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Antibody probe study of Ca^{2+} channel regulation by interdomain interaction within the ryanodine receptor

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N-terminal and central domains of ryanodine receptor 1 (RyR1), where many reported malignant hyperthermia (MH) mutations are localized, represent putative channel regulatory domains. Recent domain peptide (DP) probe studies led us to the hypothesis that these domains interact to stabilize the closed state of channel (zipping), while weakening of domain–domain interactions (unzipping) by mutation de-stabilizes the channel, making it leaky to Ca^{2+} or sensitive to the agonists of RyR1. As shown previously, DP1 (N-terminal domain peptide) and DP4 (central domain peptide) produced MH-like channel activation/sensitization effects, presumably by peptide binding to sites critical to stabilizing domain–domain interactions and resultant loss of conformational constraints. Here we report that polyclonal anti-DP1 and anti-DP4 antibodies also produce MH-like channel activation and sensitization effects as evidenced by about 4-fold enhancement of high affinity [^{3}H]ryanodine binding to RyR1 and by a significant left-shift of the concentration-dependence of activation of sarcoplasmic reticulum Ca^{2+} release by polylysine. Fluorescence quenching experiments demonstrate that the accessibility of a DP4-directed, conformationally sensitive fluorescence probe linked to the RyR1 N-terminal domain is increased in the presence of domain-specific antibodies, consistent with the view that these antibodies produce unzipping of interacting domains that are of hindered accessibility to the surrounding aqueous environment. Our results suggest that domain-specific antibody binding induces a conformational change resulting in channel activation, and are consistent with the hypothesis that interacting N-terminal and central domains are intimately involved in the regulation of RyR1 channel function.

Key words: domain–domain interaction, domain-specific antibody, excitation–contraction coupling, ryanodine receptor (RyR).

INTRODUCTION

In skeletal muscle-type e-c (excitation–contraction) coupling, muscle membrane depolarization is sensed by the dihydropyridine (DHP) receptor in the transverse tubule membrane, which then undergoes a conformational change that is associated with intra-protein charge movement [1–11]. In malignant hyperthermia (MH), the genetic sensitivity to volatile anaesthetics increases and Ca^{2+} release from the SR (sarcoplasmic reticulum) via RyR1 (ryanodine receptor 1) becomes uncontrolled, resulting in grave clinical consequences unless treated by dantrolene, which specifically inhibits SR Ca^{2+} release (for reviews, see [12–17]). In human MH, the syndrome is autosomal dominant with incomplete penetrance, and affected families have been documented to contain one of more than 50 mutations in RyR1 [13,18–27].

The exquisite control necessary for proper channel functioning requires that specific inter- and intra-protein domains transduce the necessary conformational controls during e-c coupling. In searching for such regulatory domains, we, as well as other investigators, have paid particular attention to the fact that the reported sites of MH mutations on RyR1 are not randomly distributed. Indeed, they are localized to three restricted regions: the N-terminal (Cys^{35}–Arg^{614}), central (Asp^{2129}–Arg^{2458}), and the C-terminal channel (Ile^{3916}–Ala^{4942}) domains [13,18–27]. The vast majority of MH mutations are located in the N-terminal and central domains of RyR1, while most mutations conferring susceptibility to central core disease (CCD), a rare myopathy also linked to RyR1, are located in the C-terminal channel domain [13]. The pathophysiological consequences of these MH mutations are aberrations in RyR1 Ca^{2+} channel activity, resulting in channel leakiness, hyper-activation and/or hyper-sensitization to agonists and triggering conditions. For example, muscle cells from MH-susceptible individuals have been shown to contain resting Ca^{2+} levels considerably greater than those from normal individuals, increased sensitivity of the Ca^{2+} release mechanism to electrical and pharmacological activation, and an increased open probability of RyR1 channels in single channel studies in lipid bilayers (for reviews, see [12–16]). These phenomena have been directly related to the mutations in RyR1, as experiments on the Ca^{2+} release properties of expressed RyR1 channels containing randomly selected MH mutations from the N-terminal and central domains demonstrated that these channels display similar properties of hyper-activation and hyper-sensitization [28,29]. To explain these results, we have proposed a model of channel regulation that involves inter-domain interactions between the N-terminal and central domains of RyR1 serving as a key mechanism for Ca^{2+} channel regulation [30–32].

The basic points of the hypothesis are schematically illustrated in Scheme 1. The model assumes that in the resting or non-activated state, the N-terminal and central domains make close

Abbreviations used: BAPTA, bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; Caps, 3-(cyclohexylamino)propane-1-sulphonic acid; DAB, diaminobenzidine; DHP, dihydropyridine; e-c coupling, excitation–contraction coupling; DP, domain peptide; MCA, methylcoumarinamide; MH, malignant hyperthermia; RyR, ryanodine receptor; SAED, sulphosuccinimidyl 2-(7-amino-4-methylcoumarin-3-acetamido)ethyl-1,3′-dithiopropionate; SR, sarcoplasmic reticulum.

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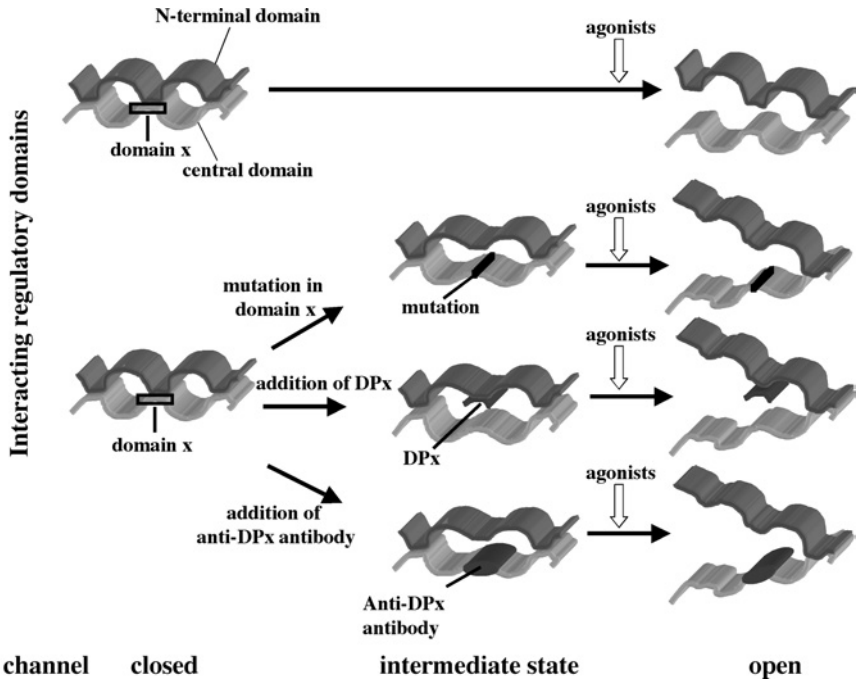

