Alamethicin Channels Incorporated into Frog Node of Ranvier

Calcium-induced Inactivation and Membrane Surface Charges

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ABSTRACT Alamethicin, a peptide antibiotic, partitions into artificial lipid bilayer membranes and into frog myelinated nerve membranes, inducing a voltage-dependent conductance. Discrete changes in conductance representing single-channel events with multiple open states can be detected in either frog node or lipid bilayer membranes. In 120 mM salt solution, the average conductance of a single channel is approximately 600 pS. The channel lifetimes are roughly two times longer in the node membrane than in a phosphatidylethanolamine bilayer at the same membrane potential. With 2 or 20 mM external Ca and internal CsCl, the alamethicin-induced conductance of frog nodal membrane inactivates. Inactivation is abolished by internal EGTA, suggesting that internal accumulation of calcium ions is responsible for the inactivation, through binding of Ca to negative internal surface charges. As a probe for both external and internal surface charges, alamethicin indicates a surface potential difference of approximately −20 to −30 mV, with the inner surface more negative. This surface charge asymmetry is opposite to the surface potential distribution near sodium channels.

INTRODUCTION

It is generally assumed that the ionic channels of excitable membranes are proteins embedded in a matrix of fluid lipid bilayer. However, virtually nothing is known about the composition and properties of lipid regions surrounding channels or about the possible influence of lipid regions on the channel mechanism. For example, lipids may influence the local environment near channels by altering the local fluidity or surface charge density. For this reason, probes of the lipid region of the membrane are needed.

Alamethicin consists of several polypeptide components with molecular weights of ~2,000 that form voltage-dependent pores across membranes. The properties of alamethicin in artificial lipid bilayer membranes of varying composition have been well characterized (Eisenberg et al., 1973; Boheim, 1974; Hall, 1975). The pores most likely consist of aggregates of alamethicin

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Volume 79 March 1982 411-436

monomers spanning the membrane. Pores form when the electric field across the membrane is increased. As a probe, alamethicin can be used to detect surface charges and, in a general way, different types of lipid content.

Alamethicin can be incorporated into frog myelinated nerve membranes at the node of Ranvier (Cahalan and Hall, 1979) or into frog skeletal muscle fibers (Sakmann and Boheim, 1979). In this paper we report on the properties of alamethicin in frog nodes of Ranvier. To a first approximation, alamethicin behaves similarly in frog node or artificial lipid bilayers. The effectiveness and time-course of channel formation are similar in node or bilayer as is the single channel conductance. In the nodal membrane with calcium ions in the external solution and chloride as the internal anion, the alamethicin conductance turns on exponentially, reaches a peak, and then inactivates. Calcium-dependent inactivation and the observed asymmetry of the alamethicin current-voltage relationship both suggest that the membrane region probed by alamethicin has internal negative surface charges. A quantitative model for calcium entry and diffusion, presented in detail in the preceding paper (Hall and Cahalan, 1982), is applied to the node results and provides an explanation for the observed calcium-induced inactivation.

METHODS

Voltage-Clamped Node of Ranvier

Single bullfrog (*Rana catesbiana*) myelinated nerve fibers from the sciatic nerve were dissected and voltage-clamped by a triple vaseline gap technique (Frankenhaeuser, 1957; Dodge and Frankenhaeuser, 1958; Hille, 1971). Sintered Ag/AgCl electrodes, 2 mm in diameter × 4 mm in length, were used to record from the four fluid-filled pools by way of 1 M KCl agar bridges. A digital computer (Nova 3; Data General Corp., Westboro, Mass.) delivered voltage steps to the preparation through a 12-bit digital-to-analog converter (MN563; Analogic Corp., Wakefield, Mass.) and stored records of the ionic current on disk using a high speed data channel. Current could be sampled up to 250 kHz using a 12-bit analog-to-digital converter with sample and hold amplifier (DAS 250B; Datel, Mansfield, Mass.). Data were sampled at a four times faster rate for the first half of the trace than the second half, normally at 10-μs intervals for recording sodium currents and at 5-ms intervals for recording the slower alamethicin-induced currents. Fortran computer programs were used to control the data collection and to analyze and display stored records. The membrane current was recorded through the axoplasmic voltage drop from the node in pool A to pool E. The E pool voltage, V_E, was amplified 10- to 100-fold and low pass filtered. For records of single alamethicin channels, the current signal was filtered with a four-pole Butterworth lowpass filter (Datel FLT-U2, corner frequency 500 Hz), and tape recorded (Tandberg 115 FM instrumentation recorder, Tandberg Radiofabrikk, Oslo, Norway) with high and low gain channels. Current was calibrated with geometrical measurements of the fiber to determine the axoplasmic access resistance, R_{ED}, from the fiber diameter, d, and the fiber length, l, from the node to pool E, according to $R_{ED} = 4\rho l/\pi d^2$, assuming an axoplasmic resistivity, ρ, of 110 Ω cm (Stämpfli, 1952; Nonner et al., 1975; Conti et al., 1976). The resistance R_{ED} averaged 7.5 MΩ for the 20-μm-Diam fibers used. All experiments were done at 22°C. The initial resting potential was assumed to be −80 mV. The membrane potentials were calibrated by checking that the sodium channel steady-state inactivation (h_∞) had a value near 0.6 at −80 mV at

the beginning of the experiment, and that the final membrane potential at the end of the experiment was near 0 mV after breaking the membrane with large voltage pulses.

Solutions

In most of the experiments, the endogenous sodium and potassium conductances were blocked by cutting the internode in end pools containing isotonic cesium solutions, by bathing the node in isotonic cesium chloride plus calcium solution with 100 nM tetrodotoxin, and by holding the membrane potential at −20–0 mV. The composition of end pool (E and C) and external (pool A) solutions is given in Table I.

Three forms of alamethicin were used in the experiments: first, a fraction of natural alamethicin purified by Dr. B. F. Gisin; second, its methyl ester derivative; and third, a component called fraction 4 purified by Dr. T. M. Balasubramanian. Each of these alamethicin fractions was active when added to the external solution at 1–5 μg/ml. The alamethicin conductance is only weakly ion selective, with all cations listed in Table I being permeant. Cesium is the major current carrier in most of the experiments; in lipid bilayer membranes, cesium and potassium are 1.7 times more permeant than sodium ions through alamethicin channels.

TABLE I

SOLUTIONS

	Na	Cs	K	Ca	Cl	EGTA	PH
				mM			
External							
Na Ringer	120	—	2	1.8	126	—	7.4
Cs, 0.2 Ca	—	120	—	0.2	120	—	7.4
Cs, 2 Ca	—	120	—	2	124	—	7.4
Cs, 20 Ca	—	120	—	20	160	—	7.4
Internal							
CsCl	—	120	—	—	120	—	7.2
Cs_2 EGTA	—	160	—	—	—	80	7.2

External solutions were buffered with 5 mM MOPS; internal solutions were buffered with 5 mM HEPES.

The magnitude of background "leakage" currents before adding alamethicin was generally small enough to neglect. However, background currents were subtracted by comparing pulses before and after adding alamethicin, or by scaling the leakage current obtained during a pulse too small to turn on alamethicin channels.

Bilayer Experiments

Asymmetric lipid bilayer membranes were formed from monolayers by a modification of the Mueller-Montal technique as described in the previous paper (Hall and Cahalan, 1982).

RESULTS

Incorporation of Alamethicin into the Node of Ranvier

Alamethicin partitions into the node membrane from the external solution, forming conducting pores as detected by an increase in current magnitudes above background leakage levels in hyperpolarized (i.e., more negative inside)

potentials. Normally, current-voltage curves in the nearly symmetrical external Cs plus 2 mM Ca and internal CsCl solution are linear, with a membrane resistance of 20–40 MΩ. Fig. 1A illustrates current-voltage curves during a slow ramp change in potential before and 10 min after adding 5 μg/ml alamethicin (Ala) to the external solution. Curve *i* is the control *I-V*, with a node membrane resistance of 20 MΩ. Curve *ii*, with alamethicin present, deviates from curve *i* at about −20 mV and also at +70 mV, with increasing current induced by alamethicin at both hyperpolarized and depolarized voltages. After subtracting curve *i* from curve *ii*, the current component due to alamethicin is obtained. Hyperpolarizing the membrane turns on conducting alamethicin pores. For increasingly hyperpolarized voltages, the current increases exponentially with an approximately e-fold change in conductance per 10 mV. At depolarized voltages, the current induced by alamethicin is much more strongly dependent on potential. When examined during step changes in membrane potential, the alamethicin-induced current has a regenerative behavior discussed at the end of Results.

