Frequency-dependent and proarrhythogenic effects of FK-506 in rat ventricular cells

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FK-506 (TACROLIMUS) is a potent macroclide widely used for primary immunosuppression or as a rescue therapy for refractory acute rejection after solid-organ transplantation. FK-506 binds with high affinity to a class of immunophilins, or protein receptors, referred to as FK-506 binding proteins (FKBPs) (15, 30). The FK-506/FKBP12.6 complex competitively binds to and inhibits the Ca2+- and calmodulin-dependent phosphatase calcineurin (16). This effect results in alterations of intracellular signaling mechanisms central to their immunosuppressive activity (29). The FK-506/FKBP12.6 complex also plays a role in the regulation of the ryanodine receptor (RyR2) and Ca2+-induced Ca2+ release in the heart (18). In addition, FK-506 has the ability to delay cardiac repolarization. This effect is manifested as QT prolongation, which provides a substrate for ventricular “torsades de pointe” (TdP) tachycardia. Several clinical cases of nearly fatal arrhythmias and TdP have been reported after the administration of FK-506 at high blood concentration (3, 13, 14, 25). Moreover, QT dispersion, a marker of risk for arrhythmia and sudden death, is elevated in kidney transplant recipients after they receive oral treatment (Prograf) (11). Quantitative relationships between the concentration of FK-506 and QT prolongation, evaluated in the guinea pig, have shown that delayed ventricular repolarization duration parallels whole blood levels of the drug (22, 23). All these electrical disturbances suggested alterations of ionic currents. Indeed, it has been shown that FK-506 has a high potential to evoke direct inhibition of outward K+ currents [namely, transient outward K+ current (Ito) and delayed rectifier K+ current (Ik)] and prolong the action potential (AP) (6–8). In the present study, we show that the effects of FK-506 are more complex and include inhibitions of L-type Ca2+ current (ICaL) and inward rectifier K+ current (Ikr). The effect on Ik has a prominent impact on the AP at low rates of stimulation, resulting in concomitant hyperpolarization of the resting membrane potential (RMP) and prolongation of the late repolarizing phase. High pacing rates dramatically enhance the effect on AP duration (APD) and, thereby, provide conditions for early afterdepolarizations (EADs). Frequency-dependent facilitation of ICaL is involved in this process.

METHODS

Animals. Six- to ten-week-old Wistar-Kyoto rats (Janvier; Le Genest-St-Isle, France) were used. This investigation conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and European directives (96/609/EEC).

QT interval measurement. A biocompatible ETA transmitter (Data Sciences; St. Paul, MN) was implanted intraperitoneally in 8-wk-old animals under slight anesthesia. In vivo Holter monitoring was performed by telemetry in untethered rats 1 wk later. FK-506 was injected at 3 mg/kg in the muscle of the left thigh 30 min after the beginning of ECG recordings. The QT interval was normalized into a rate-independent manner by telemetry in untethered rats 1 wk later. FK-506 was injected at 3 mg/kg in the muscle of the left thigh 30 min after the beginning of ECG recordings. The QT interval was normalized into a rate-independent manner.
QT/\sqrt{RR}$, where RR is the interval between two R waves. The blood concentration of FK-506 (in ng/ml) at its maximal effect on the ECG was assessed using a microparticle immunoenzymatic method (University Hospital Lapeyronie, Biochemistry Laboratory).

Cell isolation and cellular electrophysiology. Rats were heparinized and anaesthetized, and ventricular cells were enzymatically isolated as described before (10). Whole cell patch-clamp experiments were performed at room temperature (22–24°C) with an Axopatch 200A (Axon Instruments; Burlington, CA). Patch pipettes had resistance of 2 MΩ. Currents were normalized to the cell membrane capacitance (in pA/pF) measured as before (28). Series resistances were compensated before recordings.

To record APs, the pipette solution contained (in mmol/l) 130 KCl, 25 HEPES, 3 MgATP, 0.4 NaGTP, and 0.5 EGTA (unless otherwise noted); pH was adjusted 7.2 (with KOH). The bath solution contained (in mmol/l) 135 NaCl, 1 MgCl₂, 4 KCl, 11 glucose, 2 HEPES, and 1.8 CaCl₂; pH was adjusted to 7.4 (with NaOH). Li⁺ replaced Na⁺ in the Na⁺-free solution. APs were elicited by a 0.2-ms current injection suprathreshold intensity. During experiments, cells were postrested by trains of 30 stimuli at 0.1 or 3.3 Hz.

