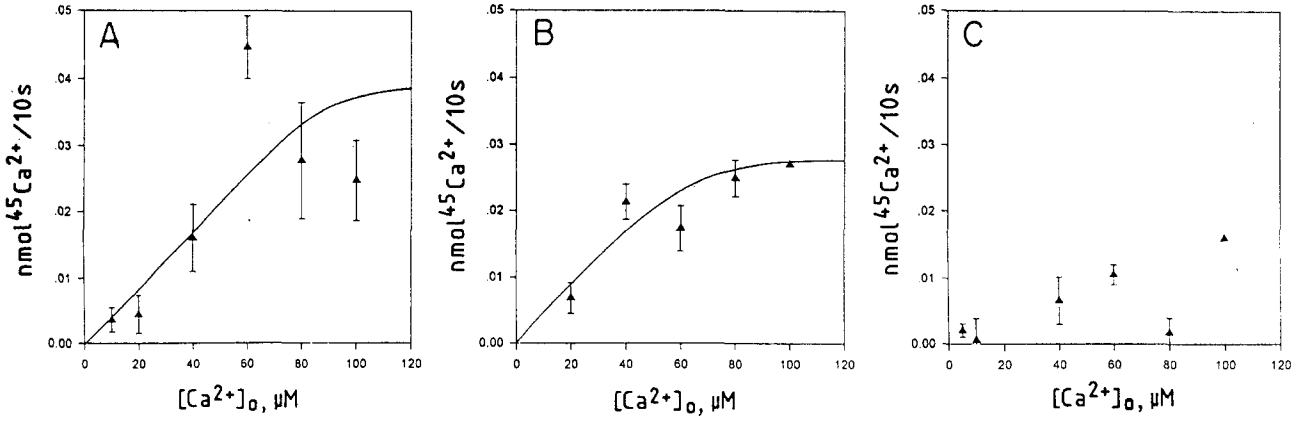

Fig. 4. Kinetics of $^{45}Ca^{2+}$ uptake in PPCT proteoliposomes. $^{45}Ca^{2+}$ uptake in PPCT proteoliposomes (40 μl) (A,B) and liposomes (40 μl) (C) (Bio-Beads reconstitution method) was assayed under conditions of voltage dependence (A) or pH gradient dependence (B,C) at the external calcium concentrations indicated. The results are means ± S.E.M. of three to five different experiments. Data were fit to the Hill equation (Curve Fitter, Scientific Graphing Software, Sigma Plot, Jandel Scientific, San Rafael, CA, USA). The *V*max, $S_{0.5}$, and Hill numbers obtained in A were 0.040 nmoles $^{45}Ca^{2+}$/10 s, 50 μM and 1.1, respectively; and 0.027 nmoles $^{45}Ca^{2+}$/10 s, 45 μM and 1.27, respectively, in B.

aration, we studied the kinetics of $^{45}Ca^{2+}$ uptake (both pH gradient- and membrane potential-dependent uptake) in PPCT proteoliposomes as a function of the calcium concentration. The calcium dependence of $^{45}Ca^{2+}$ transport (pH gradient dependent, Fig. 4B) shows saturation at about 80 μM $[Ca^{2+}]_o$ ($S_{0.5}$ of about 50 μM), a behavior not found in liposomes (Fig. 4C). The calcium dependence of the membrane potential-dependent uptake also has a saturable component in the same range of calcium concentrations (Fig. 4A) which is absent in liposomes (results not shown). These results suggest that one or more saturable components in the PPCT are able to increase the liposome $^{45}Ca^{2+}$ accumulation capacity.

3.4. Influence of the membrane potential on $^{45}Ca^{2+}$ transport in PPCT proteoliposomes

The results presented so far indicate that the driving forces for $^{45}Ca^{2+}$ uptake in valinomycin- and nigericin-dependent assays were the membrane potential and a pH gradient in the former case (Figs. 1 and 2) and only a pH gradient in the latter. Therefore, the PPCT may use either a pH gradient alone or both a pH gradient and a membrane potential as driving force.

