THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 33, pp. 23341–23348, August 18, 2006

The Sarcolemmal Calcium Pump, α-1 Syntrophin, and Neuronal Nitric-oxide Synthase Are Parts of a Macromolecular Protein Complex*

Received for publication, December 15, 2005, and in revised form, May 30, 2006 Published, JBC Papers in Press, May 30, 2006, DOI 10.1074/jbc.M513341200

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The main role of the plasma membrane Ca^{2+}/calmodulin-dependent ATPase (PMCA) is in the removal of Ca^{2+} from the cytosol. Recently, we and others have suggested a new function for PMCA as a modulator of signal transduction pathways. This paper shows the physical interaction between PMCA (isoforms 1 and 4) and α-1 syntrophin and proposes a ternary complex of interaction between endogenous PMCA, α-1 syntrophin, and NOS-1 in cardiac cells. We have identified that the linker region between the pleckstrin homology 2 (PH2) and the syntrophin unique (SU) domains, corresponding to amino acids 399–447 of α-1 syntrophin, is crucial for interaction with PMCA1 and -4. The PH2 and the SU domains alone failed to interact with PMCA. The functionality of the interaction was demonstrated by investigating the inhibition of neuronal nitric-oxide synthase-1 (NOS-1); PMCA is a negative regulator of NOS-1-dependent NO production, and overexpression of α-1 syntrophin and PMCA4 resulted in strongly increased inhibition of NO production. Analysis of the expression levels of α-1 syntrophin protein in the heart, skeletal muscle, brain, uterus, kidney, or liver of $PMCA4^{-/-}$ mice, did not reveal any differences when compared with those found in the same tissues of wild-type mice. These results suggest that PMCA4 is tethered to the syntrophin complex as a regulator of NOS-1, but its absence does not cause collapse of the complex, contrary to what has been reported for other proteins within the complex, such as dystrophin. In conclusion, the present data demonstrate for the first time the localization of PMCA1b and -4b to the syntrophin·dystrophin complex in the heart and provide a specific molecular mechanism of interaction as well as functionality.

Calcium ion (Ca^{2+}) regulation is critical for cell function and survival (1). The mechanisms that increase local calcium concentration in the heart have been extensively studied, however, less is known about the calcium extrusion mechanisms. The sarcolemmal calcium pump or plasma membrane calcium/calmodulin-dependent calcium ATPase (PMCA)[3] is expressed by most cell types, including cardiomyocytes (2). Four PMCA isoforms have been identified along with multiple splice variants; all have well defined tissue-specific expression patterns (2). In non-excitable cells, the primary function of PMCA is to expel calcium from the cytosol. In excitable cells, such as cardiomyocytes, the function of PMCA is less clear, as the sodium/calcium exchanger plays the dominant role in extrusion of Ca^{2+} across the sarcolemma. As a result, PMCA is presumed to contribute to the maintenance of low diastolic Ca^{2+} levels. In addition to their role as Ca^{2+} transporters, our group and others have identified functional interactions between PMCAs and cytoplasmic signaling proteins, suggesting a function for PMCAs as modulators of signal transduction pathways (3–8). The physiological role of PMCA in both cell signaling and regulation of calcium is emerging through the development and analysis of transgenic animals with modified PMCA expression (9–12).

Syntrophin, a member of the dystrophin protein complex that interacts with the COOH-terminal region of dystrophin, is involved in organizing functional signaling complexes at the cytoskeleton-plasma membrane (13–15). The loss of dystrophin and subsequent disruption of the dystrophin protein complex from the sarcolemma in Duchenne muscular dystrophy leads ultimately to degeneration of muscles (16). Disruption of the dystrophin protein complex has also been implicated in acquired forms of dilated cardiomyopathy (17) and as a result of viral myocarditis (18). A cardiomyopathic phenotype is observed in many patients with Becker muscular dystrophy, Duchenne muscular dystrophy, as well as other muscular dystrophies, but at the molecular level, the pathogenesis is incompletely understood (19).

Five isoforms of syntrophin have been described (α-1, β-1, β-2, γ-1, and γ-2), with different tissue distributions and developmental time courses indicating distinct functions (20–22). Each syntrophin isoform comprises four conserved domains, two pleck-

* This study was supported by an international appointee grant (awarded to L. N.) by the Medical Research Council. 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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[3] The abbreviations used are: PMCA, plasma membrane calcium calmodulin ATPase; NOS-1, neuronal nitric-oxide synthase; PH, pleckstrin homology; SU syntrophin-unique; CMV, cytomegalovirus; HEK, human embryonic kidney.

strin homology domains (PH1 and PH2), a PDZ domain, and a syntrophin unique (SU) COOH-terminal domain. PH1 and PH2, so called as they show homology to a region repeated in the protein pleckstrin, have been shown to be involved in the recruitment of proteins to the sarcolemma (23). The PDZ domain is inserted within the PH1 domain and has been shown to bind to NOS-1 in skeletal muscle (24). The SU COOH-terminal domain binds syntrophin to dystrophin (25). The fact that there are up to four syntrophin binding sites in close proximity within a single dystrophin complex (26) suggests that syntrophin may bring multiple signaling molecules together to form a large signaling machine. Such co-localization may improve the efficiency of the signaling complex or increase the specificity of the signals generated by the signaling cascade. In skeletal muscle cells, a muscle-specific isoform of neuronal nitric-oxide synthase (NOS-1), NOS-1μ, binds to α-1 syntrophin, thereby localizing NOS-1μ to the sarcolemma and the dystrophin complex (27). Our group has previously demonstrated that PMCA4b acts as a negative regulator of NOS-1 through interactions between the PDZ domain of NOS-1 and the COOH terminus of PMCA (3). We therefore wished to determine whether PMCA interacted with syntrophin, thereby linking the calcium pump to signaling from the dystrophin complex.

