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Specific Role of Neuronal Nitric-oxide Synthase when Tethered to the Plasma Membrane Calcium Pump in Regulating the β-Adrenergic Signal in the Myocardium*[S]

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The cardiac neuronal nitric-oxide synthase (nNOS) has been described as a modulator of cardiac contractility. We have demonstrated previously that isoform 4b of the sarcolemmal calcium pump (PMCA4b) binds to nNOS in the heart and that this complex regulates β-adrenergic signal transmission *in vivo*. Here, we investigated whether the nNOS-PMCA4b complex serves as a specific signaling modulator in the heart. PMCA4b transgenic mice (PMCA4b-TG) showed a significant reduction in nNOS and total NOS activities as well as in cGMP levels in the heart compared with their wild type (WT) littermates. In contrast, PMCA4b-TG hearts showed an elevation in cAMP levels compared with the WT. Adult cardiomyocytes isolated from PMCA4b-TG mice demonstrated a 3-fold increase in Ser^{16} phospholamban (PLB) phosphorylation as well as Ser^{22} and Ser^{23} cardiac troponin I (cTnI) phosphorylation at base line compared with the WT. In addition, the relative induction of PLB phosphorylation and cTnI phosphorylation following isoproterenol treatment was severely reduced in PMCA4b-TG myocytes, explaining the blunted physiological response to the β-adrenergic stimulation. In keeping with the data from the transgenic animals, neonatal rat cardiomyocytes overexpressing PMCA4b showed a significant reduction in nitric oxide and cGMP levels. This was accompanied by an increase in cAMP levels, which led to an increase in both PLB and cTnI phosphorylation at base line. Elevated cAMP levels were likely due to the modulation of cardiac phosphodiesterase, which determined the balance between cGMP and cAMP following PMCA4b overexpression. In conclusion, these results showed that the nNOS-PMCA4b complex regulates contractility via cAMP and phosphorylation of both PLB and cTnI.

Neuronal nitric-oxide synthase (nNOS)[5] is involved in a number of key processes in cardiomyocytes including calcium cycling (1), the β-adrenergic contractile response (2, 3), post-infarct left ventricular remodeling (4), and the regulation of redox equilibrium (5). Moreover, a polymorphism in an nNOS-interacting protein, CAPON, has been found to form a quantitative trait for the determination of the QT interval in humans (6), whereas a mutation in α1-syntrophin (SNTA1), another interacting partner of nNOS, has been associated with long QT syndrome (7). The signaling events downstream of the nNOS-CAPON (8) and nNOS-SNTA1 (7) complexes, which are responsible for mediating cardiac repolarization and sodium current respectively, have been elucidated. The nNOS-containing protein complex is therefore of immediate relevance to human pathology.

In recent years, we have shown that the sarcolemmal calcium pump, which ejects calcium to the extracellular compartment (reviewed in Refs. 9 and 10), is an important molecule involved in signal regulation and transmission in the heart (11). We have demonstrated that isoform 4b of the sarcolemmal calcium pump (also known as PMCA4b for plasma membrane calcium/calmodulin-dependent ATPase 4b) modulates signaling through a tight molecular interaction with nNOS, leading to the modulation of β-adrenergic responsiveness in the heart (12). However, the events following signaling through the PMCA4b-nNOS complex remain unknown.

In myocardial cells, nNOS has been localized to the sarcolemma (13), sarcoplasmic reticulum (2), and mitochondria (14), and translocation between compartments has been demonstrated (15). It has been speculated that these various localizations provide specificity to NO signaling, but the exact mechanisms have yet to be elucidated. In this study, we show a mechanism by which one fraction of nNOS serves highly specific functions through binding to PMCA4b. As PMCA4b is confined to the sarcolemma and is a calcium pump, it is the first identified protein to fulfill these aggregate functions. 1) It acts as an anchoring protein; 2) it regulates nNOS activity; and 3) it modulates a process at the plasma membrane, *i.e.* β-adrenergic signaling.

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[5] The abbreviations used are: nNOS, neuronal nitric-oxide synthase; TG, transgenic; PLB, phospholamban; cTnI, cardiac troponin I; NRCM, neonatal rat cardiomyocyte(s); sGC, soluble guanylyl cyclase; PDE, phosphodiesterase(s); PKA, protein kinase A; SMLT, S-methyl-L-thiocitrulline; DAF-FM, 4-amino-5-methylamino-2,7-difluorescein diacetate.

