Diffusion Limitation in the Block by Symmetric Tetraalkylammonium Ions of Anthrax Toxin Channels in Planar Phospholipid Bilayer Membranes

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ABSTRACT Current flow through the channel formed in planar phospholipid bilayer membranes by the PA_{AB} fragment of anthrax toxin is blocked, in a voltage-dependent manner, by tetraalkylammonium ions (at micromolar concentrations), which bind to a blocking site within the channel lumen. We have presented evidence that diffusion plays a significant role in the kinetics of blocking by tetrabutylammonium ion (Bu_{4}N\(^{+}\)) from the cis (toxin-containing) side of the membrane (Blaustein, R. O., E. J. A. Lea, and A. Finkelstein. 1990. J. Gen. Physiol. 96:921–942); in this paper we examine the implications and consequences of diffusion control for binding kinetics. As expected for a diffusion-affected reaction, both the entry rate constant (k_{\text{in}}\)' of Bu_{4}N\(^{+}\) from the cis solution to the blocking site and the exit rate constant (k_{\text{out}}\) of Bu_{4}N\(^{+}\) from the blocking site to the cis solution are reduced if the viscosity of that medium is increased by the addition of dextran. In conformity with both thermodynamics and kinetic arguments, however, the voltage-dependent equilibrium binding constant, \(K_{\text{eq}}\) (=k_{\text{out}}\)/k_{\text{in}}\)' is not altered by the dextran-induced viscosity increase of the cis solution. The entry rate constants (k_{\text{in}}\) for tetrapentylammonium (Pe_{4}N\(^{+}\)), tetrahexylammonium (Hx_{4}N\(^{+}\)), and tetraheptylammonium (Hp_{4}N\(^{+}\)) are also diffusion controlled, and all of them, including that for Bu_{4}N\(^{+}\), attain a voltage-independent plateau value at large positive cis voltages consistent with diffusion limitation. Although the plateau value of k_{\text{in}}\) for Hx_{4}N\(^{+}\) is only a factor of 3 less than that for Bu_{4}N\(^{+}\), the plateau value for Hp_{4}N\(^{+}\) is a factor of 35 less. This precipitous fall in value indicates, from diffusion-limitation theory, that the diameter of the channel entrance facing the cis solution is not much larger than the diameter of Hp_{4}N\(^{+}\), i.e., \(~\)12 Å.

INTRODUCTION

Theories developed to describe diffusion-controlled reactions have helped clarify a wide variety of biologically relevant processes, including enzyme kinetics (Alberty and Hammes, 1958; Schurr, 1970b), receptor–ligand interactions (Berg and Purcell,
1977; DeLisi and Wiegel, 1981; Shoup and Szabo, 1982), and the growth of polymer chains, colloids, and crystals (Calef and Deutch, 1983). (See the monograph by Rice [1985] for discussion of theoretical and experimental aspects of diffusion-controlled reactions.) The effect of diffusion on the permeability characteristics of ion-conducting channels has been considered by several authors (e.g., Läuger [1976]; Latorre and Miller [1983]; Yellen [1984]) and has been demonstrated by Andersen (1983) for the case of ion movement through gramicidin A channels in planar phospholipid bilayer membranes.

In the preceding paper we presented evidence that diffusion plays a significant role in the kinetics of the blocking by tetrabutylammonium ions (Bu4N+), from the cis (toxin-containing) side of the membrane, of current flow through anthrax toxin channels (Blaustein et al., 1990). We argued that both the plateauing of the blocking rate at positive voltages (on the Bu4N+ side) and the value of the blocking rate constant are consistent with diffusion-limited movement of Bu4N+ ions to the channel entrance. In this paper we strengthen this argument by demonstrating that the voltage-independent component of the blocking rate of Bu4N+ is slowed when the Bu4N+-containing cis solution is made more viscous by the addition of dextran. We further find that at positive voltages <100 mV, for which there is a significant probability that Bu4N+ exits to the cis solution from the blocking site within the channel, and at negative voltages, where Bu4N+ is essentially in equilibrium between the cis solution and the blocking site, and where the contribution of diffusion to blocking is still significant, the unblocking rate is also slowed in the presence of dextran. As expected, however, the equilibrium constant for blocking is not affected.

By comparing the diffusion-limited blocking rates of symmetric tetraalkylammonium ions ranging from tetrabutyl- to tetraheptylammonium, we can infer the size of the channel entrance. Assuming that these ions diffuse to a disk (or hemisphere) with an effective capture radius (r_c) of r_disk = r_ion, we conclude from these blocking rates that the diameter of the channel entrance facing the cis solution is slightly larger than that of a tetraheptylammonium ion, i.e., ~12 Å.