EXPERIMENTAL PROCEDURES

Cell Culture—HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.

Plasmids—The expression vectors pCMV-hPMCA4b and pMM2-hPMCA1b containing the sequence from hPMCA4b and hPMCA1, respectively, were a gift from Prof. E. Strehler (Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN).

The generation of pBThPMCA4b-(652–840), pFLAG-hPMCA4b-(652–840), and pFLAG-hPMCA4b-(749–840) has been described previously (5). The plasmid pcDNA3SYN was constructed by cloning a NotI and XhoI fragment containing the α-1 syntrophin full-length mouse cDNA into pcDNA3 (α-1 syntrophin mouse cDNA was a generous gift from Prof. S. Froehner, Department of Physiology and Biophysics, University of Washington, Seattle, WA). The pFLAG-mSYN constructs (numbered according to GenBank™ NM_009228) were generated by 35 cycles of PCR (the conditions were: denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s, and extension at 72 °C for 1 min) using the oligonucleotides mSYN81 sense (5′-TCTTCCGGAATTCCGTGACGGTGCGCAAGGCCG-3′), mSYN160 antisense (5′-TCTTCGCGGATCCTCAAACCTCCAACACAACCTCCTTGCCTG-3′), mSYN292 sense (5′-TCTTCCGGAATTCCGGCTGGCTGACAGAACAGTTGCCC-3′), mSYN399 antisense (5′-TCTTCGCGAATTCTCAATATGACAGCCATCCACCAACTGTCGGGT-3′), mSYN399 sense (5′-TCTTCCGGAATTCCATCGGGCTGCTGAAGGCATAC-3′), mSYN447 antisense (5′-TCTTCGCGGATCCTCACATGGGCTGTCGGAGCAGCATGG-3′), mSYN447 sense (5′-TCTTCCGGAATTCCCCTTCGAGAAACTTCAGATGTCATCAGATG-3′), and mSYN503 antisense (5′-TCTTCGCGGATCCTCACTAGGCCAAGAGCCCCAAGCGG-3′).

Amplified products were cloned into the EcoRI-BamHI sites of plasmid pFLAG-CMV7.1 (Sigma-Aldrich) and sequenced. The resulting plasmids pFLAG-mSYN-(81–160), -(292–503), -(292–447), -(399–503), and -(447–503) encode FLAG-tagged syntrophin-truncated proteins containing amino acids 81–160, 292–503, 292–447, 399–503, and 447–503, respectively.

Bacterial Two-hybrid Screening—A Human Fetal Heart cDNA library (Stratagene) was screened using the BacterioMatch™ two-hybrid system vector kit. The library ($\sim 2 \times 10^6$ independent cDNA clones) was assayed for resistance to carbenicillin and for β-galactosidase expression. Positive clones were sequenced following standard procedures.

Immunoprecipitation in HEK293 Cells and Heart Tissue of PMCA/Syntrophin/NOS-1—The protocol for immunoprecipitations using transfected HEK293 cells has been described previously (5). Mouse heart tissue was lysed using radioimmune precipitation assay buffer (1× phosphate-buffered saline, 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). Precleared extracts were incubated overnight with the corresponding antibody; either 5F10 (an anti-PMCA monoclonal antibody, Upstate Biotechnology), a rabbit polyclonal anti-PMCA4b antibody (Swant), a rabbit polyclonal anti-PMCA1 (Upstate Biotechnology), a rabbit polyclonal anti-α-1 syntrophin (Sigma), a rabbit polyclonal anti-NOS-1 (Affinity Bioreagents), or a rabbit polyclonal anti-luciferase antibody (Promega). Immunoprecipitated proteins were recovered by incubation with protein A-agarose beads and analyzed by Western blot.

Transient Transfections—The protocol for transient transfection of HEK293 cells has been described previously for immunoprecipitation experiments and functionality testing (5). Cells were co-transfected with 1.0 μg of pCMV-NOS-1 (27) or the corresponding empty vector (pcDNA3) and the expression vectors pCMV-hPMCA4b and/or pcDNA3SYN, encoding human PMCA4b and mouse α-1 syntrophin, respectively. cGMP activity was assessed 24 h after transfection using cGMP (low pH) colorimetric competitive enzyme-linked immunosorbent assay (R & D Systems) to determine NO-dependent cGMP production as described previously (3).

Pmca4$^{-/-}$ Mice—We have previously described the generation of the PMCA4 null mutant mice (10). All animal procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Tissue was collected from PMCA4 mutant mice and wild-type litter mates at 12 weeks of age. Western blot analysis was undertaken using anti-α-1 syntrophin polyclonal antibodies (Sigma) and a secondary anti-rabbit IgG antibody horseradish peroxidase-conjugated (Jackson ImmunoResearch Laboratories). Glyceraldehyde-3-phosphate dehydrogenase was used for the protein loading control, and blots were stripped after initial protein identification using Restore™ Western blot stripping buffer (Pierce) and probed with anti-glyceraldehyde-3-phosphate dehydrogenase (Advanced Immunochemical) primary antibody and then labeled with peroxidase-conjugated goat anti-mouse IgG (Dako).

