Protein Kinase C Activates the Plasma Membrane Ca\(^{2+}\) Pump Isoform 4b by Phosphorylation of an Inhibitory Region Downstream of the Calmodulin-binding Domain*  

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The carboxyl-terminal region of the plasma membrane Ca\(^{2+}\) pump isoform 4b contains two autoinhibitory domains which keep the pump inactive in the absence of activators such as calmodulin. One of these regions is approximately coterminal with the calmodulin-binding domain, while the second region is downstream (Verma, A. K., Enyedi, A., Filoteo, A. G., and Penniston, J. T. (1994) J. Biol. Chem. 269, 1687–1691). The carboxyl-terminal region has also been identified as the site for phosphorylation of this isoform by protein kinase C (Wang, K. K. W., Wright, L. C., Machan, C. L., Allen, B. G., Conigrave, A. D., and Roufogalis, B. D. (1991) J. Biol. Chem. 266, 9078–9085). Using constructs lacking various numbers of residues at the carboxyl terminus, we studied the degree of phosphorylation by protein kinase C and the resultant activation of Ca\(^{2+}\) transport. The results showed that the most specific and easy phosphorylation occurred in a region of about 20 residues with the carboxyl terminus of the calmodulin-binding domain, and that the downstream inhibitory domain had also about the same size and location. Phosphorylation partially activated the pump by removing only the inhibition due to this region. Binding of calmodulin to the calmodulin-binding domain activated the pump more fully by removing the inhibition due to both regions, regardless of the state of phosphorylation at the downstream inhibitory region.

The plasma membrane Ca\(^{2+}\) pump is an important element in regulating the intracellular Ca\(^{2+}\) concentration, which removes the excess Ca\(^{2+}\) from cells during intracellular Ca\(^{2+}\) signaling. Four genes and alternative splicings at two different sites produce more than 20 isoforms of this pump. Among these isoforms probably the most common is hPMCA4b1 which has been extensively studied in the last two decades. The carboxyl terminus of this pump is a multifunctional regulatory region which serves as an inhibitor of the enzyme, contains a high affinity calmodulin-binding site, binds acidic phospholipids, and is a target for protein kinase phosphorylation. The calmodulin-binding domain has been identified as a 28-residue sequence within this region which is also an autoinhibitory domain (1–3). Recent results, however, have shown that other regions downstream of the calmodulin-binding domain must also be involved in autoinhibition (4).

Stimulation of the pump by protein kinase C has been described in whole erythrocytes (5), rat arterial endothelial (6, 7), and smooth muscle cells (8), dog heart muscle (9), bovine heart muscle (10), bovine aortic smooth muscle (11), erythrocyte membrane preparations (12) and solubilized, purified erythrocyte Ca\(^{2+}\) pump (13). In the latter study, the site of phosphorylation of the isolated erythrocyte pump was located in the carboxyl-terminal region, involving both serine and threonine residues. That study used calpain digestion of the phosphorylated Ca\(^{2+}\) pump and phosphorylation of a synthetic peptide to suggest that one of the phosphorylation sites could be the threonine residue in the middle of the calmodulin-binding domain. However, no direct evidence for the phosphorylation of this residue in the enzyme still in its original membrane has been presented. In a subsequent study (14), a synthetic peptide representing the calmodulin-binding domain phosphorylated at the threonine in question was studied. This phosphopeptide failed to bind calmodulin and was much less effective in inhibiting the fully active pump than the unphosphorylated peptide. Based on the peptide data, phosphorylation would be expected to prevent binding of calmodulin to the pump and to cause a substantial activation similar to that observed with calmodulin. In contrast, Smallwood et al. (12), who studied the pump in its native membrane, found only partial activation by protein kinase C and that stimulation by protein kinase C and calmodulin were additive. Wang et al. (13), on the other hand, found that purified type III protein kinase C antagonized the calmodulin activation at low Ca\(^{2+}\) concentrations. These conflicting results could have various causes, such as the use of different Ca\(^{2+}\) pump preparations, different conditions, and/or different protein kinase C isoforms and concentrations, or they might arise from the attempt to compare data on a short peptide with data on the intact pump.

