Intramolecular Fluorescence Resonance Energy Transfer between Fused Autofluorescent Proteins Reveals Rearrangements of the N- and C-terminal Segments of the Plasma Membrane Ca\(^{2+}\) Pump Involved in the Activation*

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The blue and green fluorescent proteins (BFP and GFP) have been fused at the N- and C-terminal ends, respectively, of the plasma membrane Ca\(^{2+}\) pump (PMCA) isoform 4xb (hPMCA4xb). The fusion protein was successfully expressed in yeast and purified by calmodulin affinity chromatography. Despite the presence of the fused autofluorescent proteins BFP-PMCA-GFP performed similarly to the wild-type enzyme with respect to Ca\(^{2+}\)-ATPase activity and sensitivity to calmodulin activation. In the autoinhibited state BFP-PMCA-GFP exhibited a significant intramolecular fluorescence resonance energy transfer (FRET) consistent with the location of the fluorophores at an average distance of 45 Å. The FRET intensity in BFP-PMCA-GFP decreased when the enzyme was activated either by Ca\(^{2+}\)-calmodulin, partial proteolysis, or acidic lipids. Moreover, FRET decreased and became insensitive to calmodulin when hPMCA4xb was activated by mutation D170N in BFP-PMCA(D170N)-GFP. The results suggest that the ends of the PMCA are in close proximity in the autoinhibited conformation, and they separate or reorient when the PMCA achieves its final activated conformation.

The homeostasis of the intracellular Ca\(^{2+}\) is crucial for cell function. The Ca\(^{2+}\) ATPases from plasma membrane (PMCA) isoforms participate in the modulation of Ca\(^{2+}\) signals and are responsible for the long term maintenance of the low concentration of intracellular Ca\(^{2+}\) (1). The PMCA isoforms belong to the P2-type ATPase superfamily of ion pumps and form an aspartyl phosphate intermediate during the transport cycle (2). Another essential feature of these ATPases is their ability to switch between two different conformational states from E\(_2\) to E\(_1\) in the presence of the transported ion.

Human PMCAs are encoded by four separate genes, and additional variants are generated via alternative splicing of primary gene transcripts. PMCA4 is found virtually in all human tissues, and the splice variant xb is the most studied isoform. Computer modeling and sequence comparisons indicate that the overall structure of the PMCAs closely resembles that of other P-ATPases. Following the domain organization proposed for the SERCA (3, 4), the PMCA would contain a transmembrane region of 10 segments (M1–M10) and three major catalytic domains exposed to the cytosol. The nucleotide-binding (N) and the phosphorylation (P) domains contain the ATP binding site and the aspartate residue that forms the acyl phosphate intermediate, respectively, whereas the actuator domain (A) plays an essential role in the long range transmission of the conformational changes occurring during the transport cycle.

Despite the clear overall homology, certain amino acid segments of the PMCA protein are not found in SERCA. The existence of these segments in the PMCA molecule has been generally associated with the extensive regulatory mechanisms that are known to alter the function of the PMCA. Indeed, the major difference between the two calcium pumps is the long C-terminal segment (C region) of the PMCA following M10, and this region is involved in the so-called intrasteric inhibition of the pump (5). This type of control of enzymatic activity by inhibitory sequences acting directly at the active site of the enzyme is characteristic of protein kinases and phosphatases in which the autoinhibitory sequences resemble their substrates (6). In analogy with activation mechanism of these enzymes, it is proposed that in the resting state the autoinhibitor interacts with the catalytic region maintaining the PMCA inhibited. Calmodulin is the main activator of the pump, but a variety of mechanisms, including proteolysis, binding of acidic lipids, and phosphorylation by protein kinases, have been shown to switch partially or totally the activity of the PMCA back on. In the hPMCA4xb isoform the C-terminal autoinhibitor region has been found to contain two domains. A sequence of 28 amino acids identified as domain “C” (residues 1086–1113) binds calmodulin and acidic lipids and is responsible for ~80% of the inhibition. The binding of calmodulin to domain C enhances both the Ca\(^{2+}\) sensitivity and the turnover of the pump. Alternative splicing at the so-called alternative splice C modifies the calmodulin–binding autoinhibitory region leading to isoforms with different
responsiveness toward calmodulin activation (7). Full inhibition has been found to require a segment further downstream the calmodulin binding site (8). This second inhibitory sequence, which has been called domain “I,” encompass residues 1114–1157. Phosphorylation by protein kinase C of domain I residues has been shown to result in a partial activation of the pump in the absence of calmodulin (9).

