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TWO PURIFIED FRACTIONS OF ALAMETHICIN HAVE DIFFERENT CONDUCTANCE PROPERTIES

IGOR VODYANOY [a], JAMES E. HALL [a], T.M. BALASUBRAMANIAN [b] and GARLAND R. MARSHALL [b]

[a] *Department of Physiology and Biophysics, University of California, Irvine, Irvine, CA 92717, and* [b] *Department of Physiology and Biophysics, Washington University, St. Louis, MO 63110 (U.S.A.)*

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The properties of two purified alamethicin fractions, Fraction 4 and Fraction 6, have been studied in phosphatidylethanolamine (PE) membranes and phosphatidylserine (PS) membranes. Membranes doped with Fraction 4 show well-defined single channel conductance (mean lifetime about 20 ms). The autocorrelation function of the current fluctuations has one relaxation time of the same order as the mean lifetime of the single channels, and the current response to a voltage pulse follows an exponential with only one time constant. The conductance of a membrane doped with Fraction 6 has a voltage-independent part and a current-voltage curve with a slope that is half the slope of the Fraction 4 current-voltage curve. In the presence of Fraction 6, PS membranes and PE membranes both have symmetrical current-voltage curves even with Fraction 6 added to only one side. We did not detect any well-defined single channel levels in the presence of Fraction 6, and autocorrelation analysis of the current due to Fraction 6 gave two characteristic correlation times: a fast time (about 5 ms) and a slow time (about 50 ms). High current level kinetics of Fraction 6 also show two time constants. A possible explanation for the differences between the two fractions is that Fraction 6 monomers have a lower dipole moment than those of Fraction 4. The difference in channel stability can be explained by a lowered tendency of the monomers to line up parallel to the field. The negative branch and voltage-independent conductance can be explained by lowered energy of insertion of monomers into the membrane, and lowered energy of interaction between the monomers and the electric field.

Introduction

Alamethicin (antibiotic U 22324), made by the fungus *Trichoderma viride* [1] was first used as a model of excitability in lipid bilayers by Mueller and Rudin [2]. Considerable additional work has been done since to understand the mechanism of alamethicin conductance (for review, see Ref. 3). Natural alamethicin shows rather sophisticated conductivity behavior dependent on kind of lipid, membrane thickness, temperature, current and membrane potential. But lack of well-characterized purified derivatives had led to difficulties in interpreting some experiments. Because alamethicin provides a simple example of protein-lipid interaction, the removal of this difficulty is important to the study of lipid protein interactions. We have initiated a study of the behavior in lipid bilayers of two recently available fractions of purified alamethicin. The structure of Fraction 4 is already known [4,5] and when that of Fraction 6 is available, it promises to shed light on the molecular basis of alamethicin's ability to induce a strongly voltage-dependent conductance.

Methods

The method of membrane formation was similar to that descibed by Montal and Mueller [6] using a teflon chamber similar to a design by Schindler and Feher [7].

Temperature was controlled to 0.5°C and was 20°C in all experiments reported. Lipid solution (10 μl of 10 mg/ml in pentane) was added to the surface of each chamber using a glass microliter pipet. Then a membrane was formed by raising the levels of the two aqueous solutions separated by the thin teflon partition. A small drop of squalene was placed in the hole in this partition before raising the water levels. An alamethicin derivative was added to one side of the chamber after the membrane was formed. The side opposite the alamethicin was called ground. Phosphatidylethanolamine (PE) from *Escherichia coli* was purchased from Avanti Lipids, Inc. (Birmingham, AL) Cat. No. 810027. Phosphatidylserine (PS) Cat. No. 4-6004 was purchased from Supelco, Inc. (Bellefonte, PA). Brain PE (bovine) was purchased from Sigma Chemical Company, St. Louis, MO, product number P-9137. Squalene was purchased from Albany International Chemical Division, Albany, NY. We used *n*-pentane and salts from Mallinckrodt, Inc. (St. Louis, MO). Pentane was passed through an alumina column before use to remove surface active impurities.

