The plasma membrane Ca^{2+} pump mutant lysine⁵⁹¹ → arginine retains some activity, but is still inactivated by fluorescein isothiocyanate

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Inactivation of the wild-type human plasma membrane Ca^{2+} pump (isoform 4b) by fluorescein isothiocyanate is accompanied by covalent modification of Lys⁵⁹¹. The mutation of Lys⁵⁹¹ to arginine reduced the Ca^{2+} transport activity to 35% of the wild-type, and diminished the amount of acylphosphate formed from ATP by a corresponding amount. When this mutant was treated with fluorescein isothiocyanate, the enzyme was still irreversibly inactivated, even though no reactive residue was available at

position 591. The results show that, although Ca^{2+} pump function is sensitive to the residue at position 591, Lys⁵⁹¹ is not essential for enzyme activity. They also demonstrate that irreversible inhibition of the plasma membrane Ca^{2+} pump by fluorescein isothiocyanate does not require the covalent modification of Lys⁵⁹¹. This indicates that fluorescein isothiocyanate reacts with lysine residues at other positions in addition to Lys⁵⁹¹.

INTRODUCTION

The plasma membrane Ca^{2+} pump ejects Ca^{2+} into the extracellular space. This system is one of the mechanisms used by eukaryotic cells for the precise control of the intracellular Ca^{2+} concentration. This enzyme belongs to the P-type family of ion-motive ATPases, which also includes the sarco/endoplasmic reticulum Ca^{2+} pump and the Na^+ , K^+ pump [1,2]. Fluorescein derivatives are known inhibitors of the P-type ATPases [3–5]. Inhibition of the erythrocyte plasma membrane Ca^{2+} pump by fluorescein isothiocyanate (FITC) is accompanied by modification of Lys⁵⁹¹ [6], a residue that is completely conserved among all the P-type ATPases. Because ATP prevents the covalent modification, Lys⁵⁹¹ has been considered to be part of the ATP binding site of the enzyme [7,8].

Recently it has been shown [9,10] that isothiocyanate reagents having aromatic moieties other than fluorescein also produce inactivation by labelling Lys⁵⁹¹ (or the corresponding Lys in the Na^+ , K^+ pump), suggesting that the modification of this residue suffices for the inhibition. We have examined the importance of the side chain of Lys⁵⁹¹ in Ca^{2+} pump function by replacement of Lys⁵⁹¹ by Arg. We found that this replacement reduced the activity and that FITC could still inactivate the Ca^{2+} pump irreversibly, showing that the mechanism of inactivation is not just the reaction with Lys⁵⁹¹.

MATERIALS AND METHODS

The full-length cDNA coding for hPMCA4b was constructed as described previously [11]. The plasma membrane Ca^{2+} pump is referred to by a name such as hPMCA4b, where h indicates the human species, PMCA indicates the plasma membrane calcium pump, 4 indicates the product of the fourth gene and b indicates which alternate splice was used. This isoform has also been called hPMCA4C1 [12]. Mutations were introduced using the Altered Sites *in vitro* mutagenesis system (Promega Corp.). Two oligonucleotides (ATGTACAGCAGGGGCGCCTCT and TATGTACAGCGAGGGGCGCCTC) were used for the mutants Lys⁵⁹¹

→ Arg and Lys⁵⁹¹ → Glu respectively. The mutant clones were identified by double-strand sequencing. The 426 bp *AvaI*–*AvaI* fragment containing the mutations was excised and cloned into the PMM2 vector containing hPMCA4b for expression in COS-1 cells [13]. Cells were transfected by the DEAE-dextran/chloroquine method [11]. After 48 h the cells were harvested and the microsomal fraction was isolated [14].

