Detection of isoform 4 of the plasma membrane calcium pump in human tissues by using isoform-specific monoclonal antibodies

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The epitope location and specificity of monoclonal antibodies JA9, 5F10 and JA3, raised against the human plasma membrane Ca^{2+} pump (hPMCA), were analysed by using synthetic peptides of the corresponding epitopes as well as the complete isoforms, hPMCA4b, hPMCA4a and hPMCA1b, expressed in COS-1 cells. The experiments with the peptides showed that JA9 reacted specifically with a region containing residues 51–75 of hPMCA4 (a or b), but not with the same region of isoforms 1, 2 or 3. JA3 reacted with residues 1156–1180, a region unique to hPMCA4b. 5F10 reacted in the region of residues 719–738, which is highly conserved in all PMCA isoforms. Indeed, 5F10 recognized all three isoforms expressed in COS-1 cells. JA9, in contrast, reacted with both variants a and b of hPMCA4 but not with hPMCA1, and JA3 recognized exclusively hPMCA4b. We used these

antibodies to discern the distribution of hPMCA4a and hPMCA4b in human brain, heart, kidney and lung. In Western blots of human brain samples, we could identify both hPMCA4a and hPMCA4b. Heart tissue also showed isoform 4b, and probably 4a. In contrast, kidney and lung showed primarily hPMCA4b. In brain, overlapping bands that did not correspond to either variant of hPMCA4 were detected, and in kidney a band migrating in the same position as hPMCA1b was observed. The distribution of the a and b forms of hPMCA4 at the protein level, as analysed by these antibodies, is consistent with the available data about the abundance of mRNAs for the hPMCA isoforms. The presence of hPMCA4b in all the samples supports the proposed role of this isoenzyme as a constitutive form of the pump.

INTRODUCTION

It is widely accepted that there are at least four genes that contain the code for isoforms of the plasma membrane Ca^{2+} pump (hPMCA). Each isoform has, in turn, two or more alternative splicing variants. The expression of the mRNA coding for the isoforms has been investigated [1–4], but only very recently have antibodies been made that discriminate between the isoforms at the protein level [5,6]. Measurement of expression at the protein level is very important, because sometimes the amount of mRNA does not correlate with the amount of protein, as is the case with the α and β subunits of the Na,K-ATPase [7]. We report here the characterization and application of two monoclonal antibodies that are specific for hPMCA4, and the further characterization of an antibody (5F10) that recognizes all isoforms of PMCA from many species.

Monoclonal antibodies JA9, 5F10 and JA3 were obtained in our laboratory by immunizing mice with purified human erythrocyte Ca^{2+} pump [8,9]. One of the early applications of these antibodies was in immunohistochemistry. In our initial study on kidney, JA3 worked well with human and chimpanzee kidney but not with other species, whereas JA9 did not yield good staining [9]. Antibody 5F10, in contrast, has cross-reacted with a wide variety of tissues and species including human, rat [10] and chicken [11]. These antibodies have been used in more than 24 reviewed publications, but the reasons for the differences in their specificity have not been understood. Such differences might be due to differences in the sequences of the epitopes between isoforms or between species, or to differences in the presentation of the epitope on the surface of the molecule.

The first step in defining the epitopes had been taken in a previous study, which located them approximately on the se-

quence of hPMCA4b [12] but did not establish the isoform specificity of the antibodies. That study had shown that the epitope for JA9 was in the region containing residues 17–75, for 5F10 between residues 724 and 783 and for JA3 between residues 1135 and 1205 (the sequence numbers are from isoform hPMCA4b).

The present study defines the epitopes of these antibodies more accurately and, more importantly, characterizes their isoform specificity. It also examines the distribution of hPMCA4 and its alternative splicing variants in human brain, heart, kidney and lung at the protein level by using JA3, JA9 and 5F10.

EXPERIMENTAL

Peptide synthesis

Peptides were synthesized in the Mayo Peptide Core Facility by using a 9-fluorenylmethoxycarbonyl strategy and an Advanced Chemtech multiple peptide synthesizer. Peptides were purified by reverse-phase HPLC on a Vydac 22 mm $\times$ 25 cm preparative column, with a trifluoroacetic acid/acetonitrile buffer system. Peptide integrity was monitored by amino acid analysis or mass spectrometry.

