The Sarcolemmal Calcium Pump Inhibits the Calcineurin/Nuclear Factor of Activated T-cell Pathway via Interaction with the Calcineurin A Catalytic Subunit*

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Received for publication, February 4, 2005, and in revised form, June 9, 2005
Published, JBC Papers in Press, June 14, 2005, DOI 10.1074/jbc.M501326200

The calcineurin/nuclear factor of activated T-cell (NFAT) pathway represents a crucial transducer of cellular function. There is increasing evidence placing the sarcolemmal calcium pump, or plasma membrane calcium/calmodulin ATPase pump (PMCA), as a potential modulator of signal transduction pathways. We demonstrate a novel interaction between PMCA and the calcium/calmodulin-dependent phosphatase, calcineurin, in mammalian cells. The interaction domains were located to the catalytic domain of PMCA4b and the catalytic domain of the calcineurin A subunit. Endogenous calcineurin activity, assessed by measuring the transcriptional activity of its best characterized substrate, NFAT, was significantly inhibited by 60% in the presence of ectopic PMCA4b. This inhibition was notably reversed by the co-expression of the PMCA4b interaction domain, demonstrating the functional significance of this interaction. PMCA4b was, however, unable to confer its inhibitory effect in the presence of a calcium/calmodulin-independent constitutively active mutant calcineurin A suggesting a calcium/calmodulin-dependent mechanism. The modulatory function of PMCA4b is further supported by the observation that endogenous calcineurin moves from the cytoplasm to the plasma membrane when PMCA4b is overexpressed. We suggest recruitment by PMCA4b of calcineurin to a low calcium environment as a possible explanation for these findings. In summary, our results offer strong evidence for a novel functional interaction between PMCA and calcineurin, suggesting a role for PMCA as a negative modulator of calcineurin-mediated signaling pathways in mammalian cells. This study reinforces the emerging role of PMCA as a molecular organizer and regulator of signaling transduction pathways.

The essential role of Ca2+ in signal transduction pathways is well established (1). Critical to the coupling of Ca2+ signals to cellular responses is the serine-threonine protein phosphatase, calcineurin (Cn; also called protein phosphatase 2B), which is regulated by Ca2+/calmodulin (2, 3). Its structure is highly conserved from yeast to humans, and although abundant in neural tissue, it is found widely distributed (4).

The nuclear factor of activated T-cell (NFAT) transcription factors are the best characterized substrate of calcineurin (5). The key effect of calcineurin activation is the dephosphorylation of this family of transcription factors in response to a sustained increase in intracellular Ca2+, leading to nuclear translocation of NFATs and resultant activation of gene transcription (6). The strong body of evidence implicating NFATs in the development and adaptation of a varied range of cell types underlines the importance of calcineurin function (7). The calcineurin holoenzyme is a heterodimer consisting of a 58- to 64-kDa catalytic subunit, calcineurin A (CnA), and a 19-kDa regulatory subunit, calcineurin B (CnB). CnA comprises a catalytic domain at the N-terminal region (residues 70–328) (8) and three regulatory domains at the C terminus; the CnB binding domain (8–10), the calmodulin binding domain (11), and the “autoinhibitory” domain (12). In the absence of Ca2+/calmodulin, calcineurin is inactive as the autoinhibitory domain binds in the active site cleft (12). Ca2+/calmodulin binding to the calmodulin binding domain of CnA displaces the autoinhibitory domain ceasing the inhibition (12).

We have recently reported a role for the plasma membrane Ca2+/calmodulin ATPase pump (PMCA) as a modulator of Ca2+/calmodulin-dependent enzymes (13, 14). PMCAs are enzymatic low capacity, high affinity systems involved in the extrusion of Ca2+ from the cell (15). Four different PMCA isoforms, PMCA1–4, have been identified in mammals and are encoded by four independent genes (16–21) with alternative splicing of each of the genes’ primary transcript at three independent sites (22, 23). PMCA1 and -4 are ubiquitously expressed, whereas PMCA2 and -3 show a more restricted cell- and tissue-specific pattern of expression (16, 20). The primary protein structure of PMCAs consists of 10 membrane-spanning segments, two major cytosolic loops, and N

