0026-895X/94/050970-07$03.00/0

MOLECULAR PHARMACOLOGY, 46:970–976, 1994.



# Block of the Inactivating Potassium Channel by Clofilium and Hydroxylamine Depends on the Sequence of the Pore Region

ANDREA J. YOOL

*Departments of Physiology and Pharmacology, University of Arizona College of Medicine, Tucson, Arizona 85724*

Received July 7, 1994; Accepted August 18, 1994

**SUMMARY**

Cardiac antiarrhythmic compounds are a diverse group divided into classes that differ in their mechanisms of action. Recent attention has focused on class III compounds, which prolong the action potential by blocking $K^+$ channels. The purpose of this study was to characterize the mechanisms of actions of a class III compound, clofilium, and a simple analog, hydroxylamine, on an inactivating $K^+$ channel. The defined system used a cloned inactivating $K^+$ channel (Shaker-B) expressed in *Xenopus* oocytes. This channel is similar in physiological properties and core sequence to the inactivating $K^+$ channel cloned from mammalian heart. Results presented here demonstrate that clofilium (100 μM) and hydroxylamine (10 mM) can cause use-dependent block, depending on the sequence of the pore region. A mutation of the pore known to influence selectivity and tetraethylammonium binding (threonine-441 to serine) confers use-dependent sensitivity to hydroxylamine and clofilium. Hybrid channels were formed from the coinjection of wild-type and mutant channel mRNAs; the analysis of block with the hybrid channels suggests that binding of hydroxylamine involves all subunits of the tetrameric channel, whereas clofilium affects channels containing as few as one mutant subunit. The simplest interpretation is that all four subunits contribute to an internal binding site for blockers such as clofilium and hydroxylamine and threonine-441 influences this binding site. The effectiveness of clofilium, unlike hydroxylamine, on the hybrid channels may reflect its structural complexity, which could allow interaction with a broader receptor site. Future studies will test this idea using other class III-related compounds.

In the prophylaxis of cardiac arrhythmias, the use of class I agents that target $Na^+$ channels has come into question because of an increased mortality risk, and this has spurred interest in the class III agents (for reviews, see Refs. 1 and 2). The class III drugs seem to offer the opportunity for beneficial clinical intervention, particularly if a direct use-dependent action is identified (1). The attractiveness of class III agents may be explained by the general observation that partial blockade of $K^+$ channels broadens the action potential, thus increasing the repolarization time and slowing the firing rate without completely eliminating activity. Two properties providing partial block would be expected to enhance the therapeutic value of class III drugs, i.e., use dependence and target selectivity. Use-dependent blockers increase in effectiveness with firing frequency or depolarization (3); they would exert a maximal effect during tachycardia and much less effect during normal function. Selective blockers for certain subtypes of $K^+$ channels could provide the desired partial block by removing one component of the overlapping $K^+$ channel contributions to membrane repolarization. Selective block of a subtype of channels also would imply a broader useful range of effective doses; instead of trying to titrate the ideal partial block of a single class of channels such as $Na^+$ channels, it would be feasible to completely block a single class within a redundant system such as $K^+$ channels. Use-dependent or selective block would provide the opportunity to modulate system functions such as firing rate without resorting to all-or-none block. A key to realizing this opportunity is understanding the mechanism of action of the class III compounds at the molecular channel level.

A number of $K^+$ channels are present in the heart (4–7) and have been demonstrated to co-occur in isolated myocytes (8, 9) (for review, see Ref. 10). Although class III drugs have been suggested to act at $K^+$ channels, precise studies of mechanism are made difficult by the presence of a mixture of $K^+$ channels in dissociated native cells. A defined system allows analyses of drug actions at the molecular level with an identified class of $K^+$ channels (11, 12). Until the clinical target of clofilium in human heart is identified, model systems will serve as useful tools for evaluating the types of channels that may be involved. Clearly Shaker itself is not derived from heart, but it offers two distinct advantages as a model system for investigating molecular mechanisms of drug action, (i) the Shaker-B $K^+$ channel is strikingly similar in physiological properties and in amino acid sequence (particularly in the pore region) to Shaker family homologs cloned from mammalian heart (4, 7, 13) and (ii) both

This work was supported by a Flinn Foundation Newly Independent Investigator Award from the American Heart Association Arizona Affiliate (AZFG-20–93).

**ABBREVIATIONS:** T441S mutation, mutation of threonine-441 to serine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

the wild-type and mutant forms of Shaker used in this work have been the focus of attention of a number of laboratories and hence are well characterized in terms of the molecular regions that are associated with specific biophysical and pharmacological properties, as summarized below. Channels form as tetramers of Shaker polypeptide subunits (14); the ion-selective pore is believed to be at the center of the complex, lined in part by the H5 (pore) sequence contributed by each subunit (15–18). The essential properties of the heart Kv1.4 (one class of mammalian $K^+$ channels releated to Shaker) and Shaker-B channels are fast voltage-dependent activation, inactivation, high $K^+$ selectivity, and sensitivity to 4-aminopyridine (13, 19, 20). In a model based on the kinetic properties of the Shaker channel, the fast activation involves the opening of channels after transition through a number of voltage-dependent closed states within milliseconds in response to depolarization, and a resulting outward current is carried by $K^+$ under physiological conditions (21, 22). In the model, the ensuing inactivated state results from the binding of the amino terminus, as an inactivation particle, to the internal vestibule of the pore (23, 24). At hyperpolarized potentials, channels recover from the inactivated state to the closed state and are available again for activation by subsequent depolarizing stimuli.

