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Properties of the Conductance Induced in Lecithin Bilayer Membranes by Alamethicin

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Summary. Current-voltage relations have been measured across lecithin bilayers doped with alamethicin molecules. The results show that there are two aspects of the induced conductances, a voltage-dependent and a voltage-independent conductance. Both have been characterized as a function of alamethicin and KCl concentration. The two aspects of the conductances do not show the same changes with those two variables. The voltage-independent conductance is affected very little by changes in KCl concentration, and its dependance on alamethicin concentration reveals that it is produced by two or three alamethicin molecules. The voltage-dependent conductance is shifted by the changes in KCl concentration only when the concentrations are $\geqq 100$ mM; below 100 mM KCl the slope of the log conductance-voltage curve is also reduced. The effect of changing alamethicin concentration reveals that nine or ten molecules are involved for KCl concentrations larger than 100 mM; if the KCl concentration is less than 100 mM, the effect of changing the alamethicin concentration is reduced. Time-dependent measurements have also been performed; only one time constant was found and it is strongly voltage-dependent. Also a very slow voltage-dependent absorption process is found. These results can be explained if it is assumed that pores are formed of a mixture of charged and uncharged alamethicin molecules when a voltage is applied and that uncharged alamethicin can also form pores without applying a voltage, once the absorption process has been started by previously applied voltages. The voltage dependence of the time constant seems to indicate that the voltage-dependent pore formation is produced by aggregates of charged alamethicin rather than independent molecules.

The study of ionic currents across artificial bilayer membranes containing antibiotics has aroused more and more interest since its beginning by Mueller and Rudin (1963). One of the obvious reasons for such a growing interest resides in the fact that some of these antibiotics induce properties that are quite closely related to those observed on excitable membranes of nerve and muscle cells (Baumann & Mueller, 1974). Among these antibiotics, some have been described as carriers and others as pore formers (Haydon & Hladky, 1972). Among the pore formers, alamethicin has recently attracted much interest because of the peculiar aspects of the fluctuating currents observed with low alamethicin concentrations (Gordon & Haydon, 1972; Eisenberg, Hall & Mead, 1973; Boheim,

1974). Mueller and Rudin (1968) first described the general aspects of alamethicin-induced conductances in bilayers. The induced conductances were exponentially dependent on membrane voltage; they also varied with the sixth power of alamethicin and salt concentration in solution. Following the application of a voltage step, induced conductances increased to a new value with a rather long time constant, which was also voltage-dependent. More recently, Cherry, Chapman and Graham (1972) have reported additional results on these induced conductances in lecithin bilayers. In particular, they observed a voltage-independent conductance which was dependent on alamethicin concentration in the aqueous solutions.

In order to explain their observed data, Mueller and Rudin (1968) assumed that alamethicin monomers are complexed with a cation in solution and penetrate the membrane under the influence of the voltage to form conducting oligomers. Cherry *et al.* (1972) proposed that charged monomers diffuse across the membrane under the influence of the voltage, and conducting oligomers are formed at each membrane surface. Such a model could explain their voltage-independent conductance.

This paper reports our observations on the voltage-dependent and -independent conductances induced by different concentrations of alamethicin and the effects of low and high salt concentrations on these conductances. Also the time-dependent changes of the conductance have been measured for a constant applied potential. The results are discussed in terms of the actually available models.

Materials and Methods

L-α lecithin was purchased from Sigma Corporation (No. L0127) who specified that it was obtained from egg yolk extracts and purified following the method of Singleton, Gray, Brown and White (1965). The fatty acid composition of this lecithin has been specified by Singleton *et al.* (1965). It contains mostly palmitic (37.7%) and oleic (32.9%) acids, with a smaller amount of linoleic (17.0%), stearic (9.2%) and palmitoleic (3.2%) acids. The lecithin was received in hexane solutions and used without further purification. Membranes were formed from a solution of 35 mg/ml of this L-α lecithin in *n*-decane. A small amount of this solution was introduced with a Pasteur pipette on a small hole (1 mm^2) drilled through a teflon wall separating two compartments of 25 ml each. Each compartment was filled with KCl solutions and contained two Ag-AgCl electrodes, one for measuring the voltage and the other for passing the current. No buffers were introduced. The voltage difference across the membrane was measured through high impedance amplifiers having a gain of one. The currents were measured with a Keithley electrometer. Both current and voltage output were connected to an *x-y* recorder for tracing directly the current-voltage curves. The signal source was provided by a waveform generator with a variable frequency

and amplitude. The current-voltage curves were measured using a symmetrical triangular wave as a source (positive and negative ramps); the frequency during measurements was 10^{-3} cps. At this low frequency, there was very little hysteresis, about 10–20%, being more pronounced at the bottom of the current-voltage curves where the currents start to rise sharply. Alamethicin was kindly provided by the Upjohn Company (Dr. G.B. Withfield, Jr.) and was used without further purification. Stock solutions of 5, 2.5 and 1.25 mg per ml of ethanol were made. Small amounts of these solutions were added after the membranes were formed. The experiments were performed at room temperature (23–24 °C). The solutions on each side of the membrane were under continuous stirring provided by small teflon-coated magnetic bars driven by a small motor.

