An RYR1 mutation associated with malignant hyperthermia is also associated with bleeding abnormalities

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Malignant hyperthermia is a potentially fatal hypermetabolic disorder triggered by halogenated anesthetics and the myorelaxant succinylcholine in genetically predisposed individuals. About 50% of susceptible individuals carry dominant, gain-of-function mutations in RYR1, whereas they have normal muscle function and no overt clinical symptoms. RyR1 mutations have also been found in skeletal muscle in patients with malignant hyperthermia susceptibility (MHS), but these mutations have no apparent clinical significance. MHS patients are susceptible to the development of a rhabdomyolysis–metabolic acidosis syndrome after exposure to halogenated anesthetics and muscle relaxants. The diagnosis of MHS is based on the detection of genetic mutations in RYR1, which encodes the skeletal muscle calcium release channel RYR1.

The MHS mutation associated with malignant hyperthermia (MHS RYR1Y522S) has been reported in isolated patients with MH susceptibility (MHS) (10). Dominant RYR1 mutations are commonly associated with malignant hyperthermia (MH), a severe pharmacogenetic reaction to halogenated anesthetics and muscle relaxants; exertional rhabdomyolysis/myalgia (ERM) (5); and the congenital myopathy central core disease (CCD). Recessive RYR1 mutations have been associated with the congenital myopathies multi-minicore disease (MmD), centronuclear myopathy (CNM), and congenital fiber type disproportion (6-8). Bleeding abnormalities have been reported in isolated patients with MH susceptibility (MHS) (9-11), and homozygous mouse embryos knocked in for the MHS RYR1 mutation Y522S (RYR1Y522S) display massive edema and subcutaneous blood effusions at birth, suggestive of a severe bleeding disorder with antenatal onset (12).

Vascular smooth muscle cells play an important role in primary hemo-

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INTRODUCTION

Hemostasis is a stepwise process that causes bleeding to stop and can be grossly divided into primary and secondary hemostasis and three distinct stages. Primary hemostasis comprises (i) an instant vascular response to injury, leading to transient vasoconstriction limiting immediate blood loss, and (ii) platelet activation and aggregation, prompted by plasma von Willebrand factor (vWF) and sustained through platelet granule release that also enhances the initial vasoconstriction. Primary hemostasis results in formation of a platelet plug aimed at provisionally stopping the bleeding. Secondary hemostasis involves (iii) the sequential activation of clotting factors present in the plasma in an inactive state through a cascade of reactions, ultimately leading to the formation of a fiber-like mesh called fibrin that surrounds the provisional platelet plug and causes definite blood coagulation (1).

Bleeding disorders are clinically and genetically heterogeneous and may affect all aspects of hemostasis. The most common causes are partial deficiencies of coagulation factors and fibrinolytic proteins, deficiencies in vWF, and platelet or connective tissue disorders (2). Nevertheless, mild bleeding disorders are common in the general population, but their precise incidence and their genetic background often remain unresolved (2,3).

Mutations in RYR1, the gene encoding the skeletal muscle Ca2+ channel ryanodine receptor type 1 (RyR1), have a calculated frequency of 1:2000 to 1:3000 (4). Dominant RYR1 mutations are commonly associated with malignant hyperthermia (MH), a severe pharmacogenetic reaction to halogenated anesthetics and muscle relaxants; exertional rhabdomyolysis/myalgia (ERM) (5); and the congenital myopathy central core disease (CCD). Recessive RYR1 mutations have been associated with the congenital myopathies multi-minicore disease (MmD), centronuclear myopathy (CNM), and congenital fiber type disproportion (6-8). Bleeding abnormalities have been reported in isolated patients with MH susceptibility (MHS) (9-11), and homozygous mouse embryos knocked in for the MHS RYR1 mutation Y522S (RYR1Y522S) display massive edema and subcutaneous blood effusions at birth, suggestive of a severe bleeding disorder with antenatal onset (12).
mechanism regulating their relaxation and contraction (13). In addition to InsP₃Rs, smooth muscle cells also express the three different RyR isoforms (13-17), and their Ca²⁺ homeostasis is more elaborate compared to many other excitable cell types. Whereas InsP₃Rs act as channels releasing Ca²⁺ from the endo(sarcoplasmic reticulum leading to smooth muscle cell contraction, the specific role of RyRs is more complex, and in contrast to striated muscle, RyRs are involved in smooth muscle cell relaxation (13, 18, 19). We hypothesized that there may be a correlation between the presence of dominant MH-related RyR1 mutations and mild bleeding abnormalities.

