Channel Forming Activity of an Anionic Amphiphilic Sequential Polypeptide in a Cationic Bilayer Membrane

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A channel forming activity of hydrophobic polypeptide, hydrophobic and anionic random copolypeptide, and anionic amphiphilic sequential copolypeptide, respectively, was investigated in a cationic bilayer membrane composed of dimethyldioctadecylammonium chloride (DOACl), and correlated with their location in the membrane estimated by fluorescence spectroscopic and microscopic measurements. A pure hydrophobic polypeptide, poly(γ-methyl L-glutamate) (PMG), was incorporated into DOACl bilayer membrane resulting from their hydrophobic interaction. The incorporation, however, allowed PMG to exhibit very little channel forming activity for sodium ion. A hydrophobic and anionic random copolypeptide, composed of γ-methyl L-glutamate and L-glutamic acid containing 30 mol% of L-glutamic acid (70/30 MG/GA) could hardly penetrate the bilayer membrane and was almost localized at the cationic membrane surface because of Coulombic interaction. 70/30 MG/GA did not effectively control the sodium ion permeability through the membrane. On the other hand, an anionic amphiphilic sequential polypeptide composed of γ-methyl L-glutamate and L-glutamic acid (am. -MG/GA) was incorporated into the cationic DOACl membrane to form the transmembrane bundle ca. 40 Å in diameter. This transmembrane bundle consisting of am. -MG/GA acted as a channel for sodium ion. However, lithium ion having larger hydration size than sodium ion could not penetrate through the channel.

It has been recognized that the amino acid sequence of membrane proteins in the biological membrane is closely related to the localization and function of the membrane proteins. For example,[1–7] integral membrane protein channels spanning the lipid bilayer were shown to consist of several parallel α-helices. And each helix contains both hydrophobic and hydrophilic amino acid side chains, periodically arranged so that all side chains facing the outside are hydrophobic, while those on the inside are hydrophilic. Studies of the channel forming synthetic polypeptide are important to the understanding general mechanism of structural and functional properties of biological membranes.

In a previous study,[8] we found an unique and simple technique for the preparation of an amphiphilic sequential polypeptide (am.-MG/GA), whose sequence is

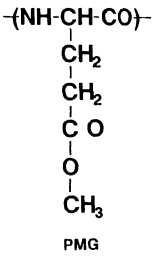
$$-(G_AG_AM_GM_GG_AM_GM_GM_GG_AM_GM_GG_AM_GM_GM_GG_AM_GM_G)_n-$$

where M_G and G_A denoted γ-methyl L-glutamate and L-glutamic acid residue, respectively. This method involves the formation of a solid condensed monolayer of poly(γ-methyl L-glutamate) (PMG) at an air-water interface and the selective saponification of the PMG side chains hydrated in the aqueous phase, keeping the remaining side chains oriented away from the aqueous phase unreacted. As a result, the α-helix of am.-MG/GA obtained is anionic on one face (G_A) and hydrophobic on the opposite face (M_G).

We report here, a channel forming activity of the anionic amphiphilic sequential polypeptide, am.-MG/GA, in the bilayer membrane and compared with those of the hydrophobic polypeptide, PMG, and a hydrophobic and anionic random copolypeptide consisting of M_G and G_A whose amino acid composition is about the same as that of am.-MG/GA.

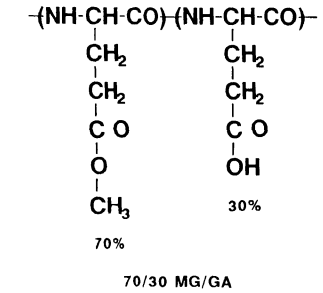
Experimental

Materials. Polypeptides; Poly(γ-methyl L-glutamate) (PMG) was obtained by polymerization of *N*-carboxyanhydride of L-glutamic acid γ-methyl ester in 1,2-dichloroethane solution with hexylamine as an initiator.[9] The molar ratio of the anhydride to initiator was 75. Polymerization occurred at room temperature for 24 h. The PMG obtained was precipitated in dry methanol. A molecular weight of 4400 was estimated from the viscosity measurements in dichloroacetic acid.



PMG

Poly(γ-methyl L-glutamate-co-L-glutamic acid)s with 3 mol% (97/3 MG/GA), 30 mol% (70/30 MG/GA) of L-glutamic acid residues were prepared by the homogeneous saponification of the PMG obtained in 1,2-dichloroethane solution.[10] The degree of polymerization did not decrease by saponification.



