Bis-Quaternary Ammonium Blockers as Structural Probes of the Sarcoplasmic Reticulum K+ Channel

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ABSTRACT   A series of n-alkyl-bis-α,ω-trimethylammonium (bisQn) compounds was synthesized, and their ability to block K^+ currents through a K^+ channel from sarcoplasmic reticulum was studied. K^+ channels were inserted into planar phospholipid membranes, and single-channel K^+ currents were measured in the presence of the blocking cations. These bisQn compounds block K^+ currents only from the side of the membrane opposite to the addition of SR vesicles (the *trans* side). The block is dependent on transmembrane voltage, and the effective valence of the block (a measure of this voltage dependence) varies with the methylene chain length. For short chains (bisQ2–bisQ5), the effective valence decreases with chain length from 1.1 to 0.65; it then remains constant at ~0.65 for bisQ5 to bisQ8; the effective valence abruptly increases to 1.2–1.3 for chains of nine carbons and longer. For the compounds of nine carbons and longer, the discrete nature of the block can be observed directly as "flickering noise" on the open channel. The kinetics of the block were studied for these long-chain blockers. Both blocking and unblocking rates of the blockers vary with chain length, with the blocking rate showing the strongest variation—an increase of 2.8-fold per added methylene group. All of the voltage dependence of the binding equilibrium resides in the blocking rate, and none in the unblocking rate. The results imply that 65% of the voltage drop within the channel occurs over a distance of 6–7 Å, and that the short-chain blockers bind in an extended-chain conformation, whereas the long-chain blockers bind in a bent-over conformation with both charges deeply inside the channel.

INTRODUCTION

The study of ionic channels is functionally sophisticated and structurally primitive. Although some of the most powerful theoretical and experimental approaches to be found in biophysics have been applied to the study of these membrane proteins and have generated an impressive understanding of their ion-transporting functions, our view of their molecular structures remains clouded. Very little opportunity exists to apply to membrane proteins the high-resolution structural methods that have led to the detailed mechanistic pictures of the operation of many water-soluble enzymes.

One approach that has provided some structural picture of channels is the

study of interactions of ammonium-derived "organic" cations with these ion-diffusion pathways. Hille (1971, 1973, 1975) originally used the technique to determine the cross sections of the narrowest parts of the Na^+ and K^+ channels of frog nerve by measuring the permeabilities of organic cations small enough to traverse the channel's entire diffusion pathway. The method has since been applied to a variety of ionic channels (Huang et al., 1978; Dwyer et al., 1980; Colombini, 1980; Schindler and Rosenbusch, 1978; McKinley and Meissner, 1978; Coronado and Miller, 1982). This method has led to estimates of channel cross sections from low values of 9 $Å^2$ for the axon K^+ channel (Hille, 1975) to high values of ~1,200 $Å^2$ for the mitochondrial porin channel (Colombini, 1980). A related approach, initiated in the squid axon K^+ channel by Armstrong (1975), uses organic cations that block K^+ conduction. This technique has also been widely applied (Farley et al., 1981; Lo and Shrager, 1981; Kirsch et al., 1980; French and Shoukimas, 1981; Hermann and Gorman, 1981; Swenson, 1981; Coronado and Miller, 1980, 1982). This approach has provided information about the dimensions of the wider parts of the channel conduction pathways, which have been termed "mouths" or "vestibules." All channels studied in this way have been viewed as containing quite wide mouths (>100 $Å^2$) opening out into the aqueous solutions on one or both sides of the membrane (Armstrong, 1975; French and Shoukimas, 1981; Swenson, 1981; Farley et al., 1980; Horn and Stevens, 1980; Rojas and Rudy, 1976; Coronado and Miller, 1982).

In this laboratory, we have been studying the conduction properties of a K^+-selective channel from mammalian sarcoplasmic reticulum (SR) (Coronado and Miller, 1979, 1980, 1982; Coronado et al., 1980; Miller, 1982). The channel is studied by inserting SR vesicles into planar phospholipid bilayers by membrane fusion, and measuring currents through single channels under a variety of conditions. We have applied the two approaches above to conclude that for this channel the narrowest cross section is ~20 $Å^2$, and that a "mouth" of at least 50 $Å^2$ opens out towards the trans aqueous phase, i.e., the side of the membrane opposite to the addition of SR vesicles.

We previously found that the SR K^+ channel is blocked from the trans side by decamethonium and hexamethonium, two divalent organic cations (Coronado and Miller, 1980). These two structurally related compounds were found to block the channel in different ways. Hexamethonium, like all the monovalent blockers studied (Coronado and Miller, 1982), merely reduces the single-channel conductance in a voltage-dependent manner, but decamethonium causes the open channel to "flicker" between fully open and fully blocked states. Furthermore, the voltage dependence of the decamethonium block was found to be twice as strong as for the hexamethonium block.

To investigate this apparent difference in more detail, a series of bis-quaternary ammonium blockers, the "methoniums"—n-alkanes with a tri-methylammonium group on each end—is studied here. The voltage dependence of the blocking reaction varies with methylene chain length in a way that strongly suggests that 65% of the total potential drop inside the channel occurs over a distance equivalent to the length of only four to five CH_2 groups,

i.e., 6–7 Å. Furthermore, for chain lengths of nine carbons and longer, the blocking kinetics become slow enough so that the blocked state of the channel can be observed directly at the 1-ms time resolution of the single-channel current measurements. The results are consistent with a model in which the bisQn blockers of short chain lengths (less than nine carbons) block in a linear, extended chain conformation, and those with chains of nine carbons and longer block in a bent-over conformation with both quaternary ammonium groups binding at the 65% site.

METHODS

Chemicals

Preparation of SR vesicles from rabbit back and leg muscle has been described (Miller and Rosenberg, 1979). Phospholipids used to make bilayers were phosphatidylcholine (PC) or phosphaditylethanolamine (PE), prepared from rabbit muscle or egg yolk by silicic acid and hydroxyapatite chromatography (Labarca et al., 1980). Quaternary ammonium compounds were synthesized from the alkyl bromides, generally following the method of Burns and Verrall (1973). Alkyl bromide (or *bis*-bromoalkane), obtained from Aldrich Chemical Co., Milwaukee, WI, or Tridom Chemical Co., Hauppauge, NY, was dissolved in acetonitrile (0.2 M final concentration), and then trimethylamine (0.5 M final concentration) was added from a 3-M solution in ethanol. The mixture was refluxed for 5–20 h, and the reaction was followed by measuring Br^- released, using a Br^--selective electrode, and by reversed-phase thin-layer chromatography (TLC) on KC18 plates (Pierce Chemical Co., Rockford, IL), developed with methanol/0.2 M KCl in water, 1/1 (vol/vol), and visualized in iodine vapor or by charring. The solvent was evaporated when the reaction was complete, and the white powder was dissolved in appropriate mixtures of hot ethanol/isobutanol, from which the final product was recrystallized twice. The only exception to this procedure was the ethane derivative, which was synthesized by reacting tetramethylethylenediamine (redistilled immediately before use) with methyl bromide under conditions similar to those above. Products were at least 98% pure, as judged by the absence of contaminating spots in the TLC system.