In most artificial lipid bilayer membranes, the alamethicin conductance varies steeply with voltage with about an e-fold change in conductance for a 4-mV change in potential. Fig. 1B illustrates a typical current-voltage curve for methylester alamethicin (MeAla) in a symmetrical egg lecithin membrane. 10 min after adding MeAla to one side only, current is observed for both positive and negative voltages and is nearly symmetrical around 0 mV, indicating nearly equal partitioning of MeAla at both membrane surfaces. Although lipid composition is known to alter the steepness of the alamethicin *I-V* relation somewhat (Donovan and Latorre, 1979), we attribute most of the differences between the node *I-V* curve at hyperpolarized voltages (e-fold per 10 mV) and the bilayer *I-V* curve (e-fold per 4 mV) to the presence of external calcium ions in the node experiments and to an internal negative surface charge, as discussed in the section on calcium-induced inactivation and in the preceding paper.

The incorporation of alamethicin into the nodal membrane appears to reach an equilibrium level after ~20 min. Upon washing away the alamethicin, the conductance disappears with a similarly slow time-course. These rates of equilibration are somewhat slower than in pure lipid bilayer experiments.

Single-Channel Properties

For low doses of alamethicin, a strong hyperpolarizing potential is required to activate the conductance. With 1–2 μg/ml alamethicin near the normal resting potential of −80 mV, fluctuations in current due to small numbers of conducting alamethicin channels can be clearly seen, and current through individual channels can be resolved. Fig. 2 A illustrates traces recorded at −90 mV after the addition of 1.5 μg/ml fraction 4 alamethicin. Step changes in current are observed corresponding to at least three conductance levels of approximately 90, 240, and 390 pS. There may well be other states of conductance below the resolution of these measurements. Thus in the Ranvier

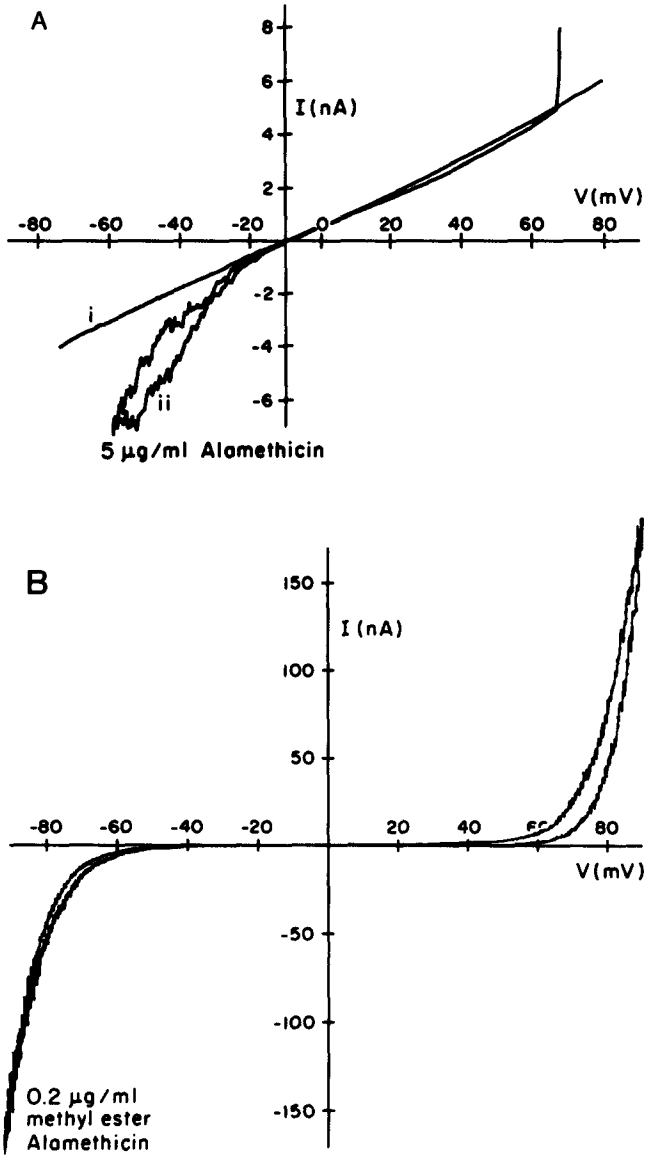


FIGURE 1. Alamethicin current-voltage relationships in node (A) and lipid bilayer (B) membranes. (A) The holding potential was −10 mV for a node bathed in CsCl plus 2 mM $CaCl_2$ with 120 mM CaCl internally. The membrane potential was varied at 5 mV/s first in the hyperpolarizing direction, then depolarizing. The records were taken with and without 5 µg/ml alamethicin in the external solution. (B) 0.2 µg/ml methylester alamethicin was added to the front compartment of a symmetrical egg lecithin membrane in 0.1 M KCl buffered to pH 7.0 with 5 mM HEPES.

node, as in artificial bilayer membranes and in rat skeletal muscle (Sakmann and Boheim, 1979), alamethicin forms multi-state pores of large dimension through discrete transitions. Alamethicin in bilayers expresses several characteristic conductance levels, each with a particular average lifetime. In the node, three conductance levels are easily observed, though the smallest level seen in bilayer membranes and in skeletal muscle with a patch electrode would probably be below the level of background noise. Thus, in the node, we

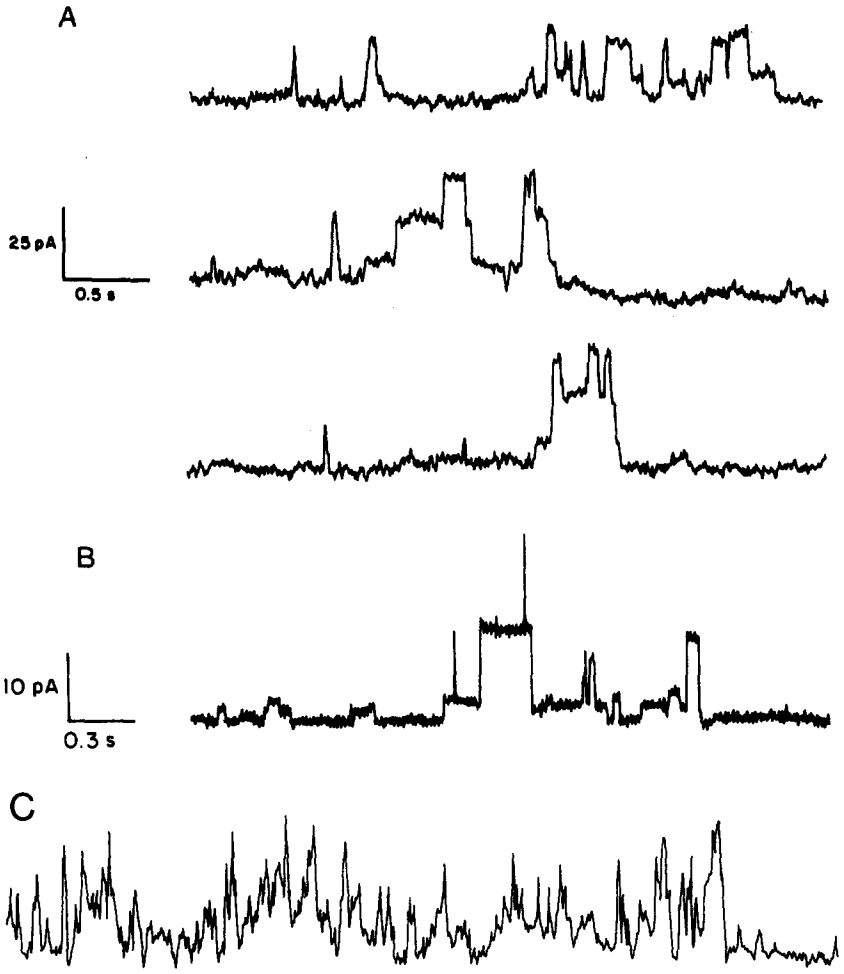


FIGURE 2. Alamethicin single channel records in node (A) and lipid bilayer (B) membranes. All records were taken at −90 mV membrane potential. (A) Node with 1.5 μg/ml fraction 4 alamethicin in CsCl plus 2 mM $CaCl_2$ and 80 mM Cs_2 EGTA internally. (B) PE bilayer with 0.087 μg/ml fraction 4 alamethicin. The solutions were internal and external node solutions described in the legend of Fig. 1. The alamethicin was added to the "external" solution side. (C) "Noisy" channels in node, same conditions and scales as in A.

may be observing levels 2, 3, and 4, or even 3, 4, and 5. By dividing the variance of alamethicin-induced conductance by the mean for higher levels of current, an estimate of the average single-channel conductance of ~0.6 nS was obtained.

