Role of plasma membrane Ca\(^{2+}\)-ATPase in contraction-relaxation processes of the bladder: evidence from PMCA gene-ablated mice

Li Liu, Yukisato Ishida, Gbolahan Okunade, Gary E. Shull, and Richard J. Paul

1Department of Molecular and Cellular Physiology and 2Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, Cincinnati, Ohio

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Liu, Li, Yukisato Ishida, Gbolahan Okunade, Gary E. Shull, and Richard J. Paul. Role of plasma membrane Ca\(^{2+}\)-ATPase in contraction-relaxation processes of the bladder: evidence from PMCA gene-ablated mice. Am J Physiol Cell Physiol 290: C1239–C1247, 2006. First published November 16, 2005; doi:10.1152/ajpcell.00440.2005.—We investigated the roles and relationships of plasma membrane Ca\(^{2+}\)-ATPase (PMCA), sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)\(_2\), and Na\(^{+}/\)Ca\(^{2+}\) exchanger (NCX) in bladder smooth muscle contractility in Pmca-ubanded mice: Pmca-null mutant (Pmca\(^{-/-}\)) and heterozygous Pmca\(^{+/−}\) and homozygous Pmca\(^{-/-}\) double gene-targeted (Pmca1\(^{-/-}\)/Pmca4\(^{-/-}\)) mice. Gene manipulation did not alter the amounts of PMCA1, SERCA2, and NCX. To study the role of each Ca\(^{2+}\) transport system, contraction of circular ring preparations was elicited with KCl (80 mM) plus atropine, and then the muscle was relaxed with Ca\(^{2+}\)-free physiological salt solution containing EGTA. We measured the contributions of Ca\(^{2+}\) clearance components by inhibiting SERCA2 (with 10 µM cyclopiazonic acid) and/or NCX (by replacing NaCl with N-methyl-d-glucamine/HCl plus 10 µM KB-R7943). Contraction half-time (time to 50% of maximum tension) was prolonged in the gene-targeted muscles but marginally shortened when SERCA2 or NCX was inhibited. The inhibition of NCX significantly inhibited this prolongation, suggesting that NCX activity might be augmented to compensate for PMCA4 function in the gene-targeted muscles under nonstimulated conditions. Inhibition of SERCA2 and NCX as well as gene targeting all prolonged the relaxation half-time. The contribution of PMCA to relaxation was calculated to be ~25–30%, with that of SERCA2 being 20% and that of NCX being 70%. PMCA and SERCA2 appeared to function additively, but the function of NCX might overlap with those of other components. In summary, gene manipulation of PMCA indicates that PMCA, in addition to SERCA2 and NCX, plays a significant role in both excitation-contraction coupling and the Ca\(^{2+}\) extrusion-relaxation relationship, i.e., Ca\(^{2+}\) homeostasis, of bladder smooth muscle.

ATP2B; sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase 2; Na\(^{+}/\)Ca\(^{2+}\) exchanger; homeostasis

SMOOTH MUSCLE CONTRACTILE activity is dependent on intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), which is regulated by the activities of channels, transporters, and pumps at the plasma-lamellar and organellar membranes. The plasma membrane Ca\(^{2+}\)-ATPase (PMCA), sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), and plasmalemmal Na\(^{+}/\)Ca\(^{2+}\) exchanger (NCX) are responsible for lowering [Ca\(^{2+}\)]\(_i\), which leads to relaxation of smooth muscle. The roles of NCX and SERCA have been investigated with specific inhibition of NCX (Na\(^{+}\)-free medium) or inhibitors [cyclopiazonic acid (CPA) and thapsigargin] as well as gene manipulation of SERCA. On the other hand, the role of PMCA in Ca\(^{2+}\) homeostasis is difficult to assess because of the lack of specific inhibitors. However, the generation of PMCA gene-targeted mice has recently provided a way to investigate the role of PMCAs in cellular function (19, 26).