Experimental Observations

Many experiments were performed to observe the effect produced by adding alamethicin to only one of the two aqueous solutions. The current-voltage curves were measured, considering the voltage and the current positive on the alamethicin-containing side and negative on the opposite side. It was found that the current-voltage curve was asymmetric initially but it could not be maintained in a stable steady-state, particularly the negative current; this current slowly increases and eventually reaches the same amplitude as the positive current. A few curves are shown in Fig. 1; these were taken at 5-min intervals, about 1 hr after alamethicin was added. The positive portion of the curves had just reached a stable state. Although it might appear that the negative portion of the curve is stable, and linear over a certain range (scale 10.0), it can be observed that this is not the case at a higher resolution (scale 1.0). For concentrations of 1 μg/ml of alamethicin or more, there is no linear relation between current and voltage when a steady state has been reached. For concentrations of 0.25 μg/ml or less, a stable linear relation could be observed over a short range of voltage in the positive and the negative regions. These observations imply that negative current measurements in this asymmetric system are not really valid, since standard conditions cannot be clearly defined to reproduce the measurements.

In order to be sure that the measured currents had really reached a steady state, it was decided to do the experiments with equal amounts of alamethicin on each side of the bilayer. In doing these experiments, it was found that it could take as long as 2 hr before a stable current-voltage curve could be obtained. Normally, the voltage was applied immediately after the alamethicin was added. Initially, the current was very small and the effect of alamethicin on the current could be observed only at large voltages (100–150 mV). As the voltage was maintained, the current-

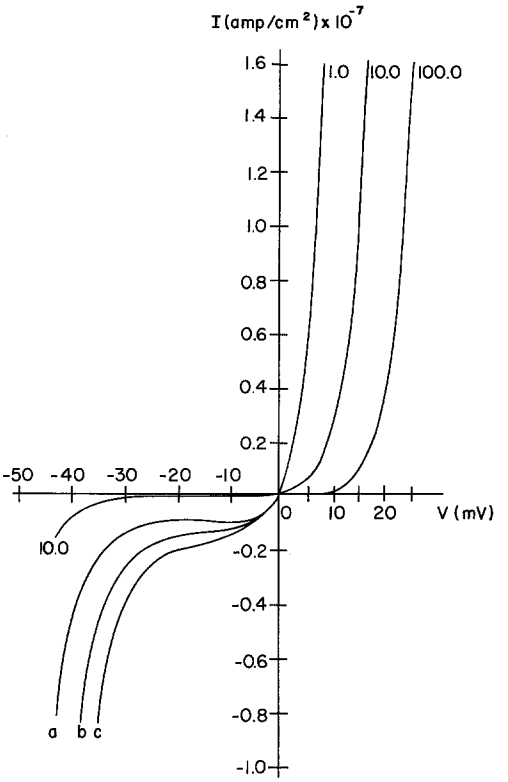

Fig. 1. Current I *vs.* voltage V measured across a lecithin bilayer. The solutions contained 100 mM KCl; 1 μg/ml of alamethicin was added on one side only. The time between curves *a*, *b* and *c* is 5 min. The numbers 1.0, 10.0 and 100.0 attached to the curves mean that the vertical scale should be multiplied by these numbers

voltage curve shifted as shown in Fig. 2. When the current became larger, the amplitude of the voltage ramp was reduced, otherwise the membrane would break. If the membrane breaks during this slow process, another one is made. The level of current is often almost the same as it was before and it keeps increasing until it reaches a stable state; if the membrane breaks three or four times, a new experiment must be started. In a few cases, alamethicin was added and no voltage was applied for about an hour. Then the voltage ramp was started; the process was the same as if alamethicin had just been added. It seems that this slow phenomenon is voltage-dependent; the current-voltage curve reaches a stable state only under a maintained voltage, and the position of the stable current-voltage curve changes if the amplitude of the voltage ramp is changed.

