BINDING CONSTANTS OF Li^+, K^+, AND Tl^+ IN THE GRAMICIDIN CHANNEL DETERMINED FROM WATER PERMEABILITY MEASUREMENTS

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ABSTRACT In an open circuit there can be no net cation flux through membranes containing only cation-selective channels, because electroneutrality must be maintained. If the channels are so narrow that water and cations cannot pass by each other, then the net water flux through those "single-file" channels that contain a cation is zero. It is therefore possible to determine the cation binding constants from the decrease in the average water permeability per channel as the cation concentration in the solution is increased. Three different methods were used to determine the osmotic water permeability of gramicidin channels in lipid bilayer membranes. The osmotic water permeability coefficient per gramicidin channel in the absence of cations was found to be 6×10^{-14} cm^3/s. As the cation concentration was raised, the water permeability decreased and a binding constant was determined from a quantitative fit to the data. When the data were fitted assuming a maximum of one ion per channel, the dissociation constant was 115 mM for Li^+, 69 mM for K^+, and 2 mM for Tl^+.

INTRODUCTION

Gramicidin is a pentadecapeptide (Sarges and Witkop, 1965) and is thought to form narrow, dimeric channels (Urry, 1971; Urry et al., 1971; Weinstein et al., 1980) that provide a pathway for the movement of cations and water across bimolecular lipid membranes (BLMs). It is widely accepted that the channel is filled with water molecules (Levitt et al., 1978; Rosenberg and Finkelstein, 1978 *a, b*) and is highly cation selective (Myers and Haydon, 1972). Aside from gating, gramicidin shows much of the complex behavior of the K^+ channel (Hille, 1975; Armstrong, 1975; Hille and Schwarz, 1978; Hagglund et al., 1979; Andersen

[1]Rosenberg and Finkelstein (1978*b*) have made similar measurements in 10 mM NaCl + 100 mM choline chloride and obtained a water permeability about six times smaller than reported here. When they made the measurement, the single channel conductance in 10 mM NaCl was not available. Therefore, to determine the number of channels in their BLMs they divided the conductance in 100 mM NaCl by 10 to obtain the conductance in 10 mM NaCl. The conductance data of Neher et al. (1978) indicate that this would cause about a 40% underestimation of the water permeability per channel. Rosenberg and Finkelstein did not correct for unstirred layer effects in their osmotic volume-flux experiments. This also would cause a small underestimation of the water permeability, but there is still a large discrepancy between the results. Possibly the difference in the lipids used to form the BLMs could account for some of this discrepancy (Bamberg et al. 1976; Frohlich, 1979). A difficult problem faced by these authors (which we avoided by using a microelectrode) was that, in the presence of excess inert electrolyte (100 mM choline chloride), severe diffusion polarization would occur during the membrane resistance determination. Thus, a correct extrapolation of the resistance measurement to zero time was vital in their experiments.

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and Procopio, 1980) found in muscle and nerve membranes. Therefore, gramicidin provides a reasonable model for such biological channels.

In this study an osmotic pressure was used to produce water flux through BLMs containing gramicidin channels. Measurements of the water flux were used to determine the osmotic (hydraulic) water permeability of the channel and to examine the equilibrium binding of cations in the channel.

Stratagem

Our approach was based on the idea that the gramicidin channel is so narrow (3.8–4 Å Diam, Urry et al., 1975; Koeppe et al., 1979) that cations and water molecules cannot pass by each other. The channel walls constrain the molecules so that they move through the channel in a "single file" procession. Since gramicidin excludes anions, a cation that enters the channel cannot pass through under open-circuit conditions because electroneutrality must be maintained in the solutions on either side of the membrane. Thus, one would expect a cation in the channel to block water transport through the channel.

The same principles apply in BLMs that contain many gramicidin channels. Because electroneutrality must be maintained, when a cation moves through a channel in one direction, another cation must move through another channel in the opposite direction. Each cation that is transported is coupled to the same number of water molecules moving in a row in front of the cation. The expected result is no net water transport through channels that contain a cation. If the premises are correct, it is possible to determine the partitioning of cations into the channels from measurements of the water permeability of the channels as a function of cation concentration. As the cation concentration is increased, a larger proportion of channels will contain a cation, and the water permeability per channel will decrease.

METHODS

Three different types of experiments were performed to examine cation blockage of water flux through the gramicidin channel. In each approach the hydraulic water permeability of multichannel BLMs was determined. In this section the water permeability measurement will be explained. Specific procedures used in each of the three types of experiments will then be presented in turn.

Hydraulic Water Permeability Measurement

BLMs were formed from a mixture of 1.5% glycerol monoolein (GMO) (Sigma Chemical Co., St Louis, Mo.) in hexadecane (City Chemical Corp., N.Y.) containing gramicidin (ICN Pharmaceuticals, Inc., Cleveland, Ohio) dissolved in methanol. Electrolyte solutions were prepared using twice glass distilled water and reagent grade salts. All glassware was cleaned in dichromic acid.

