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Structural Determinants of Quaternary Ammonium Blockers for Batrachotoxin-Modified Na^+ Channels

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SUMMARY

Amphipathic quaternary ammonium (QA) compounds are potent blockers of batrachotoxin (BTX)-modified Na^+ channels incorporated into planar lipid bilayers. To examine the topology of the QA binding site, we selected two series of QA compounds as structural probes. One series contains two separate hydrophobic moieties but with a common hydrophilic dimethyl QA ion. Most of the QAs within this group bind to BTX-modified Na^+ channels with relatively high affinities. For example, benzyldimethyldodecyl ammonium ions, when applied internally, block single, muscle, BTX-modified Na^+ channels in bilayers with a one-to-one relationship and display an equilibrium dissociation constant (K_d) of 0.2 μM at +50 mV. Furthermore, the QA dwell times appear to correlate with QA hydrophobic interactions with the channel. These results indicate that there are two large hydrophobic binding domains within the QA binding site. The QAs in the second series contain a hydrophilic head group (trialkylammonium) of variable size but with a common dodecyl hydrophobic tail. Tripropyldodecyl QAs block BTX-modified Na^+ channels more effectively (K_d = 0.4 μM at +50 mV) than do trimethyl- and triethyldodecyl QAs, suggesting that the internal Na^+ permeation pathway is at least 9 Å wide. However, tributyl- and tripentyldodecyl QAs show much lower affinities for BTX-modified Na^+ channels at comparable concentrations. These drugs are cut off from binding, probably as a result of the size of their hydrophilic heads (>10 Å), which may be too large to fit in the QA binding site and too bulky to travel freely within the internal permeation pathway. Under whole-cell voltage-clamp conditions, we have further found that BTX-modified Na^+ currents in clonal GH_3 cells can be blocked by these two series of QA ions, albeit only when the activation gate is open. Closed channels at rest do not bind appreciably with these QA ions. Binding of QA ions is reduced by external Na^+ ions in GH_3 cells in a manner indicating that external Na^+ ions can clear the bound QA ions from the Na^+ pore. These results from GH_3 cells mirror those obtained with QA blockers in K^+ channels of squid axons and suggest that the QA binding domains in BTX-modified Na^+ channels and K^+ channels may be structurally conserved.

QA compounds are structural probes useful in mapping the physical dimensions of the permeation pathway of various K^+ channels (for review, see Refs. 1 and 2). Through the pioneering work of Armstrong (3, 4), who examined the internal QA block of delayed rectifier K^+ currents in squid axons, several critical concepts regarding the permeation pathway of the K^+ channel have emerged. First, the internal QA compounds are active only when the channel is open. These QA compounds are thus classified as open-channel blockers for K^+ channels. Second, the inner vestibule of the K^+ pore is relatively large, because bulky QA ions (such as nonyltriethylammonium and tetrapentylammonium ions) have access to this vestibule through the internal mouth. Third, small QA compounds, such as methylammonium ions, cannot pass through the narrowest region of the K^+ channels in squid giant axons; as a result, external hydrophilic QA ions cannot reach the inner vestibule of the K^+ channel. Finally, the fact that inflowing K^+ ions can remove the bound QA ions from the channel is consistent with the current hypothesis that the QA binding site is located between the narrowest region of the K^+ channel (presumably the selectivity filter) and the physical gate (for details, see Ref. 1).

Information about the QA block of K^+ channels has far-reaching implications regarding the permeation pathway of ion channels in general. Recent advances in molecular cloning of Na^+, K^+, and Ca^{2+} channels have revealed that these voltage-gated ion channels all belong to a superfamily of genes and may have similar tertiary structures within the membrane (for review, see Ref. 5). A QA binding site similar to the one found in K^+ channels may therefore be found in other ion channels.

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ABBREVIATIONS: QA, quaternary ammonium; BTX, batrachotoxin; TEA, tetraethylammonium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; TMA, tetramethylammonium; benzyl-C_{12}-QA, benzyldimethyldodecylammonium bromide; C_{12}-QA, dodecyltrimethylammonium bromide; $(C_{12})_2$-QA, didodecyldimethylammonium bromide; benzyl-C_{14}-QA, benzyldimethyltetradecylammonium chloride; $(C_8)_2$-QA, dioctyldimethylammonium chloride; $(C_{18})_2$-QA, dimethyldioctadecylammonium bromide; TLC, thin layer chromatography; LA, local anesthetic.

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Indeed, internal TEA and its derivatives have been shown to be active blockers of Na^+ channels in protease-treated squid axons (6). More recently, *n*-alkyltrimethyl QA compounds were shown to block both normal and BTX-modified Na^+ channels with relatively high affinities (7, 8).

We have further examined the structural aspects of this QA binding site. Our results indicate that the QA site has two large hydrophobic binding domains and a hydrophilic binding domain, all of which can be defined through the use of various QA ions. In addition, we have demonstrated that, under whole-cell voltage-clamp conditions, these QA compounds are pure open-channel blockers of BTX-modified Na^+ channels in GH_3 cells. Finally, we have found that external Na^+ ions reduce QA binding affinity in a manner suggesting that Na^+ ions and internal QA ions encounter each other within the pore.

Materials and Methods

Chemicals. BTX was a generous gift from Dr. John Daly (National Institutes of Health, Bethesda, MD). Phosphatidylethanolamine and phosphatidylcholine were purchased from Avanti Polar Lipids (Birmingham, AL); (−)-cocaine-HCl from Mallinkrodt, Inc. (St. Louis, MO); TEA chloride from Sigma Chemical Co. (St. Louis, MO); benzyl-C_{12}-QA, domiphen bromide, didecyldimethylammonium bromide, and (−)-*N*-dodecyl-*N*-methylephedrinium bromide from Aldrich Chemical Co. (Milwaukee, WI); benzyl-C_{14}-QA and $(C_{12})_2$-QA from Fluka Chemical Corp. (Ronkonkoma, NY); $(C_8)_2$-QA from Pfaltz & Bauer, Inc. (Waterbury, CT); $(C_{18})_2$-QA from Eastman Kodak Co. (Rochester, NY); and myristoylcholine chloride from Research Organics, Inc. (Cleveland, OH). Chemical structures of several QA compounds are shown in Fig. 1. Amphipathic QA compounds were dissolved in either dimethylsulfoxide or ethanol, at a 100 mM stock concentration. All other compounds were reagent grade from commercial sources and were used without further purification.

