A variably spliced region in the type 1 ryanodine receptor may participate in an inter-domain interaction

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The aim of the present study was to examine residues that are variably spliced in the juvenile and adult isoforms of the skeletal-muscle RyR1 (type 1 ryanodine receptor). The juvenile ASI(–) splice variant is less active than the adult ASI(+) variant and is overexpressed in patients with DM (myotonic dystrophy) [Kimura, Nakamori, Lueck, Pouliquin, Aoike, Fujimura, Dirksen, Takahashi, Dulhunty and Sakoda (2005) *Hum. Mol. Genet.* **14**, 2189–2200]. In the present study, we explore the ASI region using synthetic peptides corresponding to rabbit RyR1 residues Thr³⁴⁷¹–Gly³⁵⁰⁰ either containing [PASI(+)] or lacking [PASI(–)] the ASI residues. Both peptides increased [³H]ryanodine binding to rabbit RyR1s, increased Ca²⁺ release from sarcoplasmic reticulum vesicles and increased single RyR1 channel activity. The peptide PASI(–) was more active in each case than PASI(+). [³H]Ryanodine binding to recombinant ASI(+)RyR1 or ASI(–)RyR1 was enhanced more by PASI(–) than PASI(+), with the

greatest increase seen when PASI(–) was added to ASI(–)RyR1. The activation of the RyR channels is consistent with the hypothesis that the peptides interrupt an inhibitory inter-domain interaction and that PASI(–) is more effective at interrupting this interaction than PASI(+). We therefore suggest that the ASI(–) sequence interacts more tightly than the ASI(+) sequence with its binding partner, so that the ASI(–)RyR1 is more strongly inhibited (less active) than the ASI(+)RyR1. Thus the affinity of the binding partners in this inter-domain interaction may determine the activities of the mature and juvenile isoforms of RyR1 and the stronger inhibition in the juvenile isoform may contribute to the myopathy in DM.

Key words: alternative splicing, inter-domain interaction, myotonic dystrophy, single channel recording, skeletal ryanodine receptor, synthetic peptide.

INTRODUCTION

The skeletal RyR1 (type 1 ryanodine receptor) Ca²⁺ release channel is the major player in releasing Ca²⁺ ions from intracellular Ca²⁺ stores in the SR (sarcoplasmic reticulum) of skeletal muscle and is essential for skeletal muscle EC (excitation–contraction) coupling. Mutations in RyR1 have been linked to four genetic disorders: MH (malignant hyperthermia), CCD (central core disease), multimincore disease and nemaline rod myopathy [1]. In addition, a splicing defect in RyR1 has recently been identified in DM1 (myotonic dystrophy type 1) [2]. DM1 is the most common type of muscular dystrophy in adults. The dystrophy is a dominantly inherited multi-systemic disorder caused by unstable expansion of CTG trinucleotide repeats in the DMPK (DM protein kinase) gene [3], which disrupts the mRNA splicing of several genes [4–9]. RyR1 has two variably spliced regions; ASI and ASII [10] (Figure 1), with splice variants characterized by the presence or absence of the ASI or ASII exon. The amount of the juvenile variant, ASI(–)RyR1, lacking residues 3481–3485, is significantly increased in skeletal muscles from adult DM1 patients. In contrast with ASI(–), the expression of the second juvenile splice variant of RyR1 [ASII(–)] which lacks residues 3865–3870 is unaltered in DM1 [2]. ASI(–)RyR1 channels when heterologously expressed in HEK-293T cells [human embryonic kidney 293 cells expressing the large T-antigen of SV40

(simian virus 40)] show decreased affinity for but similar Ca²⁺ dependence on [³H]ryanodine binding and decreased single channel activity, when compared with wild-type ASI(+)RyR1 [2]. Similarly, in intact myotube studies, a decreased incidence of Ca²⁺ oscillations was observed when RyR1-knockout myotubes expressing ASI(–)RyR1 were exposed to caffeine, when compared with myotubes expressing ASI(+)RyR1, which was indicative of reduced RyR1 activity [2].

Since exclusion of residues 3481–3485 in the juvenile splice variant significantly alters RyR1 activity, it is likely that the region encompassing these residues is an important determinant of RyR1 channel gating. Thus it is possible that the ASI region of RyR1, like regions supporting MH and CCD mutations [11–13], is involved in an inter-domain interaction. Most MH and CCD mutations are clustered in N-terminal, central and C-terminal regions of RyR1 (Figure 1). Mutations in N-terminal and central domains produce a similar phenotype with excess activation in response to compounds such as caffeine and halothane that activate Ca²⁺ release [14]. Ikemoto and co-workers [11–13] showed that synthetic peptides corresponding to regions in the central and N-terminal domains activated RyR1 in a similar manner to the MH mutations. They proposed that these regions contribute to an inter-domain interaction that inhibits channel activity and that the mutations interrupt the inhibitory interaction, resulting in channel activation [11–13].

