pH-Sensitive, Cation-Selective Channels Formed by a Simple Synthetic Polyelectrolyte in Artificial Bilayer Membranes†

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ABSTRACT: A synthetic amphiphilic polymer, poly(2-ethylacrylic acid), induces pore formation in artificial phospholipid bilayers under mildly acidic conditions. This process is pH-controllable and produces cation-selective ion channels of at least three unique large conductances with differing Na^+/Cl^- permeability ratio values. Two general types of channel gating kinetics are observed, one flickering and irregular and one regular and discrete. The discrete channel activity resembles that of well-characterized biological and synthetic peptides. Given the heterogeneity of polymer structure and conformation, this observation raises questions about the mechanism of polymer channel formation and the nature of these channels in lipid bilayer membranes.

Introduction

Much of our knowledge of the properties of ion permeation in natural and artificial membranes derives from studies of polypeptide-based channels. However, other macromolecules have been shown to form ion-permeable pores as well. Recent work has shown that a poly(*β*-hydroxybutyrate)/polyphosphate complex found in *E. coli* plasma membranes forms voltage-gated, calcium-selective ion channels.[1] Platelet activating factor, an alkyl ether phospholipid, has also been shown to have channel-like activity with modest cation selectivity.[2] An increase in membrane ion permeability in the presence of anionic, cationic, and nonionic detergents has been characterized by Seufert,[3] and discrete channels induced by Triton X-100 have been observed by Schlieper and de Robertis.[4] In the present report, a synthetic anionic polyelectrolyte, poly(2-ethylacrylic acid) (PEAA; number-average molecular weight, $\bar{M}_n = 2 \times 10^4$), is shown to form discrete, cation-selective ion channels in artificial membranes. We show further that the formation and disassembly of the PEAA channels is pH sensitive.

Several recent efforts have been directed toward the design and preparation of synthetic ion channels. Lear, Wasserman, DeGrado, and co-workers have taken a "minimalist" approach to synthesize a series of simple amphiphilic 21-residue peptides, using only leucine and serine residues, and have characterized their ability to form ion channels of different single-channel conductances and ion selectivities.[5] Ghadiri and co-workers have focused on the synthesis of cyclic peptides with eight or more amino acid residues in each cycle, the amino acids alternating between D- and L-stereoisomers.[6,7] These cyclic peptides self-assemble in membranes into tubes that act as gated ion channels of about 50 pS unitary conductance in 0.5 M NaCl.[7] Oliver and Deamer have shown that poly(L-leucine) and poly(L-alanine) form proton-selective ion channels in artificial membranes.[8] Montal and co-workers have synthesized ion-conducting protein pores based on the acetylcholine receptor/channel[9] as well as on other receptor/channels.[10] A substituted cyclodextrin with three metal ion binding sites and four hydrophobic tails has been shown to transport ions into artificial liposomes at a rate faster than is possible via a carrier mechanism,[11] suggesting that the cyclodextrin functions as an ion channel. A similar approach based on macrocyclic crown ethers or dextrins with poly(oxyethylene) or poly(methylene) side chains has also been shown to promote channel-like flux in liposomes.[12]

What is unique about PEAA is its capacity to alter lipid vesicle structure *in a pH-dependent manner*, e.g., to trigger reorganization of phospholipid vesicles into small disk-shaped micelles of average thickness 55 Å and diameter 160 Å upon a pH shift from 7.0 to 6.5.[13,14] In addition to its ability to reorganize lipid vesicles at high concentration, at low PEAA concentration the aqueous phase contents of large vesicles can be released without reorganization of vesicles into micelles;[14,15] similar vesicle leakage in the absence of catastrophic rupture events has also been reported in a detergent−vesicle system.[16] As the results reported herein demonstrate, PEAA induces pore formation in bilayers at low [PEAA] (<20 μg/mL) in a pH-dependent manner. Discrete events resembling those characteristic of biological channels are observed even though the polymer is stereoirregular and lacks any regular secondary structure.[17] PEAA channels are found to be cation-selective, as demonstrated by comparing the relative Na^+ and Cl^- permeabilities of the channels, but the Na^+/Cl^- permeability ratio spans a wide range, indicating the existence of channels of different ion selectivities. Point amplitude histogram analysis of one type of channel event reveals at least three separate single-channel conductance states. Taken together, these findings demonstrate that irregular polyelectrolyte chains can form several regular structures in lipid bilayer membranes.

