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Mechanism of Clofilium Block of the Human Kv1.5 Delayed Rectifier Potassium Channel

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SUMMARY

The effect of clofilium on potassium conductance was studied in excised membrane patches from Chinese hamster ovary cells stably transfected with the Kv1.5/hPCN1 delayed rectifier K^+ channel gene. Bath application of clofilium resulted in current inhibition, displaying concentration-dependent acceleration of the apparent channel inactivation in both outside-out and inside-out patches. The steady state half-inhibition concentration in inside-out patches was 140 ± 80 nM (n = 10), which was less than the half-inhibition concentration of 840 ± 390 nM (n = 10) observed in outside-out patches. Clofilium accelerated apparent current inactivation but did not influence the kinetics of current activation or deactivation. The rate of onset of channel block induced by clofilium was not voltage dependent. In contrast, the rate of recovery from channel block was slower at more hyperpolarized membrane potentials. Elevation of extracellular K^+ levels accelerated recovery from channel block without influencing the rate of onset of block. These data suggest that clofilium may induce channel block by an "activation trap" mechanism. Clofilium may be trapped near the conductivity pore so that permeating K^+ ions promote recovery from clofilium-induced block.

Clofilium is a quaternary ammonium compound that has been shown to have class III antiarrhythmic activity (1–6). The effect of clofilium administration in prolonging the cardiac action potential (1–6) is a result of the ability of clofilium to block multiple K^+ channel types (7–14).

An open-channel mechanism of action for clofilium was deduced from its effect on the transient K^+ conductance in rat ventricles (8). Although clofilium is a positively charged compound that is soluble in aqueous solutions, X-ray diffraction studies have shown that clofilium can partition into cell membranes (15). Castle (8) suggested that externally applied clofilium blocked the transient K^+ current in rat ventricles by partitioning into the plasma membrane and approaching its binding site from the plasmalemmal internal surface.

Recent progress in the molecular biology of voltage-dependent K^+ channels has facilitated the study of drug interactions with cloned channels, the deduced amino acid sequence of which is known. Cloned channels can be expressed at high levels in a mammalian environment without significant contamination from endogenous conductances, thereby presenting a convenient system for studying channel/drug interactions.

We used the voltage-dependent K^+ channel Kv1.5/hPCN1 (16) to study its interaction with clofilium. The genomic sequence of hPCN1 is intronless, and it is the human genomic sequence that we have previously expressed in *Xenopus* oocytes (16) and in a mammalian cell line (17). Human Kv1.5/hPCN1 cDNA was originally cloned from a human insulinoma cell line, but it is also expressed in human heart (18). The rat isoform of Kv1.5 is also expressed in heart and brain (19, 20).

This study found that the Kv1.5/hPCN1 channel is more sensitive to clofilium applied from the intracellular side of the plasma membrane, suggesting that the binding site for clofilium is in an intracellular domain. The kinetics of channel/drug interactions revealed that clofilium interacts predominantly with the open-channel conformation and that the rate of onset of channel block was voltage independent. However, analysis of recovery from current inhibition revealed a voltage dependence for recovery from channel block. The voltage dependence of the recovery rate suggests that clofilium could be trapped in the closed channel, as was first proposed for Na^+ channel block by local anesthetics ("activation trap" hypothesis) (21). Elevation of $[K^+]_o$ during recovery from channel block revealed that K^+ ions promoted clofilium release from this trap. The effect of K^+ permeation on the kinetics of recovery from the channel block suggests that the binding site for clofilium is near the channel pore and that repulsion between positively charged clofilium and permeable K^+ ions may be responsible for relief from the trap.

Materials and Methods

Construction of the stably Kv1.5/hPCN1-transfected cell line. We have recently described the electrophysiological and immu-

This work was done while A.A.M. was a Research Associate Fellow at the University of Chicago.

ABBREVIATIONS: $[K^+]_o$, extracellular K^+ concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CHO, Chinese hamster ovary; CMV, cytomegalovirus.

nochemical characteristics of CHO cells stably expressing the Kv1.5 channel (17). Briefly, CHO cells were transfected with the Kv1.5 channel driven by the CMV early promoter, as follows. The hPCN1/Kv1.5 cDNA was excised from the *Xenopus* oocyte expression vector pSP64T at the upstream *Sph*I site (to remove an ATG not in the reading frame) and the downstream *Eco*RV site, which had been previously converted to a *Bgl*II site, and was subcloned into the pCMV5 plasmid (22). Co-transfection of the DG-44 DHFR$^-$ line of CHO cells with the pCMV-hPCN1 and pSV2-neo plasmids was performed using a modification of the calcium phosphate precipitation method (Stratagene, La Jolla, CA). The positive clones were selected by growth in the presence of 400 μg/ml gentamicin (GIBCO-BRL, Grand Island, NY).

Current recording from transfected CHO cells. Inside-out and outside-out current recordings were made according to the methods of Hamill *et al.* (23). In most cases, the output signal from a List EPC-7 amplifier (Medical System Corp., Darmstadt, Germany) was filtered at 500 Hz with an eight-pole low-pass Bessel filter (series 902; Frequency Devices, Haverhill, MA) and sampled at 1 kHz using an IBM-compatible computer and pCLAMP software (Axon Instruments). To resolve the time course of channel activation and deactivation, the output signal was filtered at 2 kHz and sampled at 5 kHz. The leak and capacitance currents in the case of simple voltage protocols used to determine current-voltage relationships, time courses for channel activation, and deactivation were compensated with the $P/4$ procedure. In the case of the complicated prolonged voltage protocols (for recovery from current inhibition, steady state inactivation, and steady state block), leak current was eliminated by a subtraction procedure (see descriptions in the Results section). Data were processed with pCLAMP, SigmaPlot (Jandel Scientific, Corte Madera, CA), and EXCEL (Microsoft) software packages. The data are presented as mean ± standard deviation, where n, the number of experiments, is given in parentheses.

