Overexpression of FK-506–Binding Protein 12.0 Modulates Excitation–Contraction Coupling in Adult Rabbit Ventricular Cardiomyocytes

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Abstract—The effect of the 12-kDa isoform of FK-506–binding protein (FKBP)12.0 on cardiac excitation–contraction coupling was studied in adult rabbit ventricular myocytes after transfection with a recombinant adenovirus coding for human FKBP12.0 (Ad-FKBP12.0). Western blots confirmed overexpression (by 2.6±0.4 fold, n=5). FKBP12.0 association with rabbit cardiac ryanodine receptor (RyR2) was not detected by immunoprecipitation. However, glutathione S-transferase pull-down experiments indicated FKBP12.0–RyR2 binding to proteins isolated from isolated rabbit and rabbit but not dog myocardium. Voltage-clamp experiments indicated no effects of FKBP12.0 overexpression on L-type Ca2+ current (I_{Ca,L}) or Ca2+ efflux rates via the Na+/Ca2+ exchanger. Ca2+ transient amplitude was also not significantly different. However, sarcoplasmic reticulum Ca2+ load was ~25% higher in myocytes in the Ad-FKBP12.0 group. The reduced ability of I_{Ca,L} to initiate sarcoplasmic reticulum Ca2+ release was observed over a range of values of sarcoplasmic reticulum Ca2+ content, indicating that overexpression of FKBP12.0 reduces the sensitivity of RyR2 to Ca2+. Ca2+ spark morphology was measured in β-escin–permeabilized cardiomyocytes. Ca2+ spark amplitude and duration were significantly increased, whereas frequency was decreased in cells overexpressing FKBP12.0. These changes were accompanied by an increased sarcoplasmic reticulum Ca2+ content. In summary, the effects of FKBP12.0 overexpression on intact and permeabilized cells were similar to those of tetracaine, a drug known to reduce RyR2 Ca2+ sensitivity and distinctly different from the effects of overexpression of the FKBP12.6 isomer. In conclusion, FKBP12.0-RyR2 interaction can regulate the gain of excitation–contraction coupling. (Circ Res. 2007;101:1020-1029.)

Key Words: calcium signaling ■ excitation–contraction coupling

Ca2+ release via the sarcoplasmic reticulum (SR) Ca2+ release channel (ryanodine receptor [RyR2]) is modulated by regulatory proteins interacting with both the cytoplasmic and luminal aspects of RyR2. One such cytosolic protein is FK-506–binding protein (FKBP), which binds to the RyR2 with a maximum stoichiometry of 4 FKBP:1 RyR2.1 Two members of the FKBP family are expressed within mammalian ventricular cardiomyocytes: FKBP12.0 (12.0 kDa) and FKBP12.6 (12.6 kDa). The cytosolic concentration of FKBP12.0 is almost 10-fold higher than FKBP12.6.1,2 In some species (eg, canine), the affinity of FKBP12.0 for RyR2 is ~500 times lower than for FKBP12.6, hence binding is negligible under physiological conditions.1 In others (including rabbit and human), the affinity of FKBP12.0 for RyR2 is only ~7 times lower than for FKBP12.6; therefore, it is conceivable that FKBP12.0–RyR2 interaction occurs in these species under physiological conditions.1 The consequences of FKBP12.0 binding on RyR2 function are unclear; currently, no pharmacological intervention can differentiate between the effects of FKBP12.0 and -12.6. FKBP12.0-null mice showed RyR2 dysfunction1 either resulting from the absence of RyR2-FKBP12.0 binding or as an indirect consequence of the accompanying developmental defects. FKBP12.6-null mice lack developmental defects, supporting the view that the FKBP12.0 isoform is indispensable for normal cardiac development. In the current study, adenoviral-mediated transfection of the human FKBP12.0 gene was used to overexpress FKBP12.0 in isolated rabbit ventricular cardiomyocytes over 48 hours. The choice of rabbit heart tissue was based on the similarities to human heart in terms of relative affinities of FKBP12.0 and FKBP12.6 for RyR2.
FKBP12.0 Overexpression in Cardiomyocytes

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Materials and Methods

Recombinant Adenovirus Vector Construction

Full-length cDNA of the human FKBP12.0 gene was cloned by polymerase chain reaction (PCR) from human heart muscle–specific cDNA samples by the use of PCR primers that span the entire coding region of FKBP12.0 cDNA. This sequence was inserted downstream from a cytomegalovirus promoter into vector pACCMV-pP.A, and recombinant with vector pM17 was performed in HEK293 cells. The production, purification, and titration of adenovirus containing the FKBP12.0 gene (Ad-FKBP12.0) were performed according to standard procedures.4 Previous studies have used an adenovirus containing the human FKBP12.6 gene.2,5 At 100 multiplicities of infection, this Ad-FKBP12.6 vector caused an overexpression of FKBP12.6 of ~6-fold normal values after 48 hours of incubation.

Ventricular Cardiomyocyte Isolation and Transfection

New Zealand White rabbits (2 to 2.5 kg) were euthanized by administration of an intravenous injection of 50 IU heparin, together with an overdose of sodium pentobarbitone (100 mg·kg−1). Hearts were removed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and conformed to the Guide for the Care and use of Laboratory Animals (NIH publication no. 85-23, revised 1985). Ventricular cardiomyocytes were then isolated as described previously.3 Adenoviral infection with a multiplicity of infection of 100 was performed to produce 2 populations of adenovirus-transfected cardiomyocytes: (1) overexpressing FKBP12.0 (Ad-FKBP12) and (2) expressing β-galactosidase as control (Ad-LacZ). Infected cardiomyocytes were subsequently cultured in supplemented medium M199 (Sigma) for 48 hours. Western blot analysis indicated the level of FKBP12.0 overexpression to be 3.0 ± 0.9-fold (n = 5, P < 0.05) higher compared with nontransfected control cells.

Glutathione S-Transferase Pull-Down Assay

Prepared cell lysates and cardiac homogenates were incubated with 30 μg of glutathione S-transferase (GST)-FKBP12.0 or GST-FKBP12.6 immobilized on glutathione–sepharose for 5 hours at 4°C. Human FKBP12-pGEX-3X or FKBP12.6-pGEX-3X constructs (generously provided by Dr Wayne Chen, Department of Physiology and Biophysics, University of Calgary, Canada) were used to express and purify the GST-FKBP12.0 and GST-FKBP12.6 according to the instructions of the manufacturer (Amersham). After washing the glutathione–sepharose precipitates 3 times in solubilization medium, the GST-tagged protein and any associated proteins were competitively eluted with 10 mmol/L reduced glutathione in 50 mmol/L Tris–HCl, pH 8.0, for 15 minutes at room temperature. Incubation of samples with immobilized GST (Sigma) and elution with 60 μmol/L FK-506 or vehicle alone served as controls.

