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The N-terminal region of the plasma membrane Ca^{2+} pump does not separate from the main catalytic fragments after proteolysis

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Abstract

The purified plasma membrane Ca^{2+} pump (PMCA) was digested with trypsin, and the proteolytic products were identified by immunoblotting with monoclonal antibodies JA9 or 5F10 directed against the extreme N-terminal segment and the central portion of the molecule, respectively. After a short treatment with low concentrations of the protease, JA9 reacted predominantly with a peptide of 35 kDa whereas 5F10 detected a peptide of 90 kDa. The trypsin cut leading to the production of these fragments had no effect on the maximal activity of the enzyme. At higher concentrations of trypsin, JA9 detected a main fragment of 33 kDa and smaller fragments of 19 and 15 kDa. The persistence of fragments reacting with JA9 indicates that the N-terminal region containing its epitope (residues 51–75) was not easily accessible to the protease in the native PMCA. However, the reactivity with JA9 was rapidly lost during proteolysis of the denatured protein. The passage of the mixture of PMCA fragments through a calmodulin-Sepharose column resulted in the retention of the N-terminal 35 kDa fragment together with that of 90 kDa, despite the fact that only the latter binds calmodulin. The ethylenediaminetetraacetic acid (EDTA) eluate, which contained about equal amounts of both fragments, had a Ca^{2+} ATPase activity similar to that of the intact enzyme. The tight association between the two peptides was evidenced by the fact that concentrations of polyoxyethylene 10 lauryl ether ($C_{12}E_{10}$), sodium dodecyl sulfate (SDS) high enough for inactivating the enzyme and dissociate the pump from calmodulin were unable of breaking the interaction between the 35 and 90 kDa fragments. Altogether, these results show that after digestion with trypsin, the N-terminal portion of the PMCA, including the extreme N-terminal segment, remains part of a fully functional catalytic complex.

Keywords: Plasma membrane Ca^{2+} pump; Ca^{2+} pump; Ca^{2+} ATPase; Limited proteolysis

1. Introduction

The calcium transporter of plasma membranes (plasma membrane Ca^{2+} pump (PMCA)) is a P-type ATPase which is essential for control of the intracellular Ca^{2+} concentration. The PMCA consists of a single polypeptide of about 134 kDa which would transverse 10 times the membrane bilayer (transmembrane segments M1–M10) [1]. It has been reported that a PMCA mutant of 105 kDa truncated at its N-terminus is inactive [2]. Furthermore, we have shown that deletion of residues 19–75 from the extreme N-terminal segment suffices for inactivation [3]. These findings suggest that the N-terminal

Abbreviations: PMCA, plasma membrane Ca^{2+} pump; $C_{12}E_{10}$, polyoxyethylene 10 lauryl ether; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate

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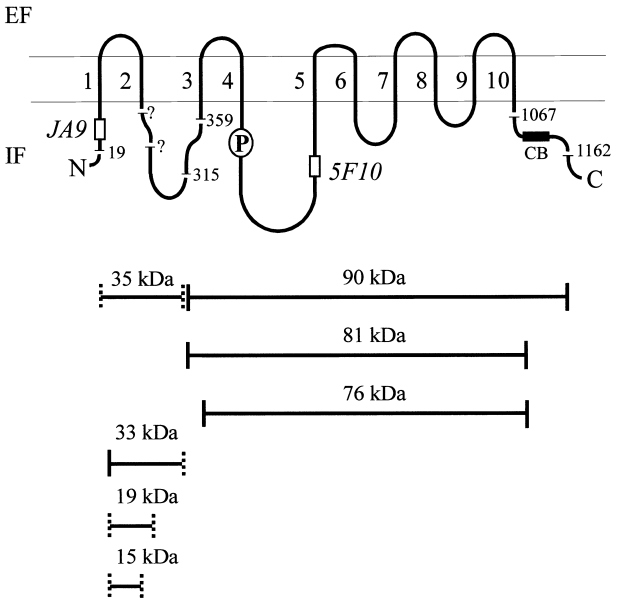