Borosilicate glass capillaries (WPI, Sarasota, FL) were used to fabricate the recording pipettes. The pipettes were drawn in a four-stage process using a horizontal pipette puller (model P87; Sutter Instruments, Novato, CA). The resistance of the filled pipettes was in the range of 4–8 MΩ. The seal resistance measured at the beginning of the experiment over the voltage range of −100 to −70 mV was in the range of 20–100 GΩ.

Two primary solutions, "intracellular" solution (140 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM EGTA-KOH, 10 mM HEPES, pH 7.2) and "extracellular" solution (140 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4), were used. In the experiments using inside-out patches, the pipettes were filled with extracellular solution, and the recording chamber was filled with intracellular solution. In the experiments using outside-out patches, the pipettes were filled with intracellular solution, and the cells were bathed in extracellular solution. In experiments with high $[K^+]_o$, the extracellular solution had the following composition: 150 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.4. In experiments in which $[K^+]_o$ = 0 mM, an additional extracellular solution was used, of following composition: 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4. Clofilium tosylate was obtained from RBI (Natick, MA). Stock solutions of 1 mM clofilium were used to obtain the final concentrations indicated.

The CHO cells were plated and grown on plastic dishes (Corning) in α-minimum essential medium (GIBCO-BRL) supplemented with 10% fetal calf serum, 1% streptomycin-penicillin, and 200 mg/ml gentamicin (GIBCO-BRL). A specially designed plastic insert, which minimized the effective volume of the dish, was used to accelerate external solution exchange. The external solution could be changed in <1 min. The cells were washed with extracellular (140 mM NaCl) solution (see above) for 25–30 min before the experiments. All experiments were performed at room temperature (22–24°).

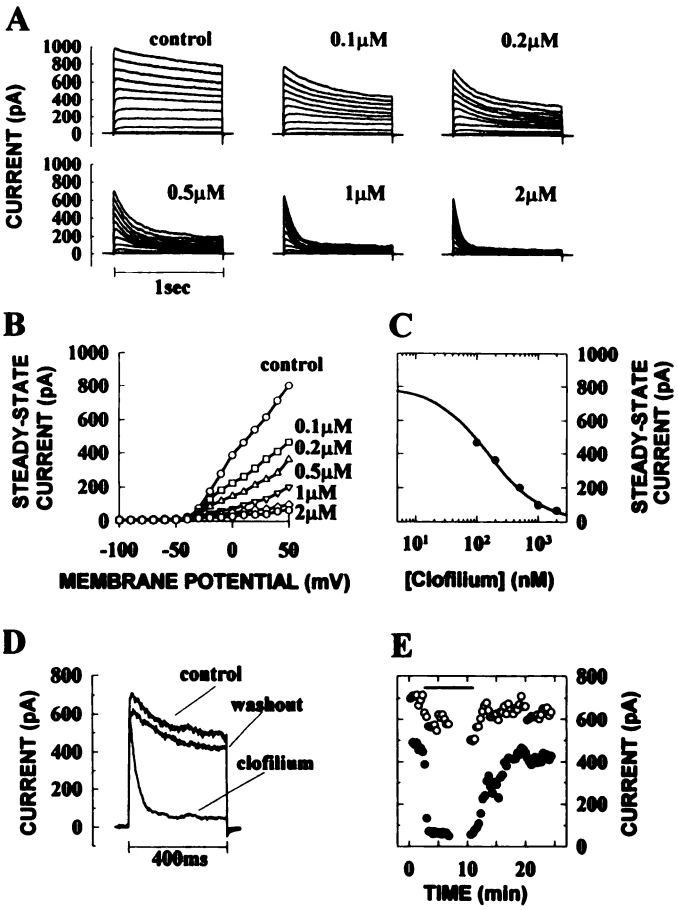

Fig. 1. A, Example of current records from an inside-out patch excised from a CHO cell transfected with the Kv1.5/hPCN1 channel. The membrane patch was held at a holding potential of −70 mV and the potential was stepped to values between +50 and −100 mV for 1 sec at 15-sec intervals. Bath concentrations of clofilium are indicated. The $[K^+]_o$ was 5.4 mM. B, Current-voltage relationships for the current traces shown in A. The current was measured at the end of a 1-sec pulse (steady state current). C, Example of the fit of the steady state current measured in response to membrane depolarization to +50 mV, with the function $y = a/(1 + [\text{clofilium}]/b)$. D, Current records obtained before, during, and after the application of 1 μM clofilium to the intracellular side of an inside-out membrane patch. The current traces were recorded in response to membrane depolarization to +50 mV from a holding potential of −70 mV. E, Time course of onset of the current inhibition and washout of clofilium. ○, Peak current induced in response to membrane depolarization to +50 mV; ●, current measured at the end of the 0.4-sec pulses (steady state current). The $[K^+]_o$ was 5.4 mM. *Horizontal bar*, application of 1 μM clofilium.

