Artificial Non-Peptide Single Ion Channels

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Abstract: We report the synthesis of amphiphilic molecules composed of glycolate ethers of monoalkyloligo(1,4-butylene glycol) complexed with dioctadecyldimethylammonium cation. When inserted into a bilayer lipid membrane, these molecules exhibited several properties characteristic of single ion channels. Stable and constant conductance levels were observed, with transitions between open and closed states at a millisecond to second range. The conductance levels were on the order of 10^1–10^3 pS, and the channels were classified into three types on the basis of their conductances and gating properties. The channels were cation selective over anion by a factor of approximately 5. In channels having the largest conductance, reversible and repeatable inactivation occurred with transmembrane voltages greater than 70 mV, while the other types of channels did not show voltage-dependent gating. We tentatively interpret these *single* ion channel properties to result from the formation of a molecular aggregate wherein the polar oligoether–carboxylates are covered with the hydrophobic alkyl groups of the ammonium in the bilayer membrane.

Introduction

Ion channel proteins play important roles in the generation of electrical signals in the nervous system and in a variety of signal transduction systems.[1] A number of important channel proteins, including Na,[2] Ca,[3] and K[4] channels as well as a nicotinic acetylcholine receptor channel,[5] were recently cloned and sequenced. On the basis of the information on the primary sequence, higher order structures of these proteins in the membrane have been proposed. According to these models, the channel proteins are commonly composed of four or five homologous transmembrane subunits, each of which is composed of six repeating transmembrane α-helices. One of these helices is very characteristic of the subunit's amphiphilic nature, that is, hydrophilic amino acid residues are lined up on one side and hydrophobic residues on the other side. It is supposed that this helix from each subunit associates to form an ion-conducting pore by facing its hydrophilic side.[6] If this model is true, amphiphilic bundle proteins that can span the lipid bilayer, even synthetic ones, would associate with themselves to form channel-like structures in the membrane. Actually, a synthetic polypeptide[7] and the channel-forming sequence[8] from native proteins have been prepared and demonstrated to show characteristic single ion channel properties.

Synthetic channel-forming molecules are interesting because they provide a deeper understanding of the ion channel in the membrane and permit the design of nanoscale transducers,[9] based on the controllable structure–function relationships of much simpler structures. Except for the proposed α-helical bundles of polypeptides,[7,8] none of the previously claimed channel molecules[10] were successful in demonstrating the properties specific to membrane spanning channels. Herein we report the preparation of totally synthetic oligoether amphiphiles that enter into the planar bilayer membrane and show several intrinsic single ion channel characteristics, including duration of constant conductance levels, cation selectivity over anion, and transitions between open and closed states. The artificial channel reported herein is characterized by extreme simplicity of structure and a function remarkably similar to that of natural single ion channels.

Experimental Section

General Procedure. ^{1}H NMR spectra were recorded with a JEOL EX 90 spectrometer at 90 MHz. The chemical shifts are reported on the δ (ppm) scale downfield from tetramethylsilane. Coupling constants (J) are reported in hertz (Hz). Infrared (IR) spectra were measured with a Hitachi 260-50 spectrophotometer. Mass spectral data were obtained on a JEOL JMS-DX300 either by field desorption or by electron impact ionization. Elemental analytical data were obtained at The Micro Analytical Center at Kyoto University.

Thin layer chromatography (TLC) was performed on glass plates coated with 0.25-mm silica gel 60 F_{254} (Merck). Column chromatography was performed on a column packed with 0.063–0.200-mm silica gel 60 (Merck). Ion-exchange chromatography was carried out on a column packed with an anion-exchange resin, Bio-Rad AG1-X8.

Soybean lecithin, type II-S, was a product of Sigma Chemical Co. Ltd. Octadecyltrimethylammonium chloride (>97%) and dioctadecyldimethylammonium chloride (>95%) were obtained from Tokyo Kasei Kogyo Co. Ltd. Ultrapure water was used for all the measurements of the membrane current.

