Plasma membrane Ca\(^{2+}\) ATPases are P-type pumps important for intracellular Ca\(^{2+}\) homeostasis. The extreme C termini of alternatively spliced “b”-type Ca\(^{2+}\) pump isoforms resemble those of K\(^{+}\) channels and N-methyl-D-aspartate receptor subunits that interact with channel-clustering proteins of the membrane-associated guanylate kinase (MAGUK) family via PDZ domains. Yeast two-hybrid assays demonstrated strong interaction of Ca\(^{2+}\) pump 4b with the PDZ1+2 domains of several mammalian MAGUKs. Pump 4b and PSD-95 could be co-immunoprecipitated from COS-7 cells overexpressing these proteins. Surface plasmon resonance revealed that a C-terminal pump 4b peptide interacted with the PDZ1+2 domains of hDlg with nanomolar affinity (KD = 1.6 nM), whereas binding to PDZ3 was in the micromolar range (KD = 1.2 μM). In contrast, the corresponding C-terminal peptide of Ca\(^{2+}\) pump 2b interacted weakly with PDZ1+2 and not at all with PDZ3 of hDlg. Ca\(^{2+}\) pump 4b bound strongly to PDZ1+2+3 of hDlg on filter assays, whereas isoform 2b bound weakly, and the splice variants 2a and 4a failed to bind. Together, these data demonstrate a direct physical binding of Ca\(^{2+}\) pump isoform 4b to MAGUKs via their PDZ domains and reveal a novel role of alternative splicing within the family of plasma membrane Ca\(^{2+}\) pumps. Alternative splicing may dictate their specific interaction with PDZ domain-containing proteins, potentially influencing their localization and incorporation into functional multiprotein complexes at the plasma membrane.

Temporal and spatial control of intracellular Ca\(^{2+}\) concentrations is essential for eukaryotic cell physiology. Plasma membrane Ca\(^{2+}\) ATPases (PMCA) represent a ubiquitous, high affinity system for the expulsion of Ca\(^{2+}\) from the cell and are thought to be responsible for the long-term setting and maintenance of intracellular Ca\(^{2+}\) levels. Mammalian PMCA isoforms are encoded by a multigene family consisting of four members termed PMCA1–4. Additional isoform diversity is generated via alternative RNA splicing (3). Alternative splicing of the C-terminal tail has been shown to alter the regulatory properties of PMCA isoforms, particularly with respect to phosphorylation and calmodulin stimulation (5–10). Many PMCA isoforms and splice variants are expressed in a tissue- and cell type-specific manner (11–17), and in several cell types, the PMCA has been shown to be concentrated in specific membrane domains by immunocytochemical analyses. For example, in kidney and intestinal epithelia involved in transcellular Ca\(^{2+}\) flux, the pump is generally localized to the basolateral membrane (18). Using immunoelectron microscopy, the PMCA was recently detected at the plasma membrane surrounding the soma, as well as at the dendrites and spines of cerebellar Purkinje cells where it co-localized with P-type Ca\(^{2+}\) channels (19). Taken together, these studies indicate that different isoforms of the PMCA may play an active role in the local control of Ca\(^{2+}\) signaling and the dynamic regulation of Ca\(^{2+}\) microdomains (7, 20). However, the mechanisms by which PMCA isoforms are localized to specific regions of the plasma membrane are unknown.

Recent studies have shown that several membrane receptors and channels are clustered into multiprotein complexes linked to the cytoskeleton via interactions of their C-terminal cytosolic tails with a novel protein interaction module called the PDZ domain. PDZ domains are found in a large variety of multifunctional proteins (21, 24) and act as mediators of protein-protein interactions. Several of these PDZ domain-containing proteins, including PSD-95, Chapsyn-110, and hDlg (29–31), are members of the membrane-associated guanylate kinase (MAGUK) protein family (32). A short, consensus C-terminal sequence motif (E/T/SXV*, where * represents the stop codon) has been shown to be critical for the interaction between membrane proteins (such as Shaker-type K\(^{+}\) channels and NMDA receptor NR2 subunits) and the PDZ domains present in the PSD-95 family of MAGUKs (24, 29, 33–35).

