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Identification of functional type 1 ryanodine receptors in human dendritic cells

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Abstract

Ryanodine receptor (RyR) is a Ca^{2+} channel that mediates Ca^{2+} release from intracellular stores. Altered Ca^{2+} homeostasis in skeletal muscle which usually occurs as a result of point mutations in type 1 RyR1 (RyR1) is a key molecular event triggering malignant hyperthermia (MH). There are three RyR isoforms, and we herein show, for the first time, that human dendritic cells (DCs) preferentially express RyR1 mRNA among them. The RyR activator, 4-chloro-*m*-cresol (4CmC), induced Ca^{2+} release in DCs, and this response was eliminated by dantrolene, an inhibitor of the RyR1, and was unaffected by xestospongin C, a selective inhibitor of IP_3 receptor. Activation of RyR1 reduced LPS-induced IL-10 production, promoted the expression of HLA-DR and CD86, and thereby exhibited an improved capacity to stimulate allogeneic T cells. These findings demonstrate that RyR1-mediated calcium signaling modifies diverse DC responses and suggest the feasibility of using DC preparations for the diagnosis of MH.

Keywords: Ryanodine receptor; Dendritic cells; Malignant hyperthermia

Dendritic cells (DCs) are the most potent antigen-presenting cells and play a crucial role in the induction and regulation of immune responses [1]. DCs are widely distributed in tissues, where they form a sentinel network that detect pathogen entry through an array of pattern recognition receptors. The signals through these receptors induce maturation of DCs, transforming them from cells specialized in antigen uptake to cells that migrate into regional lymph nodes to stimulate T cells. The potency of mature DCs (mDCs) for the presentation of antigens stems from their increased expression of the major histocompatibility complex–peptide complex and costimulatory (CD80 and CD86) molecules. In addition, DCs carry signals, which determine the polarization of naive T helper (Th) cells into various effector subsets characterized by their distinct cytokine profiles and distinct functions [2].

In DCs, Ca^{2+} signaling has been implicated in various physiological responses such as maturation, migration, and cytokine secretion [3]. Although, IP_3 formation and their interaction with IP_3 receptor (IP_3R) are important factors regulating Ca^{2+} release, ryanodine receptor (RyR); a major intracellular Ca^{2+} release channel, also

Abbreviations: RyR, ryanodine receptor; DC, dendritic cells; Th, T helper; MLR, mixed leukocyte reaction; MH, malignant hyperthermia; MHS, malignant hyperthermia susceptible; 4CmC, 4-chloro-*m*-cresol; CICR, Ca^{2+} -induced Ca^{2+} release.

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contributes to the Ca^{2+} signaling pathways [4]. Three isoforms of RyRs (RyR1-3), which appear to be encoded by different genes, have been reported. RyR1 and RyR2 are expressed predominantly in skeletal muscle and heart, respectively, and third type of RyR (RyR3) is expressed in the central nervous system as well as in a variety of other tissues [5–7]. The expression of RyR isoforms in immune cells has also been demonstrated [8]. Human B cells express a functional RyR1 that is involved in Ca^{2+} signaling in conjunction with the IP_3 receptors [9]. In addition, the expression of RyR3 has been found in human Jurkat T cells, where cyclic adenosine diphosphoribose (cADPR) induces intracellular Ca^{2+} release via RyR3 by a process referred to as Ca^{2+}-induced Ca^{2+} release (CICR) [10]. Despite the important role of Ca^{2+} signaling in DC functions, the existence of RyRs in human DCs and RyR-mediated Ca^{2+} movement has not been characterized [11].

Malignant hyperthermia (MH) is a pharmacogenetic disease triggered by inhalational anesthetics or depolarizing muscle relaxants that results from mutations in RyR1 [12,13]. Excessive Ca^{2+} mobilization from the sarcoplasmic reticulum (SR) appears to be the underlying defect of this syndrome. The diverse mutations of the RyR1 gene in MH-susceptible (MHS) individuals and the vast size of this gene have limited their mutation-based screening [14–16]. Moreover, the incidence and distribution of mutations along the RyR1 gene in MHS individuals is highly variable among populations [17]. The definitive diagnosis of MHS individuals is made by means of the caffeine-halotane contracture test (CHCT), *in vitro* contracture test (IVCT), or detection of an enhancement of CICR on biopsied muscle [12,18,19]. However, because of the invasive nature of these tests, the establishment of a more efficient and less invasive diagnostic methods would thus have greatly help to elucidate the etiology of MH and in the development of preventative and therapeutic strategies. Although, the use of human B cells that preferentially express RyR1 has been reported as a diagnostic tool [20], the characterization of the RyRs expression and their function in human DCs will expand the possibility for new diagnostic methods. The current study will demonstrate that human DCs preferentially express RyR1 that function as Ca^{2+} release channels. In addition, RyR-stimulating agent 4-chloro-*m*-cresol (4CmC) induces calcium mobilization, and in turn, promotes maturation. These observations suggest the possibility that RyR1-mediated Ca^{2+} mobilization can modify the diverse DC responses and the availability of the DC preparation for the diagnosis of MH.