Fig. 1 shows a schematic drawing of the apparatus, adapted from earlier versions (Hanai and Haydon, 1966; Holz and Finkelstein, 1970) and used to make the permeability measurements. The "front" chamber was formed from a 1.5-ml hole in a Teflon block, and the "back" chamber was a closed volume of ~50 μl formed by connecting a Teflon tube to a hollow Ag/AgCl cylinder and a 10-μl Hamilton syringe. The BLM was formed on the 1.06-mm Diam hole at the end of the Teflon tube that formed the back chamber. The hydraulic water permeability coefficient (P) of BLMs containing gramicidin channels was determined by measuring the volume flux (J_v) that resulted from infusing a solution containing an impermeant (usually mannitol) into the front chamber. Since the water permeability of the membrane varied with electrolyte concentration, the impermeant concentration that was used ranged from 0.2 to 1 M to keep J_v at $\sim 7 \times 10^{-5}$ ml/s cm^2. The volume change of the back chamber (see Fig. 2) was measured by holding the BLM at a fixed position by observing "glimmers" of light on the

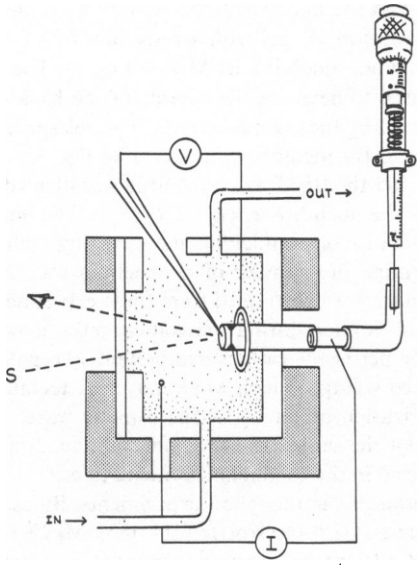

FIGURE 1

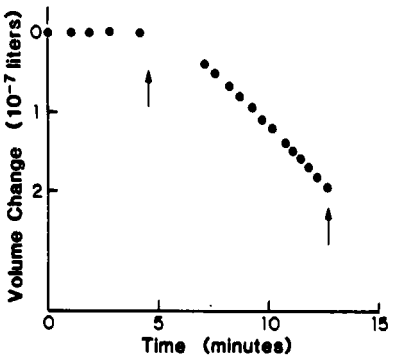

FIGURE 2

FIGURE 1 Schematic drawing of the apparatus used to make the water permeability measurements. The BLM is shown slightly back in the Teflon tube that forms part of the closed back chamber, and the tip of the voltage (V) microelectrode is positioned just inside the Teflon tube. The ports used to change the solution in the front chamber are shown. The current electrode (I) in the front chamber was larger than depicted here.

FIGURE 2 Each point represents one volume determination during the water permeability measurement. The volume of the back chamber decreases as a result of the water flux produced by the osmotic pressure difference across the BLM. At about 4 min the solution containing the impermeant was infused, and at just <13 min the experiment ended.

membrane (with a 40× microscope) and adjusting the back chamber volume using a micrometer. Volumes as small as 10^{-9} liter could be measured. The overall permeability was calculated from the volume flux using Eq. 1:

$$P = J_v/(A\bar{V}_w\Delta c) \quad (1)$$

where $\bar{V}_w$ is the molar volume of water, A is the area of the BLM (usually ~0.6 mm^2) determined from a photograph, and Δc is the impermeant concentration difference across the membrane. Appendix A describes how this Δc was determined by correcting for solute polarization in the unstirred layers next to the BLM, using the open circuit potential which developed during the volume flux. The hydraulic water permeability of BLMs without channels was determined separately and was subtracted from the overall permeability (P) to obtain the permeability of the channels (P_F) in a BLM.

TYPE I: MULTICHANNEL CONDUCTANCES AND PERMEABILITIES In these experiments the resistance of the membranes was measured just before and just after the volume flux measurement. Two pairs of electrodes were used (see Fig. 1), one pair to pass a 2-ms constant current pulse and the other to record the resulting potential difference. The current step and the potential difference were amplified (differential amplifier model 603J, Analog Devices, Norwood, Mass.) and displayed on an oscilloscope. Because of the very low resistance of the multichannel membranes, the voltage electrodes had to be placed very close to the BLM to reduce the resistance contributed by the solution. Therefore, a microelectrode that could be positioned within 30 μm of either side of the BLM was used to make the measurement.

To determine the resistance, the BLM was formed and the microelectrode (coated with silicone and filled with 3 M tetramethylammonium chloride (TMACl) in 1% gel with a resistance of ~1 MΩ) was positioned so its tip just entered the Teflon tube but did not touch the BLM (see Fig. 1). The constant current pulses were begun and the micrometer was used to decrease the volume of the back chamber causing the BLM to move forward until it was punctured by the microelectrode. The voltage deflection then showed a step increase due to the voltage drop across the membrane, as shown in Fig. 3.