Four major isoforms of PMCA are encoded by different genes in mammals: PMCA1 and PMCA4 are ubiquitously expressed, including in smooth muscle cells, whereas PMCA2 and PMCA3 are mainly present in neuronal cells (29). Zacharias and Kappen (34) also reported that the expression of PMCA4 mRNA increases in mouse bladder and spinal cord just before birth. Expression levels of PMCA have been reported to be inversely correlated with the expression levels of SERCA and NCX. Overexpression of the PMCA4 isoform in Chinese hamster ovary cells (8) or the PMCA1 isoform in rat aortic endothelial cell line (17) resulted in a reduction in the expression of SERCA2. When SERCA protein was reduced by deletion of the SERCA2 gene in cardiomyocytes, NCX was upregulated for compensation of Ca\(^{2+}\) homeostasis (1, 13).

In addition, expression of human Pmca4db was reported to augment the contractility of aortic smooth muscle, presumably because of downregulation of nitric oxide synthase (25). Therefore, it may be possible that PMCA gene manipulation alters expression of other Ca\(^{2+}\) transporter proteins responsible for lowering [Ca\(^{2+}\)]\(_i\).

In this study, we investigated the role of PMCA compared with NCX and SERCA in contraction and relaxation of bladder smooth muscle. We utilized PMCA4-null (Pmca4\(^{-/-}\)) and PMCA1-PMCA4 double gene-targeted (Pmca1\(^{+-}\)/Pmca4\(^{-/-}\)) mice in combination with inhibition of NCX and SERCA; PMCA1-null mutant mice could not be obtained, because the deletion is embryonically lethal (19). PMCA, SERCA, and NCX were also measured to assess the potential for compensation at the protein level. Our data indicate that PMCA plays an important role in the regulation of bladder smooth muscle contractility.

MATERIALS AND METHODS

Animals. Nineteen mice were used: nine wild-type (WT), five Pmca4\(^{-/-}\), and five Pmca1\(^{+-}\)/Pmca4\(^{-/-}\). All mice were male, except for two WT and one Pmca4\(^{-/-}\). Mice were between 4 and 8 mo of age, with the majority being 6 mo old. For each mouse type studied, only age-matched and gender-matched pairs of WT and gene-targeted mice were used.

Tissue preparation. Mice were euthanized in a precharged CO\(_2\) chamber. Urinary bladders were dissected from WT, Pmca4\(^{-/-}\), and Pmca1\(^{+-}\)/Pmca4\(^{-/-}\) mice, rinsed with physiological salt solution (PSS; in mM: 120 NaCl, 4.7 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 1.1 NaH\(_2\)PO\(_4\), 23.8 NaHCO\(_3\), and 11.2 glucose), and bubbled with 95%
O₂-5% CO₂, pH 7.4. The mucosal layer in the lumen was removed. For Western blot analysis, mucosa-denuded bladders were frozen in liquid nitrogen and stored at −80°C. For measurement of contractile activity, the ring preparations were made as described below. Animal treatment and experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Genotyping mouse tail DNA. PCR genotyping of tail DNA from Pmca1*/− heterozygotes, Pmca4*/− homozygotes, and Pmca1*/− Pmca4*/− double gene-targeted mice was performed as described previously (19). Briefly, WT and mutant alleles were amplified with specific forward and reverse primers for Pmca1 and Pmca4, and the PCR products were separated on 2% agarose gels and visualized with ethidium bromide.

RT-PCR analysis of PMCA mRNA. RNA was isolated and reverse transcribed, and the cDNA was amplified by RT-PCR using specific primers for Pmca1 and Pmca4, as described by Okunade et al. (19). Briefly, the resulting PCR products were cut with the specific restriction enzyme Clal (cleaves Pmca1 and leaves Pmca4 intact) and BglII (cleaves Pmca4 and leaves Pmca1 intact), and the digestion products were separated on ethidium bromide-stained 1.5% agarose gels and quantified with ImageQuant 5.2 software (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ).

SDS-PAGE. Frozen bladder tissues were homogenized (Polytron; Kinematica, Lucerne, Switzerland) in RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5% deoxycholate, 0.1% SDS, 0.1% Nonidet P-40, and 1 mM 2-mercaptoethanol containing protease inhibitors (30 μL/100 mg tissue, cocktail for mammalian cells; Sigma, St. Louis, MO), and phosphatase inhibitors (20 μL/100 mg tissue, cocktails I and II; Sigma). After centrifugation, the supernatant of each homogenized sample was stored at −80°C. The protein concentration was determined using the Bio-Rad procedure (Hercules, CA) with bovine serum albumin as a standard. The same amount of each protein (10 μg) was boiled for 5 min in SDS loading buffer (2% SDS, 10% glycerol, 0.5% bromophenol blue, 62.5 mM Tris·HCl, pH 6.8), and the proteins were separated on a 12%–14% Tris·glycine gel (Invitrogen, Carlsbad, CA) at 250 V for 2.5 h.

