BLOCK OF SODIUM CHANNELS BY INTERNAL MONO- AND DIVALENT GUANIDINIUM ANALOGUES

Modulation by Sodium Ion Concentration

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ABSTRACT We have investigated the block of squid axon sodium channels by mono- and divalent guanidinium analogues. The action of these compounds on steady state sodium currents was independent of the presence or absence of the normal inactivation process. Block by both mono- and divalent analogues was voltage-dependent, but was a steeper function of potential for divalent molecules. The voltage-dependence could not, in general, be reproduced by a simple model based on Boltzmann's equation. Inhibition of steady state currents by guanidinium ions with 50 mM internal sodium was reasonably well described by a 1:1 drug/channel binding function. Increasing the internal sodium ion concentration increased both the degree and voltage-dependence of current inhibition. This is in sharp contrast to the decrease in inactivation caused by internal sodium. Changes in the external sodium concentration had very little effect on drug block. These results are consistent with a model of the sodium channel as a multi-ion pore. Only a small increase in block can be produced by increased internal sodium in a three-barrier two-site model, but a four-barrier three-site model can reproduce these experimental findings. The implications of these results for physical models of inactivation are discussed.

INTRODUCTION

The inactivation process of sodium channels has been investigated by both electrical and biochemical techniques. The biochemical data have indicated the involvement of the amino acid arginine on the internal side of the membrane in the inactivation of sodium channels. These data include the removal of inactivation by purified alkaline protease b from Pronase (Rojas and Rudy, 1976) and by trypsin (Carbone, 1982). Eaton et al. (1978) showed that inactivation could also be removed by arginine-specific reagents.

Since arginine contains a guanidinium group, several laboratories have investigated the action of internal guanidinium analogues on sodium channel function. Morello et al. (1980) showed that internal application of nonyl guanidine induced a time- and voltage-dependent block of sodium channel currents. External application of this and all other analogues was without effect. Kirsch et al. (1980) used several *n*-alkylguanidines and found a frequency and time-dependent block of peak sodium current produced by long chain compounds. Reduction of steady state currents was weakly voltage-dependent and not a function of stimulation frequency. Lo and Shrager (1981 *a*, *b*) extensively analyzed the effects of *n*-propyl guanidinium on sodium channel currents. They found a decrease in peak sodium channel current and an increase in the rate of current decay. These effects were more pronounced when the normal sodium concentration gradient was reversed.

Since these guanidinium compounds have such specific effects on sodium channel currents, we intended to use divalent analogues to probe the spatial dependence of the electric potential through the pore as Miller (1982) has done for a potassium channel isolated from sarcoplasmic reticulum blocked by divalent quaternary compounds. Such an analysis requires that the voltage dependence of the interaction of the probe with the pore be entirely due to its interaction with the electric field. This condition is not met in multi-ion pores where the voltage dependence of a blocking ion includes contributions from the interaction of both the blocker and the permeant ions with the electric field (Hille and Schwarz, 1978; Begenisich and Smith, 1984).

We find that increasing the sodium ion concentration increases the block produced by both mono- and divalent quanidinium analogues. The actions of these compounds are rather insensitive to changes in external sodium con-

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centration. We show here that these results are consistent with the behavior of multi-ion pores. Earlier work (Begenisich and Cahalan, 1980*a*, *b*) showed that Na channels have other properties in common with multi-ion pores. Consequently, the voltage dependence of the block produced by these molecules cannot (easily) be used to determine the spatial dependence of the electric field.

We also find that the block produced by the divalent analogues is a steeper function of voltage than is the block by monovalent ones. This suggests that both charges on these compounds are within the membrane electric field but this conclusion must be tempered by the complications of the multi-ion nature of the pore.

A preliminary account of the results was presented at the annual meeting of the Biophysical Society (Smith et al., 1983).

METHODS

Biological Preparation

The data in this report were obtained with giant axons from the squid *Loligo pealei* available at the Marine Biological Laboratory, Woods Hole, Massachusetts (where some of the experiments were done), and from squid delivered to the University of Rochester.

Voltage-clamp and Internal Perfusion

The axons used in this study were internally perfused and voltage-clamped using techniques that have previously been described in detail (Begenisich and Lynch, 1974; Busath and Begenisich, 1982). All voltages have been corrected for the junction potential between the internal 0.56 M KCl electrode and the internal solutions. External potentials were measured with an agar-filled, saturated KCl electrode. A temperature of 10°C was used in these experiments.

Membrane currents were measured with a 12-bit analog/digital converter controlled by a microcomputer of our own design. Current was sampled at 20 μs intervals. In most of the experiments reported here the voltage-clamp pulses were generated by the microcomputer at 2 s intervals. In a few experiments a separate digital pulse generator was used. A typical voltage waveform consisted of a 50 ms, 30 mV hyperpolarizing pulse from a holding potential near -70 mV, then a 300 μs return to the holding potential. This was followed by a depolarizing test pulse. Potassium channel currents were eliminated by using internal Cs or Na ions. Sodium channel currents were separated from the remaining currents by using identical pulse patterns with external solutions containing 300 nM tetrodotoxin (TTX).

Solutions

The standard artificial sea water (ASW) solution contained (in millimoles per liter) 440 NaCl, 10 $CaCl_2$, 50 $MgCl_2$, 10 HEPES buffer, pH near 7.4. Tetramethylammonium or Tris was used to replace Na in 25% or zero Na ASW solutions. Two internal solutions were used. 50 Na SIS consisted of 50 mM Na and 150 mM Cs. In 200 Na SIS 150 mM Na replaced Cs for a total Na content of 200 mM. Both of these solutions contained 50 mM F, 100 mM glutamate, ~700 mM glycine (for osmotic balance), and 25 mM phosphate buffer (pH near 7.5).

The actions of several mono- and divalent guanidinium analogues were studied. The most commonly used monovalent compound was *n*-propyl guanidinium, abbreviated as C3 (Vega Biochemicals, Tucson, AZ). The monovalent compounds C4, C6, and C9 were kindly provided by Dr. Juta Reed of the University of Toronto. The most frequently used divalent analogue was 1,2 bis-guanidino-*n*-ethane (bisC2). This and the compounds bisC3, bisC4, bisC6, bisC8 were all gifts from Dr. Christopher Miller of Brandeis University.

