Vol. 88, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1979 Pages 147-153

INCREASED CA^{2+}-ATPASE ACTIVITY ASSOCIATED WITH METHYLATION OF PHOSPHOLIPIDS IN HUMAN ERYTHROCYTES

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Received March 15,1979

ABSTRACT

Ca^{2+}-ATPase activity in human erythrocytes is increased by the enzymatic methylation of membrane phospholipids. Erythrocyte membranes incubated in the presence of the methyl donor, S-adenosyl-L-methionine, demonstrate increased Ca^{2+} stimulated ATP hydrolysis, increased [$^{45}Ca^{2+}$] efflux from erythrocyte ghosts and synthesis of phosphatidyl-N-monomethylethanolamine. The increase in Ca^{2+}-ATPase activity is due to an increase in Vmax, and not due to changes in affinity for ATP or Ca^{2+}. The concentration of S-adenosyl-L-methionine needed to stimulate Ca^{2+}-ATPase closely matches that needed for the methylation of phosphatidylethanolamine. Both the stimulation of Ca^{2+}-ATPase and the methylation of phospholipids are inhibited by the methyltransferase inhibitor, S-adenosyl-L-homocysteine. Membrane fluidity is increased by phospholipid methylation, which may be the mechanism for Ca^{2+}-ATPase stimulation.

INTRODUCTION

Calcium ATPase hydrolyzes ATP and transports Ca^{2+} across the plasma membrane (1,2). The enzyme is embedded in an annulus of phospholipids which is essential for its function (3). The isolated Ca^{2+} transport protein can be reactivated by the addition of phospholipids after inactivation by removal of lipids (4). Previous studies from this laboratory have demonstrated two methyltransferases involved in the synthesis of membrane phosphatidylcholine from phosphatidylethanolamine (5,6). Because the phospholipid environment is important in Ca^{2+}-ATPase activity, we examined the effects of methylation of membrane phospholipids on the activity of the enzyme.

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0006-291X/79/090147-07$01.00/0

Phospholipid methyltransferase I, located on the cytoplasmic surface of the erythrocyte membrane, transfers a methyl group from the methyl donor, S-adenosyl-L-methionine, to phosphatidylethanolamine to form phosphatidyl-N-monomethylethanolamine. The second enzyme, located on the exterior surface of the membrane, adds two additional methyl groups to form phosphatidylcholine. The first methyltransferase has a high affinity (Km = 1.4 μM) for S-adenosyl-L-methionine, and requires Mg^{2+}. The second enzyme has a low affinity (Km = 100 μM) for S-adenosyl-L-methionine (5,6). Data presented here indicate that in the human erythrocyte, phospholipid methylation increases Ca^{2+}-ATPase mediated hydrolysis of ATP and transport of Ca^{2+}.

MATERIALS AND METHODS

Materials: S-adenosyl-[methyl-^{3}H]-L-methionine was from New England Nuclear, Boston, Mass.; $^{45}CaCl_2$ was from Amersham Corporation, Arlington Heights, Illinois. S-adenosyl-L-methionine and S-adenosyl-L-homocysteine were from Boehringer Mannheim Biochemicals, Indianapolis, Indiana. Adenosine triphosphate, from equine muscle (vanadium free), Tris (hydroxymethyl) aminomethane chloride and glycylglycine were from Sigma Chemical Company, St. Louis, Missouri. Phospholipid standards were from Grand Island Biological Company, Grand Island, New York. Silica gel G chromatography plates were from Analtech, Inc., Newark, Delaware.

