Biochem. J. (1992) 283, 355–359 (Printed in Great Britain)

New Ca^{2+} pump isoforms generated by alternative splicing of *rPMCA2* mRNA

Hugo P. ADAMO and John T. PENNISTON*
Department of Biochemistry and Molecular Biology, Mayo Clinic, 200 First Street South West, Rochester, MN 55905, U.S.A.

Alternative splices capable of generating proteins with altered functions were found (by PCR) in isoform 2 of the rat plasma membrane Ca^{2+} pump. These splices were concentrated in two hypervariable regions. One of these regions, near the *N*-terminus and the lipid-binding region, could be altered by the insertion of either or both of inserts x and y. Insertion of both x and y would add 45 amino acids to the molecule. The y insert causes the appearance of a rather hydrophobic stretch of amino acids in the middle of a highly polar region. The second variable region begins in the middle of the calmodulin-binding domain. Insertion of 229 nucleotides at this point of the message converts the b form to the a form, which has an altered (and shorter) *C*-terminus. The calmodulin-binding domain of this shortened form has a less basic character, which would decrease the affinity for calmodulin. The b form of isoenzyme 2 contains relatively weak protein kinase A substrate sequences, such as KQNSS and KNNS. These sequences are eliminated in form a, and a strongly activated kinase substrate sequence, RRQSS, appears in a different place. Different tissues use different combinations of alternative splices, with heart and brain showing the greatest diversity.

INTRODUCTION

The precise control of the intracellular free Ca^{2+} concentration is a prerequisite for the signalling function of Ca^{2+}. Ca^{2+} pumps of the plasma membrane play a key role in this process by their removal of Ca^{2+} from the cells against very large concentration gradients (Rega & Garrahan, 1986). The regulated expression of structurally distinct, tissue-specific and developmentally regulated protein isoforms is an essential characteristic of eukaryotic cell differentiation. Previous studies (Shull & Greeb, 1988; Verma *et al.*, 1988; Greeb & Shull, 1989; Strehler *et al.*, 1990) involving molecular cloning methods have shown that the plasma membrane Ca^{2+} pump belongs to a multigene family. Four different genes (*PMCA1*, *PMCA2*, *PMCA3* and *PMCA4*) have been found. Isoform diversity appears to be further increased via alternative RNA splicing of the primary transcripts of the *PMCA* genes. In the case of human *PMCA1* (*hPMCA1*), this process involves a 154 bp exon which can be spliced alternatively to produce four isoforms differing only in the *C*-terminal regulatory region, which contains the calmodulin-binding domain and the consensus site for phosphorylation by the cylic AMP-dependent protein kinase (Strehler *et al.*, 1989). Similar slicing events were proposed to occur in the rat *PMCA1* (*rPMCA1*) and *rPMCA3* genes (Shull & Greeb, 1988; Greeb & Shull, 1989), and in pig *PMCA1* (De Jaegere *et al.*, 1990) and rabbit *PMCA1* (Khan & Grover, 1991), PCR studies have shown such events to occur. cDNA cloning results and sequence comparisons have suggested four possible sites (named A, B, C and D) for alternative splicing in the *PMCA* primary transcripts (Strehler, 1991).

We have investigated, by reverse transcription and PCR, the alternative splicings of *rPMCA2*. We found two mRNAs produced by inclusion or exclusion of an exon of 229 bp at the same position as the alternatively spliced 154 bp *PMCA1* exon, showing that the splicing site C is operative in both genes. In addition, we also found that different isoforms of rPMCA2 originate by alternative splices at site A which would cause the insertion of 0, 14, 31 or 45 amino acid residues near the lipid-binding domain of the molecule. PMCA2z, x, y and w are used here to designate the variants of isoform 2 of the plasma membrane Ca^{2+}-ATPase that arise as a result of the insertion of 0, 42, 93 or 135 bp respectively at splicing site A.

