Role of FKBP12.6 in cADPR-induced activation of reconstituted ryanodine receptors from arterial smooth muscle

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Tang, Wang-Xian, Ya-Fei Chen, Ai-Ping Zou, William B. Campbell, and Pin-Lan Li. Role of FKBP12.6 in cADPR-induced activation of reconstituted ryanodine receptors from arterial smooth muscle. Am J Physiol Heart Circ Physiol 282: H1304–H1310, 2002; 10.1152/ajpheart.00843.2001.—cADPR ribose (cADPR) serves as second messenger to activate the ryanodine receptors (RyRs) of the sarcoplasmic reticulum (SR) and mobilize intracellular Ca2+ in vascular smooth muscle cells. However, the mechanisms mediating the effect of cADPR remain unknown. The present study was designed to determine whether FK-506 binding protein 12.6 (FKBP12.6), an accessory protein of the RyRs, plays a role in cADPR-induced activation of the RyRs. A 12.6-kDa protein was detected in bovine coronary arterial smooth muscle (BCASM) and cultured CASM cells by being immunoblotted with an antibody against FKBP12, which also reacted with FKBP12.6. With the use of planar lipid bilayer clamping techniques, FK-506 (0.01–10 μM) significantly increased the open probability (NO) of reconstituted RyR/Ca2+ release channels from the SR of CASM. This FK-506-induced activation of RyR/Ca2+ release channels was abolished by pretreatment with anti-FKBP12 antibody. The RyRs activator cADPR (0.1–10 μM) markedly increased the activity of RyR/Ca2+ release channels. In the presence of FK-506, cADPR did not further increase the NO of RyR/Ca2+ release channels. Addition of anti-FKBP12 antibody also completely blocked cADPR-induced activation of these channels, and removal of FKBP12.6 by preincubation with FK-506 and subsequent gradient centrifugation abolished cADPR-induced increase in the NO of RyR/Ca2+ release channels. We conclude that FKBP12.6 plays a critical role in mediating cADPR-induced activation of RyR/Ca2+ release channels from the SR of BCASM.

Recent studies (18, 21, 23) have indicated that cADPR induces Ca2+ release through the activation of the ryanodine receptors (RyRs) of the sarcoplasmic reticulum (SR). However, the mechanism by which cADPR activates RyRs on the SR is poorly understood.

There is increasing evidence indicating that FK-506 binding proteins (FKBP) play an important role in the regulation of the SR and the resulting Ca2+ release from the SR (5, 6, 13). FKBP12 is a ubiquitous 12-kDa cytosolic protein. As an accessory protein, each FKBP12 can bind to one RyR monomer (1, 12, 32, 37, 38) and its activity can be inhibited by the immunosuppressants FK-506 and rapamycin (2, 7, 10, 11). It has been demonstrated (4, 39) that the Ca2+ release from the SR was inhibited when FKBP12 bound to the RyR and vice versa. FK-506, as a ligand, binds to and consequently dissociates FKBP12 from the RyRs, resulting in the RyRs activation and Ca2+ release (3). A recent study (35) reported that cADPR is able to bind to FKBP12.6-like FK-506 and results in activation of the RyRs.

In vascular smooth muscle cells (SMCs), the RyRs have been reported (15–17, 30, 31) to mediate Ins(1,4,5)P3-independent Ca2+ release from the SR and in this way participate in the control of vascular tone. Recent studies in our laboratory and by others have demonstrated that cADPR serves as an endogenous activator of the RyRs to stimulate Ca2+ release from the SR in vascular SMCs. However, the mechanism by which cADPR activates the RyRs on the SR has yet to be clarified. The present study was designed to test the hypothesis that cADPR produces Ca2+ release from the SR by binding to FKBP12.6 and consequently activating RyR/Ca2+ release channels in bovine coronary arterial smooth muscle (BCASM).