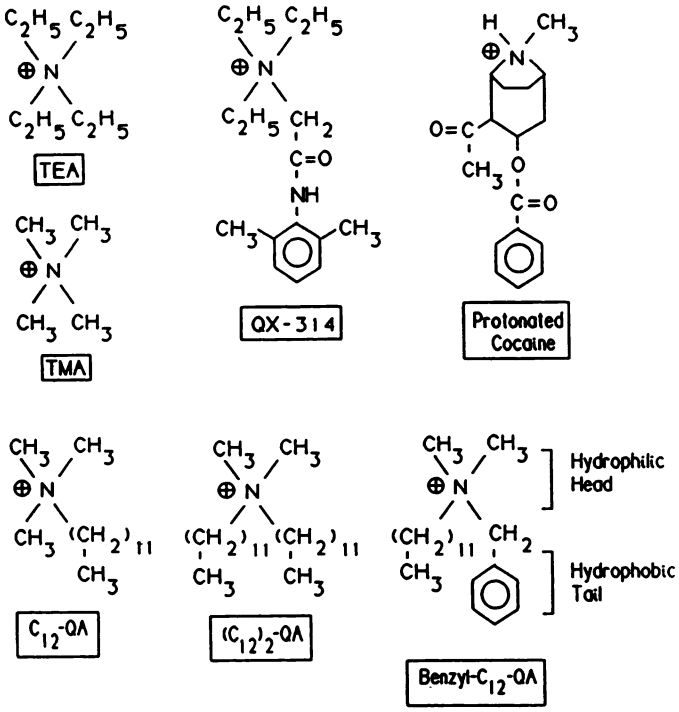

Fig. 1. Chemical structures of some representative compounds, i.e., TEA and TMA ions (hydrophilic ions), QX-314 (a quaternary derivative of lidocaine), cocaine in protonated (charged) form, C_{12}-QA, $(C_{12})_2$-QA, and benzyl-C_{12}-QA. TEA, TMA, and QX-314 are not active when applied externally. The hydrophilic head consists of a QA ion that is permanently charged.

Organic synthesis of trialkyldodecyl QAs. Triethyl-, tripropyl-, tributyl-, and tripentyldodecyl QA compounds were synthesized from 1-bromododecane (Aldrich) and the corresponding trialkylamines (Pfaltz & Bauer). An approximately 1:1 molar ratio of 1-bromododecane and the appropriate trialkylamine were refluxed at 60–80° in absolute ethanol for 1–3 days. The reaction was followed by TLC on normal phase TLC plates (Fisher Scientific, Pittsburgh, PA) developed with chloroform/ethyl acetate (13:1, v/v), ethanol, or ethanol/0.8 M NH_4Cl (4:1, v/v) or on reverse phase TLC plates (Fisher) developed with chloroform/methanol/ammonium hydroxide (30:15:1, v/v), with visualization under an UV lamp, in iodine vapor, or by charring. After the reaction was stopped, excess ethanol was evaporated until two phases were observed. The upper phase containing unreacted trialkylamine and 1-bromododecane was discarded. The lower layer of QA compound was washed several times with hexane and dried under vacuum. The yields for triethyl-, tripropyl-, tributyl-, and tripentyldodecyl QAs were 20%, 18%, 67%, and 10%, respectively. The products were >97% pure, as judged by TLC systems.

Planar bilayer experiments. Plasma membrane vesicles were isolated from rabbit skeletal muscle as described by Moczydlowski and Latorre (9). Planar lipid bilayers were formed on ~200-μm holes in polyvinylchloride partitions from a solution of 13.4 mg of phosphatidylethanolamine and 6.7 mg of phosphatidylcholine/ml of decane. Na^+ channel incorporation by vesicle fusion was monitored by constant alternative pulses of ±50 mV, each for 5 sec, in a symmetrical solution containing (in mM) 200 NaCl, 0.5 Na-EGTA, and 10 HEPES-NaOH, pH 7.4 (standard NaCl solution). Muscle vesicles at a final protein concentration of 10–20 μg/ml and BTX at 0.1 μM were applied to the *cis* chamber. The orientation of Na^+ channels in the membrane was determined by the Na^+ channel gating behavior at the beginning of experiments and was routinely confirmed by tetrodotoxin block at the end of experiments (10). The voltage assignment defines the external surface of Na^+ channels as ground. Bilayer voltage-clamp was achieved with a Warner PC501 patch clamp device (Hamden, CT).

Records of single BTX-modified Na^+ currents were generally filtered at 125 Hz (Krohn-Hite Corp., Avon, MA), digitized at 2 msec/point, stored in an IBM-AT computer, and later analyzed using pClamp software (Axon Instruments, Burlingame, CA). When the mean closed time of QA ions was >0.4 sec, the current records were digitized at 10 msec/point. For these QA ions, short closures of ≤30 msec were not counted, to reduce the intrinsic closing events occurring in BTX-modified Na^+ channels (11). All current records were displayed as upward deflections, for comparison. Bilayers with more than one channel were not used.

Whole-cell patch-clamp experiments. The whole-cell variant of the patch-clamp method (12) was applied to the measurement of macroscopic Na^+ currents in rat GH_3 pituitary clonal cells. GH_3 cells were maintained in culture flasks (Falcon Plastics, Oxnard, CA) and grown in culture dishes (Falcon) according to the method of Cota and Armstrong (13). After 1–4-day replating, each dish (2.5 cm in diameter) was used directly as a recording chamber (~1.0 ml) that was perfused continuously, at a flow rate of 1 ml/min, with an external solution containing (in mM) 150 choline chloride, 0.2 $CdCl_2$, 2 $CaCl_2$, and 10 HEPES, adjusted to pH 7.4 with TMA-OH.

Micropipettes were filled with an internal solution containing (in mM) 100 NaF, 30 NaCl, 10 EGTA, 0.005 BTX, and 10 HEPES, adjusted to pH 7.2 with CsOH. The holding potential was set at −100 mV. QA drugs were applied externally to cells via a series of narrow-bore capillary tubes positioned to within 200 μm of the cell. The external Na^+ ion concentration was raised when necessary by one of the capillary tubes containing (in mM) 130 NaCl, 2 $CaCl_2$, 0.2 $CdCl_2$, 20 choline chloride, and 10 HEPES-TMA-OH, pH 7.4.

Results

Effects of internal benzyl-C_{12}-QA and C_{12}-QA on single BTX-modified Na^+ channels. Both benzyl-C_{12}-QA (with two hydrophobic tails) (Fig. 1) and its parent compound C_{12}-

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QA (with only one hydrophobic tail) induce closures of BTX-modified Na^+ channels, but with very different blocking kinetics. Internal C_{12}-QA induces channel closures with relatively fast kinetics. The mean closed time for C_{12}-QA at +50 mV is about 28 msec (Fig. 2A, *middle*; Table 1). The mean closed time for internal benzyl-C_{12}-QA at +50 mV is about 15 times longer, with a value of 450 msec (Fig. 2A, *bottom*). This result indicates that the hydrophobic benzyl group of benzyl-C_{12}-QA has a drastic effect on the binding characteristics of this QA compound.

Benzyl-C_{12}-QA block of the open channel. Benzyl-C_{12}-QA, with two hydrophobic tails, appears to bind to the BTX-modified Na^+ channel with a one-to-one relationship. Fig. 3A shows the current traces of the BTX-activated Na^+ channel in the presence of various concentrations of benzyl-C_{12}-QA. An increase of benzyl-C_{12}-QA concentration leads to a more frequent block of the channel. Fig. 3B shows that the fractional open time (f_o) is inversely related to the benzyl-C_{12}-QA concentration. This dose-response curve can be well fitted by the Langmuir isotherm (Fig. 3B, *solid line*) with an equilibrium dissociation constant (K_d) of 204 nM; this result suggests that one benzyl-C_{12}-QA ion blocks one Na^+ channel.

