W-7 modulates K\textsubscript{r,4.3}: pore block and Ca\textsuperscript{2+}-calmodulin inhibition

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Qu Y-J, Bondarenko VE, Xie C, Wang S, Awayda MS, Strauss HC, Morales MJ. W-7 modulates K\textsubscript{r,4.3}: pore block and Ca\textsuperscript{2+}-calmodulin inhibition. Am J Physiol Heart Circ Physiol 292: H2364–H2377, 2007. First published January 12, 2007; doi:10.1152/ajpheart.00409.2005.—Ca\textsuperscript{2+}-calmodulin (Ca\textsuperscript{2+}/CaM)-dependent protein kinase II (Ca\textsuperscript{2+}/CaMKII) is an important regulator of cardiac ion channels, and its inhibition may be an approach for treatment of ventricular arrhythmias. Using the two-electrode voltage-clamp technique, we investigated the role of W-7, an inhibitor of Ca\textsuperscript{2+}-occupied CaM, and KN-93, an inhibitor of Ca\textsuperscript{2+}/CaMKII, on the K\textsubscript{r,4.3} channel in Xenopus laevis oocytes. W-7 caused a voltage- and concentration-dependent decrease in peak current, with IC\textsubscript{50} of 92.4 \mu M. The block was voltage dependent, with an effective electrical distance of 0.18 ± 0.05, and use dependence was observed, suggesting that a component of W-7 inhibition of K\textsubscript{r,4.3} current was due to open-channel block. W-7 made recovery from open-state inactivation a biexponential process, also suggesting open-channel block. We compared the effects of W-7 with those of KN-93 after washout of 500 \mu M BAPTA-AM. KN-93 reduced peak current without evidence of voltage or use dependence. Both W-7 and KN-93 accelerated all components of inactivation. We used wild-type and mutated K\textsubscript{r,4.3} channels with mutant CaMKII consensus phosphorylation sites to examine the effects of W-7 and KN-93. In contrast to W-7, KN-93 at 35 \mu M selectively accelerated open-state inactivation in the wild-type vs. the mutant channel. W-7 had a significantly greater effect on recovery from inactivation in wild-type than in mutant channels. We conclude that, at certain concentrations, KN-93 selectively inhibits Ca\textsuperscript{2+}/CaMKII activity in Xenopus oocytes and that the effects of W-7 are mediated by direct interaction with the channel pore and inhibition of Ca\textsuperscript{2+}/CaM, as well as a change in activity of Ca\textsuperscript{2+}/CaM-dependent enzymes, including Ca\textsuperscript{2+}/CaMKII.

Potassium channel; inactivation gating; KN-93; transient outward potassium current; calmodulin kinase II

CALMODULIN (CaM) is a ubiquitous Ca\textsuperscript{2+}-binding protein that plays an important role in the Ca\textsuperscript{2+}-signaling pathways of eukaryotic cells (37, 48, 59). Ca\textsuperscript{2+}/CaM-dependent protein kinase II (Ca\textsuperscript{2+}/CaMKII) is among the many kinases and phosphatases responsive to the Ca\textsuperscript{2+}-bound CaM (Ca\textsuperscript{2+}/CaM) (31a, 34, 49, 56, 64, 71). Recent studies have implicated increased Ca\textsuperscript{2+}/CaMKII activity in the pathophysiology of different types of heart disease, including ventricular arrhythmias (4, 5, 26, 36, 45, 46). These data have shown that agents that lead to a decrease in Ca\textsuperscript{2+}/CaMKII activity, such as W-7 and KN-93, are effective in different animal models of ventricular arrhythmias (4, 26, 36, 45, 46). Despite their inhibition of a common final target, Ca\textsuperscript{2+}/CaMKII, W-7, which inhibits Ca\textsuperscript{2+}/CaM, could also modulate additional enzymes and/or ion channels (4, 37, 38, 48, 59, 75). W-7 binds to CaM and blocks access to the activation site exposed when the Ca\textsuperscript{2+}-binding sites in CaM are occupied (28, 56), thereby blocking the Ca\textsuperscript{2+}/CaM effects. KN-93 competitively inhibits Ca\textsuperscript{2+}/CaM binding to Ca\textsuperscript{2+}/CaMKII (5, 7, 31a). Although prior studies examined the effects of W-7 and KN-93 on the native Ca\textsuperscript{2+} and K\textsuperscript{+} currents in cardiac myocytes, the effects of these two agents on the gating and permeation properties of K\textsubscript{r,4.3} channels have not been analyzed in detail (5, 19, 38, 42, 60, 69).

One target of these drugs is the rapidly inactivating, voltage-dependent K\textsuperscript{+} current (I\textsubscript{to}) in cardiac myocytes, which is regulated by Ca\textsuperscript{2+}-dependent kinases (5, 16, 17, 53, 67, 69). Cardiac I\textsubscript{to} makes important contributions to the early and subsequent stages of repolarization in cardiac, especially atrial, myocytes (12, 16). The consensus is that the K\textsuperscript{+} voltage-dependent K\textsubscript{r,4.2} and K\textsubscript{r,4.3} channels serve as pore-forming molecular substrates for the native I\textsubscript{to} in cardiac myocytes (13, 22, 35, 52, 53, 77). In addition, the effects of KN-93 on the A-type current in the central nervous system and smooth muscle of the gastrointestinal tract have been studied (1, 2, 11, 35, 39, 40).

