

Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes

(neutrophils/macrophages/oxygen-independent killing/cytotoxicity)

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ABSTRACT Defensins are cationic, cysteine-rich peptides ($M_r = 3500\text{--}4000$) found in the cytoplasmic granules of neutrophils and macrophages. These peptides possess broad antimicrobial activity *in vitro* against bacteria, fungi, tumor cells, and enveloped viruses, and they are believed to contribute to the “oxygen-independent” antimicrobial defenses of neutrophils and macrophages. Pathophysiologic studies *in vitro* have pointed to the plasma membrane as a possible target for the cytotoxic action of defensins. We report here that defensins form voltage-dependent, weakly anion-selective channels in planar lipid bilayer membranes, and we suggest that this channel-forming ability contributes to their antimicrobial properties observed *in vitro*.

Defensins are a family of small cationic peptides ($M_r = 3500\text{--}4000$) found in phagocytic cells of rabbits (1–3), guinea pigs (4), rats (5), and humans (6, 7). These peptides possess broad antimicrobial activity *in vitro* and are thought to constitute a key element in the nonoxidative killing of foreign organisms (for review, see ref. 8). Three lines of evidence implicate the plasma membrane as the locus of defensin action: (i) defensins induce leakage of K^+ and other cellular constituents (9, 10), (ii) membrane-depolarizing agents such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP) protect cells from defensins (11), and (iii) defensins are active against prokaryotic cells, eukaryotic cells, and enveloped viruses, but they are not active against nonenveloped viruses (12, 13). Furthermore, features *i* and *ii* of defensin killing are highly reminiscent of the bacterial cell killing induced by the channel-forming protein toxins known as colicins (14). We therefore sought to examine the interactions of the defensins with membranes. Employing “solvent-free” planar phospholipid bilayer membranes (15) as a model system, we report here that defensins can form voltage-dependent, weakly anion selective channels in lipid bilayer membranes. We present data obtained for neutrophil peptide 1 (NP-1), a rabbit defensin. Qualitatively similar results were found with the human defensin HNP-1.

MATERIALS AND METHODS

Membranes were formed at room temperature from the union of two monolayers of phospholipid (Avanti Biochemicals; usually soybean phosphatidylethanolamine, soybean phosphatidylcholine, and bovine phosphatidylserine in a weight ratio of 2:2:1) over a 100- to 200- μm hole in a Teflon partition separating two aqueous phases. The hole was precoated with a 2% solution of squalane (Fluka) in pentane. The salt solutions contained 50 mM KCl, 2 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)], and

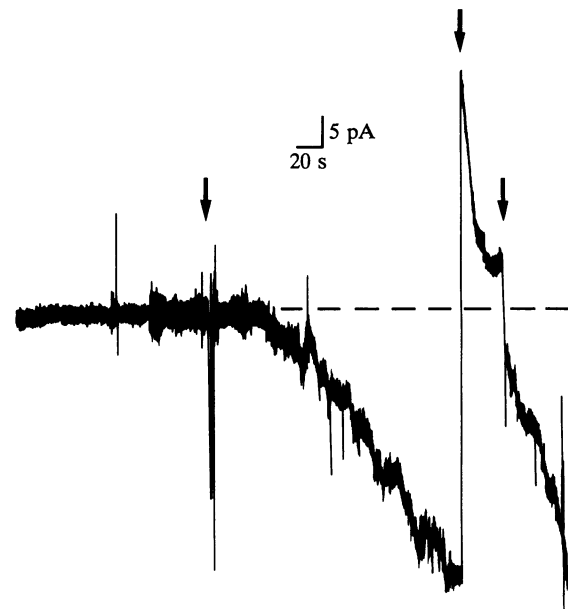


FIG. 1. Effect of NP-1 on lipid bilayer membranes. The current through a lipid bilayer voltage clamped to -90 mV is shown as a function of time. Initially, the current was nearly zero, reflecting the low conductance (permeability to ions) of the membrane. At the first arrow from the left, NP-1 was added to one side of the membrane to a final concentration of $50 \mu\text{g/ml}$. The current (and therefore conductance) began to increase rapidly (downward deflection since the current is in the “negative” direction). The voltage was then reversed to $+90$ mV (second arrow) and instantaneously the current flow was of equal magnitude but opposite sign. The current then rapidly decayed to a value close to zero (broken line). Upon switching back to -90 mV (third arrow), the current began to increase again in the negative direction. This “turning on” and “turning off” of the NP-1 induced conductance could be repeated indefinitely.

