Guanidinium Analogues as Probes of the Squid Axon Sodium Pore

Evidence for Internal Surface Charges

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ABSTRACT We have investigated the reduction of steady state sodium channel currents by a monovalent and a divalent guanidinium analogue. The amount of block by the divalent compound at a constant membrane potential was dramatically reduced by an increase in the internal salt concentration. Channel block by the monovalent molecule was a less steep function of salt concentration. These results would be expected if there were negative charges near the sodium pore that produced a local accumulation of the cationic blocking ions. According to this view, the ionic strength dependence of block results from changes in surface potential. The divalent blocker would be expected to be more sensitive to ionic strength owing to its larger valence. Our results can be quantitatively described by a simple ionic double-layer model with an effective surface charge density of about $-1 \text{ e/250 Å}^2$ in the vicinity of the pore.

INTRODUCTION

The action potential in many nerve and muscle cells results from voltage-dependent changes in the conformation of an Na ion-specific membrane protein. There are many different conformations of this protein: several nonconducting (closed) states, one or more conducting (open) states, and at least one refractory (inactivated) state. The sodium channel protein has been isolated from a variety of excitable tissues, including electric eel electroplax (Miller et al., 1983), rat skeletal muscle (Barchi, 1983), and rat brain (Hartshorne and Catterall, 1981). These studies have identified the channel as a membrane-bound glycoprotein of molecular mass near 250,000 daltons. The amino acid sequence of the sodium channel from eel electroplax has been deduced from the cDNA coding for this protein (Noda et al., 1984).

In spite of these biochemical advances, the three-dimensional structure of the sodium channel and the functionally important amino acid residues remain...
obscure. However, the results of several studies have implicated the involvement of the amino acid arginine on the internal side of the membrane in the inactivation process (Rojas and Rudy, 1976; Eaton et al., 1978; Brodwick and Eaton, 1978). Since arginine contains a guanidinium group, several laboratories have investigated the actions of internal guanidinium analogues on sodium channel function (Morello et al., 1980; Kirsch et al., 1980; Lo and Shrager, 1981a, b). Internally applied monovalent and divalent guanidinium analogues inhibit sodium channel current. The block is increased by positive membrane potentials and by increases in the internal Na⁺ concentration (Danko et al., 1986), which indicates that these guanidinium compounds enter and block the pore.

These properties of the guanidinium analogues suggest that they may be useful in testing for the presence of surface charges near the inner entrance to the sodium pore. Negative surface charges could accumulate guanidinium and Na ions near the pore entrance. An increase in the internal electrolyte concentration would electrostatically "screen" the charges and decrease the local concentration of both blocking and Na ions, resulting in a decrease in the inhibition of sodium channel current. Furthermore, because of the larger valence, the decrease in block by divalent guanidinium analogues should be a steeper function of electrolyte concentration than block by monovalent analogues.

We found that the block of sodium channels produced by internal application of both a monovalent and a divalent guanidinium analogue was reduced by increases in the internal electrolyte concentration. Block by the monovalent compound was less affected by electrolyte concentration than that by the divalent molecule. We showed these results to be quantitatively consistent with a simple surface-charge, channel-block model with an effective surface-charge density of approximately $-1 \text{e}/250 \, \text{Å}^2$.

A preliminary report of these results was presented at the annual meeting of the Biophysical Society (Smith et al., 1984).

**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, MA.

**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 (Begenisch and Lynch, 1974; Busath and Begenisch, 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. These experiments were done at a temperature of 10°C. Series resistance compensation was used and was adjusted appropriately for the solutions of different salt concentrations (and resistivities) as described below.

Membrane currents were measured with a 12-bit analog-to-digital converter controlled by a microcomputer of our own design. Current was sampled at 20-μs intervals. The voltage-clamp pulses were generated by the microcomputer at 2-s intervals. A typical
voltage waveform consisted of a 50-ms, 30-mV hyperpolarizing pulse from a holding potential near −60 or −70 mV, and then a 500-μs return to the holding potential. This was followed by a depolarizing test pulse. Potassium channel currents were eliminated by using 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 actions of two internally applied guanidinium analogues were studied with several different internal salt concentrations. The monovalent compound was n-propyl guanidine (abbreviated as C3) from Vega Biochemicals, Tucson, AZ. The divalent analogue used was 1,2-bis-guanidino-n-ethane (bisC2), a gift from Dr. Christopher Miller (Brandeis University, Waltham, MA).

