Malignant hyperthermia mutation sites in the Leu^{2442}–Pro^{2477} (DP4) region of RyR1 (ryanodine receptor 1) are clustered in a structurally and functionally definable area

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To explain the mechanism of pathogenesis of channel disorder in MH (malignant hyperthermia), we have proposed a model in which tight interactions between the N-terminal and central domains of RyR1 (ryanodine receptor 1) stabilize the closed state of the channel, but mutation in these domains weakens the inter-domain interaction and destabilizes the channel. DP4 (domain peptide 4), a peptide corresponding to residues Leu^{2442}–Pro^{2477} of the central domain, also weakens the domain interaction and produces MH-like channel destabilization, whereas an MH mutation (R2458C) in DP4 abolishes these effects. Thus DP4 and its mutants serve as excellent tools for structure–function studies. Other MH mutations have been reported in the literature involving three other amino acid residues in the DP4 region (Arg^{2452}, Ile^{2453} and Arg^{2454}). In the present paper we investigated the activity of several mutants of DP4 at these three residues. The ability to activate ryanodine binding or to effect Ca^{2+} release was severely diminished for each of the MH mutants. Other substitutions were less effective. Structural studies, using NMR analysis, revealed that the peptide has two α-helical regions. It is apparent that the MH mutations are clustered at the C-terminal end of the first helix. The data in the present paper indicates that mutation of residues in this region disrupts the interdomain interactions that stabilize the closed state of the channel.

Key words: domain–domain interaction, excitation–contraction coupling, malignant hyperthermia, ryanodine receptor.

INTRODUCTION

The RyR (ryanodine receptor) plays a central role in e–c (excitation–contraction) coupling in both skeletal and cardiac muscle. In the case of skeletal muscle-type e–c coupling, the voltage sensor-mediated activation signal received by the RyR is recognized by the Ca^{2+} pore located at the opposite end of the RyR by an as yet unidentified mechanism. As shown in cryo-electron microscope studies, there are appreciable differences in the three-dimensional image between non-activated and activated states of the RyR [1]. Presumably, a number of domains and sub-domains within the RyR are working in a co-ordinated manner to perform the necessary conformational control of RyR Ca^{2+} channels. Therefore in order to understand better the mechanism of channel regulation in normal and diseased states it is important to identify and characterize the domains involved in this conformational control.

In searching for these regulatory domains we, as well as other investigators, have paid particular attention to the fact that the reported sites of disease-linked mutations such as MH (malignant hyperthermia) and CCD (central core disease) are localized to three restricted regions of the RyR: the N-terminal, the central and the C-terminal regions. The vast majority of MH mutations are located in the N-terminal and central domains [2], whereas most mutations conferring susceptibility to CCD are located in the C-terminal region, especially within the transmembrane Ca^{2+} channel domain [3]. MH mutations in these domains cause aberrations in RyR1 channel function, such as hyper-activation of the channel by, and hyper-sensitization of the channel to, various physiological and pharmacological agonists, resulting in a leaky Ca^{2+} channel. This finding immediately suggests that these MH domains are involved in the conformational control of RyR Ca^{2+} channels. Based on several pieces of evidence, we have proposed a 'domain-switch' hypothesis. In the non-activated state, the N-terminal and central domains make close contact via several sub-domains; this 'zipped' state stabilizes the closed state of the channel. Under normal stimulating conditions, the inter-domain contact is weakened leading to an 'unzipped' state, which is recognized by the channel as an activation signal. MH mutation causes partial domain unzipping, resulting in a lowering of the energy barrier necessary for channel opening, causing the hyper-activation/hyper-sensitization effects observed in the diseased states.

In our recent studies we have tested this hypothesis using synthetic peptides corresponding to the putative critical MH domains [DPs (domain peptides)]. The underlying assumption in rationalizing the use of the DPs as functional probes is that they are capable of mimicking native conformations in the *in vitro* solution. For instance, DPy (DP matching sub-domain y) binds to its mating domain, sub-domain x, in competition with sub-domain y, resulting in domain unzipping [sub-domain x−DPy]/[sub-domain y] and consequently in channel activation, as if the domain unzipping would have been produced by a MH mutation in sub-domain y. An excellent test of the physiological relevance of the DP is to make an MH mutation within DPy (DPy-mut). Since sub-domain y-mut does not bind tightly with sub-domain x as described above,

Abbreviations used: BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid; CCD, central core disease; CTC, chlortetracycline; DP, domain peptide; DPy, DP matching sub-domain y; DTT, dithiothreitol; e–c, excitation–contraction; MH, malignant hyperthermia; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