Crude microsomal membranes were isolated from COS-1 cells by a procedure very similar to that previously described [14]. Briefly, approx. 3.5×10^7 COS-1 cells were harvested in 10 ml of PBS containing 1 mM EDTA and 0.1 mM PMSF. Cells were collected by centrifugation (2000 g, 15 min) at 4 °C and resuspended in 3 ml of a solution containing 10 mM Tris/HCl (pH 7.7 at 4 °C), 1 mM MgCl_2 , 0.1 mM PMSF, 4 µg/ml aprotinin, 1 µg/ml leupeptin and 2 mM dithiothreitol. The cells were swollen for 20 min on ice and then homogenized with 20 strokes in a Dounce homogenizer. The homogenate was diluted with 3 ml of 0.5 M sucrose, 0.3 M KCl, 2 mM dithiothreitol and 10 mM Tris/HCl (pH 7.7 at 4 °C), homogenized again with 15 strokes in the Dounce homogenizer and centrifuged at 5000 g for 15 min. The supernatant was collected, and 1.5 ml of 2.5 mM KCl and 30 µl of 0.5 mM EDTA were added. The microsomal fraction was sedimented at 100 000 g for 40 min. The final pellet was resuspended in 0.25 M sucrose, 0.15 M KCl, 2 mM dithiothreitol, 10 mM Tris/HCl (pH 7.7 at 4 °C) and 20 µM CaCl_2 , and stored in liquid nitrogen until needed.

SDS/PAGE and immunoblotting were carried out as previously described [15]. For staining, biotinylated anti-(mouse IgG) and avidin–(horseradish peroxidase) conjugate were used. The expressed Ca^{2+} pump protein was quantified by a sandwich ELISA as described previously [14].

Calcium influx into microsomal vesicles was measured as described [14]. The reaction mixture contained 28 mM KCl, 25 mM Tris/HCl (pH 7.5 at 37 °C), 5 mM NaN_3 , 4 µg/ml oligomycin, 0.5 mM ouabain, 400 nM thapsigargin, 20 mM KH_2PO_4 , 7 mM MgCl_2 , 0.1 mM CaCl_2 (labelled with ⁴⁵Ca), 250 nM calmodulin and enough EGTA to give the desired free Ca^{2+} concentration. The ATP-dependence of the Ca^{2+} transport

Abbreviations used: FITC, fluorescein isothiocyanate; hPMCA4b, human plasma membrane Ca^{2+} pump (isoform 4b).

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was measured in the same medium with the addition of 1 mM phosphocreatine, 5 units/ml creatine kinase and 0.1 mg/ml BSA. In order to ensure a constant level of free Mg^{2+} , the concentration of $MgCl_2$ in the absence of ATP was 4 mM, and ATP was added together with $MgCl_2$ in equimolar amounts. Unless stated otherwise, ATP-dependent Ca^{2+} uptake proceeded for 5 min, and the reaction was terminated by filtering the samples through a 0.45 μ m-pore-size Millipore filter. The ^{45}Ca taken up by the vesicles was determined by counting of radioactivity in a scintillation counter.

In the experiments using FITC, 20 μ g of microsomes was resuspended at 37 °C in Tris/HCl (pH 8.0) with or without 6 mM ATP. The labelling was initiated by adding 20 μ M FITC and terminated after 2 min by the addition of Ca^{2+} uptake medium (containing ATP and $^{45}Ca^{2+}$). The phosphorylation reaction was carried out at 4 °C in a medium containing 28 mM KCl, 25 mM Tris/HCl (pH 7.5 at 4 °C), 5 mM NaN_3 , 4 μ g/ml oligomycin, 0.5 mM ouabain, 400 nM thapsigargin, 20 mM KH_2PO_4 and 250 nM calmodulin, in the presence of 0.05 mM EGTA or of 0.02 mM $CaCl_2$ plus 0.02 mM $LaCl_3$. The reaction was initiated by adding 15 μ M [γ - ^{32}P]ATP and terminated after 60 s with 10% trichloroacetic acid, 10 mM P_i and 1 mM unlabelled ATP. The denatured enzyme was pelleted by centrifugation at 20000 g for 10 min, washed once and separated by acidic SDS/PAGE [16]. The protein concentration was estimated using the reagent from Bio-Rad based on the method of Bradford [16a], using BSA as standard.