MATERIALS AND METHODS

Preparation of the SR membrane and removal of FKBP12.6 from bovine coronary arteries. Coronary arteries were dissected from the bovine heart, and the SR-enriched micro-

CADDRISE (cADPR), a specific metabolite of nicotinamide adenine dinucleotide (NAD), mobilizes intracellular Ca2+ by a mechanism completely independent of d-myoinositol 1,4,5-trisphosphate [Ins(1,4,5)P3]. This cADPR-mediated Ca2+ signaling participates in the regulation of a variety of physiological functions in different tissue and cells (8, 9, 14, 20, 22, 24, 28).

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somes (SR membrane) of these arteries were prepared as described previously (26). Briefly, the dissected coronary arteries were cut into very small pieces and homogenized with Tenbroeck tissue grinders in ice-cold 3-(N-morpholino)propanesulfonic acid (MOPS) buffer containing 0.9% NaCl, 10 mM MOPS (pH 7.0), 2 μM leupeptin, and 0.8 μM benzamidine. The homogenate was centrifuged at 4,000 g for 20 min at 4°C and the supernatant was further centrifuged at 8,000 g for 20 min at 4°C. The supernatant was then collected and centrifuged at 40,000 g for 30 min, and the pellet, termed “SR membrane,” was resuspended in buffer A composed of 0.9% NaCl, 0.3 M sucrose, 5 μg/ml leupeptin, and phenylmethylsulfonyl fluoride (PMSF). These SR membranes were aliquoted, frozen in liquid N₂, and stored at −80°C until used.

To remove FKBP12.6 from the RyRs, the SR membranes were incubated with FK-506 (10 μM) in buffer A at 37°C for 15 min. FK-506 incubation mixtures were then centrifuged at 54,000 g for 15 min to remove the FK-506-FKBP12.6 complex in the supernatant and washed once by recentrifugation using buffer A. The pellet was resuspended in buffer A at a protein concentration of 2 mg/ml, termed “FKBP12.6-stripped SR” (1, 2). To determine whether cADPR can induce dissociation of FKBP12.6 from the RyRs, cADPR was used to dissociate with SR of BCASM and the resultant pellet was termed “cADPR-FKBP12.6-stripped SR.”

Western blot analysis. Western blot analysis was performed as described in our previous studies (25, 27). Briefly, 40 μg of proteins from cultured SMCs and SR from BCASM and FKBP12.6-stripped SR were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% running gel) after being heated at 100°C for 5 min. The proteins were electrophoretically transferred at 30 V overnight onto a nitrocellulose membrane. The membrane was incubated with a polyclonal antibody against the synthetic FKBP12 peptide (ABR) for 6 h at room temperature. After being washed three times with Tris-buffered saline-Tween 20, the membrane was incubated for 1 h with 1:1,000 horseradish peroxidase-labeled goat anti-rabbit IgG. To detect immunoreactive bands, 2 ml of enhanced chemiluminescence detection solution (ECL; Amersham) were added directly to the blots on the surface-carrying proteins, and the membrane was wrapped in Saran Wrap and then exposed to Kodak Omn film.

Reconstitution of RyR/Ca²⁺ into planar lipid bilayer. The coronary arterial SR membranes enriched in RyR/Ca²⁺ release channels were reconstituted into planar lipid bilayers as we have previously described (26). In brief, phosphatidyl ethanolamine and phosphatidyl-serine (1:1) were dissolved in decane (25 mg/ml) and used to form a planar lipid bilayer in a 250-μm aperture between two chambers filled with cis and trans solutions, respectively. The SR membranes (50–100 μg protein) were added into the cis solution, which corresponded to the cytosolic side of the SR RyR/Ca²⁺ release channels. The trans solution represented the luminal side of these SR RyR/Ca²⁺ release channels. The recording solution in the cis chamber was 300 mM Cs⁺ methanesulfonate and 10 mM MOPS (pH 7.2). The trans solution was the same as cis solution except that Cs⁺ methanesulfonate was 50 mM before fusion and 300 mM after fusion. In this configuration, Cs⁺ flows from the luminal (trans) to the cytosolic (cis) side at negative holding potentials. Cs⁺ was chosen instead of Ca²⁺ as the charge carrier to precisely control [Ca²⁺] around the channel, to increase the channel conductance (g_Ca²⁺/g_Ca²⁺ = 2), and to avoid interference from K⁺ channels present in the SR membrane. The Cl⁻ channels were blocked by the replacement of Cl⁻ with the impermeant anion methanesulfonate. RyR/Ca²⁺ release channel activity was detected in a symmetrical Cs⁺ methanesulfonate solution (300 mM) in all experiments. To increase the channel activity, 1 μM free Ca²⁺ in the cis solution was adjusted by adding Ca²⁺ standard solution containing CaCl₂ and EGTA as previously described (26, 30).

