Distinct Regulation of Cytoplasmic Calcium Signals and Cell Death Pathways by Different Plasma Membrane Calcium ATPase Isoforms in MDA-MB-231 Breast Cancer Cells*

Merril C. Curry†, Nicole A. Luk‡, Paraic A. Kenny‡, Sarah J. Roberts-Thomson‡, and Gregory R. Monteith++

From the †School of Pharmacy, The University of Queensland, Brisbane, Queensland 4072, Australia and ++Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Background: The roles of different PMCA isoforms are not fully understood particularly in cell death.

Results: PMCA4 and PMCA1 silencing has differential effects on Ca\(^{2+}\) signaling and caspase-dependent and -independent cell death in MDA-MB-231 cells.

Conclusion: PMCA isoforms have distinct roles in the control of cell death pathways.

Significance: Inhibition of PMCA4 may increase the effectiveness of some cancer therapies.

Plasma membrane calcium ATPases (PMCA) actively extrude Ca\(^{2+}\) from the cell and are essential components in maintaining intracellular Ca\(^{2+}\) homeostasis. There are four PMCA isoforms (PMCA1–4), and alternative splicing of the PMCA genes creates a suite of calcium efflux pumps. The role of these different PMCA isoforms in the control of calcium-regulated cell death pathways and the significance of the expression of multiple isoforms of PMCA in the same cell type are not well understood. In these studies, we assessed the impact of PMCA1 and PMCA4 silencing on cytoplasmic free Ca\(^{2+}\) signals and cell viability in MDA-MB-231 breast cancer cells. The PMCA1 isoform was the predominant regulator of global Ca\(^{2+}\) signals in MDA-MB-231 cells. PMCA4 played only a minor role in the regulation of bulk cytosolic Ca\(^{2+}\), which was more evident at higher Ca\(^{2+}\) loads. Although PMCA1 or PMCA4 knockdown alone had no effect on MDA-MB-231 cell viability, silencing of these isoforms had distinct consequences on caspase-independent (ionomycin) and -dependent (ABT-263) cell death. PMCA1 knockdown augmented necrosis mediated by the Ca\(^{2+}\) ionophore ionomycin, whereas apoptosis mediated by the Bcl-2 inhibitor ABT-263 was enhanced by PMCA4 silencing. PMCA4 silencing was also associated with an inhibition of NF\(\kappa\)B nuclear translocation, and an NF\(\kappa\)B inhibitor phenocopied the effects of PMCA4 silencing in promoting ABT-263-induced cell death. This study demonstrates distinct roles for PMCA1 and PMCA4 in the regulation of calcium signaling and cell death pathways despite the widespread distribution of these two isoforms. The targeting of some PMCA isoforms may enhance the effectiveness of therapies that act through the promotion of cell death pathways in cancer cells.

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† To whom correspondence should be addressed: School of Pharmacy, The University of Queensland, Brisbane, Queensland 4072, Australia. Tel.: 61-7-334-61855; Fax: 61-7-3346-1999; E-mail: gregm@uq.edu.au.
‡ Significance: Inhibition of PMCA4 may increase the effectiveness of some cancer therapies.
§ The abbreviations used are: PMCA, plasma membrane calcium ATPase; [Ca\(^{2+}\)]\(_{CYT}\), cytoplasmic free calcium; ER, estrogen receptor; NF\(\kappa\)B, nuclear factor of activated T-cells; Bcl-2, B-cell lymphoma-2; siPMCA1, PMCA1 siRNA; siPMCA4, PMCA4 siRNA; siNT, nontargeting siRNA; CPA, cyclopiazonic acid; PMA, phorbol 12-myristate 13-acetate; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; ANOVA, analysis of variance.
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EXPERIMENTAL PROCEDURES

Cell Culture—The human breast cancer cell line MDA-MB-231 (American Type Culture Collection) was cultured in high glucose DMEM (Sigma Aldrich) supplemented with 10% FBS and 4 mM l-glutamine (Invitrogen) at 37 °C/5% CO₂ in a humidified air incubator. All cultures were periodically screened for mycoplasma infection.

siRNA-mediated Knockdown of PMCA4 and PMCA1 Gene Expression—MDA-MB-231 cells were seeded into 96-well plates and allowed to adhere overnight. DharmaFECT 4 (0.1–0.2 µl/well, Dharmacon) was used to transfect cells with Dharmacon ON-TARGETplus™ SMARTpool siRNA, which consists of four pooled siRNA sequences rationally designed to minimize off-target effects (25, 26). Transfection media was removed 72 h post-siRNA transfection. Transfection media was replaced with physiological salt solution containing bovine serum albumin (BSA, 0.3%). [Ca²⁺]_{CYT} was measured in Ca²⁺-probed loaded cells using a fluorescence imaging plate reader (28) (FLIPRTETRA, Molecular Devices Corporation) using an excitation intensity of 470–495 nm and a 515–575 nm emission filter. Fluorescence was normalized to base-line values to assess relative [Ca²⁺]_{CYT}.