Consistent with the one-to-one binding relationship, both the open- and closed-time distributions can be well fitted by single-exponential functions (Fig. 4, A and B). As expected for an open-channel blocker, the $1/\tau_o$ of benzyl-C_{12}-QA is concentration dependent, whereas the $1/\tau_c$ is not (Fig. 4C), although the $1/\tau_c$ value of the individual channel does scatter from 1.52 to 3.85 sec^{-1}. These results can be described by the following kinetic scheme:

$$O + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} O{\cdot}L$$

where O is the open Na^+ channel, L is the benzyl-C_{12}-QA drug, O·L is the nonconducting open channel-drug complex, and k_1 and k_{-1} represent the on- and off-rate constants, respectively. According to this scheme, the kinetic constants should be as follows:

$$k_1 = 1/(\tau_o[L]) \quad (1)$$

$$k_{-1} = 1/\tau_c \quad (2)$$

$$K_d = k_{-1}/k_1 \quad (3)$$

The calculated k_1 and k_{-1} values are 15×10^6 M^{-1} sec^{-1} and 3 sec^{-1}, respectively. The calculated K_d from Fig. 4C is 200 nM, which is close to the value determined from the dose-response curve in Fig. 3B. We conclude, therefore, that benzyl-C_{12}-QA ions bind to a single site and, when bound, block Na^+ channel conductance. With this conclusion, we can proceed to define the topology of the benzyl-C_{12}-QA binding site as we did before for other QA ions with one hydrophobic moiety (8).

Effects of external benzyl-C_{12}-QA and C_{12}-QA on single BTX-modified Na^+ channels. Previously, we found that external application of QA compounds produces block less effectively than internal application (8). Results were similar for benzyl-C_{12}-QA ions. Fig. 2B shows the current traces in the presence of 300 μM external C_{12}-QA and 10 μM external benzyl-C_{12}-QA. The mean dwell times of blocked events for external benzyl-C_{12}-QA and C_{12}-QA remain about the same with internal application of these drugs. However, the calculated K_d values for external benzyl-C_{12}-QA and C_{12}-QA are 3.6 μM and 1.7 mM, respectively, or about 20- and 420-fold higher than the internal QA values. The reduced potency of the QAs applied externally is mainly due to their reduced on-rate constants, equivalent to 7% and 0.2%, respectively, of the values for the QAs applied internally. The lesser reduction of the on-rate constant for benzyl-C_{12}-QA ions is perhaps because of their greater hydrophobicity. Our results are consistent with the notion that external QA ions need to pass through the bilayer membrane to reach the internal QA binding site, whereas internal QA ions do not need to enter the membrane to exert their blocking effects. These results therefore suggest that the hydrophobic pathway for QA ions through the membrane phase alone is not a significant route to the drug binding site. If benzyl-C_{12}-QA ions interact with the hydrophobic α-helices adjacent to the lipid phase, then these interactions probably do not induce closures of the Na^+ channel.

Voltage-dependent binding of benzyl-C_{12}-QA to single BTX-modified Na^+ channels. To quantitate the voltage dependence of benzyl-C_{12}-QA binding, we measured its blocking kinetics at various voltages (Fig. 5A, *current traces*). Evidently, the more the membrane is depolarized, the stronger the binding of benzyl-C_{12}-QA. Fig. 5B shows the k_1 and k_{-1} values plotted against the voltage. Both k_1 and k_{-1} values appear to be voltage dependent; depolarization favors the on-rate for binding and

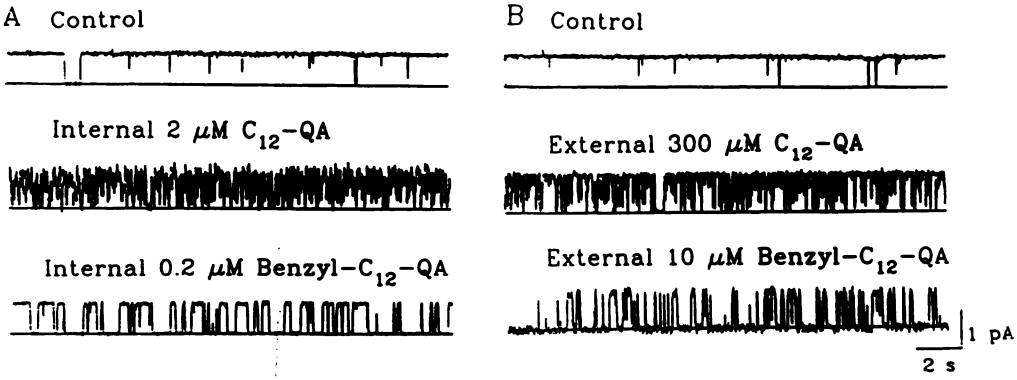

Fig. 2. QA compound-induced closures of single BTX-modified Na^+ channels. Examples of *current traces* recorded at +50 mV are shown in the absence and presence of internally (A) or externally (B) applied C_{12}-QA and benzyl-C_{12}-QA compounds. *Solid lines drawn within current traces*, zero current level. Notice the differences of >40-fold in potency between internal and external applications of QA compounds. Both *cis* and *trans* sides of the bilayer contained a standard 200 mM NaCl solution.

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TABLE 1

Binding kinetics of various QA compounds with muscle BTX-modified Na^+ channels at +50 mV

QA compound[a]	No. of events	τ_c	k_{-1}	k_1	K_d
		msec	sec^{-1}	$\mu M^{-1}\ sec^{-1}$	μM
C_{12}-QA	>1000	28	35.34	9.50	3.72
Benzyl-C_{12}-QA	478	364	2.75	15.10	0.18
Benzyl-C_{14}-QA	161	640	1.56	1.19	1.31
$(C_8)_2$-QA	256	2,495	0.41	0.85	0.47
$(C_{12})_2$-QA[b]	24	~21,900	~0.046	~0.44	~0.10
$(C_{16})_2$-QA[c]	NA[d]	NA	NA	NA	≫2.0
Domiphen	256	127	7.87	5.89	1.34
Ephedrinium	136	342	2.92	1.64	1.78
Myristoycholine	531	127	7.87	15.30	0.52

[a] Domiphen has the chemical formula $CH_3(CH_2)_{11}N(CH_3)_2(CH_2CH_2OC_6H_5)Br$, and ephedrinium represents the stereoisomer of (−)-*N*-dodecyl-*N*-methylephedrinium, $C_6H_5CH(OH)CH(CH_3)N(CH_3)_2[(CH_2)_{11}CH_3]Br$. Myristoylcholine chloride has the chemical structure $C_{13}H_{27}COOCH_2CH_2N(CH_3)_3Cl$.

[b] $(C_{12})_2$-QA induced ultra-long closures, many lasting >1 min. A 10-min recording session yielded only 24 events; the binding kinetics were estimated by assuming τ_c = average closed time and τ_o = average open time. All other kinetic data were estimated according to eqs. 1–3 (see Results), each from one representative channel with a 5–10-min recording.

[c] $(C_{16})_2$-QA at 2 μM did not induce long-lasting channel closures.

[d] NA, not available.

