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Identification of a 20-kDa Protein with Calcium Uptake Transport Activity. Reconstitution in a Membrane Model

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This paper presents results of experiments designed to further purify the membrane system involved in mitochondrial calcium transport. A partially purified extract, which transported calcium with a specific activity of 1194 nmol $^{45}Ca^{2+}$/mg protein/5 min, was used to obtain mouse hyperimmune serum. This serum inhibited calcium uptake both in mitoplasts and in vesicles reconstituted with mitochondrial proteins containing cytochrome oxidase. Western blot analysis of the semipurified fraction showed that the serum recognized specifically two antigens of 75 and 20 kDa. Both antibodies were purified by elution from the nitrocellulose sheets and their inhibition capacity was analyzed. The antibody that recognized the 20-kDa protein produced a higher degree of inhibition than the other one.

KEY WORDS: Calcium uptake; uniporter; mitochondria.

INTRODUCTION

It has been well established that Ca^{2+} regulates several intramitochondrial functions (Gunter and Pfeiffer, 1990; Denton and McCormack, 1985; Hansford, 1985); therefore, it is important to elucidate the mechanisms through which Ca^{2+} is taken up by mitochondria. It is commonly assumed that the energy-linked transport of Ca^{2+} is accomplished through a specific membrane transport system. Since the pioneering reports of Lehninger (1971) and subsequent workers (Sottocasa *et al.*, 1971; Gòmez Puyou *et al.*, 1972; Blondin, 1974; Carafoli and Sottocasa, 1974; Jeng and Shamoo, 1980; Mironova *et al.*, 1982; Ying *et al.*, 1991), considerable effort has been devoted to the search and characterization of the calcium uniporter. Sottocasa *et al.* (1971) isolated a glycoprotein from the intermembrane space which bound Ca^{2+} with high affinity. A regulatory role upon mitochondrial Ca^{2+} homeostasis has recently been attributed to that glycoprotein by Panfili *et al.* (1980). Carafoli's group has described a glycoprotein located in the inner membrane which transports Ca^{2+} and increases electric conductance in lipid bilayers (Carafoli and Sottocasa, 1974). Recent work by Miranova *et al.* (1982) describes a glycoprotein and peptide isolated from heart mitochondria capable of transporting Ca^{2+}. Their reported molecular weights are 40 and 2 kDa, respectively. This Ca^{2+} transporting activity is inhibited by ruthenium red at concentrations varying from 1 to 10 μM.

An attempt to characterize the mitochondrial Ca^{2+} transport system was carried out recently in our laboratory. We reported (Zazueta *et al.*, 1991) that mitochondrial extracts, reconstituted in cytochrome oxidase vesicles (COV), transported Ca^{2+} in an energy-dependent fashion and that this Ca^{2+} accumulation was sensitive to ruthenium red. The crude extract exhibited the following selectivity for divalent cations: $Ca^{2+} > Sr^{2+} \gg Mg^{2+}$. The present work describes the results of further purification and

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inmunochemical identification of proteins involved in mitochondrial Ca^{2+} transport. It was found that polyclonal antibodies induced against a semipurified extract recognized three specific membrane proteins, corresponding to molecular weights of 75, 70, and 20 kDa. An important feature of this work is the fact that the antibody associated with the 20-kDa protein inhibited calcium transport by 70%.

MATERIALS AND METHODS

Mitochondria from rat kidney cortex were prepared as described (Chávez *et al.*, 1985) in 0.25 M sucrose, 10 mM Tris,* and 1 mM EDTA, pH 7.0, as isolation medium. Submitochondrial particles were obtained by the method of Lee and Ernster (1965). Semipurified extracts were obtained after solubilization of SMP with 1.2% sodium cholate in a sucrose medium. This sample was 50% saturated with ammonium sulfate and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was then 90% saturated with ammonium sulfate and centrifuged at 20,000 *g* for 15 min at 4°C.