Immunoblotting—Cells were plated at 5.0 × 10³ cells per well into 96-well plates and siRNA-transfected. Cell extracts were harvested 72 h post-transfection in protein lysis buffer supplemented with protease inhibitor mixture (Roche Applied Science) as described previously (24). Proteins were separated using gel electrophoresis and transferred onto a polyvinylidene fluoride membrane as described previously (29). Membranes were probed with monoclonal anti-PMCA4 antibody (1:1000, JA9, Pierce Antibodies), monoclonal anti-PMCA antibody (1:2000, 5F10, Pierce Antibodies) and monoclonal anti-β-actin antibody (1:10,000, Sigma Aldrich). Anti-mouse horseradish peroxidase-conjugated secondary antibody (1:10000, Bio-Rad) was used to visualize protein bands by chemiluminescence using Super-Signal West Dura (Pierce). All antibodies were diluted in PBST (0.1% Tween 20 in PBS) with skim milk powder (2.5%). Images were acquired and analyzed by densitometry using a VersaDoc instrument and Quantity One Analysis software (Bio-Rad), respectively. All data were normalized to the β-actin loading control and are presented relative to siNT.

Assessment of Cell Viability—MDA-MB-231 cells were plated at 5.0 × 10³ cells per well into 96-well plates and siRNA-transfected. At 72 h post-transfection, medium was removed, and cells were treated with ABT-263 (Selleckchem), ionomycin (Enzo Life Sciences), Z-VAD-FMK (Enzo Life Sciences), IMD-0354 (Sigma-Aldrich), or dimethyl sulfoxide (up to 1%) for 48 h in phenol red-free DMEM containing FBS (8%). Live cells were stained at 37 °C for 15 min with Hoechst 33342 (10 µg/ml, Invitrogen) and propidium iodide (1 µg/ml, Invitrogen). Images were acquired using an ImageXpress micro auto-

References:
- The complete list of references and citations is provided in the full text of the article.
mated epifluorescence microscope (Molecular Devices Corp.) with a 10× objective, a system previously used for cell death assays (30–32). Four images were automatically acquired per well in the DAPI and Cy3 channel for Hoechst 33342 and propidium iodide, respectively. The multiwavelength cell scoring application module (MetaXpress, version 3.1.0.83; Molecular Devices Corp.) was used to segment cell nuclei to calculate the average Hoechst 33342 integrated intensity and the average propidium iodide intensity emitted per cell. Criteria for viable, apoptotic and necrotic (and secondary apoptosis) are shown graphically in figures and validated using the caspase inhibitor Z-VAD-FMK.

Assessment of NFκB Nuclear Translocation—MDA-MB-231 cells were plated at 3.5 × 10⁵ cells per well into 96-well plates and transfected with siRNA. Post-siRNA transfection (48 h), medium was removed, and cells were serum-reduced (2% FBS) for 12 h, prior to the addition of phorbol 12-myristate 13-acetate (PMA, 50 nM, Sigma) or dimethyl sulfoxide (0.05%) for 1 h in DMEM (10% FBS) (33). Cells were fixed (4% paraformaldehyde in PBS), blocked (PBS, 5% normal goat serum and 0.3% Triton X-100), and then stained with the polyclonal anti-NFκB p65 antibody (Cell Signaling Technologies) diluted 1:100 in PBS containing BSA (1%) and Triton X-100 (0.3%). NFκB was visualized using anti-rabbit IgG (H+L), F(ab´)2, fragment Alexa Fluor® 555 conjugate secondary antibody (1:500, Cell Signaling Technologies). Cell nuclei were stained with DAPI (40 nM, Invitrogen), and images were acquired using an ImageXpress microautomated epifluorescence microscope (Molecular Devices Corp.) with a 10× objective as described above. The ImageXpress platform has been utilized in translocation studies previously (34), and the basis of the translocation assay has been reviewed (35). NFκB translocation was assessed using the translocation package (MetaXpress, version 3.1.0.83; Molecular Devices Corp.).

