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## ALTERNATIVE SPLICE VARIANTS OF PLASMA MEMBRANE CALCIUM-ATPases IN HUMAN CORNEAL EPITHELIUM

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### Abstract

Plasma membrane calcium-ATPases (PMCA) play a critical role in regulating intracellular calcium concentration. Four genes encode PMCA proteins with alternative splicing of transcripts at three sites (A, B and C) serving to increase isoform diversity. Our previous work shows that all four PMCA are expressed and have specific locations in human corneal epithelium (hCE). The present work examined which splice variants of PMCA are expressed in hCE. Total RNA was extracted from hCE scraped from cadaver corneas of five different donors (two females and three males, age range 55 to 76 years). RT-PCR was performed using PMCA isoform-specific primers designed to amplify transcripts that included either splice site A or splice sites B and C. PMCA cDNAs were sequenced or cloned, and then sequenced. There was uniformity in the PMCA1 and PMCA4 expression profile among the five donors. Specifically, every donor expressed PMCA4 transcripts (4x at site A and 4b at site B/C). Every donor also expressed PMCA1 transcripts at sites B/C, specifically PMCA1b and PMCA1kb. In contrast, PMCA2 and PMCA3 expression varied; PCR DNAs were detected in two of five donors. One donor expressed PMCA2a and a novel PMCA2 variant we termed PMCA2<sub>(i)</sub>. PMCA3a transcript was demonstrated in a different donor. Finally, for all the donors, bands encoding site A transcripts for PMCA4 were obtained but no PCR transcripts were detected at site A for PMCA1, PMCA2 and PMCA3. This investigation showed that hCE expressed multiple splice variants of PMCA isoforms. Furthermore, this study documented the expression of the PMCA1k variant (PMCA1kb) previously only described in intestine and pancreatic beta cells and describes a novel PMCA2<sub>(i)</sub> variant. Finally, this study suggests that the molecular configuration of PMCA 1, 2 and 3 in the region of splice site A in hCE must be different than in other tissues since the same primers that produced site A transcripts in several other tissues were ineffective in priming PCR in hCE.

### Keywords

PMCA; cornea; epithelium; calcium; alternative splice variants; PCR

### INTRODUCTION

The cornea comprises the anterior surface of the eye and is its principle refracting structure. Corneal epithelium (CE) is the outermost layer of the cornea and forms a barrier against intraocular penetration of airborne pathogens and tear fluids. Additionally, CE plays a role in

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dehydration of the underlying stroma and the maintenance of corneal transparency (c.f. (Connon et al. 2006; Klyce 1977; Reinach 1985; Shurman et al. 2005). CE is a stratified epithelium comprised of morphologically and functionally distinct epithelial cell types that represent stages in a continuum of transitional forms ranging from stem cells to surface epithelial cells (Cenedella and Fleschner 1990; Hanna et al. 1961). As CE undergoes renewal, there is a constant production of cells that differ in proliferative capacity and differentiated properties and that vary in  $\text{Ca}^{2+}$ -handling properties (Duncan and Collison 2002; Kimura et al. 1999). The processes of CE renewal and wound healing involve a coordinated sequence of physiological events including cell migration, proliferation and differentiation (Thoft and Friend 1975), all of which depend on calcium-mediated processes (Cox and Huttenlocher 1998; Hazelton et al. 1979; Shurman et al. 2005).

The plasma membrane calcium ATPase (PMCA) is one of the enzymes responsible for maintaining low cytosolic calcium ( $\text{Ca}^{2+}$ ) concentration through transmembrane transport of  $\text{Ca}^{2+}$  against a large electrochemical gradient (Carafoli 1994; Strehler et al. 2007). PMCA is a member of the P class of ion-motive ATPases forming an acylphosphate intermediate as part of its reaction mechanism (Dunham and Glynn 1961). PMCA is composed of a single, large polypeptide chain. Topographically, approximately 80% of the PMCA pump mass protrudes into the cytoplasm, with short, extracellular loops connecting ten transmembrane (TM) domains. A multigene family consisting of at least four genes termed ATP2B1, ATP2B2, ATP2B3 and ATP2B4 encode PMCA proteins termed PMCA1, PMCA2, PMCA3 and PMCA4, respectively. Further diversity is generated at the protein level by alternative splicing of primary transcripts at three different sites termed A, B, and C (Figure 1) (Carafoli 1994; Howard et al. 1993; Kamagate et al. 2000; Strehler et al. 2007). Recent studies suggest that alternative splicing at these sites results in up to 30 different PMCAs each with unique targeting, membrane localization, functional characteristics and signaling properties c.f. (Chicka and Strehler 2003; Domi et al. 2007; Strehler et al. 2007).

PMCA isoforms and variants exhibit tissue- and cell-specific patterns of expression. PMCA1 and PMCA4 are expressed ubiquitously, whereas PMCA2 and PMCA3 are more prevalent in excitable tissues or in cells that exhibit  $\text{Ca}^{2+}$  oscillations or sudden  $\text{Ca}^{2+}$  influxes (for refs see, (Strehler et al. 2007)). For example, PMCA2 and PMCA3 are prevalent in brain (Stauffer et al. 1993), but are also found in pancreatic beta cells (Kamagate et al. 2000). PMCA2 expression is high in neurosensory hair cells of the inner ear (Dumont et al. 2001; Yamoah et al. 1998). These PMCA2-containing cells sustain sudden influxes of  $\text{Ca}^{2+}$  upon stimulation that necessitates rapid extrusion to maintain responsiveness (Yamoah et al. 1998). PMCA3 is found in fast skeletal muscle and in brain with high levels of expression cerebellum (Eakin et al. 1995) and in the choroid plexus (Borke et al. 1989).

Both activity- and localization studies have documented the presence of PMCAs in cornea. Specifically, Reinach, et al. characterized  $\text{Ca}^{2+}$ -stimulated,  $\text{Mg}^{2+}$ -dependent ATPase activity and  $\text{Ca}^{2+}$  transport in bovine CE (Reinach and Holmberg 1987; Reinach et al. 1991). Their work described a calmodulin-sensitive  $\text{Ca}^{2+}$ -stimulated,  $\text{Mg}^{2+}$ -dependent ATPase activity in CE that could maintain cytosolic  $\text{Ca}^{2+}$  in the submicromolar range. Previous work in our lab documented the expression and differential distribution of PMCA isoforms among the different cell types in native hCE (Talarico et al. 2005).