Tissue sources

Samples of human tissues were obtained from autopsies performed in the Department of Anatomy, Mayo Clinic, Rochester, MN. Samples (obtained about 6 h after death) came from a 62-year-old female, a 71-year-old male and a 20-year-old male. No significant differences were found between preparations from these three samples.

Abbreviations used: h, human; PMCA, plasma membrane Ca^{2+} pump.

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Expression of hPMCA isoforms

Expression of the hPMCA isoforms 1b, 4a and 4b in COS-1 cells and the isolation of the microsomal fraction of these cells were performed as described previously [13–15]. The hPMCA1b expression was done with the version of hPMCA1b that contained six point mutations [13]. None of these mutations was near the epitopes of the antibodies studied here.

Preparation of microsomal fractions

Microsomal fractions from the different tissues were prepared by following a single protocol. The tissues were collected and immediately placed and kept in liquid nitrogen until processing. The frozen tissue was broken into smaller pieces and washed in PBS containing 1 mM EDTA. After equilibration for 15 min in hypotonic solution of 10 mM Tris/HCl, pH 7.5, containing 1 mM $MgCl_2$, 0.1 mM PMSF, 4 $\mu g/ml$ aprotinin, 1 $\mu g/ml$ leupeptin, 2 mM dithiothreitol and 1 mM EDTA, the tissue was homogenized with a Potter–Elvehjem homogenizer. An equal volume of 10 mM Tris/HCl, pH 7.5, containing 0.3 M KCl, 0.5 M sucrose, 2 mM dithiothreitol and 1 mM EDTA was added to the homogenate and the mixture further homogenized. Unbroken cells, tissue debris and nuclei were removed by centrifugation at 4000 g for 15 min at 4 °C. The supernatant was finally centrifuged at 100000 g for 40 min at 4 °C and the pellet resuspended in 10 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 0.15 M KCl, 2 mM dithiothreitol. The fraction was made to 4 $\mu g/ml$ in aprotinin and 1 $\mu g/ml$ in leupeptin and kept in small aliquots in a freezer at –70 °C.

ELISA experiments

Two types of ELISA experiment were performed.

Competition experiments

Plates were coated overnight with 0.1 $\mu g/ml$ purified red cell Ca^{2+} pump dissolved in 0.5 M Na_2CO_3 , pH 9.6. The next day, the plates were washed three times with a washing solution containing 150 mM NaCl, 0.05 % Tween-20, 0.01 % chicken ovalbumin and 20 mM Tris/HCl, pH 7.4. Subsequently, antibodies and peptides were premixed and added to the wells. The final concentration of peptides is indicated in the legends to the figures. The antibody concentrations were: for JA9, ascites fluid diluted 1:15000; for 5F10, ascites fluid diluted 1/60000; for JA3, 0.4 $\mu g/ml$ purified antibody. The antibodies were diluted with 150 mM NaCl, 20 mM Tris/HCl, pH 7.4.

These concentrations were just above the concentration giving half the maximal ELISA signal for each antibody. They were chosen because they were high enough to give accurate readings and low enough to allow a sensitive competition assay. After 1 h of incubation at room temperature, the plates were washed three times with washing solution and the assay continued as described previously [16].

Direct binding

In the experiments in which direct binding of the antibodies to the peptides or the Ca^{2+} pump was measured, the plates were coated with 1 $\mu g/ml$ of either purified Ca^{2+} pump or peptides in 0.5 M Na_2CO_3 , pH 9.6. After an overnight incubation the assay was assessed as described previously [16].

Electrophoresis in slab gels and immunoblots were performed as described [12]. Production and characterization of the JA9, 5F10 and JA3 were described previously [9,16].