5,10-Dioxatetradecane-1,14-diol. Sodium metal (3.29 g, 0.14 mol) was added in small pieces to 50 g (0.56 mol) of 1,4-butanediol. After complete disappearance of the metal pieces, 12.0 g (56 mmol) of 1,4-dibromobutane was added dropwise with stirring at 100 °C over a period of 5 h. Since tris(butylene glycol) gave an almost 1:1 azeotropic mixture with 1,4-butanediol, the product mixture was acetylated, separated from the acetate of the starting material, and hydrolyzed to regenerate the alcohol. The reaction mixture was neutralized with 6 N HCl after being heated for 14 h and was concentrated to about one-half volume. Ethyl acetate was added to precipitate the inorganic salt. After separation of the salt by filtration, ethyl acetate was evaporated to leave an oil, to which was added 15 mL of acetic anhydride. The mixture was heated

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at 100 °C for 12 h. Excess acetic anhydride was removed, and the residue was poured into H_2O. The aqueous layer was extracted with Et_2O (50 mL × 4) and dried (anhydrous Na_2SO_4). 1,4-Diacetoxybutane was removed by distillation in vacuo. Crude 1,14-diacetoxy-5,10-dioxatetradecane was hydrolyzed in 70 mL of 2 N NaOH for 12 h. The solution was neutralized with 6 N HCl and concentrated to about one-half volume. The product was extracted with Et_2O (50 mL × 4), dried (anhydrous Na_2SO_4), and concentrated to leave a yellow oil (8.1 g, 62%). NMR: 1.55 (t, 12 H, J = 6.0 Hz, CCH_2C), 3.25 (m, 14 H, OCH_2 and OH). IR (film): 3450 (s, br), 2930 (s), 2850 (s), and 1110 (s) cm^{-1}.

5,10,15-Trioxanonadecan-1-ol. To 2.0 g (8.5 mmol) of 5,10-dioxatetradecane-1,14-diol in 20 mL of dry tetrahydrofuran was added 450 mg of NaH dispersion (50% in mineral oil, 10 mmol), and the solution was heated under reflux for 30 min. To this suspension was added 2.5 g (17 mmol) of 1-bromobutane over a period of 1 h, and the mixture was heated under reflux for 10 h. Solvent was removed, and the residue was neutralized with 1 N HCl, extracted with Et_2O (30 mL × 3), and dried (anhydrous Na_2SO_4). Ether was removed, and the product was isolated through column chromatography (benzene/ethyl acetate (1:1) as eluent). Yield: 1.17 g (47%). NMR: 0.86 (t, 3 H, J = 6.0 Hz, CH_3), 1.50 (m, 14 H, CCH_2C), 2.38 (s, 1 H, OH), 3.20 (t, 14 H, J = 6.6 Hz, OCH_2). IR (film): 3425 (s, br), 2940 (s), 2850 (s), 1370 (s), 1110 (s), and 750 (m) cm^{-1}.

