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

Mamta H. Buch‡§, Adam Pickard‡, Antonio Rodriguez¶, Sheona Gillies‡, Alexander H. Maass‖, Michael Emerson‡, Elizabeth J. Cartwright‡, Judith C. Williams‡, Delvac Oceandy‡, Juan M. Redondo¶, Ludwig Neyses‡‡‡, and Angel L. Armesilla‡****

From the ‡Division of Cardiology, The University of Manchester, Stopford Bldg., Oxford Road, Manchester M13 9PT, United Kingdom, the ¶Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, Madrid 28049, Spain, and the ‖Department of Medicine, University of Wuerzburg, Wuerzburg 97080, Germany

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 Ca^{2+} in signal transduction pathways is well established (1). Critical to the coupling of Ca^{2+} signals to cellular responses is the serine-threonine protein phosphatase calcineurin (Cn^1; also called protein phosphatase 2B), which is regulated by Ca^{2+}/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 Ca^{2+}, 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) (8). 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 Ca^{2+}/calmodulin, calcineurin is inactive as the autoinhibitory domain binds in the active site cleft (12). Ca^{2+}/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 Ca^{2+}/calmodulin ATPase pump (PMCA) as a modulator of Ca^{2+}/calmodulin-dependent enzymes (13, 14). PMCAs are enzymatic low capacity, high affinity systems involved in the extrusion of Ca^{2+} 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

[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 Ca^{2+}/calmodulin ATPase; NFAT, nuclear factor of activated T-cell; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PMCA, plasma membrane Ca^{2+}/calmodulin ATPase; RASSF1, Ras-associated Factor 1; CMV, cytomegalovirus; TBS, Tris-buffered saline; Io, calcium ionophore A23987; MCIP, modulatory calcineurin-interacting protein.
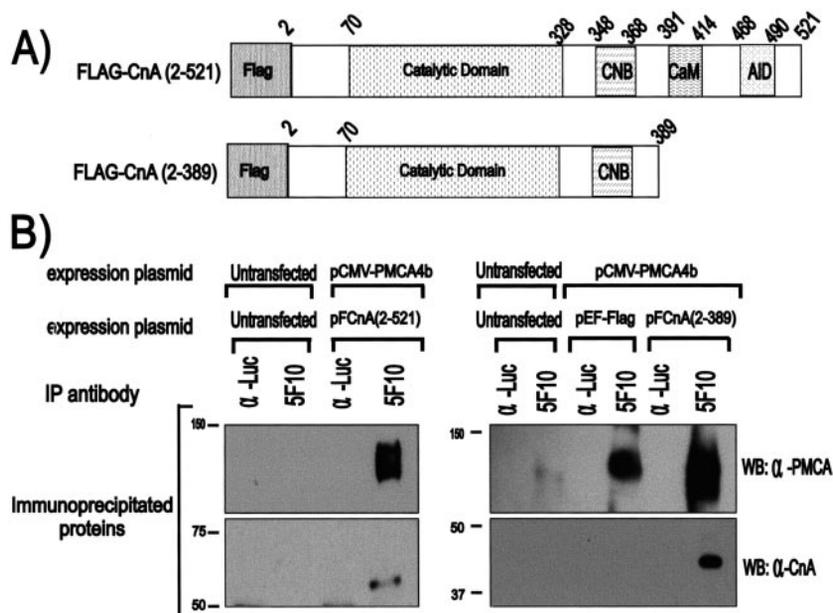
FIG. 1. **Human PMCA4b and calcineurin A interact in mammalian cells.** *A*, domain structures of FLAG-tagged full-length calcineurin A-(2–521) and a constitutively active mutant version calcineurin A-(2–389). *CNB*, calcineurin B binding domain; *CaM*, calmodulin binding domain; *AID*, autoinhibitory 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 α, were co-transfected in HEK 293 cells. Protein lysates were incubated with an anti-PMCA monoclonal antibody (*5F10*), or an irrelevant antibody raised against firefly luciferase (α-*Luc*), and the immunoprecipitated proteins were probed with the JA3 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.

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 α was amplified by 40 cycles of PCR (the conditions were: denaturation 94 °C for 60 s, annealing 58 °C for 60 s, and extension 72 °C for 2 min) using the oligonucleotides as follows: hCnA2Sense (5′-CGGGATCCGAGC-CCAAGGCAATTGATC-3′) and hCnA521Anti (5′-GCTCTAGAAGTG-GTCACTGAATATTGCTGCTATTAC-3′). The amplified product was di-

gested 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 2–397 from human calcineurin A α 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 60 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 GCA ATT GAT CCC-3′) and hCnA397 Anti (5′-TCG CGA CCT TAT CAC TTT CCG GGC-3′). The PCR product was first cloned into pGEM-T easy vector (Promega) and the BglII-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 pFCnA-(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 α was subcloned in-frame into BamHI-blunt digested pEF-FLAG expression vector.

The region encoding amino acids 3–143 of human calcineurin α was amplified by 35 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′-TCTTCCCAAgCTTgAgC-CCAAggCAATTgATCCC-3′) and BamHhCnA143Anti (5′-TCTTCg-CggATCCTCATgTTTTggggTAgAgAATTTTC-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 30 s, and extension 72 °C for 30 s) using the oligonucleotides HindhCnA3Sense and BamHhCnA97Anti (5′-TCTTCgCggATCCTC-AATCAAAgAATTgTCCATgAATg-3′) and BamHhCnA57Anti (5′-TC-TTCgCggATCCTCATTCCAgCCTTCCCTCCTTCATA-3′), respectively. The amplified products were digested with HindIII and BamHI and cloned into the HindIII and BamHI sites of plasmid p3xFLAG-CMV7.1 (Sigma) to generate plasmids pFCnA-(3–143), pFCnA-(3–97), and pFCnA-(3–57), respectively.