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DPy-mut does not bind with sub-domain x either; consequently, a mutation made in DPy abolishes the activating function of DPy. One of our DPs, DP4, corresponding to the Leu^{2442}–Pro^{2477} region of the central domain of the RyR1 worked exactly as predicted from the above hypothesis. Thus it bound to the N-terminal domain [4], produced domain unzipping as determined by spectroscopic means [4,5], caused activation of ryanodine binding and SR (sarcoplasmic reticulum) Ca^{2+} release [6], enhanced force response to caffeine and to sub-maximal depolarization in mechanically skinned fibres [7], increased the frequency of Ca^{2+} sparks in saponin-permeabilized fibres [8] and increased open probability in single-channel conductance measurements [8]. Importantly, a point mutation made in DP4 mimicking the R2458C MH mutation almost completely abolished the activating function of DP4 in each of these assays.

Several other MH/CCD mutations have been reported in the DP4 region of RyR1 [9–13]. Interestingly, these reported mutations are clustered in a rather confined area of the peptide, suggesting the possibility that this region may play a critical role in inter-domain interaction. The aims of the present paper were to investigate the effect of each of these MH/CCD mutations and to gain more information on the domain structure critical for domain–domain interaction. In the present paper we show that the reported MH/CCD mutations have a severe impact on the function of DP4. In contrast, amino acid replacements made in RyR3-type DP4 (natural mutations) and designed mutations produced either no or modest impact on the function of DP4. The disposition of the critical residues in the structure of the peptide suggests the involvement of an α-helical structure in the inter-domain interaction.

EXPERIMENTAL

Membrane preparation

SR membrane vesicles were prepared from rabbit back and hind limb skeletal muscles (Pel-Freez) by differential centrifugation of muscle homogenates [14].

Peptide synthesis

Peptides were synthesized on an Applied Biosystems model 431A synthesizer, purified by reversed-phase high-pressure liquid chromatography and evaluated by MS.

Ca^{2+} release assay

MgATP (0.4 mM) was added to a stirred solution containing SR vesicles (0.2 mg/ml of protein), 0.15 M KCl and 20 mM Mops (pH 7.2). External Ca^{2+} was clamped at the required concentration using BAPTA [bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid]-calcium buffers. The time course of Ca^{2+} uptake was monitored in a PerkinElmer LS55 luminescence spectrometer using CTC (chlortetracycline, 40 μM; excitation 392 nm, emission 536 nm) as a luminal Ca^{2+} indicator. After Ca^{2+} uptake had reached a plateau, the appropriate agonist was added, and the resultant Ca^{2+} release was monitored. Changes in luminal Ca^{2+} are expressed as changes in the intensity of CTC fluorescence (in arbitrary units). In control experiments, SR vesicles were equilibrated with various concentrations of Ca^{2+} (from 0.1 mM to 1.0 mM) by overnight incubation at 4 °C. Immediately after dilution with BAPTA-calcium buffer (0.1 μM free Ca^{2+}), fluorescence intensity was determined. The CTC fluorescence was a linear function of Ca^{2+} up to 0.5 mM, including the range of luminal Ca^{2+} concentrations achieved in subsequent active loading experiments (0.3–0.5 mM). A CTC fluorescence change of 100 units corresponded to a change in the luminal Ca^{2+} concentration of 0.15 mM.

[^{3}H]Ryanodine binding assay

SR vesicular aliquots (50 μg of protein) were incubated with 10 nM [^{3}H]ryanodine (68.4 Ci/ml) and the desired concentration of peptide for 90 min at 37 °C in 100 μl of solution containing 150 mM KCl and 20 mM Mops (pH 7.2). Specific binding and non-specific binding (in the presence of 10 μM ryanodine) were each determined in duplicate. Binding reactions were terminated by rapid filtration onto GF/B filters (Whatman). Bound [^{3}H]ryanodine was then quantified in a liquid-scintillation counter.

NMR methods

DP4 was dissolved in 10 % ^2H_2O/90 % ^1H_2O to a final concentration of approx. 2 mM with 200 mM NaCl and adjusted to pH 5.0 with small additions of dilute HCl or NaOH. NMR spectroscopy and subsequent analyses were then performed as described previously in [15]. All spectra were acquired on a Bruker 800 MHz spectrometer using a spectral width of 8000 Hz, a 90° pulse width of 12.6 μs, collecting 2048 data points and 512 increments of 96 transients.

