GRAMICIDIN-MEDIATED CURRENTS AT VERY LOW PERMEANT ION CONCENTRATIONS

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ABSTRACT Current–voltage relations have been measured for the fluxes of caesium ions through pores formed by gramicidin in lipid bilayer membranes. The ionic currents have been separated from capacitative currents using a bridge circuit with an integrator as null-detector. The conductances during brief voltage pulses were small enough to avoid the effects of diffusion polarization and the ionic strength was raised using choline chloride or magnesium sulfate to reduce the effects of double-layer polarization. Under these conditions the current–voltage relations have the same shape at 0.1 and 1 mM, but different shapes for higher concentrations. These data demonstrate that the fluxes do not obey independence for concentrations above 10 mM, but they cannot be used in isolation to support a particular value of the binding constant. The shape observed at low concentrations suggests that entry of ions into the pore remains weakly potential dependent even at 300 mV.

INTRODUCTION

Gramicidin A makes lipid bilayer membranes permeable to small monovalent cations and water (for reviews, see Finkelstein and Andersen, 1981; Hladky and Haydon, 1984). For sufficiently low permeant ion concentrations the rate of caesium ion movements across the membrane is limited by the rate of entry into the pores (Hladky and Haydon, 1972; Eisenman et al., 1980; Andersen, 1983*c*; Hladky and Haydon, 1984). Since the pores are then only rarely occupied, the conductance should increase linearly with concentration at all voltages, and thus the shape of the current–voltage relation should be independent of ion concentration. Above 10 mM the single-channel conductances are not linear with concentration and the shape of the current–voltage relation varies (Neher et al., 1978; Urban et al., 1980, Andersen, 1983*a*). These and other data suggest a first ion binding constant for Cs^+ in the region of 100 M^{-1} (Neher et al., 1978; Hladky and Haydon, 1984) though they do not exclude higher values (Urban et al., 1980; Eisenman et al., 1983). Eisenman and co-workers (Eisenman and Sandblom, 1983*a, b,* 1984; Eisenman et al., 1980, 1982, 1983) have recorded many-channel currents using a ramp protocol and report a significant change in shape of the current vs. voltage curve between 1.0 and 0.1 mM for Cs^+. They conclude that this variation implies strong binding and have provided estimates for the first ion binding constant between 660 and 2,500 M^{-1} (Eisenman et al., 1983).

Here current–voltage relations have been measured for low concentrations of CsCl using brief constant voltage pulses and a bridge circuit with an integrator as null detector. This technique allows the separation of capacitative and ionic currents and the detection of diffusion polarization. We find that the ionic currents with 1 mM CsCl increase less rapidly with potential than those with 10 mM CsCl, but that the current–voltage relations for 0.1 and 1 mM CsCl are indistinguishable. The shape observed for low concentrations suggests that the entry process remains weakly potential dependent even at high potentials.

METHODS

Films were formed across a hole in a ptfe support using standard techniques (see Fettiplace et al., 1975) from a 10 mM solution of mono-oleyl 1-rac glycerol (glyceryl mono-oleate; Sigma Chemical Co., Poole, England) in *n*-hexadecane (Koch-Light Laboratories Ltd., Colnbrook, England). The *n*-hexadecane had been passed through an alumina column to remove polar impurities. Membranes ranged from 0.1 to 1.0 mm in diameter; a typical capacitance was 1.5 nF. All experiments were performed at ~20°C. Identical salt solutions were present on both sides of the membrane.

Distilled water was obtained from a commercial still in which all components containing plasticizers were replaced with ptfe. Caesium chloride (Analar, BDH Chemicals Ltd, Poole, England) was roasted at ~400°C overnight before use. This treatment had no apparent affect on the current–voltage relation but it significantly improved membrane stability with 100 mM CsCl solutions. Choline chloride (Sigma Chemical Co., crystalline grade) was purified by recrystallization from ethanol and washing with acetone. The dried powder had no odor. It was stored as a concentrated aqueous solution at 4°C. All other reagents were analytical reagent grade. Apparatus that came into contact with the aqueous or lipid solutions was cleaned before use with a dichromate-sulfuric acid mixture.

