Study of Calmodulin Binding to the Alternatively Spliced C-Terminal Domain of the Plasma Membrane Ca^{2+} Pump†

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Received November 21, 1991; Revised Manuscript Received September 4, 1992

ABSTRACT: The C-terminal regions of the four human plasma membrane Ca^{2+} pump isoforms 1a–d generated from alternatively spliced RNA have been expressed in *Escherichia coli*, and the recombinant proteins have been purified to a very high degree. The C-termini of isoforms 1a, 1c, and 1d contain an insert encoded by an alternatively spliced exon which is homologous to the calmodulin binding domain of isoform 1b. In isoforms 1c and 1d (29 and 38 amino acid insertions, respectively), subdomain A of the original calmodulin binding site of isoform 1b is followed by the spliced-in domain, which is then followed by subdomain B of the original calmodulin binding site. The positive charges of histidine residues at positions 27, 28, and 38 of the alternatively spliced sequence are likely to be responsible for the observed pH-dependent calmodulin binding to the novel "duplicated" binding site. The affinity of calmodulin for the C-terminal domains of isoforms 1a, 1c, and 1d, which contain the histidine-rich inserts, is much higher at pH 5.9 than at pH 7.2. A synthetic peptide (I31) containing 31 amino acids of the alternatively spliced sequence (from residue 9 to 40) also binds calmodulin with strong pH dependency. Alternative splicing in the C-terminal domain is proposed to confer pH dependence to the regulation of the activity of Ca^{2+} pump isoforms.

The primary structures of plasma membrane Ca^{2+} ATPase (PMCAs)[1] pumps from several mammalian species have been recently deduced from their cDNAs (Shull & Greeb, 1988; Verma et al., 1988; Greeb & Shull, 1989; Strehler et al., 1990; DeJaegere et al., 1990). The C-terminal region of the pump, corresponding to about 120 amino acids, contains regulatory domains, e.g., the calmodulin binding site and—at least one isoform—a substrate site for the cAMP-dependent protein kinase [see Carafoli (1991) and Strehler (1991) for recent reviews]. It also contains substrate sites for protein kinase C (Wang et al., 1991). In the absence of calmodulin, the C-terminal domain acts as an internal inhibitor of the calcium pumping activity (Carafoli et al., 1987; James et al., 1989; Enyedi et al., 1989; Falchetto et al. 1991).

Alternative RNA splicing involving a single 154-bp exon procedures four variants (1a–d) of the human plasma membrane Ca pump isoform 1 (Strehler et al., 1989; Strehler, 1991). Differential use of two internal donor splice sites leads to an insertion of either 87 or 114 nucleotides into the mRNA. The corresponding proteins (hPMCA1c and hPMCA1d), thus, contain either 29 or 38 additional amino acids between residues 1117 and 1118 (numbering of the pPMCA1b isoform). The site of "insertion" coincides with the boundary between subdomains A and B of the calmodulin binding domain, which has been previously identified by chemical labeling methods (James et al., 1988; Enyedi et al., 1989; Vorherr et al., 1990). Inclusion of the whole 154-bp exon leads instead to a mRNA species that would encode a protein (PMCA1a) differently not only in the calmodulin binding domain but also in the remainder of the C-terminal amino acid sequence; i.e., it would lack the cAMP-dependent phosphorylation site due to a shift in the reading frame (Shull & Greeb, 1988; Strehler et al., 1989). The primary structure of the calmodulin binding site is thus different in isoforms 1a, 1b, and 1c/d. The 10 N-terminal amino acids of the alternatively spliced sequence replace amino acids 19–28 (subdomain B) of the calmodulin binding domain of isoform hPMCA1b. Since the replacement is highly conservative, a new subdomain B is potentially formed. As recently shown, this substitution may change the calmodulin binding and the autoinhibitory properties of the corresponding peptide (Enyedi et al., 1991).

To study the possible functional divergence between the different regulatory domains encoded by the alternatively spliced isoforms hPMCA1 mRNAs, the ≈ 150 residue C-terminal portion of isoforms hPMCA1a–d has been expressed in *E. coli*. The purified 1a, 1c, and 1d peptides, which potentially possess an additional calmodulin binding site as compared to the 1b isoform, bind calmodulin in a pH-dependent way; the presence of histidine residues in the alternatively spliced sequence is apparently responsible for the pH dependency of the binding. A synthetic peptide consisting of 31 amino acids of the altenatively spliced sequence, including the His residues, also exhibits pH-dependent calmodulin binding. The results suggest a role for alternative splicing in conferring pH dependency to the calmodulin regulation of hPMCA1 isoform subtypes.

MATERIALS AND METHODS

Source of cDNAs Corresponding to hPMCA isoforms 1a–d. The original hPMCA1 cDNA isolated from a human teratoma NTera2D cell library (Verma et al., 1988) codes for the isoform 1b subtype. Two cDNAs isolated from a skeletal muscle library, originally termed hskm-1.1 and hskm-1.2 (Strehler et al., 1989), correspond to hPMCA isoforms 1c

† This work was supported by grants from the Swiss National Science Foundation (31-25285.88 and 31-28772.90) and a grant from the Roche Research Foundation. E.E.S. is the recipient of a START research career development award from the Swiss National Science Foundation (31-27103.89).

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[1] Abbreviations: CaM, calmodulin; DC, dansyl calmodulin; kb, kilobase(s); EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PAGE, polyacrylamide gel electrophoresis; PMCA, plasma membrane Ca^{2+} ATPase; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; RT, room temperature.

0006-2960/92/0431-11785$03.00/0

and 1d, respectively [see Strehler (1991) for a description of the nomenclature]. A cDNA coding for the isoform subtype 1a was isolated according to published procedures (Maniatis et al., 1982) from a human fetal brain cDNA library (in λgt11) kindly provided by Dr. D. M. Kurnit (University of Michigan Medical Center, Ann Arbor, MI). A 2.0-kb internal *Pst*I fragment of a hPMCA2-specific cDNA (Heim et al., 1992) was employed as radiolabeled probe (Feinberg & Vogelstein, 1983) to screen 7×10^5 plaque-forming units of this library under low stringency conditions: Hybridization was for 24 h at 37 °C in 25% formamide, 5 × SSPE (1 × SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA), 5 × Denhardt's [1 × Denhardt's is 0.02% (w/v) each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 0.1% SDS, 100 μg/mL denatured herring sperm DNA, and 5×10^5 cpm/mL of heat-denatured radiolabeled probe. Final washing of the phage replica filters was for 50 min in 1 × SSC/0.1% SDS at 42 °C. One cDNA clone (hfb 15.1) isolated by this screening procedure was found to carry a 1.1-kb *Eco*RI insert corresponding to the hPMCA 1a isoform subtype [positions 2630–3578 in Verma et al. (1988) but containing in addition the 154-bp insertion encoded by a separate, alternatively spliced exon (Strehler et al., 1989) after position 3351]. All *Eco*RI insert fragments of the hPMCA1 cDNA clones were subcloned in pUC18/19 vectors (Pharmacia-LKB Biotechnology Inc., Uppsala, Sweden), and plasmid DNA was prepared as described (Maniatis et al., 1982).