Analysis of PMCA1 and PMCA4 Levels in Published Data Sets—Gene expression data for breast cancer cell lines in three-dimensional culture (36) or for breast tumors (37) were obtained from online repositories (ArrayExpress accession no. E-TABM-244 (cell lines) and NCBI GEO accession no. GSE2034 (tumors)). Affymetrix probes for PMCA1 (215716_s_at) and PMCA4 (212136_at) were shared between these data sets. Samples were stratified based on their reported annotations (basal versus luminal for cell lines, and ER− versus ER+ for tumors). Normalized gene expression levels (log₂) were plotted, and median levels of each gene were compared between groups (Mann-Whitney test). For relapse-free survival analysis, tumors were stratified into groups corresponding to the upper and lower quartiles and the interquartile range for Kaplan-Meier analysis.

Statistical Analysis—All statistical tests were performed as described in the figure legends using GraphPad Prism (version 5.04) for Windows.

RESULTS

siRNA-mediated Knockdown of Specific PMCA Isoforms in MDA-MB-231 Breast Cancer Cells—There was a significant (p < 0.05) reduction in PMCA4 (Fig. 1A) and PMCA1 (Fig. 1B) mRNA levels in cells transfected with PMCA4 siRNA or PMCA1 siRNA, respectively, compared with the siNT control. PMCA4 protein expression was also significantly (p < 0.05) reduced by PMCA4 siRNA (Fig. 1, C and D) but was not associated with changes in PMCA1, total PMCA protein levels were reduced by PMCA1 siRNA (Fig. 1, C and E; p < 0.05). This was not associated with changes in PMCA4 expression (Fig. 1, C and D).

Consequences of PMCA1 or PMCA4 Knockdown on [Ca²⁺]₅ₒᵤ₅ in MDA-MB-231 Breast Cancer Cells—In the absence of extracellular Ca²⁺, increases in [Ca²⁺]₅ₒᵤ₅ elicited by the sarcoplasmic-endoplasmic reticulum Ca²⁺-ATPase inhibitor cyclopiazonic acid (CPA, 10 μM) were markedly altered with siRNA-mediated knockdown of PMCA1 but not by PMCA4 knockdown (Fig. 2, A–D). PMCA1 silencing significantly (p < 0.05) delayed the time to reach the maximal Ca²⁺ response (Fig. 2B), increased the half-peak decay time (Fig. 2C) and increased the area under the curve (Fig. 2D) compared with siNT. The effect was isoform-specific, as PMCA4 knockdown did not alter the nature of the CPA-induced Ca²⁺ response when compared with siNT (Fig. 2, A–D).
The purinergic receptor agonist ATP (100 μM) induced a rapid rise in [Ca\(^{2+}\)]\(_{\text{CYT}}\) (Fig. 2E), with no significant difference in the peak maximum between siRNA treatments (mean ± S.D.; siPMCA1, 2.56 ± 0.23; siPMCA4, 2.68 ± 0.31; siNT, 2.99 ± 0.39). However, PMCA1 knockdown significantly altered the recovery of [Ca\(^{2+}\)]\(_{\text{CYT}}\) after ATP stimulation (Fig. 2F-H) in comparison with siNT, resulting in a slower rate of [Ca\(^{2+}\)]\(_{\text{CYT}}\) decay in the absence of extracellular Ca\(^{2+}\) (Fig. 2F), a delay in half-peak decay time (Fig. 2G) and an increase in the area under the curve (Fig. 2H). These effects were isoform-specific as similar changes were not seen with PMCA4 silencing (Fig. 2, E–H). To assess the consequences of isoform-specific PMCA knockdown with [Ca\(^{2+}\)]\(_{\text{CYT}}\) increases of a greater magnitude, we compared the effects of PMCA4 or PMCA1 siRNA on [Ca\(^{2+}\)]\(_{\text{CYT}}\) changes mediated by the Ca\(^{2+}\) ionophore ionomycin in the presence of extracellular Ca\(^{2+}\) (1.8 mM).