† Dedicated to Professor Virgil Percec on the occasion of his 50th birthday.

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Experimental Section

Polymer. Poly(2-ethylacrylic acid) was prepared by radical polymerization with azobis(isobutyronitrile) as initiator; radical polymerization of ethylacrylic acid yielded a stereoirregular polymer of weight-average molecular weight ($\bar{M}_w$) of 3.1×10^4, $\bar{M}_w/\bar{M}_n \cong 1.6$ (triad tacticity, %, isotactic:heterotactic:syndiotactic = 16:44:40).[18] Previous studies have shown a small effect of tacticity on polymer−lipid complexation.[18b]

Single-Channel Recording on "Tip-Dip" Patches. The "tip-dip" patch formation technique used for patch clamp experiments has been described in detail elsewhere.[19] All chemicals and solvents used were of the highest purity from either Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) unless specified otherwise. Milli-Q^{uv}-Plus (Millipore Corp., Bedford, MA) filtered and purified water was used for all experiments. Solutions of 200 mM NaCl, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), and 10 mM succinic acid were used in both the electrode and bath solutions; three buffers were used for the sole purpose of covering a wide range of solution pH. Fifteen microliters of 2.5 mg/mL diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Inc., Alabaster, AL) in hexane/ethanol, 9/1 (v/v), was spread on an aspirated surface of approximately 3 mL of bath solution in a 35 × 10 mm tissue culture dish (Falcon 3001, Becton Dickinson, Lincoln Park, NJ). Freshly pulled borosilicate capillaries (1B150-4, World Precision Instruments, Inc., Sarasota, FL; P-87 micropipet puller, Sutter Instrument Corp., Novato, CA), with tip resistance between 10 and 30 MΩ, were cleaned by a brief dip in chloroform/methanol solvent, 2/1 (v/v), just before monolayer spreading. The glass electrodes were not fire polished, and no Sylgard (Dow Corning Corp., Midland, MI) coating was applied. Spread monolayers were formed at least 10 min before "tip-dip" patch formation, and lipids were aspirated from the surface after patch formation; bilayer patches obtained by this method were essentially solvent free because of the 10 min evaporation step. Patches with seal resistance greater than 10 GΩ were used for experiments.

PEAA stock solution was prepared by dissolving 10 mg of PEAA in 1.96 mL of bath solution plus 40 μL of 5 M NaOH. This stock solution was then diluted with bath solution to various working concentrations, and 10−15 μL of the desired working solution was injected into the bath after patch formation. There was no appreciable pH change (<0.03 pH unit) of the bath solution after injection. Alternatively, a pipet flow system was used to introduce PEAA into the bath solution after patch formation. The flow system is described later in the pH sensitivity section. The PEAA stock solution described above was used to prepare flow solutions of various working concentrations. The flow system was the more reliable method for introducing PEAA to the patches.