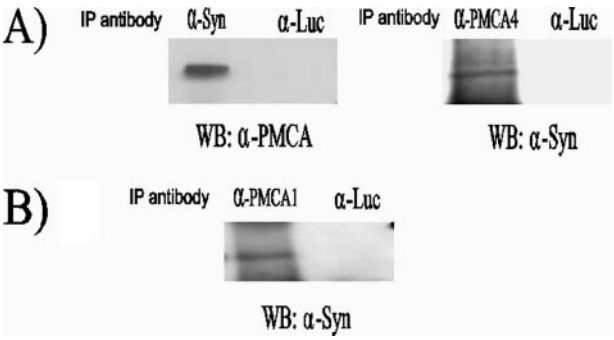


FIGURE 1. **Identification of a physical interaction between α-1 syntrophin and PMCA in mammalian cells.** *A*, HEK293 cells were co-transfected with expression vectors pCMV-hPMCA4b and pcDNA3SYN encoding human PMCA4b and mouse α-1 syntrophin, respectively. Protein lysates were incubated with a polyclonal anti-α-1 syntrophin antibody (Sigma) or a rabbit polyclonal antibody specific for PMCA4 (Swant) prior to precipitation with protein A-agarose beads. Western blots (*WB*) of the immunoprecipitated (*IP*) proteins were probed with either the JA3 (Neomarkers) monoclonal anti-PMCA4b antibody (*left panel*) or anti-α-1 syntrophin antibody (Sigma) (*right panel*) to detect the presence of human PMCA4b or α-1 syntrophin, respectively. Co-precipitation of ectopically expressed human PMCA4b and mouse α-1 syntrophin suggests the physical interaction of these two proteins in mammalian cells. Immunoprecipitations carried out with an irrelevant antibody (α-*Luc*) raised against the firefly luciferase ruled out the possibility of nonspecific binding of immunoprecipitating antibodies to protein A-agarose beads. *B*, HEK293 cells were transfected with expression vectors pMM2-hPMCA1b and pcDNA3SYN encoding human PMCA1b and mouse α-1 syntrophin, respectively. Protein lysates from transfected cells were incubated with an anti-PMCA1 rabbit polyclonal antibody (Upstate Biotechnology). Western blot analysis of the precipitated proteins using an anti-α-1 syntrophin rabbit polyclonal antibody (Sigma) indicated co-precipitation of PMCA1b and α-1 syntrophin. Immunoprecipitations carried out with an irrelevant antibody (α-*Luc*) raised against the firefly luciferase ruled out the possibility of nonspecific binding of immunoprecipitating antibodies to protein A-agarose beads. These results demonstrate the interaction in mammalian cells of recombinant α-1 syntrophin and isoforms 1b and 4b of the plasma membrane calcium pump.

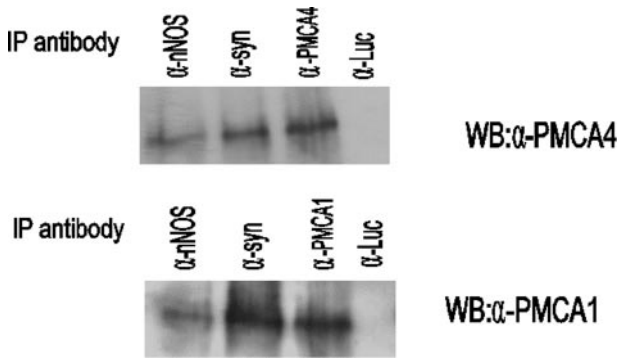


FIGURE 2. **Endogenous PMCA, α-1 syntrophin, and NOS-1 physically interact in cardiac cells.** Mouse heart proteins were incubated with a polyclonal antibody raised against either α-1 syntrophin (α-*syn*) or NOS-1 (α-*nNOS*). Immunoprecipitation (*IP*) was carried out with protein A-agarose beads. Western blot (*WB*) of the immunoprecipitated proteins was probed with a polyclonal antibody against either PMCA4 (Swant) (*upper panel*) or PMCA1 (Upstate Biotechnology) (*lower panel*). Endogenous α-1 syntrophin and NOS-1 co-precipitated with both PMCA4 and PMCA1, suggesting the physical interaction of these proteins within a macromolecular complex in cardiac cells. Control immunoprecipitations carried out with antibodies raised against PMCA4 (α-*PMCA4*) (*upper panel*) or PMCA1 (α-*PMCA1*) (*lower panel*), respectively, were included as positive controls. Control immunoprecipitations performed with an irrelevant polyclonal antibody raised against the firefly luciferase (α-*Luc*) ruled out nonspecific binding of the immunoprecipitating antibodies.