* This work was supported in part by the Medical Research Council (International Appointee Grant G00000201 to L. N.) and by the Deutscher Forschungsgemeinschaft (Grant Ma2185/1-1 to A. H. M., Grant SAP2003-02920 to J. M. R., and Grant FIS-031474 to A. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Cn, calcineurin; CASK, calcium/calmodulin-dependent serine protein kinase; ERK, extracellular signal-regulated protein kinase; Hek, human embryonic kidney; hPMCA, human plasma membrane Ca2+/calmodulin ATPase; NFAT, nuclear factor of activated T-cell; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PMCA, plasma membrane Ca2+/calmodulin ATPase; RASSF1, Ras-associated Factor 1; CMV, cytomegalovirus; TBS, Tris-buffered saline; I0, calcium ionophore A23987; MCIP, modulatory calcineurin-interacting protein.
and C cytoplasmic tails. PMCA4b has been shown to inhibit the activity of the Ca\(^{2+}\)/calmodulin-dependent protein neuronal nitric-oxide synthase (nitric-oxide synthase I) (13) and calcium/calmodulin-dependent serine protein kinase or CASK (14), by tethering them to low Ca\(^{2+}\)/calmodulin cellular microdomains via interaction between the C-terminal end of PMCA4b and PDZ domains located in the partner proteins.

Recent findings in our laboratory have demonstrated the interaction of other intracellular regions of PMCA with proteins that do not contain a PDZ domain (24). This, and the calcium/calmodulin-dependent nature of calcineurin, raised the possibility that PMCA may regulate the activity of calcineurin (a protein without PDZ-domain) through interaction with a region other than the C terminus. In keeping with this hypothesis, a number of calcineurin-binding proteins have been reported to inhibit its phosphatase activity via physical interaction with the calcineurin A catalytic subunit (25).

Here we characterize PMCA as a novel inhibitory protein of endogenous calcineurin in mammalian cells. We show that the physical interaction between PMCA and calcineurin is required to inhibit calcineurin activity. This study provides new insight into the regulation of the calcineurin/NFAT signaling pathway and suggests a role for PMCA as an upstream regulator of this pathway in the differentiation and adaptation of varied tissue types.

MATERIALS AND METHODS

Cell Culture—HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.

Plasmids—pCMV-hPMCA4b contains the human PMCA4b cDNA and was a gift from Prof. E. Strehler (Dept. of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN).

The region encoding amino acids 2–521 of human calcineurin A was amplified by 40 cycles of PCR (the conditions were: denaturation 94 °C for 30 s, annealing 56 °C for 1 min, and extension 72 °C for 1 min) using the oligonucleotides HindhCnA3Sense (5’-CGGGATCCGGCCCAAGGCAATTGATC-3’) and hCnA521Anti (5’-GCTCTAGACTAGTTCTTCCCAAgCTTgAgC-3’). The amplified products were digested with BamHI and XbaI and cloned into the BamHI-XbaI sites of plasmid pEF-FLAG (26) to generate plasmid pFCnA-(2–521).

The region encoding amino acids 3–143 of human calcineurin was amplified from total RNA amplified from total RNA extracted from Jurkat cells by 30 cycles of PCR (the conditions were: denaturation 94 °C for 30 s, annealing 62 °C for 60 s, and extension 72 °C for 1 min for the first 10 cycles, followed by 20 cycles in the same conditions except for the annealing temperature that was raised up to 68 °C) using the oligonucleotides hCnA397 Sense (5’-AGA TCT GAG CCC AAG GTA AT TAT CTT CTT CTT CTT CTT CTT-3’) and hCnA397 Anti (5’-TCG CGA CCT TAT CAC TTT CCG GGC-3’). The PCR product was cloned into pGEM-T easy vector (Promega) and the BglIII-PvuII DNA fragment, coding for a constitutively active deletion mutant (amino acids 2–389), was further “in-frame” subcloned into BamHI-blunt (EcoRI plus fill-in with Klenow) digested pEF-FLAG expression vector to generate the pCnA-(2–389) construct.

To generate plasmid pFCnA-(2–173) a BglII-blunt (MluI plus fill-in with Klenow) cDNA fragment from pGEM-CnA-(2–397) and encoding the region encompassing amino acids 2–173 of human calcineurin A was subcloned in-frame into BamHI-blunt digested pEF-FLAG expression vector.