Results

Differences in dose-response curves obtained with inside-out and outside-out patch configurations. To investigate the mechanism of delayed rectifier K^+ channel inhibition by clofilium, CHO cells stably expressing the Kv1.5/hPCN1 channels (8C4 line) were used. The effect of the drug on the total ionic conductance was studied in excised (outside-out or inside-out) patches, due to high levels of channel expression in the 8C4 cells (17). An example of current records obtained in an inside-out excised patch in the presence of various concentrations of clofilium is shown in Fig. 1, A-C. Bath application of the drug to either inside-out or outside-out patches induced a reversible apparent current inactivation. The peak current amplitude was affected much less than the current amplitude

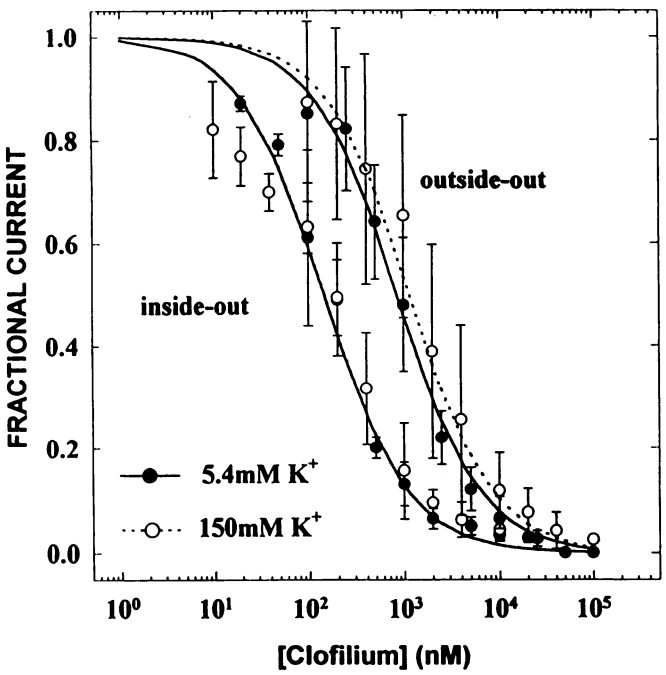

Fig. 2. Dose-response curves for the channel block in inside-out and outside-out patches, obtained at low (5.4 mM) and high (150 mM) $[K^+]_o$. *Points*, normalized averaged values of steady state current measured in response to 1-sec pulses to +50 mV from a holding potential of −70 mV. *Error bars*, standard deviations. Data were obtained from eight and five experiments for inside-out patches (at low and high $[K^+]_o$, respectively) and from eight and six experiments for outside-out patches (at low and high $[K^+]_o$, respectively). *Smooth curves* were drawn using the equation $y = 1/[1 + ([\text{clofilium}]/K_d)]$, where K_d is the average value of IC_{50} obtained in the experiments.

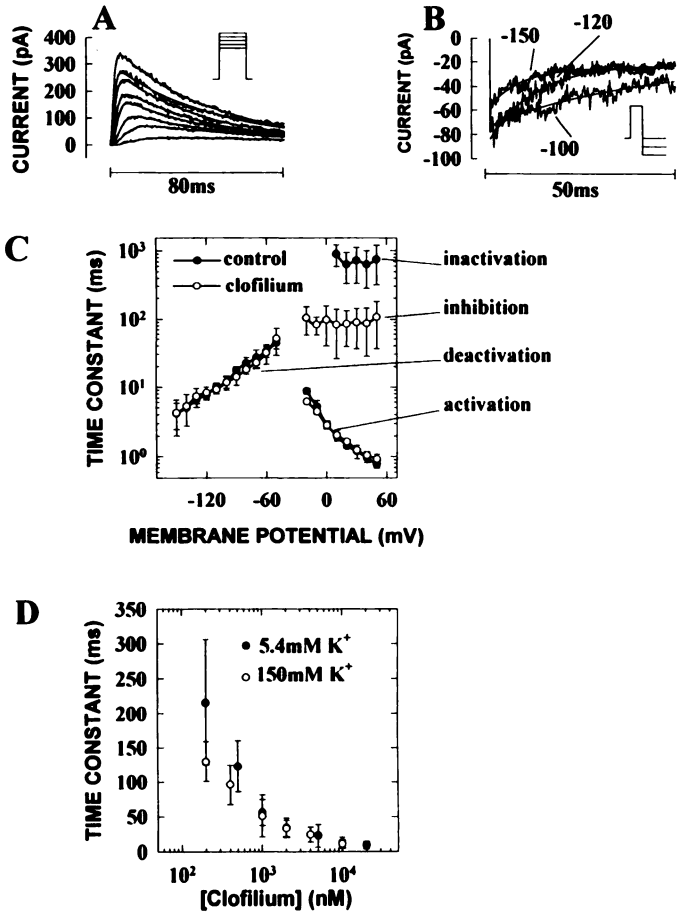

Fig. 3. A, Time course of channel activation in the presence of 1 μM clofilium at the intracellular membrane surface. The inside-out patch was held at −70 mV and depolarizing steps were applied to between +50 and −30 mV, in 10-mV decrements, for 80 msec at 15-sec intervals. Current traces were fit as described in the text. *Smooth curves*, fits to the data. $[K^+]_o$ was 5.4 mM. B, Example of current records obtained in tail current experiments (channel deactivation). First, to activate the channels the membrane was stepped to +50 mV for 250 msec from a holding potential of −70 mV and, second, the membrane potential was jumped to variable levels from −150 mV to −50 mV, in 10-mV increments. The current decay during the second pulse was fit with a monoexponential function. *Traces*, currents obtained at potentials of −150, −120, and −100 mV. *Smooth curves*, fits to the data. The records presented were obtained from an inside-out patch at high $[K^+]_o$ (150 mM), in the presence of 1 μM clofilium. C, Average time constants for channel activation, deactivation, and inactivation in the absence and in the presence of 1 μM clofilium on the intracellular membrane surface. ●, Control measurements; ○, data obtained during exposure to 1 μM clofilium. *Error bars*, standard deviations ($n = 4$). D, Dependence of the time constants for onset of channel block on concentration of clofilium. Data presented are average data obtained in inside-out patches in low ($n = 6$) and high ($n = 5$) $[K^+]_o$ (5.4 mM and 150 mM, respectively).