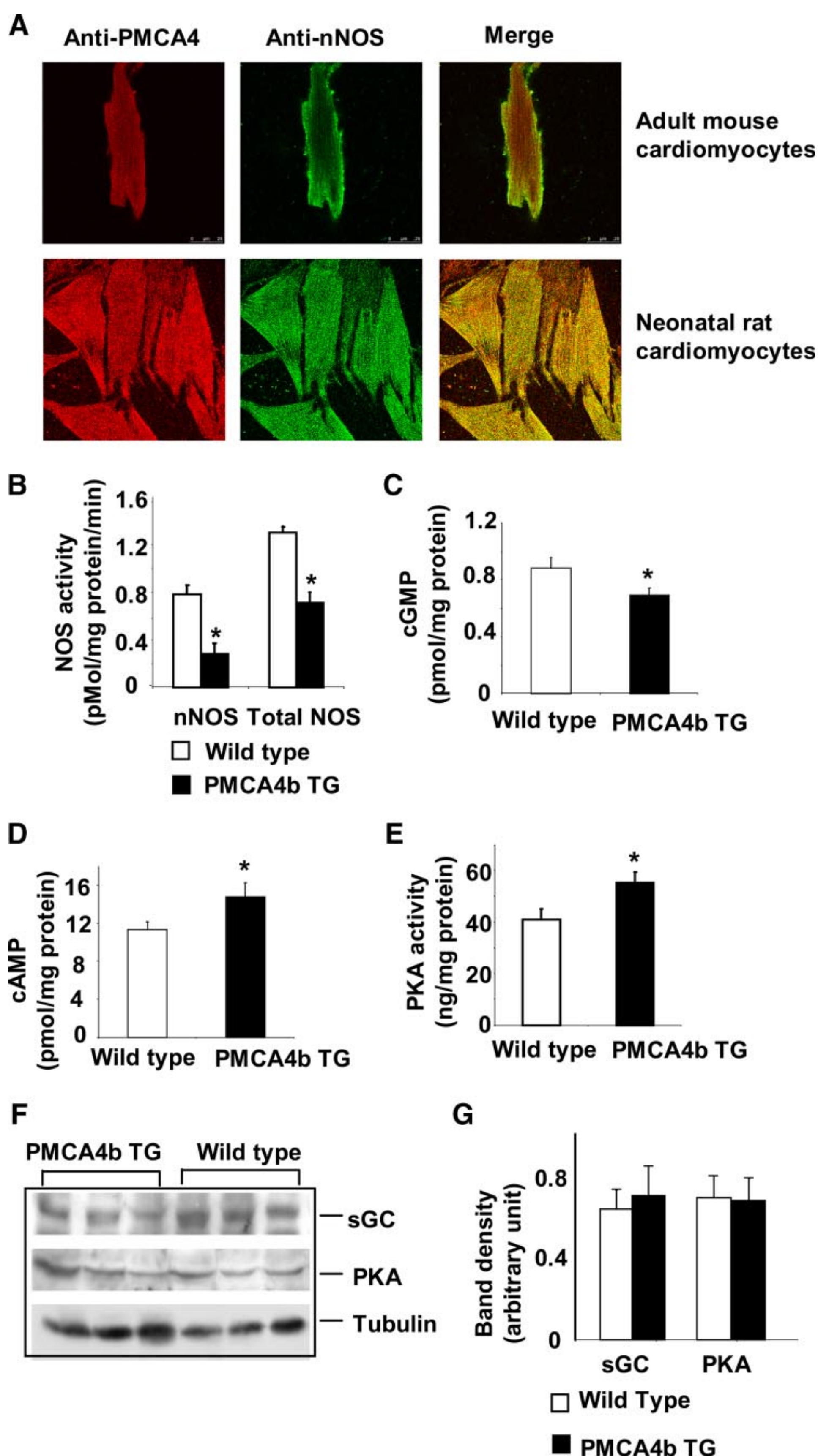
EXPERIMENTAL PROCEDURES

Animals—We used transgenic mice with cardiac-specific overexpression of human PMCA4b under the control of the MLC-2v promoter as described previously (12). See supplemental "Methods" for hemodynamic analysis and NOS activity assay in the heart.

Primary Cardiomyocyte Isolation—Adult cardiomyocytes were isolated from 3–4-month-old animals using methods described previously (12). Neonatal rat cardiomyocytes (NRCM) were isolated from 1–3-day-old Sprague-Dawley rat neonates using an established protocol (16). NRCM were maintained in medium supplemented with 1% serum prior to adenovirus infection. See supplemental "Methods" for detailed cell isolation protocol, intracellular calcium transient measurements, as well as cell-shortening measurements.

Adenovirus—An adenovirus expressing human PMCA4b was a kind gift from Dr. Alex Maass (Wüerzburg, Germany). An nNOS-expressing virus was generated by cloning nNOS cDNA (a gift from Dr. David Bredt, San Fransisco, CA) to the pAd/CMV/V5-DEST vector (Invitrogen) using the Gateway pENTR vector as the shuttle system (Invitrogen) following the manufacturer's recommended methods. An adenovirus expressing LacZ under the control of the human cytomegalovirus promoter was used as the control.

Western Blotting—Heart tissues or primary cardiomyocytes were homogenized in 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, and 500 ng/ml pepstatin) and centrifuged at 700 × *g* for 5 min to remove nuclear debris. Protein concentrations were determined using a BCA protein assay kit (Pierce). 30 μg of protein extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Primary antibodies



used for the Western blot analysis were as follows: polyclonal anti-PMCA4 (SWANT); monoclonal anti-PMCA (5F10), anti-soluble guanylyl cyclase (sGC), anti-protein kinase A (PKA), anti-cardiac troponin I, anti-phospho-Ser^{22}/Ser^{23} troponin I, anti-PDE2, anti-PDE3, and anti-PDE4 (Abcam); anti-phospholamban and anti-phospho-Ser^{16} phospholamban (Upstate); and anti-nNOS (Affinity Bioreagents). Tubulin expression (detected using an antibody from Calbiochem) was used as a loading control. Levels of expression were determined using NIH ImageJ software. See supplemental "Methods" for the *in vivo* hemodynamic analysis, NOS activity assay, cGMP and cAMP measurements, immunofluorescence analysis, PKA activity assay, determination of intracellular nitric oxide bioavailability, and phosphodiesterase (PDE) activity assay.

Data Analysis and Statistics—Data are presented as mean ± S.E. Statistical analysis has been carried out using Student's *t* test or one-way analysis of variance, where appropriate, using SPSS statistical software. Values were considered significantly different when $p < 0.05$.

RESULTS

Regulation of nNOS and Cyclic Nucleotides by PMCA4b in the Heart—We have described the interaction of PMCA4b and nNOS using a cellular model (17) and *in vivo* in mouse hearts (12, 18). Immunofluorescence analysis was used to further confirm the co-localization of PMCA4b and nNOS in the neonatal rat cardiomyocytes and adult mouse cardiomyocytes (Fig. 1*A*). We used transgenic mice overexpressing PMCA4b (PMCA4b-TG) to examine the regulatory role of PMCA4b in the nNOS-mediated signaling pathway in the heart. Total NOS and nNOS activity in the hearts of PMCA4b-TG mice and their wild type (WT) littermates were assessed by measuring the rate of conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in the presence or absence of either 1 mM *N*-ω-nitro-L-arginine methyl ester, a global NOS inhibitor, or 1 μM *S*-methyl-L-thiocitrulline (SMLT), a specific nNOS inhibitor. Total NOS and nNOS activities were significantly reduced in protein extracts isolated from PMCA4b-TG mice by 50 and 75%, respectively, compared with the wild type ($n = 8$; $p < 0.05$) (Fig. 1*B*). However, the nNOS protein expression remained unchanged in PMCA4b-TG mouse hearts compared with their WT littermates (supplemental Fig. 1, *A* and *B*).

sGC, which is responsible for converting GTP to cGMP, is known to be one of the major downstream effectors of nNOS, but a variety of NO effectors have been identified. We investigated whether overexpression of PMCA4b affects the cGMP level in the heart. Enzyme-linked immunosorbent assay detection of cGMP in cardiac tissue extracts demonstrated a significantly lower level of cGMP in protein extracts from PMCA4b-TG mice (Fig. 1*C*).

