Purification of the synaptosomal plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase from pig brain

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The Ca^{2+}-ATPase from the synaptosomal plasma membrane has been purified nearly to homogeneity from pig brain by a new procedure involving the calmodulin-affinity-chromatography technique. This is a convenient alternative to the standard methods for the purification of the plasma membrane Ca^{2+}-ATPase from different sources that were unsuitable to purify the enzyme from pig brain. The main feature of this procedure is the use of 15% (v/v) glycerol as stabilizing agent, instead of acidic phospholipid. By using this protocol the enzyme was purified 36-fold with respect to the plasma membrane vesicle fraction, showing a specific activity of 2.3 i.u. in the presence of acidic phospholipid. In SDS/PAGE, it appears as a single protein band around M_r 140000 that can be phosphorylated by [γ-^{32}P]ATP in the presence of La^{3+} and recognized by specific antibodies against the plasma membrane Ca^{2+}-ATPase from pig antral smooth muscle. Calmodulin activates the enzyme 1.5–1.8-fold in the presence of phosphatidylcholine but not in the presence of phosphatidylserine.

INTRODUCTION

The Ca^{2+} concentration in the nerve cell is a key factor in the control of neurotransmitter release. The arrival of action potentials at the nerve terminal elicits an increase in the Ca^{2+} concentration that triggers the neurotransmitter exocytosis. Removal of Ca^{2+} from the nerve terminal is then required to terminate the process of neurotransmitter release.

Nerve terminals have different ion transport systems that contribute to the regulation of the low intracellular free Ca^{2+} concentration. The neuronal plasma membrane contains mainly a Ca^{2+}-ATPase that pumps Ca^{2+} to the extracellular medium and a Na^+/Ca^{2+} exchanger antiporter that can affect the intracellular Ca^{2+} concentration by modifying the Na^+ gradient across the membrane. Within the synaptic terminal, Ca^{2+} can also be removed from the cytosol to the endoplasmic reticulum by a Ca^{2+}-ATPase [sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) type] or to the mitochondria by a Ca^{2+} uniporter [1]. The plasma membrane Ca^{2+}-ATPase (PMCA) is a member of the P-type ATPase family, that can be phosphorylated at an aspartic residue of the cytoplasmic domain as part of the ion transport mechanism. Indeed, the red blood cell has been for many years the system of choice for studying the plasma membrane, mainly because of the simple cell structure and the availability of good techniques for the isolation and manipulation of the plasma membrane. By contrast, the isolation of pure plasma membrane from other sources such as synaptosomes is a more difficult task due to the presence of subcellular organelles that also contain Ca^{2+}-transporting systems and/or hydrolyse ATP in a Ca^{2+}-dependent way [2]. Despite such a problem the enzyme has been purified from a number of different systems [3–6] by following the standard procedure established for the purification of the erythrocyte PMCA [7,8]. Since the application of this protocol to the purification of the enzyme from pig brain did not provide good results, we report here a modification of the standard method to purify the synaptosomal PMCA from pig brain.

MATERIALS AND METHODS

Calmodulin, calmodulin–agarose and phosphatidylcholine (types XI-E and II-S) were obtained from Sigma, St. Louis, MO, U.S.A. Phosphatidylserine was purchased from Boehringer Mannheim, Germany and [γ-^{32}P]ATP was a product of Dupont–NEN, Belgium. All other reagents were of the highest purity available.

