Inhibitory Effects of Vesnarinone on Cloned Cardiac Delayed Rectifier K⁺ Channels Expressed in a Mammalian Cell Line

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ABSTRACT

Vesnarinone, a phosphodiesterase inhibitor, prolongs cardiac action potential duration by inhibiting the delayed rectifier K⁺ current, \( I_{Kr} \). We examined the effect of this agent on human ether-a-go-go related gene (HERG) and KvLQT1/minK K⁺ channels heterologously expressed in human embryonic kidney 293T cells with the whole-cell patch-clamp technique. HERG channel current was inhibited by vesnarinone in a concentration-dependent manner, whereas KvLQT1/minK current was hardly affected by the drug. The inhibition of HERG current by vesnarinone became more prominent and faster as the membrane potential was more depolarized. The properties of inhibition could be described by a first order reaction between the drug and the channel that was apparently independent of HERG channel gating. Although the unbinding rate constant of the drug was constant, the apparent binding rate constant increased as the membrane was more depolarized and the drug concentration was raised. This model also could explain the fast recovery from the drug’s effect at hyperpolarized potentials and its rate-dependent inhibition of HERG. Therefore, the effect of vesnarinone on the HERG-K⁺ current could be adequately described by a simple kinetic model of drug-channel interaction.

Vesnarinone is a phosphodiesterase (PDE) inhibitor and used for treatment of patients with congestive heart failure (Taïra et al., 1984; Yamashita et al., 1984). Although most other PDE inhibitors improved the morbidity rate but not mortality rate of congestive heart failure patients, it was reported that vesnarinone improved both morbidity and mortality rates when orally administered at low doses (Feldman et al., 1993). In addition to PDE inhibition, vesnarinone prolongs the cardiac action potential duration by inhibiting the delayed outward K⁺ currents, which may be partly responsible for the positive inotropic and negative chronotropic effects of the drug (Taïra et al., 1984; Iijima and Taïra, 1987; Rapundalo et al., 1988; Asanoi et al., 1989). Therefore, this drug is thought to improve not only the contraction force but also the relaxation of the diseased heart. The delayed outward K⁺ current, \( I_{Kr} \), initiates repolarization of the cardiac action potential and thus controls its duration. In most mammalian species, including guinea pig and humans, \( I_{Kr} \) is made up of two distinct components, the rapidly and slowly activating K⁺ currents, \( I_{Kr} \) and \( I_{Ks} \), respectively (Sanguinetti and Jurkiewicz, 1990, 1991; Wang et al., 1994; Li et al., 1996). The ether-a-go-go related gene in humans (HERG) expresses a K⁺ channel current with biophysical characteristics similar to those of \( I_{Kr} \) (Sanguinetti et al., 1995; Trudeau et al., 1995). Although recently it was indicated that a minK-related peptide 1 (MiRP1) coassembles with HERG to reconstitute \( I_{Kr} \), the kinetic properties of HERG current with and without the protein do not largely alter (Abbott et al., 1999). However, the coexpression of minK with KvLQT1 is mandatory to reconstitute \( I_{Kr} \) (Barhanin et al., 1996; Sanguinetti et al., 1996; Yang et al., 1997).

In this study, we examined the effects of vesnarinone on the currents flowing through HERG and KvLQT1/minK channels expressed in HEK293T cells. HERG current was inhibited by vesnarinone with an IC₅₀ of 1.1 μM, whereas KvLQT1/minK current was not significantly depressed by the drug even at 30 μM. These results suggest that vesnarinone would prolong the cardiac action potential by specifically inhibiting \( I_{Kr} \). Detailed examination of the kinetics of the effect of vesnarinone has revealed that this compound inhibits HERG current in a voltage- and time-dependent manner. The recovery from inhibition at the resting level was relatively rapid, which caused the steady-state level of block to become more prominent at higher stimulation frequency.

ABBREVIATIONS: PDE, phosphodiesterase; \( I_{kr} \), cardiac delayed rectifier K⁺ current; \( I_{kr} \), rapidly activating component of cardiac delayed rectifier K⁺ current; \( I_{ks} \), slowly activating component of cardiac delayed rectifier K⁺ current; HERG, human ether-a-go-go related gene; MiRP1, minK-related peptide 1; I-V, current-voltage.
These properties could be explained exclusively by the voltage- and time-dependent kinetics of the drug-channel interaction.

