"Reversed" alamethicin conductance in lipid bilayers

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ABSTRACT   Alamethicin at a concentration of 2 μg/ml on one side of a lipid bilayer, formed at the tip of a patch clamp pipette from diphytanoyl phosphatidylcholine and cholesterol (2:1 mol ratio) in aqueous 0.5 M KCl, 5 mM Hepes, pH 7.0, exhibits an asymmetric current-voltage curve, only yielding alamethicin currents when the side to which the peptide has been added is made positive. Below room temperature, however, single alamethicin channels created in such membranes sometimes survive a sudden reversal of the polarity. These "reversed" channels are distinct from transiently observed states displayed as the channel closes after a polarity reversal. Such "reversed" channels can be monitored for periods up to several minutes, during which time we have observed them to fluctuate through more than 20 discrete conductance states. They are convenient for the study of isolated ion-conducting alamethicin aggregates because, after voltage reversal, no subsequent incorporation of additional ion-conducting aggregates takes place.

INTRODUCTION

Since the discovery of its membrane-conducting properties (Mueller and Rudin 1968; Gordon and Haydon 1972; Eisenberg et al., 1973), alamethicin has been studied as a model system for ion conduction through bilayer membranes. It produces a strongly voltage-dependent conductance in lipid membranes, with single channels having multiple conductance states, giving rise to current records displaying many discrete current levels. Depending on the composition of the membrane, alamethicin added to just one side can induce quite pronounced rectifying properties (Eisenberg et al., 1973). Here we report two novel observations, viz. of prolonged conduction in a rectifying membrane when the applied potential has the polarity that normally does not give rise to any alamethicin-induced current, and of some unusually high conductance states. The conductance we report here is distinct from transiently observed conductance states observed immediately after the polarity reversal, which were first reported by Boheim et al. (1983).

MATERIALS AND METHODS

The alamethicin used in this study was the so-called RF30 fraction, purified by reverse phase HPLC (Balasubramanian et al., 1981) from the UpJohn product (UpJohn Pharmaceuticals, Kalamazoo, MI). The bilayers were formed on the tips of patch pipettes by passing the tip through a monolayer of lipid spread on the surface of an aqueous 0.5 M KCl, 5 mM Hepes, pH 7.0 solution (Coronado and Lattore, 1983). The same solution was used to fill the pipette, with the addition of 2 μg/ml alamethicin. The pipettes were fabricated by standard methods from disposible borosilicate micropipettes (Drummond Scientific, Broomall, PA) using a Kopf 700C pipette puller (David Kopf Instru-

ments, Tujunga, CA), and had open tip resistances of ~5 Mohm in 0.5 M KCl. The cell was jacketed with thermostatted water. Solutions of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Inc., Birmingham, AL) and cholesterol (chromatography standard grade, Sigma Chemical Co., St. Louis, MO) in chloroform were mixed in a 2:1 molar ratio, dried under a stream of nitrogen and redissolved in pentane to a concentration of 5 μg/ml at the moment of use. The monolayer was formed by applying 3 μl of this lipid solution to an 11 cm² surface of 5 ml of the electrolyte solution, and then allowing the solvent to evaporate. Currents were measured with a high-gain current-to-voltage converter (model 3523L; Burr-Brown Corp., Tuscon, AZ) with a 100 Mohm feedback resistance, postamplified 10-fold and digitally stored at 14 bit resolution on video tape (model VR-10; Instrutech, Mineola, NY) for later analysis. Voltages were applied from a 12 bit digital to analog (D/A) converter under computer control. All potentials refer to the potential of the Ag/AgCl electrode inside the pipette versus that of the Ag/AgCl electrode in the external solution. Alamethicin was always only added to the solution inside the pipette. A current threshold detector was employed to change the applied voltage when a chosen current level was crossed, as when a single pore made a transition into a particular conductance state.

THE "REVERSED" CONDUCTANCE

Under the conditions described above, the alamethicin currents were completely asymmetric with respect to the polarity of the applied potential. This was demonstrated in three ways. First, voltage ramps were applied, sweeping the applied voltage to both positive and negative values. An approximately exponential increase in the current was observed at positive potentials, but no increase was observed at accessible negative potentials.

