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REGULATION OF CALCIUM RELEASE BY INTERDOMAIN INTERACTION WITHIN RYANODINE RECEPTORS

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1. ABSTRACT

In excitation-contraction (E-C) coupling, various types of activation signals, which are received presumably at the bulky cytoplasmic domain of the ryanodine receptor (RyR), are translated (or transduced) into the opening of the Ca^{2+} release channel located in the trans-membrane domain of the RyR. In order to elucidate the detailed mechanism of the signal transduction process, it is essential (i) to identify various sub-domains of the RyR that are involved in the Ca^{2+} channel regulation, (ii) to characterize the events occurring in these sub-domains during the activation process, and (iii) to characterize the modes of active interactions among these sub-domains. Recent developments in the E-C coupling research have provided us with new insight into each of these aspects, as outlined in this review. Of many putative regulatory sub-domains of the RyR, two domains (designated as N-terminal domain and central domain) are particularly interesting, because disease-linked mutations that have occurred in these domains (malignant hyperthermia and central core disease in skeletal muscle, and inheritable cardiac disease) induce abnormal modes of Ca^{2+} channel regulation. Pieces of evidence accumulated to this date suggest the following hypothesis. The N-terminal and central domains form, at least partly, the interacting domain pair, and unzipping and zipping actions of such domain-pair are involved in the opening and closing actions of the Ca^{2+} channel, respectively. We also propose that there are local conformational changes in the signal reception domains (e.g. the II-III loop-binding core), and such conformational changes are coupled with the aforementioned actions of the interacting domain pair. It seems that by virtue of such a coordination of the events occurring in various regions of the RyR, the Ca^{2+} channel can recognize the activation signal received at the cytoplasmic region of the RyR.

2. EXCITATION-CONTRACTION COUPLING AND RYANODINE RECEPTORS OF SKELETAL AND CARDIAC MUSCLE

E-C coupling in skeletal muscle and cardiac muscle share some basic features underlying both tissues, and have some tissue-specific characteristics as well (1). The most important basic feature common for skeletal and cardiac muscles is that both types of E-C coupling are mediated by two key components: the dihydropyridine receptor (DHPR) and the ryanodine receptor (RyR)(2-4). The DHPR is a hetero-oligomer consisting of the $\alpha 1$, $\alpha 2$, β, γ, and δ subunits (5). Four units of the DHPR tend to form the electron microscopically visible structure referred as tetrad upon linking the DHPR with the RyR (6,

7). The formation of the tetrad is pronounced in skeletal muscle, but rarely seen in cardiac muscle, because fewer RyRs are linked with the DHPRs. The α1 subunit performs at least two important functions of the DHPR as a voltage sensor and an L-type Ca^{2+} channel (2-4). The RyR shows an electron microscopic structure initially recognized as a junctional foot (8), which actually is an assembly of the four identical ~500 kDa macro-peptide chains (9). Recent cryo-electron microscope/3D imaging studies have resolved intriguing features of the tetrameric complex of both skeletal and cardiac RyR isoforms (10, 11). Both RyR isoforms have a very high affinity for ryanodine (12, 13) and behave like a Ca^{2+} channel when incorporated into lipid bilayers (14, 15), indicating that the Ca^{2+} channel responsible for SR Ca^{2+} release resides in the transmembrane domain of the RyR.

The major differences between the skeletal and cardiac types of E-C coupling are ascribable presumably to the facts that both molecular components of E-C coupling (DHPR and RyR) are expressed by the different tissue-specific genes (16) and that the anatomical arrangements of both components are quite different as described below. In skeletal muscle, practically all DHPR tetrads are physically linked with about 50% of the total population of the RyR tetramers, leaving the remaining 50% of the RyR molecules uncoupled with the DHPR (17, 18). In cardiac muscle, the relative density of the RyR (the ratio of the RyR to the DHPR) is 5-10 fold less than the skeletal muscle (17). Consequently, practically all of the RyR tetramers are structurally uncoupled with the DHPR. The DHPR α1 subunit serves as a voltage-sensor and an L-type Ca^{2+} channel for both skeletal and cardiac E-C coupling as described above, but the mode of the DHPR-mediated regulation of the RyR is quite different between skeletal and cardiac muscle cells. In skeletal muscle, the voltage-sensing by the α1 subunit leads to its physical interaction with the RyR to activate the SR Ca^{2+} release channel, presumably by mediation of one or more cytoplasmic loops of the DHPR α1 subunit (19, 20). The above-mentioned DHPR-uncoupled RyRs are activated perhaps by the Ca^{2+} that has been released from the neighboring DHPR-coupled RyRs by the so-called Ca^{2+}-induced Ca^{2+} release mechanism (21). In cardiac muscle, the voltage-sensing immediately opens the DHPR Ca^{2+} channel, causing Ca^{2+} flux from the extra-cellular space into the cytoplasm, and the entered Ca^{2+} activates the RyR by the Ca^{2+}-induced Ca^{2+} release mechanism. Interestingly, in some cases of skeletal muscle E-C coupling (e.g. invertebrate and neonatal vertebrate skeletal muscles) the Ca^{2+} inflow across the plasma membrane is required for the activation of E-C coupling (22) like cardiac E-C coupling.