Structural modelling

Structural models of the peptides were created using the Accelrys Software Insight II and energy minimized using the program Discover, first by steepest descent and finally by the conjuage gradient method until the maximum derivative was less than 0.001 kcal/A (1 cal $\approx$ 4.184 J).

Statistical analysis

Data are reported as means $\pm$ S.E.M. for $n \geqslant 4$, or means $\pm$ S.D. for $n = 3$.

RESULTS

We tested three types of DP4 variants for their effects on the [^{3}H]ryanodine binding and Ca^{2+} release activities of the RyR1 Ca^{2+} channel. Six RyR1 mutations involving four amino acid residues have been reported in the DP4 region: R2452W (MH/CCD), I2453T (MH/CCD), R2454C (MH), R2454H (MH), R2458C (MH) and R2458H (MH). We made each of these mutations except for R2454H and examined their impact on the channel-activating function of DP4. DP4-type 3, matching the DP4 region of RyR3, shows amino acid replacement of as many as eight residues. This peptide, DP4-type 3 and its mutant R2458C, which mimics the R2458C mutant of DP4-type 1 have been tested as a control. We have designed three mutations at residues where MH/CCD mutations have not yet been reported, as another control, and we have also made an alternative mutation at the MH mutation site Arg^{2452}.

Figure 1(A) depicts the sequence of DP4 and the MH/CCD mutants. DP4 produced significant activation of the [^{3}H]ryanodine binding activity, and at near maximally activating concentrations of the peptide (e.g. 100 μM) the magnitude of activation reached approx. 400 % of control (Figure 1B). DP4(R2458C), the mutant peptide which we have used extensively as a negative control in our previous studies [6,16], and the other DP4 mutants (I2453T, R2454C and R2458H), produced negligible activation with peptide concentrations below 100 μM, although a further increase in the peptide concentration resulted in a rather steep increase in the activation curve. Interestingly, the R2452W mutant produced

