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CALCIUM-SENSITIVE UNIVALENT CATION CHANNEL FORMED BY LYSOTRIPHOSPHOINOSITIDE IN BILAYER LIPID MEMBRANES

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Summary

A calcium sensitive univalent cation channel could be formed by lysotriphosphoinositide on an artificial bilayer membrane made of oxidized cholesterol. The modified membrane was selectively permeable to univalent cations, but was only very sparingly permeable to anions or divalent cations. Selectivity sequence among group IA cations was $Rb^+ > Cs^+ > Na^+ > K^+ > Li^+$. The conductance of the membrane was increased up to a value of about 10^{-2} ohm^{-1}/cm^2 with an increase in the concentration of univalent cation, and was drastically depressed by a relatively small increase in the concentration of calcium ion or other divalent cations. The sequence of depressing efficiency among divalent cations was $Zn^{2+} > Cd^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+}$.

Introduction

Inositol phospholipids have been known to be present in a wide variety of mammalian tissues and their various physiological activities have been reviewed recently [1].

Triphosphoinositide has particularly been regarded as an important constituent of the nervous system, because of the high turnover rate of their phosphate moiety [2—4]. The predominant localization of brain triphosphoinositide in the myeline sheath has been well established [5—7]. However, the rapid post-mortem depletion of triphosphoinositide has also been reported

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[8—10] and recently Eichberg and Hauser have claimed the localization of this substance at the cell membrane of nervous systems including synapse or axon [11,12].

Triphosphoinositide has unique physicochemical characteristics. Since triphosphoinositide has five net negative charges at physiological pH, it can be dissolved into water very smoothly. On the other hand, it shows an extraordinarily high affinity for calcium ion, resulting in a highly hydrophobic complex with Ca^{2+} [13,14]. Thus, triphosphoinositide shows a drastic change in hydrophilicity, when the univalent-divalent cation exchange reaction occurs.

A hypothesis was proposed to explain the molecular mechanism of nerve excitation by Tasaki and his coworkers [15]. In their hypothesis, it was proposed that an essential process of nerve excitation is a kind of phase transition of a certain component of the excitable membrane; the phase transition is caused by desorption of calcium ion from the membrane surface. No material has, however, been proposed as the key substance which undergoes such a phase transition in the excitable membrane.

We intended to incorporate the triphosphoinositide into an artificial membrane as a component which could undergo a hydrophilic-hydrophobic micelle transition with univalent-divalent cation exchange. However, triphosphoinositide had no effect on the conductance of artificial bilayer membrane of oxidized cholesterol. In contrast, a very little amount of lysotriphosphoinositide which was an oxidative breakdown product of triphosphoinositide was found to give a remarkably large conductance to the artificial membrane. The lysotriphosphoinositide could penetrate into the membrane and formed a channel system which was selectively permeable to univalent cations. Moreover, the univalent cation conductance of the membrane was greatly influenced by the concentration of calcium ion in the aqueous phase.

We will report some features of this univalent cation channel constructed from the polar head groups of lysotriphosphoinositides.

Materials and Methods

Extraction of triphosphoinositide

Triphosphoinositide was obtained by Folch fractionation of bovine brain [16]. The crude lipid was further purified by chromatography on DEAE-cellulose as described by Hendrickson et al. [17]. This sample gave a single spot in formaldehyde-treated paper chromatography and could be stored safely in wet chloroform at $-20°C$ in the dark. The formaldehyde-treated papers were prepared by the method of Kai et al. [18].

Preparation of lysotriphosphoinositide

An aqueous solution of triphosphoinositide (3 mg/ml) in a quartz cell was irradiated with ultraviolet rays (2537 Å in wavelength from a sterilizing lamp; about 3300 erg/cm^2 per s) for a week at room temperature. The triphosphoinositide molecules autooxidized and the material obtained gave a single spot in formaldehyde-treated paper chromatography just below the spot of triphosphoinositide.

With the aim of characterizing this sample the molar ratio of ester fatty

acid to phosphorus was determined. The fatty acid ester was determined by the method of Snyder et al. [19] and the phosphorus was determined by the method of Bartlett [20]. It was found that the oxidized triphosphoinositide contained one fatty acid ester per three phosphate residues. This result implied that triphosphoinositide molecules were completely oxidized and broken down into lysotriphosphoinositide molecules.

