Biochemical and Biophysical Research Communications 425 (2012) 431–435

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of ryanodine receptor isoforms in prostate DU-145, LNCaP, and PWR-1E cells

Sarah E. Kobylewski, Kimberly A. Henderson, Curtis D. Eckhert *

University of California, Los Angeles, 650 Charles E. Young Drive South, Los Angeles, CA 90095-1772, USA

ARTICLE INFO

Article history:
Received 19 July 2012
Available online 27 July 2012

Keywords:
Ryanodine receptor
DU-145
LNCaP
PWR-1E
Prostate cancer
Isoform
Cancer models
Boric acid

ABSTRACT

The ryanodine receptor (RyR) is a large, intracellular calcium (Ca^{2+}) channel that is associated with several accessory proteins and is an important component of a cell's ability to respond to changes in the environment. Three isoforms of the RyR exist and are well documented for skeletal and cardiac muscle and the brain, but the isoforms in non-excitable cells are poorly understood. The aggressiveness of breast cancers in women has been positively correlated with the expression of the RyR in breast tumor tissue, but it is unknown if this is limited to specific isoforms. Identification and characterization of RyRs in cancer models is important in understanding the role of the RyR channel complex in cancer and as a potential therapeutic target. The objective of this report was to identify the RyR isoforms expressed in widely used prostate cancer cell lines, DU-145 and LNCaP, and the non-tumorigenic prostate cell line, PWR-1E. Oligonucleotide primers specific for each isoform were used in semi-quantitative and real-time PCR to determine the identification and expression levels of the RyR isoforms. RyR1 was expressed in the highest amount in DU-145 tumor cells, expression was 0.48-fold in the non-tumor cell line PWR-1E compared to DU-145 cells, and no expression was observed in LNCaP tumor cells. DU-145 cells had the lowest expression of RyR2. The expression was 26- and 15-fold higher in LNCaP and PWR-1E cells, respectively. RyR3 expression was not observed in any of the cell lines. All cell types released Ca^{2+} in response to caffeine showing they had functional RyRs. Total cellular RyR-associated Ca^{2+} release is determined by both the number of activated RyRs and its accessory proteins which modulate the receptor. Our results suggest that the correlation between the expression of the RyR and tumor aggression is not related to specific RyR isoforms, but may be related to the activity and number of receptors.

1. Introduction

It has been nearly 40 years since Balk and his colleagues reported a difference in calcium ion (Ca^{2+}) stimulated proliferation between transformed and non-transformed fibroblasts [1]. Since this discovery, major advances have been made in elucidating Ca^{2+}'s role in the cell cycle and proliferation [2,3]. Recently, an evaluation of breast cancer tissue arrays from the National Cancer Institute found a positive correlation between ryanodine receptor (RyR) immunostaining and tumor aggressiveness [4]. Three isoforms of the RyR are known to exist (RyR1, RyR2, and RyR3) in skeletal muscle, cardiac muscle and the brain [5], but the identification of the isoforms in ryanodine-sensitive non-excitable epithelial and exocrine cells is limited to only a few cell types [6].

The RyR is a large, transmembrane, homotetrameric Ca^{2+} channel consisting of $4 \sim 550$ kDa subunits, totaling approximately 2.3 MDa, making it the largest cellular ion channel [7,8]. It is the major Ca^{2+} channel linking the rich calcium stores of the endoplasmic reticulum (ER) and cytoplasm of non-excitable cells. A high conductance ion channel, the RyR allows for rapid and precise release of Ca^{2+} across a 20,000-fold gradient between the ER ($\sim$0.2–20 mM [Ca^{2+}]) and cytoplasm ($\sim$100 nM [Ca^{2+}]) enabling cells to activate Ca^{2+}-dependent cellular processes in response to changes in the cellular environment [9]. RyR-associated Ca^{2+} release occurs when the receptors are stimulated, either through an agonist or calcium induced calcium release (CICR) [5]. RyRs serve a major role in regulating several cellular functions including differentiation, apoptosis, secretion, muscle contraction, and neurotransmitter release [8,10].

