The plasma membrane calmodulin–dependent calcium pump: a major regulator of nitric oxide synthase I

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The plasma membrane calcium/calmodulin-dependent calcium ATPase (PMCA) (Shull, G.E., and J. Greeb. 1988. J. Biol. Chem. 263:8646–8657; Verma, A.K., A.G. Filoteo, D.R. Stanford, E.D. Wieben, J.T. Penniston, E.E. Strehler, R. Fischer, R. Heim, G. Vogel, S. Mathews, et al. 1988. J. Biol. Chem. 263:14152–14159; Carafoli, E. 1997. Basic Res. Cardiol. 92:59–61) has been proposed to be a regulator of calcium homeostasis and signal transduction networks of the cell. However, little is known about its precise mechanisms of action. Knock-out of (mainly neuronal) isoform 2 of the enzyme resulted in hearing loss and balance deficits due to severe inner ear defects, affecting formation and maintenance of otoconia (Kozel, P.J., R.A. Friedman, L.C. Erway, E.N. Yamoah, L.H. Liu, T. Riddle, J.J. Duffy, T. Doetschman, M.L. Miller, E.L. Cardell, and G.E. Shull. 1998. J. Biol. Chem. 273:18693–18696). Here we demonstrate that PMCA 4b is a negative regulator of nitric oxide synthase I (NOS-I, nNOS) in HEK293 embryonic kidney and neuro-2a neuroblastoma cell models. Binding of PMCA 4b to NOS-I was mediated by interaction of the COOH-terminal amino acids of PMCA 4b and the PDZ domain of NOS-I (PDZ: PSD 95/Dlg/ZO-1 protein domain). Increasing expression of wild-type PMCA 4b (but not PMCA mutants unable to bind PDZ domains or devoid of Ca2+-transporting activity) dramatically downregulated NO synthesis from wild-type NOS-I. A NOS-I mutant lacking the PDZ domain was not regulated by PMCA, demonstrating the specific nature of the PMCA–NOS-I interaction. Elucidation of PMCA as an interaction partner and major regulator of NOS-I provides evidence for a new dimension of integration between calcium and NO signaling pathways.

Introduction

In initial attempts to gain insight into the unknown regulatory function of the plasma membrane calcium/calmodulin-dependent calcium ATPase (PMCA)* (Carafoli, 1997; Shull and Greeb, 1988; Verma et al., 1988), we have shown previously that the PMCA is controlled by β-adrenergic stimulation (Neyses et al., 1985), that it regulated differentiation in a skeletal muscle cell line (Fammes et al., 1994, 1996), modulated hypertrophic stimuli in the myocardium of transgenic animals expressing PMCA under the control of the ventricle-specific myosin light chain-2 promoter, and was not involved in beat-to-beat regulation of heart contraction (Fammes et al., 1998). Furthermore, we and others have shown that PMCA 4 is localized to caveolae (Fujimoto, 1993; Hammes et al., 1998), membrane structures (sometimes also called “signalosomes” or microdomains) containing a wide variety of effectors of cellular signal transduction (Sargiacomo et al., 1993; Chang et al., 1994; Lisanti et al., 1994; Robbins et al., 1995; Schnitzer et al., 1995; Song et al., 1996a) including nitric oxide synthase (NOS)-I (Venema et al., 1997; Segal et al., 1999) and the cytoskeletal protein dystrophin (Song et al., 1996b). Because PMCA 4b has recently been shown to bind to members of the membrane-associated guanylate kinase protein family via their PDZ domains (Kim et al., 1998), we tested whether it interacts with NOS-I, which has been demonstrated recently to be part of a multiprotein complex largely organized by PDZ domains (Kim et al., 1998), whether it interacts with NOS-I, which has been demonstrated recently to be part of a multiprotein complex largely organized by PDZ domains (Kim et al., 1998), and whether this interaction was physiologically relevant.

Results and discussion

Physical interaction of the PMCA and NOS-I was demonstrated by conventional immunoprecipitation (Fig. 1, D and...
E) as well as glutathione S-transferase (GST) pull-down assays, using the PDZ domain of NOS-I as a bait (Fig. 1 C). “Capping” the PMCA 4b COOH terminus (carrying the PDZ domain binding sequence) with an myc/His tag resulted in a loss of physical interaction (Fig. 1 D). Expression of wild-type NOS-I and deletion constructs (overview in Fig. 1 A) was confirmed by Western blotting (Fig. 1 B). This was also observed in extracts of rat brain. Using PMCA-specific antibodies demonstrating interaction of PMCA and NOS-I in brain. Antibodies specific for NOS-I (lane 3), PMCA (monoclonal, clone 5F10, lane 4, rabbit polyclonal "2A", lane 5), as well as calmodulin beads (CaM beads, lane 2) were capable of precipitating NOS-I from rat brain extracts. First lane, control using irrelevant antibodies; last lane, rat brain extract (input).