3,8,13,18-Tetraoxadocosanoic Acid (1). Sodium monochloroacetate was prepared from 0.62 g (65 mmol) of monochloroacetic acid and 0.45 g of sodium hydride (50% in mineral oil, 10 mmol) in 5 mL of dry dioxane with ice-cooling. To this solution was added 0.38 g (1.3 mmol) of 5,10,15-trioxanonadecan-1-ol dropwise with ice-cooling. The bath temperature was raised to room temperature and kept at that temperature for 1 h. The solution was finally heated under reflux for 8 h. Dioxane was removed, and water was added to the residue. The aqueous solution was extracted with Et_2O (20 mL) first under alkaline conditions to remove nonacidic components. The solution was then acidified, extracted with Et_2O (20 mL × 3), dried, and evaporated to leave 0.41 g of an oil, to which were added 8 mL of ethanol and a few drops of concentrated H_2SO_4. The solution was heated under reflux for 12 h, neutralized with 1 N Na_2CO_3, and concentrated. The product was extracted with Et_2O (20 mL × 4), dried, and evaporated to leave 0.32 g of a brown oil. The ester was isolated through column chromatography (benzene/ethyl acetate (5:2) as eluent). Yield: 170 mg (26%). NMR: 0.85 (t, 3 H, J = 5.9 Hz, CH_3), 1.20 (t, 3 H, J = 6.6 Hz, CCH_3), 1.55 (m, 16 H, CCH_2C), 3.25 (t, J = 5.7 Hz, OCH_2), 3.90 (s, OCH_2CO), 4.10 (q, J = 6.6 Hz, $COOCH_2$). IR (film): 2950 (s), 2860 (s), 1750 (s), and 1110 (s) cm^{-1}. Mass spectrum: m/z = 376 (M^+). The ethyl ester was hydrolyzed by being stirred with 3 mL of 2 N NaOH at room temperature for 9 h. After acidification, the carboxylic acid was extracted with Et_2O (20 mL × 4). Combined Et_2O extracts were dried and evaporated to leave a pale yellow oil (160 mg, 24% based on the starting monoalcohol). NMR: 0.86 (t, 3 H, J = 5.7 Hz, CH_3), 1.55 (m, 16 H, CCH_2C), 3.31 (t, 14 H, J = 6.6 Hz, OCH_2), 3.95 (s, 2 H, OCH_2CO), 7.68 (s, 1 H, $HOCO$). IR (film): 3400 (s, br), 2940 (s), 2860 (s), 1740 (s), and 1110 (s) cm^{-1}. Mass spectrum: m/z = 348 (M^+). Anal. Found (Calcd) for $C_{18}H_{36}O_6$: C, 61.75 (62.04); H, 10.70 (10.41); O, 27.77 (27.55).

Dioctadecyldimethylammonium 3,8,13,18-Tetraoxadocosanoate (3a). Dioctadecyldimethylammonium chloride (DODAC, 0.40 g, 0.68 mmol) was washed with ether (20 mL × 5) to remove impurities. Ion-exchange resin (Bio-Rad, AG1-X8, 8.53 g) was packed into a column, treated with 1 N NaOH (10 mL × 5), and washed with distilled water until the washing was not alkaline. DODAC in methanol applied at the top of the column was eluted with H_2O. Earlier alkaline fractions were discarded, and later fractions were collected. Evaporation of the solvent left 320 mg of a colorless solid. Dioctadecyldimethylammonium hydroxide (21.5 mg, 37.8 nmol) thus obtained was dissolved in 2 mL of methanol and mixed with 13.2 mg (37.8 nmol) of 3,8,13,18-tetraoxadocosanoic acid. After evaporation of the solvent, the residue was dried over P_2O_5 in vacuo for 3 h to afford 33 mg (97%) of a colorless solid.

Dioctadecyldimethylammonium 3,8,13,18,23-Pentaoxaheptacosanoate (4a). 3,8,13,18,23-Pentaoxaheptacosanoic acid (**2**) was prepared from 5,10,15-trioxanonadecan-1-ol and 5-oxanonyl bromide in a similar manner. NMR: 0.86 (t, 3 H, J = 6.0 Hz, CH_3), 1.56 (m, 20 H, CCH_2C), 3.35 (t, J = 5.6 Hz, 18 H, OCH_2), 3.98 (s, 2 H, OCH_2CO), 6.90 (s, 1 H, $HOCO$). IR (film): 3440 (s, br), 2940 (s), 2860 (s), 1730 (s), and 1110 (s) cm^{-1}. Mass spectrum: m/z = 420 (M^+). Anal. Found (Calcd) for $C_{22}H_{44}O_7$: C, 63.06 (62.83); H, 10.82 (10.54); O, 26.41 (26.63). The dioctadecyldimethylammonium salt was prepared in a manner similar to that described above.

Octadecyltrimethylammonium Salts of Oligoether-Carboxylates 3b and 4b. 3,8,13,18-Tetraoxadocosanoic acid (**1**) and 3,8,13,18,23-pentaoxaheptacosanoic acid (**2**) were converted to the corresponding salts using octadecyltrimethylammonium hydroxide in a manner similar to that described for the dioctadecyldimethylammonium salts.