Recordings of RyR/Ca²⁺ release channel currents. An Integrating Bilayer Clamp Amplifier (model BC-525C; Warner) was used to record single channel currents in the bilayer. The amplifier output signals were filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices). Currents were digitized at a sampling rate of 10 kHz and stored on a Micron Pentium III computer for off-line analysis. Data acquisition and analysis were performed with pCLAMP software (version 8, Axon Instruments). The open probability (N_Po) of the channels in the lipid bilayer was determined from recordings of 3–5 min, as described previously in our patch-clamp studies (26, 29). All lipid bilayer experiments were performed at room temperature (~20°C).

With the use of this bilayer preparation, the effects of FK-506 on the activity of RyR/Ca²⁺ release channels were first determined. After a 3-min control recording period at holding potentials of −40 mV, a series of concentrations of FK-506 was added into the bath solution, and RyR/Ca²⁺ release channel currents were recorded at each 3-min interval in the presence or absence of anti-FKBP12 antibody. Addition of IgG in the bath solution served as a negative control.

In another series of experiments, FK-506 was substituted for cADPR to determine the role of FKBP12.6 in cADPR-induced activation of RyR/Ca²⁺ release channels. The effects of cADPR on RyR/Ca²⁺ release channel activity were also examined in the absence or presence of PK-506 in the cis solution. To further address whether cADPR-induced activation of the RyR/Ca²⁺ release channels is associated with FKBP12.6, the FKBP12.6-stripped SR or cADPR-FKBP12.6-stripped SR was reconstituted into a planar lipid bilayer, and the activity of RyR/Ca²⁺ release channels was recorded in the presence of PK-506, cADPR, or ryanodine, respectively. All these compounds used in these experiments were added into the cis solution, and currents were recorded at holding potentials at −40 mV. The concentrations of FK-506, cADPR, and other compounds were chosen based on previous studies showing that they effectively altered the RyRs activity (26, 31, 34, 35).

Statistics. Data are presented as means ± SE. The significance of the differences in mean values between and within multiple groups was examined using analysis of variance for repeated measures, followed by Duncan’s multiple-range tests. Student’s t-test was used to evaluate statistical significance of differences between two paired observations. P < 0.05 was considered statistically significant.

RESULTS

Identification of FKBP12.6 in BCASM. To confirm the presence of FKBP12.6, Western blot analysis was performed on lysates of cultured SMCs, SR from BCASM, and FKBP12.6-stripped SR using a polyclonal antibody to FKBP12.6. As shown in Fig. 1A, one 12.6-kDa protein was recognized by this antibody in both SMC lysates and SR of BCASM. However, this 12.6-kDa protein band was completely blocked in the FKBP12.6-stripped SR (Fig. 1). These results suggest that FKBP12.6 was present in BCASM.
Effect of FK-506 on activity of reconstituted RyR/Ca\textsuperscript{2+} release channels from BCASM. Representative recordings of RyR/Ca\textsuperscript{2+} release channels before and after the addition of FK-506 into the cis solution are presented in Fig. 2A. FK-506 significantly increased the activity of these channels and resulted in subconductance opening of these channels. As shown in Fig. 2B, FK-506 at concentrations of 0.1–100 μmol/l produced a 1.8- to 7.1-fold increase in the NP\textsubscript{O} of RyR/Ca\textsuperscript{2+} release channels. A significant increase was seen at the lowest concentration of FK-506 studied (0.1 μmol/l) (P < 0.05).