The region encoding amino acids 3–143 of human calcineurin was amplified by 35 cycles of PCR (the conditions were: denaturation 94 °C for 30 s, annealing 66 °C for 1 min, and extension 72 °C for 2 min) using the oligonucleotides HindhCnA3Sense (5’-TCTTCTCCAAGGTTAgG-A CCAgGAATTTgATCC-3’) and BamHhCnA143Anti (5’-TCTTCTC-GgATGCTCCATATgTTTTggTgAgATTTT-3’). The regions encoding amino acids 3–97 and 3–57 were amplified by 35 cycles of PCR (the conditions were: denaturation 94 °C for 30 s, annealing 54 °C for 5 s, and extension 72 °C for 30 s) using the oligonucleotides HindhCnA3Sense and BamHhCnA97Anti and HindhCnA3Sense and BamHhCnA57Anti (5’-TCTTCTGGATGCTCCATATgTTTTggTgAgATTTT-3’), respectively. The amplified products were digested with HindIII and BamHI and cloned into the HindIII and BamHI sites of plasmid pEF-FLAG-CMV7.1 (Sigma) to generate plasmids pCnA-(3–143), pCnA-(3–97), and pCnA-(3–57), respectively.

Fig. 1. Human PMCA4b and calcineurin A interact in mammalian cells. A domain structures of FLAG-tagged full-length calcineurin A (2–521) and a constitutively active mutant version calcineurin A (2–389). CB, calcineurin B binding domain; CaM, calmodulin binding domain; AID, autoinhibitory domain. B, human PMCA4b and full-length, or constitutively active calcineurin A, co-precipitate in mammalian HEK 293 cells. Expression vectors encoding human PMCA4b and a FLAG-tagged version of full-length, or constitutively active calcineurin A, were co-transfected in HEK 293 cells. Protein lysates were incubated with an anti-PMCA monoclonal antibody (2F/10), or an irrelevant antibody raised against firefly luciferase (α-Luc), and the immunoprecipitated proteins were probed with the J3A3 anti-PMCA4b monoclonal antibody or the M2 anti-FLAG monoclonal antibody to detect PMCA4b (WB:α-PMCA) or FLAG-tagged calcineurin A (WB:α-CnA), respectively. Cells transfected with empty vector (pEF-FLAG), or left untransfected, were used as negative controls.
Inhibition of Calcineurin by PMCA

27 °C for 30 s) using the oligonucleotides ECOPMCA4bSense (5'-TC- 
TTCCggAATTCTgAaCgACCTACgACgTGTCg-3') and BAMP3CA- 
4bAnt192 (5'-TTCTgCGATCCCTCAgTTTATTCTGACTGTggg-3') for 
region 1–92, oligonucleotides ECOPMCA4bSense428 (5'-TTCTCggA- 
ATTCgCTgTCACCTCCTACgTTgCgCTCgCTCCT-3') and BAMP3CAbAnt4137 (5'-TTCTCggAGATCCCTCAgTTTATTCTGACgAAgAAg-3') for region 
428–500, and oligonucleotides ECOPMCA4bSense1137 (5'-TTCTCggA- 
GATCCCTCAgTTTATTCTGACTGTggg-3') and BAMP3CAbAnt1205 (5'-TTCTCggAGATCCCTCAgTTTATTCTGACTGTggg-3') for region 
428–500 and oligonucleotides ECOPMCA4bSense428 (5'-TTCTCggAATTCTgAaCgACCTACgACgTGTCg-3') and BAMP3CA4bAnt675 (5'-TTCTCggAGATCCCTCAgTTTATTCTGACTGTgg-3') and BAMP3CAbAnt500 (5'-TTCTCggAGATCCCTCAgTTTATTCTgAGTTTg-3') and BAMP3CAbAnt1205 (5'-TTCTCggAGATCCCTCAgTTTATTCTGACTGTggg-3') for region 428–500. The amplified products were cloned into the EcoRI and BamHI sites of plasmid pBxFLAG-CMV7.1 to generate plasmids pP-M 
4CAb-(428–575) and -(428–500). The fidelity of all PCR amplified 
products was confirmed by sequencing.

