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ORIGINAL PAPER

Ryanodine Receptor Expression Correlates with Tumor Grade in Breast Cancer

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Abstract Ryanodine receptors (RyRs) have been previously implicated in the proliferation of human T-lymphocytes and melanocytes as well as in the motility of astrocytes. We have examined RyR expression in 57 ductal, human breast cancer specimens, by immunohistochemistry. Moderate to high RyR immunostaining was found in 47 (82%) of the specimens. There was a direct correlation between RyR levels and tumor grade (r=0.48, p=0.0002). We have also examined the effect of the RyR agonist 4-chloro-*m*-cresol on the in-vitro growth of two human breast cancer cell lines, MCF-7 and MDA-MB-231. Treatment with 4-chloro-*m*-cresol inhibited the growth of both breast cancer cell lines, in a dose-dependent manner, with half-maximal inhibition observed at 30 to 50 μg/mL (210–351 μM). Our data suggest that RyR could serve as prognostic indicator and/or as a target for breast cancer treatment.

Keywords Ryanodine receptor · Breast cancer · Tumor grade · 4-chloro-*m*-cresol · MCF-7 · MDA-MB-231

Abbreviations

Ca2+	calcium ion
RyR	ryanodine receptor
Bca	breast cancer
PBS	phosphate buffered saline
RT	room temperature
HRP	horseradish peroxidase
DAB	3,3′-diaminobenzidine
L	Leibovitz's
ER+ve	estrogen receptor alpha positive
ER−ve	estrogen receptor alpha negative

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Introduction

Intracellular calcium ion (Ca2+) signaling regulates many cellular processes, including proliferation, motility, gene expression, membrane potential, secretion, necrosis and apoptosis [1]. The primary Ca2+ storage/release organelle in most cell types is the endoplasmic reticulum which contains specialized Ca2+ release channels. There are two families of Ca2+ release channels: the ryanodine receptor (RyR) and the inositol-triphosphate receptor [2]. RyR Ca2+ release-channels, of the sarcoplasmic reticulum, play a key role in muscle contraction. In addition to muscle cells, other cell types such as neuronal cells utilize RyRs in mobilizing intracellular Ca2+ [3]. The three RyR isoforms in mammals, RyR1 (skeletal muscle), RyR2 (cardiac muscle) and RyR3 (brain), are encoded by distinct genes [3]. Binding of the alkaloid ryanodine, induces complex changes in all three subtypes of RyR channels [3].

RyRs have been previously implicated in the proliferation of human T-lymphocytes and melanocytes as well as in the motility of astrocytes [4–6]. RyR3 was found in human Jurkat T-lymphocytes and ryanodine stimulated the proliferation as well as altered the growth pattern of cultured human T-cells [4]. RyR1 is expressed in cultured human melanocytes and ryanodine inhibited the proliferation as well as stimulated the pigmentation of human melanocytes [5]. Mouse astrocytes express RyR3 (but not RyR1 or RyR2) and an antagonizing dose of ryanodine (200 μM) strongly inhibited astrocyte motility, in vitro [6]. RyR1 and

RyR2 mRNAs have been detected in the LNCaP human prostate cancer cell line [7]. To our knowledge, RyR expression in breast cancer (Bca) has not been previously reported. We report here that RyR expression occurs frequently in Bca and correlates with tumor grade.

Materials and Methods

Immunohistochemistry Breast cancer (Bca) specimens on a tissue microarray slide were obtained from the Cooperative Human Tissue Network under the tissue array program of the National Cancer Institute, The National Institutes of Health (Bethesda, MD, USA). Sections were deparaffinized in xylene and hydrated through graded alcohol series. After quenching endogenous peroxidase with 0.3% hydrogen peroxide in methanol, rinsing with phosphate buffered saline (PBS), permeabilization with 0.1% Triton X-100 for 2 min and blocking with 3% bovine serum albumin in PBS, tissue sections were incubated in primary antibody, 6 μg in 300 μL PBS (anti-RyR goat polyclonal which recognizes all three isoforms RyR1, RyR2 and RyR3, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at room temperature (RT) in a humidified chamber. Sections were then rinsed with PBS and incubated with horseradish peroxidase (HRP)-conjugated, anti-goat antibody (Santa Cruz Biotechnology) at a 1:300 dilution for 30 min at RT. Freshly prepared 3,3′-diaminobenzidine (DAB, Sigma Fast Tablets, St. Louis, MO) was used as the substrate for HRP. Incubation of tissue specimens with secondary antibody alone gave no staining. Immunostaining levels were determined by visual scoring of the brown product of the

