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Ca^{2+}- OR Mg^{2+} -DEPENDENT ATPase IN PLASMA MEMBRANE OF CULTURED ENDOTHELIAL CELLS FROM BOVINE CAROTID ARTERY

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ATPase was found in plasma membrane of cultured endothelial cells from bovine carotid artery. The activity of the enzyme solubilized by octaethyleneglycol mono-*n*-dodecyl ether was enhanced by the addition of Ca^{2+} or Mg^{2+} and was not affected by F-actin and ouabain. V_{max} was 2.8 and 10.0 μmol P_i/mg protein per h for Ca^{2+}- and Mg^{2+}-dependent activity, respectively, and the corresponding K_m was $4.8 \cdot 10^{-4}$ M and $3.2 \cdot 10^{-4}$ M. Molecular weight of the protein was estimated to be approx. 250000, as determined by activity-staining electrophoresis with polyacrylamide gels.

Introduction

Recently, studies on cultured endothelial cells have been accumulated in relation to plasminogen activator [1,2], prostacyclin (PGI_2) [3,4], angiotensin converting enzyme [4,5] and blood coagulation factor VIII antigen [6]. No information about ATPase in plasma membrane of endothelial cells has been obtained as yet. ATPase activity enhanced with Ca^{2+} or Mg^{2+} was found in various plasma membranes including human erythrocyte [7–9], rat liver cell [10], rat hepatoma cell [11] and human granulocyte [12]. Some of these enzymes were partially purified after solubilization and their physicochemical properties were revealed.

The present paper deals with Ca^{2+}- or Mg^{2+}-dependent ATPase found in plasma membrane of cultured carotic endothelial cells from bovine.

Materials and Methods

Fetal bovine serum, antibiotics (penicilline-streptomycin solution) and Eagle's minimum essential medium were purchased from GIBCO New York. 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.

Endothelial cell culture

Endothelial cells were cultured by the method of Gospodarowicz et al. [13] as follows. Fresh carotid arteries were obtained from adults of bovine species in a slaughther house. The carotid arteries were opened lengthwise with a scalpel and endothelial cell layer was obtained by gently scraping the intimal surface with a scalpel. Endothelial cells were cultured in plastic petri dishes in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, streptomycin (50 μg/ml) and penicillin (50 units/ml) in an atmosphere of 95% air/5% CO_2 at 37°C. Cells in passage 13–15 to confluency were used in the present study. Endothelial cells were dissociated from the dish surface by 0.05% trypsin/0.02% EDTA/137 mM NaCl/4 mM KCl/0.5 mM $Na_2HPO_4 \cdot 12H_2O$/0.15 mM KH_2PO_4/11 mM glucose (pH

7.0). The number of cells in a petri dish (55 cm^2) was approx. $1.5 \cdot 10^7$.

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 [14]. Plasma membrane proteins were solubilized from the beads by dropping a mixture of 0.1 M KCl/20% glycerol/0.3 M sucrose/0.05–0.2% octaethyleneglycol mono-*n*-dodecyl ether ($C_{12}E_8$) dissolved in 10 mM Tes-NaOH buffer (pH 7.5) to the beads at 4°C over a period of 1 h [15].

Measurement of ATPase activity

Ca^{2+}- or Mg^{2+}-dependent 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)/3 mM ATP/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 [16]. $(Na^+ + K^+)$-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)/5 mM $MgCl_2$/140 mM NaCl/14 mM KCl/0.5 mM EDTA/3 mM ATP in the presence and the absence of 0.2 mM ouabain [17]. 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. [18].

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 h 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 $CaCl_2$)/20 mM Tris-maleate (pH 8.0) for 12 h at room temperature. The position of the enzyme was stained with 0.5% PbS [7,19].

Results and Discussion

Bovine endothelial cells in tissue culture in passage 15 shown in Fig. 1 have a characteristic morphology, so that they are not contaminated with smooth muscle cells.

Before solubilizing ATPase from beads, its activity was assessed with membrane-bound beads in the presence of monovalent and divalent cations. The activity was very small in the presence of EDTA and was markedly enhanced by the addition of Ca^{2+} or Mg^{2+}. The membrane bound to beads had also ouabain-inhibitable $(Na^+ + K^+)$-ATPase activity. The latter enzyme is known as a marker enzyme of plasma membrane [20].

