K⁺ currents responsible for repolarization in mouse ventricle and their modulation by FK-506 and rapamycin

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We recently reported the surprising finding that FK-506, an agent that acts primarily through interactions with modulatory proteins (14), decreased the magnitude of the rat ventricular K⁺ currents, I_{to} and I_{K} (10, 12). All other known effects of FK-506 result from interactions with intracellular receptors known as FK-506-binding proteins or FKBP12 (14). Of these, the best understood is FKBP12. In T lymphocytes, FK-506 targets FKBP12 to calcineurin, a Ca²⁺- and calmodulin-independent protein phosphatase. The resulting inhibition of calcineurin blocks the activation of T cells in response to an immune system challenge (18). FKBP12 is also an integral component of at least three membrane-bound protein complexes: 1) the striated muscle sarcoplasmic reticulum Ca²⁺-release channel (8, 24), 2) the inositol 1,4,5-trisphosphate (IP₃) receptor (9), and 3) the transforming growth factor-β (TGF-β) receptor (21, 27). In each of these systems, FK-506 binds with high affinity to FKBP12 and removes it from the protein complex.

FKBP12 also interacts with the immunosuppressant rapamycin. Like FK-506, rapamycin removes FKBP12 from the sarcoplasmic reticulum Ca²⁺-release channel (8), the IP₃ receptor (9), and the TGF-β receptor (21). However, the FKBP12-rapamycin complex does not target calcineurin but instead inhibits other enzymes, known in mammalian systems as RAFTs (rapamycin and FKBP12 targets) (23) or FRAPs (FKBP12-rapamycin-associated proteins) (31). This dichotomy between the actions of FK-506 and rapamycin was exploited in the present study to examine the role of FKBP12 in regulating cardiac K⁺ currents.

The studies were conducted in mouse ventricular myocytes to take advantage of the recently developed ability to manipulate the murine genome (4). However, there is variation from species to species in the K⁺ currents that define the action potential (6). In fact, there is uncertainty regarding the individual K⁺ currents present in adult mouse ventricle. Zhou et al. (32) recently reported that 50 µM 4-aminopyridine (4-AP) blocked a slowly inactivating K⁺ current, I_{slow}. However, Fiset et al. (13) reported that only a sustained current (I_{sust}) was blocked under the same conditions. Furthermore, although it is known that a portion of the mouse ventricular K⁺ current is sensitive to the K⁺ channel blocker tetraethylammonium (TEA), the specific current or currents have not yet been characterized (15, 32). Consequently, initial experiments were undertaken with the K⁺ channel blockers 4-AP and TEA, as well as a transgenic mouse model (7), to reveal and characterize the individual mouse ventricular K⁺ currents.

The results indicate that three pharmacologically and kinetically distinct currents, I_{to}, I_{slow}, and I_{K}, are the main components of the mouse ventricular K⁺ current. I_{to} and I_{slow} comprise the transient component of the current, whereas I_{slow} and I_{K} make up the sustained component. The results also show that the effects of FK-506 previously observed in rat ventricle (10, 12), action potential prolongation, inhibition of I_{K}, and prolonged recovery from inactivation of I_{to} are recapitated in the mouse. Finally, based on the remarkably similar effects of FK-506 and rapamycin on
K⁺ currents, we conclude that FKBP12 is an important modulator of the cardiac action potential through its interaction with either K⁺ channel proteins or associated regulatory components.

METHODS

Cardiac myocyte preparation. Hearts were removed from adult CD-1 mice after deep anesthesia was induced by 30 mg/kg ip pentobarbital sodium (Abbott Laboratories, North Chicago, IL). The aorta was quickly cannulated for Langen- dorff perfusion, and ventricular myocytes were isolated by a standard enzymatic technique using a HEPES-buffered solution containing (in mM) 130 NaCl, 5 KCl, 25 HEPES, 0.33 NaH₂PO₄, 1.0 MgCl₂, 20 d-glucose, 3.0 Na-pyruvate, and 1.0 lactate, pH to 7.4 with NaOH. The coronary arteries were perfused with this solution containing, in addition, 50 μM CaCl₂, 2.4 mg/ml collagenase (type II; Worthington Biochemical, Lakewood, NJ), and 0.08 mg/ml protease (type XIV). After 5 min of perfusion, the ventricles were cut down, minced, and then gently agitated for 4 min in digestion buffer containing 100 μM Ca²⁺ and 1.0% BSA. The cells were filtered through nylon mesh (300 μm) and then washed and resuspended, in succession, in enzyme-free buffer (1.0% BSA), containing 250 μM and 500 μM Ca²⁺. After the final wash the cells were resuspended at room temperature in HEPES-buffered DMEM with 10% fetal calf serum.

