Guanidine Block of Single Channel Currents Activated by Acetylcholine

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ABSTRACT The acetylcholine-activated channel of chick myotube was studied using the patch-clamp method. Single channel current amplitudes were measured between -300 and +250 mV in solutions containing the permeant ions Cs+ and guanidine (G+). G+ has a relative permeability, PG/PC., of 1.6, but carries no more than half the current that Cs+ does, with an equivalent electrochemical driving force. Experiments using G+ revealed an asymmetry of the acetylcholine-activated channel, with G+ being more effective at reducing Cs+ currents when added to the outside than when added to the inside. The block caused by outside, but not inside, G+ was evident for both inward and outward currents. The block caused by outside G+ was voltage dependent, first increasing and then being partially relieved when the driving force was made more negative. Experiments with mixtures of Cs+ and G+ revealed anomalously low magnitudes for reversal potentials, relative to predictions based on the Goldman-Hodgkin-Katz equation. These findings are consistent with a two-well, three-barrier Eyring rate model for ion flow, and demonstrate that a highly permeant ion, guanidine, can block asymmetrically by acting from within the voltage field of the acetylcholine-activated channel.

INTRODUCTION
Channels activated by acetylcholine (ACh) at the neuromuscular junction or in embryonic skeletal muscle are permeable to a variety of metal and organic cations (Fatt, 1950; Maeno et al., 1977; Dwyer et al., 1980; D. J. Adams et al., 1980). Two measures exist that gauge the ease with which ions traverse a channel: first, the relative permeability as determined from the shift of the reversal potential when a test ion replaces the reference ion, and second, the magnitude of the conductance caused by the test ion. Shifts in reversal potential can be accurately measured from macroscopic currents activated by ACh and recorded with a standard voltage clamp (Takeuchi and Takeuchi, 1960; Dwyer et al., 1980). The amplitudes of currents through a single open channel can now be measured directly using the patch clamp (Hamill et al., 1981). The patch-clamp method can also be used to estimate the reversal potential.

Measurements by the first of these methods can be used to predict the results of the second by using the independence principle. If the movement of an ion through the channel is unaffected by the presence of other ions, there should be
good agreement between the observed data and the theory (Hodgkin and Huxley, 1952). Many cations, although small enough to pass easily through the selectivity filter, do yield less current than predicted; Na⁺ itself is such an example (Horn and Patlak, 1980; Neher and Steinbach, 1978). According to the Eyring rate theory (Eyring et al., 1949), a current may be smaller than predicted because the channel is blocked for very brief periods of time, periods so brief that the individual intervals cannot be recorded by present methods. Such a mechanism of channel block can explain how the presence of one ion species can block the current carried by another and so violates the independence principle.

The amplitude of macroscopic ACh-activated currents measured by standard voltage-clamp techniques can also be decreased by the addition of noncompetitive blockers to the solution; these chemicals belong to the family of compounds generally termed local anesthetics (del Castillo and Katz, 1956). Typically, such molecules are too large to pass through the channel, although procaine is actually small enough physically to fit through the selectivity filter. When permanently charged, these molecules are able to act only from the outside (Horn et al., 1980; Farley and Narahashi, 1983), and act by blocking the open channel with brief but measurable occupancies, which results in a rapid chopping of the currents (Neher and Steinbach, 1978).

Intermediate in structure between the alkali metals on one extreme and local anesthetics on the other are small permeant cations that share the characteristics of both alkali metal ions and local anesthetics. The addition of many small organic ions to the outside solution will decrease the macroscopic ACh-activated current (Farley et al., 1981), as well as the microscopic single channel current (D. J. Adams et al., 1981; Farley et al., 1986), particularly if the ion has a hydrophobic side chain, unsaturated bonds, or delocalized electrons.

The guanidinium ion, [(NH₂)₂==C==NH₂]⁺, is an example of such an ion because it does have a delocalized electron pair shared by the three nitrogens surrounding the carbon. Moreover, the guanidinium group is important in pharmacological terms because it is present in tetrodotoxin, a specific blocker of the Na channel, amiloride, a blocker of Na channels in epithelia, streptomycin, an antibiotic that can cause neuromuscular blockade, and guanethidine, a post-ganglionic blocker. The barbituric acid ring is similar in part to G⁺, except that one nitrogen of G⁺ is replaced by an oxygen or sulfur (Gilman et al., 1980). Indirect studies have shown that the family of G⁺ derivatives acts to decrease current through the ACh-activated channel; these molecules are better able to block when added to the outside than when added to the inside (D. J. Adams et al., 1981). One member of this family, the amino acid arginine, is a monovalent cation at physiological pH. This ion does block currents at the ACh-activated channel in frog and chick embryo when applied externally (Dwyer et al., 1980; Dwyer and Farley, 1984), but block of ACh-activated currents by permeant metal ions such as Cs⁺ is relieved when arginine replaces an equal amount of Cs⁺ on the inside of a muscle cell (Dwyer et al., 1980).