Here, we investigated the potential relationship between a mild bleeding disorder and the presence of dominant, gain-of-function RyR1 mutations; we then used the heterozygous RYR1Y522S mouse model to study the mechanism linking prolonged bleeding time to Ca²⁺ homeostasis in isolated smooth muscle cells. Our results indicated that gain-of-function RyR1 mutations mainly affected Ca²⁺ homeostasis of smooth muscle cells by increasing “Spark” (spontaneous calcium release events) activity. We also demonstrated that administration of the specific RyR1 antagonist dantrolene, which is clinically approved for the treatment of MH reactions (20, 21), reversed the bleeding phenotype by decreasing spark activity in vascular smooth muscle cells.

RESULTS

Human subjects carrying RyR1 mutations have a mild bleeding abnormality

To investigate bleeding in RyR1-associated myopathies, we invited RyR1-mutated individuals and their nonmutated relatives to complete a standardized questionnaire [molecular and clinical markers for the diagnosis and management of type 1 von Willebrand’s disease (MCDM1-VWD)] validated for the evaluation of bleeding disorders (Table 1). Patients had neuromuscular features of MH, ERM, and a congenital myopathy (CCD or MmD), or a combination of these features, associated mainly with dominant heterozygous RyR1 missense mutations. A proportion of patients were compound heterozygous for two allelic RyR1 mutations, reflecting that some RyR1 mutations can be dominant with regard to MH but can be recessive with regard to the congenital myopathy phenotype. The neuromuscular features of patients from families 1, 2, 4, 6, 7, and 8 and of their relatives have been previously reported (5, 22, 23). MCDM1-VWD bleeding questionnaires were obtained from 24 RyR1-mutated patients (12 females, 12 males) and 14 relatives without the familial RyR1 mutation (4 females, 10 males). Symptoms of abnormal bleeding were common in RyR1-mutated individuals, characterized by severe menorrhagia and postpartum hemmorhage in females and milder symptoms of epistaxis and easy bruising in males. One male patient reported spontaneous hematomas in association with muscle cramps, whereas one female patient reported an additional history of recurrent and unexplained miscarriages. Improvement of menorrhagia was reported in one female (patient 4.2) after prescription of sodium dantrolene, the specific RyR1 antagonist used clinically to reverse acute MH reactions (20, 21), for her severe ERM. Baseline hematological studies including evaluation of clotting factors and platelet aggregation studies were normal in all patients with symptoms of increased bleeding, except in patients 2.6 and 2.7 in whom abnormal vWF levels were found, and patient 5.1 who had evidence of abnormal platelet function; bleeding scores from these patients were excluded from further statistical analysis because an alternative hematological diagnosis could not be confidently ruled out. Eight of 21 (38%) patients included in the statistical analysis had a pathological bleeding score (≥4) on the questionnaire compared to 0 of 14 (0%) of controls (P = 0.0118, two-tailed Fisher’s exact test). The mean bleeding score for patients is shown in Fig. 1A.

A mouse model knocked in for a human RyR1 mutation associated with MHS shows prolonged bleeding times

To gain mechanistic insight linking RyR1 mutations to bleeding, we used the heterozygous RYR1Y522S MH-susceptible mouse (12). Applying a standardized test to accurately determine bleeding times in mice (24), we demonstrated that bleeding times in RYR1Y522S mice were two to three times longer than in their wild-type littermates (Fig. 1B, bleeding times in male mice; fig. S1, bleeding times in female mice). Intraportal dantrolene administration to wild-type mice before the bleeding test did not affect bleeding times. However, pretreatment of RYR1Y522S mice with dantrolene, but not with vehicle alone, reduced bleeding to the same times as those seen in their wild-type littermates (Fig. 1B).