MATERIALS AND METHODS

The formation of planar diphytanoylphosphatidylcholine (DPhPC) phospholipid bilayer membranes separating symmetric 0.1 molal KCl/1–5 millimolal EDTA, pH 6.6 solutions, the incorporation into them of a single channel formed by the PA65 fragment of anthrax toxin, the analyses of the data for the times spent by the channel in the tetraalkylammonium ion–induced blocked and unblocked states, and all other general aspects of the methodology were as described in the preceding paper (Blaustein et al., 1990). In experiments performed with dextran present in either the cis or trans compartment, a PA65 channel was first incorporated into the membrane under our usual conditions, and the solution in the appropriate compartment was then replaced (via perfusion) with the dextran-containing solution. As before, transmembrane voltages (V) are those of the cis compartment (the compartment to which PA65 was added) relative to that of the trans compartment, which is taken as zero. Solution viscosities and conductivities were measured with an Ostwald viscometer and a conductivity meter (Radiometer America Inc., Westlake, OH), respectively. Tetrabutylammonium (Bu4N+) bromide (puriss grade), tetrapentylammonium (Pe4N+) bromide (purum grade), tetrahexylammonium (Hex4N+) bromide (purum grade), and tetraheptylammonium (Hp4N+) bromide (purum grade) were purchased from Fluka Chemical Corporation (Ronkonkoma,
The activity of Bu₄N⁺ in a 38% (wt/vol) solution of dextran (average Mr ~ 10,000) in 0.1 molal KCl (50 g dextran dissolved in 100 ml of 0.1 molal KCl) was determined using an ion-selective electrode made from a 2% solution of potassium tetra-kis (p-chlorophenyl) borate in 1,2-dimethyl-3-nitrobenzene (Corning Medical, Medfield, MA). Such an electrode should be sensitive to Bu₄N⁺ in the micromolar range in the presence of 0.1 molal KCl (Oehme and Simon, 1976); in our hands it was sensitive to Bu₄N⁺ in the millimolar range. A calibration curve was constructed with dextran-free solutions, and from this the activity of Bu₄N⁺ in the dextran solution was calculated. The activity coefficient of Bu₄N⁺ in the dextran solution, relative to that in the dextran-free solution, turned out to be 1.77; in other words, 13 micromolar Bu₄N⁺ in a 38% dextran solution (which is 10 micromolar Bu₄N⁺) has an effective concentration of 23 micromolar. Using the same electrode as a K⁺-sensitive electrode (in the absence of Bu₄N⁺), we found no significant difference in K⁺ activity between the 0.1 molal KCl dextran solution and the 0.1 molal KCl dextran-free solution.

THEORY

We consider the case of a neutral phospholipid bilayer membrane containing a single PA₆₅ channel separating 0.1 molal KCl salt solutions. Assuming that blocker is present at a concentration [B⁺] in only one of the two solutions separated by the membrane, we can write two kinetic schemes to describe the blocking of the channel by a B⁺ ion. In the first (A) blocking results from the presence of B⁺ in the cis (PA₆₅-containing) solution, and in the second (B) it results from the presence of B⁺ in the trans solution:

\[
\begin{align*}
\text{cis} & \quad \text{trans} \\
B^+ + \text{OPEN} & \xrightarrow{k_{\text{cis}}^\text{open}} \text{BLOCKED} \xrightarrow{k_{\text{trans}}^\text{trans}} \text{OPEN} \\
& \quad \text{(Scheme A)} \\
\text{OPEN} & \xrightarrow{k_{\text{cis}}^\text{trans}} \text{BLOCKED} \xrightarrow{k_{\text{trans}}^\text{trans}} \text{OPEN} + B^+.
\end{align*}
\]

(Scheme B)

In these schemes the blocked state (which results from the occupation of a binding site in the channel by a B⁺ ion) is nonconducting, and, although not explicitly written, it is understood that the k's may be voltage dependent. From the blocked state, B⁺ can exit the channel to either the cis or trans solution. In the following discussion, however, we shall focus on the case of blocker in the cis solution (case A), and limit our analysis to situations in which a B⁺ ion entering the binding site from

1 The effect of dextran on Bu₄N⁺ activity is probably a consequence of the binding of water by the carbohydrate. This bound water is not available for Bu₄N⁺ to dissolve in, and hence its effective concentration (i.e., its activity) is increased. On the other hand, the smaller K⁺ ion, with its more intense electric field, is more hydrophilic than dextran and can successfully compete with it for water; therefore, the water "bound" by dextran is available for K⁺ to dissolve in, and hence its activity is not increased.
the cis solution almost always exits back to that cis solution; that is, $k_{-1}^{mu} \gg k_{-1}^{mu}$ (e.g., negative voltages for cis Bu$_4$N$^+$; see preceding paper [Blaustein et al., 1990]). The kinetics of block are then well described by a modification of scheme A:

$$B^+ + OPEN \xrightleftharpoons{\frac{k_1^{ou}}{k_{-1}^{ou}}} BLOCKED.$$  

(Scheme A')

In this case we can consider $B^+$ to be in equilibrium between the cis solution and the blocking site, and can therefore write an equation for the corresponding (voltage-dependent) equilibrium constant:

$$K_{eq} = \frac{k_1^{ou}}{k_{-1}^{ou}}.$$  

(1)

Experimentally, we measure distributions of blocked and unblocked times at a particular voltage and calculate the rate constants from the relations

$$k_1^{ou} = \frac{1}{\tau_u^{ou} \cdot [B^+]},$$  

(2)

$$k_{-1}^{ou} = \frac{1}{\tau_b},$$  

(3)

where $\tau_u^{ou}$ and $\tau_b$ are the mean unblocked and blocked times, respectively. In the following analysis we consider the effects that diffusion of $B^+$ to the channel entrance has on the blocking kinetics, treating first the blocking rate constant $k_1^{ou}$ (and the corresponding unblocked time constant, $\tau_u^{ou}$), and then considering the unblocking rate constant $k_{-1}^{ou}$ (and the corresponding blocked time constant, $\tau_b$).