Previous work[1] demonstrated an effect of hydroxylamine on modified Shaker-B channels. This blocking effect depended on two distinct regions of the ion channel protein, i.e., the gate that inactivates the channel and the pore through which the $K^+$ ions move. These regions, with hydroxylamine, interacted to create a stable blocked state. The involvement of the pore region was suggested by the dependence of the blocking effect on a site thought to be near the internal mouth of the channel, threonine-441. This residue, when mutated to serine (the T441S mutation), was shown previously to affect channel selectivity (17) and the binding of internally applied tetraethylammonium (16). Although any site-directed mutation has the potential to produce unknown structural consequences, the effects of this mutation appear to be quite selective; other properties of the channel, such as voltage-dependent activation, macroscopic inactivation, and sensitivity to externally applied pharmacological agents, appear to be unaffected (16, 17). Single-channel recordings showed that hydroxylamine bound in the pore of the mutant channel and stabilized a long-lived nonconducting state (the inactivated state). The involvement of inactivation was determined from a second mutation that deleted the essential amino-terminal sequence. In the amino terminus-deleted channels, hydroxylamine was completely ineffective regardless of the pore sequence. These observations suggested that hydroxylamine acted to stabilize the binding of the inactivation gate and caused use-dependent block by a mechanism analogous to that described for the effects of local anesthetics on $Na^+$ channels. The T441S mutation influences interactions with several amine-based compounds; it increases permeability to ammonium ions, alters pore block by the ammonium derivative tetraethylammonium, and stabilizes inactivation at a site near the mouth of the pore in the presence of the ammonium deriviative hydroxylamine. This study tests the hypothesis that another amine-based compound, clofilium, which is thought to act at $K^+$ channels, might also be affected by the nature of the threonine-441 site.

The action of compounds such as clofilium on inactivating $K^+$ channels has not been previously evaluated (10). This study demonstrates that clofilium causes use-dependent block of the inactivating $K^+$ channel and influences the inactivation process. A similar mechanism occurs with hydroxylamine, a simple chemical that, like clofilium, has an amine-based structure. The results also show that the use-dependent block by clofilium is enhanced by the same mutation of the pore region (T441S) that is required for effective block by hydroxylamine. In combination with the results presented here, it seems that the threonine-441 site (which is highly conserved among $K^+$ channels) is important in interactions with amine-based compounds, a feature of the class III drugs as well as other compounds. Furthermore, this study serves as a model for evaluating possible mechanisms of use-dependent blocking actions of class III antiarrhythmic agents. The blocking effects are analyzed in wild-type, mutant, and hybrid $K^+$ channels.

## Materials and Methods

**Molecular techniques and oocyte preparation.** A site-directed T441S mutation was generated in the *Shaker*-B cDNA sequence. The *Shaker* gene was carried in a commercial plasmid vector (Bluescript KS) that was modified by substitution with the 3′ untranslated region of *Xenopus* β-globin to enhance expression in oocytes, as described previously (17). The mutation was confirmed by DNA sequencing. To synthesize RNA *in vitro*, wild-type and mutant cDNA constructs (3–5 μg) were linearized with *Eco*RI in the 3′ polylinker and transcribed with T3 RNA polymerase. Oocytes from anesthetized mature female *Xenopus laevis* were obtained by the surgical removal of several lobes of ovary and were prepared by techniques similar to those published previously (21). Follicular cell layers were removed by treatment with collagenase (type IA, 1.5 mg/ml; Sigma) for 2–3 hr in calcium-free preparation medium (82 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 5 mM HEPES, pH 7.6). Prepared oocytes were injected with 10–25 ng of RNA in 50 nl of sterile water and were incubated for ≥2 days at 18° in culture medium (96 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM HEPES, pH 7.6) before recording. The mRNA was diluted with sterile water (1/50 to 1/100) to yield expressed currents in the range of 3–6 μA. The preparation and culture media contained the antibiotics penicillin (100 units/ml) and streptomycin (100 μg/ml), without sodium pyruvate.

**Electrophysiological recording.** The composition of the recording saline solution was 100 mM NaCl, 4.3 mM $MgCl_2$, and 5 mM HEPES, pH 7.3. Calcium was omitted (21) to eliminate the calcium-dependent $Cl^-$ current endogenous to oocytes (25). Two-electrode voltage-clamp recordings at room temperature used two intracellular electrodes (0.5–1.5 MΩ) filled with 3 M KCl. Hydroxylamine-containing saline solution was made as 10 mM hydroxylamine HCl, 90 mM NaCl, with 4.3 mM $MgCl_2$ and 5 mM HEPES; the pH of the substituted solution was adjusted to pH 7.3. Clofilium was stored frozen in small aliquots as a 10 mM solution and was diluted to a final concentration of 100 μM in standard-$Na^+$ bath saline solution on the day of use. Test and control saline solutions were applied to oocytes at the final concentration by perfusion with a volume ≥5 times that of the recording chamber; excess saline solution was removed by aspiration. In all recordings, the voltage-gated $K^+$ channels were activated with step protocols from holding potentials of −80 or −100 mV. Data were recorded with a GeneClamp (Axon Instruments), filtered at 2–5 kHz, digitized at 100–200 μsec (in all cases except for voltage-clamp traces of >100-msec duration, for which the sampling rate was slower), and stored on a Dell 386 computer hard disk for subsequent analysis with pClamp software (Axon Instruments).

[1] A. J. Yool and T. L. Schwarz. Interactions of the H5 pore region and hydroxylamine with N-type inactivation in the Shaker $K^+$ channel. Submitted for publication.