Measurement of action potentials and membrane K⁺ currents. The electrophysiological experiments were carried out on a Nikon Diaphot 300 inverted microscope mounted on a vibration isolation table (Technical Manufacturing, Peabody, MA). The voltage-clamp amplifier was an Axopatch 200A with a CV202A headstage (Axon Instruments, Foster City, CA), and all experiments were conducted under computer control (pClamp software, Axon Instruments). All of the electrophysiological recordings were made using patch-type microelectrodes (0.7–2.0 MΩ) pulled from borosilicate glass (TW150F, WPI, Sarasota, FL) using a programmable pipette puller (model P87, Sutter Instrument, Novato, CA). The pipette filling solution consisted of (in mM) 130 KCl, 15 HEPES, 1 MgCl₂, 5 MgATP, pH adjusted to 7.2 with KOH. Because very low intracellular Ca²⁺ concentration was required for the action potential experiments, 10 mM EGTA was included in the pipette filling solution, with KCl reduced to 115 mM to compensate for the additional KOH required to obtain a pH of 7.2. The extracellular buffer consisted of (in mM) 137 NaCl, 5 KCl, 20 HEPES, 15 d-glucose, 1.3 MgSO₄, 1 NaH₂PO₄, pH adjusted to 7.4 with KOH. Extracellular CaCl₂ concentration was 0.25 mM for all voltage-clamp experiments and 1.0 mM for all action potential experiments.

Whole cell K⁺ currents were recorded from mouse ventricular myocytes without including EGTA in the pipette filling solution. Na⁺ currents were blocked with 10 μM extracellular tetrodotoxin (Calbiochem, La Jolla, CA). Ca²⁺ currents were minimized with the combination of 0.5 μM nifedipine and low (0.25 mM) extracellular Ca²⁺ concentration. This regimen was used because inorganic Ca²⁺ channel blockers, such as Co²⁺ or Cd²⁺, change the voltage dependence of steady-state inactivation of the transient outward K⁺ current, Iₒ (2). It has been shown that nifedipine blocks certain K⁺ currents, in particular the current carried through Kv1.5 channels (30). In that study the peak amplitude of the current was not affected by 0.5 μM nifedipine. However, the drug did slightly accelerate the inactivation of the current at this concentration. Thus the use of nifedipine in the present study will not prevent the observation of Kv1.5 K⁺ current but may influence its apparent kinetics. These conditions (normal extracellular Na, reduced extracellular Ca, normal intra- and extracellular K, no intracellular Ca buffering) preserve, as much as possible, the normal physiological environment of the cell and the properties of the voltage- and time-dependent K⁺ currents, while also assuring adequate block of overlapping inward currents. All electrophysiological recordings were low-pass filtered at 2 kHz, digitized at 5 kHz, and stored on a computer for subsequent analysis. The magnitude of the transient portion of the current was calculated as the difference between the fast peak of outward current and the current remaining after 300 or 1,000 ms of voltage-damp depolarization. The magnitude of the sustained current was calculated as the difference between the holding current and the current remaining at either of these two times. All currents were normalized to cell capacitance to account for differences in cell size, and the average data are reported as current densities (pA/pF). Cell capacitance was calculated by integrating the uncompensated capacity transients elicited by 10 ms hyperpolarizing pulses from −70 to −80 mV. Action potentials were stimulated under current clamp by a small depolarizing current pulse through the pipette.

Unless otherwise indicated, the reagents used for all of the procedures described herein were obtained from Sigma Chemical (St. Louis, MO). Nifedipine, FK-506 (gift of Fujisawa), and rapamycin were added to the extracellular buffer from stock solutions (in ethanol); Pharmco Products, Brookfield CT) of 20, 25, and 10 mM, respectively. Control and experimental buffers always contained identical concentrations of ethanol and the total concentration never exceeded 0.1%. All experiments were carried out at 32°C within 8 h of cell isolation. All experimental values are expressed as the means ± SE, with statistical significance (P < 0.05) assessed using Student’s paired t-test.