Because double-layer polarization produces significant distortions in the shape of current–voltage relations at ionic strengths below 100 mM (Andersen, 1983*b*; Hainsworth and Hladky, 1987), a supporting electrolyte, either choline chloride or magnesium sulfate, was added to the

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solutions. These additions also ensured that the membrane capacitance could be charged in <100 μs.

Gramicidin (Koch-light) was stored as a $\sim 10^{-5}$ M solution in ethanol at 4°C. Aliquots of this stock solution were evaporated to dryness and either dissolved in methanol and added to the aqueous phases (either or both), or taken up directly as a suspension in the lipid-hexadecane solution.

Conductances were measured with a bridge circuit, using an integrator as a null-detector, as described in detail elsewhere (Hainsworth and Hladky, 1987). Briefly a series of voltage pulses is applied to one electrode and the current required to keep the other electrode at 0 mV is measured using the integrator. Typically after a reset of the integrator to its reference value (hereafter called "zero"), the membrane voltage is stepped as follows: (*a*) 0 mV for 10 ms; (*b*) + V for 10 ms; (*c*) 0 mV for 40 ms; (*d*) − V mV for 10 ms; (*e*) 0 mV for ~700 ms. The integrator keeps a "running total" of the net charge movement resulting from ionic and capacitative currents across the membrane. In the absence of rectification these currents sum to zero over one cycle, and the integrator output in phases (*a*) and (*e*) is the same. Because the capacitative currents that charge and discharge the membrane just before, during, and just after phase (*b*) also sum to zero, the integrator output during phase (*c*) will be a constant determined by the average ionic current during phase (*b*). The phase (*c*) level may be brought to zero without disturbing the level in phase (*e*) by injecting into the integrator input, for the duration of phases (*b*) and (*d*), current pulses equal in magnitude to the membrane ionic currents but of opposite sign. This is achieved by inverting the pulse sequence and applying a variable fraction of it to one end of a known resistor. The other end is attached to the summation point of the integrator. The average ionic current may then be calculated from the fraction and the known resistance.

Applied potentials cause time-dependent changes in membrane capacitance corresponding to electrostriction and area changes. These are observed as relaxations (i.e., a time varying current approaching a final value) during, but not after, phases (*b*) and (*d*). Either diffusion or electrode polarization produces current relaxations at both the onset and the removal of applied potentials. Electrode polarization was avoided by using large area silver–silver chloride electrodes and sufficiently low membrane conductances. The membrane conductance was always <2% of the "open hole" conductance. Diffusion polarization was troublesome at very low (0.1 mM) Cs^+ concentrations. To allow data to be recorded with at least 1% accuracy using the present apparatus, the conductance at 25 mV must be $>10^{-8}$ S. For 0.1 mM CsCl to minimize diffusion polarization these conductances were also kept below 5×10^{-8} S with 10 ms pulses and 1.5×10^{-7} S with 2 ms pulses.

When gramicidin was not added to the system, films that could be seen to have "blackened" had no measurable ionic currents (steady-state conductance $<10^{-10}$ S), but displayed capacitative currents similar to those seen in the presence of gramicidin.

To correct for changes in the number of functional gramicidin channels in a membrane over the time required to record currents using many different pulse amplitudes, the membrane conductance is first recorded at 25 mV, then the test potential, and finally 25 mV again. Our results are expressed as $G(V)/G(25)$, where $G(V)$ is the average conductance during the test pulse at potential V and $G(25)$ is the average conductance during the reference pulses before and after the test pulse. Rectification if present leads to a difference in level between phases (*a*) and (*e*). The currents corresponding to the two polarities can be determined by bringing the level in phase (*c*) first to zero and then to the level in phase (*e*). We rejected all data for which the ratios $G(V)/G(25)$ for the two polarities differed by 10% or more.

Control experiments were performed using 100 mM cholineCl or 9 mM $MgSO_4$ without CsCl. Significant conductances were observed in both cases, though these were poorly reproducible. Subsequent addition of ~1 mM CsCl produced an immediate conductance increase of 10–100 ×. We assume that the currents in the absence of CsCl resulted from hydrogen ion conduction because they were greatly reduced by increasing the pH from the unbuffered value between 5 and 5.8, to ~6.8 with Tris base (Tris[hydroxymethyl]aminomethane; Sigma Chemical Co.). To reduce the contribution of hydrogen ion conduction in the experiments with $MgSO_4$, the solutions were adjusted to ~pH 6.8 by adding ~0.1 mM Tris base. Results were obtained for 0.1 mM CsCl plus 99.9 mM cholineCl either buffered or unbuffered.

