Protein Kinase C Phosphorylates the “a” Forms of Plasma Membrane Ca\(^{2+}\) Pump Isoforms 2 and 3 and Prevents Binding of Calmodulin*  

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Phosphorylation by protein kinase C of the “a” and “b” variants of plasma membrane Ca\(^{2+}\) pump isoforms 2 and 3 was studied. Full-length versions of these isoforms were assembled and expressed in COS cells. Whereas the “a” forms were phosphorylated easily with PKC, isoform 2b was phosphorylated only a little, and isoform 3b was not phosphorylated at all. Phosphorylation of isoforms 2a and 3a did not affect their basal activity, but prevented the stimulation of their activity by calmodulin and their binding to calmodulin-Sepharose. This indicated that phosphorylation prevented activation of these isoforms by preventing calmodulin binding. Based on these results, phosphorylation of the pump with PKC would be expected to increase free intracellular Ca\(^{2+}\) levels in those cells where isoforms 2a and 3a are expressed.

The plasma membrane Ca\(^{2+}\) pump is an important element in removing Ca\(^{2+}\) from the cell during intracellular signaling and in maintaining the very low resting level of cytosolic Ca\(^{2+}\) of the unstimulated cell. The pump is known to be activated in many different ways: by calmodulin, acidic phospholipids, phosphorylation with protein kinases, proteolysis, and dimerization. Although the mechanisms of all of these regulations have not been determined, the way calmodulin stimulates the pump is fairly well understood. Calmodulin binds tightly to a specific domain that is 92 amino acids upstream of the carboxyl terminus of hPMCA4b (1, 2). This domain is about 28 residues long in hPMCA4b and constitutes part of an autoinhibitory region (3, 4). Binding of calmodulin to the autoinhibitor region prevents the inhibition and activates the pump. Although phosphorylation and/or stimulation of the pump with PKC has been described in various cells and tissues (5–11), details of the molecular basis of PKC action have been difficult to obtain. Until very recently the subjects available for study have been intact cells or biological membranes of unknown isoform composition; only in the case of the erythrocyte membrane had the isoform composition of the pump been determined, consisting primarily of hPMCA4b.

Cloning of the pump has revealed the existence of at least four different genes coding for the plasma membrane Ca\(^{2+}\) pump. PMCA1 and PMCA4 are widely expressed, whereas PMCA2 and PMCA3 are more specialized forms which are expressed primarily in brain, skeletal muscle, and heart. Alternative splices at two different sites raise the number of possible pump isoforms to more than 20. One of the alternative splices occurs at the C hot spot near the middle of the calmodulin-binding domain; this splice changes the carboxyl-terminal third of the calmodulin-binding domain and the rest of the regulatory region (12). We have shown that this alternate splice in hPMCA4 changed the structure of the calmodulin-binding domain and the autoinhibitory region, and as a result, hPMCA4a had a higher basal activity and a much lower calmodulin affinity than hPMCA4b (13, 14).

Studies on the erythrocyte Ca\(^{2+}\) pump (which is mainly hPMCA4b) indicated that phosphorylation by PKC occurs at its carboxyl terminus (10). These studies inferred that threonine 1102, in the middle of the calmodulin-binding domain, was one of the sites of phosphorylation. Subsequent studies on synthetic peptides suggested that phosphorylation of this residue would prevent binding of calmodulin to the pump while it would cause a calmodulin-like activation (15, 16). Since the part of the calmodulin-binding domain that contains this threonine residue is conserved in all isoforms, this model predicted that PKC would regulate the more than 20 isoforms uniformly.

Recently, however, we showed (utilizing truncated mutants) that the site in hPMCA4b most readily phosphorylated by PKC occurs in a different location, downstream of the calmodulin-binding domain (11). The region where PKC phosphorylated the pump under mild conditions was part of the autoinhibitory region but was not involved in calmodulin binding. Thus, phosphorylation at this site caused partial activation of the pump, and full activation occurred when calmodulin bound to the phosphorylated enzyme. A construct that lacked this site but contained the calmodulin-binding domain became phosphorylated only at a relatively high PKC concentration. Whether this phosphorylation occurred at threonine 1102 is being studied by further mutations. Another important finding of our study was that phosphorylation did not prevent binding of calmodulin, i.e. the phosphorylated form of hPMCA4b bound to calmodulin-Sepharose.

