Plasma Membrane Ca\(^{2+}\) Pump Isoforms 2a and 2b Are Unusually Responsive to Calmodulin and Ca\(^{2+}\)*

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The full-length a and b variants of the rat plasma membrane calcium pump, isoform 2 (rPMCA2a and rPMCA2b), were constructed and expressed in COS-7 cells. To characterize these isoforms, calcium transport was determined in a microsomal fraction. Both rPMCA2a and rPMCA2b had a much higher affinity for calmodulin than the corresponding forms of hPMCA4, and rPMCA2b had the highest affinity among the isoforms that have been tested so far. When analyzed at a relatively high calmodulin concentration, rPMCA2b and, to a lesser extent, rPMCA2a showed higher apparent calcium affinity; i.e., they were more active at lower Ca\(^{2+}\) concentrations than hPMCA4b. This indicates that these two variants of rat isoform 2 will tend to maintain a lower free cytosolic Ca\(^{2+}\) level in cells where they are expressed. Both variants also showed a higher level of basal activity (in the complete absence of calmodulin) than hPMCA4b, a property which would reinforce their ability to maintain a low free cytosolic Ca\(^{2+}\) concentration. Experiments designed to determine the source of the higher apparent Ca\(^{2+}\) affinity of rPMCA2b showed that it came from the properties of the carboxyl terminus, rather than from any difference in the catalytic core.

The plasma membrane Ca\(^{2+}\) pump plays a key role in controlling the intracellular Ca\(^{2+}\) concentration. This P-type ATPase is regulated by calmodulin and is responsible for the ATP powered removal of Ca\(^{2+}\) from eukaryotic cells (1). The plasma membrane Ca\(^{2+}\) pump (PMCA)\(^1\) has a low level of activity in the absence of calmodulin. Calmodulin binds to an autoinhibitory domain (the C domain), and increases both the maximum velocity of the pump and the apparent Ca\(^{2+}\) affinity.

To date, at least four different genes have been found which encode for PMCA (2). Additional variability is obtained by alternate splices occurring at two sites in the pump (3–7). In each of the four genes, the alternative splice sites (8) are located in the middle of the cytosolic loop between transmembrane domains 2 and 3 (splice site A) (9) and downstream of the last transmembrane domain, in the middle of the calmodulin-binding domain (splice site C) (9–11). The first 18 amino acids of the calmodulin-binding domain are conserved for all PMCA isoforms, but the presence of the alternative RNA splice site in the middle of this region (at splice site C) changes the remainder of the calmodulin-binding domain as well as the carboxyl terminus (10). The isoforms whose mRNA contains a spliced-in exon are called “a,” while those isoforms lacking the additional exon are called “b.” The a variants of the isoforms have a less basic calmodulin-binding domain as well as a different carboxyl terminus than the b variants. When synthetic peptides corresponding to representative a and b forms of the calmodulin-binding domain were compared, the b form of the peptide showed a 10-fold higher affinity for calmodulin than the a form of the peptide (12). Additionally, full-length isoforms hPMCA4a and hPMCA4b were overexpressed in COS-1 cells and the calmodulin-response curves were analyzed. As expected, the hPMCA4b isoform had a higher affinity for calmodulin than the hPMCA4a isoform (13).

In this study, we have compared isoforms 2a and 2b of the plasma membrane Ca\(^{2+}\) pump of the rat with the most widely studied isoform to date, isoform 4b from human tissues. The exact isoforms utilized were rPMCA2ax and rPMCA2bx. In this nomenclature, the last letter (z in this case) refers to a splice at site A which changes only a small region of the enzyme because it does not involve a frameshift (5). We did not compare different alternatively spliced products in this region, and so we will not discuss its properties further. In the remainder of this paper we will omit the z since both enzymes studied were of this form. We utilized the rat message instead of human partly because the DNAs were more easily available, but also because we anticipate that many future studies in different laboratories will be carried out with rat tissues. Therefore, utilizing the rat enzymes will allow the development of antibodies and other reagents which will contribute to a coordinated attack on discovering the properties of the different isoforms of the pump in rat. In the case of the present study, the rat enzymes are a good substitute for the human ones since their amino acid sequences are nearly the same. rPMCA2ax and hPMCA2ax are 97.9% identical when aligned, and rPMCA2bx and hPMCA2bx are 98.1% identical. Thus, almost all of the differences in properties can be attributed to the differences in the two genes which are being compared, rather than to the differences in species. This is evident when one observes that rPMCA2ax is only 75.7% identical with hPMCA4b and that rPMCA2bx is 75.4% identical with hPMCA4b. Because of these relationships, we will generally not mention the species differences in our discussion, but will focus on the different gene products and the alternative