RESULTS

A previous study [12] located the epitopes of antibodies JA9, JA3 and 5F10 within regions of less than 100 amino acid residues each. To locate the epitopes in the primary structure of hPMCA4b more precisely, peptides were synthesized that represented different segments of the regions containing the epitopes. As shown in Figure 1, the peptides, 25 residues long, overlapped by at least five residues to minimize the chances of splitting the epitopes. Each peptide was tested for its ability to serve as an epitope for the antibody by competition experiments with the purified red cell Ca^{2+} pump. Figure 2 shows these competition experiments for all three antibodies. All the peptides indicated in Figure 1 were tested. Those not showing any significant reaction were not included in Figure 2 for the sake of clarity. In each case it was possible to localize the epitope in a region of not more than 25 residues. For antibody JA9, Figure 2A shows that peptide 3-JA9 was an effective competitor with an IC_{50} of 2 μM . Peptide 2-JA9 showed a very weak reaction and 1-JA9 did not compete at all (results not shown). These results indicate that the epitope for JA9 involves residues 51–75. With antibody 5F10, of the originally synthesized peptides, only 1-5F10 competed and it did so

Figure 1 Sequences of peptides representing hPMCA4b in the vicinity of the epitopes of JA9, 5F10 and JA3

The peptides were all 25 residues long and included both of the residues marked by the vertical lines at the ends of the arrows. The region containing the epitope for each antibody is indicated by the asterisks above each sequence.

Figure 2 Localization of the epitopes by competition experiments

Peptides competed with purified Ca^{2+} pump (bound to the multi-well plate) for the antibody. The peptides used for the competition are identified in the figure. Those not clearly identified are: (B) ▲, 4-5F10; ●, 0-5F10; (C) ▲, 3-JA3; ●, 1-JA3; ×, 4-JA3.

Figure 3 Direct binding of antibodies to peptides or ATPase

In **A** and **B**, antibody concentration is given in terms of the fraction of ascites fluid present in the medium. In **C**, a purified antibody was used and the concentration is given in $\mu\text{g/ml}$. For all panels, the circles and solid line represent purified ATPase. In **A** the triangles represent peptide 3-JA9. In **B** the crosses represent peptide 0-5F10 and the triangles peptide 4-5F10. In **C** the triangles and dashed line represent peptide 2-JA3.

with low affinity. This suggested that the originally defined region (residues 724–783) did not contain the complete epitope. Therefore peptides 0-5F10 and 4-5F10, containing some sequence upstream of 1-5F10, were synthesized. Both of them proved to be very effective competitors, with an IC_{50} of approx. 10 nM, as shown in Figure 2B. Because both of these peptides were equally effective, this indicated that the epitope was in the region common to both of them (residues 719–738). Addition of residues FLCLE to the N-terminus of peptide 1-5F10 (to produce peptide 0-5F10) increased markedly the affinity of the peptide–antibody binding, indicating that these residues were very important in determining the affinity of this monoclonal antibody for the antigen. In the study of the region containing the epitope for JA3 (Figure 2C) only peptide 2-JA3 competed and it did so with high affinity (IC_{50} 30 nM). This indicated that the epitope for JA3 was in the region 1156–1180, although the weak effects of peptides 3-JA3 and 4-JA3 at much higher concentrations (results not shown) suggested that residues beyond 1180 might also contribute to the binding of the antibody.

Direct binding experiments (Figure 3) showed that peptides 3-JA9, 0-5F10 and 4-5F10 bound antibodies with about one-fifth of the strength that the pump did, but its affinity for antibody JA3 was still very high (K_d 3.2 nM).

Figure 4 shows the sequences of all known rat and human PMCA in the regions containing the epitopes for the three antibodies. Examination of the sequences showed that the epitope for JA3 is unique to hPMCA4b. Isoforms 1, 2 and 3 do not contain any region that is similar to the epitope for JA3 and even rat PMCA4b has only nine identical residues within the epitope. The 'a' variants of the isoforms are not included in this figure, but they also contain no region that is similar to the epitope for JA3. On the contrary, within the 20 residues containing the epitope for 5F10 an extremely high degree of conservation exists in all of the known isoforms. Only three residues have any variation between isoforms. With JA9 some similarity exists between the known isoforms in the region of the epitope, but with numerous differences in amino acids between one isoform and the next.