The current-voltage curves were transformed into conductance-voltage curves, in order to separate the process of pore formation from the diffu-

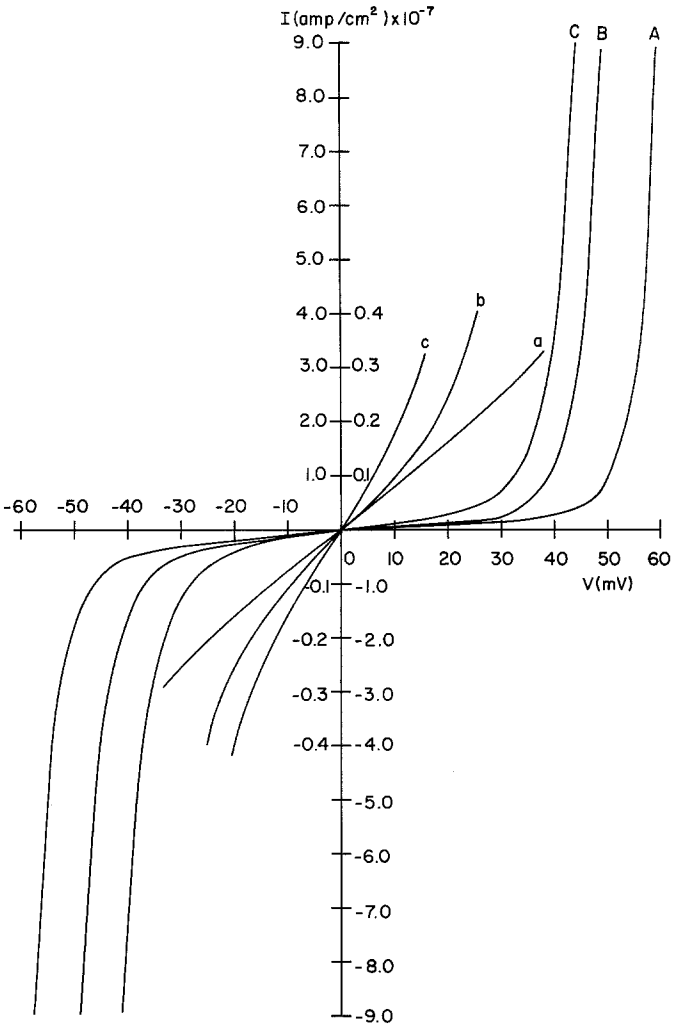

Fig. 2. Current I *vs.* voltage V obtained with an equal concentration of alamethicin of 0.5 μg/ml on both sides of the bilayer; the solutions contained 100 mM KCl. Curves A and a were measured 15 min after the addition of alamethicin, curves B and b, 50 min and curves C and c, 75 min. Curves a, b and c represent a magnification by a factor of ten of the low amplitude section of curves A, B and C

sion of ions through the pores; this is valid since the pore current is linear for voltages less than 100 mV (Eisenberg *et al.*, 1973). For higher voltages, it would be necessary to introduce a nonlinear pore current. Out of the many measured conductance-voltage curves, those shown in Fig. 3 are the ones that gave the largest steady-state amplitudes for the given concentrations of alamethicin in the aqueous solutions. Also each curve was obtained on bilayers that did not break during the slow process;

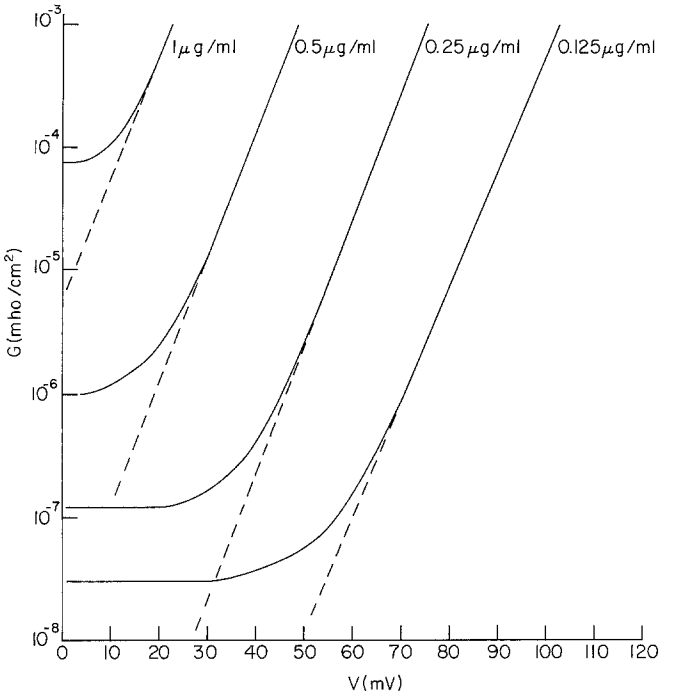

Fig. 3. Conductance G *vs.* voltage V for different concentrations of alamethicin introduced in equal amounts on each side of the bilayer; the solutions contained 100 mM KCl. The basic bilayer conductance without alamethicin was not more than 5×10^{-9} mho/cm^2. The slope of the straight lines is given by $\alpha_v FV/RT$, where $\alpha_v = 5$