\(^1\) The plasma membrane calcium pump is referred to by a name such as hPMCA4b, where h indicates the species, in this case human, PMCA indicates the plasma membrane calcium pump, 4 refers to the product of the fourth gene, and b indicates which alternate splice was used. A recently proposed system (22) describes alternate splices by capital letter, denoting which site (A or C) is spliced, and by Roman numeral I and II, indicating which axon is inserted. For this paper, we will not be using this proposed system.

\(^2\) The abbreviations used are: PMCA, plasma membrane Ca\(^{2+}\) pump; PCR, polymerase chain reaction; bp, base pair(s).

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splice in the downstream region.

Because rPMCA2a and rPMCA2b come from a different gene than hPMCA4, some of the differences between 2 and 4 are scattered throughout the molecule. However, large stretches of the molecule are very highly conserved and the specific sequence differences are concentrated in three regions: the amino terminus, the carboxyl terminus, and a region near the upstream alternative splice.

A recent paper (14) described the expression of hPMCA2b in insect cells and the effect of an alternate splice at site A. Although the authors found substantial differences between gene products 2 and 4 they did not find any difference in the characteristics of the isoforms of hPMCA2 produced by this alternate splice. In the present paper, we report the overexpression of full-length rPMCA2b and rPMCA2a isoforms from rat in COS-7 cells. rPMCA2a is produced by an alternate splice at site C which changes the calmodulin-binding domain and the rest of the carboxyl terminus of rPMCA2 and is expected to have specific regulatory characteristics. Unlike the case for the alterations at site A, we find substantial differences in properties caused by the alternate splice at site C. This report will focus on the functional properties of these two isoforms and how they compare with the most widely studied isoform to date, isoform 4b from human.

MATERIALS AND METHODS

Chemicals—**CaCl**₂ was purchased from NEN Life Sciences Products, LipofectAMINE, Opti-MEM, and restriction enzymes were obtained from Life Technologies, Inc. Calmodulin was purchased from Calbiochem. LipofectAMINE, Opti-MEM, and restriction enzymes were obtained from Life Technologies, Inc. DNA-LiptofectAMINE complex for each flask was prepared by incubating 8 µg of DNA and 100 µl of LipofectAMINE in 3.6 ml of serum-free Opti-MEM for 30 min at room temperature. The cells were incubated with the DNA-LiptofectAMINE complex in 14.5 ml of serum-free Opti-MEM for 5 h at 37 °C, then supplemented with serum and the incubation continued for a total of 24 h. The DNA-LiptofectAMINE-containing medium was then replaced with fresh tissue culture medium with 10% serum and the cells were incubated at 37 °C for an additional 24 h.

Isolation of Microsomes from COS Cells—Crude microsomal membranes were prepared as described by Verma et al. (15). To study the ATP-dependent Ca²⁺ transport activity of rPMCA2a and rPMCA2b in COS Cells, rPMCA2a and rPMCA2b isoforms, the full-length clones were overexpressed in COS cells. These isoforms are identical up to residue 1095, but downstream of this residue the alternative splice changes the calmodulin-binding domain for each isoform. The carboxyl terminus of rPMCA2a is shorter, so that the protein produced has 1154 amino acids, while the rPMCA2b protein has 1195 amino acids (16).

Crude membranes from COS cells transfected with cDNA encoding hPMCA4a, hPMCA4b, rPMCA2a, and rPMCA2b were prepared, solubilized, and their protein (1 µg) separated by 7.5% SDS-polyacrylamide gel. Fig. 1 shows a Western blot using the monoclonal antibody 5F10 to visualize rPMCA2a, rPMCA2b, hPMCA4a, and hPMCA4b. The estimated molecu-
lar weight for isoforms rPMCA2a and rPMCA2b corresponded to the expected molecular mass for each isoform based on their protein sequences. The level of expression was nearly the same for all four isoforms.