Previous work suggests that PMCAs are involved in CE renewal and wound healing. Specifically, Amino, et al. (Amino et al. 1997) showed that during corneal epithelial cell migration, at the wound margin, PMCAs redistribute from caveolae to membranous structures within the cytoplasm. After the denuded area closes, PMCAs return to cell membrane caveolae. This study did not determine which PMCA isoform(s) were “relocated” during the wound

healing process, nor were changes in  $[Ca^{2+}]_i$  measured. However, the subcellular redistribution of PMCAs suggests that calcium homeostasis is altered during CE wound healing.

PMCA dysregulation has been described in pathological conditions including sensorineural deafness, diabetes and hypertension (Lehotsky et al. 2002). Targeted ablation of PMCA1 in mice leads to embryonic lethality, whereas PMCA4 knockout in mice leads to infertile males (Prasad et al. 2007). PMCA2 mutations or knockout cause deafness and ataxia in several animals including humans (Ficarella et al. 2007; Kozel et al. 1998; Street et al. 1998). To date, PMCA deficits have not been documented in any corneal pathology. However, imbalances in calcium homeostasis (a process that involves PMCAs) do occur in corneal disease. For example, band keratopathy manifests as the deposition of calcium salts in the corneal basement membrane leading to epithelial breakdown, ocular surface instability and keratocyte loss (Anderson et al. 2001; Dark and Proctor 1982). Work that is more recent demonstrates that calcium-activated chloride channel and mRNA expression are elevated during corneal epithelium stratification suggesting a role for this transporter (and calcium) in the growth and maintenance of a multi-layered corneal epithelium (Connon et al. 2006). These studies suggest that dysfunction in  $Ca^{2+}$  homeostasis can lead to disturbances in desquamation, migration, proliferation and/or the barrier function of corneal epithelial cells. Thus, determining the molecular identities of PMCAs can contribute to understanding the role(s) of PMCAs in corneal epithelial biology. Previously, our lab documented the expression of all four PMCA isoforms in hCE (Talarico et al. 2005). The present work extends these observations and demonstrates splice variants of PMCAs in native hCE. Specifically, in native hCE, all donors examined expressed the PMCA1b variant at the C splice site. Additionally, hCE from all donors expressed both PMCA1k and non-k variants at the B splice site, a novel splicing event previously described in intestine and pancreatic beta cells for PMCA1 (Howard et al. 1993; Kamagate et al. 2000) and in heart for PMCA4 (Santiago-Garcia et al. 1996). PMCA2 transcripts from a single donor included PMCA2a and a *novel variant*, we termed PMCA2<sub>(i)</sub>. The splice variant for PMCA3 at the C site was PMCA3a. For PMCA4, 4x was the splice variant at site A and 4b at site C.

We suggest that expression of multiple PMCA isoforms and variants in hCE plays a role in enabling differences in calcium handling by the different cell types in this continuously regenerating epithelium.

## MATERIALS AND METHODS

Experiments were conducted according to the guidelines of the Institutional Review Board of the Indiana University School of Medicine, and in compliance with federal regulations governing the use and protection of human subjects in research.

### Sources of Human Donor Eyes

Corneas from human cadaver donors used in this study were from the Lions Eye Institute for Transplantation and Research (Tampa, FL). The age and cause of death of each donor, presence of ocular pathology, and the time to RNA preservation are reported in the appropriate sections that follow.

### Use of Gene-Specific Primers

Primers for molecular studies were from *Invitrogen (Life Technologies, Grand Island, NY)*. Primer sets were based on published PMCA sequences and were initially designed to study PMCA mRNA expression in retinal pigment epithelium (RPE) (Shen et al. 1999). The primers were gene-specific and selected to flank alternative splice sites A and B/C (Table 1). All primer

sets yielded transcripts in our prior research examining RPE PMCA isoform expression. PCR size markers were from *Promega* (Madison, WI).

### Total RNA Preparation

CE was collected from five donors ranging in age from 55 - 76 years (03-029, 03-030, 03-031, 03-055, and 03-065). The cause of death for donors was gastrointestinal and intracranial hemorrhage, chronic obstructive pulmonary disease and hypertension, lung cancer, cardiac arrest, and liposarcoma, respectively. One 55-year-old female donor (03-055) also suffered from insulin-dependent diabetes mellitus and had a two-year history of bilateral cataract prior to death for which she did not receive surgical intervention. One 76-year-old male donor (03-029) also had a prior history significant for left iridectomy and intraocular lens implant (date of procedure unknown). The remaining three donors had no known history of ocular disorders or pathology, or systemic disease that might affect vision.

CE from each donor cornea was scraped as described by Talarico, et al. (Talarico et al. 2005) and placed into RNALater™ (*Ambion*, Austin, TX). Total RNA for each donor was extracted using RNAqueous-4PCR (*Ambion*), and included DNase treatment of the final RNA pellet. RNA yield was assessed spectrophotometrically.

### Reverse Transcription and Polymerase Chain Reaction

Total RNA extracts were reverse transcribed using the SuperScript™ First Strand Synthesis for RT-PCR Kit (*Invitrogen*, Carlsbad, CA) and oligo dT primers. Controls included omission of reverse transcriptase for determination of genomic DNA contamination and running control RNA (*Invitrogen*) to confirm enzyme effectiveness.

PCR amplification was performed using a GeneAmp PCR System 2700 (*Applied Biosciences*), 1  $\mu$ l cDNA template, 1  $\mu$ l each of 10  $\mu$ M forward and reverse PMCA isoform, gene-specific primers, and the High Fidelity PCR Master Kit (*Roche*). The final reaction volume was adjusted to 25  $\mu$ l with sterile, nuclease-free water. Cycling conditions were as follows: initial denaturation at 94 °C for 2 min, then 35 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min followed by a final extension at 72 °C for 7 minutes. Second round PCR was routinely performed with 1  $\mu$ l of the first round PCR product in TE Buffer, pH 8.0. GAPDH gene-specific primers were used with templates from each sample as positive controls and for relative quantization. PCR DNAs were stored at -20 °C. PCR DNAs were analyzed using a Rapid Agarose Gel Electrophoresis System (RGX60, *Biokey American Instrument, Inc.*, Portland, OR) in 2.0% agarose gels in 1X TAE buffer (8 min at 210 V).