the measurements were taken about 1.5 to 2.0 hr after alamethicin was added. These measurements were chosen because they were among the ones that really had attained a steady-state. It is important to have reached a real steady state for each conductance-voltage curve in order to determine correctly the effect of alamethicin concentration. The results shown in Fig. 3 could be repeated only a few times, because of the difficulty of maintaining the membrane long enough. But in many other experiments in which the membrane broke and was reformed two or three times, the results were smaller than those shown in Fig. 3 by at most a factor of two.

According to the results of Fig. 3, there are at least two different alamethicin concentration effects on the bilayer conductance. The most important one is already known and corresponds to the linear portion (on a log scale) of the curves. Each curve differs only by a factor of two in alamethicin concentration; there is a factor of about 500 between each curve in the linear section, meaning that the conductance changes are proportional to the ninth power of alamethicin concentration. At

lower voltages there is a constant conductance for two of the curves. These two constant conductances differ by a factor of about four, meaning that they are proportional to at least the second power of alamethicin concentration. For the other two curves (1.0 and 0.5 μg/ml) there is no clear constant conductance. The small horizontal portions for voltages less than 5 mV are given by the slope of the current-voltage curves at zero voltage; these are not considered as constant conductances in the same sense as the ones above for alamethicin concentrations of 0.25 and 0.125 μg/ml. But it can be observed that the rounded portions of the conductance curves differ by factors that are intermediate between the extremes of four and five hundred, and consequently depend on intermediate powers of alamethicin concentrations.

Although these experiments to determine quantitatively the effect of alamethicin concentration on the conductance of lecithin bilayers were done in as standardized conditions as possible, the results are not very accurate. There could be an error of about a factor of two in the evaluation of the concentration effects. Part of this error is due to the impossibility of measuring each of these curves under the same maximum voltage. There is a 25 mV difference between each curve and this difference was observed to modify the conductance curve by about a factor of two. Consequently, instead of the ninth power of alamethicin concentration, the conductance could depend on the tenth power in the linear portion, and instead of the second power it could be the third power in the constant conductance portion. For concentrations of alamethicin ≤ 0.06 μg/ml, the voltage-independent conductance is given by the basic bilayer conductance ($\approx 5.0 \times 10^{-9}$ mho/cm^2) and the voltage-dependent conductance has the same characteristic as those of Fig. 3.

These results are different from those obtained by Cherry *et al.* (1972), where a voltage-independent conductance proportional to the same power of alamethicin concentration as the voltage-dependent conductance was observed. One major reason for this difference is the different conditions in which the experiments were performed. It was shown above that it was not possible with our lecithin bilayers to have stable conditions when alamethicin was added only on one of the solutions. Although an apparently linear and stable negative current was observed 20–30 min after alamethicin was added, it was found that this current was not linear and not stable if it was measured at a high resolution. Consequently, the experiments of Cherry *et al.* (1972) were not repeated in detail, because of these problems. It seems that conditions of symmetric concentrations are more reliable to determine the effects of alamethicin concentrations.