Bilayers

SR K^+ channels were incorporated into bilayers composed of PE (80%)/PC (20%), cast from decane solutions as described (Miller, 1978; Coronado et al., 1980). Only a single fusion event was allowed to occur in a given experiment, so that a small number of channels (one to five) could be routinely inserted into the bilayers. Single-channel current fluctuations were then recorded on chart paper or FM tape for later analysis. Aqueous phase was 0.15 M K^+ (as the SO_4^- salt), 10 mM MOPS, 0.1 mM EDTA, pH 7.0, and also contained the appropriate concentration of blocker. In some cases, the K^+ concentration was varied as indicated.

The *cis* side of the bilayer is defined as the side to which SR vesicles are added. The opposite side, the *trans* side, is defined as zero voltage. Block by quaternary ammonium compounds was studied by observing the effect of added blocker on currents through single channels. The blocking effect was manifested in one of two ways. If the blocking reaction is fast with respect to the time resolution of the current amplifier, the blocker simply reduces the apparent open-channel conductance; in this case, the amplifier is effectively filtering the rapid transitions between fully open and fully blocked states,

so that we measure the time average of the current between these two states. All monovalent blockers, and bisQn blockers of chain lengths shorter than nine carbons, were studied by this method. For bisQn blockers of nine carbons or longer, the blocking kinetics were slow enough so that the blocked state could be observed directly, and the time-averaged current was calculated in one of two ways. In one method, a direct measure of time average was made by determining the total times in the fully open and fully blocked states, using data taken at high time resolution (1–2 ms per point); typically, 300–2,000 blocking-deblocking transitions were analyzed, using a MINC 11/23 computer (Digital Equipment Co., Marlboro, MA). Alternatively, the channel fluctuations for these blockers were low-pass-filtered at 0.3 Hz to obtain a direct measurement of the time-averaged channel current, as with the fast blockers. In all cases for which the comparison could be done, the time-averaged conductance measured in these two ways agree to within 5%. These measurements were performed under conditions where only a single channel was open at a time. Typically, channel open times were 1–5 s, much longer than blocking times (1–20 ms).

The amplifier used to obtain 0.4-ms time resolution of the single channels was a modification of a design kindly supplied by Dr. Jim Donovan. The *trans* chamber of the bilayer was connected to the virtual ground input of an LF157 operational amplifier (National Semiconductor, Santa Clara, CA), and the 10-gigohm feedback resistor was capacitance-compensated by mounting it in a hole through a grounded copper plate; the effective feedback capacitance was ~30 fF. Data using this system were usually filtered at 1 kHz; it was necessary to form the bilayers on small holes (300 μm Diam) in order to reduce the membrane capacitance below 400 pF.

Except where noted otherwise, temperature was controlled at 25°C by seating the bilayer chambers in a temperature-controlled brass block.

Nomenclature

Throughout this paper, the proper but cumbersome names for these *bis*-quaternary ammonium compounds will be avoided. Instead, a code will be used to label the monovalent and divalent compounds as a function of methylene chain length. All compounds using this code are derived from n-alkanes, with trimethylammonium groups on one or both ends. The terminology "Qn" will refer to a monovalent compound with a trimethylammonium "head" and an alkyl "tail" of n-methylene groups; similarly, "bisQn" will refer to an n-chain alkane with a trimethylammonium group on each end. Thus, Q6 refers to hexyltrimethylammonium, whereas bisQ10 refers to n-decane-1, 10-N,N,N,N',N',N'-hexamethyl-*bis*-ammonium (commonly called decamethonium). Other compounds will be called by their proper names.

RESULTS

Fig. 1 illustrates the two types of blocking behavior to be documented here: the "quiet" block seen for the shorter chain compounds (eight carbons or less), and the "flickering" block, seen for the longer chain compounds (nine carbons or more). The effect of bisQ8 is to reduce the height of the channel; no discrete blocking events are seen, even at the 1-ms time resolution of this trace. The longer chain compound bisQ9, on the other hand, causes a channel, once opened, to flicker between two conductance states, which will be called "open" (high conductance) and "blocked" (low conductance). The blocked-state conductance is indistinguishable from the low or zero conductance of the closed state of the channel. In a flickering channel, as in a quiet channel, the

time-averaged conductance $\langle\gamma\rangle$ is also reduced, but now we can directly observe the kinetics of blocking that give rise to the reduced time average. All blocking effects to be reported were seen only when the blocker was added to the *trans* side of the membrane. No block could be observed with *cis* additions of blockers even at 10 times higher concentrations than used for the experiments reported here (data not shown).

Equilibrium Behavior of Block

The effectiveness of blocking by the bisQn compounds depends on the voltage across the membrane, and now, in contrast to the monovalent blockers, the voltage dependence of the block varies with the structure of the blocker. In
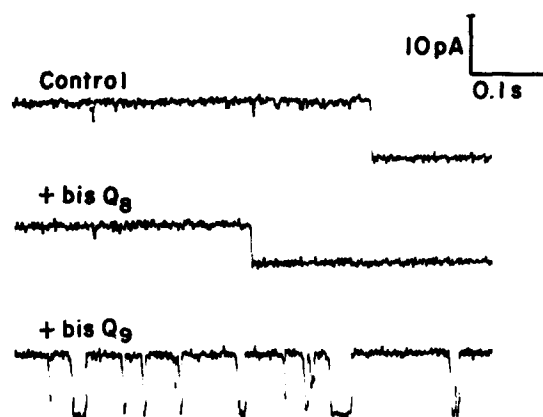
FIGURE 1.   Effects of bisQn blockers on SR K^+ channel. Records of representative channel fluctuations are shown for K^+ channels in the absence or presence of bisQn blockers. Top trace: a control channel in the absence of blocker is shown closing (downward transition). Middle trace: another closing event is shown, but in the presence of 0.7 mM bisQ8; note that the effect of the blocker is to reduce the open-channel current level by ~40% under these conditions. Lower trace: flickering noise induced in an open channel by 0.1 mM bisQ9; here the channel is open during the entire trace. Holding voltage was −50 mV in all traces. Records were filtered at 800 Hz.

Fig. 2 we see that in the presence of blocker, the channel's K^+ conductance depends upon voltage. Several points should be emphasized. First, in the absence of blocker, the channel conductance varies <2% over the range of voltage shown here (Coronado et al., 1980). Second, in all of these experiments, blocker has been added symmetrically to both sides of the membrane, even though the blocking effect is seen only with additions to the *trans* side; the reason for doing this is to avoid the possibility of inducing asymmetric surface potentials by addition of blockers, some of which are hydrophobic and could bind to the membrane (Donovan and Latorre, 1979). Third, it is important to remember that $\langle\gamma\rangle$ is a time-averaged conductance, an average of the fully open and fully blocked values, and as such represents an equilibrium between

open and blocked states of the channel (Woodhull, 1973; Coronado and Miller, 1982).