Fig. 1. Schematic representation of the hPMCA4 with the location of the point of cleavage by trypsin. The N-terminal positions of the trypsin cuts leading to the main proteolytic fragments are indicated according to [7]. The proposed location of additional cuts in the N-terminal domain is denoted by question marks. The epitopes of antibodies 5F10 and JA9 are represented by open boxes. Transmembrane segments are numbered 1–10; P, site of the aspartylphosphate formation; CB, calmodulin binding site; N, N-terminus; C, C-terminus; IF, intracellular face; EF, extracellular face. The lower part of the figure shows the extension and the size of the proteolytic fragments. A dashed line at the start or at the end of the fragment indicates that the terminal sequence of the fragment is not known.

region of the pump is needed for a functional enzyme. However, they are in contrast with the results of earlier studies of limited proteolysis of the PMCA which have suggested that PMCA peptides lacking the N-terminal portion of the pump are fully functional [4–6].

The trypsin cuts leading to the major proteolytic products of the hPMCA4b, the human isoform 4b, have previously been mapped [7] (Fig. 1). A peptide of 90 kDa was shown to be produced by attack at the N-terminal region (arginine 314-threonine 315) and at the C-terminal region (lysine 1161-phenylalanine 1162). Thus, this fragment contains the central portion of the molecule including part of the autoinhibitory domain involved in calmodulin binding. The subsequent degradation of the 90 kDa peptide produces fragments of 81 kDa and 76 kDa which have lost the C-terminal calmodulin binding site. In addition, two tryptic N-terminal fragments of 35 kDa and 33.5 kDa probably containing residues 1–314 and 19–314 were described [7]. These fragments include part of the region between transmembrane segments M2 and M3 which is highly conserved in all P-type ATPases.

In the present study, we examined the effect of trypsin on the N-terminal region of the PMCA. We took advantage of antibody JA9 that reacts with residues 51–75 of the hPMCA4 [8], allowing us to specifically follow the proteolytic processing of the N-terminal region of the protein. In agreement with previous studies [4–6,9], we found that the first cytosolic loop of the PMCA was very sensitive to proteolysis and the trypsin cleavage in this region led to the rapid accumulation of a 35 kDa peptide containing the N-terminal portion of the pump. This peptide, which included the extreme N-terminal segment, was relatively resistant to proteolysis and remained tightly associated to the main fragment of 90 kDa in a fully functional catalytic complex.

2. Materials and methods

Trypsin (T-8003), aprotinin, leupeptin, ATP, calmodulin, calmodulin-Sepharose, asolectin (P-3644), sodium dodecyl sulfate (SDS), polyoxyethylene 10 lauryl ether ($C_{12}E_{10}$) and other reagents were from Sigma. [γ-^{32}P]ATP was from New England Nuclear. Immobilon-P PVDF membranes were from Millipore. Immunochemicals were from Vector Labs.

The PMCA was purified from human red cell membranes deficient in calmodulin as described previously [10] except the use of $C_{12}E_{10}$ instead of Triton X-100.

Limited proteolysis of the purified PMCA was carried out at 4°C, in a reaction medium containing 20 mM Tris–HCl (pH 7.5 at 4°C), 0.5 mM EGTA, 0.05% $C_{12}E_{10}$, 0.1% asolectin, 50 ng/μl PMCA and either 2.5 ng/μl trypsin (trypsin:PMCA ratio of 1:20 w/w) or the trypsin needed to attain the ratio of trypsin:PMCA indicated in each experiment. The reaction was arrested by the addition of 20 ng/μl aprotinin and 5 ng/μl leupeptin. Controls were carried out without adding trypsin. When indicated, the PMCA was denatured by heating at 100°C for 3 min and rapidly cooled on ice before the addition of trypsin.

