Plasma Membrane Ca\(^{2+}\) Pump Isoform 3f Is Weakly Stimulated by Calmodulin

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Isometric 3f of the plasma membrane Ca\(^{2+}\) pump is a major isoform of this pump in rat skeletal muscle. It has an unusual structure, with a short carboxy-terminal regulatory region of only 33 residues when compared with the 77 to 124 residues found in the other isoforms. Also, whereas the regulatory regions of the other isoforms, downstream of the alternative splice, consist of two homologous groups, the sequence of 3f is not related to either group. A synthetic peptide representing the calmodulin binding domain of isoform 3f had a much lower calmodulin affinity (with a \(K_d\) of 15 nM) than the corresponding peptide of isoform 2b (\(K_d\) value was 0.2 nM). The characteristics of this domain were further studied by making chimeras of the 3f regulatory region with the catalytic core of isoform 4 and by making the full-length isoform 3f. Both constructs bound to calmodulin-Sepharose. The chimera was fully active without calmodulin, showing no stimulation of activity when calmodulin was added. The full-length isoform 3f was slightly activated by calmodulin. These data show that the regulatory region of isoform 3f is only a weak autoinhibitory domain of the enzyme, in contrast to the properties of all the other isoforms studied so far. Rather, this isoform is a special-purpose, constitutively active form of the enzyme, expressed primarily in skeletal muscle and as a minor isoform in brain.

In the regulatory region of the plasma membrane Ca\(^{2+}\) pump (PMCA), a common pattern relates all of the major isoforms: the regulatory regions are at the carboxyl terminus of the molecule, preceded by over 1000 upstream residues. Removal of the regulatory region activates the pump so that it requires no effector for activity (1). Each of the regulatory regions begins with a calmodulin-binding domain (2), followed by additional regulatory regions which include autoinhibitory domains (3), sites for phosphorylation by protein kinases (4), sites for protease digestion (5), and sites for interaction with PDZ domains (6). These different domains and sites may overlap or occur in different orders, but they generally make up a regulatory region of 77 to 124 amino acid residues, counting from the beginning of the calmodulin-binding domain (4, 7, 8). Contrasting with this picture is the situation in isoform 3f, the product of an unusual alternate splice that results in a novel calmodulin-binding domain.

Fig. 1 shows an alignment of the carboxyl terminus of rPMCA3f with the corresponding portions of the common isoforms of the rat plasma membrane Ca\(^{2+}\) pump. This alignment starts at the beginning of the calmodulin-binding domain and all of the sequences are identical for the first 18 residues, except that isoform 4 substitutes a valine for the phenylalanine at the ninth residue. At this point an alternate splice results in two isoforms (a and b) for each gene product. The downstream structures of these isoforms all differ, but they are all long complex regulatory regions which extend further than is shown in Fig. 1. The calmodulin-binding domains of isoforms 4a and 4b have been investigated. The currently accepted picture is that 4b has a domain of about 28 residues (3), whereas 4a has a longer domain (9) which is interrupted by a nonbinding region (10). These regions are double underlined in Fig. 1. Isoforms 4a and 4b also have autoinhibitory domains that make the native form of the enzyme relatively inactive. In isoform 4b there are two such domains, one of which is coterminous with the calmodulin-binding domain and the second of which is a separate, downstream domain. In isoform 4a, the autoinhibitory domain is included within the calmodulin-binding domain. Isoform 3f has a much shorter region downstream of the alternative splice, and that region has a sequence that is not related to those found in either the a or the b forms.

The message for isoform 3f is present in rat at a high level in skeletal muscle and at a lower level in brain (11). The message for a very similar human isoform was found in cerebral cortex (12). The unusual structure of this isoform has compelled us to investigate its enzymic properties. These properties have indeed also proved to be unusual. We show here that isoform 3f is expressed in rat skeletal muscle at a higher level than that of other PMCA isoforms. It still binds to calmodulin despite its unusual calmodulin binding domain. In contrast to the low to medium activity level seen in the other isoforms in the absence of calmodulin, 3f is already almost fully active.

MATERIALS AND METHODS

\(^{45}\text{Ca}\) and \([\gamma-\text{32P}]\text{ATP}\) were purchased from NEN Life Science Products. Calmodulin and calmodulin-Sepharose were obtained from Sigma. LipofectAMINE and Optimem media were obtained from Life Technologies, Inc. All other chemicals used for this study were of reagent grade. The standard ABC Kit and Universal IgG used for immunostaining were from Vector Laboratories (Burlingame, CA). rPMCA3a cDNA in pBR322 vector was a gift from Dr. G. Shull (University of Cincinnati).