Because G⁺ is the simplest member of the family, it was chosen to test the block and relief of block caused by permeant ions at the ACh-activated channel. In this article, G⁺ is shown to be a permeant cation that acts within the channel
to reduce the current carried by other permeant cations and is more effective when acting from the outside of the membrane than from the inside. Preliminary results have appeared elsewhere (Dwyer and Farley, 1985).

METHODS

Protocol

Experiments were performed as follows: a coverslip bearing 10–21-d-old cells cultured from chick embryonic muscle was placed in a chamber and maintained at 11°C. Inside-out patches were made by the technique of Hamill et al. (1981), and single channel currents were amplified by a List (Greenvale, NY) EPC-5 voltage clamp and stored by an FM tape recorder. The amplitude measurements were made with current records stored on a digital oscilloscope. For recording of currents at membrane potentials more extreme than ±150 mV, the potential was held at ±100 mV and stepped to the test voltage for no longer than 10 s. Data could be recorded until the extreme voltage caused membrane breakdown. Usually, more pulses were needed for positive potentials because many of the single channel events were too brief to yield reliable amplitude measurements at these voltages (Magleby and Stevens, 1972). Further details are given in Dwyer and Farley (1984). In all, 69 patches were used to obtain >8,000 single channel currents.

Solutions

All solutions were buffered with 5 mM HEPES; in addition, 2 mM BaCl₂ was added to the external solution, except where noted. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO), except CsOH, which was obtained from Cerac, Inc. (Milwaukee, WI). “Pure” solutions are those containing a single permeant ion, the buffer HEPES, plus BaCl₂ for pipette (outside) solutions. The concentrations given in the text include the amount of cation that was added to neutralize the HEPES buffer.

Voltage and Plotting Conventions

Membrane voltages (E) are given as inside minus outside. All voltages were corrected for liquid junction potentials, which were measured to be ≤0.5 mV. The amplitudes of single channel currents (i) are positive when they are outward. All binding constants were calculated for currents measured 100 mV away from the reversal potential, based on ion concentrations and assuming a single binding site unaffected by the composition of the opposite solution.

To directly compare absolute currents measured in two situations with opposite ionic gradients, both the amplitudes plotted as open triangles in Figs. 1, 3, and 5 and their holding potentials were multiplied by −1.0 before plotting. As a result, the upper right-hand quadrants of Figs. 3 and 5, for instance, plot currents originating from the G⁺-containing solution, regardless of whether that solution was internal or external.

Analyses

The line drawn in Fig. 1 is the result of passing the observed data, rank-ordered by holding potential, through a seventh-order monotonic digital filter (Hamming, 1983). It is the nature of this filter to eliminate six points at each extreme; as a result, the line passes only through the points whose value is known with the greatest confidence.

Equations for the three-barrier, two-well rate model were written by the algorithm given in Hille (1975). A simulator program was written in the C language for an IBM PC, with the results of the simulation displayed on the computer screen, along with the
experimental data points and the values of the parameters. The fit was obtained by adjusting the parameters one at a time and judging the results by eye. Such an approach allows for a rapid development of alternative models and the quick appreciation of the relative importance of individual variables. Extending the precision of the parameters' values beyond the first decimal place resulted in little visible change when viewed on the scale available for publication, and so all values are reported with that precision.

RESULTS

Single Channel Current Amplitudes in Single Cation Solutions

Solutions made up of a single metal cation yield ACh-activated single channel currents whose magnitudes are symmetrical with respect to membrane sidedness. Fig. 1 illustrates the simplest case, with Cs⁺ on both sides of the membrane. The

![Figure 1: Symmetry of currents in pure Cs⁺ solutions.](image)

open circles give currents recorded with 83/363 (external/interal solutions in millimolar) CsCl. In an experiment with the reverse ionic gradient, 363/83 CsCl (plusses), currents of very similar magnitudes were recorded with corresponding electrochemical gradients. The solid line is the result of applying a digital filter to the data points (Hamming, 1983). This procedure is used here because it yields a model-independent estimate of the shape of the i-E curve. In Fig. 1, the lines are essentially the same and cannot be distinguished.