Effect of anti-FKBP12 antibody on FK-506-induced activation of reconstituted RyR/Ca\textsuperscript{2+} release channels from BCASM. FK-506 increased the activity of RyR/Ca\textsuperscript{2+} release channels in the absence of the antibody against FKBP12 (1:1,000) or rabbit IgG (Fig. 3). Pretreatment of the SR membrane with an antibody against FKBP12.6 markedly inhibited FK-506-induced activation of RyR/Ca\textsuperscript{2+} release channels. In contrast to marked effect of anti-FKBP12, rabbit IgG had no effect on FK-506-induced activation of RyR/Ca\textsuperscript{2+} release channels. Figure 3B summarizes the effect of anti-FKBP12 and IgG on FK-506-induced increase in the NP\textsubscript{O} of RyR/Ca\textsuperscript{2+} release channels from BCASM. The anti-FKBP12 antibody substantially blocked the stimulatory effect of FK-506 on RyR/Ca\textsuperscript{2+} release channels.

Effect of anti-FKBP12 antibody on cADPR-induced activation of reconstituted RyR/Ca\textsuperscript{2+} release channels from BCASM. As shown in Fig. 4, cADPR increased the activity of RyR/Ca\textsuperscript{2+} release channels from the SR in a concentration-related manner. It was found that pretreatment of the SR membrane with an antibody against FKBP12 markedly inhibited cADPR-induced activation of RyR/Ca\textsuperscript{2+} release channels (Fig. 4A).

Fig. 2. Effect of FK-506 on reconstituted ryanodine (Rya) receptors (RyR)/Ca\textsuperscript{2+} release channel activity of coronary arterial smooth muscle. A: representative recording of reconstituted Ca\textsuperscript{2+} release channels in the presence or absence of FK-506. B: summarized data showing the effect of FK-506 on the open probability (NP\textsubscript{O}) of reconstituted RyR/Ca\textsuperscript{2+} channels from coronary arterial smooth muscle. *P < 0.05, significant difference from control (C); n = 11–28.

Fig. 3. A: effect of FK-506 on reconstituted RyR/Ca\textsuperscript{2+} channel activity of coronary arterial smooth muscle in the absence or presence of anti-FKBP antibody. B: summarized data showing that the effect of FK-506 on the NP\textsubscript{O} of reconstituted RyR/Ca\textsuperscript{2+} channel in the absence or presence of anti-FKBP antibody. Pretreatment of the SR membrane with IgG served as the negative control. *P < 0.05, significant difference from control; n = 11–31.

Fig. 1. Western immunoblot showing FK-506 binding protein 12.6 (FKBP12.6) in the lysate of cultured smooth muscle cells (SMC) and the sarcoplasmic reticulum (SR) of bovine coronary arterial smooth muscle (BCASM). Immunoblot band of FKBP12.6 was not shown in FKBP12.6-stripped SR; n = 2 cells.
However, rabbit IgG had no effect on cADPR-induced activation of these channels. Figure 4B summarizes the effect of cADPR on the NP₀ of reconstituted RyR/Ca²⁺ release channels from the SR pretreated with anti-FKBP12 antibody or IgG. A concentration-dependent increase in the NP₀ of RyR/Ca²⁺ release channels was markedly blocked by anti-FKBP12 antibody.

**Effect of FK-506 on cADPR-induced activation of reconstituted SR Ca²⁺ release channels from BCASM.** Figure 5 shows the effects of cADPR on the SR RyR/Ca²⁺ release channels in the presence or absence of FK-506 (10 μM). cADPR significantly increased the activity of these channels in a concentration-dependent manner in the absence of FK-506. In the presence of FK-506, however, cADPR-induced increase in the NP₀ of RyR/Ca²⁺ release channels was completely abolished, suggesting that cADPR may activate RyR/Ca²⁺ release channels through the same mechanism as FK-506.