FIG. 2 Interaction with PMCA4b maps to the region 58–143 of 
calcineurin A. A, schematic representation of FLAG-tagged C-termi 
nal deletion mutants of calcineurin A. CNB, calcineurin B binding 
domain. B, expression vectors encoding human PMCA4b and FLAG- 
tagged C-terminal deletion mutants of calcineurin A encoding amino acids 2–389, 127–143, 2–143, 2–97, or 2–57, were co-transfected in HEK 
293 cells. Cells left untransfected and those transfected with the empty 
vector (pEF-FLAG) were used as negative controls. Protein lysates 
were incubated with the anti-PMCA monoclonal antibody (5F10). Immuno 

c precipitated proteins (upper Western blots) and the expression levels 
of the recombinant proteins prior to immunoprecipitation (lower Western 
blots) were probed with the M2 anti-FLAG monoclonal antibody. The 
arrowhead indicates the decrease in the interaction of mutant FLAG- 
CN-4b-(3–97) (upper right hand panel) and its expression levels (lower 
right hand panel), suggesting that the region 98–143 plays a major role 
in the interaction. The arrow denotes the absence of interaction of 
mutant FLAG-CN-4b-(3–57) (upper right hand panel) in the context of 
equivalent expression levels (lower right hand panel). Interaction with 
PMCA4b thus maps to the region 58–143 of calcineurin A.

twice with 1 ml of cytosolic lysis buffer. Samples were analyzed by 
Western blot.

Western Blot—Samples were boiled and resolved, under reducing 
conditions, by SDS-PAGE (6 or 12% polyacrylamide for human PMCA4b or 
FLAG-tagged calcineurin A detection, respectively). The gels were trans 
ferred onto nitrocellulose membranes and Western blot performed as 
previously described (24) using a 1:1000 solution of J3 monoclonal 
antibody (Neomarkers) in PBS-T for PMCA4b detection, a 1:1000 solution 
of anti-FLAG M2 peroxidase-conjugate monoclonal antibody (Sigma 
Aldrich) in PBS-T, for FLAG-epitope detection, a 1:1000 solution of 
5F10 anti-PMCA monoclonal antibody (Abcam) in PBS-T or a 1:1000 solution 
of anti-calcineurin A monoclonal antibody (BD Bioscience) in PBS-T. Where 
relevant, developed films were scanned, and band densitometry was 
calculated on a densitometer (Gene Tools, Syngene, Europe).

RESULTS

PMCA Interacts with the Catalytic Subunit of Calcineurin in 
Mammalian Cells—HEK 293 cells were co-transfected with the 
expression plasmids pCMV-IP-PMCA4b (encoding human 
PMCA4b), and pFCN (2–521) (encoding full-length human 

calcineurin A (α) or pFCnA-(2–389) (encoding a constitutively active version lacking the calmodulin-binding domain and the autoinhibitory domain) (Fig. 1A). Protein extracts were immunoprecipitated with the anti-PMCA antibody 5F10, and probed by Western blot to demonstrate co-precipitation of PMCA with FLAG-tagged calcineurin A full-length, FLAGCnA-(2–521) (Fig. 1B, left panel) or the constitutively active mutant, FLAGCnA-(2–389) (Fig. 1B, right panel). The specificity and selectivity of the interactions were confirmed by the absence of co-precipitation in cells transfected with the pEF-FLAG empty vector (Fig. 1B, right panel) and immunoprecipitations performed with an irrelevant antibody (anti-Luciferase), respectively (Fig. 1B). These results thus demonstrate a physical interaction between PMCA4 and calcineurin A in mammalian cells.

The Region 58–143 of Calcineurin A Is Critical for Interaction with PMCA4b—To finely map the minimal interaction domain of calcineurin A responsible for the interaction with PMCA, a series of FLAG-tagged C-terminal deletion mutants of calcineurin A were generated (Fig. 2A) and then assayed by immunoprecipitation for their ability to interact with human PMCA4b.
HEK 293 cells were co-transfected with pCMV-hPMCA4b and plasmids encoding pFCnA-(2–389), pFCnA-(2–173), pFCnA-(3–143), pFCnA-(3–97), or pFCnA-(3–57). Protein extracts were immunoprecipitated with the anti-PMCA4 monoclonal antibody 5F10 and probed by Western blot to demonstrate co-precipitation of PMCA. The levels of co-precipitation were significantly reduced when amino acids 143–98 were removed, reflected by the results of mutant FLAG-CnA-(3–97) (Fig. 2B, right upper panel). Furthermore, the removal of amino acids 97 to 58 in pFCnA-(3–57) completely prevented co-precipitation with PMCA (Fig. 2B, right upper panel). Immunoprecipitations with an irrelevant antibody (anti-Luciferase) did not precipitate any protein (data not shown) demonstrating the specificity of the interaction. Untransfected cells, or cells transfected with the pFLAG empty vector were used as negative controls (Fig. 2B, upper panels). Levels of the FLAG proteins prior to immunoprecipitation were analyzed by Western blot to ensure differences were not attributable to differential expression (Fig. 2B, lower panels). These results indicate that the region 98–143 of calcineurin A is necessary for interaction with PMCA4b, whereas amino acids 58–97 appear to contribute to the interaction.