JA9, NH2-terminal region	
rPMCA1	lrlkIqesYgD vvgiCtkLKT SPnEGLsgnp adLErReaVF GknFI
hPMCA1	lrlkIqesYgD vvgiCtkLKT SPnEGLsgnp adLErReaVF GknFI
rPMCA2	vvkIketYgD teslCrrLKT SPVEGLppta pDLEkRrqIF GqNFI
hPMCA2	vvkIketYgD teaiCrrLKT SPVEGLppta pDLEkRrqIF GqNFI
rPMCA3	lqkIqeaYgD vsqICrrLKT SPTEGLadnt nDLEkRrqIY GqNFI
hPMCA4	ltqInvhYgG vqnlCsrlLKT SPVEGLsgnp adLEkRrqVF GhNVI
Consensus	---I---YG---C---LKT SP-EGL---DLE-R---G-N-I
5F10 Central cytoplasmic domain	
rPMCA1	RAIAaKCGiI hPGEDFLCLE GKDFNRIrRN EKGEIEQeri DKIWp
hPMCA1	RAIAaKCGiI hPGEDFLCLE GKDFNRIrRN EKGEIEQeri DKIWp
rPMCA2	RAIAaKCGiI hPGEDFLCLE GKDFNRIrRN EKGEIEQeri DKIWp
hPMCA2	RAIAaKCGiI hPGEDFLCLE GKDFNRIrRN EKGEIEQeri DKIWp
rPMCA3	RAIAaKCGiI qPGEDFLCLE GKDFNRIrRN EKGEIEQeri DKWp
hPMCA4	RAIAaKCGiI tPGDDFLCLE GKDFNRIrRN EKGEIEQeri DKIWp
Consensus	RAIAaKCGiI -PGEDFLCLE GKDFNRIrRN EKGEIEQeri DKIWp
JA3, COOH-terminal region, Alternate splice b	
rPMCA1b	iedDsephipL idDtdaesda ptkrnsspp.psp NkNnNAVDsg
hPMCA1b	iedDsephipL idDtdaesda ptkrnsspp.psp NkNnNAVDsg
rPMCA2b	iedDsephipL idDtdleeda alkqnspp.ssl NkNnNAVDsg
hPMCA2b	iedDsephipL idDtdleeda alkqnspp.ssl NkNnNAVDsg
rPMCA3b	indYthnlpL idDtdvdene erlrapppp.pp NqNnNAVDsg
rPMCA4b	adDemsqsfl nqEesss... lasksrtrtr lsdactvsqn NtNnNAVDch
hPMCA4b	ieDelprtpL ldEeeenpd kaskfgtrvl lldgevtppya NtNnNAVDcn
Consensus	--D-----L --D-----N-NN-AVD--

Figure 4 Peptides used to determine isoform specificity of antibodies JA9, 5F10 and JA3

Note that the peptides, designated above by names such as '3-JA9', here and in Figure 5 have 'H4' added to show that they represent human isoform 4.

Figure 5 Determination of isoform specificity by peptides

Competition experiments were done as for Figure 2. Peptides used were: (A) ●, 3-JA9-H4; ▲, JA9-R1; ×, JA9-R2; ★, JA9-R3; (B) ●, 4-5F10-H4; ★, 5F10-R1; ×, 5F10-R2; ▲, 5F10-R3; (C) ●, 2-JA3-H4; ★, JA3-R1; ▲, JA3-R2; ×, JA3-R3; ◆, JA3-R4.

To examine the specificity of these antibodies we tested them against a set of peptides (whose sequences are shown in Figure 4) representing the different isoforms, and also against PMCA of

Figure 6 Determination of isoform specificity with intact pump expressed in COS-1 cells

Western blot of hPMCA4a (molecular mass 129304 Da), hPMCA4b (133929 Da) and hPMCA1b (134708 Da) developed with the three antibodies. The lanes marked pMM2 represent controls using COS-1 cells transfected with empty plasmid. Abbreviation: kD, kDa.

Figure 7 Constructs of hPMCA stained with antibodies JA3, JA9 and 5F10

Membranes containing the constructs indicated at the top of each lane (1 μ g of protein per lane) were stained with the antibodies indicated on the bottom of each lane. The position of constructs ct24 and ct71 are indicated by arrows.

three different types expressed in COS-1 cells. Although isoforms 1, 2 and 3 are different in the JA3 region, peptides representing approximately corresponding portions of the molecule were also tested. Figures 5A and 5C show the specificity with which antibodies JA9 and JA3 interact with the corresponding peptides. In both cases the only peptide that reacts with the antibody is that derived from hPMCA4. With JA3 it was also possible to rule out interaction of the antibody with rat PMCA4b. In contrast, peptides representing all four isoforms compete with equal affinity for 5F10 (Figure 5B).