Western blot analysis. Proteins were transferred from the gels onto wet nitrocellulose membranes at 350 mA for 3 h. The nitrocellulose membranes were incubated in 5% nonfat milk in Tris-buffered saline plus 0.1% Tween 20 for 1 h to block nonspecific binding. The following antibodies were used to detect PMCA isoforms: mouse monoclonal anti-PMCA clone 5F10 (ABR, Golden, CO), mouse monoclonal anti-PMCA4 clone JA9 (Abcam, Cambridge, UK), and rabbit polyclonal anti-PMCA1 (Abcam). Monoclonal anti-SERCA2 ATPase (ABR) and monoclonal anti-NCX (R3F1, SWANT, Bellinzona, Switzerland) were used to detect SERCA and NCX, respectively. Mouse anti-actin monoclonal antibody C4 (a kind gift from Dr. James Lessard, Children’s Hospital, Cincinnati, OH) was used as a loading control. Primary antibodies were detected using goat anti-mouse IgG and goat anti-rabbit IgG horseradish peroxidase-coupled secondary antibodies (1:20,000). ECL Western blot analysis detection reagents (Amersham Biosciences) were used to determine protein levels according to the manufacturer’s protocols.

Measurements of contraction and relaxation rates. The middle part of the mucosa-denuded bladder was cut into an equatorial ring of 1.5-mm width. The ring preparation was placed into an organ bath containing PSS continuously bubbled with 95% O₂-5% CO₂ (37°C). The muscle ring was stretched three times to a peak force of 15 mN and reached nearly twice the initial length, setting the length in the range for optimal force generation. The muscle was then equilibrated for at least 30 min and exposed to 80 mM KCl for 10 min to obtain steady contractile activity.

All solutions used for testing the contraction and relaxation rates of the muscle contained atropine (1 μM) to avoid the effects of cholinergic stimulation. To observe the contractile response, KCl (80 mM) was administered for 10 min in normal PSS containing 2.5 mM Ca²⁺.

For the relaxation response, the muscle was exposed to Ca²⁺-free PSS with 3 mM EGTA for 10 min. The muscle was again exposed to normal PSS to observe the contraction and relaxation responses to another challenge of KCl under conditions inhibiting SERCA, NCX, or both transporters simultaneously to investigate the roles of SERCA and NCX in relaxation. SERCA was inhibited by the addition of CPA (10 μM), NCX was inhibited by replacing Na⁺ and NaHCO₃ with equimolar N-methyl-d-glucamine and choline bicarbonate (Na⁺-free solution, pH adjusted 7.4 with HCl) containing the reverse-mode NCX inhibitor KB-R7943 (KBR; 10 μM) (11, 32). Half-times of contraction and relaxation responses to KCl were measured as an index of contraction and relaxation rates. From the relaxation half-times, the relative contribution of PMCA, SERCA, and NCX for relaxation was estimated as described in RESULTS.

Data analysis. Statistical analyses were performed by ANOVA with the Holm-Sidak multiple-comparison test. A value of P < 0.05 was taken as indicative of a statistically significant value. All values are expressed as means ± SE; n represents the number of mice.

RESULTS

Genotyping and RT-PCR analysis of PMCA mRNA. We genotyped mice by performing PCR of tail biopsies with specific primers for Pmca1 and Pmca4 as indicated in Fig. IA, which shows WT (+/+), heterozygous (+/−), and homozygous (−/−) offspring. To estimate the relative amount of PMCA1 and PMCA4 mRNA in WT mouse bladder, quantitative RT-PCR was used as described in MATERIALS AND METHODS. PCR products were digested with restriction enzymes Clal and BglII. Clal cleaves the Pmca1 fragment into 395- and 634-bp fragments (lane 3); BglII cleaves the Pmca4 fragment into 495- and 516-bp pieces (lane 2), as shown in Fig. 1B. On the basis of the staining intensity of the PCR products, the Pmca1-to-Pmca4 mRNA ratio was 0.81 ± 0.03 (n = 5).