### Purification of PCR DNAs

When PCR yielded a single band, PCR DNAs were purified using the Wizard® PCR Preps DNA Purification System (*Promega*) or the QIAquick PCR Purification Kit (*Qiagen*, Valencia, CA). If PCR yielded two (or more) bands, then individual bands were excised and gel-purified using the QIAquick Gel Extraction Kit (*Qiagen*). Quality and relative quantity of the purified DNA were evaluated by agarose electrophoresis.

### Cloning of PCR Products

In the case of PMCA2 PCR transcripts in hCE, bands visualized via electrophoresis were too tightly spaced to successfully isolate the separate reaction products. Therefore, PMCA2 PCR products were cloned using the TOPO TA Cloning® Kit (*Invitrogen*™ Life Technologies). Plasmid DNA was purified using Wizard® Plus SV Minipreps DNA Purification System (*Promega*). Digestion was done on eluted DNA using EcoR1 restriction endonuclease.

Aliquots from purified and extracted samples were electrophoresed, and purified DNA products were stored at  $-20^{\circ}\text{C}$ .

### Sequence Determination of PMCA Alternative Splice Variants

PMCA PCR DNAs and clones were submitted for sequencing to the Cancer Research Center DNA Sequencing Facility at the University of Chicago (Chicago, IL). Chromatograms were analyzed using Chromas V2.11 (*Technelysium*). Edited sequences were aligned and translated to determine amino acid sequences using the Baylor College of Medicine Search Launcher-Multiple Sequence Alignment Program (Baylor College of Medicine at <http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>) and MatchBox. DNA and amino acid sequences were compared to known sequences using the National Center for Biotechnology Information (NCBI) Nucleotide Blast and Protein/Translated Sequence Blast, respectively. Sequences were further analyzed using NCBI Evidence Viewer. ExPasy was also used in the determination of translated sequences. Both nucleic acid and amino acid sequences were analyzed to compare expression of splice variants. TOPO2, Transmembrane protein display software by S.J. Johns (<http://www.sac.ucsf.edu/TOPO2>) (Johns 2005) and SWISS-MODEL (<http://swissmodel.expasy.org/swiss-model.html>) were used to construct the PMCA protein diagrams.

## RESULTS

### Alternative Splice Variants of PMCA

The possible splice variants for PMCA at the A, B and C splice sites and the genetic structure of PMCA genes are illustrated in Figure 1. The results obtained with RT-PCR analysis of hCE are summarized in Table 2. Representative gels of PCR products from the various reactions are presented in Figure 2. For PMCA1 (site B/C amplification) and PMCA4 (both sites A and B/C), PCR DNAs were routinely obtained on first-round PCR of all donors. This was in contrast to amplification of PMCA2 and PMCA3 cDNAs that required second-round PCR to detect product and only yielded clear bands in individual donors. PCR amplification was done on hCE cDNAs using A-site primers for all four PMCA. With all donors, several attempts to amplify DNAs encoding PMCA1, PMCA2 and PMCA3 at the site A were unsuccessful. This was despite using primers that yielded transcripts at the A site in other tissues including RPE (Shen et al. 1999). Bands of the appropriate size were readily detected in all donors for PMCA4 at site A. Figure 2D, lanes 1 and 2 show results with two of the donors.

PCR amplification of cDNA from four donors (one donor was not evaluated) using the primer set hPMCA1P/hPMCA1M that flanked the B/C sites in PMCA1 yielded two bands of 690 bp and 552 bp for all donors. Figure 2A presents representative results from one donor. Four of five samples of hCE cDNA were PCR amplified using two different sets of primers that flanked sites B/C in PMCA2. These primer sets were 2cap/2cam and P2pBeWo/2cam (Table 1). For every instance using 2cap/2cam, no product was detected. In one donor (03-031), two products were detected at the expected size with the primer set P2pBeWo/2cam (Figure 2B). The primer set 3cap/3cam (PMCA3 at site B/C) was tested in four of the five donors. Reaction products were detected in donor 03-030 (Figure 2C). Bands of the appropriate size were detected in four donors (one not tested) for PMCA4 at the B/C site (Figure 2D, lanes 3 and 4). The identity of PMCA splice variants in native hCE was determined by direct sequencing of purified PCR DNAs and two PMCA2 clones.

### PMCA1

Alternative splicing of PMCA1 at site C leads to the inclusion or removal of a single 154-bp exon that contains internal donor sites from which inserts of 87-, 114- or 152 bp originate (Figure 1B). This results in at least five possible spliced forms designated PMCA1a – PMCA1e

(Keeton et al. 1993). For PMCA1, PCR amplification of hCE cDNA from the four donors tested using primer pair, hPMCA1P/hPMCA1M, yielded two DNA fragments of approximately 690 and 552 bp (Figure 2A shows representative results from one of the donors). The two fragments were 100% identical to the reference sequence (NCBI NM 001682.2). Sequencing determined the absence of the 154 bp exon at site C in both PCR products of each donor sample of hCE. Thus, the splice variant at site C in native hCE is PMCA1b (NCBI AY740526).

Because the binding site for primer hPMCA1P is upstream of the proposed B splice site (Figure 1A), PCR amplification resulted in fragments that contained sequence from both sites B and C. For site B, the inclusion or exclusion of a 108 bp exon in rat pancreatic islets and intestine (Figure 1) has been shown (Howard et al. 1993; Kamagate et al. 2000). Deletion of this 108 bp exon at site B is termed the “k” variant (Figure 1B), and inclusion of the 108 bp is not given a “letter” designation (Kamagate et al. 2000). To determine if the sequences for hCE PMCA1 DNAs (i.e., the 690 and 552 bp fragments) contained or lacked this exon, the hCE sequences were aligned and blasted against a known sequence lacking the 108 bp exon from rat pancreatic islets (NCBI AF076783) (Kamagate et al. 2000). In each case, the 690-bp fragment from hCE, included the 108-bp exon. In contrast, the 108-bp exon was absent in the 552-bp fragment from hCE. Thus, the splice variants for PMCA1 in native hCE are PMCA1b (NCBI AY740526) and PMCA1kb (NCBI DQ201776). The nucleotide and amino acid sequences of these products are shown in Figure 3.

