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The 12 kDa FK506-binding protein, FKBP12, modulates the Ca^{2+}-flux properties of the type-3 ryanodine receptor

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Summary

We have characterised the functional regulation of the type-3 ryanodine receptor by the 12 kDa FK506-binding protein. Wild-type type-3 ryanodine receptor and mutant type-3 ryanodine receptor in which the critical valine at position 2322 in the central 12 kDa FK506-binding protein binding site was substituted by aspartate, were stably expressed in human embryonic kidney cells. In contrast to the wild-type receptor, the mutant receptor was strongly impaired in binding to immobilised glutathione S-transferase 12 kDa FK506-binding protein. Caffeine-induced $^{45}Ca^{2+}$-efflux was markedly increased in cells expressing mutant type-3 ryanodine receptor whereas the maximal-releasable Ca^{2+} was not affected. Confocal Ca^{2+} imaging provided clear evidence for a much higher sensitivity of the mutant receptor, which showed global Ca^{2+} release at about 20-fold lower caffeine concentrations than the wild-type receptor. Spontaneous Ca^{2+} sparks were observed in both wild-type- and mutant-expressing cells but the number of sparking cells was about 1.5-fold higher in the mutant group, suggesting that the degree of FK506 binding controls the stability of the closed state of ryanodine receptor channels. Furthermore, overexpression of 12 kDa FK506-binding protein decreased the number of sparking cells in the wild-type-expressing cells whereas it did not affect the number of sparking cells in cells expressing the mutant receptor. Concerning spark properties, the amplitude and duration of Ca^{2+} sparks mediated by mutant channels were significantly reduced in comparison to wild-type channels. This suggests that functional coupling between different mutant type-3 ryanodine receptor channels in a cluster is impaired. Our findings show for the first time that the central binding site for the 12 kDa FK506-binding protein of type-3 ryanodine receptor, encompassing the critical valine proline motif, plays a crucial role in the modulation of the Ca^{2+} release properties of the type-3 ryanodine receptor channel, including the regulation of both global Ca^{2+} responses and spontaneous Ca^{2+} sparks.

Key words: 12 kDa FK506-binding protein, Ryanodine receptor type 3, Ca^{2+} signalling

Introduction

Release of Ca^{2+} from intracellular Ca^{2+} stores is mainly mediated by two distinct families of tetrameric intracellular Ca^{2+}-release channels, ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (Ins(1,4.5)P_3Rs) (Berridge et al., 2000). Both channels are encoded by three different genes, leading to the expression of three different isoforms. The activity of these channels is tightly regulated by a plethora of cellular factors, such as Ca^{2+}, pH, redox state, ATP and associated proteins (Mackrill, 1999; Patel et al., 1999; Bultynck et al., 2003).

The type-1 RyR (RyR1) is predominantly expressed in skeletal muscle cells and is physiologically activated upon depolarisation of the plasma membrane by direct interaction with the dihydropyridine receptor. The type-2 RyR (RyR2) is predominantly expressed in cardiac muscle cells and is physiologically activated by Ca^{2+} influx through voltage-dependent L-type channels. The type-3 RyR (RyR3) is ubiquitously expressed in many cell types at very low levels, with the exception of diaphragm, neurons and skeletal muscle cells. It is physiologically activated by Ca^{2+}-induced Ca^{2+} release. The Ca^{2+}-flux properties of the RyR1 and RyR2 are modulated through association with FK506-binding proteins (FKBPs), which belong to the class of immunophilins (Ondrias et al., 1998). It was shown that the 12 kDa FKBP (FKBP12) strongly associated with RyR1 with a stoichiometry of 4 FKBP12 molecules per tetrameric channel (Jayaraman et al., 1992). FKBP12 was essential for the cooperativity among the four different subunits of the RyR1 channel and for the stabilisation and activation of the channel (Timerman et al., 1993; Brillantes et al., 1994). Furthermore, Marks and co-workers demonstrated that FKBP12 was also involved in

the coupled gating between neighbouring RyR1 channels, inducing the simultaneous opening and closing of adjacent channels (Marx et al., 1998). Valine proline residues at position 2461-2462 appear to be key residues for the binding of FKBP12 to the RyR1 (Gaburjakova et al., 2001). Mutation of valine to glutamate at this site inhibited FKBP12 binding and subsequently changed the single-channel properties of the RyR1 channel.

Similarly to RyR1, FKBP12.6, a related immunophilin isoform, was shown to be crucial for the proper functioning of the RyR2 channel and the coupled gating among neighbouring channels (Timerman et al., 1994; Marx et al., 2001; George et al., 2003). Using a yeast-two hybrid screening with short fragments of RyR2, Marx and co-workers found that a similar isoleucine proline dipeptide (position 2427-2428) in RyR2 was crucial for FKBP12.6 binding (Marx et al., 2000). Chen and co-workers, however, recently showed that mutations in this isoleucine proline motif neither abolishes FKBP12.6 binding nor affects Ca^{2+}-flux properties. They found that the region between amino acids 307 and 1937 was indispensable for FKBP12.6 binding to RyR2, indicating that the major FKBP12.6 binding site for RyR2 was located in the N-terminal part of the receptor (Masumiya et al., 2003). Very recently, these authors postulated that a region between residues 1815 and 1855, adjacent to divergent region 3 in RyR2, is crucial for FKBP12.6 binding to RyR2 (Zhang et al., 2003). In addition to these findings, Zissimopoulos and Lai found that a large C-terminal fragment, encompassing all 10 transmembrane-spanning domains of RyR2, was able to interact with FKBP12.6, whereas other fragments were unable to do so (Zissimopoulos and Lai, 2002).

Recently, we have demonstrated that the RyR3, which is involved in many cellular processes, including the development of neonatal skeletal muscle, astrocyte migration and spatial learning, was a target for FKBP12 and FKBP12.6 (Bultynck et al., 2001a; Bultynck et al., 2001b). We found that a valine proline dipeptide in RyR3 (position 2322-2323) was crucial for FKBP12 and FKBP12.6 binding to the channel, but the significance of these residues for Ca^{2+} release through RyR3 was not investigated (Bultynck et al., 2001b). Taking into account the controversial data on the functional FKBP12.6-binding site on RyR2 and the limited amount of data on RyR3, the functional role of the proposed FKBP12 binding site for Ca^{2+} release through the RyR3 has yet to be defined.

In this study, we have investigated the modulation of the Ca^{2+}-flux properties of the RyR3 by FKBP12 through mutation of the high-affinity FKBP12 binding site (V2322D). This single point mutation in the FKBP12-binding motif is sufficient for the loss of the high-affinity interaction between RyR3 and FKBP12 and is accompanied with altered Ca^{2+}-release properties of the RyR3 channel.