An L/M-EPC 7 amplifier (List-Electronic, D-6100 Darmstadt/Eberstadt, West Germany) driven by an Atari MEGA ST4 computer (Atari Corp., Sunnyvale, CA) through an ITC-16 interface (Instrutech Corp., Great Neck, NY) was used for voltage clamp experiments. Signals were filtered with an 8 pole Bessel filter (Frequency Devices, Haverhill, MA) and stored on videotapes by a VR-10B digital data recorder (Instrutech). Ag/AgCl wires were used as the electrode and ground wires unless specified otherwise; silver wires (0.250 mm, AGW1030, World Precision Instruments) were first sanded, then treated briefly with concentrated nitric acid, followed by a rinse with water, and finally immersed in bleach (4−6% NaOCl) overnight. Gramicidin A (GA, G5002, Sigma) single-channel events, mock injections, and undisturbed patches were used for control experiments. An unbuffered 1 M CsCl solution was used for GA recording. A single-channel conductance of 37 pS was observed (data not shown), in good agreement with published GA single-channel conductances recorded using similar conditions.[20] Mock injection solutions were prepared the same way as PEAA stock solutions in the absence of PEAA. Both mock injections and undisturbed patches were tested at pHs ranging from 5.5 to 7.0. The voltages applied to the patches were in the range of −150 to +150 mV. Occasionally, large discrete conductance transitions (>100 pS) were observed immediately prior to breakage of a patch under high voltages (>+125 or <−125 mV) in these control experiments. Low-level (18 pS) discrete conductance steps were sometimes observed in control patches at holding voltage >+100 mV (or <−100 mV). These low-level events were probably due to contamination in the system and were not large enough to obscure the large PEAA channel events. The observed GA channels were not due to contamination in the 1 M CsCl solution; open GA channels appeared only after the addition of GA. Some current transitions displayed an overshoot current that was observed with or without capacitance compensation. The observed overshoot current is unlikely to be caused by poorly compensated series resistance because the voltage difference across the patch due to a sudden large current flow is not sufficient to account for the amplitude of the overshoot. We do not have an explanation for this phenomenon at the present time.

Ion Selectivity. Transbilayer Na^+ and Cl^- concentration gradients were used to assay PEAA channel ion selectivity. The bath solution contained 450 mM NaCl and 10 mM MES. The tip-filling solution contained 50 mM NaCl, 10 mM MES, and 800 mM sucrose (Fisher Scientific, Pittsburgh, PA), for balancing osmotic pressure, at pH 6.10. Fifteen microliters of 0.45 mg/mL PEAA stock solution was injected into the bath after patch formation. The final [PEAA] was approximately 2.25 μg/mL. The bath solution was grounded through an agar bridge (1.5% agar in 3 M NaCl) with the same filling solution as the tip solution. No detectable junction potential developed between the tip and the bath. In these experiments, the bilayer voltage was held at 0 mV, with a 100 ms prepulse of −100 mV followed by a 250 ms test pulse; the prepulse was used to increase the probability of channel activity during the test pulse. The test pulse was stepped from −50 to +100 mV in 5 mV steps. The prepulse-test pulse sequence was repeated at 200 ms intervals.

pH Sensitivity. A pipet flow system was constructed for delivering solutions of different pH. The flow pipet tips were about 20 μm in diameter. Solution flow out of the pipet was driven by pneumatic pressure. The initial bath and pipet solutions contained 200 mM NaCl and 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) adjusted to pH 7.6. Both pH 7.6 and pH 6.0 flow solutions containing 17.5 μg/mL PEAA and 200 mM NaCl were used. These solutions were buffered by 10 mM HEPES and 10 mM MES, respectively. The final bath solution pH dropped to about 7.5 when pH 6.0 solution was injected into the bath during the experiments. Control experiments in which no channel activity was detected were performed under the same conditions as above except in the absence of PEAA.

Results

Single-Channel Recording. Modes of PEAA channel activity include discrete conductance steps, irregular open channel events, and fast flickering spikes (Figures 1 and 2). PEAA open channel current noise is much larger than the closed channel noise ($I_{rms,open} = 2.2$ pA, calculated from one discrete open event in Figure 1; $I_{rms,closed} = 0.2$ pA; 2 kHz filtering bandwidth) as compared to gramicidin A ($I_{rms,open} = 0.5$ pA; $I_{rms,closed} = 0.4$ pA; 2 kHz filtering bandwidth). Biological channels have well-characterized conductance transitions owing to the uniform compositions and lengths of the peptides that make up the channels.[21,22] PEAA, on the other hand, is polydisperse in chain length, stereoirregular, and devoid of regular secondary structure.[17] The observation of discrete conductance states such as those shown in Figure 1 was therefore quite unexpected. Figure 2 shows an example of open channel events that were not characterized by clear stepwise transitions but rather by their irregular appearance where the transitions between conductance states or channel events appeared to be gradual or less well-defined; similar erratic current activity has also been reported in a

