論文 / 著書情報
Article / Book Information

題目(和文)	
Title(English)	Studies on Endothelial cell -Ca²⁺ - or Mg²⁺ - ATPase and Plasminogen Activator-
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出典(和文)	学位:理学博士, 学位授与機関:東京工業大学, 報告番号:甲第1527号, 授与年月日:1984年3月26日, 学位の種別:課程博士, 審査員:
Citation(English)	Degree:Doctor of Science, Conferring organization: Tokyo Institute of Technology, Report number:甲第1527号, Conferred date:1984/3/26, Degree Type:Course doctor, Examiner:
学位種別(和文)	博士論文
Type(English)	Doctoral Thesis

H-25

Studies on Endothelial cell

-Ca^{2+}- or Mg^{2+}-ATPase and Plasminogen Activator-

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CONTENT

1. SUMMARY.

The author has succeeded in culture of endothelial cells from bovine carotid artery. Using these cultured endothelial cells, two enzymes, ATPase and plasminogen activator were studied and the following results were obtained.

1. The activity of ATPase enhanced independently either with Ca^{2+} or Mg^{2+} was found in plasma membrane of cultured endothelial cells. The values of V_{max} were 2.8 and 10.0 μmol Pi/mg protein.hr for Ca^{2+}- and Mg^{2+}-ATPase activity, respectively, and the corresponding K_m values were 4.8×10^{-4} M and 3.2×10^{-4} M. The molecular weight of these ATPase was estimated to be approximately 250,000 in both cases by activity-staining electrophoresis with polyacrylamide gels. The Ca^{2+}-ATPase activity was enhanced with calmodulin and was not affected by chemical modification of SH-groups with 5,5'-dithiobis(2-nitrobenzoic acid)[DTNB] and o-iodosobenzoate. On the other hand, Mg^{2+}-ATPase activity was not affected by calmodulin but was inhibited by DTNB and o-iodosobenzoate. Calcium antagonists including nifedipine, verapamil, diltiazem and SP-294 inhibited Ca^{2+}- and Mg^{2+}-ATPase activities but other vasodilating agents did not inhibit these activities at all. This kind of studies may give a clue to clarify the physiological function of the enzyme.

2. The endothelial cell is a rich source of plasminogen activator that is associated with fibrinolytic activity in blood vessel. The author demonstrated stimulative effect of sitosterol on the production of plasminogen activator in cultured endothelial cells. Addition of sitosterol to the culture medium of the cells gave rise to a marked increment of the activity of plasminogen activator in the culture medium and in the cells. Removal of sitosterol from the culture medium resulted in a decrease of plasminogen activator activity

back to normal level. A similar phenomenon was observed with fucosterol but not with other steroids such as cholesterol, stigmasterol, isofucosterol, brassicasterol and 5-androsten-3β-ol. This finding may suggest that sitosterol and fucosterol make a novel agent for prevention and therapy of patients with thrombosis.

2. INTRODUCTION.

Blood vessels are classified into artery, vein and capillary. Carotid artery consists of three different layers, tunica intima(A), tunica media(B), and tunica externa(C), which are mainly made of endothelial cells(D), smooth muscle cells(F) and fibroblast cells, respectively(Fig. 1-a). Endothelium consists of monolayer of flattened, fairly uniform, polygonal and elongated cells(endothelial cell(D)) that are approximately 25 to 50 μ in length and 10 to 15 μ in width, and the layers cover the inner face of the blood vessel wall(Fig. 1-b). Main physiological functions of endothelial cells, as far as I know, are as follows(1,2).

a) blood barrier. The layer of these cells selectively regulates transfer of substances of varied molecular size between the circulating blood and the surrounding tissues.

b) thromboresistant surface. These cells prevent the adhesion of platelets and leucocytes to blood vessel wall, and inhibit the activation of blood coagulation system. The cells contribute to prevent from a number of diseases of blood vessel walls such as atherosclerosis, thrombosis and disseminated intravascular coagulation(DIC).

Since endothelial cell was first cultured in an artificial medium by Jaffe et al.(3,4), extensive studies on endothelial cells from various species and organs have been accumulated. They include human umbilical cord vein(5,6) and pulmonary(7), rabbit aorta(8) and vena cava(9), bovine aorta(10) and porcine aorta(11). In recent years, it has become evident that the endothelial cell plays an important role in the synthesis of several biologically important substances, for example, plasminogen activator(12,13), prostacyclin

(PGI_2)(14,15), angiotensin converting enzyme(15-17), blood coagulation factor VIII antigen(2,3,18) and von Willebrand factor(19). Plasminogen activator is an enzyme which converts plasminogen to plasmin and activates the fibrinolytic activity in blood. Prostacyclin(PGI_2) prevents the aggregation of platelets and dilates the blood vessel. Angiotensin converting enzyme regulates the blood pressure. Blood coagulation factor VIII antigen and von Willebrand factor are necessary for normal platelet adhesion and bleeding time. Also, factor VIII antigen is now considered as a specific marker for identification of endothelial cells.

In this dissertation, the author presented results on the investigations about the following subjects using cultured endothelial cells from bovine carotid artery.

1. The properties of Ca^{2+}- or Mg^{2+}-ATPase found in plasma membrane of these cells.
2. The effect of phytosterol, sitosterol and fucosterol, on the production and secretion of plasminogen activator from cells.