A four electrode (chlorided silver wire) system was used for measuring current-voltage and current-time curves. Two electrodes drove an electrometer, a third electrode was connected to virtual ground, the summing point of an AD 42K operational amplifier. (The minimum bandwidth in all measurements was 5000 Hz.) The last electrode was connected to a voltage source. Voltage was generated by a computer-controlled 12 bit DAC (AD 5782) buffered by an AD 514 operational amplifier, or by a battery driven potentiometer. We used an *X-Y* recorder (HP 7034A) to record current-voltage curves and current-time course. For single channel analysis, we used a TIR 115 instrumentation tape recorder (Tandberg), a computer (System Z-2D, Cromemco, Inc.) and a correlation analyzer SAI-43A (Honeywell, Saicor).

Results

The conductances induced by Fraction 4 and Fraction 6 differ significantly. Fraction 6 induces a voltage-independent conductance at zero volts and a voltage-independent conductance in both quadrants, even when added to only one side of the membrane. Fraction 4 induces neither a voltage-independent zero voltage conductance nor a voltage-dependent conductance for negative voltages in bacterial phosphatidylethanolamine membranes. In other membrane systems, Fraction 4 induces a voltage-dependent conductance for negative voltages, but no voltage-independent conductance at zero volts. Sample current-voltage curves illustrating these results are shown in Fig. 1. The dependence of conductance on voltage was exponential for all fractions tested and could be expressed as

$$G = G_0 \exp(V/V_0)$$

where V_0 was 3.9–5.5 mV for three of the fractions, including Fraction 4, but was 10–12 mV for Fraction 6.

The Fraction 4 and Fraction 6 give very differ-

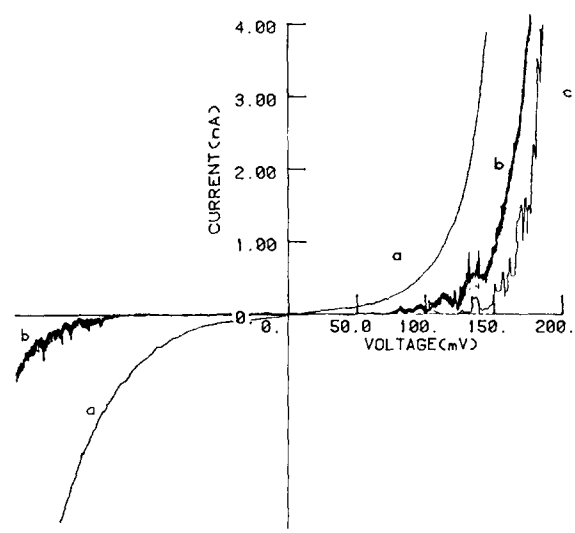


Fig. 1. Voltage-current curves in 1 M KCl unbuffered (pH 5.5). Antibiotic was added to positive side of membrane, voltage sweep rate 10 mV/s. a, PS membrane with $4 \cdot 10^{-7}$ g/ml of Fraction 6. b, PE membrane with $2 \cdot 10^{-8}$ g/ml of Fraction 6. c, PE membrane with $7 \cdot 10^{-8}$ g/ml of Fraction 4.