The skeletal RyR isoform (RyR1) and the cardiac RyR isoform (RyR2) show a ~60% homology (23, 24). An early analysis (25) identified the three major divergent (non-homologous) regions; the so-called D1, D2 and D3 regions as indicated in Figure 1. Figure 1 also shows a heterogeneity map we constructed on the basis of the residue distance score of individual corresponding residues of the two isoforms. As seen, there are several more divergent regions in the RyR. The researchers have correlated the isoform-specific structural characteristics with functional characteristics, and attempted to identify various domains involved in the regulation of the RyR as described below.

3. VARIOUS REGULATORY DOMAINS OF THE RYR

3.1. Domains involved in the physical interaction with the DHPR

As described above, voltage-dependent activation of skeletal muscle-type E-C coupling is mediated by physical interaction between the DHPR and the RyR, presumably by mediation of one of the cytoplasmic loops of the DHPR α1 subunit called the II-III loop (18, 19, 25). On the other hand, Ca^{2+} mediates cardiac E-C coupling as a chemical mediator. Then, which portions of the RyR are involved in such a physical interaction in the case of the RyR1? This important question has been addressed by several groups, yielding rather controversial results. The chimera approach by Nakai *et al*. (27) suggested that the critical region is in a rather long stretch encompassing the residues 1635-2636. On the other hand, the II-III loop affinity column assay by Leong and MacLennan (28) suggested a short 1076-1112 segment. Using deletion strategy, Yamazawa *et al*. (29) identified the residues 1303-1406 region (D2 region) as a critical region for skeletal E-C coupling. Interestingly, according to the recent studies of immuno-localization of anti-D2 antibody in the 3D image, the site of antibody reaction is located in the so-called clamp region, which is regarded as the area for the interaction with the DHPR (30, 31). Thus, the critical regions suggested in the literature are spread in a relatively wide region of the primary structure encompassing residues #1076-2636.

The above fact that the suggested DHPR-binding regions are distributed in a broad region of the RyR polypeptide chain would indicate that the putative DHPR-interaction domain is constructed by a number of small sub-domains derived from different regions of the RyR chain. We recently initiated a new approach to test this hypothesis. Using some of the II-III loop peptides, we localized their binding sites within the RyR primary structure. Using the peptide-mediated and site-directed probe-labeling technique, we could fluorescently label the binding sites of peptide A and peptide C (the peptides corresponding to the Thr^{671}-Leu^{690} and the Glu^{724}-Pro^{760} regions of the II-III loop, respectively) on the RyR. The A site and C site are located at the opposite sides of the major calpain cleavage site (residue #1400: interestingly this is right in the D2 region described above) (32, 33). The A and C sites appear to be very close to each other in the quaternary structure (our preliminary data). We tentatively propose that the putative II-III loop binding core of the RyR consists of at least two non-covalently but tightly associated domains flanking the D2 region.

3.2. Domains involved in the regulation of channel gating

In the case of skeletal muscle-type E-C coupling, the activation signal received at the signal reception domain

