Quaternary Ammonium Compounds as Structural Probes of Single Batrachotoxin-activated Na⁺ Channels

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ABSTRACT Quaternary ammonium (QA) blockers are well-known structural probes for studying the permeation pathway of voltage-gated K⁺ channels. In this study we have examined the effects of a series of n-alkyl-trimethylammonium compounds (Cₙ-QA) on batrachotoxin (BTX)-activated Na⁺ channels from skeletal muscle incorporated into planar lipid bilayers. We found that these amphipathic QA compounds (Cₙ-QA where n = 10–18) block single Na⁺ channels preferentially from the internal side with equilibrium dissociation constants (Kₐ) in the submicromolar to micromolar range. External application of amphipathic QA compounds is far less effective, by a factor of > 200. The block can be described by a QA molecule binding to a single site in the Na⁺ channel permeation pathway. QA binding affinity is dependent on transmembrane voltage with an effective valence (δ) of ~ 0.5. QA dwell times (given as mean closed times, τc) increase as a function of n-alkyl chain length, ranging from ~13 ms for C₁₀-QA to 500 ms for C₁₈-QA at +50 mV. The results imply that there is a large hydrophobic region within the Na⁺ channel pore which accepts up to 18 methylene groups of the Cₙ-QA cation. This hydrophobic domain may be of clinical significance since it also interacts with local anesthetics such as cocaine and mepivacaine. Finally, like BTX-activated Na⁺ channels in bilayers, unmodified Na⁺ channels in GH₃ cells are also susceptible to QA block. Amphipathic QA cations elicit both tonic and use-dependent inhibitions of normal Na⁺ currents in a manner similar to that of local anesthetic cocaine. We conclude that amphipathic QA compounds are valuable structural probes to study the permeation pathway of both normal and BTX-activated Na⁺ channels.

INTRODUCTION

A wide variety of quaternary ammonium (QA) compounds have been shown to block different types of voltage-gated K⁺ channels, including delayed rectifier K⁺ channels, Ca²⁺-activated K⁺ channels, and the sarcoplasmic reticulum K⁺ channel (for review, see Yellen, 1987). Some amphipathic QA compounds are potent blockers of these channels, exhibiting equilibrium dissociation constants (K₀) in the micromolar or...
submicromolar range. These compounds have since been used as structural probes to map the physical dimensions of the permeation pathway of K⁺ channels (e.g., Armstrong, 1966; French and Shoukimas, 1981; Swenson, 1981; Miller, 1982).

Although Na⁺ currents in excitable membranes have been traditionally isolated from K⁺ currents by the use of the hydrophilic QA cation tetraethylammonium (TEA) (Hille, 1984), earlier studies indicated that QA compounds may also block Na⁺ channels (Armstrong, 1966; Curtis and Scurlock, 1981). Indeed, Rojas and Rudy (1976) showed that when the Na⁺ channel inactivation of squid axons is completely removed by protease treatment, several QA cations, including TEA and nonyltriethylammonium, could effectively block the Na⁺ currents from the internal side. Their results demonstrate that there is a QA binding site within the Na⁺ channel pore.

Like protease, batrachotoxin (BTX) also removes Na⁺ channel inactivation (for review, see Hille, 1984). In addition, BTX shifts the threshold for Na⁺ channel activation by >30 mV in the hyperpolarizing direction. As a result, BTX-activated Na⁺ channels remain open most of the time at voltages greater than −60 mV in

![Chemical structures of representative organic cations. All compounds contain a hydrophilic head with a QA. Except cocaine (pK_a = 8.5), all have a permanent positive charge. Amphipathic QA compounds such as C_{14}-QA also contain a long hydrophobic tail.](figure1.png)
bic region of lipid bilayers. In contrast, cocaine in its neutral form can easily cross the membrane and amphipathic QA compounds can incorporate themselves into the planar lipid bilayers. The diffusion rate of amphipathic QAs in lipid bilayers is likely to be slower than that of neutral drugs. For example, QX-572 does give an appreciable block of Na⁺ channels in the node of Ranvier with external application (Hille, 1977a). However, it takes >25 min to reach the steady-state block. Most tertiary LAs, on the other hand, reach their steady-state block within 1–2 min after external application.

In this study, we have examined the effect of a series of amphipathic QA compounds on the BTX-activated Na⁺ channel from rabbit skeletal muscle in order to investigate the possible presence of a high affinity QA binding site. We found that these amphipathic QA compounds induce Na⁺ channel closures at concentrations in the submicromolar to micromolar range. Furthermore, the QA dwell times can be correlated with the hydrophobic interactions of QA with BTX-activated Na⁺ channels. This hydrophobic binding site is located near or along the Na⁺ permeation pathway and is relatively large, accepting up to 18 methylene groups. This QA binding site (perhaps some portion of it) also interacts with local anesthetics such as cocaine and mepivacaine. Finally, amphipathic QA compounds are also able to block normal Na⁺ currents in GH₃ cells in a manner similar to that of LAs.