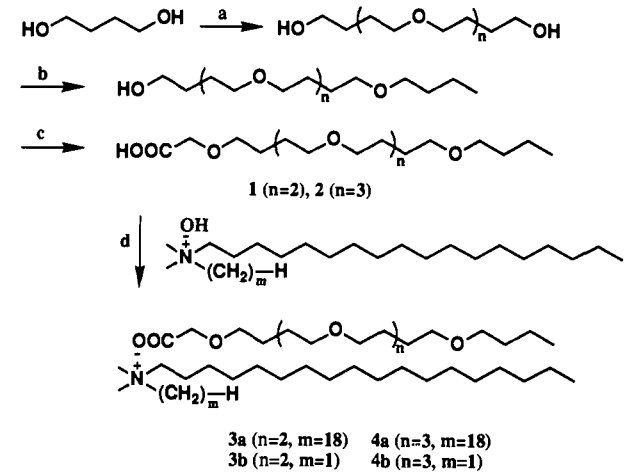

Figure 1. Synthetic scheme of amphiphilic ion pairs between oligoether-carboxylates and mono- or dioctadecylammoniums.

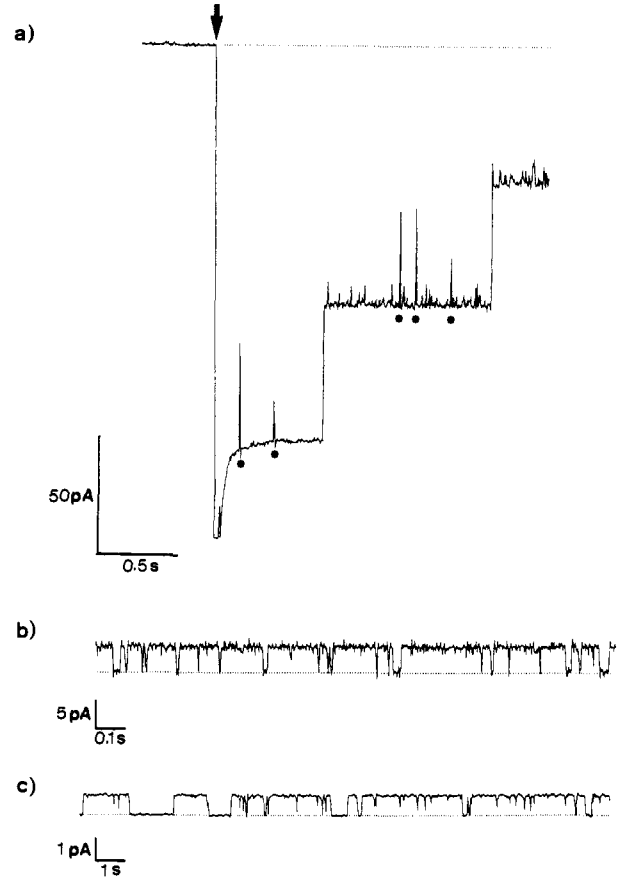

Figure 2. (a) Inactivation of type I (700 pS) channels at a high voltage (−80 mV). (b) Typical records of a type II (97 pS) channel at +47 mV. (c) Records of a type III (13.5 pS) channel at +60 mV. Currents increase downward in step a and upward in steps b and c. The dotted line in each panel indicates the closed level of the channel. All the data were taken with symmetric bath solutions (500 mM KCl, 5 mM HEPES-Tris pH 7.2).