kDa; RU, resonance units; PAGE, polyacrylamide gel electrophoresis; ZO-1, zona occludens protein-1; β-Gal, β-galactosidase.
In the PMCA isoforms, the C-terminal sequence of all "b" type alternative splice variants contains the consensus -ETSL* or -ETSV* which is similar to the -E(T/S)XV* motif found in Shaker K⁺ channel and NR2 NMDA receptor proteins. Human PMCA1b, -2b, and -3b end with the sequence -ETSL*, whereas hPMCA4b ends with the sequence -ETSV* (3). Based on this observation, we reasoned that the intracellular C-terminal tail of hPMCA4b may interact with MAGUKs via their PDZ domains. To test this hypothesis, we analyzed the interaction of hPMCA4b with the PDZ domains of several MAGUKs using a variety of binding assays. The results documented in this paper provide the first evidence of direct binding of the PMCA4b isoform to MAGUKs and suggest a novel mechanism by which alternative splicing generates Ca²⁺ pump isoforms that may be differentially recruited to multifunctional protein complexes involved in Ca²⁺ regulation.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—A cDNA fragment encoding the C-terminal 71 residues (amino acids 1135 to 1205) of hPMCA4b was cloned into pbHA (LexA fusion vector) and used in yeast two-hybrid assays with various PDZ domains of the PMCA isoforms. The pGAD10 constructs encoding each individual PDZ domain, or PDZ1 + 2 of PSD-95 fused to the GAL4 activation domain have been described (29), as has the construct encoding all three PDZ domains of hDlg (clone 4 (29)). PDGAD plasmids carrying the corresponding domains of rat Chapsyn-110 (30) were similarly constructed by PCR amplification of the desired cDNA fragments followed by subcloning into pGAD10 (PDZ1, aa 77–193; PDZ2, aa 187–300; PDZ3, aa 418–503; PDZ1 + 2, aa 77–300). pGEX-2T constructs expressing the PDZ1 + 2 domains (aa 216–413) and the PDZ3 domain (aa 457–552) of hDlg as glutathione S-transferase (GST) fusion proteins and the pRSETA vector encoding PDZ1 + 2 + 3 of hDlg with an N-terminal His-tag have been described (36, 37). Constructs expressing the C-terminal sequences of hPMCA2b (aa 1126–1168), -2b (aa 1141–1212), -4b (aa 1115–1170), and -4b (aa 1135–1205) as GST fusion proteins with a protein kinase A phosphorylation site were made by PCR amplification of the appropriate cDNA fragments and subcloning them in-frame into pGEX-2TK (Pharmacia). The mammalian expression vectors for full-length hPMCA4b and PSD-95 have been described (29, 38).

Synthetic Peptides—Peptides CT2b and CT4b corresponding to the C-terminal 21 and 30 residues of hPMCA2b (SPHSSLTEVS and hPMCA4b (SSLQSLETSV), respectively, were chemically synthesized in the Mayo Clinic Core Facility and were N-terminally coupled to biotin via a 3,000 CI/mmol, Amersham) (42). The labeled fusion proteins were eluted from the washed beads in 50 mM reduced glutathione (Sigma) and were used to probe the nitrocellulose membrane using standard Western blotting techniques (41).

Surface Plasmon Resonance Measurements—The interaction between the PDZ domains of hDlg and the C-terminal peptides of hPMCA2b and -4b was studied by surface plasmon resonance measurements using a BIAcore instrument (Biacore, Inc.) as described (36). 204 RU of CT2b and CT4b were captured on the streptavidin surface of a SA sensor chip (research grade). The purified recombinant proteins GST, GST-PDZ2 + 1, and GST-PDZ3 were exposed to the immobilized peptides at a concentration of 410 μg/ml. To quantify the affinity of the interaction of CT4b with PDZ1 + 2 of hDlg, 82 RU of peptide CT4b were immobilized on the SA sensor chip surface and increasing concentrations of GST-PDZ1 + 2 (ranging from 12.4 nM to 978 nM) were passed over the immobilized peptide surface. To quantify the interaction of CT4b with PDZ1 + 2 and PDZ3 domains of hDlg, 394 RU of CT4b were captured on the surface of a SA sensor chip, and the immobilized peptide was exposed to increasing concentrations of GST-PDZ3 (0.83 μM to 65.2 μM).