Immunoblot Analysis

FKBP12.0 from cell lysates was analyzed by 15% SDS-PAGE. Eluates obtained during GST pull-down assay were size fractionated on 4% to 20% SDS-PAGE. The immunoblotting procedure was performed as described previously.2

Electrophysiological Measurements in Rabbit Cardiomyocytes

The isolated cardiomyocytes were superfused with a Krebs–Henseleit solution with additional 5 μmol/L tetrodotoxin, 0.1 mmol/L niflumic acid, and 5 mmol/L 4-aminopyridine at 19 to 20°C in a chamber mounted on the stage of an inverted microscope (see the online supplement at http://circres.ahajournals.org for details about solutions, including pipette solutions). Cytosolic loading of Fura-2 was achieved by incubating cardiomyocytes with 5 μmol/L Fura-2/acetoxymethyl ester at room temperature for 12 minutes.

Excitation–Contraction Coupling Protocol

Using an Axon Instruments Switch-clamp (2B), cardiomyocytes were held at −80 mV and the voltage was stepped to −40 mV (50 ms) to inactivate the remaining inward Na+ current, stepping to 0 mV (150 ms) before returning to −80 mV. This protocol was repeated every 2s for 80s to achieve steady-state Ca2+ transients. SR Ca2+ content and Na+/Ca2+ exchanger (NCX) activity were then estimated by rapidly switching to 10 mmol/l caffeine to cause SR Ca2+ release. In the continued presence of caffeine, the SR is unable to reaccumulate Ca2+ and elimination of Ca2+ is mainly attributable to NCX. The time course of the decay of [Ca2+]i and iNCX represent rates of extrusion of Ca2+ from the cell predominately by NCX.6 These signals were fitted to exponential decays >80% of their amplitude.

The relationship between SR Ca2+ content and Ca2+ transient amplitude at low SR Ca2+ loads was investigated by manipulating SR Ca2+ content. Reduction of SR Ca2+ content was achieved by superfusing cardiomyocytes for set periods of time with thapsigargin (5 μmol/L).3 Increased SR Ca2+ content was achieved with a holding potential of −50 mV between voltage-clamp pulses.

Ca2+ Spark Measurements in Permeabilized Cardiomyocytes

Isolated rabbit cardiomyocytes were superfused with a mock intracellular solution and permeabilized using β-escin (Sigma) as detailed previously.5 Fluo-3 (10 μmol/L) in the perfusing solution was excited using a confocal microscope. Confocal line scan images were recorded using a Bio-Rad Radiance 2000 confocal system, further details of which have been published previously.5 All Ca2+ spark measurements were made within 2 to 3 minutes of cell permeabilization.

Statistics

Data were expressed as means ± SEM. For ionic currents, intracellular [Ca2+]i, and Ca2+ spark parameters, comparisons were performed by using the unpaired Student’s t test; otherwise, the paired Student’s t test was used. Differences were considered significant when P < 0.05. ANOVA statistics with a Tukey post test were used in cases of multiple comparisons.

Results

Overexpression of FKBP12.0 Within Isolated Adult Rabbit Ventricular Cardiomyocytes

Transgene expression at mRNA and protein levels were verified by means of RT-PCR and immunoblotting, respectively, both of which revealed a specific dose-dependent increase of exogenous FKBP12.0 (Figure 1A through 1C). To ensure effective overexpression of the transgene, cells were transfected at a multiplicity of infection of 100, which provides a moderate increase of the exogenous FKBP12.0 to 2.6 ± 0.4-fold (n = 5) fold above normal.

[Ca2+]i Measurements in Voltage-Clamped Rabbit Cardiomyocytes

No significant change in Ca2+ transient amplitude (231 ± 15 versus 238 ± 19 nmol/L; Ad-LacZ, n = 33 cells versus Ad-FKBP12, n = 20 cells) was observed in voltage-clamped rabbit cardiomyocytes (Figure 2A and 2C, i). Similarly, no significant difference between the Ca2+ decay of the Ca2+ transient was noted between the 2 populations of cells, suggesting no effect of Ad-FKBP12.0 overexpression on SERCA-mediated SR Ca2+ uptake (Figure 2C, ii). This was supported by measurements of SERCA-mediated Ca2+ uptake in oxalate equilibrated permeabilized cardiac myocytes after FKBP12.0 overexpression (data not shown). As illustrated in Figure 2A,
SR Ca\(^{2+}\) Content as Assessed by Rapid Application of Caffeine

Application of caffeine caused a rapid increase of [Ca\(^{2+}\)], as a result of SR Ca\(^{2+}\) release, the subsequent reduction of which results from extrusion of Ca\(^{2+}\) across the sarcolemma mainly via the NCX. This extrusion of Ca\(^{2+}\) via NCX generates a transient inward current, the amplitude and time course of which was monitored together with the Ca\(^{2+}\) transient (Figure 3A and 3B). As shown in Figure 3A (mean values shown in Figure 3B, i), the amplitude of the caffeine-induced Ca\(^{2+}\) release was significantly larger in Ad-FKBP12.0–transfected cardiomyocytes, suggesting an increased SR Ca\(^{2+}\) content (762±42 versus 910±74 nmol/L; Ad-LacZ [n=26 cells] versus Ad-FKBP12 [n=24 cells]). As described previously, the time integral of the current can be used as a measure of the amount of Ca\(^{2+}\) extruded by NCX during a caffeine application (an indicator of the SR Ca\(^{2+}\) content). As shown in Figure 3B, iii, the mean integral of the NCX-mediated inward current (I\(_{\text{NCX}}\)) in the Ad-FKBP12.0–transfected group was ∼25% higher than the control (Ad-LacZ) group (normalized to cell capacitance), supporting the conclusion that SR Ca\(^{2+}\) content was significantly higher in the FKBP12.0–overexpressing cardiomyocytes.