Preparation of a Specific Antibody Directed Against rPMCA3f—The polyclonal antibody named CR3F was generated following the protocol described in Filoteo et al. (13). The synthetic peptide, which was used to immunize New Zealand White rabbits, followed the sequence of the last 15 residues (VCWDGKKMLRITTEVG) of rPMCA3f. It was synthesized.
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**Construction of Full-length rPMCA3f and of the Chimeras of the h4b Catalytic Region** (ct120) with Different Lengths of the r3f Carboxyl Terminus—Using standard ligation and transformation procedures, full-length r3f was made by ligating an amplified rat 3f PCR product into rPMCA3a 5′-cloning sites (15). The amplified PCR product corresponds to the carboxyl terminus of rPMCA3f. Full-length r3f was made by ligating an amplified rat 3f PCR product into rPMCA3a DNA in pSP72 using ClaI and BamH I sites (15). The amplified PCR product corresponds to the carboxyl terminus of rPMCA3f which was prepared using GeneAmp RT-PCR kit (PerkinElmer Corp.) with rat skeletal muscle mRNA as template. The sequence of the full-length 3f was verified before the correct clone was excised and ligated into the expression vector PMM2 using SalI and ClaI sites.

The chimeras ct120–3f28 and ct120–3f33 were prepared using H4b (taken out of pSP72-h4b) as template. Appropriate primers were used in PCR reactions to synthesize NsiI-KpnI pieces corresponding to ct120–3f28 and ct120–3f33. The clones with the correct sequence were selected and used to replace the original NsiI-KpnI portions in pSP72-h4b. Full-length ct120–3f28 and ct120–3f38 were cut out of pSP72 vector and transferred into the expression vector PMM2 using SalI and ClaI sites. A Clal–BamHI piece corresponding to r3f was synthesized by PCR reaction and replaced into rPMCA3a to obtain full-length rPMCA3f.

**Calculation of $K_i$ ($K_f$) from Peptide Competition Experiments**—These experiments were set up with constant concentrations of calmodulin and purified erythrocyte Ca$^{2+}$ pump, and the concentration of peptide was varied. As the peptide concentration was increased, it competed more effectively for calmodulin, removing calmodulin from the pump. This effect was analyzed quantitatively by the method previously described (16). Note that the equation as printed in that reference contained an error in sign; the second bracket should begin with $C_i$ minus $E_f$ instead of plus. The correct equation is given below.

$$I_\ell = \left[ \frac{K_i (1 - f)}{K_f} + 1 \right] \left[ C_i - E_f - \frac{K_i}{1 - f} \right]$$  
\(\text{Eq. 1}\)

**Transfection of COS-1 Cells**—Transfection was carried out using LipofectAMINE (Life Technologies, Inc.) based on the protocol as described by the manufacturer and in Enyedi et al. (15). Briefly, transfection was initiated when the cells were 70 to 80% confluent in 150-cm$^2$ 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 was 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 cells were prepared as described by Enyedi et al. (17). Ca$^{2+}$ Transport Assay—As Ca$^{2+}$ 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 (15, 17). The reaction mixture contained 100 mM KCl, 25 mM TES-triethanolamine, pH 7.2, 40 mM KH$_2$PO$_4$-K$_2$HPO$_4$, pH 7.2, 200 mM thapsigargin, 5 mM Na$_2$ATP, 4 μM myo-inosine, 7 mM MgCl$_2$, 100 μM CaCl$_2$ (labeled with $^{45}$Ca, specific activity 100,000–150,000 cpm/mmol), and enough EGTA to obtain the desired free Ca$^{2+}$ concentration. Microsomes at 10–20 μg/ml concentration were preincubated in the presence or absence of 2 μM calmodulin for 2 min at 37 °C and Ca$^{2+}$ uptake by the vesicles was started by the addition of 6 μM ATP. The reaction was terminated by rapid filtration of the microsomes using Millipore membrane filters.

**Peptide Competition, Measurement of the Ca$^{2+}$ ATPase Activity of the Purified Erythrocyte PMCA**—This was measured by monitoring the release of inorganic $^{32}$P from [γ-$^{32}$P]ATP (18). Briefly, 0.8 to 1.1 ng of purified enzyme was incubated for 30 min at 37 °C in a medium containing 160 mM KCl, 25 mM TES-triethanolamine, pH 7.2, 2 mM MgCl$_2$, 0.1 mM EGTA, and enough CaCl$_2$ to give 10 μM free Ca$^{2+}$. The reaction was started by the addition of ATP to give a final concentration of 0.5 mM. The calmodulin concentration was chosen to give the maximum velocity. To avoid nonspecific absorption of the peptides to the walls of the tubes, peptide solution in 160 mM KCl was introduced directly into the reaction medium beneath the liquid surface.