Ca\textsuperscript{2+}/CaMKII activation and inhibition have been shown to modulate A-type current in colonic and gastric myocytes and in cardiac myocytes (1, 2, 19, 39, 40). Sergeant et al. (63) demonstrated that KN-93 and a constitutively active Ca\textsuperscript{2+}/CaMKII modulated inactivation, recovery from inactivation, and the steady-state inactivation relation of the heterologously expressed K\textsubscript{r,4.3} channel. However, although the role of CaM in modulation of the K\textsubscript{r,4.3} channel is poorly understood, it is likely to be more complex, inasmuch as this ubiquitous Ca\textsuperscript{2+}-binding protein modifies the function of numerous target enzymes (31a, 34, 37, 48, 49, 59, 71, 75). One approach that has helped answer this question has been the use of small organic molecules, such as W-7, which serve as inhibitors of Ca\textsuperscript{2+}/CaM activity (56). Although W-7 has been shown to inhibit I\textsubscript{to} (4, 5, 26, 36, 38, 42, 46), the underlying mechanism is not fully understood, as prior studies have not considered the possibility that W-7 might directly interact with K\textsubscript{r,4.3} and cause open-channel block and use dependence. Therefore, elucidation of the mechanisms of the effects of W-7 on the K\textsubscript{r,4.3} channel is highly desirable.

We set out to examine the effects of W-7 and KN-93 on the K\textsubscript{r,4.3} channel. Our first goal was to establish the extent to which either or both agents affected the permeation and gating properties of the K\textsubscript{r,4.3} channel. Second, we assessed the extent to which inhibition of Ca\textsuperscript{2+}/CaM and inhibition of Ca\textsuperscript{2+}/CaM binding to Ca\textsuperscript{2+}/CaMKII produced similar effects on open-
state inactivation and recovery from inactivation. Changes in open-state inactivation and recovery from inactivation in the presence of BAPTA were used as markers of changes of Ca\(^{2+}/\text{CaMKII}\) activity (19, 63). We demonstrate that W-7 and KN-93 modified the gating properties of the K\(_{4.3}\) channel. W-7, but not KN-93, showed evidence of direct interaction with the inner pore region of the K\(_{4.3}\) channel, as only W-7 showed use-dependent block of K\(_{4.3}\). Both W-7 and KN-93 accelerated all three components of open-state inactivation and slowed recovery from inactivation in wild-type (WT) channels. However, our data indicate that specific concentrations of KN-93 had selective effects on open-state inactivation kinetics that are consistent with a decrease in Ca\(^{2+}/\text{CaMKII}\) activity. On the other hand, W-7 had selective effects on recovery from inactivation but nonselective effects on inactivation, suggesting that its effects on inactivation kinetics were not solely mediated by a decrease in Ca\(^{2+}/\text{CaMKII}\) activity. Hence, our data suggest that the proposed effects of W-7 on the K\(_{4.3}\) channel are mediated by a combination of channel pore blockade and change in activity of different Ca\(^{2+}/\text{CaM}\)-dependent enzymes, including Ca\(^{2+}/\text{CaMKII}\).

**MATERIALS AND METHODS**

**RNA preparation and channel expression.** WT eDNA of K\(_{4.3}\) (short form) was a gift of Dr. David McKinnon (State University of New York at Stony Brook), and its use has been previously described (73). Synthetic mRNA was prepared using an mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX). A K\(_{4.3}\) channel with mutated Ca\(^{2+}/\text{CaMKII}\) consensus phosphorylation sites (S516A and S550A) in the COOH terminus (K\(_{4.3}\)[S516A, S550A]) was prepared by PCR-based site-directed mutagenesis, as previously described (63). *Xenopus laevis* were handled in compliance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols were approved by the University at Buffalo, State University of New York Institutional Animal Care and Use Committee. Mature female *X. laevis* (Xenopus One, Ann Arbor, MI) were anesthetized by immersion in a solution of 1.5% ethyl)\(-(4-methoxybenzenesulfonyl)\]amino-(4-chlorocinnamyl)-(4-chlorocinnamyl)-(4-methylbenzylamine), and water-soluble KN-92 \([2-(N-(4)-methoxybenzenesulfonyl)]aminono-(N-(4)-chlorocinnamyl)]-N\]methylbenzylamine], and water-soluble KN-92 \([2-(N-(4)-methoxybenzenesulfonyl)]aminono-(N-(4)-chlorocinnamyl)]-N\]methylbenzylamine] were obtained from Calbiochem and dissolved in ND-96 solution before use. KN-92 is an inactive structural analog of KN-93 (24). BAPTA-AM (Invitrogen) was dissolved in ethanol. The IC\(_{50}\) of KN-93 on Ca\(^{2+}/\text{CaMKII}\) activity was reported to vary from 11.1 to 20 \(\mu\)M (33, 50).

To evaluate the effects of W-7 and KN-93 in the presence of BAPTA, we used BAPTA-AM dissolved in 100% ethanol and diluted in ND-96 solution to yield a final BAPTA-AM dilution of 1:1,000 (vol/vol).

**Electrophysiological techniques.** Oocytes were voltage clamped using a two-microelectrode “bath-clamp” amplifier (model OC-750-F, WPI) using a two-stage puller (model L/M-3 P-A, Adams & List, Great Neck, NY) filled with 3 M KCl; resistances were 0.6–1.5 MΩ. During recordings, oocytes were continuously bathed in control ND-96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), and 10 mM HEPES, with pH adjusted to 7.4 with NaOH). Currents were recorded at room temperature (21–23°C) and filtered at 2.5 kHz.

**Experimental protocols.** To evaluate the effects of W-7 and KN-93 on heterologously expressed K\(_{4.3}\) channels, we performed voltage-clamp protocols before and after exposure to W-7 or KN-93 in ND-96 solution. Oocytes were superfused with 100 \(\mu\)M BAPTA-AM for 30 min (58, 61, 65) or 500 \(\mu\)M BAPTA-AM for 60 min. Thereafter, the cells were washed and incubated in ND-96 solution for 30 min. Voltage-clamp protocols were then implemented to obtain experimental data after washout of BAPTA-AM. Parallel experiments were performed in WT and double-mutant K\(_{4.3}\) channels.

**Data analysis.** Digitized data were collected and analyzed using pCLAMP software (Axon Instruments) and stored in a computer. Unless otherwise stated, neither leakage nor capacitance was subtracted from the raw data traces from the two-microelectrode voltage-clamp recordings. The pulse protocols and the equations that best fit the data are presented in each case. Values are means ± SE. Confidence levels were calculated using Student’s t-test.