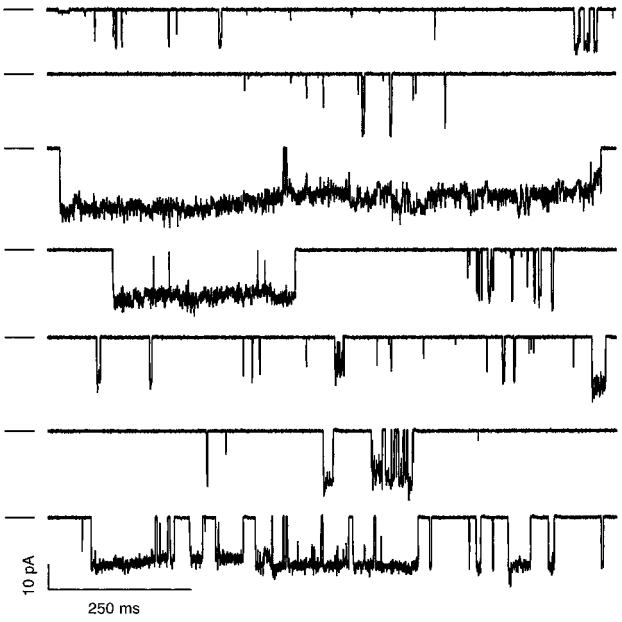

Figure 1. Well-resolved discrete conductance steps recorded at −50 mV holding potential with 0.5 μg/mL PEAA in 200 mM NaCl solution at pH 5.95, filtered at 2 kHz, and digitized at 11.8 kHz. The short horizontal bars at the left of the traces indicate the current level of the closed state.

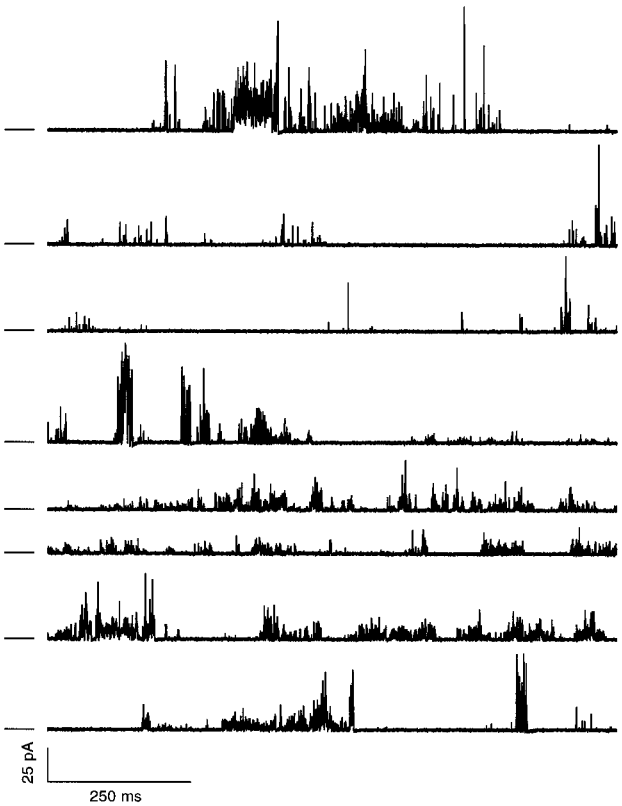

Figure 2. Irregular and rapid channel events recorded at 110 mV holding potential with 0.5 μg/mL PEAA in 200 mM NaCl solution at pH 5.95, filtered at 5 kHz, and digitized at 23.6 kHz. The short horizontal bars at the left of the traces indicate the current level of the closed state.