**Effect of FKBP12.6-depletion from SR membranes on cADPR- or FK-506-induced activation of RyR/Ca²⁺ release channels.** As shown in Fig. 6A, removal of FKBP12.6 from the SR preparation by FK-506 binding and high-speed centrifugation completely abolished the stimulatory effect of both FK-506 and cADPR. Even the highest concentration of FK-506 or cADPR had no effect on the activity of RyR/Ca²⁺ release channels in the FKBP12.6-stripped SR preparation. However, a low concentration of ryanodine (0.1 μM) mark-

![Fig. 4. A: effect of cADP ribose (cADPR) on reconstituted RyR/Ca²⁺ channel activity of coronary arterial smooth muscle in the absence or presence of anti-FKBP antibody. B: summarized data showing the effect of cADPR on the NP₀ of reconstituted RyR/Ca²⁺ channel in the absence or presence of anti-FKBP antibody. Pretreatment of the SR membrane with IgG served as the negative control. *P < 0.05, significant difference from control; n = 11.](image)

![Fig. 5. Effect of pretreatment of the SR membrane with FK-506 on cADPR-induced activation of reconstituted RyR/Ca²⁺ release channels of coronary arterial smooth muscle. *P < 0.05, significant difference from cADPR treatment only; n = 15.](image)

![Fig. 6. A: effect of cADPR (10 μM), FK-506 (10 μM), and ryanodine (0.1 μM) on the activity of RyR/Ca²⁺ release channels of FKBP12.6-stripped SR membrane from coronary arterial smooth muscle. B: summarized data showing that the effect of cADPR, FK-506 and ryanodine on the NP₀ of reconstituted RyR/Ca²⁺ channel. *P < 0.05, significant difference from control; n = 8–12.](image)
edly increased RyR/Ca\(^{2+}\) release channel activity in this FKBP12.6-stripped SR preparation. The typical stimulatory effect of ryanodine with subconductance opening was observed. The \(N_P\) of these SR RyR/Ca\(^{2+}\) release channels, including subconductance, was markedly increased (Fig. 6B). As shown in Fig. 7A, removal of FKBP12.6 from the SR preparation by cADPR also completely abolished both FK-506- and cADPR-induced activation of RyR/Ca\(^{2+}\) release channels. However, a low concentration of ryanodine (0.1 \(\mu\)M) still markedly increased RyR/Ca\(^{2+}\) release channel activity in this cADPR-FKBP12.6-stripped SR preparation. The \(N_P\) of these SR RyR/Ca\(^{2+}\) release channels, including subconductance was markedly increased (Fig. 7B).

DISCUSSION

In the present study, Western blot analysis was performed to confirm the presence of FKBP12.6, an accessory protein of the RyRs, in BCASM. A 12.6-kDa immunoreactive band was detected in both smooth muscle cell lysates and coronary arterial SR. Preincubation of SR with FK-506 completely blocked this 12.6-kDa immunoreactive band. These results provide direct evidence that FKBP12.6 is present in coronary arterial smooth muscle and the presence of FKBP12.6 may serve as a regulatory protein to control the activity of the RyRs on the SR of these SMCs.

To determine whether FKBP12.6 is involved in regulation of the RyRs on the SR of BCASM, we used lipid bilayer channel reconstitution technique to examine the effects of FK-506, a FKBP ligand on the activity of reconstituted RyR/Ca\(^{2+}\) release channels from the SR of BCASM. FK-506 was found to concentration-dependently increase the activity of these channels, indicating that FK-506 activates the RyRs in coronary arterial smooth muscle. These results suggest that activation of FKBP12.6 is of importance in the regulation of activity of RyR/Ca\(^{2+}\) release channels in BCASM.

