Distinct Mechanisms of Block of Kv1.5 Channels by Tertiary and Quaternary Amine Clofilium Compounds

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ABSTRACT The quaternary ammonium compound clofilium and its tertiary amine derivative LY97241 were used to analyze mechanisms of block in a voltage-gated potassium channel. Wild-type and mutant Kv1.5 channels expressed in *Xenopus* oocytes were recorded by two-electrode voltage clamp. Open-channel block to 20% of the control current amplitude was induced reversibly by 50 μM clofilium or 200 μM LY97241, and was seen as an acceleration of the macroscopic current decay. Although blockers remained present after application, channels recovered from block during each interpulse interval. The optimum voltage for recovery (−45 mV at pH 7.3) at the threshold for channel activation indicated that clofilium block and recovery occurred principally through the open channel state. In contrast, LY97241 appeared to exit from the closed state and the open state. In an acid-tolerant Kv1.5 mutant channel (H452Q), external pH was used to titrate LY97241. At low pH, which protonates the LY97241 amine group, recovery from block at hyperpolarized potentials was impaired in a manner similar to that seen with clofilium. Recovery from clofilium block was reduced at negative potentials independent of pH, an effect attributed to trapping of the permanently charged compound within the closed channels.

INTRODUCTION

Voltage-gated potassium channels undergo use-dependent block by quaternary ammonium (QA) ions such as tetraethylammonium (Armstrong, 1969; Choi et al., 1993). These compounds bind at receptor sites near the channel pore and physically occlude the ion permeation pathway (Armstrong, 1971; Choi et al., 1993). Potassium channel block by cytoplasmic QA ions is state-dependent; channel opening is required for the drug to gain access to the internal receptor site (Armstrong, 1971). When potassium channels close with the QA ion still bound to the internal receptor site, the blocker can become "trapped" within the channel. Hyperpolarization enhances trapping by decreasing the probability of channel opening. Trapping of TEA in a mutant *Shaker* channel has been attributed to physical blockade by an internal potassium channel gate (Holmgren et al., 1997).

The blockade of voltage-gated sodium channel by local anesthetics shows an analogous use-dependence (reviewed by Hille, 1992). Trapping of lidocaine and its derivatives in the closed state depends on the charged state and hydrophobicity of the drug. For example, with the quaternary amine derivative of lidocaine, QX-314, little recovery from block occurs at hyperpolarized membrane potentials. Conversely, trapping of the tertiary amine lidocaine is less evident at hyperpolarized potentials. The difference in trapping propensity for tertiary versus quaternary amine local anesthetics has been postulated to arise from the coexistence of hydrophilic and hydrophobic pathways for drug access to its receptor site in the Na^+ channel. In this model, the pore of the open sodium channel provides for hydrophilic access to the receptor site, whereas the hydrophobic pathway is restricted to neutral tertiary amines capable of moving past barriers that trap charged compounds.

We were interested in determining if a class III antiarrhythmic QA compound clofilium and its tertiary amine derivative (LY97241) could be used to identify analogous hydrophilic and hydrophobic pathways for receptor access in an identified voltage-gated potassium channel (Kv1.5). Although LY97241 is known to inhibit cardiac potassium currents in vitro (Arena and Kass, 1988; Castle, 1991; Zhang and Steinberg, 1995), the blocking properties of this compound have not been characterized in depth previously. As reported here, we compared block of Kv1.5 by clofilium to that induced by LY97241. We confirmed prior work (Malayev et al., 1995) by showing that clofilium undergoes processes of block and recovery principally through the open channel state, and provide new data showing that, in contrast, the tertiary amine LY97241 appears to exit the blocking site from the closed state and the open state in Kv1.5 channels. Using external pH to titrate the charge of LY97241, results presented here demonstrate that the recovery from block for Kv1.5 channels in the closed state depends on the neutral conformation of the blocker. Recovery of Kv1.5 channels from block by clofilium is reduced at negative membrane potentials, presumably due to trapping of the charged compound within closed channels.

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MATERIALS AND METHODS

Molecular techniques

The gene for Kv1.5 cloned from rat was provided by Dr. R. Swanson (Swanson et al., 1990). We transferred the cDNA for Kv1.5 into a pBluescript KS vector (Stratagene, La Jolla, CA) modified by the insertion of the 5′ untranslated region of *Xenopus* β-globin to enhance expression in oocytes. Plasmid DNA was linearized at a BstXI site in the polylinker region and used to synthesize RNA in vitro with T3 RNA polymerase (Boehringer Mannheim, Indianapolis, IN). Construction and characterization of a pH-tolerant mutant, Kv1.5 H452Q, has been described previously (Steidl and Yool, 1999).

