ON THE PHYSIOLOGICAL SIGNIFICANCE OF POLYPHOSPHOINOSITIDES

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CONTENTS

PREFACE

A group of quantitatively minor phospholipids in the plasma membrane, i.e. polyphosphoinositides (PPIs) has become of interest to cell biologists in recent years. Substantial evidence has accumulated to show that the PPIs play a crucial role in information transmission through the plasma membrane. The PPIs consist of two components, i.e., tri- and di-phosphoinositide (TPI and DPI). Particularly, the cleavage of the TPI by receptor-mediated phospholipase-C is considered to be the primary response in the plasma membrane of a certain type of cells to extracellular informations such as chemical transmitters, hormones or various growth factors. PPIs not only have such an informational role, but they seem to have more general and various roles in the plasma membrane. Ca^{2+}-pump ATPase is proposed to be regulated by the TPI-level in the plasma membrane. Very recently, it has also been shown that tumor virus alters the PPI metabolism by producing phosphatidylinositol kinase as the oncogene product. This suggests that PPIs have close relationship to various cellular functions which are changed by oncogene product during malignant transformation.

Although the role of PPIs in the plasma membrane has recently been intensively studied, characteristics and the physiological role of PPIs in the plasma membrane has not been fully understood. In this thesis, I will deal with two different aspects of the investigation in which I have aimed to clarify the function of the PPIs in the plasma membrane. The

first is on the properties of the PPI synthesizing enzymes in the acetylcholine receptor (AChR)-rich membranes. The AChR-rich membranes are known to be one of the most highly purified post-synaptic plasma membranes. I used these membranes prepared from the electroplax of electric fish (Narke japonica). I found that these membranes possessed both PI and DPI kinase activities. The role of these enzymes is to produce the PPIs in the membranes by phosphorylating phosphatidylinositol. As will be mentioned in this thesis, I found that the membrane-bound DPI kinase was activated by calcium ion and calmodulin, though the soluble-type DPI kinase was inhibited by calcium ion as had already been reported. There has been no data which shows that the nicotinic cholinergic receptor is the Ca^{2+}-mobilizing receptor or that the receptor membranes possess the agonist sensitive pool of TPI. However, if such a membrane-bound DPI kinase is commonly present in the other plasma membranes, the property of the membrane-bound DPI kinase, i.e. calcium and calmodulin-sensitivity, seems to be suitable to maintain the TPI level in the plasma membrane as the TPI is consumed by the Ca^{2+}-mobilizing receptors.

It is very important that the oncogene product of Rous sarcoma virus (RSV), i.e. $p60^{src}$ reveals PI- and DPI-kinase activity. Therefore, these PI and DPI kinase enzymes in the AChR-rich membranes may possibly be the products of cellular oncogene, c-src. In order to clarify the mechanism of malignant transformation, the study on the PI and DPI kinase in a normal plasma membrane such as an AChR-rich membrane is very much needed. On the other hand, it has been reported that the cluster of AChR on the cultured muscle cell is dispersed by the

infection of Rous sarcoma virus (Anthony et al., 1984). Therefore, it can be speculated that TPI in the plasma membrane has a close relationship with the cytoskeletal system. The calcium and calmodulin-sensitivity of the membrane-bound DPI kinase may also play an important role in such membrane-cytoskeletal systems *in vivo*.

The second is on the ion channel made of lysopolyphosphoinositide on artificial bilayer lipid membranes. This study has been conducted by the author and a coworker Masahiro Sokabe (Osaka Univ.). So far we could not identify the lysopolyphosphoinositide *in vivo*. However, there is substantial phospholipase A_2 activity in the plasma membrane, and they can hydrolyze the polyphosphoinositides. Therefore, the instantaneous occurence of lysopolyphosphoinositides in biological membrane can not be ruled out. We originally intended to examine whether the polyphosphoinositides, which has been suggested to play an important role in nerve excitation, can make the ion channel in a bilayer or not. It was found that not PPI but lysopolyphosphoinositides (LPPI) could form a channel-type ion permeating mechanism in a bilayer membrane. We also succeeded in observing the single channel conductance fluctuation of the lysotriphosphoinositide-channel. This ion channel is the first and only example of a channel made of phospholipid.

POLYPHOSPHOINOSITIDES

The polyphosphoinositides (PPIs) are quantitatively minor and highly acidic phospholipids in the plasma membrane (Michell, 1975). They consist of two components, i.e., phosphatidylinositol-4- phosphate (DPI) and phosphatidylinositol-4,5-bisphosphate (TPI). Fig.1 shows the structure of TPI.

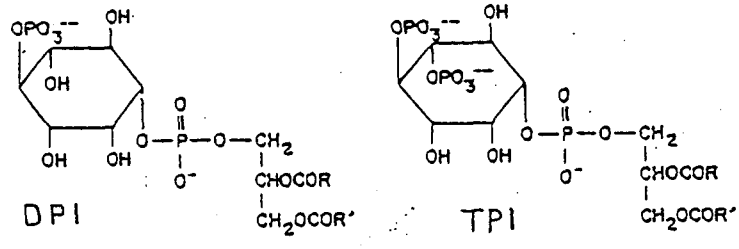

Fig.1

Chemical structure of triphosphoinositide (TPI) and diphosphoinositide (DPI).

They were discovered by Folch about 36 years ago (Folch, 1949). Dawson was the first to show how rapidly they were labelled with ^{32}P in comparison with the other phospholipid of brain (Dawson, 1954). PPIs probably occur in all eukaryotic cells. Since extraction is best done with acidified chloroform/methanol mixture, PPIs are thought to form tight complex with each other or with membrane proteins via Ca^{2+}-bridges. It is noteworthy that TPI has extraordinarily high affinity for Ca^{2+} and shows a marked change in hydrophobicity during uni- and di-valent cation exchange (Dawson, 1965). Its univalent cation salt is highly soluble in water, while its calcium salt is completely insoluble.

A consensus about the physiological significance of the PPI in various information transmission has emerged only within about the past one year. Although the inositol containing phospholipids in receptor function were implicated some 30 years ago by

Hokin and Hokin (Hokin and Hokin, 1953), the study on the inositol phospholipids has been in ernest from 1975, at which R.H. Michell proposed "Ca^{2+}-gating theory" (Michell, 1975). He proposed that increased phosphatidylinositol (PI) turnover generally accompanies the activation of calcium-linked receptors, and is the cause, not the result, of the increased intracellular calcium ion concentrations. He speculated that the PI-breakdown directly opens the Ca^{2+} gate on the plasma membrane. This theory was very influential , and stimulated the study on the inositol phospholipids. However, some of the objections to the theory were claimed , and soon it was found that the PI-breakdown is the secondary event caused by preceding PPI-breakdown. Abdel-Latif and his colleagues (Abdel-Latif et al., 1977; Abdel-Latif, 1983) showed that acetylcholine stimulation of iris smooth muscle enhances the breakdown of the PPI, and that this PPI-effect preceds the PI-breakdown. They also showed that the enzyme phospholipase C catalyzes the PPI hydrolysis. Creba and his colleagues (Creba et al., 1983) also found that PPI breakdown is faster than the PI breakdown when liver cells are treated with vasopressin. Substantial evidence has been accumulated to show that the PPI-breakdown is the primary event caused by receptor activation (Berridge, 1983). In addition, experimental evidences have suggested that cell stimulation rather mobilize Ca^{2+} from intracellular stores than opening calcium gate in the plasma membrane. The discovery that the Ca^{2+} are released first from internal stores suggested that there had to be a transmitter from the outer cell membrane to the interior Ca^{2+} stores (Heymans, et al., 1983; Joseph et al., 1984). The intracellular Ca^{2+} store is

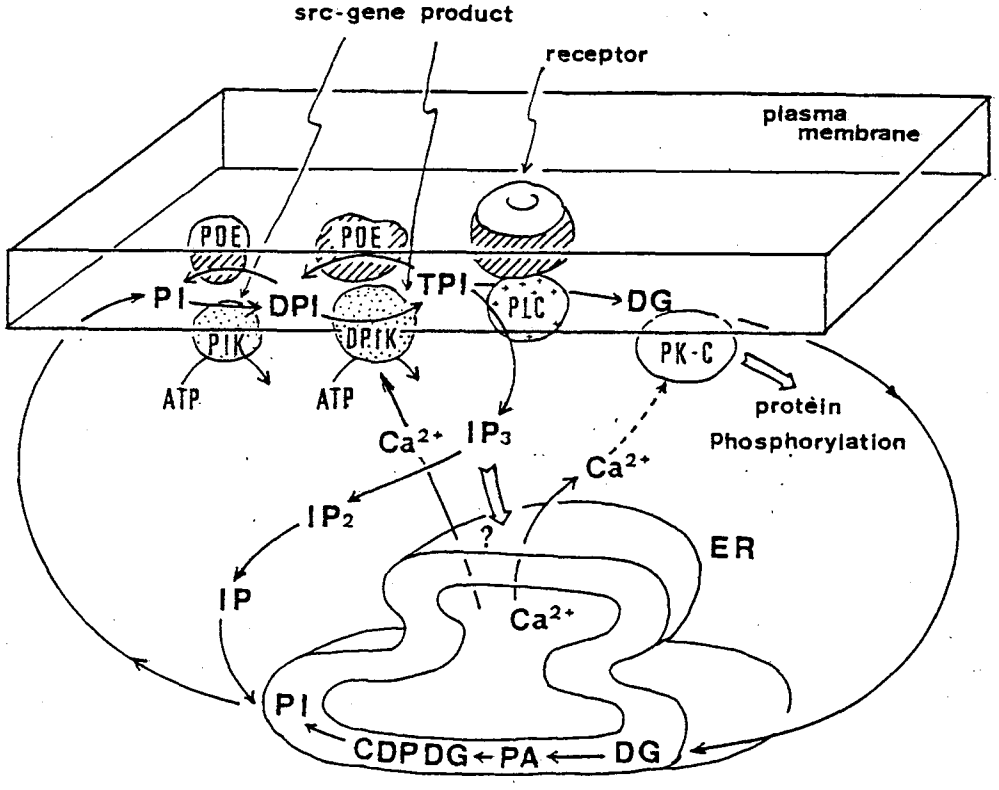

Fig.2 Proposed model for the role of the polyphosphoinositides in receptor activation. Receptor activation makes phospholipase C cleaves TPI into two hypothetical 2-nd messengers, i.e. inositol-1,4,5-trisphosphate (ITP) and diacylglycerol (DG). DG activates Ca^{2+}-dependent protein kinase (PK-C), and the PK-C phosphorylates various functional proteins to modify their activity. Water soluble ITP goes to the cytoplasmic Ca^{2+} reservoir, endoplasmic reticulum (ER) and mobilizes Ca^{2+}. PA, phosphatidic acid; CDP-DG, CDP-diacylglycerol; PDE, phosphodiesterase. Metabolic pools of DPI and TPI in the plasma membrane are maintained by the sequential phosphorylation of PI by PI kinase and DPI kinase. Very recently, an oncogene product ($pp60^{src}$) in the plasma membrane was found to have both kinase activities (We also confirmed this). We found that Ca^{2+} activates not only C-kinase but also DPI kinase in the membrane coworking with calmodulin. Activated DPI kinase replenishes the TPI to the membrane and make the PPI system to be able to liberate the ITP signal to the intracellular space.

thought to be the endoplasmic reticulum but not the mitochondria. Inositol trisphosphate (ITP), which is one of the two TPI-breakdown products, is a feasible candidate of the second messenger which releases Ca^{2+} from intracellular stores. On the other hand, the other product, diacylglycerol is known as an potent activator of a certain Ca^{2+}-dependent protein kinase (protein kinase C) discovered by Nishizuka (Nishizuka, 1983).

Although the receptor-mediated PPI breakdown has been extensively investigated, the precise nature of PPI synthesizing enzymes have scarcely been studied. In order to maintain the system responsible for the external stimuli, powerful backup mechanism for the TPI pool is essentially needed. Such replenishment of TPI is performed by the sequential phosphorylation of PI. PI is phosphorylated to DPI, and DPI is phosphorylated to TPI by PI and DPI kinase, respectively. It has recently been reported that the receptor-stimulated liberation of ITP and DG from TPI system is highly sensitive to the intracellular ATP level (Downes and Wusteman, 1983). It is also reported that the rate of formation of these second messengers depends not only upon the activity of the receptor-stimulated phospholipase C but also upon the rate at which the PPIs can be resynthesized by PI and DPI kinase. Therefore, it is suggested that there is also a high degree of control of the PPI system (perhaps secondary to control of the phospholipase C) at the level of PI and DPI kinases. The importance of our study on the phosphorylation of DPI in the AChR-rich membrane is that the membrane-bound DPI kinase was found to be activated by Ca^{2+} and calmodulin , while

the soluble DPI kinase is known to be inhibited by Ca^{2+} (Kai et al., 1968). This characteristic seems to be pertinent for maintaining the TPI level after the TPI-breakdown followed by increase in intracellular Ca^{2+} concentrations.

On the other hand, it has recently been suggested that the TPI has an ability to activate the Ca^{2+}-pump ATPase in the plasma membrane (Penniston, 1982). He and his coworkers observed an effect of TPI on the Ca^{2+}-pump ATPase purified from rat brain synaptosome plasma membranes. Low concentrations of TPI caused a stimulation of the ATPase activity of this enzyme, which was greater than that produced by saturating levels of calmodulin. The concentration of this lipid required for maximum stimulation is about 2 uM. The low amount of lipid required for this response argues for a potential physiological regulation of this enzyme by TPI. The Ca^{2+}-pump ATPase has been know to be regulated by Ca^{2+} and calmodulin (Niggli et al., 1979-a and b). Chemical analysis has shown that the purified ATPase contains no carbohydrates, but contains about 7 mol of phosphate per mol of protein (Graf et al, 1982). The nature of the phosphorus in the enzyme is not known. However, Carafoli et al. suggest that the phosphorus belongs to phospholipids tightly associated with the enzyme (Carafoli et al., 1982). The acidic phospholipids such as phosphatidylserine has been known to be able to activate the Ca^{2+}-pump ATPase purified from erythrocyte ghost in the place of calmodulin (Carafoli et al., 1982). Therefore, it seems likely that the Ca^{2+} and calmodulin-dependent production of TPI in the plasma membrane, such as I have been observed in the AChR-rich membrane, regulates the Ca^{2+}-pump ATPase.

Very recently, a dramatic development has been seen in the study of the phosphoinositide kinases. Two groups of investigators have suggested that both DPI and PI kinase are the products of oncogene (src and ros) (Sugimoto et al., 1984; Macara et al., 1984). Although these products of oncogenes are known to be both tyrosin kinases, enzymes that can attach phosphate groups to tyrosin residues in proteins, the new works show that these enzymes can also phosphorylate PI and DPI. Thus, these oncogenes may control the effectiveness of hormones, transmitters or growth factors to cells through their effects on the concentration of PPI in the plasma membranes. Although the fruit of the study in this line is still obscure, it has been increasingly suggested that the PPI system including PI kinase and DPI kinase have a general and essential role in cellular functions.

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PART - 1

CALCIUM- AND CALMODULIN- DEPENDENT PHOSPHORYLATION OF DIPHOSPHOINOSITIDE IN ACETYLCHOLINE RECEPTOR-RICH MEMBRANES FROM ELECTROPLAX OF NARKE JAPONICA

Abbreviations used: AChR, acetylcholine receptor; TPI, triphosphoinositide; DPI, diphosphoinositide; PI, phosphatidylinositol; BuTX, α-bungarotoxin; PMSF, phenylmethylsulfonylfluoride; SDS, sodiumdodecylsulfate; CPZ, chlorpromazine; TFP, trifluoperazine; W-7,N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

ABSTRACTS

The phosphorylation of phosphoinositides in the acetylcholine receptor (AChR)-rich membranes from the electroplax of electric fish, Narke japonica, has been examined. When the AChR-rich membranes were incubated with [γ-^{32}P]ATP, ^{32}P was incorporated into only two inositol phospholipids, i.e. tri- and di-phosphoinositide (TPI and DPI). Even after the alkali-treatment of the membrane, AChR-rich membranes still showed a considerable DPI kinase activity upon addition of exogeneous DPI. It is likely that the ^{32}P-incorporation into these lipids was realized by the membrane-bound DPI kinase and phosphatidylinositol (PI) kinase. Such a membrane-bound DPI kinase was activated by Ca^{2+} (10^{-6} M), while the PI kinase appeared to be inhibited by Ca^{2+}. The effect of Ca^{2+} on the DPI phosphorylation was further enhanced by the addition of ubiquitous Ca^{2+}-dependent regulator protein, calmodulin. Calmodulin antagonists such as chlorpromazine (CPZ), trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) inhibited the phosphorylation of DPI in the AChR-rich membranes. It is suggested that the small pool of TPI in the plasma membrane is replenished by such Ca^{2+}- and calmodulin-dependent DPI kinase responding to the changes in the intracellular Ca^{2+}-level. Calmodulin-binding proteins were found in the AChR-rich membranes by the radioiodinated calmodulin over-lay technique applied on SDS PAGE profile of proteins in the AChR-rich membrane. Calmodulin primary bound to doublet proteins (Mr > 100 k dalton).

