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Replacement of Val^{674} by Pro increases the sensitivity of the plasma membrane Ca^{2+} pump to inhibition by Mg^{2+}

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Abstract

A cDNA encoding a plasma membrane Ca^{2+} pump mutant $V^{674}P$(ct120) was constructed and expressed in COS-1 cells. Immunoblots of transfected COS-1 membranes showed that the $V^{674}P$(ct120) and the wild-type hPMCA4b(ct120) proteins were expressed at similar levels. The change of Val^{674} to Pro reduced the activity of the hPMCA4b(ct120) to an extent similar to that observed previously in the full-length Ca^{2+} pump (Adamo et al. (1995) J. Biol. Chem. 270, 30111–30114). Despite its lower activity, the apparent affinity for Ca^{2+} of the $V^{674}P$(ct120) enzyme was at least as high as that of hPMCA4b(ct120), indicating that substitution of Val^{674} by Pro did not impair the interaction of the enzyme with Ca^{2+}. The sensitivity of the $V^{674}P$(ct120) enzyme to inhibition by vanadate was not significantly different from that of the hPMCA4b(ct120), supporting the idea that the mutation did not alter the equilibrium between E_2–E_1. The study of the Mg^{2+} dependency of the Ca^{2+} transport showed that the $V^{674}P$(ct120) mutant reached maximum activation at 100 μM Mg^{2+} in contrast with 500 μM in the hPMCA4b(ct120). Furthermore, while at 2 mM Mg^{2+} the hPMCA4b(ct120) showed no sign of inhibition, the activity of the mutant decreased to less than 50% of the maximum activity observed at 100 μM Mg^{2+}. These results indicate that the decrease in the activity observed upon substitution of Val^{674} by Pro was due to a higher sensitivity to Mg^{2+} as inhibitor.

Keywords: Calcium ion pump; Ca ATPase

1. Introduction

The plasma membrane Ca^{2+} pump (PMCA), a member of the P-type ion transport ATPases [2] plays a key role in the control of intracellular Ca^{2+}. Val^{674} is part of the conserved region of the P-type ion pumps proposed as 'nucleotide binding domain' [3]. We have previously found that substitution of Val^{674} by Pro causes a substantial reduction in the enzyme's activity, despite the fact that Val^{674} is replaced by Pro in other P-type ion pumps [4]. Since mutant

Abbreviations: SERCA, sarco-endoplasmic reticulum Ca^{2+} pump; hPMCA4b, human plasma membrane Ca^{2+} pump isoform 4b, which has also been called hPMCA4CI [1]; hPMCA4b(ct120), hPMCA4b lacking the C-terminal 120 amino acids; $V^{674}P$, hPMCA4b mutant with Valine 674 replaced by Proline; $V^{674}P$(ct120), $V^{674}P$ lacking the C-terminal 120 amino acids

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$V^{674}P$ is capable or reacting with low concentrations of ATP to form a phosphoenzyme, and concentrations of ATP much higher than those needed to saturate its site in the wild-type, does not overcome the inhibition of the $V^{674}P$ enzyme, the effects of the alteration of Val^{674} to Pro seem unrelated to ATP binding [4].

This study was undertaken with the aim of finding the cause of the lower activity of the enzyme following substitution of Val^{674} by Pro. After knowing that removal of the C-terminal 120 amino acid residues of hPMCA4b results in a mutant enzyme called hPMCA4b(ct120) which has higher activity independently of calmodulin [5], we constructed mutant $V^{674}P$(ct120). We found that the activity of the $V^{674}P$(ct120) enzyme was higher than that of the $V^{674}P$, but still lower than that of hPMCA4b(ct120) thus making the use of $V^{674}P$(ct120) advantageous for studying the effects of the $V^{674}P$ mutation.

2. Materials and methods

2.1. Mutagenesis of the hPMCA4b cDNA and expression in COS-1 cells

The construction of the hPMCA4b full-length cDNA and the mutant $V^{674}P$ were described previously [6,4]. To obtain the $V^{674}P$(ct120) mutant, the 426 bp *Ava*I–*Ava*I fragment was removed from $V^{674}P$ and cloned into h4PMCA(ct120) [5]. The wild-type and mutant cDNAs were cloned into the pMM_2 vector [6]. For protein expression, COS-1 cells [7] were transfected by the DEAE–dextran–chloroquine method [6] and harvested after 48 h. The microsomal fraction was isolated as described previously [4]. Protein concentration was estimated by means of the Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA), with bovine serum albumin as a standard.