Sarcolemmal Ca\(^{2+}\) Efflux Rates in Rabbit Cardiomyocytes

Sarcolemmal flux rates can be estimated from the time course of the inward current decay and the corresponding decrease in [Ca\(^{2+}\)] after rapid application of 10 mmol/L caffeine. As shown in Figure 3C, i and ii, both I\(_{\text{NCX}}\) and [Ca\(^{2+}\)] decayed with a similar time course in Ad-FKBP12.0–transfected cardiomyocytes. These decays were fitted to a single exponential function, and mean rate constants were calculated. The rate constants for both parameters were not significantly different in the 2 experimental groups, suggesting that the rate of extrusion of Ca\(^{2+}\) via the NCX was not affected by FKBP12.0 overexpression.

Relationship Between SR Ca\(^{2+}\) Content and Ca\(^{2+}\) Transient Amplitude

To determine the relationship between the SR Ca\(^{2+}\) content and Ca\(^{2+}\) transient amplitude, measurements were made using thapsigargin to progressively decrease SR Ca\(^{2+}\) content. A holding potential of −50 mV was used to increase SR Ca\(^{2+}\) content. As shown in Figure 4, the amplitude of I\(_{\text{cml}}\) was not altered by this range of experimental conditions. The plot of the I\(_{\text{NCX}}\) integral and Ca\(^{2+}\) transient amplitude for the Ad-LacZ group generated an approximately exponential relationship. The data from the Ad-FKBP12.0 group was shifted to the right and described an exponential curve that was significantly different from that required to fit the Ad-LacZ data (Figure 4). Analysis of I\(_{\text{cml}}\) indicated that there were no significant changes in the amplitude or time course of this current in any of the datasets. Therefore, the exponential relationship described by the Ad-LacZ data represents the relationship between SR Ca\(^{2+}\) content and the ability of I\(_{\text{cml}}\) to trigger Ca\(^{2+}\) release from the SR, ie, excitation–contraction (E–C) coupling "gain." Overexpression of FKBP12.0 moved this curve to the right, indicating a reduced gain of E–C coupling. A similar but larger effect was observed in a separate group of Ad-LacZ cells superfused with 100 μmol/L tetracaine (a drug known to decrease the Ca\(^{2+}\) sensitivity of RyR2). As can be seen in Figure 4, this caused an increase in SR Ca\(^{2+}\) content (2.2±0.14 versus 3.78±0.33 coulombs/farad; Ad-LacZ [n=6 cells] versus Ad-LacZ+tetracaine [n=6 cells]) but no increase in Ca\(^{2+}\) transient amplitude (231±14 versus 232±53 nmol/L; n=6 cells). Data obtained from parallel studies using a previously characterized Ad-FKBP12.6 virus\(^{5}\) are shown in gray. As reported previously, FKBP12.6 overexpression significantly increased the Ca\(^{2+}\) amplitude and SR Ca\(^{2+}\) content,\(^{5}\) with no obvious shift from the
normal relationship between SR Ca\(^{2+}\) content and Ca\(^{2+}\) transient amplitude. This suggests that, in contrast with FKBP12.0 overexpression, FKBP12.6 does not alter RyR2 Ca\(^{2+}\) sensitivity.

Interaction of FKBP12.0 and FKBP12.6 With RyR2
Previously published work based on immunoprecipitation experiments suggests interaction of RyR2 with FKBP12.6.\(^9\) The present study used similar immunoprecipitation techniques to confirm FKBP12.6 binding but could not detect specific interaction of FKBP12.0 with RyR2 (Figure I in the online data supplement). To further examine the interaction between FKBP12.0 and RyR2, GST pull-down assays were performed. GST fusion FKBP12.0 and FKBP12.6, immobilized on glutathione–sepharose beads, were allowed to interact with solubilized proteins of cell lysates and cardiac homogenates. The resulting complexes were then visualized by immunoblotting and silver staining. Figure 5A shows the extraction of rabbit RyR2 using GST-FKBP12.0 and GST-FKBP12.6 immobilized on glutathione–sepharose. RyR2, shown in lanes 3 and 4, was precipitated on the basis of its association with GST-FKBP12.0 and GST-FKBP12.6, respectively. The retained RyR2 was specifically bound to GST-FKBP12.0 because GST alone did not bind any RyR2 (Figure 5A, i and iii, lane 5). Furthermore, from 1 of the precipitates formed after interaction with GST-FKBP12.0, RyR2 was eluted with 60 μmol/L FK-506 (instead of 10 mmol/L glutathione) as a specific competitor (Figure 5A, i and iii, lane 7). To clarify whether FK-506 removed all of RyR2 from the immobilized FKBP12.0 (Figure 5C, i and ii, lane 1), GST-FKBP12.0, still bound to the glutathione–sepharose after application of FK-506, was eluted with reduced glutathione (Figure 5C, lane 2). The absence of RyR2 in this fraction, as revealed by silver staining (Figure
confirmed that in FKBP12.0-immobilized complex, there was no RyR2 bound in a nonspecific manner, ie, independent of FK-506 treatment. Similarly, GST-FKBP12.0 displayed a specific FK-506–displaceable interaction with human RyR2, with efficiency similar to GST-FKBP12.6 (Figure 5B).

To exclude the possibility of detecting RyR1 instead of RyR2 when GST-FKBP12.0 is used as the ligand, the high-molecular-weight proteins from each species recognized by anti-RyR antibody and precipitated with GST-FKBP12.0 were subjected to MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectrometry. Samples from both species were identified as the cardiac isoform of RyR (see supplemental Figures II through IV).

Canine RyR2 Interacts Exclusively With the FKBP12.6 Isoform

When subjected to the GST pull down, canine RyR2 was able to interact with GST-FKBP12.6 (Figure 5D, i and iii, lane 6) but not with GST-FKBP12.0 (Figure 5D, i and iii, lane 3), whereas rabbit RyR2, probed in parallel, interacted with both GST-FKBP12.6 (Figure 5D, i and iii, lane 8) and GST-FKBP12.6 (Figure 5D, i and iii, lane 9). Analysis of the same precipitates by Western blot revealed that all canine RyR2 molecules subjected to interaction with GST-FKBP12.0 remained unabsorbed, because it was completely recovered in the flow-through fraction (Figure 5D, iii, lane 1). In contrast, GST-FKBP12.6 provided efficient extraction of RyR2 from the crude homogenate (Figure 5D, iii, lanes 4 and 6).