## PMCA2

Electrophoresis of PCR fragments using the primer pair P2pBeWo/2cam resulted in two faint bands in a single donor (all 5 donors tested) that were too close in size to be cleanly isolated for sequencing. Thus, PMCA2 PCR DNAs were cloned. Transcripts of approximately 1640 bp and 1147 bp were detected (Figure 2B). Because the P2pBeWo is upstream of the B site, the transcripts included sequence within both the B and C alternative splice sites.

The genomic structure at site C of PMCA2 includes two exons of 172 bp and 55 bp, respectively (Figure 1B). The 172 bp exon or both exons (227 bp) can be included or excluded (Adamo and Penniston 1992; Brandt et al. 1992; Kamagate et al. 2000; Keeton et al. 1993). Sequencing of the 1640 bp hCE PCR DNA showed that both exons were included; this sequence exhibited 99% identity with the human PMCA2a reference sequence (NCBI NM 001001331). Two nucleotide differences in the hCE PMCA2a transcript led to amino acid changes (proline to leucine; valine to alanine, identified by arrows in the Figure 4 inset). A proline to leucine substitution involves the loss of a hinge configuration that proline imparts to proteins. Valine and alanine are both nonpolar amino acids. Because of these changes, the chromatograms were re-evaluated to check for possible sequence errors, however, it was determined that these base differences in hCE were not sequencing artifacts. These differences for PMCA2a in native hCE (NCBI DQ201777) require functional studies to understand their significance in hCE.

Sequence analysis of the 1147 bp hCE PCR DNA revealed the absence of a 905 bp fragment that was present in PMCA2a transcript. Comparison to the reference sequence showed that this PCR DNA lacked part of exon 15, exons 16, 17, 18, 19, and a portion of exon 20 (Figure 4). This shortened hCE DNA lacks much of the ATP “pocket” within the third intracellular domain that is required for PMCA activity and is a splicing option that has not been previously described. We term this novel splice option PMCA2<sub>(i)</sub> (NCBI DQ201778).

## PMCA3

Samples from five donors were analyzed for PMCA3 expression. No site A PCR products were detected despite several attempts and varying the cycling conditions. One sample (03-030)

analyzed using the 3cap/3cam primer pair that flanks splice site C yielded a band of approximately 632 bp (Figure 2C). The genomic structure of the PMCA3 gene at site C is composed of three exons (68 bp, 154 bp and 88 bp) each of which can be inserted or excluded (Figure 1B) (Brown et al. 1996). Sequence analysis of the hCE PMCA3 DNA revealed that this fragment lacked the 154 bp exon and exhibited 100% identity with the sequence of PMCA3a from human brain (NCBI U57971). Thus, the splice variant at the C site in native hCE is PMCA3a (NCBI DQ201779).

## PMCA4

Using hCE cDNA of five donors, PCR was done using the primer pair PMCA4apa/PMCA4ama that flank site A, and using hCE cDNA of four donors amplification was done using the primer pair 4cap/4cam that flank sites B/C (Table 1). In all of the samples, PCR amplification yielded a band of approximately 336 bp at site A and a band of approximately 805 bp at sites B/C. Representative data from two donors are shown in Figure 2D. For PMCA4 at site A, a 36 bp exon can be included or excluded (Figure 1). Sequencing of the PMCA4 site-A products from all donors revealed that the 36 bp exon was inserted and was designated as PMCA4x (NCBI DQ201781). The hCE PMCA4x had 100% identity with PMCA4x of the sequence NCBI NM 001001396 (GI 48255958).

For PMCA4, splicing variants at site C result from the insertion or exclusion of a 178-bp exon (Figure 1). This exon may be completely spliced in (4a) or spliced out (4b). An internal donor site located approximately 108 bp from the beginning of this exon may also be used (4d) (Santiago-Garcia et al. 1996). As with PMCA1, a 108-bp exon can be spliced in or out at site B (Kamagate et al. 2000; Santiago-Garcia et al. 1996). Direct sequencing of the 805-bp fragment determined that the 178-bp exon was excluded and the 108 bp exon was included. Thus, the splice configuration was determined to be PMCA4b (NCBI DQ201781). This sequence determined from native hCE at the C site had 100% identity with a known PMCA4b sequence from cardiac muscle (NCBI U42026) (Santiago-Garcia et al. 1996). Thus, in native hCE PMCA4xb is the isoform expressed at the A, B and C sites.

## DISCUSSION

This study showed that the splice variants of PMCAs expressed in native human corneal epithelium were: PMCA1b and PMCA1kb (at splice sites B and C); PMCA4xb (at sites A and C); PMCA2a (at site C) and a *novel* PMCA2<sub>(i)</sub>; and PMCA3a (at site C) Our prior work documented expression of all PMCA isoforms using pooled samples of CE (Talarico et al. 2005). In the present study, hCE collected from five different donors was analyzed. The pattern of PMCA1 and PMCA4 isoform/splice variant expression in hCE was the same among all donors examined. In contrast, PMCA2 and PMCA3 expression was variable with transcripts detected in individual hCE samples. PMCA2 transcript was detected in one donor while PMCA3 transcript was detected in a different donor. Additional CE samples must be analyzed to evaluate whether diversity of PMCA2 and PMCA3 expression has functional significance.

Studies show that alternative splicing at the A-splice site located in the first intracellular loop, affects membrane targeting of PMCA variants (Chicka and Strehler 2003; Strehler et al. 2007). For example, insertion of extra amino acids at site A such as occurs in PMCA2w that includes all spliced exons targets the pump to the apical cell membrane in cochlear hair bundles. In contrast PMCA2x and z variants that containing a partial insert- or no insert, respectively, are directed to the basolateral membrane (Chicka and Strehler 2003).

