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FK506 (Tacrolimus) Increases Halothane-induced Ca^{2+} Release from Skeletal Muscle Sarcoplasmic Reticulum

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Background: **FK506 binding protein is closely associated with the sarcoplasmic reticulum ryanodine receptor–channel and can modulate its function. The ryanodine receptor is stabilized by its association with FK506 binding protein. The immunosuppressant drugs FK506 (tacrolimus) and rapamycin can promote dissociation of FK506 binding protein from the ryanodine receptor 1 and by this mechanism increase sensitivity of ryanodine receptor 1 to agonists such as caffeine. Furthermore, it was shown recently that treatment of normal human skeletal muscle with FK506 and rapamycin increased halothane-induced contracture. The authors investigated the effect of the immunosuppressants FK506 and rapamycin on halothane-induced Ca^{2+} release in skeletal muscle sarcoplasmic reticulum vesicles.**

Methods: **Skeletal muscle terminal cisterns were isolated from New Zealand White rabbits. Ca^{2+} uptake and release was monitored in skeletal muscle sarcoplasmic reticulum vesicles using the fluo-3 fluorescent technique. Western Blot analysis of FK506 binding protein was performed using standard protocol.**

Results: **The authors observed that treatment of skeletal muscle sarcoplasmic reticulum vesicles with FK506 and rapamycin enhances halothane-induced Ca^{2+} release by about five times. Furthermore, the Ca^{2+} release induced by halothane in the presence of FK506 was inhibited by several antagonists of the ryanodine receptor, such as ruthenium red, spermine, and Mg^{2+}.**

Conclusion: **Dissociation of FK506 binding protein from its binding site in skeletal muscle sarcoplasmic reticulum vesicles can modulate halothane-induced Ca^{2+} release through the ryanodine receptor. Data are discussed in relation to the role of the FK506 binding protein in modulating the effect of halothane on the ryanodine receptor and the development of malignant hyperthermia phenotype. (Key words: Immunosuppression; malignant hypothermia, ryanodine channel.)**

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SKELETAL muscle contraction is triggered by Ca^{2+} release from the sarcoplasmic reticulum[1] and is mediated by the ryanodine receptor (RyR) channel. The majority of skeletal muscle RyRs consists of RyR type 1 (RyR1).[1,2] The receptor has been cloned, and its molecular weight as deduced from the primary amino acid sequence, is 565,000 $d^{1,2}$; native RyR1 is a homotetramer.[1,2] Recently, the function of RyR1 was shown to be regulated by several accessory proteins.[1–4] *Malignant hyperthermia* (MH) is a pharmacogenetic skeletal muscle disease that is characterized by sustained muscle contraction and abrupt increase in body temperature during general anesthesia.[5–7] Dysfunction of the skeletal muscle Ca^{2+} release system in response to volatile anesthetics, such as halothane, is a key point in the pathogenesis of MH.[5–7] Linkage of the MH phenotype with the *RyR1* gene has been described[5,6]*; however, mutations in RyR1* account for approximately 50% of the human cases of MH.[6] It is possible that modulatory proteins, such as FK506 binding protein (FKBP), that regulate the function of RyR also may be involved in the pathogenesis of a number of genetic variants of MH.

FK506 binding protein 12 is closely associated with the sarcoplasmic reticulum RyR[2–4] and can modulate its function.[2–4] If FKBP is bound to the RyR, its Ca^{2+} channel is stabilized in the closed position and is less likely to permit leakage of Ca^{2+} from sarcoplasmic reticulum.[2–4] The immunosuppressant drugs FK506 (tacrolimus) and rapamycin can promote dissociation of FKBP from the RyR1[2–4] and, by this mechanism, can increase sensitivity of the RyR1 to agonists such as caffeine.[2–4] Furthermore, it was shown recently that treatment of normal human skeletal muscle with FK506 and rapamycin increases halothane-induced contracture.[8] In fact, FK506- and rapamycin-treated human skeletal muscle behaves in a manner similar to MH-susceptible skeletal muscle.[8] No direct evidence of regulation of halothane-induced Ca^{2+} release by FK506 or rapamycin, however, has been presented to date. In view of these findings, we investigated

the effect of FK506 and rapamycin on halothane-induced Ca^{2+} release in skeletal muscle sarcoplasmic reticulum. We found that dissociation of FKBP from its binding site at the sarcoplasmic reticulum vesicles, induced by FK506 and rapamycin, can increase halothane-triggered Ca^{2+} release mediated through the RyR.