Construction of Bacterial Expression Vectors. The vectors pKK233-2 and pJLA502 were from Pharmacia-LKB Biotechnology Inc., Uppsala, Sweden, and from Medac GmbH, Hamburg, Germany, respectively. Prior to construction of a hPMCA1b expression vector, a 522-bp *Rsa*I–*Eco*RI fragment isolated from cDNA clone t19c [positions 3057–3578 in Verma et al. (1988)] and a 249-bp *Eco*RI–*Hin*dIII fragment from clone t6 [positions 3579–3827 in Verma et al. (1988)] were ligated into pUC18 cut with *Sma*I and *Eco*RI to create plasmid pTRH. A 613-bp *Dra*I fragment corresponding to positions 3198–3810 of the hPMCA1b cDNA sequence (Verma et al., 1988) was then isolated from pTRH and ligated to synthetic, phosphorylated *Nco*I linkers (5′-pd[GCCATGGC]-3′, Pharmacia LKB Biotechnology, Inc.). After digestion with *Nco*I, the designed fragment was separated from excess linkers by agarose gel electrophotoresis, purified by the Gene-clean procedure (BIO 101 Inc., LaJolla, CA), and ligated into *Nco*I-cut pKK233-2 and pjLA502. Plasmids containing the hPMCA1b fragment in the correct orientation with respect to the bacterial P_{trc} (pKK233-2) or P_RP_L (pJLA502) promoter were identified by *Eco*RI digestion followed by agarose gel electrophoresis. Expression vectors specifying the C-terminal region of hPMCA1a, -1c, and -1d were constructed by a cassette replacement approach whereby the 124-bp *Sau*I–*Nsi*I fragment of pKK233-hPMCA1b [positions 3246–3369 in Verma et al. (1988)] was first replaced by the "corresponding" 278-, 211-, and 238-bp *Sau*I–*Nsi*I fragment isolated from the hPMCA1a, -1c, and -1d cDNAs, respectively. This created vectors pKK233-hPMCA1a, pKK233-hPMCA1c, and pKK233-hPMCA1d. The inserts of these vectors were then isolated by *Nco*I digestion and ligated into *Nco*I-digested pJLA502 to create expression vectors pJLA502-hPMCA1a, pJLA502-hPMCA1c, and pJLA502-hPMCA1d. The integrity of the constructs was confirmed by nucleotide sequencing (Sanger et al., 1977) either directly on double-stranded plasmid DNA (Zhang et al., 1988) or on single-stranded template DNA after subcloning of appropriate fragments into M13mp18/19 vectors (Messing, 1983). "Sequenase" version 2 DNA polymerase, dGTP, or dITP reaction mixes and "universal" M13 or hPMCA1-specific primers were used as suggested by the supplier of the sequencing kit (United States Biochemical Corp., Cleveland, OH). PMCA1-specific oligodeoxynucleotide primers RMP-1 5′-d[CGTAGTTCTTTATATG]-3′ [positions 3373–3388 in Verma et al. (1988); sense stand] and RMP-2 (5′-d[GCTCTGAATCTTCTATCCTA]-3′ [positions 3450–3469 in Verma et al. (1988); antisense strand] were obtained from S. Keller (Institute for Cell Biology, ETH, Zurich, Switzerland) and were purified by denaturing polyacrylamide gel electrophoresis (Ausubel et al., 1987) prior to use.

Expression of Recombinant Proteins. pJLA502-derived expression plasmids were used to transform the protease-deficient *E. coli* strain *lon-hptr-ter*^{R} (Goff et al., 1984). Overnight cultures of individual clones were grown in LB-medium containing 13 μg/mL of tetacycline and 50 μg/mL of ampicillin at 30 °C. For large-scale protein expression, 1 L of the same medium was inoculated with 10 mL of the desired overnight culture. Cells were incubated in a shaker at 30 °C and 250 rpm until an OD_{550nm} of approximately 0.7 was reached. The cells were then shifted to growth at 42 °C, and incubation was continued for 12 h. Cells were finally spun down in an IEC PR-6000 centrifuge at 4000 rpm.

Protein Purification. A pellet corresponding to 500 mL of cell culture was resuspended in a volume of 25 mL lysis solution containing 8 M urea, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5 mM $CaCl_2$, 0.1% Triton X-100, and 0.1 mM PMSF. The resuspended cells were lysed in a French press (American Instrument Co., Silver Spring, MD) at 16000 psi. The lysed material was briefly spun down, and the supernatant was applied to a calmodulin–Sepharose 4B column at a flow rate of 1mL/h. The column was washed with 150 column vol of a buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH7.5, and 0.5 mM $CaCl_2$. Bound proteins were eluted with 150 mM NaCl, 20 mM Tris-HCl, pH7.5, and 2 mM EGTA. Protein-containing fractions were pooled, and the solution was brought to a final concentration of 4 M guanidinium-HCl and 20% 2-propanol. This solution was injected into a 300-7-C4 HPLC column. Protein was eluted with a linear gradient running from 100% buffer A (0.1% TFA acid) to 100% buffer B (0.08% TFA, 70% acetonitrile). Fractions containing protein were pooled and lyophilized. The purified protein was dissolved in 20 mM Hepes, pH 7.2. The yield and purity of protein preparations was routinely monitored by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970).