Fragments of the human PMCA4b encompassing amino acids 1–92 (N-terminal cytoplasmic), 428–658 (part of the big intracellular loop domain between transmembrane regions 4 and 5), and 1137–1205 (C-terminal cytoplasmic, numbering according to accession number NM_001684) were amplified by 35 cycles of PCR (the conditions were: denaturation 94 °C for 30 s, annealing 62 °C for 30 s, and extension

72 °C for 30 s) using the oligonucleotides ECOPMCA4b1Sense (5′-TC-TTCCggAATTCATgACgAACCATCAgACCgTg-3′) and BAMHPMCA-4bAnti92 (5′-TCTTCgCggATCCTCAAgTCTgggCTTTTTgggggggg-3′) for region 1–92, oligonucleotides ECOPMCA4bSense428 (5′-TCTTCCggA-ATTCCgCTgTCACCTCTCACTggCCT-3′) and BAMHPMCA4bAnti651 (5′-TCTTCgCggATCCTCAAAgAggCTCTgTgTCATCgAA-3′) for region 428–651, and oligonucleotides ECOPMCA4bSense1137 (5′-TCTTCCg-gAATTCCgCCATAgAggAggAg-3′) and BAMHPMCA4bAnti1205 (5′-T-CTTCgCggATCCTCAAACTgAgTCTCTAggC-3′) for region 1137–1205. The amplified products were digested with EcoRI and BamHI and cloned into the EcoRI and BamHI sites of plasmid p3xFLAG-CMV7.1 to generate plasmids pF-PMCA4b-(1–92), -(438–651), and -(1137–1205).

Fragments of the human PMCA4b encoding amino acids 428–500 and 428–575 (part of the big intracellular loop between transmembrane regions 4 and 5) were amplified by 35 cycles of PCR (the conditions were: denaturation 94 °C for 1 min, annealing 58 °C for 1 min, and extension 72 °C for 1 min 30 s) using the oligonucleotides ECOPMCA-4BSense428 (5′-TCTTCCggAATTCCgCTgTCACCTCTCACTggCCT-3′) and BAMHPMCA4bAnti575 (5′-TCTTCgCggATCCTCACATTgACTTg-CgCACTgAgTT-3′) for region 428–575 and oligonucleotides ECOPMC-A4BSense428 (5′-TCTTCCggAATTCCgCTgTCACCTCTCACTggCCT-3′) and BAMHPMCA4bAnti500 (5′-TCTTCgCggATCCTCAgACTTTgg-gCAggAagACATCA-3′) for region 428–500. The amplified products were digested with EcoRI and BamHI and cloned into the EcoRI and BamHI sites of plasmid p3xFLAG-CMV7.1 to generate plasmids pF-P-MCA4b-(428–575) and -(428–500). The fidelity of all PCR amplified products was confirmed by sequencing.

Transient Transfections—For immunoprecipitation experiments HEK 293 cells were plated in 100- × 20-mm tissue culture dishes (4.5 × 10^6 cells/plate) the day before transfection. Transfections were performed with 10 μg of the relevant expression plasmid using Lipofectamine 2000 reagent (Invitrogen) as previously described (24).

For Luciferase assays, HEK 293 cells were plated in 6-well tissue culture plates (7 × 10^5 cells/well) the day before transfection. Cells were transfected with the relevant expression vectors, which included 4 μg of expression vector and 1 μg of the luciferase reporter vector pNFAT-TA-Luc (Clontech). Lipofectamine 2000 reagent (Invitrogen) was used to transfect the vectors according to the manufacturer's instructions. Cells were incubated with DNA-Lipofectamine complexes for 24 h, washed with PBS, and then incubated overnight in 5 ml of fresh Dulbecco's modified Eagle's medium-10% fetal calf serum. Where relevant, the cells were stimulated with PMA (20 ng/ml) and the calcium ionophore A23187 (1 μM) prior to incubation. The following morning, cells extracts were prepared, and luciferase activity was determined with a luciferase assay system (Promega). Transfection efficiency was normalized by co-transfection of plasmid pEF-LacZ carrying the β-galactosidase gene under the control of the EF1α promoter, and determination of β-galactosidase activity (27).

Immunoprecipitation—Transfected HEK 293 cells or rat neonatal cardiomyocytes were lysed with radioimmune precipitation assay buffer (1× PBS, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 20 μM phenylmethylsulfonyl fluoride, 500 ng/ml leupeptin, 1.0 μg/ml aprotinin, 500 ng/ml pepstatin) and protein extracts immunoprecipitated with the corresponding antibodies as described. Immunoprecipitated proteins were analyzed by Western blot.

Cellular Fractions—HEK 293 cells were plated in 100- × 20-mm tissue culture dishes (4.5 × 10^6 cells/plate), and transfected the following day. Transfections were performed with 10 μg of the relevant expression plasmid using Lipofectamine 2000 reagent (Invitrogen) as previously described (24).