Abbreviations used: CCD, central core disease; DHP, dihydropyridine receptor; DM1, myotonic dystrophy type 1; DP, domain peptide; EC, excitation–contraction; HEK-293T cells, human embryonic kidney 293 cells expressing the large T-antigen of SV40 (simian virus 40); MH, malignant hyperthermia; PCCD, wild-type CCD peptide; PCCDM, mutant CCD peptide; RyR1, type 1 ryanodine receptor; SERCA, sarcoplasmic/endoplasmic-reticulum Ca²⁺-ATPase; SR, sarcoplasmic reticulum.

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Figure 1 Schematic diagram of relevant sites on RyR1

(A) Exon/intron layout on the RyR1 gene. Exons are represented as boxes with exon number and horizontal lines indicate introns. Lines with letters represent different splice modes. Mature splice variants, ASI(+) and ASII(+), include exon 70 and exon 83 respectively, while juvenile variants, ASI(-) and ASII(-), lack each exon. (B) The location of the ASI and ASII regions and the MH/CCD mutation sites that are mostly included in three clustered regions (MH1–MH3). There is an isolated distinct mutation site, P3527S. (C) Sequences of peptides used in the present study and the locations of corresponding residues in the rabbit skeletal-muscle RyR1. PASI(+) and PASI(-) are shown with the variably spliced residues included in PASI(+) underlined. Next is the PASI(-) mutant peptide, PASI(-)M, with the mutated residues highlighted. This is followed by the CCD peptides, the wild-type (PCCD) and the mutant (PCCDM), with the wild-type and mutant residues underlined respectively. Finally, the PASII(+) and PASII(-) peptides are shown with the variably spliced residues included in PASII(+) underlined.

The ASI region is also likely to be important in regulating RyR1 activity. This is indicated by the functional changes that occur when the ASI residues are excluded from RyR1 and by two other observations. The first is that, although ASI is not located in the MH-susceptible regions of RyR1, a homozygous CCD mutation has been identified within the exon (P3527S, exon 71) adjacent to that of ASI (exon 70) [15] (Figure 1). Secondly, residues 3495–3502, close to the ASI residues, form the binding site for the β_{1a} subunit of the DHP (dihydropyridine receptor), which is an important determinant of EC coupling in skeletal myotubes [16]. We tested the hypothesis that the ASI region may be involved in an inter-domain interaction using peptides corresponding to the sequences surrounding the variably spliced regions. The results showed peptide PASI(+), which includes residues within the spliced exon, and peptide PASI(-), which lacks residues within the spliced exon, activated RyR1 in a sequence-specific and peptide-specific manner. Interestingly, PASI(-) activated native and purified RyR1, and recombinant ASI(-)RyR1 and ASI(+)RyR1, more than PASI(+). In contrast, peptides corresponding to the second variably spliced region, PASII(+) and PASII(-), had no effect on RyR1 activity.

EXPERIMENTAL

Materials

The peptides listed in Figure 1(C) were synthesized by the JCSMR Biomolecular Resource Facility using an Applied Biosystems 430A peptide synthesizer and were purified to 98–100% using HPLC and mass spectroscopy. Stock peptide solutions (5 mM) were prepared in water and frozen in 20 μ l aliquots. Residue numbering in Figure 1 and throughout the paper is for rabbit RyR1.

RyR1 purification

Preparation of SR vesicles from rabbit skeletal muscle, Ca^{2+} release from SR, single channel techniques [17], purification of RyR1 [18,19], transfection of rabbit ASI(+)/ASI(-)RyR1 to HEK-293T cells, preparation of microsomal protein and [^3H]ryanodine binding [2] have been described previously. Native RyR1 in SR vesicles were examined in [^3H]ryanodine binding, Ca^{2+} release and single channel experiments. Recombinant ASI(+)/ASI(-)RyR1 and purified RyR1 were used in [^3H]ryanodine binding experiments. To purify RyR1 from native SR vesicles, the SR vesicle membrane was solubilized in Buffer A containing 1 M NaCl, 25 mM Pipes (pH 7.4), 1 mM DTT (dithiothreitol), 0.1 mM EGTA, 0.092 mM CaCl_2 , 0.5 mM AMP, 0.5% CHAPS, 0.25% phosphatidylcholine and protease inhibitors (1 mM benzamidine, 0.5 mM PMSF, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 1 $\mu\text{g}/\text{ml}$ leupeptin and 1 $\mu\text{g}/\text{ml}$ aprotinin) for 1 h. After centrifugation at 100 000 g for 30 min, the supernatant was applied to a linear sucrose gradient [5–20% (w/v) in Buffer A] with centrifugation at 53 000 g for 16 h at 4°C. Fractions containing the RyR1 were identified by SDS/PAGE and concentrated.

