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_{65} 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_4N^+) 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_1^{cis}) of Bu_4N^+ from the *cis* solution to the blocking site and the exit rate constant (k_{-1}^{cis}) of Bu_4N^+ 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_{eq} ($=k_{-1}^{cis}/k_1^{cis}$), is not altered by the dextran-induced viscosity increase of the *cis* solution. The entry rate constants (k_1^{cis}) for tetrapentylammonium (Pe_4N^+), tetrahexylammonium (Hx_4N^+), and tetraheptylammonium (Hp_4N^+) are also diffusion controlled, and all of them, including that for Bu_4N^+, attain a voltage-independent plateau value at large positive *cis* voltages consistent with diffusion limitation. Although the plateau value of k_1^{cis} for Hx_4N^+ is only a factor of 3 less than that for Bu_4N^+, the plateau value for Hp_4N^+ 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_4N^+, 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, 1970*b*), receptor–ligand interactions (Berg and Purcell,

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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 (Bu_4N^+), 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 Bu_4N^+ side) and the value of the blocking rate constant are consistent with diffusion-limited movement of Bu_4N^+ ions to the channel entrance. In this paper we strengthen this argument by demonstrating that the voltage-independent component of the blocking rate of Bu_4N^+ is slowed when the Bu_4N^+-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 Bu_4N^+ exits to the *cis* solution from the blocking site within the channel, and at negative voltages, where Bu_4N^+ 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 PA_{65} 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 PA_{65} 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 PA_{65} 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 (Bu_4N^+) bromide (puriss grade), tetrapentylammonium (Pe_4N^+) bromide (purum grade), tetrahexylammonium (Hx_4N^+) bromide (purum grade), and tetraheptylammonium (Hp_4N^+) bromide (purum grade) were purchased from Fluka Chemical Corporation (Ronkonkoma, NY); dextran T10 (average $M_r \sim 10{,}000$ [light scattering]) was from Pharmacia (Uppsala, Sweden).

The activity of Bu_4N^+ in a 38% (wt/vol) solution of dextran (average $M_r \sim 10{,}000$) 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 Bu_4N^+ in the micromolar range in the presence of 0.1 molal KCl (Oehme and Simon, 1976); in our hands it was sensitive to Bu_4N^+ in the millimolar range. A calibration curve was constructed with dextran-free solutions, and from this the activity of Bu_4N^+ in the dextran solution was calculated. The activity coefficient of Bu_4N^+ in the dextran solution, relative to that in the dextran-free solution, turned out to be 1.77; in other words, 13 *micromolal* Bu_4N^+ in a 38% dextran solution (which is 10 *micromolar* Bu_4N^+) has an effective concentration of 23 *micromolal*. Using the same electrode as a K^+-sensitive electrode (in the absence of Bu_4N^+), 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.[1]

THEORY

We consider the case of a neutral phospholipid bilayer membrane containing a single PA_{65} 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* (PA_{65}-containing) solution, and in the second (B) it results from the presence of B^+ in the *trans* solution:

cis *trans*

$$B^+ + \text{OPEN} \underset{k_{-1}^{cis}}{\overset{k_1^{cis}\cdot[B^+]}{\rightleftharpoons}} \text{BLOCKED} \xrightarrow{k_{-1}^{trans}} \text{OPEN}$$

(Scheme A)

$$\text{OPEN} \xleftarrow[k_{-1}^{cis}]{} \text{BLOCKED} \underset{k_1^{trans}\cdot[B^+]}{\overset{k_{-1}^{trans}}{\rightleftharpoons}} \text{OPEN} + B^+.$$

(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

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[1] The effect of dextran on Bu_4N^+ activity is probably a consequence of the binding of water by the carbohydrate. This bound water is not available for Bu_4N^+ 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, $k_{-1}^{cis} \gg k_{-1}^{trans}$ (e.g., negative voltages for *cis* Bu_4N^+; see preceding paper [Blaustein et al., 1990]). The kinetics of block are then well described by a modification of scheme A:

$$B^+ + \text{OPEN} \underset{k_{-1}^{cis}}{\overset{k_1^{cis}\cdot[B^+]}{\rightleftharpoons}} \text{BLOCKED}.$$