MATERIALS AND METHODS

Planar Bilayer Experiments

Plasma membrane vesicles were prepared from rabbit skeletal muscle and incorporated into planar lipid bilayers as previously described (Moczydlowski and Latorre, 1983; Wang, 1988). Planar lipid bilayers were formed on ~200-μm holes in polyvinylchloride partitions from decane solution containing 13.4 mg/ml phosphatidylethanolamine (PE) and 6.7 mg/ml phosphatidylcholine (PC). Both PE and PC were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Incorporation of Na⁺ channels by vesicle fusion was monitored by constant alternative pulses of +50 mV and −50 mV each for 5 s in a symmetrical solution containing (in mM): 200 NaCl, 0.5 EGTA-Na⁺, and 10 HEPES-NaOH, pH 7.4 (standard NaCl solution). The vesicles at a final concentration of 10–20 μg/ml were applied to the cis chamber, which also included 0.1 μM BTX. The orientation of Na⁺ channels in the membrane was determined by the Na⁺ channel gating behavior, which occurred around −100 mV (Krueger et al., 1983). The external side of Na⁺ channels contained a tetrodotoxin (TTX)-binding site and was routinely confirmed at the end of experiments by observing TTX-induced block after the addition of toxin to this side. The voltage assignment is conventional; external surface of Na⁺ channels is defined as ground. Bilayer voltage clamp was achieved by a List EPC-7 clamp (Greenvale, NY) or Warner PC501 clamp (Hamden, CT). Records of single Na⁺ channel currents were filtered at 125 Hz, digitized at 2 ms per point (i.e., 500 Hz), stored in an IBM-AT computer, and later analyzed using pClamp software (Axon Instruments, Inc., Foster City, CA). As a rule, internal QA elicited its blocking effect rapidly after solution stirring, whereas external QA acted much more slowly (>5 min) and required high concentrations (>10 μM). In the bilayer system, prolonged incubation with high concentrations of external QA was technically difficult due to membrane instability caused by unwanted fusion events during recording. Dwell times of QA-induced blocked and unblocked events were measured by the Fetchan program and analyzed using the pStat program. Dwell times at +50 mV for C₁₂ to C₁₆-QA can be accurately measured, whereas dwell times for C₁₄-QA are only crude estimates due to its fast unblocking rate. No attempt was made to correct for missed events. All current records were displayed upward for comparison. Bilayers with more than one channel were not used in this study.
Whole-Cell Patch Clamp Experiments

The whole-cell variant of the patch-clamp method (Hamill et al., 1981) was used to measure Na⁺ currents in rat GH₃ pituitary clonal cells (also by List EPC-7 clamp). GH₃ cells were grown in culture dishes according to Cota and Armstrong (1989) and transferred to a recording chamber which was perfused continuously at a flow rate of 1.0 ml/min with an external solution containing (in mM): 150 choline-Cl, 0.2 CdCl₂, 2 CaCl₂, and 10 HEPES adjusted to pH 7.4 with TMA-OH. A stock of GH₃ cells was originally purchased from American Type Culture Collection (Rockville, MD). Micropipettes were fabricated from borosilicate capillary tubing and had tip resistances of ~ 1 MΩ when filled with an internal solution containing (in mM): 100 NaF, 30 NaCl, 10 EGTA, and 10 HEPES adjusted to pH 7.2 with CsOH. Because there was no external Na⁺ present, only outward Na⁺ currents were detected. The advantages of using high internal Na⁺ ions have been described previously (Cota and Armstrong, 1989). The holding potential was set at -100 mV. Most of the leak and capacitance currents were subtracted by analog circuitry and further reduced by P/4 protocol using the pClamp software. Drugs were applied externally to cells via a series of narrow bored capillary tubes positioned to within 200 μm of the cell. Internal applications of QA drugs directly within the micropipettes were unsuccessful in forming stable GΩ seals with GH₃ cells. To overcome this difficulty, the tips of micropipettes were first filled with normal internal solution by capillary action and back-filled with internal QA solution. The blocking effects of internal or external QA cations continued to increase over a time period of 20-40 min. The equilibrium conditions could not be ascertained during this time. The relative potency of internal and external drugs in GH₃ cells was compared after 20-30 min of incubation.

Organic QA Compounds and Local Anesthetics

All QA·Br or QA·Cl compounds were obtained from commercial sources without further purification. C₁₀-QA (Br), C₁₂-QA (Cl), and C₁₄-QA (Br) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI), C₁₆-QA (Br) from Sigma Chemical Co. (St. Louis, MO), and C₁₈-QA (Br) from Fluka Chemical Corp. (Ronkonkoma, NY). All the QA concentrations tested in this report are far lower than the QA critical micellization concentrations (Tanford, 1973). A stock solution of 100 mM QA was dissolved in dimethylsulfoxide (DMSO) and a serial dilution of stock could he made subsequently using the standard NaCl solution. It was not possible to dissolve amphipathic QA in decane solution, even after heating. This insolubility prevented us from applying QA compounds to the lipid phase directly. Thus, all drugs used in bilayer experiments were applied to the aqueous phase, which was then stirred vigorously for at least 30 s before recording was begun. Stock of BTX at 0.5 mM was dissolved in DMSO and applied directly to the solution in the cis-side chamber. Stocks of (-)mepivacaine and (-)cocaine at 100 mM were dissolved in standard NaCl solution. Mepivacaine and cocaine were obtained from Astra Pharmaceutical Products, Inc. (Worcester, MA) and the National Institute of Drug Abuse (Bethesda, MD), respectively. All experiments were performed at a room temperature of 23 ± 2°C. QA drugs were applied internally unless otherwise stated.

RESULTS

Equilibrium QA Binding Indicates a Single Binding Site Present in the Na⁺ Channel

Single BTX-activated Na⁺ channels incorporated into planar lipid bilayers have previously been used to examine the action of TTX and LAs (e.g., Moczydlowski et al., 1984, 1986; Wang, 1988). The bilayer system is particularly suitable for such
studies because (a) the ligand-induced block can be conveniently characterized when
the channel is in its open state most of the time, and (b) both sides of the membrane
are accessible to drugs. We therefore used the bilayer system to study the QA effect
on BTX-activated Na\(^+\) channels under 200 mM NaCl symmetrical conditions.
Amphipathic QA compounds, such as myristyltrimethylammonium bromide (C\(_{14}\)-QA), induce channel closures at internal concentrations ranging from 0.316 to 3.16
\(\mu\)M (Fig. 2). Control current traces show that at −50 mV and +50 mV BTX-activated
Na\(^+\) channels are indeed open most of the time (Fig. 2, A and B, upper traces). The
blocking effect of C\(_{14}\)-QA is both concentration and voltage dependent (Fig. 2, A and
B). The dose–response curve shows that the fractional open time at various C\(_{14}\)-QA
concentrations can be well described by the Langmuir isotherm (Fig. 2 C), suggesting
a bimolecular interaction of C\(_{14}\)-QA with the Na\(^+\) channel. At +50 mV the concentra-
tion that blocks 50% of the channel activity (KD) is ~ 0.66 \(\mu\)M, whereas at −50 mV the
KD is ~ 4.2 \(\mu\)M. Evidently, the QA binding is strongly voltage dependent.