Fig. 1-a

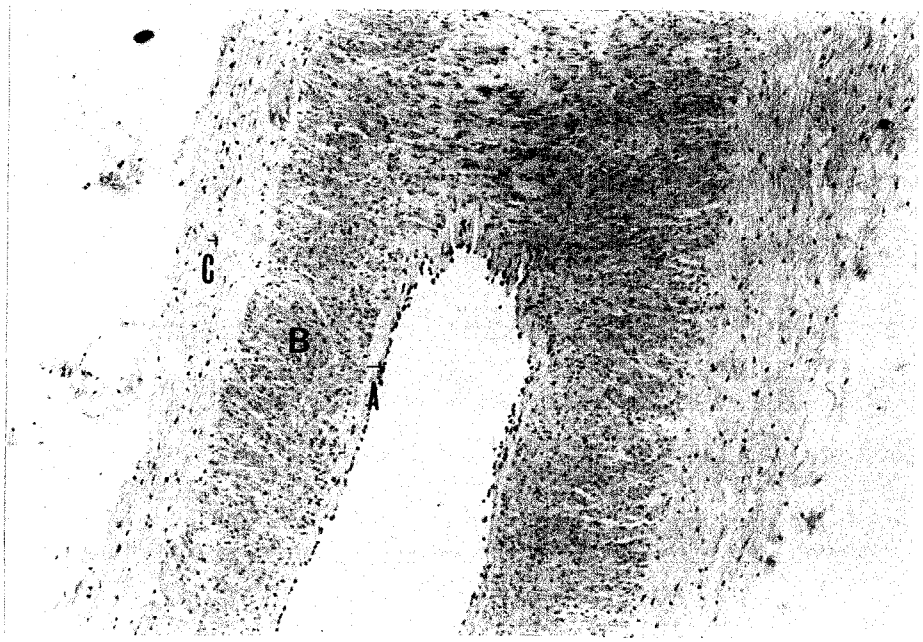


Fig.1-b

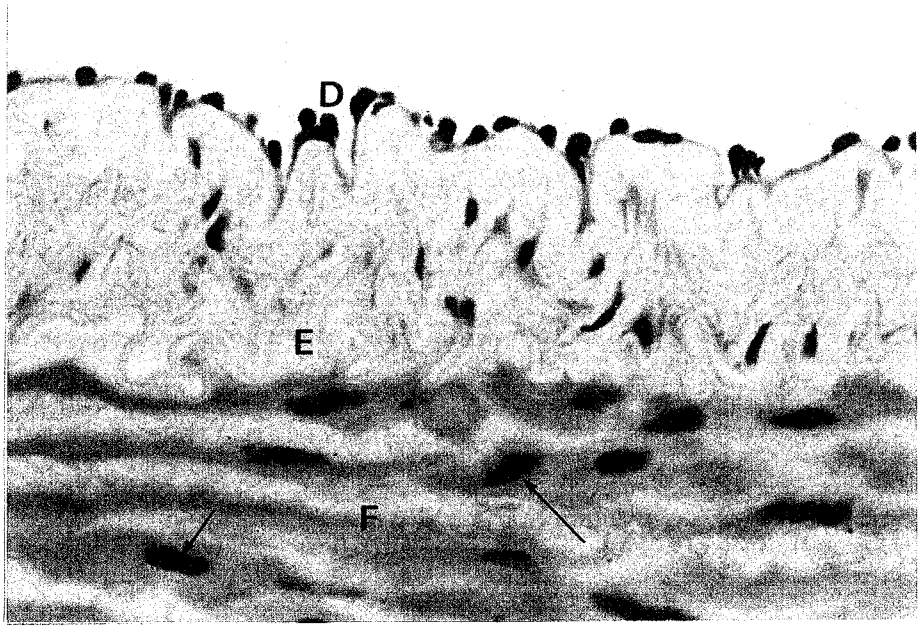


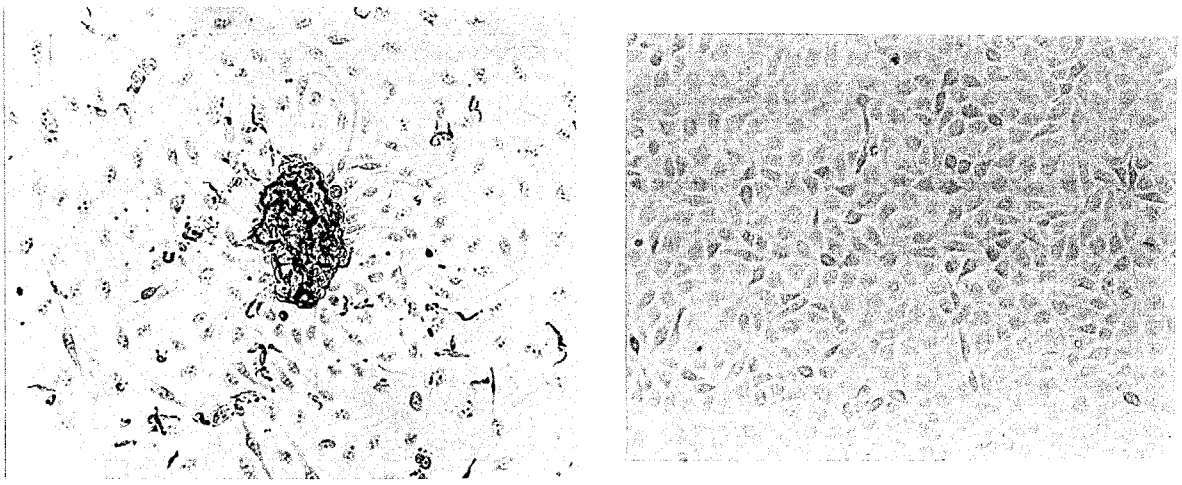
Fig. 1
Micrograph of bovine carotid artery.
After being treated by 2.5% glutaraldehyde at 4°C for 24 hr, it was stained by haematoxylin-eosin(H-E stain). a) (x 40) A; tunica intima, B; tunica media, C; tunica externa. b) (x 400) D; endothelial cell, E; basement membrane, F; tunica media(arrow is smooth muscle cell).

CHAPTER I

ENDOTHELIAL CELL CULTURE

3. ENDOTHELIAL CELL CULTURE.

Endothelial cell culture was carried out as follows(18). Bovine carotid arteries were obtained fresh at a local slaughter house. They were opened lengthwise, and endothelial cell monolayer was obtained by gently scraping the intimal surface with a scalpel. The endothelial cells were transferred to a $9cm^2$ plastic petri dish, and primary culture was carried out in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin(50 µg/ml) in an atmosphere of 95%air-5%CO_2 at 37°C(shown in Fig. 2-a). Fetal bovine serum, antibiotics(penicillin-streptomycin solution) and Eagle's minimum essential medium were purchased from GIBCO New York. Endothelial cells were dissolved from the petri dish surface by 0.05% trypsin solution containing 0.02% EDTA, 137 mM NaCl, 4 mM KCl, 0.5 mM $Na_2HPO_4 \cdot 12H_2O$, 0.15 mM KH_2PO_4 and 11 mM glucose(pH7.0). The cells were subcultured in a $55cm^2$ petri dish at a rate of one generation per



(a) (b)

Fig. 2
a) The proliferation of endothelial cells from bovine carotid artery attached to petri dish(primary culture). b) Confluent monolayer of endothelial cells in 16th passage(17 generations from primary culture).

one passage in the same conditions as primary culture, and were routinely subcultured and fed with fresh medium every 2 days.

Bovine endothelial cells which had been passaged 16 times(17 generations) are shown in Fig. 2-b. The number of cells in a petri dish(55cm^2) was approximately 1.5 x 10^7, when they reached to a confluency. Since they have a characteristic morphology of endothelial cells they are not contaminated with smooth muscle cells or other cells. The growth curve of endothelial cells in medium with 10% fetal bovine serum is shown in Fig. 3.

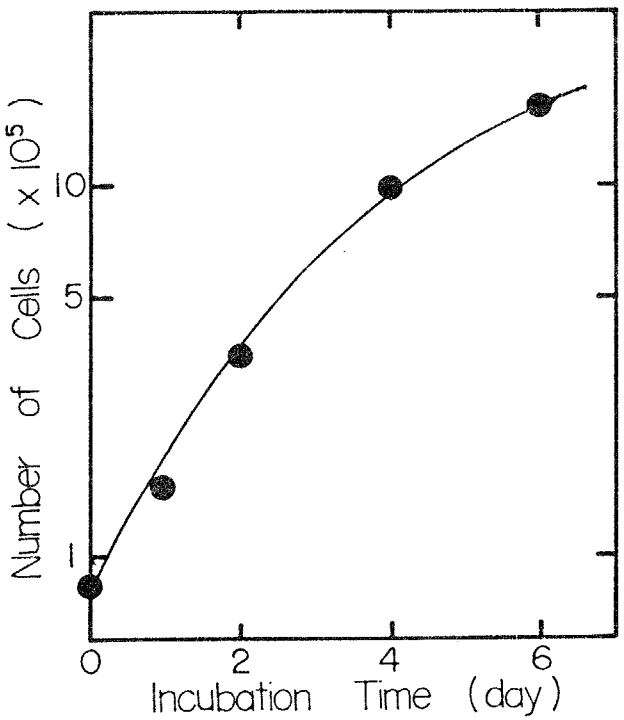


Fig. 3
The proliferation of endothelial cells from bovine carotid artery in tissue culture.
The culture was carried out with 9cm^2 petri dish.

CHAPTER II

ADENOSINE TRIPHOSPHATASE IN PLASMA MEMBRANE OF ENDOTHELIAL CELL

4. CHAPTER II.

ADENOSINE TRIPHOSPHATASE IN PLASMA MEMBRANE OF ENDOTHELIAL CELL

4-1. Summary.

ATPase was found in plasma membrane of cultured endothelial cells from bovine carotid artery. The activity in the membrane-bound beads or in the membrane solubilized by octaethyleneglycol mono-n-dodecyl ether was enhanced by the addition of Ca^{2+} or Mg^{2+}, while not by other divalent cations. It was found that the activities were independently enhanced by Ca^{2+} and Mg^{2+}, and that they were additive. This indicates either that there are two different kinds of ATPase molecules, or that there is only one kind of the enzyme but with two different kinds of active sites. This ATPase was affected by F-actin nor by ouabain.

Moreover, the ATPase activity was selectively inhibited by calcium antagonists including a new compound, 6-ethoxycarbonyl-5,7-dimethyl-1-[3-(4-phenethyl-1-piperazinyl)propyl]-3-(α,α,α-trifluoro-o-tolyl)-2,4(1H,3H)-quinazolinedione dihydrochloride, while not by the other hypotensive drugs .

V_{max} was 2.8 and 10.0 μmol Pi/mg protein·hr for Ca^{2+}- and Mg^{2+}-dependent activity, respectively, and the corresponding K_m was 4.8×10^{-4} M and 3.2×10^{-4} M. The Molecular weight of ATPase activated by Ca^{2+} or Mg^{2+} was estimated to be approximately 250,000, by activity-staining electrophoresis with polyacrylamide gels. The activity of Ca^{2+}-ATPase inhibited by treatment with Glycoletherdiaminetetraacetic acid[EGTA] was enhanced back to the normal level by the addition of calmodulin. The modification of SH-groups of proteins by DTNB and o-iodosobenzoate completely inhibited Mg^{2+}-ATPase but did not affect Ca^{2+}-ATPase.

4-2. Introduction.

Recently, studies on cultured endothelial cells have been accumulated in relation to plasminogen activator(12,13), prostacyclin(PGI_2)(14,15), angiotensin converting enzyme(15-17) and blood coagulation factor VIII antigen(3,4,18). No information about ATPase in plasma membrane of endothelial cells, however, has yet been obtained. This is probably because it is difficult to obtain enough materials for experiments. ATPase(ATP phosphohydrolase[EC 6.3.1.3]) activity, which hydrolizes ATP to ADP, was found in various plasma membrane including human erythrocyte(21-23), human granulocyte(24), rat liver cell(25) and rat hepatoma cell(26). These ATPases are generally divided into two groups; One group is Ca^{2+}-ATPase(27,28) or Mg^{2+}-ATPase(21,22,26) activated with only Ca^{2+} or Mg^{2+}, respectively, and other group includes (Ca^{2+} + Mg^{2+})-ATPase(23,29) activated with the simultaneous addition of Ca^{2+}(10^{-7}-10^{-6} M) and Mg^{2+}(10^{-3} M). Some of these enzymes were partially purified after solubilization and their physicochemical properties were revealed, but the physiological functions of these enzymes were not necessarily clear yet. In order to clarify the physiological functions of the ATPase in plasma membrane of endothelial cells, inhibition test of the enzyme was carried out by hypotensive drugs .

