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Distinct mechanisms for dysfunctions of mutated ryanodine receptor isoforms

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Abstract

Ryanodine receptor (RyR) is the Ca^{2+}-induced Ca^{2+} release channel in cells. RyR1 and RyR2 are its isoforms expressed in the skeletal and cardiac muscles, respectively. Their missense mutations, which are clustered in three regions that correspond to each other, cause hereditary disorders such as malignant hyperthermia and central core disease in skeletal muscle and catecholaminergic polymorphic ventricular tachycardia in cardiac muscle. Their pathogeneses, however, are not well understood. The following hypotheses are favorably discussed in this article: phenotypes with RyR1 and RyR2 mutations are mainly caused by dysregulations of their functions through the interdomain interaction and luminal Ca^{2+}, respectively.

Keywords: Ryanodine receptor; RyR1; RyR2; Hereditary diseases; MH; CCD; CPVT; ARVD2; Interdomain interaction; Luminal calcium

Professor Ebashi suggested the following two projects to me in 1965 when I joined his laboratory as a postgraduate. The first was whether the rate of muscle relaxation could be explained by the uptake rate of Ca^{2+} by the sarcoplasmic reticulum (SR) ("Ca^{2+} binding rate" in his words). Notably, the relaxation rate from twitch is about several times faster than that from the tetanus. The second was to investigate Ca^{2+} release. At that time, a hypothesis that Ca^{2+} release would be the reversal of Ca^{2+} uptake was prevalent. Professor Ebashi, in contrast, had claimed that Ca^{2+} release and Ca^{2+} uptake should be independent analogous to Na^{+} channel vs. Na^{+} pump [1]. This has been proved true by the isolation of purified ryanodine receptors (RyRs) as Ca^{2+}-induced Ca^{2+} release (CICR) channels from skeletal and cardiac muscles by Fleischer and his colleagues [2]. Since then, great advancements have been made together with the accumulation of a great number of findings [3,4]. Malignant hyperthermia (MH) and central core disease (CCD) linked to a missense mutation of the RyR isoform in the skeletal muscle (RyR1) have now been reported, whereas catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular dysplasia type 2 (ARVD2) caused by mutated RyR isoform in the cardiac muscle (RyR2) are also well known [5–8]. However, the precise mechanism by which channel function is altered by disease-linked mutations is poorly understood. Because this is my current interest, I will focus on this aspect in this article.

Brief overview of RyR and mutation-linked diseases

In vertebrates, three genetically distinct isoforms of RyR are detected: RyR1-3. RyR1 and RyR3 are expressed in almost equal amounts in many vertebrate skeletal muscles, but RyR3 is mostly degenerated and disappears in adult mammalian skeletal muscles, except diaphragm [9–12]. Therefore, it may be concluded that RyR1 is the main isoform expressed in the mammalian skeletal muscles, whereas RyR2 is its counterpart in cardiac muscles.

RyR isoforms show ~70% overall sequence identity and structural similarities. The monomer of RyR is a polypeptide

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of about 5000 amino acid residues with a MW of about 560 kDa [9–12]. Residues 1–4000 and the C-terminus lie in the cytoplasm, and only the one tenth or one fifth in the C-terminal exists within the membrane (4–8 transmembrane segments) and the lumen. Residues 4894–4917 in RyR1 and 4820–4829 in RyR2 form the channel pore and ion filter. The Ca^{2+}-release channel in the SR is composed of the homotetramer of RyR and some accessory proteins: calsequestrin, triadin, calmodulin (CaM), FK506 binding proteins 12/12.6 (FKBP 12/12.6) and others. The cytoplasmic part, referred to as the foot, appears to be a square or rhomboid of about $27 \times 27 \times 10$ nm, connected to a smaller transmembrane part ($12 \times 12 \times 7$ nm). The two parts are skew to each other. All three isoforms can be activated to open the gate by Ca^{2+}, giving rise to CICR. Only RyR1 can also be activated directly by a conformational change of dihydropyridine receptor (DHPR) upon depolarization of the T-tubule membrane in skeletal muscle. In other words, DHPR in the skeletal muscle works as a voltage sensor, whereas DHPR in the cardiac muscle works exclusively as a voltage dependent Ca^{2+} channel. CICR plays the pivotal role in various Ca^{2+} signaling processes, including cardiac muscle contraction. Although it is controversial how CICR is involved in the physiological skeletal muscle contraction, it clearly plays the primary role in the pharmacological contracture caused by caffeine, halothane, and other drugs.

