Calcineurin regulates ryanodine receptor/Ca^{2+}-release channels in rat heart

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The present study was undertaken to examine the physical and physiological interaction of protein phosphatase 2B, calcineurin, with the ryanodine receptor (RyR) in rat cardiac tissue and neonatal cardiomyocytes. The presence of calcineurin, the RyR and FK506-binding protein (FKBP)12.6 in rat cardiac sarcoplasmic reticulum (SR) was identified by Western blot analysis. The possible interactions between calcineurin, the RyR and FKBP12.6 were further studied by co-immunoprecipitation using CHAPS-solubilized cardiac-membrane fractions (CSMFs) or SR preparations. Physical interactions between the RyR and calcineurin were found in the CSMF in the presence of added 100 μM Ca^{2+}; however, the interactions were interrupted in the presence of 20 mM EGTA, 1 μM rapamycin or 1 μM FK506, suggesting that the interaction is Ca^{2+}-dependent, and is mediated by FKBP12.6. The Ca^{2+}-dependent interaction between FKBP12.6 and the RyR was also found by co-immunoprecipitation. Effects of calcineurin inhibitors were tested on neonatal-rat-heart cardiomyocytes. Treatment of neonatal cardiomyocytes with 20 μM deltamethrin, 10 μM cyclosporin A (CsA), or 10 μM FK506 led to Ca^{2+} oscillations in originally quiescent cardiomyocytes. Preincubation of cardiomyocytes with 20 μM rapamycin which dissociates FKBP12.6 from the RyR, evoked Ca^{2+} oscillations, probably due to the leakiness of the RyR. However, Ca^{2+} oscillations by rapamycin were not further affected by 10 μM CsA or 10 μM deltamethrin, suggesting that only RyR-associated calcineurin could regulate the channel activities. In spontaneously Ca^{2+}-oscillating cardiomyocytes, CsA or FK506 treatments increased the frequency of oscillations. In 10 μM ryanodine-treated cardiomyocytes, CsA failed to induce Ca^{2+} oscillations. These data show evidence that calcineurin associated with the RyR could modulate Ca^{2+} release in rat heart.

Key words: cardiomyocytes, cyclosporin A, phosphatase, phosphorylation, sarcoplasmic reticulum.

INTRODUCTION

Calcineurin, a hetero-dimeric serine/threonine phosphatase, is known to have a variety of cellular functions in different types of cells [1,2]. The function of calcineurin in T-cell activation has been clearly identified by the immunosuppressant drugs, cyclosporin A (CsA) and FK506. For example, upon binding of FK506 to its target proteins, FK506-binding protein (FKBP) and calcineurin, the phosphatase activity of calcineurin is sterically inhibited and phosphorylated calcineurin substrates are accumulated [3]. One of these substrates, nuclear factor of activated T-cells transcription factor ('NFAT'), in its phosphorylated form is unable to be translocated to the nucleus where it regulates the expression of interleukin-2, critical for T-cell activation [4]. Although the pharmacological actions of the immunophilins are readily explained by the above model, the physiological roles have remained to be solved despite their wide distribution and abundance in various tissues. Recent studies have shown evidence that FKBP12 and FKBP12.6 are associated with the ryanodine receptor (RyR) in skeletal and cardiac sarcoplasmic reticulum (SR) respectively [5–8].

In cardiac muscle, influx of extracellular Ca^{2+} through the dihydropyridine receptor in sarcolemma causes further release of intracellular Ca^{2+} from the SR through the RyR resulting in muscle contraction [9,10]. The native RyR complex is composed of homo-tetramers of the 565-kDa protein, which is associated with FKBP12 in a 1:1 molar ratio [6]. When FKBP12 was dissociated from the RyR–FKBP12 complex by treatment with FK506 or rapamycin, the RyR became active at lower concentrations of caffeine [6,11] or Ca^{2+} [12,13], whereas a higher concentration of Mg^{2+} was required for inactivation [13]. Evidence has also shown that calcineurin regulates the Ins(1,4,5)P_3 receptor [Ins(1,4,5)P_3R]-mediated Ca^{2+} release via modulation of the phosphorylation state of Ins(1,4,5)P_3R in rat brain microsomal preparations [14,15]. Using calcineurin inhibitors and constitutively active calcineurin mutants, we have found that intracellular Ca^{2+}-release through the Ins(1,4,5)P_3R is modulated by phosphatase activity of calcineurin *in vivo* [16]. We also found that up-regulated calcineurin in CsA-treated rat heart could alter the functions of the cardiac RyR, although the underlying molecular mechanism has remained to be solved [17]. The present study was undertaken to investigate whether calcineurin interacts with the RyR in rat cardiac tissue and regulates Ca^{2+} release in cultured cardiomyocytes. The present results show that calcineurin in cardiac SR interacts specifically with the RyR via FKBP12.6, and inhibition of endogenous calcineurin leads to substantial Ca^{2+} oscillations in isolated cardiomyocytes.

MATERIALS AND METHODS

Materials

Sprague–Dawley rats weighing 150–200 g were used for this study. The antibodies used for the present study were obtained from the following companies: mouse monoclonal anti-(RyR

Abbreviations used: AM, acetoxymethyl ester; CSMF, CHAPS-solubilized cardiac-membrane fraction; CsA, cyclosporin A; FS, final supernatant; FKBP, FK506-binding protein; Ins(1,4,5)P_3R, Ins(1,4,5)P_3 receptor; KRB, Krebs–Ringer buffer; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; SERCA2, sarco/endoplasmic-reticulum Ca^{2+}-ATPase type 2; WH, whole homogenate; PSR, highly purified cardiac SR.

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type 2) and anti-[sarco/endoplasmic-reticulum Ca^{2+}-ATPase type 2 (SERCA2)] antibodies were from Affinity Bioreagents (Golden, CO, U.S.A.); mouse monoclonal anti-calcineurin antibody was from Sigma Chemical Co. (St. Louis, MO, U.S.A.); goat polyclonal anti-calcineurin and anti-FKBP12 (C-terminal) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Protein A–Sepharose was obtained from Pharmacia (Uppsala, Sweden). Rapamycin and cyclophilin were from Sigma Chemical Co. CsA, deltamethrin and FK506 were purchased from Calbiochem (La Jolla, CA, U.S.A.). Fluo-3 acetoxymethyl ester (AM) was obtained from Molecular Probes (Eugene, Oregon, U.S.A.). All other chemicals purchased from Sigma were of analytical grade.

Preparation of whole homogenate (WH) and SR

WH preparation was carried out according to the published procedure [18]. The rat cardiac SR was prepared as described previously [18], with some modifications. Briefly, pieces of the excised heart were homogenized in 4 vol. of extraction buffer A containing 20 mM imidazole (pH 7.0), 0.3 M sucrose and 0.6 M KCl. The homogenate was diluted with 3 vol. of extraction buffer B containing 20 mM imidazole (pH 7.0) and 0.3 M sucrose, and centrifuged at 5500 ***g*** for 10 min. The supernatant was re-centrifuged at 11000 ***g*** for 20 min. The second supernatant was then centrifuged at 130000 ***g*** for 30 min in a Beckman ultra-centrifuge (XL-70; Beckman Instruments Inc. Spinco Division, Palo Alto, CA, U.S.A.). The resulting pellets were suspended in the final buffer containing 20 mM Mops (pH 6.8), 0.15 M KCl and 0.3 M sucrose with various protease inhibitors [18]. The final supernatant (FS) was also collected to conduct the present study.

Western blot analysis

Protein samples in SDS sample buffer [19] were run on SDS/PAGE gels and the proteins on the gel were electrophoretically transferred to nitrocellulose membranes. The transferred proteins on the membranes were incubated with blocking solution containing 5% (w/v) BSA and 0.1% (v/v) Tween 20 in Tris-buffered saline, pH 7.6, for 2 h at 4 °C. After blocking, the membrane was treated with primary antibody overnight at 4 °C. The membrane was washed twice in Tris-buffered saline, pH 7.6, with 0.1% (v/v) Tween 20, and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody for 2 h at room temperature. The immunoreactive proteins were developed with Nitro Blue Tetrazolium. Band intensities on the nitrocellulose membranes were quantified by densitometry scanning.