The magnitude of the positive potential at which the first alamethicin channels opened was not constant during an experiment, but tended to decrease with time, presumably due to continued incorporation of additional alamethicin into the bilayer; however, the asymmetry was maintained. Second, even when the concentration of alamethicin at the inner surface was sufficient to give a large alamethicin current at potentials between 0 and +50 mV, no alamethicin current was observed when the voltage was stepped from 0 to −160 mV and held there for up to 20 min. This experiment was repeated with many different membranes, always with the same result. Third, again using membranes that gave large alamethicin currents at potentials between 0 and +50 mV, the voltage was stepped from 0 to −160 mV for 0.5 s, then back to 0 mV. This was repeated over 500 times, but again no alamethicin current was observed.

We have found that, when the applied voltage is suddenly reversed from a positive value to −160 mV, at temperatures below 20°C, while an alamethicin pore is open, one of two things will happen. Either the pore will close within a few milliseconds, often within the capacitive transient associated with the potential step, or it will continue to fluctuate through its various conductance states for periods up to several minutes, even though the current through it now flows in the opposite direction.

Because no new pores can be opened at this negative potential, the single "reversed" ion-conducting aggregate can be observed for a long period. When the potential is stepped to 0 mV for several seconds while such a "reversed" channel is open, and is then returned to −160 mV, the channel will have closed, and will not return even if the potential is left at −160 mV for several minutes. This method, then, provides a convenient means of observing single alamethicin channels, unencumbered by subsequent opening of additional alamethicin channels.

To assess how likely a single ion-conducting aggregate is to survive a potential step to −160 mV at 19°C, we did the following experiment. Single alamethicin pores were observed at +100 mV, and after the current crossed +200 pA (which, under these conditions, would happen as the aggregate crossed into the fourth conductance state) the voltage was stepped to −160 mV. After 2 s the voltage was stepped to 0 mV for 4 s, then stepped again to +100 mV to await the opening of another pore and the repeat of the same cycle. Fig. 1 shows the superposition of the current traces while the potential was at −160 mV of 20 such trials. In only one of these the pore stayed open for the duration of the −160 mV pulse; in all the others the current returned to baseline. A total of 225 such trials were performed using three different pipettes, and seven times a channel remained open at
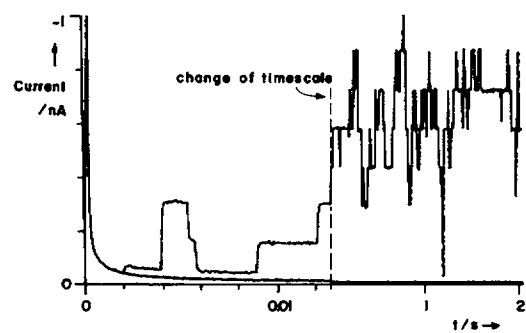

FIGURE 1 The superposition of current traces from 20 consecutive experiments, at 19°C, in which the voltage was stepped from +100 to −160 mV at the moment the current exceeded 200 pA; at +100 mV this occurs as a single alamethicin aggregate make a transition from the third to the fourth conductance state. Note the time scale change on the x-axis. Time t = 0 corresponds to the moment the voltage is stepped to −160 mV. In one trial shown here the channel continued to conduct for the 2 s duration of the negative pulse; in the other 19 cases, the channel closed within the first millisecond after the potential step, and remained closed for the duration of the pulse.

−160 mV, i.e., 3% of the total. In those cases the conductance persisted for the entire 2 s of the negative pulse. In another series of 97 trials, the potential was held at +50 mV until the channel entered the fifth conductance state. Under these conditions, the channel remained open four times, i.e., four percent of the total.