(Scheme A′)

In this case we can consider B^+ to be in equilibrium between the *cis* solution and the blocking site,[2] and can therefore write an equation for the corresponding (voltage-dependent) equilibrium constant:

$$K_{eq} = \frac{k_{-1}^{cis}}{k_1^{cis}}. \qquad (1)$$

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

$$k_1^{cis} = \frac{1}{\tau_u^{cis}\cdot[B^+]} \qquad (2)$$

$$k_{-1}^{cis} = \frac{1}{\tau_b}, \qquad (3)$$

where τ_u^{cis} and τ_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^{cis} (and the corresponding unblocked time constant, τ_u^{cis}), and then considering the unblocking rate constant k_{-1}^{cis} (and the corresponding blocked time constant, τ_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, τ_u^{cis} reaches a voltage-independent, diffusion-limited value called $\tau_u^{cis}(\text{diff})$. The corresponding diffusion-limited blocking rate constant $k_1^{cis}(\text{diff})$, is related to this mean unblocked time by the expression

$$k_1^{cis}(\text{diff}) = \frac{1}{\tau_u^{cis}(\text{diff})\cdot[B^+]}. \qquad (4)$$

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

$$k_1^{cis} = k_1^{cis}(\text{diff}) \tag{5}$$

and (diffusion limit)

$$\tau_u^{cis} = \tau_u^{cis}(\text{diff}). \tag{6}$$

(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 $\tau_u^{cis}(\text{diff})$ and $k_1^{cis}(\text{diff})$ contribute negligibly to τ_u^{cis} and k_1^{cis}, respectively. In this range, τ_u^{cis} and k_1^{cis} 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

$$\tau_u^{cis} = \tau_u^{cis}(V) \tag{7}$$

and (nondiffusion limit)

$$k_1^{cis} = k_1^{cis}(V). \tag{8}$$

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^{cis} can be written as

$$k_1^{cis} = \frac{k_1^{cis}(\text{diff}) \cdot k_1^{cis}(V)}{k_1^{cis}(\text{diff}) + k_1^{cis}(V)} \tag{9}$$

(Noyes, 1961; Schurr, 1970*a*). Taking the reciprocal of both sides, we get for τ_u^{cis}:

$$\tau_u^{cis} = \tau_u^{cis}(\text{diff}) + \tau_u^{cis}(V). \tag{10}$$

Effect of Diffusion on the Unblocking Rate

The effect of diffusion on the unblocking rate is perhaps less obvious than its effect on the blocking rate. Mechanistically it arises from the possibility that a B^+ that leaves the blocking site will be recaptured before it has a chance to diffuse away, with the extent to which this occurs being a function of how diffusion-controlled the blocking reaction is. (See the excellent paper by Schurr [1970*a*] for what is, to our knowledge, the first discussion of the effect of diffusion on the kinetics of dissociation in a bimolecular reaction, and for a derivation of Eq. 11, which follows.) It can be shown from kinetic arguments that

$$k_{-1}^{cis} = \frac{k_1^{cis}(\text{diff}) \cdot k_{-1}^{cis}(V)}{k_1^{cis}(\text{diff}) + k_1^{cis}(V)}, \tag{11}$$

where $k_{-1}^{cis}(V)$ is the "fully" voltage-dependent unblocking rate, defined analogously to $k_1^{cis}(V)$ above.

Notice that if Eqs. 9 and 11 are substituted into Eq. 1, we get:

$$K_{\mathrm{eq}} = \frac{k_{-1}^{cis}}{k_1^{cis}} = \frac{k_{-1}^{cis}(V)}{k_1^{cis}(V)} . \tag{12}$$

Thus, the equilibrium constant for the reaction depicted in scheme A′ is independent of the value of k_1^{cis}(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_1^{cis}(diff) (because the diffusion coefficient of B^+ is decreased), and hence a decrease in the value of k_1^{cis}. 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_1^{cis} [Eq. 9], must produce a corresponding decrease in k_{-1}^{cis} [Eq. 11] for K_{eq} to remain unaltered.)