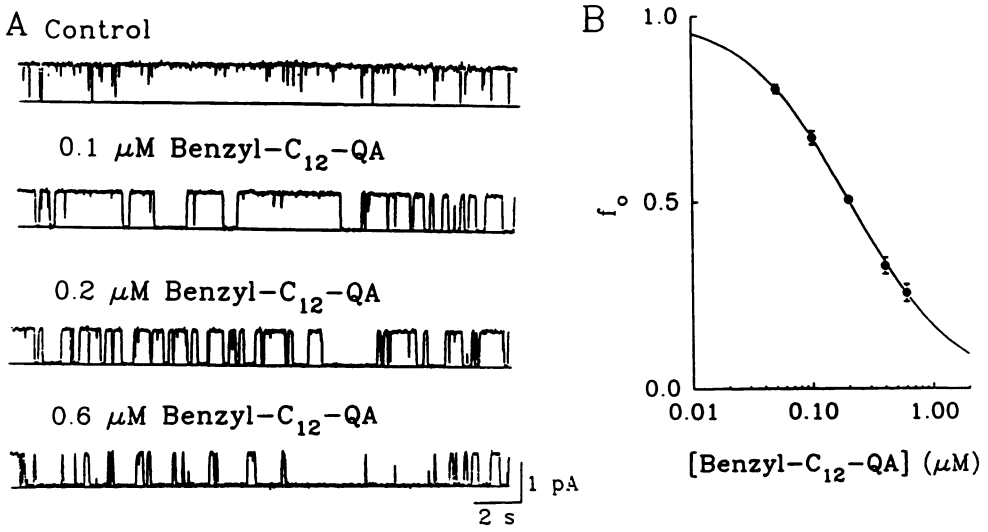

Fig. 3. Concentration-dependent blocking effect of benzyl-C_{12}-QA. A, Examples of single-channel *current traces* at +50 mV are shown for various internal benzyl-C_{12}-QA concentrations. B, The fractional open time (f_o) was measured in a 5–10-min record and then plotted against the benzyl-C_{12}-QA concentration. Two to six channels were used to determine the f_o at each concentration. *Bar*, standard error. *Solid line* was drawn according to the Langmuir isotherm, $f_o = K_d/(K_d + [L])$, where K_d is the drug concentration at which $f_o = 0.5$ and [L] is the drug concentration. The estimated K_d value of benzyl-C_{12}-QA is 0.20 μM at +50 mV. Both *cis* and *trans* sides of the bilayer contained a standard 200 mM NaCl solution.

prolongs drug dwell time, so that k_{-1} values are decreased. As a result, the calculated K_d shows a steeper voltage dependence (Fig. 5C).

We then estimated the equivalent valence of the voltage dependence of benzyl-C_{12}-QA by the following equation:

$$K_d(\Delta V) = K_d(0\ \text{mV}) \cdot \exp(-\delta \Delta V e/kT) \qquad (4)$$

where K_d(0 mV) is the estimated K_d at 0 mV, δ is an equivalent valence, ΔV is the applied voltage, e is the elementary charge, k is Boltzmann's constant, and T is the temperature, in degrees Kelvin (14). The δ value is estimated to be ~0.6, and the K_d(0 mV) value is 0.9 μM. According to Woodhull's model (14), a δ value of 0.6 suggests that the QA charge moiety enters 60% of the membrane electrical field from the internal mouth of the Na^+ channel.

Binding competition among benzyl-C_{12}-QA, TEA, and cocaine. It was not clear whether small hydrophilic QA ions such as TEA (although they also exhibit voltage-dependent binding with a δ value of 0.6) (15) can bind to the same site as large amphipathic benzyl-C_{12}-QA ions. This possibility was directly tested in competition studies. Fig. 6A shows the current traces in the presence of benzyl-C_{12}-QA, which induces discrete closures of single BTX-modified Na^+ channels. The addition of 90 mM internal TEA has little effect on the closed time of the channel but reduces the current amplitude of the unblocked events (Fig. 6B). This reduction of apparent single-channel conductance is due to the very fast block of TEA (15), with a mean dwell time of <10 msec. The concentration required to reduce 50% of single-channel conductance is estimated to be 50 mM TEA. In contrast, the open time appears to be lengthened by TEA. Fig. 6, C and D, shows that the open-time constant (τ_o) is prolonged by TEA from 92 msec to 239 msec. Results are similar for TEA in cocaine-treated BTX-modified Na^+ channels (Fig. 7).

In theory, if the benzyl-C_{12}-QA (or cocaine) and TEA drugs bind to the same site, the $\tau_{o(obs)}$ should be equal to $\tau_o(1 + [L]/$

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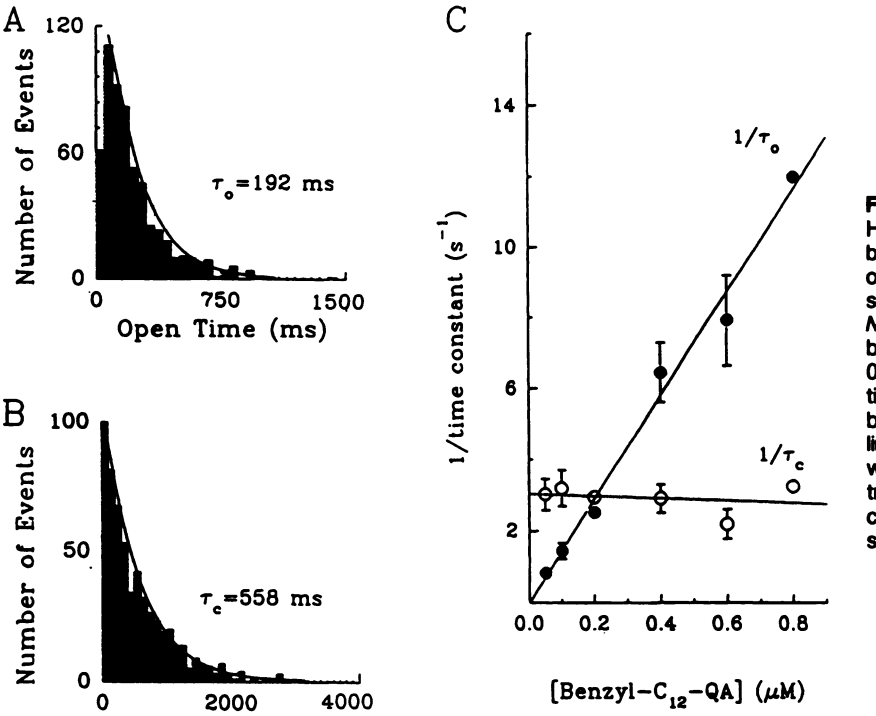

Fig. 4. Kinetics of benzyl-C_{12}-QA binding at +50 mV. Histograms of open-time (A) and closed-time (B) distributions for BTX-modified Na^+ channels in the presence of 0.6 μM internal benzyl-C_{12}-QA are shown. The observations were fitted to a single-exponential function, $N(t) = N \cdot \exp(-t/\tau)$, where $N(t)$ is the number of events/bin, N is the number of events in the population at t = 0, and τ is the mean open time (τ_o) (A) or mean closed time (τ_c) (B). C, Plot of $1/\tau_o$ (●) and τ_c (○) versus internal benzyl-C_{12}-QA concentration at +50 mV. τ_o decreases linearly with internal benzyl-C_{12}-QA concentration, whereas τ_c appears to be independent of QA concentration. *Bar*, standard error with data from three to 13 channels except at 0.8 μM, where a single channel was studied.