These compounds all reduced sodium channel currents when added to the internal perfusion solutions; external application had no effect. We did not find any qualitative differences among the various mono- and divalent analogues. The potency of these molecules increased with increasing hydrocarbon chain length, as was described by Kirsch et al. (1980).

Data Analysis

In a simple model, in which a sodium channel is conducting unless it is blocked by internal application of a drug, the fraction of channels not blocked is given by

$$F = \frac{1}{1 + \frac{[D]}{K_d}}, \tag{1}$$

where K_d is the dissociation constant and [D] the drug concentration. If the blocking compound is charged (with valence z) and binds to a site within the membrane electric field, then the dissociation constant will be a function of membrane potential, V_m

$$K_d = K_0 \exp(-\theta z V_m F/RT), \tag{2}$$

where K_0 is the dissociation constant at zero applied voltage, θ is the fraction of the membrane electric field at the binding site (measured from the internal membrane surface). R, T, and F have their usual thermodynamic meanings.

These two equations can be combined to yield an expression for the fraction of channels not blocked by the drug as a function of drug concentration and membrane voltage

$$F = \frac{1}{1 + \frac{[D]}{K_0}\exp(\theta z V_m F/RT)}. \tag{3}$$

This equation is not expected to be valid if there are several binding sites that can be simultaneously occupied by drug molecules and permeant ions, which appears to be the case for sodium channels. This more complicated situation requires models of the type described below. However, some useful information can be gained from applying Eq. 3 to the data, as will be seen in Results.

Fits of Eqs. 1 and 3 to the data used the nonlinear least squares method of Marquardt described in Bevington (1969). Fits of Eq. 3 to the data yield estimates of K_0 and θ; however, θ is only well-defined for a point charge. Since the guanidinium analogues, especially the divalent molecules, do not fit this description, the concept of "effective valence" (Hille and Schwarz, 1978; Hille, 1984) will be used here. The effective valence, z', is defined as $z\theta$.

Multi-ion Permeation/Block Models

We have examined the ability of two different sodium pore models to reproduce the experimental data in this study. The first is the three-barrier two-site model described in detail in Begenisich and Cahalan (1980*a*, *b*). The other is a four-barrier three-site model similar to that used in Begenisich and Smith (1984) for potassium channels. In these models the ion permeation pathway consists of a series of energy barriers and wells. The permeating ions "jump" (according to a Poisson process) from well to well with a rate that decreases with the height of the intervening energy barrier. An ion can be a blocker if one of its energy barriers is such that after entering the pore, the ion can leave only by returning to the solution from which it came. No ions can traverse the pore while it is occupied by the impermeant blocking ion.

The calculations of the current through these model pores were performed using techniques described in Begenisich and Cahalan (1980*a*). The computations for the three-barrier model were done with our laboratory microcomputer in compiled BASIC. Calculations for the four-barrier model were done using a Nova 3 minicomputer (Data General Corp., Westboro, MA) in FORTRAN or on an IBM PC/XT computer (with an Intel 8087 math coprocessor) in PASCAL.

RESULTS

Block Is Independent of Inactivation

The left panel of the inset of Fig. 1 shows families of Na channel currents with normal inactivation in response to a series of voltage clamp steps in control, in the presence of 50 μM bisC8, and after removal of the drug. Also shown (right panel) are these currents in the same axon after most of the inactivation process was removed with the proteolytic enzyme Pronase. Only the control and drug containing records were obtained. The drug is seen to block sodium channel currents with and without the endogenous inactivation process. The time dependence of the block is apparent after removal of inactivation.

A quantitative measure of the amount of current reduction can be obtained by computing the ratio of current at the end of a pulse obtained in the presence of the drug to the average of the control and recovery values. Using the current values at the end of the pulses assures that enough time has elapsed for the block to reach a steady level. The figure shows this ratio as a function of membrane potential for voltages more positive than zero. Only data from positive potentials are presented for several reasons. The most important one is to provide for the rapid opening of all available channels. Otherwise, the voltage dependence of channel opening might be coupled with drug block and so distort its voltage dependence. Also, at these positive potentials the endogenous inactivation process is rapid enough so that it is complete (or as complete as it gets) by the end of the pulses (usually 4 ms) used here.

The open circles in this figure represent the fraction of channels not blocked by 50 μM bisC8 with inactivation intact. The data represented by the filled circles were obtained from the same axon after inactivation was removed. In this case the ordinate is the ratio of currents obtained with the drug present to those obtained before its application. This is one of only a few experiments in which recovery data were not obtained and was due to experimental difficulties associated with axons with the inactivation process removed. Unless otherwise noted, all current ratio values in the remainder of this report include recovery data.