Mammalian RyRs exist in three distinct isoforms, approximately 70% homologous in sequence, and residing on three different chromosomes [8,10]. Isoforms 1 and 2 function in excitation–contraction coupling to stimulate contraction of striated muscle [11]. RyR1 is the primary isoform in skeletal muscle and RyR2 in cardiac muscle [8,10]. RyR3 is preferentially expressed in the brain, especially in the hippocampus and striatum [12].

RyRs are associated with a variety of diseases. Defects in RyR1 cause malignant hyperthermia and a spectrum of myopathies in skeletal muscle [13]. RyR2 dysregulation can result in fatal cardiac

* Corresponding author. Address: 650 Charles E. Young Drive South, Room 76-080, Los Angeles, CA 90095-1772, USA. Fax: +1 310 476 6545.
E-mail addresses: sarahkoby@gmail.com (S.E. Kobylewski), hndkim@aol.com (K.A. Henderson), ceckhert@ucla.edu (C.D. Eckhert).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.07.119

arrhythmias and heart failure [13]. RyR3 knockout mice are hyperactive and exhibit decreased social contact with other mice [14]. Altered RyR gating (opening and closing of the channel's pore) has been implicated in a range of other diseases, including epilepsy, neurodegeneration, pain, and cancer [13]. Consequently, these channel complexes represent potential therapeutic targets for treatment of numerous diseases, including cancer.

RyRs interact with a range of substances of public health concern and are more susceptible to modulation in tumor cells than normal cells [15]. In differentiated normal cells, Ca^{2+} is highly regulated both spatially and temporally, but in tumor cells there is a sustained elevation in whole cytoplasmic Ca^{2+} (global Ca^{2+} signaling) [16,17]. RyRs, unlike the inositol triphosphate receptor (IP3R), are modulators of global Ca^{2+} [18]. Normal and tumor models of prostate cancer have been reported to respond differently to RyR agonists and inhibitors [19]. This may indicate a potential mechanism of tumorigenicity.

Several cell models are used to study prostate cancer. Among the most common are DU-145 and LNCaP tumors cells and PWR-1E non-tumor cells. DU-145 is an epithelial, androgen-independent line originally derived from the brain tumor of a man with prostate cancer. It was the first cell line used to study prostate cancer *in vitro* and is still widely used today [20]. LNCaP cells are an androgen-dependent prostate cancer epithelial cell line. It was originally derived from the lymph nodes of a prostate cancer patient [20]. PWR-1E cells are an immortalized, non-tumorigenic, epithelial prostate cell line that is used to provide a normal prostate cell control [21]. The objective of this study was to identify RyR isoforms in DU-145, LNCaP, and PWR-1E cell lines in order to determine if RyR isoform expression differs between these cell models.

2. Materials and methods

2.1. Cell culture

DU-145 prostate cancer cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA), were maintained in RPMI Media 1640 (Gibco-Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (200 mM) (Gemini Bio-Products, Sacramento, CA). LNCaP cells were obtained from ATCC and cultured without androgen supplementation in the same media described for DU-145. When LNCaP cells are grown in androgen-depleted media they are referred to as subline LNCaP CL1 [20]. PWR-1E cells were obtained from ATCC, maintained in KSFM media supplemented with bovine pituitary extract (0.05 mg/ml), human recombinant epidermal growth factor (5 ng/ml) (Gibco-Life Technologies), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were plated on 10 cm plates (Corning Life Sciences, Corning, NY) incubated at 37 °C in a humidified chamber containing 5% CO_2 and 95% air and grown to 80% confluency.