**RESULTS**

**Effects of W-7 and KN-93 on peak K\(_{4.3}\) current.** We established a dose-response relation from an analysis of changes in peak current at +50 mV during exposure to different concentrations of W-7 (Fig. 1). W-7 at >100 \(\mu\)M produced oocytes with a large leak conductance, rendering them unsuitable for use in lengthy experiments. The dose-response relation was fit to the following function: \(f(D) = I([1 + (D/IC50)]^{-n})\), where D is W-7 concentration. The optimal fit yielded an IC\(_{50}\) of 92.4 \(\mu\)M and a power coefficient (\(n\)) of 3.5 (Fig. 1B). The effects of 85 \(\mu\)M W-7 on peak current-voltage relations of K\(_{4.3}\) channels expressed in *Xenopus* oocytes were determined by comparison of the control data with the data in the presence of W-7 (Fig. 2). W-7 reduced the magnitude of peak K\(_{4.3}\) current (\(I_{K_{4.3}}\)) and accelerated inactivation during the depolarizing pulses (Fig. 2, A–C). The peak current at +50 mV was reduced to 55.5 ± 4.3% of the control value at 85 \(\mu\)M W-7 (\(n = 7\)). In a separate set of experiments, we determined that the effects of W-7 on peak current were largely reversible (Fig. 3). W-7 at 85 \(\mu\)M decreased peak current to 54.2 ± 4.4% of the control value; peak current returned to 92.4 ± 0.5% of the control value at the end of a 30-min washout.

To ascertain whether the reduction in peak current was consistent with open-channel block, we examined the voltage dependence of block of peak current and calculated the fractional electrical distance of open-channel block (74, 76) as determined from the slope of the normalized current-reduction-voltage relation. A nonzero value reflects the movement of a charged blocking compound in the permeation pathway and places W-7 at a binding site in the transmembrane electrical field. The fractional block of peak currents in the presence of...
W-7 plotted against test potential in Fig. 2D shows a relation with typical voltage dependence (74). The fraction of the electrical field (δ) sensed by W-7 during a pulse train is 0.18 ± 0.05 (n = 7; Fig. 2D). These data are consistent with open-channel block and the δ value that closely approximates the values reported for quinidine block of Kv1.4 and Kv1.5 channels (67, 69, 74), which suggests that W-7 interacts directly with the Kv4.3 channel through the binding site on the cytoplasmic side of the pore region.

Although the effects of KN-93 on the Kv4.3 channel were reported to modify kinetics of inactivation and recovery (19, 63), these studies did not address whether some of the effects of KN-93 on peak current were also due to open-channel block, i.e., were voltage dependent. KN-93 at 35, 50, and 100 μM reduced peak current at +50 mV by 4.9 ± 3.5%, 14.4 ± 5.9%, and 30.3 ± 3.5%, respectively (Fig. 4). However, there was no evidence for voltage-dependent block, as the calculated value of δ was close to 0 for each of the three concentrations of KN-93. Hence, although KN-93 decreased the peak current, this effect cannot be attributed to open-channel block on the cytoplasmic side of the channel. KN-92, the inactive congener of KN-93, at 100 μM was used as a control and had no effect on the peak current-voltage relation (data not shown).

Use dependence. The differences in voltage-dependent block with W-7 and KN-93 predicted that we would see use-dependent block during exposure to W-7, but not KN-93.

Therefore, we examined the effects of W-7 and KN-93 on the Kv4.3 channel after the onset of a pulse train consisting of 8-ms depolarizing pulses to +50 mV at 2 Hz (Fig. 5). We selected an 8-ms pulse to minimize inactivation during the pulse and concentrations of W-7 and KN-93 that produced a clear-cut reduction in peak current (Figs. 1 and 4). As shown in Fig. 5A, after onset of a 2-Hz pulse train, the normalized currents showed nominal use-dependent block under control conditions. However, exposure to W-7 caused a progressive reduction in magnitude of the peak current until a new steady-state value was reached, and the magnitude of the reduction in the peak current under steady-state conditions was much greater than under control conditions. In contrast, 100 μM KN-93 did not progressively reduce the normalized peak currents after the first pulse, reflecting the absence of use dependence (Fig. 5B). With 35 μM KN-93, the observations were similar (data not shown).

Fig. 1. Dose-response relation for W-7 block of the Kv4.3 channel. A: current recordings from 2-electrode voltage-clamp experiments on Xenopus oocytes at 0 (control), 25, 75, and 100 μM W-7 during a 0.033-Hz train consisting of 2,000-ms depolarizing pulses between −90 and +50 mV (HP = −90 mV) in control and at 85 μM W-7. B: normalized peak current–voltage (I-V) relations in control and at 85 μM W-7. C: current recordings during a 0.033-Hz pulse train consisting of 2,000-ms depolarizing pulses to −40 mV from a holding potential (HP) of −90 mV. D: dose-response relation for W-7. Normalized Kv4.3 currents (I_{Kv4.3,W-7}/I_{Kv4.3,control}) are plotted as a function of test-pulse voltage. Equation that best fits data is as follows: f(V) = K_{D,0 mV}/(K_{D,0 mV} + [D]exp(\delta F V/R T)), where \( z \), \( F \), \( R \), and \( T \) have their usual meanings, \( D \) is W-7 concentration, \( \delta \) is fraction of electrical field sensed by W-7 during the pulse train, and \( K_{D,0 mV} \) is apparent dissociation constant at 0 mV (\( \delta = 0.18 \pm 0.05 \)).

Fig. 2. W-7 block of Kv4.3 channel. A and B: current recordings during a 0.033-Hz pulse train consisting of 2,000-ms depolarizing pulses between −90 and +50 mV (HP = −90 mV) in control and at 85 μM W-7. W-7 decreased peak currents at potentials positive to −40 mV. C: normalized peak current–voltage (I-V) relations in control and at 85 μM W-7. D: voltage dependence of W-7 block of Kv4.3 channel. Normalized Kv4.3 currents (I_{Kv4.3,W-7}/I_{Kv4.3,control}) are plotted as a function of test-pulse voltage. Equation that best fits data is as follows: f(V) = K_{D,0 mV}/(K_{D,0 mV} + [D]exp(\delta F V/R T)), where \( z \), \( F \), \( R \), and \( T \) have their usual meanings, \( D \) is W-7 concentration, \( \delta \) is fraction of electrical field sensed by W-7 during the pulse train, and \( K_{D,0 mV} \) is apparent dissociation constant at 0 mV (\( \delta = 0.18 \pm 0.05 \)).