Because the major cardiac phenotype of the PMCA4b-TG mice was the reduction of the β-adrenergic contractile response (12), we then analyzed the levels of cAMP, which is the major regulator of the β-adrenergic pathway in cardiomyocytes. Interestingly, contrary to the cGMP level, the cAMP level was significantly elevated in PMCA4b-TG mice (Fig. 1*D*). Consistent with that finding, the activity of the cAMP-dependent protein kinase (PKA) was increased in the transgenic hearts (Fig. 1*E*). The modifications of cGMP levels and PKA activity were likely due to modulation of enzyme activities rather than regulation of their expression, as expression levels of sGC and PKA were not altered in the transgenic mice (Fig. 1, *F–G*).

Differential Activation of Phospholamban and Cardiac Troponin I Reduces the β-Adrenergic Contractile Response in PMCA4b-TG Mice—We examined the activation of phospholamban (PLB) and cardiac troponin I (cTnI), the two main targets of PKA phosphorylation. The levels of Ser^{16}-phosphorylated phospholamban in isolated adult cardiomyocytes were examined in response to isoproterenol stimulation. The results shown in Fig. 2 suggest a difference in β-adrenergic responsiveness between PMCA4b-overexpressing and wild type myocytes. PMCA4b-overexpressing myocytes exhibited higher levels of phosphorylated PLB (Fig. 2, *A–C*) and cTnI (*D–E*) than wild type cells, mostly in unstimulated cells. However, the -fold increase of phosphorylation in both proteins in response to isoproterenol was significantly reduced in PMCA4b-TG myocytes, suggesting no further increase in β-adrenergic responsiveness.

Alteration of Calcium Dynamics in PMCA4b-TG Myocytes—PMCA4b-TG myocytes displayed a slightly higher calcium amplitude and faster calcium decay rate at base line (Fig. 3, *A–C*). However, in response to isoproterenol, the transgenic myocytes showed no further increase in calcium amplitude (−2.2% in transgenic (TG) *versus* +45.5% in the WT) and in calcium decay (+5% in TG *versus* +32% in the WT).

In contrast to the calcium measurement, the basal contractility and relaxation were not affected by PMCA4b overexpression as indicated by myocyte shortening (Fig. 3*D*) and *in vivo* hemodynamic (Fig. 3*E*) analyses. Similarly, the PMCA4b-TG mice demonstrated an attenuated inotropic response to the β-adrenergic agonist *in vivo*. As shown in Fig. 3*F*, following an increasing dose of isoproterenol infusion, PMCA4b-TG mice exhibited an attenuated increase in dP/dt_{max}, which is an index of contractility. These changes were not due to alterations in the expression of other major calcium transporters because the expression of the sodium calcium exchanger, SERCA (sarcoplasmic reticulum calcium ATPase), and L-type calcium channel in the heart of PMCA4b-TG animals remained the same as their WT littermates (supplemental Fig. 1, *C* and *D*).

FIGURE 1. *A*, shown is an image of nNOS co-localization to PMCA4b. Isolated adult mouse cardiomyocytes (*upper panel*) and neonatal rat cardiomyocytes (*lower panel*) were co-stained with anti-PMCA4 and anti-nNOS antibodies. *B*, nNOS-specific activity and total NOS activity were significantly reduced in heart extracts from PMCA4b-TG mice compared with wild type littermates ($n = 8$; *, $p < 0.05$). *C*, cGMP levels were also significantly decreased in heart homogenates from PMCA4b-TG mice compared with wild type controls ($n = 8$; *, $p < 0.05$). However, cAMP levels (*D*) as well as PKA activity (*E*) were significantly increased in PMCA4b-TG mice *versus* wild type ($n = 8$; *, $p < 0.05$ *versus* WT). *F*, Western blot analyses of sGC and PKA in the heart extracts are shown. *G*, a quantification of band density after normalization with α-tubulin expression suggested that the expression levels of these proteins were not altered in PMCA4b-TG mice ($n = 8$).