2.2. Semi-quantitative PCR

Semi-quantitative PCR used primers designed by the authors using Primer Express 3.0 (Applied Biosystems (ABI), Foster City, CA) and specific for each isoform (Table 1). RNA was isolated from cells using an RNeasy mini kit (Qiagen, Valencia, CA). Total RNA (2 μg) was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) with random hexamer primers (Invitrogen) at a final volume of 20 μl at 25 °C, 10 min (10:00); 50 °C, 45:00; and 70 °C, 15:00. PCR reactions for RyR isoform identification were 50 μl total: 5 μl cDNA, 10 μl 5× green GoTaq® reaction buffer, 5 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTPs, 0.25 μl 100 μM forward primers, 0.25 μl 100 μM reverse primers, 0.25 GoTaq® Flexi DNA polymerase (Invitrogen). Reactions were run on a PTC-200 (MJ Research, Ramsey, MN) using the following protocol: 95 °C, 2:00; 95 °C, 0:30–1:00; 55–60 °C, 0:30–1:00; 72 °C, 1:00; repeat steps 2–4 35 times; 72 °C, 5:00; 4 °C, forever.

2.3. Enzyme digestion

PCR products were digested with a restriction enzyme chosen for the gene of interest (Table 1). Digestion reaction consisted of 20 μl PCR product, 2 μl enzyme, 2 μl corresponding reaction buffer, and 6 μl autoclaved deionized water. Two microliters of enzyme were replaced by autoclaved deionized water as a negative control. Digestion products were run on 2% agarose gels pre-stained with ethidium bromide. Products were run adjacent to a 1 Kb Plus DNA ladder (Invitrogen). The gel was run at 100 V for 1 h using an EC4000P power source (Thermo Electron Corporation, Waltham, MA). Gels were viewed on a Typhoon 9410 Variable Mode Imager (Amersham Biosciences, Piscataway, NJ).

2.4. Real-time PCR (RT-PCR)

cDNA was created from total cellular RNA according to the above protocol. Primers specific to RyR isoforms were designed using Primer Express 3.0 (ABI) and are listed in Table 2. Ten microliters of reactions included 5 μl RT^2 Real Time™ SYBR Green/ROX PCR master mix (SA Biosciences, Frederick, MD), 3 μl cDNA, 0.9 μl forward primer, 0.9 μl reverse primer (Invitrogen), and 2 μl autoclaved ddI water. Reactions were added to MicroAmp™ fast optical 96-well reaction plates (ABI). Each plate contained reactions for the gene of interest as well as the internal housekeeping gene, GAPDH. Plates were covered with MicroAmp™ optical adhesive film (ABI) and read by a 7500 Fast Real Time PCR System using the 7500 Fast System Software v1.4.0 (ABI). The delta delta ct method was used to analyze the data.

2.5. Measuring and analysis of Ca^{2+} release

Ca^{2+} release was measured as previously described by Henderson et al. [22]. Ca^{2+} was monitored using the Ca^{2+}-sensitive dye, Rhod-2, AM ester (Biotium, Hayward, CA). This dye was chosen because it compartmentalizes well into cellular organelles. ER tracker green (Molecular Probes, Carlsbad, CA) was used to stain the ER and identify areas of ER Ca^{2+}. Ca^{2+} changes were measured by selecting regions of interest in cells where the dyes overlapped. Rhod-2, AM ester was prepared as a 1 mM stock solution in DMSO and diluted in cellular media. Cells were incubated with 5 μM Rhod-2, AM ester and 0.5 μM ER Tracker for 30 min at 37 °C. Caffeine (Sigma, St. Louis, MO) was diluted in Ringers solution at a final concentration of 20 mM. Images were collected with a Zeiss 510 LSM 5 Pascal mounted to an upright microscope (Zeiss Axioplan 2) equipped with an Axoplan X63 (NA 0.95) water immersion objective. A HeNe laser was used to excite Rhod-2, AM ester at 543 nm. ER Tracker was excited at 488 nm from a laser diode. The emission was collected on a photomultiplier tube through a 560 nm LP filter (Rhod-2) and a 505 LP filter (ER Tracker). Additional magnification, time series, and background subtraction were controlled using Zeiss LSM acquisition software. All images were acquired as 12 bit.