**Kinetics of QA-induced Na\(^+\) Channel Closures**

The one-to-one relationship for the interaction of the QA drug with the Na\(^+\) channel
is similar to that found for TTX and several LA drugs (e.g., Green et al., 1987b;
Wang, 1988, 1990). To examine further whether the QA-induced block can be
modeled by a single-site binding reaction, we tested the following scheme:

\[
\text{O} + \text{QA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{O} \cdot \text{QA} \quad \text{(blocked)}
\]

where O is the open Na\(^+\) channel, QA is the quaternary ammonium cation, \(k_1\) is the
association (on) rate constant for QA binding, and \(k_{-1}\) is the dissociation (off) rate
constant for open channel and QA complex, O-QA. As is predicted from this scheme,
the open and closed time distributions of BTX-activated Na\(^+\) channels in the
presence of C\(_{14}\)-QA drug are well described by a single exponential at +50 mV (Fig.
3, A and B). Furthermore, the reciprocal mean dwell time in state O, \(1/\tau_o\), is a linear
function of QA concentration (Fig. 3 C), whereas the reciprocal mean dwell time in
state O-QA, \(1/\tau_o\) is independent of QA concentration (Fig. 3 D). These results
provide a strong support for a model in which the dwell times of the QA drug in the
O-QA complex correspond to the actual time that a single QA molecule occupies a
specific binding site on the channel. According to the proposed scheme, it is possible
to derive the QA on-rate constant, \(k_1\), as \(1/(\tau_o[QA])\) and the QA off-rate constant, \(k_{-1}\),
as \(1/\tau_o\). Using these relationships we obtain a \(k_1\) value of \(1.52 \times 10^7\) s\(^{-1}\) M\(^{-1}\), a \(k_{-1}\)
value of 12.5 s\(^{-1}\), and an equilibrium dissociation constant \(K_D\) of \(k_{-1}/k_1 \approx 0.82\) \(\mu\)M at
+50 mV under symmetrical 200 mM NaCl conditions. This \(K_D\) value is similar to that
determined from the dose–response curve (Fig. 2 C).

**Voltage Dependence of QA Binding**

A unique feature of the QA channel interactions found in SR K\(^+\) channels is the
strong voltage dependence of the QA on-rate constant (Miller, 1982) but not the
off-rate constant. To quantify the voltage dependence of QA binding in Na\(^+\)
channels, we measured the binding kinetics at various membrane potentials. We
FIGURE 2. C₁₄-QA induces closures of single BTX-activated Na⁺ channels. (A) Examples of current traces recorded at −50 mV are shown in the absence (control) and presence of various concentrations of internal C₁₄-QA. The downward current traces are displayed upward to facilitate the comparison with the current traces recorded at +50 mV (B). The same channel was used in A and B. Solid lines drawn within current traces indicate the zero current level. Control current traces with brief spontaneous closures are purposely chosen for baseline determination. (C) The fractional open time (fₒ) is plotted against the C₁₄-QA concentration. Two to five channels were used to determine the fₒ at each concentration. Error bar represents the standard error. Solid and dashed lines are drawn according to the Langmuir isotherm, fₒ = Kₒ/(Kₒ + [L]), where Kₒ is the drug concentration at which fₒ = 0.5 and [L] is the drug concentration. The Kₒ value of C₁₄-QA is 0.66 μM at +50 mV and 4.2 μM at −50 mV.
found that the on-rate constants at various voltages do display strong voltage
dependence (Fig. 4A, open circles); however, so do the off-rate constants (Fig. 4A,
closed circles). The QA interaction with the Na\(^+\) channel therefore differs from that
of the SR K\(^+\) channel in terms of the voltage dependence of the \(k_\text{on} \) values. The
voltage dependence of \(K_\text{b} \) values is relatively steep; about an eightfold difference in
the \(K_\text{b} \) is found when the voltage is switched from -50 to +50 mV (Fig. 4 B). This

![Figure 3. Kinetics of C\(_{14}\)-QA binding at +50 mV. Histograms of open (A) and closed (B) time
distributions of C\(_{14}\)-QA induced events in BTX-activated channels at +50 mV are fitted to a
single exponential: \(N(t) = N \exp(-t/\tau)\), where \(N(t)\) is the number of events per 20-ms bin and
\(N\) is the number of events in the population at \(t = 0\). \(\tau\) is the mean open time (\(\tau_0\) in A) or the
mean closed time (\(\tau_c\) in B). The bilayer was formed in standard solution and 1 \(\mu\)M C\(_{14}\)-QA was
applied to the internal side of the Na\(^+\) channel. The reciprocal mean dwell times in the open
(C) and closed (D) states were plotted against various internal C\(_{14}\)-QA concentrations at +50
mV (open circles) and -50 mV (closed circles). \(1/\tau_0\) follows a linear relationship with the internal
C\(_{14}\)-QA concentration with a slope of 15.2 s\(^{-1}\) \(\mu\)M\(^{-1}\) for +50 mV and 5 s\(^{-1}\) \(\mu\)M\(^{-1}\) for -50 mV.
\(1/\tau_c\) appears to be independent of C\(_{14}\)-QA concentration with an average value of 12.5 s\(^{-1}\) for
+50 mV and 41 s\(^{-1}\) for -50 mV. The concentrations of C\(_{14}\)-QA tested are far less than CMC.