70/30 MG/GA

A preparation of an amphiphilic sequential copolypeptide consisting of γ-methyl L-glutamate and L-glutamic acid (am.-MG/GA) has been already reported in a previous study.[8] A solid condensed state of PMG monolayer was formed by using a L-B trough of moving wall type[11] (Nippon Laser & Electronics Lab., NL-LB240-MWA). When the area per monomer residue of PMG reached to 15 Å, a 2.5 M aqueous solution (1 M=1 mol dm^{-3}) of potassium hydroxide (KOH) was added in large excess into the aqueous phase beneath the solid condensed monolayer. The molar ratio of KOH to γ-methyl L-glutamate residue was 10^5. After ca. 10 min, excess hydrochloric acid was added to convert the copolypeptide to its acidic form. The resulting copolypeptide with 34 mol% of G_A residues, am.-MG/GA, whose sequence was

$$-(G_AG_AM_GM_GG_AM_GM_GM_GG_AM_GM_GG_AM_GM_GM_GG_AM_GM_G)_n-.$$

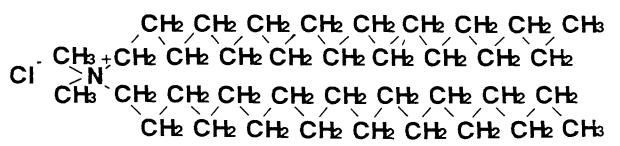
The am.-MG/GA formed an amphiphilic helix which is anionic on one face consisting of L-glutamic acid side chains (G_A) and hydrophobic on the opposite face comprising of γ-methyl L-glutamate side chains (M_G).[8]

Fluorescent Probes; PMG containing 2.7 mol% of 1-amino-8-anilinonaphthalene (AN) in the side chain (AN–PMG) was obtained by the condensation reaction of poly(γ-methyl L-glutamate-co-L-glutamic acid) containing 3 mol% of L-glutamic acid with AN in *N,N*-dimethylformamide using dicyclohexylcarbodiimide (DCC) and 1-hydroxy-1*H*-benzotriazole (HOBt) for 24 h at room temperature.[12] 70/30 MG/GA and am.-MG/GA with 3 mol% of AN in the side chains were obtained in the same way as AN–PMG. It should be noted that, in the fluorescent modification of am.-MG/GA, AN group was necessarily introduced to the anionic face of the amphiphilic α-helix rod.

The content of AN in the polypeptides was determined by fluorescence spectroscopic analysis.

N-(8-Anilino-1-naphthyl)propionamide (Pro-AN) as a model compound of the AN modified polypeptides was synthesized by the condensation reaction between propionic acid and AN using DCC and HOBt. DOACl vesicle solution containing am.-MG/GA labeling NH_2-terminal with 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD)[13] for fluorescence microscopic measurements was prepared as follows. DOACl vesicle solution, 10 ml, containing am.-MG/GA (6.0 μg) was incubated at 60 °C with 10 μl ethanol solution of NBD (10^{-2} M) for 1 min. After the reaction, this solution was rapidly quenched to 0 °C. The strong fluorecence could be observed with the resulting DOACl vesicle containing am.-MG/GA carrying NBD at the NH_2-terminal.

Amphiphile; Dimethyldioctadecylammonium chloride (DOACl) was kindly provided from Kao Co., Ltd.



DOACl

Vesicles; The DOACl vesicles containing polypeptides (PMG, 70/30 MG/GA and am.-MG/GA) were prepared as follows. PMG and 70/30 MG/GA, respectively, were dissolved in chloroform with DOACl. On the other hand, am.-MG/GA was dissolved in *N,N*-dimethylformamide with DOACl. These solutions of polypeptides and DOACl were poured into a glass flask, respectively, and then a thin film was formed in the inner surface of the flask by evaporating the solvent. Tris-HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffer solution (50 mM, pH 6.8) was added to this flask, and it was sonicated at 0 °C under a stream of nitrogen by using a Bionic model 7250B ultrasonic processor (SEIKO I & E/Sonic & Materials), for 10 min to prepare the vesicles. The phase-transition temperature of DOACl vesicle was 40 °C from differential scanning calorimetry.[14]

Methods. Spectroscopic Measurements; Fluorescence spectra of Pro-AN and AN modified polypeptides were obtained with a spectrofluorophotometer (Shimadzu, RF-540). The excitation wavelength of AN was 350 nm and the concentration of DOACl was 0.2 mg ml^{-1}. The molar ratio of polypeptides to DOACl was fixed at 4.2×10^{-4}.

