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EVIDENCE FOR A FOURTH RAT ISOFORM OF THE PLASMA MEMBRANE CALCIUM PUMP IN THE KIDNEY

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Summary: This study was conducted to identify plasma membrane Ca^{2+}-transporting ATPases present in rat kidney. Characterization of the cDNAs of the plasma membrane Ca^{2+}-ATPases revealed a family of proteins with regions of highly conserved amino acid sequence. To examine the extent of the diversity of rat renal plasma membrane Ca^{2+}-ATPases, we used the polymerase chain reaction to detect additional gene products in rat kidney mRNA that shared these conserved regions. Sequences corresponding to three previously known rat plasma membrane Ca^{2+}-ATPases were obtained. In addition, we found sequence corresponding to a new putative plasma membrane Ca^{2+}-ATPase. Our results demonstrate that the rat kidney contains at least four different plasma membrane Ca^{2+}-ATPases and the complexity of this multigene family is greater than previously thought.

ATP-dependent Ca^{2+} transport is believed to play an important role in the renal reabsorption of Ca^{2+} (1). This active process has been identified in proximal and distal convoluted tubules as assessed by ATP-dependent Ca^{2+} uptake (2), and along the nephron as assessed by Ca^{2+} and Mg^{2+} dependent ATP hydrolysis (3). In addition, studies by Borke et al (4), using monoclonal antibodies to the human erythrocyte plasma membrane Ca^{2+}-ATPase, have identified a cross-reacting epitope along the basolateral membrane of the distal convoluted tubule of the human kidney. However, no cross-reacting material was identified in any other nephron segment, raising the question as to the molecular nature of the Ca^{2+}-transporting ATPases in the other nephron segments.

Most of our knowledge regarding Ca^{2+}-transporting ATPases comes from studies on these transporters from sarcoplasmic reticulum (5) and plasma membranes (6). These Ca^{2+}-transporting ATPases are members of the aspartylphosphate family of cation-transporting ATPases. The Ca^{2+}-transporting ATPases form a family of structurally

Abbreviations: PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; RBC, red blood cell.

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related proteins that demonstrate molecular heterogeneity in their structure depending on whether they come from the sarcoplasmic reticulum or the plasma membrane. Furthermore, transporters from the sarcoplasmic reticulum demonstrate structural heterogeneity as do transporters from the plasma membrane. Thus it is possible that the Ca^{2+}-ATPase activity identified in nephron segments other than the distal convoluted tubule may represent different molecular forms of Ca^{2+}-transporting ATPase.

The present study was intended to identify and characterize the plasma membrane Ca^{2+}-ATPases present in the rat kidney. Using PCR , we have demonstrated the presence of four different plasma membrane Ca^{2+}-ATPases in rat kidney. Three correspond to previously cloned plasma membrane Ca^{2+}-ATPases and the fourth represents a new putative plasma membrane Ca^{2+}-ATPase.

MATERIALS AND METHODS

Materials: The GeneAmp RNA PCR kit was purchased from Perkin Elmer Cetus (Norwalk, CT). Sequenase was from United States Biochemical Corp. (Cleveland, OH). All other enzymes were from Boehringer Mannheim (Indianapolis, IN). [alpha-^{35}S]dATP (>1000 Ci/mmol) was from Amersham (Arlington Heights, IL). Seakem agarose was from FMC Bioproducts (Rockland, ME). Sodium dodecyl sulfate, acrylamide, N,N'-methylene-bis-acrylamide, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine and bromophenol blue were purchased from Bio-Rad Laboratories (Richmond, CA.). All other reagents were from either Boehringer Mannheim or Sigma (St. Louis, MO).

Sequence comparisons: The Feng-Doolittle analysis (7) of the EuGene software program on Baylor College of Medicine's Molecular Biology Information Resource System was used to compare the amino acid sequences for the cloned vertebrate Ca^{2+}-ATPases for which full length sequence was available. The sequences compared and their GenBank/EMBL Data Bank accession numbers are shown in Figure 1.