Plasma membrane fractions were obtained using a previously described method based on biotin and avidin interaction (28). Briefly, cells were harvested after 72 h. Cells were washed with PBS twice, and 27 × 10^6 cells resuspended in 1 ml of PBS before biotinylation with 10 mM Sulfo-NHS-biotin (Pierce) for 30 min at room temperature. After biotinylation, cells were washed three times with PBS to remove excess biotin reagent and byproducts. Crude plasma membranes were then prepared. The cells were collected and incubated in 1 ml of cytosolic lysis buffer (10 mM Hepes, 10 mM NaCl, 5 mM NaHCO3, 1 mM CaCl2, 0.5 mM Mg Cl2, 1 mM phenylmethylsulfonyl fluoride, aprotinin, 5 mM EDTA) for 5 min on ice. The cells were then homogenized and centrifuged at 3000 × g, at 4 °C, to pellet nuclei and intact cells. To prepare purified plasma membranes, the supernatant, containing the biotinylated crude plasma membranes, was then incubated with 100 μl of avidin beads (Immunopure Immobilized Monomeric Avidin, Pierce) for 1 h at room temperature. The purified plasma membranes were separated from the cytoplasmic fraction by centrifuging at 3000 × g to pellet the beads. The beads, containing the bound membranes, were washed
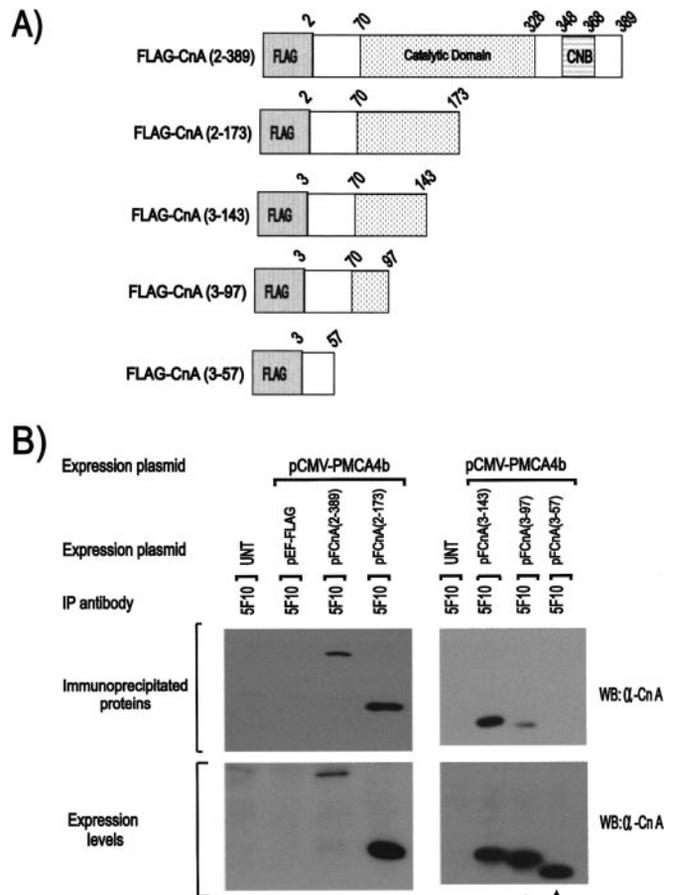
FIG. 2. **Interaction with PMCA4b maps to the region 58–143 of calcineurin A.** *A*, schematic representation of FLAG-tagged C-terminal deletion mutants of calcineurin A. *CNB*, calcineurin B binding domain. *B*, expression vectors encoding human PMCA4b and FLAG-tagged C-terminal deletion mutants of calcineurin A α encoding amino acids 2–389, 2–173, 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*). Immunoprecipitated 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-CnA-(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-CnA-(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 transferred onto nitrocellulose membranes and Western blot performed as previously described (24) using a 1:1000 solution of JA3 monoclonal antibody (Neomarkers) in TBS-T for PMCA4b detection, a 1:1000 solution of anti-FLAG M2 peroxidase-conjugate monoclonal antibody (Sigma-Aldrich) in TBS-T, for FLAG-epitope detection, a 1:1000 solution of 5F10 anti-PMCA monoclonal antibody (Abcam) in TBS-T or a 1:1000 solution of anti-calcineurin A monoclonal antibody (BD Bioscience) in TBS-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-hPMCA4b (encoding human PMCA4b), and pFCnA-(2–521) (encoding full-length human