The Region 501–575 of PMCA4b Is Essential for Interaction with Calcineurin A—To identify the region of PMCA4b involved in the interaction with calcineurin A, we generated FLAG-tagged fusion proteins containing either the N terminus, part of the catalytic domain, or the C terminus intracellular domain of PMCA4b (Fig. 3A).

HEK 293 cells were transfected with plasmids pF-PMCA4b-(1–92), pF-PMDCA4b-(428–651), or pF-PMCA4b-(1137–1205). Commercially available calcineurin was added to the protein lysates, which were then immunoprecipitated with a monoclonal anti-calcineurin A antibody, and probed by Western blot. FLAG-PMCA4b-(1–92) and PMCA4b-(428–651) strongly co-precipitated with calcineurin (Fig. 3B, upper panel). However, no precipitation was detected when FLAG-tagged proteins containing the N or C termini intracellular domains of PMCA4b were used in the assay (Fig. 3B, upper panel). Cells transfected with empty vector p3xFLAG-CMV7.1 were used as a negative control (Fig. 3B, upper panel). Cells transfected with empty vector p3xFLAG-CMV7.1 were used as a negative control (Fig. 3B, upper panel). The FLAG-tagged PMCA4b proteins were expressed at roughly equivalent levels, thus ruling out the scenario that poor expression led to lack of interaction (Fig. 3B, lower panel).

To determine the domain within the region 428–651 of PMCA4b that interacts with calcineurin, a further series of FLAG-tagged deletion mutants covering this region was generated, pF-PMCA4b-(428–575) and pF-PMCA4b-(428–500) (Fig. 3C). The removal of amino acids 501–575 completely prevented the interaction, as evidenced by the absence of co-precipitated FLAG-PMCA4b-(428–500) with calcineurin (Fig. 3D). In summary, these results demonstrate that the region 501–575 of the catalytic large intracellular loop of PMCA4b is necessary for the interaction with calcineurin.

PMCA4b Significantly Inhibits NFAT Transcriptional Activity—Previously reported interactions between calcineurin and partner proteins have been shown to inhibit the activity of calcineurin (25). We tested for a functional role of the PMCA-calcineurin interaction as a modulator of calcineurin activity, by assaying the effect of hPMCA4b expression on the transcriptional activity of NFAT, one of the best characterized calcineurin substrates.

HEK 293 cells were transfected with the NFAT-dependent luciferase reporter vector pNFAT-TA-Luc (Clontech) and stimulated with PMA and the calcium ionophore A23187. 4 µg of pCMV-hPMCA4b (encoding hPMCA4b) or control vector pEGFP, and 1 µg of the NFAT-dependent luciferase reporter vector pNFAT-TA-Luc (Clontech) were co-transfected in HEK 293 cells. Cells were stimulated with PMA (20 ng/ml) plus Io (1 µM) for 16 h. Luciferase activity is expressed as fold induction over the value of the reporter vector in unstimulated cells. *, statistically significant (p < 0.05, according to Student’s t test) 60% inhibition of the pNFAT-TA-Luc reporter vector activity as a result of co-expression of human PMCA4b. Means ± S.E. of three independent experiments are shown. B, co-expression of PMCA4b and the PMCA4b interaction domain (428–651) reverses the inhibitory effect of PMCA4b. 3 µg of the expression vector, pCMV-hPMCA4b encoding hPMCA4b and 2 µg of pFLAG-hPMCA4b (428–651) encoding hPMCA4b (428–651), was co-transfected together with 1 µg of the NFAT-dependent luciferase reporter vector pNFAT-TA-Luc in mammalian HEK 293 cells. Cells were stimulated with PMA (20 ng/ml) plus Io (1 µM) for 16 h. Luciferase activity is expressed as fold induction over the value of the reporter vector in unstimulated cells. *, statistically significant (p < 0.05, according to Student’s t test) 60% inhibition of the pNFAT-TA-Luc reporter vector activity as a result of co-expression of human PMCA4b, which is reversed by the co-expression of pFLAG-hPMCA4b-(428–651).

control vector pEGFP (Fig. 4A). However co-transfection with pCMV-hPMCA4b, an expression plasmid encoding hPMCA4b, significantly reduced (60% inhibition, p < 0.05) PMA plus Io-dependent activation of the luciferase reporter vector (Fig. 4A). These results suggest that PMCA negatively regulates the activity of endogenous calcineurin in mammalian cells.