## Results

**Effects of clofilium and hydroxylamine.** Fig. 1 illustrates the wild-type and mutant $K^+$ channel currents recorded with two-electrode voltage-clamp techniques and shows the blocking effects of clofilium and hydroxylamine. The voltage-dependent currents were evoked by repeated steps to +40 mV from a holding potential of −80 or −100 mV. Repetitive pulses every 15 sec were applied for intervals of 30 min or longer, to analyze the rates of change in peak current amplitude for control and experimental treatments. For clarity, traces are shown only for three consecutive steps immediately before perfusion and for three steps (steps 1, 6, and 11, at 15, 90, and 165 sec, respectively) after perfusion of the test compound (Fig. 1). For wild-type channels, clofilium at 100 μM causes a slowly developing block (Fig. 1B*1*) that is most apparent when analyzed over a prolonged time (Fig. 2A). For the mutant channels, the use-dependent blocking effect of clofilium is much more pronounced (Fig. 1B*2*); the peak current declines steadily with time (Fig. 2A). Although hydroxylamine has no effect on the wild-type channel (Fig. 1A*1*), it produces significant block of the mutant channel (Fig. 1A*2*). The blocking effect of hydroxylamine requires activation of the channels and does not occur if the channels are held in the closed state by a negative potential.[1] Clofilium similarly was found to have no effect on wild-type or mutant channels held at −80 mV during periods of up to 15 min of exposure to the test compound (data not shown). This requirement is characteristic of use-dependent blockers and shows that the exposure of the cells to the test compounds alone does not alter cell viability or channel integrity. Recovery from both blockers appears to be very slow.

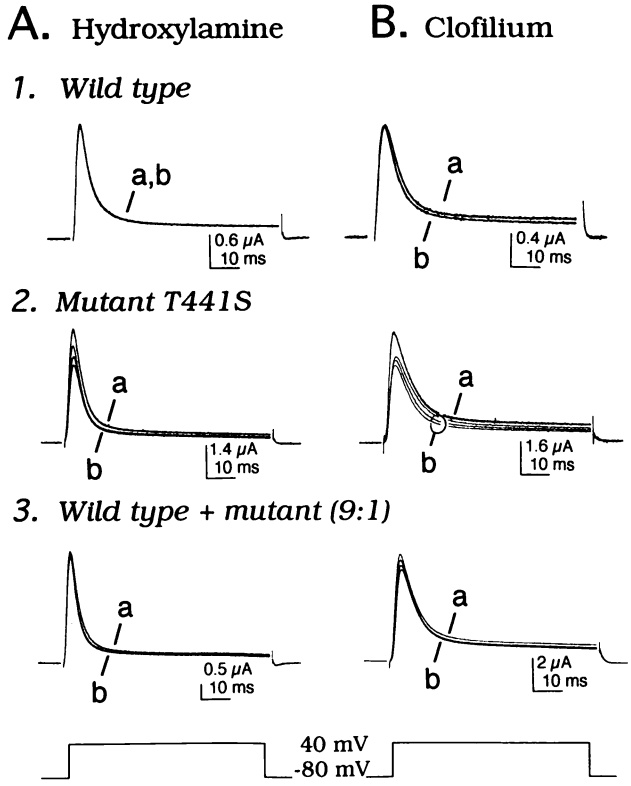


**Fig. 1.** Effects of clofilium and hydroxylamine on wild-type and mutant $K^+$ channel currents. After an initial control recording in bath saline solution, oocytes were perfused with either hydroxylamine at 10 mM (A) or clofilium at 100 μM (B), in saline solution. The currents were expressed in *Xenopus* oocytes injected with wild-type mRNA (*1*), mutant T441S mRNA (*2*), or wild-type and mutant mRNAs mixed at a ratio of 9:1 (*3*). Traces are shown for repeated steps to +40 mV from a holding potential of −80 mV for three consecutive traces immediately before perfusion (*a*) and for three traces (at 15, 90, and 165 sec) after perfusion with the test compound (*b*). The control traces are superimposable in all treatments; test traces show a progressive decline in peak current amplitude for the mutant channels in the presence of hydroxylamine and clofilium and for the hybrid wild-type plus mutant channels in the presence of clofilium. Repeated steps were applied at 15-sec intervals. Note the smaller scale bar for A*3*. Capacitance and leak currents were subtracted using a *P*/5 protocol, in which five voltage steps at one-fifth the amplitude of the test voltage pulse were summed to serve as a template for passive membrane properties. Saline solution compositions are described in Materials and Methods.

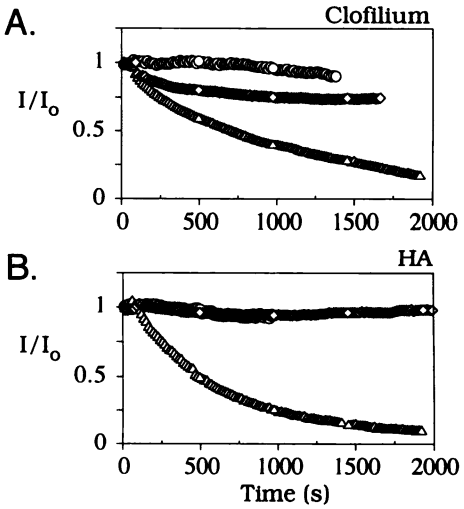


**Fig. 2.** Time-dependent effects of clofilium and hydroxylamine on wild-type and mutant channels during repetitive stimulation. Peak outward currents were measured for repeated steps to +40 mV applied using the same protocol described in Fig. 1 and are plotted versus recording time. The initial seven points in each time series are for the control condition; subsequent steps are in the continuous presence of clofilium at 100 μM (A) or hydroxylamine (*HA*) at 10 mM (B). Peak current amplitudes for the test condition (*I*) were standardized to the initial control amplitude ($I_o$). The plots are for data from wild-type (○), mutant (△), and hybrid wild-type plus mutant channels combined in a ratio of 9:1 (◇). In B, the symbols for the wild-type and hybrid channels are superimposed.