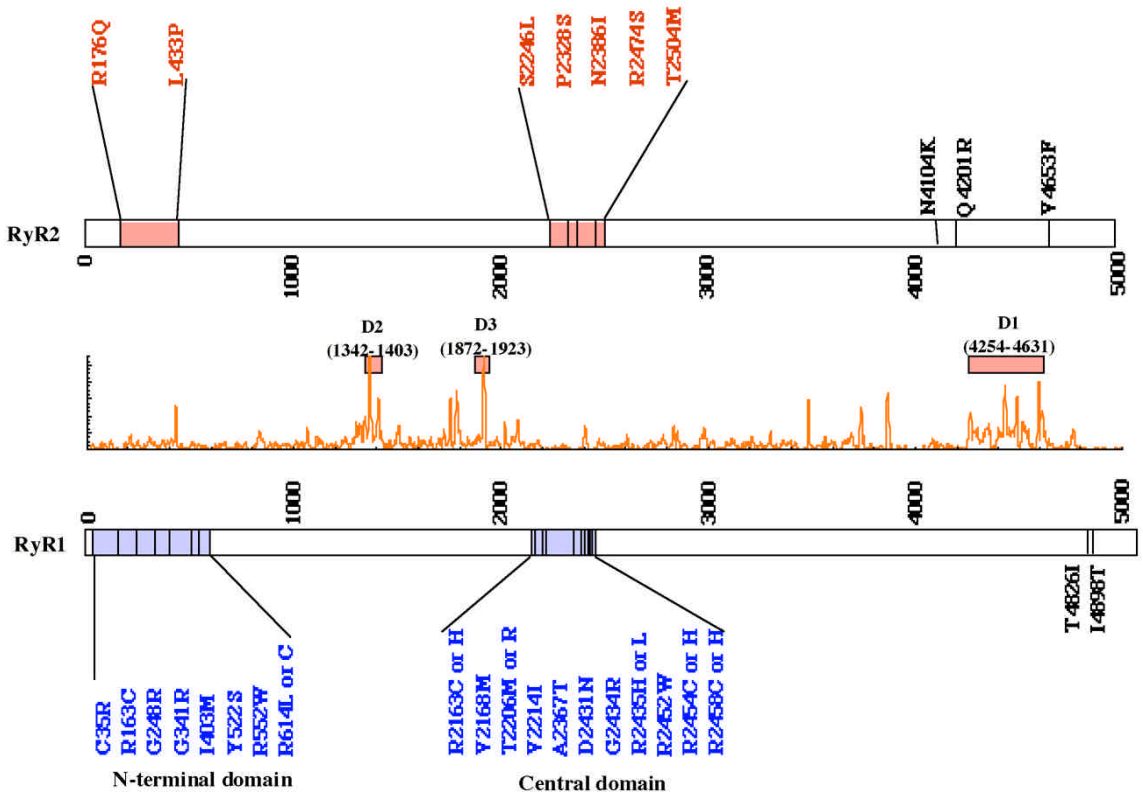

Figure 1. Comparison of the primary structures of the RyR1 and the RyR2 and the locations of MH/CCD mutation sites (RyR1) and cardiac ventricular tachycardia/ myopathy (ARVD) mutation sites (RyR2). Most of the MH/CCD mutations are found either in the regions, which we designate N-terminal domain and central domain, suggesting that these domains are the prime candidates for the putative regulatory or signal transduction domains. Recently reported cardiac mutations are also localized in the regions of the RyR2, which correspond approximately to the N-terminal and central MH/CCD domains of the RyR1. As a reference, the heterogeneity plot (the averaged residue score per every 30 residues versus the residue number) is shown.

(i.e. the II-III loop binding core described above) is recognized by the Ca^{2+} channel located at the opposite end of the RyR. There must be a relay switch-like mechanism, in which a number of putative regulatory domains and their intricate interactions are involved. In searching for such regulatory domains, we as other researchers have paid a particular attention to the fact that the sites of RyR1 mutations in the MH and CCD patients are localized in three major areas. As shown in Figure 1, most mutations are found in either N-terminal region (designated as N-terminal domain) or central region (central domain)(Refs. 34-52). Few mutations were found in the transmembrane Ca^{2+} channel domain, presumably in the channel pore region (53, 54). As widely recognized, mutations in either N-terminal or central domain produce abnormal modes of regulation of the RyR Ca^{2+} channel, as generally characterized as hyper-activation and hyper-sensitization effects (reviews: 55-57). In the other words, the channel tends to be activated more than the normal channel by the applied activation signal, and the sensitivity of the RyR to the activation signal increases. Thus, it appears that the two domains (the N-terminal domain and the central domain) represent the prime candidates for the regulatory domains involved in the relay switch mechanism described above.

The primary structure of the RyR2 corresponding to both of the skeletal N-terminal and central domains are relatively well conserved (Figure 1, heterogeneity map). We would propose that the cardiac domains corresponding to these N-terminal and central domains also play a key role for the following reasons. Recently several RyR2 mutations have been reported related to some inheritable cardiac diseases (58-60). Seven out of total eleven mutations shown here are located in either of the predicted N-terminal or central domain of the RyR2 (see red regions, Figure 1); two out of seven being in the N-terminal domain, and five in the central domain region. Of particular interest is that one of the cardiomyopathy (ARVD2) mutations in the N-terminal domain of the RyR2, Arg176Gln, corresponds exactly to the Arg163Cys human MH mutation of the RyR1. It is also noted that the amino acid residues of the RyR2 corresponding to those of the RyR1 at the potential MH and CCD mutation sites (21 in total) are exactly identical without exception. Thus, it is very likely that the essentially identical sets of regulatory domains are operating for the signal transduction (relay switch) mechanism in both RyR1 and RyR2.

3.3. Other important regulatory domains

Both RyR isoforms share some common mechanisms as described above, but their functional

properties are differentiated in many aspects. It is worthwhile to correlate the structural divergence with the characteristic functional properties seen in the cardiac RyR. For instance, the D1 region corresponds approximately to the predicted 'modulatory' domain (61) and in fact the putative Ca^{2+}-modulatory 4485-4494 segment (PEPEP sequence) is located in the region of the RyR1 corresponding to the D1 region (62). The RyR2 was reported to be more sensitive to the activating concentration of the cytoplasmic Ca^{2+}; for instance, the threshold of $[Ca^{2+}]_{cys}$ for channel opening is about 0.1 μM for the RyR2, while it is about 1.0 μM for the RyR1 (63, 64). According to the recent reports, experimental Glu3987Ala mutation in the RyR2 reduced the Ca^{2+}-sensitivity considerably in the mouse RyR2 (65). The effect of the corresponding skeletal mutation, Glu4032Ala, on the Ca^{2+}-sensitivity of the RyR1 was not observed in an earlier study (66), but according to the recent study (67) this mutation reduced the Ca^{2+}-sensitivity in the RyR1 as well. This would indicate that the residue Glu^{3987} is at least a part of the high-affinity Ca^{2+}-sensing device of both RyR2 and RyR1.