To clarify the membrane potential dependence of PPCT-mediated calcium uptake, we have studied the effects of the uncoupler FCCP on valinomycin-stimulated $^{45}Ca^{2+}$ uptake. Fig. 5B (trace a) shows a time course of $^{45}Ca^{2+}$ uptake in the presence of a K^+ diffusion potential. When FCCP was added together with valinomycin (to collapse the membrane potential), $^{45}Ca^{2+}$ uptake was stimulated (Fig. 5B, trace b) over the value obtained with valinomycin alone, and similar to that obtained with nigericin (Fig. 5A). Under these conditions, the collapse of the membrane potential is associated with an increased pH gradient [29]. These results indicate that $^{45}Ca^{2+}$ uptake in PPCT proteoliposomes is not electrophoretic. Thus, the main contributor to $^{45}Ca^{2+}$ uptake in PPCT proteoliposomes is not a voltage-dependent calcium uniporter, but a pH gradient-dependent uptake. The absence of an active calcium uniporter in the PPCT is consistent with the lack of effect of the specific inhibitor ruthenium red, at the concentrations where it effectively blocks calcium uptake in mitochondria (in the 10 nM range, results not shown).

3.5. Effect of the pH gradient on $^{45}Ca^{2+}$ uptake

To further analyze the pH gradient dependence of $^{45}Ca^{2+}$ uptake, we have studied the effect of different pH gradients imposed by the suspension of PPCT proteoliposomes in HKM adjusted to different pH from 7.4 to 12. $^{45}Ca^{2+}$ accumulation increased with increasing pH (Fig. 6A), a result consistent with the presence of a H^+/Ca^{2+} exchanger in the PPCT.

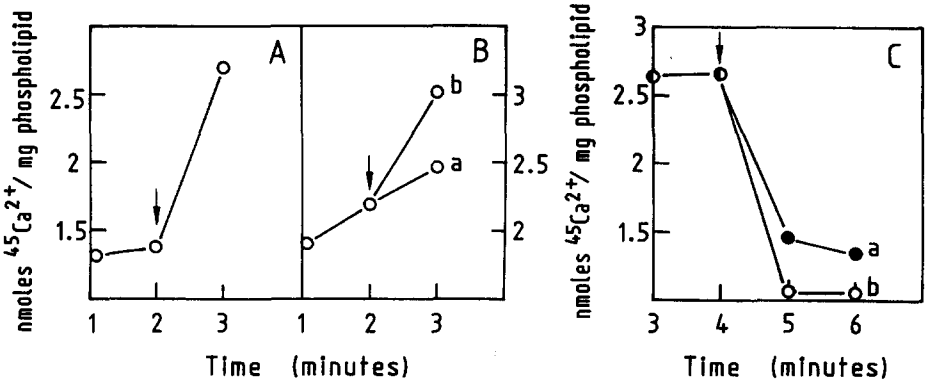

Fig. 5. Time course of $^{45}Ca^{2+}$ uptake in PPCT proteoliposomes. (A,B) Proteoliposomes (4 μl) prepared by the dialysis method were suspended in LowKM, and assayed for $^{45}Ca^{2+}$ accumulation at the times indicated. The following additions were made (arrows): 1.34 μM nigericin (A), 5 nM valinomycin or 5 nM valinomycin plus 1 μM FCCP (B). (B) Traces a and b represent $^{45}Ca^{2+}$ uptake in the presence of valinomycin alone, or valinomycin plus 1 μM FCCP, respectively. (C) Proteoliposomes were incubated in LowKM for 2 min and 1.34 μM nigericin was then added. Two minutes later (arrow) 320 μM EGTA (trace a) or 320 μM EGTA plus 300 nM A23187 (trace b) were added and $^{45}Ca^{2+}$ accumulation was determined at the times indicated. The results correspond to a representative experiment.