INTRODUCTION

Polyphosphoinositides are quantitatively minor and highly acidic phospholipids in the plasma membrane (for review, see Michell, 1975) They consist of two components, i.e. phosphatidylinositol-4-phosphate (diphosphoinositide;DPI) and phosphatidylinositol-4,5-bisphosphate (triphosphoinositide;TPI). They are produced by stepwise phosphorylation from phosphatidylinositol (PI). These phosphorylation reactions occur in a variety of tissues, and the phosphorus groups involved or the inositol phosphate head groups undergo rapid turnover (Michell, 1975; Hawthorne and White, 1975). The functional role of the polyphosphoinositides has remained obscure, but many substantial evidences have recently been accumulated to show that the polyphosphoinositides are of major importance for the information transduction mechanism in the plasma membranes (Michell, 1975; Hawthorne, 1983). Recent findings suggest that the primary response of Ca^{2+}-mobilizing receptors, which use Ca^{2+} as the second messenger, to hormones or transmitters is to stimulate the hydrolysis of TPI (Michell et al., 1981; Michell, 1982; Creba et al., 1983; Berridge, 1983)

It can be expected that the replenishment of TPI is essential for the maintenance of the information transmission mechanism. Moreover, the energy-dependent small pool of TPI itself has been proposed to be a regulatory mechanism which determines the effectiveness of membrane receptors (Berridge, 1983). Therefore, the knowledge about their phosphorylation steps as well as the breakdown processes is necessary for

understanding the functional role of polyphosphoinositides. However, the studies of these processes have been hampered by complexity of the metabolic pathways and difficulty in obtaining membrane samples for the experiment in vitro. Surprisingly little is known about the individual steps of phosphoinositide phosphorylation in the plasma membranes, even lesser in excitable membranes. For example, even the presence of the polyphosphoinositide in postsynaptic or receptor-rich membranes has not been substantiated, much less their metabolism in these membranes. We already reported our preliminary results indicating the presence of polyphosphoiositides in the nicotinic acetylcholine receptor (AChR)-rich membranes prepared from the electroplax of electric fish Narke japonica (Hayashi,F. et al., 1981). The AChR-rich membrane is known to be one of the most highly purified receptor membranes, and its sufficiently large amount is obtainable for biochemical studies. The AChR-rich membranes have the crystalline array of transmitter reception sites and also ion channels controlled by the former (Heidman and Changeux, 1978; Kistler and Stroud, 1981). In this respect, the AChR-rich membranes are unique samples for studying the phosphorylation of phosphoinositides in chemically excitable plasma membranes.

In this thesis, I will report several characteristics of the phosphoinositides phosphorylation in the AChR-rich membranes. We found that the phosphorylation of DPI on the membrane was enhanced by Ca^{2+} and calmodulin. Such Ca^{2+}- activated phosphorylation of DPI has not been reported before. Further, this may be the first report about the calmodulin-mediated phosphorylation

of phospholipids. Calmodulin is known as a Ca^{2+}-dependent modulator protein which modifies various cellular activities in response to the intracellular Ca^{2+} level (Kakiuchi, 1970; Cheung, 1980). The electric organ of an electric fish is rich in calmodulin (Childers and Siegel, 1977). Certain protein constituents of the AChR-rich membrane are phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase (Smilowitz et al., 1981; Huganir and Greengard, 1983; Palfre et al., 1983). Moreover, calmodulin antagonists partially block the information transmission through the neuromuscular junction, suggesting the importance of calmodulin in the post synaptic region (Carp, 1983).

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (7,000 Ci/mmole) was synthesized and purified by the method of Johnson and Walseth (Johnson and Walseth, 1979) with a minor modification (Shinozawa and Hayashi, 1983). Calmodulin was purchased from Boehringer Mannheim Yamanouchi (B.M.Y) and from Amano Chemical Co. α-Bungarotoxin (BuTX) was purchased from B.M.Y. and labeled with [^{125}-I]-iodine (Amersham) by the chloramine-T method (Hunter et al., 1962) and purified by a sephadex-G 20 column. TFP and CPZ were purchased from Sigma Chemical Co. W-7 was purchased from Rikaken Co., Ltd. Polyphosphoinositides were prepared from fresh bovine brain by the method of Hendrickson and Ballou (Hendrickson and Ballou, 1964). All other chemical reagents were of the highest purity commercially available.

Preparation of AChR-membranes

Narke japonica was kindly supplied by Misaki Marine Biological Station, University of Tokyo (Misaki, Miura Point, Kanagawa Prefecture). Electric fishes were struck to concussion of brain and the electric organs were excised quickly and then they were quickly frozen in liquid nitrogen. They were stored in liquid nitrogen reservoir (Daia Freezing ,SR-31). Experiments were performed within 6 months. AChR-rich membranes were prepared from the electric organ by the method of Kistler and Stroud (Kistler and Stroud, 1981) with a little modification. All the procedures were performed at 0° C and all the buffer

solutions were argonized. These solutions were prepared by using "pure" water produced by Milli-RQ (Millipore Co.). Fifty grams of electric organ was quickly thawed in buffer-A (400 mM NaCl / 5 mM EDTA / 5 mM EGTA / 0.1 mM phenylmethylsulfonylfluoride (PMSF) / 10 mM Na-phosphate, pH 7.4) by heating in 37°C water bath. Almost thawed electric organs were washed twice with ice-cold buffer-A. The homogenization was performed by a Virtis "45" macrohomogenizer (30 sec, 8 times with 30 sec cooling intervals) at the top speed at ice-cold temperature under the argon atmosphere. After centrifugation (5,500 x g, 10 min) the supernatant was recentrifuged at a higher speed (80,000 x g, 1 hour) and the pellet was resuspended with a Teflon-glass homogenizer (clearance 0.25 mm) in 5 ml buffer-B (1 mM EGTA/1 mM EDTA/0.1 mM PMSF/10 mM Na-phosphate, pH 7.4). The remaining cellular debris was spun out (5,500 x g, 10 min) and the membranes in the supernatant was pelleted by a high speed centrifugation (80,000 g, 1 hour). One additional cycle of low speed/high speed centrifugation in buffer-B was performed before the pellet was resuspended in 5 ml buffer-B containing 20 % (wt/wt) sucrose. The suspension was overlayered onto 28-40 % (wt/wt) linear sucrose gradients in buffer-B and centrifuged overnight in a Hitachi RPS 25-2 rotor at 25,000 rpm ; the density region showing the refractive index from 1.392 to 1.397 was fractionated as the AChR-rich membranes. Membranes were washed twice with Tris-HCl buffer (62.5 mM Tris-HCl, pH 6.8 / 0.1 mM PMSF) by the centrifugation and suspended in 1-ml of the same buffer. Samples were kept at 0-4°C throughout. Experiments were performed within 3 days from the start of membrane preparation.

Protein was determined by Lowry's method (Lowry et al, 1951) using bovine serum albumin as a protein standard. (Na^+-K^+)ATPase activity of the membranes was determined by the method of Nakao et al. (Nakao et al, 1963). Acetylcholine esterase activity was determined by the method of Ellman et al. (Ellman et al., 1961).

^{125}I-α-BuTX binding activity was assayed by the Millipore filter method according to Meunier et al. (Meunier et al., 1974) with a minor modification.

Alkali-treatment of AChR-rich membrane

Alkali-treatment (Neubig et al., 1979) of AChR-rich membrane was performed as follows. The membrane suspension in the gradient buffer was diluted into about 10 vol of distilled water, and brought at once to pH 11.0 with 0.1 N NaOH in a stirred cell. Membranes were pelleted by centrifugation for 30 min at 60,000 g at 4 °C and resuspended in 62.5 mM Tris-HCl (pH 6.8) buffer containing 0.1 mM PMSF.

SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (Laemmli,1970).

Phospholipid metabolism

The standard reaction mixture (80 ul) contained finally 120 mM KCl, 10 mM $MgCl_2$, 0.08 mM EGTA, 5 mM KF, 62.5 mM Tris-HCl (pH 6.8) , 0.005 % Triton X-100, 10 uM [γ-^{32}P]ATP (10 C_i/mmole) and AChR-rich membranes (5 ug protein). The reaction was performed in the presence or absence of Ca^{2+} (180 uM; free-Ca^{2+} concentration must be approx. 100 uM), calmodulin (0.4 uM), CPZ (100 uM) and

W-7 (100 uM). After a 30 sec preincubation at 37 °C, the reaction was started by the addition of [γ-^{32}P]ATP and the mixture was incubated at 37 °C for 1 min. The reaction was terminated by the addition of 0.6 ml of cold trichloroacetic acid (10 %) in 5 mM KH_2PO_4.

In examining the free-Ca^{2+} concentration dependency of TPI-labeling, Ca^{2+}/4 mM EGTA system in 25 mM Tris/maleate buffer (pH 6.8) was used in place of 0.08 mM EGTA and Tris/HCl buffer in the standard incubation mixture. Total Ca^{2+} concentration was determined by an atomic absorption spectrophotometer (Hitachi 180-70). Free-Ca^{2+} concentration was determined by using an apparent binding constant for Ca^{2+}-EGTA of 10^6 M^{-1} (Ogawa, 1968).

Phospholipid extraction and Thin-layer chromatography.

Phospholipids were extracted and isolated by the method of Jolles et al. (Jolles et al., 1979). To each terminated sample 20 ug bovine serum albumin and 25 ug polyphosphoinositides were added as carriers. Membranes were pelleted by mild centrifugation (300 x g, 10 min). Lipids were extracted twice from the pellet by $CHCl_3$/CH_3OH/12N HCl (200:100:0.75) and washed with 0.4 ml 1 N HCl. The lower phase was washed 3-times with 0.7 ml $CHCl_3$/ CH_3OH/1 N HCl (3:48:47) and evaporated to dryness by nitrogen gas flow. Lipid extracts were resuspended with 20 ul of $CHCl_3$/CH_3OH/ H_2O (75:25:2) and spotted on 1 % $K_2C_2O_4$-impregnated silica gel 60 HPTLC plate (Merk). Thin-layer plates were developed in $CHCl_3$/$(CH_3)_2CO$/CH_3OH/CH_3COOH/ H_2O (40:17:15:12:8). Radio labeled phospholipids were located by autoradiography using Kodak Omat AR X-ray film. Labeled phospholipids were scraped

from the plates and counted in 2-ml ACS II (Amersham) using plastic adapter by a liquid scintillation counter (Rack Beta ; LKB).

Radioiodination of Calmodulin

Calmodulin was purchased from Amano Chemical Co.Ltd. and was used without further purification. Calmodulin was iodinated by the method of Richman and Klee (1978) with the modification by Carlin et al. (Carlin et al., 1981): Iodination was performed (21°C) in a raction volume of 0.5 ml that contained 0.05 M sodium phosphate (pH 7.0), 0.5 mg calmodulin, 6 ug lactoperoxidase, and 0.4 mM $CaCl_2$. 1 mM ^{125}I-Na was added to the reaction mixture and the reaction was initiated by the addition of 2.5 ul of H_2O_2 (diluted 1:500). After 7 min, 2.5 ul of H_2O_2 (1:500) was again added to the reaction mixture and the reaction was allowed to proceed for an additional 7 min. The iodinated calmodulin was isolated as described by Richman and Klee (1978). The specific acivity of the iodinated protein was about 600,000 cpm/ug, as determined by an Autogamma (Packard).

Bindings of radioiodinated calmodulin proteins on SDS gels

The binding of ^{125}I-calmodulin to proteins on SDS gels was performed by the method of Carlin et al. (Carlin et al., 1981). Proteins in 2 % SDS were electrophoresed in 7.5 or 10 % polyacrylamide slab gels (1 mm x 15 cm x 13 cm) containig 0.1 % SDS. After electrophoresis the gels were fixed with 25 % isopropanol - 10 % acetic acid for 12 h with at least four

changes by shaking the gels in plastic trays. All washing and binding was done at 25 °C. The gels were then washed for 5-10 min in distilled water and then washed with buffer A (50 mM Tris, pH7.6; 0.2 M NaCl; 1 mM $CaCl_2$) for 12 h with at least four changes of buffer A containing 1 mg/ml bovine serum albumin (BSA). For incubation with iodinated calmodulin, the gel together with 10 ml of buffer A (no BSA) plus 10 ug iodinated calmodulin were inserted into a polyethylene bag which was then sealed with Polysealer (Fuji MFG). The bags were shaken for 12 h on a shaker. The gels were again placed in plastic trays and were washed for 36 h with buffer A with at least six changes. The gels were then stained with Coomassie Blue, and destained by usual procedure. The gels were dried and exposed at 25 °C for overnight on Kodak X-ray film Omat AR using enhancing screen.

RESULTS

AChR-rich membranes from Narke japonica.

Continuous sucrose gradient centrifugation of Narke japonica electroplax membrane particles results in a separation of particle types as shown in Fig.1. Three main fractions are observed in the gradient. The most dense fraction has a relatively low protein (approx. 2 mg from 50 g tissue) and contains approximately 70 % of the total [^{125}I]-α-BuTX binding sites. The middle fraction shows relatively high (Na^+-K^+)ATPase activity and the lightest fraction shows relatively high acetylcholine esterase activity.

The inset in Fig.1 shows Coomassie B.B. staining pattern of each membrane fraction on SDS-polyacrylamide gel electrophoresis. The AChR-rich membrane shows typical four subunits of AChR-protein of Narke, i.e. Mr 41, 48, 52 and 60 k dalton (Hayashi,K. et al, 1981), and the other polypeptides (Mr 43 k and 75 k dalton)those are usually observed in the AChR-rich membranes from electric fish (Neubig et al., 1979; Gordon et al, 1983).

The AChR-rich membrane fraction shows a high specific α-BuTX binding activity (2-3 nmole toxin binding site/mg protein), low (Na^+-K^+)ATPase activity (less than 5 nmole ATP hydrolysed/min/mg protein) and low acetylcholine esterase activity (less than 5 nmole acetylthiocholine hydrolyzed/min/mg protein).

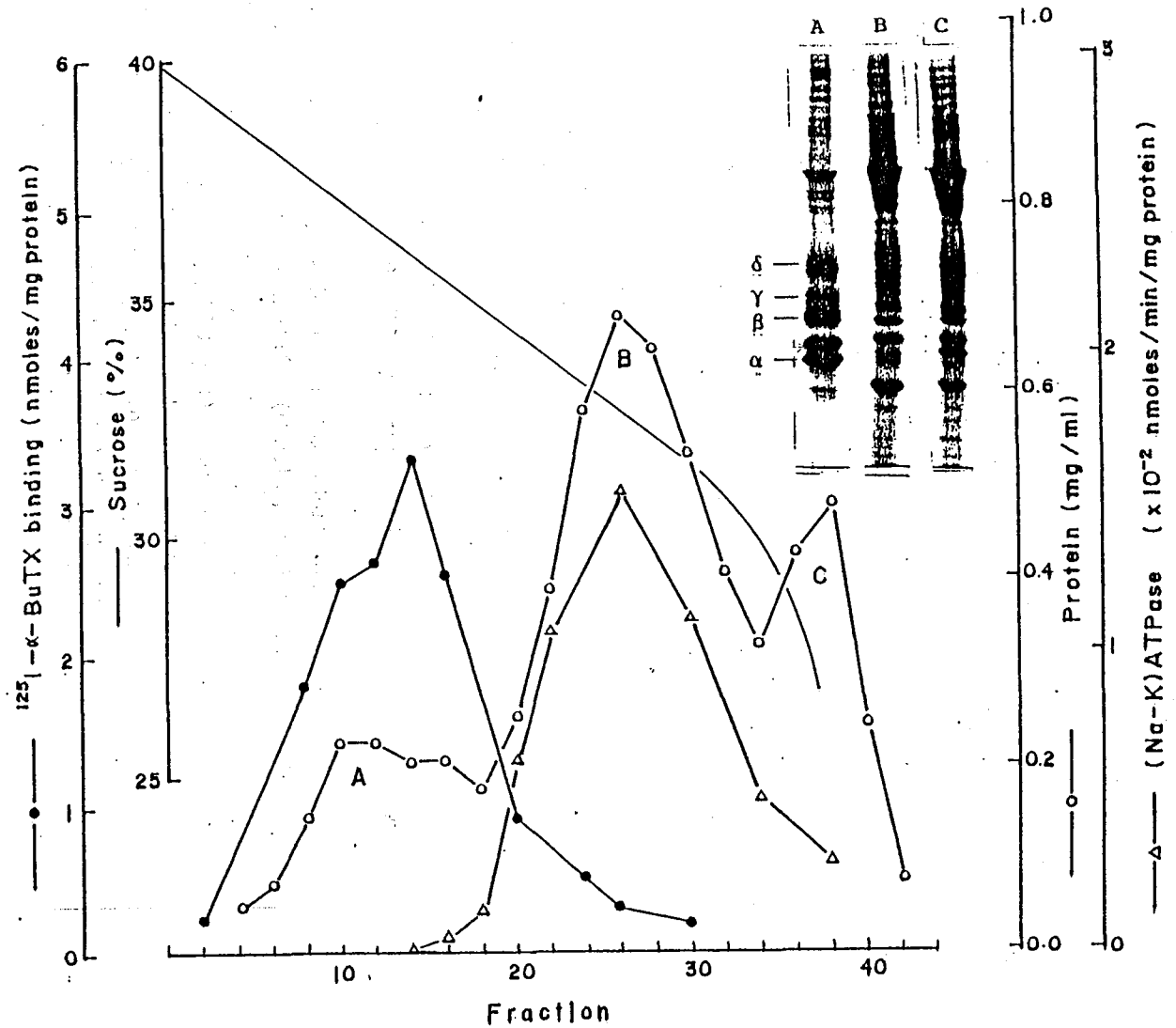

Fig.1

Sucrose density gradient separation of Narke electroplax membranes. Three diffenent fractions, A,B, and C, were obtained. (●), specific α-bungarotoxin binding activity ; (○), protein ; (△), (Na-K)ATPase activity. The inset shows the electrophoretic pattern of each fraction (A,B and C) on SDS-polyacrylamide gel (7.5%). Symbols (α,β,γ,δ) indicate the subunits of AChR.