It was also found that this oxidized sample contained other components than lysotriphosphoinositide such as free fatty acids or malonaldehyde. Free fatty acid was observed on silicic acid thin layer chromatography and also observed by colorimetric analysis [21]. Malonaldehyde which indicates the degree of lipid peroxidation was determined with a TBA test [22]. But none of them seems to contribute to following results (See Discussion).

Membrane formation and modification

Oxidized cholesterol was obtained by the method of Jain [23]. That is, a 4 percent solution of cholesterol (99% pure, Sigma Chemical Co.) in 25 ml of 1 : 1 mixture of *n*-decane and *n*-tetradecane was refluxed at boiling temperature for 20 h and stirred vigorously with a Teflon magnetic stirrer bar. The resulting solution was then cooled for overnight and a supernatant was obtained. The supernatant was useful for forming a bilayer membrane stable for about a month. The alkanes were of the best grade available commercially.

The chamber shown in Fig. 1 was used for forming membranes. The chamber had vertical septum (1.5 mm thickness) in which the membrane-forming orifice (1.3 mm diameter) was located. The volume of each compartment was about 7 ml. The chamber was made of Daiflon (a resin of trifluoromonochloroethylene, purchased from Daikin Engineering Co. Ltd.). A feeder hole (0.7 mm diameter) was drilled through the chamber septum to the membrane hole. This passage was connected externally to a microsyringe by means of a thin Teflon tube. The glass window was attached to the chamber with Araldite (Ciba-Geigy) and left for over two weeks in order to eliminate the irritating vapor from the Araldite.

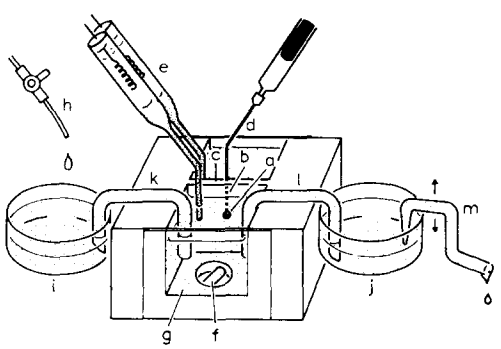

Fig. 1. Schematic diagram of apparatus for the formation of the membrane and for the perfusion of aqueous solution. a, membrane hole (1.3 mm dia.); b, feeder hole (0.7 mm dia.); c, septum (1.5 mm thickness); d, Teflon tube; e, KCl/agar bridge and Ag/AgCl electrode; f, magnetic stirrer bar; g, glass window; h, inlet of perfusing solution (Teflon tube with a stopcock); i and j, buffering vessel; k and l, siphon; m, level-controlling siphon.

Each chamber was filled with 5 ml of 10 mM Tris/Cl (pH 7.5) and a thick biconcave lens of oxidized cholesterol solution was formed at the hole by introducing an adequate volume of the solution. The oxidized cholesterol was then sucked up carefully by the syringe so that a membrane of the desired area might be formed. Lysotriphosphoinositide and $CaCl_2$ were added to both compartments through a micropipette after the membrane had become completely black. The bilayer membranes were incubated in this solution for about five minutes with gentle stirring. A small quantity of concentrated solution of electrolytes was added to either one or both aqueous phases with a micropipette in order to change the ionic composition. All electrolytes employed were the best grade available commercially, and which were dissolved in the double-distilled water.

Perfusion technique

A perfusion technique was used to eliminate the lysotriphosphoinositide from the aqueous phases. The perfusing apparatus consisted of two buffering vessels, two siphons and a level controlling siphon. All of them were made of glass. The level-controlling siphon (m) was vertically movable and had a needle tip at one end. The surface tension kept the water from flowing out when the tip was detached from the surface of the solution in the second buffering vessel (j).

The procedure of the perfusion was as follows. The tip of the level controlling siphon was lowered into the second buffering vessel in order to suck up the extensive solution as soon as a new solution ran into the first buffering vessel from the inlet. The water level of the test side compartment was kept constant by adjusting the height of the controlling siphon (m).