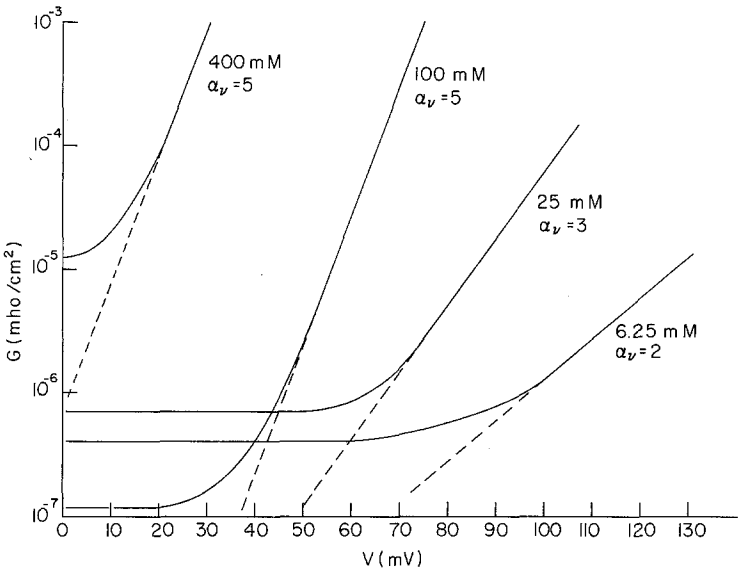

Fig. 4. Conductance G *vs.* voltage V for different concentrations of KCl in the solutions; the alamethicin concentration was 0.50 μg/ml on each side of the bilayer. The meaning of α_v is the same as in Fig. 3

Another series of conductance-voltage curves were also measured for different concentrations of KCl in the solutions. These results are shown in Figs. 4 and 5. It can be observed in Fig. 4 that the slope of the curve is much lower at 25 and 6.25 mM KCl than the slope obtained at 100 and 400 mM KCl. These effects of reducing the concentration of KCl were rather surprising, since previous results (Mueller & Rudin 1968; Eisenberg *et al.*, 1973) had demonstrated that changing alamethicin or salt concentration had similar effects. This is true in our case only for concentrations of 100 mM KCl or more. Another important observation from these results is that the voltage-independent conductance did not change too much for KCl concentrations of 25 and 6.25 mM. Normally, it would be expected that the pore conductance could be four times less at 6.25 mM than at 25 mM KCl because of a reduced ionic content; but because the maximum voltage is about 25 mV higher at 6.25 mM than at 25 mM, the curve for 6.25 mM should be reduced by a factor of about two. This leaves a net factor of two between these two constant conductances, and this corresponds to the results of Fig. 4. The constant conductance for 100 mM KCl is lower than the others. It would be expected to have a net value about two times larger than the one at 25 mM; it is actually about five times lower than expected. This discrepancy cannot be clearly explained at the moment; it is possible that reducing the ionic strength below 100 mM reduces the ratio of the voltage-dependent to the

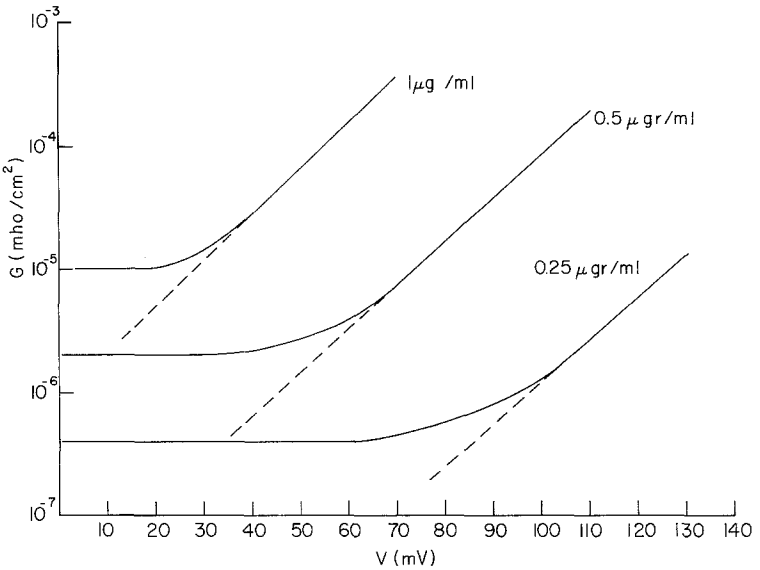

Fig. 5. Conductance G *vs.* voltage V for different alamethicin concentrations introduced in equal amounts on each side of the bilayer; the solutions contained 6.25 mM KCl. The basic bilayer conductance was not more than 5×10^{-9} mho/cm^2. The value of $\alpha_v = 2$, α_v having the same meaning as in Fig. 3

voltage-independent conductances. It should be mentioned that the voltage-independent conductance was slower to appear than the voltage-dependent conductance in the case of low KCl concentrations (25 and 6.25 mM), while at higher KCl concentrations they seemed to appear together.

The results shown in Fig. 5 demonstrate clearly that a lower KCl concentration reduces the slope of the conductance voltage-curve at any alamethicin concentrations, and for that KCl concentration the slope is always the same. Also it can be observed in Fig. 5 that the voltage-independent conductance increases again by about a factor of four between each curve. From the linear portion of the curves of Fig. 5, there is a factor of about 60 between each of them, meaning that the conductances are proportional to the sixth power of alamethicin concentration. It should be mentioned that the conductance-voltage curve obtained with 1 μg/ml of alamethicin and 6.25 mM KCl is very steep at the beginning of the experiments, and it becomes less steep as the steady-state is approached. The voltage-independent conductance is small at the beginning and becomes larger later on.