Preparation of synaptosomes and plasma membrane vesicles

The isolation of synaptosomes and plasma membrane vesicles from pig brain was based on the method of Michaelis et al. [9], modified to get maximal optimization. The summary of the complete procedure is outlined in Scheme 1. Fresh pig brain (70–80 g), obtained from a local slaughterhouse, was homogenized in 10 vol. of 10 mM Hepes/KOH, pH 7.4, 0.32 M sucrose, 0.5 mM $MgSO_4$, 0.1 mM PMSF and 2 mM 2-mercaptoethanol (buffer I), at 4 °C, in a Potter–Elvehjem glass homogenizer, by four strokes of 1000 rev./min. The homogenate was centrifuged at 1500 ***g*** for 10 min and the supernatant obtained (SN-I) was then centrifuged at 20000 ***g*** for 20 min. The pellet of the second centrifugation (P-II) was resuspended in buffer I to get a protein concentration of 32 mg/ml. Samples (800 mg) of protein (128 mg/tube) were layered on to a discontinuous gradient containing 19 ml of 40% (w/v) sucrose and 15 ml of 20% (w/v) sucrose and centrifuged at 63000 ***g*** for 45 min. Synaptosomes were obtained at the interface, and washed in 25 vol. of 10 mM Hepes/KOH, pH 7.4. After centrifugation at 20000 ***g*** for 30 min the final synaptosome fraction (P-III) was collected in the pellet and resuspended in 10 mM Hepes/KOH, pH 7.4, and 0.32 M sucrose, at a protein concentration of approx. 27 mg/ml. An aliquot (22 ml) of the synaptosome fraction was lysed by incubating at 4 °C for 40 min, in 100 vol. of 10 mM Hepes/KOH, pH 7.4, 1 mM EDTA and 2 mM 2-mercaptoethanol (lysis buffer), under continuous stirring. The lysate was centrifuged at 20000 ***g*** for 30 min and the pellet

Abbreviations used: PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid; PVDF, poly(vinylidene difluoride).

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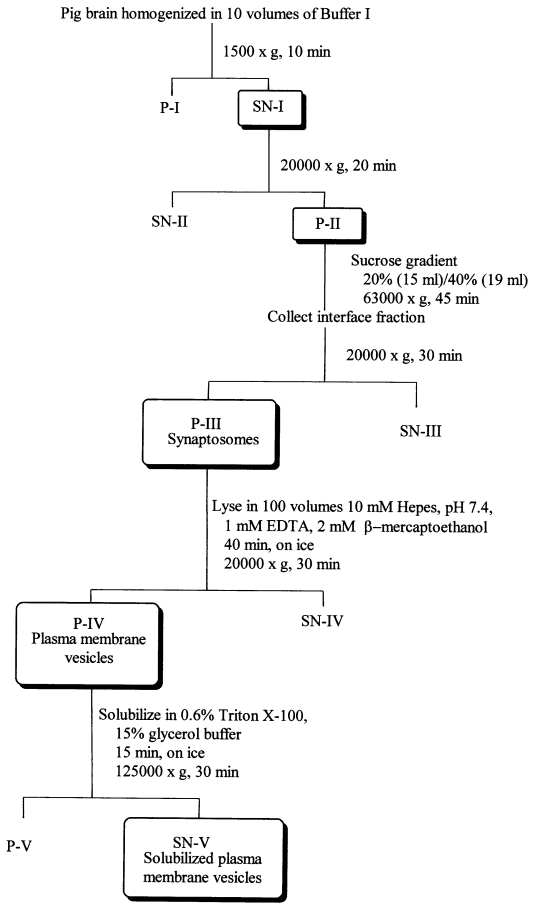

Scheme 1 Isolation procedure of synaptosomes and plasma membrane vesicles from pig brain

containing the plasma membrane vesicles was collected (P-IV). This fraction was resuspended in 27 ml of 10 mM Hepes/KOH, pH 7.4, and 0.32 M sucrose to a final concentration of 16 mg/ml, and stored at −70 °C when no further purification was required.

Solubilization of plasma membrane vesicles in Triton X-100

Plasma membrane vesicles, at a protein concentration of 6 mg/ml in a medium containing 20 mM Hepes/KOH, pH 7.4, 130 mM KCl, 0.5 mM $MgCl_2$, 50 μM $CaCl_2$, 15% glycerol and 2 mM 2-mercaptoethanol, were solubilized by the slow addition of 1 mg of Triton X-100/mg of protein (0.6%, w/v). After a 15 min incubation on ice under stirring, the sample was centrifuged at 125000 ***g*** for 30 min. The solubilized PMCA was obtained in the supernatant (SN-V) and the insoluble material (P-V) was removed.