Present study deals with Ca^{2+}- or Mg^{2+}-ATPase found in plasma membrane of cultured endothelial cells from bovine carotid artery, and the selective inhibition of ATPase by calcium antagonists having the vasodilating properties.

4-3. Materials and Methods.

Positively charged Sephadex beads(Cytodex-1) and polyacrylamide gradient gel

PAA 4/30 were obtained from Pharmacia Fine Chemicals. Octaethyleneglycol mono-n-dodecyl ether($C_{12}E_8$) was obtained from Nikko Chemical Co., Tokyo. All other reagents were of analytical grade.

Isolation of Plasma Membrane and Solubilization.

Endothelial cells were bound to cationic beads(Cytodex-1), lysed with a Vortex mixer and a Branson ultrasonic cleaner, and washed with Tris-HCl(pH 7.4) by repeated centrifugation according to the method to Gotlib(30). Plasma membrane proteins were solubilized from the beads by dropping a mixture of 0.1 M KCl, 20% glycerol, 0.3 M sucrose and 0.05-0.2% $C_{12}E_8$ dissolved in 10 mM Tes-NaOH buffer(pH 7.5) to the beads at 4°C over a period of 1 hr(31).

Measurement of ATPase Activity.

Ca^{2+}- or Mg^{2+}-ATPase activity was measured as follows. A sample solution (100 µl) was added to a buffer solution(400 µl) of 75 mM Tris-HCl(pH 8.0) containing 3 mM ATP and 5 mM $CaCl_2$(or $MgCl_2$). The mixture was incubated for 5 min for Mg^{2+}-ATPase and 15 min for Ca^{2+}-ATPase at 30°C, and then 30% trichloroacetic acid(150 µl) was added to stop the reaction. The amount of inorganic phosphate liberated from ATP was spectrophotometrically determined by the method of Martin and Doty(32). ($Na^+ + K^+$)-ATPase activity was assayed in a mixture of a sample solution(100 µl) and a buffer solution(900 µl) of 20 mM Tris-HCl(pH 7.4) containing 5 mM $MgCl_2$, 140 mM NaCl, 14 mM KCl, 0.5 mM EDTA and 3 mM ATP in the presence and the absence of 0.2 mM ouabain(33). The reaction was continued for 30 min at 37°C, and 7.5% trichloroacetic acid(2.0 ml) was added to the reaction mixture. The amount of inorganic phosphate was determined by the same method as above. Protein concentration was determined by the method of Lowry et al.(34).

Activity-Staining Gel Electrophoresis of ATPase.

The solubilized plasma membrane proteins were concentrated with Amicon CF 50A filter and were subjected to electrophoresis on polyacrylamide gel with a gradient from 4% to 30% in the absence of sodium dodecyl sulfate(SDS). After the electrophoresis for 16 hr at 4°C, the gel was washed with 20 mM Tris-maleate buffer(pH 8.0) and steeped in a mixture of 1 mM ATP, 1 mM $Pb(NO_3)_2$, 5 mM $MgCl_2$(or $CaCl_2$) and 20 mM Tris-maleate(pH 8.0) for 12 hr at room temp. The position of the enzyme was stained with 0.5% PbS(21,35).

Drugs.

Drugs employed in the inhibition test comprise chemically unrelated compounds and have different kinds of pharmacological effect; nifedipine, SP-294, verapamil·HCl and diltiazem·HCl(calcium antagonist), papaverine.HCl and hydralazine·HCl(dilation agent of smooth muscle), propranolol·HCl(beta-adrenergic blocker), clonidine·HCl(sympatholytic agent), chlorothiazide (diuretic agent) and ticlopidine.HCl(platelet aggregation inhibitor). The chemical formula of a new compound, SP-294, is 6-ethoxycarbonyl-5,7-dimethyl-1-[3-(4-phenethyl-1-piperazinyl)propyl]-3-(α,α,α-trifluoro-o-tolyl)-2,4(1H,3H)-quinazolinedione dihydrochloride, which has the properties of calcium antagonist and was kindly provided by Mr. I. Kotoku in Showa Denko K. K.(Japan).

Inhibition Test.

The inhibitory effect of hypotensive agents on ATPase activity was investigated using the membrane-bound beads and solubilized membrane proteins. The ATPase activity enhanced with Ca^{2+} was measured by the method described above in the presence and the absence of drugs. The same kind of experiment was carried out with Ca^{2+}-dependent chloroplast ATPase, chloroplast coupling

factor 1(CF_1), and alkali phosphatase. Chloroplast coupling factor 1 was prepared from spinach leaves by the method of Lien and Racker(36) and was activated by heat at 60°C for 4 min. The enzymic activity of chloroplast ATPase was determined by the method described previously(37) in the presence and the absence of drugs. Alkali phosphatase obtained from E. coli was purchased from Boehringer Mannheim Gm. and the enzymic activity was determined by the method of Garen and Levinthal(38).

4-4. Results and Discussion.

§ 1. Ca^{2+}- or Mg^{2+}-ATPase in plasma membrane of endothelial cell.

Before solubilizing ATPase from beads, its activity was assayed with membrane-bound beads in the presence of monovalent and divalent cations. The activity was markedly enhanced by the addition of Ca^{2+} or Mg^{2+}, while not by the addition of other divalent cations such as Sr^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, Pb^{2+},

TABLE 1. THE EFFECT OF DIVALENT CATIONS ON ATPase.

divalent cations (5 mM)	ATPase Activity(μmol Pi/mg protein·hr)	
	Membrane-bound beads	Solubilized proteins
Mg^{2+}	9.33	9.38
Ca^{2+}	2.73	2.50
Sr^{2+}	1.24	0.83
Co^{2+}	0.44	1.12
Mn^{2+}	0.43	0.65
Cu^{2+}	0.31	0.25
Pb^{2+}	0.20	0.18
Ba^{2+}	0.18	0.11
Cr^{3+}	0.08	0.08

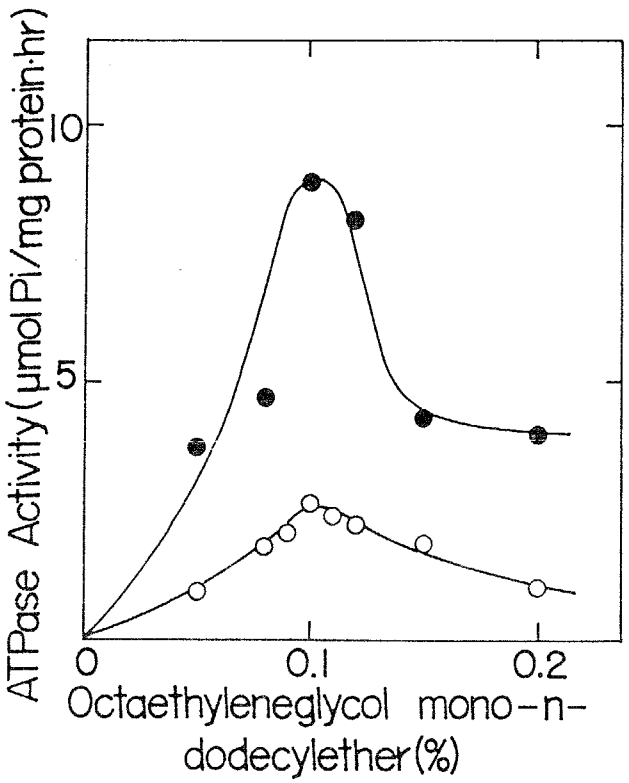


Fig. 4.
The concentration of $C_{12}E_8$ to solubilize membrane proteins from beads.
-O-O-, Ca^{2+}-ATPase activity, -●-●-, Mg^{2+}-ATPase activity.

Ba^{2+} and Cr^{3+} (shown in TABLE I). The membrane bound to beads had also ouabain-inhibitable ($Na^+ + K^+$)-ATPase activity. This enzyme is known as a marker enzyme of plasma membrane(39).