RESULTS

Because there is uncertainty regarding the K⁺ currents responsible for repolarization in mouse ventricle, the initial experiments examined the effects of the K⁺ channel blockers 4-AP and TEA on the waveform of the mouse ventricular action potential. Under control conditions (32°C, 5-Hz stimulation) the action potential duration (APD) is extremely short (APD50 = 6.52 ± 0.60 ms, APD90 = 33.9 ± 6.66 ms; n = 11). Both of the K⁺ channel blockers markedly increased the APD without changing either resting potential or overshoot (Fig. 1). In 50 μM 4-AP, APD50 and APD90 were increased from 7.1 ± 1.2 to 15.9 ± 4.4 ms and 40.0 ± 4.0 to 89.9 ± 13.7 ms, respectively (n = 4, P < 0.05). TEA (20 mM) also prolonged the average APD50 and APD90, from 7.3 ± 0.9 to 26.9 ± 8.1 ms and 40.3 ± 3.5 to 129.5 ± 14.1 ms, respectively (n = 4, P < 0.05). Note that the effects of each blocker were manifest within 1 ms of the peak of the action potential (Fig. 1, insets). For all action potential experiments, changes in intracellular Ca²⁺ activity were minimized by including 10 mM EGTA in the pipette filling solution. This assured that any increases in APD were attributable to direct effects on membrane currents and not to the secondary effects of altered intracellular Ca²⁺ concentration. These data underscore the critical role of K⁺ currents in determining the mouse ventricular action potential waveform.

Subsequent experiments were designed to characterize the composite mouse ventricular K⁺ current. The experimental conditions preserved the normal physi-
ological properties of each individual K⁺ current while at the same time minimizing contamination by other overlapping voltage- and time-dependent currents, as described in METHODS (see Ref. 10). The basic properties of the composite mouse ventricular K⁺ current measured under these conditions are presented in Fig. 2. Figure 2A shows a representative family of currents recorded during a protocol that defines the current-voltage relationship of the composite current. There is a large, rapid peak of early outward current followed by what appears to be a biphasic decay that approaches, but does not reach, a steady state by the end of the 1,000-ms pulse. In fact, this decay phase was well fit as the sum of two exponentials. For example, at +60 mV the time constant of decay was 28 ± 1.0 ms for the fast component and 427 ± 16 ms for the slow component (n = 21). Both fast and slow components of decay were independent of membrane potential between 0 and +60 mV (not shown).

In our previous studies of rat ventricular myocytes (10, 12), the magnitude of the transient component of the current was reported as the difference between the early peak of outward current and the steady-state current remaining at the end of a 300-ms voltage-clamp pulse, because there was little change in the magnitude of the sustained current beyond this point. However, it is clear that the mouse ventricular K⁺ currents continue to inactivate beyond 300 ms (Fig. 2A, first arrow). Figure 2B shows the average current-voltage relationships of the transient and sustained components of the current, measured at both 300 and 1,000 ms. The magnitude of the transient component is calculated with respect to the sustained current present at either 300 or 1,000 ms. Thus because of the slow inactivation of the composite current, there was an apparent increase in the magnitude of the transient component as depolarization duration increased. For example, at +60 mV the calculated density of the transient component is 65 ± 5.4 pA/pF at 300 ms of depolarization and 76 ± 5.9 pA/pF at 1,000 ms (P < 0.01). For the same reason, the density of the sustained component at +60 mV, which is calculated with respect to the holding current, decreased from 29.7 ± 1.6 to 17.4 ± 0.8 pA/pF between 300 and 1,000 ms.

Subsequent experiments defined the voltage dependence of steady-state inactivation of both components (Fig. 2, C and D). The magnitudes of both the transient and sustained components decrease as the cell is depolarized (Fig. 2C). To determine the voltage dependence of this effect, the densities of both current components at each holding potential were normalized to those recorded from −100 mV. The resulting inactivation curves are displayed in Fig. 2D. The curve describing the voltage dependence of steady-state inactivation of the transient component is complex. Inactivation begins at approximately −70 mV and is not complete until approximately −10 mV. In addition, the curve appears to deviate from a sigmoid relationship at approximately −50 mV. The inactivation curve of the sustained component is also complex. There are two distinct phases of inactivation separated by a region, between −80 and −50 mV, over which there is very little inactivation. In addition, the sustained current does not completely inactivate, with 20% of the total outward current remaining at −2.5 mV.

The complexity of these inactivation curves suggests that both the transient and sustained components of the mouse ventricular K⁺ current are composed of contributions from more than one K⁺ current. This hypothesis was tested by examining the effects of the K⁺-channel blockers TEA and 4-AP on each component of the composite mouse ventricular K⁺ current. Others have shown that TEA blocks the delayed rectifier K⁺ current, I_K, in rat ventricle without affecting I_to (3). As shown in Fig. 3A and Table 1, TEA (20 mM) had no effect on the magnitude of the transient component of the current. However, it markedly decreased the magnitude of the sustained component of the current at both 300 and 1,000 ms (Table 1). The TEA-sensitive difference current shown in Fig. 3A explains these results. There is fast activation to a plateau but only very slow inactivation during the voltage-clamp pulse. For this
reason, the application of TEA affects only the sustained component of the mouse ventricular $K^+$ current.