at the end of a 0.4-sec depolarizing pulse (steady state current). The rate of development of current inhibition was so fast that it could not be resolved because of the limited rate of bath solution exchange. A time course for changes in peak and steady state currents induced by addition of 1 μM clofilium to the intracellular side of the membrane and by subsequent washout of the drug is shown in Fig. 1, D and E. The slight reduction in the peak current amplitude during clofilium application could be assigned to the overlap between channel activation and development of current inhibition. Washout of the drug after 10-min exposure to clofilium resulted in 90% recovery of the peak current amplitude, showing reversibility of the effect of clofilium on the Kv1.5/hPCN1 channel ($n = 8$).

The current measured at the end of a 1-sec voltage pulse to +50 mV from a holding potential of −70 mV was used to construct dose-response curves for channel block (see an example in Fig. 1C). The average normalized current values obtained in inside-out and outside-out patches in the presence of various concentrations of clofilium are shown in Fig. 2. The drug concentration needed to induce 50% steady state current inhibition (IC_{50}) in inside-out patches was 140 ± 80 nM ($n = 10$), in comparison with 840 ± 390 nM ($n = 10$) for outside-out patches. The IC_{50} was not sensitive to changes in $[K^+]_o$ in either inside-out or outside-out patches. The average IC_{50} values obtained with $[K^+]_o = 150$ mM were 150 ± 60 nM ($n = 10$) and 1490 ± 990 nM ($n = 10$) for inside-out and outside-out patches, respectively (Fig. 2). Because the channels were more sensitive to clofilium applied to inside-out patches and the data obtained in the two configurations were qualitatively similar, further studies were carried out with inside-out patches.

Evidence that clofilium does not change parameters describing channel activation and deactivation. The time course for channel activation and channel block in the presence of clofilium was modeled as shown in eq. 1,

$$I = a + b[1 - \exp[-(t - d)/\tau_1]]^4 \exp(-t/\tau_2) \qquad (1)$$

where I is the ionic current, τ_1 is the time constant for channel activation, τ_2 is the time constant for channel inactivation or the onset of channel block, d is the delay in channel activation,

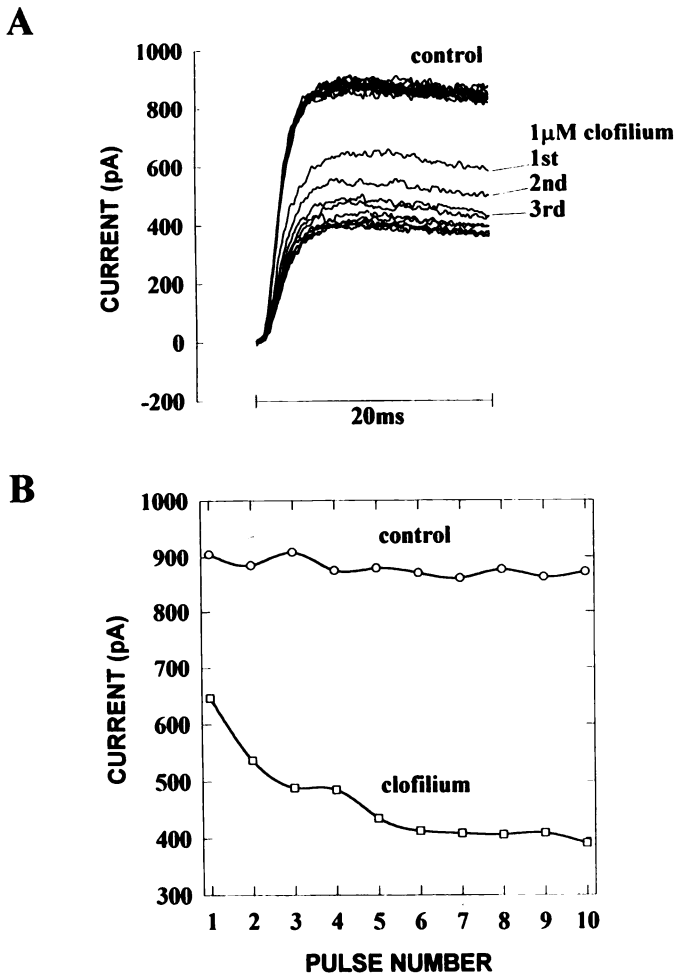

Fig. 4. Use-dependent channel block by clofilium. A, An inside-out patch was held at a holding potential of −70 mV and a train of 10 pulses to +50 mV, of 20-msec duration, at 1-Hz frequency was applied before and during the application of 1 μM clofilium. B, The peak current amplitude measured in the absence and in the presence of clofilium is presented as a function of pulse number. $[K^+]_o$ was 5.4 mM.