Steps to more negative potentials increased the probability of observing reversed conductance. Whereas none of 96 voltage steps to −50 mV, done while the
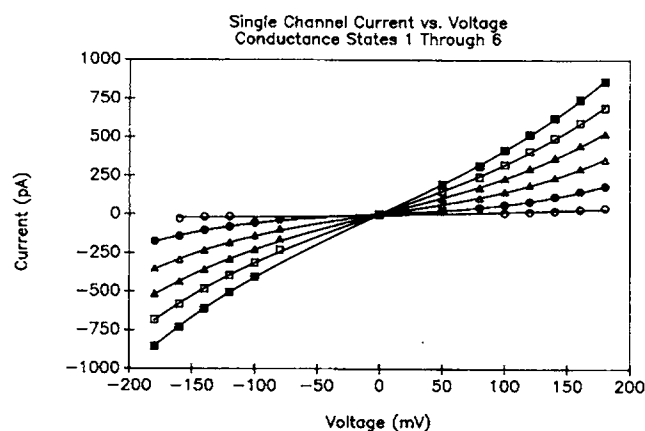

FIGURE 2 Single-channel current versus voltage for the first six conductance states in normal and reversed channels, at 19°C. The traces in the first and third quadrant are, within experimental error, identical. The lines represent third order polynomial fits to the data.

channel was in the fourth conductance state, resulted in a reversed channel, 17 out of 40 (42%) steps to −200 mV, carried out under identical conditions, did so.

We investigated the current-voltage behavior of the lower conductance states for these reversed channels by first applying a positive potential, stepping the voltage to −160 mV as soon as the current exceeded that of a single alamethicin aggregate in its fourth conductance state, then stepping to other negative values and measuring the various current levels at that potential. The current-voltage curves for the first six conductance states are identical for both polarities, see Fig. 2.

We observed reversed alamethicin channels to fluc-

tuate for over 2 min at 19°C and −160 mV. During this period, up to 18 conductance states could be resolved, as shown in Fig. 3. The current levels associated with the lower conductance states were identical in amplitude to those seen at positive potentials, whereas the higher levels were a continuation of the same progression, with each successive state (past the third level) larger than the last by 0.9 nS. The very high conductance states came in relatively short bursts, after which the more frequently seen lower states were again observed. A histogram of current versus number of data points from the digitally stored current record is shown in Fig. 4.