SDS–polyacrylamide gel electrophoresis and immunoblotting were performed as described previously [11]. Proteins were electrophoresed at 100 V for approximately 1.5 h on discontinuous 5% stacking gel and either 7.5% or 12.5% separating gels according to Laemmli [12]. Electrophoretic transfer of the proteins from the gel to Millipore Immobilon-P membranes was done as described previously [11] at a constant current of 250 mA for 1 h. Non-specific binding was blocked by incubating the membranes overnight at 4°C in a solution of 160 mM NaCl, 0.05% Tween-20 and 1% non-fat dry milk. Then the membranes were incubated with mouse ascitic fluid containing monoclonal antibody JA9 (dilution 1:1000) or 5F10 (dilution 1:2000). A biotinylated anti-mouse immunoglobulin G was used as second antibody, and avidin–horseradish peroxidase conjugate was used for detection. Staining was developed in 8.1 mM Na_2HPO_4, 2.9 mM KH_2PO_4, 136 mM NaCl, 0.003% H_2O_2, 5% $NiCl_2$ and 0.01% 3,3′-diaminobenzidine.

Calmodulin affinity chromatography of the trypsinized purified PMCA was carried out on 320 μg of PMCA digested at 4°C for 15 min at a trypsin:PMCA ratio 1:20 (w/w) in 6.4 ml of the standard proteolysis media. The reaction was stopped with 5 ml of a solution containing 20 μg/ml aprotinin, 5 μg/ml leupeptin and 0.1 mM phenylmethylsulfonyl fluoride, and diluted to 50 ml with binding buffer containing 130 mM KCl, 20 mM MOPS-K (pH 7.4 at 4°C), 1 mM $MgCl_2$, 100 μM $CaCl_2$, 0.05% $C_{12}E_{10}$, 0.1% asolectin, 2 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. The mixture was passed through a calmodulin affinity column of approximately 3 ml bed volume. After washing with 100 ml of binding buffer, the column was eluted with the same buffer except that $CaCl_2$ was omitted and 2 mM ethylenediaminetetraacetic acid (EDTA) was added. Successive eluate fractions of 250 μl were collected and the protein concentration in each fraction was determined by the Bio-Rad Protein Assay based on the method of Bradford [13]. Fractions 8 and 9 containing most of the protein were pooled.

For the study of the effects of detergents on the stability of the 35–90 kDa complex, 1 μg of trypsinized PMCA was bound to calmodulin-Sepharose (30 μl of bed volume) by incubation at 4°C for 45 min in binding buffer and then washed three times with 1 ml of binding buffer plus detergent and once more with 1 ml of binding buffer. The protein that remained bound was eluted with 50 μl of EDTA.

The phosphorylation reaction was carried out at 4°C in a medium containing 200 ng of PMCA protein, 25 mM Tris–HCl (pH 7.2 at 4°C), 50 μM $CaCl_2$, 50 μM $LaCl_3$ and different amounts of $C_{12}E_{10}$ and asolectin in a total volume of 250 μl. The reaction was initiated by adding [γ-^{32}P]ATP to a final concentration of 1 μM and terminated after 30 s with 15 μl of 100% trichloroacetic acid. After adding 30 μl of 1 mg/ml bovine serum albumin, the denatured proteins were collected by centrifugation at 5000×*g* for 20 min, washed twice with 1 ml of 7% trichloroacetic acid and once with distilled water. The samples were dissolved in 20 μl of buffer loading containing 62.5 mM Tris–HCl (pH 6.8 at 30°C), 2% SDS, 10% glycerol, 5 mM EDTA, 10 mM dithiothreitol, 125 mg/ml urea and 0.02% w/v bromophenol blue. The proteins were separated by SDS electrophoresis at pH 6.5 in a 7% polyacrylamide gel [5]. The gel was fixed for 30 min in 50% methanol, 10% acetic acid, dried and exposed to X-ray film for 24–72 h at −70°C.

The Ca^{2+} ATPase activity of the purified PMCA was measured by monitoring the [^{32}P]P_i liberated from [γ-^{32}P]ATP [14]. The reaction was carried out at 37°C for 20 min in 300 μl of a reaction medium containing 0.8 μg of protein from either intact or trypsin-digested PMCA, 50 mM Tris–HCl (pH 7.4 at 37°C), 100 mM KCl, 230 μM EDTA, 370 μM EGTA, 5 mM $MgCl_2$, 240 nM calmodulin, 70 μg/ml $C_{12}E_{10}$, 117 μg/ml asolectin, 3 mM ATP and 0.7 mM $CaCl_2$ (free [Ca^{2+}] 90 μM). Under these conditions, the [^{32}P]P_i liberated from [γ-^{32}P]ATP increased linearly with time up to 30 min.