Ca^{2+} release from SR

Extravesicular Ca^{2+} was monitored at a wavelength of 710 nm with the Ca^{2+} indicator, antipyrilazo III, using a Cary 3 spectrophotometer. Identical release experiments performed at 790 nm showed no changes in absorbance that would alter the rate of Ca^{2+} release. SR vesicles (100 μg of protein) were added to 2 ml of cuvette solution, containing (in mM): 100 phosphate buffer (pH 7), 4 MgCl_2 , 1 Na_2ATP and 0.5 antipyrilazo III.

Single-channel techniques

Artificial lipid bilayers of phosphatidylethanolamine and phosphatidylcholine (8:2, w/w) (Avanti Polar Lipids, Alabaster, AL, U.S.A.) were formed across an aperture of approx. 200 μm diameter in the wall of a 1.0 ml Delrin cup (Cadillac Plastics). SR vesicles and peptides were added to the *cis* chamber. Vesicles were incorporated into the bilayer using a *cis* solution containing: 230 mM $\text{CsCH}_3\text{O}_3\text{S}$ (caesium methyl sulfonate), 20 mM CsCl, 1.0 mM CaCl_2 , 10 mM Tes and 500 mM mannitol (pH 7.4 with CsOH) and a *trans* solution containing 30 mM $\text{CsCH}_3\text{O}_3\text{S}$, 20 mM CsCl, 1 mM CaCl_2 and 10 mM Tes (pH 7.4). Vesicles were incorporated into the bilayer with their cytoplasmic side facing the *cis* solution and their luminal side facing the *trans* solution. Following incorporation, (i) the *cis* incorporation solution was replaced with an identical solution, except that the $[\text{Ca}^{2+}]$ was 100 nM and (ii) 200 mM $\text{CsCH}_3\text{O}_3\text{S}$ was added to the *trans* chamber for symmetry. Bilayer potential was controlled at -40 mV and $+40$ mV, and channel currents were recorded over a range of peptide concentrations, using an Axopatch 200 amplifier (Axon Instruments, Foster City, CA, U.S.A.). Bilayer potential

is expressed as $V_{cis} - V_{trans}$ ($V_{cytoplasm} - V_{lumen}$). Potential was changed every 30 s and currents were recorded for 3 min under each condition. Control activity was recorded for 3 min after addition of 200 mM $CsCH_3O_3S$ and then after each aliquot of peptide (three to six different peptide concentrations were tested in each channel). Channel activity was measured as mean current (I'), i.e. the sum of all data points divided by the number of points. Peptides were perfused out of the *cis* chamber with 5 vol. of *cis* solution without peptides.

Transfection and preparation of microsomal protein

HEK-293T cells were transfected with a mammalian expression vector (pCIneo) encoding either rabbit ASI(+)/RyR1 or rabbit ASI(-)/RyR1 (40 μ g of DNA per plate) using calcium phosphate precipitation method. Cells maintained for 48 h were collected, homogenized in 20 ml of ice-cold hypo-osmotic buffer (1 mM EDTA, 10 mM Hepes, pH 7.4, and protein inhibitors) added with an equal volume of ice-cold 20% (w/v) sucrose buffer (1 mM EDTA and 10 mM Hepes, pH 7.4), and centrifuged at 110 000 *g* for 45 min at 4°C. The pellet was then resuspended in 1 ml of a buffer containing 10% (w/v) sucrose, 1 mM EDTA and 10 mM Hepes (pH 7.4) and stored at -70°C until use. [3 H]Ryanodine binding to recombinant RyR1 in this membrane fraction was examined.

[3 H]Ryanodine binding

[3 H]Ryanodine binding was performed at 37°C with 50 μ g of SR vesicles, 3 μ g of purified RyR1 or 200 μ g of microsomal protein from transfected cells, in a solution containing 100 mM KCl, 20 mM Pipes (pH 6.8), 15 nM [3 H]ryanodine, 1 mM EGTA, 200 μ M AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride] and the indicated concentrations of free Ca^{2+} . Non-specific binding was measured in the presence of 667-fold molar excess of unlabelled ryanodine. Vesicle preparations were filtered through Whatman GF(glass fibre)/B filters, which were then washed three times with 3 ml of an ice-cold solution containing 100 mM KCl. Radioactivity bound to the filters was measured by scintillation counting. Purified RyR1 was precipitated by adding 400 μ l of carrier protein solution (5 mg/ml γ -globulin, 5 mg/ml BSA and 200 mM NaCl) and 3.5 ml of 10% (w/v) poly(ethylene glycol) before filtering.