Another important observation that should be mentioned is the beginning of a saturation of the conductance when large currents could be passed through the membranes. This saturation could be observed only

a few times, because the membrane breaks at these high currents. But in all these cases, the saturation was found to be independent of alamethicin concentrations. When comparing the maximum value of the conductance before the membrane breaks with the conductance observed after the membrane is broken, it is found that they differ by a factor of about 20–40. It was then suspected that the saturation was produced by the limiting conductance of the hole without a membrane, and if the membrane could tolerate large currents, the conductance with and without a membrane would be the same. In order to test this hypothesis, large voltages were imposed with lower KCl concentrations; again the beginning of a saturation was found and it was directly proportional to the KCl concentration. Consequently, the saturation of alamethicin-induced conductances seems to be produced by the limited amount of ions in the hole and not by a limited amount of alamethicin molecules available.

Experimental results were obtained from measurements of the membrane currents in voltage clamp. The concentration of alamethicin was 1 μg/ml and the salt solution was 100 mM KCl. Typical oscilloscope traces of these currents are shown in Fig. 6. The results shown in Fig. 6 have been obtained for voltage steps applied from a zero initial voltage or voltage steps applied from a previously polarized membrane. In the latter case, the initial voltage was 50 mV and the final voltage as indicated on the curves. With this method it was possible to verify that the time constants depend only on the final voltage. This is shown in Fig. 6 with two currents measured for the same 44 mV final voltage, one of the currents being obtained from a 0 mV and the other from a 50 mV initial voltage. In addition, this method gives a more accurate measure of the time constant for very small voltages. From results such as those shown in Fig. 6, it is possible to determine the time constants of the conductance changes. The time-dependent currents were plotted on a semi-log paper, and from the slope of the curves a single time constant was obtained, as shown in Fig. 7. The results shown in Fig. 6 are not accurate for too short or too long times. From these results it seems adequate to assume that the kinetics of the currents are determinded by a single dominating time constant. The inverse of all measured time constants, τ^{-1}, has been plotted as a function of the applied voltage; the results are shown in Fig. 8. The experimental points in Fig. 8 follow a straight line on the semi-log paper, meaning that in the voltage range studied, τ^{-1} should be given by a single exponential of the membrane potential. The slope of the line is given by α^* FV/RT, where $\alpha^*=2$. These results are similar to those of Eisenberg *et al.* (1973) on phosphatidylethanolamine membranes.

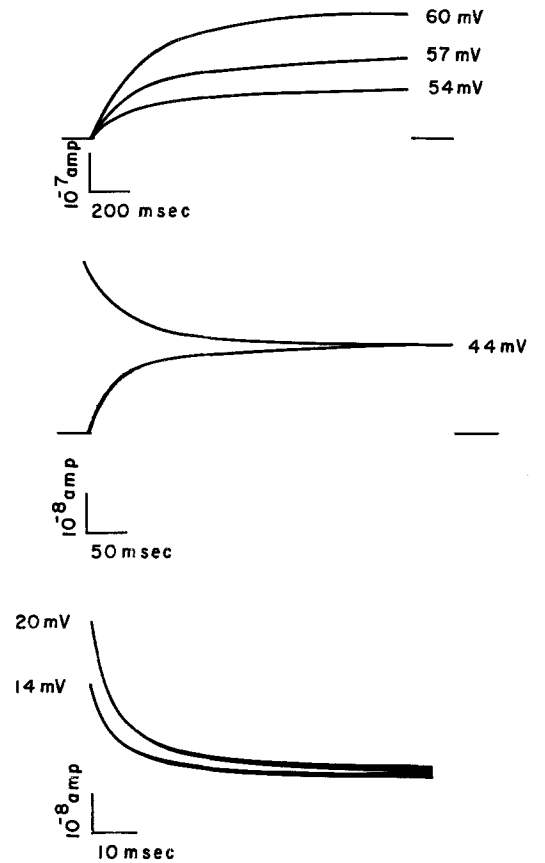

Fig. 6. Time dependence of the current measured after the application of different steps of voltage. The voltage before the application of the step was 0 or 50 mV. The numbers on the curves represent the final voltages. Alamethicin concentration was 1 μg/ml and the KCl concentration was 100 mM

