Phosphorylation by protein kinase C of isoform 4a of the human plasma membrane Ca\(^{2+}\) pump (hPMCA4a) was studied using the COS cell expression system. Phosphorylation of several truncated mutants of hPMCA4a indicated that a single phosphorylation site lies in a region between residues 1113 and 1125. This region is within the calmodulin binding domain and contains a single phosphorylatable residue, serine 1115. Converting this serine to an alanine diminished phosphorylation greatly. Phosphorylation, done in the absence of calmodulin, did not affect subsequent calmodulin binding, but previous binding of calmodulin did inhibit phosphorylation. Moreover, no significant shift in the calmodulin response curve of hPMCA4a was observed when phosphorylation was mimicked by converting serine 1115 to an acidic residue. The calmodulin binding domain of hPMCA4a is much longer than other calmodulin binding domains and has been suggested to consist of two binding lobes interrupted by a short nonbinding region. The findings of this study indicate that serine 1115 is the residue phosphorylated by protein kinase C, and that it lies within the nonbinding region of the calmodulin binding domain.

The plasma membrane Ca\(^{2+}\) pump, with its high Ca\(^{2+}\) affinity, appears to be responsible for maintaining the low resting level of Ca\(^{2+}\) in the unstimulated cell. The activity of this pump is controlled by several different means: by calmodulin, acidic phospholipids, phosphorylation with protein kinases, proteolysis, and dimerization. Most of the regulatory determinants of the enzyme are located at its carboxyl terminus. This region contains the high affinity calmodulin binding site, an autoinhibitory region, sites for phosphorylation with protein kinases C and A, and cleavage sites for proteolysis (1, 2). It is a highly variable region displaying great diversity between the different PMCA isoforms.

The pump is encoded by four different genes, and an additional variability is produced by alternative RNA splicing so that the number of possible transcripts of the pump is >20 (3).

The alternative splices at site C alone create ~10 protein variants. This site is located in the middle of the region coding for the calmodulin binding domain, and the splices involve a frameshift that changes all of the downstream residues. Because these splices are in the regulatory region, the different pump variants are expected to have different regulatory properties. In hPMCA4 such a variation has been shown to have a striking effect on the calmodulin binding and basal activity (4). These structural changes also cause a major change in the sites available for phosphorylation by protein kinases. Although all isoforms show a great proportion of serine and threonine residues at the carboxyl terminus, the locations of consensus sequences for phosphorylation are quite different from one isoform to the other (1). The alternate splice, especially, has a profound effect on the location of the phosphorylatable residues in this region.

The regulatory region of hPMCA4b begins with a 28-residue calmodulin binding domain and continues with a downstream inhibitory region, which does not contribute to calmodulin binding (5). This downstream inhibitory region contains the sites for phosphorylation with protein kinase C. Phosphorylation of these sites releases the inhibition caused by the inhibitory region but does not affect the inhibition caused by the calmodulin binding domain. Thus, phosphorylation stimulates the pump only partially, and binding of calmodulin is needed to achieve full activity of the enzyme. Because these phosphorylation sites are located downstream of the calmodulin-binding domain, phosphorylation of hPMCA4b at these sites does not affect calmodulin binding (6).

The structure of the regulatory region of hPMCA4a, the other splice variant of isoform 4, is quite different. It has a much longer calmodulin binding domain of 49 residues, which overlaps completely with the inhibitory region. We have suggested that the long calmodulin binding sequence of hPMCA4a actually consists of two binding lobes separated by a short nonbinding region (7). Because of this difference in the structure of the regulatory region, isoform 4a has a much lower affinity for calmodulin and a higher basal activity than isoform 4b (4).

The great variability between the isoforms at the carboxyl terminus has suggested that they would have different protein kinase C phosphorylation sites, and phosphorylation at these sites would have a different effect on their activity. A recent study has shown that isoforms 2b and 3b were not phosphorylated significantly by protein kinase C, whereas isoforms 2a and 3a were phosphorylated (8). Phosphorylation of 2a and 3a with the kinase reduced calmodulin binding of these forms drastically but did not affect their basal activity. Here we show that phosphorylation of hPMCA4a with protein kinase C occurs within the calmodulin binding domain but does not affect substantially either the calmodulin affinity or the basal activity of the enzyme. Using site-directed mutagenesis we have identi-
Phosphorylation of hPMCA4a with Protein Kinase C

Fig. 1. Sequence of the calmodulin binding (C) and inhibitory (I) domain of hPMCA4a. The arrows show where the truncated mutants end. Phosphorylatable residues are in bold type; basic residues are underlined; and acidic residues are boxed.