RESULTS

Conductance–voltage relations for gramicidin in glyceryl monooleate membranes and CsCl concentrations between 0.1 and 100 mM are shown in Fig. 1. Ionic strength was maintained at 100 mM using cholineCl. The curves for 1 and 0.1 mM are indistinguishable. A change in shape is apparent as the concentration is increased to 10 mM and this becomes marked for 100 mM. The curve for 100 mM is intermediate between those reported by Eisenman and Sandblom (1983*a, b;* 1984), Eisenman et al. (1983), and Andersen (1983*a*). Eisenman et al. have reported current–voltage relations for low concentrations of CsCl with 9 mM $MgSO_4$ or $MgCl_2$. Conductance ratios, $G(V)/G(25)$, measured in the present study using 9 mM $MgSO_4$, are compared with conductance ratios, $G(V)/G(0)$, calculated from their data in Fig. 2. The ratios calculated from their 0.1 mM CsCl data are lower (at every potential except 80

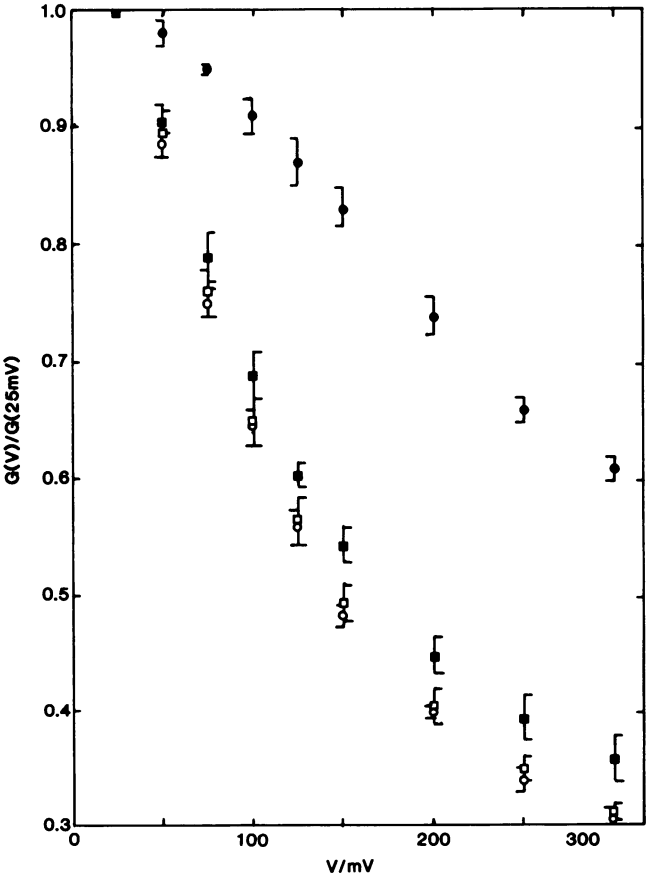

FIGURE 1 Conductance–voltage relations for CsCl at 0.1 mm, □ and [; 1 mM, ○ and] ; 10 mM, ■ and [; and 100 mM, ● and] . Ionic strength was held constant at 100 mM by additions of choline chloride. The bars indicate ± 1 SD.