**Effect of Diffusion on the Blocking Rate**

Let us consider three different situations corresponding to three ranges of voltage. Suppose, first, that at large positive voltages on the blocker side, $\tau_u^{ou}$ reaches a voltage-independent, diffusion-limited value called $\tau_u^{ou}$(diff). The corresponding diffusion-limited blocking rate constant $k_1^{ou}$(diff), is related to this mean unblocked time by the expression

$$k_1^{ou}$(diff) = \frac{1}{\tau_u^{ou}$(diff) \cdot [B^+]}.  

(4)

Thus for large positive voltages, when blocking proceeds as rapidly as diffusion will

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2 As we mentioned in the preceding paper (Blaustein et al., 1990), this is not a true equilibrium, since $K^+$ flows through the unblocked channel and thereby introduces a dissipative process into the system. Nevertheless, it is a reasonable approximation to apply equilibrium arguments to the interaction of $B^+$ with the blocking site.
allow, the blocking rate is diffusion limited, and we write

$$k_1^{u} = k_1^{u}(\text{diff})$$

(5)

and

$$r_1^{u} = r_1^{u}(\text{diff}).$$

(6)

(In terms of the barrier-well model of the preceding paper, we can imagine that the cis barrier has moved all the way to the cis side, sitting essentially at the channel entrance and "feeling" no effect of the transmembrane potential.)

At the other extreme, i.e., at large negative voltages, suppose that $r_1^{u}(\text{diff})$ and $k_1^{u}(\text{diff})$ contribute negligibly to $r_1^{u}$ and $k_1^{u}$, respectively. In this range, $r_1^{u}$ and $k_1^{u}$ are "fully" voltage dependent. (In terms of our barrier-well model, the cis barrier has already moved from its diffusion-limited location at the channel entrance to a stable location within the channel.) In this limit we write

$$r_1^{u} = r_1^{u}(V)$$

(7)

and

$$k_1^{u} = k_1^{u}(V).$$

(8)

At intermediate voltages there will be both a diffusional and a voltage-dependent contribution to blocking. (The barrier, in this case, sits in the channel somewhere [as a function of voltage] between the two previous extremes.) Under these circumstances, $k_1^{u}$ can be written as

$$k_1^{u} = \frac{k_1^{u}(\text{diff}) \cdot k_1^{u}(V)}{k_1^{u}(\text{diff}) + k_1^{u}(V)}$$

(9)

(Noyes, 1961; Schurr, 1970a). Taking the reciprocal of both sides, we get for $r_1^{u}$:

$$r_1^{u} = \frac{1}{r_1^{u}(\text{diff}) + r_1^{u}(V)}.$$
Notice that if Eqs. 9 and 11 are substituted into Eq. 1, we get:

$$K_{eq} = \frac{k_{eq}^{\text{out}}}{k_{eq}^{\text{in}}(V)} = \frac{k_{eq}^{\text{in}}(V)}{k_{eq}^{\text{out}}(V)}.$$ (12)

Thus, the equilibrium constant for the reaction depicted in scheme A' is independent of the value of $k_{eq}^{\text{out}}(\text{diff})$. This result, which follows from the kinetic analysis, is demanded by thermodynamics, as can be seen from the following consideration. If the viscosity of the cis solution is increased by the addition of a solute (such as dextran, in the experiment described in Results), it will produce a decrease in the value of $k_{eq}^{\text{out}}(\text{diff})$ (because the diffusion coefficient of B+ is decreased), and hence a decrease in the value of $k_{eq}^{\text{in}}$. But the equilibrium constant, $K_{eq}$, will not change, since it is a function only of thermodynamic parameters such as temperature and pressure, and therefore cannot be affected by a kinetic parameter such as viscosity. (The increase in viscosity, in addition to decreasing $k_{eq}^{\text{out}}$ [Eq. 9], must produce a corresponding decrease in $k_{eq}^{\text{in}}$ [Eq. 11] for $K_{eq}$ to remain unaltered.)

