Ca^{2+}-Dependent Interaction between FKBP12 and Calcineurin Regulates Activity of the Ca^{2+} Release Channel in Skeletal Muscle

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ABSTRACT Calcineurin is a Ca^{2+} and calmodulin-dependent protein phosphatase with diverse cellular functions. Here we examined the physical and functional interactions between calcineurin and ryanodine receptor (RyR) in a C2C12 cell line derived from mouse skeletal muscle. Coimmunoprecipitation experiments revealed that the association between RyR and calcineurin exhibits a strong Ca^{2+} dependence. This association involves a Ca^{2+} dependent interaction between calcineurin and FK506-binding protein (FKBP12), an accessory subunit of RyR. Pretreatment with cyclosporin A, an inhibitor of calcineurin, enhanced the caffeine-induced Ca^{2+} release (CICR) in C2C12 cells. This effect was similar to those of FK506 and rapamycin, two drugs known to cause dissociation of FKBP12 from RyR. Overexpression of a constitutively active form of calcineurin in C2C12 cells, ΔCnA(391–521) (deletion of the last 131 amino acids from calcineurin), resulted in a decrease in CICR. This decrease in CICR activity was partially recovered by pretreatment with cyclosporin A. Furthermore, overexpression of an endogenous calcineurin inhibitor (cain) or an inactive form of calcineurin (ΔCnA(H101Q)) in C2C12 cells resulted in up-regulation of CICR. Taken together, our data suggest that a trimeric-interaction among calcineurin, FKBP12, and RyR is important for the regulation of the RyR channel activity and may play an important role in the Ca^{2+} signaling of muscle contraction and relaxation.

INTRODUCTION

In skeletal muscle, coupling of electrical excitation to intracellular Ca^{2+} release and mechanical contraction (E-C coupling) occurs at the triad junction between the transverse tubular invagination of plasma membrane and the terminal cisternae of sarcoplasmic reticulum (SR) (Rios and Pizarro, 1991). The ryanodine receptor (RyR) located in the SR membrane is key to this process, functioning as a Ca^{2+} release channel that mediates the mobilization of Ca^{2+} from the SR store (Franzini-Armstrong and Joregensen, 1994; Meissner, 1994). The Ca^{2+} release channel is a homotetramer of the 565-kDa RyR polypeptide. Each RyR subunit is bound by one FK506-binding protein (FKBP12) (Timerman et al., 1993; Jayaraman et al., 1992). Ligands that dissociate the interaction of RyR with FKBP12, such as FK506 or rapamycin, can enhance the activity of the Ca^{2+} release channel (Ahern et al., 1994; Brillantes et al., 1994; Chen et al., 1994; Ma et al., 1995). Dissociation of FKBP12 from RyR results in a Ca^{2+} release channel that is activated by lower concentrations of caffeine (Timerman et al., 1993) or lower concentration of Ca^{2+} (Timerman et al., 1995), compared with the normal channel. These biochemical and functional data suggest that FKBP12, as an accessory partner of RyR, plays a key role in the function of the Ca^{2+} release channel.

Calcineurin is a ubiquitous cytoplasmic serine/threonine protein phosphatase (Cameron et al., 1995; Clipstone et al., 1994; Lai et al., 1998) that is present at ~10-fold higher concentration in brain and muscle than in other cell types (Klee et al., 1988). Calcineurin has been shown to transduce hypertrophic signals leading to cardiac growth (Molkentin et al., 1998; Sussman et al., 1998), to mediate the effect of insulin-like growth factor 1 in skeletal muscle (Musaro et al., 1999; Semesarian et al., 1999), and to control gene regulation and cell differentiation in different subtypes of skeletal muscle (Chin et al., 1998; Dunn et al., 1999; Abbott et al., 1998). However, whether calcineurin regulates RyR directly or indirectly in skeletal muscle and how calcineurin participates in the physiological regulation of the RyR channel activity remain largely unknown.

In this study, we investigated whether the RyR channel activity in skeletal muscle can be regulated by calcineurin in vivo using a mouse skeletal myogenic cell line, C2C12 (Airey et al., 1991; Lorenzon et al., 2000). We tested the physical and functional interaction among RyR, FKBP12, and calcineurin in C2C12 and Chinese hamster ovary (CHO) cells. A Ca^{2+}-dependent interaction between FKBP12 and calcineurin was identified in C2C12 cells, which mediates calcineurin-dependent regulation of the Ca^{2+} release channel by dephosphorylating RyR. Overexpression of cain, an endogenous inhibitor of calcineurin (Lai et al., 1998), or an inactive form of calcineurin, ΔCnA(H101Q), in C2C12 cells leads to significant enhancement of the RyR channel activity. Our data suggest that calcineurin may play important roles in the Ca^{2+} signaling cycle of muscle contraction and relaxation.

Submitted November 13, 2001, and accepted for publication July 2, 2002.

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0006-3495/02/11/2539/11 $2.00

MATERIALS AND METHODS

Cell culture

C2C12 cells (American Type Culture Collection, Manassas, VA) were cultured according to established procedures of Airey et al. (1991). Briefly, C2C12 myoblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. After 2 days, myoblast differentiation was induced by replacing the medium with Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 1% penicillin-streptomycin. Experiments were performed on C2C12 myotubes expressing RyR (i.e., from fourth day of culture in differentiation medium), when it is possible to select myotubes with mature skeletal-type E-C coupling. CHO cells stably transfected with the rabbit skeletal muscle RyR were cultured at 37°C and 5% CO_2 in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.1 mg/mL G418 (Bhat et al., 1997).

Immunoprecipitation and Western blot

Confluent C2C12 myotubes or CHO cells growing in 100-mm dish were harvested and washed twice with ice-cold phosphate-buffered saline, and lysed in radioimmuno precipitation assay buffer (1 $\times$ phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.1 mM phenylmethylsulfonyl fluoride, 1.0 μM pepstatin, 1 mM benzamidine, 10 μM leupeptin, 1 μg/mL aprotinin). Immunoprecipitation was performed with monoclonal anti-RyR antibody (Affinity BioReagents, Golden, CO), or polyclonal anticalcineurin antibody or polyclonal anti-FKBP12 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Negative controls were done with mouse or rabbit preimmune serum purchased from Sigma (St. Louis, MO). Immunoprecipitates were recovered with protein G-agarose (Boehringer Mannheim, Indianapolis, IN) and separated by 3% to 15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane and probed with the above antibodies. The protein-antibody complexes were then probed with a horseradish peroxidase-linked secondary antibody and the signal detected on Kodak films using enhanced chemiluminescence assay (Pierce, Rockford, IL).

Molecular cloning and mutagenesis

A 1.2-kb *Eco*R1 fragment containing the amino-terminal portion of calcineurin, (ΔCnA(amino acid 391–512), deletion of 1173–1563 bp of calcineurin) was cloned into the pCMS-EGFP expression vector (Clontech, Palo Alto, CA), to obtain pCMS-EGFP(ΔCnA). The pCMS-EGFP plasmid contains two separate promoters that drive the transcription of green-fluorescent protein (GFP, under SV40 promoter) and the gene of interest (i.e., ΔCnA, under cytomegalovirus promoter) (Pan et al., 2000). The ΔCnA cDNA fragment was also ligated to the 5′ end of GFP, to create a ΔCnA-GFP fusion construct. Polymerase chain reaction was used to amplify the cDNA encoding amino acids 1881 to 2173 of cain, an endogenous inhibitor of calcineurin. The polymerase chain reaction product of cain was ligated to the 3′ end of GFP, to create the GFP-c-cain fusion construct.

The QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to mutate a histidine residue into glutamine (H101Q) in the pCMS-EGFP(ΔCnA) plasmid to obtain ΔCnA(H101Q). Oligonucleotides containing the H101Q mutation were synthesized as follows: forward primer, 5′-GTTTGCGGGGACATCCAGGGACAATTCTTTGAC-3′ and reverse primer, 5′-GTCAAAGAATTGTCCCTGGATGTCCCCGCAAAC-3′.

After polymerase chain reaction amplification using PfuTurbo DNA polymerase, the methylated, nonmutated parental DNA template was digested with *Dpn*I. The mutated, nicked dsDNA was transformed into XL-1 Blue supercompetent *Escherichia coli*. Point mutation of ΔCnA(H101Q) was confirmed by automated sequencing (Cleveland Genomics, Cleveland, OH).

Lipofectamine-mediated gene transfection

The various cDNA plasmids (i.e., pCMS-EGFP, pCMS-EGFP(ΔCnA), pCMS-EGFP(ΔCnA-H101Q), ΔCnA-GFP, GFP-c-cain) were introduced into the 1.5-day proliferating C2C12 myoblasts, using the Lipofectamine Plus reagent according to manufacturer's instructions. To enhance the transfection efficiency, the incubation time for DNA/lipofectamine complex with cells was set at 6 h. Differentiation of myoblasts was induced by 2% horse serum and 1% penicillin-streptomycin 12 h after transfection. Myotubes transfected with the genes of interest were selected based on the appearance of green fluorescent signal monitored with an upright fluorescence microscope equipped with filter settings for GFP (excitation wavelength 488 nm and emission filter set at 510 nm) (Pan et al., 2000).

For subcellular localization of ΔCnA-GFP, mature myotubes expressing GFP alone or ΔCnA-GFP were fixed with 4% paraformaldehyde and mounted on glass slides (coated with 0.5% collagen). The GFP signal was visualized with a Zeiss laser scanning confocal microscope using a 63$\times$ oil immersion objective (Pan et al., 2000).

Intracellular Ca^{2+} measurement in single cells

C2C12 mytobues were grown in ΔTC3 dishes (Bioptechs, Inc., Butler, PA) and incubated for 45 min at 37°C in balanced salt solution (BSS): 140 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.2, containing 5 μM Fura 2-AM, and then washed with BSS solution to allow deesterification of the dye. Using a dual-wavelenghth spectrofluorometer, with excitation wavelengths at 340 and 380 nm and emission at 510 nm, fluorescence measurements were performed at 37°C in a temperature-regulated chamber, mounted on the stage of an inverted fluorescence microscope (Olympus IX-70). Single-cell fluorescence spectra were continuously monitored at a sampling frequency of 50 Hz and collected with a PTI spectrofluorometer (Photon Technology International, Monmouth Junction, NJ) (Pan et al., 2000). The release of intracellular Ca^{2+} in individual cells was measured following exposure to 5 mM caffeine in absence (Ca^{2+} free-BSS containing 0.5 mM EGTA) or presence of extracelluar Ca^{2+} (Ca^{2+}-BSS containing 2 mM Ca^{2+}). Myotubes expressing GFP were selected under fluorescence filter 488 nm.

Statistical analysis

Values are represented as mean $\pm$ SE. Significance was determined by Student's *t*-test or analysis of variance. A value of $p < 0.05$ was used as criterion for statistical significance.

RESULTS

Calcium-dependent interaction between RyR and calcineurin

The interaction between calcineurin and RyR was examined by testing the ability of an anticalcineurin antibody to co-immunoprecipitate RyR in differentiated C2C12 cells. The presence of RyR in the immunoprecipitate was examined by Western-blot analysis using anti-RyR antibody (Fig. 1 *A*, top). Because calcineurin is regulated by Ca^{2+}, we investigated whether calcineurin interacts with RyR in a Ca^{2+}-dependent manner. In the presence of 10 mM EGTA, the association between calcineurin and RyR was diminished

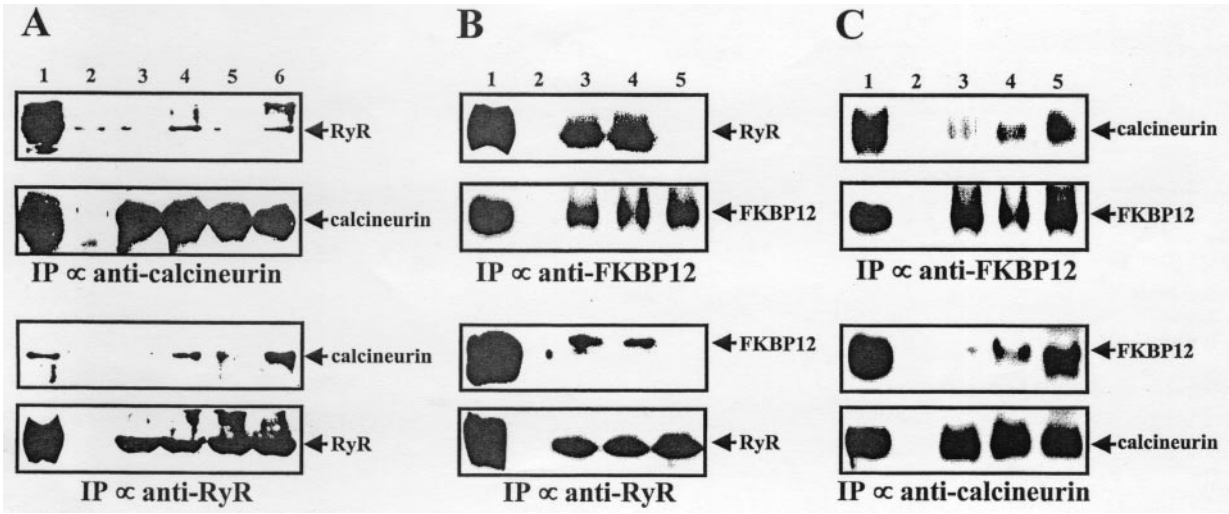

FIGURE 1 Ca^{2+}-dependent interaction among RyR, FKBP12, and calcineurin in C2C12 myotubes. (*A*) Cell lysates from differentiated C2C12 myotubes were incubated with 10 mM EGTA (*lane 3*), 10 μM Ca^{2+} (*lane 4*), 10 μM Ca^{2+} plus 1 μM FK506 (*lane 5*), 10 μM Ca^{2+} plus 1 μM cyclosporin A (*lane 6*) for 2 h at 4°C, and subjected to immunoprecipitation (IP) with anticalcineurin (*top*), or antiryanodine receptor (RyR1) antibody (*bottom*). Top and bottom panels represent Western blot probed with anti-RyR or anticalcineurin antibodies, respectively. (*B*) Coimmuniprecipitation of RyR and FKBP12 in C2C12 myotubes. Top panel shows IP with anti-FKBP12 and Western blot with anti-RyR. Bottom panel shows IP with anti-RyR and Western blot with anti-FKBP12. (*Lane 3*) 10 mM EGTA; (*lane 4*) 10 μM Ca^{2+}; (*lane 5*) 10 μM Ca^{2+} plus 1 μM FK506. (*C*) Ca^{2+}-dependent interaction between calcineurin and FKBP12. IP with anticalcineurin (*top*) or anti-FKBP12 antibody (*bottom*) was performed with buffers containing 10 mM EGTA (*lane 3*), 1 μM Ca^{2+} (*lane 4*), and 10 μM Ca^{2+} (*lane 5*). Western-blot analysis was performed with anti-FKBP12 (*top*) or anticalcineurin antibody (*bottom*), respectively. In all panels, whole cell homogenates of C2C12 myotubes are shown in lane 1 (positive control), and preimmune serum-treated immunoprecipitates are shown in *lane* 2 (negative control). Equal amounts of protein were loaded in each panel.

