Effects of FK506 on \([\text{Ca}^{2+}]_{\text{i}}\) Differ in Mouse and Rabbit Ventricular Myocytes

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ABSTRACT

FK506 binding proteins (FKBPs 12 and 12.6) interact with ryanodine receptor (RyR) and modulate its functions. FK506 binds to and reverses effects of FKBP on RyR, thus increasing RyR sensitivity to Ca\(^{2+}\), decreasing RyR cooperativity, and increasing RyR open probability. FK506 would thus be expected to have an effect on excitation-contraction coupling, but which of these FK506 effects predominates and how the [Ca\(^{2+}\)] transient would be altered are difficult to predict. FK506 has been reported to increase the [Ca\(^{2+}\)] transient in rat myocytes, but effects in other species have not been described. We compared the effects of FK506 on [Ca\(^{2+}\)] transients, L-type Ca\(^{2+}\) channel and Na/Ca exchange currents, membrane potential, and sarcoplasmic reticulum (SR) Ca\(^{2+}\) content in adult mouse and rabbit ventricular myocytes (VM). FK506 (10 \(\mu\)M) increased the [Ca\(^{2+}\)] transient in mouse VM (656 \pm 116 to 945 \pm 144 nM, \(p < 0.001\)) but decreased the amplitude of [Ca\(^{2+}\)] transients in rabbit VM (627 \pm 61 to 401 \pm 37 nM, \(p < 0.001\)). Similar effects were observed with rapamycin. The effects of FK506 and rapamycin on [Ca\(^{2+}\)] transients in VM of both species were reversible upon washout. FK506 did not alter SR Ca\(^{2+}\) content in mouse VM (0.79 \pm 0.1 versus 0.78 \pm 0.1 pC/pF) but reduced the SR Ca\(^{2+}\) content in rabbit VM (0.43 \pm 0.05 versus 0.30 \pm 0.04 pC/pF, \(P < 0.05\)) [pC = the integral (a - s) of the caffeine-induced inward \(I_{\text{Na/Ca}}\) normalized by cell capacitance (pF)]. FK506 had no effects on membrane potential, \(I_{\text{Ca,L}}\) and outward \(I_{\text{Na/Ca}}\) in either mouse or rabbit VM. These results indicate that alteration of the functions of RyR by FK506-mediated dissociation of FKBP from RyR has different species-dependent effects on SR Ca\(^{2+}\) load and thus [Ca\(^{2+}\)] transients. This difference may result from the fact that [Na\(^+\)] is low in rabbit myocytes, allowing extrusion by Na\(^+\)/Ca\(^{2+}\) exchange of Ca\(^{2+}\) released by FK506-induced dissociation of FKBP12.6 from SR RyR.

FK506 is a potent immunosuppressant that has been used in the prophylaxis of allograft rejection in organ transplantation. The immunosuppressant actions of FK506 are mediated by its cytosolic receptors, FKBPs (FKBP12 or FKBP12.6) which are members of a family of the immunophilin proteins (Marks, 1996). The immunophilin proteins are highly conserved and widely distributed in virtually all cell types (Schreiber, 1991). The drug-immunophilin complex of FK506-FKBPs binds to calcineurin and inhibits its phosphatase activity resulting in the inhibition of T-lymphocyte activation.