Cross-linking experiments using a synthetic peptide C28W made after the sequence of the autoinhibitory domain C of hPMCA4xb revealed that the N-terminal half of this sequence interacts with the N domain (Cys337–Thr544), whereas the C-terminal part interacts with the A domain (Ile206–Val271) of the catalytic core (10, 11). It has been hypothesized that the binding of calmodulin to the C28W sequence in the pump induces a conformational change in the autoinhibitory domain that disrupts its interaction with the catalytic domain, thereby activating the pump. Consistently with this model it has been shown that the C-terminal segment of the hPMCA4xb is more accessible to proteases when the pump in an activated open state than in the inhibited closed state (12). Furthermore, the activating effect of mutations of Asp1080 has been suggested to arise from the impaired function of the region preceding the autoinhibitory sequence as a hinge between the regulatory and catalytic regions (13).

Although the concept that activation of the PMCA involves conformational changes associated with the removal of the autoinhibitory domain from the active site is widely accepted there have been no direct measurements of the rearrangements that take place in the PMCA during the activation. Moreover, the degree in which the C-terminal sequence downstream domain C participates in the conformational changes associated, which would occur during activation, is not clear at this time. In this study we have explored the possibility of obtaining a functional PMCA labeled with two autofluorescent proteins and its potential use as a reporter of conformational changes associated with the activity of the enzyme.

**MATERIALS AND METHODS**

**Chemicals**—Polyoxyethylene-10-laurylether (C12E10), t-α-phosphatidylcholine Type X-E (P-5394) from dried egg yolk, phosphodiesterase 3′,5″-cyclic nucleotide activator (calmodulin) from bovine brain, calmodulin-agarose, calcimycin (A23187), trypsin, ATP (disodium salt, vanadum-free), SDS, yeast synthetic drop-out media supplement without leucine, yeast nitrogen base without amino acids, dextrose, enzymes and cofactors for the synthesis of 32P]ATP, and all other chemicals were obtained from Sigma. Tryptone and yeast extract were from Difco. Carrier-free [32P]H3PO4 was provided by PerkinElmer Life Sciences (Boston, MA). [γ-32P]ATP was prepared by the method of Glynn and Chappell (14) except that no unlabeled orthophosphate was added to the incubation medium. Salts and reagents were of analytical reagent grade.

**Constructions of the DNA Coding for BFP-PMCA-GFP**—The DNA coding for PMCA-GFP was obtained by a two-step PCR in a standard megaprimer protocol (15) using Pfu DNA polymerase and primers A (5′-GCCCATGGCGCTGTAGGACT3′), B (5′-GTCAAAGGCCTCACATAGTGGAGGTCCAGTAACAGG-3′), and C (5′-TATTTAGGCCCCCATGATGTTGATGACAG3′). Primer C contains a restriction site for nuclease Apal at its 3′ position, whereas primer A anneals to the hPMCA4xb DNA upstream of a naturally occurring BspEI unique site. During the first PCR a fragment was amplified using the SuperGloGFP cDNA (Qiogene) as template and primers B and C. The 750-bp B–C product was isolated by electrophoresis in a 1% agarose gel and extracted using DNA QIAEX II (Qiagen). The purified BC fragment was used in the next PCR step along with primer A using the cDNA coding for hPMCA4xb as template. The 2144-bp A–B fragment was digested with BspEI and Apal and subcloned into the corresponding position of hPMCA4xb inserted into the Xhol–Apal sites of the pMPl625 vector (16).

