Voltage-dependent Block of Anthrax Toxin Channels in Planar Phospholipid Bilayer Membranes by Symmetric Tetraalkylammonium Ions

Effects on Macroscopic Conductance

ROBERT O. BLAUSTEIN and ALAN FINKELSTEIN

From the Departments of Physiology & Biophysics and Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT In a recent paper (Blaustein, R. O., T. M. Koehler, R. J. Collier, and A. Finkelstein. 1989. *Proc. Natl. Acad. Sci. USA.* 86:2209–2213) we described the general channel-forming properties of the PA_{65} fragment of anthrax toxin in planar phospholipid bilayer membranes. In the present paper we extend our previous studies of the permeability properties of this channel, using a series of symmetric tetraalkylammonium (TAA) ions. Our main finding is that at micromolar concentrations on either the *cis* (toxin-containing) or *trans* side of a membrane containing many (>1,000) channels, these ions, ranging in size from tetramethylammonium to tetrahexylammonium, induce a voltage-dependent reduction of membrane conductance. (We attribute a similar voltage-dependent reduction of membrane conductance by millimolar concentrations of HEPES to a cationic form of this buffer present at micromolar concentrations.) In going from large negative to large positive voltages (on the TAA side) one sees that the conductance first decreases from its value in the absence of TAA, reaches a minimum, and then rises back at larger positive voltages toward the level in the absence of TAA. Our interpretation of this behavior is that these symmetric TAA ions block the cation-selective PA_{65} channel in a voltage-dependent manner. We postulate that there is a single site within the channel to which TAA ions can bind and thereby block the passage of the major current-carrying ion (potassium). A blocking ion is driven into the site by modest positive voltages, but is driven off the site and through the channel by larger positive voltages, thus explaining the relief of block. (In the accompanying paper [Blaustein, R. O., E. J. A. Lea, and A. Finkelstein. 1990. *J. Gen. Physiol.* 96:921–942] we confirm this interpretation of the data by analysis at the single-channel level.) This means that these blocking ions can pass through the channel; the permeability to tetrahexylammonium, the largest ion studied, implies that the narrowest part of the channel has a diameter of at least 11 Å.

Address reprint requests to Dr. Alan Finkelstein, Department of Physiology and Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461

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INTRODUCTION

We have earlier recounted (Blaustein et al., 1989) the channel-forming properties in phospholipid bilayers of "anthrax toxin," a tripartite toxin elaborated by the bacterium *Bacillus anthracis,* the causative agent of the disease anthrax. (For a general review of anthrax toxin see Leppla et al. [1985].) In that study we examined the three separate proteins that make up anthrax toxin—protective antigen (PA), edema factor, and lethal factor—and found that the trypsin-cleaved 65-kD COOH-terminal portion of PA, called PA_{65},[1] forms voltage-dependent and pH-dependent cation-selective channels in planar phospholipid bilayers, whereas the other toxin components are devoid of channel-forming activity. We also reported that the "instantaneous" current through PA_{65}-treated membranes saturates at voltages above +50 mV and below −80 mV.

Subsequent analysis of this saturation phenomenon, reported in this paper, shows that it results from a voltage-dependent block of the channel by micromolar amounts of a cationic form of HEPES buffer present in the experiments. We also find that micromolar amounts of symmetric tetraalkylammonium ions, when added to either side of the membrane, produce a voltage-dependent block that is relieved by large voltages of the appropriate sign; it is the interaction of these tetraalkylammonium ions with the channel that is the main focus of the present study. We argue that the voltage-mediated relief of block implies that the blocking ions can go through the channel, thus providing us with a novel way to determine the diameter of the channel lumen. All of the experiments described in this paper were with membranes containing many channels, and thus deal with "macroscopic" effects; the following paper (Blaustein et al., 1990) addresses these same phenomena at the single-channel level.

MATERIALS AND METHODS

PA of anthrax toxin was a generous gift from Dr. T. M. Koehler and Dr. R. J. Collier of the Department of Microbiology and Molecular Genetics, Harvard Medical School. The protein was purified and trypsin-cleaved, as previously described, to yield PA_{65} (Blaustein et al., 1989). It was stored in 0.35 M NaCl/20 mM ethanolamine HCl, pH 9.0, as frozen aliquots; once thawed, it was kept at 4°C. At a concentration of ~600 μg/ml, pH 9.0, and 4°C, PA_{65} showed little loss of channel-forming capability after storage for several months.

All experiments were performed on planar phospholipid bilayer membranes formed at room temperature by the brush technique of Mueller et al. (1963) across a 1-mm-diam hole in a Teflon partition separating two Lucite compartments, each containing 3 ml of salt solutions that could be stirred with small magnetic stir bars. Generally, the salt solutions were 100 mM KCl/1 mM EDTA, pH 6.6, and the membranes were formed from a 3% solution of diphytanoyl phosphatidylcholine (DPhPC) in *n*-decane; bi-ionic potential measurements were on membranes formed from a 5% solution of asolectin in *n*-decane. After the membranes were completely black, PA_{65} was added to one compartment (defined as the *cis* compartment) to concentrations ranging from 1 ng/ml to 1 μg/ml, and records were then taken.

Experiments were done under voltage-clamp conditions with a single pair of Ag/AgCl electrodes that made electrical contact with the solutions in the compartments via agar salt bridges (usually 3 M KCl). The member conductance (g) in symmetric salt solutions is defined as the current (I) flowing through the membrane divided by the transmembrane voltage (V),

[1] Recently renamed PA_{63} (Singh et al., 1989).

i.e., $g = I/V$, where V is the potential of the *cis* compartment relative to that of the *trans*, which is taken as zero; before addition of PA_{65}, membrane conductance was <20 pS. The applied voltages and the current responses were displayed simultaneously on a physiograph chart recorder (Narco Bio-Systems, Houston, TX) and a Hitachi V-212 oscilloscope, and, when desired, were digitized using an Instrutech VR-10 digital data recorder and stored on VHS tape using a Panasonic PV-2700 video cassette recorder.

