PMCA2 silencing potentiates MDA-MB-231 breast cancer cell death initiated with the Bcl-2 inhibitor ABT-263

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Abstract

PMCA2 overexpression in some breast cancers suggests that this calcium pump isoform may play a role in breast pathophysiology. To investigate PMCA2 as a potential drug target for breast cancer therapy, we assessed the functional consequence of PMCA2 silencing on cell death pathways and calcium signals in the basal-like MDA-MB-231 breast cancer cell line. Silencing PMCA2 expression alone has no effect on MDA-MB-231 cell viability, however, PMCA2 silencing promotes calcium-induced cell death initiated with the calcium ionophore ionomycin. Assessment of cytoplasmic calcium responses generated with various agents including ionomycin demonstrates that in MDA-MB-231 cells, PMCA2 does not play a major role in shaping global calcium signals. We also examined the ability of PMCA2 silencing to modulate caspase-dependent cell death triggered by a Bcl-2 inhibitor that is in clinical development for the treatment of various cancers, ABT-263 (Navitoclax). Despite the lack of effect on global calcium responses, PMCA2 silencing augmented Bcl-2 inhibitor (ABT-263)-mediated MDA-MB-231 breast cancer cell death. These studies provide evidence that PMCA2 inhibitors could sensitize PMCA2-positive breast cancers to cell death initiators that work through mechanisms involving the Bcl-2 survival pathway.

Keywords

Abbreviations

Ca^{2+}, calcium; $[Ca^{2+}]_{CYT}$, cytoplasmic free calcium; FBS, Fetal Bovine Serum; PMCA, plasma membrane calcium ATPase; Bcl-2, B-cell lymphoma-2; siPMCA2, plasma membrane calcium

ATPase siRNA; siNT, non-targeting siRNA; CPA, cyclopiazonic acid; ANOVA, analysis of variance.

Introduction

The calcium efflux pump, plasma membrane calcium ATPase (PMCA) isoform 2 has been proposed as potential target for the treatment of some breast cancers [1]. Aberrant overexpression of PMCA2 in some breast cancer cell lines [2] and the positive correlation between PMCA2 protein expression and tumor characteristics including tumor grade and HER2 status in clinical breast cancer cases [3] suggests that PMCA2 could facilitate disease progression [3]. More recently, PMCA2 mRNA levels were assessed in breast cancers grouped by molecular subtype [4]. PMCA2 mRNA was higher in basal breast cancers compared to HER2, luminal A and luminal B subtypes [4]. Although a feature of some breast cancers, only a few studies have evaluated the functional significance of PMCA2 upregulation in the context of cell death in breast cancer; and detailed assessment of PMCA2 through overexpression or silencing techniques has been limited to luminal-like breast cancer cell lines that express at least one hormone receptor (estrogen or progesterone receptors) or those with HER2 amplification [3, 5]. Heterologous PMCA2 expression in T47D cells attenuates global cytosolic free Ca^{2+} levels ($[Ca^{2+}]_{CYT}$) and protects T47D cells from calcium-induced cell death initiated with ionomycin [3]. Assessment of PMCA2 silencing in the context of HER2 positive breast cancers has been examined in the SKBR3 and BT474 breast cancer cell lines [5], both of which are positive for HER2 amplification [6]. PMCA2 knockdown in SKBR3 promotes ionomycin cell death and downregulates HER2 signaling [5].

Until recently the consequence of PMCA2 silencing in a basal-like breast cancer cell line had not been evaluated. One cell line widely used as a model for basal breast cancer subtypes, which overlaps with breast cancers that are negative for hormone receptor expression and HER2 amplification is the MDA-MB-231 breast cancer cell line [6]. Studies in this cell line demonstrate PMCA2 silencing attenuates proliferation and potentiates the anti-proliferative action of low concentrations of the cytotoxic doxorubicin [4]. Since neither PMCA1 nor PMCA4 silencing produced effects on cell proliferation, PMCA-mediated regulation of MDA-MB-231 cell growth appears to be PMCA2 specific in this breast cancer cell line. This and other studies emphasize that PMCA isoforms are functionally distinct, even when expressed in the same cell type.