The difference in the voltage deflection before and after the BLM was punctured was divided into the constant current pulse to give the conductance of the membrane ($G = I/\Delta V$). The membrane conductance was divided by the single channel conductance of gramicidin (measured by Neher et al., 1978) at the same electrolyte concentration to determine the number of channels in the membrane ($\sim 10^{11}/cm^2$). The average water permeability per channel was determined from these experiments.

The magnitude of diffusion polarization that should develop during the voltage pulse is derived in Appendix B. It is shown that for the case where only permeant cations are present, the polarization voltage at 2 ms should contribute ~2% of the measured voltage, which would not be detectable in our experiments (see Fig. 3). Because of the solute polarization produced by the osmotic water flux, the cation concentrations at the membrane surfaces are not the same as in the bulk solution. Appendix A describes the small correction for this effect that was used in the conductance calculations.

Several tests were conducted to check for possible problems in the type I experiments. By using a low concentration of permeant cation (1 mM KCl) and excess inert electrolyte (100 mM $MgCl_2$) to lower the solution resistance, it was found that the microelectrode arrangement and large Ag/AgCl electrodes both gave the same value for the membrane conductance. In another control, the water permeability of membranes was unchanged after they were punctured four times by the microelectrode, indicating that puncturing the BLM did not alter its permeability characteristics. In addition, when inert electrolytes were used they were tested at higher concentrations to be sure they did not contribute to the conductance. Experiments conducted at one concentration of conducting cation in the presence and absence of inert electrolyte (up to 50 mM $MgCl_2$ with 10 mM KCl) indicated that a surface potential on the BLM did not effect the results, and the inert electrolyte was not blocking conductance (Bamberg and Lauger 1977) at the concentrations that were used (see Table I).

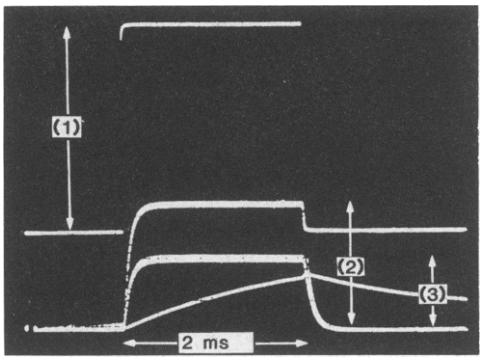

FIGURE 3 Photograph of the oscilloscope traces used to determine membrane conductance in 0.1 M LiCl. From top to bottom the traces show (1) the 2-ms current deflection (25 μA); (2) the voltage deflection when the BLM is punctured by the microelectrode; (3) the voltage deflection due to the resistance of the solution, obtained while the BLM is not punctured; and, in the bottom curve, the voltage deflection when the microelectrode tip is partially plugged with lipid. The difference between 2 and 3 is the voltage drop across the BLM (14 mV). Traces 2 and 3 represent the superposition of about five deflections taken while the BLM was being moved to see that the position of the membrane did not affect the resulting voltage deflection. Usually when the microelectrode tip passed through the membrane, it became partially plugged with lipid. The tip was cleared by putting a 1 V, 900 kHz sine wave across the voltage electrodes.

TABLE I
PERMEABILITY PER CHANNEL IN 10 mM KCl AND INERT ELECTROLYTE

P_F/channel	Inert electrolyte
(10^{-14} $cm^3/s \pm SE$)	(mM)
4.6 ± 0.5 (3)*	0
6.1 ± 1.7 (3)	10 $MgSO_4$
5.7 ± 0.3 (2)	10 $MgCl_2$
3.9 ± 0.3 (3)	50 $mgCl_2$
4.7 ± 0.5 (5)	20 TMACl
5.0 ± 0.3 (6)	60 TMACl

*Numbers in parentheses indicate number of experiments.

The major weakness of these measurements is the inherent assumption that the conductance of the gramicidin channel in a membrane that contains $\sim 10^{11}$ channels/cm^2 is the same as in a membrane that contains only one channel. We feel that this is a reasonable assumption because the measurements were not extended to the very high cation concentrations where channel interaction is more likely (Kolb and Bamberg, 1977), and the conductance could be measured at early times (<0.5 ms) before diffusion polarization became important (see Appendix B and Fig. 3). As an additional check, we performed the following two types of experiments that do not require this assumption.

TYPE II: PERMEABILITY WITH A CONSTANT NUMBER OF CHANNELS The purpose of these experiments was to try and add the gramicidin to the bilayer in a well controlled, reproducible manner so that it could be assumed that each membrane had the same number of channels and the relative variation in water permeability per membrane could be directly related to the fraction of the channels that were blocked (contained a cation). The mixture used to form the BLM was 10 μl of 10 mg gramicidin dissolved in 1 ml of methanol added to 100 μl of 1.5% GMO-hexadecane. Just before a bilayer was formed, the vial containing this mixture was vortexed to obtain a uniform suspension. About 1 μl of the lipid mixture was placed on the Teflon tube (Fig. 1) and was agitated with a pipette tip to initiate bilayer formation at the same time for each membrane. The vial containing the membrane-forming mixture was only opened a few times before the contents were discarded, to insure that the amount of methanol did not decrease by evaporation.