RESULTS AND DISCUSSION

The b splice forms of the human PMCA isoforms terminate in the conserved sequence -S/LV/ETS(L/V)* that matches the minimal -T/SXV* consensus motif for PDZ domain interaction (29, 33). The sequence -ETS(L/V)* of hPMCA4b matches the consensus precisely whereas that of all other PMCA b splice forms (-ETSL*) deviates conservatively from the consensus at the last residue. To test if hPMCA4b can interact with PDZ domains of known channel/receptor clustering molecules, we performed yeast two-hybrid assays using the C-terminal 71 residues of hPMCA4b fused to the LexA DNA binding domain as "bait," and various PDZ domains fused to the GAL4 activating domain as potential partners. The interaction was assayed semiquantitatively based on the degree of HIS3 and β-Gal reporter gene induction (29). Fig. 1 shows that no reporter gene activity was detected when the control plasmid pGAD10 was transformed into yeast expressing the LexA-PMCA4b fusion protein from the pbHA-PMCA4b bait construct. By contrast, both reporter genes were activated when the transformation was performed with the PDZ domain-expressing constructs. A very strong induction was observed when the combined PDZ1 + 2 domains of either PSD-95 or Chapsyn-110 or all three PDZ domains of hDlg were expressed together with the PMCA4b fusion protein (Fig. 1). As has been shown for the Kv1.4 potassium channel (29) and the NR2 subunits of NMDA receptors (33, 43), interaction of the PMCA4b C-terminal sequence was strongest with the individual PDZ domain 2 of the MAGUKs tested. However, an interaction with the PDZ1 and PDZ3 domains was also observed (Fig. 1). It is noteworthy that a weak but significant interaction was observed of the hPMCA4b with the PDZ3 domain of PSD-95 and Chapsyn-110. This is in contrast to the Kv1.4 potassium channel that showed no affinity for PDZ3 even in the sensitive two-hybrid assay (29). The crystal structures of the PDZ2 domain of hDlg alone and of PSD-95 complexed with a peptide ligand ending in the sequence -QTSV* were recently reported (37), rationalizing the importance of the C-terminal Val and of the Thr residue at position –2. Because the extreme C termini of hPMCA4b (which can bind to PDZ2) and of Kv1.4 (which does not bind to PDZ2) differ only at position –1 (Ser in the PMCA, Asp in the Kv1.4), it is likely that the residue at this position (and/or additional more N-terminally located residues) also play a role in determining the binding affinity of a C-terminal peptide to the PDZ3 domain.

Gel Overlays—Aliquots of purified His-tagged hDlg-PDZ1 + 2 + 3 protein (1 μg each) were run on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane using standard Western blotting techniques (41). GST-PMCA fusion proteins and GST alone (control) were expressed in E. coli JM101 from the appropriate pGEX-2TK vector and were phosphorylated while bound to glutathione–Sepharose beads using bovine heart muscle protein kinase A (Sigma) and γ-[32P]ATP (3,000 Ci/mmol, Amersham) (42). The labeled fusion proteins were eluted from the washed beads in 50 mM Tris-HCl, pH 8.0, containing 10 μM reduced glutathione (Sigma) and were used to probe the nitrocellulose membranes essentially as described (42).
Interaction of hPMCA4b C-terminal domain with PDZ domains as determined by semiquantitative yeast two-hybrid assays. Top panel, schemes of the bait construct and the interacting MAGUKs. The bait consists of the LexA DNA binding domain (LexA DB) fused to residues 1135–1205 of hPMCA4b. The MAGUKs contain three PDZ domains, a Src Homology 3 (SH3) domain and a guanylate kinase-like domain (GUK). Bottom panel, the interaction of various test proteins (consisting of the Gal4 activation domain (Gal4 AD) fused to PDZ domains of various MAGUKs) with the PMCA bait was assayed semiquantitatively based on the degree of induction of the two reporter genes HIS3 and β-Gal. HIS3 activity was determined by the percentage of yeast colonies growing on histidine-lacking medium: ++, >80%; +++, 30–60%; --, 10–30%; --, no significant growth. β-Gal activity was estimated from the time taken for colonies to turn blue in 5-bromo-4-chloro-3-indolyl β-D-galactoside filter lift assays at room temperature: ++, <45 min; +++, 45–90 min; +, 90–240 min; --, no significant activity.