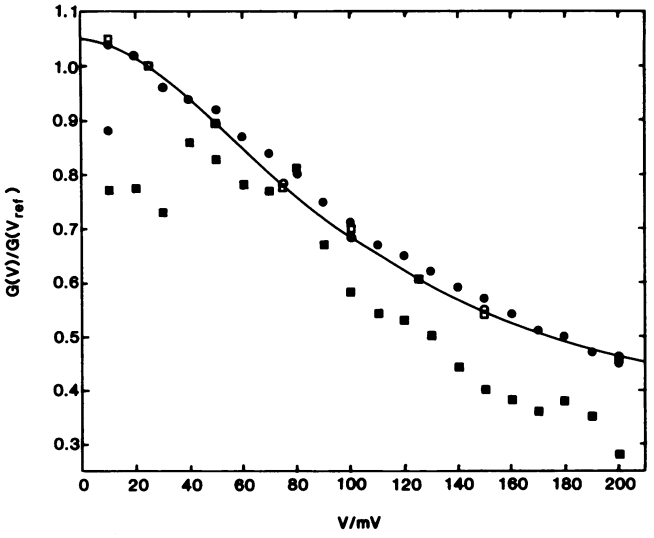

FIGURE 2 Conductance–voltage relations for CsCl at 0.1 mM (□ and ■ and 1 mM (○ and ●). 9 mM $MgSO_4$ has been added to keep the ionic strength constant. □ and ○, pulse data as $G(V)/G(25)$; ■ and ●, ramp data as $G(V)/G(0)$ (Eisenman et al., 1983). For these conditions, $G(0)$ is expected to exceed $G(25\text{ mV})$ by 4–5%. The curve has no theoretical significance except that it has the correct limiting behavior at $V = 0$. It connects together the pulse data.

mV) than the ratios calculated from their 1 mM data. By contrast the conductance ratios measured using brief pulses are the same for 0.1 and 1 mM CsCl.

DISCUSSION

In the experiments reported here the shape of the current–voltage relation is the same for 0.1 and 1.0 mM CsCl but varies as the Cs concentration is increased beyond 10 mM. The variation suggests that above 10 mM, ions interact during the conduction process. The current–voltage relations provide no evidence for or against competition between ions at concentrations below 10 mM. If the first ion binding constant to the pore is ~100 M^{-1} as inferred from the conductance–activity relations above 1 mM (Neher et al., 1978; Hladky and Haydon, 1984), then the observation of only a small change in the shape of the current–voltage relation below 10 mM (at constant ionic strength) implies that the rate-limiting processes when pores are rarely occupied and when they are occupied about half the time have a similar, weak potential dependence.

An estimate of the potential dependence of the access process for empty pores (but in the presence of 100 mM cholineCl) can be obtained from the shape of the current–voltage curve at low concentrations. If it is assumed that the rate-limiting step varies exponentially with some small fraction, n, of the applied potential, then

$$I(V)/I(V_{ref}) = \exp[ne(V - V_{ref})/kT] \quad (1)$$

$$\simeq [1 + ne(V - V_{ref})/kT]. \quad (2)$$

The curves for 1.0 and 0.1 mM Cs (Fig. 3) are roughly linear between 125 and 300 mV. From these curves, n is ~0.045. Part of this dependence will be a consequence of the small residual effect of double-layer polarization that has not been eliminated by raising the ionic strength to 100 mM (Andersen, 1983*b*). From the equations presented elsewhere (Hainsworth and Hladky, 1987), this effect could account for a value of $n \sim 0.008$. A weak but finite potential dependence is expected for access to the pore even if it is limited by steps occurring at or just outside the mouth of the pore (Jordan, 1982; Andersen, 1983*c*; Hainsworth and Hladky, 1987).

In contrast to these results obtained with the pulse technique, Eisenman and Sandblom (1983*a*, *b*; 1984) and Eisenman et al. (1983) using a sawtooth ramp technique observe a difference between the shapes of the current–voltage relations for 0.1 and 1 mM CsCl. The relation for 0.3 mM was intermediate between the other two. The origin of the discrepancy between their results and ours is unclear, but there are at least four factors that may contribute.