voltage dependence is similar to that determined independently in the dose–
response curve (Fig. 2 C). Quantitatively, the binding affinity vs. voltage relationship
can be described by the following equation:

\[
K_\text{b}(\Delta V) = K_\text{b}(0 \text{ mV}) \cdot \exp(-\delta \cdot \Delta V / kT)
\]
FIGURE 4. Voltage-dependent binding of C_{14}QA. (A) On-rate constant \( k_1 \) (open circles) and off-rate constant \( k_{-1} \) (closed circles) are plotted against voltage. \( k_1 \) and \( k_{-1} \) were measured according to \( 1/(\tau_o[QA]) \) and \( 1/\tau_r \), respectively. \( C_{14}QA \) at 2 \( \mu M \) was applied internally. (B) \( K_D \) values are plotted against voltage, where \( K_D = k_{-1}/k_1 \). Both \( k_{-1} \) and \( k_1 \) were taken from A. The line is drawn according to a linear regression of all data points. A single channel was used to determine the binding kinetics of this figure. The \( \delta \) value determined in this figure is \( \sim 0.42 \) (see text).
where $K_0 (0 \text{ mV})$ is the estimated $K_0$ at $0 \text{ mV}$, $\delta$ is an equivalent valence that quantifies how the applied voltage ($\Delta V$) affects QA binding, $e$ is the elementary charge, $K$ is Boltzmann's constant, and $T$ is the temperature in degrees Kelvin. The $\delta$ value is estimated to be 0.42 for $C_{14}\text{-QA}$. We have noticed that the $\delta$ value varies considerably from channel to channel. The main error source is probably from the fast kinetics at the negative voltages due to the short dwell time of $C_{14}\text{-QA}$. Nevertheless, this $\delta$ value is similar to the voltage-dependent binding of local anesthetics such as QX-314 and cocaine (Wang, 1988). The theoretical implication of this $\delta$ value will be discussed later.

The Longer the Alkyl Chain, the Longer the QA Dwell Time

The binding affinity of internal $C_{14}\text{-QA}$ appears to be several orders of magnitude stronger than that of internal TMA and TEA, which have $K_0$ values of $>10 \text{ mM}$ at $+50 \text{ mV}$ (Moczydlowski et al., 1986; Green et al., 1987a). This difference in binding affinity may be due to the presence of 9–13 additional methylene groups. To test this possibility, we used a series of $n$-alkyl trimethylammonium compounds with $n$-alkyl chain length ranging from 10 to 18. Fig. 5A shows that increasing the number of methylenes in the $C_n\text{-QA}$ structure results in a longer drug dwell time at $+50 \text{ mV}$. This correlation is clearly shown in the $k_-$ vs. carbon number plot in Fig. 5B. The $k_-$ value for $C_{10}\text{-QA}$ at $+50 \text{ mV}$ is estimated to be 75 s$^{-1}$, corresponding to a $\tau_-$ value of $\sim 13 \text{ ms}$, a relatively short dwell time that is difficult to measure accurately in bilayers. However, the $k_-$ values for $C_{12}$ to $C_{18}\text{-QA}$ can be reasonably well determined. We conclude that the dwell times of QA compounds are primarily governed by the hydrophobic interactions between QA and its binding site. In contrast, the graph of the on-rate constant, $k_+$ vs. carbon number shows a bell-shaped relationship with a maximal value of $1.92 \times 10^7$ M$^{-1}$ s$^{-1}$ corresponding to $C_{14}$ at $+50 \text{ mV}$ (Fig. 5B). The $K_0$ values at $+50 \text{ mV}$ and $\Delta G_{eq}^{\infty}$ of QA compounds are plotted against carbon number (Fig. 5C). The calculated $\Delta G_{eq}^{\infty}$ contributed by each methylene group from $C_{10}$ to $C_{14}$ is $\sim 540 \text{ cal/mol}$ at $+50 \text{ mV}$ (Fig. 5C, dashed line). The $\Delta G_{eq}^{\infty}$ values of $C_{16}$ and $C_{18}\text{-QA}$ deviate from 540 calories per methylene group mainly due to their much smaller $k_+$ values, suggesting that QA compounds with $C_n \geq 16$ may encounter steric hindrance within the channel due to their bulky hydrophobic tail. All these QA compounds have relative high critical micellization concentration (CMC) values in aqueous solution as compared with the concentrations of QA used in this study. For example, $C_{16}\text{-QA}$ has a CMC value of 1 mM in 50 mM NaCl solution (Tanford, 1973; Calbiochem catalog), a value that far exceeds the micromolar range used in this report. It is therefore unlikely that the low $k_+$ values of $C_{16}\text{-QA}$ and $C_{18}\text{-QA}$ in QA-Na$^+$ channel interactions are due to the QA concentration reaching its CMC value. It is noteworthy that, like $C_{14}\text{-QA}$, all amphipathic QA compounds shown in Fig. 5 also display strong voltage dependence in their binding affinity; $\delta$ values range from 0.41 to 0.58 with a mean of 0.49 (Table I), indicating that these cations sense $\sim 50\%$ of the membrane electric field.