DPhPC was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL), asolectin was lecithin type IIS from Sigma Chemical Company (St. Louis, MO) and was purified by the method of Kagawa and Racker (1971), and *n*-decane (99+% pure) was from Aldrich Chemical Company (Milwaukee, WI). Tetramethylammonium (Me_4N^+) bromide (purum grade), tetraethylammonium (Et_4N^+ or TEA^+) bromide (puriss grade), tetrapropylammonium (Pr_4N^+) bromide (purum grade), tetrabutylammonium (Bu_4N^+) bromide (puriss grade), tetrapentylammonium (Pe_4N^+) bromide (purum grade), tetrahexylammonium (Hx_4N^+) bromide (purum grade), tetraheptylammonium (Hp_4N^+) bromide (purum grade), and tetraoctylammonium (Oc_4N^+) bromide (purum grade) were purchased from Fluka Chemical Corporation (Ronkonkoma, NY) and used as provided. Tetraethylammonium chloride was purchased from Eastman Chemical Company (Rochester, NY) and was treated several times with activated charcoal and filtered.

Selectivity Measurements

In the single-salt experiments the membrane was formed in symmetric (e.g., 0.1 M KCl) solutions, and, after the establishment of a significant PA_{65}-induced conductance ($>10^{-8}$ S), gradients were generated by additions of concentrated salt solutions (e.g., 3 M KCl) to the *cis* side. After each addition, the reversal potential (i.e., the potential at which $I = 0$) for the existing gradient was determined. In the bi-ionic experiments the membrane was formed in the presence of the asymmetric salt solutions (e.g., 0.1 M KCl vs. 0.1 M TEA·Cl), and, after its treatment with PA_{65}, the reversal potential was determined; because of the large (3 ml) volumes of the compartments, there was minimal mixing of solution contents before painting the membrane-forming solution across the 1-mm-diam hole.

Current–Voltage Relations

To generate "instantaneous" *I-V* characteristics, PA_{65}-treated membranes were pulsed for 0.25 s, at 5- or 10-mV increments, to voltages ranging from −150 mV to +150 mV; currents were measured 10–20 ms after the beginning of each pulse to minimize the effect of gating at large negative voltages. (At voltages below −50 mV, half of the channels close within a few seconds [Blaustein et al., 1989].) After each pulse the voltage was returned to 0 mV for 0.5 s to reopen any channels that might have closed during the previous pulse (see Fig. 3). To assess the effect of HEPES or a given tetraalkylammonium ion on a PA_{65}-treated membrane, we first generated the "instantaneous" *I-V* characteristic in the absence of the agent, and then subsequently in its presence at two to three different concentrations; this complete set of recordings generally required 3 min. To minimize the effect of the linear increase with time (for periods of up to 1 h) of the PA_{65}-induced conductance (Blaustein et al., 1989), we began these measurements ~40 min after the addition of PA_{65}. Consequently, the number of channels present in the membrane throughout the course of the recordings remained essentially constant to within 10%.

Binding Isotherms

The equilibrium dissociation constant for the binding, at 0 mV, of a particular tetraalkylammonium ion to a site(s) in the channel was determined by measuring the small signal current (ΔI, resulting from a voltage pulse between +5 mV and −5 mV) in the absence of this

blocking ion and then in its presence at equal concentrations on the two sides of the membrane. ΔI in its presence divided by ΔI in its absence gives the normalized conductance, g_{norm}; thus $1 - g_{norm}$ is the fraction of conductance blocked (f). This quantity was determined on a single PA_{65}-treated membrane at several different (symmetric) concentrations of the blocking ion, and a binding isotherm was thereby constructed. To calculate the dissociation constant, data were fit, using a nonlinear least-squares algorithm, to the equation $f = [TAA]/([TAA] + K_d)$, where TAA represents a tetraalkylammonium ion. As with the *I-V* experiments, measurements were begun ~40 min after addition of PA_{65} to maintain an essentially constant number of channels in the membrane during this period.

RESULTS

Selectivity

We previously reported that the PA_{65} channel is much more permeable to K^+ than to Cl^-, and ideally selective for tetrapropylammonium (Pr_4N^+) over Br^- (Blaustein et al., 1989). These data, as well as those from subsequent reversal potential measurements with TEA·Cl, are shown in Fig. 1. The channel is also permeable to the divalent cations Mg^{2+} and Ca^{2+}. An activity ratio of 4.5 for $MgCl_2$ (500 mM vs. 100 mM) gives a reversal potential of 13.4 mV (ideal Mg^{2+} permeability is −19.3 mV; ideal Cl^- permeability is 38.7 mV); an activity ratio of 1.8 for $CaCl_2$ (186 mM vs. 100 mM) gives a reversal potential of 3.3 mV (ideal Ca^{2+} permeability is −7.3 mV; ideal Cl^- permeability is 14.5 mV).

The channel's permeability to Pr_4N^+ places a lower limit of 9 Å on its diameter. We had hoped to continue sizing the channel with similar selectivity experiments using larger symmetric tetraalkylammonium ions (Bu_4N^+, Pe_4N^+, etc.), but unfortunately the intrinsic lipid bilayer permeabilities of these ions are so large that interpretation of such experiments is impossible at the concentrations necessary for ion selectivity measurements.

In the course of measuring reversal potentials, we noticed that the conductances induced by given concentrations of PA_{65} were orders of magnitude less in Me_4N·Br, Et_4N·Br, and Pr_4N·Br than in KCl or NaCl, suggesting that these ions might block the channel. (This led us to the study, described in the next section, of the blocking action of these, and larger ions in the series, at micromolar concentrations.) Surprisingly, however, bi-ionic potential measurements of KCl vs. TEA·Cl indicate that the channel is more permeable to TEA^+ than to K^+. For a PA_{65}-treated asolectin membrane separating 0.1 M KCl *cis* from 0.1 M TEA·Cl *trans*, the reversal potential is ~30 mV (KCl side positive).