Marks and colleagues (Jayaraman et al., 1992; Timerman et al., 1993; Brillantes et al., 1994; Marx et al., 1998) have demonstrated that FKBP12 is tightly associated with the ryanodine receptor (RyR) of skeletal muscle and modulates its functions by increasing RyR subunit cooperativity, decreasing open probability, and increasing RyR/Ca\(^{2+}\) release channel conductance. Another FK506 binding protein, FKBP12.6, is associated with the RyR of cardiac muscle sarcoplasmic reticulum (Sewell et al., 1994; Timerman et al., 1994; Lam et al., 1995; Kaftan et al., 1996). The actions of FKBP12.6 on cardiac RyR (RyR2) are similar to the actions of FKBP12 on skeletal RyR (RyR1). Whereas FK506 stimulates the binding of FKBPs to calcineurin, FK506 dissociates FKBPs from RyR. The dissociation of FKBPs from the RyR-FKBP complex by treatment with FK506 causes increased sensitivity of RyR to Ca\(^{2+}\), more frequent openings, and the appearance of subconductance states of the calcium release channel in isolated SR vesicles or the purified RyR reconstituted into planar lipid bilayers (Jayaraman et al., 1992; Brillantes et al., 1994; Lam et al., 1995; Kaftan et al., 1996). Thus, removal of FKBPs from the RyR-FKBP complex could cause the calcium release channels to become more sensitive to Ca\(^{2+}\), producing a positive inotropic effect; or “leaky”, causing a negative inotropic effect by depleting the SR of Ca\(^{2+}\).
Ca2+ (Marks, 1997). FK506 has been reported to increase the [Ca2+]i transient in rat cardiac myocytes (McCall et al., 1996; duBell et al., 1997; Xiao et al., 1997), but effects of FK506 on [Ca2+]i, transients in other species have not been described. Since there are substantial species-dependent variations in Ca2+-handling components (Bassani et al., 1994; Sham et al., 1995; Su et al., 1999a,b), it is possible that the effects of FK506 on [Ca2+]i, transients are species-dependent. Therefore, we compared the effects of FK506 on [Ca2+]i, transients in adult rabbit and mouse ventricular myocytes.

Materials and Methods

Dissociation of Myocytes. Adult rabbit and mouse ventricular myocytes were isolated as described previously (Su et al., 1999a). Animals were anesthetized with pentobarbital sodium (65 mg/kg i.v. for rabbit, i.p. for mouse), Collagenase D (Roche Diagnostics, Indianapolis, IN) was used for mouse myocyte isolation and type 1 collagenase (Worthington Biochemicals, Freehold, NJ) for rabbit myocyte isolation. All experiments using single myocytes were performed at 25°C.

Measurement of [Ca2+]i. The [Ca2+]i was measured as previously described (Yao et al., 1998). Fluo-3-loaded myocytes were illuminated by a 485-nm excitation light through an epifluorescence attachment (510-nm dichroic mirror; Omega Opticals Inc., Brattleboro, VT) and a x40 Fluor oil objective lens. The resulting fluorescence signals at 530 nm (DF30, Omega) were detected with a photomultiplier (SFX-2; Solamere Technology Group, Salt Lake City, UT). Fluo-3 fluorescence was transformed to [Ca2+]i, by a pseudoratio method (Cheng et al., 1995). [Ca2+]i = Kd(F/FP) / Kd / [Ca2+]i rest + 1 / (FP), where Kd is the dissociation constant for Fluo-3 (493 nM at 25°C), F the fluorescence intensity, Frest the intensity at rest, and [Ca2+]i rest the [Ca2+]i at rest and assumed to be 100 and 150 nM for mouse and rabbit myocytes, respectively, based on previous calibrated measurements of [Ca2+]i, in these species in our laboratory (Yao et al., 1998a,b).

Measurements of Na+/Ca2+ Exchange and L-type Ca2+ Currents and Membrane Potential. The exchange current (Iex), was measured by means of a whole-cell voltage clamp technique and a rapid solution switching technique (Su et al., 1999a). The voltage-clamped (~40 mV) cell was initially superfused in a switcher microstream in which Li+ replaced Na+ (from ASPET Journals on July 11, 2017)
tolic [Ca\(^{2+}\)]\textsubscript{i} in mouse ventricular myocytes (Fig. 1A). The alteration of the peak of the [Ca\(^{2+}\)]\textsubscript{i} transient is similar to that previously observed in rat myocytes (McCall et al., 1996; Xiao et al., 1997). However, FK506 decreased the diastolic and peak systolic [Ca\(^{2+}\)]\textsubscript{i} in rabbit ventricular myocytes (Fig. 1B). The effects of FK506 on [Ca\(^{2+}\)]\textsubscript{i} transients in ventricular myocytes of both species were noted within 3 to 4 min, were stable by 10 min of exposure, were completely reversible upon washout of FK506, and were reproducible after a second application of FK506 in the same myocyte (data not shown). As shown in Table 1, there was no effect of FK506 on resting membrane potential or on action potential characteristics under these experimental conditions. Although duBell et al. (2000) have reported that FK506 reduces action potential duration (APD) in mouse myocytes at 32°C with 5 Hz stimulation frequency, electrophysiologic effects of FK506 do not appear to account for the alterations in [Ca\(^{2+}\)]\textsubscript{i} we have observed under our experimental conditions.

