Water Permeability of Gramicidin A-Treated Lipid Bilayer Membranes

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ABSTRACT In membranes containing aqueous pores (channels), the osmotic water permeability coefficient, P_f, is greater than the diffusive water permeability coefficient, P_d. In fact, the magnitude of P_f/P_d is commonly used to determine pore radius. Although, for membranes studied to date, P_f/P_d monotonically declines with decreasing pore radius, there is controversy over the value it theoretically assumes when that radius is so small that water molecules cannot overtake one another within the channel (single-file transport). In one view it should equal 1, and in another view it should equal N, the number of water molecules in the pore. Gramicidin A forms, in lipid bilayer membranes, narrow aqueous channels through which single-file transport may occur. For these channels we find that $P_f/P_d \approx 5$. In contrast, for the wider nystatin and amphotericin B pores, $P_f/P_d \approx 3$. These findings offer experimental support for the view that $P_f/P_d = N$ for single-file transport, and we therefore conclude that there are approximately five water molecules in a gramicidin A channel. A similar conclusion was reached independently from streaming potential data. Using single-channel conductance data, we calculate the water permeability of an individual gramicidin A channel. In the Appendix we report that there is a wide range of channel sizes and lifetimes in cholesterol-containing membranes.

INTRODUCTION

The relation between the water permeability of cell membranes as measured, on the one hand, by tracer diffusion experiments (P_d) and, on the other hand, by osmotic or hydrodynamic experiments (P_f) has interested physiologists for over 40 years (Hevesy et al., 1935). It is generally recognized that $P_f/P_d = 1$, if water crosses the membrane by a solubility diffusion mechanism through a water-poor region such as a lipid bilayer (see Cass, 1968), whereas $P_f/P_d > 1$ if water moves through aqueous pores (Mauro, 1957). The inequality arises from the difference between laminar (or quasi-laminar) flow through a pore, which occurs when a hydrostatic or osmotic pressure difference exists, and simple diffusion, which takes place when isotopic water (e.g., THO) exchanges with unlabeled water (Mauro, 1957). From the magnitude of P_f/P_d, the "equivalent pore radius" of aqueous channels in biological membranes is calculated (Solomon, 1968).

For macroscopic systems, hydrodynamic theory establishes that P_f/P_d declines as pore radius decreases. Robbins and Mauro (1960) extended this formulation

to pores with radii of tens of angstroms. For nystatin and amphotericin B pores, whose estimated radii are 4 Å, $P_f/P_d \approx 3$ (Holz and Finkelstein, 1970). P_f/P_d therefore appears to approach 1 as the pore radius approaches that of the solvent molecule. This intuitive feeling was apparently given a theoretical basis in the analyses by Longuet-Higgins and Austin (1966)[1] and by Manning (1975) of diffusion and osmosis for single-file transport; i.e., diffusion and osmosis through pores so narrow that solvent molecules cannot pass one another within the pore. Implicit, however, in the analyses of Hodgkin and Keynes (1955), Lea (1963), and Heckmann (1972), and explicit in the analyses of Dick (1966), and Levitt (1974), is that P_f/P_d is *not* equal to 1 for single file transport but equal to N, the number of water molecules in the channel.

The gramicidin A channel offers an opportunity to determine experimentally P_f/P_d in a very narrow pore. Its permeability to water but not to urea (Finkelstein, 1974) along with molecular model building (Urry, 1972) suggest that the radius of the channel is about 2 Å and that transport occurs via a single-file process. In this paper we show that $P_f/P_d \approx 5$ for gramicidin-treated membranes, and from comparison with the electrokinetic results in the preceding paper (Rosenberg and Finkelstein, 1978) we argue that 5 is approximately the number of water molecules in the pore. We discuss the interesting implications of this result for water transport through biological channels, particularly those induced by antidiuretic hormone (ADH) in toad urinary bladder and mammalian collecting tubles.

MATERIALS AND METHODS

Strategy

There are two major problems in measuring water permeability of gramicidin A channels: (a) the significant water permeabilities of unmodified bilayers; and (b) the very low resistances at which gramicidin-induced water permeability becomes significant.