The standard artificial seawater (ASW) solution contained (millimolar): 440 NaCl, 10 CaCl₂, 50 MgCl₂, 10 HEPES buffer, pH ~7.4. The sodium channel reversal potential is ~50 mV with this ASW and our standard internal solution, which contains 50 mM Na⁺. The block of sodium channel current by these guanidinium compounds is most conveniently studied in the range of membrane potentials from 0 to 120 mV. The presence of the reversal potential in the middle of this range restricts the amount of data obtainable. Consequently, in all the experiments with bisC2, an external solution without Na ions was used. Tetramethylammonium (TMA) or Tris was used to replace Na⁺. A zero-Na⁺ ASW was also used in most of the experiments with C3, but some data were obtained in 25 or 100% Na⁺ ASW solutions. There is little effect of external Na⁺ on the block produced by these and other guanidinium analogues (Danko et al., 1986).

The standard internal solution (50 Na SIS) consisted of 50 mM NaF and 150 Cs glutamate. Several other internal solutions used also contained 50 mM NaF, but had Cs glutamate concentrations ranging from 0 to 350 mM. These solutions are described by their total salt concentration. Since Cs ions are only sparingly permeant through the sodium pore (Chandler and Meves, 1965), Na ions are the major permeant ions in these solutions. In addition, the solutions also contained HEPES buffer (pH ~7.5) and glycine for osmotic balance. In a few early experiments with C3, a solution consisting of 25 mM Na₂HPO₄, 150 mM CsF was used.

Data Analysis

The block of sodium channel currents by guanidinium compounds is time dependent (Morello et al., 1980; Kirsch et al., 1980). Consequently, we estimated steady state block using currents measured at the end of 4-ms voltage-clamp pulses. This is sufficient for block and sodium channel inactivation to reach steady state at potentials >0 mV. In some of these experiments, inactivation was removed by proteolytic enzymes, but in most of the experiments, endogenous steady state currents were used. We have previously shown (Danko et al., 1986) that block of steady state sodium channel currents by guanidinium analogues is independent of the inactivation process. The use of pulses to positive potentials also assures rapid opening of all available channels. If this were not the case, then channel block might have been coupled to channel opening.

Surface-Charge Model

We investigated the ability of a simple model to account for the effects of internal salt concentration (ionic strength) on sodium channel block by C3 and bisC2. A basic assumption of this model is that a negative charge near the inner surface of the sodium pore accumulates both Na and guanidinium ions. The surface potential, \( V_s \), is computed
as a function of salt concentration from the Gouy-Chapman (Gouy, 1910; Chapman, 1913) double-layer theory with the surface-charge density, \( \sigma \), as an adjustable parameter:

\[
V_s = (2kT/e) \sinh^{-1} \left[ e \left( \frac{\sigma}{(2\pi n kT)} \right) \right],
\]

(1)

where \( k \) is Boltzmann's constant, \( T \) is the absolute temperature, \( e \) is the charge on the electron, \( n \) is the salt concentration, and \( \epsilon \) is the dielectric constant of the aqueous solution.

As discussed in Danko et al. (1986), the fraction, \( f \), of sodium channels blocked by the guanidinium compounds can be described by the simple equation:

\[
f = \frac{B_s}{(B_s + K)},
\]

(2)

where \( K \) is the effective dissociation constant and \( B_s \) is the surface concentration of the guanidinium ions, which is related to the bulk concentration, \( B \):

\[
B_s = B \exp\left(-\frac{z_i eV_s}{kT}\right).
\]

(3)

\( z_i \) is the valence of the appropriate guanidinium ion: 1 for C3 and 2 for bisC2.

Eq. 1 is appropriate only for solutions containing monovalent salts. The presence of the divalent bisC2 ions might be expected to invalidate the use of this simple equation; however, these ions were used at low concentrations. Calculations with the more complete Grahame equation (Grahame, 1947) demonstrated that including 0.5 mM divalent bisC2 produced only a 1–5-mV change in the surface potential as computed from Eq. 1 for the surface-charge densities and salt concentrations used here.

