Postulated role of interdomain interaction between regions 1 and 2 within type 1 ryanodine receptor in the pathogenesis of porcine malignant hyperthermia

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We have demonstrated recently that CICR (Ca^{2+}-induced Ca^{2+} release) activity of RyR1 (ryanodine receptor 1) is held to a low level in mammalian skeletal muscle ('suppression' of the channel) and that this is largely caused by the interdomain interaction within RyR1 [Murayama, Oba, Kobayashi, Ikemoto and Ogawa (2005) Am. J. Physiol. Cell Physiol. **288**, C1222–C1230]. To test the hypothesis that aberration of this suppression mechanism is involved in the development of channel dysfunctions in MH (malignant hyperthermia), we investigated properties of the RyR1 channels from normal and MHS (MH-susceptible) pig skeletal muscles with an $Arg^{615} \rightarrow Cys$ mutation using [^{3}H]ryanodine binding, single-channel recordings and SR (sarcoplasmic reticulum) Ca^{2+} release. The RyR1 channels from MHS muscle ($RyR1_{MHS}$) showed enhanced CICR activity compared with those from the normal muscle ($RyR1_N$), although there was little or no difference in the sensitivity to several ligands tested (Ca^{2+}, Mg^{2+} and adenine nucleotide), nor in the FKBP12 (FK506-binding protein 12) regulation. DP4, a domain peptide matching the Leu^{2442}–Pro^{2477} region of RyR1 which was reported to activate the Ca^{2+} channel by weakening the interdomain interaction, activated the $RyR1_N$ channel in a concentration-dependent manner, and the highest activity of the affected channel reached a level comparable with that of the $RyR1_{MHS}$ channel with no added peptide. The addition of DP4 to the $RyR1_{MHS}$ channel produced virtually no further effect on the channel activity. These results suggest that stimulation of the $RyR1_{MHS}$ channel caused by affected interdomain interaction between regions 1 and 2 is an underlying mechanism for dysfunction of Ca^{2+} homoeostasis seen in the MH phenotype.

Key words: calcium release channel, malignant hyperthermia, ryanodine receptor type 1, sarcoplasmic reticulum, skeletal muscle.

INTRODUCTION

In skeletal muscle, RyR1 (type 1 ryanodine receptor) is a Ca^{2+} release channel of the SR (sarcoplasmic reticulum) and plays an important role in excitation–contraction coupling [1,2]. Ca^{2+} release through the RyR1 channel can be activated by two distinct modes: DICR (depolarization-induced Ca^{2+} release) and CICR (Ca^{2+}-induced Ca^{2+} release). DICR is triggered by conformational change of the dihydropyridine receptor upon depolarization of the T tubular membrane [3,4]. CICR is a ligand-gated mode in which Ca^{2+} itself regulates the channel activity: micromolar or more concentrations of Ca^{2+} activate the channel, whereas millimolar Ca^{2+} concentrations inactivate it [5]. In addition, 'gain' is an important determinant for the CICR activity which determines the maximal activity under respective conditions independently of Ca^{2+}-sensitivity [6,7]. The gain is affected by various CICR modulators, e.g. adenine nucleotides increase the gain, whereas procaine decreases it.

RyR1 is the major target for MH (malignant hyperthermia), an autosomal-dominant pharmacogenetic disorder triggered by volatile anaesthetics such as halothane, and to date more than 80 mutations have been identified in RyR1 of MH patients [8,9]. An enhanced CICR activity with higher sensitivity to caffeine or halothane was consistently reported in MH-mutated RyR1 [10]. Sensitization of DICR activity was also reported [11,12]. Thus it is widely accepted that MH mutations cause hyperactivation/hypersensitization of the Ca^{2+} release channel, resulting in abnormal Ca^{2+} homoeostasis in skeletal muscle. However, it remains to be elucidated how these mutations cause such dysfunctions in the Ca^{2+} release channel. Most known MH mutations are located in one of three 'hot spots', i.e. the N-terminal region (region 1, amino acids 35–614), the central region (region 2, amino acids 2163–2458) and the C-terminal transmembrane region (region 3, amino acids 3916–4973). Ikemoto and Yamamoto [13] have found that several short synthetic peptides corresponding to sequences within regions 1 and 2 of RyR1 activate the Ca^{2+} release channel. Among them, a 36-residue peptide corresponding to the Leu^{2442}–Pro^{2477} region, designated DP4, potently activated RyR1 [14–16]. Importantly, introduction of an MH mutation in DP4 (DP4-mut, mimicking an $Arg^{2458} \rightarrow Cys$ mutation) totally abolished these effects. Similar activation was induced by site-specific antibodies directed to the N-terminal (region 1) and the central (region 2) domains [17]. Further studies demonstrated that DP4 binds with the N-terminal portion of region 1 [18], and causes unzipping between regions 1 and 2 [19]. On the basis of these findings, it was hypothesized (i) that the two domains of RyR1 normally interact with each other to stabilize the closed state of the channel, (ii) that MH mutation in either domain weakens this interdomain interaction, resulting in destabilization of the channel, which erroneously increases channel activity, and (iii) that exogenous

Abbreviations used: AMPPCP, β,γ-methylene adenosine triphosphate; CICR, Ca^{2+}-induced Ca^{2+} release; DICR, depolarization-induced Ca^{2+} release; FKBP, FK506-binding protein; MH, malignant hyperthermia; MHS, MH-susceptible; Mopso, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; P_o, mean open probability; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

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domain peptides (e.g. DP4) mimic the phenotype of MH mutation by competitively interfering with the interdomain interaction [13].

We have shown previously that [^{3}H]ryanodine binding to RyR1 is significantly lower than that of RyR3 (type 3 ryanodine receptor), another isoform in skeletal muscle, suggesting that the CICR activity of RyR1 is 'suppressed' to a low level in skeletal muscle SR [20–22]. This suppression is attributed to a reduced gain of the CICR without significant changes in the sensitivity to known CICR ligands and drugs (e.g. Ca^{2+}, Mg^{2+}, adenine nucleotides and caffeine) [20,21]. The suppression effect is produced by two independent factors: protein-bound FKBP12 (FK506-binding protein 12) and the interdomain interaction within RyR1; the latter mechanism accounts for $\sim$70 % of the suppression [21,22]. We claimed that suppression of the RyR1 channel is important in Ca^{2+} homoeostasis in skeletal muscle, and aberration in this mechanism thus causes dysfunctions of RyR1 channels as seen in some muscle diseases such as MH. However, this remains to be elucidated.