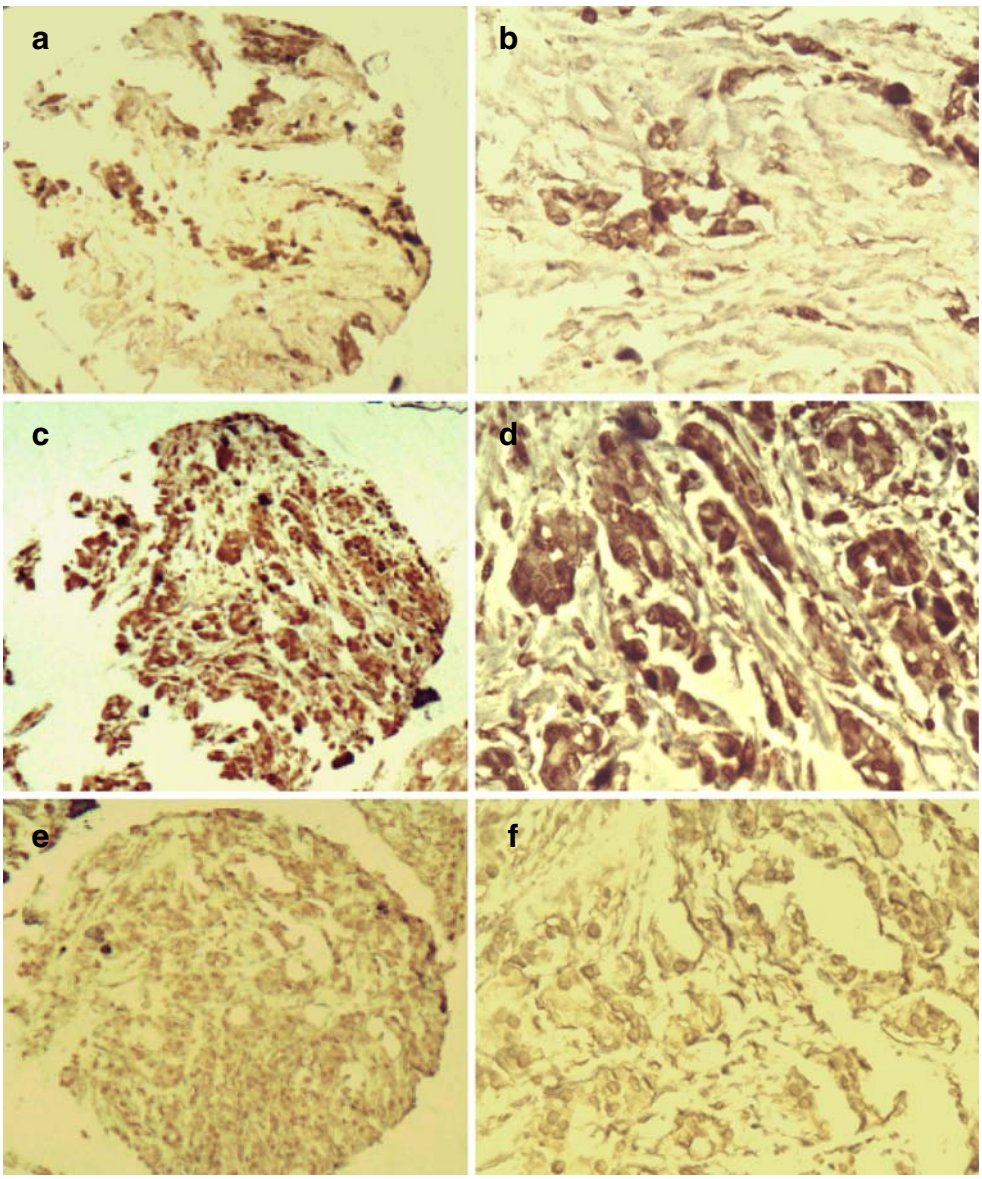

Fig. 1 RyR immunostaining in the epithelium of three representative human breast cancer specimens (**a**, **c**, **e**). Specimens **a** and **c** display high staining, whereas specimen **e** shows low RyR immunostaining. Images **b**, **d** and **f** are high magnification images of **a**, **c** and **e**, respectively. Magnification is 100× (**a**, **c**, **e**) and 400× (**b**, **d**, **f**). No counterstain

peroxidase substrate DAB, in the absence of counterstain. Statistical analysis, to determine the correlation coefficient (Pearson r), was done using Statistica (Statsoft Software, Tulsa, OK, USA).