In order to obtain further information about ATPase in the membrane, I tried to solubilize proteins from the membrane with $C_{12}E_8$. Varing the concentration of $C_{12}E_8$ to solubilize membrane proteins from beads, it was found that 0.1% gave the optimal Ca^{2+}- or Mg^{2+}-ATPase activity as shown in Fig. 4. Using the solubilized membrane proteins, the effect of divalent cations on ATPase activity was tested(shown in TABLE I). ATPase activity was enhanced by the addition of Ca^{2+} and Mg^{2+}. The effect of the concentration of Ca^{2+} or Mg^{2+} on ATPase activity was shown in Fig. 5. The activity

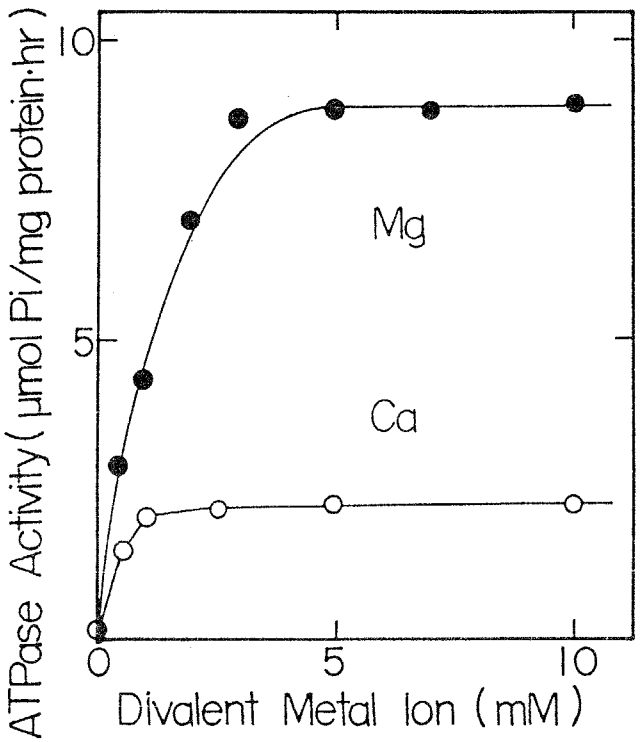


Fig. 5.
Effect of divalent cations on solubilized ATPase activity obtained from cultured endothelial cells from bovine carotid artery.
-O-O-, in the presence of Ca^{2+}. -●-●-, in the presence of Mg^{2+}.

was enhanced by increasing the concentration of Ca^{2+} or Mg^{2+} and reached a constant value at 3 mM of these divalent cations. The ATPase activity stimulated by Mg^{2+} was approximately 4-fold greater than that by Ca^{2+}.

The enzymic activity of the solubilized membrane proteins was measured using various substrates, such as AMP, ADP, p-nitrophenyl phosphate and glucose 6-phosphate. The results are shown in TABLE II. No hydrolysis of p-nitrophenyl phosphate, which is known as a substrate of phosphatase(40), was observed in the presence of Ca^{2+} or Mg^{2+}. Hydrolysis of glucose 6-phosphate occurred slightly, suggesting the existence of glucose 6-phosphatase in the solubilized membrane proteins. The enzyme, glucose 6-phosphatase, is mainly

TABLE II. HYDROLYSIS OF PHOSPHATE ESTERS BY SOLUBILIZED PROTEINS FROM PLASMA MEMBRANE OF CULTURED ENDOTHELIAL CELLS.

Substrate	Relative Hydrolysis (%)	
	5 mM Ca^{2+}	5 mM Mg^{2+}
3 mM ATP	100**	100*
3 mM ADP	3.5	1.4
3 mM AMP	2.3	1.0
3 mM p-nitrophenyl phosphate(PNPP)	0	0
3 mM glucose 6-phosphate(G-6-P)	12.1	13.4
3 mM ATP plus F-actin(1.8μg) from rabbit skeletal muscle	106.9	99.5
3 mM ATP plus 0.2 mM Ouabain	100.0	94.7

* Mg^{2+}-ATPase Activity 100%; 9.3 μmol Pi/mg protein·hr
** Ca^{2+}-ATPase Activity 100%; 2.4 μmol Pi/mg protein.hr
ADP, AMP, PNPP and G-6-P were added instead of ATP to reaction medium of ATPase activity(see in Materials and Methods).

found in endoplasmic reticulum(41). The liberation of inorganic phosphate from AMP or ADP took place only to a small extent in the presence of Ca^{2+} or Mg^{2+}. The effect of F-actin from rabbit skeletal muscle on the Ca^{2+}- or Mg^{2+}-ATPase activity was tested. No stimulative effect on the ATPase obtained from carotid endothelial cells is quite different from myosin ATPase from muscle. The Ca^{2+}- or Mg^{2+}-ATPase was not inhibited by ouabain, an inhibitor of $(Na^{+} + K^{+})$-ATPase.

$ 2. Inhibition of Ca^{2+}- or Mg^{2+}-ATPase by calcium antagonists.

Fig. 6 shows inhibition curves of Ca^{2+}-ATPase activity of membrane-bound beads from endothelial cells by various concentrations of calcium antagonists,

nifedipine(-●-●-), and SP-294(-O-O-) together with propranolol(-▲-▲-). The ATPase activity decreased markedly by incresing nifedipine concentration and reached a constant level(80% inhibition) above 25 μM. A similar inhibition curve was obtained in the case of SP-294 and the ATPase activity decreased to approximately 36% of the original activity above 25 μM. The complete loss of the ATPase activity did not occur by increasing the concentration of nifedipine or SP-294. A similar incomplete inhibition had been observed for inhibition studies on chloroplast ATPase by basic polypeptides(42), E. coli ATPase by troponin component TN-I(43) and by mitochondrial ATPase inhibitor(F_1I)(43), although the reasons were unclear. No inhibitory effect was observed by propranolol, one of beta-adrenergic blocker(-▲-▲-).

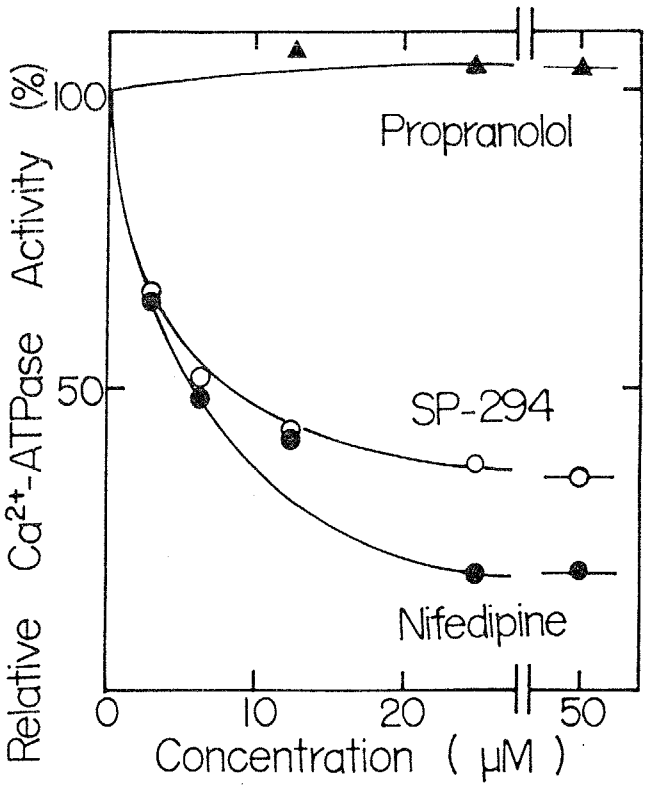


Fig. 6.
Inhibition of Ca^{2+}-ATPase of membrane-bound beads from endothelial cells by nifedipine, SP-294 and propranolol.
-●-●-, nifedipine, -O-O-, SP-294, -▲-▲-, propranolol.

TABLE III. INHIBITION OF Ca^{2+}-ENDOTHELIAL ATPase, Ca^{2+}-CHLOROPLAST ATPase AND ALKALI PHOSPHATASE BY HYPOTENSIVE DRUGS.

DRUGS (25 μM)	Ca^{2+}-Endothelial ATPase Activity (%) Membrane-bound beads	Solubilized protein	Ca^{2+}-Chloroplast ATPase Activity (%)	Alkali Phosphatase Activity (%)
none	100	100	100	100
Nifedipine	15.0 ± 5.0	43.0 ± 3.7	99.3 ± 5.9	100.2 ± 4.4
SP-294	36.9 ± 5.0	45.5 ± 5.0	98.0 ± 5.0	101.4 ± 0.1
Verapamil	64.7 ± 4.0	84.7 ± 0.6	137.0 ± 5.8	94.4 ± 4.0
Diltiazem	80.3 ± 3.0	84.8 ± 1.0	107.0 ± 9.6	100.1 ± 6.2
Papaverine	90.7 ± 3.0	86.6 ± 1.5	110.4 ± 3.6	101.8 ± 4.2
Hydralazine	100.1 ± 5.0	87.1 ± 3.7	95.4 ± 6.6	104.8 ± 8.6
Clonidine	101.2 ± 5.0	105.8 ± 6.0	96.7 ± 2.6	106.0 ± 5.8
Propranolol	110.6 ± 6.0	97.3 ± 11.3	94.7 ± 8.4	102.3 ± 5.0
Chlorothiazide	105.5 ± 5.0	95.0 ± 7.1	104.8 ± 4.0	93.8 ± 6.8
Ticlopidine	108.1 ± 2.0	107.1 ± 0	112.4 ± 4.7	100.8 ± 4.7

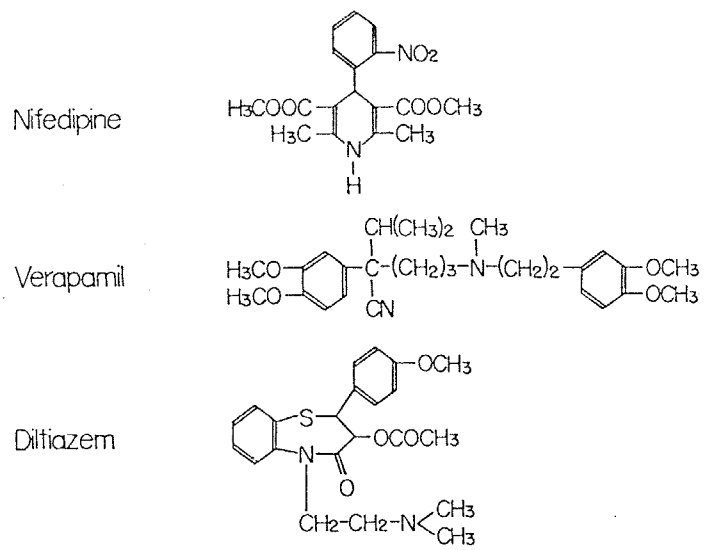


Fig. 7.
Structure of calcium antagonists.