Hybrid channels were formed by coinjection of wild-type and mutant mRNAs. The mRNAs were tested independently in several batches of oocytes, diluted as needed to yield currents equivalent in peak magnitude, and then mixed at a ratio of 9 parts wild-type channel to 1 part mutant channel. The contrasting effects of clofilium and hydroxylamine on the hybrid channels are shown in Figs. 1 and 2. Hydroxylamine is not effective on channels in the hybrid mixture (Fig. 1A*3*), whereas clofilium causes use-dependent block (Fig. 1B*3*). These data are plotted in Fig. 2. The lack of sensitivity to hydroxylamine is apparent in Fig. 2B. In contrast, clofilium causes a biphasic effect on the hybrid channels, consisting of an initial phase of strong block followed by a much slower phase that approximately parallels the sensitivity of the wild-type channels but at a lower level (Fig. 2A). In the presence of clofilium, the residual current remaining after the fast block is complete is about 70–80% of the amplitude of the wild-type channel current, a level that approximately matches the percentage of channels in the mixture that are predicted to be homomeric wild-type channels (see Discussion).

**Rates of block by clofilium and hydroxylamine.** To

quantify the rates of block in wild-type and mutant channels, histograms were compiled for the net changes between consecutive steps in control bath saline solution and in saline solutions containing hydroxylamine or clofilium. The histograms showed single gaussian distributions and were used to evaluate mean values for the net change per step (Table 1). The net change per step was calculated as the difference in peak amplitude between each pair of consecutive steps within a series and was standardized as a percentage of the initial peak control amplitude for that series. For wild-type channels, the control and hydroxylamine values overlap and show little evidence of any net change per step. A small negative percentage change with clofilium is consistent with the slowly developing block of wild-type channels illustrated in Fig. 2A. Over the prolonged recording periods used to analyze the slow development of block, a minor amount of cumulative inactivation of the mutant channels is observed.[1] However, for the mutant channels, both hydroxylamine and clofilium produce a substantially greater decrease in peak amplitude per step, consistent with the faster development of block illustrated in Fig. 2.

**Effect of clofilium on recovery from inactivation.** The evaluation of recovery from inactivation in the presence of clofilium suggests that both the time course and voltage dependence of the recovery process are affected by this compound (Fig. 3). This result is consistent with the hypothesis that clofilium may act in a manner similar to that of hydroxylamine, which was proposed in previous work to create use-dependent block by stabilizing inactivation.[1] The time course of recovery from inactivation was evaluated with a double-pulse protocol that used a variable duration for the interpulse interval, at a constant potential of −100 mV (Fig. 3A). Clofilium slows the recovery from inactivation for both wild-type and mutant channels. The voltage dependence of the recovery process was evaluated with a double-pulse protocol that used a variable potential for recovery, at a constant duration of 300 msec (Fig. 3B). As expected, both wild-type and mutant channels show more recovery after interpulse intervals at negative potentials and a small decrease in the amount of recovery at more depolarized potentials. Clofilium exacerbates the decrease in recovery for both wild-type and mutant channels at depolarized potentials, although the effect is most dramatic for the mutant channel. The greater effect of clofilium at depolarized potentials classifies it as a blocker that shows true use dependence

TABLE 1

**Mean values for the percentage change in peak current amplitude per step**

The net change per step was calculated as the difference in peak current amplitude between each pair of consecutive steps within a series of repeated steps to +40 mV, in control bath saline solution and in the presence of clofilium (100 μM) or hydroxylamine (10 mM). The difference in peak amplitude was standardized as a percentage of the initial peak amplitude of the first step in each series. The percentage change values are presented as mean values ± standard errors. A net negative change indicates blocking. The total number of events compiled and the number of oocytes per treatment are also shown.

| Preparation | Treatment | Net change/step | No. of events | No. of oocytes |
|---|---|---|---|---|
| | | % | | |
| Wild-type | Control | 0.037 ± 0.11 | 199 | 18 |
| | Hydroxylamine | −0.100 ± 0.05 | 248 | 3 |
| | Clofilium | −0.188 ± 0.04 | 237 | 11 |
| *T441S* | Control | −0.62 ± 0.04 | 302 | 32 |
| | Hydroxylamine | −1.57 ± 0.13 | 316 | 8 |
| | Clofilium | −1.35 ± 0.09 | 127 | 21 |