Phosphorylation of phosphatidylinositol and DPI

Fig.2 shows an autoradiograph of the phospholipids of AChR-rich membranes separated on 1 % $K_2C_2O_4$-impregnated silicagel 60-HPTLC plate. Quantitative results are shown in Fig.3. The membranes (10 μg protein) were incubated in the assay medium containing 10 μM [γ-^{32}P]ATP for 1 min at 37°C. Only two of phospholipids, TPI and DPI, were labeled with ^{32}P. Fig.2 and Fig.3A,B show the effects of various reagents on the ^{32}P-incorporation into TPI and DPI in the presence of Mg^{2+}. It is indicated that the phosphorylation of DPI was enhanced by the addition of Ca^{2+} (100 μM), and the effect of Ca^{2+} on the phosphorylation of DPI was further enhanced by the addition of bovine brain calmodulin (0.4 μM). It is also indicated that the calmodulin can not affect the DPI-phophorylation without the presence of Ca^{2+} in the incubation medium. In addition, it is

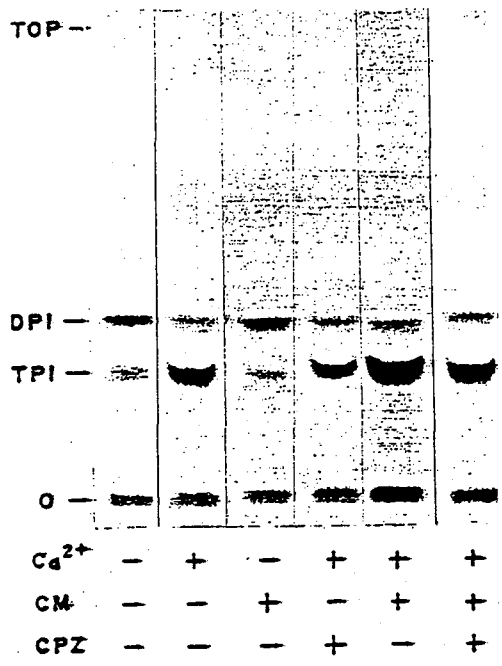

Fig.2

Autoradiograph of thin-layer chromatography showing the effects of Ca^{2+}, calmodulin, chlorpromazine and W-7 on the ^{32}P-incorporation into di- and triphosphoinositide (DPI and TPI) in AChR-rich membranes. Aliquotes (10 μg protein) were incubated for 1 min after addition of 10 μC_i of [γ-^{32}P]ATP (5 C_i/mmol) in the presence (+) or absence (-) of reagents described above. If present, addition to the incubation mixture were Ca^{2+} ($CaCl_2$, 180 μM), CM (calmodulin, 0.4 μM), CPZ (chlorpromazine, 100 μM) and W-7 (100 μM). Incubation medium always contained 80 μM EGTA , 100 mM KCl, 10 mM $MgCl_2$, 0.005 % Triton X-100 and 62.5 mM Tris-HCl (pH6.8).

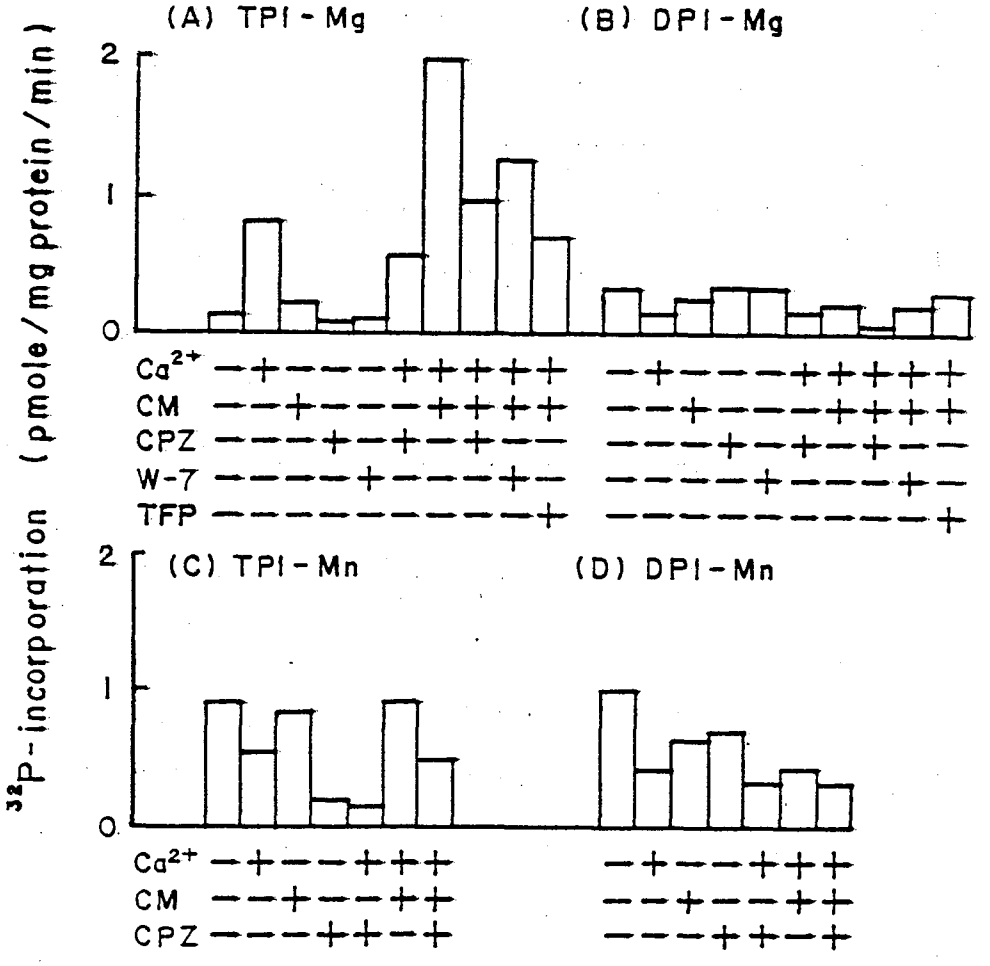

Fig.3

Effects of Ca^{++}, calmodulin (CM), chlorpromazine (CPZ), trifluoperazine (TFP) and W-7 on the ^{32}P-incorporation into tri- and di-phosphoinositide in the AChR-rich membranes. (A),(B): tri- and di-phosphoinositide in the presence of 10 mM $MgCl_2$, respectively. (C),(D): those in the presence of 0.5 mM $MnCl_2$ insteade of $MgCl_2$. Symbols are the same as the legend of Fig.2 except for TFP (trifluoperazine, 100 μM). The data shown are averages of duplicate incubation with assay variation less than 5 % and are representative of four experiment.

indicated that calmodulin antagonists such as CPZ, TFP and W-7 depress the both Ca^{2+}-activated and Ca^{2+}/calmodulin-activated DPI phosphorylation. It is noteworthy that Ca^{2+}, calmodulin and its antagonists can affect mostly the ^{32}P-incorporation into TPI, and on the contrary, PI phosphorylation was less sensitive to Ca^{2+} and calmodulin. The PI phosphorylation rather tends to decrease with

the increase in Ca^{2+} concentration (See Fig.2 and Fig.3B).

We also found that the Mn^{2+}, which can partially bind with calmodulin(Wolff et al, 1983), affected the phosphorylation of PI and DPI in the AChR-membranes. Fig.3(C,D) shows the phosphorylation of PI and DPI in the Mn^{2+}-medium which contains 0.5 mM $MnCl_2$ in place of $MgCl_2$. Mn^{2+} enhanced the basal phosphorylation of PI and DPI, and these activities were depressed by the addition of the calmodulin antagonists. It is worth noting that Ca^{2+} depresses the phosphorylation of DPI in the Mn^{2+}-medium.

Fig.4 indicates the time course of the phosphorylation of phosphoinositides in the AChR-rich membrane in the presence of Ca^{2+} and calmodulin. These polyphosphoinositides incorporated ^{32}P almost monotonically within 2-min. After that, ^{32}P-incorporation did not reach to saturation even after 20 min incubation, though the slope of the phosphorylation slowly decayed.

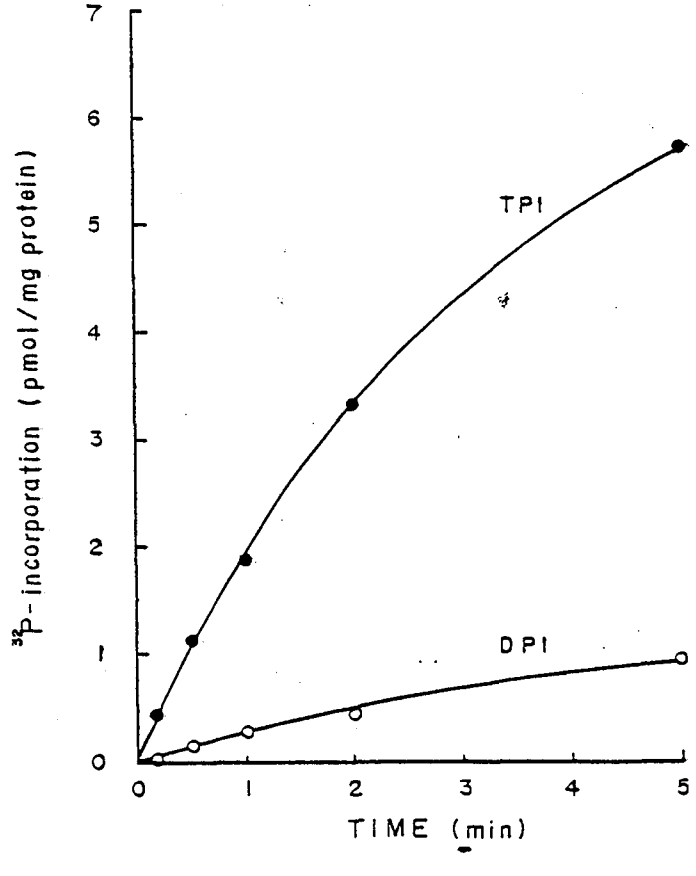

Fig.4
Time course of ^{32}P-incorporation into TPI and DPI in the AChR-rich membrane in the presence of Ca^{2+}(180 μM in the presence of 80 μM EGTA) and calmodulin (0.4 μM). The membranes were incubated under the standard condition. Open circle, TPI; closed circle, DPI. The data shown are average of duplicate incubation with assay variation less than 5 %.

Fig.5 shows the effect of various concentrations of Ca^{2+} on the phosphorylation of DPI in the presence or absence of 0.4 uM calmodulin. The DPI phosphorylation in the AChR-rich membrane was activated by Ca^{2+} concentration range beyond 10^{-6} M, irrespective of the presence or absence of bovine brain calmodulin. The addition of exogeneous calmodulin increased the maximum rate of ^{32}P incorporation into TPI. The Ca^{2+} concentration which gives the half maximum activity seems unchanged by the addition of bovine brain calmodulin.

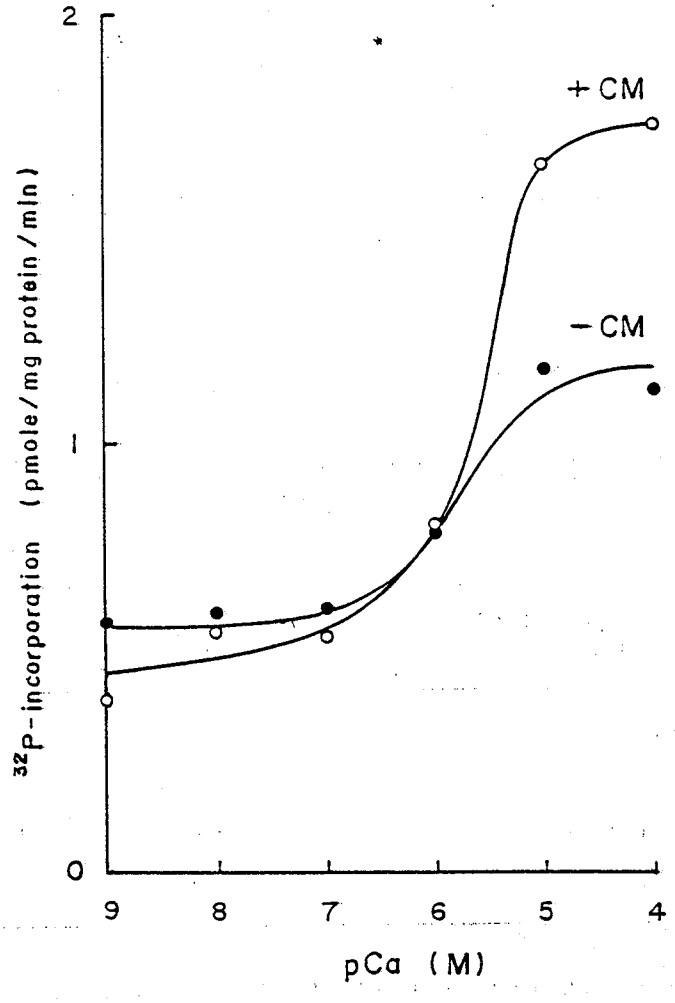

Fig.5

Effect of various Ca^{2+} concentrations on the phosphorylation of DPI in the AChR-rich membrane in the presence or absence of calmodulin. The membranes were incubated with or without 0.4 μM calmodulin under the condition indicated in Materials and Methods. Free-Ca^{2+} concentration was determined by using an apparent binding constant for Ca^{2+}-EGTA of 10^6 M^{-1} (Ogawa, 1968)

The alkali-treatment is known to be a useful technique to remove non-receptor, peripheral membrane proteins from the AChR membrane fractions (Neubig et al, 1979). With the use of this technique, a several major polypeptides could be solubilized from the AChR-rich membranes. Most notably, the 43 k-dalton polypeptide, which is known to be one of the major polypeptide of the AChR membrane, was practically eliminated. A number of higher molecular weight polypeptides except for 75 k-dalton were also eliminated. The specific β-BuTX binding activity of the alkali-treated membrane was about 6 nmole toxin binding site/mg protein. Table-1 indicates that the alkali-treated AChR-rich membranes could not phosphorylate the endogeneous DPI even in the presence of Ca^{2+} and calmodulin. However, upon addition of the exogeneous DPI (100 uM), the purified AChR-rich membranes showed a considerable DPI kinase activity.

Table-1

The effect of exogeneous DPI on ^{32}P-incorporation into TPI of alkali-treated and non-treated AChR-rich membranes.

membrane	exogeneous DPI	^{32}P incorporated into TPI *
AChR-rich membrane	-	1.0
	+	3.1
Alkali-treated AChR-rich membrane	-	0.0
	+	1.4

Membranes were incubated for 1 min at 37°C in the assay medium containing 100 μM Ca^{2+} and 0.5 μM calmodulin in the absence or in the presence of 100 μM DPI as indicated. *The values are the ratio of the amount of ^{32}P incorporated into TPI to that of in the absence of exogeneous DPI, and are means of duplicate determinations with deviations less than 5 % for each condition.

