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Voltage-gated channels formed in lipid bilayers by a positively charged segment of the Na-channel polypeptide

(integral membrane proteins/voltage-dependent channels/amphipathic peptides/planar bilayers)

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ABSTRACT **The Na-channel polypeptide is responsible for the voltage-gated and time-dependent ionic permeability changes that give rise to the action potential in the membranes of nerve cells. We have synthesized a 22-amino acid peptide with a sequence identical to that of the segment named S_4, repeat IV of the primary structure of the Na channel. We have found that this peptide induces a voltage- and time-dependent conductance in bilayers formed by a mixture of phosphatidylethanolamine and phosphatidylserine. This conductance is activated when the cis side is made positive, with an apparent gating charge of 3. The results are consistent with the idea that this segment plays a role in determining the voltage sensitivity of the Na channel.**

We have shown previously that certain L-amino acid peptides, which are long enough to traverse the membrane only once, can self-aggregate to form ion-permeable channels in bilayers (1–3). We have suggested that the capacity of peptides to assume amphipathic conformations allows them to self-aggregate with hydrophilic residues facing inward, to form the walls of the channel, and hydrophobic side chains facing outward, to interact with the hydrocarbon chains of the phospholipid molecules in the interior of the bilayer. Similar suggestions have been made about the probable conformation of putative intramembrane segments of channel-forming integral membrane proteins (4–6). Based on results of our work with melittin and its analogues, we have put forth the hypothesis that the voltage dependence of channels formed by peptides depends on the presence of positive charges on the side chains of some of the amino acid residues. Thus, when the primary sequence of the Na-channel protein was determined, our attention was drawn to the S_4 segment, which is highly positively charged and potentially amphipathic. S_4 is one of six segments (S_1–S_6) present in each of the four homologous domains of the Na-channel polypeptide. Moreover, it has been suggested that this segment is somehow involved in the voltage gating of the Na channel (4, 7, 8). We decided to synthesize this segment, which has the following structure: Arg-Val-Ile-Arg-Leu-Ala-Arg-Ile-Ala-Arg-Val-Leu-Arg-Leu-Ile-Arg-Ala-Ala-Lys-Gly-Ile-Arg. Noticeable is the fact that this segment is highly polar; it contains seven arginine and one lysine residues, each located at every third position, and the intervening residues are nonpolar. Assuming that this segment forms a 3_{10}-helical structure, these charged residues would lie on a straight line parallel with the long axis on one side of the helix, making the segment strongly amphipathic. Moreover, since the distance per residue along the axis of the helix would be about 0.15 nm, the 22-amino acid segment would be ≈3.3 nm long, which is sufficient to traverse the hydrocarbon core of lipid bilayers. In what follows, we show that the peptide incorporates into lipid bilayers and forms cation-selective channels, which have voltage-dependent characteristics.

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MATERIALS AND METHODS

Materials. Phosphatidylserine and phosphatidylethanolamine were obtained from Avanti Polar Lipids. All other chemicals were analytical grade and were purchased from Baxter (Pittsburgh).

Peptide Synthesis. The peptide was synthesized by the solid-phase method using the *tert*-butyloxycarbonyl/benzyl strategy for weak acid removal of the α-amino protecting group and strong acid removal of side-chain protecting groups (9). The peptide was purified by a preparative reversed-phase HPLC with a linear gradient of 30–43.5% acetonitrile in water containing 0.1% trifluoroacetic acid on a Waters μBondapak C_{18}, and its purity was determined through amino acid composition.

Bilayers and Peptide Incorporation. Bilayers were made by apposition of two monolayers made from phosphatidylethanolamine/phosphatidylserine, 1:1 (wt/wt) (10) in the presence of 0.5 M NaCl buffered to pH 7.4 with 0.01 M Tris/Mops. The determination of the electrical properties of the bilayers and the analysis of the results were done as described (3). The 22-mer peptide was dissolved in water, and an aliquot was added to one side of a preformed bilayer with stirring.

RESULTS AND DISCUSSION

The current response to a fixed voltage after addition of the peptide to one side of a lipid bilayer is shown in Fig. 1. In Fig. 1*A*, at a peptide concentration of 80 nM, 10–45 min after the addition of the peptide to the cis aqueous solution, there was an increase in the conductance to 300 pS with concomitant fluctuations of 200 pS (Fig. 1*A*, traces 1 and 2). Increasing the peptide concentration to 200 nM and to 400 nM (Fig. 1*A*, traces 3 and 4 and traces 5 and 6, respectively) increased the probability of the open state (cf. Fig. 1*B*).