To investigate the relevance of the interaction between the two proteins and the inhibition of calcineurin activity, we blocked the interaction by overexpressing an excess of the region encompassing amino acids 428–651 of PMCA4b (Fig. 4B). Under these conditions ectopic PMCA4b was not able to...
inhibit the activity of endogenous calcineurin, demonstrating the functional significance of the interaction. These findings were specific and not the result of generalized transcriptional repression, because β-galactosidase expression (encoded by plasmid pEF-LacZ) was not influenced by co-expression of hPMCA4b (data not shown).

**PMCA4b Is Unable to Inhibit a Calcium/Calmodulin-independent Constitutively Active Mutant of Calcineurin A**—We next assessed the effect of PMCA on the activity of FLAG-CnA-(2–389), a constitutively active mutant of calcineurin A that is known to activate NFAT in a calcium/calmodulin-independent manner. HEK 293 cells were co-transfected with the NFAT-dependent luciferase reporter vector pNFAT-TA-Luc (Clontech), pFCnA-(2–389), and either an expression vector encoding EGFP (control plasmid) or an expression vector encoding hPMCA4b (pCMV-hPMCA4b) encoding hPMCA4b, or control vector pEGFP-C1 (Clontech), and 1 μg of the NFAT-dependent luciferase reporter vector pNFAT-TA-Luc in mammalian HEK 293 cells. Cells were transfected for 24 h, washed with PBS, and incubated for 16 additional hours. Luciferase activity is expressed as fold induction over the value of the reporter vector in cells co-transfected with the empty vectors pEF-FLAG and pEGFP-C1. Transfection efficiency was normalized by co-transfection of the pEF-LacZ plasmid as internal control and measure of β-galactosidase expression. Means ± S.E. of three independent experiments are shown.

**DISCUSSION**

Through its effect on the NFAT family of transcription factors, calcineurin represents a crucial mediator of cellular function, differentiation, and adaptation (7). In this work we describe a novel role for the plasma membrane Ca\textsuperscript{2+}/calmodulin-ATPase pump, PMCA, as an inhibitor of calcineurin signaling. Calcineurin A and PMCA interacted in mammalian cells. PMCA bound equally well to full-length calcineurin A and a constitutively active deletion mutant (Fig. 1, A and B), and the interaction domain mapped to amino acids 58–143 located within the catalytic domain of calcineurin A.

The interaction of PMCA4b with the catalytic subunit of calcineurin A is similar to the interactions reported for other calcineurin interaction partners (immunophilins, AKAP79, cabin-1, MCIP1, or calsarcins) that also exert their inhibitory effect by binding to the catalytic subunit (25, 29). Our results suggest that interaction of PMCA does not require binding of calcineurin B or Ca\textsuperscript{2+}/calmodulin to calcineurin A, which is also true for modulatory calcineurin-interacting proteins (MCIPs) (30). The interaction of PMCA with calcineurin A occurs at a site distinct from the main immunophilin-immunosuppressant complex binding region (31, 32). Crystal structure studies have demonstrated that cyclophilin A-CsA and FKBP12-FK506 complexes bind to calcineurin residues within a highly conserved region that overlaps with the binding domain for CnB (31). All the known endogenous modulators of
calcineurin, with the exception of calsarcins, exert their effects through interaction with calcineurin residues that overlap with the immunophilin-binding sites (33). This is in contrast to the PMCA binding domain, although AKAP79 has been reported to bind to two regions of calcineurin (residues 30–98 and 311–336), one of which partially overlaps with the identified PMCA-binding site (32). PMCA4b is therefore likely to represent a new, non-competitive inhibitor of calcineurin.