Calmodulin-binding proteins in the AChR-rich membranes

Using the procedure in Materials and Methods, I examined the presence of calmodulin binding protein in the AChR-rich membranes. Fig.6 shows the calmodulin-binding proteins in the AChR-rich membranes which was visualized by the iodinated calmodulin overlay technique on the SDS PAGE profile of the membrane. Radio iodinated calmodulin is found to bind primarily the doublet proteins, the molecular weight of which were higher than 100,000 dalton. I believe that the calmodulin binding doublet protein of such molecular weight region is caldesmon. Caldesmon is a calmodulin-binding and F-actin-binding protein originally purified from chicken gizzerd smooth muscle. This protein binds with F-actin filaments or calmodulin in a "flip-flop" manner depending upon the presence of Ca^{2+}. Therefore, the caldesmon is thought to regulate the function of actin filaments (Kakiuchi and Sobue, 1983; Sobue et al., 1981). It has been known that all of calmodulin binding proteins are not necessarily capable of binding iodinated calmodulin on gels (Carlin et al., 1981). However, it is possible that the caldesmon-like protein indicated here have a regulatory function on the DPI kinase in the AChR-rich membranes. In addition, it is noteworthy that the calmodulin-binding protein in the AChR-rich membrane, which is the post-synaptic membrane of neuro-electroplax junction, is quite different from those observed in the post synaptic density (PSD) of rat brain (Carlin et al., 1981). Carlin et al., found that the major calmodulin-binding protein in PSD was 51 k-dalton polypeptide, and calmodulin also

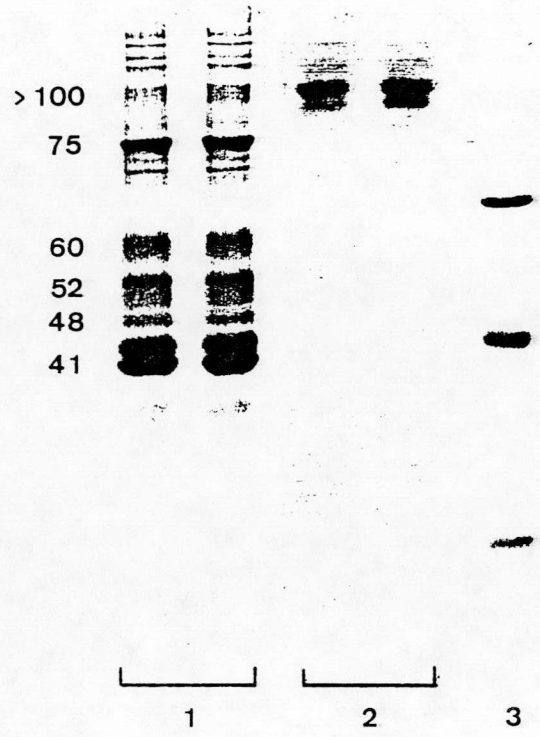

Fig.6 Binding of radioiodinated calmodulin to proteins from AChR-rich membranes separated on SDS polyacrylamide gels. The Mr of the major proteins are shown on the left (k dalton). The method of binding is given in Materials and Methods with 100 ug AChR-rich membrane proteins being used in all slots. Duplicate slots 1 show the Coomassie Blue staining of the AChR-rich membrane preparation. Slots 2 show the autoradiograph of the gel after treatment with radioiodinated calmodulin. Slot 3 shows the molecular weight marker: bovine serum albmin (66 k), Ovalbmin (45 k), trypsinogen (24 k).

bound to the polypeptides of 60, 140 and 230 k-dalton region. I could not observe low molecular weight calmodulin-binding protein in the AChR-rich membranes.

DISCUSSION

The existence of endogeneous PI-kinase and DPI-kinase is suggested from the presented results in the highly purified AChR membranes prepared from the electroplax of the electric fish. Since PI-kinase and DPI-kinase activities are retained in these membranes after the loosely bound membrane proteins were washed away with the buffer containing EGTA and EDTA, it is inferred that these enzymes are closely bound to the AChR-rich membranes. Moreover, as shown in our experiment (Table-1), the DPI kinase activity was also retained in highly purified AChR-rich membranes even after the alkali-treatment of these membranes. Alkali-treatment solubilized several major polypeptides off the membranes and approximately doubled the specific -BuTX binding activity. Nevertheless, appreciable DPI kinase activity remained in the alkali-treated AChR-rich membranes, if exogeneous DPI was added as a substrate. These results suggest that the phosphoinositides phosphorylation was performed by the membrane-bound PI- and DPI-kinase.

The PI kinase activity in the AChR-rich membranes was depressed by Ca^{2+} in the presence of Mg^{2+} (Fig.3-A). Similar characteristics of membrane-bound PI kinase have been reported by many researchers in homogenate of various tissues or membraneous subcellular fractions, i.e., rat brain microsomal fraction (Colodzin and Kennedy, 1965), rat brain homogenate (Kai et al, 1966), liver subcellular fractions (Michell and Hawthorne, 1965), rat parotid gland secretory granules (Oron et al, 1978) and rat

liver Golgi (Jergil and Sundler, 1983).

On the other hand, the membrane-bound DPI kinase has scarcely been studied. It is well established that the TPI is a component of the plasma membrane, and the location of its synthesis is the inner surface of the plasma membrane (Hawthorne, 1983). However, only the soluble DPI-kinase in the cytosol fraction has mainly been studied (Kai et al, 1966; Oron et al, 1978). Such a cytosolic DPI-kinase is known to be inhibited by Ca^{2+} in the presence of Mg^{2+} (Kai et al, 1968). On the contrary, as indicated in this thesis, the DPI-kinase on the AChR-rich membranes was activated by Ca^{2+} even in the presence of Mg^{2+}. From the circumstantial evidence, the Ca^{2+}-dependent activation of the membrane-bound DPI kinase seem to be suitable for recovery or continuous replenishment of TPI to its small pool in the plasma membrane during Ca^{2+}-mobilization caused by TPI breakdown. Therefore, I believe that the Ca^{2+} and calmodulin-dependent DPI kinase is the intact DPI kinase which functions in vivo. The soluble DPI kinase may be in some different physico-chemical state, or it may be proteolyzed DPI kinase.

The other unique characteristics of the membrane-bound DPI kinase lies in its calmodulin-sensitivity. As shown in Fig.3A, calmodulin does not affect the DPI-kinase in the absence of Ca^{2+}, suggesting that the Ca^{2+}-calmodulin complex formation is absolutely necessary for the calmodulin-effect on the enzyme. Although there are many Ca^{2+}- and calmodulin-dependent enzymes, yet the calmodulin-sensitive enzyme concerned with the phospho-lipid metabolism has not been found except for phospholipase A_2 (Wong and Cheung, 1979). Our finding suggests that the TPI

synthesis in the plasma membrane is also the target process of the calmodulin. The regulation of TPI-level in the plasma membrane in response to the intracellular Ca^{2+} level may be one of the important functions of the calmodulin in the post-synaptic region.

Calmodulin is known to regulate many cellular enzymes through its hydrophobic surface exposed by binding with Ca^{2+} (LaPorte et al., 1980; Tanaka and Hidaka, 1980). A physico-chemical characteristic such as change in hydrophobicity plays an important role in the Ca^{2+} and calmodulin-dependent regulation of enzyme activities. Since acidic phospholipids have anionic head groups and hydrophobic tails together, they have a similar physicochemical nature as the calmodulin. Polyphosphoinositides, especially TPI, have high affinity for Ca^{2+} and they show a remarkable increase in the hydrophobicity on binding with Ca^{2+}. So far, TPI-dependent enzyme activity has scarcely been substantiated in any biological system with the exception of TPI-dependent mitochondrial cholesterol side chain cleavage (Farese Sabir, 1980). With regard to the regulatory effect of calmodulin on various enzymes, it is speculated that the TPI synthesized by the membrane-bound DPI-kinase may increase the hydrophobicity in the presence of Ca^{2+}, and hence regulate the activities of some membrane enzymes or effectiveness of receptors in the vicinity of the enzyme.

Calcium and calmodulin-activated DPI phosphorylation was inhibited by the calmodulin antagonists (See Fig.3). Apart from phenothiazine-derivatives such as CPZ or TFP, the aminonaph-

thalenesulfonamide-derivative, W-7, also inhibits the DPI kinase. The W-7 is known to be a more reliable calmodulin antagonist (Hidaka et al., 1979). In addition, Fig.3 indicates that the half maximum dose of the antagonists is approximately 100 uM. Therefore, it can be said that the DPI kinase is inhibited by these antagonists at a concentration level (100 uM) lower than those needed to cause their non-specific effect on calmodulin-dependent enzymes. The brain phosphodiesterase is inhibited non specifically by higher concentration of TFP (>300 uM) or CPZ (>500 uM) (Levin and Weiss, 1975). Therefore, the effect of these drugs on the DPI kinase may be caused by their specific action to the calmodulin.

Fig.3-A and Fig.5 show that Ca^{2+} ($>10^{-6}$M) partially enhances the DPI kinase activity even in the absence of exogeneous calmodulin. Calmodulin antagonists also inhibited such a Ca^{2+}-activated DPI kinase (See Fig.3-A). These findings suggest that the Ca^{2+}-effect on the DPI kinase activity is due to the endogeneous calmodulin residing in the membrane. A considerable amount of calmodulin has been previously identified in the membranes or particulate fractions which were prepared from mammalian brain by homogenizing the tissue in the buffer containing Ca^{2+}- chelators (Kakiuchi et al., 1982). In addition, the electroplax of electric fish is known to be one of the tissues rich in calmodulin (Childers and Siegel, 1977). The DPI-kinase in the AChR-rich membrane is activated by 10^{-6} - 10^{-5} M free-Ca^{2+} in the presence of 0.4 uM calmodulin (See Fig.5). This sensitivity to Ca^{2+} makes the DPI-kinase responsive to the fluctuations in calcium levels which are believed to occur in

vivo. The basal cytoplasmic concentration of free Ca^{2+} in muscles or neurons has been estimated to range between 0.01 and 0.1 uM (Dipolo and Beauge, 1980). Following the cell stimulation, free Ca^{2+} concentration in the cell rises to between 1 and 10 uM. Therefore, it is highly likely that the activities of DPI kinase will undergo dynamic changes also in vivo.

A variety of agonists act on cell-surface receptors to cause an increase in the cytosol Ca^{2+} concentration. The same agonists also induce the TPI-breakdown, which couples the receptor activation with the Ca^{2+}-mobilization (Durrell et al., 1968; Abdel-Latif et al., 1977; Abdel-Latif and Akhtar, 1982; Kirk et al., 1981; Weiss et al., 1982; Creba et al., 1983 ; Billah and Lapetina, 1983; Rhodes et al., 1983; Thomas et al., 1983). For example, the muscarinic cholinergic receptor is known to be a Ca^{2+}-mobilizing receptor which utilizes the TPI-breakdown in its information transduction process (Abdel-Latif et al., 1977; Downes and Wusteman, 1983) . On the other hand, there is no data which shows either that the nicotinic cholinergic receptor has a Ca^{2+}-mobilizing activity or that the nicotinic receptor membranes possess the agonist-sensitive pool of TPI. Thus, the physiological function of the TPI and its synthesizing enzyme in the nicotinic AChR-rich membranes is not clarified yet. However, if the Ca^{2+} and calmodulin-dependency is common to the membrane-bound DPI kinase in various plasma membranes, these characteristics should be a suitable for replenishing the agonist-sensitive TPI-pool. As has been mentioned previously, TPI breakdown by the receptor-mediated activation of phospholipase C results in

the production of inositol-1,4,5-trisphosphate (ITP) and diacylglycerol (DG). Two groups of researchers have recently shown that the ITP could liberate Ca^{2+} from intracellular Ca^{2+} stores (Joseph et al., 1984; Streb et al., 1983). The endoplasmic reticulum is thought to be the intracellular Ca^{2+} store. Since the intracellular Ca^{2+} level is raised by the TPI breakdown, the calcium and calmodulin-dependent DPI kinase is suitable for recovering the TPI level in the plasma membrane after the TPI breakdown. Furthermore, this Ca^{2+}- and calmodulin-dependent TPI replenishing mechanism seems to make the PPI system to be able to liberate the ITP signal to the intracellular space even though an excess amount of agonist is continuously applied to the receptor membrane. Such a continuous liberation of ITP by an excess amount of agonist can be observed in vivo, and the amount of liberated ITP is in fact much greater than the pool size of TPI in the plasma membrane (Downes and Wusteman, 1983). The following reaction scheme can be expected. The TPI-breakdown causes ITP liberation from the inner surface of the plasma membrane. The ITP causes the Ca^{2+} liberation from the intracellular Ca^{2+} reservoir, and in turn the liberated Ca^{2+} activates the TPI-formation in the plasma membrane in order to support the continuous ITP liberation (See Fig.2 in the section of POLYPHOSPHOINOSITIDES).

On the other hand, it has recently been suggested that the TPI has an ability to activate the Ca^{2+}-pump ATPase in the plasma membrane (Penniston, 1982). If it is true, the Ca^{2+} and calmodulin-activated DPI kinase may also have a role in the regulation of such a Ca^{2+}-pump ATPase. The acidic phospholipids

such as phosphatidylserine has been known to be able to activate the Ca^{2+}-pump ATPase purified from erythrocyte ghost in the place of calmodulin (Carafoli et al., 1982). Therefore, it seems likely that the Ca^{2+} and calmodulin-dependently produced TPI regulates the Ca^{2+}-pump ATPase responding to the cytoplasmic Ca^{2+} concentration.

In conclusion, the membrane-bound DPI kinase in the AChR-rich membrane prepared from the electroplax of electric fish is stimulated by Ca^{2+} and calmodulin, whereas the soluble DPI kinase is inhibited by Ca^{2+}. The Ca^{2+} and calmodulin-dependent DPI kinase in the plasma membrane may have a role in the recovery of TPI level after the receptor-mediated TPI breakdown. The receptor-mediated ITP liberation, by the TPI breakdown, seems to be supported by the Ca^{2+} and calmodulin-dependent DPI kinase. The Ca^{2+}-pump ATPase in the plasma membrane is also suggested to be regulated by the Ca^{2+} and calmodulin-dependent DPI kinase.

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PART II

A CALCIUM-SENSITIVE UNIVALENT CATION CHANNEL MADE OF LYSOTRIPHOSPHOINOSITIDE

Abbreviations used: LTPI, lysotriphosphoinositide; TPI, tri-phosphoinositide; LPPI, lysopolyphosphoinositide; pS, pico Siemens (10^{-12} ohm^{-1}).

ABSTRACTS

It was found that the lysotriphosphoinositide (LTPI), a highly polar lysophospholipid having high affinity for Ca^{2+}, could form a Ca^{2+}-sensitive univalent cation channel in planar lipid bilayers. The properties of the LTPI channel were investigated by measuring the both macroscopic membrane conductance and single channel conductance. The macroscopic membrane conductance was depressed with the increase of Ca^{2+} concentration. The ion permeation mechanism induced by the LTPI was directly demonstrated to be a channel type mechanism by the observation of single channel conductance fluctuations. However, the single channel conductance, γ, was ohmic and independent of Ca^{2+} concentration. At 0.5 M KCl concentration, γ = 6 pS on both oxidized cholesterol and glycerylmonooleate bilayer membranes. The distribution of channel open times was fit by a single exponential, reflecting the existence of only one open state. Ca^{2+} depresses $\bar{t}_{do}$, the average dwell time of open state, while it increased $\bar{t}_{dc}$, that of closed state. Thus, it is highly likely that Ca^{2+} affects the gating of LTPI channel. We propose a "micelle transition model" for the LTPI channel.

INTRODUCTION

Triphosphoinositide (TPI) has unique physicochemical characteristics. Although the TPI is one of the phospholipid components in the plasma membrane, its univalent cation salt is highly soluble in water. On the other hand, the TPI can strongly bind with calcium ion forming a highly hydrophobic complex with calcium (Dawson, 1965, 1969, 1970). The TPI can show a drastic hydrophilic-hydrophobic transition depending on whether it binds monovalent cations or divalent cation.