Figure 6 shows the specificity of these antibodies for hPMCA1b, hPMCA4a and hPMCA4b expressed in COS-1 cells. The results obtained with the native enzymes were entirely consistent with those expected from the analysis of the sequences and the experiments with the peptides. Antibody 5F10 recognized bands of the correct size in Western blots from the membranes of cells expressing each of the three isoforms. Because of the high conservation of the 5F10 epitope, this antibody also detected the smaller amount of endogenous PMCA expressed in COS cells.

Figure 8 Western blot of crude microsomal preparations from (A) human brain tissue and (B) human heart tissue, immunostained with different monoclonal antibodies

For molecular mass standards (MW std), overexpressed hPMCA4a, hPMCA4b and truncated constructs ct24 and ct71 (see the Experimental section) were used.

These cells, which are derived from monkey kidney, showed a band migrating in the position of hPMCA1b when transfected with the empty plasmid (pMM2). Antibody JA9 recognized hPMCA4a and hPMCA4b but did not recognize hPMCA1b or the endogenous PMCA; JA3 recognized only hPMCA4b.

The specificity of these antibodies allowed us to analyse the distribution of hPMCA4 and its alternative splicing variants (a

Figure 9 Western blot of crude microsomal preparations from (A) human kidney tissue and (B) human lung tissue, immunostained with different monoclonal antibodies

Abbreviation: MW std, molecular mass standard.

and b) in Western blots of human tissues. Instead of using standard proteins as molecular mass markers to identify the bands, we used Ca^{2+} pump isoforms hPMCA4b (133929 Da) and hPMCA4a (129304 Da), and two constructs, ct24 (131829 Da) and ct71 (126119 Da), which were expressed in COS-1 cells. Constructs ct24 and ct71 are derivatives of hPMCA4b that lack the 24 and 71 C-terminal residues respectively. hPMCA4b and ct24, but not ct71, contain the epitope for JA3. The use of these proteins as standards and their characteristic staining with the antibodies provided us with an analytical tool in the immunoanalysis of the Western blots of the different tissues.

Figure 7 shows a Western blot of the expressed proteins, with ct24 and hPMCA4a migrating to almost the same position. As expected, hPMCA4a, ct24 and ct71 were all recognized by 5F10 and JA9, whereas JA3 recognized ct24 only (as shown before, hPMCA4b is recognized by all three antibodies). The blot also showed clearly that hPMCA4a is not recognized by JA3.

In Figure 8, Western blots of human brain (Figure 8A) and human heart (Figure 8B) microsomes were stained with antibodies 5F10, JA9 and JA3; saturating antibody concentrations were used for all the immunostaining. Negative controls were stained in the absence of primary antibody. Blots of human brain stained with 5F10 (Figure 8A) showed a broad band that is practically a continuum covering a range of molecular sizes. JA9 stained primarily a strong band that migrated in the position of ct24 and hPMCA4a. Some very faint bands can also be observed that can be ascribed to products resulting from proteolysis of hPMCA4. The JA3 blot, which we have highly overdeveloped (see molecular mass standards), showed two faint bands, one band migrating at the position of hPMCA4b and a slightly stronger band migrating at the position of ct24/hPMCA4a. The

weak staining with JA3 nevertheless indicates the presence of hPMCA4b and a proteolytic fragment of hPMCA4b that still contains the epitope for JA3. The presence of hPMCA4a, however, is also indicated. Because the antibodies were used at saturating conditions, the strongly stained band recognized by JA9 presents evidence that only a minor portion of this band is attributable to proteolysed hPMCA4b and that a large component of this band is hPMCA4a. Finally, the presence of bands of higher and lower molecular mass stained by 5F10 and not stained by JA3 and JA9 indicates the presence of other isoforms of the pump besides hPMCA4.