To record $I_{CaL}$, the bath solution contained (in mmol/l) 1.8 CaCl₂, 140 TEA-Cl, 2 MgCl₂, 10 glucose, and 10 HEPES; pH was adjusted to 7.4 (with TEAOH) (28). The pipette solution contained (in mmol/l) 130 NaCl, 10 HEPES, 10 EGTA, 0.4 NaGTP, and 3 MgATP; pH was adjusted to 7.2 (with CsOH). $I_{CaL}$ was elicited by step depolarizations (150 ms) from −80 to −10 mV at 0.1 or 3.3 Hz. $I_{CaL}$ amplitude was estimated as the difference between peak $I_{CaL}$ and the current level at the end of the pulse. The decay of $I_{CaL}$ was best fitted by the sum of two exponential components using the following formula: $I_{CaL} = A_{fast} \times \exp(-t/\tau_{fast}) + A_{slow} \times \exp(-t/\tau_{slow})$, where $A_{fast}$ and $A_{slow}$ are the current amplitudes of the fast and slow components, respectively; $t$ is time; and $\tau_{fast}$ and $\tau_{slow}$ are the related time constants of inactivation (8, 28).

K⁺ currents were recorded with the solution used for APs but with 10 μmol/l tetrodotoxin and 2 mmol/l Co²⁺ added extracellularly to block Na⁺ current and $I_{CaL}$, respectively. They were elicited from a holding potential of −80 mV by test depolarizations (1 s) varying between −50 and +70 mV at 0.1 Hz. The amplitude of $I_{K}$ was calculated as the difference between the peak of the fast $I_{K}$ and steady-state current at the end of depolarizing pulses. Amplitudes of $I_{K}$ were measured between the holding current and the steady-state current at the end of depolarizations. Quasi-steady-state current-voltage ($I$-$V$) relations of $I_{K}$ were also obtained by applying voltage ramps from −120 to +50 mV with a slope of 0.04 V/s.

Measurements of $Ca^{2+}$ transients. Cells were loaded for 30 min at room temperature with indo-1 AM (10 μmol/l, Molecular Probes). Cells were field stimulated at 0.2 and 1.0 Hz. Fluorescence was measured using a multiphotonic microscope (LSM510-LNO, Zeiss; Le Pecq, France; coupled to a 5-mW laser, TiSa Mira-Verdi, Coherent; Orsay, France). The fluorescent emission, at 405 and 480 nm after a femtosecond laser pulse excitation at 740 nm, were simultaneously recorded in the line-scan mode (1.9 ms/lane). Their ratio ($F_{405}/F_{480}$) was used to determine the qualitative variation of intracellular $Ca^{2+}$ concentration ([Ca²⁺]).

Analyses and statistics. ECGs were analyzed using automated software (Ecg Auto 1.4 rev2, Emka Technologies; Paris, France). Electrophysiological data acquisition and analyses were performed using pCLAMP (version 8.1, Axon Instruments). RMP, AP amplitude, and APDs at 20%, 50%, and 90% repolarization were measured. $Ca^{2+}$ transients were analyzed by subroutines of IDL software (RSI; Paris, France). $Ca^{2+}$ influx during activation of $Ca^{2+}$ channels was measured as the integrated surface area during the depolarization. All averaged data are presented as means ± SE. Statistics were performed using Student’s t-test (for paired or unpaired samples). Differences were considered significant with $P < 0.05$, highly significant with **$P < 0.01$, and extremely significant with ***$P < 0.001$.

**RESULTS**

Effect of FK-506 on the QT interval. Holter recordings were performed during 12 h overnight, corresponding to the active period of animals. Figure 1A shows a representative evolution of the ECG after the injection of 3 mg/kg FK-506 (see METHODS). Prolongation of the QT/QTc interval started 40 min after the injection and lasted for several hours (Fig. 1B). Similar effects were observed in four different animals. Measurements of the blood concentration of FK-506 during its maximal effect in other animals demonstrated the presence of circulating FK-506 at concentrations varying between 40 and 220 ng/ml (i.e., range 50–250 mmol/l). We concluded, therefore, that FK-506 could prolong the QTc interval also in the rat, as described previously in humans (see Introduction) and in an experimental model such as the guinea pig (22, 23). We did not detect arrhythmic events in these experiments.