RESULTS

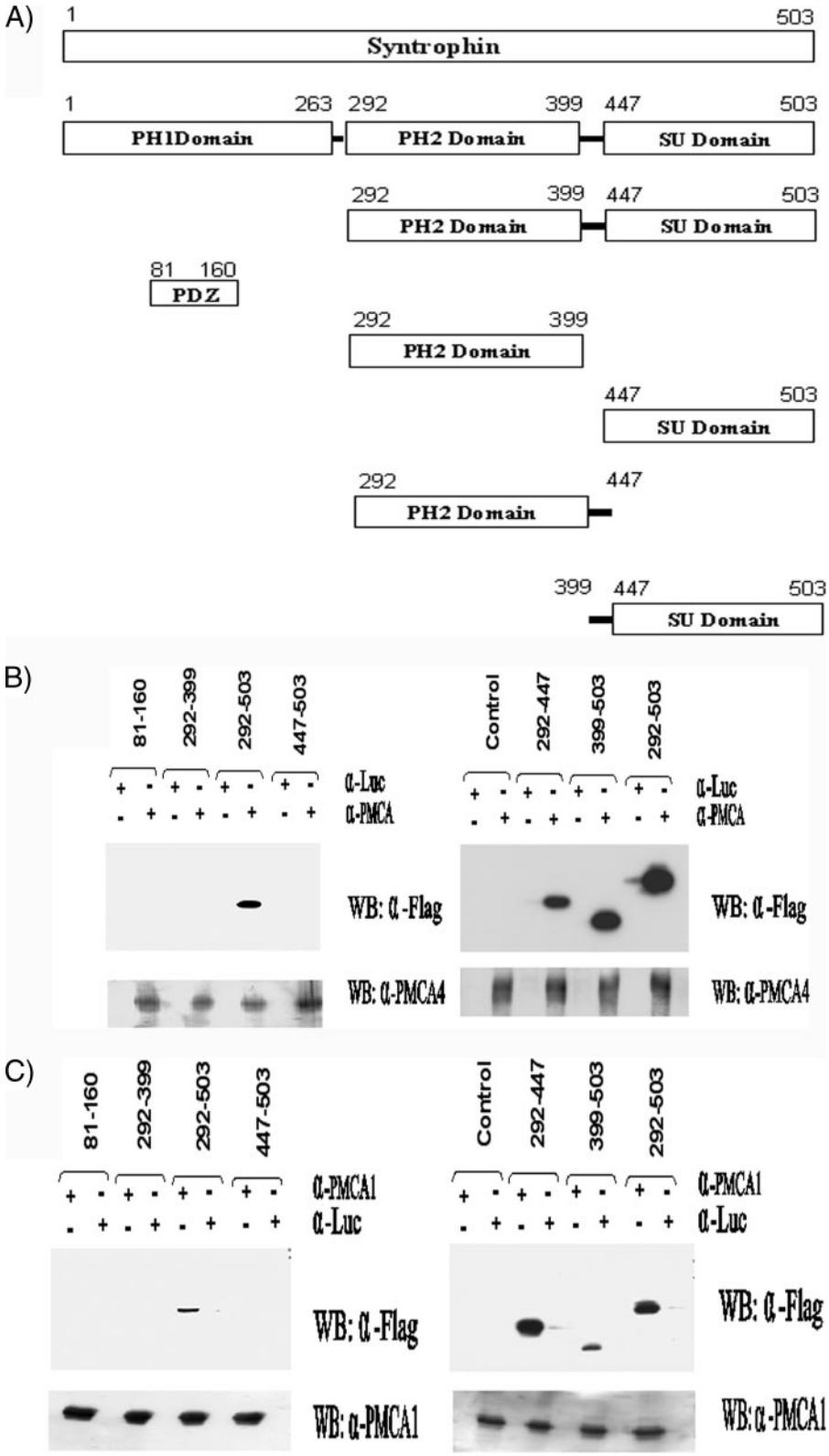
PMCA Interacts with α-1 Syntrophin and NOS-1—Previous work by our group and others has demonstrated the interaction between the COOH terminus of PMCA and PDZ domains contained in partner proteins (3, 4, 7, 8, 28). These findings raised the possibility that α-1 syntrophin interacts with PMCA4b via its PDZ domain. PMCA4b terminates with amino acids ETSV, which fits the consensus motif E(S/T)*X*V, essential for binding to the syntrophin PDZ domain (29). However, two-hybrid screening of a human fetal heart cDNA library using the COOH terminus of PMCA4b as bait failed to identify syntrophin as a potential interaction partner. Additional two-hybrid screening was then undertaken using a region of the large catalytic intracellular loop of PMCA4b to identify potential interaction partners. A construct was designed, incorporating amino acids 652–840 (GenBank™ accession number NM_001684) (5). The screening identified α-1 syntrophin as a potential interaction partner of PMCA4. Sequence analysis of the α-1 syntrophin clone revealed that the sequence encoding for the PDZ domain (81–164) was absent, and the truncated clone contained amino acids corresponding to the PH2 and SU domains 335–503 (GenBank™ accession number NM_009228).

To confirm the physical interaction between human PMCA4b and α-1 syntrophin, plasmids containing full-length hPMCA4b (pCMVhPMCA4b) and α-1 syntrophin (pcDNA3SYN) were transfected into HEK293 cells. Protein extracts were immunoprecipitated with a polyclonal anti-α-1 syntrophin antibody (Sigma). The presence of PMCA4b among the precipitated proteins was detected by Western blot with JA3 (Neomarkers), a mouse monoclonal antibody recognizing human PMCA4b specifically (Fig. 1*A*, *left panel*). Conversely, protein lysates from transfected HEK293 cells were immunoprecipitated with a rabbit polyclonal antibody raised against PMCA4 (Swant) and immunoprecipitated proteins analyzed by Western blot using a polyclonal anti-α-1 syntrophin antibody (Sigma). α-1 Syntrophin co-precipitated together with PMCA4, demonstrating the physical interaction between the two proteins (Fig. 1*A*, *right panel*).