The effects of a low dose of 4-AP (50 µM) on both components of the composite current are shown in Fig. 3B. This concentration of 4-AP has been reported by others to spare $I_{\text{slow}}$ in mouse ventricles (13), while blocking a $K^+$ current referred to as $I_{\text{trans}}$ (13, 32). As shown in Fig. 3B, the magnitudes of both the transient and sustained components of the composite mouse ventricular $K^+$ current were significantly decreased by 50 µM 4-AP when measured from 300 ms of depolarization (see Table 1). The difference current shown at the bottom of Fig. 3B explains these results. The 4-AP-sensitive current activates rapidly to a peak and then inactivates to about 50% of the peak within 300 ms. When examined with respect to a 1,000-ms depolarization, 50 µM 4-AP still decreases the transient component of the current. However, it has only a small effect on the sustained component at 1,000 ms. This indicates that inactivation of the 50 µM 4-AP-sensitive current (Fig. 3B) is nearly complete by the end of a 1,000-ms depolarization. These results show that $I_{\text{slow}}$ contributes to both components of the composite mouse ventricular $K^+$ current. However, because of its slow inactivation, the extent of its contribution to the sustained component decreases as depolarization duration increases.

Although the results indicate that $I_{\text{slow}}$ contributes to the transient component of the mouse ventricular $K^+$ current, they also reveal that most of the transient component is insensitive to the low concentration of 4-AP. For example, at +60 mV, 50 µM 4-AP decreased the magnitude of the transient component by only 15.3% (Table 1). The nature of this residual, low 4-AP-insensitive current is shown in Fig. 4. In the presence of 50 µM 4-AP and 20 mM TEA, the remaining current rises very rapidly to a peak and then decays within 50 ms to a plateau. Application of 4 mM 4-AP (Fig. 4A) almost completely blocked the remaining transient component of the current, a response ex-
pected of the cardiac transient outward current, $I_{to}$ (3). This conclusion is also supported by the properties of the difference current shown in Fig. 4B. This current, sensitive only to high concentrations of 4-AP, rises rapidly to a peak within 3 ms and decays rapidly to a plateau within 50 ms. The time course of this current is consistent with identification as $I_{to}$, although $I_{to}$ is generally thought to inactivate completely (3). These results indicate that $I_{to}$ comprises most of the transient component of the mouse ventricular $K_1$ current. Because the transient component of the current includes contributions from both $I_{to}$ and $I_{slow}$ (Fig. 3), it seemed likely that the complex steady-state inactivation curve seen in Fig. 2B could be explained by the overlapping inactivation of these two currents. As expected, TEA was without effect on the inactivation of the transient component (not shown). However, 50 µM 4-AP had a marked effect on the inactivation of the transient component of the current (Fig. 5A). 4-AP shifted the curve in the negative direction and narrowed the voltage range over which inactivation occurred. Furthermore, the deflection seen in the control curve was eliminated, resulting in a curve more characteristic of a single current. Based on these results, inhibition of $I_{to}$ should shift the inactivation curve of the transient component in the positive direction. Clearly, this cannot be accomplished with millimolar 4-AP without blocking $I_{slow}$ as well. However, we capitalized on the recent development of transgenic mice expressing a dominant-negative mutant of the Kv4.2

Table 1. Effects of 50 µM 4-AP and 20 mM TEA on magnitude of transient and sustained components of mouse ventricular $K_1^+$ current at +60 mV

<table>
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<tr>
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<td></td>
<td>300 ms</td>
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<td>Control Drug</td>
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<tr>
<td>50 µM 4A-P</td>
<td>66.1 ± 4.5</td>
<td>56.0 ± 3.2*</td>
<td>29.7 ± 1.2</td>
<td>18.2 ± 1.2*</td>
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<tr>
<td>20 mM TEA</td>
<td>55.5 ± 0.5</td>
<td>61.4 ± 7.8</td>
<td>29.9 ± 4.2</td>
<td>10.5 ± 4.2*</td>
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Values are means ± SE in pA/pF. 4-AP, 4-aminopyridine; TEA, tetraethylammonium. *Statistically significant difference from control.
As shown in Fig. 5A, the steady-state inactivation curve of the transient component of the current in these cells is indeed shifted to the right of control. These results are consistent with the hypothesis that the complex inactivation exhibited by the transient component of the current reflects the overlapping inactivation of only I_{slow} and I_{to}.