Fig. 3. Reversibility of Kv4.3 channel block by W-7. Peak I-V normalized relations are shown in control, at 85 μM W-7, and after 30 min of washout. Effects of W-7 on peak current were almost completely reversible.
Fig. 4. KN-93 block of K\(_{A.3}\) channel. A: normalized current recordings during a 0.033-Hz pulse train consisting of 2,000-ms depolarizing pulses between -90 and +50 mV (HP = -90 mV) in control and at 35 and 100 \(\mu\)M KN-93. KN-93 accelerated open-state inactivation kinetics. B: normalized peak I-V relations in control and at 35, 50, and 100 \(\mu\)M KN-93.

shown). Hence, the use-dependent reduction in the peak current during exposure to W-7 is consistent with open-channel block of the K\(_{A.3}\) channel, and the absence of similar use-dependent effects of KN-93 is consistent with the absence of open-state pore block.

**Steady-state inactivation.** Next, we examined the effects of W-7 on steady-state inactivation (Fig. 6A). Steady-state inactivation relations were determined using a 2,000-ms first pulse (P1) followed by a second pulse (P2) to +50 mV. The values were as follows: half-maximal potential, \(V_{1/2} = -46.2 \pm 1.1\) and slope factor \(k = 6.0 \pm 0.3\) mV in the presence of 85 \(\mu\)M W-7 and \(V_{1/2} = -42.8 \pm 3.2\) and \(k = 5.5 \pm 0.5\) mV (\(n = 6\)) for control (Fig. 6A), and \(V_{1/2} = -44.4 \pm 2.3\) and \(k = 5.6 \pm 0.4\) mV in the presence of 100 \(\mu\)M KN-93 and \(V_{1/2} = -44.5 \pm 2.6\) and \(k = 5.2 \pm 0.3\) mV for control (\(n = 5\); Fig. 6B). In contrast to W-7, 35 and 100 \(\mu\)M KN-93 had no effect on the steady-state inactivation relation. There was a 3.4-mV hyperpolarizing shift in \(V_{1/2}\) after exposure to W-7; however, there was no shift in \(V_{1/2}\) after exposure to KN-93. If the modest hyperpolarizing shift in steady-state inactivation in the presence of W-7 was mediated by a decrease in Ca\(^{2+}\)/CaMKII activity, then one would expect the same degree of shift in \(V_{1/2}\) in the presence of W-7 and KN-93. However, since we observed the shift in \(V_{1/2}\) only in the presence of W-7, we conclude that the hyperpolarizing shift resulted from open-channel blockade superimposed on the inactivation induced by P1 during the pulse protocols. Similar shifts in the steady-state inactivation relation have been reported for open-channel blockers of Na\(^+\) channels (8, 29, 30, 31, 66). KN-92 had no effect on the steady-state inactivation relation (data not shown).

**Open-state inactivation kinetics.** Agents that directly decrease the activity of Ca\(^{2+}\)/CaMKII have been shown to accelerate inactivation kinetics of heterologously expressed K\(_{A.3}\) channels (19, 63). W-7 inhibits Ca\(^{2+}\)/CaM effects, which in turn decrease the activity of Ca\(^{2+}\)/CaMKII as well as other kinases and phosphatases. Therefore, the effects of W-7 on inactivation kinetics might reflect its influence on these other kinases and phosphatases and, therefore, could differ from the effects of KN-93. Using a 2,000-ms depolarizing pulse, as previously described (72), we examined the effects of W-7 on the kinetics of open-state inactivation. W-7 at 50 \(\mu\)M accelerated the kinetics of each of the three components of inactivation during a 2,000-ms pulse (Fig. 7A). The effects of W-7 on open-state inactivation were concentration dependent (Table 1). The time constants of the fast (\(\tau_1\)), intermediate (\(\tau_2\)), and slow (\(\tau_3\)) components of inactivation decreased dramatically by 2.6-, 3.1-, and 4.8-fold, respectively, at the highest concentration of W-7 (Table 1). As expected, 100 \(\mu\)M KN-93 also accelerated all three components of inactivation (Fig. 7B) (19, 63). Although the effects of KN-93 on all three components of open-state inactivation were similar to those of W-7, the effects of both agents might not result from a selective reduction in Ca\(^{2+}\)/CaMKII activity in *Xenopus* oocytes (19, 63) (see below).

Since we established that the effects of W-7 on peak current were largely reversible, we also performed a separate set of experiments to determine whether the effects of W-7 on K\(_{A.3}\) channel open-state inactivation kinetics were also reversible. We used a 1,000-ms depolarization pulse, which only results in a biexponential inactivation time course (72). During exposure to 85 \(\mu\)M W-7, \(\tau_1\) and \(\tau_2\) decreased from 47.1 ± 2.7 and 322.4 ± 27.1 ms to 24.8 ± 4.2 and 123.8 ± 7.0 ms, respectively. These time constants returned to 40.5 ± 2.9 and 249.0 ± 12.8 ms at the end of a 30-min wash, which were 85.9% and 77.3% of the control values for \(\tau_1\) and \(\tau_2\). The degree of reversibility in values of inactivation kinetics and peak current after 30 min was similar.
**Closed-state inactivation kinetics.** In addition to open-state inactivation, Kv4.3 channels can inactivate from the closed state at potentials reported for myocardial cells during acute myocardial ischemia (18). Using previously described protocols (72), we examined the effects of W-7 on the kinetics of closed-state inactivation (Fig. 8). The kinetics of closed-state inactivation were evaluated using subthreshold depolarizing pulses (9, 10, 72). These protocols consisted of four pulses: P1 from −100 to +40 mV (800 ms); P2 to −100 mV (5 s); P3 to variable voltages and durations (from −80 to −40 mV and from 800 ms to 14.4 s); and P4 to +40 mV (800 ms), followed by a return to a holding potential of −100 mV. The magnitude of peak current during P4 represents the degree of inactivation that developed during P3. Closed-state inactivation was monoexponential and accelerated by 85 μM W-7 (Fig. 8). At −60 mV, W-7 decreased the time constant of inactivation by 59.2%.