The high degree of homology between the PMCA isoforms in the region encompassing amino acids 652–840 suggested that other isoforms might also interact with α-1 syntrophin. The functional significance of syntrophin in skeletal and cardiac muscle prompted us to examine its potential interaction with PMCA1, the other PMCA isoform expressed in these muscle types (2). To test this possibility, plasmids pMM2-hPMCA1b (encoding hPMCA1b) and pcDNA3SYN (encoding mouse α-1 syntrophin) were transfected in HEK293 cells. Protein extracts were immunoprecipitated with an anti-PMCA1 rabbit polyclonal antibody (Upstate Biotechnology). Co-precipitated α-1 syntrophin was detected by Western blot of the precipitated samples probed with an anti-α-1 syntrophin rabbit polyclonal antibody (Sigma) (Fig. 1*B*).

Control immunoprecipitations with an irrelevant antibody (anti-luciferase) did not precipitate any protein, ruling out the possibility of nonspecific binding of the immunoprecipitating antibodies to protein A-agarose beads (Fig. 1). These results demonstrate the physical interaction between ectopically expressed PMCA4b or PMCA1b and α-1 syntrophin and extend our initial observations from two-hybrid experiments to mammalian cells.

Endogenous Interaction in the Heart—To confirm the relevance of this interaction in the heart, the physical interaction between endogenous α-1 syntrophin and PMCA was examined. Previously reported interactions between PMCA and NOS-1 (3) and α-1 syntrophin and NOS-1 (27) suggest these proteins might be part of a macromolecular complex. In view of the functional significance of α-1 syntrophin in the heart and the expression of PMCA4, PMCA1, and NOS-1 in cardiac muscle (2, 30), we tested this hypothesis in heart tissue. Protein lysates from mouse heart tissue were immunoprecipitated with



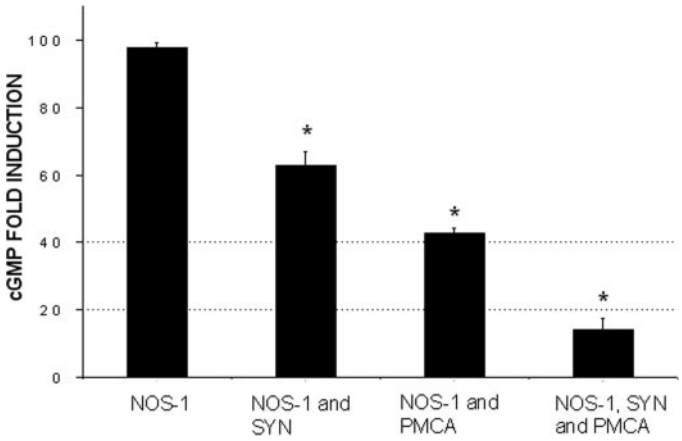


FIGURE 4. **Co-expression of α-1 syntrophin and hPMCA4b inhibits NO production.** HEK293 cells were co-transfected with either NOS-1, α-1 syntrophin, PMCA4b, or pcDNA3 empty vector or with a combination of vectors. Ectopic expression of NOS-1 enhanced cGMP production in HEK293 cells. Co-expression of mouse α-1 syntrophin or human PMCA4b, together with NOS-1, reduced the NOS-1-dependent cGMP production by 38 and 58%, respectively. When both α-1 syntrophin and PMCA4b were co-expressed in the presence of NOS-1, 84% inhibition in the production of cGMP was observed. These results suggest that the interaction of PMCA4b and α-1 syntrophin synergistically inhibit NOS-1-mediated NO production. The *asterisk* denotes statistically significant ($p \leq 0.01$, according to Student's *t* test) inhibition in NO production as observed in cGMP production. Means ± S.E. of three independent experiments are shown. Data are expressed as fold induction over the value obtained in cells transfected with the corresponding empty vector.

a polyclonal antibody raised against either α-1 syntrophin or NOS-1 (nNOS). Cardiac PMCA1 and -4 were detected in the immunoprecipitated proteins by Western blot probed with a polyclonal antibody against PMCA1 (Upstate Biotechnology) and a polyclonal antibody against PMCA4 (Swant), respectively (Fig. 2). These results demonstrate that endogenous α-1 syntrophin and NOS-1 physically interact with both PMCA4 and PMCA1 in cardiac cells, suggesting that PMCA, α-1 syntrophin, and NOS-1 are part of a macromolecular complex in heart cells.