Binding Competition between QA and LA Drugs

Several characteristics of QA binding appear to resemble those of LA binding (Wang, 1988). First, the binding is voltage dependent with a $\delta$ value of $\sim 0.5$. Second, the
dwell times of both QA and LA drugs are largely determined by hydrophobic interactions (Fig. 5 B; Wang, 1990). To examine whether these similarities are due to a common binding site for both QA and LA drugs, we performed competition experiments. Fig. 6 A shows that (-)cocaine induces channel closures in the absence and the presence of C_{10}^\text{QA}. In the (-)cocaine-treated Na^+ channel, C_{10}^\text{QA} induces flickering current noise, just as it does alone (Fig. 5 A). In addition, the open times of the cocaine unblocked events are significantly lengthened by C_{10}^\text{QA}, whereas the closed times are not. Similar observations have been made in the competition experiments between C_{10}^\text{QA} and (-)mepivacaine (Fig. 6 B). The result of the cocaine plus C_{10}^\text{QA} competition study is therefore consistent with the following
According to this kinetic scheme, the QA and LA drugs will block the Na⁺ channel in a mutually exclusive manner. When (-)cocaine binds, it induces channel closures with discrete dwell times ($\tau_c = 515$ ms). In contrast, when C₁₀-QA binds, it induces flickering current noise since its dwell time is <15 ms. In the presence of C₁₀-QA, the 

$$\tau_c$$ for (-)cocaine-induced closures remains essentially the same ($\tau_c = 492$ vs. 515 ms) as expected if C₁₀-QA cannot bind to the occupied site. In contrast, the open times that show frequent flickering noise due to the C₁₀-QA binding are lengthened since (-)cocaine cannot bind to the QA occupied site (Fig. 6, C and D). Quantitatively, the $\tau_{o(\text{obs})}$ should be equal to $\tau_o (1 + [C_{10-}\text{QA}]/K_D)$, where $\tau_o$ and $\tau_{o(\text{obs})}$ are open time constants in the presence of (-)cocaine and (-)cocaine plus C₁₀-QA, respectively, $[C_{10-}\text{QA}]$ is the concentration of C₁₀-QA, and $K_D$ is the dissociation constant of C₁₀-QA. The calculated $K_D$ from this method is 25 µM for C₁₀-QA, a value similar to that measured directly (Fig. 5 C). Our results thus clearly show a mutually exclusive feature of LA and QA drugs and suggest that these drugs share a common binding area in BTX-activated Na⁺ channels.

### Table 1

<table>
<thead>
<tr>
<th>C₁₀-QA</th>
<th>$k_+$ (0 mV)</th>
<th>$k_-$ (0 mV)</th>
<th>$K_D$ (0 mV)</th>
<th>$\delta$</th>
<th>$n$</th>
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<td>C₁₀-QA</td>
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<td>19.3</td>
<td>9.6</td>
<td>0.58</td>
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<td>6.0</td>
<td>0.8</td>
<td>0.41</td>
<td>3</td>
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<tr>
<td>C₁₀-QA</td>
<td>0.19</td>
<td>5.0</td>
<td>26.4</td>
<td>0.57</td>
<td>2</td>
</tr>
</tbody>
</table>

Mean = 0.49

The values for $k_+$ (0 mV), $k_-$ (0 mV), and $K_D$ (0 mV) were determined as described in Fig. 3 except for C₁₀-QA. $n$ represents the number of channels used for this determination. Data were pooled and the kinetic constants at 0 mV were determined by the best fitted line. In the case of C₁₀-QA, current-voltage plots were derived from data recorded before and after C₁₀-QA application. These data were used to calculate the $K_D$ value at various voltages according to the equation

$$K_D = \frac{[\text{C}_{10-}\text{QA}]}{\text{i}_0/\text{i}_b}$$

where $[\text{C}_{10-}\text{QA}]$ is 30 µM and $\text{i}_0$ and $\text{i}_b$ are the averaged current amplitude before and after C₁₀-QA application, respectively. The $K_D$ (0 mV) was then determined as shown in Fig. 3 B. The equivalent valence, $\delta$, was determined by an equation described in the text.
Direct application of QA cations to the lipid phase was not feasible (see Materials and Methods) since they are not soluble in decane solution. However, an amphipathic QA cation in aqueous solution can easily incorporate its hydrophobic tail into a lipid bilayer (Tanford, 1973). To determine whether this hydrophobic pathway through the lipid phase is important for QA binding, we tested the blocking effect of external

\[ \text{A} \quad 300 \text{ \textmu M (-)cocaïne} \]
\[ \quad \text{300 \textmu M (-)cocaïne plus 30 \textmu M C}_{10}\text{-QA} \]

\[ \text{B} \quad 0.3 \text{ \textmu M C}_{16}\text{-QA} \]
\[ \quad 0.3 \text{ \textmu M C}_{16}\text{-QA plus 5 mM (-)mepivacaine} \]

FIGURE 6. Direct binding competition between QA and LA drugs at +50 mV. (A) Examples of single channel records at +50 mV in the presence of 300 \textmu M (-)cocaïne and 300 \textmu M (-)cocaïne plus 30 \textmu M C\textsubscript{10}-QA. Both drugs were applied internally to the same channel. (B) A similar experiment as shown in A, except 0.3 \textmu M C\textsubscript{16}-QA and 5 mM (-)mepivacaine were used. Zero current baseline is indicated by a solid line. Open time distributions in the presence of 300 \textmu M (-)cocaïne (C) and 300 \textmu M (-)cocaïne plus 30 \textmu M C\textsubscript{16}-QA (D) show that there is a two-fold increase of the mean open time when both QA and LA drugs are present (118 vs. 258 ms). In contrast, \( v_c \) values remain unchanged (515 vs. 492 ms). The \( \tau_0 \) value for 0.3 \textmu M C\textsubscript{16}-QA alone is measured at 205 ms, which is subsequently lengthened by 5 mM (-)mepivacaine to 405 ms, whereas the \( \tau_0 \) value remains the same (159 vs. 167 ms). A cut-off closed time of 30 ms (C) or 50 ms (D) was used to eliminate the possible contamination of flickering events.