**Effects of Cyclosporin A on [Ca\(^{2+}\)]\textsubscript{i} Transients.** Calcineurin is also one of the major protein phosphatases in cardiac muscle. To examine whether calcineurin inhibition is responsible for the different effects of FK506 on [Ca\(^{2+}\)]\textsubscript{i} transients in rabbit and mouse ventricular myocytes, we observed the effects of cyclosporin A (CsA) on [Ca\(^{2+}\)]\textsubscript{i} transients in both species. Although CsA and FK506 are structurally unrelated and bind to distinct immunophilins, both drugs are potent inhibitors of calcineurin (Liu et al., 1991). Figure 2 shows that CsA had no effects on [Ca\(^{2+}\)]\textsubscript{i} transients in either rabbit or mouse ventricular myocytes. These results indicate

**Fig. 2.** Effects of cyclosporin A on [Ca\(^{2+}\)]\textsubscript{i} transients in rabbit and mouse ventricular myocytes. Fluo-3-loaded myocytes were field-stimulated at 0.5 Hz and treated with cyclosporin A (10 μM) for 10 min. Cyclosporin A had no effect on [Ca\(^{2+}\)]\textsubscript{i} transients (shown by Fluo-3 fluorescence) in either rabbit (panel A) or mouse (panel B) ventricular myocytes. Similar results were observed in six rabbit myocytes and seven mouse myocytes.

**Fig. 3.** A, effects of rapamycin on [Ca\(^{2+}\)]\textsubscript{i} transients in mouse ventricular myocytes loaded with Fluo-3. Left panel: representative traces of the steady-state [Ca\(^{2+}\)]\textsubscript{i} transients in a field-stimulated (0.5 Hz) single mouse ventricular myocyte in the absence (control) or presence of 10 μM rapamycin. The myocytes were treated with 10 μM rapamycin for 10 min. Right panel: average values of diastolic and peak systolic [Ca\(^{2+}\)]\textsubscript{i} before and after exposure to rapamycin (means ± S.E.M., n = 12, *, p < 0.05, **, p < 0.01 compared with control). B, effects of rapamycin on [Ca\(^{2+}\)]\textsubscript{i} transients in rabbit ventricular myocytes loaded with Fluo-3. Left panel: representative traces of the steady-state [Ca\(^{2+}\)]\textsubscript{i} transients in a field-stimulated (0.5 Hz) single rabbit ventricular myocyte in the absence (control) or presence of 10 μM rapamycin. The myocytes were treated with 10 μM rapamycin for 10 min. Right panel: average values of diastolic and peak systolic [Ca\(^{2+}\)]\textsubscript{i} before and after exposure to rapamycin (means ± S.E.M., n = 5 cells, **, p < 0.01 compared with control).
that calcineurin inhibition is not responsible for the different effects of FK506 on \([\text{Ca}^{2+}]_{i}\) transients in rabbit and mouse ventricular myocytes.

**Effects of Rapamycin on \([\text{Ca}^{2+}]_{i}\) Transients.** Rapamycin is a new immunosuppressive drug that is a structural analog of FK506 and binds to the same immunophilins (FK-BPs) (Schreiber, 1991; Marks, 1996). The complexes of rapamycin and FK-BPs bind to calcineurin but do not change its phosphatase activity (Marks, 1996). Therefore, we also observed the effects of rapamycin on \([\text{Ca}^{2+}]_{i}\) transients in mouse and rabbit ventricular myocytes. As shown in Fig. 3, A and B, rapamycin enhanced the amplitude of \([\text{Ca}^{2+}]_{i}\) transients in mouse myocytes but reduced the amplitude of \([\text{Ca}^{2+}]_{i}\) transients in rabbit myocytes, effects that are similar to those of FK506.