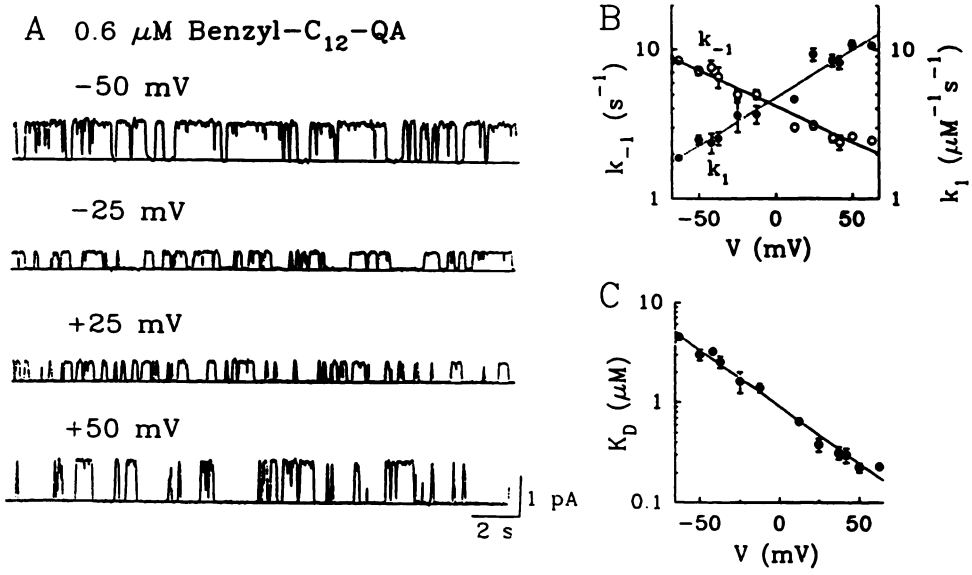

Fig. 5. Voltage dependence of benzyl-C_{12}-QA binding. A, Single-channel *current traces* at various voltages with the internal application of 0.6 μM benzyl-C_{12}-QA are shown. The QA potency increases as the voltage becomes more positive. *Solid lines drawn within current traces*, zero current level. B, On-rate constant (k_1) (●) and off-rate constant (k_{-1}) (○) are plotted against voltage. The rate constants were calculated from τ_o and τ_c values using eqs. 1 and 2 (see Results). C, K_d values are plotted against voltage. *Bar*, standard error for data from two to nine channels. The K_d values were calculated using eq. 3. The δ value from these data is 0.6 (see Results). Both *cis* and *trans* sides of the bilayer contained a standard NaCl solution.

K_d), where τ_o and $\tau_{o(obs)}$ are open-time constants in the presence of benzyl-C_{12}-QA (or cocaine) and benzyl-C_{12}-QA plus TEA, respectively, [L] is the concentration of TEA, and K_d is the equilibrium dissociation constant of TEA. The calculated K_d from this method is 56 mM for TEA in the presence of benzyl-C_{12}-QA and 53 mM in the presence of cocaine. The calculated K_d value is consistent with the measured K_d value (50 mM) based on the reduction of single-channel conductance by TEA ions (Figs. 6 and 7). Our results therefore demonstrate that TEA and benzyl-C_{12}-QA are mutually exclusive at the QA binding site and that this binding site overlaps with the LA binding site.

Structural determinants of QA blockers. From the results described thus far, it is evident that the addition of a benzyl group to C_{12}-QA enhances the binding affinity of the drug. To determine the involvement of other functional groups in blocking single BTX-modified Na^+ channels, we surveyed a series of amphipathic QA blockers in planar lipid bilayers. Fig. 8 shows representative current traces in the presence of these QA blockers. It is apparent that substitution of a methyl group at the hydrophilic head of QA compounds with an *n*-alkyl group increases the binding affinity drastically [e.g., $(C_{12})_2$-QA] (Table 1). However, substitution of both hydrophobic tails with two bulky 18-carbon chains does not work well, perhaps because of the limited dimension of the Na^+ pore and/or the limited hydrophobic domain at the QA binding site. The compounds with optimal on-rate constants in this survey are benzyl-C_{12}-QA and myristoylcholine (15 μM^{-1} sec^{-1}; Table 1).

It is interesting to observe the sequence of mean dwell times of QAs in Table 1, as follows: C_{12}-QA < benzyl-C_{12}-QA < benzyl-

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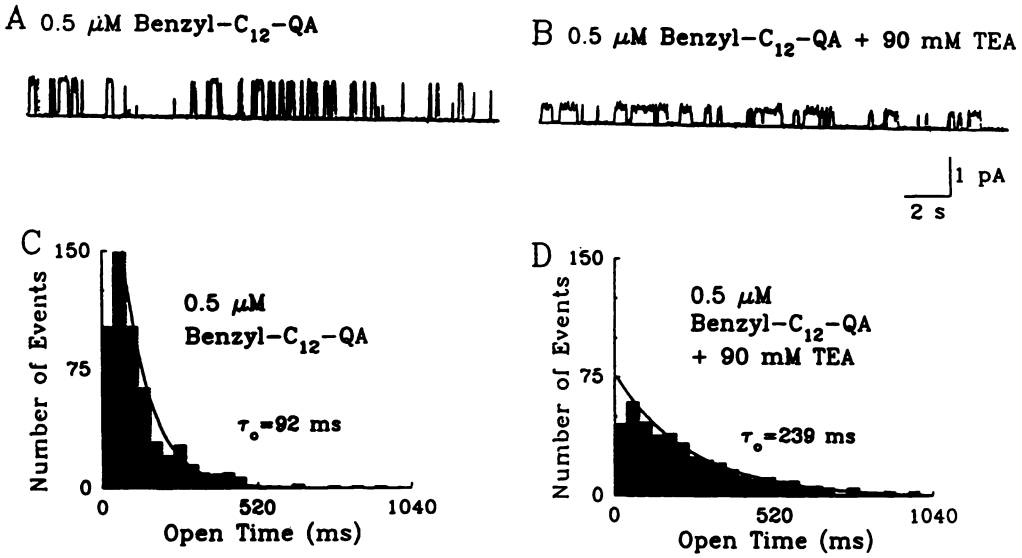

Fig. 6. Binding competition between hydrophilic and amphipathic QA drugs. Single-channel *records* at +50 mV in the presence of 0.5 μM benzyl-C_{12}-QA (A) and 0.5 μM benzyl-C_{12}-QA plus 90 mM TEA (B) are shown. Both drugs were applied internally to the same channel. Open-time distributions of benzyl-C_{12}-QA alone (C) and benzyl-C_{12}-QA plus TEA (D) reveal an increase in mean open time when both drugs are present (92 msec versus 239 msec).

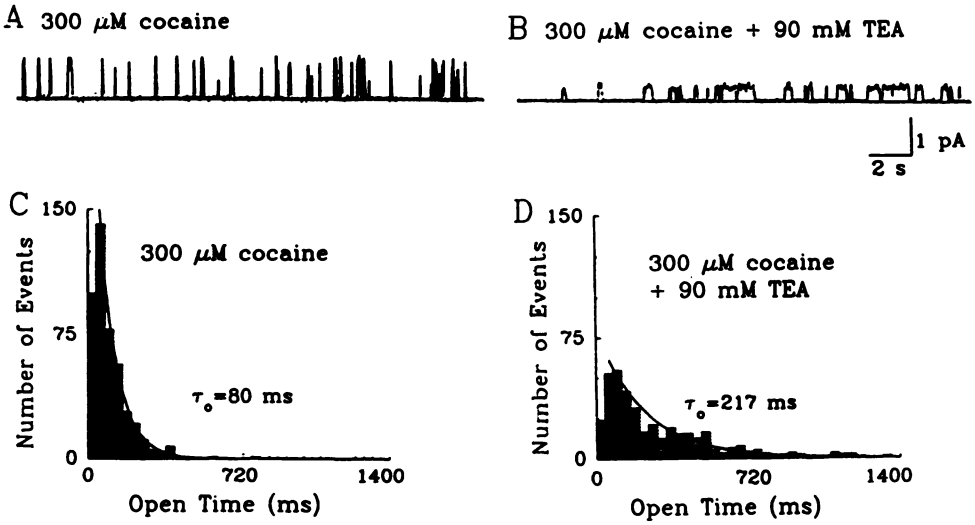

Fig. 7. Binding competition between LA and QA drugs. Single-channel records at +50 mV in the presence of 300 μM cocaine (A) and 300 μM cocaine plus 90 mM TEA (B) are shown. Both drugs were applied internally to the same channel. Open-time distributions of cocaine alone (C) and cocaine plus TEA (D) show an increase in mean open time when both drugs are present (80 msec versus 217 msec).