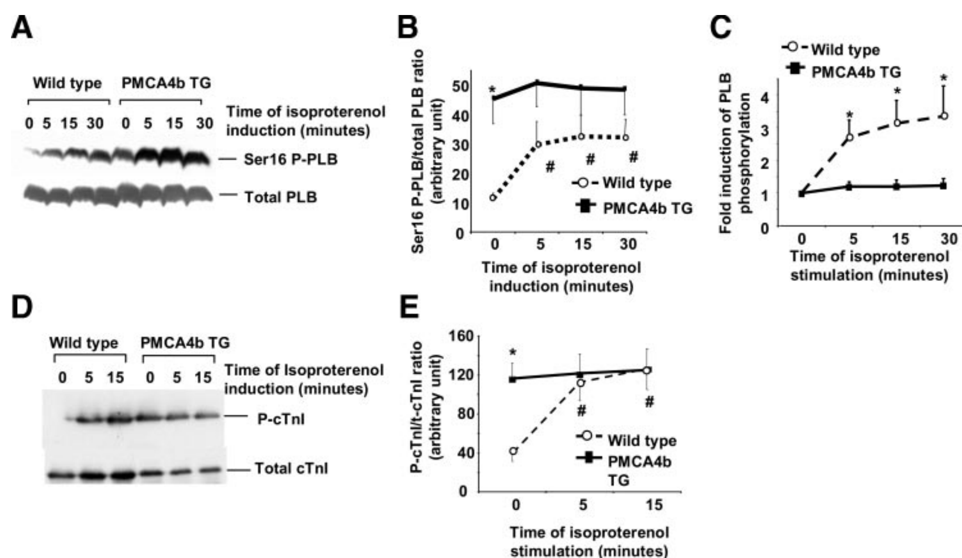


FIGURE 2. *A*, shown are representative Western blots of Ser[16]-phosphorylated phospholamban (*Ser16 P-PLB*) and total PLB from adult cardiomyocytes isolated from PMCA4b-TG mice and wild type controls at base line and after stimulation with 2 μM isoproterenol for 5–30 min. *B*, the ratio of Ser[16]-phosphorylated PLB/total PLB was calculated by measuring the band density from five pairs of transgenic and wild type littermates. The ratio of Ser[16] -phosphorylated PLB/total PLB was significantly higher in PMCA4b-TG *versus* wild type at base line (*, $p < 0.05$). However, in response to isoproterenol stimulation, no significant changes were observed in PMCA4b-TG, whereas in wild type mice, the ratio of Ser[16]-phosphorylated PLB/total PLB was significantly increased following isoproterenol induction (#, $p < 0.05$ *versus* base-line level). *C*, in transgenic cardiomyocytes overexpressing PMCA4b, phosphorylation of phospholamban at Ser[16] in response to isoproterenol stimulation was not increased relative to the basal level, whereas in wild type cells, similar treatment resulted in a 2.5–3-fold induction of Ser[16] PLB phosphorylation ($n = 5$; *, $p < 0.05$). *D*, shown are the representative Western blots of Ser[22]/Ser[23]-phosphorylated troponin I (*P-cTnI*) and total cTnI in adult cardiomyocytes isolated from PMCA4 TG mice and their WT littermates at base line and after stimulation with 2 μM isoproterenol for 5 and 15 min. *E*, a quantification of the Ser[22]/Ser[23]-phosphorylated cTnI/total cTnI ratio shows that there is no further increase in phosphorylated cTnI in response to isoproterenol stimulation in cardiomyocytes derived from PMCA4b-TG mice ($n = 6$; *, $p < 0.05$ *versus* WT; #, $p < 0.05$ *versus* basal level).

PMCA4b Inhibits Nitric Oxide (NO) and cGMP Production in Isolated Cardiomyocytes—To further study the mechanism by which the PMCA4b-nNOS interaction mediates β-adrenergic responsiveness, we generated a cellular model using isolated NRCM. An adenovirus system was used to overexpress PMCA4b and nNOS in NRCM. Fig. 4*A* shows overexpression of PMCA4b and nNOS in adenovirus-infected cells detected by Western blotting. Staining with the NO-sensitive dye DAF-FM demonstrated that cardiomyocytes overexpressing PMCA4b displayed significantly reduced NO levels compared with cells infected with the LacZ-expressing virus, which was used as a control. Furthermore, coexpression of PMCA4b blunted the increase in NO levels in cells overexpressing nNOS (Fig. 4, *B–C*). In keeping with this finding, we also demonstrated that the cGMP level was significantly lower in PMCA4b-overexpressing cardiomyocytes compared with controls (Fig. 4*D*). To examine whether this effect was due to modulation of sGC activity or its expression level, we determined sGC levels by Western blotting. No difference in expression level was observed in PMCA4b-overexpressing cells compared with controls (Fig. 4*E*).

β-Adrenergic Response Is Altered in NRCM Overexpressing PMCA4b—To examine the β-adrenergic responsiveness in NRCM infected with the PMCA4b-expressing virus, the cells were treated with 2 μM isoproterenol, and the levels of PLB phosphorylation as well as cTnI phosphorylation were detected after 5 and 15 min of stimulation. Under no stimulation, PMCA4b-overexpressing cardiomyocytes exhibited significantly higher levels of Ser[16] PLB phosphorylation as well as cTnI phosphorylation compared with the control cells (Fig. 5, *A–D*). However, similar to the data from PMCA4b-TG mice, no further elevation of either PLB or cTnI phosphorylation was observed in response to β-adrenergic stimulation in PMCA4b-overexpressing NRCM.

nNOS Is Involved in Regulation of β-Adrenergic Responsiveness—To independently ascertain that modulation of nNOS is capable of modifying β-adrenergic signaling, we treated cardiomyocytes with the specific nNOS inhibitor SMLT (1 μM) for 60 min (before the addition of isoproterenol). In control cells at base line, specific inhibition of nNOS increased phospholamban phosphorylation to a level comparable with PMCA4b-overexpressing cells (Fig. 5*E*). Furthermore, the response to isoproterenol treatment was also attenuated in SMLT-treated control cells, which emulated the condition in PMCA4b-overexpressing cells. In addition, no further significant changes were observed in PMCA4b-overexpressing NRCM after treatment with SMLT. These findings suggested that the effect of PMCA4b overexpression on phospholamban phosphorylation is mainly due to an nNOS-dependent signaling cascade.

PDE Activity Is Reduced in PMCA4b-overexpressing Cells—To elucidate the link between PMCA4b-nNOS/cGMP signaling and the β-adrenergic pathway in PMCA4b-overexpressing myocytes, we investigated the role of PDE. PDE are responsible for the degradation of cyclic nucleotides including cAMP and cGMP. We measured PDE activity in NRCM overexpressing PMCA4b. Compared with control cells, PMCA4b-overexpressing NRCM showed a significant reduction in PDE activity (Fig. 6*A*). Expression of PDE2, PDE3, and PDE4 were not different between cardiomyocytes overexpressing PMCA4b and control cells (data not shown), suggesting that the difference in PDE activity was due to modulation of its activity and not its expression level.