PMCA2 silencing-mediated effects on breast cancer cell death have focused on ionomycin [5], which can trigger cell death through sustained $[Ca^{2+}]_{CYT}$ increases of high magnitude [5, 7, 8]. The consequence of PMCA2 knockdown on B-cell lymphoma-2 (Bcl-2) inhibitor-mediated apoptosis has not been assessed. Bcl-2 is a pro-survival oncogene overexpressed in 75% of breast cancers [9]. ABT-263 (Navitoclax) is a Bcl-2 inhibitor undergoing clinical trial assessment for various cancer indications including lymphoma, prostate and colon cancer [10]. Studies of PMCA knockdown-mediated effects on Bcl-2 inhibitor (ABT-263)-induced apoptosis have only so far been examined for PMCA1 and PMCA4 isoforms [7]. PMCA1 regulates global $[Ca^{2+}]_{CYT}$ signals, however, PMCA1 knockdown had no effect on Bcl-2 inhibitor-mediated cell death [7]. Whereas, PMCA4 silencing promoted apoptosis in MDA-MB-231 cells initiated with the Bcl-2 inhibitor ABT-263, without significant effects on global calcium signals [7]. Isoform-specific PMCA knockdown in MDA-MB-231 cells produces very different outcomes on

$[Ca^{2+}]_{CYT}$, and on the cancer-relevant pathways proliferation and apoptosis. However, the consequence of PMCA2 silencing on Bcl-2 inhibitor-mediated cell death has not been evaluated.

In this current study, we examined the effects of PMCA2 silencing in MDA-MB-231 breast cancer cells on global calcium signals and in the modulation of cell death initiated with ionomycin and the Bcl-2 inhibitor ABT-263. Our findings suggest that PMCA2 inhibitors, in combination with anti-cancer agents that work through the Bcl-2 pathway, could be effective for the treatment of PMCA2-positive breast cancers; including breast cancer cases that are typically unresponsive to targeted therapies such as those targeting HER2 (e.g. trastuzumab) and the estrogen receptor (e.g. tamoxifen) [11, 12].

Materials and Methods

Cell culture

Human MDA-MB- 231 breast cancer cells (American Type Culture Collection, Rockville, MD, USA) were maintained in high glucose DMEM (D6546, Sigma Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum and 4 mM L-glutamine (25030081, Invitrogen, Carlsbad, CA, USA) at 37°C/5% CO_2 in a humidified air incubator. All cultures were periodically screened for mycoplasma infection.

RNA interference

PMCA2 levels were downregulated in MDA-MB-231 cells according to methods previously described [4, 7] using ON-TARGET*plus*™ Human SMARTpool siRNAs (Thermo Scientific, Waltham, MA, USA). These siRNAs consist of four pooled sequences rationally designed to

minimize off-target effects [13-15]. The ON-TARGET*plus*™ Human SMARTpool siRNA sequences used were PMCA2 (siPMCA2, L-006116-00) and the non-targeting control siRNA (siNT, D-001810-10). For all experiments knockdown of PMCA2 (>70%) was confirmed by real time RT-PCR at 120 h post-siRNA transfection.

Real time RT-PCR

At 48 h or 120 h post-siRNA transfection quantitative real time RT-PCR was performed as previously reported [7]. Total RNA was isolated (74134, RNeasy Plus mini kit; Qiagen, Hilden, Germany) and then reverse transcribed (205111, Omniscript RT kit; Qiagen). The target cDNAs were amplified using the TaqMan Fast Universal PCR Master Mix (4352043, Applied Biosystems, Carlsbad, CA, USA) and TaqMan Gene Expression assays (PMCA2, Hs00155975_m1 and 18S rRNA, 4319413E; Applied Biosystems) under universal cycling conditions with a StepOnePlus real time PCR system (Applied Biosystems). Target mRNA levels were quantified by the comparative Ct method as described previously [16], normalizing to 18S rRNA.

Cytoplasmic free calcium measurements

At 120 h post-siRNA transfection, media was removed from MDA-MB-231 cells and replaced with culture medium containing Fluo-4 AM (4 μM; Molecular Probes, Eugene, OR, USA) or Fluo-4FF AM (4 μM; Molecular Probes). Using these Ca^{2+} indicators changes in $[Ca^{2+}]_{CYT}$ were recorded using a fluorescence imaging plate reader [17] (FLIPR^{TETRA}, Molecular Devices, Sunnyvale, CA, USA) as previously described [7]. To assess relative $[Ca^{2+}]_{CYT}$, fluorescence values were normalized to initial baseline values.

Assessment of cell viability

MDA-MB-231 cells were siRNA-transfected for 72 h, and then treated for an additional 48 h with ionomycin (Enzo Life Sciences, Farmingdale, NY, USA), ABT-263 (S1001, Selleckchem, Houston, TX, USA), or dimethyl sulfoxide (up to 0.33%). Cells were then stained with Hoechst 33342 (10 μg/mL; H3570, Invitrogen) and propidium iodide (1 μg/mL; P3566, Invitrogen) at 37°C for 15 min and cell death assessed using an ImageXpress micro automated epifluorescence microscope (Molecular Devices Corporation) and the multi-wavelength cell scoring application module (MetaXpress v3.1.0.83; Molecular Devices) as previously described [7]. Cell viability was determined by combining propidium iodide positive cells and those identified as apoptotic to calculate the total number of dead cells [7].