Tissue culture Two human Bca cell lines, MCF-7 and MDA-MB-231 (American Type Culture Collection), were studied. Cell lines were maintained in Leibovitz's (L) medium (Gibco, Rockville, MD, USA) containing 100 units/mL penicillin G, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B and supplemented with 20% Hams F-12, 5 μg/mL insulin, 100 μg/mL transferrin, 30 nM sodium selenite, 5% fetal bovine serum. To study the effect of the RyR agonist 4-chloro-*m*-cresol on cellular proliferation, Bca cells lines were plated at 15,000 cells per well in 96-well plates in supplemented L-medium. The following day cells were treated with the RyR agonist in unsupplemented L-medium. After 5 days of incubation, cell numbers were determined using calcein AM (Molecular Probes, Eugene, OR). Data was plotted using GraphPad Prism and statistical analysis was done using GraphPad InStat (GraphPad Software, San Diego, CA, USA).

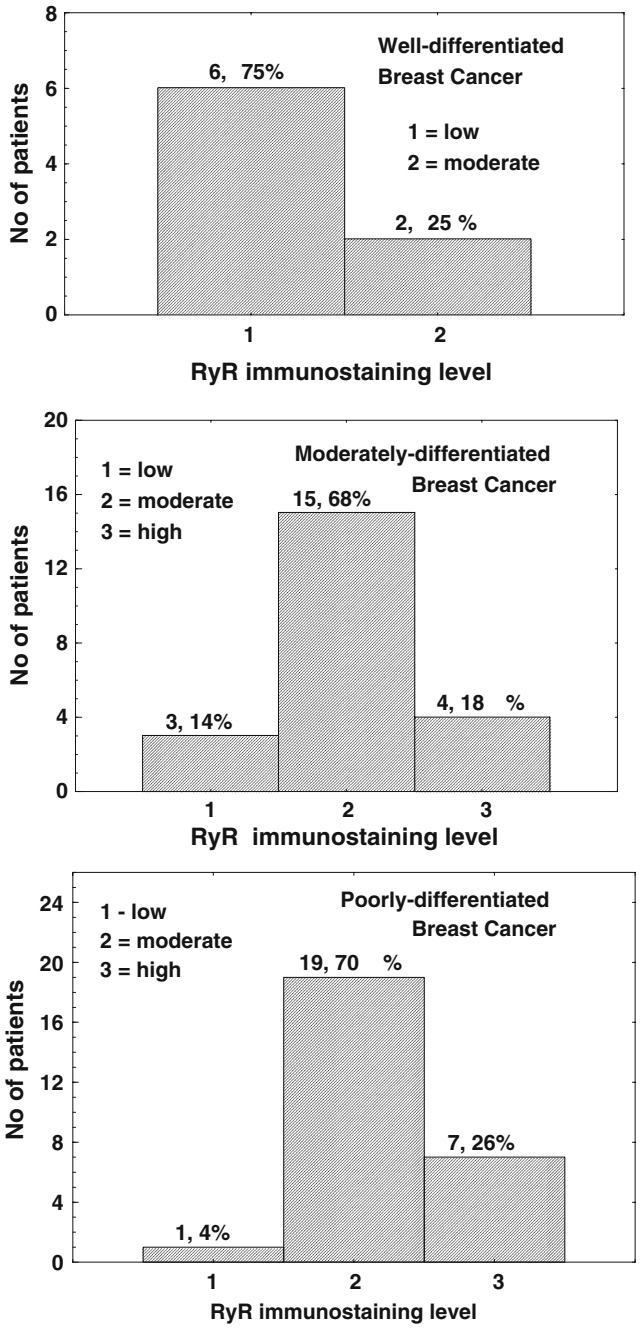

Fig. 2 Relationship between tumor grade and ryanodine receptor (RyR) levels in 57 human breast cancer specimens. There was a significant, direct correlation between tumor grade and RyR levels (Pearson r=0.48, p=0.0002, n=57)

Results

RyR immunohistochemistry showed low levels of RyR in 10 (18%), moderate levels in 36 (63%) and high levels in 11 (19%) of the Bca specimens (Fig. 1). Tumor grade was well-differentiated in 8 (14%), moderately-differentiated in 22 (39%) and poorly-differentiated in 27 (47%) of the ductal Bca specimens studied. We found a good correlation between RyR levels and tumor grade (r=0.48, p=0.0002, n=57). Examination of RyR levels in Bca specimens on the basis of tumor grade (Fig. 2), shows that majority (75%) of those with well-differentiated tumors had low RyR levels. In contrast, majority of the specimens that were moderately-differentiated (86%) or poorly-differentiated (96%) had moderate to high RyR levels (Fig. 2).