Western blots of human heart microsomes (Figure 8B) showed two broad bands with 5F10, the upper band migrating with ct24 and hPMCA4a, and the lower band in the same position as ct71. In addition, 5F10 stained a fuzzy band of smaller molecular size than ct71. Staining with JA9 showed essentially the same pattern as with 5F10, suggesting that the main isoform present in human heart is hPMCA4 and proteolytic fragments derived from it. JA3 also recognized a band at the ct24/hPMCA4a position but the band was always thinner than that observed with JA9. This difference is not as clearly evident in a reproduction such as Figure 8. Direct examination of three different Western blots stained with JA9 clearly showed a fainter and less sharp flanking band on the top side of the main band, which was absent from the blots stained with JA3. Taking into account that JA9 recognizes hPMCA4a as well as hPMCA4b, and JA3 recognizes hPMCA4b only, a plausible interpretation of these results is that human heart contain both a and b variants of hPMCA4.

Figure 9 shows the Western blot of human kidney and human lung microsomes. In Western blots of kidney microsomes (Figure 9A), 5F10 stained four bands. One was a strong, non-specific, fast migrating band that was stained by all three antibodies and was also visible in the negative control. Two bands migrated with hPMCA4b and ct24 respectively. These two bands were also recognized by JA9 and JA3, indicating the presence of hPMCA4b and its degradation product in kidney tissue. Unlike in brain, the two bands in kidney were stained equally by JA9 and JA3, clearly showing the presence of hPMCA4 (alternative splicing variant b). The slowest migrating faint band was recognized only by 5F10, indicating the presence of an isoenzyme other than hPMCA4. This band migrated similarly to the endogenous pump present in COS-1 cells, which, as mentioned before, migrates with hPMCA1b. It is highly probable that this band is hPMCA1b because the message for this isoform has been found by PCR to be present in almost all tissues including kidney [6]. We conclude from these results that hPMCA4b and possibly hPMCA1b are present in kidney.

A Western blot of human lung microsomes (Figure 9B) stained with 5F10 showed three bands. The upper two bands migrated with hPMCA4b and ct24 respectively, whereas the third band migrated faster than ct24. All three bands were also stained by JA9. JA3 stained only the upper two bands, with an intensity equal to that of JA9. Because the bands were stained by JA3 as well as JA9, they must represent hPMCA4b. The third band, which has an average calculated molecular mass of 124515 Da, therefore does not contain the epitope for JA3. It could conceivably come also from the variant a. However, because PCR data showed no evidence of the message for hPMCA4a in lung tissue [4], it most probably came from hPMCA4b.

DISCUSSION

Location and specificity of the antibodies

In this paper we have discussed three kinds of evidence for the specificity of JA9, JA3 and 5F10. These are: (1) the comparison

of the sequences similar to the defined epitope; (2) comparison of the reactivity of peptides representing the different isoforms, and (3) reaction with three expressed intact Ca^{2+} pumps in COS cells.

Despite the fact that we do not know all the sequences of all of the isoforms of the pump, it is possible to reach conclusions about the specificity of the antibodies. The evidence clearly shows that 5F10 reacts equally well with all of the known isoforms of PMCA. The high degree of sequence conservation of the epitope with which 5F10 reacts reinforces the notion that this reactivity also extends to the unknown isoenzymes. Our previous experience with this antibody shows that this general reactivity of 5F10 extends beyond the human and rat pumps analysed here, because 5F10 also reacts appropriately with cat [8], hamster [17], chicken [11], and even with trypanosomatids [18]. Despite its wide range of applicability, 5F10 seems to be highly specific for the PMCA. This is borne out not only by our experience with this antibody but also by a sequence analysis. A search of the Swiss protein database (release 31.0, May 1995) showed that the non-PMCA sequence most closely related to the epitope defined here occurs in a DNA repair protein from yeast (accession number P25301), which has only 10 identical residues scattered over the 20 residue sequence, so that even this protein would not be likely to react with the antibody. The only non-specific reaction that we have detected is that the prestained carbonic anhydrase in molecular mass standards is moderately stained in Western blots performed with 5F10. This may be due to an alteration in its antigenicity caused by the prestaining, because we have never seen any evidence that carbonic anhydrase is stained in Western blots of cell extracts or under any of the other circumstances in which this antibody has been employed.