Taken together, our data indicate that the central valine proline motif at position 2322-2323 is indispensable for the proper regulation of RyR3 activation.

Materials and Methods

Cloning of the wild-type type-3 ryanodine receptor (^{wt}RyR3)-, mutant type-3 ryanodine receptor (^{V2322D}RyR3)- and FKBP12-cDNA, cell culture and transfection

Cloning of the ^{wt}RyR3 and ^{V2322D}RyR3 cDNAs into the pcDNA3.1 mammalian expression vector and culture of human embryonic kidney cells (HEK293 cells) were performed as described previously (Bultynck et al., 2001b; Rossi et al., 2002). FKBP12-cDNA was subcloned into the *Nhe*I- and *Bam*HI-digested pcDNA3.1/Zeo(+) mammalian expression vector. DNA transfection of the ^{wt}RyR3 and ^{V2322D}RyR3 cDNA was carried out using the calcium phosphate method. Cells (8×10^5) were plated on a 100 mm^2 tissue culture dish 24 hours before transfection. One hour before DNA addition, fresh medium was added to the cells. RyR3 expression vector (10 μg) was diluted in HBS (5 g/l Hepes, 8 g/l NaCl, pH 7.1) supplemented with 0.7 mM Na_2HPO_4, 0.7 mM NaH_2PO_4, 120 mM $CaCl_2$ and incubated for 30 minutes. For stable transfection, geneticin sulphate G418 (Life Technologies, Groningen, The Netherlands) was added 48 hours after transfection, at a final concentration of 800 μg/ml. Single colonies were transferred to a 96-well multiplate, expanded and tested for RyR expression. ^{wt}RyR3- and ^{V2322D}RyR3-expressing cells were further transfected using Fugene (Roche, Mannheim, Germany) with FKBP12 cDNA containing pcDNA3.1/Zeo(+) vector. Permanent cell lines were obtained by subculturing the transfected cells in the presence of 250 μg/ml Zeocin (Invitrogen, Carlsbad, CA). For Ca^{2+} fluxes, HEK293 cells were seeded in gelatine-coated 12-well plates (Costar, MA; 4 cm^2) at a density of 10^5 cells/well and were analysed 5 days after seeding. For Confocal Ca^{2+} measurements, cells were plated on 2-well chambered coverglasses (Labtek, Nunc Inc., NY) at a density of 5000-15,000 cells/well and used between 4 and 9 days after plating.

Microsomal proteins preparation

Cells were resuspended in homogenisation medium, containing 10 mM Tris-HCl pH 7.4, 1 mM EGTA and protease inhibitors (0.83 mM benzamidine, 0.23 mM PMSF, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.5 μg/ml pepstatin A), sonicated with a probe sonicator (MSE, Crawley, Surrey, UK) on ice for three times 20 seconds at an amplitude of 15 μm and centrifuged at 125,000 ***g*** for 25 minutes. Microsomal pellets were resuspended in 20 mM Tris-HCl pH 7.4, 300 mM sucrose and protease inhibitors. Protein concentrations were determined by the Lowry assay (Lowry et al., 1951) and samples were stored at –80°C. Treatment of RyR3-expressing microsomes with 25 μM FK506 was performed at 37°C for 40 minutes and terminated by centrifugation (25 minutes, 125,000 ***g***). The supernatant and the microsomal pellet were further analysed by western blotting.

Western blot analysis

For immunological detection of RyR3 proteins, microsomal preparations were separated on Tris-acetate 3-8% NUPAGE® gels in Tris-acetate buffer (Invitrogen, Merelbeke, Belgium) by running the samples for 100 minutes at 150 V. Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA) using transfer buffer (Invitrogen, Merelbeke, Belgium) for 2 hours at 30 V. For immunological detection of FKBP12, microsomal preparations were separated on Bis-Tris 4-12% NUPAGE® gels in MES-SDS buffer by running the samples for 35 minutes at 200 V. Proteins were transferred to Immobilon P membranes, using transfer buffer (Invitrogen, Merelbeke, Belgium) supplemented with 20% (v/v) methanol for 75 minutes at 30 V. Membranes were blocked for 1 hour with PBS supplemented with 0.1% (v/v) Tween 20 and 5% (w/v) non-fat milk powder. After subsequent incubation with the primary antibodies and alkaline phosphatase-linked secondary antibodies, proteins were visualised by incubation with Vistra™ ECF substrate (Amersham Biosciences, Uppsala, Sweden) and scanned with the FluorImager Storm 840 (Molecular Dynamics, Uppsala, Sweden). RyR3 proteins were detected with m34C, a mouse monoclonal antibody against RyR proteins (Affinity BioReagents, Golden, CO), whereas FKBP12 proteins were detected with a rabbit polyclonal FKBP12 antibody (Affinity BioReagents, Golden, CO).

Construction of glutathione S-transferase (GST) fusion proteins, encoding FKBP12 and GST-FKBP12 affinity chromatography

cDNA encoding human FKBP12 was cloned in the *Bam*HI and *Eco*RI sites of the pGEX-2T plasmid as described previously (Bultynck et al., 2001a). GST-FKBP12 was purified by glutathione-Sepharose 4B chromatography as described previously (Bultynck et al., 2001a). The GST-FKBP12 and GST-FKBP12.6 affinity-chromatography assay was done as described previously (Bultynck et al., 2001a), except that 80 μg GST or GST-FKBP12 was used for each pull-down experiment.

$^{45}Ca^{2+}$ fluxes on non-permeabilised HEK293 cells

Experiments were carried out with confluent monolayers of HEK293 cells on the fifth day after plating. The 12-well plates were fixed on a thermostatic plate at 25°C. The culture medium was aspirated, and the cells were washed with modified Krebs solution (135 mM NaCl, 5.9 mM KCl, 0.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.6 mM Hepes, 11.5 mM glucose, pH 7.3). Ca^{2+} loading was performed in modified Krebs solution, supplemented with $^{45}Ca^{2+}$ (23 μCi/ml) for 45 minutes at room temperature. After the $^{45}Ca^{2+}$-loading phase, cells were rapidly washed with 1 ml Ca^{2+}-free efflux medium, i.e. modified Krebs solution, supplemented with 1 mM EGTA. This medium (500 μl) was replaced every 2 minutes. At the end of each experiment, the $^{45}Ca^{2+}$ remaining in the cells was measured by incubating the cells with 1 ml of 2% (w/v) SDS solution for 30 minutes. The time course of the $^{45}Ca^{2+}$ efflux was calculated by summing, in reverse order, the amount of $^{45}Ca^{2+}$ remaining in the cells at the end of the efflux and the amounts of $^{45}Ca^{2+}$ collected during the successive time intervals. Data were plotted as fractional loss, i.e. the amount of $^{45}Ca^{2+}$ present in the efflux medium during a 2-minute time period, divided by the total $^{45}Ca^{2+}$ content of the cell at that time.