In order to obtain further information about ATPase in the membrane, we 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 Mg^{2+}-dependent ATPase activity. Using the solubilized membrane proteins, the effect of divalent cations on ATPase activity was tested. The results are shown in Fig. 2. The activity was enhanced by increasing the concentration of Ca^{2+} or Mg^{2+} and reached a constant activity at 3 mM divalent cation. The ATPase activity stimu-

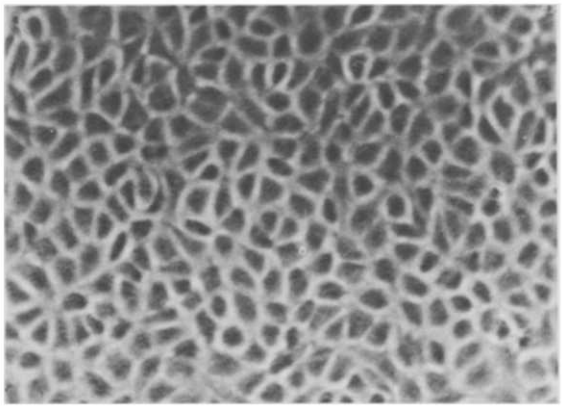

Fig. 1. Micrograph (×200) of cultured endothelial cells from bovine carotid artery.

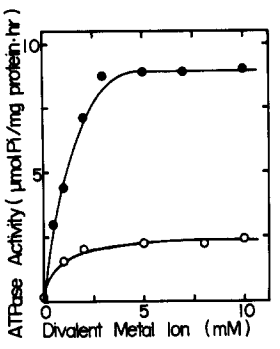

Fig. 2. Effect of divalent cations on solubilized ATPase obtained from cultured endothelial cells from bovine carotid artery. ○——○, in the presence of Ca^{2+}; ●——●, in the presence of Mg^{2+}.

lated by Mg^{2+} was approx. 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 I. No hydrolysis of *p*-nitrophenyl phosphate, which is known as a substrate of phosphatase [21], 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 found in endoplasmic reticulum [22]. The liberation of inorganic phosphate from AMP or ADP took place to only 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+}-dependent ATPase activity was tested. No stimulative effect on the ATPase activity was observed in the presence of Ca^{2+} or Mg^{2+}. This indicates that the ATPase obtained from carotic endothelial cells is quite different from myosin ATPase from muscle. The Ca^{2+}- or Mg^{2+}-dependent ATPase was not inhibited by ouabain, an inhibitor of $(Na^+ + K^+)$-ATPase.

TABLE I

SUBSTRATE SPECIFICITY OF AND THE EFFECT OF F-ACTIN AND OUABAIN ON THE SOLUBILIZED ATPase IN THE PRESENCE OF Ca^{2+} AND Mg^{2+}

ADP, AMP, *p*-nitrophenyl phosphate and glucose 6-phosphate were added instead of ATP to a reaction medium (see Materials and Methods).

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

[a] Ca^{2+}-ATPase activity, 100%; 2.4 μmol P_i/mg protein per h.
[b] Mg^{2+}-ATPase activity, 100%; 9.3 μmol P_i/mg protein per h.

Using double-reciprocal plots it was found that V_{max} was 2.8 and 10.0 μmol P_i/mg protein per h for Ca^{2+}- and Mg^{2+}-dependent ATPase, respectively, and the corresponding K_m was $4.8 \cdot 10^{-4}$ M and $3.2 \cdot 10^{-4}$ M.

The molecular weight of a protein(s) with Ca^{2+} or Mg^{2+}-dependent ATPase activity was determined by gel electrophoresis with histochemical activity staining. A sharp major band caused by the precipitation of lead sulfide appeared on electrophoretic gels. The position of the band obtained for Ca^{2+}-dependent ATPase was in good agreement with that obtained for Mg^{2+}-dependent ATPase. The molecular weight of the band was estimated to be approx. 250000. Although these results may suggest that ATPase present in plasma membrane of carotic endothelial cells stimulated by Ca^{2+} or Mg^{2+} is a single protein with molecular weight of 250000, it remains to be seen whether they are identical in many other aspects.

Questions as to the physiological roles played by the enzyme(s) and detailed study about the divalent metal ion reqirement are currently under investigation.

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