Bleeding times depend on the contraction of injured blood vessels, platelet number and function, and the activation of circulating clotting factors. We have previously not found a difference in the number and function of circulating platelets between wild-type and RYR1Y522S mice (25). RyR1 is not detectable in platelets, virtually excluding disturbed platelet function as a potential cause for the bleeding disorder. Because wild-type and RYR1Y522S knock-in littermates only differ in their RyR1, we hypothesized that these differences in bleeding times could be due to changes in the contraction and relaxation properties of the smooth muscle cells lining the blood vessels. Vessel reactivity can be assessed using a vessel injury model; however, because of their small size (with a diameter of 0.26 ± 0.03 mm), we could not accurately measure changes in the diameter of the tail artery, which would be a direct indication of contraction/relaxation. Furthermore, because of their predisposition to MH, deep anesthesia was not possible in the RYR1Y522S knock-in mice, excluding the possibility of performing injury experiments in larger blood vessels. The fact that direct measurements could not be performed constitutes a limitation to our study. To demonstrate a cause-effect relationship between RyR1 mutations and vessel response, we used the pulsed wave Doppler and measured velocity of blood flow (26, 27), at a point 1 cm upstream from the incision of the tail artery, at a constant blood pressure (fig. S2). Velocity of blood is inversely related to vessel diameter (28), and therefore, changes in velocity reflect changes in the contraction and relaxation status of smooth muscle cells. Under baseline conditions, there were no differences in blood flow velocity through the caudal artery between wild-type and RYR1Y522S mice (Fig. 1C). However, in response to injury and more specifically at 8 to 10 min after incision, the velocity of blood flow in the tail artery of wild-type mice was significantly faster than in that of RYR1Y522S mice. This result indirectly indicates that vasoconstriction occurs to a larger extent in the tail arteries of wild-type than in RYR1Y522S mice.

Quantitative polymerase chain reaction (qPCR) experiments confirmed the expression of RyR1 in primary arterial vascular smooth muscle cells, which was normalized to DES, which encodes desmin, a skeletal and smooth muscle cell–specific protein (29). Cells in mouse aorta and tail artery express the RyR1 transcript, though as expected, to a much lower extent than skeletal muscle (Fig. 2A), and the presence of the RYR1Y522S mutation in the heterozygous state does not affect the expression of RyR1 (Fig. 2B) or of RyR2 and RyR3 (fig. S3). Reverse transcription PCR (RT-PCR) on mRNA isolated from tail arteries confirmed the expression of a mutated transcript in the heterozygous RYR1Y522S mice (Fig. 2C). Confocal immunohistochemical analysis on isolated primary vascular smooth muscle cells confirmed the presence of the specific smooth muscle marker actin (Fig. 2D, central panels). Analysis of the subcellular distribution of RyR1 using an antibody that, under our experimental conditions, does not cross-react with RyR2 (fig. S4) showed that RyR1s are localized close to the plasma membrane (Fig. 2E, red). This distribution is similar to that of the voltage-sensing dihydropyridine receptor Ca1.2, which is found on the plasma membrane of vascular smooth muscle cells (Fig. 2E, green) (30–32).
The subcellular distribution of RyR1 in isolated primary vascular smooth muscle cells is similar to that reported in pulmonary artery smooth muscle cells and in vas deferens (32–34); in the latter cells, localized RyR1-mediated calcium release from subplasmalemmal stores causes activation of BK channels, resulting in cellular hyperpolarization, leading to vasodilation (34, 35).

Table 1. Genetic details, neuromuscular features, MCMDD-1VWD bleeding questionnaire scores, and bleeding phenotypes from patients with RYR1-related myopathies and their healthy relatives. Abnormal MCMDD-1VWD bleeding scores (>4) are highlighted in red. Patient 1.1 was deceased, and the MCMDD-1VWD bleeding questionnaire was completed retrospectively on the basis of the information in the patient medical notes. Two dominant heterozygous MHS-related RYR1 mutations were running independently in families 5, 7, and 8. RYR1-mutated individuals in families 2 and 5 reported additional symptoms suggestive of smooth muscle involvement concerning both bladder (urinary incontinence, vesicoureteral reflux, and "spastic colon"). Neuromuscular features from families 1, 2, 4, 6, 7, and 8 have been previously reported. F, family; P/C, patient/control; S, sex; I, inheritance; NM, neuromuscular phenotype; RYR1 +/-, RYR1 mutation carrier state; BS, MCMDD-1VWD bleeding score; AR, autosomal recessive; AD, autosomal dominant.