TABLE III represents drug-induced inhibition of ATPase activity of endothelial membrane-bound beads and solubilized membrane proteins. The selective inhibition of ATPase activity of membrane-bound beads or solubilized membrane proteins was observed by calcium antagonists, nifedipine, SP-294, verapamil or diltiazem among hypotensive drugs. Among them, nifedipine and SP-294 were the most effective to inhibit the ATPase activity. The other hypotensive drugs such as propranolol, clonidine and chlorothiazide as well as an inhibitor of platelet aggregation, ticlopidine, have no inhibitory action of endothelial ATPase. The extent of inhibition is different between membrane and solubilized proteins, which may be due to alteration of protein conformation by solubilization with $C_{12}E_8$. A similar drug-induced inhibitory effect was also observed with endothelial ATPase enhanced by Mg^{2+}(data not shown). However, Ca^{2+}-dependent chloroplast ATPase and alkali phosphatase were not inhibited at all by any of these drugs.

Fleckenstein introduced in 1969 the term calcium antagonist as a novel drug designation(44) and an extensive study has been accumulated from the viewpoint of biochemical and pharmacologic aspects(45). Although many important questions remain to be answered concerning the action of calcium antagonists, it is known to block the calcium slow channel for the entry of Ca^{2+} into the cells. Nifedipine, verapamil and diltiazem had been defined as calcium antagonists(46). A new vasodilating drug, SP-294, dilated selectively cerebral and coronary arteries in comparison with peripheral ones in anesthetized dogs. SP-294 inhibited the contraction induced by Ca^{2+} in depolarized arterial smooth muscle and taenia caecum. The mode of the inhibition was non-competitive in arterial smooth muscle and competitive in taenia caecum which was the

same properties as those observed for nifedipine(47,48). It was felt, therefore, that SP-294 has a calcium antagonistic properties although further experiments are needed. Verapamil inhibited Ca^{2+}-ATPase activities of cardiac salcolemmal preparation(49) as well as rat aorta microsomes(50). The present study revealed selective inhibition of ATPase in plasma membrane of endothelial cells by only calcium antagonists including nifedipine, SP-294 and diltiazem among hypotensive drugs. This finding may give a clue for the clarification of the physiological functions of adenosine triphosphatase in plasma membrane of endothelial cells.

\$ 3. Properties of Ca^{2+}- or Mg^{2+}-ATPase.

The properties of Ca^{2+}- or Mg^{2+}-ATPase prepared from endothelial cells were studied. Using double-reciprocal plots it was found that V_{max} was 2.8 and 10.0 μmol Pi/mg protein·hr for Ca^{2+}- and Mg^{2+}-ATPase, respectively, and the corresponding K_m was 4.8×10^{-4} M and 3.2×10^{-4} M as shown in Fig. 8.

The molecular weight of protein(s) with Ca^{2+} or Mg^{2+}-ATPase activity was determined by gel electrophoresis with histochemical activity staining. The results are shown in Fig. 9. A sharp major band caused by the precipitation of lead sulfide appeared on electrophoretic gels. The position of the band obtained for Ca^{2+}-ATPase activity was in good agreement with that obtained for Mg^{2+}-ATPase activity. The molecular weight was estimated to be approximately 250,000.

The next series of experiments were carried out to see the different properties of ATPase activity stimulated with Ca^{2+} or Mg^{2+}. At first, it was found that ATPase obtained was independently activated with Ca^{2+} and Mg^{2+}.

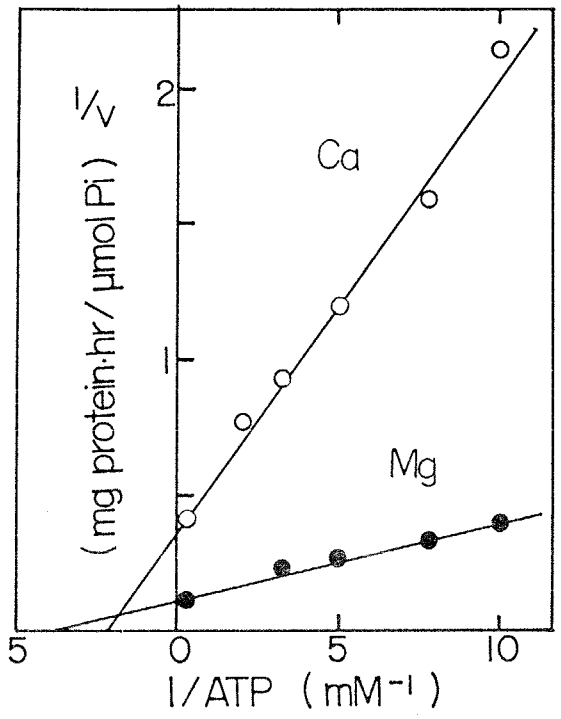


Fig. 8.
Lineweaver-Burk plots of Ca^{2+}-ATPase activity(-O-O-) and Mg^{2+}-ATPase activity (-●-●-) of solubilized proteins from plasma membrane of the cells.

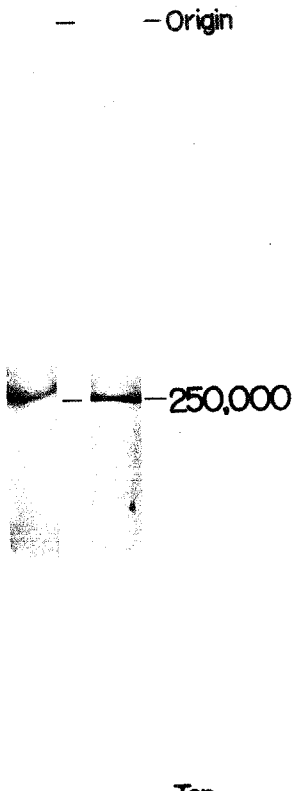


Fig.9.
The patterns of polyacrylamide gel electrophoresis of Ca^{2+}- or Mg^{2+}-ATPase activity.
The method is described in Materials and Methods.
A; Ca^{2+}-ATPase activity.
B; Mg^{2+}-ATPase activity.

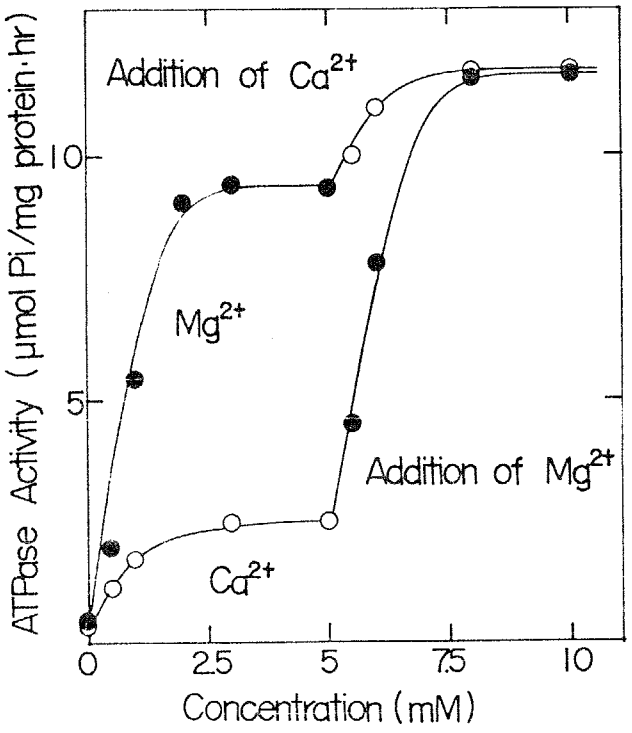


Fig. 10.
Effect of divalent cations on ATPase activity of solubilized membrane proteins. 10 mM ATPwas used in this experiment. -●-●-, Mg^{2+}-ATPase. -0-0-, Ca^{2+}-ATPase.

It was shown in Fig. 10. ATPase activities enhanced with Ca^{2+} (-0-0-) and Mg^{2+} (-●-●-) reached to constant levels(9.2 and 2.6 μmol Pi/mg protein·hr, respectively) above 2.5 mM. The addition of various concentrations of Ca^{2+} in addition to 5 mM Mg^{2+} increased ATPase activities still further to the level comparable to the sum of the maximum activities obtained independently by these two ions. This additive effects of these ions were also observed when various concentration of Mg^{2+} were added on top of 5 mM Ca^{2+}. This indicates that Ca-ATP and Mg-ATP bind to different active sites on ATPase molecule.