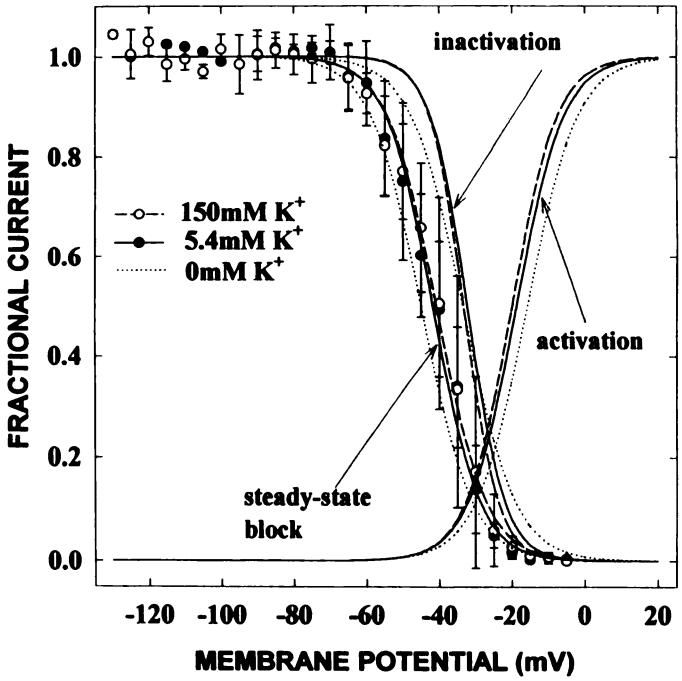

Fig. 5. Voltage dependence of steady state channel block by clofilium at various $[K^+]_o$ values. To obtain the voltage dependence for the steady state block, an inside-out patch was held for 25 sec at variable conditioning potentials and the peak current induced by the test pulse to +50 mV at the end of a conditioning pulse was measured. The average normalized current (see text for detailed measurement procedure) is presented as a function of conditioning potential. Clofilium was applied at a concentration of 1 μM. The *smooth curves* were drawn using the Boltzman equation with average parameters obtained in the experiments (Table 1). The *curves* for channel inactivation are also presented and were drawn using average parameters for the steady state channel inactivation in the absence of clofilium. The voltage protocol to obtain the parameters for the steady state channel inactivation was the same as for the steady state channel block. Voltage dependence for channel activation in the absence of clofilium is also given. The *curves* were drawn using the average parameters for the voltage dependence of channel activation (Table 1). To obtain the parameters for channel activation, a double-pulse protocol was used. An excised patch was held at −70 mV. The channels were activated by application of a short depolarizing pulse to values between +50 and −60 mV, at 5-mV decrements. The activating pulse in the first double-pulse step of this protocol was 10 msec. To reflect the changes in activation time for less depolarized potentials, the activating pulse in subsequent steps was 2 msec longer, and so on. The second pulse stepped the membrane potential to −40 mV, and the current value just after this step was measured. The normalized current value was considered to be an estimate for channel activation. The dependence of channel activation on the magnitude of the first pulse, E, was fit with the function $y = 1/[1 + \exp[(E - E_{0.5})/k]]$, where k is the slope factor and $E_{0.5}$ is the midpoint.

b is the current amplitude, and a is the current offset due to the uncompensated leak conductance. The experimental data were well described by this model. An example of the fit to the experimental data is presented in Fig. 3A.

Parameters for channel deactivation were obtained from tail current experiments in high $[K^+]_o$ (150 mM). The tail current traces were fit with a monoexponential function. An example of the fit is presented in Fig. 3B. The average time constants for current activation, deactivation, and onset of channel block, in the absence and presence of clofilium, obtained in four different experiments are shown as a function of membrane potential in Fig. 3C. The measurements of tail currents showed that in the presence of clofilium the rate of current deactivation was not different from that obtained under control conditions. The time constants for channel activation were also unaffected by clofilium. The time constants for the onset of channel block were voltage independent within the voltage range from −30 to +50 mV but were concentration dependent. The dependence of the time constants for onset of channel block on the concentration of clofilium is shown in Fig. 3D, which illustrates averaged data obtained from eight experiments in low $[K^+]_o$ (5.4 mM) and from five experiments in high $[K^+]_o$ (150 mM). The elevation of $[K^+]_o$ did not influence the rate of onset of channel block.

Application of short (20-msec) depolarizing pulses to +50 mV from a holding potential of −70 mV at 1 Hz revealed the use dependence of channel block. The parameters describing the time course for channel activation did not change during the development of use-dependent block (n = 4) (data not shown). An example of use-dependent block induced by clofilium is shown in Fig. 4.

Open-channel block of Kv1.5 channels. To investigate the efficacy of clofilium in inducing channel block at hyperpolarized membrane potentials, a double-pulse voltage protocol was used. The membrane patch was held at conditioning potentials for 25 sec and the peak current, I, in response to a test

TABLE 1

Comparison of parameters for steady state inactivation, channel block, and channel activation at different $[K^+]_o$ values

The data were fit with the function $y = 1/[1 + \exp[(E - E_{0.5})/k]]$, where E is the membrane potential; $E_{0.5}$ is the half-point of the curve, and k is the slope factor.