2 mM BaCl₂ was regularly added to the outside solution to block the Ca-activated K channel (Meech, 1978). However, Ba²⁺ is in fact able to interact with
the ACh-activated channel; not only is \( \text{Ba}^{2+} \) permeant (D. J. Adams et al., 1980), but it is also able to block current carried by other ions (Dani and Eisenmann, 1985). The relative importance of the block by \( \text{Ba}^{2+} \) for the solutions used for this article is illustrated by the solid diamonds in Fig. 1, which give the amplitudes of the ACh-activated channels at +100 and −100 mV in a \( \text{Ba}^{2+} \)-free experiment with \( 83/363 \) CsCl. Each diamond represents the average of the mean currents measured in three different patches. The block by \( \text{Ba}^{2+} \) reduced inward currents by an average of 33% and outward currents by an average of 16%.

The guanidinium ion (\( \text{G}^+ \)) is a more permeant ion than \( \text{Cs}^+ \), since the current was seen to be outward at 0 mV with \( 123 \text{ Cs}^+//123 \text{ G}^+ \) (Fig. 2). The reversal potential was −11 mV, which indicates that \( P_{\text{G}}/P_{\text{Cs}} \), the relative permeability, was 1.6. However, the net outward current carried by \( \text{G}^+ \) was smaller than the net inward current carried by \( \text{Cs}^+ \), with conductances of 23 and 31 pS, respectively. When \( \text{Cs}^+ \) is the inside ion, both inward and outward currents are larger, with chord conductances of 38 and 39 pS (Dwyer and Farley, 1984). This reduction in conductance is not due to intermittent block of the channel on a millisecond time scale, since there is no increased noise or spiking closures during the period of time the channels are open. Using the model of D. J. Adams et al. (1981) to describe the block of outward current, the apparent one-sided binding constant of \( \text{G}^+ \) was 51 mM, using a \( K_D \) of 135 mM for \( \text{Cs}^+ \) (Dwyer and Farley, 1984).

In sharp contrast to \( \text{Cs}^+ \) and the simple amines (Fig. 1; Dwyer and Farley, 1984), pure \( \text{G}^+ \) carries less current for a given driving force when it is the outside cation than when it is the inside cation. Fig. 3 shows results from a series of experiments that illustrate this behavior; plotted in this figure are the amplitudes of currents recorded in \( 83 \text{ Cs}^+//103 \text{ G}^+ \) (circles) as well as the converse, \( 103 \text{ G}^+//83 \text{ Cs}^+ \) (triangles) (with the current and voltage both negated). The reversal potentials for these two solutions were −15 and +17, and so were close to the predicted values of ±16.8 mV as calculated from the observed relative permeability of 1.6. Outside \( \text{G}^+ \) (triangles) reduced the amplitudes of both the \( \text{G}^+ \) and \( \text{Cs}^+ \) currents to a greater extent than did \( \text{G}^+ \) added to the inside (circles).

**Channel Block in Mixtures of Permeant Ions**

With an apparent dissociation constant of 51 mM, 100 mM \( \text{G}^+ \) should block an appreciable fraction of the single channel current when added to a solution containing the more permeant ion \( \text{Cs}^+ \). This was indeed the case when \( \text{G}^+ \) was added to the internal solution. In the experiments of Fig. 4A, \( \text{G}^+ \) was added to 363 mM \( \text{Cs}^+ \). A low concentration of \( \text{G}^+ \) (20 mM) had no appreciable effect, but the addition of 100 mM \( \text{G}^+ \) reduced the outward single channel currents to 0.7 of that seen in 363 mM \( \text{Cs}^+ \) alone, as measured at 100 mV from the reversal potential. Inward currents were little affected.

\( \text{G}^+ \) added to the external solution also caused a block of ACh-activated single channel currents but the block differed from that caused by inside \( \text{G}^+ \) in three important ways (Fig. 4B). First, net currents carried in part by \( \text{G}^+ \) were blocked more effectively with \( \text{G}^+ \) on the outside than on the inside. As little as 20 mM external \( \text{G}^+ \) caused a noticeable block of inward current. The block by 20 and 100 mM \( \text{G}^+ \) was consistent with an apparent \( K_D \) of 29 mM at −100 mV. Second,
net currents carried by Cs⁺ alone were more effectively blocked by external G⁺. Outward Cs⁺ currents were reduced in size by more than half when 100 mM G⁺ was added to the outside solution, whereas the inward Cs⁺ currents discussed in the previous paragraph were little changed when G⁺ was the inside cation (Fig.