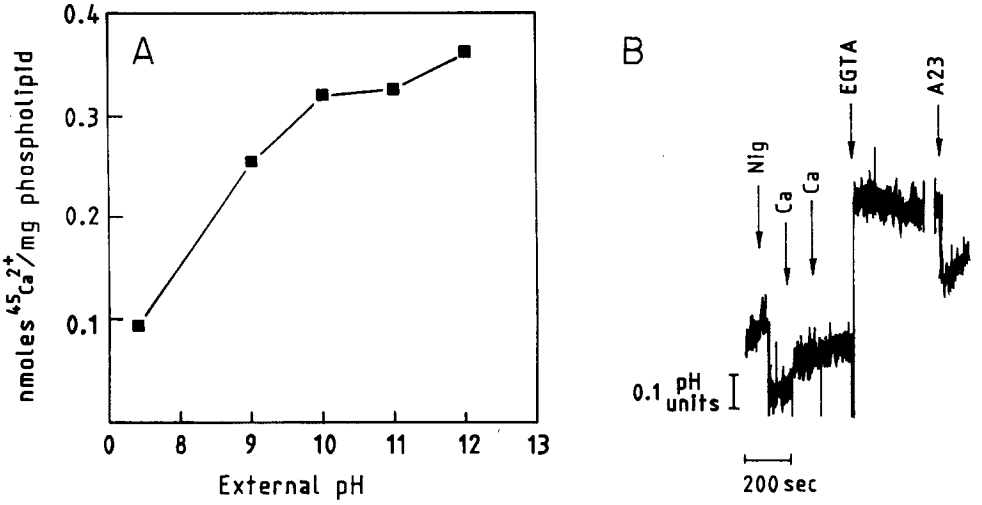

Fig. 6. pH dependence of $^{45}Ca^{2+}$ transport and calcium-dependent pH_i changes. (A) PPCT proteoliposomes or liposomes (4 μl) (reconstituted with the dialysis method) were suspended in HKM adjusted at the pH indicated, and $^{45}Ca^{2+}$ accumulation after 30 s was determined. The results show $^{45}Ca^{2+}$ accumulation in proteoliposomes after correction for that obtained in liposomes, and correspond to a representative experiment. (B) Pyranine-loaded PPCT proteoliposomes (dialysis reconstitution) were suspended in LowKM and the following additions were made: Nig, 1.34 μM nigericin; Ca, 80 μM $CaCl_2$; EGTA, 800 μM EGTA; A23, 1 μM A23187.

3.6. Coupling of H^+ and Ca^{2+} fluxes

To study whether Ca^{2+} fluxes are coupled to H^+ fluxes, pH_i was determined in pyranine-loaded proteoliposomes (Fig. 6B). Nigericin addition to PPCT proteoliposomes suspended in LowKM results in a rapid and transient pH drop (reflected as a downward deflection in fluorescence). The subsequent addition of Ca^{2+} was followed by a gradual increase in pH_i, indicative of coupled Ca^{2+} influx-H^+ efflux. The Ca-dependent increase in intravesicular pH was not produced in HKM (results not shown). Furthermore, the addition of EGTA reversed the change in pH_i (Fig. 6B). The magnitude of the Ca^{2+}-dependent pH_i increase is small (about 0.15 units in 2 min), especially when compared to the pH_i changes induced by A23187 (see Fig. 6B), but it reflects an increase in internal H^+ concentration of 407 μM (assuming that the internal concentration of Tris is 20 mM, which may be underestimated [30], and an influx of 1.46 nmol H^+/mg phospholipid (in 2 min), i.e., within the range of PPCT-dependent Ca^{2+} fluxes.

3.7. Protein bands in PPCT preparations

The PPTC contained major bands at about 66 kDa, a diffuse band of higher molecular weight (at 80 kDa), and a group of bands of smaller molecular weight, at around 50 kDa (Fig. 7). After reconstitution in proteoliposomes this pattern was consistently altered: the high molecular weight bands disappeared, and the smear of smaller molecular weight bands becomes an intense band of 55 kDa. Thus, the reconstitution procedure by itself appears to behave as a purification step, in that it provides a selective enrichment of some bands, in particular the 66 kDa and 55 kDa bands. Moreover, we have consistently observed a total decrease of Coomassie blue stained material after the reconstitution step (see Fig. 7).

