ULTRA-DEEP BLOCKADE OF Na⁺ CHANNELS
BY A QUATERNARY AMMONIUM ION: CATALYSIS BY A
TRANSITION–INTERMEDIATE STATE?

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SUMMARY

1. Individual Na⁺ channels from isolated guinea-pig ventricular heart cells were studied using the patch-clamp technique. To localize the selectivity region of the channels we investigated their blockade by a permanently charged quaternary ammonium ion (QX-314, 2-(triethylamino)-N-(2,6-dimethylphenyl)acetamide, 0–5 mM) that was applied to the cytoplasmic side of the channel.

2. Resolution of individual blocking events was enhanced by covalent removal of fast inactivation following brief internal exposure to the enzyme papain. The improved resolution reveals the existence of two distinct modalities of blockade: reduction of unitary current, and millisecond interruptions of current.

3. Both modes of internal block could be potentiated by lowering external Na⁺ concentration. This finding argues that the two corresponding sites of interaction are both located within the channel pore.

4. Analysis of the voltage dependence of block placed both binding sites deep within the pore, at 70% of the electric field from the cytoplasmic entrance. Combined with recent studies localizing block by external Cd²⁺, the present results argue that the selectivity region of Na⁺ channels is quite narrow (spanning about 10% of the electric field), and located near the external side of the channel.

5. The manner in which the two blocking processes interact, along with the physical proximity of their binding sites, leads us to propose that the block configuration responsible for the reduction in unitary current serves as a transition intermediate that catalyses formation of the discrete-block complex.

INTRODUCTION

The interaction of permanently charged quaternary ammonium ions (QA) with voltage-gated channels has proven a valuable biophysical property with which to probe structural features of the permeation pathway (Armstrong, 1971; Miller,

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1982). Recent work has combined site-directed mutagenesis and QA blockade of channels to implicate the H5 region as a deep part of the K^+ channel pore (Yellen, Jurman, Abramson & MacKinnon, 1991; Hartmann, Kirsch, Drewe, Taglialatela, Joho & Brown, 1991), and to establish the M2 region as a major pore-lining domain in the nicotinic acetylcholine receptor (Leonard, Labarca, Charnet, Davidson & Lester, 1988).

In the case of Na^+ channels, QA may also prove one of the most promising tools for mapping out the conduction pathway (Lester, 1991), but key properties of QA blockade must first be clarified at the level of single channels. While there has been extensive characterization of QA effects upon macroscopic Na^+ current (Frazier, Narahashi & Yamada, 1970; Strichartz, 1973; Hille, 1977; Cahalan, 1978; Cahalan & Almers, 1979; Wang, Brodwick, Eaton & Strichartz, 1987), the interpretation of these data is complicated by the interplay between channel gating and block (Strichartz, 1973; Cahalan, 1978; Wang et al. 1987). Single-channel studies could resolve these ambiguities, but most published reports of this type have used toxins to facilitate resolution of QA blocking events (e.g. Moczydlowski, 1986). While elegant, these results may reflect QA interaction with toxins as well as with the channel itself (Postma & Catterall, 1984).

To avoid these difficulties, we here examine QX-314 (a permanently charged QA analogue of lidocaine) association with single cardiac Na^+ channels in which fast inactivation has been covalently (enzymatically) removed to enable resolution of blocking events. We find that internal block occurs on two very different time scales: one so rapid that it manifests itself as a reduction of unitary current, and the other producing millisecond interruptions of current. Remarkably, the estimated location of QX-314 binding in either mode of block is very deep: 70% of the electric field in from the cytoplasmic side of the channel. Less than 10% of the electric field separates this from the site of external blockade of the channel by cations like H^+ (Woodhull, 1973) and Cd^{2+} (Ravindran, Schild & Moczydlowski, 1991; Backx, Yue, Lawrence, Marban & Tomaselli, 1992). Hence, QX-314 binding probably reports interactions right up to a very narrow region of the channel that comprises the selectivity filter (Hille, 1975; Heinemann, Terlau, Stühmer, Imoto & Numa, 1992). QA blockade therefore promises to be a useful biophysical assay to combine with site-directed mutagenesis in the search for deep pore-lining regions of the channel.

Another notable finding is that appearance of the short-lived quaternary ammonium complex modulates the formation of the longer-lived block state, as if the former serves as a "transition–intermediate" state that catalyses generation of the latter. A preliminary report of this work has appeared as an abstract (Gingrich, Beardsley & Yue, 1991).

**METHODS**

*Cell isolation and electrophysiology*

Guinea-pigs were killed by cervical dislocation according to Johns Hopkins Medical Institution protocol GP93M66. A mid-line thoracotomy was then rapidly performed, and the heart immediately removed and digested enzymatically as described previously (Yue & Marban, 1990). Individual ventricular cardiomyocytes were then placed in a bath solution containing 5 mM KCl, 20 mM potassium taurine, 120 mM potassium glutamate, 2 mM MgCl_2, 1 mM adenosine-5'-triphosphate (calcium salt), 2 mM EGTA, 10 mM Hepes–KOH, pH 7.4, at room temperature (20–22 °C). Thick-walled, borosilicate glass pipettes were pulled, Sylgarded and fire-polished to tip
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diameters near 1 \( \mu \)m. The patch pipettes were filled with a solution containing 170 mM NaCl, 2 mM CaCl\textsubscript{2}, 10 mM Hepes-TEA-OH, at pH 7.3, unless otherwise noted. Excised, inside-out patches were formed and moved into the mouth of a large-bore perfusion tube, through which various solutions containing papain or QX could be conducted. Single-channel current records were obtained with an Axopatch 1C amplifier and CV-3 headstage (Axon Instruments, Foster City, CA, USA), low-pass filtered at 1–2 kHz (−3 dB, 4-pole, Bessel) and digitally sampled at 100 \( \mu \)s intervals. Na\textsuperscript{+} channel activity was elicited every 1 s by voltage steps lasting 180 ms.

Removal of fast inactivation

Fast inactivation was removed by passing a bath solution containing papain through the large-bore perfusion tube for several minutes. Papain was used at 2 units/ml after 30 min of activation with 3 mM cysteine (both from Sigma), at room temperature. As soon as channel gating became modified, the large-bore perfusion pipette was switched to a normal bath solution containing 0–5 mM QX.

Data analysis

Records had leak and capacity currents eliminated by subtracting smooth functions, and were idealized by half-height criteria. Idealized records were used to construct the ensemble-average currents shown.

