Calcineurin-independent inhibition of Kv1.3 by FK-506 (tacrolimus): a novel pharmacological property

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FK-506 (TACROLIMUS) is a potent immunosuppressant that is widely used to prevent rejection after organ transplantation and in the treatment of various autoimmune diseases (12, 26). FK-506 binds to FK-506 binding protein (FKBP), a cytosolic protein, and the resulting FK-506-FKBP complex inhibits the action of calcineurin, a Ca2+/calmodulin-dependent phosphatase 2B, subsequently preventing calcineurin-dependent interleukin (IL)-2 transcription and T lymphocyte activation (24, 36, 45). Thus the mechanism of immunosuppressive action in these cells is thought to be mediated by a calcineurin-dependent mechanism.

Kv1.3, a member of the Shaker family of voltage-gated K+ channels, is a delayed-rectifier channel (41). Kv1.3 currents display characteristic gating kinetics: under whole cell patch-clamp conditions, Kv1.3 is activated within a few milliseconds in response to depolarizing pulses and undergoes a slow C-type inactivation during prolonged depolarization (9). Because Kv1.3 recovers from inactivation extremely slowly, cumulative inactivation occurs after a train of repetitive pulses (25). Kv1.3 is found in many tissues, specifically in lymphocytes and the brain (41). In human T lymphocytes, it sets the cell membrane potential and controls the efflux of K+ necessary to maintain Ca2+ influx (3). Therefore, Kv1.3 is a primary regulator of T lymphocyte activation and is widely recognized as a potential target for immunotherapy (14).

Calcineurin is known to have a variety of cellular functions in different types of cells such as neurotransmitter release, the activity of several ion channels, synaptic plasticity, and nerve regeneration (37, 47). Immunosuppressants such as FK-506 and cyclosporin A (CsA), calcineurin inhibitors, also regulate the activity of ion channels. For example, FK-506 and CsA block high-voltage-activated Ca2+ channels in cultured hippocampal neurons via a calcineurin-dependent mechanism (27). It has been reported that both drugs act in a similar manner on voltage-activated Ca2+ channels in hippocampal neurons and coronary arterial smooth muscle cells (29, 48). In addition, the inhibition of calcineurin by FK-506 and CsA causes the upregulation of cell surface functional Na+ channels in adrenal chromaffin cells (35). In contrast, CsA reduces the functional expression of K+ channels and nicotinic and 5-HT3 receptors to a significant extent (4, 16, 17). These results are mediated by inhibition of the activation of the calcineurin-dependent pathway. However, several studies have alluded to the possibility that FK-506 has a direct and nonspecific effect on membrane structures and function in different cells. FK-506 inhibits outward K+ currents that are responsible for the repolarization of the action potentials in rat ventricular myocytes (10, 11) and modulates the single-channel activity of Ca2+-dependent K+ channels via a calcineurin-independent mechanism (44). In addition, FK-506 blocks the voltage-gated K+ current, resulting in a time-dependent and rapid membrane depolarization in human T lymphocytes by a yet-to-be-defined mechanism (31). These results also raise the possibility that FK-506 modulates ion channel activity via mechanisms that do not involve calcineurin.
not involve effects on calcineurin. Although these observations suggest the possibility of the direct blocking action of FK-506 on voltage-gated K⁺ channels, the mechanism responsible and the effect on the kinetics of the channels are not well understood. Therefore, in the present study, we examined the effects of FK-506 on the cloned K⁺ channel Kv1.3 to investigate the direct action and detailed kinetics between the drug and the channel.

**EXPERIMENTAL PROCEDURES**

**Stable transfection and cell culture.** Chinese hamster ovary (CHO) cells (American Type Culture Collection, Rockville, MD) were maintained in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum, 0.1 mM hypoxanthine, and 0.01 mM thymidine in a humidified 5% CO₂ incubator at 37°C. The CHO cells used stably expressed Kv1.3, Kv1.5, or Kv4.3 channels as previously described (5, 7, 28). The cultures were exchanged at 2- to 3-day intervals with fresh IMDM containing 0.3 mg/ml of genetin (Invitrogen) and passed every 2–3 days with the use of a brief trypsin-EDTA treatment. The trypsin-EDTA-treated cells were seeded onto glass coverslips (diameter 12 mm, Fisher Scientific, Pittsburgh, PA) in a petri dish 24 h before use. For electrophysiological recordings, coverslips with attached cells were transferred to a continually perfused recording chamber (RC-13, Warner Instrument, Hamden, CT).