Scheme 1 Interactions between N-terminal and central domains of RyR are involved in Ca^{2+} channel regulation

A close interaction between the N-terminal and central domains of RyR1 (zipping) stabilizes the closed state of Ca^{2+} channel. Mutation in sub-domain x, addition of the peptide corresponding to sub-domain x (DPx) and addition of an antibody raised against sub-domain x, produce partial unzipping of the interacting domains, which result in hyper-activation and hyper-sensitization effects.

contact at several as yet undetermined sub-domains. The conformational constraints imparted by the 'zipped' configuration of these two domains stabilize and maintain the closed state of the Ca^{2+} channel. The model proposes that stimulation via e-c coupling or pharmacological agents weakens these critical inter-domain contacts, resulting in loss of conformational constraints, thereby lowering the energy barrier for Ca^{2+} channel opening. Weakening of these inter-domain interactions may also occur via mutation (as in MH) or with the use of synthetic domain peptides, as we have recently shown [30–34]. For instance DP4, the domain peptide corresponding to Leu^{2442}–Pro^{2477} of the central MH region of RyR1, binds to the N-terminal region and produces MH-like effects on Ca^{2+} channel function and e-c coupling events [31]. Spectroscopic studies are consistent with the hypothesis that DP4 produces unzipping of interacting domains [31]. Significantly, introduction of a single amino acid substitution corresponding to an MH mutation in DP4 results in the peptide losing its channel-activating capacity. This supports the hypothesis that an MH mutation critically reduces the ability of either the native or synthetic sequence to interact with its mating domain. We have extended these results by demonstrating that the synthetic, N-terminal domain peptide, DP1, corresponding to Leu^{590}–Cys^{609} of RyR1, also produces MH-like hyper-activation/hyper-sensitization effects on RyR1 channels [30]. Furthermore, this peptide also contains the binding site for dantrolene, the drug that suppresses both normal and aberrant intracellular Ca^{2+} release, used to treat MH [36,37]. Surprisingly, DP1 is recognized by monoclonal antibody anti-RyR1 raised to native rabbit RyR1, and this antibody inhibits dantrolene binding to RyR1 [37]. These data present valuable evidence that synthetic domain peptides are capable of mimicking native conformations, and that experimental data obtained with them are physiologically relevant.

The above model predicts that antibodies directed against key sub-domains will disrupt the interaction of native paired domains and, consequently, sensitize the domain switch to activating stimuli. If antibodies raised against synthetic domain peptides are shown to have effects on RyR1 channel function similar to the parent antigen, this would be further evidence both for the conformational significance of the particular native domain to channel function, and for conformational mimicry of the synthetic domain peptide. In the present study, we tested this prediction by using two domain-specific polyclonal antibodies, anti-DP4 and anti-DP1, raised against their corresponding synthetic peptides. As will be shown below, both antibodies enhanced [^{3}H]ryanodine binding to SR in a concentration-dependent manner, and increased the sensitivity of SR Ca^{2+} release to the RyR1 agonist polylysine, indicating that these antibodies are capable of sensitizing RyR1 to activating stimuli in a manner consistent with Scheme 1. After introducing a site-directed conformationally sensitive fluorescent probe into RyR1, our fluorescence quenching studies showed that these antibodies do produce the domain 'unzipping' postulated above.

MATERIALS AND METHODS

Preparation of triad-enriched microsomes

Triad-enriched microsomal fractions were prepared from frozen rabbit back paraspinous and hind leg skeletal muscles (Pel-Freez Biologicals, Rogers, AR, U.S.A.) using a method of differential centrifugation as described previously [38]. Microsomes from the final centrifugation were homogenized in a sample solution containing 0.3 M sucrose, 0.15 M KCl, proteolytic enzyme inhibitors (0.1 mM PMSF, 1 μg/ml leupeptin, 2.0 μg/ml soybean trypsin inhibitor), 20 mM Mes, pH 6.8, to a final concentration of 20–30 mg/ml, frozen immediately in liquid N_2 and stored at -78 °C.

Domain peptides used and their synthesis

We used two domain peptides, DP1 and DP4, to raise the antibodies used in this study. We also used seven other RyR1 domain

Table 1 Domain peptides used in this study

Domain peptide	Sequence
DP9	W^{147}WTMHPASKQRSEGEKVRVGDDLILVSVSSE^{177}
DP3	D^{324}TAPKRDVEGMGPPEIKYGESLCFVQHV^{351}
DP7	N^{543}LDWVVSKLDRLEASSGILEVLYCVLIESPEVLN^{576}
DP1	L^{590}DKHGRNHKVLDVLCSLCVC^{609}
DP1-scramble	DCHHNVLDKLCKVGRLVLSC
DP6	L^{2038}LAHCGIQLEGEEEEPEEETSLSSRLRSLL^{2067}
DP5	V^{2149}EDTMSLLECLGQIRSLLIVQMGPQEENLMIQSIGNI^{2185}
DP8	G^{2407}EEPPEENRVHLGHAIMSFYAALIDLLGRCAPEMHLIQAGK^{2447}
DP4	L^{2442}IQAGKGEALRIRAILRSLVPLDDLVGIISLPLQIP^{2477}
DP4-scramble	ALDLPSPIGRALLQLGVIGDIEVPKSILRQIILALR
DP10	S^{2493}FVPDHKASMVLFLDRVYGIENQDFLLHVLDVGFLP^{2528}

peptides (as described in Table 1), corresponding to various regions of the RyR (DP9, DP3, DP7, DP6, DP5, DP8, and DP10), as controls in the ELISA assay.