Statistics

Average data are given as means $\pm$ S.E.M. Difference between values was evaluated by paired or unpaired Student's *t* test as appropriate. Difference was considered to be significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

Peptides PASI(+) and PASI(-) increase [3 H]ryanodine binding to rabbit RyR1

Both of the PASI peptides significantly increased [3 H]ryanodine binding to native SR vesicles over a range of Ca^{2+} concentrations from 100 nM to 10 μ M (Figure 2). The increase in [3 H]ryanodine binding produced by peptide PASI(-) was significantly greater than that by PASI(+) ($P < 0.05$). The percentage increase induced by both peptides was greatest with 100 nM Ca^{2+} and declined with increasing Ca^{2+} concentrations. For example, 100 μ M PASI(-) increased the binding to $331 \pm 7\%$ of control with 100 nM Ca^{2+} , but only to $195 \pm 8.81\%$ with 10 μ M Ca^{2+} . Neither of the peptides affected the Ca^{2+} dependence of [3 H]ryanodine binding (Fig-

Figure 2 Peptides PASI(+) and PASI(-) increase [3 H]ryanodine binding to native and purified RyR1 with no change in Ca^{2+} dependence

(A–C) Dose-dependent effects of peptides PASI(+), PASI(-) and the mutant PASI(-)M [PASI(-)M] on relative [3 H]ryanodine binding (% of control). Binding was carried out with various concentrations of the DPs (described in the Experimental section) in the presence of 100 nM (A), 1 μ M (B) and 10 μ M (C) free Ca^{2+} . The binding activities without peptides were 0.077 ± 0.008 , 0.758 ± 0.098 and 2.60 ± 0.15 pmol/mg of protein with 100 nM, 1 μ M and 10 μ M Ca^{2+} respectively. (D) Ca^{2+} dependence of [3 H]ryanodine binding to native RyR1 without (control) or with 100 μ M PASI(+) or PASI(-). (E) Dose dependence of relative [3 H]ryanodine binding to purified RyR1 is shown for peptides PASI(+), PASI(-) and PASI(-)M (see Figure 1). The values are means $\pm$ S.E.M. for three to five observations under each condition.

ure 2D). The activating effect of the peptides on ryanodine binding was sequence-specific because the peptide failed to enhance [3 H]ryanodine binding when two alanine residues and a leucine were substituted for three of the basic residues in the PASI(-)M (Figure 2C). The activation was also peptide-specific since neither PASII(+) nor PASII(-) had any effect on [3 H]ryanodine binding. The binding was $110 \pm 2\%$ of control with 10 μ M PASII(+) and $100 \pm 2\%$ of control with 100 μ M PASII(+) and,

similarly, $114 \pm 5\%$ of control with $10 \mu\text{M}$ PASII(−) and $90 \pm 2\%$ of control for $100 \mu\text{M}$ PASII(−) ($n = 3$ in each case).

To determine whether the PASI peptides acted directly on the RyR or on a major associated protein, we examined their actions on purified RyR1. Sucrose gradient purification removed calsequestrin, junctin and triadin but not FKBP12 (FK506-binding protein 12) from RyR1. As with native RyR1, both PASI(+) and PASI(−) significantly increased [^3H]ryanodine binding and the increase by PASI(−) was significantly greater than that by PASI(+) (Figure 2E). The activation of the purified RyR1 was significantly greater than that of the native RyR1. For example, $100 \mu\text{M}$ of PASI(−) activated native RyR1 to $195 \pm 9\%$ of control (Figure 2C), but activated purified RyR1 to $858 \pm 89\%$ of control (Figure 2E). This may have been because the associated proteins such as calsequestrin, junctin and triadin inhibited the activation of the native channels by the peptides, or masked their binding sites. These results are consistent with the hypothesis that the ASI region participates in an inter-domain interaction in RyR1 and that the ASI peptides unzip the endogenous interaction by binding to the ASI binding partner.

Peptides corresponding to the sequence surrounding the CCD mutation were also examined. The PCCD (wild-type CCD peptide) and PCCDM (mutant CCD peptide) increased [^3H]ryanodine binding; however, there was no significant difference between PCCD and PCCDM (results not shown). With $100 \mu\text{M}$ peptide, [^3H]ryanodine binding was $158 \pm 4.0\%$ of control for the wild-type peptide and $148 \pm 4\%$ of control for the mutant peptide ($n = 3$ in each case).