To test the hypothesis, in the present study, we investigated the properties of the RyR1 channel with the SR vesicles isolated from skeletal muscles of normal and MHS (MH-susceptible) pigs using [^{3}H]ryanodine binding assays, single-channel current recordings and caffeine-induced Ca^{2+} release measurements. MHS pigs carry an $Arg^{615} \rightarrow Cys$ mutation in the RyR1 and are known to be an excellent animal model for human MH [10]. Here we report that affecting the N-terminal and central domain interactions by the aforementioned domain peptide (DP4) produced a significant channel activation (i.e. removal of suppression) in the RyR1 from normal pigs ($RyR1_N$), but it produced no further effect on the channel function of the RyR1 from MHS pigs ($RyR1_{MHS}$). These results suggest that the suppression is largely impaired in $RyR1_{MHS}$ in comparison with $RyR1_N$, and that this impaired suppression is caused primarily by the affected interdomain interaction within the $RyR1_{MHS}$.

EXPERIMENTAL

Materials

Peptides (DP4 and DP4-mut) were synthesized on a synthesizer (Applied Biosystems model 431 A) employing Fmoc (fluoren-9-ylmethoxycarbonyl) as the α-amino-protecting group and purified by reverse-phase HPLC [14]. [^{3}H]Ryanodine (50–60 Ci/mmol) was purchased from NEN Life Science Products. All other reagents were of analytical grade.

Animals and preparation of SR vesicles

All of the experiments were carried out in accordance with Juntendo University Ethics Committee guidelines. Homozygous MHS pigs for the RyR1 $Arg^{615} \rightarrow Cys$ mutation and homozygous normal pigs were obtained by breeding heterozygous parent pigs that had been maintained in the Miyazaki Station, National Livestock Breeding Center (Miyazaki, Japan). Animals used for the experiments were 2-month-old littermates that had been tested for the presence of the normal and MHS RyR1 alleles. Animals were killed using an intravenous injection of pentobarbital overdose. Back muscles were excised immediately and frozen rapidly in liquid nitrogen. Crude SR vesicles were prepared from the muscle by the method of Murayama and Ogawa [23] and terminal cisterna-rich fractions were obtained by sucrose-density-gradient ultracentrifugation [21]. The vesicles were quickly frozen with liquid nitrogen, and stored at -80 °C until use.

[^{3}H]Ryanodine binding

The [^{3}H]ryanodine binding assay was carried out as described previously [22]. Briefly, the SR vesicles (100 μg of protein) were incubated with 8.5 nM [^{3}H]ryanodine for 5 h at 25 °C in a 100 μl solution containing 0.17 M NaCl, 20 mM Mopso [3-(*N*-morpholino)-2-hydroxypropanesulfonic acid], pH 6.8, 2 mM dithiothreitol, 1 mM AMPPCP (β,γ-methylene adenosine triphosphate) and various concentrations of Ca^{2+} buffered with 10 mM EGTA (calculated using the value of 8.79×10^5 M^{-1} as the apparent binding constant for Ca^{2+} of EGTA [24]) unless otherwise indicated. The protein-bound [^{3}H]ryanodine was separated by filtering through polyethyleneimine-treated Whatman GF/B filters. Nonspecific radioactivity was determined in the presence of 20 μM unlabelled ryanodine. The [^{3}H]ryanodine binding data (B) obtained under various assay conditions in the presence of a fixed concentration of [^{3}H]ryanodine (8.5 nM throughout these experiments) were expressed in relative values to the maximal binding sites for the ligand (B_{max}); thus B/B_{max} reflected an apparent averaged activity of individual Ca^{2+} release channels. B_{max} was determined on the Scatchard plot for [^{3}H]ryanodine binding in a medium containing 1 M NaCl and various concentrations of [^{3}H]ryanodine. Apparent dissociation constants for Ca^{2+} of the Ca^{2+}-activation site ($K_{A,Ca}$) and Ca^{2+}-inactivation site ($K_{I,Ca}$) and those for Mg^{2+} ($K_{A,Mg}$ and $K_{I,Mg}$) were estimated according to the method of Murayama et al. [25].

Single-channel recordings

Single-channel recordings were carried out as described previously with CHAPS-solubilized SR protein as a material [26,27]. Lipid bilayers consisting of a mixture of L-α-phosphatidylethanolamine, L-α-phosphatidyl-L-serine and L-α-phosphatidylcholine (5:3:2 by weight) in n-decane (40 mg/ml) were formed across a hole of $\sim$250 μm in diameter in a polystyrene partition separating *cis* and *trans* chambers. Channel currents were recorded in symmetrical solutions of 250 mM caesium methanesulfonate buffered at pH 6.8 with 20 mM Hepes/Tris at the holding potential of -40 mV (*cis*). Experiments were carried out at 18–22 °C. Bilayers containing only a single channel were used for analysis. Channel currents amplified by an Axopatch 1D patch clamp amplifier (Axon Instruments) were filtered at 1 kHz using an eight-pole low-pass Bessel filter and collected at 5 kHz for analysis. Mean open probability (P_o) was calculated from the records of duration >2 min by 50 % threshold analysis using pClamp (version 6.0.4) software.

Ca^{2+} release measurements

Ca^{2+} release from the isolated SR vesicles was fluorometrically measured by monitoring free Ca^{2+} concentration in the solution [22]. Briefly, SR vesicles (80 μg) were incubated at 30 °C in a fluorimeter cuvette containing 400 μl of 0.17 M KCl, 20 mM Mopso, pH 6.8, 1 mM $MgCl_2$, 5 mM potassium phosphate, 10 mM phosphocreatine, 2 units/ml of creatine kinase and 2 μM fura 2. Fluorescence was measured in a Hitachi F-4500 fluorescence spectrophotometer with wavelength settings of 340 and 380 nm for excitation (alternating) and 510 nm for emission. Active loading of the SR vesicles with Ca^{2+} was started by addition of 100 μM MgATP and 20 μM $CaCl_2$. Free Ca^{2+} concentration in the cuvette declined with time and reached a steady state within 5 min. At this point, caffeine was added and the changes in fura 2 fluorescence were recorded. For some experiments, 30 μM DP4 was added before the start of Ca^{2+} loading. Calibration of fura 2 signals was carried out with 100–400 nM free Ca^{2+} buffered with 1 mM EGTA in the reaction medium.