The peptides were synthesized on an Applied Biosystems model 431A synthesizer employing Fmoc (fluoren-9-ylmethoxycarbonyl) as the α-amino protecting group. The peptides were cleaved and de-protected with 95% trifluoroacetic acid and purified by reversed-phase HPLC.

Preparation and characterization of anti-domain peptide (DP) antibodies

Polyclonal antibodies were raised commercially in rabbits using DP4 or DP1 as an antigen (Bio-Synthesis, Inc., Lewisville, TX, U.S.A.). Anti-DP1 and Anti-DP4 antibodies were purified by subjecting the immune serum to DP1- and DP4-affinity column chromatography in the following way. First, in order to prepare the DP-affinity column, 2 ml of 4% cross-linked beaded agarose (AminoLink Immobilization Kit, Pierce) was incubated with 2 ml solution containing 1–2 mg/ml of DP4 or DP1 in the presence of 0.1 M sodium cyanoborohydride ($NaCNBH_3$) with a rocking motion overnight at 22 °C. After washing the DP-affinity column with 1 M NaCl and then with PBS, 1.5 ml of immune serum was applied to the column. The effluent was collected and re-applied to the column. This procedure was repeated at least two more times in order to ensure maximum binding of anti-DP antibody to the column. The column was then washed with 12 ml of PBS. The column-bound antibody was then eluted with 8 ml of 0.1 M glycine buffer (pH 2.5) and 1 ml fractions were collected. In order to minimize the time of exposure of the antibody to acidic conditions, 50 μl of 1 M Tris buffer (pH 9.0) was added to the effluent collection tubes before glycine buffer elution, so that the pH of the eluted antibody solution was neutralized as soon as the eluted fraction was collected in the tube [39]. Eluted fractions were pooled and concentrated by centrifugation in Centricon 30 (Amicon, Danvers, MA, U.S.A.).

Antibody concentration was determined using a dot blotting technique. A series of dilutions were done for anti-DP1 and anti-DP4 immune sera, purified anti-DP1 and anti-DP4 antibodies, and pre-immune sera. Rabbit IgG of a known concentration was used as a standard and was also used to make the series of dilutions. A 1 μl portion of each diluted serum with rabbit IgG was blotted on the 0.45 μm nitrocellulose membrane (Osmonics, Minnetonka, MN, U.S.A.), which contained a grid. The membrane was dried and blocked with 5% non-fat dried milk for 1 h. Next, the membrane was washed with PBS and incubated in a peroxidase-conjugated anti-rabbit IgG solution (1000× dilution) for 3 h at 22 °C. The membrane was then washed again with PBS and the colour was developed in a PBS solution containing 0.05% Tween-20 (PBS/Tween solution), 0.8 mg/ml DAB (diaminobenzidine), and 0.012% H_2O_2. The IgG concentrations of the anti-DP immune sera, purified anti-DP antibodies, and pre-immune sera were determined by calibration with the standard rabbit IgG.

ELISA assay was used to determine the immuno-reactivity of anti-sera and purified antibodies. For this purpose, 0.25 nmol of DP-1 or DP4 was attached to each well of the MicroWell plate. After washing the plate with the PBS/Tween solution three times, the plate was treated with 5% non-fat dried milk (100 μl/well) on the shaker for 60 min, followed by washing three times with the PBS/Tween solution. Various concentrations of anti-DP sera, purified anti-DP antibodies, and pre-immune sera were added to the plate (50 μl/well), and incubated for 3 h at 22 °C under agitation on the shaker. After washing, 50 μl of peroxidase-conjugated anti-rabbit IgG solution (1000× dilution) was added to each well and incubated for 1 h at 22 °C. The plate was washed, followed by colour development with a solution containing 0.4 mg/ml OPD (*o*-phenylenediamine) and 0.013% H_2O_2. The colour development was stopped by adding 50 μl of 3 M H_2SO_4 to each well, and the colour developed (A_{450}) was measured using a micro-plate reader.

For dot blotting of various domain peptides with anti-DP1 and anti-DP4 antibodies, 250 pmol fractions of various peptides were added to nitrocellulose membranes (Osmonics Minnetonka, MN, U.S.A.). The membranes were then dried and incubated in a 5% non-fat milk solution for 1 h. For colour development of the blots, the membranes were treated with primary antibodies (anti-DP4 or anti-DP1 immune serum, 10 μg/ml IgG; purified anti-DP4 or anti-DP1 antibody, 0.2–0.4 μg/ml IgG) and peroxidase-conjugated anti-rabbit IgG, followed by washing with PBS and colour development with DAB.

[^{3}H]Ryanodine binding assay

Rabbit skeletal (0.5 mg/ml) microsomes were incubated in a 0.1 ml reaction solution containing 10 nM [^{3}H]ryanodine (the original stock solution was 1.786 pmol/ml, 68.4 Ci/ml; PerkinElmer Life Science, Norwalk, CT, U.S.A.), 0.15 M KCl, 10 μM $CaCl_2$, 20 mM Mops, pH 7.2, for 13–15 h at 22 °C in the presence of various concentrations of purified anti-DP antibodies and/or polylysine ($M_r = 14600$). Samples were filtered on to glass fibre filters (Whatman GF/A) and washed twice with 5 ml of distilled water. The filters were then placed in scintillation vials containing 10 ml of scintillation cocktail Ecoscint A and counted in a Beckman LS 3801 counter. Specific binding was calculated as the difference between the binding in the absence (total binding) and in the presence (nonspecific binding) of 10 μM non-radioactive ryanodine. Assays were carried out in duplicate and each datum point was obtained by averaging the duplicates [40].

Assays of Ca^{2+} release

Rabbit skeletal microsomes (0.2 mg/ml) were incubated in 1 ml of solution containing 0.15 M KCl, 2.0 μM fluo-3, 20 mM Mes, pH 6.8. Ca^{2+} uptake reaction was initiated by the addition of 1 mM MgATP into the cuvette, and the time course of Ca^{2+} uptake was monitored in a spectrophotometer (Luminescence Spectrometer, LS50B, PerkinElmer) using fluo-3 as a Ca^{2+} indicator (excitation at 488 nm, emission at 525 nm), as described previously [41]. In control experiments, after Ca^{2+} uptake had reached a plateau, various concentrations of polylysine ($M_r = 14600$) were added, and the resultant Ca^{2+} release was monitored. To examine the effect of antibodies on Ca^{2+} release, after Ca^{2+} uptake had reached a plateau, 10 μg/ml anti-DP4 or anti-DP1

antibody were added. Approx. 1 min after the addition of antibody various concentrations of polylysine ($M_r = 14600$) were added, and the resultant Ca^{2+} release was monitored. The amounts of Ca^{2+} released were plotted as a function of the concentration of polylysine added.