The study of sodium channel block by these guanidinium analogues showed that the effective dissociation constant in Eq. 2 is a function of membrane voltage (Danko et al., 1986). If changes in the surface potential also altered the voltage at the blocking site, then Eq. 2 should be modified to reflect this situation. However, because of the rather weak voltage dependence of \( K \) (e.g., see Fig. 3), any such modification would be small. Consequently, in the absence of data on this issue and to keep the numerical analysis tractable, \( K \) is assumed to be independent of changes in surface potential. Danko et al. (1986) also showed that block by these compounds is increased by internal Na ions. This observation was incorporated in the analysis by making \( K \) inversely proportional to the surface concentration of Na ions. This set of equations was fitted to the data using the nonlinear fitting algorithm called "simplex" (Caceci and Cacheris, 1984), with the residuals weighted appropriately for the propagation of experimental errors. There are two adjustable parameters: the surface-charge density, and a parameter proportional to the effective dissociation constant.

The limitations of the Gouy-Chapman double-layer theory are well known (see, e.g., Aveyard and Haydon, 1973; Davies and Rideal, 1963). Nevertheless, this theory has been quite successful in simulating a wide variety of surface-charge phenomena, no doubt because many of the approximations inherent in the theory produce errors of comparable magnitude in opposite directions.

One of the important assumptions of the double-layer theory is that the ions in solution can be considered point charges. This assumption may not be appropriate for divalent ions whose charge separation is comparable to the Debye length of the salt solution being considered (Carnie and McLaughlin, 1983; Alvarez et al., 1983). The Debye length in the solutions used in this study change from just over 13 Å (50 mM salt) down to ~6 Å (250 mM). The charge separation of bisC2 is ~6.5 Å. Consequently, deviations from the simple theory might be expected only at high salt concentrations. However, experiments with the 10-Å hexamethonium ion showed that the deviations are not large, even when the Debye length is similar to the charge separation (Alvarez et al., 1983). In view of the many other approximations already inherent in the Gouy-Chapman model, we used this
RESULTS

Block Is a Function of Internal Salt Concentration

The effect of changing the internal salt concentration on the block of sodium channel current by internally applied bisC2 is illustrated in Fig. 1. The top row shows that in a 200 mM salt solution, 0.5 mM bisC2 has only a small effect on the currents. There is a much larger reduction of current in 100 mM salt (middle row) and almost complete block in 50 mM (bottom row). It is apparent from these data that the effects of this compound are readily reversible. Negligible effects of this concentration of bisC2 were observed in experiments with salt concentrations of 350 and 400 mM.

For the reasons discussed in the Methods, we were interested in the effects of the guanidinium compounds on steady state currents. Consequently, currents measured at the end of the 4-ms test pulses to several potentials are plotted in Fig. 2. Only positive test potentials were used, so that block would not be coupled to channel gating (see Methods). Fig. 2A illustrates that there was little effect of 0.5 mM bisC2 on these steady state currents with a 250 mM internal salt solution. A much larger effect was seen in Fig. 2B at all voltages at the lower salt concentration of 100 mM. The effects of bisC2 are reversible.
Fig. 3 illustrates the voltage dependence of current block by 0.5 mM bisC2 at several internal salt concentrations. These data were obtained from the currents of Fig. 1. The fraction of sodium channels blocked is estimated from the amount of current reduction relative to the average of the "control" and "recovery"}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Sodium channel current-voltage relations. Effects of 0.5 mM bisC2 in 250 mM salt (A) and in 100 mM salt (B). External solution was zero-Na\(^+\) (TMA) ASW. The solid lines are cubic spline fits to the data. Axon SQDSX.}
\end{figure}
currents. As was apparent from the previous figures, block at all potentials is a function of internal salt concentration: it is small at high concentrations and large at low concentrations.

The Effects of Salt Concentration Are Consistent with a Surface-Charge Model

Data from several experiments with 0.5 mM bisC2 are illustrated in Fig. 4. Block at 53 mV (A) and 113 mV (B) is plotted at several internal salt concentrations. Also shown in this figure are fits of the surface-charge, channel-block model described in the Methods.

The solid line in Fig. 4A represents the best fit of the model, and the associated surface-charge density is $-1 \, e/235 \, \text{Å}^2$. The figure also shows the fit of the model with the charge density fixed as $-1 \, e/300 \, \text{Å}^2$ (dashed line); only the effective binding constant was adjustable. This fit is inferior to the best fit at high concentrations. A surface-charge density larger than $-1 \, e/235 \, \text{Å}^2$ produces a visibly poorer fit at low concentrations. Consequently, a rough estimate of the accuracy associated with the fitted surface-charge density is $\sim 25\%$.