Overexpression of hPMCA4b in COS-1 cells has made it possible to study its regulatory region in more detail. In this report, we describe the expression of truncated constructs in COS-1 cells and the study of their phosphorylation in the native environment of the membrane. The shortest construct was ct92, which contained all 28 residues of the calmodulin-binding domain (including the putative phosphorylation site) but lacked everything downstream of it. We had previously shown that it had full calmodulin affinity, but that in the absence of calmodulin it had higher activity than the full-length enzyme (4). When compared to the full-length pump, ct92 did not show significant phosphorylation by protein kinase C. The longest construct (ct48) had, in the absence of calmod-
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Phosphorylation of the Microsomal Membrane Protein with Protein Kinase C—10 μg of microsomal membrane isolated from cells transfected with the appropriate construct was phosphorylated with rat brain protein kinase C at 37°C in a medium containing 100 mM KC1, 25 mM Tris-triethanolamine, pH 7.2, 1 mM MgCl\(_2\), 5 mM dithiothreitol, 0.1 mM sodium orthovanadate, 100 μM NaCl, and enough EGTA to obtain 10 μM Ca\(^{2+}\)-free concentration. Protein Kinase C (7–144 milliunit, 0.014–0.29 μg/μmol) and 100 μM TPA were added and the reaction was started by the addition of 200 μM [γ-\(^{32}\)P]ATP. After 2–30 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 phosphate. After the addition of 50 μg of bovine serum albumin, the precipitated protein was collected by centrifugation and washed three times with the same buffer. The precipitates were 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, 100 mM dithiothreitol. An aliquot of this solution containing 2–4 μg of microsomal membrane protein was applied to each track of a SDS-polyacrylamide gel. If the phosphorylated proteins were used for calmodulin-Sepharose binding instead of electrophoresis the reaction was terminated by putting the samples on ice. Protein kinase C was activated with an excess amount of TPA instead of using phosphatidyserine and diacylglycerol because phosphatidyserine is a well known activator of the pump that would interfere with the protein kinase C effect.

Binding of the Calcium Pump and the Transfected Mutants to Calmodulin-Sepharose—To 200 μl of ice-cold phosphorylation medium containing 1 μg of the phosphorylated microsomal protein, 40 μL of 5 × concentrated extraction solution containing 5 mM MgCl\(_2\), 5 mM CaCl\(_2\), 2% Triton X-100, 1.5 mM sucrose, and 0.8 mM NaCl, and 50 mM TES-triethanolamine, pH 7.2, was added. The mixture was incubated on ice for 15 min. A 200-μl aliquot of this mixture was introduced to 50 μL of calmodulin-Sepharose 4B beads that were previously equilibrated in 1 × concentration of the above buffer to which 2 mM dithiothreitol, 9 μg/ml aprotinin, and 1 μg/ml leupeptin was added. Binding was allowed to proceed on ice for 90 min. The unbound protein mixture was removed from the beads by centrifugation at 3800 rpm for 5 min at 4°C and by washing the beads sequentially with 200 μl of buffer used for equilibration, 400 μl of the same buffer containing 8-fold lower detergent concentration, and 0.2 mM ATP, and lastly, with 200 μl of the last buffer minus ATP.

The pump was eluted stepwise by incubating the beads twice, each time using 100 μL of low detergent buffer containing 2 mM EDTA instead of CaCl\(_2\). The eluates were pooled, and the pump was trichloroacetic acid-precipitated in the presence of 50 μg of bovine serum albumin as carrier. The precipitates were dissolved in the same electrophoresis sample buffer as above and applied to a SDS-polyacrylamide gel.

**RESULTS**

To identify the region which contains the site(s) for protein kinase C phosphorylation, we used constructs having different portions of the carboxyl terminus. The carboxyl-terminal sequences of hPMCA4b and of these constructs are shown in Fig. 1. Ct92 contains all 28 residues of the calmodulin-binding domain of hPMCA4b but lacks everything downstream of it. Ct71 and ct48 contain additional regions of the carboxyl terminus and are 71 and 48 residues shorter than the full-length enzyme. With these constructs, pumps of exactly known length can be studied in situ in the original membrane. The amount of expression and the size of the mutants were analyzed by SDS-gel electrophoresis followed by immunoblotting. A typical picture is shown in Fig. 2A.