The most readily phosphorylated region of hPMCA4b is located downstream of the “hot spot” where the alternate splice of the mRNA occurs. This region of the molecule is highly variable. Inspection of the sequences in the corresponding regions of isoforms 2 and 3 of PMCA indicates that they have different candidate sequences for phosphorylation with protein kinases and that phosphorylation of them might have different consequences on their activity.

Among the pump isoforms, hPMCA4b has been studied ex-
tensely, but little is known about the other forms. Other isoforms of the pump have been expressed in COS cells only recently, which has made it possible to study their unique properties (17, 18). In the study presented here, we examined the phosphorylation with PKC of isoforms rPMCA2a, -2b, -3a, and -3b. While isoforms rPMCA2a and 3a, like hPMCA4b, appeared to be very sensitive to PKC phosphorylation, rPMCA2b was phosphorylated only a little and rPMCA3b was not phosphorylated at all. Unlike the case of hPMCA4b, phosphorylation of rPMCA2a and -3a prevented their binding to calmodulin-Sepharose. While phosphorylation with PKC did not affect the basal activity of rPMCA2a and -3a, it prevented stimulation of the activity by calmodulin.

MATERIALS AND METHODS

**45Ca and [γ-32P]ATP** were purchased from NEN Life Science Products. Calmodulin and calmodulin-Sepharose were obtained from Sigma. PMA and rat brain PKC (containing isoforms α, β, β2, and γ) were purchased from Calbiochem. The specific activity of the PKC preparation was 1130 units/mg of protein. LipofectAMINE™, Opti-MEM, and restriction enzymes were obtained from Life Technologies, Inc.

**Construction of the Full-length rPMCA3a—**Full-length versions of rPMCA2a were constructed as described in Elwess et al. (18). The full-length rPMCA2a isoform in the pBR322 vector was a gift from Dr. G. Shull (University of Cincinnati). It was cloned into the expression vector pMM2 also as described by Elwess et al. (18).

**Construction of the Full-length rPMCA3a—**The full-length rPMCA3a isoform in the pBR322 vector was also a gift from Dr. Shull. A 5′ forward polymerase chain reaction primer (GGGATCATAGGTGTC- GACCCGCCTC) containing a SalI site and a 3′ reverse primer (CTGAAGGAGTTACCTGTGTTG) containing a KpnI site amplified the rPMCA3a cDNA. This was done using the GeneAmp kit (Perkin-Elmer); a total volume of 100 μl was used with 900 ng of template being added per sample. The reaction (in a Perkin-Elmer 9600 thermal cycler) was initiated with a 2-min melting step at 94 °C for 1 min, 52 °C for 2 min, and 72 °C for 2 min, with a final 5-min extension step at 72 °C. The expected product was ~2.9 kilobases. The full-length rPMCA3a DNA was cloned into the pMM2 expression vector at the SalI and KpnI sites. **Construction of the Full-length rPMCA3b—**rPMCA3a cDNA was digested with SalI and BamHI and ligated into the pUC19 vector at these sites; XL-1 Blue competent cells (Stratagene) were used for the transformation. Amplification was done to produce the carboxyl terminus of the pump, the “a” and “b” forms of the pump, the “a” and “b” forms of the pump, was also expressed. The full-length rPMCA2 and rPMCA3 clones were generously given to us by Dr. G. Shull, University of Cincinnati, and we incorporated them into the expression vector pMM2. Figure IA shows an immunoblot of the expressed proteins. To visualize the pump isoforms we used monoclonal antibody 5F10, which has been recognized to recognize all isoforms of the PMCA family. As judged from the staining intensity and the migration pattern of the isoforms, the level of expression was nearly the same and the size corresponded well to the expected molecular mass which has been calculated from the protein sequences of each isoform. An endogenous PMCA, isoform 1b, is also present in COS cell membranes, but it does not interfere with any of the measurements reported here. It doesn’t interfere because, in electrophoresis, it migrates slower than any of the other isoforms and is separated from the bands corresponding to the overexpressed pump proteins. That no staining at the position of PMCA1b is seen shows that it represents only a minor component of these membranes.