RESULTS

The Effect of Viscosity on the Mean Unblocked Time (τ_u^{cis})

With Bu_4N^+ in the *cis* solution, the mean unblocked time of the channel, τ_u^{cis}, is only weakly voltage dependent, and plateaus at large positive voltages to a value of ~6.75 ms at a Bu_4N^+ concentration of 1 micromolal (Blaustein et al., 1990).[3] We interpreted this to mean that τ_u^{cis} is diffusion controlled by the rate at which Bu_4N^+ 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 Bu_4N^+ in solution, should increase the value of τ_u^{cis}. 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 PA_{65} channel (Blaustein et al., 1990), we wanted to insure that any observed effect on τ_u^{cis} could not be attributed to the molecule entering the channel.

Fig. 1 *A* compares a plot of τ_u^{cis} (times [Bu_4N^+]) vs. voltage obtained in symmetric 0.1 molal KCl solutions with that obtained when the 0.1 molal KCl *cis* solution also contained 38% (wt/vol) dextran. (The conductance of the PA_{65} channel fell ~10% when the solution of 0.1 molal KCl plus 38% dextran was perfused into the *cis* compartment to replace the 0.1 molal KCl dextran-free solution.) Note that the plateau value of τ_u^{cis} (for [Bu_4N^+] = 1 micromolal) at large positive voltages in the dextran experiment is ~17.8 ms, 2.6 times larger than the 6.75 ms value in the

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[3] Since τ_u^{cis} is inversely proportional to the Bu_4N^+ concentration, it is convenient in discussing it to consider its value at a given concentration of Bu_4N^+, which we have chosen as 1 micromolal. In the preceding paper we chose this concentration as 1 micromolar; for the 0.1 molal KCl 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 (in 0.1 molal KCl), there is a 30% difference in molar and molal concentrations. Since it is the molal concentration that is thermodynamically relevant, we express the Bu_4N^+ concentrations in molal units throughout this paper. The issue of molal and molar concentration units is considered further in the Discussion.