(a) If the water permeability of the unmodified bilayer is too great, it is very difficult to measure gramicidin A-induced P_d, because unstirred layer corrections are so large that impossible accuracies are required for meaningful data. To minimize this problem, we initially chose a high cholesterol-containing membrane-forming solution (lecithin: cholesterol molar ratio of 1:4), because cholesterol greatly reduces bilayer water permeability (Finkelstein and Cass, 1967). After completing a series of experiments, we were chagrined to discover an enormous spread in single channel sizes and lifetimes with membranes formed from this mixture.[2] Because this obscured any interpretation of the results, we turned instead to cholesterol-free bacterial phosphatidylethanolamine (PE), whose membranes yield classical single-channel behavior in the presence of gramicidin A. The resultant water permeability, although considerably larger than that of the lecithin-cholesterol membranes, is still low enough that P_d measurements on gramicidin-treated membranes are feasible. Unstirred layer corrections were maximally a factor of 2, which was acceptable, inasmuch as they could be determined simultaneously with P_d (see "Tactics").

(b) When membrane resistances are small relative to the access resistance (the resistance

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[1] This paper is frequently cited as implying that $P_f/P_d = 1$. It is not clear to us, however, that this is a correct implication.

[2] This phenomenon, occurring in cholesterol-rich bilayers, is described in the Appendix.

measured in the absence of a membrane), they cannot be evaluated accurately, because their determination involves subtraction of two nearly identical numbers; under inauspicious circumstances, for example, a 10% error in the total resistance can result in a fivefold error in membrane resistance. We minimized this problem by our choice of salt solution—0.01 M NaCl plus 0.1 M choline chloride. The 0.1 M choline chloride reduced the access resistance by about a factor of 10 from that in 0.01 M NaCl alone, without altering channel resistance, in that choline is an impermeant of the gramicidin channel,[3] and thus we could easily determine membrane resistance with reasonable accuracy. (Access resistance was 1200 Ω; the lowest measured resistance was 1700 Ω, thus giving a membrane resistance of 500 Ω.)

Tactics

All membranes described in the body of this report were formed from bacterial phosphatidylethanolamine (2.5% PE in 2,2,4,6,6-pentamethylheptane). The aqueous solutions bathing the membrane were 0.01 M NaCl + 0.1 M choline chloride + 10^{-4} M EDTA (pH 7). The gramicidin A used in most experiments was a sample obtained from the late Dr. Lyman Craig; similar results were obtained with gramicidin purchased from ICN Pharmaceuticals, Inc. (Irvine, Calif.), a mixture of 72% A, 9% B, and 19% C (Glickson et al., 1972). Bacterial PE was obtained from Supelco, Inc. (Bellafonte, Pa.); 2,2,4,6,6-pentamethylheptane was from Analabs, Inc. (North Haven, Conn.).

TRACER EXPERIMENTS (P_d) Membranes were formed at room temperature ($23° \pm 2°$ C) by the brush technique (Mueller et al., 1963) across a 0.78 mm^2 circular hole in a Teflon partition (125 μm thick) separating two lucite compartments each containing 3 ml of solution. After the membrane formed, gramicidin was added (from stock methanol or ethanol solutions) to one or both compartments to a final concentration of from 0.2 to 1 μg/ml. In some instances the membrane was formed in the presence of gramicidin in the aqueous solutions. When conductance became stable, THO was added to one compartment and its flux, Φ^*, measured as described previously (Holz and Finkelstein, 1970). (Sometimes Φ^* was first measured for an unmodified membrane, gramicidin A then added, and Φ^* again determined.) The observed permeability coefficient, $(P_d)_{obs}$, was calculated from the equation:

$$\Phi^* = - (P_d)_{obs}A\Delta c^*, \tag{1}$$

where A is the membrane area and Δc^* is the difference in concentration of isotope in the two compartments; it was corrected for unstirred layers as described previously (Holz and Finkelstein, 1970). The unstirred layer thickness was found, using [^{14}C]butanol (Holz and Finkelstein, 1970), to be 100 μm; in some experiments THO and [^{14}C]butanol fluxes were determined simultaneously. Both compartments were stirred continuously with magnetic fleas.