Materials and Methods

**45Ca** and [γ-32P]ATP were purchased from NEN Life Science Products. Calmodulin and calmodulin-Sepharose were obtained from Sigma. Phorbol 12-myristate 13-acetate and rat brain protein kinase C (con-actus). Calmodulin and calmodulin-Sepharose were obtained from Sigma. The specific activity of the protein kinase C preparation was 1130 units/mg protein. LipofectAMINE and Optimem media were obtained from Life Technologies, Inc. All other chemicals used for this study were of reagent grade.

**Construction of the hPMCA4a Mutants**—4a-c156 and 4a-c44 were constructed as described before (4, 7). Mutations of serine 1115 of the protein kinase C preparation was 1130 units/mg protein. LipofectAMINE and Optimem media were obtained from Life Technologies, Inc. All other chemicals used for this study were of reagent grade.

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**Isolation of Microsomes from COS Cells**—Crude microsomal membranes from COS-1 cells were prepared as described by Enyedi et al. (6). Briefly, transfection was initiated when the cells were 70–80% confluent in 150-cm² flasks. The cells were incubated at 37 °C with the DNA-LipofectAMINE complex (formed by incubating 8 ml of serum-free Optimem medium. After 5 h of incubation, the DNA-LipofectAMINE complex was added to each sample, and binding was allowed to proceed on ice for 90 min. The unbound proteins were removed by washing the beads four times with 200 ml of 5 × diluted extraction buffer. The proteins bound to the calmodulin-Sepharose were then eluted with the blunit PCR cloning kit from Invitrogen and sequenced by the Mayo Molecular Biology Core Facility. The NsiI-KpnI piece was cut out and placed into the wild-type hPMCA4a in the vector psp72. The full-length SfiI/KpnI piece was then cut out of psp72 and ligated into the expression vector pMM2.

**Transfection**—Transfection was carried out using LipofectAMINE based on the protocol as described by the manufacturer and by Enyedi et al. (8). Briefly, transfection was initiated when the cells were 70–80% confluent in 150-cm² flasks. The cells were incubated at 37 °C with the DNA-LipofectAMINE complex (formed by incubating 8 μg of DNA and 100 μl of LipofectAMINE in 3.6 ml of serum-free Optimem medium) in 14.5 ml of serum-free Optimem medium. After 5 h of incubation, the cells were supplemented with serum, and incubation continued for a total of 24 h. The medium containing DNA-LipofectAMINE complex was then replaced with fresh tissue culture medium with 10% serum, and the cells were cultured for an additional 24 h.

**Isolation of Microsomes from COS Cells**—Crude microsomal membranes from COS-1 cells were prepared as described by Enyedi et al. (6). Cr⁺⁺: Transport Assay—Cr⁺⁺ uptake by microsomal vesicles was carried out in a 200-μl reaction mixture and assayed by rapid filtration through Millipore membrane filters (0.45 μm pore size, type HA) as described (6, 8). The reaction mixture contained 100 mM KCl, 25 mM TES-triethanolamine, pH 7.2, 40 mM KH₂PO₄/K₂HPO₄, pH 7.2, 200 mM theophosphagin, 5 mM NaN₃, 4 μg/ml oligomycin, 7 mM MgCl₂, 100 mM NaCl, (labeled with [45Ca; specific activity, 100,000–150,000 cpm/nmol), and enough EGTA to obtain 1.2 μM free Ca⁺⁺ concentration. Micro- somes at a 10–20 μg/ml concentration were preincubated in the presence of the appropriate calmodulin concentration for 2 min at 37 °C, and Ca⁺⁺ uptake by the vesicles was started by the addition of 6 mM ATP. When the effect of protein kinase C on the calmodulin-activity was studied, Ca⁺⁺ uptake by the vesicles was started in the presence of 20 millimol (0.0857 μg/ml) of PKC and 100 mM phorbol 12-myristate 13-acetate in the absence of calmodulin. 250 nM calmodulin was added where appropriate, and the reaction was continued for an additional 5 min. The reaction was terminated by separating the microsomes with filtration through the Millipore membrane filters.