Peptides PASI(+) and PASI(−) release Ca^{2+} from SR vesicles

The ability of the PASI peptides to release Ca^{2+} from native SR vesicles was examined. In these experiments, the vesicles were loaded with Ca^{2+} by four additions of Ca^{2+} to final concentrations of $7.5 \mu\text{M}$ Ca^{2+} (arrows 2–5, Figure 3A). When 200 nM thapsigargin was added to block Ca^{2+} uptake by the SERCA (sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase; arrow 6), there was a slow resting Ca^{2+} release ($\sim 53 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$) through the RyR1. Addition of $100 \mu\text{M}$ peptide PASI(+) (arrow 7) increased the rate of Ca^{2+} release to approx. $317 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ and $20 \mu\text{M}$ PASI(+) induced a smaller increase in Ca^{2+} release (compare Figures 3A and 3B). PASI(−) at $20 \mu\text{M}$ produced the same release as $100 \mu\text{M}$ PASI(+) (compare Figures 3A and 3C). In marked contrast, $100 \mu\text{M}$ PASII(+) did not change Ca^{2+} release from the SR vesicles (Figure 3D). The average data (Figure 3E) show that, as with [^3H]ryanodine binding, the PASI peptide increases Ca^{2+} release in a dose-dependent manner, with PASI(−) releasing significantly more Ca^{2+} at peptide concentrations between 10 and $50 \mu\text{M}$ ($n = 3$ – 5 , $P < 0.05$). Both PASI(+) and PASI(−) were clearly more potent activators than 1 mM caffeine (Figure 3E).

Peptides PASI(+) and PASI(−) activate native RyR1 channels

The PASI peptides were added to the cytoplasmic (*cis*) solution bathing native RyR1 channels incorporated into bilayers from SR vesicles that were added to *cis* solution. The cytoplasmic *cis* Ca^{2+} concentration was 100 nM so that control channel activity was low and the activating effects of the peptides could be clearly seen. PASI(+) increased the activity of RyR1 channels at -40 mV without any change in the maximum single channel conductance (Figures 4A and 5A). The increase in mean current at -40 mV was, on average, significant with 100 nM or more PASI(+). At $+40 \text{ mV}$, however, there was a trend towards activation that was strongest with $1 \mu\text{M}$ PASI(+).

Figure 3 Peptides PASI(+) and PASI(−) release Ca^{2+} from SR vesicles

(A–D) Records of absorbance changes at 710 nm , with changes in extravesicular $[\text{Ca}^{2+}]$ measured using antipyrilazo III ($500 \mu\text{M}$). (A) A full Ca^{2+} release experiment with an addition of SR vesicles (first arrow), four additions of Ca^{2+} to final concentrations of $7.5 \mu\text{M}$ with subsequent Ca^{2+} uptake into the SR (four short arrows), an addition of $1 \mu\text{M}$ thapsigargin (thap; 6th arrow) to block SERCA, an addition of $100 \mu\text{M}$ PASI(+) (7th arrow), $5 \mu\text{M}$ Ruthenium Red (RR; 8th arrow) and $1.5 \mu\text{g/ml}$ Ca^{2+} ionophore A23187 (last arrow). Ruthenium Red clearly blocked Ca^{2+} release when added before release was complete, at earlier times or with lower concentrations of the PASI peptides (results not shown). Release with $20 \mu\text{M}$ PASI(+) is shown in (B), $20 \mu\text{M}$ PASI(−) in (C), and $100 \mu\text{M}$ PASII(+) in (D). (E) Average rates of Ca^{2+} release ($\text{nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$) induced by PASI(+) (■), PASI(−) (●), PASII(+) (□) and 1 mM caffeine (▲). The rates shown are the initial rate of release with peptide minus the preceding rate with thapsigargin. The values are means $\pm$ S.E.M. for four to six observations under each condition.