**Electrophysiological recordings.** Currents were recorded at room temperature (22–24°C) with the whole cell and inside-out configuration of the patch-clamp technique with an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA). Micropipettes were pulled from PG10165-4 glass capillary tubing (World Precision Instruments, Sarasota, FL) and had resistances of 2–3 MΩ when filled with internal pipette solution. The liquid junction potentials between the external bath solution, FK-506 (1, 3, 10, and 30 μM) was maintained, as described previously (7, 25). When applied to the external bath solution, FK-506 (1, 3, 10, and 30 μM) not only reduced the peak amplitude of the current but also altered the time course for current decay, increasing the rate of current decay during depolarization at the concentrations used. Under control conditions, the current decay of Kv1.3 was well fitted to a single exponential function with a time constant of 166.3 ± 9.5 ms (n = 11). After the addition of FK-506, the apparent inactivation of Kv1.3 was accelerated with time constants of 141.5 ± 7.5, 114.5 ± 8.6, 75.5 ± 7.6, and 38.8 ± 4.3 ms (n = 11) for 1, 3, 10, and 30 μM, respectively. Thus the peak amplitude of the current was affected much less than the steady-state current amplitude at the end of the 200-ms depolarizing pulse. The current amplitude measured at the most hyperpolarized preconditoning pulse and \( V_{1/2} \) represents the current measured at the most hyperpolarized preconditioning pulse and \( I_0 \) represents a nonzero current that was not inactivated at the most depolarized 30-s preconditioning pulse. We eliminated this nonzero residual current by subtracting it from the actual value. The resulting steady-state inactivation data were fitted to the Boltzmann equation \( y = I/I_0 = 1/(1 + \exp(V - V_{1/2})/k) \), where \( V \) is the potential corresponding to the half-inactivation point (in mV) and the slope value (in mV), respectively. The time courses of current inactivation during the depolarizing pulses were fitted to a single exponential function. Data are expressed as means ± SE. One-way analysis of variance, followed by Bonferroni test, was used to evaluate the statistical significance of the observed differences (46). Statistical significance was considered at \( P < 0.05 \) with Origin 7.0 software (OriginLab).

**RESULTS**

**Concentration-dependent inhibition of Kv1.3 by FK-506 and rapamycin.** In the whole cell configuration, no appreciable endogenous currents were detected in nontransfected CHO cells, as described previously (6, 33). Figure 1A shows the superimposed Kv1.3 current traces as the result of a 200-ms depolarizing pulse to +40 mV under control conditions and in the presence of FK-506. In the absence of the drug, the Kv1.3 currents were rapidly activated, reached a peak value, and then were slowly inactivated while the depolarizing pulse was maintained, as described previously (7, 25). When applied to the external bath solution, FK-506 (1, 3, 10, and 30 μM) not only reduced the peak amplitude of the current but also altered the time course for current decay, increasing the rate of current decay during depolarization at the concentrations used. Under control conditions, the current decay of Kv1.3 was well fitted to a single exponential function with a time constant of 166.3 ± 9.5 ms (n = 11). After the addition of FK-506, the apparent inactivation of Kv1.3 was accelerated with time constants of 141.5 ± 7.5, 114.5 ± 8.6, 75.5 ± 7.6, and 38.8 ± 4.3 ms (n = 11) for 1, 3, 10, and 30 μM, respectively. Thus the peak amplitude of the current was affected much less than the steady-state current amplitude at the end of the 200-ms depolarizing pulse. The current amplitude measured at the end of the 200-ms depolarizing pulse to +40 mV was used as an index of the inhibition. A nonlinear least-squares fit of the Hill equation to the concentration-response data yielded an IC₅₀ value of 5.6 ± 0.7 μM and \( n_H \) of 1.5 ± 0.1 (n = 11) for FK-506 at +40 mV.