Phosphorylation of each of these constructs is shown in Fig. 2B. Prior to loading the membrane samples on the SDS gel,
they were subjected to phosphorylation by protein kinase C in the presence of \([\gamma^32P]ATP\) at 37°C for the time periods indicated. In the phosphorylation medium, 100 nM TPA was used to ensure the complete activation of the protein kinase at a low Ca\(^{2+}\) concentration and in the absence of phosphatidylserine and diacylglycerol. When compared to the control membrane isolated from cells which were transfected with the empty plasmid, pMM2, each mutant was expressed at a high level. They also migrated with the expected size, which was in between full-length hPMCA4b and ct120. Ct120 is 28 residues shorter than ct92 and is used here as a control construct lacking both the whole calmodulin-binding domain and the rest of the carboxyl terminus. On the autoradiograms strong phosphorylated bands were found associated with the expressed hPMCA4b, ct48, and ct71. At the position of ct92 and ct120, however, only a fuzzy band was found which was formed with a slow time course than the others. This broad band occurred in the same position whether ct92, ct120, or no enzyme (pMM2) was expressed. The lack of specific phosphorylation in ct92 was rather surprising since the threonine in the calmodulin-binding domain had been proposed as being a protein kinase C phosphorylation site. In contrast to the slowly formed bands seen at the position of ct92 and ct120, the phosphorylation of hPMCA4b, ct48, and ct71 was very fast, a strong phosphorylated band corresponding to these constructs appeared within 2 min and reached a maximum by 10 min. The phosphorylation of the membrane proteins was strongly dependent on the presence of protein kinase C and no phosphorylated bands at the position of the hPMCA4b constructs appeared when the kinase was omitted from the phosphorylation medium (a typical experiment is shown in Fig. 6B). In order to prevent the formation of the phosphorylated intermediate of the pump, vanadate was always present when phosphorylation was performed for electrophoretic analysis.

The phosphorylation of the full-length hPMCA4b as compared to that of ct92 and ct120 was further analyzed as a function of protein kinase C concentration. Fig. 3 shows that increasing the amount of protein kinase C in the assay mixture from 7 to 144 milliunits (14–290 ng/ml) produced only a small increase in the phosphorylation of the full-length hPMCA4b. The enzyme was easily phosphorylated with the kinase and a nearly maximum phosphorylation occurred even at the lowest kinase concentration applied. Similar results were obtained when the experiment was repeated with ct48 (not shown). These experiments demonstrated the presence of a high sensitivity phosphorylation site in hPMCA4b. In contrast, the band at the position of ct92 and ct120 did not show significant phosphorylation at low kinase concentrations and a more pronounced increase in their phosphorylation could only be seen at a rather high protein kinase C concentration (144 milliunits, 32463

**Fig. 1.** Carboxyl-terminal sequences of hPMCA4b and the truncated mutants. The mutants are named according to the number of residues cut off at the carboxyl terminus of hPMCA4b.

**Fig. 2.** Phosphorylation of hPMCA4b and the truncated mutants ct48, ct71, ct92, and ct120 with protein kinase C. Panel A shows an immunoblot and panel B shows an autoradiogram of the same immunoblot. In panel A, the faint upper band is the plasma membrane Ca\(^{2+}\) pump endogenous to the COS-1 cells. Since it is the same in all the membranes, it serves as a reference marker. 10 μg of microsomal membrane protein isolated from COS-1 cells transfected with the appropriate construct was phosphorylated in the presence of 36 milliunits of protein kinase C and 100 nM TPA in a 200-μl reaction mixture at 37°C for 2, 10, and 30 min as indicated. PMM2 represents the control membranes that were isolated from cells transfected with empty plasmid. ct120 is a truncated mutant of hPMCA4b which lacks the entire region shown in Fig. 1 and ends at position 1085 before the sequence LRGG (15). In the positions of hPMCA4b, ct48, and ct71, distinct sharp radioactive bands can be seen. These bands match exactly with the bands seen on the corresponding immunoblots. In the positions of ct92 and ct120 fuzzy bands were observed that were indistinguishable from the band seen at the same position of the autoradiogram of control membranes. The phosphorylation pattern shown here is typical of several separate experiments on different membrane preparations.
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Fig. 3. hPMCA4b, unlike ct92 and ct120, is easily phosphorylated with protein kinase C. 10 \(\mu\)g of microsomal membrane isolated from COS-1 cells transfected with hPMCA4b, ct92, or ct120 was phosphorylated with various doses of protein kinase C (7–144 milliunits/200-\(\mu\)l assay) in the presence of 100 nm TPA for 10 min at 37°C. The phosphorylated proteins were separated on SDS-polyacrylamide gel electrophoresis and then immunoblotted. Only the autoradiogram of an immunoblot is shown. The sharp radioactive band in the position of hPMCA4b could be seen at the lowest dose of protein kinase C.