The TPI had been considered to play a crucial role in ion transport across the plasma membrane (Michell, 1975). We tried to incorporate the TPI into the artificial planar bilayer membranes. However, TPI itself had no effect on the conductance of the artificial bilayer membrane. In contrast, we found that a very little amount of lysotriphosphoinositide (LTPI), which was an autoxidation product of TPI, gave a markedly large conductance to the artificial bilayer membrane (Hayashi,F., 1978). Originally, we found that a long-stocked aqueous solution of TPI could induce an ion permeability to the artificial planar bilayer membrane. The membrane-active substance in the oxidized TPI gave a single spot on paper chromatogram (see Fig.1) and was highly likely to be a lyso-like substance which was produced by the degradation of unsaturated acylchain of TPI during the autoxidation. The β acylchain of PPI is known to be highly unsaturated. In addition, it was also found that the anti-TPI antibody could selectively block the ion channel made of autoxidized TPI (Sokabe

et al., personal communication). Almost all the data which will be indicated here were obtained by use of the autoxidized TPI. Later, we could determine that the LTPI, which was obtained from TPI by the phospholipase A_2 treatment, could induce a similar ion permeability to the membrane. I call hereafter the autoxidized TPI as LTPI.

The LTPI could penetrate the membrane and form a channel structure which was selectively permeable to univalent cations. Moreover,the univalent cation conductance of the membrane was greatly influenced by the concentration of calcium ions in the aqueous phase. Masahiro Sokabe (Osaka Univ.) and I tried to observe the single channel current through LTPI channel. The amplitude of the conductance jump was fairly uniform suggesting that the LTPI channel had two conductance states corresponding to "open" and "closed" states. The single channel conductance was constant both in the oxidized cholesterol and glycerylmonooleate membranes, i.e., 6 pS in the presence of 500 mM KCl. As mentioned above, one of the unique features of the membrane which is modified by the LTPI lies in the fact that the macroscopic univalent cation conductance is strongly depressed by calcium ion. From our single channel experiments, it was found that Ca^{2+} ion does not affect the amplitude of single channel conductance . On the contrary, the macroscopic membrane conductance depression by calcium was ascribed to the effect of calcium on the channel gating kinetics. The mean dwell time for the "open" state of the channel and the transition frequency of the conductance jumps decreased with the increase in the calcium concentration in the bathing solution. That is, calcium ion affects the gating

kinetics of the channel and decreases the average number of open channel. This is ascribable from the fact that the calcium ion stabilized the configuration of the LTPI channel to its closed state and raised the activation energy barrier.

MATERIALS AND METHODS

Preparation of triphosphoinositide (TPI)

TPI was obtained from bovine brain by the method of Hendrickson and Ballou (1964). This sample gave a single spot on formaldehyde-treated paper chromatograph or $K_2C_2O_4$-impregnated silica gel 60 high performance thin layer chromatograph , and could be stored safely in wet chloroform at -80°C. The formaldehyde-treated papers were prepared by the method of Kai and Hawthorne (1966). Thin layer chromatography was performed according to Jolles et al. (1979).

Preparation of lysotriphosphoinositide (LTPI)

LTPI was prepared from pure triphosphoinositide by the following two methods. Almost all the experiments were done by the use of autoxidized TPI. However, we confirmed that both LTPI and lysoPPI obtained from TPI and PPI by the treatment of phospholipase A_2 were able to induce the Ca^{2+} sensitive univalent cation permeability to the planar bilayers in the same manner as the autoxidized TPI does. Therefore, I will call the membrane-active substance in the autoxidized TPI as LTPI.

1) Autoxidation Method: An aqueous solution of TPI (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 sec) for a week at room temperature. The autoxidized TPI gave a single spot in formaldehyde-treated paper chromatography just below the spot of TPI (see Fig.1).

It was also found that this oxidized sample contained other

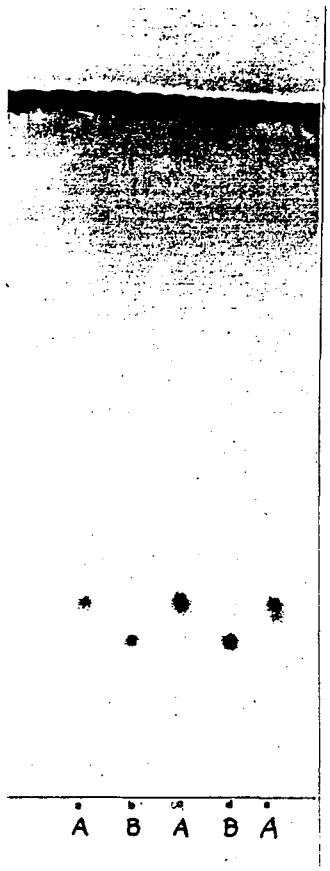

Fig.1 Autoxidized TPI visualized on formadehyde-treated paper chromatogram. Lane A, TPI; B, autoxidized TPI. Phospholipids were visualized by Nile blue in 0.001 % H_2SO_4.

components than LTPI such as free fatty acids or malonaldehyde. Free fatty acid was observed on silicic acid thin layer chromatography and also observed by colorimetric analysis (Kushiro, 1970). Malonaldehyde which indicates the degree of lipid peroxidation was determined with a TBA test (Dahle, Hill and Holman, 1962). However, the LTPI which was obtained from TPI by phospholipase A_2 treatment could also induce the same kind of ion permeability to the membrane. Therefore, the other products contained in the oxidized sample than LTPI do not seem to contribute to the ion permeation.

2) Phospholipase A_2 treatment: the beta-acyl chain of TPI was cleaved off by the phospholipase A_2 by the method of Thompson (1969). TPI (1 umole) was incubated with 2.0 ml, 0.02 % aqueous solution of bee venom (Sigma, lyophylized whole bee venom) in 0.01

M Tris-HCl (pH 7.2). After addition of 3 ml diethylether, the two-phase system was vigorously mixed and kept at room temperature for 1-2 hours in a stoppered tube. The fatty acids were eliminated by repeated washing with diethylether, and the aqueous lower phase was evapolated to dryness. A small volume of $CHCl_3/CH_3OH/H_2O$ (75:25:2) was added to the dried material to dissolve the LTPI and the insoluble materials were eliminated by centrifugation. The clean supernatant was dried with nitrogen gas flow, and the dried material was dissolved with a small quantity of double distilled water and was served for use.

Membrane materials

Planar lipid bilayers were formed from oxidized cholesterol or glycerylmonooleate. The oxidized cholesterol was obtained by the method of Jain et al.(1973) or Robinson and Strickholm (1978) with a minor modification.

Cholesterol and glycerylmonooleate were purchased from Sigma Chemical Co.. Alkans were purchased from Tokyo Kasei Co. Ltd. and passed through active-almina column.

Membrane formation and electric measurements

1) Macroscopic conductance measurement

Macroscopic membrane conductance was measured by use of the membrane-forming apparatus as shown in Fig.2-A, B. In the early stage of our investigation, we used the A-type chamber. The planar bilayer membrane was formed in the hole (1.3 mm diameter) on the chamber septum. Each chamber was filled with 5 ml of 10 mM Tris/HCl(pH 7.5). LTPI and $CaCl_2$ were added to both compartments after the membrane had become completely black. A small quantity of concentrated solution of electrolytes was added to either one or both aqueous phases in order to change the ionic composition. The composition of the aqueous phase could also be changed by a perfusion technique.

In order to perfuse both bathing solutions more completely, we improved the membrane forming chamber as shown in Fig.2-B. This apparatus consists of three main parts i.e. the front and back chambers, and the partition film (0.2 mm in thickness) with a membrane hole (1.3 mm in diameter). These parts were made of Daiflon (trifluoromonochloroethylene; purchased from Daikin Engineering Co.Ltd.). The volume of each chamber was approx. 300 ul. the front chamber had a glass window. The back chamber was equipped with a Teflon-coated stainless pipe connected to micro-syringe which supplied the membrane forming material. The back chamber was also equipped with a Daiflon brush which could be manipulated from the out side. These three parts were attached tightly with metal screws and fluorine-contained rubber was used as a shielant. Having been supplied oxidized cholesterol solution to the membrane hole, bilayer membrane was formed by a stroke of

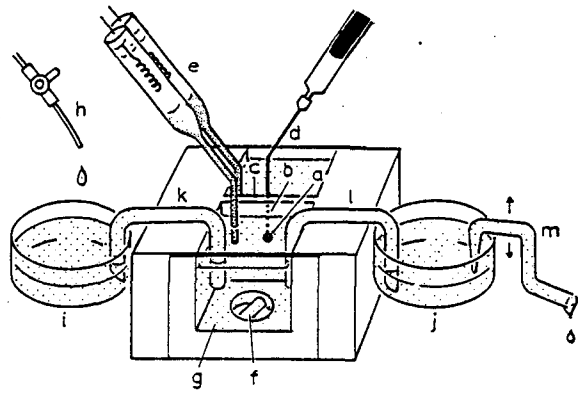

Fig.2-a Schematic diagram of apparatus for the formation of the membrane and for the perfusion of aqueous solution. a, membrane hole (1.3 mm dia.); b, feeder hole (0.7 mm dia.); c, septum (1.5 mm thickness); d, Teflon tube; e, KCl/agar bridge and Ag/AgCl electrode; f, magnetic stirrer bar; g, glass window; h, inlet of perfusing solution (Teflon tube with a stopcock); i and j, buffering vessel; k anl, siphon; m, level-conrolling siphon.

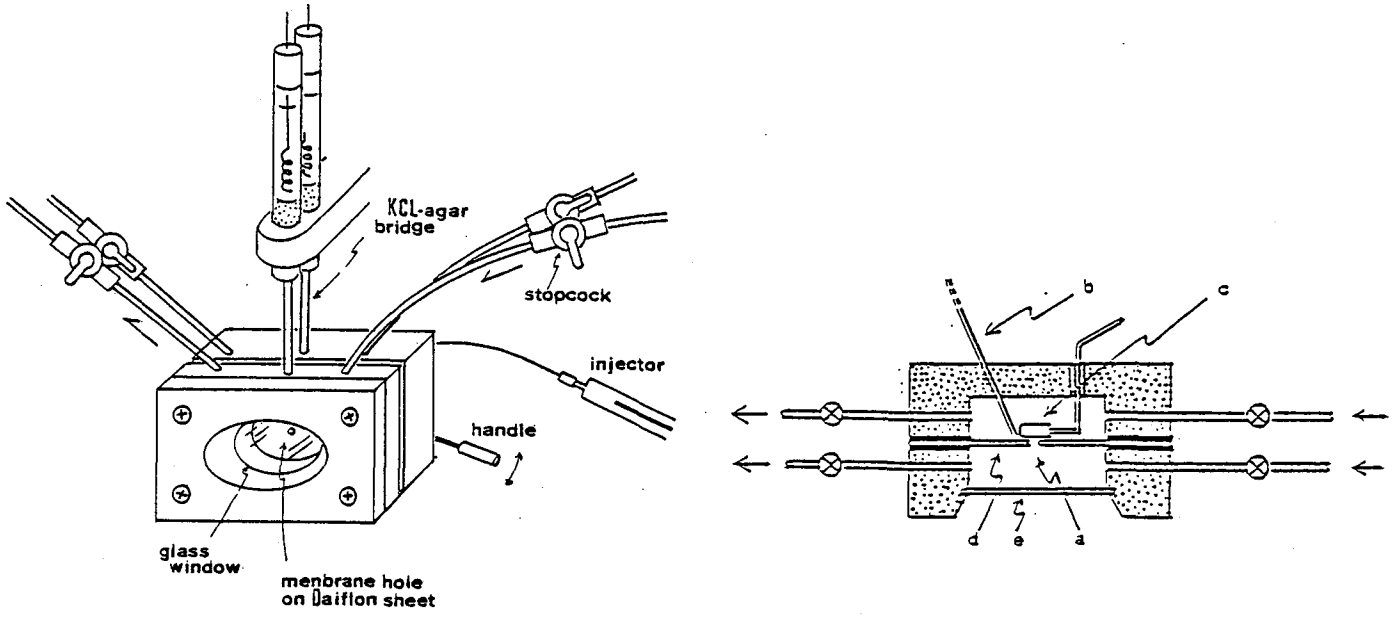

Fig.2-b Schematic diagram of improved apparatus for the formation of bilayers and for the perfusion of bulk solutions (sketch and top view). a, membrane hole (1.3 mm dia.); b, feeder tube; c, Daiflon blush; d, Daiflon sheet (0.2 mm thickness); e, glass window.

the brush. Each chamber was perfused with solutions through two Teflone tubes (2 mm in diameter). When the stopcocks of one chamber were closed, the opposite compartment could be safely perfused with arbitrary solutions without destruction of the membrane. We could change the solution within 30 sec completely.

The zero current conductance of the membrane was obtained by applying a 10 mV rectangular voltage of 0.5 sec duration and by measuring the corresponding current using a low-drift FET input operational amplifier (Teledyne-Philbrick, 1023). The measurements were done at room temperature.

2) Microscopic conductance measurement

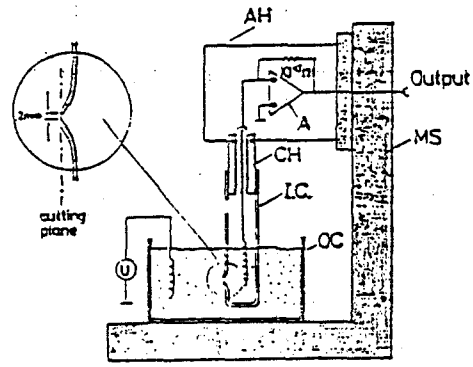

Fig.3 Membrane formation apparatus for the single channel experiments. OC, outer chamber; IC, inner chamber; CH, chamber holder; A, amplifier; AH, amplifier headstage; MS, micromanipulator stand. (Neher et al., 1978).

Fig.3 shows the apparatus for measuring the single channel conductance. Membrane forming apparatus and current measuring procedure were identical to those reported by Neher et al.(1978). The planar membrane was formed in a small hole (0.2 mm in dia-

meter) on the side-wall of a 10 ml disposable polypropylene test-tube. The test-tube was dipped in the solution containing 1.6-2 nM of LTPI, 50-500 mM KCl, 10^{-6}-10^{-2}M $CaCl_2$ and 1 mM Tris/HCl (pH 7.2) in a 50 ml glass beaker. The membrane forming material was applied to the membrane hole with a glass micropipette. After thinning the membrane, current fluctuations were measured at constant voltage of different levels (0-150 mV). The current amplifier connected through coaxial connector to the test-tube was constructed from an electrometer amplifier (teledyne 1035) equipped with 10^{10} ohm glass enclosed resistor and 10 pF titanium capacitor in the feedback circuit. With this configuration, the limited bandwidth and electrical noise of the measuring system (including membrane) was about 8 Hz and lower than 50 fA respectively. Ag-AgCl electrodes were used for current and voltage measurement. Most of the experiments were carried out with applying $\pm$ 50 mV dc-voltage (the inner side of the tube was a virtual ground) through pulse generator (Nihon Koden, SEN 3101).

RESULTS

[1] MACROSCOPIC MEMBRANE CONDUCTANCE INDUCED BY LYSOTRIPHOSPHOINOSITIDE (LTPI)

LTPI induced univalent cation conductance in the presence of Ca^{2+}.

Although our main purpose was to examine the ability of TPI in modifying the ion permeability of the membrane, no change in the membrane conductance could be induced by the simple addition of TPI. On the contrary, we found that the LTPI could induce the univalent cation permeability to the planar bilayer membrane. Fig.4 shows a typical time course of the membrane conductance change induced by the LTPI. The conductance of oxidized cholesterol bilayer membrane was 3-7 x 10^{-8} $S \cdot cm^{-2}$. Upon addition of 10^{-6} g/ml LTPI and 1 mM $CaCl_2$ in final concentration to the both side of a black membrane, an increase in the membrane conductance was observed (Fig.4). Calcium ion was necessary to incorporate the LTPI molecules into bilayer membranes. The conductance gradually increased from about 10^{-8} $S \cdot cm^{-2}$ to about 10^{-6}

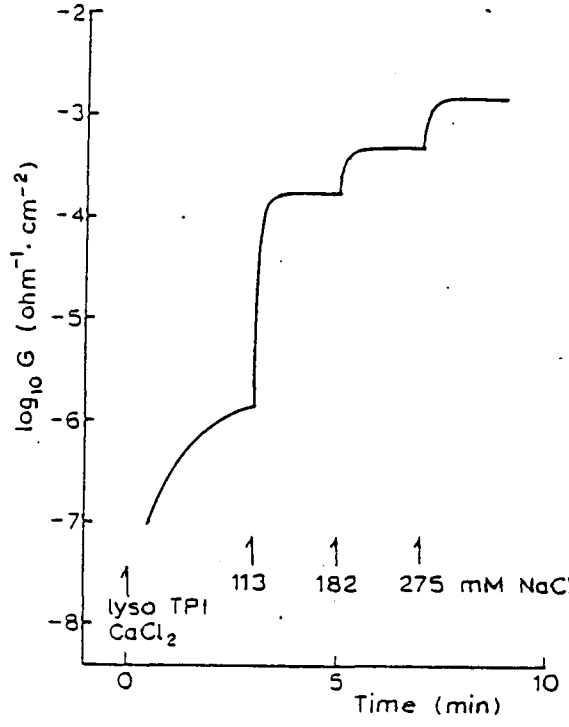

Fig.4 Time course of the conductance changes of a black membrane modified with 10^{-6}g/ml LTPI and 1 mM $CaCl_2$. The first arrow indicates the time at which LTPI and $CaCl_2$ were added to both the aqueous phases containing 10 mM Tris-HCl (pH 7.5). Aliquots (100 µl) of 2 M NaCl were added to both phases.