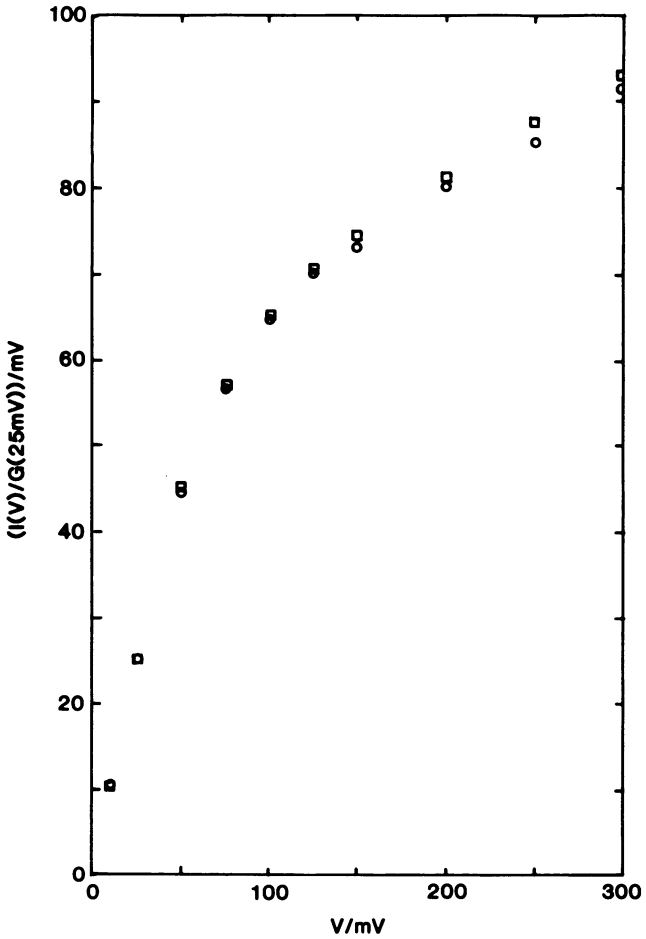

FIGURE 3 Current–voltage relations for 0.1 mM (□) and 1 mM CsCl (○) with ionic strength held constant at 100 mM by addition of choline chloride.

(*a*) In the pulse experiments (with $MgSO_4$) the pH was 6.8, while in the ramp experiments it was 5.8–6.0. However, this factor is unlikely to be important because in pulse experiments with 0.1 mM CsCl plus 99.9 mM cholineCl the current–voltage relations that we obtained with buffered (pH 6.8) and unbuffered solutions (pH 5–5.8) could be superimposed within the size of the squares shown in Fig. 1.

(*b*) By definition the ratio $G(V)/G(0)$ should approach 1 as the test potential, V, approaches 0. Furthermore for a symmetrical system the curve should initially be flat, i.e., $d[G(V)/G(V)_{ref}]/dV \rightarrow 0$ as $V \rightarrow 0$. Using smooth curves that obey this latter condition, the values of $G(V)/G(0)$ calculated from the data of Eisenman et al. for 1 mM CsCl appear to approach a value near 1.05, while those for 0.1 mM CsCl appear to approach a value between 0.8 and 0.9 (see Fig. 2). A 5% increase in the value of $G(0)$ used to normalize the data for 1 mM, a 2% decrease for 0.3 mM, and a 10–15% decrease for 0.1 mM, would allow all three sets of ramp data to be described by the same curve as the pulse data for potentials below 100 mV.

(*c*) The total current flowing across the membrane in ramp or pulse experiments is the sum of the ionic and capacitative currents. In the pulse records capacitative currents have been eliminated by using an integrator to detect the current as described in the methods. In the ramp method capacitative currents measured using membranes with very low conductances have been subtracted from the total currents to yield the ionic current (Eisenman et al., 1983). Apparently to obtain sufficient accuracy in the values after subtraction, the conductances must be substantially higher than are required using the pulse technique.

(*d*) Diffusion polarization occurs whenever the applied field can push ions across the membrane as rapidly as they can diffuse up to the membrane through the aqueous phases. It becomes more prominent as the membrane conductance increases and as the bulk concentration of permeant ion decreases. In the pulse experiments diffusion polarization is easily recognized because it causes a decrease in current with time after the start of the pulse and a current in the reverse direction after its end. A quantitative estimate of these effects is given in the Appendix. Both theoretically and experimentally diffusion polarization has a negligible effect on the results reported here. For 0.1 mM CsCl and 10-ms pulses polarization is predicted and was noticed for ratios of the conductance to the capacitance, G/C, exceeding 50 s^{-1} (see Appendix).