As a check that the membranes contained the same number of channels, membrane conductances were measured in separate experiments with BLMs formed in the same manner using two different gramicidin concentrations. The number of channels was determined from the single-channel conductances (Neher et al., 1978). As shown in Fig. 4 *a* and *b*, there was no significant variation in the number of channels for KCl concentrations varying from 10 mM to 2 M. Although the apparent constancy in the number of channels could result from a cancellation of a variation in channel number by a coincidentally appropriate channel interaction, this seems unlikely.

TYPE III: PERMEABILITY OF A MEMBRANE AT TWO ELECTROLYTE CONCENTRATIONS The water permeability of a multichannel BLM was determined at a given cation concentration. Then a second concentration of the same electrolyte species was infused into the front chamber and the permeability of the same BLM was determined again. Infusions of the two electrolyte concentrations were altered for a minimum of three measurements. It is assumed that, since the same BLM was used for the two different cation concentrations, the change in membrane water permeability represents a change in water permeability per channel. The Nernst potential resulting from the concentration change in the front chamber was <100 mV and should not alter the number of channels in the BLM (Bamberg and Benz, 1976). In addition, because the concentration of gramicidin in the BLM is high, most of the gramicidin is in the form of dimerized channels (Bamberg et al., 1977). Therefore, voltage changes cannot induce a

significant increase in the number of channels. When there is a different electrolyte concentration on the two sides of the membrane, it is shown in Appendix D that it is a good approximation to use the average activity as that which governs partitioning of cations into the channel.

RESULTS

In 12 experiments the average water permeability of the BLM without gramicidin channels was determined to be $3.7 \pm 0.3 \times 10^{-3}$ cm/s (SE). Fig. 5 illustrates the data obtained in the type I experiments. The water permeability of membranes vs. their conductance is shown in various concentrations of LiCl. The decrease in the slopes of the lines as the concentraton is increased indicates a lower water permeability per channel. Similar results were also obtained for KCl and TlCl. The hydraulic water permeability per channel determined from the type I experiments is plotted as a function of cation activity in Fig. 6. The permeability of the ion-free channel ($\sim 6 \times 10^{-14}$ cm^3/s) was determined by extrapolating the lower limit KCl and LiCl data back to zero cation concentration.

The theoretical fits to the data were obtained from the following equation (Appendix C):

$$P(a)/P(0) = (1 + a/K_1 + a^2/K_1K_2 + \ldots)^{-1} \quad (2)$$

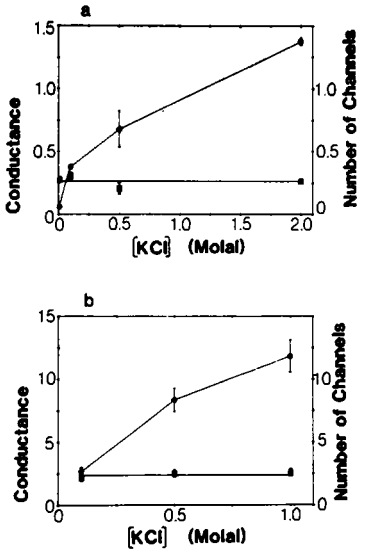

FIGURE 4

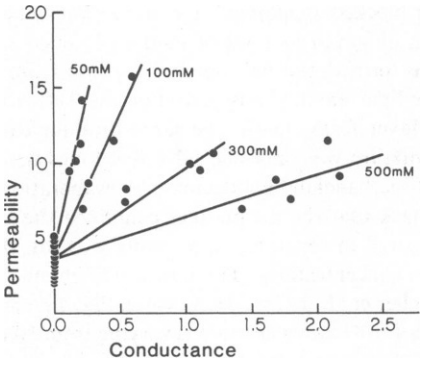

FIGURE 5

FIGURE 4 The conductance (●, S/cm^2 ± SE) and number of channels (■, 10^{11}/cm^2 ± SE) per membrane is shown vs. concentration. The BLMs were formed in a well-controlled reproducible manner from a lipid mixture containing gramicidin (type II experiments). In the two series of experiments (*a* and *b*) the number of channels per membrane (membrane conductance/single channel conductance) remained constant, independent of the KCl concentration. A higher concentration of gramicidin was used to form the BLMs in the series of experiments shown in (*b*).

FIGURE 5 Each data point represents one experiment in which the water permeability (10^{-3} cm/s) and the conductance (S/cm^2) of a membrane were determined in an electrolyte solution of 50, 100, 300, or 500 mM LiCl. The scatter in the points is represented by standard error bars in Fig. 6. At each concentration the points are fitted by a linear regression line all of which intercept the *y*-axis at ~3.7. The points on the *y*-axis were determined by using BLMs without any gramicidin channels.