Peptide PASI(−) activated the channels at both potentials and activated at lower concentrations (with higher affinity) than PASI(+). PASI(−) produced a significant increase in channel activity at -40 mV at all concentrations between 1 nM and $10 \mu\text{M}$ (Figure 5B), and activation at $+40 \text{ mV}$ was significant ($P < 0.008$) with 10 and 100 nM peptide. These results were in contrast with PASI(+) which failed to activate at concentrations of 1 or 10 nM . Curiously, the inhibitory action of high concentration of the PASI(−) peptide was also stronger and apparent at both potentials. Activity fell when PASI(−) was increased from 10 to $100 \mu\text{M}$ ($P = 0.026$ for -40 mV and $P = 0.012$ for $+40 \text{ mV}$). The lower affinity inhibitory action of the peptides was seen only in single channel activity and was most apparent at $+40 \text{ mV}$. This voltage-dependent inhibition is likely to be due to

Figure 4 Peptides PASI(+) and PASI(−) activate and then inhibit single native RyR1 channels

(A, B) Representative records of control activity with 100 nM Ca^{2+} in the *cis* chamber (first trace); after addition of 10 nM peptide (second trace); 1 μM or 10 μM peptide (third trace) and then 100 μM peptide (fourth trace) and after removing peptide by perfusion (fifth trace). (A) PASI(+). (B) PASI(−). Channel openings are downward deflections at −40 mV (left) and upward deflections at +40 mV (right). The continuous line in each record shows the closed channel current (labelled C). The broken line shows the maximum open channel conductance, labelled O in (A) or O1, O2 or O3 in (B), to show the level for a maximum single channel opening (O1) or two or three channels opening simultaneously (O2 or O3). Note the substantial increase in activity with PASI(−) at a concentration of 10 nM.

a non-specific pore block by the basic sequence in the C-terminal half of the PASI peptides (Figure 1). A similar block is seen with DHPR II–III loop A peptide, which contains a similar basic sequence [17].

The higher affinity activation by PASI(−) was consistent with the ryanodine binding and Ca^{2+} release results. The higher affinity inhibition was not seen in the ryanodine binding and Ca^{2+} release data. This was most likely due to the fact that the concentrations of peptides required to alter single RyR1 channel activity were consistently lower than those required to alter [^3H]ryanodine binding or Ca^{2+} release from SR vesicles. Thus it is possible that the concentration of peptides could not be increased sufficiently to see inhibition of [^3H]ryanodine binding or Ca^{2+} release. These technique-dependent concentration differences have also been

Figure 5 Average effects of peptides PASI(+) and PASI(−) on relative mean current flowing through single native RyR1 channels at −40 mV (left) and +40 mV (right)

Data were obtained with 100 nM *cis* Ca^{2+} . Relative mean current (where the mean current with peptide is expressed relative to the mean current under control conditions – see the Experimental section) is plotted against peptide concentration. (A) Peptide PASI(+) ($n=6$). (B) Peptide PASI(−) ($n=6$). The first symbol (C) on *x*-axis shows the control value which is 1. (C) Dilution of 100 μM of the PASI peptides by perfusion with 5 vol. of *cis* solution activates single native RyR1 channels at −40 mV. Histograms of the average relative mean current before peptide addition (control), with 100 μM peptide PASI(+) (black bars, $n=6$) and peptide PASI(−) (cross-hatched bars, $n=3-5$), after perfusion with 5 vol. of *cis* solution at −40 mV (left) and +40 mV (right). Asterisks (*) indicate that the mean value is significantly different from that obtained under control conditions, $P < 0.05$. The hash symbols (#) indicate that the mean value is significantly different from that obtained in the presence of 100 μM of each peptide, $P < 0.05$. Note that under most conditions activity at 100 μM peptide was less than at lower peptide concentrations and was less than control with PASI(−) at +40 mV (see A, B).

noted with the DHPR II–III loop and with the DHPR II–III loop peptide A [17,20].

Many native RyR1 channels were further activated at −40 mV after removing much of the 100 μM of peptides PASI(+) and PASI(−) by perfusion with 5 vol. of *cis* solution (Figure 5C and see also Figure 4). This perfusion would have reduced the peptide concentration 20-fold to approx. 5 μM [21]. Overall, the washout data illustrate the rapid reversibility of the inhibitory effect of the peptides. However, the channel activity after washout is more than that might be expected from the concentration dependence shown previously in Figures 5(A) and 5(B). This observation can be explained by a hypothesis in which (i) activation and inhibition are independent processes, (ii) the lower affinity inhibitory effect is reversible, while activation is irreversible (within the time frame of the experiment) and (iii) strong irreversible activation occurring at higher peptide concentrations was masked by inhibition

Figure 6 Peptides PASI(+) and PASI(-) increase relative [^3H]ryanodine binding to recombinant ASI(+)RyR1 and ASI(-)RyR1

The dose-dependent effects of PASI(+) and PASI(-) on recombinant ASI(+)RyR1 and ASI(-)RyR1 as indicated to the right of each curve. Binding was carried out with $10\ \mu\text{M}$ Ca^{2+} . The values are means $\pm$ S.E.M. for six observations under each condition.

in the presence of the peptide, but revealed when the inhibitory effect was removed. The one exception was with PASI(+) at +40 mV where washout activation was not seen. However, this was not surprising since activation by this peptide was weak at +40 mV.