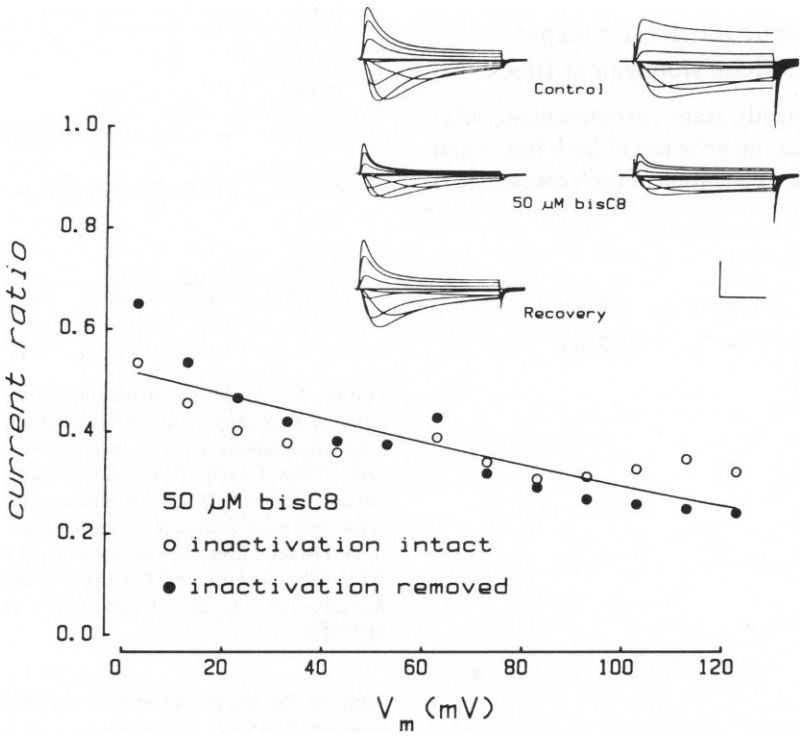

FIGURE 1 Action of bisC8 with and without inactivation. *Inset* Families of currents in response to a series of depolarizations of 0, 20, 40, 60, . . . , 180 mV from a holding potential of −68 mV. Left hand panel shows currents before, during, and after application of 50 μM bisC8 to an axon with inactivation intact. The right hand panel shows currents from the same axon after inactivation was removed by enzyme treatment. Calibration: 2 mA/cm^2, 1 ms. *Graph* Fraction of steady state sodium channel current remaining in the presence of bisC8 with intact inactivation (O) and with inactivation removed (●) as a function of membrane potential. The solid line was obtained by fitting Eq. 3 to the data from which values of 0.24 and 56 μM for z' and K_0 were obtained. Axon SQDHI.

Fig. 1 shows that the reduction of steady state sodium channel currents by bisC8 is the same, regardless of whether or not inactivation has been removed. All the results described here are from measurements of steady state sodium channel currents and were found to be independent of the degree of inactivation. No distinction will be made between axons with inactivation intact and those with inactivation removed.

The data in this figure and in several others are a bit scattered near 50 mV. This reflects the difficulty of measuring the small steady state sodium channel currents near the reversal potential.

Drug Block Appears to Have a 1 Molecule : 1 Channel Stoichiometry

Fig. 2 illustrates the concentration dependence of reduction of steady state sodium channel current by bisC8 at membrane potentials of 23 mV (open circles) and 103 mV (filled circles). All of these measurements were obtained from a single axon. The solid line represents the best fit of Eq. 1 to these data. The K_d values are 30.3 and 7.1 μM at 23 and 103 mV, respectively, and reflect the voltage dependence of drug block (see below). This equation is a reasonable representation of the data at both potentials. This result is consistent with a 1:1 drug molecule : Na channel stoichiometry.

Divalent Analogue Block is a Steeper Function of V_m Than Monovalent Block

Fig. 3 *A* compares the steady state current-voltage relations in the absence and in the presence of both bisC3 and C3. The recovery after the application of each compound is seen to be good. There is much more block at positive potentials than at negative values—a reflection of the voltage dependence of the action of these compounds. The currents in the presence of these two compounds are about the same at potentials more negative than 60 mV, but there is progressively more current reduction by bisC3 than by C3 at more positive potentials. This suggests that block by the divalent molecule is a steeper function of voltage than

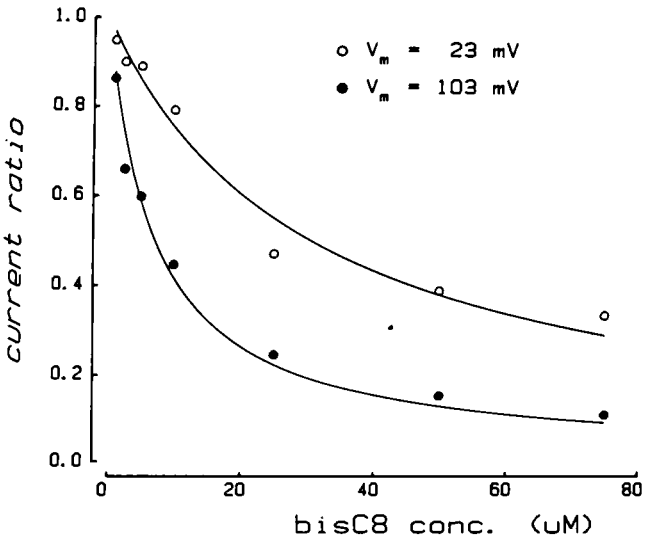

FIGURE 2 Steady state sodium channel current remaining as a function of the concentration of bisC8. Data at two membrane potentials are shown. The solid lines are fits of Eq. 1 with K_d values of 30.3 and 7.1 μM at 23 (O) and 103 mV (●), respectively. Axon SQDHM.