The effect of calmodulin on Ca^{2+}- or Mg^{2+}-ATPase treated with EGTA was studied using calmodulin prepared from bovine brain by the method of Dedman et al.(51)(shown in Fig. 11). It has been reported that calmodulin regulates Ca^{2+}-ATPase activity while not Mg^{2+}-ATPase activity, and that calmodulin is

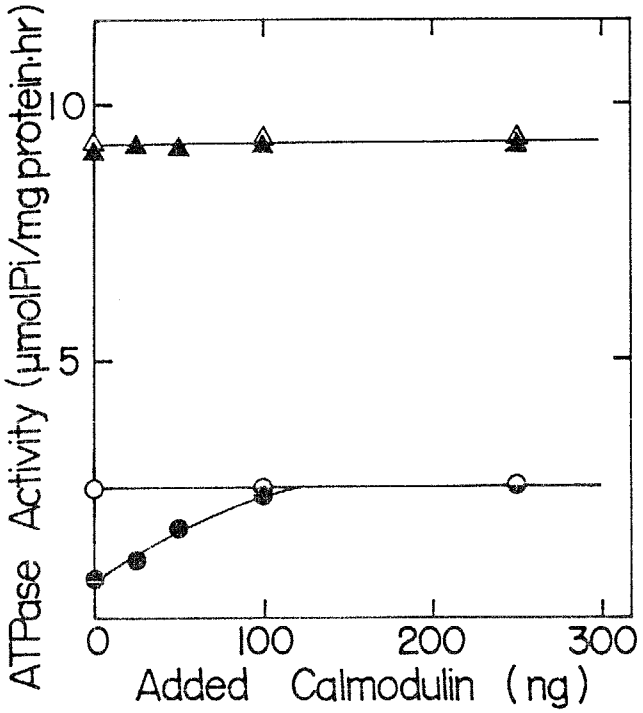


Fig. 11.
The activation of ATPase activity by calmodulin.
Two samples of solubilized proteins from plasma membrane of endothelial cells used in this experiment were prepared as follows. Membrane-bound beads obtained were washed by Tris-HCl buffer(pH 7.4) with and without 1 mM EGTA two times, and proteins were solubilized from membrane by 0.1% $C_{12}E_8$. The ATPase activities enhanced with Ca^{2+} and Mg^{2+} were measured by the addition of various concentrations of calmodulin. -O- and -●-, Ca^{2+}-dependent activity of ATPase untreated and treated by EGTA, respectively. -△- and -▲-, Mg^{2+}-dependent activity of ATPase untreated and treated by EGTA, respectively.

liberated from ATPase molecule by the treatment of EGTA. Ca^{2+}-ATPase activity treated by EGTA was 0.76 μmol Pi/mg protein·hr, which was 1/3 of that obtained for untreated ATPase. By the addition of 100 ng calmodulin, Ca^{2+}-ATPase activity was recovered to normal level(2.55 μmol Pi/mg protein·hr). Mg^{2+}-ATPase activity was not affected by EGTA treatment and calmodulin.

To see further different properties of ATPase enhanced with Ca^{2+} or Mg^{2+}, the chemical modification of SH-groups in ATPase molecule was carried out using DTNB and o-iodosobenzoate. As it is shown in Fig. 12, Mg^{2+}-ATPase activity was inhibited by the modification of SH-groups by DTNB and o-iodoso-

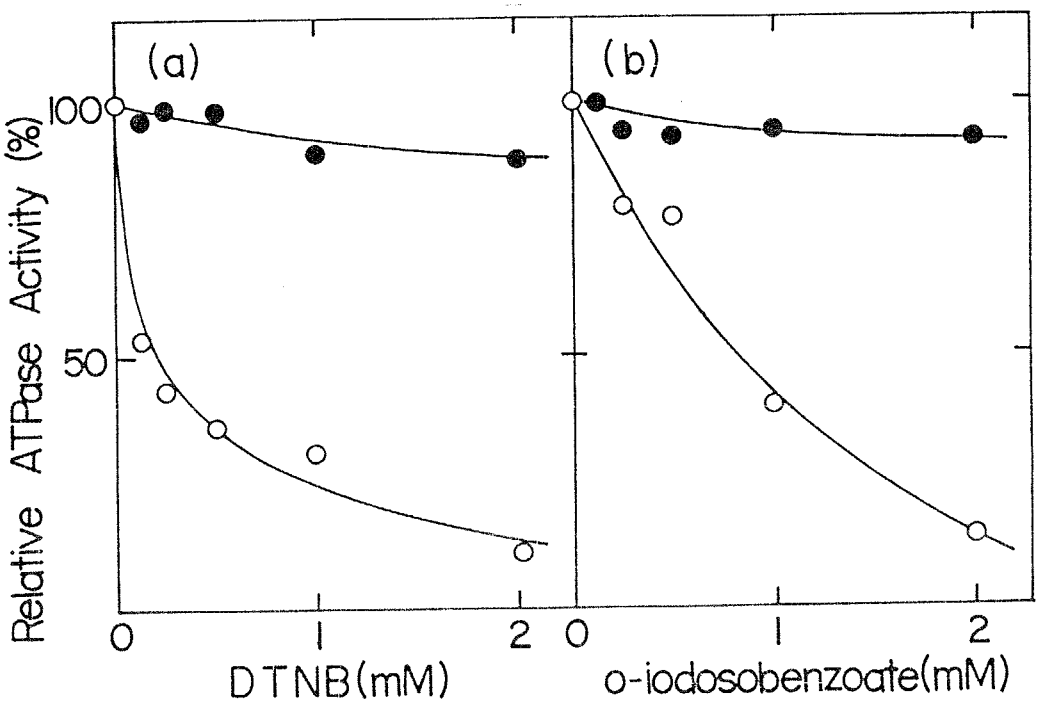


Fig. 12.
The chemical modification of SH-groups in ATPase by DTNB(a) and o-iodosobenzoate (b).
The chemical modification was carried out as follows(52); Membrane-bound beads prepared from endothelial cells were incubated with various concentrations of DTNB and o-iodosobenzoate at 25°C for 1 hr, and then membrane proteins were solubilized from membrane-bound beads by 0.1% $C_{12}E_8$. Mg^{2+}-ATPase activity 100%; 9.3 μmol Pi/mg protein·hr. Ca^{2+}-ATPase activity 100%; 2.4 μmol Pi/mg protein·hr. -●-●-, Ca^{2+}-ATPase activity. -O-O-, Mg^{2+}-ATPase activity.

benzoate, but Ca^{2+}-ATPase activity was not affected by the modification. This indicates that reactive SH-groups are located in the vicinity of the active center of Mg^{2+}-ATPase.

From these results, it was demonstrated that ATPase enhanced with Ca^{2+} or Mg^{2+} may have a different active centers although it is unclear yet whether these two enzymic activities are ascribed to one molecule.

CHAPTER III

PHYTOSTEROL-STIMULATED PRODUCTION OF PLASMINOGEN ACTIVATOR BY CULTURED ENDOTHELIAL CELLS

5. CHAPTER III.

PHYTOSTEROL-STIMULATED PRODUCTION OF PLASMINOGEN ACTIVATOR BY CULTURED ENDOTHELIAL CELLS.

5-1. Summary.

The endothelial cell is a rich source of plasminogen activator that is associated with fibrinolytic activity in blood vessel. Addition of sitosterol, one of plant sterol, to the culture medium of endothelial cells from bovine carotid artery gave rise to a marked increment in the extracellular and intracellular activities of plasminogen activator. Removal of sitosterol from the culture medium resulted in a decrease of plasminogen activator activity back to normal level. A similar phenomenon was observed with fucosterol, which is obtained from algae, but not with other steroids such as cholesterol, stigmasterol, isofucosterol, 5-androsten-3β-ol and others.

5-2. Introduction.

Since endothelial cells were cultured in an artificial medium(3,4), extensive studies have been accumulated for plasminogen activator which is an enzyme that converts plasminogen to plasmin. Loskutoff and Edgington reported the synthesis of a plasminogen activator and an inhibitor of plasminogen activator by endothelial cells from rabbit aorta(53,54). Levin and Loskutoff(13) demonstrated two types of plasminogen activator from bovine aortic endothelial cells; urokinase- and tissue-type plasminogen activators. Bykowska et al.(12) partially purified a plasminogen activator secreted by cultured endothelial cells and clarified physicochemical properties of the enzyme.

Sitosterol was first isolated in 1926 from wheat germ oil by Anderson et al.

(55) and is commonly found in leaves of plants but not in animals. Hamamura et al.(56) pointed out the significance of sitosterol in food selection by silkworm and called it the biting factor. Intraperitoneal injection of sitosterol in rats caused a lowering of cholesterol and lipid concentrations in tissue and enhanced biosynthesis and oxidative degradation of these lipids in tissues(57,58). Several investigators reported that plant sterols including sitosterol have an ability to lower cholesterol level in patients with hypercholesterolemia(59,60).

Fucosterol was first isolated in 1934 from the brown alga Fucus vesiculosus by Heilbron et al.(61).