$S \cdot cm^{-2}$ within 5 minutes. The subsequent additions of $CaCl_2$ did not induce any further increase in the conductance (see broken line in Fig.5). Upon addition of a small aliquot of a concentrated solution of univalent electrolyte into both aqueous phases (indicated by arrows in Fig.4), the marked increase in the membrane conductance was observed. 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} $S \cdot cm^{-2}$ (see the upper solid line in Fig.5).

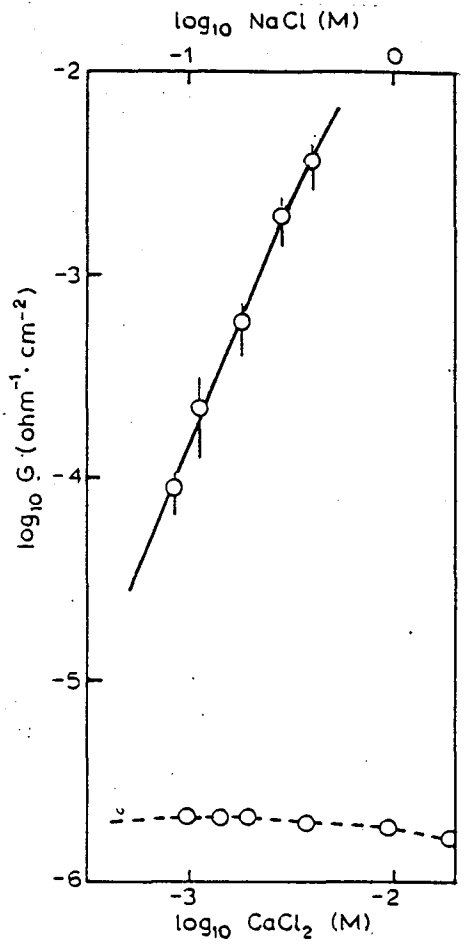

Fig.5 Plot of membrane conductance vs. concentration of electrolyte solution. Upper curve: NaCl in the presence of 1 mM $CaCl_2$. Lower curve: $CaCl_2$ in the absence of group IA cation. The data represent mean values (±S.D.) of at least four membranes.

The univalent cation conductance was greatly influenced by the addition of calcium ion. However the effect of calcium ion was highly complex if the LTPI was remaining in the bathing solution. In order to examine the effect of LTPI in the aqueous phase, we compared the calcium effect on the membrane conductance of the perfused membrane to that of the non-perfused one. Fig.6 shows the changes in the membrane conductance when $CaCl_2$ was added to one side of the membrane in

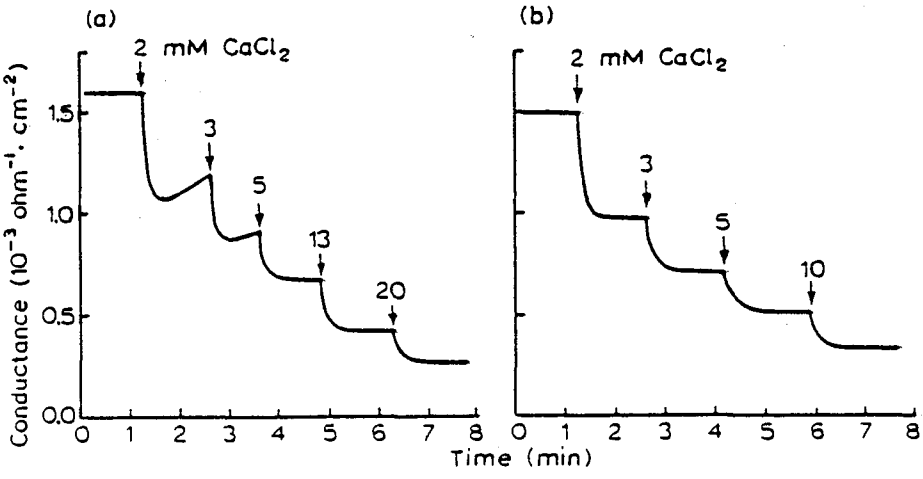

Fig.6 Time course of the membrane conductance when $CaCl_2$ was added to one side of compartment (a) in the prensece and (b) in the absence of LTPI. Both aqueous phases contained initially 10^{-6}g/ml LTPI, 1 mM $CaCl_2$, 275 mM NaCl and 10 mM Tris-HCl (pH 7.5). In the case (b), the aqueous phase was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

the presence (a) and in the absence (b) of LTPI in the bathing solution. As shown in Fig.6(a), there were two opposite aspects in the effect of calcium ion on the membrane conductance. The one is a rapid decrease just after the injection of calcium ion, and the other is a subsequent gradual increase in the conductance. However, when the LTPI was eliminated from the bulk solution by perfusion, the later conductance increase disappeared (see Fig.6(b)). The presence of LTPI in the aqueous phases is the cause for the latter effect of calcium ion on the membrane conductance. The mechanism of the later increase in the conductance may be explained as follows. The hydrophobicity of LTPI in the aqueous phases increases with the increase in the concentration of calcium ion. Thereby, incorporation of LTPI into the membrane will be accelerated by the addition of $CaCl_2$. The absorbed LTPI molecules immediately form the univalent cation channels, resulting in the increase of the number of channels and

therefore the membrane conductance. On the other hand, as shown in Fig.7, the rate of conductance increase by the addition of $CaCl_2$ had a peak value at about 2-3 mM $CaCl_2$. This also may be due to the decrease in the rate of LTPI incorporation into the membrane. Such a decrease in the rate of incorporation at the $CaCl_2$ concentration range beyond 3 mM seems to be due to the increase in the hydrophilicity of the LTPI molecule in the aqueous phase. The increase in the hydrophilicity at high calcium ion concentration is ascribable to the reversal of the surface charge of the LTPI micelle by binding with excess calcium ion. Dawson and Hauser (1970) 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 LTPI micelle in the water, their hydrophobicity will become maximum when the charge of the micelle is neutralized by calcium ions.

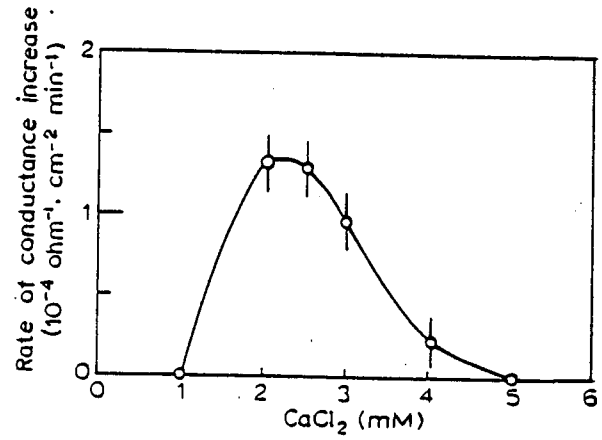

Fig.7 Effect of Ca^{2+} concentration on the rate of conductance increase due to LTPI adsorption to the membrane. Both aqueous phases contained 1 μg/ml LTPI, 1 mM $CaCl_2$, 275 mM NaCl and 10 mM Tris-HCl (pH 7.5). The data reprensent mean values (± S.D.) of at least three membranes.

Ion Selectivity of The LTPI Channel

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.8, the membrane potential could be fitted successfully with

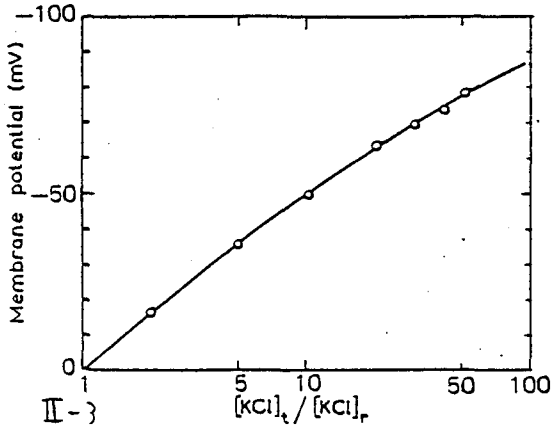

Fig.8 Membrane potential generated by the concentration gradient of KCl. The concentration of KCl at the reference side was fixed at 8 mM. The subscripts "t" and "r" represent the two aqueous phases separated by the membrane. The solid line indicates the membrane potential calculated from the Goldman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

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 $CaCl_2$ (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.9 presents the "fingerprint" characteristic of the univalent cation permeability of the membrane modified with LTPI. The logarithm of the experimentally observed permeability ratio

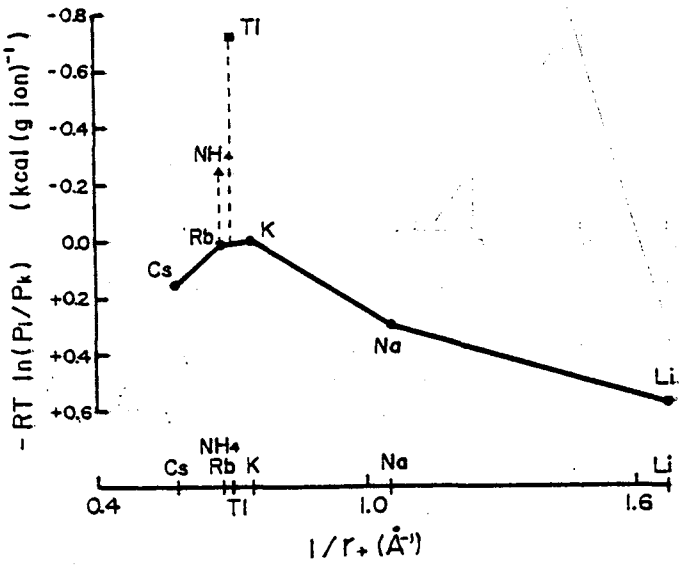

Fig.9 Selectivity "fingerprint" for permeation through the channel made of LTPI. The logarithm of the permeability ratios relative to K^+ determined from zero-current potential measurements of a bilayer membrane is plotted against the reciprocal of the naked cation radius. The ionic strength of the each aqueous phase was equalized. Reference side: 400 mM KCl + 40 mM MeCl (Me: group IA cation). Test side: 40 mM KCl + 400 mM MeCl.

(Szabo et al., 1969) has been plotted as a function of the reciprocal of the naked cation radius (Pauling,1960). The selectivity sequence among group IA cations was $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$, Both NH_4 and Tl^+ showed higher permeability than K^+ which has almost the same naked cation radius to those ions. The pattern of this fingerprint is less in magnitude but very similar to that of the K^+-channel of nerve with the exception of NH_4^+ (Eisenmann and Krasne, 1973).

Univalent and Divalent Cation Exchange and Macroscopic Membrane Conductance.

After the LTPI had been eliminated from the bathing solution, the effect of univalent and divalent cation concentration on the membrane conductance was examined. Fig.10 shows the membrane conductance as a function of the potassium ion concentration in the presence of various concentrations of calcium ion. At the low concentrations of divalent cation, the membrane conductance shows the saturation behaviour against the univalent cation concentration

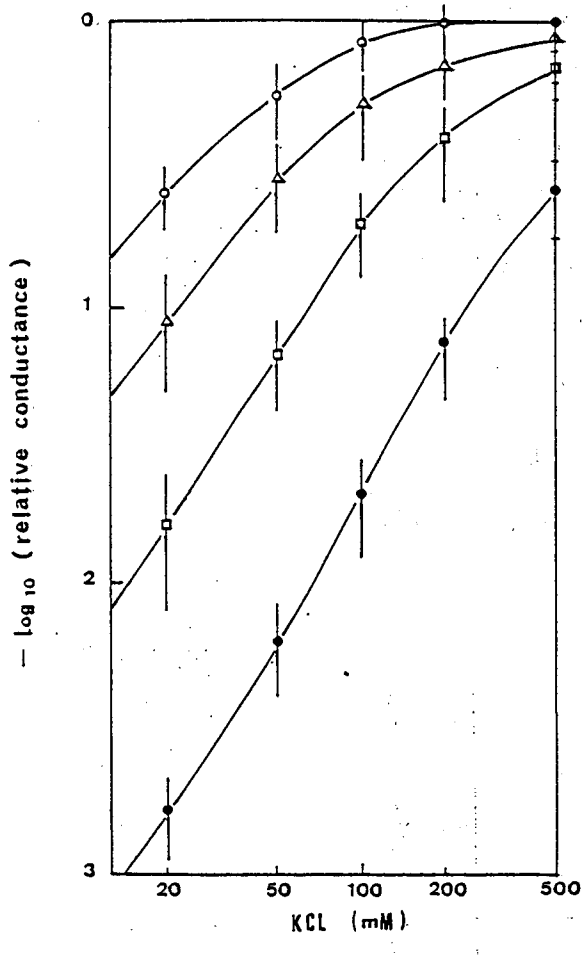

Fig.10 Membrane conductance as a function of KCl concentration in the presence of various concentration of CaCl. (○), 10^{-6}M; (△), 10^{-5}M; (□), 10^{-4}M; (●), 10^{-3}M.

increase. The divalent cation seems to shift the curve to higher univalent cation concentration range.

Fig.11 illustrates the relative conductance as a function of the concentration of various divalent cations. The relative conductance in this figure means the ratio of the membrane conductance to that in the absence of $CaCl_2$ in the both aqueous phases. Both aqueous phases always contained 300 mM KCl. The order of the efficiencies between these divalent cations was La^{2+} >> Zn^{2+} > Cd^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+}. Such an effectiveness seems to depend on the affinity of divalent cation to the head group of

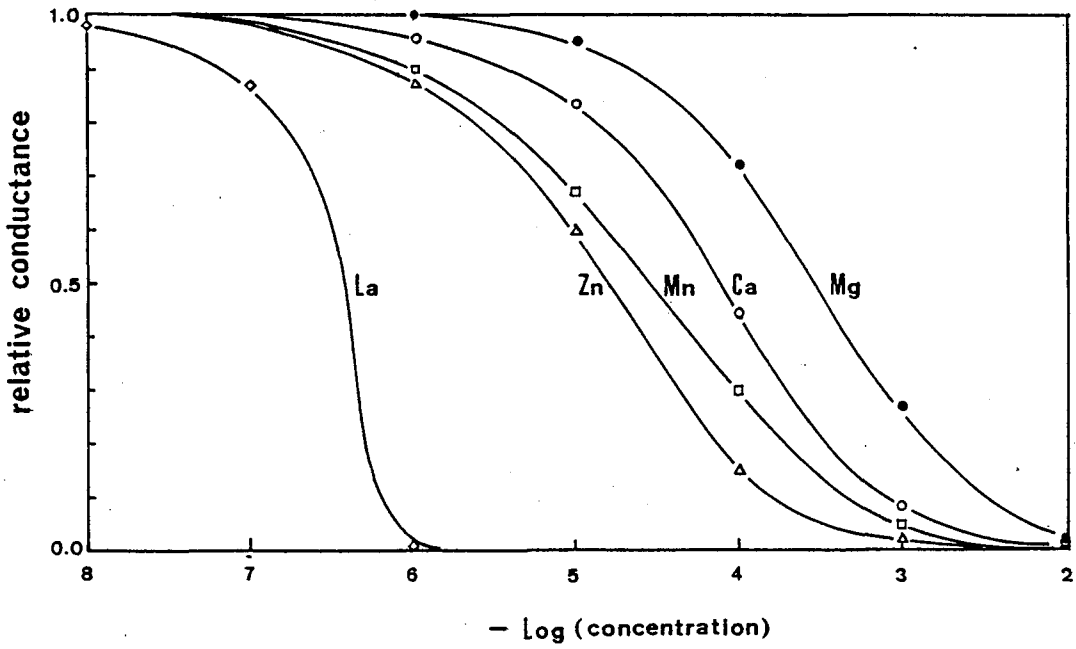

Fig.11 Plot of relative membrane conductance vs. concentration of divalent cation added to both sides. Oxidized cholesterol membrane was incubated in the modifying solution containing 1 nM LTPI, 1 mM $CaCl_2$ and 10 mM Tris-HCl (pH 7.5) for 5 min. After the addition of 300 mM KCl, LTPI and Ca^{2+} were eliminated from bathing solutions by the perfusion with LTPI- and Ca^{2+}-free 300 mM KCl solution. The divalent cation concentration was changed by perfusion. Perfusing solution always contained 300 mM KCl and 10 mM Tris-buffer.