C_{14}-QA < $(C_8)_2$-QA < $(C_{12})_2$-QA. This sequence indicates that, the more hydrophobic the drug, the longer the dwell time. The benzyl group is indeed less hydrophobic than the *n*-alkyl group with the same carbon number (16). However, because it has a planar shape, this group may have easier access to its binding site, as indicated by the high k_1 value for benzyl-C_{12}-QA. Other substitutions, such as the introduction of an ether bond in front of an aromatic ring (domiphen) (Fig. 8E) or a hydroxy group in the methylene chain [(−)-*N*-dodecyl-*N*-methylephedrinium] (Fig. 8F), reduce binding affinity but do not appear to drastically affect the off-rate constant (Table 1). With the insertion of an ester bond within the *N*-alkyl chain, myristoylcholine retains its binding affinity in the submicromolar range (Fig. 8G; Table 1). Together, our results suggest that the hydrophobic binding domain within the QA binding site can tolerate some hydrophilic functional groups.

Amphipathic QA compounds with hydrophilic domains of variable sizes as structural probes. We have synthesized a series of *n*-trialkyldodecyl QA compounds to address the role of the hydrophilic region in binding. French and Shoukimas (17) applied a similar approach in studies of delayed rectifier K^+ channels in squid axons, using tetra-*n*-alkylammonium ions. Unfortunately, both tetrapentyl and tetrahexyl QA ions disrupt the planar lipid bilayers. In addition, tetrabutyl and tetrapropyl QA ions are weaker blockers (K_d > 1 mM) that do not elicit discrete blocking events in our preparation. In contrast, we found that tripropyldodecyl QA ions are potent blockers of BTX-modified Na^+ channels. Fig. 9C shows current traces in the presence of this compound at 2 μM. The binding of this compound is again strongly voltage dependent (Fig. 9C, *left* versus *right*). Both the open- and closed-time distributions can be well fitted with single-exponential functions. Kinetic data are presented in Table 2. The dwell times of this series of QA ions are highly dependent on the length of the trialkyl chain (Fig. 9), ranging from ~30 msec (trimethyldodecyl QA) to 2.2 sec (tripropyldodecyl QA). However, as the hydrophilic head group becomes too large, the dwell times decrease to ~0.4 sec and ~0.3 sec for tributyldodecyl and

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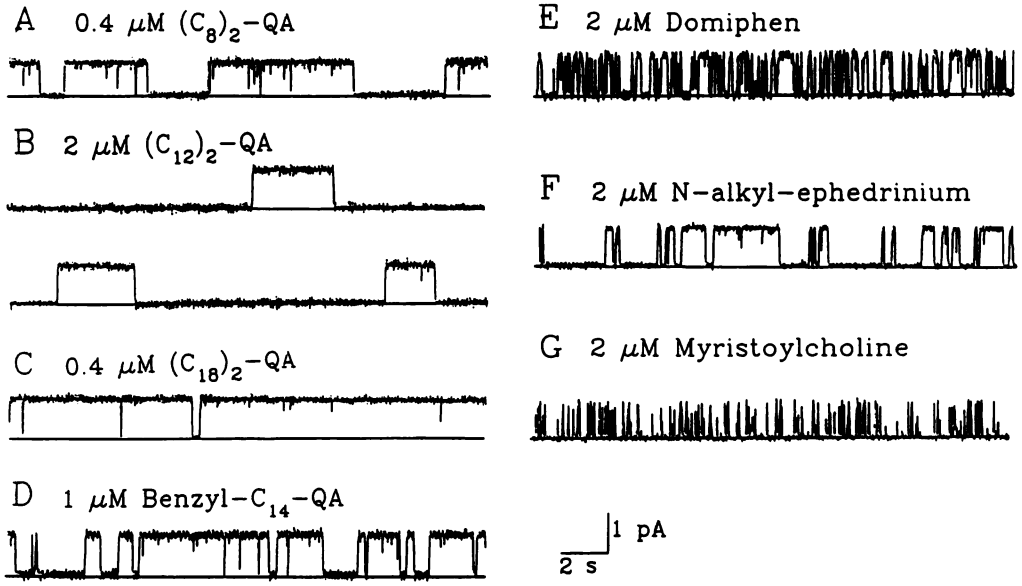

Fig. 8. Elicitation of different blocking effects by different QA compounds. Examples of *current traces* recorded at +50 mV for single, muscle, BTX-modified Na^+ channels in the presence of the indicated internally applied compounds are shown. Both *cis* and *trans* sides of the bilayer contained a standard 200 mM NaCl solution. *Solid lines drawn within current traces*, zero current level.

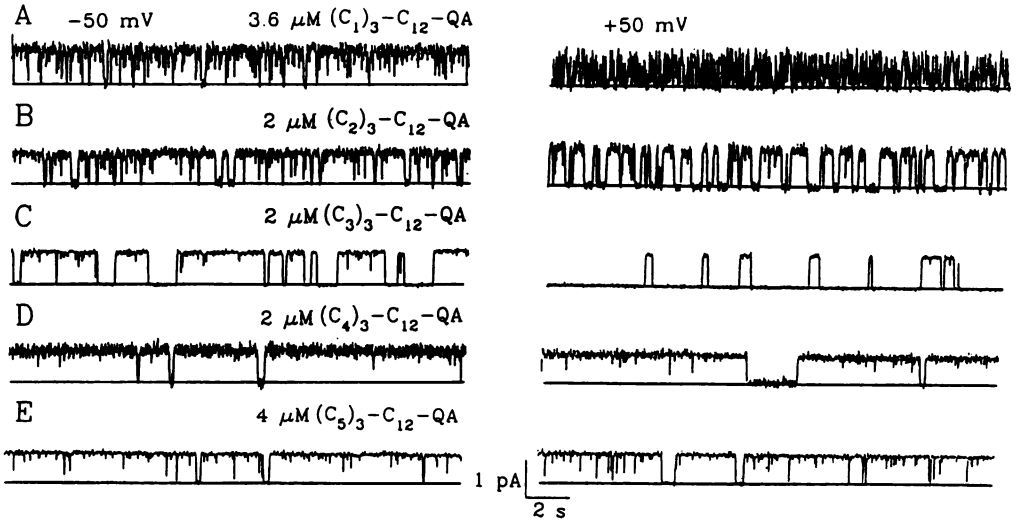

Fig. 9. Blocking effect of trialkyldodecyl QA compounds in single BTX-modified Na^+ channels. Trialkyldodecyl QAs ranging from trimethyl to tripentyl were each applied to the internal side of single BTX-modified Na^+ channels at the concentration indicated. *Left*, *current traces* recorded at −50 mV; *right*, *current traces* recorded at +50 mV. Five different channels (A through E) were used. Notice that the blocking effect is strongly voltage dependent and that tripropyldodecyl QA ions elicit the longest dwell time. Inward Na^+ current traces at −50 mV are displayed as upward deflections for comparison.