3. Results

Fig. 2A shows the effect of digesting the purified PMCA with trypsin (trypsin:PMCA ratio of 1:20 w/w) at 4°C for increasing times. The peptides produced were separated by SDS–polyacrylamide gel electrophoresis and identified by immunoblotting with antibodies 5F10 and JA9. Antibody 5F10 reacts between residues 719 and 738 while antibody JA9 reacts between residues 51 and 75 near the N-termi-

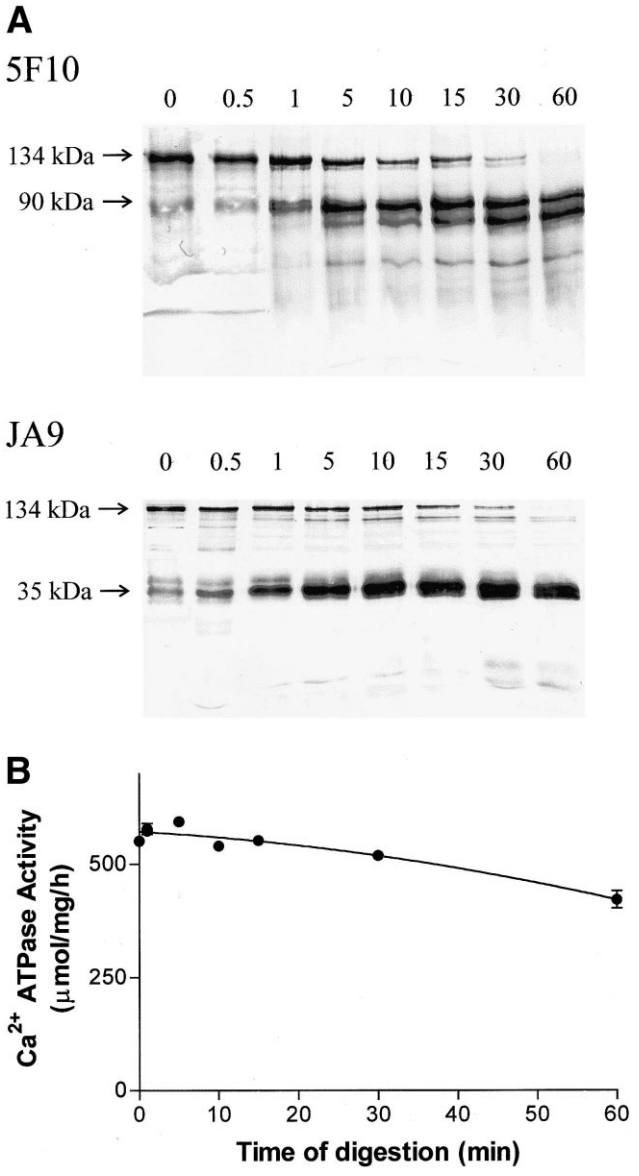

Fig. 2. Proteolysis of the purified PMCA by trypsin. A: Immunoblots of native and trypsin-digested PMCA. Digestion was carried out at 4°C at a trypsin:PMCA ratio of 1:20 (w/w). The numbers on top of each lane indicate the duration in minutes of proteolysis. 95 ng of the PMCA protein was loaded per lane and submitted to electrophoresis in a 7.5% (top) or 12.5% (bottom) SDS–polyacrylamide gel and the peptides were identified by using monoclonal antibodies 5F10 or JA9. B: Effect of trypsin digestion on the Ca^{2+} ATPase activity. The Ca^{2+} ATPase activity of the trypsinized enzyme was measured at 37°C in the presence of 240 nM calmodulin as described in Section 2.