2.2. Detection of expressed Ca^{2+} pump protein

SDS-electrophoresis and immunoblotting were carried out as previously described [9]. Proteins were electrophoresed on a 7.5% acrylamide gel according to Laemmli [10] and subsequently transferred to Millipore Immobilon membranes. Non-specific binding was blocked by incubation overnight at 4°C in a solution of 160 mM NaCl, 0.05% Tween-20 and 1% non-fat dry milk. The membranes were incubated at 37°C for 1 h with 5F10 monoclonal antibody from ascitic fluid (dilution 1:1000). For staining, biotinylated anti-mouse immunoglobulin G and avidin–horseradish peroxidase conjugate were used.

To estimate the level of expression of the $V^{674}P$(ct120) Ca^{2+} pump, COS-1 membranes containing the mutant and different amounts of membranes containing the hPMCA4b(ct120) enzyme were loaded in parallel wells of the same gel. The immunoblots were digitally scanned and the intensity of the bands were quantified using the program Sigma Scan (Jandel Sci.).

2.3. Ca^{2+} transport assay

Ca^{2+} uptake assays were performed as described previously [5]. The reaction mixture contained 100 mM KCl, 50 mM Tris-HCl (pH 7.3 at 37°C), 5 mM NaN_3, 400 nM thapsigargin, 20 mM sodium phosphate, 95 μM EGTA, 6 mM ATP and enough $MgCl_2$ and $CaCl_2$ to give the desired concentrations of free Mg^{2+} and free Ca^{2+}. The free concentration of Mg^{2+} and Ca^{2+} were estimated using the program of Fabiato and Fabiato [8]. Unless stated otherwise, free Ca^{2+} was 1 μM and free Mg^{2+} 0.8 mM. Vesicles (5–10 μg of protein) were preincubated at 37°C for 5 min and the reaction initiated by the addition of ATP. The reaction was terminated after 5 min by filtering the samples through a 0.45-μm filter. The ^{45}Ca taken up by the vesicles was then determined by counting in a liquid scintillation counter. The activity of the expressed Ca^{2+} pump was estimated after subtracting the activity of the endogenous enzyme from COS-1 cells transfected with the empty plasmid pMM_2 and corrected by the variability in expression in each membrane preparation.

2.4. Dephosphorylation

For the dephosphorylation experiments, 10 μg of microsomal protein were phosphorylated at 4°C for 30 s in 0.25 ml of a mixture of 100 mM KCl, 25 mM Tris-HCl (pH 7.4 at 4°C), 400 nM thapsigargin, 0.02 mM $CaCl_2$ and 5 mM $MgCl_2$ and 1 μM [γ-^{32}P]ATP, and the reaction was then interrupted by the addition

of a chase solution containing cold ATP to give a final concentration of 0.6 mM. After 5, 10 or 15 s the dephosphorylation was terminated by adding 1 ml of 10% trichloroacetic acid, 10 mM P_i and 1 mM cold ATP (stopping solution) at 4°C. After adding 50 μl of 1 mg/ml of bovine serum albumin the denatured proteins were collected by centrifugation at 20 000 × *g* for 10 min, washed once more with stopping solution and once with distilled water. Labeled proteins were separated by SDS-electrophoresis in a 7% acrylamide gel according to Sarkadi [11]. After drying the gel, autoradiographs were produced.

3. Results

The immunoblot in Fig. 1 shows that the V^{674}P(ct120) Ca^{2+} pump could readily be detected in transfected COS-1 cell membranes as a single band with the expected migration according to its molecular mass. To compare the expression of the V^{674}P(ct120) protein with that of hPMCA4b(ct120), a standard curve was constructed by plotting the intensity of the hPMCA4b(ct120) as a function of the amount of membrane protein loaded in each lane. Using this plot the intensity of the V^{674}P(ct120) from 2 μg of membranes was similar to that of hPMCA4b(ct120) from 1.7 μg of membranes, indicating that the expression of the V^{674}P(ct120) enzyme was about 85% of that of hPMCA4b(ct120). This value compares satisfactorily with that of 83% obtained previously by means of enzyme-linked immunosorbent assay [4] for the expression of the V^{674}P mutant. These results indicate that mutants containing the substitution V^{674}P are expressed at levels close to that of the wild-type Ca^{2+} pump.

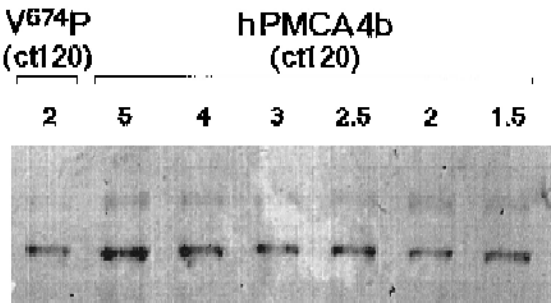

Fig. 1. Immunoblot of wild-type hPMCA4b(ct120) and V^{674}P(ct120) mutant Ca pumps expressed in COS-1 cells. Monoclonal antibody 5F10 was used. The number on top of each lane indicates μg of membrane protein loaded.