Fig. 1. PCR genotype and RT-PCR analysis of plasma membrane Ca²⁺-ATPase (PMCA) mRNA. PCR products were separated by agarose gel electrophoresis and visualized using ethidium bromide staining. A: PCR genotyping of tail DNA from wild-type (WT, +/+), PMCA4-heterozygous (+/−), and PMCA4-homozygous (−/−) mice. B: RT-PCR analysis of mRNA levels of Pmca1 and Pmca4 in WT mouse bladder. RT-PCR products were digested without restriction enzymes (uncut); with Clal, which cleaves only Pmca1 (Clal); with BglII, which cleaves only Pmca4 (BglII); or both enzymes (BglII/Clal), which cleave both Pmca1 and Pmca4.
SDS-PAGE and Western blot analysis. Because PMCA, SERCA, and NCX are the major Ca\textsuperscript{2+} transport proteins responsible for the regulation of [Ca\textsuperscript{2+}], we investigated the effects of PMCA gene manipulation on the levels of these proteins using Western blot analysis. Figure 2 shows the levels of PMCA isoforms in bladders of WT, Pmca1\textsuperscript{+/+}, Pmca4\textsuperscript{+/+}, and Pmca\textsuperscript{1+/+Pmca4\textsuperscript{+/+}} mice. A pan-specific antibody (clone 5F10) against PMCA1 detected expression of PMCA1 in both WT and Pmca\textsuperscript{1+/+Pmca4\textsuperscript{+/+}} mice, as expected. However, no signal for PMCA4 was observed in either WT or Pmca\textsuperscript{1+/+Pmca4\textsuperscript{+/+}} mice. In contrast, a pan-specific antibody (clone JA9) against PMCA4 detected expression of PMCA4 in both WT and Pmca\textsuperscript{1+/+Pmca4\textsuperscript{+/+}} mice, as expected. However, no signal for PMCA1 was observed in either WT or Pmca\textsuperscript{1+/+Pmca4\textsuperscript{+/+}} mice. These results clearly indicate that ablation of the Pmca4 gene abolished expression of the PMCA4 isoform in the bladder.

Western blot analysis using antibodies against SERCA and NCX was used to compare the levels of these proteins among bladders from PMCA gene-ablated mice (Fig. 3). SERCA was detected with an anti-SERCA2b antibody because SERCA2b is the predominant isoform in smooth muscle (4, 5, 21). A pan-specific NCX antibody was used to determine the total amount of NCX (23). There were no significant differences in the levels of SERCA or NCX among bladders of WT, Pmca\textsuperscript{1+/+}, Pmca4\textsuperscript{+/+}, and Pmca\textsuperscript{1+/+Pmca4\textsuperscript{+/+}} mice (n = 4).

Contractile responses. The bladder contracted to carbachol (1 \mu M) with an early phasic component and a sustained component; the sustained component was oscillatory (Fig. 4).

Fig. 2. Western blot analysis of bladder smooth muscle PMCA isoforms. Tissue extracts (10 \mu g) from WT, Pmca1\textsuperscript{+/+}, Pmca4\textsuperscript{+/+}, and Pmca\textsuperscript{1+/+Pmca4\textsuperscript{+/+}} mice were subjected to SDS-PAGE (4–12%). Proteins were transferred onto nitrocellulose membranes and probed with the following antibodies: pan-specific monoclonal anti-PMCA (clone 5F10, ABR; A), polyclonal anti-PMCA1 (Abcam; B), isoform-specific monoclonal anti-PMCA4 (clone JA9, Abcam; C), and mouse anti-actin monoclonal antibody C4, which served as a loading control (D).

Fig. 3. Western blot analysis of sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) in bladder smooth muscle. Tissue extracts (10 \mu g) from WT, Pmca1\textsuperscript{+/+}, Pmca4\textsuperscript{+/+}, and Pmca\textsuperscript{1+/+Pmca4\textsuperscript{+/+}} mice were subjected to SDS-PAGE (4–12%). Proteins were transferred onto nitrocellulose membranes and probed with the following antibodies: anti-SERCA2b (ABR; A), monoclonal anti-NCX antibody (R3F1, SWANT; B), and mouse anti-actin monoclonal antibody C4, which served as a loading control (C).