We further examined the effects of another immunosuppressant, rapamycin, on Kv1.3 currents (Fig. 1B). Similar to FK-506, rapamycin enhanced the rate of current decay, resulting in a decrease in current amplitude at the end of a 200-ms depolarizing pulse. Figure 1B shows the concentration dependence for the rapamycin-induced inhibition of Kv1.3 measured at the end of the 200-ms depolarizing pulse. A nonlinear least-squares fit of the Hill equation to the concentration-response data yielded an IC₅₀ value of 6.7 ± 0.8 μM and \( n_H \) of 1.3 ± 0.1 (n = 6) at +40 mV. Thus the potency of rapamycin was similar to that of FK-506 in inhibiting the Kv1.3 current.
To assess the reversibility of the effect of the drug, the single depolarizing pulse was repeated while 5 μM FK-506 was applied. As shown in Fig. 2, when solutions were switched to solutions containing the drug, steady-state inhibition of KV1.3 was reached within 2 min. The washout of FK-506 by perfusion with a drug-free solution was also complete within 3 min. The currents recovered to 93.1 ± 2.4% (n = 9) of the control from the steady-state inhibition by FK-506. Therefore, the inhibition was reversible on washout, with little rundown in the current being observed under these conditions.

Effects of calcineurin inhibitors on inhibition of KV1.3 by FK-506. The KV1.3 channel can be phosphorylated and dephosphorylated (8, 32, 40), and FK-506 has been reported to function as a phosphatase inhibitor (45). To determine whether calcineurin is involved in the FK-506-induced inhibition of KV1.3, we investigated the effects of other calcineurin inhibitors, such as cypermethrin in the bath solution and calcineurin autoinhibitory peptide in the pipette solution. Figure 3A shows the effects of cypermethrin on the inhibition of KV1.3 by FK-506. After a 30-min preincubation with 40 nM cypermethrin, 5 μM FK-506 inhibited the steady-state current of KV1.3, measured at the end of a depolarizing pulse of +40 mV, by 59.3 ± 0.9% (n = 4). Figure 3B shows the effects of 100 μM calcineurin autoinhibitory peptide in the pipette solution. Over a 10-min period after membrane rupture, cell dialysis with a pipette solution containing calcineurin autoinhibitory peptide produced no effect on the amplitude or kinetics of KV1.3 compared with the control measured immediately after membrane rupture. With addition of 5 μM FK-506 to the bath solution, the steady-state current of KV1.3 was decreased by 50.7 ± 1.2% (n = 7). The lack of effect of cypermethrin and calcineurin autoinhibitory peptide on the FK-506-induced inhibition of KV1.3 strongly indicates that FK-506 directly inhibits KV1.3 via a calcineurin-independent mechanism.

Voltage-dependent inhibition of KV1.3 by FK-506. Figure 4 shows the effect of FK-506 on the current-voltage (I–V) relationship. Under control conditions, the I–V relationship was almost linear for depolarizing pulses between −40 and +40 mV (Fig. 4, A and C). In the presence of 5 μM FK-506, the steady-state currents were inhibited over the entire voltage range in which KV1.3 was activated, as shown in Fig. 4, B and C. In a plot of the relative current (I_{FK-506}/I_{control}) versus potential (Fig. 4D), the inhibition of KV1.3 by FK-506 increased steeply between −30 and −10 mV, which corresponded with the voltage range for channel activation (F_{2,21} = 11.7, P < 0.01). However, the inhibition in the voltage range between 0 and +40 mV, where the channels are fully activated, did not show any voltage dependence. The linear curve fitting of the data at potentials >0 mV (Fig. 4D) yielded a value approximately equal to zero for the slope of the line: 49.5 ± 4.2% of the control value at 0 mV and 46.8 ± 3.2% of the control at +40 mV (F_{4,35} = 0.09, P > 0.05).

Use-dependent inhibition of KV1.3. Twenty repetitive 200-ms depolarizing pulses of +40 mV from a holding potential of −80 mV were applied at two different frequencies, 1 and 2 Hz (Fig. 5). Under control conditions, the peak amplitude of KV1.3 decayed progressively, eventually reaching a steady state, and was decreased by 55.3 ± 3.6% and by 71.0 ± 3.9%
pulses. The extent of the steady-state inhibition was 88.0 ± 2.9% and 94.7 ± 1.6% (n = 6) at 1 and 2 Hz, respectively. Thus FK-506 exhibited a strong use-dependent inhibition of Kv1.3.

