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Block of Single Batrachotoxin-Activated Na^+ Channels by Clofilium

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SUMMARY

The effects of clofilium on single batrachotoxin-activated Na^+ channels from rabbit skeletal muscle, incorporated into planar bilayers, were studied under symmetrical 200 mM NaCl conditions. Internally applied clofilium (0.3–30 μM) induced long lasting closures, with a mean duration of ~450 msec at +50 mV. The fraction of time spent in the clofilium-induced closed state was concentration dependent, with an equilibrium dissociation constant (K_d) of 3.4 μM at +50 mV. Kinetic analysis showed that both open and closed time distributions were well described by single exponentials, with respective time constants of τ_o and τ_c. As expected for an open channel blocker, $1/\tau_o$ increased linearly with increasing clofilium concentration, whereas $1/\tau_c$ remained relatively constant. Inhibition of batrachotoxin-activated Na^+ channels by clofilium exhibited a strong voltage dependence. The binding affinity of clofilium increased about 10-fold upon depolarization from −50 mV to 50 mV. Competition studies using quaternary and tertiary local anesthetics showed that clofilium and local anesthetics probably share a common receptor site located about halfway across the membrane electrical field. Together, our results demonstrate that clofilium is a potent Na^+ channel blocker in bilayers and its action is similar to that of other local anesthetics characterized previously.

Clofilium is an antiarrhythmic drug that prolongs the cardiac action potential duration at submicromolar concentrations (1). The physiological target site of clofilium is not yet known, although blockade of K^+ channels has been implicated (2, 3). Clofilium at ≤1 μM appears to have little effect on the rising phase of cardiac action potentials, suggesting that cardiac Na^+ channels are insensitive to clofilium (4). However, this insensitivity may be due to the limited concentration range tested.

The structure of clofilium consists of two relatively large hydrophobic domains attached to a quaternary ammonium, which carries a permanent positive charge (Fig. 1). A comparison of the structure of clofilium with that of QX-314, a quaternary derivative of lidocaine (Fig. 1), reveals several similarities. Both compounds contain an aromatic ring and a quaternary ammonium that are separated by a linker group with a length of ~4–5 Å. The major difference between clofilium and QX-314 is that clofilium has a long alkyl chain of seven carbons attached to the quaternary ammonium, versus two carbons in QX-314. Such a difference can affect the binding affinity of local anesthetics to BTX-activated Na^+ channels, because the larger the hydrophobic domain around the amine group, the stronger the binding between local anesthetics and BTX-activated Na^+ channels (5). Based on this structural information, we would predict that clofilium will have a stronger binding affinity for Na^+ channels than QX-314. To test this prediction, we have studied the effects of clofilium on BTX-activated Na^+ channels from skeletal muscle, which were incorporated into planar bilayers. The detailed action of various local anesthetics on muscle BTX-activated Na^+ channels has been previously characterized (5–7) and can, thus, be compared with the results for clofilium. In addition, the location of the clofilium binding site may be determined, because both internal and external solutions are accessible for drug applications.

Several questions concerning the effects of clofilium on Na^+ channels have come to our attention. First, does clofilium block BTX-activated Na^+ channels, as other local anesthetics do? Second, is there a single clofilium binding site on Na^+ channels? Third, where is this binding site located? Fourth, given the similarity in structure, do clofilium and local anesthetics share a common binding site? Fifth, is clofilium binding voltage dependent? Our results show that there is a single binding site for clofilium in BTX-activated Na^+ channels. This binding site is probably identical to or overlapping with the local anesthetic site. The long dwell time of clofilium in BTX-activated Na^+ channels suggests further that the long alkyl hydrophobic domain is involved in the binding interactions, a finding that is consistent with previous studies on the structure-activity relationship of local anesthetics (5).

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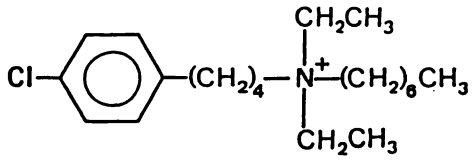
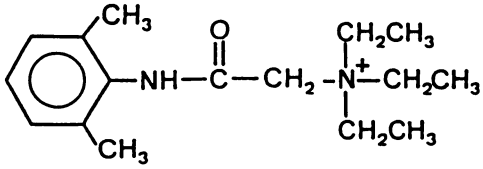
ABBREVIATIONS: BTX, batrachotoxin; EGTA, ethylene-bis(oxyethylenenitrilo)tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.



Clofilium



QX−314

Fig. 1. Chemical structures of clofilium and QX-314. Clofilium and QX-314 are both permanently charged quaternary ammonium compounds.