**Recovery from open-state inactivation.** Our data suggest that the changes in $I_{Kv4.3}$ during exposure to W-7 are due to the simultaneous effect of open-channel block and possible inhibition of Ca$^{2+}$/CaMKII activity and that KN-93 acts solely through inhibition of Ca$^{2+}$/CaMKII activity. To further validate this interpretation, we examined recovery from inactivation in the absence and presence of W-7 (Fig. 9) and KN-93, since KN-93 was shown to prolong recovery from inactivation (63). Our data suggested that the association kinetics of W-7 were fast. Hence, for P2, we selected a 10-ms pulse to minimize association of W-7 during P2 in the recovery protocol.

**Fig. 7.** Effects of W-7 on kinetics of open-state inactivation of Kv4.3 channels. Inactivation kinetics were measured during 2,000-ms pulses between −20 and +50 mV (HP = −90 mV) under control conditions and in 50 μM W-7 or 100 μM KN-93. Fitting function for analysis of inactivation is as follows: $f(t) = \sum_{i=1}^{N} A_i \exp(-t/\tau_i)$, where $N = 3$. Data for inactivation time constants were obtained from 6 cells. W-7 and KN-93 caused an acceleration of all 3 components of inactivation.

Under control conditions, recovery from inactivation was monoexponential, with time constant, $\tau_{rec} = 294 ± 34$ ms. Interestingly, 75 μM W-7 changed recovery kinetics, which were best described by two components ($\tau_{rec1} = 399 ± 44$ ms and $\tau_{rec2} = 8,110 ± 505$ ms). The time constant of the first component was increased by 35.7% compared with control values (Fig. 9). The appearance of a second extremely slow component of recovery from inactivation confirmed the presence of open-channel block, representing dissociation of W-7 from the inner channel pore. The y-axis intercept value for the second component is 0.21 ± 0.05, which corresponds to the degree of open-channel block during P1 (Fig. 1). In the presence of 100 μM KN-93, recovery from inactivation remained monoexponential and $\tau_{rec}$ increased by 22.5% from

**Table 1. Concentration-dependent effects of W-7 on open-state inactivation kinetics of Kv4.3 channel**

<table>
<thead>
<tr>
<th>Conc, μM</th>
<th>$\tau_1$, ms</th>
<th>$\tau_2$, ms</th>
<th>$\tau_3$, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control) (n = 7)</td>
<td>53.9 ± 4.6</td>
<td>192 ± 19</td>
<td>1,126 ± 127</td>
</tr>
<tr>
<td>5 (n = 6)</td>
<td>46.0 ± 4.5</td>
<td>157 ± 15</td>
<td>983 ± 52</td>
</tr>
<tr>
<td>25 (n = 6)</td>
<td>41.6 ± 4.7</td>
<td>136 ± 16</td>
<td>782 ± 82</td>
</tr>
<tr>
<td>50 (n = 6)</td>
<td>34.1 ± 5.5</td>
<td>114 ± 26</td>
<td>515 ± 52</td>
</tr>
<tr>
<td>85 (n = 4)</td>
<td>20.4 ± 1.4</td>
<td>62.5 ± 6.2</td>
<td>237 ± 12</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n$, number of cells; $\tau_1$, $\tau_2$, and $\tau_3$ time constants of fast, intermediate, and slow components of inactivation. Inactivation kinetics were determined at a test potential of +50 mV.
338 ± 42 to 414 ± 45 ms (P < 0.03, paired t-test). We suggest that the slowing of the first component of recovery in the presence of W-7 reflects a decrease in CaMKII activity, inasmuch as $\tau_{\text{rec}}$ in the presence of W-7 is similar to $\tau_{\text{rec}}$ in the presence of KN-93 (63). KN-92 had no effect on recovery from inactivation (data not shown).

To exclude the possibility of association of W-7 with the Kv4.3 channel in the inactive state, we examined the amplitude of the second component of recovery from inactivation as a function of pulse duration. We utilized a standard recovery protocol for determination of recovery kinetics, except for changes in P1 duration. Pulses (P1) of 10, 67, and 2,000 ms to +50 mV were applied in the presence of 35 μM W-7. Our data indicate that the amplitude of the second component of recovery from inactivation was independent of pulse duration. The amplitude of the second component of recovery was 7.93 ± 0.92% at 10 ms, 7.88 ± 1.04% at 67 ms, and 6.78 ± 0.86% at 2,000 ms. These data indicate that there is no significant association of W-7 with the Kv4.3 channel during the inactive state.

Interventions designed to identify selective inhibition of $\text{Ca}^{2+}$/CaMKII kinase activity. If changes in the Kv4.3 channel inactivation kinetics are due to $\text{Ca}^{2+}$-dependent phosphorylation, then a reduction of intracellular $\text{Ca}^{2+}$ concentration ([$\text{Ca}^{2+}$]) as seen after washout of 500 μM BAPTA-AM should cause these changes. To ascertain whether the effects of W-7 and KN-93 on Kv4.3 channel open-state inactivation kinetics were independent of changes in [$\text{Ca}^{2+}$], we evaluated the effects of W-7 and KN-93 on Kv4.3 channels in oocytes after washout of 500 μM BAPTA-AM (see MATERIALS AND METHODS). These experiments showed that $\tau_1$ decreased by 9.8 ± 3.2%, $\tau_2$ by 16.3 ± 5.3%, and $\tau_3$ by 0.3 ± 4.6% after washout of 500 μM BAPTA-AM (Fig. 10, Table 2). To establish whether these changes were mediated by a decrease in $\text{Ca}^{2+}$/CaMKII activity, we utilized a double-mutant Kv4.3 channel with modified CaMKII consensus phosphorylation sites, Kv4.3[S516A, S550A] (see MATERIALS AND METHODS) (63). In contrast to the WT channel, inactivation kinetics were unchanged in the mutated channel after washout of 500 μM BAPTA-AM (Fig. 10, Table 2). These data suggest that BAPTA decreased inactivation time constants through a partial decrease in $\text{Ca}^{2+}$/CaMKII activity.