LTPI. Blaustein and Goldman (1968) 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. (1975) also showed similar sequence or the affinities of divalent cations to negative surface charge near sodium channels of nerve. These sequences are same as that of LTPI channel.

[2] SINGLE CHANNEL CONDUCTANCE OF LYSOTRIPHOSPHOINSOITIDE

In the absence of LTPI in the bathing solutions, the apparent membrane conductance of oxidized cholesterol or glyceryl-monooleate bilayers was about 20-50 pS (3-7.5 x 10^{-8} $S \cdot cm^{-2}$) in the presence of 500 mM KCl, and no conductance fluctuations were observed at constant voltages below 100 mV. However, upon addition of trace amounts of LTPI (approx. 2 nM) to the bathing solutions, well defined discrete jumps were frequently observed.

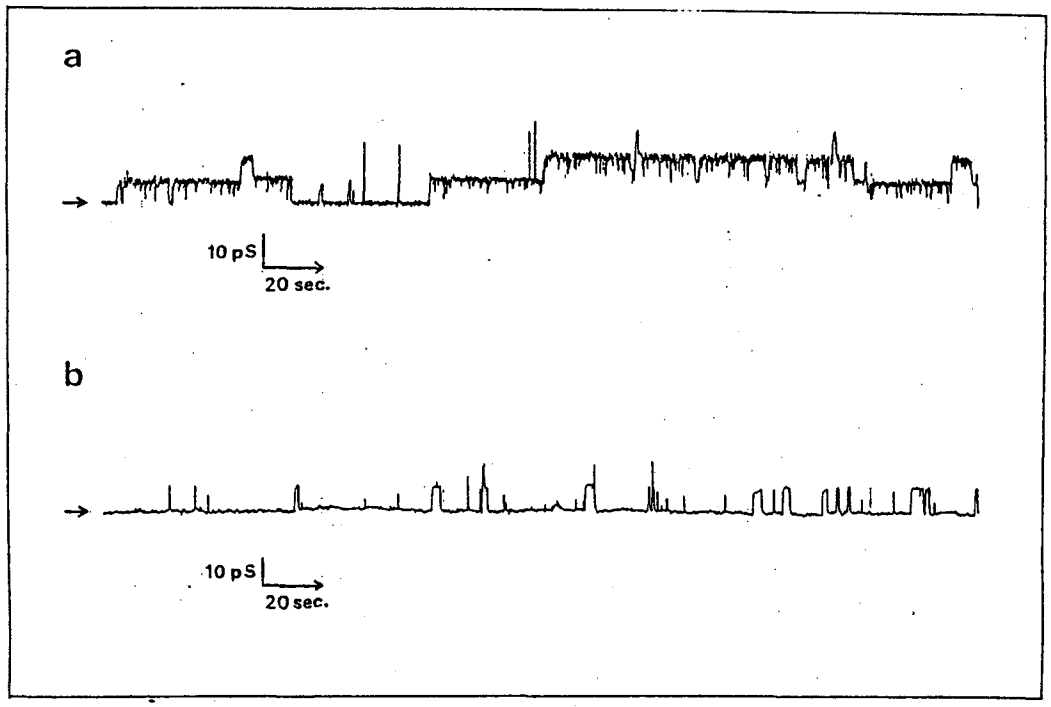

Fig.12 Conductance fluctuations of LTPI channels in (a) oxidized cholesterol and in (b) glycerolmonooleate bilayer; electrolytes, 500 mM KCl, 1 mM $CaCl_2$, 1 mM Tris-HCl (pH 7.2), 1.6 nM LTPI, T=24°C, V=50 mV.

Fig.12-a shows the typical conductance fluctuations induced by LTPI on an oxidized cholesterol bilayer membrane. The magnitude of the most frequent conductance jumps was approx. 6 pS in the presence of 500 mM KCl. Fig.12-b shows the conductance fluctua-

tions induced by LTPI on a glycerylmonooleate bilayers. Current fluctuation in the glycerylmonooleate membrane was somewhat different from that in the oxidized cholesterol membrane. However, unit conductance jump was about 6 pS which was identical to that observed in oxidized cholesterol membranes. Since this unit conductance was much higher than that for carrier mechanism (Hladky and Haydon, 1972; Läuger, 1972), it is highly likely that LTPI forms a channel structure in both kinds of lipid bilayers. The magnitude of each conductance change was uniform even in multi-step changes as shown in Fig.12-a and b, suggesting that the channel had two conductance states corresponding "open" and "closed".

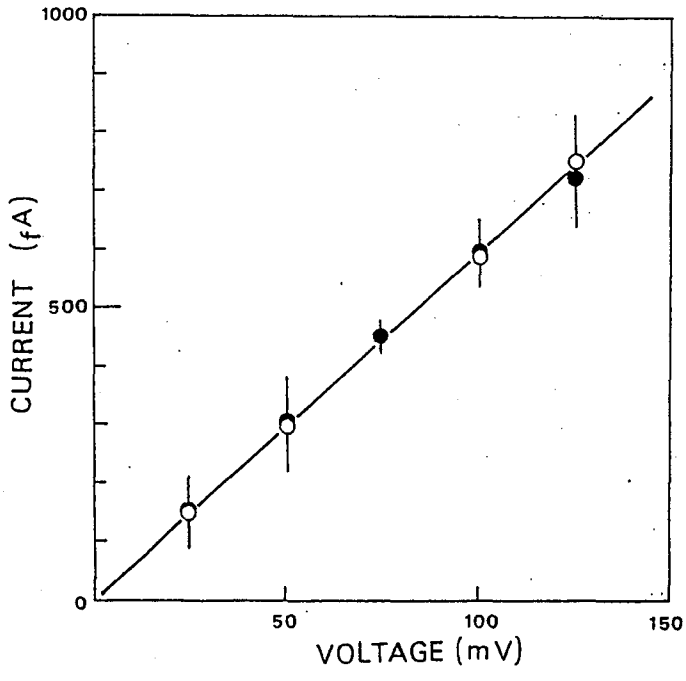

Fig.13 Steady state current-voltage curves of the single LTPI channel in (●) oxidized cholesterol and (○) glycerylmonooleate bilayers in the presence of 500 mM KCl and 10^{-6} M $CaCl_2$. Data represent mean values (± S.D.) of at least 10 events from four membranes.

Fig.13 indicates the I-V characteristics of the single channel of LTPI in oxidized cholesterol and glycerylmonooleate membranes. Identical linear relationships were obtained in both

membranes in the voltage range of 0-125 mV under symmetrical electrolyte condition.

Fig.14 shows the single channel conductance of LTPI as a function of KCl concentration. The single channel conductance appears to approach a limiting value. The single chanel conductance of LTPI channel is saturated by lower concentration of KCl than that reported for the other ionophores such as EIM or alamethicin (Bean, 1973; Eisenberg et al., 1973). Similar saturation phenomenon was reported for gramicidin A channel, which was explained in terms of the mutual competition between permeant ions for the binding sites of the channel (Neher et al., 1978). It is expected that the same kind of mechanism as that in the gramicidin-A channel is limitting the rate of ion permeation through the LTPI channel.

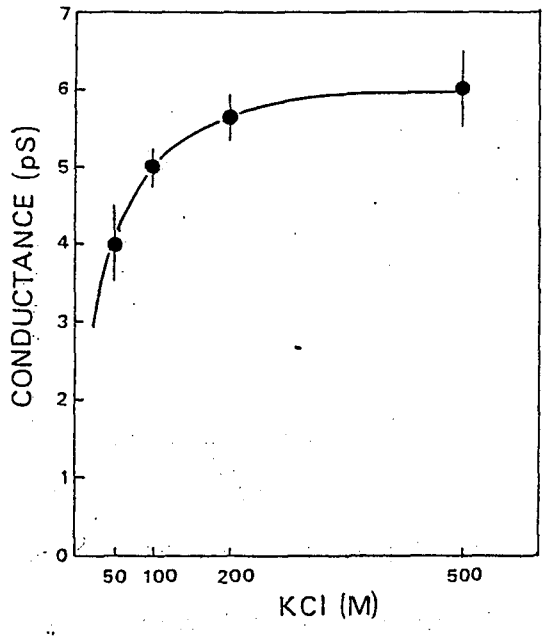

Fig.14 Dependency of the single channel conductance on KCl concentration in oxidized cholesterol bilayers at 50 mM in the presence of 10^{-6}M $CaCl_2$. Data represent mean values ($\pm$ S.D.) of at least five events from three membranes.

EFFECT OF Ca^{2+} ON THE SINGLE CHANNEL CONDUCTANCE

As previously mentioned, a small amount of Ca^{2+} strongly reduces the macroscopic univalent cation conductance induced by LTPI. Therefore, it is of special interest to investigate the underlying mechanism of the effect of Ca^{2+} on the LTPI channel. Fig.15 demonstrates that no measurable single conductance change was provoked by Ca^{2+}. The I-V relationships were also unaffected by Ca^{2+} concentration in both oxidized cholesterol and glyceryl-monooleate membranes (data were not shown).

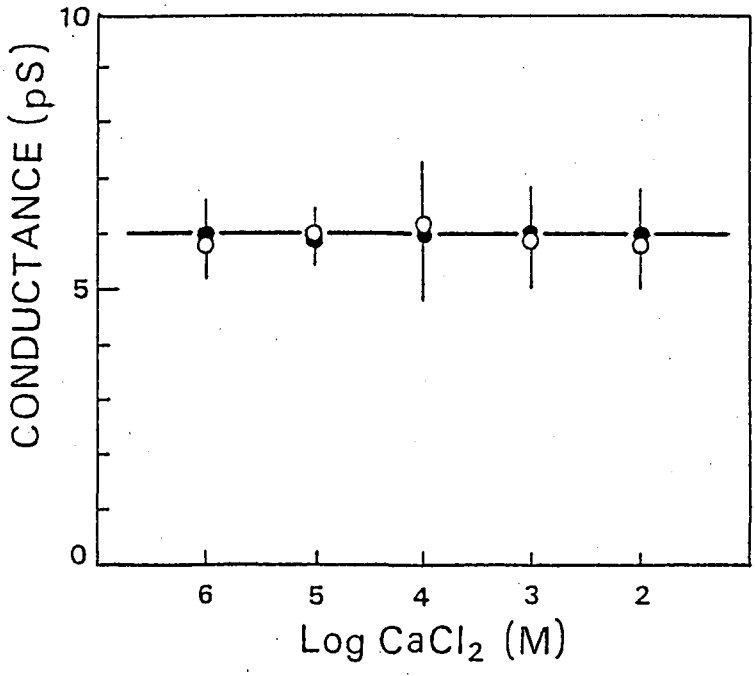

Fig.15 Single channel conductance as a function of $CaCl_2$ concentration in (●) oxidized cholesterol and in (○) glyceryl-monooleate bilayers at 50 mV in the presence of 500 mM KCl.

EFFECT OF Ca^{2+} ON THE OPENING AND CLOSING KINETICS

The single channel conductance of LTPI was not affected by Ca^{2+}. However, Ca^{2} affected the kinetics of its opening and closing reaction. In order to investigate the Ca^{2+}-effect on the conductance fluctuation, we carried out a seriese of experiments in the presence of various concentration of Ca^{2+} using both oxidized cholesterol and glycerylmonooleate membranes. However,

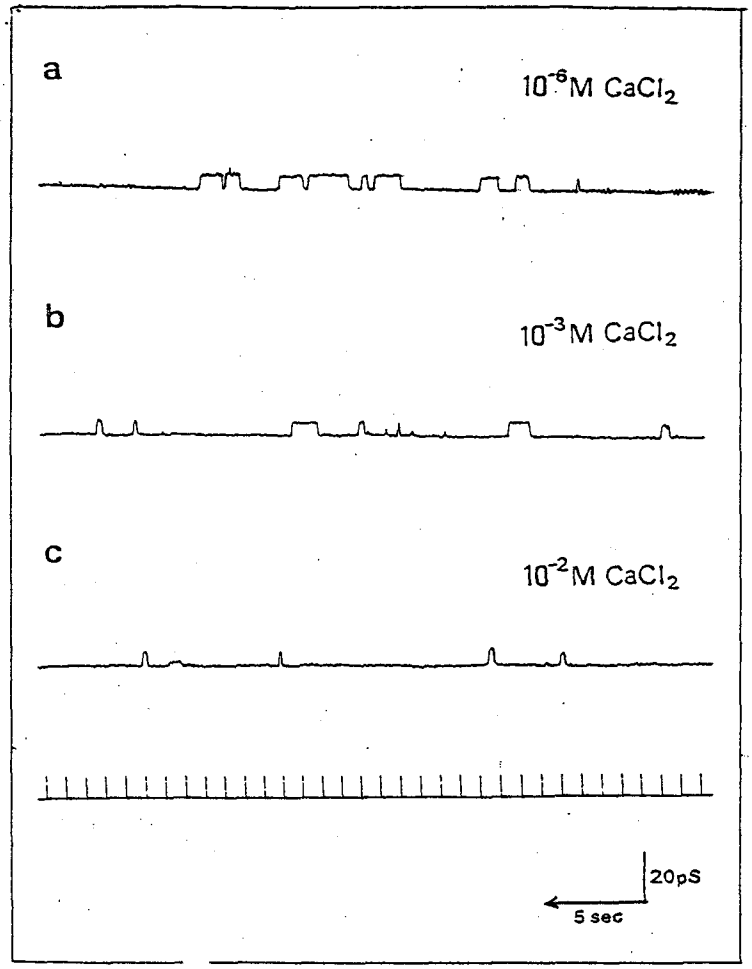

Fig.16 Conductance fluctuations of the single LTPI channel in glyceryl-monooleate bilayers at various $CaCl_2$ concentrations; (a) 10^{-6} M, (b) 10^{-3}M, (c) 10^{-2}M, in the presence of 500 mM KCl. Fluctuations were recorded with high speed recorder (response time; 10 msec.). A few very small size jumps are seen on the charts, but they were neglected in the statistics shown in Fig.17. The lowest record is a timing marker.

in the oxidized cholesterol membranes, a high speed switching was observed in the open state. Because of the limited bandwidth of our measuring system, we could not estimate the effect of Ca^{2+} on the time structure of the conductance fluctuations in the oxidized cholesterol membranes. On the other hand, in glyceryl-monooleate membranes, the open state of the channel was relatively stable so that we could estimate the dwell time of both open and closed states of the channel. Fig.16 indicates a typical time structure of the conductance fluctuation on a glycerylmonooleate membrane in the presence of various Ca^2

concentrations. It is shown that Ca^{2+} reduces the dwell times for the open states, whereas it increases the dwell times for the closed states.