Materials and Methods

Chemicals. The synthetic phospholipids phosphatidylcholine and phosphatidylethanolamine were purchased from Avanti Polar Lipids (Birmingham, AL). BTX was a generous gift of Dr. John Daly, Laboratory of Bioorganic Chemistry, National Institutes of Health (Bethesda, MD). Clofilium tosylate was kindly provided by Eli Lilly (Indianapolis, IN). QX-314·Cl was supplied by Dr. Bertil Takman of Astra Pharmaceutical Products (Worcester, MA). Tetrodotoxin was obtained from Calbiochem-Behring (San Diego, CA). All other chemicals were reagent grade from commercial sources, used without further purification. Stock solutions of 0.5 mM BTX and 100 mM clofilium tosylate were dissolved in dimethyl sulfoxide, and stock solutions of 200 mM QX-314 and 100 mM (+)-mepivacaine were dissolved in standard aqueous solution (see below). These drug solutions were stored at −70°. All experiments were performed at room temperature (23 ± 2°). Standard aqueous solution contained 200 mM NaCl, 0.2 mM EGTA, and 10 mM HEPES-NaOH, pH 7.4. The final concentration of dimethyl sulfoxide applied during experiments was ≤1%, which had no effect on channel activity.

Membrane preparation. Plasma membrane vesicles were prepared from rabbit skeletal muscle, as described before (8, 9). Light vesicles banding on a cushion of 30% sucrose (w/v) were pelleted, resuspended at ~10 mg of protein/ml in 300 mM sucrose, and stored at −70°. The membrane preparation remained suitable for bilayer studies for more than 1 year.

Planar bilayers and Na^+ channel insertion. Planar bilayers were cast on 100–200-μm holes in polyvinyl chloride partitions, from decane solutions containing phosphatidylethanolamine (26.8 mg/ml) and phosphatidylcholine (6.7 mg/ml). Ionic currents were monitored at a constant holding voltage, using a List EPC-7 voltage clamp (Medical Systems Corp., Great Neck, NY). In general, plasma membrane vesicles (~10 μg of protein/ml, final concentration) were added to the *cis* (ground) side of the bilayer, and the voltages at the *trans* side were alternated from −65 mV to 65 mV every 10 sec, to facilitate the incorporation. Insertion of Na^+ channels could be detected in the presence of 100 nM BTX added to the *cis* side of the bilayer, essentially as described by Krueger *et al.* (10) and Green *et al.* (11). Under symmetrical 200 mM NaCl conditions, single BTX-activated Na^+ channels gave rise to about ±0.9-pA current amplitude deflection at $V = \pm 50$ mV. Both inward and outward current traces have been displayed upward in the figures, to facilitate comparison. The BTX-activated Na^+ channels were activated around −100 mV and could be blocked by tetrodotoxin in a voltage-dependent manner. All voltages are defined as 'intracellular' voltage, and the external face of Na^+ channels is defined as zero voltage.

Data acquisition and analysis were performed on an IBM-AT computer, using a Labmaster interface (40 KHz; Scientific Solutions, Solar, OH) and pClamp software (Axon Instruments, Foster City, CA). Curve fitting was performed using the Levenberg-Marquardt algorithm (12). Currents were filtered at 100 Hz (corner frequency at −3 db), recorded at a 100-Hz sampling rate, stored, and later analyzed on an AT computer as described (7). Because of the long closure times induced by clofilium, a sampling rate of 100 Hz was purposely chosen to reduce the events of the intrinsic short closures. For the same reason, closing events of ≤30 msec were ignored in the analysis.

Results

Induction of long lasting closures of BTX-activated Na^+ channels by internal clofilium. Application of clofilium to the internal side of the BTX-activated Na^+ channels caused discrete closing events, as shown in Fig. 2A. The frequency of closing events increased with increasing clofilium concentrations. Quantitative analysis showed that the fractional closed time (f_c) increased with clofilium concentration, in a manner that could be described by the Langmuir isotherm (Fig. 2B),

$$f_c = 0.97 \cdot [L]/([L] + K_d) \quad (1)$$

where K_d is the equilibrium dissociation constant, $[L]$ is the drug concentration, and f_c is the fractional closed time (determined over a period of 5–10 min, during which there were 300–600 closing events). The correction factor of 0.97 was included in the fitting of Eq. 1 in order to compensate for the spontaneous closing events that could be seen in the control records (9). The least squares estimate of K_d was 3.4 μM at +50 mV.