Figure 3. Caffeine-induced SR Ca²⁺ release and the corresponding membrane currents. A, [Ca²⁺]i and membrane current recorded on application of 10 mmol/L caffeine in Ad-LacZ–transfected (i) and Ad-FKBP12.0–transfected (ii) cardiomyocytes. B, Ca²⁺ transient amplitude for Ad-LacZ and Ad-FKBP12.0 groups (i); I_{NCX} amplitude for Ad-LacZ and Ad-FKBP12.0 groups (ii); I_{NCX}time integral for Ad-LacZ and Ad-FKBP12.0 groups (iii). C, Mean ± SEM values: rate constant for the caffeine-induced Ca²⁺ transient for Ad-LacZ and Ad-FKBP12.0 (i) and rate constant for the recovery of membrane current in response to caffeine for the Ad-LacZ and Ad-FKBP12.0 groups (ii).

Figure 4. E–C coupling gain. Plot of I_{Ca,L} amplitude (top) and Ca²⁺ transient amplitude (bottom) vs I_{NCX} integral (an index of SR Ca²⁺ content). The data are from cardiomyocytes from the control group (Ad-LacZ) (n = 10); 5 μmol/L thapsigargin, 20-second exposure (n = 8); 40-second exposure (n = 8); 100-second exposure (n = 8); holding potential of −50 mV (n = 8); and Ad-FKBP12 (n = 10); 5 μmol/L thapsigargin, 20-second exposure (n = 8); 40-second exposure (n = 7); 100-second exposure (n = 8); holding potential of −50 mV (n = 8). Gray symbols are the data from parallel experiments in an adenoviral vector for FKBP12.6 (Ad-FKBP12.6) and the corresponding Ad-LacZ data (n = 10 and n = 12.0, respectively). Solid lines are least-squares best-fit exponential relationship (Y = exp[−X×X]), where Y is the Ca²⁺ transient amplitude and X is the I_{NCX} integral. The best-fit value of coefficient G for the FKBP12.0 dataset was significantly lower than LacZ (1.69 ± 0.11 vs 1.09 ± 0.05; P < 0.01).
Fractions eluted from the matrix using 60 μmol/L FK-506 and glutathione–sepharose, respectively. Lanes 7 and 8, bound to immobilized GST-FKBP12.0 and GST-FKBP12.6, immobilized on glutathione–sepharose. Lanes 3 and 4, RyR2, the supernatant after washing RyR-GST-FKBP12.0 complex by glutathione–sepharose. Lane 1, The supernatant after adsorption by silver staining (A, i) or Western blot analysis using anti-RyR antibody (A, iii, and B). Lane 2, The supernatant after adsorption by silver staining (C, i) or Western blot analysis using anti-RyR antibody (C, ii). Lanes 1 and 3, RyR2 eluted from the matrix using 60 μmol/L FK-506 or vehicle alone, respectively. Lanes 2 and 4, Fractions bound to immobilized GST-FKBP12.0 after treatment with 60 μmol/L FK-506 or vehicle alone, respectively, D, Extraction of RyR2 from canine cardiac homogenates. Electrophoresis on 4% to 20% linear gradient SDS-PAGE and RyR detected by silver staining (C, i) or Western blot analysis using anti-RyR antibody (C, ii). Lanes 1, The supernatant after adsorption by silver staining (A, i) or Western blot analysis using anti-RyR antibody (A, iii, and B). Lane 1, The supernatant after adsorption of RyR-GST-FKBP12.0 complex by glutathione–sepharose. Lane 2, The supernatant after washing RyR-GST-FKBP12.0 complex immobilized on glutathione–sepharose. Lanes 3 and 4, RyR2 bound to immobilized GST-FKBP12.0 and GST-FKBP12.6, respectively. Lanes 5 and 6, Fractions bound to immobilized GST and glutathione–sepharose, respectively. Lanes 7 and 8, Fractions eluted from the matrix using 60 μmol/L FK-506 or vehicle alone, respectively. C, Analysis of immobilized FKBP12.0 complex after treatment with FK-506. Extraction of RyR2 with GST-FKBP12.0 from rabbit cardiac homogenate, 4% to 20% SDS-PAGE stained with Coomassie blue; different electrophoretic mobilities of GST-FKBP12.0 and GST-FKBP12.6. The data shown are representative of 3 similar experiments.

**Figure 5.** Affinity purification of RyR2 using GST-FKBP12.0 and GST-FKBP12.6. A and B, Extraction of RyR2 from rabbit (A) and human (B) cardiac homogenates. Electrophoresis on 4% to 20% linear gradient SDS-PAGE (A, i and iii, and B) and RyR detected by silver staining (A, i) or Western blot analysis using anti-RyR antibody (A, iii, and B). Lane 1, The supernatant after adsorption of RyR-GST-FKBP12.0 complex by glutathione–sepharose. Lane 2, The supernatant after washing RyR-GST-FKBP12.0 complex immobilized on glutathione–sepharose. Lanes 3 and 4, RyR2 bound to immobilized GST-FKBP12.0 and GST-FKBP12.6, respectively. Lanes 5 and 6, Fractions bound to immobilized GST and glutathione–sepharose, respectively. Lanes 7 and 8, Fractions eluted from the matrix using 60 μmol/L FK-506 or vehicle alone, respectively. C, Analysis of immobilized FKBP12.0 complex after treatment with FK-506. Extraction of RyR2 with GST-FKBP12.0 from rabbit cardiac homogenate, 4% to 20% SDS-PAGE stained with Coomassie blue; different electrophoretic mobilities of GST-FKBP12.0 and GST-FKBP12.6. The data shown are representative of 3 similar experiments.