**Figure 2.** Currents recorded in isotonic Cs⁺//G⁺. (A) The amplitude of ACh-activated single channel currents in 125 mM CsCl (outside) and 125 mM GCl (inside) is plotted against the holding potential. Each point is the average of ~10 individual current amplitudes; the standard deviation is typically smaller than the symbol. The curves numbered 1 and 2 are computed from the GHK equations; curve 3 is computed from the two-well, three-barrier rate model. (B) Examples of individual currents are provided with the holding potential given on the right. The recordings at +61 and −61 mV are from patch 011985A and the remaining currents are from patch 011985G.
Third, the block of inward currents by external G\(^+\) was relieved by increasing the driving force: the slope conductances from the reversal potential to \(-60\) mV for 0, 20, and 100 mM G\(^+\) were 39, 26, and 15 pS, respectively, whereas the slope conductances from \(-60\) to \(-120\) mV were 40, 37, and 36 pS, respectively.

The increased ability of G\(^+\) to block from the outside is shown in Fig. 5A. This figure is plotted in the same way as Fig. 1 and shows the effect of 100 mM G\(^+\) added to 365 mM Cs\(^+\) in the inside and outside (current and voltage negated) solutions; the upper right quadrant contains the currents from the G\(^+\) compartment for both protocols. This asymmetry of block was also seen when 100 mM G\(^+\) was added to 83 mM Cs\(^+\) (Fig. 5B). The extent of the asymmetry was similar for 83 and 363 mM Cs\(^+\), with an outside addition of G\(^+\) reducing the currents to an amplitude of 0.5 (363 mM Cs\(^+\)) and 0.6 (83 mM Cs\(^+\)) of that seen for an inside addition of G\(^+\).

Reversal Potentials

On the basis of the value of \(P_G/P_Cs\) obtained from the experiment using 123 Cs\(^+\)//123 G\(^+\), reversal potentials \(V_r\) can easily be calculated based on the Goldman-Hodgkin-Katz (GHK) equation (Hodgkin and Huxley, 1952). Good agreement exists for protocols using a single species of cation, as shown in Table I. Thus, in protocols 4 and 5, which use solutions that contain only Cs, \(V_r\) values
were -36 and 36 mV, respectively; theory predicts ±36.1 mV. In solutions 2 and 3, which have either Cs⁺ or G⁺ but not both, Vᵢ was -17 and 18 mV, respectively, compared with the predicted value of ±16.6 mV.

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**FIGURE 4.** Guanidine added to the inside and outside solutions. (A) The i-E relation for 83//363 CsCl with 0 (○), 20 (△), and 100 (□) mM G⁺ added internally is plotted in the standard way. The theoretical curves are calculated from the two-well, three-barrier model; there is little difference among the three solutions. (B) The i-E relation for 363//83 CsCl with 0, 20, and 100 mM G⁺ added externally (same symbols as in A). The solid lines emphasize the block at negative potentials.
In experiments where solutions contained mixtures of Cs\(^+\) and G\(^+\), there was a systematic deviation from the \(V_\text{r}\) predicted by the GHK equation (Table I). In each case, the magnitude of \(V_\text{r}\) was smaller than predicted, and the difference

**Figure 5.** Asymmetry of block with the addition of G\(^+\). (A) The \(i-E\) relation for 83 Cs\(^+\)/\(363\) Cs\(^+\) plus 100 G\(^+\) (O) is plotted in the standard way; the \(i-E\) relation for 363 Cs\(^+\) plus 100 G\(^+\)/83 Cs\(^+\) (\(\Delta\)) is plotted with the values negated. These data are also plotted in Fig. 4, but are replotted here to offer a direct comparison. (B) Same experiment as in A, except that the G\(^+\) was added to 83 mM Cs\(^+\), not 363 mM. In both panels, the extra block caused by external application of G\(^+\) is relieved by increasing the driving force only when that current is carried in large part by G\(^+\) itself.
was greatest when G⁺ was added to the outside solution. Increasing the concentration of G⁺ from 20 to 100 mM increased the deviation, with the \( V_r \) of protocols 6 and 7 being 4 and -7 mV and that of protocols 8 and 9 being 5 and -12 mV away from predicted values, respectively. Decreasing the concentration of Cs from 363 to 83 mM decreased but did not abolish the effect.