RNA Isolation, cDNA Synthesis and PCR: Total RNA was isolated from male Sprague-Dawley rat kidney using a modification of the guanidine isothiocyanate/CsCl method (8). Poly(A)$^+$ RNA was purified by chromatography over oligo(dT)-cellulose (9). First strand cDNA synthesis was carried out in total reaction volume of 20 ul on 0.5 ug of poly(A)$^+$ RNA using 2.5 uM random hexamers, Moloney murine leukemia virus reverse transcriptase, and "Master Mix" ($MgCl_2$, dNTPs, RNase inhibitor and buffer) provided in the GeneAmp RNA PCR kit. The mixture was incubated for 10 min at room temperature (to extend the hexamers) followed by 15 min at 42°C. The reaction was stopped by incubation at 99°C for 5 min followed by 5 min at 4°C. The PCR reaction was carried out in the same tube containing the cDNA. The cDNA mixture was supplemented with 2.5 units of Thermus aquaticus DNA polymerase (AmpliTaq), PCR buffer and $MgCl_2$ provided in the kit, 25 pmol of each primer and water to bring the final volume to 100 ul. The reaction mixture was overlaid with mineral oil and incubated using a PTC-100 programmable thermal cycler (MJ Research, Watertown, MA) programmed to repeat the following cycle 35 times: 30 sec at 94°C, 30 sec at 54°C, and 60 sec at 72°C. All PCR primers were synthesized on an Applied Biosystems model 381A DNA synthesizer, removed from the resin with ammonium hydroxide, and purified through 20% polyacrylamide-urea gels.

Subcloning of PCR Products and Sequencing: The PCR products were purified through a 0.7% agarose gel and were subcloned into the SmaI site of M13mp19 by blunt end ligation. The ligation mixture was used to transform competent TG-1 Escherichia coli (9). Individual white plaques were isolated. Single-stranded DNA was sequenced with [alpha-^{35}S]dATP and the dideoxy protocol described by the manufacturer for use with Sequenase version 2.0 (U.S. Biochemical Corp.) using as first primer the univeral M13 primer and then 21-mers synthesized on the basis of information obtained from previous sequencing. Sequences were confirmed by at least three independent readings in both directions.

RESULTS AND DISCUSSION

The Ca^{2+}-ATPases involved in active Ca^{2+} reabsorption in the kidney should belong to the plasma membrane variety. Therefore, to select primers to be used in the PCR reaction, the deduced amino acid sequences of the cloned plasma membrane Ca^{2+}-ATPases were compared. Areas of amino acid identity and difference were identified. Within areas of amino acid identity that bordered areas of variability, stretches of eight to nine amino acids with the lowest codon redundancies were identified. These areas were examined because priming from areas of identity across areas of variability would allow for the detection of sequence that would be diagonistic for a given Ca^{2+}-ATPase isoform and allow for the detection of potential new members of this gene family. The area chosen to be amplified corresponds to amino acids 438-701 in the human RBC Ca^{2+}-ATPase sequence (10). This area includes the aspartate phosphorylation site (11) as well as the FITC binding region (12). Primer 1 is a sense primer composed of a mixture of 32 different oligonucleotides while Primer 2 is an antisense primer composed of a mixture of 768 oligonucleotides (see Fig. 1 for primer design). We decided to synthesize completely redundant primers to increase the likelihood of isolating previously unidentified gene products for plasma membrane Ca^{2+}-ATPases. The predicted amplified product should be 790 bp.

Upon amplification of cDNA synthesized from poly(A)$^+$ RNA isolated from rat kidney three prominent products were amplified (Fig. 2). One of which was of the expected size (790 bp) as well as two smaller products that were 600 and 460 bp long. Product formation was found to be sensitive to $MgCl_2$ concentration and optimal amplification was obtained with 4.5 mM $MgCl_2$. Higher $MgCl_2$ concentrations reduced the amount of product formed (not shown). Elimination of either poly(A)$^+$ RNA, reverse transcriptase, or Taq polymerase from the reaction mixture resulted in no product formation (not shown).