RESULTS

The Effect of Viscosity on the Mean Unblocked Time ($\tau_u^{\text{out}}$)

With Bu4N+ in the cis solution, the mean unblocked time of the channel, $\tau_u^{\text{out}}$, is only weakly voltage dependent, and plateaus at large positive voltages to a value of ~6.75 ms at a Bu4N+ concentration of 1 micromolar (Blaustein et al., 1990). We interpreted this to mean that $\tau_u^{\text{out}}$ is diffusion controlled by the rate at which Bu4N+ can diffuse (outside the electric field) from solution to the channel entrance. If this is correct, then increasing the viscosity of the cis solution, and thereby decreasing the diffusion constant of Bu4N+ in solution, should increase the value of $\tau_u^{\text{out}}$. We chose a large molecule, dextran ($M_r \approx 10,000$), rather than a small molecule such as glycerol, glucose, or sucrose, to increase the viscosity, because given the size of at least 12 Å inferred for the diameter of the PA65 channel (Blaustein et al., 1990), we wanted to insure that any observed effect on $\tau_u^{\text{out}}$ could not be attributed to the molecule entering the channel.

Fig. 1A compares a plot of $\tau_u^{\text{out}}$ (times [Bu4N+]) vs. voltage obtained in symmetric 0.1 molar KCI solutions with that obtained when the 0.1 molar KCI cis solution also contained 38% (wt/vol) dextran. (The conductance of the PA65 channel fell ~10% when the solution of 0.1 molar KCI plus 38% dextran was perfused into the cis compartment to replace the 0.1 molar KCI dextran-free solution.) Note that the plateau value of $\tau_u^{\text{out}}$ (for [Bu4N+] = 1 micromolar) at large positive voltages in the dextran experiment is ~17.8 ms, 2.6 times larger than the 6.75 ms value in the

5 Since $\tau_u^{\text{out}}$ is inversely proportional to the Bu4N+ concentration, it is convenient in discussing it to consider its value at a given concentration of Bu4N+, which we have chosen as 1 micromolar. In the preceding paper we chose this concentration as 1 micromolar; for the 0.1 molar KCI solution used in all the experiments reported there, molar and molal concentrations are essentially the same. Here, where we also report experiments using a 38% (wt/vol) dextran solution (0.1 molar KCI), there is a 30% difference in molar and molal concentrations. Since it is the molal concentration that is thermodynamically relevant, we express the Bu4N+ concentrations in molal units throughout this paper. The issue of molal and molar concentration units is considered further in the Discussion.
FIGURE 1. Effect of cis solution viscosity on the rate of blocking of PA65 channels by Bu₄N⁺. A, Linear plots of the mean time (τ) spent by a channel in the unblocked state (times [Bu₄N⁺]) as a function of voltage, with Bu₄N⁺ in the cis solution. The closed circles are from three separate experiments done in symmetric 0.1 molal KCl solutions. (These are the same data that are shown in Fig. 7 of the preceding paper [Blaustein et al., 1990]). The open circles are from an experiment in which the 0.1 molal KCl cis solution also contained 38% (wt/vol) dextran (average Mr ~ 10,000). (The concentrations of Bu₄N⁺ in the dextran-free experiments were 15 and 37 micromolar; its concentration in the dextran experiment was 34.5 micromolar.) The curve through the closed circles is Eq. 7 (times [Bu₄N⁺]) of Blaustein et al. (1990). The curve through the open circles is an upward parallel displacement of the lower curve by 17.8 × 10⁻⁹ molal/s. (The amount of displacement was determined by eye to give the best fit to the open circles.) B, Linear plots of kₐ as a function of voltage. The closed circles are from three separate experiments done in symmetric 0.1 molal KCl solutions and the open circles are from an experiment in which the 0.1 molal KCl cis solution also contained 38% (wt/vol) dextran (average Mr ~ 10,000). These are the same experiments as described in A; the values of the data points are simply the reciprocals of those shown there. The curve through the closed circles is Eq. 7 of Blaustein et al. (1990).
absence of dextran. (The corresponding plateau value of $k_t'$ decreases 2.6-fold from its value of $1.48 \times 10^8$ m$^{-1}$ s$^{-1}$ in the absence of dextran to $0.56 \times 10^8$ m$^{-1}$ s$^{-1}$ in the dextran experiment [Fig. 1 B].) In addition, the entire $r_u'$ vs. $V$ curve obtained in the presence of dextran is shifted upward (at [Bu$_4$N$^+$] = 1 micromolar) by ~11.0 ms (17.8 to 6.75 ms) from the curve obtained in its absence. This is just what one expects (see Eq. 10) if $r_u'$ is diffusion affected; that is, an increase in viscosity increases $r_u'(\text{diff})$, and this in turn adds linearly to the value of $r_u'$.

The magnitude of the increase in the plateau value of $r_u'$ produced by dextran is remarkably consistent with the magnitude of the increase in microviscosity it produces. The 38% dextran solution used in our experiments has about a 32-fold larger viscosity than that of water, as determined by flow rates in an Ostwald viscometer. This macroscopic measure of viscosity, however, is not what is relevant to the effect of viscosity on the diffusion coefficient of a molecule such as Bu$_4$N$^+$, which is small relative to $M_r = 10,000$ dextran. A Bu$_4$N$^+$ molecule wandering around in solution experiences a microviscosity much smaller than the viscosity determined from bulk flow of the dextran solution. Instead of measuring the diffusion coefficient (or mobility) of Bu$_4$N$^+$ in the dextran solution, however, which is not a simple task, we took as an approximation of dextran's effect on Bu$_4$N$^+$ mobility its effect on K$^+$ and Cl$^-$ mobility as determined from a conductivity measurement. The conductivity of the 0.1 molal KCl solution containing 38% dextran was 2.7-fold smaller than the conductivity of dextran-free 0.1 molal KCl. This implies a microviscosity effect of the dextran on the diffusion coefficient of Bu$_4$N$^+$ of ~2.1-fold, which is in good agreement with its 2.6-fold effect on the plateau value of $r_u'$.