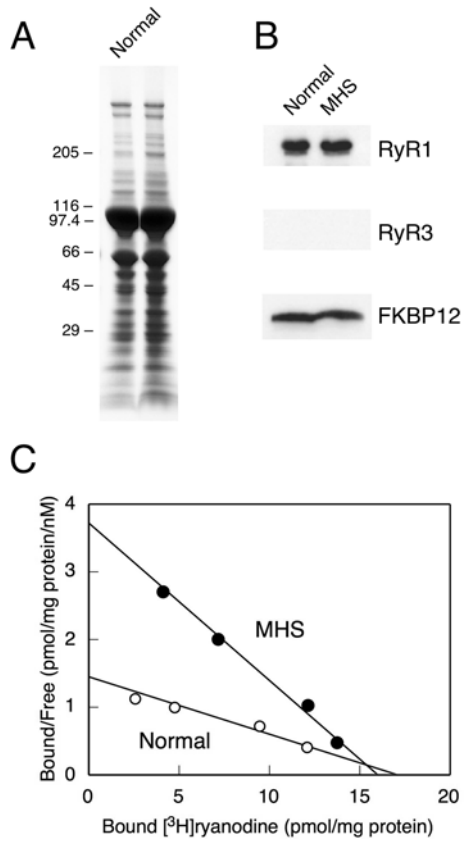

Figure 1 Protein compositions and the RyR1 content in SR vesicles isolated from normal and MHS pigs

(**A**) SR vesicles (30 μg) from normal (left) and MHS (right) pigs were subjected to SDS/PAGE and stained with Coomassie Brilliant Blue. The molecular markers (in kDa) are shown to the left of the gel. (**B**) Western blotting with antibodies against RyR1, RyR3 and FKBP12. Note that the normal and MHS SR preparations contain similar amounts of RyR1 and FKBP12, but no detectable RyR3. Data are representative of three experiments. (**C**) Scatchard plot analysis of [^{3}H]ryanodine binding to SR vesicles. Assays were carried out as outlined in the Experimental section, except that 1 M NaCl instead of 0.17 M NaCl was used. K_d and B_{max} values were 11.8 $\pm$ 1.8 nM and 17.1 $\pm$ 1.1 pmol/mg of protein and 4.3 $\pm$ 0.4 nM and 16.0 $\pm$ 4.6 pmol/mg of protein for normal and MHS pigs respectively. Results are means $\pm$ S.E.M. for three experiments.

Statistics

Data are given as means $\pm$ S.E.M. for n repeated experiments. To determine the significance of the difference between mean values, Student's unpaired t test was applied.

RESULTS

Protein compositions and the RyR1 content in the SR vesicles isolated from normal and MHS pigs

The protein compositions of SR vesicles isolated from normal and MHS pig skeletal muscles are shown in Figure 1. Both SR preparations show similar protein compositions in the Coomassie Brilliant Blue-stained SDS/polyacrylamide gel (Figure 1A). Western blots with the isoform-specific antibodies show that these SR preparations contain comparable amounts of RyR1 and FKBP12, an accessory protein of RyR1 (Figure 1B). There was no detectable RyR3 (Figure 1B). Fractions of active channel in the SR vesicles were determined by Scatchard plot analysis of [^{3}H]ryanodine binding. The B_{max} of the MHS vesicles (16.0 $\pm$ 4.6 pmol/mg of protein) was comparable with that of the normal vesicles (17.1 $\pm$ 1.1 pmol/mg of protein) (Figure 1C), indicating

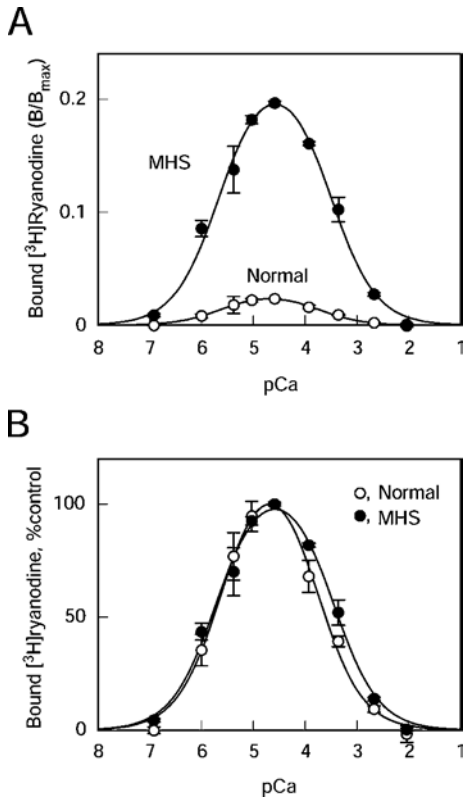

Figure 2 Ca^{2+}-dependence of the [^{3}H]ryanodine binding activity of $RyR1_N$ and $RyR1_{MHS}$

[^{3}H]Ryanodine binding was carried out as outlined in the Experimental section in 0.17 M NaCl, 20 mM Mopso, pH 6.8, 2 mM dithiothreitol, 1 mM AMPPCP and various free Ca^{2+} concentrations buffered with 10 mM EGTA. (**A**) Results are expressed as B/B_{max}. $RyR1_{MHS}$ (●) showed 8-fold greater amplitude of ryanodine binding as compared with $RyR1_N$ (○). (**B**) Activity was normalized by respective peak values. Although Ca^{2+}-sensitivity was not different for activation, $RyR1_{MHS}$ showed a slight reduction in Ca^{2+}-sensitivity for inactivation (approx. 2-fold increase in IC_{50}). Results are means $\pm$ S.E.M. ($n = 3$).

that the two SR preparations contain very similar amounts of active RyR1 channels. However, the K_d for [^{3}H]ryanodine was 3-fold smaller (4.3 $\pm$ 0.4 nM) in MHS SR than in normal SR (11.8 $\pm$ 1.8 nM), indicating that the affinity of ryanodine binding is significantly higher in MHS pigs than in normal pigs.