Since heat shock proteins could copurify with the mitochondrial calcium transporters [31], in further experiments we assayed the PPCT for immunoreaction with antibodies (anti-DR-1) recognizing the heat shock mitochondrial proteins of mammalian cells, p54 and p74 [32] and the related proteins p56 and p71 in *Drosophila melanogaster* [33]. Western blot analysis showed that these antibodies did not recognize any of the bands of the PPCT (results not shown). Thus, it appears that mitochondrial heat shock proteins are not present in the PPCT.

3.8. Antibodies

Our attempt to raise polyclonal antibodies against the PPCT proved to be successful. The α-PPCT anti-

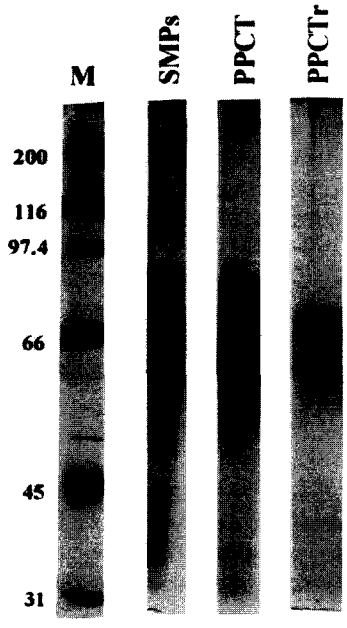

Fig. 7. Affinity chromatography purification of mitochondrial inner membrane proteins. Electrophoretic pattern of proteins in SDS-PAGE (10% acrylamide) stained with Coomassie blue. Lane SMPs corresponds to 3% Triton X-100 extract of inner membrane proteins (50 μl); lanes PPCT and PPCTr correspond to 1/5 of the final eluted PPCT fraction (14.6 μg protein), before (PPCT) or after (PPCTr) reconstitution in proteoliposomes. Molecular weight markers are also shown (M).

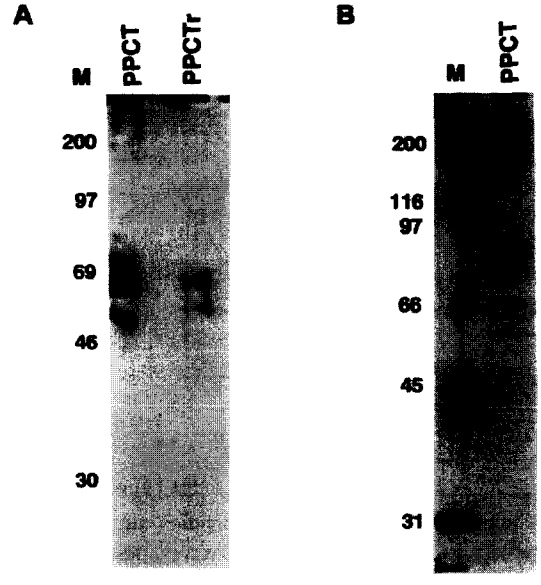

Fig. 8. Immunoreactivity of the antibodies against PPCT. (A) Western blot analysis of the PPCT and the PPCTr with purified antibodies (1/1000) raised against PPCT. (B) SDS protein pattern of the PPCT. Molecular weight markers are also shown (M).

bodies recognized predominantly the 66 kDa major band present in PPCT proteoliposomes showing a weaker reactivity towards the other bands with slower or faster (55 kDa) electrophoretic mobility (Fig. 8) that are still highly visible in the PPCT.

The α-PPCT antibodies were incubated with the PPCT in order to immunoprecipitate the 66 kDa and 55 kDa bands. The immunoprecipitates were separated by centrifugation and the supernatants reconstituted in proteoliposomes. The results from these experiments (Table 2) indicate that different batches of α-PPCT antibodies were able to eliminate the calcium transport activity of the PPCT, albeit with different potency. Therefore, one or both of these protein bands is likely to be responsible for the calcium transport activity. On the other hand, we were unable to show any inhibition of calcium transport after incubation of PPCT proteoliposomes with these antibodies.