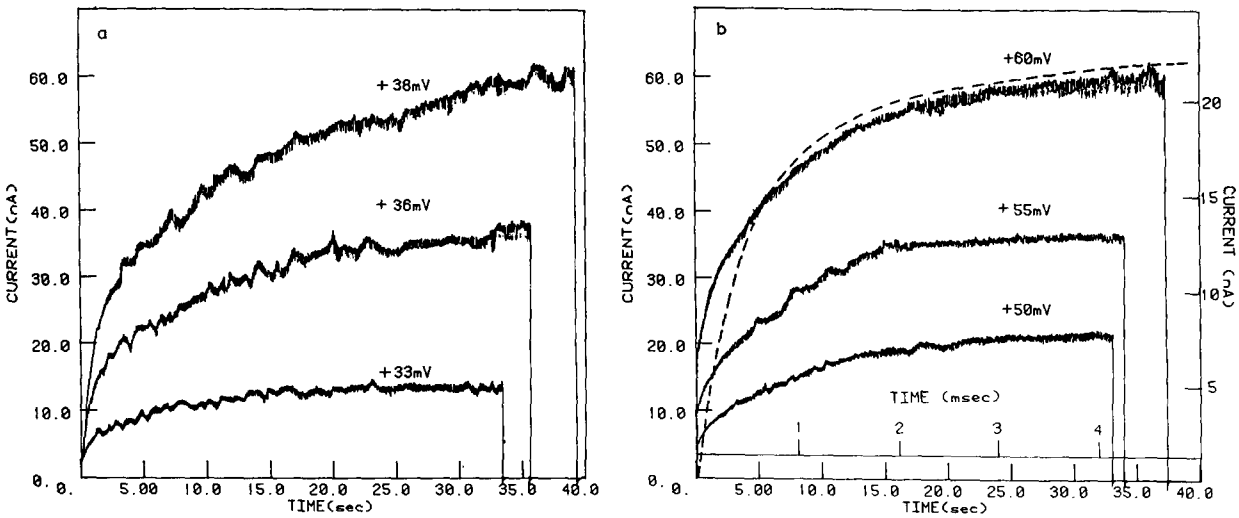


Fig. 2. Membrane current responses to a positive voltage step (amplitude of voltage shown on the curves). (a) PE membrane with $4 \cdot 10^{-7}$ g/ml of Fraction 4. (b) PE membrane with $5 \cdot 10^{-7}$ g/ml of Fraction 6. The dotted line represents the fast initial transient after subtraction of the slow current response. (Top time scale and right current scale for the dotted line.)

ent current responses to voltage steps in PE membranes. Current relaxation in the presence of Fraction 4 shows only one characteristic time of about 10 s (Fig. 2a). The current response in presence of Fraction 6 shows (Fig. 2b) two-exponential processes with a fast relaxation time of about 1 ms and a slow time of about 10 s. Fraction 6 shows no well-defined single channel levels, but Fraction 4 shows well defined single channel levels with a mean lifetime of about 20 ms at 20°C.

Samples of current records at low current level for membranes doped with Fraction 4 and Fraction 6 are shown in Fig. 3.

To measure dose-response curves, we chose a fixed conductance value of 30 $\mu S/cm^2$ (approximately one order of magnitude larger than bare membrane conductivity [8]) and define a 'characteristic voltage' as the voltage at which this conductance is reached. Characteristic voltage dependence on alamethicin fraction concentration is shown in Fig. 4. The slope of the logarithmic dependence was ~ 38 mV per e-fold change in concentration for all the fractions studied. Thus, while the single channel, kinetic and current-vs.-voltage behavior of two fractions are notably different, their dose-response curves are quite similar.

The implication is that the basic peptide-lipid interaction responsible for creating a pore is the

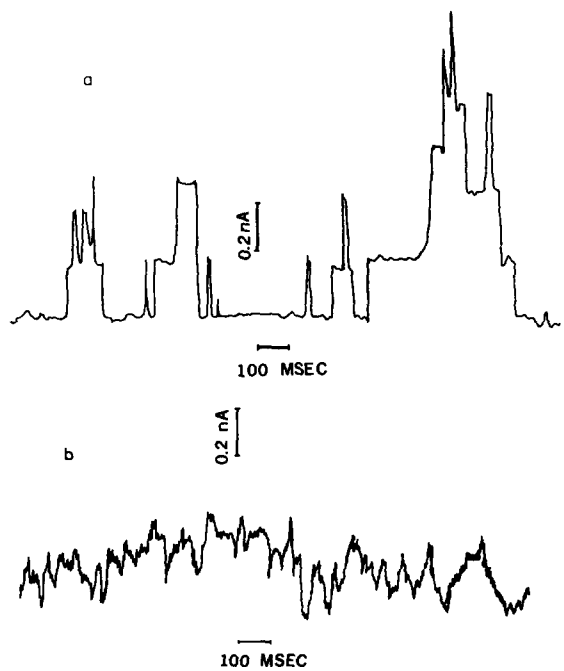


Fig. 3. Current fluctuations of Fraction 4 and Fraction 6 doped PE bacterial membranes formed in 1 M KCl (unbuffered) solution at 20°C. Transmembrane voltage is 120 mv. Fluctuations were tape recorded and reproduced later by strip chart recorder. (a) Current fluctuations of a Fraction 4 doped PE bacterial membrane. Concentration of Fraction 4 is $2 \cdot 10^{-8}$ g/ml. (b) Current fluctuations of a Fraction 6 doped PE bacterial membrane. Concentration of Fraction 6 is $2 \cdot 10^{-8}$ g/ml.