Confocal Ca^{2+} imaging

RyR3-expressing HEK293 cells were loaded with the fluorescent Ca^{2+} indicator fluo-4 by incubating the cells in standard salt solution containing 10 μM of the acetoxymethyl (AM) ester of the dye (fluo-4 AM; Molecular Probes, Leiden, The Netherlands) for 45 minutes at room temperature. The standard salt solution contained 150 mM NaCl, 6 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes and 10 mM glucose, pH 7.4. After loading, the cells were washed three times with the standard saline and transferred to a laser-scanning confocal system equipped with an inverted microscope (Noran Oz®; Nikon TE 300) with a 40× oil-immersion objective (NA 1.3). Fluo-4 was excited with the 488 nm line of an argon ion laser and the emitted fluorescence was measured at wavelengths >500 nm. Data were acquired at a frequency of 7.5 (global Ca^{2+} responses) and 30 Hz (elementary Ca^{2+} signals). Data were stored directly on the hard disk of a computer. The confocal slit was set to 50 μm. All experiments were carried out at room temperature and caffeine was applied using a fast perfusion system.

Fluorescence signals collected from regions covering the cell soma or fluorescence changes in small regions of interest were analysed off-line. Background-corrected fluorescence traces were calculated as % ΔF/F, i.e. fluorescence increase divided by average pre-stimulus fluorescence. Amplitude and rate of rise are expressed as mean±s.e.m. (*n* is the number of cells). Statistical significance was determined by an independent Student's *t*-test. Values were considered significantly different when $P \leq 0.05$. For spark amplitude and spark duration, *n* is the number of sparks, whereas for spark frequency, *n* is the number of fully analysed traces. To allow direct comparison between ^{wt}RyR3- and ^{V2322D}RyR3-transfected cells, cells were grown under identical conditions including cell density and passage number.

Results

Expression of ^{wt}RyR3 and ^{V2322D}RyR3 in HEK293 cells, and interaction with FKBP12

Expression vectors containing ^{wt}RyR3 or ^{V2322D}RyR3 cDNAs were transfected into HEK293 cells and different stable clones were obtained following geneticin selection. Expression was verified in all selected clones by western blot analysis using isoform-specific RyR antibodies. Fig. 1A shows that the microsomes prepared from ^{wt}RyR3- and ^{V2322D}RyR3-expressing clones had similar expression levels of RyR3 protein and that there was no indication of significant degradation of the expressed proteins. These clones were used for further functional characterisation. In agreement with previous reports (Tong et al., 1999), untransfected HEK293 cells did not show expression of endogenous RyR and were used as a negative control. Using western blot analysis with an antibody against FKBP12 (Fig. 1A), we found that FKBP12 levels in microsomes from ^{wt}RyR3-expressing cells were

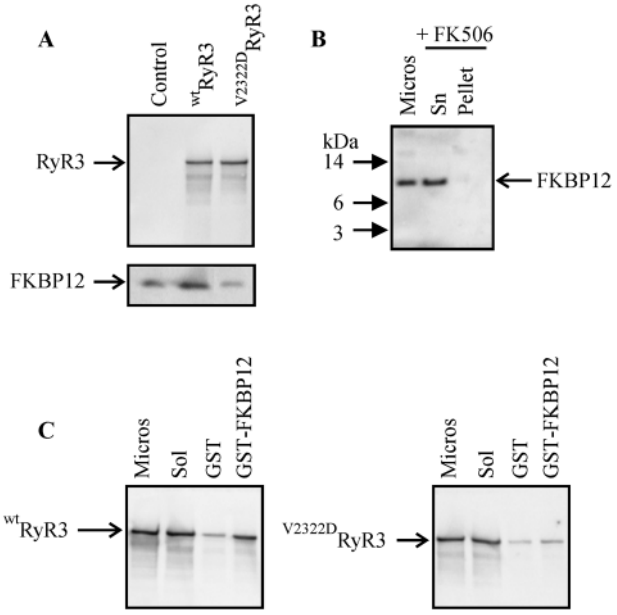

Fig. 1. Expression and FKBP12-binding properties of ^{wt}RyR3 and ^{V2322D}RyR3 channels. (A) RyR3-expression levels (upper blot) and FKBP12-expression levels (lower blot) were determined in the microsomal fraction of control, ^{wt}RyR3- and ^{V2322D}RyR3-overexpressing HEK293 cells (2 μg protein/lane and 25 μg protein/lane, respectively) by western blot analysis, using the monoclonal m34C RyR antibody (1/4000) and the polyclonal FKBP12 antibody (1/1000), respectively. (B) FKBP12 expression was detected in the microsomal fraction of ^{wt}RyR3-overexpressing cells (Micros) by western blot analysis, using the polyclonal anti-FKBP12 antibody (1/1000). Microsomes were treated with 25 μM FK506 and collected by centrifugation. The supernatants (Sn) of this fraction and the FK506-treated microsomes (Pellet) were analysed for FKBP12 expression. All lanes contained 25 μg protein. (C) Immunoblot (m34C RyR antibody; 1/3000) showing RyR3 in the microsomes of HEK293 cells (Micros; 2 μg) expressing either ^{wt}RyR3 or ^{V2322D}RyR3, the solubilised fraction (Sol; 8 μg) and the fraction retained after incubation with GST or GST-FKBP12 immobilised on glutathione-Sepharose 4B (GST or GST-FKBP12; 16 μg). Localization of RyR3 and of molecular mass markers are indicated.

increased in comparison with the FKBP12 levels found in microsomes from control cells or ^{V2322D}RyR3-expressing cells, suggesting that FKBP12 is endogenously associated with the ^{wt}RyR3. In addition, FKBP12 was completely removed from the microsomal fraction of ^{wt}RyR3-expressing cells by incubation with 2 the immunosuppressant FK506 (5 μM), since FKBP12 disappeared from the pellet into the supernatant (Fig. 1B). FKBP12-binding properties of both clones were further analysed by GST-FKBP12 affinity chromatography (Fig. 1C). Using this assay, we found that about 30% of the solubilised ^{wt}RyR3 protein was specifically retained by GST-FKBP12. This value correlates well with our previous observations (Bultynck et al., 2001a) and reflects the strong interaction between RyR3 and FKBP12. With the ^{V2322D}RyR3 protein, the amount of solubilised ^{V2322D}RyR3 protein retained was only slightly above the background level found for the GST control and was less than 5%. This finding strongly suggests that Val^{2322} Pro^{2323} is involved in the high-affinity binding of FKBP12 to the channel and that mutation of Val to Asp strongly decreased this binding. Next, we investigated whether the altered FKBP12-binding properties of the mutated channels resulted in altered Ca^{2+}-flux properties.