In a simple working model, a PMCA molecule interacting with the PMCA rather than some other yet unknown module of the molecule that regulates NOS-I activity: Asp672 →Glu, thereby reducing the ATPase activity to ~10% (Adamo et al., 1995). In pCMV–hPMCA 4b cap the stop codon is mutated and the myc/His epitope, prevented interaction of PMCA and NOS-I (penultimate lane), although NOS-I was present in the cells (last lane). (C) GST pull-down assay (Brenman et al., 1995) demonstrating specific interaction of PMCA 4b with PDZ domain of NOS-I. Lysate of pCMV–hPMCA 4b–transfected HEK293 cells was used as expression control (first lane), GST alone did not bind PMCA 4b (second lane), whereas GST–PDZ fusion protein was able to bind PMCA 4b (last lane). (D) NOS-I–PMCA 4b interaction in cotransfected HEK293 cells. In the first lane NOS-I coprecipitation by a hPMCA 4b–specific antibody (JA3) from lysates of cotransfected cells is shown. In the third lane, lysates of NOS-I/PMCA–transfected cells were used as expression controls. Irrelevant antibody (anti-FLAG) or protein G beads alone showed no binding of NOS-I (second and fourth lane). Cotransfection with pCMV–hPMCA 4b cap, containing an additional COOH-terminal Myc/6×His epitope, prevented interaction of PMCA and NOS-I (penultimate lane), although NOS-I was present in the cells (last lane). (E) Coimmunoprecipitation using NOS-I-specific antibodies. The NOS-I–specific polyclonal antibody (ABR) coprecipitated the PMCA from lysates of cotransfected HEK293 cells (lane 4). Negative controls: unlabeled beads (lane 1), beads plus irrelevant antibodies (lane 2). Positive controls: calmodulin–coated beads (CaM beads, lane 3), anti-PMCA–specific coated beads (with polyclonal antibody “2A,” lane 5, and lysates of pCMV–hPMCA 4b–transfected HEK293 cells (lane 6). (F) Immunoprecipitation using rat brain extracts and different PMCA-specific antibodies demonstrating interaction of PMCA and NOS-I in brain. Antibodies specific for NOS-I (lane 3), PMCA (monoclonal), clone 5F10, lane 4, rabbit polyclonal “2A,” lane 5), as well as calmodulin beads (CaM beads, lane 2) were capable of precipitating NOS-I from rat brain extracts. First lane, control using irrelevant antibodies; last lane, rat brain extract (input).
Interaction of PMCA and NOS-I

Schuh et al. 203

cotransfected with NOS-I. No regulation of NOS activity by this construct was observed (Fig. 2 A, last column). An NOS-I mutant carrying a deletion of the PDZ domain showed no regulation by PMCA 4b (Fig. 2 B). Endothelial NOS (eNOS or NOS-III) is also regulated by calcium/calmodulin, but carries no PDZ domain, and we have been unable to coprecipitate NOS-III with PMCA (not shown). In keeping with the absence of a physical interaction between PMCA 4b and NOS-III, the activity of this enzyme was not regulated by PMCA 4b (Fig. 2 C).

The potential physiological relevance of NOS-I regulation by PMCA 4b was tested in neuro-2a neuroblastoma cells, a commonly used model system in neuronal biology (Olmsted et al., 1970). Similarly to HEK293 cells, tight functional coupling of PMCA4b and NOS-I was observed: NOS-I activity was strongly downregulated by the PMCA4b (Fig. 3 A). This effect was reversed by the NO donor NOC-18 (2,2'-[hydroxynitrosohydrino]bis-ethanamine), suggesting
that interaction of these proteins not only occurs in HEK293 cells, but also in a neuroblastoma-derived cell line.

These results show that the plasma membrane calmodulin-dependent calcium pump 4b is an interaction partner and a major regulator of neuronal NOS-I and also that this regulation very likely is of physiological relevance. The strategic localization to caveolae (Fujimoto, 1993; Hammes et al., 1998) also suggests that local control of calcium and/or NO might have further regulatory effects in caveolae-mediated signal transduction.