(lane 3) compared with that in the presence of 10 μM Ca^{2+} (lane 4). This suggests that the interaction between the two proteins requires Ca^{2+}. Immunosuppressant drugs FK506 and rapamycin regulate the function of Ca^{2+} release channel by binding to FKBP12 and causing its dissociation from RyR (Cameron et al., 1995; Brillantes et al., 1994; Snyder et al., 1998). The interaction between RyR and calcineurin was abolished in the presence of FK506 (Fig. 1 *A*, lane 5) but not by cyclosporin A, another immunosupressant drug that does not affect the physical interaction between inositol 1,4,5-trisphosphate receptor (IP_3R) and FKBP12 (9) (lane 6). Reverse immunoprecipitation experiments were also performed using anti-RyR antibody to pull down calcineurin (Fig. 1 *A*, bottom). Similarly, the association between RyR and calcineurin required the presence of Ca^{2+} and was diminished by FK506 and EGTA.

Calcineurin is known to be indirectly associated with RyR through FKBP12 in brain (Cameron et al., 1995) and heart cells (Bandyopadhyay et al., 2000a). To investigate the basis of Ca^{2+}-dependent interaction between RyR and calcineurin in skeletal muscle, we first performed immunoprecipitation experiments with anti-FKBP12 or anti-RyR antibody in the presence or absence of Ca^{2+}, followed by Western-blot assay (Fig. 1 *B*). No significant effect of Ca^{2+} was observed in the association between RyR and FKBP12, as there was no change in the amount of immunoprecipitated proteins in the presence or absence of Ca^{2+} (Fig. 1 *B*, lanes 3 and 4). As a control, the interaction between RyR and FKBP12 was completely abolished by FK506 (lane 5).

We next performed a series of experiments to test the Ca^{2+}-dependent interaction between calcineurin and FKBP12 (Fig. 1 *C*). Anti-FKBP12 antibodies (top) or anti-calcineurin (bottom) were used in the immunoprecipitation assay with the buffer containing 10 mM EGTA (lane 3), 1 μM Ca^{2+} (lane 4), or 10 μM Ca^{2+} (lane 5). The presence of FKBP12 or calcineurin in the immunoprecipitates was probed with the corresponding antibodies. As Ca^{2+} concentration increased, more FKBP12 or calcineurin appeared in the immunoprecipitates (Fig. 1 *C*). This suggests a strong Ca^{2+}-dependence in the interaction between FKBP12 and calcineurin. Taken together, our data show that the Ca^{2+}-dependent association between RyR and calcineurin originates from the Ca^{2+}-dependent interaction between FKBP12 and calcineurin.

Effect of calcineurin inhibitors on Ca^{2+} release from SR

To examine the functional effects of calcineurin and FKBP12 on the RyR/Ca^{2+} release channel, we examined the effect of cyclosporin A (CsA), FK506, or rapamycin, on caffeine-induced Ca^{2+} release in differentiated C2C12 cells. All experiments were performed in Ca^{2+}-free medium to avoid the interference from extracelluar Ca^{2+}. We had

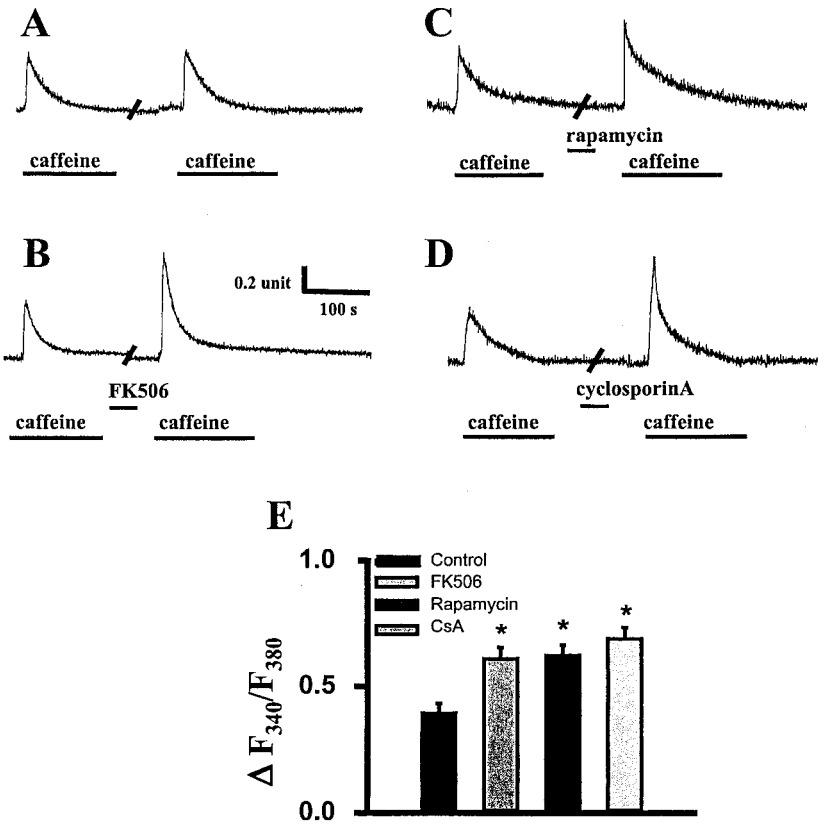

FIGURE 2 Effects of calcineurin inhibitors on caffeine-induced Ca^{2+} release in C2C12 cells. Mature C2C12 myotubes were loaded with Fura-2 AM Ca^{2+} indicator in 2 mM Ca^{2+}-BSS for 10 min. Ca^{2+}-release from SR under control condition was measured after first application of 5 mM caffeine to a Ca^{2+}-free BSS. After the first caffeine-induced Ca^{2+} release, myotubes were reloaded with 2 mM Ca^{2+}-BSS, with or without drugs for 10 min to ensure complete loading of Ca^{2+} into SR. The bath solution was then changed to Ca^{2+}-free BSS, followed by a second addition of 5 mM caffeine. Incubation of 10 μM FK506 (*B*, $n = 6$), 1 μM rapamycin (*C*, $n = 5$), or 1 μM cyclosporin A (*D*, $n = 5$), to the bath solution significantly enhanced the second caffeine-induced Ca^{2+} release, compared with the control (*A*, $n = 7$). Solid bars indicate the presence of caffeine, FK506, rapamycin, cyclosporin A, and reloaded Ca^{2+}. Data from multiple experiments are summarized in *E* as mean ± SE. *, significance difference $p < 0.05$.

previously reported that both CsA and FK506 enhance Ca^{2+} release through the IP_3R in COS-7 cells (Bandyopadhyay et al., 2000b). Caffeine, an effective activator of RyR channels in skeletal muscle, was found to induce Ca^{2+} release in a dose-dependent manner (Bhat et al., 1997). At a concentration of 10 mM or higher, caffeine usually depletes the SR Ca^{2+} stores in skeletal myotubes. Therefore, we used a submaximal concentration of caffeine (5 mM) in all subsequent functional measurements of intracellular Ca^{2+} release in C2C12 cells. The following experimental protocol was used. First, mature myotubes were treated with 5 mM caffeine to establish the SR Ca^{2+} release under control condition. Then, CsA (1 μM), FK506 (10 μM), or rapamycin (1 μM) were incubated to the bath solution for 10 to 15 min, and the changes in SR Ca^{2+} release were assayed following stimulation with 5 mM caffeine. The longer incubation time for these various compounds were necessary for their complete effect on the function of the RyR channel, because shorter incubation times, e.g., 1 min or 5 min, produced either no effect or variable results.