FIG. 3. **The region 501–575 of human PMCA4b interacts with calcineurin.** *A*, schematic representation of FLAG-tagged proteins containing the N-terminal (amino acids 1–92) intracellular region of human PMCA4b, the region 428–651 of its catalytic domain, and the fragment 1137–1205 of the C-terminal intracellular region. The *numbers* correspond to human PMCA4b amino acids according to accession number NM_001684. *B*, immunoprecipitation analysis of the interaction between calcineurin and FLAG-tagged proteins containing the regions 1–92, 428–651, or 1137–1205 of human PMCA4b. Expression vectors encoding FLAG-tagged PMCA4b-(1–92), -(428–651), or -(1137–1205) were transfected in HEK 293 cells. Empty vector (p3xFLAG-CMV7.1, Sigma) was used as a negative control. Protein lysates were incubated with commercial calcineurin (20 nM, Sigma), and then complexes precipitated with an anti-calcineurin monoclonal antibody (BD Biosciences). FLAG-tagged immunoprecipitated proteins were detected by Western blot with M2 anti-FLAG peroxidase monoclonal antibody (*upper Western blots*). Expression levels of the FLAG proteins prior to immunoprecipitation were analyzed by Western blot (*lower Western blots*). The region 428–651, located within the catalytic big intracellular loop of PMCA4b specifically interacts with calcineurin. *C*, schematic representation of FLAG-tagged C-terminal deletions of the region 428–651 generated to determine the minimal region interacting with calcineurin. *D*, expression vectors encoding FLAG-tagged versions of the regions 428–651, 428–575, or 428–500 of human PMCA4b were transfected in HEK 293 cells. Protein lysates were immunoprecipitated as described in Fig. 3*B*. Western blots were then probed with the M2 anti-FLAG monoclonal antibody to detect immunoprecipitated proteins (*upper Western blot*) and expression levels (*lower Western blot*). FLAG proteins containing amino acids 428–651 or 428–575 of PMCA4b both co-precipitated with calcineurin. FLAG-PMCA4b-(428–500), however, failed to interact with calcineurin, narrowing down the interaction domain to the region 501–575 of human PMCA4b.
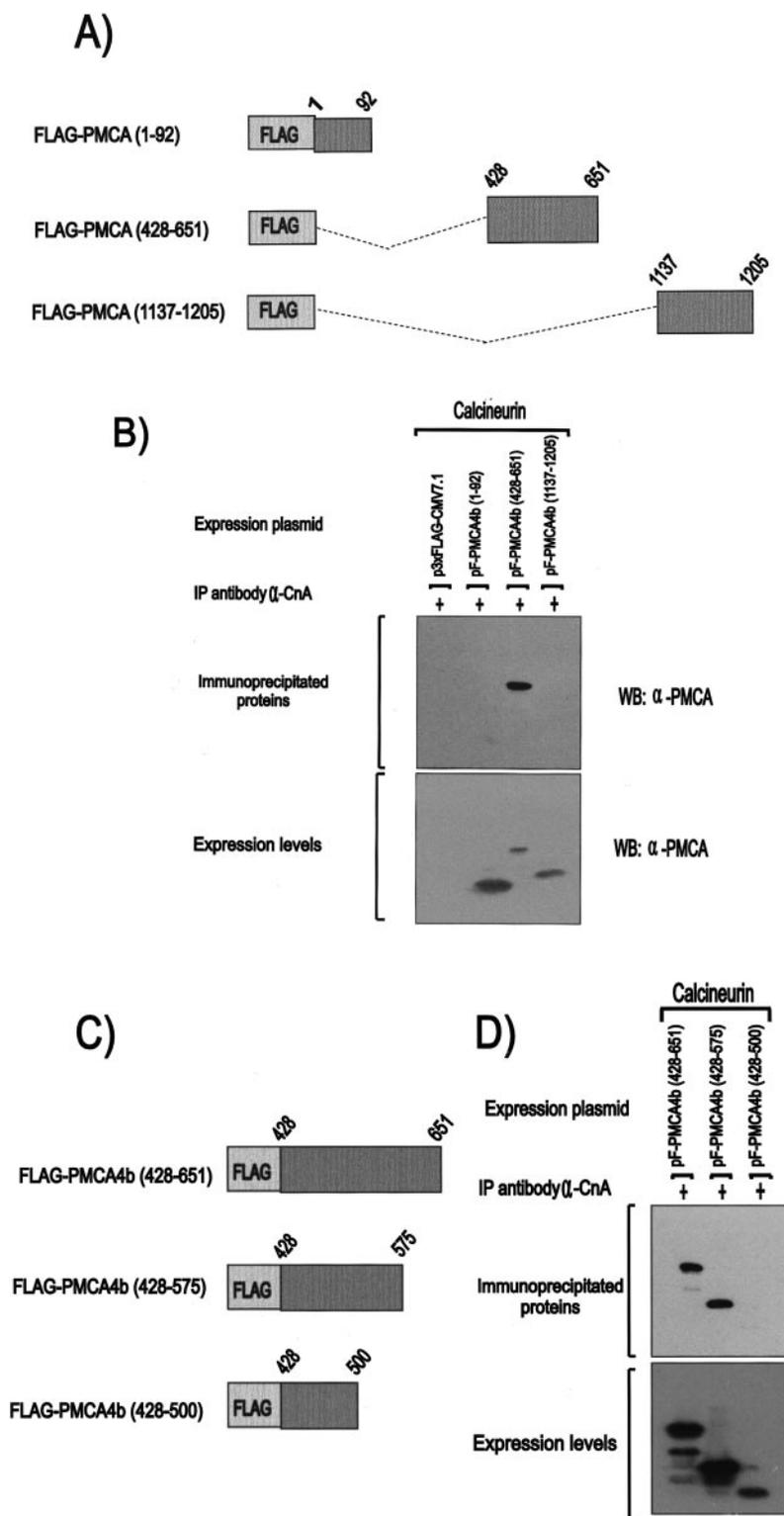
calcineurin A α) or pFCnA-(2–389) (encoding a constitutively active version lacking the calmodulin-binding domain and the autoinhibitory domain) (Fig. 1*A*). Protein extracts were immunoprecipitated with the anti-PMCA antibody 5F10, and probed by Western blot to demonstrate co-precipitation of PMCA with FLAG-tagged calcineurin A full-length, FLAGCnA-(2–521) (Fig. 1*B*, *left panel*) or the constitutively active mutant, FLAGCnA-(2–389) (Fig. 1*B*, *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. 1*B*, *right panel*) and immuno-

precipitations performed with an irrelevant antibody (anti-Luciferase), respectively (Fig. 1*B*). 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 α were generated (Fig. 2*A*) and then assayed by immunoprecipitation for their ability to interact with human PMCA in mammalian cells.