It is conceivable that the effect of dextran on $r_u'$ is unrelated to its effect on the diffusion coefficient of Bu$_4$N$^+$ in solution, but is instead a consequence of some direct action of dextran on the PA$_{a5}$ channel. The effect of dextran on the mean blocked time ($r_b$), described in the next section, argues against this. As we shall see, the equilibrium constant of Eq. 1 is not affected by dextran; this is exactly what is predicted if dextran acts only through its effect on solution “viscosity,” but it is not anticipated if dextran alters the intrinsic properties of the channel. Also arguing against an effect of dextran on intrinsic channel properties is the result of our “control” experiment in which the trans rather than the cis compartment contained the solution of Bu$_4$N$^+$ in 38% dextran. As we showed previously (Blaustein et al., 1990), the mean unblocked time with Bu$_4$N$^+$ in the trans compartment ($r_u'$ trans), unlike $r_u'$ cis, is not diffusion limited, and hence $r_u'$ trans should not be increased by dextran. Indeed, with the 38% dextran solution in the trans compartment, there was only an ~30% increase of $r_u'$ trans values in the voltage range $-60$ to $-100$ mV, in contrast to the 2.6-fold increase in the plateau value of $r_u'$ with the dextran solution in the cis compartment. (We noted previously [Blaustein et al., 1990] that there should be some contribution of diffusion to $r_u'$ trans for voltages less than $-60$ mV, although we

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4 The 2.7-fold reduction in conductivity of the 0.1 molal KCl solution is not entirely attributable to the increase in microviscosity produced by dextran, since a 1.3-fold reduction occurs simply because of the volume occupied by dextran, which excludes KCl and thereby reduces the number of K$^+$ and Cl$^-$ ions present per unit volume of solution. In other words, the conductivity of a 38% dextran solution of 0.1 molal KCl is 2.1-fold (not 2.7-fold) less than that of dextran-free 0.1 molal KCl. This point is considered further in the Discussion.
were unable to resolve it; the small effect on $r_u^{\text{trans}}$ values, produced by dextran in the trans compartment, may therefore be real; that is, it may be consistent with dextran's increasing $r_u^{\text{trans}}(\text{diff})$.

The Effect of Viscosity on the Unblocking Rate Constant ($k_{-1}^{\text{cis}}$)

As noted in the Theory section, if $k_0^{\text{cis}}$ is diffusion affected, $k_1^{\text{cis}}$ is similarly affected; therefore, the expected reduction of $k_0^{\text{cis}}$ produced by an increase in viscosity of the cis solution should be accompanied by a comparable reduction of $k_{-1}^{\text{cis}}$. This prediction is strikingly confirmed in Figs. 2 and 3. Fig. 2 compares a plot of $k_{ex}$ vs. voltage obtained in symmetric 0.1 m KCl solutions with that obtained when the 0.1 m KCl cis solution also contained 38% (wt/vol) dextran. $k_{ex}$ is the exit (or unblocking)

![Figure 2. Effect of cis solution viscosity on the exit or unblocking rate constant ($k_{ex}$) of Bu$_4$N$^+$ from the blocking site in a PA$_{65}$ channel. The closed circles are from a number of experiments done in symmetric 0.1 molal KCl solutions, with Bu$_4$N$^+$ in either the cis or trans solution at concentrations ranging from 15 micro-molal to 2.5 millimolal. (These are the same data that are presented in Fig. 5 of the preceding paper [Blaustein et al., 1990], except that the reciprocal of those values are plotted here.) The open circles are from the same experiment described in Fig. 1 A, in which the 0.1 molal KCl cis solution also contained 38% (wt/vol) dextran (average $M_r \sim 10,000$).

rate constant of Bu$_4$N$^+$ from the blocking site and is equal to the sum of the exit rate constants to the cis and trans solutions in schemes A and B:

$$k_{ex} = k_{-1}^{\text{cis}} + k_{-1}^{\text{trans}}.$$ (13)

(For those who prefer to think in terms of the mean dwell time, $\tau_b$, of the channel in the blocked state, recall that $k_{ex}$ is simply the reciprocal of $\tau_b$.) At large positive

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5 Whereas in describing the effect of viscosity on blocking rate kinetics it is advantageous to focus on $\tau_b$ rather than $k_0^{\text{cis}}$, because of the simpler form of Eq. 10 compared with that of Eq. 9 (compare also Fig. 1 A to Fig. 1 B), there is no similar advantage gained in choosing $\tau_b$ over $k_0^{\text{cis}}$. Since subsequent discussions of $K_m$ of Eq. 1 deal with the $k's$ rather than with the $r's$, we describe the results in this section in terms of $k_0^{\text{cis}}$ instead of $\tau_b$. 