To obtain the cDNA coding for BFP-PMCA first a unique site for nuclease Mlu was introduced at position 4 of the cDNA of hPMCA4xb. To this end a DNA fragment was amplified by PCR using oligonucleotides CA2 (5′-TGCAAGTCCGACCATGGACCGTCCATCCAGACCTGTT-3′) and 308 (5′-TCTCCAGGATGATGACCTGGCATC-3′) digested with Sall and DraII and cloned back at the corresponding position of hPMCA4xb. A DNA fragment was amplified by PCR using the SuperGlo BFP cDNA (Qiogene) as template and the pair of primers, 5′-BGMLUI and 3′-BGMLUI. Primer sequences were as follows: 5′-BGMLUI, 5′-ATAATACGCGTGAGGTGGAGCTAGCAAAGGTTCCATCGATGTTGATGACAGTTCCATCCA-3′; and 3′-BGMLUI, 5′-AAATATACGCGTCCGCTCCCATCGATGTTGTACAGTTCTATCCA-3′. Primers 5′-BGMLUI and 3′-BGMLUI included a restriction site for nuclease Mlu. The product 5′-BGMLUI/3′-BGMLUI was isolated by electrophoresis in a 1% agarose gel and extracted using DNA QIAEX II (Qiagen). The isolated fragment was digested with MluI and subcloned into the hPMCA4xb cDNA containing a MluI site at position 4. The DNA coding for mutant BFP-PMCA-GFP was obtained by replacing the wild-type Xhol–BspEI fragment of PMCA-GFP with the Sall and BspEI fragment of BFP-PMCA.

**Yeast Strain, Transformation, and Growth Media—Saccharomyces cerevisiae** strain DBY 2062 (MATa his4–619 leu2–3,112) (17) was used for expression. Yeast cells were transformed with the pMPl625 vector containing a Leu1+ marker and the PMAI promoter. For transformation with the plasmid construct a lithium acetate/polyethylene glycol method was utilized (18). The cells were grown in complete media (0.75% yeast extract, 1.13% Tryptone, 2.2% dextrose), and transformants were selected for their ability to grow in the absence of leucine on plates containing 6.7% yeast-nitrogen base without amino acids (YNB), 0.67% complete supplemented medium minus Leu (Leu-), 2.2% dextrose, and 1.5% agar.

**Membrane Isolation and Purification of Recombinant BFP-PMCA-GFP**—Four liters of yeasts expressing the BFP-PMCA-GFP protein were grown in YNB Leu– media at 28 °C in glass flasks with agitation, and, after the culture reached an approximate A600 of 1.5, 4 liters of complete media was added, and the incubation continued for 6 h (A600 = 4.0–5.0). Total yeast membranes were solubilized with C12E10 and the PMCA was purified by calmodulin-affinity chromatography as described previously (19). ~400 μg of purified PMCA was obtained from one batch of yeast. For the calculation of the molar concentration values the Mr of BFP-PMCA-GFP was
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taken as 190,000 and that of the single labeled PMCAs as 162,000.

Protein Assay—The protein concentration was initially estimated by the method of Bradford (20) using bovine serum albumin as a standard. To achieve a better assessment of the content of PMCA protein in each preparation, the samples were analyzed by SDS-PAGE using PMCA purified from porcine red cells as standard, and the intensity of the bands was compared after staining the gels with Coomassie Blue.

Reconstitution of BFP-PMCA-GFP in Proteoliposomes—Proteoliposomes containing the BFP-PMCA-GFP protein were obtained as described by Hao et al. (21) with a slight modification. Briefly, 1 ml of purified BFP-PMCA-GFP (~100 µg of protein) was added to 250 µl of a previously sonicated mixture of 29 mg/ml phosphatidylcholine and 57 mg/ml C12E10. The detergent/protein/phospholipid mixture was kept for 5 min under gentle stirring, and then the detergent was partially removed by adding 0.25 g of prewashed wet SM-2 Bio-Beads (Bio-Rad). The mixture was stirred at room temperature for 30 min, and the Bio-Beads were separated by filtration.

Ca2+ -ATPase Activity—The Ca2+ -ATPase activity was estimated from the release of [32P]P, from [γ32P]ATP at 37 °C (22) in 0.3 ml of ATPase reaction medium containing 50 mM HEPES-K (pH 7.00 at 37 °C), 100 mM KCl, 4 mM MgCl2, 500 µM EGTA, 2 µM A23187, 3 mM [γ32P]ATP and enough CaCl2 and calmodulin to give the Ca2+ and calmodulin concentrations indicated in each experiment. The reaction was initiated by the addition of 2 µg of BFP-PMCA-GFP reconstituted in proteoliposomes and was terminated by acid denaturation after 30 min. During this period the amount of P, liberated from ATP increased linearly with time. The free Ca2+ concentration in reaction medium was calculated using the Fabiato and Fabiato program (23) using the Blinck constants for EGTA/Ca2+, EGTA/Mg2+, ATP/Ca2+, and ATP/Mg2+.