Electrical measurement

The current-voltage curve of this membrane was linear provided that the applied voltage was less than 20 mV. Therefore, zero current conductance of the membrane was obtained by applying a 10 mV rectangular voltage of 0.5-s duration and by measuring the corresponding current using a low-drift FET input operational amplifier (Teledyne-Philbrick, 1023). Rectangular voltage was applied to the membrane by using a stimulator (Nihonkoden, MSE-3R) and isolater (Nihonkoden, MSE-JM) followed by a low output impedance operational amplifier, 1009. A two channel recorder (National, VP-6521-A) connected to the FET input operational amplifier (Teledyne-Philbrick, 1009) through one channel allowed the recording of the voltage applied or the membrane potential. The other channel was connected to the output of the ammeter operational amplifier to record the corresponding current.

An Ag/AgCl electrode was employed for electrical measurement. It made contact with the saturated KCl solution in the shank of the glass tube which was connected to the aqueous phase of the cell by means of a saturated KCl/agar bridge (See Fig 1.).

The measurements were done at room temperature ($20 \pm 2^{\circ}C$).

Results

The basic observation is shown in Fig. 2. Upon addition of 10^{-6} g/ml lysotriphosphoinositide and 1 mM $CaCl_2$ in final concentration to both sides of

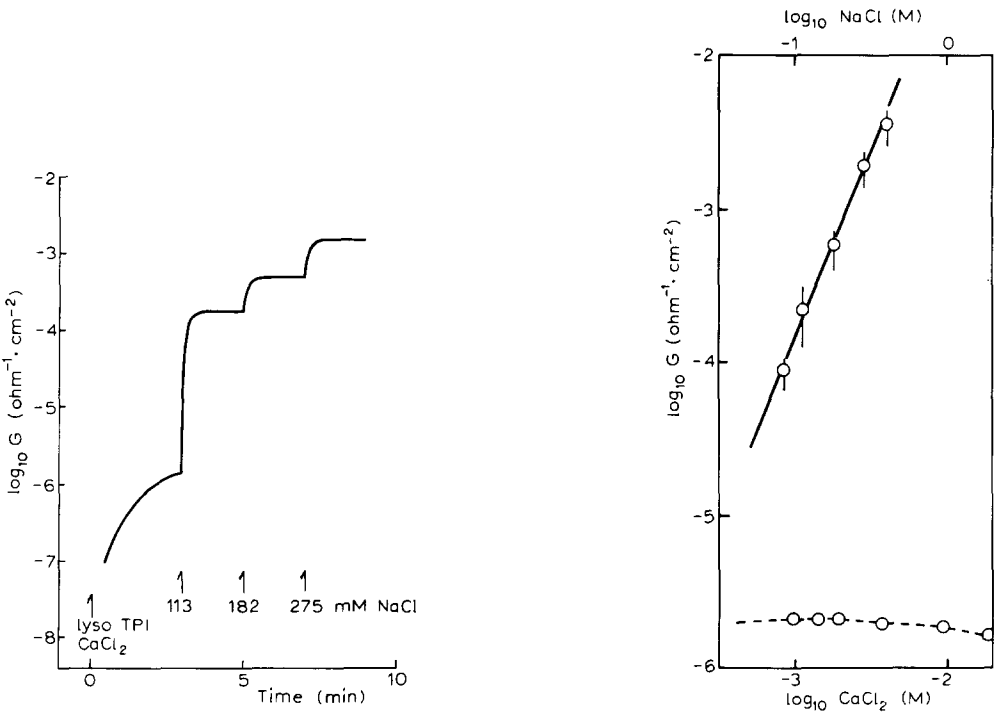

Fig. 2. Time course of the membrane conductance change of a black membrane modified with 10^{-6} g/ml lysotriphosphoinositide and 1 mM $CaCl_2$. The first arrow indicates the time at which lysotriphosphoinositide and $CaCl_2$ were added to both the aqueous phases containing 10 mM Tris/Cl (pH 7.5). Aliquots (100 μl) of 2 M NaCl were added to both aqueous phases. Final concentrations are indicated in the figure.