An interesting suggestion that the highly negatively charged region of the RyR1 at positions 1873-1903 may be involved in the low-affinity Ca^{2+}/Mg^{2+} binding was made by Laver et al. (68). In agreement with this suggestion, the RyR1 mutant, in which the D3 (Ile^{1641}-Ala^{2437}) region was deleted, showed a considerably reduced (~10 fold) sensitivity to the inhibitory Ca^{2+} or Mg^{2+} (69). The considerably reduced sensitivity to the inhibitory Mg^{2+} or Ca^{2+} is the widely recognized property characteristic for the RyR2. Therefore, the structural difference in the acidic region present in the D3 region (highly acidic in the RyR1 but less acidic in the RyR2) might be the cause for the difference in the sensitivity to the inhibitory Mg^{2+} or Ca^{2+}. The structural modification in the D3 region could also be the cause for the above-mentioned loss of the DHPR-binding ability in the RyR2 (see section 3.1.).

Both RyR1 and RyR2 have two kinds of tightly associated proteins that are involved in 'external' regulation of the skeletal and cardiac Ca^{2+} channels. One is a ubiquitous regulatory protein calmodulin (CaM) and the other is the FK506-binding protein (FKBP). The fact that both proteins bind to the RyR with a very high affinity and in a strictly stoichiometrical amount (one mol per one mol of the 550 kDa RyR monomer) has suggested an essential requirement of these proteins for the RyR regulation. This also suggests that the regions of the RyR to which these proteins bind must be considered as the regulatory domains of the RyR.

Calmodulin (CaM) binding domain: CaM has interesting Ca^{2+}-dependent dual effects on the RyR1, but produces somewhat different effects on the RyR2. Namely, at lower Ca^{2+} (< 0.1 μM) CaM activates the RyR1, but it inhibits at higher Ca^{2+} (70-72). In the case of the RyR2, CaM produces no effect at lower Ca^{2+}, but it inhibits at higher Ca^{2+} as in the case of the RyR1 (73). Earlier studies suggested several potential CaM binding regions of the RyR1 (74, 75). According to the more recent information, however, only one of these predicted CaM binding regions, which corresponds to the residue 3614-3642 region (23), seems to play a major role at least for the CaM binding to the RyR1 at higher Ca^{2+} (e.g. the 3614-3643 region, Refs. 76, 77)). At lower Ca^{2+}, CaM binding to the RyR1 takes place to about the same region, although in the strict sense the CaM binding site at low Ca^{2+} is somewhat shifted to the C-terminal side of the RyR1 polypeptide chain (77). Also in the case of the RyR2, one of the major predicted (from the overlay assay) CaM binding region corresponds to the region encompassing the residues 3298-3961 (corresponding to the skeletal sequence: 3336-4005) (78). Thus, it appears that both RyR1 and RyR2 share at least one common CaM-binding domain.

FKBP binding domain: The potential role of FKBP in the regulation of the RyR has been investigated chiefly by dissociation/reconstitution and knock-out experiments (79-83). Although not generally agreed, several important functions could be assigned to the FKBP. FKBP may stabilize the RyR channel in the closed state. Thus, dissociation of the protein-bound FKBP by FK506 or rapamycin (79-81) or protein kinase A-mediated phosphorylation (in case of the RyR2, Ref. 84) increased the Po and the channel open time (81-84). An important recent finding in this context is that experimental induction of cardiomyopathy by pacing decreased the amount of RyR2-associated FKBP, which in turn caused the leakiness of the Ca^{2+} channel (85, 86). FKBP may also be involved in the mediation of coupled gating of the multiple number of neighboring RyRs (83). With regard to the putative FKBP binding domain, the recent report by Gaburjakova et al (87) is particularly interesting. According to this study, mutation of Val^{2461} of the RyR1 produced a severe effect on the ability of FKBP to bind to the RyR, suggesting that this region represents the FKBP binding domain. Furthermore, replacement of that Val to Ile, just as seen in the corresponding amino acid residue of the RyR2, conferred the RyR2-like specific FKBP12.6 binding (88) to the RyR1. The above-mentioned Val^{2461} is positioned just adjacent to the Pro^{2462} that has previously suggested as a critical residue for FKBP binding (89). It is interesting to point out that this putative FKBP binding domain is located in the C-terminal region of the 'central domain' described in section 3.2. Since we are proposing in the next section (4.) that the tight interaction between the 'N-terminal domain' and the 'central domain' stabilizes the closed state of the channel, it is tempting to speculate that FKBP binding to the RyR may be facilitating the tightening of the intra-molecular domain-domain interaction.