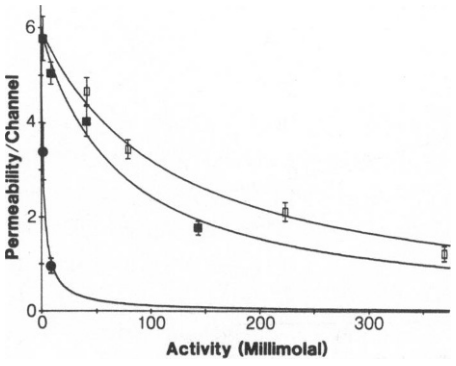

FIGURE 6

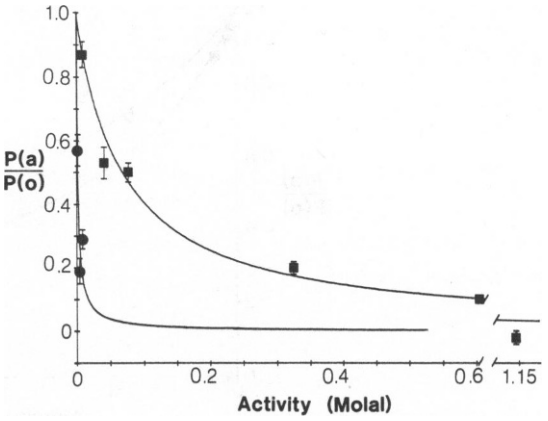

FIGURE 7

FIGURE 6 Type I experiments. The water permeability per channel ± SE (P_f) (10^{-14} cm^3/s) is plotted vs. the activity of LiCl (□), KCl (■), and TlCl (●). The data are fitted by theoretical curves (see text) with K_1 = 115 mM for Li^+, 60 mM for K^+, and 2 mM for Tl^+. The water permeability of the ion-free channel (the *y*-intercept) was determined by extrapolation of the Li^+ and K^+ data from the lowest concentrations and is 6×10^{-14} cm^3/s.

FIGURE 7 Type II experiments. The hydraulic water permeability at activity *a* relative to that in the absence of cations [$P(a)/P(0)$] is plotted vs. the activity of KCl (■) and TlCl or TlF (●). The theoretical curves are for the same values of K_1 as were used in Fig. 6.

where $P(a)/P(0)$ is the ratio of the water permeability at cation activity a to that in the absence of cations and K_1 and K_2 are the equilibrium dissociation constants that describe the binding of the first and second cation in the channel. The theoretical lines shown in Fig. 6 are for a K_2 of infinity and a K_1 equal to 115 mM for Li^+, 69 mM for K^+, and 2 mM for Tl^+. Good fits to the data (within the SE) were obtained by using just the first binding constant.

In Fig. 7 the results of the type II experiments are plotted as the fraction of ion-free channels [$P(a)/P(0)$] at the given cation activity. In Fig. 8 the type III experiments are displayed in the same manner. In both cases the theoretical curves are the same as those in Fig. 6.

DISCUSSION

The basic assumption of this approach for determining ion binding constants is that the gramicidin channel is so narrow that an ion and water cannot get past each other. Finkelstein and Anderson (1981) have argued that a cation at a channel binding site has almost no effect on the water movement through the channel. This is inconsistent with the findings of this study, and the incompatibility may in some part arise from the difference in lipids used.

A great deal of evidence has accumulated that indicates the channel is single file. There is general agreement that the "static" diameter of the channel is only ~4 Å (Urry et al., 1975; Koeppe et al., 1979). For a K^+ ion (2.7 Å in diameter) and water (~3 Å in diameter) to get around each other, the gramicidin channel would have to flex or "breath" ~1.7 Å, an event that should be very improbable. Even if this event occurred with a low probability, it would not significantly alter our conclusions which depend only on the assumption that the resistance

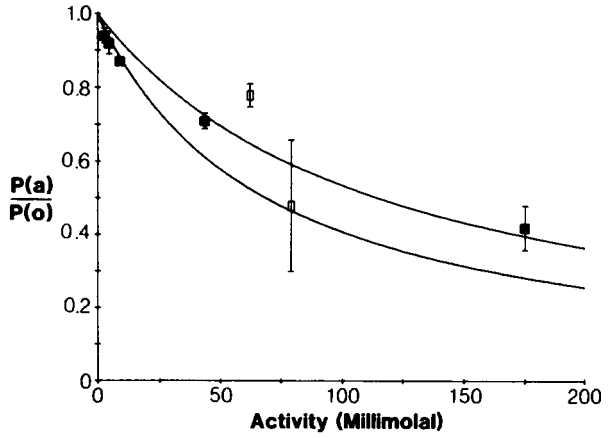

FIGURE 8 Type III experiments. The value of $P(a)/P(0)$ is plotted vs. the activity of LiCl (□) and KCl (■). The theoretical curves are for the same values of K_1 as were used in Fig. 6.

to movement of water around an ion is much larger than the resistance in the absence of the ion. The streaming potential measurements which show that about nine water molecules are coupled to the transport of a cation through the channel (Levitt et al., 1978 and unpublished results) is consistent with both the single-file arrangement of the water molecules and the inability of the water and ion to get around each other. Finally, the most direct evidence for this assumption is the data of Figs. 6 and 7 that show that the water permeability per channel approaches zero at high K^+ and Tl^+ concentrations.