Spectroscopic monitoring of domain unzipping

Site-specific fluorescent labelling of the DP binding sites on RyR1 in skeletal muscle triads was performed using the cleavable heterobifunctional cross-linking reagent, SAED [sulphosuccinimidyl 2-(7-amino-4-methylcoumarin-3-acetamido)ethyl-1,3′-dithiopropionate] [42] as follows. Peptide–SAED conjugate was formed by incubating 0.5 mM DP4 with 0.5 mM SAED in 20 mM Hepes (pH 7.5) for 60 min at 22 °C in the dark. The reaction was quenched by 20 mM lysine. Free SAED was removed using Sephadex G15 gel filtration. DP4–SAED conjugate retained essentially the same activities as the unmodified peptide. The peptide–SAED conjugate (5 μM final concentration) was mixed with 2 mg/ml triad protein in sample solution (see above) containing 1 mM BAPTA [bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid]/calcium buffer (1.0 μM free Ca^{2+}) in the dark for 1 min, and photolysed with UV light in a Pyrex tube placed at 5 cm distance from the light source, at 4 °C for 2 min. 2-Mercaptoethanol was added (100 mM, final concentration) to cleave the disulphide bond of SAED. After incubation on ice for 1 h, the mixture was centrifuged at 100000 ***g*** for 15 min, and the sedimented vesicles were resuspended in the sample solution to a final protein concentration of approx. 20 mg/ml.

To make a macromolecular collisional quencher, the quenching reagent, QSY® 7 carboxylic acid ($M_r = 791.32$), was conjugated to BSA by incubating 5 mM QSY® 7 carboxylic acid with 0.5 mM BSA in 20 mM Hepes (pH 7.5) for 60 min at 22 °C in the dark. Unreacted QSY® 7 carboxylic acid was removed by means of Sephadex G-50 gel filtration. Fluorescence quenching by both QSY® 7 carboxylic acid–BSA conjugate (a macromolecular quencher) and unconjugated QSY® (a small-size quencher) were performed by measuring steady state fluorescence of labelled MCA (methylcoumarinamide) (excitation at 348 nm, emission at 445 nm) in the presence or absence of 20 μg/ml anti-DP4 or anti-DP1 antibody (Luminescence Spectrometer, LS50B, PerkinElmer). The data were analysed using the Stern–Volmer equation [43]

$$F_o/F = 1 + K[Q] \quad (1)$$

where F and F_o are fluorescence intensities in the presence and in the absence of added quencher; K, quenching constant, which is the measure of the accessibility of the protein-bound probe to the quencher; [Q], the concentration of quencher (in the present case, the concentration of QSY®).

RESULTS

Characterization of anti-DP4 and anti-DP1 antibodies

We carried out immunoblot testing of the reactivity of anti-DP4 and anti-DP1 immune sera and affinity-purified anti-DP4 and anti-DP1 antibodies against nine domain peptides corresponding to various regions of RyR1 listed in Table 1. Starting from the N-terminus, these are: DP9, DP3, DP7, DP1, DP6, DP5, DP8, DP4 and DP10. Both the anti-DP1 and andi-DP4 immune sera, and their respective purified antibody, reacted only with their corresponding antigens; namely DP1 and DP4 respectively (results not shown). Neither of these antibodies reacted with their corresponding peptides if the sequences were scrambled (see Materials and methods section). Pre-immune serum showed no reactivity with any of these domain peptides (results not shown). These results indicate that these antibodies recognize the specific sequence of their corresponding antigens.

We then sought to determine whether these antibodies recognized their target sequences in native RyR1 in Western blots. Figure 1(A) depicts Coomassie Blue and Western-blot staining patterns of skeletal muscle SR before and after digestion of RyR1 with calpain II. Calpain digestion cleaves the 565 kDa RyR1 monomer primarily between amino acids 1400 and 1401, yielding two fragments of approx. 410 kDa and 155 kDa [11,36,44]. Polyclonal anti-RyR1 antibody immuno-staining identified these three RyR1 bands before experimental digestion, indicating partial cleavage prior to addition of exogenous calpain II. Given the locations of epitopes of our synthetic domain peptides on RyR1 (DP1, Leu^{590}–Cys^{609}; DP4, Leu^{2442}–Pro^{2477}), we predicted that anti-DP1 would react with the RyR1 565 kDa monomer and the 155 kDa calpain fragment, and that anti-DP4 antibody would react with the RyR1 monomer and its 410 kDa calpain fragment.

Our results (Figure 1A) demonstrate that these predictions are experimentally validated. Anti-DP4 antibody reacted with the 565 kDa RyR1 monomer and with its 410 kDa fragment, while anti-DP1 reacted with the N-terminal, 155 kDa fragment, as well as its parent monomer. As suggested from the above results, anti-DP4 and anti-DP1 antibodies react with the native RyR1 domains from which DP4 and DP1 are derived.

For the sensitive *in vitro* assays described below, we affinity-purified the antibodies in each serum using the corresponding domain peptide as antigen, and the immunoreactivity of the purified antibodies towards these antigens was compared with parent serum in ELISA assays (Figure 1B). As can be seen, purification of anti-DP4 antibody resulted in a significant decrease in the half maximal concentration of IgG necessary for immuno-staining (0.33 ± 0.06 μg/ml) when compared with that of anti-DP4 immune serum (8.74 ± 3.32 μg/ml). These results indicate that affinity chromatography of these anti-sera yielded an approx. 27-fold ($P < 0.001$) purification for anti-DP4 antibody. Similar results were obtained with anti-DP1 antibody. Thus, the half maximal concentration of IgG necessary for immuno-staining was 0.34 ± 0.08 μg/ml with purified anti-DP1 antibody, and it was 9.22 ± 2.52 μg/ml with anti-DP1 immune serum, indicating an approx. 27-fold ($P < 0.001$) purification for anti-DP1 antibody. Pre-immune sera showed no appreciable reactivity towards DP4 and DP1.