The fraction of time that the 500-pS channel spends in the open or in the closed state is not affected by voltage, and the histograms of dwell times can be fitted by one exponential at voltages less than 40 mV (a fit to two exponentials is necesary at higher voltages, suggesting that there are more than one open and one closed states; see below). As illustrated in Fig. 1*C*, the time constant for the open state increases from 8 ms (at 80 nM) to 20 ms (at 400 nM), whereas the time constant for the closed state decreases from 10 ms to about 1 ms.

The conductance induced by the peptide closes at negative voltages (Fig. 2). Maintaining the voltage on (for about 3 min after the conductance turns off), turning the voltage off, or going to a lower, negative voltage fails to reopen the conductance (data not shown). Application of a positive voltage does reopen the channel as indicated in Fig. 2. We have not observed closures of the type depicted at negative voltages when we applied positive voltages of the same magnitude and

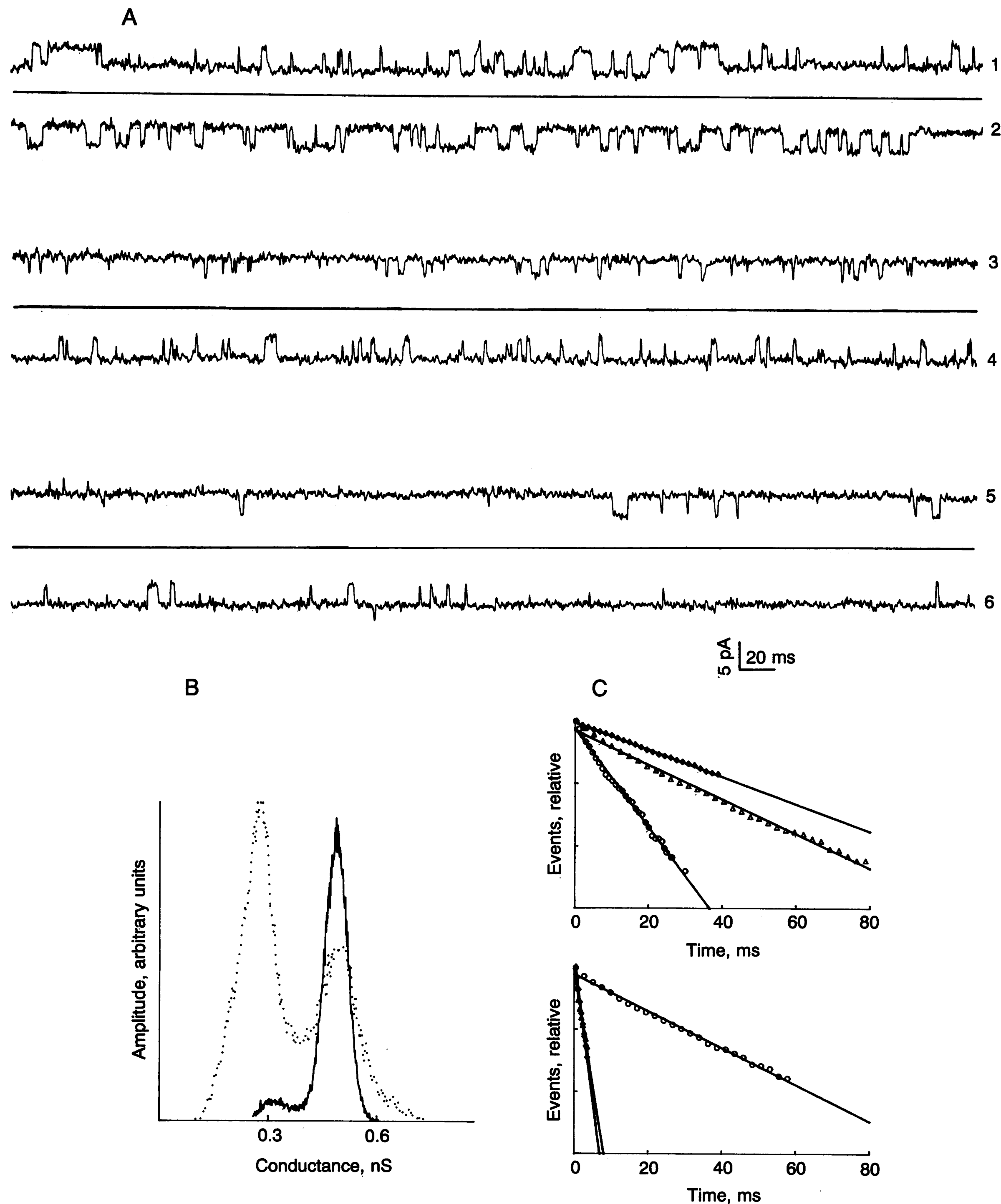

FIG. 1. Concentration dependence of the kinetic characteristics of the single-channel conductance. (*A*) Single-channel records at V = +20 mV (traces 1, 3, and 5) and at V = −20 mV (traces 2, 4, and 6) in the presence of 80 nM (traces 1 and 2), 200 nM (traces 3 and 4), and 400 nM (traces 5 and 6) peptide added to the aqueous solution bathing one side of a lipid bilayer. The continuous line indicates the level of the current at zero applied potential. (*B*) Single-channel conductance histograms corresponding to the data partially shown in traces 1 and 2 and also traces 3 and 4. The origin of the abscissa was set to correspond to zero voltage, zero current. · · ·, 80 nM peptide; —, 200 nM peptide. (*C*) Dwell time distributions for the data partially depicted in *A*. (*Upper*) Open dwell time. (*Lower*) Closed dwell time. Solid lines are the least-squares fit to the points. Dwell times for peptide concentrations of 80 nM (○), 200 nM (◇), and 400 nM (△) are shown.