Immunoblot Analysis. Proteins were separated on 15% SDS–polyacrylamide gels and transferred to nitrocellulose as described (Towbin et al., 1979; Ausubel et al., 1987). Nonspecific binding was blocked by shaking the filter for ≥1 h in 10 mM Na_2HPO_4, pH7.0, and 140 mM NaCl (PBS) containing 2% nonfat milk powder. The nitrocellulose was incubated for 1 h at RT with rabbit polyclonal antibody against hPMCA peptide I31 (see Table I) diluted 1:100 in PBS. The antibody against peptide I31 (see below) was prepared using the procedure for the antibody against peptide C20W (Vorherr et al., 1991). After washing the nitrocellulose filter three times in PBS and 2% milk powder, the filter was incubated for 1 h with peroxidase-coupled goat anti-rabbit IgG diluted 1:1000 in PBS and 2% milk powder. The blot was rinsed three times in PBS and developed in PBS containing 0.015% hydrogen peroxide, 0.05% chloronaphthol, and 17% methanol.

Calmodulin Overlay. The above-described Western-blotting protocol was followed up to the blocking step. Nitrocellulose filters were then incubated for ≥1 h in a solution

Table I: Sequences of Calmodulin Binding Sites in hPMCA Isoforms[a]

Site 1:	
hPMCA1b	LRRGQILWFRGLNRIQTQ IRVVNAFRSS
hPMCA1a/c/d	LRRGQILWFRGLNRIQTQ MDVVNAFQSG
	1 10
Site 2:	
hPMCA1c	LRRQPSIASQHHD IRVVNAFRSS
	17 29
hPMCA1d	LRRQPSIASQHHDVTNISTPTH IRVVNAFRSS
	17 38
hPMCA1a	LRRQPSIASQHHDVTNISTPTHVVFSSSTASTTVG
	17 51
Peptide I31:	
	SGSSIQGALRRQPSIASQHHDVTNISTPTHI
Peptide C28Y:	
	LRRGQILYFRGLNRIQTQIKVVNAFSSS

[a] Basic residues are printed in boldface letters; insertions due to alternative splicing are underlined and are numbered beginning with residue Met-1.

containing 10 nM ^{125}I-labeled calmodulin, prepared using 'enzymobeads' according to Marcholonis (1969), in PBS, 0.1% milk powder, and 0.5 mM $CaCl_2$. Nitrocellulose filters were washed three times in the same buffer without calmodulin and exposed to Kodak-X-Omat XAR-5 films with intensifying screens.

Synthesis of Peptide I31. Amino acid derivatives, resins, and reagents for peptide synthesis were obtained from Novabiochem (Läufelfingen, Switzerland). The synthesis of the peptide I31 S-G-S-S-I-Q-G-A-L-R-R-Q-P-S-I-A-S-Q-H-H-D-V-T-N-I-S-T-P-T-H-I (corresponding to amino acids 1126–1156 in isoform 1d) was performed starting with 0.3 mmol of *p*-hydroxymethylphenoxymethyl-polystyrene resin (1% divinylbenzene, 104 mM/g) and a 4-fold excess of amino acid derivative using DCCD/HOBT (dicyclohexylcarbodiimide/1-hydroxybenzotriazole) (1:1) activation and the F moc/tBu (9-fluorenylmethoxycarbonyl/*tert*-butyl) strategy. The Pmc (2,2,5,7,8-penamethylchroman-6-sulfonyl) protecting group for Arg and trityl side chain protection for the His residues were used. Cleavage of the peptide was performed in a mixture of 4 mL of TFA, 30 μL of ethanedithiol, 90 μL of dimethylsulfide, and 90 μL of thioanisol for 1.5 h at room temperature. Crude product (94 mg) was obtained from 198 mg of peptide resin after precipitation and washing with ethyl ether. Preparative and analytical HPLC were carried out using Nucleosil reversed-phase materials packed in Macherey & Nagel columns (Oensingen, Switzerland). The reversed-phase buffers were as follows: A, 0.1% TFA in water; B, 50% *n*-propanol in water and 0.1% TFA. The peptides were purified on a 250 × 55 mm C_8-column (10 μm, 300 Å) by applying a linear gradient from A to B. The flow rate was 21 mL/min. Purified peptide (13.6 mg) was obtained from 94 mg of crude material after preparative HPLC. Analytical control was performed on a 125 × 2.1 mm C_{18}-column (3 μm, 120 Å) with buffer: A, 0.1% TFA in water; and B, 70% acetonitrile and 0.1% TFA in water. Chromatography was carried out using Applied Biosystems equipment (Foster City, CA). UV detection for analytical control was performed at 210 nm. Sequencing was carried out using an Applied Biosystems 470A sequencer with 120A on-line phenylthiohydantoyl detection. An Applied Biosystems derivatizer 420A and on-line phenylthiocarbamoyl detection with the Model 130A Applied Biosystems analyzer was used for the derivatization, separation, and identification of the amino acids. The amino acid analysis agreed with the expected ratios. C28Y was synthesised as described in Vorherr et al. (1990).

Fluorescence Measurements. Fluorescence measurements were performed with a SPEX Fluorolog 1680 (Metuchen, NJ) double-wavelength spectrometer connected to a DM1B coordinator. Quartz cuvettes with a path length of 10 mm and a volume of about 3.5 mL were used. Dilution effects were less than 5%. The fluorescence emission spectra of dansyl calmodulin, prepared according to Vorherr et al. (1990), were recorded after excitation of the probe at 340 nm. The system was buffered with 20 mM Hepes, pH 7.2 or pH 5.9, containing 0.5 mM $CaCl_2$. Proteins and peptides dissolved in 20 mM Hepes, pH 7.2, were added to the medium containing dansyl calmodulin. The samples were stirred, and spectra were recorded from 400 to 550 nm. The titrations of fluorescence enhancement or quenching were performed by recording the fluorescence emission at 497 nm after equilibration of the mixture.

Determination of Affinity Constants. Data points were calculated according to the method of Stinson and Holbrook (1978).

Ca-ATPase Competition Assay. The ATPase activity was measured by the method of Lanzetta et al. (1979). The reactions were carried out at 37 °C in 20 mM Hepes, pH 5.5 or 7.26, 50 mM KCl, 5 mM $MgCl_2$, and 10 μM $CaCl_2$. Peptides and calmodulin were first added to the reaction buffer and incubated for 5 min at 37 °C. Then the ATPase was added, and preincubation was continued for 5 min at 37 °C. Reaction was started by the addition of 1 mM ATP, carried out for 10 min at 37 °C, and stopped by the addition to the acidic color reagent.