The pivotal role of NOS-I in neuronal tissue is well established, exemplified by the observation that NOS-I deficiency leads to reduced susceptibility to cerebral ischemic damage (Huang et al., 1994). Its function in other excitable cells has been extensively characterized in recent years (Christopherson and Bredt, 1997). In contrast, the specific role of the PMCA, and Bredt, 1997). In contrast, the specific role of the PMCA, has been extensively characterized in recent years (Christopherson and Bredt, 1997). In contrast, the specific role of the PMCA, and also that this regulation very likely is of physiological relevance. The strategic localization to caveolae (Fujimoto, 1993; Hammes et al., 1998) also suggests that local control of calcium and/or NO might have further regulatory effects in caveolae-mediated signal transduction.

The pivotal role of NOS-I in neuronal tissue is well established, exemplified by the observation that NOS-I deficiency leads to reduced susceptibility to cerebral ischemic damage (Huang et al., 1994). Its function in other excitable cells has been extensively characterized in recent years (Christopherson and Bredt, 1997). In contrast, the specific role of the PMCA, beyond the general concept of a calcium transporter, has been elusive. Our present results assign a function to isoform PMCA 4b, i.e., regulation of NOS-I activity. In a simple model (see Fig. 4), NOS-I, a Ca\(^{2+}\)/calmodulin-dependent isoform of NOS, is regulated by the PMCA through adjustment of local calcium concentration. Both interact either directly or through an adapter protein via the PDZ domain of NOS-I and the COOH-terminal amino acids of PMCA 4b. Our results are in line with two previous reports showing the interaction of COOH-termini of different PMCA "b" splice variants (PMCA 2b and 4b) with PDZ domains and PDZ domain containing proteins of the MAGUK (membrane-associated guanylate kinase) protein family, whereas the splice variants 2a and 4a failed to bind to PDZ domains (Kim et al., 1998; DeMarco and Strehler, 2001). Indeed, it is conceivable that the predominantly neuronal isoform 2b regulates NOS-I in the context of (at least some specialized) neurons in vivo. This important issue will need to be addressed in further studies. These results and the reduced inhibition of NOS-I activity by the mutated PMCA (PMCAmut, Fig. 2, in which the Ca\(^{2+}\)-transporting and ATPase activity are reduced) strongly suggest that it is the calcium transport function of the PMCA which affects regulation of NOS-I. However, it cannot be entirely excluded that the COOH-terminal peptide of the PMCA 4b, interacting with the PDZ domain of NOS-I, mediates inhibition directly; in this case one would have to postulate that the PMCAmut (Asp\(^{672}\) mutation) undergoes a conformational change conducive to reduced interaction with NOS-I. A definitive answer to this and other unlikely possibilities may have to await direct measurements of calcium concentrations in the vicinity of the pump which is currently not possible.

The coupling of interaction and function of the two proteins is in close analogy to the observation that loss of the PDZ-binding COOH-termini of NMDA receptors results not only in loss of interaction, but also in complete loss of receptor function (Sprengel et al., 1998). Different PMCA isoforms and their splice variants (overview in Strehler and Zacharias, 2001) are expressed ubiquitously and NOS-I is a widespread isoform of NOS in neurons and in excitable tissues (Christopherson and Bredt, 1997). Therefore, the physical and functional interaction of PMCA isoforms and NOS-I or other PDZ domain-containing proteins is most likely relevant in a large variety of cells. These enzymes may define a modular signal transduction system which subserves different functions in different cellular contexts.

**Materials and methods**

**Immunoprecipitations and GST pull-downs**

Samples were lysed in RIPA buffer (1× PBS containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS and Complete \(^{TM}\) protease inhibitor (Roche Diagnostics). Debris was removed by centrifugation (10,000 × g, 5 min, 4°C). After a precleaving step with 0.5 μg irrelevant antibody (anti-FLAG epitope, Neomarkers) and 25 μl of protein G-Sepharose \(^{TM}\) (~50% slurry; Amersham Pharmacia Biotech) 500 μg remaining protein lysate was coimmunoprecipitated together with 3 μg of precipitating antibodies (polyclonal rabbit anti-NOS-I, Affinity Bioreagents catalog no. PA3-032A; monoclonal mouse anti-hPMCA4b, clone J3; and monoclonal mouse anti-PMA, clone 5F10, Neomarkers, catalog no. MS-1305-P1 and MS-1304-P1; polyclonal rabbit anti-hPMCA4b was generated using a peptide spanning the 15 COOH-terminal amino acids of the PMCA4b polyclonal rabbit anti-PMA ("2A") was a gift from D. Guerini, ETH Zürich, Switzerland) and 25 μl protein G-Sepharose \(^{TM}\) at 4°C for 2 h. Calmodulin-Sepharose beads (CalBeads; Amersham Pharmacia Biotech) served as control. After washing four times with RIPA buffer (above beads) were resuspended in above 100 μl sample buffer and boiled. 20 μl of each sample was loaded and 5 μg (1/100 input) protein extract served as control. NOS-I was detected using a monoclonal antibody (clone "16"; Transduction Laboratories; catalog no. N31020) in a dilution of 1:1,000, and transfected hPMCA4b was detected using the PMCA \(^{TM}\) antibody (clone "16"; Transduction Laboratories; catalog no. N31020) in a dilution of 1:1,000, and transfected hPMCA4b was detected using the PMCA 4 isoform-specific antibodies J3 (1:1,000; Figs. 1, C and E, 2 A, and 3 B) and SF10 (dilution 1:1,000, Neomarkers), necessary to detect appropriate expression of PMCA(c120). Immunodetection of proteins on membrane was done using the ECL \(^{TM}\) system according to the manufacturer's protocol (Amersham Pharmacia Biotech).