Caffeine-induced Ca^{2+} release in HEK293 cells expressing ^{wt}RyR3 or ^{V2322D}RyR3

$^{45}Ca^{2+}$ fluxes on non-permeabilised HEK293 cells expressing either the ^{wt}RyR3 or ^{V2322D}RyR3 were performed. Fig. 2 shows that non-permeabilised HEK293 cells loaded to steady state with $^{45}Ca^{2+}$, slowly lost their accumulated $^{45}Ca^{2+}$ during incubation in a Ca^{2+}-free efflux medium. Addition of caffeine (10 mM) induced a transient increase in the fractional loss in both the ^{wt}RyR3- and ^{V2322D}RyR3-expressing cells (Fig. 2A). In ^{wt}RyR3-expressing cells caffeine enhanced fractional loss by a factor of 4.75±0.63, whereas in the ^{V2322D}RyR3-expressing cells this factor amounted to 7.37±0.89 (P<0.01, n=5). Thus, the caffeine-induced fractional loss from the ^{V2322D}RyR3-expressing cells was about 1.5-fold higher than that from the ^{wt}RyR3-expressing cells. The fractional loss induced by the Ca^{2+}-ionophore A23187 (10 μM), which is a reflection of the total releasable Ca^{2+} content, was similar in both cells (Fig. 2B). The latter finding indicates that there are no significant differences in the luminal Ca^{2+} content between ^{wt}RyR3- and ^{V2322D}RyR3-expressing cells.

Caffeine-induced Ca^{2+}-release events were also measured in individual cells using confocal Ca^{2+} imaging. Untransfected HEK293 cells did not respond to caffeine (Tong et al., 1999; Rossi et al., 2002). When ^{wt}RyR3- and ^{V2322D}RyR3-expressing HEK293 cells were exposed to 1 mM caffeine, almost all cells exhibited a large and transient change in cytosolic free [Ca^{2+}]. Fig. 3A shows representative traces of caffeine responses. Fig. 3B shows the corresponding first differentiate dA/dt. Analysis of a large number of observations revealed that both the mean amplitude and the mean rate of rise of the caffeine response were significantly larger in the ^{V2322D}RyR3-expressing cells (Fig. 3C,D).

When a dose-response curve for caffeine was constructed (Fig. 4) it became clear that ^{V2322D}RyR3-expressing HEK293 cells displayed a much higher sensitivity for caffeine than ^{wt}RyR3-expressing HEK293 cells. Even at supramaximal concentrations (60 mM), the peak amplitude of the caffeine response was twice as large in the ^{V2322D}RyR3 group. The estimated EC_{50} values amounted to 0.3 mM for ^{V2322D}RyR3s and to 5.2 mM for ^{wt}RyR3s. Thus, ^{V2322D}RyR3s were about 20 times more sensitive to stimulation by caffeine than ^{wt}RyR3s. Since ^{V2322D}RyR3s are no longer capable of binding FKBP12, it appears that FKBP12 is an important endogenous regulator of RyR3.

In agreement with previous reports in frog skeletal muscle and rat hippocampal neurons (Mironov and Hermann, 1996; Oba et al., 1997), we also found that low doses of ethanol greatly potentiated caffeine-induced Ca^{2+} release in RyR3-expressing HEK293 cells (Table 1). In accordance with the caffeine effect, the effect of ethanol was also much more pronounced in ^{V2322D}RyR3- than in ^{wt}RyR3-expressing cells.

These findings indicate that in the absence of FKBP12

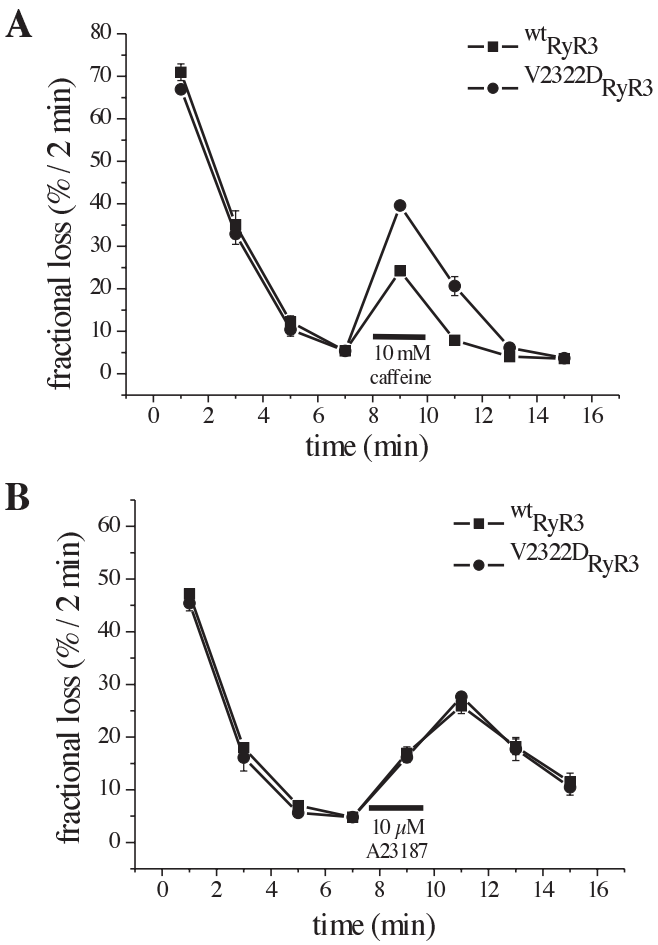

Fig. 2. Effect of caffeine and A23187 on Ca^{2+} efflux from non-permeabilised ^{wt}RyR- and ^{V2322D}RyR3-expressing HEK293 cells. (A) $^{45}Ca^{2+}$ efflux was measured in ^{wt}RyR3 (squares) and ^{V2322D}RyR3-expressing cells (circles) in Ca^{2+}-free medium. After 8 minutes, cells were challenged for 2 minutes with 10 mM caffeine. (B) The total-releasable Ca^{2+} was measured by challenging the cells with 10 μM A23187 in ^{wt}RyR3- (squares) and ^{V2322D}RyR3-expressing cells (circles) in the same medium. The results are plotted as fractional loss as a function of time. Representative traces from five independent experiments are shown. Data points are given as mean±s.d.