The resultant pellet was homogenized with 50 mM KH_2PO_4, pH 7.0, and dialyzed against the same buffer in a 1:250 proportion (F90). This fraction was extracted with 60% chloroform: 40% *n*-butanol and separated by centrifugation at 1,500 *g* for 10 min. The organic phase was evaporated to dryness under a nitrogen current. The resultant dry extract was dissolved in 50 mM KH_2PO_4, 0.8% sodium cholate, pH 7.0 (F_0). Protein was determined by a modified Lowry method (Nakamura *et al.*, 1983). The extracts were reconstituted into COV to test their calcium transporting activity, as follows: Dried lipids were sonicated to clarity in 50 mM H_3PO_4–TEA, pH 7.0. Cytochrome oxidase was added to a final concentration of 0.25 mg/ml and incorporated by simply mixing it with the liposomes as described by Ramírez *et al.* (1987). Mitochondrial extracts solubilized with 0.8% sodium cholate were incorporated to COV after a gentle sonication for 10 sec and dialyzed overnight at 4°C against 250 volumes of 50 mM KH_2PO_4, pH 7.0. The next day they were passed through a G-50 Sephadex column pre-equilibrated with the same buffer. The vesicles were collected by centrifugation at 100,000 *g* for 1.5 h and assayed for calcium uptake.

Hyperimmune sera was obtained from female Balb-c mice, 6–8 weeks old, immunized every three weeks by subcutaneous or intraperitoneal injection of 40 μg of the semipurified fraction (F90) emulsified in incomplete Freund's adjuvant. Sera from immunized mice were stored at −20°C until required. The specificity of the antisera against the F90 protein components was determined by Western blot, whereas the optimal titration was determined by enzyme-linked inmunoabsorbent assay (ELISA). (Engvall and Perlmann, 1971). The serum from each mouse was tested separately, and only active antisera were pooled (AntiUni-Ca^{2+}).

Inhibition of calcium transport by antisera was assayed in two different systems: in liposomes with incorporated mitochondrial proteins and in mitoplasts. Mitoplasts were freshly prepared each day as follows: Digitonin, 1.2% (w/v) was added dropwise to 10 mg of mitochondrial protein. The resulting suspension was incubated for 10 min at 5°C and centrifuged at 15,000 *g* for 10 min. The mitoplast pellet was resuspended and an outer membrane preparation was obtained by ultracentrifugation of the supernatant at 144,000 *g* for 10 min at 5°C. Most of the activity of monoamine oxidase, assayed by the method of Schnaitman and Greenawalt (1968), was found associated with the outer membrane preparation, (88 ± 12 nmol/min/mg protein). The inner membrane-matrix fraction retained its morphological integrity and exhibited a high respiratory rate when assayed in a sucrose/HEPES medium containing EDTA. Calcium transport in mitoplasts was followed by dual-wavelength spectroscopy at 675–685 nm, with the methallochromic dye Arsenazo III (Kendrick, 1976). Quantification of calcium uptake was obtained by a filtration technique using $^{45}CaCl_2$ (specific activity 1000 cpm/nmol) in reconstituted liposomes and in mitoplasts. Changes in the transmembrane potential were followed by dual-wavelength spectroscopy, at 511–533 nm, using safranine as reported (Rottenberg, 1979).

Polyacrilamide gel electrophoresis was performed in the presence of 2% sodium dodecyl sulfate, as described by Laemmli (1979). The proteins were electroblotted onto nitrocellulose (Towbin *et al.*, 1979), and then visualized for specific antibody

* Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS PAGE, sodium dodecyl polyacrylamide gel electrophoresis; EDTA, ethylendiaminetetraacetic; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TEA, triethanolamine; COV, cytochrome oxidase vesicles.

Table I. Calcium Uptake Activity of Semipurified Mitochondrial Extracts[a]

Solublized extract co-reconstituted with cytochrome oxidase	Total protein (mg)	Specific activity (nmol/mg/5 min)	Yield purification (%)	
Mitochondria	2900.00	151.88	100	1.00
Inner membrane proteins	1315.00	127.33	40	0.83
50% ammonium sulfate precipitation	659.10	—	—	—
90% ammonium sulfate precipitation	44.20	443.00	2.7	2.91
Organic-extraction ($CHCl_3$/*n*-butanol)	2.70	1194.00	0.9	7.80

[a] COV (1.5 mg phospholipids) with incorporated protein from semipurified extracts were added to an incubation medium containing 50 mM KH_2PO_4, 7.5 mM ascorbate, 0.75 mM TMPD, 150 μg cytochrome *c* (pH 7.0), and 0.5 μM $^{45}CaCl_2$ (specific activity 1000 cpm/nmol). The samples were incubated during 5 min, and an aliquot filtered through a 0.45 μM Millipore filter using the protamine aggregation/filtration technique. Specific activity values represent the difference between the energized and nonenergized uptake rates. Under all conditions the calcium transport activity was inhibited by ruthenium red. Final volume 1 ml; temperature 25°C.

binding by the inmunoperoxidase method, using diaminobenzidine tetrahydrochloride.