Statistical Analysis

All statistical tests were performed as described in the figure legends using GraphPad Prism (version 6.07) for Windows (GraphPad Software, Inc., La Jolla, CA, USA).

Results

PMCA2 silencing and consequences on MDA-MB-231 breast cancer cell viability in the absence and presence of the calcium ionophore ionomycin

To explore the functional significance of PMCA2 in a basal like breast cancer cell line, we silenced PMCA2 gene expression in MDA-MB-231 cells. Non-targeting (siNT) and PMCA2 (siPMCA2) siRNAs were transfected into MDA-MB-231 cells and PMCA2 silencing was confirmed by mRNA analysis. Cells transfected with siPMCA2 showed a significant ($P < 0.05$)

downregulation in PMCA2 mRNA, at both time points 48 h and 120 h post-transfection, compared with siNT controls (Fig. 1A).

Using this model, we assessed siPMCA2-mediated regulation of cell viability. With no stimulus, PMCA2 had no effect on MDA-MB-231 cell viability compared with the siNT control (Fig. 1B and C). However, in the presence of sub-maximum (3 µM) ionomycin, siPMCA2 significantly ($P < 0.05$) reduced cell viability compared to the siNT control (Fig. 1C). No measurable effect ($P > 0.05$) on cell viability was detected at the maximum (10 µM) ionomycin concentration, comparing siPMCA2 with the siNT control (Fig. 1C). These findings suggest that PMCA2 silencing without a stimulus does not trigger cell death, although, siPMCA2 can modestly promote Ca^{2+}-induced MDA-MB-231 cell death initiated by sub-maximal concentrations of ionomycin.

Effects of PMCA2 silencing on ionomycin-induced cytoplasmic calcium signals in MDA-MB-231 cells

To further evaluate the relationship between PMCA2 and ionomycin, high magnitude $[Ca^{2+}]_{CYT}$ signals generated using ionomycin were measured in siRNA-transfected MDA-MB-231 cells using the low affinity Ca^{2+} indicator Fluo-4FF AM, in the presence of extracellular calcium. As reflected by assessment of the calcium transient parameter, area under the curve (Fig. 2B and D), calcium signals at sub-maximum (3 µM; Fig. 2A and B) and maximum (10 µM; Fig. 2C and D) ionomycin concentrations were not significantly ($P > 0.05$) altered by PMCA2 silencing, compared with the siNT controls. Although significance was not detected, at sub-maximal ionomycin, siPMCA2-mediated regulation of area under the curve approached statistical

significance ($P = 0.0693$). These data indicate that siPMCA2 does not elicit profound effects on $[Ca^{2+}]_{CYT}$ signals generated with ionomycin in MDA-MB-231 breast cancer cells.

Consequences of PMCA2 silencing on CPA, ATP and trypsin induced calcium signals in MDA-MB-231 breast cancer cells

The potential of PMCA2 to shape bulk $[Ca^{2+}]_{CYT}$ increases of more moderate magnitudes was also examined using the high affinity Ca^{2+} indicator Fluo-4 AM. In siRNA-transfected MDA-MB-231 cells, $[Ca^{2+}]_{CYT}$ signals were monitored in the presence of the Ca^{2+} chelator BAPTA (100 µM) in nominal free Ca^{2+} physiological salt solution, following the addition of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor cyclopiazonic acid (CPA, 10 µM, Fig. 3A), the purinergic receptor agonist ATP (100 µM, Fig. 3D) or the protease activated receptor activator trypsin (0.1 µM, Fig. 3G). Assessment of the calcium transient parameters, half peak decay time (Fig. 3B, E and H) and area under the curve (Fig. 3C, F and I) demonstrated that compared with the siNT control, PMCA2 silencing did not significantly ($P > 0.05$) shape Ca^{2+} responses associated with either CPA (Fig. 3B and C), ATP (Fig. 3E and F), or trypsin (Fig. 3H and I). These data support the conclusion that PMCA2 in MDA-MB-231 cells is not a key regulator of bulk $[Ca^{2+}]_{CYT}$ signals.