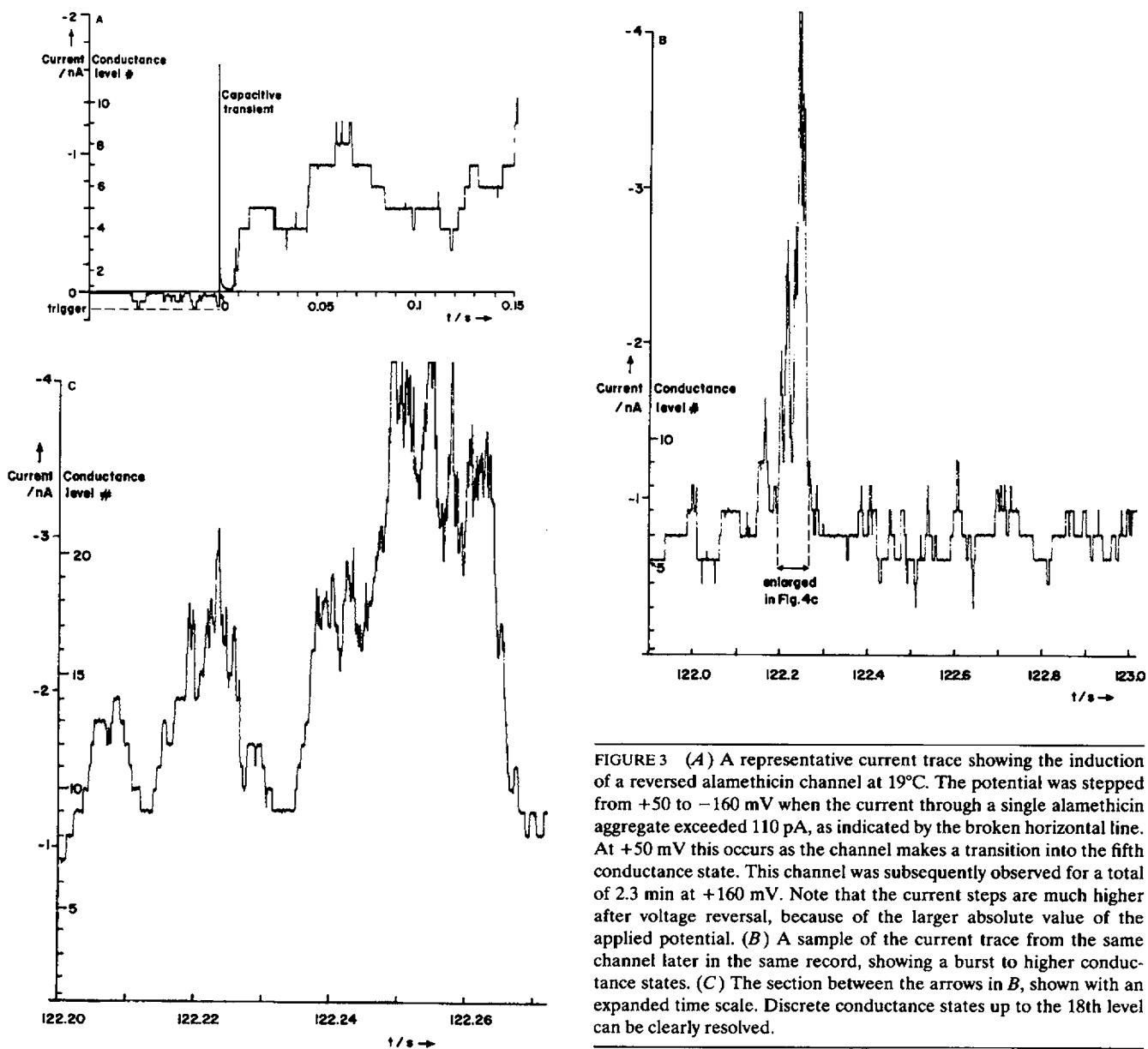Similar behavior was also observed using membranes

FIGURE 3 (A) A representative current trace showing the induction of a reversed alamethicin channel at 19°C. The potential was stepped from +50 to −160 mV when the current through a single alamethicin aggregate exceeded 110 pA, as indicated by the broken horizontal line. At +50 mV this occurs as the channel makes a transition into the fifth conductance state. This channel was subsequently observed for a total of 2.3 min at +160 mV. Note that the current steps are much higher after voltage reversal, because of the larger absolute value of the applied potential. (B) A sample of the current trace from the same channel later in the same record, showing a burst to higher conductance states. (C) The section between the arrows in B, shown with an expanded time scale. Discrete conductance states up to the 18th level can be clearly resolved.
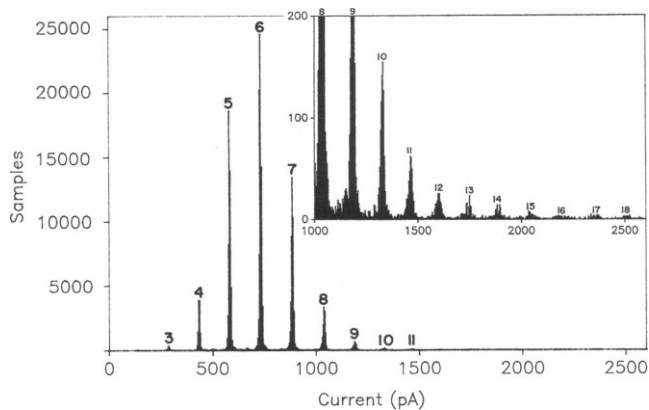
FIGURE 4 Histogram of frequency-of-occurrence versus current for the entire record of the reversed channel shown in Fig. 3. The record was digitized at a rate of one sample per 730 μs, and the data were sorted into bins with a width of 4 pA. The current was most frequently found in the sixth conductance level, as indicated in the graph. The insert shows the portion of the histogram above 1,000 pA, redrawn with an expanded vertical scale. Numbers with the peaks indicate conductance levels.