Materials and methods

Antibodies and reagents. Recombinant human (rh) IL-4, GM-CSF, and TNFα were purchased from Primmune (Osaka, Japan). Fluorescein isothiocyanate (FITC)-conjugated anti-human HLA-DR (G46-6) and CD86 (FUN-1) mAb, FITC-mouse IgG_1 (MOPC-21), FITC-mouse IgG_{2b} (27–35), and 7-AAD were from BD Biosciences (San Diego, CA). FITC-annexin V was from Sigma Chemical (St. Louis, MO). CD14 and CD45RO microbeads was from Miltenyi Biotec (Bergisch, Gladbach, Germany). 4-Chloro-*m*-cresol (4CmC), adenosine 5′-triphosphate (ATP), and dantrolene were from Calbiochem (Darmstadt, Germany). Xestospongin C was from Wako (Osaka, Japan).

Induction of human monocyte-derived dendritic cells (Mo-DCs). Buffy coats of blood were obtained from healthy volunteers (Japanese Red Cross Society, Saitama, Japan) and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (GE Healthcare). $CD14^+$ monocytes were purified and cultured at 1.0×10^6 cells/ml in RPMI (Sigma) containing 5% human serum in the presence of rhGM-CSF and rhIL-4 (50 ng/ml each). On days 2 and 3, the DC cultures received an additional dose of rhGM-CSF and rhIL-4 (50 ng/ml each). On day 6, nonadherent DCs were harvested and served as immature DCs (iDCs). Further differentiation into mature DCs (mDCs) was induced by treatment with 100 ng/ml LPS from *Escherichia coli* (serotype 055:B5, Sigma) or 1 μM prostaglandin E2 (Sigma) plus 20 ng/ml TNFα for 48 h. The DC phenotype was determined by flow cytometry (FACScan; BD Biosciences). The presence of dead cells was excluded by running parallel 7-AAD-stained samples. For apoptosis detection, the cells were stained for 10 min with FITC-annexin V (Sigma) and analyzed by flow cytometry without dead-cell exclusion.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). Genomic DNA was digested and removed using an RNase-Free DNase kit (Qiagen). First strand cDNA was synthesized using oligo $(dT)_{12-18}$ primer (Invitrogen) and the Omniscript RT kit (Qiagen). Synthesized cDNA was amplified by PCR using a primer set that selectively amplifies the specific isoforms of the RyR as previously described [8]. The PCR conditions were: 95 °C for 10 min, followed by 35 cycles at 95 °C for 1 min, 62 °C for 1 min, 72 °C for 2 min, followed by a 5 min extension at 72 °C (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA). PCR using cDNA-specific β-actin primers was done as control [21]. cDNA from the human brain was purchased from OriGene Technology (Rockville, MD).

Real-time quantitative RT-PCR. Transcripts were quantified by real-time quantitative PCR using an ABI PRISM 7900 Sequence Detector (Applied Biosystems) with Applied Biosystems predesigned TaqMan Gene Expression Assays (RYR1; Hs00166991_m1) and reagents according to the manufacturer's instructions. Real-time fluorescence measurements were taken, and the threshold (C_T) cycles of amplification, where the plots crossed a defined baseline, were determined for each sample. For relative quantification, the gene expression in samples was normalized by comparison with the GAPDH expression using the ddC_T method.

$[Ca^{2+}]_i$ measurement. Cells (1.0×10^6 cells/ml) were loaded with 5 μM Fura2/AM (Biotium, Hayward, CA) in loading-buffer for 30 min at 4 °C. (Loading-buffer: 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM glucose, 1 mM $CaCl_2$, and 10 mM Hepes). Where indicated, $BAPTA_{-AM}$ was loaded simultaneously into the cells. Fura2/AM-loaded cells were washed twice with loading-buffer or Ca^{2+}-free loading-buffer containing 5 mM EGTA (ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) and were plated on 35 mm glass base dish (Iwaki, Chiba, Japan). The cells were incubated for 10 min at 37 °C before stimulation for monitoring the intracellular Ca^{2+} concentration. Fluorescence changes (340/380 nm wavelength excitation ratio at an emission wavelength of 505 nm) were measured every 5 s using fluorescent microscopy ECLIPSE TE2000 and AquaCosmos software (Hamamatsu Photonics, Hamamatsu, Japan).

Mixed leukocyte reaction (MLR). $CD4^+CD45RO^-$ (naive) Th cells were isolated from PBMCs using a $CD4^+$ T cell isolation kit II (Miltenyi Biotec) and CD45RO microbeads. Differentially stimulated DCs were irradiated (3000 cGy) and cultured with allogeneic naive Th cells. After 5 days of culture, the cells were pulsed with [^{3}H]-thymidine (1 μCi/well) for 16 h and proliferative responses were measured as [^{3}H]-thymidine incorporation.

Measurement of cytokines. The cytokine levels in the culture supernatants were evaluated with ELISA (R&D systems, Minneapolis, MN).

Statistical analysis. Statistical analyses were performed using Student's *t*-test. Values were considered statistically significant at a value of $^*p < 0.05$.