1 mM MgCl_2 , all adjusted to pH 7.0. The solutions were stirred continuously with magnetic stir bars. Membrane formation was monitored by measuring membrane capacitance. Voltages were applied by using a signal generator or a battery-driven stimulator. Currents were measured with a Keithly current amplifier and recorded on an oscilloscope and chart recorder. Ag/AgCl electrodes were used routinely to impose voltages and record currents across the membrane. Agar salt bridges were employed for salt gradient experiments. Membrane conductance (g) was calculated from Ohm's law, $g = I/V$, in which I is current and V is voltage. NP-1 and HNP-1 were purified to homogeneity as previously described (6, 16).

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Abbreviations: NP-1, rabbit neutrophil peptide 1; HNP-1, human neutrophil peptide 1.

RESULTS

Fig. 1 shows the effects of adding NP-1 (final concentration 50 $\mu\text{g/ml}$) to one side of a planar lipid bilayer composed of a mixture of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (2:2:1, wt/wt). The trace shows membrane current as a function of time, with a voltage of -90 mV imposed across the membrane. (In our sign convention, the side to which NP-1 was added is taken as ground. The voltages given refer to the voltage of the side *opposite* to NP-1.) Initially, there was little current flow, due to the inherent impermeability to ions of lipid membranes, but after addition of NP-1 (arrow) the current (and therefore conductance, $g = I/V$) increased dramatically. When the voltage was held constant at -90 mV, the current continued to increase rapidly until the membrane broke. Conductances as high as 10^5 picosiemens (pS) were observed under these conditions (data not shown). In the absence of defensins, g was approximately 5 pS. Reduced and carboxymethylated NP-1 (1), which is nontoxic (unpublished data), had no effect on the membrane. Since membrane conductance reflects the permeability to ions in the bathing solution such as Na^+ , K^+ , or Cl^- , we can conclude that NP-1 was able to increase the ionic permeability of lipid bilayer membranes by at least four orders of magnitude. NP-1 needed only to be added to one side of the bilayer to exert this effect. Concentrations of NP-1 required for lipid bilayer membrane activity (0.1–50 $\mu\text{g/ml}$) are quite comparable to those required for *in vitro* antimicrobial activity (1–100 $\mu\text{g/ml}$). In the second half of Fig. 1 (second arrow), the sign of the membrane voltage was reversed to $+90$ mV. Instantaneously, the current through the membrane had equal magnitude, but opposite sign. Rapidly, the change in voltage stopped the increase in current and caused a precipitous decrease in the membrane current. When the voltage was returned to -90 mV, the current began to increase again. This reversible “turning on” and “turning

off” of the NP-1-induced conductance could be repeated many times. Fig. 2 *Left* shows the current response of an NP-1-treated membrane to a series of various voltage pulses. The conductance was voltage dependent in two ways: (i) The conductance appeared and increased only at negative voltages. The conductance decreased at positive voltages. (ii) The steady-state conductance increased exponentially with increasingly negative values of the membrane voltage (see Fig. 2 *Right*). For example, the steady-state conductance at -100 mV was approximately 2.5 times the steady-state conductance at -80 mV. The NP-1-induced conductance usually reached a steady state within 15–30 min after the addition of NP-1 to the chamber. To achieve a steady state, the membrane had to be held for a prolonged period at a negative voltage of sufficient magnitude to induce channel formation (e.g., -70 to -90 mV).

The NP-1 conductance is due to formation of ion-permeable channels (Fig. 3). The single-channel conductance is quite heterogeneous (Fig. 3), ranging from 10 to 1000 pS. While certain conductance sizes appear more frequently, a consistent observation has been that the mean single-channel conductance appeared to increase with time after NP-1 addition. This may have reflected the formation of NP-1 channels containing progressively larger numbers of molecules of NP-1. Similar single-channel heterogeneity has been observed for other small peptide channel formers such as mellitin (18), nisin (19), and Pep 5 (20).

NP-1-induced channel formation appeared to be a steep function of NP-1 concentration (Fig. 4). When conductance was plotted as a function of concentration on log-log axes, slopes of 2–4 were obtained, suggesting that a multimer of NP-1 is likely to form the channel.