**Effects of FK506 on L-type Calcium Currents and Na\(^+/\text{Ca}^{2+}\) Exchange Currents.** To examine the possible mechanisms for the different effects of FK506 on \([\text{Ca}^{2+}]_{i}\) transients in mouse and rabbit ventricular myocytes, we investigated the influence of FK506 on L-type calcium currents and Na\(^+/\text{Ca}^{2+}\) exchange currents in both mouse and rabbit ventricular myocytes. As shown in Fig. 4, A and

![Fig. 4. Effects of FK506 on L-type calcium current (\(I_{\text{Ca,L}}\)) in rabbit (A) and mouse (B) ventricular myocytes. \(I_{\text{Ca,L}}\) density was measured as described in Methods. FK506 (10 \(\mu\)M) did not alter the \(I_{\text{Ca,L}}\) densities and current-voltage relationships in either rabbit (n = 11–15, Panel A) or mouse (n = 8–13, Panel B) ventricular myocytes. Examples of current traces are shown in the inserts.](image-url)
B, FK506 did not alter the $I_{Ca,L}$ densities and current-voltage relationships in either rabbit or mouse ventricular myocytes. This is consistent with the findings of McCall et al. (1996) and Xiao et al. (1997) that FK506 did not affect the amplitude and kinetics of the L-type Ca$^{2+}$ channel currents in rat ventricular myocytes. Figure 5, A and B, shows a similar lack of effect of FK506 on outward $I_{Na/Ca}$ density (in rabbit myocyte) measured as described under Materials and Methods. The pipette [Na$^+$] was 10 mM. B, average results expressed as means ± S.E.M. FK506 (10 μM) did not alter the $I_{Na/Ca}$ densities in either rabbit ($n = 11–15$) or mouse ($n = 8–13$) ventricular myocytes. #, $p < 0.05$ compared with control mouse values.

**Fig. 5.** Effects of FK506 on Na/Ca exchange current ($I_{Na/Ca}$) in rabbit and mouse ventricular myocytes. A, example of effects on outward $I_{Na/Ca}$ density (in rabbit myocyte) measured as described under Materials and Methods. The pipette [Na$^+$] was 10 mM. B, average results expressed as means ± S.E.M. FK506 (10 μM) did not alter the $I_{Na/Ca}$ densities in either rabbit ($n = 11–15$) or mouse ($n = 8–13$) ventricular myocytes. #, $p < 0.05$ compared with control mouse values.

SR Ca$^{2+}$ Load. Because of the lack of effects of FK506 on L-type calcium currents and Na$^+$/Ca$^{2+}$ exchanger currents, we concluded that the different effects of FK506 on [Ca$^{2+}$]$_i$ transients in rabbit and mouse ventricular myocytes are not due to the variable effects of FK506 on calcium channel currents and Na$^+$/Ca$^{2+}$ exchanger currents. In addition to $I_{Ca,L}$ and $I_{Na/Ca}$, the amplitude of [Ca$^{2+}$]$_i$ transients is also very sensitive to the Ca$^{2+}$ loading status of SR. We therefore examined the SR Ca$^{2+}$ content in rabbit and mouse ventricular myocytes when they were exposed to FK506. Figure 6, A and B, shows that FK506 treatment did not alter the SR Ca$^{2+}$ content in mouse ventricular myocytes but reduced the SR Ca$^{2+}$ content in rabbit ventricular myocytes. It is also interesting to note that rabbit ventricular myocytes have a significantly smaller SR Ca$^{2+}$ content than mouse myocytes.