RESULTS

Construction of Expression Vectors for C-Termini of hPMCA1 Isoforms. The 10th hydrophobic transmembrane domain of the hPMCA1b isoform is thought to be located between residues 1036 and 1054 of the 1220-residue molecule (Verma et al., 1988). To avoid potential problems with hydrophobic sequences, it was decided to construct expression vectors containing only the portion of the pump C-terminal to the last membrane-spanning region. A conveniently located *Dra*I restriction site cutting within the codon for residue 1066 (TTA, Leu-1066) was chosen as the 5′ end for the construction; however, because of the *Nco*I linker addition necessary to provide the coding sequence of the recombinant protein with its own initiation codon and because of the predetermined reading frame, the two N-terminal amino acids of the expressed protein were Met-Ala rather than the original Arg^{1065}-Leu^{1066} (Figure 1). The remaining sequence was identical to the C-terminal sequence of the corresponding hPMCA1 isoforms. All constructs were first made in the IPTG-inducible vector pKK233.2. Although the yields of protein expression from this vector were inferior to those obtained with the heat-inducible pJLA502 vector, the pKK233.2-based hPMCA1b construct proved to be ideal for the construction of the additional hPMCA1 vectors. In contrast to pJLA502, pKK233.2 contains neither *Nsi*I nor *Sau*I restriction sites, thus facilitating the construction of hPMCA1a, -1c, and -1d vectors by an approach exploiting the possibility of isolating the various alternatively spliced DNA regions on a *Nsi*I–*Sau*I fragment (see the Materials and Methods section). The complete coding sequence of each isoform variant could then be easily cloned into the pJLA502 vector on a *Nco*I fragment. The expected protein products generated by the four expression

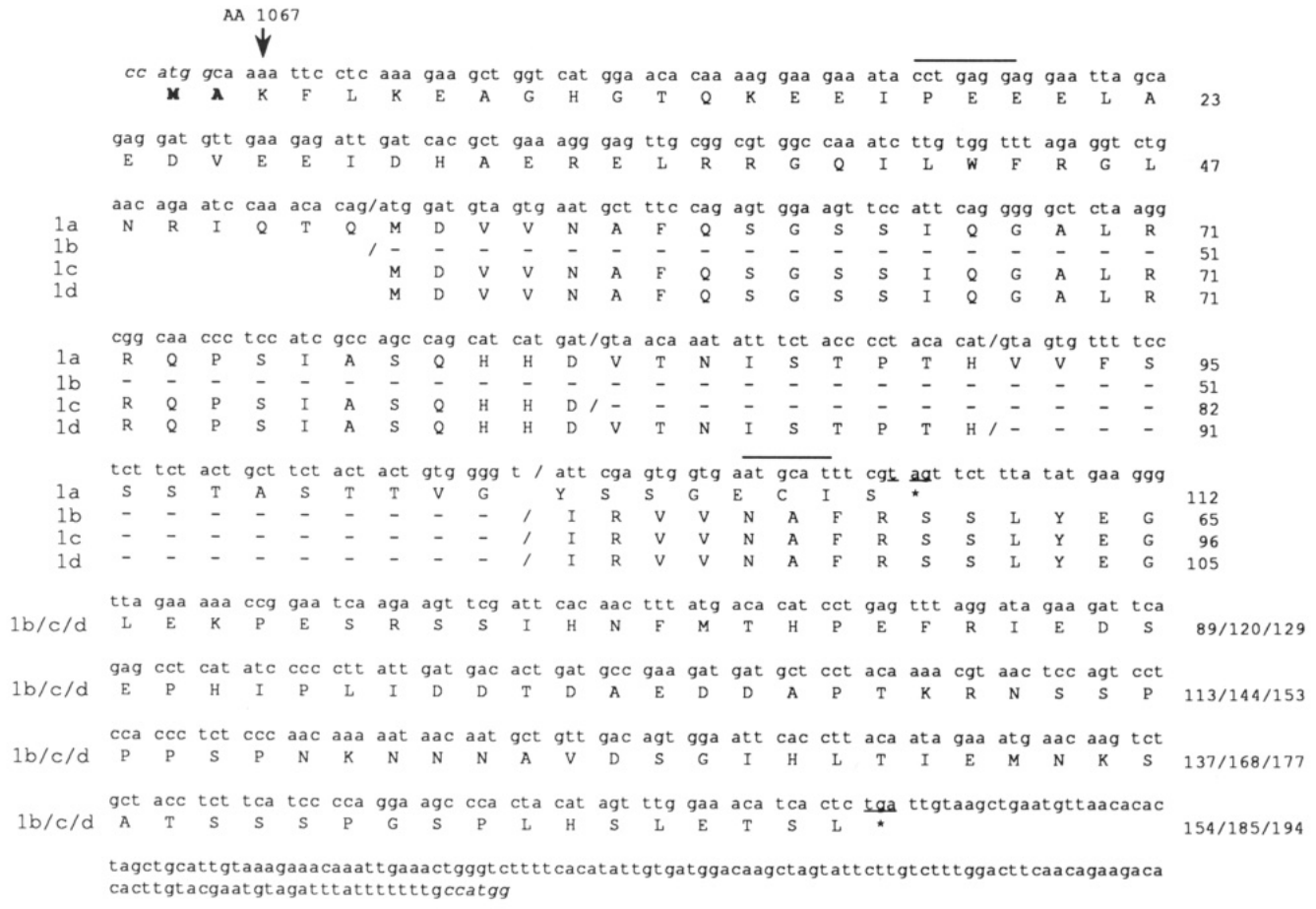

FIGURE 1: Sequence of the hPMCA1 C-terminal coding fragments used for vector constructions. The nucleotide and encoded amino acid sequence of the DNA fragments inserted into the expression vectors is shown. The *Nco*I sites created by linker addition to the ends of the fragments and the two N-terminal residues not present in the original sequence of hPMCA1 are in boldface letters. Lys-3 of the recombinant proteins corresponds to residue 1067 of the full-length hPMCA1 sequence. The unique *Sau*I and *Nsi*I recognition sequences used for vector constructions by cassette replacement are overlined, and the TAG and TGA translational stop signals for isoforms 1a and 1b–d, respectively, are underlined. The boundaries of alternatively spliced sequences are indicated by slashes; amino acids lacking in isoforms 1b–d are represented by hyphens.

vectors contained 112 (isoform 1a), 156 (1b), 185 (1c), and 194 (1d) residues, respectively (Figure 1).