Regulation of ABT-263-mediated cell death by PMCA2 silencing in MDA-MB-231 cells

PMCA4 potentiates caspase-dependent cell death, initiated with the Bcl-2 inhibitor ABT-263 in MDA-MB-231 cells [7]. We therefore investigated whether PMCA2 silencing modulates ABT-263-mediated death responses. At 1 µM, ABT-263-mediated cell death was almost doubled by knocking down PMCA2 (mean ± S.D.; 36 ± 11% for the siNT control *versus* 68 ± 5% for siPMCA2; Fig. 4A and B). To characterize this effect, cell viability was measured in siRNA-

transfected MDA-MB-231 cells, treated at increasing concentrations of the Bcl-2 inhibitor ABT-263 (0.1 – 10 µM; Fig. 4C). Compared to the siNT control, siPMCA2 significantly ($P < 0.05$) reduced cell viability in the presence of ABT-263. This was evidenced by a 4.5-fold leftward shift in the half maximal inhibitory concentration (IC_{50}), from 2.2 µM for siNT *versus* 0.48 µM for siPMCA2. These data show that PMCA2 silencing sensitizes the MDA-MB-231 cell line to caspase-dependent ABT-263-mediated cell apoptosis.

Discussion

This report extends previous studies of PMCA1 and PMCA4 in MDA-MB-231 breast cancer cells [7], and evaluated PMCA2-mediated effects on cell death and calcium signaling in this cell line using targeted siRNAs to silence PMCA2 gene expression. Initial experiments showed that PMCA2 silencing alone does not alter MDA-MB-231 cell viability. Since PMCA isoforms are known regulators of calcium-induced cell death [3, 5, 7], we assessed the consequence of PMCA2 silencing on ionomycin death responses. Our results indicated that PMCA2 silencing modestly promoted ionomycin MDA-MB-231 cell death at submaximal concentrations. This is consistent with other studies that demonstrate through overexpression or downregulation systems, that PMCA2 modulates ionomycin cell death in the T47D and SK-Br-3 breast cancer cell lines [3, 5].

We found that PMCA2 is not a key regulator of global $[Ca^{2+}]_{CYT}$ signals generated with ionomycin, or those associated with physiologically relevant mediators ATP and trypsin, which increase $[Ca^{2+}]_{CYT}$ via G protein-coupled receptor activation. This result may be surprising, however, not entirely unexpected because PMCA2 expression is relatively low in MDA-MB-231 cells compared with other isoforms [4]. PMCA1, which is the predominate isoform expressed in

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MDA-MB-231 cells, when knocked down has a pronounced effect on the shape of global $[Ca^{2+}]_{CYT}$ signals generated with many stimuli [4, 7].

Although reported for PMCA1 and PMCA4 [7], PMCA2-mediated regulation of Bcl-2 inhibitor-mediated cell death has not been previously examined. Our data demonstrated that despite no significant effects on global calcium signaling, PMCA2 silencing augmented ABT-263-mediated MDA-MB-231 cell death. This finding is analogous to PMCA4, where knocking down PMCA4 sensitized MDA-MB-231 cells to ABT-263 initiated cell death, independent of global calcium [7]. While PMCA4 and PMCA2 distinctly regulate cell proliferation [4], these isoforms appear to have related effects on the Bcl-2 survival pathway.

Growing evidence indicates that PMCA isoforms regulate calcium-dependent signal transduction through protein-protein interactions, and fine-tuning of localized calcium signals [4, 7, 18-20]. The ability for PMCA2 silencing to promote ABT-263-mediated cell death, is likely the result of disrupting pro-survival protein-protein interactions through processing localized calcium signals. PMCA2 interacts with the calcium-dependent signaling molecule calcineurin, and this interaction can regulate cell death [21, 22]. Preventing the interaction between PMCA2 and calciuneurin, upregulates pro-apoptotic protein expression via the nuclear factor of activated T cell (NFAT) transcriptional pathway [22]. This intervention reduces cell viability across a panel of breast cancer cell lines, and enhances paclitaxel-induced cytotoxicity in MCF-7 and ZR-75-1 breast cancer cells [22]. PMCA2-mediated regulation of the Bcl-2 survival pathway may also involve another calcium-dependent transcription factor nuclear factor-kappa B (NFκB). We previously demonstrated that PMCA4 silencing inhibits NFκB nuclear translocation, and that pharmacological inhibition of NFκB phenocopies the promotion of ABT-263-mediated cell death

produced by PMCA4 silencing [7]. Mechanistic studies are now required to clarify the importance of the calcineurin/NFAT and/or NFκB transcription factor pathways in the promotion of Bcl-2 inhibitor cell death produced by PMCA2 silencing in MDA-MB-231 breast cancer cells.