It is evident that the blocking effect becomes increasingly pronounced as voltage is made increasingly negative; at highly positive voltages, the time-averaged channel conductance approaches its unblocked value, which is ~150 pS under these conditions. This is the polarity of the blocking effect to be expected if the cationic blocker can enter the channel only from the *trans* side of the membrane, since the voltage convention defines the *trans* side as zero
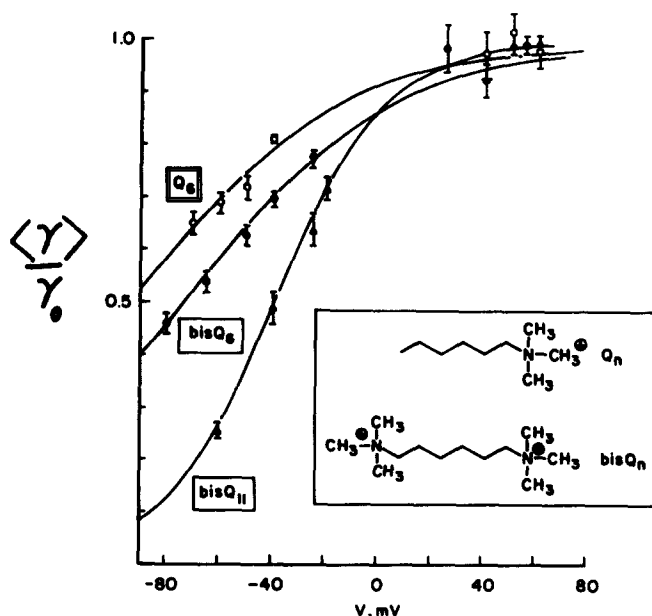

FIGURE 2. Voltage dependence of bisQn block. Time-averaged channel conductance, $\langle\gamma\rangle$, was calculated as a function of voltage, and was normalized to the unblocked conductance, γ_o (140–150 pS), for channels in the presence of either Q6 (4 mM), bisQ6 (4.5 mM), or bisQ11 (0.16 mM). Solid curves are drawn according to Eq. 2, with the following parameters: $Q6-K(0) = 53$ mM, $z\delta = 0.66$; $bisQ6-K(0) = 30$ mM, $z\delta = 0.66$; $bisQ11-K(0) = 1.2$ mM, $z\delta = 1.26$. These parameters were determined by least-squares regression lines of a linearized form of Eq. 2 (Coronado and Miller, 1979, 1980).

voltage. This sort of behavior has been seen in the channel previously (Coronado and Miller, 1980, 1982), and can be described in terms of a simple scheme in which blockers can exert their effects only on the open channel:

$$\text{CLOSED} \underset{\mu}{\overset{\lambda}{\rightleftarrows}} \text{OPEN} \underset{\beta}{\overset{\alpha}{\rightleftarrows}} \text{BLOCKED.} \tag{1}$$
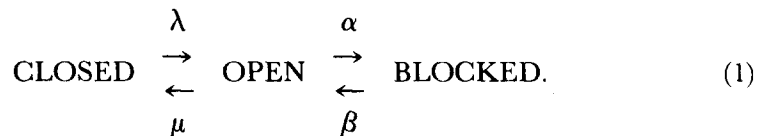
Here, α is the second-order rate constant of entry of the blocker into the channel, and all other rate constants are first order. In general, all rate constants are voltage dependent. The time-averaged channel conductance can be written as (Coronado and Miller, 1979, 1980, 1982; White and Miller, 1981):

$$\langle\gamma\rangle = \gamma_0[1 + [B]/K(0)\exp(Fz\delta V/RT)]^{-1}, \tag{2}$$

where $[B]$, z, and $K(0)$ are the concentration, valence, and zero-voltage dissociation constant of the blocker, respectively, γ_0 is the value of the unblocked channel conductance, and δ is the fraction of the total voltage drop
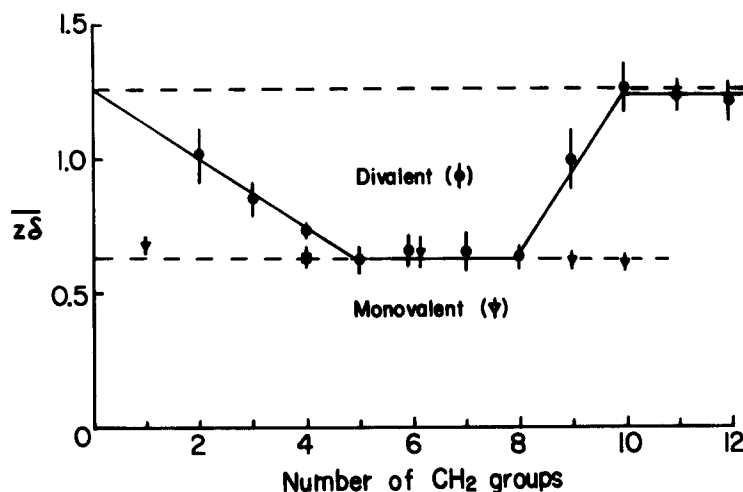

FIGURE 3. Effective valence of Qn and bisQn blockers. The effective valence of block, $z\delta$, was determined for monovalent Qn and divalent bisQn compounds as in Fig. 2. Blockers were used at concentrations giving ~50% block at −40 mV. Filled points represent quiet blockers, and open points represent flickering blockers; the single filled-square point represents the triple-bonded butyne derivative (compound I in the text). Errors correspond to the maximum range of parameters obtained by rocking the least-squares regression line through the points by eye.

V seen at the blocking site. The quantity $z\delta$, which measures the voltage dependence of the block, is usually called the "effective valence" of the blocking reaction.

In Fig. 2, the three blockers Q6, bisQ6, and bisQ11 all follow a blocking reaction according to Eq. 2. The divalent blocker bisQ6 displays an effective valence identical to that of the monovalent blocker Q6, and this has the value seen for virtually all monovalent blockers, i.e., 0.65 (Coronado and Miller, 1982). In contrast, the voltage-dependence of bisQ11 block is twice as steep as that for the two others, i.e., $z\delta = 1.2$–1.3.

The above assay can be conveniently used to measure the blocking parameters for the Qn and bisQn blockers. These are shown in Figs. 3 and 4 for

chain lengths of 2–12 carbon atoms. As expected, the monovalent Qn blockers all yield the same effective valence, 0.63 (average value), as though, regardless of size, they all interact with a site located 63% of the way down the voltage drop from the *trans* side of the membrane. In contrast, the divalent bisQn blockers display a complicated variation of effective valence with chain length. For short chain compounds, bisQ2–bisQ5, $z\delta$ decreases smoothly as chain length increases, from 1.1 for bisQ2 to 0.65 for bisQ5. Note that the rigid four-carbon analogue of bisQ4, with a C2–C3 triple bond (2-butyne,N,N,N,N',N',N'
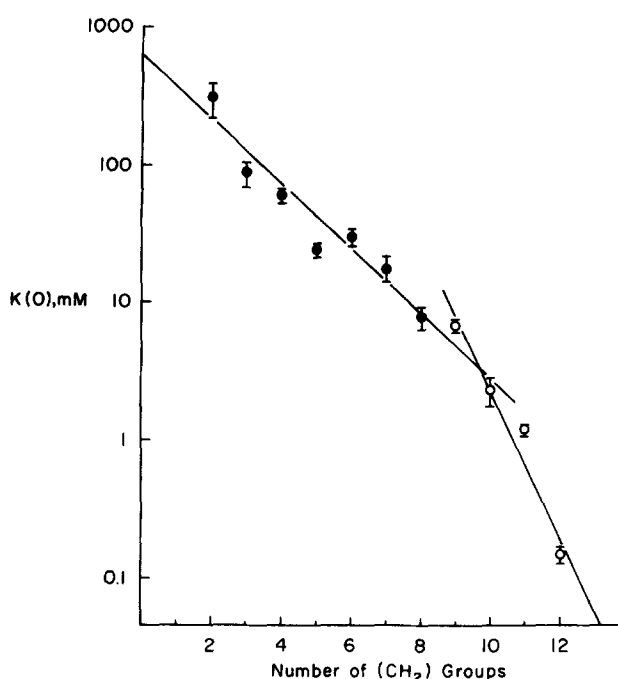