Methods:

Preparation of human erythrocyte membranes: Thirty ml of heparinized blood was obtained by venipuncture, washed twice by centrifugation and resuspended in 0.9% NaCl. Membranes were prepared by hemolyzing the washed erythrocytes in 40 volumes of 5 mM Tris(hydroxymethyl) aminomethane (Tris) phosphate buffer with 1 mM $MgCl_2$. The membranes were centrifuged and resuspended in 5 volumes 0.5 mM Tris-glycylglycine, pH 8.0, with 1 mM $MgCl_2$. The membranes were then centrifuged and resuspended in 3 volumes 25 mM Tris-glycylglycine, pH 8.0, with 5 mM $MgCl_2$. Membranes were either used on the day of preparation or frozen and stored at -20°.

Determination of Ca^{2+}-ATPase activity: Ca^{2+}-ATPase activity was determined by measuring free inorganic phosphate released by the hydrolysis of ATP, using a spectrophotometric assay (7). Erythrocyte membranes (180 μl) were incubated for 20 minutes at 38° in the presence of 2.5 mM ATP, with and without 0.1 mM $CaCl_2$. Ca^{2+} stimulated ATPase was defined as the difference in hydrolysis of ATP in the presence and absence of Ca^{2+}. Assays were run in triplicate.

Determination of phospholipid methylation: Erythrocyte membranes were incubated 20 minutes at 38° in the presence of S-adenosyl-[methyl-^{3}H]-methionine. The [^{3}H] methyl groups incorporated into the lipid fraction were measured by extraction with 3 ml of chloroform/methanol/2 N HCl

(6/3/1, v/v) as described previously (5,6). The chloroform phase was washed twice with 1.5 ml of 0.1 N KCl in 50% methanol. To separate and quantitate the various methylated phospholipids, the chloroform phase was dehydrated with anhydrous sodium sulfate and evaporated under a stream of N_2 gas. The residue was dissolved into a small volume of chloroform-methanol mixture (1/1, v/v) and applied on a silica gel G Plate. The chromatography was developed with a solvent system of propionic acid/n-propyl alcohol/ chloroform/water (2/2/1/1, v/v). The front migrated approximately 16 cm.

Determination of [$^{45}Ca^{2+}$] efflux from erythrocyte ghosts: Erythrocyte ghosts were prepared from heparinized human blood, using a technique described previously (8). Ghosts (125 µl) were incubated overnight at 4° in 50 mM Tris-glycylglycine, 5 mM $MgCl_2$, pH 8.0, with 5 mM ATP, and 0.9 ml of 1 mM $CaCl_2$ and 0.1 ml $^{45}CaCl_2$ (0.75 mCi), with and without 50 µM S-adenosyl-L-methionine. After the ghosts were incubated overnight, they were washed in 5 volumes 0.9% NaCl with 5 mM $MgCl_2$ and 1 mM $CaCl_2$. The pellet was washed again in 3 ml of 100 mM Tris-glycylglycine, 5 mM $MgCl_2$ and 1 mM $CaCl_2$ and resuspended in 1.0 ml. The reaction was started by transferring the ghosts from 4° to 24°. Fifty µl aliquots were withdrawn and centrifuged for 30 seconds in a Beckman Microfuge B. Thirty µl of supernatant were removed, and counted by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

To determine whether phospholipid methylation altered Ca^{2+}-ATPase activity, human erythrocyte membranes were preincubated for 20 minutes with varying concentrations of S-adenosyl-L-methionine (0-10 µM) and 0.1 mM $MgCl_2$. Ca^{2+}-ATPase activity was then determined by measuring Ca^{2+} stimulated ATP hydrolysis as described in Methods. S-adenosyl-L-methionine increased ATP hydrolysis in a concentration dependent manner with maximal stimulation observed at 4 µM S-adenosyl-L-methionine (Fig. 1A). In six experiments, methylation increased Ca^{2+}-ATPase hydrolysis of ATP by 0.054 µmoles Pi/ mg protein/20 min, from a basal activity of 0.126 $\pm$ .010 to a stimulated activity of 0.180 $\pm$ .011 µmoles Pi/mg protein/20 min. The stimulation of Ca^{2+}-ATPase took place at a concentration of S-adenosyl-L-methionine that synthesized phosphatidyl-N-monomethylethanolamine but only negligible amounts of phosphatidylcholine (Fig. 1A). To further evaluate the role of phospholipid methylation, the effect of the methyltransferase inhibitor, S-adenosyl-L-homocysteine, was examined. Membranes were preincubated for 10 minutes in the presence of 10 µM S-adenosyl-L-methionine and various concentrations (0-300 µM) of S-adenosyl-L-homocysteine. S-Adenosyl-L-homocysteine decreased both phospholipid methylation (5,6) and Ca^{2+}-ATPase