**Phosphorylation of Microsomal Membrane Proteins with Protein Kinase C**—10 μg of microsomal membranes isolated from COS-1 cells transfected with the appropriate construct were phosphorylated with rat brain protein kinase C basically as described (6, 8). A 200-μl reaction mixture contained 100 mM KCl, 25 mM TES-triethanolamine, pH 7.2, 1 mM MgCl₂, 5 mM dithiothreitol, 0.1 mM sodium orthovanadate, 100 μM CaCl₂, and 90 μM EGTA. This mixture was preincubated for 3 min with 20 millimol (0.0875 μg/ml) of protein kinase C and 100 mM phorbol 12-myristate 13-acetate, and the reaction was started by the addition of 20 μl (10⁻²³P)ATP. After 5 min of incubation the reaction was terminated by the addition of 1 ml of ice-cold 6% trichloroacetic acid containing 1 mM ATP and 10 mM inorganic phosphate. The precipitate was supplemented with 50 μg of bovine serum albumin, washed three times with the same trichloroacetic acid solution, and then dissolved in the electrophoresis sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5 mM EDTA, 125 mg/ml urea, and 100 mM dithiothreitol. An aliquot of this solution containing 2 μg of membrane protein was applied to each track of an SDS-polyacrylamide gel.

**Binding of the Phosphorylated hPMCA4a Constructs to Calmodulin-Sepharose**—This was done as described previously (6, 8). Briefly, the phosphorylation reaction described above was terminated by the addition of 1 ml of ice-cold 6% trichloroacetic acid containing 1 mM ATP and 10 mM inorganic phosphate. The precipitate was supplemented with 50 μg of bovine serum albumin, washed three times with the same trichloroacetic acid solution, and then dissolved in the electrophoresis sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5 mM EDTA, 125 mg/ml urea, and 100 mM dithiothreitol. An aliquot of this solution containing 2 μg of membrane protein was applied to each track of an SDS-polyacrylamide gel.

**Gel Electrophoresis, Electrotransfer, and Autoradiography**—The radioactive samples were electrophoresed on 4–15% gradient acrylamide gel following Laemmli’s procedure (13). The samples were subsequently electroblotted, and the blots were immunostained with monoclonal antibody 5F10 (10) before autoradiography for 2–3 days. The amount of phosphorylation was quantitated using the Bio-Rad GS-363 Molecular Image System and related to the amount of pump protein of the same immunoblot. The amount of the constructs was determined by antibody binding using the enhanced chemiluminescence Western blot detection system and was quantitated using the Bio-Rad Molecular Image System.

**RESULTS**

We studied the phosphorylation of hPMCA4a expressed in the COS cell system with protein kinase C. To determine the region where phosphorylation occurs, we followed the same strategy as we had previously used for hPMCA4b (6); i.e. we used truncated mutants of hPMCA4a called 4a-c156 and 4a-c44 expressed in COS cells. Fig. 1 shows the sequence of the calmodulin binding domain at the carboxyl terminus of hPMCA4a, and the arrows show where the truncated ends. Using these constructs, we have been able to determine the boundaries of the calmodulin binding domain and the autoinhibitory region (7). Phosphorylation of the membranes containing the expressed construct was performed in the presence of protein kinase C and [γ-32P]ATP. Fig. 2A shows an immunoblot of the phosphorylated membrane samples stained with monoclonal antibody 5F10, whereas Fig. 2B is an autoradiogram of the same immunoblot. Fig. 2A shows that hPMCA4a and the truncated were expressed in the COS cell membrane equally well. On the autoradiogram shown in Fig. 2B strong phosphorylated bands could be seen at the position of full-length hPMCA4a and 4a-c44, whereas the phosphorylation was much weaker at the position of 4a-c156.

To analyze binding of the phosphorylated proteins to calmodulin, the samples were loaded onto calmodulin-Sepharose in

![Phosphorylation of hPMCA4a with Protein Kinase C](https://example.com/image.png)
the presence of calcium followed by extensive washings to remove the loosely bound proteins. The protein bound to the Sepharose beads was analyzed by gel electrophoresis and immunoblotting (Fig. 3). It is evident from Fig. 3 that all constructs bound to the calmodulin-Sepharose and that strong phosphorylation of hPMCA4a and 4a-ct44 could be seen. This experiment allowed us to see more clearly the lack of labeling of 4a-ct56. This was evident because only the pump bound to the column with high affinity, greatly improving the labeling/background ratio in the phosphorylation experiment. Because 4a-ct56 was not labeled, our data suggested that the phosphorylatable site in hPMCA4a resides between residues 1113 and 1125, within a region that is common to both hPMCA4a and 4a-ct44 but missing in 4a-ct56. The only residue that could become phosphorylated within this region is the serine at position 1115. Although this region lies within the calmodulin binding domain of hPMCA4a, phosphorylation did not affect calmodulin binding drastically; i.e. the phosphorylated samples bound to calmodulin-Sepharose. On the other hand, including excess calmodulin in the phosphorylation medium effectively inhibited the labeling of the pump (Fig. 4).