The selectivity of KN-93 for CaMKII has been reported to be concentration dependent, which suggests that its effects on WT and Kv4.3[S516A, S550A] channels should also be concentration dependent (19, 63). The concentration of KN-93 that accelerates inactivation kinetics of WT, but not mutated, channels represents the concentration that selectively decreases $\text{Ca}^{2+}$/CaMKII activity. We examined the effects of 35 and 50 μM KN-93 on Kv4.3 inactivation kinetics in oocytes pretreated for 60 min with 500 μM BAPTA-AM (see MATERIALS AND METHODS) (5, 31a, 19, 33, 63). The changes in the time constants of the three components of open-state inactivation during 2,000-ms pulses at 35 and 50 μM were compared with the kinetics measured after washout of BAPTA-AM and are summarized in Figs. 10 and 11 and Table 2. KN-93 had a concentration-dependent effect on Kv4.3 channel inactivation kinetics (35 vs. 50 μM). Only 35 μM KN-93 caused a significant decrease in each of the three components of inactivation without changing any of the components of inactivation of Kv4.3[S516A, S550A] channel (Fig. 11, Table 2). KN-93 at 35 μM decreased $\tau_1$, $\tau_2$, and $\tau_3$ of WT channels by 7.2 ± 1.3%, 8.5 ± 1.5%, and 13.9 ± 3.5%, respectively. Even at 50 μM KN-93, the decrease in Kv4.3 channel inactivation time constants was much greater in WT than in mutant channels (Fig. 11, Table 2). On the basis of the changes in Kv4.3 channel inactivation kinetics, we conclude that 35 μM KN-93 selectively inhibited $\text{Ca}^{2+}$/CaMKII activity, while additional effects at 50 μM were mediated by other enzymes. We examined these effects at test potentials of +20 and +50 mV and found that the data were voltage insensitive.

To determine whether the effects of W-7 were also a result of a selective decrease in $\text{Ca}^{2+}$/CaMKII activity, we compared the effects of 15, 25, and 50 μM W-7 on WT and mutant channels after pretreatment with 500 μM BAPTA. Exposure to 15, 25, and 50 μM W-7 resulted in a significant acceleration of all three components of inactivation in WT channels (Figs. 10 and 12, Table 3). W-7 at 50 μM decreased $\tau_1$ by 39.3 ± 8.8%, $\tau_2$ by 52.0 ± 5.9%, and $\tau_3$ by 62.3 ± 5.5% (Fig. 12, Table 3).
However, there was no significant difference in inactivation kinetics between WT and mutant channels at any concentration tested. In sum, the W-7-induced changes in inactivation kinetics are inconsistent with selective decrease of Ca\(^{2+}\)/CaMKII activity.

A selective decrease in Ca\(^{2+}\)/CaMKII activity results not only in an acceleration of open-state inactivation kinetics, but also in a slowing of recovery from inactivation (63). As a result, one would predict that this effect on recovery kinetics in the BAPTA-pretreated oocytes would occur in the WT, but not the mutated, K\(_v4.3\) channel. Indeed, the time constant of recovery after washout of BAPTA-AM increased by 23.7 ± 5.7% and 3.6 ± 6.4% in the WT and mutant channels, respectively (Fig. 13, Table 4). We also predict that the effects of W-7 on the first component of recovery kinetics would occur after pretreatment with BAPTA-AM. Hence, we examined the effects of W-7 on the kinetics of inactivation recovery in the WT channel to determine whether these effects were concentration dependent and whether there was a comparable effect in the mutated K\(_v4.3\) channel (Fig. 13, Table 4). Recovery kinetics in the WT channel were selectively slowed at 15 μM W-7. In the mutant channel, W-7 had no or little effect on the first component of recovery kinetics (Fig. 13, Table 4). There were significant differences in the recovery kinetics of WT and
The inhibition of Ca$^{2+}$, W-7, a commonly used inhibitor of Ca$^{2+}$, types of heart disease, including ventricular arrhythmias (2, 4, 6, 7), has been shown to suppress ventricular arrhythmias in animal models (4, 26, 36, 45, 46). To gain an understanding of the mechanisms underlying the effects of W-7, we examined the effects of W-7 and KN-93 on the Kv4.3 voltage-gated K$^+$ channel, which plays an important role in cardiac repolarization in atria and ventricles (13, 16, 53).

The goal of our study was to examine the effects of W-7 on the permeation and gating properties of heterologously expressed Kv4.3 channels in Xenopus oocytes. Comparison of the effects of W-7 with those of KN-93 at a concentration that selectively reduces Ca$^{2+}$/CaMKII activity should help establish whether the inhibition of Ca$^{2+}$/CaM produces effects similar to those attained by competitive inhibition of Ca$^{2+}$/CaM binding to CaMKII. Decreased Ca$^{2+}$/CaMKII activity has been shown to accelerate open-state inactivation and slow recovery from inactivation in HEK-293 cells (19, 63). However, no comparable data are available for such studies performed in Xenopus laevis oocytes. Our data demonstrate voltage-dependent pore block by W-7, but not KN-93. In addition, we have shown that the effects of W-7 did not solely result from inhibition of Ca$^{2+}$/CaMKII activity.