Kinetic analysis of the open and closed time distribution. The time distributions of open and closed events, plotted as histograms, could be well fit by single exponentials (Fig. 3A). The time constant for open events (τ_o) was found to be inversely proportional to the clofilium concentration (Fig. 3B, *left*), whereas the closed time constant (τ_c) appeared to be independent of drug concentration (Fig. 3B, *right*). These results are consistent with a simple bimolecular interaction between clofilium and the BTX-activated Na^+ channel and can be described by the following kinetic scheme (for details, see Ref. 13):

$$O + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} O \cdot L$$

where O is the open Na^+ channel, L is the clofilium molecule, and $O \cdot L$ is the drug-bound nonconducting channel. k_{on}, k_{off}, and K_d are the association and dissociation rate constants and equilibrium dissociation constant, respectively, where

$$k_{on} = 1/(\tau_o \cdot [L]) \quad (2)$$

$$k_{off} = 1/\tau_c \quad (3)$$

$$K_d = k_{off}/k_{on} \quad (4)$$

The k_{on} value was given by the slope of the fitted regression in Fig. 3B, which yielded a value of 7.1×10^5 M^{-1} sec^{-1}. The average k_{off} value determined from Fig. 3B equalled 2.3 sec^{-1}. The estimated K_d from 17 separate channels was 3.2 μM, which is very similar to the value derived from a fit of the fractional closed time (3.4 μM in Fig. 2B).

Voltage-dependent binding of clofilium. An important

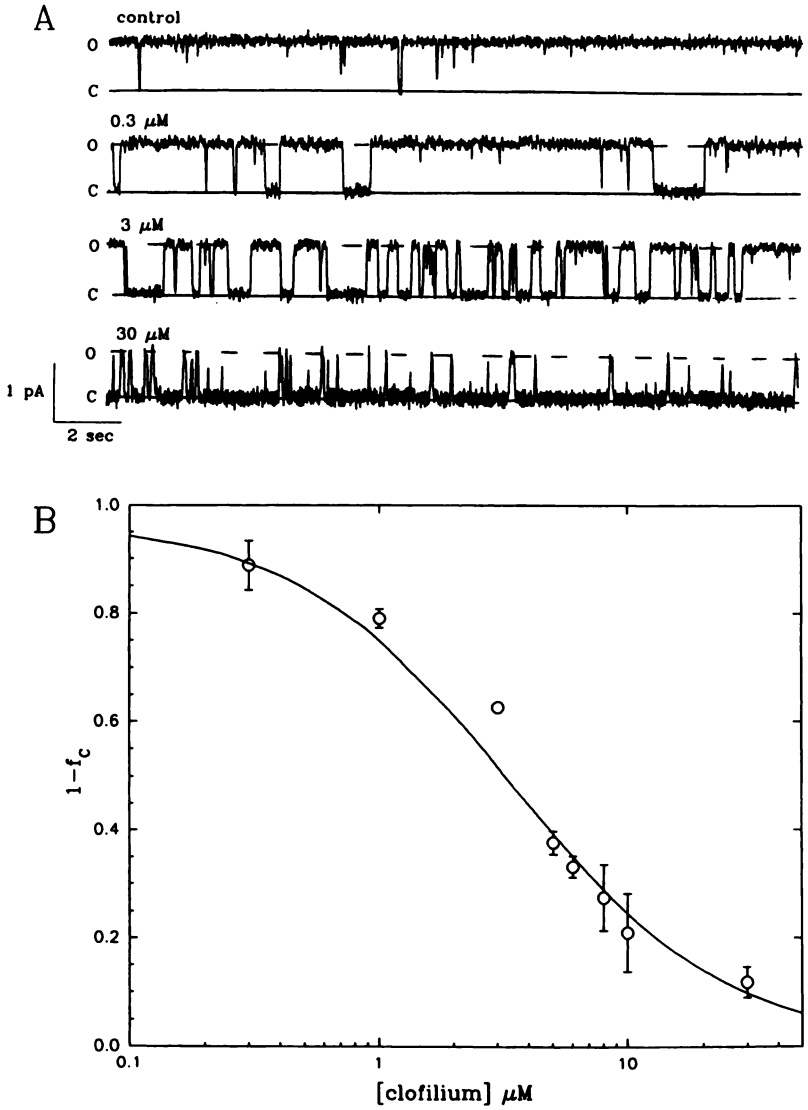


Fig. 2. Effect of clofilium on single BTX-activated Na^+ channels. A, Set of 20-sec current traces from two separate channels (first three from the same channel) held at +50 mV. Traces were recorded after application of various concentrations of clofilium to the internal side of the Na^+ channel. The control trace (*top*) in the absence of clofilium shows that the channel remains open (*O*) (– – –) most of the time (~97%). In the presence of clofilium, channel closures (*C*) (——) are frequent. B, The fractional closed time, f_c, was measured from current traces of Na^+ channels held at +50 mV and was plotted against internally applied clofilium concentration. The plot was subsequently fit by a Langmuir isotherm (Eq. 1) to obtain a value for K_d of 3.4 μM. Data for this plot are from 18 separate channels. Values are mean ± standard error from two to seven measurements. All experiments were performed under symmetrical 200 mM NaCl conditions.