To verify that phosphorylation was indeed occurring at position 1115, this residue was modified. Fig. 4 shows that converting serine 1115 to an alanine or an aspartate in the full-length hPMCA4a blocked phosphorylation completely, in good accord with the results obtained with the truncates. These experiments confirmed that serine 1115 is indeed a potential phosphorylation site in hPMCA4a and that this site lies within a region that is involved in calmodulin binding.

Then we tested the effect of phosphorylation with protein kinase C on the Ca\(^{2+}\) transport activity of hPMCA4a. Microsomal membrane vesicles were preincubated with protein kinase C and phorbol 12-myristate 13-acetate in the presence of ATP, and Ca\(^{2+}\) uptake was initiated by the addition of labeled Ca\(^{2+}\). Phosphorylation did not affect either the basal or the calmodulin-stimulated activity of the enzyme (data not shown). Moreover, the activity of the mutant in which phosphorylation was mimicked by adding a fixed negative charge by replacing serine 1115 with an aspartate was equal to that of the wild type or to the mutant in which the serine was replaced by an alanine.

Although phosphorylation did not abolish calmodulin binding, a shift in the calmodulin affinity would be expected because of the introduction of a negative charge within the calmodulin binding domain. Thus, we examined whether the negative charge added by phosphorylation of serine 1115 affected the calmodulin affinity of hPMCA4a. When calcium transport by membrane vesicles expressing the wild-type hPMCA4a was tested, we were unable to detect any effect of phosphorylation on the calmodulin concentration required for half-maximal stimulation of the enzyme (not shown). Because calmodulin may interfere with the phosphorylation reaction, in
transfection to the other, because of a variation in transfection level ranging from 4 to 6 nmol mg of membrane protein \(-1\) min \(^{-1}\). \(V_c\) was calculated from an initial hyperbolic fit of the data, and the calculated value was used for the second fit, which is shown. Data points are the average of three independent determinations on three different preparations. The data from hPMCA4a are shown by the filled circles and the solid line, from 4a S1115D by the open circles and line of short dashes, and from 4a S1115A by the open triangles and line of long dashes.

Fig. 5 we compared the calmodulin response curves of the mutants in which serine 1115 was replaced by an aspartate or by an alanine. These mutants mimic the totally phosphorylated and nonphosphorylated forms of the pump, respectively. No substantial shift in the calmodulin response curves of the mutants compared with that of the wild type could be detected, indicating that phosphorylation of hPMCA4a at serine 1115 does not affect the calmodulin affinity of the enzyme.

**DISCUSSION**

Protein kinase C is a key element of intracellular signaling, which phosphorylates and regulates a wide variety of intracellular targets. Many of these targets are calmodulin-binding proteins in which phosphorylation occurs in or near the calmodulin binding domain; therefore, phosphorylation of these proteins affects (usually decreases) calmodulin binding (11, 12). It has been accepted that one of these targets is the plasma membrane Ca\(^{2+}\) pump and that the phosphorylation sites of the enzyme also lie near its calmodulin binding domain. The isoform diversity of this pump has caused us to ask whether the different isoforms are all regulated in the same way, or whether the regulation also shows diversity among the isoforms.

Close inspection of the sequences of the isoforms has indicated that they should have different phosphorylation sites. Indeed, our recent experiments have demonstrated that among five different isoforms (rPMCA2b, rPMCA3b, hPMCA4b, rPMCA2a, and rPMCA3a) expressed in the COS cell system, only three are potential targets for protein kinase C phosphorylation (hPMCA4b, rPMCA2a, and rPMCA3a), and that phosphorylation affects their activities differently. In the case of hPMCA4b, protein kinase C partially increases the basal activity and does not affect calmodulin binding (6).

Although the sequences of the regulatory regions of rPMCA2a and 3a resemble each other rather closely, the sequence of the same region of the other "a" forms is different. In the case of hPMCA4a the serine and threonine residues of this region are much less abundant than in the case of rPMCA2a and 3a, with fewer putative phosphorylation sites. The regulatory sequence and the location of the putative phosphorylation sites of hPMCA4a shows even less resemblance to hPMCA4b.