To determine if hPMCA4b can interact with a PDZ domain protein when both are expressed as full-length proteins in a mammalian cell, COS-7 cells were transfected with expression vectors for hPMCA4b and PSD-95. Co-immunoprecipitation experiments were performed using either a polyclonal antibody against PSD-95 or a monoclonal antibody against PMCA4b. Both proteins were abundantly expressed upon transient transfection of COS-7 cells with the appropriate vectors (Fig. 2, lanes 1–3). PSD-95 and PMCA4b were effectively co-immunoprecipitated in reciprocal immunoprecipitation experiments using cells that had been co-transfected with the two expression plasmids (Fig. 2, lanes 4–7). Neither of the two antibodies showed nonspecific cross-reactivity with the partner protein as demonstrated by the inability of anti-PSD-95 to precipitate the PMCA from cells transfected with PMCA alone (Fig. 2, lane 5) and the inability of anti-PMCA4b to precipitate significant amounts of PSD-95 from cells expressing only PSD-95 (Fig. 2, lane 7, compare with lane 2). The small amount of PSD-95 immunoprecipitated by the anti-PMCA4b antibody from cells expressing PSD-95 (Fig. 2, lane 7) could be due to the presence of endogenously expressed PMCA4b in the COS-7 cells. However, under our immunoblotting conditions the endogenous PMCA4b remained below the detection limit (see Fig. 2, lanes 2 and 7). These data, therefore, show that full-length PMCA4b can interact with full-length PSD-95 in a cellular environment.

Based on the yeast two-hybrid analysis, this interaction is likely mediated by binding of the C-terminal sequence of the pump to a PDZ domain (probably PDZ2) of its partner PDZ domain-containing protein (PSD-95/Chapsyn-110/hDlg).

To quantify the interaction of the PMCA4b C terminus with the PDZ domains and to determine comparative values for a possible interaction of other PMCA b splice forms with the same PDZ domains, we performed surface plasmon resonance measurements using synthetic PMCA peptides and purified GST fusion proteins of the various PDZ domains of hDlg. When recombinant GST, GST-PDZ1+2, and GST-PDZ3 were exposed to the immobilized peptide CT4b (corresponding to the C-terminal 10 residues of hPMCA4b), both GST-PDZ1+2 and GST-PDZ3 fusion proteins bound to the peptide (Fig. 3A) whereas no binding was observed with GST alone. Interestingly, when the same experiment was performed with immobilized peptide CT2b (corresponding to the C-terminal 10 residues of hPMCA2b), binding was only evident to GST-PDZ1+2. No binding was observed with either GST-PDZ3 or GST alone (Fig. 3B). The affinity of the interaction of CT4b with the combined PDZ1+2 domains of hDlg was quantified by passing increasing concentrations of GST-PDZ1+2 fusion protein over the CT4b peptide surface (Fig. 3C). Analysis of the association and dissociation rates of the sensorgrams yielded an overall dissociation constant, $K_d$, of $1.64 \text{ nM}$ ($k_a = 1.16 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ and $k_d = 1.9 \times 10^{-3} \text{ s}^{-1}$), indicative of a high affinity interaction between CT4b and the combined PDZ1+2 domains of hDlg. The interaction between CT4b and PDZ3 of hDlg was similarly analyzed. A $K_d$ of $1.22 \mu \text{M}$ was calculated from the association ($k_a = 3.59 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) and dissociation ($k_d = 4.38 \times 10^{-3} \text{ s}^{-1}$) rate constants (Fig. 3D), indicating a relatively low affinity interaction between CT4b and the PDZ3 domain of hDlg. These results fully agree with the two-hybrid interaction data. The binding affinities measured for PDZ domains of hDlg should be broadly extrapolatable to PSD-95 and Chapsyn-110, since the equivalent PDZ domains of these closely related proteins are highly conserved and no differences in binding specificity between the different proteins have been detected to date (29, 43). Quantification of the interaction between peptide CT2b and PDZ1+2 of hDlg was not attempted due to the relatively low affinity of the interaction (compare Fig. 3, A and B) as well
as technical difficulties in capturing sufficient amounts of the CT2b peptide on the biosensor chip surface.