Removal of suppression in $RyR1_{MHS}$ as shown in the [^{3}H]ryanodine binding assay

Figure 2 shows the Ca^{2+}-dependence of the [^{3}H]ryanodine binding to the SR vesicles from normal and MHS pig skeletal muscle. The activity is expressed as $B/B_{max,}$ in which the ryanodine binding values (B) were corrected for the maximum number of binding sites (B_{max}), thus reflecting the apparent averaged activity of the individual channels (Figure 2A). The B/B_{max} value of RyR1 in normal SR ($RyR1_N$) at the optimum Ca^{2+} (0.023 $\pm$ 0.002; $n = 3$) was comparable with the values from bovine and rabbit skeletal muscle SR vesicles under similar conditions [21,22], suggesting that $RyR1_N$ is suppressed to the same extent as those of the other mammalian species. RyR1 in MHS SR ($RyR1_{MHS}$) showed 8-fold greater magnitude of [^{3}H]ryanodine binding than $RyR1_N$ at all Ca^{2+} concentrations examined ($B/B_{max} = 0.18 \pm 0.02$; $n = 3$). As shown in the normalized plot (percentage of the peak value), both RyR1s show almost identical Ca^{2+}-dependence in the activating range of Ca^{2+}: the K_d values for Ca^{2+} of the Ca^{2+} activating site ($K_{A,Ca}$) were 2.5 $\pm$ 0.4 μM and 2.0 $\pm$ 0.3 μM for $RyR1_N$ and

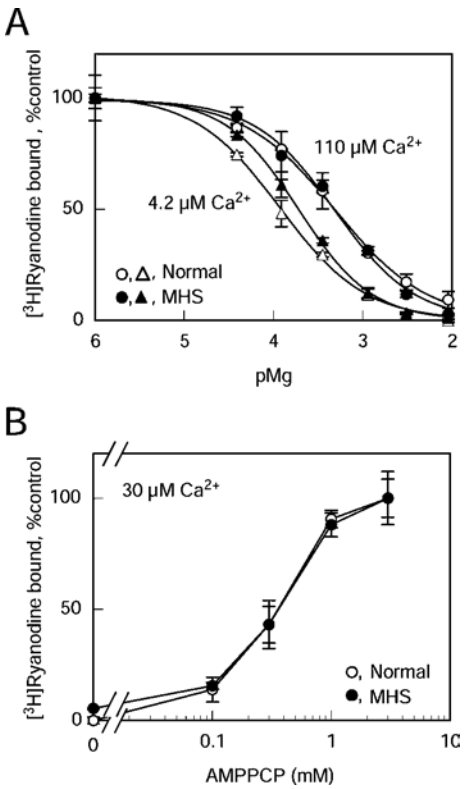

Figure 3 Effects of Mg^{2+} and AMPPCP on the [^{3}H]ryanodine binding of $RyR1_N$ and $RyR1_{MHS}$

(**A**) Inhibition by Mg^{2+}. Experiments were carried out as described in Figure 2 at 110 μM (circles) or 4.2 μM (triangles) Ca^{2+}. $RyR1_N$ (open symbols) and $RyR1_{MHS}$ (closed symbols) show similar sensitivity to Mg^{2+} inhibition at 110 μM Ca^{2+}, whereas $RyR1_{MHS}$ is less sensitive to Mg^{2+} than $RyR1_N$ at 4.2 μM Ca^{2+}. The B/B_{max} values in the absence of Mg^{2+} (corresponding to 100 %) for $RyR1_N$ and $RyR1_{MHS}$ were 0.017 and 0.17 at 110 μM Ca^{2+} and 0.018 and 0.15 at 4.2 μM Ca^{2+} respectively. Results are means $\pm$ S.E.M. ($n = 3$). (**B**) Activation by AMPPCP. Experiments were carried out as described in Figure 2 at 30 μM Ca^{2+}. The B/B_{max} values in the presence of 3 mM AMPPCP (corresponding to 100 %) for $RyR1_N$ and $RyR1_{MHS}$ were 0.016 and 0.17 respectively. Results are means $\pm$ S.E.M. ($n = 3$).

$RyR1_{MHS}$ respectively ($n = 3$). In the inhibitory concentration range, $RyR1_{MHS}$ showed 2-fold lower affinity than $RyR1_N$: K_d values for Ca^{2+} of the Ca^{2+}-inactivating site ($K_{I,Ca}$) were 0.17 ± 0.03 mM and 0.35 ± 0.06 mM ($n = 3, P < 0.05$) for $RyR1_N$ and $RyR1_{MHS}$ respectively (Figure 2B).

Mg^{2+} is known to inhibit the RyR1 channel by two distinct mechanisms: it acts as a competitive antagonist for the Ca^{2+}-activating site and as an agonist for the Ca^{2+}-inactivating site [25,28,29]. There was no substantial difference in Mg^{2+} inhibition between $RyR1_N$ and $RyR1_{MHS}$ at higher Ca^{2+} (110 μM) where Mg^{2+} acts exclusively as an agonist for the to Ca^{2+}-inactivation site (Figure 3A, circles): K_d for Mg^{2+} of the Ca^{2+}-inactivation site ($K_{I,Mg}$) was estimated to be 0.26 ± 0.04 mM and 0.30 ± 0.06 mM for $RyR1_N$ and $RyR1_{MHS}$ respectively. On the other hand, there was a slight reduction in the Mg^{2+} inhibition of $RyR1_{MHS}$ at lower Ca^{2+} (4.2 μM) where the inhibitory effect of Mg^{2+} at the Ca^{2+}-activation site becomes more involved (Figure 3A, triangles). K_d for Mg^{2+} of the Ca^{2+}-activation site ($K_{A,Mg}$) of $RyR1_{MHS}$ (0.40 ± 0.02 mM; $n = 3$) was significantly ($P < 0.05$) greater than that of $RyR1_N$ (0.21 ± 0.02 mM; $n = 3$), indicating that Mg^{2+}-sensitivity for the Ca^{2+}-activation site is somehow reduced in $RyR1_{MHS}$. There was no appreciable difference between $RyR1_N$ and $RyR1_{MHS}$ in the sensitivity to the agonist AMPPCP, a non-hydrolysable analogue of ATP: EC_{50} values were $\sim$0.3 mM

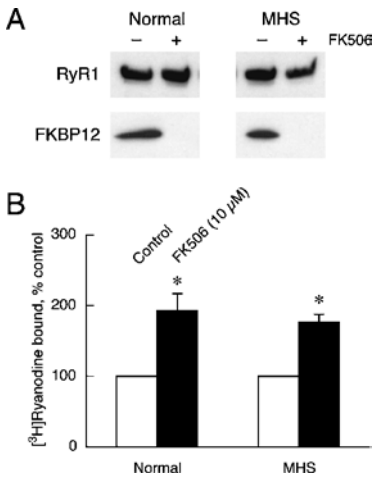

Figure 4 Unaltered regulation by FKBP12 in $RyR1_{MHS}$

(**A**) Binding of FKBP12 to RyR1. CHAPS-solubilized SR proteins from normal (left-hand panel) and MHS (right-hand panel) pigs were immunoprecipitated with anti-RyR1 antibody in the presence (+) and absence (−) of 10 μM FK506. RyR1 and FKBP12 in the precipitate were detected by Western blotting. A similar amount of FKBP12 was co-precipitated with $RyR1_N$ and $RyR1_{MHS}$. FK506 at 10 μM (+FK506) completely prevented the FKBP12 binding. (**B**) Effect of FK506 on [^{3}H]ryanodine binding. Experiments were carried out as described in Figure 2 at 30 μM Ca^{2+} with (closed bars) and without (open bars) 10 μM FK506. The 100 % values for $RyR1_N$ and $RyR1_{MHS}$ were 0.013 and 0.13 in B/B_{max} respectively. Results are means $\pm$ S.E.M. ($n = 3$). $^*P < 0.05$.