RESULTS

Calcium Uptake Activity of Semipurified Extracts

As described in the preceding section, partially purified extracts were obtained and reconstituted into vesicles to test their ability to accumulate calcium in similar conditions as those reported for whole mitochondria extracts (Zazueta *et al.*, 1991). The results are presented in Table I. As indicated, after elimination of external membrane and matrix space constituents, inner membrane proteins still transported calcium with a specific activity of 127.3 nmol $^{45}Ca^{2+}$/mg protein/5 min. The ammonium sulfate fractionation step provided two extracts; the one obtained after precipitation at 50% saturation completely lost the ability to accumulate the cation regardless of its considerable amount of protein content.

The second extract (F90), once reconstituted, restored calcium uptake with a specific activity of 443 nmol $^{45}Ca^{2+}$/mg protein/5 min. Finally, this fraction was extracted with chloroform:*n*-butanol and reconstituted into the vesicle system (F_0); again calcium transport with a specific activity of 1194 nmol $^{45}Ca^{2+}$/mg/5 min was observed. Figure 1 shows the electrophoretic pattern of the extracts with calcium transport activity, from left to right, duplicates of 10 and 20 μg protein: submitochondrial particles (SMP), fraction precipitated with 90% ammonium sulfate (F90), and fraction extracted with organic solvents (F_0). We also explored the ability of the F90 fraction to promote calcium movements through an organic phase. As seen in Fig. 2, calcium was translocated from a hydrophilic medium, through the hydrophobic phase, to an aqueous solution containing a calcium chelating buffer. This movement was 50% inhibited by 5 μM ruthenium red. The fraction assayed contained an excess of lipids, which was quantified as 0.654 μmol Pi/mg protein. In the organic phase experiment, the possible calcium transporting activity of phospholipids was also

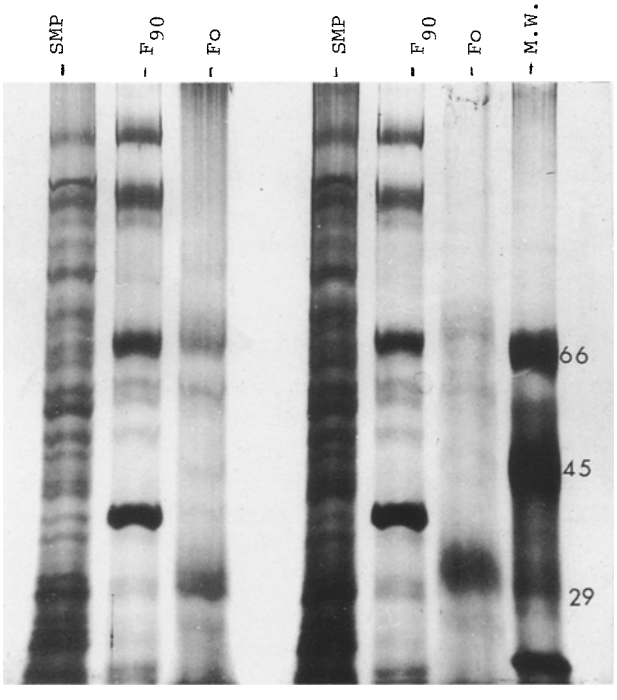

Fig. 1. SDS-PAGE (10% gel) of mitochondrial extracts reconstituted into COV with calcium transport activity. From left to right: submitochondrial particles; F90 (fraction precipitation with 90% ammonium sulfate) and F_0 (fraction obtained after organic solvent extraction).