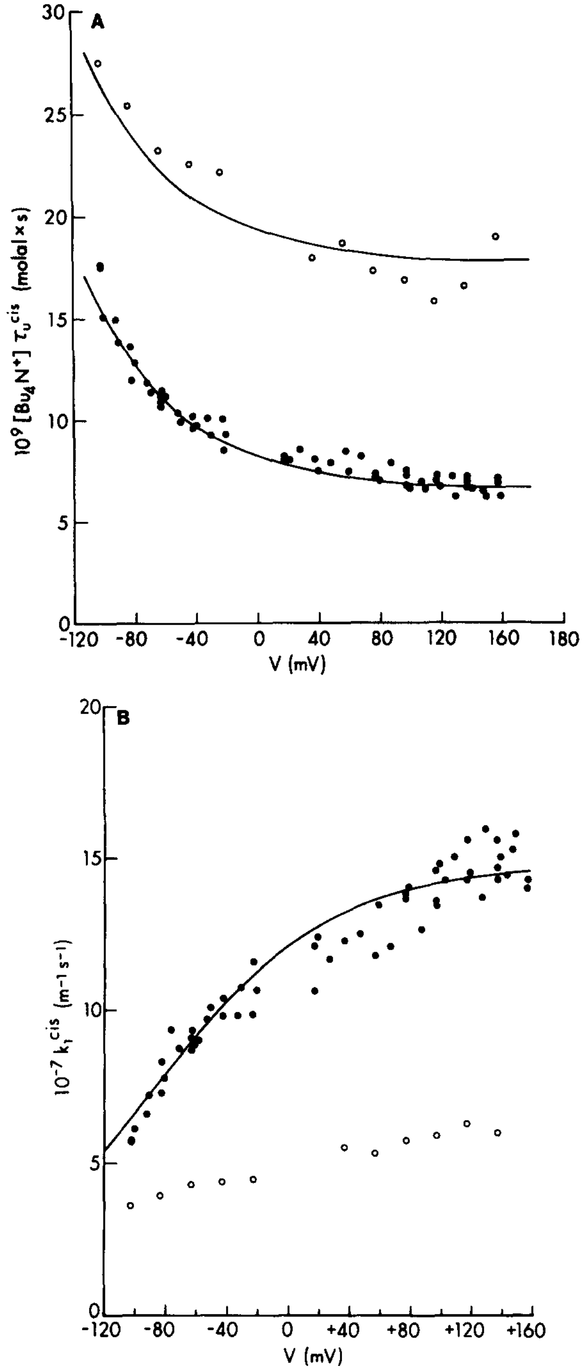

FIGURE 1. Effect of *cis* solution viscosity on the rate of blocking of PA_{65} channels by Bu_4N^+. *A*, Linear plots of the mean time (τ_u^{cis}) spent by a channel in the unblocked state (times $[Bu_4N^+]$) as a function of voltage, with Bu_4N^+ in the *cis* solution. The closed circles are from three 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 $M_r \sim 10{,}000$). (The concentrations of Bu_4N^+ in the dextran-free experiments were 15 and 37 micromolal; its concentration in the dextran experiment was 34.5 micromolal.) The curve through the closed circles is Eq. 7 (times $[Bu_{44}N^+]$) 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 k_1^{cis} 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 $M_r \sim 10{,}000$). 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_1^{cis} decreases 2.6-fold from its value of 1.48×10^8 m^{-1} s^{-1} in the absence of dextran to 0.56×10^8 m^{-1} s^{-1} in the dextran experiment [Fig. 1 *B*].) In addition, the entire τ_u^{cis} vs. *V* curve obtained in the presence of dextran is shifted upward (at $[Bu_4N^+] = 1$ micromolal) 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 τ_u^{cis} is diffusion affected; that is, an increase in viscosity increases τ_u^{cis}(diff), and this in turn adds linearly to the value of τ_u^{cis}.

The magnitude of the increase in the plateau value of τ_u^{cis} 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_4N^+, which is small relative to M_r 10,000 dextran. A Bu_4N^+ 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_4N^+ in the dextran solution, however, which is not a simple task, we took as an approximation of dextran's effect on Bu_4N^+ 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_4N^+ of ~2.1-fold,[4] which is in good agreement with its 2.6-fold effect on the plateau value of τ_u^{cis}.

It is conceivable that the effect of dextran on τ_u^{cis} is unrelated to its effect on the diffusion coefficient of Bu_4N^+ in solution, but is instead a consequence of some direct action of dextran on the PA_{65} channel. The effect of dextran on the mean blocked time (τ_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_4N^+ in 38% dextran. As we showed previously (Blaustein et al., 1990), the mean unblocked time with Bu_4N^+ in the *trans* compartment (τ_u^{trans}), unlike τ_u^{cis}, is *not* diffusion limited, and hence τ_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 τ_u^{trans} values in the voltage range −60 to −100 mV, in contrast to the 2.6-fold increase in the plateau value of τ_u^{cis} with the dextran solution in the *cis* compartment. (We noted previously [Blaustein et al., 1990] that there should be some contribution of diffusion to τ_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 *molar* KCl is 2.1-fold (not 2.7-fold) less than that of dextran-free 0.1 *molar* KCl. This point is considered further in the Discussion.

were unable to resolve it; the small effect on τ_u^{trans} values, produced by dextran in the *trans* compartment, may therefore be real; that is, it may be consistent with dextran's increasing τ_u^{trans}(diff).)