RESULTS

Figure 1 shows a Western blot of membranes from COS-1 cells expressing the wild-type hPMCA4b or the mutants $Lys^{591} \rightarrow Glu$ or $Lys^{591} \rightarrow Arg$. The migration of the mutant proteins was indistinguishable from that of the wild-type, indicating that they had the same size. The low intensity of the $Lys^{591} \rightarrow Glu$ band showed that its expression was lower. The amount of expression was tested more quantitatively by means of ELISA. The results confirmed the lower expression of $Lys^{591} \rightarrow Glu$, which was 30% of the wild-type. Because of its lower expression the activity remaining in the $Lys^{591} \rightarrow Glu$ mutant was too small to study, so it was not included in subsequent experiments. The amount of $Lys^{591} \rightarrow Arg$ protein expressed was 88% of the wild-type, so this mutant was chosen for further study.

Mutation of Lys^{591} to Arg reduced the activity of the enzyme, as measured by Ca^{2+} transport. In five independent experiments the average activity of the $Lys^{591} \rightarrow Arg$ mutant was $35 \pm 10\%$

Figure 1 Immunoblot of wild-type and mutant hPMCA4b

A sample of 40 μ g of purified erythrocyte Ca^{2+} pump was loaded into the first lane, and membranes from non-transfected COS cells into the second lane. The remaining lanes contained membranes from COS cells expressing the constructs indicated.

Figure 2 Phosphorylation of hPMCA4b and of the mutant $Lys^{591} \rightarrow Arg$

Samples of 25 μ g of COS cell membrane protein were loaded into each lane, and La was present except where otherwise indicated. The first two lanes contained membranes from COS cells transfected with the empty plasmid pMM2, and the remaining lanes contained the constructs indicated.

Figure 3 Activity of hPMCA4b and of the mutant $Lys^{591} \rightarrow Arg$: dependence on ATP concentration

The activity of a blank containing membranes from COS cells transfected with pMM2 was subtracted from each value.

of the wild-type. The activity was linear for at least 20 min in both the wild-type and the mutant.

The ability of the $Lys^{591} \rightarrow Arg$ mutant to form the acylphosphate intermediate from ATP was also decreased, by a similar amount. The phosphorylation reaction was carried out in the presence of Ca^{2+} and La^{3+} to facilitate observation of the acylphosphate. As shown in Figure 2, under these conditions mutant $Lys^{591} \rightarrow Arg$ was able to form an acylphosphate, although the amount was much smaller than that obtained with the wild-type enzyme.

Since Lys^{591} has been considered to form part of the ATP site, the lower activity and decreased phosphorylation of the mutant could be due to a lowered affinity for ATP. To investigate this possibility the activity of the mutant was measured as a function of ATP concentration. Figure 3 and Table 1 show that the dependence of the activity of the mutant on ATP was essentially identical to that of the wild-type. Both had the same effective affinity for ATP (Figure 3), and the activity of mutant $Lys^{591} \rightarrow Arg$ remained lower than that of the wild-type even at 24 mM ATP (Table 1), indicating that even very high ATP concentrations did not overcome the inhibition.

In the mutant, the residue that normally forms a covalent bond with FITC has been altered to a residue that will not be

Table 1 Activity of mutant and wild-type enzymes at high ATP levels

The Ca^{2+} uptake activity in membranes from COS cells transfected with the empty plasmid pMM2 was $0.52 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $0.58 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ at 6 mM and 24 mM ATP respectively, and was subtracted from each value.

	Ca^{2+} uptake ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	
	6 mM ATP	24 mM ATP
hPMCA4b	1.54	1.50
Lys ⁵⁹¹ → Arg	0.90	0.88

Table 2 Effect of FITC on the activity of hPMCA4b and the mutant Lys⁵⁹¹ → Arg

The activity of a control (membranes from COS cells transfected with empty plasmid) was $0.48 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ when ATP was added before FITC, and $0.35 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ when FITC was added first. These values were subtracted from each point.

	Ca^{2+} uptake ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	
	ATP followed by FITC	FITC followed by ATP
hPMCA4b	1.63	0.23
Lys ⁵⁹¹ → Arg	0.55	0.07

reactive. If the inhibition of the Ca^{2+} pump by FITC were due to the covalent modification of Lys⁵⁹¹, FITC should not inhibit the residual activity of the Lys⁵⁹¹ → Arg mutant. The results in Table 2 (column 2) show that FITC inactivated the mutant Lys⁵⁹¹ → Arg just as it did the wild-type, and that the inactivation was not reversed by ATP. The control experiment shown in column 1 of Table 2 demonstrates that the full activity of the enzyme was retained in the presence of FITC if ATP was added first, as expected [6].