for both SR preparations (Figure 3B). Taken together, these results indicate that the gain of the Ca^{2+}-dependent activation of [^{3}H]ryanodine binding is considerably increased in $RyR1_{MHS}$ without substantial alterations in sensitivity to the CICR ligands. Since the reduced gain of CICR activity is the sign of a suppressed Ca^{2+} channel [21,22], these results suggest that suppression is largely impaired in the $RyR1_{MHS}$.

Unaltered regulation by FKBP12 in $RyR1_{MHS}$

We showed previously that suppression of the RyR1 channel is exerted partly by FKBP12 and partly by some mechanisms involving the interdomain interaction [21,22]. FKBP12 is thought to stabilize a closed state of the RyR channel [30] and accounts for $\sim$30 % of the suppression [21]. Therefore we investigated regulation of $RyR1_N$ and $RyR1_{MHS}$ by FKBP12. As shown in Figure 4(A), comparable amounts of FKBP12 were co-precipitated with $RyR1_N$ and $RyR1_{MHS}$, indicating that the $RyR1_{MHS}$ retains capability to bind FKBP12. Furthermore, 10 μM FK506, which dissociated the bound FKBP12 from RyR1 (Figure 4A), increased the [^{3}H]ryanodine binding of $RyR1_N$ and $RyR1_{MHS}$ to almost the same extent (1.9- and 1.8-fold for $RyR1_N$ and $RyR1_{MHS}$ respectively) (Figure 4B). Thus we can exclude the possibility that the FKBP12-mediated regulation might be involved in the channel stimulation seen in the $RyR1_{MHS}$ pigs.

Differential effects of domain peptides on $RyR1_N$ and $RyR1_{MHS}$

The involvement of the interdomain interaction in the impaired suppression of $RyR1_{MHS}$ was examined using the domain peptide approach [14,22]. In the interdomain interaction hypothesis, the two domains (regions 1 and 2) of RyR1 should interact with each other to stabilize the closed state of the channel, and MH mutation in either domain should weaken the interaction, resulting in activation of the Ca^{2+} release channel. Similar activation can be produced by an exogenous domain peptide (e.g. DP4) which is a part of the mating domain and is reasonably hypothesized

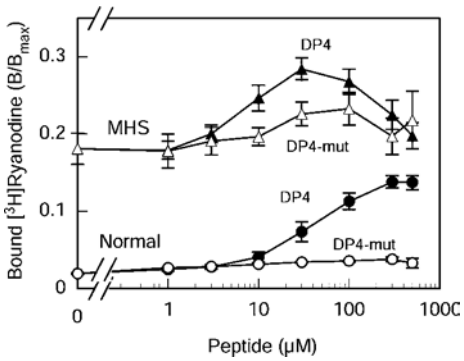

Figure 5 Effects of DP4 and DP4-mut on [^{3}H]ryanodine binding to RyR1$_N$ and RyR1$_{MHS}$

Experiments were carried out as described in Figure 2 with 0–500 μM DP4 (closed symbols) or DP4-mut (open symbols) at 30 μM free Ca^{2+}. Circles, RyR1$_N$; triangles, RyR1$_{MHS}$. Note that DP4 greatly activated RyR1$_N$ in a concentration-dependent manner, whereas it produced only small activation of RyR1$_{MHS}$. DP4-mut had little activating effects. Results are means $\pm$ S.E.M. ($n = 3$–4).

to competitively interfere with the interdomain interaction [13]. Thus, if the impaired suppression seen in the RyR1$_{MHS}$ is caused by the weakened interdomain interaction, we expect that the addition of such domain peptides will produce significant activation in RyR1$_N$, but will produce little or no activation in RyR1$_{MHS}$, because the interaction has already been weakened in the latter. The most effective way to test the physiological significance and specificity of the domain peptide is to introduce an MH mutation into the peptide (e.g. DP4-mut). The mutation should weaken or abolish the ability of the peptide to interfere with the interdomain interaction (cf. the hypothesis), and the mutated peptide has little or no activation effect [13].

The results of this test are shown in Figure 5. As can be seen, DP4 increased the [^{3}H]ryanodine binding to RyR1$_N$ in a concentration-dependent manner and reached a plateau at approx. 300 μM with the maximal enhancement of 7-fold (from 0.019 ± 0.002 to 0.14 ± 0.02; $n = 4$). DP4-mut produced only slight activation (1.9-fold, from 0.019 ± 0.002 to 0.036 ± 0.003; $n = 3$). These results are consistent with previous reports with rabbit [14] and bovine [22] skeletal muscle SR vesicles. However, the addition of DP4 to RyR1$_{MHS}$ produced only a very small increase of the ryanodine-binding activity with a peak value of 1.6-fold (from 0.18 ± 0.02 to 0.28 ± 0.03 at 30 μM; $n = 4$), which is comparable with a negligible extent of activation by DP4-mut (1.3-fold, from 0.18 ± 0.02 to 0.23 ± 0.02 at 100 μM; $n = 3$). Thus DP4 had only a little additional effect on RyR1$_{MHS}$, in which the suppression had already been removed.