for the same amount of time. Shown also in Fig. 2 is the fact that when the 500-pS open state closes at negative voltages, it does so to a state with a conductance of about 70 pS, as shown in Fig. 3*B*.

The results are consistent with a picture of a channel with three open states (conductances of 500, 300, and 70 pS). Fluctuations between the 500-pS and 300-pS states and between the 70-pS state and the closed state are rapid (in the millisecond range), whereas fluctuations between the 500-pS or the 300-pS states and the closed states are infrequent. It is possible that these different conductance states are due to different quaternary structures of the S_4 aggregates, as has been suggested recently for short- and long-lived gramicidin channels (11) or that each of the states is due to a different aggregation number.

Increasing the peptide concentration to 1000 nM results in a multichannel conductance that is voltage- and time-

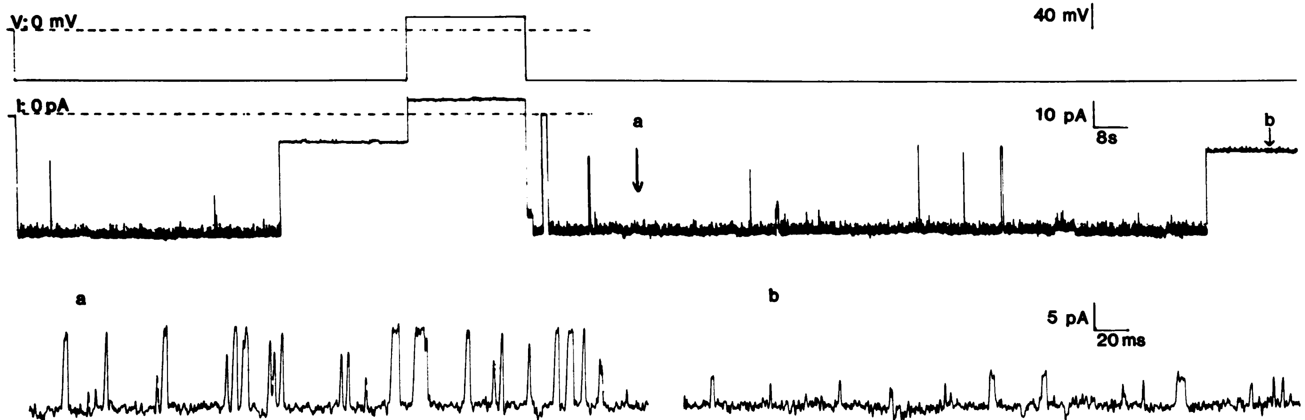

FIG. 2. Voltage dependence of the single-channel conductance. The top trace in the figure indicates the time course of the applied voltage (V). The position of zero voltage is indicated by the broken line. The middle trace is the current (I) response to the voltage pattern depicted in the top trace. The current level that corresponds to zero applied voltage is indicated by the broken line. The two traces at the bottom of the figure correspond to segments a and b in the middle trace, shown at higher time and amplitude resolution (upward deflection corresponds to channel closing). The peptide concentration was 30 nM.

dependent, as shown in Fig. 3. The conductance for positive potentials increased *e*-fold for every 10-mV increase in potential, indicating that there are three apparent gating charges per conducting unit. The conductance at negative voltages is independent of voltage, within the range studied.