Blocking[2]

HEPES. With 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 6.5–7.5) on both sides of a PA_{65}-treated membrane, the "instantaneous" current saturates, and even declines, at voltages greater than +50 mV and less than −80 mV (see Fig. 5 of Blaustein et al., 1989); in the absence of

[2] Although the effects of HEPES and tetraalkylammonium ions reported in this section are on macroscopic conductances, we describe them as resulting from "blocking" of the PA_{65} channel by these ions. This is the most obvious interpretation of the data, and it is confirmed by the single-channel results reported in the following paper (Blaustein et al., 1990).

buffer, or with MES (2-[*N*-morpholino]ethanesulfonic acid) buffer in that pH range, the *I-V* characteristic is fairly linear (Fig. 2 *A*). It appears that HEPES can block the channel in a voltage-dependent fashion. At first glance this is paradoxical, since the channel is strongly cation selective, and at neutral pH HEPES exists chiefly in two forms: anionic and neutral. (In fact, the HEPES anion has been shown to block anion-selective channels [Yamamoto and Suzuki, 1987; Soejima and Kokubun,

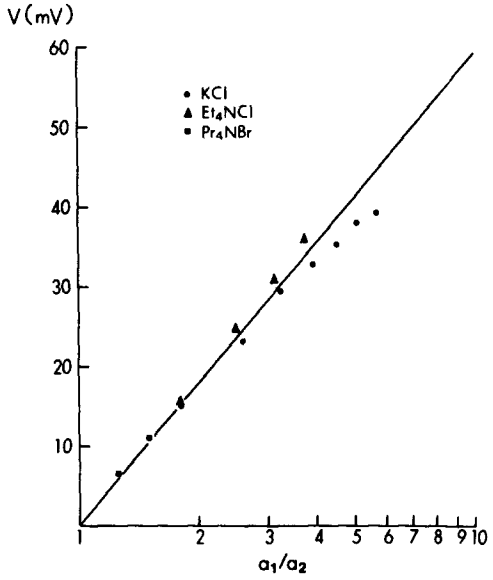

FIGURE 1. Plot of membrane potential (at $I = 0$) vs. the logarithm of the ratio of salt activity across PA_{65}-treated membranes. DPhPC membranes were formed at room temperature across a 1-mm-diam hole separating unbuffered symmetric 0.1 M solutions of either KCl, $Et_4N \cdot Cl$, or $Pr_4N \cdot Br$. After the membranes were completely black, PA_{65} was added to the solution on one side (the *cis* side) to a concentration of ~2 ng/ml. Membrane conductance increased linearly with time, and after it had reached at least 10^4 pS, salt gradients were established across the membrane by additions of concentrated salt solutions to the *cis* side, and the resulting membrane potential at $I = 0$ (the reversal potential) was recorded. The potentials plotted in the figure are those of the dilute, 0.1 M *trans* solution with respect to the more concentrated *cis* solution. The line drawn is that for ideal monovalent cation selectivity (59 mV per 10-fold activity ratio). Data points for each of the three salts tested were obtained on a single membrane. PA_{65}-treated membranes are ideally selective for Et_4N^+ and Pr_4N^+ with respect to Cl^- and Br^-, respectively, but there is clear deviation from ideality for KCl. (It is conceivable, but unlikely, that the deviation from ideality for KCl is totally a consequence of a combination of polarization potentials and streaming potentials resulting from the osmotic gradient of KCl across the membrane. The next to the last point has already been corrected for at least polarization effects, as urea [a permeant solute through the PA_{65} channel] was added to the *trans* side to balance the osmotic gradient. [This increased the measured potential by 1.0 mV.] Independent streaming potential measurements in 0.1 M KCl gave a value of 2.9 mV for a two osmolal gradient of sucrose [an almost impermeant solute], which would add another 1.5 mV to the point, still leaving it 2 mV short of the ideal value. Note also that the last point deviates proportionately even further from ideality.) Activity coefficients for KCl were obtained from Robinson and Stokes (1959) and those for $Et_4N \cdot Cl$ and $Pr_4N \cdot Br$ were from Lindenbaum and Boyd (1964).

1988].) However, aqueous titration of HEPES buffer reveals a group, presumably the second nitrogen of the piperazine ring, that is titrated at a pK around 4.0–4.5 (unpublished observations). Therefore, at neutral pH roughly one-thousandth of the HEPES (i.e., ~10 μM in our experiments) exists as a cation, and it is this species that we believe blocks the channel. There are three observations consistent with this assumption: (*a*) as noted above, with MES buffer, which lacks a second nitrogen and