Preparation of the CHAPS-solubilized cardiac-membrane fraction (CSMF)

Rat heart was homogenized in homogenization buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mg/ml leupeptin, 1 mg/ml pepstatin and 0.1 mg/ml PMSF [14]. The homogenate was centrifuged at 45000 ***g*** for 10 min and the pellets were resuspended in homogenization buffer without 2-mercaptoethanol. The resulting suspension was then solubilized in 1% (v/v) CHAPS for 20 min and centrifuged at 45000 ***g*** for 20 min. The supernatant contained the CSMF.

Co-immunoprecipitation

An aliquot of mouse monoclonal anti-RyR (1 μg,10 μl) or goat polyclonal anti-calcineurin (3 μg, 15 μl) was added to 300 μg (500 μl) of the CSMF and incubated for 4 h at 4 °C. Protein A–Sepharose was then used to precipitate the antibodies. The immunoprecipitates were washed sequentially with 1% (v/v) CHAPS in PBS, 0.5 M NaCl in PBS and PBS. The immunoprecipitates with anti-RyR were subjected to Western blot analysis using anti-calcineurin or anti-RyR antibodies.

Primary cell culture

Primary culture of ventricular cardiomyocytes was conducted from 2-day-old neonatal rats by methods described previously [20], with minor modifications. The ventricular tissue from 14 pups was transferred to a Petri dish and minced into small pieces in Krebs–Ringer buffer (KRB)-1 containing 1 mg/ml BSA, 2 mg/ml glucose and 100 units/ml penicillin/streptomycin in KRB basic solution (136 mM NaCl, 28.6 mM KCl, 1.9 mM $NaHCO_3$, 0.08 mM Na_2HPO_4, pH 7.4). Stepwise enzymic digestion in KRB-2 (100 units/ml collagenase, 10 mg/ml BSA, 2 mg/ml glucose and 100 units/ml penicillin/streptomycin in KRB) was performed at 37 °C for 10 min each under continuous mixing. The first tissue digest that consisted mainly of cell debris and mesenchymal cells was discarded. The two to five supernatants obtained after each 10 min digestion period were centrifuged for 5 min at 1000 ***g***. The cell pellets were resuspended in 5 ml of KRB-3 (10 mg/ml BSA, 2 mg/ml glucose and 100 units/ml penicillin/streptomycin in KRB). The suspension was filtered through a 250 μm nylon filter into Dulbecco's modified Eagle's medium and the cells were concentrated by centrifugation for 10 min at 1000 ***g***. The cells were resuspended in complete medium containing 8% (w/v) horse serum, 5% (w/v) fetal bovine serum and 100 units/ml penicillin/streptomycin in Dulbecco's modified Eagle's medium. For enrichment of cardiomyocytes, the cells were pre-plated on to a 100-mm dish for 1 h at 37 °C. The resultant suspension of cardiomyocytes was plated on to collagen-coated coverslips at a density of 10^5 cells/cm^2 and maintained at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2; the medium was replaced after 24 h. The majority of the cells were cardiomyocytes, as assessed by immunofluorescence with a monoclonal antibody specific to sarcomeric myosin (MF-20).

Immunofluorescence

To distinguish cardiomyocytes from non-cardiomyocytes, immunofluorescence cytochemistry with MF-20 was conducted as described previously [16]. Cardiomyocytes on coverslips were washed with PBS and fixed with 3.5% (v/v) paraformaldehyde for 10 min. The cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed with PBS and incubated with MF-20 for 40 min at room temperature. The cells were washed three times with PBS and then stained with rhodamine-isothiocyanate-conjugated goat anti-mouse immunoglobulin for 40 min at room temperature. To stain nuclei, the coverslips were incubated with PBS containing bis-benzimide (Hoechst 33258; Sigma Chemical Co.) and washed twice with PBS alone. The coverslips were mounted and examined under a Leica DMRBE microscope equipped with a 63X PLAPO objective and a HBO 100 W mercury lamp (Leica, Heidelberg, Germany). Images were photographed with T-max 3200 ASA film (Kodak, Rochester, NY, U.S.A.).

Measurement of transient Ca^{2+}-release by confocal microscopy

The free cytosolic Ca^{2+} was monitored in primary cardiac-muscle cells using the fluorescent Ca^{2+} indicator, fluo-3/AM, as described previously [21]. Briefly, cells grown on glass coverslips were

loaded with 5 μM fluo-3/AM at 30 °C in Krebs–Ringer solution containing 140 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 5.5 mM Hepes, 10 mM glucose and 1.2 mM $CaCl_2$, pH 7.4. After 60 min, cells were rinsed with the Krebs–Ringer solution, to remove unhydrolysed indicator, and transferred to a chamber where drugs were added, as indicated in Figure 5. Deltamethrin (20 mM) and FK506 (2.4 mM) were dissolved in DMSO to make stock solutions. CsA was dissolved in ethanol to make a 16 mM stock solution. DMSO (0.1 %) or ethanol (0.06 %) at the highest final concentrations used had no effect on Ca^{2+} release (results not shown). The experiments were performed using a laser scanning confocal microscope (Leica). Fluo-3 was excited with light at 488 nm and fluorescence was measured at 515 nm. Fluo-3 fluorescence was expressed as normalized increase in fluorescence compared with the resting level (F/Fo). The ratio of fluorescence, F/Fo, was produced by dividing the fluorescence intensity (F) of each pixel in the original fluorescence image by its intensity at the beginning of the images (defined as Fo), during the time when the cell was assumed to be in the resting state. Data analyses were performed using a workstation with Microsoft Windows NT software. After trial experiments, 10 μM CsA, 20 μM deltamethrin and 10 μM FK506 were found to be maximally effective in inducing Ca^{2+} release in resting cardiomyocytes. To see the effects of calcineurin inhibitors on spontaneous Ca^{2+} release, the same group of isolated single cardiomyocytes was scanned before and 10 min after the addition of the drugs. To examine the effect of CsA or deltamethrin on FKBP12.6-dissociated RyR, cardiomyocytes were pretreated with 20 μM rapamycin for 30 min before monitoring for spontaneous Ca^{2+} oscillations (see Figures 6E and 6F).

Miscellaneous

Ryanodine binding to WH, FS and SR was conducted as described previously [18]. Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, U.S.A.). The data are presented as means ± S.E.M. Statistical significance was evaluated with the Student's unpaired t test. P values < 0.05 were considered significant.

Table 1 Quantification of total proteins and ryanodine-binding activities in WH, SR and FS of cardiac tissue during SR preparation

The values are means ± S.E.M. for three independent experiments. Ryanodine binding was carried out using 20 nM [^{3}H]ryanodine.

Sample	Total protein (μg/mg of tissue)	Ryanodine binding (pmol/mg of protein)
WH	149.0 ± 35.8	0.264 ± 0.010
SR	1.9 ± 0.1	1.434 ± 0.180
FS	102.0 ± 14.3	0.006 ± 0.005

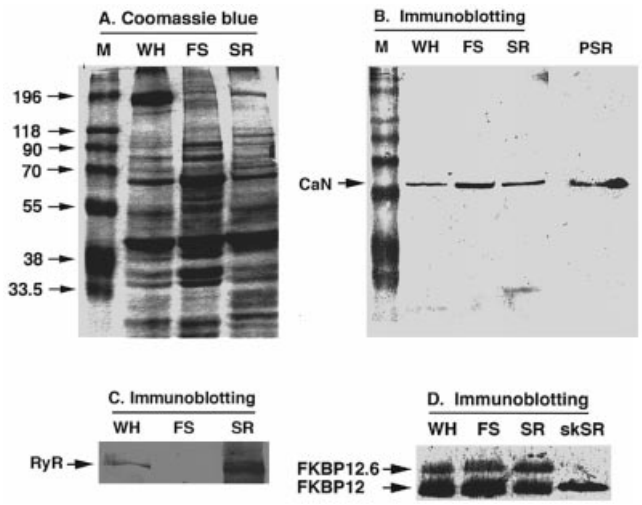

Figure 1 Identification of calcineurin (CaN), FKBP and the RyR in WH, FS, SR and PSR by Western blot analysis