Oocyte preparation

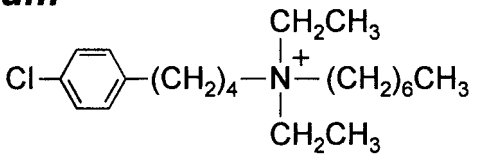
Oocytes from anesthetized mature female *Xenopus laevis* were obtained by surgical removal of several lobes of ovary. Follicular cell layers were removed by treatment with collagenase (type I, 1.5 mg/ml; Worthington Biochemical, Freehold, NJ) and trypsin inhibitor (type III-O chicken egg white, 0.5 mg/ml, Sigma, St. Louis, MO) for ~2 h in nominally calcium-free medium (104 mM NaCl, 3.3 mM KCl, 1.3 mM $MgCl_2$, 6.3 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, pH 7.6). Prepared oocytes were injected with ~0.5–1 ng of mRNA in 50 nl of sterile water and incubated for 1–7 days at 18°C in culture medium (ND96: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM pyruvic acid, 100 units/ml penicillin, 100 μg/ml streptomycin, pH 7.6) before recording.

Electrophysiological recording

The composition of the recording saline was 100 mM NaCl, 4.3 mM $MgCl_2$, 2.0 mM KCl and 5.0 mM HEPES, pH 7.3 for characterization of block of Kv1.5 by clofilium and LY97241 at neutral pH. The pH-dependence of recovery of Kv1.5 H452Q channels from block was tested with a bath saline composed of 100 mM NaCl, 5 mM $MgCl_2$, 2 mM KCl, 2 mM $CaCl_2$, 2 mM EGTA, and either 5 mM 2-[N-Morpholino]ethane-sulfonic acid (MES, pH 5.3 and pH 6.3), 5 mM HEPES (pH 7.3), or 5 mM Trizma (pH 8.3). Two-electrode voltage-clamp recordings at room temperature used two electrodes (0.5–2.0 MW) filled with 3 M KCl. Clofilium (Research Biochemicals Incorporated, Natick, MA) and LY97241 (a gift from Lilly Research Laboratories, Greenfield, IN) were stored frozen in small aliquots as 5- or 10-mM solutions and diluted to a final concentration with recording saline on the day of use. Test saline solutions were applied to oocytes by perfusion with a volume ~5 times that of the recording chamber; recordings were done in a static bath. Voltage-gated potassium currents were activated with step protocols from a holding potential of −80 mV. The expressed currents ranged from 2 to 8 μA. Data were recorded with a GeneClamp (Axon Instruments, Foster City, CA), filtered at 2 kHz, digitized at 200-4000 μsec, and analyzed with pClamp software (Axon Instruments).

Time constants for recovery from block by clofilium were determined by fitting the percent recovery plotted as a function of time with a single exponential equation, $y = A_0 + A_1\exp(-t/\tau_1)$, where τ is the calculated time constant, A is amplitude, and t is time. Recovery from block by LY97241 was better described by a double exponential function, $y = A_0 + A_1\exp(-t/\tau_1) + A_3\exp(-t/\tau_2)$. Analysis of residuals demonstrated a substantial improvement for a double exponential fit of data for LY97241, as compared with a single exponential fit. In contrast, data for clofilium showed an equivalent distribution of residuals for both single and double exponential fits. To confirm the validity of using a single exponential for clofilium data, we compared the results of a double exponential fit. Double exponential fits of clofilium data yielded a major component with a τ value comparable to that seen for the single exponential fit, and a second meaningless component with a small amplitude.

Clofilium



LY97241

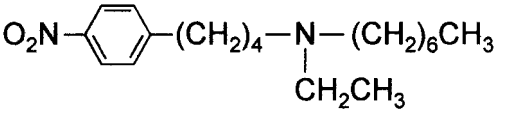


FIGURE 1 Structures of clofilium (Research Biochemicals Incorporated) and the p-nitro tertiary amine analog LY97241 (Lilly Research Laboratories).

RESULTS

The structures of clofilium and the tertiary ammonium analog LY97241 are shown in Fig. 1. Clofilium carries a permanent positive charge in a quaternary amine group. LY97241 differs from clofilium in having a tertiary amine that allows it to exist in a neutral or charged state depending on protonation state, and also has a substitution of a para-nitro group for the para-chloro group on the aromatic ring. The compound LY97542 (not shown) has a permanent positive charge, and a para-nitro group on the aromatic ring. The effects of pH and channel open state on the blocking properties of these compounds were tested for Kv1.5 channels expressed in *Xenopus* oocytes.

Open channel block

Oocytes expressing wild type Kv1.5 channels were stepped repeatedly from a resting membrane potential of −80 mV to a test potential of +40 mV for 1600 ms to elicit outward potassium currents. Properties of endogenous slow inactivation were first analyzed under control conditions, and then salines containing either LY97241 or clofilium at final concentration were perfused into the recording chamber. After perfusion of the blocker in the bath saline, a persistent reduction in peak current amplitude occurred, referred to as initial block. After the rapid initial block, the remaining current showed a stable state-dependent process of recovery and reblock that stayed relatively constant throughout the experiment. The state-dependent channel block by clofilium (Fig. 2 *A*) and LY97241 (Fig. 2 *B*) was evident in an acceleration of the macroscopic decay phase at depolarized potentials. This fast decay component was larger in amplitude and faster in time course than the inherent slow inactivation