Reversal potential experiments in gradients of NaCl and KCl indicated that the NP-1 channel favored Cl^- over Na^+ and K^+ , although not exclusively so. A tenfold gradient of NaCl (500 to 50 mM) resulted in a diffusion potential of 18 mV

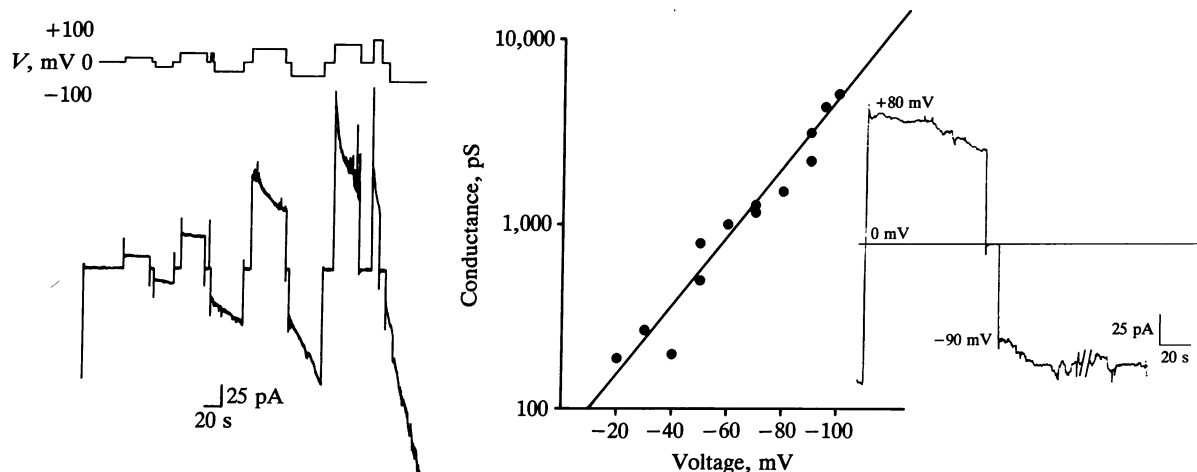


FIG. 2. (*Left*) Voltage dependence of currents induced by NP-1. Currents in response to a series of voltage pulses (± 20 , ± 40 , ± 60 , ± 80 mV) are shown for a membrane to which NP-1 had been added 20 min earlier to a final concentration of 50 $\mu\text{g/ml}$. Note that current “turns on” at negative voltages and “turns off” at positive voltages. Note also that the rate of current turn-on was a steep function of voltage, increasing sharply as the voltage became more negative. Similarly, the rate of turn-off increased as the voltage became more positive. Although current could be completely turned off at early times after addition of NP-1, a voltage-independent current gradually developed. At long times (greater than 60 min) after NP-1 addition, a voltage-dependent inactivation of current could sometimes be seen (data not shown) that was similar to the inactivation observed with the ionophore monazomycin (17). This “inactivation” may represent reverse turn-off of channels that have been translocated to the opposite side of the membrane as postulated for monazomycin. (*Right*) Semilogarithmic plot of steady-state conductance induced by NP-1 as a function of voltage. At low concentrations (0.1–10 $\mu\text{g/ml}$) of NP-1, a steady-state conductance can be achieved. Data from such a membrane are plotted. The linearity of these data shows that the steady-state conductance induced by NP-1 was an exponential function of voltage, increasing e -fold for every 23.4 mV. This implies that NP-1 channels have an “apparent gating charge” of about 1, according to the Boltzmann relation (14). Conditions were as in Fig. 1. (*Inset*) Current response to a membrane about 25 min after the addition of NP-1 to a final concentration of 5 $\mu\text{g/ml}$. Note that the current at -90 mV reaches a steady state within 5 min (the break in the record represents about 2 min of the trace which has been left out for compactness). The turn-off at $+80$ mV is also much slower and less complete than when the NP-1 was first added. These are typical features of NP-1-induced conductances which develop about 30 min after addition of NP-1. The mechanism of this time-dependent change is unknown.