To confirm that the effects of FK-506 on RyR/Ca\(^{2+}\) release channel activity of the SR are associated with FKBP12.6, a specific anti-FKBP12 antibody was added into the cis solution to examine whether FK-506-induced activation of RyR/Ca\(^{2+}\) release channels can be blocked. As predicted, the anti-FKBP12 antibody substantially blocked FK-506-induced activation of RyR/Ca\(^{2+}\) release channels, whereas IgG had no effect on the FK-506-induced activation of these channels. These findings further demonstrate that FKBP12.6 is the isoform of FKBPs that regulate the activity of RyR/Ca\(^{2+}\) release channels in BCASM. Recent studies (32) have indicated that there are five types of FKBPs in different mammalian cells, including FKBP12, -12.6, -13, -25, -38, and -52. FKBP12 binds to the RyRs and thereby participates in the regulation of the RyRs activation on the SR of myocardial or skeletal muscle cells (12, 32, 33, 37). It has been reported (3) that RyR/Ca\(^{2+}\) release channels on the SR can be inhibited when FKBP12 binds to the RyRs. FK-506, a ligand of FKBPs can bind to both FKBP12 and FKBP12.6, dissociate and release these proteins from the RyRs, whereby the RyRs are activated and Ca\(^{2+}\) released from the SR (35). This study demonstrates for the first time that this accessory protein is also present in vascular smooth muscle and it is functioning as an inhibitory protein of the RyRs. However, the endogenous ligand or activator of this accessory protein remains unknown. Because cADPR-induced Ca\(^{2+}\) release is associated with the activation of RyR/Ca\(^{2+}\) release channels (14, 15, 19, 26, 34–36) and cADPR cannot directly bind to the RyRs (35), we were wondering whether cADPR serves as an endogenous ligand of FKBP12.6 in vascular SMCs.

To answer this question, a series of experiments were performed to determine the role of FKBP12.6 in cADPR-induced activation of reconstituted RyR/Ca\(^{2+}\) release channels from BCASM. It was found that addition of cADPR into the cis solution significantly increased the \(N_P\) of these Ca\(^{2+}\) release channels. In the presence of anti-FKBP12 antibody, cADPR-induced activation of these channels was significantly blunted. Similarly, in the FK-506-treated bilayer, cADPR was without further effect on RyR/Ca\(^{2+}\) release channel activity. These results suggest that cADPR may dissociate FKBP12.6 from the RyRs and thereby activate these SR receptors. Because cADPR is produced within vascular SMCs, it may be an endogenous ligand for

\[\text{Fig. 7. A: effect of cADPR (10 \(\mu\)M), FK-506 (10 \(\mu\)M), and ryanodine (0.1 \(\mu\)M) on the activity of RyR/Ca\(^{2+}\) release channels of FKBP12.6-stripped SR membrane from coronary arterial smooth muscle. B: summarized data showing that the effect of cADPR, FK-506 and ryanodine on the \(N_P\) of reconstituted RyR/Ca\(^{2+}\) channel. \(*P < 0.05,\) significant difference from control; } n = 8–12.\]
FKBP12.6, dissociate this protein from the RyRs, and activate RyR/Ca\(^{2+}\) release channels. To further confirm the role of FKBP12.6 in cADPR-induced activation of RyR/Ca\(^{2+}\) release channels, FKBP12.6-stripped SR membranes prepared by prebinding of FK-506 and subsequent high-speed centrifugation were used to reconstitute the RyR/Ca\(^{2+}\) release channels on the lipid bilayer, and the effects of cADPR on the activity of RyR/Ca\(^{2+}\) release channels were examined. In this FKBP12.6-stripped SR membrane, both FK-506 and cADPR had no effect on the NP\(_O\) of RyR/Ca\(^{2+}\) release channels. This blockade of FK-506 or cADPR effect is associated with the removal of FKBP12.6 because ryanodine, a direct binding ligand of the RyRs, still activates these RyR/Ca\(^{2+}\) release channels. We also found that removal of FKBP12.6 from the SR membrane by cADPR completely abolished both FK-506- and cADPR-induced activation of RyR/Ca\(^{2+}\) release channels, but had no effect on ryanodine-induced activation of these channels. These results strongly indicate that cADPR does not work to release Ca\(^{2+}\) from the SR by direct binding to the RyRs and FKBP12.6 is the target of cADPR in coronary arterial SMCs. In this regard, a recent study using ligand-binding techniques also demonstrated that cADPR is able to bind to FKBP12.6 in islet microsomes. The present study further provides functional evidence that cADPR activates the RyRs to release Ca\(^{2+}\) through FKBP12.6.

In summary, the present demonstrated that FKBP12.6 is present in BCASM and cultured SMCs and that cADPR activated RyR/Ca\(^{2+}\) release channels through FKBP12.6 on the SR of BCASM. These results suggest that cADPR produces Ca\(^{2+}\) mobilization from the SR in BCASM by stimulation of FKBP12.6 dissociation from the RyRs.

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