Increases in [Ca\(^{2+}\)]\(_{\text{CYT}}\) mediated by ionomycin (3 μM) were significantly (p < 0.05) increased with PMCA1 or PMCA4 knockdown compared with siNT (Fig. 3, A and B). These effects were more pronounced (p < 0.05) with PMCA1 knockdown compared with PMCA4 knockdown (Fig. 3, A and B). Silencing of PMCA1 or PMCA4 also augmented increases in [Ca\(^{2+}\)]\(_{\text{CYT}}\) mediated by 10 μM ionomycin; however, in contrast to 3 μM ionomycin, there were no differences in effect between the two isoforms (Fig. 3, C and D).

**Assessment of PMCA1 or PMCA4 Knockdown on Cell Viability in MDA-MB-231 Breast Cancer Cells**—No effect on cell viability was detected with knockdown of PMCA4 (Fig. 4, A, B, and D) or PMCA1 (Fig. 4, A, C, and D) compared with siNT. PMCA1 and PMCA4 were effectively silenced at the time of the assessment of cell viability (Fig. 4, E and F). Reduced Ca\(^{2+}\) efflux mediated by either PMCA1 or PMCA4 silencing was therefore insufficient for the activation of cell death pathways in MDA-MB-231 cells.

**Mechanism of Ionomycin and ABT-263 Activated Cell Death in MDA-MB-231 Cells**—To assess whether reduced PMCA1 or PMCA4 expression could alter the responsiveness of breast cancer cells to different cell death mechanisms, we first characterized cell death in MDA-MB-231 cells initiated by the Ca\(^{2+}\) ionophore ionomycin (Fig. 5, A and C) and the Bcl-2 inhibitor, ABT-263 (Fig. 5, A and E). Ionomycin (10 μM) and ABT-263 (10 μM) both initiated significant (p < 0.05) increases in cell death compared with control (Fig. 5, G and H). The nature of the initiated cell death was probed using the pan-caspase inhibitor, Z-VAD-FMK (50 μM). Consistent with a necrotic mechanism (13) ionomycin-induced (10 μM) cell death was not affected by Z-VAD-FMK (Fig. 5, C, D, and G), whereas cell death initiated by ABT-263 was almost entirely prevented by caspase inhibition (Fig. 5, E, F, and H), indicating an apoptotic pathway (38).

**Consequences of PMCA1 or PMCA4 Silencing on Ionomycin-induced Necrosis in MDA-MB-231 Breast Cancer Cells**—Necrosis mediated by submaximal ionomycin (3 μM) was not significantly affected by PMCA4 knockdown (Fig. 6, A, B, and G); however, it was significantly (p < 0.05) augmented with PMCA1 silencing (Fig. 6, A, C, and G). Additionally, the level of necrosis was significantly (p < 0.05) higher in cells treated with PMCA1 siRNA compared with PMCA4 siRNA (Fig. 6G) at 3 μM.

For the higher ionomycin concentration (10 μM), knockdown of both PMCA4 (Fig. 6, D, E, and G) and PMCA1 (Fig. 6, D, F, and G) significantly (p < 0.05) elevated the proportion of necrotic cells compared with siNT. However, similar to their
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**FIGURE 3. Ionomycin-evoked \([\text{Ca}^{2+}]_{\text{CYT}}\) signals in the presence of PMCA1 and PMCA4 silencing.**

MDA-MB-231 breast cancer cells were transfected with siPMCA1, siPMCA4, or siNT, and changes in \([\text{Ca}^{2+}]_{\text{CYT}}\) were assessed after the addition of ionomycin (3 \(\mu M\) A) or 10 \(\mu M\) C). \([\text{Ca}^{2+}]_{\text{CYT}}\) transient parameters. All data were pooled from nine individual wells, from three independent experiments performed in triplicate. *\(p < 0.05\), one-way ANOVA, Bonferroni post hoc analysis.

Effects on \([\text{Ca}^{2+}]_{\text{CYT}}\), the isoform-specific differences seen with submaximal ionomycin concentration (3 \(\mu M\)) were not detected at higher \([\text{Ca}^{2+}]_{\text{CYT}}\) loads associated with 10 \(\mu M\) ionomycin (Fig. 6G).

**Consequences of PMCA1 or PMCA4 Silencing on ABT-263-induced Apoptosis in MDA-MB-231 Breast Cancer Cells—** Submaximal ABT-263-induced (3 \(\mu M\)) apoptosis was significantly \((p < 0.05)\) augmented by PMCA4 siRNA compared with siNT (Fig. 7, A, B, and G). This effect was isoform-specific, as PMCA1 knockdown did not alter apoptosis compared with siNT (Fig. 7, A, C, and G). In contrast, ABT-263-mediated (10 \(\mu M\)) cell death was not modulated by either PMCA4 siRNA or PMCA1 siRNA compared with siNT (Fig. 7, D–G).