FIGURE 4.   Zero-voltage dissociation constants of bisQn blockers. The zero-voltage blocking constants, $K(0)$, were determined for bisQn blockers as in Fig. 2. Filled points represent quiet blockers, and open points represent flickering blockers. Solid lines, drawn by eye through each set of blockers, correspond to incremental free energies of binding of 310 cal/mol CH_2 for quiet blockers, and 720 cal/mol CH_2 for flickering blockers.

hexamethyl-1,4-*bis*-ammonium, compound I), shows a slightly lower effective valence (0.67) than does bisQ4 (0.74).

$$(CH_3)_3\overset{+}{N}CH_2\text{—}C\equiv C\text{—}CH_2\text{—}\overset{+}{N}(CH_3)_3$$

I
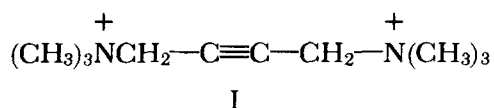
The effective valence remains constant at 0.63 (average value) as the chain length increases from five to eight carbons. These bisQn compounds are

indistinguishable in their voltage dependence from the monovalent Qn compounds. All blockers with chain lengths of eight carbons or less are "quiet" blockers (Fig. 1), i.e., they do not induce detectable flickering noise on the open channel.

For bisQn compounds of 9–12 carbons, the behavior abruptly changes. Here, the effective valence suddenly jumps to 1.2–1.3, twice the value for the monovalent Qn compounds and for the intermediate-length bisQ5–bisQ8 blockers. Furthermore, all of these long-chain bisQn compounds show clear flickering noise, as shown for bisQ9 in Fig. 1.

The equilibrium binding affinity, as determined from the zero-voltage dissociation constants, increases for the bisQn blockers as the chain length increases. For the quiet blockers of eight carbons or less, $K(0)$ decreases by 1.7-fold for each methylene group added to the chain, whereas for the long-chain flickering blockers, the variation is twice this (3.5-fold per methylene). The monovalent Qn blockers ($n = 1, 6, 9, 10$) show a variation in $K(0)$ similar to the shorter chain bisQn compounds, i.e., a 1.5–2-fold decrease in $K(0)$ per methylene (Coronado and Miller, 1982).

Blocking Kinetics for Long-Chain Compounds

BisQn compounds of chain lengths longer than eight carbons exert their effects on the channel by segmenting the duration of the open channel into short-lived dwell times in the fully conducting and nonconducting states. This "flickering" phenomenon was first seen by Neher and Steinbach (1978) with local anaesthetics acting on the acetylcholine-gated channel of muscle; each downward transition was interpreted to represent the diffusion into the channel of a single molecule of blocker, which would then remain on its blocking site for a randomly distributed length of time. The molecule would then leave the site and allow the channel to conduct cations once again, before the next blocker molecule enters.

The observation of flickering behavior for the long-chain bisQn compounds immediately allows the measurement of the kinetic constants of the blocking reaction on the single-channel level. The dwell-times in the blocked and unblocked states of a flickering channel were found to be exponentially distributed, as is shown in Fig. 5 for bisQ10. Thus, the kinetic constants of the blocking reaction in scheme 1 can be measured by the mean open and mean blocked times, $\bar{\tau}_o$ and $\bar{\tau}_b$ as:

$$\bar{\tau}_o = 1/(\mu + \alpha[B]) \tag{3a}$$

$$\bar{\tau}_b = 1/\beta. \tag{3b}$$

Here, $[B]$ represents the blocker concentration. For all experiments here, the closing rate, μ, is <3% of the blocking rate, $\alpha[B]$; therefore, the mean open time provides a good approximation to the rate constant for blocking:

$$\bar{\tau}_o \cong 1/\alpha[B]. \tag{3c}$$

In Fig. 6 we see that the essential requirements of scheme 1 hold: the mean open time varies inversely with blocker concentration, whereas the mean
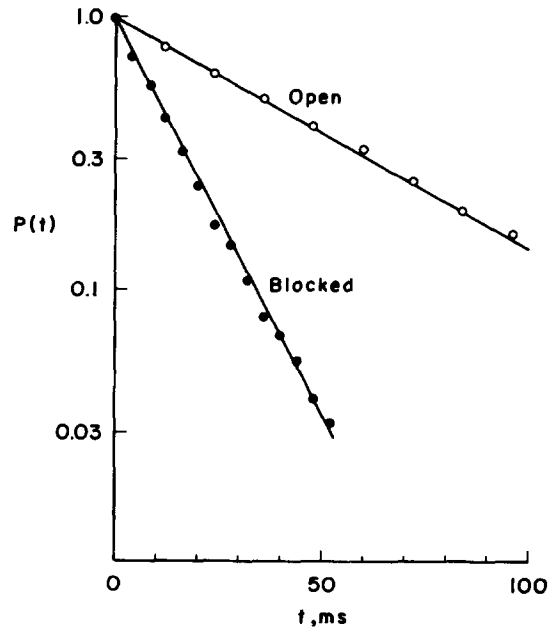
FIGURE 5. Time distribution of open and block times of a flickering channel. The statistical distributions of open or blocked dwell times were measured for a channel in the presence of 70 μM bisQ10, at −50 mV. The FM tape record was sampled at 500 points per second, and the blocking or unblocking transitions were found by a pattern-recognition program, using a MINC 11/23 computer. The probability that a given dwell time is longer than t, $P(t)$, was calculated from 35 s of data involving 1,045 transitions. In this experiment, the mean open time was 51 ms, and the mean block time was 16 ms.
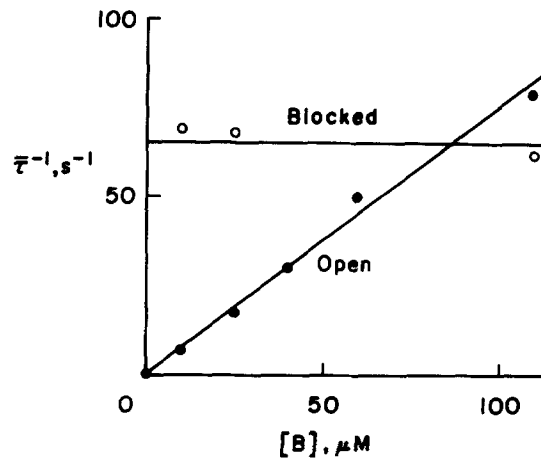

FIGURE 6. Variation of flickering kinetics with blocker concentration. Channel flickering was analyzed as in Fig. 5 at −50 mV. Reciprocals of open or blocked times, determined as in Fig. 5, are plotted as a function of the concentration of blocker, [B], which in this experiment was bisQ10. Each point represents a single analysis of 600–2,000 transitions.

