The Ca\(^{2+}\) Affinity of the Plasma Membrane Ca\(^{2+}\) Pump Is Controlled by Alternative Splicing*

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The plasma membrane Ca\(^{2+}\) pump is a calmodulin-regulated P-type ATPase that is an essential element in controlling intracellular Ca\(^{2+}\) concentration. Studies on the gene structure of this pump have revealed an alternate splice option that changes the structure of the calmodulin-binding domain. This change in the structure of the enzyme results in a reduced calmodulin affinity. Tests of the enzyme's activity in the presence of a high calmodulin concentration, approximating that found inside living cells, show that this reduced calmodulin affinity causes a reduced apparent affinity of the enzyme for Ca\(^{2+}\). This shift in the Ca\(^{2+}\) activation occurs in a Ca\(^{2+}\) concentration range crucial to cellular function and is probably the physiologically important consequence of the alternate splice.

The plasma membrane Ca\(^{2+}\) pump is the sole high affinity pump for Ca\(^{2+}\) in the cytosol to the extracellular space. The activity of this pump is stimulated by direct interaction with calmodulin, which increases both the \(V_{\text{max}}\) and the apparent affinity for Ca\(^{2+}\). In the presence of Ca\(^{2+}\), calmodulin binds to an autoinhibitory domain (C domain), which is a 28-residue region near the carboxyl terminus of the enzyme. Since calmodulin binds tightly only when it is loaded with Ca\(^{2+}\), stimulation of the enzyme by calmodulin will be regulated by changes in the intracellular concentration of Ca\(^{2+}\). Because of this, an alteration in the affinity of the pump for Ca\(^{2+}\)-calmodulin might lead to an enzyme with altered Ca\(^{2+}\) sensitivity under the conditions in a living cell.

Such a change in the calmodulin affinity can be obtained by the utilization of an alternate splice. At least four different genes code for this pump, and alternate splices affecting at least two areas of the pump introduce additional variation in its properties (1). In all four genes, an alternative splice option exists in the middle of the sequence coding for the C domain, which causes a substantial change in the structure of the downstream portion of the domain. The most common form of the C domain is the \(b\) form, which has a net plus charge of 6 or 7, while the \(a\) form, expressed in a limited range of tissues, has a net plus charge of 3 or 4. Little is known about the properties of the proteins coded for by the different genes and splices, and most of the available information has been gained by inference, by experiments using synthetic peptides (2) and inactive products from short genetic constructs (3). Studies utilizing synthetic peptides corresponding to the C domain showed that the peptide representing the \(a\) form has about a 10-fold lower affinity for Ca\(^{2+}\)-calmodulin and was also about 10-fold less effective as an inhibitor of the activated Ca\(^{2+}\) pump (2). Recent successes at overexpression of the Ca\(^{2+}\) pump in COS cells (4, 5) and the development of a useful assay for the enzyme expressed in COS cells (6) have opened the way to studies relating the structure of the pump to its biochemical activity. We will focus here on the functional consequences of an alternative splice in the middle of the sequence coding for the C domain.

MATERIALS AND METHODS

Chemicals—\(45\)CaCl\(_2\) was obtained from DuPont NEN. Calmodulin was purchased from Sigma. All other chemicals used in this study were of reagent grade.

Construction of Full-length hPMCA4b and hPMCA4a and Their Truncated Versions, 4b(ct56) and 4a(ct56)—Full-length hPMCA4b was put together from the partial clones (4) and cloned into expression vector pMM2. A NsiI and KpnI fragment of hPMCA4a obtained from a partial clone was substituted for the 3' end of hPMCA4b to obtain a full-length hPMCA4a. 4b(ct92) and 4a(ct56) were constructed by the strategy described for dl20 (6).

Transfection—Transfection of COS-1 cells was performed as described (6) except that the M6 isolate of COS-1 cells obtained from the Genetech Institute, Cambridge, MA was used in the transfection and 15 x 10\(^5\) cells were used per 150-cm\(^2\) flask.

Isolation of Microsomes from COS-1 Cells—Crude microsomal membranes were prepared as described (6).

