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Resveratrol inhibits plasma membrane Ca^{2+}-ATPase inducing an increase in cytoplasmic calcium

Joshua Allen Peterson, Richard Vernon Oblad, Jeffrey Chad Mecham, Jason Donald Kenealey *

[a] Department of Nutrition, Dietetics and Food Science, Brigham Young University, ESC S-127, Provo, UT, United States

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ABSTRACT

Plasma membrane Ca^{2+}-ATPase (PMCA) plays a vital role in maintaining cytosolic calcium concentration ($[Ca^{2+}]_i$). Given that many diseases have modified PMCA expression and activity, PMCA is an important potential target for therapeutic treatment. This study demonstrates that the non-toxic, naturally-occurring polyphenol resveratrol (RES) induces increases in $[Ca^{2+}]_i$ via PMCA inhibition in primary dermal fibroblasts and MDA-MB-231 breast cancer cells. Our results also illustrate that RES and the fluorescent intracellular calcium indicator Fura-2, are compatible for simultaneous use, in contrast to previous studies, which indicated that RES modulates the Fura-2 fluorescence independent of calcium concentration. Because RES has been identified as a PMCA inhibitor, further studies may be conducted to develop more specific PMCA inhibitors from RES derivatives for potential therapeutic use.

1. Introduction

Plasma membrane Ca^{2+}-ATPase (PMCA) plays a critical role in maintaining the cytosolic calcium concentration ($[Ca^{2+}]_i$) in cells by extruding calcium from the cytosol into the extracellular space utilizing energy from ATP hydrolysis. PMCA consists of 10 transmembrane α-helices and 2 large cytosolic loops [1]. PMCA is part of a series of pumps and channels that maintain a calcium concentration gradient with a homeostatic $[Ca^{2+}]_i$ of 100 nM. In contrast to the cytosol, the endoplasmic reticulum (ER) and extracellular milieu have calcium concentrations of approximately 1 mM. The elevated ER calcium concentration is sustained by the sarcoendoplasmic reticular Ca^{2+}-ATPase (SERCA), which hydrolyzes ATP to transfer calcium from the cytosol to the ER. Channels on the ER membrane (inositol 1,4,5-trisphosphate (IP_3) and ryanodine receptors) and plasma membrane (voltage-gated calcium channels, store-operated channels, and receptor-operated channels), when open, enable calcium to move down the concentration gradient from the ER or extracellular space into the cytosol. When $[Ca^{2+}]_i$ rises above the resting concentration, PMCA expels calcium from the cytosol into the extracellular space. Together, these pumps and channels facilitate the use of Ca^{2+} as an important signaling mechanism inside the cell [2].

Changes in $[Ca^{2+}]_i$ regulate critical cellular functions such as apoptosis, muscle contraction, neuronal synapse firing, and cellular motility [2]. The cellular response to $[Ca^{2+}]_i$ modulation is dictated by the duration, magnitude, subcellular location, and the Ca^{2+}-binding protein expression profile of each cell [3,4]. PMCA activation controls the location, duration and magnitude of the changes in $[Ca^{2+}]_i$, and thereby helps determine the cellular response to the calcium flux.

Because PMCA is a critical component in defining $[Ca^{2+}]_i$, genetic mutations, overexpression, down-regulation, dysregulation, and inhibition of PMCA have an array of biological effects in mammalian cells. Alzheimer's disease, hypertension, male infertility, cardiovascular disease, deafness, diabetes, and cancer have all been correlated with altered PMCA activity [5–9]. More specifically, some of these diseases are characterized by modulated expression or activity of the four separate PMCA isoforms. For example, upregulation of PMCA is found in several gastric (KATO-III) and colon (HT-29, Caco-2, DLD-1, LS-174 T) cancer cell types during cell differentiation as well as in several breast cancer cell types (MDA-MB-231, T47D) [6,10,11]. Therapeutic options aiming to inhibit PMCA could offer new treatment options for cancers and other diseases with upregulated PMCA profiles as PMCA inhibition