$[K^+]_o$	Steady State Block		Steady State Inactivation		Activation	
	$E_{0.5}$	k	$E_{0.5}$	k	$E_{0.5}$	k
mM	mV	mV	mV	mV	mV	mV
150	−41 ± 6 (n = 5)	6.7 ± 0.9	−33 ± 5 (n = 8)	4.7± 1.0	−20 ± 1 (n = 7)	−6.2 ± 0.8
5.4	−42 ± 5 (n = 5)	6.4 ± 0.7	−32 ± 3 (n = 16)	5.1 ± 0.9	−19 ± 3 (n = 19)	−6.5 ± 0.9
0	−45 ± 4 (n = 2)	6.9 ± 0.5	−34 ± 6 (n = 4)	7.1 ± 1.5	−16 ± 3 (n = 4)	−6.9 ± 0.5

pulse to +50 mV was measured (Fig. 5). To minimize the channel block during the test pulse, the duration of the pulse was 30 msec. The current measured for the most hyperpolarized conditioning potential, I_{first}, was used as a control value. The current measured for the most depolarized conditioned potentials, I_{min} (when steady state inactivation and current inhibition occurred and the residual current was the leak current), was subtracted from the actual value to eliminate leak current contamination. The data presented in Fig. 5 were calculated as shown in eq. 2.

$$I_{fractional} = (I - I_{min})/I_{first} - I_{min}) \quad (2)$$

Peak current inhibition could be observed when the membrane was depolarized at levels higher than −65 mV. The peak current inhibition calculated with eq. 2 and presented as a function of conditioning potential E could be fitted with the function y shown in eq. 3,

$$y = 1/[1 + \exp[(E - E_{0.5})/k]] \quad (3)$$

where k is a slope factor for the curve and $E_{0.5}$ is the membrane potential at which half-inhibition occurs. The average parameters for the function obtained in the presence of 1 μM clofilium were 6.4 ± 0.7 mV for the slope factor and −42 ± 5.0 mV (n = 5) for the half-inhibition point, in comparison with k = 5.1 ± 0.9 and $E_{0.5}$ = −32 ± 3.0 mV (n = 16) for steady state inactivation and k = −6.5 ± 0.9 mV and $E_{0.5}$ = −19 ± 3 (n = 19) for channel activation. Also shown in Fig. 5 are data obtained with $[K^+]_o$ = 150 mM and $[K^+]_o$ = 0 mM. The parameters for channel activation, inactivation, and channel block obtained with different $[K^+]_o$ values are summarized in Table 1. The changes in $[K^+]_o$ did not greatly influence the parameters for steady state block, channel activation, or inactivation. The observation that peak current did not change during sustained membrane hyperpolarization suggests that, even if closed-channel block occurred, it was much slower than open-channel block. The voltage dependences of steady state block and channel activation were similar; however, they differed in the midpoints and thresholds. The difference between the thresholds for steady state block (−65 mV) and for channel opening and inactivation (−50 mV) suggests that the changes in channel conformation due to the membrane depolarization may occur at potentials lower than the threshold for channel opening. The binding site for clofilium would then be exposed at a potential more negative than that required for channel opening and subsequent inactivation.

Slow recovery from channel block. The time course for recovery from current inhibition was determined using triple-pulse protocols. Examples of the time course for recovery in the presence and absence of clofilium are shown in Fig. 6, A and B. The degree of recovery was estimated by calculating the value $I_{fractional}$ as shown in eq. 4,

$$I_{fractional} = (I_{peak,test} - I_{steady\ state})/(I_{peak,first} - I_{steady\ state}) \quad (4)$$

where $I_{peak,first}$ is the peak current induced in response to the first 1-sec pulse to +50 mV, $I_{peak,test}$ is the peak current induced in response to a test pulse to +50 mV, which was applied after the first pulse and separated from the first pulse by a conditioning pulse, and $I_{steady\ state}$ is the current measured at the end of the first pulse. By subtraction of $I_{steady\ state}$ from $I_{peak,first}$ and from $I_{peak,test}$, the leak current and current through the channel not blocked by clofilium were eliminated. The value of $I_{fractional}$ was dependent on the level, E, and the length, t, of a conditioning pulse between the first and test pulses. The interval between the sequences of these pulses was 25 sec, to allow for complete recovery from current inhibition. The time course for recovery could be fitted with a double-exponential function y (eq. 5),

$$y = 1 - A\ \exp(-t/\tau_1) - B\ \exp(-t/\tau_2) \quad (5)$$

where τ_1 and τ_2 represent time constants for the fast and slow components, respectively. Recovery from channel inactivation in the absence of clofilium was 95 ± 3% in 3 sec (n = 3) at a conditioning potential of −100 mV, in comparison with recovery from channel block by clofilium of 48 ± 8% (n = 13) obtained under the same conditions. A comparison of the time course obtained in the absence and in the presence of clofilium demonstrates that recovery from channel block by clofilium was only slightly contaminated by recovery from channel inactivation.

Two components in the time course for recovery from channel block were observed in the range of membrane potentials of −150 to −70 mV (see example in Fig. 6C). The voltage dependence of the recovery time constants is shown in Fig. 6D. The time constants for the slow component increased as membrane potential became more hyperpolarized; however, the time constants for the fast component decreased with membrane hyperpolarization. The average relative amplitude of the slow component is presented as a function of membrane potential in Fig. 6E. The relative amplitude was between 0.7 and 0.85 as the membrane potential was changed from −150 to −70 mV. The predominance of the slow component was the reason that the recovery was slower at more hyperpolarized potentials. Analogous voltage dependence of the time course for recovery from channel block was observed previously for the squid axon sodium channel blocked with local anesthetics (24). To explain this phenomenon, the "m-gate trap" (or "activation trap") hypothesis was put forward (21).