**Phosphorylation of Microsomal Membrane Proteins with PKC—**10 μg of microsomal membrane was phosphorylated basically as described (11). The 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 milliunits (0.0875 μg/ml) of PKC and 100 mM PMA, and the reaction was started by the addition of 20 μl [γ-32P]ATP. After a 5-min incubation the reaction was terminated by the addition of 1 ml of ice-cold 6% trichloroacetic acid containing 1 mM ATP and 10% 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, 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 PMCA Isoforms to Calmodulin-Sepharose—**This was also done as described previously (11), with some modifications. Briefly, the phosphorylation reaction described above was terminated by putting the samples on ice, and subsequently, 40 μl of extraction solution (11) was added. The mixture was incubated on ice for 15 min. Then, 50 μl of calmodulin-Sepharose beads were introduced 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 μl of 5 mM Tris-HCl, pH 7.2, 0.8 M NaCl, 0.1% SDS. That no staining at the position of PMCA1b is seen shows that it represents only a minor component of these membranes.

**Gel Electrophoresis, Electrottransfer, and Autoradiography—**This was done as described (11). Western blots were immunostained using antibody 5F10 which reacts with all of the pump isoforms (21).
Phosphorylation of rPMCA2a and -3a with Protein Kinase C

Fig. 1. Phosphorylation of the "a" and "b" versions of rPMCA2 and -3 with PKC. 10 µg of microsomal membrane proteins isolated from COS cells transfected with isoforms 4b, 2b, 2a, 3b, and 3a were phosphorylated with PKC. 2 µg of each sample were separated on SDS-polyacrylamide gel and immunoblotted. Panel A shows 5F10 staining and panel B an autoradiogram of the same blot. In panel C, the amount of phosphorylation is quantitated using the Molecular Dynamics STORM system and expressed as percent of the phosphorylation of hPMCA4b which is used in these experiments as control. The phosphorylation pattern shown in this figure is typical of three different experiments.

Fig. 2. Binding of the phosphorylated isoforms 2a, 3a, and 4b to calmodulin-Sepharose. Phosphorylation of 10 µg of membrane samples was carried out as described in the legend of Fig. 1 and binding of the enzyme to calmodulin-Sepharose as described under "Materials and Methods." The bound material was removed from the calmodulin-Sepharose by incubating the beads with the electrophoresis sample buffer, and the beads were separated from the samples by centrifugation. An aliquot of each sample was applied onto the SDS-polyacrylamide gel, and the proteins were electrophoresed and electroblotted. The blots were immunostained with monoclonal antibody 5F10 (panel A) and autoradiographed (panel B). The amount of phosphorylation was quantitated using the Molecular Dynamics STORM system, and the data are shown in panel C. These data are expressed as percent of the phosphorylation of hPMCA4b and are typical of three independent determinations.

Fig. 1B is an autoradiogram of that immunoblot. In panel C of Fig. 1 the amount of phosphorylation is related to that of hPMCA4b as control. Strong phosphorylated bands were found associated with the expressed hPMCA4b, rPMCA2a, and rPMCA3a isoforms. In contrast, the phosphorylation of rPMCA2b was much weaker, and no phosphorylated band at the position of rPMCA3b was found. It is important to emphasize that no additional phosphorylation of the pump isoforms was observed even at much higher PKC concentrations, at which the phosphorylation of the other membrane proteins became more pronounced (not shown).

Whether phosphorylation of 2a and 3a with PKC affects calmodulin binding was tested by loading the phosphorylated proteins onto calmodulin-Sepharose in the presence of Ca2+. Whether phosphorylation of 2a and 3a with PKC affects calmodulin binding was tested by loading the phosphorylated proteins onto calmodulin-Sepharose in the presence of Ca2+. Whether phosphorylation of 2a and 3a with PKC affects calmodulin binding was tested by loading the phosphorylated proteins onto calmodulin-Sepharose in the presence of Ca2+. Whether phosphorylation of 2a and 3a with PKC affects calmodulin binding was tested by loading the phosphorylated proteins onto calmodulin-Sepharose in the presence of Ca2+. Whether phosphorylation of 2a and 3a with PKC affects calmodulin binding was tested by loading the phosphorylated proteins onto calmodulin-Sepharose in the presence of Ca2+. Whether phosphorylation of 2a and 3a with PKC affects calmodulin binding was tested by loading the phosphorylated proteins onto calmodulin-Sepharose in the presence of Ca2+. Whether phosphorylation of 2a and 3a with PKC affects calmodulin binding was tested by loading the phosphorylated proteins onto calmodulin-Sepharose in the presence of Ca2+. Whether phosphorylation of 2a and 3a with PKC affects calmodulin binding was tested by loading the phosphorylated proteins onto calmodulin-Sepharose in the presence of Ca2+.
beads. That a substantial amount of nonphosphorylated 2a and 3a could be recovered from the calmodulin-Sepharose beads shows that phosphorylation of the isoforms was not complete. From a previous experiment (22), we concluded that a large proportion of the membrane vesicles have a right-side-out orientation. In these vesicles, PKC cannot reach the cytoplasmically oriented regions of the pump, leaving a substantial nonphosphorylated portion of isoforms 2a and 3a. When solubilized, these regions become readily accessible and will bind to the calmodulin-Sepharose beads.