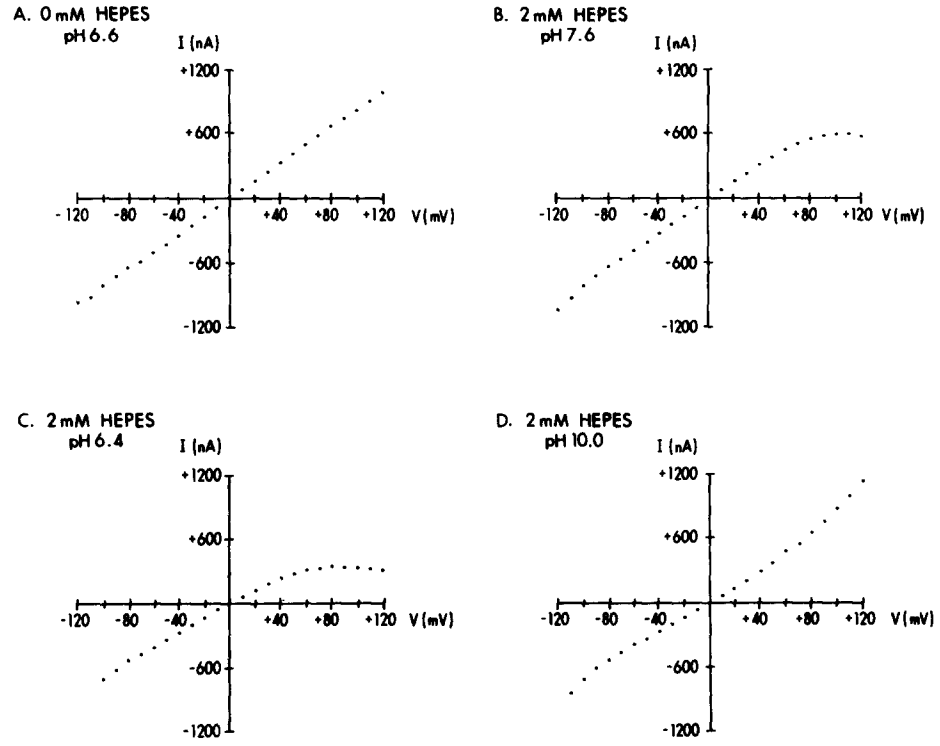

FIGURE 2. Voltage-dependent block of current through a PA_{65}-treated membrane upon addition of HEPES buffer. A DPhPC membrane was formed at room temperature across a 1-mm-diam hole separating symmetric 0.1 M KCl/1 mM EDTA, pH 6.6 solutions. Once the membrane was completely black, PA_{65} was added to one solution (defined as the *cis* solution) to a concentration of 18 ng/ml, while the membrane voltage was clamped at +20 mV. Membrane conductance rose linearly with time; after 40 min the membrane was pulsed from −120 mV to +120 mV in 10-mV increments (see Fig. 3), and the resulting current responses were recorded. HEPES buffer was then added to the *cis* solution to a concentration of 2 mM, raising the pH of the solution to 7.6, and the membrane was pulsed as described above. The pH of the *cis* solution was then lowered to 6.4 by addition of HCl and the membrane was once again pulsed as above. Finally, KOH was added to the *cis* solution, raising the pH to 10.0, and the membrane was pulsed as above. The entire procedure took less than 3 min, so that the number of channels present during the course of these measurements remained essentially constant to within 10%. The *cis* solution was stirred continuously throughout the course of the experiment. "Instantaneous" (within 10–20 ms) currents for each set of voltage pulses were determined and plotted vs. voltage. *A*, No HEPES, pH 6.6. *B*, 2 mM HEPES, pH 7.6. *C*, 2 mM HEPES, pH 6.4. *D*, 2 mM HEPES, pH 10.0.

hence does not exist in a cationic form, the "instantaneous" current does not exhibit saturation and decline with voltage; (*b*) if HEPES is present only on the *cis* side of the membrane, current saturation and decline occur at positive, but not negative, voltages (Fig. 2 *B*), where the converse occurs with HEPES present only on the *trans* side (data not shown), thus indicating that a cationic species is being driven into the

channel; (*c*) if, in the presence of HEPES, the pH is lowered from 7.6 to 6.4 during the course of an experiment, the current saturation and decline are enhanced (compare Fig. 2 *B* and *C*); (*d*) if the pH is then raised to 10.0, the *I-V* characteristic once again becomes linear (Fig. 2 *D*). Rather than study the effect of $HEPES^+$, which is an esoteric cation, we chose instead to probe the channel's structure with a series of symmetric tetraalkylammonium ions which, like $HEPES^+$, exhibit voltage-dependent blockade of the channel.

Tetraalkylammonium ions. Symmetric quaternary ammonium ions, ranging in size from tetramethylammonium to tetrahexylammonium, block the PA_{65} channel in a voltage-dependent fashion when added to either the *cis* or *trans* compartment. Fig. 3 shows current responses of PA_{65}-treated membranes to voltage pulses in the absence and presence of Bu_4N^+; *I-V* curves derived from these records are shown in Fig. 4 *A*. Note that these are "instantaneous" *I-V* curves (see Materials and Methods). In Fig. 4 *B* these *I-V* curves have been converted to *g-V* curves, where conductances in the presence of Bu_4N^+ have been normalized to the conductances just before its addition. Similar results are obtained with the other tetraalkylammonium ions, representative examples of which are shown in Fig. 5. (In these experiments the intrinsic bilayer tetraalkylammonium conductances were negligible.)

There are several notable features of these curves: (*a*) The tetraalkylammonium ions block from both the *cis* and *trans* sides. Although a possible interpretation of this result is that these ions bind to a single site in the channel accessible from either side, implying that they are permeant, it is not possible from this fact alone to rule out two (or more) different sites separated by an essentially infinite potential energy barrier. However, (*b*) at sufficiently large positive voltages on the blocking-ion side there is relief of block. This is evident in the shape of the *I-V* curves and is even more apparent from the presence of a minimum in the *g-V* curves. The most obvious interpretation of this behavior is that these ions are permeant, and are driven through the channel by large voltages of the appropriate sign. (*c*) These blockers are more potent from the *cis* side; ~20-fold higher concentrations are needed on the *trans* side to achieve comparable effects.[3] (*d*) The *I-V* and *g-V* curves generated with blocker on the *cis* side are roughly mirror images of the curves generated with *trans* blocker.

Binding

Results of equilibrium binding measurements (i.e., at $V = 0$) with several tetraalkylammonium ions are summarized in Table I. The binding data are well fit by Langmuir adsorption isotherms, with a characteristic dissociation constant (K_d) for each ion; a representative isotherm is shown in Fig. 6. Excluding Pr_4N^+, which is slightly anomolous, the ions appear to block more potently with increasing size up to Pe_4N^+, whose K_d is about fourfold smaller than that of Bu_4N^+ and about twofold smaller than that of Hx_4N^+; K_d values range from 1.6 mM for Me_4N^+ down to 2 μM

[3] An identical asymmetry in blocker efficacy is always seen at the single-channel level (Blaustein et al., 1990); that is, PA_{65} channels always insert with the same orientation.