Materials and Methods

Isolation of Sarcoplasmic Reticulum Vesicles

These experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Mayo Clinic Institutional Animal Care Committee. Sarcoplasmic reticulum terminal cisternae vesicles (SRVs) were isolated from New Zealand White rabbit skeletal muscle, as described previously.[9] Predominant white muscle of the hind leg was separated from muscle that was pink and from connective tissue. Dissection was performed on a glass tray placed on packed ice. The muscle was ground and a 50-g portion was homogenized in 250 ml homogenizing media (0.3 [mscap]m sucrose, 5 mM imidazole–HCl [pH 7.4]), using a blender at maximum speed for 1 min. The SRVs were isolated by differential centrifugation and sucrose gradient, as described by Saito *et al.*[9] After isolation, the SRVs were suspended in homogenization medium, quick-frozen, and stored at −70°C. The Ca^{2+} loading and release properties of the SRV were not found to be compromised by storage at −70°C. After Ca^{2+} loading, the baseline Ca^{2+} levels were approximately 27.8 ± 6 mmol in three different preparations and were consistent from day to day.

Ca^{2+} Release Assay

Frozen sarcoplasmic reticulum was thawed in a 37°C water bath and diluted to 1.0 mg/ml in an intracellular media solution that contained 250 mM *N*-methyl glucamine, 250 mM potassium gluconate, 20 mM HEPES buffer (pH 7.2), 1 mM $MgCl_2$, and 20 mM potassium phosphate. For Ca^{2+}-release experiments, sarcoplasmic reticulum vesicles were loaded actively with Ca^{2+} with an adenosine triphosphate–regenerative system consisting of 2 U/ml creatine kinase, 4 mM phosphocreatine, and 1 mM adenosine triphosphate. Ca^{2+} uptake and release were monitored as described previously using 3-μM fluo-3.[10] Fluo-3 fluorescence was monitored at 490-nm excitation and 535-nm emission in a 250-μl cuvette at 37°C with a circulating water bath and continuously mixed with a magnetic stirring bar using a spectrofluorometer (F-2000; Hitachi, San Jose, CA).[10] We observed no changes in the properties of fluo-3 (emission, excitation, or response to exogenous Ca^{2+}) in the presence of all tested compounds. In addition, no significant photobleaching was observed. The addition of stock solutions of various substances did not exceed 1% of homogenate volume in the cuvette. Furthermore, in control experiments, FK506, rapamycin, or halothane treatments did not have any significant effect on the rate of Ca^{2+} uptake of SRVs measured in the presence of ruthenium red. In these control experiments, ruthenium red was used to promote inhibition of the RyRs and prevent interference of halothane, FK506, and rapamycin on the Ca^{2+} channel leaking pathway.

Western Blot Analysis of FK506 Binding Protein

Soluble FKBP supernatant was diluted to 1 mg/ml in extraction buffer that contained 0.3 M sucrose and 5 mM imidizole–HCl at a pH of 7.4. Samples were diluted at a concentration of 1:2 with sample buffer, and 20 μg was resolved in a 15% tris–HCl gel (Bio-Rad 161-1157) in tris–glycine–SDS (Bio-Rad 161-0732, Hercules, CA). Protein was transferred to nitrocellulose membrane (Bio-Rad 162-0145) in a minitransblot apparatus at 200 mA for 1 h in 25 mM tris, 192 mmM glycine, and 20% (vol/vol) methanol (pH 8.3) surrounded by ice. The blotted membrane was blocked overnight at 4°C in tris buffer solution with Tween, containing 0.1% Tween 80 and 5% (wt/vol) powdered milk, and probed 1 h with anti–FKBP-12 goat antiserum (Santa-Cruz Biotechnology, Santa-Cruz, CA) diluted at a concentration of 1:200 in blocking buffer. After repetitive washes with tris–buffer solution with Tween, the membrane was probed with secondary antibody (antigoat immunoglobulin G-horseradish peroxidase) (Piscataway, NJ) for 45 min and developed using Amershams ECL detection reagents. Films were analyzed on a Bio-Rad Fluor S Multi imager set to use radiographic film.