**PMCA4 but Not PMCA1 Knockdown Inhibits NFkB Translocation in MDA-MB-231 Breast Cancer Cells—** Nuclear factor-\(\kappa\)B (NFkB) is a \([\text{Ca}^{2+}]_{\text{CYT}}\)-dependent transcription factor (39–41), which is implicated in breast cancer progression (42) and the resistance to cancer therapies (43, 44). PMCA4 is linked to the regulation of \([\text{Ca}^{2+}]_{\text{CYT}}\)-dependent transcription factors such as NFAT, independent of bulk \([\text{Ca}^{2+}]_{\text{CYT}}\) changes (10). We assessed the effects of PMCA4 and PMCA1 silencing on nuclear translocation of NFkB induced by PMA (50 \(nM\)) (33). PMA-induced NFkB nuclear translocation (Fig. 8, A and B) was not affected by PMCA1 silencing but was significantly \((p < 0.05)\) reduced by PMCA4 siRNA relative to siNT.

**Effects of NFkB Inhibition on ABT-263-induced MDA-MB-231 Cell Death—** To further explore the possible link between PMCA4 and NFkB on MDA-MB-231 cell death, we assessed whether NFkB inhibition could mimic PMCA4 siRNA by augmenting cell death induced by ABT-263 (3 \(\mu M\)). The NFkB inhibitor IMD-0354 (10 \(\mu M\)) (45) did not induce cell death alone (Fig. 8, C–F); however, it did significantly \((p < 0.05)\) augment ABT-263-mediated apoptosis (Fig. 8, C–I), suggesting that IMD-0354 phenocopies the effect of PMCA4 siRNA on ABT-263-mediated cell death.

**PMCA1 and PMCA4 in Clinical Breast Cancers—** To determine the distribution of PMCA1 and PMCA4 in breast cancer, we first examined their expression in a panel of breast cancer cell lines grown in three-dimensional culture (36). Expression varied widely between cell lines, with variation between the highest and lowest measurements being 18.9- and 12.8-fold for PMCA1 and PMCA4, respectively (Fig. 9, A and B). PMCA1 expression measurements were found throughout this range in both luminal and basal cell lines (Fig. 9A); however, PMCA4 was most highly expressed in the basal cell lines (Fig. 9B, \(p = 0.007\)). We next evaluated the level of these genes in a cohort of 286 node-negative breast cancer patients described by Wang and co-workers (37). Consistent with the non-significant trend suggested in the cell line data (Fig. 9A), PMCA1 was expressed at higher levels in the ER+ tumors (Fig. 9C, \(p = 0.007\)). Median PMCA4 levels were higher in the ER-negative tumors (Fig. 9D, \(p = 0.028\)). No differences were observed in patient outcomes when tumors were stratified by PMCA1 (Fig. 9E, \(p = 0.92\)) or PMCA4 (Fig. 9F, \(p = 0.76\)) levels.