**Ca2⁺ Sparks and Caffeine-Induced Ca2⁺ Release in Permeabilized Cardiomyocytes Overexpressing FKBP12.0**

An investigation of the direct effects of FKBP12.0 overexpression on Ca2⁺ sparks in intact rabbit cardiomyocytes is complicated by the rapid sarcolemmal extrusion of intracellular Ca2⁺ and reduction of resting [Ca2⁺]i and SR Ca2⁺ content during the quiescent periods required for Ca2⁺ spark recording. For this reason, sarcolemmal fluxes were functionally bypassed by the acute permeabilization of the sarcolemma with β-escin and the cytosolic [Ca2⁺]i maintained at 155 to 165 nmol/L. Ca2⁺ spark activity and SR Ca2⁺ content was monitored by the inclusion of 10 μmol/L Fluo-3 in the perfusing solution. SR Ca2⁺ content was assessed at the end of spark recording by rapid application of 10 mmol/L caffeine; the mean values are shown in Figure 6B. SR Ca2⁺ content was significantly higher in cells transfected with Ad-FKBP12. (627±94 versus 1901±338 nmol/L; Ad-LacZ [n=8 cells] versus Ad-FKBP12 [n=6 cells]). To quantify Ca2⁺ spark activity, the line scan images (Figure 6A) were analyzed using an automated spark detection program.10 The results collated from a number of cardiomyocytes are shown in Figure 6C. Increases in mean values of peak (1.96±0.03 versus 2.14±0.09 F/F0) and duration (40.4±1.5 versus 47.3±2.1 ms) were observed (Ad-LacZ: n=18 cells, 1742 events; versus Ad-FKBP12: n=10 cells, 626 events). Spark frequency was significantly reduced (0.064±0.005 versus 0.047±0.005 events · μm⁻¹ · sec⁻¹). Incubation of cells with rapamycin (3 μmol/L) during the 48 hours of quiescent culture inhibited the ability of Ad-FKBP12.0 to alter Ca2⁺ spark parameters (Figure 6D). To support the conclusion that these Ca2⁺ spark characteristics were attributable a decreased Ca2⁺ sensitivity of RyR2, Ad-LacZ transfected cells were equilibrated with 50 μmol/L tetracaine. Under these conditions the SR Ca2⁺ content in these cells increased to similar levels observed in FKBP12.0 overexpressing cells (720±116 versus 1640±320 nmol/L, Ad-LacZ, n=10cells versus Ad-LacZ+Tetracaine, n=10cells) as shown in Figure 7B. Ca2⁺ spark parameters were also affected in a similar manner with increases in Ca2⁺ spark peak (1.95±0.03 versus 2.05±0.04 F/F0), duration (36.7±0.9 versus 40.58±1.8 ms), Ad-LacZ n=25 cells 2971 events; Ad-LacZ versus Ad-LacZ+ tetracaine.
n = 11 cells (1646 events) and a decrease in Ca\(^{2+}\) spark frequency (0.062 ± 0.004 versus 0.047 ± 0.004 events/μm\(^2\)·sec\(^{-1}\)). To compare the effects FKBP12.0 and tetracaine with previously published data on FKBP12.6 overexpression,\(^5\) the relative effects of these interventions on peak, duration, width, and frequency of Ca\(^{2+}\) sparks is shown in Figure 7D. The data are expressed relative to the corresponding Ad-LacZ (control) data. FKBP12.0 and tetracaine both caused an increase in peak and duration to ≈115% of control. As published previously, FKBP12.6 overexpression caused a decrease in peak and duration to ≈90% of control. All interventions decreased Ca\(^{2+}\) spark frequency.

**Discussion**

**Relationship Between Ca\(^{2+}\) Transient Amplitude and SR Ca\(^{2+}\) Content**

This study is the first to show that overexpression of FKBP12.0 increases SR Ca\(^{2+}\) content (by ≈25%) but does not alter Ca\(^{2+}\) transient amplitude (Figures 2 and 3). Previous studies have shown an approximately exponential relationship between SR Ca\(^{2+}\) content and Ca\(^{2+}\) transient amplitude.\(^1\) In the present study, the Ad-LacZ group demonstrated a similar exponential relationship (Figure 4), indicating that an ≈25% increase in SR Ca\(^{2+}\) content would be expected to increase Ca\(^{2+}\) transient amplitude by ≈100%. The absence of any significant effect on the Ca\(^{2+}\) transient amplitude suggests that the sensitivity of the Ca\(^{2+}\)-induced Ca\(^{2+}\) release process is reduced by FKBP12.0 overexpression.\(^1\) In support of this conclusion, tetracaine, a drug known to reduce the Ca\(^{2+}\) sensitivity of RyR2, produced a similar effect on E–C coupling.

A previous study has shown that overexpressing FKBP12.6 increases Ca\(^{2+}\) transient amplitude and SR Ca\(^{2+}\) content.\(^5\) Similar effects shown in the present study indicated that FKBP12.6 overexpression does not significantly affect E–C coupling gain (Figure 4). In contrast, overexpression of FKBP12.0 reduced E–C coupling gain, suggesting that the 2 isoforms of FKBP may modulate RyR2 activity in different ways. This is reinforced by the contrasting effects of FKBP12.0 and 12.6 on Ca\(^{2+}\) sparks. A more detailed study comparing the 2 isoforms is required to clarify this point further.

**Verification of RyR2-FKBP12.0 Interaction**

Previously, Jeyakumar et al used an exchange binding assay with soluble [\(^{35}\)S]FKBP12.0 and [\(^{35}\)S]FKBP12.6 to demonstrate the ability of both FKBP isoforms to bind to RyR2.\(^1\) This work provided the first evidence that RyR2-FKBP12.0 interaction may occur in cardiac muscle in some vertebrates. The current study used immunoprecipitation techniques to demonstrate RyR2/FKBP12.6 association in rabbit heart cell lysates as previously reported in myocardium from rat and mice.\(^9\) However, this technique failed to show RyR2/FKBP12.0 association. A GST pull-
down assay involving purified proteins subsequently showed that both GST-FKBP isoforms were capable of binding with rabbit and human RyR2 (Figure 5A and 5B). In the immunoblot analysis that followed GST pull down, the anti-RyR antibody used could not discriminate between the 2 RyR isoforms. Recent data have demonstrated the coexpression of 3 different RyR isoforms in both healthy and failing human hearts.12 In addition, possible contaminations of starting material with RyR1-containing cells/tissue may also occur. MALDI-TOF mass spectrometry confirmed that the high-molecular-weight proteins precipitated with GST-FKBP12.0 was the cardiac isoform (RyR2). The reason for the discrepancy between immunoprecipitation and GST pull-down assays with regard to FKBP12.0/12.6-RyR2 interactions is unclear. The dichotomy would indicate that the nature of the interaction of the 2 FKBP isoforms with RyR2 is different. The association of FKBP12.6 appears to be more stable and capable of surviving the preparation of the tissue for biochemical studies. Although the functional data strongly indicate that FKBP12.0 affects RyR2 function, and the GST pull down indicates that direct FKBP12.0-RyR2 interaction is possible, the nature of the association may not be sufficiently strong to survive tissue preparation for immunoprecipitation studies.