The effect of salt concentration on bisC2 block at a membrane potential of 113 mV is illustrated in Fig. 4B. These data are similar to those of Fig. 4A, except that there is generally more block, reflecting the voltage dependence of the action of this compound. The solid line is the best fit of the model to these data and represents a surface-charge density of $-1 \, e/260 \, \text{Å}^2$. This value is close to the best-fit value of $-1 \, e/235 \, \text{Å}^2$ for the 53-mV data of Fig. 4A. Fits of
the surface-charge, channel-block model to data obtained at potentials of 33, 73, and 93 mV yielded surface-charge densities of $-1 \text{ e/190 } \text{Å}^2$, $-1 \text{ e/300 } \text{Å}^2$, and $-1 \text{ e/270 } \text{Å}^2$, respectively. This range is consistent with the estimated 25% error in the determination of values for this parameter.

![Graph](image)

**Figure 4.** Effects of internal salt concentration on block of sodium channels by 0.5 mM bisC2. Block was at membrane potentials of 53 mV (A) and 113 mV (B). Data are from six axons. Each point represents a single measurement. One of the points at 100 mM has been displaced slightly. Lines are as described in the text.
It is apparent that the effect of salt concentration on block of sodium channels by bisC2 is quantitatively consistent with the predictions of the surface-charge, channel-block model described in the Methods, with an effective surface-charge density of about \(-1 \text{ e/250 } \text{Å}^2\). There are no significant differences among the best-fit surface-charge densities over an 80-mV range of membrane potential.

**Divalent Blockers Are More Sensitive to Salt Concentration than Monovalent Ones**

The block of sodium channels by the monovalent guanidinium compound C3 is also affected by the internal salt concentration. Fig. 5 shows the block produced by 1.25 mM C3 as a function of membrane voltage in internal salt concentrations of 75 mM (filled squares) and 200 mM (open squares). There is clearly less block in 200 mM salt than in 75 mM.

Also shown in Fig. 5 are data obtained from the same axon with 0.5 mM bisC2 at internal salt concentrations of 75 mM (open circles) and 200 mM (filled circles). The block produced by the divalent molecule is somewhat more voltage dependent than that by the monovalent ion, in agreement with our previous report (Danko et al., 1986). With an internal salt concentration of 75 mM, there is a similar degree of block exhibited by both compounds. There is, however, a large difference in the block at the higher salt concentration, which shows that the divalent compound is more sensitive to changes in ionic strength.
Data from several experiments with 1.25 mM C3 are illustrated in Fig. 6. Block of sodium channels at 111 mV is plotted as a function of internal salt concentration. The dashed line in this figure represents the block by bisC2 at a similar potential (solid line in Fig. 4B). Even though there is some scatter in the individual measurements, the C3 data are consistently below the dashed line at low concentrations and above it at high concentrations. This supports the results of Fig. 5, which showed that the monovalent compound C3 is less affected by salt concentration than the divalent molecule bisC2.

Also shown in Fig. 6 is the fit of the surface-charge, channel-block model to the C3 data (solid line). For this computation, the surface-charge density was fixed at the value obtained from the bisC2 data of Fig. 4B ($-1\, \text{e/260}\, \text{Å}^2$). Also, the valence was set at 1, which is appropriate for the monovalent C3 ion. The only adjustable parameter, then, was the effective binding constant. The quality of the fit demonstrates that, with a fixed surface-charge density, the model can successfully predict the behavior of both monovalent and divalent blocking molecules.

**DISCUSSION**

The results presented here show that the internal salt concentration has a profound effect on the ability of internal monovalent and divalent guanidinium ions to block sodium channels. A simple interpretation of these results is that there are surface charges near the inner surface of the sodium pore. These charges accumulate ions near the mouth of the pore. High electrolyte concentrations electrostatically "screen" the charges and produce less local accumulation of both blocking and permeant ions. Consequently, channel block is expected to decrease with increasing electrolyte concentration.

As illustrated in Fig. 4, such a model is quantitatively consistent with the bisC2 data. One prediction of this model is that divalent cations would be more affected by ionic strength changes than would monovalent ions. Not only is this qualitative
prediction found experimentally (Figs. 5 and 6), but the C3 data can be quanti-
tatively described by the same surface-charge density that fits the bisC2 data
(Fig. 6).