In conclusion, this study provides further evidence supporting the view that PMCA2 inhibitors may be a novel treatment for PMCA2-positive breast cancers. This approach may be most effective in basal-like subtypes of breast cancers when delivered in combination with agents that initiate cell death via effects on the Bcl-2 cell survival pathway.

Acknowledgements

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Figure Legends

Fig. 1. PMCA2 silencing in MDA-MB-231 breast cancer cells and the consequence of silencing on cell viability and ionomycin-induced cell death. PMCA2 expression was silenced using siRNA and then cell viability assessed in the absence and presence of the calcium ionophore ionomycin, comparing siPMCA2 with the siNT control. (A) PMCA2 mRNA levels 48 h and 120 h post-siRNA, *$P < 0.05$, two-way ANOVA, Bonferroni post hoc analysis. (B) Representative dot plots show cell viability with siNT and siPMCA2. Dot plots are Hoechst 33342 and propidium iodide fluorescence measured in random cells (10 000 cells/dot plot)

selected from three experiments ($n = 3$). (C) Cell viability following siNT and siPMCA2 transfection, either in the absence (0 µM) or presence (3 µM or 10 µM) of ionomycin. *$P < 0.05$, repeated-measures, two-way ANOVA, Bonferroni post hoc analysis. All bar graphs show mean ± S.D obtained from three independent experiments ($n = 3$), performed in duplicate or triplicate wells.

Fig. 2. Effect of PMCA2 silencing on ionomycin-induced calcium signals in MDA-MB-231 breast cancer cells. $[Ca^{2+}]_{CYT}$ signals generated with the calcium ionophore ionomycin at (A) 3 µM and (C) 10 µM were recorded in cells transfected with siPMCA2 or siNT. Calcium traces depict mean relative $[Ca^{2+}]_{CYT}$ fluorescence. Area under the curve for the (B) 3 µM and (D) 10 µM ionomycin Ca^{2+} signals. Bar graphs are mean ± S.D, *$P < 0.05$, unpaired two-tailed student's *t* test. All data were pooled from three independent experiments ($n = 3$), performed in triplicate wells.

Fig. 3. Effect of PMCA2 silencing on various agents that increase global cytoplasmic free calcium. siRNA-transfected MDA-MB-231 breast cancer cells were treated with the calcium mobilizing agents (A) CPA (10 µM), (D) ATP (100 µM), and (G) trypsin (0.1 µM) in the presence of extracellular BAPTA (100 µM) in nominal free calcium. Ca^{2+} traces depict mean relative $[Ca^{2+}]_{CYT}$. Half peak decay time calculated for the (B) CPA, (E) ATP and (H) trypsin Ca^{2+} responses. Area under the curve calculated for the (C) CPA, (F) ATP and (I) trypsin responses. Bar graphs quantitate calcium transient parameters as mean ± S.D, *$P < 0.05$, unpaired two-tailed student's *t* test. All data were pooled from three independent experiments ($n = 3$), performed in triplicate wells.

Fig. 4. Effects of PMCA2 silencing of MDA-MB-231 breast cancer cell death initiated by the Bcl-2 inhibitor ABT-263. (A) Representative dot plots show cell viability detected in cells transfected with siNT or siPMCA2 and treated with 1 μM ABT-263. Dot plots are Hoechst 33342 and propidium iodide fluorescence measured in an equal cell number (10,000 cells/dot plot), randomly selected from three independent experiments (*n* = 3). (B) Effect of siPMCA2 and siNT on cell death at 1 μM ABT-263. Bar graph shows mean ± S.D, **P* < 0.05, paired two-tailed student's *t* test (C) Concentration-response curves show effects of siNT and siPMCA2 on cell death initiated at various ABT-263 concentrations (0.01 – 10 μM). Values plotted are mean ± S.D, **P* < 0.05, siNT compared to siPMCA2 at the indicated ABT-263 concentration, repeated measures two-way ANOVA, Bonferroni post hoc test. All data were obtained from three independent experiments (*n* = 3), performed in triplicate wells.

A

siNT + 1 µM ABT-263   siPMCA2 + 1 µM ABT-263

Dead 37%   Dead 68%

Propidium iodide intensity

10^3

10^2

Viable 63%   Viable 32%

10^4 10^5 10^6 10^4 10^5 10^6

Hoechst 33342 integrated intensity

B

% Cell death

80

60

40

20

0

*

siNT   siPMCA2

C

% viable cells

100

80

60

40

20

0

-8   -7   -6   -5

ABT-263 [Log M]

● siNT
■ siPMCA2

19