TABLE 2

Binding kinetics of QA compounds with hydrophilic head groups of various sizes with BTX-modified Na^+ channels at +50 mV

All kinetic data were estimated according to eqs. 1–3 (see Results). Each value was determined from a pool of two to four channels (except for R = CH_3, which was taken from Table 1), with a 10–20-min total recording time.

$(R)_3N^+C_{12}H_{25}$	No. of events	τ_c	k_{-1}	k_1	K_d
		msec	sec^{-1}	$\mu M^{-1}\ sec^{-1}$	*μM*
R = CH_3	>1000	28	35.34	9.50	3.72
R = C_2H_5	2047	196	5.11	1.42	3.60
R = C_3H_7	2017	2213	0.45	1.05	0.43
R = C_4H_9	370	443	2.26	0.12	20
R = C_5H_{11}	1552	318	3.14	0.09	33

tripentyldodecyl QA ions, respectively. There appears to be a cutoff phenomenon for the binding of tributyldodecyl QA and tripentyldodecyl QA, due to a 5–7-fold increase in k_{-1} and a 10-fold decrease in k_1, compared with values for tripropyldodecyl QA ions. As a result, binding affinity is reduced by 50–70-fold (Table 2). Clearly, the hydrophilic head group in its extended configuration cannot exceed 10 Å in size if it is to freely enter the permeation pathway and efficiently interact with the channel.

Failure of amphipathic QA ions to block BTX-modified Na^+ channels at rest. To determine whether amphipathic QA ions can block the closed BTX-modified Na^+ channels at rest, we used the whole-cell configuration of the patch-clamp technique on GH_3 cells. Na^+ channels were first treated with

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internal 5 μM BTX and then activated by +50 mV pulses (with a duration of 50 msec) repetitively at 2 Hz. Up to 60–95% of Na^+ channels are converted to BTX-modified channels within 5–10 min, as manifested by the appearance of the maintained Na^+ current during a 5-sec pulse. Upon application of external 1 μM benzyl-C_{12}-QA, a block of the maintained Na^+ current develops that increases with time during the period of depolarization (Fig. 10). Such a time-dependent block during depolarization has not been studied before in bilayers because of their large capacitance artifact. It is noteworthy that benzyl-C_{12}-QA ions do not act as quickly as LA drugs in GH_3 cells, which usually reach their steady state block within 2 min. It takes at least 10–30 min for QA ions to reach steady state when they are applied at micromolar levels; it appears that amphipathic QA ions must move across the cell membrane and accumulate within the cell to block the Na^+ channels. The time required to reach steady state varies considerably from cell to cell. The effect of this drug in GH_3 cells is practically irreversible: washing of the external surface of GH_3 cells with drug-free solution does not significantly decrease the degree of the time-dependent block. Because little or no block of BTX-modified Na^+ current occurs at the beginning of depolarization by external 1 μM benzyl-C_{12}-QA, whereas >75% of the maintained current is blocked at the end of the 5-sec pulse (Fig. 10), we conclude that benzyl-C_{12}-QA is an open-channel blocker in BTX-modified Na^+ channels. As in the QA block of K^+ channels (1), the QA binding site in the BTX-modified Na^+ channel probably becomes accessible to the QA drug only when the physical gate is open. This result implies the lack of a direct hydrophobic pathway by which QA ions can reach their binding site.

Na^+-dependent binding of QA ions to BTX-modified Na^+ channels. External Na^+ ions reportedly can antagonize LA action on Na^+ channels (11, 18). Because LA and QA compounds appear to share a common binding site, it is likely that external Na^+ ions also antagonize QA action. This was found to be the case in GH_3 cells. After treatment with benzyl-C_{12}-QA ions, only ~10% of BTX-modified Na^+ current remains in the absence of external Na^+ ions and ~23% remains in the presence of 130 mM external Na^+ ions (Fig. 11A). Results are similar for tripropyldodecyl QA ions (Fig. 11B). Hence, external Na^+ ions antagonize the blocking effect of QA ions in BTX-modified Na^+ channels. These patch-clamp experiments (Figs. 10 and 11) demonstrate that, 1) as in bilayers, QA ions are open-channel blockers in intact cells and 2) QA ions can be a general tool for studying different subtypes of Na^+ channels (i.e., skeletal muscle or GH_3 cells). However, single-channel analyses of QA action on BTX-modified GH_3 cell Na^+ channels incorporated into planar bilayers will be needed to obtain detailed kinetic information.

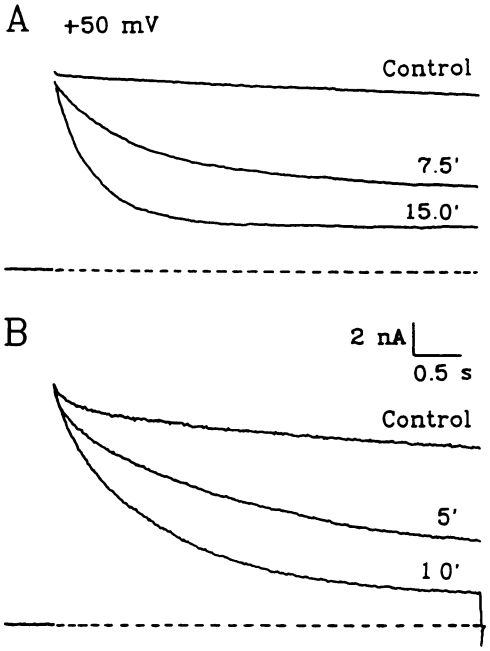

Fig. 10. Elicitation of a time-dependent block by QA ions of the BTX-modified Na^+ current in GH_3 cells. *Traces* of BTX-modified Na^+ currents at +50 mV are superimposed before (control) and after treatment with external benzyl-C_{12}-QA at 1 μM for 7.5 and 15 min (A) or external tripropyldodecyl QA at 2 μM for 5 and 10 min (B). Notice that a rapid time-dependent block occurs during prolonged depolarization after the treatment with QAs. E_{hold} = −100 mV; $E_{prepulse}$ = −130 mV for 5 sec. External solution contained no NaCl.

Discussion

The novel findings of this report are that 1) the QA binding site contains two apparent, large, hydrophobic binding domains, 2) small hydrophilic QA ions, such as internal TEA, can reach and bind to the same QA binding site as do large amphipathic QA ions, 3) the hydrophilic binding domain in the QA binding site is able to accept ions as large as tripropylammonium ion (~9 Å in extended form), and 4) QA ions do not interact with closed BTX-modified Na^+ channels but instead bind only when the channel is in its open conformation.