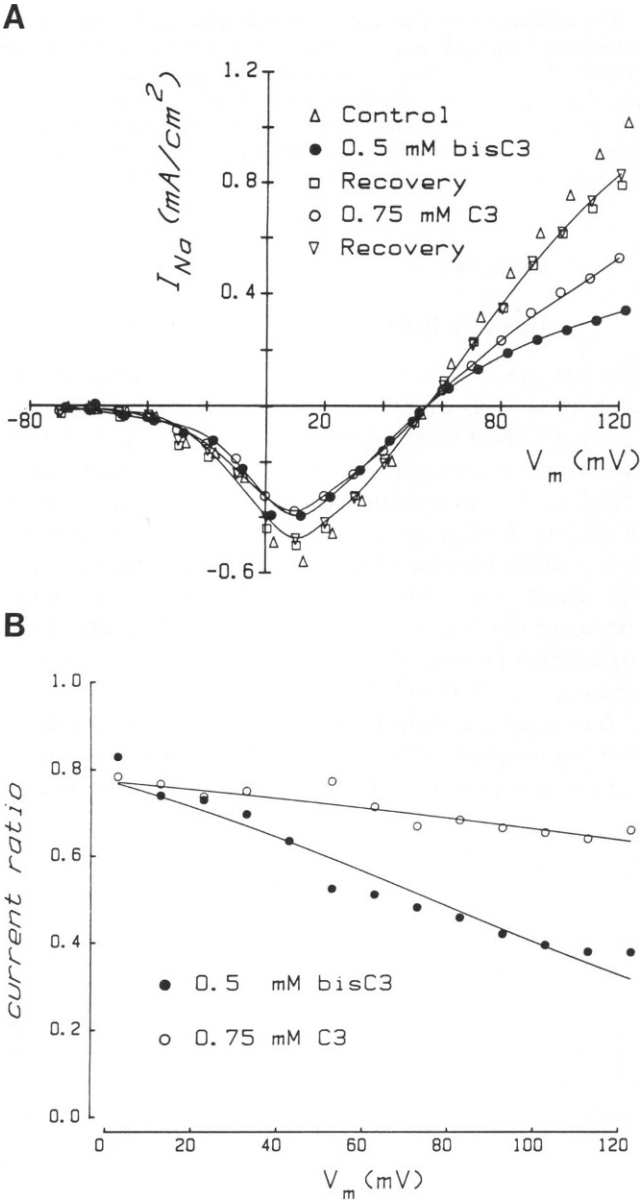

FIGURE 3 Comparison of the voltage dependence of block by C3 and bisC3. (*A*) Steady state sodium channel current-voltage relations in drug-free solutions (Δ, □, ▽) and in the presence of 0.5 mM bisC3 (●) and 0.75 mM C3 (O). (*B*) Fraction of current remaining in 0.5 mM bisC3 (●) and 0.75 mM C3 (O) as a function of membrane potential. Currents before and after each application of drug were averaged to compute the ratio. The solid lines are from fits of Eq. 3 to the data from which values for z' of 0.14 and 0.4 for C3 and bisC3 were obtained. The corresponding K_0 values are 2.6 and 1.7 mM for C3 and bisC3, respectively. Axon SQDMW.

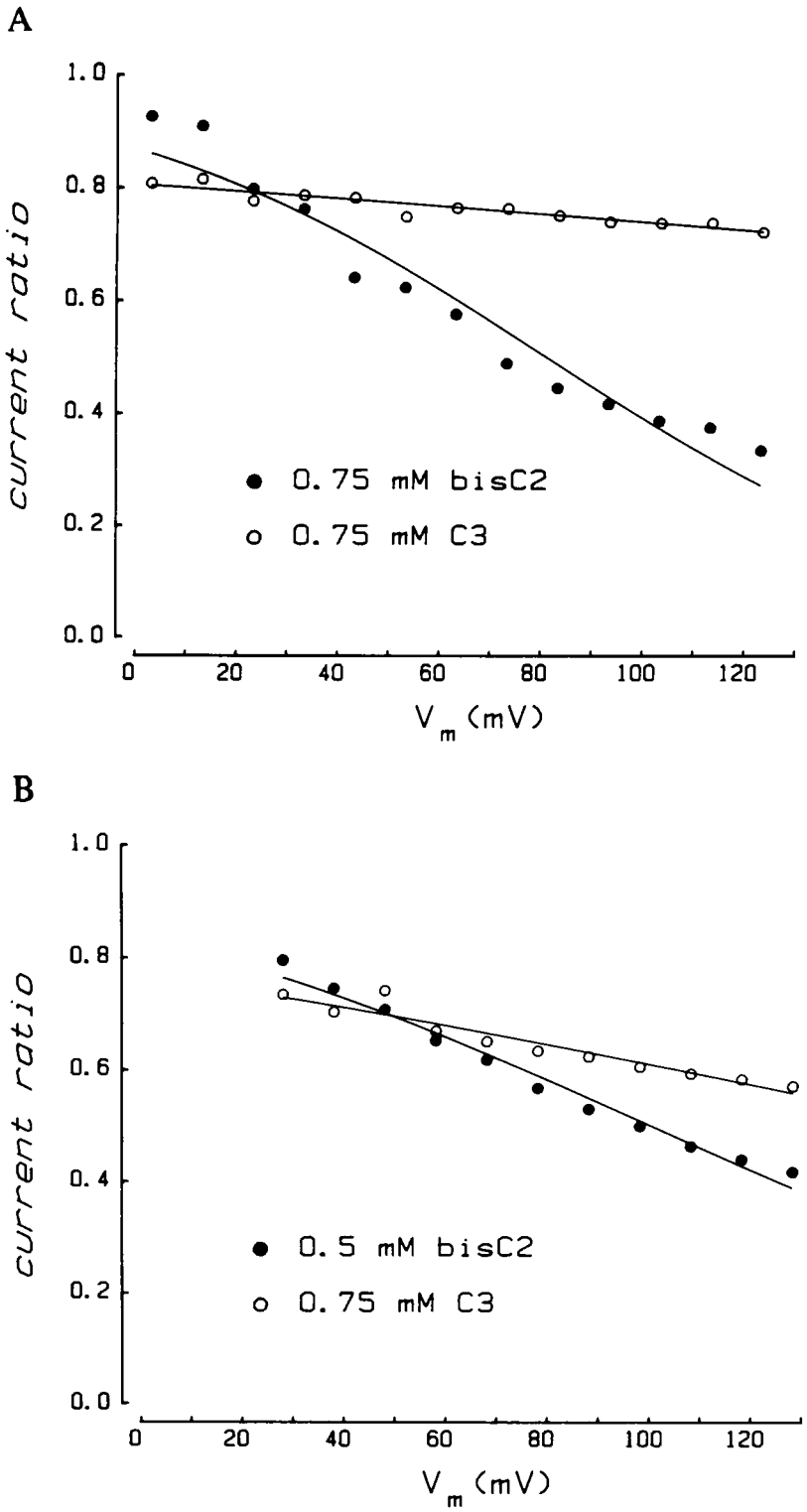

FIGURE 4 Comparison of the voltage dependence of block by C3 and bisC2. (*A*) Fraction of current remaining in 0.75 mM bisC2 (●) and C3 (○) as a function of membrane potential. Fits of Eq. 3 (solid lines) to these data yield values for z' and K_0 of 0.44, 0.09, and 5.0, 3.1 mM for bisC2 and C3, respectively. Axon SQDMI. (*B*) Similar to (*A*) but data from axon SQDOT with 0.5 mM bisC2 (●) and 0.75 mM C3 (○). The solid lines are from Eq. 3 with values for z' and K_0 of 0.4, 0.19, and 2.6, 2.5 mM for bisC2 and C3, respectively.

block by the monovalent one. The current ratio data of Fig. 3 *B* confirm this suggestion.