Alamethicin channels have a longer open lifetime in the node than in lipid bilayer membranes. The average lifetime for the middle conductance level observed in the node is ~30 ms at −90 mV using fraction 4 alamethicin. Fig. 2B illustrates records at −90 mV in a PE membrane with fraction 4 alamethicin. In bilayer membranes formed from PE at −90 mV, the average lifetime is 15 ms. Thus in frog node, as well as in frog and rat skeletal muscle (Sakmann and Boheim, 1979), the open alamethicin channels last longer than in PE lipid bilayer membranes.

In the node membrane, as in lipid bilayer membranes, alamethicin sometimes behaves in a "noisy" manner, lacking clean open channel transitions. The channel conductance levels seem to be destabilized later in the experiment. Records of "noisy" alamethicin channels are shown in Fig. 2 C.

Kinetics and Dose-dependence of Channel Formation

Fig. 3 illustrates a family of voltage clamp steps for alamethicin in node and lipid bilayer membranes. In the node, hyperpolarization induces channel

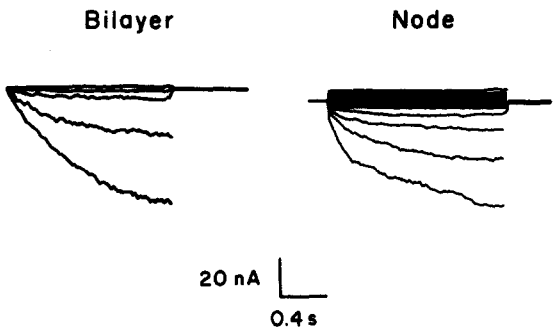


FIGURE 3. A comparison of alamethicin-induced current in a PE lipid bilayer and in a node. Membrane potentials ranged from −70 to +50 mV in 10-mV increments from a holding potential of 0 mV in the node with 2 μg/ml externally applied alamethicin. The node was bathed in 120 mM CsCl with 20 mM Ca and the internodal segment was cut in 120 mM CsCl. In the bilayer experiment, voltage pulses of 54, 60, 65, 72, and 76 mV were applied, with 0.89 μg/ml alamethicin in 120 mM CsCl plus 20 Ca.

formation with similar kinetics to the opening of channels in bilayer membranes. The voltage dependence is less steep in the node, and current-voltage properties will be discussed in the next section.

A detailed study of the concentration dependence of alamethicin in the node has not been attempted, although there is clearly a steep dependence of conductance on alamethicin concentration, as there is in bilayer membranes. The conductance induced by 2 μg/ml alamethicin in the node in similar in

magnitude to that in PE lipid bilayer membranes at the same membrane potential.

The general conclusion thus far is that alamethicin acts qualitatively the same in both node and artificial lipid bilayer membranes, forming a voltage-dependent conductance through discrete pores of large diameter. Two differences have been noted. In the node with internal chloride solutions, the conductance varies less steeply with membrane potential and the channel open lifetimes are several times longer than alamethicin channels in lipid bilayer membranes. The next section considers "inactivation" of the alamethicin conductance in the node, due to the entry and internal accumulation of calcium ions.

Ca-induced Inactivation of Alamethicin: Nodes with Internal Cl

During a hyperpolarizing pulse lasting several seconds, the alamethicin-induced current initially rises along an exponential time-course as channels form. In nodes with CsCl inside and Cs plus 0.2 mM Ca outside, the time constant for forming channels is ~0.3 s at −70 mV. Fig. 4 illustrates currents at −70 mV for three different external calcium concentrations. At 0.2 mM

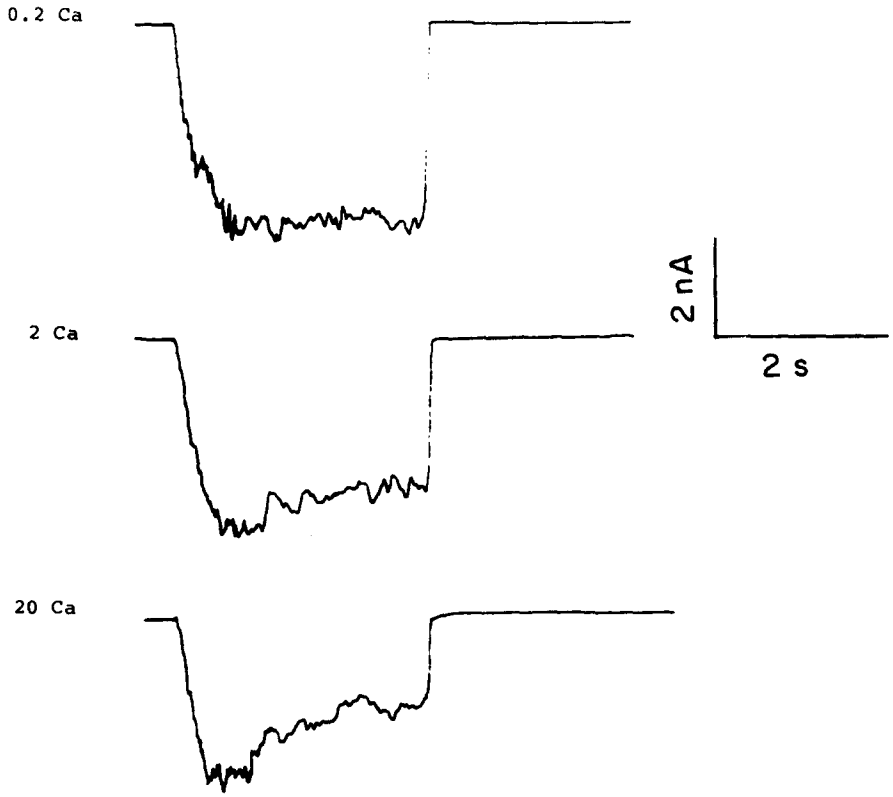


FIGURE 4. Alamethicin kinetics with 0.2, 2, and 20 mM Ca externally, each with 120 mM CsCl outside, and 120 mM CsCl inside three separate nodes.

Ca, the current rises and reaches a steady level. For 2 and 20 mM Ca, the current reaches a peak and then declines or inactivates. The degree of inactivation is sensitive to the external calcium concentration. Kinetic inactivation is never observed in 0.2 mM Ca, always observed with 2 mM Ca, and often, though not always, observed with 20 mM Ca. Fig. 5 illustrates currents during hyperpolarizing pulses to −60 and −70 mV. At −60 mV, the current levels out at roughly −2 nA. When the membrane is hyperpolarized to −70, the current reaches a peak and then inactivates. Thus, the degree of inactivation depends on the magnitude of the inward current, as well as the external calcium concentration.

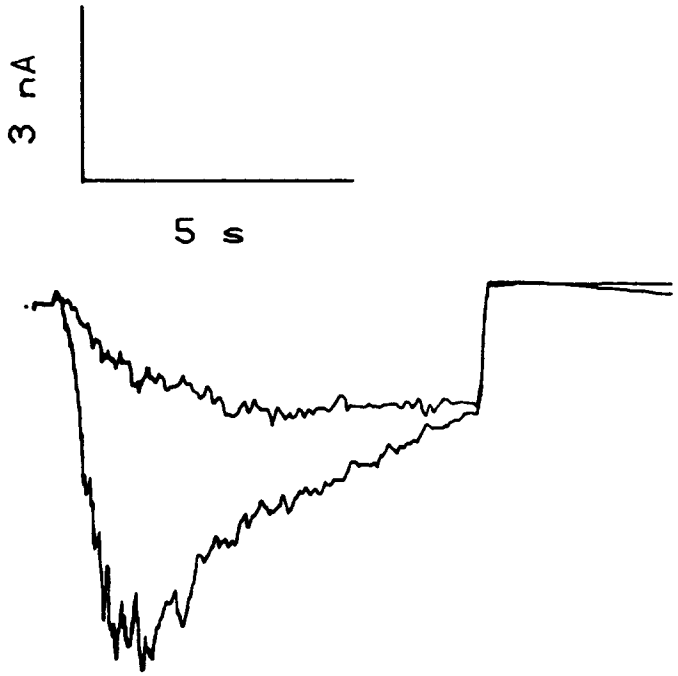


FIGURE 5. Alamethicin kinetics at two potentials. Node holding potential = 0 mV. Pulses are to −60 and −70 mV. 2 μg/ml methylester alamethicin in 20 mM Ca external solution, 120 mM CsCl inside.