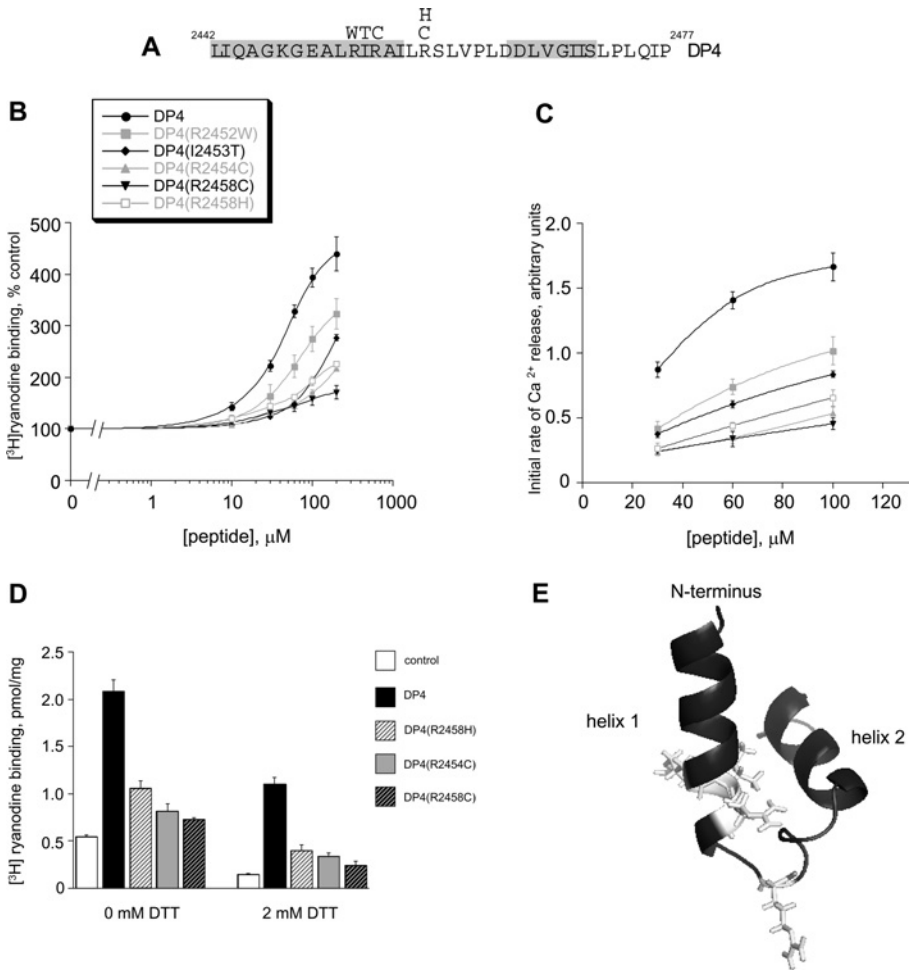

Figure 1 Effect of MH mutations on peptide function

(**A**) Sequence of DP4, corresponding to RyR1 residues 2442–2477. MH mutations are indicated above the sequence. Shaded boxes indicate helical regions (see **E**). All MH mutations made in DP4 had a severe impact on the channel-activating function of the wild-type DP4. (**B**) [^{3}H]Ryanodine binding versus [peptide], $n = 4$–13. (**C**) Initial rate of Ca^{2+} release, $n = 5$–17. (**D**) Effects of 100 μM wild-type DP4 and DP4 mutants on the [^{3}H]ryanodine binding activity in the absence and in the presence of 2 mM DTT, $n = 5$–13. (**E**) Structural model of DP4. The critical residues involved in MH mutations (labelled white with side chain) are clustered in the region encompassing the C-terminal end of helix 1 and the consecutive N-terminal end of the loop connecting helix 1 and helix 2.

appreciable activation in the concentration range below 100 μM, but this activation was still significantly smaller than wild-type DP4. Because of the solubility limits of the peptides, the binding curves do not extend beyond 200 μM. The lack of full saturation therefore limits quantitative analysis of the data. The curves for the more hydrophobic peptides such as DP4(R2452W) do appear to plateau, and this is in sharp contrast with the peptides with polar substitutions (particularly the isoleucine to threonine residue mutant). Although it is possible that some of the peptides are acting as partial agonists, we are reluctant to draw conclusions about the mechanism of action due to the poor aqueous solubility and the likelihood of non-specific effects at high peptide concentrations.

We followed the time course of SR Ca^{2+} release induced by DP4 and its mutants using CTC as a luminal Ca^{2+} indicator while clamping the extra-vesicular [Ca^{2+}] at 0.1 μM [16]. The initial rates of Ca^{2+} release determined from the release time course were plotted as a function of peptide concentration (Figure 1C). The general trend of the effects of these mutations is similar to that seen in the [^{3}H]ryanodine binding assay. Each of the reported MH/CCD mutations made in DP4 resulted in a severe loss of the channel activating functions of DP4, although the effect of one mutation, R2452W, was less severe compared with the rest of the group.

The most severe mutation, R2458C, introduced a cysteine residue into the peptide. To examine the possibility of dimerization of this cysteine mutant, we performed [^{3}H]ryanodine binding assays in the presence of 2 mM DTT (dithiothreitol) and compared the results with those obtained in the absence of added DTT. As shown in Figure 1(D), the ryanodine binding activity was diminished in the presence of DTT in all peptides tested, although the general pattern of the effects of these mutants remained unchanged, thus excluding the possible involvement of peptide aggregation in the critical effect of the arginine to cysteine residue mutations. An interesting finding in this experiment was that in the presence of 2 mM DTT the extent of channel activation by DP4 was approx. 750 % of control, which was almost twice as large as the value in the absence of DTT.

NMR techniques were used to examine the structure of the DP4 peptide. The mutant peptides were not assessed owing to poor solubility and possible aggregation at the concentrations required for NMR analysis. ^{1}H assignments were made using standard two-dimensional methods [17], i.e. a TOCSY [18] experiment to identify spin types and a NOESY [19] experiment to make sequence specific assignments. Short and medium range NOE (nuclear Overhauser effect) patterns suggest the presence of a weakly helical secondary structure in the DP4 peptide. The