Fig. 3. Plot of membrane conductance vs. concentration of electrolyte solution. Upper curve was obtained when the concentration of NaCl was increased in the presence of 1 mM $CaCl_2$. Lower curve was obtained when the concentration of $CaCl_2$ was increased in the absence of group IA cation. The data represent mean values (±S.D.) of at least four membranes.

a black membrane made of oxidized cholesterol, an increase in the membrane conductance was observed. The conductance gradually increased from about 10^{-9} ohm^{-1}/cm^2 to about 10^{-6} ohm^{-1}/cm^2 within 5 minutes. However, no further increase in conductance was found by the subsequent additions of $CaCl_2$ (lower curve in Fig. 3).

The next great step of increase in the membrane conductance was observed when a small aliquot of a concentrated solution of uni-univalent electrolyte was microinjected into both aqueous phases (indicated by arrows in Fig. 2). The increase in the conductance caused by the addition of uni-univalent electrolyte depended on the concentration of the electrolyte, and the conductance could be increased to as large as the order of 10^{-2} ohm^{-1}/cm^2. As shown in Fig. 3, the conductance was proportional to about the second power of the concentration of the uni-univalent electrolyte (upper curve in Fig. 3).

No change in the membrane conductance was observed when the membrane was incubated in the solution containing triphosphoinositide and $CaCl_2$. That is, triphosphoinositide which was not subjected to autoxidation was unable to modify the oxidized cholesterol membrane. When the membrane was

not black, i.e. colored membrane or oil film, no conductance change was generated even with the lysotriphosphoinositide.

In order to know the species of ion which could permeate this membrane, membrane potential was measured first against the concentration gradient of KCl across the membrane. As shown in Fig. 4, the membrane potential could be fitted successfully with the Goldman equation, if the ratio of the permeability coefficient (P_{Cl^-}/P_{K^+}) was assumed to be 0.03.

On the other hand, a concentration-gradient of $CaCl_2$ generated no membrane potential. Concentration gradient of $CaCl_2$ (1 : 20 mM) did not influence the membrane potential which was generated by univalent cation. These results implied that this membrane was highly selectively permeable to the univalent cation, and is only very sparingly permeable to both calcium ion and anion.

Fig 5 presents the "fingerprint" characteristic of the membrane modified with lysotriphosphoinositide. The logarithm of the experimentally observed permeability ratio [24] has been plotted as a function of the reciprocal of the naked [25] cation radius. Selectivity sequence among group IA cations was $Rb^+ > Cs^+ > Na^+ > K^+ > Li^+$, and NH_4^+ was markedly "supra-IA" [26].

No effect on the membrane conductance was observed by the elimination of lysotriphosphoinositide in the aqueous phase by perfusing the solution with a lysotriphosphoinositide-free saline solution. This fact suggested that the lysotriphosphoinositide molecule once incorporated in the membrane could never be dissolved into the aqueous phase.

The univalent cation conductance was greatly influences by calcium or other divalent cations. Fig. 6 shows the change in membrane conductance when $CaCl_2$ was added to one side in the presence (Fig. 6a) and in the absence (Fig. 6b) of lysotriphosphoinositide in the aqueous solution. As shown in

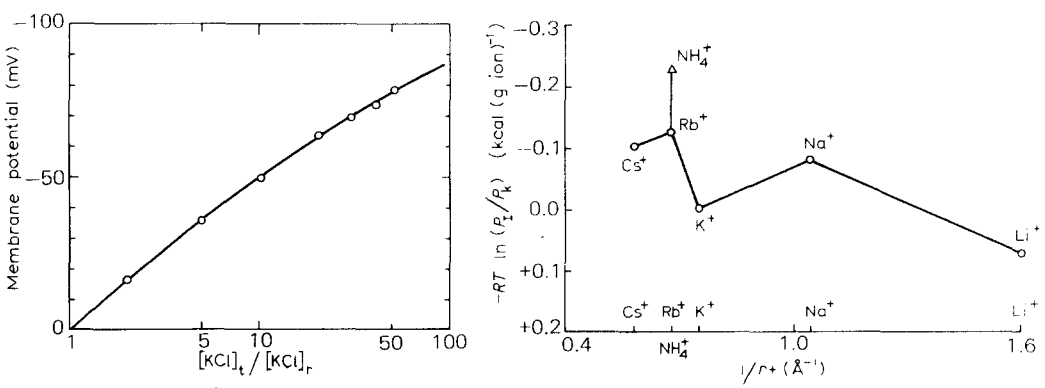

Fig. 4. Membrane potential generated by the concentration gradient of KCl. The concentration of KCl at the reference side was fixed at 8 mM. The subscripts "t" and "r" represent the two aqueous phases separated by the membrane (test and reference side). The solid line indicates the membrane potential calculated from the Goldman equation by assuming $P_{Cl^-}/P_{K^+} = 0.03$.