Anti-DP4 and anti-DP1 antibodies produce MH-like hyper-activation and hyper-sensitization effects

We have postulated that the regions of RyR1 corresponding to DP1 and DP4 are involved in inter-domain stabilization of the resting state of the channel. If this is true, then antibodies directed at these domains should disrupt these conformationally critical interactions and produce hyper-activation and hyper-sensitization. Therefore, we examined the effects of anti-DP1 and anti-DP4 on measures of channel activation including [^{3}H]ryanodine binding and sensitivity of RyR1 to the agonist polylysine, which is a potent trigger of SR Ca^{2+} release.

We also show the effects of affinity-purified anti-DP antibodies on [^{3}H]ryanodine binding to skeletal muscle SR (see Figure 4). These results are compared with those obtained for pre-immune sera and non-specific IgG. As can be seen, purified antibody, but not pre-immune sera or non-specific IgG, produced significant, enhancement of [^{3}H]ryanodine binding activity at high IgG concentrations. Purified anti-DP4 antibody enhanced maximal [^{3}H]ryanodine binding to approx. 400% of control, with an

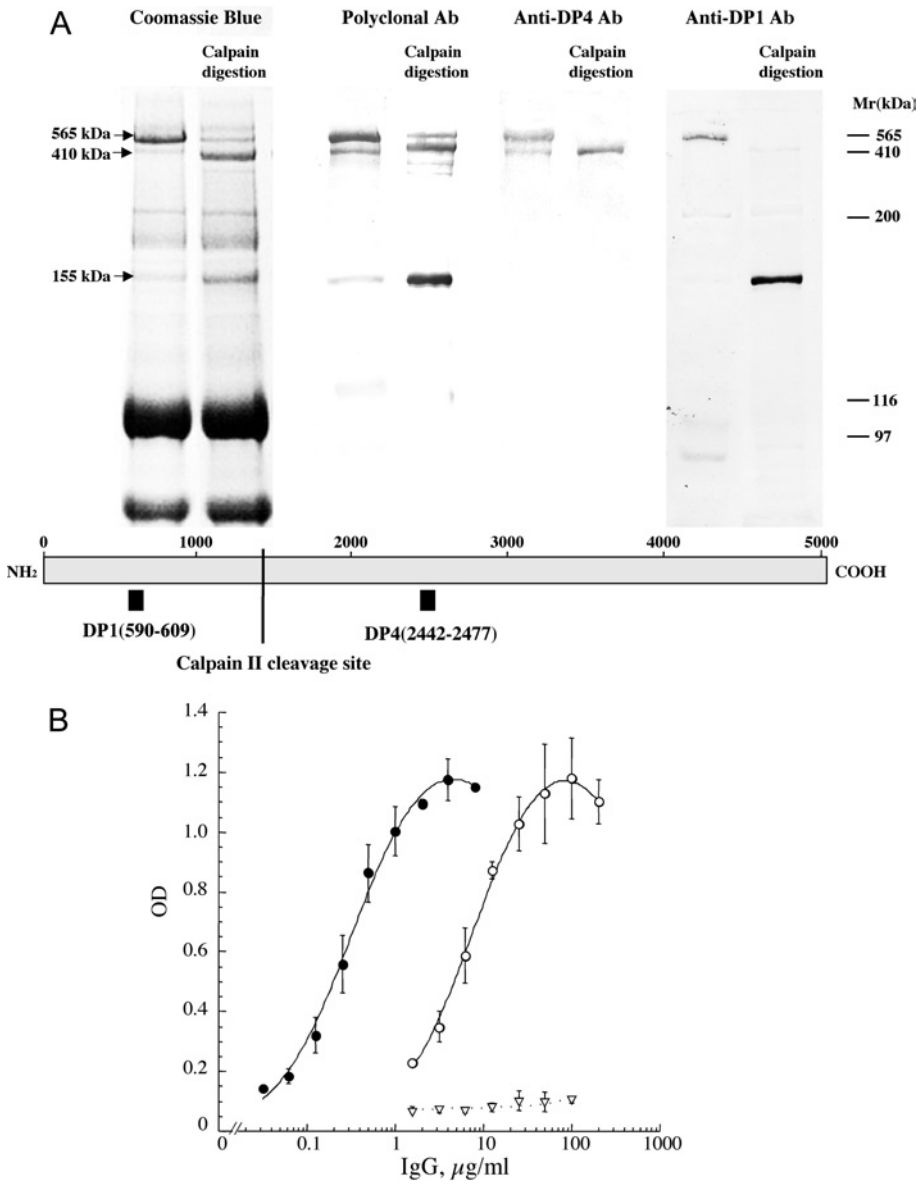

Figure 1 Anti-DP4 antibody binds to a C-terminal 410 kDa calpain fragment, while anti-DP1 antibody binds to an N-terminal 155 kDa fragment

(**A**) The left-hand two lanes are Coomassie Blue stained gels from SR or SR treated with exogenous calpain II. Polyclonal Ab denotes anti-RyR1 antibody. For digestion with calpain II, microsomes (2 mg/ml) were mixed with recombinant calpain II at a ratio of 6 units calpain to 1 mg SR protein in a solution containing 150 mM NaCl and 20 mM Mops (pH 7.2). Digestion was started by adding 3 mM $CaCl_2$. After digestion for 6 min at 22 °C, the reaction was stopped by adding 3 mM BAPTA. For Western blotting, protein bands electrophoretically separated by SDS/PAGE (6 % gels) were transferred to PVDF membranes (Immobilon-P, Millipore) for 12–15 h at 90 V in 10 % methanol, 10 mM Caps (pH 11.0). The membranes were treated with primary antibodies at appropriate dilution/concentration (purified anti-DP1 antibody, 8 μg/ml IgG; purified anti-DP4 antibody, 8 μg/ml IgG), followed by the peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized with DAB. (**B**) ELISA assay showing the concentration dependence of immunoreactivity of anti-DP immune sera and purified antibodies with corresponding domain peptides. Anti-DP4 immune serum (○) and purified antibody (●) reactivity towards DP4. Pre-immune serum (▽) was used as a control. Data were fitted to the equation: $y = [aK_1x/(1 + K_1x)] \times [1 - bK_2x/(1 + K_2x)] + c$ and the EC_{50} values were calculated as $1/K_1$. Each data point represents the mean ± S.D. of at least five experiments carried out in duplicate. A paired *t* test was employed to determine the statistical significance of the data.