Analogous experiments were done using the same K+ channel blockers to test the hypothesis that the complex inactivation of the sustained component results simply from the individual voltage-dependent inactivation properties of I_{slow} and I_{K} (Fig. 5, B and C). Recall that inactivation of the sustained component occurred over two distinct voltage ranges, with an intervening plateau over which there was no inactivation (Fig. 2D). The contribution of I_{slow} to the inactivation curve is revealed when 20 mM TEA is added to block I_{K} (Fig. 5B). Note the similarity between this TEA-insensitive curve (Fig. 5B) and the 4-AP-sensitive “difference” curve in Fig. 5C. The I_{slow} inactivation curve has a relatively simple voltage dependence, with little inactivation between −100 and −50 mV, and then inactivation to a plateau at about −20 mV. Thus it would appear that I_{slow} is responsible for the more positive of the two inactivating regions. However, when 50 µM 4-AP is used to block I_{slow}, the 4-AP-insensitive inactivation curve retains the complex character of the control curve (Fig. 5C). That the 4-AP-insensitive inactivation curve represents I_{K} is supported by the similarity between it and the TEA-sensitive “difference” curve (Fig. 5B). Thus both I_{K} and I_{slow} contribute to the inactivation that occurs between −50 and −20 mV. Furthermore, the fact that the TEA-sensitive current inactivates over two distinct regions suggests that I_{K} may contain contributions from two distinct TEA-sensitive channels.

We previously showed that the immunosuppressant FK-506 prolonged the rat ventricular action potential by inhibiting the K+ currents I_{to} and I_{K}. However, because of the large diversity of K+ channels (6), the question remained as to whether the mouse ventricular action potential is also modulated by immunosuppressants. Figure 6 shows that 5 µM FK-506 does indeed prolong the mouse ventricular action potential. After 2 min of exposure, the APD_{50} had increased by 22.6% (n = 3) from 4.73 ± 0.03 to 5.8 ± 0.2 ms (P = 0.03) and the APD_{90} had increased by 66.7% from 17.1 ± 0.32 to 28.5 ± 2.4 ms (P = 0.046).

In the next series of experiments, the effects of FK-506 on mouse ventricular K+ currents were examined in detail (Fig. 7). We examined whether the FK-506-sensitive difference current exhibits only slight inactivation over the course of the 300-ms pulse (Fig. 7A). Average data (Fig. 7B) reveal that 5 µM FK-506 has no effect on the magnitude or voltage dependence of the transient component of the K+ current. However, the magnitude of the sustained component is significantly decreased (i.e., by 48% at +60 mV) at all potentials positive to −30 mV (P < 0.05). As in the rat (10), FK-506 did not affect the voltage dependence of steady-state inactivation of the transient component of the current (data not shown).

The other effect of FK-506 observed in rat ventricle was prolongation of the recovery from inactivation of I_{to}...
This was difficult to address directly in the mouse because the transient component of the current consists of both $I_{\text{to}}$ and $I_{\text{slow}}$. However, Fig. 7E shows that the recovery from inactivation of the transient component of the mouse ventricular $K^+$ current is prolonged by FK-506. These experiments were conducted by applying pairs of 200-ms pulses with the interval between the pulses ranging from 20 to 200 ms. The magnitude of the current elicited by the conditioning pulses ($I_0$), i.e., the fully recovered current, was not affected by 5 µM FK-506, with an average density of 58.1 ± 3.3 pA/pF in control and 54.6 ± 2.8 pA/pF in FK-506 ($P = 0.426$). However, at 20 ms the amount of recovery, calculated as $I_{\text{test}}/I_0$, decreased from 0.29 ± 0.01 to 0.18 ± 0.02 ($P = 0.012$), and at 200 ms the amount of recovery decreased from 0.72 ± 0.06 to 0.53 ± 0.07 ($P = 0.005$) (Fig. 7F). To better isolate the effect of FK-506 on mouse ventricular $I_{\text{to}}$, we also measured its effects on the recovery from inactivation of the transient component in the presence of 50 µM 4-AP. Under these conditions the transient component should consist almost entirely of $I_{\text{to}}$. As under control conditions, FK-506 did not decrease the magnitude of $I_{\text{to}}$. However, in the presence of 50 µM 4-AP, FK-506 still decreased the extent of recovery at all times between 60 and 200 ms ($P < 0.05$, $n = 3$). For example, at 200 ms the value was decreased from 0.89 ± 0.05 to 0.72 ± 0.03. These data indicate that the effects of FK-506 on mouse ventricular $K^+$ currents are the same as those in rat ventricle: voltage- and time-independent inhibition of $I_K$ and prolongation of recovery from inactivation of $I_{\text{to}}$.

An important question arises concerning the mechanisms through which FK-506 modulates these repolarizing $K^+$ currents. All of the previously known cellular effects of FK-506 involve interactions with cognate binding proteins (14), or FKBP12, which is highly expressed in heart (5, 25). Figure 8 depicts the two major pathways through which FK-506/ FKBP12 is known to act. As depicted schematically in Fig. 8A, FK-506 can remove or “strip-away” FKBP12 from integral membrane proteins (5, 8, 9, 21, 24). Alternatively, as depicted in Fig. 8B, FK-506 binds to
FKBP12, thereby targeting this complex to modulate the function of other cellular targets (18).