Expression of C-Termini of hPMCA1 Isoforms in E. coli. The protease-deficient *E. coli* strain *lon-hptr-tet*^{R} yielded the best results for the expression of the C-termini from the pJLA502-derived vector constructs allowed. The presence of a heat-sensitive repressor of the P_L promoter in *E. coli* transformed with pJLA502-derived constructs allows a stringent control of the expression of the recombinant protein. Once optical density at 30 °C had reached 0.7, cell cultures were shifted to 42 °C in order to induce vector-driven protein expression. However, the newly expressed proteins were not abundant enough to be visible on Coomassie Blue-stained SDS–polyacrylamide gels of total *E. coli* cell extracts. They were thus detected by the sensitive ^{125}I-labeled calmodulin overlay technique on total *E. coli* cell extracts after 3.5 h of induction (Figure 2).

Purification of Recombinant Proteins. Calmodulin affinity chromatography of total *E. coli* cell extracts resulted in the isolation of a highly purified protein for every construct as visualized by SDS–PAGE (Figure 3). The apparent molecular masses of the purified calmodulin binding peptides were significantly higher than expected from the sequences of the different C-terminal domains (Table II) but were identical to those of the heat-shock-induced calmodulin binding proteins in total *E. coli* extracts (Figure 2). Somehow then, in the SDS–PAGE system used, the mobility of the expressed peptide was lower than expected from the calculated molecular mass

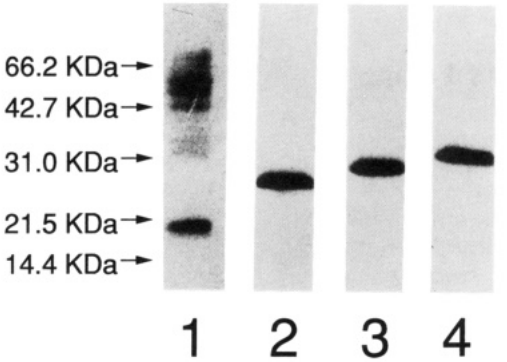

FIGURE 2: ^{125}I-Labeled calmodulin overlay to detect expressed C-terminal hPMCA1 domains in total *E. coli* extracts. Induced cell cultures (100 μL) were pelleted and dissolved in 30 μL of SDS–PAGE sample buffer. Proteins were separated by SDS–PAGE and blotted onto nitrocellulose. Unspecific binding was quenched by incubation for ≥1 h in PBS solution (see the Materials and Methods section) containing 2% nonfat milk powder solution. The nitrocellulose was then incubated for ≥1 h in PBS solution containing 0.1% nonfat milk powder, 0.5 mM $CaCl_2$, and 10 nM ^{125}I-labeled calmodulin. After three washings (see the Materials and Methods section), the nitrocellulose was exposed to X-ray film. Lane 1, cells expressing construct 1a; lane 2, cells expressing construct 1b; lane 3, cells expressing construct 1c; lane 4, cells expressing construct 1d.

(the reasons for this behavior are analyzed in the Discussion section). Mass spectrometry was used to establish that the molecular mass of the bands was that expected from the sequence. Peptide 1b was used as a test: the mass spectrometry

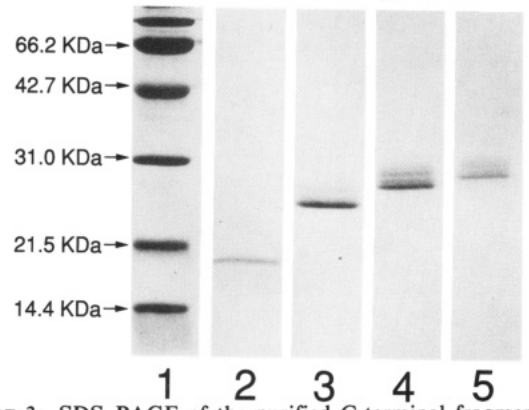

FIGURE 3: SDS–PAGE of the purified C-terminal fragments of hPMCA1 isoforms. 1 μg of each purified C-terminal domain was run on a 15% polyacrylamide gel and then stained and Coomassie Blue. Experimental details are described in the Materials and Methods section. Lane 1, molecular mass standards in kilodaltons; lane 2, 1 μg of 1a; lane 3, 1 μg of 1b; lane 4, 1 μg of 1c; lane 5, 1 μg of 1d.

Table II: Molecular Masses of Expressed C-Terminal Portions of hPMCA1 Isoforms

	calculated masses (Da)	apparent masses (Da)
1a	12 408	20 300
1b	17 567	26 000
1c	20 696	28 000
1d	21 647	29 500

runs showed that the mass of the peptide indeed corresponded exactly to that calculated from the sequence. The minor bands associated with the main expressed products (Figure 3) could be due to proteolytic activity that would result in several forms of the peptide with different N- and/or C-termini. They could, however, also represent different conformational states of the peptides, probably because of some calcium bound to them. Independent evidence in this laboratory has indeed shown that the C-terminus of the pump contains calcium binding sites resistant to SDS–PAGE conditions (Hofmann et al., unpublished).

Characterization of Purified hPMCA1 Isoform C-Termini. As expected, the purified calmodulin binding proteins interacted with ^{125}I-labeled calmodulin in overlay experiments (Figure 4). The low CaM concentration employed (10 nM) indicates that the recombinant proteins bound calmodulin with high affinity. Bands running at the apparent molecular masses listed in Table II most likely represent monomers of each C-terminal domain. Strong binding occurring in a region of the gel corresponding to about twice the respective molecular masses are likely to represent dimers of the recombinant proteins. Such dimers have been proposed to be formed by the intact pump (Kosk-Kosicka et al., 1989) and have been shown to involve the calmodulin binding domain (Vorherr et al., 1991). Dimerization has been shown to occur easily in the low nanomolar concentration range of the protein (Kosk-Kosicka et al., 1990). Bands running at even higher molecular masses are likely to be oligomers. Aggregate bands are particularly evident for the calmodulin binding protein expressed from the hPMCA1a construct and could be detected even in total *E. coli* extracts (see Figure 2). As discussed above, the dimers and oligomers appear very clearly in the ^{125}I-labeled calmodulin overlays, but are hardly visible in Coomassie Blue stained gels. This could be due to their very low concentration or, alternatively, to their weak ability to bind Coomassie Blue.