Materials

Fluo-3 was purchased from Molecular Probes (Eugene, OR), FK506 from Biomol (Plymouth Meeting, PA). All other reagents, the most pure available, were supplied by Sigma (St. Louis, MO). The reported experiments were repeated at least three to six times in three different preparations.

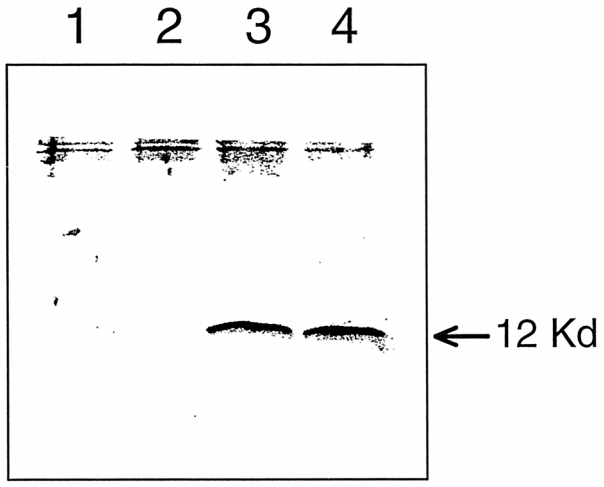

Fig. 1. Dissociation of FK506 binding protein from sarcoplasmic reticulum terminal cisternae vesicles (SRVs). One milligram SRVs per milliliter was incubated at 37°C for 40 min without or with 25 μM FK506. After incubation, SRVs were centrifuged for 30 min at 50,000*g* in a Beckman 50.4 Ti rotor (Palo Alto, CA) at 4°C, and the supernatant was collected. The figure shows the SDS-PAGE and western blot analysis for FK506 binding protein 12 in the collected supernatant of control (lanes 1 and 2) and FK506-treated (lanes 3 and 4) SRVs.

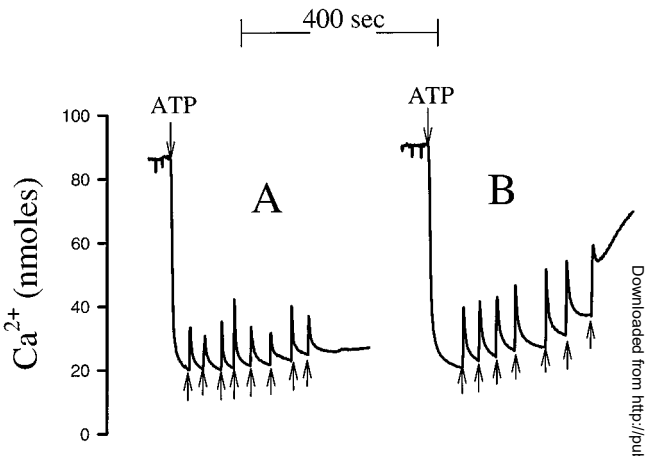

Fig. 2. Effect of FK506 on Ca^{2+} release induced by Ca^{2+}. One milligram sarcoplasmic reticulum terminal cisternae vesicles (SRVs) per milliliter was loaded actively with Ca^{2+} by addition of 1 mM adenosine triphosphate in the presence of an adenosine triphosphate–regenerative system. Before Ca^{2+} loading and challenging with sequential additions of Ca^{2+}, SRVs were incubated at 37°C for 40 min in the absence (*A*) or presence (*B*) of 25 μM FK506. After active Ca^{2+} uptake, the extravesicular concentration of Ca^{2+} was essentially the same for control and FK506-treated SRVs. The lower arrows indicate single additions of 10 nmol Ca^{2+}. The Ca^{2+} release induced by seven sequential additions of 10 nmol Ca^{2+} was observed in approximately 5 of 12 (40%) SRVs treated with FK506. In contrast, up to eight sequential additions of 10 mmol Ca^{2+} produced no Ca^{2+}-induced release of Ca^{2+} in control SRVs (0 of 14).