**DISCUSSION**

Despite the proposed differential contributions of PMCA isoforms in \([\text{Ca}^{2+}]_{\text{CYT}}\)-dependent cellular processes, few studies have evaluated the specific roles of different PMCA isoforms in shaping intracellular \([\text{Ca}^{2+}]_{\text{CYT}}\) signals and cellular responses in the same cell types. PMCA1 and PMCA4 have a broad tissue distribution and frequently co-express within the same cell (46). We compared the consequences of knockdown of these two PMCA isoforms on \([\text{Ca}^{2+}]_{\text{CYT}}\) signals generated by various \([\text{Ca}^{2+}]_{\text{CYT}}\)-mobilizing agents. Pronounced effects with PMCA1 knockdown but not with PMCA4 knockdown were seen on the nature of CPA- and ATP-induced \([\text{Ca}^{2+}]_{\text{CYT}}\) transients. PMCA1 siRNA reduced the rate of \([\text{Ca}^{2+}]_{\text{CYT}}\) decay and prolonged the ATP \([\text{Ca}^{2+}]_{\text{CYT}}\) response. Previous studies overexpressing PMCA isoforms in CHO cells found that some variants not only alter \([\text{Ca}^{2+}]_{\text{CYT}}\) homeostasis within the cytoplasm but also within subcellular compartments such as the endoplasmic reticulum (2). Increases in \([\text{Ca}^{2+}]_{\text{CYT}}\) mediated by sarcoplasmic endoplasmic reticulum \([\text{Ca}^{2+}]_{\text{ER}}\) and ATPase inhibitors have been reported to correlate with the calcium content of the endoplasmic reticulum (47). Hence, increases in the CPA-mediated \([\text{Ca}^{2+}]_{\text{CYT}}\) transient upon PMCA1 silencing, in addition to being a consequence of reduced \([\text{Ca}^{2+}]_{\text{ER}}\) efflux, may also involve increases in endoplasmic reticulum \([\text{Ca}^{2+}]_{\text{ER}}\) levels.
Our study suggests that in MDA-MB-231 breast cancer cells, the PMCA1 isoform is the major regulator of global $[\text{Ca}^{2+}]_{\text{CYT}}$ increases, such as those generated by phosphatidylinositol 1,4,5-trisphosphate-mediated $\text{Ca}^{2+}$ release after G protein-coupled receptor activation. The significance of PMCA4 in MDA-MB-231 cells may become apparent during very high $[\text{Ca}^{2+}]_{\text{CYT}}$ loads. Indeed, during the high magnitude increases in $[\text{Ca}^{2+}]_{\text{CYT}}$ initiated by $3\mu$M ionomycin, a contribution for PMCA4 was identified. However, this PMCA4 siRNA-mediated effect was not as pronounced as seen with PMCA1 knockdown. This finding is also consistent with a predominant role for PMCA1 in the regulation of global $[\text{Ca}^{2+}]_{\text{CYT}}$ signals in this cell type and is consistent with previous studies, demonstrating distinct phenotypes in PMCA1 and PMCA4 knock-out animals (5, 7, 8). PMCA1 ablation is lethal during embryogenesis, indicative of its vital role in the maintenance of $[\text{Ca}^{2+}]_{\text{CYT}}$. 

**FIGURE 4.** Cell viability in the presence of siRNA-mediated silencing of PMCA1 and PMCA4 gene expression. MDA-MB-231 breast cancer cells were transfected with siPMCA4, siPMCA1, or siNT, incubated for a further 48 h and then assessed for cell viability. Cell viability for siNT (A), siPMCA4 (B), and siPMCA1 (C) presented as dot plots of Hoechst 33342 and propidium iodide fluorescence. Each dot plot shows an equal cell number (10,000 cells) randomly selected from three independent experiments performed in triplicate wells. D, bar graph showing the mean ± S.D. of the proportion of viable cells 120 h post-siRNA transfection from three independent experiments. *, $p > 0.05$, one-way ANOVA, Bonferroni post hoc analysis. PMCA4 mRNA (E) and PMCA1 mRNA (F) levels, 120 h post-siRNA transfection with siPMCA4, siPMCA1, or siNT. Real time RT-PCR data were pooled from six individual wells, from three independent experiments performed in duplicate. *, $p < 0.05$, one-tailed Student’s $t$ test.

**FIGURE 5.** Effects of the caspase inhibitor Z-VAD-FMK on ionomycin and ABT-263-mediated cell death in MDA-MB-231 breast cancer cells. A–F, dot plots for control, ionomycin, or ABT-263 either in the absence or presence of Z-VAD-FMK. Each dot plot represents an equal number of total cells (10,000 cells) selected at random from three independent experiments. G and H, the pooled data for the effect of Z-VAD-FMK on the proportion of dead cells induced by ionomycin or ABT-263 (mean ± S.D.) from three independent experiments. *, $p < 0.05$, repeated measures two-way ANOVA, Bonferroni post hoc analysis.
homeostasis in an array of cell types (5). Although PMCA4 has a broad tissue distribution, PMCA4 null mice reach adulthood and exhibit tissue-specific phenotypes such as male infertility (5, 7, 8). These differences suggest that PMCA1 adopts a vital housekeeping function by regulating global Ca²⁺/H¹¹001 homeostasis and supports the involvement of PMCA4 in Ca²⁺/H¹¹001-dependent signal transduction and cell processes by shaping of Ca²⁺/H¹¹001 signals within subcellular domains (5, 7, 8).