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voltages, where Bu₄N⁺ almost always exits to the trans compartment (and hence $k_{ex} = k_{-1}^{trans}$), the values of $k_{ex}$ in the dextran and dextran-free experiments are the same. In contrast, for modest positive voltages, at which Bu₄N⁺ can exit to either compartment, and for negative voltages, where Bu₄N⁺ almost always exits to the cis solution (and hence $k_{ex} \approx k_{-1}^{cis}$), the values of $k_{ex}$ in the dextran experiment are substantially lower than those in the dextran-free experiments. In Fig. 3 we see that the effect on $k_{ex}$ of dextran in the cis compartment is precisely that anticipated from its effect on $k_{1}^{cis}$ described in the previous section. Namely, at negative voltages, where Bu₄N⁺ is essentially in equilibrium between the cis compartment and the blocking site, and $k_{ex} = k_{-1}^{cis}$, $K_{eq}$ (which is equal to $k_{-1}^{cis}/k_{1}^{cis} \approx k_{ex}/k_{1}^{cis}$) is unaltered by the dextran-induced viscosity increase of the cis solution.

In contrast to the above-mentioned effects produced by dextran in the cis compartment, $k_{ex}$ was totally unaffected by dextran in the trans compartment, our “control” experiment. In particular, at large positive voltages with Bu₄N⁺ in the cis compartment and the 38% dextran solution in the trans compartment, the values of $k_{ex} (= k_{-1}^{trans})$ were the same as in the absence of dextran. This result is in complete accord with expectations; since $k_{1}^{trans}$ is diffusion independent (Blaustein et al., 1990),

**Figure 3.** Demonstration that the equilibrium that exists (at negative voltages) for Bu₄N⁺ between the cis solution and the blocking site in the PA₆₅ channel is not a function of cis solution viscosity. This is a semilogarithmic plot of $k_{ex}/k_{1}^{cis}$ vs. voltage, where $k_{ex}$ is the exit rate constant of Bu₄N⁺ from the blocking site in the channel, and $k_{1}^{cis}$ is its entry rate constant from the cis solution. At the negative voltages shown, Bu₄N⁺ almost always exits to the cis solution, so that $k_{ex} = k_{-1}^{cis}$, and hence the ordinate is the equilibrium constant $K_{eq} (= k_{-1}^{cis}/k_{1}^{cis})$ of Eq. 1 of the text. The closed circles are calculated from the average values of all $k_{ex}$ and $k_{1}^{cis}$ experiments done in symmetric 0.1 molal KCl solutions; these values were read off, at voltages corresponding to those used in the dextran experiment, from Figs. 5 and 7 of Blaustein et al. (1990). The open circles are from an experiment in which the 0.1 molal KCl cis solution also contained 38% (wt/vol) dextran (average $M_r \sim 10,000$). Their values were calculated from the corresponding points for $k_{ex}$ and $k_{1}^{cis}$ in Figs. 2 and 1 B, respectively.
an increase in viscosity of the \textit{trans} solution should not (and does not) decrease $k_{-1}^{\text{trans}}$.

**The Effect of Tetraalkylammonium Size on $\tau_u^{\text{diff}}$**

If the plateau value of $\tau_u^{\text{diff}}$ obtained at large positive voltages with $\text{Bu}_4\text{N}^+$ in the \textit{cis} compartment is indeed a consequence of diffusion limitation, an interesting prediction can be made about the dependence of this value on tetraalkylammonium size. The plateau value [$\tau_u^{\text{diff}}$] should be inversely proportional to both the diffusion coefficient of the ion ($D_{\text{ion}}$) and its capture radius ($r_c$). If we model the channel entrance as a circular disk, then the capture radius is proportional to the difference between the disk radius ($r_{\text{disk}}$) and the ion radius ($r_{\text{ion}}$). Thus,

$$\frac{1}{D_{\text{ion}}(r_{\text{disk}} - r_{\text{ion}})}.$$ \hspace{1cm} (14)

In going from tetrabutylammonium to tetraheptylammonium, $r_{\text{ion}}$ increases from ~5 Å to 6 Å (Robinson and Stokes, 1959). This means that there is an ~20% difference between $D_{\text{Bu}_4\text{N}^+}$ and $D_{\text{Hp}_4\text{N}^+}$, which should have, according to Eq. 14, a comparably small effect on $\tau_u^{\text{diff}}$. On the other hand, as the radius of the symmetric tetraalkylammonium ion approaches that of the disk, the difference between them becomes very small, and consequently, according to Eq. 14, $\tau_u^{\text{diff}}$ should increase enormously. Thus, if at some point in going from $\text{Bu}_4\text{N}^+$ to $\text{Hp}_4\text{N}^+$ the value of $\tau_u^{\text{diff}}$ takes off, the radius of the ion that produces this explosion closely approximates the radius of the entrance to the PA65 channel.