Role of the hydrophobic moiety in QA blockers. Our

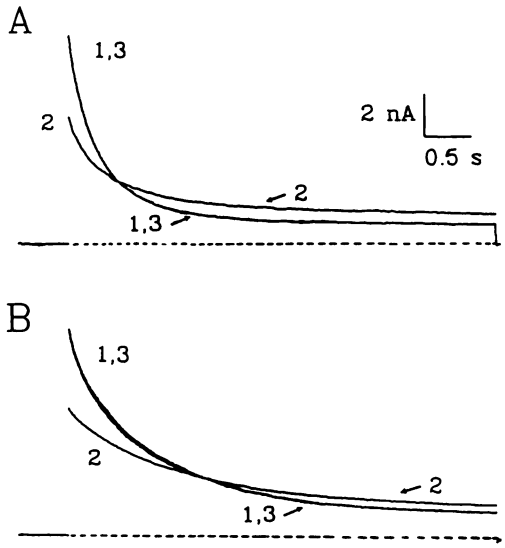

Fig. 11. Reduction of QA block by external Na^+ ions. *Traces* of BTX-modified Na^+ currents at a test pulse of +50 mV in the absence (*trace 1*) and presence (*trace 2*) of external 130 mM NaCl are superimposed for comparison. Cells were first treated either with external 1 μM benzyl-C_{12}-QA ions (A) or with 2 μM tripropyldodecyl QA ions (B) for 15–20 min and were then superfused with a drug-free solution for 10 min. Notice that the time-dependent block, as seen in Fig. 10, remains after washing (*trace 1*). Na^+ ions apparently reduce the binding affinity of benzyl-C_{12}-QA ions, because the degree of the time-dependent block with external 130 mM NaCl (*trace 2*) is much less than that at 0 mM NaCl (*trace 1*). Subsequent removal of the external Na^+ ions results in restoration of the original binding affinity (*trace 3*). This experiment was repeated in four separate cells for each QA compound. A high concentration of Na^+ ions reduces QA affinity without exception. E_{hold} = −100 mV; $E_{prepulse}$ = −130 mV for 5 sec.

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results demonstrate that the QA binding site in BTX-modified Na^+ channels contains two hydrophobic binding domains, each accepting up to 12 methylene groups of an *n*-alkyl chain. It is interesting that this QA binding site seems able to interact with most of the QA compounds tested; this observation suggests that the receptor site may be relatively "flexible" and that both the receptor and the ligand may align themselves in optimal orientations for binding (for the theory underlying this idea, see Ref. 19). A benzyl group is a good substitution for the *n*-alkyl chain, although this planar substitution is actually less hydrophobic. An additional ester bond, ether bond, or hydroxy bond between the hydrophobic chain and the hydrophilic head group does not greatly interfere with QA binding. The exact configuration of these two hydrophobic binding domains within the Na^+ channel was not determined in our study.

In general, the larger the two hydrophobic chains of the QA compound, the longer the dwell time (Fig. 8; Table 1). The only exception to this general rule is $(C_{18})_2$-QA (Table 1). The reason why $(C_{18})_2$-QA does not induce ultra-long-lasting block is not clear. Amphipathic C_{18}-QA does induce long-lasting block but with a much slower on-rate than that of C_{16}- or C_{14}-QA; this difference suggests that C_{18}-QA ions encounter some steric hindrance within the channel pore, perhaps due to the fixed dimension of the pore (8). A similar explanation may account for the results obtained with the $(C_{18})_2$-QA ion, which appears to have little access to its binding site. In aqueous solution the two hydrophobic tails are likely paired and interweaved to form a ball-like structure, so that the hydrocarbon-water interface is reduced (16). Thus, $(C_{18})_2$-QA ions in solution may be physically too large to enter the Na^+ permeation pathway.

Role of the hydrophilic moiety in QA blockers. It is generally assumed that a positively charged ligand is likely to bind to its receptor in part through ionic charge-charge interactions. According to Coulomb's law, the electrostatic attraction between ions with net charges falls off as $1/(Dr^2)$, where D is the dielectric constant of the surrounding medium and r is the distance between ions. At present, it is difficult to quantify the binding of the QA charge moiety to its receptor, because the binding energy depends crucially on the dielectric constant of the surrounding medium. When the hydrophilic charge moiety of the QA ion is "shielded" by three alkyl chains with increasing carbon number, the QA ion appears to increase its affinity for K^+ channels in squid axons (up to six carbons) (17), as well as for BTX-modified Na^+ channels in lipid bilayers (up to three carbons) (Fig. 8). The cutoff effect in QA binding to BTX-modified Na^+ channels occurs when the trialkyl chain is larger than four carbons (Fig. 9). It is likely that the distance between net charges increases when alkyl chain length is increased, a change that, in theory, should reduce QA binding affinity. On the other hand, the local dielectric constant may decrease as a result of the additional hydrophobic carbon chain, a change that, in theory, should increase the binding affinity. Experiments dissecting these two opposing factors in the cutoff phenomenon are needed.

Another possible contribution of the QA charge moiety to binding affinity is its pivotal role in the local distribution of QA ions within the electrical field along the Na^+ permeation pathway. Strichartz (20) first examined the strong voltage-dependent binding of a quaternary lidocaine derivative and proposed that the permanent positive charge of QX-314 causes a voltage-dependent binding phenomenon because the charge ion can be driven toward or away from its binding site by voltage when the ion is within the electrical field. In our laboratory, we have found that all QA ions display similar strong voltage-dependent binding to BTX-modified Na^+ channels, with an equivalent valence of 0.5–0.6 (Figs. 5 and 9) (8). These results are consistent with the notion that the QA charge moiety affects QA binding affinity because the QA binding site is located about halfway across the electrical field.

Topology of Na^+ channels, compared with K^+ channels. The K^+ channel pore has been well defined with the use of TEA and its larger QA equivalents (1). The topology of the K^+ pore has been proposed to have the following sequence: external mouth, selectivity filter, QA binding site, activation gate, and then internal mouth. The internal vestibule with the QA binding site, which is located between the physical gate and the selectivity filter, may be relatively large (at least 10 Å in diameter), because large QA compounds appear to reach the QA binding site as readily as their smaller counterparts (17, 21).

It is interesting that the characteristics of the QA binding site within the Na^+ pore practically mirror those within the K^+ pore. This QA binding site is likely to be within the Na^+ pore, because it can be reached by internally applied hydrophilic TMA and TEA ions (Fig. 7) (15, 22) and because the activation gate, when closed, may prevent binding of amphipathic QA ions (Fig. 10). However, even small hydrophilic QA ions (1.5–2.5 Å) are not active when applied externally, presumably because they cannot pass through the narrowest region of the Na^+ selectivity filter. Finally, the inflowing Na^+ ions reduce QA binding (Fig. 11) in a manner indicating that Na^+ and QA ions encounter each other within the pore. Hence, the topology of the Na^+ pore is likely to be the same as that of the K^+ pore, i.e., external mouth, selectivity filter, QA binding site, activation gate, and then internal mouth. As in the K^+ pore, the internal vestibule between the physical gate and the selectivity filter may be relatively large, because large tripropyldodecyl QA ions (~9 Å in diameter when fully extended) can still reach their binding site. However, it is conceivable that at the binding site the *n*-alkyl chain of the QA compounds may intercalate into the cleft of two transmembrane α-helices (8, 17). If so, these binding interactions probably occur with transmembrane α-helices adjacent to the Na^+ permeation pathway.

The similar topologies of the permeation pathways of the K^+ and Na^+ channels and the presence of closely related QA binding sites in the two channels suggest that the pore configurations of the Na^+ and K^+ channels are similar. This hypothesis is consistent with the amino acid sequence information on Na^+ and K^+ channels (for review, see Ref. 5). The two types of ion channels belong to a superfamily of genes and contain extensive sequence similarities in their primary structures, particularly within the transmembrane regions. The molecular basis for the presence of similar QA binding sites within Na^+ and K^+ channels may simply be the similarities in their primary protein structures.

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