synthetic channel peptide system.[5a] Fast flickering spikes were often observed along with the other two types of events. The fast flickering was not resolved in either amplitude or time given the 5 kHz filtering bandwidth, implying that conductance transitions were less than 200 μs in duration. The fast flickering and

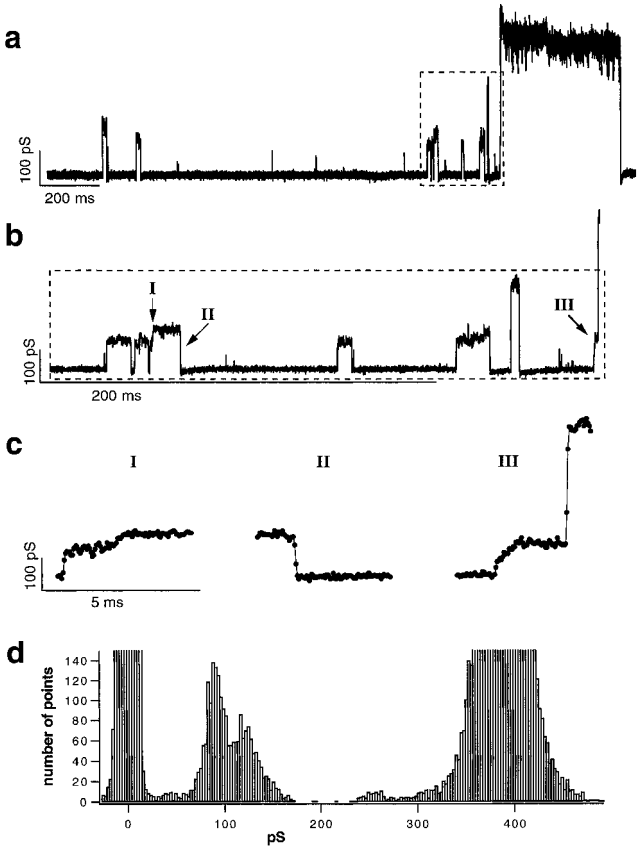

Figure 3. Point amplitude histogram analysis of a trace of discrete channel steps. (a) Discrete conductance steps recorded at −100 mV, 0.5 μg/mL PEAA, and pH 6.86. (b) The boxed region in (a) expanded over a shorter time scale. (c) An expanded view of the marked regions in (b). (d) The point amplitude histogram for the trace in (a). Bin size for the histogram is 2.6 pS. The larger peaks were truncated to show the smaller peaks. A multiple Gaussian fit to the full histogram produces five peaks centered at −1 (closed state), 87, 119, 255, and 384 pS with standard deviations of 4, 9, 16, 13, and 22 pS, respectively.

irregular open channel activities were the most common modes of behavior observed for PEAA channels.

A point amplitude histogram analysis of one sample trace showing discrete conductance transitions is illustrated in Figure 3. Figures 3a and 3b show the trace and an expanded view of one region of the trace, respectively. These data clearly demonstrate multiple, independent conductance states induced by PEAA. The point amplitude histogram of Figure 3d quantitates these different conductance states. The higher conductance states did not result from the overlapping opening of multiple channels; on the contrary, most channel opening and closing transitions clearly took place in one step. The unitary conductance values derived from Gaussian fits to the point amplitude histogram also support this argument (Figure 3 legend). However, some two-step transitions are apparent in regions I and III of Figure 3b (Figure 3c is an expanded view of the three regions marked in Figure 3b). For cases of this kind, it is not clear whether one PEAA channel was switching states or whether two channels were open during the recording, since both two-step transitions occurred during channel opening while their corresponding closing transitions took place in one step, e.g., region II of Figure 3c. Assuming Ohm's law is valid and that the channel is a homogeneous conducting cylindrical pore, single-channel conductances in the observed range of 90−400 pS in 200 mM NaCl solution give