Planar Bilayer System. Planar bilayers were formed by applying phospholipid solution (15 mg/mL soybean lecithin in *n*-decane) to a hole (200–500 μm in diameter) in a polypropylene partition separating two aqueous chambers (3 mL in volume) as described elsewhere.[11] Each chamber was connected to an Ag/AgCl electrode via a glass KCl (3 M) agar bridge. Currents across the bilayers were measured under volt-

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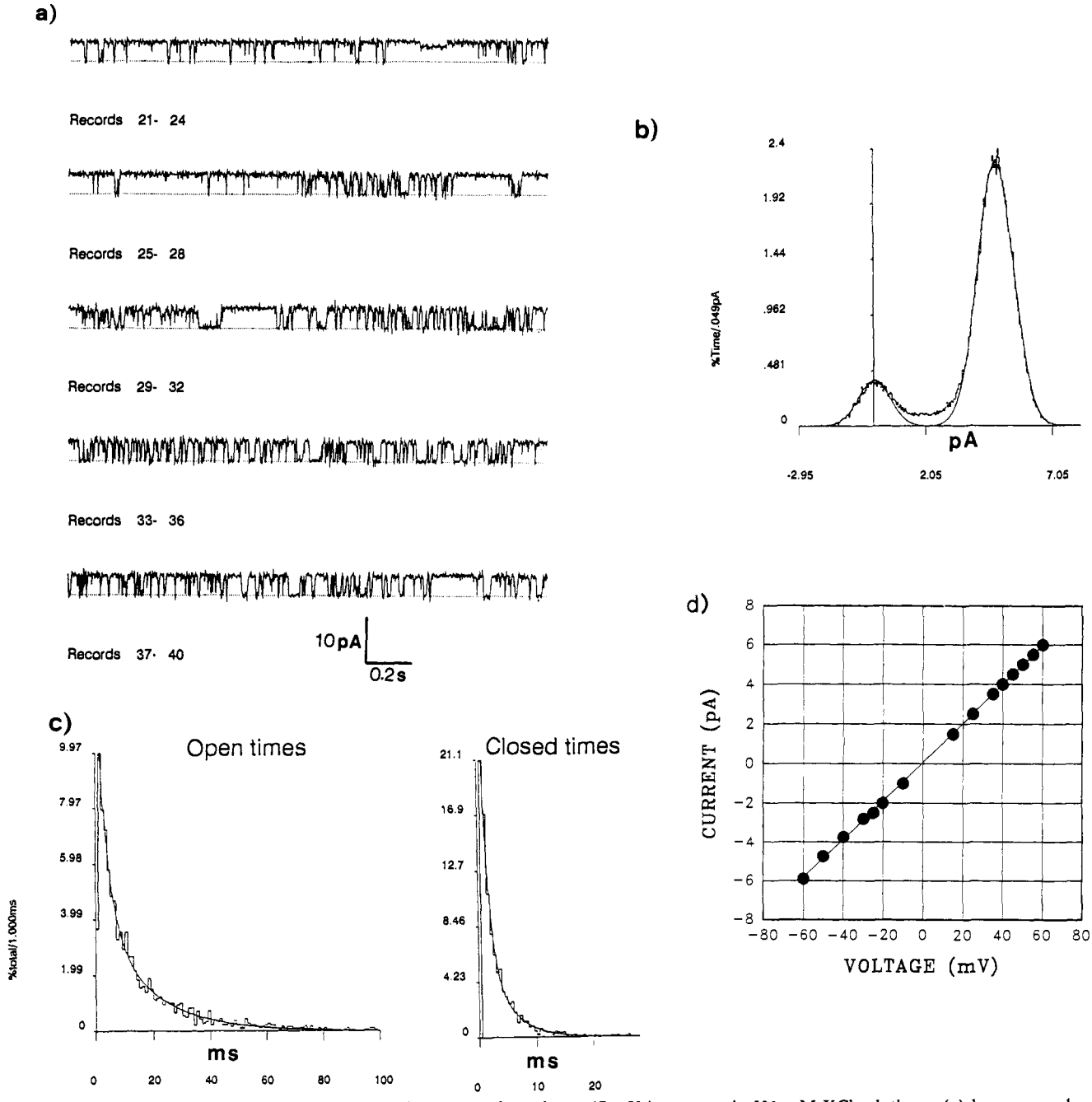