We tested the effect of phosphorylation with PKC on the activity of rPMCA2a and -3a in the presence and absence of calmodulin. Since the conditions for phosphorylation were similar to those of Ca2+ transport, phosphorylation with PKC was allowed to occur during the Ca2+ transport assay. In similar experiments we have shown that PKC activated hPMCA4b only partially and that maximum activation occurred when experiments we have shown that PKC activated hPMCA4b. Since the sequence around this threonine is conserved in all isoforms, if it were a phosphorylation site, hPMCA4b, rather phosphorylation of these isoforms prevented stimulation by calmodulin. This agreed well with the finding that phosphorylation of both rPMCA2a and -3a prevented binding by calmodulin. Since the conditions for phosphorylation were similar to those of Ca2+ transport, phosphorylation with PKC was allowed to occur during the Ca2+ transport assay. In similar experiments we have shown that PKC activated hPMCA4b. Since the sequence around this threonine is conserved in all isoforms, if it were a phosphorylation site, hPMCA4b, rather phosphorylation of these isoforms prevented stimulation by calmodulin. This agreed well with the finding that phosphorylation of both rPMCA2a and -3a prevented binding by calmodulin. Since the conditions for phosphorylation were similar to those of Ca2+ transport, phosphorylation with PKC was allowed to occur during the Ca2+ transport assay. In similar experiments we have shown that PKC activated hPMCA4b. Since the sequence around this threonine is conserved in all isoforms, if it were a phosphorylation site, hPMCA4b, rather phosphorylation of these isoforms prevented stimulation by calmodulin. This agreed well with the finding that phosphorylation of both rPMCA2a and -3a prevented binding by calmodulin.

In conclusion, we show here for the first time that PKC regulates isoforms 2a and 3a of the plasma membrane Ca2+ pump in an entirely different way from the regulation seen in isofrom 4b. The carboxyl-terminal regulatory regions of the isoforms studied are shown in Fig. 4. As marked in the figure, in hPMCA4b the most easily phosphorylatable site(s) lie outside of the 28-residue calmodulin-binding domain, and thus, phosphorylation does not affect binding of calmodulin. In hPMCA4a the calmodulin-binding domain is twice as long as in hPMCA4b (14), and the calmodulin-binding domains of the 2a and 3a isoforms are probably also long. Inspection of the sequences at the carboxyl terminus suggests that there are several candidate sequences for PKC phosphorylation in rPMCA2a and -3a within this longer domain. The phosphorylation site(s) in these isoforms are yet to be determined but, based on the interference of phosphorylation with calmodulin-binding, they are expected to lie within the calmodulin-binding domain. Little or no phosphorylation by PKC was found in the “b” forms of rPMCA2 and -3. This finding provides additional evidence against the widely accepted threonine (in the middle of the calmodulin-binding domain) as a phosphorylation site. Since the sequence around this threonine is conserved in all isoforms, if it were a phosphorylation site, hPMCA4b, rPMCA2b, and rPMCA3b would all be expected to be equally good substrates for PKC phosphorylation. Another important finding of our study was that PKC did not affect the basal activity of rPMCA2a and -3a. On the contrary, by inhibiting the binding of calmodulin it prevented calmodulin stimulation of the activity of these isoforms. Thus, PKC would inhibit the activity of these pumps in the cell. That inhibition would increase the intracellular Ca2+ during Ca2+ signaling in those cells where rPMCA2a and -3a are expressed. Since in brain both calmodulin and rPMCA2a are abundant, the unique regulation of this Ca2+ pump isoform is expected to have a great significance. Our studies indicate that the regulation of the plasma membrane Ca2+ pump with alternative RNA splicing, calmodulin, and PKC is more complex than has generally been believed.

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