Correction for missed events: open and closed durations, as detected by the half-height threshold, suffer from artifactual shortening intrinsic to this detection method (e.g. Fig. 11–11B in Colquhoun & Sigworth, 1983). These were corrected by eqn (17) in Colquhoun & Sigworth (1983) in the histograms shown (e.g. Fig. 4). Single- and biexponential probability density functions were fitted to all corrected open and closed intervals, respectively, using a maximum-likelihood criterion, as specified in eqns (58) and (60) in Colquhoun & Sigworth (1983). For purposes of display only (e.g. Fig. 4), corrected intervals and fits were plotted in the form of complemented distributions. The parameters derived from such exponential fits still, however, suffer from distortions owing to missed events (Blatz & Magleby, 1986). To account for this, we took advantage of the equations of Blatz & Magleby (1986) that predict what the observed exponential parameters, distorted by missed events, should be, given an assumed set of true rate constants for the four transitions in eqn (1) (below). Using a Levenberg–Marquardt method (Mathcad, MathSoft Inc., Cambridge, MA, USA), we could then iteratively solve for the set of rate constants (including discrete block and unblock rates) that best predicts the observed exponential parameters as distorted by missed events. We rarely observed degenerate solutions as Blatz & Magleby (1986) reported; in those rare instances, the appropriate solution was easily identified by comparing measured open probabilities with those predicted by the various solutions.

Adjustments for two-channel patches: in some patches, two channels rather than one were observed after removal of fast inactivation, as gauged by the stacking of unitary events. In such cases, first latency functions were corrected to that anticipated for a single channel by taking the square root of complemented distribution functions accumulated for two channels (Aldrich, Corey & Stevens, 1983). For the analysis of the discrete block of open channels, only open durations from non-stacked openings were considered. Also, only the intervals between openings with both channels closed were compiled for closed time analysis. As described above, such open and closed intervals were corrected for artifactual shortening, and fitted with single- and biexponential probability density functions. The good quality of the fits, along with the paucity of stacked openings, indicated that the observed openings and closings of two channels could be well approximated by the gating of an 'equivalent' single channel with \( C\rightarrow O\rightarrow OB \) kinetics. Hence, the iterative technique mentioned above (Blatz & Magleby, 1986) could be used to account for missed events in the extraction of transition rates for the equivalent one-channel scheme. These transition rates could in turn be used to predict what the open and closed time distributions for two channels would have been like in the absence of missed events. We were, however, interested in what the distributions would have been like for any of the two channels in the patch, after correction for missed events. Obtaining the one-channel open time distribution, \( P_{\text{on}}(t) \), is straightforward: since most observed openings were not stacked, the desired distribution will be virtually identical to the single-exponential distribution predicted by the equivalent one-channel model. On the other hand, obtaining the one-channel closed times is more difficult. Although the corrected transition rates for the equivalent one-channel model can be used to predict the complemented distribution of closed
times for two channels, $P_{\text{oth,2}}(t)$, this will differ significantly from the analogous distribution for one of the two channels, $P_{\text{oth,1}}(t)$. Binomial statistics, however, allow $P_{\text{oth,1}}(t)$ to be calculated from $P_{\text{oth,1}}(t)$ according to the following quadratic equation:

$$P_{\text{oth,2}}(t) = 2F_{0,1}(1 - F_{0,1})P_{\text{oth,1}}(t) + (F_{0,1})^2P_{\text{oth,1}}(t)^2,$$

where $F_{0,1}$ is the fraction of sweeps with activity for a single channel, derived from the measured overall fraction of active sweeps in the two-channel patch. Parameters for single- and biexponential fits to $P_{\text{oth,2}}(t)$ and $P_{\text{oth,1}}(t)$ can then be used to calculate the discrete block and unblock rates that would be anticipated for either one of the two channels in the patch.

Statistical tests were performed as described in the legends. One-tailed $P$ values $< 0.05$ were considered to have statistical significance.

**RESULTS**

Our initial aim was to determine the kinetics of QX-314 (QX) blockade of native Na$^+$ channels. However, this goal was untenable for the following reasons. With fast inactivation intact, QX dissociates slowly over seconds, as reflected by the well-known property of use dependence (Courtney, 1975). Consequently, for there to be appreciable opening at all, a similarly slow association rate is required, as can be achieved by using 'low' concentrations of QX ($\approx 0.1$ mM). The predominant effect under this condition is to increase the frequency of activating voltage-steps without activity, but to leave the gating kinetics unchanged when channels manage to open (Yeh, McCarthy, Quandt & Yamamoto, 1986). This comes as no surprise because QX association would then be slow compared with the kinetics of intrinsic gating.

Clear-cut observation of the interaction of QX with the channel requires slowing the rate of intrinsic gating, or speeding the kinetics of QX interaction. We therefore removed fast inactivation by enzymatic digestion with papain (Quandt, 1987), which not only slows intrinsic gating (by removal of fast inactivation), but abolishes the long-lived associations between QX and the channel permitted by the fast inactivated state (Cahalan, 1978; Yeh, 1978). The latter feature allows faster QX association rates to be observed experimentally because [QX] can now be increased without altogether eliminating channel activity.

Figure 1A shows the effects of papain treatment in an inside-out patch containing a single Na$^+$ channel. The individual records and ensemble average indicate that the channel spends most of its time open, and inactivates little over the course of more than 100 ms. Subsequent application of 2 mM QX produces two distinct effects (Fig. 1B): (1) millisecond interruptions in current consistent with a discrete blocking process, and (2) a uniform reduction in unitary current. The simplest explanation is that there are two receptor sites for QX on the channel, one for each effect. The dwell time of QX bound to the site associated with the observable blockages of current (the 'discrete site') would be in the range of milliseconds, while the kinetics of interaction with the other receptor (the 'rapid site') would be more than tenfold faster than the rise time of our recording system ($\approx 0.2$ ms). In the latter case, then, the interruptions in open channel current would be low-pass filtered to appear as a uniform reduction in unitary current (Yellen, 1984).

In contrast to the marked effects of internally applied QX, external application of upwards of 10 mM QX produced no appreciable changes in gating (not shown), consistent with single-channel studies in bilayers (Moczydlowski, 1986; but see Baumgarten, Makielski & Fozzard, 1991). Our lack of extracellular block implies
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that if internal block by QX involves the entry of a blocker molecule from the cytoplasm into the channel pore, then unblock should result mainly by the exit of QX to the cytoplasmic side as well.