These results are consistent with the hypothesis that
FK506 causes depletion of SR Ca\(^{2+}\) in rabbit ventricular myocytes, possibly by causing opening of the RyR, with subsequent extrusion of Ca\(^{2+}\) from the cell by Na\(^+\)/Ca\(^{2+}\) exchange. To test this possibility more directly, we examined the effects of exposure to FK506 on resting [Ca\(^{2+}\)]\(_i\) in rabbit myocytes in the presence of 0 Na\(^+\)/0 Ca\(^{2+}\) solution to inhibit Ca\(^{2+}\) extrusion on the exchanger. Under these conditions, resting [Ca\(^{2+}\)]\(_i\) increased from 150 to 201 nM (p < 0.05, n = 8). This contrasts with the effect of FK506 to decrease resting [Ca\(^{2+}\)]\(_i\) in rabbit myocytes under control conditions (Fig. 1B) but resembles the effect seen in mouse myocytes (Fig. 1A), in which Ca\(^{2+}\) extrusion by Na\(^+\)/Ca\(^{2+}\) exchange is somewhat limited by a high resting [Na\(^+\)].

**Discussion**

In the present study, we have demonstrated that FK506 exerts different effects on [Ca\(^{2+}\)]\(_i\) in rabbit and mouse ventricular myocytes. FK506 increased diastolic [Ca\(^{2+}\)]\(_i\), and the amplitude of [Ca\(^{2+}\)]\(_i\) transients in mouse myocytes but decreased diastolic [Ca\(^{2+}\)]\(_i\), and the amplitude of [Ca\(^{2+}\)]\(_i\) transients in rabbit myocytes (Fig. 1). Our findings of FK506 effects in mouse are similar to those reported by McCall et al. (1996), Xiao et al. (1997), and duBell et al. (1997), in rat myocytes.

duBell et al. (1997, 1998) have shown that FK506 can inhibit repolarizing K\(^+\) currents and have suggested that the resulting prolongation of APD is the cause of the increased
transient in rat myocytes. However, our results in rabbit ventricular myocytes demonstrate that the effects of FK506 on [Ca\(^{2+}\)] transient are species-dependent and, under our experimental conditions, are not associated with marked alterations of APD. Furthermore, since FK506 reduces binding of FKBP12.6 to RyR, our finding of an FK506-induced decrease in the [Ca\(^{2+}\)] transient in rabbit myocytes is consistent with the recent report by Prestle et al. (2001) that overexpression of FKBP12.6 (and presumably increased FKBP12.6-RyR interaction) increases fractional shortening and SR Ca\(^{2+}\) content in rabbit myocytes. CsA and FK506 are structurally unrelated and bind to distinct immunophilins. Both drugs are potent inhibitors of calcineurin (Liu et al., 1991). Rapamycin is a new immunosuppressive drug that is a structural analog of FK506 and binds to the same immunophilins (FKBPs), but does not inhibit calcineurin (Marks, 1996). Our results show that a concentration of CsA (10 mM) that significantly inhibits calcineurin (duBell et al., 1998) had no effects on [Ca\(^{2+}\)] transient in either mouse or rabbit ventricular myocytes (Fig. 2), and that the effects of rapamycin on [Ca\(^{2+}\)] transient in mice and rabbit (Fig. 3) were similar to those of FK506. Thus, dissociation of FKBP from RyR, not calcineurin inhibition or inhibition of K\(^{+}\) currents, appears to be responsible for the effects of FK506 on [Ca\(^{2+}\)] transient in mouse and rabbit myocytes.

To examine factors responsible for the species-dependent difference in effects of FK506, we investigated whether this species-dependent difference is due to possible different effects of FK506 on L-type Ca\(^{2+}\) channel, Na/Ca exchanger, membrane potential, or SR Ca\(^{2+}\) load. FK506 did not alter the \(I_{\text{Ca_L}}\) densities and current-voltage relationships in either rabbit or mouse ventricular myocytes (Fig. 4). FK506 also had no effects on Na\(^{+}/\text{Ca}^{2+}\) exchange currents and membrane potential in either rabbit or mouse ventricular myocytes (Fig. 5; Table 1). From these results, we can exclude the possibility that the different effects of FK506 on [Ca\(^{2+}\)] transient in mouse and rabbit myocytes are due to the variable direct effects of FK506 on sarclemmal membrane currents.