that had been cooled to 1°C. In this case, the likelihood of reversal of the alamethicin conductance was greater, doing so in 6 out of 18 trials. The conductances of the various states were smaller than those observed at higher temperature, presumably due to the reduced electrolyte conductivity at lower temperature. The average lifetimes of all the conductance states are considerably longer at the lower temperature, and the first few levels are very rarely observed. When the voltage was allowed to remain at −160 mV after the creation of a "reversed" channel, we again observed short bursts of higher conductance states. A sample of the current trace from such a channel, and the corresponding current histogram, are shown in Figs. 5 and 6, respectively. This channel was observed at −160 mV for 3.5 min, during which conductances corresponding to the fifth through at least the 25th levels were clearly resolved. The conductance fluctuated to even higher levels, where, however, the step-wise nature of the fluctuations could no longer be resolved, due to the increased noise and the short conductance state lifetimes.

THE TRANSIENT CONDUCTANCE

The above conductance states, which persist for relatively long periods, are quite distinct from a much shorter lived, "transient" conductance that can be observed immediately after the polarity reversal. The step

size between such transient states is considerably smaller than that between states observed at positive potentials, and the transitions are almost always to lower conduction states, with the result that the transient channels quickly close. Small negative potentials favor the observation of these transient closing states, whereas the "reversed" conductance is more likely to be observed at larger negative voltages.

At 19°C, the pore usually closes within the capacitive transient associated with the voltage step, as illustrated in Fig. 1. However, at lower temperatures, one can resolve discrete conductance states through which the pore passes as it closes. We obtained the current-voltage relation for some of the lower levels of such transient conductance states in a membrane cooled to 8°C, by repeatedly stepping the voltage to a series of different negative potentials, subtracting the capacitive current from the current-time record, and measuring the amplitude of the remaining current as the channel passed through its various conductance states as it closed, see Fig. 7. In almost all cases the transitions led to successively lower conductance states although, in a few instances, individual transitions to a higher state were observed. Often, not all of the states were resolved as the channel closed, and even at 8°C the channel often closed so quickly that no individual states could be resolved, see Fig. 8.

Steps to smaller negative potentials allowed the channel to remain open longer after the step than steps to larger amplitudes. For example, the average survival time of the transient conductance after a step to −160 mV, carried out while the normal channel was in the fourth conductance state, was 1.0 ms, with a standard deviation of 1.0 ms and a maximum observed in 96 trials of 4.4 ms. After a step to −50 mV, the average time before closing was 3.9 ms, with an SD of 4.4 ms, and a maximum of 20.0 ms, out of 79 trials; the survival time exceeded 10 ms in five of these latter trials. Plots of the lifetimes of the transiently observed conductance are shown in Fig. 9.