Fig. 4 shows a plot of $\tau_u^{\text{diff}}$ vs. voltage for $\text{Bu}_4\text{N}^+$, $\text{Pe}_4\text{N}^+$, $\text{Hx}_4\text{N}^+$, and $\text{Hp}_4\text{N}^+$. The curves all have a very similar shape, and all plateau at large positive voltages to a value, $\tau_u^{\text{diff}}$, characteristic for the particular ion. The trend in the values for $\tau_u^{\text{diff}}$ is striking. The value for $\text{Pe}_4\text{N}^+$ is essentially the same as that for $\text{Bu}_4\text{N}^+$, the value for $\text{Hx}_4\text{N}^+$ is ~3-fold larger, but the value for $\text{Hp}_4\text{N}^+$ is ~35-fold larger than that for $\text{Bu}_4\text{N}^+$. This is the expected trend if the radius of the channel entrance is close to that of $\text{Hp}_4\text{N}^+$. We therefore conclude that the radius of the entrance to the PA65 channel from the \textit{cis} side is ~6 Å, and by a well-known transformation, this means that the diameter is ~12 Å.

**DISCUSSION**

The voltage dependence of the entry rate of $\text{Bu}_4\text{N}^+$ into the blocking site of a PA65 channel is weak when $\text{Bu}_4\text{N}^+$ is entering the site from the \textit{cis} solution. $\tau_u^{\text{diff}}$ becomes significantly voltage dependent only for \textit{cis} voltages more negative than ~40 mV; at large positive voltages $\tau_u^{\text{diff}}$ levels off to a voltage-independent value of ~6.75 ms (for a $\text{Bu}_4\text{N}^+$ concentration of 1 micromolar). In the preceding paper (Blaustein et al., 1990) we proposed that this plateauing of $\tau_u^{\text{diff}}$ at large positive voltages results from its being diffusion limited by the rate at which $\text{Bu}_4\text{N}^+$ can diffuse from the \textit{cis} solution to the channel entrance, and we offered two arguments to support this proposal. In this paper we have presented two additional arguments that the entry rate of blocking ion from the \textit{cis} solution to the blocking site is diffusion controlled and have also stressed that a necessary consequence of this is that the exit rate of
blocking ion from the site in the channel to the cis solution must also be diffusion controlled.

Evidence for Diffusion Limitation on Entry Rate

Let us recapitulate our four arguments for diffusion limitation. (a) The voltage independence of \( \tau_u^a \) (at large positive voltages) means that the rate-limiting step for entry of \( \text{Bu}_4\text{N}^+ \) into the blocking site is occurring in a region that does not sense the electric field. The solution near the entrance of the channel, to which \( \text{Bu}_4\text{N}^+ \) must diffuse before entering the channel, is such a voltage-insensitive region. (b) The magnitude of \( 1.48 \times 10^8 \text{ mol}^{-1} \text{ s}^{-1} \) for the plateau value of the entry rate constant \( k_u' = \frac{1}{(\tau_u^a \cdot [\text{Bu}_4\text{N}^+] )} \) is consistent with diffusion limitation. (c) Raising the micro-viscosity of the cis solution by a factor of 2.1 with dextran (a large molecule that cannot enter the channel), and thereby reducing the diffusion coefficient of \( \text{Bu}_4\text{N}^+ \) in the cis solution by this amount, lowers the plateau value of \( k_u' \) by a factor of 2.6. This confirms that the rate-limiting step for \( \text{Bu}_4\text{N}^+ \) entry into the blocking site is its diffusion from solution to the channel entrance. (d) In going from tetrabutylammonium to tetrahexylammonium there is only about a threefold increase in the plateau value of \( k_u' \), but in going from there to tetraheptylammonium there is a precipitous rise in the value by another factor of 12. If the plateau value of \( \tau_u^a \) is determined by the rate at which the blocking ion can diffuse to the channel entrance from solution, it should be inversely proportional to the difference between the radius of the channel entrance and the radius of the blocking ion. One therefore anticipates that the plateau value of \( \tau_u^a \) will be relatively the same for all symmetric tetraalkylammno-
nium ions until an ion is reached whose radius is close to that of the channel entrance, at which point the plateau value increases dramatically.