Ca2+ Uptake—For measuring the Ca2+ uptake activity 272 µg of BFP-PMCA-GFP was reconstituted in proteoliposomes as described above except for that the reconstitution media contained 5 mM of potassium oxalate. The detergent was removed by incubating the protein with 0.19 g of prewashed wet SM-2 Bio-Beads. The mixture was stirred at room temperature for 30 min, then 0.19 g more of prewashed wet SM-2 Bio-Beads. The mixture was stirred at room temperature for 30 min, and the Bio-Beads were separated by filtration. The sample was diluted with 3 ml of 160 mM KCl, 20 mM Tris-HCl (pH 7.4 at 25 °C), and the proteoliposomes were collected by centrifugation at 40,000 × g for 30 min. The Ca2+ uptake was measured as described previously (24). The reaction mixture contained 100 mM KCl, 50 mM Tris-HCl (pH 7.4 at 37 °C), 1.5 mM ATP, 95 µM EGTA, 2.5 mM MgCl2, and CaCl2 (labeled with 45Ca) to give 10 µM free Ca2+. The proteoliposomes (40 µl) were incubated in the reaction mixture at 25 °C for 5 min, and the reaction was initiated by the addition of ATP. The reaction was finished by filtering the samples through a 0.2-µm filter. The 45Ca taken up by the liposomes was determined by counting in a scintillation counter. The radioactivity retained in the filter in the absence of ATP was subtracted from each data point.

Western Blotting and Protein Staining—SDS electrophoresis and immunoblotting were carried out as previously described (25). Proteins were electrophoresed on a 7.5% acrylamide gel according to Laemmli (26) and revealed by Coomassie Blue staining, or subsequently electrotransferred onto Millipore Immobilon P membranes. Nonspecific binding was blocked by incubating the membranes overnight at 4 °C in a solution of 160 mM NaCl, 0.05% Tween 20, and 1% non-fat dry milk. The membranes were incubated for 1 h with 5F10 antibody (27, 28) from ascitic fluid (dilution 1:1000). For staining, biotinylated antimouse immunoglobulin G and avidin-horseradish peroxidase conjugate were used.

Tryptic Digestion of BFP-PMCA-GFP—Digestion of BFP-PMCA-GFP with trypsin was done at 25 °C, in a medium containing 5 µg/ml of BFP-PMCA-GFP, 0.25 mg/ml C12E10, 0.12 µg/ml PC, 100 mM KCl, 50 mM Tris-HCl (pH 7.25 at 25 °C), 5 mM MgCl2, and 500 µM EGTA. The reaction was initiated by the rapid addition of 0.5 µg/ml trypsin, and the emission spectra were registered at different times as indicated in the each figure. For SDS-PAGE 19 µg of BFP-PMCA-GFP was suspended in 3.6 ml of the same media as above, and the reaction was initiated by the addition of 2 µg of trypsin. At different times 200 µl of the reaction was removed and mixed with 20 µl of 100% trichloracetic acid. The protein was precipitated and dissolved in sample buffer, and the proteolytic products were separated by electrophoresis in 9% acrylamide gel. The gel was stained with Coomassie Blue and scanned for fluorescence at 520 nm in a Storm 840 scanner.