The magnitudes of Ca^{2+} release from SR by two sequential caffeine treatments were comparable ($\Delta F_{340}/F_{380} = 0.38 \pm 0.02$ and 0.41 ± 0.02, $n = 7$, for 1st and 2nd response, respectively) (Fig. 2 *A*). However, the second caffeine-induced Ca^{2+} release after treatment with FK506 or rapamycin was significantly higher (Fig. 2, *B* and *C*). On average, the caffeine-induced Ca^{2+} releases after drug treatment were 0.63 ± 0.03 ($n = 6$) and 0.64 ± 0.03 ($n = 5$) for FK506 and rapamycin, respectively (Fig. 2 *E*). Similar to the effect of FK506 and rapamycin, pretreatment of C2C12 myotubes with CsA also resulted in an increase in the amplitude of caffeine-induced Ca^{2+} release ($\Delta F_{340}/F_{380} = 0.71 \pm 0.03$, $n = 5$) (Fig. 2, *D* and *E*).

Effect of constitutively active calcineurin on SR Ca^{2+} release

The increases in SR Ca^{2+} release function induced by CsA, FK506, and rapamycin could be due to changes in the intermolecular interaction between RyR, FKBP12, and calcineurin or could reflect the phosphorylation states of RyR as a result of changes in phosphatase activity of calcineurin. To test the latter possibility, we overexpressed a truncated, constitutively active form of calcineurin (ΔCnA) in C2C12 cells. Calcineurin contains a calmodulin-binding domain and an autoinhibitory region at the carboxyl-terminal end (Klee et al., 1988). Deletion of the carboxyl-terminal 131 amino acids from calcineurin, ΔCnA(391–521), leads to constitutively active phosphatase activity of calcineurin without the requirement for Ca^{2+} and calmodulin (Shibasaki et al., 1996). X-ray crystallographic data show that the domain of CnA interaction with FKBP12 resides within the amino-terminal portion of the protein (Griffith et al.,

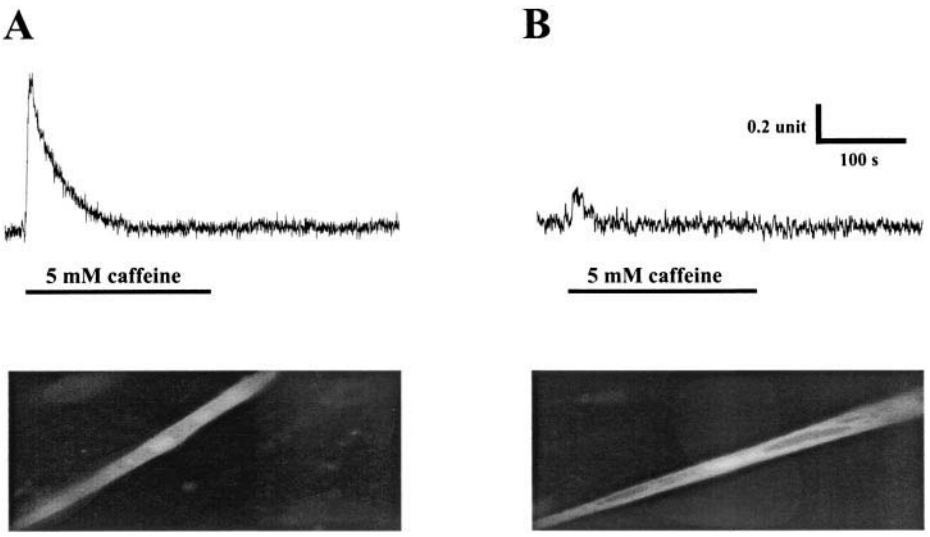

FIGURE 3 Transient expression of ΔCnA-GFP down-regulates caffeine-induced Ca^{2+} release in C2C12 cells. Five millimolar caffeine-induced Ca^{2+} release in C2C12 cells transfected with GFP or ΔCnA-GFP are shown in *A* and *B*, respectively. The traces are representative of 13 to 17 cells in six to eight independent experiments. The expression of GFP and ΔCnA-GFP can be visualized in confocal images.

1995; Kissinger et al., 1995). Therefore, the ΔCnA mutant is unlikely to alter the interaction with FKBP12, and thus it may provide a direct test to the effect of CnA-mediated dephosphorylation on the RyR function.

To visualize the expression of ΔCnA, a ΔCnA-GFP fusion construct was used in confocal imaging. As a control, myotubes were transfected with pEGFP vector encoding GFP only. The expressed ΔCnA-GFP fusion proteins in myotubes were abundantly distributed in the cytosol (Fig. 3 *B*). Based on the GFP fluorescence, we were able to select the transfected cells and measured their intracellular Ca^{2+}. The caffeine-induced Ca^{2+} transients in cells transfected with ΔCnA-GFP ($\Delta F_{340}/F_{380} = 0.16 \pm 0.01$, $n = 17$, Fig. 3 *B*) were significantly smaller than those expressing GFP alone ($\Delta F_{340}/F_{380} = 0.37 \pm 0.01$, $n = 13$, Fig. 3 *A*). This decreased Ca^{2+} release may result from dephosphorylation of RyR by the constitutively active calcineurin (ΔCnA); or alternatively, overexpressed ΔCnA may influence the expression level of RyR in C2C12 cells, as calcineurin is known to regulate the transcription of several genes (Abbott et al., 1998; Olson and Williams, 2000). It is also possible that the ΔCnA-GFP fusion protein may behave differently from ΔCnA.

To further test the effect of ΔCnA on the RyR/Ca^{2+} release channel, we cloned the ΔCnA cDNA into a pCMS-EGFP vector. The pCMS-EGFP(ΔCnA) vector contains an additional GFP cDNA driven by a separate promotor, thus allowing visualization of myotubes transfected with ΔCnA using green fluorescence. Immunoblot analysis revealed the expression of both exogenous ΔCnA (48 kDa) and endogenous wild-type calcineurin (61 kDa) in C2C12 cells (Fig. 4 *A*). Typically, only 5% to 10% of myotubes can be trans-

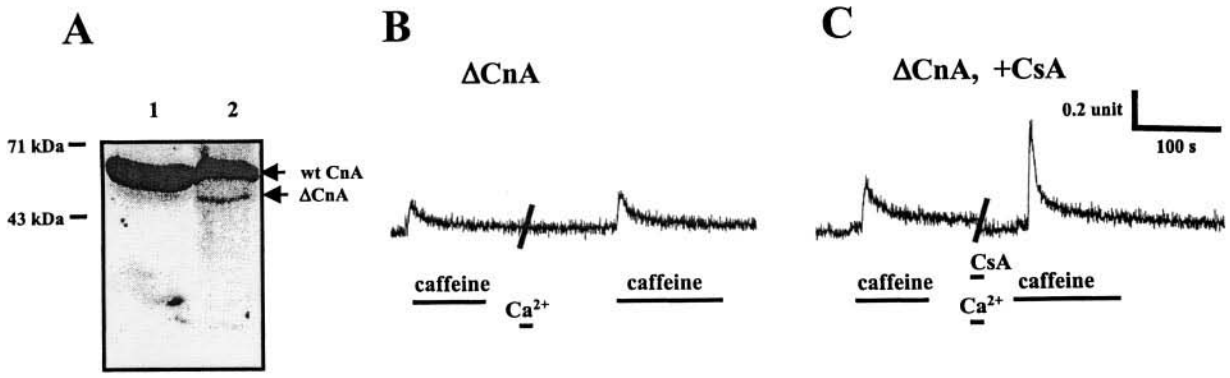

FIGURE 4 Reversible regulation of caffeine-induced Ca^{2+} release in C2C12 cells by ΔCnA. Transient overexpression of ΔCnA in C2C12 myotubes were detected with anticalcineurin Pan A antibody (*A*). (*Lane 1*) Cells transfected with pCMS-EGFP; (*lane 2*) cells transfected with pCMS-EGFP(ΔCnA). Arrowheads designate the endogenous calcineurin (61 kDa) band and overexpressed ΔCnA (48 kDa) band. The low intensity of the ΔCnA band reflects the low transfection efficiency of ΔCnA in C2C12 cells. Myotubes overexpressing ΔCnA exhibited low activity of caffeine-induced Ca^{2+} release (*B* and *C*). After the first caffeine stimulation, cells were incubated with either vehicle (*B*) or 1 μM CsA (*C*) in 2 mM Ca^{2+}-BSS for 10 min. Incubation with 1 μM CsA lead to partial reversal of the inhibitory effect of ΔCnA on the RyR channel during the second application of caffeine. The traces are representative of five independent experiments. Solid bars indicate the presence of caffeine, cyclosporin A, and Ca^{2+}.

fected with exogenous genes using the lipofectamine reagent. Therefore, the low level of ΔCnA expression is likely due to the intrinsic low transfection efficiency of genes in the myotubes.