factor that governs the binding kinetics of clofilium appears to be the membrane potential. Fig. 4 shows current records of the same channel in the presence of 8 μM internal clofilium, taken at four different membrane potentials. As the membrane potential was made more depolarized, the time that the channel spent in an open conducting state decreased. Kinetic analysis showed that both the k_{on} and k_{off} values were dependent on membrane potential; k_{on} increased, whereas k_{off} decreased with increasing depolarization (Fig. 5, A and B). This resulted in an even steeper voltage dependence of the K_d. Thus, the binding affinity for clofilium increased 10-fold when the membrane potential was switched from −50 mV to +50 mV (Fig. 5C).

According to the Woodhull hypothesis (14), which models the voltage-dependent binding of ligands to Na^+ channels, our data can be explained by the charged drug, clofilium, being driven into or out of the binding area by the electric field applied to the membrane. The relationship between voltage and drug potency can be described by the following equation:

$$K_d(V) = K_d(0) \cdot \exp(-\delta Ve/kT) \qquad (5)$$

where $K_d(0)$ is the estimated K_d at 0 mV, V is the applied voltage, e is the elementary charge, k is the Boltzman constant, T is the absolute temperature in degrees Kelvin, and δ is the equivalent valence. The δ value quantifies how strongly the applied voltage (V) affects clofilium potency. From Fig. 5C, the δ value was found to be 0.58. This value is similar to those reported for local anesthetics (5, 7). The similarity in δ values suggests that the clofilium binding site in BTX-activated Na^+ channels is located at a similar position within the membrane electric field as the site for other known local anesthetics.

Probable common receptor site for clofilium and local anesthetics. Previous studies have shown that internal QX-314 and (+)-mepivacaine can both compete directly with other local anesthetics, such as (−)-cocaine and (−)-bupivacaine, suggesting that these amine local anesthetics compete for a common binding site in BTX-activated Na^+ channels (5, 7). In order to examine whether clofilium and local anesthetics share a common binding site, we have determined the clofilium dwell time in the absence and presence of QX-314 and (+)-mepivacaine (Fig. 6). Both QX-314 and (+)-mepivacaine are weak blockers of BTX-activated Na^+ channels. Inhibition of BTX-activated Na^+ currents by these compounds is characterized not by discrete closing events, as seen with clofilium, but by a flickering block that gives the appearance of a reduction in the