Alternative splicing of all four PMCAs at site A has been demonstrated in several other tissues e.g., (Heim et al. 1992; Kamagate et al. 2000; Stauffer et al. 1993; Zacharias et al. 1995). In the present study, PCR for PMCA1, PMCA2 and PMCA3 using primer sets designed to amplify

across the A site (pmca-A1P/pmca-A1M, pmca-2AAP/pmca-2AAM and pmca-A3P/pmca-A3M) did not produce transcripts using hCE from any of the donors. This was despite several attempts and varying PCR cycling conditions. These same primers successfully amplified site-A PMCA1-3 DNAs from several other tissues including human RPE (Shen et al. 1999). In contrast, PCR for site A of PMCA4 yielded abundant transcript for all donors (e.g., Figure 2D, lanes 1 and 2). By contrast, PCR of the same hCE cDNAs with primers that flanked the B/C splice sites (Table 1) yielded transcripts for PMCA1, PMCA2 and PMCA3 showing the presence of sequence for these respective PMCA. This suggests that there is some sequence difference in the region of splice site A that is unique to CE such that the current primers cannot effectively “prime” PCR.

The finding of splicing at site B for PMCA1 in hCE (Figure 3) is only the fourth time that site B splicing has been demonstrated. Previously, PMCA1 (intestine and pancreatic beta cells) and PMCA4 (fetal cardiac muscle) cDNAs were identified in which splicing at site B leads to the inclusion or exclusion (k variant) of a 108-bp exon (Howard et al. 1993; Kamagate et al. 2000; Santiago-Garcia et al. 1996). Some have suggested that site-B splicing results from aberrations in the splicing mechanism (Howard et al. 1993; Keeton et al. 1993; Preiano et al. 1996). However, here we demonstrated PMCA1b and PMCA1kb transcripts in hCE from four different donors. The present findings obtained under different PCR conditions and in a different tissue suggest that splicing at site B is not due to aberrant splicing. Exclusion of the 108-bp exon at site B leads to deletion of the 10<sup>th</sup> and possibly the 9<sup>th</sup> transmembrane domains (Howard et al. 1993; Preiano et al. 1996). This alteration could lead to the “k” variant having a regulatory site on the external side of the plasma membrane that would enable response to external Ca<sup>2+</sup> (Preiano et al. 1996). Functional significance of this variant in any tissue remains to be determined.

The hCE PMCA2a determined in this study contained two base changes. The first was a change from C to T that would result in a residue change from proline to leucine in exon 21; the second was a change from T to C that would yield a residue change from valine to alanine in exon 22 (Figure 4 and insert). Leucine is a hydrophobic/nonpolar amino acid, whereas proline is a nonpolar amino acid that adds a kink or hinge in the polypeptide chain. Alanine and valine both are nonpolar/hydrophobic amino acids. It is unclear what the results of these changes would be in hCE PMCA2a, however, lack of a kink may affect the three-dimensional configuration near the end of the third-intracellular loop altering activity or autoinhibition by the CaM-binding domain.

The results also revealed a *novel* PMCA2 splice variant, termed PMCA2<sub>(i)</sub> in hCE. The significance of the deletion of a portion of exon 15, exons 16, 17, 18, 19, and part of exon 20 is the absence of much of the tertiary structure necessary to constitute the pocket of the active site for PMCA activity (Carafoli 1994). This suggests that PMCA2<sub>(i)</sub> would lack ATPase activity. The fact that PMCA2<sub>(i)</sub> was detected only in one donor and with very low abundance at the mRNA level suggests a low physiological importance of this variant in *normal* CE Ca<sup>2+</sup> homeostasis. PMCA2 transcripts were found in a single donor (03-031), a 56-year-old male Caucasian, with a primary cause of death noted as lung cancer (type unspecified). Immediately prior to the time of death (TOD), this donor was medicated with several drugs including, zincef, heparin, Xanax, Zoloft, propofol, lidocaine and fentanyl, but the affect of these drugs on PMCA expression or splicing is unknown. With respect to other donors regarding gender, age, race, cause of death, TOD to preservation of tissue in Optisol, TOD to preservation of CE in *RNALater*, and total time in Optisol, the only factor that stood out for donor 03-031 was age. Specifically, this donor was 14-to-20 years younger than all other donors. Clearly, additional CE samples must be analyzed to assess the physiological significance of this new splicing option.

Splice site C is located within a region that encodes a CaM-binding domain near the C-terminus (Figure 1) that regulates PMCA activity (for refs see (Strehler et al. 2007)). Alternative splicing at site C alters the configuration of the CaM-binding domain thus affecting the CaM affinity and extent of activation by CaM. For example, studies show a-splice variants have approximately a 10-fold lower CaM affinity but higher basal (CaM-independent) activity than the “b” variants. Inclusion of all exons at splice-site C results in a full-length transcript (a-splice form), but a shortened protein with a truncation in the CaM domain because of a stop codon. In contrast, splicing out of exons at site C leads to a shortened mRNA transcript but a longer protein product (b-splice form) with a fully functional CaM domain. Studies show that in the presence of Ca<sup>2+</sup> alone, ATPase activity of the 4a and 4b variants was equal, whereas ATPase activity in the presence of Ca<sup>2+</sup> and CaM was higher with 4b than 4a (Preiano et al. 1996;Varadi et al. 1996). The present study showed that hCE expressed three different b-splice forms (PMCA1b, PMCA1kb; and PMCA4xb) that would be predicted to handle slow, tonic Ca<sup>2+</sup> changes in CE cells and two a-splice forms (PMCA2a and PMCA3a -- albeit low-abundance forms) that would be predicted to handle rapid Ca<sup>2+</sup> changes (c.f. Strehler et al. 2007;Domi et al., 2007).

In summary, this study identified alternative splice variants of PMCA isoforms expressed in human corneal epithelium. Understanding which PMCA splice variants are expressed and identifying where within the continuum of cells that comprise the CE these splice variants are expressed lays the foundation for future studies that will characterize their physiological role (s) in corneal biology.