Effects of domain peptides on single Ca^{2+} release channel currents of normal and MHS RyR1 channels

Single Ca^{2+} channel currents through the RyR1 were recorded in symmetrical solutions containing 250 mM caesium methanesulfonate, 20 mM Hepes/Tris, pH 6.8, and 30 μM free Ca^{2+} at a holding potential of -40 mV (Figure 6). The RyR1$_N$ channel displayed low P_o under control conditions and DP4 increased P_o in a concentration-dependent manner; the P_o value at 30 μM DP4 was approx. 6-fold higher than the control level (Figure 6A, left). In contrast, the P_o of the RyR1$_{MHS}$ channel was already 7.4-fold higher than the control level of RyR1$_N$ even in the absence of DP4, and addition of DP4 (10 and 30 μM) produced virtually no further increase in P_o (Figure 6A, right). A summary of these experiments is presented in Figure 6(B). The RyR1$_{MHS}$

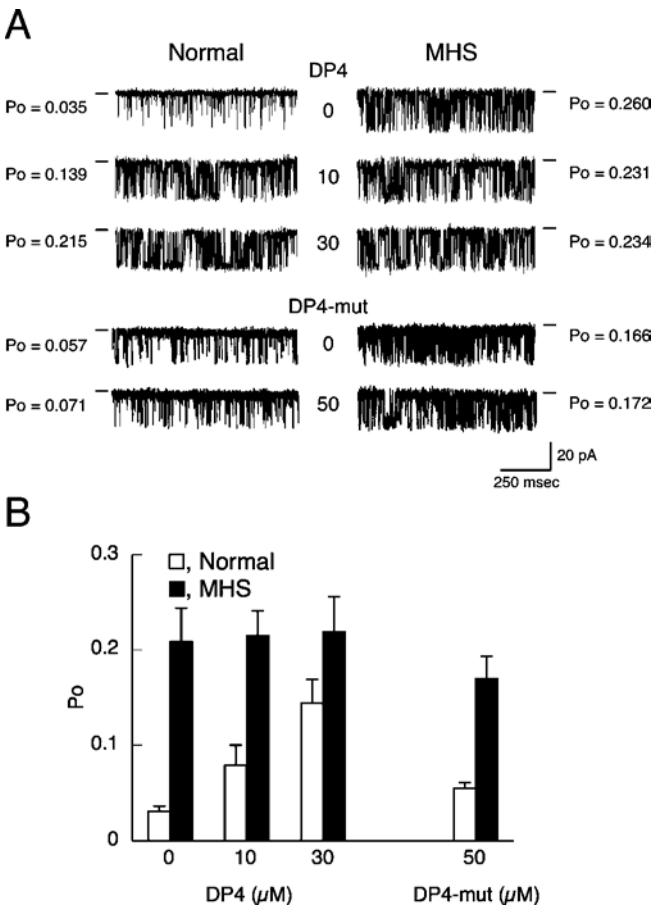

Figure 6 Single Ca^{2+} release channel currents through RyR1$_N$ and RyR1$_{MHS}$ and the effect of DP4

Single Ca^{2+} channel currents through RyR1$_N$ and RyR1$_{MHS}$ were recorded at a holding potential of -40 mV (*cis*) in a symmetrical solution containing 250 mM caesium methanesulfonate buffered with 20 mM Hepes/Tris, pH 6.8, at 30 μM Ca^{2+}. (**A**) Representative traces of the current recordings through RyR1$_N$ (left-hand panels) and RyR1$_{MHS}$ (right-hand panels) channels in the presence and absence of DP4 (upper six panels) or DP4-mut (lower four panels). The conductance level of the closed channel is shown by a short line on the side of each current recording. (**B**) Summary of the P_o data collected in the presence of 0–30 μM DP4 or 50 μM DP4-mut. Results are means $\pm$ S.E.M. ($n = 3$–10).

channel showed nearly 7-fold higher P_o (0.20 ± 0.03; $n = 10$) than RyR1$_N$ channel (0.030 ± 0.006; $n = 10$) in the absence of DP4, but the difference became smaller in the presence of 30 μM DP4 ($P_o = 0.14 \pm 0.03$ and 0.22 ± 0.04 for RyR1$_N$ and RyR1$_{MHS}$ respectively; $n = 4$). DP4-mut (50 μM) produced no activating effect on either RyR1$_N$ ($P_o = 0.055 \pm 0.011$; $n = 5$) or RyR1$_{MHS}$ ($P_o = 0.17 \pm 0.02$; $n = 5$) (Figure 6).

Effects of domain peptides on caffeine-induced Ca^{2+} release from normal and MHS SR vesicles

Ca^{2+} release from the isolated SR vesicles was fluorimetrically monitored using fura 2 as a fluorescent Ca^{2+} probe [22]. The SR vesicles were actively loaded with Ca^{2+} using MgATP in a solution containing 0.17 M KCl and 1 mM free Mg^{2+}, and Ca^{2+} release was induced by caffeine. With normal SR vesicles, 5 mM caffeine induced a small Ca^{2+} release (Figure 7A, left-hand panel). Addition of 30 μM DP4 at the onset of Ca^{2+} loading greatly increased the subsequent Ca^{2+} release. With MHS SR vesicles, in contrast, Ca^{2+} release by 5 mM caffeine was greater than that with normal SR, but was not increased substantially

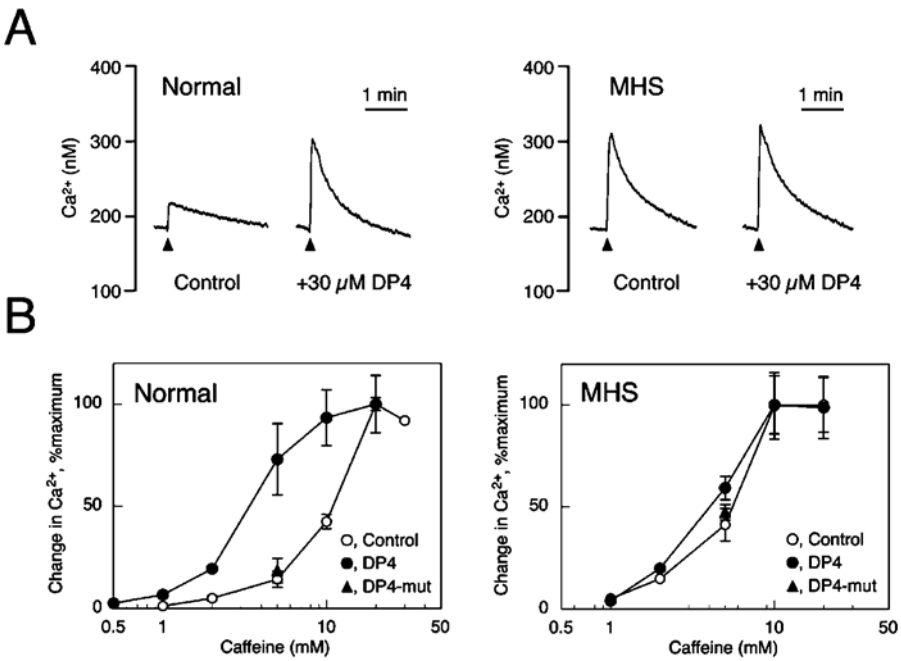

Figure 7 Caffeine-induced Ca^{2+} release from the isolated SR vesicles and the effect of DP4