Figure 3. A typical example of the basic behaviors of a type II channel at +47 mV in symmetric 500 mM KCl solutions: (a) longer records of single-channel currents; amplitude histogram of the single-channel currents (b) and histograms of open and closed times (c) from 288-s continuous records; (d) current–voltage relationship. The estimated single-channel conductance from the slope was 97 pS.

age-clamp conditions by means of a patch-clamp amplifier (Nihon Koden Model S-3666). All experiments were carried out at room temperature (22–25 °C). The side to which compounds were added was defined as "cis". The opposite side was defined as "trans", and the voltage was referenced to the cis side with respect to the trans side. The buffer solutions used were composed of symmetrical 500 mM KCl, 5 mM HEPES, pH 7.2 adjusted with Tris base unless otherwise noted.

Measurement of Membrane Current and Analysis. The artificial ion channel compounds were premixed with phospholipid solutions, and bilayers were formed from the mixed solution. Alternatively, a small aliquot (10–30 μL) of the compounds dissolved in DMSO (5 mg/mL) was added to the cis side with gentle stirring. In either case, the compounds were successfully incorporated into bilayers, as confirmed by the observation of changes in bilayer conductances. All the data presented here were obtained by the latter method. The currents across the bilayer were fed into the patch-clamp amplifier, low-pass filtered at 0.1–1 kHz using a four-pole Bessel filter, and recorded on a PCM recorder. Current recordings obtained at each voltage were digitized at 0.5–5 ms/point by means of an analog to digital converter (Data translation Model 2801A) controlled by a 386-based PC/AT-compatible computer. Single channel current amplitude was measured as peak-to-peak distance (each peak represents open and closed levels) on the amplitude histogram constructed by the software PAT V. 6.1.[12] Open- and closed-time distributions were analyzed by the same program.

Results

Design and Synthesis of Artificial Ion Channels. The molecules designed are shown in Figure 1. As the ionophoric unit, ether oxygen atoms were incorporated regularly into fatty acid chains separated by a tetramethylene unit as in **1** and **2**. The molecular lengths, adjusted to fit the lipid monolayer in extended or helical conformations, were approximately 24 and 30 Å for **1** and **2** in an extended form, respectively, as estimated from the CPK models. The oxa chain is considered moderately hydrophilic, and the

(12) Written by Dr. John Dempster, University of Strachclyde, Glasgow, U.K.

carboxylic acid was converted to salts **3** and **4** with ammoniums containing one or two long alkyl chains to obtain hydrophobicity for incorporation into the lipid membrane.

Tris(1,4-butylene glycol) was obtained from 1,4-butylene glycol and its bromide. One of the alcoholic terminals was alkylated with either *n*-butyl bromide or *n*-butoxybutyl bromide. The other alcoholic terminal was alkylated with sodium monochloroacetate to afford oligoether–glycolates. The carboxylic acid was converted to the ethyl ester for the purpose of purification through silica gel column chromatography and was then regenerated by alkaline hydrolysis. Octadecyltrimethyl- or dioctadecyldimethylammonium chloride was passed through an anion-exchange resin, followed by the addition of a stoichiometric amount of the above oligoether–carboxylic acids to produce ion pair amphiphiles **3a,b** and **4a,b**.

Single-Channel Measurements. Recording currents across the bilayer at the single-channel level is the best way to test whether the compounds can form ionic channels. We used two methods to incorporate compounds **3a** and **4a** into the bilayer. Initially, the compounds were premixed with phospholipid solutions, and bilayers were formed from the mixed solution. Later, the compounds, dissolved in DMSO (5 mg/mL), were added to the cis side with gentle stirring. As the latter method was a bit more successful, we employed this method in most experiments. Usually, a few minutes or a few tenths of a minute after addition of the compounds, there appeared one of three types of conductance levels. The occurrence of each level was almost random, and coexistence of different levels was frequently observed. As each level, once apparent, was very stable, the levels seemed to be independent of one another.