Materials and Methods

Transfection and Cell Culture. HERG and human KvLQT1 cDNAs were kindly provided by Drs. M. T. Keating and M. C. Sanguinetti (University of Utah, Salt Lake City, UT). The human minK cDNA was obtained by polymerase chain reaction from genomic DNA of human whole blood (Murai et al., 1989). They were each subcloned into an expression vector pcDNA3 (Invitrogen, San Diego, CA). HEK293T cells were transfected with pcDNA3-HERG or cotransfected with pcDNA3-KvLQT1 and pcDNA3-minK by using LipfectAMINE (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. Electrophysiological measurements were usually conducted 2 to 4 days after transfection.

Electrophysiological Recordings and Analysis. The channels expressed in HEK293T cells were recorded with the whole-cell variant of the patch-clamp method. Experiments were conducted at room temperature (22–25°C). A dish containing cells was placed on the stage of an inverted microscope and superfused continuously with the standard extracellular solution. The electrodes filled with the internal solution had a resistance of 2 to 5 MΩ after coating with silicon and being fire polished. Currents were measured with a patch-clamp amplifier (Axopatch 200A; Axon Instruments, Inc., Foster City, CA) and monitored with an analog-storage oscilloscope (dual beam storage oscilloscope; Tektronix, Inc., Beaverton, OR). For subsequent analysis, currents were recorded on videocassette tapes by using a PCM converter (VR-10B; Instrutech Corp., Mineola, NY). For analysis, the data were reproduced, low-pass filtered at 1.0 kHz (−3 decibels) with an 8-pole Bessel filter (Frequency Devices, Hatfield, MA), and digitized at 3 or 5 kHz with an analog-to-digital converter (ITC-16; Instrutech Corp.). These data were analyzed offline on a computer (Macintosh Quadra 700; Apple Computer Inc., Cupertino, CA) with commercially available programs, i.e., Pulse Program (HEKA Electronik, Lambrecht, Germany) and Patch Analyst Pro (MT Corporation, Hyogo, Japan). A microperfusion system allowed local application and rapid change of the different experimental solutions. HEK293T cells per se expressed a voltage-dependent Kv current, which was insensitive to dofetilide. Because dofetilide (3 μM) completely suppressed HERG current (data not shown), we applied the drug to the cells at the end of each experiment. The HERG currents were defined as the dofetilide-sensitive components of the membrane currents in the HEK293T cells transfected with HERG cDNA. Results were expressed as mean ± S.E. Student’s t test was used to compare differences between mean values, with a value of P < .05 being considered significant.

Solutions and Chemicals. For whole-cell recording, the pipettes were filled with “internal” solution containing 140 mM KCl, 2 mM MgCl₂, 5 mM ATP, 5 mM EGTA-KOH, and 5 mM HEPES-KOH (pH 7.3 with KOH). The bath was perfused with a control bath solution containing 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.53 mM MgCl₂, 5.5 mM glucose, and 5.5 mM HEPES-NaOH (pH 7.4 with NaOH). Vesnarinone was a gift from Otsuka Pharmaceutical Co. (Tokyo, Japan) and dissolved at 30 mM in dimethyl sulfoxide. Dofetilide was a gift from Pfizer Pharmaceutical Inc. (New York, NY) and dissolved at 10 mM in dimethyl sulfoxide. Other chemicals and materials were obtained from commercial sources. Dimethyl sulfoxide, at the concentrations used herein and which never exceeded 1:10,000, had no significant effect on any of the parameters measured in these studies (n = 3).