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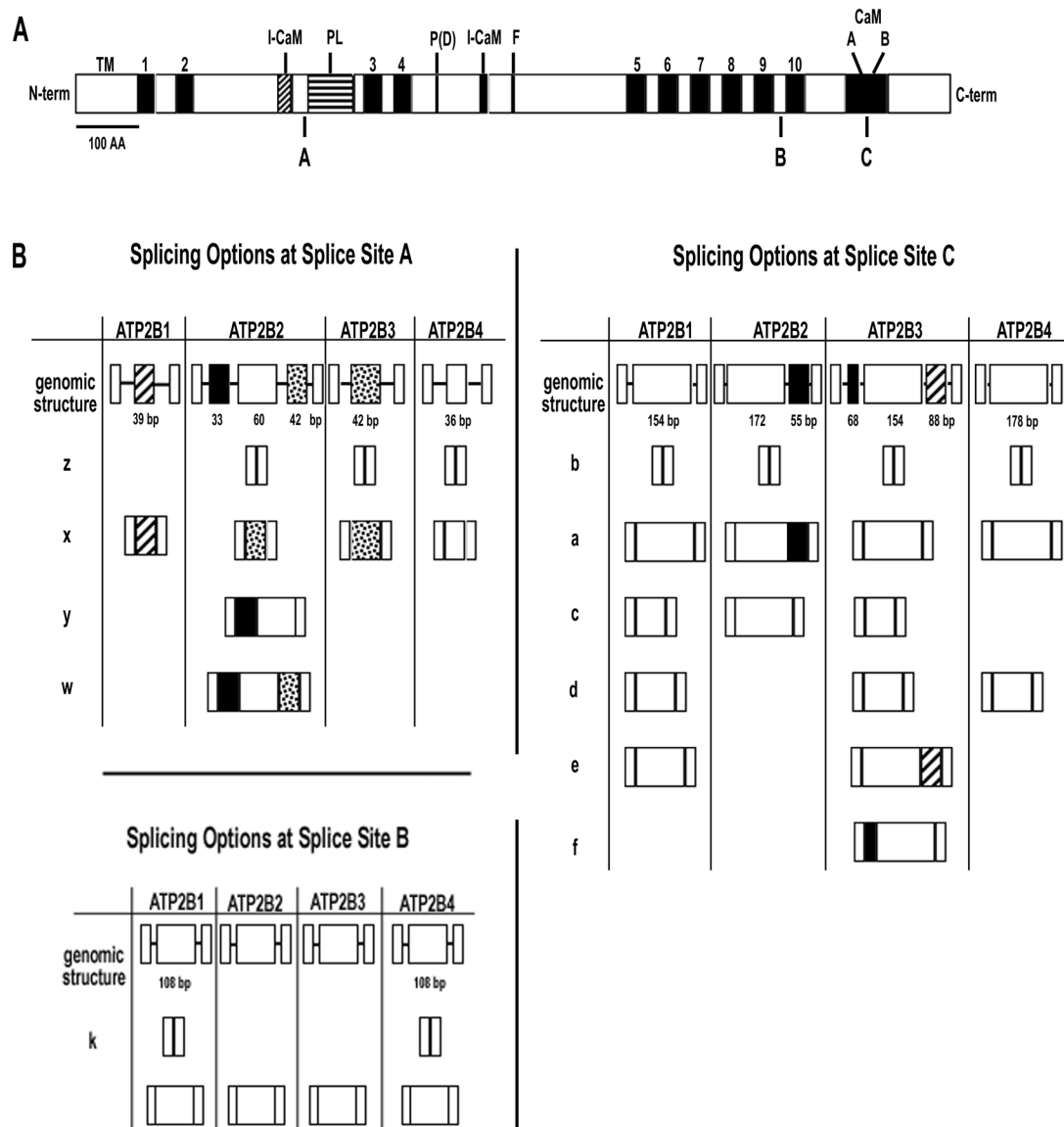
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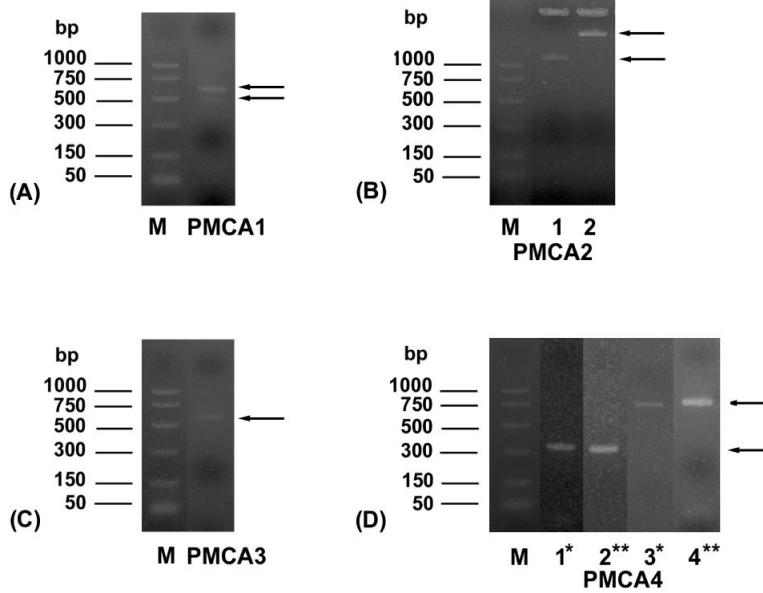
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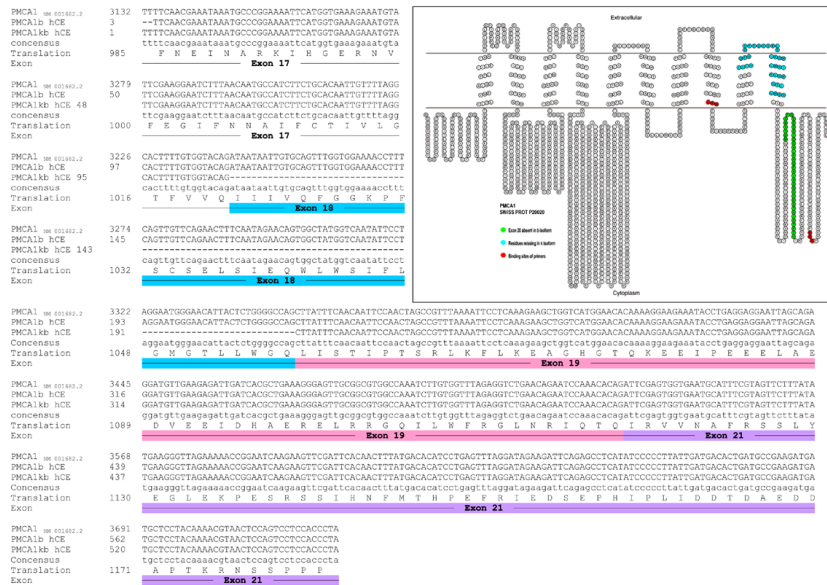
**Figure 1. Location of Splice Sites A, B and C Relative to the PMCA Domain Structure and Overview of Possible Splice Variants Generated at Each of These Sites.**