The voltage dependence of the current remaining in the presence of bisC3 and C3 is illustrated in Fig. 3 *B*. Membrane potential clearly has a more pronounced effect on current reduction by bisC3 than by C3. These data are reasonably well fit by Eq. 3 from which a value of the effective valence, z', of 0.14 was obtained for C3. For the bisC3 data a z' value of 0.4 was obtained. That is, bisC3 acts like a monovalent ion binding to a site 40% of the way down the membrane electric field from the inner surface or like a divalent ion with a site 20% down the field.

Similar results with bisC2 and C3 are shown in Fig. 4 *A*. The open circles in this figure represent the fraction of channels not blocked by 0.75 mM C3 and filled circles are with 0.75 mM bisC2. It is apparent that the divalent cation block is a steeper function of voltage. This is reflected in the values of z' obtained by fitting Eq. 3 to these data (solid lines). Values of 0.09 and 0.44 were obtained for C3 and bisC2, respectively.

These same conclusions are supported by similar data in Fig. 4 *B*. The block caused by bisC2 in this figure is smaller than that in Fig. 4 *A* consistent with the lower concentration. The corresponding z' values are 0.19 and 0.4 for C3 and bisC2, respectively.

The results of Figs. 3 and 4 suggest that both charges of the divalent molecules bisC2 and bisC3 are within the membrane electric field when these ions are bound to their binding sites. It is tempting to use the z' values obtained from the data of these figures to determine the relationship between electrical distance (θ) and spatial distance as Miller (1982) has done for a potassium channel isolated from sarcoplasmic reticulum. For such an analysis to be valid the pore must not contain more than one ion at a time, as is believed the case with the sarcoplasmic reticulum K channel. Begenisich and Cahalan (1980*a*, *b*) and McKinney et al. (1985) provide evidence that this condition may not be met for squid sodium channels. The next section provides more direct data on this point.

Internal Sodium Ions Increase Drug Block

Drug block of a pore that can contain only one ion should be independent of permeant ion concentration (Eq. 3) or should decrease at elevated permeant ion concentrations due to competition for the pore (Hille and Schwarz, 1979). As seen in Fig. 5, increasing the internal sodium concentra-

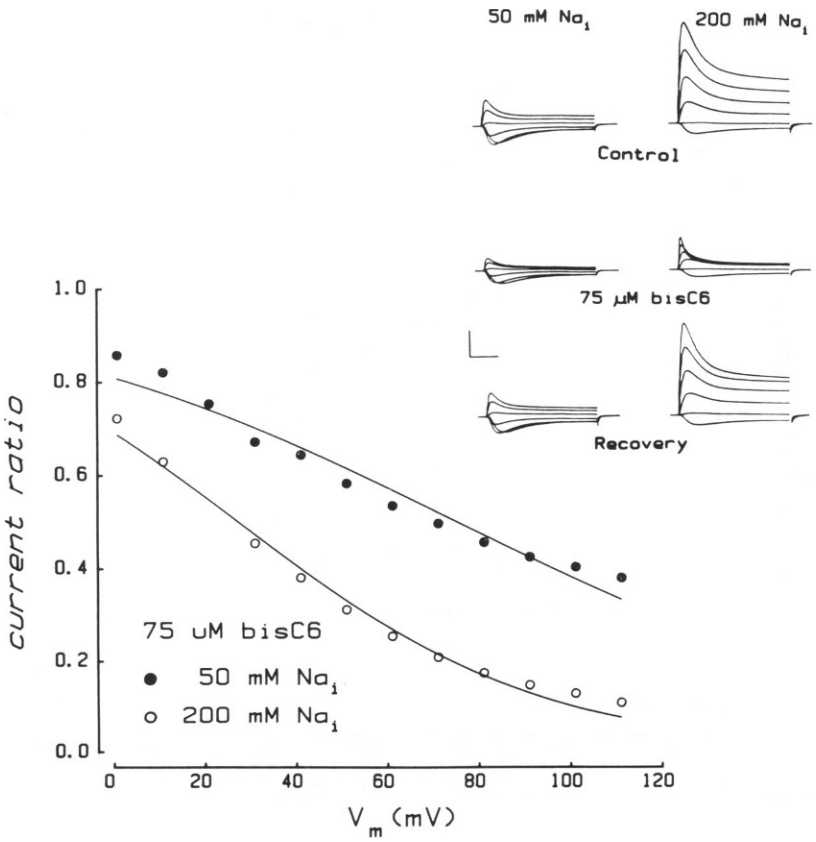

FIGURE 5 Effects of intracellular sodium on block by bisC6. *Inset* Families of sodium channel currents in response to membrane depolarizations to 11, 31, . . . , 111 mV with internal sodium concentrations of 50 mM (*left panel*) and 200 mM (*right panel*). Records are shown before, during, and after application of 75 μM bisC6. Calibration is 5 mA/cm^2 and 1 ms. Figure shows fraction of current remaining in this compound with 50 mM (●) and 200 mM (○) internal Na. Solid lines are computed from Eq. 3 using values for z' and K_0 of 0.48, 0.72, and 0.32 mM, 0.17 mM for 50 and 200 mM Na, respectively. Axon SQDRV.