GST pull-down assays were performed using purified GST-PDZ (NOS-I) fusion protein, containing the PDZ domain of NOS-I (Brennan et al., 1995, 1996) bound to glutathione-Sepharose \(^{TM}\) 4B beads (Amersham Pharmacia Biotech). Pelleted beads were washed four times with RIPA buffer, resuspended in electrophoresis sample buffer, boiled for 3 min, and proteins were separated by 10% SDS-PAGE and blot to Hybond \(^{TM}\) nitrocellulose membrane (Amersham Pharmacia Biotech).

**Construction of DNA vectors**

Expression vector pCMV-hPMCA 4b has been described previously (pCMV-hPMCA4C; Hammes et al., 1996). The point mutation Asp\(^{672}\) Glu was inserted with the QuikChange \(^{TM}\) site-directed mutagenesis kit (Stratagene) using the hPMCA 4b-modifying primers TGC GCT GAG GAG CTC GAT GGC AGG CAC GAG TGT GGC ACT CTC TGG GCC GCC CAC AGG CTC CTT AAT GCC CAC. The pCMV-hPMCA4b cap vector was constructed by insertion of hPMCA 4b cDNA in BamHI-KpnI sites of pcDNA3.1(−)/Myc-His vector (Invitrogen) and subsequent mutation of

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**Figure 4. Model for the regulation of NOS-I by PMCA 4b.** NO production by NOS-I is reduced through Ca\(^{2+}\) extrusion via the PMCA, resulting in alteration of downstream signaling.
the hPMCA 4b stop codon to obtain a c-myc/His tag at the PMCA COOH terminus. The constitutively active form of the PMCA 4b (pPMCA4b[c][2b]; Adamo et al., 1995), was a gift of E. Strehler (Mayo Clinic, Rochester, NY). NOS-I expression plasmid (pCMV-NOS-I; Brennan et al., 1996) and pGST-PDZ of NOS-I (Brennan et al., 1993) were a gift of David S. Bredt (University of California, San Francisco, CA). The NOS-III cDNA (Lamas et al., 1992; EMBL/GenBank/DDB accession no. M89952) was cloned in the EcoRI site of pcDNA3 (Invitrogen), similar to the NOS-I expression construct (see above). The ANOS-I vector, coding for NOS-I and lacking the PDZ domain, was constructed by excision of nucleotides 1–975 of the rat cDNA (numbering according to EMBL/GenBank/DDB accession no. X59949), resulting in a truncated NOS-I, which is expressed in transfected cells and exhibits NO synthase activity.

Transfections and cGMP measurement

HEK293 and Neuro-2a cells (DSMZ) were grown in 90% DME/10% fetal bovine serum/1× nonessential amino acids (Sigma-Aldrich) to a density of 50–70% before transfection. HEK293 and neuro-2a cells were transfected using the LipofectaminPLUS™ system according to the manufacturer’s protocol (Life Technologies). After medium replacement cells were incubated overnight. cGMP was measured using cGMP (low pH) colorimetric competitive ELISA (R&D Systems). Cells were transfected with L-arginine (30 min, 1 mM) and, 5 min before lysis, nascent NO was stabilized by addition of superoxide dismutase (100 U/ml culture medium; Roche Diagnostics). In parallel, 3-isobutyl-1-methylxanthine (IBMX, 1 mM; Sigma-Aldrich) was added to the culture 5 min before lysis (to inactivate phosphodiesterase activity) and NO synthesis was induced by addition of 0.5 μM monomycin 3 min before lysis. Subsequent determination of NO-dependent cGMP production was estimated in relation to untransfected control cells.

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