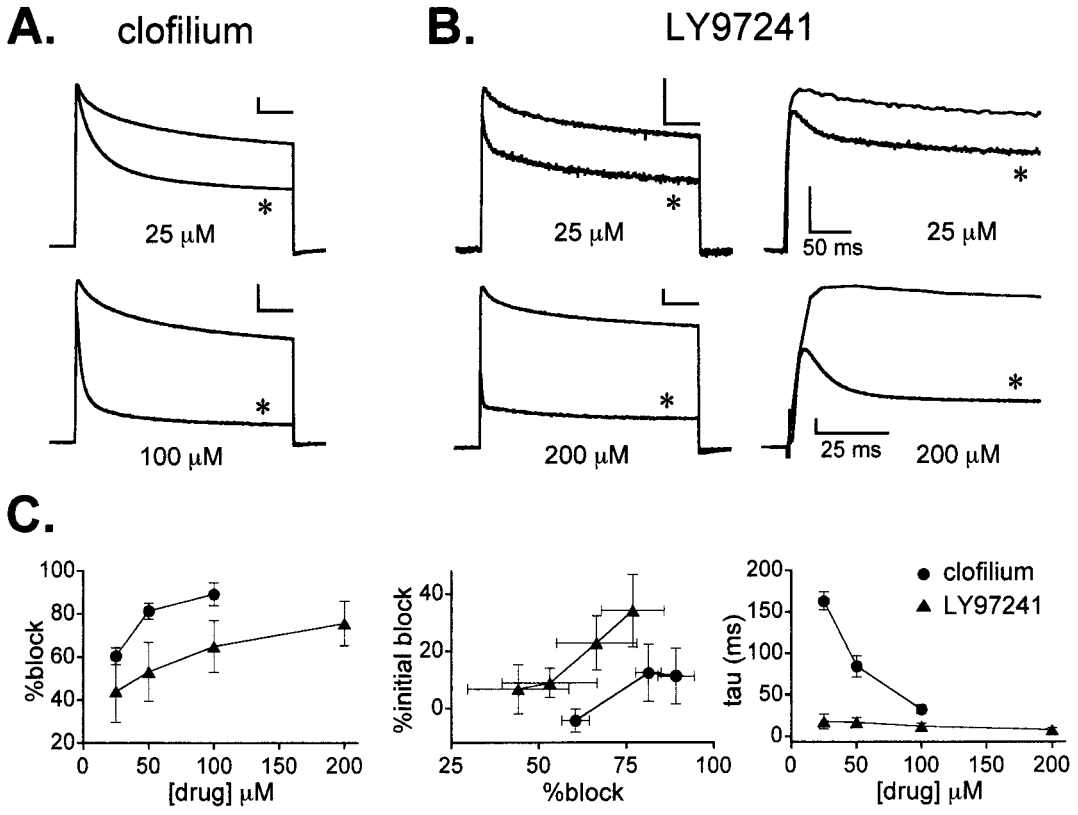


FIGURE 2 Block of Kv1.5 with clofilium and LY97241. Kv1.5 currents recorded with two-electrode voltage clamp are shown for steps to +40 mV from a holding potential of −80 mV before and after (∗) perfusion of recording bath with (*A*) clofilium or (*B*) LY97241. Examples illustrate the lowest and highest concentrations of blocker tested. Data for LY97241 also are shown on an expanded time scale (*B, far right*). The amplitudes of the peak control current and apparent steady state (SS) current with blocker were determined, and percent SS block was calculated as 100 × (peak control − SS drug)/peak control and plotted as a function of drug concentration (*C, left panel*). Initial block was defined as (peak control − peak drug) and plotted as a function of SS block (*C, middle panel*). To determine the time constants for onset of block *t*, the current in the presence of blocker was expressed moment to moment as a fraction of the current in the absence of blocker, and the apparent decay phase was fit with a single exponential equation, $y = A_0 + A_1 e - t/\tau$. τ is plotted as a function of drug concentration (*C, right panel*). Data points are mean ± SD for 4 to 12 oocytes per treatment. Scale bars for traces are 0.5 μA and 250 ms unless otherwise indicated.

process. The blocking compounds remained present after application; however, the channels recovered from block during each interpulse interval (30 s at −80 mV), such that the traces evoked by repetitive voltage steps were superimposed. The magnitude of the initial block of the peak current was more pronounced with LY97241 than with clofilium (Fig. 2 *C, middle*). The initial block measured as the decrease in peak current amplitude at +40 mV after addition of clofilium was 12 ± 10% at 50 μM ($n = 4$) and 11 ± 10 at 100 μM ($n = 5$). The initial block with LY97241 was 9 ± 5% at 50 μM ($n = 9$), 23 ± 9% at 100 μM ($n = 10$), and 34 ± 13% at 200 μM ($n = 10$). Data are mean ± SD.