\[ \text{C} \quad 300 \text{ \textmu M (-)cocaïne} \]
\[ \quad \tau_0 = 118 \text{ ms} \]

\[ \text{D} \quad 300 \text{ \textmu M (-)cocaïne plus 30 \textmu M C}_{10}\text{-QA} \]
\[ \quad \tau_0 = 258 \text{ ms} \]

FIGURE 7. A shows an example of this study. The estimated \( K_D \) for the external C\textsubscript{16}-QA is \( \sim 200 \text{ \mu M} \), or <1% of the binding affinity of the internal C\textsubscript{16}-QA. Kinetic analysis shows that the main difference is due to the on-rate constant of the external QA, which is \( \sim 200\)-fold smaller than that of the internal QA. Both the off-rate constant and the voltage dependence of external QA block remain the same (data not shown). Subsequent application of internal C\textsubscript{16}-QA at 2 \textmu M gives rise to a much stronger blocking effect by this compound (Fig. 7 B). This result indicates that the
hydrophobic pathway for the external QA compounds is a rather poor pathway to reach the QA binding site. Instead, it suggests that the binding site must be readily accessible to the QA compounds through the internal hydrophilic pathway. Indeed, we have also observed that when the lipids in the annulus (a mass at least a million times that of the bilayer) were allowed to equilibrate with amphipathic QA compound before channel insertion and solution exchange, a very minor QA effect could then be detected in the subsequently incorporated channel. This result implies that amphipathic QA compounds in the annulus do not have easy access to their binding site through the lipid phase.
QA Compounds Act as Local Anesthetics in Blocking Normal Na⁺ Channels

One of the disadvantages of using the planar bilayer technique to study Na⁺ channels is that it requires BTX to remove the inactivation process. It remains to be seen, therefore, whether QA compounds can block normal Na⁺ channels in intact cells. To address this question, we applied either external or internal C₁₄-QA to GH₃ cells in the absence of BTX. Fig. 8, A and B, shows that the peak Na⁺ currents at a test potential of +30 mV were inhibited by ~50% (i.e., tonic inhibition) after 20 min exposure to external 10 μM C₁₄-QA. When the cell was stimulated at 2 Hz, Na⁺ currents were progressively reduced (i.e., use-dependent inhibition; Fig. 8 B) to reach a steady-state level of ~15% of the control peak Na⁺ current after 30

\[
K_0 = \frac{[C_{14} - QA]}{[C_{14} - QA] + K_D}
\]

where \([C_{14} - QA]\) is 10 μM, \(I_o\) is the control peak current, and \(I_b\) is the peak current in the presence of \(C_{14} - QA\) after repetitive pulses. The apparent \(K_0\) was estimated to be 2.0 μM (2.6 ± 1.0 μM; mean ± SE, \(n = 3\)). For comparison, experiments with an identical pulse protocol were performed in the absence (C) and presence (D) of external 100 μM (-)cocaine. Both tonic and use-dependent block reached their steady-state level within 2 min of drug application. Tonic block was measured at ~55%. More than 65% of the remaining current could be further blocked by repetitive pulses.

FIGURE 8. External C₁₄-QA compound and (-)cocaine block normal Na⁺ channels in a similar manner. (A) Control Na⁺ currents in the absence of BTX were recorded when the GH₃ cell was stimulated at 2 Hz with a total of 30 pulses (see bottom; +30 mV each for 50 ms). All current traces were superimposed. Holding potential was set at −100 mV. (B) After 20 min treatment of 10 μM C₁₄-QA, Na⁺ currents were recorded again. Peak Na⁺ current was reduced by ~45%. Repetitive depolarizations of the cell further reduced the peak current amplitude to a level of ~15% of the control peak current amplitude. Notice that in A little use-dependent inhibition is present in the control Na⁺ current. The dashed line is the zero current baseline. The apparent \(K_0\) of external C₁₄-QA was calculated according to the following equation:

\[
K_0 = \frac{[C_{14} - QA]}{[C_{14} - QA] + K_D}
\]
consecutive pulses. Control currents in the absence of QA drug, on the other hand, were little reduced by repetitive stimulations (Fig. 8A). Such tonic and use-dependent inhibitions of Na⁺ currents are well known for LA drugs (Fig. 8, C and D; also Hille, 1977a, b), although external LA drugs such as cocaine normally act rapidly

FIGURE 9. Internal C₁₄-QA compound blocks Na⁺ channels in the absence of BTX. (A) Na⁺ currents were recorded with repetitive pulses as shown at the bottom (+30 mV each for 50 ms). The pipette tip contained 1 μM C₁₄-QA compound (see Materials and Methods). The current traces were superimposed and were taken ~5 min after the break-in of the pipette. Repetitive pulses reduced the peak current by ~25%. The dashed line is the zero current baseline. (B) Na⁺ currents were recorded following the same pulse protocol. The traces were taken ~30 min after the pipette break-in. The tonic block was ~30% as determined by the reduction of the peak current amplitude between the first trace of A and B. This reduction of the peak current developed continuously over a time period of 30–40 min. After repetitive pulses, the peak current was further reduced by 55%. The calculated apparent Kᵦ (see Fig. 8 legend) yielded a value of 0.48 μM (0.36 ± 0.08; mean ± SE, n = 3). This value should be considered as an upper limit for the Kᵦ value since we could not determine the control peak current in the absence of C₁₄-QA. Instead, we arbitrarily used the peak current amplitude of the first trace in A as the control, which was the earliest stable current trace we could record.
and reversibly, whereas QA compounds act slowly over a time span of 30 min and take tens of minutes of perfusion to partially remove their effects (data not shown).