Abbreviations: RES, resveratrol; $[Ca^{2+}]_i$, cytosolic calcium concentration; PMCA, plasma membrane Ca^{2+}-ATPase; EGCG, epigallocatechin gallate; SERCA, sarcoendoplasmic reticular Ca^{2+}-ATPase; Fura-2, Fura-2-Acetoxymethyl ester; TG, thapsigargin; ROI, region of interest; HBSS, Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BAPTA, BAPTA-Acetoxymethyl ester; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle medium; ER, endoplasmic reticulum

* Corresponding author.
E-mail address: jason_kenealey@byu.edu (J.D. Kenealey).

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could generate the high $[Ca^{2+}]_i$ typically associated with apoptosis [2]. Current inhibitors of PMCA have significant off-target effects due in part to a lack of isoform-specific inhibition [7]. The discovery of an additional PMCA inhibitor is an important step in the development of a more isoform-specific treatment for diseases exhibiting modulated PMCA profiles.

Resveratrol (trans-3, 4′, 5-trihydroxystilbene) (RES), a polyphenolic, cell-permeable phytoalexin found in grapes, peanuts and berries, exhibits chemotherapeutic, anti-aging, and cardioprotective properties [12–14], while exhibiting minimal toxicity in humans and animal models [15,16]. One possible explanation for the pleiotropic biologic effects of RES is that RES modulates calcium signaling. RES has been shown to increase $[Ca^{2+}]_i$ in breast cancer cells [17] and modulate the magnitude and duration of the calcium signal in excitable cells, such as neurons and muscle [18]. However, the mechanism by which the increase in $[Ca^{2+}]_i$ is induced is unclear. In this study, we use a PMCA activity assay [19] to assess if RES directly inhibits PMCA and thereby induces increases in $[Ca^{2+}]_i$ in both normal and cancerous cell lines. This study demonstrates that RES inhibits PMCA activity in both normal primary dermal fibroblasts (PDF) and MDA-MB-231 (MDA) breast cancer cells.

2. Materials and methods

The MDA-MB-231 human breast cancer (HTB-26) (MDA) and human primary dermal fibroblast (PCS-201–012) (PDF) cell lines were purchased from ATCC (Manassas, VA). The passage numbers of MDA cells used in experiments ranged from 10 to 35. Fura-2-Acetoxymethyl ester (14591) (Fura-2), thapsigargin (10522) (TG), (-)-epigallocatechin gallate (70935) (EGCG), quercetin (10005169), trans-resveratrol (70675) (RES), and BAPTA-Acetoxymethyl ester (15551) (BAPTA) were purchased from Cayman Chemical (Ann Arbor, MI). Lanthanum (III) chloride heptahydrate ($LaCl_3$) (262072) was purchased from Sigma-Aldrich (St. Louis, MO). Fura-2, pentasodium salt (50032) was purchased from Biotium (Hayward, CA).

2.1. Cell culture

Both MDA and PDF cells were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic-antimycotic. Cells were cultured at 37° C in 5% CO_2.