4. POSTULATED INTERDOMAIN INTERACTIONS WITHIN THE RYR

4.1. Global conformational change of the RYR

The classic 'plunger' hypothesis proposed by Schneider and Chandler (90) predicted a global change in the RyR as a mechanism for the Ca^{2+} channel opening. As shown in the recent cryo-electron microscope studies, there seem to be appreciable differences in the 3D image between inactive and active states of the RyR (91, 92). Thus, in the activated state, the portion of the cytoplasmic domain (the generally called 'clamp' region) extends towards the T-tubule with conspicuous changes in its

configuration (91, 92). Also there is a slight rotation of the channel domain relative to the cytoplasmic domain with concomitant appearance of a pore-like structure in a way reminiscent of the action of opening a camera aperture (91).

No information is available about dynamic changes occurring in the global structure of the RyR, which are presumably occurring during the E-C coupling process. We have been trying to follow rapid local conformational changes occurring in different regions of the RyR. Site-directed incorporation of the fluorescence conformational probe using various RyR-specific ligands as site-directing carriers permitted us to introduce the probe to the designated regions (93). For instance, the probe that had been introduced into the channel domain by mediation of neomycin reported the gating behavior during the activation by T-tubule depolarization and the agonist polylysine (94). As described in the following sections (4.2. and 4.3.), this site-specific conformational probe approach has provided some new insights into the dynamic conformational changes occurring in several regulatory domains during T-tubule depolarization-induced activation process.

4.2. LOCAL CONFORMATIONAL CHANGES IN THE II-III LOOP PEPTIDE BINDING DOMAIN

As described above (27-29), the literature suggests that the RyR domains interacting with the DHPR II-III loop span over a very broad 1076-2636 region (1561-residue long) of the RyR polypeptide chain. Since the total length of the II-III loop is only about 120-residue long, the putative II-III loop binding domain must be confined in a small area forming the II-III binding core, which presumably consists of multiple numbers of small segments derived from different regions of the RyR polypeptide chain. Unfortunately, only very limited amount of information is available about the structure and function of the putative DHPR-interacting domain. Moreover, there is a considerable amount of controversy in the interpretations about which parts of the DHPR are responsible for the voltage-regulated interaction with the RyR (19, 20, 95-104). However, we would make the tentative proposal described below.

In case of the RyR1, the two kinds of II-III loop peptides, peptide A (Thr^{671}-Leu^{690}) and peptide C (Phe^{725}-Pro^{742}), bind to the C-terminal and N-terminal sides of the major calpain cleavage site (at the vicinity of residue 1400), respectively (32, 33). The location of this region corresponds approximately to the IP_3-binding region of the IP_3 receptor. Furthermore, in the postulated IP_3-binding core, the critical basic residues for the IP_3 binding are located at both sides of the site, which is highly susceptive to the proteolytic digestion. This shows a striking similarity to the postulated structure of the II-III loop-binding core described above. We hypothesize the II-III binding core will close upon voltage-mediated binding of the DHPR in a similar manner as the closing of the IP_3 binding core upon the ligand binding (cf. ref. 105). Such conformational changes in the II-III binding core would be recognized as the activation signal by the Ca^{2+} channels. Our recent observation that the fluorescence conformational probe attached to the II-III loop binding region increased upon T-tubule depolarization (32) is consistent with this idea.

4.3. Conformational changes in the signal transduction domains

The physiological activation signal, which is received at the II-III loop binding core of the RyR1, is translated into the opening action of the Ca^{2+} channel located at the other side of the RyR. Presumably a number of regulatory domains, including those listed in Section 3, are involved in the signal transduction mechanism, the mechanism by which the signal received is translated into the channel opening action. Recent studies began to reveal how intra-molecular interactions of these domains are involved in the signal transduction mechanism.

According to an earlier study of Zorzato *et al* (106), an antibody raised against the region containing Gly^{341} human MH mutation site produced a considerable increase in the rate of Ca^{2+}-induced Ca^{2+} release, and shifted the concentration for half-maximal activation (AC_{50}) of Ca^{2+} from 1.2 to 0.1 μM; the same type of effects as seen in MH (i.e. hyper-activation and hyper-sensitization effects). Furthermore, the peptide containing Gly^{341} bound to the 3010-3225-residue and the 799-1172-residue regions of the RyR1, suggesting that the N-terminal region interact with central regions of the RyR and that such interdomain interactions may be involved in the Ca^{2+} channel regulation.