MH is a hereditary disorder characterized by an abrupt rise of body temperature and high fever on exposure to volatile anesthesias. The disorder accompanied by muscle contracture has been a preferred target for study [13]. Although the genetic locus is of various origins, a missense mutation of RyR1 is well studied. Without such an episode as a surgical operation, however, many patients would not acknowledge themselves to be unusual. CCD is characterized by hypotonia and proximal muscle weakness together with characteristic histology of amorphous central areas (cores) in type 1 muscle fibers which consist of unstructured myofibrils and lack of mitochondria and oxidative enzyme activity. CCD is also linked to mutations in the RyR1 gene. In some cases, it is accompanied by MH. All of the identified MH and CCD point mutations in RyR1 are clustered in three regions: the N-terminal region or region1 (residues 35–614), the central region or region2 (residues 2129–2458), and the C-terminal membrane-associated region or region3 (residues 3916–4973) [5–8]. Mutations in region1 and region2 are common among MH, whereas region3 is often related with CCD [14]. CPVT and ARVD2 are exercise and stress induced hereditary ventricular tachycardia (VT) which is linked to missense mutations of RyR2. The mutated sites are also clustered in three regions, notably being homologous to each within RyR1: residues 77–466 (region1), 1724–2958 (region2), and 3778–4959 (region3) [7]. Mutations in region3 are frequently identified in the inherited VT.

The cytoplasmic Ca^{2+} concentration is largely regulated by Ca^{2+} release from and Ca^{2+} uptake by SR and extrusion of Ca^{2+} out of cells by the Na^{+}/Ca^{2+} exchange (NCX) reaction [15]. Extremely low permeability to Ca^{2+} of the SR membrane at rest is a unique characteristic of *adult* skeletal muscle. Application of CPA or thapsigargin alone does not induce Ca^{2+} leakage from the SR in *adult* skeletal muscle. Stimulation of Ca^{2+} release is necessary for depletion of the SR [16,17]. This property is in marked contrast to the cases with other materials including the primary cultured cells from skeletal muscle or myotubes, cardiac myocytes and a wide range of other kinds of cells where instantaneous massive Ca^{2+} leakage can be observed [5,6,18]. Whereas skeletal muscle shows a low NCX activity, great NCX activity in the cardiac myocytes strongly buffers cytoplasmic Ca^{2+} in spite of Ca^{2+} release, resulting in depletion of the Ca^{2+} store. This is the reason why ryanodine exerts negative inotropy on cardiac muscle, whereas the reagent causes marked contracture with skeletal muscles. Dirksen and his colleagues [5,6] have proposed "leaky-channel model" and "EC uncoupling model" for the pathophysiology underlying MH and CCD, using dyspedic myotubes homologously expressing recombinant RyR1. These models would offer understandable explanations of the underlying processes. The actual events in the *adult* skeletal muscle, however, may be different. Abrupt rise in the cytoplasmic Ca^{2+} would lead to the muscle contraction in skeletal muscle, and slow increase may result in degeneration of mitochondria, because metabolic processes are affected at lower Ca^{2+} than the mechanical system. It also should be noted that NCX is electrogenic. VT, a leading cause of sudden death, is believed primarily to be caused by delayed afterdepolarizations, which are produced by spontaneous Ca^{2+} release from the SR during Ca^{2+} overload and electrogenic NCX activity. Ca^{2+} activated chloride current and inward rectifier potassium current may also be involved in change in the membrane potential. Physical and emotional stress, resulting in tachycardia followed by Ca^{2+} overload, are critical triggers for VT. These events also activate the β-adrenergic receptor/protein kinase A signaling pathway.