Even when carbachol was removed by rinsing with PSS, the muscle relaxed in an oscillatory manner. On the other hand, the contraction to and relaxation after KCl (80 mM) were stable (Fig. 4). Receptor-mediated stimulation has been reported to elicit contraction with a more prominent Ca\textsuperscript{2+} sensitization in the [Ca\textsuperscript{2+}]-force relationship compared with KCl stimulation in smooth muscle (14, 27), including in urinary bladders (28, 30, 33). Thus the relationship between [Ca\textsuperscript{2+}], and tension may also not be as straightforward in carbachol as it is in KCl. Therefore, we used KCl to further investigate the effects of factors affecting Ca\textsuperscript{2+} clearance systems on the contraction-relaxation responses of the bladder.

Fig. 4. Comparison between contractile responses to KCl (80 mM) and carbachol (1 \mu M) of the mucosa-denuded ring preparation of the mouse urinary bladder. A: responses to KCl and carbachol. B: expanded traces of relaxation responses after carbachol or KCl with Ca\textsuperscript{2+}-free physiological salt solution (PSS) as the rinse solutions (arrowheads). Small contractile responses occurring immediately after removal of KCl or carbachol were considered to be artifacts upon rinse.
Depolarization may elicit neurotransmitter release from nerves in the tissue. Our bladder preparation responded strongly to the agonist for muscarinic receptors but not for α-adrenergic receptors (data not shown). Atropine (1 μM) inhibited the early phasic contraction to 80 mM KCl by 19.6 ± 4.0% (n = 4) and the sustained phase by 4.2 ± 2.8% (n = 4), suggesting that cholinergic effects are more prominent in the early phase of contraction than in the sustained phase. Therefore, all solutions used for subsequent investigation of contraction/relaxation rates contained atropine at 1 μM.

We investigated the contribution of PMCA, SERCA, and NCX to contraction and relaxation kinetics using the protocol shown in Fig. 5. As summarized in Fig. 6, the magnitudes of the first peak responses to KCl were 35.2 ± 8.7 mN/mm² (n = 20) in WT, 33.7 ± 6.3 mN/mm² (n = 11) in Pmca4−/−, and 25.5 ± 8.7 mN/mm² (n = 12) in Pmca1+/−/Pmca4−/− bladders. The magnitudes of the sustained responses to KCl were 23.3 ± 2.5 mN/mm² (n = 20) in WT, 27.8 ± 5.8 mN/mm² (n = 11) in Pmca4−/−, and 20.9 ± 4.8 mN/mm² (n = 12) in Pmca1+/−/Pmca4−/− bladders; these differences were not significant at P < 0.05.

When Ca²⁺ was readmitted after the exposure to Ca²⁺-free PSS, bladders from the WT mice did not produce a contractile response (Fig. 5). In contrast, the Ca²⁺ readmission elicited a small, sustained tension in Pmca4−/− muscle and a transient contraction in Pmca1+/−/Pmca4−/− muscle (Fig. 5). The magnitude of the transient contraction in Pmca1+/−/Pmca4−/− muscle (1.04 ± 0.39% of the initial peak tension to KCl; n = 12) was much lower than the sustained contraction in Pmca4−/− muscle (3.36 ± 0.97%; n = 11). Inhibition of SERCA by addition of CPA (10 μM) in normal PSS elicited a submaximal contraction that gradually relaxed in the WT muscle (Fig. 5). The contractile response to CPA was sustained in Pmca4−/− and Pmca1+/−/Pmca4−/− muscles (Fig. 5). The inhibition of NCX or both NCX and SERCA also elicited a contraction with a higher magnitude compared with those with only inhibition of SERCA in WT and PMCA gene-targeted muscles (Fig. 5).

The effects of inhibition of SERCA, NCX, or both SERCA and NCX on KCl-induced responses were also evaluated. The total peak tension in response to 80 mM KCl tended to be larger in all muscles after inhibition of SERCA by CPA. Compared with WT, PMCA gene-targeted muscles showed smaller peak tensions in the presence of CPA (Fig. 6A). The total sustained tensions in response to KCl were slightly higher in all genotypes when the muscles were treated with CPA (Fig. 6B). On the other hand, the inhibition of NCX by Na⁺-free PSS and addition of KBR apparently inhibited the magnitude of the sustained tension, but not the initial rapid tension, in both WT and PMCA gene-targeted muscles (Figs. 5 and 6). Inhibition of both SERCA and NCX also decreased the magnitude of sustained tension to KCl (Fig. 6B).