Clofilium and LY97241 were tested to determine concentrations that induced similar but subsaturating levels of steady-state block (Fig. 2 *C, left*) for further experimental comparisons. Clofilium at 50 μM induced 81 ± 4% block, and LY97241 at 200 μM induced 76 ± 10% block, (mean ± SD, $n \geq 4$). The effective blocking concentration for clofilium was higher in oocytes than that reported for Kv1.5 channels expressed in CHO cells (Malayev et al., 1995). As expected, the rate of net onset of block, approximated by exponential fits of the macroscopic decay phase, was concentration-dependent (Fig. 2 *C, right*). Time constants from exponential fits of the fast component of decay were significantly lower with LY97241 (7.4 ± 3.2 ms, $n = 10$ at 200 μM; mean ± SD) than with clofilium (84 ± 13 ms, $n = 4$ at 50 μM). This difference in the rate of onset of channel block suggested that LY97241 might have faster access to its binding site, a hypothesis we explored by analyzing the time and voltage dependence of recovery of Kv1.5 channels from block.

Time dependence of recovery from block

The time required for recovery from block was evaluated in wild-type Kv1.5 channels using a two-pulse protocol in the continuous presence of blocker (Fig. 3, Table 1). Complete

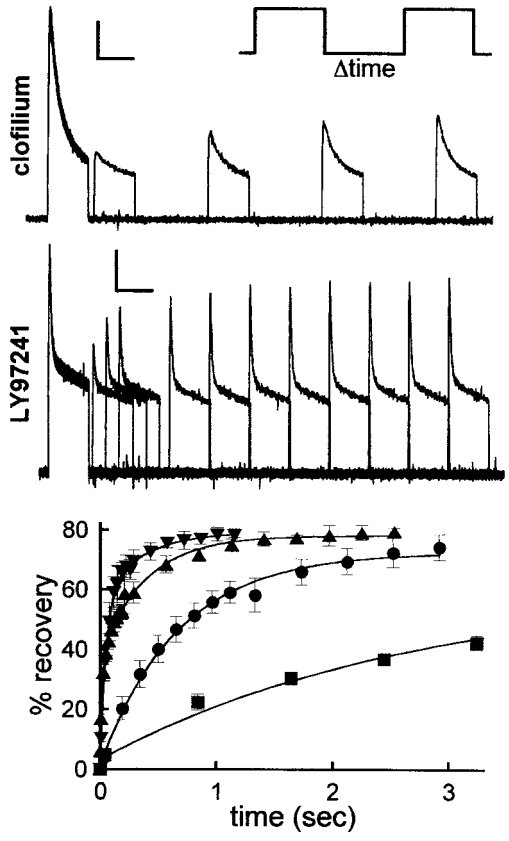


FIGURE 3 Time dependence of recovery from block in wild type Kv1.5. Recovery from block was measured with a two-pulse protocol in the continuous presence of blocking agents. A conditioning pulse to +60 mV for 288 ms was used to induce block. Recovery was tested at −45 or −80 mV for variable time intervals (2 to 7200 ms). The graph (*bottom panel*) is truncated at 3.3 s for clarity. A test pulse to +60 mV was delivered to evaluate recovery from block. Examples of recovery at −80 mV in the presence of either 50 μM clofilium or; 200 μM LY97241 are shown in the top and middle panels. The peak and apparent steady state of the conditioning pulse and the peak of the test pulse were determined ($peak_1$, steady $state_1$, and $peak_2$, respectively). Percent recovery was calculated as $100 \times (peak_2 - steady\ state_1)/(peak_1 - steady\ state_1)$ and plotted as a function of recovery time interval (*bottom panel*). Data are mean ± SD (5 to 7 oocytes) in 50 μM clofilium at recovery potentials of −45 mV (*circles*) or −80 mV (*squares*) recovery potentials, and in 200 μM LY97241 at −45 mV (*inverted triangles*) and −80 mV (*triangles*). Scale bars for traces are 0.5 μA and 250 ms.

recovery from open channel block was obtained during the 30-s interval between sequential traces, as seen in the superimposition of initial peak currents. A conditioning pulse to +60 mV was used to induce open channel block, and a subsequent test pulse to +60 mV was used to evaluate recovery from block as a function of time (2–7200 ms). Traces illustrate a substantially slower rate of recovery for clofilium (Fig. 3, *top*) as compared with LY97241 (Fig. 3, *middle*) at a recovery interval voltage of −80 mV. Recovery was standardized to the net amplitude of open channel block obtained in the first pulse, and plotted as a function of the duration of the recovery interval at −45 and −80 mV (Fig. 3, *bottom*). Data were fit with a single exponential equation for clofilium and a double exponential for LY97241, as determined by the minimum number of components needed to achieve a good fit defined by an analysis of residuals (see Methods). An additional ultraslow component of recovery (not analyzed) was present in all traces, accounting typically for 20–25% of the current amplitude, and was complete within the 30-s interval between successive traces.