The ion and water could get easily past each other if the ion binding site was located at a very superficial position near the channel mouth. Thus, we cannot rule out the possibility that there are ion binding sites of this type, since they would not alter the water permeability and could not be detected in this study. However, this seems unlikely since x-ray diffraction studies (Koeppe, et al., 1979) show the first binding site well within the channel and most kinetic studies have concluded that the ion binding has a voltage dependence (Hladky et al., 1979; Urban et al., 1980; Eisenman et al., 1980*a*, Urry et al., 1980, Anderson and Barrett, 1981), implying that the ion must be binding at some depth into the channel. In addition, the NMR studies of Cornelis and Laszlo (1979) show "partial dehydration of Na^+ upon binding," again suggesting a site within the channel.

The fall in water permeability per channel with increasing ion concentraton provides a direct equilibrium measurement of ion binding. The only adjustable parameters are the values of the binding constants in Eq. 2. The data in Figs. 6–8 were fitted (within the statistical errors) on the basis of the assumption that there was a maximum of one ion per channel. Since the fits could be improved (although without statistical significance) by the use of two binding constants, these experiments do not provide a definitive test for the number of ions in the channel at high concentrations. If the channel is perfectly single file, the K_1 determined in Figs. 6–8 (assuming only one ion per channel) represents a lower limit for the dissociation constant of the first ion. As is evident from Eq. 2, the same block of water permeability would be obtained at a larger K_1 if the number of binding constants used in the fitting procedure were increased. Thus, the minimum value for the dissociation constant for the binding of the

first ion is 115 mM for Li^+, 69 mM for K^+ and 2 mM for Tl^+. If there is at most one ion per channel or if the second ion has a much lower affinity than the first, then these numbers are accurate values for the dissociation constant of the first ion.

Table II lists some previous determinations of the equilibrium binding constants of the gramicidin channel, mostly from measurements of transport properties. Estimates based on transport properties are indirect and are critically dependent on the detailed theoretical model that is used to describe the results and the relative weighting that is given to the different transport experiments.

The estimates of the different authors vary over a wide range. In particular Urban et al. (1980) have estimated equilibrium constants about fifty times smaller than those obtained in this study. The main evidence for these high affinities is the inequality of the permeability and conductance ratios of Na^+ and K^+ at concentrations of ~10 mM. This inequality implies ion interaction and, therefore, a high fraction of occupied channels. As Urban et al. (1980) state, when ions move independently of each other, permeability and conductance ratios are equal if they are measured at the same potential. Since the difference in the two ratios is not large (at 10 mM the permeability ratio is ~2.9, corresponding to a membrane potential of 27 mV while the conductance ratio is ~2.4 when measured at a potential of 100 mv, Urban et al., 1980), it is possible that a small voltage dependence could account for the difference.

Some of the variability in Table II could result simply from the use of different lipids and solvents to form the BLMs (Bamberg et al., 1976; Frohlich, 1979). Also, a problem that was present for all of the authors is that charged surface impurities could alter the cation concentration at the surface of the BLM.

Our results are consistent with the conductance data. As discussed above, our values for the dissociation constant of the first ion (K_1) are correct if the second ion binds with a much lower affinity than the first. For this case, an Eadie-Hofstee plot in the low concentration range of the conductance vs. the conductance/activity should provide an estimate of K_1 (Levitt, 1978;

TABLE II
ESTIMATED DISSOCIATION CONSTANTS FOR THE FIRST ION IN THE CHANNEL

LI^+	Na^+	K^+	Tl^+	Reference
	(*mM*)			
	303	232		Lauger, 1973
	322		1.3	Neher, 1975
		5	1	Eisenman et al., 1976
		4	0.3	Sandblom et al., 1977; Eisenman et al., 1978*b*
111	50	25	0.9–3.2	Eisenman et al., 1978*a*
169	345	25	0.9	Neher et al., 1978
	350	365	1–1.3	Levitt, 1978
	4	1.2	0.056	Urban et al., 1980
			0.6	Eisenman et al., 1980*a*
	5			Urry et al., 1980
	300			Andersen and Procopio, 1980
	>17		0.5–1	Veatch and Durkin, 1980
115		69	2	This study