Ca^{2+} release from the SR vesicles was fluorimetrically determined using fura 2 in a solution containing 0.17 M KCl, 20 mM Mopso, pH 6.8, and 1 mM $MgCl_2$. The vesicles were actively loaded with Ca^{2+} by addition of 100 μM MgATP and 20 μM $CaCl_2$, and then Ca^{2+} release was triggered by caffeine. (**A**) Representative traces of Ca^{2+} release induced by 5 mM caffeine (arrowhead) with (+30 μM DP4) or without added peptide (Control). DP4 was added to the solution just before the start of Ca^{2+} loading. Left, normal SR; right, MHS SR. (**B**) Caffeine-concentration-dependence of Ca^{2+} release in the presence (●) and absence (○) of 30 μM DP4. Left, normal SR; right, MHS SR. The change in Ca^{2+} concentration was normalized at the maximal values at each condition; 100 % denotes 240, 180, 240 and 150 nM for Normal-Control, Normal-DP4, MHS-Control and MHS-DP4 respectively. Results are means ± S.E.M. ($n = 3$–6). Note that DP4 sensitized the normal SR to caffeine in releasing Ca^{2+}, whereas it produced no sensitizing effect on the MHS SR. DP4-mut (30 μM) produced no effects on either SR (▲).

by 30 μM DP4 (Figure 7A, right-hand panel). The Ca^{2+} release data obtained at various concentrations of caffeine are depicted in Figure 7(B). DP4 increased the apparent affinity of the normal SR to caffeine; the EC_{50} of caffeine was changed from ~10 mM to ~3 mM using 30 μM DP4 (Figure 7B, left-hand panel). This is consistent with the results from bovine SR vesicles [22]. In contrast, 30 μM DP4-mut had no effect on the caffeine-induced Ca^{2+} release (Figure 7B). In the MHS SR, the sensitivity of Ca^{2+} release to caffeine was much higher (EC_{50} ~5 mM) than the normal SR, in agreement with a previous report [10] (Figure 7B, right-hand panel). The sensitivity, however, remained virtually unchanged by the addition of 30 μM DP4 or 30 μM DP4-mut.

DISCUSSION

Our previous studies demonstrated that Ca^{2+}-dependent [^{3}H]ryanodine binding was much lower in RyR1 than in RyR3 in the SR without change in their Ca^{2+} dependences, which was referred to as suppression [20–22]. This finding would be along the same lines as the effect of an adenine nucleotide on CICR, where the agent increased the rate of CICR at any given Ca^{2+} concentration without major change in the Ca^{2+} sensitivity [29]. These findings indicate that occupation by Ca^{2+} of the Ca^{2+} sites may be a necessary but not a sufficient condition for Ca^{2+} release, and necessitate the introduction of the concept of gain or 'attenuating coefficient' besides the occupation of the Ca^{2+} sites. We proposed a hypothesis that the gain or the attenuating coefficient is mainly regulated by the interdomain interaction between region 1 and region 2 within RyR 1 [30]. Therefore we could paraphrase the previous results [30–32] as follows: the attenuating coefficient for RyR1 in the SR membrane is 1/7–1/8, whereas it is unity for RyR3. Using MHS pigs with the $Arg^{615} \rightarrow Cys$ mutation in RyR1 in the present study, we showed that the attenuating factor was near to unity with the mutated RyR1 in the SR (Figure 2). We advanced further our hypothesis that aberration in the interdomain interaction may be the main causes of channel dysfunctions seen in RyR1-linked muscle diseases such as MH.

Normal suppression is impaired in the RyR1 channels of the MHS pig model

Extensive studies have been carried out on the MHS pig model, and a considerable amount of information has been accumulated in the literature concerning the altered properties of RyR1 in MH. It is widely recognized that there is a massive increase in the CICR activity in the $RyR1_{MHS}$ channel, as shown using various types of assay, such as Ca^{2+} release experiments with skinned fibres [33] or isolated SR vesicles [34], [^{3}H]ryanodine binding assay [31,32] and single-channel recordings [35] (for a review, see also [10]). These results may be consistent with the hypothesis that defectiveness in the channel suppression mechanism is a causative mechanism of abnormal activation of the $RyR1_{MHS}$ channel. Some results, however, claimed a different mechanism: a reduced sensitivity to Ca^{2+}/Mg^{2+} inhibition. This hypothesis was based on the finding that $RyR1_{MHS}$ is less sensitive to Ca^{2+} inactivation or Mg^{2+} inhibition than is $RyR1_N$ [36–40]. The extent of reduction in the Mg^{2+}-sensitivity was in the range 1.5–3-fold in these studies. We also found in the present study that there is a 2-fold reduction in the apparent affinity for Ca^{2+} of the Ca^{2+}-inactivation site (Figure 2B) and for Mg^{2+} of the Ca^{2+}-activation site (Figure 3A). However, the magnitude of the change in the

Ca^{2+}- or Mg^{2+}-sensitivity is too small to explain the increase in the CICR (8-fold) (Figure 2A). Thus it appears that the reduction in Ca^{2+}/Mg^{2+}-sensitivity may contribute little to the development of the pig MH phenotype.

However, there are controversial reports that no such increase in the CICR activity was observed in $RyR1_{MHS}$ channels of the pig model [37,40,41]. This reported claim is at least partly due to a higher pH used in these studies, as mentioned below. The dramatic effect of pH on normal and MHS RyR1 channels was demonstrated by Shomer et al. [35,41]. For instance, single $RyR1_{MHS}$ channels showed much larger P_o ($\sim$0.2) at optimum Ca^{2+} concentrations ($\sim$10 μM) than the $RyR1_N$ channel ($P_o = \sim 0.05$) at pH 6.8, whereas there was virtually no difference between the normal and MHS channels at pH 7.4 ($P_o = 0.4$ for both channels), indicating that the higher pH preferentially activated $RyR1_N$. Balog et al. [40] reported almost the same peak [^{3}H]ryanodine binding activity at pH 7.4, which contrasts with the previously reported results showing a significant difference between the normal and MHS channels at pH 7.0 [33,34] and the present data at pH 6.8. Thus it seems that higher pH (e.g. pH 7.4) preferentially activates the $RyR1_N$ channels; consequently the difference in the CICR activity between $RyR1_N$ and $RyR1_{MHS}$ channels becomes undetectable. We have found that such an alkaline pH as pH 7.4 lessened the magnitude of the suppression (T. Murayama, unpublished work). Considering that the intracellular pH in skeletal muscle is estimated to be $\sim$7.0 [42,43] and that metabolic acidosis precedes the muscle contracture in the episode of MH [10], it is quite likely that $RyR1_{MHS}$ channels are in the non-suppressed state unlike the suppressed $RyR1_N$. The impaired suppression mechanism in the $RyR1_{MHS}$ channels will then cause erroneously enhanced CICR and cause dysfunction of Ca^{2+} homoeostasis in MHS pigs.