Equal amounts (80 μg) of protein from WH, FS and SR were resolved in a 12% (w/v) polyacrylamide/SDS gel in duplicate. (**A**) Coomassie Blue-stained gel showing the profiles of proteins in WH, FS and SR. M, molecular-mass markers. (**B**) Western blot analysis of the companion gel shown in (**A**) with anti-calcineurin antibody. (**C**) Western blot analysis of the proteins from rat cardiac WH, FS and SR resolved in a 6% (w/v) polyacrylamide/SDS gel with anti-RyR antibody. (**D**) Western blot analysis of proteins from rat cardiac WH, FS, SR and rabbit-skeletal SR separated in a 15% (w/v) polyacrylamide/SDS gel with the antibody raised against the C-terminus of FKBP12 of human origin, which could detect both isoforms. To distinguish the mobility of FKBP12 isoforms, gels were polymerized with excess *N,N,N′,N′*-tetramethylethylenediamine ('TEMED'), as described previously [7]. Note that both FKBP12 and FKBP12.6 isoforms exist in cardiac-tissue fractions, while only FKBP12 was detected in skeletal SR (SkSR).

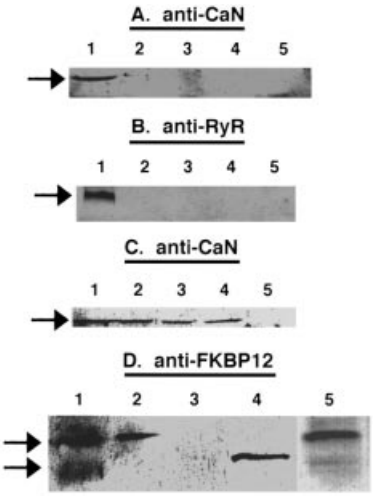

Figure 2 Association of calcineurin with the RyR in the CSMF

(**A**, **B** and **C**) The CSMF was treated with 100 μM Ca^{2+} (lanes 1), 20 mM EGTA (lanes 2), 100 μM Ca^{2+} plus 1 μM rapamycin (lanes 3) or 100 μM Ca^{2+} plus 1 μM FK506 (lanes 4) for 4 h at 25 °C [14], and subjected to immunoprecipitation with anti-RyR (**A**) or anti-calcineurin (anti-CaN; **B**) antibodies. Detection of calcineurin (**A** and **C**) or RyR (**B**) bands was conducted by Western blot analysis with anti-calcineurin or anti-RyR antibodies respectively. As a negative control, anti-SERCA2 antibody was used to immunoprecipitate the CSMF sample in the presence of 100 μM Ca^{2+} (lanes 5 in **A**, **B** and **C**). (**D**) Interaction of FKBP–calcineurin or FKBP–RyR in the CSMF was examined by immunoprecipitation with anti-calcineurin antibody followed by detection with anti-FKBP12 antibody (lane 1) or by immunoprecipitation with RyR antibody followed by detection with FKBP12 antibody (lane 2) in the presence of 100 μM Ca^{2+}. Note that both FKBP isoforms were detected in lane 1, but only FKBP12.6 was detected in lane 2. The CSMF was treated with 100 μM Ca^{2+} plus 1 μM rapamycin (lane 3) or 100 μM Ca^{2+} plus 1 μM CsA/cyclophilin (lane 5) and subjected to immunoprecipitation with anti-RyR antibody and detection with anti-FKBP12 antibody. Rabbit-skeletal SR (lane 4) was used to detect the presence of the FKBP12 isoform as a positive control.

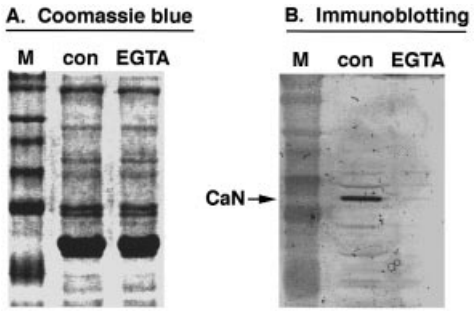

Figure 3 Preparation of calcineurin-devoid SR by EGTA

Equal amounts of protein (60 μg) from control SR (con) or calcineurin-devoid SR (EGTA), prepared using 20 mM EGTA, were resolved in a 12% (w/v) polyacrylamide/SDS gel and analysed by Coomassie Blue staining (**A**) and Western blot analysis with anti-calcineurin antibody (**B**). The procedure for preparation of the calcineurin-devoid SR is described in the Materials and methods section.

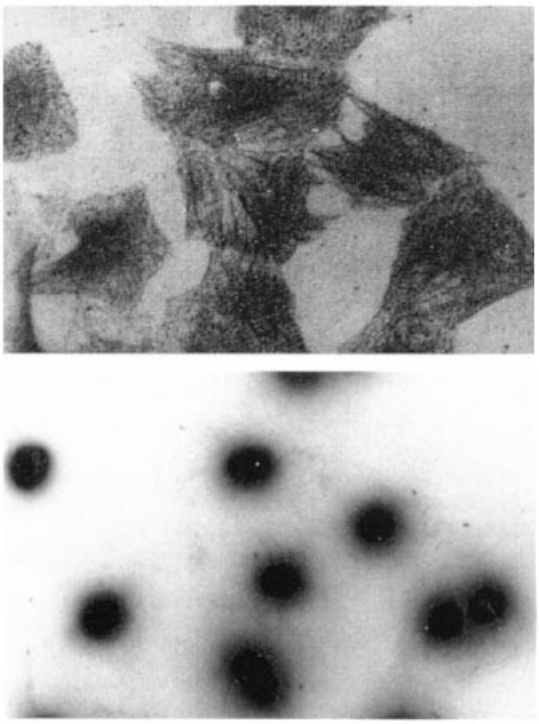

Figure 4 Identification of cardiomyocytes by immunufluorescence with MF-20

Representative images of cardiomyocytes identified by immunofluorescence with MF-20 (upper panel) and the nuclei in the same field stained with bis-benzimide (lower panel). Note that all the cells shown in the picture are cardiomyocytes.

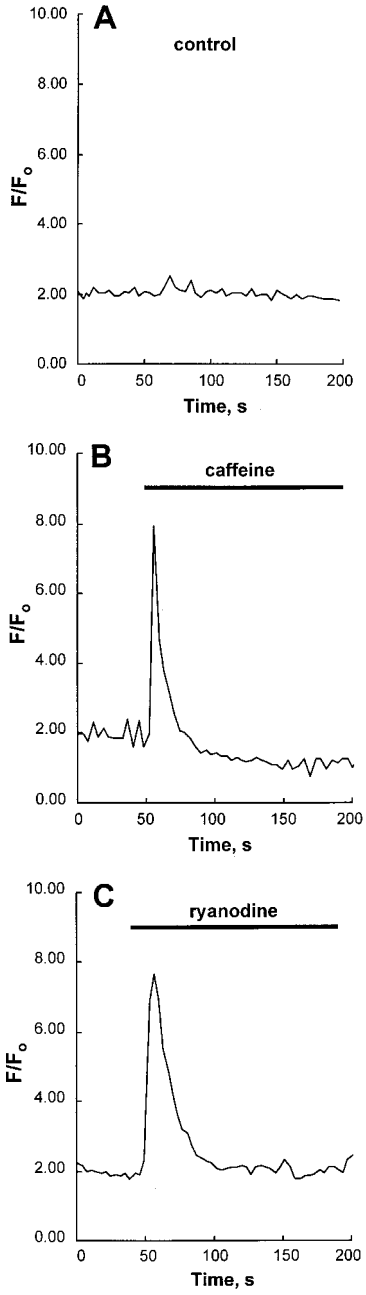

Figure 5 Transient Ca^{2+}-release in non-beating cultured cardiomyocytes by caffeine or ryanodine

Fluo-3/AM-loaded individual cardiomyocytes were examined for their ability to release Ca^{2+} from the SR by the application of caffeine or ryanodine. Fluorescence ratio (F/Fo), derived from one selected pixel of line scan images, is plotted as a function of time. The basal and the transiently increased fluorescence level in control (**A**), 10 mM caffeine (**B**) and 10 μM ryanodine (**C**) treated cardiomyocytes are shown. The data are representative of five independent experiments with more than five cells in each experiment.