Effect of FK-506 on single cells. Prolongation of the QTc interval is considered to be a hallmark of increased susceptibility to arrhythmias. At the cellular level, this is related to AP prolongation. Figure 2A,a shows the effect of FK-506 (25 mmol/l) on the AP of a cardiomyocyte recorded at 0.1 Hz. FK-506 induced both a shortening of the AP and a hyperpolarization. Steady state was achieved after 8 min. APD₂₀₀ was increased from 25.6 ± 3.8 to 35.5 ± 4.5 ms (Fig. 2A,b) with a mean prolongation of 20.9 ± 5.0%. RMP hyperpolarized from −80.1 ± 0.3 (control) to −83.9 ± 0.6 mV (**$P < 0.001$). Increasing the pacing rate from 0.1 to 3.3 Hz in the presence of FK-506 caused further prolongation of the AP, considerably exceeding that observed in control cells (Fig. 2B,b)}
2B,a and b). This effect was accompanied by a depolarization of the RMP to \(-78.8 \pm 0.4\) mV (**P < 0.001). Furthermore, high pacing rates triggered EADs (Fig. 2B,a) in 59\% of cells (n = 17). EADs developed at plateau potentials. All these effects were reversible upon washout of FK-506 for at least 10 min. They were also observed at 5 \(\mu\)mol/l, but they were less pronounced than at 25 \(\mu\)mol/l. Hence, most of the study was conducted using this latter concentration (see also Refs. 6–8).

**Effect of FK-506 on I_{CaL}.** An increase in the APD is expected to reflect an increase in an inward current or/and a decrease in an outward current. We (10) previously related frequency-dependent changes of rat ventricular AP duration to changes in I_{CaL}. We reasoned that I_{CaL} could be involved in the pacing-dependent effects and in the EADs induced by FK-506. This hypothesis was further supported by the fact that the Ca\(^{2+}\) channel blocker nifedipine (2 \(\mu\)mol/l) prevented the occurrence of EADs in all four cells tested. This is illustrated in Fig. 3A. Because nifedipine abolishes the whole cardiac excitation-contraction coupling process, i.e., Ca\(^{2+}\) entry via I_{CaL}, subsequent sarcoplasmic reticulum Ca\(^{2+}\) release, and Ca\(^{2+}\)-activated currents, we investigated the effect of FK-506 directly on I_{CaL} recorded in voltage-clamp conditions. Surprisingly, FK-
506 did not increase but rather decreased $I_{CaL}$ peak amplitude (Fig. 3B) with no change in the $I-V$ relationship (Fig. 3C) and $I_{CaL}$ decay kinetics (Fig. 3D,a) and with no use dependence. The decrease was unrelated to spontaneous rundown of $I_{CaL}$ peak amplitude because recovery was observed after washout of the drug (Fig. 3E). Detailed analysis showed that $A_{fast}$ (see methods), the $Ca^{2+}$-dependent fast inactivating component (1), was decreased with no change in the slow component $A_{slow}$ (Fig. 3C,b), which resulted in a decrease of the $[A_{fast}/(A_{fast} + A_{slow})]$ ratio. This decrease of $A_{fast}$ therefore, accounted for the decrease in global peak $I_{CaL}$ (Fig. 3D,b). Importantly, the frequency-dependent facilitation of $I_{CaL}$, which we routinely assessed by changing the rate from 0.1 to 3.3 Hz (10), persisted consistently (Fig. 3E). In the examples shown in Fig. 3E, $Ca^{2+}$ entry, measured as integrated surface area during depolarizations, was increased by 55% after the change from 0.1 to 3.3 Hz in control conditions (top). In the presence of FK-506 (Fig. 3E, middle), the increase was still important (40%) and exceeded largely the reduction induced by FK-506 (6% of $Ca^{2+}$ entry during the depolarization) that occurred mainly on current peak amplitude. Therefore, we concluded that the slowing of $I_{CaL}$ decay kinetics can clearly be a major factor in the triggering of EADs.