As also depicted, a related immunosuppressant, rapamycin, should be a valuable probe to distinguish between these two possible signaling mechanisms (1, 8, 28). Thus if FK-506 modulates \( K^+ \) currents by removing FKBP12 from the \( K^+ \) channel or an associated regulatory protein, then the effects of rapamycin on \( K^+ \) currents should converge with those of FK-506 (Fig. 8A). Alternatively, if FK-506 acts through the targeting model, then the effects of FK-506 and rapamycin should diverge (Fig. 8B). The results of the experiments done to test this idea are shown in Fig. 9. Like FK-506, 5 \( \mu M \) rapamycin decreased only the magnitude of the sustained component of the current recorded during current-voltage protocols (Fig. 9A) without decreasing the magnitude of the transient component. Importantly, the rapamycin-sensitive difference current (Fig. 9A) is very similar to the FK-506-sensitive difference current (Fig. 7A). Furthermore, 5 \( \mu M \) rapamycin also prolonged the recovery from inactivation of the transient component of the current (Fig. 9B). The plots of average \( I_{\text{slow}}/I_{\text{to}} \) (\( n = 5 \)) reveal inhibition comparable to that produced by 5 \( \mu M \) FK-506. Recovery at 20 ms decreased from 0.40 \( \pm \) 0.06% to 0.29 \( \pm \) 0.03% (\( P = 0.036 \)) and at 200 ms from 0.82 \( \pm \) 0.05% to 0.63 \( \pm \) 0.04% (\( P = 0.013 \)). Thus the effects of rapamycin on the mouse ventricular \( K^+ \) current are virtually identical to the effects of FK-506.

**DISCUSSION**

In the present study we have used pharmacological techniques as well as a genetically engineered mouse (7) to examine the idea that the immunophilin FKBP12 modulates mouse ventricular \( K^+ \) currents and thus the cardiac action potential. In the process, we have resolved the three time- and voltage-dependent \( K^+ \) currents from the composite mouse ventricular \( K^+ \) current, showing that both \( I_{\text{to}} \) and \( I_{\text{slow}} \) contribute to the transient component of the current, whereas \( I_{\text{slow}} \) and \( I_K \) comprise the sustained component. We also demonstrate that the previously observed effects of FK-506 on rat ventricular \( I_{\text{to}} \) and \( I_K \) (10) are recapitulated in the mouse. Finally, based on the remarkably similar effects of both FK-506 and rapamycin, we provide evidence that FKBP12 is an important regulatory component in the control of at least two cardiac \( K^+ \) channels.

Characterization of the composite mouse ventricular \( K^+ \) current. There have been several recent studies describing individual components of the mouse ventricular \( K^+ \) current (7, 15, 19, 26, 32) and a very recent study reporting four kinetically distinct mouse ventricular \( K^+ \) currents (29). However, none of these has been carried out under nearly physiological conditions. The experimental strategy employed in the present study focused on accurately measuring each individual current in its physiological setting, while at the same time minimizing other overlapping time- and voltage-dependent currents. This approach included the use of near physiological temperature and minimal intracellular \( Ca^{2+} \) buffering. Nifedipine was used as a \( Ca^{2+} \) channel blocker, rather than \( Co^{2+} \) or \( Cd^{2+} \), which evoke marked shifts in the voltage dependence of inactivation of \( I_{\text{to}} \) (2). Nifedipine can block current through the Kv1.5 channel (30), the channel identified by others as responsible for \( I_{\text{slow}} \) in mouse (13, 32). However, at the concentration used in the present study, nifedipine only accelerates inactivation of the Kv1.5 current without affecting its peak magnitude or voltage dependence (30). Thus the selected conditions represent a compromise that concedes minor effects on \( I_{\text{slow}} \) but preserves the normal properties of \( I_{\text{to}} \).