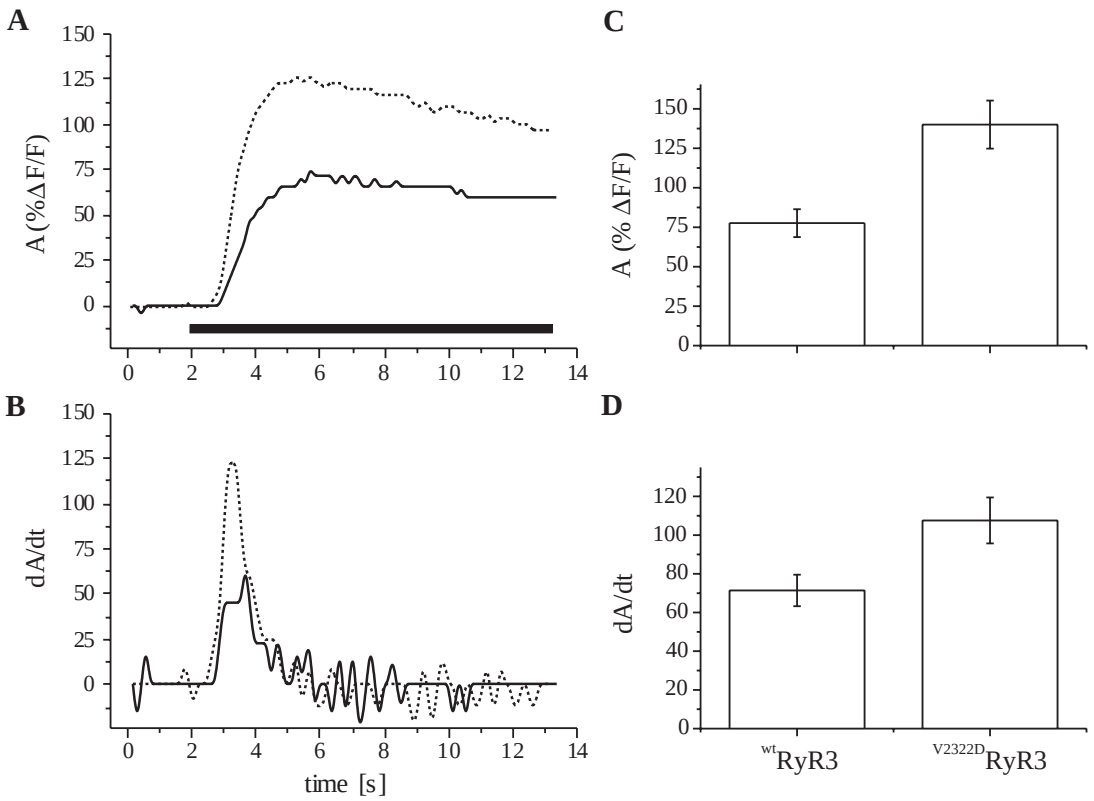

Fig. 3. Cytosolic Ca^{2+} signals evoked by 1 mM caffeine in ^{wt}RyR3- and ^{V2322D}RyR3-expressing HEK293 cells. (A,B) Ca^{2+} responses and the corresponding first differentiate dA/dt of ^{wt}RyR3- (continuous line) and ^{V2322D}RyR3-expressing (dotted line) HEK293 cells stimulated with 1 mM caffeine as indicated by the black horizontal bar. (C,D) Ca^{2+} transients in ^{V2322D}RyR3-expressing HEK293 cells were characterised by a larger amplitude and a faster rate of rise than in ^{wt}RyR3-expressing cells. Amplitude (A) expressed as % ΔF/F: 77.6±8.8, *n*=62 versus 140.0±15.2, *n*=48 in the ^{wt}RyR3- and ^{V2322D}RyR3-expressing cells (P<0.001), respectively. Rate of rise (dA/dt): 71.4±8.1, *n*=62 versus 107.6±11.9, *n*=48 in the ^{wt}RyR3- and ^{V2322D}RyR3-expressing HEK293 cells (P<0.01), respectively.

binding the RyR3 channel is much more susceptible to activation by compounds such as caffeine and ethanol.

Spontaneous Ca^{2+}-release events in ^{wt}RyR3- and ^{V2322D}RyR3-expressing HEK293 cells

As previously published (Rossi et al., 2002), local, spontaneous Ca^{2+}-release events or Ca^{2+} sparks were observed in ^{wt}RyR3-expressing cells. Although the RyR3 expression levels (Fig. 1A) and the maximally releasable Ca^{2+} were similar between the 2 groups (Fig. 2B), the number of cells displaying spontaneous Ca^{2+} sparks was much higher for ^{V2322D}RyR3-expressing cells (68%) than for ^{wt}RyR3-expressing cells (44%) (Fig. 5C).

Ca^{2+} sparks in both the ^{wt}RyR3- (Fig. 5A) and ^{V2322D}RyR3-expressing (Fig. 5B) HEK293 cells showed a great variability in amplitude, frequency and duration (Rossi et al., 2002). When data from individual sparking sites were pooled, mean spark amplitude and duration was significantly smaller in the ^{V2322D}RyR3-expressing cells, whereas the frequency was not significantly affected (Fig. 5D-F).

Since the occurrence of spontaneous Ca^{2+} sparks in HEK293 cells was independent of RyR3 expression levels (Rossi et al., 2002), we propose that FKBP12 may be a critical component in the regulation of RyR3-mediated spontaneous Ca^{2+} sparks.