The amplified 790bp product was isolated, subcloned into the SmaI site of M13 and sequenced to determine the identity of the amplified products. Out of 30 clones isolated and sequenced, we obtained sequence that is diagnostic for the three rat brain plamsa membrane Ca^{2+}-ATPases that have previously been cloned (Fig. 1). We also obtained sequence for a new putative plasma membrane Ca^{2+}-ATPase, designated rat PMCA4 *, that has yet to be described. Rat PMCA4 has the highest homology with the human RBC Ca^{2+}-ATPase (85.2% amino acid identity, Table 1). Homology of rat PMCA4 with the three previously cloned rat brain plasma membrane Ca^{2+}-ATPases is lower, ranging from

*The nomenclature used for the plasma membrane Ca^{2+}-ATPase (PMCA) isoforms was introduced in ref. 13. The numbering of the isoforms rat PMCA1, rat PMCA2 (13), rat PMCA3 (14), and human PMCA4 (10) is according to the order in which these cDNAs were identified. Each protein seems to be encoded by a separate gene. We have designated the new putative Ca^{2+}-ATPase cDNA that we have identified as rat PMCA4 since it has the highest homology to the human PMCA4 (10) and it is the fourth rat isoform identified. However, as indicated in the text, this may represent a new isoform different from human PMCA4.

```
      431    PRIMER 1    .         .PHOS SITE        .         .         .
 hum4: ISLAYSVKKMMKDNNLVRHLDACETMGNATAICSDKTGTLTMNRMTVVQAYIGGIHYRQIPSPDVFLPKV
 hum1: ----------------------------------------------------NEK--KKV-E-EAIP-NI
 por1: ----------------------------------------------------NEK--KK--E-EAIP-NI
 rat1: ----------------------------------------------------NEK--KKV-E-EAIP-NI
 rat2: ---------------------------------------T---------V-DV--KE--D-SSINA-T
 rat3: ---------------------------------------T-------S-L-DT--KE--A-SALT--I
*rat4: ------------------------------------------------T------K--DLP-N-
      501       .         .         .         .         .         .         .
 hum4: LDLIVNGISINSAYTSKILPPEKEGGLPRQVGNKTECALLGFVTDLKQDYQAVRNEVPEEKLYKVYTFNS
 hum1: -SYL-T---V-C----------------H----------LLL---R---D----I---A---------
 por1: -SYL-T---V-C----------------H----------LLL---R---D----I---A---------
 rat1: -SYL-T---V-C----------------H----------LL---R---D----I---A---------
 rat2: -E-L--A-A------T---------A----------G-----L--R---EP--SQM-------------
 rat3: ---L-HA--------T---------A----------IL---R-F-P--EQI--DQ--------------
*rat4: ------S-C-------------------------G----------S-M-----F-------
      571       .         FITC SITE .         .         .         .         .
 hum4: VRKSMSTVIRNPNGGFRMYSKGASEIILRKCNRILDRKGEAVPFKNKDRDDMVRTVIEPMACDGLRTICI
 hum1: --------LK-SD-SY-IF---------K--FK--SAN---KV-RPR----I-K-------SE------L
 por1: --------LK-SD-SY-IF---------K--FK--SAN---KV-RPR----I-K-------SE------L
 rat1: --------LK-SD-S--IF---------K--FK--SAN---KV-RPR----I-K-------SE------L
 rat2: ---------KM-DES-----------V-K--CK--SGA--PRV-RPR---E--KK--------------V
 rat3: ----------M-D----LF-------L-K--TN--NSN--LRG-RPR------KKI--------------
*rat4: ----------K-E----VF-------M----V---NKE-GI----T----N---N------SE-----G-
      641       .         .         .         .         .    PRIMER 2    .
 hum4: AYRDFD..DTEPSWDNENEILTELTCIAVVGIEDPVRPEVPDAIAKCKQAGITVRMVTGDNINTARAIA
 hum1: -F---PagEP--E-----D-V-G--------------------K--QR--------------------
 por1: -F---PagEP--E-----D-V-G--------------------K--QR--------------------
 rat1: -F---PagEP--E-----DVV-G------------------E--K--QR--------------------
 rat2: -----Pss.P--D-----D--N-----C-------------E--R--QR--------------------
 rat3: -----SaiQ.--D------VVGD-----------------E--R--QR--------------------
*rat4: ------.gE.----E-----F-G-V----------------N---RV------------
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Primer 1 - sense                    Primer 2 - antisense

5'-AAAAAAATGATGAAAGACAATAA-3'       5'-TTTATGTTGTCTCCTGTTACCAT-3'
    G  G       G  T  C                   A  A  A  A  A  A
                                          G        C  C  C
                                                   G  G  G
```