Fluorescence Measurements—The fluorescence measurements were performed in an SLM-Aminco-Bowman spectrophotometer (Spectronic Instrument Inc., Rochester, NY) using a 3-× 3-mm quartz cuvette. The media contained 5 µg/ml PMCA-fused fluorescent protein, 0.25 µg/ml C12E10, 0.12 µg/ml PC, 100 mM KCl, 50 mM Tris-HCl (pH 7.25 at 25 °C), 5 mM MgCl2, and 500 µM EGTA. The final volume was 150 µl. The temperature was set at 25 °C. The excitation and emission bandwidths were set at 4 nm. Appropriate blanks corresponding to the reaction media were subtracted from the measurements to correct for background fluorescence.
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The efficiency of energy transfer ($E$) was estimated as the fraction of donor molecules de-excited via energy transfer to the acceptor, $E = (1 - I_d/I_0)$, where $I_d$ and $I_0$ are the intensity of emission of the donor (BFP) in the presence and in the absence of acceptor, respectively. To achieve a more precise determination of the values of $I_d$ and $I_0$ the integral of the area under the curve between 430 nm and 470 nm was taken. The estimation of the distance between the fluorophores was done according to the Förster equation, $E = 1/(1 + r_{da}^6/R_0^6)$, where $r_{da}$ is the distance between the donor (BFP) and acceptor (GFP) and $R_0$ (Förster distance) is the distance at which 50% the energy is transferred. $R_0$ depends on the quantum yield of the donor ($\Phi_d$), the extinction coefficient of the acceptor ($\varepsilon_a$), the overlap of the donor emission and acceptor excitation spectra ($I_{da}$), and the mutual orientation of the chromophores ($\kappa^2$) (29). A Förster distance ($R_0$) of 40 Å was used for the fluorophore pair BFP-GFP (30, 31), and a random orientation ($\kappa^2 = 2/3$) was assumed for distance calculations.

RESULTS

We have previously found that the extreme N-terminal segment of the hPMCA4xb (residues 1–6) is not critical for the activity of the PMCA (32). Accordingly, the BFP was fused at the N-terminal segment of after Thr5. The GFP was fused after the last C-terminal residue at ~100 residues downstream of the calmodulin binding-autoinhibitory sequence. A Gly-Gly-Gly spacer was introduced between the PMCA and the fluorescent proteins to increase the chance that the fluorophores would exhibit a random orientation as assumed for distance calculations from the FRET efficiency.

Total membranes from yeast cells transformed with the expression vector pMP625 containing the DNA coding for the autofluorescent PMCA fusions were isolated and solubilized by the detergent C12E10, and the dual- or single-labeled PMCA proteins were purified by calmodulin-affinity chromatography following the same protocol used for the purification of the wild-type PMCA. As shown in Fig. 1 the SDS-PAGE of the purified proteins revealed the presence of one major polypeptide, which migrated according to the expected size.

Results in Fig. 2A show that BFP-PMCA-GFP was capable of hydrolyzing ATP. The Ca$^{2+}$-ATPase activity of BFP-PMCA-GFP increased with increasing concentrations of Ca$^{2+}$ reaching a maximal activity of 0.4 μmol/mg/min at ~10 μM Ca$^{2+}$. The addition of 200 nm calmodulin increased the maximal activity of BFP-PMCA-GFP and reduced the concentration of Ca$^{2+}$ needed for half-maximal activity from 0.5 to 0.1 μM Ca$^{2+}$. As shown in Fig. 2B, half-maximal activation of BFP-PMCA-GFP was attained at 5 μM calmodulin. In the presence of 1 μM Ca$^{2+}$ the activity of the BFP-PMCA-GFP enzyme in the absence of calmodulin was ~40% of that attained at saturating concentrations of calmodulin. This value of basal activity is similar to that of the recombinant wild-type hPMCA4xb enzyme obtained from yeasts under similar conditions (19). These results suggest that the inhibitory function of the C-terminal regulatory proteoliposome suspension of an experiment conducted in triplicate is shown. The continuous line represents the best fit given by the equation, $v = V(1 - e^{-kt})$, with $V = 983$ cpm/40 μl and $k = 0.015$ s$^{-1}$.
domain was preserved despite the addition of the GFP. In addition results in Fig. 2C show that after reconstitution in proteoliposomes BFP-PMCA-GFP supported the ATP-dependent uptake of Ca\(^{2+}\), further reinforcing the idea of the functional competence of the PMCA fusion protein.

The BFP-PMCA-GFP protein exhibited maximum excitation and emission peaks at near the same wavelength that the isolated fluorescent proteins. Fig. 3A shows the fluorescence emission spectra of BFP-PMCA and BFP-PMCA-GFP when the BFP fluorophore was excited at 387 nm and that of PMCA-GFP excited at 474 nm. The fluorescence emission of BFP-PMCA-GFP was notably lower than that of BFP-PMCA at the BFP emission wavelength, and the fluorescence intensity increased in the region of the spectra corresponding to the GFP emission. This was indicative of the existence of energy transfer between the two fluorescent proteins.