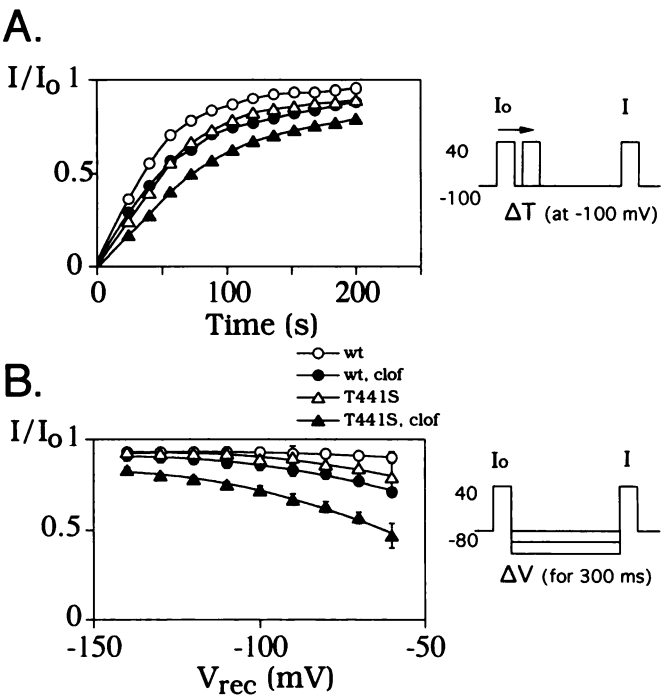


**Fig. 3.** Effect of clofilium on the time and voltage dependence of recovery from inactivation. Double-pulse protocols were used to evaluate the time dependence (A) and voltage dependence (B) of the recovery of wild-type and mutant channels from inactivation. For each data point, inactivation was induced at +40 mV during the first pulse ($I_o$) and the recovery process was assessed in the second pulse ($I$), as the proportion of channels available for activation. *Diagrams at right*, voltage protocols. A, Data are plotted for representative cells expressing wild-type (*wt*) or mutant channels tested before and after perfusion with 100 μM clofilium (*clof*). B, Data show $I/I_o$ values calculated as mean ± standard error ($n$ = 5 for wild-type channel control, $n$ = 3 for wild-type channel with clofilium, $n$ = 2 for mutant channel control, and $n$ = 3 for mutant channel with clofilium). Clofilium impedes recovery from inactivation for both wild-type and mutant currents.

(rather than the "reverse" use dependence described for non-inactivating $K^+$ channels, for which block decreases with depolarization) (1). In both the time- and voltage-test protocols, each test peak amplitude ($I$) is standardized to the amplitude of the immediately preceding conditioning peak ($I_o$), so the possible contributions of cumulative inactivation or use-dependent block to the analyses described above are negligible.

**Lack of effect of clofilium on activation and steady state inactivation.** Activation and steady state inactivation properties of wild-type and mutant channels are not affected by 100 μM clofilium (Fig. 4). Activation properties determined from conductance-voltage relationships are comparable for both channel types, with midpoints at −10 to −20 mV in the presence or absence of clofilium (Fig. 4A). Values for peak current amplitude were standardized to the peak current amplitude evoked by the step to +40 mV. The fitted values for the mutant channel with clofilium were corrected for the effect of cumulative block. Uncorrected values also are shown in Fig. 4. Steady state inactivation was determined with a prepulse protocol in which the cell was held for 5 sec at the prepulse potential and the proportion of noninactivated channels was assessed by a subsequent test step to +40 mV. Values for peak current amplitude were standardized to the peak current amplitude evoked after a prepulse step of −80 mV. As expected, the noninactivated currents are maximal after negative pre-

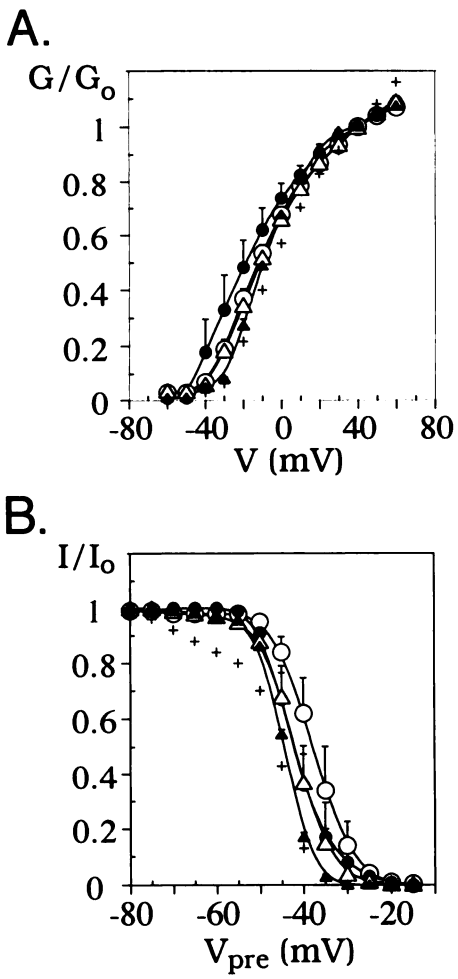


**Fig. 4.** Lack of effect of clofilium on activation and steady state inactivation properties. Conventional plots of activation (A) and steady state inactivation (B) properties of the currents show voltage-dependent properties typical of the inactivating Shaker channel. The relationships are comparable for wild-type (*circles*) and mutant (*triangles*) channels in the absence (*open symbols*) and presence (*closed symbols*) of 100 $\mu$M clofilium. A, Data show the conductance ($G$) at each step voltage standardized to the conductance at +40 mV ($G_o$); conductance was calculated assuming a reversal potential of −80 mV. B, Data show the current activated by a test step to +40 mV after a 5-sec conditioning prepulse at different voltages; the peak current amplitude in the test step ($I$) was standardized to the peak amplitude measured after a prepulse at −80 mV ($I_o$) and is plotted against prepulse voltage ($V_{pre}$). Values are mean ± standard error of an average of four cells (range, two to six cells) per treatment. The values for the mutant channel with clofilium were corrected for cumulative block at 2%/trace; uncorrected values are shown without fitted curves (+).

pulse potentials and decrease at more depolarized prepulse potentials, reflecting the shift of channels at equilibrium into the inactivated state. The inactivation midpoints are comparable for wild-type and mutant channels, ranging from −35 to −45 mV in the presence or absence of clofilium.