Resistance ($R = \Delta V/\Delta I$) was measured using two pairs of Ag/AgCl electrodes, one pair to apply a current step, ΔI, and the other to record the resulting initial[4] potential difference, ΔV. The recording electrodes were connected to a high input impedance amplifier, and its output was displayed on an oscilloscope face. The membrane

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[3] Gramicidin-treated membranes separating 0.1 M choline chloride solutions had a conductance of about 7% that occurring in 0.01 M NaCl. This conductance was produced by a contaminant (possibly NH_4^+) in the choline.

[4] At the high conductances of most experiments, the response to a step of current was an initial jump of voltage followed by a further rise with time to some steady-state value. This further rise was a polarization voltage resulting from the accumulation of NaCl at one interface and its depletion from the other. The initial jump measures the membrane (plus access) resistance.

resistance, R_m, was found by subtracting the access resistance from the measured resistance. The membrane conductance, G_m, is by definition $1/R_m$.

THO (5 mCi/ml) and $[1\text{-}^{14}C]n$-butanol (3.7 mCi/mmol) were obtained from New England Nuclear (Boston, Mass.)

OSMOTIC EXPERIMENTS The net flow of water produced by a concentration difference of solute (urea) was measured as described by Holz and Finkelstein (1970); the present arrangement, however, contained two pairs of Ag/AgCl electrodes (one for stimulating and the other for recording). Membrane area was 1.27 mm²; electrical resistance was monitored continually as described for the tracer experiments.

Membranes were formed at 23° ± 2°C. After they were completely black, stirring of the solution in the outer chamber began, and gramicidin was added from stock solutions to a final concentration of from 0.05 to 0.15 μg/ml. When the membrane resistance attained a constant value, a small volume of 8 M urea (in the same salt solution already present) was added to the outer chamber to a concentration of between 0.43 to 1.64 osmolality, and the movement of water was recorded as described previously (Holz and Finkelstein, 1970). The osmotic permeability coefficient, P_f, was calculated from the equation:

$$\Phi_w = P_f A \varphi \Delta c_s,\tag{2}$$

where Φ_w is the flux of water (in moles per unit time) across a membrane of area A in the presence of a concentration difference, Δc_s, of impermeant solute (urea); φ is the osmotic coefficient of urea (≈ 0.93), obtained from the Handbook of Chemistry and Physics, 57th Edition.

RESULTS

P_f and P_d each increase linearly with conductance (Fig. 1), and the increase is attributed to water permeation through gramicidin channels (see below). The slopes calculated from Fig. 1 give the proportionality of gramicidin-induced water permeability to gramicidin-induced conductance (ion permeability). From them we see that $P_f/P_d = 5.3$ for gramicidin channels.[5]

DISCUSSION

Water and Ions Share a Common Pathway through Gramicidin-Treated Membranes

The analysis of the data in this paper rests on the assumption that increased water permeability after membranes are treated with gramicidin results from water flux through the ion-permeable gramicidin channels. Several lines of evidence strongly support this assumption:

(a) Water permeability is a linear function of conductance; this is predicted if water and ions share a common pathway.

(b) If gramicidin-induced water permeability occurred through the bilayer

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[5] The osmotic experiments were performed under open-circuited conditions. In such circumstances the osmotic flow of water is opposed by an electroosmotic backflow induced by the streaming potential. It is easily shown from the data in this and the preceding paper (Rosenberg and Finkelstein, 1978), however, that this effect is small in the present case (0.01 M NaCl), so that there is no significant difference in the values of P_f obtained under open-circuited and short-circuited conditions. What this means is that there is little coupling between ion and water flow; this is to be expected in 0.01 M NaCl, because at any instant most channels have no ion in them.

proper (e.g., if gramicidin acted as a detergent and increased membrane fluidity), it should be accompanied by a proportional increase in nonelectrolyte permeability (Finkelstein, 1976). We found, however, that neither n-butyramide nor urea permeability is significantly increased by gramicidin action.

(c) If gramicidin-induced water permeability were through the bilayer proper, then P_f/P_d should equal 1. Inasmuch as P_f/P_d is much greater than 1 (≈ 5), this provides strong evidence that the gramicidin-induced water permeability occurs through pores.