Ca\(^{2+}\) Transport Assay—Calcium influx into microsomal vesicles was measured at \(37^\circ\)C by rapid filtration through Millipore membrane filters (0.45-\(\mu\)m pore size, type HA) essentially as described in the previous paper (6) except that the higher specific activity of the microsomes allowed reduction of their concentration to 4–7 \(\mu\)g/ml. The vesicles were preincubated at \(37^\circ\)C in the presence of the appropriate concentrations of calmodulin for 3 min, and Ca\(^{2+}\) uptake was initiated by the addition of ATP. Controls from pMM2-transfected cells were subtracted from each data point.

RESULTS AND DISCUSSION

We have overexpressed in COS cells the full-length \(a\) and \(b\) versions of the human plasma membrane Ca\(^{2+}\) pump, isofrom 4, which are called hPMCA4a and hPMCA4b, and also the truncated versions 4a(ct56) and 4b(ct92). These truncated versions contain the 28 residues of the C domain, but all residues downstream of that are omitted. Fig. 1 shows a Western blot of membranes from COS cells expressing these four constructs, showing that enzymes of the expected molecular weight are produced. All four constructs express proteins that are identical up to residue 1104. Downstream of this residue, the structures of the \(a\) and \(b\) isoforms are different because of the insertion of a 178-base pair sequence into the mRNA of the \(a\) form. Fig. 1 (lower panel) shows the sequences of the carboxyl-terminal portions of all four constructs used in this study.

When hPMCA4b and hPMCA4a were expressed in COS cells and their activity measured as a function of calmodulin con-
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COOH-terminal sequences of constructs used in this study

|    | hPMCA4b     | hPMCA4a     |
|----|-------------|-------------|
| 4b(ct92) | LERGQILNFRGLNIQTVKVEAFSS | LERGQILNFRGLNIQTVKVEAFSS |
| 4a(ct56) | LERGQILNFRGLNIQTVKVEAFSS | LERGQILNFRGLNIQTVKVEAFSS |

Fig. 1. Immunoblot of microsomes prepared from COS-1 cells transfected with plasmid encoding full-length hPMCA4b or hPMCA4a or their truncated versions, 4b(ct92) or 4a(ct56). 4b(ct92) and 4a(ct56) contain all 28 residues of the C domain and are named according to the number of residues cut off at the carboxyl terminus. Upper panel, 3 μg of membrane protein was applied on each lane of a 7.5% polyacrylamide gel. Immunoblots using monoclonal antibody 5F10 are shown. Lower panel, the exact carboxyl-terminal sequences of each construct are shown.

centration, it was found that hPMCA4a was less responsive to calmodulin than hPMCA4b. As shown in Fig. 2, the concentration of calmodulin required for half-maximal activation of hPMCA4a was about 7 times higher than that required to activate hPMCA4b. A 3–5-fold stimulation by calmodulin was observed under these conditions.

Another function of the pump is its Ca\(^{2+}\) affinity under conditions approximating those in a living cell. The total calmodulin concentration is about 3.6 μM in red cells (7) and about 2 μM in thyroid and in skeletal muscle (8). In the tissues where its most abundant (brain, testis) it approaches 30 μM (8). We measured the dependence of Ca\(^{2+}\) uptake on free Ca\(^{2+}\) at two high free calmodulin concentrations that might be encountered in living cells. Fig. 3 shows the Ca\(^{2+}\) response of hPMCA4b and hPMCA4a at 0.3 and 1.5 μM calmodulin. Under both circumstances, hPMCA4b was more responsive to Ca\(^{2+}\) than hPMCA4a. At 1.5 μM calmodulin the K\(_d\) for Ca\(^{2+}\) was 0.25 μM for hPMCA4b and 0.54 μM for hPMCA4a. Since these differ in a range crucial to cellular function, we can expect these two isoforms to regulate intracellular Ca\(^{2+}\) differently.

To determine which portion of the pump's carboxyl-terminal sequence was responsible for the difference in Ca\(^{2+}\) affinity, we also measured the Ca\(^{2+}\) response of the truncated mutants in the presence of 0.3 μM calmodulin (Fig. 4). The difference in the K\(_d\) for Ca\(^{2+}\) was still present, indicating that the difference in Ca\(^{2+}\) affinity came from the 9 residues in the C domain, which is the only difference between 4b(ct92) and 4a(ct56).