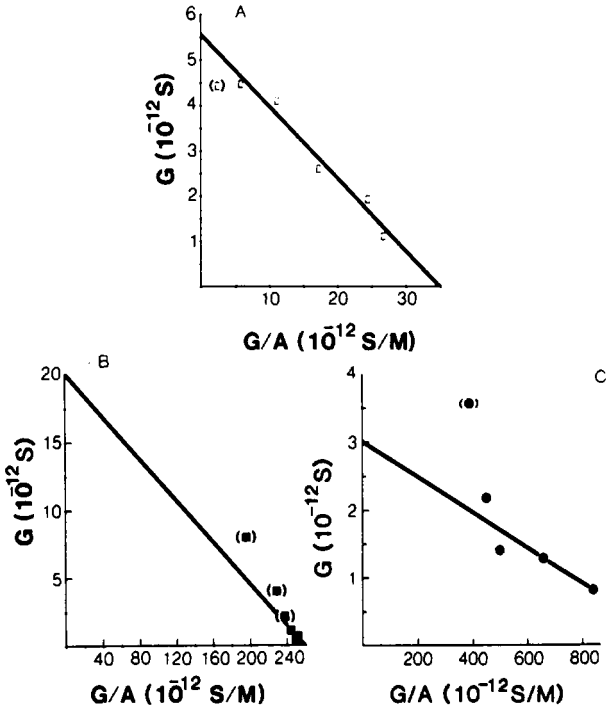

FIGURE 9 Eadie-Hofstee type plots of single channel conductance (G) vs. conductance/activity (G/A) in (A)LiCl, (B)KCl and (C)TlCl. A least-square linear regression line is drawn through the low concentration points. The higher concentration points that were not used in the fit are indicated by parentheses. The slopes of the lines (which should be equal to K_1 for the first ion) are 158 mM for Li^+, 77 mM for K^+ and 3 mM for Tl^+. (Data from Neher et al., 1978).

Eisenman et al., 1978*a*; Urban et al., 1980). These plots are shown in Fig. 9 for LiCl, KCl, and TlCl using the conductance data of Neher et al. (1978). The dissociation constant determined from a least-square fit to the data is 158 mM for Li^+, 77 mM for K^+, and 3 mM for Tl^+. This is in very good agreement with our results. At the higher K^+ and Tl^+ concentrations, the data points deviate from the straight line, implying that another type of binding site (binding of the second ion) is becoming important.

APPENDIX A

Correction for Solute Polarization

During the volume-flux experiments, there is solute polarization in the unstirred layers (USL) next to the membrane, so that the effective osmotic driving force is less than that of the bulk solutions. The electrolyte is dragged up to the BLM surface on one side, and swept away from the other side of the BLM. In the steady state, the ratio of the concentration in the bulk solution (C_b) to the concentration at the surface of the membrane (C) is given by

$$C/C_b = \exp(-J_v L/DA) \qquad \text{(A1)}$$

where J_v is the volume flux, L is the thickness of the USL, D is the diffusion coefficient of the solute and A is the area of the BLM.[2]

The following experiment showed that, due to the geometry of the apparatus (Fig. 1), the USL in the front chamber was negligible. The water permeability of a membrane formed in distilled water was determined at several different volume fluxes by changing the impermeant concentration in the front chamber. There was no possibility of solute polarization in the back chamber since it did not contain any solutes. If there was a significant USL in the front chamber, the volume flux should have reduced the effective impermeant concentration at the membrane surface (in accordance with Eq. A1); the apparent water permeability (obtained using the bulk impermeant concentration) should decrease as J_v was increased. In five experiments in which J_v was changed by a factor of two or more, there was no significant change in the water permeability, indicating that USL effects were not significant in the front chamber.

In the back chamber the USL was significant, and a correction was made. During the volume-flux measurements, the open circuit potential difference Ψ was recorded. This potential is the sum of two terms:

$$\Psi = \Psi_{st} + (RT/F) \ln (K_1^+/K_2^-). \tag{A2}$$

The first term is the streaming potential, and the second is the Nernst potential due to the K^+ concentration difference across the membrane which results from the polarization in the back chamber. Since the value of Ψ_{st} was known from a separate set of experiments (Levitt et al. 1978; and unpublished results), the K^+ concentration at the back surface of the membrane could be determined from Eq. A2, and the appropriate correction made to obtain the net osmotic driving force. This correction ranged from <1% at 10 mM KCl to a high of ~25% in 2 M KCl. When the cation and anion had different mobilities (i.e., LiCl), a diffusion potential term had to be added to Eq. A2.

Because of the small polarization of electrolyte in the back chamber, the conductance is being measured at a cation concentration that differs slightly from that of the bulk solution. This was also corrected for. The relation between the concentration on the two sides of the membrane and the conductance depends on the specific model that is used for the channel. However, for small changes, the ratio of the conductance G_1 (when the back chamber concentration is equal to the bulk concentration c_1) to the conductance G_2 (when the back concentration is equal to c_2 (while the front concentration remains c_1 since the USL in the front chamber is negligible, see above) is (Dani, 1980):

$$G_1/G_2 = (c_1/c_2)^{1/2}.$$

This correction was always <17%.

APPENDIX B

Diffusion Polarization During Conductance Measurements

As current is passed, the permeant cation is depleted from the USL on one side of the BLM, and accumulates in the USL on the other side of the BLM. This polarization which is produced by the short current pulses that are used to measure the membrane conductance has two effects. First, the cation gradient across the membrane produces a Nernst potential that adds to the IR voltage drop that was used to determine the membrane conductance. Second, the change in cation concentration alters the membrane conductance. This second effect is negligible relative to the first, because the increase in

[2]This simple analysis of solute polarization is not exact, but it does give a semi-quantitative approach for predicting the effects of USLs. Everitt and Haydon (1969) have analyzed USL effects on osmotic flow in detail.

concentration at one side of the membrane is approximately equal to the decrease on the other side (Dani, 1980).