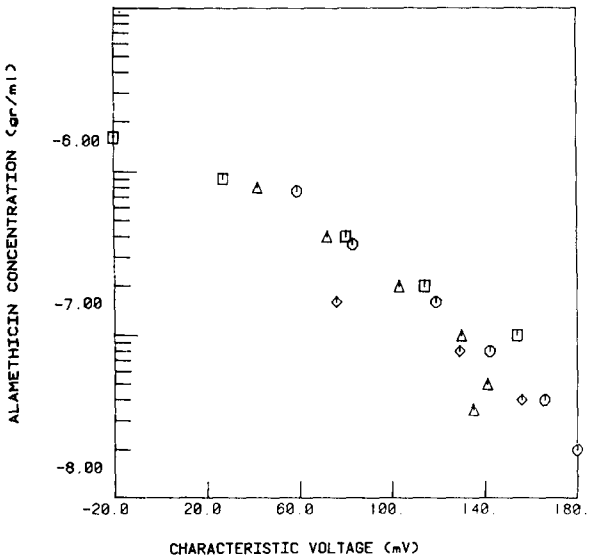
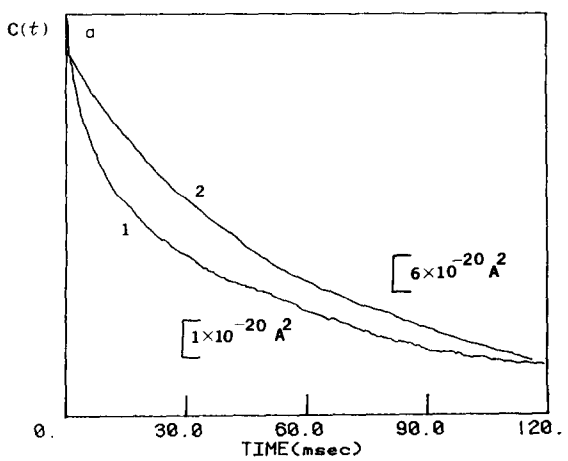
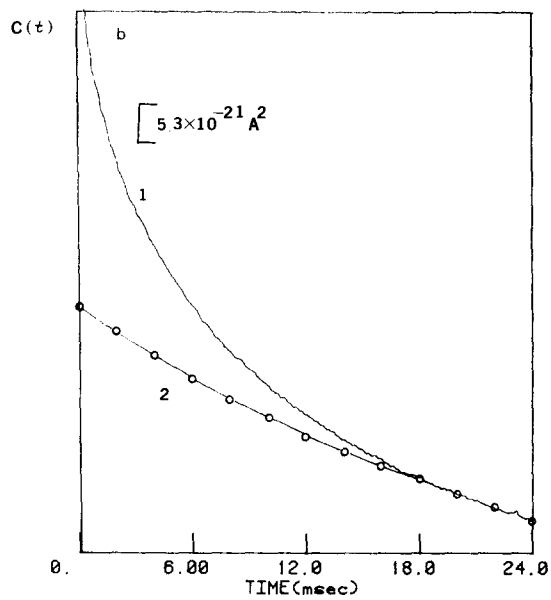


Fig. 4. Characteristic voltage to produce a conductance of 30 $\mu S/cm^2$ in PE membranes in 1 M KCl unbuffered (pH 5.5) as function of the alamethicin concentration: □, Fraction 4; ○, Fraction 6; △, Fraction 5; ◇, Fraction 7.

same for both fractions, but the mechanisms of conductance change within a pore differs between the two fractions. This view is supported by autocorrelation analysis.