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*Three patients (2.6, 2.7, and 5.1) were excluded from further statistical analysis because of abnormal vWF levels (2.6 and 2.7) and evidence of abnormal platelet function (5.1), respectively. †Patient 4.2 reported improvement of bleeding symptoms after commencement of dantrolene treatment, which was prescribed for exertional myalgia.
Calcium homeostasis in vascular smooth muscle cells isolated from the RYR1Y522S mouse is significantly different compared to that of their wild-type littermates

To investigate how RYR1 mutations affect Ca\textsuperscript{2+} homeostasis, we isolated primary vascular smooth muscle cells from mouse tail arteries. We used this cellular model, because bleeding times have been assessed on this tissue and these cells are responsible for the control of the vasomotor activity in the tail (35). The resting Ca\textsuperscript{2+} concentration was similar in primary vascular smooth muscle cells isolated from wild-type and RYR1Y522S mice (Fig. 3, A and B), though the latter cells showed significantly smaller total rapidly releasable Ca\textsuperscript{2+} stores compared to those isolated from wild-type littermates (Fig. 3C). In smooth muscle cells, local calcium release events termed “Ca\textsuperscript{2+} sparks” have been ascribed to the opening of single or clusters of RyR channels. Although they have little or no direct effect on contraction, they indirectly lead to vasodilatation through activation of BK channels (13, 18). We studied spark activity in isolated primary vascular smooth muscle cells isolated from tail arteries loaded with the fast calcium dye Fluo-4. The frequency of calcium sparks is significantly higher in cells from the RYR1Y522S mice than in those from their wild-type littermates (Fig. 3, D and E). Representative videos of sparks occurring in cells from wild-type and RYR1Y522S are shown in videos S1 and S2, respectively. Detailed analysis of spark amplitude and kinetics revealed small but significant differences between cells isolated from wild-type and RYR1Y522S knock-in mice (table S1).

We next used several drugs including RyR1 blockers and the InsP\textsubscript{3} receptor antagonist xestospongin C to determine the origin of these spontaneous Ca\textsuperscript{2+} release event. Sparks were extinguished in wild-type and RYR1Y522S primary vascular smooth muscle cells by preincubation with ryanodine (Fig. 3E and video S3). Additionally, preincubation with dantrolene significantly decreased spark frequency in both wild-type and RYR1Y522S cells (Fig. 3E and video S4). This effect was not observed when cells were incubated with the InsP\textsubscript{3} antagonist xestospongin C (video S5). These results indicated that RYR1 was present in primary vascular smooth muscle cells, that RYR1 mutations leading to MHS were not only present in skeletal muscle but also in arterial smooth muscle cells, and that the RYR1Y522S mutation increased the frequency of spark events. In skeletal muscle, RYR1 mutations associated with MHS are gain-of-function mutations leading to an increased sensitivity to activating stimuli, resulting in prolonged and sustained muscle contractions (37–40). Our results suggested that a similar mechanism did not operate in smooth muscle cells, because RYR1 mutations caused prolonged rather than shorter bleeding times, which would be expected if mutated RyR1 caused a gain of function in primary vascular smooth muscle cells. In skeletal muscle, excitation-contraction coupling depends on the mechanical interaction between the voltage-sensing dihydropyridine receptor and RyR1 (41), whereas in smooth muscle cells the functional unit is made up of dihydropyridine receptors, ryanodine receptors, and BKCa channels (13, 18). RyR-dependent Ca\textsuperscript{2+} sparks activate BKCa channels causing plasma membrane hyperpolarization, thereby decreasing Ca\textsuperscript{2+} influx through the dihydropyridine receptor and leading to smooth muscle relaxation (13, 18).

We found that vascular smooth muscle cells from RYR1Y522S mice were significantly more hyperpolarized than those from wild-type mice, which had membrane potentials in line with previous reports (Fig. 4A) (18). Furthermore, the membrane potential of cells from the RYR1Y522S mice was reverted to control values after treatment with dantrolene (Fig. 4A).