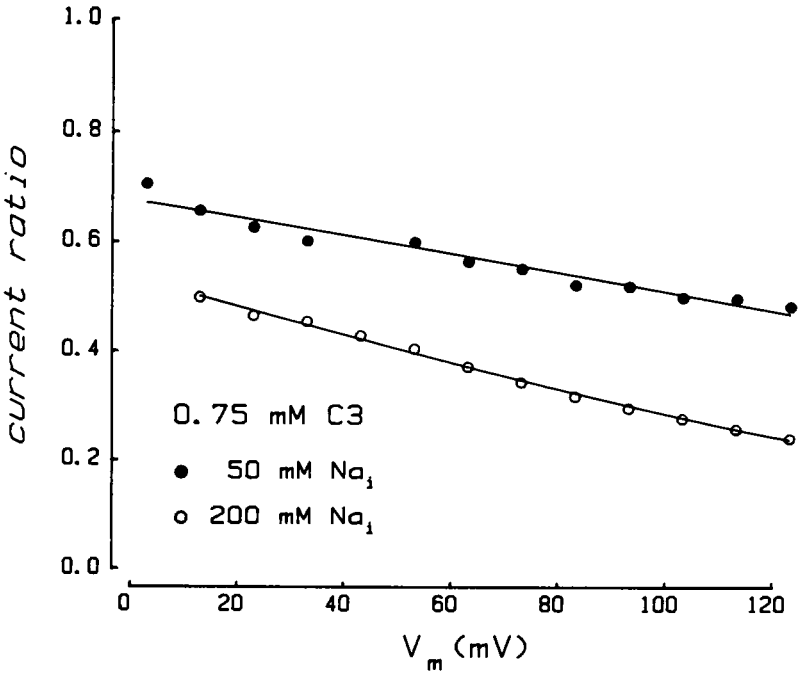

FIGURE 6 Effects of internal sodium on block by C3. Similar to Fig. 5 but data for 0.75 mM C3. Fits of Eq. 3 to the data (*solid lines*) yield values for z' and K_0 of 0.17, 0.26, and 1.6 and 0.86 mM for 50 and 200 mM internal Na, respectively. Axon SQDNB.

tion produces a dramatic increase in block—just the opposite expected for one-ion pores.

The inset of Fig. 5 illustrates the reduction of sodium channel current by 75 μM bisC6 in both 50 and 200 Na SIS. The enhanced block in 200 mM Na is clearly apparent. A quantitative analysis of this effect is shown in the figure where relative current remaining in this drug is plotted as a function of membrane potential for both 50 and 200 mM Na_i. Not only is there more block in the elevated Na concentration, but the block is a steeper function of voltage. The lines in this figure are fits of Eq. 3 to the data from which values of z' and K_0 of 0.48 and 0.32 mM are obtained for the 50 mM solution and 0.72 and 0.17 mM for the 200 mM data.

This same result is demonstrated in Fig. 6 for C3. The values of z' and K_0 obtained from fitting Eq. 3 to the 50 and 200 mM data are 0.17, 1.6 mM, and 0.26, 0.86 mM, respectively. The increase in voltage dependence in 200 mM Na is reflected in the change in z'. The K_0 values obtained from the data of this figure and Fig. 5 indicate that a fourfold increase in intracellular Na concentration produces an approximately twofold increase in blocking potency at zero mV. There is an even larger enhancement of block at positive potentials due to the increased voltage dependence in 200 mM Na_i.

External Na Has Little Effect on Block

In contrast to the large enhancement of block produced by increasing the internal sodium ion concentration, changes in external sodium have little effect. The average current ratio values from three experiments in which both 100% and 25% Na ASW were used are plotted in Fig. 7. The compound used in the experiments of this figure was C3 (1.25 mM), but similar results were obtained for bisC2 and C9. The overlap of the SEM limits in this figure indicates the lack of a significant difference between drug block with these two solutions.

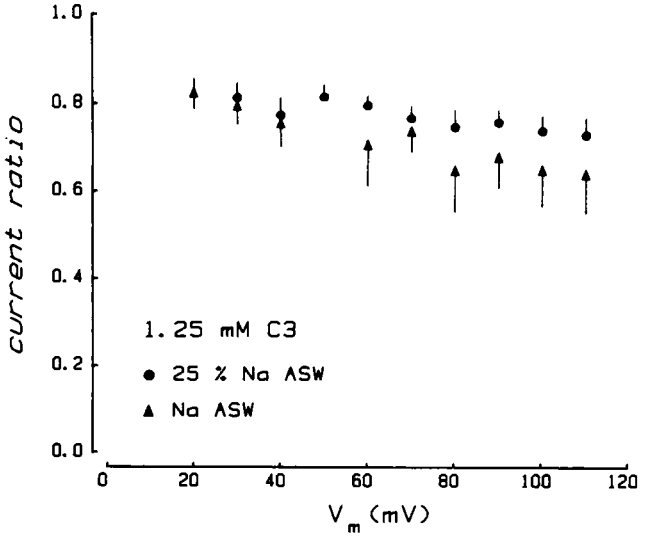

FIGURE 7 Effects of extracellular Na on block by C3. Average fraction of current remaining in the presence of 1.25 mM C3 with normal ASW (▲) and in 25% Na ASW (●) plotted (with SEM limits) against membrane potential. Axons SQDRM, SQDRR, and SQDRT.

DISCUSSION

Comparison to Previous Results

Our results with the monovalent guanidinium analogues are in general agreement with earlier studies of the actions of these compounds on nerve sodium channels (Morello et al., 1980; Kirsch et al., 1980; Lo and Shrager, 1981*a*, *b*). However, unlike Kirsch et al. (1980), but in agreement with Lo and Shrager (1981*a*), we find a time dependence to the block produced by compounds with hydrocarbon chains shorter than C5. Kirsch et al. (1980) also report no voltage dependence to the block in contrast to data in this report, Morello et al. (1980), and Lo and Shrager (1981*a*). The voltage dependence of block by the monovalent derivatives is small with "normal" (50 mM) concentrations of internal Na (Figs. 3, 4, 6, and 7). Most of the measurements of Kirsh et al. (1980) were of peak currents, the block of which is less voltage dependent than the block of steady state currents. In most of the experiments in Lo and Shrager (1981*a*) elevated internal Na concentrations were used, which enhances the voltage-dependent block (Figs. 5 and 6).

Lo and Shrager (1981*a*, *b*) investigated the actions of C3 under two sets of ionic conditions. In the first case the external Na concentration was 200 mM with 220 mM K and 15 mM Na inside the fiber. The second set of solutions contained 2.3 mM Na outside and 235 mM Na inside the axon. The effects of C3 were greatly enhanced in the latter situation. These authors suggested that this might be explained by external Na ions preventing the drug from occupying its usual binding site and when the external sodium concentration was lowered, the drug was able to block the channels.

Our results show that it is not the decrease in external Na that alters guanidinium block; it is the increase in internal Na. These results support the idea that the guanidinium compounds block Na pores by occluding the ion permeation pathway.