α-1 Syntrophin Interacts with PMCA via a Unique Binding Domain—Truncated fusion proteins linking the FLAG epitope to individual, conserved domains of α-1 syntrophin were expressed to identify the binding domain responsible for the interaction between α-1 syntrophin and PMCA. The bacterial two-hybrid screen identified the COOH-terminal region of α-1 syntrophin, corresponding to amino acids 335–503, as an interaction partner of PMCA4. The COOH terminus of PMCA4b contains the consensus motif essential for interaction with syntrophin PDZ domains (29), and initially it had been presumed that PMCA interacted weakly with syntrophin via a PDZ ligand-PDZ domain interaction (2). To determine whether α-1 syntrophin could bind simultaneously to the large intracellular loop of PMCA4b and the COOH terminus, FLAG-tagged truncated proteins were constructed for the PDZ domain (pFLAG-mSYN-(81–160)), PH2 domain (pFLAG-mSYN-(292–399)), a region spanning the PH2 and SU domains (pFLAG-mSYN-(292–503)), and the SU (pFLAG-mSYN-(447–503)) domain of α-1 syntrophin (Fig. 3*A*). These plasmids were transfected in HEK293 cells and assayed by immunoprecipitation for their abilities to interact with ectopically expressed human PMCA4b. The region spanning the PH2 and SU domains (pFLAG-mSYN-(292–503)) co-precipitated with PMCA4b (Fig. 3*B*, *left upper panel*). However, no precipitation was observed with the PDZ, PH2, or SU domains (Fig. 3*B*, *left upper panel*). This result indicated that either both the PH2 and SU domains were important for interaction with PMCA4b to take place or that a linker region (amino acids 399–447) between the two domains was critical. To resolve this, new constructs encoding FLAG-tagged fusion proteins were generated containing either the PH2 domain with the linker region (pFLAG-mSYN-(292–447)) or the SU domain with the linker region (pFLAG-mSYN-(399–503)). Co-precipitation experiments confirmed that both plasmids interacted with PMCA4b (Fig. 3*B*, *right upper panel*), thereby establishing that amino acids 399–447 are critical for α-1 syntrophin to interact with PMCA4b. Western blot analysis of the immunoprecipitated proteins probed with a polyclonal antibody specific for PMCA4 (Swant) showed comparable levels of PMCA4 immunoprecipitation in all cases, thus ruling out the possibility of poor immunoprecipitation as the reason for the lack of interaction (Fig. 3*B*, *lower panels*).

Having previously demonstrated the interaction of α-1 syntrophin with both the PMCA4 and -1 isoforms, we wished to determine whether the same interaction domain of α-1 syntro-

FIGURE 3. **α-1 Syntrophin interacts with both PMCA4b and -1b via a unique binding domain.** *A*, schematic diagram of the α-1 syntrophin domains used to generate α-1 syntrophin truncated FLAG-tagged fusion proteins. The numbers correspond to amino acids, according to GenBank™ accession number Q61234. *B*, amino acids 399–447 of α-1 syntrophin are critical for its interaction with PMCA4b. HEK293 cells were co-transfected with an expression vector encoding PMCA4b (pCMV-hPMCA4b) and the corresponding construct expressing FLAG-syntrophin truncated proteins as indicated *above* the *lanes*. Proteins were immunoprecipitated with the anti-PMCA 5F10 monoclonal antibody and the presence of co-immunoprecipitated FLAG-syntrophin proteins detected by Western blot (*WB*) using the M2 anti-FLAG peroxidase-conjugated monoclonal antibody. This experiment shows that the peptide corresponding to the PH2 and SU domains (pFLAG-mSYN-(292–503)) interacts with hPMCAb (*left upper panel*). The truncated proteins corresponding to the PDZ domain (pFLAG-mSYN-(81–160)), PH2 domain (pFLAG-mSYN-(292–399)), and SU domain (pFLAG-mSYN-(447–503)) do not interact with full-length hPMCA4b (*left upper panel*). The truncated proteins corresponding to the PH2 domain, including the linker region (pFLAG-mSYN-(292–447)), and the SU domain, including the linker region (pFLAG-mSYN-(399–503)), interacted with full-length PMCA4b (*right upper panel*). These results demonstrate that the linker region (amino acids 399–447) is critical for the interaction between α-1 syntrophin and PMCA4b. Levels of immunoprecipitated PMCA4b in the different transfection experiments were confirmed to be comparable by Western blot using a polyclonal antibody specific for PMCA4 (Swant) (*lower panels*), thus ruling out the possibility of poor immunoprecipitation as the reason for lack of interaction. Control immunoprecipitations using an antibody against firefly luciferase (+ α-Luc) were carried out to rule out nonspecific binding of antibodies to protein A-agarose beads. *C*, the domain of α-1 syntrophin involved in the interaction with PMCA4b also mediates the interaction with PMCA1b. HEK293 cells were co-transfected with pMM2-hPMCA1b, an expression vector encoding PMCA1b, and the plasmids encoding the FLAG-syntrophin truncated fusion proteins described in *B*. As reported for PMCA4b, immunoprecipitation experiments demonstrated that domain 399–447 of α-1 syntrophin is also critical for the interaction with PMCA1b (*upper panels*). Levels of immunoprecipitated PMCA1b in the different transfection experiments were confirmed to be comparable by Western blot using a polyclonal antibody specific for PMCA1 (Upstate Biotechnology) (*lower panels*), thus ruling out the possibility of poor immunoprecipitation as the reason for lack of interaction. Control immunoprecipitations using an antibody against firefly luciferase (+ α-Luc) were carried out to rule out nonspecific binding of antibodies to protein A-agarose beads.