2.2. Intracellular calcium imaging

Cells were prepared for imaging by seeding at a density of 20,000 (PDF) or 30,000–40,000 (MDA) cells per well in an 8-well chamber Lab-Tek #1.0 Borosilicate Coverglass (Thermo Scientific, Rochester, NY). Cells were grown for 48–72 h after initial seeding. No significant changes were recorded as a result of variation in initial cell density or time allowed for cells to grow. Prior to imaging, cells were washed twice with 1:1 FBS:PBS solution. Cells were then incubated in Ringer solution (NaCl 150 mM, glucose 10 mM, HEPES 5 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, pH 7.4) with 8 μM Fura-2 for 15 min at 37° C in 5% CO_2. Where indicated, 20 μM BAPTA was co-loaded with Fura-2. The Fura-2/Ringer solution was then removed, and cells were incubated in fresh Ringer solution for 30 min at 37° C in 5% CO_2. Ringer solution was then removed and replaced with a Ca^{2+}-free buffer which was also used as vehicle for treatments. The Ca^{2+}-free buffer used was either Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) or, if treating with $LaCl_3$, a Ca^{2+}, Mg^{2+}, and phosphate-free HEPES buffer (NaCl 150 mM, glucose 10 mM, KCl 5 mM, HEPES 5 mM, 10 mM EGTA, pH 7.4) to prevent La^{3+} ions from complexing with phosphate ions in HBSS. Cells were imaged following Fura-2 loading with an Olympus IX51 inverted microscope. All treatments were manually pipetted into the wells in 100 μL increments at the indicated time points. In all trials, cells were imaged for 1 min before the first treatment was added in order to establish a baseline. Subsequently, the cells were treated with 5 μM TG. In all trials, RES, $LaCl_3$ and EGCG were dissolved in 1% DMSO in vehicle. Quercetin was dissolved in 2% DMSO in vehicle. Other details concerning specific treatments are included in the Results section. Data acquired from intracellular calcium imaging was analyzed with CellSens software by Olympus. Regions of interest (ROI) representing 1 cell each were selected from each individual experiment and analyzed for changes in $[Ca^{2+}]_i$. The resulting data from at least 10 ROIs per trial were then normalized ($n \geq 3$).

2.3. Emission spectra

Spectra measurements were taken by exciting at 340 nm and 380 nm while collecting the emission spectrum from 400 to 650 nm in Ca^{2+}-free and 1 mM Ca^{2+} PBS buffers. RES and Fura-2 fluorescence were measured on the BMG LABTECH FLUOstar OPTIMA plate reader (BMG LABTECH Inc., Cary, NC). Triplicate 100 μL solutions were made and placed on a Greneir Bio-One Cellstar 96-well plate. Measurements of the Ca^{2+}-containing solution (1 mM $CaCl_2$ in PBS), and Ca^{2+}-free solution (10 mM EGTA in PBS) with DMSO concentrations of 0.001%, 0.01%, 0.025%, 0.05%, 0.075%, or 0.1% were used as blanks. The Ca^{2+}-containing solution and Ca^{2+}-free solution were then treated to make up a 5 μM Fura-2-pentasodium salt solution with either 0, 1, 10, 25, 50, 75 or 100 μM RES. Spectra measurements were taken by exciting at 340 nm and 380 nm and collecting emissions at 510 nm.

2.4. Statistical analysis

All P values were calculated using a two-tailed *t*-test. P values from intracellular calcium imaging experiments were calculated by comparing the peak magnitude of $[Ca^{2+}]_i$ of each treatment to the vehicle control. Statistical significance was determined by $p < 0.01$.

3. Results

3.1. RES inhibits PMCA leading to an increased $[Ca^{2+}]_i$

We tested RES-induced PMCA inhibition because of the potential RES has as a therapeutic for PMCA-modulated diseases. Our interest in MDA-MB-231 cells stems from previous research that indicated PMCA upregulation in this cell type [6]. Previously, it was demonstrated that in MDA cells, RES had an indirect effect on PMCA by activating its degradation [17]. Additionally, RES has been shown to increase $[Ca^{2+}]_i$ in many cell types, including MDA cells [17,20–22]. Because of evidence supporting interactions between RES and PMCA, in this study, we use a PMCA activity assay to explore the RES-induced calcium signal [19]. We studied MDA cells while using PDF cells as a control to determine the direct effect of RES on PMCA inhibition in cells with and without a mutated PMCA profile.

As described in a previous study, we isolated endogenous PMCA activity [19]. We used Ca^{2+}-free media to eliminate the possibility that changes in $[Ca^{2+}]_i$ were due to an influx of Ca^{2+} from the extracellular space. The SERCA pump inhibitor thapsigargin (TG) was used to induce a rise in $[Ca^{2+}]_i$ (Fig. 1). The TG-induced increase in $[Ca^{2+}]_i$ is due to Ca^{2+} leaking from the ER, paired with the inhibited reuptake of Ca^{2+} by SERCA pumps. The