blocked time remains constant. Similar experiments performed with bisQ9 and bisQ12 lead to the same conclusion: that the apparent rate constant for blocking increases linearly with blocker concentration, with no indication of saturation, even up to 85% block (data not shown). The second-order rate constant for bisQ10 blocking at 25°C is 6×10^5 $M^{-1}s^{-1}$, several orders of magnitude below a diffusion-controlled rate.
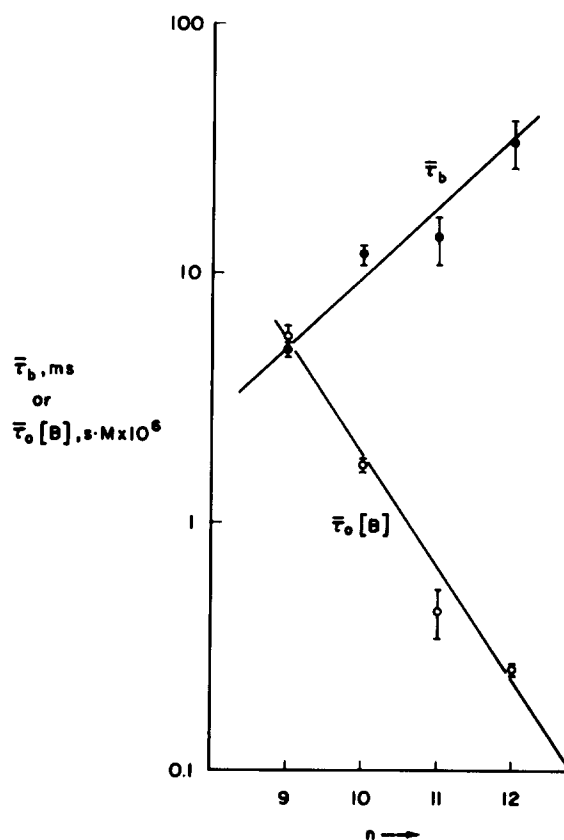

FIGURE 7.   Variation of blocking kinetics with chain length. Blocking kinetics were analyzed for bisQn compounds of varying chain length ($n = 9, 10, 11, 12$), at -50 mV, as in Fig. 5. The plot shows the mean block time, $\bar{\tau}_b$, and the product of the blocker concentration, $[B]$, and the mean open time, $\bar{\tau}_o$; this latter product is the reciprocal of the second-order rate constant of blocking. Each point represents mean $\pm$ SE of four separate determinations of mean dwell times, at blocker concentrations such that mean open times would be between 6 and 150 ms.

We know from Fig. 4 that the equilibrium binding of the bisQn blockers becomes stronger as chain length increases. In Fig. 7 we see that both the blocked and the open times vary with chain length in such a way as to enhance the equilibrium binding. Whereas the mean dwell time of the blocker in the channel increases with chain length about 1.4-fold per methylene group,

the major effect of chain length is to decrease the mean open time 2.8-fold per methylene. This is a somewhat counter-intuitive result showing that the "on" rate constant of blocking increases as the blockers are made larger.

We can examine the voltage dependence of the open and blocked times. Fig. 8 shows traces for a fixed concentration of bisQ10 at three different voltages; the enhancement of block at negative voltage is apparent. An analysis of traces like these presents us with a remarkable result: that only the mean open time is sensitive to voltage (Fig. 9). Over a range of voltage leading to a variation of the blocking rate constant by a factor of >2,000, no variation at all (i.e., <20%) can be discerned in the mean block time (the inverse unblocking rate constant).

The blocking kinetics are also sensitive to temperature, as shown in Fig. 10 for bisQ10. Both open and blocked times decrease with increasing temperature, but by far the stronger variation is in the open time. The best lines of the Arrhenius plot give activation enthalpies of 20 and 11 kcal/mol for the
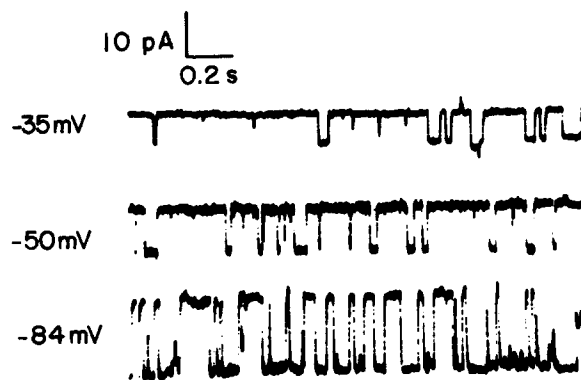

FIGURE 8. Voltage dependence of channel flickering. Representative traces are shown at voltages indicated for a channel in the presence of 2 μM bisQ12.

blocking and unblocking rate constants, respectively (Q_{10} of 3.2 and 1.8). The corresponding Van't Hoff plot constructed from these data for the equilibrium binding shows that the blocker binding reaction is entropy driven; the standard enthalpy of binding is highly unfavorable ($\Delta H^\circ = +9$ kcal/mol), whereas the standard binding entropy is favorable ($\Delta S^\circ = +20$ cal/mol-K).

Previous work on this channel (Coronado and Miller, 1982) showed that the blocking equilibrium for organic cationic blockers is competitive with K^+. According to a single-ion blocking scheme, a more stringent prediction is made: that all the competition with K^+ must reside in the inward rate constant, i.e., the mean open time. In other words, if only one ion can occupy the channel at a time, then the probability of a blocker entering will be decreased by raising K^+ concentration, but this should have no effect on the probability of the blocker leaving the channel. Thus, neglecting the closing rate as before, we can write that the mean open time should increase linearly with $[K^+]$:

$$\bar{\tau}_0[B] = (1 + [K^+]/K_K)/\alpha^*, \tag{4}$$

where K_K is the dissociation constant for K^+, and α^* is the rate constant for blocking in the limit of zero $[K^+]$.

The prediction above holds (Fig. 11). The kinetics of bisQ10 block were measured in the range of 75–500 mM $[K^+]$. The mean open time was found to increase linearly with $[K^+]$, whereas the block time remained constant. Furthermore, the value of K_K calculated from the blocking kinetics, 60 mM, is in good agreement with that measured directly by the variation of channel conductance with $[K^+]$, 70 mM (Coronado et al., 1980; C. Miller and M. Barroll, unpublished data). These results are consistent with the notion of a
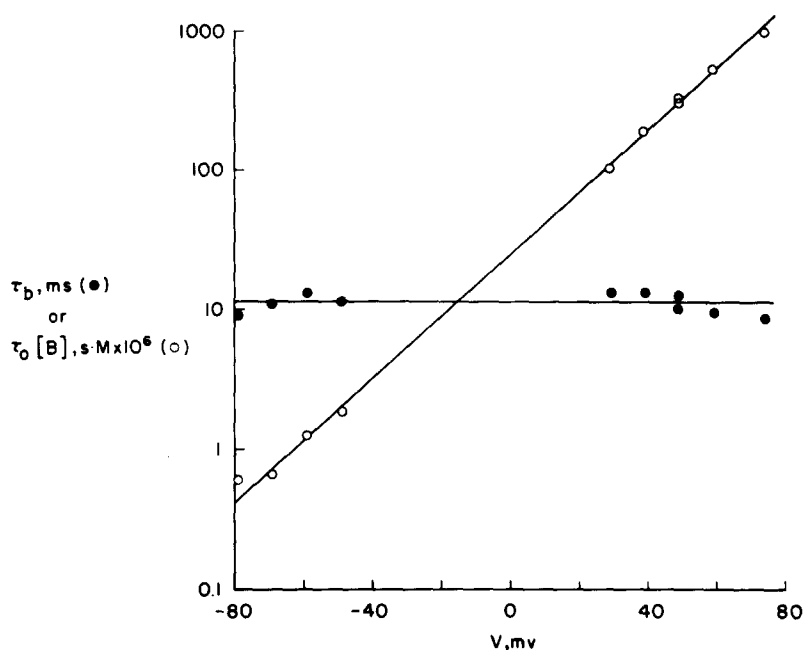

FIGURE 9. Voltage dependence of bisQ10 kinetics. Blocking kinetic parameters as in Fig. 7 were determined as a function of voltage in the presence of bisQ10. For negative voltages, 10 μM bisQ10 was used, whereas observation of block at positive voltages required much higher concentrations, 2.5 mM. Least-squares lines are shown, giving a 10-fold increase in $\bar{\tau}_o[B]$ per 46 mV; slope of least-squares line for $\bar{\tau}_b$ was not significantly different from zero.

channel operating by the single-ion rule (Coronado et al., 1980): the channel can be occupied either by a K ion or by a blocker, but not by both simultaneously.