Rates of contraction and relaxation. The half-time (τ₁) for the initial rapid phase of contraction to KCl was taken as an...
index of the contraction rate. Figure 7A shows an expanded scale of the time course for development of force for the control KCl contractions in Fig. 5, top, illustrating measurement of the half-times. Figure 8 summarizes the data from these types of experiments. When WT bladders were exposed to KCl in normal PSS, tension increased with a half-time of 7.4 ± 1.2 s (n = 20). In the WT and PMCA gene-targeted bladders, inhibition of SERCA tended to shorten the half-time of contraction, although this was not significant. The inhibition of NCX also shortened the half-time in WT and PMCA gene-targeted muscles but was only significant in the Pmca1+/−/Pmca4−/− mice. Inhibition of both NCX and SERCA significantly shortened the half-time in PMCA gene-ablated muscles but not in WT muscles. On the other hand, when values were compared between muscle genotypes, half-times for contraction in Pmca4−/− and Pmca1+/−/Pmca4−/− muscles

Fig. 6. Summary of data from KCl (80 mM)-induced contraction after inhibition of SERCA, NCX, or both in bladder smooth muscle ring preparations from WT, Pmca4−/−, and Pmca1+/−/Pmca4−/− mice. A: peak tensions. B: sustained tensions. y-Axis: tension responses under each inhibitory condition were normalized by responses without inhibition of SERCA and/or NCX. Data are means ± SE. Na-0, Na1-free PSS; KBR, KB-R7943.

Fig. 7. Control KCl contraction/relaxation on expanded time scale showing protocol for measurement of half-times. A: contraction half-time. Arrow and dotted line indicate the time of KCl addition. Horizontal line drawn at 50% of the developed force indicates the magnitude of the calculated contraction half-time. B: relaxation half-time. Arrow indicates the initiation of the rinse to Ca2+−free PSS. To correct for the artifact attributable to the solution change, we first calculated the magnitude of relaxation from the initial force, shown at the arrow. The time to reach this original force level was subtracted from the calculated half-time, starting at the arrow. Horizontal line at 50% force indicates the calculated relaxation half-time.
were two to three times longer than that in the WT muscle. This trend toward longer half-times in the PMCA gene-targeted muscles was observed even when SERCA and NCX were inhibited. These results suggest that the inhibition of SERCA and NCX increases the contraction rate in either WT or PMCA gene-ablated bladder muscles, whereas the gene manipulation of Pmca4/H11002/H11002 and Pmca1/H11001/H11002 reduced the contraction rate.

Similarly, the relaxation half-time (τ) after KCl stimulation was measured when the muscle was rinsed with Ca²⁺-free PSS containing 3 mM EGTA as shown in Fig. 7B, and these data are summarized in Fig. 9. Inhibition of SERCA slightly increased the half-time of relaxation, but not significantly, in muscles of WT and PMCA gene-ablated mice. The inhibition of NCX significantly prolonged the half-time by more than three times that of the control for each genotype. Inhibition of both NCX and SERCA prolonged the relaxation half-time further, although marginally so. Comparison of genotypes revealed Pmca4⁻/⁻ bladders exhibited a significantly prolonged relaxation half-time in NCX-inhibited conditions. The half-time in both control and SERCA-inhibited conditions was only slightly, although significantly, prolonged. Pmca1⁺/⁻/⁻Pmca4⁻/⁻ half-times were prolonged under each treatment, which was significant when both SERCA and NCX were inhibited. These results suggest that the relaxation rate is slowed moderately by the inhibition of PMCA and SERCA and markedly by the inhibition of NCX.