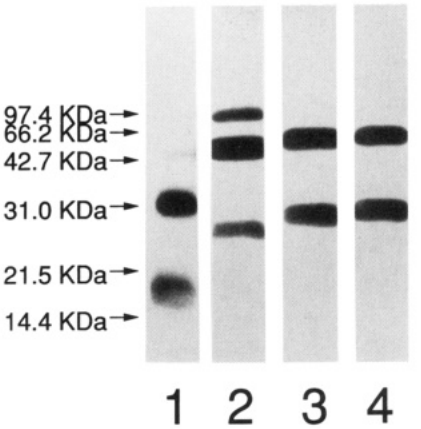

FIGURE 4: ^{125}I-Labeled calmodulin overlay of the purified C-terminal domains of hPMCA1 isoforms. 1 μg of each purified C-terminal domain was run on a 15% polyacrylamide gel and blotted onto nitrocellulose. After saturation in a 2% milk powder solution in PBS, the nitrocellulose was incubated in a solution containing 10 nM ^{125}I-labeled calmodulin. The nitrocellulose was washed and exposed to X-ray film. Experimental details are described in the Materials and Methods section. Lane 1, 1 μg of peptide 1a; lane 2, 1 μg of peptide 1b; lane 3, 1 μg of peptide 1c; lane 4, 1 μg of peptide 1d.

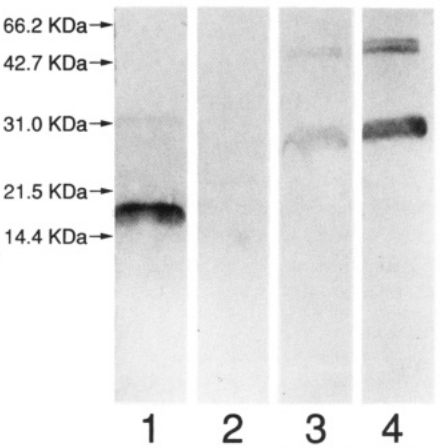

FIGURE 5: Western blot of the expressed peptides with anti-peptide I31 antibody. 1 μg of each purified C-terminal domain was separated on a 15% polyacrylamide gel and blotted onto nitrocellulose. After saturation in a milk powder solution, the nitrocellulose was incubated in a solution containing affinity purified anti-peptide I31 antibody. Experimental details are described in the Materials and Methods section. Lane 1, 1 μg of peptide 1a; lane 2, 1 μg of peptide 1b; lane 3, 1 μg of peptide 1c; lane 4, 1 μg of peptide 1d.

Peptide I31 corresponds to a region of the spliced-in sequence present in isoforms 1a and 1d (and partially in 1c; Table I). Purified calmodulin binding proteins derived from constructs 1a, 1c, and 1d containing alternatively spliced sequences reacted with affinity-purified anti-peptide I31 antibodies (Figure 5). As expected, the protein derived from construct 1b (which lacks the alternatively spliced exon coding for the I31 sequence) showed no reaction. Weak antibody staining was present at twice the apparent molecular mass seen on SDS–PAGE gels (Figure 3). These findings are consistent with those from the ^{125}I-labeled calmodulin overlay experiments and corroborate the suggestion of dimerization/oligomerization of the domains.

Calmodulin Binding Properties of Recombinant Proteins. To obtain more detailed information on the calmodulin binding properties of the various C-terminal domains, dansyl calmodulin was used. For these experiments the calmodulin binding peptides produced from the vectors for isoforms 1b–d were further purified by reversed-phase chromatography. Further purification of the calmodulin binding peptide 1a by

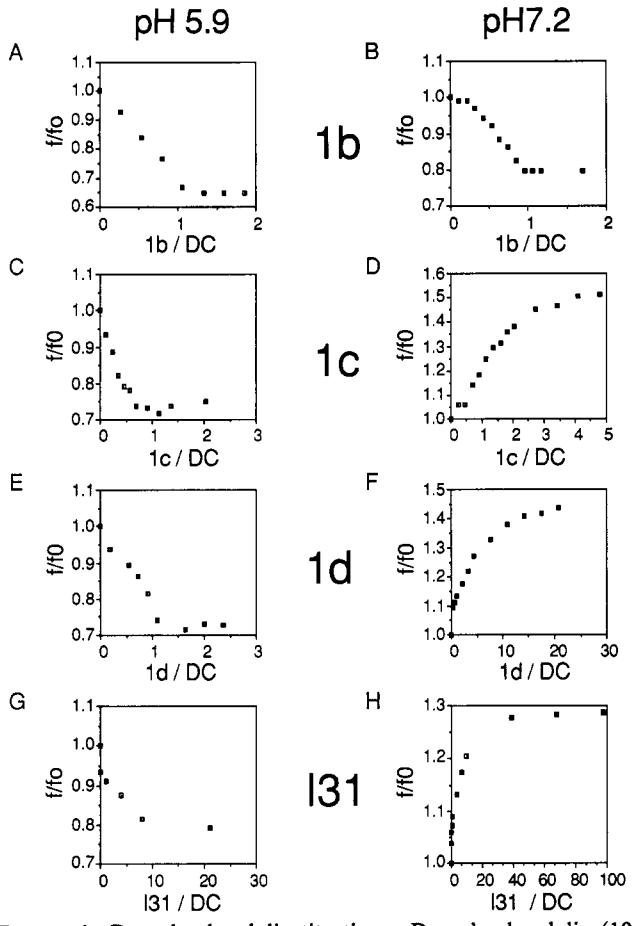

FIGURE 6: Dansyl calmodulin titrations. Dansyl calmodulin (10 nM) was titrated with calmodulin binding domains and with peptide I31 in the presence of Ca^{2+}. Excitation was at 340 nM. The relative fluorescence intensities (f/f_0) are plotted against the ratio of the total concentration of C-terminal domains or of peptide I31 to the concentration of dansyl calmodulin (protein/DC), as given by one representative experiment. The data points were recorded as outlined in the Materials and Methods section. (Panels A and B) titration with domain 1b, (panels C and D) titration with domain 1c, (panels E and F) titration with domain 1d, (panels G and H) titration with peptide I31. Titrations were performed at pH 5.9 (panels A, C, E, and G) and at pH 7.2 (panels B, D, F, and H).