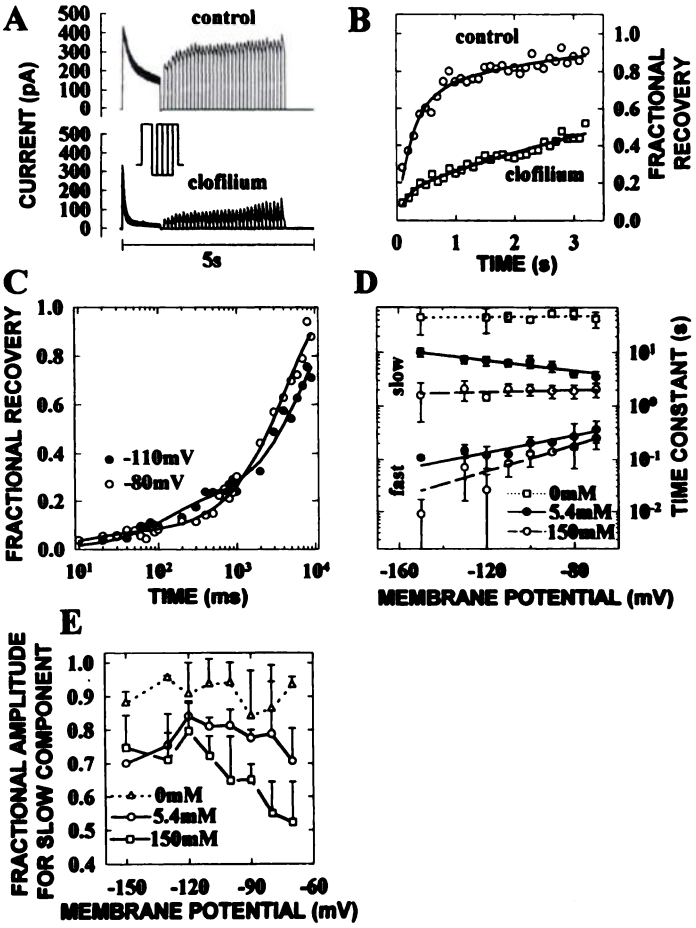

Fig. 6. Recovery from channel block induced by clofilium. A, Comparison of current traces recorded in a control experiment and in the presence of 1 μM clofilium. The current inhibition was induced by application of a depolarizing pulse to +50 mV for 1 sec, from a holding potential of −70 mV. Current inhibition was reversed at a conditioning potential of −100 mV for variable times. The degree of recovery was estimated by measuring the peak current in response to a test pulse to +50 mV applied at the end of a conditioning pulse. $[K^+]_o$ was 5.4 mM. B, Normalized peak current, presented as a function of the duration of the conditioning pulse. The experimental data were fit with a double-exponential function. *Smooth curves*, results of the fit. C, Illustration that the time course for recovery from the channel block cannot be described with a monoexponential function. An inside-out patch was exposed to 1 μM clofilium. The $[K^+]_o$ was 5.4 mM. The time course was obtained for two different conditioning potentials, −80 and −110 mV. The experimental data were fit with a double-exponential function. *Smooth curves*, results of the fit. D, Average time constants for the fast and slow components, obtained at various $[K^+]_o$ values, shown as a function of membrane potential. Standard deviations are also presented (n = 4 for $[K^+]_o$ = 0 mM, n = 4 for $[K^+]_o$ = 5.4 mM, and n = 4 for $[K^+]_o$ = 150 mM). *Solid lines*, fits of the experimental data with the function $y = \exp[k(E - E_0)]$. E, Amplitudes of the slow component for recovery from the channel block induced by application of 1 μM clofilium. The presented data were obtained from the same patches as in D. The average amplitudes of the slow components obtained at various $[K^+]_o$ values are presented as a function of membrane potential. The standard deviations are also shown.

Effect of $[K^+]_o$ on recovery from channel block. If positively charged clofilium is trapped inside the channel, it could interact with K^+ ions and this interaction could cause changes in the observed time course for recovery from channel block. To test this assumption, experiments on recovery from channel block were performed at high $[K^+]_o$ (150 mM) and in K^+-free solutions. The averaged time constants for both components of recovery are presented in Fig. 6D. An elevation in $[K^+]_o$ resulted in acceleration of the recovery, which was seen as a decrease in the time constants for both components. Although the time constant for the slow component did not reveal obvious voltage dependence in the case of high $[K^+]_o$, the relative amplitude of the slow component was higher at more hyperpolarized potentials (Fig. 6E).

Withdrawal of external K^+, in contrast, resulted in slowing of the recovery. The relative amplitude of the slow component in the absence of extracellular K^+ ($[K^+]_o$ = 0 mM) was in the range of 0.85–1 (Fig. 6E). The fast component in the absence of extracellular K^+ could not be resolved in 36% of the measurements performed (n = 28). The lack of voltage dependence for both the time constant and the relative amplitude of the slow component in the case of $[K^+]_o$ = 0 mM could be the result of inherent limitations in the duration of the triple-pulse voltage protocols. The estimate of 45 sec for the time constant means that the interval between the series of pulses should be at least several minutes to provide for complete recovery from channel block. This would require that the entire voltage protocol be 0.5–1-hr long, which is beyond the life-time of excised patches used in these studies.