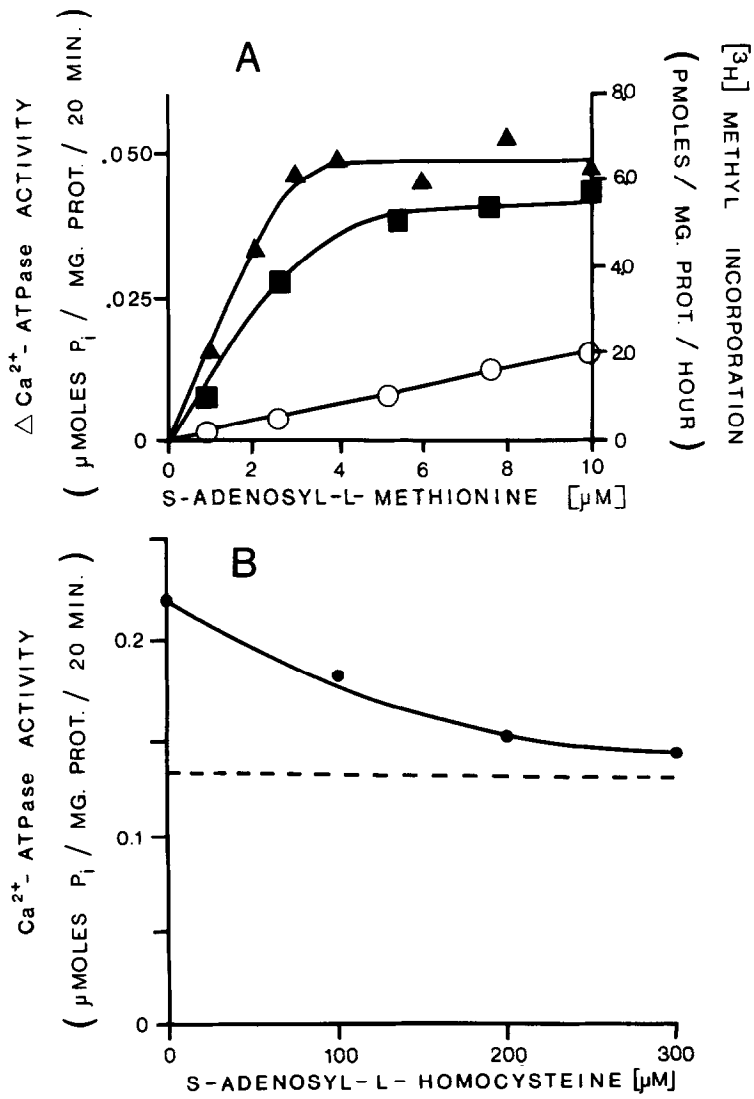

Fig. 1A. Effect of varying concentrations of the methyl donor S-adenosyl-L-methionine on Ca^{2+}-ATPase activity and phospholipid methylation. Erythrocyte membranes were incubated with varying concentrations of [^{3}H]-S-adenosyl-L-methionine, and the synthesis of phosphatidyl-N-monomethylethanolamine (■——■) and phosphatidylcholine (○——○) was determined by thin layer chromatography. Ca^{2+}-ATPase activity (▲——▲) was determined by measuring the hydrolysis of ATP in the presence and absence of Ca^{2+}. Ca^{2+}-ATPase is expressed as the change in Ca^{2+}-ATP activity in the presence of S-adenosyl-L-methionine. Each point represents the mean of triplicate determinations in one experiment. The experiment was performed three times with similar results.