Fig. 5. Selectivity "fingerprint" for permeation through the channel made of lysotriphosphoinositide. The logarithm of the permeability ratios relative to K^+ determined from zero-current potential measurements of a bilayer membrane is plotted against the reciprocal of the naked cation radius. The ionic strength of the each aqueous phase was equalized. Reference side: 400 mM KCl + 40 mM MeCl (Me : group IA cation). Test side: 40 mM KCl + 400 mM MeCl.

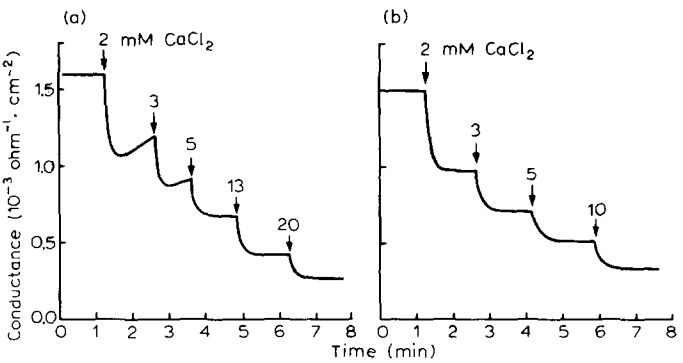

Fig. 6. Time course of the membrane conductance when $CaCl_2$ was added to one side of compartment (a) in the presence and (b) in the absence of lysotriphosphoinositide. Both aqueous phases contained initially 10^{-6} g/ml lysotriphosphoinositide, 1 mM $CaCl_2$, 275 mM NaCl and 10 mM Tris/Cl (pH 7.5). In the case (b), the aqueous phase was perfused with the lysotriphosphoinositide-free solution containing same salt contents, and then 10 μl aliquots of 500 mM $CaCl_2$ were added successively to one side through a micropipette at the time indicated.

Fig. 6a, there were two opposite aspects in the effect of calcium ion in the membrane conductance. The one is a rapid decrease just after the microinjection of calcium ion, and the other is a subsequent gradual increase. However, if the lysotriphosphoinositide was eliminated from the bulk solution by per-

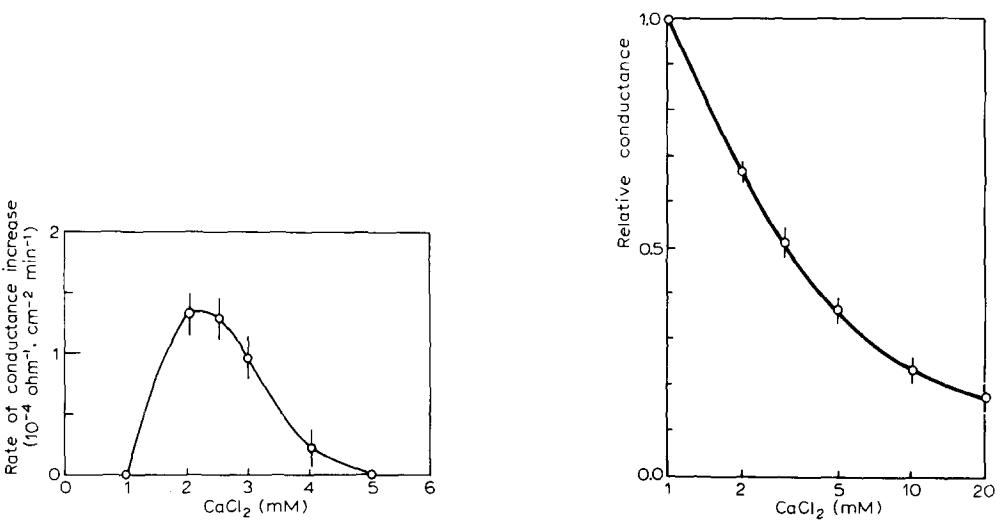

Fig. 7. The dependence of the rate of conductance increase to the concentration of calcium ion added to one side. Both aqueous phases contained 10^{-6} g/ml lysotriphosphoinositide, 275 mM NaCl, 1 mM $CaCl_2$ and 10 mM Tris/Cl (pH 7.5) initially. The data represent mean values (±S.D.) of at least three membranes.