MATERIALS AND METHODS

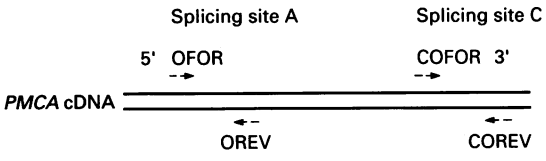
Female white rats were anaesthetized before removing brain,

Table 1. Primers bracketing splicing sites A and C

The primers bracketing site A were altered from the original sequence to create new restriction sites. The base which was in the original sequence is written below the altered base, and the restriction site is underlined.

Primer	Sequence (5′ → 3′)
Splice site A	
OFOR.R1	CGAGAATTCGTTGACTGGC G *Eco*RI
OREV.R1	CCCTTCTTCACTCTGCAGA T *Pst*I
OFOR.R2	TGAAAGCTTGCTCACAGGG C *Hind*III
OREV.R2	TCAGAGGCTGCAGTTCCATAG T *Pst*I
Splice site C	
COFOR.R1	CCCTGAGGAGGAATTGGCG
COREV.R1	GATTGAACTTCTTGATTCT
COFOR.R2	CCTGAATCGGATCCAGACA
COREV.R2	GAACGCCTTCACGACGCGG

* To whom correspondence should be addressed.



Scheme 1. Strategy for detection of alternative splices by PCR

In this schematic representation of *PMCA* cDNA, the location of the primers used for amplifying regions of potential alternative splicing are shown.

heart and kidneys as whole organs. The myometrium from the uterus was dissected out. Total RNA was prepared from these tissues. Primers shown in Table 1 were synthesized and purified using OPC cartridges (Applied Biosystems). These primers were designed to bracket the expected splicing sites (see Scheme 1). PCR products obtained with oligonucleotides COFOR.R2 and COREV.R2 were cloned using a pre-existing *Bam*HI site in the 5′ end and with a 3′ blunt end. Restriction sites for *Hin*dIII, *Pst*I and *Eco*RI were created in OFOR.R2, OREV.R2, OFOR.R1 and OREV.R1 to facilitate the cloning of the PCR products into the sequencing vectors. Reverse transcription and PCR were performed using RNA GeneAMP (Perkin–Elmer–Cetus). Random hexamers were used as primers to transcribe 1 μg of total RNA according to the manufacturer's instructions. Following the synthesis of the first DNA strands, the PCR was carried out in the same tube using 100 ng of each appropriate pair of primers and 3 units of AmpliTaq polymerase in a volume of 100 μl, and incubating at 94 °C (30 s), 60 °C (45 s) and 74 °C (60 s) for 35 cycles. The samples were electrophoresed in an agarose gel (Nusieve agarose, 2%; Seakem agarose, 1%), stained with ethidium bromide and photographed under u.v. light. Each band was excised from the gel, purified, digested with restriction enzymes and cloned into M13mp18 and M13mp19 using standard protocols. For sequencing, Sequenase version 2.0 was used.

RESULTS

Alternatively spliced transcripts of *rPMCA1* and *rPMCA2*

The alternative splicing of a 154 bp exon of the *hPMCA1* gene has been reported (Strehler *et al.*, 1989). To investigate the PMCA1 transcripts in rat tissues, total RNA from brain, heart, kidney and uterus was reverse-transcribed and the resulting first strands were amplified by PCR using primers COFOR.R1 and COREV.R1. This resulted, as expected, in several products (Fig. 1*a*). Brain showed primarily a band at 335 bp, with faint bands at 268 and 181 bp. Heart showed the 181 bp and 268 bp bands, and a faint one at 335 bp. Kidney and uterus showed only the shortest band of 181 bp. These sizes (335 bp, 268 bp and 181 bp) agree with the expected results for the fragments produced from rat isoforms rPMCA1a, rPMCA1c and rPMCA1b. This confirms that the *rPMCA1* gene has an exon similar to that of the human gene and indicates that it is alternatively spliced in a similar process. Even though a rigorous quantification of the PCR products was not made, the relative band intensities observed suggest that the rPMCA1a transcript is more abundant in brain, rPMCA1b and rPMCA1c in heart, and rPMCA1b in kidney and in uterus.