As discussed, FK506-induced dissociation of FKBP12.6 from RyR2 in ventricular myocytes could produce an increased sensitivity of RyR to Ca\(^{2+}\), which could cause an increase in the [Ca\(^{2+}\)] transient, or decreased RyR cooperativity and increased open probability. Both these effects could cause a decrease in the [Ca\(^{2+}\)] transient, the latter by causing a "leak" of Ca\(^{2+}\) from the SR, which could deplete SR Ca\(^{2+}\) stores (Marks, 1997). Indeed Yano et al. (2000) have shown that defective interaction of FKBP12.6 with RyR in a canine model of pacing-induced heart failure leads to an abnormal SR Ca\(^{2+}\) leak, which may contribute to impaired function of the myocardium.

Previous work in our laboratory has shown that the Na\(^{+}\), in rabbit myocytes is 4 to 5 mM (Yao et al., 1998b), whereas in mouse and rat myocytes, Na\(^{+}\) is much higher, in the range of 12 to 15 mM (Yao et al., 1998). Since Na\(^{+}\) is a major determinant of the rate at which [Ca\(^{2+}\)] can be extruded by forward Na\(^{+}/\text{Ca}^{2+}\) exchange, we postulate that in mouse (and rat) myocytes, an increased leak of Ca\(^{2+}\) from SR induced by FK506 does not cause Ca\(^{2+}\) depletion. Since Ca\(^{2+}\) is not extruded from the cell by Na\(^{+}/\text{Ca}^{2+}\) exchange, the diastolic [Ca\(^{2+}\)] is high and the Ca\(^{2+}\) is taken back up into the SR by the Ca\(^{2+}\)-ATPase. In this situation, SR Ca\(^{2+}\) loading is maintained, and the effect of FK506 to increase sensitivity of RyR to Ca\(^{2+}\) could predominate, resulting in an increase in the [Ca\(^{2+}\)] transient. On the other hand, in rabbit myocytes with a low Na\(^{+}\), Ca\(^{2+}\) leaking from SR could be extruded by Na\(^{+}/\text{Ca}^{2+}\) exchange, resulting in a lower diastolic [Ca\(^{2+}\)] and sufficient SR Ca\(^{2+}\) depletion to cause a decrease in the [Ca\(^{2+}\)] transient despite an increase in sensitivity of RyR to Ca\(^{2+}\). The SR Ca\(^{2+}\) content measurements are consistent with this hypothesis, as is the effect of exposure to zero Na\(^{+}\), on the change in resting [Ca\(^{2+}\)] in rabbit myocytes induced by FK506. This idea is also consistent with the observations of JANIak et al. (1996). These investigators showed that exposure of guinea pig ventricular myocytes to 1.0 \(\mu\)M ryanodine, which induces an SR Ca\(^{2+}\) leak, caused a decrease in resting [Ca\(^{2+}\)], that was dependent on function of the Na\(^{+}/\text{Ca}^{2+}\) exchanger.

These findings may be relevant to the observation of Atkinson et al. (1995) who reported that hypertrophy and heart failure were induced in five pediatric patients undergoing immunosuppression with FK506 after organ transplantation. Reduced SR Ca\(^{2+}\) content and release induced by FK506 in young myocardium could potentially induce hypertrophy and failure (Meyer et al., 1998). Although the Na\(^{+}\), in human myocardium is not defined, rabbit myocardium is more similar to human myocardium than are rat and mouse. Thus, the effects of FK506 we note in rabbit myocytes may better predict effects in human myocardium. It is also clear that dissociation of FKBP from RyR induced by hyperphosphorylation of RyR was predicted to have a negative inotropic effect in rabbits, and perhaps also in larger mammals. This would be consistent with the proposal by Marx et al. (2000) that protein kinase A-induced hyperphosphorylation of RyR may contribute to the progression of heart failure.

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References


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