These conclusions require the assumption that Cs ions are inert. For example, it could be argued that in our 50 mM Na solution, Cs ions prevent the guanidinium compounds from blocking the channel. Increasing the Na concentration to 200 mM represents a removal of Cs and the guanidinium ions could then inhibit Na channel currents. We cannot unequivocally reject this alternate interpretation, but it seems unlikely that the relatively impermeant Cs ions could prevent block by the guanidinium molecules but not block the Na channel themselves. Furthermore, Oxford and Yeh (1985) have demonstrated that several properties of Na channel function, particularly the relative amount of steady state current, are little affected by internal Cs. Also, the similar results reported in Lo and Shrager (1981*a*, *b*) were obtained without the use of Cs.

An analogous enhancement of block by increasing permeant ion concentration has been reported for the inward rectifier K channel in starfish and tunicate egg cells (Hagiwara et al., 1976; Ohmori, 1980) and in skeletal muscle (Senyk, 1985). The block of these channels by external Cs is increased when the external concentration of K is increased.

Kirsch et al. (1980) found a strong correlation between the alkyl chain length of the monovalent guanidinium analogues and blocking potency. This suggests the existence of a hydrophobic binding site near the inner mouth of the sodium pore. We also find that longer chain length monovalent molecules are more effective blockers than shorter chain compounds. In addition, our results show that this same observation applies to the divalent molecules as well. Since both charges are influenced by the membrane field, both are probably within the pore. The dependence of block on hydrocarbon chain length, then, might be explained by some hydrophobicity within the pore, not just near the entrance.

A Permeation/Drug Block Model

A three-barrier two-site model has previously been described that accounted for much of the squid axon permeation data (Begenisich and Cahalan, 1980*a*, *b*). This model subsequently could reproduce measurements of unidirectional fluxes and external hydrogen ion block (Begenisich and Busath, 1981; Busath and Begenisich, 1982; Begenisich and Danko, 1983). Senyk (1985) used a three-barrier two-site model (with parameters appropriate for the inward rectifier K channel in skeletal muscle) to reproduce enhancement of block by increases in permeant ion concentration. We find that this model cannot account for the large effect observed in this study (2–2.5-fold increased block at ~100 mV; see Figs. 5 and 6). The largest enhancement in the three-barrier two-site model was found to be ~1.3-fold.

We next examined a four-barrier three-site model. Computations from this model simulating our experimental conditions are illustrated in Fig. 8. The model parameters are simple extensions of the three-barrier model parameters preserving the overall energy profile. The exact parameter values are given in the figure legend. In this four-barrier model there is a substantial enhancement of block produced by an increase in internal Na (Fig. 8 *A*). Changing external Na (Fig. 8 *B*) has little effect on the computed block in agreement with our data.

Calculations from this model are generally consistent with the other known permeation properties of sodium channels (see Begenisich, 1982, for a summary) but we have not made quantitative comparisons. Of special interest is the sodium flux ratio exponent, n', since this parameter is influenced by the number of ions that a pore can contain (Hille and Schwarz, 1978). The four-barrier model used here yields values of n' in 50 and 200 mM Na_i of 1.1 and 1.2, which are consistent with the observations of Begenisich and Busath (1981).

The computations from this model displayed in Fig. 9 help provide an understanding of the mechanism by which

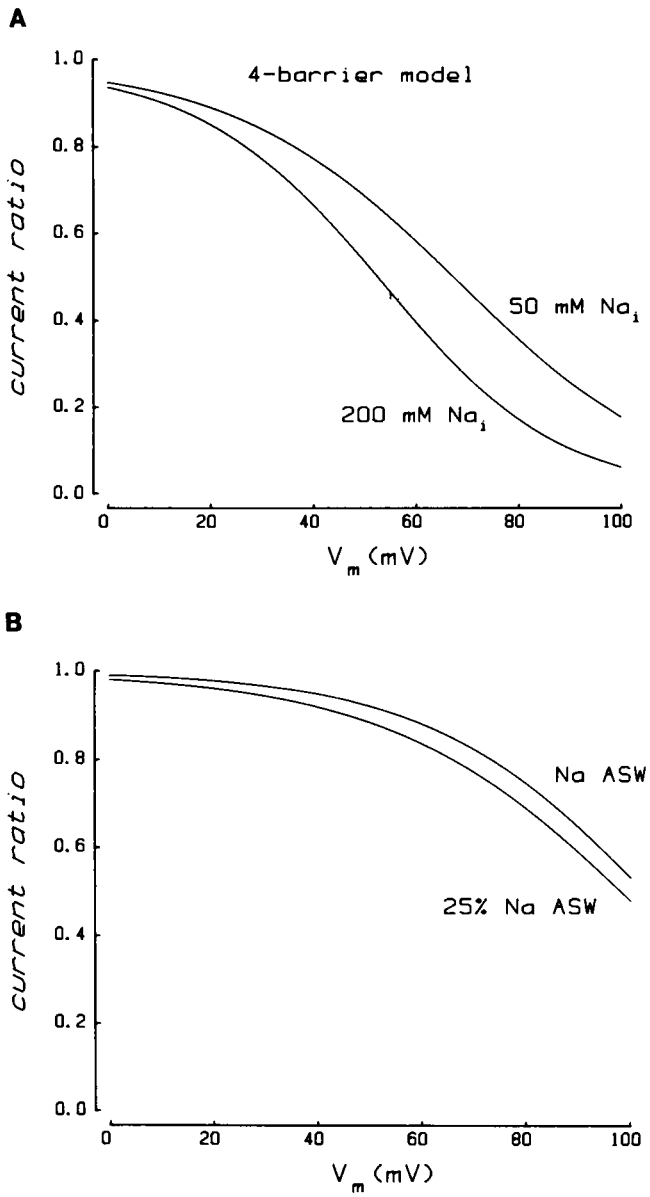

FIGURE 8 Computations from the four-barrier three-site model. (*A*) Fraction of current remaining as a function of potential for 50 and 200 mM Na_i. For these computations the wells were equally spaced and the barriers were symmetrically shaped. For sodium ions the barriers were (in *RT* units from outer to inner membrane surface): 10.5, 7, 7, 10.5, with well depths of 0, −0.75, −0.75. The blocking ion (taken to have a valence of one) barrier values were 20, 4, 4, 6, with well depths of −7, −3.4, −5.5. For this simulation the external Na concentration was 440 mM and the internal concentration of the blocking ion was 100 μM. (*B*) Similar to (*A*) but with 20 μM of blocker and with external concentrations of Na of 440 (Na ASW) and 110 mM (25% Na ASW). The internal Na concentration was 50 mM. All other parameters as in (*A*).