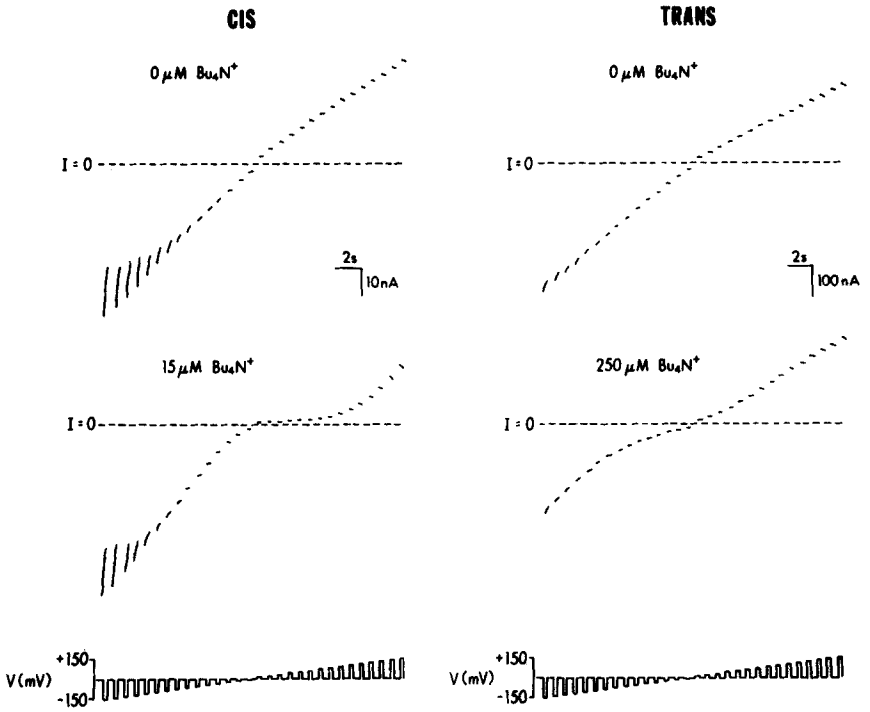

FIGURE 3. Voltage-dependent block of current through PA_{65}-treated membranes upon addition of Bu_4N^+ to either the *cis* or *trans* compartment. DPhPC membranes were formed at room temperature across a 1-mm-diam hole separating symmetric 0.1 M KCl/1 mM EDTA, pH 6.6 solutions. After the membranes were completely black, PA_{65} was added to one solution (the *cis* solution) to a concentration of 8 ng/ml (left records) or 30 ng/ml (right records). After 40 min the voltage was pulsed from −150 mV to +150 mV, in 10-mV increments as shown, and the resulting current responses were recorded (two upper records). Bu_4N^+ was then added either to the *cis* compartment (lower left record) to a concentration of 15 μg/ml, or to the *trans* compartment (lower right record) to a concentration of 250 μg/ml, and the resulting current responses were recorded. The transients, particularly prominent at large negative voltages, result from rapid gating of the PA_{65} channels. The number of channels present during the course of these measurements remained essentially constant to within 10%. Solutions were stirred continuously throughout the course of the experiments.

for Pe_4N^+. The effects with ions larger than Hx_4N^+ were uninterpretable,[4] making it difficult to assess any continuing trend. In agreement with the blocking data of the previous section, almost all of the decrease in membrane conductance occurred upon each addition of tetraalkylammonium ions to the *cis* side during the course of

[4] Upon addition of Me_4N^+ through Hx_4N^+ to the *cis* and *trans* solutions, the small signal conductance fell to a new equilibrium value within seconds—the mixing time of the compartments. With Hp_4N^+, however, conductances fell continuously for several minutes, never reaching a stable value. With Oc_4N^+, conductances first decreased somewhat and then rose to much larger values; we interpret this increase as resulting from the intrinsic bilayer permeability of Oc_4N^+.