As shown in Fig. 4 *B*, caffeine-induced Ca^{2+} release was significantly reduced in myotubes overexpressing ΔCnA. But the ΔCnA-mediated reduction of SR Ca^{2+} release could be partially recovered by pretreatment of cells with 1 μM CsA (for 10 min) ($\Delta F_{340}/F_{380} = 0.33 \pm 0.04$, $n = 5$, Fig. 4 *C*). These results indicate that the inhibitory effect of ΔCnA on RyR is due to a direct interaction with the Ca^{2+} release channel, rather than down regulation of the RyR protein expression.

Up-regulation of SR Ca^{2+} release by overexpression of cain and ΔCnA (H101Q)

Cain, also known as cabin-1, is an endogenous noncompetitive inhibitor of calcineurin (Lai et al., 1998; Molkentin et al., 1998; Sun et al., 1998). It is known to bind calcineurin at a site distinct from the FK506/FKBP12 binding sites. The calcineurin binding domains of cain are located at amino acid residues 1881 to 2173 (c-cain), and this portion of cain can specifically inhibits the phosphatase activity of calcineurin (Lai et al., 1998; Sun et al., 1998). The calcineurin-inhibitory domain of cain (c-cain, nucleotide 5643–6519) was fused with GFP at the 3′ end to create the GFP-c-cain fusion construct. The formation of myotubes from myoblasts transfected with GFP-c-cain seemed to be delayed compared with cells transfected with GFP alone (data not shown). This observation is in agreement with recent reports demonstrating that cain decreases myogenic differentiation, myotube fusion, resulting in a decreased number of multinucleated myotubes (Delling et al., 2000; Friday et al., 2000). The few multinucleated myotubes expressing GFP-c-cain were stimulated with 5 mM caffeine in the absence of extracellular Ca^{2+}. The amplitude of peak Ca^{2+} transients was higher in GFP-c-cain-expressing myotubes ($\Delta F_{340}/F_{380} = 0.63 \pm 0.03$, $n = 12$, Fig. 5, bottom) than that in control cells ($\Delta F_{340}/F_{380} = 0.37 \pm 0.02$, $n = 13$, Fig. 5, top), suggesting that inhibition of endogenous calcineurin phosphatase activity can lead to up-regulation of RyR/Ca^{2+} release function.

Mutation of one of the three conserved histidine residues (H101, H160, or H290) in the active site of calcineurin results in a catalytically inactive form of calcineurin (ΔCnA(H101Q)) (Shibasaki et al., 1996). ΔCnA(H101Q) lacks phosphatase activity of calcineurin without changing its native structure. Site-directed mutagenesis was used to generate this catalytically inactive form of ΔCnA by changing histidine at position 101 into glutamine. Myotubes transfected with ΔCnA(H101Q) exhibited an enhanced caffeine-induced Ca^{2+} release ($\Delta F_{340}/F_{380} = 0.71 \pm 0.06$, $n = 12$, Fig. 6 *B*, bottom) compared with control myotubes ($\Delta F_{340}/F_{380} = 0.37 \pm 0.02$, $n = 13$, Fig. 6 *B*, top). The

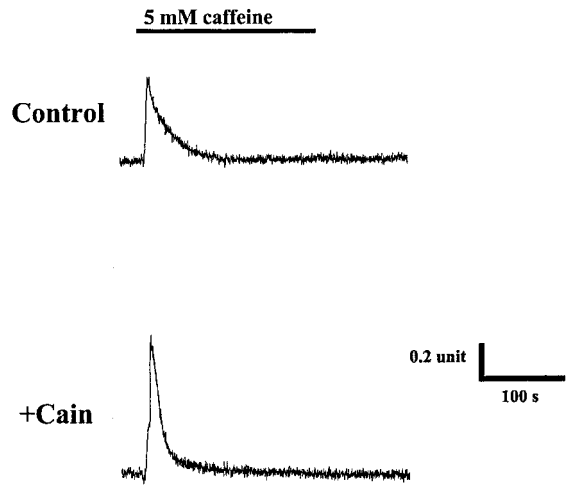

FIGURE 5 Up-regulation of caffeine-induced Ca^{2+} release in C2C12 cells by GFP-c-cain. GFP (as control) or GFP-c-cain was transiently expressed in C2C12 myotubes. Cells expressing GFP-c-cain contained higher activity of caffeine-induced Ca^{2+} release compared with those expressing GFP alone. Each trace is representative of 12 to 13 independent experiments.

effect was similar to that of cain. Overexpression of ΔCnA (H101Q) may in principal compete with the endogenous calcineurin for binding with RyR and, therefore, prevents dephosphorylation of RyR by calcineurin.

The results from multiple experiments are summarized in Fig. 7 *A*. The effects of calcineurin-related proteins on

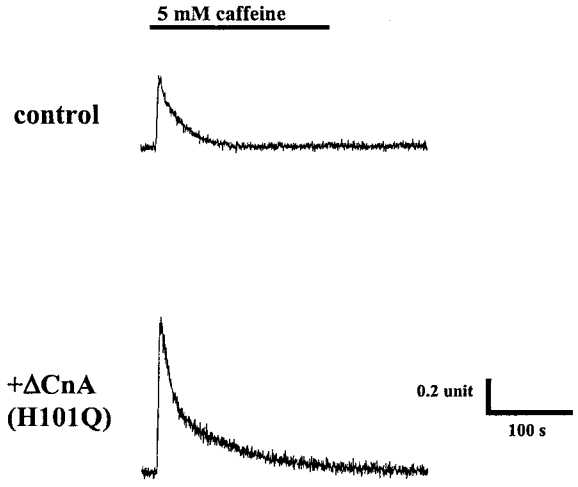

FIGURE 6 Up-regulation of caffeine-induced Ca^{2+} release in C2C12 cells by ΔCnA(H101Q). C2C12 myoblasts were transfected with pCMS-EGFP(ΔCnA(H101Q)) and allowed to differentiate into mature myotubes. Myotubes overexpressing ΔCnA(H101Q) were selected based on the appearance of green fluorescence. Those nongreen cells in the same dish were used as controls. Five millimolar caffeine induced higher activity of SR Ca^{2+} release in cells expressing ΔCnA(H101Q) (*bottom trace*) compared with the controls (*top trace*). Notice that the kinetics of decay in Ca^{2+} transients were similar between the two groups of cells. The traces are representative of 12 to 13 independent experiments.