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. 2*B*, *right upper panel*). Furthermore, the removal of amino acids 97 to 58 in pFCnA-(3–57) completely prevented co-precipitation with PMCA (Fig. 2*B*, *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. 2*B*, *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. 2*B*, *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 regions of PMCA4b (Fig. 3*A*).

HEK 293 cells were transfected with plasmids pF-PMCA4b-(1–92), -(428–651), or -(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-(428–651) strongly co-precipitated with calcineurin (Fig. 3*B*, *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. 3*B*, *upper panel*). Cells transfected with empty vector p3xFLAG-CMV7.1 were used as a negative control (Fig. 3*B*, *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. 3*B*, 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. 3*C*). 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. 3*D*). 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 A23987 (Io) to induce endogenous calcineurin activation and consequent NFAT transcriptional activity. NFAT-dependent luciferase activity was induced ~8.5-fold in cells co-transfected with the
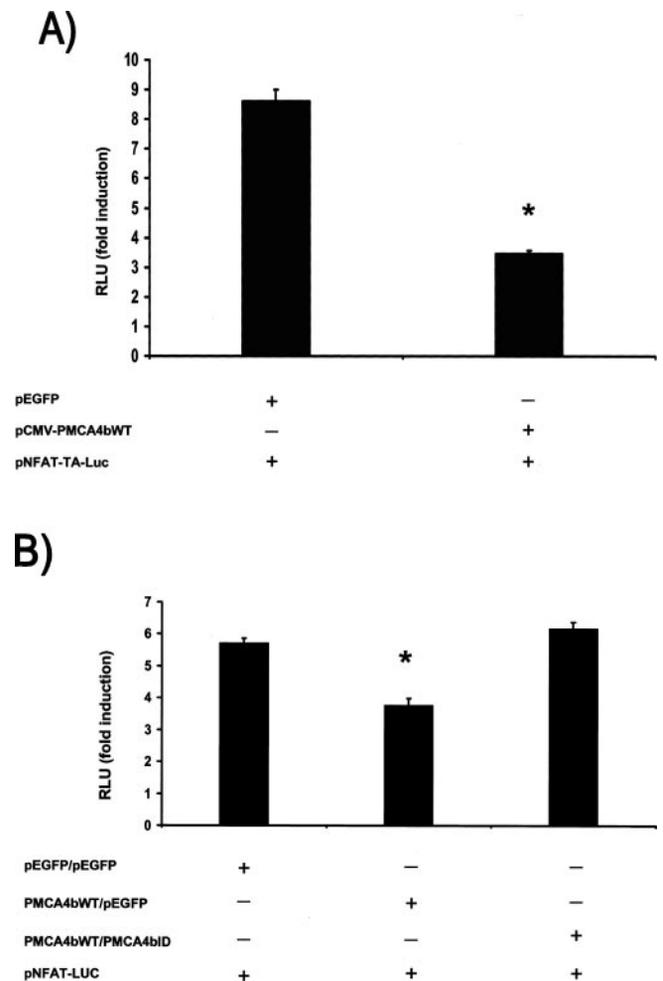
FIG. 4. **PMCA4b significantly inhibits NFAT transcriptional activity.** *A*, PMCA4b inhibits the activation of an NFAT-dependent luciferase reporter vector in response to PMA plus the calcium ionophore (*Io*) A23187. 4 μg of pCMV-hPMCA4b (encoding hPMCA4b) or control vector pEGFP, and 1 μg of the NFAT-dependent 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 ($p \leq$ 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 $\pm$ 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 ($p \leq$ 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. 4*A*). However co-transfection with pCMV-hPMCA4b, an expression plasmid encoding hPMCA4b, significantly reduced (60% inhibition, $p \leq$ 0.05) PMA plus Io-dependent activation of the luciferase reported vector (Fig. 4*A*). 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. 4*B*). Under these conditions ectopic PMCA4b was not able to
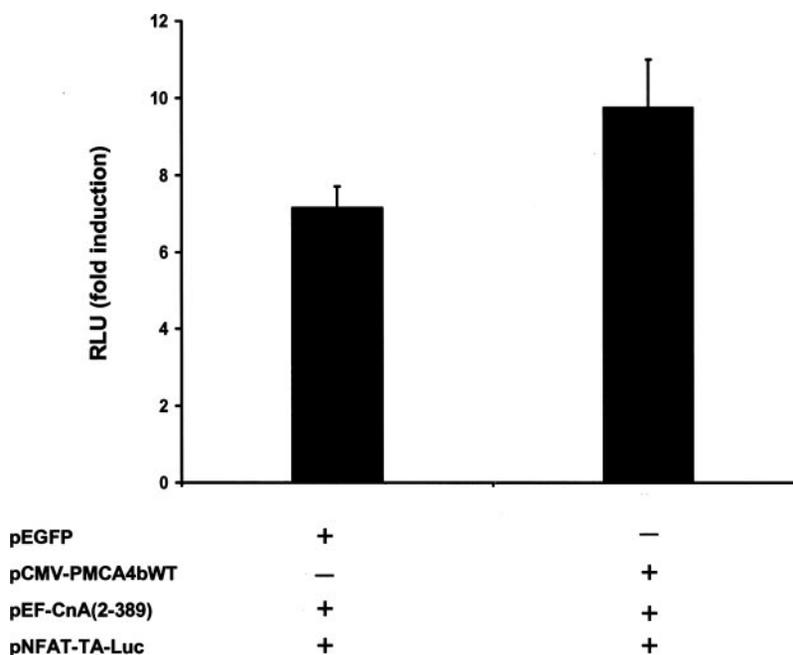
FIG. 5. **PMCA4b is unable to inhibit the activation of an NFAT-dependent luciferase reporter vector in response to pFCnA-(2–389), a constitutively active deletion mutant of calcineurin A.** 3 μg of a expression vector, pFCnA-(2–389), encoding a constitutively active deletion mutant of calcineurin A or its corresponding empty vector, pEF-FLAG, was co-transfected together with 1.5 μg of the expression vector 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 $\pm$ S.E. of three independent experiments are shown.