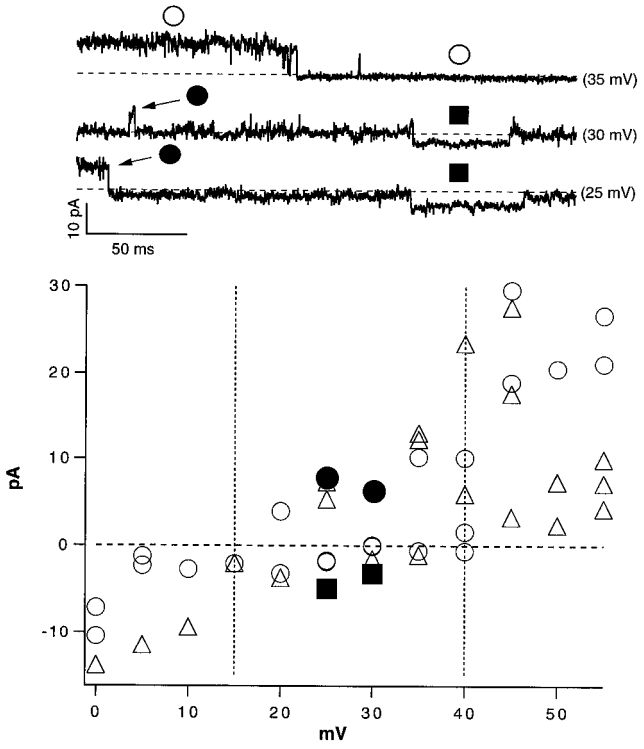

Figure 4. Cation selectivity of PEAA. The current−voltage plot of data taken from two consecutive groups of voltage sweeps at pH 6.10 and pipet to bath [NaCl] ratio of 1/9. The top three traces are current traces from consecutive voltage sweeps demonstrating how the points on the $I-V$ plot correspond to the actual current traces. The positions of circles, filled circles, and filled squares correspond to where their current values are averaged. Triangles are data taken from a different group of sweeps. The current traces are leak-corrected and offset for clarity. The sweep voltages are indicated on the right. The horizontal dashed lines mark the zero-current level of each trace. The two vertical dashed lines on the $I-V$ curve roughly define the region where current values cross over from negative to positive. The fact that all crossovers from negative to positive currents occur at positive sweep voltages indicates that PEAA channels preferentially select for Na^+ over Cl^-.