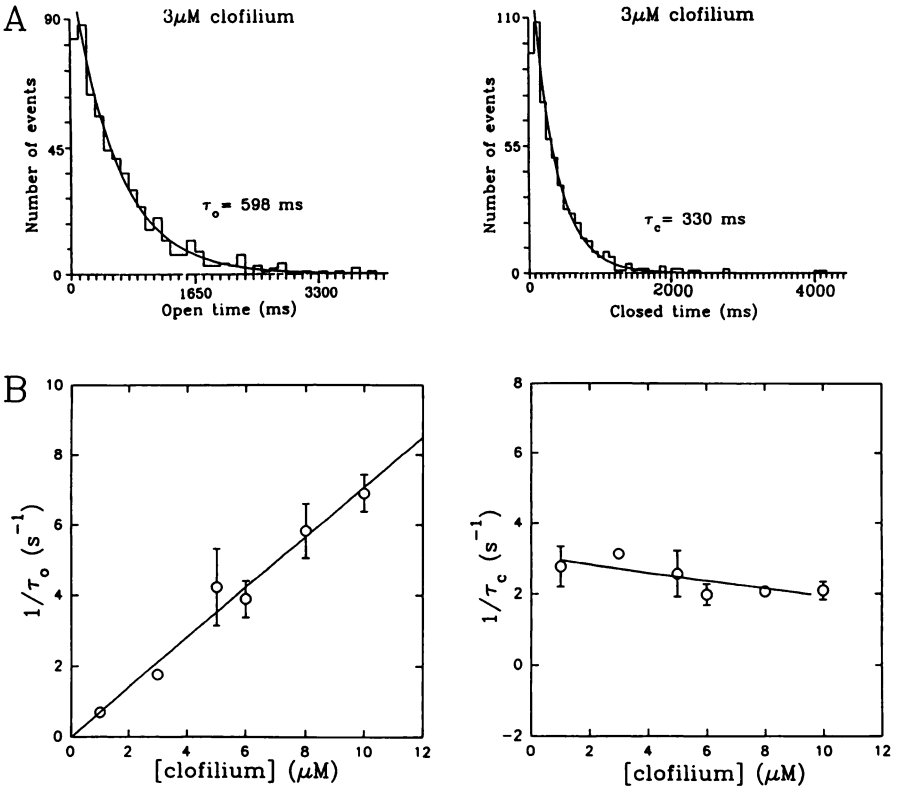


Fig. 3. A, Open and closed time histograms of clofilium-induced closures. Open and closed time durations were measured from current traces and binned into histograms. In order to determine the open (τ_o) and closed (τ_c) time constants, the histogram was fit to $N_t = N_0 \exp(-t/\tau)$, where N_t is the number of events of time length t, N_0 is the number of events at time 0, and τ is the time constant of the fit. *Left*, τ_o = 598 msec, N_0 = 109. *Right*, τ_c = 330 msec, N_0 = 146. All data are from the same channel, in the presence of 3 μM clofilium at +50 mV. B, Concentration dependence of open and closed time constants. Shown are plots of $1/\tau_o$ (*left*) and $1/\tau_c$ (*right*) versus clofilium concentration. Each plot was fit with a first-order linear regression. $1/\tau_o$ showed a linear dependence on clofilium concentration, with a slope of 0.71. On the other hand, $1/\tau_c$ was not concentration dependent (slope = −0.11, not significantly different from zero slope; $p < 0.05$, Student t test). The average $1/\tau_c$ value from all points was 2.3 ± 0.1 sec^{-1}. The time constants, τ_c and τ_o, were determined as in A, from 17 separate single channels held at +50 mV, with increasing amounts of internally applied clofilium. Each *point* represents the average of at least two values; the *error bars* are the standard error.

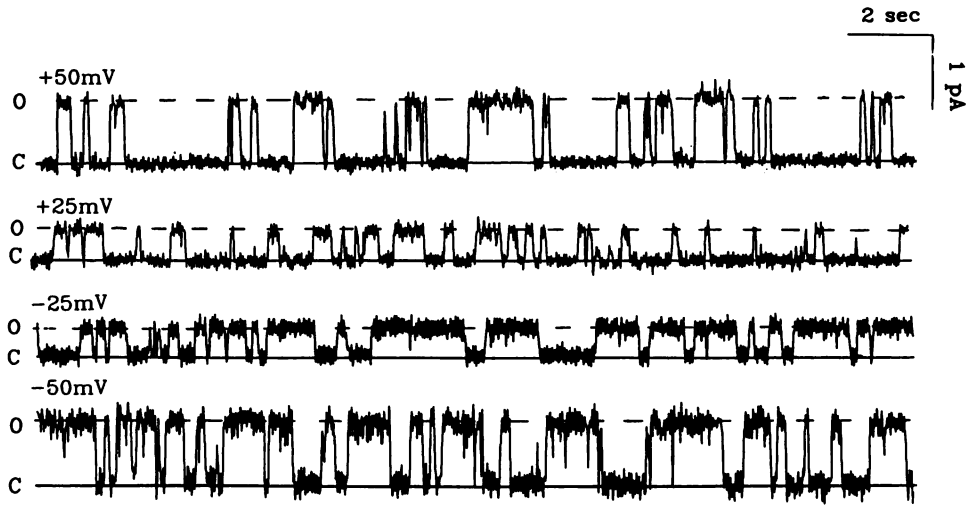
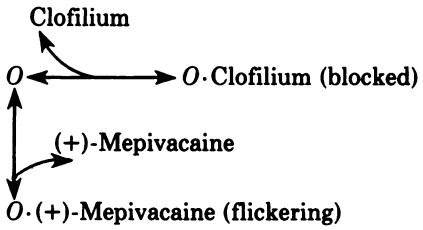


Fig. 4. Voltage dependence of clofilium binding. Current traces are from the same BTX-activated Na channel, with 8 μM clofilium applied internally, taken at four different holding potentials under symmetrical 200 mM NaCl conditions. Holding potential for each trace is shown directly above each trace. In the absence of clofilium, the channel is open approximately 97% of the time at voltages greater than or equal to −50 mV. (For control trace, see Fig. 2.) All traces are 20 sec long and are shown with currents deflected upward for comparison.

current amplitude and an increase in open channel noise (5). Thus, it was possible to distinguish blocking events induced by clofilium from those induced by QX-314 and (+)-mepivacaine.