The possible existence of a corresponding exon of the *rPMCA2* gene, which would also be alternatively spliced, was investigated. Using primers COFOR.R2 and COREV.R2 the amplification of a 43 bp fragment was expected, according to the published sequence of rPMCA2 (Shull & Greeb, 1988). Fig. 1(*b*) shows that a fast-migrating fragment of the predicted size was produced in all of the tissues tested. However, a second fragment of 272 bp was also amplified from brain, heart and uterus. Both fragments from brain were cloned and sequenced. The resulting sequence showed that an insertion of 229 bp occurs in the *rPMCA2* transcript (Fig. 1*c*). The inclusion of this sequence results in the shorter isoform rPMCA2a, with a *C*-terminal region analogous to that of PMCA1a. The insertion of 229 bp in *rPMCA2* occurs at the same point (in the middle of the calmodulin-binding region), as does the insertion of the 154 bp splice in *rPMCA1* and *rPMCA3*. In the case of *rPMCA2*, the absence of other bands indicates the isoforms generated by partial inclusion of this exon are not present.

A new splicing site in *rPMCA2*

Comparisons of nucleotide sequences from different cDNAs have suggested the existence of four possible sites for alternative RNA splicing in *PMCA* primary transcripts. However, only the alternative splicing of the 154 bp exon of *hPMCA1* (splicing site C) has been demonstrated to be operative in generating different *PMCA* mRNAs. The alternative splicing at the proposed splicing site A, which would produce isoforms differing in the *N*-terminal half of the molecule, was investigated. According to the known sequence of the *rPMCA2* cDNA (Shull & Greeb, 1988), using primers OFOR.R2 and OREV.R2 a fragment of 242 bp was

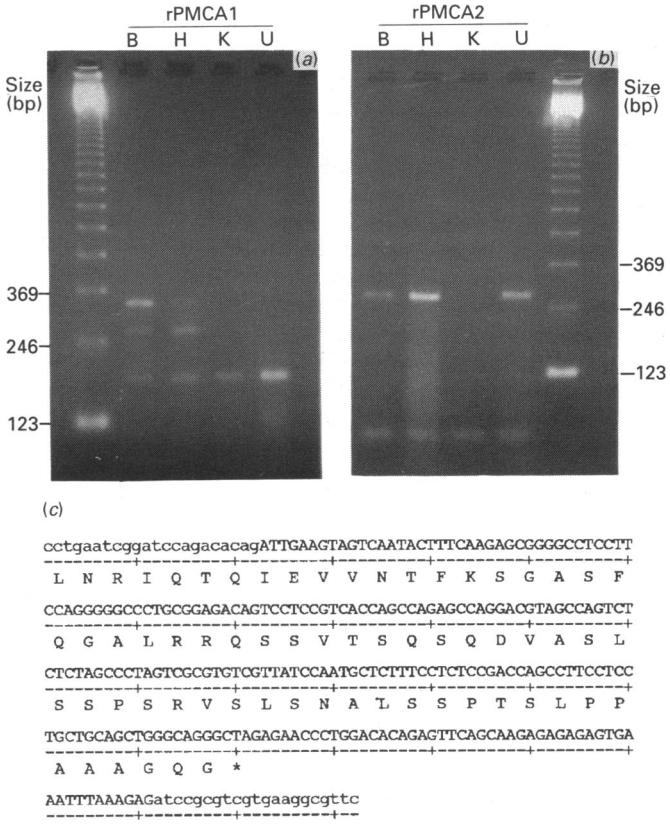


Fig. 1. rPMCA transcripts generated by alternative splicing at site C

(*a*) Electrophoresis of PCR products obtained with COFOR.R1 and COREV.R1 (rPMCA1). (*b*) Electrophoresis of PCR products obtained with COFOR.R2 and COREV.R2 (rPMCA2). Total RNA was transcribed from: B, brain; H, heart; K, kidney; U, uterus. The positions and sizes (in bp) of the markers are indicated. (*c*) *C*-Terminal splice in isoform 2: sequence of the 272 bp fragment from rPMCA2 amplified by PCR using COFOR.R2/COREV.R2. The 229 bp inserted exon is shown in upper case letters, and the encoded amino acids in the one-letter code below.