External calcium alters the steepness of the current-voltage relationship for alamethicin, as well as modifying the kinetics during a voltage-clamp pulse. A family of voltage clamp pulses in 20 mM calcium is illustrated in Fig. 6A. Alamethicin-induced current is just barely visible for hyperpolarizing pulses to −30 mV in both 20 mM and 2 mM Ca solutions in this node; calcium ions seem not to alter the potential for activating conductance for a low density of channels. The fact that a 10-fold change in calcium concentration produces no shift in the potential to activate alamethicin conductance may be explained by postulating that the external surface potential is near zero. Although the currents do not appear to inactivate during the pulse, the current-voltage

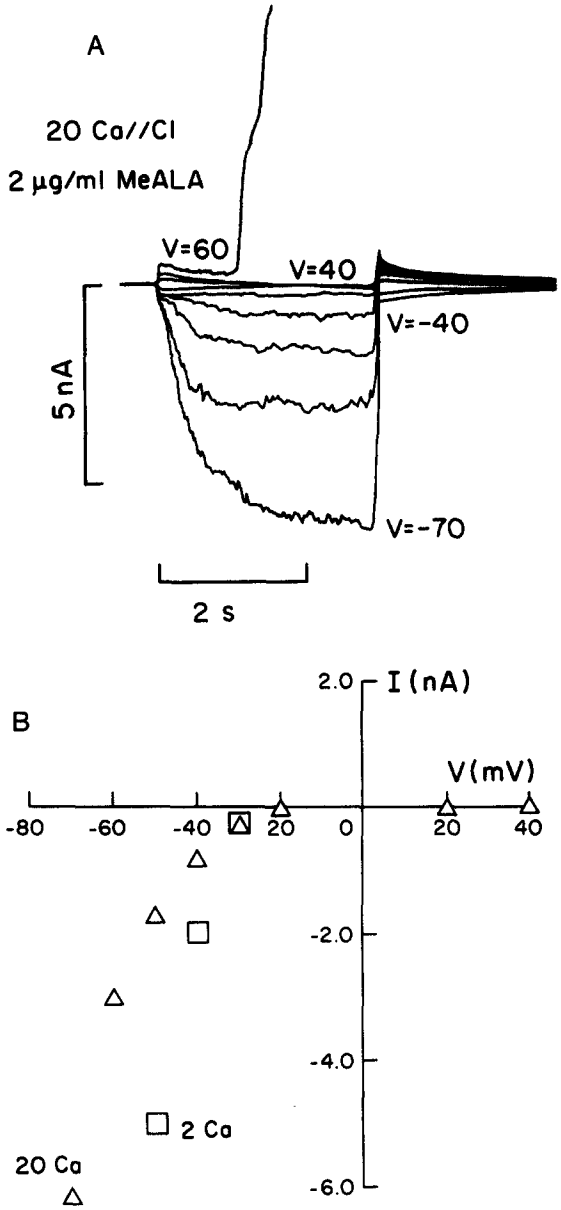


FIGURE 6. Current-voltage characteristics with chloride inside. (A) Voltage-clamp pulses were given from a holding potential of 0 mV to the potentials indicated, with 10-mV increments of potential. 2 μg/ml methylester alamethicin was present externally in 20 mM Ca solution, with 120 mM CsCl inside. At +60 mV, a regenerative current response occurred as described in Results. (B) *I-V* curve determined at the end of a 3-s pulse, with 2 μg/ml methylester alamethicin in 2 and 20 mM Ca external solution; 120 mM CsCl inside.

curve is less steep than in lower calcium media, which indicates that inactivation has occurred. Fig. 6 B illustrates the *I-V* curves determined at the end of 3-s pulses to varying potentials with 2 and 20 mM external calcium. In 20 mM Ca, the *I-V* curve is less steep. In this node, calcium ions appear to reach the site for inactivation (at the inner membrane surface) before the current reaches a peak value, resulting in a decrease in steepness of the current voltage curve. Recovery from calcium-induced inactivation is quite variable from node to node with half-times ranging from ~2 to 30 s, perhaps because diffusion of calcium is expected to be dependent on metabolically active processes, which could vary from fiber to fiber.

Internal EGTA Prevents Inactivation

In fibers with end pools containing 80 mM EGTA, a calcium chelating agent, inactivation of the alamethicin conductance is never observed, regardless of the external calcium concentration or current magnitude. A family of voltage-clamp current records is shown in Fig. 7 A. The current activates more slowly and is much more voltage-dependent than the records of Fig. 6 A with chloride inside the fiber. The current-voltage relation is illustrated in Fig. 7 B.

These results are consistent with the idea that calcium entry through the alamethicin pores and internal accumulation result in inactivation of the current in nodes with low calcium-buffering capacity. If the inner surface of the nodal membrane has negatively charged groups, the field within the membrane during a hyperpolarizing pulse will initially bias the channels toward pore formation, resulting in an inward current carried by Cs and Ca. Later on, as calcium enters through alamethicin channels and accumulates, the field sensed by alamethicin will decrease, tending to bias the channels to the closed position. Internal EGTA will bind calcium ions as soon as they enter, thereby preventing kinetic inactivation and steepening the voltage dependence.

Alamethicin as a Probe of Surface Potentials at the Node

The alamethicin conductance is extremely sensitive to membrane potential in both node and bilayer membranes. Alamethicin behaves as a dipole that senses the field within the membrane, and is therefore a sensitive probe for alterations in surface charge density. In lipid bilayer experiments, calcium ions resulted in a substantial shift in the alamethicin current-voltage curve in membranes containing negatively charged PS, but no shift in membranes formed from neutral PE (Hall and Cahalan, 1982). Because of shape changes in the *I-V* curve resulting from Ca-induced inactivation, the shift is most accurately measured for very low current densities to determine the "switching voltage," the potential that is just barely adequate to turn on a detectable number of channels. In the node of Ranvier, the switching voltage changed by less than 10 mV in six experimental determinations for a calcium concentration change of 0.2 to 2 mM in one experiment, and from 2 to 20 mM in five other experiments. When external calcium is varied, the *I-V* relationship

changes in steepness if chloride is the internal anion, but the switching voltage does not change, as illustrated in Fig. 6 B. Thus, alamethicin is not influenced by adjacent *external* negative surface charges in the node. The sodium channel has quite a different local surface potential than the region of membrane

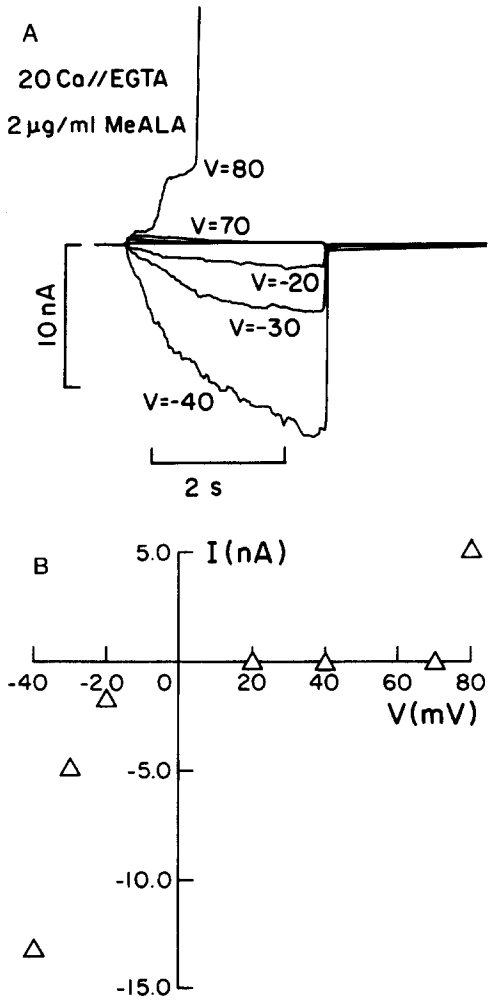


FIGURE 7. Current-voltage characteristics with EGTA inside. (A) Voltage-clamp pulses delivered from 0 mV holding potential in 10-mV increments. 2 µg/ml methylester alamethicin added to 20 mM Ca external solution, with 80 mM Cs_2 EGTA inside. A regenerative current response occurred at +80 mV. (B) *I-V* curve determined at the end of a 3-s pulse, same conditions as in A.

probed by alamethicin. The external surface charge density near sodium channels is about 1 e per 100–200 $Å^2$ (Hille et al., 1975); a 10-fold change in Ca concentration from 2 to 20 mM results in a 20–25 mV shift of the permeability-voltage relation.