The effects of diffusion polarization in ramp experiments are more difficult to take into account. Polarization should produce hysteresis, and different sawtooth frequencies should lead to variations in the conductance at zero voltage, the ionic current vs. voltage curve, or both. The data for low concentrations of CsCl have not been analyzed for these effects and the actual conductances of the membranes were not reported. However, experimental records have been presented for 0.1 mM KCl (Eisenman et al., 1980) and 0.2-mM HCl (Eisenman et al., 1983) in which G/C exceeds 200 s^{-1} and 1,000 s^{-1}, respectively. Furthermore the concentrations of gramicidin used in the ramp experiments with 0.1 mM CsCl (up to 10^{-4} M in the lipid) far exceeded those used in the pulse experiments (usually $<10^{-7}$ M). Thus it is likely that the actual membrane conductances were substantially higher in the ramp experiments than in the pulse experiments. These higher conductances should produce significant diffusion polarization. It would be very interesting to know the ionic currents after step changes in potential for the membranes and solutions used in the ramp experiments as such pulse records would be much easier to interpret.

APPENDIX

The extent to which diffusion polarization alters the currents after a step change in potential can be calculated using equations given by Carslaw and Jaeger (1959) and applied to ion transport across lipid membranes by Neumcke (1971). For the present case of sufficiently low concentrations that the fluxes obey independence, the current per unit area of membrane can be written as

$$I/A = zF(k'c' - k''c''), \tag{3}$$

where c' and c'' are the concentrations at the left and right surfaces and k' and k'' are the rate constants for movements in the two directions. The membrane area A can be calculated as the ratio of the actual capacitance, C_m, to the capacitance per unit area, C_a. Immediately after a potential is applied, $c' = c''$ and the current per unit can be expressed as

$$I_0 C_a/C_m = zFc(k' + k'') \tanh (zFV/2RT). \tag{4}$$

For

$$t(k' + k'')^2/D \ll 1, \tag{5}$$

polarization will lead to a fall in current following

$$I_t = I_0[1 - \{2(k' + k'')/D\}\{Dt/\pi\}^{1/2}]. \tag{6}$$

(For the general equation and expression for $t[k' + k'']^2/D \gg 1$ see Neumcke, 1971). Thus from Eqs. 4 and 6, diffusion polarization will produce a fractional error in the current at time t after the potential is applied,

$$\begin{aligned} E &= [I_t - I_0]/I_0 \\ &= \{[2I_0C_a/C_m]/[zFc \tanh (zFV/2RT)]\}\{t/D\pi\}^{1/2}, \end{aligned} \tag{7}$$

and a fractional error in the average current over a pulse of length t

$$EA = \int_0^t E dt/t = 2E/3. \tag{8}$$

Thus to keep the error in the average current below 1% it is necessary to use conductances and pulse durations that satisfy

$$G_0 t^{1/2}/C_m < 10^{-2}\, 3zFc \tanh (zFV/2RT)\{D\pi\}^{1/2}/4C_aV. \tag{9}$$

For 0.1 mM CsCl, 200 mV, and glyceryl monooleate + *n*-hexadecane membranes, $c = 10^{-7}$ mol/cm^3, $F = 10^5$ C/mol, $V = 0.2$ V, $\tanh (zFV/2RT) = 1$, $D\pi = 6 \times 10^{-5}$ cm^2/s, $C_a = 0.6$ μF/cm^2, and

$$G_0 t^{1/2}/C_m < 4.8\ s^{-1/2}. \tag{10}$$

For a 10-ms pulse

$$G_0/C_m < 48\ s^{-1}. \quad (11)$$

In the present experiments with 0.1 mM CsCl when the conductance/capacitance ratio reached this level, there was a noticeable fall in current (~2%) during a 10-ms pulse at 200 mV. The data reported here were obtained for $G_0 t^{1/2}/C_m < 5\ s^{-1/2}$.

In a ramp which sweeps from a previously constant value of 0 to 200 mV in 2.5 ms, the error in the current at the end of the sweep will be roughly the same as the error in the current at the end of a 200-mV pulse of slightly shorter duration. For a 2-ms pulse, 1% accuracy in the current at the end of the pulse requires,

$$G_0/C_m < 70\ s^{-1}. \quad (12)$$

We thank George Eisenman for providing us with the ramp data in tabular form together with unpublished information about the experimental conditions and for his constructive criticism during the drafting of this report. We thank Lucille Vaughan for help with the manuscript.

This work was supported by a grant from the Research Fund of Jesus College, Cambridge.

Received for publication 17 September 1986 and in final form 26 February 1987.

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