DISCUSSION

There is now ample evidence to conclude that organic cations exert their blocking effects on the SR K^+ channel by diffusing into the normal K^+ conduction pathway of the channel and thus preventing K^+ permeation by virtue of the channel's "single-ion rule:" that at most one ion at a time may

occupy the channel. That blocking scheme 1 holds has been shown by the simple nature of the block by monovalent organic cations (Coronado and Miller, 1982), including the demonstration that the channel must open before it can be blocked. That the blocked state of scheme 1 literally represents an open channel occluded by the blocking ion (as opposed to an open channel with a blocker occupying a site other than inside the K^+ diffusion pathway)
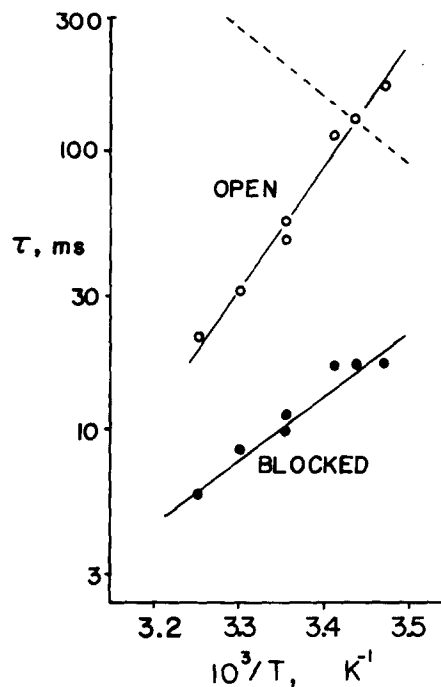

FIGURE 10. Temperature dependence of blocking kinetics. Channels were inserted into bilayers with temperature control set to 35°C, in the presence of 10 μM bisQ10. Mean open and block times were determined at −50 mV as in Fig. 7. Ice was then added to the circulating water bath, and the bilayer temperature was allowed to drop during the subsequent 10–20 min, while records were collected and the temperature was monitored. Data shown here represent three separate runs, each on a different bilayer. Solid lines represent activation enthalpies of 11 and 20 kcal/mol for the mean blocked and open times, respectively. Dashed line shows the ratio of blocked time (in milliseconds) to open time (in seconds), and corresponds to a binding equilibrium with ΔH° = +9 kcal/mol.

is suggested strongly by two separate observations: (a) that the blocking equilibrium and kinetics are competitive with K^+, and (b) that some of the smaller monovalent blockers (e.g., methylammonium, guanidinium, and others) actually permeate the channel in a manner similar to the ion from which they are structurally derived, ammonium, which itself permeates very similarly to K^+ (Coronado and Miller, 1982; Coronado et al., 1980).

In addition, it is pertinent to note that the blocking behavior supports the single-ion rule for this channel proposed earlier on the basis of conduction by alkali metal cations (Coronado et al., 1980). If multiple occupancy of the channel could occur (either two blockers or a K^+ and a blocker), then we would expect that the effective valence of the block should depend on the concentration of both blocker and $[K^+]$, and that the blocking effectiveness should depend not solely on applied voltage, but also on the magnitude and direction of the K^+ current; all of these indications of multiple occupancy have been observed in the K^+ channel of squid axon (Armstrong, 1975; Adelman and French, 1978; Hille and Schwarz, 1978), and none is observed here. In this regard, it is striking that the blocking rate constant is purely
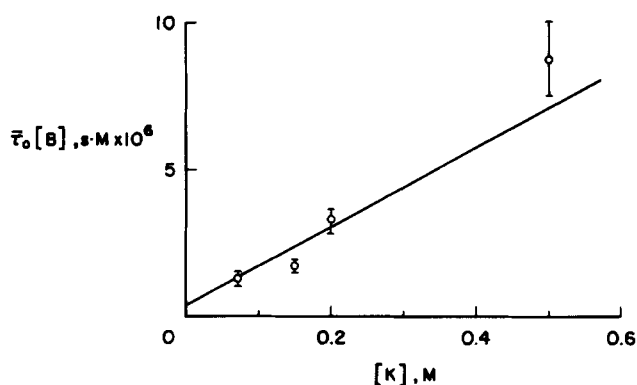

FIGURE 11.   Competition of blocking kinetics with K^+. Blocking kinetics were determined as a function of K^+ concentration, using bisQ10 as blocker, under conditions of Fig. 7. Each point represents the mean ± SE of four to six separate determinations, each at a different blocker concentration. For the lowest $[K^+]$, bisQ10 concentrations were in the range 10–160 μM, whereas for the highest $[K^+]$, 50–1,000 μM concentrations were required. Line drawn through data according to a simple competitive scheme, Eq. 4, corresponds to $\alpha^* = 1.5 \times 10^6$ $M^{-1}s^{-1}$, and $K_K = 60$ mM. The average block time in these experiments was 12.7 ms, and did not vary significantly with $[K^+]$.

voltage dependent and that the unblocking rate is utterly independent of voltage (Fig. 9), regardless of whether the blockers are moving upstream or downstream in the K^+ current; in other words, there is not even a hint of "knock-on" or "knock-off" behavior (Armstrong, 1975) over a very wide range of K^+ currents. It is therefore reasonable to suggest that the blockers are not, in fact, swimming in a stream of K^+ at all; rather, a channel occupied by a blocker refuses entry to a K ion, and vice versa.

Finally, the flickering kinetics for the long-chain bisQn compounds are readily explained by the notion that each flickering transition represents the movement of a single blocking molecule into or out of the channel, and that a given dwell time in the blocked state represents the time that a single blocker

molecule is actually residing inside the channel. These ideas are supported by the well-behaved nature of the flickering kinetics: (*a*) both open and blocked dwell times are exponentially distributed; (*b*) the mean open time varies inversely with blocker concentration, whereas the mean block time is independent of concentration; (*c*) the voltage dependence of the probability of being in the blocked state follows Eq. 2.

Variation of Effective Valence of bisQn Blockers

Having reviewed the arguments to support a very simple picture of blocking by organic cations in this channel, I will now rely on this picture to interpret the results on the variation of blocking behavior with blocker structure. The most obviously complicated behavior, and the central point to be addressed here, is the variation of effective valence of the bisQn compounds with chain length (Fig. 3). This result is all the more striking because of past experience with this channel (Coronado and Miller, 1982), which showed that the effective valence of monovalent blockers is virtually invariant with blocker structure: that, although the affinities of these blockers may vary over orders of magnitude, the effective valence is always within a few percent of 0.65. This result led to the postulate that within this channel there exists a site that can bind a quaternary ammonium or guanidinium group, and that this site is located ~65% of the way down the electric potential drop, from the *trans* side of the channel.