Peptides were synthesized with 28 or 33 residues; each peptide began with the first residue of the calmodulin binding domain. The peptides were named to reflect the isoform being represented, i.e. C28R2b represents isoform r2b and C28R3f represents isoform r3f. In previous papers (19, 20), C28R2b was called C28R2.

**Binding of Constructs to Calmodulin-Sepharose**—This was done as described previously (15, 17) with some modifications. Briefly, 10–20 μg samples were placed in a buffer containing 100 mM KCl, 25 mM TES-triethanolamine, pH 7.2, 1 mM MgCl$_2$, 5 mM dithiothreitol, 100 μM CaCl$_2$, and 90 μM EGTA in a 200-μl volume. The reaction was terminated by putting the samples on ice, and subsequently, 40 μl of extraction solution containing 5 mM MgCl$_2$, 5 mM CaCl$_2$, 2% Triton X-100, 1.5 mM succrose, 0.8 mM NaCl, and 50 mM TES-triethanolamine, pH 7.2 was added. The mixture was incubated on ice for 15 min. Then, 200 μl of this mixture was added to 50 μl of a suspension of calmodulin-Sepharose beads previously equilibrated in the same buffer. The binding was allowed to proceed on ice for 90 min. Unbound proteins were removed by washing the beads four times with 200 μl of 5× diluted extraction buffer. The proteins bound to the calmodulin-Sepharose were removed by incubating the beads with the electrophoresis sample buffer described above, which contained SDS-urea. The beads were separated from the samples by centrifugation, and an aliquot of each sample was applied to an SDS-polyacrylamide gel.

**Preparation of the Plasma Membrane Fraction from Rat Skeletal Muscle**—Plasma membrane vesicles were prepared from the white muscles of legs of 3–5 adult rats. The procedure followed was that described by Seiler and Fleischer (21). When needed, a final enrichment of the plasma membrane calcium pump was done by the use of calmodulin-Sepharose beads as described above.

**Gel Electrophoresis, Electotransfer, and Immunostaining**—The samples were electrophoresed on 7.5% acrylamide gel following Laemmli’s procedure except that the sample buffer contained 100 mM dithiothreitol and 125 mg/ml urea. The samples were subsequently electrolotted and the blots were immunostained with monoclonal antibody 5F10 (22) and/or with polyclonal antibodies NR3 (13) and CR3F as needed. 5F10 antibody was used at a 1:1000 dilution, and both NR3 and CR3F were used at 1:400. A universal second antibody that includes both anti-mouse and anti-rabbit IgG was used.

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RESULTS

Fig. 2 shows a test of the specificity of antibody CR3F. In this Western blot, three antibodies were tested for their reactivity with four isoforms of PMCA expressed in COS cells. The COS cells express isoform 1b spontaneously (13), and this batch of COS cells was transfected with plasmids coding for isoforms 3a, 3f, and 4a. As expected, antibody 5F10 reacted with all of the isoforms present (lane 1). Antibody NR3, which was already known to react with isoform 3a (13), also reacted with 3f (lane 2). This result was expected because NR3 is directed against the amino terminus of isoform 3, which is identical in 3a and 3f. Lane 3 shows that antibody CR3F was specific for isoform 3f, not reacting with any of the other isoforms present.

The same set of antibodies was then used to probe skeletal muscle plasma membranes, and the results are shown in Fig. 3. Each panel contains a 3f standard in the adjacent track to assure the positive identification of 3f in these high-resolution blots. The first panel shows the results obtained with 5F10, which is known to react with all isoforms of the pump (13, 22). The pattern obtained is consistent with the presence of isoform 4a or 3a (upper band), 3f (middle band), and smaller proteolyzed isoform(s) which are difficult to identify (lower band). The second panel shows results with CR3F and confirms that the middle band of the first panel is isoform 3f, and that a small amount of proteolytically degraded 3f was present (faint lower band). Because the gel which produced the middle blot had better focused bands, the 3f band looks much narrower than the middle band in the first panel, but it was clear from comparison of a number of similar experiments that 3f accounted for essentially all of that middle band. The lower band of the second panel did not correspond in size to the lower band of the first panel, showing that another isoform is present. This must be a proteolyzed isoform because it is smaller than any of the known full-length isoforms of PMCA. The third panel, with antibody NR3, shows that the top band was primarily isoform 3a and reconfirmed the identity of the middle band.