Results

Effect of FK506 on Caffeine- and Ca^{2+}-induced Ca^{2+} Release

Dissociation of FKBP from SRVs by FK506 was confirmed by western blot analysis using an antibody to FKBP. Figure 1 clearly indicates that treatment of SRVs with FK506 results in the appearance of FKBP in the supernatant fraction, indicating that, in our preparation, treatment with FK506 promotes dissociation of FKBP from SRVs. We also confirmed previous results that indicated FKBP modulates agonist-induced Ca^{2+} release through the RyR.[2–4] As previously described, treatment of SRVs with FK506 increases the rate of Ca^{2+} release induced by caffeine.[2–4] We observed that pretreatment of SRVs with 25 μM FK506 increased the Ca^{2+} release induced by 1 mM caffeine approximately fivefold (from 7.8 ± 2.7 to 35.5 ± 5.3 nmol Ca^{2+} per 2 min). Furthermore, we observed that SRVs treated with FK506 were more sensitive to Ca^{2+} release induced by Ca^{2+} itself. In SRVs not treated with FK506, serial additions of Ca^{2+} by itself were not sufficient to cause Ca^{2+} release. In approximately 40% of SRVs treated with FK506, however, serial additions of Ca^{2+} were able to promote Ca^{2+} release (fig. 2).

Effect of FK506 on Halothane-induced Ca^{2+} Release

We also tested the effect of FK506 on Ca^{2+} release induced by halothane. As shown in figure 3, treatment of SRVs with 0.4% (vol/vol) halothane produced a slow rate of Ca^{2+} release, which is in agreement with previously published observations.[7] In SRVs pretreated with FK506 the rate of Ca^{2+} release induced by halothane was increased approximately fivefold (fig. 3B). Furthermore, the binding of halothane appears to be increased by treatment of SRVs with FK506 (fig. 3). To determine whether Ca^{2+} release induced by halothane, in the presence of FK506, was mediated by activation of RyRs, we investigated the effect of several inhibitors of RyRs.[11] As previously described, Ca^{2+} release through RyRs can be inhibited by ruthenium red, Mg^{2+}, and spermine. As shown in figure 4, halothane-induced Ca^{2+} release in SRVs pretreated with FK506 was abolished if the vesicles were treated with the three inhibitors of RyR. These data indicate that the effect of halothane observed here is mediated through activation of RyR. It was reported previously that the immunosuppressant rapamycin, by promoting dissociation of FKBP from its binding site at

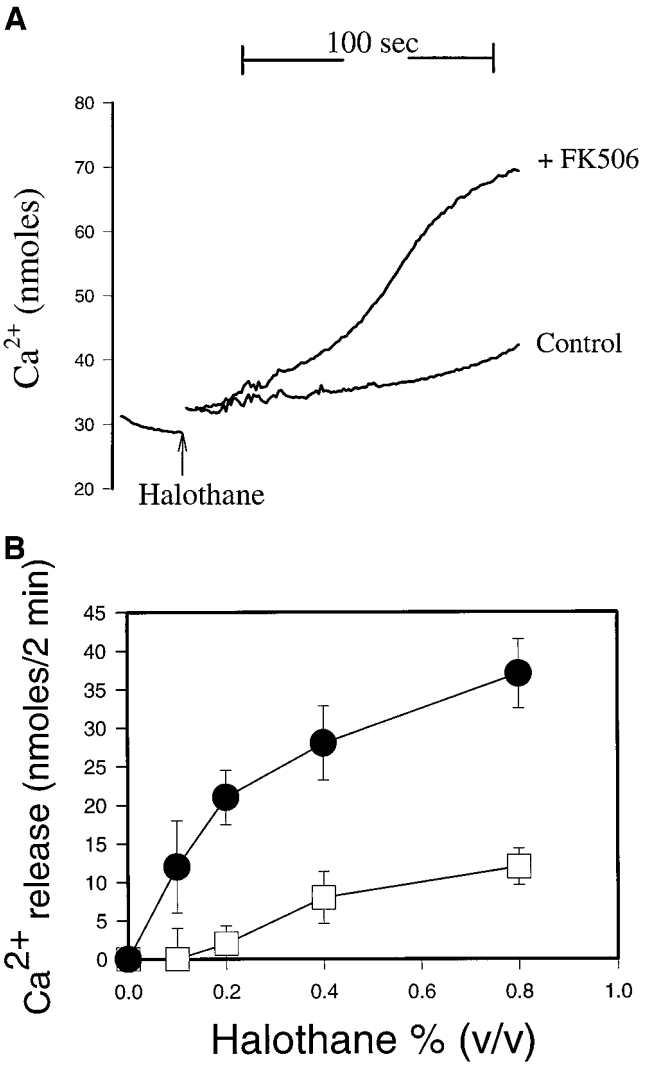

Fig. 3. Effect of FK506 on halothane-induced Ca^{2+} release. Ca^{2+} release in 1.0 mg sarcoplasmic reticulum terminal cisternae vesicles/ml was monitored using fluo-3 as a Ca^{2+} indicator. (*A*) Ca^{2+} release initiated by addition of 0.4% (vol/vol) halothane. (*B*) The rate of Ca^{2+} release induced by different concentrations of halothane in control vesicles (□) or pretreated with 25 μM FK506 (●) as described in figure 2. The data are representative of four independent experiments. The data represent the mean ± SD; n = 4 independent experiments.