**DISCUSSION**

Here, we demonstrate that gain-of-function RYR1 mutations associated with MH and/or exertional rhabdomyolysis cause a mild but distinct bleeding disorder in humans and a corresponding phenotype in a murine model of MH, by affecting vascular smooth muscle cell calcium homeostasis. A model showing how RYR1 mutations affect smooth muscle cell contraction is shown in Fig. 4B. Characteristic features of the human bleeding phenotype were female preponderance, presentation with marked menorrhagia, postpartum bleeding, and a history of miscarriages in the context of normal coagulation tests. Although in our study we evaluated...
Fig. 2. RyR1 is present at the mRNA and protein level in aortae and tail arteries and is localized close to the plasma membrane. (A) RyR1 expression in skeletal muscle (SKM), aorta (Ao), tail (TA), and mesenteric artery (Ma) was normalized to DES content, which is present in skeletal and smooth muscle cells (29). The numbers below the bars indicate number of mice used for the analysis. (B) The presence of the Y522S mutation does not affect RyR1 expression as determined by qPCR. The numbers below the bars indicate number of mice used for the analysis. (C) Total RNA was extracted from purified aortas, and the expression of RyR1 was evaluated by RT-PCR. Digestion of the amplified RyR1 cDNA (complementary DNA) from WT mice yielded the uncut band of about 376 base pairs (bp). Digestion of the cDNA from heterozygous RyR1<sub>Y522S</sub> mice yielded two bands of 276 and 100 bp plus the uncut 376-bp band from the WT allele. Image is representative of two independent experiments, with two mice analyzed per genotype. (D) Phase contrast of an isolated primary vascular smooth muscle cell (left); confocal immunofluorescence on the same cell using a mouse antibody specific for smooth muscle actin followed by anti-mouse Alexa Fluor 568 (middle) or DAPI (4′,6-diamidino-2-phenylindole) to localize the nucleus (right). Images are representative of cells from four mice per genotype. (E) Phase contrast on an isolated primary vascular smooth muscle cell (leftmost image). Confocal immunofluorescence of the same cell stained with mouse monoclonal anti-RyR1 antibody followed by anti-rabbit Alexa Fluor 488 (green), and DAPI (blue). Composite image showing co-localization of RyR1 and Ca<sub>2+</sub>1.2 (rightmost image). Images are representative of cells from four mice per genotype. Scale bars, 10 μm.

a relatively small sample of RyR1<sub>-mutated</sub> patients and controls, the clinical data are consistent with the data obtained from the corresponding mouse model carrying the murine equivalent of a human MH-related RyR1 mutation (12). The large variation in the bleeding times of RyR1<sub>Y522S</sub> mice may reflect different extents of contraction of the skeletal muscles within the mouse tail when placed at 37°C. Alternatively, it may be due to intrinsic structural properties of the tetrameric RyR1 complex, which may or may not contain RyR1 mutants. A variation in the proportion of protomers encoded by wild-type and mutated alleles within the tetrameric RyR1<sub>Ca<sup>2+</sup></sub> channel complex affects the biophysical properties of the RyR1 complex (42) and therefore may account for the variability of the calcium signals responsible for vascular responses. This explanation agrees with the data obtained from wild-type mice, which exhibit more consistent bleeding times, presumably because of the homogeneous protomeric composition within the RyR1 tetrameric protein complex. Our mouse data are also consistent with our findings from humans, in that higher mean bleeding scores correlated with greater variability in RyR1<sub>-mutated</sub> patients compared to relatives without the familial RyR1 mutation. Prolonged bleeding times in the RyR1<sub>Y522S</sub> mice and in one patient were reversed by administration of the specific RyR1 antagonist dantrolene, supporting an association between the observed bleeding phenotype and the RyR1 mutant state. These results suggest that dantrolene could be administered as a pharmacological treatment for RyR1-related bleeding disorders and, potentially, other causes of prolonged bleeding due to impaired vascular smooth muscle cell contractility rather than primary platelet or coagulation factor abnormalities. Considering that carriers of MH-associated RyR1 mutations are frequent (1:2000 to 1:3000) (4) but often asymptomatic unless exposed to triggering agents, such mutations may account for common mild bleeding abnormalities in humans that are currently without genetic explanation. Notably, we found evidence of abnormal platelet function and borderline reduced vWF levels in one and two RyR1-mutated patients within our cohort, respectively. These patients were not considered for the final statistical analysis, because we could not exclude a primary platelet or vWF abnormality as an alternative explanation for the observed bleeding phenotype. However, similar findings of mildly abnormal platelet function and vWF levels have been reported in other nonhematological disorders associated with prolonged bleeding such as collagen disorders (43) or Noonan syndrome (44), where the primary defect does not concern platelets or coagulation factors and may therefore well be a secondary feature also in the RyR1-related bleeding disorder reported in this study.