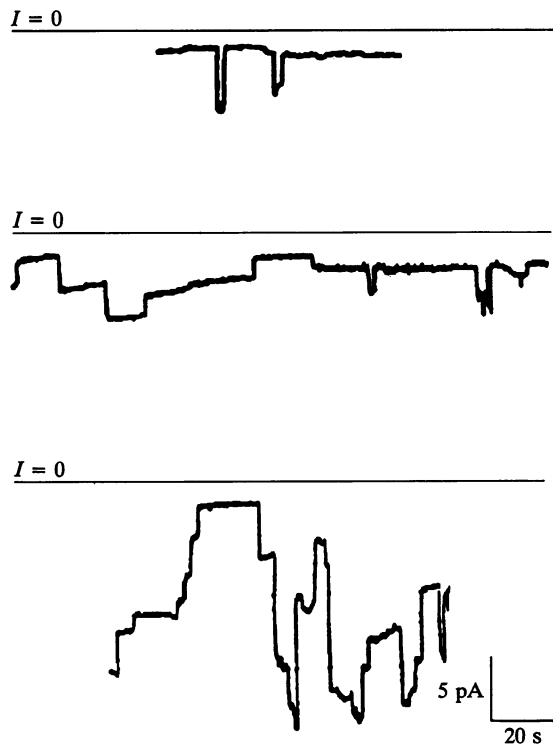


FIG. 3. Single-channel currents induced by NP-1. The conductance induced by NP-1 in lipid bilayers is due to the formation of ionic channels. At low concentrations of NP-1 or during the final stages of conductance turn-off, single-channel currents could be observed. A heterogeneous population of single-channel currents was observed and no single unitary size stood out. The traces show single-channel currents from a single membrane held at -80 mV (top trace), -90 mV (middle trace), and -100 mV (bottom trace). Channel openings are seen as downward deflections in current. Note that the size of single-channel events varies over a wide range, with larger events predominating at higher voltages. One possible explanation of this behavior is that channels may be formed by multimers of NP-1 molecules, and these multimers may have a wide variety of conductance levels. Lipids and solutions were the same as in Fig. 1. The concentration of NP-1 was $5 \mu\text{g/ml}$.

(dilute side negative), indicating a permeability ratio of about 2.4:1 (Cl^- to Na^+). A tenfold gradient of KCl (500 to 50 mM) resulted in a diffusion potential of 22 mV (dilute side negative). NP-1 was able to form channels in membranes formed from phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, or mixtures of these purified lipids, indicating that there is no apparent specific lipid requirement for activity.

DISCUSSION

The evidence demonstrating that the peptides are the active principals consists of the following. First, no channel activity was observed in the absence of defensins. Each membrane was stirred and held at -90 mV for 10 min before the addition of peptides to assure a quiet, steady baseline current. Second, the defensin preparations used were extremely pure as judged by reverse-phase high-pressure liquid chromatography, amino acid analysis, and polyacrylamide gel electrophoresis (e.g., see ref. 4). Third, reduced and carboxymethylated NP-1, which is non-toxic to cells, had no effect on the membranes. Fourth, at least two different defensins, NP-1 (rabbit) and HNP-1 (human), can induce similar conductances and similar channels in lipid bilayer membranes. The voltage dependence and concentration dependence of channel activity were also similar in these two distinct peptides. Fifth, the concentrations of defensins required to obtain

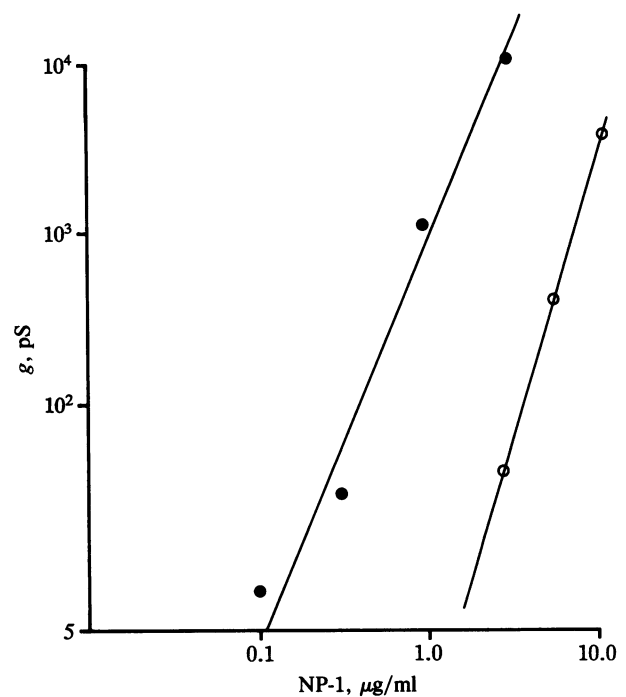


FIG. 4. Conductance vs. concentration plot for NP-1. The conductance induced by NP-1 in a lipid bilayer is shown as a function of the NP-1 concentration for two different membranes. The membranes were held at a constant voltage with continuous stirring. NP-1 was added to one side of the membrane and the conductance was measured 7 min later. Low concentrations of NP-1 (0.1 – $10.0 \mu\text{g/ml}$) were used so that a steady-state conductance could be achieved in a relatively short time. The data on the left (●) are from a membrane held at -90 mV. The data on the right (○) are from a membrane held at -50 mV. In the absence of a negative voltage no NP-1-induced conductance was observed. The slopes of the lines are 2.3 and 3.2, respectively. Several other experiments all gave slopes between 2 and 4, suggesting that the NP-1 channel is formed by a multimer containing 2–4 molecules of NP-1. Conditions were as in Fig. 1.

channel activity are nearly identical to the concentrations required for cell killing *in vitro*. Taken together, these observations clearly indicate that the defensin peptides themselves are responsible for the observed channel activity.