In summary, the results so far show that the PASI peptides increase [^3H]ryanodine binding to SR vesicles, Ca^{2+} release from SR vesicles and single RyR channel activity (more so at -40 mV than at +40 mV). As mentioned above, one interpretation of these observations is that the ASI peptides, like the MH DPs (domain peptides), interrupt an inhibitory inter-domain interaction in which the ASI region binds to a partner domain elsewhere in RyR1. The action of PASI(-) suggests that this peptide binds to RyR1 with higher affinity (or more strongly) than PASI(+). When extrapolated to the RyR1 protein, this suggests that the ASI region in the ASI(-)RyR1 may bind more strongly to its partner site than the ASI region in the ASI(+)RyR1. Thus a working hypothesis is that our previous observation [2] that the ASI(-)RyR1 channel is less active than the ASI(+)RyR1 channel can be explained by an inhibitory inter-domain interaction that is stronger in the ASI(-)RyR1 channel than it is in the ASI(+)RyR1 channel. In other words, our hypotheses are first that the PASI(-) peptide is more effective at interrupting an inter-domain interaction than the PASI(+) peptide and secondly that ASI(-)RyR1 is less active than ASI(+)RyR1 because the ASI(-) sequence forms a stronger inhibitory interaction with its *in situ* binding partner in RyR1.

Effects of the PASI peptides on [^3H]ryanodine binding to recombinant ASI(+)RyR1 and ASI(-)RyR1

Both RyR1 isoforms were activated by both peptides in a dose-dependent manner (Figure 6), but the relative activation of each isoform by PASI(-) was significantly greater than by PASI(+) ($n = 6$, $P < 0.05$). It might have been expected that higher concentrations of both peptides would be required to interrupt the *in situ*

interaction between the ASI sequence in ASI(-)RyR1 (see previous section), if this interaction was stronger in ASI(-)RyR1 than in ASI(+)RyR1. However, in contrast with this expectation, the data in Figure 6 suggest that PASI(-) in fact activated ASI(-)RyR1 at lower concentrations than ASI(+)RyR1. This result suggested that, if the inhibitory interaction was in fact stronger in ASI(-)RyR1, then the concentration dependence is limited by a separate variable such as accessibility to the binding site. For example increased accessibility of the smaller PASI(-) peptide to the binding site in ASI(-)RyR1 could contribute to enhanced activation of ASI(-)RyR1 at lower peptide concentrations. We have some evidence that the ASI site in RyR1 is relatively inaccessible because antibodies that recognized PASI(+) with high titre cross-reacted with peptide ASI(+) in ELISA, but not with whole RyR1 and failed to alter [^3H]ryanodine binding or Ca^{2+} release from SR.

It is also clear in Figure 6 that the relative increase in [^3H]ryanodine binding with $100\ \mu\text{M}$ PASI(-) was significantly greater for the ASI(-)RyR1 than for ASI(+)RyR1 [ASI(+)RyR1: $189 \pm 6\%$; ASI(-)RyR1: $267 \pm 11\%$, $P = 0.032$, $n = 6$]. There was also a trend towards greater activation of ASI(-)RyR1 than ASI(+)RyR1 by the PASI(+) peptide, but the differences in the individual points were not statistically significant. It might be expected that the increase in activity in the ASI(-)RyR1 would be greater than that in the ASI(+)RyR1 if the activity of the channels was similar once the inhibitory interaction was removed, since ASI(-)RyR1 was initially less active than the ASI(+)RyR1.

Inter-domain interactions

Regions of RyR1 associated with MH mutations have been previously implicated in inter-domain interactions. Two peptides corresponding to residues in the MH domains of RyR1, DP1 and DP4, also activate RyR1 (see references in the Introduction section). Although the possibility that DP1 and DP4 bind to RyR1 in a non-specific manner cannot be excluded, it is notable that the peptides cause changes that are remarkably similar to changes in channel activity seen in MH. Additional experiments with antibody and fluorescence probes confirmed this hypothesis [22,23]. Our studies with the PASI peptides are consistent with the ASI region also participating in an inter-domain interaction and we propose the following model.

(i) An inter-domain interaction between the ASI region and a binding partner stabilizes the closed state of the RyR1 channel. Exogenous peptides PASI(+) or PASI(-) bind to the binding partner for, and 'unzip', the ASI interaction, resulting in channel activation.

(ii) PASI(-) interacts with the binding partner in RyR1 more tightly than PASI(+).