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), an expression vector encoding FLAG-CnA-(2–389), and either an expression vector encoding EGFP (control plasmid) or an expression vector encoding hPMCA4b-(pCMV-PMCA4b). The constitutively active calcineurin A mutant increased NFAT-dependent luciferase activity around 6.5-fold in the presence of the EGFP protein (Fig. 4B). Co-expression of hPMCA4b, however, was not able to inhibit this constitutively active calcineurin A mediated induction of NFAT-dependent luciferase activity (Fig. 5).

PMCA4b Recruits Endogenous Calcineurin from the Cytoplasm to the Plasma Membrane—A negative modulatory effect of PMCA4b on calcineurin via direct physical interaction requires the localization of calcineurin in the plasma membrane. Moreover, we would expect to observe an effect of PMCA4b on the calcineurin distribution.

Western blot analysis of a 1:3 dilution of the total plasma membrane-bound protein fraction and a 1:100 dilution of the total cytoplasmic protein fraction revealed the presence of both cytoplasmic and plasma membrane-associated calcineurin (Fig. 6A). When HEK 293 cells were transfected with an expression vector encoding hPMCA4b (pCMV-PMCA4b), a reduced level of cytoplasmic calcineurin and increased level of calcineurin in the

plasma membrane was observed. Transfection of an expression vector encoding EGFP (control plasmid) did not affect calcineurin distribution (Fig. 6, A and B). Quantification of plasma membrane calcineurin by densitometry confirmed a 28% increase in calcineurin localization at the plasma membrane fraction in the presence of ectopic PMCA4b (Fig. 6B). Conversely, in the cytoplasmic fraction, a 38% reduction in calcineurin level was observed in the pCMV-PMCA4b-transfected cells compared with the pEGFP-transfected cells (Fig. 6B).

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^{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^{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

A)
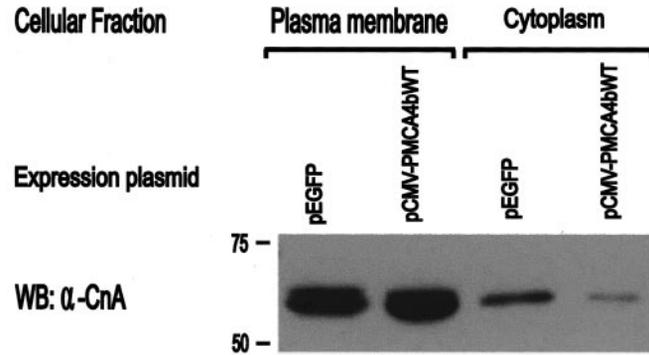
Fig. 6. **PMCA4b recruits endogenous calcineurin from the cytoplasm to the plasma membrane.** *A*, HEK 293 cells were transfected with an expression vector encoding hPMCA4b (pCMV-PMCA4b) or control vector pEGFP. A 1:3 dilution of the total plasma membrane-bound proteins and a 1:100 dilution of the total cytoplasmic fraction were analyzed by Western blot, using an anti-calcineurin monoclonal antibody (BD Bioscience), to examine the effect of PMCA4b expression on endogenous calcineurin localization. Calcineurin is seen to be present in both the plasma membrane and the cytoplasmic fractions. In the presence of ectopic PMCA4b, there is a reduced level of calcineurin in the cytoplasmic fraction and an increased level of calcineurin in the plasma membrane fraction compared with the control pEGFP. *B*, quantification of calcineurin localization by densitometry, expressed as percentage of calcineurin levels in control pEGFP-transfected cells. Increased calcineurin levels in plasma membrane fractions and reduced calcineurin levels in the cytoplasmic fractions of cells as a result of co-expression of human PMCA4b are observed. Means of two independent experiments are shown.