**Effect of FK-506 on $I_{to}$ and $I_K$.** FK-506 decreased both $I_{to}$ and $I_K$ (Fig. 4A,a). Steady-state inhibition of $I_K$ was obtained within <2 min, whereas steady-state inhibition of $I_{to}$ needed >10 min (Fig. 4A,b). FK-506 blocked $I_K$ nearly completely at all voltages, whereas $I_{to}$ was much less sensitive (Fig. 4B,a and b). Inhibition of $I_{to}$ was slightly enhanced by higher pacing rates (<10% of the remaining peak current, data not shown), as reported previously (7).

**Effect of FK-506 on $I_{K1}$.** FK-506 also blocked $I_{K1}$. We used 1 mmol/l $Ba^{2+}$ to block, and thereby isolate, $I_{K1}$ (39). Quasi-steady-state $I-V$ relations were studied by applying voltage ramps from $-120$ to $+50$ mV with a slope of 0.04 V/s. Figure 5A shows the traces obtained before and after perfusion of $Ba^{2+}$ and the subtracted current reflecting pure $Ba^{2+}$-sensitive $I_{K1} (I_{K1,Ba})$. Figure 5B summarizes the effect of FK-506 on $I_{K1,Ba}$. FK-506 reduced both the inward and outward components of $I_{K1}$, and a leftward shift was observed ($-4.8 \pm 0.9$ mV, $n = 6$). This shift may partly account for the RMP hyperpolarization induced by FK-506 (Fig. 2A,a). Block of the
outward current occurred at voltages corresponding to AP repolarization (between −40 mV and RMP).

Role of extracellular Na$^+$ and intracellular Ca$^{2+}$ in the genesis of EADs. In the presence of FK-506, high pacing rates triggered EADs in 59% of single cells dialyzed with 0.5 mmol/l EGTA in the patch pipette (Fig. 2B,a). Interestingly, EADs occurred in only 25% of cells (n = 12) dialyzed with 10 mmol/l EGTA and never in cells (n = 7) dialyzed with 20 mmol/l BAPTA (data not shown). Hence, the occurrence of EADs was related to [Ca$^{2+}$]$_i$. We further assessed this hypoth-

Fig. 4. FK-506 decreases transient outward K$^+$ current ($I_{to}$) and blocks delayed rectifier K$^+$ current ($I_K$). A: decreasing effect of FK-506 (25 μmol/l) on both $I_{to}$ and $I_K$ recorded at +50 mV from a HP of −80 mV at 0.1 Hz. A:a: original data traces with arrows showing how the transient $I_{to}$ and sustained $I_K$ were measured. A:b: time course of effects. B: $I-V$ curve of K$^+$ currents recorded between −50 and +70 mV (protocol in inset). B:a: original data traces. B:b: averaged data (n = 12). $I_{to}$ and $I_K$ were measured as shown in A,a.

Fig. 5. FK-506 decreases inward rectifier K$^+$ current ($I_{K1}$). A: quasi-steady-state $I-V$ relations of pure Ba$^{2+}$-sensitive $I_{K1}$ ($I_{K1-Ba}$) before (control, $I_{cont}$) and after the addition of 1 mmol/l Ba$^{2+}$ ($I_{Ba}$) in the bath; the difference current ($I_{cont} - I_{Ba}$) determines $I_{K1-Ba}$. B: effect of FK-506 (25 μmol/l) on $I_{K1-Ba}$ between −120 and 0 mV. Reversal potential ($E_{rev}$) was shifted from −83 to −90 mV in this cell (same as in A).
esis in intact cells loaded with indo-1 and stimulated at 1.0 Hz. The results revealed two populations of cells. In one-half of the cells, FK-506 had no effect on $[\text{Ca}^{2+}]_{i}$. In the other one-half, FK-506 generated doublets of $\text{Ca}^{2+}$ transients (Fig. 6A,a) that were associated with increased diastolic (Fig. 6A,b) and systolic $\text{Ca}^{2+}$ (Fig. 6A,c). Therefore, increased $[\text{Ca}^{2+}]_{i}$ caused by FK-506 may contribute to the genesis of EADs. Because $\text{Ca}^{2+}$ overload will favor $\text{Na}^{+}$/$\text{Ca}^{2+}$ exchanger activity and, thereby, induce inward $\text{Na}^{+}$/$\text{Ca}^{2+}$ exchanger current ($I_{\text{NaCa}}$), we replaced $\text{Na}^{+}$ by equimolar $\text{Li}^{+}$ to block $I_{\text{NaCa}}$. EADs were abolished in this condition, although rapid pacing-induced prolongation of the AP was still observed (Fig. 6B).