In human MH, more than 80 mutations have been found in the *RyR1* gene [8,9]. An important question is then whether the conclusions deduced here from the particular pig MH mutation can be applied to the other MH mutations. With biopsied muscle fibres, Endo and colleagues [44,45] showed an abnormal enhancement of the CICR activity in MH patients at all of the Ca^{2+} concentrations examined. A similar observation was made in the [^{3}H]ryanodine binding assay with SR vesicles from a MH patient carrying a $Gly^{2434} \rightarrow Arg$ mutation [46]. Thus it appears that the impaired channel suppression is a general mechanism for the pig and human MH phenotype. Various studies of human MH mutations using cultured cells expressing the mutated RyR1 [12,47,48] have shown the increased sensitivity of MH mutants to caffeine or halothane. Yang et al. [12], in particular, reported that RyR1 channels carrying six human MH mutations show reduced Ca^{2+} inactivation and Mg^{2+} inhibition of [^{3}H]ryanodine binding, as also observed in the present study. Unfortunately, only a limited amount of quantitative data are available about the extent of suppression of the channel and the CICR activity of these expressed mutants, probably owing to the limitation in the quantity of the expressed protein. However, the similarity in the phenotype of these expressed mutants suggests that all of these MH mutations share a common mechanism involved in their pathogenic process.

Mechanism of impaired suppression of the $RyR1_{MHS}$ channel

We have shown recently that the 'suppression of CICR gain' is controlled by two independent factors: the RyR1-bound FKBP12 and the tight interdomain interaction within RyR1; the latter mechanism accounts for $\sim$70% of the suppression [21,22]. In the present study, there was no appreciable change in the bound FKBP12 and its regulation in MHS pig skeletal muscle SR (Figure 4). This excludes the possibility that FKBP12 might be one of the major factors involved in the channel suppression mechanism. On the other hand, there is a clear difference in the effect of DP4 on $RyR1_N$ and $RyR1_{MHS}$ (Figures 5–7), indicative of the major contribution of the altered interdomain interaction to the mechanism of the impaired suppression in the $RyR1_{MHS}$ channel.

An increasing body of evidence supports the interdomain interaction hypothesis that the two domains of RyR1 harbouring many of the reported MH mutations, the N-terminal domain (region 1) and the central domain (region 2), interact with each other, and that tight interaction stabilizes the closed state of the Ca^{2+} channel and weakened interaction destabilizes the channel. Domain peptides that bind specifically to these domains produced channel destabilization effects as shown in several reports [14–17,22]. For example, DP4 that corresponds to the Leu^{2442}–Pro^{2477} segment of region 2, binds with the N-terminal portion of region 1 [18]. This causes local conformational changes in RyR1 [19], unzips the interaction between regions 1 and 2 [19] and activates the Ca^{2+} release channel [15,16,22,49]. Furthermore, an antibody raised against DP4 was found to produce the same effects as DP4 (domain unzipping and channel activation) by reacting with its epitope located in region 2 [17]. These findings strongly support the hypothesis that the RyR Ca^{2+} channels are regulated in fact by mediation of the interaction between region 1 and region 2, and channel activation (i.e. removal of suppression or impaired suppression) by DP4 is caused by the effect of the peptide to interfere with the domain–domain interaction.

According to the interdomain-interaction hypothesis, MH mutation in either of these domains will weaken the interdomain interaction and will destabilize the channel. In fact, DP4-mut, a peptide carrying an $Arg^{2458} \rightarrow Cys$ MH mutation in DP4, produced no activating effect on the RyR channel, presumably due to the reduced or lost ability to interfere with the domain–domain interaction [14,19]. However, this hypothesis has not yet been tested using an actual disease model. In the present study, we demonstrated that DP4 greatly activated $RyR1_N$, but produced a little or no activation of $RyR1_{MHS}$, as shown in the [^{3}H]ryanodine binding assay (Figure 5), single-channel recordings (Figure 6) and Ca^{2+} release measurements (Figure 7). These findings well fit the hypothesis that the $RyR1_{MHS}$ channel is already activated by the weakened interdomain interaction. Furthermore, the level activated by DP4 in $RyR1_N$ was comparable with that in $RyR1_{MHS}$ without DP4, indicating that addition of DP4 to $RyR1_N$ could experimentally mimic the phenotype of $RyR1_{MHS}$. The results of the present study provide the first direct evidence that the weakened interdomain interaction induced by a mutation in region 1 is the primary cause for the impaired suppression in MH.

It should also be noted that the effectiveness of DP4 varied with the type of measurements: more potent on single-channel recordings than in the [^{3}H]ryanodine binding assay. The reason for this remains unclear, but it is suggested that the accessibility of the peptide to its reactive site may have varied depending on the assay conditions.

It is of interest to know whether affected interdomain interaction can explain the human MH phenotype. An $Arg^{614} \rightarrow Cys$ mutation, equivalent to $Arg^{615} \rightarrow Cys$ in MHS pigs, has also been found in humans [8]. A recent study with the domain peptides demonstrates that MH mutations within the DP4 peptide ($Ile^{2453} \rightarrow Thr$, $Arg^{2454} \rightarrow Cys$, $Arg^{2458} \rightarrow Cys$ and $Arg^{2458} \rightarrow His$) reduce its activating effect on the RyR1 channels [50]. Thus the affected interdomain interaction might also be applicable to some human MH mutations. Several human MH mutations have also been found in the C-terminus region (region 3), where many mutations for CCD (central core disease) as well as MH

are reported [51]. We tentatively propose that the information concerning the changes in the mode of interaction between region 1 and region 2 (e.g. weakening of the interaction) is transmitted to the transmembrane channel-forming segments by mediation of some regulatory domains, such as region 3. Both the functional and structural studies of the RyR1 channels with different MH mutations will help us to understand better the mechanism of dysfunction of Ca^{2+} release and the postulated involvement of the affected interdomain interaction in the mechanism of pathogenesis of human MH.

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