It is possible to qualitatively account for the effects of salt concentration on
the block produced by these guanidinium compounds as a direct effect of Cs⁺.
In this view, Cs ions compete with the blocking ions, so that an increase in the
electrolyte (Cs⁺) concentration would then decrease the amount of block. While
such an interaction cannot be unequivocally rejected without a totally benign
(and so far unknown) replacement cation, many of the properties of sodium
channels, including steady state current (Oxford and Yeh, 1985) and block by
guanidinium analogues (Lo and Shrager, 1981a, b), are independent of the
presence of Cs ions.

It seems unlikely that the relatively impermeant Cs ions could prevent block
by the guanidinium molecules and not also block the channels. Furthermore,
this type of model cannot explain the difference in sensitivity to salt concentration
changes exhibited by the monovalent and divalent guanidinium ions. Conse-
quently, the presence of surface charges near the inner surface of the sodium
pore represents a simple interpretation that is quantitatively consistent with the
data.

Surface Charges and Permeation

The presence of surface charges near the inner surface of the sodium pore might
be expected to influence many of the permeation properties, including ion
current and block by cationic drugs like local anesthetics and H ions. In general,
the negative surface charges will produce surface concentrations of cations higher
than bulk solution values. Consequently, the affinity of the pore for blocking
cations will be underestimated. For example, the pKₐ for internal H ion block of
the squid axon sodium pore has been reported as 5.8 (Wanke et al., 1980). The
surface pH, computed with a surface-charge density of -1 e/250 Å², is 0.8 lower
than that in the bulk solution, which implies that the actual pKₐ would be 5. A
more complex computation would be required if H ions also titrated the surface
charges or if the surface potential affected the voltage at the binding site. This
difference between bulk and surface pH is particularly important when apparent
pKₐ values are interpreted as evidence for the involvement of particular chemical
groups.

Changes in the surface potential near the pore will change the local accumu-
lation of Na⁺ and hence the ion current. Consequently, lowering the ionic
strength of the internal solution should increase the outward Na⁺ current. Fig.
1 shows that this indeed occurs (at least for a change from 200 to 100 mM).
However, the experiment illustrated in this figure was a particularly long one
and was not specifically designed to address the issue of the ionic strength
dependence of the current. In three other experiments in which an ionic strength
change from 250 to 100 mM was made without introducing any guanidinium
ions, an average increase of current (at 95 mV) of 1.77 ± 0.14 (SD) was obtained.
The change in bulk ionic strength will increase the activity of Na ions, but only
by a factor of 1.17 (Kielland, 1937).
The surface concentrations of Na⁺ in 250 and 100 mM salt solutions (50 mM Na salt and 200 or 50 mM inert salt) computed from the surface-charge model (−1 e/250 Å³) are 340 and 700 mM, respectively, or activities of 235 and 430 mM. The ratio of the activities is 1.85, not much more than the current ratio of 1.77 described above. This small difference between the increase in surface activity of Na⁺ and the increase in current is consistent with the large apparent dissociation constant for saturation of current through the sodium pore. Begenisich and Cahalan (1980) determined that a bulk activity of ~600 mM (at 90 mV) is required to half-saturate the sodium pore of squid axons.

It is difficult to predict the effect surface potentials would have on the sodium channel reversal potential without detailed information on the structure of the pore and the permeation mechanism. However, the available data suggest that for bionic conditions (Na⁺ outside/K⁺ inside), changes in ionic strength have no effect on the sodium channel reversal potential (Cahalan and Begenisich, 1976). Consequently, any models for ion permeation through this pore would need to accommodate the presence of surface charges and provide for reversal potentials that are independent of surface potential.

There is some evidence for the presence of negative charges on the external surface of the sodium pore (Sigworth and Spalding, 1980; Green and Andersen, 1986). The external and internal surface charges described here may be necessary for proper functioning of the pore. These charges may provide the selectivity against anions characteristic of this channel (Cahalan and Begenisich, 1976). They may also provide high current flow by accumulating permeant ions at the entrance to the pore as described above. Indeed, the addition of negative charges to the ends of the pore-forming peptide gramicidin increases the current severalfold over the native molecule. Furthermore, the current through the modified pore increases as the ionic strength is lowered, similar to our results described above (Apell et al., 1977).

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