Specificity and Reliability of GST Biochemical Data
To validate the specificity of the GST pull-down assay, canine RyR2 was used as a negative control. When subjected to the GST pull-down assay, canine RyR2 was able to interact with GST-FKBP12.6 but not with GST-FKBP12.0 (Figure 5D). This result supplements previously published data suggesting that canine RyR2 has a higher specificity for FKBP12.6 over FKBP12.0 compared with other mammals.1

Effect of FKBP12.0 Overexpression on Ca²⁺ Sparks
Steady-state Ca²⁺ spark activity and SR Ca²⁺ load was measured in permeabilized cardiomyocytes at a standardized bathing [Ca²⁺] (155 to 165 nmol/L).13,14 Previous work has established that Ca²⁺ spark characteristics (amplitude, time course, and frequency) in permeabilized cells are indistinguishable from those observed in intact cells14 and regulated by known modulators of RyR2 activity (Ca²⁺–calmodulin and cyclic ADPribose).14 It is unlikely that there was any loss of FKBP from permeabilized preparations because previous work on isolated SR vesicles indicates minimal FKBP loss even after vigorous homogenization procedures.15 FKBP12.0 overexpression resulted in a significantly increased steady-state SR Ca²⁺ load in permeabilized cells. This confirmed the
conclusion that FKBP12.0 overexpression acted directly on the SR and not via sarcolemmal-based processes. The relative effect of FKBP12.0 overexpression on SR content was greater than that seen in intact cells. This may reflect the higher cytoplasmic [Ca\textsuperscript{2+}] and higher SR loads observed in the permeabilized cell experiments.

Ca\textsuperscript{2+} sparks were significantly increased in amplitude (\(\approx 110\%\)) and duration (\(\approx 114\%\)), but frequency was reduced (\(\approx 73\%\)) compared with control. These effects were prevented by preincubation with rapamycin. The changes in Ca\textsuperscript{2+} spark characteristics are unlikely to be a simple consequence of the increased SR Ca\textsuperscript{2+} content because manipulations that increase SR Ca\textsuperscript{2+} content by stimulating the SR Ca\textsuperscript{2+} pump increase both Ca\textsuperscript{2+} spark size and frequency.\textsuperscript{13} In the present study, SR Ca\textsuperscript{2+} content was increased by inhibition of RyR2 using tetracaine (50 \(\mu\text{mol/L}\)); this increased Ca\textsuperscript{2+} spark amplitude (\(\approx 106\%\)) and duration (\(\approx 111\%\)) but reduced Ca\textsuperscript{2+} spark frequency (\(\approx 76\%\)). The similarity of the changes in Ca\textsuperscript{2+} spark parameters produced by tetracaine to those of the Ad-FKBP12.0 group supports the conclusions from the E–C coupling studies (Figure 4), namely that FKBP12.0 overexpression acts to reduce the overall Ca\textsuperscript{2+} sensitivity of RyR2. This effect is distinct from the effect of FKBP12.6 overexpression reported earlier\textsuperscript{10} and summarized in Figure 7D, ie, a decrease in spark peak and duration to \(\approx 90\%\) of control. This reinforces the point that the two forms of FKBP have differing effects on RyR2.

**Does FKBP12.0 Influence E–C Coupling Under Physiological Conditions?**

In rabbit cardiomyocytes, FKBP12.0 protein expression is \(\approx 10\) times higher than that of FKBP12.6.\textsuperscript{2} The affinity of FKBP12.0 for rabbit RyR2 was estimated as \(\approx 7\) times lower than FKBP12.6;\textsuperscript{1} therefore, under physiological conditions, FKBP12.0 bound to RyR2 may be comparable to FKBP12.6. Under resting conditions, FKBP12.6 knockout mice appear to have a functional Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism.\textsuperscript{16} However, work on isolated RyR2 in lipid bilayers has shown that complete absence of FKBP12.6 and 12.0 from RyR2 causes dramatic increases in open probability and uncoupling of channels,\textsuperscript{17} behavior that is not compatible with normal E–C coupling. This paradox may be explained by the retention of the influence of FKBP12.0 on RyR2 in animals without the FKBP12.6 isoform.

**Conclusions**

This study provides the first evidence that FKBP12.0 influences RyR2 function in a distinct fashion from that observed with FKBP12.6. The differences are summarized as follows. (1) Unlike FKBP12.6, the association of FKBP12.0 to RyR2 could not be detected by immunoprecipitation but was evident in GST pull-down assays. (2) FKBP12.0 overexpression reduces the gain of E–C coupling, an effect not observed with FKBP12.6 overexpression. (3) FKBP12.0 overexpression increases Ca\textsuperscript{2+} spark amplitude, the opposite from that observed with FKBP12.6. The molecular actions of FKBP on RyR are not clear; changes in isolated RyR2 channel kinetics have been observed as a result of FKBP12.6 binding.\textsuperscript{18} FKBP12.6 is also thought to mediate the coupled gating observed in RyR2 channel clusters.\textsuperscript{19} These data suggest that the net effect of FKBP12.6 on a cluster of RyR2s in the dyad is therefore a combination of at least 2 effects. One possible explanation for the distinct effects of FKBP12.0 observed in this study is a different balance of effects on the single-channel kinetics and interchannel cooperatively. But there is, as yet, no evidence to suggest that FKBP12.0 and FKBP12.6 act on the same site on RyR2. Further studies of FKBP12.0– and FKBP12.6–RyR2 interactions would benefit from a conditional FKBP12.6/FKBP12.0 knockout to circumvent the developmental cardiac defects observed in FKBP12.0 knockout mice and allow the effects of FKBP12.6 and FKBP12.0 to be studied in isolation.

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**Disclosures**

None.

**References**


Overexpression of FK-506–Binding Protein 12.0 Modulates Excitation–Contraction Coupling in Adult Rabbit Ventricular Cardiomyocytes
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Preparation of cell lysates
For immunoblotting, 5 x 10^5 cardiomyocytes were homogenized by sonication in lysis buffer containing (in mmol/L): 20 Tris-HCl, 250 NaCl, 3 EDTA, 0.5 EGTA, 20 β-glycerophosphate, 1% NP-40, pH 7.4, and supplemented with protease inhibitors (0.2 mmol/L pefabloc SC, 100 mmol/L aprotinin, 1 µmol/L leupeptin, 1 µmol/L pepstatin A, 1 mmol/L benzamidine, 1 µmol/L of calpain inhibitor I, and 1 µmol/L of calpain inhibitor II). Crude homogenates were centrifuged for 5 min at 10000 g (4°C). Protein concentrations of the supernatants were determined by the BCA method (Pierce) and samples were stored at –80°C.