Previous studies have shown that the purified hPMCA solubilized with detergents undergoes a reversible self-association (33–35). To investigate if association between BFP-PMCA-GFP molecules was causing FRET, the emission of 18 nM BFP-PMCA was titrated with increasing amounts of PMCA-GFP either in the presence of 0.5 mM EGTA or 1 \(\mu\)M Ca\(^{2+}\). Fig. 3B shows that in the absence of Ca\(^{2+}\) the emission spectra of mixtures of BFP-PMCA and PMCA-GFP up to 30 nM were similar to that of the BFP-PMCA alone. At higher concentrations of PMCA-GFP the relative fluorescence at 509 nm increased suggesting that at these concentrations energy was transferred between BFP-PMCA and PMCA-GFP. In the presence of Ca\(^{2+}\) (Fig. 3C) the enhancement of the PMCA-GFP fluorescence was even more evident in agreement with the idea that the oligomerization of the enzyme is favored by Ca\(^{2+}\) (36).

The results shown in Fig. 3 suggest that, at the concentration of BFP-PMCA-GFP used in Fig. 3, FRET arises from the interaction between fluorophores belonging to the same molecule. The efficiency transfer estimated by comparing the emission of BFP-PMCA-GFP and BFP-PMCA at 450 nM was 52%. If a free orientation of donor-acceptor pair is assumed, the calculated average distance between BFP and GFP in the BFP-PMCA-GFP protein was 45 Å.

It has been previously shown that a limited exposure of the PMCA to trypsin leads primarily to the removal of peptides from both ends of the molecule (37). Fig. 4A shows the effect of the treatment with trypsin on the BFP-PMCA-GFP emission spectra. As the time of proteolysis increased, the relative emission at 509 nm decreased, consistently with the idea that the cleavage of the fluorescent proteins from the PMCA increased the separation between them. Interestingly the decline of FRET with the time of proteolysis was biphasic (Fig. 4B) with a fast component accounting for \(\approx 25\%\) of the total and a larger component decaying much slower. Proteolysis of BFP-PMCA-GFP was also followed by SDS-PAGE. Fig. 4C shows the production of proteolytic fragments revealed by Coomassie Blue staining, subtracted from the measurements to correct for background fluorescence, and the fluorescence intensities were normalized to the maximum emission of the BFP fluorophore. C, titration of BFP-PMCA emission by PMCA-GFP in the presence of Ca\(^{2+}\). The experiment was conducted as in B, except that CaCl\(_2\) was added to the media to give 10 \(\mu\)M Ca\(^{2+}\).
whereas Fig. 4D shows the scan of the same gel for GFP fluorescence. As the time of proteolysis increased the BFP-PMCA-GFP was digested to a peptide of apparent molecular mass of 170 kDa and then to other peptides of lower apparent molecular mass. Because the 170-kDa peptide did not exhibit GFP fluorescence the conversion of BFP-PMCA-GFP to the 170-kDa peptide must have involved the cleavage of the GFP moiety. At the longest digestion times all the detected fluorescence was in a peptide of apparent molecular mass ~35 kDa probably corresponding mostly to the free GFP. The time course of the appearance of the 170-kDa fragment is in good agreement with the fast phase of FRET decay. Interestingly, the amount of 170-kDa fragment began to decrease after 2 min of proteolysis following a time course similar to the slow phase of FRET decay. Interestingly, the amount of 170-kDa fragment began to decrease after 2 min of proteolysis following a time course similar to the slow phase of FRET decay. The excitation wavelength was 387 nm. The fluorescence intensity without added protease was taken as 100%. The line is the best fit to the data given by a double exponential equation using the following parameters: $A_f = 24 \pm 4\%$, $k_f = 1.2 \pm 0.8$ min$^{-1}$, $A_s = 76 \pm 3\%$, and $k_s = 0.024 \pm 0.001$ min$^{-1}$, where $A_f$ and $A_s$ represent the maximum amplitude of the fast and slow phase, respectively. C, SDS-PAGE analysis of the proteolytic digestion of BFP-PMCA-GFP. The BFP-PMCA-GFP protein was submitted to proteolysis with trypsin as described under "Materials and Methods." The protein was electrophoresed in 9% acrylamide gel. M, molecular mass marker. The peptides were visualized by Coomassie Blue staining. D, GFP fluorescence of proteolytic fragments of BFP-PMCA-GFP. The same gel shown in C was scanned for fluorescence emission at 520 nm.