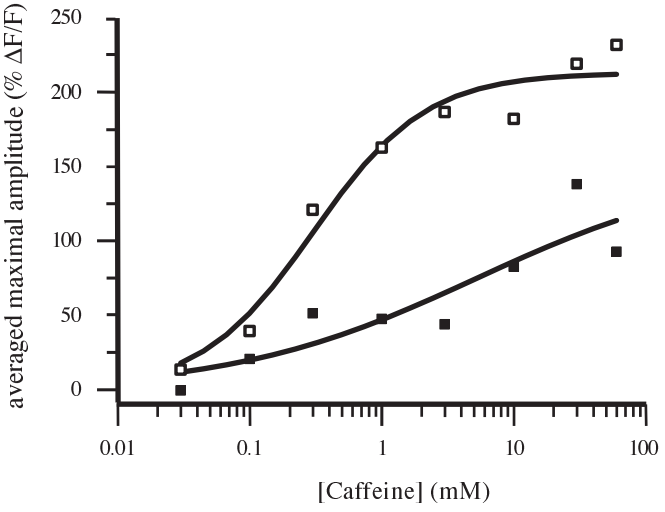

Fig. 4. Dose-response curve for the caffeine-induced cytosolic Ca^{2+} signals. ^{wt}RyR3- (filled squares) and ^{V2322D}RyR3- (open squares) expressing HEK293 cells were stimulated with increasing caffeine concentrations. Each data point is the averaged maximal amplitude of two to three independent experiments. EC_{50} values amounted to 0.3 mM and 5.2 mM for the ^{wt}RyR3 and the ^{V2322D}RyR3 group, respectively. Sigmoidal fitting was performed using Microcal^{TM} Origin (version 6.0) software.

Table 1. Effect of ethanol on caffeine-induced Ca^{2+} release

	^{wt}RyR3		^{V2322D}RyR3	
	Control	+Ethanol	Control	+Ethanol
	(*n*=62)	(*n*=47)	(*n*=48)	(*n*=52)
A	77.6±8.8	104.3±7.4	140.0±15.2	217.2±10.8
dA/dt	71.4±8.1	93.6±7.6	107.6±11.9	234.1±13.3

HEK-RyR3 cells were exposed to 1 mM caffeine in the presence or absence of 0.05% (v/v) ethanol. Ethanol significantly enhanced the mean amplitude (A expressed as % ΔF/F) and the mean rate of rise (dA/dt) in both ^{wt}RyR3- (P<0.05 and P<0.05, respectively) and ^{V2322D}RyR3-expressing HEK293 cells (P<0.001 and P<0.001, respectively) but the effect was much more pronounced in the ^{V2322D}RyR3 group.

Therefore, we overexpressed FKBP12 in ^{wt}RyR3- and ^{V2322D}RyR3-expressing HEK293 cells. Western blot analysis of homogenized fractions of these cells showed that the FKBP12-expression levels were about twofold higher than those in the ^{wt}RyR3-expressing cells (Fig. 6A). Analysing the number of sparking cells revealed that overexpression of FKBP12 significantly decreased the number of sparking ^{wt}RyR3-expressing cells, whereas it did not affect the number of sparking ^{V2322D}RyR3-expressing cells (Fig. 6B). These data confirm that the FKBP12 can bind with high affinity and saturate the binding sites on ^{wt}RyR3 channels, and that the higher number of sparking ^{V2322D}RyR3 cells may be due to the inability of ^{V2322D}RyR3 to bind FKBP12 with high affinity. Furthermore, these findings indicate that the endogenous FKBP12 expression levels are insufficient for all RyR3 channels to be occupied in the RyR3-expressing HEK293 cells. Taking together, our observations reveal that the ability of ^{wt}RyR3 channels to provoke spontaneous Ca^{2+} sparks critically depends on the presence of FKBP12.

Discussion

FKBPs are important enzymes for the fluent restructuring of the peptide backbone during protein synthesis and for the folding of proteins, because of their characteristic peptidyl-prolyl isomerase activity (rotamase). Besides their transient, catalytic role in protein folding, FKBPs are also involved in regulatory interactions with mature proteins, thereby acting as a stabilising adapter protein or as a linker protein for other proteins, such as the Ca^{2+}/calmodulin-dependent serine-threonine phosphatase calcineurin. Different studies showed that FKBP12 tightly associates with at least RyRs (Jayaraman