Whether the phosphorylation of the constructs corresponds to the pump protein was examined by loading the phosphorylated samples onto calmodulin-Sepharose in the presence of calcium and eluting the bound pump protein with excess EGTA. Using this method, we could isolate the constructs which have the ability to bind to calmodulin. Fig. 4 shows an immunoblot (panel A) with the corresponding autoradiogram (panel B) of the constructs that bound to the calmodulin-Sepharose and were eluted with EGTA. All constructs except ct120 bound to calmodulin as expected; the failure of ct120 to bind was also expected since it lacks the calmodulin-binding domain. Phosphorylation did not prevent their binding. At an intermediate protein kinase C concentration (36 milliunits) discrete radioactive bands corresponding to hPMCA4b, ct48, and ct71 were seen whereas little or no phosphorylation of ct92 could be detected. At a high kinase concentration ct92 was also labeled. Since ct120 could not be separated from the other membrane proteins by calmodulin-Sepharose, we cannot tell whether under these conditions ct120 was also labeled and whether the labeling of ct92 occurred within the calmodulin-binding domain or somewhere else in the molecule. We can tell, however, that the phosphorylation of this site requires a much higher protein kinase C concentration than the labeling which occurs in ct71, ct48, and hPMCA4b. The labeling in the latter constructs appears to be at a highly specific site in a region downstream of the calmodulin-binding domain.

We then tested the effect of phosphorylation with protein kinase C on the activity of hPMCA4b as compared to that of ct92. Ca\(^{2+}\) uptake by microsomal vesicles was monitored as a function of protein kinase C concentration. Increasing concentrations of the kinase stimulated the basal activity of hPMCA4b and in the presence of a maximum concentration of the kinase the level of the activity of ct92.

Phosphorylation did not prevent binding of the constructs to calmodulin-Sepharose. Microsomal membrane proteins were phosphorylated in the presence of 36 or 144 milliunits of protein kinase C for 10 min at 37°C. Then the samples were cooled down on ice and were incubated with calmodulin-Sepharose 4B in the presence of 1 mM CaCl\(_2\) and 0.4% Triton X-100 and the constructs were eluted with 2 mM EDTA as described under “Materials and Methods.” The eluted proteins were precipitated with trichloroacetic acid and the samples were subjected to SDS-gel electrophoresis and immunoblotting. Panel A shows the immunoblot and panel B shows the autoradiogram of that immunoblot. A typical experiment is shown. Ct120 is used here as a negative control which does not adsorb to the calmodulin-Sepharose.

Fig. 4. Phosphorylation did not prevent binding of the constructs to calmodulin-Sepharose.

Fig. 5. Protein kinase C increases the activity of hPMCA4b to the level of the activity of ct92. Ca\(^{2+}\) uptake by microsomal vesicles isolated from COS-1 cells transfected with hPMCA4b or ct92 was measured as a function of protein kinase C concentration in the presence of 100 nm TPA at 8.1 \(\mu\)M free Ca\(^{2+}\) for 10 min at 37°C. The amount of protein kinase C varied between 7 and 144 milliunits/200-\(\mu\)l assay mixture. Ca\(^{2+}\) uptake by control membrane vesicles isolated from PM2 transfected cells was subtracted from each data point. The maximum Ca\(^{2+}\) uptake was determined in the presence of enough calmodulin (119 nm) to fully activate the enzyme; this activity was between 6 and 10 nmol of Ca\(^{2+}\) (mg of membrane protein)\(^{-1}\), min\(^{-1}\). Data points are average of two independent determinations.

with the kinase was partial and maximum activation occurred only when calmodulin was present. In the presence of calmodulin, on the other hand, no additional stimulation with protein kinase C occurred.