Recently we synthesized several peptides corresponding to different regions of the N-terminal and central MH/CCD mutation domains (cf. 3.2.), designated as domain peptides (DP) (100, 107, 108). One of these, DP4, which corresponds to the Leu^{2442}-Pro^{2477} region containing three potential MH/CCD mutation sites, was found to bind to the N-terminal region of the RyR, as indicated by the DP4-mediated site-specific MCA labeling study (109). Thus, it appears that the two MH/CCD mutation domains, i.e. the N-terminal and central domains, come close to each other, at least partially, in the quaternary structure of the RyR.

As seen in a number of case reports (review: 57), any MH/CCD mutations occurred in either N-terminal or central domain seem to produce more or less identical type of effects on the mode of channel regulation (namely, an increased response to the RyR-agonist caffeine, Ref. 110), regardless of the position of the mutation site. In this context, it is important to refer to the work of Tong et al (111). They produced mutations corresponding to the 15 human MH/CCD mutations (9 in the N-terminal domain; 6 in the central domain) in a full-length rabbit RyR1 cDNA, and transfected wild-type and mutant cDNAs into HEK-293 cells. Ca^{2+} release in the cells expressing MH/CCD mutant RyRs was significantly more sensitive to caffeine than the wild-type for all mutations tested. This indicates that all mutations that occurred in either of these domains have a basically identical contribution to the production of the abnormal mode of channel regulation regardless of the

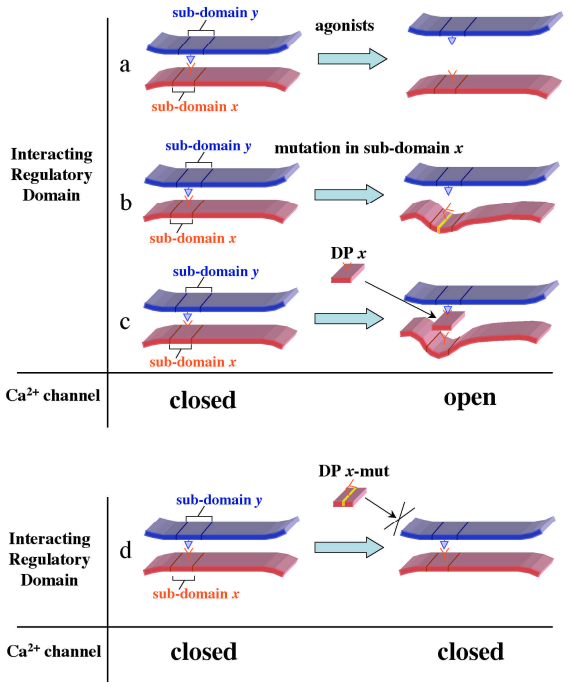

Figure 2. Hypothetical model showing how the changes in the mode of interaction between the two major MH/CCD domains (N-terminal domain and central domain) control the state of the Ca^{2+} channel of the RyR1. Probably the same or similar mechanism operates for the regulation of cardiac Ca^{2+} channel. a. The model assumes that a close contact between the N-terminal domain and the central domain (zipping) stabilizes the closed state of the Ca^{2+} channel, and the removal of the close contact (unzipping) de-blocks the channel to open. Such an unzipping action is produced by the activation signal received by the RyR (e.g. T-tubule depolarization). b. MH/CCD mutations in either of the N-terminal or the central domain cause weakening of the interaction between these domains, resulting in the unzipping and channel activation. c. Domain peptide (in this example, the peptide corresponding to sub-domain *x* of the central domain; namely domain peptide *x* or DP*x*) binds to sub-domain *y* of the N-terminal domain. As a result of competition between DP*x* and sub-domain *x* for their binding to sub-domain *y*, the interaction between sub-domains *x* and *y* (hence the interaction between the N-terminal and central domains) is weakened. This causes unzipping of the interacting domain pair and activation of the channel. d. MH/CCD-like mutation in DP*x* abolishes its ability to bind to sub-domain *y*, resulting in the loss of the activating function of DP*x*.

position of mutation sites. The hypothetical model illustrated in Figure 2 provides the simplest and the most straightforward explanation for such situations. In this model we assume that a close contact between the N-terminal domain and the central domain (*zipping*) stabilizes the closed state of the Ca^{2+} channel. The activation signal applied to the RyR removes the close contact, which causes *unzipping* of the interacting domain pair and Ca^{2+} channel opening. A mutation that has occurred in either of these interacting domains will weaken the affinity of the interdomain interaction, resulting in the increased tendency of unzipping and consequently in the hyper-activation/hyper-sensitization effects. Therefore, the degree of unzipping (hence the extent of channel-activation) caused by mutations is more or less the same regardless of the location the mutation has taken place.