## Discussion

Results presented in this study demonstrate that the class III compound clofilium and a simple analog, hydroxylamine, serve as use-dependent blockers of the inactivating Shaker $K^+$ channel. This finding is particularly exciting in light of a convincing perspective on the need for direct use-dependent blockers of $K^+$ channels that was presented by Hondeghem and Snyders (1). They pointed out that many antiarrhythmic compounds such as quinidine show reverse instead of direct use dependence and thus may be proarrhythmic. Direct use dependence entails an increase in effectiveness as stimulation frequency or depolarization increases; in contrast, reverse use dependence involves a decrease in effectiveness with depolarization. It is interesting to note that studies on class III compounds have emphasized their effects on noninactivating $K^+$ currents (for review, see Ref. 10). However, it seems advantageous to explore the effects on inactivating $K^+$ channels as well, especially given the current interest in identifying direct use-dependent blockers. An inactivating type of $K^+$ channel is a logical choice for study because (i) other use-dependent blockers (class I) act on the $Na^+$ channel by stabilizing the inactivated state (26) and (ii) hydroxylamine serves as a use-dependent blocker of the $K^+$ channel only when the inactivation process is intact.[1] As described in this paper, direct use-dependent block by clofilium is evident in two ways. First, $K^+$ channels held in the closed state at −80 mV for long periods of exposure to clofilium remain unaffected; block requires activation of the channel and occurs in the open or inactivated state. Second, clofilium has a greater effect on the recovery from inactivation at more depolarized potentials. Thus, some of the desirable features envisioned for the ideal antiarrhythmic drug (1) appear to exist in currently available class III compounds, when they are matched with an appropriate target.

The use-dependent effects of hydroxylamine and clofilium suggest the intriguing possibility that class III-like compounds may act on the inactivating $K^+$ channel in a manner analogous to the action of local anesthetics on the $Na^+$ channel. In the $K^+$ channel, block by hydroxylamine and clofilium involves the inactivation process and the pore region. In the $Na^+$ channel, local anesthetics appear to stabilize inactivation, and the use-dependent block depends on the frequency of activation (3, 26–28). A single local anesthetic binding site in the pore of the $Na^+$ channel may cause a block of conduction and the stabilization of inactivation (26). Local anesthetics, hydroxylamine, and class III compounds are similar in their structural dependence on an amine group.

The mechanisms of $K^+$ channel block, although generally similar for hydroxylamine and clofilium, differ in detail. Both hydroxylamine and clofilium influence the inactivation process. Clofilium slows the recovery from inactivation, particularly at more depolarized potentials, but does not appreciably affect steady state inactivation. Hydroxylamine also slows recovery from inactivation but produces a small shift in the inactivation midpoint to a more negative potential.[1] This may reflect different effects of the two compounds on the relative rate of entry into as well as recovery from the inactivated state. The T441S mutation enhances the effectiveness of both hydroxylamine and clofilium. Hydroxylamine does not block the wild-type channel and appears to require the mutant pore sequence at multiple subunits of the tetrameric channel. In contrast, clofilium does block the wild-type channel, albeit weakly, and the rate of block is enhanced in the pore mutant channel even when fewer than four of the subunits carry the mutation. The greater blocking ability of clofilium may reflect its more complex structure, which could allow interactions of the compound with an extended region of the channel vestibule or adjoining areas. For example, hydrophobic interactions around the internal mouth of the pore have been implicated in mediating block

by quaternary ammonium derivatives (for review, see Ref. 29) and could also contribute to binding of the hydrophobic moieties of class III compounds. Ongoing studies will analyze other, structurally similar compounds as potential blockers of the inactivating $K^+$ channel.

The threonine-441 site is suggested to be located at the internal mouth of the pore, because it influences the binding of internally applied but not externally applied tetraethylammonium (16) and ionic selectivity (17). In a hypothetical model, threonine-441 has been proposed to form the innermost ion binding site in the pore (30). Single-channel data suggested that the T441S mutation induced a greater affinity for hydroxylamine and that hydroxylamine stabilized inactivation by binding at the inner mouth of the pore and there interacting with the amino-terminal particle.[1] Hydroxylamine is likely to access the binding site from the cytoplasmic side; it is effective in producing use-dependent block when applied to the internal face in inside-out patches.[1] A similar strategy for the rat Kv1.4 channel expressed in oocytes showed that quinidine was effective on inside-out patches, suggesting a cytoplasmic site of action (12). The access of an externally applied drug to the inside face must involve transit across the membrane. Presumably, the compound partitions first into the membrane phase and then into the cytoplasmic compartment. For clofilium and hydroxylamine, the loading appears to occur quickly; the gradually developing block begins before the perfusion with the bath saline solution is complete (<45 sec). The poor reversibility of the blocked state seems consistent with a multiphasic pathway for drug entry and exit. Poor reversibility also has been described for clofilium effects on the delayed rectifier current in isolated guinea pig ventricular cells (31).