RESULTS

Identification of calcineurin, FKBP12 and the RyR in rat cardiac SR

To examine whether calcineurin interacts with the RyR in cardiac SR, the presence of endogenous calcineurin in SR and in the cytosol of rat heart was first examined by Western blot analysis. Equal amounts (80 μg) of WH, SR and FS proteins were loaded on to and separated in 12% (w/v) polyacrylamide/SDS gels. The Coomassie Blue-stained gel showed the profiles of the separated proteins in the three different samples (Figure 1A), whereas the Western blot result showed the presence of calcineurin in all of the three samples (Figure 1B). The relative amount of calcineurin in the FS, as determined by densitometry scanning, was approx. 1.5-fold higher than that in the SR and 2-

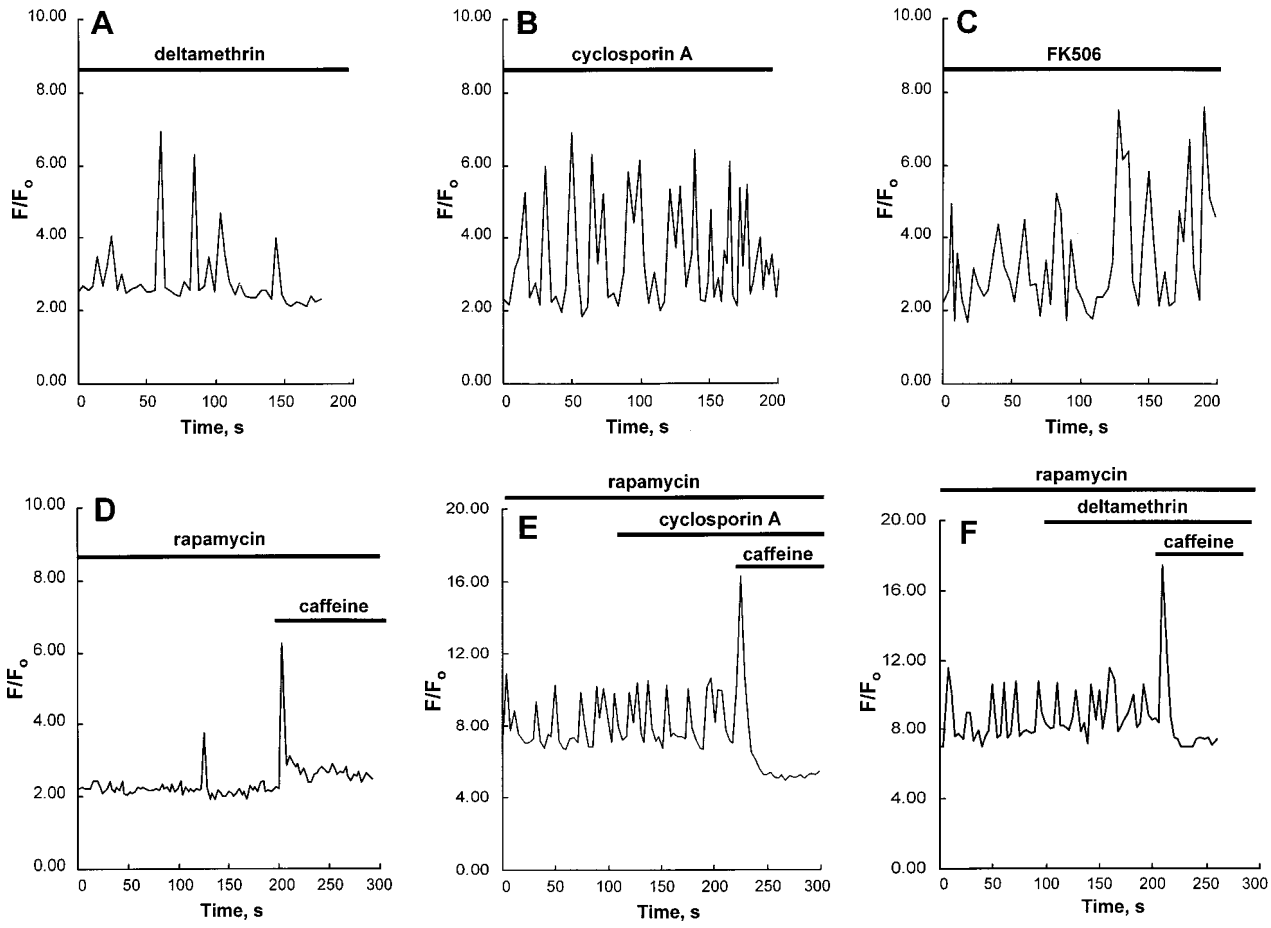

Figure 6 Effect of calcineurin inhibitors on Ca^{2+} oscillations in non-beating cultured cardiomyocytes

The effects of various calcineurin inhibitors on Ca^{2+} oscillations in fluo-3/AM-loaded individual cardiomyocytes are shown by confocal line scan, as described in the legend to Figure 5. (**A**–**D**) Profiles of the oscillating fluorescence in cardiomyocytes 10 min after treatment with the calcineurin inhibitors 20 μM deltamethrin (**A**), 10 μM cyclosporin A (**B** and **E**), 10 μM FK506 (**C**) and 10 μM rapamycin (**D**). (**E**, and **F**) Oscillating fluorescence 30 min after treatment with 20 μM rapamycin. During the periods indicated by solid lines, 10 μM CsA, 10 μM deltamethrin and 10 mM caffeine were applied. The data are representatives of three independent experiments with more than four cells in each experiment.

fold higher than that in WH. Since the total protein yield in the FS was much higher than in the SR (Table 1), the total amount of calcineurin in the cytosol must be considerably larger than that in the SR [22]. In order to check whether calcineurin found in the SR was a simple contamination from the cytosolic calcineurin, SR vesicles were washed twice more by repeating the centrifugation steps (see the Materials and methods section), and Western blot analysis was conducted. Similar amounts of calcineurin were found in the washed SR (results not shown), indicating that calcineurin is tightly bound to SR membranes. In highly purified cardiac SR (PSR) prepared by sucrose-density-gradient centrifugation [23], the presence of calcineurin was also observed (Figure 1B, lane PSR) further confirming the association of calcineurin with the SR.

In light of the evidence that FKBP12 is associated with the RyR in heart [7,8], the presence of FKBP12 in the SR was examined by Western blot analysis with the antibody raised against the C-terminal domain of FKBP12. The results show that the rat cardiac SR contained both FKBP12 and FKBP12.6 immunoreactive bands, whereas in the rabbit skeletal SR, only the FKBP12 band was detected (Figure 1C).

The relative amounts of the RyR in the cardiac tissue fractions (WH, SR, FS) were also examined by Western blot analysis using anti-RyR antibody in 6% (w/v) polyacrylamide gels. The RyR was present both in SR and WH, but was absent from the FS (Figure 1D). The relative amounts of the RyR in the three cardiac-tissue fractions were also examined by equilibrium [^{3}H]-ryanodine binding (Table 1). Consistent with the Western blot data (Figure 1D), the ryanodine-binding activity was enriched in the SR (1.434 pmol/mg of protein), while negligible amounts of ryanodine binding were observed in the FS (Table 1).