Neumcke (1971) has derived an expression for the polarization at the membrane surface for the case of a constant current (used in this study) and no potential gradients in the bulk solution:

$$c(t)/c(0) = 1 + 2[J(0)/c(0)]\ (t/\pi D)^{1/2} \tag{A3}$$

where $c(t)$ is the surface concentration at time t, $c(0)$ is the bulk concentration, $J(0)$ is the initial cation flux and D is the diffusion coefficient of the cation. This case (no bulk solution voltage gradients) should be applicable when there is an excess of inert electrolyte (Lauger, 1976). When there is no inert electrolyte, there are voltage gradients in the bulk solution (Lauger, 1976), and Eq. A3 must be slightly modified (Dani, 1980):

$$c(t)/[c(0)] = 1 + [J(0)/c(0)]\ (t/\pi D)^{1/2} \tag{A4}$$

The Nernst potential (Ψ_N) that results from this polarization is given by:

$$\Psi_N = (RT/F) \ln [(c_2(t)/c_1(t)] \tag{A5}$$

where c_2 and c_1 are the concentrations at the two membrane surfaces determined from Eq. A4. Eqs. A4 and A5 can be used to estimate the error introduced by this polarization effect. For a representative experiment at 10 mM KCl, Ψ_N is ~0.04 mV at 2 ms, which is only 2% of the total voltage deflection (2mV) and could probably not be detected in our measurements (see Fig. 3). Thus polarization effects were not important in our experiments. (Since $J[0]/c[0]$ decreases as the concentration is raised, the correction at concentrations >10mM is <2%, see Eq. A4).

APPENDIX C

Calculation of Dissociation Constants for Channel

The water permeability data were fitted using an equilibrium analysis that is model independent. The equilibrium binding was analyzed using the following state diagram:

$$f_0 \underset{K_1}{\longleftrightarrow} f_1 \underset{K_2}{\longleftrightarrow} f_2 \cdots$$

where K_1 and K_2 are the dissociation constants for the binding of the first and second cation, respectively. The probability of the channel containing no cations is f_0, one cation is f_1, two cations is f_2, etc:

$$\begin{aligned} f_1 &= a f_0/K_1 \\ f_2 &= a^2 F_0/K_1K_2 \\ &\cdot \\ &\cdot \\ &\cdot \\ f_0 + f_1 + f_2 + \ldots &= 1 \\ f_0 = (1 + a/K_1 + a^2/K_1 K_2 \ldots.)^{-1} & \end{aligned} \tag{A6}$$

where a is the activity of the permeant cation. Since there is a net water flux only through the channels

that do not contain an ion,[3] the water permeability of the channels at activity a relative to that in the absence of cations $[P(a)/P(0)]$ is:

$$P(a)/P(0) = (1 + a/K_1 + a^2/K_1K_2 \ldots)^{-1}. \quad \text{(A7)}$$

APPENDIX D

Type III Experiments

In these experiments the concentration of the permeable cation on the two sides of the membrane was not always the same. Therefore, the symmetrical concentration at which the same fraction of channels would be occupied had to be determined. These experiments were conducted in an open circuit, so that there was no current flow, and the ends of the channel were in equilibrium with the adjacent bulk solutions. The cation binding is described by the following state diagram:

$$f_{1,0} \overset{a_1}{\underset{K}{\leftrightarrow}} f_{0,0} \overset{a_2}{\underset{K}{\leftrightarrow}} f_{0,1}$$

where K is the dissociation constant for the binding of an ion at either end of the channel (which is assumed to be symmetrical) and a_1 and a_2 are the cation activities on the two sides. The channel can exist in three states: no cations ($f_{0,0}$), cation bound at end 1 ($f_{1,0}$), or end 2 ($f_{0,1}$). The fraction of channels that do not contain an ($f_{0,0}$) ion and therefore are permeable to water, is given by:

$$P_F(a_1, a_2)/P_F(0) = [1 + (a_1 + a_2)/K]^{-1} \quad \text{(A8)}$$

where $P(a_1, a_2)$ is the permeability for cation activities a_1 and a_2. For the symmetrical case $(a_1 = a_2 = a)$;

$$P_F(a)/P_F(0) = (1 + 2\,a/K)^{-1} \quad \text{(A9)}$$

comparing Eqs. A8 and A9, it can be seen that the equivalent symmetrical cation concentration (a) is related to the concentration on the two sides by:

$$a = (a_1 + a_2)/2. \quad \text{(A10)}$$

The major assumptions in this derivation are that the concentrations are low enough that multiple occupancy can be neglected and that the voltage drop between the binding site and the bulk solution is negligible. If there is a significant voltage drop it must be included in the equilibrium binding constant.

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[3]This is not strictly true if the channels can contain more than one ion. There will be a small circulation of ions going one direction through one ion channels and the opposite direction through two ion channels. Since there is about one less water molecule transported per ion in the two-ion channel (as determined by streaming potential measurements) this will produce a small net water flux. However this effect is negligible (and undetectable) because the water permeability of the channel that contains either one or two ions is so much smaller than that of the cation-free channel (Dani and Levitt, 1981).

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