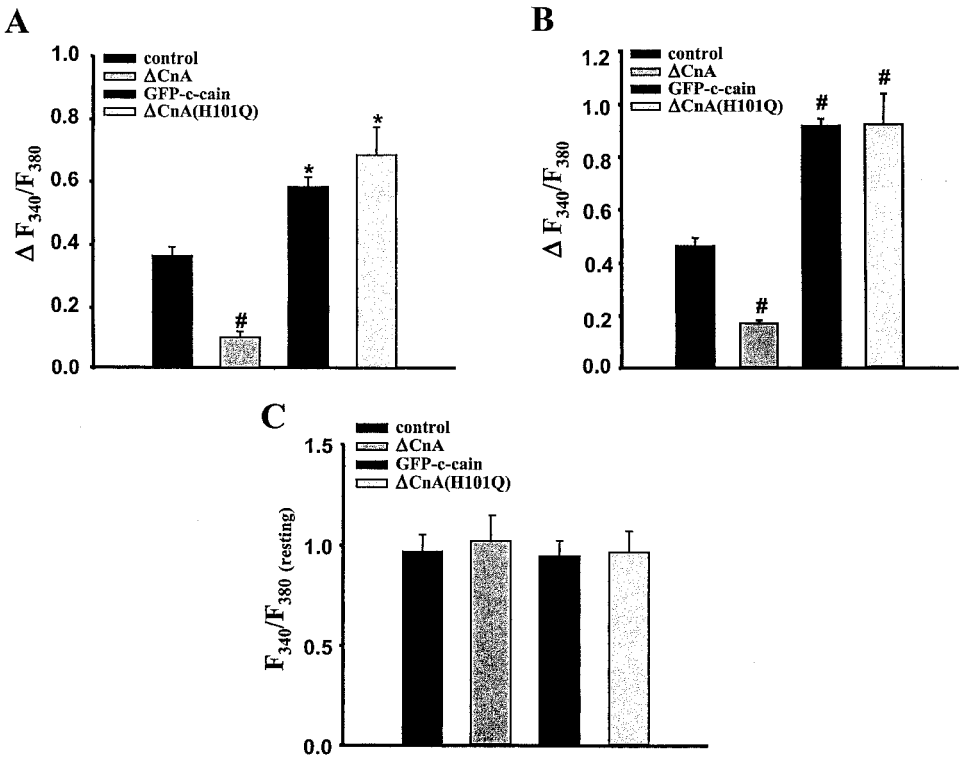

FIGURE 7 Effects of ΔCnA, ΔCnA(H101Q), and GFP-c-cain on Ca^{2+} release in C2C12 myotubes. (*A*) Data from multiple experiments with caffeine-induced Ca^{2+} release in C2C12 cells expressing ΔCnA, GFP-c-cain, or ΔCnA(H101Q) is presented. The experiments were performed with 0 Ca^{2+} present in the bath solution. (*B*) Experiments with caffeine-induced Ca^{2+} release in C2C12 cells with 2 mM Ca^{2+} present in the bath solution. Data were averaged from 5 to 10 independent experiments. *, Significance difference with $p < 0.05$; #, significance difference with $p < 0.01$, compared with control. (*C*) Measurement of resting [Ca^{2+}] in cells expressing ΔCnA, GFP-c-cain, or ΔCnA(H101Q). Data were averaged from 7 to 14 independent experiments.

caffeine-induced Ca^{2+} transients were also studied in the presence of extracellular Ca^{2+} (2 mM). Fig. 7 *B* shows that ΔCnA reduced caffeine-induced Ca^{2+} release ($\Delta F_{340}/F_{380} = 0.18 \pm 0.01$, $n = 10$), whereas c-cain and ΔCnA (H101Q) caused significant increase in caffeine-induced Ca^{2+} release ($\Delta F_{340}/F_{380} = 0.92 \pm 0.01$, $n = 5$; and 0.93 ± 0.01, $n = 6$, respectively) compared with control ($\Delta F_{340}/F_{380} = 0.47 \pm 0.01$, $n = 6$). Clearly, overexpression of ΔCnA down-regulates the SR Ca^{2+} release channel function in C2C12 cells, and overexpression of ΔCnA(H101Q) and GFP-c-cain up-regulates the function of RyR in skeletal muscle.

Effects of rapamycin on myotubes overexpressing ΔCnA, GFP-c-cain, or the ΔCnA (H101Q) proteins

To further test the role of protein-protein interaction in the function of the Ca^{2+} release channel, we studied the effect of rapamycin on myotubes transfected with ΔCnA, GFP-c-cain, ΔCnA(H101Q), respectively. Our data shown in Fig. 2 *E* demonstrate that rapamycin can enhance the Ca^{2+} release channel function in C2C12 cells. It may be possible that rapamycin increases caffeine-triggered Ca^{2+} release by dissociating FKBP12 without affecting the phosphorylation state of RyR, or that rapamycin causes the dissociation of FKBP12 and thus the dissociation of CnA. The dissociation of CnA would allow kinases to rephosphorylate the RyR, resulting in activation of RyR and hence an increase in caffeine-triggered Ca^{2+} release, assuming that CnA can only dephosphorylate RyR when it is bound to the RyR.

As shown in Fig. 8 *A*, treatment of myotubes with rapamycin (1 μM) could reverse the ΔCnA-mediated reduction of caffeine-induced SR Ca^{2+} release ($\Delta F_{340}/F_{380} = 0.35 \pm 0.04$, $n = 6$, +rapamycin). This effect is similar to that observed with CsA (Fig. 4 *C*), indicating that the action of the ΔCnA required FKBP12 association with RyR. Interestingly, rapamycin also enhanced the caffeine-induced Ca^{2+} release in myotubes overexpressing GFP-c-cain (Fig. 8 *B*) and ΔCnA(H101Q) (Fig. 8 *C*), respectively. Following 10-min incubation with 1 μM rapamycin, the caffeine-induced Ca release was significantly increased in myotubes transfected with GFP-c-cain ($\Delta F_{340}/F_{380} = 0.87 \pm 0.02$, $n = 7$) and with ΔCnA(H101Q) ($\Delta F_{340}/F_{380} = 0.93 \pm 0.06$, $n = 5$).

Because the phosphatase activity of endogenous calcineurin is effectively blocked by overexpression of GFP-c-cain or ΔCnA (H101Q), the additive effect of rypamycin

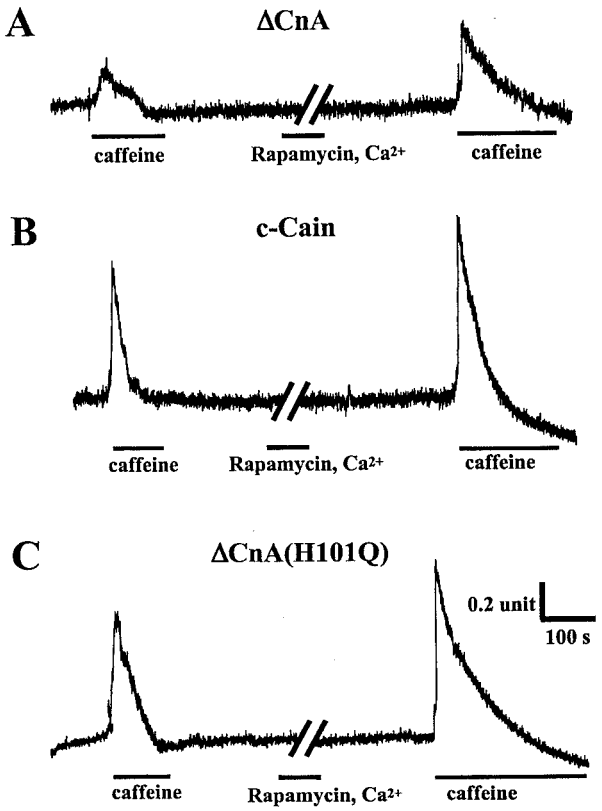

FIGURE 8 Effect of rapamycin on caffeine-induced Ca^{2+} release in myotubes overexpressing ΔCnA, ΔCnA(H101Q), or GFP-c-cain. The ΔCnA, ΔCnA(H101Q), or GFP-c-cain cDNAs cloned into the pCMS-EGFP plasmid were introduced into C2C12 cells using the lipofectamine reagent. Ca^{2+}-release from SR under control condition was measured after first application of 5 mM caffeine to a Ca^{2+}-free BSS. Following the initial caffeine-induced Ca^{2+} release, myotubes were reloaded with 2 mM Ca^{2+}-BSS, plus 1 μM rapamycin for 10 min, to ensure complete loading of Ca^{2+} into SR. The bath solution was then changed to Ca^{2+}-free BSS, followed by a second addition of 5 mM caffeine. Representative traces with caffeine-induced Ca^{2+} release in C2C12 cells expressing ΔCnA (*A*), GFP-c-cain (*B*), or ΔCnA(H101Q) (*C*) are plotted. Each trace is representative of five to seven independent experiments. The averaged data are presented in the text.

on intracellular Ca release suggests that dissociation of FKBP12 from RyR by rapamycin and hyperphosphorylation of RyR through inhibition of calcineurin by GFP-c-cain or ΔCnA(H101Q) can act synergistically to enhance the Ca^{2+} release channel activity of RyR in C2C12 cells.