Association of calcineurin with the RyR in the CSMF

To examine whether the interaction of calcineurin with the cardiac RyR is Ca^{2+}-dependent, the CSMF was treated with 100 μM Ca^{2+} or 20 mM EGTA for 4 h at 25 °C [14]. The RyR was then immunoprecipitated by the anti-RyR antibody and the presence of calcineurin in the immunoprecipitate was examined by Western blot analysis using anti-calcineurin antibody. Pre-treatment of the CSMF with 100 μM Ca^{2+} led to immunoprecipitation of calcineurin with the RyR antibody (Figure 2A,

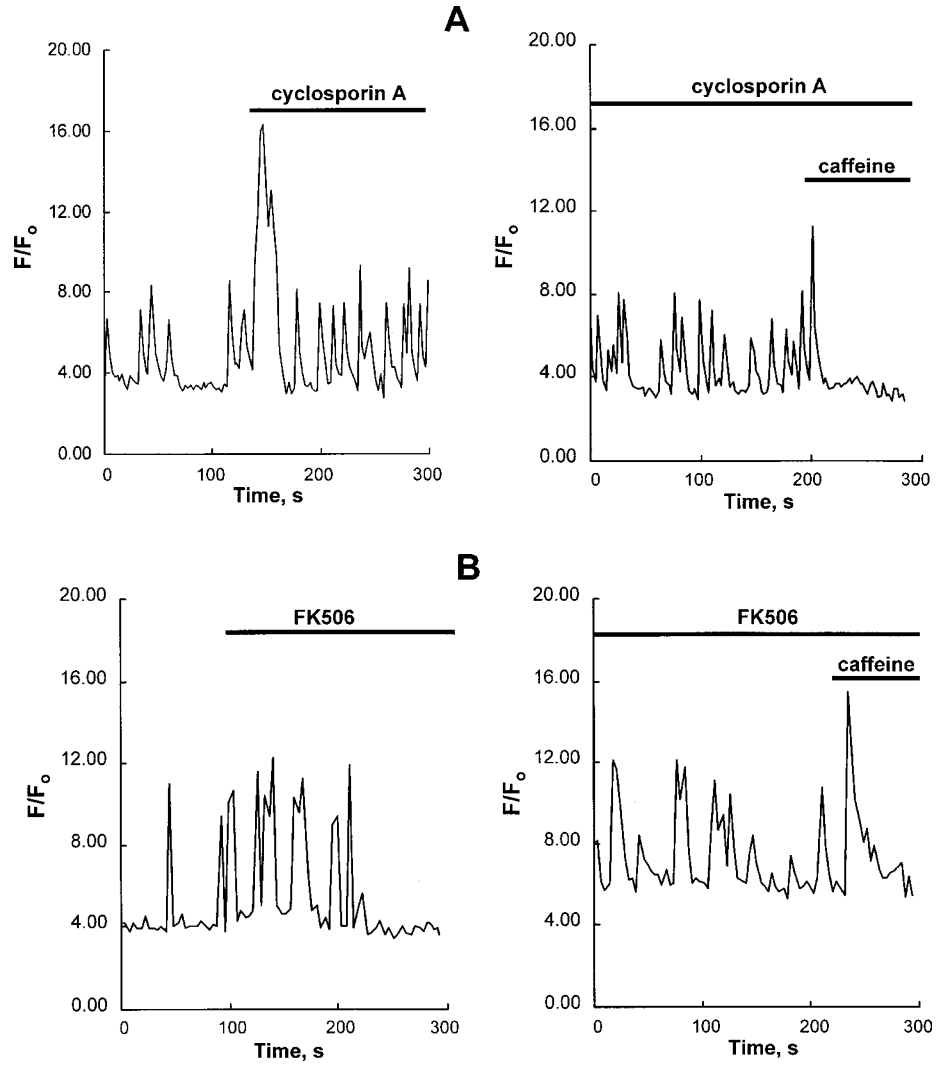

Figure 7 Effect of CsA and FK506 on transient Ca^{2+}-release in spontaneously oscillating cardiomyocytes

Fluorescence intensity of fluo-3/AM-loaded spontaneously Ca^{2+}-oscillating cardiomyocytes was examined under a laser scanning confocal microscope and treated with 10 μM cyclosporin (**A**) or 10 μM FK506 (**B**) at the indicated times (left panels). Approx. 15 min later, 10 mM caffeine was added into the chamber in the presence (solid line) of CsA or FK506 (right panels). Experiments were conducted four times and the fluorescence intensity was recorded in three cells in each experiment.

lane 1). In contrast, pretreatment of the CSMF with 20 mM EGTA abolished the appearance of calcineurin in the immunoprecipitate, suggesting that the interaction between calcineurin and the RyR requires Ca^{2+}. The hypothesis that the association of calcineurin with the RyR is anchored by FKBP12.6 was tested by incubating the CSMF with 100 μM Ca^{2+} plus 1 μM rapamycin (Figure 2A, lane 3), or 100 μM Ca^{2+} plus 1 μM FK506 (Figure 2A, lane 4) for 4 h at 25 °C. The co-immunoprecipitation results showed that both rapamycin (lane 3) and FK506 (lane 4) abolished the interaction between calcineurin and the RyR.

To confirm the above results, experiments were also conducted to immunoprecipitate calcineurin using the anti-calcineurin antibody and to examine the presence of the RyR by Western blot analysis using the RyR antibody (Figure 2B). Similarly, the RyR was co-immunoprecipitated with calcineurin using the anti-calcineurin antibody when the CSMF was preincubated with 100 μM Ca^{2+} (lane 1), but not with 20 mM EGTA (lane 2), 1 μM rapamycin (lane 3) or 1 μM FK506 (lane 4). As expected, calcineurin was present in all of the above samples (Figure 2C, lanes 1–4). However, calcineurin or the RyR were not co-immunoprecipitated with anti-SERCA2 antibody (Figures 2A and 2B, lane 5), further confirming that the calcineurin–RyR interaction is specific.

To examine the direct interaction between FKBP12.6 and the RyR, the CSMF was incubated with 100 μM Ca^{2+} or 1 μM rapamycin for 4 h at 25 °C and then subjected to immunoprecipitation with anti-RyR antibody, as described above. In agreement with earlier reports [6–8], FKBP12.6, but not FKBP12, was detected in the immunoprecipitate in the presence of Ca^{2+} (Figure 2D, lane 2), while no bands were detected when the CSMF was treated with rapamayin (Figure 2D, lane 3). Treatment of the CSMF with 1 μM cyclosporin A/cyclophilin did not affect immunoprecipitation of FKBP12.6 by anti-RyR antibody (Figure 2, lane 5). On the other hand, both FKBP12 and FKBP12.6 were detected when the CSMF was immunoprecipitated with anti-calcineurin antibody (Figure 2D, lane 1).