nus of hPMCA4b [8]. Even before trypsin was added, antibody 5F10 detected a small amount of a 90 kDa fragment in addition to the 134 kDa band corresponding to the intact PMCA, whereas JA9 detected the intact protein and a triplet of bands of approximately 37, 35 and 33 kDa. As the trypsin digestion progressed, the amount of intact protein decreased and the bands of 90 kDa and 35 kDa became more intense. The amount of 90 kDa fragment peaked at about 10–15 min and then began to drop concomitantly with the increase of the intensity of major fragments of 81–76 kDa which have previously been described [4–7,9]. Similarly, the band of 35 kDa reached its maximum intensity at about 10 min of digestion but then it remained relatively stable and only showed signs of degradation at long diges-

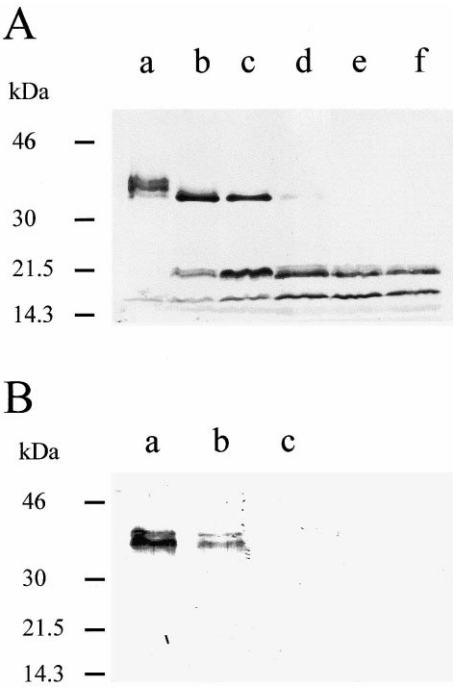

Fig. 3. N-terminal fragments produced by digestion of the native and denatured PMCA with trypsin. Immunoblots with antibody JA9 of the fragments at 4°C with increasing concentrations of trypsin or during different times. 50 ng of trypsinized PMCA was loaded per lane and submitted to electrophoresis in a 12.5% SDS–polyacrylamide gel. A: The trypsin:PMCA ratio (w/w) and the digestion times were: lane a, 1:20, 15 min; lane b, 1:2, 15 min; lane c, 1:0.2, 15 min; lane d, 1:0.1, 15 min; lane e, 1:0.1, 20 min; lane f, 1:0.1, 30 min. B: The PMCA was denatured by heating for 3 min at 100°C and then digested at 4°C with trypsin (trypsin:PMCA ratio w/w, 1:2) for different times. Lane a, 0.5 min; lane b, 1 min; lane c, 10 min.

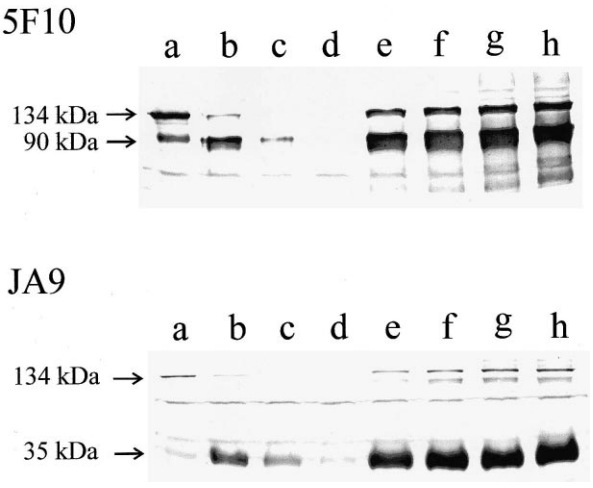

Fig. 4. Co-purification of the 90 kDa and 35 kDa proteolytic fragments by calmodulin affinity chromatography. 320 μg of purified PMCA was digested at 4°C for 15 min under the standard conditions described in Fig. 1. The trypsinized PMCA was diluted to 50 ml with binding buffer containing 100 μM $CaCl_2$ and passed through a calmodulin affinity column. The column was washed with more of the binding buffer and eluted with 2 mM EDTA. Fractions of the sample at each step of the purification were submitted to electrophoresis in a 7.5% (top) or 12.5% (bottom) SDS–polyacrylamide gel and developed using antibodies 5F10 or JA9, respectively. Lanes a, 100 ng of native PMCA; b, 15 μl of diluted proteolysed PMCA (~100 ng); c, 15 μl of the proteolysis mix after passing through the column; d, 15 μl of the Ca^{2+} wash; lanes e, f, g and h, 100, 150, 200 and 300 ng of protein from the EDTA eluate.