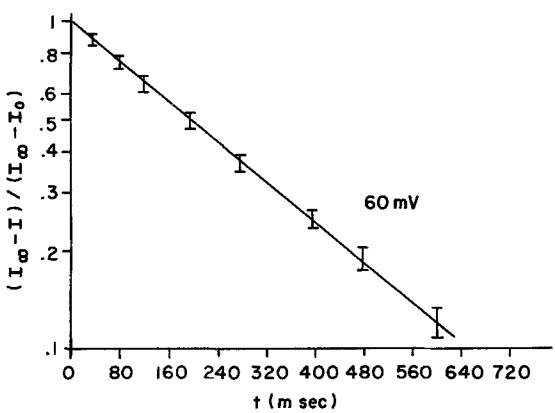

Fig. 7. Semi-logarithmic plot of one of the time-dependent curves shown in Fig. 6. A single time-constant is obtained from the slope of the line; its value is 275 msec

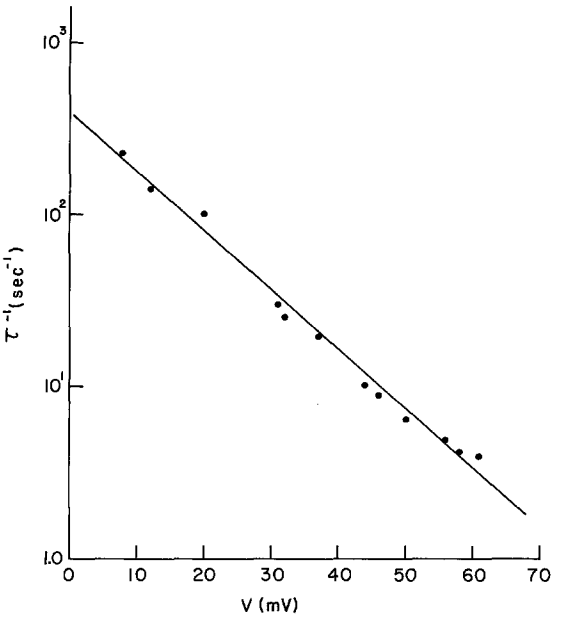

Fig. 8. Voltage dependence of τ^{-1}; the time constant τ is obtained from the time dependence of the currents. The slope of the line is given by α^*FV/RT, where $\alpha^* \approx 2$.

Discussion

The measurements of fluctuating currents by Gordon and Haydon (1972), Eisenberg *et al.* (1973) and Boheim (1974) have provided many details regarding the mechanisms of alamethicin-induced conductances. From the results obtained by these authors, alamethicin molecules form conducting pores, and the conductance of single pores is very similar to the conductance of ions in water. The statistical analysis of the discrete steps of conductance changes has given much insight into the mechanism of pore formation. According to Eisenberg *et al.* (1973) there are two aspects of the fluctuating currents: fluctuations related to pore formation and fluctuations of conductance within a single pore. These authors have concluded from their observations that only the fluctuations related to pore formation are voltage-dependent, and that can explain the voltage dependence of the average conductance. On the other hand, Boheim (1974) has found that the discrete levels of conductance in a single pore are voltage-dependent. His model assumes that pores are formed by the aggregation in the membrane of many alamethicin-ion complexes. The different steps of aggregation could be measured by Boheim (1974) for different voltages, and each step was found to have a similar voltage dependence.

Boheim (1974) concluded that alamethicin-ion complexes are rotated by the electric field and are associated with other alamethicin-ion complexes to form pores of different sizes; the maximum number of alamethicin in a pore is around nine.

In order to explain our results, it seems necessary to introduce an additional hypothesis, which was not considered in the previous models: alamethicin molecules not complexed with a positive ion can also form pores, and they can also participate in the formation of the voltage-dependent pores with the alamethicin-ion complexes. The slope of the conductance-voltage curve is explained more adequately if it is assumed that among the nine (or ten) monomers forming the pores, some are charged and some are not charged. In the linear portion (on a log scale) of the conductance-voltage curves, a factor α_v can be introduced to represent the slope; if this slope is $\alpha_v FV/RT$, the value of $\alpha_v \simeq 5$ for the curves of Fig. 3. The results of Fig. 4 give $\alpha_v \sim 3$ for 25 mM KCl and $\alpha_v \sim 2$ for 6.25 mM KCl. In order to give a more detailed interpretation of these results, it would be necessary to introduce a model. Since the model proposed by Boheim (1974) seems to have good experimental support, some of its features will be used to explain our observations.