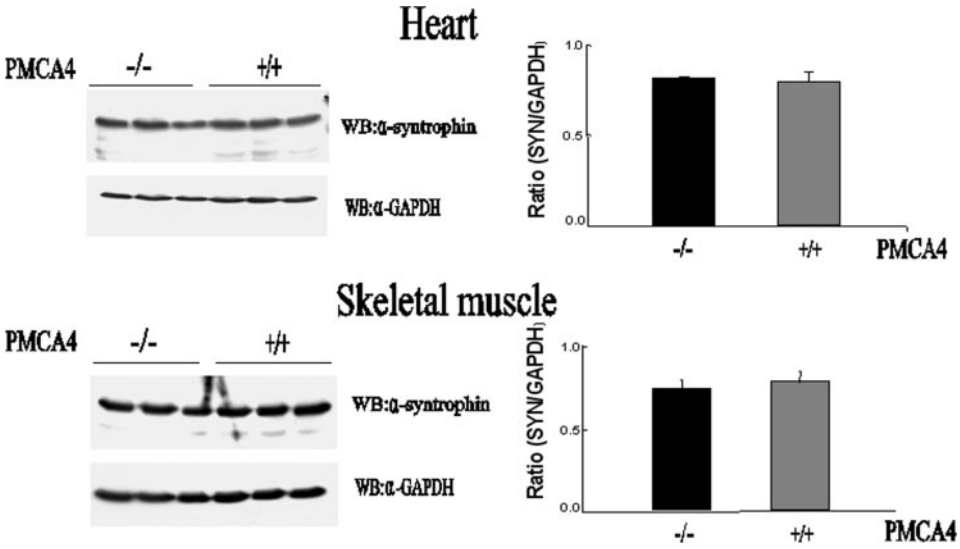


FIGURE 5. **α-1 Syntrophin protein levels are unaffected in PMCA4$^{-/-}$ mice.** Expression levels of α-1 syntrophin in hearts or skeletal muscle of 3-month-old wild-type or PMCA4 null mice were analyzed by Western blot (*WB*) using a polyclonal anti-α-1 syntrophin antibody (Sigma). Levels of α-1 syntrophin were compared with those of the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) detected with an anti-GAPDH antibody (Advanced Immunochemical). Data from the densitometry analysis of Western blots from different animals ($n = 3$) showed no alterations in the levels of α-1 syntrophin between wild-type and PMCA4$^{-/-}$ animals.

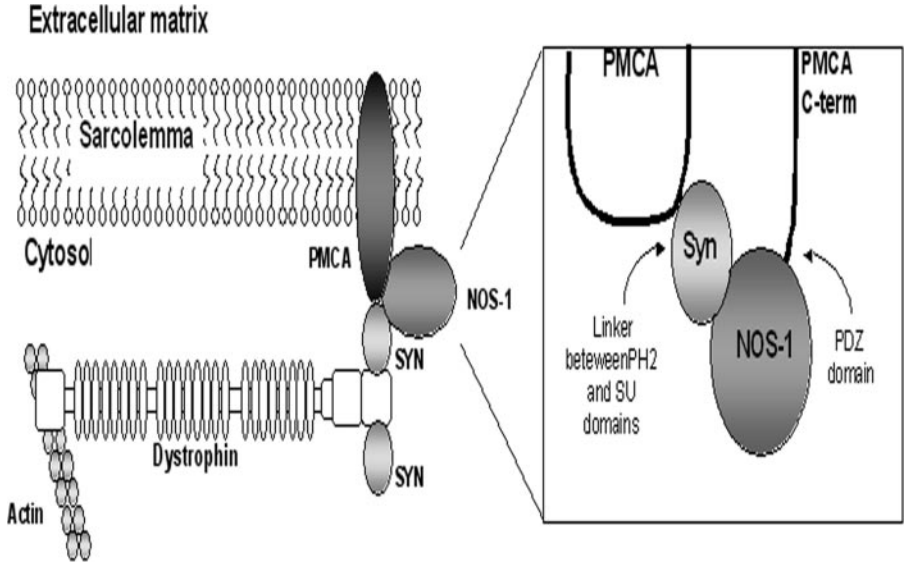


FIGURE 6. **Diagrammatic representation of the interaction between PMCA, α-1 syntrophin, and NOS-1.** The diagram highlights the interaction between PMCA via syntrophin and NOS-1 to dystrophin. The expanded diagram provides a more detailed illustration of the binding of PMCA via the intracellular loop located between transmembrane domains 4 and 5 to syntrophin and via the COOH terminus to the PDZ domain of NOS-1.

phin was responsible for interaction with PMCA4 and -1. HEK293 cells were co-transfected with an expression plasmid encoding PMCA1b (pMM2-hPMCA1b) and the FLAG-tagged truncated versions of α-1 syntrophin described above. Identical results to that observed for PMCA4 were obtained withPMCA1 (Fig. 3*C*), demonstrating that interaction with either PMCA1 or -4 maps to the same domain (399–447) of α-1 syntrophin.

α-1 Syntrophin Modulates NOS-1 Activity—Our group has recently shown that PMCA4b negatively regulates the activity of NOS-1 (3). The functional consequences of the interaction between α-1 syntrophin and PMCA may thus involve the synergistic action of α-1 syntrophin and PMCA in regulating NOS-1 activity. It is known that α-1 syntrophin interacts with NOS-1 (27) and that PMCA4b and NOS-1 interact; we therefore wished to determine whether the formation of the complex PMCA4b·α-1 syntrophin·NOS-1 negatively regulates NO production. Transient transfection of α-1 syntrophin in the presence of NOS-1 led to a 38% reduction in NOS-1 activity (Fig. 4). Co-expression of both proteins produced an 84% ($p = 0.05$) reduction over and above that observed by the expression of PMCA4b alone (58%) (Fig. 4). The expression of α-1 syntrophin, PMCA4b, or pcDNA3 empty vector control in the absence of NOS-1 did not alter cGMP production (data not shown). These results suggest that the interaction of PMCA4b and syntrophin synergistically inhibit NOS-1-mediated NO production, because maximum inhibition was observed in cells expressing both proteins.