The peptide probe study described in the following parts (cf. review 100) has been found to be a very effective way to test the above hypothesis and to investigate the details how the unzipping action causes channel activation. The principle of this approach is illustrated in Figure 2. Suppose that sub-domain *x* of the central domain is interacting with sub-domain *y* of the N-terminal domain, then synthetic or expressed peptide corresponding to the sub-domain *x*, i.e. domain peptide *x* (DP*x*), would bind to the sub-domain *y* in competition with sub-domain *x*. This will weaken the interaction between sub-domains *x* and *y*, causing the increased tendency of unzipping and channel activation. An excellent negative control to test the physiological relevance of the observed activation effect of DP*x* is as follows. Since mutation in sub-domain *x* weakens the interaction between sub-domains *x* and *y*, the same mutation made in DP*x* (namely DP*x*-mut) will reduce the affinity of its binding to sub-domain *y*, causing the loss of the activating function that would have present in the peptide.

Table 1 depicts the results obtained with one of such domain peptides, DP4, which corresponds to the Leu^{2442}-Pro^{2477} region of the central domain. Interestingly, DP4 and its mutant DP4-mut (see the sequence diagrams below) function exactly as predicted from the above hypotheses. Thus, DP4 enhanced ryanodine binding, induced Ca^{2+} release from the SR, induced contraction in skinned muscle fiber at an inhibitory Mg^{2+} concentration (112), increased the frequency of Ca^{2+} sparks in saponin-permeabilized fibers (113), and increased the open probability of single channels (113). DP4-mut, in which one mutation was made to mimic the Arg^{2458}-to-Cys^{2458} MH mutation (see the sequence diagram), produced no appreciable effect on any of these parameters with one exception where its effect could not be determined.

DP4: ^{2442}LIQAGKGEALRIRAILRSLVPLDDLVGIISLPLQIP^{2477}
DP4-mut: ^{2442}LIQAGKGEALRIRAILCSLVPLDDLVGIISLPLQIP^{2477}

Consistent with the hypothesis that the two MH/CCD domains (N-terminal and central domains) interact with each other, the central domain peptide DP4 binds to the N-terminal region of the RyR, as evidenced by the fact that the DP4-mediated site-directed probe labeling resulted in an exclusive fluorescence labeling of the ~150 kDa N-terminal segment of the RyR (109). Furthermore, activation of the RyR caused (a) a rapid decrease in the fluorescence intensity of the MCA probe attached to the N-terminal segment and (b) an increase in the accessibility to a large-size collisional fluorescence quencher (109). Both of these facts are consistent with the proposal that the unzipping of the two interacting domains is the causative mechanism for the Ca^{2+} channel activation. In further support of this concept, another domain peptide DP1-2

Table 1. A central domain peptide DP4 produces MH/CCD-like hyper-activation effects on the RyR Ca^{2+} channel as seen in various systems: from a level of the single channel to a level of the whole cell. Single mutation in the peptide abolishes its activating function in all cases, except for the skinned fiber experiment, in which its effect could not be determined (ND) because of the solubility problem.

System	Function	DP4	DP4-mut
Triad	Ryanodine binding[(i)]	+	-
	SR Ca^{2+} release[(i)]	+	-
	Apparent affinity to agonist[(i)]	+	-
Skinned fiber	Force response to caffeine[(ii)]	+	ND
	Force response to sub-max depolarization[(ii)]	+	ND
Permeabilized fiber	Frequency of Ca^{2+} sparks[(iii)]	+	-
Single channel	Po[(iii)]	+	-

(+):increase, (-):no change, (i) Yamamoto, T., R. El-Hayek & N. Ikemoto: Postulated role of interdomain interaction within the ryanodine receptor in Ca^{2+} channel regulation. *J Biol Chem* 275, 11618-11625 (2000), (ii) Lamb, G. D., G. S. Posterino, T. Yamamoto, & N. Ikemoto: Effects of a domain peptide of the ryanodine receptor on Ca^{2+} release in skinned skeletal muscle fibers. *Am J Physiol Cell Physiol* 281, C207-C214 (2001), (iii) Shtifman, A., C. W. Ward, T. Yamamoto, J. Wang,, B. Olbinsky, H. H. Valdivia, N. Ikemoto, & M. F. Schneider: Interdomain interactions within ryanodine receptors regulate Ca^{2+} spark frequency in skeletal muscle. *J. Gen. Physiol.* 116, 15-31 (2002)

corresponding to the Leu^{590}-Gly^{628} portion of the N-terminal domain activated the RyR and induced SR Ca^{2+} release, indicating that both central domain peptide DP4 and N-terminal domain peptide DP1-2 produced essentially identical MH/CCD-like hyper-activation effects (107, 108).

As described in 3.2., many mutations related to the inheritable cardiac myopathies occur in the regions of the RyR2 corresponding to the N-terminal and central MH/CCD domains of the RyR1 (cf. Figure 1). This suggests that these domains and their interdomain interactions play an important role also in the cardiac Ca^{2+} channel regulation. Consistent with this idea, both central domain peptide DP4 and N-terminal domain peptide DP1-2 activated the RyR2 as they activated the RyR1 (107), suggesting that that the cardiac Ca^{2+} channel is controlled by the basically identical mechanism as in the RyR1.