Xu et al. (29) have recently reported that the composite mouse ventricular \( K^+ \) current consists of four kinetically distinct components. They report a current sensitive to low concentrations of 4-AP (\( I_{K,slow} \)), a TEA-sensitive noninactivating current (\( I_{\text{to}} \)), and a transient outward current that could be attributed to either of two high 4-AP-sensitive components, \( I_{\text{to,f}} \) and \( I_{\text{to,s}} \). In most aspects, our results support these conclusions, with the current designated in the present study as \( I_{\text{slow}} \) corresponding to \( I_{K,slow} \), \( I_K \) corresponding to \( I_{\text{to,s}} \), and \( I_{\text{to,f}} \) corresponding to \( I_{\text{to,f}} \) (29). Thus it is unlikely that we would have detected it in our experiments with control mice. However, we did observe this current in our experiments with the Kv4.2 W362F transgenic mice. Despite the similarity in the main conclusions, however, there are aspects of the present study that differ from that of Xu et al. (29). For example, the decay time constants of the composite current reported here are much faster, a discrepancy that is likely related to the higher temperature (32 vs. 22°C) used in the present study. In this regard, our
values are similar to those reported by Zhou et al. (32), a study in which currents were recorded at 37°C. In addition, Xu et al. (29) report that \( I_{K_{\text{slow}}} \) may consist of multiple components, based on the complex nature of its steady-state inactivation curve. Our methodology did not enable us to directly measure the steady-state inactivation of this current so our results cannot either support or refute this observation. However, we report the possibility that \( I_{K} \) (\( I_{\text{ss}} \)) may consist of multiple components, an observation not put forward by Xu et al. (29). In general, however, the present results regarding the components of the mouse ventricular \( K^{+} \) current are in agreement with those of Xu et al. (29), with minor differences that are likely related to the different methodology employed and the very different conditions under which the experiments were carried out.

Multiple currents contribute to the transient component. We have demonstrated that the transient component of the mouse ventricular \( K^{+} \) current is composed of both \( I_{10} \) and \( I_{\text{slow}} \). Evidence for this was seen initially in the complex inactivation curve of this component (Fig. 2D), which was similar to those modeled by Po et al. (22) for systems expressing two distinct inactivating \( K^{+} \) currents. Four experimental observations confirmed this conclusion. First, a block of \( I_{\text{slow}} \) with 50 \( \mu M \) 4-AP (7) reduced the magnitude of the transient component of the current (Fig. 3B). Second, the current remaining after block of \( I_{\text{slow}} \) had the characteristic kinetics and pharmacology of \( I_{10} \) (Fig. 4). Third, the inactivation curve of the current remaining after block of \( I_{\text{slow}} \) was shifted to the left of the control curve (Fig. 5A) and was in fact very similar to that of rat ventricular \( I_{10} \) under the same conditions (10). Fourth, the inactivation curve for the transient component recorded from \( I_{10} \)-deficient transgenic mice (7) was shifted to the right of the control curve. However, this latter result may be complicated by a novel \( K^{+} \) current that is upregulated in these cells (7). Taken together, these results provide compelling evidence that \( I_{\text{slow}} \) and \( I_{\text{ss}} \) combine to produce the transient component of the mouse ventricular \( K^{+} \) current.

Multiple currents contribute to the sustained component. The complex steady-state inactivation curve of the sustained component, with two inactivating regions separated by a plateau (Fig. 2D), suggested contributions from at least two currents to this phase as well. Whereas \( I_{\text{slow}} \) contributed only to the inactivation seen between −50 and −20 mV, \( I_{K} \) exhibited inactivation over both voltage ranges (Fig. 5, B and C). It is important to note that this property is shared by rat ventricular \( I_{K} \) (10). In the present study, \( I_{K} \) was solely
K currents and the action potential. Our results (Fig. 1), and those of others (13, 32) clearly demonstrate the importance of $I_{\text{slow}}$ in controlling the waveform of the mouse ventricular action potential. In addition, Barry et al. (7) have demonstrated the importance of $I_{\text{to}}$ because the action potential is prolonged in ventricular myocytes isolated from transgenic mice in which $I_{\text{to}}$ has been functionally depressed. However, our results indicate a greater role for $I_K$ in the mouse ventricular action potential than others have previously reported (15, 32). This is probably because of the high concentration of TEA (20 mM) that we have used. It may be because of the properties of $I_K$ and the waveform of the mouse ventricular action potential, a greater degree of block of $I_K$ is required to substantially change the APD. Our results also indicate that both $I_{\text{slow}}$ and $I_K$ are critical for the rapid repolarization of the action potential because block of either produces prolongation of the action potential beginning immediately after the peak (Fig. 1).

This is probably explained by the fast activation kinetics of both of these currents (Fig. 3). It is interesting to note the importance of all three of these large, rapidly activating, repolarizing $K^+$ currents in producing the short action potential required to sustain the high heart rates exhibited by the mouse (>600 beats/min (20)).