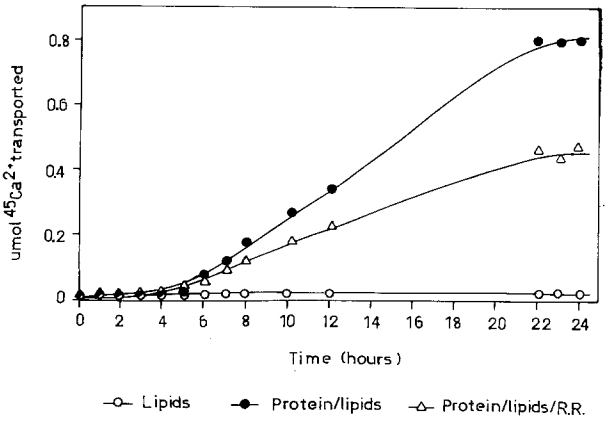

Fig. 2. Purified mitochondrial extracts induced calcium mobilization through a hydrophobic phase. An 8-ml mixture of 60% chloroform: 40% *n*-butanol containing 0.7 mg of mitochondrial protein from the F90 fraction was transferred into a U-tube (270 mm long × 7 mm diameter). In the left side, 1.0 ml of 7.5 μM $^{45}CaCl_2$ in 10 mM TRIS, pH 7.3, was layered and in the right side, 1 ml of a 5 mM EGTA solution in 10 mM TRIS, pH 7.3, was deposited (●). The same experiment was carried out in the absence of the protein (○) and in the presence of 2.5 μmol ruthenium red (△). At the indicated times, aliquots of 0.05 ml were withdrawn for radioactivity measurements.

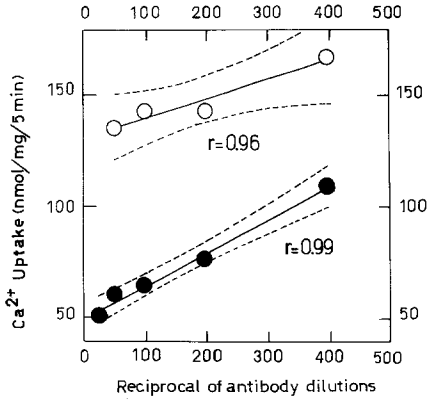

Fig. 3. Net calcium uptake in COV reconstituted with whole mitochondria proteins in the presence of preimmune serum (○) and AntiUni-Ca^{2+} (●). Proteoliposomes were incubated for 10 min at 4°C with antisera and then added to the reaction medium described in Table I. After 5 min, an aliquot was filtered and the radioactivity measured in a scintillation counter. The results are expressed as the mean of three experiments represented as a linear regression with confidence limits of 95% (dotted lines).

tested, observing that phospholipids themselves had no ionophoretic properties. The experiments with denaturalized protein added further evidence about the essential role of a protein component in the calcium transport activity. Boiled preparations reconstituted into liposomes completely lost their calcium transport activity (not shown).

Characterization of the Antibodies

Titers of Sera

Titers were determined by an indirect ELISA method. The titer is defined as the highest dilution at which the ELISA response was twice the blank value of the test. High titers were obtained in all the animals. The capacity of the antibodies to inhibit calcium uptake was tested in reconstituted liposomes and mitoplasts.

Inhibitory Action of Antiserum on Calcium Transport Activity in Reconstituted Liposomes and Mitoplasts

Control antiserum and AntiUni-Ca^{2+} were incubated with COV reconstituted with whole mitochondria proteins, for 10 min, and assayed for ^{45}calcium uptake by the filtration method and scintillation counting.

Figure 3 shows the net uptake of calcium in COV in the presence of different dilutions of control serum and AntiUni-Ca^{2+}. Both sera inhibit calcium uptake; this can be explained by a nonspecific recognition of the antibodies or of another serum component, of a similar epitope. Nevertheless, the remaining activity of calcium transport in the vesicles incubated with control serum was almost twice the observed when AntiUni-Ca^{2+} was present under the same conditions. The difference between the mean values of the two groups was statistically significant at $P < 0.001$ (Student's t test). Each value represents the difference between "energized" vesicles uptake, i.e., liposomes with a negative inner membrane potential and "nonenergized" uptake, or passive calcium accumulation and unespecific binding of the cation to the liposomes.