Results

Effects of Vesnarinone on KvLQT1/minK and HERG Channel Currents. We first compared the effects of vesnarinone on the K⁺ currents in HEK293T cells expressing KvLQT1/minK channels (Fig. 1A) and HERG channels (Fig. 1B). The cells expressing KvLQT1/minK channels were voltage clamped at −80 mV and voltage steps (7.5-s duration) were applied to potentials between −80 and +40 mV in 20-mV increments. The tail currents were recorded on repolarization to −50 mV. Under control conditions, voltage steps evoked a slowly activating outward K⁺ current (Fig. 1Aa). The threshold potential was −20 mV, and the amplitude of the currents increased linearly as the membrane was depolarized. The amplitude of the tail current increased with depolarization and reached a maximum value after a voltage step to +40 mV. These properties are the same as those previously reported for KvLQT1/minK channels (Barhanin et al., 1996; Sanguinetti et al., 1996). The KvLQT1/minK channel currents were unaffected by the application of vesnarinone (1–10 μM) to the bath. Even in the presence of 30 μM
vesnarinone, the K⁺ current was only slightly depressed without significant alteration of the activation time course (Fig. 1Ab). The amplitudes of peak tail current recorded after voltage steps to +40 mV in the control condition and in the presence of 30 μM vesnarinone were 280.6 ± 34.7 and 251.7 ± 29.0 pA, respectively (n = 4).

In contrast, vesnarinone effectively inhibited the HERG K⁺ channel currents expressed in HEK293T cells (Fig. 1B). The cells were voltage clamped at −80 mV and voltage steps (4-s duration) to potentials between −80 and +40 mV were applied in 10-mV increments, and tail currents were recorded on repolarization to −60 mV. In the control condition, a delayed outward current was evoked during voltage steps more positive than a threshold of −40 mV and reached a peak at 0 mV (Fig. 1Ba, see also open squares in Fig. 2A). Depolarizing steps to potentials more positive than 0 mV resulted in an inward rectification because of fast C-type inactivation (Smith et al., 1996). When 1 μM vesnarinone was added to the bath, HERG current was reduced by ~20% at the end of the voltage step to −40 mV, ~25% at −30 mV, ~40% at −20 mV, and ~50% at potentials more positive than 0 mV (Fig. 1Bb). The inhibition of the HERG currents was not prominent at the beginning but developed gradually during the voltage steps, which indicates that HERG block by vesnarinone is voltage- and time dependent.

The concentration-dependent effect of vesnarinone on the HERG current was assessed from the amplitude of tail currents recorded at −60 mV after the voltage step to +20 mV (Fig. 1C). The relationship between the concentration of vesnarinone and current amplitude was fitted by a Hill equation:

\[
\text{relative tail current} = \frac{1}{1 + ([\text{drug}] / IC_{50})^n}
\]

where IC₅₀ is the drug concentration for 50% block, and n is the Hill coefficient. These values were 1.06 μM and 1.22, respectively (Fig. 1C).

**Voltage Dependence of Vesnarinone Block of HERG Channel Currents.** The current-voltage relationship at the end of the 4-s depolarizing voltage steps was plotted in the absence and presence of 1, 3, or 10 μM vesnarinone (Fig. 2A). In the control, the HERG current increased with depolarization from −250 mV and reached a peak at 0 mV. Due to fast C-type inactivation, it decreased with further depolarization. In the presence of vesnarinone, the current level was suppressed at potentials more positive than −40 mV in a concentration-dependent fashion. The peak of the current-voltage (I-V) curve also slightly shifted to the left, suggesting that the effect of vesnarinone may be more potent at more depolarized potentials.

The effect of vesnarinone on the activation curve of HERG is shown in Fig. 2B. The voltage dependence of channel activation was assessed by plotting the amplitude of tail current at −60 mV as a function of the prior test potential (Fig. 2B, open squares). It could be fitted by a Boltzmann equation (Sanguinetti et al., 1995):

\[
d(V) = d_0 / [1 + \exp(-(V - V_{1/2})/k)]
\]

The control values for the half-maximum activation voltage (V₁/₂) and the slope factor (k) were −22.6 ± 1.9 mV and 8.94 ± 0.39, respectively (n = 9). These values were −28.9 ± 2.2 mV and 8.37 ± 1.67, −35.9 ± 3.50 mV and 5.73 ± 0.73, and −37.0 ± 2.7 mV and 6.21 ± 1.48 in the presence of 1, 3, and 10 μM vesnarinone, respectively (n = 3 for each). The apparent shift of V₁/₂ of HERG current in the presence of
vesnarinone may be due to the voltage-dependent nature of inhibition.

In Fig. 2C, the tail current amplitudes in the presence of 1, 3, and 10 μM vesnarinone were normalized with reference to that recorded in the same cells in the absence of the drug and plotted as the function of the potential of preconditioning pulse. Vesnarinone reduced the relative amplitudes of tail current more prominently as the membrane was depolarized, which clearly indicates that vesnarinone inhibited HERG current in a voltage-dependent manner.