The Effect of Viscosity on the Unblocking Rate Constant (k_{-1}^{cis})[5]

As noted in the Theory section, if k_1^{cis} is diffusion affected, k_{-1}^{cis} is similarly affected; therefore, the expected reduction of k_1^{cis} produced by an increase in viscosity of the *cis* solution should be accompanied by a comparable reduction of k_{-1}^{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) rate constant of Bu_4N^+ 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:

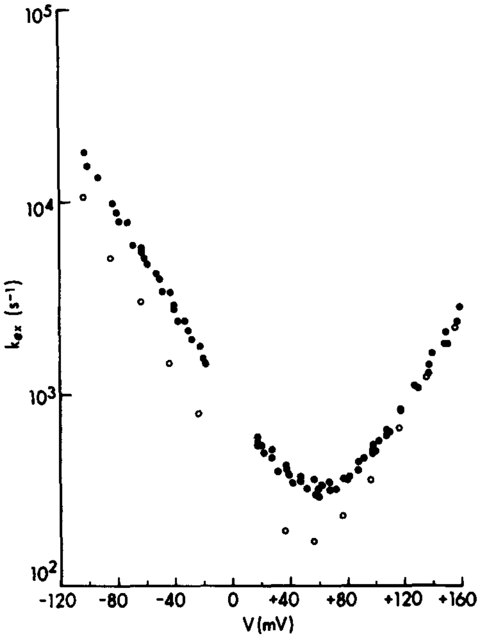

FIGURE 2. Effect of *cis* solution viscosity on the exit or unblocking rate constant (k_{ex}) of Bu_4N^+ from the blocking site in a PA_{65} channel. The closed circles are from a number of experiments done in symmetric 0.1 molal KCl solutions, with Bu_4N^+ in either the *cis* or *trans* solution at concentrations ranging from 15 micromolal 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 $M_r \sim 10{,}000$).

$$k_{ex} = k_{-1}^{cis} + k_{-1}^{trans}. \tag{13}$$

(For those who prefer to think in terms of the mean dwell time, τ_b, of the channel in the blocked state, recall that k_{ex} is simply the reciprocal of τ_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 τ_u^{cis} rather than k_1^{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 τ_b over k_{-1}^{cis}. Since subsequent discussions of K_{eq} of Eq. 1 deal with the *k*'s rather than with the τ's, we describe the results in this section in terms of k_{-1}^{cis} instead of τ_b.

voltages, where Bu_4N^+ almost always exits to the *trans* compartment (and hence $k_{ex} \approx k_{-1}^{trans}$), the values of k_{ex} in the dextran and dextran-free experiments are the same. In contrast, for modest positive voltages, at which Bu_4N^+ can exit to either compartment, and for negative voltages, where Bu_4N^+ 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 Bu_4N^+ is essentially in equilibrium between the *cis* compartment and the blocking site, and $k_{ex} \approx k_{-1}^{cis}$, 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.

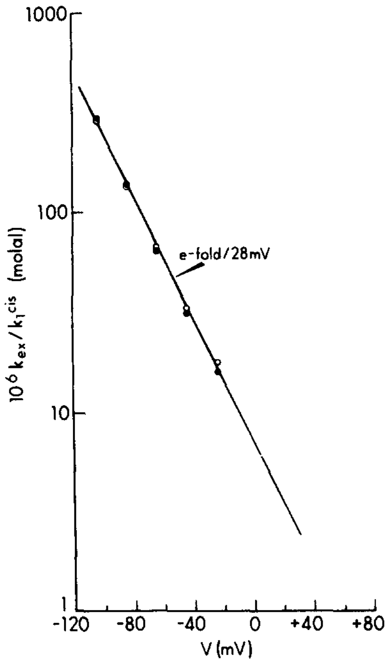

FIGURE 3. Demonstration that the equilibrium that exists (at negative voltages) for Bu_4N^+ between the *cis* solution and the blocking site in the PA_{65} channel is not a function of *cis* solution viscosity. This is a semilogarithmic plot of k_{ex}/k_1^{cis} vs. voltage, where k_{ex} is the exit rate constant of Bu_4N^+ 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, Bu_4N^+ almost always exits to the *cis* solution, so that $k_{ex} \approx k_{-1}^{cis}$, 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_{-1}^{cis} 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.