4.4. Coordination of local conformational changes

We now return to the question how the Ca^{2+} channel located in the trans-SR membrane region can sense the activating signal received at the T-tubule site of the RyR. As described in section 4.2., upon T-tubule depolarization, the putative E-C coupling activator (presumably located in the DHPR II-III loop) binds to the II-III loop binding core. This induces the change of the configuration of the II-III loop-binding core. Thus, the electric signal elicited in the DHPR is converted to the conformational signal. As described in section 4.3., upon T-tubule depolarization (or upon addition of activating domain peptides), there seems to be local conformational changes in the cytoplasmic domain of the RyR, such as the zipping action of the interacting N-terminal domain/central domain pair. T-tubule depolarization induces local conformational change also in the trans-SR membrane channel region, as demonstrated by using the fluorescence conformational probe attached to the neomycin (the Ca^{2+} channel blocker) binding region (114) of the RyR (94). We propose that all of these events occurring in these domains of the RyR take place in a highly coordinated manner upon the arrival of the activating signal to the signal reception domain. By virtue of this coordination, the conformational changes occurring in the T-tubule side and the SR-membrane side of the RyR can be coupled, permitting the Ca^{2+} channel to recognize and respond to the activation signal that has received at the opposite end of the receptor.

5. CONCLUSIONS AND PERSPECTIVES

The Ca^{2+} release channel is activated by mediation of a global conformational change of the RyR, which presumably consists of consorted local conformational changes occurring in many different places of the RyR. In this review, we dealt chiefly with two mechanisms. First, the activation signal (i.e. the voltage-dependent interaction of the DHPR with the RyR1) seems to be received at the II-III loop binding core consisting of at least two sub-domains of the RyR polypeptide chain flanking the major calpain cleavage site (at residue #1400). This produces a conformational change in the loop binding core (closing of the core), and such a change in the local structure is recognized as the activation signal by the Ca^{2+} release channel. Second, the frequent occurrence of both skeletal and cardiac myopathy mutations in the two domains of the RyR, viz. N-terminal and central domains, and the well-known functional outcome of these mutations have permitted us to identify these two domains as the prime domains involved in the Ca^{2+} channel regulation. The accumulated evidence suggests that the mode of interaction between these two domains is the important factor that controls the Ca^{2+} channel function. Thus, a close contact between these domains, *zipping*, stabilizes the closed state of the channel; while, removal of such a contact, *unzipping*, produces de-blocking, namely the activation, of the Ca^{2+} channel. The conformational change occurring in the N-terminal/central domain pair is tightly coupled with the conformational change occurring in the II-III loop-binding core described above. In summary, the voltage-dependent activation signal is translated into the local conformational change in the II-III loop-binding core. Then, this conformational information is transmitted to the Ca^{2+} channel by mediation of the coupled conformational change in the N-terminal/central domain pair.

The above mechanism provides a reasonable explanation for the pathogenesis of some RyR-linked

muscle diseases. For example, site-mutations taken place in either side of the N-terminal/central domain pair will weaken the interdomain interaction, resulting in an increased tendency of unzipping. This seems to be the major cause for the hyper-activation effects seen in the RyR1 of the patients with MH and CCD, and for the leaky Ca^{2+} channels seen in the RyR2 of cardiac myopathy patients. The fact that many of the critical cardiac mutation sites are localized in the regions of the RyR2 corresponding to the N-terminal and central domains of the RyR1 suggests that the essentially identical domain-domain interaction-mediated mechanism is operating for the regulation of both RyR1 and RyR2 Ca^{2+} channels. This idea may be tested in future studies, by producing the skeletal MH/CCD mutations in the RyR2, or alternatively by producing the cardiac myopathy mutations in the RyR1.

Most of the information described in this review has been derived from the studies with the peptides corresponding to the selected regions of the *in vivo* E-C coupling components. Needless to say, stringent tests (such as the mutation control as described here) must be carried out to verify the relevance of these peptides as a physiologically meaningful probe. Once it is done, these peptides serve as a powerful tool for many purposes; e.g. to localize the sites of peptide binding in the primary structure of the RyR, to introduce the fluorescence conformational probe to the designated sites, to monitor local conformational changes in the designated domains, etc. With the aid of a sufficiently large number of useful peptides and with finer localization of the peptide binding sites within the primary structure of the RyR, it should become possible to construct the intra-molecular or inter-molecular domain-domain interaction map. Upon accumulation of a sufficient amount of information from the *in vitro* studies, some of these key domain peptides can be tested for their effects in the *in vivo* system, as done with DP4. It would also be worthwhile to apply the domain peptide-mediated site-specific fluorescence labeling technique developed from the *in vitro* work to introduce the conformational probe to the designated domain of the RyR *in situ* and monitor the local events occurring during E-C coupling *in vivo*.

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