The autocorrelation functions of current fluctuations in bacterial PE membranes modified by Fraction 6 and Fraction 4 differ significantly (Fig. 5). The autocorrelation function for Fraction 6 is well described as the sum of two exponentials,





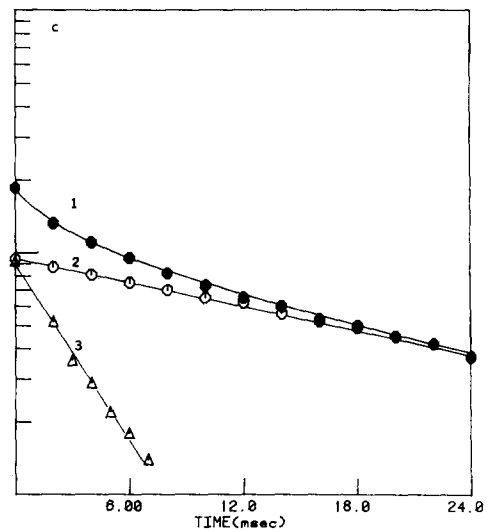


Fig. 5. Autocorrelation function, $C(t)$, of PE membrane current fluctuations in 1 M KCl (262×1024 summations) taken on a Honeywell Saicor SAI-43A autocorrelator. (a) 1, 100 mV membrane voltage ($1 \cdot 10^{-7}$ g/ml of Fraction 6). 2, 100 mV membrane voltage ($4 \cdot 10^{-8}$ g/ml of Fraction 4). (b) 1, Short-time part of curve 1 shown in (a). 2, Theoretical curve. Points were calculated for 50 ms time constant. Curve fits real autocorrelation curve at long times and extrapolated to the time 0. (c) 1, Semi-logarithmic plot of curve 1 shown in (a). 2, Semi-logarithmic plot of the theoretical curve 2 shown in (b). 3, Difference between 1 and 2 which represents fast exponential (~3.5 ms).

a fast exponential with a time constant of 3–5 ms, and a slow exponential with a time constant of about 50 ms. The autocorrelation function of Fraction 4 shows only a single slow correlation time of about 50 ms.

The fast autocorrelation decay time and the rapid relaxation component in response to a voltage pulse shown by Fraction 6 are consistent with the noisy signal seen at the single channel level. The long correlation time, also seen as a slow relaxation in response to a voltage-pulse, is similar to the single correlation time and relaxation time shown by Fraction 4. Both relaxation times measured at high conductance levels are longer than the correlation times measured at the single pore level [9,10].

Discussion

Zero voltage conductance has been seen in various membrane systems by a number of workers [2,8–12]. Since Fraction 4 does not induce a zero voltage conductance in any membrane system we have examined (PE, PS, brain PE) and Fraction 6 induces a zero-voltage conductance in all of them, we propose that the zero-voltage conductances previously observed were due to the Fraction 6 component [6], which constitutes about 25% of the mass of alamethicin supplied by Upjohn Co.

It is generally agreed that the voltage-dependent conductance of alamethicin is due to aggregation of monomers to form a pore. The greater the average number of monomers in a single pore, the steeper the current-voltage curve, but the macroscopic conductance behavior would not be much changed if the character of the single channel levels were to be altered. For these reasons, we believe the similarities in long-time steady-state properties of Fraction 4 and Fraction 6 indicate that the aggregation properties of the two molecules are similar, but not identical. Fraction 6 thus differs from Fraction 4 primarily in those factors which stabilize aggregate conformations.

The negative branch of the current-voltage curve, seen in all membrane systems with Fraction 6 and in some membrane systems with Fraction 4, has at least two possible explanations. First, alamethicin may diffuse rapidly through some kinds of membranes so that an appreciable concentration builds up on the far side. The negative branch in this scheme arises in exactly the way the positive branch does, but from alamethicin on the other side. Alternatively, alamethicin may be able to rotate into the membrane either positive end first or negative end first. These two mechanisms would be favored by different structural changes, and we cannot yet distinguish between them. Natural alamethicin is known to have a dipole moment [13], and we point out that assuming that Fraction 6 monomers have a lower dipole moment than those of Fraction 4 provides an economical explanation for all the differences between them. The difference in voltage-dependence is explained by the lowered energy of interaction between the monomers and the electric field. The difference in channel stability is explained by the lowered tendency of the monomers to line up parallel to the field, and the zero-voltage conductance and the negative branch are explained by lowered energy of insertion of monomers into the membrane. The dipole moment of Fraction 6 has not yet been measured, and other explanations are consistent with our results. Nonetheless, we feel obliged to point out the utility of this explanation even though it may prove to be incorrect.

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