### DISCUSSION

**Comparison with Previous Work**

Block by permeant metal ions. Horn and Patlak (1980) have shown that the single channel conductance of the ACh-activated channel saturates as the concentration of the inside permeant ion increases. When this saturation is significant, currents obtained in 83//363 CsCl, for instance, should be larger than predicted from the results obtained with 108//363 CsCl (Dwyer and Farley, 1984), once adjustments are made for the differences in ion concentration using the independence principle (Hille, 1984). If Cs ions moved independently, currents from these two experiments would be in a ratio of 0.9 at a holding potential of \( (V_r + 100) \) mV. Instead, the currents were measurably larger and gave a ratio of 1.3. This difference suggests that fewer channels are blocked when the concentration of Cs⁺ is lower, as if the \( K_D \) for outside Cs⁺ were changed from 135 to 250 mM by decreasing the internal Cs⁺ from 108 to 83 mM (D. J. Adams et al., 1981; Dwyer and Farley, 1984). A similar calculation can be made for the case of inside G⁺ plus either 123 or 83 mM Cs⁺ outside (Figs. 2 and 3).
In these cases, as external Cs\(^+\) was made less concentrated, the \(K_D\) for G\(^+\) increased from 51 to 70 mM.

**Permeant organic cations: homeotherms vs. poikilotherms.** By the reversal potential criterion, the present study has shown G\(^+\) to be 1.6 times more permeant than Cs\(^+\). This is similar to findings in other tissues: G\(^+\) has also been shown to be permeant at the amphibian neuromuscular junction (D. J. Adams et al., 1980), although with a smaller \(P_G/P_C\) = 1.1. A similar relationship was seen for another small organic ion, ammonia, with ratios of 1.9 and 1.3 for chick and frog (Dwyer and Farley, 1984; D. J. Adams et al., 1980). In contrast, the opposite is true for the permeability ratios of the larger organic cations. For instance, ethylamine has permeability ratios of 0.65 and 0.80 for chick and frog, respectively (Dwyer and Farley, 1984; D. J. Adams et al., 1980). This observation that permeability ratios decrease more sharply with increasing ionic diameter in chick than in frog suggests that the molecular structure about the selectivity filter is more constricting in chick than in frog.

Conversely, the single channel conductance for Cs\(^+\)/G\(^+\) was 23 pS, smaller by almost a factor of 2 than that for Cs\(^+\)/Cs\(^+\) (all at 123 mM, Fig. 2A; Dwyer and Farley, 1984). This observation is consistent with the fact that the addition of alkyl derivatives of G\(^+\) to the outside solution reduces the amplitude of nerve-evoked endplate currents in the frog (Farley et al., 1981) and the amplitude of single channel currents of chick ACh-activated channels (Farley et al., 1986). G\(^+\) itself also carries less current than Cs\(^+\) at the frog neuromuscular junction, where single channel currents resulting from iontophoresed ACh were smaller in a mixture of G\(^+\) and Na\(^+\) than in isotonic Na\(^+\) itself; analysis of the current noise was consistent with a \(K_D\) of 12 mM for G\(^+\) (D. J. Adams et al., 1980). This binding is much tighter than the \(K_D\) of 51 mM reported here for chick embryo. Other small organic cations also have larger conductances in homeotherms than in frog. For instance, ethylamine carries twice the current in chick as in frog (Dwyer and Farley, 1984). Similarly, dimethylamine has almost four times the conductance in mouse as in frog (Sanchez et al., 1986). Thus, not only does the selectivity filter differ between chick and frog, but so does the molecular structure about the channel that limits the flow of ions.

**Channel Symmetry**

As a first approximation, the channel opened by ACh at the frog neuromuscular junction or in chick embryonic muscle appears to be symmetrical with regard to the passage of ions (P. Adams, 1979). Indeed, over a range of 500 mV, the absolute amplitude of single channel currents depends only on the electrochemical gradient, and not on the side on which the solutions are placed, for solutions of Cs\(^+\) (Fig. 1). The same appears to be the case for Cs\(^+\) solutions with added arginine (Dwyer and Farley, 1984). However, this observed symmetry is surprising since the molecular structure has been shown to be markedly asymmetric (Young et al., 1985) and the pharmacological block by local anesthetics is localized to the outside mouth of the channel (Horn et al., 1980). The following paragraphs describe experiments where currents through the ACh-activated channel show
asymmetric properties that can be demonstrated in solutions of highly permeant ions.