Calmodulin affinity chromatography

A calmodulin–agarose column (10 ml) was equilibrated with 5 bed vols. of 20 mM Hepes/KOH, pH 7.4, 130 mM KCl, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 0.6% Triton X-100, 2 mM 2-mercaptoethanol and 15% glycerol. The solubilized protein (432 mg), was applied on to the column and washed with 15 bed vols. of the washing buffer, that was similar to the equilibration buffer except that the Triton X-100 concentration was 10 times more dilute (0.06%). The Ca^{2+}-ATPase protein was eluted in a buffer containing the same composition as the washing buffer except that 2 mM EDTA was substituted for Ca^{2+}. The enzymic activity and the protein content were assayed immediately after the chromatography column. Fractions containing maximum protein concentration and ATPase activity were pooled, concentrated in a Centriplus 30 from Amicon, aliquoted and stored at −70 °C.

Ca^{2+}-ATPase activity

The enzymic activity was measured following spectrophotometrically the reaction at 340 nm, using a coupled enzyme assay. Samples (40 μg) of protein from synaptosomes or plasma membrane vesicles were added into the reaction mixture containing, in a final volume of 1 ml, 50 mM Hepes/KOH, pH 7.4, 100 mM KCl, 5 mM Na_3N, 2 mM $MgCl_2$, 100 μM $CaCl_2$, 100 μM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA; 5 μM free Ca^{2+}), 0.22 mM NADH, 0.42 mM phosphoenolpyruvate, 10 i.u. of pyruvate kinase and 28 i.u. of lactate dehydrogenase. After a 4 min incubation at 37 °C the reaction was started by the addition of 1 mM ATP. The specific activity of the purified ATPase was assayed as described above after a previous incubation at 37 °C for 4 min of 7.5 μg of protein with type-II-S phosphatidylcholine, or another phospholipid when specified, at a ratio of 5.33 mg of lipid/mg of protein (equivalent to 40 μg/ml). The enzymic activity was also assayed in the presence of bovine brain calmodulin. In this case 0.42 μg/ml of calmodulin was incubated with 100 μM $CaCl_2$ for 4 min at 37 °C and then added to the standard reaction medium deprived of Ca^{2+}. The Ca^{2+}-ATPase activity was obtained after subtraction of the Mg^{2+}-dependent activity, measured in the presence of 3 mM EGTA.

Protein determination

The protein content was measured by the Bradford method [10]. The interference of Triton X-100 was eliminated by a preliminary treatment of the samples with BioBeads SM-2 from Bio-Rad, U.S.A.

SDS/PAGE and immunoblotting

Protein samples were previously concentrated by the Microcon-50 ultrafiltration system (Amicon). Electrophoresis was performed by the methods of Laemmli [11] or Weber and Osborn [12]. Transfer of proteins to a poly(vinylidene difluoride) (PVDF) membrane was done in a semi-dry system from Bio-Rad, before the autoradiographic exposure and immunoblotting. Immunoblotting was performed following the standard procedure of Towbin et al. [13].

Enzyme phosphorylation by ATP

The purified enzyme (10 μg) or the plasma membrane vesicles (100 μg) were incubated on ice in a total volume of 0.1 ml in the presence of 30 mM Mops/KOH, pH 7.0, 75 mM KCl, 50 μM $LaCl_3$ and 218 μM $CaCl_2$ (for the purified enzyme) or 20 μM $CaCl_2$ (for the plasma membrane vesicles) to give a 20 μM free Ca^{2+} concentration. The phosphorylation reaction was started by adding 5 μM [γ-^{32}P]ATP [(1–2) × 10^7 c.p.m./nmol] and stopped 1 min later by the addition of 1 ml of ice-cold 7% (w/v) trichloroacetic acid [14]. In other experiments, the plasma membrane vesicles were phosphorylated for 15 s, in the presence of 15 μM $MgCl_2$/50 μM $CaCl_2$ and in the absence of La^{3+} to evaluate the SERCA-type content [15]. Samples were washed three times with the stopping solution and then resuspended in the electrophoresis sample buffer. The ^{32}P-phosphorylated

ATPase was separated at pH 6.3 by the method of Weber and Osborn [12], using a 6.5 % polyacrylamide gel and 0.1 % lithium dodecyl sulphate. Radioactive phosphoproteins in the PVDF membrane were detected by autoradiography on Amersham Hyperfilm RPN-6 in the presence of a Lightning Plus intensifying screen. Exposure was allowed to proceed for 24 h at −70 °C.