**AP simulation.** We assessed the mechanistic details of the pacing-dependent effect of FK-506 on the AP using a theoretical model of rat ventricular cells (see METHODS). To mimic the effect of FK-506, we decreased all conductances inhibited by FK-506 consistently with experimental observations: 25% for $I_{\text{CaL}}$, 20% for $I_{\text{to}}$, 80% for $I_{\text{K}}$, and 40% for $I_{\text{K1}}$. We also shifted the reversal potential of $I_{\text{K1}}$ ($\sim 3 \text{ mV}$). At a pacing rate of 0.1 Hz, simulations resulted in RMP hyperpolarization and prolongation of the repolarizing phase of the AP (Fig. 7A,a). These effects were quite consistent with the experimental effects of FK-506 (Fig. 2A,a). The shift of $I_{\text{K1}}$ was required to produce the hyperpolarization but not sufficient per se to induce the AP prolongation (data not shown). Simulation with only a decrease in $I_{\text{K1}}$ showed that block of this current by FK-506 accounts for most of the effect of the drug on the AP at low pacing rates (data not shown). An increase in the pacing rate from 0.1 to 3.3 Hz immediately promoted a depolarization of the RMP, a marked prolongation of the AP, and occurrence of EADs, reproducing all the effects of FK-506 (Fig. 7B,a and b). These effects were antagonized by suppressing facilitation of $I_{\text{CaL}}$ (i.e., by suppressing frequency-dependent slowing of the decay of $I_{\text{CaL}}$ in the model; Fig. 7C), which indicated that this current has a central role. However, the extent of block of $I_{\text{to}}$ is also potentially critical (Fig. 7D). Finally, when $I_{\text{NaCa}}$ was set to 0, EADs were prevented, although a substantial prolongation of the AP remained (Fig. 7E), as observed experimentally (Fig. 6B).

**DISCUSSION**

FK-506 is a promising immunosuppressant agent (27). FK-506 acts primarily as an inhibitor of the T cell response by preventing dephosphorylation and translocation of the nuclear factor of activated T cells. FK-506 binds to its cellular target,
the FKBPs, among which FKBP12.6 is tightly associated to the RyR2, and plays a role in the regulation of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in the heart (18). However, large QT dispersion along with prolonged QT interval and cases of ventricular TdP tachycardia have been reported in organ recipients with high FK-506 blood levels (3, 11, 13, 14, 25). Various effects of FK-506 have also been reported before in rat ventricular cells, including AP prolongation, increased intracellular Ca\(^{2+}\) transient, Ca\(^{2+}\) release from the sarcoplasmic reticulum, effects on electrogenic \(I_{\text{NaCa}}\), and direct effects on \(I_{\text{Na}}\) and \(I_{\text{K}}\) (6, 8, 20, 34, 38). The novel findings here are as follows: 1) FK-506 also inhibits \(I_{\text{CaL}}\) and \(I_{\text{K1}}\), and 2) its effects on the AP are highly frequency dependent. The effect on \(I_{\text{K1}}\) has major electrophysiological consequences such as RMP hyperpolarization and AP prolongation. Frequency-dependent facilitation of \(I_{\text{CaL}}\) plays a permissive role in the frequency-dependent prolongation of the AP and triggering of EADs.