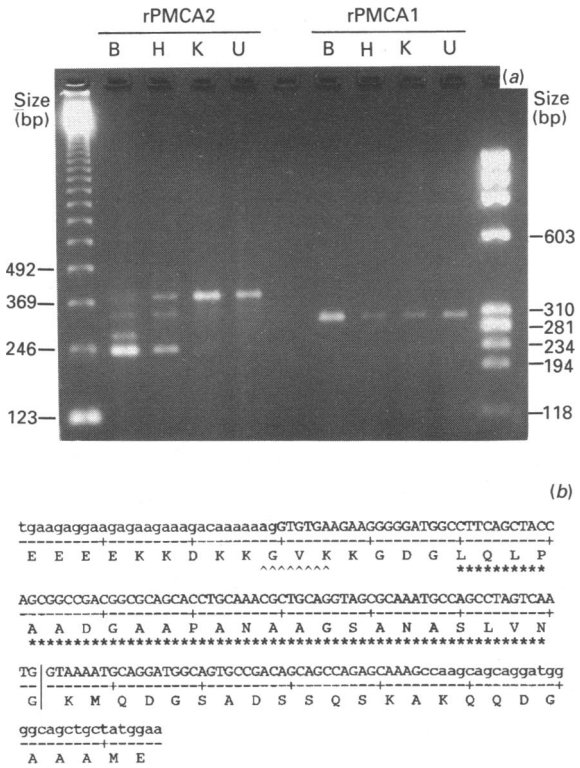


Fig. 2. rPMCA1 and rPMCA2 transcripts generated by alternative splicing at site A

(*a*) Electrophoresis of PCR obtained with OFOR.R1/OREV.R1 (rPMCA1) or OFOR.R2/OREV.R2 (rPMCA2). Total RNA was transcribed from: B, brain; H, heart; K, kidney; U, uterus. The positions and sizes (in bp) of the markers are indicated. (*b*) Sequence of splices in region A of rat isoform 2: partial sequence of the 335 bp fragment from rPMCA2. Potential exon sequences are given in upper cases letters. A consensus intron splice donor sequence is marked with ^. The site where splicing can occur within the exon is indicated in a vertical line. The segment of high hydrophobicity is marked with ★.

expected to be amplified. However, Fig. 2(*a*) shows that fragments of 284, 335 and 377 bp were also produced. Brain showed a very intense band of 242 bp, a less prominent band of 284 bp, and fainter bands of 335 and 377 bp. Heart showed bands of 242, 335 and 377 bp. The most intense band in kidney and uterus was that of 377 bp. Each one of the fragments obtained from brain and heart was cloned and sequenced.

As expected, the nucleotide sequence near the primers was identical in all of the PCR products, showing that they are alternative RNA splicings of a common primary transcript. Insertions of 0, 42, 93 or 135 bp occurred after amino acid 302 of the published sequence of the rPMCA2b Ca^{2+} pump (Shull & Greeb, 1988) (Fig. 2*b*). The splices always coded for an integral number of amino acids. An intron donor consensus sequence, (AG\gtgtga) instead of the canonical (AG\gtgagt), is present at the 5′ splice site junction flanking the longest insertion. The nucleotide sequence adjacent to the 3′ splice site does not match with the usual acceptor sequence. The 42 bp and 93 bp splices are generated by splitting the 135 bp fragment in two and inserting either the first 93 bp or the last 42 bp. No intron donor or acceptor consensus sequence can be recognized at the place where this splitting occurs. This suggests that an intron sequence interrupts the *rPMCA2* gene at this point. To see if the same splicing site was also active in generating different *rPMCA1* mRNAs, primers OFOR.R1 and OREV.R1 were used. The PCR resulted in the amplification of only one band in all of the tissues (Fig. 2*a*). The size of this fragment was estimated at 307 bp, which is the expected size for a fragment amplified from *rPMCA1*. This indicates that, in the tissues examined, splicing site A is not active in *rPMCA1*, so no further increase in the diversity due to alternative splicing at this site is produced.