Like the external QA application, internal C\(_{14}\)QA at 1 \(\mu\)M also inhibits the normal Na\(^+\) current. 5 min after the pipette break-in of GH\(_3\) cells there is a moderate use-dependent inhibition (~20\%, Fig. 9A). After 25 min the peak current is further reduced by ~15\% (tonic inhibition) and an additional 50\% of the remaining current can be blocked by repetitive depolarizations (use-dependent inhibition; Fig. 9B). These results also demonstrate that the relative potency of external and internal C\(_{14}\)QA in GH\(_3\) cells is less than a 10-fold difference (\(K_D = 2.6 \mu\)M for external vs. 0.36 \(\mu\)M for internal application), whereas in planar bilayers the difference is ~200-fold. We conclude, therefore, that the lack of strong external QA effects in bilayers is because the external QA cation cannot gain easy access to the internal hydrophilic pathway, perhaps due to its slow diffusion rate in bilayers and the presence of a large internal volume (3 ml). Our results also indicate that QA compounds can be used as structural probes for studying normal Na\(^+\) channels even in the absence of BTX. The fact that QA compounds act like local anesthetics in intact cells further strengthens the notion that QA and LA drugs share a common binding area in the Na\(^+\) channel. Detailed studies of QA effects on the gating kinetics of unmodified Na\(^+\) channels will undoubtedly provide further information on this common binding area. Such a task is currently being undertaken in our laboratory.

**DISCUSSION**

**Site of Action of Amphipathic QA Compounds**

This report demonstrates that internal amphipathic QA compounds at submicromolar to micromolar concentrations block BTX-activated Na\(^+\) channels by binding to a single site. External QA application is far less effective, by a factor of ~200 in bilayers, but is somewhat more effective in intact cells, probably because of their limited internal volume. We conclude that the QA binding site is located within the Na\(^+\) channel pore and can be reached primarily through the internal hydrophilic pathway. The hydrophobic pathway of the internal QA compound is probably not significant for QA block as suggested in the Results, but this has not been determined directly. Our conclusions are in agreement with the fact that hydrophilic QA compounds such as TEA and TMA only block BTX-activated Na\(^+\) channels internally (\(K_D \geq 10 \text{ mM at } +50 \text{ mV}; \)Green et al., 1987a), and the destruction of Na\(^+\) channel inactivation reveals a TEA receptor inside the pore (Rojas and Rudy, 1976). The destruction of Na\(^+\) channel inactivation by protease or by BTX, nevertheless, is not a prerequisite for the amphipathic QA block. Normal Na\(^+\) channels can also be blocked by amphipathic QA compounds as clearly shown in Figs. 8 and 9.

**Mechanism of Voltage-dependent Binding of Amphipathic QA Compounds**

The binding of amphipathic QA compounds is strongly voltage dependent with an effective valence equal to ~0.5. According to the Woodhull hypothesis (Woodhull, 1978), this result can be interpreted to mean that amphipathic QA compounds with a permanent positive charge are driven in and out of their binding site within the membrane electrical field. Depolarization favors the binding since the QA molecule is
repelled by the positive internal voltage and is driven toward its binding site within the Na⁺ channel pore. The equivalent valence of 0.5 suggests that the QA binding site senses ~50% of the membrane electric field and is located about halfway within this field. The binding site cannot be reached by external amphipathic QA compounds through the external mouth, presumably because it has a narrower region consisting of a “selectivity” filter (for details, see Hille, 1984).

**Hydrophobic Interactions Determine the Amphipathic QA Dwell Time**

What is the major factor that governs the relatively high binding affinity of amphipathic QA compounds? Kinetic analysis shows that the dwell times of QA compounds are largely determined by their hydrophobic interactions with the Na⁺ channel. There is a clear correlation between \( k_{-1} \) and the carbon number of the \( n \)-alkyl chain in these QA compounds. The estimate of \( \Delta G_{\text{w}} \) contributed by each methylene group from \( C_{10}^- \) to \( C_{14}^- \) QA is ~540 cal. This value is nearly the same (560 cal/mol of CH₂) as reported by Rojas and Rudy (1976), who studied the effects of TEA derivatives on Na⁺ currents in protease-treated squid axons. An obvious deviation from this relationship is that the released binding energy (\( \Delta G^0_{\text{w}} \)) of \( C_{16}^- \) and \( C_{18}^- \) QA compounds is far less than the predicted value (Fig. 5 C). This deviation is mainly because of a much smaller on-rate constant, which gives rise to a higher \( K_D \) value. The fact that the \( k_{-1} \) values of both \( C_{16}^- \) and \( C_{18}^- \) QA continue to decrease and remain within the predicted line (Fig. 5 B) suggests that their interactions with the binding site still occur normally. The smaller on-rate constants for \( C_{16}^- \) and \( C_{18}^- \) QA could be due to the fixed physical dimension of the Na⁺ channel conduction pathway; a narrow internal region may prevent the bulky QA compounds in the aqueous solution from reaching their binding site. The QA compound that has an optimal association rate constant is the \( C_{14}^- \) QA cation, which has a \( k_1 \) value of \( \approx 1.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) at +50 mV.

**A Common Binding Site for QA and LA Drugs**

Several pieces of evidence strongly indicate that the QA and LA binding sites in BTX-activated Na⁺ channels overlap with each other. First, the effective valence of ~0.4–0.6 for LA drugs (Wang, 1990) is about the same for amphipathic QA compounds. Second, the drug dwell time of LA is also determined by the hydrophobic interactions between the LA drug and the binding site (Wang, 1990). Third, the protonated form of (−)cocaine appears to be the active form (Nettleton and Wang, 1990), which is equivalent to a QA derivative. In addition, the quaternary LAs (QX-314, RAC-421) and the quaternary antiarrhythmic agent (clofilium) are also active blockers of Na⁺ channels in bilayers only when applied internally (Wang, 1990; Nettleton et al., 1991). Finally, direct competition shows that amphipathic QA and (−)cocaine mutually exclude each other from their binding site. Taken together, we conclude that QA and LA drugs share a common binding site within the Na⁺ channel pore.