Preparation of cell lysates and cardiac homogenates for GST pull-down assay
Freshly isolated rabbit ventricular cardiomyocytes (1 x 10^6) were lysed in 500 µl of solubilization medium (50 mmol/L Tris-HCl, 0.9% NaCl, 0.25% Triton X-100, 1 mmol/L NaF, 1 mmol/L Na3VO4, pH 7.4 and protease inhibitors in concentrations indicated above) and incubated on ice for 30 min. Left ventricular tissue from canine hearts and from human hearts explanted from patients with IDCMP, undergoing cardiac transplantation, was thawed and homogenized in 3 volumes of buffer for homogenization containing (in mmol/L): 50 Tris-HCl, pH 7.4, 200 NaCl, 20 NaF, 1 Na3VO4, 1 DTT and supplemented with protease inhibitors in concentrations indicated above. Cell lysates and cardiac homogenates were obtained by centrifugation for 10 min at 3000 g (4°C). Cardiac homogenates (1 mg) were further suspended in solubilization medium (0.5 mL) supplemented with protease inhibitors and incubated on ice for 30 min.

GST pull-down assay
Cell lysates and cardiac homogenates prepared as described above were incubated with 30 µg of GST-FKBP12.0 or GST-FKBP12.6 immobilized on glutathione-sepharose for 5 hr at 4°C. Human FKBP12-pGEX-3X or FKBP12.6-pGEX-3X constructs (generously provided by Dr. Wayne Chen, Department of Physiology and Biophysics, University of Calgary, Canada) were used to express and purify the GST-FKBP12.0 and GST-FKBP12.6 according to manufacturer’s instructions (Amersham). After washing the glutathione-sepharose precipitates three times in solubilization medium, the GST-tagged protein and any associated proteins were competitively eluted with 10 mmol/L reduced glutathione in 50 mmol/L Tris-HCl, pH 8.0 for 15 min at room temperature. Incubation of samples with immobilized GST (Sigma) and elution with 60 µM FK506 or vehicle alone (6% MethOH) served for controlling the specificity of interaction.

Immunoblot details
Primary antibodies used were goat polyclonal anti-FKBP12.0 (1:1000, C-19, Santa Cruz Biotechnology) rabbit polyclonal anti-RyR (1:10000, RyR2-5029, generously provided by Dr. A. Marks (Center for Molecular Cardiology, Columbia University, New York, U.S.A.)) rabbit polyclonal anti-calsequestrin (CS) (1:5000, PA1-913, Affinity Bioreagents). Secondary antibodies used were rabbit anti-goat affinity-purified IgG (1:2000, Dako) and donkey anti-rabbit whole Ig (1:10000, Amersham). The immunoreactive bands were visualized using SuperSignal® West Pico Chemiluminescent Substrate (Pierce). In addition, protein bands were visualized by silver staining using a previously published protocol.

E-C coupling studies:
Electrophysiology solutions:
Krebs Hensleit solution (mmol/L): 144 NaCl, 5.4 KCl, 1.0 MgCl2, 5.0 HEPES, 11.1 Glucose, 0.3 NaH2PO4·2H2O, 1.8 CaCl2, 0.1 niflumic acid, and 5.0 4-amino pyridine at 37°C. Tetrodotoxin (TTX, Sigma 5 µmol/L) was included in the perfusate to suppress the inward Na+ current. Voltage clamp was achieved using whole cell ruptured patch technique using an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA, USA) operated in switch clamp mode. Pipette resistance was 7-10 MΩ. The pipette solution contained (mmol/L): 20 KCl, 100 K aspartate, 20 tetraethylammonium chloride, 10 HEPES, 4.5 MgCl2, calculated free [Mg2+] ~0.9 mmol/L; 4 Na2ATP, 1 Na2 Creatine Phosphate (free Na+ = 10mM), 0.1 EGTA, pH 7.25 with KOH. No correction for liquid-junction potentials was applied, the small DC offset observed in Krebs-Henseleit solution was nulled prior to patching on to the cell.
**E-C coupling studies at a range of SR Ca\(^{2+}\) loads:**
The relationship between SR Ca\(^{2+}\) content and Ca\(^{2+}\) transient amplitude was investigated by superfusing cardiomyocytes for set periods of time with thapsigargin (5 \(\mu\)mol/L) in a manner similar to that described earlier \(^4\). This achieved a decrease in Ca\(^{2+}\) transient amplitude and SR Ca\(^{2+}\) content as a result of progressive SERCA2a inhibition \(^5\). Complete inhibition of the SR was achieved after 100 s perfusion with thapsigargin; rapidly switching to 10 mmol/L caffeine did not generate a Ca\(^{2+}\)-release or \(I_{\text{NCX}}\). Shorter periods of thapsigargin containing perfusion medium achieved intermediate caffeine responses representing intermediate SR Ca\(^{2+}\) contents. Separate groups of cells were exposed to 40 s and 80 s periods of perfusion with thapsigargin, Ca\(^{2+}\) transient amplitude was measured from the last 4 transients before caffeine application. SR Ca\(^{2+}\) content was increased by using a voltage-clamp protocol where a holding potential of –50 mV was used. Measurements of L-type Ca\(^{2+}\) channel current amplitude and the calculation of the integral of this current was used to verify that none of the above protocols caused a significant change in either of these parameters (results not shown).

**Ca\(^{2+}\) spark measurements:**
Fluo-3 was excited at 488 nm (Kr laser) and measured >515 nm using epifluorescence optics of an inverted microscope with a 60 X/1.2 NA water-immersion objective lens. Fluorescence was acquired in line-scan mode at 2 ms/line; pixel dimension was 0.29 \(\mu\)m (512 pixels/scan; zoom=1.4). The scanning laser line was oriented parallel with the long axis of the cell and placed approximately equidistant between the outer edge of the cell and the nucleus/nuclei, to ensure the nuclear area was not included in the scan line. To enable this trace to be converted to [Ca\(^{2+}\)] a series of calibration solutions were used at the end of each Ca\(^{2+}\) spark measurement period incorporating 10 mmol/L EGTA as previously described \(^6\). In all experiments concerning Ca\(^{2+}\) sparks, the [Ca\(^{2+}\)] in the test solution was 145-160 nmol/L. Ca\(^{2+}\) sparks recorded in Fluo-3 containing solutions were quantified using an automatic detection and measurement algorithm adapted from a previously published method \(^7\).
FKB12.6 but not FKB12 co-immunoprecipitates with rabbit cardiac ryanodine receptor

To examine whether the co-immunoprecipitation technique could detect the association of FKBPs isoforms with RyR2, a CHAPS-solubilized cardiac membrane fraction (CSMF) was prepared. This preparation is optimal for this type of analysis, since the CSMF is relatively easy to obtain and it is enriched with proteins of sarcoplasmic reticulum, including ryanodine receptor.