RESULTS

All the experimental manipulations during the isolation and purification procedures were performed on ice or at 4 °C. We started from a pig brain of about 80 g, equivalent to 7.2 g of total protein after homogenization. The homogenate had a Ca^{2+}-ATPase activity of 0.017 i.u. After two centrifugations at low and high speed, 89 % of the initial protein was removed, and the Ca^{2+}-ATPase activity was 0.039 i.u. The synaptosome fraction (594 mg of protein) collected from the interface after the sucrose gradient showed a value of 0.052 i.u. for the Ca^{2+}-ATPase activity. After the synaptosome lysis the plasma membrane fraction was 6 % of the homogenate total protein and the Ca^{2+}-ATPase activity was 0.063 i.u. This value corresponds to an enrichment of 371 % with respect to the homogenate enzymic activity.

The plasma membrane vesicles (432 mg of protein) were solubilized in order to obtain most of the PMCA protein in the supernatant and the enzyme was further purified by calmodulin affinity chromatography. Table 1 shows the results of a typical purification procedure obtained as described in this study. It includes the protein balance and the specific enzyme activity during the solubilization and the chromatography steps. The solubilization of the PMCA was performed in the presence of 0.6 % Triton X-100. In addition, we have included 15 % (v/v) glycerol instead of acidic phospholipid to stabilize the membrane protein. This amount of glycerol was also present in all the buffers during the chromatographic separation. After this step, 67 % of the total plasma membrane vesicle proteins were solubilized. This fraction contained 64 % of the total Ca^{2+}-ATPase activity (0.060 i.u.) with respect to that of the intact vesicles, whereas the unsolubilized fraction contained the remaining 9.8 % (0.019 i.u.). When the solubilized plasma membrane vesicles (290 mg of protein) were loaded on to a 10 ml calmodulin–agarose column and washed with a Ca^{2+}-containing washing buffer, 92.4 % of the proteins were eluted from the column. They include 46.4 % of the Ca^{2+}-ATPase activity (0.030 i.u.). By loading the column with less protein we observed that these values did not depend on the amount of protein applied to the column. When EDTA was present in the buffer instead of Ca^{2+}, the calmodulin-dependent PMCA was eluted in a fraction containing 1.67 mg of protein. The Ca^{2+}-ATPase activity in this fraction reached a value of 2.8 i.u., representing an activity yield of 26.4 % with respect to the fraction applied to the column and 17 % with respect to the intact plasma membrane vesicle fraction. The Mg^{2+}-ATPase, the other enzyme contributing to the total specific activity, was present during the isolation procedure. However, it was completely removed during the affinity chromatography step.

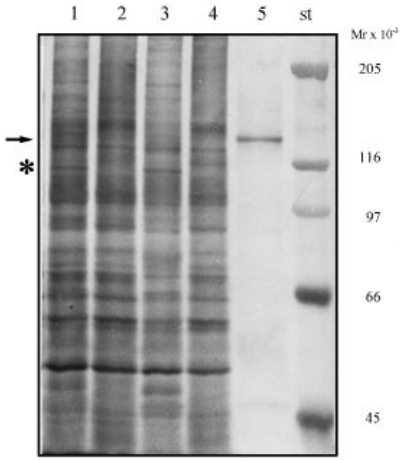

Figure 1 SDS/PAGE of the PMCA purification from plasma membrane vesicles

SDS/PAGE was performed according to Laemmli [11] in a 6.5% gel. The protein bands were stained with silver stain. Samples of 40 μg (lanes 1–4) or 30 μg (lane 5) of protein were loaded as follows: intact plasma membrane vesicles, lane 1; solubilized plasma membrane vesicles, lane 2; unsolubilized plasma membrane vesicles, lane 3; peak eluted with the Ca^{2+} buffer, lane 4; peak eluted with the EDTA buffer, lane 5; relative-molecular-mass standards, lane st. The results of a typical purification are shown. Similar results were obtained in at least four other purifications.