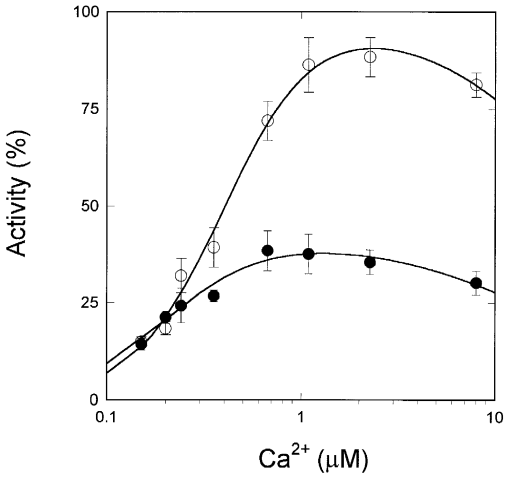

Fig. 2. Ca^{2+} dependency of Ca^{2+} transport in the hPMCA4b(ct120) and V^{674}P(ct120) Ca^{2+} pumps. The initial rates of Ca^{2+} uptake were measured as described in Section 2 in the presence of various concentrations of $CaCl_2$ to give the free Ca^{2+} indicated in the figure and 0.8 mM Mg^{2+}. The maximal activity of hPMCA4b(ct120) was taken as 100%. ○, hPMCA4b(ct120); ●, V^{674}P(ct120).

The ability of the V^{674}P(ct120) enzyme to pump Ca^{2+} was compared to that of the hPMCA4b(ct120) by measuring the rate of Ca^{2+} uptake by microsomal vesicles derived from transfected COS-1 cells. The activity of the V^{674}P(ct120) mutant was about 40% of that of the hPMCA4b(ct120), indicating that the inhibitory effect of the V^{674}P mutation previously observed on the full-length hPMCA4b [4], was not altered by the C-terminal truncation. This result supports the idea that the effects of the alteration of Val^{674} are not related to the regulation of the enzyme by calmodulin.

Because the activity of the V^{674}P(ct120) enzyme was higher than that of the V^{674}P, the former construct was used in the experiments that follow.

The dependence of the Ca^{2+} uptake by hPMCA4b(ct120) and V^{674}P(ct120) on the Ca^{2+} concentration is shown in Fig. 2. The response of the hPMCA4b(ct120) enzyme to Ca^{2+} was biphasic, the rate of Ca^{2+} uptake increased along a sigmoidal curve with $K_{1/2}$ 0.4 μM, reached a maximum at about 1 μM and slightly decreased at higher Ca^{2+}

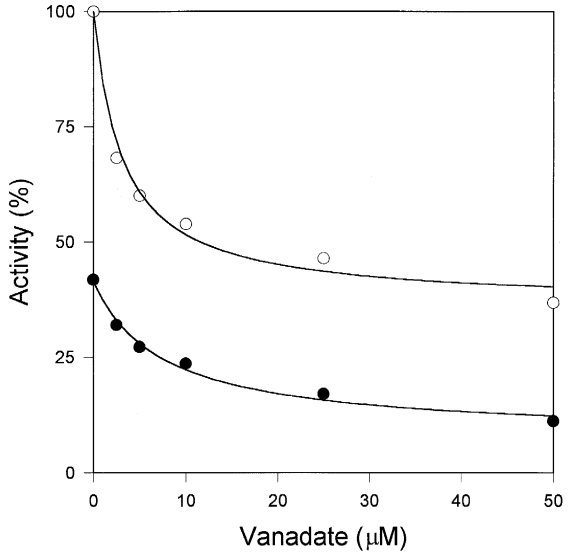

Fig. 3. Effect of vanadate on the Ca^{2+} transport by hPMCA4b(ct120) and V^{674}P(ct120) Ca^{2+} pumps. Each point in the figure is the average of two or three independent experiments. The activity of hPMCA4b(ct120) at 0 μM vanadate was taken as 100% ○, hPMCA4b(ct120); ●, V^{674}P(ct120).

concentrations. At concentrations of Ca^{2+} of 0.3 μM or less, the rate of Ca^{2+} uptake by the V^{674}P(ct120) enzyme was close to that of the hPMCA4b(ct120). As the concentration of Ca^{2+} was raised, the activity of the V^{674}P(ct120) became lower than that of hPMCA4b(ct120) and remained close to 40% of the activity of the hPMCA4b(ct120) at concentrations of Ca^{2+} higher than 1 μM. As a result, the V^{674}P(ct120) enzyme seemed to display a slightly higher apparent affinity for Ca^{2+} ($K_{1/2}$ of 0.2 μM) than the hPMCA4b(ct120). This finding indicated that the inhibition produced by the substitution of Val^{674} by Pro was not caused by a reduction in the affinity at the transport site.