The properties of the regulatory region of isoform 3f were next tested by constructing chimeras. These consisted of the catalytic core of hPMCA4 (which we call ct120 because the carboxyl-terminal 120 residues have been removed) linked to the regulatory region of 3f. Two chimeras were made, one having 28 residues from 3f added to the carboxyl terminus of ct120 and the second having all 33 residues of the regulatory region of 3f added to ct120. Both of these constructs still bound calmodulin, as was shown by their binding to a calmodulin-Sepharose column. Fig. 5 compares immunoblots of pump constructs with and without calmodulin-Sepharose treatment. It is evident from this figure that ct120, which lacks the calmodulin-binding region, did not bind to the column, but that all of the other constructs did bind. The calmodulin-binding domain of 3f, when incorporated into a pump (either chimera or full-length 3f), still had a high enough affinity to interact with calmodulin even though the peptide C28R3f had a low affinity for calmodulin.

However, the binding of calmodulin to the chimeras did not regulate their activity. These chimeric pumps were already fully activated in the absence of calmodulin. Under these circumstances they displayed the high activity and high affinity for Ca\(^{2+}\) which is normally found only when the enzyme is activated by addition of calmodulin or the regulatory region is removed. The addition of calmodulin, even at quite high concentrations, had no significant effect on the activity. Fig. 6 shows a comparison of the Ca\(^{2+}\) activation curves of the chimeras with those of the fully active truncated mutant ct120 and of the partially inhibited truncated mutant ct92. ct92 is hPMCA4b cut off at the downstream end of the 28-residue calmodulin-binding domain. The chimeras were all essentially fully activated, regardless of whether they had 28 or 33 residues from 3f and whether they had a high level of calmodulin.
or none. At very low Ca\textsuperscript{2+} concentrations, the chimeras appeared to be slightly inhibited relative to ct120, but this effect was not significant.

The situation was somewhat different for the full-length 3f, which was weakly activated by calmodulin. Fig. 7 shows the activity of 3f with and without calmodulin. It is evident from this figure that the $V_{\text{max}}$ was still not changed by the presence of calmodulin. The average activity was increased only 1.41-fold by calmodulin, and the maximum increase was 1.64-fold at 0.69 $\mu$M free Ca\textsuperscript{2+}. This contrasts strongly with the effect of calmodulin on hPMCA4b, where $V_{\text{max}}$ was increased 4.0-fold, the average activity was increased 9.0-fold, and the maximum increase was 20.5-fold at 0.27 $\mu$M free Ca\textsuperscript{2+} (25).

**DISCUSSION**

Four studies (11, 12, 26, 27) have used molecular biology methods to study the presence of the various isoforms of PMCA in many different tissues including skeletal muscle. A comparison of these studies allows us to reach some conclusions about which isoforms are present in skeletal muscle but also emphasizes the difficulties in quantitating such results. All of the studies which considered the issue agree on the near-absence of any variant of isoform 2 from skeletal muscle (11, 12, 27). With respect to the other three isoforms, all are present to some extent, but it is difficult to compare the results between the different isoforms because of the many differences in the methods used. Northern blots give the impression that isoform 3 is more abundant than 1 or 4, but differences in the probes, specific activities, and times of exposure make it difficult to compare the data. One study (12) attempts to quantitatively compare the amounts of messages of all the isoforms, but this study failed to detect any variant of isoform 3 in adult skeletal muscle. It is difficult to reconcile these results with those of the other three studies, but various things might cause this discrepancy. There are two obvious factors which confound a comparison of this study with the other three: 1) human tissue was used instead of rat and 2) the samples were from autopsy material frozen up to 24 h after death.

Unlike the molecular biology studies, the Western blots shown in Fig. 3 provide a means of comparing the amounts of all of the PMCA isoforms in a single experiment. Antibody 5F10 is directed toward an epitope which is nearly identical in all known mammalian isoforms of PMCA (22), so a Western blot based on 5F10 is equally sensitive to all of them. The left panel of Fig. 3 shows the presence of three bands, 3a, 3f, and an unidentified PMCA, and in the original blot the 3f and 3a bands were of almost equal intensity, stronger than the unidentified PMCA band. Several other Western blots utilizing 5F10 confirmed this result. This shows that the plasma membrane Ca\textsuperscript{2+} pump in skeletal muscle consist primarily of isoforms 3a and 3f, and that isoform 3f is a major isoform of the plasma membrane Ca\textsuperscript{2+} pump in skeletal muscle.