Even with this simplification, however, the presence of two QX receptors in the channel could well complicate analysis of the individual characteristics of each site, taken separately. Nevertheless, since the rate constants governing rapid block are likely to be more than tenfold faster than those for discrete block or channel closure, we can, practically speaking, ignore both of these slower processes in analysing the rapid block of open channels, although the converse would not necessarily be true. For this reason, we first consider the analysis of the rapid blocking mode.

*Rapid block is mediated by a deep site within the pore*

The reduction of unitary current by QX is shown in greater detail when individual sweeps are plotted on an expanded time base (Fig. 2A). The binding reaction underlying rapid block can be investigated by examining the dependence of

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**Fig. 1.** QX induces discrete and rapid block of single Na⁺ channels. A, individual current records of a single Na⁺ channel after removal of fast inactivation. The holding potential is −120 mV. Openings are downward to a unitary current level marked by the dashed line. The ensemble-average current (below) illustrates the successful removal of fast inactivation. B, response of the same channel with application of 2 mM QX. Individual records now show long non-conducting periods punctuated by brief openings, and the unitary current amplitude is reduced. The ensemble-average current (below) shows a marked overall inhibition of current by QX. All current records filtered at 1.25 kHz. Patch rle6.
normalized unitary current on [QX] (Fig. 2B). This relation, in effect, expresses the probability of rapid site occupation as a function of [QX]. This relation demonstrates saturation that, in conjunction with the reasonably good fit of a Langmuir isotherm relation (continuous line), argues for a one-to-one stoichiometry of binding.

Fig. 2. Graded reduction of unitary currents by QX. A, the upper trace shows the current records of a single Na+ channel during a depolarizing pulse to −50 mV in control. The lower trace shows the same patch at −50 mV, but after exposure to 2 mM QX. The clear-cut reduction in unitary current (continuous line, control; dashed line, QX) is consistent with an ultra-fast, rapid blocking process. Filtered at 1.5 kHz. B, Normalized degree of rapid block as a function of [QX] during voltage steps to −50 mV. Standard error bars are shown around means derived, in most cases, from about six patches. The data can be approximated well by the Langmuire isotherm relation shown (continuous curve, Fig. 2B), with a $K_d$ of 4.4 mM as gauged by regression of a linearized version of the data.

The location of the rapid site within the membrane electric field can be deduced from the voltage dependence of rapid block. Unitary current is steeply dependent on voltage (Fig. 3A); a Boltzmann function fit (continuous line, after Woodhull, 1973) suggests that a QX molecule traverses 73% of the transmembrane voltage to reach
the rapid site from the cytoplasmic channel entrance. The deep location according to electrical co-ordinates supports an intrapore location for the rapid site. Moreover, by reasoning along the lines of Armstrong (1971), the potentiation of rapid block by lowered external [Na⁺] with maintained ionic strength (Fig. 3B) can be taken as further independent evidence for a rapid site within the pore.

Fig. 3. Voltage dependence and sensitivity to external [Na⁺] of rapid block implicate a deep binding site in the pore. A, normalized unitary current (i) versus membrane voltage, with 2 mM QX present throughout. The reduction of unitary current is steeply voltage dependent; the Boltzmann function (continuous curve), fitted by regression of a linearized version of the data, suggests that the block site resides 73% of the electrical distance in from the cytoplasm, with a half-blocking voltage of −17 mV. Standard errors are shown bracketing, the means of at least three patches. B, potentiation of rapid block by reduced external [Na⁺]. The bar graphs demonstrate the difference in normalized unitary current with 85 mM versus 170 mM external [Na⁺] (P < 0.0005, unpaired t test). Standard deviations are shown bracketing the means of three and six patches, respectively. The voltage was −50 mV, and 2 mM QX was present. The 85 mM Na⁺ solution was identical to the standard 170 mM Na⁺ solution, except that 85 mM NaCl was replaced with TEA-Cl.
Fig. 4. Kinetics for the discrete block of open states. A, open and closed time histograms in control at $-20$ mV, plotted in the form of complemented distributions as detailed in the Methods. B, open and closed time histograms at $-20$ mV, with 2 mM QX present. In A and B, the continuous lines are single- and biexponential functions, with the parameters as shown. $Amp_1$ and $Amp_2$ are the individual exponential coefficients of biexponential
Discrete block of open channels

We now turn to the analysis of discrete block of open channels. As a first approach, we ignored possible interactions between rapid and discrete blocked states, and used the following blocking model to analyse open and closed intervals:

\[
\begin{align*}
D & \\
\downarrow & k_b \\
\downarrow & k_{ba} \\
C & \leftrightarrow O,
\end{align*}
\]

where C and O signify closed (non-conductive) and open (conductive) states that are intrinsic to native channel gating, and D denotes a state whereby an open channel has been blocked at the discrete site by QX. While this model ignores interconnections with the rapid blocked state, R, it turns out that straightforward adjustment of analyses based on eqn (1) will render quantitatively correct results for the more complicated case.

Duration histograms obtained before application of QX can validate some of the inherent features of the simple model in eqn (1). The 'control' open time histogram (Fig. 4.A, top) is distributed according to a monoexponential function, supporting the predominance of a single open state (Colquhoun & Hawkes, 1983). The control closed time histogram is well fitted by a biexponential function (Fig. 4.A, bottom), in which the faster exponential predominates and accounts from more than 90% of closures. This predominance argues that open channels close primarily to a single closed state, again in accord with the simple model above.