Although the external surface seems to lack negative charges near alamethicin, two experimental findings argue for an *internal* negative surface charge. First, the inactivation induced by internal calcium accumulation can be accounted for by a negative surface potential. Qualitatively, the inactivation observed agrees with our model for alamethicin-induced calcium entry and binding to the inner surface charges (see Discussion) and can be mimicked in asymmetric lipid bilayer membranes with calcium ions on the side opposite to negatively charged PS (Hall and Cahalan, 1982). Second, the current-voltage curve with the methylester derivative is markedly asymmetric in the node, as shown in Fig. 7 B. The switching voltage in this node was +80 mV for turning on conductance with depolarizing pulses, but only −10 to −20 mV for turning on conductance with hyperpolarizing pulses. A similar asymmetry of the current-voltage relation was illustrated in Fig. 6 B for a node with internal chloride. In lipid bilayer membranes with methylester alamethicin added to one side, the current voltage curve is within 5 mV of being symmetrical (see Fig. 1 B), indicating that this derivative partitions nearly uniformly across the membrane. The observed asymmetry in the node *I-V* relation would indicate an excess of negative charge on the inner surface compared with external surface. The asymmetry of the methylester alamethicin current-voltage curve is 40–60 mV and allows us to estimate a difference in surface potential of 20–30 mV at the inner and outer membrane surface. Taken with the Ca-induced inactivation and the minimal effect of external calcium concentration on switching voltage, a negative surface potential of roughly 20–30 mV is indicated at the inner membrane surface, with very little external negative surface charge.

Regenerative Current Responses

For depolarized voltages, the conductance induced by alamethicin appears to be regenerative in the node of Ranvier. Fig. 8 illustrates currents during pulses to +60 and +70 mV in the presence of 2 μg/ml alamethicin. At 60 mV, only background leakage current is recorded, but when the voltage is increased by 10 mV, the current suddenly activates and rises out of control to a very high level. A large tail current after the depolarizing pulse is observed if a regenerative response is elicited. This tail current representing a leaky nodal membrane is often too large for the voltage clamp to maintain a constant membrane potential. Occasionally, the node recovers after a regenerative response and the experiment can continue.

The origin of the regenerative response for depolarizing voltages, also seen in Figs. 1 A, 6 A, and 7 A, is not certain, but the response is consistent with the internal negative charge postulated above. As the voltage is made sufficiently positive to turn on a small alamethicin conductance, calcium entry and subsequent reduction of the internal negative surface potential by calcium binding would alter the electric field across the membrane. This alteration would be of the same sense as an additional depolarization and would turn on additional alamethicin conductance accelerating in turn the rate of calcium entry. The depolarizing response is thus regenerative.

Unlike the inactivating hyperpolarizing response, the depolarizing regenerative response can occur even if the fiber ends are cut in 80 mM EGTA. Fig. 7A shows a regenerative response at +80 mV with internal EGTA. This makes qualitative sense because the hyperpolarizing response is self-limited, but the depolarizing response is inherently unstable and once initiated will continue until the calcium flux ceases. This instability virtually guarantees that calcium influx will overwhelm local internal calcium buffering. Additional possible explanations for the depolarizing regenerative response include incomplete

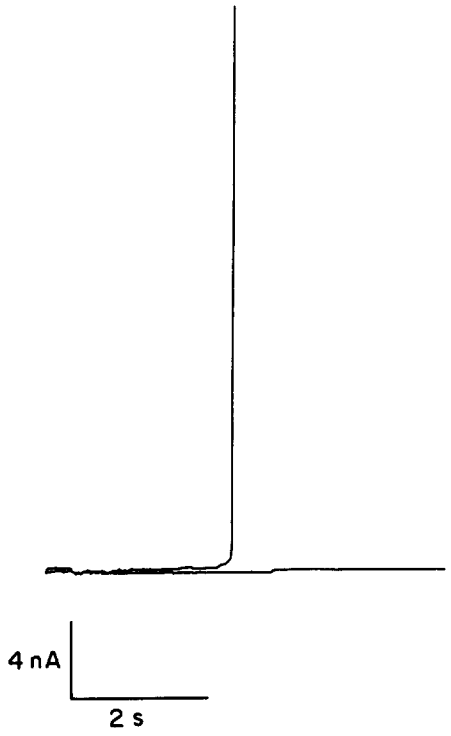


FIGURE 8. Regenerative current response at +70 mV with 2 μg/ml methylester alamethicin in 20 Ca external solution and 120 mM CsCl inside. Current at +60 mV shows no alamethicin activity. At +70 mV, the current becomes regenerative as soon as alamethicin activity begins.

equilibration of alamethicin at the inner surface of the membrane (see Schindler, 1979) or perhaps flip-flop of some lipid component induced by alamethicin leading to an increase in the field across the membrane (Hall, 1981). Regenerative responses are not observed for hyperpolarized voltages from 0 to about −100 mV. However, at more negative potentials, the current activated by alamethicin can also rise out of control. This response may represent membrane breakdown caused by large current magnitudes allowing calcium entry for several seconds.

Indirect Effect of Alamethicin on Sodium Channels

With a low concentration of alamethicin that turns on more negative than −80 or −90 mV, one can study the properties of endogenous sodium or potassium channels in the presence of nonconducting alamethicin. In this experiment, the fiber was cut in end pools containing isotonic CsCl and the node was bathed in normal Ringer solution. Fig. 9 A illustrates the steady-state voltage dependence of sodium inactivation before (open circles) and

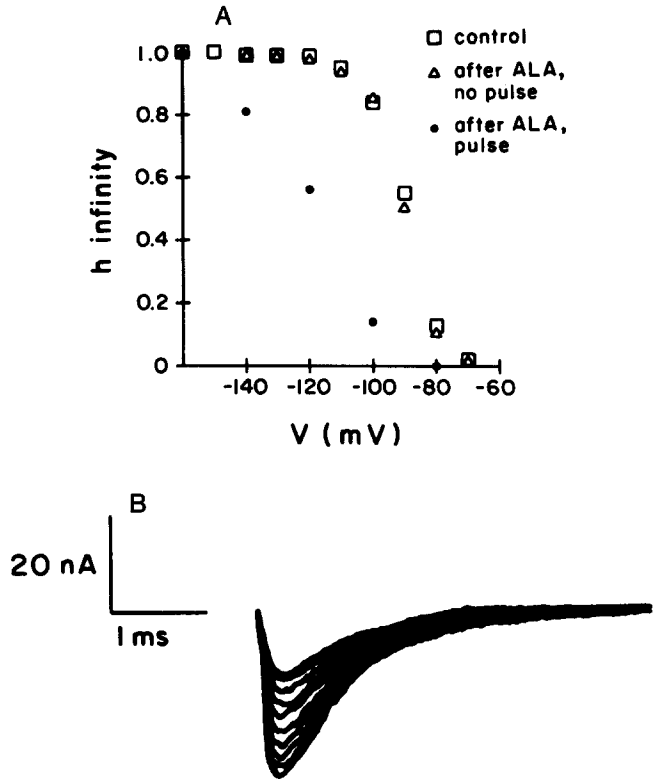


FIGURE 9. (A) Sodium channel steady-state inactivation (h_∞) curves before and after adding 1.25 μg/ml alamethicin to normal Ringer solution with 120 mM internal CsCl. The squares and triangles represent the h_∞ curve for the control and 10 min after adding alamethicin. Immediately after the second h_∞ curve was determined, a pulse to 110 mV lasting 3 s turned on an alamethicin conductance peaking at 10 nA. Then the h_∞ curve was determined again from the holding potential of −90 mV resulting in the shifted (•) curve. (B) Use-dependent block by QX314. 10 mM QX314 was added to normal Ringer solution with 1.25 μg/ml fraction 4 alamethicin. No effect of either agent on sodium channel kinetics was observed until a single pulse lasting 3 s to −110 mV activated 9 nA of inward current. The records show decreasing sodium currents during repetitive pulses to −10 mV at 2 Hz, applied immediately after the alamethicin-induced current.