B)
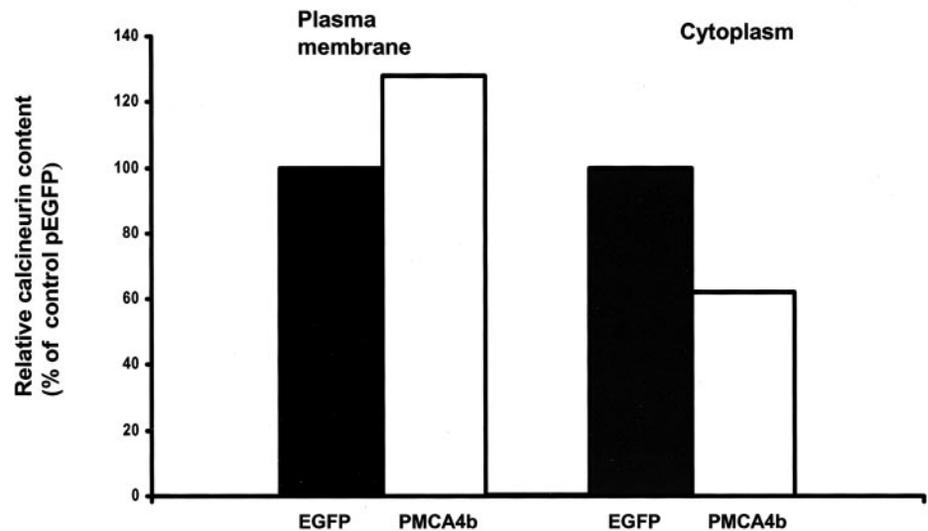
calcineurin, with the exception of calsarcins, exert their effects through interaction with calcineurin residues that overlap with the immunophilin-binding sites (33). This is in contrast to the PMCA binding domain, although AKAP79 has been reported to bind to two regions of calcineurin (residues 30–98 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.
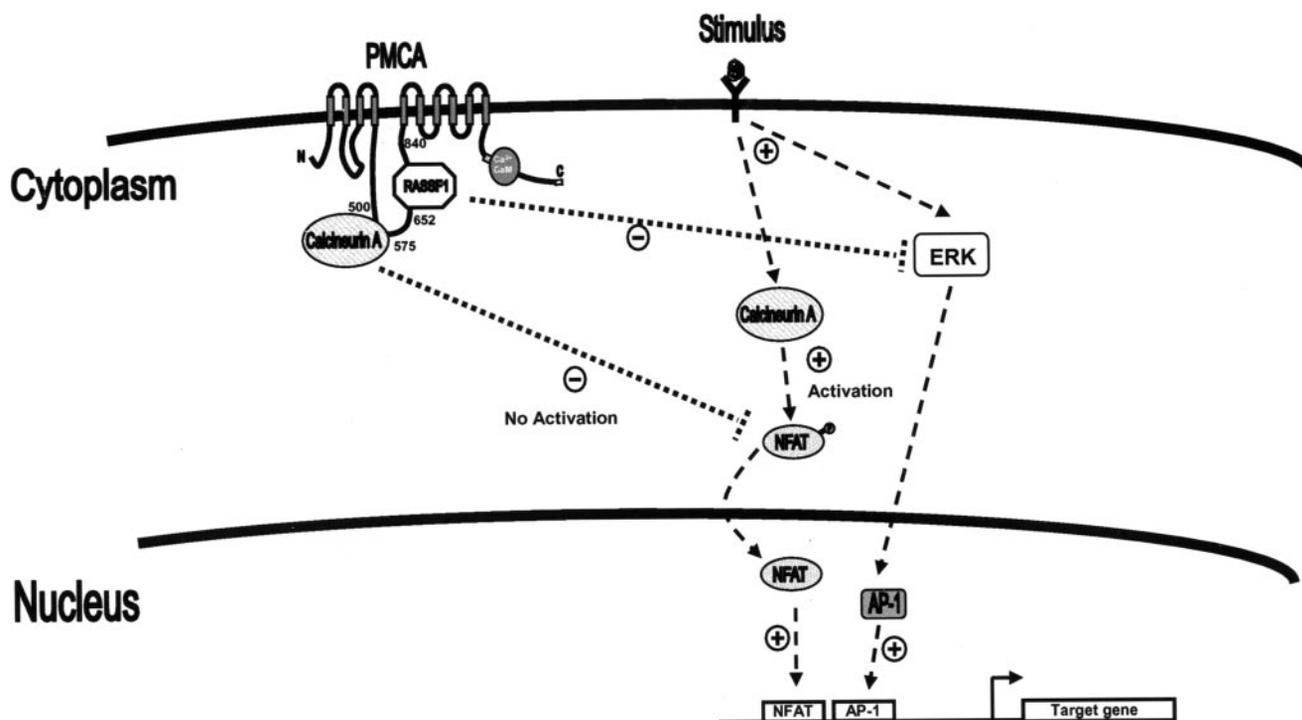
FIG. 7. **Schematic model depicting the proposed role of PMCA as an inhibitor of NFAT/AP-1-responsive genes.** The binding of an agonist to its specific receptor triggers the activation of the calcineurin/NFAT and ERK signaling pathways, and the subsequent transcriptional expression of NFAT/AP-1-responsive target genes. Interaction of calcineurin or RASSF1 with PMCA results in inhibition of the calcineurin (this report) and ERK (24) pathways, respectively, leading to a decrease in the transcriptional response to agonist stimulation.

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.