Transient expression of PMCA4b strongly reduced the response of a NFAT-dependent luciferase reporter vector to stimulation with PMA plus Io (Fig. 4A). This inhibition reflects the effect of PMCA4b on endogenous calcineurin. We suggest that this functional effect is facilitated by the recruitment of calcineurin by PMCA4b to a cellular microenvironment where the concentrations of Ca$^{2+}$ are kept low, which would thus reduce the phosphatase activity of the enzyme. This interpretation is corroborated by the reversal of inhibition observed when PMCA4b is co-expressed with its interaction domain. Furthermore, the inability of PMCA4b to confer an inhibitory effect on constitutively active calcineurin A suggests this functional interaction is dependent on calcium/calmodulin.

The intracellular domain of PMCA responsible for the interaction with calcineurin was mapped to the region containing amino acids 501–575, located within the catalytic intracellular loop of PMCA4b between trans-membrane domains 4 and 5. Structurally, the region 501–575 of PMCA4b differs from the interaction domains identified in the other endogenous proteins that also inhibit calcineurin. AKAP79, Cain, and MCIP, have been reported to contain a conserved motif (P$^X$I$^X$IT) (25), which is also present in the NFAT proteins (34). The presence of this motif in the inhibitory proteins displaces NFAT binding to calcineurin, and subsequently inhibits NFAT activation. An examination of the region 501–575 of PMCA4b did not reveal any sequence resembling the P$^X$I$^X$IT motif indicating an alternative mechanism to P$^X$I$^X$IT competition must be involved in PMCA-dependent NFAT inhibition. The mechanism responsible for this inhibition is unclear at present. In fact, BLAST searches (basic local alignment search tool) carried out with this sequence did not show any significant homology to known proteins in the data base except other PMCA isoforms. The high conservation of this sequence in the other members of the PMCA family of proteins (≥80% amino acid identity) suggests that calcineurin A may also interact with other PMCA isoforms.
This interaction is contingent upon sub-cellular localizations that place calcineurin and PMCA together. There is precedence for proposing localization of calcineurin to the plasma membrane. Calcineurin has been implicated in α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor dephosphorylation, and thus regulation at excitatory synapses (35), via a signaling complex of three second messenger-regulated signaling enzymes, including the calcineurin anchoring protein AKAP79 (36, 37), which in turn interacts with SAP97, a PDZ domain-containing protein known to interact with PMCA (38). Our findings are consistent with this. Furthermore, the data indicate a definite trend that goes one step further in suggesting PMCA4b may be involved in the physical recruitment of calcineurin from the cytoplasm to the plasma membrane where the two proteins directly interact.

Both proteins are calcium/calmodulin-dependent, and PMCA itself is a calcium-extruding pump. Proposing a calcium-dependent mechanism is not only intuitive but supported by our data. Although our work focuses on the effect of PMCA4b on calcineurin, it may be that calcineurin interaction with the PMCA catalytic domain modifies the Ca\(^{2+}\) transporting activity of the protein. Elucidating the relationship between the calcium-extruding role of PMCA4b and the recruitment of calcineurin, as well as the actual physical interaction, is a complex process and beyond the scope of this study. We are currently investigating this in our laboratory.

The diverse physical interactions of PMCA with signal transduction molecules such as calcineurin (this work), RASSF1 (24), neuronal nitric-oxide synthase (13), and CASK (14), thus provide a substantial backdrop for its prevailing role as a pivotal organizer of signal transduction complexes. Indeed, the emergent role of PMCA can be characterized nicely when this work is taken in context of our recent demonstration of a functional interaction between PMCA and the tumor suppressor protein RASSF1. This interaction leads to an inhibition of the extracellular signal-regulated kinase (ERK) pathway (24). ERK signaling activates the AP-1 transcription factor (39), and NFAT interacts with AP-1 to form a cooperative composite binding site in the promoter regions of many target genes (40). Thus both the calcineurin and ERK pathways, which lead to activation of transcription factors NFAT and AP-1, respectively, appear to have a common denominator in PMCA as an upstream regulator that inhibits these signaling pathways (Fig. 7).

In summary, our results offer strong evidence for a novel functional interaction between PMCA and calcineurin, suggesting a role for PMCA as an organizer and negative modulator of calcineurin-mediated signaling pathways in mammalian cells. The implications of these findings are potentially far-reaching, considering the ubiquitous nature of calcineurin and its key role in diverse cellular processes. Further investigations of this interaction with in vivo studies are necessary to enhance our understanding of these findings at a physiological and pathophysiological level.

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