**Determination of Ca**\(^{2+}\) **Transport Activities in Microsomes from** rPMCA2a, rPMCA2b, and hPMCA4b-transfected COS Cells—Microsomal membranes isolated from rPMCA2a, rPMCA2b, and hPMCA4b transfected cells were assayed in the presence of thapsigargin and oligomycin to inhibit the activity of the endogenous endoplasmic reticulum Ca**\(^{2+}\) pump and the mitochondrial ATPase as described (16). Table I shows the activity of Ca**\(^{2+}\)** uptake by the microsomal membranes from rPMCA2a, rPMCA2b, and hPMCA4b transfected COS cells in the absence and presence of calmodulin (540 nM). Both rPMCA2a and rPMCA2b had higher basal activity than hPMCA4b when tested in the absence of calmodulin. The activity when measured at saturating calmodulin and Ca**\(^{2+}\)** concentrations was nearly the same, with only small variations due to variations in the expression level.

The calmodulin dependence of each isoform was observed by measuring its Ca**\(^{2+}\)** transport activities at a fixed Ca**\(^{2+}\)** concentration as a function of calmodulin concentration. The calmodulin response curves of the isoforms are compared in Fig. 2. RPMCA2b showed the highest sensitivity to calmodulin; this isoform was over four times more sensitive to calmodulin than hPMCA4b. These results were consistent with those of Hilfiker et al. (13) when they studied these pumps expressed in insect cells and measured the Ca**\(^{2+}\)**-dependent ATPase activity. We also included the calmodulin response curve of isoform rPMCA2a. Although rPMCA2a had somewhat lower affinity for calmodulin than rPMCA2b, it still showed much higher affinity than the corresponding 4 isoform, hPMCA4a (see Enyedi et al. (15)).

A crucial element in the behavior of the plasma membrane Ca**\(^{2+}\)** pump is the affinity it has for calcium. Consequently, to study the characteristics of the isoforms, the Ca**\(^{2+}\)** transport activities were tested by analyzing the dependence of Ca**\(^{2+}\)** uptake on free Ca**\(^{2+}\)** in the presence or in the absence of calmodulin. In Fig. 3, the Ca**\(^{2+}\)** transport activities of rPMCA2a and rPMCA2b at 540 nM calmodulin are compared with those of hPMCA4b. Although 540 nM calmodulin gave nearly full activation at saturating Ca**\(^{2+}\)** for each isoform, the apparent Ca**\(^{2+}\)** affinities of the isoforms were different. RPMCA2b, and to a lesser extent rPMCA2a, were more responsive to Ca**\(^{2+}\)** stimulation than hPMCA4b (Fig. 3). Both rPMCA2a and rPMCA2b showed higher activities at lower Ca**\(^{2+}\)** concentrations.

To determine whether the higher apparent Ca**\(^{2+}\)** affinity of rPMCA2b came from a difference in the catalytic core of the enzyme, we investigated whether the Ca**\(^{2+}\)** responsiveness of hPMCA4b could be brought up to the level displayed by rPMCA2b. We did this by comparing ct120, a constitutively activated form of hPMCA4b, with rPMCA2b. Ct120 is made from hPMCA4b by removal of the regulatory carboxyl terminus, and is fully activated without calmodulin (16). Fig. 4A compares the Ca**\(^{2+}\)** responses of rPMCA2b and hPMCA4b, in the presence of enough calmodulin to fully activate the enzyme. The error bars represent the standard errors of the mean. The K**\(_{V_C}\)** values were: hPMCA4b, 9.8 nM; rPMCA2a, 8.4 nM; rPMCA2b, 2.1 nM.

| Type of transfection | Ca**\(^{2+}\)** transport | − Calmodulin | + Calmodulin |
|----------------------|---------------------------|-------------|-------------|
| rPMCA2a              | 3.67 ± 0.83               | 7.83 ± 1.78 |
| rPMCA2b              | 4.21 ± 1.52               | 5.87 ± 2.18 |
| hPMCA4b              | 1.45 ± 0.51               | 6.15 ± 0.71 |

**FIG. 2.** rPMCA2b is more sensitive to calmodulin stimulation than hPMCA4b. The calmodulin dependence of Ca**\(^{2+}\)** uptake by microsomal vesicles isolated from COS-7 cells transfected with hPMCA4b (open triangles), rPMCA2a (diamonds), or rPMCA2b (circles) is shown. Ca**\(^{2+}\)** uptake by vesicles made from pMM2-transfected cells has been subtracted from all results. The free Ca**\(^{2+}\)** concentration was 10 μM.