Calcium uptake by mitoplasts is illustrated in Fig. 4. Trace A shows energy-dependent calcium accumulation in mitoplasts. It can be observed that when the proton gradient was dissipated by CCCP, the cation was released by mitoplasts. Trace B shows the same experiment in the presence of control serum. In this case the antibodies had no effect on calcium movements. Trace C clearly shows the inhibitory action of AntiUni-Ca^{2+}. These results raised the question of a possible inhibitory effect upon the mechanism that induces the protonmotive force. Mitoplasts respiration was followed in an oxymeter with a Clark type electrode, revealing a high rate

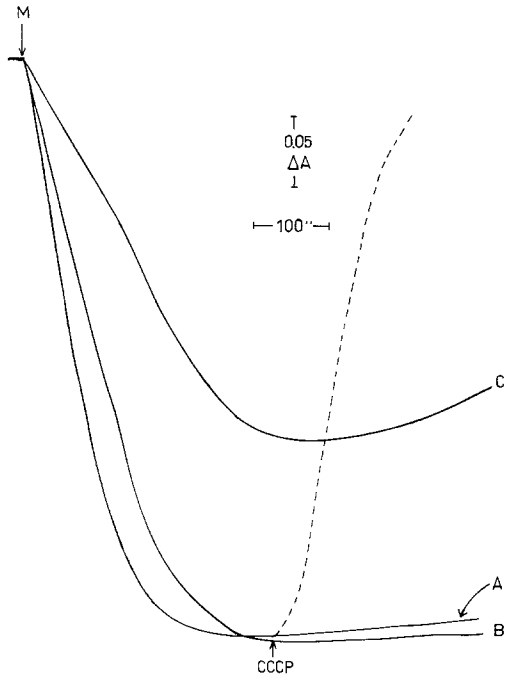

Fig. 4. Inhibition of calcium uptake by Anti-UniCa^{2+} in mitoplasts. Protein (1 mg) was added to an incubation medium containing 250 mM sucrose, 10 mM succinate, 10 mM HEPES, 50 μM $CaCl_2$, 200 μM ADP, 10 μg rotenone, 1 mM phosphate, and 50 μM Arsenazo III. Double wavelength spectrophotometric tracings were obtained at 685–675 nm. Final volume 3 ml, temperature 25°C, pH 7.3.

of respiration in the presence of the antisera (not shown).

It is known that a rapid, electrophoretic calcium uptake can decrease the internal negative membrane potential (Scarpa and Azzone, 1970; Gunter and Pfeiffer, 1990). The effect of the antisera on the mitoplasts' membrane energization was explored. Figure 5A shows the membrane potential of control mitoplasts; a partial de-energization of the membrane is induced by 50 μM calcium as a result of the cation cycling across the membrane (Lehninger *et al.*, 1967; Broekemeier and Pfeiffer, 1989; Chávez *et al.*, 1991). Once the equilibrium of positive charges is established, the transmembrane potential is restored to a value of 200 mV.

The $\Delta\psi$ decrease after addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone. In Figs. 5B and 5C, the antibodies were incubated with mitoplasts for 10 min and then added to the assay medium to measure changes in optical absorbance in the presence of safranine. As shown, mitoplasts were capable of maintaining their membrane integrity in spite of the high calcium concentration present in the serum. Only when a critical concentration of calcium has been reached inside the mitoplast did the $\Delta\psi$ begin to drop. EGTA was added to chelate the excess of external calcium.

Specificity of the Antibodies

The specificity of the antibodies was monitored using the Western blot technique. After electrophoresis

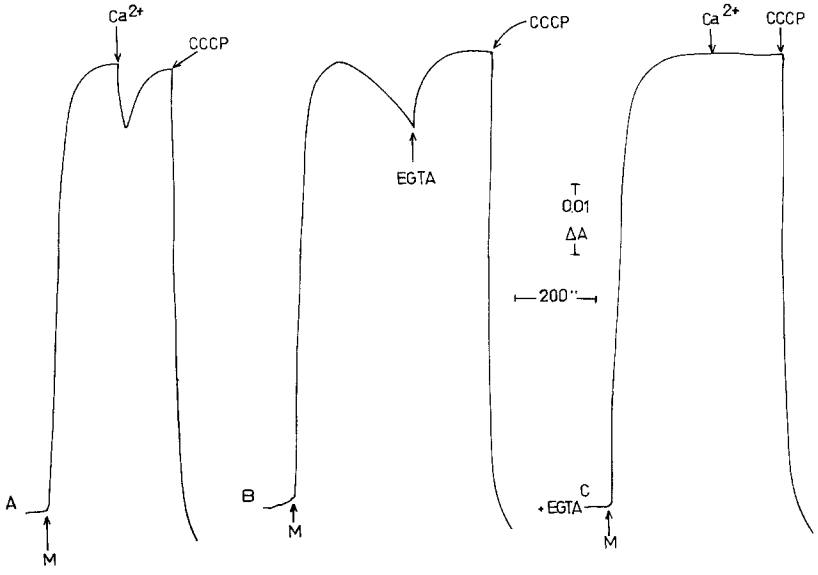

Fig. 5. Transmembrane potential of mitoplasts incubated with preimmune serum and AntiUni-Ca^{2+}. The assay medium was essentially the same as described in Fig. 4, but safranine was used instead of Arsenazo III and the changes in absorbance were followed at 533–511 nm. Calcium was added where indicated.