Figure 1 shows an SDS/polyacrylamide gel corresponding to the fractions shown in Table 1. By using the silver staining procedure a large number of proteins in all the fractions prior to the EDTA-elution in the affinity chromatography column can be seen. The PMCA band (arrow) can be observed in the intact plasma membrane vesicle fraction (lane 1). Among the contaminating proteins, there is a band around M_r 110000 (asterisk). Interestingly, this protein is absent in the Triton X-100-solubilized vesicles (lane 3). In fact, Figure 1 (lane 3) shows a Ca^{2+}-ATPase activity that can be selectively inhibited by thapsigargin or cyclopiazonic acid (results not shown). This confirms the presence

Table 1 Purification of the plasma membrane Ca^{2+}-ATPase from pig brain

Fraction	Total protein (mg)	Protein yield (%)	Total Ca^{2+}-ATPase activity (μmol · min^{-1})	Activity yield (%)	Specific (Ca^{2+}-Mg^{2+})-ATPase activity (μmol · mg^{-1} · min^{-1})	Specific Ca^{2+}-ATPase activity (μmol · mg^{-1} · min^{-1})
Plasma membrane vesicles (P-IV)	432	100	27.2	100	0.156	0.063
Triton X-100 unsolubilized vesicles	140	32.4	2.7	9.8	0.128[a,b]	0.019
Triton X-100 solubilized vesicles	290	67.1	17.4	63.9	1.105[a,b]	0.060
Peak eluted with Ca^{2+} buffer	268	62.0	8.0	64.2	0.072[a,b]	0.030
Peak eluted with EDTA buffer	1.67	0.39	4.6	17.0	2.77[b]	0

[a] The specific activity was measured without removal of Triton X-100

[b] The specific activity was measured in the presence of 0.06% Triton X-100 and phosphatidylcholine II-S (40 μg/ml).

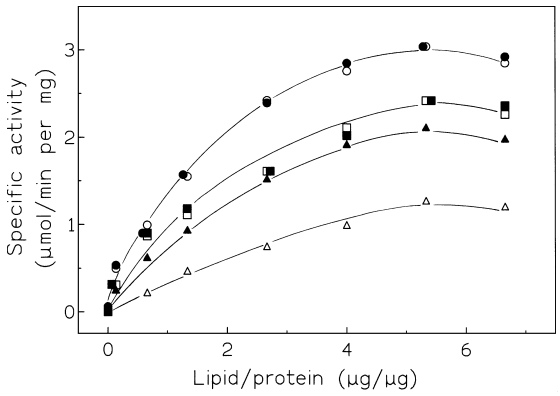

Figure 2 Effect of phospholipid on the Ca^{2+}-ATPase activity of the purified protein in the absence and in the presence of calmodulin

Aliquots (7.5 μg) of the purified Ca^{2+}-ATPase were incubated with different concentrations of phosphatidylserine (●, ○), type-II-S phosphatidylcholine (■, □), or pure phosphatidylcholine (▲, △), at the indicated ratios. The activity was measured as described in the Materials and methods section, in the absence (open symbols) or in the presence (closed symbols) of 0.42 μg of calmodulin. These results represent means of duplicates from a single experiment. Additional experiments gave similar values.

of a SERCA-type ATPase in this fraction. The calmodulin affinity chromatography was very efficient, because most of the contaminant proteins were eluted by the Ca^{2+} buffer (Figure 1, lane 4). The fraction that remained tightly bound to calmodulin was eluted by EDTA buffer and showed a single band in the SDS gel around M_r 140000 (lane 5). This value is similar to that reported for the rat synaptic PMCA [5] and that of erythrocytes [7].