Discussion

Activation trap of clofilium in the Kv1.5 channel. An important finding of this study is the discovery of slow recovery from clofilium block and its voltage dependence. The slow rate of recovery from current inhibition indicates that clofilium remains bound to the channel for a long time at potentials where the channel should be closed. Analogous behavior was observed for squid axon Na^+ channels blocked by local anesthetics (21, 24). To explain the slow rate of recovery from the channel block induced by local anesthetics, the activation trap hypothesis was put forward by Yeh (21). In this model, the drug could be trapped inside the channel by an activation gate when the channel closes. To release the drug from the channel, the channel must open. The probability for the voltage-activated channels to be open at potentials that are far beyond the threshold for activation is low. This hypothesis can explain the voltage dependence of the recovery rate. The presence of the fast component and its opposite voltage dependence in the case of the Na^+ channel blocked by local anesthetics were explained as recovery from channel inactivation (25), because the rate of recovery from inactivation also was accelerated by hyperpolarization of the membrane potential. Previous work on the interaction between the fast inactivating K^+ channel and clofilium (8) did not find a voltage dependence of recovery from channel block. Differences were also observed in the rate of development of current inhibition. To achieve steady state inhibition, the fast inactivating channels were exposed to clofilium for >0.5 hr (8), in contrast to 1 min employed in this study and in the case of noninactivating K^+ channels (7). These differences suggest that clofilium may interact with K^+ channels by several mechanisms.

Interaction between clofilium and K^+ in the Kv1.5 channel. Elevation of the concentration of permeant ions induced acceleration of recovery from the channel block by clofilium. Relief from channel block by permeant ions was observed previously for voltage-activated K^+ channels blocked by tetraethylammonium ions (26) and for Ca^{2+}-activated K^+ channels blocked by Ba^{2+} ions (27), by charybdotoxin (28), or

by Na^+ ions (29). Relief from channel block by permeant K^+ ions suggests that clofilium is trapped in the channel near the conducting pore. Filling the pore with K^+ ions permeating from the extracellular mouth of the pore might induce electrostatic repulsion between positively charged clofilium and the K^+ ions. This repulsion could accelerate the release of clofilium from the channel. Changes in $[K^+]_o$ did not change the gating parameters of the channel and did not influence the kinetics of onset of channel block. On the other hand, changes in $[K^+]_o$ not only influenced the rate of recovery from the channel block but also shifted the distribution between the slow and fast components of recovery. The fast, voltage-dependent component of recovery was present in experiments in the presence of extracellular K^+ but was only weakly observed in the absence of extracellular K^+. Although this study does not reveal the mechanism for the fast component of recovery, it is reasonable to assume that it is derived from the interaction between charged clofilium and K^+ ions. The voltage dependence of the time constants for the fast component could be the result of the voltage dependence of K^+ ion permeation.

Binding of clofilium to an intracellular domain of the Kv1.5 channel. Our results demonstrate that the Kv1.5/hPCN1 delayed rectifier channel is highly sensitive to clofilium. The IC_{50} of 140 nM is similar to 0.5 μM reported for the transient K^+ conductance in rat ventricle (8) but much less than the values reported for other K^+ and Na^+ channels (7–11, 14, 25) (Table 2). This discrepancy could be the result of measurement procedures or the presence of different K^+ channel isoforms. Recent data (15) suggest that clofilium partitions into the plasma membrane, thereby allowing entry into the cytoplasm. The discrepancy in the IC_{50} values obtained for inside-out and outside-out patches suggests that the Kv1.5/hPCN1 channel binding site for clofilium is closer to the intracellular surface. It is interesting that an analogous sidedness of effect has been reported for the class III antiarrhythmic drug tedisamil (30). Thus, current measurements in the whole-cell configuration with clofilium applied externally might not reflect direct channel/drug interaction but could be dependent on the rate of clofilium partitioning in the plasma membrane. This study performed with isolated patches showed that the block of the Kv1.5/hPCN1 channel is reversible, in contrast to the whole-cell studies (7, 8, 11).[1] The slow rate of reversal previously observed in other preparations (7, 8, 11) is probably the result of clofilium accumulation within the cells.

Open-channel block. Taken together, the shape of the voltage-dependence curve for steady state channel block and the apparent acceleration of the inactivation kinetics show that clofilium interacts with the channel in the open state conformation. The slight inhibition of the peak current is probably the result of contamination of current activation with current inhibition and some modest access of clofilium to the channel binding site at a holding potential of −70 mV. The observation that clofilium accelerates the apparent current inactivation and does not influence channel deactivation [in contrast to data reported for Kv1.5 channels exposed to quinidine (31) and polyunsaturated fatty acids (30)] suggests that clofilium does not just "plug" the open channel and "unplug" the channel during closing, and it is consistent with the conclusion that clofilium remains bound after channel closure.

TABLE 2

Comparison of IC_{50} values for clofilium shown for different K^+ and Na^+ channel isoforms

Source	IC_{50}	Ref.
Kv1.5/hPCN1 K^+ channel from insulinoma	140 nM	This study
Transient K^+ conductance from rat ventricle	0.5 μM	8
Delayed rectifier K^+ channel from mouse neuroblastoma × rat glioma hybrid (NG 108-15) cells	4 μM	11
Delayed rectifier K^+ channel from guinea pigs	50 μM	7
K^+ channel from human T lymphocytes	60 μM	10
K^+ channel from human heart	60 μM	14
K^+ channel from human T lymphocytes	80 μM	10
K^+ channel from mouse heart	100 μM	9
Na^+ channel from rabbit skeletal muscle	3.4 μM	25

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