**Amphipathic QA Cations Inhibit Normal Na⁺ Currents in GH₃ Cells**

The hallmark of the action of nearly all LAs is their ability to elicit both tonic and use-dependent inhibitions of normal Na⁺ channels (Hille, 1984). It is therefore expected that QA upon binding to the LA site will also inhibit Na⁺ currents in a
similar manner. The results shown in Figs. 8 and 9 agree well with this prediction. External $C_{14}$-QA and (-)cocaine give rise to comparable tonic and use-dependent inhibitions of $Na^+$ currents in $GH_3$ cells. As anticipated, the action of external $C_{14}$-QA is much slower than that of (-)cocaine (Fig. 8) since $C_{14}$-QA with a permanent positive charge does not pass through the membrane easily. In $GH_3$ cells, the apparent $K_D$ for external $C_{14}$-QA is estimated to be 2.6 $\mu$M at $+30$ mV, whereas in bilayers the $K_D$ is $\sim 219$ $\mu$M at $+50$ mV. Several factors together could account for this difference. First, BTX may alter the QA binding site and hence its binding affinity toward QA cations. Second, external Na$^+$ ions may reduce the QA binding affinity. A reduction in cocaine binding by external Na$^+$ ions was reported previously (Wang, 1988). In our whole-cell experiments, the external Na$^+$ ions were omitted, which may result in a stronger binding affinity of Na$^+$ channels toward QA cations. Third, the internal volume of $GH_3$ cells is rather small, whereas the internal volume of bilayers is practically unlimited by comparison. This factor may become significant, particularly when the QA diffusion rate in bilayers is small. The internal QA cations in the aqueous phase will diffuse away before they can accumulate near the bilayer. Finally, QA cations may bind more strongly to the inactivated or resting state of Na$^+$ channels than to the open state, as suggested for some local anesthetics (e.g., Courtney, 1975). In bilayer studies, we are mainly studying the open channel-QA interactions.

Paradoxically, the action of internal $C_{14}$-QA in $GH_3$ cells is also very slow (Fig. 9). This is unexpected since in bilayers the steady-state effects of internal $C_{14}$-QA can be achieved within 1 min. The slow action of internal $C_{14}$-QA indicates that the equilibrium condition for internal QA drug cannot be reached within a 30-min period. It is conceivable that a diffusion barrier may be present between the pipette and the cell membrane. First of all, the opening of the pipette tip is relatively small as compared with the internal volume of $GH_3$ cells. In addition, the pipette tip was first filled with normal internal solution (1-2 mm in length) before it was back-filled with QA containing solution (see Materials and Methods). This procedure created an additional barrier for QA to diffuse into the cells. It is also possible that some nondiffusible proteins, organelles, and cytoskeletons may bind with or uptake the QA cations while they diffuse into the cells. Despite these factors, which may influence the rate and potency of QA cations in $GH_3$ cells, unmodified Na$^+$ channels are as susceptible to QA block as BTX-activated Na$^+$ channels, if not more so.

**QA Compounds as Structural Probes**

The hydrophobic domain of the QA binding site is relatively large, accepting up to 18 methylene groups, larger than the C10 tested so far for K$^+$ channels (Swenson, 1981; Miller, 1982). How could such a large hydrophobic region exist near or along the Na$^+$ channel pore? Two possible interpretations of our results can be envisioned: 
(a) the hydrophobic region of the QA compounds may partially intercalate into the cleft of two trans-membrane $\alpha$-helices (French and Shoukimas, 1981), or alternatively, 
(b) the ion channel pore has a long stretch of hydrophobic residues along its conduction pathway. Whatever the answer may be, the presence of a large hydrophobic region near or along the conduction pathway apparently is not a feature unique to Na$^+$ channels (for review, see Miller, 1989). Both the acetylcholine receptor
(Charnet et al., 1990) and K⁺ channels (Swenson, 1981) probably also possess such a hydrophobic region along their conduction pathway, although not necessarily as large as in the Na⁺ channel. The function of this common hydrophobic motif in different channel conduction pathways deserves further investigation.

Finally, what is the role of the hydrophilic head of the amphipathic QA cations in blocking Na⁺ channels? In our study, the hydrophilic trimethylammonium portion of the amphipathic QA compounds is relatively small, with only ~6 × 6 Å in the space filling model. The 6 × 6 Å size of drug will still be too large to pass through the narrowest region of the Na⁺ pore. Hille (1971) found that organic QA cations as small as methylammonium and TMA cannot pass through the Na⁺ pore. He suggested that the narrowest region of the Na⁺ pore has a cross-section of 3.1 × 5.1 Å (Hille, 1984). Coincidentally, internal TMA ions can indeed block BTX-activated Na⁺ channels, also with a δ value of ~0.5 (Moczydlowski et al., 1986), suggesting that the small internal QA cation penetrates only as deep as the large amphipathic QA does within the Na⁺ channel pore. Together, these results raise an interesting possibility that the hydrophilic head of amphipathic QA cations may reach and block this narrowest region of the Na⁺ channel from the internal side. Near this narrowest region, the physical dimension of the interior Na⁺ pore can now be mapped with different sizes of QA compounds. Such structure–activity studies will probably provide further information on the interior architecture of the Na⁺ channel.

We thank Dr. John Daly, Bertil Takman, and Rao Rapaka for the gifts of various drugs. We are grateful to Dr. Neil Castle for comments on this manuscript and Ms. Ellen Jacobson for typing.

This work was supported by NIH grant GM-35401.

Original version received 18 March 1991 and accepted version received 21 June 1991.

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