Fig. 1B. Inhibition of Ca^{2+}-ATPase activation by the methyltransferase inhibitor, S-adenosyl-L-homocysteine. Erythrocyte membranes were pre-incubated with 10 μM S-adenosyl-L-methionine and varying concentrations of (0-200 μM) S-adenosyl-L-homocysteine, and Ca^{2+} stimulated ATP hydrolysis was measured. The dotted line indicates Ca^{2+} stimulated ATP hydrolysis in the absence of both S-adenosyl-L-methionine and S-adenosyl-L-homocysteine.

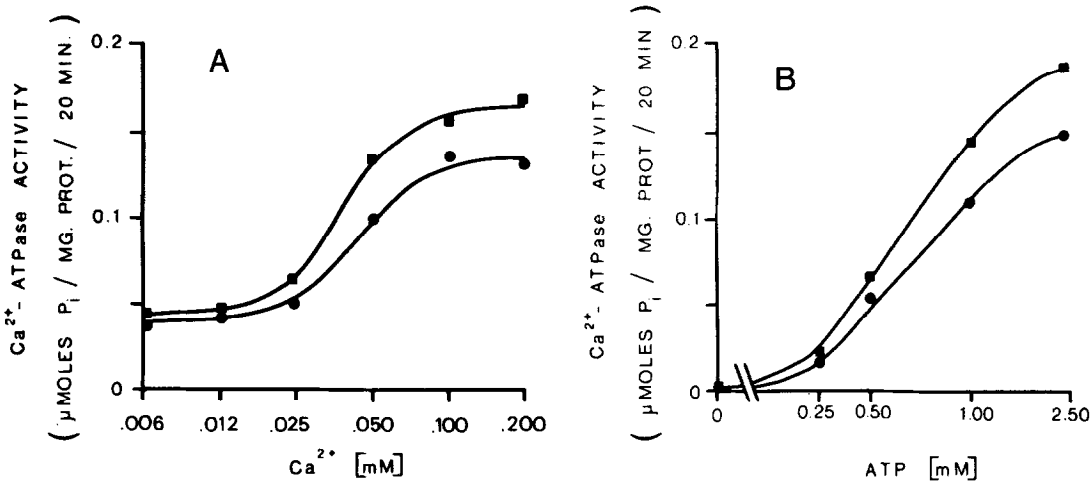

Fig. 2A. Effect of varying Ca^{2+} concentration on Ca^{2+}-ATPase with and without methyl donor S-adenosyl-L-methionine. Erythrocyte membranes were preincubated with (■——■) and without (●——●) 200 µM S-adenosyl-L-methionine. Ca^{2+}-ATPase activity was determined by measuring ATP hydrolysis in the presence of varying concentrations of $CaCl_2$ (0-0.2 mM). Results shown are from one experiment. This experiment was performed four times with similar results.

Fig. 2B. Effect of varying ATP concentrations on Ca^{2+}-ATPase with and without S-adenosyl-L-methionine. Membranes were preincubated with (■——■) and without (●——●) 200 µM S-adenosyl-L-methionine. Ca^{2+}-ATPase activity was then determined in the presence of 0.1 mM $CaCl_2$ and varying concentrations of ATP (0.25-2.0 mM). Results shown are from one experiment. The experiment was performed twice.

activity (Fig. 1B). Mg^{2+}-ATPase was not altered by phospholipid methylation (data not shown).