Vanadate is a potent inhibitor of all P-ATPases [2]. As shown in Fig. 3, the concentration of vanadate for half-maximal inhibition was not significantly different for the hPMCA4b(ct120) and the V^{674}P(ct120) enzymes. Full inhibition of Ca^{2+} uptake was not achieved even at the highest concentration of vanadate employed (50 μM). Under these conditions, vanadate had a similar effect on the activity of the V^{674}P(ct120) and that of the hPMCA4b(ct120) enzymes, reducing the activities to about 30% of those in the absence of inhibitor. Although we have not investigated this point further, lack of complete inhibition could be related to the presence of high concentrations of ATP (6 mM) and P_i (20 mM) in the assay media.

Since vanadate is believed to bind to the E_2 conformer of the pump [12,13], the similar sensitivity to vanadate of the V^{674}P(ct120) and hPMCA4b(ct120) enzymes suggest that the substitution of V^{674} by Pro did not significantly affect the equilibrium between E_2–E_1.

The response of the Ca^{2+} pump activity to Mg^{2+} concentration is biphasic, with activation in the micromolar range followed by inhibition above 5 mM Mg^{2+} [14,15]. Fig. 4 shows the dependence of the activity of the hPMCA4b(ct120) and V^{674}P(ct120) enzymes on the concentration of Mg^{2+}. The activity of the hPMCA4b(ct120) rose with increasing concentrations of Mg^{2+} reaching a maximum at about 500 μM Mg^{2+} and then remained constant without a significant decrease at the highest concentration of Mg^{2+} used (2 mM). In contrast, within the same

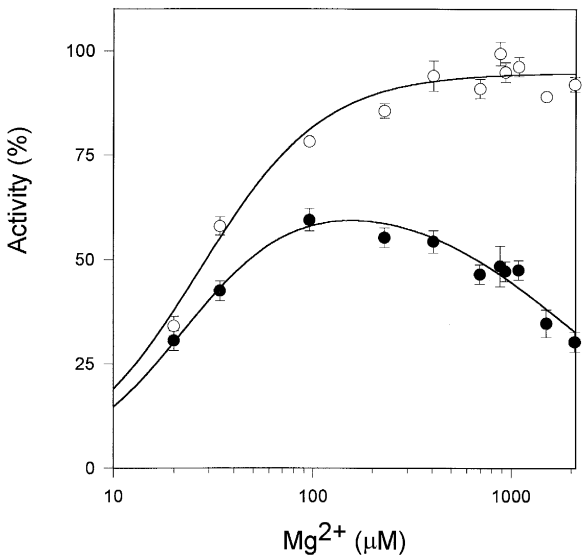

Fig. 4. Ca^{2+} transport by the hPMCA4b(ct120) and V^{674}P(ct120) Ca^{2+} pumps as a function of Mg^{2+} concentrations. Ca^{2+} uptake assays were carried out as described in Section 2 in the presence of various concentrations of Mg^{2+} given by the addition of different amounts of $MgCl_2$ to the standard media. Because Mg^{2+} was varied, the free Ca^{2+} also varied from 1.45 to 1.81 μM. All of the values shown were normalized to the activity of hPMCA4b(ct120) at 0.8 mM Mg^{2+} (3 nmol/mg/min), which was taken as 100%. ○, hPMCA4b(ct120); ●, V^{674}P(ct120).

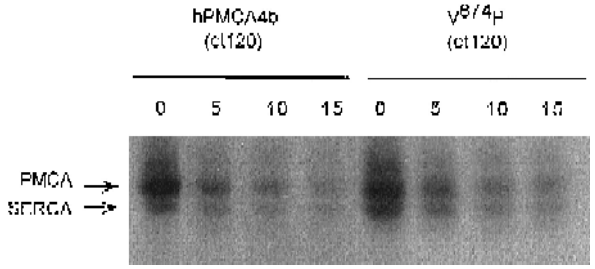

Fig. 5. Dephosphorylation of hPMCA4b(ct120) and V^{674}P(ct120). The phosphoenzyme was formed and chased with unlabled ATP as described in Section 2. 10 μg of protein were loaded per well. The number on top of each lane indicates the duration of the dephosphorylation in seconds. In addition to that of the PMCA, the phosphoenzyme formed by the endogenous SERCA is also observed.