tion times. As shown in Fig. 2B, the maximal Ca^{2+} ATPase activity of the PMCA measured in the presence of saturating amounts of calmodulin was minimally affected by the trypsin cleavage, decreasing about 20% after 60 min. Thus, the Ca^{2+} ATPase activity of the PMCA was preserved despite the cleavage of the N-terminal portion of the molecule.

The effect of higher concentrations of trypsin on the N-terminal region of the PMCA was analyzed in more detail. As shown in Fig. 3A, increasing the concentration of the protease led to the degradation of the 37 kDa and the 35 kDa peptides and the accumulation of that of 33 kDa. At even higher concentrations of trypsin, the fragment of 33 kDa was also digested to smaller peptides of approximately 19 and 15 kDa. The strong intensity of the bands suggests that the epitope for JA9 was mostly preserved even under extensive proteolysis. However, the small tryptic fragments reacting with JA9 were rapidly lost if before proteolysis the PMCA was heated at 100°C for 3 min (Fig. 3B).

The effective separation of the cleaved N-terminal region of the pump from the main catalytic fragments was investigated. The PMCA was digested with trypsin for 15 min and after diluting the mixture 10 times in a solution containing Ca^{2+}, phospholipids and detergent, it was passed through a calmodulin column, washed with 100 ml of the same solution and finally eluted with EDTA. Immunoblots in Fig. 4 show that after the treatment with trypsin, the fragments of 35 and 90 kDa were the predominant products. Only a small amount of the 90 kDa fragment was detected after passing the proteolytic mixture through the calmodulin column, indicating that, as expected, this peptide remained bound to the calmodulin-Sepharose. However, the 35 kDa fragment was also retained despite the fact that it does not bind calmodulin [1]. Both fragments were recovered in the EDTA eluate together with some intact PMCA. This result indicates that the disruption of the covalent linkage by trypsin does not suffice for the separation of the N-terminal region of the PMCA from the rest of the molecule.

The functional state of the peptides obtained after calmodulin affinity chromatography of the trypsinized PMCA was evaluated by measuring the Ca^{2+} ATPase activity and the formation of the phosphorylated intermediate. The Ca^{2+} dependent ATP hy-

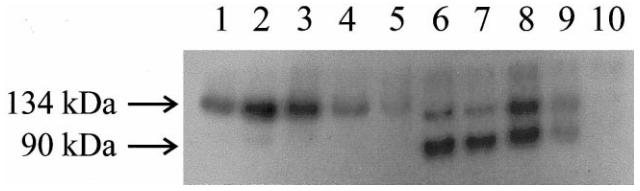

Fig. 5. Formation of the phosphorylated intermediate by the native and the trypsinized PMCA after calmodulin affinity chromatography at different concentrations of $C_{12}E_{10}$. 200 ng of protein was phosphorylated for 30 s on ice in the presence of 50 μM $CaCl_2$, 200 μM $LaCl_3$ and 1 μM [γ-^{32}P]ATP and the indicated amounts of $C_{12}E_{10}$: lanes 1 and 6, 0.001%; lanes 2 and 7, 0.034%; lanes 3 and 8, 0.5%; lanes 4 and 9, 1%; lane 5 and 10, 1.64%. Asolectin was added to the reaction media to maintain a constant ratio of asolectin:$C_{12}E_{10}$ of two. Lanes 1–5, native PMCA; lanes 6–10, calmodulin affinity-purified PMCA fragments. The denatured samples were submitted to electrophoresis in a 7% acidic SDS–polyacrylamide gel, dried and autoradiographed.