In this study we demonstrated that hPMCA4a is also a potential target for protein kinase C phosphorylation. Determination of the site of phosphorylation was obtained by analyzing several carboxyl-terminally truncated mutants of hPMCA4a and by point mutation of the full-length enzyme expressed in the COS cell system. Using this strategy, we identified serine 1115 as a single phosphorylation site in the consensus sequence SFKG. The phosphorylation was blocked when calmodulin was bound to the enzyme, in good accordance with previous findings that this sequence is part of the calmodulin binding domain.

Although binding of calmodulin inhibited phosphorylation, the phosphorylated pump still bound to calmodulin-Sepharose and was activated by calmodulin. Moreover, the mutant in

**Fig. 5.** Calmodulin response curves of the mutants 4a S1115A and 4a S1115D compared with the wild-type hPMCA4a. Calmodulin concentration dependence of Ca\(^{2+}\) uptake by microsomal membrane vesicles isolated from COS cells transfected with hPMCA4a, 4a S1115A (4a S1115 → A), and 4a S1115D (4a S1115 → D). Ca\(^{2+}\) uptake was measured at 1.2 μM free Ca\(^{2+}\) concentration and was expressed as \(f = (v - V_c)V_o/V_o - V_c\), where \(V_o\) is the activity in the absence of calmodulin, \(V_c\) is the maximum activity, and \(v\) is the activity in the presence of the appropriate calmodulin concentration. \(V_c\) varied from one transfection to the other, because of a variation in transfection level ranging from 4 to 6 nmol mg of membrane protein \(-1\) min \(^{-1}\). \(V_o\) was calculated from an initial hyperbolic fit of the data, and the calculated value was used for the second fit, which is shown. Data points are the average of three independent determinations on three different preparations. The data from hPMCA4a are shown by the filled circles and the solid line, from 4a S1115D by the open circles and line of short dashes, and from 4a S1115A by the open triangles and line of long dashes.

**Fig. 6.** Model of the calmodulin binding and autoinhibitory domain of hPMCA4a. According to the model, the calmodulin binding domain of hPMCA4a consists of two binding lobes separated by a short nonbinding region. The nonbinding region is shown as a hairpin. Phosphorylation with protein kinase C occurs within this hairpin; therefore, it does not affect calmodulin binding. The arrow indicates the position of the alternate splice that changes the structure of the calmodulin binding domain.
which phosphorylation was mimicked by converting serine 1115 to an aspartic acid also was activated by calmodulin. We were unable to detect an effect by introducing a negative charge either by phosphorylation or by mutation on the concentration of calmodulin required for half-maximal activation of hPMCA4a. These results show that serine 1115 is not involved directly in calmodulin binding but is hidden from protein kinase C phosphorylation when calmodulin is bound before phosphorylation. Recently, we have suggested that the calmodulin binding domain of hPMCA4a consists of two separate binding regions, which are interrupted by a short nonbinding loop (7). The present findings support this idea and indicate that serine 1115 is part of the loop that is folded out of the calmodulin-enzyme interaction surface. Thus, the nonbinding loop may start with the aspartic acid at position 1105 and end with the sequence surrounding serine 1115, as shown in Fig. 6.

Phosphorylation of hPMCA4b at its downstream inhibitory region caused partial activation of the enzyme. On the contrary, phosphorylation of hPMCA4a within its calmodulin binding domain (which also serves as an inhibitor) did not affect the basal activity. This suggests that the loop that is folded out from the calmodulin-enzyme interaction is not in contact with the catalytic core of the pump and therefore is not involved in self-inhibition.

In conclusion, here we have provided additional evidence for the differential regulation of the plasma membrane calcium pump isoforms with protein kinase C. It is clear that each isoform has a unique mode of regulation by protein kinase C phosphorylation, which is presumably suited to its function in the cell. Of the six isoforms studied so far, four (4b, 4a, 3a, and 2a) are phosphorylated by protein kinase C, but only one (4b) is activated by this phosphorylation. The phosphorylation of 2a and 3a prevented calmodulin binding, whereas phosphorylation of 4a and 4b had little effect on calmodulin binding. The relationship of these properties to the functions of these isoforms in the living cell remains to be determined. All of these findings are based on studies using the overexpressed pump isoforms in COS cell membranes. We cannot exclude the possibility that in other kinds of membranes unknown factors may modify the locus and the effect of protein kinase C phosphorylation, but this is the only system currently available for studying the characteristics of the individual pump isoforms.

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