Microscopic Measurements; Fluorescence microscopic measurements of DOACl vesicles containing am.-MG/GA with NBD at the NH_2-terminal, was carried out using a fluorescence microscope (Olympus Optical, Moded BHS-RFK, ×1250). The concentration of DOACl was 0.2 mg ml^{-1}. NBD was excited by a super high-pressure mercury lamp using BP490 and EY455 filters (Olympus Optical), and fluorescence emission was detected by a Olympus camera PM-10ADS. The molar ratio polypeptides to DOACl was fixed at 4.2×10^{-4}.

The shape of am.MG/GA in DOACl bilayer membrane was directly observed with a transmission electron microscope using a freeze-fracture replica technique.[15] An aliquot of the DOACl vesicle solution containing am.-MG/GA was placed in a thin gold plate, and this sample was rapidly plunged into liquid Fleon 22 (Balzers). The sample was stored in liquid nitrogen until fractured. Freeze-fracturing was carried out with a freeze-etching system (Balzers, BAF-400) at −115 °C and 10^{-6} mbar (1 bar=10^5 Pa). In order to prepare the reprica, platinum–carbon and pure carbon were evaporated at angles, 45° and 90°, respectively, to the specimen surface. Electron microscopy was carried out using a Hitachi H-500 electron microscope. The concentration of DOACl was 1.0 mg ml^{-1}. The molar ratio of polypeptides to DOACl was fixed at 9.4×10^{-4}.

Ionic Permeability; DOACl vesicle solution containing potassium gluconate in the interior was prepared as follows. DOACl (100 mg) was added to the 50 mM Tris-HEPES buffer, pH 6.8, (10 ml) containing potassium gluconate (0.1 M), and it was sonicated in a similar manner as above. The aqueous suspension of the vesicle was filtered off, and 150 mg of the residue was added to the buffer solution containing 0.1 M sodium or lithium gluconate as external solution, respectively. The total volume was 50 ml. The resulting vesicles had an ionic concentration gradient between the interior (0.1 M potassium ion) and exterior (0.1 M sodium or lithium ion) under isotonic conditions. The addition of polypeptides to the vesicle was performed by addition of *N,N*-dimethylformamide solution of polypeptides (1 mg ml^{-1}). The pure *N,N*-dimethylformamide did not induce any changes in the rate of the potassium ion permeability. The amount of polypeptide added was

6.8×10^{-9} mol. The amount of potassium ion that transported across the vesicular bilayer before and after the addition of polypeptide, was detected with an ion meter (Horiba Co., Ltd., N-7ionII) at 20 °C. In this case, because of electroneutrality the number of potassium ion transported from vesicular interior to the external solution is the same as that of sodium or lithium ion from the external to the interior. Further, the cations, sodium and lithium ions, both having larger hydration size than that of potassium ion determine the permeability across the membrane for the cationic pairs, potassium/sodium and potassium/lithium, respectively.

Results and Discussion

Location of Polypeptides in DOACl Bilayer Membrane. We have already reported [14,16] the interaction between polypeptides and a cationic bilayer membrane consisting of dimethyldioctadecylammonium chloride (DOACl), indicating that the hydrophobic polypeptide, PMG, was readily incorporated into the hydrophobic membrane interior to form membrane-spanning helix, however, the incorporation of hydrophobic and anionic random copolypeptides, MG/GA, containing more than 30 mol% of L-glutamic acid residues could not occur, and they were almost localized at the membrane surface.