After elution from the column in the absence of phospholipid the enzyme does not show Ca^{2+}-ATPase activity, but the protein could be reactivated in the presence of phospholipid. The effect of different phospholipids on the enzymic activity of the purified enzyme is shown in Figure 2. This activation is higher in the presence of phosphatidylserine (up to 3.04 i.u.) than in the presence of type-II-S phosphatidylcholine (a mixture of phosphatidylcholine and other phospholipids) or pure phosphatidylcholine. Moreover, calmodulin was not able to increase further the activity of the protein incubated in the presence of phosphatidylserine or type-II-S phosphatidylcholine, but it could further stimulate (up to 1.7-fold) the enzymic activity in the presence of pure phosphatidylcholine. The effect of calmodulin required the preincubation with Ca^{2+} before addition to the reaction medium. We should note that type-II-S phosphatidylcholine was used in all the routine assay activity experiments performed due to the efficient activating effect and the low economical cost of the phospholipid. In any case, the maximum activation of the enzyme was achieved when the purified protein was incubated with a phospholipid concentration of 40 μg/ml, equivalent to 5.33 mg of lipid/mg of protein. Higher amounts of phospholipid give rise to a decrease in the activation effect.

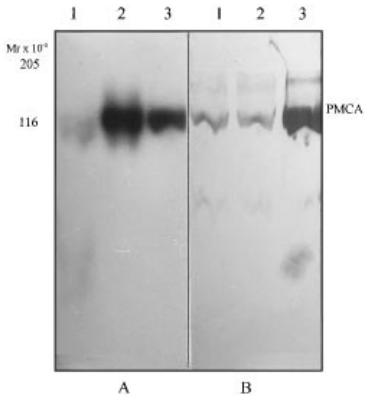

Figure 3 Phosphoprotein intermediates and their immunocharacterization

(**A**) The autoradiogram shows the phosphorylated proteins under the following conditions: plasma membrane vesicles, $-La^{3+}/+Mg^{2+}$ (lane 1); plasma membrane vesicles, $+La^{3+}$ (lane 2); purified enzyme, $+La^{3+}$ (lane 3). (**B**) The immunostaining of the semi-dry blot after autoradiography was performed in the presence of 2H9 anti-PMCA monoclonal antibody from pig antrum smooth muscle (1/1000 dilution). The results of a typical experiment are shown. Similar results were obtained in at least five more experiments, using different preparations.

The plasma membrane vesicles and the purified enzyme were subjected to phosphorylation in the presence of radioactive ATP in order to identify the PMCA protein (Figure 3). When the plasma membrane vesicles were phosphorylated in the absence of La^{3+} the autoradiogram shows the presence of a faint band at around M_r 110000 (lane 1 in Figure 3A). The presence of La^{3+} in the reaction mixture produces the appearance of a broad band in the range of M_r 110000–140000 (lane 2). However, by using the purified enzyme, the phosphorylation reaction in the presence of La^{3+} gives rise to a phosphoprotein around M_r 140000 (lane 3). This band can be clearly recognized by the 2H9 monoclonal antibody raised against the pig antrum PMCA [16] in the plasma membrane vesicles (lanes 1 and 2 in Figure 3B) and in the purified enzyme (lane 3 in Figure 3B). Similar results were obtained by using a polyclonal antibody raised against the purified protein (results not shown).

DISCUSSION

The fact that the PMCA is present in the membrane at very low amounts, about 0.1 % of the total protein [2], and the unstable character of the enzyme in the solubilized state, made the purification of the pump a difficult task. The main aim in the present work has been to establish a fast and economical procedure for the enzyme purification from pig brain, using calmodulin affinity chromatography. This is an easy and powerful way to selectively purify the PMCA from different origins. We initially tried different established methods for the purification of the PMCA from rat brain [5] or human erythrocytes [7,8]. These methods involved the addition of phospholipid to the solubilized vesicles and during the affinity chromatography step. However, the final protein content and the enzymic activity yields were very low in our preparations. In some cases, the enzyme was even not retained by the column. One possible explanation is that the lipid composition of the pig brain membrane could be different from that of rat brain. In fact the lipid composition is different in rat and mouse brain [17] and in erythrocytes [18].