**Asymmetry of Guanidine Action**

*Sidedness of block.* In all cases tested, G+ blocked more effectively when on the outside, as can be seen by directly comparing protocols 2 and 3, 6 and 7, and 8 and 9 (Figs. 3 and 5). In the first pair of protocols, outside G+ reduced both inward and outward currents relative to those seen with inside G+. As expected, adding G+ to an inside solution of 363 mM Cs+ did block current to some extent (Fig. 4A), but adding G+ to an outside solution of 363 mM Cs+ blocked the current to a much greater degree (Fig. 4B). Raising Cs+ from 83 to 363 mM had little effect on the relatively greater block seen when the contralateral G+ was external.

Two kinds of explanations exist for the different action of G+ added to the outside and inside solution. First, the presence of G+ in the outside solution may alter the structure of the channel (Gage and Van Helden, 1979; Fox and Ciani, 1985) or change the electric profile within the channel by affecting the ionic occupancy of the channel (Dani, 1986). It is possible under these circumstances that the apparent binding constant for G+ is greater when that cation is present in the outside solution.

Alternatively, there may be multiple binding sites within the channel (Hille and Schwarz, 1978; Dwyer and Farley, 1984). Such a model would have to explain two kinds of behavior. First, the binding sites would have to bind both the metal cations like Cs+ or Na+ and also the organic ions like G+ or the local anesthetics. These common sites would explain the apparent increase in the binding constant of G+ when the contralateral Cs+ is increased as described in this article, as well as the competition between metal ions and the binding of local anesthetics (Redmann, 1982). At least one of these sites must be closer to the outer edge of the channel than the selectivity filter to explain why the nonpermeant blockers are more effective when added to the outside than when added to the inside (Horn et al., 1980; Farley and Narahashi, 1983).

Second, the structure of the barriers and wells must be such as to explain the extra block seen when the highly permeant cation G+ is added to the outside, as opposed to the inside solution. This block is partially relieved at extremely negative potentials, where the driving force on G+ is the greatest. A similar result is seen for the block of the delayed rectifier K+ channel by the Na ion (French and Wells, 1977).

Finally, the model must explain the consistent deviation of the observed Vr from the value predicted from the GHK equation. The deviation is not due to a residual offset or error in gain of the patch clamp, because protocols with solutions that contain only a single ion, such as 4 and 5 and 2 and 3, give the predicted results. This lack of agreement is not likely to be due to junction potentials, ψ, because the ψ's were measured for all solutions, and E was adjusted accordingly. Moreover, the average of the magnitude of Vr for conjugate solutions (e.g., 8 and 9) is still smaller than predicted (this calculation should cancel any artifact caused by ψ). Finally, an effect on surface potential alone could not
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explain the finding, since the deviation is apparent when as little as 20 mM G⁺ is added to as much as 363 mM Cs⁺.

Other channels that show deviations from independence also show complex behavior of $V_r$, such that the relative permeability, $P_r/P_N$, appears to change, depending on the experimental conditions. In the Na channel of the squid axon, $P_{NH_4}/P_N$ falls as the inside NH₄ concentration is increased; external NH₄ does not have this effect. A two-well, three-barrier model can explain this behavior (Begenisich and Cahalan, 1980). The inwardly rectifying K⁺ channel shows an anomalous fall in $V_r$ as Ti replaces K⁺ in the outside solution (Hagiwara et al., 1977); a three-well, four-barrier model can explain this behavior (Hille and Schwarz, 1978).

Theory

The three lines in Fig. 2A illustrate three approaches to fitting theory to the observed current-voltage curve. The first two assume an independent movement of ions and use the GHK equation. One fits the equation to the reversal potential, but this results in an overestimation of the single channel currents. The second seeks to match the shape of the $i-E$ curve, but in so doing shifts the entire curve by +27 mV and gives an erroneously low relative permeability for G⁺. These two curves show that a satisfactory fit using the GHK equation is not possible. A reasonable conclusion is that permeant ions do not move in an independent manner, but must interact with the channel and each other.