DISCUSSION

Comparison of the various *PMCA* cDNA sequences predicts the presence of several splicing sites, but only one active alternative splice has been conclusively demonstrated. This site, named splicing site C (Strehler, 1991) was shown to generate four *hPMCA1* mRNAs by the alternative splicing of a 154 bp exon (Strehler *et al.*, 1989). Our investigation explored isoform diversity which originated by alternative splicing from the *PMCA2* gene. Using PCR to examine the mRNA transcripts derived from this gene, we found both splicing sites C and A to be active in generating isoform diversity. The exclusion or inclusion of a 229 bp exon at site C produced two rPMCA2 isoforms corresponding to rPMCA1b and rPMCA1a. In addition, we found that, by alternative splicing at site A, four rPMCA2 isoforms differing in the *N*-terminal region of the molecule would be generated. The actual number of rPMCA2 isoforms may increase even further due to the possible combinations of these two splicing sites. Co-ordination between the expression of the different exons may occur in order to build a Ca^{2+} pump which better fulfils specific requirements for the regulation of cytosolic Ca^{2+}, either in different tissues or at different stages of development.

Isoform variability due to alternative *C*-terminus regulatory domains

The results obtained show that the splicing site C is active in both the *rPMCA1* and *rPMCA2* genes. The complete inclusion of either the 154 bp exon in *PMCA1* or the 229 bp exon in *PMCA2* leads in both cases to a similar *C*-terminal regon, which is much shorter and quite different from that of the other isoforms. Even though the sizes of these inserts are different, when translated a stop codon comes in frame at exactly the same position, making the *C*-terminal domains of rPMCA1a and rPMCA2a equal in length. The stop codon is generated in different ways in rPMCA1 and rPMCA2. In rPMCA1, the 154 bp insert causes a frame shift and the stop codon occurs beyond the end of the insert. In rPMCA2, the stop codon is contained in the 229 bp insert. One can speculate that not only the amino acid sequence but also the length is an important feature for the proper functioning of these isoforms.

Another characteristic of these inclusions is that they occur in the middle of the calmodulin-binding domain and drastically change the *C*-terminal portion of this domain. This change in the calmodulin-binding domain of isoforms rPMCA1a and rPMCA2a is likely to alter the interactions between the calmodulin and its binding site. Recent investigations using synthetic peptides representing these two classes of calmodulin-binding domains have suggested that the type a isoforms have a lower affinity for calmodulin and a higher basal activity in the absence of activator (Enyedi *et al.*, 1991).

Both rats and human PMCA1b transcripts contain a known substrate sequence for protein kinase A, i.e. KRNSS (James *et al.*, 1989). Insertion of the 154 bp inclusion in PMCA1a eliminates this sequence. The corresponding sequence in PMCA2b is KQNSS, which would not be expected to be as good a kinase substrate. In this case, the inclusion of 229 bp in form rPMCA2a eliminates the KQNSS and creates a new strongly activated kinase site with the consensus sequence RRQSS, in a different

```
                                  *******        +++++
R1a   LRRGQILWFRGLNRIQTQ|MDVVNAFQSGGSIQGALRRQPSIASQHHDVTNVSTPTHVVFSSS
R2a   LRRGQILWFRGLNRIQTQ|IEVVNTFKSGASFQGALRRQSSVTSQSQDVASLSSPSRVSLSNA
R1b   LRRGQILWFRGLNRIQTQ|IRVVNAFRSSLYEGLEKPESRSSIHNFMTHPEFRIEDSEPHIPL
R2b   LRRGQILWFRGLNRIQTQ|IRVVKAFRSSLYEGLEKPESRTSIHNFMAHPEFRIEDSQPHIPL

R1a   TASTPVGYPSGECIS
R2a   LSSPTSLPPAAAGGG
R1b   IDDTDAEDDAPTKRNSSPPPSPNKNNNAVDSGIHLTIEMNKSATSSSPGSPLHSLETSL
R2b   IDDTDLEEDAALKQNSSPPSSLNKNNSAIDSGINLTTDTSKSATSSSPGSPIHSLETSL
                  ^^^^^
```