We extended our study to examine the consequences of PMCA4 and PMCA1 silencing on cell death in the presence of cell death stimuli. Initial studies, in the absence of any stimuli, showed that the viability of MDA-MB-231 breast cancer cells was not affected by PMCA1 or PMCA4 knockdown. In contrast, PMCA2 knockdown produces spinal cord neuronal cell death (48) in the absence of external stimuli. Assessment of PMCA1 and PMCA4 knockdown on cell death initiated by ionomycin or ABT-263 demonstrated distinct roles for PMCA1 and PMCA4 in the regulation of caspase-independent and -dependent cell death pathways, respectively. Consistent with the effects seen with ionomycin-induced [Ca²⁺]Cyto transients, PMCA1 knockdown had a more pronounced effect on ionomycin-induced cellular necrosis than PMCA4 knockdown. This result further underscores the more subtle nature of the change in calcium handling in the cell with PMCA4 knockdown. Previous results examining PMCA4 as a sensitizer of cell death in HT-29 colon cancer cells showed that PMCA4 silencing does not alter the sensitivity of HT-29 cells to tumor necrosis factor-related apoptosis inducing ligand (TRAIL) or carbonyl cyanide 3-chlorophenylhydrazone (CCCP)-induced cell death (48). Reduced expression of another PMCA isoform, PMCA2, augments ionomycin-mediated cell death in SH-SY5Y neuroblastoma cells (23), whereas its overexpression in T47D breast cancer cells bestows resistance to ionomycin-mediated cell death through the attenuation of [Ca²⁺]Cyto responses (21).

FIGURE 6. PMCA1 and PMCA4 silencing effects in promoting ionomycin-mediated cell death in MDA-MB-231 breast cancer cells. Dot plots of Hoechst 33342 and propidium iodide fluorescence in cells transfected with siNT (A and D), siPMCA4 (B and E), or siPMCA1 (C and F) following treatment with 3 μM or 10 μM ionomycin. Each dot plot represents an equal number of total cells (10,000 cells) selected at random from three independent experiments. G, the proportion of necrotic cells pooled from three independent experiments (mean ± S.D.). *, p < 0.05, repeated measures two-way ANOVA, Bonferroni post hoc analysis.

FIGURE 7. PMCA1 and PMCA4 silencing effects in promoting ABT-263-mediated cell death in MDA-MB-231 breast cancer cells. Dot plots of Hoechst 33342 and propidium iodide fluoresence in cells transfected with siNT (A and D), siPMCA4 (B and E), or siPMCA1 (C and F) following treatment with ABT-263 (ABT). Each dot plot represents an equal number of total cells (10,000 cells) selected at random from three independent experiments. G, the proportion of apoptotic cells pooled from three independent experiments (mean ± S.D.). *, p < 0.05, repeated measures two-way ANOVA, Bonferroni post hoc analysis.
potential significance of this survival advantage is reflected in the poorer prognosis of breast cancer patients with elevated expression of PMCA2 (21). Our silencing studies suggest that modulators of PMCA isoforms involved in global \([Ca^{2+}]_{CYT}\) may sensitize cells to cell death stimuli.

The Bcl-2 inhibitor (ABT-263) used in this study to induce apoptotic cell death in MDA-MB-231 breast cancer cells is progressing through clinical trials (17). A structurally related analog (ABT-737) sensitizes Bcl-2–expressing breast cancers to chemotherapies (49), highlighting the potential for Bcl-2 inhibitors as a therapeutic option for breast cancer. ABT-263–induced cell death was not affected by PMCA1 knockdown but instead was augmented upon reduced expression of PMCA4. These distinct differences in the consequences of isoform-specific PMCA knockdown on ionomycin and ABT-263 initiated cell death is not totally unexpected, considering these agents activate cell death by distinct mechanisms and have markedly different effects on \(Ca^{2+}\) signals. Ionomycin produces sustained global increases in \([Ca^{2+}]_{CYT}\) augmented by PMCA1 silencing, resulting in \(Ca^{2+}\) overload and cell necrosis. Bcl-2 inhibitors such as ABT-263 initiate caspase-dependent apoptosis by binding to and blocking the prosurvival activity of BH3 domains present within Bcl-2 proteins (14–16). Increases in intracellular \(Ca^{2+}\) signals, particularly within subcellular compartments, such as the endoplasmic reticulum and mitochondria can modulate Bcl-2–mediated survival pathways (18, 50, 51). Our identification of PMCA4 as a modulator of ABT-263–mediated cell death may be analogous to the characterized role of PMCA4 in cardiac cells, where altered PMCA4 expression appears to play little role in shifting global \([Ca^{2+}]_{CYT}\) increases (10, 52) yet is an important regulator of the \(Ca^{2+}\)-dependent transcription factor NFAT influencing outcomes such as cardiac hypertrophy (9, 10). \(Ca^{2+}\)-dependent gene transcription critically depends on localization of \(Ca^{2+}\) signals as well as \(Ca^{2+}\) oscillation frequency and amplitude (39, 40). Thus, PMCA4–mediated regulation of transcription may be mediated through the fine-tuning of \(Ca^{2+}\) signals within localized subcellular domains or through alterations in oscillation frequency. Context–dependent modulation of apoptosis by PMCAs is reflected in PMCA4 knock-out mice studies. Smooth muscle cells from the portal veins of PMCA4 knock-out mice on a mixed 129/SvJ and Black Swiss background display features of apoptosis during in vitro contraction studies (7).