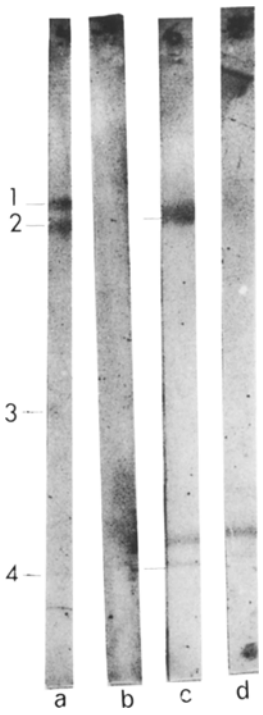

Fig. 6. Western Blot of semipurified mitochondrial extracts. From left to right: submitochondrial particles, incubated with AntiUni-Ca^{2+} (a) and with preimmune serum (b); F90 incubated with AntiUni-Ca^{2+} (c) and preimmune serum (d), developed with *o*-diaminobenzidine.

on polyacrylamide gels under denaturing conditions, the proteins were transferred to nitrocellulose paper and tested against antibodies. The fact that antibodies lost their recognition capacity for boiled, electrophoresed proteins, blotted onto nitrocellulose, suggests that the recognized epitope is conformational or situated very near to an active locus.

Figure 6 shows the patterns of transferred proteins revealed by inmunochemical detection using AntiUni-Ca^{2+} and a second antibody labeled with peroxidase. Among the numerous bands detected by protein staining (not shown) only a few reacted with the antiserum and were revealed after incubation with *o*-diaminobenzidine. Nonspecific recognition of a 25-kDa protein was observed by the control antisera incubated with SMP and F90 (lines b and d), probably by cross reaction; on the other hand AntiUni-Ca^{2+} specifically detected two bands, a 75-kDa protein and a second band of approximately 20 kDa, both present in SMP and F90 (lines a and c). In line a, AntiUni-Ca^{2+} detected another band in SMP (70 kDa), probably a degradation product of the same protein present in F90, as a single, but rather broad band.

Calcium Uptake Inhibition by Specific Antibodies

Removal of specific antibodies bound to proteins transferred onto nitrocellulose was made after careful detection. An acid elution technique was used in the presence of 0.1 M glycine solution, pH 3. After 60 sec of continuous shaking, the eluates were neutralized to pH 7.0. This was done until no proteins were detected in the eluates. Three specific antibodies and a control eluate were obtained. The specific antibodies correspond to 75-, 70- and 20-kDa proteins. A control eluate in each experiment was obtained after acid treatment of a different zone of the nitrocellulose matrix, which showed no affinity for the specific antibodies.

Table II shows the inhibitory effect of eluted antibodies on the initial rate of calcium uptake. Mitoplasts incubated with the control eluate accumulated 86.62 ± 6.34 nmol $^{45}Ca^{2+}$/mg/min. The same preparation incubated with the antibody against the 75-kDa protein of F90 reduced the uptake to 50.7 ± 12.27 nmol $^{45}Ca^{2+}$/mg/min, which represents 59% of the total calcium transport activity. Similar values were found for the antibody that recognized the 70-kDa protein, i.e., 63.57 ± 3.97 nmol $^{45}Ca^{2+}$/mg/min. In some experiments AntiUni-Ca^{2+} recognized a band of approximately 66 kDa; this antibody was eluted and its inhibitory action on calcium accumulation was tested. It did not affect the cation uptake; an activity

Table II. Eluted Antibodies Inhibited Calcium Uptake in Mitoplasts[a]

Eluted antibodies	nmol $^{45}Ca^{2+}$ transported/mg/min
Control	86.82 ± 6.34
1	50.70 ± 12.27
2	63.57 ± 3.97
3	84.35 ± 1.30
4	28.28 ± 0.78