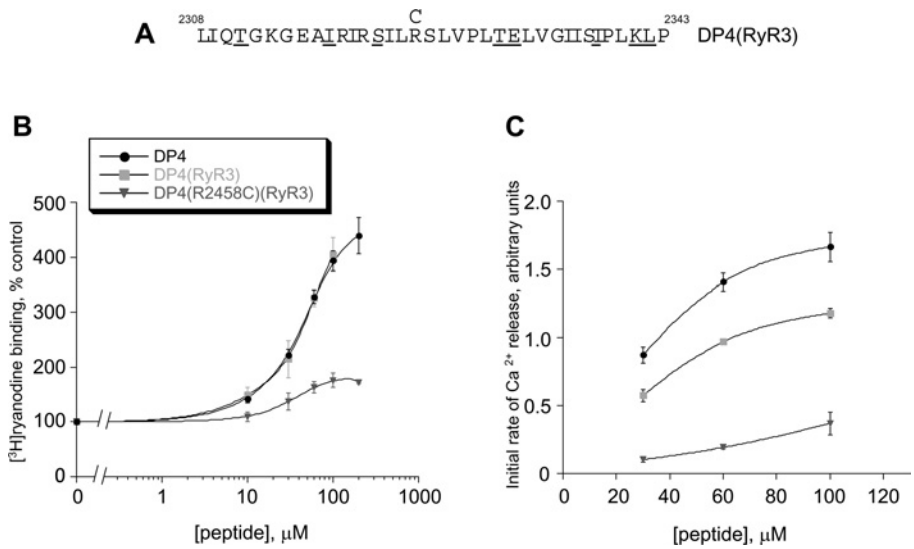

Figure 2 Effect of RyR type 3 peptides

(**A**) Sequence of DP4-type 3, corresponding to RyR3 residues 2308–2343. Residues different from the RyR1 isoform are underlined. The RyR1 MH mutation is indicated above the sequence. (**B**) DP4-type 3 shows a similar extent of activation of RyR1 as the DP4-type 1 in the [^{3}H]ryanodine binding assay, although as many as eight amino acid residues are different between the two isoforms of DP4. However, an arginine to cysteine residue mutation corresponding to the R2458C MH mutation of DP4-type 1 abolished the activating function of wild-type DP4-type 3 almost completely, $n = 3$–13. (**C**) The initial rate of Ca^{2+} release is lower for DP4-type 3, $n = 6$–17.

presence of strong and medium NH-NH connectivity patterns and weaker NHi-NHi + 2, αHi-NHi + 3 and αHi-βHi + 3 cross-peaks support the formation of a helix-like structure involving the first 15 amino acid residues (Lys^1–Ile^{15}, corresponding to Lys^{2442}–Ile^{2456} of RyR1). The NOE patterns were also suggestive of a short helix involving Asp^{24}–Ser^{30} (corresponding to Asp^{2465}–Ser^{2471} of RyR1). The remainder of the peptide was reasonably unstructured with possible short helices or turns between the two helical regions and at the C-terminus. No $^3J_{NH\text{-}H\alpha}$ coupling constants were determined due to spectral overlap and the chemical shift index data were inconclusive. Based upon the secondary structure information obtained from the NOE patterns, we created a possible structural model of DP4 (Figure 1E). This model consists of a 15-residue helix (helix 1) at the N-terminal of the peptide linked to a second shorter helical region (helix 2) by a length of random coil. The exact length of these helices and their orientation towards each other are estimates only.

DP4-type 3 has eight amino acid residues that differ from DP4-type 1 (Figure 2A). A previous study by Murayama et al. [20] suggests that DP4-type 3 is a potent activator of RyR1 as well as RyR3. In the [^{3}H]ryanodine binding experiment shown in Figure 2(B), we examined the concentration-dependence of activation of [^{3}H]ryanodine binding to the RyR1 by DP4-type 1 and DP4-type 3. Both DP4 isoforms showed similar activation profiles, suggesting that these eight amino acid residues represent 'neutral' residues of DP4, whose replacement with other amino acids produces less impact on the mechanism of channel activation, i.e. the ability to interact with its partner domain. In further support of the critical importance of the residues susceptible of MH/CCD mutations, the arginine to cysteine residue mutation made in DP4-type 3 (corresponding to the R2458C mutation in the DP4-type 1) abolished the activating function of DP4-type 3 almost completely [DP4(R2458C)(RyR3), Figure 2B]. In contrast, the initial rate of Ca^{2+} release by DP4-type 3 is significantly lower than that of DP4-type 1 (Figure 2C), indicating that activation by the type 3 variant is a somewhat lower-affinity process. As for the [^{3}H]ryanodine binding assay, the DP4-type 3 mutant is a very poor activator of the RyR.