We have studied the ion selectivity of the conductive pathway both by increasing the salt concentration on the trans side of the bilayer through addition of an aliquot of concentrated solution or by complete replacement of the solution in this compartment. We have found (to our surprise) that the pathway is selective for Na^+ over Cl^- ($P_{Na}/P_{Cl} \approx 6$) and is slightly more permeable to K^+ than to Na^+ ($P_K/P_{Na} \approx 1.2$). These results could be due to the binding of the permeant anion to the positively charged side chains, thus playing a role in the process of permeation of cations through this channel. Alternatively, the results could also be explained if the phospholipid headgroups themselves act as counterions for the side chains, with the hydrocarbon chains forming an integral part of the channel walls. The data presented here indicate that the segment S_4 from the linear sequence of the Na channel, predicted to be associated with the voltage-gating of the Na channel (4, 5, 7, 8), forms voltage-dependent channels in lipid bilayers. The peptide seems to form a channel with three open states (70-, 300-, and 500-pS conductance) with lifetimes in the range of those of the Na channel. The values for the single-channel conductance are higher than those found by Oiki *et al.* (12) for a voltage-independent channel formed in bilayers by a synthetic peptide with a sequence corresponding to that of segment S_3, repeat I of the rat brain protein (4, 8).

The voltage-dependent conductance that the S_4 segment induces in lipid bilayers (Fig. 3) increases *e*-fold for every 10 mV, a value similar to that obtained by Stimer *et al.* (13) for the voltage-dependence of the fraction of open channels in squid giant axon.

The work presented in this communication, as well as that of Oiki, has shown that amphipathic segments from the primary structure of the Na channel, which have been postulated to form part of the Na-channel lining, incorporate and form channels in lipid bilayers. Furthermore, the cationic segment that we have synthesized and incorporated into lipid bilayers forms voltage-gated channels and thus joins the ranks of other cationic, amphipathic peptides that also induce voltage-dependent, ion-permeable pathways in lipid bilayers.

Clearly more work needs to be done in order to be able to elucidate the functional significance of the various segments of channel-forming proteins.

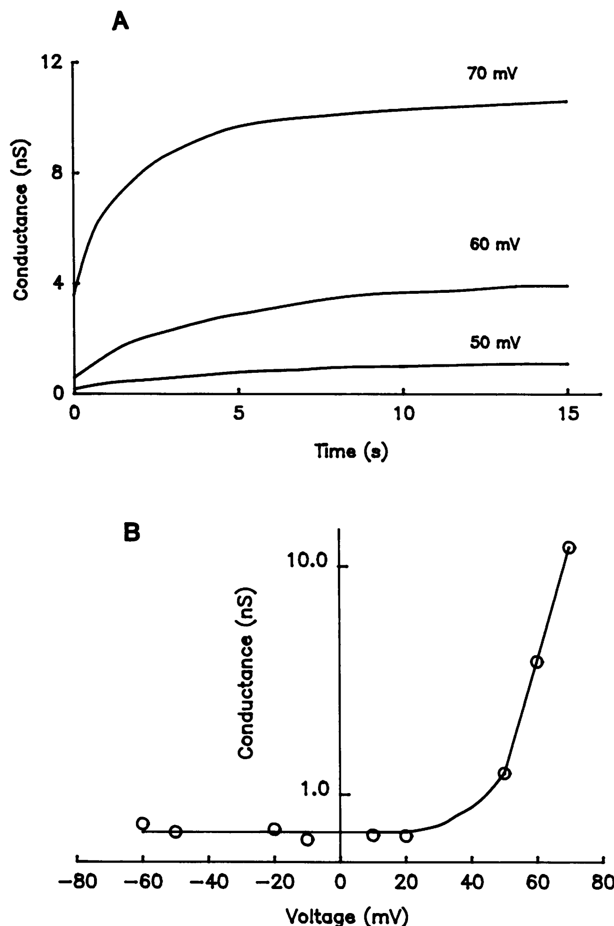

FIG. 3. Voltage and time dependence of the multichannel conductance. (*A*) Retrace of data showing the time-dependent conductance of a bilayer exposed to a peptide concentration of 1000 nM. (*B*) Plot of conductance (logarithmic units) as a function of voltage from data partially shown in *A*.

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