α-1 Syntrophin Protein Levels Are Unaffected in PMCA4$^{-/-}$ Mice—We have generated PMCA4 null mutant mice (10) to determine the role of PMCA4 in the regulation of cardiac function. Western blot analysis demonstrated that α-1 syntrophin protein expression was not altered in the heart, skeletal muscle, brain, uterus, kidney, or liver of PMCA4$^{-/-}$ mice when compared with the expression of the protein in the same tissues of wild-type littermates at three months of age ($p < 0.001$) (Fig. 5 and data not shown). The Western blots were normalized by comparing the levels of glyceraldehyde 3-phosphate dehydrogenase protein expression. These results suggest that PMCA4 is tethered to the syntrophin complex as a regulator of NOS-I, but its absence does not cause collapse of the complex, contrary to what has been reported for other proteins within the complex, such as dystrophin.

DISCUSSION

Our initial hypothesis was that syntrophin binds to PMCA4b via the PDZ binding motif and, through these interactions, links PMCA signaling to the dystrophin complex. We have previously shown that PMCA4b and NOS-1 interact via PDZ binding and that PMCA inhibits NOS-1 activity (3). α-1 Syntrophin contains a PDZ domain that could potentially bind to the ETSV consensus sequence at the COOH terminus of PMCA4b. How-

ever, in this paper, we have shown that the PDZ domain of α-1 syntrophin does not interact directly with full-length PMCA4b. We have established that α-1 syntrophin interacts with the distal region of the large intracellular loop of PMCA4. Our group has previously reported a functional interaction between this domain of PMCA4 and the Ras association factor RASSF1 (5). The interaction of another protein with the same region of PMCA highlights the importance of the large cytoplasmic loop of PMCA for associations with partner proteins. We are currently investigating the effect of this interaction on the calcium-pumping function of PMCA.

We have also determined that the linker region between the PH2 and SU domains (corresponding to amino acids 399–447) of α-1 syntrophin is crucial for the interaction to take place. This is similar to the mechanism that has been reported for α-1 syntrophin binding to utrophin, where individual PH2 or SU domains fail to bind (31) and interaction only takes place in the presence of both domains. We propose a model for a ternary interaction between PMCA, α-1 syntrophin, and NOS-1 (Fig. 6), where PMCA4b is linked to NOS-1 through interactions between the COOH-terminal tail of PMCA and the PDZ domain of NOS-1 and α-1 syntrophin is tethered to the complex through interactions between the linker region between the PH2 and SU domains and the large intracellular loop between transmembrane regions four and five of PMCA4.

There is a growing body of evidence suggesting that PMCA acts as a modulator of signal transduction pathways (3–8). In this work, we have established that PMCA and α-1 syntrophin act synergistically to negatively regulate NOS-1 activity, which has significant implications for the functional role of this interaction on nitric oxide-regulated signaling pathways. The importance of NOS-1 signaling in the heart is well established. NOS-1 ablation enhances basal contractility (33, 34), and NOS-1-derived NO increases as a consequence of experimental and pathological human heart failure (35). It has recently been shown that NOS-1 expression is up-regulated in the final stages of late phase ischemic preconditioning (36), suggesting it may be important in protecting the heart against myocardial infarction. The role of the interactions between PMCA, α-1 syntrophin, and NOS-1 on NOS-1 regulation in cardiac cells requires further investigation.

We have not detected modifications in the levels of α-1 syntrophin expressed in the hearts of transgenic PMCA4 knock-out mice. We show that PMCA1, the other isoform of PMCA present in cardiac muscle, is also able to bind to α-1 syntrophin in cardiac cells. Therefore, it is likely that the lack of PMCA4 expression can be compensated for by the presence of PMCA1 in the PMCA4$^{-/-}$ mice. Loss of PMCA1 in null mutant mice results in embryonic lethality (11), making difficult the generation of PMCA1/4 double knock-out mice required to study the physiological consequences of the disruption of the PMCA-syntrophin interaction.

Our molecular model of a PMCA·α-1 syntrophin·NOS-1 complex also brings PMCA into close contact with the dystrophin protein complex, where it could regulate signaling either through direct interactions or by alterations in Ca^{2+} and/or nitric oxide signaling. Dystrophin has been shown to co-localize with the cardiac L-type Ca^{2+} channel and inactivate channel activity (32). This inactivation was reduced in cardiac myocytes from mice lacking dystrophin (*mdx* mice) (32). One theory is that cardiac tissue becomes more susceptible to damage from Ca^{2+} loading, and cytoskeletal disruption appears to alter Ca^{2+} channel kinetics, a potential mechanism for cardiac dysfunction observed in Duchenne and Becker muscular dystrophies (32). We speculate that an analogous system may exist for the regulation of PMCA activity by dystrophin via interactions with α-1 syntrophin.

In conclusion, the present data demonstrate for the first time the localization of PMCA1b and -4b to the syntrophin·dystrophin complex in the heart and provide a specific molecular mechanism of interaction as well as functionality. We are currently investigating how this interaction regulates nitric-oxide synthase activity at a molecular level. It will be interesting to address these observations in other tissues that contain the complex, such as skeletal, smooth muscle, and brain tissues.

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