These data indicated that the phosphorylation site(s) downstream of the calmodulin-binding domain might be responsible for the partial activation observed in hPMCA4b. This hypothesis was further analyzed by studying the effect of protein kinase C phosphorylation on the activity of the different constructs. Ca\(^{2+}\) uptake by microsomal vesicles was measured under conditions identical to those used for the \(32^P\) labeling experiments except that a higher ATP concentration was used and vanadate, an inhibitor of the function of the pump, was
The activation of hPMCA4b by protein kinase C (PKC) is specific: Ca\textsuperscript{2+} uptake by microsomal vesicles was measured for 10 min at 37 °C in the presence or absence of 119 nM calmodulin, 50 nM TPA, and 92 milliunits (0.24 μg/ml) protein kinase C in a reaction mixture of 200 μl. The free Ca\textsuperscript{2+} concentration was 7 μM. The average activities (±SD) from different determinations are shown. Ca\textsuperscript{2+} uptake by microsomal vesicles made from pMM2-transfected cells has been subtracted.

| Additions     | Ca\textsuperscript{2+} uptake (nmol Ca\textsuperscript{2+} (ng membrane protein)\textsuperscript{-1} min\textsuperscript{-1}) |
|---------------|-------------------------------------------------|
| None          | 1.26 ± 0.36                                      |
| TPA           | 1.24 ± 0.08                                      |
| PKC           | 1.25 ± 0.14                                      |
| TPA + PKC     | 2.58 ± 0.23                                      |
| CaM           | 5.96 ± 1.29                                      |
| CaM + TPA + PKC | 5.66 ± 1.86                                    |

omitted. The data are summarized in Fig. 6. It is clearly shown that hPMCA4b, ct48, and ct71 were phosphorylated in a strictly protein kinase C-dependent manner (panel B) and this phosphorylation accompanied a substantial increase in the activity of these constructs (panel C). No phosphorylation or significant activation of ct92 could be seen under these conditions. Comparison of the activities obtained for the non-phosphorylated constructs gives another important result: this comparison shows that ct48 was fully inhibited whereas the activity of ct71 was somewhat higher than that of the full-length enzyme but significantly lower than that of ct92. Based on these data we can conclude that the region downstream of the calmodulin-binding domain within the length of ct48 contains the additional determinants needed for full inhibition, and that the activation of hPMCA4b with protein kinase C correlates with the phosphorylation of this downstream inhibitory region. This would explain the observation that either phosphorylation in this region or its removal by mutagenesis induced similar activation of the pump.

**DISCUSSION**

The phosphorylation and activation of the plasma membrane Ca\textsuperscript{2+} pump with protein kinase C has been demonstrated both in vivo and in vitro. The phosphorylation of the erythrocyte pump (that is mainly hPMCA4b) was shown to occur in the carboxyl terminus in a pioneering study by Wang et al. (13), who inferred that threonine 1102, in the middle of the calmodulin-binding region, was one of the sites of phosphorylation, while the other was a serine downstream of the calmodulin-binding region. Their phosphorylation by protein kinase C was carried out on the purified erythrocyte Ca\textsuperscript{2+} pump in the presence of detergent, phosphatidylserine, and a very high Ca\textsuperscript{2+} concentration (1.5 mM), and was incubated for a long time (90 min) at 30 °C. These conditions may have resulted in a less specific phosphorylation than we observed in this study. Moreover, phosphatidylserine is a known activator of the pump which may interfere with the protein kinase C stimulation observed in our study.

Two subsequent studies (14, 18) extended the idea that protein kinase C phosphorylated threonine 1102. These studies were carried out on peptides whose only serine or threonine was the threonine representing threonine 1102. When given such a peptide as substrate, protein kinase C would be expected to phosphorylate the only phosphorylatable residue, so such studies cannot be regarded as evidence that this residue is normally phosphorylated in the intact molecule. The idea that threonine 1102 is a target for protein kinase C has become widespread, and has been repeated in numerous reviews (19–23). The confusion about the subject could arise from the fact that the isolated erythrocyte pump may have different site(s) exposed for phosphorylation than the membrane-bound one and that the phosphorylation studies were done on the isolated pump (13) whereas most of the activity measurements were performed on the membrane-bound enzyme (12). An additional problem is that different protein kinase C isoforms may have different sensitivity for a given phosphorylation site.