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 Bu_4N^+ in the *cis* compartment and the 38% dextran solution in the *trans* compartment, the values of k_{ex} $(\approx k_{-1}^{trans})$ 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), an increase in viscosity of the *trans* solution should not (and does not) decrease k_{-1}^{trans}.

The Effect of Tetraalkylammonium Size on τ_u^{cis}

If the plateau value of τ_u^{cis} obtained at large positive voltages with Bu_4N^+ in the *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 [τ_u^{cis}(diff)] should be inversely proportional to both the diffusion coefficient of the ion (D_{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_{disk}) and the ion radius (r_{ion}). Thus,

$$\tau_u^{cis}(\text{diff}) \propto \frac{1}{D_{ion}(r_{disk} - r_{ion})}\,. \tag{14}$$

In going from tetrabutylammonium to tetraheptylammonium, r_{ion} increases from ~5 Å to 6 Å (Robinson and Stokes, 1959). This means that there is an ~20% difference between $D_{Bu_4N^+}$ and $D_{Hp_4N^+}$, which should have, according to Eq. 14, a comparably small effect on τ_u^{cis}(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, τ_u^{cis}(diff) should increase enormously. Thus, if at some point in going from Bu_4N^+ to Hp_4N^+ the value of τ_u^{cis}(diff) takes off, the radius of the ion that produces this explosion closely approximates the radius of the entrance to the PA_{65} channel.

Fig. 4 shows a plot of τ_u^{cis} vs. voltage for Bu_4N^+, Pe_4N^+, Hx_4N^+, and Hp_4N^+. The curves all have a very similar shape, and all plateau at large positive voltages to a value, τ_u^{cis}(diff), characteristic for the particular ion. The trend in the values for τ_u^{cis}(diff) is striking. The value for Pe_4N^+ is essentially the same as that for Bu_4N^+, the value for Hx_4N^+ is ~3-fold larger, but the value for Hp_4N^+ is ~35-fold larger than that for Bu_4N^+. This is the expected trend if the radius of the channel entrance is close to that of Hp_4N^+. We therefore conclude that the radius of the entrance to the PA_{65} channel from the *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 Bu_4N^+ into the blocking site of a PA_{65} channel is weak when Bu_4N^+ is entering the site from the *cis* solution. τ_u^{cis} becomes significantly voltage dependent only for *cis* voltages more negative than −40 mV; at large positive voltages τ_u^{cis} levels off to a voltage-independent value of ~6.75 ms (for a Bu_4N^+ concentration of 1 micromolal). In the preceding paper (Blaustein et al., 1990) we proposed that this plateauing of τ_u^{cis} at large positive voltages results from its being diffusion limited by the rate at which Bu_4N^+ can diffuse from the *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 *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 τ_u^{cis} (at large positive voltages) means that the rate-limiting step for entry of Bu_4N^+ 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 Bu_4N^+ must diffuse before entering the channel, is such a voltage-insensitive region. (*b*) The magnitude of 1.48×10^8 $molal^{-1}$ s^{-1} for the plateau value of the entry rate constant k_1^{cis} $[=1/(\tau_u^{cis} \cdot [Bu_4N^+])]$ is consistent with diffusion limitation. (*c*) Raising the microviscosity 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 Bu_4N^+ in the *cis* solution by this amount, lowers the plateau value of k_1^{cis} by a factor of 2.6. This confirms that the rate-limiting step for Bu_4N^+ 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 τ_u^{cis}, 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 τ_u^{cis} 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 τ_u^{cis} will be relatively the same for all symmetric tetraalkylammo-