**FIG. 3.** Ca**\(^{2+}\)** concentration dependence of Ca**\(^{2+}\)** transport by hPMCA4b, rPMCA2a, and rPMCA2b in the presence of 540 nM calmodulin. Ca**\(^{2+}\)** uptake by microsomal vesicles was measured as a function of free Ca**\(^{2+}\)**. The methods and symbols are the same as for Fig. 2, except that the percent of maximum activity was (v/V**\(_C\)**) × 100 and the lines represent the best fit to the data given by the Hill equation. Each point represents three to four experiments on different membrane preparations. The K**\(_{V_C}\)** values were: hPMCA4b, 0.16 μM; rPMCA2a, 0.09 μM; rPMCA2b, 0.06 μM.
in the absence of calmodulin both rPMCA2a and rPMCA2b were 3–4 times more responsive to Ca$^{2+}$ stimulation than hPMCA4b. In addition, in the absence of calmodulin, the $V_{\text{max}}$ for rPMCA2b was much higher than for rPMCA2a or hPMCA4b. It was 71% of the maximum activity whereas in the case of rPMCA2a and hPMCA4b it was 46 and 23%, respectively.

Table I also shows that the basal activity of rPMCA2b was the highest among the isoforms. In the presence of calmodulin hPMCA4b had a maximum velocity over four times above its basal level, while rPMCA2a and rPMCA2b had maximum velocities only 2 and 1.4 times, respectively, over the basal level (Fig. 5, Table I). Hilfiker et al. (14) also had similar results with the human PMCA2 isoform, which they discussed in connection with the possibility of incomplete removal of the endogenous calmodulin.

To address this concern, we did additional experiments to determine whether the endogenous calmodulin was removed during the preparation of the microsomal membranes. In Fig. 5B, the dependence of Ca$^{2+}$ uptake on free Ca$^{2+}$ by rPMCA2b in the absence of calmodulin was measured again. This time the membrane was preincubated with or without EGTA for 10 min at 37 °C prior to the start of the Ca$^{2+}$ uptake assay without calmodulin. The slight decrease in the activity of rPMCA2b indicates that only a small amount of calmodulin was removed by the EGTA treatment. The second experiment addressing the high basal level of rPMCA2b was done using synthetic peptide C28R2. This synthetic peptide corresponds to the 28 residues that make up the calmodulin-binding domain in isoform rPMCA2b, and binds calmodulin very tightly, with a $K_d$ of about 0.1 nM (12).

FIG. 4. A, a fully activated construct from hPMCA4b is equally sensitive to Ca$^{2+}$ as is rPMCA2b. The activity of rPMCA2b and hPMCA4b with calmodulin and of the construct ct120 are shown. Measurements and symbols are as in Fig. 3, except that inverted triangles represent ct120. Data points are from assays using three to four different membrane preparations. B, calmodulin dependence of rPMCA2b at 61 nM free Ca$^{2+}$. The conditions were like those used for Fig. 2, except for the much lower free Ca$^{2+}$.

FIG. 5. A, Ca$^{2+}$ concentration dependence of Ca$^{2+}$ transport in the absence of calmodulin. The $K_{i\text{Ca}}$ values were: hPMCA4b, 1.06 μM; rPMCA2a, 0.33 μM; and rPMCA2b, 0.25 μM. In the absence of calmodulin the $V_{\text{max}}$ values were: hPMCA4b 24 ± 2%; rPMCA2a, 47 ± 6%; rPMCA2b, 72 ± 3%. B, effect of EGTA incubation on Ca$^{2+}$ concentration dependence of rPMCA2b without calmodulin. Ca$^{2+}$ uptake was measured as in Fig. 3. Data points are from assays using three to four different membrane preparations. Circles, no EGTA; triangles with EGTA. The $K_{i\text{Ca}}$ values were: without incubation, 0.25 μM; with EGTA incubation, 0.24 μM. The $V_{\text{max}}$ values were 72 ± 3% without EGTA and 62 ± 3% with EGTA.