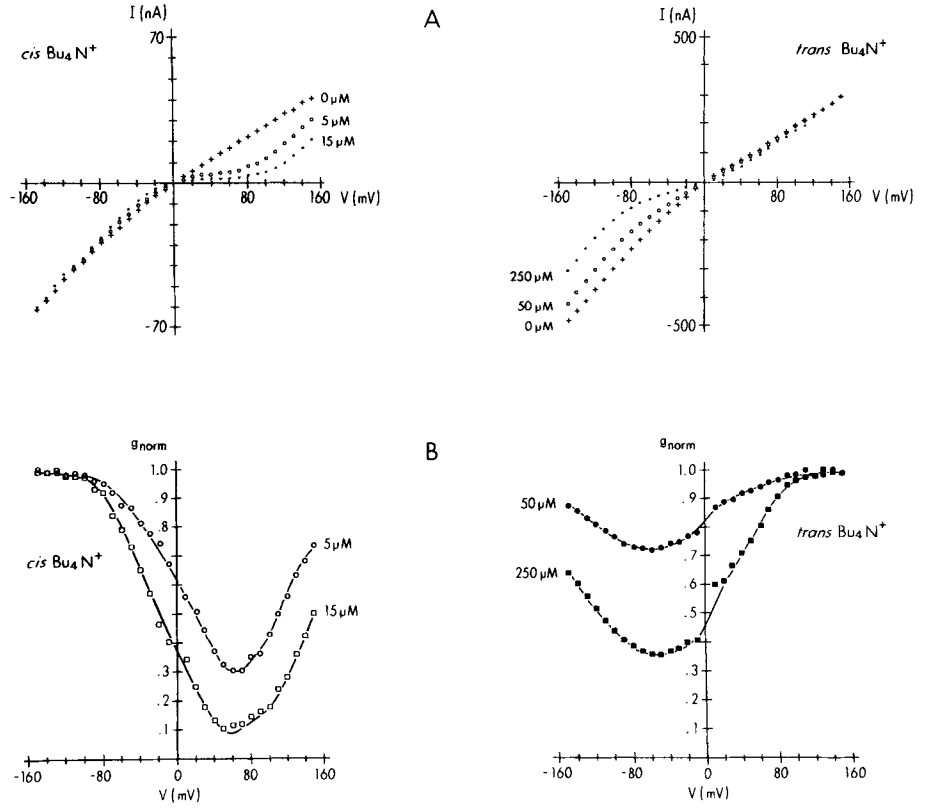

FIGURE 4. *A, I-V* relations for PA_{65}-treated membranes in the absence and presence of Bu_4N^+. "Instantaneous" currents (within 10–20 ms after application of the voltage pulse) were determined in the absence of Bu_4N^+ and in its presence in either the *cis* compartment (*left*) or the *trans* compartment (*right*). The curves are derived from the records shown in Fig. 3 and include intermediate concentrations of Bu_4N^+ not shown in that figure. *B,* Conductance–voltage relations for PA_{65}-treated membranes in the presence of Bu_4N^+. The points were generated using the data in *A;* the normalized conductance, g_{norm}, is obtained at each voltage by dividing the conductance in the presence of Bu_4N^+ by the value in its absence. Smooth lines are drawn to aid the eye. The left panel shows curves obtained with Bu_4N^+ present in the *cis* compartment, and the right panel shows curves with Bu_4N^+ present in the *trans* compartment. Note the difference in Bu_4N^+ concentrations used for the *cis* and *trans* experiments.

these equilibrium experiments; i.e., upon subsequent addition to the *trans* side no further significant decrease in conductance was seen.

DISCUSSION

The main finding reported in this paper is that at micromolar concentrations on either the *cis* or *trans* side of the membrane, symmetric tetraalkylammonium ions—tetramethylammonium ion (Me_4N^+) through tetrahexylammonium ion (Hx_4N^+)—induce a voltage-dependent block of the cation-selective channel formed

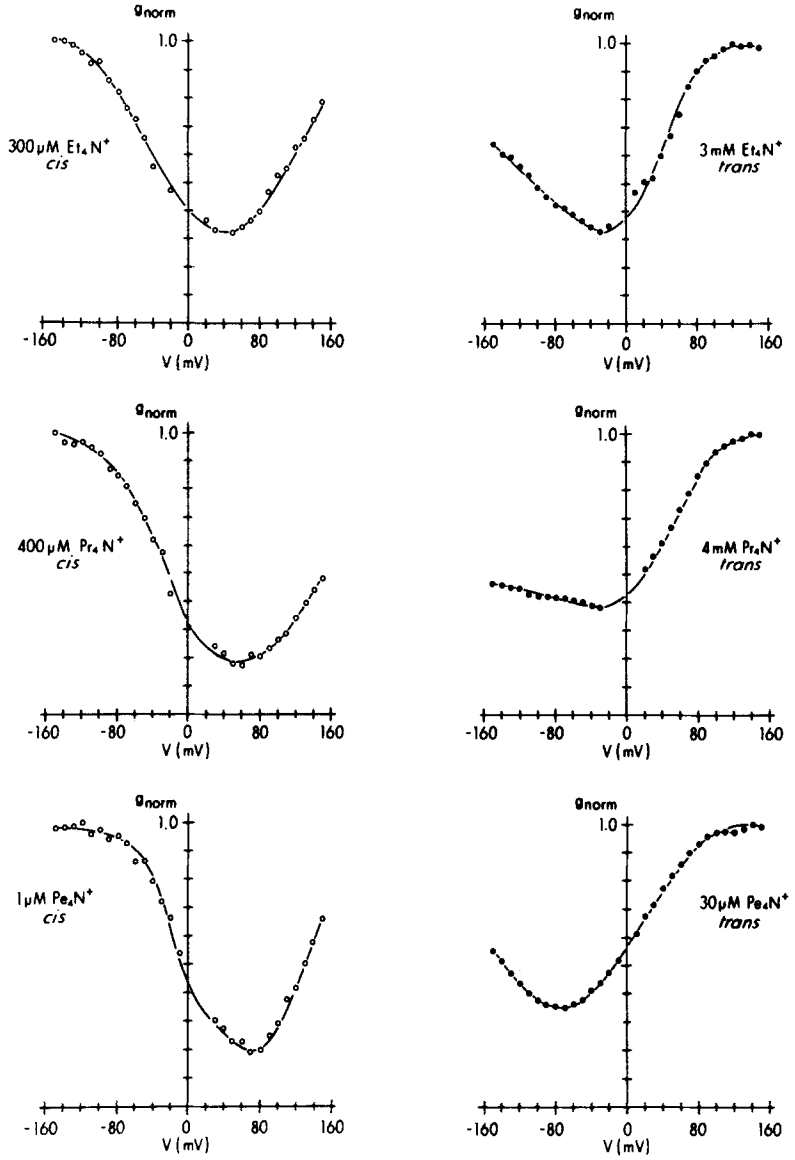

FIGURE 5. Conductance–voltage relations for PA_{65}-treated membranes with various symmetric tetraalkylammonium ions present in either the *cis* (*left*) or *trans* (*right*) compartment. The normalized conductances plotted here, g_{norm}, were obtained as described in Fig. 4 *B* from records analogous to those shown in Fig. 3 and 4 *A*. Note the differences in concentrations used for the *cis* and *trans* experiments. All experiments were performed on DPhPC membranes separating symmetric solutions of 0.1 M KCl/1 mM EDTA, pH 6.6.

in planar phospholipid bilayer membranes by the PA_{65} fragment of anthrax toxins.[5] At voltages larger than about +60 mV on the blocking-ion side there is relief of block. That is, in going from large negative voltages to large positive voltages (on the blocking-ion side), one sees the conductance first decrease from its value in the absence of blocking ion, reach a minimum at about +60 mV, and then rise back at larger positive voltages toward the level seen in the absence of blocker (Figs. 4 *B* and 5). For a given tetraalkylammonium ion, ~20-fold higher concentrations are required on the *trans* side to obtain effects comparable in magnitude to those with the ion on the *cis* side.