In this study, the dose-response curve has not been assessed for two reasons. First, it is difficult to determine quantitatively how the external concentration relates to the internal concentration across a hydrophobic membrane barrier. The doses used for clofilium (100 $\mu$M) and hydroxylamine (10 mM) are probably in excess, because the effects are initiated rapidly; other published studies using lower concentrations of clofilium employ an extended incubation period before assessment of block (32). Second, the block does not appear to achieve a steady state. Lower external concentrations create use-dependent block that develops more slowly, but ultimately the levels of block are comparable (data not shown). This is expected if the blocked state is an absorbing state from which recovery is very slow, as appears to be the case for clofilium and hydroxylamine with the mutant Shaker channel. With a slowly reversible effect, even a very low concentration is effective at creating complete block, given sufficient time.

The nature of the pore binding site was investigated with hybrid channels formed by the coinjection of wild-type and mutant RNAs. Hybrid channels formed by random subunit associations are predicted to fall into five general categories, i.e., homomeric wild-type channels (no mutant subunits) and channels with one, two, three, or four mutant subunits in the tetramer. With wild-type channels dominating the RNA mixture by 9-fold, the percentage of homomeric wild-type channels is estimated as $100(0.9)^4$, or 66%, and the percentage of channels having at least one mutant subunit is $100(1 - 0.66)$, or 34% (14). The percentage of homomeric mutant channels is very small (~0.01%). Because hydroxylamine is an effective blocker of the homomeric mutant channel but has no discernible effect on the hybrid channels, it is reasonable to conclude that hydroxylamine is effective only when at least three and probably all four subunits are mutant. If two or more mutant subunits were required, then approximately 5% of the channels should have been affected. If only one mutant subunit was sufficient to confer sensitivity, then approximately 34% of the channels in the hybrid mixture should have been affected. This does appear to be true for clofilium, however. The block of the hybrid channels by clofilium developed in a use-dependent manner until 23% of the current was blocked; the remaining 77% was more resistant to clofilium and showed a rate of decline roughly parallel to that of the wild-type homomeric control. This is consistent with the interpretation that all of the channels containing one or more mutant subunits were more rapidly blocked by clofilium and that the remaining channels were homomeric wild-type channels. In addition, the dependence of hydroxylamine activity on the sequence of multiple subunits in the tetramer suggests that the primary site of action of hydroxylamine is a site formed where all of the subunits interface. This is consistent with the idea that the binding site is in or near the mouth of the pore, and this offers additional evidence that hydroxylamine does not have nonspecific deleterious effects on the protein, as discussed in detail elsewhere.[1]

With regard to clinical relevance, it will ultimately be necessary to identify the targets of class III drug action in human heart, to test with cardiac channels the proposed mechanisms of block described here for the Shaker channel. The importance of this study is that it demonstrates that the inactivating type of $K^+$ channel is important in the consideration of clinically relevant targets of class III drugs and it identifies specific structural regions of the channel that interact to create the use-dependent blocking effect. For speculation on the implications of the work described here, it is important to acknowledge that clofilium and hydroxylamine are most effective on the mutant (T441S), and not the wild-type, form of the Shaker $K^+$ channel. However, even the modest blocking effect of clofilium on the wild-type channel could be of interest; because the rate of recovery is much slower than the rate of block, a substantial cumulative block gradually develops. Other compounds resembling clofilium or hydroxylamine may be found to block wild-type channels more rapidly. Alternatively, channels with a serine naturally occurring at the site equivalent to position 441 might be singled out for highly selective blockade, allowing the pharmacological dissection of specific components of the action potential repolarization. This study characterizes the nature of a molecular interaction that may contribute to some of the blocking effects of class III-related agents, and it identifies the inactivating type of $K^+$ channel as a potential target for use-dependent mechanisms of block.

**Acknowledgments**

Judd Rice provided technical assistance.

**References**

1. Hondeghem, L. M., and D. J. Snyders. Class III antiarrhythmic agents have a lot of potential but a long way to go: reduced effectiveness and dangers of reverse use dependence. *Circulation* **81**:686–690 (1990).
2. Grant, A. O., and D. J. Wendt. Block and modulation of cardiac $Na^+$ channels by antiarrhythmic drugs, neurotransmitters and hormones. *Trends Pharmacol. Sci.* **13**:352–358 (1992).
3. Courtney, K. R. Mechanism of frequency-dependent inhibition of sodium currents in frog myelinated nerve by the lidocaine derivative GEA 968. *J. Pharmacol. Exp. Ther.* **195**:225–236 (1975).
4. Tseng-Crank, J. C. L., G.-N. Tseng, A. Schwartz, and M. A. Tanouye.