2.6. Statistical analysis of the data

Images of semi-quantitative PCR products of all three isoforms are representative of three biological replicates. All RT-PCR data was analyzed using six biological replicates of each cell line. The

Table 1
Primers and enzymes used to identify RyR isoforms.

	Forward primer	Reverse primer	Restriction enzyme
RyR1	TGTCAAGCGCAAGGTCCTGG	TGTCCAGGAGATGGGCAGCAA	BglII (Invitrogen)
RyR2	AAGGAGCTCCCCACGAGAAGT	CAGATGAAGCATTTGGTCTCCAT	BsmI (New England Biolabs, Ipswich, MA)
RyR3	AAGAGGAAGAAGCGATGGT	CTCCAAGCTTCCAGATATGG	BglII

Table 2
RT-PCR primers.

Gene	Forward primer	Reverse primer
RyR1	GTCATCCTGTTGGCCATCATC	GGTCTCGGAGCTCACCAAAAG
RyR2	TTTTTTTGCCGCTCACCTTCT	CTGAGGACAAGATGGTTCTTAATGTC
GAPDH	CCTGTTCGACAGTCAGCCG	CGACCAAATCCGTTGACTCC

paired Student's *t*-test was used to measure significance between DU-145 and the other cell lines. Ca^{2+} release data is representative of three biological replicates and analyzed using the unpaired Student's *t*-test. Ca^{2+} levels were analyzed as a measure of fluorescence intensity (F). The formula $((F_0 - F)/F_0)$ was used to calculate Ca^{2+} level. Time points pre-treatment were chosen randomly while the treatment time was chosen using the peak value. The unpaired Student's *t*-test was used to calculate significance.

3. Results

3.1. RyR isoform identification

The expression of RyR isoforms in DU-145, LNCaP, and PWR-1E cells was determined by semi-quantitative PCR using primers specific for each isoform (Table 1). Restriction enzyme digestion was performed on PCR products in order to confirm that the band was the expected sequence. A172 human glioblastoma cells (ATCC) were used as a positive control because, like many brain cells, it expresses all three RyR isoforms [11,23]. RyR1 is only expressed in DU-145 and PWR-1E cells but not at all in LNCaP cells (Fig. 1). RyR 2 is expressed in all three cell lines (Fig. 1). RyR3 is not expressed in any of the cell lines (Fig. 1).

3.2. Relative RyR expression

We performed RT-PCR in order to determine the relative expression levels of each RyR isoform. We used SybrGreen because predesigned assays gave inconsistent results and the SybrGreen method allowed us to design our own primers. DU-145 cells were chosen arbitrarily as the "control" cell line to compare the other lines to. RT-PCR results confirmed that only DU-145 and PWR-1E cells express RyR1 (Fig. 2A) and all three cell lines express RyR2 (Fig. 2B). RT-PCR was not performed on RyR3 since semi-quantitative-PCR showed that none of the cell lines express this isoform (Fig. 1). PWR-1E cells expressed approximately 0.48 the level as DU-145 cells (Fig. 2A). LNCaP and PWR-1E cells expressed approximately 26 and 15 times the amount of RyR2 than DU-145 cells, respectively (Fig. 2B). It is clear from these results, as well as the isoform identification, that tumorigenicity was not correlated with RyR isoform in our prostate cells.

3.3. Ca^{2+} release from RyRs

In order to determine if DU-145, LNCaP, and PWR-1E cells translate RyRs into functional proteins, we used caffeine to stimulate Ca^{2+} release from the RyR-sensitive ER Ca^{2+} stores ($[Ca^{2+}]_{ER}$). Caffeine stimulated significant $[Ca^{2+}]_{ER}$ reduction in DU-145 (Fig. 3A), LNCaP (Fig. 3B), and PWR-1E (Fig. 3C) cells, showing that RyR expression resulted in functional protein in all three cell lines.