How are these observables to be explained? The simplest interpretation is that there is a single site within the PA_{65} channel to which tetraalkylammonium ions can bind, and when these ions occupy this site the channel is blocked; that is, the major current-carrying ion (K^+, present at 100-mM concentration in our experiments) cannot traverse the channel. The blocking ion is driven into the site by modest positive voltages, but is driven off the site and out through the channel by larger positive voltages, thus explaining the relief of block. In terms of the oft-used barrier-well models, this is a two-barrier, single-well model (Fig. 7), with the larger

TABLE I

Dissociation Constants for the Binding of Tetraalkylammonium Ions to PA_{65} Channels

Ion	K_d
	μM
Me_4N^+	1,600
Et_4N^+	224
Pr_4N^+	298
Bu_4N^+	7.91
Pe_4N^+	2.04
Hx_4N^+	3.70

height of the *trans* barrier accounting for the greater effectiveness of blocker from the *cis* side.

Before considering the quantitative aspects of this picture, we note an important qualitative feature of our findings. Whatever the details of the number, shape, and size of the energy barriers and wells, or the general energy profile in any continuum model of the channel, the most obvious and straightforward interpretation of relief of block at large positive voltages on the blocker side is that the blocking ion is driven through the channel.[6] In other words, the PA_{65} channel is permeable to tetraalkylam-

[5] Although the observed effects are on macroscopic currents and conductances in membranes containing many channels, we describe them in this discussion as occurring on the individual channels to avoid awkward circumlocutions.

[6] It is conceivable, though very unlikely, that the rise in conductance at large positive voltages, or for that matter, the fall in conductance at smaller positive voltages, results from a direct voltage dependence of the PA_{65} channel induced by tetraalkylammonium ions, and that it has nothing to do with blocking and unblocking. In the following paper (Blaustein et al., 1990) we directly confirm blocking and unblocking at the single-channel level.

monium ions up to the size of Hx_4N^+, implying that the narrowest part of the channel is at least 11 Å in diameter. Interestingly, even though TEA^+ blocks the channel, it is more permeant than K^+, as judged from the bi-ionic potential between 0.1 M KCl and 0.1 M TEA·Cl. Of further interest, and possibly relevant to the mechanism of ion permeation through this channel (although we don't know how), is that this basically cation-selective channel has a finite anion (Cl^-) permeability, as evidenced both by small, but real, deviations from Nernstian potentials in KCl gradients (Fig. 1) and by comparable Cl^- and Mg^{2+} (or Ca^{2+}) selectivity, manifested by the reversal potentials in $MgCl_2$ and $CaCl_2$ gradients.

Quantitatively, the most significant feature of the model in Fig. 7 that can be extracted from the macroscopic *g-V* curves is the location of the blocking site, its so-called electrical distance within the channel. At negative voltages a blocking ion that enters the site almost certainly exits to the *cis* solution, because the energy barrier from the site to the *cis* side is so much smaller than that to the *trans* side.

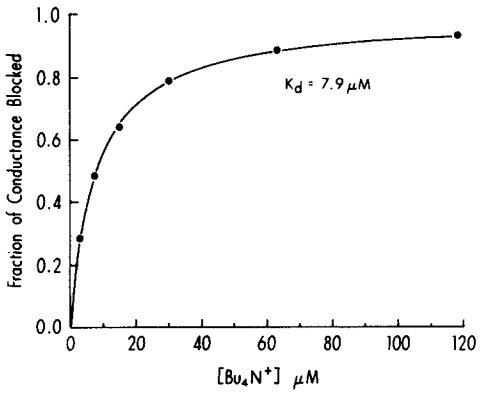

FIGURE 6. Binding isotherm of Bu_4N^+ to the PA_{65} channel. Plotted is the fraction of the PA_{65}-induced conductance that is blocked (i.e., $1 - g_{norm}$) at various symmetric concentrations of Bu_4N^+ in the *cis* and *trans* compartments. (See Materials and Methods for the experimental protocol used in obtaining the data.) The data were fit (by nonlinear least-squares fit) to a Langmuir adsorption isotherm with the equation $f = [Bu_4N^+]/([Bu_4N^+] + K_d)$, where f is the fraction of conductance blocked. The curve drawn is with a dissociation constant, K_d, equal to 7.9 μM. The experiment was performed on a DPhPC membrane separating symmetric solutions of 0.1 M KCl/1 mM EDTA, pH 6.6.

Therefore, at negative voltages with blocking ion in the *cis* solution, the blocker rarely traverses the channel, and is therefore effectively at equilibrium between the *cis* solution and the blocking site. Consequently, if we call the number of channels in the open or unblocked state n_u and the number in the closed or blocked state n_b, then at negative voltages with blocking ion in the *cis* solution we can write the Boltzmann distribution:

$$\frac{n_u}{n_b} = Ae^{-qV\delta_{cis}/kT}, \tag{1}$$

where q is the unit charge on a tetraalkylammonium ion, k is the Boltzmann constant, T is temperature in degrees Kelvin ($kT/q \approx 25.6$ mV at room temperature), V is the voltage on the *cis* side, and δ_{cis} is the electrical distance from the *cis* side to the binding site (see Fig. 7); i.e., δ_{cis} is the fraction of the total transmembrane potential, V, seen at the well. The constant A is a function of the particular blocker and its

concentration. Since the normalized conductance (g_{norm}) of Fig. 4 *B* is equal to $n_u/(n_u + n_b)$, Eq. 1 can be rewritten as:

$$\frac{g_{norm}}{1 - g_{norm}} = Ae^{-qV\delta_{cis}/kT}. \quad (2)$$

(This treatment is analogous to that used by Woodhull [1973] to analyze proton blockage of sodium channels in nerve.) A semilogarithmic plot of $g_{norm}/(1 - g_{norm})$ vs. voltage from −15 mV to −90 mV for Bu_4N^+ yields a straight line whose slope represents an *e*-fold change per 27 mV (Fig. 8 *A*); i.e., $\delta_{cis} = 0.95$. Similar plots for the other tetraalkylammonium ions in this study also yield straight lines with essentially the same slope; the range is 27–34 mV, with a mean of 31 mV ($\delta_{cis} = 0.83$).