**Time Dependence of Effects of Vesnarinone on HERG Currents.** The activation level of the HERG current was assessed from the amplitude of the tail currents at the beginning of the voltage step (Fig. 3Aa). In the presence of 1 μM vesnarinone, the activation of HERG, which was assessed by the tail current amplitude, increased in the beginning but decreased gradually as the duration of the voltage step was prolonged (Fig. 3Ab). Therefore, the decrease of the HERG current during the voltage pulse to +20 mV was due to channel inhibition by the drug.

The relative tail current amplitude in the presence of vesnarinone with reference to that in control with various durations of the voltage pulse is shown in Fig. 3B. The time course of the effect of the drug could be fitted by a single exponential curve with a time constant of 0.89 s. The relative tail current recorded at the beginning of the voltage step estimated from the fit curve was almost 1 and declined to a value of 0.57 in the steady state. This time course coincided with that obtained by dividing the outward current recorded during the voltage step to +20 mV in the presence of the drug with the control current (Fig. 4).

Figure 4 illustrates the time course of the vesnarinone-induced inhibition of the HERG current during voltage steps. This was obtained by dividing the current recorded in the presence of the drug with the control current at each potential. Figure 4A shows examples of superimposed current traces obtained during voltage steps to −20 and +20 mV in the presence and absence of vesnarinone. The time course of vesnarinone-induced inhibition at different potentials is shown in Fig. 4B. At each potential, the vesnarinone inhibition could be fitted by a single exponential curve. The fitted curves obtained at different potentials all crossed close to 1 at the beginning of the voltage steps, indicating that there had been no significant block at the holding potential of −80 mV. The decay of the fitted curves became faster and the steady-state inhibition became stronger, as the potential of the voltage step was more depolarized: The decay time constant (τ) was 1.72 ± 0.12 s at −40 mV, 1.55 ± 0.20 s at −20 mV, 1.18 ± 0.12 s at 0 mV, 0.96 ± 0.09 s at +20 mV, and 0.92 ± 0.06 s at +40 mV (n = 5 for each), and the steady-state ratio estimated from the fitted curve was 0.82 ± 0.04 s at −40 mV, 0.68 ± 0.07 s at −20 mV, 0.56 ± 0.08 s at 0 mV, 0.47 ± 0.03 s at +20 mV, and 0.49 ± 0.03 s at +40 mV (n = 5 for each). Thus, the block of HERG by vesnarinone was clearly voltage- and time dependent.

Because the time course of the effect of vesnarinone could be fitted by a single exponential curve, its block of HERG current could be analyzed by adopting the first order reaction model, which was apparently independent of HERG current kinetics:

\[
\begin{align*}
\beta^* \\
\alpha &\quad O \Leftrightarrow I
\end{align*}
\]

where O is the conducting HERG channel without vesnarinone binding, I is the nonconducting state of the channel bound by the drug, β* is the apparent binding rate constant of the drug to the channel, and α is the unbinding rate constant. In this first order reaction, because the ratio of Ivesnarinone/Icontrol is equivalent to the open probability (P_o) of this reaction, the steady-state P_o and τ could be described as \(\beta^*/(\alpha + \beta^*)\) and \(1/(\alpha + \beta^*)\), respectively. Therefore, from the steady-state P_o and τ obtained from the fitted curve (Fig. 5, A and B), the rate constants of α and β* could be calculated at
Each potential for each concentration of vesnarinone (Fig. 5, C and D).

The unbinding rate constant ($\alpha$) was constant at $-0.5$ s$^{-1}$ irrespective of the potential and the concentration of vesnarinone, whereas the binding rate constant ($\beta^*$) was clearly voltage- and concentration-dependent. $\beta^*$ increased as the membrane was depolarized. At each potential, it also increased as the drug concentration was raised. When we assumed $\beta^*$ as $\beta[D]$, $\beta^*$ could be described by the Boltzmann equation:

$$\beta = 4.06 \times 10^5/[1 + \exp((-16.5-V)/12.6)].$$

$\beta$ took a half-maximum value at $-16.5$ mV and the slope factor of the curve was 12.6.