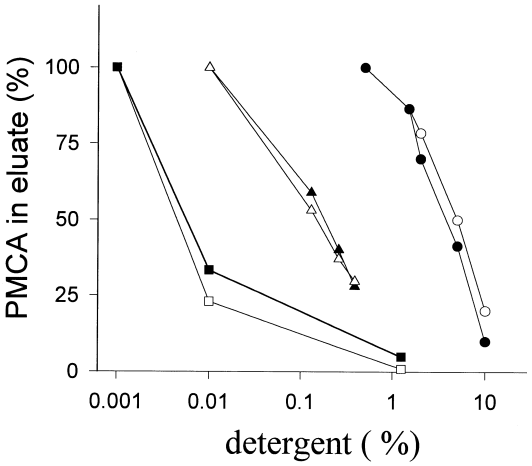

Fig. 6. Effect of detergents on the recovery of the 35–90 kDa PMCA fragments after calmodulin affinity chromatography. The trypsinized PMCA was bound to calmodulin-Sepharose and washed with solutions containing the indicated concentration of detergent as described in Section 2. The amounts of 35 kDa and 90 kDa peptides recovered in the EDTA eluate were estimated by measuring the intensity of the bands after immunoblotting with JA9 or 5F10. The intensity of the 35 and 90 kDa bands when the washing solution contained no detergent was taken as 100%. Closed symbols, 90 kDa fragment; open symbols, 35 kDa fragment. Detergent present in the washing solution: circles, $C_{12}E_{10}$; triangles, sodium deoxycholate; squares, SDS.

drolysis of the trypsinized PMCA before and after passing the proteolytic mixture through the calmodulin-Sepharose column was 529 μmol/mg/h and 495 μmol/mg/h, respectively. In addition, as shown in Fig. 5, the incubation of the tryptic peptides present in the EDTA eluate with ATP in the presence of Ca^{2+}+La^{3+} resulted in the appearance of two phosphorylated bands in SDS–polyacrylamide gels, corresponding to intact PMCA and to the 90 kDa fragment. These results indicate that the 35–90 kDa peptides present in the EDTA eluate were functionally competent. In addition, Fig. 5 shows the effect of increasing concentrations of the detergent $C_{12}E_{10}$ on the formation of phosphoenzyme by the proteolysed mixture and the intact pump. The level of phosphoenzyme did not significantly change by increasing the concentration of detergent up to 0.5%. However, at higher concentrations of $C_{12}E_{10}$, both bands of 134 and 90 kDa decreased in intensity, suggesting that high concentrations of $C_{12}E_{10}$ were inactivating the enzyme by a process not related to the separation of the 35 kDa and the 90 kDa peptides.

The interaction of the 35 and 90 kDa fragments was investigated further by analyzing the composition of the EDTA eluate obtained after binding the peptides to calmodulin-Sepharose and washing with increasing concentrations of different detergents. Results in Fig. 6 show that as the concentration of $C_{12}E_{10}$, deoxycholate or SDS in the washing solution increased, lower amounts of the fragments were recovered in the EDTA eluate, indicating that at high concentrations, these detergents disrupted the interaction of the pump with calmodulin. However, because the intensity of the 35 kDa peptide decreased in parallel with that of the 90 kDa, apparently none of the detergents was able to specifically break the association between these fragments.

4. Discussion

In this study, we used monoclonal antibodies to follow the tryptic degradation of the N-terminal portion of the pump. This allowed us to unequivocally identify the N-terminal fragments of the PMCA, and avoided the uncertainties of earlier studies that used non-specific protein stains for the detection of the proteolytic peptides.