Effects of FK-506 on steady-state inactivation of Kv1.3. To characterize the inhibitory effects of FK-506 on Kv1.3, we examined the voltage dependence of the steady-state inactivation curve of Kv1.3 (Fig. 6A). Under control conditions, the potential corresponding to the half-inactivation point (V1/2) was −42.8 ± 0.7 mV and slope factor k was 2.84 ± 0.07 mV (n = 8). FK-506 shifted the steady-state inactivation curves (V1/2) to a hyperpolarized potential in a concentration-dependent manner (−52.3 ± 0.5 mV at 5 μM, −56.9 ± 1.1 mV at 30 μM FK-506; F2,21 = 74.8, P < 0.001). In the presence of FK-506, however, the slope factor was not significantly changed (2.81 ± 0.06 mV at 5 μM, 2.85 ± 0.11 mV at 30 μM FK-506; F2,21 = 0.05, P > 0.05). The inhibition, measured as the reduction in the steady-state current in the presence of FK-506 (the IC50 value obtained from concentration-response data as shown in Fig. 1), does not represent the true affinity of the drug for the inactivated states of Kv1.3. Thus the apparent Ki for the inhibition of Kv1.3 by FK-506 in the inactivated state was estimated from the concentration-dependent shift in the steady-state inactivation curve (2). The theoretical value of Ki was calculated to be 0.37 ± 0.08 μM (n = 8; Fig. 6B). Thus the effect of FK-506 on channel inactivation is 15 times more potent than on the steady-state current.

Effects of FK-506 on recovery from inactivation of Kv1.3. Recovery from FK-506-induced inhibition was measured with a double-pulse protocol. Figure 7 shows the peak current amplitudes elicited by the test pulses as a function of time. Under control conditions, the recovery from inactivation could be described by a biexponential function to a fast time constant of 0.65 ± 0.34 ms and a slow time constant of 7.01 ± 0.55 ms (n = 5). In the presence of 5 μM FK-506, the recovery process was also best fitted with a biexponential function (fast time constant of 0.62 ± 0.19 ms, slow time constant of 18.17 ± 3.69 ms; n = 5). The fast time constant obtained in the presence of FK-506 remained unchanged, but the slow time constant was statistically different from that for the current recovery under control conditions (F1,8 = 8.97, P < 0.05).
Effects of FK-506 on Kv1.3 in inside-out patches. Figure 8 shows the effects of FK-506 on Kv1.3 currents recorded from inside-out patches. In the presence of 5 μM FK-506, inhibition of the Kv1.3 currents was also characterized by an acceleration in the apparent rate of current decay with little effect on peak amplitude, as was found in whole cell recordings (Fig. 1). FK-506 (5 μM) inhibited the steady-state current of Kv1.3 at the end of a depolarizing pulse of +40 mV by 54.9 ± 2.7% of the control value (n = 7), similar to 58.2 ± 6.7% (n = 9) of the control obtained for whole cell patches.

Concentration-dependent inhibition of Kv1.5 and Kv4.3 by FK-506. Figure 9a shows superimposed Kv1.5 current traces produced by 250-ms depolarizing pulses to +40 mV under control conditions and in the presence of various concentrations of FK-506. Similar to Kv1.3, FK-506 also induced a reduction in the steady-state current of Kv1.5 during the depolarizing pulse, with little effect on peak amplitude. The current amplitude, measured at the end of the 250-ms depolarizing pulse, was also used as an index of the inhibition. A nonlinear least-squares fit of the individual data points to the Hill equation yielded an IC50 value of 4.6 ± 0.1 μM and nH of 1.2 ± 0.1 (n = 5). We further characterized the effect of FK-506 on Kv4.3, a rapidly inactivating A-type current. As shown in Fig. 9b, FK-506 decreased the peak amplitude of Kv4.3 in a concentration-dependent manner. However, the most obvious effect of FK-506 was an acceleration in the rate of the current decay of Kv4.3. To quantify the effect of FK-506 on Kv4.3, therefore, we measured the integral of the total current over the duration of a depolarizing pulse to +40 mV and obtained an IC50 value of 53.9 ± 4.6 μM and nH of 0.9 ± 0.1 (n = 8).