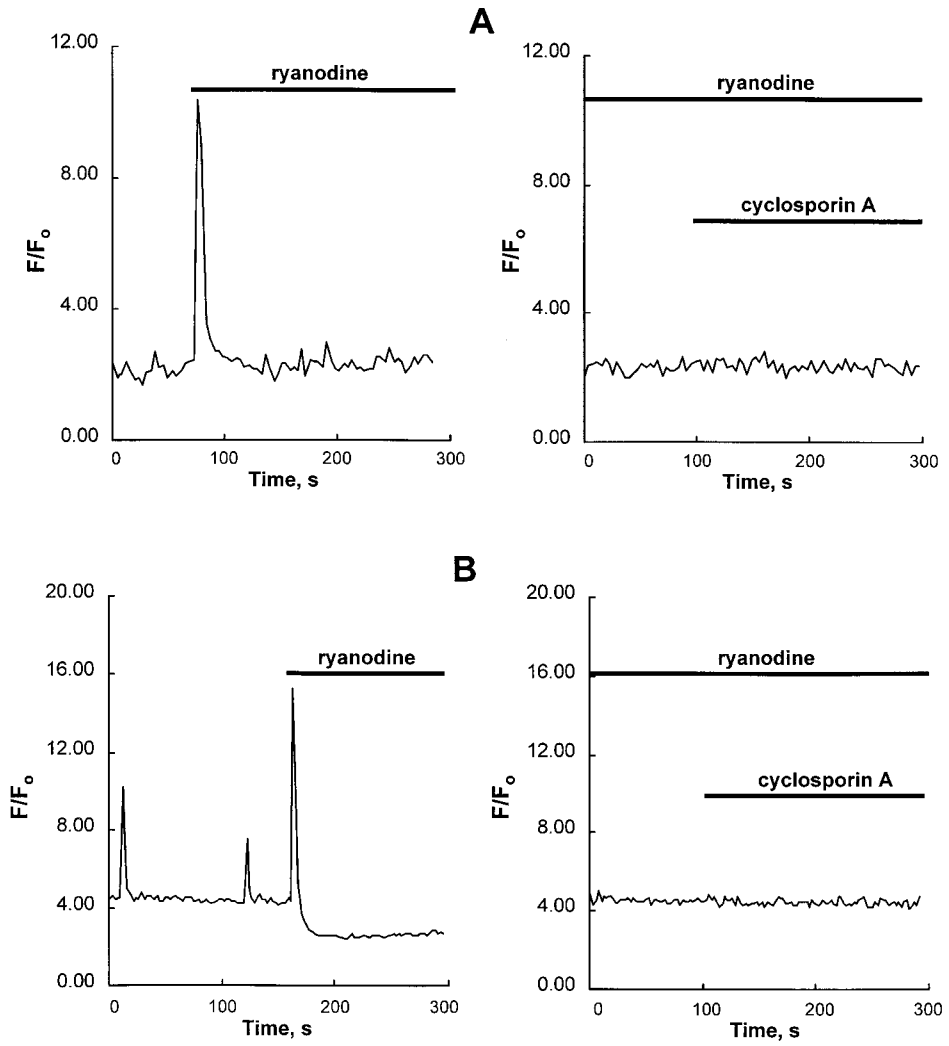

Figure 8 Effects of CsA on ryanodine-treated cardiomyocytes

Fluo-3/AM-loaded resting (**A**) and spontaneously Ca^{2+}-oscillating (**B**) cardiomyocytes were transferred to the chamber of a confocal microscope and subjected to fluorescence monitoring, where 10 μM ryanodine was applied (left panels). During the presence of ryanodine for approx. 15 min, 10 μM cyclosporin A was applied at the times indicated (right panels). Each experiment was repeated three times with five cells in each experiment. The solid line indicates the continued presence of the chemicals in the chamber.

EGTA-dependent dissociation of calcineurin from SR vesicles

Using the CSMF, we found the specific interaction between calcineurin and the RyR (Figures 1 and 2). We also examined whether treatment with EGTA dissociated endogenously associated calcineurin from SR vesicles. During the SR preparation (see the Materials and methods section), the 11000 ***g*** supernatant was treated with 20 mM EGTA for 2 h at 25 °C before ultracentrifugation at 130000 ***g***. The pellets were washed and suspended, as described in the procedure. Equal amounts of protein (60 μg/lane) for control or EGTA-treated samples were run on a 12% (w/v) polyacrylamide/SDS gel and the gel was either stained with Coomassie Blue (Figure 3A) or subjected to Western blot analysis using anti-calcineurin antibody (Figure 3B). Calcineurin was present in the SR which was prepared in the presence of endogenous Ca^{2+} (control), whereas no calcineurin band was present in the EGTA-treated SR. When the regular SR preparations were treated with EGTA and washed at 130000 ***g***, similar results were obtained (results not shown). These data show the Ca^{2+}-dependent *in vivo* association of calcineurin with SR. Furthermore, this simple procedure offers an easy way to prepare the calcineurin-devoid SR vesicles useful for the *in vitro* study of calcineurin function in excitation–contraction coupling.

Effect of calcineurin inhibitors on transient Ca^{2+}-release in cardiomyocytes

As shown in Figure 4, most of the cells were stained by MF-20, indicating that $> 90\%$ of the cells were cardiomyocytes. To investigate the possible role of calcineurin in the regulation of the cardiac RyR, transient Ca^{2+}-release in cardiomyocytes were examined in the absence or presence of calcineurin inhibitors by confocal microscopy. Figure 5(A) shows the basal level of fluorescence in fluo-3/AM-loaded non-beating cardiomyocytes. An application of 10 mM caffeine or 10 μM ryanodine to the

cells resulted in a transient rise in fluorescence intensity indicating that Ca^{2+} release was mediated by the RyR/Ca^{2+}-release channels (Figures 5B and 5C respectively). On the other hand, treatment of cardiomyocytes with various calcineurin inhibitors, such as deltamethrin (20 μM), CsA (10 μM) and FK506 (10 μM) [24–26], led to repetitive Ca^{2+} oscillations (Figures 6A–6C respectively). Rapamycin (10 μM) failed to evoke Ca^{2+} oscilliations as frequently as those observed in CsA-, deltamethrin- and FK506-treated non-beating cardiomyocytes (Figure 6D). An application of 10 mM caffeine evoked transient Ca^{2+}-release in the same cells, indicating that SR was not completely depleted due to rapamycin treatment (Figure 6D). Rapamycin also failed to influence Ca^{2+} oscillations induced by CsA when applied directly (results not shown). However, preincubation of cardiomyocytes with 20 μM rapamycin for approx. 30 min resulted in Ca^{2+} oscillations (Figures 6E and 6F). It is interesting to note that CsA and deltamethrin did not show any additional effects on the frequency and magnitude of the Ca^{2+} oscillations induced by 20 μM rapamycin (Figures 6E and 6F). These data suggest that calcineurin associated with the RyR via FKBP12.6 inhibits Ca^{2+} release in cardiac cells.

When cardiomyocytes were plated with high ($> 80\,\%$) confluency, some of the cells underwent beating, displaying Ca^{2+} oscillations. To examine the effects of calcineurin inhibitors on Ca^{2+} release in these cells, cardiomycocytes were stimulated by the application of 10 μM CsA or 10 μM FK506 (Figure 7). Figure 7(A) shows that fluo-3/AM-loaded cardiomyocytes had irregular Ca^{2+} oscillations. After CsA treatment, the irregular Ca^{2+} peaks were more frequent and sustained. Figure 7(B) shows that the infrequent Ca^{2+} oscillations in cardiomyocytes became more frequent following FK506 treatment. It appears that in the cell culture conditions, the magnitude and the frequency of spontaneous oscillations vary, even among the control cardiomyocytes. According to our statistical analysis, the initial frequency of Ca^{2+} oscillations (2.5 ± 0.6 per 100 sec, $n = 6$) became 6.6 ± 0.8 ($n = 3$) and 6.6 ± 0.3 per 100 sec ($n = 3$), after CsA and FK506 treatments respectively. The application of 10 mM caffeine to the same cells 15 min after the addition of 10 μM CsA or 10 μM FK506 resulted in transient Ca^{2+}-release (Figures 7A and 7B), with larger peaks indicating that Ca^{2+} oscillations evoked by CsA/FK506 were mediated by the RyR/Ca^{2+}-release channels.

To further examine that CsA-induced Ca^{2+} oscillations originated from the RyR, fluo-3/AM-loaded cardiomyocytes were pre-treated with 10 μM ryanodine and 10 μM CsA was added to the chamber 15 min later. As shown in Figure 8(A) ryanodine induced a transient Ca^{2+}-release in cardiomyocytes and the following treatment with CsA failed to evoke Ca^{2+} oscillations. Application of 10 μM ryanodine to spontaneously oscillating cardiomyocytes immediately caused a large transient Ca^{2+}-release and suppressed further spontaneous Ca^{2+} oscillations (Figure 8B), as described previously [27]. The addition of CsA (10 μM) to these cells did not evoke Ca^{2+} oscillations (Figure 8B). These data indicate that the Ca^{2+} oscillations triggered by CsA (Figures 6 and 7) originated from the RyR.

DISCUSSION

With the help of the immunosuppressant drugs, FK506 and CsA [3], calcineurin is now known to be involved in the regulation of Ca^{2+}-dependent transcription of a variety of genes essential for T-cell activation [28–31]. The role of calcineurin has also been implicated in other Ca^{2+}-regulated mechanisms, such as hippocampal long-term depression [32] and in the migration of neutrophils [33]. Studies *in vitro* have suggested that calcineurin could be involved in the regulation of Ca^{2+}-release channels [14]. Recently, we showed that the RyR in rat heart [17] and the Ins(1,4,5)P_3R in COS-7 cells [16] could be regulated by calcineurin through dephosphorylation of the Ca^{2+}-release channels. The present study was undertaken to further investigate the physiological role of calcineurin in the regulation of the RyR/Ca^{2+}-release channel and the molecular interaction between the proteins involved in the heart. The results from this study further show that calcineurin associated with Ca^{2+}-release channels could be an important regulator of Ca^{2+} release from the SR.