The physiologic effects of NP-1 described here seem highly relevant to the cytotoxic mechanism of action of defensins observed *in vitro*. (i) The broad spectrum of defensin antimicrobial activity supports channel-mediated membrane permeabilization as a toxic mechanism. All organisms sensitive to defensins are bounded by a plasma membrane, and non-enveloped viruses are curiously resistant. The lack of a specific lipid requirement we have observed for channel formation is consistent with the broad spectrum (bacteria, fungi, tumor cells, viruses) of defensin action. (ii) NP-1 channels form only at voltages (opposite side negative) which correspond to the voltages defensin molecules would encounter at the target cell membrane. The steep voltage dependence of channel formation in bilayers may explain the observation that *Escherichia coli* that are growing aerobically and/or have an "energized" membrane are most sensitive to defensins (11). The observed voltage requirement also explains the "protection" of *E. coli* by agents such as carbon-cyanide *m*-chlorophenylhydrazine that abolish the membrane potential of the cell (11). (iii) Channel formation readily accounts for the observed leakage of K^+ , *o*-nitrophenylgalactoside (ONPG), and other low molecular weight molecules through bacterial cell membranes after defensin treatment. Whether the lethal action of defensins consists of outward leakage of vital cellular constituents such as K^+ or Mg^{2+} , inward leakage of toxic ions such as Ca^{2+} , depolarization of

the membrane potential resulting in ATP depletion, or other membrane-mediated pathways, awaits further study. Recent experiments by Lehrer *et al.* (9) indicate that, in *E. coli*, inner membrane permeabilization by HNP-1 occurs concomitantly with loss of cell viability, suggesting that channel formation in the *E. coli* inner membrane may be the lethal event in defensin action.

An important question in the mechanism of defensin action is how these peptides might gain access to the target cell membrane. For fungi, enveloped viruses, and tumor cells, this is not a problem, since the target membrane is exposed to the environment. For Gram-negative bacteria, which possess a hydrophobic outer membrane, there is a significant barrier to defensin access to the inner membrane. The ability of defensins to permeabilize the outer membrane of Gram-negative bacteria is described elsewhere (9).

Alterations in ion permeability have long been known to be the basis of the cytotoxic action of agents such as the carrier valinomycin (21). One of us (B.L.K.) has previously proposed channel formation as the mechanism of action for the much larger ($M_r = 50,000$ – $60,000$) bacterial toxins known as colicins (14) and the yeast killer toxins ($M_r = 11,000$) (22). There is evidence for a similar mechanism of immune cell killing of foreign organisms by lymphocyte-derived perforins ($M_r = 70,000$), eosinophil cationic protein, and complement protein C9 (23, 24). A class of peptides (magainins), derived from frog skin, which have antimicrobial action and possibly comparable membrane effects has also been described (25, 26). The bacterial peptides Pep 5 and nisin ($M_r = 3500$) appear to have antibacterial and channel-forming properties resembling those of defensins (19, 20). Similar channel-forming properties can also be observed in antibacterial peptides from cecropia moths [cecropins, (27, 28)]. A more complete understanding of the role of channel-forming toxins in cytotoxicity and immune defenses will require better knowledge of how membrane permeabilization leads to cell death.

The ability of small peptides to perform complex physiologic functions such as voltage-dependent ion-selective transport has been known for some time (29), but the molecular mechanisms underlying ion transport remain obscure. Recent evidence suggests that the functioning of small peptide channels may be relevant for understanding the physiologic properties of much larger protein channels (30). Unlike other channel-forming peptides and proteins (31–41), defensins are not helical in solution, nor are they able to assume a helical conformation in the membrane (42, 43). Intriguingly, the three-dimensional structure of the human defensin contains a 10-residue stretch of antiparallel β -sheet (43, 44), a configuration which has been proposed for the channel-forming protein porin (45). The defensins' ability to form ion-permeable channels suggests that nonhelical structures may be important elements to consider in ion channel model building. The availability of detailed three-dimensional information about the defensins (43, 44, 46, 47) may help provide a deeper understanding of structure–function relationships in ionic channels.

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