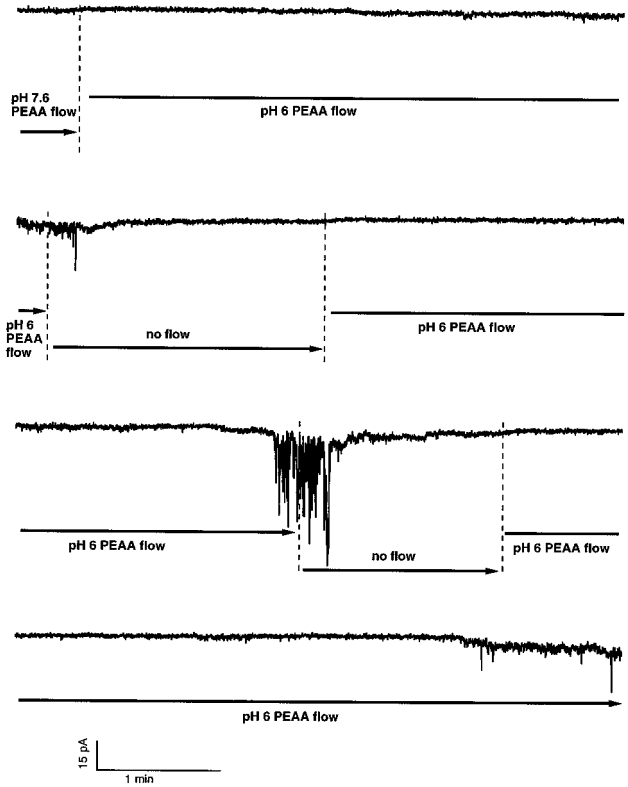

Figure 5. An example of switching on and off of PEAA channels by changing the solution pH. A continuous current trace from one experiment is presented here in four consecutive segments, each about 5 min in duration. The vertical dashed lines indicate the time of changing flow condition. The patch was held at −50 mV throughout the experiment. The patch was subjected to flow of PEAA solution at pH 7.6 for about 10 min before switching to flow of PEAA solution at pH 6 at the first dashed line, as indicated. The cycle of pH 6 PEAA flow followed by no flow was repeated twice in this experiment. The patch broke during the last flow of PEAA solution at pH 6. The final pH of the bath solution was 7.5. The trace was digitized at 10 Hz.

estimated pore diameters ranging from 5.6 to 12.2 Å (assumed pore length = 50 Å and solution resistivity = 49 Ω cm).[21,23]

Cation Selectivity. The observed PEAA channels were cation-selective. The reversal potential for PEAA channel current was found to be 0 mV as expected when the ionic compositions of the pipet and bath solutions were identical, but shifted to a positive value when $[NaCl]_{pipet}/[NaCl]_{bath} = 1/9$ (Figure 4). The observed reversal potentials, ranging from 15 to 40 mV, indicate permeability ratios of Na^+ over Cl^- to be in the range of 2−11. One particularly interesting phenomenon observed in the current traces was that different conductance levels reversed at different positive voltages. Two current traces showed events with currents going both inward and outward from the pipet at 25 and 30 mV (filled circles and squares, Figure 4).

pH Sensitivity. In order to demonstrate pH sensitivity of the PEAA-induced formation of ion channels, bilayer patches of DPhPC in pH 7.6 solution were held at −50 mV and subjected to a flow of 17.5 μg/mL PEAA solution at pH 7.6 from a pipet with a 20 μm tip diameter. After 10 min the flowing solution was changed to PEAA solution at pH 6.0. Channel activity was observed after the switch to acidic pH in 18 of the 19 patches studied while no activity was seen prior to acidification in any of the patches. The onset time of channel activity after the switch to acidic pH ranged from a few seconds to a few minutes. Two of the 18 active patches were stable enough to allow multiple PEAA flow switches. Channel activity observed under pH 6.0 PEAA flow stopped within the order of 1 min after the flow was interrupted (Figure 5). Cycling of solution pH thus produced a cycling of channel activity. This cycling of PEAA channel activity was repeated five times in the two stable patches.

Discussion

This report demonstrates that ion channels exhibiting large unitary conductance states are induced by PEAA in diphytanoyl phosphatidylcholine membranes fabricated on a pipet tip by the tip-dip method. This finding is surprising in that PEAA is polydisperse in chain length, stereoirregular, and devoid of regular secondary structure.[17] Not only does the irregular PEAA form channels, but the channels display discrete conductance levels (Figure 3). This finding suggests that the conductance pathways induced by PEAA are a subset of discrete structures and that these structures can be stable for many seconds (Figure 1). The fact that the most common channel event found in PEAA-treated membranes displays rapid gating (of the order of 100 μs channel open times in prolonged bursts lasting up to several seconds, Figure 2) indicates that the channel-forming structures are transiently stable in the open