after adding 1 μg/ml alamethicin. At the holding potential of −90 mV, ~3/4 of the sodium channels are available to be opened by brief depolarizing test pulses. Hyperpolarizing conditioning pulses lasting 50 ms remove inactivation, but do not result in alamethicin-induced influx. The presence of nonconducting alamethicin does not alter sodium channel properties. However, after activating alamethicin conductance and therefore calcium entry with a 3-s pulse to −110 mV, the steady-state inactivation curve is shifted toward more hyperpolarized potentials. This is consistent with a reduction of internal negative surface near the sodium channel gates, as a result of calcium influx induced by alamethicin.

Alamethicin can also be used to deliver normally impermeant drug molecules to the inside of the cell. Externally applied 10 mM QX-314, a charged lidocaine derivative, normally produces no effect on the sodium currents, but internal QX-314 results in accumulating "use-dependent" block of sodium current upon repetitive pulsing (Strichartz, 1973; Cahalan and Almers, 1979). The addition of external 1 μg/ml alamethicin plus QX-314 results in no alteration of sodium currents until a hyperpolarizing pulse is delivered to activate alamethicin conductance. Then, use-dependent block of sodium current is observed (Fig. 9 B), presumably due to entry of QX-314 through open alamethicin pores, resulting in axoplasmic accumulation of the drug.

DISCUSSION

Alamethicin as a Probe of Nerve Membrane Properties

Myelinated nerve fibers are specialized for rapid propagation of the action potential, with a high density of sodium channels at the nodes. There are $\sim 10^5$ sodium channels per node, each with a conductance of 7–8 pS, corresponding to an average density of ~2,000 per μm^2 (Conti et al., 1976; Sigworth, 1977). A similarly high number of potassium channels are present in frog node, though in mammalian fibers, potassium channels seem to be mainly present in the paranodal region (Chiu and Ritchie, 1980). There are very few sodium channels underneath the insulating myelin of the internode (Ritchie and Rogart, 1977). The mechanism for this uneven distribution of sodium channels in the membrane is not understood. Protein ionic channels must occupy a large fraction of the nodal surface. Nevertheless, alamethicin forms conducting channels presumably by incorporating into lipid bilayer regions of the node. The effectiveness of channel formation for a given concentration of alamethicin in either node or bilayer is similar, which suggests either that the protein does not represent a barrier to incorporation of conducting alamethicin pores in the node or that negative surface charges aid in pore formation in the node.

The properties of alamethicin in the node are similar though not identical to its behavior in artificial lipid bilayer membranes formed with a variety of lipid components. The conductance varies steeply with membrane voltage. In nodes with internal EGTA or low external calcium, conditions that avoid Ca-induced inactivation, the conductance varies e-fold in 4 mV, as it does in lipid

bilayer membranes. The kinetics of channel formation are similar in node and bilayer, with an exponential time-course requiring seconds after hyperpolarizing for a steady level in nodes with EGTA inside to prevent inactivation. Three conducting levels of individual alamethicin pores have been resolved in the node, where in bilayer membranes there are least seven levels, some of which are too small to resolve with the node voltage clamp. Qualitatively, then, alamethicin works in the node as it does in lipid bilayer membranes.

Alamethicin reports changes in membrane surface potentials. In asymmetric lipid bilayer membranes with negatively charged phosphatidyl serine on one surface and neutral phosphatidyl ethanolamine on the other surface, the alamethicin current-voltage curve is markedly asymmetric, even using the methylester derivative of alamethicin which forms nearly symmetrical *I-V* curves in symmetrical membranes. The *I-V* curve for alamethicin in the node is asymmetric; larger depolaring pulses are required to activate alamethicin conductance than hyperpolarizing pulses. This asymmetry averaged −50 mV in five determinations, and tended to be somewhat larger in nodes with internal EGTA.

The asymmetry of the alamethicin *I-V* curve could be explained in two ways. First, the methylester alamethicin may not equilibrate at the inner surface of the node, and therefore would require a larger field across the membrane to activate conductance in the depolarizing limb of the *I-V* curve. Arguing against this idea, however, is the finding that in symmetrical lipid bilayer membranes, the methylester derivative of alamethicin induces a symmetrical *I-V* curve (see Fig. 1B). Second, the asymmetry may be due to a difference in surface and/or dipole potentials between the inner and outer surface of the node membrane. An internal negative surface potential difference of −20 mV to −30 mV with no external surface potential could account for the *I-V* asymmetry. The second hypothesis is favored by our result with changes in the external calcium concentration. In lipid bilayer membranes, calcium shifts the alamethicin *I-V* curve when added to a membrane with PS, but produces no shift when added to a neutral PE membrane (Hall and Cahalan, 1982). In the node of Ranvier, the switching voltage to activate a just-detectable alamethicin conductance does not shift noticeably with a 10-fold change in external calcium. This would indicate a virtual absence of negatively charged lipid at the external surface near alamethicin. Furthermore, calcium results in a time-dependent inactivation of alamethicin during a maintained hyperpolarizing pulse, provided internal Ca chelating agents are not present. We believe that the inactivation is due to a reduction in the internal surface potential during a pulse as calcium ions accumulate.

Fig. 10 summarizes our view of the surface potential profile for lipid regions of the node of Ranvier. The solid line shows the hypothetical potential sensed by the sodium channel gating mechanism for a membrane potential of −75 mV, with the external surface potential more negative than the inside (Hille et al., 1975). The proposed surface potential profile sensed by alamethicin is represented by the dashed line, with the inner surface more negative. The field within the membrane is much higher in the lipid region than near the

sodium channel, due to the asymmetric distribution of negative charges. The identity of this charged component is not known. Measurements in the node using the lipophilic anion, dipicrylamine, as a probe also indicate an asymmetry of surface charges with a zero-field membrane potential of +30 mV (Benz and Nonner, 1981).

Lipids are known to be distributed asymmetrically in disc membranes of vertebrate rod outer segments, in red blood cell membranes, and in other types of membranes. Usually the cytoplasmic surface has a predominance of phosphatidylserine, which would contribute a bias to the field across the membrane, similar to the node (Op den Kamp, 1979). Interestingly, the sodium channel has a surface potential bias in the opposite direction (see Hille et al., 1975 and Fig. 10). The sodium channel through negatively charged

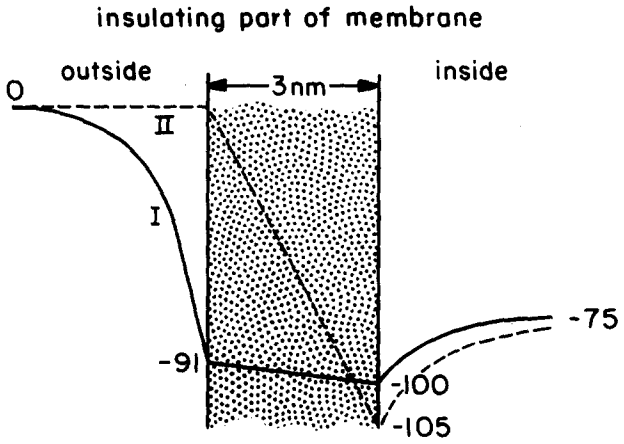


FIGURE 10. Hypothetical surface potential profile near sodium channel (solid line) and near alamethicin channels incorporated into the node (dotted line). The surface potentials that alter sodium channel gating properties were taken from Hille et al. (1975, Model I, Fig. 8), with the external surface charge density larger than the inside. The alamethicin surface charge profile is asymmetric with the inner surface more negative.

protein groups or perhaps specific boundary lipids, has a voltage dependence for gating shifted to more negative potentials, making it easier for the membrane potential to reach threshold for the action potential.