**FK-506 inhibits \(I_{\text{CaL}}\).** Our detailed analysis shows that most of the moderate effect of FK-506 on \(I_{\text{CaL}}\) reflects preferential inhibition of the magnitude of the fast inactivating component \(A_{\text{fast}}\), suggesting a link with Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels (1, 5, 32, 33). Inactivation may result from local Ca\(^{2+}\) elevation from enhanced activity of the RyR2 by FK-506, thereby decreasing the number of channels available for opening. Previous reports have concluded that FK-506 has no effect on \(I_{\text{CaL}}\) peak amplitude (8, 20, 34). A possible explanation for this discrepancy with our results is that, in these studies, \(I_{\text{CaL}}\) was evoked from a depolarized holding potential (−40 mV). Depolarizing the holding potential to this level indeed decreases \(A_{\text{fast}}\) preferentially (1, 28), which might further prevent the effect of FK-506 on \(I_{\text{CaL}}\). Importantly, despite partial inhibition of peak \(I_{\text{CaL}}\) by FK-506, high pacing-induced facilitation of \(I_{\text{CaL}}\) persisted. Facilitation compensated largely the decreasing effect of FK-506 on current peak amplitude and provided the triggering event for EADs here. Large increases in Ca\(^{2+}\) entry were indeed still promoted by high rates in the presence of FK-506. Both the nifedipine effect (Fig. 3A) and numerical simulation (Fig. 7C) demonstrated that the high rate-induced increase in Ca\(^{2+}\) entry via Ca\(^{2+}\) channels provide the depolarization required for the genesis of EADs in the presence of FK-506.

**FK-506 inhibits \(I_{\text{K1}}\).** \(I_{\text{K1}}\) has a major role in maintaining the RMP near the high negative K\(^+\) equilibrium potential in ventricular cells (24). Moreover, between the RMP and −30 mV as during the AP plateau, the outward current will contribute to phase III repolarization (21). In contrast to its effects

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**Fig. 7. Computer simulations of FK-506 effects on rat ventricular APs.** A: APs at 0.1 Hz after reducing various conductances consistently with the experimental effects of FK-506 on ionic currents (see text). B: APs at 0.1 and 3.3 Hz before (a) and after (b) simulating the effect of FK-506. C: consequence of suppressing facilitation of \(I_{\text{CaL}}\) in the model. No EADs occurred at 3.3 Hz. D: effects of different degrees of block of \(I_{\text{Na}}\) (0, 17, and 23%) on EADs in the model. Block of \(I_{\text{Na}}\) favors the occurrence of EADs at 3.3 Hz. E: role of the Na\(^+\)/Ca\(^{2+}\) exchanger. When the Na\(^+\)/Ca\(^{2+}\) exchanger current was set to 0, pacing-dependent prolongation of AP occurred, but EADs were suppressed.
on $I_{\text{Na}}$ and $I_K$, the inhibition of $I_{K_1}$ by FK-506 was diminished in the presence of intracellular Ca$^{2+}$ buffers (unpublished results), which may be interpreted as reflecting Ca$^{2+}$-dependent block of the outward flow due to increased rectification of $I_{K_1}$ channels (19, 39). The consequences of this block were primarily a prolongation of the AP late phase and a hyperpolarization of the RMP due to a leftward shift in the $I_{K_1}$-$V$ curve. Block of the outward current is indeed expected to shift the K$^+$ Nernst potential from its resting value to more hyperpolarized potentials (4). From a physiological point of view, inhibition of $I_{K_1}$ might be, at least partially, involved in the prolongation of the QTc interval observed experimentally (Fig. 1). Selective block of $I_{K_1}$ by Ba$^{2+}$ has been associated with prolonged QTc in isolated rabbit hearts (37). Similarly, in guinea pig hearts probed with Kir2.1 overexpression and dominant negative suppression, the QTc interval is prolonged by $I_{K_1}$ suppression (21).

**FK-506-induced AP prolongation at low pacing rate.** FK-506 inhibits various currents involved in the repolarizing phase of the AP. These currents have two opposite roles: depolarization ($I_{\text{Cal}}$) and repolarization ($I_{\text{Na}}$, $I_{K_1}$, and $I_K$). In theory, inhibition of $I_{\text{Cal}}$ shortens the AP plateau, whereas inhibition of $I_{\text{Na}}$, $I_{K_1}$, and/or $I_K$ delays repolarization. At a low pacing rate (0.1 Hz), FK-506 has a stronger effect on the late phase of the AP. In addition, it hyperpolarizes the RMP. The opposite effects on $I_{\text{Na}}$ and $I_{\text{Cal}}$ are probably more or less counterbalancing each other during the early phase. Because $I_K$ has very little contribution in rat ventricular cells (4, 12), we suggest therefore that most of the prolongation of the AP at the low pacing rate reflects inhibition of $I_{K_1}$. This hypothesis was confirmed by computer simulation, which reproduced the $I_{K_1}$-mediated effect of FK-506 on both APD and RMP.