Independent confirmation of the specificity of the interaction of hPMCA4b with the PDZ domains of hDlg was obtained using a filter assay. Immobilized recombinant His-tagged hDlg-PDZ1+2+3 fusion protein was probed with radiolabeled GST alone or with various GST-PMCA C-terminal fusion proteins. As shown in Fig. 4, GST-hPMCA4b bound strongly to the hDlg-PDZ1+2+3 fusion protein, whereas GST-hPMCA2b bound weakly and GST-hPMCA2a, GST-hPMCA4a and GST alone did not bind at all. These findings agree with the BIAcore measurements showing a much reduced affinity of hPMCA2b for the PDZ1+2 domains of hDlg. Importantly, the results also show that none of the alternatively spliced "a" forms of the PMCA are able to bind the PDZ domains and thus suggest a novel mechanism whereby alternatively spliced forms of the Ca\(^{2+}\) ATPase pumps are distinguished by their interaction with PDZ domain-containing target proteins.

The nanomolar affinity of hPMCA4b for the PDZ1+2 domains of hDlg suggests that hPMCA4b and hDlg may be binding partners in vivo where they are co-expressed in kidney and intestinal epithelial cells (17, 18, 29, 44–46) as well as in several regions of the brain (15, 29, 30, 47). An in vivo interaction between PMCA4b and other members of the PSD-95 family of MAGUKs may be equally plausible: PSD-95 and Chapsyn-110 have been found pre- and postsynaptically in several areas of the brain that also express PMCA4b (15, 30, 47). The large difference in binding affinity of the C termini of hPMCA4b and hPMCA2b to the PDZ1+2 domains of the PSD-95/Chapsyn-110/hDlg members of the MAGUK family underscores the importance of the carboxyl-terminal residue (Val in hPMCA4b, Leu in the other PMCA b-splice forms) in determining the specificity of this interaction. This observation is consistent with the recently demonstrated target specificity of PDZ domains using an oriented peptide library approach (35). The selectivity and specificity of PDZ domain-target sequence interactions (27, 35, 48) also suggest that other PMCA isoforms of the b splice type, such as PMCA2b, may recognize novel PDZ domain-containing proteins.
An interaction of Ca\textsuperscript{2+} pump isoforms with specific PDZ domain proteins may be relevant for the local organization of Ca\textsuperscript{2+} signaling domains at the plasma membrane and/or for anchoring Ca\textsuperscript{2+} regulatory complexes to the cytoskeleton. It is probably not coincidental that the Ca\textsuperscript{2+} pump 4b can interact with the same clustering proteins (PSD-95, Chapsyn-110) as NMDA receptors. The ability of PSD-95 MAGUKs to homo- and heteromultimerize allows them to colocalize several different ligands (40) thus allowing for co-aggregation of Ca\textsuperscript{2+} pump and NMDA receptor proteins in the same membrane microdomain. The local concentration of Ca\textsuperscript{2+} pump (e.g. in dendritic spines) would facilitate the extrusion of calcium ions admitted into the dendritic spine through activated NMDA receptors, thereby restricting the duration of the Ca\textsuperscript{2+} signal. The functional significance of these interactions is currently under investigation.

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