Table 2
Immunoprecipitation of $^{45}Ca^{2+}$ uptake activity of the PPCT

	pH gradient-dependent activity (%)
α-PPCT antibodies (1) 1/2	52.9 ± 9.0
α-PPCT antibodies (2) 1/20	90.47
α-PPCT antibodies (2) 1/50	96.2

The PPCT was incubated with different batches of antibodies (1 and 2) at the dilutions indicated and the supernatants were reconstituted in proteoliposomes (Bio-Beads reconstitution method), and assayed for $^{45}Ca^{2+}$ uptake activity in LowKM and in the presence of nigericin. The results represent the inhibition (expressed as percentage of the activity after incubation with preimmune antibodies) of the corresponding activity. The experiments were repeated 2–4 times with similar results.

4. Discussion

We have used immobilized metal affinity chromatography, with calcium ions as the immobilized cations, as a purification procedure to obtain a preparation enriched in calcium transporters from the inner membrane of rat liver mitochondria (PPCT). The PPCT were reconstituted in preformed asolectin liposomes and we studied their ability to increase the calcium uptake over that of liposomes. Since the specific calcium transporters present in rat liver mitochondria are the electrophoretic uniporter and

the H^+/Ca^{2+} or Na^+/Ca^{2+} exchangers, we studied $^{45}Ca^{2+}$ uptake under conditions where the predominant driving forces were a membrane potential (negative inside), a pH gradient (acid inside) or a Na gradient (higher inside). Our observations may be summarized as follows.

(a) The inclusion of the PPCT in proteoliposomes increases their capacity for calcium accumulation. This is not merely due to the calcium binding capacity of the PPCT but reflects real transmembrane transport since (i) the PPCT itself (not reconstituted in proteoliposomes) did not bind $^{45}Ca^{2+}$ under the conditions used for calcium transport assays (results not shown), and (ii) all of the $^{45}Ca^{2+}$ accumulated in PPCT proteoliposomes could be discharged with the calcium ionophore A23187 (Fig. 5C).

(b) The $[Ca^{2+}]_o$ dependence of calcium uptake was studied for both the voltage- and pH gradient-dependent modes. In both cases, PPCT proteoliposomes appeared to contain a saturable component (at around 80 μM calcium) with low ($S_{0.5}$ 50 μM) calcium affinity (Fig. 4). An interpretation of this result is that the saturable component is a calcium transporter present in the PPCT.

(c) $Na_i^+/^{45}Ca_o^{2+}$ exchange activity in PPCT proteoliposomes was not detectable. Since rat liver mitochondria have very low Na^+/Ca^{2+} activity and the Na^+/Ca^{2+} exchanger isolated by Li et al. [13] appears to correspond to a protein band of 110 kDa, the lack of bands of this size in the PPCT (Fig. 7) are consistent with this result.

(d) One possibility was that the PPCT catalyzes an electrophoretic uniport of calcium. This type of transport should be stimulated by a membrane potential (negative inside) and abolished in the presence of FCCP that collapses the membrane potential. Our experimental results show that while the addition of valinomycin in the presence of an outwardly directed K^+ gradient created a membrane potential (Fig. 1) and stimulated $^{45}Ca^{2+}$ uptake (Table 1), FCCP did not inhibit, but rather stimulated $^{45}Ca^{2+}$ accumulation (Fig. 5B), indicating that the PPTC catalyzes mainly a non-electrophoretic transport of calcium.

(e) An alternative possibility is that the positive charges of Ca^{2+} are compensated by a movement of a positive ion on the opposite direction. It is unlikely that the countercation is Ca^{2+} since in experiments where the dialysis reconstitution method was used, Ca_i^{2+} was lowered by washing off the calcium ions bound to asolectin and using 500 μM EGTA as an internal calcium chelator, to minimize any $^{45}Ca_o^{2+}/^{40}Ca_i^{2+}$ exchange. Since the only other Ca^{2+} exchanger present in mitochondria (besides the Na^+/Ca^{2+} exchange) is the H^+/Ca^{2+} antiporter, we have analyzed the possibility that a pH gradient (acid inside) drives $^{45}Ca^{2+}$ uptake.