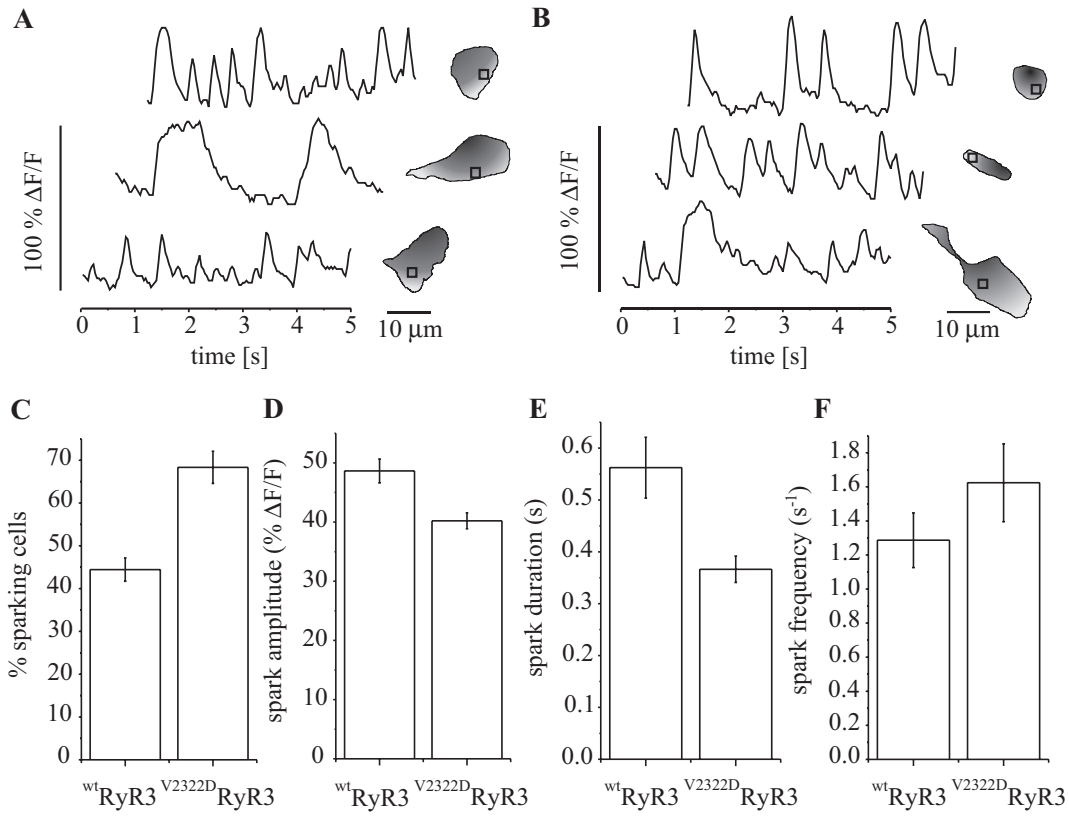

Fig. 5. Spontaneous Ca^{2+} sparks in ^{wt}RyR3- and ^{V2322D}RyR3-expressing HEK293 cells. (A,B) Individual traces show time courses of spontaneous Ca^{2+} sparks in ^{wt}RyR3- and ^{V2322D}RyR3-expressing cells, respectively. The contour images show the corresponding regions over which the relative changes in fluorescence were integrated. (C) The bar chart shows that the percentage sparking was significantly larger in the ^{V2322D}RyR3- than in the ^{wt}RyR3-expressing cells (68.3±3.8 versus 44.4±2.7, n=18, P<0.001). (D-F) The Ca^{2+}-spark properties, mean amplitude and duration were significantly smaller in the ^{V2322D}RyR3- expressing than in the ^{wt}RyR3-expressing cells whereas the spark frequency was not affected. Amplitude (A) expressed as % ΔF/F: 40.2±1.3, n=109 versus 48.6±2.0, n=82 in the ^{V2322D}RyR3- and ^{wt}RyR3-expressing cells (P<0.001), respectively. Duration (seconds): 0.37±0.02, n=109 versus 0.56±0.06, n=82 in the ^{V2322D}RyR3- and ^{wt}RyR3-expressing cells (P=0.001), respectively. Frequency (per second): 1.62±0.23, n=12 versus 1.29±0.16, n=12 in the ^{V2322D}RyR3- and ^{wt}RyR3-expressing cells, respectively (P=0.24).

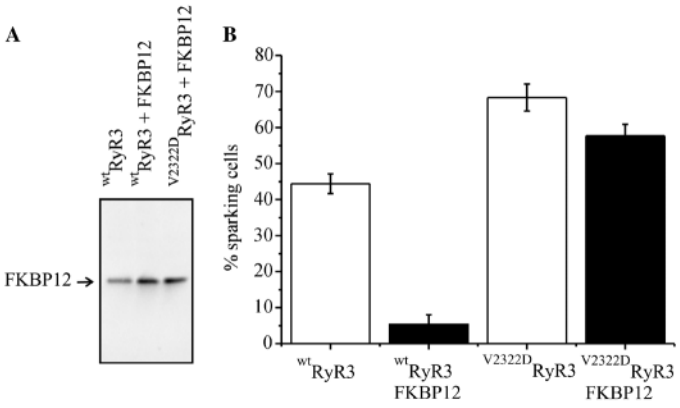

Fig. 6. FKBP12 overexpression in ^{wt}RyR3- and ^{V2322D}RyR3-expressing HEK293 cells. (A) FKBP12 expression levels were determined in the homogenate of ^{wt}RyR3- and ^{V2322D}RyR3-expressing HEK293 cells after exogenous overexpression of FKBP12 by western blot analysis, using the polyclonal FKBP12 antibody (1/1000). FKBP12 expression was increased by 2.07±0.27 (n=4) in ^{wt}RyR3-expressing cells and 1.63±0.22 (n=4) in ^{V2322D}RyR3-expressing cells. All lanes contained 30 μg protein. (B) The bar chart shows that the percentage sparking was significantly reduced in the ^{wt}RyR3 cells overexpressing FKBP12 (44.4±2.7, n=18 versus 5.3±2.7, n=7, P<0.001) while FKBP12 overexpression did not significantly affect the percentage sparking in ^{V2322D}RyR3-expressing cells (68.3±3.8, n=18 versus 57.7±3.2, n=10, P<0.068).

et al., 1992; Timerman et al., 1993; Brillantes et al., 1994; Mayrleitner et al., 1994; Timerman et al., 1994; Lam et al., 1995; Timerman et al., 1996; Qi et al., 1998), whereas the situation for Ins(1,4.5)P_3Rs is controversial (Cameron et al., 1995a; Cameron et al., 1995b; Cameron et al., 1997; Shou et al., 1998; Bultynck et al., 2000; Bultynck et al., 2001a; Bultynck et al., 2001b; Carmody et al., 2001; Dargan et al., 2002). The interaction site of FKBP12 with intracellular Ca^{2+}-release channels appears to involve a peptidylprolyl residue with the first peptidyl residue always being a hydrophobic amino acid (leucyl, isoleucyl, valyl). In RyR1, -2 and -3, the hydrophobic character appears to be critical for FKBP12 binding, since mutation to a charged amino acid disrupted the binding (Marx et al., 2000; Bultynck et al., 2001b; Gaburjakova et al., 2001). However, the molecular and functional importance of these residues for RyR2 remains a matter of debate. Chen and co-workers found that mutations in the isoleucine proline residue (2427-2428) in the full-size RyR2 sequence affected neither the FKBP12.6 binding to the channel nor the Ca^{2+}-flux properties of the channel (Masumiya et al., 2003). These authors provided evidence that the FKBP12.6 binding site was located in a region upstream of the divergent region 3 in RyR2 (Zhang et al., 2003). In addition, there was one report suggesting that the FKBP12.6 binding site is located at the C-terminal domain (Zissimopoulos and Lai, 2002). It is therefore conceivable that there are additional contact points between FKBP12.6 and RyR2, and perhaps between FKBPs and RyRs in general.

In order to address the relevance of the peptidylprolyl residue for FKBP12 binding to RyR3, we have analysed the Ca^{2+}-flux properties of a mutated RyR3, in which a valine proline residue in the presumed FKBP-binding domain was replaced by an aspartate proline residue. As shown by GST-FKBP12 affinity chromatography, this single-point mutation was sufficient to largely disrupt FKBP12 binding to the RyR3 channel. These observations fully agree with previous data for RyR1 and thus indicate that this valine proline dipeptide (2322-2323) is indispensable for FKBP12 binding to RyR3 (Gaburjakova et al., 2001).