the same procedure proved extremely difficult, presumably because of its distinct propensity to form aggregates (see also Figure 2). Dansyl calmodulin titrations were therefore performed only with the 1b–d C-terminal fragments and with peptide I31. The sequence of the insert in isoform 1d and in peptide I31, which is homologous to subdomain B of the "canonical" calmodulin binding domain of isoform PMCA1b, is identical to that of 1a (Table I); the results obtained with peptides 1d and I31 were therefore likely to be similar to those expected for 1a. The low concentration of dansyl calmodulin (10 nM) used in the titrations (shown in Figure 6A–H) was chosen to maintain the concentrations of the binding peptides at a level low enough to minimize dimerization. Since calmodulin itself prevents dimerization of the pump via its calmodulin binding domain (Vorherr et al., 1991), it is very likely that under the experimental conditions the peptides remained monomeric. The titrations showed pH dependency of calmodulin binding to the domains containing alternatively spliced sequences (1c and 1d) but not to the domain lacking them (1b). With the 1b domain, fluorescence quenching was observed at pH 5.9 (maximum of 0.75 f/f_0 at a protein/dansyl calmodulin ratio of 1) (Figure 6A), and at pH 7.2 the result was essentially identical, i.e., strong quenching reaching saturation at a low protein/dansyl calmodulin ratio (Figure 6B). Different results were obtained with the alternatively spliced domains at pH 7.2. Fluorescence enhancement was observed reaching saturation at higher protein/dansyl calmodulin ratios: for isoform 1c the enhancement reached a maximum of 1.5 at a ratio of 4 (Figure 6D) and for isoform 1d a maximum of 1.45 at a ratio of 20 (Figure 6F). In contrast, at pH 5.9 quenching was observed reaching its maximum at ratios close to 1 for both 1c and 1d (Figure 6C and 6E). Titration of the synthetic peptide I31 at pH 5.9 showed maximal quenching at a ratio of 20 (Figure 6G, f/f_0 0.8, calculated K_D for calmodulin 29.7 nM). At pH 7.2 the fluorescence enhancement reached a maximum of 1.29 at a ratio of 50 (Figure 6H), resulting in a calculated K_D of 80.4 nM.

Table III: Effects of Calmodulin Binding Peptides on Ca^{2+}-ATPase in Presence and Absence of Calmodulin[a]

additions	ATPase activity (nmol P_i mg of protein^{-1} min^{-1}) pH 5.5	pH 7.25
control	11.4	20.1
calmodulin	62.5	113.8
calmodulin and C28Y	20.0	28.9
calmodulin and I31	72.5	120.3
C28Y	13.4	25.7
I31	11.2	24.4

[a] The ATPase activity was measured as described in the Materials and Methods section. Concentrations were as follows: Ca^{2+}-ATPase, 30 nM; calmodulin, 176 nM; peptide C28Y, 1 μM; peptide I31, 1 μM. The results are the average of at least three separate experiments.

Effects of Calmodulin Binding Peptides in Absence and Presence of Calmodulin. It seemed of interest to compare the effects of synthetic peptides C28Y and I31 on the Ca^{2+}-ATPase activity of the pump in the presence and absence of calmodulin. Peptide C28Y decreased the stimulation of the ATPase by calmodulin. When added alone to the intact pump, it stimulated the ATPase slightly, an effect which could be conveniently explained with the heterodimerization of the peptide with the ATPase (Vorherr et al., 1991). As expected, peptide C28Y reduced the effect of calmodulin on the ATPase activity at both pH 5.5 and pH 7.3. However, peptide I31 produced surprising effects. At pH 5.5 the stimulation of the pump by calmodulin was increased substantially, whereas at pH 7.25 the increase was much less pronounced. At variance with peptide C28Y, at pH 5.5 peptide I31 did not affect the basal activity of the pump. At pH 7.25, however, the effect of peptide I31 was similar to that of C28Y (Table III).

DISCUSSION

The studies reported here on the alternatively spliced C-terminal domains of hPMCA1 isoform subtypes and on a peptide derived from the inserted sequences have shown that alternative splicing in the C-terminal region introduces a potential second calmodulin binding site into the corresponding isoform. The C-terminal domains were expressed in *E. coli* and purified by calmodulin affinity chromatography and by reversed-phase chromatography (peptides 1b–d only). Surprisingly, their apparent masses on SDS–PAGE were larger by about 8000 Da than expected from the calculated values. Additional protein mass accounting for this difference was ruled out on several accounts. First, the integrity of the constructs was confirmed by nucleotide sequencing. Secondly, test-digestion experiments of the expressed and purified peptide 1b with CNBr resulted in the expected fragments. Thirdly, N-terminal partial sequencing was performed to verify the amino acid sequences. Lastly, mass spectrometry tests of

peptide 1b confirmed that its molecular mass corresponded to the calculated value (data not shown). The apparent molecular mass difference could be due to the presence of highly negatively charged regions in the expressed peptides [e.g., corresponding to the domain previously defined as A18 in hPMCA1, see Verma et al. (1988)]. Electrostatic repulsion of SDS could thus result in a shift in electrophoretic mobility. Abnormal migration in SDS–PAGE has been observed for other highly charged proteins, e.g., calpastatin (Takano et al., 1988) and the nuclear histone–binding protein (Kleinschmidt et al., 1986).

^{125}I-Labeled calmodulin overlays have revealed calmodulin binding not only to the major bands corresponding to the purified C-terminal domains but also to minor components of molecular masses corresponding to dimers or oligomers. The Ca-ATPase indeed dimerizes (or oligomerizes) through its C-terminal domain (Vorherr et al., 1991). Western blotting with antibodies against peptide I31 discriminated between a contaminant and dimers (oligomers) of domains 1a, 1c, and 1d. The antibody stained the main band at twice the molecular mass of peptides 1a, 1c, and 1d but, as expected, failed to do so with peptide 1b. The dimers and oligomers of the C-terminal domains evidently did not dissociate in the SDS–PAGE system. The strong tendency of the domains to dimerize is further supported by the observation mentioned above that expressed calmodulin binding domains, additionally purified by preparative electrophoresis, showed the multimeric bands when reelectrophoresed and blotted for the ^{125}I-labeled calmodulin overlay experiment.