FK-506 and $K^+$ currents. New results presented here indicate that the effects of FK-506 on the mouse ventricular action are identical to those seen previously in rat (10, 12). Despite the species differences in the repolarizing $K^+$ currents underlying the action potential, it was interesting to discover that FK-506 regulates $I_K$ and $I_{\text{to}}$ in mouse in a manner identical to that seen in rat. Several results reveal that FK-506 inhibits $I_K$. First, FK-506 decreases the sustained component of the current without any change in the magnitude of the transient component (Fig. 7, A and B). Second, this effect is similar to that produced by the $I_K$ blocker TEA (Fig. 2B). Third, the FK-506-sensitive current (Fig. 7A) is nearly identical to the TEA-sensitive current (Fig. 3A). As in the rat ventricle, the effects of FK-506 on mouse $I_{\text{to}}$ were observed only at high stimulation rates, as the immunosuppressant prolonged its recovery from inactivation (Fig. 7). Thus FK-506 evokes characteristic and novel regulatory mechanisms on an array of $K^+$ currents in mouse myocytes as well.

The detailed characterization of $I_{\text{slow}}$ as a constituent of the transient current led to the important question as to whether or not FK-506 was regulating this
component as well. Whereas it was not possible to inhibit \( I_{\text{slow}} \) pharmacologically without also blocking \( I_{\text{K}} \), the \( K_v4.2W362F \) transgenic mouse, in which \( I_{\text{slow}} \) is functionally eliminated (7), should in principle be an ideal system to directly examine this possibility. However, many of the transgenic myocytes exhibited a large, transient current that was insensitive to 50 µM 4-AP. This is likely to be the novel upregulated current described by Barry et al. (7) and is probably the current described by Xu et al. as \( I_{\text{to,s}} \) (29). The expression of this current prevented unambiguous interpretation of recovery experiments so we were unable to examine the effects of FK-506 on \( I_{\text{slow}} \). This underscores the fact that gene expression which compensates for a knocked-out or over-expressed gene may complicate studies in transgenic hearts.

FKBP12 and \( K^+ \) currents. The intriguing physiological importance of the FK-506 effects, reported here and previously (10, 12), resides with FKBP12, the family of intracellular cognate binding proteins for this drug (14). Experiments focused on the possible role of FKBP12 because it is highly expressed in the heart (25) and is a natural component of the sarcoplasmic reticulum \( Ca^{2+} \) release channel (16). As depicted schematically in Fig. 8, all of the known effects of the FK-506/FKB12 cascade can be understood through two signaling motifs. The first is defined by FK-506-evoked removal of FKBP12 from an endogenous membrane protein such as the ryanodine receptor (17), the \( IP_3 \) receptor (9), or the TGF-β receptor (21) (Fig. 8A). In the second mechanism (Fig. 8B) FK-506 binds to FKBP12, resulting in a functional targeting of the complex to other intracellular proteins such as the phosphatase calcineurin (18). Whereas our previous results reveal that calcineurin is not involved in \( I_{\text{K}} \) channel modulation by FK-506 (10), other possible roles for FKBP12 were explored by exploiting rapamycin as a probe. As depicted in Fig. 8, rapamycin can distinguish between these motifs because it shares the ability of FK-506 to dissociate FKBP12 from complexes but diverges from FK-506 action since it targets the immunophilin differently (1). It was striking to observe that rapamycin acted exactly like FK-506 in the regulation of both \( I_{\text{K}} \) and \( I_{\text{slow}} \). These results show that in mouse, as in rat (10), the effects of FK-506 are not mediated by inhibition of calcineurin because the rapamycin-FKB12 complex is devoid of calcineurin inhibitory activity (1). Furthermore, the fact that the ability of both FK-506 and

Fig. 9. Effect of immunosuppressant rapamycin (5 µM) on mouse ventricular \( K^+ \) currents. A, top: representative rapamycin-sensitive current obtained by subtracting current elicited during current-voltage protocol in presence of rapamycin from that recorded in its absence. A, middle: plots of average (\( n = 12 \)) voltage dependence of transient component of current in absence (■) and presence (□) of 5 µM rapamycin. A, bottom: same for sustained component of current (\( n = 12 \)). B, top: representative family of currents recorded during protocol measuring recovery from inactivation in absence (left) and presence (right) of 5 µM rapamycin. B, bottom: average (\( n = 5 \)) effect of 5 µM rapamycin on recovery from inactivation of transient component of the mouse ventricular \( K^+ \) current. Normalized magnitude of current is plotted against rest interval before (■) and after (□) 5 µM rapamycin.

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rapamycin to dissociate FKBP12 from membrane-bound proteins is well established (8, 9, 17, 21) and that it is the only action they are known to share (14) strongly supports the idea that FKBP12 is a functional component of particular cardiac K⁺ channels. It is clear that further studies are needed to define a molecular mechanism for FKBP12 action in the cardiac cell. By analogy with other systems, i.e., the TGF-β receptor and the IP₃ receptor, it is possible that FKBP12 is involved in tethering important regulatory proteins to the K⁺ channels for the efficient coupling of components of a signaling cascade.

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