Using solubilized cardiac proteins, we demonstrated that calcineurin is specifically associated with the RyR (Figure 2). The association of calcineurin and the RyR is affected by rapamycin or FK506, suggesting that calcineurin and the RyR in cardiac SR are anchored by FKBP12.6. The Ca^{2+}-dependent interaction of calcineurin with the RyR is in agreement with the hypothesis that an increased level of cytosolic Ca^{2+} causes activation and association of calcineurin with the RyR [15,34]. However, in a recent report calcineurin was not co-purified with the RyR in solubilized cardiac proteins [35]. This discrepancy may be due to the absence of enough endogenous Ca^{2+} in their experimental conditions. The FKBP12 antibody used in the present study detected both isoforms in the rat SR preparations (Figure 1D). According to a recent paper by Xin et al. [36], FKBP12.6 binds to the cardiac-type RyR selectively, whereas both isoforms bind to the skeletal-type RyR. In the present study, we found that FKBP12.6, but not FKBP12, was immunoprecipitated with the RyR in rat cardiac SR (Figure 2D), whereas both FKBP isoforms were immunoprecipitated with calcineurin. It is interesting to note that in the presence of Ins(1,4,5)P_3R /RyR, FKBP12 can directly bind to calcineurin [14,15,34]. The presence of both FKBP12 isoforms in the calcineurin-immunoprecipitated samples (Figure 2D) could be due to the fact that the calcineurin-binding site is conserved for both FKBP12 and FKBP12.6. As shown in Figure 2(D), it is likely that the major FKBP isoform involved in the interaction with calcineurin and the RyR in rat heart is FKBP12.6 [7,8,36]. On the other hand, lack of FKBP12.6 in rabbit-skeletal SR suggests that FKBP12 is the major isoform involved in the interaction with the skeletal RyR.

Several *in vitro* studies have reported that dissociation of FKBP12 from the RyR by FK506 or rapamycin caused the Ca^{2+}-release channels to be more leaky in the presence of Ca^{2+}-release agonists [6,11–13,37]. In cardiomyocytes, FK506 and rapamycin caused Ca^{2+} spikes more frequently compared with the untreated cells, presumably due to the removal of FKBP12 from the RyR tetramer [37,38]. Our data also showed that FK506 (Figure 6C) or rapamycin (Figures 6E and 6F) induced Ca^{2+} oscilliations in cardiomyoctes. The occurrence of the repetitive Ca^{2+} oscillations by FK506 (Figure 6C) is likely to be due to both the dissociation of FKBP12.6/calcineurin from the RyR [6,11–13,37–39] and the inhibition of the phosphatase activity of calcineurin. Furthermore, deltamethrin and CsA which could not dissociate FKBP12/calcineurin from the RyR evoked Ca^{2+} oscillations effectively (Figures 6A and 6B), suggesting that calcineurin-mediated dephosphorylation is involved in the regulation of the RyR/Ca^{2+}-release channel activity. Addition of CsA (Figure 6E) or deltamethrin (Figure 6F) did not significantly affect rapamycin-induced Ca^{2+} oscillations. Therefore, it is likely that calcineurin associated with the RyR, but not the unbound calcineurin, could regulate the Ca^{2+}-release channel activities.

In mammalian heart, Ca^{2+} release from the SR is triggered by Ca^{2+} influx via L-type Ca^{2+} channels of the sarcolemma [40]. A signalling system with such a substantial amplification of the initial trigger exhibits some spontaneous activity of the cardio-

myocytes [27,41]. This tendency for spontaneous activity may result from the direct influx of Ca^{2+} through L-type Ca^{2+} channels and/or occasional openings of SR Ca^{2+}-release channels even under resting condition [42]. In our study, the neonatal cardiomyocytes cultured in the presence of normal concentrations of Ca^{2+} (1–2 mM), showed spontaneously active cells [40,41] with substantial Ca^{2+} oscillations, as shown in Figure 7. The further increase in the frequency of Ca^{2+} oscillations induced by CsA (Figure 7A) and FK506 (Figure 7B) is likely to be coupled to the increased tendency of the Ca^{2+}-release channel. This result is consistent with the increased contractility of cardiomyoctes by CsA or FK506 [43,44]. Taken together, these data suggest that inhibition of the endogenous phosphatase activity of calcineurin by calcineurin inhibitors leads to a higher probability of Ca^{2+}-release channel openings.

The processes of protein phosphorylation and dephosphorylation are essential for regulating various cellular functions. Evidence has suggested that activities of the cardiac RyR are increased by both cAMP and Ca^{2+}/calmodulin-dependent phosphorylation of the RyR [45–47]. From the available evidence, it is tempting to hypothesize that the increased intracellular Ca^{2+}-release from the SR could activate endogenous calcineurin and its association with the RyR followed by the termination of the Ca^{2+}-release process. This dephosphorylation-dependent regulatory process could operate to maintain the Ca^{2+} homoeostasis *in vivo*.