We elucidated the partition of amphiphilic sequential polypeptide in the cationic bilayer membrane consisting of DOACl by means of fluorescence spectroscopic measurements. It is well-known that fluorescence characteristics, such as emission maxima and fluorescence intensity, of anilinonaphthalene derivatives are very sensitive to the environmental polarity around the fluorescence probes.[17] Figure 1 shows fluorescence spectra of AN modified polypeptides (AN–PMG, AN–70/30 MG/GA and AN–am.-MG/GA) in DOACl vesicle solution. The relationship between the emission maxima, λ_{max}, of AN modified polypeptide in DOACl vesicle solution and the empirical solvent polarity, Z,[18] was shown in Fig. 2. The solid line in the figure shows the relationship between the Z value and λ_{max} of Pro-AN, the fluorescent model compound for AN modified polypeptides, in various solvents. It is apparent that the λ_{max} of Pro-AN shifts to higher wavelength with increasing solvent polarity. The λ_{max} of AN–PMG, hydrophobic polypeptide, in DOACl vesicle solution, 453 nm, corresponds to Z value of octane. However, the λ_{max} of AN–70/30 MG/GA, hydrophobic and anionic random copolypeptide, in DOACl vesicle solution, 499 nm, corresponds to Z value between methanol and water. It may say, therefore, that PMG is readily incorporated into DOACl membrane interior, whereas 70/30 MG/GA is almost localized at the cationic surface of the membrane. On the other hand, the λ_{max} of AN–am.-MG/GA in DOACl vesicle solution, 470 nm, corresponds to Z value between AN–PMG (membrane interior) and AN–70/30 MG/GA (membrane surface). As was described in the experimental section, the fluorescent AN group was localized at the anionic face of the amphiphilic α-helix rod. Therefore, the λ_{max} of 470 nm means a possibility that the anionic face of AN–am.-MG/GA is accessible to neither the membrane surface nor the hydrocarbon chains of DOACl.

To elucidate the partition of am.-MG/GA in DOACl vesicle solution more clearly, freeze-fracture electron microscopy of DOACl bilayer membrane vesicles containing am.-MG/GA was carried out. Figure 3 shows the freeze-fracture electron micrograph of DOACl bilayer membrane vesicle containing am.-MG/GA. Initial magnification was ×40000. The

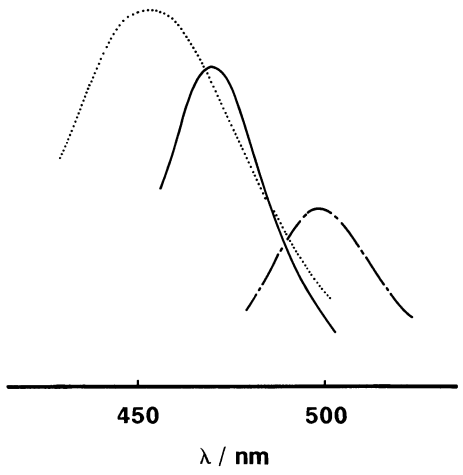


Fig. 1. Fluorescence spectra of AN modified polypeptides in DOACl bilayer membrane; (·····): AN–PMG, (—·—): AN–70/30 MG/GA, (——): AN–am.-MG/GA, [DOACl]=0.02 wt%, [polypeptide]/[DOACl]=4.2×10^{-4}.

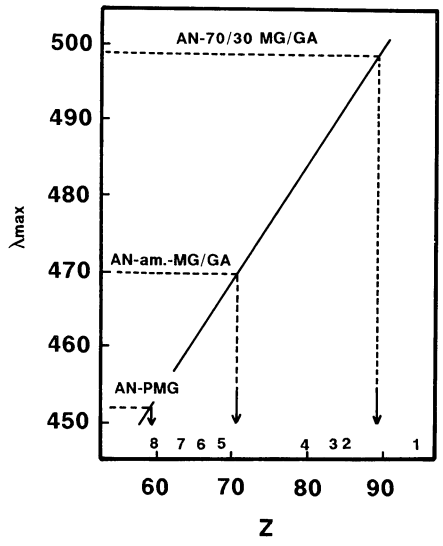


Fig. 2. Relation between fluorescent emission maxima of Pro-AN and empirical Z solvent polarity. [Pro-AN]=3.0×10^{-5} M, (1): water, (2): methanol, (3): ethanol, (4): 2-propanol, (5): *N*,*N*-dimethylformamide, (6): acetone, (7): chloroform, (8): octane.