Properties of CICR in relation to pathogeneses

It is generally assumed that CICR from mutated RyR is unusually enhanced on triggering stimuli, otherwise no abnormality could apparently be detected. CICR activity of RyR is modulated by many endogenous and exogenous agents including Ca^{2+}, Mg^{2+}, adenine nucleotides, -SH modifying agents, pH, CaM, FKBP 12/12.6, luminal Ca^{2+}, RyR domain peptides which may affect interdomain interactions within RyR molecules, and pharmacological reagents [9–15]. Ca^{2+} and Mg^{2+} are of primary importance among these factors. Ca^{2+} stimulates CICR at low concentrations (μM order), whereas it inhibits CICR at 0.1 mM or higher. This biphasic Ca^{2+} dependence is explained by the integration of two independent effects on the activating (A-site) and inactivating (I-site) Ca^{2+} sites on the RyR molecules. Mg^{2+} exerts an effect antagonistic to Ca^{2+} on the A-site and synergistic with Ca^{2+} on the I-site, shifting the

relationship between Ca^{2+} and CICR to a higher Ca^{2+} range and reducing activity. An adenine nucleotide such as ATP or a non-hydrolyzable ATP analog (AMPPCP or β, γ-methylene adenosine triphosphate) stimulates CICR in its dose-dependent manner without change in the Ca^{2+} dependence. This means that occupation by Ca^{2+} of the Ca^{2+} sites may be a necessary, but not a sufficient condition for CICR [19]. A similar situation is observed with [^{3}H]ryanodine binding, a biochemical measure of CICR, to RyR1 and RyR3 in the SR membrane. Purified RyR1 and RyR3 showed almost equivalent [^{3}H]ryanodine binding activities to each other, to which the activity of RyR3 in the SR membrane approximately corresponded [20,21]. The RyR1 in the SR membrane, in contrast, showed an activity as low as about one seventh, without the change in the Ca^{2+} dependence. This stabilized state in the SR membrane was referred to as suppression [20]. Addition of a domain peptide, DP4 (a 36-residue peptide in region2 which corresponds to 2442–2477 of RyR1 [14]) specifically increased [^{3}H]ryanodine binding in a dose-dependent manner. These findings may indicate that the channel activity of RyR1 in the SR membrane is strongly stabilized at a low activity by a certain interdomain interaction between the N-terminal and central regions within RyR1 molecules as proposed by Ikemoto and his colleagues [14], but that such regulation (suppression) is negligible with RyR3 [22]. FKBP 12 also contributes partly (about 10%) to the suppression of RyR1. RyR3, by the way, is not affected by FKBP 12, although the isoform can bind it [21]. On the other hand, the presence of a physiological cytoplasmic concentration of Mg^{2+} (0.3–1 mM) is necessary for the functional integrity of RyR2, being free of suppression [23]. Without Mg^{2+}, RyR2 in the SR membrane may be frozen to a level in activity at intermediate Ca^{2+} concentrations (10–100 μM) [23]. In conclusion, RyR1 is unique in that the isoform is stabilized at a low CICR activity in the SR membrane of the skeletal muscle, whereas RyR2 shows unsuppressed activity in the presence of about 1 mM Mg^{2+} [23]. Regulation of RyR2 through FKBP 12/12.6 is controversial [4]. Here, I would like briefly to state that FK506 treatment did not change the activity of RyR2 in our hands [23].