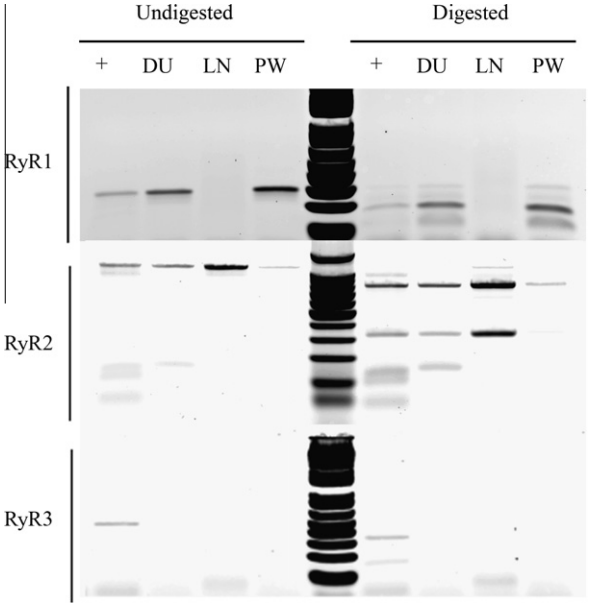

Fig. 1. Ryanodine receptor isoform identification in DU-145, LNCaP, and PWR-1E cells. The positive control (+) cell line is A172, a glioblastoma cell line. RyR1 (290 base pairs (bp) undigested; 171 and 119 bp digested) is expressed in DU-145 (DU) and PWR-1E (PW) cells (top). RyR2 (1082 bp undigested; 763 and 319 bp digested) is expressed in DU-145, LNCaP (LN), and PWR-1E cells (middle). RyR3 (477 bp undigested; 323 and 154 bp digested) is not expressed in any of the examined cell lines (bottom). Images are representative of three individual experiments.

4. Discussion

The differences in the expression of RyR isoforms was most pronounced between tumor cell lines, DU-145 and LNCaP (Figs. 1 and 2). The dominant isoform in DU-145 was RyR1 whereas RyR2 was most highly expressed in LNCaP. PWR-1E expressed both RyR1 and RyR2. RyR3 was not expressed in any of the cell lines. RyR1 has been identified in MCF-7 breast cancer carcinoma cells [24] and prostate cancer LNCaP cells [25], but this is the first time it has been shown in prostate DU-145 cancer cells and normal PWR-1E cells. RyR2 has previously been reported in HeLa [11,23] and melanoma cells [26]. Our results in LNCaP cells differ from Mariot and colleagues who reported finding RyR1 and RyR2 in LNCaP cells [25]. The difference between our results may be explained by the use of different oligoprimers. We used different forward and reverse primers to the transmembrane region of the receptor that were specific to the individual isoforms whereas they used the same reverse primer for all three isoforms. Their set of primers for RyR2 was also specific for RyR3. In addition, we used a positive control which was not mentioned in the Mariot paper. Differences in RyR isoform expression have also been reported in HeLa cells due to the use of different nucleotide sequences [11,23]. It is also possible that our laboratories used a different LNCaP cell line, as LNCaP has many sublines [20].