**DISCUSSION**

The primary focus of this study was to determine the direct effect of FK-506 on the cloned voltage-gated K+ channels Kv1.3, Kv1.5, and Kv4.3. The findings indicate that FK-506 caused a reversible time-, concentration-, and use-dependent inhibition of Kv1.3 channels. These results suggest that FK-506 inhibits Kv1.3 by mechanisms that do not involve the suppression of the protein phosphatase activity of calcineurin but by a distinct mechanism involving a direct interaction with calcineurin.

FK-506 is used as an immunosuppressant in organ transplantation and in the treatment of autoimmune diseases (13, 26, 38). The mechanism of its immunosuppressive action is thought to be mediated by a calcineurin-dependent mechanism (23, 36, 45). The findings here, however, indicate that FK-506 directly inhibits Kv1.3 currents by a mechanism that is independent of calcineurin activity, based on the following results. First, rapamycin is another macrolide immunosuppressant drug, and its mechanism of action is different from that of FK-506. Rapamycin has no effect on calcineurin but suppresses later events in signal transduction pathways that are responsible for IL-dependent T lymphocyte activation (1, 36). From an analysis of the concentration-response curve, however, the potencies of rapamycin and FK-506 in inhibiting
KV1.3 were very similar. The fact that rapamycin and FK-506 have similar structures suggests that this effect might be due to structural similarities rather than the modification of the signal transduction pathways required for T lymphocyte activation, namely, the inhibition of calcineurin activity. Thus we suggest that FK-506 and rapamycin share a common mechanism of action at the molecular level: direct interaction with the KV1.3 channel. Second, in our experiments, the presence of cypermethrin in the bath solution or calcineurin autoinhibitory peptide in the patch pipette failed to prevent the inhibition of KV1.3 by FK-506. Because the concentrations of calcineurin inhibitors used in this experiment were sufficiently high to completely inhibit calcineurin activity (12, 15), these results are incompatible with phosphorylation events due to the inhibition of the phosphatase activity of calcineurin by FK-506.

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Third, the catalytic activity of calcineurin requires a significant increase in the concentration of intracellular free Ca^{2+} (39).

Our experiments were performed in whole cell recordings in which free Ca^{2+} was buffered by EGTA (10 mM) to resting concentrations (<10 nM), and low levels of calcineurin activity would be expected in CHO cells. Thus our experimental conditions support a scenario involving the calcineurin-independent inhibition of KV1.3 by FK-506. However, we cannot...
completely rule out the possibility that calcineurin-dependent effects may still exist. Finally, in an excised inside-out patch devoid of diffusible cytosolic molecules required for phosphorylation and dephosphorylation, the rapid and reversible effect of FK-506 was also consistent with a direct interaction with the KV1.3 channel rather than through a phosphorylation-dependent mechanism. In addition, the inhibition of KV1.3 by FK-506 was voltage dependent, increasing in the voltage range for channel activation. This reflects the voltage dependence of channel inactivation but is not due to a direct voltage dependence of binding to KV1.3 channels. These effects probably result from the preferential interaction of this drug with the inactivated state of the KV1.3 channel. Consistent with this, the inhibitory effect of FK-506 was use dependent, with the effects enhanced at higher rates of channel activation. A possible mechanism of action is that FK-506 is highly lipophilic (36) and is readily partitioned into the plasma membrane, thereby modulating the inactivation kinetics of KV1.3 by an allosteric mechanism. FK-506 binding to inactivated KV1.3 channels stabilizes the inactivated state, and thus decreases the current after steady-state inactivation at any given potential. Under these conditions, the availability of KV1.3 channels is decreased when sustained depolarization occurs in the case of nonexcitable cells, such as T lymphocytes. Thus the state-dependent inhibition by FK-506 seems suited to producing a prolonged nonconducting inactivated state of KV1.3 from which they recover with a time constant of several seconds.