$EC_{50} = 29.1$ μg IgG/ml (Figure 2A); the slope of the Hill plot was 1.53. The corresponding values for purified anti-DP1 antibody were approx. 380 % of control and 48.7 μg/ml (Figure 2B); the slope of the Hill plot was 1.36. Interestingly, comparison of the data from Figures 1(B) and 2 reveals that the EC_{50} for [^{3}H]ryanodine binding (i.e. channel activation) in the presence of either of the purified antibodies is considerably higher, i.e. of lower affinity, than that for antibody binding to domain peptides, as determined by ELISA assay. This suggests that the epitopes in RyR1 *in situ* are less accessible to these antibodies than the epitopes in the peptides, or that they have somewhat differing conformations.

We then sought to determine whether these antibodies are capable of hyper-sensitizing RyR1 to agonists. Figure 3 shows the concentration dependence of polylysine-induced SR Ca^{2+} release in the absence (control) and presence of anti-DP antibodies, and in the presence of non-specific IgG. The slope of the Hill plot of these data was 6.6 in the control, 6.2 with non-specific IgG, 3.4 with anti-DP4, and 4.4 with anti-DP1. EC_{50} was 59.7 ± 1.4 nM in the control, 58.3 ± 2.3 nM with non-specific IgG, 23.7 ± 1.7 nM with

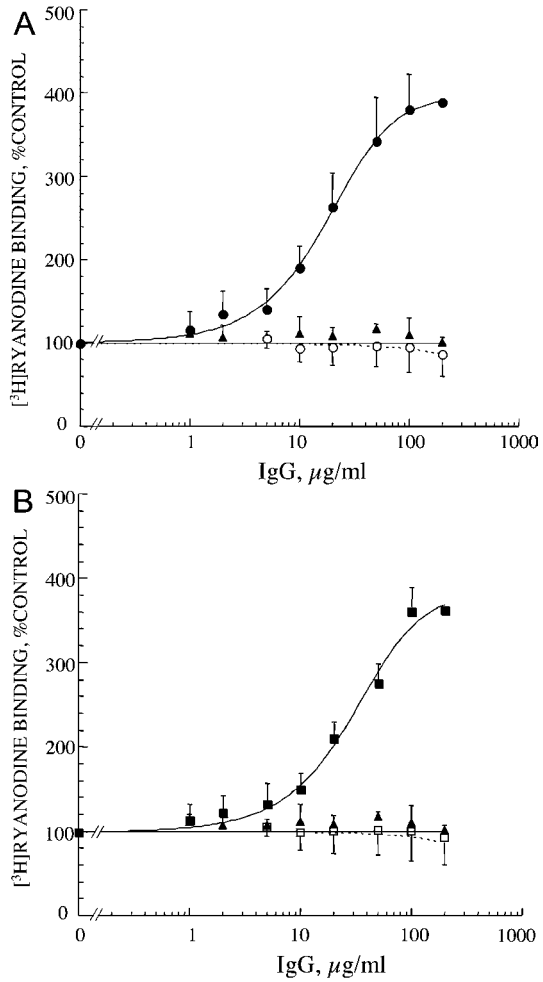

Figure 2 Purified anti-DP antibodies, but not pre-immune sera or non-specific IgG, activate RyR

(**A**) [^{3}H]Ryanodine binding as a function of purified anti-DP4 antibody (●), pre-immune serum (○), and non-specific IgG (▲) concentrations. (**B**) [^{3}H]Ryanodine binding as a function of purified anti-DP1 antibody (■), pre-immune serum (□), and nonspecific IgG (▲) concentrations. Data were fitted by an equation: $y = a(Kx + K^2x^2)/(1 + Kx + K^2x^2)$, and the EC_{50} values were calculated as 1/K. The assay conditions are described in the Materials and methods. Each data point represents the mean ± S.D. of at least four experiments carried out in duplicate.

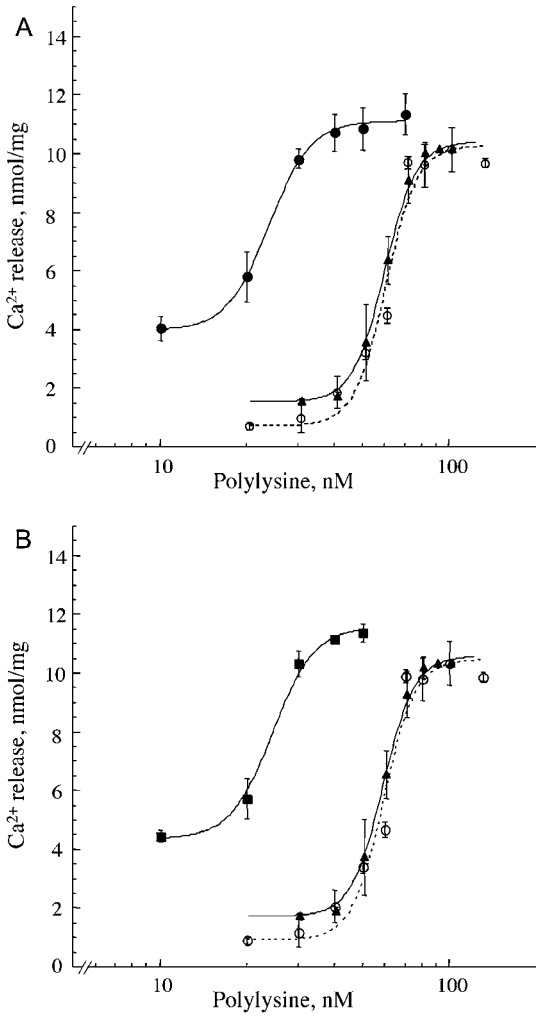

Figure 3 Anti-DP antibodies hyper-sensitize the RyR Ca^{2+} channel to the Ca^{2+} release trigger polylysine