The calmodulin binding domain of the plasma membrane Ca^{2+} pump consists of about 30 amino acids and can be conveniently divided in two subdomains. The N-terminal subdomain A (18 amino acids) has been synthesized and shown to have an affinity for calmodulin only slightly lower than that of the "full" synthetic domain (28 amino acids, containing also the C-terminal subdomain B) (Vorherr et al., 1990). Alternative splicing interrupts the "original" 28 amino acid domain of isoform 1b at the end of subdomain A, introducing a sequence whose first 10 residues are homologous to the original subdomain B (Table I). The main difference is the replacement of two arginines by an aspartate and a glutamine, leading to a change of the net charge of the domain [Table I and see Strehler et al. (1989)]. Synthetic peptides corresponding to the inserted sequences were recently studied by Enyedi et al. (1991). The introduction of acidic residues decreased their affinity for calmodulin and their inhibitory effects on the fully activated, C-terminally truncated Ca^{2+} pump. The data presented in this study show that in addition to altering the original calmodulin binding site, the alternative splicing leads to the assembly of a second, "duplicated" calmodulin binding site. This site is composed of a novel subdomain A, combined with the original subdomain B (see Table I). During RNA splicing the use of exon-internal "cryptic" donor splice sites regulates the length of subdomain A and the positioning of subdomain B in isoforms 1c and 1d. However, insertion of the complete alternatively spliced sequence (isoform 1a) leads to the loss of the original subdomain B, which would follow the insert because of additional C-terminal changes of this isoform due to a shift in reading frame. Sequence inspection of isoform 1a shows that conservation of two Val, a Phe, and two Ser residues (VVFSS) are conserved in a position where they could conceivably take over the function of subdomain B (Table I).

Electrostatic interactions are essential for calmodulin interaction with the binding domain (O'Neil & DeGrado, 1990). The positive charges required are supplied by arginine residues in the canonical binding site of the 1b isoform and in the "first" site of the alternatively spliced variants, although the two positive charges in the C-terminal portion of the domain are replaced by an acidic and an uncharged residue. In the "second" calmodulin binding site, which is present only in the alternatively spliced variants, the potential positive charges are supplied instead by histidine residues, by becoming positively charged at acidic pH they could thus confer pH dependency to calmodulin binding. Peptide I31 was a model for this type of calmodulin binding, since three out of its five positive charges are represented by histidine residues. The peptide is essentially an analogue of peptide C20 (Vorherr et al., 1990), i.e., a calmodulin binding domain lacking subdomain B and, therefore, showing lower binding affinity than the full 28-residue domain. At pH 5.9 its K_D for calmodulin was 29.7 nM, i.e., in the range found for peptide C20 (Vorherr et al., 1990). At pH 7.2, the K_D was instead 80.4 nM. In all titrations performed at acidic pH and low salt concentrations, quenching of fluorescence was observed, an effect which cannot be fully explained at the moment, but which could be due to both pH and salt concentrations. Titrations performed at physiological salt concentrations yielded the same qualitative result except that fluorescence enhancements with smaller relative effects were observed. At acidic pH, peptides 1c and 1d showed affinities in the same range (i.e., below 11 nM) (Figure 6C and 6E). Maximal fluorescence quenching was obtained in both cases at a protein to dansyl calmodulin ratio approaching 1. At neutral pH, however, isoform 1c (Figure 6D) exhibited higher affinity for calmodulin that 1d (Figure 6F), possibly due to the closer spacing of charged residues (Table I). The dansyl calmodulin titration curves of peptides 1c and 1d at pH 7.2 resembled the titration curve of peptide I31 (Figure 6H) but not that of peptide 1b (Figure 6B) at the same pH. For the peptide 1b, which contains no alternatively spliced sequences, the calmodulin binding had virtually no pH dependence (Figure 6A and 6B).

The calmodulin binding domain mediates the self-association and the resulting activation of the Ca^{2+}-ATPase (Vorherr et al., 1991). The synthetic calmodulin binding domain stimulates the ATPase activity of the intact pump, indicating the formation of heterooligomers of the peptides with the pump. In this study the effects of peptide I31 on the Ca^{2+}-ATPase in the presence and absence of calmodulin have been compared to those of peptide C28Y (Table III). Peptide I31 increased the stimulation of the ATPase activity by calmodulin, the effect being more pronounced at acidic pH. Since at acidic pH peptide I31 had no effect on the basal activity of the pump, it probably interacted directly with calmodulin, somehow facilitating its interaction with the pump. By contrast, at neutral pH peptide I31 stimulated the basal ATPase activity-like peptide C28Y, an effect which could be due to hetero-oligomerization. It is tempting to suggest that in the alternatively spliced domain in the pump this event could take place intramolecularly.

A major question concerns the stoichiometry of calmodulin binding to the alternatively spliced C-terminal peptides. Photo-cross-linking experiments with calmodulin containing a carbene precursor (Nassal, 1983) and analysis of complex formation on urea gels failed to provide evidence for the binding of two calmodulin molecules to a single alternatively spliced peptide (data not shown). In hindsight, this was not surprising, given the obvious difficulty to accommodate two calmodulin molecules within such a short stretch of amino acids. Although a 2:1 stoichiometry cannot be absolutely ruled out, the binding

of only one calmodulin to the C-terminal domain of each of the four isoforms seems most likely. Binding of one calmodulin to two separate sequences has been previously observed in the γ-subunit of phosphorylase kinase, where the two sites contributing to the binding of calmodulin are contained within a stretch of 70 amino acids (Dasgupta et al., 1989; Trewhella et al., 1990). Interestingly, the calmodulin binding domain of the calmodulin-dependent cyclic nucleotide phosphodiesterase contains a His residue (Novack et al., 1991).

Based on a 1:1 binding stoichiometry, a model for the functional role of alternative splicing in the calmodulin binding site can be proposed. The spliced domain could act predominantly as a pH-dependent regulatory element of the canonical high affinity calmodulin binding site. This "cryptic" calmodulin binding site could act through indirect means, e.g., intramolecular heterodimerization with the ATPase. At neutral pH the two calmodulin binding sites would thus be associated with each other, reducing the affinity of the pump for calmodulin. In the absence of calmodulin, the intramolecular heterodimerization would result in the observed increase in basal activity. At acidic pH in the absence of calmodulin, the basal activity of the pump would be low because the original (autoinhibitory) calmodulin binding domain would be free to interact with its binding site (Falchetto et al., 1991) on the pump. At acidic pH, the binding of calmodulin would be facilitated, explaining the observed increased activation of the ATPase. Thus, the model predicts a partially activated ATPase for the alternatively spliced isoforms at neutral pH, i.e., under the physiologically prevailing condition. At acidic pH the ATPase would become more stringently regulated by calmodulin. Whether pH values in the range found to be effective in this work are ever reached in vivo is a question that cannot be decided at the moment. It could perhaps be mentioned in this context that pH values as low as 6.1 have been measured in skeletal muscles (Hultman et al., 1985). Interestingly, the spliced isoforms 1c and 1d have been cloned from a skeletal muscle cDNA library.

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