We found that the mean closed time with clofilium remained unchanged, with or without (+)-mepivacaine or QX-314 (Fig. 6) being present, demonstrating that the off-rate constant (k_{off}) for clofilium is not affected by these agents. This result indicates that the binding site is not altered by (+)-mepivacaine and QX-314 when clofilium is bound. In contrast, the mean open time for Na^+ channels in the presence of clofilium was significantly increased in the presence of (+)-mepivacaine, as shown in Fig. 7. The fact that the mean open time of Na^+ channels in the presence of clofilium is greatly lengthened by (+)-mepivacaine and QX-314 but the mean closed time is not indicates that clofilium and these local anesthetics are mutually exclusive in their binding. These results are, therefore, consistent with the following kinetic scheme:



As predicted by this kinetic scheme, the lengthening of the

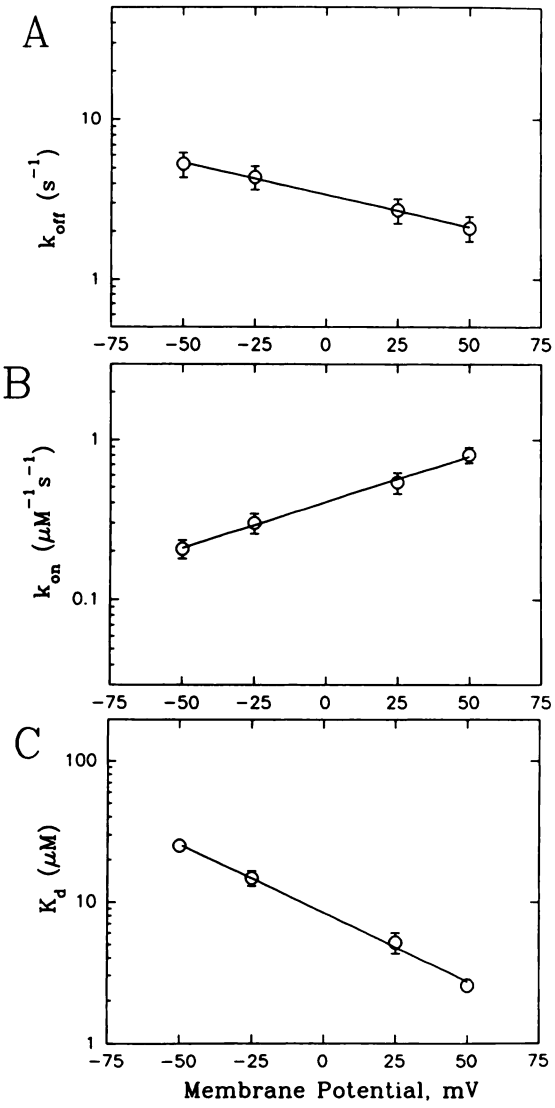


Fig. 5. Voltage dependence of rate constants. Semi-logarithmic plots of k_{on}, k_{off}, and K_d versus holding potentials are shown. Each plot was fit with a first-order linear regression. The slopes for each plot, in log unit/mV, were: A, -4.0×10^{-3}; B, 5.8×10^{-3}; and C, -9.8×10^{-3}. The rate constants k_{on}, k_{off}, and K_d were measured from four different channels, each held at +50, +25, −25, and −50 mV. Clofilium concentrations used were 5–10 μM. Each *point* represents the average value found at that holding potential; the *bars* are the standard error.

mean open time (τ_o) of Na^+ channels in the presence of clofilium by (+)-mepivacaine ($\tau_{o_{flickering}}$) should be according to the equation:

$$\tau_{0\,\text{flickering}} = \tau_0\left(1 + \frac{[(+)\text{-Mepivacaine}]}{K_D}\right) \quad (6)$$

where [(+)-mepivacaine] is the concentration of (+)-mepivacaine and K_D is the equilibrium dissociation constant of (+)-mepivacaine. Using the τ_0 values measured in Fig. 7, we calculated a K_D value of 1.6 mM for (+)-mepivacaine, a value that is close to 1.2 mM, measured directly (5). Similar conclusions can be made for the clofilium and QX-314 competition experiments.

Discussion

This paper demonstrates that clofilium is a potent blocker of Na^+ channels in muscle. The equilibrium dissociation constant of clofilium (~3 μM at +5 mV) in BTX-activated Na^+ channels is about 10-fold less than that reported to inhibit the delayed rectifier potassium currents in guinea pig ventricular myocytes (2) but 6-fold more than that required to inhibit the transient outward potassium current in rat ventricular myocytes (3). Thus, our results suggest that clofilium is a relatively nonspecific blocker of ion channels, like many local anesthetics previously studied (15).