The first channel type (type I), with a relatively high conductance level (700–1400 pS), stayed in the open state most of the time and occassionally showed brief closings (Figure 2a) and subconductance levels (data not shown). Interestingly, when the voltage across the membrane was increased beyond 70 mV, the channel was reversibly inactivated, as shown in Figure 2a, where stepwise channel closures can be seen in response to a switch of the membrane voltage from 0 to -80 mV at the time indicated by the arrow. During opening of the channel, brief, unresolved closings (indicated by asterisks (*)) are observable. The membrane contained four type I channels in this example. The multichannel incorporation should be noted as strong evidence of successful channel formation in the membrane. The second class of conductance (type II channel) (70–100 pS, 93.17 ± 8.73 pS, $n = 6$) showed repeating opening and closing in the millisecond range (Figure 2b). Finally, the third class of conductance (type III channel) (10.85 ± 1.58 pS, $n = 7$) showed relatively slow gating at the second range (Figure 2c).

A common and important feature of these conductances is that, once they appear, their amplitude remains very stable and persists for over 1 h. A typical example of longer records from a type II channel with an amplitude histogram is shown in Figure 3a,b. The histogram data have been fitted with a sum of two Gaussians (continuous line) to emphasize the two stable levels (Figure 3b). This suggests that the structure of the assembly of these compounds in the bilayer, once established, is very stable, meeting one of the criteria for the existence of an ion channel. At the same time, these channels have sufficient flexibility to exhibit transitions between open and closed states. The open- and closed-time histograms of the type II channel with fast gating kinetics were well fitted to double-exponential functions (continuous line) with time constants of 3.52 and 17.12 ms for open time and 1.11 and 3.95 ms for closed time (Figure 3c). The current–voltage relationship of all the channel types was almost linear under symmetric ionic conditions (for type II channel, see Figure 3d).

Ionic selectivity of the type I and II channels was examined under asymmetrical ionic conditions (cis, 500 mM KCl; trans, 100 mM KCl). The reversal potentials of these channels were almost equal (−23.2 mV for type I and −23.5 mV for type II). From the Goldman–Hodgkin–Katz equation, the permeability ratio between K^+ and Cl^- was calculated as approximately 5, indicating that these channels were selectively permeable to cations.

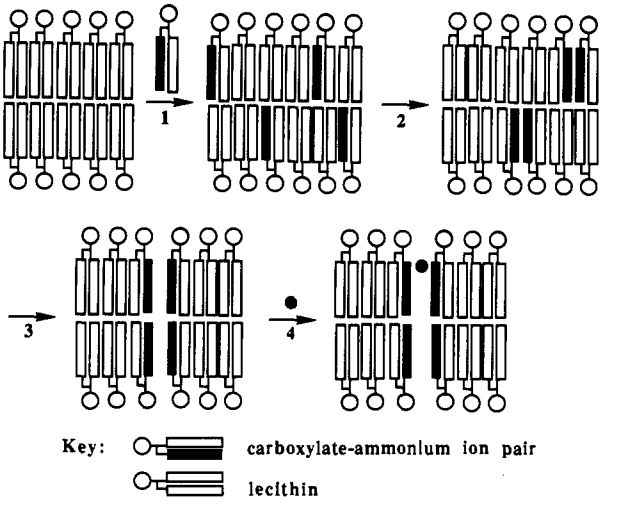

Figure 4. Hypothetical processes for the formation of an artificial channel: (1) incorporation of the ion pair amphiphile; (2) molecular recognition and assembly formation; (3) connection of the pores in different lipid layers; (4) ion movement through the channel.

As the ion-conducting pore can be considered to be formed by oligoether chains, the obtained selectivity seems reasonable. Since the single-channel conductances in 0.5 M NaCl were almost the same as those in 0.5 M KCl, these channels do not seem to discriminate among monovalent cations. Thus, we concluded that the compounds can form nonselective cation channels spanning the lipid bilayer. The shorter and the longer oligoether chain molecules **3a** and **4a** exhibited almost the same ion channel properties. In sharp contrast to this, single-alkyl-chain compounds **3b** and **4b** did not successfully form stable ionic channels. We tested oligoether–carboxylic acids **1** and **2** and the ammonium-compounds having double alkyl chains separately, but none of them could form ionic channels.