Consistent with the data obtained from PMCA4b-TG mice, cAMP levels were elevated by ~2-fold in PMCA4b-overexpressing cardiomyocytes compared with the control at base line (Fig. 6*B*). This difference was abolished in the presence of the nNOS-specific inhibitor SMLT. In addition, PKA activity was elevated in PMCA4b-overexpressing cells (Fig. 6*C*). Taken together, these data suggest that modulation of PDE activity by cGMP causes the increase in cAMP and hence increased PKA

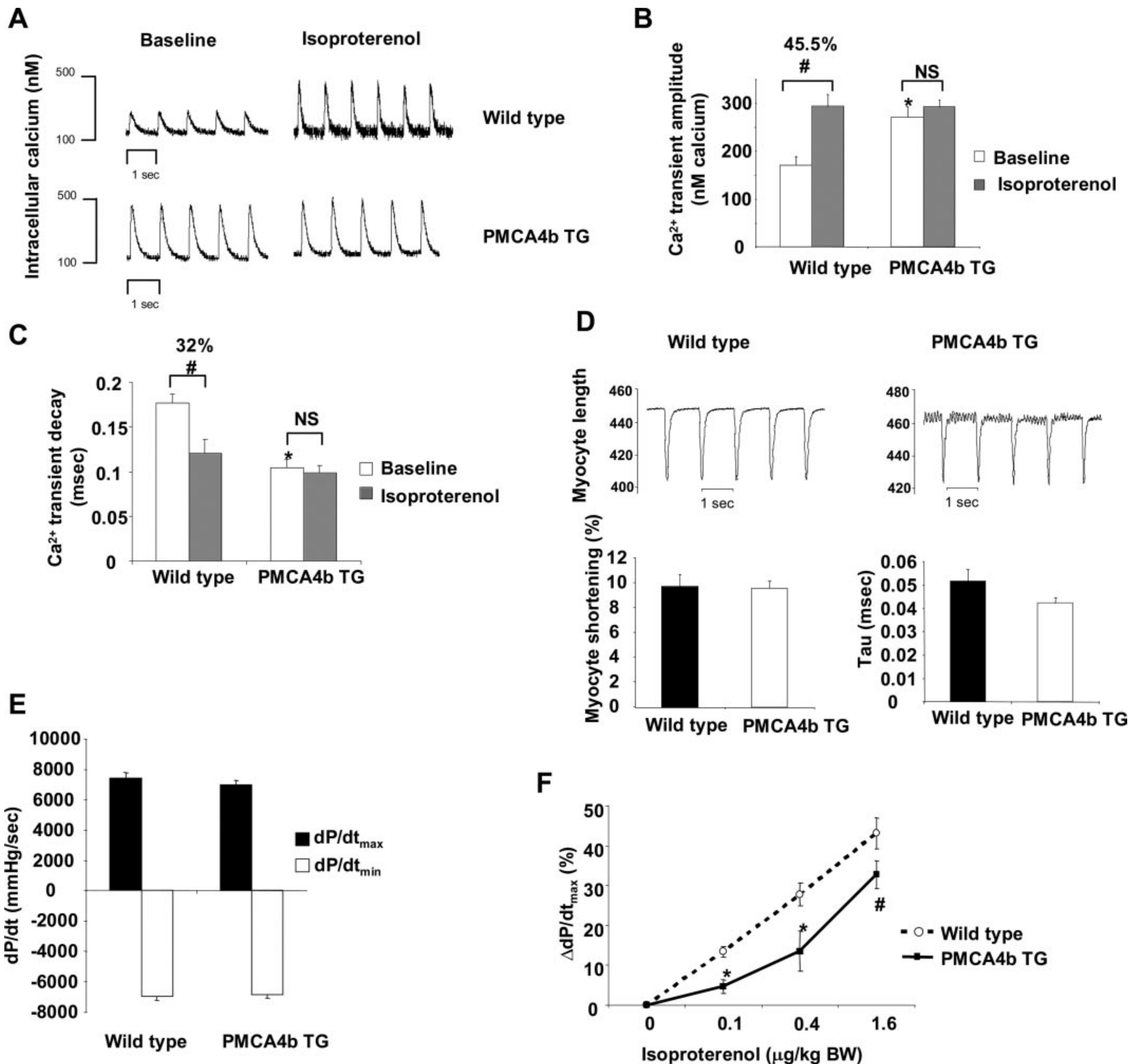


FIGURE 3. *A*, the representative calcium transient traces from isolated PMCA4b-TG and WT myocytes indicate higher calcium amplitude in PMCA4b-TG at base line but a reduced response to isoproterenol treatment. *B* and *C*, shown is the quantification of calcium transient amplitude and decay rate (*Tau*), respectively (*, $p < 0.05$ *versus* WT at base line; #, $p < 0.05$ *versus* base-line level; n = seven to eight independent animals in each group). *D*, the representative traces of myocyte shortening, quantification of myocyte shortening (%), and time constant of myocyte relaxation (*Tau*) show that there was no difference between PMCA4b-TG and wild type myocytes. *E*, *in vivo* hemodynamic analysis indicated that there was no difference in basal contractility (dP/dt_{max}) and relaxation (dP/dt_{min}) between PMCA4b-TG mice and wild type littermates (n = eight in each group). *F*, however, β-adrenergic inotropic response was significantly attenuated in PMCA4b-TG mice, as indicated by the percentage increase of dP/dt_{max} ($\Delta dP/dt_{max}$) in response to increasing isoproterenol (*Iso*) dose (*, $p < 0.05$; #, $p = 0.07$; $n = 8$).

activity in PMCA4b-overexpressing cells, and this effect is dependent on the modulation of nNOS activity by PMCA4b.

DISCUSSION

It is widely assumed that nNOS generates functionally diverse signals in the myocardium. These signals are likely dependent on its subcellular location (2, 15) and its interacting partners. However, the molecular nature of these interacting protein partners and their functions has largely remained elusive. Here, we present insight into the mechanism by which one fraction of nNOS, *i.e.* the PMCA4b-bound nNOS, mediates signaling specificity.