$^{45}Ca^{2+}$-flux experiments and confocal Ca^{2+} imaging further revealed that disrupting FKBP12-binding properties of RyR3 channels had major functional consequences. Indeed, ^{V2322D}RyR3 channels became much more sensitive to caffeine and ethanol than ^{wt}RyR3. This is compatible with previous findings showing that in the presence of the immunosuppressant FK506, which disrupts FKBP12 interaction, RyR1 and RyR2 channels become more sensitive to activators, such as caffeine, Ca^{2+} or ATP (Timerman et al., 1993; Brillantes et al., 1994; Mayrleitner et al., 1994; Ondrias et al., 1998; Gaburjakova et al., 2001). The use of FK506, however, must be viewed with caution, since this drug has multiple effects on intracellular Ca^{2+} homeostasis independent of RyRs (Bultynck et al., 2003), including effects on the sarco- and endoplasmic reticulum Ca^{2+}-ATPases (Bultynck et al., 2000; Bilmen et al., 2002), the passive Ca^{2+} leak (Bultynck et al., 2002), Ca^{2+}-influx channels (Burley and Sihra, 2000), voltage-operated Ca^{2+} channels (Yasutsune et al., 1999), Ca^{2+}-activated K^+ channels (Terashima et al., 1998) and the outward K^+ current (duBell et al., 1997). Disruption of FKBP12 interaction with ^{wt}RyR3 by FK506 treatment led, in our hands, to non-specific effects (data not shown), probably related to Ca^{2+}-pump inhibition (Bultynck et al., 2000) and to effects of the solvent ethanol on the activation of RyR3 channels (Table 1).

We further found that both ^{wt}RyR3- and ^{V2322D}RyR3-expressing HEK293 cells displayed spontaneous Ca^{2+} sparks but their rate of occurrence was significantly higher in the ^{V2322D}RyR3 group. Furthermore, exogenous overexpression of FKBP12 significantly decreased the number of sparking ^{wt}RyR3-expressing cells, whereas the number of sparking ^{V2322D}RyR3-expressing cells was not affected. The appearance of spontaneous Ca^{2+} sparks appears to be a unique property of RyR3 channels since this behaviour was not observed in HEK293 cells expressing RyR1 (Rossi et al., 2002). Previous experiments further elucidated that RyR3s are more sensitive to caffeine than RyR1s (Rossi et al., 2002). It is conceivable that the unique properties of RyR3 receptors are linked to their FKBP12-binding properties (Fig. 7). In a previous study, we have shown that the interaction of FKBP12 with RyR3 was weaker than that with RyR1, implying that the latter has a higher affinity for FKBPs (Bultynck et al., 2001a). In addition, the FKBP12 interaction with RyR1 was Ca^{2+} independent, whereas the binding efficiency of FKBP12 to RyR3 was significantly decreased at low Ca^{2+} concentrations (Bultynck et al., 2001b). Therefore, at resting levels of intracellular Ca^{2+} RyR1 tetramers may be fully occupied by FKBP12 ligands, whereas RyR3 tetramers may be only partially occupied. Assuming that the degree of FKBP saturation controls the stability of the closed state of the RyR channel, spontaneous Ca^{2+}-release events would only be associated with RyR3 channels. Our observation that cells bearing the V2322D mutation had considerably more

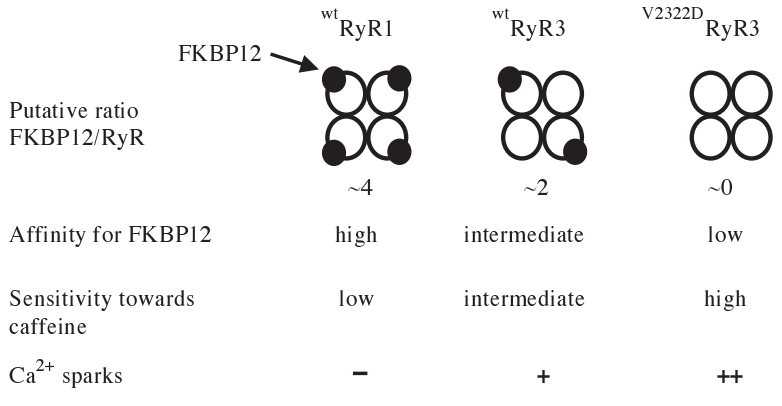

Fig. 7. Model for the generation of Ca^{2+} sparks by RyR isoforms and the regulation by FKBP12. The first line shows a schematic representation of ^{wt}RyR1, ^{wt}RyR3 and ^{V2322D}RyR3 tetramers with their putative degree of FKBP12 saturation. At resting Ca^{2+} levels, the affinity of FKBP12 for ^{wt}RyR1 is high (~4 FKBPs/RyR), for ^{wt}RyR3 is intermediate (~2 FKBPs/RyR) and for ^{V2322D}RyR3 is low (~0 FKBPs/RyR). In this model, we propose that the relative FKBP12 saturation degree determines the stability of the closed state of the RyR channel and the sensitivity to channel activators, such as caffeine. This model explains the absence of spontaneous Ca^{2+} sparks in ^{wt}RyR1-$(FKBP12)_4$-expressing cells and the increased occurrence of Ca^{2+} sparks in ^{V2322D}RyR3-expressing cells (68% of cells) as compared to ^{wt}RyR3-$(FKBP12)_2$ expressing cells (44% of cells).

spontaneous Ca^{2+} sparks and that FKBP12 overexpression reduced the number of sparking ^{wt}RyR3-expressing cells clearly demonstrate that the ability of RyR3 channels to provoke spontaneous Ca^{2+} sparks is critically dependent on the degree of FKBP12 saturation of RyR3s. Previous findings that FK506 evoked Ca^{2+} sparks in cardiomyocytes, mainly expressing RyR2 (Valdivia, 1998), and prolonged the mean open time of RyR2s reconstituted in bilayers are also compatible with a stabilising role of FKBPs on the channel activity (Xiao et al., 1997). Our finding that ^{V2322D}RyR3-expressing cells displayed the highest sensitivity to caffeine or ethanol implies that the degree of FKBP binding also controls the sensitivity to known RyR activators (Xiao et al., 1997).

In addition, Ca^{2+} sparks in ^{V2322D}RyR3-expressing cells were of significantly smaller amplitude and duration. This is in contrast to properties of RyR2-mediated Ca^{2+} sparks in cardiomyocytes treated with FK506 (McCall et al., 1996; Xiao et al., 1997; Valdivia, 1998) or results obtained from FKBP12.6 null mice (Xin et al., 2002). Nevertheless, the smaller spark amplitude and duration, which reflect a decreased amount of Ca^{2+} released from a single Ca^{2+} spark site, suggest that ^{V2322D}RyR3 channels may be partially and functionally uncoupled in a cluster. It is probable that FKBP12 is also important for coordinated openings and closings of neighbouring RyR3s and promotes coupled gating between clustered RyR3s as observed for RyR1 (Marx et al., 1998) and RyR2 (Marx et al., 2001).

Taken together, our data demonstrate that a central valine proline motif (position 2322-2323) in RyR3 is critical for its association with FKBP12. Disrupting this FKBP12-binding property increased spontaneous Ca^{2+} release, and sensitivity to caffeine or ethanol. Our data indicate that RyR3 gating is tightly regulated by FKBP12 binding.

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