Online Figure 1. Association of FKB12.6 with RyR2 in rabbit CHAPS-solubilized cardiac membrane fraction and rabbit cardiomyocytes. A and B – The CHAPS-solubilized membrane fraction from rabbit heart was subjected to immunoprecipitation with anti-RyR or anti-FKBPs Ab, the immunoprecipitates were electrophoretically separated on 5% (A) and 15% (B) SDS-PAGE; detection of RyR or FKBP bands was conducted by Western blot analysis with anti-RyR or anti-FKBPs Ab. C – Protein lysates obtained from rabbit cardiomyocytes were subjected to immunoprecipitation with anti-RyR Ab, the immunoprecipitates were electrophoretically separated on 4-20% linear gradient SDS-PAGE followed by detection with anti-FKBPs Ab. Recombinant FKB12 and FKB12.6 display evident difference in their electrophoretic mobilities (C-two last lanes and D), confirming that the resolution capacities of the used 15% (B and D) and 4-20% linear gradient (C) SDS-PAGEs are high enough to separate different isoforms of FKBP.

RyR2 was immunoprecipitated from CHAPS-solubilized cardiac membrane fraction (Online Fig. 1A, lane 1) using a mouse monoclonal anti-RyR antibody (34C clone, Affinity Bioreagents) and its presence in the immunoprecipitate was examined by Western blot analysis using another mouse monoclonal anti-RyR antibody (C3-33 clone, Affinity Bioreagents). Different antibodies were used for immunoprecipitation and immunovisualization of RyR2 to increase the reliability of specific detection of RyR.

To examine the presence of FKBPs isoforms (both or one of them), the same immunoprecipitate was blotted with anti-FKBPs12 antibody (SA-169), which cross-reacts with rabbit FKBPs12.6. SA-169 was used rather than the commercially available anti-FKBPs12 antibody C-19 because SA-169 appeared to be more sensitive to rabbit antigen. Although this antibody does not discriminate between FKBPs12 and FKBPs12.6 isoforms, it is still possible to distinguish the two FKBPs on the basis of their electrophoretic mobilities, since FKBPs12.6 migrates somewhat slower than FKBPs12 (Online Fig. 1D).
After blotting the same immunoprecipitate with SA-169 antibody, only one band appears (Online Fig. 1B, lane 1). Its electrophoretic mobility is distinctly different from that of recombinant FKBP12 (Online Fig. 1B, lane 5), which was loaded to ensure that the resolution capacity of the gel is high enough to separate the FKBP isoforms. Next to the immunoprecipitates, 12 ng of both recombinant FKBP isoforms were placed next to each other. These proteins displayed an evident difference in their electrophoretic mobilities confirming that the two co-immunoprecipitated proteins are FKBP12.6 (Online Fig. 1C, lanes 1-2). To confirm the specificity of interaction between RyR and FKBP, several negative controls were included: treatment of CHAPS solubilized cardiac membrane fraction with FK506 prior to immunoprecipitation completely abolished RyR2–FKBP12.6 interaction (Online Fig. 1B, lane 2); incubation of samples with antibody against SERCA2a, which is known neither to be associated with RyR nor with FKBP12, did not lead to precipitation of FKBP12.6 (Online Fig. 1B, lane 3); incubation of samples with protein G-sepharose beads alone also did not lead to precipitation of FKBP12.6 (Online Fig. 1B, lane 4).

Reciprocal co-immunoprecipitation using anti-FKBP antibody (C-19) revealed the presence of RyR in the immunoprecipitate (Online Fig. 1A, lane 2).
**MALDI-TOF mass spectrometry of proteins recognized by anti RyR2 antibodies binding GST-FKBP12.0 or GST-FKBP12.6**

To exclude the possibility of detecting RyR1 instead of RyR2 when GST-FKBP12.0 is used as the ligand in cardiac homogenates, the high molecular weight proteins from each species recognised by anti-RyR antibody and precipitated either with GST-FKBP12.0 or with GST-FKBP12.6 were subjected to MALDI-TOF mass spectrometry. Both were identified as the cardiac isoform of ryanodine receptor, RyR2 (Online Figs. 2,3,4 & 5). Thus, these studies provide clear evidence that both rabbit and human RyR2 are capable of binding to FKBP12.
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Online Figure 2. Protein identification by MALDI-TOF mass spectrometry. From the trypsin digestion profile, depicted as MALDI-MS spectrum (upper panel), high molecular weight protein extracted from the rabbit cardiac homogenate on the basis of its association with GST-FKBP12 (see inset depicting Coomassie Blue-stained gel, upper panel) was identified as cardiac isoform of RyR (see extract from Mascot Search Results, bottom panel).

Online Figure 3: Protein identification by MALDI-TOF mass spectrometry. From the trypsin digestion profile, depicted as MALDI-MS spectrum (upper panel), high molecular weight protein
Online Figure 4: Protein identification by MALDI-TOF mass spectrometry. From the trypsin digestion profile, depicted as MALDI-MS spectrum (upper panel), high molecular weight protein extracted from the human cardiac homogenate on the basis of its association with GST-FKBP12 (see inset depicting Coomassie Blue-stained gel, upper panel) was identified as cardiac isoform of RyR (see extract from Mascot Search Results, bottom panel).
Online Figure 5: Protein identification by MALDI-TOF mass spectrometry. From the trypsin digestion profile, depicted as MALDI-MS spectrum (upper panel), high molecular weight protein extracted from the human cardiac homogenate on the basis of its association with GST-FKBP12.6 (see inset depicting Coomassie Blue-stained gel, upper panel) was identified as cardiac isoform of RyR (see extract from Mascot Search Results, bottom panel).
References

Reference List