Boheim (1974) has found that a slope given by $\alpha FV/RT$ can be related to his observations on the voltage dependence of the conductance steps. If it is assumed as in Boheim (1974) that α is related to the movement of a single charged monomer across the membrane, the value of α_v should be given by $v\alpha$, where v represents the number of charged monomers involved in pore formation. Since the value of α obtained by Boheim (1974) is 0.9 for his di-(22:1)-lecithin membranes, it should be valid to assume a similar value in our case. That would give v between 5 and 6 for 100 mM KCl or more, v between 3 and 4 for 25 mM KCl and v about equal to 2 for 6.25 mM KCl. From the alamethicin concentration dependence of the voltage-dependent conductance of Fig. 3 and Fig. 5, it is necessary to assume that uncharged monomers are also participating in pore formation. In the case of Fig. 3, there could be about 4 to 5 uncharged alamethicin involved, in order to obtain a total of nine or ten monomers. In the case of Fig. 5, for 6.25 mM KCl, there are probably three or four uncharged monomers involved, since the total must give about six monomers.

The reduction in ionic strength from 400 to 6.25 mM KCl is probably responsible for the reduction of charged monomers forming the pores. If as many as five or six positive charges are assumed to form a pore in the way proposed by Boheim (1974), they must come in close proximity,

thus requiring a strong shielding by charges in solution. This is also why uncharged monomers would be necessary in order to provide increased separation between positive charges. If the ionic strength of the solution is reduced, the number of positively charged monomers forming the pore would also be reduced. It must also be assumed that there is a maximum size for the pores, since beyond 100 mM KCl, there is no additional increase of charged monomers. For reasons of symmetry, it would seem logical that a charged monomer is always accompanied by an uncharged monomer in the pore. It seems that uncharged monomers alone cannot form large pores and also do not form as many pores as the combination of charged and uncharged monomers do, because the application of a voltage produces a tremendous increase in the number of pores. The penetration of charged monomers in the bilayer probably increases the aggregation of uncharged monomers in the membrane.

The observation of a single relaxation time constant following the application of a constant voltage means that one of the steps of the voltage-dependent pore formation is much slower than the others. The very slow absorption processes do not appear on these time-dependent measurements; they were not perturbed by the short voltage pulses. The voltage-dependence of τ^{-1} also implies a single process but the problem with this voltage-dependence is its steep slope, τ^{-1} varying by a factor of about 7.5 for a 25 mV potential difference. If the rate constant of each step of the voltage-dependent pore formation is related to the rate of rotation of only one charged monomer across the membrane, it would be expected that τ^{-1} vary much less with voltage. In fact, the rate constants of the conductance steps observed by Boheim (1974) have a very small voltage dependence. The discrepancy between these observations could mean that voltage-dependent pore formation is indeed a process different from that observed in conductance fluctuations of a single pore as proposed by Eisenberg *et al.* (1973). It is possible that the applied voltage forces the penetration or rotation of charged oligomers, and that the observed fluctuations of conductance represent the movement of charged monomers becoming associated or dissociated from the other molecules of the pore. It is quite possible that charged alamethicin molecules are aggregated on the surface of the bilayer. Once inside the bilayer forming a pore, their aggregation could fluctuate in a voltage-dependent manner. Because of these unresolved difficulties, it is not appropriate at the moment to develop a more quantitative model.

The fact that the only saturation observed is independent of alamethicin concentration means that in the whole range of conductance measure-

ments, there is only a small fraction of monomers that aggregates to form pores. The conductances are observed in the range of 10^{-10} to 10^{-5} mhos in 100 mM KCl. The smallest conductances are observed when a single pore is formed, requiring about 10 monomers. The largest conductances would require about 10^6 monomers. Normally it would be expected that at least 10 times that amount should be available in order that no saturation is observed. This gives 10^7 monomers in a volume of about 10^{-8} cm^3 for the bilayer, or about 10^{-6} moles/liter. The largest values of the conductance are observed for alamethicin concentrations in solution as low as 10^{-8} moles/liter. This suggests that alamethicin molecules have a large partition coefficient in favor of the membrane phase, but only in the presence of an electric field. Without the electric field, alamethicin does not seem to penetrate in the membrane phase. Trying to dissolve alamethicin into the membrane-forming solution was unsuccessful.

The slow voltage-dependent shifts of the current-voltage curve cannot be explained simply for the moment. It appears like a slow absorption of alamethicin in the bilayer or at its surface; but since this phenomenon was not studied in detail, many hypotheses are possible. Further experiments on this aspect will be needed before an appropriate explanation is introduced.

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