We have demonstrated that W-7 and KN-93 reduced peak current of heterologously expressed Kv4.3 channels in Xenopus oocytes. Although both compounds reduced the peak current, only W-7 caused voltage- and concentration-dependent block. The power coefficient of 3.5 (Fig. 1) represents pore block and inhibition of Ca$^{2+}$/CaM (63, 77). The voltage-dependent effects of W-7, but not KN-93, on peak $I_{K_{A4.3}}$ the hyperpolarizing shift in steady-state inactivation relation, and the use dependence demonstrate that only W-7 fulfills the criteria for open-channel block resulting from binding of W-7 to residues in the cytoplasmic portion of S6, which faces the channel pore (8, 66, 74, 76, 77).

Analysis of recovery from inactivation in the presence of W-7 provides additional evidence for the presence of open-channel block. In the presence of KN-93, recovery was demonstrated to be a monoexponential process, with a slowing of
W-7 and KN-93 on inactivation kinetics were concentration dependent. W-7 was shown to accelerate the kinetics of closed-state inactivation. Recovery from inactivation in the presence of KN-93 was slowed but remained monoexponential, while the first component of recovery from inactivation in the presence of W-7 was slowed to a similar degree. Using WT and mutant [modified CaMKII consensus phosphorylation sites (S516A and S550A)] Kv4.3 channels, we demonstrated that the effects of KN-93 were selective in the WT channel, but only at low concentrations. The nonselective effects at higher concentrations of KN-93 suggest that these effects are mediated independently of the inhibition of CaMKII-mediated phosphorylation of Kv4.3 channels. On the other hand, W-7 did not show a selective effect on open-state inactivation. Despite the nonselectivity of effect on inactivation, the first component of recovery from inactivation (unblocked channels) was slowed selectively by W-7 in WT channels. The nonselective effects of W-7 on open-state inactivation kinetics indicate the presence of multiple Ca\(^{2+}\)-CaM-sensitive regulatory pathways.

To understand the basis of the effects of W-7 on inactivation kinetics, it is useful to recall the effects of CaM in cells. CaM, a ubiquitous Ca\(^{2+}\)-binding protein, plays an important role in the Ca\(^{2+}\)-dependent signaling pathways of eukaryotic cells (37, 48). Among the targets of Ca\(^{2+}\)/CaM are Ca\(^{2+}\)/CaM-dependent kinases and calcineurin (31a, 34, 49, 71). Ca\(^{2+}\) binding to CaM results in a transition from the closed to the open conformation, thereby creating a hydrophobic pocket on the surface of each domain, which is essential for Ca\(^{2+}\)/CaM binding to target enzymes such as Ca\(^{2+}\)/CaMKII (32, 41, 47, 48, 51). Ca\(^{2+}\)/CaM binds to the regulatory domain of Ca\(^{2+}\)/CaMKII and induces a conformational change that releases the catalytic domain from autoinhibition (31a, 44, 64). W-7 binds to Ca\(^{2+}\)/CaM, preventing the activation of its target enzymes (28, 54, 56). To further resolve the mechanism of action of W-7, we compared its effects with those produced by KN-93, an inhibitor of Ca\(^{2+}\)/CaMKII, with little or no effect on other protein kinases, such as PKA and PKC (68), in WT and mutant Kv4.3 channels. In addition, we also used KN-92, a congener of KN-93 that lacks Ca\(^{2+}\)/CaMKII inhibitory activity, as an experimental control (70, 71).

Changes in intracellular Ca\(^{2+}\) levels have also been shown to affect the properties of ion channels through a direct effect
on CaM bound to a putative binding site, e.g., the IQ motif, in an ion channel and through activation of Ca\(^{2+}\)/CaMKII or other Ca\(^{2+}\)-sensitive kinases, resulting in phosphorylation of specific residues (serine/threonine) within the NH\(_2\) and COOH termini of the channel (31a, 23, 43). There is no consensus sequence representing a CaM-binding site in the Kv4.3 channel, and no other evidence suggests that Ca\(^{2+}\)-CaM binds to this channel. Hence, it is likely that the effects of changes in intracellular Ca\(^{2+}\) on the Kv4.3 channel result from Ca\(^{2+}\)/CaMKII-mediated phosphorylation (63). Within the context of these observations and other studies on Ca\(^{2+}\)/CaMKII activity (19, 63), we can begin to understand how Ca\(^{2+}\)-CaM modulates the properties of the Kv4.3 channel in the presence of W-7.

To assess changes in Ca\(^{2+}\)/CaMKII activity, we used changes in open-state inactivation kinetics in a WT vs. a mutant [modified CaMKII consensus phosphorylation sites (S516A and S550A)] Kv4.3 channel as a reporter of the changes in Ca\(^{2+}\)/CaMKII activity after washout of BAPTA-AM. We showed that 500 \(\mu\)M, but not 100 \(\mu\)M, BAPTA-AM accelerated inactivation kinetics in the WT, but not the mutant, channel, suggesting that an appreciable reduction in [Ca\(^{2+}\)]\(_i\) was needed to decrease Ca\(^{2+}\)/CaMKII activity. We suggest that, at 100 \(\mu\)M BAPTA-AM, [Ca\(^{2+}\)]\(_i\) remained at levels that were insufficient to cause an acceleration of open-state inactivation resulting from phosphorylation of Ca\(^{2+}\)/CaMKII consensus sites in the Kv4.3 channel (63). Although our data are consistent with the view that the Kv4.3 channel is phosphorylated under resting conditions (19), the effects of 500 \(\mu\)M BAPTA-AM on gating kinetics could have been mediated also via other Ca\(^{2+}\)-sensitive kinases, such as PKC. In this situation, one would
expect that 500 μM BAPTA-AM would also have had significant effects on the inactivation kinetics of the mutant channel, which was not the case.

We observed a large change in Kv4.3 channel inactivation kinetics at 100 μM KN-93. However, the concentration employed and the magnitude of decrease were much greater than reported by others (19, 63), raising concerns about the selectivity of KN-93. As there are no data identifying a concentration at which the effects of KN-93 on Ca2+-CaM activity in oocytes, we used the approach described above to demonstrate that the effects of KN-93 on open-state inactivation kinetics were selective. We showed that 35 μM KN-93 selectively accelerated open-state inactivation kinetics. Although a higher concentration (50 μM) of KN-93 resulted in a greater acceleration of open-state inactivation kinetics in the WT channel, it also caused a modest effect on open-state inactivation in the mutant Kv4.3 channel, consistent with a decrease in Ca2+-CaM activity.