Dehydration of FRET with the concentration of calmodulin was examined in more detail in Fig. 6. The decrease of FRET produced by increasing concentrations of calmodulin occurred in parallel with the activation of the Ca$^{2+}$-ATPase. At saturating concentrations of Ca$^{2+}$-calmodulin the calculated average distance between the fluorophores in the BFP-PMCA-GFP increased to 50 Å.

It is well known that acidic lipids can mimic the activation of the PMCA produced by calmodulin (38, 39). As shown in Fig. 7, when BFP-PMCA-GFP was activated by phos- phatidylinositol the intensity of FRET was similar to that attained by the enzyme in the presence of PC plus Ca$^{2+}$-calmodulin. Moreover, in the presence of phosphatidylinositol the further addition of Ca$^{2+}$-calmodulin produced only a minor effect on the emission spectra of BFP-PMCA-GFP.

To attain a conformation with the maximal catalytic activity the PMCA is likely to undergo sequential conformational rearrangements. Thus, the most important point of interest was to investigate which step of the BFP-PMCA-GFP activation was being reported by the decrease in FRET. For this purpose we took advantage of the activating effect of mutation D170N reported previously (19). As shown in Fig. 8 in the absence of Ca$^{2+}$ FRET was lower in the mutant BFP-PMCA(D170N)GFP than in the wild-type BFP-PMCA-GFP, accounting for an efficiency transfer of ~26%. Neither the addition of Ca$^{2+}$ nor Ca$^{2+}$-calmodulin produced a significant change in the BFP-PMCA(D170N)GFP spectra.

**DISCUSSION**

FRET is a highly sensitive method used for investigating the structure and dynamics of macromolecules (40). Frequently, however, the attachment of the required fluorophores is detrimental to protein function. One of the questions posed at a beginning of this work was whether a double-labeled BFP-PMCA-GFP containing two large fluorescent protein moieties could be generated without significantly disturbing the function on the enzyme. The results reported here show that, by fusing the fluorescent proteins at both ends of the PMCA, we have succeeded in creating such a construct. We found that BFP-PMCA-GFP possesses Ca$^{2+}$-dependent ATPase and ATP-dependent Ca$^{2+}$-pumping activities. Moreover, the Ca$^{2+}$-ATPase activity of BFP-PMCA-GFP in the absence of calmodulin and its apparent affinity for calmodulin were similar to the values reported for the wild-type enzyme (19), suggesting that...
the fused proteins did not impede the autoinhibition of the enzyme.

The second concern in this study was whether the fluorophores would get close enough in the BFP-PMCA-GFP molecule to display FRET. This was indeed the case, because in the absence of Ca\(^{2+}\)/calmodulin and acidic lipids we observed significant FRET in BFP-PMCA-GFP. The PMCA purified from human red cells has been previously observed to form dimers, and the reported concentration of PMCA needed for half-maximal dimerization varies between 17 and 370 nM (33–35). Thus, FRET in BFP-PMCA-GFP could reflect both the interaction of fluorophores from the same molecule and from associated PMCA molecules. However, at a concentration of protein below 30 nM, we did not detect significant FRET when the fluorescent proteins were attached to different PMCA molecules.

Thus, at low protein concentration the FRET we observed in BFP-PMCA-GFP was due to the interaction of fluorophores from the same molecule.

The values of energy transfer have been frequently translated as distance between the probes (40). The value of FRET observed in the BFP-PMCA-GFP protein indicates that both probes would be an average of \(45 \text{ Å}\) apart. Thus, the BFP and GFP would be indeed very close in the BFP-PMCA-GFP, because both fluorophores cannot come closer than 30 Å from one another as they are each buried \(15 \text{ Å}\) within the fluorescent protein. It should be noted that the calculated distance has to be taken with caution, because it is the average of the distance
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\[
\begin{align*}
\text{CaM. PMCA(c)} & \quad \rightarrow \quad \text{CaM. PMCA(o)} \\
\text{CaM} & \quad \downarrow \quad 1 \quad \downarrow \quad 2 \quad \downarrow \quad 3 \\
\text{PMCA(c)} & \quad \rightarrow \quad \text{PMCA(o)}^+ \\
\end{align*}
\]

SCHEME 1

The purified solubilized protein should be extrapolated with caution to the PMCA in the natural membrane environment.