**High pacing rates and EADs: mechanisms.** APD is an important arrhythmogenic determinant. AP lengthening can result in the occurrence of EADs (36). In normal conditions, high pacing rates enhance APD in rat ventricular cells. This is a highly dynamic process that involves facilitation of $I_{\text{Cal}}$ and occurs through beat-to-beat adaptation (10, 35). We show here that the rapid pacing-induced increase in APD is enhanced by FK-506. Both the plateau phase and late repolarization are markedly prolonged, and EADs eventually develop at plateau potentials. Despite partial inhibition of $I_{\text{Cal}}$ by FK-506, high pacing-induced facilitation of $I_{\text{Cal}}$ persists and plays a key role (Fig. 7C). Although facilitation of $I_{\text{Cal}}$ is not enhanced per se, the slowing of the $I_{\text{Cal}}$ decay kinetics after pacing acceleration provides sufficient net depolarization during the AP plateau (Fig. 7C) and contributes to or possibly amplifies intracellular Ca$^{2+}$ overload to generate EADs. In addition, inhibition of $I_{\text{Na}}$ and $I_{K_1}$ by FK-506 contributes synergistically to this process in two different ways: 1) it slows AP repolarization within the “window” voltage range of Ca$^{2+}$ channels, thereby allowing reactivation and enhancement of $I_{\text{Cal}}$; and 2) the use-dependent block of $I_{\text{Na}}$ (see Refs. 7 and 8 and present results) causes further depolarization (Fig. 7D).

The EADs are also linked to extracellular Na$^+$ concentration and elevated [Ca$^{2+}$]. Manipulations of the Ca$^{2+}$ buffer in patch-clamped cells and direct measurements of [Ca$^{2+}$] in intact cells (Fig. 6A) both highlighted the contribution of [Ca$^{2+}$]. FK-506 increases both diastolic [Ca$^{2+}$] and the amplitude of [Ca$^{2+}$] transients, as reported by others in rat and mouse ventricular myocytes (8, 20, 34). Arrhythmia is associated with these increases (Fig. 6A). FK-506 has probably no direct effect on the Na$^+/Ca^{2+}$ exchanger (20, 34). However, our data suggest that forward $I_{\text{NaCa}}$ is important (Fig. 6B) in conjunction with Ca$^{2+}$ overload. Depolarization $I_{\text{NaCa}}$ is enhanced by AP prolongation, causing reactivation of $I_{\text{Cal}}$ and, thereby, EADs (36), which was confirmed by numerical simulations: setting $I_{\text{NaCa}}$ to zero prevents EADs (Fig. 7E). Therefore, we conclude that enhancement of $I_{\text{NaCa}}$ also contributes to acceleration-induced EADs (2) in the presence of FK-506. However, the fact that changes in Ca$^{2+}$ release function caused by FK-506 were not included in our computational modeling might limit the interpretation concerning the precise contribution of Ca$^{2+}$-dependent mechanisms.

In conclusion, this study provides explanations for the proarrhythmogenic potential of FK-506. Inhibition of repolarizing K$^+$ currents contributes to AP prolongation and disordered QT. The effect on $I_{K_1}$, responsible for prolonged terminal repolarization of the AP, is the main effect at low pacing rates, and it has probably a significant role in the QT prolongation. We demonstrated that a use-dependent increase in Ca$^{2+}$ entry, initiated by frequency-dependent facilitation of $I_{\text{Cal}}$, has a permissive effect for the occurrence of EADs with a contribution of electrogenic $I_{\text{NaCa}}$. These currents act in combination with elevated intracellular Ca$^{2+}$ induced by FK-506. This model of drug-induced long QT syndrome provides interesting insights in the cellular mechanisms involved in acceleration-induced EADs.

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