W-7, on the other hand, interacts with Ca2+-CaM and, as a result, influences multiple Ca2+-CaM-dependent pathways. Our observations illustrate the complexity of its actions. Although we were unable to show that the effects of W-7 on open-state inactivation kinetics were greater in the WT than in the mutant channel, the selective effect on the recovery from inactivation in unblocked channels at 15 μM and the greater effects at higher concentrations suggest that W-7 decreased CaMKII activity. However, the difference in selectivity of W-7 on inactivation and recovery suggests that the Ca2+-CaM-dependent pathways that modulate inactivation and recovery may be different.

Thus our data show that W-7 has five important effects on the Kv4.3 channel. 1) Pore block was voltage dependent, which is consistent with use dependence and a biexponential recovery from inactivation. 2) Inactive state block was not a factor contributing to the acceleration of open-state inactivation. 3) We established a concentration of KN-93 that selectively accelerated open-state inactivation of the WT, but not the mutant, channel in oocytes; however, we could not establish a comparable effect with W-7, suggesting that inhibition of Ca2+-CaM activity in oocytes affects multiple regulatory pathways, including Ca2+-CaMKII. The greater slowing of recovery from inactivation in the WT than in the mutant channels suggests that the selective effects of W-7 on recovery from inactivation result from a reduction in Ca2+-CaMKII activity. 4) The marked acceleration of closed-state inactivation in the presence of W-7 will result in a reduction of peak current elicited from potentials positive to −70 mV. 5) We showed that exposure of the oocytes to 500 μM BAPTA-AM significantly accelerated open-state inactivation only in the WT channel through a decrease in Ca2+-CaMKII activity.

Physiological relevance. Extrapolation of our findings to cardiac myocytes suggests that W-7 and KN-93 would accelerate Ito inactivation kinetics and reduce peak Ito, and these effects would be concentration dependent. Although W-7 and KN-93 accelerated open-state inactivation of the Kv4.3 channel, only W-7 caused open-channel block. Because temperature differences between experiments performed in oocytes and in vivo experiments would have appreciable effects on gating kinetics, our data can only be qualitatively extrapolated to in vivo settings. Since the Kv4.3 channel serves as the molecular basis of Ito in humans and most other mammals, both agents would be predicted to decrease the contribution of Ito to repolarization in the atria and ventricles. Simulations of different ionic currents in ventricular action potentials with use of in silico models have established that a reduction in Ito has its primary effects on the early part of repolarization, namely, the speed of phase 1, the magnitude of the notch, and the voltage level of phase 2 (21, 27, 55, 57). The accuracy of these model predictions for all myocytes in the ventricle is based on the assumption that the channels incorporated in these models are representative of uniform channel density in the myocardium, which we know is not the case, since heterogeneity of channel expression has been widely described (13, 14, 25, 53). The different modeling studies have also shown that the effects of a reduction in Ito or I_{Ks4.3} on simulated action potentials depend on the magnitude of the other repolarizing currents, which vary widely between different animal species (12, 27). In the atria, where heterogeneity of repolarizing currents is even more marked, two sets of simulations have shown that a reduction in Ito can lead to a lengthening of action potential duration (14, 21, 55). Experimental data support these conclusions. For example, Kirchhof et al. (36) showed that W-7 and KN-93 prolonged action potential duration in mouse ventricle, where Ito is dominant, but the
effect of W-7 was greater. We hypothesize that the greater effect of inactivation of W-7 could have been caused by acceleration of inactivation, open-channel block, and use-dependent reduction of $I_{to}$. It would then follow that the antiarrhythmic effects of W-7 and KN-93 could differ.

In general, inhibition of $I_{to}$ leads to an increase in APD, which in turn increases Ca$^{2+}$ influx into the cell and Ca$^{2+}$-induced Ca$^{2+}$ release, thereby affecting excitation-contraction coupling (62). The increase in action potential duration may also terminate reentrant arrhythmias. Inhibition of CaMKII should decrease $I_{to}$ and L-type Ca$^{2+}$ current and, thereby, reduce Ca$^{2+}$ entry into the cell (3). Since Ca$^{2+}$ overload leads to cardiac arrhythmias, KN-93 and W-7 have the potential to be better antiarrhythmic drugs than other class III antiarrhythmic drugs. In addition to the antiarrhythmic effects, alterations in $I_{to}$ resulting from a decrease in peak current and/or an acceleration of inactivation can lead to significant depolarizing shifts in notched potential and phase 2 of repolarization (45, 62).

The loss of the notch and depolarizing shift in the plateau has been shown to cause desynchronization of Ca$^{2+}$ sparks, prolongation and reduction in L-type Ca$^{2+}$ current, and intracellular Ca$^{2+}$ transients (62).

We showed that W-7 caused a marked acceleration of closed-state inactivation of $I_{Kv4.3}$. If this acceleration occurs in native channels, it would cause $I_{to}$ to be inactivated and, therefore, unavailable for opening at resting potentials more positive than those normally encountered in atrial and ventricular myocytes. Our observation is relevant to understanding electrophysiological experiments performed in isolated cardiac myocytes, where the use of holding potentials positive to approximately −65 mV fall in a range where a change in the kinetics of closed-state inactivation could result in effects that are attributed to open-state inactivation. Of more profound significance is the rapid efflux of K$^{+}$ during acute myocardial ischemia, which results in depolarization of the resting membrane potential to a range where closed-state inactivation occurs (18). Failure to routinely evaluate closed-state inactivation in the Kv4.3 channel and $I_{to}$ under different experimental conditions could result in failure to detect a change in the magnitude and kinetics of closed-state inactivation. Hence, the acceleration of closed-state inactivation would lead to a reduction of $I_{to}$ that might mistakenly be attributed to other factors such as CaMKII-mediated effects on K$^{+}$ channel subunit gene expression.

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