(d) The electrokinetic phenomena described in the preceeding paper (Rosenberg and Finkelstein, 1978) directly demonstrate that ions and water share a common pathway through gramicidin-treated membranes.
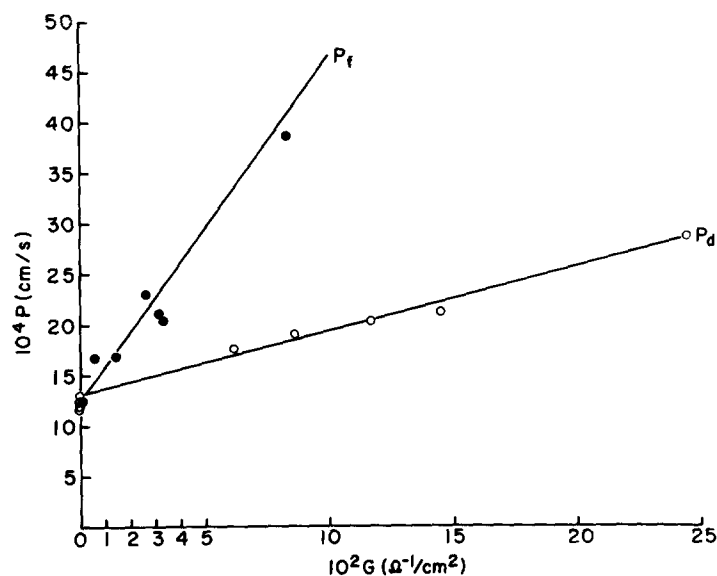

FIGURE 1. ($\bullet$) P_f and (O)P_d of gramicidin-treated membranes as a function of membrane conductance. Note that for the unmodified membrane ($G \approx 0$), $P_f = P_d$. The slopes of the P_f and P_d lines are 34.2 × 10^{-3} and 6.5 × 10^{-3} cm s^{-1}/Ω^{-1} cm^{-2}, respectively.

In summary, these considerations strongly suggest that gramicidin-induced water transport occurs through the ion-permeable gramicidin channels.

P_f/P_d for Narrow Pores

The theoretical expectation for the value of P_f/P_d in single-file transport is controversial: in one view it equals 1 (Manning, 1975), and in the other it equals N, the number of water molecules in the channel (Dick, 1966; Levitt, 1974). In our opinion, the latter view is correct, and we find Levitt's argument particularly convincing. But it is not our purpose to undertake a critique of the theoretical arguments. Our contribution is an experimental one, insofar as experiments can contribute to theory (which is a doubtful propositon).

Clearly, $P_f/P_d \neq 1$ for the gramicidin channel. By itself this result is not particularly significant. One might argue that single-file transport does not occur through the gramicidin channel, and therefore, as expected for an aqueous channel, $P_f/P_d \neq 1$. The result takes on added significance, however, when compared to that obtained for nystatin and amphotericin B channels. For these channels, with radii $\approx 4\text{Å}$, $P_f/P_d \approx 3$ (Holz and Finkelstein, 1970), whereas for the small gramicidin channel, with a radius $\leq 2\text{Å}$, $P_f/P_d \approx 5$. These results are not consistent with a theory in which $P_f/P_d = 1$ for single-file transport, because such a theory also implies that P_f/P_d decreases continuously as channel radius decreases. Hence, P_f/P_d should be smaller for the narrower gramicidin channel than for the wider nystatin and amphotericin B channels.

Our results are, however, consistent with the theory that $P_f/P_d = N$ for single-file transport. This theory also implies that P_f/P_d decreases continuously as channel radius decreases, but only until the single-file situation is approached. At this point, P_f/P_d becomes a function of an entirely new parameter, the number of water molecules in the channel (which should be proportional to the length of the channel). Thus, this theory anticipates that P_f/P_d could be larger for the 2Å radius gramicidin channel than for the 4Å radius nystatin and amphotericin B channels.

Comparison of Water Permeability with Streaming Potential Data

In the preceeding paper (Rosenberg and Finkelstein, 1978) we arrived at 6.5 for the number of water molecules in the gramicidin channel, on the assumption that an ion cannot pass a water molecule in the channel; in this paper we arrive at 5.3 for the number of water molecules in the gramicidin channel on the assumption that one water molecule cannot pass another in the channel. The agreement between these two values, obtained from completely independent experiments and theories, is remarkable.[6] The concordance of these two approaches reinforces the view that transport through the gramicidin channel is a single-file process for both ions and water.[7]

Water Permeability of Single Channels

Given P_d and P_f as functions of conductance and knowing the single-channel conductance, we can calculate the permeabilities, p_d and p_f, per channel from the relations:

$$p_d = \frac{P_d A}{n},$$ (3a)

and

$$p_f = \frac{P_f A}{n},$$ (3b)

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[6] We attach no significance to the difference between 6.5 and 5.3.