All of the above features—conductance level, lifetimes of open and closed states, and ion selectivity—are strikingly similar to those observed in the synthetic polypeptide that exactly mimics the Sc_1 segment of the natural Na^+ channel protein.[8] This indicates that the ability to form transmembrane ionic channels is not an exclusive property of channel proteins and peptides. The present results provide a vehicle for exploring in detail the structure–function relationships of ion channels through synthesis of artificial ionic channels with simple and controllable structural features.

Discussion

Formation of an Ion Channel. Although speculative at present, the transfer of ions across a membrane via a channel mechanism may be visualized as in Figure 4, where four key steps are postulated: (1) stable incorporation of an amphiphilic carboxylate–ammonium ion pair into the bilayer membrane; (2) molecular recognition of polar oligoether chains by surrounding hydrophobic lipid molecules to induce self-association; (3) connection of polar domains in different lipid layers via discrimination of the polar molecular domain from the hydrophobic lipid domain; (4) transfer of ions from the aqueous phase into the channel via ion–dipolar stabilization and movement through the polar pore.

It should be emphasized that the tendency of the hydrophilic oligoether chain to separate from the hydrophobic membrane components may be the driving force for assembly formation and, presumably, for association of two half-channels located in different lipid layers.

The model structure that can account for the observations may be schematically shown as in Figure 5. Oligoether–carboxylates, whose chains are illustrated by nonshaded circles 1, 4, and 7, are assembled to constitute the polar inner surface of the ion channel. Each polar chain is surrounded by two hydrophobic, long alkyl chains from the ammonium counterpart, whose chains 2, 3, 5, 6, 8, and 9 are illustrated by shaded circles, to constitute the outer hydrophobic wall of the ion channel. The channel here illustrated may or may not be complete, depending on the requirement of

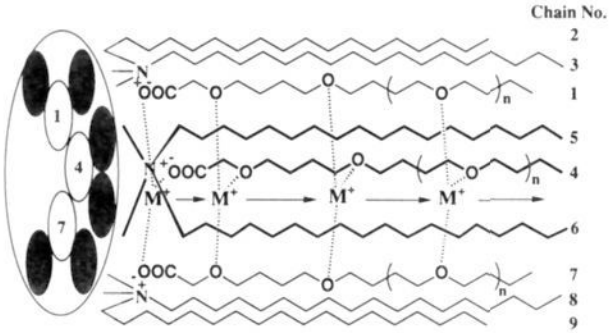

Figure 5. Schematic representation of a hypothetical half ion channel and cation movement in the channel.

the aggregation number, and should be regarded as hypothetical. The aggregation number is difficult to propose at present, but may be more than 3, considering the required three-dimensional stabilization of metal ions through coordination with neutral oxygen atoms. The existence of different conductance levels may represent different aggregation numbers as suggested in the case of synthetic polypeptides.[7,8] Metal cation M^+ is assumed to proceed, according to the membrane potential, through an inner hydrophilic cylinder by receiving successive stabilization via coordination from ether oxygens. Metal cation is then transferred to another half-channel located in the other lipid layer. The alkyl chains in the ammonium complex may make an important contribution to stabilization of the hydrophilic pore. Double alkyl chains seem to be very appropriate for shielding the inner hydrophilic pore with an outer covering that should be compatible with the surrounding hydrophobic lipid chains. As the compounds having a single alkyl chain failed to produce channel activity, the single chain seems insufficient to stabilize the pore.

Gating Mechanism. The open–closed transitions provide another interesting mechanistic problem. We postulate either (1) the association–dissociation reaction of the half-channel on each side of the bilayer suggested in the gramicidin channel[13] or (2) structural fluctuations in the proposed aggregate similar to synthetic transmembrane polypeptides.[7,8] This will be tested by using a new compound, presently being synthesized, that has a double-length oligoether chain.

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