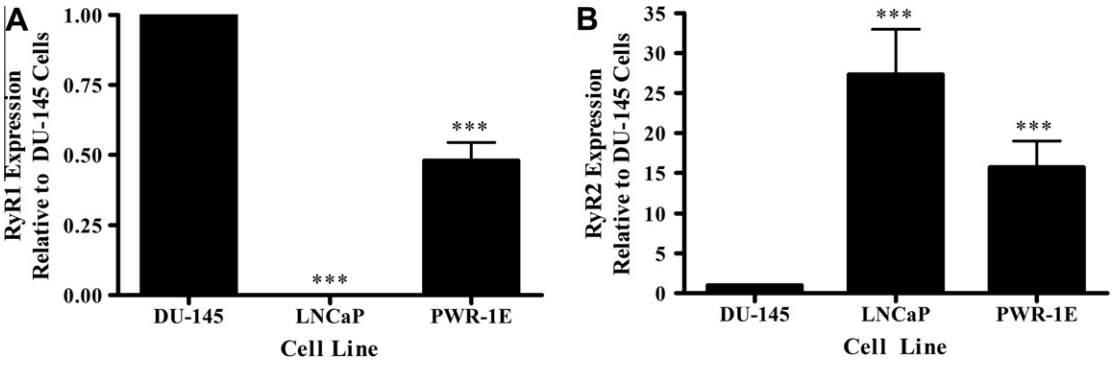

Fig. 2. Relative expression of RyR isoforms in DU-145, LNCaP, and PWR-1E cells. RT-PCR was performed on cDNA created from all three cell lines. LNCaP and PWR-1E cells were compared to DU-145. DU-145 was arbitrarily chosen as the control line. (A) RT-PCR confirmed the absence of RyR1 in LNCaP cells. PWR-1E expression was approximately 0.48 that of DU-145. (B) Expression of RyR2 in LNCaP and PWR-1E cells was approximately 26 and 15 times that of DU-145 cells, respectively. Analysis was performed on six biological replicates per cell line. RT-PCR was not performed on RyR3 as semi-quantitative PCR revealed the absence of expression in all three cell lines.