(A) Linear representation of the PMCA domain structure showing the location of splice sites A, B and C, where alternative RNA splicing may lead to changes in protein structure; transmembrane domain (TM); autoinhibitory region (I-CaM) that interacts with the calmodulin-binding domain in the absence of  $\text{Ca}^{2+}$  - CaM; putative acidic phospholipid-sensitive region (PL); site (aspartate residue) of acylphosphate formation P(D); fluorescein isothiocyanate binding site (F); calmodulin-binding regions (CaM), separated into subdomains A and B. (B) Genomic structure and summary of the alternative splice options for each of the four PMCA genes in the region of alternative splice sites A (upper left panel), B (lower left panel) and C (right panel). [Adapted from: (Kamagate et al. 2000; Zacharias et al. 1995) to include the published results from (Stauffer et al. 1993), (Heim et al. 1992), (Santiago-Garcia et al. 1996), and (Howard et al. 1993).



**Figure 2. Amplification of PMCA1, PMCA2, PMCA3 and PMCA4 PCR DNAs From Human Cornea Epithelium.**

(A) PCR amplification of cDNA using primers that flanked the B/C site for PMCA1 yielded two fragments in each tested donor of approximately 690 and 552 bp, respectively (Donor 03-030 is shown). (B) Cloning of PCR DNAs for PMCA2 in donor 03-031 (one of 5 donors tested) revealed two inserts of approximately 1640 bp and 1147 bp, respectively (lanes 1 and 2). The highest band of ~2072 bp in the two lanes is cloning vector DNA. (C) PCR amplification across the B/C site for PMCA3 yielded a band ~632 bp in one of 4 donors examined. (D) PCR amplification for PMCA4 was done in all donor samples of hCE (two different samples shown) for the A splice site (lanes 1 - 2) and in 4 of 5 donors (2 different samples shown) for the B/C splice site (lanes 3 - 4). In all donors tested, bands of ~336 bp at site A, and bands of ~805 bp at site C were detected. [Lane M in all panels shows PCR DNA size markers. In panel D, asterisks (\* and \*\*) identify sample (i.e., donor) pairs for PMCA4 analysis.]



**Figure 3. Multiple Alignment of PMCA1 at Splice Sites B and C**

The figure shows the alignment of nucleotide sequences for both PMCA1 isoforms found in native human corneal epithelium and the reference sequence (NM 001682). The consensus sequence and the corresponding peptide sequence are also shown. For orientation, a schematic of the topography of the protein structure is illustrated (insert) with color-coding of the relevant exons. The binding sites of PMCA1 isoform-specific primers are shown (red). PMCA1b and PMCA1kb both lack the 154 bp fragment that corresponds to exon 20 (green) at site C. PMCA1kb also lacks a 108 bp piece (exon 18 shown in blue) at splice site B (inset). PMCA1kb is a novel splice variant at splice site B that previously has only been described in intestine and pancreatic beta cells (Howard et al. 1993; Kamagate et al. 2000).

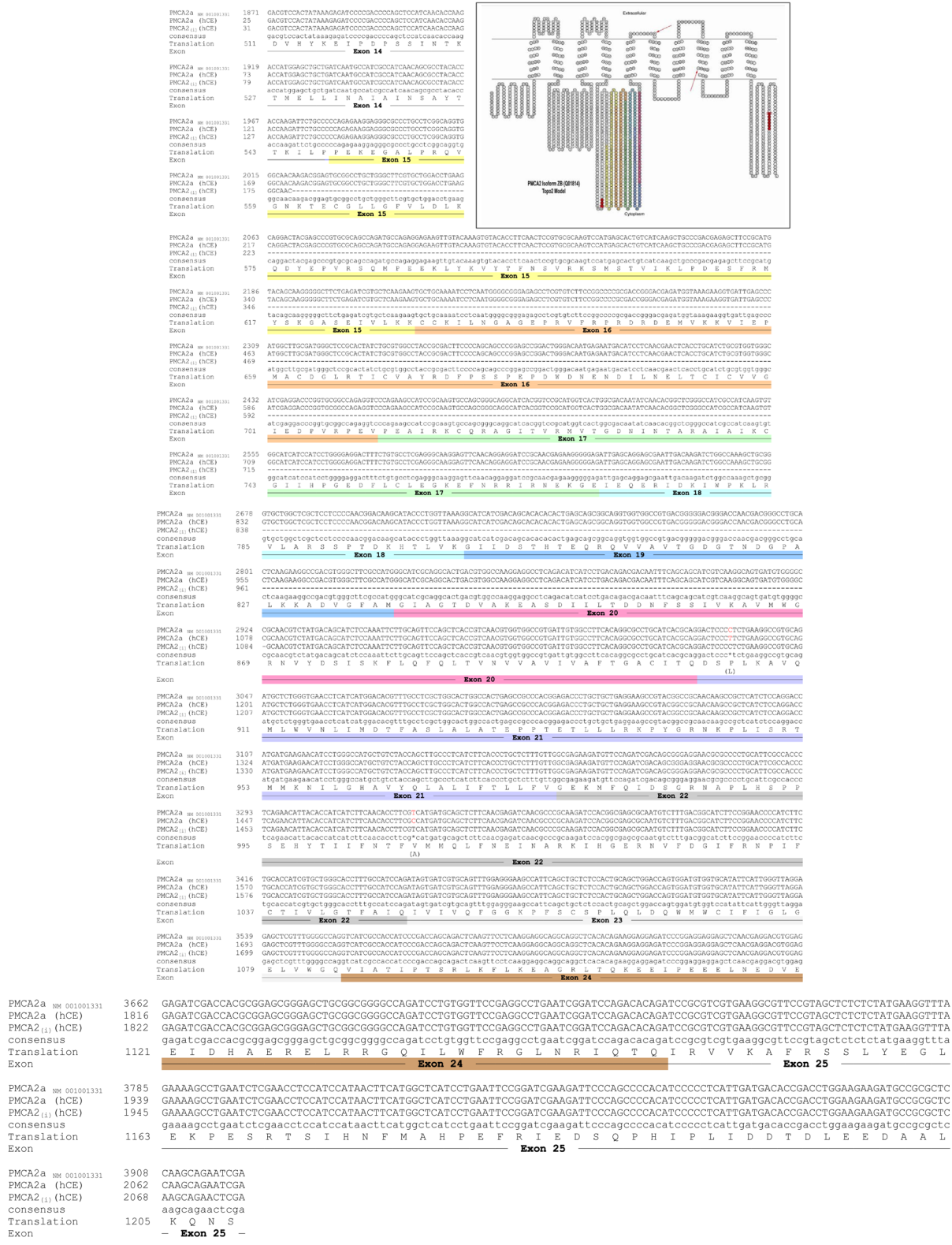


Figure 4. Multiple Alignment of PMCA2 Isoforms from Human CE at Splice Sites B and C