range of Mg^{2+} concentrations, the response of the V^{674}P(ct120) mutant was clearly biphasic. At low concentrations of Mg^{2+}, the activity of V^{674}P(ct120) was approximately equal to that of hPMCA4b(ct120). As the Mg^{2+} concentration increased, the activity of V^{674}P(ct120) increased in parallel with that of hPMCA4b(ct120), reaching a maximum activation at 100 Mg^{2+}, and then dropped to about 50% of the maximum at 2 mM Mg^{2+}. This finding indicates that the higher sensitivity to inhibition by Mg^{2+} can account for the lower activity of the V^{674}P(ct120) mutant in media with 0.8 mM Mg^{2+} (Fig. 2 and Fig. 3), a concentration which is optimal for the wild-type enzyme.

We have previously shown that substitution of Val^{674} by Pro does not impair the ability of the enzyme to form a phosphorylated intermediate from ATP [4]. To investigate whether the mutation affected the dephosphorylation step of the reaction cycle, the V^{674}P(ct120) and the hPMCA4b(ct120) enzymes were first phosphorylated during 30 s in a media containing 1 μM [γ-^{32}P]ATP, 20 μM $CaCl_2$ and 5 mM $MgCl_2$ and then chased for 5, 10 or 15 s with 0.6 mM of non-radioactive ATP. Under these conditions the disappearance of the radioactive protein was fast in both enzymes. In some experiments (Fig. 5), the decay of the V^{674}P(ct120) phosphoenzyme seemed slightly slower than that of hPMCA4b(ct120); however, we were unable to observe consistent variations.

4. Discussion

We have previously reported that substitution of Val^{674} by Pro results in a partial inhibition of the plasma membrane Ca^{2+} pump [4]. This was a rather surprising result, since the corresponding residue is Pro in other P-type ion pumps. Results in this paper show that the lower activity of the V^{674}P(ct120) mutant was the consequence of an increased sensitivity to inhibition by Mg^{2+}. Unfortunately, the mechanism of inhibition of the E_1–E_2 catalytic cycle of the wild-type plasma membrane Ca^{2+} pump by high Mg^{2+} concentration is unknown, making difficult the more precise understanding of the changes due to the substitution of Val^{674} by Pro.

The V^{674}P(ct120) and hPMCA4b(ct120) enzymes were equally susceptible to inhibition by vanadate, suggesting that the inhibition by Mg^{2+} was not caused by a shifting of the equilibrium between E_2–E_1 in favor of E_2, the conformer of the enzyme that would bind vanadate.

Our results also allow to discard a displacement of Ca^{2+} by Mg^{2+} as the mechanism of the increased inhibitory effect of Mg^{2+} on the activity of the V^{674}P(ct120) enzyme, since the apparent affinity for Ca^{2+} of V^{674}P(ct120) was equal or higher than that of hPMCA4b(ct120). Furthermore, since the lower activity of the V^{674}P(ct120) enzyme was apparent only at a non-limiting concentration of Ca^{2+}, the inhibitory effects of the mutation should take place after the binding of Ca^{2+} to the enzyme.

These observations, together with the fact that the mutation does not alter the ability of the enzyme to form the phosphorylated intermediate, suggest that Mg^{2+} inhibit the V^{674}P(ct120) enzyme by lowering the rate of a reaction occurring after phosphorylation. Interestingly, in the SERCA the replacement of Pro^{603} (which corresponds to Val^{674} in the hPMCA4b) by Leu has been shown to inactivate the enzyme by blocking the Mg^{2+}-dependent conversion of E_1P to E_2P [16].

We have shown recently that mutation of Asp^{672} to Glu, just one residue apart from Val^{674}, leads to almost complete loss of Ca^{2+} transport activity by lowering the rate of dephosphorylation [4]. The low resolution of our manual technique did not allow us to confirm differences between the rate of disappearance of the V^{674}P(ct120) and the hPMCA4b(ct120)

phosphoenzymes. Such differences may be more apparent at higher temperatures using rapid-kinetic techniques, which demand much larger amounts of the mutant enzyme than those presently available.

In conclusion, in this paper we have shown that upon substitution of Val^{674} by Pro, the plasma membrane Ca^{2+} pump becomes highly sensitive to inhibition by Mg^{2+}. Further work will be needed to establish the precise mechanisms by which Mg^{2+} leads to inhibition of the plasma membrane Ca^{2+} pump.

Acknowledgements

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