Loskutoff reported that the fibrinolytic potential of cultured endothelial cells is rapidly suppressed by trace amount of thrombin(62). A similar suppressive effect on plasminogen activator activity by steroid hormones and their derivatives were observed with macrophages(63), tumor cells(64) and slices of rat tongue(65) as well as endothelial cells(66). On the other hand, Butler et al.(67) demonstrated that the production of plasminogen activator is stimulated in human breast cancer cells by estradiol and progesterone, while it is not stimulated in normal breast cells by these steroids. Also, it was reported that glucocorticoids increase protein synthesis and content in cultured endothelial cells without affecting cell proliferation(68).

The present study deals with phytosterol-stimulated production of plasminogen activator in cultured endothelial cells from bovine carotid artery.

5-3. Materials and Methods.

Fibrinogen(93% clottable), thrombin(50 units/mg), urokinase(2400 I.U./ml)

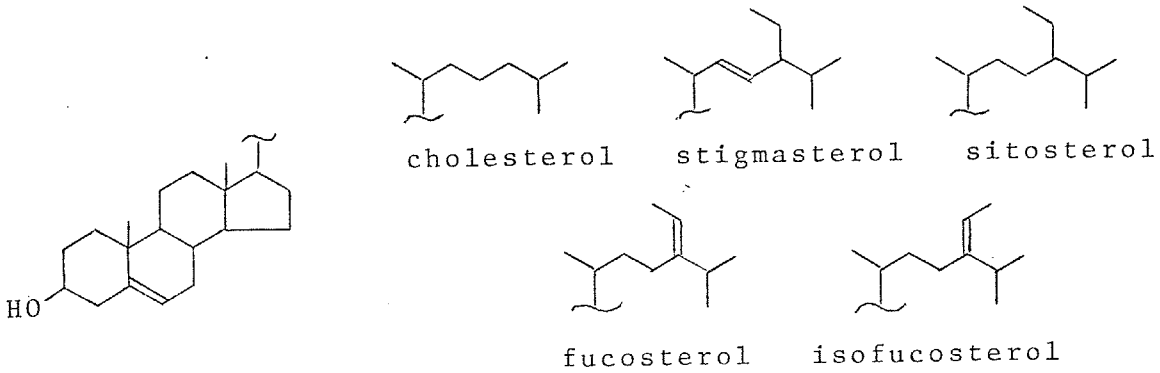


Fig. 13.
The structure of steroids used in this study.

and plasminogen(70 I.U./ml) from human were supplied by Green Cross Co., Japan. Cholesterol and 5-androsten-3β-ol were purchased from Sigma Chemical Company, St. Louis, Mo. and Steraloids, Wilton, NH., respectively. Sitosterol and stigmasterol were purified from Phellodendrom amurense(69), and fucosterol was purified from Fucus evanescens(70). Isofucosterol was prepared by the method of Dusza(71), and 20(R)-propyl-5-pregnen-3β-ol and 20(R)-heptyl-5-pregnen-3β-ol were prepared as described previously(72). The purity of these samples was more than 99%, as analyzed by gas chromatography(73) and/or high pressure liquid chromatography(74). All other reagents were of analytical grade.

Preparation of Conditioned Media and Cellular Extracts.

To see the effect of steroids on the activity of plasminogen activator in endothelial cells, the cells(13-16th passage; 14-17 generations) were subcultured with the culture medium in the presence and the absence of steroids dissolved in 1% ethanol(final concentration). This concentration of ethanol did not affect the secretion of plasminogen activator from the cells in this study. The cells which were harvested from one $55cm^2$ petri dish were seeded in 12 plates with $9cm^2$. Several days after visual confluency was reached,

the monolayers were washed with cold minimum essential medium. The washed monolayers were incubated in the serum-free medium, and at a given time incubated media(conditioned media) and cells were subjected to analysis. The conditioned media, in which plasminogen activator is secreted from endothelial cells, was used as one of samples. The cells were treated with Triton X-100 by the method of Loskutoff and Edgington(53), and cellular extracts thus obtained were used as another sample. The conditioned media and the cellular extracts were stored at -20°C untill assayed.

Assay of Fibrinolytic Activity.

Fibrinolytic activity was measured by the method which was explored by Saito et al.(75). This method is based on the nephelometric measurement of turbidity decrease of fibrin suspension, which is a natural substrate of plasmin formed from plasminogen by plasminogen activator. To 700 µl of a fibrin suspension(35 µg of fibrin) were added 100 µl of plasminogen solution(7.0 units /ml) and 200 µl of conditioned media or cellular extracts with plasminogen activator activity. Turbidity decrease caused by fibrinolysis was recorded at 37°C with time with a Kyoto Daiichi Kagaku nephelometer Model DZ-2111. The fibrinolytic activity of plasminogen activator was expressed in terms of the time required for a 20% reduction of turbidity. Standard curve was obtained by plotting the rate of the turbidity decrease against urokinase concentrations(0.005-0.08 units), so that plasminogen activator activity was expressed as urokinase units.

5-4. Results and Discussion.

Addition of sitosterol or fucosterol to the culture medium causes little

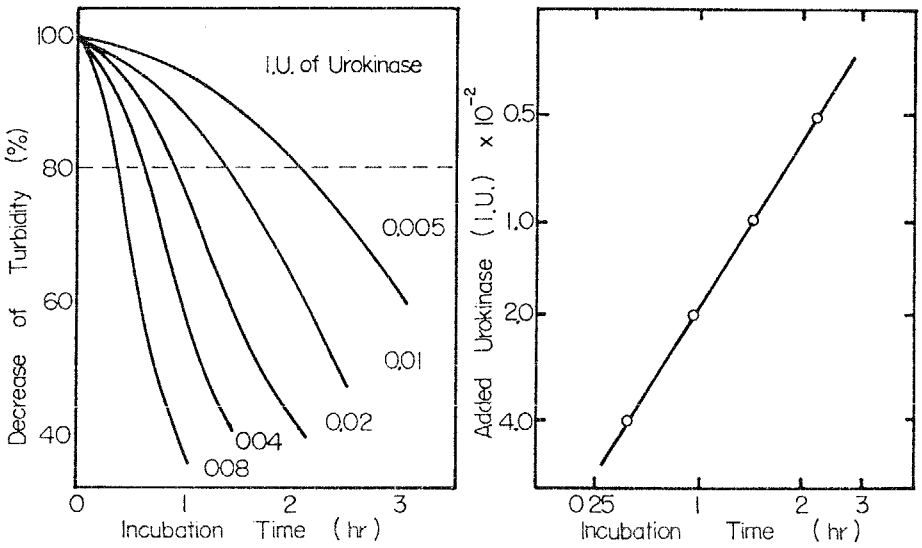


Fig. 14.
Standard curve of urokinase units using fibrin suspension.

morphological changes of cells and scarcely changes of cell number when confluency was reached, in comparison with that of cells cultured without these steroids. The rate of growth was reduced in the presence of sitosterol or fucosterol.

The fibrinolytic activity by plasminogen activator in conditioned media was measured at a given period of incubation-time. The results are shown in Fig. 15, in which each curve was obtained after cells are treated in subculture with sitosterol(-●-), cholesterol(-▲-), 5-androsten-3β-ol(-△-) and without steroid (-○-), respectively. The activity of plasminogen activator obtained from sitosterol-treated cells increased markedly with incubation-time and was 0.45 UK units/10^7 cells for 6 hr-incubation, while that obtained from untreated cells was only 0.1 UK units/10^7 cells. Moreover, the extracellular plasminogen activator activity of sitosterol-treated cells was exponentially increased with incubation-time and the activity at 8 hr-incubation reached in 1.24 UK units/10^7 cells(data not shown). The plasminogen activator activity

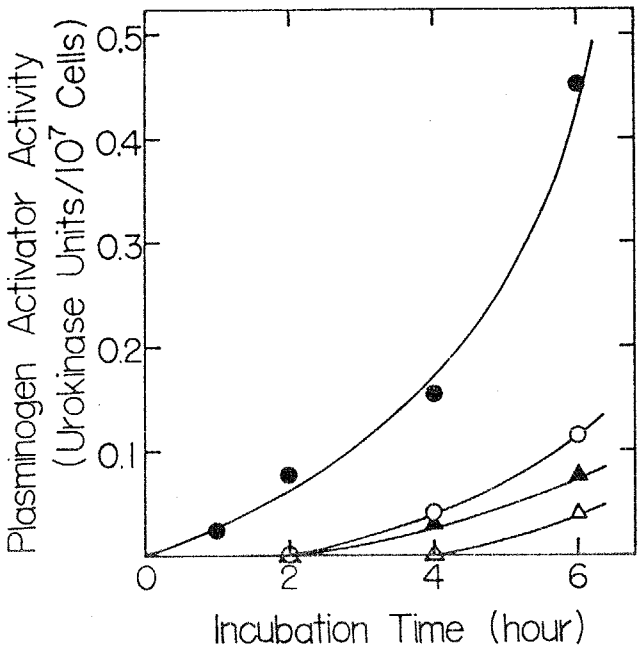


Fig. 15.
Enhancement of plasminogen activator activity in medium by sitosterol-treated cells.
Endothelial cells were subcultured in the presence of 50 μM each steroids. The details are shown in Materials and Methods. Values are represented as means of values obtained from two different samples. Sitosterol-treated, (-●-●-), untreated, (-0-0-), Cholesterol, (-▲-▲-), and 5-androsten-3β-ol, (-△-△-).

in conditioned media of sitosterol-treated cells was always 3-8 times greater than that obtained in the untreated cells. Other steroids such as cholesterol and 5-androsten-3β-ol had no ability to enhance the extracellular activity of plasminogen activator by endothelial cells. Sitosterol itself had no fibrinolytic activity and did not directly stimulate the plasminogen activator activity without endothelial cells.