The first indication that $^{45}Ca^{2+}$ uptake depends upon a pH gradient (acid inside) was obtained from the stimulation of $^{45}Ca^{2+}$ accumulation by nigericin when an outwardly directed K^+ gradient was present (Table 1). In fact, the calcium uptake activity observed in assays with valinomycin (Table 1) is probably due to the fact that a pH gradient (acid inside) is also built up in these assays (Fig. 2). In addition, $^{45}Ca^{2+}$ uptake showed a pH dependence, with increased $^{45}Ca^{2+}$ accumulation at higher external pH values (Fig. 6A). Furthermore, the enhancement of $^{45}Ca^{2+}$ accumulation produced by FCCP in the presence of valinomycin (see above and Fig. 5B) is probably also driven by the larger acidification of the internal HKM obtained upon FCCP addition [29].

All these results indicate that a pH gradient produced by various means stimulates $^{45}Ca^{2+}$ uptake in PPCT proteoliposomes. Moreover, our results indicate that proton efflux is coupled to Ca^{2+} entry (Fig. 6B), providing a stronger evidence for the occurrence of an exchange of external calcium against internal H^+.

Thus, we can conclude that a pH gradient-sensitive Ca^{2+} transporter, possibly a H^+/Ca^{2+} antiporter, is present in the PPCT. At present we do not know whether the exchanger is electroneutral ($2H^+/Ca^{2+}$) or electrogenic ($3H^+/Ca^{2+}$), as suggested for the Na^+/Ca^{2+} exchanger [11,12]. We have tried to clarify this point by studying the effect of nigericin and valinomycin on $^{45}Ca^{2+}$ accumulation in PPCT proteoliposomes incubated in HKM. This would allow to determine whether a pH gradient alone ($2H^+/Ca^{2+}$) or in combination with a membrane potential ($3H^+/Ca^{2+}$) was formed during $^{45}Ca^{2+}$ uptake. However, this was unsuccessful as we did not detect any significant calcium accumulation in the absence of a preformed pH gradient (results not shown).

The calcium uptake activity of the PPCT (100–200 nmoles/30 s/mg protein in PPCT proteoliposomes) is

about 500-fold higher than that of the native H^+/Ca^{2+} exchanger in native mitochondria (about 0.2 nmoles/mg protein in 10 s in mitochondria [21]). The PPCT preparation contains several protein bands, two of which, the 66 kDa and 55 kDa bands, are reconstituted in proteoliposomes (Fig. 7). These two bands are also recognized by the α-PPCT antibodies (Fig. 8) which are able to immunoprecipitate the calcium transport activity (Table 2). Therefore, it may be concluded that the H^+/Ca^{2+} exchanger corresponds to one of these bands. Attempts to separate these bands with the use of other purification procedures that have been widely employed to purify mitochondrial carriers [34] or cation transport systems [31] such as hydroxyapatite chromatography, for basic or acidic proteins [35], or DEAE-cellulose chromatography, have not been successful.

To the extent of our knowledge, this is the first report of a partial purification of a protein which may represent the H^+/Ca^{2+} exchanger of the inner mitochondrial membrane. This exchanger and the Na^+/Ca^{2+} antiporter appear to be separate entities, as indicated by the fact that they are affected differently by inhibitors and have unequal transport kinetics [36–38]. The absence of detectable Na^+/Ca^{2+} exchange in our PPCT preparation supports that contention. The H^+/Ca^{2+} exchange activity of mitochondria was identified as the mechanism responsible for Na^+-independent Ca^{2+} efflux (see [1] for review) and has been controversial since its discovery; (i) it could not be detected in non-respiring rat liver mitochondria, suggesting that coupling of Ca^{2+} and H^+ fluxes might be indirect via other ions [39], (ii) it has been reported that it transports calcium out of mitochondria against a Ca^{2+} concentration gradient many times greater than that possible for a passive $2H^+/Ca^{2+}$ exchanger, suggesting that it may use some form of energy source [14,40]. Thus, the present work represents an important step towards the identification of the protein(s) responsible for this activity.

Acknowledgements

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