In peptide competition experiments such as those shown in Fig. 4, the $K_i$ for inhibition by the peptide of binding of the pump to calmodulin can be calculated. These $K_i$ values are equivalent to the $K_d$ values for dissociation of the calmodulin-peptide complexes, and we will refer to them as $K_d$ values in this paper. For the peptide C28R3f, the result does not allow us to extrapolate directly to the $K_d$ of calmodulin for the full-length isoform 3f. Nonetheless, the $K_d$ for the peptide does give an indication of what the $K_d$ will be for the full-length isoform.
In the two known cases, calmodulin has about a 25–40-fold higher affinity for the peptide than for the full-length enzyme. These cases used peptides representing the calmodulin-binding domains of myosin light chain kinase (28) and PMCA (16). The $K_d$ for the myosin light chain kinase peptide is 0.006 nM, whereas that for full-length myosin light chain kinase is 0.10–0.22 nM. The $K_d$ is 0.2 nM (Fig. 4) in the case of the PMCA peptide, whereas that of the full-length enzyme is 7.6 nM (23). In our previous papers (16, 24), we have used the value of 4 nM, which was derived from equilibrium measurements of binding of iodinated calmodulin to erythrocyte membranes (29). We now use the newer value because it refers to the $K_d$ of non-modified calmodulin to a specific isoform. This results in a different calculated $K_d$ for C28R2b, which was previously calculated to be 0.1 nM (24).

The chimera experiments shown in Fig. 6 allowed us to assess the nature of the interaction of the 3f carboxyl terminus with the catalytic core of isoform 4. As the figure shows, there was no significant effect when the carboxyl terminus of 3f was added to the catalytic core of isoform 4, either in the absence or presence of calmodulin. Because the fully active ct120 (representing the catalytic core) was not inhibited by this addition, this indicates that there is no biologically significant interaction between the carboxyl terminus of 3f and the catalytic core of isoform 4. Varying the length of the carboxyl terminus of 3f had no effect on the interaction.

The full-length version of isoform 3f showed a slight activation by calmodulin (Fig. 7). This shows that the calmodulin-binding domain of 3f has a slightly inhibitory interaction with its own catalytic core, which it did not have with the core of isoform 4. This behavior contrasts with the relatively strong
Properties of rPMCA3f

inhibitory interactions between the carboxyl termini of isoforms 2a, 2b, 4a, and 4b and their own catalytic cores (3, 25). The amount of activation was much less than has been found in any of the isoforms studied to date. Its activation was compared with that of 4b under "Results"; isoforms 4a, 2a, and 2b have degrees of activation intermediate between that of 3f and 4b (9, 25). All of the other isoforms showed a decrease in $V_{max}$ upon calmodulin addition, whereas 3f showed no change. Isoform 2b showed the smallest activation of the previously studied isoforms, and it still showed a doubling in activity at 1 μM free Ca$^{2+}$, compared with the approximately 40% activation shown by 3f under similar conditions. The amount of activation of the isoforms by calmodulin is on the order of 4b > 4a > 2a > 2b > 3f. At 0.29 μM Ca$^{2+}$ in the absence of calmodulin, the percent maximal activity shown by the isoforms was: 4b, 3%; 4a, 15%; 2a, 23%; 2b, 40%; and 3f, 56%.

In preliminary experiments,2 we tested the calmodulin stimulation of isoforms 3b and 3a. These forms act like isoforms 2b and 2a: they are inhibited more strongly than isoform 3f and were stimulated by calmodulin at high levels of Ca$^{2+}$ by about 1.5- and 2.5-fold, respectively. These results indicate that the weak inhibition observed in the case of 3f was because of its unique carboxyl end and not to its catalytic core.

The unusual properties of skeletal muscle, in which control of the Ca$^{2+}$ signal is dominated by the highly organized sarcoplasmic reticulum, also require special properties of the other molecules controlling the signal. In the case of the plasma membrane Ca$^{2+}$ pump, the results of this study show that this special property is a modest activation by calmodulin. All isoforms of the plasma membrane Ca$^{2+}$ pump require calmodulin for full activity so that, for activation, a molecule of calmodulin must collide with a molecule of pump. Isoform 3f has considerable activity when calmodulin is not bound, so that it is more similar to the Ca$^{2+}$ pump of sarcoplasmic reticulum in that it is relatively independent of a separate activator molecule. Although our data do not give any information about the speed of response of PMCA to the Ca$^{2+}$ signal in muscle, these properties suggest that 3f would be more ready than other isoforms of PMCA to respond to the rapid Ca$^{2+}$ transients of this tissue.

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