In human T lymphocytes, KV1.3 is the predominant voltage-gated K⁺ channel. This channel plays an important role in the physiology of T lymphocytes, i.e., indirectly modulating Ca²⁺ signaling by regulating their membrane potential (3, 9). The selective blockade of KV1.3 inhibits T lymphocyte activation by depolarizing the membrane potential of the cells, thereby attenuating the Ca²⁺ signaling response. Previous studies have indicated other possible immunosuppressive effects of margatoxin, a specific KV1.3 blocker, such as inhibiting the production of Th-1-, Th-2-, IL-2-, and T lymphocyte-mediated cytoly, in addition to inhibiting T lymphocyte activation (22, 34). Therefore, selective blockers of KV1.3 have consistently been of interest and are considered to be an important therapeutic target for immunosuppressive drugs and autoimmune diseases (14). In the present study, FK-506 inhibited KV1.3 in a concentration-dependent manner. The depolarization of T lympho-

![Fig. 7. Effects of FK-506 on recovery from inactivation of KV1.3. Top: a double-pulse protocol was used to characterize the recovery of KV1.3 from inactivation in the absence and presence of FK-506. A 1st prepulse of a 200-ms depolarizing pulse of +40 mV from a holding potential of −80 mV was followed by a 2nd identical pulse after increasing the interpulse intervals between 10 ms and 30 s at −80 mV. Pulses were applied at intervals of 30 s. Bottom: solid lines represent the biexponential fit of the peak amplitude of KV1.3 currents as a function of the interpulse interval (n = 5). Data are expressed as means ± SE.](http://ajpcell.physiology.org/)

![Fig. 8. Effects of FK-506 on KV1.3 currents recorded from inside-out patches. Superimposed macroscopic currents of KV1.3 were elicited by applying 200-ms depolarizing pulses from a holding potential of −80 mV to +40 mV every 30 s in the absence and presence of FK-506.](http://ajpcell.physiology.org/)
cytes by FK-506 was also observed by electrophysiology in membrane potential measurements (31). Therefore, this inhibitory effect of FK-506 on KV1.3 channels, which results in the membrane depolarization of T lymphocytes, could be another underlying mechanism by which FK-506 exerts its immunosuppressive effect.

Although FK-506 is used extensively as an immunosuppressive agent in human organ transplantation, it causes numerous side effects including nephrotoxicity, hypertension, and neurotoxicity (13, 38). Moreover, secondary toxic effects have been reported in cardiac tissue, such as arrhythmia and heart failure. KV1.5 has been postulated to be the predominant delayed-rectifier K^+ current responsible for human atrial repolarization (43). KV4.3, a rapidly inactivating A-type current, is expressed at high levels in the heart and is responsible for the early repolarization of the cardiac action potential (28). Theoretically, the inhibition of KV1.5 and/or KV4.3 delays the repolarization of cardiac action potentials. Several studies have reported ventricular tachycardia in patients receiving FK-506, and its putative mechanism involves retarding ventricular repolarization (18, 19). Therefore, it would be expected that, in patients treated with FK-506, arrhythmia may result, at least partly, from the direct interaction of FK-506 with KV1.5 and/or KV4.3 of cardiomyocytes.

The therapeutic plasma concentration of FK-506 is in the range of 0.5–1.5 nM in transplant patients (20). We estimated the \( K_i \) for binding to the inactivated state of KV1.3 to be 0.37 \( \mu \)M, which is well above the therapeutic dose, and this effect appears to have no clinical relevance. However, the concentration of FK-506 in blood is up to 100-fold higher than that in plasma (21). In addition, the concentration of FK-506 in the blood does not necessarily reflect its tissue concentration, and the concentrations in target tissues themselves may be more important in terms of assessing the pharmacological action or side effects of a drug. FK-506 that is highly lipophilic and cell membrane permeant can progressively accumulate in higher concentrations in tissues including T lymphocytes (42). Moreover, the acute cardiovascular effect of FK-506 on electrophysiological and mechanical properties in the isolated guinea pig heart was reported at a micromolar concentration (30). Therefore, under this assumption, our results shed some light on the molecular mechanisms that underlie some of the cardiac toxicity observed in patients who are being administered FK-506.

In this study, we report on a novel calcineurin-independent pharmacological property of FK-506, the direct inhibitory effects of FK-506 on cloned KV1.3 channels. Our findings reveal another mechanism of action of FK-506 for immune suppression.

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GRANTS

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