The synaptic plasma membrane vesicles were prepared using a modification of the Michaelis et al. [9] procedure, in order to improve the yield and the specific activity of the ATPase from pig brain. Thus, we have prepared synaptosomes using a 20 %/40 % discontinuous sucrose gradient. Moreover, the osmotic lysis of synaptosomes to prepare plasma membrane vesicles was performed in a buffered medium containing Hepes, at pH 7.4, EDTA and 2-mercaptoethanol instead of water as a lysis buffer, to stabilize the proteins during the process.

The optimum enzyme solubilization in the plasma membrane vesicles was achieved by using a 0.6 % Triton X-100 concentration. The insoluble material also showed a Ca^{2+}-ATPase

activity, probably of the SERCA type, since the gels showed a band at approximately M_r 110000 as in the intact vesicles. This band is not present in the solubilized vesicles fraction. The phosphorylation of the intact vesicles showed a broad band that could be due to both the PMCA and the SERCA, whereas the purified protein showed a narrow band at M_r 140000. Higher Triton X-100 concentrations led to a decrease in the Ca^{2+}-ATPase activity in the solubilized vesicles. The solubilization treatment, under the conditions described here, was highly selective, allowing the recovery of the PMCA without contamination of the SERCA type.

The use of phospholipid, mainly phosphatidylserine, during the solubilization and purification of the PMCA from pig brain as done with other PMCA proteins [5,7,8], is very expensive. In addition, the yield of this method is low and therefore, it is necessary to repeat the preparation several times to get enough pure protein. A critical feature of the present method is that all the buffers used during the solubilization of the plasma membrane and the calmodulin affinity chromatography contained glycerol instead of phospholipid as stabilizing agent. Glycerol has been previously used to purify the PMCA from erythrocyte and smooth muscle [19–21] due to the following advantages: (a) the manipulation is easier and less costly than the use of phospholipids; (b) although the enzyme eluted from the column is inactive, it can be stored in the elution buffer at -70 °C and can be fully reactivated by phospholipid incubation at any time for at least 1 month; (c) the delipidated form of the enzyme allows further lipid studies to be undertaken very easily and also reconstitution with different phospholipids.

Phospholipids are able to stimulate the enzyme from pig brain as observed in erythrocytes [22]. When calmodulin was added to the purified enzyme in the presence of phosphatidylcholine the increase in activity (66%, 1.7-fold) was lower than that observed for other PMCA proteins. This is consistent with a lower percentage (7%) of acidic phospholipids such as phosphatidylserine in erythrocytes [18] compared with the 13–18% in brain [17]. Therefore, the enzyme from brain could be more activated by phospholipids in its native environment than the enzyme from erythrocytes. It cannot be ruled out that the PMCA retains a certain amount of acidic phospholipid as an annulus. In this case, the presence of endogenous phospholipid hinders the activation effect of calmodulin. The plasma membrane synaptic vesicles showed a 14% activation (1.14-fold) of the enzyme by calmodulin, whereas the erythrocyte membranes and other plasma membranes showed a higher activation [21,22]. This effect can also be explained by the membrane lipid composition [17] or by the isoform diversity of the enzyme [23].

When the synaptic plasma membrane vesicles are solubilized in the presence of glycerol, and no phospholipid is added after solubilization, the enzyme is in a delipidated state. It was observed in erythrocyte samples, by using a similar protocol [21], that the Ca^{2+}-ATPase protein presents a different tryptic pattern before and after solubilization. This fact was attributed to a possible rearrangement of the delipidated protein that did not affect the calmodulin-binding site [21]. According to this explanation we can assume that the PMCA present in the solubilized material that we applied to the calmodulin column could possibly have a different structure to that in the intact vesicles. However, the putative structural rearrangement of the delipidated protein did not affect the calmodulin-binding region since the lipid-free protein could still bind efficiently to the calmodulin column and be eluted by EDTA. Moreover, the phosphorylation and immunoblotting data, along with the activating effect shown by phospholipid and calmodulin in the presence of phosphatidylcholine, indicate that the purified protein retains the main characteristics of the PMCA protein family.

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