Molecular cloning and functional expression of a potassium channel cDNA isolated from a rat cardiac library. *FEBS Lett.* **268**:63–68 (1990).
5. Paulmichl, M., P. Nasmith, R. Hellmiss, K. Reed, W. A. Boyle, J. M. Nerbonne, E. G. Peralta, and D. E. Clapham. Cloning and expression of a rat cardiac delayed rectifier potassium channel. *Proc. Natl. Acad. Sci. USA* **88**:7892–7895 (1991).
6. Tamkun, M. M., K. M. Knoth, J. A. Walbridge, J. Kroemer, D. M. Roden, and D. M. Glover. Molecular cloning and characterization of two voltage-gated $K^+$ channel cDNAs from human ventricle. *FASEB J.* **5**:331–337 (1991).
7. Roberds, S. L., and M. M. Tamkun. Cloning and tissue-specific expression of five voltage-gated potassium channel cDNAs expressed in rat heart. *Proc. Natl. Acad. Sci. USA* **88**:1798–1802 (1991).
8. Escande, D., A. Coulombe, J. F. Faivre, E. Deroubaix, and E. Coraboeuf. Two types of transient outward currents in adult human atrial cells. *Am. J. Physiol.* **252**:H142–H148 (1985).
9. Apkon, M., and J. M. Nerbonne. Characterization of two distinct depolarization-activated $K^+$ currents in adult ventricular myocytes. *J. Gen. Physiol.* **97**:973–1011 (1991).
10. Roberds, S. L., K. M. Knoth, P. Sunny, T. A. Blair, P. B. Bennett, R. P. Hartshorne, D. J. Snyders, and M. M. Tamkun. Molecular biology of the voltage-gated potassium channels of the cardiovascular system. *J. Cardiovasc. Electrophysiol.* **4**:68–80 (1993).
11. Snyders, D. J., K. M. Knoth, S. L. Roberds, and M. M. Tamkun. Time-, voltage-, and state-dependent block by quinidine of a cloned human cardiac potassium channel. *Mol. Pharmacol.* **41**:322–330 (1991).
12. Yatani, A., M. Wakamori, G. Mikala, and A. Bahinski. Block of transient outward-type cloned cardiac $K^+$ channel currents by quinidine. *Circ. Res.* **73**:351–359 (1993).
13. Po, S., D. J. Snyders, R. Baker, M. M. Tamkun, and P. B. Bennett. Functional expression of an inactivating potassium channel cloned from human heart. *Circ. Res.* **71**:732–736 (1992).
14. MacKinnon, R. Determination of the subunit stoichiometry of a voltage-activated potassium channel. *Nature (Lond.)* **350**:232–235 (1991).
15. Hartmann, H. A., G. E. Kirsch, J. A. Drewe, M. Taglialatela, R. H. Joho, and A. M. Brown. Exchange of conduction pathways between two related $K^+$ channels. *Science (Washington D. C.)* **251**:942–944 (1991).
16. Yellen, G., M. E. Jurman, T. Abramson, and R. MacKinnon. Mutations affecting internal TEA blockade identify the probable pore-forming region of a $K^+$ channel. *Science (Washington D. C.)* **251**:939–942 (1991).
17. Yool, A. J., and T. L. Schwarz. Alteration of ionic selectivity of a $K^+$ channel by mutation of the H5 region. *Nature (Lond.)* **349**:700–704 (1991).
18. Heginbotham, L., T. Abramson, and R. MacKinnon. A functional connection between the pores of distantly related ion channels as revealed by mutant $K^+$ channels. *Science (Washington D. C.)* **258**:1152–1155 (1992).
19. Tempel, B. L., D. M. Papazian, T. L. Schwarz, Y. N. Jan, and L. Y. Jan. Sequence of a probable potassium channel component encoded at *Shaker* locus of *Drosophila*. *Science (Washington D. C.)* **237**:770–775 (1987).
20. Timpe, L. C., Y. N. Jan, and L. Y. Jan. Four cDNA clones from the *Shaker* locus of *Drosophila* induce kinetically distinct A-type potassium currents in *Xenopus* oocytes. *Neuron* **1**:659–667 (1988).
21. Zagotta, W. N., T. Hoshi, and R. W. Aldrich. Gating of single Shaker potassium channels in *Drosophila* muscle and in *Xenopus* oocytes injected with Shaker mRNA. *Proc. Natl. Acad. Sci. USA* **86**:7243–7247 (1989).
22. Zagotta, W. N., and R. W. Aldrich. Voltage-dependent gating of Shaker A-type potassium channels in *Drosophila* muscle. *J. Gen. Physiol.* **95**:29–60 (1990).
23. Hoshi, T., W. N. Zagotta, and R. W. Aldrich. Biophysical and molecular mechanisms of Shaker potassium channel inactivation. *Science (Washington D. C.)* **250**:533–538 (1990).
24. Isacoff, E. Y., Y. N. Jan, and L. Y. Jan. Putative receptor for the cytoplasmic inactivation gate in the Shaker channel. *Nature (Lond.)* **353**:86–90 (1991).
25. Barish, M. E. A transient calcium-dependent $Cl^-$ current in the immature *Xenopus* oocyte. *J. Physiol. (Lond.)* **342**:309–325 (1983).
26. Hille, B. *Ionic Channels of Excitable Membranes*, Ed. 2. Sinauer Associates, Sunderland, MA (1992).
27. Hille, B. Local anesthetics: hydrophilic and hydrophobic pathways for the drug-receptor interaction. *J. Gen. Physiol.* **69**:497–515 (1977).
28. Chernoff, D. M., and G. R. Strichartz. Kinetics of local anesthetic inhibition of neuronal sodium currents: pH and hydrophobicity dependence. *Biophys. J.* **58**:69–81 (1990).
29. Latorre, R., and C. Miller. Conduction and selectivity in potassium channels. *J. Membr. Biol.* **71**:11–30 (1983).
30. Eisenman, G., G. Appleby, and O. Alvarez. Free energy perturbation studies of ion-selective permeation in Guy-Durrell "short *beta* barrel" and "random coil" structures for the Shaker $K^+$ channel pore. *Biophys. J.* **64**:A228 (1993).
31. Arena, J. P., and R. S. Kass. Block of heart potassium channels by clofilium and its tertiary analogs: relationship between drug structure and type of channel blocked. *Mol. Pharmacol.* **34**:60–66 (1988).
32. Snyders, D. J., M. M. Tamkun, and P. B. Bennett. A rapidly activating and slowly inactivating potassium channel cloned from human heart. *J. Gen. Physiol.* **101**:513–543 (1993).

**Direct correspondence to:** Andrea J. Yool, Department of Physiology, University of Arizona College of Medicine, Tucson, AZ 85724.