Fig. 3. Alignment of the alternate splices in region C from rPMCA1 and rPMCA2

The point of insertion of the splice is marked by a vertical line. The persistent motif VV(N/K)(T/A)FXS is marked by *, the A-kinase site in splice b is marked by ^, and the probably kinase site in splice a is marked by +.

```
R2z   EEEEKKDKK.....................................................AKQQDGAAA
R2y   EEEEKKDKKGVKKGDGLQLPAADGAAPANAAGSANASLVN.....................AKQQDGAAA
R2x   EEEEKKDKK.....................................GKMQDGSADSSQSKAKQQDGAAA
R2w   EEEEKKDKKGVKKGDGLQLPAADGAAPANAAGSANASLVNGKMQDGSADSSQSKAKQQDGAAA
                  ******                          ******        ******
```

Fig. 4. Alignment of the four splices observed in region A of isoform 2

The repeated motif ZKXXDG is marked by *. Note that this motif follows immediately after the sequence EEEEKKDKK(G), regardless of which alternative splice occurs.

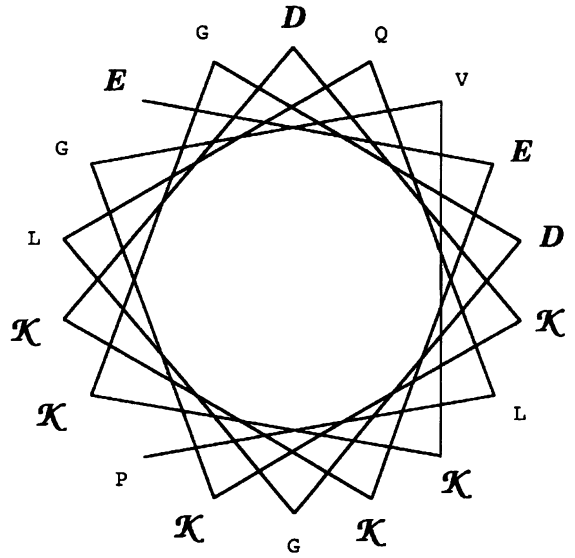


Fig. 5. Helical wheel plot of part of the isoform R2w, whose sequence is shown in Fig. 4

This plot shows the dipolar nature which this portion of the molecule would have if it were a helix: the upper side of this helix has four minus charges, while the lower side has six plus charges. The *N*-terminus of the sequences shown is the E in the upper left quadrant.

part of the molecule. In addition, of the 59 amino acids placed at the *C*-terminus of rPMCA2a by the alternative splice, 25% are serines. This may also be an indication of a high sensitivity of this isoform towards other kinases.

Second splicing site in the *PMCA2* gene

Our results show the existence of four *rPMCA2* mRNA alternative splices in the A region of the molecule. The total inclusion of a 135 bp segment in rPMCA2w results in the insertion of 45 amino acids. A closer look shows that the initial part of this segment codes a rather hydrophobic stretch of 24 amino acids (Fig. 2*b*). Partial inclusion of this segment results in the insertions of either the first 31 or the last 14 residues. In isoform rPMCA2y only the initial part of the segment is inserted, so the selective inclusion of the relatively hydrophobic region is obtained. In the case of rPMCA2x, the portion of the segment inserted corresponds to the last 42 nucleotides, which encode a polar and charged sequence indistinguishable from the rest of this region.

The relative abundance of each rPMCA2 mRNA varies among the tissues. In brain and heart rPMCA2z predominates, while rPMCA2w predominates in kidney and uterus. Brain is also the only tissue which expresses important amounts of rPMCA2x.

Significance of the variability produced by the splicing in region A

The region of the molecule where these insertions occur (between putative transmembrane helices 2 and 3) has been indicated as the location of several functionally important sites for the ion-motive ATPases (Serrano, 1988; Clarke *et al.*, 1990). We do not known exactly why the observed changes occur in PMCA2, but it is suggestive that the insertions occur just upstream of the place where phospholipids are supposed to bind to activate the Ca^{2+} pump. This portion of the molecule occurs only in the plasma membrane Ca^{2+} pump, and has no homologous region in other ion-transport ATPases (Zvaritch *et al.*, 1990). The proteolytic removal of this region causes an activation similar to that caused by acidic lipids, leading to the suggestion that this region binds acidic lipids. The different alternative splices observed in the A region of *PMCA2* may encode isoforms with different sensitivity or specificity towards lipids. It may be added that it is expected that the domain responsive to phospholipids should be able to interact closely with the membrane environment. The inclusion by alternative splicing of the relatively hydrophobic stretch into a highly charged region may have a consequence for the interaction of this isoform with the plasma membrane lipids.