increased Na produces more block. This figure shows the probability of a model pore being in certain states. The state *O* represents the condition with no ions within the pore. The state denoted by Na includes all states with only Na ions in the pore. *B* represents those states with only blocking ions present. *B*Na refers to states in which both Na and blocking ions are present within the pore, but only those with an Na ion in a site closer to the inner membrane surface than a blocking ion. That is, the states *B*,*B*,Na and *O*,*B*,Na are included but not, for example, *O*,Na,*B*. The probability of finding both Na and blocking ions in the pore but with an Na ion closer to the external surface than a blocking ion (e.g. Na,*B*,*O*) is neglible under the conditions used here and not included in the figure.

The calculations for this figure used the model parameters given in the legend of Fig. 8 with the concentration of the (monovalent) blocking ion of 100 μM. A membrane potential of 100 mV was used. The probabilities of occupancy are calculated as functions of Na_i. The external Na concentration was fixed at 440 mM. The fraction of empty channels and the fraction of channels occupied only by Na ions decreases when the concentration of intracellular Na is increased.

The most dramatic changes occur in the states represented by *B* and *B*Na. The probability of pores with only *B* ions decreases with increases in the concentration of internal Na. Increasing internal sodium increases the fraction of pores with Na ions occupying sites more toward the inner surface than those occupied by the blocking ion. Most of this increase arises from increases in the probability of the states *B*,Na,*O* and *B*,Na,Na. With 50 mM these probabilities are 0.268 and 0.08; they increase to 0.316 and 0.38 in 200 mM Na.

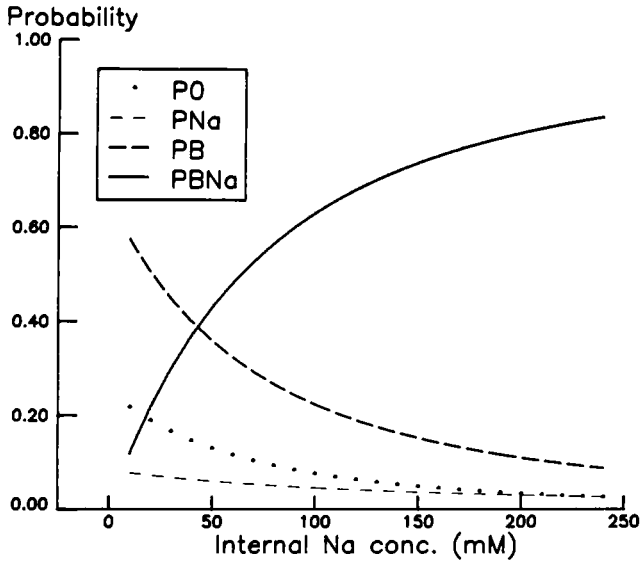

FIGURE 9 Probabilities of occupancy of certain states in the four-barrier model as functions of internal Na concentration. *O* represents an empty pore. Na represents a pore occupied only by Na ions with no distinction made about which site or sites are occupied. The state *B* means a pore with one or more blocking ions (and only blocking ions) in it. *B*Na represents states in which both Na and blocking ions are present but with Na ions always in sites closer to the inner membrane surface than the blocking ions. The model calculations were done at a membrane potential of 100 mV with the barriers and wells given in the legend of Fig. 8 and with 100 μM of the blocker present. The external Na concentration was 440 mM.

The increased probability of pores in states represented by BNa means that, in effect, the blocker is locked in place by the increase in internal Na. Not only does this produce more block, but the block is also more voltage-dependent for two reasons. First, an increased fraction of blocking ions cross more of the membrane field to reach the outer site. Second, as described by Hille and Schwarz (1978), the voltage dependence of block in multi-ion pores involves not only movements of the blocking ion, but also any other ion movements that occur—in this case the movement of Na^+ ions into more sites.

Implications for the Inactivation Mechanism

The similarity between normal inactivation and the current decay induced by exogenous blocking particles (including the guanidinium analogues used in this study) has led to a rather specific physical model for Na channel inactivation (Armstrong and Bezanilla, 1977). In this model (sometimes called the "ball and chain" model) the voltage-dependent activation process opens the pore and reveals a negatively charged binding site near the mouth of the pore. Then a tethered, positively charged inactivation molecule binds to the site, and so closes the pore in an inactivated state.

Increased internal Na causes a decrease in Na channel inactivation (Chandler and Meves, 1970; Fig. 5, here). Oxford and Yeh (1985) find that increasing the internal concentration of tetramethylammonium ions decreases the degree of inactivation similar to the effect produced by Na ions. These results are interpreted in terms of the ball and chain model as a competition between these two types of ions and the endogeneous ball (Yeh and Oxford, 1985).

Therefore, it might be expected that increased intracellular Na ions would compete with the guanidinium ions for the blocking site and decrease the amount of block. We find just the opposite. Consequently, these results demonstrate a significant difference between the behavior of the normal inactivation mechanism and one class of blocking ions that have previously been described as simulating inactivation.

Conclusions

We have examined the reduction of steady state sodium channel currents by several mono- and divalent analogues of guanidinium. The block by both types of molecules is voltage-dependent, but is a steeper function of voltage for the divalent ions. This suggests that both charges of the divalent analogues are within the membrane electric field when these compounds are at their blocking sites. Fits of Eq. 3 to the divalent ion data yielded values of z' of ~0.25 to 0.5 (values of θ between 0.125 and 0.25, $z = 2$). The distance between the charges of these molecules is ~7–14 Å. A rough calculation, then, suggests that ~15% of the membrane electric potential occurs over a distance of 10 Å.

Increasing the internal concentration of Na increases the block. These results are not consistent with one-ion pores but are consistent with multi-ion pores. A four-barrier three-site model appears to be necessary to account for the large effect of increased internal Na.

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