Figure 1. Region of the plasma membrane Ca^{2+}-ATPases selected for PCR amplification. Deduced amino acid sequences of the plasma membrane Ca^{2+}-ATPases compared using the Feng-Doolittle analysis (7). Numbering of amino acids corresponds to that of the human RBC Ca^{2+}-ATPase (hum4, 10). Also indicated is the design of the primers used to amplify rat renal poly(A)$^+$ RNA (Primer 1 and 2); the amino acid sequence they correspond to (PRIMER 1 and 2); the aspartate phosphorylation site (PHOS SITE, 11); and the FITC binding site (FITC SITE, 12). In addition to the reference, the GenBank/EMBL Data Bank accession numbers are given for each sequence analyzed. The Ca^{2+}-ATPases correspond to: hum4, human RBC (10; M25874); hum1, human teratoma (22; J04027); por1, porcine smooth muscle (23; X53456); rat1, rat brain 1 (13; J03753); rat2, rat brain 2 (13; J03754); rat3, rat brain 3 (14; J05087); *rat4, the new sequence reported here and referred to as PMCA4. Dashes represent sequence identical to hum4, letters indicate amino acid differences, dots in the sequence indicate gaps introduced to maintain the alignment.

73.1 to 74.6% amino acid identity. Rat PMCA4 appears to code for an enzyme that is different than the human RBC enzyme. This conclusion comes from comparison of the sequences of hum1, por1 and rat1 as shown on Table 1. These three sequences appear to code for the same Ca^{2+}-ATPase isoform from the three different species and demonstrate 98 to 99% amino acid identity in the amplified area. Rat PMCA4 does not appear to be an organellar Ca^{2+}-ATPase as it is only 23% homologous to an organellar rat kidney Ca^{2+}-ATPase (15), and does not contain two inserts of 20 and 18 amino acids in this area that are present in the organellar Ca^{2+}-ATPases. Most of the observed homology resides around the aspartyl phosphorylation site (not shown). Thus it appears that we have identified a new plasma membrane Ca^{2+}-ATPase.

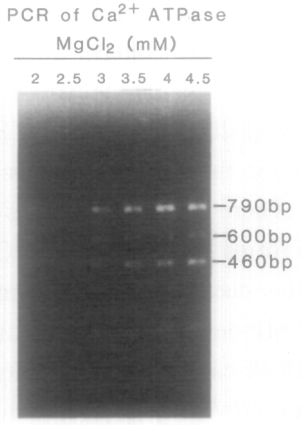

Figure 2. Effects of $MgCl_2$ on amplification of cDNA for plasma membrane Ca^{2+}-ATPases from rat kidney. Poly(A)$^+$ RNA isolated from rat kidney was subjected to reverse transcription and amplification as described under "Materials and Methods". Amplification was carried out in the presence of the indicated concentrations of $MgCl_2$. The amplified products were separated on a 0.7% agarose gel and the size of the amplified products indicated. A photograph of an ethidium bromide-stained gel is shown.