From a series of these data, dwell time histograms were obtained. Fig.17 shows the cumulative sum of the number of events of duration longer than a given dwell time (t_d) plotted as a function of t_d. The distribution of the dwell times for both the

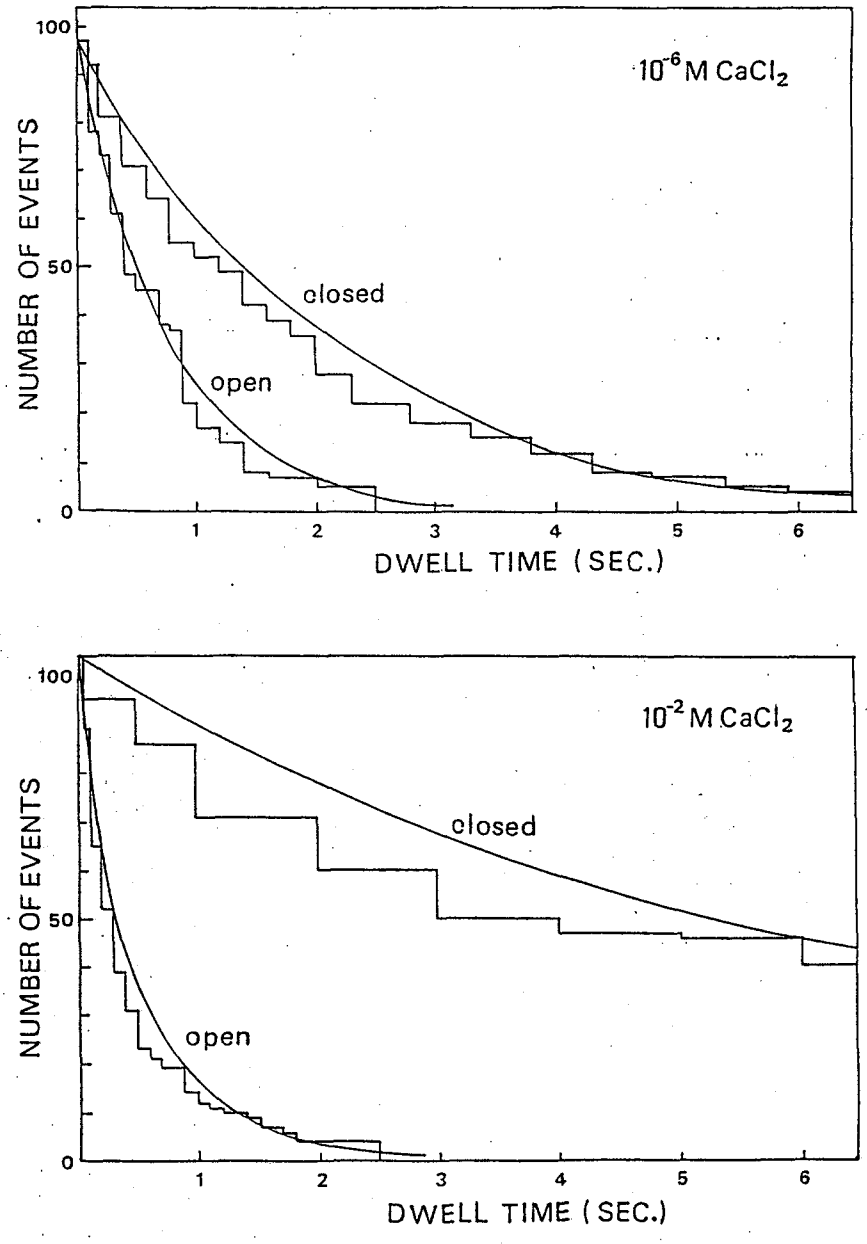

Fig.17 Distributions of the open and closed dwell times for a single channel in glycerylmonooleate bilayers at (a) 10^{-6} M $CaCl_2$ and (b) 10^{-2} M $CaCl_2$. The number of the individual dwell times which are longer than the time indicated bythe abscissa are plotted. The solid curves are single expornential functions ($n \cdot \exp[-\alpha t]$, $n \cdot \exp[-\beta t]$) calculated assuming that the rate constant is equal to the reciprocal of the average dwell time (cf. Eq.1). "n" denotes the total numbr of the events observed.

open and closed states of a single channel was well fitted by a single exponential function as shown in Fig.17. This fact means that each opening and closing event occurred independently on time, i.e. Poisson process. This allows us to describe the reaction with the first order kinetics as follows,

$$(\text{ closed }) \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} (\text{ open }) \qquad (1)$$

where α and β denote the rate constants for the opening and closing of the channel, respectively. The α and β are simply the reciprocals of the average dwell times ($\bar{t}_{dc}$, $\bar{t}_{do}$) for the closed and open states of the channel. That is,

$$\bar{t}_{do} = \frac{1}{m} \sum_{i=1}^{m} t_{doi} = \beta^{-1} \qquad (2a)$$

$$\bar{t}_{dc} = \frac{1}{m} \sum_{i=1}^{m} t_{dci} = \alpha^{-1} \qquad (2b)$$

where t_{doi} and t_{dci} are individual dwell time for the open and closed states, m is the number of jumps in a record. The rate constants calculated from the dwell times in the record of the single-channel conductance fluctuations at various $CaCl_2$ concentrations are summarized in Table.1. It is shown that Ca^{2+} decreased $\bar{t}_{do}$, whereas it increased $\bar{t}_{dc}$. Table 1 also indicates that the transition frequency (a number of events per unit time; P_t) defined as

$$P_t = (\bar{t}_{do} + \bar{t}_{dc})^{-1} = \alpha\beta / \alpha + \beta \qquad (3)$$

appears to decrease with the increase in Ca^{2+} concentration. Thus it is suggested that Ca^{2+} increased not only the free energy

difference between the open and closed states but also the the height of the activation energy barrier between these states.

Ca^{2+} EFFECT ON THE CONDUCTANCE OF MANY CHANNEL MEMBRANES

Ehrenstein et al.(1974) developed a kinetic theory for the opening and closing of the EIM, and accounted for the properties of many-channel membrane conductance. It is possible to calculate the steady state conductance of many-channel membrane using the rate constants obtained from single channel experiments after their theory. The following discussion is based on the assumption that there are no interactions between individual channels.

Let G be a steady state conductance of many-channel membrane conductance, n_o be the number of the open channel with the conductance g_o, and n_c be that of the closed channel with the conductance g_c, then

$$G = n_o g_o + n_c g_c \qquad (4)$$

We assume there are N channels in the membrane.

$$N = n_o + n_c$$

stands. From the principle of the detailed balance,

$$\bar{n}_o/\bar{n}_c = \alpha / \beta \qquad (6)$$

where the bar denote the equilibrium values. Combining Eqs.4,5 and 6, we obtain

$$G = N(\alpha g_o + \beta g_c)/(\alpha + \beta) \qquad (7)$$

We suppose g_c is negliglibly small, then Eq.7 becomes

$$G = N g_o \alpha / (\alpha + \beta) \qquad (8)$$

In order to compare the steady state conductance which was calculated from from Eq.8 with that experimentally obtained, we intoduce a relative conductance (Gr) which was difined as a ratio ofthe conductance at a given concentration of Ca^{2+} and that at 10^{-6}M Ca^{2+}. Since it could be assumed that both g_o and N were constant under different Ca^{2+} concentrations, we obtained

$$Gr = G_j / G_i = \beta_j(\alpha_i + \beta_i)/ \beta_i(\alpha_j + \beta_j) \qquad (9)$$

where the parameters subscripted i and j are at 10^{-6}M Ca^{2+} and at a given Ca^{2+} concentration, respectively. Many-channel membrane (100-1000 channels in a membrane) experiments were carried out with the same procedure as that for few-channel membrane. Calculated and experimental relative conductances were shown in Table.1. The coincidence between the calculated and the measured G_r indicates that, the conductance depression by Ca^{2+} in the glycerylmonooleate membranes was principally explaied by the change in the rate constants for the opening and closing reaction of the individual channels.

Table 1

DEPENDENCY OF THE KINETIC PARAMETERS FOR THE OPENING-CLOSING REACTION ON Ca^{++} CONCENTRATION

$CaCl_2$ (M)	Tr (sec.)	n	β^{-1} / α^{-1} (sec.)	β / α ($sec.^{-1}$)	Pt ($sec.^{-1}$)	Calculated Gr	Experimental Gr
10^{-6}	297.6	97	0.81 2.25	1.23 0.44	0.32	1	1
10^{-3}	290.6	55	0.70 4.58	1.43 0.22	0.19	0.48	0.49
10^{-2}	909.6	104	0.56 8.18	1.79 0.12	o.11	0.22	0.25

* Tr, total recording time; n, number of events; β^{-1}, mean open dwell time; α^{-1}, mean closed dwell time; β, rate constant for the closing; α, rate constant for the opening; Pt ($=\alpha\cdot\beta/\alpha+\beta \cong n/Tr$), transition frequency; Calculated Gr, relative conductance calculated by Eq.8; Experimental Gr, relative conductance of many-channel membrane experimentally obtained.

Data were obtained from at least five membranes.

DISCUSSION

From the results of our macroscopic experiments, it was speculated that the ion permeating mechanism formed from LTPI was not a carrier-type but a channel-type mechanism. Carrier-type molecules are known to be able to penetrate the hydrophobic core of the bilayer and transport the ions through the membrane even when they are applied to one side of the membrane, whereas the LTPI can not induce membrane conductance unless they are applied to both sides. This hypothesis as to the channel forming ability of LTPI molecules was directly demonstrated by the observation of the single channel conductance. As shown in Fig.12, well defined discrete conductance jumps were frequently observed upon addition of trace amount of LTPI,. The height of the most frequent jumps was about 6 pS in the presence of 500 mM KCl. This value (10^6 ions/sec/channel) seems to be higher than that predicted by the carrier mechanism (Hladky and Haydon, 1972; Laeuger, 1972).

The interesting feature of the membrane conductance induced by LTPI is the divalent cation-sensitivity, i.e. the membrane univalent cation conductance is blocked by divalent cations (See Fig.11). Single channel observation demonstrated that such a divalent cation including Ca^{2+} on the membrane conductance was due to its effect on the channel kinetics rather than the single channel conductance. Fig.15 shows that the single channel conductance of the LTPI channel remains constant even when the Ca^{2+} concentration increases from 10^{-6}M to 10^{-2}M , whereas both the average dwell time of the open state and the transition frequency ($\alpha\beta$ / α + β) are decreased with the Ca^{2+} concentration increase

(See Table.1). The reduction of the single channel conductance by the Ca^{2+} has been reported in the Ca^{2+} block of gramicidin A channel (Bamberg and Laeuger, 1977) and the Na^{+} channel in the neuroblastoma cell (Yamamoto et al., 1984). The reduction of the single channel conductance by the Ca^{2+} has been ascribed to the competitive occupation of the binding sites for the permeable cations in the channel by Ca^{2+} (Bamberg and Laeuger, 1977). On the other hand, the independence of the single LTPI channel conductance to the Ca^{2+} was demonstrated in this report. This suggests the absence of the Ca^{2+} binding site in the channel pathway. The Ca^{2+} sensitivity of the gating mechanism of the LTPI channel seems to be attributable to the physicochemical characteristics of the LTPI molecules. The LTPI has three phosphate groups which reveal five net negative charges per molecule at physiological pH. In addition, it is known, with regards to TPI, that divalent cations such as Ca^{2+} strongly binds to the phosphate moieties resulting in the drastic increase in the hydrophobicity of the molecules and cause a drastic hydrophilic-hydrophobic micelle transition. A similar hydrophilic-hydrophobic micelle transition induced by Ca^{2+} has been observed in aqueous solutions of LTPI (unpublished observation). It is highly likely that such a hydrophilic-hydrophobic micelle transition of LTPI is underlying the open-closed state transition of the LTPI channel.

In order to consider the Ca^{2+}-effect on the LTPI channel further, it is worthwhile to discuss the origin of the spontaneous conductance fluctuations of the LTPI channel. The

mechanisms speculated to be the origin of the conductance fluctuation of the various well defined channels can be divided broadly into three categories.

The first is the conductance fluctuation in the gating mechanism of channel penetrating the membrane. Many channels in biological membranes are supposed to be involved in this type of channel, and these channels are considered to be made of membrane protein (Miller and Rosenberg, 1979; Gilly and Armstrong, 1982 (a),(b)). The current fluctuation is believed to be caused by the blocking and unblocking of the channel pathways by the hypothetical gate molecule made of polypeptide.

The second is those from the monomer-dimer reaction between "half pores". The half pore penetrates its own hydrophobic tail to the hydrophobic core of the bilayer and they can freely move on the membrane surface, though the monomer cannot permeate ions by itself unless they form a dimer by binding tail-to-tail with each other. Gramicidin A, nystatin and amphotericin are thought to be in this category (Bamberg and Janko, 1977; Bamberg and Laeuger, 1973, Szabo and Urry, 1978; Finkelstein and Holtz, 1973).

The last category is those from the spontaneous phase transition of the cluster of the channeling materials. A certain channeling material having hydrophilic and hydrophobic region has the nature of forming a 2-dimensional cluster on the membrane surface and spontaneously (in general depending on the electric field in the membrane) flip their headgroups to the other surface to make a channel structure. The "barrel stave model" for alamethicin and monazomicin seem to be the typical model in this category (Baumann and Mueller, 1974; Boheim, 1974; Heyer et al.,

1976; Boheim and Kolb, 1978; Hall, 1975).

Since the LTPI channel is thought to be an assembly of LTPI molecules, we may be able to neglect the first category. We also have a contradictory view against the second category, i.e., monomer-dimer hypothesis. The fact that the single channel current fluctuation can be observed for a rather long period suggests that there is a stable channel structure which is made of LTPI and undergoes open and close transition. Therefore, the monomer-dimer hypothesis, in which the monomer is thought to be freely movable on the membrane surface , seems to be inadequate to explain our results.

It is likely that the last category is the most feasible one for the LTPI channel. Fig.18 shows a hypothetical model for the LTPI channel. The LTPI molecules incorporated into the membrane would make a cluster in the presence of Ca^{2+} in a similar manner to phosphatidylserine in neutral phospholipid membrane (Ohnishi,

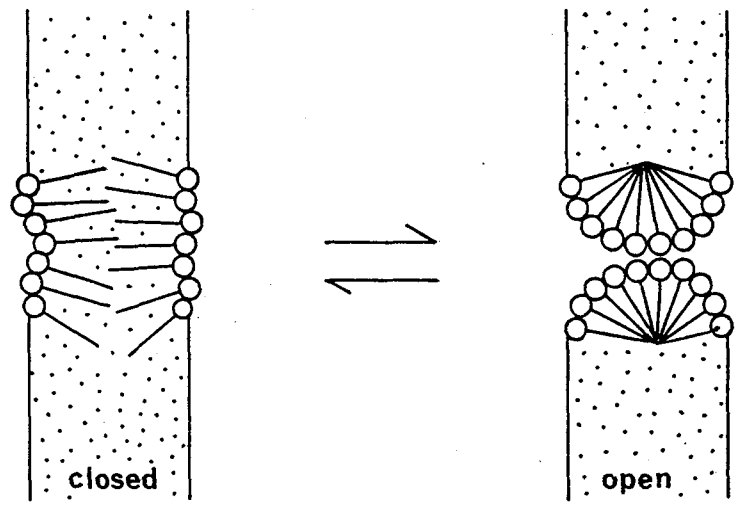

Fig.18 Model for formation of LTPI channel. LTPI molecules form a claster in bilayer, and the form of claster fluctuates between two stable states. Open circle with bar denotes one LTPI molecule. Univalent cation salt of LTPI has effective head group which has larger area than its own monoglyceride residue. This is similar to strong detergent sodium dodecylsulfate (SDS). Thus, univalent cation salt of LTPI form a pore like stracture in the bilayer. The stability of these two states is dependent on both uni- and di-valent cation concentrations.

1978). The LTPI has a highly wedge-like shape and its univalent cation salt makes very small micelles in the water. The lysophospholipid such as lysolecithin has been thought to make a pore-like structure in the membrane (Robertson, 1983; Cullis and Kruijff, 1979). The LTPI is, of cause, one of the lysophospholipids. Therefore, when the Ca^{2+} bound to the LTPI is replaced with univalent cation, it can be speculated that the micelle form of the LTPI changes and turns to be a channel like structure. Thus, the current fluctuation can be ascribed to the fluctuation of the micelle form of the LTPI cluster on the membrane. The dependency of the channel kinetics on Ca^{2+} gives us information about the channel mechanism. As summarized in Table-1, Ca^{2+} decreases the rate constant of the opening reaction (α) and increases that of the closing (β), resulting in a decrease in the transition frequency (P_t). This situation can be described in terms of the changes in the energy profile of the reaction process between the open and closed states, as shown in Fig.19. These changes in the kinetic parameters suggest that the Ca^{2+}

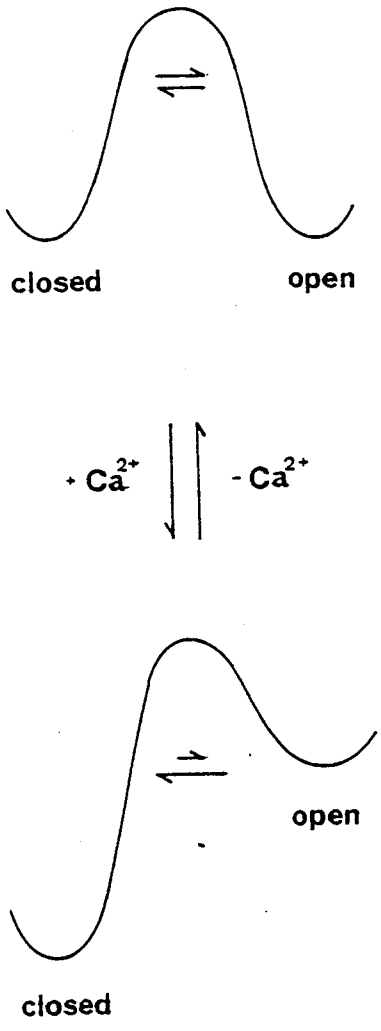

Fig.19 Effect of Ca^{2+} on the activation energy profile between closed and open state of LTPI channel. Ca^{2+} stabilizes the closed state, and decreases and increases the opening and closing rate, respectively.

increases the activation energy of the opening reaction and decreases the activation energy of the closing reaction. Ca^{2+} seems to lean the whole energy profile to stabilize the closed state. An activation energy barrier is thought to exist in the middle of the opening-closing reaction process. The phase transition hypothesis mentioned above provides a physical image for this activation energy barrier. It is obvious that LTPI molecules have to dip their hydrophilic portion into the hydrophobic core of the membrane and make a channel through this hydrophobic barrier. This hydrophobic barrier may be equivalent to the activation energy barrier in the gating reaction.

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