The final experiment of this set involved the use of an anti-calmodulin antibody to test for the presence of calmodulin in the microsomal membrane. Equal amounts of hPMCA4b and rPMCA2b microsomal fractions were immunoblotted and stained with an anti-calmodulin antibody following SDS-gel electrophoresis. No calmodulin was detected in either sample (results not shown). Unfortunately, the significance of this result was limited by the insensitivity of the anti-calmodulin
antibody. The results obtained in the experiments using EGTA incubation, C28R2, and the anti-calmodulin antibody indicated that we had been successful in removing endogenous calmodulin and that the high basal level in rPMCA2b is an intrinsic property of the enzyme.

**DISCUSSION**

This paper analyzed the characteristics of isoforms rPMCA2b and rPMCA2a and compared them to those of the more widely studied form, hPMCA4b. A recent study (14) has expressed, in insect cells, hPMCA2b (which they called PMCAIIA) and variants of gene 2 at splice site A and studied has expressed, in insect cells, hPMCA2b (which they called PMCAIIA) and variants of gene 2 at splice site A and studied was capable of a Ca\(^{2+}\) affinity just as high as that of rPMCA2b. This indicated that the difference in the Ca\(^{2+}\) affinity comes from the difference in the carboxyl terminus of these isoforms. Hilfiker et al. (14) were not able to detect any difference between the Ca\(^{2+}\) stimulation of rPMCA2b and hPMCA4b in the presence of calmodulin, perhaps because of the different expression and assay system they used. Also, this difference in the activity is seen only at low Ca\(^{2+}\) concentrations and could be overlooked.

In the absence of calmodulin, rPMCA2b showed much higher activity than hPMCA4b at each Ca\(^{2+}\) concentration. These results agreed with Hilfiker et al. (14), who discussed the higher activity of rPMCA2b in the context of the tight binding of calmodulin to the enzyme. Using several methods, our results indicated that no calmodulin remained bound to the membranes containing rPMCA2b. Recent experiments indicated that in addition to the calmodulin-binding domain, hPMCA4b has a downstream inhibitory region which is responsible for the very low activity of this isoform in the absence of calmodulin (19, 20). Since this region, between residues 1113 and 1134 of hPMCA4b, is quite different from the corresponding region of rPMCA2b, it is possible that, unlike hPMCA4b, rPMCA2b does not have an extra downstream inhibitory region.

RPMCA2a also showed higher activity in the absence of calmodulin than hPMCA4b; it resembled hPMCA4a in this respect (compare Fig. 5A with Fig. 5 from Verma et al. (15)). Recent experiments (15) have shown that hPMCA4a has a much longer calmodulin-binding domain than hPMCA4b and that the whole inhibitory region (which appears to be less effective in self-inhibition than the one in hPMCA4b) is included within this domain. The structure of the regulatory region of the rPMCA2 isoforms remains to be determined but the data on rPMCA2a indicate that the inhibitory regions of rPMCA2a and hPMCA4a might be similar.

Tissue distribution of the PMCA isoforms in human and rat has been examined by S1 nuclease protection, polymerase chain reaction, in situ hybridization, and at the protein level by Western blot analysis (8, 10, 21). These studies have shown that PMCA1 and 4 are broadly distributed, leading to the suggestion that they represent the “housekeeping” isoforms. On the other hand, PMCA2 and 3 were only detected in specialized tissues and cells: RPMCA2b mRNA has been localized to brain, heart, liver, skeletal muscle, spleen, and testes whereas rPMCA2a is found only in brain and heart. The data presented in this paper suggest that rPMCA2b will have very different properties from hPMCA4b under physiological conditions. Intracellular calmodulin concentrations are usually very high (2–5 μM in most cells, in brain about 50 μM); Figs. 3 and 4B show that rPMCA2b remains activated by moderate levels of calmodulin even below 0.1 μM intracellular Ca\(^{2+}\) concentration. This indicates that rPMCA2b is a form of the PMCA pump which is extremely effective in removing Ca\(^{2+}\) from the cytosol, a property which may make an important contribution to the physiology of cells where it is expressed.

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