In addition to these isoforms, Brandt et al (16) have isolated a clone from a bovine brain cDNA library containing the C-terminal domain of a plasma membrane Ca^{2+}-ATPase that contains amino acid sequence that is not present in any of the other full length plasma membrane Ca^{2+}-ATPases cloned to date. Furthermore, as the area we amplified by PCR is outside of this region we do not know whether rat PMCA4 codes for the same protein as the bovine cDNA. As indicated above, of the six full length plasma membrane Ca^{2+}-ATPase cDNAs cloned, one human (hum1) one porcine (por1) and one rat (rat1) sequence appear to represent forms of the same enzyme. Thus, together with the partial sequence from the bovine cDNA and rat PMCA4, and if these two partial clones code for different gene products, there appears to be at least six different plasma membrane Ca^{2+}-ATPase genes identified to date.

Northern blot analysis (14) using cDNAs for rat PMCA1, -2 and -3 under moderate stringency revealed the presence of an 8.8 kb mRNA in uterus and stomach that was not

Table 1. Amino Acid Identity Percentages Among the Plasma Membrane Ca^{2+}-ATPases Within the Amplified Area

	hum4	hum1	por1	rat1	rat2	rat3	rat4
hum4	100	75.8	76.1	75.8	76.5	76.5	85.2
hum1		100	99.6	98.5	76.5	76.5	73.9
por1			100	98.1	76.9	76.9	74.2
rat1				100	77.3	78.4	74.6
rat2					100	80.7	73.1
rat3						100	74.2
rat4							100

detected under high stringency analysis suggesting also the existence of additional isoforms for the plasma membrane Ca^{2+}-ATPase gene family.

Thus far our discussion on the diversity of the plasma membrane Ca^{2+}-ATPases has centered on different genes producing different enzymes. However, there is also evidence that alternate splicing of mRNA for these Ca^{2+} pumps can lead to the formation of additional enzymes (6, 13, 14, 17). There may be as many as five different forms of PMCA1, two of PMCA2 and -3, and three forms of the human RBC Ca^{2+}-ATPase. Interestingly, some of these alternate splice sites occur within the calmodulin binding domain of the enzyme and include a potential cAMP-dependent protein kinase phosphorylation site. This may account for the apparent lack of calmodulin-sensitivity of several plasma membrane Ca^{2+}-ATPases (18-21) as well as providing an additional level of control of pumping activity by cAMP-dependent protein kinase (6). The functional significance of each molecular form of plasma membrane Ca^{2+}-ATPase is currently unknown and must await detailed kinetic analysis of each form individually expressed in the absence of any other plasma membrane Ca^{2+} pump.

The molecular identity of the Ca^{2+}-transporting ATPases involved in Ca^{2+} reabsorption by the kidney remains unresolved. The studies by Borke et al (4) clearly demonstrate that a monoclonal antibody directed against purified human erythrocyte Ca^{2+}-ATPase recognizes an epitope present on the basolateral aspect of the distal tubule. However, the purified human erythrocyte enzyme appears to be heterogenous. Comparison of peptide sequences from purified human erythrocyte Ca^{2+} pump with that of the deduced amino acid sequence of the human cDNAs for the pump indicate that the purified enzyme represents a mixture of isoforms (10, 22). Thus, we do not know which isoform the monoclonal antibody used by Borke et al (4) recognizes. Furthermore, the nature of the plasma membrane Ca^{2+}-transporting ATPases in the other nephron segments remain to be determined. Northern blot analysis (14) of mRNA from thirteen different tissues indicate that rat PMCA1 is widely distributed. This finding led Greeb and Shull (14) to the conclusion that rat PMCA1 may represent a "house keeping" plamsa membrane Ca^{2+} pump. If this is true, it is unlikely that this isoform is specifically involved in Ca^{2+} reabsorption in the kidney, leaving PMCA2, -3 and -4 as potential candidates. Determination of which of the various molecular forms of Ca^{2+} pumps are involved in Ca^{2+} reabsorption in the kidney will require a combination of molecular biological, biochemical and physiological approaches.

In summary, our findings indicate that the rat kidney contains mRNA for at least four different plasma membrane Ca^{2+}-transporting ATPases and the complexity of this multigene family is greater than previously thought.

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