DISCUSSION

The two types of pore conductance observed after a polarity reversal described here, and labeled "reversed" and "transient," appear to be quite distinct. The transient conductance states of an alamethicin pore are only observed immediately after polarity reversal, and seem to be similar to the single transient reported by Boheim et al. (1983). These transient states are smaller in conductance than either those arising from single aggre-
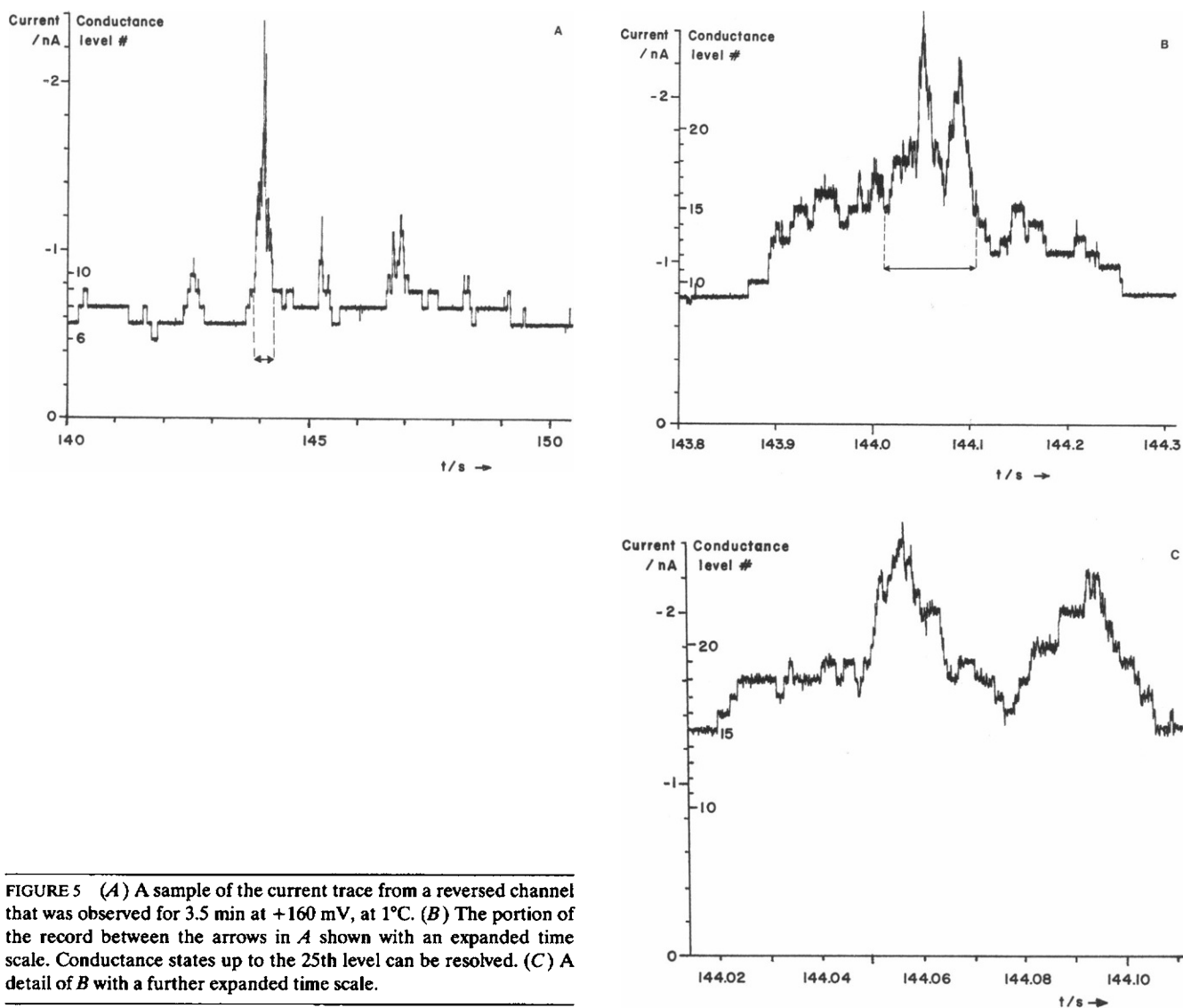
FIGURE 5 *(A)* A sample of the current trace from a reversed channel that was observed for 3.5 min at +160 mV, at 1°C. *(B)* The portion of the record between the arrows in *A* shown with an expanded time scale. Conductance states up to the 25th level can be resolved. *(C)* A detail of *B* with a further expanded time scale.

gates observed at positive potentials, or of the "reversed" channels. The transient conductance states disappear quickly after the polarity reversal, they are more frequently observed at less negative voltages, and they also last longer at less negative voltages. In contrast, the "reversed" conductance states exhibit the same conductance states as the normal alamethicin single-channel fluctuations, remain conducting for minutes, and are more frequently observed the larger the amplitude of the negative voltage.

All the conductance states observed seem to arise from a single alamethicin aggregate. First, there was only a single ion-conducting aggregate open at the time of the polarity reversal to a negative potential; subsequently, no additional channels can open. Also, the first

two current levels of an independent channel would be of the characteristically smaller sizes, but current steps corresponding to these levels were not observed. The fluctuations were discrete at all levels but a high levels the average time before a transition to a new state was considerably shorter. Furthermore, the probability of finding the channel in any particular conductance state was observed to decrease monotonically as the conductance level increased.