[7] If strict single file transport does not hold (i.e., if there is some slippage of the water molecules past one another), N obtained from P_f/P_d underestimates the number of water molecules in the channel. This is also true of N obtained from streaming potentials, if there is some slippage of ions and water molecules past one another (Rosenberg and Finkelstein, 1978).

where n is the number of channels corresponding to a given value of P. (p has the dimensions of cm^3/s rather than cm/s, since $\Phi = \pm np\,\Delta c$.) In making this calculation, we assume that channel conductances are the same in the densely channeled membranes of the water permeability experiments as in the sparsely channeled membranes of the single channel measurements.

At a conductance of 1 mho/cm^2, the gramicidin-induced water permeabilities in 0.01 M NaCl are (see Fig. 1):

$$P_d = 6.5 \times 10^{-3} \text{ cm/s}, \tag{4a}$$

and   (at 1 mho/cm^2 in 0.01 M NaCl)

$$P_f = 34.2 \times 10^{-3} \text{ cm/s}. \tag{4b}$$

Taking the conductance of a channel in 0.01 M NaCl as 2.8×10^{-13} mho (based on the value of 2.8×10^{-12} mho in 0.1 M NaCl for PE membranes [Andersen, 1978]), we obtain from eq. 3:

$$p_d = 1.82 \times 10^{-15} \text{ cm}^3/\text{s}, \tag{5a}$$

and

$$p_f = 9.58 \times 10^{-15} \text{ cm}^3/\text{s}. \tag{5b}$$

(p_d is a factor of 6 smaller than reported previously [Finkelstein, 1974]. The difference is attributable to two factors. First, the earlier experiments were in 0.1 M NaCl alone, and small errors in measuring the combined membrane and access resistance produced large errors in the membrane resistance, as discussed in Methods [see "Strategy"]. Secondly, the earlier experiments were with lecithin:cholesterol membranes, and the single-channel conductance, which at the time was thought known, is ambiguous [see Appendix].)

Biological Implications

Equivalent pore radii of channels in biological membranes are commonly calculated from values of P_f/P_d (Solomon, 1968). It is known, however, that spuriously high values can be obtained because of unstirred layer problems (Dainty, 1963). This is a recognized problem for P_f/P_d determinations in the ADH-stimulated toad bladder (Hays, 1972) and cortical collecting tubules (Schafer and Andreoli, 1972). Because the ADH-induced pores hold back urea (Levine et al., 1973; Grantham and Berg, 1966), thus implying a pore radius $\leq 2\text{Å}$, it is generally believed that if proper unstirred layer corrections were made, it would turn out that $P_f/P_d \approx 1$. The present report on the gramicidin A channel places a new light on this. Indeed, gramicidin A channels resemble ADH-induced channels phenomenologically, in that both are permeable to water but not to urea. Apparently, then, single-file transport could also occur through them, and we must therefore entertain the possibility that P_f/P_d is considerably greater than 1. A proper determination of this ratio would give the number of water molecules in ADH-induced channels. It would be interesting to see how that value compares with the one obtained for gramicidin A channels.