One can assign a relative percentage of the contribution to relaxation for each component of each Ca²⁺ extrusion element, using rates taken as the inverse of the relaxation half-times. The relaxation rate constant of WT muscle can be expressed in terms of a simple linear model as \( \tau_{WT} = \tau_{PMCA} + \tau_{SERCA} + \tau_{NCX} + \tau_{others} \). The half-time of relaxation dependent on other residual components is expressed as \( \tau_{others} \), because relaxation still occurred when PMCA, SERCA, and NCX were inhibited. The percent contribution for each component is calculated as \( i = (1 - \tau_i/\tau_{WT}) \times 100 \). This simple analysis assumes independent components (discussed below) but is useful as a first
approximation of the relative role of each Ca\(^{2+}\) clearance component in relaxation.

Table 1 presents the relaxation rate time constants and statistical analysis; Table 2 shows the percent contribution for each clearance component associated with the time constants in Table 1. From the values for WT muscles, SERCA and NCX contribute ~20% and 70% to the relaxation process, respectively. From the values for PMCA gene-targeted muscles, PMCA contributes ~25–35%. The residual components other than PMCA, SERCA, and NCX can be estimated by using values obtained from the inhibition of both SERCA and NCX in \(Pmca4^{-/-}\) and \(Pmca1^{+/+}/Pmca4^{-/-}\) mice, yielding a relative contribution of 15–20%. When SERCA and NCX were inhibited simultaneously, the percent contribution increased marginally but is unlikely to be additive in SERCA and NCX in any of the genotypes.

Table 1. Relaxation half-times in mouse bladder

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control</th>
<th>CPA</th>
<th>Na(^{+})-Free + KBR</th>
<th>CPA Na(^{+})-Free + KBR</th>
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<tbody>
<tr>
<td>WT</td>
<td>16.9±2.1</td>
<td>20.7±2.8</td>
<td>61.2±7.4*</td>
<td>66.0±4.9†</td>
</tr>
<tr>
<td>(Pmca4^{-/-})</td>
<td>25.8±3.9*</td>
<td>35.1±4.6*</td>
<td>99.6±20.0*†</td>
<td>109.2±12.0*†</td>
</tr>
<tr>
<td>(Pmca1^{+/+}/Pmca4^{-/-})</td>
<td>22.2±1.1</td>
<td>34.7±5.2</td>
<td>69.4±1.1†</td>
<td>85.4±4.5*†</td>
</tr>
</tbody>
</table>

Values (in s) are means ± SE. WT, wild type; \(Pmca\), plasma membrane Ca\(^{2+}\)-ATPase (PMCA) gene. CPA, cyclopiazonic acid; KBR, KB-R7943. *\(P < 0.05\), significant differences between genotypes in relaxation half-times on inhibition of each Ca\(^{2+}\) clearance component; †\(P < 0.05\), significant differences within a genotype on inhibition of Ca\(^{2+}\) clearance components.
genotypes. In PMCA gene-targeted muscles, the contribution of NCX increased compared with the WT value but again was not completely additive. On the other hand, comparing values among genotypes, PMCA and SERCA seemed to operate in an additive manner. Apparently, PMCA contributes to relaxation nearly as much as does SERCA. It is notable that NCX contributes more than double the contribution of PMCA or SERCA.

**DISCUSSION**

We investigated the role of PMCA in bladder smooth muscle contractility. Northern blots indicate that the level of 
Pmca1  mRNA is approximately equal to that of 
Pmca4  mRNA. However, on the basis of Western blots using the pan-specific PMCA antibody, PMCA4 appears to be the major PMCA isoform in bladder smooth muscle. The genetic alterations in 
Pmca1+/−/Pmca4−/− and 
Pmca4−/− mice did not alter the protein levels of SERCA and NCX. Interestingly, targeted ablation of one allele of 
Pmca1 did not appear to significantly reduce total PMCA (or PMCA1) protein levels. This suggests that the PMCA4 isoform is responsible for the observed alterations in contractility.

The contraction half-time for KCl tended to be shorter when SERCA and/or NCX was inhibited. This is consistent with a more rapid increase in [Ca^{2+}]i due to the loss of Ca^{2+} clearance from the cytosol by SERCA and NCX. NCX appears to be the dominant factor. The stimulation of capacitative Ca^{2+} entry (6, 10) due to the inhibition of SERCA may also be responsible for the more rapid force development.

In contrast, the half-time for force development to KCl was significantly prolonged in the PMCA gene-targeted bladders, suggesting that PMCA gene ablation may impede depolarization-induced Ca^{2+} influx. There are a number of potential mechanisms that might interfere with the depolarization-contraction coupling leading to the increased contraction half-time.