The remaining model features gain experimental support from the change in duration histograms upon application of 2 mM QX. The open time histogram remains monoexponential (Fig. 4.B, top), although shifted towards briefer openings. These results suggest strongly that open channels undergo discrete block, as the model above proposes. Closed time histograms are now dominated by a new, slow exponential component (Fig. 4.B, bottom), suggesting that QX adds a single, non-conducting state to the picture, consistent with the proposed open blocked state D.

functions. Data from patch rle6, with one channel. C, discrete (un)block rates as a function of voltage, with 2 mM QX present. The apparent discrete unblock rate (k_{un}, □) showed no statistically significant change with voltage (by linear regression analysis). The apparent discrete block rate (k_{b, apparent}, ○) changes e-fold per 58 mV, as shown by the dashed exponential curve (determined from linear regression analysis of a logarithmic version of the data). Means and standard errors from about three to four patches are shown for k_{ub} and k_{b, apparent}. The filled circles show mean values of k_{b} after normalization by i_{QX}/i_{ctrl}, as determined from the Boltzmann fit in Fig. 3.A. Linear regression of a logarithmic version of the plot reveals an e-fold change per 35.7 mV, as illustrated by the continuous exponential curve:

\[
1.88 \text{ ms}^{-1} \exp \left( \frac{-0.7 F V}{RT} \right).
\]

D, dependence of discrete (un)block rates on [QX], all at ~50 mV. The apparent discrete block rate (○) saturates with increasing [QX], while the apparent discrete unblock rate (□) shows no statistically significant change (by linear regression analysis). Standard errors are shown bracketing the means of four to five patches. The filled circles show mean k_{b} values, after normalization by i_{QX}/i_{ctrl}, as specified by the fit in Fig. 3.A. These define a linear relation with a slope of 0.25 mm^{-1} ms^{-1}.
Accordingly, in the absence of missed events (Blatz & Magleby, 1986), the reciprocal of the time constant would be equal to the unblocking rate \( (k_{ub}) \). The fast exponential component of the closed time histogram, with a time constant nearly equal to that of the predominant component in control, reflects shut events corresponding to native closures.

Having justified the coarse features of the simple blocking model, we could begin to use this framework to derive rate constants for discrete block and unblock \( (k_b \) and \( k_{ub} \), respectively). Open and closed time histograms in the presence of QX (e.g. Fig. 4B) were used to specify discrete (un)blocking rates in the following manner. Maximum-likelihood criteria were used to fit mono- and biexponential probability density functions to all open and closed intervals exceeding the dead time of our system \((≈ 0.2 \text{ ms})\). The fits yield time constants and amplitudes that differ somewhat from those that would be measured in the absence of missed opening and closing transitions inherent to the half-height detection algorithm used here. To derive the precise values for \( k_b \) and \( k_{ub} \), this distortion was accounted for by the iterative procedure of Blatz & Magleby (1986) (see Methods for details).

Figure 4C shows the results of the first-order approach to gauging discrete (un)block rates. Here, we examine the apparent voltage dependence of the associated rates, as an approximate guide to the location of the discrete site. \( k_b \) (○, Fig. 4C) increases moderately with depolarization, changing e-fold per 58 mV (dashed curve). In contrast, \( k_{ub} \) (□, Fig. 4C) is independent of voltage, thereby placing the discrete site at 43% of electrical distance inward from the cytoplasmic entrance of the channel. While this result supports an intrapore site for discrete block, the quantitative accuracy of discrete block rates must still be viewed as approximate since possible interactions with rapid block have been ignored, so far.

To assess the possible interplay of rapid and discrete block, we next considered a generalized case for such interactions:

\[
\begin{align*}
D &\xrightarrow{k_{OD}} \xleftarrow{k_{RD}} DR \\
&\xrightarrow{k_{OR}} \xleftarrow{k_{RDR}} C \\
&\xrightarrow{k_{RO}} \xleftarrow{k_{RDR}} R
\end{align*}
\]

where \( R \) is the rapid blocked state, and \( DR \) is a state in which discrete and rapid block sites of an open channel are occupied simultaneously by QX molecules. To determine which of the potential interconnections above are actually important, we examined the shape of the relationship between apparent \( k_b \) (as measured by the naive approach above) and cytoplasmic [QX]. Since this relationship reflects only those transitions that culminate in discrete block \( (D \text{ or } DR) \), analysis of the apparent \( k_b \) (henceforth denoted \( k_{b,\text{apparent}} \)) is simplified considerably. These transitions are represented below by the subscheme of eqn (2):

\[
\begin{align*}
D &\xrightarrow{k_{OD}} \xleftarrow{k_{RD}} DR \\
&\xrightarrow{k_{OR}} \xleftarrow{k_{RDR}} O \\
&\xrightarrow{k_{RO}} \xleftarrow{k_{RRO}} R
\end{align*}
\]
Reasoning according to this subscheme enables us to ascertain the chief transition pathways for discrete block. If discrete and rapid block proceed as independent processes, then rate constants in the scheme above will be constrained such that the relationship between $k_{b,\text{apparent}}$ and [QX] will be linear. If this is not the case, then elaboration of eqn (3) yields the following non-linear expression for the generalized case.

$$k_{b,\text{apparent}} = \frac{(k_{OD} k_{RO} + k_{RD} k_{OR}) [QX]}{k_{OR}[QX] + k_{RO}} + \frac{(k_{ODR} k_{RO} + k_{RDR} k_{OR}) [QX]^2}{k_{OR}[QX] + k_{RO}}. \quad (4)$$

The formation of the D state gives rise to the first set of terms, which saturates with increasing [QX] according to a Langmuir isotherm equation. The generation of DR is reflected in the second set of terms, which grows at least linearly with increasing [QX]. These features allow us to determine which of the discrete block mechanisms predominate.

Figure 4D shows data for the corresponding experiment. $k_{b,\text{apparent}}$ saturates with increasing [QX] ($\bigcirc$), suggesting the predominance of D over DR, as well as arguing against the independence of discrete and rapid blocking processes. This conclusion can be bolstered further by examining a normalized version of the apparent block rate, one that should demonstrate a linear relation to [QX] if D is the principal discrete blocked state. If we divide $k_{b,\text{apparent}}$ by the probability that a channel is open, given that it is either open or rapid blocked ($P(O|\text{O or R})$), eqn (4) yields:

$$k_{b,\text{normalized}} = \frac{(k_{OD} k_{RO} + k_{RD} k_{OR}) [QX]}{k_{RO}} + \frac{(k_{ODR} k_{RO} + k_{RDR} k_{OR}) [QX]^2}{k_{RO}}, \quad (5)$$

where $P(O|\text{O or R}) = k_{RO}/(k_{OR}[QX] + k_{RO})$. The important point of this transformation is that if DR is negligible compared with D, then the quadratic term in [QX] (relating to DR) drops out, uncovering a linear relation between $k_{b,\text{normalized}}$ and [QX]. The ratio of unitary currents in QX and control ($i_{QX}/i_{\text{ctrl}}$) provides an independent experimental measure of $P(O|\text{O or R})$, allowing us to perform the proposed normalization. The filled circles in Fig. 4D show the actual result: a remarkably linear dependence of corrected $k_{b,\text{normalized}}$ on [QX]. The demonstrated linearity quantitatively discounts any significant contribution of DR. Further support for the rarity of DR comes with the result that the apparent $k_{ub}$ is independent of [QX] (Fig. 4D, □). These two features argue that discrete block reflects a bimolecular process involving a channel and a single QX molecule. Hence, we can reduce the general blocking scheme in eqn (2) to that shown below.