It is quite possible that these high conductance states of single alamethicin channels also occur in normal (i.e., not reversed) current-time records, but that they have not (to our knowledge) been reported before because, under such circumstances, then can readily be mistaken for coincident conductances of several channels. More-
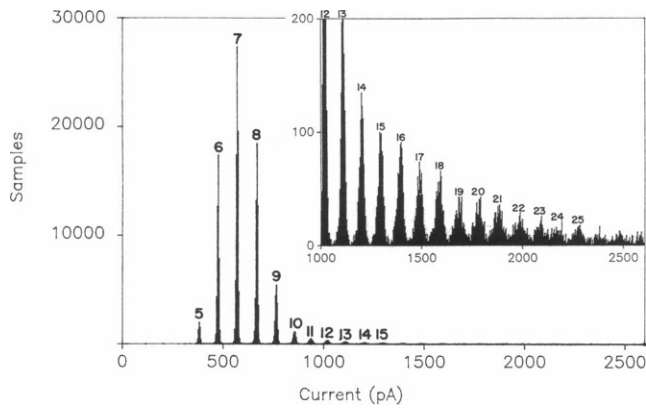
FIGURE 6 Histogram of frequency-of-occurrence versus current for the entire 3.5-min record of the channel shown in Fig. 5. The data rate was one sample per 1,200 μs, and the data were sorted into bins with a width of 4 pA. The channel in this case was found most frequently in the seventh conductance state; the four lowest conductance states were not observed at all. The insert shows the portion of the histogram above 1,000 pA, redrawn with an expanded vertical scale.

FIGURE 8 Four representative current-time records showing the transiently observed reverse conductance. The voltage was stepped from +160 to −80 mV at time $t = 0$. The temperature was 8°C. The conductance of the various transient states was measured after subtraction of the capacitive transient.

over, most studies have been confined to room temperature, whereas our observations pertain to lower temperatures.

Our observations can be summarized as follows. Under conditions where the steady-state current-voltage curve is asymmetric, reversed alamethicin conductance
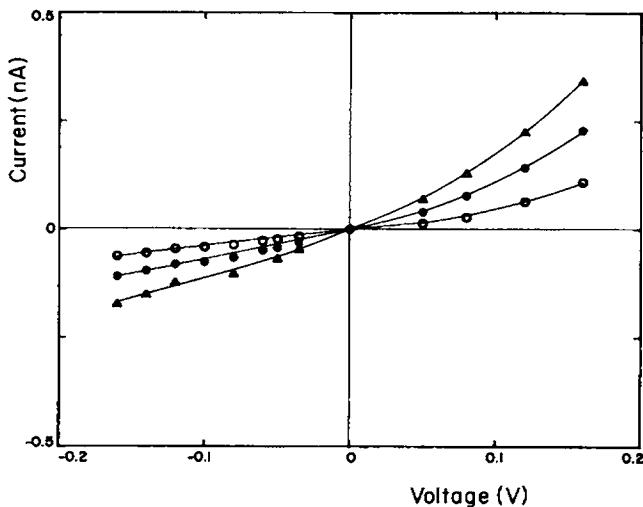

FIGURE 7 Single-channel current versus voltage for normal and transient states two, three, and four of the alamethicin channel at 8°C. The data points at positive voltages correspond to the normal conductance, whereas those at negative voltages correspond to the transiently observed conductance states observed immediately after voltage polarity reversal.
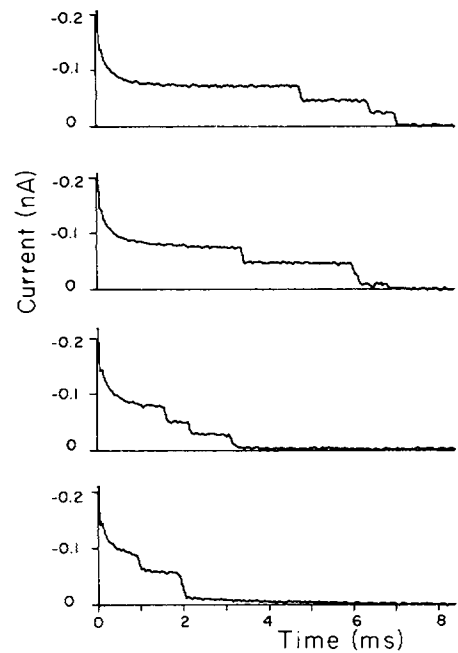
can be obtained by changing the polarity of the applied voltage while at least one alamethicin aggregate is conducting. The reversed conduction we report here is distinct from transiently observed conductance states observed immediately after a polarity reversal, in terms of duration, amplitude of the conductance states, and the magnitude of the negative voltage that favors the observation of one mode over the other.