Measurement of sustained cytosolic Ca^{2+} levels at resting state

Ca^{2+} homeostasis in muscle cells is mainly maintained by two major proteins, RyR and Ca^{2+}-ATPase (SERCA1). It is possible that some of the changes in SR Ca^{2+} release induced by calcineurin could reflect the changes in basal Ca^{2+}-ATPase activity on the SR membrane. In separate experiments, we observed that 20 mM caffeine, a concentration that maximally stimulated the Ca^{2+} release channel, released similar amount of Ca^{2+} in C2C12 cells irrespective of their transfection with GFP, ΔCnA, ΔCnA(H101Q), or GFP-c-cain. In addition, the decaying phase of caffeine-induced Ca^{2+} release was not very different among all C2C12 cells tested under the various conditions (i.e., treatment with FK506, CsA, rapamycin, or bearing transfection with exogenous genes). The results show that calcineurin is unlikely to affect the Ca^{2+} uptake process into the SR membrane in C2C12 cells.

As a further test of whether Ca^{2+}-ATPase activity is affected by calcineurin, we monitored cytosolic [Ca^{2+}] at resting state in myotubes transfected with ΔCnA, GFP-c-cain, or ΔCnA(H101Q). There were no significant differences in the resting [Ca^{2+}] among the C2C12 cells: $F_{340}/F_{380} = 0.99 \pm 0.08$ ($n = 14$) (control); 1.03 ± 0.12 ($n = 10$) (+ΔCnA); 0.96 ± 0.07 ($n = 8$) (+GFP-c-cain); 0.98 ± 0.10 ($n = 7$) (+ΔCnA(H101Q)) (Fig. 7 *C*). Together, our data suggest that calcineurin does not seem to affect the Ca^{2+}-ATPase activity in C2C12 cells.

Effect of calcineurin in CHO cells stably transfected with RyR

We have previously reported that RyR stably expressed in CHO cells (CHO-RyR) exhibits functional properties that are similar to RyR from native skeletal muscle (Bhat et al., 1997). CHO cells do not express muscle specific proteins, such as junctin, triadin, and calsequestrin, but they contain endogenous FKBP12. Pretreatment of CHO-RyR cells with 10 μM FK506 increased the magnitude of Ca^{2+} transient induced by 100 μM ATP or 5 mM caffeine compared with that of control (data not shown). Therefore, studies with CHO-RyR cells will provide additional insights on the effects of calcineurin on RyR (with respect to the role of accessory proteins and cell-type specific effect of calcineurin).

The various calcineurin-related genes were transiently transfected into CHO-RyR cells using the lipofectamine reagent. The presence endogenous calcineurin (61 kDa) in CHO-RyR cells (Taniguchi et al., 2001) and the presence of exogenous ΔCnA, ΔCnA(H101Q), and GFP-c-cain expressed in CHO-RyR cells were confirmed with Western-blot assay (Fig. 9). Individual transfected CHO cells were selected based on the appearance of green fluorescent. As shown in Fig. 10, ΔCnA decreased caffeine-induced Ca^{2+} release from the endoplasmic reticulum ($\Delta F_{340}/F_{380} = 0.15 \pm 0.03$, $n = 8$), whereas GFP-c-cain ($\Delta F_{340}/F_{380} = 0.72 \pm 0.05$, $n = 5$) and ΔCnA(H101Q) ($\Delta F_{340}/F_{380} = 0.75 \pm 0.04$, $n = 5$) significantly enhanced Ca^{2+} release compared with that of cells transfected with GFP alone (0.43 ± 0.02, $n = 5$). The results with CHO-RyR cells are similar to those obtained with C2C12 cells.

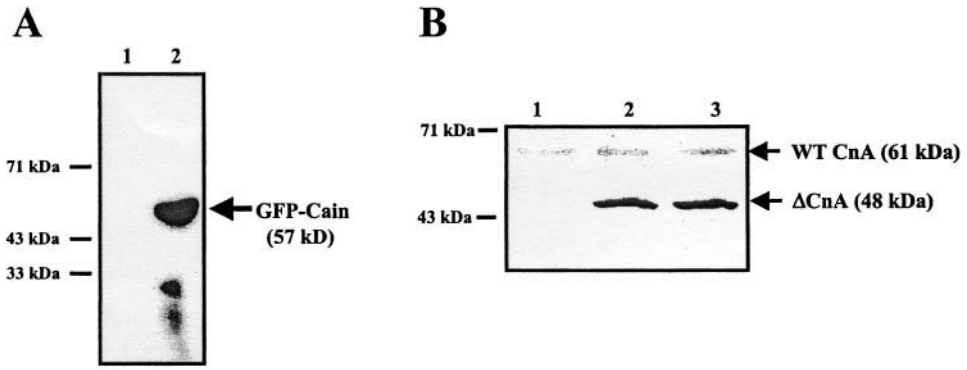

FIGURE 9 Expression of ΔCnA, ΔCnA(H101Q), or GFP-c-cain CHO cells. The ΔCnA, ΔCnA(H101Q), or GFP-c-cain cDNAs were introduced into CHO cells that are permanently transfected with the skeletal muscle RyR using the lipofectamine reagent. (*A*) Western blot with anti-GFP antibody. (*Lane 1*) Untransfected CHO-RyR cells; (*lane 2*) cells transfected with GFP-c-cain. Arrow indicates position of the 57-kDa GFP-c-cain protein. (*B*) Western blot with anticalcineurin antibody. (*Lane 1*) Cells transfected with pCMS-EGFP; (*lane 2*) cells transfected with ΔCnA; (*lane 3*) cells transfected with ΔCnA(H101Q). Arrows indicate position of the ΔCnA and endogenous CnA bands.

DISCUSSION

In the present study, we examined the effect of calcineurin on the skeletal muscle RyR using a C2C12 skeletal muscle cell line and a CHO cell line stably transfected with the skeletal muscle RyR. Coimmunoprecipitation experiments demonstrated that the association between RyR and calcineurin involves Ca^{2+}-dependent interaction between FKBP12 and calcineurin. This Ca^{2+}-dependent interaction between RyR and calcineurin is similar to previous reports with the cardiac RyR and calcineurin (Bandyopadhyay et al., 2000a), and IP_3R and calcineurin (Cameron et al., 1995). A close physical interaction between RyR and calcineurin provides the cellular basis for functional regulation of the Ca^{2+} release channel by dephosphorylation. The strong Ca^{2+}-dependent interaction between FKBP12 and calcineurin offers a unique possibility for a feedback regulation of the Ca^{2+} release channel by cytosolic Ca^{2+}. This concept is supported by the fact that maximal catalytic activity of calcineurin requires a large rise in $[Ca^{2+}]_i$ (to the micromolar range) (Stammer and Klee, 1994). Similar Ca^{2+}-dependent regulation of RyR by accessory proteins has been well documented with calmodulin (Fuentes et al., 1994; Tripathy et al., 1995; Rodney et al., 2000). It is known that Ca^{2+}-free calmodulin (apocalmodulin) activates the Ca^{2+} release channel, whereas Ca^{2+}-bound calmodulin inhibits the Ca^{2+} release channel. The conversion of calmodulin from an activator to an inhibitor is due to Ca^{2+} binding to calmodulin.