Our study identified PMCA4 siRNA–mediated inhibition of NFkB nuclear translocation. Pharmacological inhibition of NFkB phenocopied the augmentation of ABT-263–mediated apoptosis produced by PMCA4 silencing, suggesting that PMCA4 siRNA augmentation of apoptosis may be due to modulation of NFkB. The ability of PMCA4 silencing to inhibit NFkB is significant. The activity of NFkB is governed by the nature of \(Ca^{2+}\) signals (39–41), and \(Ca^{2+}\) influx mediated by the calcium channel TRPC1 can inhibit NFkB activity in an intestinal epithelial cell line (53). Breast cancers with a poor prognosis are associated with elevated constitutive activity of NFkB (43, 44). Agents that inhibit NFkB are themselves promising anti-tumor modulators for the treatment of breast cancers.

FIGURE 8. NFkB activity in the presence of PMCA1 and PMCA4 siRNA and the effect of pharmacological inhibition of NFkB on ABT-263–mediated cell death. A, MDA-MB-231 breast cancer cells stained for NFkB (orange) with DAPI (nuclei, blue) and with white arrows depicting translocation of NFkB to the nucleus in the (i) absence of PMA (50 nM), (ii) presence of PMA (50 nM), (iii) presence of siPMCA1 and PMA (50 nM), and (iv) presence of siPMCA4 and PMA (50 nM). B, percentage of cells with NFkB nuclear translocation in siRNA transfected cells following the addition of PMA (50 nM) normalized to the dimethyl sulfoxide control. C, the proportion of viable cells following IMD-0354 and/or ABT-263 (ABT) treatment. D, the proportion of apoptotic cells following IMD-0354 and/or ABT-263 treatment. E–I, dot plots of Hoechst 33342 and propidium iodide fluorescence in cells transfected with siNT or with siPMCA4 in the presence of IMD-0354 and/or ABT-263. Each dot plot represents an equal number of total cells (10,000 cells) selected at random from three independent experiments. All data were from three independent experiments and are presented as mean ± S.D. where relevant. *, \(p < 0.05\), repeated measures two–way ANOVA, Bonferroni post hoc analysis. Scale bar, 25 \(\mu m\).
and can enhance the effects of Bcl-2 inhibitors (56). Our studies comparing PMCA1 and PMCA4 in clinical samples suggest that although inhibition of PMCA4 in basal breast cancer may be an effective way to augment responsiveness to Bcl-2 inhibitors in breast cancer therapy, increases in PMCA4 (or PMCA1) expression are not a cause of general apoptotic resistance in breast cancer, as there was no association between PMCA4 (or PMCA1) levels and prognosis.

In summary, we show that PMCA1 is a major regulator of global Ca\(^{2+}\) homeostasis in MDA-MB-231 breast cancer cells and this is associated with an ability of PMCA1 silencing to augment necrotic cell death generated by high Ca\(^{2+}\) loads. Although, PMCA4 is not a key regulator of global changes in [Ca\(^{2+}\)]\(_{\text{CYT}}\) associated with many stimuli, our study demonstrates a novel relationship between PMCA4 and NFkB. Our study highlights isoform diversity between the two almost ubiquitously expressed PMCA isoforms (PMCA1 and PMCA4) and identifies PMCA4 as a potential anti-tumor modulator. Inhibitors of PMCA4 (57) may be novel therapeutics to sensitize some cancer cells to apoptotic stimuli.

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