Fig. 8. Plot of relative conductance vs. concentration of $CaCl_2$ added to one side. Both aqueous phases contained initially 10^{-6} g/ml lysotriphosphoinositide, 1 mM $CaCl_2$, 275 mM NaCl and 10 mM Tris/Cl (pH 7.5). After the test side aqueous phase was perfused with lysotriphosphoinositide-free solution containing same salt contents, 10-μl aliquots of 500 mM $CaCl_2$ were added to the test side through micropipette. The relative conductance was obtained by dividing the value of membrane conductance with that of the membrane in the initial condition (1 mM $CaCl_2$). The data represent mean values (±S.D.) of 6 membranes.

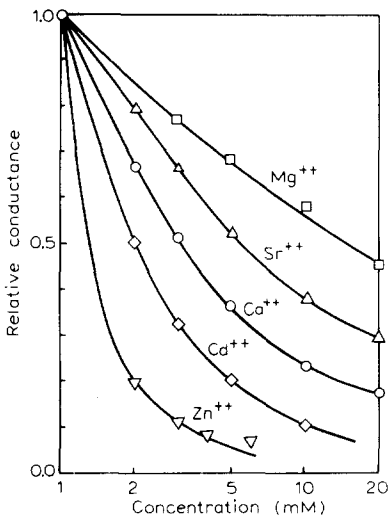

Fig. 9. Plot of relative conductance vs. concentration of divalent cation added to one side. Both aqueous phases contained initially 1 mM $CaCl_2$, 275 mM NaCl and 10 mM Tris/Cl (pH 7.5). The 10 μl aliquots of 500 mM solution of divalent cation were added to the test side after the perfusion.

fusion, the later conductance increase disappeared. The presence of lysotriphosphoinositide in the aqueous phase, therefore, is the cause for the latter effect of calcium ion on the membrane conductance.

The rate of rise in conductance changed with the concentration of calcium ion as shown in Fig. 6a. The rate was plotted against the concentration of calcium ion in Fig. 7. The curve had a peak for the $CaCl_2$ concentration at about 2—3 mM.

Fig. 8 illustrates the relative conductance as a function of the concentration of calcium ion which was added to one side of the membrane after the elimination of lysotriphosphoinositide by perfusion. The relative conductance in this figure means a value of the membrane conductance relative to that observed in the presence of 1 mM $CaCl_2$ in both compartments.

Though divalent cations other than calcium could also depress the membrane conductance, their effectiveness differed as shown in Fig. 9. A small aliquot of 500 mM solution of divalent cation was added to one side of the membrane and the amount of conductance decrease was measured. Both aqueous phases always contained 275 mM NaCl, 1 mM $CaCl_2$ and 10 mM Tris/Cl (pH 7.5). The order of the efficiencies was $Zn^{2+} > Cd^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+}$.

Discussion

It is known that a phospholipid having double bonds in its hydrocarbon chain is susceptible to the peroxidation induced by ionizing radiation, ascorbate or metal ions [22,27,32], and that the subsequent chain of metabolic conversion of phospholipid acyls thus started forms lysophospholipid and other peroxidation products [27—32]. Most of the ester fatty acid of triphosphoinositide is arachidic acid ($C_{20:4}$) at the β-position [33]. We found that the oxidized triphosphoinositide contained three phosphorus atoms per one fatty

acid ester. Therefore, it is highly likely that triphosphoinositide was broken down into lysotriphosphoinositide being subjected to peroxidation induced by ultraviolet irradiation. On the other hand, the oxidized triphosphoinositide used in this experiment contained free fatty acids, malonaldehyde and possibly short chain acyl fragments as the products of lipid peroxidation. However, we found that purified lysotriphosphoinositide obtained from DEAE-cellulose column caused similar phenomena in bilayer membranes of oxidized cholesterol (unpublished). These components other than lysotriphosphoinositide might not have a role in this ion-conducing system.