The effect of luminal Ca^{2+} on CICR activity has been controversial. The critical point is that it depends entirely on the kind of muscles. Kurebayashi and Ogawa [24] investigated Ca^{2+} influx into the empty SR in skinned frog skeletal muscle fibers and compared it with Ca^{2+} efflux from the loaded SR, i.e., Ca^{2+} release, to understand more deeply the properties of the Ca^{2+}-release channel. The major Ca^{2+} influx pathway was found to be the CICR channel. They also reached the conclusion that luminal Ca^{2+} has an inhibitory impact on CICR, although the apparent rate constant for Ca^{2+} efflux changed little when the loading level was decreased to one third. Kurebayashi and her colleagues [25] also examined the behavior of Ca^{2+} waves in multicellular preparations from guinea-pig cardiac ventricles and concluded that these waves occurred when Ca^{2+} content in the store attained a fixed level and that the majority of the releasable Ca^{2+} in the store was released in an almost all-or-none fashion. The cytoplasmic Ca^{2+} concentration cannot be the trigger, because Ca^{2+} waves occurred when it was decreasing. The stored Ca^{2+} level was dependent on the heart rate, being greater as the rate increased. Notably, the Ca^{2+} wave hardly propagated over the cell boundary, even though the wave appeared to propagate in one direction through a preparation during Ca^{2+} overload. Consistently, Chen and his colleagues [26–28] reported as follows: (1) Spontaneous Ca^{2+} releases in cardiac cells occurred more frequently as ambient Ca^{2+} concentrations increased. The amplitude of the Ca^{2+} wave, however, was kept relatively constant. (2) Unlike cardiac cells, skeletal muscle cells exhibited few such spontaneous Ca^{2+} releases. (3) HEK293 heterologously expressing RyR2 exhibited robust spontaneous Ca^{2+} waves, whereas no Ca^{2+} wave was observed in HEK293 expressing RyR1. (4) To identify the molecular determinants responsible for these differences, two chimera between RyR1 and RyR2 were examined: N-RyR1(1-4006)/C-RyR2(3962–4968) and N-RyR2(1-3961)/C-RyR1(4007-5037). The C-terminal region of RyR2 is an essential determinant of spontaneous Ca^{2+} waves and response to luminal Ca^{2+}, and the channel activity of the C-terminal region is subjected to the inhibitory impact caused by the N-terminal cytoplasmic part within RyR2, as replacement by the counterpart of RyR1 increases activity. Similar regulation between the channel part and the cytoplasmic part is also the case within RyR1. (5) RyR2 mutations linked to CPVT and ARVD2 reduced the threshold level of luminal Ca^{2+} to induce Ca^{2+} waves.

Ogawa and Kurebayashi [29] reported that the Ca^{2+} releasing action of halothane on rabbit skeletal muscle SR vesicles was greater in a higher temperature, whereas with frog skeletal muscle SR vesicles it was weaker. This result suggests that localized increase in temperature caused by muscular fasciculation accompanying administration of a depolarizing relaxant is an explanation other than the coexisting 4-chloro-*m*-cresol [30] for the reason that use of succinylcholine precipitates the induction of MH. Murayama and his colleagues [31] found that the SR from MH susceptible pig skeletal muscle with R615C mutation in RyR1 showed 8-fold greater [^{3}H]ryanodine binding compared with that from the wild type skeletal muscle, although there was only minor difference, if any, in the sensitivity to several ligands including Ca^{2+}, Mg^{2+} and adenine nucleotide or in the FKBP 12 regulation. The affinity for Mg^{2+} of the A-site and that for Ca^{2+} of the I-site were lower in the mutated RyR1. However, the combined effect of the two would amount at most to only a two-fold increase in activity in the presence of the activating Ca^{2+} concentrations and 1 mM Mg^{2+}. Therefore, the change in affinities to ligands would play only a minor part. The addition of DP4 produced only weak enhancement on the R615C mutated RyR1, whereas it stimulated the wild type RyR1 to a level comparable with that of mutated

RyR1. This is the first paper that clearly shows de-suppression of mutated RyR1 in the SR obtained from the native material. Furthermore, the SR vesicles from the mutant skeletal muscle were apparently more sensitive to caffeine in the Ca^{2+} releasing action. Murayama et al. conclude that the affected interdomain interaction between region1 and region2 is the major underlying mechanism for dysfunction observed in the MHS pig and that this hypothesis should be the case with human MH phenotype linked to mutations in region1 or region2. Interestingly, Oyamada et al. [32] reported that skinned fibers from MHS human biopsied muscle with L4838V mutation in RyR1 increased the Ca^{2+} dependent rate of CICR without change in Ca^{2+} sensitivity. This finding suggests that phenotypes linked to mutation in region3 also are probably subjected to modulation from the cytoplasmic domain, as in the case with RyR2. It also should be noted that region1 and region2 are closely localized at the ''clamp region'' in the three-dimensional structure of RyR [33,34], which are far from the pore region that is localized in the center of the structure [34].

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