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REFERENCES

1 Klee, C. B., Draetta, G. F. and Hubbard, M. J. (1988) Calcineurin. Adv. Enzymol. Relat. Areas Mol. Biol. **61**, 149–200
2 Klee, C. B., Ren, H. and Wang, X. (1998) Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J. Biol. Chem. **273**, 13367–13370
3 Liu, J., Farmer, J. D., Lane, W. S., Friedman, J., Weissman, I. and Schreiber, S. L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell (Cambridge, Mass.) **66**, 807–815
4 Crabtree, G. R. and Clipstone, N. A. (1994) Signal transmission between the plasma membrane and nucleus of T lymphocytes. Annu. Rev. Biochem. **63**, 1045–1083
5 Jayaraman, T., Brilliantes, A. M., Timerman, A. P., Fleischer, S., Erdjument-Bronage, H., Tempst, P. and Marks, A. R. (1992) FK506-binding protein associated with the calcium release channel (ryanodine receptor). J. Biol. Chem. **267**, 9474–9477
6 Timerman, A. P., Ogunbumni, E. A., Freund, E., Wiederrecht, G., Marks, A. R. and Fleischer, S. (1993) The calcium release channel of sarcoplasmic reticulum is modulated by FK506-binding protein. J. Biol. Chem. **268**, 22992–22999
7 Timerman, A. P., Jayaraman, T., Wiederrecht, G., Onoue, H., Marks, A. R. and Fleisher, S. (1994) The ryanodine receptor from canine heart sarcoplasmic reticulum is associated with a novel FK-506 binding protein. Biochem. Biophys. Res. Commun. **198**, 701–706
8 Timerman, A. P., Onoue, H., Xin, H. B., Barg, S., Copello, J., Wiederrecht, G. and Fleischer, S. (1996) Selective binding of FKBP12.6 by the cardiac ryanodine receptor. J. Biol. Chem. **271**, 20385–20391
9 Martonosi, A. N. (1984) Mechanisms of Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle. Physiol. Rev. **64**, 1240–1320
10 Feher, J. J., LeBolt, W. R. and Manson, N. H. (1989) Differential effect of global ischemia on the ryanodine-sensitive and ryanodine-insensitive calcium uptake of cardiac sarcoplasmic reticulum. Circ. Res. **65**, 1400–1408
11 Brillantes, A. B., Ondrias, K., Scott, A., Kobrinsky, E., Ondriasova, E., Moschella, M., Jayaraman, T., Landers, M., Ehrlich, B. and Marks, A. R. (1994) Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. Cell (Cambridge, Mass.) **77**, 513–523
12 Mayrleitner, M. M., Timerman, A. P., Wiederrecht, G. and Fleischer, S. (1994) The calcium release channel of sarcoplasmic reticulum is modulated by FK-506 binding protein: effect of FKBP-12 on single channel activity of the skeletal muscle ryanodine receptor. Cell Calcium **15**, 99–108
13 Timerman, A. P., Wiederrecht, G., Marcy, A. and Fleischer, S. (1995) Characterization of an exchange reaction between soluble FKBP12 and the FKBP12-ryanodine receptor complex. J. Biol. Chem. **270**, 2451–2459
14 Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnet, G. V. and Snyder, S. H. (1995) Calcineurin associated with the 1,4,5-inositol trisphosphate receptor-FKBP12 complex modulates Ca^{2+} flux. Cell (Cambridge, Mass.) **77**, 513–523
15 Cameron, A. M., Nucifora, F. C., Fung, E. T., Livingston, D. J., Aldape, R. A., Ross, C. A. and Snyder, S. H. (1997) FKBP12 binds the inositol 1,4,5-trisphosphate receptor at leucine-proline (1400–1401) and anchors calcineurin to this FK506 domain. J. Biol. Chem. **272**, 27582–27588
16 Bandyopadhyay, A., Shin, D. W. and Kim, D. H. (2000) Regulation of ATP-induced calcium release in COS-7 cells by calcineurin. Biochem. J. **348**, 173–181
17 Park, K. S., Kim, T. K. and Kim, D. H. (1999) Cyclosporin A treatment alters characteristics of Ca^{2+}-release channel in cardiac sarcoplasmic reticulum. Am. J. Physiol. **276**, H865–H872
18 Kim, D. H., Mkparu, F., Kim, C. R. and Caroll, R. F. (1994) Alteration of Ca^{2+} release channel function in sarcoplasmic reticulum of pressure-overload-induced hypertrophic heart. J. Mol. Cell. Cardiol. **26**, 1505–1512
19 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
20 Gomez, J. P., Poterau, D. and Raymond, G. (1994) Intracellular calcium transients from newborn rat cardiomyocytes in primary culture. Cell Calcium **15**, 265–275
21 Huser, J., Bers, D. M. and Blater, L. A. (1998) Subcellular properties of $[Ca^{2+}]_i$ transients in phospholamban-deficient mouse ventricular cells. Am. J. Physiol. **274**, H1800–H1811
22 Farber, L. H., Wilson, F. J. and Wolff, D. J. (1987) Calmodulin-dependent phosphatases of PC12, GH3, and C6 cells: physical, kinetic, and immunochemical properties. J. Neurochem. **49**, 404–414
23 Valdivia, C., Vaughan, D., Potter, V. L. and Coronado, R. (1992) Fast release of $^{45}Ca^{2+}$ induced by inositol 1,4,5-trisphosphate and Ca^{2+} in the sarcoplasmic reticulum of rabbit skeletal muscle: evidence for two types of Ca^{2+} release channels. Biophys. J. **61**, 1184–1193
24 Hashimoto, Y., Perrino, B. A. and Soderling, T. R. (1990) Identification of an autoinhibitory domain in calcineurin. J. Biol. Chem. **265**, 1924–1927
25 Enan, E. and Matsumura, F. (1992) Specific inhibition of calcineurin by type II synthetic pyrethroid insecticides. Biochem. Pharmacol. **43**, 1777–1784
26 Matsuda, T., Takamura, K., Asano, S., Kishida, Y., Nakamura, H., Mori, K., Maeda, S. and Baba, A. (1998) Involvement of calcineurin in Ca^{2+} paradox like injury of cultured rat astrocytes. J. Neurochem. **70**, 2004–2013
27 Lipp, P. and Niggli, E. (1994) Modulation of Ca^{2+} release in cultured neonatal rat cardiac myocytes: Insight from subcellular release patterns revealed by confocal microscopy. Circ. Res. **74**, 979–990
28 Klee, C. B., Crouch, T. H. and Krinks, M. H. (1979) Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc. Natl. Acad. Sci. U.S.A. **76**, 6270–6273
29 Stewart, A. A., Ingebritsen, T. S., Manalan, A., Klee, C. B. and Cohen, P. (1982) Discovery of a Ca^{2+}- and calmodulin-dependent protein phosphatase: probable identity with calcineurin (CaM-BP80). FEBS Lett. **137**, 80–84
30 Clipstone, N. A. and Crabtree, G. R. (1992) Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature (London) **357**, 685–697
31 O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J. and O'Neill, E. A. (1992) FK506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. Nature (London) **357**, 692–694
32 Mulkey, R. M., Endo, S., Shenoliker, S. and Malenka, R. C. (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. Nature (London) **369**, 486–488
33 Hendey, B., Klee, C. B. and Maxfield, F. R. (1992) Inhibition of neutrophil chemokinesis on vitronectin by inhibitors of calcineurin. Science (Washington, D.C.) **258**, 296–299
34 Snyder, S. H., Sabatini, D. M., Lai, M. M., Steiner, J. P., Hamilton, G. S. and Suzdak, P. D. (1998) Neural actions of immunophilin ligands. Trends Pharmacol. Sci. **19**, 21–26
35 Marx, S. O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblit, N. and Marks, A. R. (2000) PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. Cell (Cambridge, Mass.) **101**, 365–376
36 Xin, H. B., Rogers, K., Qi, Y., Kanematsu, T. and Fleisher, S. (1999) Three amino acid residues determine selective binding of FK506-binding protein 12.6 to the cardiac ryanodine receptor. J. Biol. Chem. **274**, 15315–15319
37 Chen, L., Molinski, T. F. and Pessah, I. N. (1999) Bastadin 10 stabilizes the open conformation of the ryanodine-sensitive Ca^{2+} channel in an FKBP12-dependent manner. J. Biol. Chem. **274**, 32603–32613
38 Xiao, R. P., Valdivia, H. H., Bogdanov, K., Valdivia, C., Lakatta, E. G. and Cheng, H. (1997) The immunophilin FK506-binding protein modulates Ca^{2+} release channel closure in rat heart. J. Physiol. (Cambridge, U.K.) **500**, 343–354

39 Mackrill, J. J. (1999) Protein–protein interactions in intracellular Ca^{2+} release channel function. Biochem J. **337**, 345–336
40 Wier, W. G. (1990) Cytoplasmic [Ca^{2+}] in mammalian ventricle: dynamic control by cellular processes. Annu. Rev. Physiol. **52**, 467–485
41 Hohl, C. M., Livingston, B., Hensley, J. and Altschuld, R. A. (1997) Calcium handling by sarcoplasmic reticulum of neonatal swine cardiac myocytes. Am J. Physiol. **273**, H192–H199
42 Sitsapesan, R. and Williams, A. J. (1990) Mechanism of caffeine activation of single calcium release channels of sheep cardiac sarcoplasmic reticulum. J. Physiol. (Cambridge, U.K.) **423**, 425–439
43 Hohl, C. M. and Altschuld, R. A. (1999) FK506 alters sarcoplasmic reticulum calcium release in neonatal piglet cardiac myocytes. Pediatr. Res. **46**, 316–319
44 Kolcz, J., Drukala, J., Jurkiewicz, A., Pfitzner, R., Garlicki, M., Czyz, J. and Korohoda, W. (1999) Effects of cyclosporin A on contractile activity and cytoskeleton in chick embryo cardiomyocytes. Biochem. Cell Biol. **77**, 133–140
45 Hain, J., Onoue, H., Mayrleitner, M., Fleischer, S. and Schindler, H. (1995) Phosphorylation modulates the function of the calcium release channel of sarcoplasmic reticulum from cardiac muscle. J. Biol. Chem. **270**, 2074–2081
46 Witcher, D., Strifler, B. A. and Jones, L. R. (1992) Cardiac-specific phosphorylation site for multifunctioal Ca^{2+}/calmodulin-dependent protein kinase is conserved in the brain ryanodine receptor. J. Biol. Chem. **267**, 4963–4967
47 Xu, A. and Narayanan, N. (1998) Effects of aging on sarcoplasmic reticulum Ca^{2+}-cycling proteins and their phosphorylation in rat mycocardium. Am. J. Physiol. **275**, H2087–H2094

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