REFERENCES

1. Carafoli, E. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 1115–1122
2. Klee, C. B., Crouch, T. H., and Krinks, M. H. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 6270–6273
3. Kincaid, R. (1993) *Adv. Second Messenger Phosphoprotein Res.* **27,** 1–23
4. Klee, C. B., Ren, H., and Wang, X. (1998) *J. Biol. Chem.* **273,** 13367–13370
5. Rao, A., Luo, and Hogan, P. G. (1997) *Ann. Rev. Immunol.* **15,** 707–747
6. Crabtree, G. R., and Olson, E. N. (2002) *Cell.* **109,** S67-S79
7. Horsley, V., and Pavlath, G. K. (2002) *J. Cell Biol.* **156,** 771–774
8. Hubbard, M. J., and Klee, C. B. (1989) *Biochemistry* **28,** 1868–1874
9. Watanabe, Y., Perrino, B. A., Chang, B. H., and Soderling, T. R. (1995) *J. Biol. Chem.* **270,** 456–460
10. Sikkink, R., Haddy, A., MacKelvie, S., Mertz, P., Litwiller, R., and Rusnak, F. (1995) *Biochemistry* **34,** 8348–8356
11. Kincaid, R. L., Nightingale, M. S., and Martin, B. M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8983–8987
12. Hashimoto, Y., Perrino, B. A., and Soderling, T. R. (1990) *J. Biol. Chem.* **265,** 1924–1927
13. Schuh, K., Uldrijan, S., Telkamp, M., Röthlein, N., and Neyses L. (2001) *J. Cell Biol.* **155,** 201–205
14. Schuh, K., Uldrijan, S., Gambaryan, S., Röthlein, N., and Neyses L. (2003) *J. Biol. Chem.* **278,** 9778–9783
15. Strehler, E. E., and Zacharias, D. A. (2001) *Physiol. Rev.* **81,** 21–50

16. Shull, G. E., and Greeb, J. (1998) *J. Biol. Chem.* **263,** 8646–8657
17. Greeb, J., and Shull, G. E. (1989) *J. Biol. Chem.* **264,** 18569–18576
18. Verma, A. K., Filoteo, A. G., Stanford, D. R., Wieben, E. D., and Penniston, J. T. (1988) *J. Biol. Chem.* **263,** 14152–14159
19. Strehler, E. E., James, P., Fischer, R., Heim, R., Vorherr, T., Filoteo, A. G., Penniston, J. T., and Carafoli, E. (1990) *J. Biol. Chem.* **265,** 2835–2842
20. Heim, R., Iwata, T., Zvaritch, E., Adamo, H. P., Rutishauser, B., Strehler, E. E., Guerini D., and Carafoli, E. (1992) *J. Biol. Chem.* **267,** 24476–24484
21. Brown, B. J., Hilfiker, H., DeMarco, S. J., Zacharias, D. A., Greenwood, T. M., Guerini, D., and Strehler, E. E. (1996) *Biochim. Biophys Acta* **1283,** 10–13
22. Keeton, T. P., and Shull, G. E. (1995) *Biochem. J.* **306,** 779–785
23. Stauffer, T. P., Hilfiker, H., Carafoli, E., and Strehler, E. E. (1993) *J. Biol. Chem.* **268,** 25993–26003
24. Armesilla, A. L., Williams, J. C., Buch, M. H., Pickard, A., Emerson, M., Cartwright, E. J., Oceandy, D., Vos, M. D., Gillies, S., Clark, G. J., and Neyses, L. (2004) *J. Biol. Chem.* **279,** 31318–31328
25. Martinez-Martinez, S., and Redondo, J. M. (2004) *Curr. Med. Chem.* **11,** 997–1007
26. Copeland, J. W., and Treisman, R. (2002) *Mol. Biol. Cell.* **13,** 4088–4099
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
28. deBlaquiere, J., and Burgess, A. W. (1999) *J. Biomol. Tech.* **10,** 64–71
29. Sun, L., Youn, H.-D., Loh C., Stolow, M., He, W., and Liu, J. O. (1998) *Immunity* **8,** 703–711
30. Rothermel, B., Vega, R. B., Yang, J., Wu, R., Bassel-Duby, R., and Williams, R. S. (2000) *J. Biol. Chem.* **275,** 8719–8725
31. Huai, Q., Kim, H. Y., Liu, Y., Zhao, Y., Mondragon, A., Liu, J. O., and Ke, H. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 12037–12042
32. Kashishian, A., Howard, M., Loh, C., Gallatin, W. M., Hoekstra, M. F., and Lai, Y. (1998) *J. Biol. Chem.* **273,** 27412–27419
33. Vega, R. B., Bassel-Duby, R., and Olson, E. N. (2003) *J. Biol. Chem.* **278,** 36981–36984
34. Aramburu, J., García-Cóza, R. F., Raghavan, A., Okamura, H., Rao, A., and Hogan, P. G. (1998) *Mol. Cell* **1,** 627–637
35. Lee, H. K., Barbarosie, M., Kameyama, K., Bear, M. F., and Huganir, R. L. (2000) *Nature* **405,** 955–959
36. Faux, M. C., and Scott, J. D. (1996) *Cell* **70,** 8–12
37. Carnegie, G. K., and Scott, J. D. (2003) *Genes Dev.* **17,** 1557–1568
38. DeMarco, S. J., and Strehler, E. E. (2001) *J. Biol. Chem.* **276,** 21594–21600
39. Whitmarsh, A. J., and Davis, R. J. (1996) *J. Mol. Med.* **74,** 589–607
40. Macian, F., Lopez-Rodriguez, C., and Rao, A. (2001) *Oncogene* **20,** 2476–2489