Fig. 16 shows the relation between the activity of plasminogen activator in conditioned media secreted from endothelial cells and the amount of sitosterol added to the subculture media. The activity of plasminogen activator in conditioned media was increased markedly at concentrations above 20 μM sitosterol and reached a constant level(1.5 UK units/10^7 cells) at 50 μM.

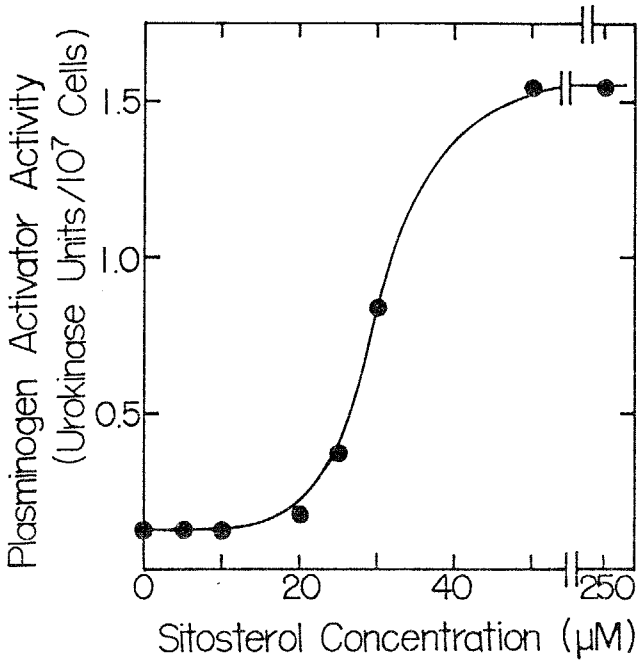


Fig. 16.
Relation between the plasminogen activator activity in conditioned media and the amount of sitosterol added.
The plasminogen activator activity in conditioned media after 6 hr-incubation was assayed. The details are shown in Materials and Methods. Values are represented as means of values obtained from two different samples.

The question arises as to whether sitosterol enhances the secretion of plasminogen activator in conditioned media from endothelial cells or the synthesis of plasminogen activator in the cells. To clarify this question, cells were subcultured in the presence and the absence of sitosterol(50 μM), and the activity of plasminogen activator was determined with two kinds of samples, conditioned media and cellular extracts. The results are shown in TABLE IV. In the sitosterol-treated cells, the activity in cellular extracts at 6 hr-incubation was 0.39 UK units/10^7 cells, which was 6.5 folds over that obtained for untreated cells(0.06 UK units/10^7 cells). The activity in conditioned media(6 hr-incubation) of sitosterol-treated cells was 0.42 UK units/10^7 cells which is 3.5 times greater than that obtained in untreated cells. This indi-

TABLE IV. EXTRACELLULAR AND INTRACELLULAR ACTIVITIES OF PLASMINOGEN ACTIVATOR IN SITOSTEROL-TREATED OR UNTREATED ENDOTHELIAL CELLS.

Culture Medium	Plasminogen Activator Activity(Urokinase Units/10^7 Cells)			
	Cellular Extracts after Incubation for		Conditioned Media after Incubation for	
	0 hr	6 hr	0 hr	6 hr
Control	0.01	0.06	0.00	0.12
+Sitosterol(50 μM)	0.19	0.39	0.00	0.42

Endothelial cell monolayers obtained in the medium with and without 50 μM sitosterol were washed and were cultured with a fresh serum-free medium. After incubation, conditioned media and cellular extracts were prepared by the method described in Materials and Methods. Values are represented as means of values obtained from different four samples.

TABLE V. TRANSFORMATION OF SITOSTEROL-TREATED CELLS INTO NORMAL CELLS BY REMOVING SITOSTEROL IN CULTURE MEDIUM.

Condition	Plasminogen Activator Activity(Urokinase Units/10^7 Cells)	
	Control	Sitosterol-treated Cells
subculture with and without sitosterol(50 μM)*	0.10	0.65
additional culture for one week in the medium without sitosterol**	0.12	0.10

* Sitosterol-treated and untreated cells were incubated for 6 hr in the serum-free medium and the plasminogen activator activity in conditioned media were assayed by the method described in Materials and Methods.

** Sitosterol-treated and untreated cells were further cultured for one week in a fresh culture medium without sitosterol, and conditioned media were assayed as above. Values are represented as means of values obtained from four different samples.

cates that sitosterol has an ability to enhance not only extracellular activity but also intracellular one of plasminogen activator.

The next series of experiments were carried out to see whether sitosterol-treated cells were transformed into normal cells by removing sitosterol in

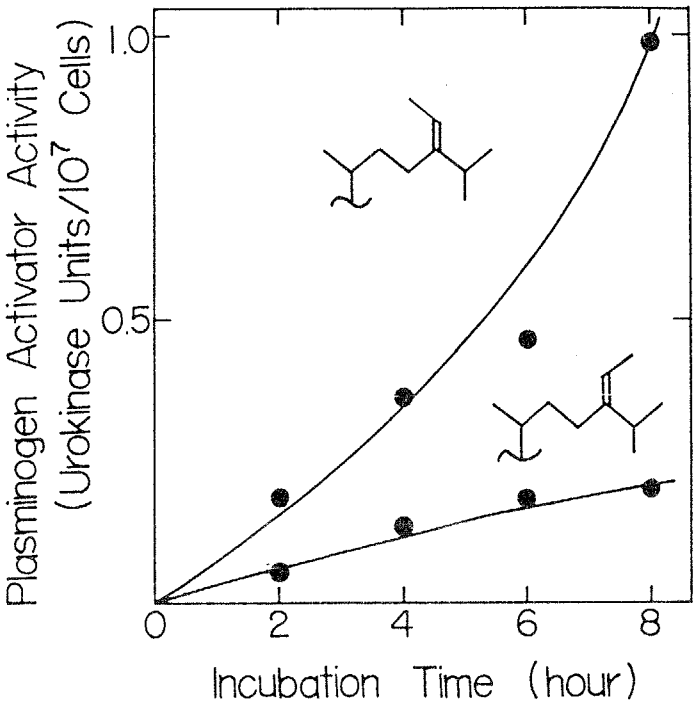


Fig. 17.
The effect of fucosterol and isofucosterol on the secretion of plasminogen activator from endothelial cells.
Endothelial cells were subcultured in the presence of 25 μM fucosterol and isofucosterol. The details are shown in Materials and Methods. Values are represented as means of values obtained from two different samples.

culture medium. Sitosterol-treated cells with high levels of plasminogen activator activity(0.65 UK units/10^7 cells) were further cultured in a fresh medium without sitosterol for one week, and the activity in these cells was determined(TABLE V). Consequently, removal of sitosterol from the culture medium resulted in a decrease of plasminogen activator activity back to normal level. This indicates that cells once stimulated with sitosterol are transformed into normal cells by the incubation with the medium in the absence of sitosterol.

The effect of other steroids such as fucosterol, isofucosterol, stigmasterol, brassicasterol, 20(R)-propyl-5-pregnen-3β-ol and 20(R)-heptyl-5-pregnen-3β-ol on the secretion of plasminogen activator from endothelial cells was

investigated. As it is shown in Fig. 17, fucosterol stimulated the production of plasminogen activator in endothelial cells. Isofucosterol and others did not affected the production of plasminogen activator in the cells.

From these results obtained above, it may be concluded that a certain plant sterol, sitosterol and fucosterol, enhances not only secretion of plasminogen activator from the cells but also synthesis of the activator in the interior of the cells, although its mechanism is unclear yet. This finding may suggest that sitosterol and fucosterol make a novel agent for prevention and therapy of patients with thrombosis.

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7. ACKNOWLEDGEMENT.

I would like to express sincere gratitude to Professor Yuji Inada for his wholehearted guidances, correct advices and encouragement throughout these studies. I would also like to thank Dr. Yuji Saito, Dr. Ayako Matsushima, Miss Yayoi Ohtsu, Mr. Motoyuki Shimonaka and colleagues in Laboratory of Biological Chemistry for their assistance as well as for their helpful discussion.

I am deeply indebted to Professor S. Murota and Dr. I.Morita in Tokyo Metropolitan Institute of Gerontology, to Professor Y. Nakamura and colleagues in School of Pharmaceutical Sciences, Showa University and to Professor N. Ikekawa and Dr. M. Morisaki in Chemistry Department, Tokyo Institute of Technology for many helpful suggestions and discussion throughout these studies.