This study demonstrates for the first time that the site in hPMCA4b which is most susceptible to protein kinase C phosphorylation is within an autoinhibitory region downstream of the calmodulin-binding domain. To demonstrate this, we used truncated mutants of isoform 4b which contains mainly isoforms α, β1, β2, and γ. We studied the phosphorylation of these mutants in parallel with the effect of this phosphorylation on their Ca\textsuperscript{2+} transport activity in the original membrane environment. Our phospo-

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**TABLE I**

| Additions     | Ca\textsuperscript{2+} uptake (nmol Ca\textsuperscript{2+} (ng membrane protein)\textsuperscript{-1} min\textsuperscript{-1}) |
|---------------|-------------------------------------------------|
| None          | 1.26 ± 0.36                                      |
| TPA           | 1.24 ± 0.08                                      |
| PKC           | 1.25 ± 0.14                                      |
| TPA + PKC     | 2.58 ± 0.23                                      |
| CaM           | 5.96 ± 1.29                                      |
| CaM + TPA + PKC | 5.66 ± 1.86                                    |

**FIG. 6.** Protein kinase C phosphorylates and activates hPMCA4b, ct48, and ct71. 10 μg of microsomal membrane protein isolated from COS-1 cells transfected with the appropriate construct was labeled with 200 μM [γ\textsuperscript{32P}]ATP in the presence or absence of 109 milliunits of protein kinase C and 100 nM TPA in a reaction mixture of 200 μl for 15 min at 37 °C. Panel A shows an immunoblot, whereas panel B shows the autoradiogram of the same immunoblot. Panel C represents Ca\textsuperscript{2+} uptake measurements on the corresponding membrane preparations. Ca\textsuperscript{2+} uptake by the microsomal vesicles was determined under the same conditions as the phosphorylation except that vanadate was omitted from the medium and the ATP concentration was 6 mM. The activity of the control membrane was subtracted from each data point. The maximum transport activity for each sample was determined in the presence of a saturating (119 nM) calmodulin concentration and the activity was expressed as a percent of this maximum. In panel C the data points are average (±S.D.) of four independent determinations on three different membrane preparations.
Phosphorylation was done under more physiological conditions than in the previous study (13) in the following ways: 1) the pump was still in its original membrane, with no detergent or additional phosphatidylserine present; 2) the Ca\(^{2+}\) concentration was much lower; and 3) the incubation time was shorter.

Under our conditions, the full-length pump, ct48, and ct71 (see Fig. 1) all exhibited strong and rapid phosphorylation with protein kinase C in the presence of TPA. The phosphorylated proteins bound to calmodulin-Sepharose and responded to calmodulin with high affinity. A complete activation of the pump occurred in the presence of excess calmodulin and the calmodulin-response curve did not change when the pump was pre-treated with TPA and protein kinase C (data are not shown in detail).

**FIG. 7.** Scheme of the modulation of hPMCA4b activity by protein kinase C. C domain represents the 28-residue calmodulin-binding domain which also serves as an autoinhibitor of the pump. The I domain is a downstream inhibitory region which is a target for protein kinase C phosphorylation. Panel A shows the location of phosphorylation and of calmodulin binding and summarizes their effect on activity. Panel B shows the constructs used in this study and their activity. Comparison of the panels shows how the constructs mimic the effects of biological activation.

**TABLE II**

Comparison of conditions required to phosphorylate hPMCA4b and other putative C-kinase target proteins

| Protein     | The amount of C-kinase used | Conditions | Incubation |
|-------------|----------------------------|------------|------------|
|             | µg/ml                      | µM         | min        | °C         |
| hPMCA4b     | 0.014–0.29                 | 7–144      | 10         | 37         |
| MARCKS, Ref. 26 | 0.19–3.9               | 1500       | 1–20       | 20         |
| Neuromodulin | 0.4                        | 400–600    | 45         | 30         |
| Ref. 27     | 0.15–8                     | EDTA       | 5–40       | 35         |
| Calcineurin, Ref. 29 | 400              | +          | 30         | 30         |