(**A**) Anti-DP4 antibody. (**B**) Anti-DP1 antibody. Note that EC_{50} of activation was decreased (the apparent affinity was increased) considerably in the presence of antibodies. Control (○), non-specific IgG (▲), anti-DP4 antibody (●), anti-DP1 antibody (■). Data were fitted by an equation: $y = aK^n x^n/(1 + K^n x^n)$, $n = 8$ for control and non-specific IgG and $n = 6$ for anti-DP4 and anti-DP1, and the EC_{50} values were calculated as 1/K. Each datum point represents the mean ± S.D. obtained from at least five different experiments. Paired t test was employed to determine the statistical significance of the data.

anti-DP4, and 24.2 ± 0.7 nM with anti-DP1. Anti-DP antibodies induced a significant left-shift of the concentration-dependence curve of Ca^{2+} release relative to that produced in the absence of anti-DP antibody (control) ($P < 0.001$ in both cases of anti-DP4 and anti-DP1). Thus, the data in Figures 2 and 3 demonstrate that anti-DP4 and anti-DP1 antibodies produce hyper-activation and hyper-sensitization effects on RyR1.

Hyper-activation and hyper-sensitization effects of anti-DP4 and anti-DP1 antibodies are produced by their domain unzipping action

We have developed a fluorescence-quenching technique to monitor the postulated zipping/unzipping action of the RyR1 domain switch, presumably involving its N-terminal and central domains (Scheme 1) [31,32]. The method involves (i) incorporation of the conformationally sensitive fluorescence probe, MCA, into a particular domain in a site-specific manner by using an appropriate domain peptide as a site-directing carrier, and (ii) examination of the accessibility of the incorporated MCA probe to a macromolecular fluorescence quencher. We predicted that if the protein-bound MCA probe is occluded between interacting domains in the 'zipped' configuration, it would then be relatively inaccessible to a macromolecular fluorescence quencher. Unzipping, however, will render the MCA probe more accessible to the quencher. A further test of the hypothesis is to use a small molecule fluorescence quencher as control, and demonstrate that no difference in the concentration dependence of fluorescence quenching occurs in the presence or absence of either antibody. In the present study, therefore, we incorporated MCA into the DP4 binding site of RyR1 using DP4 as a site-directing carrier. We then determined the degree of fluorescence quenching as a function of the concentration of the fluorescence quencher, QSY®, either alone or conjugated to BSA (see Materials and methods section).

We determined first the fluorescence intensity of RyR1-bound MCA as a function of increasing concentrations of QSY®–BSA quencher in the absence or in the presence of purified anti-DP

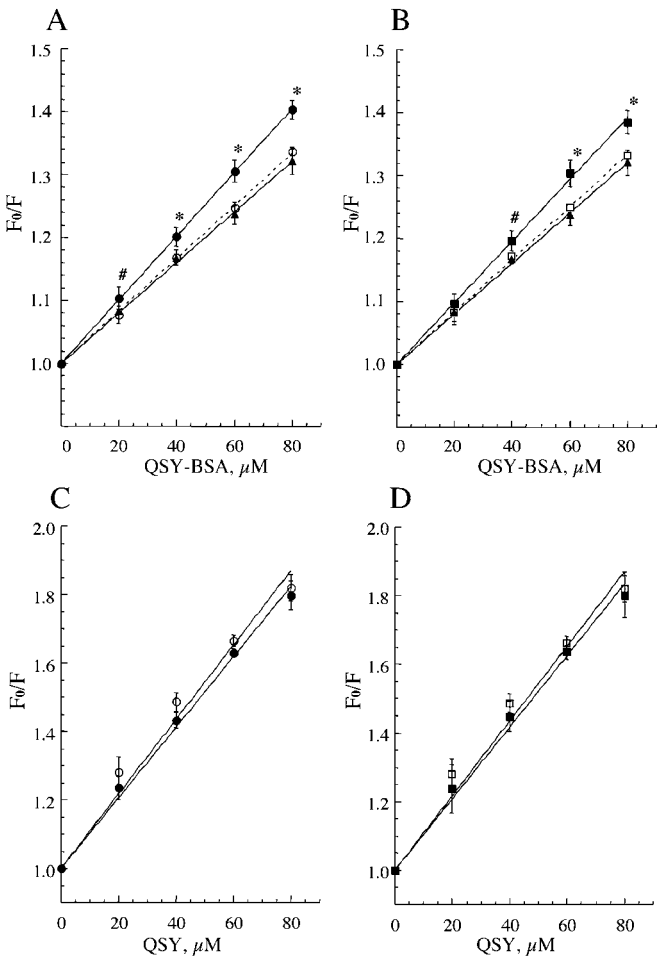

Figure 4 Anti-DP antibodies produce unzipping of the interacting domains as shown by an increased accessibility of the protein bound MCA probe to a bulky fluorescence quencher, BSA-conjugated QSY®

(**A**) Anti-DP4 antibody (●) in the presence of BSA–QSY®. (**B**) Anti-DP1 antibody (■) in the presence of BSA–QSY®. (**C**) Anti-DP4 antibody (●) in the presence of unconjugated QSY®. (**D**) Anti-DP1 antibody (■) in the presence of unconjugated QSY®. Controls are in the absence of antibody (○, □); or in the presence of non-specific IgG (20 μg/ml; ▲). There is a significant increase in the slope of Stern–Volmer quenching plot (i.e. in accessibility of the macromolecular quencher) only in the presence of anti-DP antibodies. This indicates that the domain-attached MCA probe is relatively inaccessible to the large-size quencher QSY®–BSA (i.e. 'zipped') in the absence of antibody, but upon addition of these antibodies it becomes more accessible to the QSY®–BSA conjugate ('unzipped'). Note that the probe accessibility to the unconjugated QSY® quencher is significantly higher than that of the conjugated QSY®–BSA quencher. This suggests that QSY®, because of its small size, is unhindered in its ability to diffuse into the probe-attached area regardless of 'zipped' or 'unzipped' configurations. Each datum point represents the mean ± S.D. obtained from at least four different experiments.