\[
\begin{array}{c}
\text{D} \\
\text{C} \rightarrow \text{O} \rightarrow \text{R} \\
\end{array}
\]

Discrete and rapid sites share a deep intrapore location

To pinpoint quantitatively the location of the discrete block site within the membrane electrical field, we take advantage of the simplifications afforded by the reduced system in eqn (6). It now turns out that the voltage dependence of
$k_{b, \text{normalized}}$ bears a direct relationship to the electrical distance of the discrete block site from the cytoplasmic surface ($\delta_D$). Recalling the voltage independence of $k_{ub}$ for discrete block, we can explicitly express the voltage dependence of $k_{b, \text{normalized}}$ in the simplified version of eqn (5):

$$k_{b, \text{normalized}}(V) = \left( k_{OD}(0) + \frac{k_{RD}(0)}{K_d(0)} \right) \exp \left( \frac{\delta_D F V}{RT} \right) [QX],$$  

(7)

where $V$, $F$, $R$ and $T$ have their usual meanings, $k_{OD}(0)$ and $k_{RO}(0)$ are the zero-voltage values of the corresponding rate constants, and $K_d(0)$ is the zero-voltage value for $k_{RO}/k_{OR}$. This yields a monoexponential function of voltage, the fit of which to empirically determined $k_{b, \text{normalized}}$ (Fig. 4C, ○) yields a $\delta_D$ of 0.7. Hence, the discrete site can now be placed very deep within the pore, at 70% of the electrical distance in from the cytoplasmic surface. This value is remarkable because it places the discrete site at, or very near, the same location as the rapid site.

**Rapid block serves as a transition intermediate for discrete block**

The extreme physical proximity of discrete and rapid blocking sites permits us to propose that transitions from R to D in eqn (6) should be favoured tremendously over those from O to D because of the intramolecular nature of the former reaction. The following line of reasoning develops the case explicitly. By analogy with the enzymatic enhancement of reaction rate derived from binding a substrate molecule near the catalytic group of an active site (‘togetherness’ or ‘propinquity’ effects; Jencks & Page, 1972), the R state serves to facilitate discrete block by increasing the effective concentration of QX near the discrete block site to levels perhaps as high as 100 mM (Hegarty & Bruice, 1970; Lehninger, 1975). The rate constant for R to D transitions ($k_{RD}$) would then be in the order of the rate of O to D transitions ($k_{OD}$) multiplied by an effective concentration of $10^5$ mM (i.e. $k_{RD}$ ms$^{-1}$ ≈ $k_{OD}$ ms$^{-1}$ mM$^{-1}$×$10^5$ mM). On this basis, we can calculate that the rate of discrete block from O is negligible relative to discrete block from R. According to eqns (4) and (6), the rate of formation of the discrete blocked state, given that a channel is in O or R, can be written:

$$k_{b, \text{apparent}} = \frac{k_{OD} K_d [QX]}{K_d + [QX]} + \frac{k_{RD} [QX]}{K_d + [QX]},$$  

(8)

where $K_d = k_{RO}/k_{OR}$, and the $[QX]^2$ term relating to the doubly blocked DR state has been dropped. The first term represents the rate of O to D transitions, while the second relates to R to D reactions. Substituting $k_{OD} \approx k_{RD} \times 10^{-5}$ mM$^{-1}$ yields:

$$k_{b, \text{apparent}} \approx \frac{k_{RD} \times 10^{-5}}{K_d + [QX]} K_d [QX] + \frac{k_{RD} [QX]}{K_d + [QX]} K_d [QX].$$

Recalling that $K_d$ is about 4·4 mM (Fig. 2B), this simplifies to:

$$k_{b, \text{apparent}} \approx \frac{k_{RD} [QX]}{K_d + [QX]}.$$  

(9)

This expression corresponds to a scheme in which O to D transitions are negligible.
Moreover, D to O transitions must also be insignificant, otherwise there would be a violation of microscopic reversibility. We are thus left with the following mechanism of rapid and discrete block:

\[
\begin{align*}
C & \rightleftharpoons O \rightleftharpoons R \rightleftharpoons D. \\
& \quad k_{OR} \quad k_{RD} \\
& \quad k_{RO} \quad k_{DR}
\end{align*}
\]

(10)

R can therefore be viewed as a transition–intermediate state for the formation of D.

Given this mechanism, the true rates of discrete intermediate (k_{DR}) and block (k_{RD}) can now be determined. k_{DR} is now simply equal to the apparent unblock rate (k_{ub}), which is 0.075 ms\(^{-1}\) (Fig. 4C, mean of open squares). Reasoning from eqn (9), k_{RD} can now be determined from \(k_{b,\text{normalized}}\) by the relation:

\[
k_{RD} = \frac{k_{b,\text{normalized}}K_d}{[QX]}.
\]

(11)

We can then explicitly calculate the value of \(k_{RD}\), as well as its voltage dependence, by substitution of the voltage-dependent functions for \(k_{b,\text{normalized}}\) and \(K_d\). Recall from Fig. 4C (●) that \(k_{b,\text{normalized}}(V)\) can be expressed as:

\[
k_{b,\text{normalized}}(V) = 1.88 \text{ ms}^{-1} \times \exp\left(\frac{-0.7 FV}{RT}\right).
\]

(12)

From the analysis of the voltage dependence of rapid block (Fig. 3A) and the determination of \(K_d\) at -50 mV (Fig. 2B), the expression for \(K_d(V)\) is:

\[
K_d(V) = 1.02 \text{ mm} \times \exp\left(\frac{-0.73 FV}{RT}\right).
\]

(13)

Incorporating these expressions into eqn (11) yields the remarkable result that \(k_{RD}\) is virtually independent of voltage, and it has a value of 1.02 ms\(^{-1}\).

The ability to calculate true discrete block and unblock rates also allows us to perform an independent test for the intrapore location of discrete block: examination of the effects of lowered external Na\(^+\) concentration on discrete block (Fig. 5). Representative records of such a test are shown at the top of the figure. The result is that true block rates (k_{RD}) are increased with lowered external Na\(^+\) concentrations, while discrete unblock rates (k_{DR}) are slightly decreased or unchanged. These results provide additional support for an intrapore location of the discrete site.