An alternative approach is to assume that a model for the movement of ions across the membrane can be reduced to considering only a few hops (Eyring et al., 1949). The simplest model with one barrier is not able to explain any deviation from independence; a model with two barriers and a single well (Lewis and Stevens, 1979) does not mimic the very straight $i-E$ relation of the permeant metal cations (Dwyer and Farley, 1984), nor can it correctly model the degree of voltage dependence of G⁺ block in mixtures (Dwyer and Farley, 1984; Sanchez et al., 1986). The model used in this article consists of two energy wells and three barriers.

The currents recorded in solutions of Cs⁺ alone were fit first (protocols 2 and 3, Fig. 1). The wells and barriers were kept symmetrical to best fit the observed symmetry of the single channel currents. Values of $-4\,RT$ and greater allowed for a satisfactory fit with appropriate adjustments of the barrier height; a barrier spacing of $0.09-0.12$ gave fits of equivalent quality. Thus, the minimum value of $-4\,RT$ was used throughout the calculations and all three barriers were set accordingly; there appeared to be no reason to give the barriers different heights. A better estimate for the values of these parameters is not possible without further data at more concentrations of Cs⁺.

The energy profile for G⁺ was set using the data from protocols 4 and 5, where the solutions contained either Cs⁺ or G⁺ but not both. The height of the innermost barrier had to be greater than the other two. In addition, the outer well was required to be somewhat deeper than the inner one. The wells had to be within the voltage field of the membrane for an adequate fit, and the best results were seen with the barriers being symmetrically placed 18% from either
membrane edge. This placement of the wells is very similar to that deduced by Farley et al. (1986) for channel block caused by methyl- and ethylguanidine. The actual values used for the simulations in Figs. 3 and 4 are given in Table II.

Such a model fits the data in five important ways. First, G⁺ is a permeant blocker of the ACh-activated channel. The block becomes greater with increasing concentrations of G⁺, unlike that described for the small permeant blocker dimethylamine, which has a partial blocking effect at low concentrations that does not change as the concentration is further increased (Sanchez et al., 1986). Second, increasing the driving force to 100–150 mV beyond Vᵣ can overcome the block caused by G⁺. Third, Cs⁺ competes with G⁺ for permeation and can relieve block when the concentration of Cs⁺ is increased. Fourth, the magnitudes of the reversal potentials of ion mixtures are smaller than predicted by the GHK equation, although they are not in quantitative agreement. Finally, unlike other ions tested, G⁺ and the alkylguanidines are more effective at blocking the channel when they are placed outside the membrane than when they act from the inside. Arginine is an exception to this rule and is a large enough ion that it may be sterically hindered from access to the channel sites that make this behavior possible (Dwyer and Farley, 1984).

Residual deviations between the predicted and observed single channel currents remain, partially because it was not possible to include the effect of the permeant blocking cation Ba⁺, partially because the model for Cs⁺ may be incomplete, and partially because the simplifying assumption of three barriers and two wells may itself be inadequate. The fit deviates from that observed in Fig. 4B because it underestimates the degree of relief of block at extremely negative potentials. While the asymmetry of block is correctly predicted for Fig. 5, the reversal potentials are not in quantitative agreement; by necessity, this deviation made a correct fit of the amplitudes very difficult.

**Conclusion**

Four direct lines of evidence have been presented that ACh activates a channel with an energy profile that contains multiple wells and barriers: asymmetric block, lack of independence, a relief of block with increasing driving force, and an anomalous reversal potential sequence in mixtures of small-diameter ions. An Eyring rate model is presented in this report that contains two wells and three

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**Table II**

**Energy Barrier Profile of the Two-Well, Three-Barrier Model**

|        | G₁₂ | G₂₃ | G₃₄ | G₂ | G₃ |
|--------|-----|-----|-----|----|----|
| D      | 9%  | 50% | 91% | 18%| 82%|
| Cs⁺    | 8.0 | 8.0 | 8.0 | -4.0| -4.0|
| G⁺     | 7.0 | 7.25| 8.25| -6.2| -5.1|

The electrical distances (D) for the barriers and wells is given in the first line. G⁺ has arbitrarily been given the same electrical profile as Cs⁺. G₁₂ is the outermost barrier, G₂₃ the middle, and G₃₄ the innermost barrier. G₂ is the outer well and G₃ is the inner well. Values are in RT units.
barriers; the profile is symmetrical for Cs, but asymmetric for guanidine, with lower barriers and a deeper well on the outer side of the channel.

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