Recovery from Block and Use Dependence of Vesnarinone. We examined recovery from vesnarinone-induced inhibition with a double-pulse voltage-clamp protocol (Fig. 6A). The amplitude of the tail current was examined on repolarization to $-80$ mV after the second voltage step. When the interval was 0 s, the tail current was suppressed by vesnarinone (1 $\mu$M) by 45.4 $\pm$ 1.4% ($n = 4$). When the interval was 5.12 s, it was suppressed by 16.9 $\pm$ 4.8% ($n = 4$). As shown in Fig. 6B, the amplitude of the tail current in the presence of 1 $\mu$M vesnarinone increased with a time constant of 1.75 s. This is similar to the value of $1/\alpha + \beta^*$ at $-80$ mV of 1.91 s, which was calculated from the voltage-dependent kinetics of vesnarinone block obtained in Fig. 5. This suggests that the fast recovery from vesnarinone block at $-80$ mV is derived from the same process as the inhibition of HERG current at depolarized potentials.

In Fig. 7, we examined the use dependence of inhibition of the HERG current by vesnarinone. The HERG current was elicited by 0.5-s voltage steps from $-80$ mV to +40 mV with intervals of various durations (1–12 s). Each pulse was followed by a 0.2-s step to $-100$ mV. The activation of HERG current was assessed by the amplitude of the inward tail current. In the presence of 1 $\mu$M vesnarinone, the HERG current was suppressed by 19.0 $\pm$ 1.1% ($n = 13$) during the first pulse (+40 mV for 0.2 s). When the interval between voltage steps was 12 s (Fig. 7, open squares), further suppression of HERG current was not observed ($n = 3$). At 3-s intervals (Fig. 7, open circles), the current was further inhibited during the first three depolarizing pulses to a level of 24.6 $\pm$ 2.3% ($n = 5$) and stayed at that level thereafter. When the interval was shortened to 1 s (Fig. 7, filled circles), inhibition of the current developed gradually over 40 to 50 pulses and reached a steady level of 37.0 $\pm$ 3.0% ($n = 5$). The continuous lines in the figure at each frequency were calculated from the voltage-dependent kinetics of vesnarinone inhibition obtained in Fig. 5. They closely followed the experimental results except for the initial phase of inhibition at 1-Hz stimulation.

Discussion

In this study, the effects of vesnarinone on HERG and KvLQT1/minK channel currents were examined in a mammalian cell line. We found that the drug specifically inhibited the HERG current in a concentration-dependent manner. This is consistent with the reports in native cardiac myocytes that vesnarinone inhibits $I_K$ (Iijima and Taira, 1987; Toyama et al., 1997). In rabbit cardiac myocytes, Toyama et al. (1997) showed that vesnarinone exhibited time-dependent block of $I_K$ at the depolarized potential of +10 mV ($\tau = 0.36$ s), rapid recovery from block at a holding potential of $-75$ mV ($\tau = 1.87$ s) and an increase of the block with increasing frequency of stimulation (0.2–2 Hz). All of these properties were reproduced on the HERG current in this study.

We analyzed the details of the kinetic properties of vesnarinone-induced inhibition of HERG current, which could be described as a first order reaction between the drug-free and drug-bound channels. The reaction would occur independently of the gating of the HERG channel because the time course of the effect of vesnarinone during voltage steps could be fitted by single exponential curves. The binding rate constant of the drug to the channel increased as the membrane...
was depolarized, whereas the unbinding rate constant remained the same irrespective of the membrane potential. The recovery from block- and frequency dependence of the inhibition could be approximated from the voltage- and concentration dependence of the rate constants. This may indicate that this drug can access and leave the channel irrespective of the state of channel gating, suggesting that this drug uses the hydrophobic pathway to access the channel. This is in contrast to the properties of classical \(I_{Kr}\) blockers, such as dofetilide and E-4031 (Carmeliet, 1992; Snyders and Chaudhary, 1996; Spector et al., 1996). These drugs are so-called open channel blockers. They mainly use the hydrophilic pathway and thus enter and leave the HERG channel pore only when the channel is open. Because of this property, the recovery from drug-induced channel inhibition is very slow and the steady-state inhibition is the same irrespective of the stimulation frequency. This is thought to be one of the reasons why the classical \(I_{Kr}\) blockers show reverse frequency dependence in evoking prolongation of the cardiac action potential. Because vesnarinone inhibited the HERG current more prominently as the stimulation frequency was increased, this drug may not exhibit reverse frequency dependence. In addition, the kinetics of the drug effects may indicate a 1:1 interaction between the drug and the site, which was consistent with a Hill coefficient (~1) in the concentration-dependent curve. Because it is difficult to reach a definitive conclusion merely from the electrophysiological data, further studies examining the binding of the drug to the channel are needed.