The fact that the divalent blockers behave so remarkably in contrast with the monovalent compounds leads to a simple suggestion: that perhaps this is because of the second charge on these compounds. Let us suppose that for a bisQn blocker, one of the trimethylammonium groups always binds at the 65% site. But in general, this will leave the other positive charge at a different place in the channel. What, then, do we expect the observed effective valence to be? Consider a compound containing two chemical groups of charges z_1 and z_2. Suppose that the compound blocks in a conformation with the two groups separated in space inside the channel, such that each group experiences a different fraction of the electric potential drop, δ_1 and δ_2, respectively. Then, each charge will make an independent contribution to the voltage dependence of the binding energy in proportion to its δ, so that now the observed effective valence, $z\delta$, will be:

$$\overline{z\delta} = z_1\delta_1 + z_2\delta_2. \tag{5}$$

For compounds we are studying here, $z_1 = z_2 = +1$, and so

$$\overline{z\delta} = \delta_1 + \delta_2. \tag{6}$$

If we assume that $\delta_1 = 0.63$ for *all* of these compounds, then a measurement of the effective valence leads to a calculation of the position, δ_2, of the second charge in the electric field within the channel.

What would we expect for the variation of effective valence with blocker structure from this treatment? We would expect that for charges close together,

the effective valence should be high, approaching 1.3 as the charges super-impose upon each other (such as a hypothetical doubly charged tetramethyl-ammonium ion); as the separation of the charges increases, the effective valence should drop as the second charge is left behind, less deeply inside the channel. This decrease in effective valence should occur until the second charge is left completely outside the applied electric field ($\delta_2 = 0$), though perhaps not outside the channel protein per se. At this point, the effective valence should have a value of 0.63, and the divalent blocker would appear to the channel as a monovalent cation. Any further separation of the two charges would leave the effective valence unchanged, since the second charge would already be out of the electric field.

The above description of the theory's prediction is also a precise description of the variation of effective valence for bisQn blockers in the range of two to eight methylene carbon atoms in the chain. Before exploring the consequences of accepting such an explanation, we should consider two possible objections. The first is that perhaps this behavior is merely as expression of the increasing size of these molecules: that the larger ones cannot squeeze as far into the channel as the smaller ones. This possibility is made unlikely by the observation that the *monovalent* Qn blockers (from 1–10 carbons) show a constant effective valence of 0.64 (Fig. 3), as do virtually all monovalent blockers (Coronado and Miller, 1982). A second objection to the applicability of the model above is that the methylene chains of these compounds are flexible, and that the charges would not be expected to be at fixed separations in space. There are two answers to this objection. First, in interpreting the effective valence according to the above treatment, we speak of the average position of the second charge, and do not really require that the chain be rigid. Second, it is not so unreasonable to propose that inside a channel protein, the motion of the flexible chains of the bisQn compounds may be much more severely restricted than in free solution, so that in effect they may be more rigid than they are usually imagined to be.

Let us tentatively accept this explanation, then, for the behavior of the effective valence. We can then consider the second trimethylammonium group to be a "reporter group" for the voltage inside the channel protein, and use Eq. 6 to calculate δ_2 as a function of distance between the charges. This latter measurement was made by using Corey-Pauling-Koltun models of the compounds in their most extended conformations. We see (Fig. 12) that ~65% of the voltage drop falls over a remarkably small distance, 7–8 Å. This estimate is an absolute upper limit on this length, since the compounds probably are shorter, on average, then their most extended conformations; the one truly rigid compound (the triple-bond bisQ4 analogue) supports this idea by showing a significantly lower effective valence than bisQ4. Thus, considering this rigid compound as a more reliable calibration on the charge-charge separation, we can say that 65% of the voltage drop occurs over 6–7 Å.

Since blockers act from the *trans* side, we do not know anything about the 35% of the voltage drop facing the *cis* side of the channel; but if we propose for the sake of simplicity that the electric field profile there is similar to that

in the *trans*-facing 65%, we would conclude that the region of the channel's conduction pathway over which the applied voltage drops is only ~10 Å long. If this conclusion is correct, then it is required that the channel contain a short constriction of, say, 10 Å in series with a "mouth" on one or both sides of the constriction. The mouth would provide a low-resistance pathway for ions to gain access to the constriction of the channel, in which the bulk of the ionic selectivity would take place. This mouth would have to be truly enormous, since it would need to have dimensions substantially larger than those of the constriction region, which itself must be at least 7 Å in diameter in order to accommodate some of the larger monovalent blockers (Coronado and Miller, 1982). A schematic cartoon of such a structure is shown in Fig. 13.
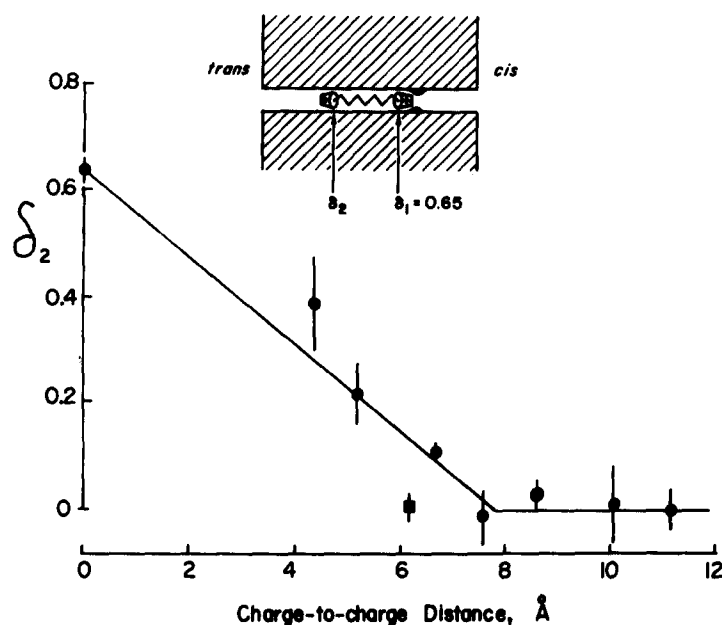

FIGURE 12. Map of potential drop inside SR K^+ channel. Effective valence data of Fig. 3 were used to calculate the position of the second charge on the bisQn blockers, δ_2, as a function of chain length, according to Eq. 6, using δ_1 = 0.63. Charge-to-charge distance was measured using CPK models of the blockers, in the most extended chain conformation. Square point represents the rigid triple-bonded butyne derivative, compound I in the text.

Long-Chain Blockers

The above discussion has been directed at the "quiet" blockers of chain lengths shorter than nine carbon atoms. For chains of nine carbons and longer, a sudden discontinuity in the blocking behavior occurs. There are three separate manifestations of this discontinuity: (*a*) the increase in effective valence to a value of 1.2–1.3, i.e., just twice that of the monovalent blockers and of the intermediate chain length bisQn blockers; (*b*) the change in the

dependence of binding affinity on chain length from $\sim$300 cal/mol CH_2 for the shorter chains to a value of 700 cal/mol CH_2; and (*c*) the sudden appearance of flickering block for the longer chains. This last manifestation is not simply an artifact of the inability of our amplifier to detect blocking events shorter than 0.5 ms; extrapolation of the block time in Fig. 7 to an eight-carbon blocker gives an expected mean block time of 3 ms, which we would be able to detect easily. But we never see any flickering events for bisQ8, and so we conclude that the mean block time for this compound must be substantially shorter than 0.1 ms. Thus, the quiet blockers follow kinetics orders of magnitude faster than the flickering blockers.