antibodies, or in the presence of non-specific IgG (Figures 4A and 4B). As can be seen, the slope of the Stern–Volmer plot (equivalent to the quenching constant), a measure of the degree of 'unzipping', was significantly greater in the presence of added antibody than in its absence. Figures 4(C) and 4(D) show the results of the same experiments performed with unconjugated QSY®, as control. The slopes of the Stern–Volmer plot in the presence or absence of anti-DP antibody are virtually identical. The slope of the plot, i.e. the quenching constant, is greater than that seen in the presence of the QSY®–BSA conjugate, indicating easier access for the low-molecular-mass quencher than for the macromolecular quencher. These results suggest that the macromolecular quencher, QSY®–BSA, is less accessible to the domain-bound MCA than is small molecule QSY®, presumably because covalently linked MCA is somewhat trapped by the 'zipped' domains. Upon the addition of critical domain-specific antibodies, occluded MCA becomes partially accessible to the macromolecular quencher because of antibody-induced domain 'unzipping'. Unlike the QSY®–BSA conjugate, the small molecule quencher, unconjugated QSY®, appears to be highly accessible to the domain-bound MCA probe, regardless of the configuration of interacting domains.

DISCUSSION

This study has tested the hypothesis described in the Introduction (see Scheme 1) that MH-mutation-rich domains of RyR1 are conformationally critical, by using a group of synthetic peptides corresponding to various mutable sub-domains of either the N-terminal or central domains (domain peptides). The most extensively studied domain peptide is DP4, corresponding to Leu^{2442}–Pro^{2477} of the central domain. We have examined [^{3}H]ryanodine binding [30], single channel conductance [33], SR Ca^{2+} release [31], Ca^{2+} sparks in permeabilized fibres [33], and caffeine-induced contraction of skinned fibres [34]. In all of these models, DP4 produced many of the hyper-activation/hyper-sensitization effects on RyR1 seen with MH mutations. Interestingly, DP4 was found to bind to the calpain-cleaved, 155 kDa N-terminal region of the RyR1 [31], which contains the dantrolene binding site (amino acids 590–609) [36,37]. These activating effects of DP4 were abolished when a single MH-type mutation was made in the peptide [30]. Similarly, DP1, the synthetic peptide corresponding to the dantrolene binding site, was also found to produce MH-like effects [30]. Thus the data we have accumulated to date, suggest that the two domains represented by DP1 and DP4 are critically important for the domain-switch-mediated channel regulation. We are as yet unable to say whether the sub-domains of RyR1 corresponding to DP4 and DP1 directly interact with each other, although this remains an experimental target.

The findings in the present study add significant support to our hypothesis that the DP1 and DP4 regions of RyR1 are critically involved in channel regulation. Both anti-DP4 and anti-DP1 antibodies activated RyR1, as indicated by significant enhancement of ryanodine binding activity, and by an appreciable left-shift of the concentration-dependence curve of the RyR-agonist polylysine to induce SR Ca^{2+} release. Both antibodies significantly enhanced the fluorescence quenching of a covalently bound, site-directed fluorescent probe to a macromolecular quencher, indicating exposure of a site access to which was previously hindered. Thus, both antibodies produced hyper-activation and hyper-sensitization of RyR1, similarly to those produced by MH mutations in the N-terminal or central domains [30]. Since DP4 binds to the N-terminal region [31] and the anti-DP4 antibody epitope is on the central domain, these results clearly support the notion that the co-ordinated action of at least these two domains is important for RyR1 Ca^{2+} channel regulation. This notion is further supported by an earlier report of Zorzato et al. [45], who noted that an antibody raised against the N-terminal domain containing a Gly^{341} human MH mutation produced a significant increase in the rate of Ca^{2+}-induced Ca^{2+} release. We do not yet know the mating domain of DP1, but it is tempting to speculate that it is near DP4. The fact that these domain-specific antibodies produce MH-like effects immediately suggests that the sub-domains of RyR1 antigenic to those antibodies play key roles not only in the basic mechanism of Ca^{2+} channel regulation, but also in the pathogenesis of Ca^{2+} channel dysfunction.

The fluorescence quenching experiments carried out in the present study have permitted a direct test of the hypothetical

domain 'unzipping' postulated for channel activation of RyR1 in Scheme 1. When coupled with the results discussed above, our fluorescence quenching results indicate that the effect of anti-DP1 and anti-DP4 antibodies is to expose a partially hidden domain within RyR1. These results further suggest that the MH-like hyper-activation/hyper-sensitization of these anti-domain antibodies are in fact produced by the ability of these antibodies to distort domain–domain interactions by virtue of their binding affinities for their conformationally significant epitopes.

We take particular notice of the homology of the three MH domains in RyR1 to the three domains of RyR2, the cardiac isoform, conferring susceptibility to arrhythmogenic right ventiricular dysplasia type 2 and catecholaminergic polymorphic (familial) ventricular tachycardia, two genetic causes of sudden cardiac death [46–52]. We have demonstrated that both dantrolene and monoclonal antibody anti-RyR1, a DP1 sequence-specific monoclonal antibody, interact with the N-terminal region of RyR1, but poorly with RyR2 in the case of dantrolene and not at all in the case of the monoclonal antibody; this despite an identical DP1 domain sequence in both isoforms [36,37,53]. These results suggest that there exist functional homologies between these regions in the two isoforms, but that they do not necessarily translate into conformational or antigenic identity.

In conclusion, the synthetic central domain peptide DP4 and N-terminal domain peptide DP1 of RyR1, which we have previously used as probes to characterize the mechanism of domain-mediated channel regulation, here served as antigens to produce antibodies directed at the corresponding *in vivo* domains of the RyR1. As we have shown here, anti-DP4 and anti-DP1 antibodies produced both hyper-activation and hyper-sensitization effects on RyR1 Ca^{2+} channel, similarly to those produced by MH RyR1 mutations. Spectroscopic studies of a fluorescence probe attached to the N-terminal, DP4-mating region showed that access to the domain-bound probe is hindered in the absence of added antibodies, but becomes accessible to the macromolecular fluorescence quencher upon addition of domain specific antibodies. These results suggest that the antibodies produced domain unzipping, thereby sensitizing Ca^{2+} channels to activation. The present study suggests that domain-specific antibodies will provide us with useful probes to identify and characterize the critical sites involved in the pathogenic mechanisms of channel disorders.

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