DISCUSSION

Lys⁵⁹¹ has received much attention because it is completely conserved among the P-type ATPases and because it is covalently modified by FITC, a reagent whose binding competes with that of ATP [4,6]. This modification is one of the reasons that the region containing Lys⁵⁹¹ has been considered to be a nucleotide binding fold. Conversion of Lys⁵⁹¹ into Glu caused the enzyme to be expressed at a very low level, possibly due to the effect of this drastic amino acid change on the protein structure. Alteration of Lys⁵⁹¹ to Arg did not change the level of expression, but reduced the activity to 35 % of that of the wild-type. Similar reductions in activity upon conversion of the corresponding Lys residue to Arg were reported for the sarco/endoplasmic reticulum Ca^{2+} pump [17] and for the H^{+} pump from yeast [18]. Thus this residue is of substantial importance for the activity of the enzyme but a misfitting substitute does not destroy the activity, suggesting that the ϵ -amino group of Lys⁵⁹¹ is not directly involved in coordination to ATP.

We found that mutant Lys⁵⁹¹ → Arg was able to react with ATP to transport Ca^{2+} and form the acylphosphate intermediate, although the amounts of both were diminished. The ability of the enzyme to bind ATP did not appear to be altered, since the ATP-dependence of Ca^{2+} uptake remained unchanged. This is consistent with our results with other mutants containing changes in the region of the putative nucleotide binding fold [19]. Instead,

the parallel reduction of activity and acylphosphate formation suggests that inhibition of the reactions leading to the formation of the acylphosphate is the cause of the low Ca^{2+} transport activity of the mutant. Mutation of the corresponding Lys in the sarco/endoplasmic reticulum Ca^{2+} pump had no effect on phosphorylation, which may indicate a different function of the two enzymes [20].

Our data show that the inactivation of hPMCA4b by FITC is irreversible even when Lys⁵⁹¹ has been converted into Arg. In this mutant and in the wild-type, ATP added before FITC prevented inactivation, but ATP had no effect when added after FITC.

FITC reacts predominantly with Lys⁵⁹¹ of the wild-type plasma membrane Ca^{2+} pump [6]. In the mutant Lys⁵⁹¹ → Arg this reaction is not possible, so the inactivation of the mutant by FITC indicates that FITC can also inactivate by reacting with Lys residues other than Lys⁵⁹¹. Since the protective effect of ATP was preserved in the mutant, these other residues must also be accessible from the ATP site. This result is consistent with the report of Xu [21], who observed that although one molecule of FITC inactivated one $\text{Na}^{+}/\text{K}^{+}$ -ATPase molecule, only 50 % of the total FITC was bound to Lys⁵⁰¹ (which corresponds to Lys⁵⁹¹ in the hPMCA4b). The rest of the FITC was bound to Lys⁴⁸⁰ and Lys⁷⁶⁶.

Further protein chemistry work may be needed to establish the residues that react with FITC in the Lys⁵⁹¹ → Arg mutant. One possibility which cannot be excluded at the moment is that the alteration of Lys⁵⁹¹ increases the reactivity of FITC with other Lys residues which are less reactive in the wild-type enzyme. The results in the present paper demonstrate that modification of the side chain of Lys⁵⁹¹ is not in itself the cause of the inhibition produced by chemical reagents targeting this residue.

Crystallographic studies have shown that, in a variety of nucleotide binding enzymes, the fluorescein moiety of fluorescein derivatives fills the adenosine binding region [22]. Our results support the idea that these reagents prevent ATP binding by steric interactions. It has been reported that pyridoxal 5'-phosphate inhibits the sarco/endoplasmic reticulum Ca^{2+} pump and the $\text{Na}^{+}/\text{K}^{+}$ pump by labelling Lys⁴⁹² in the former and Lys⁴⁸⁰ in the latter [23,24]. These residues were also shown not to be essential for activity by mutagenesis studies [25]. Thus it would seem that reagents of different chemical structure and size are effective in preventing the entry of ATP to its site.

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