Our observations are consistent with emerging knowledge concerning the dynamic and complex role of BK<sub>Ca</sub> channels and RyR in tissue-specific vasoregulation. Pressure-induced RyR activation in mesenteric resistance arteries has a strong vasodilatory effect, mediated by Ca<sub>2+</sub>2-induced activation of BK<sub>Ca</sub> channels and, possibly, Ca<sub>2+</sub>2-induced inactivation of L-type voltage-dependent Ca<sub>2+</sub> channels (45). Considering that RyR2 rather than RyR1 is the predominant RyR isoform in murine mesenteric arteries (17), the RyR1-associated arterial smooth muscle cell phenotype seems unexpected.
However, in this context, a distinct spatial distribution may be more important than a relative amount of RyR isoforms. As we have demonstrated in murine arterial smooth muscle cells, RyR1s are predominantly found subsarcolemmally, and altered activity of the mutant RyR1s may be more consequential because of their close proximity to BKCa channels. We also cannot exclude the possibility that the release of Ca\textsuperscript{2+} from mutated RyR1 channels may facilitate the opening of adjacent RyR2 channels through the regenerative Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism (46).

Our study reports a nonneuromuscular phenotype, that is, abnormal bleeding, in RYR1-related myopathies and is likely to inform the surveillance and anticipatory management of patients affected by these conditions. Considering that RyR1 is found in many types of tissues, non-neuromuscular manifestations of RYR1 mutations are not unexpected but may have been overlooked, because they are mild and/or involve symptoms that are not necessarily considered part of a neuromuscular disorder. The marked female preponderance in our series with severe menorrhagia and/or postpartum hemorrhage raises the question of more extensive smooth muscle cell involvement in RYR1-related myopathies. This additional smooth muscle cell involvement may confound the observed bleeding phenotype, in a gender-specific manner in particular on the uterine level, considering that smooth muscle cells also play an important role in uterine vasoregulation and myometrial contraction (47, 48).

Patients from two of our families reported prominent bowel and bladder involvement (urinary incontinence, vesicoureteral reflux, and spastic colon), suggesting a more widespread smooth muscle cell involvement, also in keeping with a role of both RyRs and BKCa channels in bladder smooth muscle signaling (49). Although it is beyond the goal of the present study to specifically investigate bleeding abnormalities in patients with RYR1 mutations, these observations should prompt future investigations into...
Membrane potential was significantly different between WT and RYR1Y522S mice. (were either left untreated (control) or pretreated with 20 mM dantrolene (10 mg/kg) or vehicle alone (saline solution) an hour before bleeding time determination. The resting membrane potential was measured using the potentiometric probe bis-oxonol in vascular smooth muscle cells from WT mice (seven controls, two +dantrolene) and RYR1Y522S mice (four controls and two +dantrolene) (see also fig. S5). n indicates the number of individual cells that were assessed. Cells were either left untreated (control) or pretreated with 20 mM dantrolene for 30 min. *P < 0.05, Student's t test, mean resting membrane potential was significantly different between WT and RYR1Y522S mice. (B) Schematic representation of the mechanism leading to prolonged bleeding time due to RYR1 mutations. In normal conditions (left panel), early vasoconstriction occurring immediately after injury reduces bleeding (in coordination with platelets and coagulation factors not shown here). Mutations in RYR1 (right panel) result in hyperpolarization of the smooth muscle cell (SMC) membrane potential, reducing the ability of vessels to contract and ultimately increasing the bleeding time. PM, plasma membrane; SR, sarcoplasmic reticulum; BK, calcium-activated potassium channel. Blue balls, potassium; peach balls, calcium sparks; RyR1Y522S, MH knock-in.

the association between RYR1 mutations and a more generalized smooth muscle dysfunction on both the clinical and the cellular level.