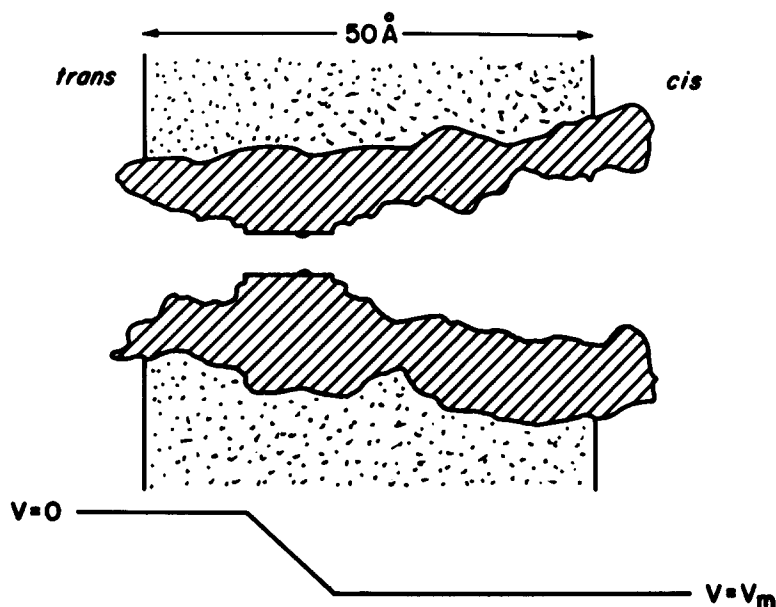These three examples of a discontinuous change at nine carbons strongly

FIGURE 13. Cartoon of SR K^+ channel. A plausible picture of SR K^+ channel is presented to emphasize the major conclusion of the present results: that the applied voltage appears to fall over a small distance. In this picture, a longitudinal section of the channel is depicted, embedded in a 50-Å-thick membrane.

indicate that the long-chain blockers bind in a conformation different from that of the shorter chain blockers. Taking into account the increased flexibility and hydrophobicity of the longer chains, the constancy of the 65% site for all monovalent blockers, and, most of all, the value of the effective valence for the flickering blockers—1.2–1.3, just twice that of the monovalent and intermediate-length bisQn blockers—I propose that these long-chain bisQn blockers bind in a bent-over conformation, with *both* charges located close to the 65% site inside the channel, as has been previously suggested for bisQ10 (Coronado and Miller, 1980). Why this change should occur at nine carbons and why it should be so sudden is not clear, but there is no question that the

discontinuity is a fact. It is certainly unappealing to have to place both charges deeply inside the channel structure, but this seems to be the least offensive explanation of the high effective valence, given the generally simple nature of the interactions of organic cations with this channel. This picture of the long-chain blockers would require the existence of several negatively charged groups at the 65% site; otherwise, the positioning of the two positively charged head groups in such close proximity would be forbidden energetically. The postulate of multiple negative charges inside a channel is not a new one, but has been proposed to explain various aspects of the conduction and selectivity properties of the Na^+ channel (Hille, 1975).

Binding Affinity

For the bisQn blockers, as for the Qn blockers, the blocking affinity increases as the alkyl chain is made longer (Fig. 4). For the monovalent and quiet divalent blockers, this represents an enhanced chemical affinity of the blocker for the channel of ~300 cal/mol per added methylene group; for the flickering bisQn blockers, the value is approximately twice this, 700 cal/mol per added methylene. Although there is little that can be said specifically from this result, these values are in the range of a classic hydrophobic effect (Tanford, 1980), and may suggest that inside the channel there is a "hydrophobic patch" that stabilizes these blockers. This kind of conclusion, which is quite unexpected for a protein whose job it is to provide a hydrophilic diffusion pathway for an ion, has also been reached for the Na^+ and K^+ channels of axons as well (Armstrong, 1975; Swenson, 1981; French and Shoukimas, 1981; Hermann and Gorman, 1981; Rojas and Rudy, 1976). That it is the hydrophobicity, and not merely the length of the chain which stabilizes the binding, is corroborated by the blocking behavior of compound II, a hydrophilic analogue of bisQ8. The affinity of this compound

$$\overset{+}{(CH_3)_3N}\text{—}CH_2\text{—}CH_2\text{—}O\text{—}CH_2\text{—}CH_2\text{—}O\text{—}CH_2\text{—}CH_2\text{—}\overset{+}{N(CH_3)_3}$$

II
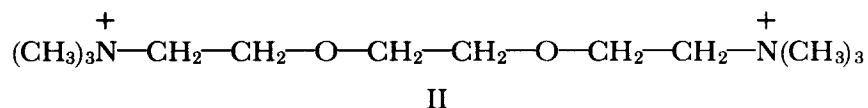
is about 30-fold lower than that of bisQ8 (data not shown), although the lengths and flexibilities of the two compounds are identical. A hydrophobic interaction inside the channel is also made plausible by the large favorable entropy and large unfavorable enthalpy of binding seen for bisQ10; according to such an explanation, a major driving force for binding of the blocker would be the entropy increase resulting from release of "ice-like" water surrounding the bisQ10 chain in free solution, and, possibly, surrounding the hypothetical hydrophobic patch inside the channel.

Kinetics of the Flickering Blockers

The main purpose of presenting the blocking kinetics here is to show that the flickering of the long-chain blockers is a well-behaved process easily understood in terms of the basic model for blocking by occlusion of the channel. There are several noteworthy features of the kinetics, however, which warrant special

attention. A general rule emerging from this work is that whenever a parameter is varied (chain length, voltage, temperature), the main effect is upon the mean open time, i.e., on the blocking rate constant; the mean closed time, a measure of the unblocking rate, is relatively insensitive to variation in conditions. Of particular note is the fact that *all* of the voltage dependence of the binding of the blockers is found in the blocking rate, and none in the unblocking rate (Fig. 9). This result has palpable meaning: that there is virtually no electric potential drop between the binding site and the transition state of the rate-limiting step for blocking-unblocking kinetics. In other words, the charge configurations of the blocker in the binding site and in the transition state are nearly identical.

A speculation to explain this would be that the rate-limiting step for blocking is the bending over of the second charge into the proposed horseshoe conformation of the long-chain blockers. If the energetically unfavorable step were the movement of the second charge into a side-by-side conformation with the first, then most of the voltage dependence would be manifested in the blocking rate. This speculation is also consistent with the fact that as the chains become longer, the blocking rate speeds up: a longer chain would have more flexibility and could bend over with less strain. Obviously, more work will be required to test this idea, as well as others arising out of this study, and to assess the relative contributions of steric and hydrophobic effects in the blocking reaction; but the results here have at least made clear the kinds of variations in blocker structure and chemistry that need to be made.

The major structural conclusion of this study is that the applied voltage falls inside the SR K^+ channel over a remarkably small distance, ~ 10 Å. This conclusion leads to a picture of the channel as containing a very short "ion selectivity constriction" in series with wide "mouths" opening out to the aqueous solutions. This kind of picture is not new in the study of ion channels. Similar proposals have been made for the Na^+ and K^+ channels of axons, as well as for the acetylcholine receptor channel (Hille, 1975; Armstrong, 1975; Horn and Stevens, 1980). The one novel aspect of this analysis is the possibility of using a thermodynamic measurement—that of effective valence—to obtain a quantitative estimate for the physical length of the constriction region of an ion-conducting channel protein. This estimate should be considered only tentatively valid at this point; it will require further work involving blockers of known, locked-in conformations to test the ideas proposed here. But if future work does validate this idea for the SR K^+ channel, it may be of interest to attempt to study the effects of *bis*-cationic blockers as a general method for mapping the electric field within the diffusion pathway of other ionic channels.

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