One clofilium blocks one BTX-activated Na^+ channel. The block of BTX-activated Na^+ channels induced by internal clofilium appears to be due to interactions of clofilium with a single binding site in the channel. Two lines of evidence support this conclusion; (a) the dose-response curve can be described by the Langmuir isotherm, and (b) the open and closed time distributions of clofilium in BTX-activated Na^+ channels can be well described by single exponentials. The clofilium binding site is readily accessible from the internal surface but not from the external surface of Na^+ channels. Although clofilium is known to penetrate into the membrane phase (16), it may not reach the internal surface at high enough concentrations to exert its blocking effect, because of a large volume of internal solution. In the bilayer system, prolonged incubation with high concentrations of external clofilium was technically difficult, due to membrane instability caused by unwanted fusion events.

Clofilium and local anesthetics share a common binding site. Voltage-dependent binding of local anesthetics to BTX-activated Na^+ channels is a common phenomenon in bilayer systems (4). The quantitative analysis of this phenomenon indicates that the local anesthetic binding site is located about halfway across the membrane electrical field. This study shows that clofilium also senses about 50% of the membrane electrical field upon binding.

Furthermore, the local anesthetics QX-314 and (+)-mepivacaine lengthen τ_o in clofilium-treated Na^+ channels but have little effect on τ_c, indicating that when clofilium is bound local anesthetics are excluded from their binding site, and vice versa. The simplest explanation for the similarity in the voltage dependence of binding and the mutually exclusive binding is that local anesthetics and clofilium share a common binding site.

Hydrophobic interaction is an important determinant for the clofilium dwell time in BTX-activated Na^+ channels. Clofilium has two large hydrophobic domains, which appear to be an important factor for its long dwell time (τ_c) in BTX-activated Na^+ channels, as compared with those of QX-314 and (+)-mepivacaine [QX-314 and (+)-mepivacaine, $\tau_c <$ 10 msec; clofilium, τ_c = 450 msec]. This notion is consistent with the previous report (17) that the environment near the local anesthetic binding site is relatively hydrophobic. Nettleton and Wang (17) showed that the pK_a of the local anesthetic cocaine near its binding site is 1.4 units lower than that in bulk solution (7.1 versus 8.5), indicating that the protonation of cocaine is not favored near the binding site, probably because of a hydrophobic environment.

The direct contribution of the hydrophobicity of local anesthetics to their binding affinity has also been demonstrated (5). Removal of the benzoic moiety from (−)-cocaine destroys its blocking activity completely, indicating that the hydrophobic aromatic ring is required for the local anesthetic binding. Furthermore, increasing the number of methylene groups on the alkyl chain attached to the tertiary amine of bupivacaine hom-