*a One unit is defined as the amount of enzyme that will phosphorylate H1-histone at 1 nmol/min at 22 °C (Calbiochem). The specific activity of protein kinase C varied between 1530 and 2480 units/mg protein. The milliunit given is the amount of enzyme used in 200 µl of assay mixture.
Ct92, a construct that contained the whole calmodulin-binding domain but lacked everything downstream of that domain, was not phosphorylated under similar conditions. A weak phosphorylation of ct92 occurred only at a relatively high concentration of protein kinase C. Interestingly, the phosphorylated form of ct92 also bound to calmodulin-Sepharose and was also stimulated with calmodulin. This contrasted with the behavior of a peptide representing the calmodulin-binding domain phosphorylated on its threonine, which failed to bind calmodulin (14). At present, we are not certain whether the phosphorylation of ct92 occurred within the calmodulin-binding domain or somewhere else in the molecule. The second possibility seems more likely, because a band at the position of ct120 (lacking the whole calmodulin-binding domain) was also phosphorylated at high protein kinase C concentrations, and because of the binding of phosphorylated ct92 to calmodulin.

These data indicate that the most specific phosphorylation site of hPMCA4b must reside within a region that is common to ct71, ct48, and the full-length enzyme, but is missing in ct92. Within this region, that is located downstream of the calmodulin-binding domain between residues 1113 and 1134, there are three serines and a threonine residue which might serve as substrates for protein kinase C. Although no exact consensus phosphorylation site sequences have been defined for protein kinase C, basic residues on either side and a hydrophobic residue on the carboxyl-terminal side of the phosphorylated residue appear to have an important influence on the site specificity of protein kinase C. Serine 1126 may satisfy these criteria in hMPCA4b. Although ct92 did not show significant phosphorylation with protein kinase C we cannot exclude that one of the serine residues at the carboxyl-terminal boundary of the calmodulin-binding domain might act as a substrate in the native enzyme. Studies on the myosin light chain kinase (24) and on a glycogen synthase peptide (25) have indicated that deletion of residues on the carboxyl-terminal side of the phosphorylation site may dramatically reduce the sensitivity of the phosphorylation site. In order to identify the specific phosphorylation site(s) in hPMCA4b, point mutations are being made within the region in question.

Although the phosphorylation did not affect the binding of calmodulin to the pump, it did result in a partial activation of the enzyme when its activity was tested in the absence of calmodulin. Maximum activation could only be achieved when excess calmodulin was added. A scheme explaining the possible mechanism by which protein kinase C and calmodulin regulate hPMCA4b is shown in Fig. 7. As indicated in this figure, the phosphorylation site in hPMCA4b corresponds to an autoinhibitory region (I-domain) that is separate from the C domain. The properties of the truncated mutants, i.e. that ct48 is fully inhibited whereas ct92 is only partially, suggest that this autoinhibitory region is located downstream of the C domain between residues 1113 and 1157. As previous work (4) has indicated, this region has no essential role in calmodulin binding; it rather strengthens the inhibition caused by the C domain and thus, modifies the basal activity of the enzyme. Removal of the I-domain, producing the construct ct92, mimicked the effect of phosphorylation and a partial activation occurred. This observation is consistent with the proposal that phosphorylation by protein kinase C causes the release of the I domain from its inhibition without affecting the inhibition by the C domain. A partial activation of the pump by protein kinase C and TPA has been reported (12) but these authors reported that the activation by protein kinase C and calmodulin were additive. According to the scheme presented here maximum activation occurs when both the C and I domains are removed from inhibition. This state is represented by ct120, a truncated mutant that lacks these regions. Alternatively, binding of calmodulin alone removes both the C and I domains from their inhibition and causes the same activation as phosphorylation with protein kinase C followed by calmodulin binding.

Finally, many protein phosphorylation studies have used a large amount of protein kinase, a long incubation time and/or a high substrate or activator concentration, which allowed the phosphorylation of many targets which may or may not represent physiological substrates. Therefore, we compared the conditions needed for phosphorylation of hPMCA4b and other putative protein kinase C target proteins (Table II). Although the conditions used for phosphorylation of these proteins are quite different, it should be obvious from the table that among them hPMCA4b is the most susceptible target for protein kinase C phosphorylation.

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