Such a lipid peroxidation has been observed in some biological membranes. For example, a light-dependent lipid peroxidation was observed in rod outer segment of retina [34] and in the solution of chlorophyll [35]. Also, such a lipid peroxidation was observed in mitochondria [36—38] and microsomes [28,30,32], when they were incubated with ascorbate of NADPH or Fe^{+}. The lipid peroxidation in these membranes may bring about a formation of lysophospholipid including lysotriphosphoinositide.

It is well known that lysophospholipid such as lysolecithin behaves as a detergent and has lytic activity to the membrane. Relatively low concentrations cause complete disorganization and disruption of lipid bilayers [39—41]. On the contrary, lysotriphosphoinositide did not disrupt bilayer membranes even if its concentration was high. Lysotriphosphoinositide caused several order higher conductance than lysophosphatidylcholine and the conductance was susceptible to the concentration of calcium ion.

As mentioned in the Results, lysotriphosphoinositide could not induce ionic conductance in the thick (colored) membrane which had a bulk oil phase between two oil-water interfaces. This result implied that the lysotriphosphoinositide could not carry ions through the oil phase as a "carrier". Therefore, it is suggested that they formed a ion conducting channel mechanism in the membrane.

In this experiment, calcium ion caused two opposite effects on the membrane conductance in the presence of a relatively high concentration of univalent cation (Fig. 6a). As previously mentioned, the conductance increased at a constant rate by addition of calcium ion just after the initial decrease. This latter increase could not be observed in the absence of lysotriphosphoinositide in the aqueous phase (Fig. 6b). Therefore, the increase in conductance can be attributed to the additional incorporation of lysotriphosphoinositide from the bulk solution into the membrane. Abramson et al. [42] showed that the hydrophobicity of acidic phospholipid in the presence of calcium ion was reduced by coexistence of a relatively high concentration of univalent cation. Therefore, the hydrophobicity of lysotriphosphoinositide in 1 mM $CaCl_2$ solution should be depressed to considerably low value by addition of enough amount of Na^{+}. Thus, the mechanism of the later increase in the conductance be explained as followings. The hydrophobicity of lysotriphosphpinositide increases with increase in the concentration of calcium ion (1 mM to several mM). Thereby, incorporation of lysotriphosphoinositide into the membrane will be accelerated. The absorbed lysotriphosphoinositide molecules immediately form univalent cation channels, resulting in the increase of the membrane conductance.

The rate of conductance increase by addition of $CaCl_2$ had a peak value at about 2—3 mM $CaCl_2$ (Fig. 7). If the conductance increase is due to the incorporation of lysotriphosphoinositide from the aqueous phase as mentioned before, the rate of its incorporation will increase with its hydrophobicity. From turbidity measurement, we have also observed a maximum hydrophobicity of lysotriphosphoinositide in an aqueous medium at a defined concentration of $CaCl_2$ (unpublished). Such a decrease of hydrophobicity may be caused by the charge reversal of its micelle by binding with excess calcium ion. Dawson et al. [43] showed that the excess calcium ion reversed the sign of the surface potential of an acidic phospholipid monolayer on an air/water interface. If a similar phenomenon occurs at the surface of lysotriphosphoinositide micelle in the water, their hydrophobicity will become maximum when the charge of the micelle is neutralized by calcium ions.

On the other hand, the conductance decrease by addition of calcium ion could not be interpreted simply in terms of electrostatic concepts, since the effectiveness for decreasing the membrane conductance differed among divalent cations (Fig. 9). Such an effectiveness seems to depend on the affinity of divalent cation to the head group of lysotriphosphoinositide. Blaustein and Goldman [44] studied the shift of activation of sodium channel of nerve with changes of external divalent cation concentration by means of voltage clamp. With regard to the effectiveness for the shift, they ranked divalent cations in the following sequence: $La^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+} > Cd^{2+} > Ba^{2+} > Ca^{2+} > Mg^{2+}$. Hille et al. [45] also showed similar sequence or the affinities of divalent cations to negative surface charge near sodium channels of nerve. These sequences are same as ours.

Our results show that calcium ion having high affinity for lysotriphosphoinositide has extremely low permeability in the membrane, whereas univalent cation having relatively low affinity for the lysotriphosphoinositide has very high permeability. These results suggest that calcium ion causes some modification of the channel made of lysotriphosphinositide and blocks the channel passway.

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