Both reversed and transient conduction have been observed only when normal alamethicin conductance was present at the moment of voltage reversal; the probability of observing reversed conduction increases sharply with decreasing temperature, and increasing negative voltage. Occasional bursts of high-level fluctuations exhibit, within experimental accuracy, the same additional conductance steps as those of the lower-level fluctuations, but they have considerably shorter average residence times at each conductance level.

The origin of these occasional bursts is not understood at present. Even disregarding these occasional short bursts of high-level fluctuations, it is not clear how the observation of reversed conduction fits the existing models of alamethicin-induced conductance (Baumann and Mueller, 1974; Fox and Richards, 1982; Boheim et al., 1983; Hall et al., 1984, Cascio and Wallace, 1988).
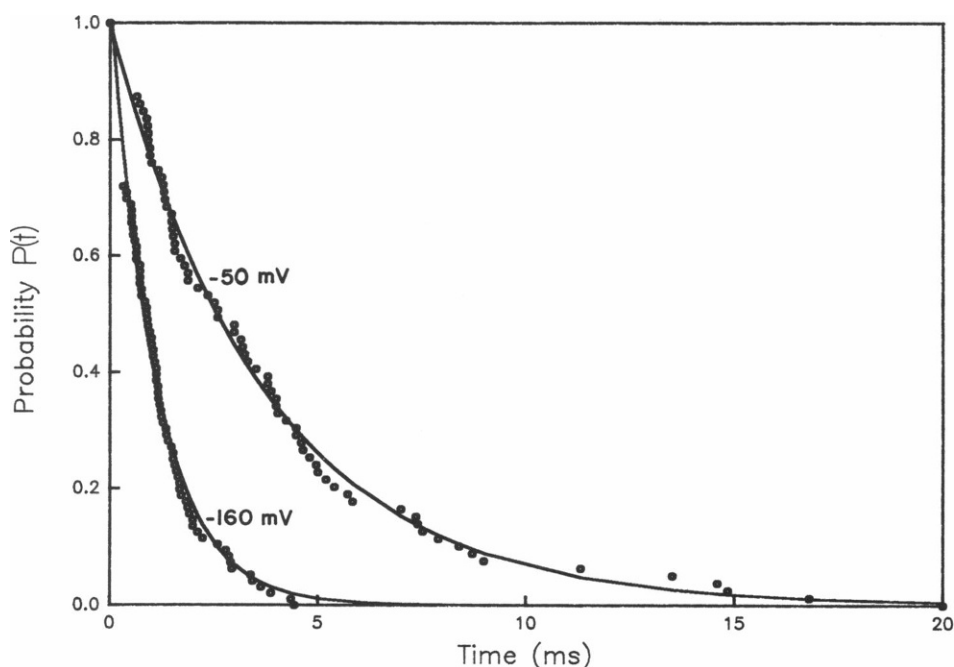
FIGURE 9 Cumulative lifetime probability distributions for the transiently observed conductance, after the voltage was stepped to −160 or −50 mV, at 8°C. The applied voltage was changed from +100 mV when a single channel made a transition into the fourth conductance state. The line represents a weighted least-squares fit to the equation $P(t) = \exp(-kt)$. For steps to −160 mV, $k = 0.90/\text{ms}$, (96 events), and for steps to −50 mV, $k = 0.27/\text{ms}$, (79 events).

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