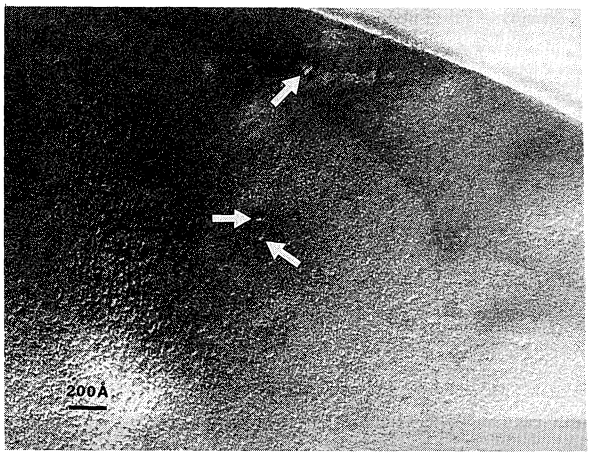


Fig. 3. Transmission electron micrograph of DOACl bilayer vesicle prepared by freeze-fracture, initial magnification×40000, [DOACl]=0.1 wt%, [am.-MG/GA]/[DOACl]=9.4×10^{-4}.

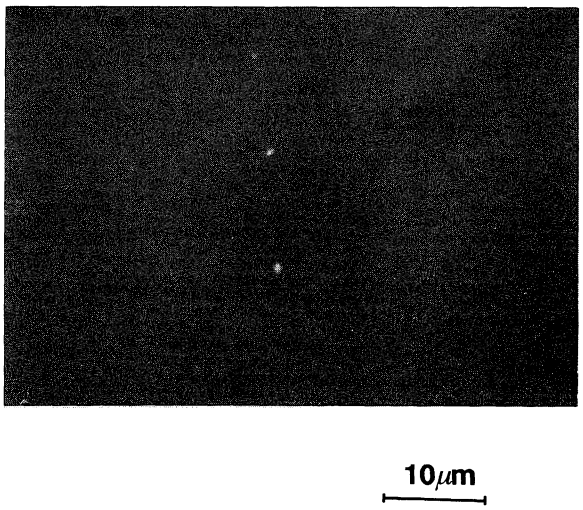


Fig. 4. Fluorescence micrograph of DOACl vesicles containing am.-MG/GA labeled at the NH_2-terminal with NBD, initial magnification×1250, [DOACl]=0.02 wt%, [am.-MG/GA]/[DOACl]=4.2×10^{-4}.

intramembranous particles were clearly observed in the electron micrograph (Fig. 3). These particles displayed annular shapes ca. 40 Å in diameter. The size of the particles and their annular nature suggests that the intramembranous particles consist of cylindrical aggregate of several am.-MG/GA helical rods, since a single helical rod of polypeptide would not be large enough to make the annular structure seen in Fig. 3. Further, in the fluorescence micrograph of DOACl vesicles containing am.-MG/GA labeled with NBD, the fluorescence emission of NBD could be observed as the spherical particles (vesicles) on the dark background (aqueous phase) (Fig. 4). This may also support the incorporation of am.-MG/GA into the cationic bilayer membrane. These results suggest

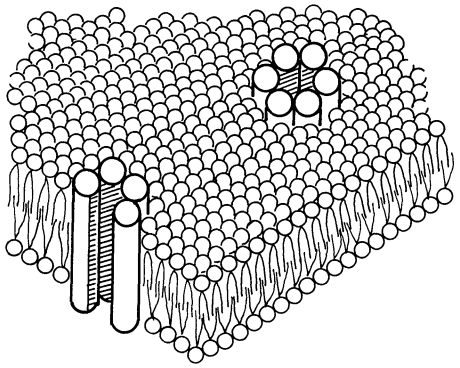


Fig. 5. A proposed structure of am.-MG/GA–DOACl membrane system. am.-MG/GA aggregates consist of bundles of paralleled α-helices oriented perpendicular to DOACl bilayer membrane surface. Shaded surfaces represent location of hydrophilic amino acid side chains and unshaded surfaces are hydrophobic.

the following possibilities concerning the partition of am.-MG/GA in DOACl bilayer membrane. The am.-MG/GA can be incorporated into the DOACl membrane resulting from the formation of transmembranous bundles composed of several am.-MG/GA molecules. The hydrophobic residues of each am.-MG/GA are on the exterior surface of the transmembrane bundle to contact with the hydrocarbon region of DOACl membrane. While, the hydrophilic (anionic) faces, which are accessible to neither the membrane surface nor the hydrocarbon region of the membrane, are in contact with each other in the interior of the transmembrane bundle. These speculations may give a visual model shown in Fig. 5 as a possible structure of the membrane system.