Vesnarinone inhibited the HERG current more effectively...
Vesnarinone Inhibits HERG K⁺ Channel Current

![Diagram showing use-dependent block of HERG current](https://example.com/diagram)

Fig. 7. Use-dependent block of HERG current was tested at intervals of 1, 3, and 12 s (□, and ▲, respectively). The voltage-clamp protocol is illustrated schematically. Ratios of peak tail current in the presence of 1 μM vesnarinone compared with those of the control were plotted against time after the first pulse. The final amount of steady-state block was lower at lower frequencies than at higher frequency. The limits of S.E.M. did not exceed 5% and are not depicted for the sake of clarity.

as the membrane was depolarized. Because vesnarinone exists mostly in a neutral form at physiological pH (Shimizu et al., 1984), this voltage dependence cannot be explained by an interaction between a charged blocker and a binding site in the channel pore and other possibilities should be considered. Because the voltage dependence of the binding rate constant, \( V_{1/2} \), of -16.5 mV and \( k \) of 12.6, and that of HERG activation, \( V_{1/2} \), of -22.9 mV and \( k \) of 8.94, were similar, there may be some correlation between the two. However, because the drug-induced inhibition of the current could be described by a first order reaction that was independent of gating, the activation gating of the HERG channel itself seems not to be involved. Furthermore, it is likely that the drug exclusively uses the hydrophobic pathway to access to the channel. To explain these properties of vesnarinone-inhibition, we propose that it is possible that a HERG channel whose activation gate is open has a conformation that allows the drug to bind to the channel. This hypothesis would explain why the voltage dependence of the binding rate constant of the drug to the channel coincides with that of activation gating of the HERG channel, without direct interaction of the drug with the channel gate. Further studies are needed to examine the validity of this hypothesis.

Recently, it was shown that E-4031 inhibits the channels formed by coassembly of MiRP1 with HERG with a biphasic time course similar to that in native cardiac \( I_{K_C} \) channels, whereas this drug inhibits the channels formed by HERG alone in a monophasic time course (Abbott et al., 1999). However, the kinetics of vesnarinone inhibition of the homomeric HERG channel current obtained in this study was very similar to that reported in \( I_{K_C} \) in native cardiac myocytes (Toyama et al., 1997). Therefore, it is expected that the properties of vesnarinone inhibition of HERG current would not be significantly altered by coexpression of MiRP1.

Concentrations of vesnarinone in clinical use range from 4.9 to 10.1 mg/l, i.e., from 12.4 to 25.5 μM (Feldman et al., 1988), and 80% of vesnarinone are bound by plasma proteins (Miyamoto and Sasabe, 1984). The IC₅₀ value for vesnarinone inhibition of HERG channels is ~1 μM. The IC₅₀ for vesnarinone inhibition of PDE is reported to be ~300 μM (Taira et al., 1984). Therefore, the plasma concentration of the drug in clinical use is not high enough to cause PDE inhibition but sufficient to inhibit \( I_{K_C} \) and the expressed HERG current. This suggests that in the clinical use the inhibition of \( I_{K_C} \), but not an increase of the inward Ca²⁺ current, may be mainly responsible for the inotropic action of this drug (Iijima and Taira, 1987).

In summary, this study showed that the prolongation of the cardiac action potential induced by vesnarinone can be attributed to its block of HERG channels, but not \( K_vLQT1/minK \) channels. The characteristics of this compound are not associated with reverse use dependence, which might be beneficial to the antiarrhythmic effect. In addition, kinetic analysis indicates that vesnarinone may access the HERG channel via the hydrophobic pathway and recognize the open-state conformation. This might be the reason why vesnarinone shows a voltage dependence of its effect in spite of its uncharged form.

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