(iii) The interaction between the endogenous ASI region and its binding partner is tighter in ASI(-)RyR1 than in ASI(+)RyR1 so that the ASI(-)RyR1 tends to dwell more in the closed state.

(iv) When the PASI peptides are added to the ASI(-)RyR1, the removal of this stronger inhibition results in greater relative channel activation than that seen when the peptides are added to ASI(+)RyR1.

The inhibitory effects of high concentrations of PASI(+/-) on channels is not considered in this model because they are likely to be non-specific actions of the peptides (see the 'Peptides PASI(+) and PASI(-) activate native RyR1 channels' section).

Where is the partner binding region for ASI? Yamamoto and Ikemoto [23] hypothesized that N-terminal and central domains interact with each other to stabilize the closed state of the RyR1, since there was competition between DP4 and DP1 in their activating function [12]. DP4 has also been shown to interact with the

N-terminal region of RyR1 using a conformation-sensitive fluorescence probe [23]. The ASI region is downstream from the central MH-susceptible region (towards the C-terminal part of RyR1) and thus could interact with the same N-terminal or central domains to which DP4 and DP1 bind. This is unlikely however because DP1 and DP4 sensitize RyR1 to Ca^{2+} -activation, while neither of the PASI peptides change Ca^{2+} dependence, nor do the splice variants of RyR1 differ in their Ca^{2+} dependence [2]. These differences indicate that different regulatory mechanisms are associated with the interactions, and hence different interaction sites may be involved. In support of this conclusion, we have preliminary data showing that DP4 does not compete with the actions of PASI(–) (T. Kimura, N. Ikemoto and A. F. Dulhunty, unpublished work).

The peptides corresponding to the ASII region have no effect on channel activity suggesting either (i) that this region does not participate in an inter-domain interaction, or (ii), if it does, that the site is inaccessible to the peptides or the interaction is too strong to be interrupted. The DP corresponding to the region encompassing the CCD mutation P3527S did activate RyR1, but the mutant and wild-type peptides had the same effect on channel activity. Thus the CCD region, which is in an exon adjacent to ASI, may also be involved in an inter-domain interaction within RyR1, but the mutation does not appear to alter this interaction under our conditions (with $10\ \mu\text{M}\ \text{Ca}^{2+}$).

Inhibition of RyRs by high concentrations of the PASI peptides

Higher concentrations of PASI(+) inhibited RyR1 only at +40 mV, while PASI(–) inhibited at both –40 mV and +40 mV. This inhibitory effect was rapidly removed after washout, while the higher affinity activating effect remained. These results suggest that RyR1 has distinct sites for activation and inhibition. The stronger inhibitory effect at +40 mV is reminiscent of the voltage-dependent inhibitory action of high concentrations of the positively charged DHPR segment, peptide A [17]. The ASI peptides contain a positively charged sequence ($\text{K}^{3495}\text{KKRR}^{3499}$) adjacent to the ASI site that is similar to the basic sequence in peptide A. The positively charged residues in peptide A are likely to bind to negative charges close to the channel pore and block the channel [17,24]. The positively charged ASI peptides might also be pushed into the channel pore at positive potentials. Charge density is critical for inhibition by peptide A [24] and the stronger inhibition by PASI(–) [than by PASI(+)] may be explained by a higher positive charge density in that peptide. The PASI(+) sequence includes a negatively charged aspartate residue, absent from PASI(–), that would reduce the overall positive charge on PASI(+). In addition the five basic residues would impose a higher positive charge density on the smaller 25-residue PASI(–) peptide than on the larger 30-residue PASI(+) peptide. Thus the higher positive charge density of PASI(–) could account for its stronger inhibition.

In conclusion, we show for the first time that peptides PASI(+) and PASI(–), corresponding to sequences including the variably spliced ASI region of RyR1, increase the [^3H]ryanodine binding to native, purified and recombinant RyR1s in a dose-dependent manner without altering the Ca^{2+} dependence of [^3H]ryanodine binding. We also showed that the peptides enhance Ca^{2+} release from the SR and activate single RyR1 channels, particularly at –40 mV. PASI(–) had stronger activating effects than PASI(+) in all experiments. Both recombinant ASI(–)RyR1 and ASI(+)RyR1 were activated by the ASI peptides, but ASI(–)RyR1 was activated more than ASI(+)RyR1 by PASI(–). We previously showed that ion channels formed by the ASI(–)RyR1 splice variant are less active than those formed by

the ASI(+)RyR1 [2]. Our results in the present study suggest that a stronger inhibitory inter-domain interaction involving the ASI region is a possible mechanism for the lower activity of ASI(–)RyR1.

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