A Channel Forming Activity of Polypeptides Incorporated into DOACl Bilayer Membrane Vesicle. Figure 6 shows the rate of sodium ion permeation through the bilayer membrane of DOACl vesicle. The permeabilities of sodium ion through the bilayer membrane were measured before and after addition of polypeptides, am.-MG/GA, 70/30 MG/GA and PMG, respectively, at 20 °C. The sodium ion permeability was almost independent on the addition of 70/30 MG/GA, since 70/30 MG/GA,[16] as is noted above, could hardly penetrate the bilayer membrane and were almost localized at DOACl membrane surface owing to the electrostatic interaction. Furthermore, it is found that the addition of PMG can induce little increase in the sodium ion permeability. The little increase in permeability through the membrane with PMG, in spite of the easy incorporation of PMG into the membrane, is associated with the absence of the permeation site for cation in PMG.[16] On the other hand, the degree of increase in the permeability induced by the addition of am.-MG/GA was much larger than that by the addition of the other polypeptides (PMG

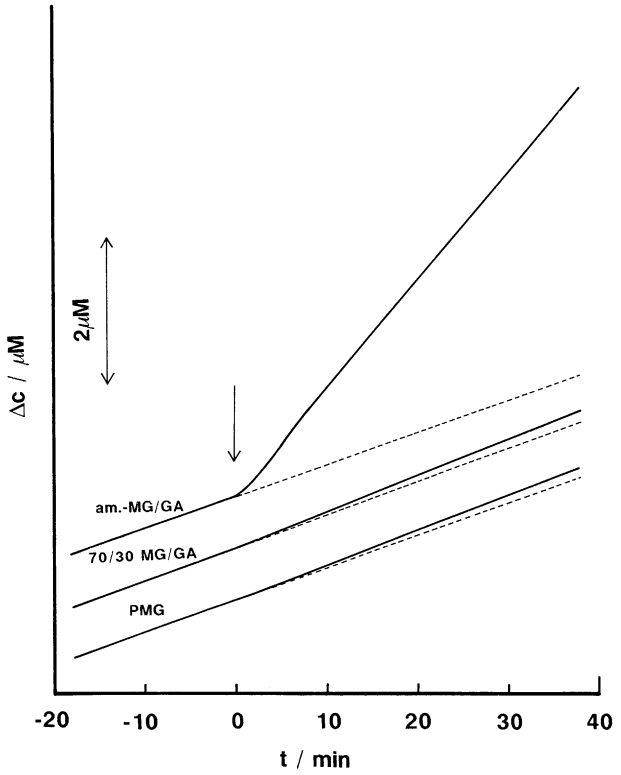


Fig. 6. Effect of polypeptides, PMG, 70/30 MG/GA and am.-MG/GA, addition on the rate of sodium ion permeation through the bilayer membrane consisting of DOACl at 20 °C. The arrow marks the time at which the polypeptides were introduced.

and 70/30 MG/GA). This result may be explained in terms of the hypothetical molecular arrangement of am.-MG/GA in the bilayer membrane based on the fluorescent and microscopic studies. That is, am.-MG/GA aggregates each other to form transmembrane bundle having the hydrophobic exterior and hydrophilic inner pore surrounded by anionic surface. Through the anionic pore the cation may be transported.

It was found, furthermore, that the ionic permeability through the ion channel formed by the am.-MG/GA aggregate in DOACl bilayer membrane was strongly dependent on a permeant ion size. That is, lithium ion was used as the external cation in the place of sodium ion, the channel-forming activity of am.-MG/GA was disappeared. Table 1 shows the ratio of the permeability coefficient, P/P_0, (P and P_0 are the permeability coefficients of the bilayer membrane with and without am.-MG/GA, respectively.), and the Stokes' radius, r_{is}, of external cation. The large hydrated ion, lithium ion hardly permeates through the ion channel composed of several am.-MG/GA molecules, however, sodium ion having smaller hydration size than that of lithium ion, easily permeates through the channel. Thus, this result shows that the ion channel formed by transmembranous bundle composed of several am.-MG/GA molecules in DOACl bilayer membrane recognizes the cation by its hydrated size.

Table 1. The Ratio of Permeability Coefficient, P/P_0, and the Stokes' Radius, r_{is}, of External Cation

External cation	Sodium	Lithium
r/Å	1.83	2.37
P/P_0	3.4	1.0

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