MATERIALS AND METHODS

Patients
Patients were identified through the participating tertiary neuromuscular and MH centers. Genetic testing and hematological studies including evaluation of clotting factors and platelet aggregation were performed as part of the routine diagnostic workup. Patients were invited to complete the MCMDM-1VWD bleeding questionnaire, a validated and widely used diagnostic tool in the evaluation of bleeding disorders (50). MCMDM-1VWD bleeding questionnaires were scored independently by two clinicians blinded to the genetic status (mutated or nonmutated) of the proband. The study received UK Research Ethics Committee approval (15/WS/204, granted by the West of Scotland REC 5). Patients gave informed consent for anonymized publication of their clinical information.

Animal model
Experiments were carried out on 7- to 12-week-old heterozygous RYR1Y522S knock-in mice and their wild-type littermates. The mouse model was generated by Chelu et al. (12) and was a gift from S. Hamilton (Baylor College of Medicine, Houston, TX). Experimental procedures were approved by the Veterinary Cantonal Authorities (permit numbers 1728 and 1729).

Bleeding time assay
Bleeding time was determined according to Liu et al. (24). Intraperitoneal injections with dantrolene (10 mg/kg) or vehicle alone (saline solution) were administered 1 hour before bleeding time determination.

Pulsed wave Doppler
Male mice (15 to 20 weeks old) were lightly anesthetized with ketamine/xylazine (ketamine (95 mg/kg) + xylazine (10 mg/kg)) and placed on a heated blanket at 26°C. Blood flow velocity in the tail artery was measured using a pulsed wave Doppler ultrasound system (Vevo 2100, VisualSonics Inc.) at high frequency (32 MHz, MS-550D transducer). The probe was positioned where a consistent signal could be detected at the proximal end of the tail, about 1 cm from the base of the tail. Velocity of blood flow was measured before and at 6 time points after an incision was made on the tail artery; measurements were made 1 cm upstream from the incision site. The velocity was measured during five consecutive cardiac cycles, and the mean blood flow velocity per mouse was plotted at each time point before and after incision. The experimenters were blinded and had no previous knowledge of the genotype of the mice. Data were analyzed and plotted using the GraphPad Prism V 6.0 and OriginPro 8.6.0 softwares.

Blood pressure measurements
Systolic blood pressure was monitored in conscious mice using a noninvasive tail-cuff photoplethysmography system (BP-2000 Blood Pressure Analysis System, Visitech Systems). Blood pressures were assessed in a quiet environment at the same time of day, for a period of 7 days before the actual measurements, to allow the mice to adjust to the protocol. Each symbol represents the blood pressure value of individual mice.

Isolation of single smooth muscle cells
Primary vascular smooth muscle cells were isolated essentially as described (51) using an enzymatic cocktail containing papain (9.6 U/ml) (Sigma, #P4762), collagenase (1200 U/ml) (Worthington, #LS004176), elastase (2.58 U/ml) (Worthington, #LS002292), 0.6% bovine serum albumin, and soybean trypsin inhibitor (1 mg/ml) (Worthington, #LS003587).

Calcium imaging and Spark analysis
The resting calcium fluorescence was measured using the calcium indicator Fura-2 AM (Invitrogen) (25), and the total amount of Ca2+ present in the rapidly releasable intracellular stores was determined as previously described (52). Calcium sparks were measured in cells loaded with 5 µM Fluo-4 using a Nikon A1R laser-scanning confocal microscope (Nikon Instruments Inc.) with a 60× oil immersion Plan Apo VC Nikon objective (numerical aperture, 1.4). Five-second-duration linescan images (x,t) were acquired in resonant mode at super high temporal resolution (76800 fps) with 512 pixels (0.05 µm/pixel) in the x direction and 39936 pixels (0.126 ms/pixel) in the t direction using a pinhole size of 72.27 µm. Four

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to five images were taken at different positions across each cell. Fluo-4 was excited with a laser at 487 nm, and the fluorescence emitted at 525 ± 25 nm was recorded. Images were analyzed using the open-source image processing software Fiji (53). After binning 4× in the temporal axes, linescan images were run with the automated sparks processor plug-in SparkMaster (54).