It is evident that the neutral mutation sites are distributed throughout the entire region of DP4, whereas the critical mutation sites are clustered in a confined area from Ile^{2453} to Arg^{2458}. In order to test the impact of mutations at other residues in this hot spot, we made three designed mutations: I2456T, L2457V and V2461G (Figure 3A). The isoleucine to threonine residue and leucine to valine residue modifications represent possible/allowed MH mutations that result from substitution of a single base in a triplet codon, and have previously been reported at other sites in the RyR. The V2461G mutation has previously been demonstrated to increase channel open probability in RyRs incorporated in planar bilayers [21]. The [^{3}H]ryanodine binding data for these designed mutants are shown in Figure 3(B). L2457V, having a mutation at the residue adjacent to the most critical MH mutation site (Arg^{2458}), produced virtually no impact on the activating function of DP4. The other two mutants, I2456T and V2461G, had a moderate impact on the function of DP4, but much less severe than that produced by MH/CCD mutations (I2453T, R2454C, R2458C and R2458H). The results suggest that these designed mutations produce either no or modest effects. Interestingly for DP4(L2457V) and DP4(V2461G), the initial rates of release (Figure 3C) were markedly lower than the activation as measured by [^{3}H]ryanodine binding. It is worth noting that there are several differences in the experimental conditions used in the two assays. In particular, the binding experiments involve incubation for 90 min whereas the Ca^{2+} release experiments last only a few minutes. The structural data suggest that Leu^{2457} and Val^{2461} lie within a region of the peptide that is already flexible. This may make the region more sensitive to the temperature at which the experiments were conducted (37 °C for binding, 22 °C for Ca^{2+} release). An increase in temperature may allow the peptide to adopt an active conformation more quickly. The possibility of Ca^{2+}-dependence was excluded; we performed Ca^{2+} release experiments at 10 μM Ca^{2+} and found that release by the mutants

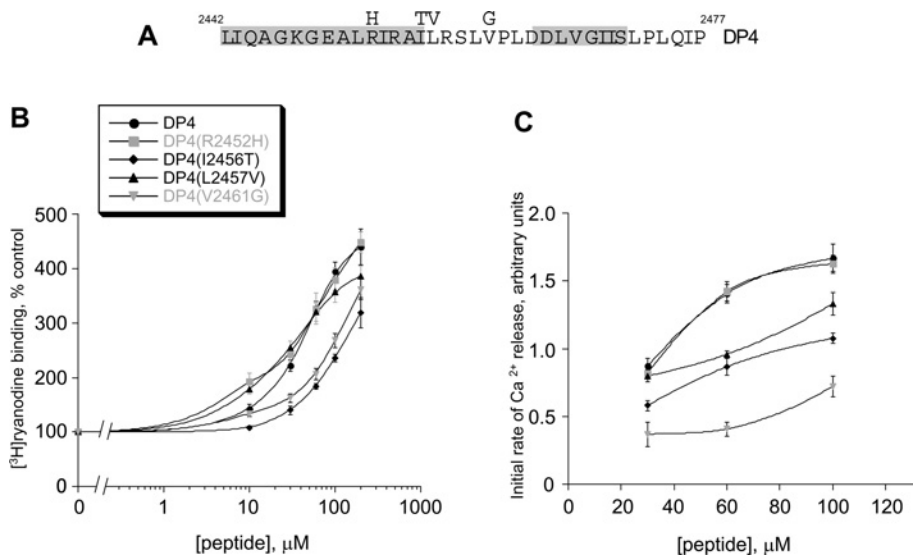

Figure 3 Effect of designed mutations on peptide function

(**A**) Sequence of DP4-type 1, with designed mutations indicated above the sequence. (**B**) The designed mutations I2456T, L2457V and V2461G made in the neighbourhood of the critical MH mutations produced less impact on the [^{3}H]ryanodine binding compared with the MH mutations reported in the literature, $n = 4$–13. (**C**) The designed mutation, V2461G produced a more severe impact on the Ca^{2+} release activity compared with [^{3}H]ryanodine binding. The mutation R2452H did not reduce the activity of the wild-type peptide, $n = 3$–17.

(relative to that of DP4) was equivalent at 10 and 0.1 μM Ca^{2+} (results not shown). On the other hand, the different effects on Ca^{2+} release-inducing ability and [^{3}H]ryanodine binding could imply a degree of uncoupling between channel opening and [^{3}H]ryanodine binding. Ryanodine is employed as a probe for the open state of the RyR, with binding assumed to occur only when the channel is open, but this may not necessarily always be the case (see also [22]). For example, channel opening may involve a sequence of changes in pore structure, of which exposure of the ryanodine binding site is only one. Thus the DPs could prove highly useful in a different context in analysing structure–function relationships in RyR gating.