TABLE 1 Time constants for recovery of Kv1.5 currents from block by clofilium and LY97241, analyzed at two recovery voltages

Blocker	Dose (μM)	Voltage (mV)	τ_1 (ms)	τ_2 (ms)	n
Clofilium	50	−45	671 ± 149	—	4
	50	−80	2795 ± 236	—	7
LY97241	200	−45	51.5 ± 9.2	387 ± 68	3
	200	−80	16.3 ± 3.3	395 ± 118	5

Data for clofilium were well fit by a single exponential; data for LY97241 required a double-exponential fit (see Methods for details). Data shown are mean ± SD; n is the number of oocytes used.

The exponential fits were used to estimate time constants for the net recovery process (Table 1). Recovery from block by LY97241 was substantially faster than that with clofilium. For LY9721, the fast component accounted for 36% of the recovery at −80 mV, and 57% of the recovery at −45 mV. The slow time constant for recovery from block with LY97241 was more than two-fold faster than the time constant for clofilium at the optimal recovery voltage of −45 mV, and seven-fold faster at a recovery potential of −80 mV. Similar results were observed at concentrations of drug that blocked ~65% of the current (25 μM clofilium, and 100 μM LY97241; data not shown). Recovery from block with clofilium showed a strong voltage dependence, with −45 mV being four-fold more effective than −80 mV. In contrast, recovery from block with LY97241 was comparable at both voltages.

Voltage dependence of recovery from block

The voltage dependence of recovery from open channel block (Fig. 4) was analyzed further with a constant duration two-pulse protocol in wild-type Kv1.5 channels. An initial step to +60 mV was used to induce open channel block, and a second step evaluated recovery after a constant time interval (1150 ms) at different voltages (0 to −120 mV). The percent recovery in the second step was standardized to the net amplitude of open channel block obtained in the first step. The greatest recovery from block was achieved at −45 mV, which provided 75 ± 3% (mean ± SD; n = 9) recovery of the current in 50 μM clofilium, and 94 ± 4% (n = 10) recovery in the presence of 200 μM LY97241. An interesting difference was observed at voltages more negative than −45 mV. Recovery from block by clofilium was impaired at hyperpolarized potentials, giving a bell-curve

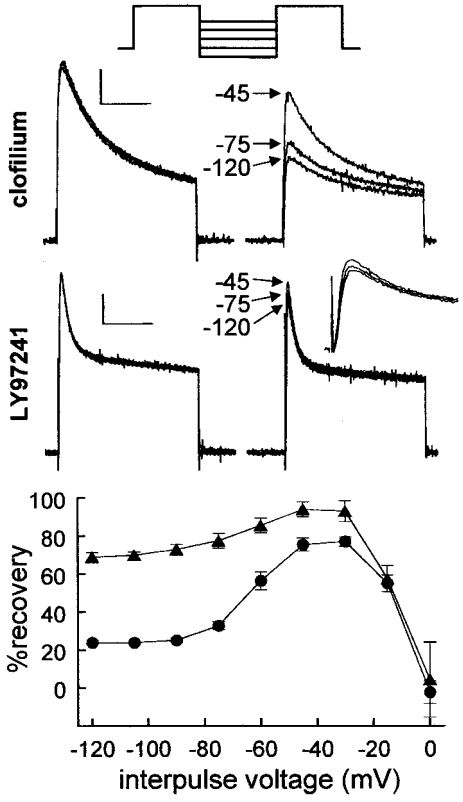


FIGURE 4 Voltage dependence of recovery from block in wild-type Kv1.5. A two-pulse protocol in the continuous presence of blocking agent was used to analyze voltage-dependent recovery. The conditioning pulses (*left*) and test pulses (*right*) were each +60 mV for a duration of 288 ms. Recovery interval potentials were 0 to −120 mV (−15-mV increments) at a constant duration (1150 ms). Traces are shown for interval potentials of −45, −75, and −120 mV, with 50 μM clofilium or 200 μM LY97241. For clarity, 1000 ms of the recovery interval has been omitted. An expanded time scale (*inset*) for LY97241 shows recovery currents were adequately resolved. Complete recovery of peak current during the 30-s interval at −80 mV was evident in the superimposition of sequential responses to the conditioning pulse. Percent recovery was calculated as $100 \times (\text{peak}_2 - \text{steady state}_1)/(\text{peak}_1 - \text{steady state}_1)$ and plotted as a function of the voltage of the recovery interval (graph). Data are mean ± SD for 9–10 oocytes per treatment in 50 μM clofilium (*circles*) or 200 μM LY97241 (*triangles*). Scale bars are 0.5 μA and 100 ms (8 ms for inset).

shape to the plot of percent recovery as a function of interpulse voltage; a bell curve was less apparent for Kv1.5 channels blocked by LY97241 (Fig. 4, *graph*). Differences in blocking effects were most pronounced at voltages favoring the closed channel state. At positive potentials (above threshold for channel opening), voltage-dependent suppression of recovery from block was similar with either compound. The impaired recovery from block at hyperpolarized potentials was also seen with LY97542 (data not shown), which has a permanent positive charge similar to that of clofilium but carries a para-nitro group on the aromatic ring. The voltage-dependent trapping effect seen with LY97542 demonstrates that it is the positively charged nature of the compound, and not the substituent group on the aromatic ring that determines the state-dependent blocking effects.