Antibody JA3 is certainly specific for hPMCA4b and reacts weakly or not at all with other species or isoforms. The other isoforms and variants of the pump do not contain any sequence similar to the epitope of hPMCA4b. Rat PMCA4b does contain a partly similar region, but with so many important differences from hPMCA4b that the peptide derived from rat PMCA4b did not cross-react. In the experiments with the expressed protein, the specificity of this antibody for isoform 4b as opposed to 4a was demonstrated, as was the fact that it did not react with hPMCA1b. This specificity of JA3 is consistent with our early studies [9] in which this antibody reacted with human and chimpanzee kidney but not with rat, monkey or dog kidney in immunohistochemical staining. In another study, JA3 reacted with the pump in a Western blot of rat myometrium, but the reaction was very weak compared with that of 5F10 in the same tissue (results not shown).

Antibody JA9 also seems to be isoform specific, in this case for hPMCA4. As was shown in Figure 6, it reacts with both isoforms 4a and 4b, which was expected because these two products of an alternative splice are identical in the N-terminal portion of the pump where this antibody reacts. Its failure to react with hPMCA1b expressed in COS-1 cells and with peptides from rat isoforms 1, 2 or 3 (whose sequences are nearly identical with the corresponding regions of the human isoforms) suggests that it is specific for isoform 4, a result consistent with the sequence analysis, which shows considerable substitution of amino acids in this region.

The epitopes defined by these peptide studies seem to account for almost all of the capacity of these antibodies to bind to the Ca^{2+} pump. Only JA3 showed a tighter binding to intact pump than it did to the peptide containing the epitope, but still the affinity of the antibody for the peptide was in the nanomolar range. With the other two antibodies, JA9 and 5F10 (despite a weak reaction of antibody JA9 with peptide 2-JA9), the curves

for the peptide containing the epitope and for pure ATPase were superimposable (Figure 3) so that one need not look further for additional determinants of these antibodies.

Distribution of hPMCA4a and hPMCA4b in human tissues

The precise information gathered about the epitope location and specificity of these antibodies allowed us to use them to discern the distribution of hPMCA4 and its alternative splicing variants at site C, in human brain, heart, lung and kidney. The use of the isoforms of the pumps expressed in COS-1 cells as standards was also essential for the proper identification of the bands present in the Western blots. While this paper was being completed, a study with polyclonal antibodies against the four different gene products of hPMCA appeared [6]. This study characterized the distribution of the four isoforms in various human and rat tissues. Because the polyclonal antibodies were raised against an N-terminal portion of the pump, that study gave no information about the alternative splice at site C. The information reported here about the distribution of the alternative splices at site C is particularly important because it has been demonstrated that the affinity for calmodulin, and hence for Ca^{2+} , of hPMCA4 is markedly different according to which of the variants is expressed [15]. This fact is likely to be relevant for the Ca^{2+} handling by any given cell type.

Western blots of human brain showed that both hPMCA4a and b are present in this tissue. In addition, staining with 5F10 showed extra bands that have to be attributed to isoenzymes of the pump other than hPMCA4 because they are not revealed with JA3 or JA9. In contrast, lung showed hPMCA4b only. The heart was an intermediate case, because evidence for both hPMCA4a and 4b was found in this tissue. In kidney, the presence of hPMCA4b was found. There was evidence that hPMCA1b might be present as well. As a whole, these results agree with the reverse transcriptase-PCR results obtained from human and rat kidney [1–4] and with the results of the study with polyclonal antibodies [6].

The presence of degradation products has to be taken into account for the interpretation of the results. Specifically, a band near 132 kDa was consistently seen in all tissues. On the basis of two pieces of evidence we identified this band as a degradation product of hPMCA4b: (1) it was stained with JA3; (2) the construct ct24, which lacks the 24 C-terminal residues of hPMCA4b, but still contains the JA3 epitope, migrated in the same region and was stained by the antibodies in the same way as the proteolytic product observed in the tissues. Although the occurrence of proteolysis of the pump *in vivo* cannot be ruled out, the presence of this product is most probably related to the fact that 6 h elapsed between death and the preservation of the tissues used in this study.

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