To determine whether phospholipid methylation increased Ca^{2+}-ATPase activity by altering the affinity for Ca^{2+} or ATP, Ca^{2+}-ATPase activity was measured in varying concentrations of $CaCl_2$ (0-0.2 mM) or ATP (0.25-2.0 mM) following preincubation with 200 µM S-adenosyl-L-methionine. The apparent affinity for Ca^{2+} (Km = 0.04 mM) and for ATP (Km = 0.65 mM) was unaltered by the absence or presence of S-adenosyl-L-methionine. Increased Ca^{2+}-ATPase activity was observed at saturating concentrations of either Ca^{2+} (Fig. 2A) or ATP (Fig. 2B), indicating that increased activity of Ca^{2+} ATPase was due to changes in maximal rate (Vmax) but not to changes in affinity (Km) for Ca^{2+} and ATP.

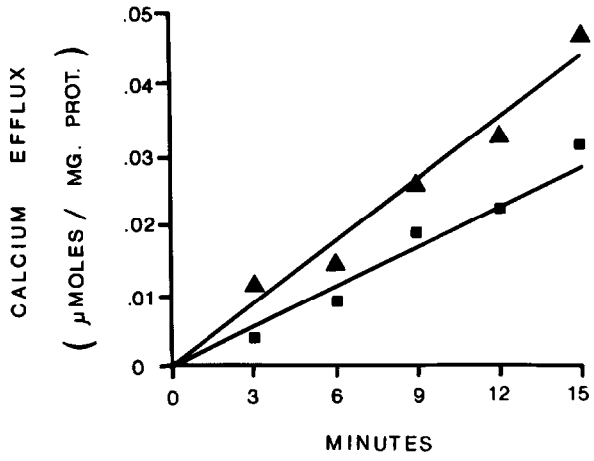

Fig. 3. Effect of S-adenosyl-L-methionine on calcium efflux from erythrocyte ghosts. Ghosts were prepared containing 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.75 mCi $^{45}CaCl_2$, with (▲——▲) and without (■——■) 50 μM S-adenosyl-L-methionine. $^{2}[^{45}Ca^{2+}]$ efflux was measured at various times during incubation at 24°C. Each point represents the mean of duplicate determinations. The experiment was performed four times with similar results.

Since phospholipid methylation increased the Ca^{2+}-ATPase hydrolysis of ATP, its effect on transport of Ca^{2+} in human erythrocyte ghosts was studied. Ca^{2+} efflux was measured by preparing erythrocyte ghosts containing $^{45}CaCl_2$, $MgCl_2$, and ATP with and without 20 μM S-adenosyl-L-methionine. Ghosts containing S-adenosyl-L-methionine demonstrated increased efflux of $^{45}Ca^{2+}$ (Fig. 3). The passive exchange of $^{45}Ca^{2+}$ from ghosts, determined by omitting ATP, was not altered by methylation (data not shown).

The mechanism by which phospholipid methylation increases Ca^{2+}-ATPase activity is not known. Methyltransferase I synthesizes phosphatidyl-N-monomethylethanolamine, a phospholipid that increases membrane fluidity (9). Stimulation of Ca^{2+}-ATPase activity by methyltransferase I may be due to the synthesis of a phospholipid that enhances enzyme activity or due to changes in the physical properties of the membrane. Other studies have shown that Ca^{2+}-ATPase activity in sarcoplasmic reticulum can be decreased by reducing membrane fluidity with exogenous phospholipids (10). Increased membrane fluidity due to methyltransferase I would permit increased ro-

tational mobility and might directly facilitate Ca^{2+} transport through the membrane. The amount of phospholipid synthesized by transmethylation is small, compared to the total phospholipid content of the membrane. However the turnover of phospholipids in this pathway is approximately fifty times of the phospholipid turnover in the CDP pathway (unpublished data). Phospholipid domains surrounding transport proteins might profoundly alter transport function with relatively little synthesis. In addition to modulating Ca^{2+}-ATPase activity, phospholipid methylation affects many other membrane functions such as β-adrenergic receptor-adenylate cyclase coupling (8), availability of β-adrenergic receptors (11), mast cell secretion and lymphocyte mitogenesis (unpublished data).

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