These experiments demonstrate that the alteration of a few residues in a region not directly involved in Ca\(^{2+}\) transport can cause a substantial change in the effective Ca\(^{2+}\) affinity. This alteration in the enzyme structure directly affected only the

**Calmodulin, nM**

**Fig. 2. hPMCA4b is more sensitive to calmodulin stimulation than hPMCA4a.** The calmodulin concentration dependence of Ca\(^{2+}\) uptake by microsomal vesicles isolated from COS-1 cells transfected with hPMCA4b or hPMCA4a is shown. The free Ca\(^{2+}\) concentration was 1.2 μM. The vesicles were preincubated at 37° C in the presence of calmodulin for 3 min, and Ca\(^{2+}\) uptake was initiated by the addition of ATP. Ca\(^{2+}\) uptake was expressed as f = (v - V\(_o\))/V\(_m\) - V\(_o\)), where V\(_o\) is the activity in the absence of calmodulin, v is the activity in the presence of the appropriate calmodulin concentration, and V\(_m\) is the maximum Ca\(^{2+}\) uptake, which was determined in the presence of enough calmodulin to fully activate the enzyme. V\(_o\) varied from one transfection to the next, ranging from 5 to 8 nmol (mg membrane protein\(^{-1}\) min\(^{-1}\). Similar variations in V\(_o\) gave activities of 1–2 nmol (mg membrane protein\(^{-1}\) min\(^{-1}\). Data are from two independent determinations on two different preparations. The K\(_d\) values ± standard deviation were: hPMCA4b, 17.8 ± 1.7 nm; hPMCA4a, 125.9 ± 15.9 nm.
related to the existence of higher average Ca\(^{2+}\) levels in some of the cell types in these tissues. The presence of hPMCA4a in them may be primarily in brain, smooth muscle, and perhaps heart (10, 11). Since these tissues make a particularly heavy use of the intracellular Ca\(^{2+}\) signal, the presence of hPMCA4a in them may be related to the existence of higher average Ca\(^{2+}\) levels in some of the cell types in these tissues.

It has recently been reported that alternative splicing of two different receptors produces four or five different isoforms, which couple via different G proteins (12, 13). Thus, alternative splicing of mRNA, generating multiple isoforms from a single gene, appears to be a general mechanism for the fine tuning of intracellular signaling.

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**FIG. 3.** A change in the affinity of Ca\(^{2+}\)-calmodulin for the Ca\(^{2+}\)-pump leads to a change in the apparent affinity of the pump for calcium. Ca\(^{2+}\) uptake by microsomal vesicles from hPMCA4b- and hPMCA4a-transfected COS-1 cells was measured as a function of free Ca\(^{2+}\). Maximum activities of the enzymes were determined at a saturating free Ca\(^{2+}\) (14 µM) and calmodulin concentration, and Ca\(^{2+}\) uptake activities were expressed as a percent of this maximum activity. The lines represent the best fit to the data given by the Hill equation. Where shown, the error bars represent the average of two independent determinations on two different preparations. The values for Ca\(^{2+}\) activation standard deviation were: hPMCA4b, 0.29 ± 0.05 µM; hPMCA4a, 0.84 ± 0.06 µM; lower panel, hPMCA4b, 0.25 ± 0.04 µM; hPMCA4a, 0.54 ± 0.07 µM.

**FIG. 4.** The differences in the kinetic behavior of hPMCA4b and hPMCA4a reside in their alternatively spliced calmodulin-binding domain. Ca\(^{2+}\) uptake by microsomal vesicles from 4b(ct92)- and 4a(ct56)-transfected COS-1 cells was measured as a function of free Ca\(^{2+}\). The activities are expressed as a percent of the maximum determined at saturating Ca\(^{2+}\) and calmodulin concentrations. Data points are from two independent determinations on two different membrane preparations. The lines represent the best fit to the data given by the Hill equation. The values for Ca\(^{2+}\) activation standard deviation were: 4b(ct92), 0.29 ± 0.02 µM; 4a(ct56), 0.63 ± 0.05 µM.