Our key finding is that in cardiomyocytes, the PMCA4b-nNOS complex governs cyclic nucleotide production, protein kinase A activity, and, ultimately, phosphorylation of proteins involved in the excitation-contraction coupling process such as PLB and cTnI. These biochemical changes led to alteration of calcium dynamics and reduction of β-ad-

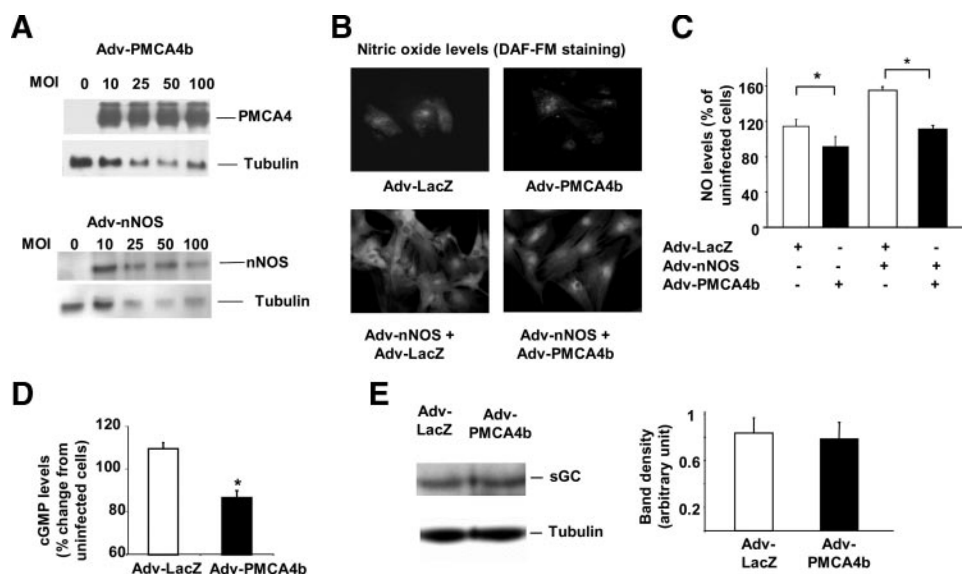


FIGURE 4. *A*, shown is a Western blot analysis showing overexpression of PMCA4b and nNOS in neonatal rat cardiomyocytes infected with the corresponding adenovirus (*Adv*)-overexpressing construct. *MOI*, multiplicity of infection. *B*, intracellular nitric oxide level was assessed in NRCM overexpressing PMCA4b or control (LacZ) with or without nNOS coexpression by staining with NO-sensitive dye (DAF-FM). *C*, measurement of fluorescence intensity showed that PMCA4b-overexpressing cells exhibited a significantly reduced intracellular NO level with or without coexpression of nNOS ($n = 64$ cells from six independent cell preparations in each group; *, $p < 0.05$). *D*, cGMP levels were also reduced in NRCM overexpressing PMCA4b compared with control ($n = 6$; *, $p < 0.05$). *E*, the sGC expression level was not changed in PMCA4b-overexpressing cells, as indicated by quantification of Western blot analysis ($n = 5$).

renergic contractile response, as demonstrated in isolated myocytes as well as in intact animals, despite no alterations in SERCA, sodium calcium exchanger, or L-type calcium channel expression levels.

In this study, by using PMCA4b-TG mice, as well as NRCM overexpressing PMCA4b, we demonstrated that both nNOS activity (but not the protein expression) and NO levels were decreased following PMCA4b overexpression. nNOS has been shown to be a powerful regulator of the β-adrenergic contractile response; deficiency of nNOS leads to reduction of the β-adrenergic contractile response (2, 3). Our data are in line with these findings, as PMCA4b overexpression leads to a reduction in nNOS activity, reduced NO levels, and a subsequent reduction of β-adrenergic responsiveness. Conversely, recent experiments in our laboratory have demonstrated that knockdown of PMCA4 using RNA interference in NRCM increases NO production; nNOS activity was also increased in PMCA4 knockout hearts compared with their WT littermates (data not shown).

In line with the notion that sGC is the primary effector of NO from the nNOS-PMCA4b complex, our data demonstrate that overexpression of PMCA4b led to a reduction of cGMP production, both in intact hearts as well as in NRCM. We cannot completely exclude that other effectors of NO action, *e.g.* oxygen radical production and various protein nitrosylation reactions (reviewed in Ref. 19) also play a role, but the canonical pathway via sGC (20) is sufficient to explain our findings both *in vivo* and in isolated cardiac cells. It is therefore likely that this is the predominant effector of NO generated from the interaction of PMCA4b and nNOS in the heart.

In our models, cAMP levels were increased in intact hearts as well as in NRCM overexpressing PMCA4b. cAMP is the major second messenger in the β-adrenergic signaling pathway and activates PKA. We hypothesized that the well described cross-talk between cGMP and cAMP signaling (21, 22) might be crucial in determining the downstream effects of the PMCA4b-nNOS interaction. A growing body of evidence suggests that cGMP alters cAMP signaling by modulating PDE activity (22, 23). PDE are a family of enzymes responsible for the degradation of cyclic nucleotides including cAMP and cGMP, though with varying affinity for each compound (21, 24). Recent findings have demonstrated that some PDE isoforms such as PDE2 and PDE3 are regulated by cGMP, and this mechanism has been suggested as the link between cGMP and cAMP signaling (23, 25). PDE2 is stimulated by cGMP (reviewed in Ref. 22); therefore, the reduction in cGMP levels in our PMCA4b-overexpressing models provides a straightforward explanation for the decrease in PDE2 and hence total PDE activity. PDE2 has been shown to mainly control compartmentalized cAMP degradation in caveolae (26), where PMCA4b is localized (27, 28). PDE3, which is inhibited by cGMP (22), is also active in the myocardium. One possible explanation for our data is that NO and cGMP signals from the PMCA4b-nNOS complex are confined to a space not accessible to PDE3. This is in line with recent concepts of highly compartmentalized roles for PDE isoforms (21). This issue is currently being addressed in our laboratory using fluorescence resonance energy transfer techniques, by which we will be able to demonstrate the activity of each individual PDE isoform at the membrane and the cytoplasmic compartments.