In agreement with previous studies [4–7,9], we found that a short exposure of the pump to trypsin results in the cleavage of a 35 kDa peptide. This fragment, which has been proposed to encompass residues 1–314 [7], was the main N-terminal proteolytic product. A small amount of this peptide and traces of others of 37 and 33 kDa were also detected in undigested preparations of the purified PMCA, together with similar amounts of the 90 kDa fragment. A similar 90 kDa peptide has been observed in undigested preparations of inside-out vesicles from red cell membranes, and its presence was attributed to the degradation of the pump by endogenous proteases [5]. These observations suggest that the region of the PMCA between transmembrane segments M2 and M3 is not only rapidly cleaved by trypsin but is also highly sensitive to endogenous proteolysis. The results presented here indicate that the 35 and 90 kDa fragments are present in purified preparations of PMCA because once produced, they co-purify with the intact pump. Furthermore, they are fully functional despite the covalent break.

In the native enzyme, the proteolytic fragment of 35 kDa containing the N-terminal portion of the pump was relatively stable and only became degraded to smaller fragments at high concentrations of trypsin or prolonged time of digestion, by further proteolysis at its C-terminus. The low sensitivity of the N-terminal segment of the PMCA to digestion by trypsin may reflect structural constrains which make this region less susceptible to the attack of proteases [15]. The results of limited proteolysis have frequently been used to define domain boundaries in proteins. On the basis of the sensibility to proteolysis, the N-terminal domain of the PMCA may be identified with the portion of the molecule containing the two first transmembrane segments, part of the cytosolic loop between M2 and M3 and at least a portion of the N-terminal segment which includes residues 51–75.

It has been reported that the EDTA eluate obtained by a calmodulin affinity purification of a briefly trypsinized PMCA possesses a low level of ATPase activity [4]. This capability of hydrolyzing ATP was attributed either to a small amount of undigested pump present in the eluate [2] or to the 90 kDa peptide [4]. At variance with [4], we found that the ATPase activity of the trypsin-treated PMCA after affinity chromatography was similar to that of the undigested enzyme despite the fact that the 90 kDa fragment was the major PMCA peptide. This result indicates that the remains of intact enzyme cannot account for the ATPase activity of the EDTA eluate. In agreement with this idea, after incubation with Ca^{2+}, La^{3+} and ATP, we detected two phosphorylated bands, one corresponding to the intact pump and the other to the 90 kDa fragment.

We found that the EDTA eluate from the calmodulin affinity column contained not only the 90 kDa fragment and some undigested PMCA but also the N-terminal 35 kDa portion of the molecule, indicating that the two peptides did not separate during the affinity chromatography. The strong interaction between the 35 and 90 kDa fragments was evidenced by the fact that high concentrations of $C_{12}E_{10}$, deoxychyolate or SDS, which disrupted the interaction of the C-terminal domain of the pump with calmodulin, were ineffective for separating the fragments of 35 and 90 kDa. It may be difficult to brake up the tight interaction between these two fragments without compromising the structural integrity of other regions of the protein. The 35 and 90 kDa fragments effectively separate during the SDS–polyacrylamide gel electrophoresis. However, SDS is highly denaturing and concentrations as low as 0.005% have been shown to completely inactivate the PMCA from red blood cell membranes [16], thus making the use of this detergent unsuitable for the isolation of the proteolytic fragments in a functional form.

The studies of PMCA mutants with deletions in the N-terminal region have indicated that this portion of the molecule is essential for a functional enzyme [2,3] and hence it is likely that the 90 kDa is not active by itself. Studies of other closely related P-type pumps have shown that after limited proteolysis, they do not disassemble and are still partially or totally functional [17]. Consistently with this idea, the results presented here demonstrate that the 35 kDa proteolytic peptide containing the N-terminal region of the pump including residues 51–75 remains non-covalently associated to the 90 kDa fragment in a fully functional catalytic complex. A major site of trypsin cleavage has also been located in the M2–M3 connecting segment of the Na^+–K^+ and SERCA pumps (arginine 262 and 198, respectively), which is consistent with the idea that despite the variability of amino acid sequence, the P-type pumps share a similar tridimensional structure [18]. Furthermore, these sites have been shown to be differentially exposed during the conformational changes associated with ion transport [19]. Therefore, the interaction between the N-terminal and the catalytic domain of the pump should be taken into account when analyzing the functional properties of mixtures of proteolytic PMCA fragments.

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