We also examined the effects of making an alternative MH-like mutation at a known MH mutation site. Since R2452W was the least effective of the mutations, we substituted a histidine residue instead of a tryptophan residue. DP4(R2452H) was not so different from wild-type DP4 in Ca^{2+} release and [^{3}H]ryanodine binding assays, indicating that the arginine residue corresponding to residue 2452 is not required for the activating function of DP4, and that the reduced activation seen for the tryptophan mutant may be due to steric effects. An alternative explanation is that a protonated histidine residue side chain may mimic the positively charged arginine residue therefore maintaining the ionic interaction with its binding partner. Tryptophan on the other hand has only a non-charged indole proton to form this contact, resulting in a diminished interaction.

Table 1 summarizes the severity of the effect of these mutations on the DP4 activation of the [^{3}H]ryanodine binding and Ca^{2+} release activities: $1 - [A(DP4\text{-}mut) - A0]/[A(DP4) - A0]$, where A(DP) is the activity with peptides and A0 is the activity in the absence of peptide. It appears that we can categorize the tested mutations into three groups according to the severity of their effects: 'severe' being greater than a 50 % reduction in both [^{3}H]ryanodine binding and Ca^{2+} release activities; 'modest' being a reduction of less than 50 % in [^{3}H]ryanodine binding, but a substantial impact on the Ca^{2+} release activity; and 'negligible' having little or no impact on either [^{3}H]ryanodine binding or Ca^{2+} release activities. All MH/CCD mutations were categorized as 'severe', although one of the five mutations tested, R2452W, produced a lesser effect than the remainder of this group. The designed mutations we generated in the putative hot spot of the DP4 peptide were categorized as 'modest'. The eight amino acid replacements found naturally in the DP4 region of RyR3 produced little or no impact on the function of DP4. It is noteworthy that the majority of the mutations that fall into the most severe class involve arginine residues, suggesting that the interaction is governed by electrostatic forces. Mutations classed as 'modest', on the other hand, generally involve changes in hydrophobic residues. All critical MH/CCD mutation sites are localized in the region constituting the C-terminal end of helix 1 and the adjacent unstructured region, suggesting that this region plays a key role in domain–domain interaction within the RyR1.

Table 1 Summary of the effect of mutations on DP4 activation

*1 − [A(DP4-mut) − A0]/[A(DP4) − A0], where A(DP) is the activity with peptides (mutated or wild-type) and A0 is the activity in the absence of peptide.

DP4-mut (100 μM)	Loss of activation of [^{3}H]ryanodine binding*	Loss of activation of Ca^{2+} release*
R2452W (MH/CCD)	0.40	0.39
I2453T (MH/CCD)	0.69	0.50
R2454C (MH)	0.77	0.68
R2458C (MH)	0.80	0.73
R2458H (MH)	0.68	0.61
A2445T, L2451I, A2445S, D2464T, D2465E, L2472I, Q2475K, I2476L (type 3)	−0.04	0.29
R2458C (type 3)	0.74	0.78
R2452H (designed)	0.05	0.02
I2456T (designed)	0.53	0.35
L2457V (designed)	0.13	0.20
V2461G (designed)	0.43	0.56

DISCUSSION

Studies on the Ca^{2+} release properties of heterologously [23] or homologously [24] expressed RyR1 channels containing randomly selected MH mutations from the N-terminal and central domains demonstrated that these channels in fact display the same type of phenotype of RyR1 Ca^{2+} channels (hyper-activation and hyper-sensitization) as reported in the MH animal model [25] and human patients [26]. Importantly, it was found that the phenotype of the mutated channels was more or less identical for all MH mutations tested. This indicates that all mutations in either of these domains make an equivalent contribution to the production of the abnormal mode of channel regulation regardless of the position of mutation sites. Thus these expression studies not only confirmed that the diseased states of the channel are in fact caused by these mutations, but also suggested that the channel disorder may be caused by a somewhat global mechanism involving, for instance, domain or domains of the RyR, rather than specific residues or sites. The inter-domain interaction hypothesis we have described offers a simple and straightforward explanation for the general effects of mutations elucidated in the above studies with *in vivo* mutants. However, testing of this hypothesis under experimentally controllable conditions is not readily possible with the expressed mutant approach alone. The DP approach provides a considerable amount of new information about the role of the interaction between the N-terminal domain and the central domain in channel regulation, in both normal and diseased states. In particular, DP4, a DP corresponding to the Leu^{2442}–Pro^{2477} region of the central domain-bound with the 1–450 region of the N-terminal domain [4], thus unzipping the interacting N-terminal and central domains, as shown by the fluorescence quench technique [4,5], and activating the Ca^{2+} channel, as determined using different preparations ranging from a single molecule, the isolated SR, to the skinned or permeabilized muscle fibres [5–8]. Importantly, the activation effects of DP4 mimic the MH phenotype as illustrated by the following facts. First, DP4 produced MH-like hyper-activation of the channel of normal porcine [or normal RyR1 expressed in HEK (human embryonic kidney) cells], whereas it produced virtually no additional effect on the channel of MH-susceptible porcine (or expressed MH mutant RyR1) [27]. Secondly, dantrolene, a drug used for the treatment of MH patients, stabilized the zipped state of the interacting N-terminal and central domains, and prevented the abnormal channel activation from occurring [28]. Thirdly, a mutation made in DP4, mimicking the R2458C MH mutation [13], almost completely abolished all of the aforementioned activation effects of wild-type DP4 [5–8,16].