As discussed in detail above, the PMCA4b-nNOS complex regulated the production of NO, cGMP, and cAMP. We subsequently examined the activity of PKA and the phosphorylation of both PLB and cTnI, which are the end targets of β-adrenergic stimulation.

Increased PKA activity, as well as increased levels of phosphorylated PLB and cTnI, have been shown both in transgenic animals and in cardiomyocytes overexpressing PMCA4b at base line (before β-adrenergic stimulation). Interestingly, PLB phosphorylation and cTnI phosphorylation in response to isoproterenol induction were attenuated in PMCA4b-overexpressing myocytes, which is in agreement with the *in vivo* data showing that a blunted β-adrenergic contractile response in PMCA4b-TG mice. Because the increase of PLB phosphorylation and cTnI phosphorylation at base line was considerable, most of the PLB and cTnI in transgenic myocytes were phosphorylated at base line, and so no significant relative increase in phosphorylation was detectable following isoproterenol stimulation. This phenotype resembles PLB knock-out mice, in

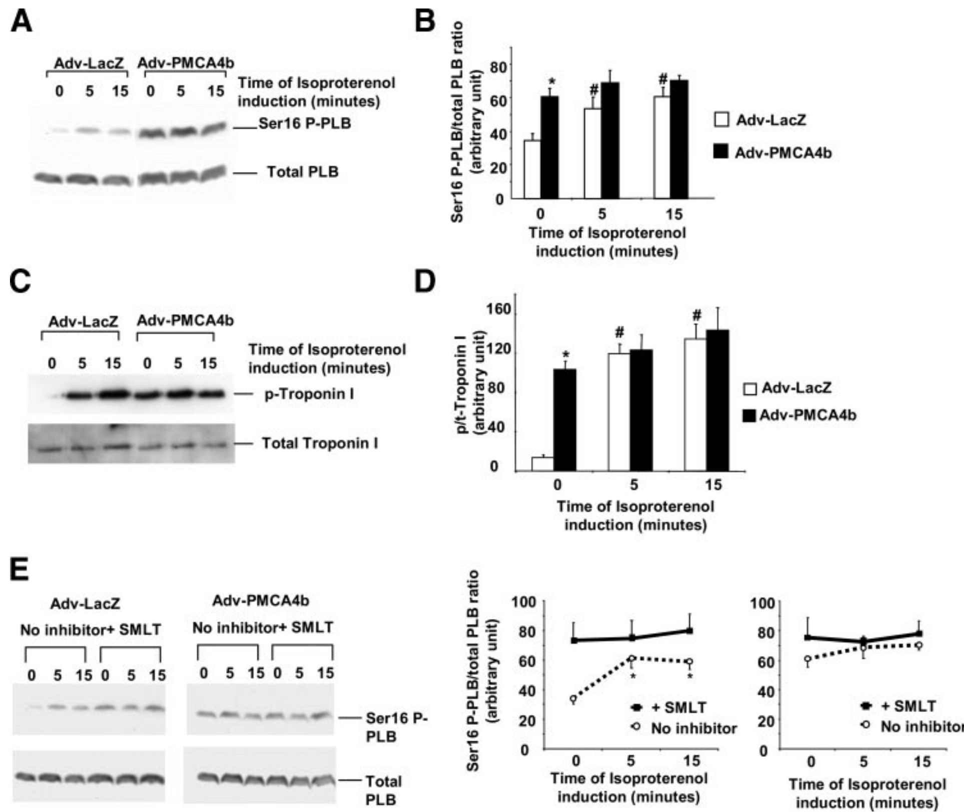


FIGURE 5. *A*, representative Western blots of Ser[16]-phosphorylated PLB and total PLB from neonatal rat cardiomyocytes overexpressing PMCA4b and control cells (NRCM expressing LacZ) at base line and after stimulation with 2 μM isoproterenol for the indicated times. *B*, measurement of band density indicated that the ratio of Ser[16]-phosphorylated PLB/total PLB was significantly increased in cardiomyocytes overexpressing PMCA4b compared with the controls. However, after isoproterenol treatment, no significant increase in phosphorylated PLB was observed in PMCA4b-overexpressing cells, whereas control cells displayed significant elevation of PLB phosphorylation. ($n = 5$; *, $p < 0.05$ *versus* control at base line; #, $p < 0.05$ *versus* unstimulated cells). *C*, Western blot detection of Ser[22]/Ser[23]-phosphorylated cTnI and total cTnI in NRCM overexpressing PMCA4b and control cells at base line and after stimulation with 2 μM isoproterenol for 5 and 15 min. *D*, phosphorylation of cTnI at Ser[22] and Ser[23] in response to isoproterenol stimulation was blunted in cardiomyocytes overexpressing PMCA4b, whereas in control cells similar treatment resulted in a 3–4-fold induction of Ser[22]/Ser[23] troponin I phosphorylation ($n = 6$; *, $p < 0.05$ *versus* control at base line; #, $p < 0.05$ *versus* unstimulated cells). *E*, treatment with the specific nNOS inhibitor SMLT abolished the difference in PLB phosphorylation caused by PMCA4b overexpression. NRCM overexpressing PMCA4b, and LacZ as a control, were incubated with SMLT (1 μM for 60 mins) and then stimulated with isoproterenol for the indicated times. Levels of Ser[16]-phosphorylated PLB and total PLB were detected by Western blot. Densitometric analysis showed that at base line the ratio of Ser[16]-phosphorylated PLB/total PLB was elevated in control cells after treatment with SMLT to a level comparable with that of PMCA4b overexpressing cells. Hence, in response to isoproterenol induction the phosphorylation level was not elevated in SMLT-treated cells. No changes were observed in PMCA4b-overexpressing cells after treatment with SMLT both at base line and after isoproterenol induction ($n = 5$, *, $p < 0.05$). *Adv*, adenovirus.