APPENDIX

We attempted to measure single-channel conductances in gramicidin-treated lecithin:cholesterol membranes.[8] Fig. 2 A (top) represents a typical record in 2
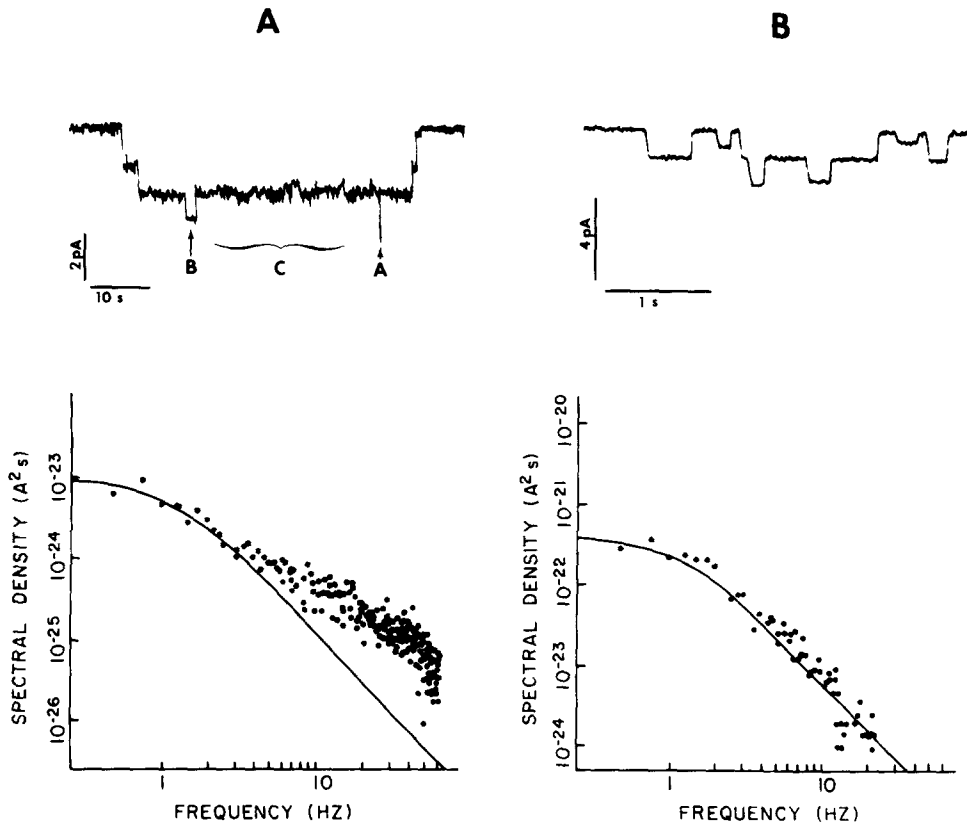

FIGURE 2. (Top) single channel records of gramicidin-treated membranes containing few channels, and (bottom) current-noise power spectra of gramicidin-treated membranes containing many channels. In the top records, downward deflections represent increases in current. (A) Lecithin:cholesterol membranes. Applied voltages are 175 and 100 mV for the top and bottom records, respectively. (B) PE membranes. Applied voltages are 80 and 50 mV for the top and bottom records, respectively. Note that the power spectrum for the PE membrane is fit by a single Lorentzian curve, whereas the power spectrum for the lecithin:cholesterol membrane is more complicated.

M NaCl. One component of the record is a type of current jump indicated by the arrow at A; such jumps, viewed on an oscilloscope face, have a mean lifetime of 50 ms and a mean size of 12×10^{-12} mho — typical of gramicidin channels observed by others (e.g., Hladky and Haydon, 1972; Zingsheim and Neher, 1974; Andersen, 1978). The event at B and the two current jumps preceeding it

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[8] The membrane-forming solution was 2% egg lecithin plus 4% cholesterol in n-decane.

are representative of another component of the record. These jumps have very long lifetimes, ranging from seconds to minutes, and a size about two-thirds that of the A component. The component at C, which merges into the membrane noise, is unresolvable with respect to size and lifetime; the conductances are smaller than 2×10^{-12} mho. The B and C components are not typically observed in cholesterol-free membranes. The activity of all components increases with gramicidin concentration, and the heterogeneity persists at macroscopic conductances (Fig. 2 A, bottom). All three components are cationic selective. Cholesterol-containing PE and PG membranes demonstrate a similar heterogeneity of channel sizes and lifetimes. The degree of this heterogeneity increases with cholesterol concentration; at the low concentrations used by Zingsheim and Neher (1974), it is minimal.

Our attempts to demonstrate single channels in PE membranes were more successful (Fig. 2 B, top). In 2 M NaCl the channels have a mean lifetime of 200 ms and a mean size of 15×10^{-12} mho. Records are clearly constituted out of a single component of current jump narrowly distributed around the mean size. This uniformity persists at macroscopic conductances (Fig. 2 B, bottom).

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