In summary, our results showed a rapid recovery from block with LY97241 that remained relatively efficient even at negative potentials, whereas clofilium showed optimal recovery at a voltage near the activation threshold and reduced recovery at negative potentials. These results suggested that the tertiary amine LY97241 might have an alternate pathway for escape when in a neutral nonprotonated state. In contrast, the permanent positive charge carried by clofilium correlated with dependence on the open state of the channel; escape of the blocker was impaired at negative voltages that favor the closed state of the channel. These observations suggested that the recovery from block with LY97241 should be sensitive to protonation state, an idea that we tested experimentally by altering pH.

Reduced pH alone caused a reversible block of the Kv1.5 channel by promoting slow C-type inactivation, but this pH sensitivity was removed by mutagenesis of a key histidine (H452) in the extracellular loop that links S5 to the P pore domain (Steidl and Yool, 1999). The mutation of H452 did not affect voltage-dependent activation of Kv1.5, or the pH sensitivity of activation, seen as a depolarizing shift in the midpoint of the conductance–voltage plot with decreased external pH. Creation of a pH-tolerant Kv1.5 channel enabled our analysis of the effects of acidic pH on the block induced by LY97241. Kv1.5 channels, in which histidine 452 was substituted by glutamine (H452Q), were functional over a broad range of pH values, unlike the wild-type channel (Steidl and Yool, 1999). The effect of pH on the voltage dependence of recovery from block was evaluated with a double-pulse protocol, as described for Fig. 4. At pH 7.3, the bell-curve plots of recovery in clofilium were comparable for H452Q (Fig. 5 *A*) and wild-type Kv1.5 (Fig. 4, *graph*). Similarly, at pH 7.3, the blocking effects of LY97241 were comparable for the wild-type (Fig. 4) and mutant H452Q Kv1.5 channels (Fig. 5 *B*). These data showed that the mutation itself did not affect block of the channel by either compound.

Bath salines with pH values of 5.3 to 8.3 were constructed with buffering agents appropriate to each pH range, as described in the Methods. The effect of external pH on the recovery from block was evaluated by a double-pulse protocol for clofilium (50 μM) and LY97241 (200 μM), and plotted as a function of the recovery interval voltage (Fig. 5, *A* and *B*). At all pH values with clofilium, recovery from block was suppressed to approximately 20% at negative voltages (−90 to −120 mV). The optimal voltage for recovery (the peak in the bell curve) showed a pH-dependent displacement toward positive voltages as pH was decreased. The voltage required for maximal recovery correlated directly with the voltage threshold for activation seen in the conductance–voltage plot (Fig. 5 *C*). This positive shift in $V_{1/2}$ value for activation in acidic pH has been