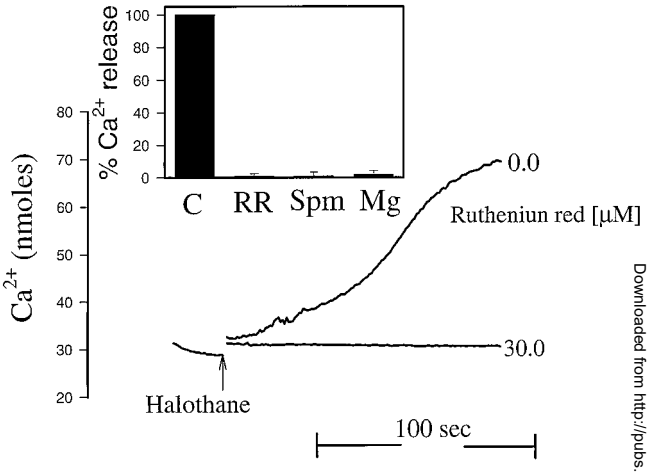

Fig. 4. Effect of inhibitors on halothane-induced Ca^{2+} release. Before Ca^{2+} loading and release, sarcoplasmic reticulum terminal cisternae vesicles were preincubated with 25 μM FK506, as described in figure 2. Furthermore, before the addition of 0.4% (vol/vol) halothane, Ca^{2+}-loaded vesicles were incubated for 1 min alone (control) or with 30 μM ruthenium red. (*Inset*) Effects of several inhibitors on 0.4% halothane-induced Ca^{2+} (taken as 100%) with no further addition (control; C) or incubated for 1 min with 30 μM ruthenium red (RR), 3 mM spermine (Spm); or 10 mM $MgCl_2$ (Mg) in Ca^{2+}-loading vesicles. The data represent the mean ± SD; n = 3 independent experiments.

Discussion

Treatment of SRVs with the immunosuppressants FK506 and rapamycin can increase halothane-induced Ca^{2+} release. We also observed that SRVs treated with FK506 are more sensitive to Ca^{2+}-induced release of Ca^{2+}. The results observed here probably are related to dissociation of FKBP from RyRs. As described previously, dissociation of FKBP from RyRs increases sensitivity of RyRs to agonists.[2–4] Also, Ca^{2+} release induced by halothane, as observed here, appears to be mediated by RyR because inhibitors of RyR, such as ruthenium red, spermine, and Mg^{2+}, were able to block the effect of halothane.

It was shown previously that treatment of normal skeletal muscle with FK506 and rapamycin can produce an MH-like phenotype, as observed in muscle contracture studies.[8] No direct evidence indicating modulation of Ca^{2+} release in response to halothane, however, was observed.[8] It is possible that *in vivo* dissociation of FKBP 12 from RyR1 may be an important component in the pathophysiology of MH. As discussed previously, in some instances, the cellular defect responsible for development of MH has not been determined.[5,6,8] Mutations in FKBP or RyR that can modify the interaction between

the SRV, also can potentiate Ca^{2+} release induced by caffeine through RyRs.[4] Finally, we observed that rapamycin also was able to increase halothane-induced Ca^{2+} release in SRVs from 7.8 ± 2.7 to 24.7 ± 5.6 nmol of Ca^{2+} per 2 min. Furthermore, pretreatment of SVRs with 20 μM cyclosporine for 40 min before Ca^{2+} loading had no effect on halothane-induced Ca^{2+} release.

these two proteins might result in MH phenotypes. No such mutations, however, have been described to date. Finally, it is possible that by modulating the RyR, not only in skeletal muscle, but also in cardiac and smooth muscle, the clinical use of these immunosuppressants may have an important influence on the anesthetic management of patients.

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