In comparing the behavior of the probe in biological membranes and lipid bilayer membranes, the presence of negative surface charges must be taken into account. For example, the effective field biasing the alamethicin conductance at a membrane potential of −90 mV would be −120 mV if there is a negative internal surface potential of −30 mV. The added bias would lengthen the open time for conducting channels and tend to make alamethicin more effective for a given aqueous concentration. The unusually long open-channel time in both skeletal muscle (Sakmann and Boheim, 1979) and nerve (see Fig. 2) at −90 mV should be compared with bilayer results at a more

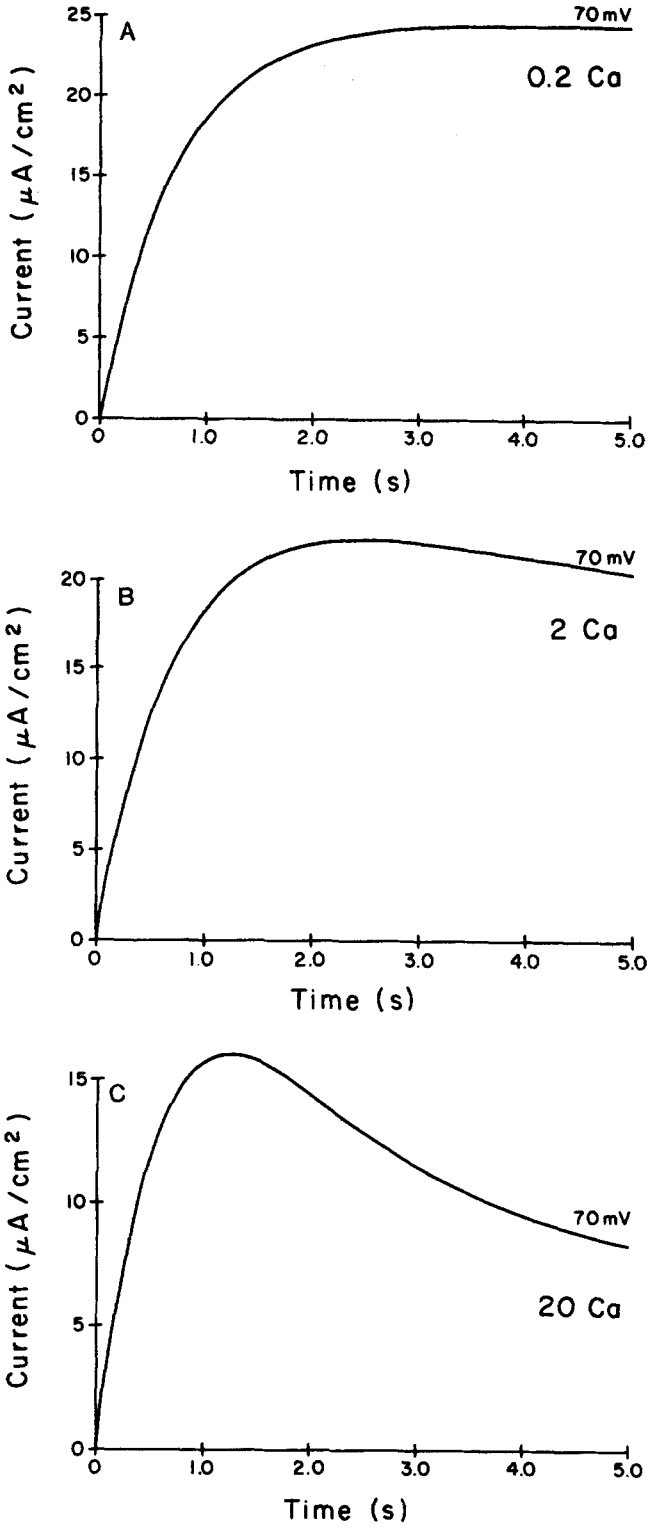
hyperpolarized potential. Then, the discrepancy between the channel lifetimes in biological and lipid bilayer membranes would be reduced because channels tend to remain open longer for stronger fields across the membrane. Thus, surface potentials in the node could account for some of the lengthening of channel lifetimes in biological membranes compared to lipid bilayer membranes. The presence of cholesterol may also contribute to longer channel lifetimes (Latorre and Donovan, 1980). If we attempt to correct the dose-response properties of alamethicin in node for an internal negative surface potential, we come to the tentative conclusion that a major fraction of the nodal area is not available for alamethicin to form channels. This conclusion would agree with the results of Benz and Nonner (1981), who found ~10% of the expected current carried by the lipophilic anion, dipicrylamine, in the node of Ranvier.

Ca-induced Inactivation

The decline in alamethicin-induced current during a maintained hyperpolarizing pulse (Figs. 4 and 5) is due to calcium entry through open alamethicin channel and accumulation inside the node. The degree of inactivation is modulated both by external calcium concentration (Fig. 4) and by the internal calcium buffering capacity of the axoplasm (Figs. 6 and 7). We can account for this inactivation of alamethicin-induced conductance by postulating the presence of negative surface charges on the inside of the membrane to which calcium ions may adsorb and/or bind. A quantitative model for this mechanism applicable to lipid bilayers is presented in the preceding paper (Hall and Cahalan, 1982). The model takes into account calcium diffusion within unstirred layers on either side of the membrane, calcium flux through conducting alamethicin channels and calcium binding to negative surface charges. Normally the calcium concentration inside the node is probably very low, perhaps 10^{-7}–10^{-9} M, resulting in near maximal negative surface potential. When alamethicin channels open in response to a change in membrane potential, calcium ions enter the node through the open alamethicin channels. Inside the node, the calcium ions bind to negative surface charges, reducing the electric field across the membrane and inactivating the alamethicin conductance induced by hyperpolarizing potentials.

The bilayer model, although qualitatively consistent with our observations in node, must be modified to give the proper degree of inactivation at nodal current densities. We have found two plausible modifications of the model that give adequate quantitative agreement. The diffusion coefficient inside the node can be reduced by two orders of magnitude in accord with estimates of calcium diffusion in axoplasm (Baker et al., 1971), or a calcium-reflecting barrier can be put ~4 μm away from the membrane to approximate the nodal geometry. Both these procedures increase the amount of calcium accumulated at the surface for a given calcium influx. With the calcium-reflecting barrier, the degree of inactivation in the model is far less sensitive to changes in calcium diffusion coefficient than in its absence.

The predictions of the model for a surface charge density of 1 electronic


A
70 mV
0.2 Ca
Current (μA/cm²)
Time (s)
B
70 mV
2 Ca
Current (μA/cm²)
Time (s)
C
70 mV
20 Ca
Current (μA/cm²)
Time (s)

charge every 500 Å^2 (corresponding to a surface potential of −30 mV) are shown in Figs. 11 and 12. Fig. 11 shows the degree of inactivation at three different calcium concentrations (cf. Fig. 4 for corresponding node results). In both the model and the node, inactivation is more pronounced at higher external concentrations, a consequence of more rapid calcium entry.

Fig. 12 shows the degree of inactivation predicted for two different membrane potentials −60 and −70 mV (cf. Fig. 5 for node data). With 20 mM external calcium, the calcium concentration near the membrane surface (but far enough away to be unaffected by the negative charge) rises rapidly in the model, reaching ~0.15 mM at the pak of the alamethicin-induced conductance and reaching 0.4 mM after 5 s. The calcium concentration at the membrane surface would be ~10 mM because of the negative surface charge.

Similar inactivation has been demonstrated in lipid bilayers. Alkylammonium derivatives can press through the monazomycin channel, and by binding to the membrane alter the surface potential (Heyer et al., 1976). Local anesthetics and pancuronium pass through the alamethicin channel and alter surface potential again by binding to the surface (Donovan and Latorre, 1979). Our proposed mechanism for inactivation is similar to inactivation by these hydrophobic compounds, but requires the presence of negative surface charges that can bind calcium.