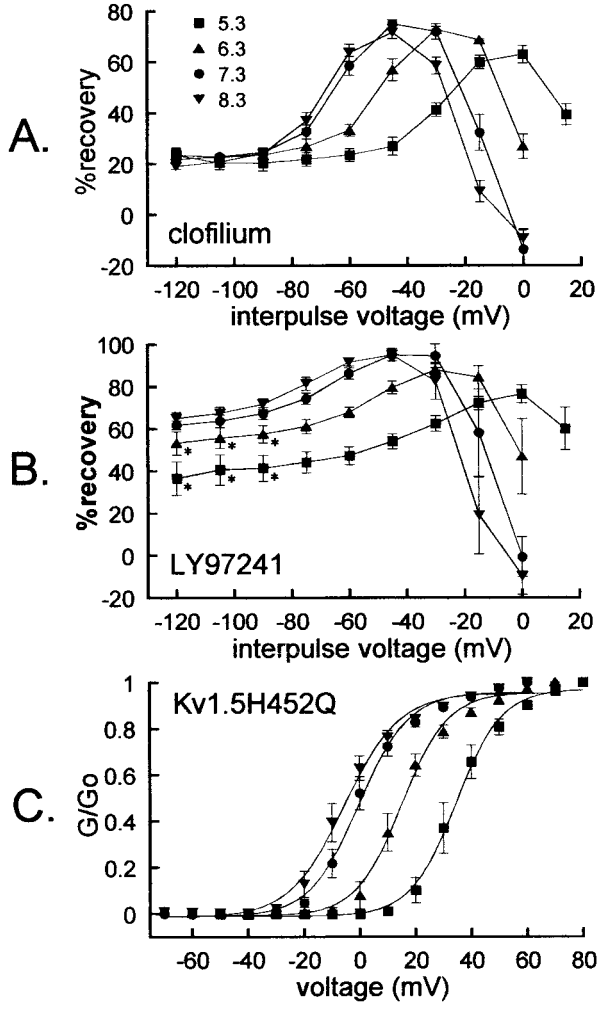


FIGURE 5 The pH dependence of recovery from block in Kv1.5 H452Q channels. Recovery of pH-tolerant Kv1.5 H452Q channels from block by (*A*) 50 μM clofilium or (*B*) 200 μM LY97241 was evaluated at external pHs of 5.3, 6.3, 7.3, and 8.3 (mean $\pm$ SD; $n = 4$–11 oocytes per treatment). The double pulse protocol was as described in Fig. 4. The sample means at the four different pH levels were compared using a one-way analysis of variance at the -90, -105, and -120 mV recovery interval potentials. A significant difference between the means was detected by ANOVA for LY97241 ($p < 0.01$), and paired means were compared post hoc using the Bonferroni *t*-test. Statistically significant differences ($p < 0.01$) from the pH 7.3 mean data are indicated with an asterisk. (*C*) Voltage-dependent activation was pH-sensitive. Normalized conductance (G/G_O) was plotted as a function of voltage (-70 to $+80$ mV, 10 mV increments). The holding potential was -80 mV. Data for mean $\pm$ SD ($n = 5$ oocytes) were fit by Boltzmann equations (*solid lines*). Half-maximal activation voltages were: 34.4 $\pm$ 3.4 mV at pH 5.3; 15.0 $\pm$ 2.4 mV at pH 6.3; -0.26 ± 2.6 mV at pH 7.3; and -5.5 ± 2.9 mV at pH 8.3.

described previously for Kv1.5 channels in *Xenopus* oocytes, and is mediated by a mechanism separate from the C-type inactivation enabled by H452 (Steidl and Yool, 1999). The correlation between optimal recovery from block and activation threshold supported the hypothesis that clofilium exits from its binding site primarily through the

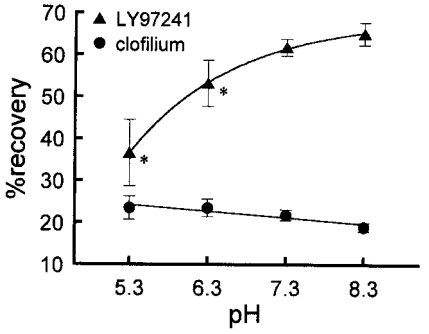


FIGURE 6 Relationship between external pH and the percent recovery from block in Kv1.5 H452Q channels. Data show the percent recovery from block at an interpulse recovery voltage of -120 mV using the double-pulse protocol with two-electrode voltage clamp recording as described in Fig. 4. Data are mean $\pm$ SD for 4–6 oocytes for each treatment group. Clofilium was 50 μM, and LY97241 was 200 μM. Statistically significant differences ($p < 0.01$) from the pH 7.3 mean data are indicated with an asterisk (ANOVA, post hoc Bonferroni *t*-test)

open state. For clofilium, recovery at hyperpolarized potentials was insensitive to pH, as expected in the absence of a tertiary amine moiety.

The same pH range was tested with LY97241 (Fig. 5 *B*). In contrast to results with clofilium, the recovery from LY97241 block was sensitive to pH at all voltages below the threshold for channel activation. Recovery from block was greatest at pH 7.3 and 8.3, and reduced at acidic pH. For example, recovery at an interpulse voltage of -120 mV was 65 $\pm$ 3% at pH 8.3, as compared to 37 $\pm$ 8% at pH 5.3. These data supported the hypothesis that the recovery from block in the closed state was possible for neutral LY97241, but less likely for charged clofilium. At voltages above activation threshold, both clofilium and LY97241 showed similar voltage-dependent effects on net recovery, indicating that the access of the compounds through the open state was comparable.

Figure 6 shows the relationship between external pH and the percent recovery from block at an interpulse voltage of -120 mV. The data were obtained using the pH-resistant channel Kv1.5 H452Q to assess the effects of pH on the charged state of the blocking compound. As expected, the recovery from block by clofilium is independent of pH. In contrast, the recovery from block by LY97241 shows a dose-dependent relationship with pH. The reduction in recovery from block at acidic pH is consistent with the shift of the LY97241 tertiary amine into a protonated charged state similar to clofilium that can be trapped in the channel at negative potentials.

DISCUSSION

To explore the possibility of multiple pathways by which clofilium derivatives can block Kv1.5 channels, we compared the voltage and pH dependence of recovery from

block with clofilium and its tertiary amine derivative LY97241. The rapid state-dependent processes of blocking and unblocking seen with these compounds produced an intriguing cycle of recovery and reblock in response to voltage, despite the constant presence of the blocking agent. Block of Kv1.5 channels by clofilium and by LY97241 was use dependent; current activation induced by membrane depolarization was followed by a macroscopic decay phase that showed a dose-dependent rate. Both onset and recovery of block for Kv1.5 channels were found to be substantially faster for LY97241 than for clofilium. Our results contradict the claim that LY97241 blocks HERG channels exclusively (Suessbrich et al. 1997); however, the higher doses of LY97241 used in our work suggest that this compound may be a more potent blocker of HERG than Kv1.5 channels.