This seems to imply that the blocking site is close to the *trans* side, since its electrical distance from the *cis* side is over four-fifths of the total electrical distance (assuming $\delta_{total} \equiv \delta_{cis} + \delta_{trans} = 1.0$). However, if experiments with Bu_4N^+ present in the *trans* solution are analyzed in a similar manner at large positive voltages (where Bu_4N^+ is effectively in equilibrium between the blocking sites and the *trans*

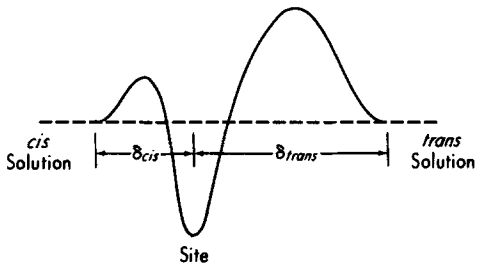

FIGURE 7. Free energy diagram, at 0 mV, of the proposed energy barriers and well for tetraalkylammonium ions in the PA_{65} channel. Note that the well is closer to the *cis* side and that the *trans* barrier is higher than the *cis* barrier.

solution),[7] we obtain an *e*-fold change per 17 mV (Fig. 8 *B*), which gives $\delta_{trans} = 1.51$, thus making $\delta_{total} = 2.5$. The usual interpretation of $\delta_{total} > 1$ is that there is multiple ion occupancy, in which case voltage dependence of blocking involves movement not only of the blocking ion, but also of the other charges (potassium ions) in the channel (see Hille, 1984). Our data (with $\delta_{total} = 2.5$) therefore indicate that in addition to a single blocking ion there are also one to two potassium ions in the channel. Since the voltage dependence of blocking from the *trans* side is roughly twice as steep as that from the *cis* side, the blocking site is electrically closer to the latter, being about one-third the distance from the *cis* side and two-thirds the distance from the *trans* side.

Consistent with the notion of multiple ion occupancy of the channel is our finding that δ_{total} in 0.01 M KCl is smaller than in 0.1 M KCl. Specifically, semilogarithmic plots of $g_{norm}/(1 - g_{norm})$ vs. voltage (analogous to those shown in Fig. 8) in 0.01 M

[7] Because the barrier is larger on the *trans* side than on the *cis* side, one must go to voltages greater than 80 mV to be in the range where exit from the well is predominantly to the *trans* side. For g_{norm} to be measurably less than 1.0 at these voltages, relatively large concentrations of blocker in the *trans* solution are required.

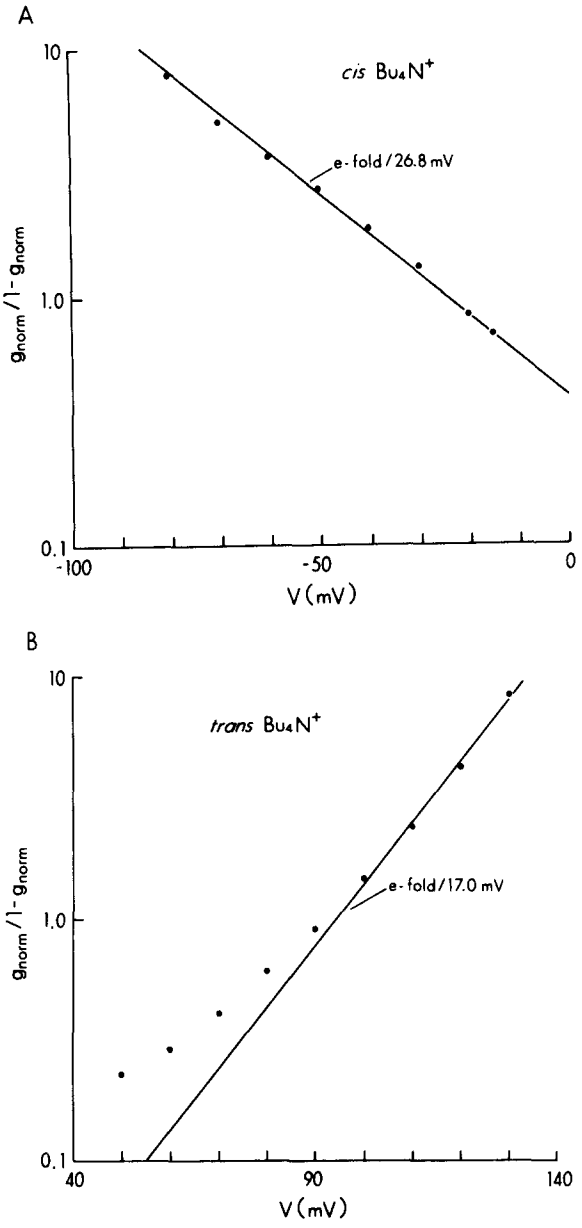

FIGURE 8. Semilogarithmic plot of $g_{norm}/(1 - g_{norm})$ vs. voltage for a PA_{65}-treated membrane in the presence of Bu_4N^+ in the *cis* compartment (*A*) or the *trans* compartment (*B*). g_{norm} was calculated as described for Fig. 4 *B*. The concentration of Bu_4N^+ in *A* was 15 μM; its concentration in *B* was 2 mM. Data were fit for exponential voltage dependence using a nonlinear least-squares fit; note in *B* that only the points for voltages ≥100 mV were used for the fit (see Discussion). In both experiments the membrane was formed from DPhPC and separated symmetric solutions of 0.1 M KCl/1 mM EDTA, pH 6.6.

KCl for *cis* and *trans* Bu_4N^+ yield slopes representing *e*-fold changes per 29 and 22 mV, respectively. These translate into $\delta_{cis} = 0.88$ and $\delta_{trans} = 1.16$, thus making $\delta_{total} = 2.0$ in 0.01 M KCl, in contrast to its value of 2.5 in 0.1 M KCl. Quantitative interpretation of the effect of KCl concentration on both δ_{total} and single-channel conductance is model dependent, extremely complicated, and beyond the scope of our present study.

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