**Immunofluorescence**

Cells were fixed with ice-cold methanol/acetoaceton (1:1) for 30 min and processed as previously described (52). The primary antibodies used were mouse anti–smooth muscle actin (Sigma, #A2228), mouse anti-RyR1 (Thermo Scientific, #MA3-925), and rabbit anti-Cav1.2 (Santa Cruz Biotechnology, #sc-25686). The secondary conjugates were anti-mouse Alexa Fluor 568 and chicken anti-rabbit Alexa Fluor 488 (LuBioScience). Nuclei were counterstained with DAPI (25 to 30 μM) for 5 min before mounting. Cells were examined by confocal microscopy using a Nikon A1R laser-scanning confocal microscope with a 60× oil immersion Plan Apo objective.

**Real-time qPCR and RT-PCR**

Total RNA was extracted and treated with deoxyribonuclease I (Invitrogen) as previously described (25). After RT using 1000 to 1500 ng of RNA, cDNA was amplified by real-time qPCR with a 7500 Fast Real-Time PCR system (Applied Biosystems) using SYBR Green technology (Fast SYBR Green Master Mix, Applied Biosystems) and the following primers: *RyR1*, 5′-CTACTCTCAATGGAAAGCAG-3′ (forward) and 5′-AGCAGATGAGCATAACGAA-3′ (reverse); *DES*, 5′-TGAGACCATCGGCGCTAA-GA-3′ (forward) and 5′-GGTCGGTATTCATCCTCC-3′ (reverse). Gene expression was normalized to desmin (*DES*) content, because this marker is specifically expressed in muscle cells (29, 55). *RyR1* expression levels in mouse aorta, tail artery, and mesenteric artery were compared to *RyR1* expression in aorta was also investigated by semiquantitative RT-PCR as previously described (25).

**Membrane potential measurements**

Membrane potential measurements were performed using the potentiometric fluorescence dye bis-oxonol as described (56). Briefly, isolated arterial smooth muscle cells were plated on laminin/gelatin (1:10 ratio) precoated coverslips and allowed to attach for 20 min in a modified Krebs-Ringer solution (140 mM NaCl, 0.5 mM CaCl₂, 5 mM KCl, 1 mM MgSO₄, 10 mM Hepes, 1 mM Na₂HPO₄, 5.5 mM glucose, and 1% albumin). Cells were loaded with the potentiometric fluorescence dye bis-oxonol (Molecular Probes, #B3438) in the presence or absence of 20 μM dantrolene at room temperature for 30 to 40 min. To calibrate the membrane, potential cells were incubated with the Na⁺ ionophore gramicidin (10 μM) (Sigma, #G-5002) and then exposed to different Na⁺-containing solutions (fig. S5). The concentration ratios of Na⁺ and choline were varied to maintain [Na⁺] + [choline] = 144 mM. Theoretical values for membrane potential were calculated according to the formula Em = 60 log((Na⁺) + [K⁺]) / ((Na⁺) + [K⁺]) and considering the internal concentration of Na⁺ and K⁺ as described by Nelson et al. (18). Fluorescence was recorded every 0.5 min with a Nikon A1+ confocal microscope, using a 60× oil objective. Samples were illuminated with a sapphire laser at 488 nm, and the fluorescence emitted at 525/50 nm was recorded. Fluorescence images were processed using the open-source software Fiji.

**Statistical analysis and graphical software**

Statistical analysis on the MCDM-1VWD bleeding questionnaires was performed by converting raw bleeding scores to binary numbers for use in Fisher's exact test with two-tailed *P* value (negative screening test ≤3, positive screening test ≥4). Mean scores were compared using Student's *t* test.

For all other experiments, statistical analysis was performed using the Student’s *t* test; means were considered statistically significant when the *P* value was <0.05. Data were processed, analyzed, and plotted using the software OriginPro 8.6.0 (OriginLab Corporation). Images were assembled using Adobe Photoshop CS (version 8.0).

**SUPPLEMENTARY MATERIALS**

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**REFERENCES AND NOTES**


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An RYR1 mutation associated with malignant hyperthermia is also associated with bleeding abnormalities
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