Recovery of Kv1.5 channels from block by clofilium was optimal at −45 mV (at pH 7.3), near the threshold for channel activation. When the voltage dependence of activation was shifted by changing external pH, the optimal voltage for recovery shifted accordingly. Recovery in clofilium was reduced at hyperpolarized potentials, suggesting that the compound was trapped in the closed channel state. It is possible that the large size of clofilium precluded sustained trapping of this molecule inside an activation gate, as has been described for internal Ba^{2+} block of K_{Ca} channels (Miller et al., 1987). With clofilium, the recovery process may occur through a brief reopening of the channel despite the negative potential, as has been suggested for recovery from inactivation in *Shaker* K channels (Demo and Yellen, 1991). The slow recovery of HERG K^+ channels from block by MK-499 (methanesulfonanilide) was attributed to trapping of the compound in the inner vestibule by closure of the activation gate; channel reopening allowed recovery from block (Mitcheson et al., 2000).

In contrast, recovery of Kv1.5 channels from block in LY97241 remained efficient even at hyperpolarized potentials. This phenomenon is most readily explained by postulating that the neutral compound escapes through a hydrophobic pathway even when channels are in the closed state. The nature of the hydrophobic pathway is not known, but might involve hydrophobic membrane-spanning domains adjacent to the pore, or simply a hydrophobic gate within the ion-conduction pathway. Access to the binding site in the closed channel state would be consistent with the large initial block in peak current amplitude seen with LY97241 (Fig. 2). This reduction in peak current amplitude in the presence of the LY97241, as compared to initial control current from the same oocyte, suggested that some block could occur in the closed state before channel opening. Less initial block was seen with clofilium, consistent with reduced access of the charged compound to the closed state. However, it is equally possible that all or part of the initial block in peak amplitude may be due to rapid open channel block, if the on rates of LY97241 and clofilium binding are fast with respect to the rate of voltage-dependent activation of the channels.

We propose that, in the neutral state, LY97241 can transverse a hydrophobic pathway in the closed channel to access and leave its putative receptor site. This hydrophobic pathway would be less accessible to clofilium. The permanently charged amine group would act to limit the blocking and unblocking processes mainly to the open channel state. Both compounds efficiently access the receptor site at depolarized potentials via an open channel state that constitutes a hydrophilic pathway. The suggestion for a dual hydrophobic and hydrophilic pathways for channel blockers has precedent in studies on sodium channels. Block by the local anesthetic lidocaine and its quaternary derivative, QX-314, is use dependent (reviewed by Hille, 1992). QX-314 is permanently charged and is limited to open channel block, whereas lidocaine is a tertiary amine compound that can access its receptor site in both the open and closed channel states. The charged state of the blocker modulates its access to sodium channel hydrophilic and hydrophobic pathways (Hille, 1977), in that recovery of sodium channels from block by lidocaine is reduced at acidic pH and enhanced at basic pH. Acceleration of block and recovery for the tertiary amine compound may indicate the existence of alternative pathways through the channel protein that are not available to quaternary amines. Ragsdale et al. (1994) demonstrated that the mutation of an isoleucine to an alanine at position 1760 in rat brain type IIA Na^+ channels created a pathway to allow QX-314 access to its putative receptor site in both closed and open channel states by removing a critical hydrophobic barrier.

The correlation between the optimal voltage for recovery from block and the activation threshold supports the hypothesis that clofilium exits from its binding site primarily when the channel is in the open state. Recovery appears to be most favored at a voltage that supports transient channel openings. Higher levels of activation driven by more depolarized potentials are expected to allow recovery, but also would enable rapid reblocking of the open channels because the probability of channel opening is greater. Thus, the enhanced blocking effect at positive voltages, forming the right flank of the bell curve, is most reasonably attributed to reblocking of the open channels. Use-dependent block induced by LY97241 was dramatically faster than that induced by clofilium. Recovery of Kv1.5 channels from block in LY97241 was 2.5–9-fold faster (depending on voltage) than recovery from block in clofilium. These data imply that the tertiary amine nature of LY97241 conferred accelerated access to and from the Kv1.5 receptor site. Changes in external pH were effective in altering the rates of recovery from block in LY97241, when analyzed in a pH-tolerant Kv1.5 channel (H452Q; Steidl and Yool, 1999). Because it has been proposed that the binding site for clofilium is on the internal face of the pore (Yool, 1994), the rapid and reversible effect of external pH might be explained if

LY97241 can be protonated while bound in the channel (Woodhull, 1973), assuming that the putative binding site is located in the conduction pathway. Consistent with this model, prior work showed that block is destabilized by increased external K^+ presumably via a knock-off mechanism (Malayev et al., 1995), which would indicate the binding site is in the conduction pathway for potassium ions. Results presented here characterize the distinct features of use-dependent block of Kv1.5 channels by clofilium and a tertiary amine analog, and show that the charged state of the amine blocker is an important determinant for access to the putative quaternary ammonium receptor site.

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