A simple idea may be a reduction of the transmembrane [Ca^{2+}] gradient due to an increase in sub-plasma membrane [Ca^{2+}], in the 
Pmca-ablated muscles. Furthermore, the increased near-membrane [Ca^{2+}] in the 
Pmca-ablated muscles may stimulate the forward mode of NCX activity, without an elevation of NCX protein expression to compensate for the loss of PMCA function. This might counter the initial Ca^{2+} influx, resulting in a slower contraction. Inhibition of NCX with Na^{+}-free medium plus KBR in 
Pmca4−/− and 
Pmca1+/−/Pmca4−/− bladders significantly shortened the contraction half-time to a level similar to the half-time of the WT bladder, supporting this conjecture.

Another potential candidate for the lower rate of contraction in PMCA gene-ablated bladders may be hyperpolarization caused by activation of the electrogenic Na^{+}-K^{+}-ATPase by increases in intracellular Na^{+} concentration due to increased NCX activity. Also, Ca^{2+}-activated K+ channels may contribute to a slowdown in the contractile response, as the elevation of sub-plasma membrane [Ca^{2+}] could activate these K+ channels, leading to hyperpolarization (9, 20). A coupling of this type between PMCA inhibition and Ca^{2+}-activated K+ channel activation was reported in responses of sickle cells to oxidants (7).

Relaxation in Ca^{2+}-free PSS likely involves all components of Ca^{2+} clearance, such that inhibition of any extrusion component would be expected to impede relaxation. PMCA gene ablation and inhibition of SERCA and/or NCX all slowed the relaxation. The observed contractile response to CPA is likely due to the inhibition of SERCA and increased [Ca^{2+}], as reported in many smooth muscles (15) as well as in urinary bladders (12, 18). The more sustained contractile response to CPA in PMCA-ablated bladder compared with that in WT muscle may be due to the additional inhibition of both PMCA and SERCA activity. Because readmission of Ca^{2+} after exposure to Ca^{2+}-free PSS in 
Pmca-ablated (but not in WT) muscle caused a small contraction, it appears that PMCA may play a role by continuously extruding Ca^{2+} from the cytosol.

We estimated the relative capacity of each component of Ca^{2+} extrusion to relax by comparing relaxation half-times. Assuming the independence of components, the relative prolongation of the half-times provides an index of the contribution of each element to relaxation: 25–30% for PMCA, 20% for SERCA, 70% for NCX, and 15–20% for all “other” components. Mitochondrial Ca^{2+} uptake and Ca^{2+} leak pathways are included in the “other” component category. When all component values are summed, the value ranges from 125% to 135%. The excess beyond 100% may not be due simply to experimental error but may be due to the assumption that the components are independent. In the PMCA gene-ablated muscle, the percent contribution of SERCA increased considerably over that which could be attributed to the loss of PMCA alone. On the other hand, the combined inhibition of NCX and SERCA increased the contribution by <5% in each phenotype tested. Because the cooperativity between SERCA and NCX was observed in WT muscle, these functions could be related. This cooperativity could arise in series form whereby Ca^{2+} uptake by the sarcoplasmic reticulum is extruded out of the cell through the operation of NCX, as proposed by van Breemen and colleagues (16, 22). On the other hand, the combined inhibition of PMCA and NCX increased by 10% in 
Pmca4−/− muscle and 2% in 
Pmca1+/−/Pmca4−/− muscle, suggesting that the inhibition is not completely additive. This apparent cooperativity between PMCA and NCX may arise partly from the increase in NCX activity on ablation of the PMCA4 isoform, as discussed above. The present study shows that NCX plays the largest role in Ca^{2+} homeostasis under our conditions. However, the removal of Na+ from PSS changes the ionic environment and perturbs many cellular functions (31). Therefore, a better idea of the role of NCX in Ca^{2+} homeostasis may be obtained when a specific inhibitor is found.
for NCX. Importantly, the present experiments using gene-ablated PMCA isoforms indicate that PMCA4 is an important factor in both excitation-contraction coupling and Ca\(^{2+}\) extrusion-relaxation of the bladder smooth muscle.

**REFERENCES**