This diagram shows the alignment of the nucleotide sequences for both PMCA2a and PMCA2<sub>(i)</sub> found in native human corneal epithelium and the reference sequence derived from trophoblasts isolated from human term placenta (NCBI NM **001001331**). The consensus sequence, corresponding peptide sequence and protein structure (insert) are also shown. Exons are color coded in the nucleotide sequence alignment. In the inset, color coding identifies the exons/amino acids that are omitted (i.e., spliced out) in the PMCA2<sub>(i)</sub> variant. Red-shaded amino acids in the inset identify beginning and ending positions of the flanking primers. PMCA2a is the splicing option that includes a 108-bp exon (exon 23) at site B and both 172- and 55 bp exons at site C (exon 24 and part of exon 25, respectively). Two base differences in the hCE PMCA2a sequence when compared with reference sequence (shown in red in the nucleotide sequence alignment) led to amino acid differences in hCE (proline to leucine and valine to alanine). Red arrows in the inset identify the positions of the two amino acids that are changed. PMCA2<sub>(i)</sub> is a “novel” PMCA2, where part of exon 15, exons 16, 17, 18, 19, and a portion of exon 20, that together comprise most of the third intracellular domain responsible for Ca<sup>2+</sup>-ATPase activity are missing (insert).

TABLE 1

PMCA Gene-Specific Primers Used for RT-PCR.

<u>A Site Primers</u>	Primer Name	Direction	(5' to 3') Primer Sequence	Starting Position	Possible Variants
ATP2B1	pmca-A1P	forward	5' - gctgacggcacaacttcaagg - 3'	857	1x
	pmca-A1M	reverse	5' - ggagacatcaacagaccctgc - 3'	1327	
ATP2B2	pmca-AA2P	forward	5' - tgaactctgtgggtfg - 3'	1394	2x; 2z; 2w
	pmca-AA2M	reverse	5' - cagcttgggagctgfc - 3'	1620	
ATP2B3	pmca-A3P	forward	5' - acattgcccaggccaagtagc - 3'	770	3x; 3z
	pmca-A3M	reverse	5' - ttccgactgcacggctag - 3'	1244	
ATP2B4	pmca-4apa	forward	5' - tactcttgggggcaatga - 3'	1736	4x
	pmca-4ama	reverse	5' - ccattgcttgcgattataca - 3'	2066	
<u>B/C Site Primers</u>	Primer Name	Direction	(5' to 3') Primer Sequence	Starting Position*	Possible Variants
ATP2B1	hPMCA1P	forward	5' - ttcaacagaataatgcccg - 3'	3134	1k and non-k
	hPMCA1M	reverse	5' - aggtggaggactggagttagc - 3'	3726	1a, 1b, 1c, 1d, 1e
ATP2B2	2cap 2cam	forward reverse	5' - agatccacggcgagcgcaat - 3' 5' - cgagtctgttgagcgcg - 3'	3485 4039	2a, 2b, 2c
	P2pBeWo 2cam	forward reverse	5' - acagtgtacagccctatgctg - 3' 5' - cgagtctgttgagcgcg - 3'	1712 3784	2k and non-k 2a, 2b, 2c
ATP2B3	3cap 3cam	forward reverse	5' - tgtccacagaacagtggtc - 3' 5' - atgccctgtctatggctt - 3'	3226 3849	3a, 3b, 3c, 3d, 3e, 3f
ATP2B4	4cap 4cam	forward reverse	5' - caetccgaagatccatg - 3' 5' - tggtaacacagcagctgac - 3'	3812 4611	4k and non-k 4a, 4b, 4d

\* Primer sequences were from Shen, et al. 1999 except P2pBeWo which was from Moreau, et al., 2003. Reference Sequences from the National Center for Biotechnology Information for starting position of primers: pmca-A1P/pmca-A1M and hPMCA1P/hPMCA1M (NM 001682); pmca-AA2P/pmcaAA2M and 2cap/2cam (NM 001683); pmca-A3P/pmca-A3M and 3cap/3cam (G2-600); pmca-4apa/pmca-4ama and 4cap/4cam (NM 001684).

TABLE 2

RNA Yield and PCR Results by Donor.

Donor	Total RNA Yield (µg/µl)	260/280 Ratio	PCR Products								
			PMCA1		PMCA2		PMCA3		PMCA4		
			A	B/C	A	B/C*	A	B/C	A	B/C	
03-029	16.47	2	-	+	-	-/-	-	-	-	+	+
03-030	18.9	2	-	+	-	-/-	-	-	+	+	+
03-031	5.86	1.98	-	+	-	-/+	-	-	-	+	+
03-055	9.9	2	-	+	NE	-/-	-	-	-	+	+
03-065	20	1.7	-	NE	-	-/-	-	-	NE	+	NE

Splice variants of PMCA5 expressed in hCE were determined by PCR amplification of hCE using PMCA isoform gene-specific primers that flanked splice sites A and B/C. NE indicates a sample was not evaluated secondary to insufficient amount of sample. (-) indicates that no PCR products were detected. (+) indicates PCR products were detected; bands were purified and their identity as PMCA5 determined by sequencing.

\* PCR at splice site B/C for PMCA2 was done using two different primer pairs (2cap/2cam and P2pBeWo/2cam). In four donors, no reaction products were detected using either primer set (-/-). PCR using the primer pair P2pBeWo/2cam yielded products of the expected size in Donor 03-031.(-/+).