**Effect of Solution Viscosity on Kinetics**

In discussing diffusion limitation above, and in particular in discussing the effect of cis solution viscosity on the channel-blocking action of Bu₄N⁺, we have focused on the blocking rate constant \( k_{\text{on}} \) (or, equivalently, the mean unblocked time, \( r_{\text{on}} \)) because diffusion limitation is usually thought of in terms of the "forward" reaction in a bimolecular reaction (in our case, the association of Bu₄N⁺ with a site in the channel); it is obvious that if the diffusion coefficients of the reacting species in a diffusion-affected reaction are decreased by raising the viscosity of the medium, the forward rate constant is reduced. As we emphasized in the Theory section, however, thermodynamics demands (and kinetic analysis confirms) that the "back" reaction (in our case, the dissociation of Bu₄N⁺ from the channel site) must be equally affected by a change in the viscosity of the medium. Thus in our system a change in viscosity of the cis solution must decrease \( k_{\text{on}} \) (the exit rate constant of Bu₄N⁺ from the channel site to the cis solution) to the same extent that it decreases \( k_{\text{on}} \). We see this clearly confirmed in Figs. 2 and 3. (At negative voltages and modest positive voltages, when the exit of Bu₄N⁺ from the channel site is almost always to the cis solution, \( k_{\text{on}} \) is essentially equal to \( k_{\text{on}} \), the unblocking [or exit] rate constant shown in Figs. 2 and 3.) The kinetic explanation for the effect of diffusion on the back reaction is that the dissociated reactants can reassociate before they have diffused apart (Schurr, 1970a). In our system this means that a Bu₄N⁺ exiting the blocking site can be recaptured before it has a chance to diffuse away. In principle, there is blocking and unblocking flickering (or excess noise) associated with recapture, but we have made no attempt to resolve it.

The quantitation of the effect of the dextran-induced increase of cis solution viscosity on the kinetics of channel blocking and unblocking by Bu₄N⁺ raises theoretical and practical issues of a general nature, not confined to this particular system, that are worth airing. In comparing the data obtained in the presence of dextran with those obtained in its absence, there is no question that from a thermodynamic viewpoint the comparison should be made at the same molar activity of Bu₄N⁺, rather than at the same molal activity. There the symmetry of the effect of viscosity on \( k_{\text{on}} \) and \( k_{\text{on}} \), demanded by thermodynamics (see Theory), is apparent, and serves as a check that the effects of dextran on the k's are not a consequence of some modification by dextran of intrinsic channel properties. (In fact, it was an apparent violation of this symmetry that made us suspect that dextran substantially increased the activity coefficient of Bu₄N⁺ in solution, and therefore prompted us to measure Bu₄N⁺ activity with an ion-selective electrode [see Materials and Methods].) From a kinetic viewpoint, however, in terms of the effect of viscosity on the diffusion coefficient of Bu₄N⁺ in solution, molal rather than molal activity is the appropriate concentration unit.

To estimate the effect of the dextran-induced viscosity increase on the diffusion coefficient of Bu₄N⁺, we measured its effect on the diffusion coefficient of KCl (which is equal to that of K⁺) by a conductivity measurement. (This measures the electrical mobilities of ions, which in turn are directly related to their diffusion
coefficients via the Nernst-Einstein relation.) The conductivity of 0.1 molal KCl in our 38% (wt/vol) dextran solution was 2.7-fold less than that of 0.1 molal KCl (~0.1 molar KCl) in the absence of dextran. Making the comparison at equal molarities, the factor is 2.1. (In 38% dextran the molarity of KCl or Bu4N+ is 1.3 times smaller than its molality.) This is probably an underestimation of dextran’s effect on the diffusion coefficient of Bu4N+, since this ion should feel a greater microviscosity than the smaller K+.

How large an effect does the dextran-induced increase of *cis* solution viscosity produce in the kinetics of channel block by Bu4N+? The diffusion-limited value of $k_{1}^w$ in the dextran solution was a factor of 2.6 smaller than its dextran-free value at the same *molarity*. At the same *molarity*, which is the kinetically relevant term, this becomes a factor of 2.0, in good agreement with the factor of 2.1 from the conductivity measurement (with, of course, the caveat that the effect of viscosity on the diffusion coefficient of Bu4N+ is probably greater than that on the diffusion coefficient of K+). The effect on $k_{-1}^{n}$ is somewhat larger. (Note that in considering $k_{-1}^{n}$, the issue of molarity and molality does not enter, since its value is independent of the concentration of Bu4N+ in solution.) At a voltage of −23 mV, where the k’s are not even completely diffusion limited, the value of $k_{-1}^{n}$ in the dextran experiment is a factor of 2.3-fold less than that in the dextran-free experiment. We believe that this greater effect of dextran on $k_{-1}^{n}$ compared with its effect on $k_{1}^{n}$ using *molar concentrations* is real and is a necessary consequence of the required symmetry of the effect of dextran on $k_{-1}^{n}$ compared with its effect on $k_{1}^{n}$ using *molal concentrations*.

**The Size of the PA65 Channel**

On the basis that tetraheptylammonium ion can traverse the PA65 channel (Blaustein et al., 1990), we can conclude that the channel has a diameter of at least 12 Å. To the extent that the alkyl tails of this ion can insinuate themselves in hydrophobic regions of the channel wall, this may be an overestimate, and the channel diameter available to more hydrophilic ions could be less. It is interesting, however, that through the analysis of the diffusion-limited rate constant $k_{1}^{w}$ we have an independent estimate of the diameter of the channel entrance (at the *cis*-facing end) that is in good agreement with the above value. Namely, the precipitous decrease of $k_{1}^{w}$ in going from tetrahexyl- to tetraheptylammonium (Fig. 4) indicates that the difference between the diameter of the channel entrance and the diameter of Hp4N+ (which is ~12 Å) is very small. Thus the diameter of the entrance to the channel (at the *cis* side) appears to be not much larger than 12 Å.

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