It seems possible that this type of internal surface charge-calcium ion interaction could have important physiological consequences in regulating membrane excitability, neurosecretion, and other functions where calcium may have a role in intracellular signalling. Voltage-dependent conductances at nerve terminals or rod outer segments, for example, would be altered when the local internal calcium concentration changed as a result of activity. A requirement for this mechanism to operate is a sufficiently rapid calcium entry or release from an internal store into a limited volume to allow calcium to accumulate near a membrane with negative surface charges.

Endogenous voltage-dependent calcium channels have been studied using voltage clamp techniques in squid presynaptic terminals (Llinas et al., 1976), molluscan neurons (see review by Adams et al., 1980), skate electroreceptors (Clusin and Bennett, 1977), skeletal (Almers and Palade, 1981) and cardiac

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FIGURE 11. (*opposite*) Simulations of calcium-induced inactivation generated by the model described in the companion paper (Hall and Cahalan, 1982). The surface charge inside the node is set at 0.0020 electronic charges/Å^2. The outside diffusion constant of calcium is 6.2×10^{-6} cm^2/s. The inside diffusion constant is 6.2×10^{-8} cm^2/s. The unstirred layer thickness is 50 μm. The calcium-reflecting barrier is 10 μm from the inside membrane surface. We have used 12 liters/mol as the binding constant, the value for calcium binding to phosphatidyl serine (McLaughlin et al., 1980). The alamethicin conductance and time constant were adjusted for best fit, but the voltage dependence of the alamethicin conductance parameters was determined by measurement and was such that an e-fold rate of change in initial conductance occurred for a 6.9-mV voltage change and an e-fold change in time constant occurred for a 10-mV voltage charge. External calcium varied from 0.2 (A) to 2 (B) to 20 mM (C).

muscle (see review by Reuter, 1972), and neuroblastoma cells in culture (Moolenaar and Spector, 1979). Because of the difficulty in separating the many components of ionic current sometimes including calcium-activated

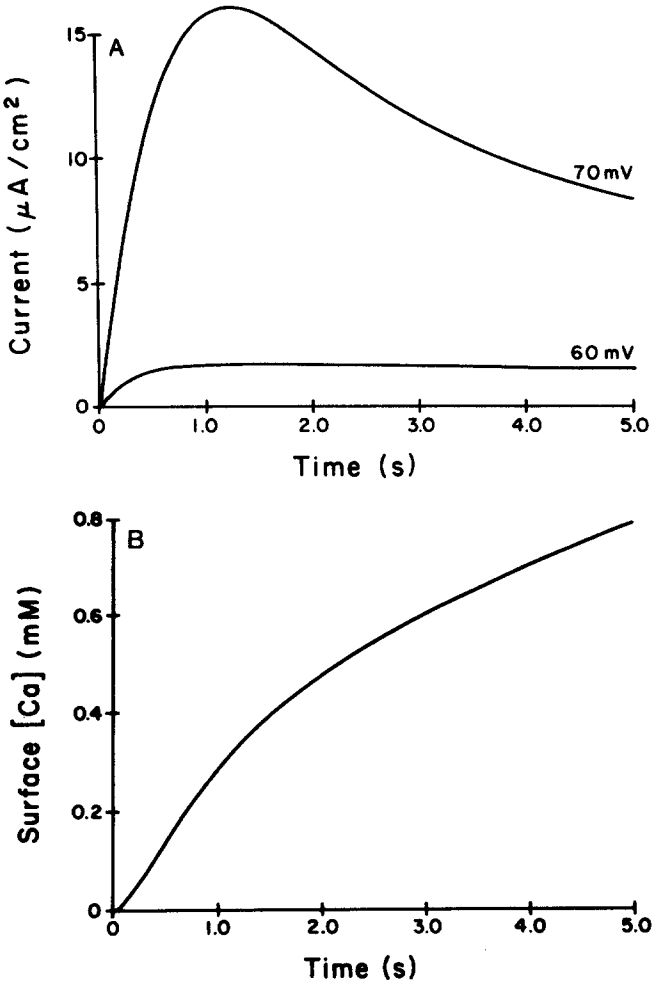

FIGURE 12. (A) Simulations of calcium-induced inactivation for voltage pulses of 60 and 70 mV. The parameters are the same as those of Fig. 11, except that the alamethicin concentration and time constant were charged to give the best fit to experimental data. (B) Plot of the dependence of calcium concentration, several debye lengths from the interior aspect of the membrane as a function of time for a 70 mV voltage pulse applied at $t = 0$. The parameters match those in A. The concentration near the charges would be about 10 mM, taking into account the 30-mV surface potential due to the surface charge.

potassium currents in these preparations, the kinetics of calcium channel gating remain controversial. Inactivation of calcium current has been described in some preparations, but has been found to be slower or incomplete

in cells that have been injected with calcium chelating agents (see Adams et al., 1980). Indeed, inactivation of calcium channels may depend directly upon calcium entry and internal accumulation (Tillotson, 1979). The surface charge mechanism that we propose for the inactivation of alamethicin-induced conductance in nodes could not account for calcium channel inactivation by shifting the voltage dependence for the activation gates of calcium channels. Rather, internal accumulation of calcium would enhance activation, because calcium binding to an internal negative surface charge would mimic further depolarization. In this way, the activation curves for calcium channels could be steepened as a result of calcium entry. However, if there is an endogenous voltage-dependent inactivation gate that closes the calcium channel during prolonged depolarization, its action would be enhanced by the surface charge mechanism we describe. Recovery from inactivation of calcium channels has a slow component lasting seconds (Adams and Gage, 1979; Tillotson and Horn, 1978); perhaps this component represents unbinding of calcium and diffusion away from a charged membrane component that modulates inactivation.

Potential Applications of Alamethicin in Cell Membranes

We have shown that alamethicin provides a useful probe to investigate membrane surface charges. Charge asymmetries in lipid bilayer regions can be revealed from the current-voltage characteristics of alamethicin. As more is understood about the influence of different lipid composition on the alamethicin channels, alamethicin will be used to determine the nature of lipid environments surrounding ionic channels. These studies could be of use in selecting a membrane for reconstitution of isolated channels into lipid bilayers. Alamethicin channels have a prolonged lifetime in both nerve (see Fig. 2) and muscle (Sakmann and Boheim, 1979) membranes. In part, this may be due to negative surface charges on the inside of the membrane, biasing channels on. The long channel lifetimes may also be due to a particular phase for lipids surrounding channels or perhaps a requirement for cholesterol or some other lipid constituent that helps to stabilize conducting channels. The sodium channel has a bias potential opposite to that which alamethicin senses. The increased external negative surface charge density near sodium channel gates could indicate a requirement for particular negatively charged boundary lipids to surround the sodium channel or it could represent the existence of numerous acidic groups on the channel protein. Tetrodotoxin binding sites isolated from eel are highly acidic proteins with a requirement of specific lipids for stability (Agnew and Raftery, 1979). The chemical nature of surface charges that influence sodium channel gating is still unknown.

Alamethicin should also be useful as a means of loading cells with interesting substances under membrane potential control. The open alamethicin channel is a very large pore through which monovalent cations, divalent cations, local anesthetics, lipids, and even ATP can permeate. We have shown that calcium ions and a charged local anesthetic derivative can be introduced inside the node through alamethicin channels. The ionophore nystatin has been used to

replace internal potassium with cesium in molluscan nerve cell bodies (Russel et al., 1977; Tillotson, 1979). Alamethicin could provide certain advantages over nystatin, because its conductance is voltage-dependent and activates during long duration hyperpolarizing pulses. With a low dose of alamethicin (1 μg/ml in frog node) the resting potential would be normal and alamethicin would be nonconducting over the depolarized range of membrane potentials. Alamethicin could then be used to deliver calcium, monovalent ions, or metabolically active compounds to the inside of cells. There has been considerable interest in treating cells with vesicles or lysolecithin to introduce substances into cells (Baserga et al., 1979). Alamethicin provides an alternative approach: a voltage-dependent conductance activated by membrane hyperpolarization.

We would like to thank Drs. F. Bezanilla, J. Vergara, and J. Fernandez for advice on the computer interface, and T. Boles for technical assistance. We are indebted to Mary Ann Tacha and Karen Jessup for secretarial assistance and to Maureen Killackey for several illustrations. Supported by National Institutes of Health grants NS 14609 and HL 23813 and by Research Career Development Award HL 00579 to J.E.H.

Received for publication 20 August 1981 and in revised form 30 November 1981.

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