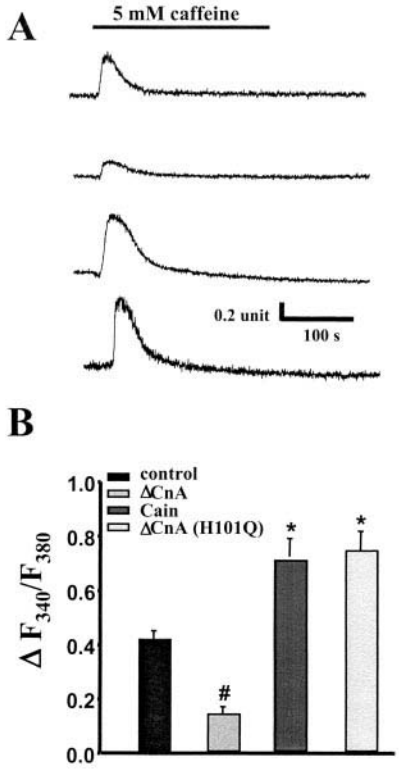

FIGURE 10 Effects of ΔCnA, ΔCnA(H101Q) or GFP-c-cain on RyR stably expressed in CHO cells. (*A*) Representative traces of Ca^{2+} release triggered by 5 mM caffeine in CHO-RyR cells transiently expressing GFP (*control*), ΔCnA, GFP-c-cain, or ΔCnA(H101Q) (from *top* to *bottom*). (*B*) Data from multiple experiments are summarized as mean ± SE. Data are from five to eight independent experiments. *, $p < 0.05$ compared with control; #, $p < 0.01$ compared with control.

FK506 and rapamycin, ligands that dissociate FKBP12 from RyR, significantly enhanced the caffeine-induced Ca^{2+} release in C2C12 cells. Cyclosporin A, a direct inhibitor of calcineurin had similar effect on caffeine-induced Ca^{2+} release. Since the major effect of cyclosporin A is to inhibit the phosphatase activity of calcineurin, the increased activity of caffeine-induced Ca^{2+} release likely reflects the reduced dephosphorylation of RyR. Our experiments were performed at resting status, suggesting that some of the endogenous calcineurin are active or are associated with RyR via FKBP12 at resting status, since blocking the en-

dogenous CnA activity by pharmacological agent (CsA), by competitive protein inhibitor (GFP-c-cain), or by overexpression of a dominant negative mutant (ΔCnA(H101Q)) all leads to up-regulation of caffeine-induced Ca^{2+} release in C2C12 cells. The ΔCnA-mediated reduction of caffeine-induced Ca^{2+} release, on the other hand, was unlikely due to an effect on the expression level of RyR (Genazzani et al., 1999), because pretreatment with CsA and rapamycin can partially reverse the inhibitory effect of ΔCnA on RyR.

Regulations of RyR by various protein kinases and phosphatases have been extensively examined by in vitro studies of lipid bilayer reconstitution of single RyR/Ca^{2+} channel, radioligand binding of [^{3}H]ryanodine to the isolated RyR protein, or biochemical assays of RyR phosphorylation by exogenous or endogenous protein kinases or phosphatases (Hermann-Frank and Varsanyi, 1993; Hain et al., 1994; Lokuta et al., 1995; Witcher et al., 1991). Our study with the C2C12 cells represents the first systematic assay of calcineurin effect on skeletal RyR in native cells. In intact rat cardiomyocytes, β-adrenergic agonists increase PKA activity, and the ensuring phosphorylation of RyR in cardiac muscle is responsible for the ionotropic effect of β-adrenergic agonists on changes in cytosolic $[Ca^{2+}]_i$ (Hain et al., 1994; Takasago et al., 1991; Allen and Blinks, 1978). A recent study by Marx et al. (2000) showed that PKA hyperphosphorylation of cardiac RyR could result in an increased Ca^{2+} sensitivity for activation and elevated single channel activity by causing dissociation of FKBP12.6 from RyR. Our data show that inhibition of endogenous calcineurin by CsA, GFP-c-cain, or ΔCnA (H101Q) leads to up-regulation of caffeine-induced Ca^{2+} release in skeletal muscle. Presumably, inhibition of the phosphatase activity of calcineurin could lead to hyperphosphorylation of the skeletal muscle RyR and increase in the Ca^{2+} release channel function.

CHO cells transfected with RyR do not express muscle specific proteins, such as triadin, junctin, or calsequestrin. Therefore, the use of CHO cells helped us rule out the possibility that calcineurin interacts other accessory proteins to regulate the activity of RyR. RyR/Ca^{2+} release channel expressed in CHO cells can be significantly up-regulated through transient expression of GFP-c-cain or ΔCnA(H101Q), and overexpression of ΔCnA leads to down-regulation of RyR. These data are consistent with the observation in C2C12 cells that calcineurin can reduce the activity of Ca^{2+} release channel.

Overexpression of ΔCnA, ΔCnA(H101Q), or GFP-c-cain in C2C12 cells does not appear to affect the function of the Ca^{2+}-ATPase located on the SR membrane, because the maximal caffeine-releasable Ca^{2+} pool in the SR remained unchanged, and the Ca^{2+} uptake process following caffeine-induced Ca^{2+} release is not significantly affected in both C2C12 and CHO cells. In addition, the resting cytosolic $[Ca^{2+}]$ is not changed after overexpression of calcineurin-related genes. These results are in agreement with our previous studies with CsA on the function of Ca^{2+}-ATPase in rat heart or fast-twitch skeletal muscles (Park et al., 1999). The difference in oxalate-supported Ca^{2+} uptake between control and chronic CsA-treated rat heart or fast-twitch white muscles was negligible, indicating that Ca^{2+}-ATPase activity was not altered by CsA treatment.

Rapamycin, a drug that causes dissociation of FKBP12 from RyR without affecting the phosphatase activity of calcineurin, was found to enhance the caffeine-induced Ca^{2+} release in controls myotubes, as well as in myotubes over expressing ΔCnA, GFP-c-cain, or ΔCnA(H101Q). These data together demonstrate an important role of protein-protein interaction in the regulation of intracellular Ca^{2+} release in muscle cells. Presumably, association of FKBP12 with RyR exerts an inhibitory role in the function of the Ca^{2+} release channel, which may or may not dependent on the phosphorylation status of RyR. Anchoring of calcineurin to RyR via FKBP12 provides spatial coupling for controlling activity of the Ca^{2+} release channel via protein dephosphorylation. Also, activation of calcineurin by Ca^{2+} release from the SR may help to terminate further intracellular Ca^{2+} release through a negative feedback inhibition via dephosphorylation of RyR.

We thank Dr. Takayoshi Kuno and his colleagues for providing cDNA encoding the rat calcineurin, and Dr. Solomon Snyder for providing the cain cDNA. We are grateful to Dr. Derek Damron for his generous help with the Ca^{2+} measurements and to Dr. Salim Hayek, Mr. J.I. Lee, and Mr. Y. Abdellatif for helpful discussions in preparing the manuscript. This work was supported by the National Institutes of Health Grants RO1-AG15556, RO1-CA95739, and RO1-HL69000 (to J.M.), by the Korea Ministry of Science and Technology Critical Technology 21, 00-J-LF-01-B-54, by the Korea Science and Engineering Foundation Basic Research Program, 1999-1-20700-002-5, and by the Brain Korea 21 Project (to D.H.K.).

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