It is well known that the PMCA can be activated by acidic lipids. It has been proposed that the activation by acidic lipids is in part due to their binding to the calmodulin-binding site mimicking the effects of calmodulin (38, 39). Consistently with this idea phosphatidylinositol-activated BFP-PMCA-GFP showed low FRET similar to that attained by the enzyme activated by \(\text{Ca}^{2+}\)-calmodulin in the presence of PC. This result suggests that the activation by acidic lipids, like that produced by calmodulin, involves a change in the relative position of the N- and C-terminal end of the protein.

Although the BFP and GFP proteins are relatively resistant to proteolytic attack, the PMCA is highly sensitive to cleavage at the N- and C-terminal regions. The decrease of the BFP-PMCA-GFP FRET with the extent of proteolysis was consistent with the liberation of the fluorescent proteins from the PMCA, and it pointed out the essential role of the PMCA in bringing the two fluorescent proteins together. We found that the decrease of FRET with the time of proteolysis was biphasic. ~25% of the total FRET intensity decayed rapidly concomitantly with the removal of the GFP from the BFP-PMCA-GFP while the remainder 75% decayed with a time course compatible with the removal of the N-terminal region containing the BFP. The persistence of FRET after the removal of GFP from BFP-PMCA-GFP may reflect the interaction of a proteolytic fragment of BFP-PMCA and the cleaved C-terminal part of the pump containing GFP. In fact the C28W peptide resembling the calmodulin-binding autoinhibitory domain is capable of inhibiting the pump activated by proteolysis but with less efficiency than the complete C-terminal autoinhibitory sequence when it is part of the PMCA molecule (42).

The precise structural rearrangements of the PMCA molecule involved in the activation process are not known. Recently, a model for the activation of the PMCA by calmodulin has been recently proposed (43). According to this model the preferred route leading to activation involves (i) the binding of calmodulin to domain C of an inhibited conformation of the pump, which has the C domain interacting with the catalytic region; (ii) a conformational change in the autoinhibitor promoted by the binding of calmodulin and the detachment of the autoinhibitor from the catalytic site; and (iii) a further rearrangement of the open conformation, which stabilizes the pump in an activated state (Scheme 1).

We have previously shown that the mutation D170N activates the pump and renders it almost insensitive to further activation by calmodulin (19). Here, we found that BFP-PMCA(D170N)-GFP exhibited less FRET than the wild type in
agreement with the high activity of D170N mutant of the PMCA in the absence of calmodulin.

The investigation of FRET in BFP-PMCA(D170N)-GFP allowed a better characterization of the relationship between the conformational change reported by FRET and the activation of the PMCA. Because FRET in BFP-PMCA(D170N)-GFP was not changed by calmodulin, the decrease in FRET is not likely to be caused by the conformational changes produced during the activation step 1 (Scheme 1). We have previously found that, despite the high activity of mutant D170N, the rate of proteolysis of its C-terminal segment was still significantly accelerated by calmodulin, suggesting that the mutation did not disrupt the interaction between the autoinhibitor and its acceptor site (19). Because the BFP-PMCA(D170N)-GFP FRET was insensitive to calmodulin the observed changes in FRET did not seem to be related to the opening of the pump (step 2, Scheme 1). Following this line of reasoning we propose that the changes in FRET in BFP-PMCA-GFP were caused by the interaction between the N and C segments during the final activated state of the PMCA (step 3). The results obtained with BFP-PMCA(D170N)-GFP suggest that the change in the relative location of the ends of the PMCA, while occurring during activation, may take place independently of the major autoinhibitory region. This finding is in line with our previous proposal that the activated state may be attained without a full disengagement of the autoinhibitory domains from the catalytic core. The finding that the relative location of the ends of the PMCA molecule are sensitive to the activation state of the enzyme seems surprising given the fact that neither the amino acid segment near the N nor the C terminus has been reported to have a functional role (8, 9, 32).

Constructions of the type of BFP-PMCA-GFP characterized here seem a promising tool for more detailed studies of the activation process of PMCA. Furthermore, this strategy may allow analysis of PMCA activation in its specific environment in live cells by FRET microscopy. In particular its application to other PMCA isoforms may help to clarify recent doubts as to the actual importance in vivo of the differences in their calmodulin regulation deduced from in vitro assays (44).

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