**Discrete block of closed channels**

The remaining concern is whether a closed channel itself is subject to discrete block ('closed-blocked state'). Should this be the case, our analysis of the conclusions about discrete block of open channels, which did not include the possibility of closed block, could require revision. The slow exponential component of closed time histograms (Fig. 4B) might not exclusively reflect lifetimes in the open–blocked state (D), but may report dwell times in a closed–blocked state (CD) as well. Hence, our estimates for k_{DR}, the presumed unblock rate for transitions from D to R (eqn (10)), might be in error.
First-latency histograms offer an approach to detecting closed-blocked states. Figure 6A plots such a histogram before application of QX. The graph expresses the probability that a channel will first open at a time greater than that specified on the abscissa. For the control condition, this relation demonstrates a precipitous decay,

Fig. 5. Potentiation of discrete block by reduced external [Na⁺]. The traces at the top illustrate how altered external [Na⁺] affects discrete block by 2 mM QX at -50 mV (patches r1e111 and r1e7 for 170 and 85 mM [Na⁺]). Lowering external [Na⁺] increases the degree to which openings are shortened by QX. Traces filtered at 1 kHz. These trends are confirmed below by the bar graph summaries of \( k_{RD} \) (corrected for rapid block interactions) and \( k_{DR} \). Each bar represents the means of about four patches, with bracketing standard errors shown. The potential was -50 mV, and 2 mM QX was present in all cases. Lowering external [Na⁺] produced a highly significant increase in \( k_{RD} \) (\( P < 0.01 \)), but only a slight decrease in \( k_{DR} \) (n.s.). The value of \( K_d \) used for calculations in the lower external [Na⁺] was 3.13 mM, derived from the mean \( i_{QX}/i_{Ctrl} \) under these conditions in Fig. 3B. The solutions are as described in the legend for Fig. 3, and the Methods.

reflecting the rapid kinetics of intrinsic channel activation (e.g. Fig. 2A). The non-zero plateau represents sweeps without any activity (‘blanks’); these probably reflect sojourns in slow inactivation states (Quandt, 1987), distinct from the fast inactivation states already removed by enzymatic treatment. QX (2 mM) adds a new, slow exponential component (Fig. 6B) that reflects the occurrence of very late first openings (e.g. Fig. 1B). The kinetics of the slow component are steeply voltage dependent, as the contrast between Fig. 6B and C, and the derived time constants

\[ [Na^+]_o \begin{array}{ll}
170 \text{ mM} \quad & \text{Control} \\
85 \text{ mM} \quad & +QX-314
\end{array} \]

\[ 1 \text{ pA} \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad 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ULTRA-DEEP BLOCKADE OF Na⁺ CHANNELS

Fig. 6. Discrete unblock of closed channels. A–C, complemented distribution of first latencies. The graphs show the probability that the first opening will occur at a time greater than that specified on the abscissa. All data from patch r4e108. A, control data after removal of fast inactivation. B, data from the same patch at -50 mV, but in the presence of 2 mM QX. QX introduces a new slow exponential component consistent with discrete unblock of closed channels. The continuous curve plots the maximum likelihood fit of a biexponential function: 0.07 \exp (-t/1.4) + 0.23 \exp (-t/41.1) + 0.69. C, data from the same patch with 2 mM QX present, but at -20 mV. The slow component persists, but is considerably faster at -20 mV. The continuous curve plots the maximum likelihood fit of a biexponential function: 0.06 \exp (-t/0.9) + 0.27 \exp (-t/9.1) + 0.67. D, potentiation of unblock from closed blocked states with increasing depolarization. The unblock rate is gauged by the reciprocal of the slow time constant of biexponential fits, as illustrated in A–C. The means of reciprocal time constants from four patches demonstrate a marked increase with voltage (P < 0.05, paired t test). Standard error bars are shown. E, no change (n.s., paired t test) in the fraction of blank sweeps upon addition of 1–3 mM QX to patches depolarized to -50 mV. Means and standard errors are shown for twelve patches. Multichannel data have been corrected to one-channel analogues before averaging. F, proposed model to account for first-latency kinetics in the presence of QX. (Fig. 6D) from exponential fits (Fig. 6B and C, continuous curves), illustrate. Finally, there is little change in the plateau value with QX (compare Figs. 6A–C), as confirmed by averages of the fraction of blank sweeps (Fig. 6E).
These results have definite implications for the existence of closed–blocked states. The insensitivity of blank sweeps to QX argues against closed–blocked states with lifetimes longer than the 1 s repetition interval for voltage steps (Moczydlowski, 1986); the existence of these would increase the frequency of blank sweeps with QX. On the other hand, the slowing of first latencies suggests the existence of short-lived closed–blocked states, as follows.

The slowing of first latencies may be explained by discrete unblock of channels that have either: (1) become newly blocked between sweeps or before the first opening in a given sweep, or (2) remained blocked from a previous sweep. While our data do not distinguish between the possibilities, discrete closed–blocked states are likely to be involved in either case. The first explanation directly requires their existence. The second explanation requires uninterrupted QX binding throughout the period between voltage steps. The long interruptions in current that such a slow dissociation entails are not apparent in either single-channel records (e.g. Fig. 1B) or the detailed closed time analyses (Fig. 4B) at voltages of $-50$ mV or greater. By exclusion, the slow dissociation rate must only be manifest during the hyperpolarization between test pulses. QX bound to open channels could not serve as the long-lived complex during this period: dissociation of QX from an open channel would be speeded by hyperpolarization. The only remaining possibility is that hyperpolarization causes channels to close upon and trap QX molecules until the next depolarizing step (Miller, Latorre & Reisin, 1987). Hence, if the second explanation is true, there must also be closed–blocked states.

As mentioned earlier, closed–blocked states could cause $k_{DR}$, the calculated rate of unblock from D to R, to be in error, thereby challenging our quantitative conclusions regarding the discrete block of open channels. We do not, however, believe such a complication exists for two reasons. First, QX introduces only a single distinct exponential in the closed time histogram (Fig. 4B). This finding argues against significant closed channel unblock except in the unlikely event that its rate closely matches that of the unblock of open channels. Second, there is the marked acceleration of the slow phase of first latencies with voltage (Fig. 6D). This feature stands in sharp contrast to the lack of voltage dependence of calculated $k_{DR}$ (□, Fig. 4C). If calculated $k_{DR}$ were to reflect unblock from both D and CD states, it should also manifest voltage dependence like the slow component of first latencies (Fig. 6B–D). Since this is not observed, it seems that transitions from CD states contribute little to the determination of $k_{DR}$. Hence, our conclusions about the discrete block of open channels are likely to stand.