[a] A 50-μg preparative SDS-PAGE was blotted onto a nitrocellulose membrane and then incubated for 3 h at room temperature with a 1:25 dilution of the hyperimmune sera. At the end of the incubation period, strips were cut of the regions where the 75-, 70- and 20-kDa antigens were located. One strip obtained from the blotted membrane, developed with a peroxidase-labeled rabbit antimouse IgG and diaminobenzidine, was used as control. Each strip was immediately incubated for 2 min with 1 ml of 0.1 M glycine in distilled water and the pH adjusted to 7.0 with KOH. Mitoplasts were incubated for 10 min at 4°C with the specific antibodies and then added to the assay medium, described in Fig. 4, which contained 50 μM $^{45}CaCl_2$. Radioactivity measurements were carried out after 1 min of incubation.

of 84.35 ± 1.30 nmol $^{45}Ca^{2+}$/mg/min was found. The antibody associated to the 20-kDa protein inhibited almost 67% of the calcium transport in mitoplasts, i.e., 28.28 ± 0.78 nmol $^{45}Ca^{2+}$/mg/min. It should be noted that mitoplasts accumulated 85.53 ± 2.3 nmol $^{45}Ca^{2+}$/mg/min under normal conditions.

DISCUSSION

In previous study we showed that a crude extract of proteins isolated from the inner mitochondrial membrane had the ability to transport Ca^{2+}, provided a membrane potential was built up (Zazueta *et al.*, 1991). The present work reports data on further purification of a calcium carrier uniporter. As shown, extraction of the F90 fraction with chloroform–butanol conduces to the isolation of four protein bands, identified in SDS-PAGE, and with molecular weights ranging between 20–70 kDa (F_0).

Interestingly, this extract was able to transfer Ca^{2+} from a hydrophilic phase to another, separated by a hydrophobic phase. It should be noted that this membrane extract, due to the form of extraction, contained phospholipids. In this regard, Sokolove and Brenza (1983) showed that phosopholipids may act as ionophores for Ca^{2+}. However, this possibility was discarded, since, as shown in Fig. 2, when using phospholipids as controls, no calcium transport was observed. Further evidence on the essential role of the protein component was provided by the experiment showing that after boiling the extract, its Ca^{2+} transporting activity was completely lost.

An important finding of this work was that the polyclonal antibodies prepared with an inner membrane extract (F90) identified three proteins with apparent molecular weights of 75, 70, and 20 kDa. It is relevant to note that the antibody reacting against the 20-kDa protein almost completely inhibited Ca^{2+} accumulation in mitoplasts (Table II). These data strongly suggest that this 20-kDa protein is a necessary component of the Ca^{2+} uniporter. Saris *et al.* (1993) reported that antibodies induced against a Ca^{2+}-binding mitochondrial glycoprotein inhibited the uniporter-mediated transport of calcium in mitoplasts prepared from rat liver mitochondria. The protein was reported to consist of a glycoprotein part and a small putative channel peptide that might be dissociated from it. Due to its elusive character, identification of the calcium transport system is a difficult challenge that remains open to all possibilities. It can be inferred that the 20-kDa protein could be the calcium uniporter *per se*, or at least a fundamental constituent of the transporting proteins. Although we used polyclonal antibodies, the antibody directed against the 20-kDa protein was indeed selectively purified, since it was eluted after specific recognition of this protein. This specificity was observed even in a very complex mixture of proteins, i.e., submitochondrial particles. Finally, the dissociation of respiratory activity from inhibition upon initial calcium influx, in mitoplasts, supports the notion that the AntiUni-Ca^{2+} acts on calcium translocation at the uniporter level. It has been proposed that the possible role of the high-affinity Ca^{2+}-binding proteins could consist in being a "recognition site" for superficial binding of calcium, not involved in membrane translocation of the cation, but essential for the transport process (Carafoli, 1975; Prestipino *et al.*, 1974). Remarkably, the calcium transport activity observed with the extract used to induce antibodies was not a binding activity, but an energy-dependent Ca^{2+} uptake, depending on a selective and differential energization. This energization method has been widely described in the literature (Horstman and Racker, 1970; Skulachev, 1974; Racker, 1974; Carrol and Racker, 1977; Kessler *et al.*, 1977).

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