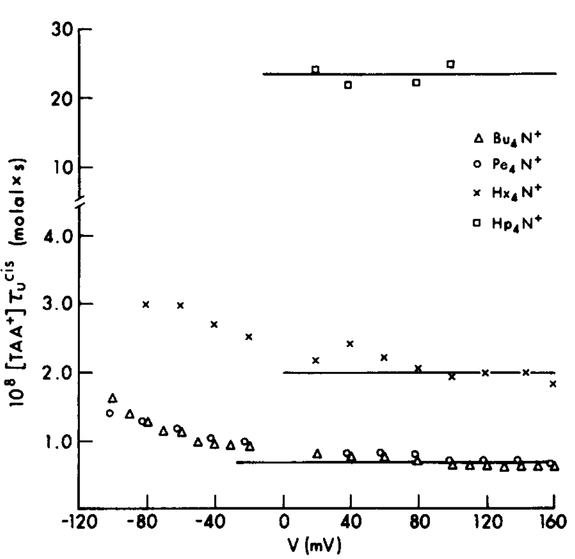

FIGURE 4. The effect of tetraalkylammonium (TAA^+) size on the mean time (τ_u^{cis}) spent by a channel in the unblocked state with TAA^+ in the *cis* solution. These are linear plots of τ_u^{cis} (times [TAA^+]) vs. voltage. Focusing on the plateau values reached at large positive voltages, note that they are essentially the same for Bu_4N^+ and tetrapentylammonium (Pe_4N^+), ~3-fold larger for tetrahexylammonium (Hx_4N^+), and ~35-fold larger for tetraheptylammonium (Hp_4N^+). The data for Bu_4N^+, which are from one of the experiments in Fig. 1 *A*, were obtained at a Bu_4N^+ concentration of 15 micromolal; the data for Pe_4N^+ and Hx_4N^+ were obtained at Pe_4N^+ and Hx_4N^+ concentrations of 10 micromolal; the data for Hp_4N^+ were obtained at an Hp_4N^+ concentration of 21 micromolal.

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 Bu_4N^+, we have focused on the blocking rate constant k_1^{cis} (or, equivalently, the mean unblocked time, τ_u^{cis}) because diffusion limitation is usually thought of in terms of the "forward" reaction in a bimolecular reaction (in our case, the association of Bu_4N^+ 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 Bu_4N^+ 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_{-1}^{cis} (the exit rate constant of Bu_4N^+ from the channel site to the *cis* solution) to the same extent that it decreases k_1^{cis}. We see this clearly confirmed in Figs. 2 and 3. (At negative voltages and modest positive voltages, when the exit of Bu_4N^+ from the channel site is almost always to the *cis* solution, k_{-1}^{cis} is essentially equal to k_{ex}, 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, 1970*a*). In our system this means that a Bu_4N^+ 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 Bu_4N^+ 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 *molal* activity of Bu_4N^+, rather than at the same *molar* activity. There the symmetry of the effect of viscosity on k_1^{cis} and k_{-1}^{cis}, 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 Bu_4N^+ in solution, and therefore prompted us to measure Bu_4N^+ 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 Bu_4N^+ in solution, *molar* rather than *molal* activity is the appropriate concentration unit.

To estimate the effect of the dextran-induced viscosity increase on the diffusion coefficient of Bu_4N^+, 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 Bu_4N^+ is 1.3 times smaller than its molality.) This is probably an underestimation of dextran's effect on the diffusion coefficient of Bu_4N^+, 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 Bu_4N^+? The diffusion-limited value of k_1^{cis} in the dextran solution was a factor of 2.6 smaller than its dextran-free value at the same *molality*. 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 Bu_4N^+ is probably greater than that on the diffusion coefficient of K^+). The effect on k_{-1}^{cis} is somewhat larger. (Note that in considering k_{-1}^{cis}, the issue of molarity and molality does not enter, since its value is independent of the concentration of Bu_4N^+ in solution.) At a voltage of -23 mV, where the *k*'s are not even completely diffusion limited, the value of k_{-1}^{cis} 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}^{cis} compared with its effect on k_1^{cis} *using molar concentrations* is real and is a necessary consequence of the required symmetry of the effect of dextran on k_{-1}^{cis} compared with its effect on k_1^{cis} *using molal concentrations.*

The Size of the PA_{65} Channel

On the basis that tetraheptylammonium ion can traverse the PA_{65} 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^{cis} 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^{cis} in going from tetrahexyl- to tetraheptylammonium (Fig. 4) indicates that the difference between the diameter of the channel entrance and the diameter of Hp_4N^+ (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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