The results in this section suggest the following for voltage steps to potentials of $-50$ mV, or greater. Immediately upon depolarization, a small fraction of channels remain unblocked and thereby activate quickly along the top row of closed states leading to the open state (Fig. 6F). These channels give rise to the residual fast component of first latencies with QX (Fig. 6B and C). A large fraction of channels are, however, initially detained in closed–blocked states (CD, in Fig. 6F), perhaps by QX trapping following the previous step. These channels only gradually become unblocked, along either, or both, of the dashed pathways; this process underlies the predominant slow component of first latencies with QX. The voltage dependence of activation makes it so that once a channel opens, sojourns to closed states that can
be blocked to form CD are too brief or rare for there to be appreciable reappearance of CD. By analogy to the reluctance for closure, transitions from D to CD are probably nil. The only reason first latencies are slow is because channels happen to start out in CD. Once opening occurs, however, the channel rarely comes back to CD for the remainder of the depolarization epoch.

**DISCUSSION**

*Deep blockade of Na*⁺ *channels by QX-314*

One main result is that Na*⁺* channel inhibition by an internally applied quaternary ammonium ion, QX-314, involves a very deep blockade of the channel pore: in reaching the block site, we argue that the quaternary ammonium moiety of QX traverses 70% of the electrical distance from the cytoplasmic entrance.

Several features of our localization of the QX binding site are unique, and enable us to propose such a deep blocking site. First, our conclusions are based upon single-channel data; these have exposed two modes of blockade, and their interactions. On the other hand, analysis of macroscopic currents alone would have lumped both blocking processes into one, possibly leading to a different calculated localization of QX binding sites. Second, fast inactivation was removed, facilitating direct observation of QX blocking kinetics by slowing intrinsic gating and permitting us to speed *k*ₐ,apparent with higher [QX]. With native Na*⁺* channels, we reasoned in the Results that [QX] would be limited to ≈ 0.1 mM if a workable amount of activity were to be preserved. Our block analysis (Fig. 4D) predicts an apparent QX association rate of 0.025 ms⁻¹ for this [QX], about two orders of magnitude slower than intrinsic gating. It comes as no surprise, then, that the effect of QX on native channels is to increase the fraction of blank sweeps without appreciable change in the gating of active sweeps (Yeh et al. 1986; and our unpublished work); accordingly, determination of QX association and dissociation properties would have been derived from model-dependent analysis that presumes the validity of a modulated-or guarded-receptor model (Hille, 1977; Starmer, Grant & Strauss, 1984). Third, fast inactivation was permanently removed by proteolysis, not reversibly inhibited by a toxin (Moczydlowski, 1986; Wang, 1988). Hence the observed effects could definitely be attributed to interactions between QX and the channel, as opposed to between QX and the toxin (Postma & Catterall, 1984). Finally, the bandwidth of our single-channel data obtained with patch pipettes (≈ 2 kHz) is about tenfold larger than is generally feasible with bilayer recording, thereby facilitating the analysis of distinct blocking modes. This may explain why rapid and discrete modes of block were not apparent in earlier bilayer data (Moczydlowski, 1986); quite possibly both modes of blockade were low-pass filtered to appear as one.

There remains, however, a potential limitation to the quantitative accuracy of our blocker localization. Our quantitative estimates, derived from analysis of the voltage dependence of block, implicitly ignore possible interactions between Na*⁺* ions and QX molecules in the permeation pathway (as did Woodhull, 1973). Specifically, we disregard voltage-dependent redistribution of Na*⁺* ions in the pore as a potential factor contributing to the overall voltage dependence of QX blockade. While this simplification could require quantitative revision of our localization, our conclusions
are based on qualitative outcomes of our analysis about the proximity of various blocking ions.

Localization of a very narrow selectivity region

Hille (1971, 1978) was among the first to propose that the selectivity region of Na\(^+\) channels is short, perhaps only a few angstroms in length. The proposal was motivated by the need to explain the high throughput rate of Na\(^+\) channels. Our present result that QX traverses 70% of the electrical distance from the cytoplasmic entrance provides more direct biophysical evidence that the selectivity region is, in fact, quite narrow. Recent single-channel studies demonstrate that externally applied Cd\(^{2+}\) blocks Na\(^+\) channels at a site located 20% of the electrical distance from the external entrance (Ravindran et al. 1991; Backx et al. 1992). Hence, the selectivity region of the Na\(^+\) channel can span no more than 10% of the electrical distance, the dimension bracketed by internal and external blocking ions.

If we interpret the restricted electrical dimension of the selectivity region as tantamount to a restricted physical dimension, we can take advantage of recent work with site-directed mutagenesis to make physical proposals about the location of the QX binding site. The first step in such molecular triangulation arises from two phenotypically unique properties of the cardiac isoform of Na\(^+\) channels: high-affinity block by external Cd\(^{2+}\), and low-affinity block by external tetrodotoxin (TTX). Both of these properties have recently been shown to depend critically upon a single cysteine residue located in the first of four so-called H5 motifs (Backx et al. 1992; Satin et al. 1992). These results argue strongly that the binding site for external Cd\(^{2+}\) is physically very close to the TTX receptor. A second clue comes from the idea that each voltage-gated channel has four H5 motifs that approximate each other like the staves of a barrel to form the selectivity region of the pore (Yellen et al. 1991; Hartmann et al. 1991; Yool & Schwarz, 1991; Heinemann et al. 1992). Residues located within a few amino acids of the critical cysteine (Noda, Suzuki, Numa & Stühmer, 1989), or in the analogous stretches within all three other H5 regions, are also critical to determining TTX affinity (Terlau, Heinemann, Stühmer, Pusch, Conti, Imoto & Numa, 1991). Hence, the external Cd\(^{2+}\) site must be physically close to all four H5 regions in the channel. The final link in our reasoning draws upon the proposed secondary structure of H5 motifs in Shaker K\(^+\) channels. In Shaker, H5 regions may form a hairpin structure that spans the permeation pathway of the channel, with the sites for internal and external block by tetraethylammonium (TEA) located in the centre and arms of the hairpin, respectively (Yellen et al. 1991). If Na\(^+\) and K\(^+\) channels share a similar design, then the electrical proximity of Cd\(^{2+}\) and QX sites gives reason to propose that certain residues in the H5 motifs will comprise the QX binding site. In fact, if the quaternary ammonium moiety in QX is co-ordinated in Na\(^+\) channels, as is TEA in K\(^+\) channels, threonine (Yellen et al. 1991) or the aromatic amino acids phenylalanine and tyrosine (Heginbotham & MacKinnon, 1992) could well be involved. A recent alignment of H5 domains in the rat brain II Na\(^+\) channel clone reveals more than five threonines and five aromatic amino acids in the relevant regions (Terlau et al. 1991). Negatively charges residues in this region could also contribute to QX co-ordination.