As it is clear from the principle of the DP probe strategy, wild-type DP mimics the MH-phenotype by causing domain unzipping, and the peptide mutant mimics the state of the corresponding *in vivo* domain where the mutation has occurred. The lack of effect of the mutated peptide corresponds to the reduced or lost ability of its corresponding mutated *in vivo* domain to interact with its mating domain. Thus we anticipated that thorough investigation of the structure and function of DP and its mutants would provide information about the molecular mechanism underlying critical domain–domain interactions.

A total of six MH/CCD mutations involving four residues are known in the DP4 region of the RyR1. One of the most important findings in the present paper is that each of these mutations made in DP4 produced severe loss of the ability of DP4 to activate [^{3}H]ryanodine binding and induce SR Ca^{2+} release, although there was a small variation in the severity of the effects among these mutants. This suggests that the loss of activating function in the DP4(R2458C) peptide we have used extensively in our recent studies as a negative control, is not a special case. Rather, all critical, disease-causing mutations share a common mechanism of pathogenesis, that is, the weakening or the loss of the ability of the peptide to mimic the *in vivo* domain–domain interaction. Numerous mutations must have occurred in 'healthy' RyRs and perhaps only the critical mutations amongst these have been identified in the MH/CCD patients. For this reason, it would be of great interest to investigate naturally occurring, healthy mutations. According to the recent study of Murayama et al. [20], DP4 matching the corresponding sequence of the RyR3, DP4-type 3, produced significant activation of RyR1 as well as RyR3, suggesting that this may provide us with a model of naturally occurring, healthy mutations. DP4-type 3 has eight amino acid replacements compared with the DP4-type 1. In agreement with Murayama et al. [20], DP4-type 3 produced significant activation of RyR1. In fact, the present [^{3}H]ryanodine binding data have shown that both isoforms of DP4 produced almost identical activation patterns. Importantly, a single R2458C RyR1-type MH mutation made in the DP4-type 3 virtually ablated the activating function of DP4-type 3, again in the same way as its effect on the RyR1 peptide. This finding suggests that the unzipping mechanism may be a common feature amongst all three RyR isoforms. The results obtained with the three designed non-MH/CCD mutations were somewhat complex, although intriguing. Since the critical MH/CCD mutations are clustered in a limited area of the peptide, we expected that some of the designed mutations might produce severe effects comparable with those of the critical mutations. Two (I2456T and V2461G) of the three produced severe damage on the Ca^{2+} release-inducing ability of DP4, but only modest effects on the [^{3}H]ryanodine binding activity. However, one (L2457V) of the three produced little effect on the [^{3}H]ryanodine binding activity, although this residue is located within the putative critical portion of the peptide.

The present NMR analysis has clearly indicated that DP4 has a defined three-dimensional structure that is characterized by helix 1–loop (turn)–helix 2. Since DP4 seems to retain several important functional properties ascribable to its corresponding *in vivo* domain as described above, it is not unreasonable to assume that the structure of the Leu^{2442}–Pro^{2477} region of RyR1, to which DP4 corresponds, has a similar or identical structure with that shown in the structural model of DP4. As shown in the model, the critical residues are located in a particular area in the helix-coil region. We tentatively propose that this portion plays a critical role in the domain–domain interaction.

We thank Dr Renne C. Lu, Dr Paul Leavis, David Schrier and Elizabeth Gowell (all from Boston Biomedical Research Institute, Watertown, MA, U.S.A.) for synthesis and purification of the peptides. This work was supported by NIH (National Institutes of Health) Grants AR 16922 from NIAMS (National Institute of Arthritis and Musculoskeletal and Skin Diseases) and HL072841 from NHLBI (National Heart, Lung and Blood Institute).

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Received 14 June 2006/25 July 2006; accepted 7 September 2006
Published as BJ Immediate Publication 7 September 2006, doi:10.1042/BJ20060902