We have studied the effect of the RyR agonist 4-chloro-*m*-cresol on the growth of two human breast cancer cell lines: estrogen receptor alpha positive (ER+ve) MCF-7 and ER−ve MDA-MB-231. Concentration-dependent, inhibito-

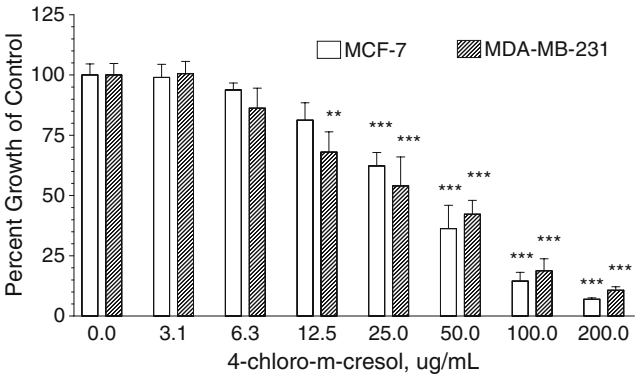

Fig. 3 Inhibitory effect of a ryanodine receptor agonist on the in-vitro proliferation of two human breast cancer cell lines. Experiments were repeated five times and Mean+SD is indicated. ***p<0.001, **p< 0.01, significantly different from untreated control, Student's t test

ry effect of 4-chloro-*m*-cresol on the in-vitro proliferation of the two Bca cell lines is shown in Fig. 3.

Discussion

There are very few reports of RyR expression in cancer tissue. Functional RyRs have been found in the LNCaP human prostate cancer cell line [7]. LNCaP cells responded to caffeine and 4-chloro-*m*-cresol by mobilizing Ca2+ and treatment with caffeine had a small stimulatory effect on apoptosis [7]. The Hungarian National Cancer Consortium recently reported that in malignant melanoma several novel markers were tested, among which RyR appeared to be promising [8]. Using an antibody that recognizes all three isoforms of RyR, we found that RyR is commonly expressed in Bca. Moreover, RyR levels correlated directly with tumor grade, suggesting that high RyR levels may indicate a poor prognosis. RyR expression in normal breast tissue remains to be examined. In similarity to the findings with LNCaP prostate cancer cells [7], modulation of RyR activity resulted in growth-inhibition in ER+ve as well as ER−ve human Bca cell lines. Further studies of RyR expression and activity in normal and neoplastic breast tissue are warranted.

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References

1. Bootman MD, Lipp P, Berridge MJ (2001) The organization and functions of local Ca(2+) signals. J Cell Sci 114:2213–2222
2. Mackrill JJ (1999) Protein–protein interactions in intracellular Ca2+-release channel function. Biochem J 337:345–361
3. Fill M, Copello JA (2002) Ryanodine receptor calcium release channels. Physiol Rev 82:893–922
4. Hakamata Y, Nishimura S, Nakai J et al (1994) Involvement of the brain type of ryanodine receptor in T-cell proliferation. FEBS Lett 352:206–210
5. Kang HY, Kim NS, Lee CO et al (2000) Expression and function of ryanodine receptors in human melanocytes. J Cell Physiol 185:200–206
6. Matyash M, Matyash V, Nolte C et al (2002) Requirement of functional ryanodine receptor type 3 for astrocyte migration. FASEB J 16:84–86
7. Mariot P, Prevarskaya N, Roudbaraki M et al (2000) Evidence of functional ryanodine receptor involved in apoptosis of prostate cancer (LNCaP) cells. Prostate 43:205–214
8. Timar J, Kasler M, Katai J et al (2006) Developments in cancer management by innovative genomics: 2006 report of the National Cancer Consortium. Hungarian Oncology 50:349–359