If QX binding is in fact mediated by H5 residues in the Na\(^+\) channel, the resultant proposal that H5 spans the selectivity region would be less controversial than the
current scheme in K⁺ channels. One of the unusual implications of the H5 structure in K⁺ channels is that the hairpin must span 80% of the electrical distance. In order for the eight-amino-acid arm of the hairpin to span the distance, the proposal was advanced that the hairpin is comprised of β-strands (Yellen et al. 1991), a somewhat unusual proposal in this context. By contrast, the H5 hairpin need only span 10% of the electrical distance in Na⁺ channels; even traditional α-helices might cover the requisite distance.

Do all Na⁺ channel isoforms share the same pore geometry? Earlier work with macroscopic currents in squid axons suggests that there is little voltage dependence of QX block after proteolytic removal of fast inactivation (Cahalan, 1978; Yeh, 1978). On the other hand, our work here with the cardiac isoform, and that of Moczydlowski (1986) in single Na⁺ channels from rat skeletal muscle, demonstrate significant voltage dependence of QX block. It is conceivable that this apparent inconsistency could derive from unanticipated factors in the interpretation of the macroscopic current blockade. More likely, perhaps, is the existence of a genuine difference between squid neuronal and mammalian cardiac Na⁺ channels. Such a striking divergence in behaviour would be one of only a few qualitative phenotypic differences among the structurally similar family of voltage-gated Na⁺ channels; once the DNA clone for the squid Na⁺ channel is available, this distinction could help to implicate the amino acid residues that co-ordinate QX-314 in the pore.

**Proposed mechanism for blockade**

Although the existence of a transition intermediate in the formation of a discrete block state is not unusual, the electrophysiological resolution of such a species is, to our knowledge, unprecedented. This resolution has afforded us a rare opportunity to examine features of the detailed reaction mechanism of drug block.

What might be the physical correlate to the reaction scheme in eqn (10)? The usual conception of QX binding in the pore is shown in Fig. 7A (adapted from Schwarz, Palade & Hille, 1977). A QX molecule enters the channel from the bulk solution to become tightly bound at two positions: one channel domain co-ordinates the quaternary ammonium moiety, while another domain forms a hydrophobic pocket for the lipophilic tail. The shielding of the aromatic moiety from the hydrophilic cytoplasmic environment makes an important contribution to the stability of the complex. Dual co-ordination of the QX molecule in this specific manner could result in a high-affinity state, giving rise to the millisecond lifetimes of discrete block (state D).

Our data lead us to suggest the following reaction mechanism by which this overall sequence of events in Fig. 7A occurs (Fig. 7B). The first step involves rapid entry of a QA molecule into the pore to form a transition–intermediate state (R). To account for the closely similar electrical distances associated with R and D states, we propose that the charged quaternary ammonium moiety is co-ordinated by the identical domain here as in the high-affinity D state, although the rotational orientation of the charged group may differ in the two cases. It follows that the distinctive properties of the transition–intermediate state should, by exclusion, arise from the manner in which the lipophilic tail is stabilized by the channel. One possibility would be that the lipophilic tail is not stabilized at all in the R state. This case seems unlikely because even 10 mM internal TEA produces no appreciable change in unitary current...
Thus, the R state probably involves low-affinity interactions between the lipophilic tail and the channel. Figure 7B illustrates one plausible configuration for the R state; the proximity of the lipophilic tail to the watery cytoplasmic environment explains the relative instability of the R state. In order for the QA molecule to proceed to the high-affinity D state, the lipophilic tail must find its way into a more avid configuration, perhaps by entry into a specific hydrophobic pocket as diagrammed. The pathway to the D state is presumably very restrictive because the probability of a QA molecule proceeding to state D on a given sojourn to state R is low: $k_{RD}$ is about 1 ms$^{-1}$, while $k_{RO}$ is at least tenfold faster. However, because there are so many sojourns to the R state in a given period of time, the overall rate of formation of state D is appreciable. In this manner, state R ‘catalyses’ the formation of state D. Without state R, the equilibrium occupancy of state D would...
not be altered, but the time scale, on which equilibrium could be achieved (if ever) would be slowed enormously.

Our proposed mechanism raises two points. First, drug efficacy might not only be determined by the overall affinity of the final drug–channel complex, but also by the properties of transition–intermediate states that determine the kinetics of interaction. Second, since we postulate two separate domains for co-ordination of the aromatic group, it should be possible to create mutant channels in which one mode of block is destabilized without affecting the other (cf. work with the Shaker K⁺ channel by Choi, Mossman, Aubé & Yellen (1993)).

Are there other examples of observable transition intermediates among the many instances of QA blockade of ionic channels? In native cardiac Na⁺ channels with intact fast inactivation, we also detect a reduction in unitary currents with QX (not shown). Without our inactivation-free results as a guide, this reduction might be overlooked because the decrement is small at the lower [QX] required in the native condition (5% reduction at −50 mV, 0·25 mM [QX]). But once attention is appropriately focused, the diminished unitary current in native Na⁺ channels can easily be discerned. Na⁺ channel blockade by internal pentacainide reveals no obvious transition–intermediate states (unchanged unitary currents) with fast inactivation removed by DPI 201–106 (Carmeliet, Nilius & Vereecke, 1989) or chymotrypsin (Gruber, Vereecke & Carmeliet, 1991). It may be that pentacainide has much more lipophilic mobility than does QX-314, such that access to state D is much less restrictive (kRD larger). This accords with the lower concentrations that had to be used (3–60 μM). The result might have been that the reduction in unitary current would be undetectable in this concentration range. In many channels, the single-channel block properties of QA remain to be investigated. It will be interesting to determine whether a transition–intermediate state can be visualized in these cases.

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ULTRA-DEEP BLOCKADE OF Na+ CHANNELS