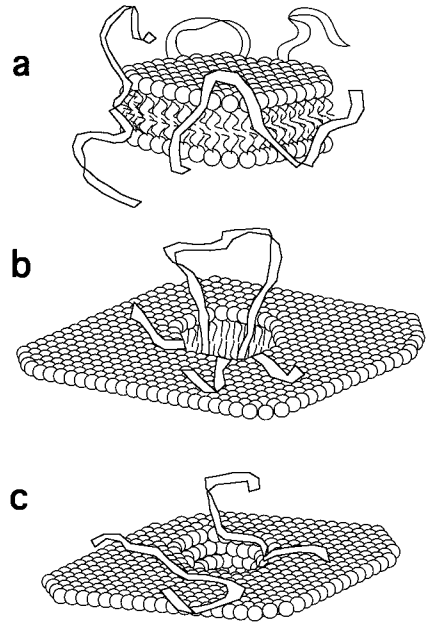

Figure 6. Hypothetical models of PEAA−lipid structures. (a) Disk-shaped micelle with PEAA stabilizing its free edge. (b) "Inverse" structure of disk-shaped micelle with PEAA stabilizing the free edge of the pore. (c) Polymer-induced saddle-surface pore with phospholipid head groups lining the pore. Circles represent phospholipid head groups. Short wiggly lines are hydrocarbon tails. Ribbons represent the PEAA polymer. The models are not drawn to scale.

pore state while the overall channel-forming structure remains intact in or on the membrane for a much longer time. This observation is consistent with the formation of a PEAA-induced channel by a single molecule or an oligomer of PEAA, although it does not differentiate between these possibilities.

This report also demonstrates that the channel behavior induced by PEAA is due to the formation of conducting pathways by pores rather than carriers. The smallest PEAA single-channel conductance from Figure 3, 87 pS at 100 mV holding potential, corresponds to a current of 8.7 pA or an ion flow rate of 5.4×10^7 ions/s, which is much faster than the ion transfer rate of any known carrier.[21]

One can envision two simple models of pore formation by PEAA. One model is similar to postulated biological channel structures where the pores are lined by polypeptides of well-defined structures.[21,22] Extending this idea to PEAA, one can imagine that segments of the PEAA chain could align with their main axes spanning the bilayer in a staved barrel form with the polymer stabilizing the inner surface of the pore (Figure 6b). The staves of the PEAA barrel channel could be contributed from a single or multiple polymer molecule(s). This structure can also be viewed as the "inverse" form of the disk-shaped PEAA−lipid mixed micelle where the free edge of the micelle is stabilized by the polymer (Figure 6a).[24] The negatively charged polymer molecules forming the pores (the degree of ionization of PEAA at pH 6.5 is 0.35 in 0.4 M NaCl)[25] are likely to make the channels cation-selective, which is in good agreement with the data presented in Figure 4.

Another possible model of PEAA pore formation has the conducting pathways lined by lipids, similar to the lipid-lined pores postulated in electroporation-induced bilayer breakdown (Figure 6c).[26] In this model, the adsorbed polymers would induce a change in local membrane curvature,[27] either spontaneously producing saddle-surface holes in the bilayer or substantially lowering the transmembrane voltage required to create such holes. Negatively charged PEAA chains localized near the mouths of the phospholipid-lined pores would still be expected to confer cation selectivity. We are unable to rule out either model at this time.

The cation selectivity of PEAA channels appears to differ for different channel conductance states as is shown in Figure 4. Although more experiments will be required to clarify the relationship between channel conductance and ion selectivity, the findings to date suggest that this relationship is an intrinsic property of PEAA channels. Whether this relationship has to do with a variation in the pore size or in the configuration of negatively charged groups within the pore remains to be determined. It is possible that modification of the charged group distribution on PEAA or changes in the ionic composition of the bathing solutions could be used to regulate the selectivity of PEAA channels. Such regulation could prove useful in the application of PEAA ion channels in drug delivery or targeted cell killing.

A critical result demonstrated here is that PEAA ion channels will form under specific, controllable conditions. Bilayers exposed to PEAA at pH 7.6 show no channel activity. When the pH is reduced to 6.0 at the same concentration of PEAA, the polymer-induced channel activity begins within seconds to minutes. Raising the pH back to 7.6 stops the channel activity (Figure 5). Thus, the activity of PEAA channels can be regulated by controlling the pH of the medium in which PEAA is introduced to membranes. This property is unique and suggests that PEAA may be useful in the design of liposome-based drug delivery systems, in the modulation of endosome-mediated ligand internalization, and in other applications in which regulatable ion permeability of membranes is required.[28]

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