An interesting feature shared by all of the *PMCA* splices generated, whether at splicing site C or A, is that the amino acid sequence just downstream of the splicing site is conserved, in spite of the inclusions produced from each splice variant (Figs. 3

and 4). The initial part of all the possible peptides resulting from the alternative splicing are conserved, even though they are encoded by different parts of the mRNA. In the case of the splices in region C, the conserved region is VV(N/K)(T/A)FXS, where X = R, K or Q. In this case, we have some direct evidence about the function of this domain, since its presence promotes binding to calmodulin (Enyedi *et al.*, 1991). In the case of the splice in the A region of rPMCA2, the conserved region is ZKXXDG, where Z = G, V or A and X = K, G, M or Q. Such a sequence follows a 9-residue sequence of charged amino acids in each of the possible isoforms (Fig. 4). Since this sequence occurs at the start of each insert, the number of times it occurs increases with the length of the insert, up to three times in rPMCA2w. The region of the molecule where these insertions take place is noticeably polar, and stretches of negatively charged residues alternative with positive ones. In a helical structure this would create an asymmetric distribution in which the negative and positive charge lie on the opposite sides of the helix. This dipolar arrangement occurs in all of the alternate splices, and is particularly prominent in rPMCA2w (Fig. 5). This feature may reflect a specific organization of this region related to its function. A better understanding of the function of this region of the Ca^{2+} pump molecule, and of the importance of the different isoforms, will be one of the most interesting areas of future research.

We thank Dr. Anil Verma for the initiation in molecular biology techniques and Dr. Maria Magocsi for the help in the RNA preparation.

Received 27 August 1991/24 October 1991; accepted 4 November 1991

This work was supported in part by grant GM 28835 from the U.S. National Institutes of Health.

REFERENCES

Clarke, D. M., Loo, T. W. & MacLennan, D. H. (1990) J. Biol. Chem. **265**, 14088–14092

De Jaegere, S., Wuytack, F., Eggermont, J. A., Herboomen, H. & Casteels, R. (1990) Biochem. J. **271**, 655–660

Enyedi, A., Filoteo, A. G., Gardos, G. & Penniston, J. T. (1991) J. Biol. Chem. **266**, 8952–8956

Greeb, J. & Shull, G. E. (1989) J. Biol. Chem. **264**, 18569–18576

James, P. H., Pruschy, M., Vorherr, T. E., Penniston, J. T. & Carafoli, E. (1989) Biochemistry **28**, 4253–4259

Khan, I. & Grover, A. K. (1991) Biochem. J. **277**, 345–349

Rega, A. F. & Garrahan, P. J. (1986) in The Ca^{2+} Pump of Plasma Membranes (Rega, A. F. & Garrahan, P. J., eds.), pp. 13–56, CRC Press Inc., Boca Raton, FL

Serrano, R. (1988) Biochim. Biophys. Acta **947**, 1–28

Shull, G. E. & Greeb, J. (1988) J. Biol. Chem. **263**, 8646–8657

Strehler, E. E. (1991) J. Membr. Biol. **120**, 1–15

Strehler, E. E., Strehler-Page, M.-A., Vogel, G. & Carafoli, E. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6908–6912

Strehler, E. E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A. G., Penniston, J. T. & Carafoli, E. (1990) J. Biol. Chem. **265**, 2835–2842

Verma, A. K., Filoteo, A. G., Stanford, D. R., Wieben, E. D., Penniston, J. T., Strehler, E. E., Fischer, R., Heim, R., Vogel, G., Matthews, S., Strehler-Page, M.-A., James, P., Vorherr, T., Krebs, J. & Carafoli, E. (1988) J. Biol. Chem. **263**, 14152–14159

Zvaritch, E., James P., Vorherr, T., Falchetto, R., Modyanov, N. & Carafoli, E. (1990) Biochemistry **29**, 8070–8076