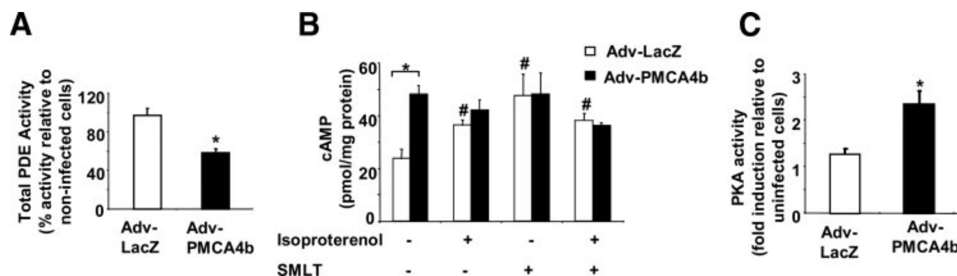


FIGURE 6. *A*, PDE activity was measured in NRCM overexpressing PMCA4b and in control cells. A significant reduction in total PDE activity was observed in PMCA4b-expressing cells ($n = 5$; *, $p < 0.05$). *B*, cAMP levels were significantly increased in PMCA4b-overexpressing cardiomyocytes; however, the difference was abolished in the presence of SMLT. Control cells exhibited an increase in cAMP in response to isoproterenol stimulation, whereas both PMCA4b-overexpressing cells and control cells treated with SMLT displayed no further cAMP elevation in response to isoproterenol stimulation ($n = 5$; *, $p < 0.05$). *C*, PKA activity was significantly increased in NRCM overexpressing PMCA4b ($n = 6$; *, $p < 0.05$).

which ablation of PLB led to the attenuation of the β-adrenergic contractile response by a conceptually similar mechanism (25). These data are also in agreement with results from Burkard *et al.* (29) showing a significant decrease in Ser[16] PLB phosphorylation in conditional cardiac-specific nNOS overexpressing mice.

On the other hand, a recent paper demonstrated that in nNOS knockout mice, PLB phosphorylation was reduced (30). These data are not contradictory, as in nNOS knockout mice, nNOS was deleted from all localizations, whereas our studies specifically addressed the sarcolemma-bound nNOS-PMCA4b complex.

As a result of increased Ser[16] PLB phosphorylation in PMCA4 TG mice, it was expected that the baseline intracellular calcium transients as well as the contractility would be elevated. Transgenic cardiomyocytes overexpressing PMCA4b indeed showed an increase in baseline calcium transient amplitude. However, cell shortening and *in vivo* contractility showed no significant difference compared with the WT. This means that PMCA4b-overexpressing cardiomyocytes did not respond to the increased calcium transient. This might be explained by the increased phosphorylation of cTnI. It is well known that PKA-dependent phosphorylation of cTnI reduces myofibril sensitivity to calcium (31). Moreover, two independent groups have reported that transgenic replacement of native cTnI with the β-adrenergic phospho-mimetic cTnI (to mimic constitutive phosphorylation) resulted in the reduction of cardiomyocyte calcium sensitivity (32, 33). In addition, it has been reported that increased PKA-dependent phosphorylation of cTnI leads to a reduction in myofilament calcium sensitivity and blunted β-adrenergic response (34). Therefore, the increased phosphorylation of cTnI in PMCA4b-TG hearts may desensitize the myofibril to calcium and may compensate for the effect of the

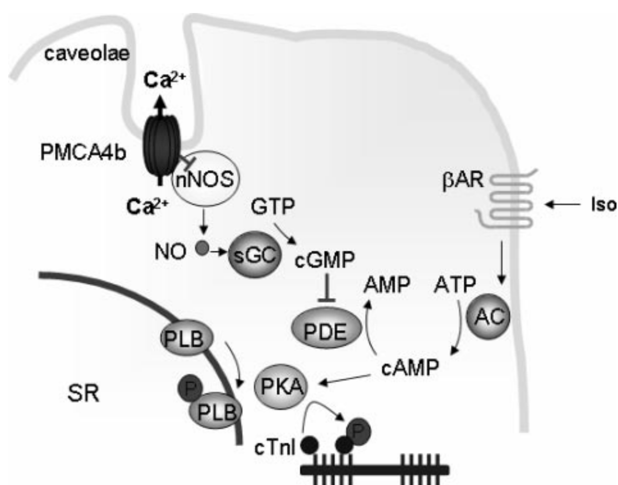


FIGURE 7. **Schematic diagram shows the signaling cascade downstream of the PMCA4b-nNOS complex and its role in regulating the β-adrenergic response.** PMCA4b inhibits nNOS, localized to the plasma membrane compartment, by altering local calcium concentration. Reduced nNOS activity leads to a reduction in cGMP production by sGC. Decreased cGMP levels will result in the reduction of PDE activity. This will prevent cAMP degradation and hence increase PKA activity, which will finally lead to increased phosphorylation of PLB as well as phosphorylation of cTnI.

small increase in calcium transient (for more explanation, see supplemental "Discussion"). Moreover, as we have previously suggested that PMCA4 regulates local calcium levels (reviewed in Ref. 11), the global calcium transient may not reflect its true role, and we are currently developing PMCA4-based local calcium sensors to address this question.

In summary, our present data unravel a novel molecular mechanism by which spatially confined nNOS signal specificity is achieved. PMCA4b anchors nNOS to caveolae and reduces nNOS activity. This leads to the reduction of cGMP, an increase in cAMP generation, and a subsequently altered β-adrenergic responsiveness (Fig. 7). In light of these and other recent data (1, 2, 15), it appears likely that nNOS molecules in other subcellular compartments (*i.e.* sarcoplasmic reticulum and mitochondria) also fulfill highly specialized and spatially restricted functions. Furthermore, nNOS interaction with non-PMCA proteins, such as CAPON and α1-syntrophin (7, 8), has been described to have significant functional relevance, thus adding a novel level of complexity to nNOS signaling in the heart.

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