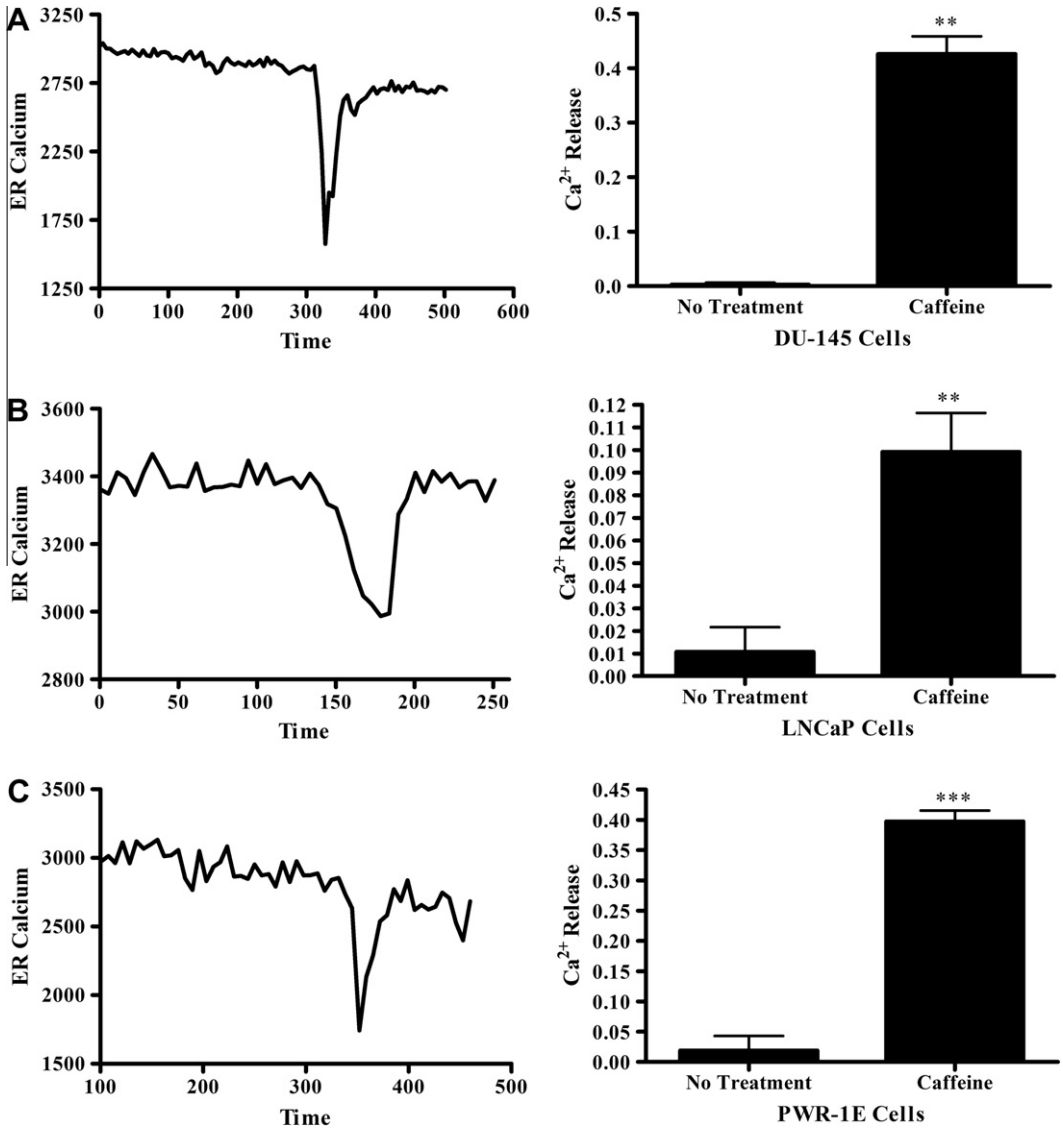

Fig. 3. Calcium release from functional RyRs in DU-145, LNCaP, and PWR-1E cells. Caffeine stimulated ER calcium release from RyRs in (A) DU-145, (B) LNCaP, and (C) PWR-1E cells. Graphs are representative of three experiments.

In tumor cells there is a shift from low to high and sustained global cellular Ca^{2+} concentrations compared to normal cells [17]. Both the IP3R and RyR control spatially localized Ca^{2+} but Westcott has reported that only the RyRs control global Ca^{2+} concentrations [18]. Inhibition of DU-145 and LNCaP cell proliferation has been reported to be associated with an inhibition of RyR Ca^{2+} release

[22,27,28]. The chemopreventative agent boric acid inhibits RyR Ca^{2+} release in DU-145 and LNCaP cells and slows proliferation within a concentration range that can be achieved by a boron rich diet. However, it requires a 4-fold higher concentration to inhibit the proliferation and 15-fold higher concentration to inhibit RyR Ca^{2+} release in non-tumorigenic PWR-1E cells [19,22]. We hypothesized that this difference in boric acid sensitivity between the tumor cell lines and a normal prostate cell line was due to the expression of different RyR isoforms or to differences in the response of one of the accessory proteins that modulate the activity of the receptor. The results of the present study rule out the first possibility since the major difference in RyR1 and RyR2 expression occurred between tumor cell lines, whereas PWR-1E cells, the non-tumorigenic and least sensitive to boric acid, expressed both RyR 1 and RyR2. The second hypothesis now seems the most likely to explain the differences in boric acid response. The RyR is a macromolecular complex that serves as a scaffold for proteins that modulate Ca^{2+} channel function [13,29] and offer attractive targets that may explain boric acid's unique chemopreventive effect.

Acknowledgments

The work for this Grant was funded by personal funds (CD) and in part by the University of California Toxic Substances Research and Training Program (TSR&TP) for partial support of KH and SK. SK was partially funded by the UCLA Dissertation Fellowship. CE is the doctor mentor for KH and SK. TSR&TP and Dissertation Fellowship funding was for student support and had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors would also like to thank Kristin Yamada for her help with editing this manuscript.

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