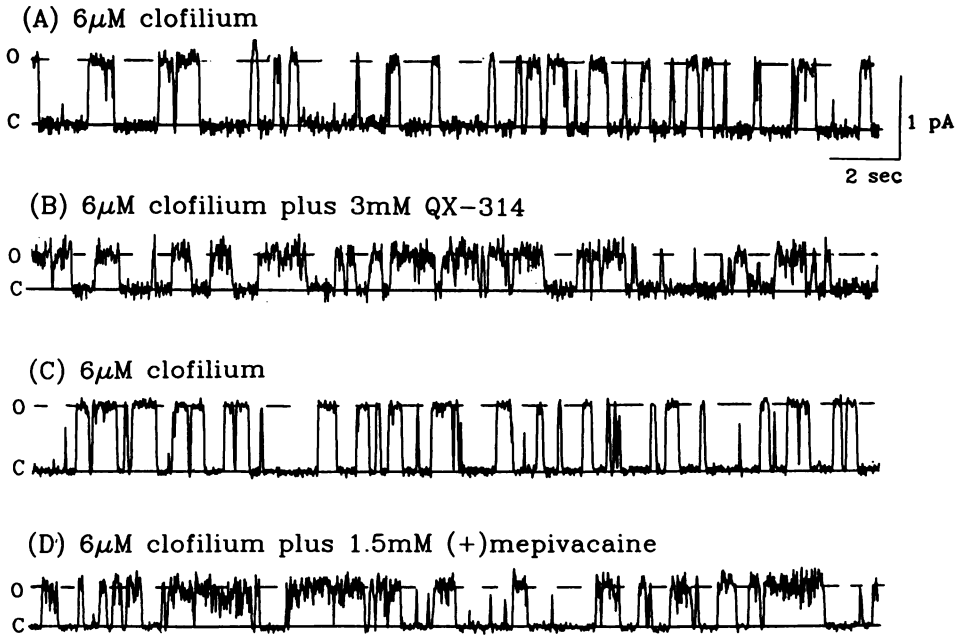


Fig. 6. Current traces showing competition between clofilium and QX-314 and between clofilium and (+)-mepivacaine. Data were recorded for 5 min after 6 μM clofilium was applied (A). QX-314 (3 mM) was then applied to the same channel and an additional 5 min of channel activity were recorded (B). The addition of QX-314 lengthened the open time, whereas the closed time remained relatively unchanged. (A, k_{on} = 1.1 × 10^6 M^{-1} sec^{-1}, k_{off} = 2.4 sec^{-1}; B, k_{on} = 4.6 × 10^5 M^{-1} sec^{-1}, k_{off} = 2.6 sec^{-1}). The same effect could be seen with (+)-mepivacaine, in C and D. (C, k_{on} = 9.2 × 10^5 M^{-1} sec^{-1}, k_{off} = 2.5 sec^{-1}; D, k_{on} = 4.7 × 10^5 M^{-1} sec^{-1}, k_{off} = 2.5 sec^{-1}). For all experiments, drugs were applied internally and the holding potential was +50 mV. In the analysis of B and D, all closings less than 50 msec were ignored, in order to exlude closings caused by QX-314 or (+)-mepivacaine.

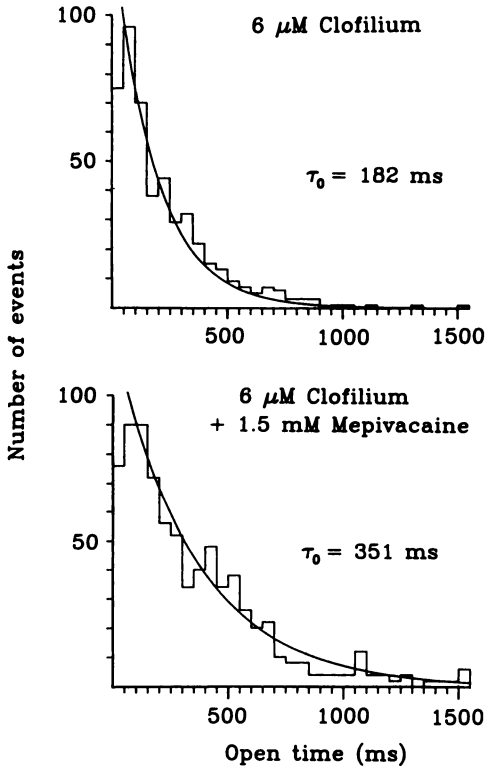


Fig. 7. Open time histograms of the clofilium-treated BTX-activated Na^+ channel in the absence and presence of (+)-mepivacaine. Open time durations were measured from current traces, as shown in Fig. 6, C and D. Both clofilium and (+)-mepivacaine were applied internally, at 6 μM and 1.5 mM, respectively. All data are from the same channel. Each histogram was fit to $N_t = N_0 \exp(-t)/\tau_o)$, as described in Fig. 3. The τ_o value is given in the figure. The τ_c values in the absence and presence of (+)-mepivacaine were 394 and 396 msec, respectively.

ologues results in an increase in binding affinity, with each methylene group contributing about 450 cal/mol. This increase in binding affinity is mainly due to the increase of τ_c. Thus, we conclude that the hydrophobic interaction between clofilium and BTX-activated Na^+ channels is the dominant factor for the long dwell time of clofilium.

Is the intermediate amide (or ester) bond of local anesthetics essential for their action? With the exception of benzocaine, which lacks an amine group, clinically used local anesthetics consist of an aromatic ring, an intermediate amide or ester bond, and a tertiary amine attached to alkyl groups (18). The involvement of the intermediate amide or ester bond in local anesthetic binding to Na^+ channels is not yet clear. Courtney and Etter (19) proposed that the amide bond causes some steric hindrance in the binding interaction, as compared with the ester bond. Our results show that, without the ester or amide bond, clofilium can still effectively block the Na^+ channels, suggesting that the intermediate amide or ester bond is not essential for the binding of local anesthetics to Na^+ channels. This would explain why clofilium, despite its permanent positive charge, is such a potent blocker of Na^+ channels in the bilayer, because it replaces the intermediate bond with four hydrophobic methylene residues. The calculated binding energy contributed by the three substituted methylene groups in the intermediate bond and five additional methylene groups in the alkyl chain attached to the amine is about 3.6 kcal/mol [450 cal/mol per methylene group (5)]. The amount of energy released upon binding would decrease the equilibrium dissociation constant by about 300-fold, as calculated by the equation $\Delta G^0_{eq} = -RT \cdot \ln(K_d/K_d')$ (see Ref. 5). This would account for the most of the K_d difference between QX-314 ($K_d(0)$ = 5.8 mM) (6) and clofilium ($K_d(0)$ = 8.4 μM).

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